STUDIES ON INTERSPECIFIC AND INTRASPECIFIC VARIATION IN THE GENUS *PLUMBAGO* (PLUMBAGINACEAE) FROM SOUTH INDIA

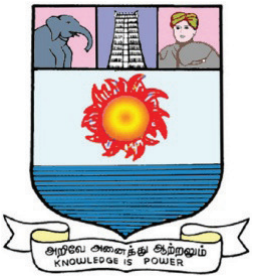
THESIS SUBMITTED TO
MANONMANIAM SUNDARANAR UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

By

RENISHEYA JOY JEBA MALAR. T

(Reg. No.5682)



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CERTIFICATE

This thesis entitled **"STUDIES ON INTERSPECIFIC AND INTRASPECIFIC VARIATION IN THE GENUS *PLUMBAGO* (PLUMBAGINACEAE) FROM SOUTH INDIA"** submitted by **Mrs. T. RENISHEYA JOY JEBA MALAR** for the award of Degree of Doctor of Philosophy in Biotechnology of Manonmanium Sundaranar University, Tirunelveli is a record of bonafide research work done by her and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University/Institution.

Station: Palayamkottai **Signature of the Guide**

Date :

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DECLARATION

I hereby declare that the thesis entitled **" STUDIES ON INTERSPECIFIC AND INTRASPECIFIC VARIATION IN THE GENUS *PLUMBAGO* (PLUMBAGINACEAE) FROM SOUTH INDIA"** submitted by me for the Degree of Doctor of Philosophy in Biotechnology is the result of my orginal and independent research work carried out under the guidance of **Dr. M. Johnson**, Assistant Professor, Department of Botany, St. Xavier's College (Autonomous), Palayamkottai and it has not been submitted for the degree, diploma, associateship, fellowship of any University or Institution.

Place: Palayamkottai **T. Renisheya Joy Jeba Malar**

Date:

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T. Renisheya Joy Jeba Malar

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Table – 24: Compounds identified in the ethanolic extracts of *Plumbago zeylanica* by GC-MS

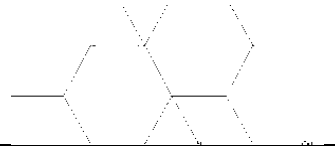
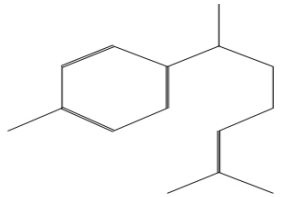
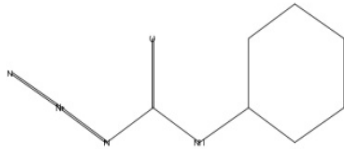
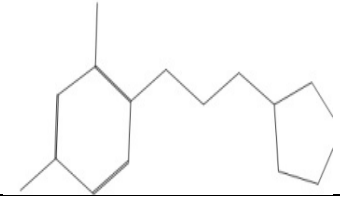
Si. No	Name of the compound	RT	Peak area %	Mol. formula	Mol. wt	Biological activity	Structure of compound
1.	**(1R,2S,4aR,6R,8aR)-1,2,4a,5,6,7,8,8aOctahydro-2,6-dimethyl-1-(hydroxymethyl)naphthalene**	14.04	0.83	C13H22O	194	Respiratory failure Euphoria, Dermatitis, Conjunctivitis, interleukin antagonist, siberoblastic	
2.	**3-Tetradecene, (E)-**	14.57	0.77	C14H28	196	JAK2 expression inhibitor,Thrombocytopoies inhibitor, Diarrhea, neurotrophic	
3.	**Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- (CAS)**	16.51	0.59	C15H22	202	Demyelination,Thioredoxin inhibitor, Xerostomia, Alpha-N acetyl glucosaminidase inhibitor	
4.	**Cyclohexyl Carbamoyl Azide**	18.65	1.34	C7H12N4O	168	HERG channel blocker, Potassium channel blocker and interleukin antagonist.	
5.	**Benzene, 1-(3-cyclopentylpropyl)-2,4-dimethyl- (CAS)**	20.32	0.89	C16H24	216	Contd., JAK2 expression inhibitor, Hypercholesterolemic, UGT1A4 substrate	

Table – 24: Compounds identified in the ethanolic extracts of *Plumbago zeylanica* by GC-MS Contd.,

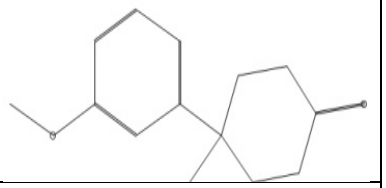
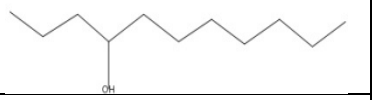
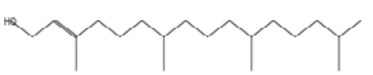
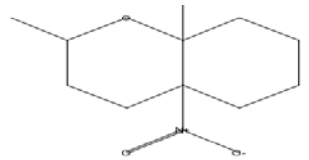
6.	**4-(3-Methoxyphenyl)-4-methylcyclohexanone**	22.29	0.63	C14H18O2	218	Occular toxicity, optic neuropathy, optic neuritis, Respiratory analeptic	
7.	**Undecanol-4**	22.56	0.83	C11H24O	172	Ocular toxicity, Hypercholesterolemic, Behavioral disturbance.	
8.	**1,2-Octadecanediol (CAS)**	23.29	0.50	C18H38O2	286	Stroke treatment, Antiischemic, cerebral, Acute neurologic disorders treatment, Thioredoxin inhibitor.	
9.	**2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS)**	30.12	3.90	C20H40O	296	Lacrimal secretion stimulant, Anaemia, Thioredoxin inhibitor, Respiratory failure, Ataxia, Euphoria, Sensitization, Antiprotozoal (Leishmania)	
10.	**Hexadecanoic acid, methyl ester (CAS)**	26.23	8.87	C17H34O2	270	Antineoplastic reproductive dysfunction, Thromboxane synthase stimulant, JAK2 expression inhibitor, Fibrillation, atrial.	
11.	**t-3-Methyl-c-6-nitro-2-oxabicyclo[4.4.0]decan-r-1-ol**	26.63	1.32	C10H17NO4	215	Ataxia, Diarrhea, Amyloid beta precursor protein antagonist, Anemia, Dyskinesia, GST P1-1 substrate.	

Table – 24: Compounds identified in the ethanolic extracts of *Plumbago zeylanica* by GC-MS Contd.,

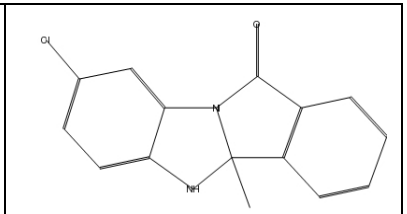
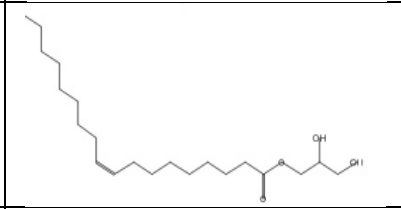
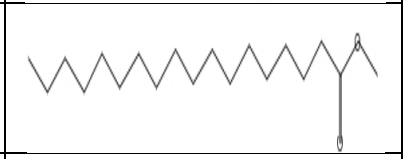
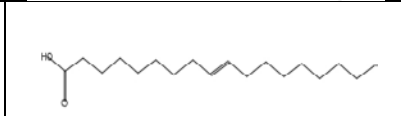
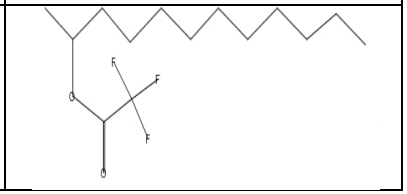
12.	**4b,5-Dihydro-4b-methyl-8-chloro-11H-isoindolo[2,1-a]benzimidazol-11-one**	27.10	26.90	C15H11ClN2O	270	Laryngospasm, Reproductive dysfunction, Demyelination, Anti-hepatitis.	
13.	**9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester**	29.89	15.54	C21H40O4	356	Optic neuritis, analeptic and optic neuropathy.	
14.	**Octadecanoic acid, methyl ester (CAS)**	30.50	0.41	C19H38O2	298	GST A substrate, Euphoria, Apnea, Conjunctivitis, Muscle weakness.	
15.	**9-Octadecenoic acid (Z)-(CAS)**	30.87	11.90	C18H34O2	282	Stroke treatment, acute neurologic disorder treatment, respiratory failure and anemia,	
16.	**2-Trifluoroacetoxydodecane**	31.44	0.91	C14H25F3O2	282	Euphoria, Respiratory analeptic, JAK2 expression inhibitor	

Table – 24: Compounds identified in the ethanolic extracts of *Plumbago zeylanica* by GC-MS Contd.,

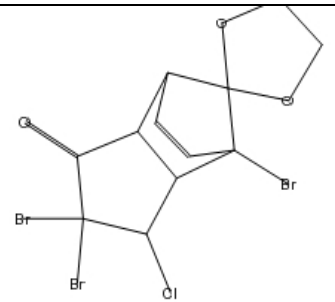
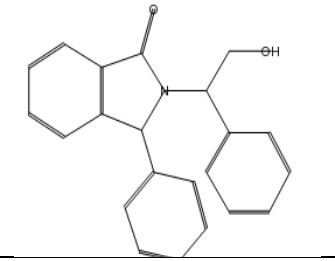
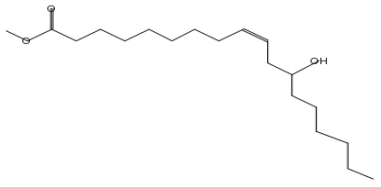
17.	**1-(2,3,5-Tribromophenyl)-2-(tert-butyl)diazene**	32.95	1.02	C10H11Br3N2	396	Visual acuity impairment, Lacrimal secretion stimulant, Fibrillation, atrial, Acidosis, metabolic, Reproductive dysfunction.	
18.	**(3S)-3-Phenyl-2-[(1S)-2-hydroxy-1-phenylethyl]-2,3-di hydro-1H-isoindol-1-one**	33.26	0.56	C22H19NO2	329	GST A substrate, Euphoria, Apnea, Conjunctivitis, Muscle weakness, Acidosis.	
19.	**Methyl ricinoleate**	34.07	0.89	C19H36O3	312	Catalase inhibitor, Tetany, Pulmonary edema, Fibrillation, atrial, sideroblastic, Hypercholesterolemic	
20.	**6-Chlorohexylhydroperoxide**	34.68	2.48	C6H13ClO2	152	Antineoplastic, Reproductive dysfunction, Aldehyde oxidase inhibitor, JAK2 expression inhibitor	

Table – 24: Compounds identified in the ethanolic extracts of *Plumbago zeylanica* by GC-MS Contd.,

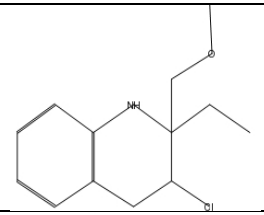
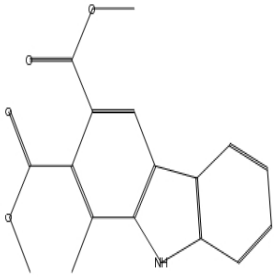
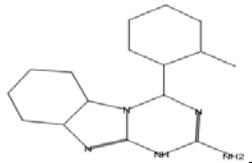
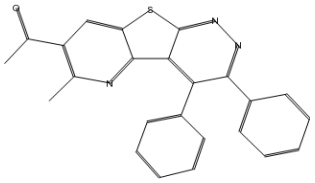
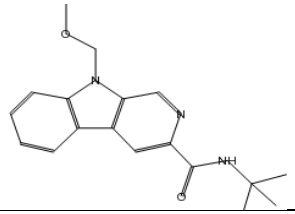
21.	**(2S*,3R*)-3-Chloro-2-ethyl-2-(methoxymethyl)-1,2,3,4-tetrahydroquinoline**	38.70	0.64	C13H18ClNO	239	GST A substrate, Conjunctivitis, Hepatotoxic, Neurotoxic	
22.	**bis(methyl) 1-methyl9H-carbzole-2,3 dicarboxylate**	41.18	1.87	C17H15NO4	297	Dyspnea, Reproductive dysfunction, Thioredoxin inhibitor, Apnea, platelet aggregation inhibitor, ulceration	
23.	**[1,3,5]Triazino[1,2a][1,3]benzimidazol-2-amine,1,4 dihydro-4-(2-methylphenyl)-**	42.26	0.82	C16H15N5	277	Antiischemic, Antidiabetic, Antidiabetic (type 2), Thioredoxin inhibitor.	
24.	**7-Acetyl-6-methyl-3,4-diphenylpyrido[2',3':4,5]thieno[2,3-c]pyridazine**	42.59	0.92	C24H17N3OS	395	Antialcoholic, Signal transduction pathways inhibitor, sideroblastic, Interleukin antagonist.	
25.	**N"-tert-Butyl-9 methoxymethyl-á-carboline-3-carboxamide**	43.38	0.91	C18H21N3O2	311	Flavin-containing monooxygenase substrate, Neuropeptide Y2 antagonist, Fasciculation, HCV IRES inhibitor,	

Table – 24: Compounds identified in the ethanolic extracts of *Plumbago zeylanica* by GC-MS Contd.,

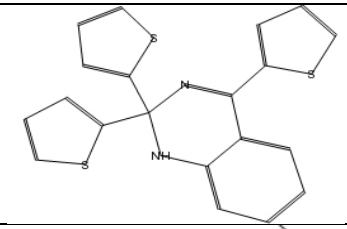
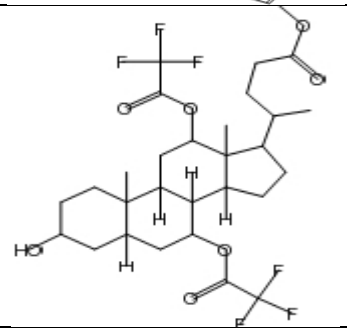
26.	**2,2,4-tri(2-thienyl)-1,2dihydroquinazoline**	44.36	6.79	C20H14N2S3	378	Antineoplastic, Retinoic acid receptor antagonist, Antiviral	
27.	**Methyl ester of (3à,5á,7à,12à)-3-hydroxy-7,12bis[(trifluoroacetyl)oxy]cholan-24-oic acid**	45.60	1.21	C29H40F6O7	614	Prostacyclin antagonist, Retinoic acid receptor.	

Table – 25: Compounds identified in the ethanolic extracts of *P. auriculata* by GC-MS

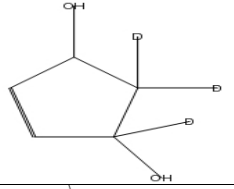
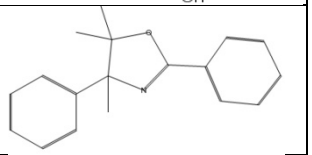
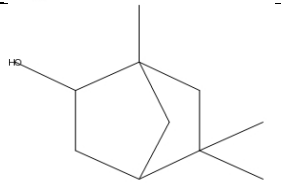
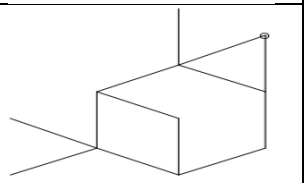
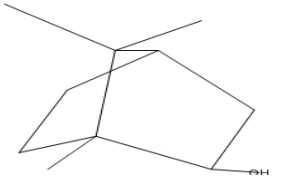
SINO	Name of the compound	RT	Peak area %	Mol. form	Mol.wt	Biological activity	Structure of compound
1.	3,4,4-D3-Trans-3,5-Dihydroxy-cyclopentene	3.04	0.06	C5H5D3O2	100	Pulmonary edema, Antiinflammatory, Respiratory failure, Thrombocytopoiesis inhibitor, Xerostamia	
2.	4,5,5-Trimethyl-2,4-diphenyl-2-oxazoline	3.86	0.20	C18H19NO	265	HERG channel blocker, Hypercholesterolemic, UGT1A4 substrate, potassium channel blocker.	
3.	Bicyclo[2.2.1]heptan-2-ol,1,5,5-trimethyl- (CAS)	8.55	1.09	C10H18O	154	Optic neuropathy, Hypercholesterolemic, JAK2 expression inhibitor.	
4.	3Oxatricyclo[4.1.1.0(2,4)]octane, 2,7,7-trimethyl-	9.32	0.57	C10H16O	152	Respiratory analeptic	
5.	Isoborneol	9.73	86.99	C10H18O	154	Optic neuropathy, analeptic, tetany, Respiratory failure, Optic neuropathy, reproductive dysfunction	

Table – 25: Compounds identified in the ethanolic extracts of *P. auriculata* by GC-MS

Contd.,

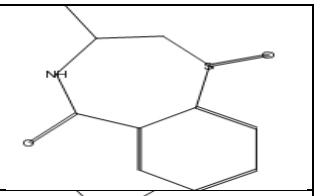
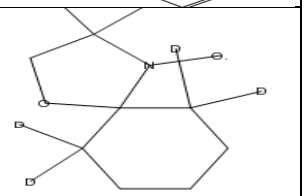
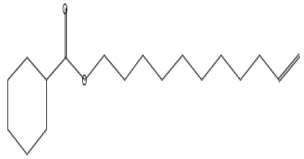
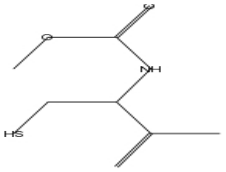
6.	3-Methyl-2,3-dihydro-1,4-benzothiazepin-5(4h)-one	10.36	6.21	C10H11NO2S	209	Antiiischemic, interleukin 1b antagonist, antithrombotic, antiepileptic, insulin inhibitor, Optic neuropathy.	
7.	3,3-Dimethyl-1-oxa-4-Azaspiro(4.5) Dec-4-y loxy-6,6,10,10-D4	14.07	0.09	C10H14D4NO2	184	Thioredoxin inhibitor Shivering	
8.	2-Methyl-n-1-tridecene	14.56	0.19	C14H28	196	Lacrimal secretion stimulant, Antineoplastic, embryotoxic, ataxia and Demyelination, apoptosis agonist	
9.	Cyclohexanecarboxylic acid, undec-10-enyl ester	14.97	0.04	C18H32O2	280	Apnea, Thioredoxin inhibitor, Anemia, Antiprotozoal, ataxia Lacrimal secretion stimulant, Euphoria	
10	5-Octadecene, (E)-	18.65	0.23	C18H36	252	Vanilloid 1 agonist,Visual acuity impairment, Reproductive dysfunction, Endocannabinoid uptake inhibitor .	
11	1-mercapto-2 methoxycarbonylamino 3-methylbut-3-ene	20.36	0.08	C7H13NO2S	175	Lipoprotein lipase inhibitor, Lacrimal secretion stimulant, Laryngospasm, Spermicide, Thrombocytopoiesis	

Table – 25: Compounds identified in the ethanolic extracts of *P. auriculata* by GC-MS

Contd.

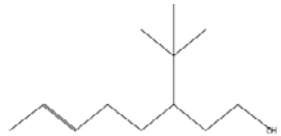
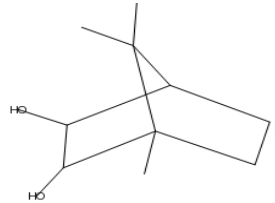
						inhibitor	
12	(RS)-n-Dodecyl trifluoromethyl carbinol	8.45	0.16	C14H27F3O	268	Lipoprotein lipase inhibitor,spermicide, Hematotoxic Thrombocytopoiesis inhibitor, Dyskinesia Reproductive dysfunction, Tetany	
13	4-Hexenoic acid, 2,2,5-trimethyl-, ethyl ester	24.24	0.15	C12H24O	184	Tetany, Hypercholesterolemic, Euphoria, Respiratory analeptic, JAK2 expression inhibitor, optic neuritis	
14	Bicyclo[2.2.1]heptane-2,3-diol, 1,7,7-trimethyl-, (exo,exo)-	24.79	0.08	C10H18O2	170	Gastrointestinal hemorrhage, Tetany, Visual acuity impairment, Lacrimal secretion stimulant, BRAF expression inhibitor, Fibrillation, spermicide	
15	10-Nonadecanone	25.09	0.16	C19H38O	282	Hyperthermic, Pulmonary edema, Reproductive dysfunction, Psychoses, Myoclonus, GABA aminotransferase, Hypotonia.	

Table – 25: Compounds identified in the ethanolic extracts of *P. auriculata* by GC-MS

Contd.,

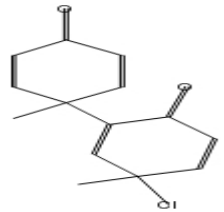
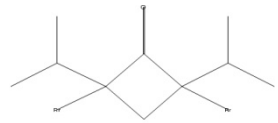
16	Hexadecanoic acid, methyl ester (CAS)	26.21	0.73	C17H34O2	270	Succinate dehydrogenase inhibitor, peroxidase inhibitor, myoclonus, Aldehyde oxidase inhibitor, catalase inhihitor.	
17	n-Hexadecanoic acid	27.01	0.84	C16H32O2	256	Optic neuritis, antineoplastic, reproductive dysfunction, thioredoxin inhibitor, antineoplastic	
18	2-[4'-Methyl-1'-oxocyclohexa-2',5'-dien-4'-yl]-4-methyl-4-chloro-1-oxocyclohexa-2,5diene	27.66	0.04	C14H13ClO2	248	Antiasthmatic, antiallergic, Antiinflammatory, optic neuropathy, hepatotoxic, pneumotoxic	
19	Methyl dodecadienoate	29.84	0.33	C13H22O2	210	Apoptopsis agonist, spermicide, anti-inflammatory, hypercholesterolemic, reproductive dysfunction	
20	2,4-Dibromo-2,4-diisopropylcyclobutane	30.08	0.08	C10H16Br2O	310	Antidiabetic, Antidiabetic (type 2), Thioredoxin inhibitor, neurotoxic, dyskinesia	
21	Pentadecanoic acid, 15-bromo-, methyl ester	30.49	0.08	C16H31BrO2	334	Atherosclerosis treatment, pulmonary edema, ABCA1 expression enhancer, visual acuity inhibitor	

Table – 25: Compounds identified in the ethanolic extracts of *P. auriculata* by GC-MS

Contd.

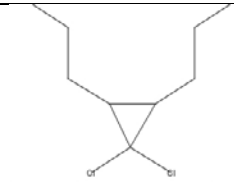
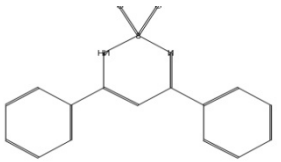
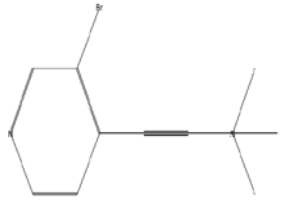
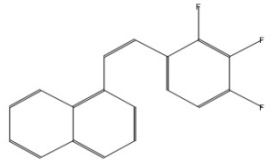
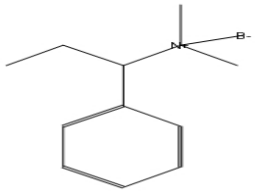
22	Ethyl 2-heptynoate	30.94	0.28	C9H14O2	154	Antidiabetic (type 2) pulmonary edema, thioredoxin inhibitor	
23	3,5-Diphenyl-2H-1,2,6-thiadiazine 1,1-dioxide	31.47	0.06	C15H12N2O2S	284	Antialcoholic, sideroblastic, antineoplastic (endocrine cancer), nootropic, signal transduction pathway inhibitor.	
24	3-Bromo-4 (trimethylsilylethynyl) pyridine	33.24	0.14	C10H12BrNSi	253	Lacrimal secretion stimulant, HCV IRES inhibitor, Superoxide dismutase inhibitor, Aldehyde oxidase inhibitor, Thrombocytopoiesis inhibitor.	
25	(Z)-1-(2,3,4-Trifluorophenyl)-2-(naphthyl)ethene	34.02	0.11	C18H11F3	284	Platelet aggregation inhibitor, FMO1 substrate, ulceration, Antineoplastic (lymphocytic leukemia), Hypocalcaemic, Neuropeptide Y2 antagonist	
26	à-(Ethyl) benzyldimethylamine borane complex	38.66	0.19	C11H20BN	177	Antineoplastic, Retinoic acid receptor antagonist, Antiviral, antiischimic.	

Table – 25: Compounds identified in the ethanolic extracts of *P. auriculata* by GC-MS

Contd.,

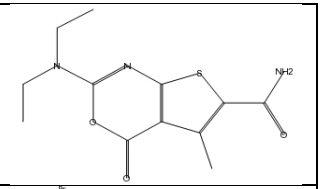
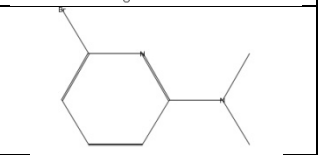
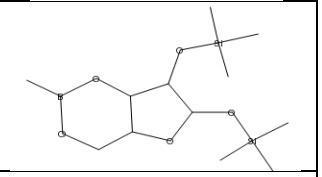
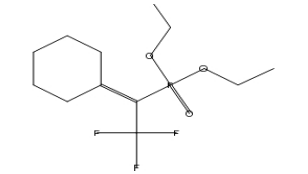
27	6-Carbamoyl-2-(diethylamino)-5methyl-4H-thieno[2,3-d] [1,3]oxazin-4-one	41.13	0.07	C12H15N3O3S	281	Pulmonary edema, Visual acuity impairment.	
28	6-bromo-2-(dimethylamino)pyridine	42.49	0.33	C7H9BrN2	200	Prostacyclin antagonist, Shivering.	
29	á-D-Xylofuranose,1,2bis-O-(trimethylsilyl),cyclic methylboronate (CAS)	43.39	0.16	C12H27BO5Si2	318	Antineoplastic inhibitor, cell adhesion inhibitor, sideroblasic	
30	5,6-Epoxy6,10Dimethyl-3-methoxymethyl-2-oxo-2,3,3A,4,5,6,7,8,12,15,15A-Dodecahydro cyclotetradeca(B)furan14-ol	45.04	0.27	C21H32O5	364	Aldehyde oxidase inhibitor, GST A substrate, Dyskinesia, Reproductive dysfunction	

Table – 26: Compounds identified in the ethanolic extracts of *Plumbago rosea* by GC-MS

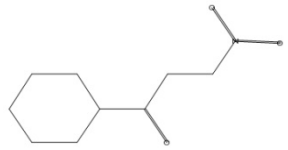
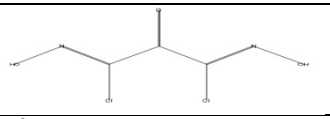
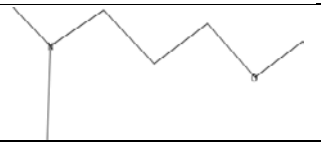
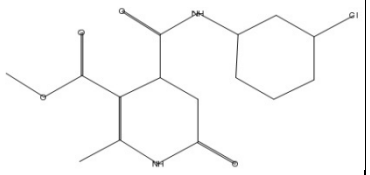
SI.NO	Name of the compound	RT	Peak area %	Mol.frm	Mol. wt	Biological activity	Structure of compounds
1.	**Propan-1-one, 3-nitro-1-phenyl-**	3.86	9.48	C9H9NO3	179	Superoxide dismutase inhibitor, Thyroid hormone alpha antagonist, Hypothermic, Extra pyramidal effect, neurotoxicity	
2.	**Excitability 1,3-Dichloro-1,3-dioximino-2-propanone**	4.26	1.38	C3H2Cl2N2O3	184	Peroxidase inhibitor, Shivering	
3.	**N,N-Dimethyl-3-methoxypropylamine**	10.31	1.20	C6H15NO	117	Spasmolytic, Muscle weakness, Laryngospasm, visual acuity inhibitor, occular toxicity, Cyanosis.	
4.	**1-Tetradecene**	14.55	2.07	C14H28	196	Pulmonary edema, Lacrymal secretion stimulant, GABA aminotransferase inhibitor Hypothermic, CYP2E1 substrate, Thioredoxin inhibitor.	
5.	**3-Pyridinecarboxylic acid, 4-[[(3 chlorophenyl)amino]carbonyl]-1,4,5,6-tetrahydro -2-methyl-6-oxo-, methyl ester**	18.12	1.03	C15H15ClN2O4	322	Anti anginal, Heart failure treatment, anti nephritic, antiviral, Photo allergy dermatitis, RNA-directed RNA polymerase inhibitor, Calcium channel activator.	
6.	**1-Hexadecene**	18.65	3.63	C16H32	224	Conjunctivitis, GABA aminotransferase inhibitor, Nicotinic alpha 4 beta2 receptor antagonist, Hepatotoxic, lipoprotein lipase inhibitor.	

Table – 26: Compounds identified in the ethanolic extracts of *Plumbago rosea* by GC-MS

Contd.

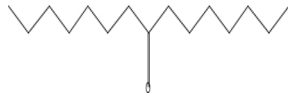
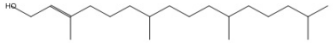
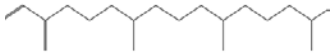
7.	**8-Pentadecanone (CAS)**	20.38	0.42	C15H30O	226	Conjunctivitis, GABA aminotransferase inhibitor, Nicotinic alpha4beta2 receptor antagonist, Hepatotoxic, Demyelination.	
8.	**14,16-Dioxa-4-heptadecanol 91.47**	22.56	0.97	C15H32O3	260	Tetany, Anemia, GST A substrate, Vasodilator, peripheral, Conjunctivitis, Shivering, Spermicide ,Apnea	
9.	**1-Eicosanol**	23.27	1.95	C20H42O	298	Dermatitis, CCL2 expression inhibitor, lacrymal secretion stimulator, occular toxicity, pulmonary edema, spermicide, Tetany, TP53 expression enhancer,spermicide.	
10.	**2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS) 6.50**	24.25	11.69	C20H40O	296	Reproductive dysfunction, Respiratory failure, Dyspnea, Asthma.	
11.	**Neophytadiene**	25.21	4.99	C20H38	278	Lipoprotein lipase inhibitor, Peroxidase inhibitor, Superoxide dismutase inhibitor.	
12.	**Hexadecanoic acid, methyl ester (CAS)**	26.21	6.24	C17H34O2	270	Shivering, GST A substrate, Euphoria, Apnea, Conjunctivitis, Muscle weakness, Acidosis, metabolic, GABA aminotransferase inhibitor.	

Table – 26: Compounds identified in the ethanolic extracts of *Plumbago rosea* by GC-MS

Contd.

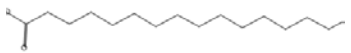
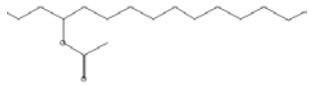
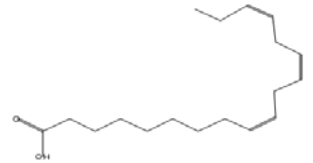
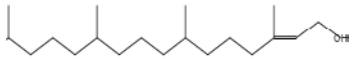
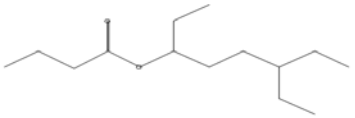
13.	**n-Hexadecanoic acid**	27.00	14.19	C16H32O2	256	Antiulcerative, CCL2 expression inhibitor, Hematotoxic, sideroblastic, Nephrotoxic, Embryotoxic.	
14.	**4-Acetoxypentadecane**	27.65	1.09	C17H34O2	270	Miotic, Pulmonary edema, Anti inflammatory, Lacrymal secretion stimulant, Hepatitis, nephrotoxic	
15.	**9,12,15-Octadecatrienoic acid, (Z,Z,Z)-**	29.83	6.64	C18H30O2	278	Neurotoxic, sensitization, ataxia.	
16.	**Phytol**	30.08	7.22	C20H40O	296	Ocular toxicity, Conjunctivitis, GABA amino transferase inhibitor, TP53 expression enhancer Antiulcerative, hypotonia	
17.	**6,9,12,15-Docosatetraenoic acid, methyl ester (CAS)**	30.79	6.23	C23H38O2	346	Succinate dehydrogenase inhibitor, Optic neuropathy, Lipoprotein lipase inhibitor, Catalase inhibitor, GABA aminotransferase inhibitor.	
18.	**(5à,6à)-4,5-epoxy-3-acetoxymorphinan-6-one**	31.36	2.05	C18H19NO4	313	Anti ischemic, Anti inflammatory, Lacrymal secretion stimulant, Dermatitis, BRAF expression inhibitor.	

Table – 26: Compounds identified in the ethanolic extracts of *Plumbago rosea* by GC-MS

Contd.

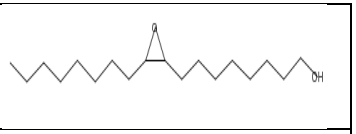
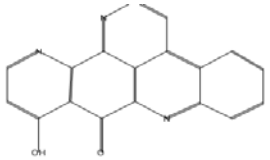
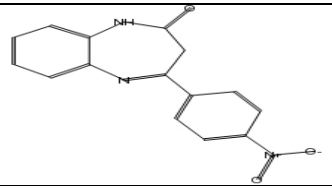
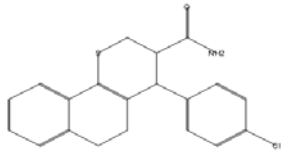
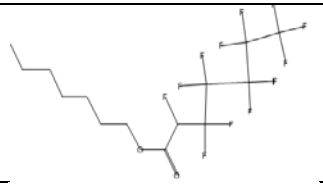
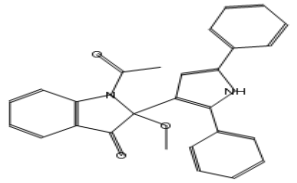
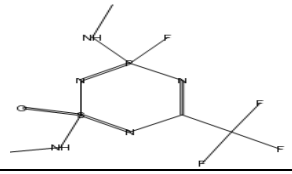
19.	**cis-9,10-Epoxyoctadecan-1-ol**	32.07	2.92	C18H36O2	284	Anti neoplastic (bladder cancer, colorectal cancer, colon cancer, lung cancer).	
20.	**11-Hydroxyascididemin**	32.71	0.83	C18H9N3O2	299	Acidosis, metabolic Shivering, Euphoria, Superoxide dismutase inhibitor, Lipoprotein lipase inhibitor, GST A substrate.	
21.	**2-(4-hydroxyphenyl)-3-phenylindenone**	38.33	1.00	C21H14O2	298	TP53 expression enhancer, Gastrointestinal hemorrhage, peroxidase inhibitor, nephrotoxic.	
22.	**3-Carbamoyl-4-(p-chlorophenyl)-5,6-dihydrobenzo[h]thiochroman**	38.65	0.44	C20H18ClNOS	355	Endo cannabinoid uptake inhibitor, Vasodilator, Inflammation, Antithrombotic	
23.	**heptyl 2-hydroperfluoroheptanoate**	40.49	0.96	C14H16F12O2	444	Glutamate receptor antagonist, teratogen, hypolipemic, GST A substrate	
24.	**1-Acetyl-2-(2,5-diphenylpyrrol-3-yl)-2-methoxy-1,2-dihydro-3H-indol-3-one**	41.36	3.06	C27H22N2O3	422	Visual acuity impairment, Gastrointestinal hemorrhage, Spermicide, Allergic contact dermatitis, Apnea.	
25.	**5-Chloro-3-(3,4-dimethoxyphenyl)-6-methyl-2H-1,4-oxazin-2-one**	42.08	1.24	C13H12ClNO4	281	Anti neoplastic (bladder cancer, colorectal cancer, colon cancer and lung cancer)	

Table – 26: Compounds identified in the ethanolic extracts of *Plumbago rosea* by GC-MS

Contd.

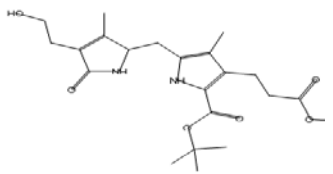
26.	**Methyl ester of 5'-Tert-Butoxycarbonyl-2,5-Dihydro-4-(2H ydroxyethyl)-3,3'-Dimethyl-5-oxo-2,2'-dip yryl methan-4'-propionic acid**	42.79	1.37	C22H32N2O6	420	Antineoplastic (cervical cancer), Thioredoxin inhibitor, Panic, NOS2 expression inhibitor, Photosensitizer, Superoxide dismutase inhibitor, Apoptosis agonist, Antineoplastic.	
27.	**Tricyclo[4.4.1.0(1,6)]undeca-3,8-dien-11,11-dimethanol**	45.54	0.73	C13H18O2	206	RNA-directed RNA polymerase inhibitor, Inflammation, Neuroleptic malignant syndrome, Anti ischemic, Ocular toxicity, Asthma.	
28.	**D:A-Friedoolean-18-ene (CAS)**	45.81	1.12	C30H50	410	Superoxide dismutase inhibitor, Anti neoplastic alkaloid, Radiosensitizer, Uterine relaxant, Stomatitis	

Table – 13: FTIR peak values for the selected *Plumbago* species and Plumbagin

Peak Values	Functional Groups	Peak range (nm)	Plum	*P. zeylanica*		*P. auriculata*		*P. rosea*	
				Powder	Ethanolic extract	Powder	Ethanolic extract	Powder	Ethanolic extract
514.96	Bromides (C-Br str.)	**500-600**	+	-	-	-	-	-	-
555.46			+	-	-	-	-	-	-
599.82			+	-	-	-	-	-	-
615.25	Halides C-C1str	**800-600**	+	+	-	+	-	+	-
665.40			+	+	-	+	+	+	+
754.12			+	-	-	-	+	-	-
806.19	Benzene ring containing two adjacent H atoms (C-H def)	**870-800**	+	-	-	+	-	+	-
835.12			+	-	-	-	-	-	-
898.77	Tetra or penta-substituted benzene containing 1 free H (C-H def)	**900-860**	+	-	-	-	-	-	-
985.56	Monosubstituted alkenes ($RCH=CH_2$) C-H def.	**995-985**	+	-	-	-	-	-	-
1031.85	Sulfoxides	**1050-990**	+	-	+	-	+	-	+
1116.71	Secondary alcohols (C-OH str.)	**1120-1100**	+	+	-	+	-	+	-
1193.85	Esters (RCOOR)	**1190-1200**	+	+	-	+	-	+	-
1228.57	Aromatic ethers(R-O-Ar Str.)	**1260-1200**	+	-	-	-	-	-	-
1257.50	Aromatic primary amines (C-N vib.)	**1340-1250**	+	-	-	-	-	-	-
1334.65	Sulfones, Sulfonamides (S=O str.)	**1300-1350**	+	+	+	+	+	+	+
1363.58	Sulfites (S=O Str.)	**1430-1350**	+	-	-	-	-	-	-
1400.22	Phenols, tert carboxyl	**1430-1400**	+	+	-	+	-	+	+
1454.23	Alkanes ($-CH_2-$, C-H def)	**1445-1485**	+	+	+	+	+	+	-
1554.52	Nitrogen heterocycles, combination (C=C and C=N Str.)	**1580-1550**	+	-	-	-	-	-	-

Contd……

Table – 13: FTIR peak values for the selected *Plumbago* species and plumbagin
Contd.....

Peak Values	Functional Groups	Peak range (nm)	Plum	*P. zeylanica*		*P. auriculata*		*P. rosea*	
				Powder	Ethanolic extract	Powder	Ethanolic extract	Powder	Ethanolic extract
1645.17	Intra molecular H-bounded aldehydes (C=O Str)	**1670-1645**	+	-	+	-	+	-	+
1666.38	α, β Unsaturated Acyclic or 6 ring ketones (C=O Str.)	**1695-1660**	+	+	--	+	-	+	-
1755.10	Ureas (-CO-NH-CO-) amide 1(C=O Str.)	**1790-1720**	+	-	-	-	-	-	-
1805.25	Alkenes ($CHR=CH_2$) C-H str.	**1850-1800**	+	-	-	-	-	-	-
1841.89			+	-	-	-	-	-	-
1880.47	Amino acids	**1945-1835**	+	-	-	-	-	-	-
1901.68			+	-	-	-	+	-	-
1930.61			+	-	-	-	-	-	-
2171.70	Boron compounds	**2220-1600**	+	-	+	-	+	-	+
2314.42			+	-	-	-	+	-	+
2349.14	Charged amines ($C=NH^+$)	**2500-2325**	+	-	-	-	+	-	-
2376.14	Phosphorus compounds P-OH (P-H str.)	**2440-2350**	+	+	-	+	-	+	-
2600.04	Organo –phosphorus compounds (O-H Str.)	**2700-2560**	+	-	+	-	+	-	+
2879.52	Alkanes $-CH_3$-(C-H Str., asym)	**2975-2860**	+	-	-	-	-	-	-
2929.67			+	-	-	-	+	-	-
2968.24			+	-	-	-	-	-	-
3195.83	Carboxylic acid bonded OH (O-H Str.)	**3333-2500**	+	+	-	+	+	+	-
3315.41	Alcohols and phenols chelate compounds (O-H Str.)	**3200-2500**	+	+	-	+	+	+	+
3569.99	Alcohols and phenols single bridge comp (O-H Str.)	**3570-3450**	+	-	-	-	+	-	-
3631.71	Alcohols and phenols free –OH (O-H Str.)	**3665-3590**	+	-	-	-	-	-	-
Total			**40**	**11**	**6**	**12**	**15**	**12**	**9**

Table – 36: Nucleotide 16 paired statistical data of *P. zeylanica*

Main	ii	Si	Sv	R	TT	TC	TA	TG	CT	CC	CA	CG	AT	AC	AA	AG	GT	GC	GA	GG	Total
Pz3 and Pz5	1095	0	onc	-	309	0	0	0	0	225	0	0	0	0	309	0	0	0	0	252	**1095**
Pz1 and Pz7	185	147	287	0.5	60	36	52	32	38	34	27	33	52	30	52	36	29	32	37	39	**619**
Pz2 and Pz6	368	234	427	0.5	103	64	73	55	60	67	36	49	67	56	115	46	59	32	64	83	**1029**
Average	252	195	391	0.5	79	50	62	50	47	44	46	38	62	45	76	50	52	38	48	53	**838.7**

Table – 37: Nucleotide paired statistical data of *P. zeylanica*

Nucleotide pair statistical data of *P. zeylanica*	**Over all statistical data**	**Accessions Pz3 and Pz5**	**Accessions Pz1 and Pz7**	**Accessions Pz2 and Pz6**	**Accessions Pz4 and Pz2**
No of conserved region	14	1095	185	368	299
No of variable region	1081	nil	434	661	730
No. of parsimony informative sites	770	nil	nil	nil	Nil
No. of singleton sites	331	nil	nil	nil	Nil
Total no of nucleotides	**1127**				

Table – 38: Maximum composite likelihood estimate pattern of nucleotide substitution in *P. zeylanica*

Base pair	**A**	**T/U**	**C**	**G**	**Tv/Ts ratio**		
					Purines K_1	**Pyrimidines K_2**	**Over all (Tv/Ts bias) R**
A	-	8.73	6.41	**14.85**	**2.202**	**0.465**	**0.604**
T/U	8.17	-	**2.98**	6.74			
C	8.17	**4.06**	-	6.74			
G	**18**	8.73	6.41	-			
Nucleotide frequencies	0.272	0.291	0.213	0.224			

Table – 40: Amino acid composition of *P. zeylanica*

Accessions	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Pz1	7.32	1.95	5.85	6.34	4.88	8.78	0.98	3.41	4.88	8.78	0.98	2.93	7.32	3.90	5.85	4.39	7.80	5.85	1.95	5.85	**205**
Pz2	3.98	2.14	2.75	3.06	3.36	4.89	3.06	8.26	6.12	16.21	2.45	5.20	5.81	3.36	5.20	6.42	6.42	7.03	2.14	2.14	**327**
Pz3	2.94	2.65	3.53	3.82	4.12	5.00	2.35	6.47	6.76	15.88	3.53	5.59	4.41	3,24	4.71	7.06	5.59	8.24	2.35	1.76	**340**
Pz4	4.32	3.75	3.17	3.17	5.19	5.76	2.02	7.20	2.88	8.07	2.59	2.59	4.90	4.90	10.09	11.53	5.19	4.03	2.88	5.76	**347**
Pz5	2.94	2,65	3.53	3.82	4.12	5.00	2.35	6.47	6.76	15.88	3.53	5.59	3.24	3.24	4.71	7.06	5.59	8.24	2.35	1.76	**340**
Pz6	3.78	1.74	4.07	5.23	6.10	7.85	3.20	7.56	5.52	11.05	1.16	3.49	4.07	4.07	7.85	7.27	4.36	5.52	1.45	3.20	**344**
Pz7	5.66	2.83	3.77	5.66	3.30	3.77	0.94	4.25	5.66	15.57	2.36	3.30	1.89	1.89	5.19	4.25	9.91	8.02	3.30	4.72	**212**
Average	**4.16**	**2.55**	**3.69**	**4.26**	**4.49**	**5.82**	**2.27**	**6.52**	**5.53**	**13.14**	**2.46**	**4.21**	**3.59**	**3.59**	**6.34**	**7.19**	**6.10**	**6.67**	**2.32**	**3.40**	**302.1**

Table – 41: Tajima's neutrality test for *P. zeylanica*

M	S	p_s	Θ	Π	D
7	619	1.00000	0.408163	0.707362	4.318566

Table – 43: Nucleotide 16 paired statistical data of *P. auriculata*

Main	ii	Si	Sv	R	TT	TC	TA	TG	CT	CC	CA	CG	AT	AC	AA	AG	GT	GC	GA	GG	Total
Pa2 and Pa3	173	138	292	0.5	56	35	56	32	34	35	25	35	49	35	44	32	36	24	37	38	**603**
Pa1, Pa2 and Pa3	166	145	297	0.5	56	37	50	37	35	33	31	31	45	32	46	39	42	28	35	31	**619**
Pa4 and Pa5	165	154	283	0.5	55	36	51	35	41	32	30	25	48	39	38	35	32	23	42	40	**602**
Average	**164**	**144**	**298**	**0.5**	**54**	**37**	**50**	**36**	**36**	**31**	**32**	**31**	**48**	**33**	**45**	**36**	**40**	**28**	**35**	**34**	**605.9**

ii = Identical Pair, **si** = Transitionsal Pair, **sv** = Transversional Pair, **R** = si/sv

Table – 44: Nucleotide paired statistical data of *P. auriculata*

Nucleotide pair statistical data of *P. auriculata*	Over all statistical data	Accessions Pa2 and Pa3	Accessions Pa4 and Pa5	Accessions Pa1, Pa2 and Pa3
No of conserved region	13	190	165	58
No of variable region	607	429	437	561
No. of parsimony informative sites	290	Nil	Nil	nil
No. of singleton sites	316	Nil	Nil	555
Total no of nucleotides	**641**			

Table – 45: Maximum composite likelihood estimate pattern of nucleotide substitution in *P. auriculata*

Base pair	A	T/U	C	G	Tv/Ts ratio		
					Purines K_1	Pyrimidines K_2	Over all (Tv/Ts bias) R
A	-	9.95	7.26	**9.61**	**1.262**	**0.654**	**0.436**
T/U	9.04	-	**4.75**	7.62			
C	9.04	**6.51**	-	7.62			
G	**11.4**	9.95	7.26	-			
Nucleotide frequencies	0.267	0.294	0.214	0.225			

Table – 46: Composite distance pattern of *P. auriculata*

Accessions	Pa1	Pa2	Pa3	Pa4	Pa5
Pa1	1.000				
Pa2	0.017	1.000			
Pa3	0.025	0.005	1.000		
Pa4	0.005	0.012	0.018	1.000	
Pa5	0.005	0.008	0.022	0.008	1.000

Table – 47: Amino acid composition of *P. auriculata*

Accessions	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Pa1	3.23	6.45	1.61	2.69	4.84	4.84	1.08	5.91	1.61	8.06	2.15	2.69	6.45	3.23	12.90	12.90	4.84	3.23	3.23	8.06	**186**
Pa2	2.94	2.94	2.45	3.43	3.43	3.92	2.94	6.37	7.84	21.57	3.43	6.37	5.39	2.45	3.43	3.43	5.88	7.35	2.94	1.47	**204**
Pa3	3.14	6.28	1.57	2.62	5.76	4.71	1.05	5.76	2.09	8.38	2.09	3.14	6.28	3.14	13.09	12.57	4.19	3.14	3.14	7.85	**191**
Pa4	3.24	6.49	1.62	2.70	5.41	4.86	1.08	5.95	1.62	7.57	2.16	2.70	7.03	3.24	12.97	12.97	4.32	2.70	3.24	8.11	**185**
Pa5	3.17	6.35	1.59	2.65	5.29	4.76	1.06	5.82	1.59	8.47	2.12	2.65	6.88	3.17	13.23	12.70	4.23	3.17	3.17	7.94	**189**
Average	**3.14**	**5.67**	**1.78**	**2.83**	**4.92**	**4.61**	**1.47**	**5.97**	**3.04**	**10.99**	**2.41**	**3.56**	**6.39**	**3.04**	**10.99**	**10.79**	**4.71**	**3.98**	**3.14**	**6.60**	**191**

Table – 48: Tajima's neutrality test for *P. auriculata*

M	S	p_s	Θ	π	D
5	596	0.991681	0.476007	0.731448	4.102591

Table – 50: Nucleotide 16 paired statistical data of *P. rosea*

Main	ii	Si	Sv	R	TT	TC	TA	TG	CT	CC	CA	CG	AT	AC	AA	AG	GT	GC	GA	GG	Total
Pr1 and Pr3	1025	30	40	0.8	297	7	8	3	5	213	3	7	4	6	289	7	8	1	11	226	**1095**
Average	734	11	14	0.8	214	3	3	1	2	156	1	3	1	2	201	2	3	1	4	48	**758.3**

Table – 51: Nucleotide paired statistical data of *P. rosea*

Nucleotide paired statistical data of *P. rosea*	Over all statistical data	Accessions Pr1 and Pr3
No of conserved region	1023	1025
No of variable region	72	70
No. of parsimony informative sites	Nil	nil
No. of singleton sites	2	nil
Total no of nucleotides	**1126**	

Table – 54: Amino acid composition of *P. rosea*

Accessions	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Pr1	2.59	3.17	2.88	3.46	5.76	5.76	2.88	6.63	6.92	14.70	2.59	5.19	4.61	3.75	3.75	8.36	7.78	6.63	1.44	1.15	**347**
Pr2	2.66	3.19	2.66	3.19	3.19	3.72	2.66	6.38	7.45	22.87	3.72	5.32	5.85	2.13	3.19	3.72	6.91	6.91	2.66	1.60	**188**
Pr3	2.59	2.88	2.59	4.03	5.76	6.05	2.88	6.34	6.63	15.27	2.31	4.90	4.61	4.61	4.32	6.34	7.78	7.49	1.44	1.15	**347**
Average	**2.61**	**3.06**	**2.72**	**3.63**	**5.22**	**5.44**	**2.83**	**6.46**	**6.92**	**16.67**	**2.72**	**5.10**	**4.88**	**4.88**	**3.85**	**6.58**	**7.60**	**7.03**	**1.70**	**1.25**	**294**

Table – 55: Tajima's neutrality test for *P. rosea*

M	S	p_s	Θ	π	D
3	590	0.947780	0.6318	0.755295	477.40565

Table – 56: Nucleotide composition of *Plumbago* species

Localities with accession number	***P. zeylanica***	***P. auriculata***	***P. rosea***
GenBank accession number	**KF233552**	**KF233547**	**KF 261598**
Length of the base pair	1095	613	1114
Total % of Thymine	28.2	29.4	28.6
Total % of guanine	23	22.8	22.7
Total % of cytosine	20.5	21.4	20.9
Total % of adenine	28.2	26.4	27.7

Table – 57: Nucleotide 16 paired statistical data of *Plumbago* species

Main	**ii**	**Si**	**Sv**	**R**	**TT**	**TC**	**TA**	**TG**	**CT**	**CC**	**CA**	**CG**	**AT**	**AC**	**AA**	**AG**	**GT**	**GC**	**GA**	**GG**	**Total**
P. zeylanica* and *P. auriculata	15	161	301	0.5	46	44	51	39	40	29	32	30	48	27	46	41	43	31	36	30	**613**
Average for *Plumbago* species	190	200	384	0.5	63	49	62	50	51	31	43	38	60	44	57	49	47	38	50	39	**773.7**

ii = Identical Pair, **si** = Transitionsal Pair, **sv** = Transversional Pair, **R** = si/sv

Table – 58: Nucleotide paired statistical data of *Plumbago* species

Nucleotide paired statistical data of *Plumbago* species	**Over all statistical data**	**Accessions *P. zeylanica* and *P. auriculata***
No of conserved region	146	151
No of variable region	949	462
No. of parsimony informative sites	Nil	nil
No. of singleton sites	581	nil
Total no of nucleotides	**1114**	

Table – 59: Maximum composite likelihood estimate pattern of nucleotide substitution in *Plumbago* species

Base pairs	**A**	**T/U**	**C**	**G**	**Tv/Ts ratio**		
					Purines K_1	**Pyrimidines K_2**	**Over all (Tv/Ts bias) R**
A	-	3.16	2.32	17.73	**7.164**	**7.287**	**3.274**
T/U	2.89	-	**16.88**	2.48			
C	2.89	**23**	-	2.48			
G	20.71	3.16	2.32	-			
Nucleotide frequencies	0.266	0.293	0.218	0.223			

Table – 60: Amino acid composition of *Plumbago* species

Accessions	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
P. zeylanica	2.94	2.65	3.53	3.82	4.12	5.00	2.35	6.47	2.35	15.88	3.53	5.59	4.41	3.24	4.71	7.06	5.59	8.24	2.35	1.76	**340**
P. auriculata	3.17	6.35	1.59	2.65	5.29	4.76	1.06	5.82	1.06	8.47	2.12	2.65	6.88	3.17	13.23	12.70	4.23	3.17	3.17	7.94	**189**
P. rosea	3.39	2.26	2.54	3.11	3.95	5.08	3.11	5.08	3.11	17.23	2.82	5.93	3.95	1.98	6.21	8.47	5.93	7.63	2.82	2.54	**354**
Average	**3.17**	**3.28**	**2.72**	**3.28**	**4.30**	**4.98**	**2.88**	**4.98**	**2.38**	**14.84**	**2.94**	**5.10**	**4.76**	**2.72**	**7.13**	**8.83**	**5.44**	**6.91**	**2.72**	**3.40**	**294.3**

Table – 61: Tajima's neutrality test for *Plumbago* species

M	S	p_s	Θ	π	D
3	581	0.947798	0.631865	0.751495	47732284.405865

Table – 20: HPTLC- Amino acid profile of *Plumbago* species

	Pz	Pa	Pr	Standard Aminoacids																	
Rf				**GIL**	**GIA**	**GIG**	**GIM**	**GIIP**	**GIIS**	**GIIC**	**GIITY**	**GIITR**	**GIIIH**	**GIIIA**	**GIIIAS**	**GIIIT**	**GIIIL**	**GIVG-**	**GIVA**	**GIVV**	**GIVI**	**GIVP**
0.17	+	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
0.20	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.22	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
0.27	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.34	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
0.36	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.38	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-
0.40	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
0.45	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.46	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
0.56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.58	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.61	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	--	-	-
0.65	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
0.67	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	**7**	**8**	**8**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

Table - 1: Locality and accession numbers of the three studied *Plumbago* species

Locality	Site code	Voucher no (XCH.NO)	Geographic Location	Locality	Site code	Voucher no (XCH.NO)	Geographic Location	Locality	Site code	Voucher no (XCH.NO)	Geographic Location
Rasthacadu **(Tamil Nadu)**	**Pz1**	**28095**	**8°2377'N 77°3324'E**	Mulakumoodu **(Tamil Nadu)**	**Pa1**	**28094**	**8°27358'N 77°36473'E**	Cheruvarankonam **(Kerala)**	**Pr1**	**28102**	**8°3310495N 77°1513E**
Palayamkottai **(Tamil Nadu)**	**Pz2**	**28099**	**8°7166'N 77°73333'E**	Tenkasi **(Tamil Nadu)**	**Pa2**	**28093**	**8°58'N 77°18'E**	Dana **(Tamil Nadu)**	**Pr2**	**28101**	**8°43'3"N 77°22'29"E**
Bangalore **(Karnataka)**	**Pz3**	**28098**	**12°97°N 77°56'E**	Perunthurai **(Tamil Nadu)**	**Pa3**	**28092**	**11°27'N 77°58'E**	Bangalore **(Karnataka)**	**Pr3**	**28102**	**12°97°N 77°56'E**
Papanasam **(Tamil Nadu)**	**Pz4**	**28089**	**10°9333'N 79°2823'E**	Kattakadu **(Kerala)**	**Pa4**	**28091**	**8°30'15''N 77°4'49'E**				
Tiruppur **(Tamil Nadu)**	**Pz5**	**28096**	**11°1075'N 77°3398'E**	Mysore **(Karnataka)**	**Pa5**	**28090**	**12°2958'N 76°63938'E**				
Kuttichal **(Kerala)**	**Pz6**	**28097**	**8°51757'N 76°93685'E**								
Coimbatore **(Tamil Nadu)**	**Pz7**	**28100**	**11°0'45'N 76°58'17'E**								

Table – 2: Preliminary phytochemical analysis using Harborne method (1999)

Test	Observation	Inference
Test solution + chloroform + 3 drops of acetic anhydride + 1 drop of concentrated sulphuric acid.	Purple change into blue or green colour	**Steroids**
Test solution + 1ml chloroform + 1ml acetic anhydride	Cherry red	**Terpenoids**
Test solution + 2N HCl aqueous layer formed. Add 2 drop of Mayer's reagent.	White turbidity	**Alkaloids**
Test solution in alcohol + 1 drop of ferric chloride	Intense colour	**Phenolic groups**
Alcoholic test solution + bit of magnesium + 2 drops of concentrated HCl & heat.	Red or orange colour	**Flavonoids**
Test solution + water + shake.	Foamy layer	**Saponins**
Test solution + water + lead acetate	White precipitate	**Tannins**
Test solution + magnesium acetate solution	Pink colour	**Anthraquinone**
Alcoholic test solution + 1 drop of ferric chloride + 2 ml glacial acetic acid + H_2SO_4	Yellow colour	**Cardiac glycosides**
Alcoholic test solution + sodium hydroxide	Bluish green	**Coumarin glycosides**
Test solution + 5 drop α- Naphthol + 2ml alcohol+1ml Concentrated sulphuric acid.	Violet ring	**Carbohydrates**
Alcoholic test solution + Ehrlich reagent + concentrated HCl	Pink colour	**Catechin**
Test solution + 1% ninhydrin in alcohol	Blue or violet colour	**Amino acids**

Table – 3: Standards used for HPTLC- Amino acid quantification

Group I	Group II	Group III	Group IV
Lysine	Proline	Histidine	Glycine
Asparagine	Serine	Arginine	Alanine
Glutamine	Cystine	Aspartic acid	Valine
Glutamic acid	Tyrosine	Threonine	Isoleucine
Methionine	Tryptophan	Leucine	Phenyl alanine

Table – 4: Mobile phase and spraying reagents employed for HPTLC Analysis

Compounds	Mobile phase	Spraying reagent	Detection
Alkaloids	Ethyl acetate-Methanol-Water **(10: 1.35: 1)**	Dragendorff's reagent followed by 10% ethanolic Sulphuric acid reagent	Yellow, Orange yellow, Brownish yellow coloured
Phenolic groups	Toluene-Acetone-Formic acid **(4.5: 4.5 : 1)**	20% Sodium carbonate reagent followed by Folin Ciocalteu reagent.	Blue, Brown coloured
Tannins	Toluene-Ethyl acetate-Formic acid-Methanol **(3 : 3 : 0.8 : 0.2)**	5% Ferric chloride reagent	Green, Blue, Greenish brown coloured
Flavonoids	Ethyl acetate Butanone-Formic acid-Water **(5 : 3 : 1 : 1)**	1% ethanolic Aluminium chloride reagent	Yellow, Blue, Bluish yellow coloured
Plumbagin	Toluene-Formic acid **(9.9: 0.1)**	10% methanolic KOH Reagent	Pink coloured zone
Amino acids	n-Butanol-Acetic acid-Water **(3 : 1 : 1)**	Ninhydrin reagent prepared freshly	Violet-pink, Yellow coloured zones

Table – 5: Stock solutions and chemical reagents used for SDS-PAGE electrophoresis

Acrylamide stock 30%	**:**	29.2g acrylamide + 0.8g bis-acrylamide + 100ml water
Lower Tris	**:**	pH 8.8 for separating gel 36.34g of Tris made upto 200ml
Upper Tris	**:**	pH 6.8 for stacking gel 12.1g Tris made upto 200ml
Ammonium persulphate	**:**	0.05g in 0.5ml distilled water.
Running Gel Buffer(5X)	**:**	Tris 15.0 g + glycine 720 g in 1000 ml distilled water. dilute 300 ml 5X stock with 1200 ml distilled water. Do not adjust the pH.
Sample Buffer	**:**	7.25 ml distilled water +1.25 ml stocking gel buffer +1ml of glycerol and bromo phenol blue.

Table – 6: Chemical composition of SDS-Poly Acrylamide Gel Electrophoresis (Anbalagan, 1999)

SEPARATING GEL		
10% Running gel: (Lower Tris)		
30% Acrylamide	-	10ml
Lower Tris(8.8)	-	7.5ml
Distilled water	-	12.3ml
Total	-	29.8ml
APS	-	150µl
TEMED	-	50µl
Total	-	30 ml
6% Stacking gel: (Upper Tris)		
30% Acrylamide	-	2ml
Upper Tris(6.8)	-	3ml
Distilled water	-	4.9ml
Total	-	9.9ml
APS	-	75µl
TEMED	-	25µl
Total	-	30 ml
STOCK		
Acrylamide	-	30%
Upper Tris)	-	6.8pH
Lower Tris	-	8.8pH
TEMED	-	

Table – 7: Reagents required for silver staining in SDS-PAGE

(Sorensen *et al.*, 2002 and Mortz *et al.*, 2001)

Reagents		Preparation of chemicals
Fixation solution	**:**	50% ethanol or methanol + 12% glacial acetic acid + 0.05% formalin + 50µl formalin+ 100 ml distilled water
Washing solution	**:**	20% ethanol + 100 ml distilled water.
Sensitizing solution	**:**	0.02% sodium thio sulphate + 100 ml distilled water
De ionized water	**:**	100 ml distilled water for washing
Staining	**:**	0.2% silver nitrate + 0.076% formalin + 100 ml distilled water.
Developing solution	**:**	6% sodium carbonate + 0.0004% sodium sulphate + 0.05% formalin + 100 ml distilled water.
Terminating solution	**:**	12% acetic acid + 100 ml distilled water
Drying solution	**:**	20% ethanol+ 100 ml distilled water

Table – 8: Reagents required for DNA isolation

(Murrey and Thompson, 1980)

Extraction Buffer (pH 8.0)		
CTAB	-	2.0 gm
1M Tris HCl	-	10.0 ml
0.5 M EDTA	-	4.0 ml
1.4 M NaCl	-	8.2 gm
PVP	-	1.2%
Made up to 100 ml with distilled water		
Precipitation Buffer (pH 8.0)		
CTAB	-	1.0 gm
50 mM Tris HCl	-	5.0 ml
10 mM EDTA	-	2.0 ml
Made up to 10 ml with distilled water		
Stock Precipitation Buffer (TE Buffer)		
1M Tris HCl	-	100 ml
0.5M EDTA	-	50 ml
1X TE Buffer		
1M Tris HCl	-	1.0 ml
0.5 M EDTA	-	0.2 ml
Made up to 100 ml with distilled water		
High Salt TE		
1M NaCl	-	5.85 gm
10mM Tris HCl (pH 8.0)	-	1.0 ml
0.1mM EDTA (pH 8.0)	-	20µl
Made up to 100 ml with distilled water		
10X TBE (pH 8.0)		
Tris base	-	21.6 gm
Boric acid	-	11.0 gm
0.5 M EDTA (pH 8.0)	-	8.0 ml
Made up to 200 ml with distilled water and the pH was adjusted with NaOH		
CTAB/ NaCl (10 % CTAB/0.2M NaCl)		
CTAB	-	5.0 gm
NaCl	-	2.04 gm
Made up to 50 ml with distilled water		

Table – 9: Reagents required for Polymerase chain reaction

Components		**Quantity**
PCR vial Master mix	-	**20 µl**
*rbc*L Primer mix (5pmoles/µl)	-	**2 µl**
Genomic DNA	**-**	**2 µl**
Water, nuclease free	-	**6 µl**
Total volume	-	**30 µl**

Table - 10: UV-Vis absorption values for different extracts of *Plumbago zeylanica*

Plumbago zeylanica									
Pet ether		**Chloroform**		**Acetone**		**Ethyl acetate**		**Ethanol**	
nm	**abs**	**nm**	**Abs**	**Nm**	**abs**	**nm**	**abs**	**nm**	**abs**
668	0.437	1091	0.059	651	0.381	665	0.334	662	0.136
608	0.109	1021	0.062	604	0.124	607	0.088	605	0.046
533	0.138	952	0.065	532	0.107	605	0.046	532	0.056
505	0.155	667	1.022	403	1.336	534	0.118	502	0.073
409	1.037	608	0.29			504	0.139	401	0.741
		538	0.354			410	1.019	295	1.497
		505	0.392			319	0.611		
		416	2.659						

Table - 11: UV-Vis absorption values for different extracts of *Plumbago auriculata*

Plumbago auriculata									
Pet ether		**Chloroform**		**Acetone**		**Ethyl acetate**		**Ethanol**	
nm	**abs**	**nm**	**abs**	**Nm**	**abs**	**nm**	**abs**	**nm**	**abs**
669	0.818	1052	0.119	665	2.482	665	0.390	1086	0.21
611	0.121	929	0.126	606	0.55	606	0.110	1009	0.222
533	0.163	667	1.455	533	0.69	534	0.144	872	0.241
502	0.238	609	0.482	503	0.829	410	1.155	656	0.38
407	2.237	538	0.556	400	3.675	303	1.202		
		507	0.59						
		415	3.436						

Table - 12: UV-Vis absorption values for different extracts of *Plumbago rosea*

Plumbago rosea									
Pet ether		**Chloroform**		**Acetone**		**Ethyl acetate**		**Ethanol**	
Nm	**abs**	**nm**	**abs**	**Nm**	**abs**	**nm**	**abs**	**nm**	**Abs**
665	0.329	992	0.118	665	1.020	666	0.879	664	0.982
669	0.971	666	0.384	606	0.220	607	0.178	610	0.299
610	0.193	605	0.259	533	0.271	558	0.099	532	0.385
533	0.231	503	0.334	504	0.321	411	2.451	320	4.000
503	0.311	415	1.110	411	2.683				
407	2.466								

Table - 14: TLC - Phenolic profile of *Plumbago* species

Rf value	P			C			A			EA			E		
	Pz	Pa	Pr	Pz	Pa	Pr	Pz	Pa	Pr	Pz	Pa	Pr	Pz	Pa	Pr
0.10	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
0.20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
0.22	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-
0.25	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
0.27	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
0.29	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
0.30	-	-	-	-	-	+	-	+	+	-	-	-	-	-	+
0.33	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+
0.35	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-
0.38	-	-	-	-	+	+	+	+	-	-	+	-	+	+	+
0.40	-	-	+	+	+	+	-	-	-	+	-	-	+	-	-
0.42	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
0.44	+	-	-	-	-	-	-	+	-	+	+	-	+	+	-
0.45	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+
0.48	-	-	-	+	+	-	+	-	-	+	+	-	-	-	-
0.50	+	+	+	-	-	-	-	+	-	-	-	+	+	-	-
0.53	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
0.56	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
0.58	-	-	+	+	+	+	-	-	+	-	-	-	+	-	+
0.60	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-
0.62	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.65	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-
0.70	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
0.72	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	4	4	3	5	5	5	4	5	5	5	5	4	7	3	7

Pz- *P. zeylanica*; Pa- *P. auriculata*; Pr- *P. rosea*; P- Petroleum ether extract; C- Chloroform extract; A- Acetone extract; EA-Ethyl acetate extract; E- Ethanolic extract; Aq- Aqueous extract.

Table - 15: TLC - Steroidal profile of *Plumbago* species

Rf value	P			C			A			EA			E		
	Pz	Pa	Pr	Pz	Pa	Pr	Pz	Pa	Pr	Pz	Pa	Pr	Pz	Pa	Pr
0.03	-	+	-	-	-	-	+	-	-	+	-	-	+		-
0.05	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
0.07	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
0.09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.12	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+
0.14	+	-	+	-	+	-	+	-	-	-	-	+	+	-	-
0.16	-	+	-	+	-	+	+	-	-	-	-	-	-	-	+
0.18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.20	-	-	-	-	-	-	-	+	-	+	-	+	+	-	+
0.23	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+
0.25	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-
0.30	-	-	-	-	+	-	-	+	+	+	-	+	-	-	+
0.32	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
0.38	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
0.41	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
0.44	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
0.46	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0.5	+	+	-	-	-	-	-	-	+	+	-	+	-	-	-
0.52	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
0.55	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.57	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
0.58	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
0.60	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
0.63	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0.65	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-
0.68	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+
0.70	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
0.75	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
0.78	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
0.81	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
0.90	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-
Total	7	8	5	3	9	4	4	4	6	5	4	7	4	1	8

Pz- *P. zeylanica*; Pa- *P. auriculata*; Pr- *P. rosea*; P- Petroleum ether extract; C- Chloroform extract; A- Acetone extract; EA-Ethyl acetate extract; E- Ethanolic extract; Aq- Aqueous extract.

Table – 16: HPTLC- Phenolic profile of *Plumbago* species ethanolic extract

Rf value	Pz	Pa	Pr	AS	Que
0.05	-	+	+	k	
0.08	+	-	-	k	
0.10	-	+	+	Un	
0.14	-	-	+	Un	
0.16	+	+	-	Un	
0.19	+	-	-	K	
0.20	-	+	-	Un	
0.23	-	+	-	Un	
0.26	-	-	+	K	
0.32	+	-	-	Un	
0.36	-	+	+	K	
0.40	-	-	+	Un	
0.45	+	-	+	k	
0.47	+	-	-	k	
0.51	-	-	+	k	
0.61	-	-	-		+
0.62	+	+	+	k	
0.73	+	+	+	Un	
0.94	+	+	+	Un	
Total	9	9	11	17	

Pz- *Plumbago zeylanica*; Pa- *Plumbago auriculata*; Pr - *Plumbago rosea*

Un- Unknown Phenol; K – Known Phenol; Que – Quercitin; AS – Assigned Substance

Table – 17: HPTLC- Tannin profile of *Plumbago* species ethanolic extract

Rf value	Pz	Pa	Pr	AS	Gal
0.04	-	+	+	k	
0.10	-	-	+	k	
0.16	-	-	+	Un	
0.17	+	-	-	Un	
0.23	-	-	+	Un	
0.32	+	-	-	Un	
0.33	-	-	+	Un	
0.41	-	-	+	Un	+
0.53	+	-	-	Un	
0.54	-	-	+	Un	
0.57	-	+	-	Un	
0.58	+	-	-	Un	
0.60	-	-	+	Un	
0.62	+	+	+	Un	
0.70	+	-	-	Un	
0.73	-	+	-	Un	
0.77	+	-	+	k	
0.79	-	-	+	Un	
0.80	+	-	-	Un	
0.94	+	+	+	k	
Total	9	5	12	20	1

Pz- *Plumbago zeylanica*; Pa- *Plumbago auriculata*; Pr - *Plumbago rosea*

Un- Unknown Tannin; k – Known Tannin; Gal– Gallic acid; AS – Assigned Substance

Table – 18: HPTLC- Alkaloid profile of *Plumbago* species ethanolic extract

Rf value	Pz	Pa	P r	AS	Col
0.01	+	-	+	Un	
0.11	+	+	+	Un	
0.14	-	-	+	un	
0.15	-	+	-	un	
0.22	+	-	-	k	
0.23	-	-	+	k	
0.26	-	+	-	k	
0.33	+	+	+	k	
0.36	-	+	-	k	
0.42	-	-	-		+
0.45	+	+	+	Un	
0.52	-	-	+	k	
0.53	+	-	-	k	
0.61	-	-	+	Un	
0.68	-	-	+	Un	
0.73	+	-	-	Un	
0.91	+	+	+	Un	
Total	8	7	10	16	

Pz- Plumbago zeylanica*; *Pa – Plumbago auriculata*; *Pr - Plumbago rosea

Un- Unknown Alkaloid; k- Known Alkaloid; Col – Colchicine; AS - Assigned Substance

Table – 19: HPTLC- Flavonoid profile on ethanolic extract of *Plumbago* species

Rf value	Pz	Pa	Pr	AS	Rutin
0.07	+	+	+	k	
0.17	-	-	+	Un	
0.23	-	-	+	k	
0.36	-	+	-	k	
0.37	-	-	+	k	
0.42	+	-	-	k	
0.46	-	-	+	k	
0.48	-	-	-		+
0.51	+	+	-	Un	
0.60	-	+	+	k	
0.64	+	-	-	k	
0.66	-	+	-	k	
0.68	-	-	+	k	
0.77	+	-	-	k	
0.81	-	-	+	k	
0.88	-	-	+	k	
0.94	+	+	-	Un	
0.96	-	-	+	Un	
Total	6	6	10	17	

Pz- *Plumbago zeylanica*; Pa- *Plumbago auriculata*; Pr - *Plumbago rosea*,

Un- Unknown- Flavonoids; k- Known Flavonoids; Standard-Rutin;

AS - Assigned Substance

Table – 21: HPTLC- Quantification amino acid profile of *P. zeylanica*

Rf	Assigned substance	Area (in AU)	Conc (in µg)	Content (in %)
0.17	Lysine + Histidine + Proline	8059.5	0.4 + 2.0 + 1.3	1.0 + 4.8 + 3.1
0.22	Glycine + Arginine	6757.0	1.1 + 0.4	2.6 + 1.0
0.36	Serine + Glutamine	9034.2	0.4 + 0.4	1.0 + 1.0
0.40	Cystine + Aspartic acid + Alanine	14679.6	0.7 + 2.8 + 0.7	1.7 + 6.7 + 1.7
0.45	Threonine + Valine + Glutamic acid	12317.0	0.6 + 0.6 + 1.2	1.4 + 1.4 + 2.9
0.58	Isoleucine + Methionine	4728.3	0.2 + 0.2	0.5 + 0.5
0.67	Phenyl alanine + Leucine + Tryptophan	12968.6	0.5 + 0.4 + 1.2	1.2 + 1.0 + 2.9
0.27	Asparagine	1856.5	0.5	1.0

Table – 22: HPTLC- Quantification amino acid profile of *P. auriculata*

Rf	Assigned substance	Area (in AU)	Conc (in µg)	Content (in %)
0.20	Lysine + Histidine + Proline	9977.4	1.6 + 2.5 + 1.7	3.8 + 6.0 + 4.1
0.22	Glycine + Arginine	7909.2	1.3 + 0.4	3.1 + 1.0
0.27	Asparagine	1856.5	0.4	1.0
0.36	Serine + Glutamine	10310.7	0.4 + 0.4	1.0 + 1.0
0.40	Cystine + Aspartic acid + Alanine	19157.5	0.9 + 3.6 + 0.9	2.2 + 8.7 + 2.2
0.45	Threonine + Valine + Glutamic acid	15709.0	0.8 + 0.7 + 1.5	1.9 + 1.7 + 3.6
0.58	Isoleucine + Methionine	7175.3	0.3 + 0.4	0.7 + 1.0
0.67	Phenyl alanine + Leucine + Tryptophan	16579.7	0.6 + 0.6 + 1.5	1.4 + 1.4 + 3.6

Table – 23: HPTLC- Quantification amino acid profile of *P. rosea*

Rf	Assigned substance	Area (in AU)	Conc (in µg)	Content (in %)
0.17	Lysine + Histidine + Proline	8500.7	0.5 + 2.1 + 1.4	1.2 + 5.0 + 3.4
0.22	Glycine + Arginine	7316.5	1.2 + 0.4	2.9 + 1.0
0.27	Asparagine	2434.2	0.5	1.2
0.36	Serine + Glutamine	10126.3	0.4 + 0.4	1.0 + 1.0
0.40	Cystine + Aspartic acid + Alanine	15123.2	0.7 + 2.8 + 0.7	1.7 + 6.7 + 1.7
0.46	Threonine + Valine + Glutamic acid	11936.1	0.6 + 0.6 + 1.1	1.4 + 1.4 + 2.6
0.58	Isoleucine + Methionine	5112.9	0.2 + 0.3	0.5 + 0.7
0.67	Phenyl alanine + Leucine + Tryptophan	9512.8	0.3 + 0.3 + 0.9	0.7 + 0.7 + 2.2

Table – 27: MW-Rf values and protein profile of *Plumbago zeylanica*

MW-Rf	Positions	Regions	*Plumbago zeylanica*						
			Pz1	**Pz2**	**Pz3**	**Pz4**	**Pz5**	**Pz6**	**Pz7**
0.20	PP1[1]	**1**	-	-	-	-	-	+	-
0.24	PP1[2]		+	+	+	+	+	+	-
0.34	PP2[1]	**2**	+	+	+	+	-	+	-
0.44	PP3[1]	**3**	+	+	+	-	-	-	+
0.50	PP4[1]	**4**	-	+	+	+	+	+	+
0.55	PP4[2]		+	-	-	-	-	-	-
0.58	PP4[3]		-	+	+	+	+	+	+
0.64	PP5[1]	**5**	-	-	+	-	-	-	+
0.70	PP6[1]	**6**	+	+	+	+	+	+	+
0.78	PP6[2]		-	+	-	-	-	-	+
0.81	PP7[1]	**7**	-	-	+	+	+	+	+
0.91	PP8[1]	**8**	-	+	+	+	+	+	+
0.98	PP8[2]		-	-	-	-	-	-	+
	Total		**5**	**8**	**9**	**7**	**6**	**8**	**9**

Table – 28: MW- Rf values and protein profile of *Plumbago auriculata*

MW-Rf	Positions	Regions	*Plumbago auriculata*				
			Pa1	**Pa2**	**Pa3**	**Pa4**	**Pa5**
0.07	PP1[1]	**1**	+	+	+	+	+
0.12	PP2[1]	**2**	+	-	+	+	+
0.32	PP3[1]	**3**	-	-	-	-	+
0.40	PP4[1]	**4**	+	+	+	+	+
0.50	PP5[1]	**5**	-	-	+	-	-
0.57	PP5[2]		-	-	+	+	+
0.60	PP6[1]	**6**	-	-	-	-	+
0.65	PP6[2]		+	+	+	+	-
0.70	PP7[1]	**7**	+	-	+	+	+
0.78	PP7[2]		+	-	+	-	+
0.82	PP8[1]	**8**	-	+	+	+	+
0.85	PP8[2]		+	+	-	+	-
	Total		**7**	**5**	**9**	**8**	**9**

Table – 29: MW-Rf values and protein profile of *Plumbago rosea*

MW-Rf	Positions	Regions	*Plumbago rosea*		
			Pr1	**Pr2**	**Pr3**
0.04	PP1[1]	**1**	+	+	+
0.28	PP2[1]	**2**	+	+	-
0.34	PP3[1]	**3**	+	+	+
0.40	PP4[1]	**4**	-	+	+
0.44	PP4[2]		-	+	+
0.50	PP5[1]	**5**	-	+	+
0.53	PP5[2]		-	+	-
0.58	PP5[3]		+	+	+
0.67	PP6[1]	**6**	+	-	+
0.84	PP7[1]	**7**	-	-	+
	Total		**5**	**8**	**8**

Table – 30: MW-Rf values and protein profile of *Plumbago* species

MW-Rf	Positions	Regions	*Plumbago* species		
			P. zeylanica	***P. auriculata***	***P. rosea***
0.04	PP1[1]	**1**	-	-	+
0.07	PP1[2]		-	+	-
0.12	PP2[1]	**2**	-	+	-
0.24	PP3[1]	**3**	+	-	-
0.32	PP4[1]	**4**	-	+	-
0.34	PP4[2]		+	-	+
0.40	PP5[1]	**5**	-	+	+
0.44	PP5[2]		+	-	+
0.50	PP6[1]	**6**	+	-	+
0.57	PP6[2]		+	+	+
0.60	PP7[1]	**7**	-	+	-
0.64	PP7[2]		+	-	-
0.67	PP7[3]		-	-	+
0.70	PP8[1]	**8**	+	+	-
0.78	PP8[2]		-	+	-
0.82	PP9[1]	**9**	+	+	-
0.84	PP9[2]		-	-	+
0.91	PP10[1]	**10**	+	-	-
	Total		**9**	**9**	**8**

Table – 31: Mass spectral (m/z) values of *P. zeylanica*

m/z peak values						
Pz1	**Pz2**	**Pz3**	**Pz4**	**Pz5**	**Pz6**	**Pz7**
753	**655**				**655**	
821	805				**821**	
				965		
1032	**1036**			972	**1032**	1197
	1289	1242	**1289**		1335	1483
1450		**1454**				
1689	**1524**		**1547**			
	1800	**1724**	**1735**		**1767**	**1712**
	2018	**2047**	**2038**		2078	**2019**
		2446	**2473**		**2361**	**2318**
	2554					**2581**
		2829	2690		**2859**	2914
	3060				3005	
	3134	**3111**	**3104**	3218		
					3429	**3470**
		3367	**3344**	**3313**		
		3602	3681			3577
					4056	**4056**
		4124	**4170**		**4200**	
	4432	4365	**4451**			4412
		4785				4913
		5293	5379			5207
		5708	5791			5614
		6254	6358			**6245**
		6821	**6833**			**6973**
		7479	**7202**			**7323**
			8159			8247
			8609			8264
		9062	**9777**			**9743**
		10441	11161			
			21348			
			28596			
			30414			
		39429	37559			
			53293			
			75317		72493	
			80916			83978

Table – 32: Mass spectral (m/z) values of *P. auriculata*

***P. auriculata* m/z peak values**				
Pa1	**Pa2**	**Pa3**	**Pa4**	**Pa5**
659				
821		**822**		
1034	**2056**		**2032**	2246
1535		1987	**1573**	1422
2601			**2663**	
	2956			2883
4167	**4104**	**4164**	**4164**	**4167**
4042			4576	6198
	10583			10254
	20981			
	28723			
	31288			
	31243			
		46880	47609	
		54046		56644
	81137			82260
	86132			92970

Table – 33: Mass spectral (m/z) values of *P. rosea*

***P. rosea* m/z peak values**		
Pr1	**Pr2**	**Pr3**
823		**822**
	1730	
	2186	
	2657	
3339	3793	
4067	**4059**	**4058**
	4985	
	55036	
	64379	
	92000	

Table - 34: Mass spectral (m/z) values of *Plumbago* species

***Plumbago* species m/z peak values**		
P. zeylanica	***P. auriculata***	***P. rosea***
1242		822
1454	**1422**	
1724		
2047		
2446	2246	
2829	**2883**	
3111		
3367		
3602		
4124	**4167**	4058
4365		
4785		
5293		
5708		
6254	**6198**	
6821		
7479	10254	
9062	56644	
10441	82260	
39429	92970	

Table – 35: Nucleotide composition of *P. zeylanica* collected from various sites

Localities with accession number	**Pz1**	**Pz2**	**Pz3**	**Pz4**	**Pz5**	**Pz6**	**Pz7**
GenBank accession number	**KF 233546**	**KF 233551**	**KF 233552**	**KF 233545**	**KF 233544**	**KF 233543**	**KF 233542**
Length of the base pair	619	1029	1095	1127	1095	1064	661
Total % of Thymine	28.9	28.1	28.2	28.6	28.2	28.0	29.0
Total % of guanine	22.6	22.6	23	22.9	23.0	22.9	22.5
Total % of cytosine	21.3	21.3	20.5	20.7	20.5	20.5	21.3
Total % of adenine	27.1	28.0	28.2	27.9	28.2	27.7	27.1
Total nucleotide composition	**619**	**1029**	**1095**	**1127**	**1095**	**1064**	**661**

Table – 39: Composite distance pattern of *P. zeylanica*

Accessions	Pz1	Pz2	Pz3	Pz4	Pz5	Pz6	Pz7
Pz1	1.000						
Pz2	0.003	1.000					
Pz3	0.005	0.011	1.000				
Pz4	0.008	0.008	0.010	1.000			
Pz5	0.005	0.011	0.000	0.010	1.000		
Pz6	0.026	0.016	0.050	0.027	0.050	1.000	
Pz7	0.011	0.005	0.026	0.010	0.026	0.005	1.000

.

Table – 42: Nucleotide composition of *P. auriculata* collected from various sites

Localities with accession number	**Pa1**	**Pa2**	**Pa3**	**Pa4**	**Pa5**
GenBank accession number	**KF 193871**	**KF 233550**	**KF 233549**	**KF 233548**	**KF 233547**
Length of the base pair	604	640	619	602	613
Total % of Thymine	29.1	29.5	29.4	29.2	29.4
Total % of guanine	22.7	22.2	22.5	22.4	22.8
Total % of cytosine	21.4	21.1	21.3	21.6	21.4
Total % of adenine	26.8	27.2	26.8	26.7	26.4

Table – 49: Nucleotide composition of *P. rosea* collected from various sites

Localities with accession number	**Pr1**	**Pr2**	**Pr3**
GenBank accession number	**KF 261596**	**KF 261597**	**KF 261598**
Length of the base pair	1107	590	1114
Total % of Thymine	28.8	29.2	28.6
Total % of guanine	22.1	22.2	20.9
Total % of cytosine	20.6	22.0	20.9
Total % of adenine	28.5	26.6	27.7

Table – 52: Maximum composite likelihood estimate pattern of nucleotide substitution in *P. rosea*

Base pairs	A	T/U	C	G	Tv/Ts ratio		
					Purines K_1	Pyrimidines K_2	Over all (Tv/Ts bias) R
A	-	7.3	5.44	5.44	**0**	**3.929**	**0.88**
T/U	6.64	-	**21.38**	5.57			
C	6.64	**28.7**	-	5.57			
G	0	7.3	5.44	-			
Nucleotide frequencies	0.266	0.293	0.218	0.223			

Table – 53: Composite distance pattern of *P. rosea*

Accessions	Pr1	Pr2	Pr3
Pr1	1.000		
Pr2	0.005	1.000	
Pr3	0.000	0.005	1.000

Fig. 61: Antibacterial activity of *P. zeylanica*

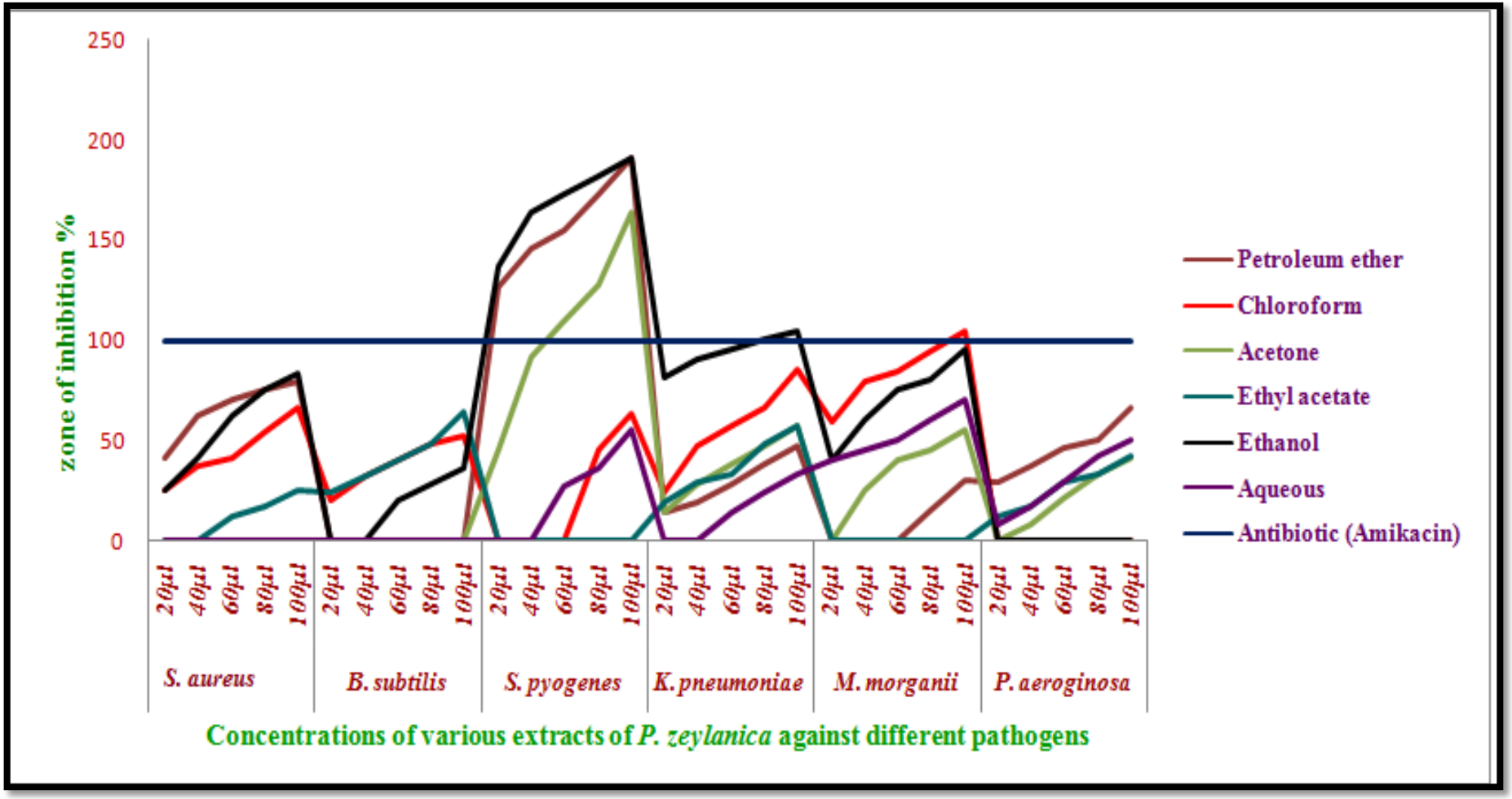


Fig. 62: Antibacterial activity of *P. auriculata*

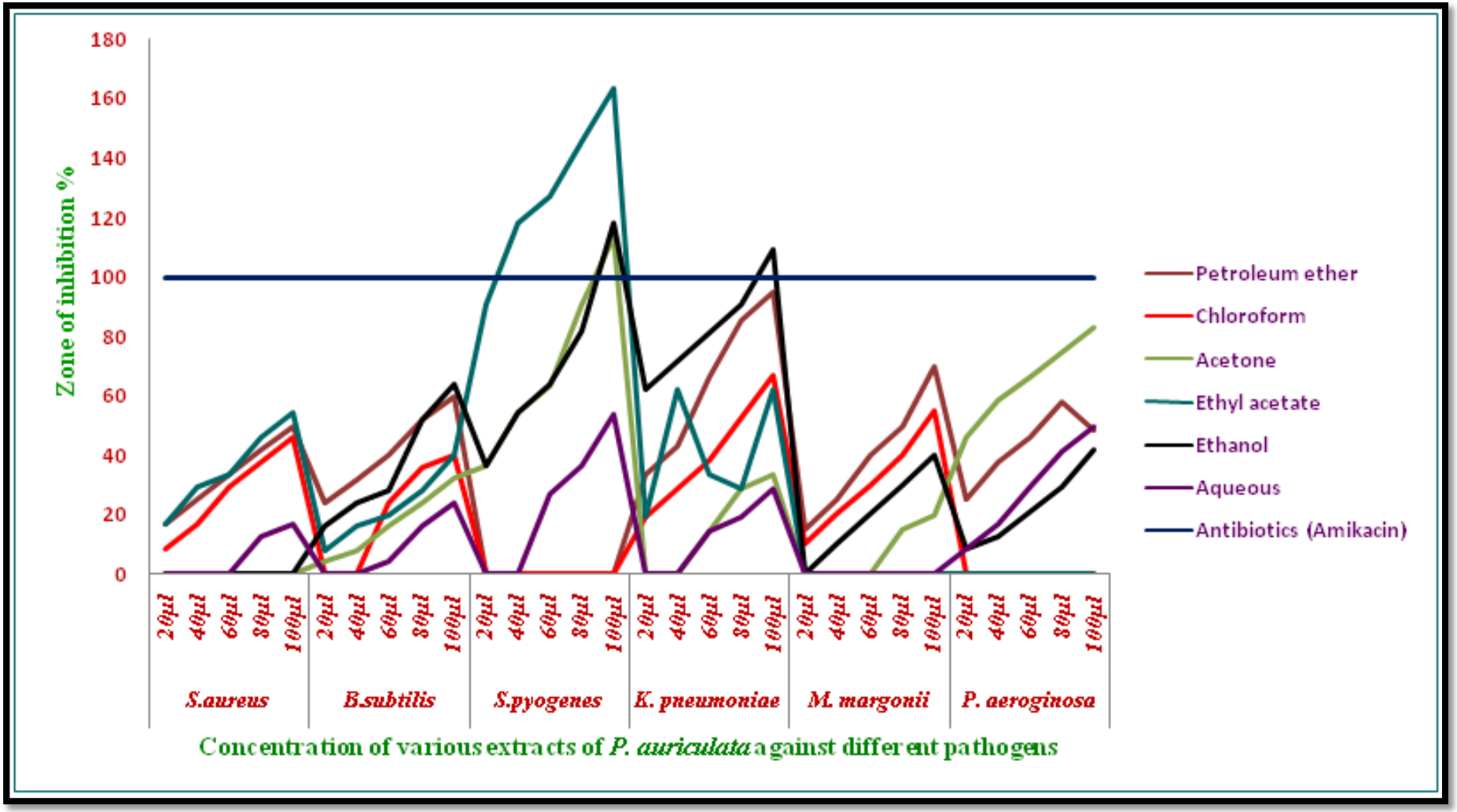


Fig. 63: Antibacterial activity of *P. rosea*

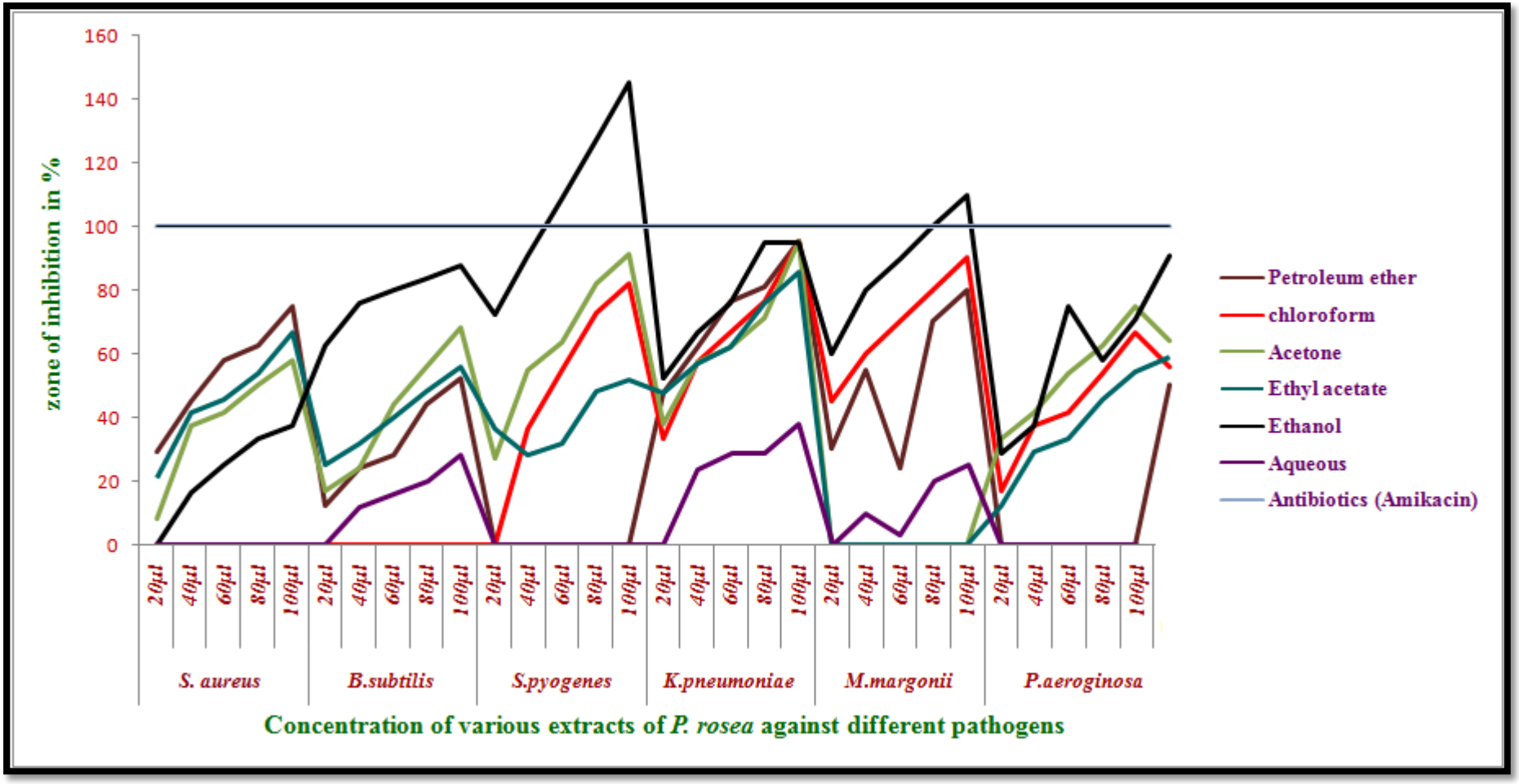


Fig. 2: Percentage of phytochemicals present in different extracts of *Plumbago* species

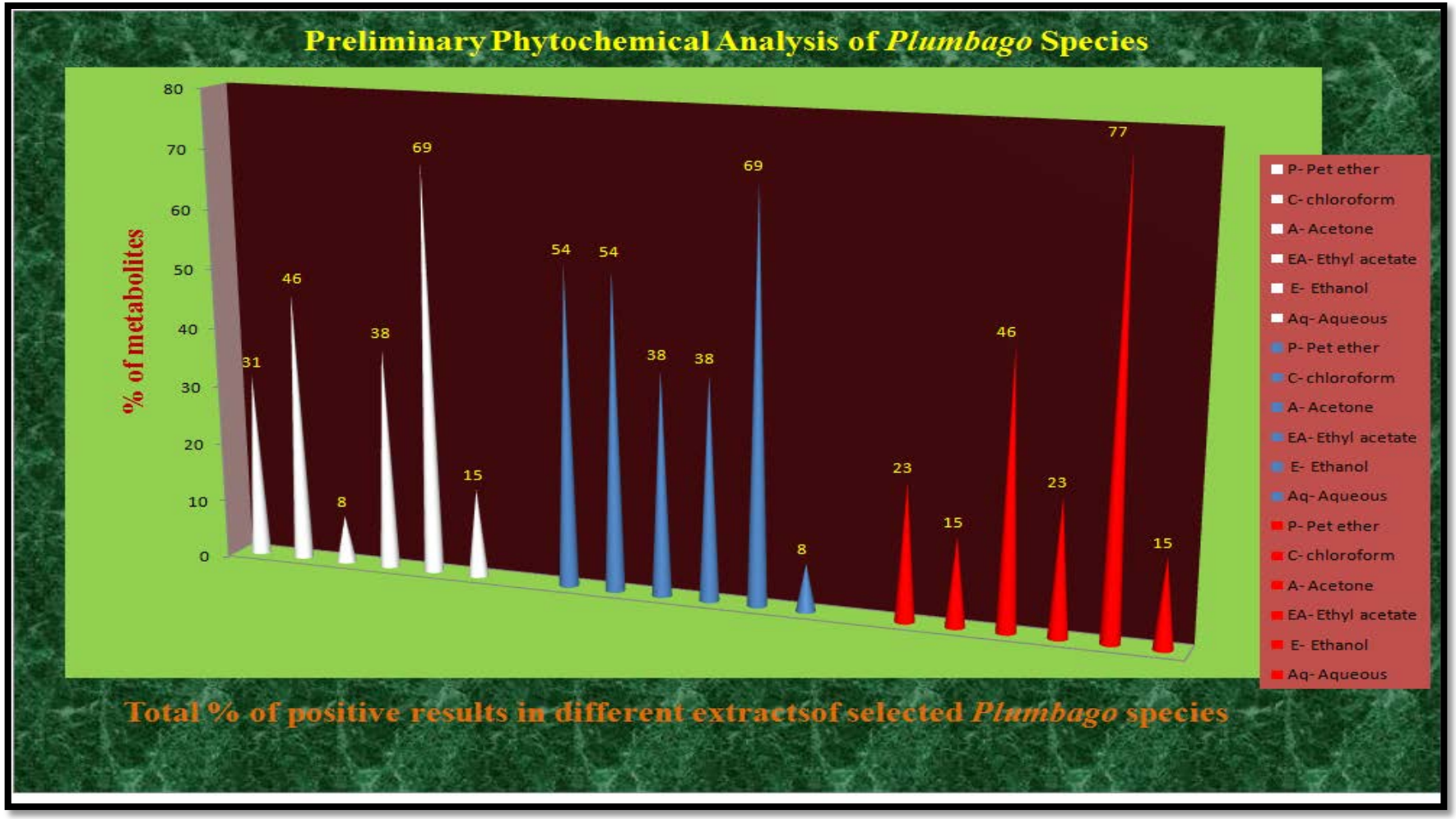


Fig. 22: Chromatogram of the barcoded sequences of *P. zeylanica* collected from Rasthakadu (Pz1)

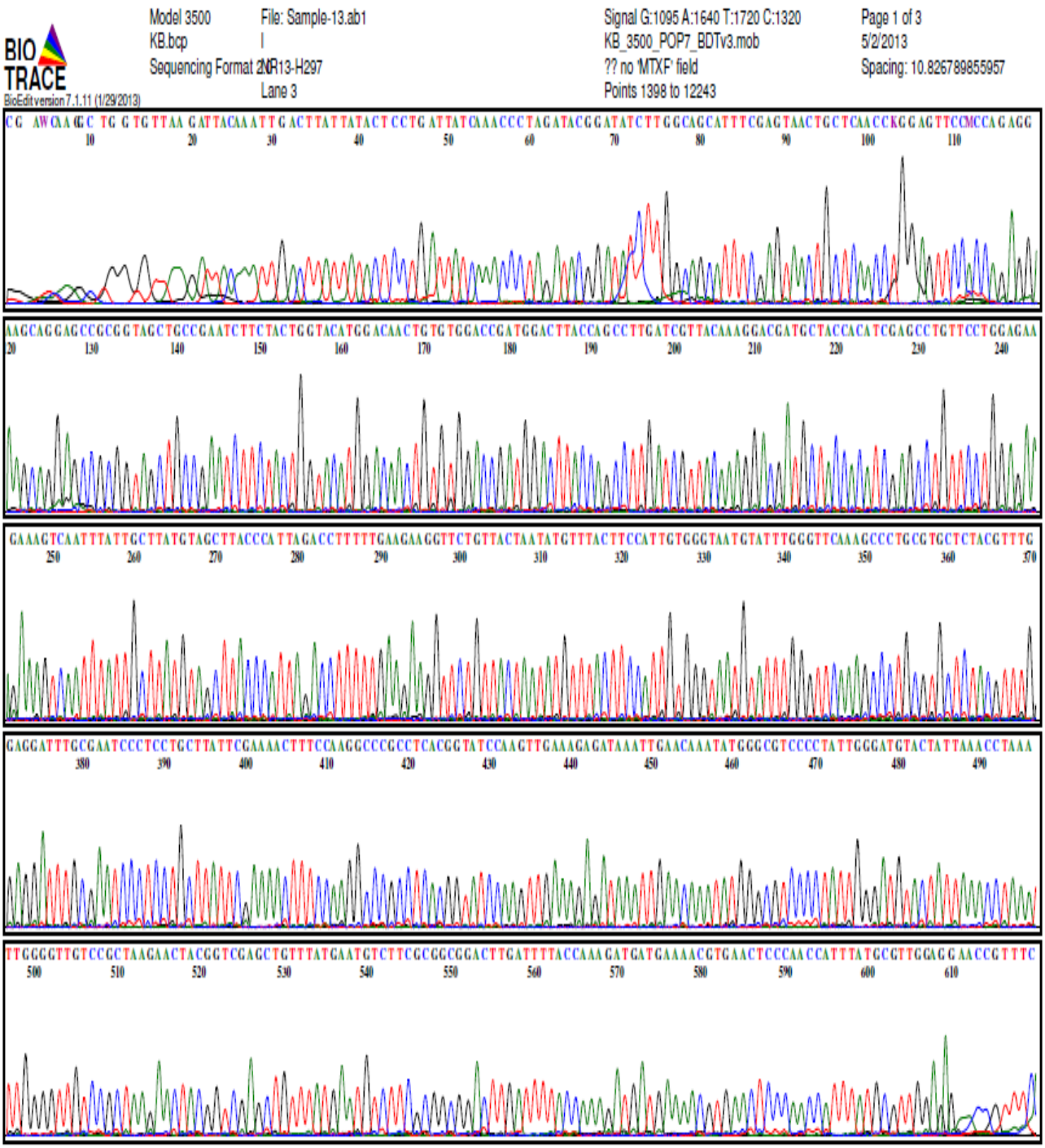


Fig. 23: Chromatogram of the barcoded sequences of *P. zeylanica* collected from Palayamkottai (Pz2)

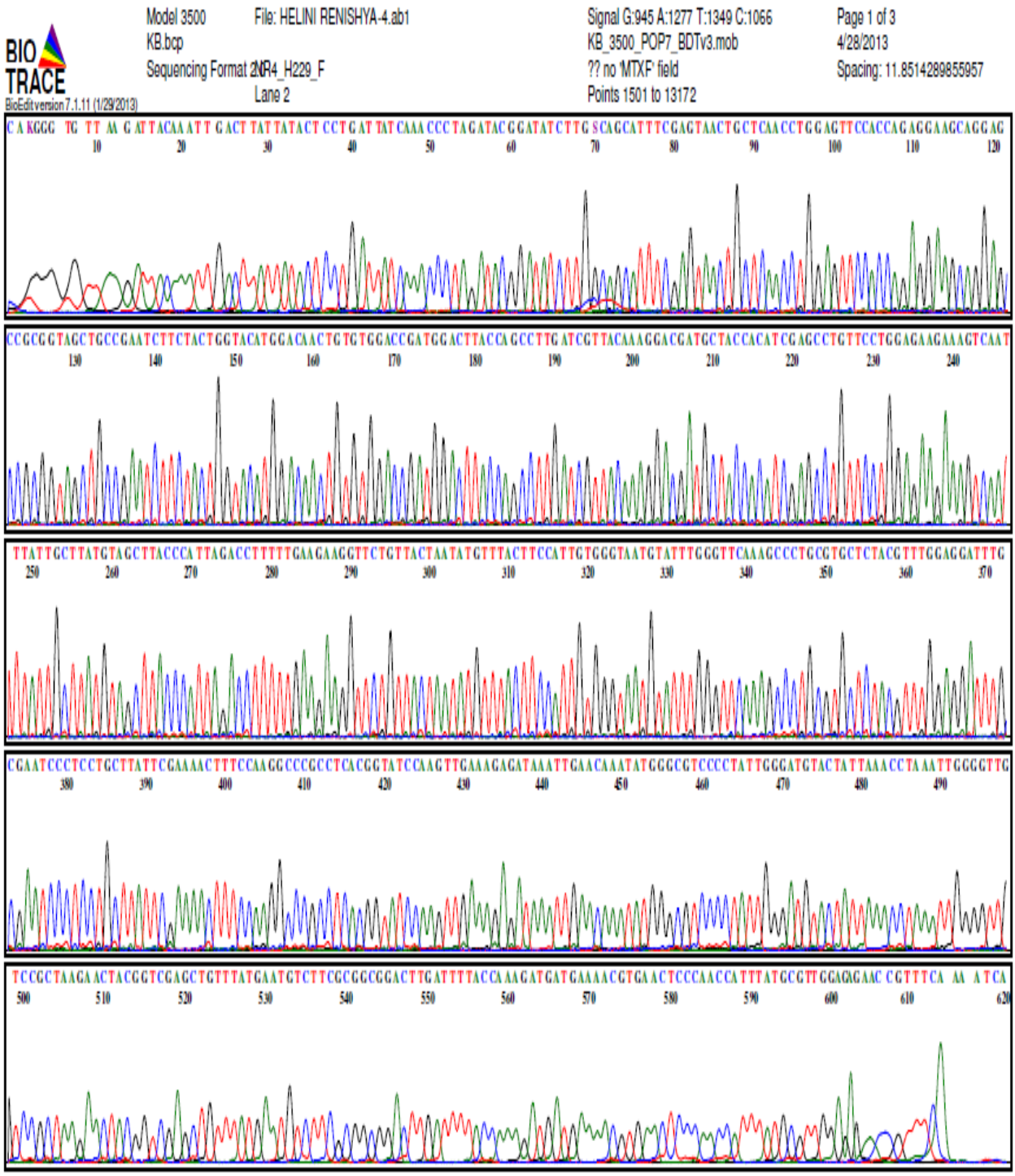


Fig. 24: Chromatogram of the barcoded sequences of *P. zeylanica* collected from Bangalore (Pz3)

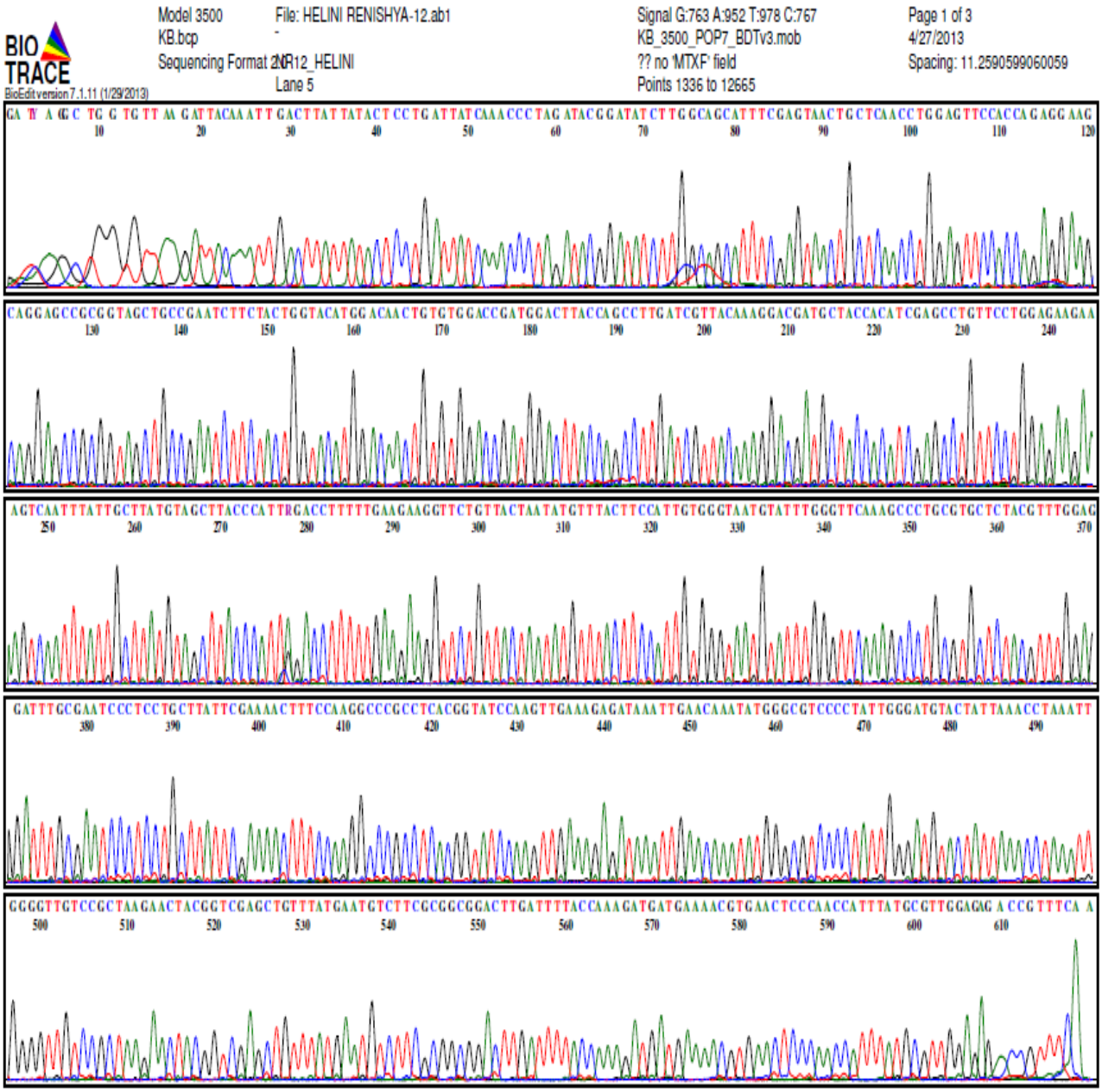


Fig. 25: Chromatogram of the barcoded sequences of *P. zeylanica* collected from Papanasam (Pz4)

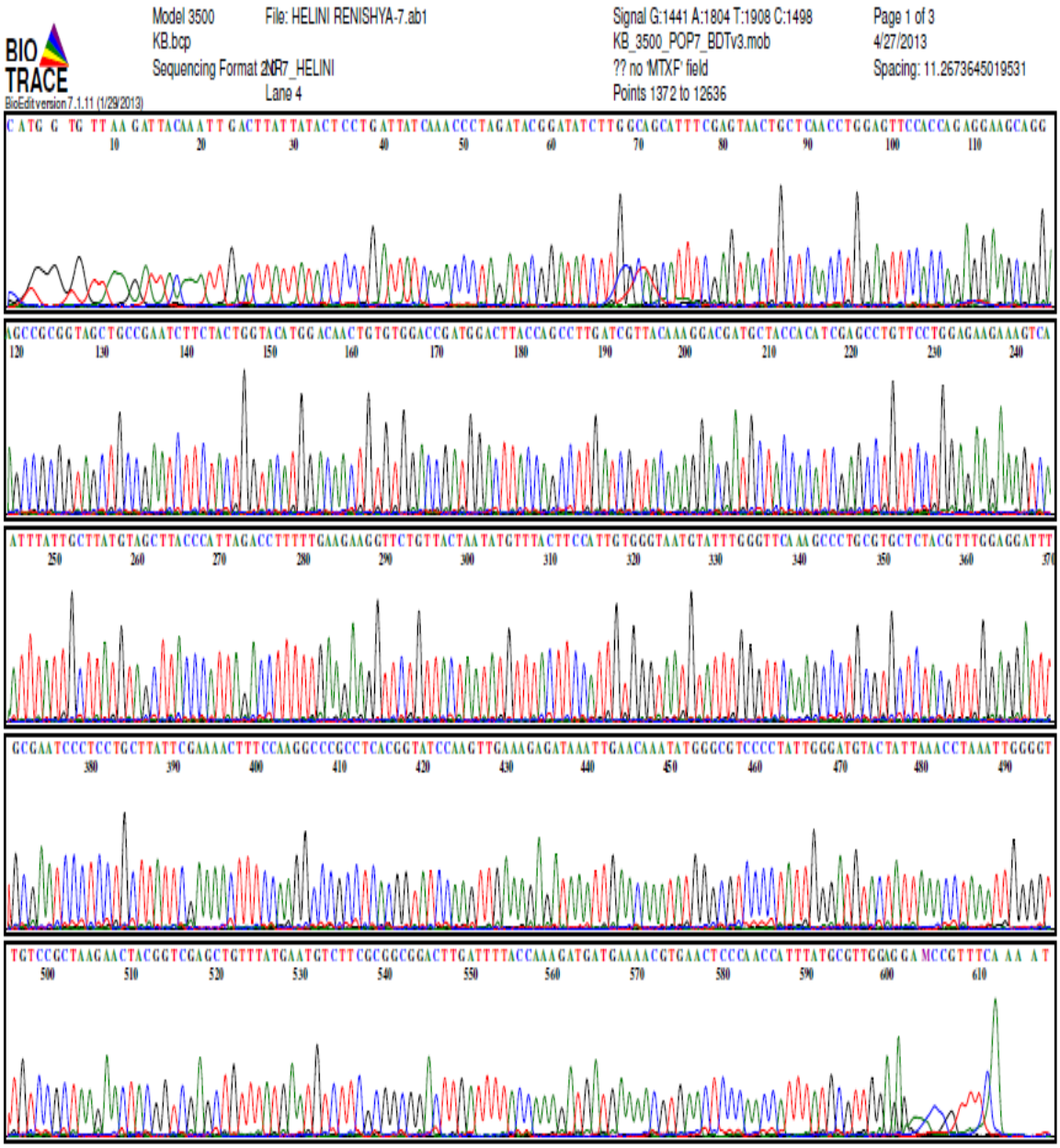


Fig. 26: Chromatogram of the barcoded sequences of *P. zeylanica* collected from Tiruppur (Pz5)

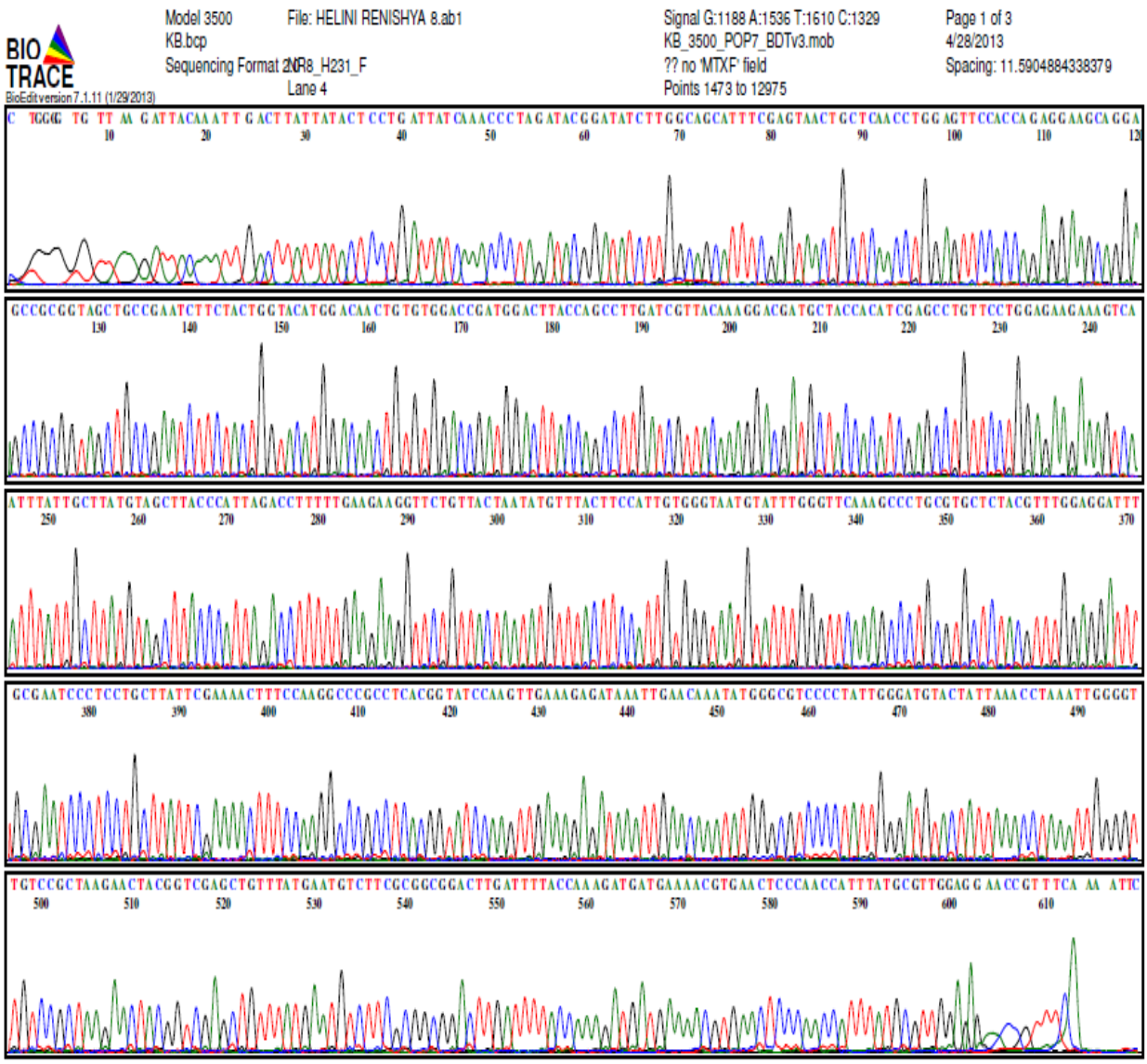


Fig. 27: Chromatogram of the barcoded sequences of *P. zeylanica* collected from Kuttichal (Pz6)

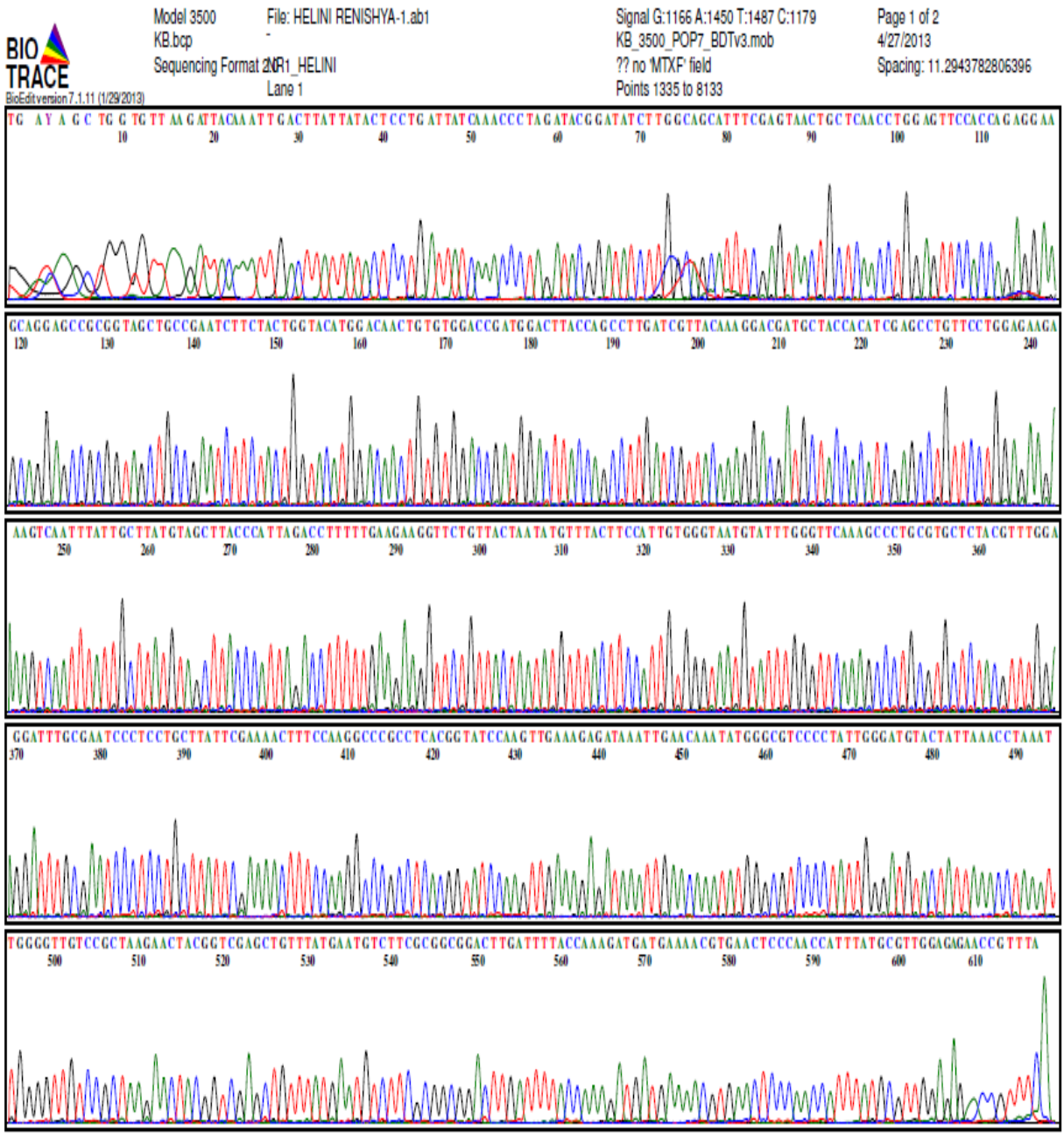


Fig. 28: Chromatogram of the barcoded sequences of *P. zeylanica* collected from Coimbatore (Pz7)

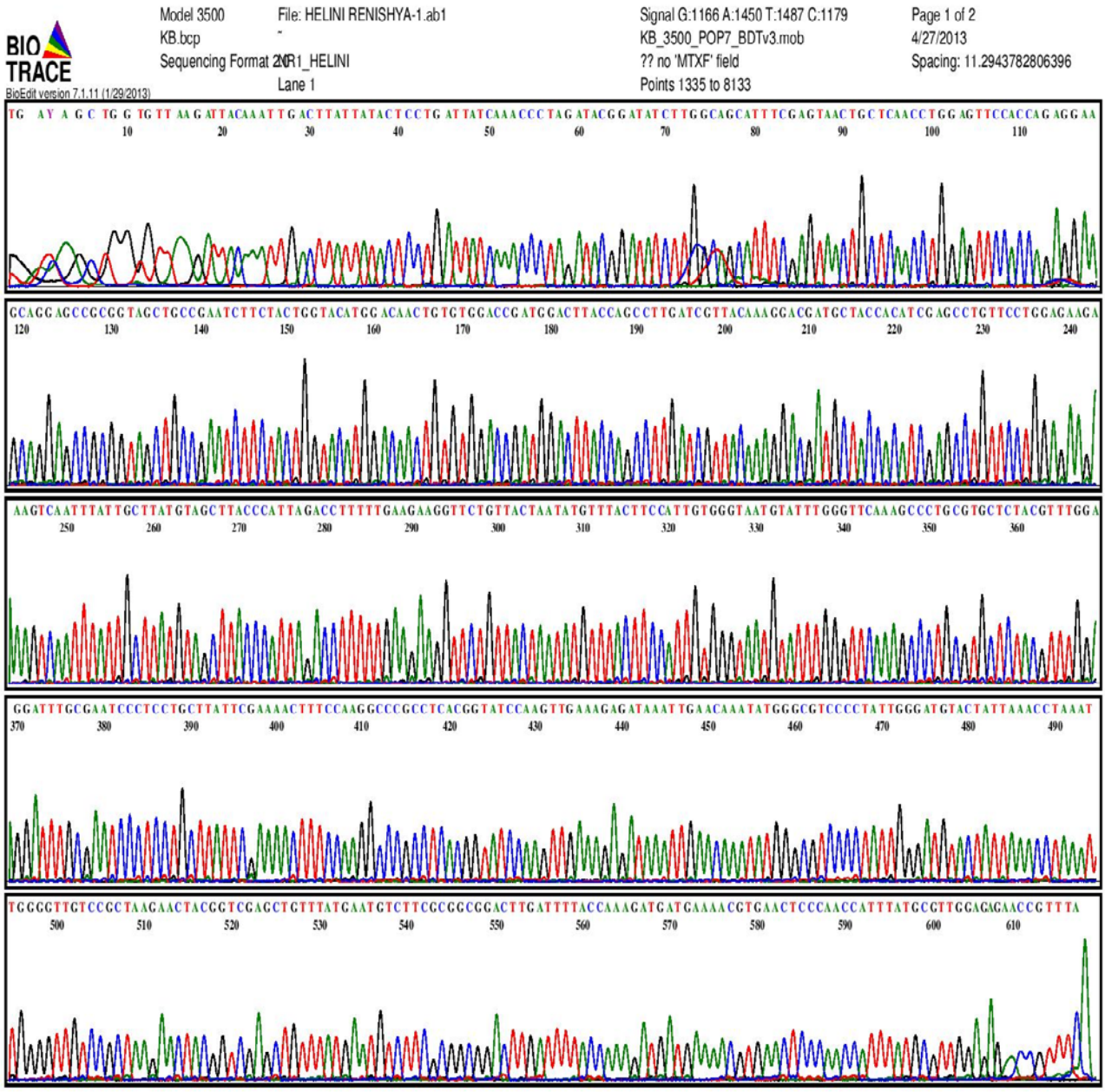


Fig. 29: Aligned sequences of *P. zeylanica* by MULTALIN

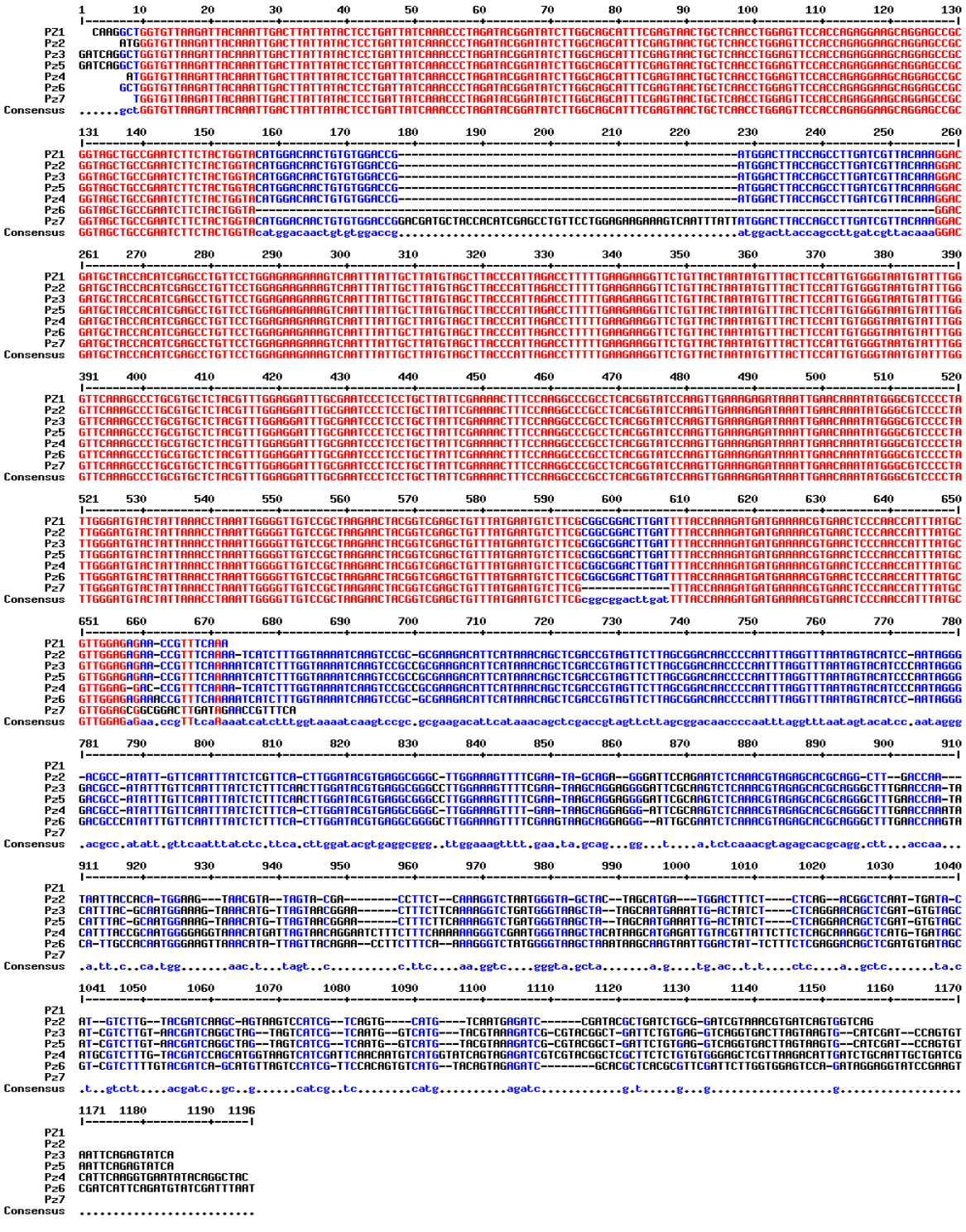


Fig. 36: Chromatogram of the barcoded sequences of *P. auriculata* collected from Mulakumoodu (Pa1)

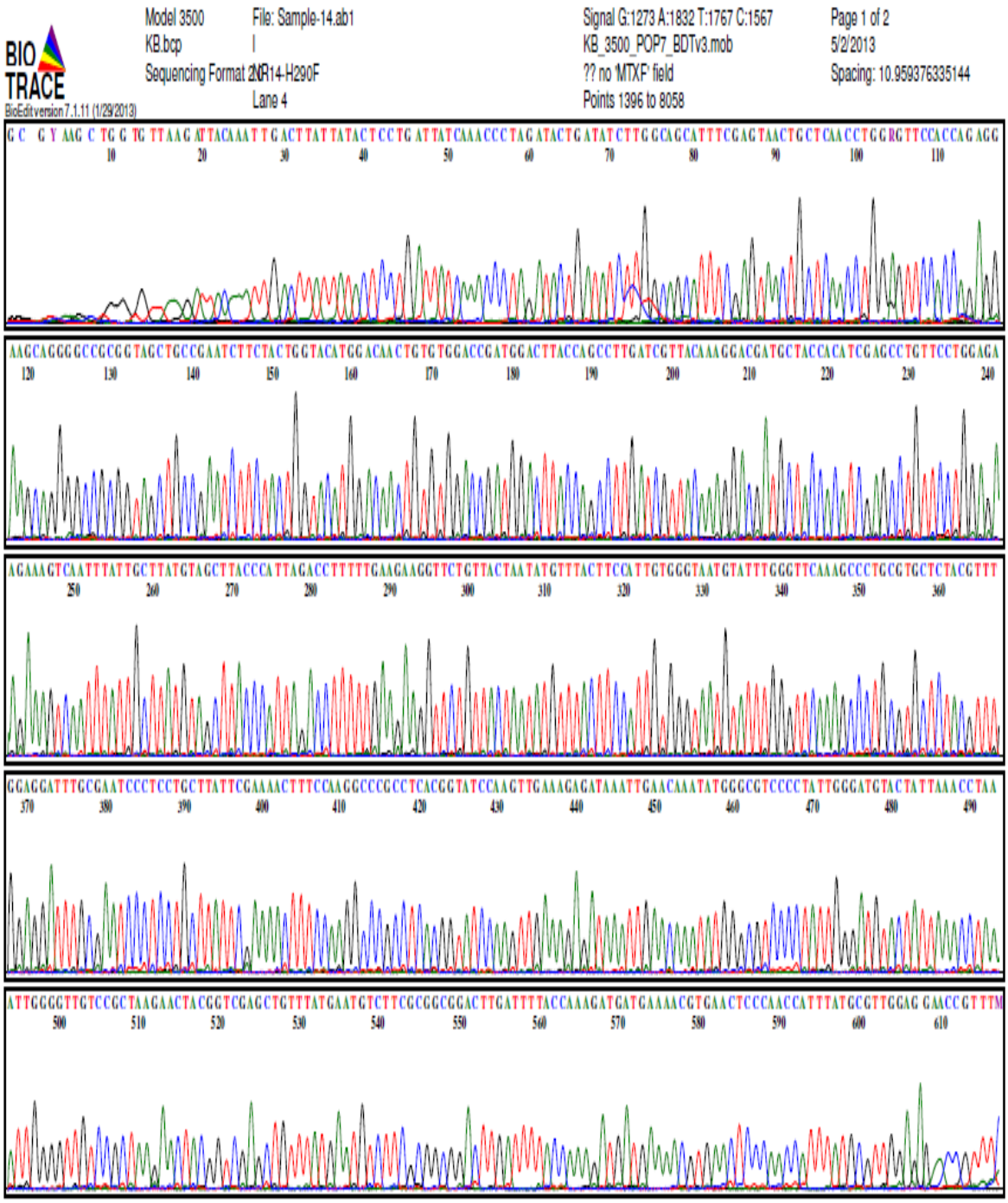


Fig. 37: Chromatogram of the barcoded sequences of *P. auriculata* collected from Tenkasi (Pa2)

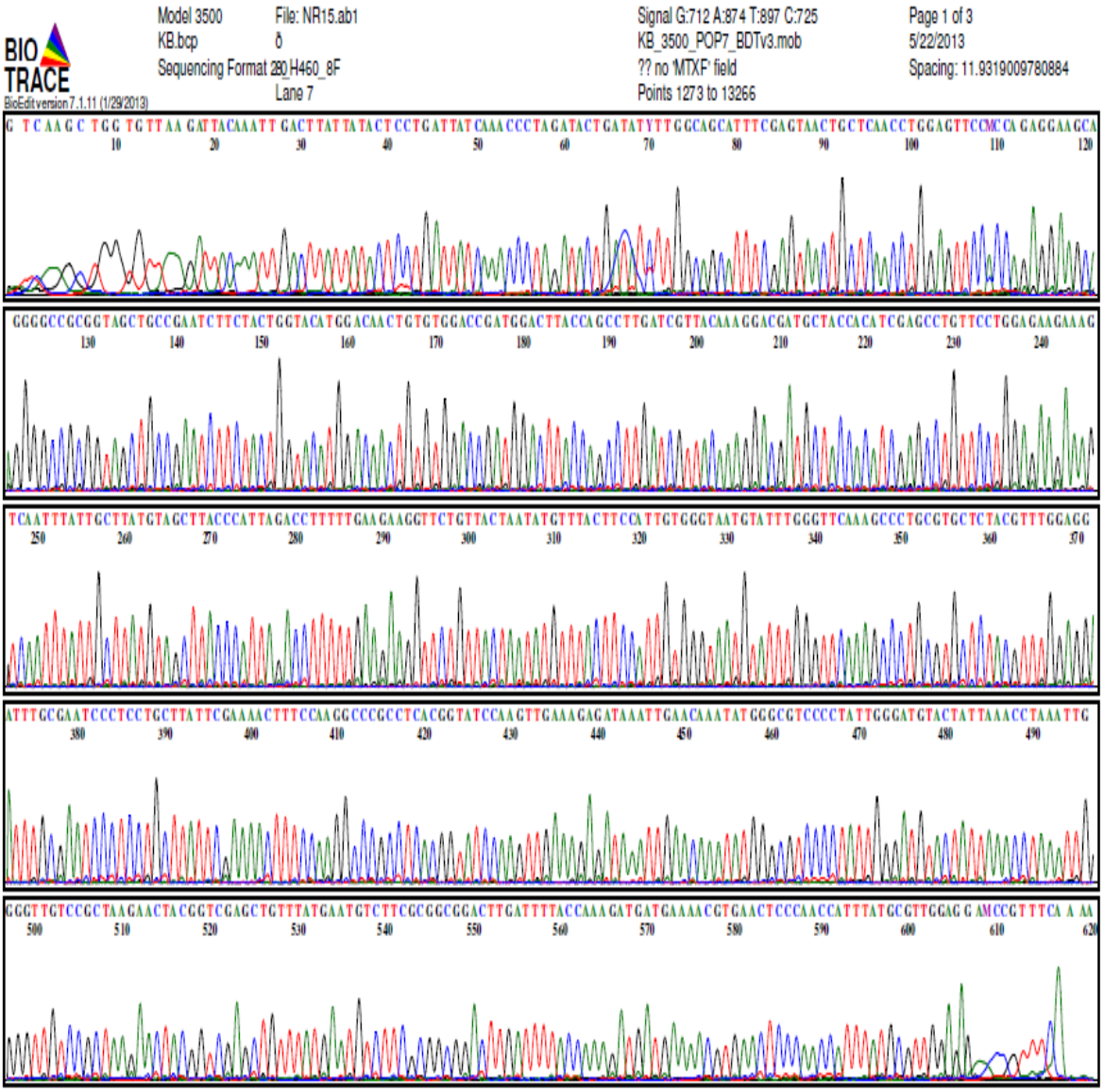


Fig. 38: Chromatogram of the barcoded sequences of *P. auriculata* collected from Perunthurai (Pa3)

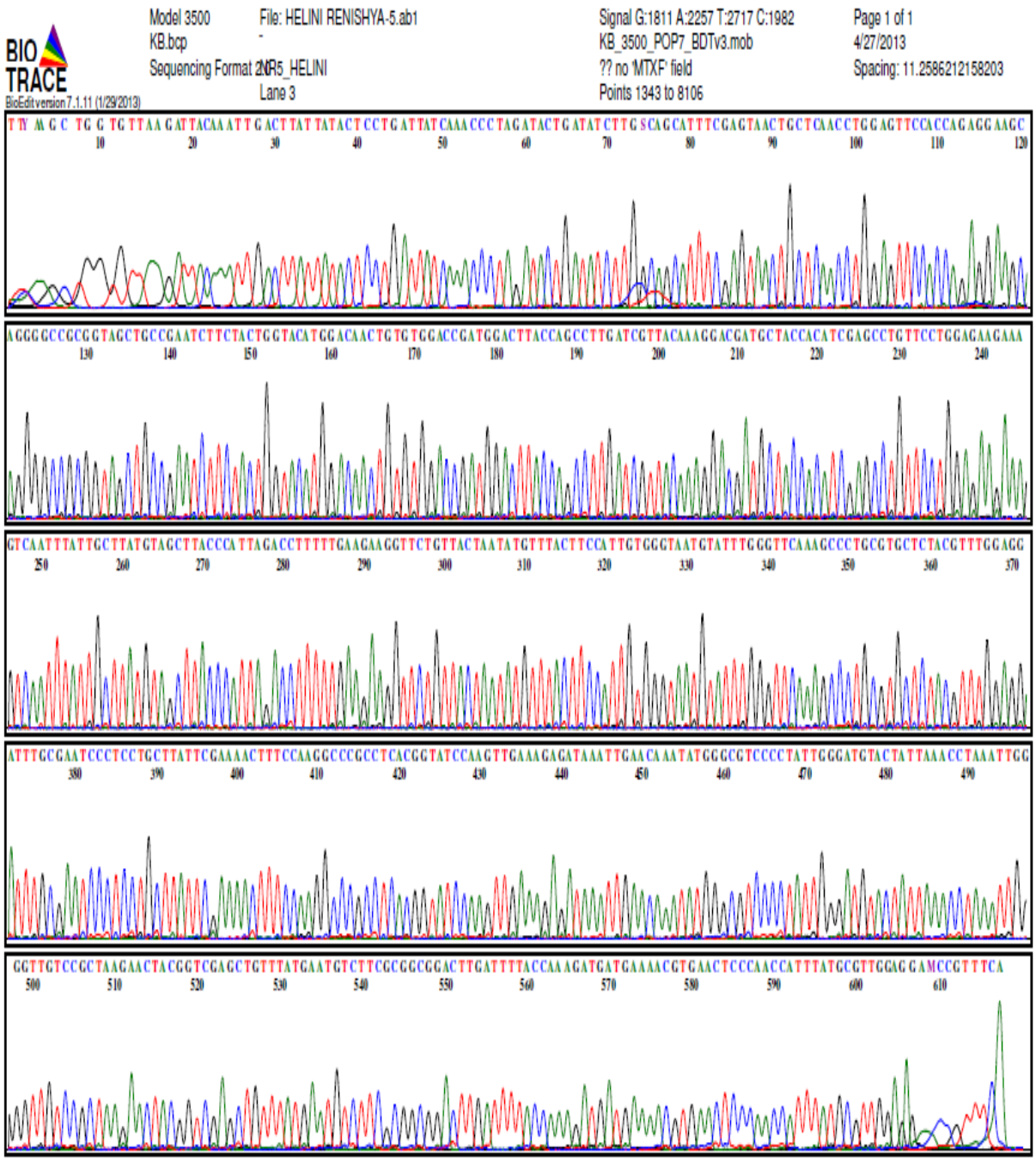


Fig. 39: Chromatogram of the barcoded sequences of *P. auriculata* collected from Kattakadu (Pa4)

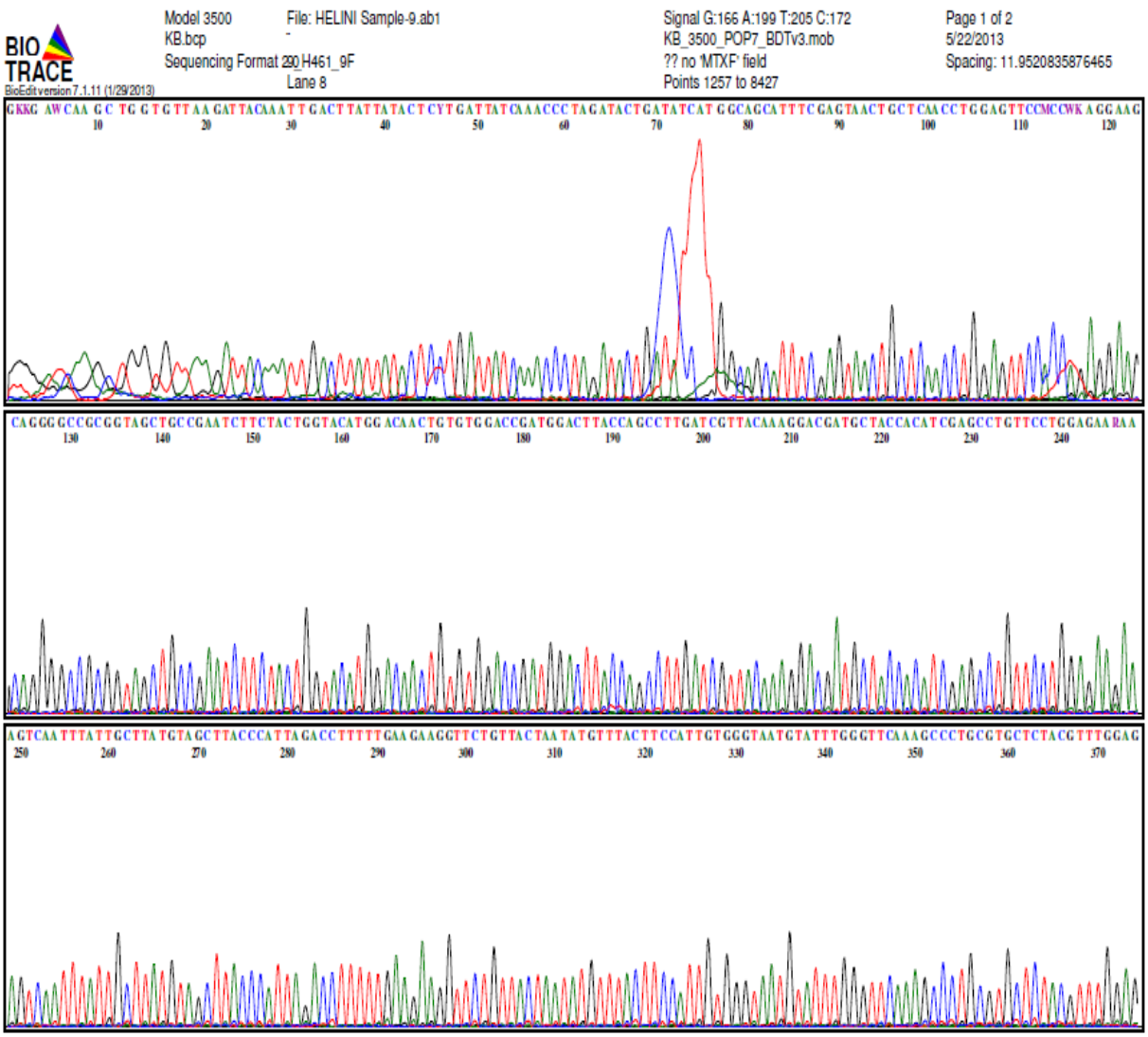


Fig. 40: Chromatogram of the barcoded sequences of *P. auriculata* collected from Mysore (Pa5)

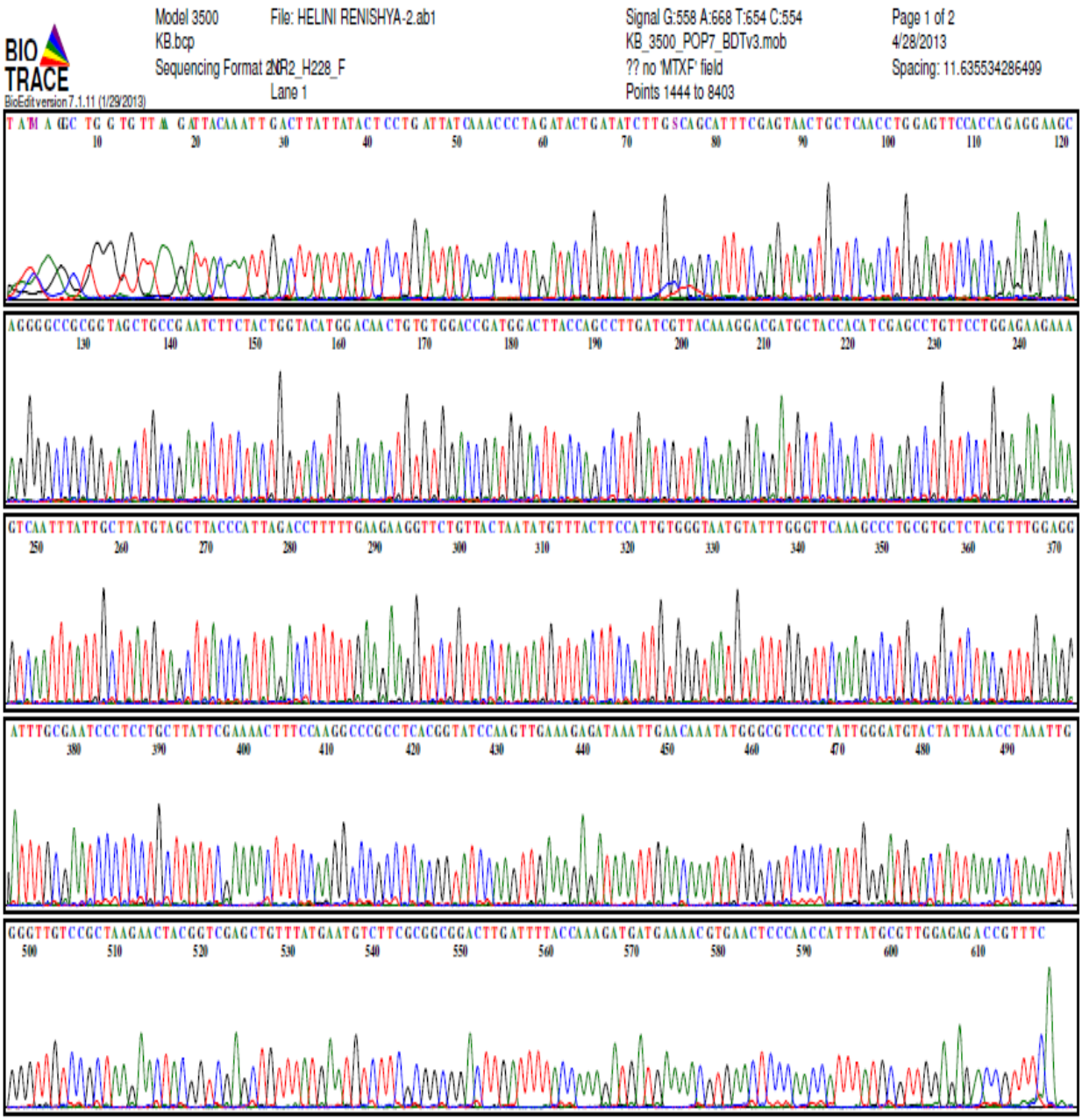


Fig. 41: Aligned sequences of *P. auriculata* by MULTALIN

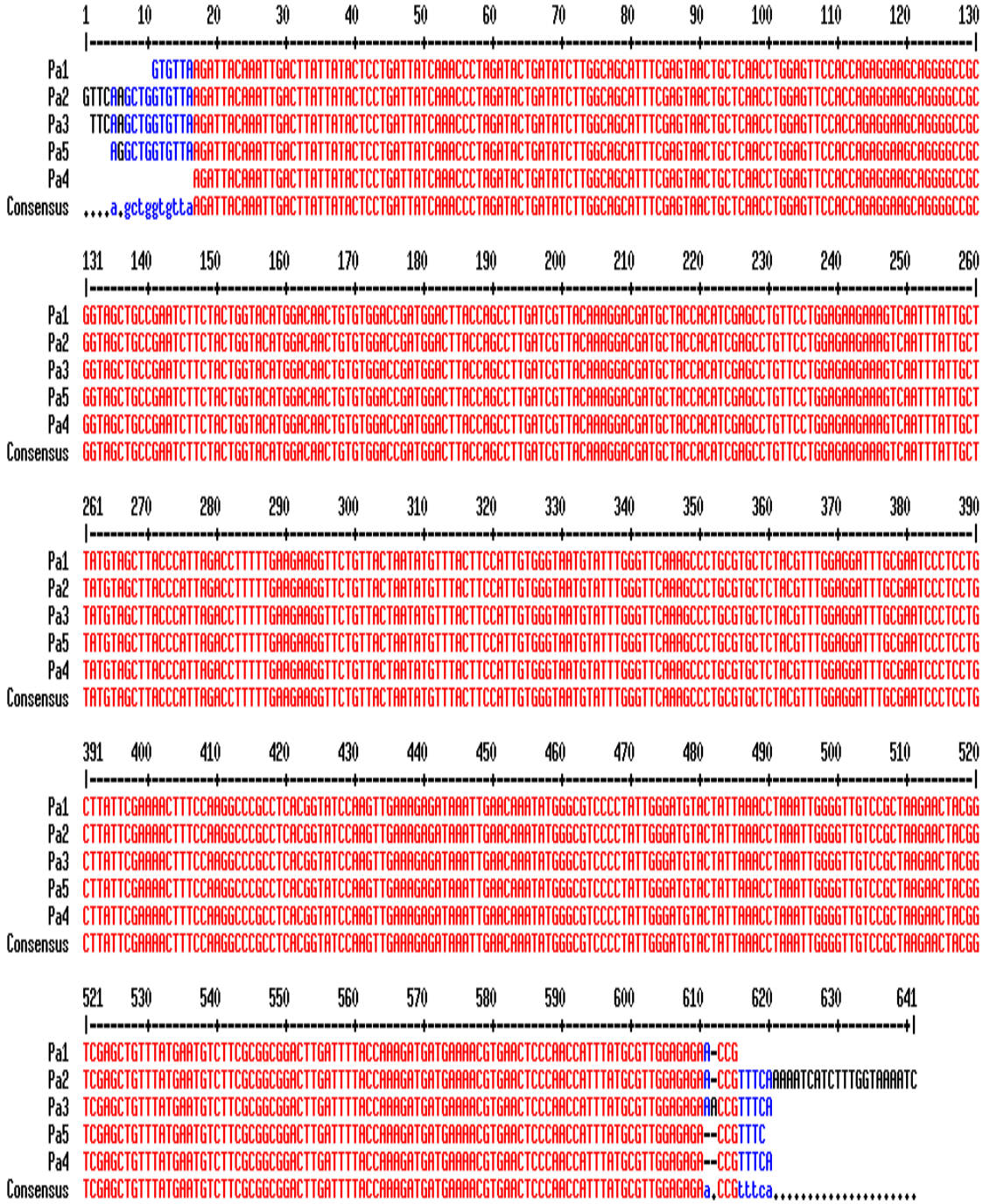


Fig. 48: Chromatogram of the barcoded sequences of *P. rosea* collected from Cheuvarankonum (Pr1)

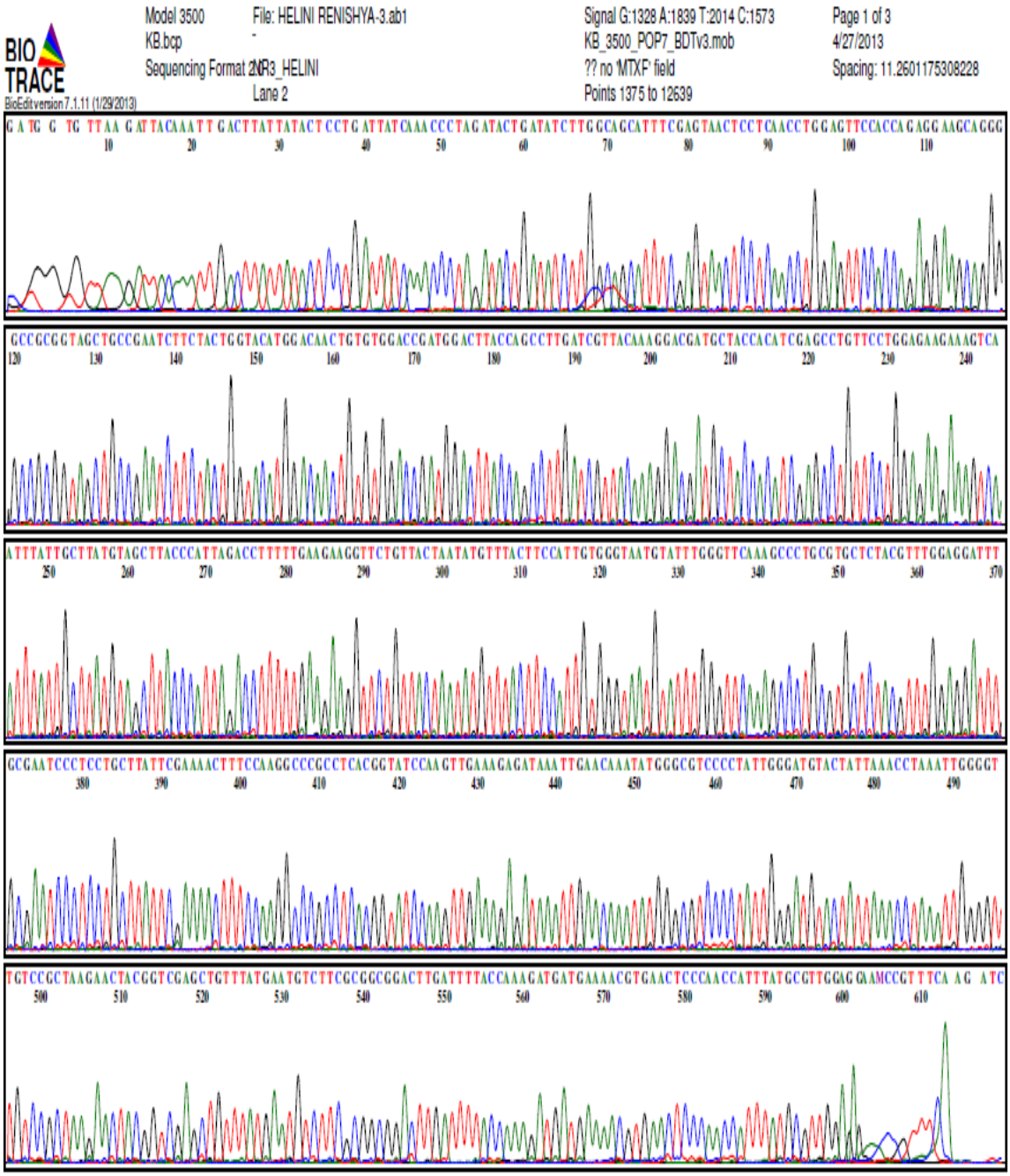


Fig. 49: Chromatogram of the barcoded sequences of *P. rosea* collected from Dana (Pr2)

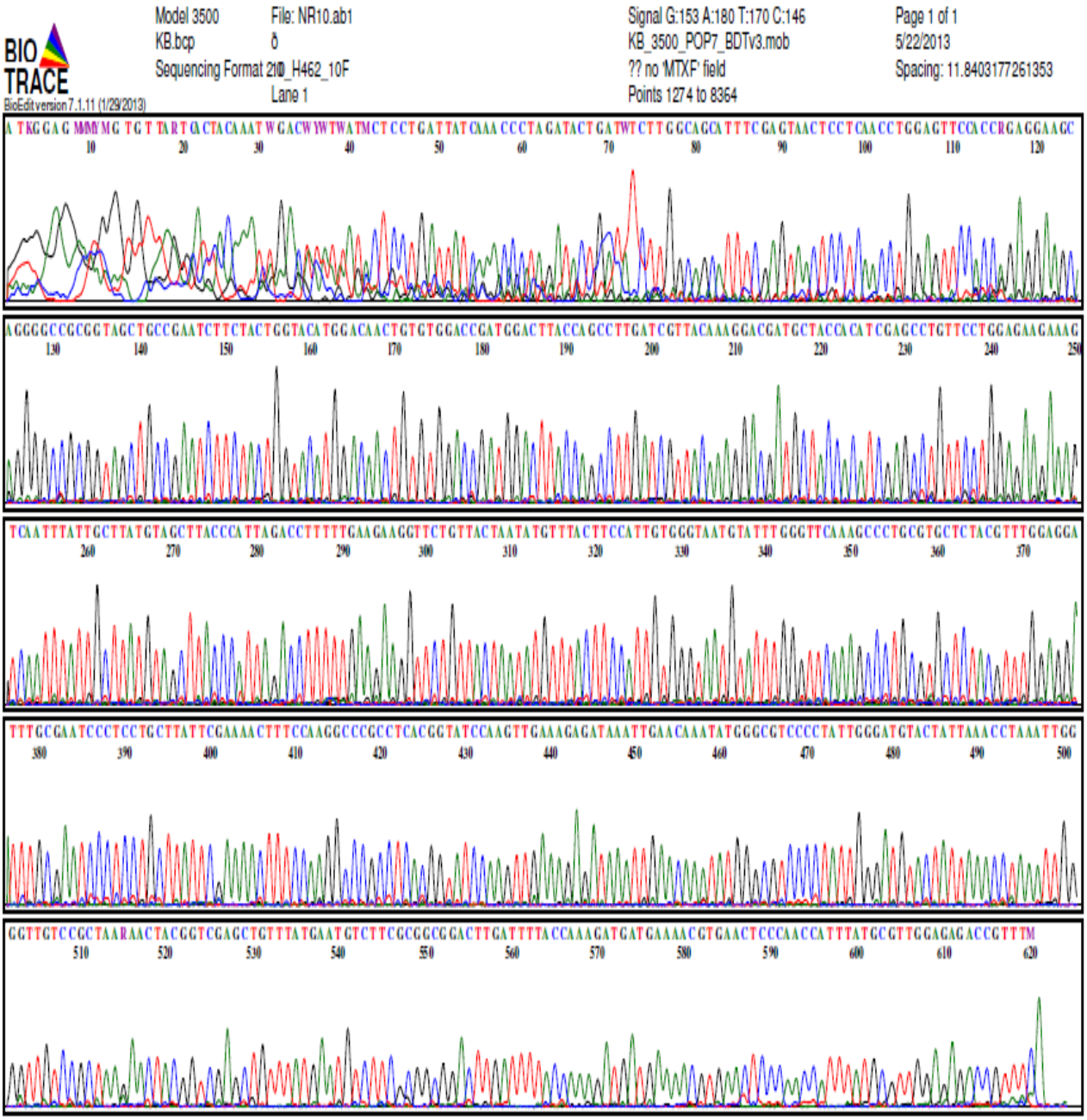


Fig. 50: Chromatogram of the barcoded sequences of *P. rosea* collected from Bangalore (Pr3)

Fig. 51: Aligned sequences of *P. rosea* by MULTALIN

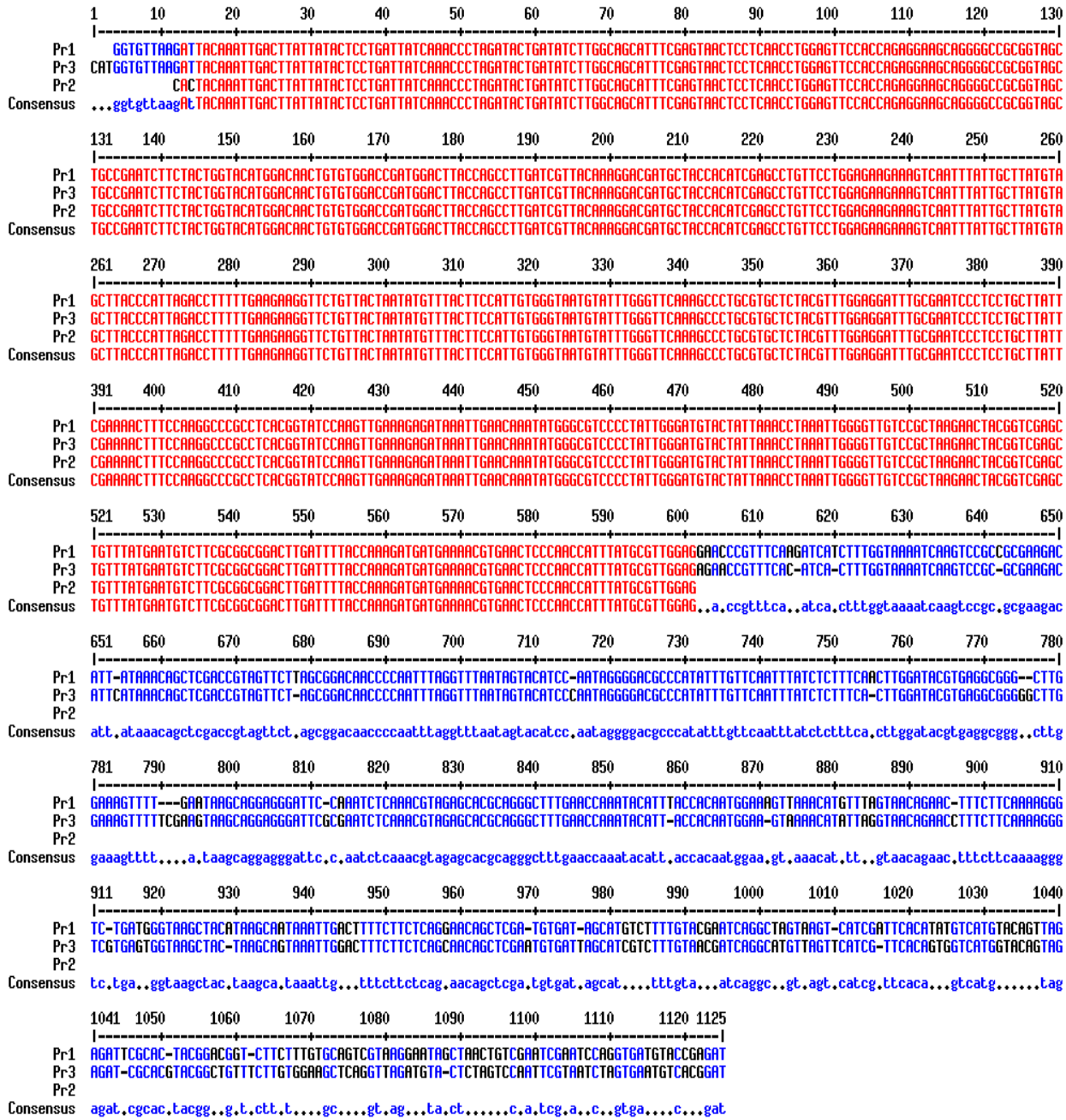


Fig. 57: Aligned sequences of *Plumbago* species by MULTALIN

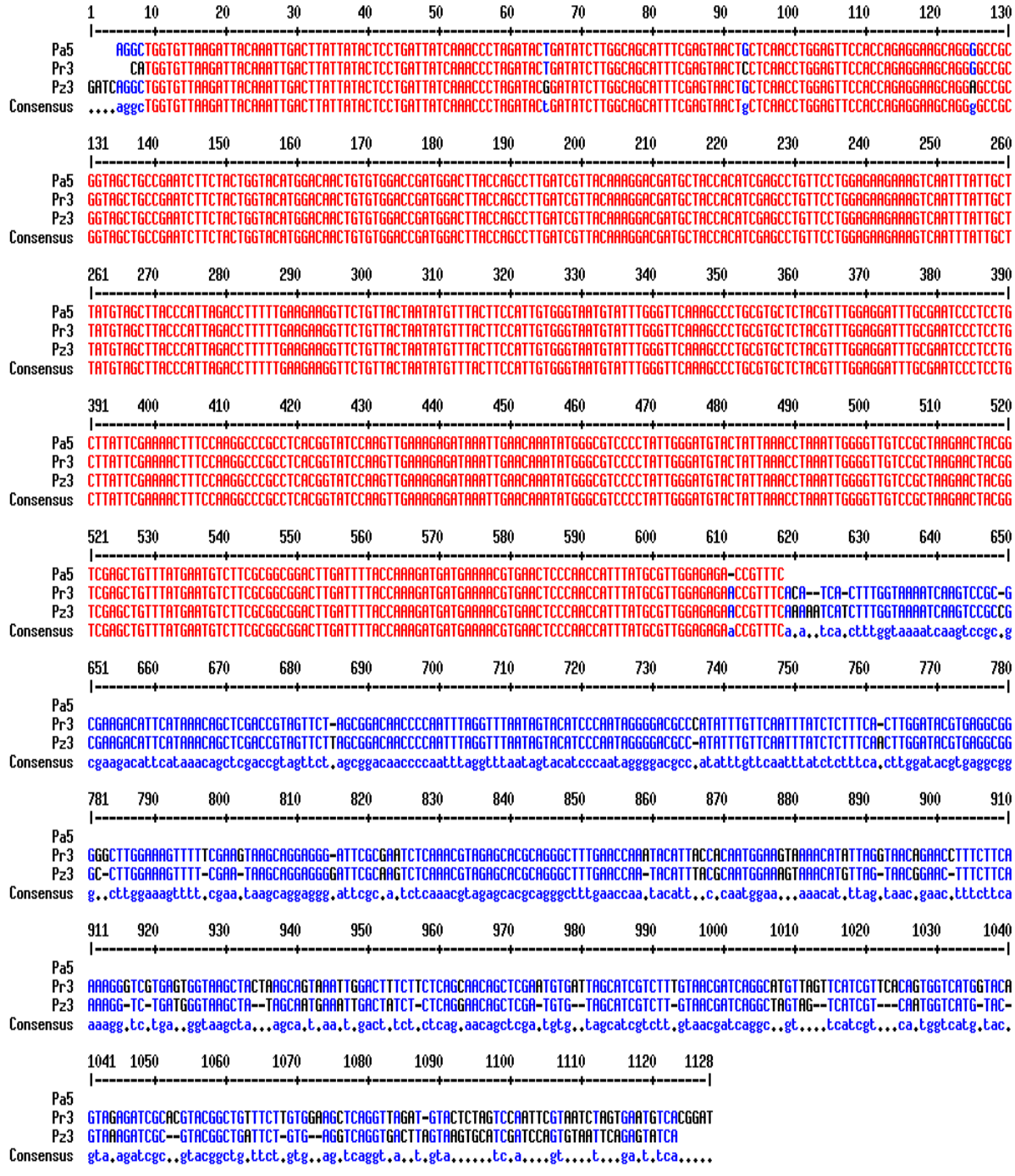
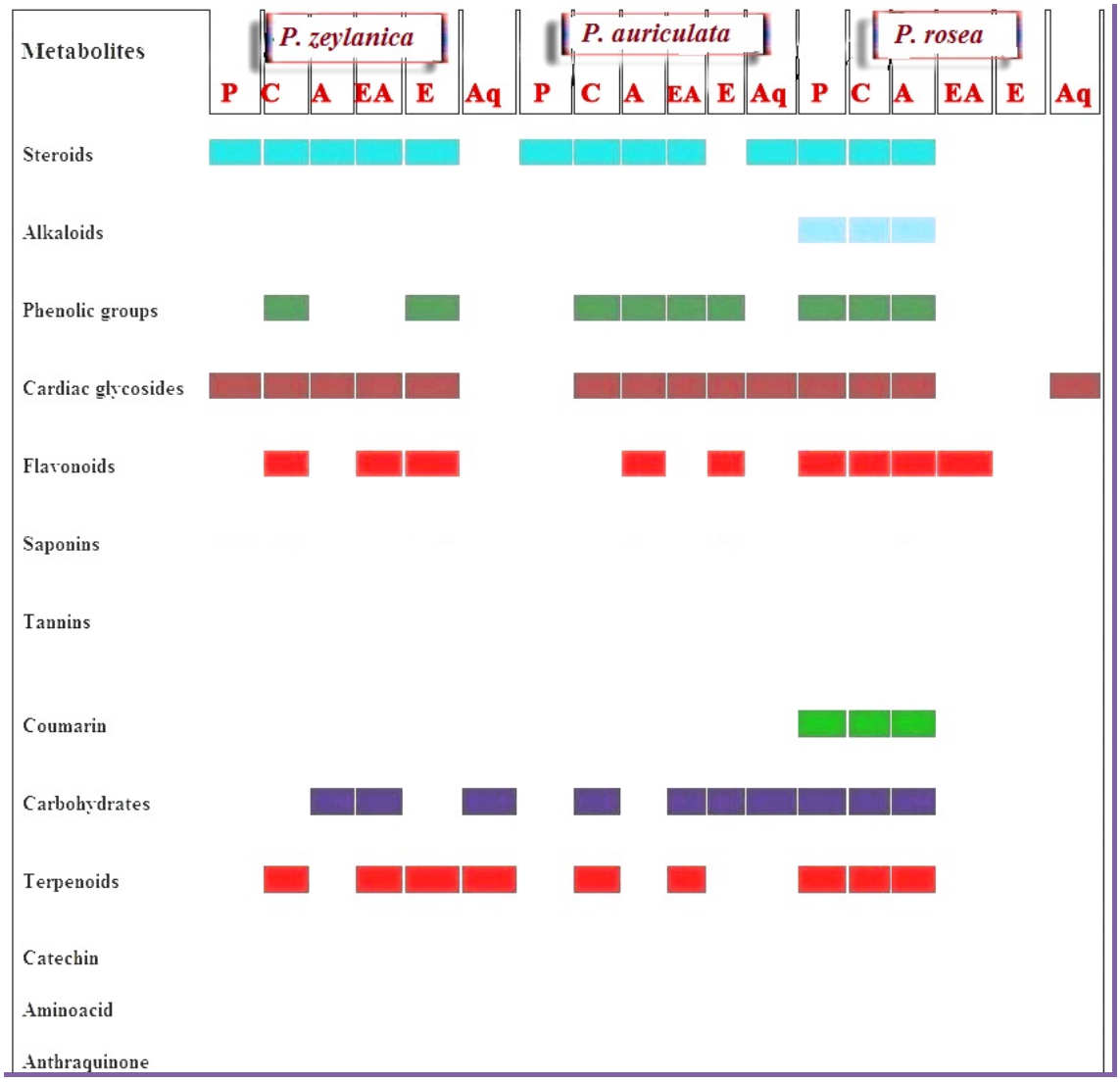


Fig. 1: Preliminary phytochemical screening of various extracts of the selected *Plumbago* species



- **Petroleum ether extract**
- **C- Chloroform extract**
- **A- Acetone extract**
- **EA- Ethyl Acetate**
- **E- Ethanolic extract**
- **Aq- Aqueous extract**

Fig. 4. Flourescence characteristics of the *Plumbago* species

	Day light			UV-Light					
				265 nm			365 nm		
	P. zeylanica	*P. auriculata*	*P. rosea*	*P. zeylanica*	*P. auriculata*	*P. rosea*	*P. zeylanica*	*P. auriculata*	*P. rosea*
Crude powder	Pale green	Light green	green	Dark green	green	Dark green	Dark brown	Dark green	Dark green
Petroleum ether	Pale yellow	Yellow	Pale yellow	Yellow	Dark yellow	Yellow	Brown	Brown	Green
Chloroform	Dark green	Green	Green	Dark green	Dark green	Brown	Dark brown	Dark brown	Brown
Acetone	Green	Light green	Dark yellow	Pale green	Green	Green	Brown	Red	Dark brown
Ethyl acetate	Dark yellow	Dark green	Dark green	Green	Red	Red	Dark green	Brown	Brown
Ethanol	Yellow	Yellow	Yellowish green	Dark yellow	Green	Green	Dark green	Dark green	Dark green
Aqueous	Light yellow	Light yellow	Light yellow	Pale yellow	Yellow	Yellow	Yellow	Light green	Green
50% H_2SO_4	Brown	Dark green	Green	Brown	Brown	Dark green	Red	Dark brown	Brown
1N HCl	Pale brown	Brown	Brown	Brown	Dark brown	Dark brown	Black	Red	Dark brown
NaOH	Yellow	Dark yellow	Yellow	Yellowish green	Yellowish green	Green	Green	Black	Yellowish green
50% Nitric acid	Red	Green	Green	Dark red	Green	Dark green	Brown	Dark green	Brown

ABBREVIATIONS

°C	Degree Celsius
µL	Micro litre
cm	Centimetre
CTAB	Cetyl Tri Methyl Ammonium Bromide
DNA	Deoxy Ribo Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic acid
EtOH	Ethanol
Fig	Figure
GC-MS	Gas Chromatography / Mass Spectrometry
gm	gram
HCl	Hydro Chloric Acid
Hex	Hexane
HPLC	High-performance Liquid Chromatography
L	Lane
Mol. Formula	Molecular Formula
M	Molar
Min	Minute
Mil	Milli litre
mM	Milli molar
Mol. Wt	Molecular weight
MALDI -TOF MS	Matrix-Associated Laser Desorption Ionization Time Of Flight Mass Spectrometry
SDS-PAGE	Sodium Do decyl Sulphate Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
Pet. Ether	Petroleum ether

rpm	Revolutions per minute
***rbc*L**	Ribulose,1-5,bisphosphate large subunit
RT	Retention Time
TEMED	N,N,N',N'-Tetra Methyl Ethylene Diamine
TLC	Thin Layer Chromatography
Tris HCl	Tris (Hydroxymethyl) amino methane hydrochloride
UV	Ultra Violet
HPTLC	High-performance Thin Layer chromatography
β ME	β -mercapto ethanol
Pz	*Plumbago zeylanica*
Pa	*Plumbago auriculata*
Pr	*Plumbago rosea*
FTIR	Fourier transform infrared spectroscopy
UV-Vis region	Ultra Violet- Visible region
m/z values	Mass spectral values
kDa	Kilo Daltons
NaoH	Sodium hydroxide
UPGMA	Un weighed pair group mean Average
NTSYSpc- 2.0 software	Numerical Taxonomy System, Version 2.2 for Windows XP
v/v	Volume/volume
w/v	Weight/volume
Rf	Resolution factor
SCL	System ControLler
***VP* system**	Validation and Productivity
NIST library	National Institute of Standards and Technology mass spectral library
PASS	Prediction Activity Spectra for Substances
MTCC	Microbial Type Culture Collection

µg	Micro gram
%	percentage
ACN/TFA	Acetonitrile/ Trifluroacetic acid
kV	kiloVolts
Nacl	Sodium chloride
TBE buffer	Tri-Borate-EDTA buffer
TE buffer	Tris EDTA buffer
dNTP	Deoxy nucleotide tri phosphate
KCl	Potassium chloride
(NH4)2SO4	Ammonium sulphate
MgSO4.7H2O,	Magnesium sulphate
Taq DNA	*Thermus aquaticus* DNA polymerase
CLUSTAL W	CLUSTAL with a command line interface
MULTALIN	Multiple sequence alignment by floresence carpet
NCBI	National Center for Biotechnology Information
MEGA(6.0)	Molecular Evolutionary Genetic analysis (version 6.0 software)
NJ Tree	Neighbor Joining tree
GenBank	Comprehensive databank
CNI algorithm	Close-Neighbor-Interchange
ME tree	Minimum Evolutionary tree
Rt	Retention time
MW-Rf values	Molecular Weight- Resolution front
ITS	Internally Transcribed Spacer
GIL	Group I Lysine
GIA	Group I Asparagine
GIG	Group I Glutamine

GIM	Group I Methionine
GIIP	Group II Proline
GIIC	Group II Cystine
GIIS	Group II Serine
GIITY	Group II Tyrosine
GIITR	Group II Tryptophan
GIIIH	Group III Histidine
GIIIA	Group II Arginine
GIIIAS	Group III Aspartic acid
GIIIT	Group IV Threonine
GIIIL	Group IV Leucine
GIVG	Group IV Glycine
GIVA	Group IV Alanine
GIVV	Group IV valine
GIVI	Group IV Glycine
GIVP	Group IV phenyl alanine
AU (Peak area)	Arbitrary Units
pH	power of Hydrogen Ion concentration
***mat*K**	maturase K

Life on earth mainly depends on plants and it is very important for the survival of human beings. Biodiversity is the vast array of all flora and fauna inhabiting the earth either in aquatic or the terrestrial habitats. It provides enormous direct and indirect essential services through natural ecosystem function and stability to humankind. Plants and plant products have been the primary sources upon which the modern civilization has been built. In the tropics alone it has been estimated that 25,000 - 30,000 plants are in use (Shanley and Luz, 2003). Recent studies have reported that out of the total 4, 20, 000 flowering plants in 440 families of plants from the world more than 50,000 are used for medicinal purposes (Schippmann *et al.,* 2002). India is one of the twelve mega-biodiversity centres of the world having rich vegetation with a wide variety of plants with medicinal value (Patrick, 2002). India is also the largest producer of medicinal herbs and is appropriately called the botanical garden of the world. In India, more than 43% of the total flowering plants are reported to be of medicinal importance (Pushpangdan, 1995). The exploitation of plants for medicinal purposes in India has been documented long back in ancient literature (Charak and Drdhbala, 1996). In rural India, 70 percent of the population is dependent on traditional systems of medicine. The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000 plants (Alam, 2008).

Economical, social, ecological, cultural and aesthetic cases have been made for identification, quantification and understanding the distribution and relationship of biological diversity (Kunin and Lawton, 1996). Biological diversity may be assesed at three diferent levels: the community, the species and the gene (Frankel *et al*., 1995). Efficient utilization, improvement and conservation of taxa must be based on a sound understanding of phylogeny,

the amount and distribution of genetic variation. Genetic markers that are observable traits are classified into five broad groups viz., morphological, cytological, chemical, protein and DNA (Szmidt and Wang, 2000).

Nature has myriads of life forms on this planet among which variations are of ubiquitous occurrence. Variations between individuals of the species have occured the millennia and are considered as real "hotspot" of evolution. Down the years, variations among plants have always fascinated the inquistive mind and helped an evolutionary biologist or a breeder to select a desirable variant or breed a new form of greater agronomic value. In the wild, plants grow in extreme situations along longitudinal, latitudinal and temperature gradients. Therefore variations within and between populations of a species are not uncommon (Lei *et al.,* 2006). Much of the variations in phenotype observed in natural populations of a species were earlier attributed to environmental influences (Briggs and Walters, 1984). Many botanists reasoned that distinct intra-specific variations of plants were merely due to habitat modifications and adaptation to environment was by phenotypic plastic responses. Phenotype was accepted as a resultant product of interaction between genotype, environment and different phenotypes. In certain plants developmental variations were observed as evident from differential morphological characterstics of the juvenile and adult forms (Smila *et al.,* 2007; Johnson, 2007). The problem of variations are further compounded in medicinal plants which apart from displaying visible interactions, synthesize and accumulate an array of plant specific chemicals. These compounds are biosynthetically derived from primary metabolism and accumulated by certain plants or group of plants in trace quantities. A study of variation in the active principles is often an important element in the investigation of variation in such plants.

A wide spectrum of simple and overlapping variations is now documented in plants (Connely *et al.,* 1993; James and Ashburner, 1997; Sen *et al.,* 2009). All observed variations are broadly grouped into two categories: epigenetic and genetic. Genetic variations in plants are strictly heritable. They occur invariably due to alterations in the genetic material and may affect both phenotypic and chemical characteristics of a medicinal plant. In general the variations in plants are classified as inter-specific and intra-specific variations. Inter-specific variation means the variations which are found between the populations, i.e. between the different species of a single genus or among different genera themselves. Intra-specific variation is the variations within populations which are found within a single species of a particular genus. Intra-specific and inter-specific variations are the building blocks for trait/character improvement through breeding or genetic selection and hence has become very important in botanical breeding, plant science research and gene bank activities related to preservation of integrity in viable seed materials (Mercandante and Pfander, 1998). Chemo-profiling and morphological evaluation is routinely used for the identification of genotypic variation. In addition to variation in chemical production resulting from environmental and temporal conditions, many studies have looked at the variation of chemical groups within genetically related taxa as well. For example, polyacetylenes and related compounds have been shown to segregate in various intra-generic groups within *Artemisia* (Greger *et al.,* 1982; Albasini *et al.,* 1983). Variation in chemical production by non-specific individuals has also been documented in wild *Artemisia dracunculus*. Both French and wild tarragon have been analyzed to determine if there are differences in their phytochemical compositions (Balza *et al.,* 1985; Deans *et al.,* 1988).

Secondary metabolites can be derived from any part of the plant like bark, leaves, flowers, seeds, etc (Cragg and Newman, 2001). It is necessary to check the quality of the

material by estimating the phytomarkers available in the plant parts. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the production of complex chemical constituents. Secondary metabolite screening in various plants was reported by many workers (Siddiqui *et al.,* 2009; Chitravadivu *et al.,* 2009; Ashok Kumar *et al.,* 2010). Chemical complexity and lack of therapeutic markers are some of the limitations associated with the identification of genotype. Chemical profiling establishes a characteristic chemical pattern for a plant material. Identification of DNA markers that can co-relate DNA fingerprinting data with quantity of selected phytochemical markers associated with that of particular plant would have extensive applications in quality control of raw materials (Lee *et al.,* 1990; Sikorska *et al.,* 2001; Joshi *et al.,* 2004).

In pharmacognosy, chemoprofiling and marker compound analysis was carried out using modern analytical techniques such as fluorescence, UV-Vis, FTIR, TLC, HPLC, HPTLC and GC-MS. These have become firmly established as a key technological podium for secondary metabolite profiling in both plant and other species (Fernie *et al.,* 2004; Biren Shah, 2009). Gas Chromatography is a very good method for separating components of a mixture and mass spectrometry is a powerful technique for identifying components in a mixture. The combination of these two techniques may be considered as a natural marriage (Merlin *et al.,* 2009).

With the advancement in the separation and isolation of natural product chemistry scientists have shown that phytochemical constituents can be used to distinguish, illustrate and classify species into taxa. Correlations between traditional morphological and chemical classifications can be traced as early as 1699 (Fairbrothers, 1968). However, a real interest in the understanding of possible relationships between plant constituents and systematics is more recent. Interest in this aspect of systematics has increased with the development of rapid and

precise analytical techniques, and there is a consensus that data from as many sources as possible should be employed in plant classification (Stace, 1980; Datta, 1988). Evidence from chemical constituents has already led to the reconsideration of many plant taxa. For example a number of taxonomically difficult families have been successfully grouped on the basis of their secondary metabolite profiles. At lower taxonomic levels, several metabolites have proved useful in establishing taxonomic relationships (Waterman, 1998).

Molecular markers have provided a powerful new tool for breeders to search for new sources of variation and to investigate genetic factors controlling quantitatively inherited traits. The molecular approach for the identification of plant varieties/genotypes seems to be more effective than traditional morphological markers because it allows direct access to the hereditary material and makes it possible to understand the relationships between individuals (Paterson *et al.,* 1991; Sangwan *et al.,* 2001). References for genetic variation among medicinal plants are scanty although analysis of such variation holds great promise owing to the location specific attributes of the herbs and the attendant diversity of plant specific compounds. It is also now understood that the genetic variation within a given species is usually much more serious and occurs much earlier than the total extinction of the species itself (He and Sheng, 1997). For efficient conservation and management of the selected medicinal plants diversity, the genetic and phytochemical composition of species needs to be assessed. Based on morphology, it is very difficult to identify the medicinal plants in the form of crude drug and juvenile stage.

Genetic diversity among inter and intra-specific populations can be determined using morphological, phytochemical, cytological and molecular markers. Phenotypic characters have limitations since they are influenced by environmental factors and the developmental stage of the plant. Measurement of morphological traits alone may not serve as a useful criterion for

assessing genetic diversity of plant germplasm. The environmental influence on these traits may sometimes render this measure relatively insensitive particularly where differences are very small. However, several molecular and biochemical analyses make it possible to establish differences at various taxonomic levels which in turn helps the researchers to assess genetic diversity in the investigated germplasm (Vaughan, 1983; Rabbani *et al.,* 2010; Pervaiz *et al.,* 2010). Electrophoretic separation methods are increasingly playing an important role in genetic diversity analysis and conservation of plant genetic resources. These methods are being used as complementary strategies to traditional approaches for assessment of genetic diversity. This can be performed at any growth stage using any plant part and it requires only small amount of materials. SDS-PAGE is a practical and reliable method for species identification because seed storage proteins are largely independent of environmental fluctuation (Gepts, 1989). Molecular markers, based on DNA sequence polymorphisms, are independent of environmental conditions and show higher levels of polymorphism (William *et al.,* 1990). The basic premise is that variation in the nucleotide sequence of DNA can be exploited to produce characteristic fingerprints (Mace *et al.,* 1999). The barcode of life is a short DNA sequence from a uniform locality on the genome, which is used for identifying species (Schori and Showalter, 2011). DNA barcoding has rapidly achieved recognition as an important tool with the power to aid many basic research and applied endeavors in taxonomy and species identification (Savolainen *et al.,* 2005; Hajibabaei *et al.,* 2007).

In the present study three medicinally important *Plumbago* species were selected to reveal the inter and intra-specific variation. The roots of *Plumbago zeylanica* extract has antiplasmodial (Simonsen *et al.,* 2001), antimicrobial (Ahmad *et al.,* 2000), antifungal (Mehmood *et al.,* 1999), anti inflammatory and anticancer (Oyedapo, 1996), antihyperglycemic (Olagunju *et al.,* 1999),

hypolipidaemic and anti atherosclerotic activities (Sharma *et al.,* 1991). The aerial parts or roots of *Plumbago auriculata* is taken to treat black water fever (Dorni *et al.,* 2006). Various parts of *P. auriculata* contain the naphthoquinone plumbagin (2-methyl juglone), which blisters the skin. *P. auriculata* has insecticidal properties as an antifeedant and as a moulting inhibitor (Paiva *et al.,* 2003). Plumbagin is also a yellow pigment, occurring in a colourless combined form in the plant and is liberated by acid treatment. *P. auriculata* contains an antifungal protein that inhibits spore germination in *Macrophomina phaseolina* (Gangopadhyay *et al.,* 2011). *Plumbago rosea* also contains the napthoquinone plumbagin. Plumbagin possess several pharmacological activities i.e. antimicrobial, anticancer, cardiotonic, anti rheumatism and anti fertility actions (Lal *et al.,* 1993). It is also a powerful irritant. In small doses, the compound is asudorific and it stimulates the central nervous system; large doses may cause death from respiratory failure and paralysis, anti implantation and abortive activity. The ethanolic leaf extract of *P. rosea* is active against herpus simplex virus type I. The root of *P. rosea* was used to treat digestive problems, dyspepsia, colic cough and bronchitis (Dinda *et al.,* 1997).

In addition, previous studies on the *Plumbago* species were focused on the roots only, very few reports are available on the preliminary phytochemical analysis on *P. zeylanica* and aerial parts of the selected *Plumbago* species. To supplement the previous observations, an attempt has been made to reveal the phytochemical properties of the aerial parts of three *Plumbago* species and find out the inter-specific and intra-specific variation among the selected three species of *Plumbago* using preliminary phytochemical screening, spectroscopic, chromatographic and electrophoretic (protein and DNA) analysis. These phytochemical and molecular analyses of intra and inter-specific variation in particular may find application in resolving disputes of taxonomic identities, relations and authentication of the species in the pharmaceutical industries.

With this knowledge the present investigation aims to reveal the phytoconstituents present in the aerial parts of medicinally important *Plumbago* species viz., *P. zeylanica* L. *P. auriculata* Lam. and *P. rosea* L. In addition it also aimes to assess the variation at genus and species level (inter and intra-specific) among the selected three *Plumbago* species (*P. zeylanica, P. auriculata* and *P. rosea*) using phytochemical (UV-Vis qualitative analysis, FTIR analysis, TLC, HPLC, HPTLC and GC-MS analysis. The biochemical and molecular variation among the selected three species was studied using SDS-PAGE, MALDI-TOF MS and DNA barcoding.

Variations among the individuals belonging to the same species are accumulated over the millennia and the mutations are considered as the real reasons for the process of evolution (Rafi, 2010) Variation in medicinal plants is often noticed at chemical level which is due to the synthesis and accumulation of a wide variety of biochemicals that are often plant-specific. These compounds collectively grouped as secondary metabolites are 'high-value, low-volume' compounds biosynthetically derived from primary metabolism which help to defend, tolerate, adapt and adjust themselves against abiotic and biotic stresses including insect pests, fungal and other pathogenic diseases. Some of these agents can also act within the human body against various microorganisms and other causes of disease (Arunachalam, 2009).

Plant-derived substances have recently become a great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al.,* 2008). Plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80%. Angiosperms are the most diverse group of the plant kingdom comprising of about 4, 20, 000 species in 440 families (Schippmann *et al.,* 2002). Flowering plants are by far the most numerous, diverse and successful extant plant group containing well over 95% of all land plant species alive today (Simpson, 2006). Of the 4, 20,000 higher plant species on earth, more than 80,000 are medicinal. India is one of the world's 12 mega biodiversity centres with the presence of over 45,000 different plant species. India's diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes (habitats of specific

species). Of these, about 15,000 - 20,000 plants have good medicinal value. However, traditional communities use only 7000 - 7500 species for their medicinal values (Joy *et al.,* 2003; Kubmarawara *et al.,* 2007; Krishnaiah *et al.,* 2009).

Pharmacognosy is a simple and reliable tool by which complete information of the crude drug can be obtained (Mukherjee, 2002; Trease and Evans, 2002). Chemosystematics has been used to distinguish plants and other organisms. This knowledge has led to insights into taxonomy of these plants, animals and micro-organisms. Advances in analytical instrumentation, in particular chromatography, followed by electronic detection methods, have speeded these studies, culminating in metabolic profiling, "metabolomics" (Reynolds, 2007). In pharmacognosy, the phytochemical assessment is one of the important and vital tools for quality assessment, which includes preliminary phytochemical screening, fluorescence, UV-Vis, FTIR, TLC, HPLC, HPTLC and GC-MS analysis.

Spectroscopic methods have become firmly established as a key technological platform for differentiating, classifying and discriminating closely related plants (Lu *et al.,* 2004; Bisht *et al.,* 2009; Biren, 2009). Spectroscopic (UV-Vis, FTIR) methods together or separately can be used in this sense as well as predictable methods for detection of biomolecular composition (Socaciu *et al.,* 2005; Schultz *et al.,* 2007; Ibrahim *et al.,* 2008; Fernanda *et al.,* 2012). Fourier Transform Infrared Spectroscopy (FTIR) is a rapid, non invasive, high-resolution analytical tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular fingerprint (Langergraber *et al.,* 2002; Ellis *et al.,* 2003; Baranska and Schultz, 2006; Kaiser *et al.,* 2012).

Chromatographic screening methods could provide the needed chemical and pharmacological investigations of various medicinal plants (Aparicio *et al.,* 2000). TLC is a type

of liquid chromatography in which the stationary phase is a thin (~ 0.25 mm) uniform coating of a solid fine material spread, on a glass plate, aluminium foil, or plastic sheet (Sherma and Fried, 2005; Zhang *et al.,* 2008). TLC has the special ability to assay many samples at the same time on a single plate (Li *et al.,* 2004; Fodor *et al.,* 2006; Talukdaar *et al.,* 2010; Mohammad *et al.,* 2010; Gujjet *et al.,* 2013). It is recommended as an effective method for identification of plant derivatives by Chinese, American and European Pharmacopoeias (Fuzzati, 2004). HPTLC has been widely used for the phytochemical evaluation of the herbal drugs, due to its simplicity and minimum sample clean up requirement (Vinatoru, 1997). HPTLC has become a routine analytical technique due to its advantages of reliability in quantification of analytes at micro and nanogram levels (Pant and Rajasekaran, 2011). Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity (Gupta *et al.*, 1996, 1999; Saxena *et al.*, 2000; Srivastava *et al.*, 2000; Sasikumar *et al*., 2010; Khan *et al.,* 2009).

High performance liquid chromatography (HPLC) is an important qualitative and quantitative technique, generally used for potency/purity/performance assays, pharmacokinetics / bioanalytical testing, purification, high-throughput screening (HTS), In Process Control (IPC) monitoring, Quality Control (QC) testing, estimation of pharmaceutical and biological samples (Snyder *et al.,* 2009; Ahuja *et al.,* 2010; Guillarme *et al.,* 2012). It is the most versatile chromatographic technique for the quality control of drug components (Chen *et al.,* 2007; Li *et al.,* 2011; Ranjith *et al.,* 2013; Wang *et al.,* 2013).

The determination of phytoconstituents is largely performed by relatively expensive and often laborious techniques such as gas (GC) and liquid (LC) chromatography combined with specific detection schemes (Uzer *et al.,* 2005; Eisenhauer *et al.,* 2009). Analysis of small

amounts of chemicals has become easier and more cost-effective owing to the development of hyphenated chromatographic techniques such as GC or LC-MS. GC-MS analysis can identify pure compounds present at less than 1 ng (Liebler *et al.,* 1996; Adams, 2004; Delazar *et al.,* 2009). In the last few years, GC-MS has become firmly established as a key technological platform for secondary metabolite profiling in both plant and non-plant species (Delazar *et al.,* 2004; ; Fernie *et al.,* 2004; Robertson, 2005; Kell *et al.,* 2005; Abdelwahab *et al.,* 2009; Ilavenil *et al.,* 2010; Suresh *et al.,* 2010).

Phytochemical constituents may also reflect the genetic diversity or epigenetic responses or both. Integration of chemotype fingerprinting with genotype driven molecular technique is necessary for optimal characterization of plant species (Lee *et al.,* 1990).

Since the 1930s, electrophoresis in conjunction with the zymogram technique has been used as a tool for the study of heritable variation. Proteins are widely used because of their relative efficiency and cost effectiveness, particularly in studies of intra and inter-specific variation (Sabu *et al.,* 2001 and Smila *et al.,* 2007). Proteins are practically useful genetic and biochemical markers as well as good estimators of genetic variability in plant populations (Hamrick and Godt, 1997). Recently, studies on protein and isoenzyme homologies have been carried out along with the improved biochemical techniques. The analysis of protein patterns has become a useful tool for the investigation of variations in plant populations (Onus and Pickergill, 2000; Zeildler, 2000; Siva and Krishnamurthy, 2005).

Molecular markers are more promising tools to study genetic variation as any change in the protein sequence would be brought about by a mutation in its DNA sequence. DNA markers are not typically influenced by environmental conditions and therefore can be used to help describe patterns of genetic variation among plant populations and to identify duplicated

accessions within germplasm collections (Sen and Sharma, 1982; James and Ashburner, 1997; Ram *et al.,* 2008). The phenotypic correlation between any two characters is the net result of both genetic and environmental correlation between these characters (Adams, 1991). During the last twenty years, the classical methods to evaluate genetic diversity have been complemented by molecular techniques. Molecular markers consist of DNA segments at specific portions of chromosomes or their products (proteins). Proteomic studies are considered more reliable than morphological or cytological evidences (Yadav, 2008). Proteins are physiologically stable and easy to handle (Ghafoor and Arshad, 2008). They operate at the level of gene product where the environment has very little influence (Yousaf *et al.,* 2008; Khalid *et al.,* 2012). Protein electrophoresis is increasingly being utilized as an additional approach for species identification and as a useful tool for tracing back the evolution of various groups of plants (Sher *et al.,* 2010; Bhat and Kudesia, 2011).

Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF MS) has been suggested as a fast and reliable method for plant species identification, based on the characteristic protein profiles for each plant species. Using this technology it has been estimated that up to 99% of species tested are correctly identified when comparing with commercial phenotypic identification panels or gene sequencing (Seng *et al.,* 2009; Bizzini *et al.,* 2010; Cherkaouii *et al.,* 2010; Giebel *et al.,* 2010). MALDI-TOF analysis is extremely fast with regard to data acquisition, requires little expertise, tolerant to contaminants such as salts and detergents, easy to automate and allows the analysis of large number of samples in a short period of time. Protein identification relies purely on matching of the peptide masses accurately, and it can be relatively inexpensive (Lambert *et al.,* 2005; Karlova *et al.,* 2006; Schulze and Usadel 2010; Kaspar *et al.,* 2011; Pflieger *et al.,* 2011).

DNA barcoding uses short regions of DNA to identify species by assigning individuals to known taxa through comparison of their barcodes with a reference library. DNA barcoding will provide a quick, simple and economic tool for identifying and discovering biological diversity. Ideally, a DNA barcode would require only small amounts of possibly poor quality tissue to be easily amplified and sequenced using universal primers. DNA barcoding provides rapid species identification; its accuracy relies on PCR technology by using a standardized DNA region as a tag (Hebert and Gregory, 2005; Savolainen *et al*. 2005; Kress and Erickson, 2008; Gao *et al.,* 2010; Arca *et al.,* 2012; Cai *et al.,* 2012; Yang *et al.,* 2012). *rbc*L has been suggested as a primer for plant barcoding, even though it has generally been used to determine evolutionary relationships at the generic level and above (Luo *et al.,* 2010; Groot *et al.,* 2011; Du *et al.,* 2011).

Phytochemical properties

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties (Raj, 2010). Plants produce these chemicals to protect themselves, but recent research demonstrates that many phytochemicals can protect humans against diseases (Kubmarawa *et al.,* 2008). According to Pawar *et al.* (2010), the physiological action on the human body can be determined by phytochemicals. Many of the plant extracts have proven to possess pharmacological action due to the presence of various phytochemicals.

Various phytochemical, pharmacognostical and pharmacological studies were carried out on *Plumbago* species. Subhash *et al*. (2012) revealed the presence of cardiac glycosides, alkaloids, tannins, steroids, flavonoids, saponins and anthraquinones in the ethanolic extract of *P. zeylanica* collected from Alagar hills, Madurai. Devi *et al.* (2012) determined the extractive and ash value on dried root, stem and leaves of *P. zeylanica* from Tirumala, Andhra Pradesh. Saha *et al.* (2012) studied the fluorescence characters in the methanolic extracts of *P. indica* (*P. rosea*)

under visible and UV light by treatment of various chemical reagents collected from Chittagong, Bangladesh. Rao *et al.* (2012) carried out preliminary phytochemical analysis in the petroleum ether, benzene, chloroform, ethanolic and aqueous extracts of *P. zeylanica*. Ethanolic extracts of *P. zeylanica* revealed higher number of phytoconstituents compared to other extracts. Kanungo *et al.* (2012) estimated the primary and secondary metabolites present in the ethanolic extracts of *in vivo* and *in vitro* grown *P. zeylanica* leaves collected from Odisha, India.

Ibrahim *et al.* (2012) determined the presence of alkaloids, steroids, saponins and reducing sugar in the methanolic extract of *P. indica* collected from Naramuk, Bangladesh. Lenora *et al.* (2012) compared the chemical profile of *in vitro* and *in vivo* grown roots of *P. indica* collected from India and Srilanka using preliminary phytochemical methods. Ajayi *et al.* (2011) identified the phytochemical constituents present in the ethanolic extract of *P. zeylanica* and showed the presence of alkaloids, tannins, steroids, flavonoids, saponins, anthraquinones, cardiac glycosides, phlobatannins and carbohydrates. Ravikumar and Sudha (2011) analysed the preliminary phytochemical compounds in petroleum ether, ethanolic and aqueous extracts of stem and leaves of *P. zeylanica* collected from Anaikatti hills, Coimbatore. Kodati *et al.* (2011) performed preliminary phytochemical analysis in the petroleum ether, chloroform, methanolic and ethanolic extracts of *P. zeylanica* root collected from Waranghi, India. Arunachalam *et al.* (2010) screened the phytochemicals in dichloromethane extract of *P. zeylanica* root from Katankulathur, Tamil Nadu and confirmed the presence of terpenoids and flavonoids. Kumar *et al.* (2009) screened the phytoconstituents present in the petroleum ether, hexane, chloroform and methanolic extracts of aerial parts of *P. zeylanica*. Raj *et al.* (2008) studied the preliminary phytochemical analysis on the methanolic root extracts of *P. zeylanica* collected from SRM University Campus, Kattankulathur.

A wide range of researches have been conducted to study the UV and IR spectral analysis in various *Plumbago* species. Cong *et al.* (2013) elucidated eleven new guanidine alkaloids, plumbagines and plumbagosides from the aerial parts of *P. zeylanica* using 2D-NMR, IR and MS spectroscopic studies. Subash *et al.* (2012) confirmed the presence of flavonoids in the ethanolic extracts of *P. zeylanica* using FTIR spectroscopic study. Skaar *et al*. (2012) isolated six new anthocyanins from *P. auriculata* flowers. Their structures were identified by 2D NMR and high-resolution MS. Lenora *et al.* (2012) compared the chemical compounds and quantified the plumbagin content in *P. indica* grown under *in vitro* and *in vivo* grown plants. Maximum plumbagin content was observed in roots of *in vitro* field grown plants followed by roots of *in vivo* hydroponically grown plants and callus samples. Among the different growing systems of *P. indica*, maximum plumbagin content was observed in roots of *in vitro* field grown plants. Kishore *et al.* (2012) isolated the novel naphthoquinone from the methanolic extracts of *P. zeylanica* roots. Their structures were determined by UV, IR, MS, H and C-NMR.

Bothiraja *et al.* (2011) isolated plumbagin from roots of *P. zeylanica* using cold maceration method. The cold maceration yielded 1.2% (w/w) fine crystalline orange needle of plumbagin. It showed melting point at 78.4°C, Rf value at 0.64 in n-hexane: benzene (1:9) solvent system, UV absorption maxima at 417 nm. Ariyanathan *et al.* (2010) isolated capensisone, isoshinanolone, diomuscinone, α-amyrin acetate, plumbagin, α-amyrin, β-sitosterol and β-sitosterol-3β-D-glucoside from the methanolic root extracts of *P. capensis*. Kishore *et al.* (2010) characterized difuranonaphthoquinones from *P. zeylanica* roots by spectral analysis (UV, IR, 1D and 2D NMR, MS) and identified naphthoquinones, lapachol, plumbagin, 2-isopropenyl-9-methoxy-1,8-di-oxa-di cyclopenta (b,g) naphthalene-4,10-dione, 9-hydroxy-2-isopropenyl-1,8-dioxa-dicyclopenta (b,g) naphthalene-4,10-dione, 2-(1-hydroxy-1-methyl-ethyl)-9-methoxy-1,8-

dioxa-dicyclopenta (b,g) naphthalene-4,10-dione and 5,7 -dihydroxy-8-methoxy-2-methyl-1,4-naphthoquinone from the roots of *P. zeylanica*. Nile and Khobragade (2010) quantified the phenolic and flavonoid content of *P. zeylanica* ethanolic root extracts using UV-Vis spectroscopic study.

Ariyanathan *et al.* (2010) isolated and identified two flavonoids and two carboxylic acids using IR, NMR and MS spectroscopic studies from the ethyl acetate extracts of *P. rosea*. Maniafu *et al.* (2009) isolated and determined various naphthoquinones from different extracts of *Plumbago* species viz., *P. zeylanica, P. dawei* and *P. stenophylla* using 1D and 2D NMR spectroscopic method. Higher concentration of compounds was identified in methanolic extract of *P. stenophylla* collected from Kibwezi, University of Nairobi. Chao *et al.* (2006) isolated and identified the quinones from *P. zeylanica* ethanolic extracts. Isolated orange needle-like crystals, according to physical and chemical properties, thin-layer chromatography and spectral data analysis revealed the presence of *P. zeylanica* quinone. Chuntaratin (2006) isolated and estimated plumbagin from the stems, leaves and root of *in vivo* and *in vitro* grown *P. indica*. The highest plumbagin content was observed in the roots followed by the stems and leaves of *Plumbago s*pecies. Nayana *et al.* (2005) isolated plumbagin from the roots of *P. indica* by an efficient method involving column chromatography. The compound was further confirmed by melting point data, UV, IR and mass spectral data. Nguyen *et al.* (2004) reported the bioassay-guided fractionation in aerial parts of *P. zeylanica* using dichloromethane extract. This led to the isolation of beta-sitosterol, beta-sitosteryl-3beta-glucopyranoside,beta-sitosteryl-3beta-glucopyranoside-6'-O-palmitate, lupenone, lupeol acetate, plumbagin and trilinolein. Paiva *et al.* (2003) isolated and identified naphthoquinones from the chloroform root extract of *P. scandens* using ^{1}H and ^{13}C NMR method.

TLC enables reliable separation and analysis of compounds from a wide variety of classes in many types of biological samples (Sherma and Fried, 2005). Lenora *et al.* (2012) identified the presence of the naphthoquinone plumbagin in the root extracts of conventionally field grown, tissue cultured field grown and *in-vitro* developed callus from leaf explants. Maximum plumbagin was present in tissue cultured field grown *P. indica*. Gupta *et al.* (2010) evaluated the phytochemical and TLC profile on the root of *P. zeylanica*. Phytochemical analysis of *P. zeylanica* root exhibited the presence of terpenes, glycosides and sugars. TLC studies on petroleum ether extracts of *P. zeylanica* using the mobile phase benzene: methanol (19:1) as mobile phase showed nine spots. In chloroform extracts, eight spots were identified using benzene: ethyl acetate (4:1) as mobile phase and in ethanolic extracts only four spots was observed using chloroform: methanol (93:7) as mobile phase. Kanchana and Sadiq (2011) isolated and determined plumbagin from the petroleum ether root extract of *P. zeylanica* collected from Adhiparasakthi Agricultural College, Kalavai. Maniafu *et al.* (2009) isolated and determined various naphthoquinones from different extracts of *Plumbago* species viz., *P. zeylanica, P. dawei* and *P. stenophylla* using TLC method. Higher concentration of compounds was identified in methanolic extracts of *P. stenophylla.* Yogananth and Basu (2009) identified the presence of the bioactive compound plumbagin in the hairy root culture of *P. rosea* using TLC method. Nandhini and Raj (2008) identified the presence of bioactive compounds in methanolic extract of *P. zeylanica* using TLC method and demonstrated the Rf value of 0.678 and 0.565. Jeyachandran *et al.* (2009) isolated and separated plumbagin from the chloroform extract of *P. zeylanica* by TLC method using chloroform: methanol as a mobile phase and ethanolic KOH solution as a spraying reagent. Meyer *et al.* (2007) identified the chemical compound plumbagin from the dichloromethane extract of *P. auriculata* by TLC method using

hexane: ethyl acetate (5:2) as mobile phase. Panichayayupakaranant and Tewrakul (2002) determined the plumbagin content in the crude methanolic root extracts of *P. rosea* using TLC densitometric method.

Several chromatographic methods have been reported to estimate the various phytocompounds in *Plumbago* species; of which the HPLC was adopted for the quantitative estimation of plumbagin and other phytoconstituents present in the roots and leaves extracts of *P. zeylanica*. In addition, the chemical profile of *P. zeylanica* collected from Tirupathi, Andhra Pradesh was quantified by HPLC (Rao *et al.,* 2012). Kaewbumrung and Panichayupakaranant (2012) isolated three naphthoquinones viz., plumbagin, 3, 3'-biplumbagin and elliptinone from *P. indica* roots collected from Prince of Songkla University, Thailand. RP-HPLC was also established for the simultaneous determination of the naphthoquinones in *P. indica* methanolic root extracts. Muhammad *et al.* (2009) extracted and quantified plumbagin from ethyl acetate root and leaves extracts of *Plumbago europaea* using HPLC analytical column. Meyer *et al.* (2007) estimated the chemical compound plumbagin from the dichloromethane *P. auriculata* extract by HPLC method using 62:55 acetonitrile and 5% aqueous acetic acid as mobile phase. Gopinath *et al.* (2009) quantified the plumbagin content in various extracts of *P. zeylanica* using HPLC and found that maximum percentage of plumbagin was found in methanolic extract of *P. zeylanica* compared to other extracts. Aphacha *et al.* (2007) estimated the plumbagin in the aerial parts and roots of wild and propagated *P. indica* using HPLC analysis. They observed higher plumbagin content in the root extracts of wild *P. indica* compared to the wild and in-vitro grown *P. indica.* Rodrigues *et al.* (2006) isolated and determined the solubility of 1,4-naphthoquinone, plumbagin, lawsone and juglone from *P. scandens* using supercritical carbon dioxide and spectroscopic methods.

Liu *et al.* (2006) used HPLC method for determination of plumbagin in *P. zeylanica*. Yuan *et al.* (2006) determined the plumbagin content in the ethanolic extract of *P. zeylanica* collected from different areas using HPLC method. The concentration range was 0.0104-0.3328 µg of plumbagin in the ethanolic extract of *P. zeylanica.* Wang and Huang (2005) quantified and compared the plumbagin content in the ethyl acetate extract of *P. zeylanica* from 13 different regions of China. Paiva *et al.* (2003) identified four different napthoquinones viz., plumbagin, *epi*-isoshinanolone, palmitic acid and sitosterol by four different extraction methods in *P. scandens* using HPLC method. Higher plumbagin content was observed in methanolic extract compared to other extraction method. Satheesh Kumar and Seeni (2002) quantified the level of plumbagin in the callus and cell suspension cultures of *P. indica* using HPLC method.

An accurate, precise and specific HPTLC method was developed to quantify the chemical marker plumbagin by Patel *et al*. (2008). Saraswathy *et al.* (2011) studied the variation in the plumbagin content in *P. zeylanica* due to change in season using HPTLC. They observed more concentration of plumbagin constituent during February followed by January. The content was found to be very less in April. Sasikumar *et al*. (2010) evaluated the plumbagin content of *P. zeylanica* roots procured from Trichy and Chennai market as well as fresh samples collected from gardens at Chennai using HPTLC analysis. The results revealed that the sample collected from Trichy market had produced higher plumbagin content.

Pawar *et al.* (2010) determined the plumbagin in different samples of *P. zeylanica* chloroform root extract collected from Ghaziabad, Uttar Pradesh, India by HPTLC method using Toluene: Ethyl acetate (3:1) v/v as mobile phase and anisaldehyde – sulphuric acid as spraying reagent. The chloroform extract of *P. zeylanica* root samples were quantified using UV detector at wavelength of 270 nm. The content of marker compound plumbagin was found similar in the

chloroform root extract of *P. zeylanica*. Lin *et al.* (2003) isolated two plumbagic acid glycosides (3′-O-β-glucopyranosyl plumbagic acid and 3′-O-β-glucopyranosyl plumbagic acid methylester), naphthoquinones (plumbagin, chitranone, maritinone, elliptinone and isoshinanolone) and five coumarins (seselin, 5-methoxyseselin, suberosin, xanthyletin and xanthoxyletin) from the roots of *P. zeylanica* using HPTLC method. Verma *et al.* (2002) identified the presence of plumbagin in *A. rhizogenes* mediated hairy root cultures of *P. zeylanica* using HPTLC method.

Ajayi *et al.* (2011) confirmed the presence of alkaloids, tannins, steroids, flavonoids, saponins, anthroquinones, cardiac glycosides, phlobatannins and carbohydrates in the ethanolic root extract of *P. zeylanica*. They identified the major compound phenol,2,4-Bis(1,1-dimethylethyl) with RT 6.796% using GC-MS analysis. Ming *et al*. (2011) isolated the chemical constituents from *P. zeylanica* by various column chromatographic methods and their structures were elucidated as plumbazeylanone, plumbagic acid, β sitosterol, lupeol, lup-20(29)-en-3,2 1-dione, norcanelilline, 3-O-glucopyranosyl plumbagic acid methylester, uridine and daucosterol. Paiva *et al.* (2004) identified the presence of plumbagin in the chloroform extract of *P. scandens* collected from Rio de Janeiro, Brazil using GC-MS method.

Various pharmacological studies have been reported on different *Plumbago* species viz., anti fertility (Sheeja *et al.,* 2009; Gupta *et al.,* 2011), anti-diabetic (Vijay Kumar 2006; Sunil *et al.,* 2012), anticancer and anti tumour activity in *P. zeylanica* (Lin *et al.,* 2003; Nguyen *et al.,* 2004; Tilak *et al.,* 2004; Zahin *et al.,* 2009; Chen *et al.,* 2009; Nazeem *et al.,* 2009; Hirangi *et al.,* 2011; Poosarla *et al.,* 2011). Devi *et al.* (1994) and Solomon *et al.* (1993) revealed the anticancer potential of *P. rosea*. Antioxidant properties of *Plumbago* species were also studied by many workers (Siddiqui *et al.,* 2011; Nile and Khobragade, 2010; Vishnukanta and Rana, 2010; Maryam *et al.,* 2009; Jeyachandran *et al.,* 2009). Pharmacological studies also confirmed

the anti ulcer (Kakjing *et al.,* 2012), antihelminthic (Desai *et al.,* 2012), anti sickling (Olufunmilayo *et al.,* 2010), anti inflammatory (Kantha *et al.,* 2010), anti leishmanial (Bhuwan *et al.,* 2012) and dermatotoxic (Teshome *et al.,* 2008) properties of *Plumbago* species.

UV-Vis spectroscopic analysis on ethanolic extract of *Gymnema kollimalayanum* was evaluated and the peak values were 413.77, 469.15 and 664.43 and also studied the electron transition due to OH group (Ramachandran and Viswan, 2011). Sethiya *et al.* (2010) investigated the comparative pharmacognostical and phytochemical properties of petroleum ether, benzene, chloroform, ethyl acetate, ethanol and aqueous extract of medicinally important plants viz., *Evolvulus alsinoides, Convolvulus pluricaulis, Canscora decussata* and *Clitoria ternatea.* Umamaheshwari *et al.* (2010) determined the preliminary phytochemical analysis in the chloroform, ethanol and ethyl acetate extract of *Boerhaavia diffusa*. The results revealed the presence of proteins, carbohydrates, glycosides, furanoids, tri terpenoids, flavonoids and amino acids in *B. diffusa* and maximum numbers of phyto constituents were present in the ethyl acetate extract of *Boerhaavia diffusa* species. Sule *et al*. (2010) revealed the phytochemical analysis in the methanolic, ethanolic and petroleum ether extracts of *Senna alata* leaves. The results obtained from the biochemical analysis determined the presence of maximum number of phytoconstituents in the ethanolic extract of *S. alata* which showed the presence of alkaloids, saponins, tannins, anthraquinones and carbohydrates.

Mishra *et al.* (2010) reported a new anthraquinone, 1-methyl-2-(3′-methylbut-2′-enyloxy)-anthraquinone from the seeds of *Aegle marmelos* and it was characterized on the basis of spectral analysis (UV, IR, 1H NMR, 13C NMR, 2D NMR and mass spectroscopy). Kumar and Prasad (2010) used the FTIR technique to understand the composition, chemical structure and discrimination of bio molecules in *Tephrosia tinctoria* and *Atylosia albicans.* IR spectrum in

the mid infrared region (4000-400 cm^{-1}) was used for discriminating and identifying various functional groups present in two different species of medicinal plants belonging to the family Leguminosae. The results showed that *T. tinctoria* and *A. albicans* are rich in phenolic compounds. FTIR analysis of ethanolic extract of *G. kollimalayanum* confirmed the presence of the carboxylic acid and alkenes-CH_2; CH_3 aromatic stretching the major peaks at 1019.87 and 2922.33 cm (Ramachandran and Viswan, 2011). Maoela *et al.* (2009) reported the UV-Vis absorption spectra of *Coprinus mellei* and *Coprinus quadrifidus* overlap with that of catechin, which confirms the presences of the catechin. The UV-Vis absorption spectra of *C. mellei* and *C. quadrifidus* showed two absorption bands, a strong one at 218 nm (Band II) and a weak one at 282 nm (Band I). Muruganantham *et al.* (2009) determined the functional groups present in the leaves, stem and root of crude powder of *Eclipta alba* and *Eclipta prostrate* using FTIR spectrophotometry.

Anubhati and Pratibha (2013) collected five different varieties of *Cyamopsis tetragonaloba* (RGC-936, 1002, 1003, 1031 and 1017) from Krishi, Banasthali and demonstrated the intra-specific variation by quantifying the polyphenols using HPLC analysis. Sharma *et al*. (2012) compared and quantified iricoid glycosides viz., picroside I and picroside II in *Picrorhiza kurroa* collected from North Indian higher altitude Himalayas to study the intra-specific phytochemical variation using HPLC method. Jeetrandra *et al.* (2009) determined the oleanolic acid in methanolic extract of *Achranthes aspera* roots and leaves using toluene: ethyl acetate: formic acid (4.5:0.5:0.1) as mobile phase. The oleonoic acid concentration was found to be 0.37% and 0.13% w/w in roots and leaves. Gopinath *et al.* (2009) identified the embelin content in various extracts of *Embelia ribes* using HPLC method. Among these soxhlet methanolic extract produced higher concentration of embelin compared to other extracts. Raghu *et al.* (2007)

made an attempt to analyze the variability among five accessions of *Tribulus terrestris* collected from different geographical regions of South India using HPLC (Malappuram, Kerala; Coimbatore, Tamil Nadu; Palakkad, Kerala; Erode, Tamil Nadu and Thrissur, Kerala). All the five accessions showed different levels of morphological and phytochemical variability. The leaf area showed the maximum morphological variability. In phytochemical analysis**,** the accession collected from Malappuram, Kerala, showed the highest chemical affinity (70.58%) compared to the accession from Coimbatore. Among the six morphometric characters studied, all the characters except inter-nodal length showed significant positive correlation between them. Olszewska and Wolbis (2002) isolated and identified two new flavonol glycosides, quercetin 3-o-(2-o-D-glucopyranosyl)-a-L-arabinofuranoside and kaempferol 3-O-(2-O-E-Pcoumaroyl)- a-L-arabinofuranoside-7-o-a-L-rhamnopyranoside from the leaves of *Prunus spinosa* using HPLC.

Selvamaleeswaran *et al.* (2013) studied the alkaloid content in the methanolic extract of *Clitoria ternatea* seed, stem and leaves by HPTLC method using ethyl acetate: methanol: water (100:13.5:10) as mobile phase. Alkaloid content was higher in seeds (10) followed by leaves (9) and stem (7). Ganatra *et al.* (2012) screened the phytochemicals using TLC method in different extracts of *Cyamopsis tetragonoloba*. Methanolic extract of *C. tetragonoloba* reported more number of compounds compared to other extracts. Different solvent systems chloroform:n-hexane (7.5:2.5), ethyl acetate:methanol (1:1), Chloroform:Glacial acetic acid:Methanol:Water (60:32:12:8) were used to differentiate the varied phytochemicals present in different extracts of *C. tetragonoloba.*

Devi *et al.* (2012) reported the phenolic compound ursolic acid in the ethanolic extract of *Clerodendrum inerme* by HPTLC method using the mobile phase toluene: ethyl acetate: formic acid (9:1:0.5) at 254 nm and 366 nm. Gambhir *et al.* (2011) quantified the vitamin C content in

methanolic extract of *Pithecellobium dulce* by HPTLC method using ethanol: water (2:1) as mobile phase. Sarkar *et al.* (2011) carried out TLC in the chloroform extract *of Psidium guajava* leaves extracts. Gupta *et al.* (2011) recorded the TLC profiling in various extracts of *Ocimum gratissimum* using toluene: ethyl acetate (93:7) and cyclo hexane: ethanol: diethylamine (7:2:1) as mobile phase. Ushir *et al.* (2011) studied the phytoconstituents (quercitin, β-sitosterol, stigmasterol, catechin and ovatodiolide) present in two *Anisomeles* species (*A. indica* and *A. malabarica*) by HPTLC method using the mobile phase toluene: ethyl acetate: formic acid (5:4:1), chloroform: methanol (8:0.6), toluene: ethyl acetate: methanol (4:3:3) and toluene: ethyl acetate: formic acid (7:3.1) and categorized at 365 nm. Rajkumar and Sinha (2010) studied the chromatographic finger print analysis of the alkaloid budmunchiamines in *Albizia amara* by HPTLC method. The plates were developed using a mobile phase of chloroform: diethylamine (86:22 v/v) and characterized at 236 nm.

Paramasivam *et al.* (2009) determined the phytoconstituents curcumin, demethoxycurcumin and bis-demethoxycurcumin in *Curcuma longa*. The solvent system consisted of chloroform: methanol (48:2 v/v) at 425 nm. This system gave compact spots with Rf value of 0.66, 0.48 and 0.30 for curcumin, demethoxycurcumin and bisdemethoxycurcumin respectively. Rakesh *et al.* (2009) quantified the compound quercetin in the hydroalcoholic extract of dried flowers of *Nymphaea stellata* using toluene: ethyl acetate: formic acid (5:4:0.2) as mobile phase. Vijayalakshmi *et al.* (2012) recognised the presence of gallic acid in the ethanolic root extract of *Aerva lanata* and *Diospyrus ferrea* by employing HPTLC profiling using Toluene - ethyl acetate - formic acid - methanol (3:3:0.8:0.2) as mobile phase at 277 nm. Sharma *et al*. (2012) quantified the compounds hyoscyamine and scopolamine in the methanolic extract of *Datura metal.* Densitometric evaluation was achieved by using chloroform: acetone:

diethyl amine (50:40:10 v/v/v) as a mobile phase at 530 nm. Tripathi *et al.* (2006) estimated the compound phyllanthin and hypophyllanthin in the hexane, chloroform, ethyl acetate and methanolic extract of *Phyllanthus* species using HPTLC method and eluted with the mobile phase hexane: acetone: ethyl acetate (74:12:8) and visualised at 580 nm. Among all the *Phyllanthus* species methanolic extract revealed maximum amount of phyllanthin and hypo phyllanthin content compared to other extracts of *P. amarus.*

Rosy and Rosakutty (2012) compared the phytoconstituents present in methanolic extract of *Cissus xavierensis, C. quadrangularis* var. *rotundus* and *C. vitiginea* by GC-MS analysis. The major compound present in *C. xavierensis* was hexane (peak area. 35.09%), *C. quadrangularis* var. *rotundus* was 1, 3, 2-oxazaborolane (peak area. 14.29%) and *C. vitigenea* was Dibutyl phthalate, 1,2-benzenedicarboxylic acid (peak area. 16.18%). Naher *et al.* (2012) compared the essential oils present in two varieties of the Aniseed (*Pimpinella anisum*) in Bangladesh using GC-MS analysis. The oil rich compound cis-Anethole (69.404%) was present in higher concentration. Mohan *et al.* (2012) carried out GC-MS analysis on the ethanolic extract of *Acalypha indica.* Gyawali and Kim (2012) identified the compounds viz., camphor, borneol, capric acid, furfural, myrtanal, β-pinene, β-terpeniol, perillaldehyde, 2-carene, butyrophenones, furfural, β-caryophyllene, 2-nitropropane using GC-MS analysis in the methanolic extract of *Asparagus racemosus, Bergenia ciliate* and *Terminalia chebula.*

Aziz *et al.* (2012) identified and compared the oily compounds present in two varieties of ginger (*Zingiber officinale*) collected from Chittagong using GC-MS analysis. Ezhilan and Neelamegam (2012) compared the phytocompounds present in *Polygonum chinense* collected from Mekkari, Chenkottai Taluk, Tamil Nadu. The most prevailing compounds were squalene, a triterpinoid compound (47.01%). Gopalakrishnan and Vadivel (2011) carried out the GC-MS

analysis in the ethanolic extract of *Mussaenda frondosa* and identified the presence of eight different compounds.

Azizi *et al.* (2009) revealed the intra-specific variation among *Origanum vulgarae* collected from 42 accessions using GC-MS analysis. Totally, 42 constituents were identified for the essential oil: 36 constituents for *O. vulgare* ssp. *hirtum*, 30 constituents for *O. vulgare* var. *creticum* and 27 constituents for *O. vulgare* var. *samothrake.* Carvacrol content was the dominant constituent of the essential oil for all three populations tested, ranging from 70.0% to 77.4%. Din *et al.* (2009) collected five locally available *Solanum nigrum* complexes (*S. americanum, S. chenopodioides, S. nigrum, S. villosum* and *S. retrofexum*) from Botanical Garden of GC University Lahore, Pakistan. The compounds present in them were identified using GC-MS analysis to study the chemotaxanomic significance among the selected *Solanum* species. The significant distance found between *S. americanum, S. chenopodioides, S. nigrum* and *S. villosum* indicated them as distinct species. But *S. retrofexum* did not show such a marked difference and hence might be regarded as a variety or subspecies of *S. nigrum.* Uma *et al.* (2009) identified 38 different compounds in the methanolic extract of *Cinnamon zeylanicum* by GC-MS analysis. The studies on the active principles content in *C. zeylanicum* by GC-MS shows the presence of 38 components. The most identified compound was monoterpenes, sesquiterpenes, aromatic aldehydes and ketones. Cinnamaldehyde was identified as the major compound (peak area 68.41%), followed by benzaldehyde.

Variation study

Variety and variability are the essence of life. It forms the basic characteristic of living things. Among the innumerable number of species available on the phase of the earth, variation is the noticeable one. Apart from species richness, variations among the individuals belonging to

the same genus are observed over the millennia and were considered real reasons for the process of evolution (Bharat, 1997). Apart from visible variations, the synthesis, accumulation of species-specific chemicals make the phenomenon a further complex one. Considerable work has been carried out to correlate DNA markers with phytochemical compositions among closely related species (Fico *et al.*, 2003; Li *et al.*, 2003). Merging of these profiles will certainly help in developing a comprehensive understanding of a species.

Phytochemical variation study on *Plumbago* species

Identification and characterization of active principles in medicinal plants can be used in generating a species specific fingerprint (Anandjiwala *et al.,* 2007). Therefore, the current trend on chemotype-based fingerprint to support the genotype based molecular markers helps in the proper structuring of a species beyond any level of ambiguity (Fico *et al.,* 2003; Li *et al.,* 2003). Variation in chemical profile is highly subjected to extrinsic factors such as cultivation, harvesting, drying and storage conditions. Therefore the marker based on secondary metabolites should be able to discriminate one species from another species and one accession from other accessions. Such markers may not necessarily be a therapeutically important active principle. Any gene product that is neutral and unaffected by environmental changes can function as a phytochemical marker (Joshi *et al.,* 2004). In early 1960s, scientists were interested in exploring the variation among plants based on their secondary chemical compounds. Various techniques has been employed in detection and quantification of chemicals. Stebbins *et al*. (1991) used chromatographic evidence to study the tetraploid *Viola quercetorum* hybrid derivative of the cross between the diploid taxa *V. purpurea* subsp. *purpurea* and *V. aurea* subsp. *mohavensis*. Bose and Frost (1967) detected phenolic compound variations in *G. pubescens* and *G. speciosa* and their presumed polyploid derivative *G. tetrahit*.

Perusal of literature regarding phytochemical variation studies on *Plumbago* species have been attempted by different workers. Gala *et al.* (2012) compared the anatomical features of leaves, stems and roots of *P. auriculata, P. indica* and *P. zeylanica* using light microscopy. Ariyanathan *et al.* (2010) analyzed the physico chemical parameters of roots of three *Plumbago* species, *P. capensis*, *P. rosea* and *P. zeylanica*. Attempt has also been made to estimate the biologically active chemical plumbagin present in them. They observed that the plumbagin content was in decreasing order *P. rosea* (0.17%), *P. auriculata* (0.04%) and *P. zeylanica* (0.01%). These studies also revealed the presence of some phytoconstituents in the ethanolic extract of *Plumbago* species and found that *P. zeylanica* showed maximum percentage (12.83) of phytoconstituents followed by *P. auriculata* (9.14%) and *P. rosea* (5.57%).

Israni *et al.* (2010) determined the plumbagin content in the roots of *P. zeylanica, P. capensis* and *P. rosea* employing the UV-Vis spectrophotometric analysis. The study revealed that the bioactive marker was found to be maximum in *P. rosea* (0.424) followed by *P. zeylanica* (0.416) and *P. capensis* (0.281) at 520 nm. The plumbagin content in the roots of *P. zeylanica* and *P. indica* were analysed using RP-HPLC method and HPTLC method using hexane: ethyl acetate (8:2) as the mobile phase at 265 nm. The content of plumbagin in *P. indica* and *P. zeylanica* was found to be 0.2001 and 0.1601% respectively (Unnikrishnan *et al.* 2008). Dorni *et al.* (2007) compared the quantity of plumbagin present in the hydro alcoholic extract of three *Plumbago* species viz., *P. zeylanica, P. capensis, P. rosea* was 0.247%, 0.429% and 0.569% using HPTLC analysis. Paiva *et al.* (2004) identified the presence of four different compounds viz., plumbagin, epi-isoshinanolone, palmitic acid and sitosterol in various extracts of *P. scandens* and *P. auriculata* using GC-MS analysis. Among these, chloroform extract showed higher percentage of plumbagin compared to other extracts. Mallavadhani *et al.* (2002)

quantitatively screened the bioactive marker plumbagin by HPTLC in the selected species of *Plumbago* viz., *P. auriculata, P. zeylanica* and. *P. rosea*. They found the maximum plumbagin accumulations in the roots.

Kurian *et al.* (2001) collected 45 accessions of *P. rosea* from different parts of Kerala State and evaluated their variability in morphological and plumbagin content. Significant variation was evident for all the characters studied except leaves size indicating wide variability in the accessions. Arya *et al.* (1999) studied the genotypic and phenotypic coefficient of variation on *P. indica* collected from seven accessions and *P. zeylanica* from College of Horticulture, Vellanikkara, Kerala. The plant cuttings were treated with six doses of gamma rays (0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 kR) and M_1V_1 generation was evaluated. Pollen germination was not noticed in *P. rosea* whereas the pollen grains of *P. zeylanica* were germinated. All characters studied had significantly high genotypic correlation with yield. High estimates of heritability were observed for all characters studied. High estimates of heritability coupled with high genetic advance was observed for plant height and root length which indicate that direct selection for improvement of these traits will be effective. Path coefficient analysis showed that dry root yield had maximum direct effect on plumbagin content of roots and plant height had maximum direct effect on fresh and dry root yield. The index score for selection was highest for *P. zeylanica* followed by the ecotype of *P. rosea* from Kottayam.

The phytochemical content would not change under cultivated conditions if it is genetically determined. 28 genotypes of *Cassia angustifolia* recorded the highest accumulation of sennosides A and B in the leaves (3.51%) whereas cultivated accessions showed only (0.89%) which is almost 4 times less (Seeni *et al.,* 1998). These reports were used to locate secondary plant compounds that have systematic variation rather than medicinal importance.

Molecular variation study

Genetic diversity has ranged from classical strategies such as morphological analysis to biochemical and molecular techniques (Demissie *et al*., 1998). Several molecular approaches have been used to identify, diagnose, delimit species and assess phylogenetic relationships between different cultivars. Three molecular methods viz., random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and more recently DNA sequencing, have been the most extensively applied.

Although, morphological trait can be used for assessing genetic diversity but it is often influenced by the environmental factors (Siddiqui and Naz, 2009). Biochemical markers such as proteins and isozymes have served as an important tool to detect genetic relationships in plants (Mukhlesur *et al.,* 2004). Protein polymorphism serves as genetic markers as they are direct products of active genes and are quite polymorphic and generally heritable (Gepts, 1990). The polymorphism observed in the protein profiles reflects the changes in the active part of the genome. Although protein polymorphism can be analysed through a variety of techniques, Polyacrylamide Gel Electrophoresis (PAGE) is generally favoured technique for rapid analysis (Fergusan and Grabe, 1986; Smith and Smith, 1986; Ratmond and Bauer, 2001) due to its validity and simplicity for describing genetic variations (Ahmed and Slinkard, 1992). This technique has been used effectively to interpret genetic diversity among/ between genotypes in different plant species (Cook, 1984; Mukherjee and Datta, 2008).

Proteomic studies

Proteomics has become an important research tool to study complex biological systems in the post-genomics era and the large-scale systematic analysis of tissue and organelle specific proteins. Proteomics approach has been employed in revealing phylogenetic relationship among

various species by many taxonomists (Ishtiq *et al.,* 2007). The standard method for quantitative proteome analysis is the combination of high resolution (isoelectric focusing / SDS-PAGE) two dimensional gel electrophoresis (2D-GE) with mass spectrometric (MS) or tandem (MS/MS) identification of selected protein spots. This technique can provide insight on the specialized biochemistry of distinct tissues, protein localization, protein - protein interactions, enzymatic complexes, protein-metabolite complexes, post-translational modifications and cellular signaling (Kersten *et al.,* 2002; Baginsky, 2009).

Johnson *et al.* (2010) studied the genetic affinities and variation among three *Plumbago* species using esterase, peroxidase and polyphenol oxidase. A total of 32 bands were observed in the isozyme system of *Plumbago* species. Rout *et al.* (2010) studied the protein content using SDS-PAGE analysis and found that *P. zeylanica* contained several protein bands of molecular weights viz., 50.08, 41.25, 38.41, 36.21, 28.74 and 25.52 kDa. Chuntaratin (2006) studied the plumbagin protein conjugated glutaraldehyde reaction in *P. indica* using SDS-PAGE. The molecular weight of native BSA and plumbagin-BSA was 66564.232 kDa and 72817.704 kDa respectively. The respective molecular weight of plumbagin linked protein was 188.2 kDa.

Bompalli and Nallabilli (2013) studied the genetic variation among eleven *Ocimum* spp. collected from Northern region of India by SDS-PAGE. Nine molecular weight protein bands ranging from 11.3 – 33.3 KDA were totally observed in the eleven spp. collected from Northern region of India. Among these more number of protein bands was identified in the accession collected from Jungli Tulsi, Sikandra, Uttar Pradesh. The samples collected from Agra District and Rajasthan was illustrated in cluster 1(C_1) and cluster 2(C_2) represents Haryana and New Delhi showing closest similarity compared to other localities. Sharawy (2013) examined the relationship between the taxa belonging to the subfamily Asclepiadoideae (Apocyanaceae)

collected from their natural habitats in Egypt and Saudi Arabia using SDS-PAGE. A total of 52 protein bands have been revealed in the electrophoretic profiles. Majority of the bands has been found polymorphic across the examined taxa. In cluster analysis, the two samples of *Leptadenia pyrotechnica* are clearly distinguished from the other species at a UPGMA distance coefficient of about 1.20. Omaonhinmin and Ogunbodede (2013) studied the diversity among the seed storage protein of 20 Fabaceae species, 4 grain legumes and 16 non-pulses of 16 genera using SDS-PAGE analysis to estimate the genetic relationship. 28.3% similarity and 71.7% proteomic polymorphism was scored for the species.

Britto *et al.* (2012) studied the protein profiles of the few medicinal plants in Apocynaceae family using SDS-PAGE. The number of protein bands varied from 7 to 13. More number of peptide bands was produced in *R. tetraphylla* and less number of bands was observed in *N. indicum*. Molecular weight of the five plants varied from 1.26 to 89.12 kDa. Highest molecular weight protein was observed in *N. indicum* (89.12 kDa). *R. tetraphylla* showed the low molecular weight (1.26 kDa) protein. Khalik *et al.* (2012) elucidated genetic relationship between seed proteins of ten *Ipomoea* species using SDS-PAGE. The results revealed a close relationship between the subgenus *Ipomoea* and *Quamoclit.* Masoumi *et al.* (2012) revealed the genetic relationship of the selected medicinal plants viz., *Cuminum cyminum*, *Foeniculum vulgare* and *Falcaria vulgaris* belonging to Apiaceae family. Gairola *et al.* (2012) studied the intra-specific genetic variability in the seed sources of *Jatropha curcus* collected from 7 geographical locations of Central Himalaya using SDS-PAGE. Among all the seed sources the protein sources of the accessions collected from Kalachauna and Saknidhar had highest similarity with other sources whereas Gandhari had the lowest similarity.

Yousaf *et al.* (2011) studied the seed protein profiles of 54 accessions of *Solanum* and *Capsicum* belonging to the family Solanaceae by SDS-PAGE. Alishah *et al.* (2011) studied the variation in seed protein of 14 wild rice species (*Oryza* spp.) along with cultivated rice (*Oryza sativa*) collected from International Rice Gene bank Collection (IRGC), Philippines using SDS-PAGE protein profiling studies. Totally 34 bands were observed in the selected *Oryza* species. Britto *et al.* (2011) investigated the protein profiling of the selected Asclepidaceae species viz., *Daemia extensa*, *Calotropis gigantea*, *Tylophora indica*, *Cryptolepis buchanani* and *Gymnema sylvestre* using SDS-PAGE analysis and their relative similarity between the species were estimated by Jaccard's similarity index and UPGMA method. A total of 41 bands with molecular weight between 125.89 and 1.58 kDa were detected.

Dhabhai and Batra (2011) investigated and compared the *in vivo* and *in vitro* protein profiles of *Acacia nilotica* using SDS-PAGE. *A. nilotica* contained several protein bands of molecular weight 54.3, 44.1, 42.7, 40.1, 35.6, 31.2, 28.6, 24.7 and 19.5 kDa. These results indicate that the intensity of protein bands was high in *in vitro* plant sample compared to *in vivo* protein samples of *Acacia nilotica*. Alfy *et al.* (2011) revealed the proteomic variation between *Celtis australis* and *Celtis occidentalis* by SDS-PAGE. Low banding variation was observed between the two *Celtis* species. The molecular weight observed within the studied samples ranges between 223.89 and 9.45 kDa. The total number of bands recorded in both samples was 18, among these four are common to both *Celtis* species. Analysis of seed SDS-PAGE revealed that the distance between the two studied *Celtis* species did not exceed 27.5%, which indicates little variation amongst their SDS-PAGE profiles.

Raj *et al.* (2011) revealed the seed protein profile on 9 species of *Crotalaria* collected from 12 accessions of South India (Rapinat Herbarium, Tiruchirappalli). *C. grahamiana* and *C.*

beddomeana were collected from the Western Ghats of South India at high altitudes. Similarly *C. pallida* was collected from foothills of Kolli hills and Puliyancholai hills respectively. The other accessions were from plains of Central Tamil Nadu to study the inter and intra-specific relationship among the selected *Crotalaria* species using SDS-PAGE. Most species had distinctly different protein patterns but close association was found between *C. pallida* and *C. laburnifolia.* The morphological variation observed between the two specimens of *C. grahamiana* collected from two different geographical regions showed good variations in their protein profiles that are enough to give it the rank of sub species of *C. grahamiana***.** Negi *et al*. (2011) revealed the intra-specific diversity among the endangered tree (*Tecomella undulata*) collected from various localities of Rajasthan using SDS-PAGE proteomic study. A total of 9 protein bands were observed. Eight bands were common between all the three morphotypes. Ninth band, just heavier than the bottom two lighter bands was absent in the red morphotype and was found present in orange and red morphotypes. These distinct characters suggest existence of atleast two morphotypes in *Tecomella undulata* growing in Rajasthan. One with yellow flowers and the other with red flowers, the third morphotype with intermediate flower colour might be an intra-specific hybrid of the two morphotypes.

Johnson (2010) evaluated the intra-specific biochemical variation of *Aegle marmelos* species collected from Thrissur, Dharmapuri, Tenkasi, Salem, Attur and Kolli Hills using SDS-PAGE profiling. The protein gel system revealed a total of forty six bands with eight active zones / regions and twenty one positions were observed in the protein system of *A. marmelos*. Highest percentage (60%) of similarity was observed between the accession from Tenkasi and Salem. Uysal *et al.* (2010) determined the proteomic relationship between 47 *Centaurea* species collected from different parts of Turkey by SDS-PAGE protein banding pattern. The results

displayed in dendrogram differentiated in to 2 main groups; the first group was divided further into 3 subgroups, with genetic distances between 0% and 90%. The upper cluster consisted of *Rhaponticodes mykalea, R. iconiensis* (genus *Rhaponticoides), Centaurea balsamita,* and *C. coronopifolia*. The middle cluster consisted of *C. polyclada, C. yozgatensis, C. pinetorum, C. lycaonica, C. hierapolitana, C. inexpectata and C. macrocephala.* The lower cluster included *C. cheirolopha, C. cheirolepidoides, C. cf. isaurica, C. isaurica, C. drabifolia, C. drabifolia, cappadocica, C. kotschyi* var. *kotschyi, C. kotschyi* var. *persica, C. kotschyi* var. *decumbens, C. derderiifolia, C. deflexa, C. nivea, C. sericea, C. cankiriensis, C. armena, C. glastifolia, C. pterocaula, C. spectabilis* var. *spectabilis,* and *C. spectabilis* var. *microlophus*. SDS-PAGE facilitated important and applicable selection in the sectional or inter species level within *Centaurea*, particularly for *Cheirolepis* and its relatives.

Faisal *et al.* (2009) identified the seed protein present in *Glycine max* collected from 92 accessions of Pakistan, USA, North Korea and Japan by SDS-PAGE. The results showed 26 reproducible bands which are used to study the intra-specific genetic variation. On the basis of the relative mobility of seed proteins on the gel, totally 26 bands were detected, among these ten major bands were recorded out of total 26 bands detected, while 50% of total were polymorphic. The band frequencies in these samples ranged from 0.03 to 0.99 with a mean of 0.53. The banding pattern revealed three regions. Region I comprised of 11 bands of above 66.0 kDa Region II consisted of nine bands lying between 45.0 and 66.0 kDa, while Region III had six bands between 24.0 kDa and 45.0 kDa. Johnson *et al.* (2008) characterized the fourteen *Capsicum* species using SDS-PAGE. A total of 153 protein bands were obtained in nine active regions of the proteomic profile. Yousaf *et al.* (2008) investigated the SDS-PAGE protein profile of 42 accessions belonging to 7 species of 4 different genera – *Datura, Hyoscyamus, Withania*

and *Atropa* to discriminate the inter and intra-specific variation among the selected genera. The dendrogram based on UPGMA revealed close association between *Withania / Datura* and *Atropa / Hyoscyamus*.

Ahmad *et al*. (2007) studied the eight ecotypes of *Elaeagnus umbellata* for comparing their relationship and evolution based on SDS-PAGE. The total seed proteins showed each autumn olive population could be distinguished by their own specific protein bands with reference to a molecular marker. Emre *et al*. (2006) studied the taxonomic relationship among the seed cotyledons protein of *Lathyrus* species belonging to 9 geographical regions by SDS-PAGE profiling. Protein banding pattern was higher in *L. spathudatus* and lowest banding profile in *L. brachypterus.* Mohamed *et al.* (2006) recognised the protein profile of 19 species representing 14 genera of Araceae family plants using SDS-PAGE analysis expressing 57 protein bands. Among these, 9 species of Araceae are identified as novel using proteomic studies.

Pereira *et al.* (2004) investigated the proteins from young unexpanded leaves of seven cassava cultivars *Manihot esculenta* through SDS-PAGE. The comparison was made through the protein patterns obtained, and their relative amounts determined in plants leaves infected or not with bacteria *Xanthomonas axonopodis* pv. *manihotis*. The electrophoretic protein pattern obtained from the investigated cultivars, showed a polypeptide subunit present extensively in the "Fecula Branca" cultivars, with a molecular weight of 93.5 kDa. The protein fractions were more intensely stained in young leaves of *M. esculenta* plants infected with bacteria. The 93.5 kDa protein fractions can be used as a molecular marker for the "Fecula Branca" cultivars. Masood *et al.* (2004) observed the inter-specific and intra-specific variation studies on seed proteins of *Aegilops* and *Hordeum* species using SDS-PAGE protein profiling. The studies reported the presence of proteins with molecular weight range higher than 24 kDa.

Sinha *et al.* (2001) identified soluble proteins from the tuberous roots of *Momordica dioica* by SDS-PAGE to compare the protein profiles of the sex forms. Twenty eight bands with molecular masses ranging from approximately 15 kDa to > 94 kDa, proteins were found to be common in both staminate and pistillate plants. The pistillate plant only had 22 kDa polypeptide which was not detected in staminate ones.

Sammour (1999) used SDS-PAGE for the extraction and characterization of six proteins with molecular weights ranging from 41 - 55 kDa in *Linum usitatissimum*. Ona e*t al.* (1995) studied the genetic variability among five species of *Solanum* species viz., *S. aethiopicum, S. macrocarpon, S. nigrum, S. melongena* and *S. surattense* collected from Philippines using SDS-PAGE protein profiles. Among the five *Solanum* species, *S. melongena* was closely related to *S. aethiopicum.* Norman and Russell (1988) isolated three fractions of *P. zeylanica* pollen protein (male germ unit rich, cytoplasmic-particulate and water-soluble proteins) using one and two dimensional polyacrylamide gels and identified 427 spots > 33 kDa. Przybylska (1986) carried out electrophoretic protein analysis for the systematic description of *Pisum* genetic resources. The electrophoretic patterns of the legumin fraction obtained by means of Urea-PAGE and Iso electro focussing were helpful in discriminating the genetic resources samples.

MALDI-TOF MS Analysis

MALDI was introduced in 1988 by Karas and Hillenkamp as a revolutionary method for ionizing and mass-analysing large biomolecules (Karas and Hillenkamp, 1988). MALDI-TOF MS was first reported in 1994 (Spengler *et al.,* 1994) and has been applied to visualise peptides and proteins since 1997 (Caprioli *et al.,* 1997). MALDI-TOF MSI has become a powerful technique that enables the identification and localisation of biological compounds directly on tissue surfaces. The predominant method used for imaging protein was MALDI-TOF Mass

Spectrometry (MS). Sinapinic acid is the matrix of choice for MALDI-MSI of proteins in tissue sections (Chaurand *et al.,* 2008). Several studies have been reported on a successful reversible coupling of a MALDI source to an ESI tandem quadrupole TOF mass spectrometer (Shevchenko *et al.,* 2000). MALDI-TOF MS has been suggested as a fast and reliable method for plant species identification, based on the characteristic protein profiles for each species. Using this technology it has been estimated that up to 99% of species tested are correctly identified when comparing with commercial phenotypic identification panels or gene sequencing (Chen *et al.,* 2009; Bizzini *et al.,* 2010; Chekaoui *et al.,* 2010; Giebel *et al.,* 2010). Chuntaratin (2006) studied the plumbagin protein conjugated glutaraldehyde reaction in *P. indica* using MALDI-TOF MS analysis.

Zhang *et al.* (2013) identified a hepatoprotective compound, scoparone through protein – protein interaction by MALDI-TOF MS analysis. Zhao *et al.* (2012) differentiated the expression of proteins in the rice (*Oryza sativa*) leaves and roots using MALDI-TOF MS analysis. A total of 16 proteins were expressed in the rice leaves and 25 proteins were expressed in the rice roots. Wang *et al*. (2012) differentiated the two closely related pathogenic bacteria *Acidovorax oryzae* and *Acidovorax citrulli* using MALDI-TOF MS analysis. 22 peaks were present in *A. oryzae* and 18 peaks were specific to *A. citrulli*. Ferreira *et al*. (2011) validated 56 rhizobial species belonging to the family Rhizobiaceae using MALDI-TOF MS. Spectra were recorded in the linear, positive mode at a laser frequency of 200 Hz within a mass range from 2,000 to 20,000 kDa. Que *et al.* (2011) compared the two protein sugarcane varieties (NCo376 and Ya71-374) using MALDI-TOF MS analysis and expressed 23 differential proteins. Ishtiaq *et al.* (2010) studied the phylogenetic relationship on the seven species of *Clematis* collected from Tian Mu Shan Biosphere Reserve, China by MALDI-TOF MS analysis. The protein profile of *C.*

armandii and *C. finetiana* species are related to each other and the species *C. chinensis* was present in a distinct cluster and is congruently differentiated from other allied species.

Xiang *et al.* (2010) elucidated cyclotides, a mini protein present in *Viola tianshanica*. It was spectaculated at a mass range of 3,000 – 3,500 kDa using MALDI- TOF MS analysis. The purified cyclotides were reduced with DTT, alkylated with iodoacetamide and then were cleaved with endoproteinase Glu-C, endoproteinase Lys-C and Trypsin separately. The digested peptides were purified on RP-HPLC and analyzed on MALDI-TOF analyzer. A new cyclotide, cycloviolacin T1 was systemically determined using MALDI -TOF system. Shin *et al.* (2010) studied the proteomic profiling of *Fagopyrum esculentum* and *Fagopyrum tataricum* using MALDI-TOF MS analysis. The results identified 166 unique proteins and six common proteins available in both *Fagopyrum* species in which the proteins 79 and 81 exclusively belonged to *F. tataricum*. Respinis *et al.* (2010) investigated the proteomic profile of 129 morphologically and genetically well-characterized strains of *Hypocrea* and *Trichoderma* belonging to 25 species in 8 phylogenetic clades by MALDI-TOF MS. It allows quick species identification which represents a valid alternative to gene sequencing for species diagnosis of *Trichoderma* and other fungal taxa.

Pradeep and Kumari (2009) identified the variation of protein expression in *in vitro* and *in vivo* grown plants of *Artemisia vulgaris* using MALDI-TOF MS analysis. Savithry *et al.* (2009) investigated the major proteins present in the soya been seeds using MALDI-TOF MS analysis. Kamal *et al.* (2009) compared the proteins present in two varieties of pre-harvest sprouting wheat cultivators (Jinpum - susceptible and Keumgang - resistant) using MALDI-TOF MS analysis. Variation between normal and drought resistant protein of *Helianthus annus* was studied using MALDI-TOF MS analysis (Castillego *et al.,* 2008). Wu *et al.* (2007) determined

and compared the alkaloids present in *Aconitum carmichaeli* collected from four accessions of China for herb differentiation and explanation of the significant difference in their toxicities by MALDI-TOF MS analysis.

Jacobs *et al.* (2005) identified 88 protein spots representing a novel protein involved in the biosynthesis of alkaloid from *Catharanthus roseus* using MALDI-TOF MS analysis. Carter and Thornburg (2004) discriminated an array of nectarin proteins present in the ornamental tobacco (*Nicotiana langsdorffii* x *Nicotiana sanderae*) and found five discrete 61 - 65 kDa proteins. Caldos *et al.* (2004) identified 23 protein data sets to study the genetic diversity using seed proteome analysis in 12 genotypes of *Capsicum* using MALDI-TOF MS, presented a variation pattern of expressed proteins within the mass range value of (1,500 to 15,000 kDa. Creer *et al.* (2002) characterized the pit viper venom protein in *Trimeresurus stejnegeri* collected from diverse geographical location of Taiwan to reveal the intra-specific variation using MALDI-TOF MS analysis.

Variation within nucleotides can be detected at very narrow levels by sequencing. Sequencing uses minimal amount of DNA to develop accurate results. Sequencing can also reveal whether the variation is due to substitution or rearrangement. Since this method involves money, labour and time a few loci of importance are sequenced for developing markers. Very specific genes such are phytochrome, heat shock proteins and nuclear rRNA gene families have been sequenced for marker generation (Soltis and Soltis, 1995).

Various protocols for DNA extraction have provided DNA suitable for several kinds of analyses (Wang and Taylor, 1993; Ziegenhagen and Scholz, 1998). Doyle and Doyle (1987) have reported the protocol for isolation of DNA as well as the optimization of the PCR

conditions required. DNA extraction was improved by modifying some of the steps in the original CTAB DNA isolation protocol.

DNA-based techniques have been successfully applied for differentiation and authentication of plant species of medicinal importance which are often adulterated by morphologically similar species such as *Lycium barbarum* (Zhang *et al*., 2001), *Echinacea* species (Wolf *et al*., 1999), *Taraxacum mongolicum* (Cao *et al*., 1997), *Cuscuta reflexa* (Khan *et al*., 2010). Nathar and Gadge (2013) isolated and quantified the DNA in *P. zeylanica, P. auriculata* and *P. rosea* using CTAB method and found the DNA concentration as 4.40, 4.55 and 4.65 µg/ µl in the leaves samples. Dharmar and Britto (2012) estimated the genetic diversity of *P. zeylanica* collected from five accessions of Southern Western Ghats viz., Marunthuvalmalai, Courtallum, Achankoil, Maruthamalai hills and Anchetty. Of the 12 primers screened, 6 produced highly polymorphic DNA fragments. The total number of amplified DNA fragments was 35. The P3 (Achankoil) population was found to be least diverse (0.2023) and displayed the high level of variability (0.2978) and the P2 (Courtallam) population revealed intermediate diversity (0.2671). Overall genetic diversity was 0.2194 and percentage of polymorphic loci was 54.29.

Ali *et al.* (2013) studied the genetic variability in Kaempferol, a chemical constituent in eleven accessions of *Clitoria ternatea* collected from different regions of the India (New Delhi, Haryana, Uttar Pradesh, Rajasthan, Madhya Pradesh and Tamil Nadu) using 25 RAPD primers and HPLC analysis. A total of 71 reproducible bands of which 32 (45%) were polymorphic. The genetic distance ranged from 0.02 to 0.28. A dendrogram based on UPGMA clustering method revealed two major clusters. Cluster 1 comprises of the accessions of North India while cluster 2 includes accessions of Central and South India. The Kaempferol content was 9.31–20.01 mg/g,

being maximum in the accession of Haryana and minimum in Bhopal. Hu *et al.* (2007) compared the genetic diversity and chemical profile of *Vitex rotundifolia* from different locations of China. The HPLC data showed considerable variation of chemical constituents among the *V. rotundifolia* populations. The hierarchical clustering analysis further revealed four major groups based on their chemotype variation. Abundant genetic diversity was detected among the *V. rotundifolia* populations that was also clustered into four groups based on their ISSR data This provides a solid basis that the genetic variation pattern revealed by molecular markers was closely associated with that indicated by chemical constitutes in the fruits of *V. rotundifolia*.

DNA based molecular markers have been extensively utilized in the fields like taxonomy, physiology, embryology, genetics, etc. for authentication of different plant species. DNA markers have been considered as the most reliable polymorphism because the genetic composition is inimitable for each species and is not affected by age, physiological conditions as well as environmental factors (Chan and Choy, 2003). A more recently employed approach in plant systematic and population biology is DNA barcoding study. The purpose of the study is to permit the identification of taxa and the determination of phylogenetic relationships. The use of such techniques for germplasm characterization facilitates the conservation and utilization of plant genetic resources, permitting the identification of unique accessions or sources of genetically diverse germplasm (Kapteyn and Simon, 2002).

The initial goal of the DNA barcoding process is to construct on-line libraries of barcode sequences for all known species that can serve as a standard to which DNA barcodes of any identified or unidentified specimens can be matched (CBOL, 2009). This can alleviate several inherent problems associated with traditional taxonomic identification, based on morphological characters, such as wrong identification of species due to phenotypic plasticity and genotypic

variability of the characters, overlooking cryptic taxa, difficulty in findings reliable characters due to long maturity period etc (Ratnasingham and Hebert, 2007)

A DNA barcode uses genes to identify a species. It can be developed at various sites in a plant such as nuclear, mitochondrial and chloroplast sequences. However, the designed barcode should be in a region of the genome which is variable but conserved enough to design primers that can amplify short regions of 100-150 bp. Since cp DNA evolves faster than mitochondria and shows considerable mutation rate, it is considered a better choice for developing a barcode for angiosperms (Clegg, 1993). The sequence of chloroplast gene, *rbc*L has been used as a marker in many phylogenetic studies (Clegg and Zurawki, 1992). *rbc*L has been used very extensively in plant phylogenetic studies. More than 10,000 *rbc*L sequences are already available in Gen Bank (Chase, 2007; Kress *et al.,* 2007). Most of the phylogenetic studies suggest that *rbc*L is best studied to reconstruct the relationships down to the generic levels, but it is not useful for specific levels (Newmaster *et al.,* 2006; Albert *et al.,* 1994).

Barcoding studies in national level

Chandramohan *et al.* (2013) barcoded and identified *Croton bonplandianus* procured from Red hills, Chennai by *mat*K primer. The evolutionary analysis Nucleotide BLAST (blastn) shows 97% maximum identity to *Croton lachnocarpus* and *Croton stellatopilosus* which reveal that *Croton bonplandianum* are closely related to them and 94% similarity to *Jatropha podagrica*. Gurudeeban *et al.* (2013) described the phylogenetic relationships among Rhizophoraceae genus *Rhizophora*, *Ceriops* and *Bruguiera* using tRNA Leu (UAA) intron sequences as a molecular marker by DNA sequencing analysis. The results revealed congeneric relationship between *R. apiculata, R. mucronata, B. gymnorhiza* indicating a high degree of gene flow within them. Mahadani *et al.* (2013) tested the efficiency of matK and trnH-psbA for

differentiation of selected ethnomedicinal plants of Apocynaceae. Evidently, *matK* sequence information could help in correct species identification for medicinal plants.

Selvaraj *et al*. (2012) identified and distinguished four species of *Boerhavia* (*B. diffusa*, *B. erecta, B. repanda, B. verticillata*) collected from the regions of Western Ghats, Coimbatore, India. Among the four species, *B. diffusa* was differentiated from its closely-related species. Phylogenetic analysis was carried out for the four species of *Boerhavia* using nuclear ribosomal DNA regions ITS, ITS1, ITS2 and the chloroplast plastid gene *psb*A-*trn*H. Sequence alignment revealed 26% polymorphic sites in ITS, 30% in ITS1, 16% in ITS2 and 6% in *psb*A-*trn*H respectively. Phylogenetic tree was constructed for the related *Boerhavia* species using ITS sequences which clearly distinguished *B. diffusa* from the other species. The ITS1 demonstrates a higher transition/transversion ratio, percentage of variation and pairwise distance which differentiate *B. diffusa* from other species of *Boerhavia*.

Singh *et al.* (2012) compared the amplification, sequencing and species discrimination success rates among multiple accessions of 36 *Dendrobium* species procured from different geographical locations of India viz., Pachmarhi (Madhya Pradesh), Nainital, Dehradun, Mussoorie and adjoining areas (Uttarakhand), Kolhapur and adjoining areas (Maharashtra), Kalimpong (West Bengal), Tropical Botanic Garden and Research Institute (TBGRI), Thiruvananthapuram (Kerala), Dibrugarh University (Assam), Bio-Resource Development Center (BRDC), Shillong (Meghalaya) and Botanical Survey of India (BSI), Shillong (Meghalaya) with *mat*K, *rbc*L*, rpo*B, *rpo*C1, *trn*H-*psb*A spacer and ITS loci. Among the tested loci, ITS recommended as a possible barcode for plants, provided 100% species identification. Another locus, *mat*K*,* also recommended as a universal barcode for plants, resolved 80.56% species. Two-locus combination of *mat*K + *rbc*L could discriminate 86.11% of 36 species.

Among the recommended combinations, the barcode based on three loci - *matK, rpoB* and *rpoC1*- resolved maximum number of species. Raj *et al.* (2012) barcoded *H. indicus* and *D. hamiltonii* roots collected from medicinal plants garden in Bangalore and roots of *A. gonoclados* and *A. racemosus* collected from Thirumala Hills (Andhra Pradesh) and Denkanikotta (Tamil Nadu) by ITS2 primer. The results showed that the ITS2 region can effectively discriminate *Asparagus racemosus* and *Hemidesmus indicus* from its substitute samples and hence can resolve species admixtures in raw samples.

Ragupathy *et al*. (2009) carried out DNA barcoding to discriminate a new cryptic species *Tripogon* located within the Velliangiri Hills, part of the Nilgiri Biosphere Reserve hill tribes. DNA barcode regions *rbc*L, *mat*K and *trn*H-*psb*A showed distinct sequence variations among the closely related ethnotaxa. DNA barcoding discriminates the cryptic species *Tripogon cope* from the morphologically similar species of *Tripogon wightii*. Although there were no differences in the *rbc*L sequences for these two cryptic species, the *mat*K and *trn*H-*psb*A sequences were consistently different. Inter-specific variation among all eight species ranged from (*p*-distance 0.002 – 0.003). Intra-specific *p*-distance was 0.00 for all regions within all eight species.

Barcoding studies in international level

Saarela *et al.* (2013) identified the 490 vascular plant species, representing half of the Canadian Arctic flora using the core plastid barcode loci (*rbc*L and *mat*K). Sequence recovery was higher for *rbc*L than *mat*K (93% and 81%). Distance-based and sequence-similarity analyses of combined *rbc*L + *mat*K data discriminate 97% of genera, 56% of species, and 7% of intra-specific taxa. Ashfaq *et al.* (2013) differentiated eight diploid genome groups and five allotetraploid species of the genus *Gossypium* which are morphologically similar. The

effectiveness of three widely used markers (*mat*K, *rbc*L and ITS2) was used in the discrimination of 20 diploid and five tetraploid species of cotton. Sequence recovery was high ranging from 92% to 100% with mean pair wise inter-specific distance for ITS2 (3.68%) and lowest for *rbc*L (0.43%).

Enan *et al.* (2012) studied the intra-specific variation within the morphologically similar *Ricinus communis* among eight different accessions of Egypt and United Arab Emirates using *mat*K and ITS 2 primers. Jeanson *et al.* (2012) determined the variation in the 48 species of Caryoteae family using *mat*K, *rbc*L and *psb*A- *trn*H markers. The combination of three markers discriminated 92% adequate variation compared to the DNA barcodes produced by the individual markers. Bafeel *et al.* (2012) used *rbc*L gene sequences to identify twelve arid wild plants belonging to diverse families. The results showed 92% variation in genus level and 17% in species level.

Wang *et al.* (2012) used *mat*K, *rbc*L*, trn*H- *psb*A and ITS primers to differentiate nine species belonging to Nyssaceae family collected from three different accessions. The results showed that ITS loci were the best performing single locus, although *mat*K + *rbc*L might be used as the core barcodes for land plants. Li *et al.* (2012) studied the inter and intra-specific divergences among 755 species of *Ficus* using five plastid loci (*rbc*L, *mat* K, *trn*H – *psb*A, psbK – *psb*I and *atp*F – *atp*H). The results demonstrated that ITS have the most variable sites, greater inter and intra-specific divergences (72%) compared to other primer loci. Vere *et al.* (2012) barcoded the native flowering plants and conifers of Wales using *rbc*L and *mat*K primers. The results provide relative discrimination levels of 69.4 to 74.9% of all species and 98.6 to 99.8% of genera using both markers.

Bruni *et al.* (2012) identified vascular plant specimens from the local flora of Mt. Valerio, a small hill near the centre of Trieste (NE Italy) using core barcode markers (*rbc*L and *mat*K) plus the additional *trn*H- *psb*A region. The core barcode markers univocally identify most species of the local flora (96%). The trnH - *psb*A data improve the discriminating power of DNA barcoding among closely related plant taxa. Arca *et al.* (2012) studied the genetic variability of *Fraxinus* species using several loci of DNA barcodes (*rpl*32 – *trn*L, *mat*K, *rpo*B, *rpo*C1 and *trn*H– *psb*A). The intergenic spacer *trn*H- *psb*A was the best performing locus compared to other barcoding loci. Li *et al.* (2012) revealed the intra-specific genetic variation of *Achyranthes bidentata* (Amaranthaceae) collected from 58 accessions of India, China and Japan by DNA barcoding analysis using ITS primer.

Burgess *et al*. (2011) distinguished 436 plant species from Jokers Hill, Southern Ontario, Canada using the barcoding primers *rbc*L and *mat*K. The combination of *rbc*L and *mat*K showed 92.7% of sequencing success. Xiang *et al.* (2011) barcoded 9 genera and 54 species of Juglandaceae family to investigate the utility of the four potential barcoding loci viz., *rbc*L, *mat*K, *trn*H- *psb*A and ITS. The result revealed that primer ITS appeared tier priority for species identification and *mat*K for first tier priority for genus discrimination. Yan *et al.* (2011) distinguished 48 individuals representing 12 species of *Primula* L. sect. *Proliferae* pax belonging to Primulaceae family using DNA barcoding. The combination of *rbc*L + *mat*K gave higher genetic diversity among the *Primula* species. Ali *et al*. (2011) barcoded 20 populations of *Diplocyclos palmatus* belonging to five different geographical locations (Bihar, Jharkhand, Maharashtra, Madhya Pradesh, and Tamil Nadu) using Internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Analysis of nucleotide sequences reveals total variance of 3.260.

Fu *et al.* (2011) used *rbc*L, *mat*K, *trn*H-*psb*A and ITS as DNA barcodes to distinguish 23 species of *Tetrastigma* collected from different localities of China and Thailand. The results indicated that the best barcode was ITS, which showed significant inter-specific genetic variability. Multiple loci also provided a greater ability to distinguish species than single loci. Among the 23 species, *Tetrastigma hemsleyanum* was identified precisely using DNA barcoding methods. Costion *et al.* (2011) estimated species diversity of trees in a tropical rain forest by DNA barcoding studies using three different primers *rbc*L, *mat*K, *trn*H –*psb*A. The combination of the *rbc*L and *trn*H-*psb*A loci performed better than any two-locus combination that included *mat*K.

Xue and Li (2011) carried out DNA barcoding using ITS for the differentiation of *G. paludosa* from the adulterant species (*Gentianopsis barbata*, *G. contorta, G. grandis, Halenia elliptica, Lomatogonium macranthum, L. rotatum, Swertia angustifolia, S. bifolia* and *S. erythrosticta).* The data showed that the ITS regions differ significantly between *G. paludosa* and all nine adulterant species. Guo *et al*. (2011) barcoded 25 *Hedyotis* taxa using the plastids (*mat*K, *trn*H–*psb*A, *pet*D, and *rbc*L) and nuclear ITS of rDNA. ITS showed the best species discrimination by resolving 23 of the species as exclusive lineages with no shared alleles between any of the 24 distinct species. *H. assimilis* and *H. mellii* are not supported as distinct species. *rbc*L resolved 10 species as exclusive lineages and 10 species with shared alleles. Wang *et al.* (2011) barcoded two species of *Pugionium* (Brassicaceae) using DNA regions (*rbc*L, *mat*K, and *trn*H-*psb*A) and nuclear ribosomal ITS to study the inter and intra-specific variation. Among all the tested primers, ITS region had completed inter and intra-specific sorting possibly compared to other barcodes.

Li *et al.* (2011) studied 57 gymnosperm species representing 40 genera, 11 families and four subclasses to evaluate the universality of nine *mat*K primers and one *rbc*L primer. A specific *mat*K primer for *Ephedra* was newly designed which performed well on the sampled species. Shi *et al.* (2011) barcoded 260 species belonging to 30 genera in Zingiberaceae using ITS2, *rbc*L, *mat*K, *psb*K–*psb*I, *trn*H-*psb*A and *rpo*B. The discrimination ability of the ITS2 locus was 99.5% at the genus level and 73.1% at the species level. Yu *et al.* (2011) developed a new pair of *mat*K primers to barcode angiosperm species that resulted in success for both amplification and sequence analysis. Dong *et al*. (2011) used *rbc*L, *mat*K and ITS to evaluate the five species of *Pterygiella* (Orabanchaceae) and found that ITS was successfully used to identify all species of this genus.

Bafeel *et al.* (2011) barcoded *Anthemis deserti, Pulicaria undulata, Sonchus oleraceus, Malva parviflora* and *Salsola imbricate* using *mat*K and *rbc*L primers. The success rate of sequencing was higher for *rbc*L (88%) compared with *mat*K (69%). Jiang *et al.* (2011) barcoded ten species of *Epimedium* using four DNA barcodes (*psb*A-*trn*H, ITS, *rbc*L, *mat*K). *psb*A-*trn*H region was the better barcode for the genus *Epimedium*. Gu *et al.* (2011) differentiated the species in the genus *Ligustrum* using four potential DNA barcodes viz., nuclear ribosomal ITS and three cpDNA regions (*rbc*L, *mat*K and *trn*H-*psb*A). The discriminating power of *rbc*L, *mat*K, *trn*H-*psb*A and ITS based on neighbor-joining (NJ) trees was 36.8%, 38.9%, 77.8%, and 80% respectively.

Chen *et al.* (2010) studied the discrimination ability of ITS2 in more than 6600 plant samples belonging to 4800 species from 753 distinct genera. The combination of *rbc*L + *mat*K was successfully discriminated among 907 samples from 550 species at the species level with a probability of 72%. The rate of successful identification with the ITS2 was 92.7% at the species

level. Yao *et al*. (2010) downloaded 50,790 plant and 12,221 animal ITS2 sequences from GenBank to evaluate sequence length, GC content, intra and inter-specific divergence, and efficiency of identification. The success rates to identify dicotyledons, monocotyledons, gymnosperms, ferns, mosses and animals were 76.1%, 74.2%, 67.1%, 88.1%, 77.4% and 91.7% at the species level.

Steven *et al.* (2009) discriminated multiple populations of 1355 *Acacia* species collected from different biogeographical patterns of India, Africa and Australia using *rbc*L, *trn*H-*psb*A and *mat*K. Selvaraj *et al.* (2008) selected 47 genera from Zingerabaceae family and carried out DNA sequencing using *mat*K gene. The sequence alignments were performed by Clustal X, transition/transversion rates were predicted by MEGA and phylogenetic analyses were carried out by PHYLIP package. The result indicates that the genus *Afromonum, Alpinia, Globba, Curcuma* and *Zingiber* shows poly phylogeny. The overall variants between the species are 24% and transition/ transversion rate is 1.54. Sun *et al.* (2002) sequenced 7 species and 8 subspecies of *Hippophae* genus using (ITS) region of nuclear ribosomal DNA (nrDNA). Parsimony analysis and NJ methods suggested that the species *H. tibetana, H. neurocarpa* and *H. salicifolia* were distinct from other species of *Hippophae*. Downie *et al.* (2000) revealed *rbc*L as a good barcode for *Acacia nilotica*, with a 100% match to multiple vouchers (representing different subspecies).

Antibacterial activity

Medicinal plants represent a rich source of antimicrobial agents. Wide range of different parts of medicinal plants was used for extract as raw drugs and they possess varied medicinal properties. Some of these raw drugs are collected in smaller quantities for local use while many other raw drugs are collected in smaller quantities and traded in market as raw material for many herbal industries (Parekh *et al.,* 2006). The increasing failure of chemotherapeutics and antibiotic

resistance exhibited by pathogenic microbial infections agents have led to the screening of several medicinal plants for their potential antimicrobial activity (Uniyal *et al.,* 2006). Silver and Bostian (1993) documented the use of natural products as new antibacterial drugs. There is an urgent need to identify novel substances active towards highly resistant pathogens (Recio 1989; Cragg *et al.,* 1997). In an effort to discover new compounds, many research groups screen plant extracts to detect secondary metabolites with the relevant biological activities (Hostettmann, 1991).

Antimicrobial activities of *Plumbago* species have been reported by many workers, Ibrahim *et al*. (2012) evaluated the antibacterial activity using the methanolic extracts of *P. indica* against *S. aureus, S. typhi, S. dysenteriae, B. cereus P. aeruginosa, S. sonnei, V. cholera* and *E. coli.* The highest susceptibility was observed against *S. aureus, E. coli* and *S. typhi* compared to other pathogens ranged from 15-27 mm. Devi and Krishna (2012) studied the antibacterial activity on methanolic leaf root and stem extract of *P. zeylanica* against *Bacillus subtilis*. The results concluded higher antibacterial activity on *P. zeylanica* root extract. Vishnukanta and Rana (2011) studied the antibacterial activity in two different extracts of *P. zeylanica* against *S. gallinarium, E. coli, P. vulgaris, S. typhimurium, P. aeruginosa* and *S. aureus*. Among the two extracts methanolic extract exhibited higher antibacterial activity against all the pathogenic bacteria. Antibacterial activity of methanolic and chloroform extracts of *P. zeylanica* against five different organisms viz., *S. pyogenes*, *S. aureus*, *Bacillus* sp., *P. aeruginosa* and *E. coli* were studied using disc diffusion method. The methanolic extracts were more active against all the tested organisms (Devi and Thenmozhi, 2011).

Jetty *et al.* (2010) evaluated the antimicrobial properties of compounds such as neoisoshinanolone and 1-epineo-isoshinanolone isolated from the roots of *P. zeylanica*. Among

these 1-epineo-isoshinanolone is more active with a MIC of 12.5-25 µg/mL whereas neoisoshinanolone has recorded a MIC of 50-100 µg/mL. The activities are compared with plumbagin (0.78-3.13 µg /mL) and standards streptomycin for bacteria and nystatin for fungi.

Jeyachandran *et al.* (2009) revealed the minimum inhibitory concentration of methanolic, chloroform and aqueous extract of *P. zeylanica* root against *E. coli*, *S. typhi* and *S. aureus*. Inhibition against *K. pneumoniae*, *S. marcescens* and *B. subtilis* was moderate, and lower against *Proteus vulgaris* and *Pseudomonas aeruginosa*. The methanolic extract exhibited moderate activity and the aqueous extract weak activity against the bacterial strains as assessed by disc diffusion assays.

Rahman and Anwar (2007) studied the antimicrobial activities of ethanolic extracts of *P. zeylanica* root against 11 human pathogenic bacteria and 6 phytopathogenic fungi using disc diffusion method. *V. cholerae* was found to be the most sensitive. Parekh *et al.* (2006) studied the antibacterial potential of *P. zeylanica* using agar disc diffusion method and agar well diffusion method against five bacterial strains viz*., B. cereus, S. aureus, K. pneumoniae, E. coli* and *P. pseudoalcaligenes*. Preliminary screening revealed that methanolic extracts were more potent than the aqueous extracts. Wang and Huang (2005) revealed the anti-*H. pylori* activity of ethanolic, ethyl acetate, acetone and aqueous extracts of *P. zeylanica*. All the tested extracts showed higher anti-*H. pylori* activity except the aqueous extracts. The ethyl acetate extracts exhibited the lowest MIC against five *H. pylori* strains followed by acetone and ethanolic extracts.

Paiva *et al.* (2003) evaluated the antimicrobial activity in the plumbagin isolated from the chloroform extract of *P. scandens against S. aureus*, *P. aeruginosa*, *B. subutilis*, *P. vulgaris*, and against the yeast *C. albicans*. Plumbagin exhibited relatively specific antimicrobial activity.

The growth of *S. aureus* and *C. albicans* was completely inhibited. Antibacterial activity of *P. zeylanica* alcoholic root extracts was studied against multidrug-resistant clinical isolates of bacteria (*S. paratyphi*, *S. aureus*, *E. coli*, *S. dysenteriae*. R-plasmid-harbouring standard strain, *E. coli* x^+). The extracts exhibited strong antibacterial activity against all test bacteria irrespective of their antibiotic resistance behaviour (Beg and Ahmad, 2000). Perumalsamy and Ignacimuthu (2000) studied the antibacterial properties of 30 Indian folklore medicinal plants used by the tribal healers. Among the 30 plant species the extracts of *Cassia occidentalis a*nd *Cassia auriculata* exhibited significant broad spectrum of activity against *B. subtilis* and *S. aureus*.

To fulfill the objectives of the present study, the following *Plumbago* species viz., *Plumbago zeylanica* Linn., *Plumbago auriculata* Lam. and *Plumbago rosea* Linn. were collected from various localities of South India. The preliminary phytochemical analysis was performed by the method described by Harborne (1999). To reveal the inter and intra-specific variation among the selected *Plumbago* species, the qualitative phytochemical analysis viz., UV-Vis, FTIR, TLC, HPLC, HPTLC, GC-MS analysis and genetic studies viz., protein profiling using SDS-PAGE, MALDI-TOF MS and DNA Barcoding was performed. To know the antibacterial potential of the selected *Plumbago* species, antibacterial activity was conducted using well diffusion method.

The detailed methodologies of present investigation are as follows.

Collection of materials

The *Plumbago* species viz., *Plumbago zeylanica* Linn., *Plumbago auriculata* Lam. and *Plumbago rosea* Linn. were collected from various localities of South India. *P. zeylanica* was collected in maximum of seven localities due to the common availability of species, whereas *P. auriculata* (5) and *P. rosea* (3) were collected from limited localities only due to their limited distribution (Table - 1; Plate I). The *P. zeylanica, P. auriculata* and *P. rosea* collected from different sites of South India were displayed in Plate II, III and IV.

Family: Plumbaginaceae

Botanical name: *Plumbago zeylanica* Linn.

Hindi: Chira; Tamil: Vellai Kodiveli, Chitramoolam

Classification

Kingdom	-	Plantae
Subkingdom	-	Tracheobionta
Super division	-	Spermatophyta
Division	-	Magnoliophyta
Class	-	Magnoliopsida
Subclass	-	Caryophyllidae
Order	-	Caryophyllales
Family	-	Plumbaginaceae
Genus	-	*Plumbago*
Species	-	*zeylanica*

Distribution and Morphology of *Plumbago zeylanica*

It is a perennial sub-scandent shrub, grows throughout India, especially in Bengal, Uttar Pradesh, South India and Sri Lanka, in moist places. The plant grows 0.5 - 1.0 meters in height. Flowers are white in colour, 10-25 cm long, inodorous, inbracteate, auxillary and terminal elongated spikes, and bisexual. Calyx densely covered with stalked, sticky glands. Corolla is white, very slender, and tubular and Stamens 5, free. Ovary superior, 5-gonous, one celled, ovule one basal**.** Leaves are simple, alternate, 8 cm long and 3 cm broad, ovate or oblong, petiole narrow, amplexicaul at the base and often dilated into stipule like auricles. Stems are somewhat woody, spreading, terate, striate, glabous. Roots are 30 cm or more in length, 6 mm or more in diameter, stout, cylindrical, friable, blackish red in colour, light yellow coloured when fresh, reddish brown when dry, straight unbranched or slightly branched with or without secondary

roots, with uniform and smooth texture. It has characteristic odour with acrid and bitter taste. Bark is thin and brown in colour**.** Capsule oblong, 4-5 mm long, glabrous. Seeds are oblong, dark purplish, and 4 mm long.

***Plumbago auriculata* Lam.**

Botanical name: *Plumbago capensis* Thunb.

Common name: Cape, Leadwort, Blue *Plumbago,* Cape *Plumbago*

Hindi: Chitra; Tamil: Neela Kodiveli, Chitramoolam

Classification

Kingdom	**-**	Plantae
Subkingdom	**-**	Tracheobionta
Super division	**-**	Spermatophyta
Division	**-**	Magnoliophyta
Class	**-**	Magnoliopsida
Subclass	**-**	Caryophyllidae
Order	**-**	Caryophyllales
Family	**-**	Plumbaginaceae
Genus	**-**	*Plumbago*
Species	**-**	*auriculata*

Distribution and Morphology of *Plumbago auriculata* Lam.

A perennial, evergreen, semi-climbing shrub, native to South Africa often diffusely branched, glabrous except the inflorescence. Leaves elliptic to elliptic-spathulate or elliptic-ovate, 3-7 cm long, obtuse and shortly mucronate, tapering into a short petiole. Flowers in compact panicle. Bracts ovate-mucronate. Calyx tubular, 2-3 times shorter than the corolla, finely pubescent with stalked glands on the upper part, limb 5-fid. Corolla gamopetalous, tube 2 cm long; limb usually 5-lobed. The flowers are pale blue in colour, rounded clustered at the stem of tips. The individual flowers are tubular, 1 inch long and have five spreading round tipped

lobes. These are sticky, glandular hairs at the base of the flowers. The flowers are followed by sticky seed capsules.

***Plumbago rosea* Linn.**

Botanical name: *Plumbago indica* Linn.

Hindi: Chitra; Malayalam: Chettikkoduveli, Sevapu koduveli

Classification

Kingdom	-	Plantae
Subkingdom	-	Tracheobionta
Super division	-	Spermatophyta
Division	-	Magnoliophyta
Class	-	Magnoliopsida
Subclass	-	Caryophyllidae
Order	-	Caryophyllales
Family	-	Plumbaginaceae
Genus	-	*Plumbago*
Species	-	*rosea*

Distribution and morphology of *Plumbago rosea* Linn.

It is a perennial small shrub up to 2 m tall. The stems are erect, trailing or climbing, simple or branched from the base, sometimes rooting at the nodes. The leaves are alternate, simple and entire while the stipules are absent. The petiole is short and the auricles are absent. The blade narrowly ovate to elliptical ovate in shape measuring 5-15 cm × 2-8 cm. The base rounded to obtuse in shape, apex acute and papery. The inflorescence an elongated spike is raceme, many- flowered, measuring 10-30 cm long and glabrous. The bracts ovate in shape measuring 2-3 mm long, peduncle measures 2-10 cm long. The flowers bisexual, regular and pentamery. The pedicel measures 0-1 mm long, apex acuminate. The calyx tubular in shape measuring 8-9 mm long, glandular and red in colour. The corolla tube measures 2.5-4.5 cm long.

The lobes obovate in shape measuring 1.5- 3 cm in diameter, apex rounded, muctonate, purple to red in colour; stamens free, exserted, ovary superior, ellipsoid ovaid, 1- celled, style filiform, stigma lobes 5.

Preparation of extracts

For phytochemical analysis, the aerial parts of *P. zeylanica*, *P. auriculata* and *P. rosea* were collected from Papanasam (Tamil Nadu), Tenkasi (Tamil Nadu) and Dana (Tamil Nadu) respectively. The collected species of *Plumbago* were thoroughly washed with tap water followed by distilled water. The washed *Plumbago s*pecies were blotted on the blotting paper and spread out at room temperature in shade to remove the excess water contents. The shade dried plant samples were ground to fine powder using mechanical grinder. The powdered samples were stored at 4°C for further use. The dried and powdered aerial parts of *Plumbago* species were extracted successively with 30 g of plant powder and 180 ml of petroleum ether, chloroform, acetone, ethyl acetate, ethanolic and aqueous solvents by using soxhlet extractor for 8 hr at a temperature not exceeding the boiling point of the solvent. The extracts were filtered using Whattman filter paper (No.1) and then concentrated in vacuum at 40ºC using rotary evaporator. The residues obtained were stored in a freezer until further tests.

Fluorescence analysis

To reveal the physico-chemical properties of selected *Plumbago* species, the crude powder and various extracts of *P. zeylanica*, *P. auriculata* and *P. rosea* were examined under visible and UV light. The crude powder of *P. zeylanica*, *P. auriculata* and *P. rosea* were also treated with various reagents such as 50% nitric acid, 50% sulphuric acid, 1N HCl and 1N NaOH and changes in colour were recorded.

Preliminary phytochemical analysis

Petroleum ether, chloroform, acetone, ethyl acetate, ethanolic and aqueous extracts of selected *Plumbago* species were tested for the presence or absence of steroids, phenolic compounds, alkaloids, cardiac glycosides, saponins, tannins, flavonoids, carbohydrates, terpenoids, aminoacids, coumarin glycosides and anthraquinones according to the method described by Harborne (1999). The methodology for preliminary phytochemical analysis was described in Table - 2. To reveal the inter-specific variation among the selected *Plumbago* species, the preliminary phytochemical profile was converted into "1" and "0" matrix, to indicate the presence or absence of the phytoconstituents similarities, respectively. Phytochemical similarities were estimated according to Nei and Li (1979). To demonstrate the inter-specific relationship, a cladogram was constructed by UPGMA using NTSYSpc- 2.0 software.

UV-Visible spectral analysis

To find out the metabolites and functional group presence in the crude extracts, the UV-Vis spectroscopic analysis was carried out using Shimadzu spectrophotometer. For UV-Vis spectrophotoscopic analysis, the extracts were centrifuged at 3000 rpm for 10 min and filtered through Whatmann No. 1 filter paper by using high pressure vacuum pump. The sample was diluted to 1:10 with the same solvent. The filtered extracts of *P. zeylanica, P. auriculata* and *P. rosea* were scanned in the wavelength ranging from 200 - 1100 nm using Shimadzu spectrophotometer and the characteristic peaks were observed and recorded. The UV-Vis analysis was repeated twice and confirmed the spectrum. The peak values of UV-Vis spectra were used to distinguish the selected *Plumbago* species. To reveal the inter-specific similarities among the selected *Plumbago* species, the UV-Vis spectral profile was converted in to "1" and "0" matrix, to indicate the presence or absence of the absorbance in the particular nanometer,

respectively. UV-Vis spectral similarities were estimated according to Nei and Li (1979). To demonstrate the inter-specific relationship, cladogram was constructed by UPGMA using NTSYSpc- 2.0 software.

FTIR analysis

To know the functional groups, the FTIR analysis was performed using Shimadzu 8400S Spectrophotometer system. Infrared spectra of the test plants were measured using the methods cited in Harborne (1999). About 1.0 mg of the crude powder of the three *Plumbago* species were separately made into thin discs with 10 - 100 mg of potassium bromide using a mould and pressed under anhydrous conditions. The pellets were measured in an automatic recording IR spectrophotometer (Shimadzu 8400S) in the range of 667 to 4000 cm^{-1}. In addition, the crude ethanolic extracts of three *Plumbago* species were scanned. The crude extracts were measured in an automatic recording IR spectrophotometer (Shimadzu 7600) in the range of 500 to 4000 cm^{-1}. The peak values of the FTIR were recorded. Based on the Mistry (2009) guidelines, the functional groups were predicted.

To reveal the inter-specific similarities among the studied *Plumbago* species, the FTIR profile was converted in to "1" and "0" matrix, to indicate the presence or absence of peak values, respectively. FTIR similarities were estimated according to Nei and Li (1979). To demonstrate the inter-specific relationship among the selected *Plumbago* species, a cladogram was constructed by UPGMA using NTSyspc- 2.0 software.

TLC analysis

To reveal the phenolic and steroidal profile of *P. zeylanica, P. auriculata* and *P. rosea* Thin layer chromatography (TLC) was performed on 10×20 cm silica gel plates (Merck, Germany). An aliquot of various extracts of *P. zeylanica, P. auriculata* and *P. rosea* were

spotted on the silica gel plate with a developing solvent system of chloroform/methanol (9:1 v/v) for phenol and benzene and methanol (9:1 v/v) for steroid. The spots were visualized under a UV detector before and after spraying the plates with spraying reagents viz., folin-ciocalteau reagent for phenol and 5% alcoholic sulphuric acid for steroid (Harborne, 1999). The phenolic and steroidal profiles were recorded, the Rf values of the individual spots was calculated. To reveal inter-specific similarities among the selected *Plumbago* species, the phenolic and steroidal chromatographic profile was converted in to "1" and "0" matrix, to indicate the presence or absence of phenols and steroids, respectively. Phytochemical similarities were estimated according to Nei and Li (1979). To illustrate the inter-specific relationship, a cladogram was constructed by UPGMA using NTSypc- 2.0 software.

Preparation of sample solutions for HPLC / HPTLC / GC-MS analysis:

Based on the preliminary phytochemical and TLC analysis results, the ethanolic extracts of the selected *Plumbago* species was subjected to HPLC, HPTLC and GC-MS analysis. The ethanolic extracts of the selected *Plumbago* species was centrifuged at 3000 rpm for 10 min and the supernatant was used for further phytochemical analysis.

HPLC analysis

To know the functional compounds present in the ethanolic extract of the selected *Plumbago* species viz., *P. zeylanica, P. auriculata* and *P. rosea*, HPLC analysis was performed on a Shimadzu LC-10AT VP HPLC system, equipped with a model LC-10AT pump, UV-Vis detector SPD-10AT, Rheodyne injector fitted with a 20 µl loop and auto injector SIL-10AT. A Hypersil BDS C-18 column (4.6 × 250 mm, 5 µm size) with a C-18 guard column was used. The elution was carried out with gradient solvent systems with a flow rate of 1 ml min^{-1} at ambient temperature (25-28°C). The mobile phase consisted of 0.1% v/v methanol (solvent A) and water

(solvent B). The mobile phase was prepared daily, filtered through a 0.45 µm and sonicated before use. Total running time was 15 min. The sample injection volume was 20 µl while the wavelength of the UV-Vis detector was set at 254 nm (Sharanabasappa *et al*., 2007; Mallikharjuna *et al*., 2007).

Instrumentation

An isocratic HPLC (Shimadzu HPLC class *VP* series) with two LC- 0 AT *VP* pumps (Shimadzu), variable wave length programmable photo diode array detector SPD-M10A *VP* (Shimadzu), CTO- 10AS *VP* column oven (Shimadzu), SCL-10A *VP* system controller (Shimadzu) and reverse phase Luna 5µ C18 (2) Phenomenex column (250 mm X 4.6 mm) was used. The mobile phase components ethanol: water (45:55) were filtered through 0.2 µ membrane filter before use and were pumped from the solvent reservoir at a flow rate of 1 ml/min which yielded column backup pressure of 260-270 kgf / cm^2. The column temperature was maintained at 27°C. 20 µl of respective sample was injected by using Rheodyne syringe (Model 7202, Hamilton).

The standard plumbagin was also dissolved in ethanol and injected along with the ethanolic extracts of selected *Plumbago* species and the peak obtained in the standard was compared with the plant extracts which was used as a spectroscopic tool to study the inter-specific variation among the selected *P. zeylanica, P. auriculata* and *P. rosea.*

HPTLC analysis

Test solution preparation

50 mg aerial parts of *P. zeylanica, P. auriculata* and *P. rosea* dried ethanolic extracts were weighed in an electronic balance (Afcoset) and dissolved in 1 ml of ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solution for phenol, tannin, flavonoid,

and alkaloid. In the case of plumbagin standard, 50 mg of plumbagin dissolved in 1 ml of ethanol and centrifuged at 3000 rpm for 5 min.

Sample application

3 µl of test solutions and 2 µl of standard solution were loaded as 5 mm band length in the 7 x 10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase (phenol, tannin, flavonoid, plumbagin and alkaloid) and the plate was developed in the respective mobile phase up to 90 mm.

To reveal the amino acid composition of the selected *Plumbago* species, 100 mg aerial parts powder was weighed accurately in an electronic balance and transferred into labelled glass test tubes (BOROSIL). 1 ml of 6 M Hydrochloric acid solution was added with sample in specified test tubes. These test tubes were sealed at the top under vacuum by high temperature gas flame, conducted triplicates of samples. All the sealed tubes were kept in a hot air oven at 110^{o}C for 48 hrs continuously.

Test solution preparation

After completion of digestion, the tubes were broken at the top and the digest was transferred into glass beaker (borosil). The tubes were rinsed 5 times with distilled water. The acid in the digest was evaporated to core dry under vacuum using Roto-vac evaporator. The residual content was dissolved with distilled water and made-up to 2.4 ml in a centrifuge tubes.

This solution contains 41.6 µg raw sample in 1 µl distilled water and used as test solution for amino-acid profile analysis by HPTLC technique.

Sample and Standard amino acid loading

1 µl of each test solution was loaded as 4mm band in pre-coated Silica gel $60F_{254}$ TLC plate (5 cm x 10 cm) using 100 µl Hamilton syringe and CAMAG-LINOMAT 5 instrument. 1 µl of each Group I, II, III and IV standards (Table - 3) were loaded in the plate for analysis as separate tracks. The mobile phase and spraying reagents used to detect the phenolic, tannin, flavonoid, alkaloid and amino acids profile in the selected *Plumbago* species are explained in the Table - 4.

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured at visible light, UV 254 nm and UV 366 nm.

Derivatization

The developed plate was sprayed with respective spraying reagent (phenol, tannin and alkaloid) and dried at 100°C in Hot air oven. The plate was photo-documented in Visible light and UV 366nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 500 nm. The peak table, peak display and peak densitogram were noted. The software used was Win CATS 1.3.4 version.

To reveal the inter-specific similarities among the selected *Plumbago* species, the phenolic, alkaloid, amino acids, flavonoid, plumbagin and tannin chromatographic Rf values

were converted in to "1" and "0" matrix, to indicate the presence or absence of phytoconstituents respectively. Phytochemical similarities were estimated according to Nei and Li (1979). To display the inter-specific relationship, a cladogram was constructed by UPGMA using NTSyspc-2.0 software.

GC-MS analysis

To reveal the chemical constituents present in the aerial parts of the selected *P. zeylanica, P. auriculata* and *P. rosea*, the GC-MS analysis was performed using the Clarus 500 GC-MS (Perkin Elmer). 2 µl of selected *P. zeylanica, P. auriculata* and *P. rosea* ethanolic extracts were injected for GC-MS analysis (Merlin *et al.,* 2009).

The Clarus 500 GC used in the analysis employed a fused silica column packed with Elite-1 (100% dimethyl poly siloxane, 30 nm × 0.25 nm ID × 1 µm df) and the components were separated using helium as carrier gas at a constant flow of 1 ml/min. The 2 µl sample extract injected into the instrument was detected by the Turbo gold mass detector (Perkin Elmer) with the aid of the Turbo mass 5.1 software. During the 36th minute GC extraction process, the oven was maintained at a temperature of 110°C with 2 minutes holding. The injector temperature was set at 250°C (mass analyser). The different parameters involved in the operation of the Clarus 500 MS, were also standardized (Inlet line temperature: 2000°C; source temperature: 2000°C). Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 kDa. The MS detection was completed in 36 minutes.

Identification of components

The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The detection employed the NIST (National Institute of Standards and Technology) Ver.2.0 - Year 2005 library (Sangeetha and Vijayalakshmi, 2011).

Interpretation of GC-MS was conducted using the database of NIST having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components present in the test materials were ascertained. The compound biological activity prediction is based on Dr. Duke's Phytochemical and Ethnobotanical Databases by Dr. Jim Duke of the Agricultural Research Service/USDA. The biological activity spectrum of PASS was designed according to the algorithm specified below. For the compound under prediction structural descriptors are generated.

For each activity the following values are calculated.

$u_j = a_i \, \mathrm{ArcSin}\{r_i(2p_{ij}-1)\}$, $u_{0j} = a_i \, \mathrm{ArcSin}\{r_i(2p_j-1)\}$

$S_j = \mathrm{Sin}\,(u_j/m)$, $S_{0j} = \mathrm{Sin}\,(u_{0j}/m)$

$P_{rj} = (1 + (s_j - s_{0j})/ (1 - s_j s_{0j}))/2$

Antibacterial activity

Preparation of the test organisms

Staphylococcus aureus (MTCC 737)*, Streptococcus pyogenes* (MTCC 1928)*, Bacillus aureus* (MTCC 6633), *Klebsiella pneumoniae* (MTCC 109), *Morganella morganii* (MTCC 662) and *Pseudomonas aeruginosa* (MTCC 1688) were commercially purchased from Institute of Microbial Technology, Chandigarh, India. Stock cultures of different bacteria were grown in nutrient broth at 30^{o}C and were sub-cultured and maintained in nutrient broth at 4^{o}C. Before swabbing, each culture was diluted (1:10) with fresh sterile nutrient broth.

Antibacterial assay

The antibacterial activity was determined by the agar well diffusion method (Parekh and Chand, 2007). The Muller Hinton agar medium was prepared, sterilized and transferred to sterile

petriplates and allowed for solidification. A suspension of the culture organism was swabbed above the solidified Muller Hinton agar medium at 45°C. Wells were made using sterile cork borer under aseptic condition. The plant extracts were added to the wells in various concentrations viz., 20 µl, 40 µl, 60 µl, 80 µl and 100 µl. The antibiotic amikacin (30 µg /disc) was used as a standard to compare its effect on test organisms with the plant extracts. The plates were kept at room temperature for 2 h to allow diffusion of the test solution into the agar then they were incubated for 24 h at 37°C. After the incubation period, the plates were observed and zone of inhibition was measured (mm) and the activities were recorded.

Macromolecular studies

Protein isolation

To reveal the inter and intra-specific variation of the selected *Plumbago* species, the young leaves of *P. zeylanica, P. auriculata* and *P. rosea* collected from various localities were washed once in deionised water. For protein analysis, the young leaves of the selected *Plumbago* species were mashed in a pre-chilled mortar in 500 µl of phosphate buffer (pH 7.0). The resultant slurry was centrifuged at 10,000 rpm for 10 min at 4°C in a Micro 22 R centrifuge and the supernatant was stored at 4°C before use.

Protein separation (Anbalagan, 1999)

SDS-PAGE was carried out at 25°C in the air conditioned room. Separation of protein was carried out at 50V till the tracking dye reaches the separating gel and at 100V thereafter for 3-5 hours or until the tracking dye had migrated to the bottom of the gel. After electrophoresis, the gels were carefully removed from the mold and stained.

Gel mold

Two precisely cutted glass plates were clamped together with Vaseline coated 1.0 mm spacers on each side and sealed on both sides and bottom.

Gel preparation

Reagents for Gel preparation

Solution A - 30 g Acrylamide and 0.8 g Bis acrylamide in 100 ml of distilled water.

Solution B - Tris HCl 1.5M, pH 8.8

Solution C - Tris HCl 0.5M, pH 6.8

Solution D - 10% (w/v) Ammonium per sulphate

10% (v/v) separation gel consisted of 3.3ml of solution A, 2.5ml of solution B, 100µl of solution D, 4.1ml of distilled water and 10µl TEMED. The solution was mixed and poured in up to three fourth of the mold and allowed to polymerize. A thin layer of distilled H_2O was dispersed on top of the acrylamide solution to level the surface. 6% (v/v) stacking gel consisted of 0.6 ml of solution A, 1.25 ml of solution C, 50 µl of solution D, 2.1 µl distilled H_2O and 6 µl TEMED. The solution was mixed and added to the top of the separating gel after the removal of water topped over the separating gel. The comb was inserted into the top layer of the solution before it began to polymerize. The gel was allowed to polymerize and the comb was removed immediately after polymerization. The molded gel was clamped on to a vertical slab gel electrophoresis unit. The upper and lower reservoirs of the electrophoresis unit were filled with required quantity of 1.5 M Tris-glycine (pH 8.3) tank buffer (Table - 5; Table - 6).

Sample loading

50-60 µl of each sample was loaded directly into a well using a syringe. Care was taken to avoid mixing of the sample with the reservoir buffer and also to avoid cross contamination of samples. After electrophoresis the gels were stained using the method described by Sorensen *et al*. (2002) and Mortz *et al*. (2001).

Silver staining:

Methodology for silver staining:

- After electrophoresis, the gel was removed from the cassette and placed into a tray containing appropriate volume of fixing solution then the gel was soaked in this solution app. 2 h.
- The fixative solution was discarded and the gel was washed in 20% ethanol for 20 min. the solution was changed three times to remove the remaining detergent ions as well as fixation acid from the gel.
- The ethanolic solution was discarded and enough volume of the sensitizing solution was added and incubated for 2 min with gentle rotation.
- The sensitizing solution was discarded and the gel was washed twice, 1 min each time, in deionized water. Then the water was discarded.
- The cold silver staining solution was added and shaked for 20 min to allow the silver ions to bind to proteins.
- After staining, the staining solution was poured off and the gel was rinsed with a large volume of de ionized water to 20-60 sec to remove the excess of unbound silver ions. Repeat the washing once more.
- Again the gel was shortly rinsed with the developing solution and then the solution was discarded.

- To stop the reduction reaction 50 ml of terminating solution was added directly to the gel that is still immersed into developing solution. The gel was gently agitated for 10 mins. As soon as "baubling" of the solution is over, the development is stopped.
- Moist gels was kept in 12% acetic acid at 4 ^{o}C in sealed plastic bags or placed in the drying solution for 2 hr prior to vacuum drying.
- After staining the gel was viewed using a Vilber Loubermat gel documentation system and banding profiles of protein was determined by the migration from the origin towards the anode (Gromova and Celis 2006). The chemicals needed for silver staining were prepared as illustrated in Table - 7.

To reveal the inter and intra-specific similarities among the selected *Plumbago* species, the protein banding pattern of selected *Plumbago* species converted in to "1" and "0" matrix, to indicate the presence or absence of bands, respectively. Genetic similarities (GS) were estimated according to Nei and Li (1979). To illustrate inter and intra-specific relationship, a cladogram was constructed by UPGMA using NTSyspc- 2.0 software.

MALDI-TOF MS analysis

MALDI spectrum of *Plumbago* species were recorded using Applied Biosystems MALDI-TOF Voyager De-Pro spectrometer. The MALDI sample was prepared by mixing 1µL of protein sample solution and sinapic acid matrix solution (5 mg/mL sinapic acid in 50% ACN/0.1% TFA). 0.75µL of the resulting mixture was spotted onto a freshly cleaned stain less steel MALDI target plate. After air drying, the crystallized spots were processed with a MALDI-TOF mass spectrometer (Voyager DE PRO) (Applied Biosystem). MS was recorded in the positive and negative mode within a mass range from 500 to 100 kDa, using a nitrogen laser (337 nm). The acceleration voltages applied for MS was 25 kV.

DNA barcoding

For the present study, seven accessions of *P. zeylanica*, five accessions of *P. auriculata* and three accessions of *P. rosea* were examined. The plant specimens used in this study are summarized in Table - 1.

DNA was extracted from (0.01 – 0.05 g) leaves using a modified Cetyl Trimethyl Ammonium Bromide CTAB) extraction method as described in Murray and Thompson, 1980.

Procedure:

Step I

- 1 gm of *Plumbago* leaves samples were weighed and taken for the isolation of DNA.
- The young leaves samples were grounded using liquid nitrogen at -196°C to a fine powder by using mortar and pestle.
- 1% mercaptoethanol was added to the extraction buffer and warmed at 65°C for 5-10 mins.
- 1 ml of warm extraction buffer was added to the ground sample.
- Then it was thoroughly mixed to make it slurry and transferred to 3 ml eppendorf tubes.
- These tubes were incubated at 65°C for 1-2 hrs.

Chloroform extraction

- Chloroform: isopropanol mixture was prepared in 24:1 ratio and it was added in equal volume to the slurry.
- The slurry was centrifuged at 10,000 rpm for 10 min at 4°C.
- Supernatant was taken and $1/10^{th}$ volume of CTAB/NaCl and equal volume of chloroform was added.
- It was centrifuged at 10,000 rpm for 5 min at 4°C.

- To the clear (greenish yellow) supernatant, double the volume of CTAB precipitation buffer was added.
- The mixture was incubated at 37°C overnight in a water bath.

Step II

- The precipitation mixture was centrifuged at 8000 rpm for 8-10 min at 4°C.
- The pellet was collected and the supernatant was discarded.
- 1 ml of high salt TE was added to re-suspend the pellet.
- The suspended pellets were transferred to glass tubes.
- 1.0 ml (0.6 volumes) of isopropanal was added and refrigerated at -20°C for 30 min to get the pellets.
- After the precipitates were observed, the tubes were spun at 8000 rpm for 5 min at 4°C.
- The pellets were washed with 1ml of 80% ethanol by spinning at 10,000 rpm for 5 min at 4°C to remove the residual CTAB.
- The pellets were re suspended in 0.5 ml 1xTE.
- The suspended pellets were collected in micro-vials.

Step III

- The overnight incubated vials were spun at 12,000 rpm for 15 min at 4°C.
- The pellets were collected and washed with 0.5 ml of 70% ethanol.
- Centrifugation was done at 10,000 rpm for 5 min at 4°C.
- The pellets were re-suspended in 100 µl 1X TE.
- The re-suspended pellets were collected and stored at -20°C.

Verification of DNA levels in Agarose Gel Electrophoresis

- The sides of the gel tray were tapped to hold the gel while setting and well forming combs were placed with tray.
- 0.8% agarose gel was prepared by mixing 1.5 g agarose with 150 ml 1X TBE Buffer. It was boiled in a microwave oven until the agarose dissolves (1-2 minutes).
- Cooled down by holding under a running cold tap; once cooled and 6 µl of ethidium bromide was added (make sure to work in the fume hood) and swirlled to mix.
- Then the gel was poured into the tray and allowed to stand for at least 30 minutes before loading the samples. The combs are removed and placed the gel into the electrophoresis tank.
- 5 µl of Loading/Tracking dye - sucrose mixture (Bromophenol blue and Xylene cyanol) was spotted for each 25 µl of the DNA sample on a strip of parafilm.
- 5 µl of DNA was mixed using a pipette with the loading buffer and the mix was loaded

  In to a well on the gel and allowed to run for about 30 minutes at 110 Milli Amps.
- After electrophoresis the gel was viewed under the ultraviolet light box and the gel was photographed.

The chemicals required for DNA Isolation and Identification are depicted in Table - 8.

PCRs were conducted in a total reaction volume of 30 µL containing 6 µL of autoclaved ion-exchanged water, 5µL of dNTP mixture (stock of 10 mM of each dNTP), 13µL of 10 X Taq reaction buffer [200 mM Tris pH 8.8, 100 mM KCl, 100 mM (NH4)2SO4, 20mM MgSO4.7H2O, 1% (v/v) Triton X-100, 50% (w/v) sucrose, 0.25% (w/v) cresol red], 2µL of rbCL primer (0.67 mM final concentration), 2µL of Taq DNA polymerase and 2 µL of genomic DNA. The amplicon size of *rbc*L ranges from 654 bp; primers used (5'–3') were ATGTCACCACAAACAGAGACTAAAGC and GAAACGGTCTCTCCAACGCAT (Kress and

Erickson, 2007; Fazekas *et al.,* 2008). PCR Samples are loaded after mixing with gel loading dye along with 10 µl of DNA Ladder. (100 bp DNA Ladder: (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp). The chemicals required for DNA isolation and amplification (Table - 9)

The PCR for *rbc*L was performed with an initial denaturation of 30 sec at 94 °C followed by 35 cycles under the following conditions: 94°C for 30 seconds, 58°C for 30 sec and 72°C for 30 sec, terminated by an extension of 72°C for 5 min.

Cycle Sequencing

PCR *rbc*L products were sequenced after simple purification and the DNA samples were sequenced according to the method originally described by Sanger's method (1977) on an ABI Prism 3100-Avant Genetic Analyzer (USA) The electrophoresis was runned at 50V till the dye reaches three fourth distance of the gel.

Gel viewed in UV Transilluminator and the banding pattern was observed and the obtained sequence was aligned using the BioEdit sequence alignment editor version 7.0.4.1.

Sequencing and Alignment

The sequences from each DNA region were aligned by CLUSTAL W and MULTALIN software tool. The nucleotide sequence data of the partial *rbc*L sequence spacer were deposited in the GenBank nucleotide sequence databases (NCBI) and the accession numbers are given in the result. The genetic distance and phylogenetic trees were calculated and constructed using MEGA 6.0 software. The construction of phylogenetic tree was based on the (Tamura *et al.,* 2013) genetic distance model.

Phylogenetic analysis

Phylogenetic analysis involved the nucleotide sequences of three selected *Plumbago* species collected from different localities of South India. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 619, 602, and 590 in the final dataset of intra-specific sequence analysis of *P. zeylanica, P. auriculata, P. rosea.* The inter-specific sequence analysis of the selected *Plumbago* species showed 613 positions between three *Plumbago* species. The evolutionary distances were computed using the Maximum Ccomposite likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA 6 (Tamura *et al.,* 2013) in four different methods viz., Neighbor Joining method, maximum parsimony, minimum evolution method and UPGMA method.

Neighbor joining method

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.00340044 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Maximum parsimony analysis

The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 1722 is shown. The Maximum parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates).

Minimum evolution method

The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The optimal tree with the sum of branch length = 6.15550592 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree.

UPGMA method

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 6.13286675 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

To reveal the inter-specific variation among the three selected species of *Plumbago* viz., *P. zeylanica, P. auriculata* and *P. rosea* the phytochemical analysis was carried out. The phytochemical studies include the preliminary phytochemical analysis, fluorescence analysis, spectroscopic and chromatographic analysis such as UV-Vis, FTIR, TLC, HPLC, HPTLC and GC-MS. In addition, the biochemical and molecular analysis viz., SDS-PAGE, MALDI-TOF MS and DNA barcoding were also performed to disclose inter and intra-specific variation among the selected *Plumbago* Species. The antibacterial activity was studied to compare the bioactive potential of the three studied *Plumbago* species.

Preliminary phytochemical analysis

Among the six solvents used for extraction of metabolites in *P. zeylanica*, the phytochemical analysis revealed the presence of more number of metabolites in ethanolic extract of *P. zeylanica* (69%) followed by chloroform extract (46%) and ethyl acetate extract (38%). The least percentage of metabolites (8%) was observed in acetone extract of *P. zeylanica* (Fig. 1). Among the tested extracts of *P. auriculata,* the ethanolic extracts of *P. auriculata* showed highest number of metabolites (69%) existence. Subsequently, chloroform and petroleum ether extract showed the occurrence of metabolites with 54% and lowest percentage (8%) of metabolites presence was reported in the aqueous extract of *P. auriculata* (Fig. 1).

Among the six screened extracts of *P. rosea*, maximum frequency (77%) of metabolites were observed in the ethanolic extract of *P. rosea*, the acetone extract showed forty six percentage of occurence, followed by petroleum ether and ethyl acetate extracts (23%). The chloroform and aqueous extracts illustrated the least percentage (15) of metabolites presence

(Fig. 2). The cladogram constructed based on the existence of phytoconstituents showed two clusters, of which Cluster 1 (C_1) was shared by *P. zeylanica* and *P. auriculata*. Cluster 2 (C_2) includes *P. rosea* only and showed 100% of divergence from other two studied species (Fig. 3).

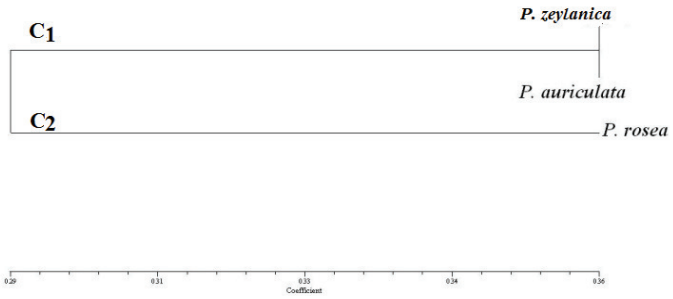


Fig. 3: UPGMA cladogram based on the preliminary phytochemical analysis of the selected *Plumbago* species

Fluorescence analysis

The fluorescent characteristics of the *Plumbago* species were observed under UV and Day light. The results are depicted in the Fig. 4.

UV-Vis analysis of *Plumbago zeylanica*

UV-Vis spectrum showed two diverse regions in the visible and UV region. UV region ranges from 200- 340 nm where as visible region ranges from 380- 900 nm (Siek *et al.,* 1976). Here the qualitative UV-Vis absorption profile of different extracts of *Plumbago* species was selected at the wavelengths from 190 to 900 nm due to sharpness of the peaks and proper baseline. The absorbance reveals the concentration of compounds present in the expressed nanometer.

Among the six different extracts examined, chloroform extract of *P. zeylanica* exhibited eight peaks which denotes the presence of greatest number of compounds compared to other extracts followed by ethyl acetate extract (7), ethanolic extract (6), petroleum ether extract (5) and acetone extract showed least number of (4) peaks compared to other extracts.

Chloroform extract of *P. zeylanica* showed highest absorbance (2.659) in the visible region at 416 nm and the lowest absorbance was observed in the ethanolic extract (0.046) at 605 nm in the visible region. The ethyl acetate extract of *P. zeylanica* represented highest absorbance 1.497 at 295 nm in the UV region and lowest absorbance 0.611 in the UV region at 319 nm (Table - 10; Plate V).

UV-Vis analysis of *Plumbago auriculata*

Chloroform extract of *P. auriculata* expressed more peaks (8), next to that petroleum ether, acetone and ethyl acetate extracts of *P. auriculata* illustrated (5) peaks. The ethanolic extract depicted least number of peaks (4) compared to other extracts. Acetone extract of *P. auriculata* represented highest absorbance of 3.675 at 400 nm in the visible region and the lowest absorbance of 0.21 at 1086 nm in the visible region. Ethyl acetate extract of *P. auriculata* depicted absorbance of 1.202 at 303 nm in the UV region (Table - 11; Plate VI).

UV-Vis analysis of *Plumbago rosea*

Petroleum ether extract of *P. rosea* displayed more number of peaks (6) followed by chloroform and acetone extracts which showed 5 peaks. Ethyl acetate and ethanolic extracts expressed the least number of peaks (4) ethanolic extract of *P. rosea* showed highest absorbance value of 4.000 at 320 nm in the UV region. The acetone extract of *P. rosea* showed highest absorbance of 2.683 at 411 nm and the ethyl acetate extract illustrated lowest absorbance of 0.099 at 558 nm in the visible region compared to other *P. rosea* extracts (Table - 12; Plate VII).

UV-Vis analysis of plumbagin

The petroleum ether, chloroform, acetone, ethyl acetate and ethanolic extract spectrum of plumbagin showed only one peak. The peaks are observed in the nm viz., 406, 408, 409, 411 and 416 with 2.111, 1.668, 2.352, 1.928 and 1.384 respectively (Plate VIII). The plumbagin

dissolved in aqueous extract failed to show the absorption at UV-Visible regions. The UV-Vis absorption spectra of *P. auriculata, P. rosea* aerial parts petroleum ether, chloroform, acetone, ethyl acetate overlap withthat of plumbagin, which confirms the presence of plumbagin. The UV-Vis absoption spectra of *P. zeylanica* aerial parts of *P. zeylanica* aerial parts petroleum ether, chloroform, acetone, ethyl acetate and overlap with that of plumbagin, which confirms the plumbagin presence in the aerial parts of *P. zeylanica.*

Similar to the preliminary phytochemical analysis, the cladogram constructed based on UV- Vis spectrum also showed two clusters, of which Cluster 2 (C_2) includes only one species viz., *P. rosea* showed 100% of divergence from the other two species under study. Cluster 1 (C_1) showed two nodal (N) branches, (C_1N^1 and C_1N^2). C_1N^1 was *P. zeylanica* and C_1N^2 was *P. auriculata* (Fig. 5).

The result of UV-Vis spectroscopic analysis directly coincides with the results of the preliminary phytochemical analysis and confirms the relationship between the presence of metabolites and number of peaks present.

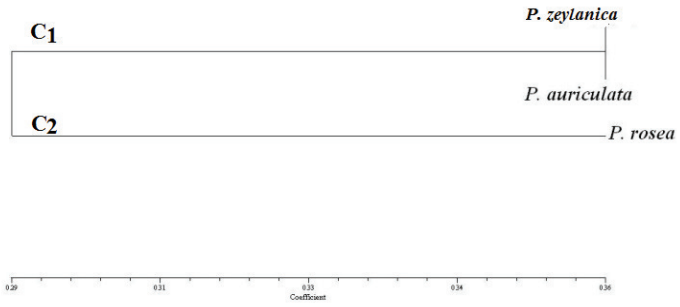


Fig. 5: UPGMA cladogram based on the UV-Vis spectroscopic profile of the selected *Plumbago* species

FTIR spectroscopic profile

The FTIR spectrum and peak values were used to identify the functional group of the active metabolites present in the crude powder / extracts. The crude powder and the ethanolic

extract of *P. zeylanica, P. auriculata* and *P. rosea* were passed into the FTIR and the functional groups of the components were identified based on its peak ratio and compared with the standard plumbagin. The results are demonstrated in Table - 13 and Plate IX.

In the ethanolic extract of selected *Plumbag*o species the presence of 40 different peak values ranging from 514.96 - 3631.71 were observed (Table - 13). The maximum number of functional groups has been observed in ethanolic extract of *P. auriculata* when compared to the other extracts. The peak values 1454.23 and 1334.65 with the functional group alkanes and sulfones showed their presence in plumbagin; alkanes and sulfones were observed in the ethanolic extracts and crude powder of *P. zeylanica, P. auriculata* and *P. rosea*. The ethanolic extracts of *P. auriculata* and *P. rosea* mutually shared the peak values 665.40, 2314.42 and 3315.41 which was present in the standard plumbagin but absent in the ethanolic extract of *P. zeylanica*.

The crude powder of selected *Plumbago* species showed different peak values viz., 3313.48, 3195.76, 2360.71, 1668.31, 1450.37, 1400.22, 1334.65, 1195.78, 1112.85,752.19, 653.82 and 605.61 (Plate VIII). This confirmed the existence of various functional groups such as amides, phosphorus compounds, carbonyl compounds, phenols, carboxylic acid, alcohols, unsaturated aliphatic carbonyl compounds, sulphur compounds and halogen compounds. *P. auriculata* and *P. rosea* showed an additional peak at 806.19 confirming the presence of a benzene ring with two adjacent H atoms which was absent in *P. zeylanica.*

The ethanolic extract of *P. zeylanica*, *P. auriculata* and *P. rosea* showed different peak values 1031.85, 1334.65, 1454.23, 1645.17, 2171.70 and 2600.04 (Table - 13). The peak values presence confirmed the existence of functional groups viz., sulfoxides, sulfones, sulfonamides (S=O Str.), alkanes (-CH_2-, C-H DEF), intra molecular H-bounded aldehydes (C=O Str.), boron

compounds and organo-phosphorus compounds (O-H Str.). The functional groups such as halides, amino acids, charged amines, alkanes, alcohols and phenols single bridge compound (O-H Str.) showed their unique presence only in the ethanolic extract of *P. auriculata* which was absent in other two studied *Plumbago* species viz*., P. zeylanica* and *P. rosea.*

The cladogram constructed based on FTIR analysis showed two clusters, of which Cluster 2 (C_2) represents *P. auriculata* which showed 100% of divergence from the other two studied species. Cluster 1 (C_1) was shared by *P. zeylanica* and *P. rosea.* The similarity indices showed that *P. auriculata* represented varied functional groups while *P. zeylanica* and *P. rosea* showed similar functional groups (Fig. 6).

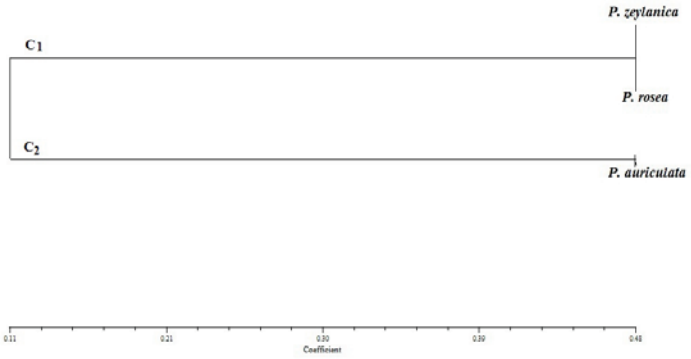


Fig. 6: UPGMA cladogram based on the FTIR spectroscopic profile of the selected *Plumbago* species

TLC studies on *Plumbago* species

The selected *Plumbago* species totally expressed 80 phenolic bands; among these the maximum numbers of bands (55) were observed in *P. rosea* followed by *P. auriculata* (50) and *P. zeylanica* (47) with varied Rf values ranging from 0.10 to 0.99 (Table – 14; Plate X). The chromatographic profile showed the similarities and variation between the three species. Similar to the phenolic profile, the steroid TLC system of selected *Plumbago* species also showed 72 bands. Of these, 31 bands were observed in the ethanolic extract of *P. rosea*, followed by *P.*

auriculata with 28 bands and *P. zeylanica* with 22 bands and varied Rf values ranged from 0.03 to 0.99. Among the various solvents screened, ethanolic and petroleum ether extracts of *Plumbago* species showed higher number of phenolic (13) and steroidal (21) bands with varied Rf values ranged from 0.03 to 0.99. The results of phenolic and steroids profile clearly bring out the similarities and variation among the three *Plumbago* species under study.

The selected *Plumbago* species expressed eighty phenolic bands with different Rf values. The petroleum ether extract of *Plumbago* species expressed eleven phenolic bands. *P. zeylanica* illustrated three unique bands with Rf values 0.35, 0.44 and 0.62. Similarly *P. auriculata* exhibited three distinct phenolic bands with Rf values 0.60, 0.65 and 0.72 and *P. rosea* depicted three restricted phenolic bands with Rf values viz., 0.40, 0.53 and 0.58. The phenolic band with Rf value 0.50 was shared by the three studied *Plumbago* species.

The chloroform extracts of three *Plumbago* species revealed fifteen phenolic bands with varied Rf values. *P. zeylanica* showed three unique bands with Rf values 0.25, 0.33 and 0.35. *P. auriculata* expressed two isolated bands with Rf values 0.10 and 0.53. *P. rosea* determined two distinct bands with Rf values 0.30 and 0.45. The phenolic bands with Rf values 0.40 and 0.58 were commonly present in the three studied *Plumbago* species. The phenolic band with Rf value 0.48 was shared by *P. zeylanica* and *P. auriculata* whereas the universal band with Rf value 0.38 was jointly shared by *P. auriculata* and *P. rosea.*

The acetone extract of three *Plumbago* species revealed fourteen phenolic bands. *P. zeylanica* showed three distinct bands with Rf values 0.22, 0.29 and 0.48. *P. auriculata* expressed three exclusive bands with Rf values 0.44, 0.50 and 0.56. *P. rosea* depicted three unique bands with Rf values 0.27, 0.45 and 0.58. The phenolic band 0.38 was shared by *P.*

zeylanica and *P. auriculata*. Similarly, the phenolic band with Rf value 0.30 showed its presence in *P. auriculata* and *P. rosea.*

The ethyl acetate extracts of three *Plumbago* species expressed fourteen phenolic bands with varied Rf values. *P. zeylanica* showed three unique bands with Rf values 0.22, 0.35 and 0.40. *P. auriculata* expressed two distinct bands with Rf values 0.38 and 0.65. *P. rosea* determined three individual bands with Rf values 0.42, 0.50 and 0.70. The phenolic bands with Rf values 0.44 and 0.48 showed its presence jointly in *P. zeylanica* and *P. auriculata* whereas the band with Rf value 0.60 was shared by *P. auriculata* and *P. rosea.*

The ethanolic extracts of three *Plumbago* species depicted seventeen phenolic bands. *P. zeylanica* showed two distinct bands with Rf values 0.40 and 0.50. *P. auriculata* expressed a single band with Rf values 0.60. *P. rosea* revealed four individual bands with Rf values viz., 0.20, 0.30, 0.42 and 0.45. The phenolic band with Rf values 0.44 was commonly present in *P. zeylanica* and *P. auriculata.*

The phenolic bands with Rf values 0.30, 0.33 0.53 and 0.58 showed its presence jointly presence in *P. zeylanica* and *P. rosea*. The phenolic band with Rf value 0.38 was commonly present in all the three studied *Plumbago* species. The results are illustrated in Table – 14; Plate X.

A total of seventy two steroidal bands were observed in the steroidal TLC system of the *Plumbago* species (Table – 15; Plate XI). The petroleum ether extracts of *Plumbago* species depicted twenty steroidal bands with varied Rf values. *P. zeylanica* illustrated four distinct bands with Rf values 0.12, 0.41, 0.55 and 0.58 while *P. auriculata* showed seven different bands with Rf values 0.03, 0.16, 0.26, 0.60, 0.68, 0.81 and 0.90. *P. rosea* revealed three unique steroidal bands with Rf values 0.05, 0.46 and 0.63. The steroidal band with Rf value 0.50 showed its

presence jointly in *P. zeylanica* and *P. auriculata*. The steroidal band with Rf value 0.14 was shared by *P. auriculata* and *P. rosea.* The steroidal band with Rf value 0.25 was showed its presence in all the three studied *Plumbago* species.

The chloroform extracts of three *Plumbago* species revealed sixteen steroidal bands with different Rf values. *P. zeylanica* showed a single distinct band with Rf value 0.78. *P. auriculata* expressed eight different steroidal bands with Rf values 0.07, 0.14, 0.23, 0.30, 0.44, 0.57, 0.90 and 0.75. *P. rosea* depicted two separate bands with Rf values 0.25 and 0.41. The steroidal band with Rf value 0.65 was shared by *P. zeylanica* and *P. auriculata* whereas the band with Rf value 0.68 showed it's jointly presence in *P. auriculata* and *P. rosea.* The steroidal band with Rf value 0.16 was common to *P. zeylanica* and *P. rosea*.

The acetone extracts of three *Plumbago* species showed the presence of fourteen steroidal bands with varied Rf value. *P. zeylanica* illustrated four distinct bands with Rf values 0.03, 0.14, 0.16 and 0.60, *P. auriculata* expressed three restricted bands with Rf values 0.20, 0.32 and 0.68 and *P. rosea* depicted five discrete bands with Rf value 0.05, 0.12, 0.23, 0.25 and 0.50. The steroidal band with Rf value 0.30 showed its presence in *P. auriculata* and *P. rosea.*

The ethyl acetate extracts of three *Plumbago* species illustrated sixteen steroidal bands with different Rf values. *P. zeylanica* showed three unique bands with Rf values 0.03, 0.32 and 0.38. *P. auriculata* expressed four separate steroidal bands with Rf values 0.52, 0.57, 0.58 and 0.65 and *P. rosea* depicted four individual bands with Rf values 0.14, 0.70, 0.81 and 0.90. The steroidal bands 0.20, 0.30 and 0.50 were showed their common existence in *P. zeylanica* and *P. rosea.*

Seventeen steroidal bands were reported in the ethanolic extracts of the selected *Plumbago* species. *P. zeylanica* represented with two unique bands 0.03 and 0.14. *P. rosea*

revealed six individual bands with Rf values 0.07, 0.12, 0.16, 0.23, 0.30 and 0.44. The steroidal band with 0.68 of showed its occurrence in all the three studied *Plumbago* species. The steroidal band with Rf value 0.20 was shared by *P. zeylanica* and *P. rosea* (Table – 15; Plate XI).

The cladogram constructed based on the phenolic and steroidal TLC banding profile of *Plumbago* species showed two clusters. Cluster 1 (C_1) was shared by *P. auriculata* and *P. zeylanica* and Cluster 2 (C_2) includes only one species viz., *P. rosea* showed 100% of divergence from other two studied species (Fig. 7). The phenolic and steroid profile showed the relationship and variation of the *Plumbago* species. These profiles can act as pharmacognostical marker in the pharmaceutical industries to distinguish the medicinal plants from its adulterants.

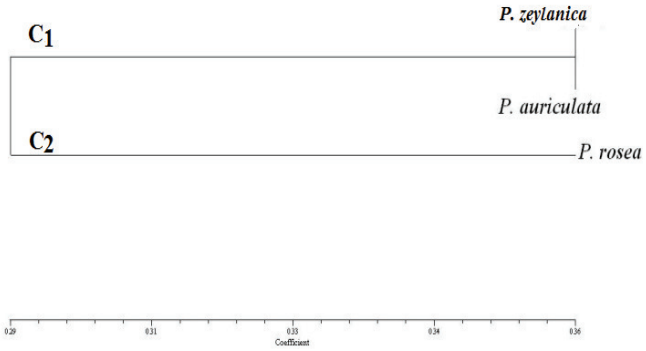


Fig.7: UPGMA cladogram based on the TLC banding pattern of the selected *Plumbago* species

HPLC Analysis

The HPLC chromatogram of ethanolic extracts of *P. zeylanica, P. auriculata* and *P. rosea* was selected based on sharpness of the peaks and proper baseline. Ethanolic extract prepared by hot extraction method was subjected to HPLC for the separation and identification of constituents present in the selected *Plumbago* species. Two compounds were separated in *P. zeylanica* at different retention time's viz., 2.167 and 2.717 min respectively. In *P. auriculata,* two compounds was separated at different retention times i.e. 1.397 and 3.140 min respectively.

Two compounds were separated at different retention times in *P. rosea* viz., 1.357 and 3.147 min respectively. HPLC chromatogram of ethanolic extract of standard plumbagin showed a prominent peak at a retention times 3.127 min (Plate XII) which was used as a marker to compare the presence of plumbagin in the ethanolic extract of the selected *Plumbago* species. Finally, compared to the three *Plumbago* species, the peak with retention time 3.127 was expressed in the ethanolic extract of standard plumbagin spectrum illustrating the highest peak area of 43.321. Similarly, the spectrum with retention time 3.140 and 3.147 was expressed in the ethanolic extract of *P. auriculata P. rosea* and *P. zeylanica* depicting the peak area of 10.942, 6.984 and 1.245 which was more are less similar to the peak retention time of standard plumbagin (3.127 RT). From the HPLC chromatogram of the selected *Plumbago* species with the marker plumbagin showed that the compound plumbagin was uniquely present in *P. auriculata* and *P. rosea* but low in *P. zeylanica.*

The cladogram constructed based on the HPLC chromatographic profile of *Plumbago* species showed two clusters. Cluster 1 (C_1) was shared by *P. auriculata* and *P. rosea* and Cluster 2 (C_2) includes only one species viz., *P. zeylanica* showed 100% of divergence from other two studied species (Fig. 8). These profiles can act as chromatographic marker in the pharmaceutical industries to distinguish the medicinal plants from its adulterants.

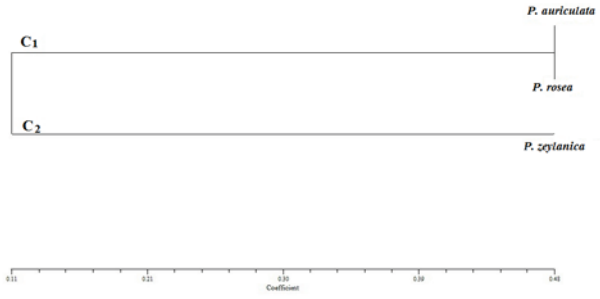


Fig. 8: UPGMA cladogram based on the HPLC banding pattern of the selected *Plumbago* species

HPTLC - phenolic banding profile of *Plumbago* species

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using Toluene-Acetone-Formic acid (4.5:4.5:1) as the mobile phase (Table - 4). The ethanolic extracts of *P. zeylanica, P. auriculata* and *P. rosea* showed the presence of 29 diverse type of phenols with 17 different Rf values ranged from 0.04 to 0.94 (Table – 16; Plate XIII). In general more degree of phenol diversity was observed in ethanolic extract of *P. rosea.* Ethanolic extract of *P. zeylanica* (9) and *P. auriculata* (9) was displayed in Table 16. Among the 29 different types of phenols, three phenols (0.62, 0.73 and 0.94) showed their presence in all the studied species (*P. zeylanica, P. auriculata* and *P. rosea*). The phenol with values 0.16 showed its presence commonly in the ethanolic extracts of *P. auriculata* and *P. rosea*. The phenols with Rf value 0.05, 0.10 and 0.36 were shared by the ethanolic extract of *P. zeylanica* and *P. auriculata* (Table - 16).

HPTLC - Tannin banding profile of *Plumbago* species

In order to obtain high resolution and reproducible peaks, various compositions of the mobile phase were tested. The mobile phase Toluene-ethyl acetate-formic acid-methanol (3:3:0.8:0.2) showed high resolution compared to other compositions (Table - 4). The ethanolic extract of *Plumbag*o species showed the presence of 26 different types of tannins with 20 different Rf values ranged from 0.04 to 0.95 (Table – 17; Plate XIV). In general more degree of tannins diversity was observed in ethanolic extract of *P. rosea* when compared to the other species. The maximum number of 12 tannins has been observed in the ethanolic extract of *P. rosea* (Table - 17) followed by ethanolic extract of *P. zeylanica* (9). Among the 26 different types of tannins, the Rf value 0.62 showed its presence in all the three examined species viz., *P. zeylanica, P. auriculata* and *P. rosea* (Table - 17). Tannin with Rf value 0.04 showed its

presence jointly in the ethanolic extracts of *P. auriculata* and *P. rosea*. The tannin with Rf value 0.94 was shared by the ethanolic extract of *P. zeylanica* and *P. auriculata.* The tannins with Rf value 0.32, 0.53, 0.58 and 0.94 displayed their occurrence only in the ethanolic extract of *P. zeylanica*. The tannin profile of three *Plumbago* species displayed both known and unknown tannin in the chromatographic system (Table - 17).

HPTLC - Alkaloid Banding profle of *Plumbago* species

Mobile phases of different compositions were tested in order to obtain high resolution and reproducible peaks. The desired aim was attained using Ethyl acetate-methanol-water (10:1.35:1) as the mobile phase (Table - 4). The ethanolic extract of *Plumbag*o species showed the presence of 25 diverse type of alkaloids with 16 different Rf values ranged from 0.01 to 0.91 (Table – 18; Plate XV). In general more degree of alkaloid diversity was observed in ethanolic extract of *P. rosea* when compared to the other species. Maximum number (10) of alkaloids was observed in ethanolic extract of *P. rosea* (Table - 18), followed by *P. zeylanica* and *P. auriculata* with 7 and 8 alkaloid bands. Among the 25 different types of alkaloids, two alkaloids (0.44 and 0.91) showed their presence in all the three *Plumbago* species. The alkaloid (0.10) showed its presence jointly in the ethanolic extracts of *P. auriculata* and *P. rosea* (Table - 18). The alkaloid profile of three *Plumbago* species displayed both known and unknown alkaloids in the chromatographic system.

HPTLC- Flavonoid banding profle of *Plumbago* species

The high resolution and reproducible peaks were obtained by using the mobile phase Ethyl acetate-Butanone-formic acid-water (5:3:1:1). The ethanolic extract of *Plumbago* species showed the presence of 22 different types of flavonoids with 17 different Rf values ranged from 0.07 to 0.96 (Table – 19; Plate XVI). In general more degree of flavonoids diversity was observed in

ethanolic extract of *P. rosea* when compared to the other species. Maximum number (10) of flavonoids was illustrated in ethanolic extract of *P. rosea* (Table - 19; Plate XVI) followed by ethanolic extract of *P. zeylanica* (6) and *P. auriculata* (6). Among the 22 different types of flavonoids, flavonoid with Rf value 0.07 showed its presence in all the three tested species of *Plumbago* viz., *P. zeylanica, P. auriculata* and *P. rosea*; flavonoid bands with Rf values 0.51 and 0.94 were mutually shared by the ethanolic extract of *P. zeylanica* and *P. auriculata* (Table - 19). Similar to that, the ethanolic extracts of *P. auriculata* and *P. rosea* also shared the flavonoids (0.60 and 0.94). The flavonoid with Rf value 0.32, 0.53 and 0.58 displayed their presence in the ethanolic extract of *P. zeylanica*. The flavonoid profile of three *Plumbago* species were displayed both known and unknown flavonoids in the chromatographic system (Table - 19).

HPTLC - Aminoacid profle of *Plumbago* species

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using n-Butanol-Acetic acid-water (3:1:1) as the mobile phase (Table - 4), Ninhydrin was used as a spraying reagent and the amino acids lysine, asparagine, glutamine, glutamic acid, methionine, proline, serine, cystine, tyrosine, tryptophan, histidine, arginine, aspartic acid, threonine, leucine, glycine, alanine, valine, isoleucine and phenyl alanine were employed as standard (Table - 3). *P. zeylanica, P. auricuata* and *P. rosea* showed the presence of diverse type of 20 amino acids with 17 different Rf values ranged from 0.17 to 0.67 (Table - 20). In general more amino acid diversity was observed in *P. auriculata* and *P. rosea* when compared to the *P. zeylanica*. Maximum number (8) of amino acids were illustrated in *P. auriculata* and *P. rosea* (Table - 20; Plate XVII) followed by *P. zeylanica* (7). Among the 20 different types of amino acids, five amino acids showed their presence in all the three *Plumbago* species. The amino acids with Rf

value (0.45) jointly showed its presence in *P. zeylanica* and *P. auriculata.* The amino acid with Rf value (0.27) demonstrated its presence in *P. rosea* and *P. auriculata*. In addition to the qualitative analysis, they were estimated quantitatively and the results were tabulated in Table 21, 22 and 23.

HPTLC - Plumbagin Banding profle of *Plumbago* species

To identify the plumbagin present in the aerial parts of the selected *Plumbago* species viz., *P. zeylanica, P. auriculata* and *P. rosea,* a specific HPTLC analysis was performed using the mobile phase toluene - formic acid and 10% methanolic acid as spraying reagent. The presence of plumbagin was confirmed by the presence of similar Rf value of 0.52 in the standard plumbagin and all the three ethnalolic extract of *Plumbago* species viz., *P. zeylanica, P. auriculata* and *P. rosea* with varying percentage of plumbagin (0.48%, 0.76% and 1.399%) which represents the quantity of plumbagin presence in the ethanolic extract of the studied *Plumbago* species. Finally it was identified, that the ethanolic extract of *P. rosea* (1.399%) revealed the highest amount of plumbagin (Plate XVIII).

The cladogram based on HPTLC fingerprint profile showed two clusters, of which Cluster 2 (C_2) includes only one species viz., *P. rosea* showed 100% divergence from other two studied species. Cluster 1 (C_1) was shared by *P. zeylanica* and *P. auriculata* (Fig. 9). The similarity indices showed that *P. zeylanica* and *P. auriculata* showed similar metabolite expression where as *P. rosea* showed high percentage of variation.

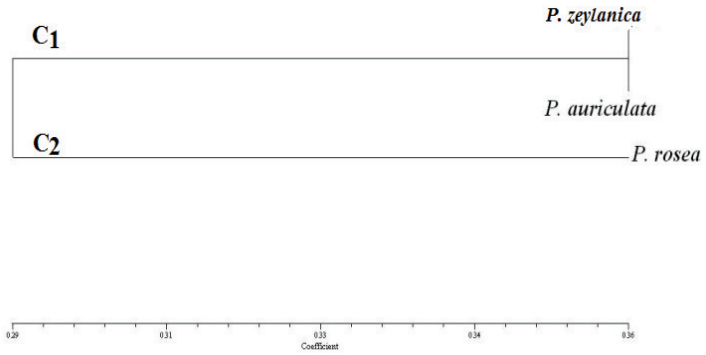


Fig. 9: UPGMA cladogram based on the HPTLC banding pattern of the selected *Plumbago* species

GC-MS analysis of *Plumbago zeylanica* ethanolic extracts

The results of GC-MS analysis leads to the identfitication of 27 different phytocompounds in the ethanolic extracts of *P. zeylanica.* Wide range of RT ranged from 14.04 - 45.60 min and their structures and nature were identified based on the mass spectrometry and FTIR peak values. The identified compound bioactivities were predicted based on online PASS Prediction. The results of *P. zeylanica* ethanolic extracts GC-MS analysis revealed the presence of few prominent compounds viz., Dihydro-4b-methyl-8-chloro-11H-isoindolo [2, 1-a] benzimidazol-11-one, 2, 3-dihydroxypropyl ester, 9-Octadecenoic acid (Z)-(CAS) and Hexadecanoic acid, methyl ester (CAS) with high percentage of peak area 26.90, 15.54, 11.90 and 8.87 respectively. The online PASS prediction results revealed the laryngospasm activity, reproductive dysfunction, anti hepatitis activity and demyelination potentials for Dihydro-4b-methyl-8-chloro-11H-isoindolo [2, 1-a] benzimidazol-11-one, optic neuritis, analeptic and optic neuropathy for 2, 3-dihydroxypropyl ester, stroke treatment, neurologic disorder treatment respiratory failure and anaemia for 9-Octadecenoic acid (Z)-(CAS) and antineoplastic, reproductive dysfunction, thromboxane synthase stimulant and JAK2 expression inhibitor for

Hexadecanoic acid, methyl ester (CAS). The other phytoconstituents and their bio-potentials were represented in Table - 2; Fig.10.

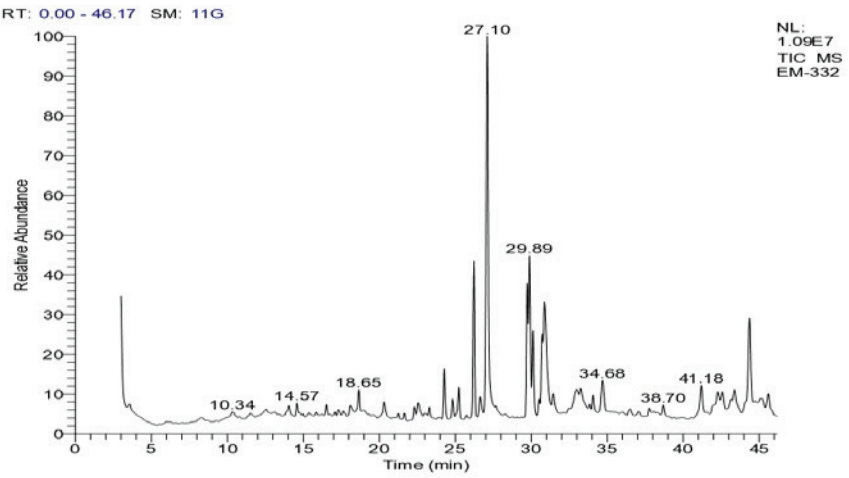


Fig.10: GC-MS chromatogram of *Plumbago zeylanica* ethanolic extract

GC-MS analysis of *Plumbago auriculata* ethanolic extracts

The results of GC-MS analysis leads to the identfitication of 27 different phytocompounds in the ethanolic extracts of *P. auriculata.* Wide range of RT ranged from 3.04 - 45.04 min and their structures and nature were identified based on the mass spectrometry and FTIR peak values ranged from and their structures and nature were identified based on mass spectrometry and FTIR peak values. The bioactivities of the compound identified were predicted based on online PASS prediction. The results of *P. auriculata* ethanolic extracts GC-MS analysis revealed only one compound isoborneol with prominent percentage of peak area 86.99. The online PASS prediction results on isoborneol revealed the optic toxicity, hypercholesteromic, analpetic, tetany, respiratory failure, optic neuropathy and reproductive dysfunction potentials. The 3- methyl-2,3- dihydro-1,4-benzothiazepin-5(4h)-one illustrated with moderate percentage of peak area with 6.21 and the online PASS prediction disclosed the anti ischemic, interleukin 1b

antagonist, antithrombotic, antiepileptic, insulin inhibitor and optic neuropathy potentials. The other identified phytoconstituents and their bio-potentials were presented in Table - 25; Fig. 11.

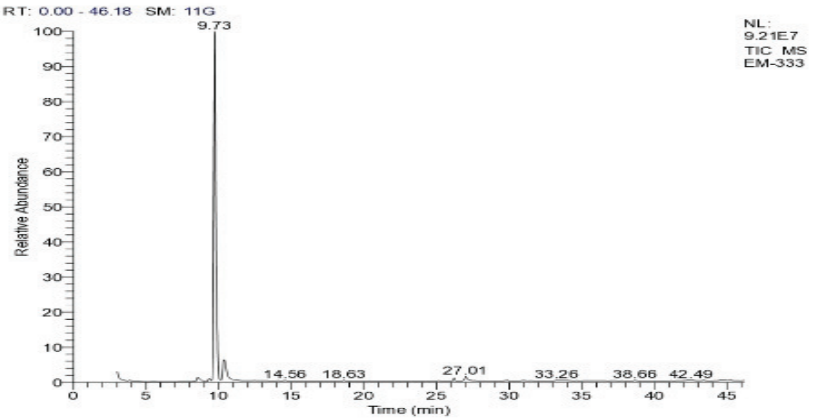


Fig.11: GC-MS chromatogram of *Plumbago auriculata* ethanolic extract

GC-MS analysis of *Plumbago rosea* ethanolic extracts

The results pertaining to GC-MS analysis on the ethanolic extracts of *P. rosea* guides to the identification of 28 different compounds with wide range of retention time (RT) ranged from 3.86 - 45.81 min and their structures and nature were recognized based on the mass spectrometry and FTIR peak values. The bioactivities of the compound identified were predicted based on Online PASS Prediction. The results of *P. rosea* ethanolic extracts GC-MS analysis revealed n-Hexadecanoic acid, phytol, 9,12,15-Octadecatrienoic acid (Z,Z,Z) and 6,9,12,15-Docosatetraenoic acid methyl ester, (CAS) presence with perdominant peak area 14.19, 7.22, 6.64 and 6.23% respectively. The online PASS prediction results disclosed the antiulcerative, CCL2 expression inhibitor, hematotoxic, sideroblastic, nephrotoxic and embryotoxic potentials for n-Hexadecanoic acid, ocular toxicity, conjunctivitis, GABA aminotransferase inhibitor, TP53 expression enhancer, antiulcer and hypotonia properties for phytol, neurotoxic, sensitization, ataxia for 9, 12, 15-Octadecatrienoic acid (Z, Z, Z) and succinate dehydrogenase inhibitor, optic neuropathy, lipoprotein lipase inhibitor, catalase inhibitor, GABA aminotransferase inhibitor for

6, 9, 12, 15-Docosatetraenoic acid methyl ester, (CAS). The other recognised phytoconstituents and their bio-potentials were illustrated in Table - 26: Fig. 12.

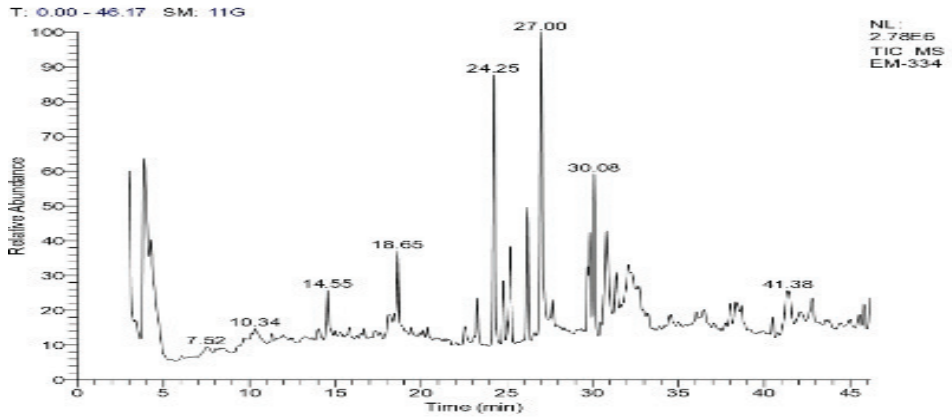


Fig.12: GC-MS chromatogram of *Plumbago rosea* ethanolic extract

The GC-MS analysis on the ethanolic extract of three selected *Plumbago* species revealed the similarities and variation among the three studied species. Out of 85 compounds identified, Hexadeconoic acid methyl ester (CAS) showed its presence in all the three studied *Plumbago* species. Whereas, n-Hexadeconoic acid displayed its occurrence in the ethanolic extract of *P. auriculata* and *P. rosea.*

Molecular Variation studies

SDS-PAGE Protein Profiling

Intra-specific protein variation in *Plumbago zeylanica*

A total of 52 bands with multiple regions of activity and MW-Rf values were observed in the protein electrophoretic system of *Plumbago zeylanica* collected from various localities of South India viz., Rasthakadu (Pz1), Palayamkottai (Pz2), Bangalore (Pz3), Papanasam (Pz4), Tiruppur (Pz5), Kuttichal (Pz6) and Coimbatore (Pz7). The *P. zeylanica* collected from Rasthakadu (Pz1), showed 5 bands, Palayamkottai (Pz2) - 8 bands, Bangalore (Pz3) - 9 bands , Papanasam (Pz4) - 7 bands, Tiruppur (Pz5) - 6 bands, Kuttichal (Pz6) - 8 bands and Coimbatore

(Pz7)- 9 bands. Among the localities, *P. zeylanica* collected from Bangalore (Pz3) and Coimbatore (Pz7) displayed maximum number of bands (9). The *P. zeylanica* collected from Rasthakadu (Pz1) showed lowest number of bands (5). The results obtained through the electrophoretic separation paved a way to identify the intra-specific similarities and variation among the studied *P. zeylanica*. The protein with MW-Rf 0.2, 0.55 and 0.98 showed its unique presence in the accessions of *P. zeylanica* collected from Kuttichal (Pz6), Rasthakadu (Pz1) and Coimbatore (Pz7) respectively. The protein bands with MW-Rf value 0.64 was shared by the accessions of *P. zeylanica* collected from Bangalore (Pz3) and Coimbatore (Pz7). The protein with MW-Rf value 0.78 showed its common presence in the accession of *P. zeylanica* collected from Palayamkottai (Pz2) and Coimbatore (Pz7). The protein with MW-Rf values 0.24 was present commonly in all the studied accessions except Coimbatore (Pz7). The protein with MW-Rf values 0.5, 0.58 and 0.91 displayed their presence in all the studied accessions except Rasthakadu (Pz1). Each region is occupied by different protein in the form of bands and is representative of the expression of a particular gene / allele of *P. zeylanica* collected from the various localities. These protein profiles can be used as a biomarker to identify the superior accession of *P. zeylanica* in the pharmaceutical industries (Table - 27; Plate XIX)

The cladogram constucted based on the protein profiles clearly explained the similarities and variation among the studied accessions of *P. zeylanica*. The cladogram constucted based on the protein profile showed two clusters (Cluster 1 and Cluster 2). The cluster 1(C_1) includes only the accession collected from Rasthakadu (Pz1) which shows 100% divergent from all the other accession of *P. zeylanica*. The cluster 2 (C_2) was further divided into two nodes viz., C_2N^1 and C_2N^2. The node C_2N^2 incorporated only the accession collected from Coimbatore (Pz7). The node C_2N^1 was further branched into two viz., $C_2N^1B_1$ and $C_2N^1B_2$. The branch $C_2N^1B_1$ included

only the accession collected from Palayamkottai (Pz2). The branch $C_2N^2B_2$ was subdivided into two separate sub nodes viz., $C^2N^1B_2b_1$ and $C_2N^1B_2b_2$. The sub node $C_2N^1B_2b_2$ illustrated only the accession of Tiruppur (Pz5). The sub node $C^2N^1B_2b_1$ was divided into two sub branches viz., $C^2N^1B_2b_1A$ and $C^2N^1B_2b_1B$. $C^2N^1B_2b_1A$ displayed the accession of Bangalore (Pz3), whereas $C^2N^1B_2b_1B$ was commonly shared by the accessions collected from Papanasam (Pz4) and Kuttichal (Pz6). The UPGMA based cluster analysis clearly showed the variation and similarity among the various accession of *P. zeylanica* (Fig. 13). The accession collected from Rasthakadu (Pz1) expressed highest variation among the studied accessions, this may be due to the ecological differences, the Rasthakadu is located nearby Kanyakumari seashore and other studied accessions are collected from near hill stations or plains.

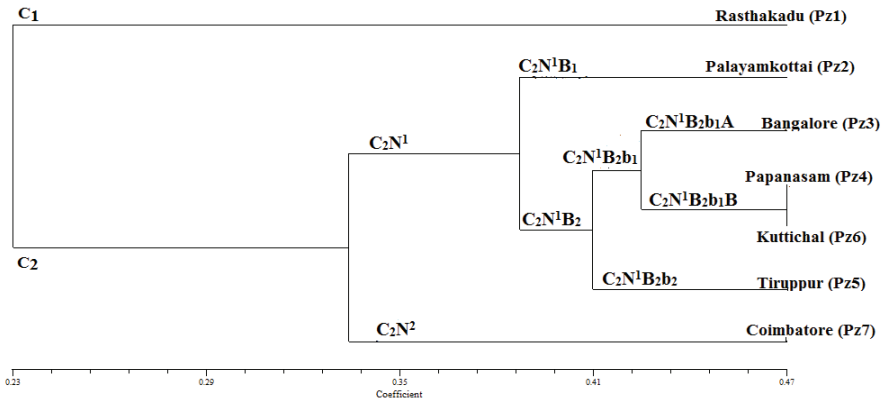


Fig.13: UPGMA cladogram based on the SDS-PAGE protein profile of *P. zeylanica*

Intra-specific protein variation in *Plumbago auriculata*

A total of 38 bands with multiple regions of activity and MW-Rf values were obtained in the protein electrophoretic system of *Plumbago auriculata* collected from various localities of South India. The *P. auriculata* collected from five different localities viz., Mulakumoodu (Pa1), Tenkasi (Pa2), Perunthurai (Pa3), Kattakadu (Pa4) and Mysore (Pa5). The *P. auriculata* collected from Mulakumoodu (Pa1) demonstrated with 7 bands, Tenkasi (Pa2) with 5 bands,

Perunthurai (Pa3) - 9 bands, Kattakadu (Pa4) - 8 bands and Mysore (Pa5)-9 bands. Among the studied localities, *P. auriculata* collected from Perunthurai (Pa3) and Mysore (Pa5) demonstrated maximum number of bands (9). The *P. auriculata* collected from Tenkasi (Pa2) expressed the lowest number of bands (5). The results obtained through the electrophoretic separation paved a way to identify the intra-specific similarities and variation among the *P. auriculata*. The proteins with MW-Rf value 0.32 and 0.60 displayed their unique presence in the accessions of *P. auriculata* collected from Mysore (Pa5). The protein with MW-Rf value 0.65 showed its presence commonly in all the accessions of *P. auriculata* except in Mysore (Pa5). The protein with MW-Rf value 0.12 and 0.70 represented their presence in all the studied accessions except in Tenkasi (Pa2). The proteins with MW- Rf 0.07 and 0.40 were expressed in *P. auriculata* collected from all the accessions. The recorded data are used to identify the similarity and variations among the *P. auriculata* collected from various localities. Each region is occupied by different protein in the form of bands and is representative of the expression of a particular gene/allele of *P. auriculata* collected from various localities. These protein profiles can be used as a biomarker to identify the superior accessions of *P. auriculata* in the pharmaceutical industries (Table – 28; Plate XIX)

The cladogram constructed based on the protein profiles visibly explained the similarities and variation among the studied accessions of *P. auriculata.* The cladogram showed two clusters (Cluster 1 and Cluster 2). Cluster 2 (C_2) includes only the accession collected from Tenkasi (Pa2) which shows 100% divergent from all the other accessions of *P. auriculata*. The cluster 1 (C_1) was further divided into two nodes viz., C_1N^1 and C_1N^2. The node C_1N^1 was further branched into two branches viz., $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ included only the accessions collected from Mulakumoodu (Pa1). The node C_1N^2 showed the individual presence

of the accession collected from Mysore (Pa5). The branch $C_1N^1B_2$ was includes Perunthurai (Pa3) and Kattakadu (Pa4). The UPGMA based cluster analysis clearly showed the variation and similarity among the various accession of *P. auriculata* (Fig. 14). The accession collected from Tenkasi (Pa2) expressed highest variation among the studied accessions; this may due to ecological differences.

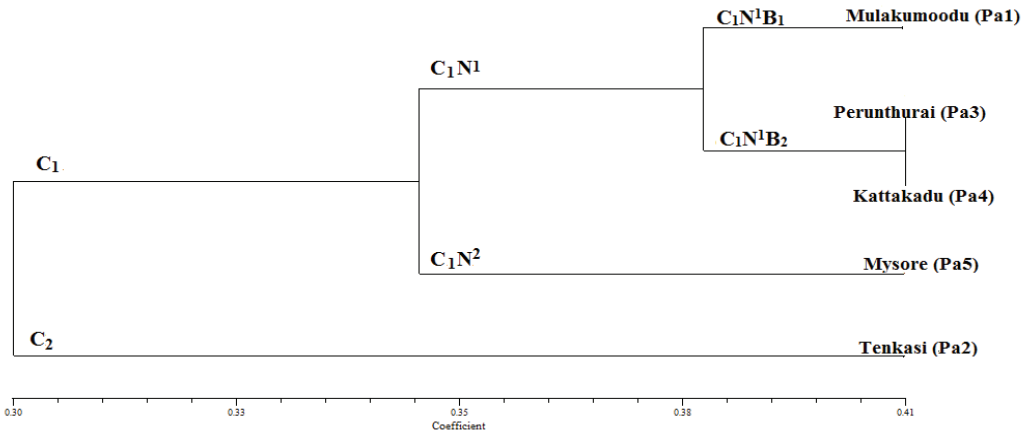


Fig. 14: UPGMA cladogram based on the SDS-PAGE protein profile of *P. auriculata*

Intra-specific protein variation on *Plumbago rosea*

A total of 21 bands with multiple regions of activity and MW-Rf values were obtained from protein electrophoretic system of *Plumbago rosea* collected from various localities of South India viz., Cheruvarankonam (Pr1), Dana (Pr2) and Bangalore (Pr3). The *P. rosea* collected from Cheruvarankonam (Pr1) demonstrated with 5 bands, Dana (Pr2) and Bangalore (Pr3) illustrated with 8 bands. The protein with MW-Rf 0.53 and 0.84 showed its unique presence in Dana (Pr2) and Bangalore (Pr3) respectively. The Protein bands with MW-Rf value 0.4, 0.44 and 0.5 illustrated their distinctive presence in Dana (Pr2) and Bangalore (Pr3). The protein with MW-Rf value 0.28 was mutually shared by the accessions collected from Cheruvarankonam (Pr1) and Dana (Pr2). The proteins with MW-Rf values 0.04, 0.34 and 0.58 displayed their common

presence in all the three studied localities. Each region is occupied by different protein in the form of bands and is representative of the expression of a particular gene / allele of *P. rosea* collected from the various localities. These protein profiles can be used as a biomarker to identify the superior accession of *P. rosea* in the pharmaceutical industries (Table – 29; Plate XIX).

The cladogram constructed based on the protein profiles of *P. rosea* explained the similarities and variation among the studied accessions of *P. rosea.* The cladogram constructed based on the protein profile showed two clusters (Cluster 1 and Cluster 2). The Cluster 1 (C_1) includes only the accession collected from Cheruvarankonam (Pr1) which shows 100% divergent from the other two accessions of *P. rosea.* The cluster 2 (C_2) was equally shared by the accessions collected from Dana (Pr2) and Bangalore (Pr3). The UPGMA based cluster analysis clearly showed the variation and similarity among the various accession of *P. rosea* (Fig. 15). The accession collected from Cheruvarankonum (Pr1) reported highest variation among the studied accessions, this may due to the ecological differences, Cheruvarankonum was located in Kerala which was located in the hill station and other two accessions are located in plain surface.

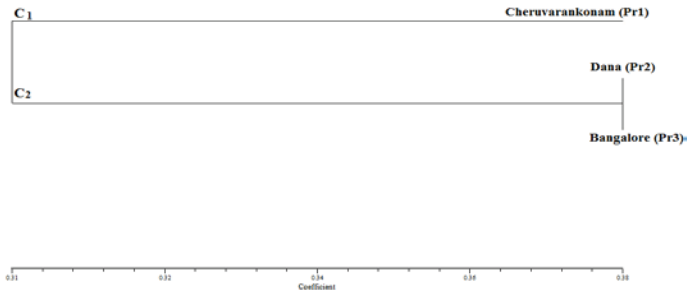


Fig.15: UPGMA cladogram based on the SDS-PAGE protein profile of *P. rosea*

Inter-specific protein variation in *Plumbago* species

A total of 26 bands with multiple regions of activity with varied MW-Rf values were observed in the protein electrophoretic system of *Plumbago* species viz., *P. zeylanica* (Pz3), *P.*

auriculata (Pa5) and *P. rosea* (Pr3) collected from Karnataka. The *P. zeylanica* and *P. auriculata* collected from Karnataka displayed with 9 bands and *P. rosea* demonstrated with 8 bands respectively. The results obtained through the electrophoretic separation revealed inter-specific variation of *Plumbago* species and paved a way to distinguish the studied three *Plumbago* species. The proteins bands with MW-Rf values 0.07, 0.12, 0.32, 0.6 and 0.78 showed their unique presence in *P. auriculata.* The protein with MW-Rf values 0.24, 0.64 and 0.91 displayed their distinctive presence in *P. zeylanica*. The protein with MW-Rf values 0.04, 0.84 and 0.67 recognized their individual presence in *P. rosea*. The protein bands with MW-Rf value 0.70 and 0.82 was commonly shared by *P. zeylanica* and *P. auriculata.* The proteins with MW-Rf value 0.34, 0.44 and 0.5 was shared by *P. zeylanica* and *P. rosea.* Protein bands with Rf value 0.57 illustrated its common presence in all the three *Plumbago* species. Each region is occupied by different protein in the form of bands and is representative of the expression of a particular gene/allele of *Plumbago* species (*P. zeylanica, P. auriculata* and *P. rosea*) collected from Karnataka. These protein profiles can be used as a biomarker to identify the superior accession of *Plumbago* species in the pharmaceutical industries (Table – 30; Plate XIX).

The cladogram constructed based on the protein profiles clearly explained the similarities and variation among the *Plumbago* species. The cladogram constructed based on the protein profile showed two clusters (Cluster 1 and Cluster 2). The cluster 1(C^1) was shared between *P. zeylanica* (Pz3) and *P. rosea* (Pr3). Cluster 2 (C^2) demonstrated the unique presence of *P. auriculata* (Pa5) which showed 100% divergence from the other two species (Fig. 16).

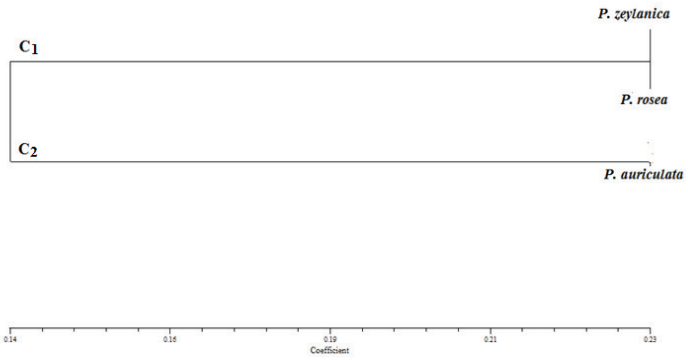


Fig. 16: UPGMA cladogram based on the SDS-PAGE protein profile of *Plumbago* species

MALDI-TOF MS analysis of *Plumbago zeylanica*

MALDI-TOF MS characterization of *P. zeylanica, P. auriculata, P. rosea* collected from various accessions showed a proximate spectra of varied ion peaks (m/z) ranged from 0 - 1, 00, 000 kDa. The results of MALDI-TOF MS analysis showed both positive and negative peaks; to reveal the intra-specific similarity and variation between the accessions, the positive peaks were selected. The obtained spectral profiles were further screened for the presence of recurring peaks or biomarker ions specific for all the species. Based on the unique spectral values the cladogram was constructed.

Totally 94 m/z values were selected for the MALDI-TOF MS characterization of *P. zeylanica* and summarized in Table - 31. Among the seven accessions of *P. zeylanica,* the accession Papanasam (Pz4) showed maximum number of (27) m/z peaks ranged from 1289 to 80916 m/z values from of which fourteen specific peaks were observed only in the accession Papanasam. Next to that, the accessions Bangalore (Pz3) and Coimbatore (Pz7) represented twenty one m/z peaks ranged from 1242-39429 and 1197-83978 m/z values respectively. Out of 21 spectral peaks, nine unique peaks were reported in the Bangalore accession (Pz3). Similarly the accession collected from Coimbatore (Pz7) depicted ten unique spectral values. The accession collected from Kuttichal (Pz6) displayed thirteen positive spectral peaks, of which five

unique peaks were recorded. *Plumbago zeylanica* collected from Palayamkottai (Pz2) showed eleven positive spectral peaks among these only one individual spectral peak was showed its presence. The lowest numbers of peaks (5) were observed in the accession collected from Rasthakadu (Pz1) and Tiruppur (Pz5) with two and three restricted peaks respectively (Plate XX). These ionic unique spectral peaks of the *Plumbago zeylanica* collected from various accessions offer a strong proof in differentiating the selected accession and paved a way to study the similarity and variation among the accession using MALDI-TOF MS analysis.

The cladogram constructed based on the MALDI-TOF-MS analysis and the results revealed the intra-specific variations and similarities among the morphologically similar *P. zeylanica* accessions collected from seven different localities of South India viz., Rasthakadu (Pz1), Palayamkottai (Pz2), Bangalore (Pz3), Papanasam (Pz4), Tiruppur (Pz5), Kuttichal (Pz6) and Coimbatore (Pz7). The cladogram distinguished two clades viz., C_1 and C_2 based on the m/z peak values. Clade$_1$ (C_1) includes the accessions Rasthakadu (Pz1), Palayamkottai (Pz2), Bangalore (Pz3), Kuttichal (Pz6) and Coimbatore (Pz7). Clade1 was further divided into two nodes C_1N^1 and C_1N^2. C_1N^1 demonstrated two branches $C_1N^1B_1$ Rasthakadu (Pz1) and $C_1N^1B^2$ Palayamkottai (Pz2). C_1N^2 was branched into $C_1N^2B_1$ and $C_1N^2B_2$. $C_1N^2B_1$ was further branched into two sub branches viz., $C_1N^2B_1b_1$ Bangalore (Pz3) and $C_1N^2B_1b_2$ Kuttichal (Pz6). $C_1N^2B_2$ showed the individual presence of the accession Coimbatore (Pz7). Clade 2 (C_2) was mutually shared by Papanasam (Pz4) and Tiruppur (Pz5). The distinctive presence of Pz4 and Pz5 in a separate clade 2 represented the presence of some unique m/z peaks compared to other accessions (Fig. 17). These MALDI-TOF MS spectroscopic profile can act as a biological spectroscopic tool to study the intra-specific variation and similarity of selected *P. zeylanica* accessions collected from seven different localities of South India.

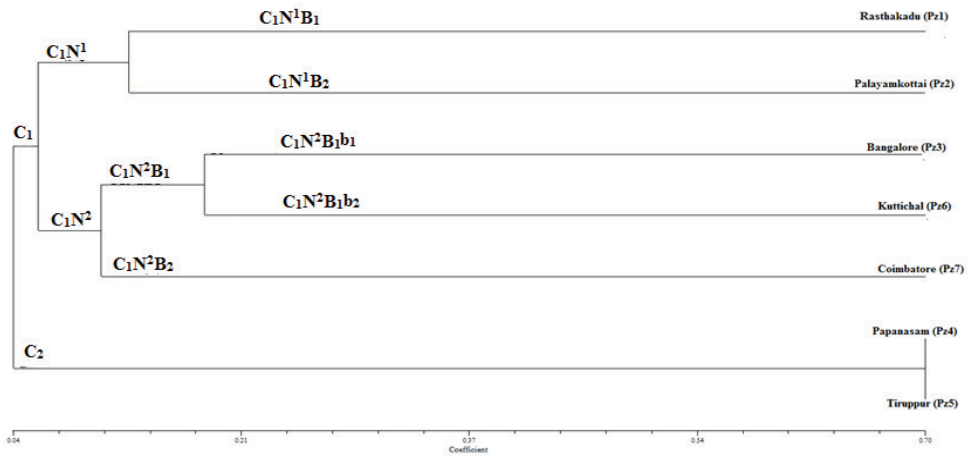


Fig.17: Cladogram based on MALDI-TOF MS m/z values of *P. zeylanica*

MALDI TOF MS analysis of *Plumbago auriculata*

MALDI-TOF MS characterization of *P. auriculata* collected from various accessions showed totally 32 m/z values and the spectral values were summarized in Table - 32. Among the five accession of *P. auriculata*, maximum number (10) of m/z peaks ranged from 2055 to 86132 m/z values were observed in the *P. auriculata* collected from Tenkasi (Pa2); of which seven specific peaks were observed only in the Tenkasi accession. Next to that, the accessions Mysore (Pa5) represented nine m/z peaks ranged from 1422-92969 respectively. Out of nine spectral peaks, eight distinctive peaks were reported in the Mysore accession (Pa5). Similarly the accession collected from Mulakumoodu (Pa1) depicted seven spectral values demonstrating three isolated peaks. The accession collected from Kattakadu (Pa4) displayed six positive spectral peaks, of which two peaks are unique. The lowest numbers of peaks were renowned in the accession collected from Perunthurai (Pa3) showed five positive spectral peaks among these three individual spectral peaks were displayed (Plate 21). These ionic unique spectral peaks of the *Plumbago auriculata* collected from various accessions offer a strong proof in differentiating

the selected accession and paved a way to study the similarity and variation among the accession using MALDI-TOF MS analysis.

The cladogram constructed based on the MALDI-TOF MS analysis and the results revealed the intra-specific variations and similarities among the morphologically similar *P. auriculata* accessions collected from five different localities of South India viz., Mulakumoodu (Pa1), Tenkasi (Pa2), Perunthurai (Pa3), Kattakadu (Pa4) and Mysore (Pa5). The cladogram distinguished two clades viz., C_1 and C_2 based on the m/z peak values. Clade I (C_1) includes the accessions Mulakumoodu (Pa1), Tenkasi (Pa2), Perunthurai (Pa3) and Kattakadu (Pa4). Clade 1 was further divided into two nodes C_1N^1 and C_1N^2. C_1N^1 demonstrated two branches $C_1N^1B_1$ and $C_1N^1B_2$. $C_1N^1B_1$ was shared by the accessions Mulakumoodu (Pa1) and Kattakadu (Pa4). $C_1N^1B_2$ illustrated the unique presence of the accession Perunthurai (Pa3). C_1N^2 represented the individual presence of Tenkasi (Pa2). Clade 2 (C_2) showed the distinctive presence of the accession Mysore (Pa5). The unique presence of the accession Mysore (Pa5) in a separate clade 2 represented the presence of some unique m/z peaks compared to other accessions (Fig. 18). These MALDI-TOF MS spectroscopic profile can act as a biological spectroscopic tool to study the intra-specific variation and similarity of selected *P. auriculata* accessions collected from five different localities of South India.

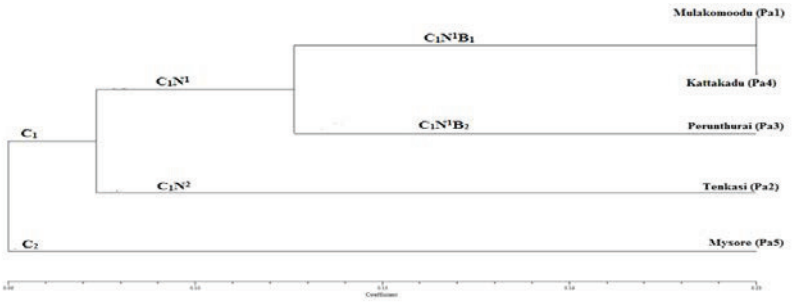


Fig.18: Cladogram based on MALDI-TOF MS m/z values of *P. auriculata*

MALDI -TOF MS analysis of *Plumbago rosea*

MALDI-TOF MS characterization of *Plumbago rosea* collected from various accessions showed 12 m/z values and the selected spectral values were summarized in Table - 33. Among the three accession of *Plumbago rosea* maximum number (9) of m/z peaks ranged from 1730 to 92,000 m/z values were observed in the *Plumbago rosea* collected from Dana (Pr2); of which seven specific peaks were observed only in the accession Dana. Next to that, the accessions Cheuruvarankonam (Pr1) represented three m/z peaks ranged from 823-4067 respectively. Out of three m/z peaks, only one unique peak was demonstrated in the accession Cheruvarankonum (Pr1). Similarly the accession collected from Bangalore (Pr3) depicted two spectral values but no unique peaks were observed in the accession Pr3. These ionic unique spectral peaks of the *Plumbago rosea* collected from various accessions offer a strong proof in differentiating the selected accession and paved a way to study the similarity and variation among the accession using MALDI-TOF MS analysis (Plate XXII).

The cladogram constructed based on the MALDI-TOF MS analysis and the results revealed the intra-specific variations and similarities among the morphologically *P. rosea* accessions collected from three different localities of South India viz., Siruvarankonum (Pr1), Dana (Pr2) and Bangalore (Pr3). The cladogram distinguished two clades viz., C_1 and C_2 based on the m/z peak values. $Clade_1$ (C_1) was shared by the accessions Siruvarankonum (Pr1) and Bangalore (Pr3). $Clade_2$ (C_2) showed the distinctive presence of the accession Dana (Pr2). The unique presence of the accession Dana (Pr2) in a separate $clade_2$ represented the presence of some unique m/z peaks compared to other accessions (Fig. 19). These MALDI TOF MS spectroscopic profile can act as a biological spectroscopic tool to study the intra-specific

variation and similarity of selected *P. rosea* accessions collected from three different localities of South India.

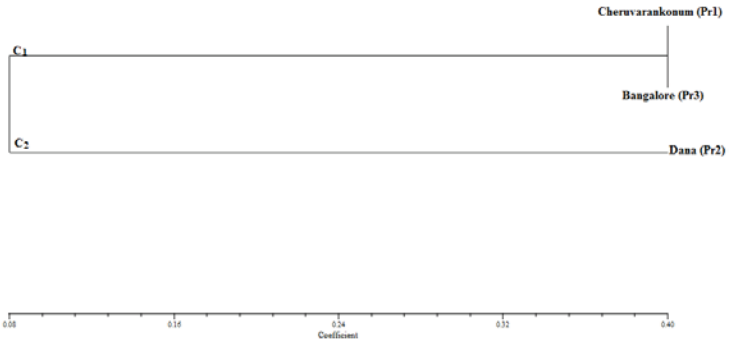


Fig.19: Cladogram based on MALDI-TOF MS m/z values of *P. rosea*

MALDI-TOF MS analysis of *Plumbago* species

MALDI-TOF MS characterization of *Plumbago* species collected from Karnataka showed a proximate spectra of varied ion peaks m/z ranged from 0 - 1,00,000 kDa. The results of MALDI-TOF MS analysis showed both positive and negative peaks; to reveal the inter-specific similarity and variation between the *Plumbago* species, the positive peaks were selected. The obtained spectral profiles were further screened for the presence of recurring peaks or biomarker ions specific for all the species. Based on the unique spectral values the cladogram was constructed. Totally 31 spectral peak values / m/z values were selected and summarized in Table – 34; Plate XXIII. Among the three *Plumbago* species, *Plumbago zeylanica* represented maximum number (21) of m/z peaks ranged from 1242 to 39429 m/z values; of which sixteen specific peaks were observed only in the *P. zeylanica*. Next to that, the *Plumbago auriculata* demonstrated nine m/z peaks ranged from 1422-92970 respectively. Out of nine peaks, only five unique peaks were observed in *P. auriculata*. Similarly, *Plumbago rosea* depicted two distinct spectral values 822 and 4058 respectively. These ionic unique spectral peaks of the *Plumbago* species collected from various accessions offer a strong proof in differentiating the selected

accession and paved a way to study the similarity and variation among the accession using MALDI-TOF MS analysis (Plate XXIII).

The cladogram constructed based on the MALDI TOF-MS analysis and the results revealed the intra-specific variations and similarities among the *Plumbago* species collected from South India viz., *Plumbago zeylanica, Plumbago auriculata* and *Plumbago rosea.* The cladogram distinguished two clades viz., C_1 and C_2 based on the m/z peak values. Clade 1 (C_1) was shared by *P. zeylanica* and *P. auriculata*. Clade 2 (C_2) showed the individual presence of the *P. rosea*. The exclusive presence of the *P. rosea* in a separate clade$_2$ represented the presence of some unique m/z peaks compared to other accessions (Fig. 20). These MALDI-TOF MS spectroscopic profile can act as a biological spectroscopic tool to study the inter-specific variation and similarity of selected *Plumbago* species collected from South India.

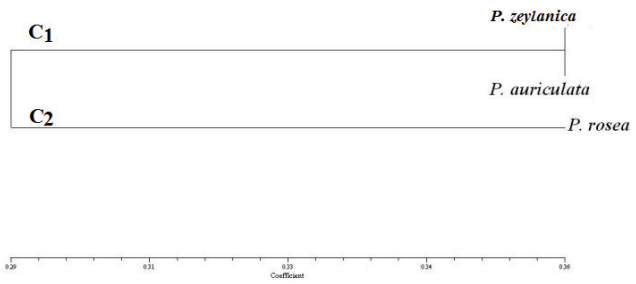


Fig. 20: Cladogram based on MALDI-TOF MS m/z values of *Plumbago* species

DNA Barcoding to reveal the intra-specific variation among the selected *P. zeylanica* species

The genomic DNA isolated from the *P. zeylanica* species collected from seven different accessions were amplified using universal primer performed for the gene *rbc*L and the base pairs of the isolated DNA of the seven accessions was identified in agarose gel electrophoresis using 100 base paired DNA ladder as shown in Plate XXIV. The PCR amplification efficiency was good for the selected accessions and the amplicons were sequenced. The obtained sequences

were annotated and submitted in GenBank viz., KF 233546, KF 233551, KF 233552, KF 233545, KF 233544, KF 233543 and KF 233542. Multiple sequence alignment was carried out for *rbc*L region using CLUSTAL W and MULTALIN. The sequence alignment and Phylogenetic analysis performed by minimum evolution method using MEGA 6.0 (Molecular Evolutionary Genetic analysis) discriminated the intra-specific variation among the studied accessions of *P. zeylanica*. To know the molecular similarity and variation, it is necessary to know some basic statistical similarities such as nucleotide frequencies, codon frequencies and transition / transversion ratios (Tamura *et al.,* 2013).

Alignment result

The result of multiple sequence alignment using MULTALIN is summarized in Fig. 21. The phenogram clearly demonstrated the intra-specific similarity and variations among the studied accession of *P. zeylanica*. The phenogram displayed two clades, C_1 and C_2. The clade 1 (C_1) displayed two nodes C_1N^1 and C_1N^2. The node C_1N^1 includes two accessions Kuttichal (Pz6) and Rasthakadu (Pz1).The node C_1N^2 includes only one accession Palayamkottai (Pz2). Similarly the clade 2(C_2) displayed two nodes; the node C_2N^1 includes the accessions Papanasam (Pz4) and Coimbatore (Pz7). The node C_2N^2 includes the accessions Bangalore (Pz3) and Tiruppur (Pz5). The phenogram displayed the genetic closeness and variations among the studied accessions of *P. zeylanica* (Fig. 21). The chromatogram of the sequenced DNA using ABI Prism 3100 genetic analyser was displayed in Fig. 22 to Fig. 28. The sequences of *P. zeylanica* aligned by MULTALIN were displayed in Fig. 29.

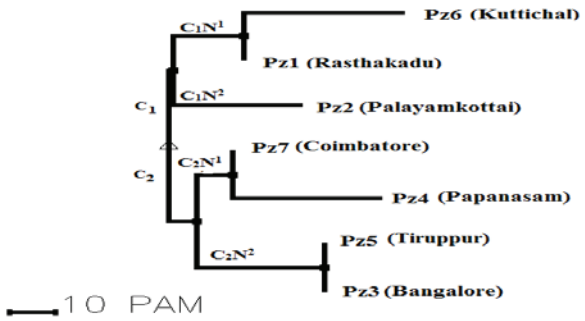


Fig. 21: Phenographic representation of *P. zeylanica* based on Multiple Sequence Alignment

Variation analysis using nucleotide composition

The nucleotide composition of the seven aligned sequences of *P. zeylanica* were analysed using the MEGA software version 6.0 and the results were displayed in Table - 35. Among the seven accessions more number of 1127 nucleotides was observed in *P. zeylanica* collected from Papanasam (Pz4). Next to that, the accessions collected from Bangalore (Pz3) and Coimbatore (Pz7) showed 1095 number of nucleotides. Lowest number of 619 nucleotides was displayed in the accession collected from Rasthakadu (Pz1). The total percentage of thymine content was higher (29%) in the accession collected from Coimbatore (Pz7) whereas the accessions collected from Bangalore (Pz3) and Tiruppur (Pz5) showed the similar thymine content of 28.2%. Higher percentage of guanine (23%) was observed in the accessions collected from Bangalore (Pz3) and Tiruppur (Pz5). The nucleotide cytosine content was higher in the accessions collected from Rasthakadu (Pz1), Palayamkottai (Pz2) and Coimbatore (Pz7) with 21.3%; Bangalore (Pz3), Tiruppur (Pz5) and Kuttichal (Pz6) showing 20.5% of cytosine content. Adenine content was higher (28.2%) in Bangalore (Pz3) and Tiruppur (Pz5) accessions of *P. zeylanica*. Based on the nucleotides composition it was demonstrated that the *rbc*L sequences of Bangalore (Pz3) and

Tiruppur (Pz5) accessions recognized highly similar nucleotide composition whereas Rasthakadu (Pz1) and Papanasam (Pz4) showed an isolated number of nucleotide composition.

The accession collected from Bangalore and Tiruppur showed the similar nucleotide composition compared to other accessions. The accessions collected from Rasthakadu and Papanasam showed variation among the nucleotide composition.

Nucleotide directional 16 pair frequencies

The percentage of nucleotide substitution was analysed using MEGA 6.0 software and displayed in Table - 36. The average transversional pairs (391) were higher when compared to transitional pairs (195) and the average identical pairs are 252 among the aligned sequences. The AT content was 67%, 52% and 0% for the paired sequences of the accessions Pz2 and Pz6: Pz1 and Pz7: Pz3 and Pz5 whereas the overall percentages for the seven sequences are 62%. The GC content was 32 for the paired accessions Pz2 and Pz6: Pz1 and Pz7 and 0% for the accession Pz3 and Pz5. The overall percentage of GC content for all the selected accessions was 38%. The average of TT base pair was higher (309) in the paired accessions Pz3 and Pz5 following these accessions Pz2 and Pz6: Pz1 and Pz7 showed 103 and 64. The total average of TT base pairs for all the seven accessions of *P. zeylanica* was 79%. The R value for the paired sequences Pz1 and Pz7: Pz2 and Pz6 were 0.5 and the overall average R value was 0.498. But the paired sequences of the accession Pz3 and Pz5 showed “0” R value. The average of total nucleotide pair frequencies of the accessions Pz3 and Pz5: Pz2 and Pz6: Pz1 and Pz7 was 1095, 1029 and 619.The average of the total nucleotide pair frequencies of the seven selected accessions of *P. zeylanica* was 838.7.

Nucleotide paired statistical data

The nucleotide sequences of *P. zeylanica* collected from seven different accessions were compared in the MEGA version 6.0. This gives the comparative nucleotide frequencies among the seven accessions. The total numbers of base pairs are 1127. Among these, 14 conserved regions, 1081 variable regions, 331 singleton sites and 770 parsimonial sites were identified in the aligned sequences of *P. zeylanica* collected from seven varied accessions. The accessions Bangalore (Pz3) and Tiruppur (Pz5) recorded highest number of 1095 conserved regions. The lowest number of 185 conserved region. 434 variable region was observed among the accessions Pz1 (Rasthakadu) and Pz7 (Coimbatore). The accession Palayamkottai (Pz2) and Kuttichal (Pz6) revealed the highest number of variable region (661) showing 368 conserved regions but no variable, parsimony and singleton sites were observed in the paired accessions. The results were displayed in Table - 37.

Maximum composite likelihood estimate of the pattern of nucleotide substitution

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in normal in the Table - 38. The nucleotide frequencies are 0.272 (A), 0.291 (T/U), 0.213 (C) and 0.224 (G). The transition / transversion rate ratios are k_1 = 2.202 (purines) and k_2 = 0.465 (pyrimidines). The overall transition / transversion bias is R = 0.604, where R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 619 positions in the final dataset. All calculations were conducted in MEGA 6.0 (Tamura *et al.,* 2007).

Composite distance pattern

The composite distance pattern measures the differences in the nucleotide distance among the sequences of the selected accessions. Among the seven accessions lowest composite

pattern (0.000) was observed between the accessions Bangalore (Pz3) and Tiruppur (Pz5). Highest nucleotide composite pattern (0.050) was observed between the accessions Bangalore (Pz3) and Kuttichal (Pz6); Tiruppur (Pz5) and Kuttichal (Pz6). The overall mean distance of maximum likelihood distance method was 2.201. The composite distance pattern between the selected accessions of *P. zeylanica* was displayed in Table - 39.

The cladogram was constructed based on composite distance pattern using NTSys and the cladogram was displayed in Fig. 30. The cladogram consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1 (C_1) was branched into two nodes C_1N^1 and C_1N^2**.** The node C_1N^1 includes the accession collected from Rasthakadu (Pz1). The node C_1N^2 was branched into two branches $C_1N^2B_1$ and $C_1N_2B_2$. The branch $C_1N^2B_1$ was divided into $C_1N^2B_1b_1$ and $C_1N^2B_1b_2$. $C_1N^2B_1b_1$ includes the accessions Bangalore (Pz3) and Tiruppur (Pz5) Which shared the common node and demonstrated highest variability from other accessions. $C_1N^2B_1b_2$ was divided into two sub branches $C_1N^2B_1b_2A$ and $C_1N^2B_1b_2B$ which represents the accessions Palayamkottai (Pz2) and Kuttichal (Pz6). The Branch $C_1N^2B_2$ showed the individual presence of the accession Coimbatore (Pz7). The clade 2 (C_2) illustrated the unique presence of the accession collected from Papanasam (Pz4) which was highly distinct from other accessions.

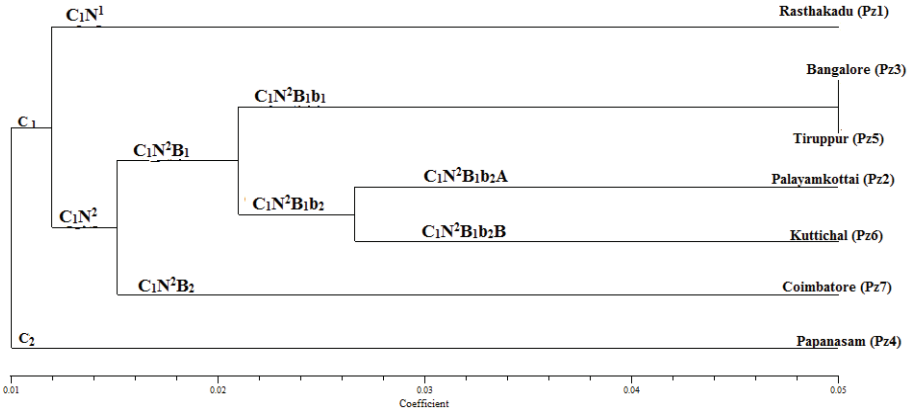


Fig. 30: Composite distance pattern between the selected accessions of *P. zeylanica*

Amino acid composition

The amino acid compositions of the selected accessions are analysed using MEGA 6.0 and the results were displayed in Table - 40. The accession collected from Papanasam (Pz4) represented maximum number of 347 amino acids. Followed by this, the accession collected from Bangalore (Pz3) and Tiruppur (Pz5) showed 340 amino acids. The accession collected from Rasthakadu (Pz1) displayed least number of 205 amino acids. Among the twenty amino acids, leucine showed highest percentage of 13.14% occurrence. The accession collected from Tiruppur displayed the maximum percentage of 15.88% leucine existence, followed by the accession collected from Coimbatore (15.57 %). The amino acid histidine was present in least percentage of 2.27. The overall amino acid composition was 302.1.

Tajima's neutrality test

Tajima's neutrality test is a statistical method used for testing the neutral mutation hypothesis of *P. zeylanica* species collected from seven different accessions. All positions containing gaps and missing data were eliminated from the dataset. The abbreviations used are as follows: **m** = number of sites, **S** = Number of segregating sites, $\mathbf{p_s}$ = S/m, $\Theta = p_s/a_1$, $\boldsymbol{\pi}$ = nucleotide diversity and **D** is the Tajima test statistic. The Tajima's neutrality test was depicted in the Table - 41.

Evolutionary relationships using different types of Bootstrap analysis

The phylogenetic analysis of *P. zeylanica* collected from seven different accessions of South India were analysed using four methods viz., Neighbor joining method, maximum parsimony, minimum evolution method and UPGMA method. The percentage of similarity and evolutionary distance was calculated using the branch length of the cladogram presented.

Bootstrap Neighbor joining method

The Phylogenetic tree based on Bootstrap Neighbor joining method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1 was detached into two nodes C_1N^1 and C_1N^2**.** The node C_1N^1 was further separated into two branches viz., $C_1N^1B_1$ and $C_1N^1B_2$ represents the accession Rasthakadu (Pz1) and Coimbatore (Pz7). Both the accessions showed 72% similarity with the varied distance value of 1.0548 and 0.8090 respectively and the distance value of 0.2469 from other accessions. The node C_1N^2 includes the accessions of Bangalore (Pz3) and Tiruppur (Pz5) with a branch length of 0.0000. They shared the common node and demonstrated highest variability from other sequences with highest distance value of 1.1707 compared to the other accessions. The result of NJ analysis revealed the maximum percentage of 95% similarity between Bangalore (Pz3) and Tiruppur (Pz5). The clade 2 was divided into two nodes viz., C_2N^1 and C_2N^2. The node C_2N^1 represents the accession Papanasam (Pz4) demonstrated its isolated variability from other accessions with the distance value of 1.1111 and similarity percentage of 78%. The node C_2N^2 branched into two branches, viz., $C_2N^2B_1$ Palayamkottai (Pz2) and $C_2N^2B_2$ Kuttichal (Pz6) with 68% similarity. The accessions Palayamkottai (Pz2) and Kuttichal (Pz6) showed 0.3754 and 0.6049 diverse distance value between them and the distance of 0.5128 from other accessions (Fig. 31).

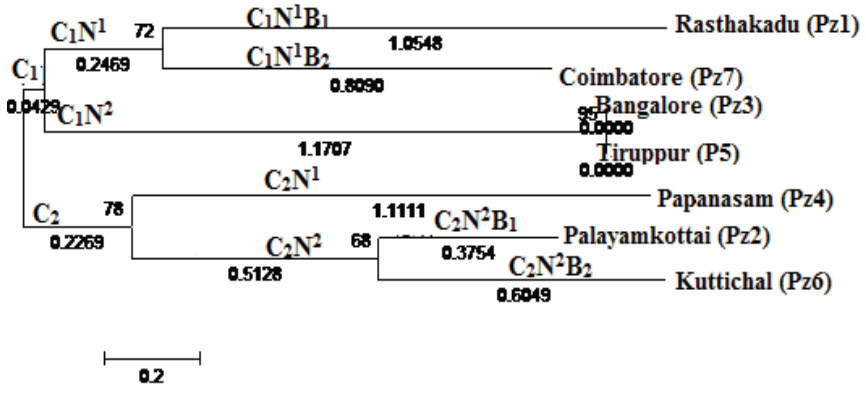


Fig. 31: Phylogenetic tree constructed using Bootstrap Neighbor joining method

Bootstrap minimum evolution

The Phylogenetic tree based on Bootstrap minimum evolution method consists of two clades viz., Clade 1 (C_1) and Clade 2 (C_2). Clade 1 was divided into two nodes C_1N^1 and C_1N^2. The node C_1N^1 was branched into two branches viz., $C_1N^1B_1$ (Rasthakadu -Pz1) and $C_1N^1B_2$ (Coimbatore - Pz7). They showed 79% of identical similarity with the distance value of 1.0108 between them and the distance of 0.2343 from other accessions. The node C_1N^2 includes the accessions Bangalore (Pz3) and Tiruppur (Pz5) with maximum percentage of similarity (94%) and 0.0000 distance value. The accessions of Bangalore (Pz3) and Tiruppur (Pz5) showed the distance of 1.2451 from other accessions. The clade 2 was divided into two nodes viz., C_2N^1 and C_2N^2. The node C_2N^1 recorded the discrete presence of the accession Papanasam (Pz4) with highest distance value of 1.1464 and 74% of similarity compared to other sequences. The node C_2N^2 was branched into two branches $C_2N^2B_1$ and $C_2N^2B_2$ viz., $C_2N^2B_1$ includes the accession collected from Palayamkottai (Pz2) and $C_2N^2B_2$ represents Kuttichal (Pz6). They shared the conserved distance value of 0.5613 and showed lowest percentage of similarity (71%) between them. The accession Palayamkottai (Pz2) and Kuttichal (Pz6) illustrated the lowest distance value with 0.5851 (Fig. 32)

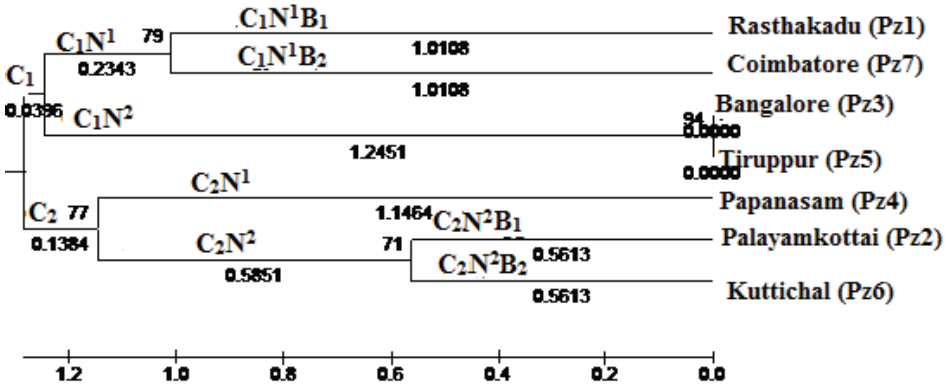


Fig. 32: Phylogenetic tree constructed using Bootstrap minimum evolution method

Bootstrap maximum parsimony

Similar to Bootstrap minimum evolution, the phylogenetic tree based on Bootstrap maximum parsimony method also showed two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1 (C_1) was divided into two nodes viz., C_1N^1 and C_1N^2**.** The node C_1N^1 was branched into two branches viz., $C_1N^1B_1$ represents the accession collected from Rasthakadu (Pz1) and $C_1N^1B_2$ showed the accession Coimbatore (Pz7) with 95% and the distance value of 233.8216 between them and 65.6085 from other accessions. The node C_1N^2 includes the accessions collected from Bangalore (Pz3) and Tiruppur (Pz5) with 0.0000 values. They displayed maximum of percentage of similarity with less distance between them and the distance value of 299.4301 from other accessions. The clade 2 (C_2) was divided into two nodes viz., C_2N^1 and C_2N^2. The node C_2N^1 recorded the discrete presence of the accession collected from Papanasam (Pz4) with highest distance value of 279.2704. The node C_2N^2 branched into two branches viz., $C_2N^1B_1$ and $C_2N^2B_2$. The branch $C_2N^1B_1$ includes the accessions Palayamkottai (Pz2) and $C_2N^2B_2$ Kuttichal (Pz6). They shared the conserved distance value of 193.3772 and denote 71% of similarity between them and the distance of 85.8932 from other accessions (Fig. 33).

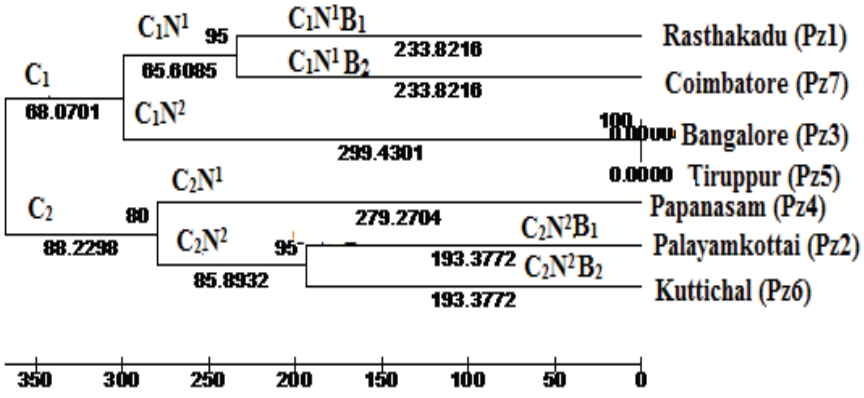


Fig. 33: Phylogenetic tree constructed using Bootstrap maximum parsimony method

Bootstrap UPGMA analysis

The Phylogenetic tree based on Bootstrap UPGMA analysis displayed two clades viz., Clade 1 (C_1) and Clade 2 (C_2). Clade 1 (C_1) includes two nodes C_1N^1 and C_1N^2**.** The node C_1N^1 was branched into two branches viz., $C_1N^1B^1$ represented the accession collected from Rasthakadu (Pz1) and $C_1N^1B^2$ displayed the accession Coimbatore (Pz7). Both the accessions showed similarity 82% of with the distance value of 0.9319 between them and 0.2429 distances from other accessions. The node C_1N^2 includes the accessions collected from Bangalore (Pz3) and Tiruppur (Pz5) with 0.0000 distance value and 99% similarity between them and the distance value of 1.1748 from the other accessions. The clade 2 was divided into two nodes viz., C_2N^1 and C_2N^2. The node C_2N^1 recorded the discrete presence of the accession Papanasam (Pz4) with the distance value of 1.0570. The node C_2N^2 was branched into two branches $C_2N^2B_1$ and $C_2N^2B_2$ viz., $C_2N^2B_1$ includes the accession Palayamkottai (Pz2) and $C_2N^2B_2$ represents Kuttichal (Pz6). Both the accessions shared the conserved distance value of 0.4902 with 99% between them and distance value of 0.5669 from other accessions. The Bootstrap UPGMA analysis suggests that accessions collected from Palaymkotttai (Pz2) and Kuttichal (Pz6) posses least evolutionary distance compared to other accessions whereas Bangalore (Pz3) and Tiruppur (Pz5) displayed highest evolutionary distance from the studied accessions (Fig. 34).

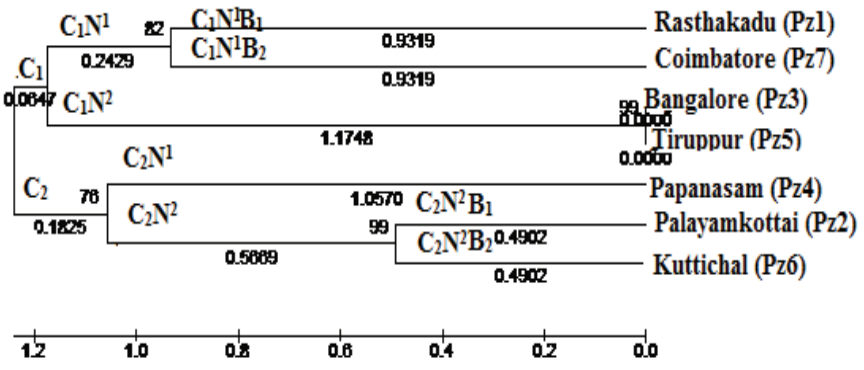


Fig. 34: Phylogenetic tree constructed using Bootstrap UPGMA method

The accessions collected from Palayamkottai (Pz2) and Kuttichal (Pz6) illustrated the lowest distance value of 0.5128, 0.5851, 0.5669 and 193.3772 in Bootstrap Neighbor - Joining method, minimum evolution method, UPGMA method and maximum parsimony method (Fig. 34).

The above multiple sequence alignment, phylogenetic analysis, amino acid composition and statistical parameters carried out by MEGA 6.0 version software for the barcoded *rbc*L sequences of *P. zeylanica* revealed that the sequences of the accessions collected from Bangalore (Pz3) and Tiruppur (Pz5) are highly conserved and identical. Following these the accessions collected from Palayamkottai (Pz2) and Kuttichal (Pz6); Rasthakadu (Pz1) and Coimbatore (Pz7) showed moderate similarity in their aligned sequences whereas the sequence of the accession Papanasam (Pz4) showed its unique presence and confirmed the variations among the seven *P. zeylanica* sequences retrieved from different accessions of South India.

Phylogenetic analysis to reveal the intra-specific variation among the selected *P. auriculata* species

The genomic DNA isolated from the *P. auriculata* species collected from five different accessions were amplified using universal primer carried out for the gene *rbc*L and the base pairs of the isolated DNA of the five accessions was identified in agarose gel electrophoresis using 100 base paired DNA ladder as shown in Plate XXIV. The PCR amplification efficiency was good for the selected accessions and the amplicons were sequenced. The obtained sequences were annotated and submitted in GenBank viz., KF 193871, KF 233550, KF 233549, KF 233548 and KF 233547. Multiple sequence alignment was performed for *rbc*L region of *P. auriculata* using CLUSTAL W and MULTALIN. The sequence alignment discriminated the intra-specific variation among the studied accessions of *P. auriculata*. Phylogenetic analysis was performed by

Minimum evolution method using MEGA 6.0 (Molecular Evolutionary Genetic analysis). To know the molecular similarity and variation, it is necessary to know some basic statistical similarities such as nucleotide frequencies, codon frequencies and transition / transversion ratios (Tamura *et al.,* 2013). The chromatogram of the sequenced DNA using ABI Prism 3100 genetic analyser was displayed in Fig. 36 to Fig. 40. The sequences of *P. auriculata* aligned by MULTALIN were displayed in Fig. 41.

Alignment result

The results of multiple sequence alignment using MULTALIN are summarized in Fig. 41. The phenogram displayed that, *P. auriculata* accessions collected from Perunthurai (Pa3) and Tenkasi (Pa2) occupied the same clade 1 (C_1) and shared the same node (C_1N^1) which represent the close similarity between the sequences. The accession collected from Mulakumoodu (Pa1) was denoted in a separate node (C_1N^2) this shows its diversity from the other accessions. Similarly, the accessions collected from Mysore (Pa5) and Kattakadu (Pa4) was represented in clade 2 (C_2) in the nodes C_2N^1 and C_2N^2. These phenogram shows the variations and similarities among the DNA sequences of *P. auriculata* collected from different accessions of South India (Fig. 35).

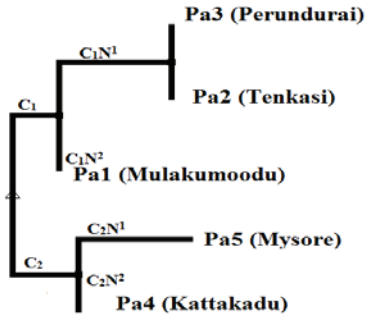


Fig. 35. Phenographic representation of *P. auriculata* based on Multiple Sequence Alignment

Variation analysis using nucleotide composition

The nucleotide composition of the five aligned sequences of *P. auriculata* were analysed using the statistical tool of MEGA 6.0 and the results were displayed in Table - 42. Among the five accessions more number of 640 nucleotides was observed in *P. auriculata* collected from Tenkasi (Pa2). Next to that the accessions collected from Perunthurai (Pa3) showed 619 numbers of nucleotides. Lowest number of 602 nucleotides was displayed in the accession collected from Kattakadu (Pa4). The total percentage of thymine content was higher (29.5%) in the accession collected from Tenkasi (Pa2) whereas the accessions collected from Perunthurai (Pa3) and Mysore (Pa5) revealed 29.4% of thymine content. Higher percentage of guanine (22.8%) was observed in the accessions collected from Mysore (Pa5). The nucleotide cytosine content (21.6%) was higher in the accession collected from Kattakadu (Pa4); Mulakumoodu (Pa1) and Mysore (Pa5) showed 21.4% of cytosine content. Adenine content was higher (27.2%) in Perunthurai (Pa2) accessions of *P. auriculata*.

The accession collected from Mulakumoodu (Pa1) and Perunthurai (Pa3) showed the similar nucleotide composition compared to the other accessions.

Nucleotide directional 16 pair frequencies

The percentage of nucleotide substitution was analysed using MEGA 6.0 version software and the results were displayed in Table - 43. The average transversional pairs (Si) and transitional pairs (Sv) were 144 and 298. The average identical pairs (ii) are 164 among the aligned sequences. The AT content was 56% for the paired sequences of the accessions Tenkasi (Pa2) and Perunthurai (Pa3). The accessions Mulakumoodu (Pa1), Tenkasi (Pa2) and Perundurai (Pa3): Kattakadu (Pa4) and Mysore (Pa5) represented the AT content of 55%and the GC content were 35, 31 and 25 for the paired accessions Pa2 and Pa3: Pa1, Pa2 and Pa3: Pa4 and Pa5. The

overall percentage of GC content for all the five accessions was 31. The average of TT base pair was higher (56) in the paired accessions Pa2 and Pa3: Pa1, Pa2 and Pa3 following these accessions Pa4 and Pa5 showed 55. The total average of TT base pairs for all the five accessions of *P. auriculata* was 54. The R value for all the paired sequences of *P. auriculata* was 0.5. The average of total nucleotide pair frequencies of the accessions Pa2 and Pa3: Pa1, Pa2 and Pa3: Pa4 and Pa5 were 603, 619 and 602.The average of the total nucleotide pair frequencies of the five studied accessions of *P. auriculata* was 605.9.

Nucleotide paired statistical data

The nucleotide sequences of *P. auriculata* collected from five different accessions were compared in the MEGA version 6.0. This gives the comparative nucleotide frequencies comparing among the five accessions. The total number of base pairs is 641. Among these, 13 conserved regions, 607 variable region, 290 singleton sites and 316 parsimonial sites were identified in the aligned sequences of *P. auriculata* collected from five varied accessions. Following these highly similar accessions Tenkasi (Pa2) and Perunthurai (Pa3) recorded highest numbers of 190 conserved regions and lowest number of 429 variable regions were recorded. The lowest number of conserved region 58 was observed among the accessions Mulakumoodu (Pa1), Tenkasi (Pa2) and Mysore (Pa3) with 561 variable regions and 555 singleton sites. The accession Pa4 and Pa5 revealed 165 conserved region and 437 variable regions. The results were displayed in Table - 44.

Maximum composite likelihood estimate of the pattern of nucleotide substitution

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in normal in the Table - 45. The nucleotide frequencies are 0.267 (A), 0.294 (T/U), 0.214 (C), and 0.225 (G). The transition/transversion rate ratios are

k_1 = 1.262 (purines) and k_2 = 0.654 (pyrimidines). The overall transition / transversion bias is R = 0.436, where R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 597 positions in the final dataset in aligned sequences of *P. auriculata*. All calculations were conducted in MEGA 6.0 (Tamura *et al.,* 2007).

Composite distance pattern

The composite distance pattern measures the differences in the nucleotide distance among the sequences of the selected accessions of *P. auriculata*. Among the five accessions lowest composite pattern (0.005) was observed between the accessions Mulakumoodu (Pa1) and Kattakadu (Pa4): Tenkasi (Pa2) and Perunthurai (Pa3): Mulakumoodu (Pa1) and Mysore (Pa5). Highest nucleotide composite pattern (0.025) was observed between the accessions Mulakumoodu (Pa1) and Perunthurai (Pa3). The overall mean distance of maximum likelihood distance method was 2.201. The composite distance pattern between the selected accessions of *P. auriculata* was displayed in Table - 43.

The cladogram constructed based on composite distance pattern using NTSys was displayed in Fig. 42. The cladogram consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1 (C_1) was divided into two nodes C_1N^1 and C_1N^2**.** The node C_1N^1 was shared by the two accessions collected from Mulakumoodu (Pa1) and Perunthurai (Pa3). The node C_1N^2 includes the accession collected from Mysore (Pa5). The clade 2 (C_2) was separated into two nodes viz., C_2N^1 and C_2N^2 which includes the accessions Tenkasi (Pa2) and Kattakadu (Pa4). The composite distance was demonstrated in the Fig. 42.

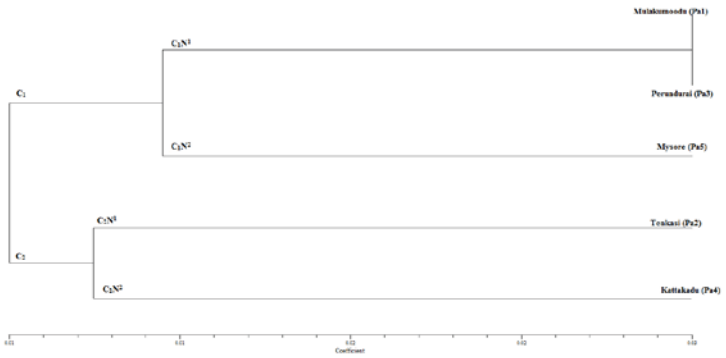


Fig. 42: Composite distance pattern between the selected accessions of *P. auriculata*

Amino acid composition

The amino acid compositions of *P. auriculata* was analysed using MEGA 6.0 and the results were displayed in Table - 47. The accession collected from Tenkasi (Pa2) represented maximum number of 204 amino acids. The accession collected from Kattakadu (Pa4) displayed least number of 185 amino acids. Among the twenty amino acids, leucine and arginine showed highest percentage of occurrence (10.99%). The accession collected from Tenkasi (Pa2) displayed the maximum percentage 21.57% of leucine and the least percentage of leucine (7.57%) was observed in the accession collected from Kattakadu (Pa4). The amino acid arginine recorded the highest percentage of 13.23% in the accession collected from Mysore (Pa5) and the lowest percentage (3.43%) of arginine was observed in the accession collected from Tenkasi (Pa2). The amino acid histidine was present in least percentage of 1.47%. Comparing the total number of amino acids, the accession Tenkasi (Pa2) represented of 204 amino acids followed by the accessions Mulakumoodu (Pa1) and Kattakadu (Pa4) experienced more or less similar number of amino acids 186 and 185. The overall amino acid composition was 191.

Tajima's neutrality test

Tajima's neutrality test is a statistical method used for testing the neutral mutation hypothesis of *P. auriculata* species collected from five different accessions. All positions

containing gaps and missing data were eliminated from the dataset. The abbreviations used are as follows: **m** = number of sites, **S** = Number of segregating sites, $\mathbf{p_s}$ = S/m, $\Theta = p_s/ a_1$, $\boldsymbol{\pi}$ = nucleotide diversity and **D** is the Tajima test statistic. The Tajima's neutrality test was depicted in the Table - 48.

Evolutionary relationships using different types of Bootstrap analysis

The phylogenetic analysis of *P. auriculata* collected from five different accessions of South India were scrutinized using four methods viz., Neighbor Joining method, maximum parsimony, minimum evolution method and UPGMA method. The percentage of similarity and evolutionary distance was calculated using the branch length of the cladogram presented.

Bootstrap Neighbor joining method

The Phylogenetic tree based on Bootstrap neighbor joining method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1 was detached into two nodes C_1N^1 and C_1N^2**.** The node C_1N^1 was further separated into two branches viz., $C_1N^1B_1$ and $C_1N^1B_2$ represented the accessions Mulakumoodu (Pa1) and Mysore (Pa5). They showed 30% similarity with the varied distance value of 0.8926 and 0.8266 respectively and the distance value of 0.0333 from other accessions. The node C_1N^2 includes the accessions collected from Kattakadu (Pa4) with a branch length of 0.7957. The clade 2 was divided into two nodes viz., C_2N^1 and C_2N^2 showing 55% similarity. The node C_2N^1 includes the accession collected from Tenkasi (Pa2) with the distance value of 0.8736 and the similarity percentage of 55%. The node C_2N^2 includes the accession Perunthurai (Pa3) representing the distance value of 0.6376. The phylogenetic tree based on Bootstrap neighbor joining method suggests that Mulakumoodu (Pa1) and Mysore (Pa5) expressed least evolutionary distance of 0.0333 compared to other accessions where as the

accessions collected from Tenkasi (Pa2) and Perundurai (Pa3) showed highest evolutionary distance 0.0829 from all the studied accessions of *Plumbago auriculata* (Fig. 43).

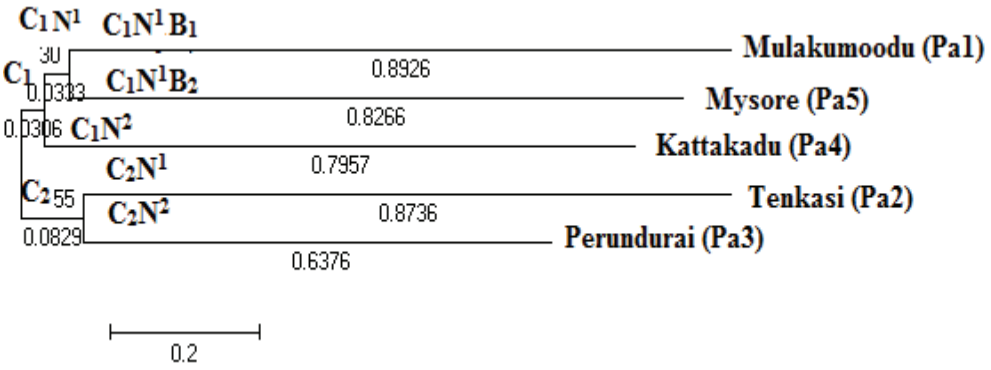


Fig. 43: Phylogenetic tree constructed using Bootstrap Neighbor joining method

Bootstrap minimum Evolution

The Phylogenetic tree based on Bootstrap minimum evolution method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1(C_1) was detached into two nodes C_1N^1 and C_1N^2**.** The node C_1N^1 was further separated into two branches viz., $C_1N^1B^1$ and $C_1N^1B^2$ represented the accessions Mulakumoodu (Pa1) and Mysore (Pa5) showing 22% similarity. They showed varied distance value of 0.8854 and 0.8338 and the distance value of 0.0333 from other accessions. The node C_1N^2 includes the accessions collected from Kattakadu (Pa4) with a branch length of 0.7957 and the distance of 0.0342 from other accessions. The clade 2 (C_2) was divided into two nodes viz., C_2N^1 and C_2N^2 showing 55% similarity. The node C_2N^1 includes the accession Tenkasi (Pa2) with the distance value of 0.8736. The node C_2N^2 includes the accession Perunthurai (Pa3) representing the distance value of 0.6376. The similarity percentage was 55% and the distance value of 0.0793 compared to other sequences (Fig. 44).

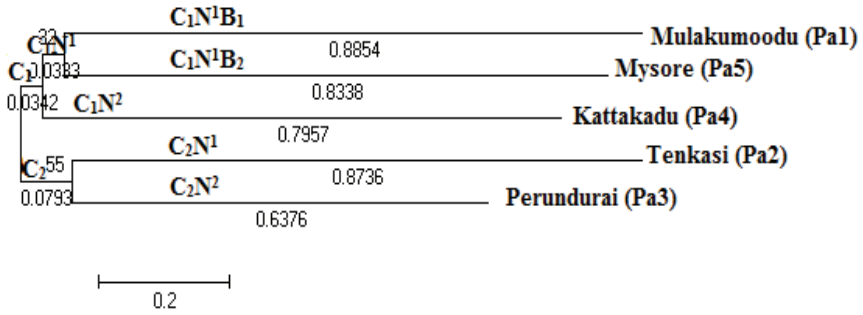


Fig. 44: Phylogenetic tree constructed using Bootstrap minimum evolution method

Bootstrap Maximum Parsimony

The Phylogenetic tree based on Bootstrap maximum parsimony method consists of two clades viz., Clade 1 (C_1) and Clade 2 (C_2). Clade 1 (C_1) was separated into two nodes C_1N^1 and C_1N^2. The node C_1N^1 was further separated into two branches viz., $C_1N^1B_1$ and $C_1N^1B_2$ represents the accessions Tenkasi (Pa2) and Perunthurai (Pa3) with 51% similarity and the similar distance value of 0.7556 between them and 0.0982 from other accessions. The node C_1N^2 was divided into two branches viz., $C_1N^2B_1$ and $C_1N^2B_2$ includes the accessions collected from Kattakadu (Pa4) and Mysore (Pa5) with the common distance value of 0.8170 and similarity percentage of 47%. The clade 2 (C_2) recorded unique presence of the accession Mulakumoodu (Pa1) with the distance value of 0.8761 (Fig. 45).

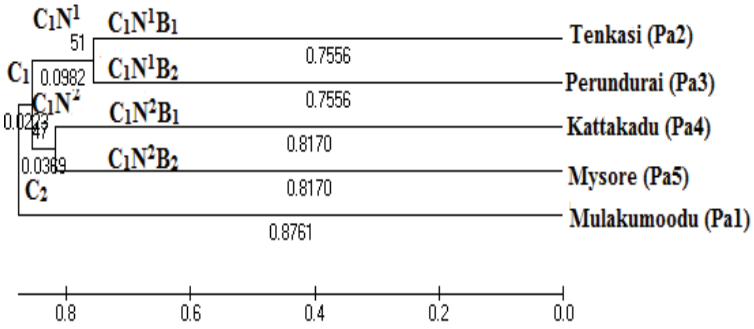


Fig. 45: Phylogenetic tree constructed using Bootstrap maximum parsimony method

Bootstrap UPGMA analysis

The Phylogenetic tree based on Bootstrap UPGMA method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1 (C_1) was separated into two nodes C_1N^1 and C_1N^2. The node C_1N^1 was further separated into two branches viz., $C_1N^1B_1$ and $C_1N^1B_2$ represents the accessions Tenkasi (Pa2) and Perunthurai (Pa3) with 51% similarity and the similar distance value of 0.7556 and 0.0982 from other accessions. The node C_1N^2 was divided into two branches viz., $C_1N^2B_1$ and $C_1N^2B_2$ includes the accessions Kattakadu (Pa4) and Mysore (Pa5) with the common distance value of 0.8170 and similarity percentage of 50%. The clade 2 (C_2) includes the accession collected from Mulakumoodu (Pa1) with the distance value of 0.8761 (Fig. 46).

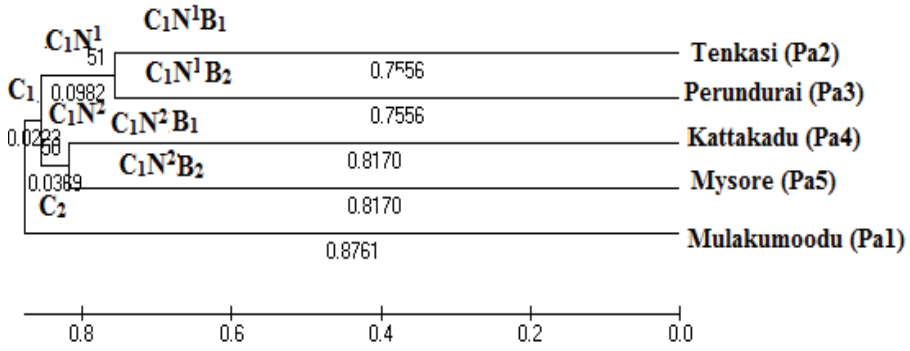


Fig. 46: Phylogenetic tree constructed using Bootstrap UPGMA method

The accessions collected from Mulakumoodu (Pa1) and Mysore (Pa5) illustrated the lowest distance value 0.0333 and the accession collected from Tenkasi (Pa2) and Perunthuurai (Pa3) demonstrated highest evolutionary distance 0.0829 in the phylogenetic tree based on Bootstrap Neighbor joining method and bootstrap minimum evolution method. The Phylogenetic tree based on Bootstrap maximum parsimony method and UPGMA method suggest that the accession collected from Tenkasi (Pa2) and Perunthurai (Pa3) expressed highest evolutionary distance of 0.0982 and lowest distance value of 0.0369 in the accession collected from Kattakadu (Pa4) and Mysore (Pa5) compared to other accessions. The accessions collected from Tenkasi

(Pa2) and Perunthurai (Pa3) showed highest evolutionary distance among the studied accessions of *P. auriculata.*

The above multiple sequence alignment, phylogenetic analysis, amino acid composition and statistical parameters carried out by MEGA 6.0 version software for the barcoded *rbc*L sequences of *P. auriculata* revealed that the sequences of the accessions collected from Tenkasi (Pa2) and Perundurai (Pa3) are highly conserved and identical. Following these the accessions collected from Kattakadu (Pa4) and Mysore (Pa5): Mulakumoodu (Pa1), Tenkasi (Pa2) and Perunthurai (Pa3) recognised moderate similarity in their aligned sequences.

DNA Barcoding to reveal the intra-specific variation among the selected *P. rosea* species

The genomic DNA isolated from the *P. rosea* species collected from three different accessions were amplified using universal primer performed for the gene *rbc*L and the base pairs of the isolated DNA of the three accessions was identified in agarose gel electrophoresis using 100 base paired DNA ladder as shown in Plate XXIV. The PCR amplification efficiency was good for the selected accessions and the amplicons were sequenced. The obtained sequences were annotated and submitted in GenBank viz., KF 261596, KF 261597 and KF 261598. Multiple sequence alignment analysis was performed for *rbc*L region using CLUSTAL W and MULTALIN. The sequence alignment discriminated the intra-specific variation among the studied accessions of *P. rosea*. Phylogenetic analysis analysis was performed by Minimum evolution method by using MEGA 6.0 (Molecular Evolutionary Genetic analysis). To know the molecular similarity and variation, it is necessary to know some basic statistical similarities such as nucleotide frequencies, codon frequencies and transition / transversion ratios (Tamura *et al.,* 2013). The chromatogram of the sequenced DNA using ABI Prism 3100 genetic analyser was

displayed in Fig. 48 to Fig. 50. The sequences of *P. rosea* aligned by MULTALIN were displayed in Fig. 51.

Alignment result

The results of multiple sequence alignment using MULTALIN are summarized in Fig. 51. The phenogram displayed two clades viz., clade 1 (C_1) and clade 2 (C_2) clade 1 (C_1) was separated into two nodes C_1N^1 and C_1N^2 includes the accessions collected from Cheruvarankonum (Pr1) and Bangalore (Pr3). The accession collected from Dana (Pr2) showed its unique presence in the clade 2 (C^2). These phenogram shows the variations and similarities among the DNA sequences of *P. rosea* collected from different accessions of South India (Fig.47).

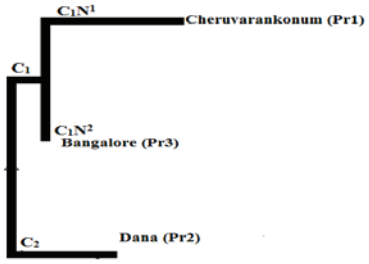


Fig. 47: Phenographic representation of *P. rosea* based on multiple sequence alignment

Variation analysis using Nucleotide composition

The nucleotide composition of the three aligned sequences of *P. rosea* were analysed using the statistical tool of MEGA 6.0 and the results were displayed in Table - 49. Among the three accessions more number of 1107 nucleotides was demonstrated in *P. rosea* collected from Cheruvarankonum (Pr1). Next to that the accession Bangalore (Pr3) showed 1095 numbers of nucleotides. Lowest number of 590 nucleotides was displayed in the species *P. rosea* collected from Dana (Pr2). The total percentage of thymine content was higher (29.2%) in the *P. rosea* collected from Dana (Pr2) followed by the accession collected from Cheruvarankonum (Pr1) and

Bangalore (Pr3) showing thymine content of 28.8% and 28.6% respectively. Higher percentage of guanine (22.7%) was observed in the accession collected from Bangalore (Pr3). The nucleotide cytosine content (20.9%) was higher in the accession collected from Bangalore (Pr3). Adenine content was higher (28.5%) in the accession collected from Cheuvarankonum (Pr1).

Nucleotide directional 16 pair frequencies

The percentage of nucleotide substitution analysed using MEGA 6.0 software was illustrated in Table - 50. The average transversional pairs, transitional pairs and identical pairs were 14, 11 and 734 among the three aligned sequences of *P. rosea*. The AT and GC content was 8 and 7 for the paired sequences of the *P. rosea* accessions collected from Bangalore (Pr3) and Cheruvarankonum (Pr1). The overall percentage of GC and AT content for all the selected accessions of *P. rosea* was "3". The average of TT base pair was 297 in the paired accessions Cheruvarankonum (Pr1) and Bangalore (Pr3). The total average of TT base pairs for all the three selected accessions of *P. rosea* was 214. The R value for all the paired sequences of *P. rosea* was 0.8. The average of total nucleotide pair frequencies of the accessions Cheruvarankonum (Pr1) and Bangalore (Pr3) was 1095. The average of the total nucleotide pair frequencies of the three selected accessions of *P. rosea* was 758.3.

Nucleotide paired statistical data

The nucleotide sequences of *P. rosea* collected from three different accessions were compared in the MEGA version 6.0 and it gives the nucleotide frequencies comparing the three accessions. Among the three accessions the total number of base pairs is 1126. Among these 1023 conserved regions, 72 variable region, 2 singleton sites and "0" parsimonial sites are identified in the aligned sequences of *P. rosea* collected from three varied accessions. Following

these the highly similar accessions Cheruvarankonum (Pr1) and Bangalore (Pr3) recorded 1025 conserved regions and 70 variable regions. The results were displayed in Table - 51.

Maximum composite likelihood estimate of the pattern of nucleotide substitution

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in normal in the Table - 52. The nucleotide frequencies are 0.266 (A), 0.293 (T/U), 0.218 (C), and 0.223 (G). The transition/transversion rate ratios are k_1 = 0 (purines) and k_2 = 3.929 (pyrimidines). The overall transition / transversion bias is R =0.88, where R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 590 positions in the final dataset in aligned sequences of *P. rosea*. All calculations were conducted in MEGA 6.0 (Tamura *et al.,* 2007).

Composite distance pattern

The composite distance pattern measures the differences in the nucleotide composition among the sequences of the selected accessions. Among the three accessions lowest composite pattern (0.000) was observed between the accessions Cheruvarankonum (Pr1) and Bangalore (P3). The similar nucleotide distance pattern (0.005) was observed between the accessions Cheruvarankonum (Pr1) and Dana (Pr2): Dana (Pr2) and Bangalore (Pr3). The overall average composite pattern was 0.003. The composite distance was demonstrated in the Table - 53.

The cladogram constructed based on composite distance pattern using NTSys and the cladogram was displayed in Fig.52. The cladogram consists of two clades viz., Clade 1 (C_1) and Clade 2 (C_2). Clade 2 (C_2) was shared by the two accessions collected from Dana (Pr2) and Bangalore (Pr3). The clade 1 (C_1) represents the accessions of *P. rosea* collected from Cheruvarankonum (Pr1). The cladogram for composite distance was illustrated in Fig. 52.

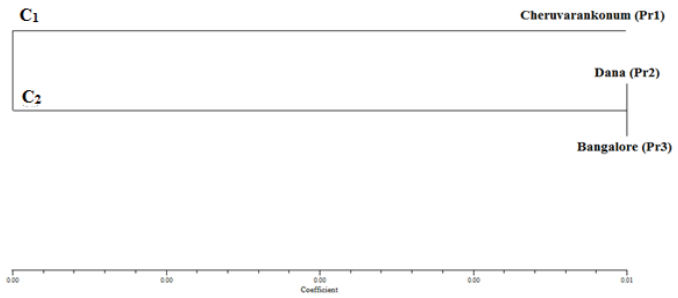


Fig. 52: Composite distance pattern between the selected accessions of *P. rosea*

Amino acid composition

The amino acid compositions of the selected accessions of *P. rosea* was analysed using MEGA 6.0 and the results of amino acid composition were displayed in Table - 54. The accession collected from Cheruvarankonum (Pr1) and Bangalore (Pr3) represented maximum number of 347 amino acids. The accession collected from Dana (Pr2) displayed least number of amino acids (188). Among the twenty amino acids, leucine represented highest percentage of occurrence (16.67%). In comparing the leucine content in the three accessions, the accession collected from Dana (Pr2) displayed the maximum percentage of 22.87% leucine and the least percentage of 14.70% leucine was observed in the accession collected from Cheruvarankonum (Pr1). The amino acid tyrosine was present in least percentage of 1.25%. The accessions collected from Cheruvarankonum (Pr1) and Dana (Pr3) experienced similar number of amino acids 347. The overall amino acid composition was 294.

Tajima's neutrality test

Tajima's neutrality test is a statistical method used for testing the neutral mutation hypothesis of *P. rosea* species collected from three different accessions. All positions containing gaps and missing data were eliminated from the dataset. The abbreviations used are as follows: **m** = number of sites, **S** = Number of segregating sites, $\mathbf{p_s}$ = S/m, $\Theta = p_s / a_1$, $\boldsymbol{\pi}$ = nucleotide

diversity and **D** is the Tajima test statistic. The Tajima's neutrality test was depicted in the Table 55.

Evolutionary relationships using different types of Bootstrap analysis

The phylogenetic analysis of *P. rosea* collected from three different accessions of South India were analysed using four methods viz., Neighbor joining method, maximum parsimony, minimum evolution method and UPGMA method. The percentage of similarity and evolutionary distance was calculated using the branch length of the cladogram presented.

Bootstrap Neighbor joining method

The Phylogenetic tree based on Bootstrap Neighbor joining method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1 was detached into two nodes C_1N^1 and C_1N^2. The node C_1N^1 represented the accessions Cheruvarankonum (Pr1) with a distance value of 0.000849 and the node C_1N^2 includes the accessions collected from Bangalore (Pr3) with a branch length of 0.000429. The clade 2 (C_2) consists of the *P. rosea* accession collected from Dana (Pr2) with the branch length of 0.001983 (Fig. 53). The accessions Pr1 and Pr3 showed close similarity showing the distance value of 0.001559 from the main clade.

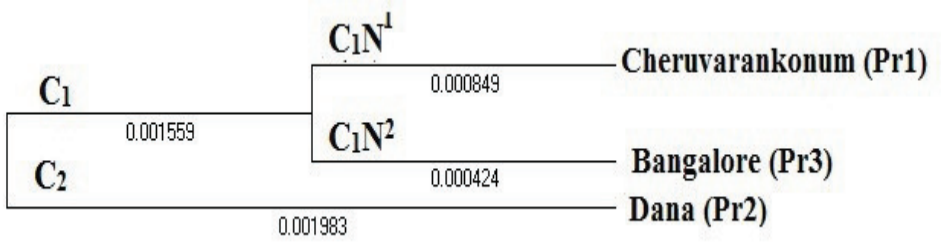


Fig. 53: Phylogenetic tree constructed using Bootstrap Neighbor joining method

Bootstrap minimum Evolution

The Phylogenetic tree based on Bootstrap minimum evolution method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1(C_1) was detached into two nodes viz., C_1N^1

and C_1N^2. The node C_1N^1 consists of the accession collected from Cheruvarankonum (Pr1) illustrating the distance value of 0.00000 and the node C_1N^2 represents the accession collected from Bangalore (Pr3) showing the distance value of 0.0000. The clade 2 (C_2) consists of the accession collected from Dana (Pr2) suggesting the distance value of 0.001983. The *P. rosea* accession collected from Cheruvarankonum (Pr1) and Bangalore (Pr3) revealed 70% similarity and revealed 0.000849 from the main clade (Fig. 54).

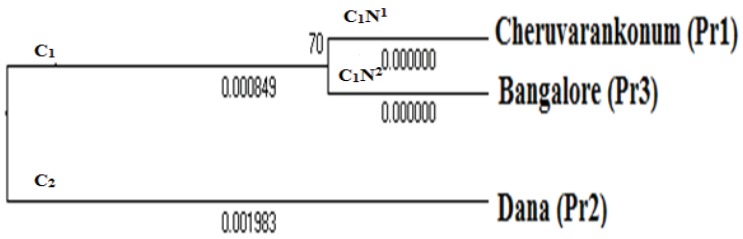


Fig. 54: Phylogenetic tree constracted using Bootstrap minimum evolution method

Bootstrap Maximum Parsimony

There is no parsimonial informative sites in the aligned sequences of *P. rosea* collected from three different accessions of South India.

Bootstrap UPGMA analysis

The Phylogenetic tree based on UPGMA method consists of two clades viz., Clade 1 (C_1) and Clade 2 (C_2). Clade 1 (C_1) was shared by the accession collected from Cheruvarankonum (Pr1) and Bangalore (Pr3) showing the distance value of 0.00000. The clade 2 (C_2) consists of the accession collected from Dana (Pr2) suggesting the distance value of 0.001983. The *P. rosea* accession collected from Cheruvarankonum (Pr1) and Bangalore (Pr3) revealed 71% similarity with 0.000849 distance value from the main clade (Fig. 55).

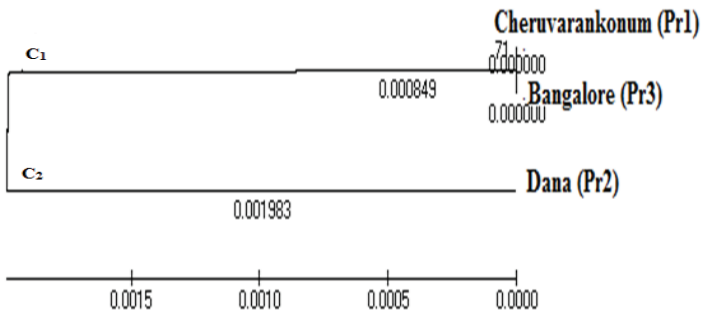


Fig. 55: Phylogenetic tree constructed using Bootstrap UPGMA method

The accessions collected from Cheruvarankonum (Pr1) and Bangalore (Pr3) illustrated the lowest distance value compared to the accession collected from Dana (Pr2) demonstrated the distance value of 0.000829 in the phylogenetic tree based on Bootstrap Neighbor joining method. The Phylogenetic tree based on Bootstrap minimum evolution method and UPGMA method suggested the distance value of 0.00000. The accession *P. rosea* collected from Dana (Pr2) showed its unique presence in the clade 2 (C_2).

The above multiple sequence alignment, phylogenetic analysis, amino acid composition and statistical parameters carried out by MEGA 6.0 version software for the barcoded *rbc*L sequences of *P. rosea* revealed that the sequences of the accessions collected from Cheruvarankonum (Pr1) and Bangalore (Pr3) are highly conserved and identical. The accession collected from Dana (Pr2) represented an isolated clade.

DNA Barcoding to reveal the inter-specific variation among the selected *Plumbago* species

The genomic DNA isolated from the *Plumbago* species viz., *P. zeylanica, P. auriculata* and *P. rosea* collected from Karnataka were amplified using universal primer performedfor the gene *rbc*L and the base pairs of the isolated DNA of the three species was identified in agarose gel electrophoresis using 100 base paired DNA ladder as shown in Plate XXIV. The PCR amplification efficiency was good for the selected accessions and the amplicons were sequenced.

The obtained sequences were annotated and submitted in GenBank viz., KF233552, KF233547 and KF261598. Multiple sequence alignment was performed for *rbc*L region using CLUSTAL W and MULTALIN. The sequence alignment discriminated the inter-specific variation among the *Plumbago* species collected from Karnataka. Phylogenetic analysis was performed by Minimum evolution method using MEGA 6.0 (Molecular Evolutionary Genetic analysis). To know the molecular similarity and variation, it is necessary to know some basic statistical similarities, such as nucleotide frequencies, codon frequencies and transition / transversion ratios (Tamura *et al.,* 2013). The sequences of *Plumbago* species aligned by MULTALIN were displayed in Fig. 57.

Alignment result

The results of multiple sequence alignment using MULTALIN are summarized in Fig. 60. The phenogram displayed two clades viz., clade 1(C_1) and clade 2(C_2) clade 1(C_1) was separated into two nodes C_1N^1 includes the species *P. zeylanica* and C_1N^2 denotes *P. auriculata*. The species *P. rosea* showed its unique presence in the clade 2 (C_2). This shows the variation among the three *Plumbago* species. These phenogram shows the variations and similarities among the DNA sequences of *Plumbago* species collected from Karnataka (Fig. 56).

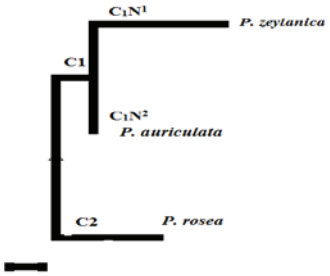


Fig. 56. Phenographic representation of *Plumbago* species based on Multiple Sequence Alignment

Variation analysis using nucleotide composition

The nucleotide composition of the three aligned sequences of *Plumbago* species were analysed using the statistical tool of MEGA 6.0 and the results were displayed in Table - 56. Among the three *Plumbago* species more number of nucleotides (1114) was demonstrated in *P. rosea*. Next to that *P. zeylanica* showed 1095 numbers of nucleotides. Lowest number of nucleotides (613) was observed in the species *P. auriculata*. The total percentage of thymine content was higher (29.4%) in the species *P. auriculata* followed by the species *P. rosea* and *P. zeylanica* showing thymine content of 28.6% and 28.2% respectively. Higher percentage of guanine (23%) was observed in the species *P. zeylanica*. The nucleotide cytosine content (21.4 %) was higher in the species *P. auriculata*. Adenine content was higher (28.2%) in the species *P. zeylanica*.

Nucleotide directional 16 pair frequencies

The percentage of nucleotide substitution analysed using MEGA 6.0 software was displayed in Table - 57. The average transversional pairs, transitional pairs and identical pairs were 200, 384 and 190 among the three selected *Plumbago* species. The AT and GC content was 51 and 30 for the paired sequences of *P. zeylanica* and *P. auriculata*. The overall percentage of GC and AT content for all the selected *Plumbago* species was 38 and 62. The average of TT base pair was 46 in the paired sequences of *P. zeylanica* and *P. auriculata*. The total average of TT base pairs for all the three selected *Plumbago* species was 63. The R value for all the paired sequences of *Plumbago* species was 0.5. The average of total nucleotide pair frequencies of *P. zeylanica* and *P. auriculata* was 613. The average of the total nucleotide pair frequencies of the three selected *Plumbago* species was 773.7.

Nucleotide paired statistical data

The nucleotide sequences of *Plumbago* species viz., *P. zeylanica, P. auriculata* and *P. rosea* were compared in the MEGA version 6.0 and it gives the nucleotide frequencies comparing the three accessions. Among the three *Plumbago* species the total numbers of base pairs are 1114. Among these 146 conserved regions, 949 variable region, 581 singleton sites and "0" parsimonial sites are identified in the aligned sequences of *Plumbago* species. Following these highly similar species *P. zeylanica* and *P. auriculata* recorded 151 conserved regions and 462 variable regions. The results were displayed in Table - 58.

Maximum composite likelihood estimate of the pattern of nucleotide substitution

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in normal in the Table - 59. The nucleotide frequencies are 0.267 (A), 0.291 (T/U), 0.214 (C), and 0.228 (G). The transition/transversion rate ratios are k_1 = 7.164 (purines) and k_2 = 7.287 (pyrimidines). The overall transition / transversion bias is R = 3.274, where R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 613 positions in the final dataset in aligned sequences of *Plumbago* species. All calculations were conducted in MEGA 6.0 (Tamura *et al.,* 2007).

Composite Distance Pattern

There is no variation based on composite distance pattern among the selected *Plumbago* species.

Amino acid composition

The amino acid compositions of the selected *Plumbago* species was analysed using MEGA 6.0 and the results were displayed in Table - 60. *P. rosea* represented maximum number of 354 amino acids. *P. auriculata* recognized least number of 189 amino acids. Among the

twenty amino acids, leucine represented highest percentage of 14.84%. Among the studied *Plumbago* species maximum percentage of 17.23% leucine was observed in *P. rosea* and the least percentage of leucine (8.47%) was observed in *P. auriculata.* The amino acid histidine was present in least percentage of 2.38%. The *Plumbago* species *P. zeylanica* and *P. rosea* experienced more or less similar number of amino acids 340 and 354. The overall amino acid composition was 294.3.

Tajima's neutrality test

Tajima's neutrality test Statistical method for testing the neutral mutation hypothesis of *Plumbago* species collected from Karnataka using MEGA 6.0. All positions containing gaps and missing data were eliminated from the dataset. The abbreviations used are as follows: **m** = number of sites, **S** = Number of segregating sites, $\mathbf{p_s}$ = S/m, $\Theta = p_s/ a_1$, $\boldsymbol{\pi}$ = nucleotide diversity and **D** is the Tajima test statistic. The Tajima's neutrality test was depicted in the Table 61.

Evolutionary relationships using different types of Bootstrap analysis

The phylogenetic analysis of *Plumbago* species collected from Karnataka were analysed using four methods viz., Neighbor joining method, maximum parsimony, minimum evolution method and UPGMA method. The percentage of similarity and evolutionary distance was calculated using the branch length of the cladogram presented.

Bootstrap Neighbor joining method

The phylogenetic tree based on Bootstrap neighbor joining method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1(C_1) was detached into two nodes C_1N^1 and C_1N^2. The node C_1N^1 represented *P. zeylanica* with a distance value of 5.350 and the node C_1N^2 includes the *P. auriculata* with a branch length of 7.968. The clade 2 (C_2) consists of *P. rosea*

with the branch length of 25.983 (Fig. 58). The species *P. zeylanica* and *P. auriculata* showed the distance value of 18.015 from the main clade.

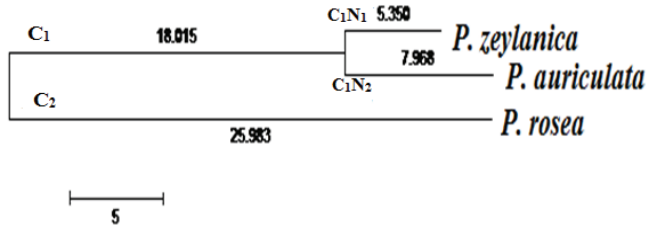


Fig. 58: Phylogenetic tree constructed using Bootstrap Neighbor joining method

Bootstrap minimum evolution

The phylogenetic tree based on Bootstrap minimum evolution method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1(C_1) was detached into two nodes C_1N^1 and C_1N^2. The node C_1N^1 represented *P. zeylanica* with a distance value of 0.001663 and the node C_1N^2 includes the *P. auriculata* with the similar distance value of 0.001663. The clade 2 (C_2) consists of *P. rosea* with the branch length of 0.002496 (Fig. 59). The species *P. zeylanica* and *P. auriculata* represented the distance value of 0.000830 from the main clade.

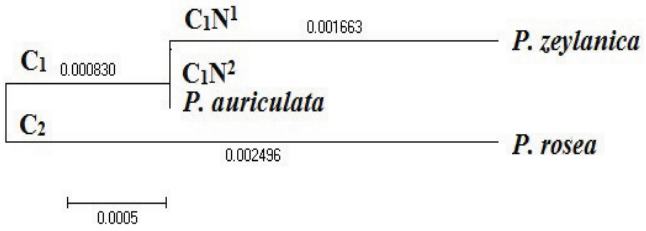


Fig. 59: Phylogenetic tree constructed using Bootstrap minimum evolution method

Bootstrap maximum parsimony

There are no parsimonial sites in the aligned sequences of three *Plumbago* species.

Bootstrap UPGMA analysis

The phylogenetic tree based on Bootstrap UPGMA method consists of two clades viz., Clade 1(C_1) and Clade 2 (C_2). Clade 1(C_1) was detached into two nodes C_1N^1 and C_1N^2. The node C_1N^1 represented *P. zeylanica* and the node C_1N^2 includes the *P. auriculata* with a similar branch length of 6.659. The clade 2 (C_2) consists of *P. rosea* with the branch length of 25.329 (Fig. 60). The species *P. zeylanica* and *P. auriculata* illustrated the distance value of 18.670 from the main clade.

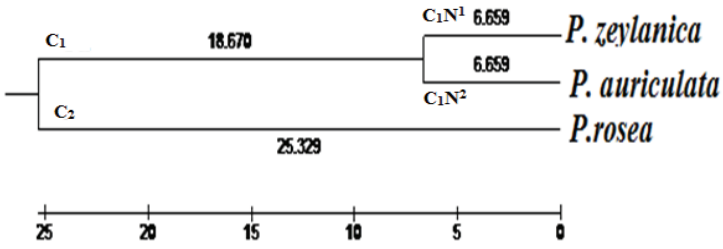


Fig. 60: Phylogenetic tree constructed using Bootstrap UPGMA method

P. zeylanica and *P. auriculata* illustrated the lowest distance value compared to *P. rosea* demonstrated the distance value of 18.015 in the phylogenetic tree based on Bootstrap Neighbor joining method. The Phylogenetic tree based on Bootstrap minimum evolution method and UPGMA method suggested the distance value of 0.000830 and 18.670 from the main clade. *P. rosea* revealed its unique presence in the clade 2 (C_2) in all the bootstrap phylogenetic tree methods.

From the above multiple sequence alignment, phylogenetic analysis, amino acid composition and statistical parameters carried out by MEGA 6.0 version software on the barcoded *rbc*L sequences of *Plumbago* species revealed that *P. zeylanica* and *P. auriculata* are highly conserved and identical whereas *P. rosea* was present in a separate clade illustrating the variation in the sequences.

Antibacterial activity

Antibacterial activity of *P. zeylanica, P. auriculata* and *P. rosea* were screened in six different extracts with five different concentrations (20 - 100 µg/ml) against six different pathogens viz., *S. aureus, B. subtilis, S. pyogenes* (Gram positive), *K. pneumoniae, M. morganii, P. aeroginosa* (Gram negative) using well diffusion method.

Among the eighteen different extracts of three different *Plumbago* species, highest degree of antibacterial activity (54%) was recorded in *P. rosea*. The range of antibacterial activity of various extracts in different concentration of the selected *Plumbago* species are as follows: *P. rosea* (54%) > *P. zeylanica* (49%) > *P. auriculata* (40%).

Major antibacterial activities were observed predominantly in ethanolic extracts (85%) of *P. zeylanica.* However the range of the inhibition zone varied with test organisms and based on different concentration of extracts. Highest antibacterial activity was observed in ethanolic extract at 100 µg/ml followed by petroleum ether and chloroform extract. There was minor difference on the size of the inhibition zone between ethanolic and other extracts of *P. zeylanica.* The range of inhibitory activity was less at lower concentration of extracts (Fig. 61 / Table - 62).

Ethanolic extracts of *P. zeylanica* was active against all the selected bacterial pathogens except *B. subtilis* which represents no zone of inhibition at 20 - 40 µg/ml concentration. Compared to standard amikacin (30 µg), 100 µg/ml of ethanolic extract showed more antibacterial (90% and 5%) activity against *S. pyogenes* and *K. pneumoniae* respectively (Plate XXVI). The pathogens *K. pneumonia* and *S. pyogenes* showed higher antibacterial activity to all the tested extracts of *P. zeylanica.*The petroleum ether extract of *P. zeylanica* expressed maximum zone of inhibition (20 ± 0.5 mm) against *S. pyogenes.*

The acetone and aqueous extracts of *P. zeylanica* failed to show the antibacterial activity against *S. aureus*. Similar to that, the petroleum ether, acetone and aqueous extracts of *P. zeylanica* were also unsuccessful against *B. subtilis*. The pathogen *S. pyogenes* doesnot show inhibition against 20 – 100 µg/ml of ethyl acetate, 20, 40 and 60 µg/ml of chloroform extracts and 20 & 40 µg/ml of aqueous extracts. The ethanolic extracts of *P. zeylanica* displayed higher antibacterial activity against *S. pyogenes* with 21 ± 0.3 mm zone of inhibition. Except 20 - 40 µg/ml aqueous extract of *P. zeylanica* all the other extracts of various concentrations (20-100 µg/ml) showed activity against *K. pneumoniae* and 100 µg/ml of *P. zeylanica* ethanolic extracts showed maximum zone of inhibition with 22 ± 0.3 mm. No antibacterial activity was recognized in 20 – 60 µg/ml of petroleum ether extract and 20 -100 µg/ml ethyl acetate extracts of *P. zeylanica* against *M. morganii.* 20 -100 µg/ml of *P. zeylanica* chloroform and aqueous extract failed to show inhibition against *P. aeruginosa* (Fig. 61/ Table 62).

The highest zone of inhibition was observed in 100 µg/ml of *P. zeylanica* ethanolic extracts compared to other tested extracts. The antibacterial activity of *P. zeylanica* extracts at different concentrations are arranged as follows: ethanolic extracts (85%) > petroleum ether (69%) > chloroform (62%) > acetone (53%) > ethyl acetate (34%) > aqueous extract (31%). Ethanolic extracts of *P. zeylanica* showed 91% percentage of activity against Gram positive pathogens and 66% of activity against Gram negative pathogens. The pathogens illustrating highest zone of inhibition was displayed in Plate XXV.

Similar to *P. zeylanica*, highest activity was observed in ethanolic extracts (70%) of *P. auriculata* followed by petroleum ether and chloroform extracts. However the range of the inhibition zone varied with test organisms and based on the concentration of extracts. There was distinguished difference on the size of the inhibition zone between ethanolic and other extracts of

P. auriculata. The range of inhibition was directly coinincided with the concentrations of extracts tested, less inhibition was observed at lower concentration of extracts and maximum inhibition was obtained in higher comcentrations (Fig. 62 / Table - 63).

S. aureus was unsuccessful to show inhibitory activity at 20 - 60 µg/ml of aqueous, 20 - 100 µg/ml of acetone and ethanolic extract of *P. auriculata*. 20 and 40 µg/ml of chloroform and aqueous extracts were failed to show inhibiton against *B. subtilis*. 20 and 40 µg/ml of aqueous, 20- 100 µg/ml of petroleum ether and chloroform extracts of *P. auriculata* were unsuccessful against *S. pyogenes*. The pathogen *K. pneumoniae* depicted antibacterial activity against all the tested extracts except 20 and 40 µg/ml of acetone and aqueous extracts of *P. auriculata*. The ethanolic extracts of *P. auriculata* demonstrated maximum zone of inhibition (23 ± 0.3 mm) *M. morganii* was incapable to demonstrate inhibitory activity against 20 - 60 µg/ml of acetone, 20 - 100 µg/ml of ethyl acetate and aqueous extracts of *P. auriculata* (Fig. 62 / Table - 63).

The *P. auriculata* ethanolic extracts was active against all the examined pathogens except *S. aureus.* Compared to all the tested pathogens, *K. pneumoniae* was highly sensitive to all the screened extracts of *P. auriculata.* The 40 -100 µg/ml of ethyl acetate extracts represented more percentage of activity (18, 27, 45 and 63%) against *S. pyogenes* than the standard amikacin. 100 µg/ml ethanolic extracts represented 18% and 9% of more antibacterial activity against *S. pyogenes* and *K. pneumoniae* than the standard amikacin. Similar to ethyl acetate and ethnaolic extracts, acetone extracts also showed more activity against *S. pyogenes* than the standard amikacin.

The significant antibacterial activity was observed in ethanolic extracts compared to other tested extracts of *P. auriculata*. The activity of *P. auriculata* extracts as follows: Ethanolic extracts 70% > Petroleum ether 54%, > Ethyl acetate 53%> Acetone 48% > Chloroform 34%>

Aqueous extract 31%. The ethanolic extracts of *P. auriculata* showed above 50% activity against Gram positive pathogens and 26% activity against Gram negative pathogen. The pathogens illustrating highest zone of inhibition was displayed in Plate XXVI.

In *P. rosea,* highest activity was observed in ethanolic extracts (91%) followed by acetone (64%) and chloroform extracts (59%). The ethanolic extracts 100 µg/ml showed 45% higher antibacterial activity against *S. pyogenes* and 10% more activity against *M. morganii* than the standard amikacin. There was prominent difference on the size of the inhibition zone between ethanolic and other extracts of *P. rosea*. *K. pneumoniae* recognized antibacterial activity to all the screened extracts of *P. rosea* (Fig. 63 / Table 64).

The ethanolic extracts of *P. rosea* were active against all the selected bacterial pathogens except 20 µg/ml of ethanolic extract. Highest antibacterial activity was observed in ethanolic extracts against *B. subtilis* and *M. morganii* with 22 ± 0.3 and 22 ± 0.7 mm zone of inhibition.The results showed that acetone and aqueous extracts failed to show inhibition against *S. aureus.* Ethanolic extracts with 20 µg/ml and 20 -100 µg/ml of chloroform extracts of *P. rosea* were unsuccessful to show inhibiton against *B. subtilis*. The pathogen *S. pyogenes* was unable to show inhibition in 20-100 µg/ml of *P. rosea* petroleum ether and aqueous extracts and 20 µg/ml of *P. rosea* chloroform extracts. Except 20 µg/ml of aqueous extracts, all other extracts of *P. rosea* with various concentrations (20 - 100 µg/ml) should activity against *K. pneumonia* and maximum zone of inhibition (20 ± 0.3 mm) was observed in ethanolic extracts of *P. rosea.* The 100 µg/ml of *P. rosea* ethanolic extracts displayed higher activity against *M. morganii* with 22 ± 0.5 mm zone of inhibition. All the concentrations of ethyl acetate & acetone extracts of *P. rosea* and 20 µg/ml of *P. rosea* ethanolic extracts unsuccessful to show the inhibition against *M. morganii. P. aeruginosa* was ineffective to show antibacterial activity against petroleum ether

and aqueous extracts of *P. rosea.* The pathogens illustrating highest zone of inhibition was displayed in Plate XXVII.

Similar to other two studied plants, the highest zone of inhibition was observed in ethanolic extracts compared to other tested extracts of *P.rosea.* The activity of *P. rosea* extracts as follows: Ethanolic extracts possess 91% >Acetone 64% > Chloroform 56% > Ethyl acetate 52% > Petroleum ether 50% > Aqueous extract 15%. Ethanolic extract showed 99% activity against Gram positive pathogens and 89% activity against Gram negative pathogens.

Plants grow in populations in difficult situations along gradients of latitude, longitude and temperature. Therefore variations within and between populations are not uncommon. Earlier, much of the variation in phenotype was attributed to environmental influences. Many botanists reasoned that distinct intra-specific and inter-specific variations of plants were merely habitat modifications and adaptation to environment was by phenotypic plastic response (Bodekar, 1997). Different environmental conditions can also affect the chemical composition of the plants. Variations observed in plants can be grouped into two simple categories, epigenetic and genetic. Genetic variations are a result of alterations in genetic material that may have taken place during the course of time course and now are strictly heritable. These variations influence the phenotypic and chemical characteristics of the medicinal plants and do not change under conditions of cultivation (Chew and Rodman, 1979; Johnson and Scriber, 1994). The epigenetic variations on the other hand are solely dependent on the environment in which the plants are growing and are also partially affected by developmental efforts. However, methylation based epigenetic alterations often have phenotypic consequences without changes in the underlying DNA sequences and are stably transmitted from parents to offsprings in plants. Therefore a great deal of information-morphological, biochemical, physiological and genetic is necessary before the observed pattern of variation may be interpreted. It is also true that beneath these intra-specific variations, there exists a fixed unchangeable spectrum of characteristics which make up the species (Khan *et al*., 2010).

Pharmacognosy is the scientific study of crude drugs which originated from different natural sources namely plants, animals, minerals and metals. It is estimated that 90% of the crude

drugs are originated from plant sources (Joy *et al*., 1998). Pharmacognosy provides the basis for the study of secondary metabolites, which are beneficial for their ecological, medicinal, gustatory or other functional properties. The field of Pharmacognosy is not limited to special area and is constantly being reinvigorated by input from time to time by new developments in scientific fields and the development of new technologies (Samuelsson, 2004; Taylor *et al*., 2001). Pharmacognosy research areas are continuing to expand and now include aspects of cell and molecular biology in relation to natural products, ethanobotany and phytochemistry (Cooper, 2002).

Systematics is an important tool in pharmacognostical practice and research. This is due to the fact that related families often contain similar types of compounds. The systematic position of a particular plant helps to have an idea about the probable presence of secondary metabolites in it (Vijayakumari *et al.,* 2008; Khatoon *et al.,* 2008). Progress in pharmacognosy has capitalized on the anatomical and chemical profiles as markers for authentication. The advancement of techniques in molecular biology has provided new means for examining genotypic characteristics (Nambiar *et al.,* 2000; Syamasundar *et al.,* 1999). Therefore using newer analytical techniques as markers can be generated for the researches as a chain of markers for use of the common man to evaluate the quality of herbal drug and also to incorporate the knowledge obtained in pharmacopoeias. In the present study also the spectroscopic and chromatographic profiles were used to reveal inter and intra-specific variation between the *Plumbago* species. The results of the present study are supplemented as markers to evaluate the quality of herbal products.

Plants as master chemists accumulate a wealth of intricate secondary metabolites, which further deliver effective treatments for a plethora of human diseases. However, the concentration

and composition of these secondary metabolites varies in nature amongst the members of same species (Arora *et al.,* 2005; Artyukova *et al.,* 2004). Phytochemicals are chemical compounds formed during the plants normal metabolic processes. Pharmacological activities of a drug or plant are contributed only by the presence of these secondary metabolites such as alkaloids, phenols, terpenoids, glycosides, saponins, tannins, anthraquinones and flavonoids which varies from plant species to species (Menkovic *et al*., 2000; Kubmarawa *et al.,* 2008; Shalini and Sampathkumar, 2012). The medicinal value of the plant is directly dependent on the level of therapeutically active molecules engendered by it. Natural populations of the plant species may have superior and inferior chemotypes on the basis of the content of active phytochemicals. This variation is again orchestrated by either intrinsic (genetic) or extrinsic (environmental) factors or the interaction between the two.

The plant material may be subjected to preliminary phytochemical screening for the detection of various plant constituents (Kokate, 2001).The present study was designed to interpret the possible correlation between the phytochemical concentrations with their genetic structure. The results revealed inter and intra-specific variation among the selected *Plumbago* species. The results supplemented the observations made by Arora *et al.,* 2005 and Artyukova *et al.,* 2004.

According to Pawar *et al*. (2010), the physiological action on the human body can be determined by these phytochemicals. Many of the plant extracts have proven to possess pharmacological action due to the presence of various phytoconstituents. Plants produce a large variety of secondary products that contain a phenolic group, a hydroxyl functional aromatic phenol ring, a chemically heterogeneous group. They could be an important part of the plants defense system against pests and diseases including root parasitic nematodes (Wuyts *et al*.,

2006). Terpenoids constitute the largest class of secondary metabolites and are united by their common biosynthetic origin from acetyl-coA or glycolytic intermediates (Gerhenzon *et al*., 2000; Grayson, 1998). Terpenoids are presumed to be involved in plant defense as toxins and feedants to a large number of plant feeding insects and mammals (Gershenzon and Croteau, 1991). Flavonoids protect plants against various biotic and abiotic stresses and exhibit a diverse spectrum of biological functions and play an important role in the interaction between plants and their environment (Pourcel *et al.,* 2007). Flavonoids have been considered to have an effect on human nutrition and health as it shows antioxidant activity and their mechanism of action are through scavenging or the chelating process (Anpin *et al.,* 2010).

Alkaloids are a large family of nitrogen containing secondary metabolites found in approximately 20% of the species of vascular plants (Hegnauer *et al*., 1988). Most of them, including the pyrrolizidine alkaloids (PAs) are toxic to some degree and appear to serve primarily in defense against microbial infection and herbivoral attack. Tannins are one of the major phytochemicals found in many higher plants. Tannins have major impact on animal nutrition, including inhibition of growth rate digestive enzymes (Bennick, 2000). Tannins and related polyphenols have been implicated to various pharmacotherapeutic effects (Ferreira *et al.,* 2008). In particular, the tannin containing remedies are in use as antihelmintics (Ketzis *et al.,* 2006), antioxidants (Koleckar *et al*., 2008), antimicrobials and antivirals (Buzzini *et al.,* 2008), cancer treatment (Chung *et al*., 1998) and to chelate dietary iron (Clauss *et al.,* 2007).

Saponins are secondary plant metabolites that occur in a wide range of plant species (Hostettmann and Marston, 1995). They are stored in plant cells as inactive precursors but are readily converted into biologically active antibiotics by plant enzymes in response to pathogen attack (Osbourn, 1996). The natural role of saponins in plants is thought to be protection against

attack by pathogens (Morrissey and Osbourn, 1999). Plants produce a variety of different sterols. In parallel to the functions of other sterols in animals, plant sterols are membrane constituents (Hartmann, 1998) as well as precursors for plant hormones and other secondary metabolites, i.e., substances interfering with pathogens and insects (Hartmann, 1998; Lindsey *et al.,* 2003; Schaller, 2003;).

The presence of several secondary metabolites and carbohydrates in *Plumbago* species (*P. zeylanica, P. auriculata* and *P. rosea*) suggests that these plants are considered as the potential sources of drugs, which could be used for the preparation, formulation and delivery of medicines and thus it forms one of the important medicinal plants. Krishnaswamy and Purushothaman (1980), Bhargava (1984), Itoigawa *et al.* (1991), Dinda *et al.* (1997), Likhitwitayawuid *et al.* (1998), Sugie *et al.* (1998) and Wang and Huang (2005) have reported various pharmacological properties and role of phytoconstituents present in the *Plumbago* species for anti cancerous and anti-microbial activities, anti-HIV-1, anti-malarial ,cardiotonic, anti-fertility action, anti-*Helicobacter pylori* activity and anti-intestinal carcinogenesis. Plumbagin is the main chemical constituent present in *Plumbago* species which act as chemical marker for the identification of *Plumbago* species.

In the present investigation, the presence of these chemical constituents has been evaluated by qualitative estimation (preliminary phytochemical analysis) in the selected *Plumbago* species. This can be also be used as a chemical tool to reveal the inter-specific variation among the closely related *Plumbago* species. The results indicate that all the tested phytoconstituents are present to the maximum in the ethanolic extract and to the minimum in aqueous extracts of the selected *Plumbago* species. However tannins which are present in the all the tested extracts of *Plumbago* species except ethyl acetate extract.

The results of the inter-specific variations based on the existence of phytoconstituents among the *Plumbago* species are displayed in UPGMA cladogram. The cladogram demonstrates two clusters, of which Cluster 1 (C_1) was shared by *P. zeylanica* and *P. auriculata*. Cluster 2 (C_2) includes *P. rosea* only and showed 100% of divergence from other two studied species. The results suggest that the number of phytoconstituents varied from other two *Plumbago* species. Thus preliminary phytochemical analysis was used as a tool to study the inter-specific variation among the selected *Plumbago* species and it can be used as a pharmacognostical marker for the qualitative analysis.

In the current study, a total of 83 chemical compounds were identified from the three different species of *Plumbago*, Similarly, Ravikumar and Sudha (2011) reported fourteen phyto constituents in the aerial parts of *P. zeylanica* petroleum ether, ethanol and aqueous extracts. Among these ethanolic extracts represented the maximum number of nine compounds compared to other extracts. Haribabu *et al.* (2012) reported that the phytoconstituents presence in the root ethanolic extracts of *P. zeylanica*. Ibrahim *et al.* (2012) identified the presence of alkaloids, tannins, steroids, saponins, glycosides and reducing sugar in the methanolic extract of *P. indica.* The results of the present study also coincide with the observations of Ibrahim *et al.* (2012). *P. zeylanica* and *P. rosea* demonstrated higher phytoconstituents presence in ethanolic extracts.

The complexity of the mixture of compounds and the presence of several compounds in small concentrations can make the isolation and identification of the substances present in this genus very laborious. It has been established that the choice of solvent in the isolation of compounds is very crucial (Calixto *et al*., 1998; Khan *et al.,* 2010). Thus in the present observation, it is concluded that the ethanolic extract had the maximum number of chemical constituents in all the three *Plumbago* species. So the *Plumbago* ethanolic extract can be chosen

as a key solvent to identify and compare the different phytoconstituents using advanced instrumental analysis.

The fluorescence phenomenon exhibited by plant powder or extracts is primarily due to its chemical composition. The same material may appear to be dissimilar in different wavelength of light. Some constituents of the extract show fluorescence in the visible range in daylight while some florescence only in Ultra Violet light. If substances do not show fluorescence phenomena, then they may be made fluorescent by applying various reagents to their decomposition products or their derivatives. Fluorescence study therefore can be used as a finger print for crude drug and plant identification (Ansari *et al.,* 2006; Reddy and Chaturvedi, 2010). In the present study also, fluorescence analysis was used as a pronounced pharmacognostic marker to differentiate and characterize the inter-specific variation among the selected *Plumbago* species. The results based on fluorescence pharmacognostic analysis showed minute variations between the crude powder and various extracts of *Plumbago* species. Similarly, Saha and Swati (2012) studied the fluorescence analysis on powder and aqueous extracts of aerial parts of *P. indica.* Their results also showed varied colour, odour and taste in the aerial parts powder and aqueous extract of *P. indica* treated with different chemicals recognising diverged colours under visible and UV light. Similarly in the present study also, the fluorescence analysis recognised interspecific variations among the selected species.

Ultra Violet Visible (UV-Vis) spectroscopy is used as an analytical technique for two reasons. First, it can be used to identify certain functional groups in molecules, and second, it can be used for assaying the chemical constituents. UV-Vis analysis is an analytical method based on the property of an ion or molecular species to absorb at certain wavelengths of UV-Vis radiation. In the process of absorbing the radiation, the energy of the photons is transferred to the

molecules of the medium under analysis to cause electron transitions associated with vibrational and rotational transitions (Israni *et al.,* 2010). The chemical constituents of the three selected *Plumbago* species was analysed by UV-Vis spectroscopic analysis in six different extracts of three *Plumbago* species, which revealed the presence of various phytoconstituents and the occurrence of plumbagin was identified. Plumbagin was higher in chloroform extract of *P. rosea* displaying 3.436 abs at 415nm followed by acetone extract of *P. auriculata* showing 2.683 abs at 411 nm and chloroform extract of *P. zeylanica* recognising 2.659 abs at 416 nm. Israni *et al.* (2010) studied the presence of plumbagin in the acetone root extract of different Plumbaginales viz., *P. zeylanica, P. auriculata* and *P. rosea* using UV-Vis spectrophotometric method at 520 nm wavelength and determined that *P. rosea* showed maximum absorbance with (0.424 abs) followed by *P. zeylanica* (0.416 abs) and *P. auriculata* (0.281 abs). They observed maximum absorbance in acetone root extract of *P. rosea* followed by other two *Plumbago* species. Similarly in the present research, maximum absorbance of plumbagin was expressed in the acetone extract of *P. rosea* at 400 nm followed by the other two *Plumbago* species. Ariyanathan *et al.* (2010) recorded the presence of two flavonoids and two carboxylic acids in the ethyl acetate extract of *P. rosea* using UV-Vis analysis. Polyphenols are normally characterized by their specific chromatographic behaviours and distinctive UV-Vis spectra. Nile and Khobragade (2010) quantified the phenolic and flavanoid content in the ethanolic root extract of *P. zeylanica* at 750 and 510 nm using UV-Vis spectroscopic study. In the present study also, different compounds were revealed in varied nanometers, Similar to to the work of Nile and Nil and Khobragade (2010) the results of the present analysis showed the absorbance in the range of 503-532 nm which recognized the presence of flavonoid in all the extracts of three *Plumbago* species. Among these the acetone extracts of *P. rosea* reported maximum absorbance of 0.829 at 503 nm

and the least absorbance was noted in ethanolic extract of *P. zeylanica* showing the absorbance of 0.056 at 532 nm. From the present study, it was concluded that UV-Vis analysis can be used as a spectroscopic tool to study the variation between the biologically related *Plumbago* species. The amalgamated cladogram based on preliminary phytochemical and UV-Vis analysis on the selected *Plumbago* species characterized similar results classifying *P. rosea* in an isolated clade which showing its distinct features from *P. zeylanica* and *P. auriculata* shared by a common clade.

FTIR is one of the most widely used methods to identify the chemical compounds structures, functional groups and has been used as a requisite method to identify pharmacopoeia in many countries (Liu *et al.,* 2006). FTIR spectroscopy was useful for the compound identification and when run under IR region in the range of 400 – 4000 cm^{-1}. FTIR allows detecting the whole range of infrared spectrum simultaneously providing speed and accuracy in measurements of biological compounds (Thenmozhi *et al.,* 2011). FTIR method has been successfully utilized in the characterization of bacterial, fungal and plant species (Helm *et al.,* 1991; Timmins *et al.,* 1998). Many workers applied the FTIR spectrum as a tool for differentiating, classifying and discriminating closely related plants and other organisms (Rebuffo *et al.,* 2006; Thenmozhi *et al.,* 2011). Cong *et al.* (2013) elucidated eleven new guanidine alkaloids, plumbagines and *Plumbago*sides from the aerial parts of *P. zeylanica* using IR spectroscopic studies. Subhash *et al.* (2012) investigated the presence of flavonoid in the ethanolic extract of *P. zeylanica* and identified the presence of functional groups, phenolic groups, C=C stretching aromatic group and C=H bending ring. In comparison to the work of Subhash *et al.* (2012), the FTIR spectroscopic profile of the present study also showed the presence of phenolic groups in the powder and ethanolic extracts of all the selected *Plumbago*

species. Sreelatha *et al.* (2010) determined the presence of six known compounds and two new naphthoquinones in the chloroform root extracts of *P. capensis* using IR spectroscopic data analysis. Ariyanathan *et al.* (2010) isolated and identified two flavanoids and two carboxylic acids using IR spectroscopic studies from the ethyl acetate extracts of *P. rosea* collected from Erode. Hazhar *et al.* (2009) studied the spectroscopic data (IR, 1H NMR, and 13C NMR) of *P. europaea* which confirmed the chemical structure of the isolated constituent (A) to contain the important functional groups of plumbagin. The FTIR spectrum of the crude powder and extracts of *Plumbago* species randomly coincided with the spectral profile of standard plumbagin which recognised the presence of plumbagin in the selected *Plumbago* species. The inter-specific variations among these selected species were recognised by comparing the peak value and the functional groups among them. The results illustrated some functional groups are commonly shared among these species

Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment of traditional systems of medicine throughout the world (Halinski *et al.,* 2009). The optimized chromatographic finger print is not only an alternative analytical tool for authentication, but also an approach to express the various patterns of chemical ingredients distributed in the herbal drugs and to preserve such "database" for further multifaceal sustainable studies (Sweta *et al.,* 2011; Yadav *et al.,* 2011). TLC has been used to detect active principles in plant-based and synthetic drug compounds and is often recommended due to its simplicity, low cost and sensitivity (Oprean *et al.,* 1998; Pascual *et al.,* 2002). Coupled with selective GC-MS to identify pre-screened compounds, TLC could be used to rapidly survey wild populations, allowing researchers to identify: 1) the spectrum of chemo types present, 2) the range of these chemo types, and 3) their prevalence in natural and cultivated populations. TLC method outlined

in this work captured intra-specific variation within and among populations of *Plumbago* species and is a low-cost means to survey wild populations for known and novel chemo types that requires very little time or analytical equipment. Lenora *et al.* (2012) studied the TLC chemical profile on conventionally grown and cultivated *P. indica.* They observed four prominent spots with Rf values viz., 0.16, 0.35, 0.55, 0.93 in conventionally field grown and tissue cultured field grown *P. indica* root samples using methanol: ethyl acetate: cyclo hexane (0.1: 3.9: 1) as mobile phase. Ariyanathan *et al.* (2011) also reported the TLC profiling of *P. auriculata* collected from Arumbakkam, Chennai, which showed fluorescent yellow spots with Rf value of 0.4 with toluene: ethyl acetate as mobile phase. Kantha *et al.* (2010) subjected TLC profiling of dichloromethane extract of *P. zeylanica* using Hexane: Ethyl acetate (7:3) and Methanol: Ethyl acetate (2:8) as mobile phase. The appearance of blue coloured spots indicated the presence of organic molecules. In the present study, TLC profile of the selected *Plumbago* species was carried out for the specific metabolites phenol and steroids in the crude extracts of studied *Plumbago* species. Their Rf values were used as the pharmacognostic marker to identify the intra-specific variations. The chemical profile of the selected *Plumbago* populations using the TLC fingerprint analysis revealed negligible variations in the studied *Plumbago* species.

Pawar *et al.* (2010) revealed TLC profiling of plumbagin in chloroform root extract of *P. zeylanica* which resolved as a dark grey colour band with Rf. 0.84. In the present study also, the plumbagin was identified by the presence of a single yellowish orange spot using Folin-Ciocalteau reagent and the results showed more or less similar Rf values for all the standard plumbagin extracts viz., petroleum ether extracts with Rf value 0.68: chloroform (0.67), acetone (0.61), ethyl acetate (0.65), ethanolic extract (0.62) and aqueous extract (0.61). TLC is used as a

pharmacognostical tool to authenticate the chemical constituents present in the selected *Plumbago* species.

High performance liquid chromatography (HPLC) techniques are the most widely used methods of analysis in the field of phytochemistry. They have been successfully employed in the discovery and identification of new biologically active secondary metabolites. HPLC has been used for the chemical identification and discrimination of many closely related herbs (Warburton *et al.,* 1998; Dumarey *et al.,* 2008; Djabou *et al.,* 2011). Arunachalam *et al.* (2009) identified the compound Guggultetrol-18-ferrulate showing the retention time 4.913 min in the *P. zeylanica* dichloromethane crude extract using HPLC technique. Hazhar *et al.* (2009) extracted plumbagin from ethyl acetate extract of *P. europaea* and quantitative and qualitative study of plumbagin in the roots and leaves extracts was carried out by HPLC technique using analytical column. The percentage of plumbagin in the root and leaves extracts was recorded to be (1.9%) and (1.5%) respectively. Unnikrishnan *et al.* (2008) develop a reverse phase HPLC method with UV detection has been developed and validated in order to quantify the plumbagin present in *P. indica* and *P. zeylanica*. Rodrigues *et al.* (2006) determined the solubility of 1, 4 naphthoquinone, plumbagin, lawsone and juglone isolated from *P. scandens* using HPLC.

In the present study also, HPLC profiling was carried out in the ethanolic extract of the selected *Plumbago* species along with the standard plumbagin, which revealed some similar and distinct peaks. The chromatogram with the retention time 3.147, 3.140 and 3.147 min was observed in the aerial parts of *P. zeylanica*, *P. auriculata* and *P. rosea* which were more or less similar to the retention time of standard plumbagin (3.127 RT) showed the presence of plumbagin. The highest peak area of 10.942 was observed in the ethanolic extract of *P. auriculata* and followed by *P. rosea* but the particular chromatogram representing the retention

time of plumbagin was present in very least amount with the lowest peak area in aerial parts of *P. zeylanica*. Thus, HPLC is used as a chromatographic marker to investigate the variation and similarity among the aerial parts of selected *Plumbago* species.

HPTLC is the fingerprint marker to study the biological complexity. A fingerprint is the individual chromatographic track representing a mixture of organic substances. By the HPTLC fingerprint approach, it is possible to obtain a proper identification of the chemical constituent in a plant material, but also determine and accept the limits of the biological changes. Variations in HPTLC tracks of the same species are mainly quantitative, not qualitative (Mordue and Blackwell, 1993). The use of HPTLC fingerprint allows obtaining an easy identification of the different species, certifying in some cases also the geographic origin, as reported in the HPTLC volume of the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2009).

Ariyanathan *et al.* (2010) recognized the plumbagin content in the ethanolic root extracts of *P. rosea*, *P. capensis* and *P. zeylanica* by HPTLC technique using toluene: ethyl acetate (4:1) as the mobile phase at 254 and 366 nm. The percent content of plumbagin was found to be 0.17% in *P. rosea*, 0.04% in *P. capensis* and 0.01% in *P. zeylanica.* (Galal *et al.*, 2012) distinguished the presence of plumbagin in the root, stem and leaves extract of *P. zeylanica, P. auriculata* and *P. rosea* using UPLC-UV and HPTLC technique. UPLC-UV analysis showed the highest concentration of plumbagin in the roots of *P. zeylanica* (1.62% w/w) followed by the roots of *P. indica* (0.97% w/w) and then *P. auriculata* (0.33-0.53% w/w). In contrast, plumbagin was not detected in the stems and leaves of *P. indica* and in the leaves of *P. auriculata*, whereas very low concentrations (<0.02% w/w) of plumbagin were detected in the stems and leaves of *P. zeylanica* and in the stems of *P. auriculata.* HPTLC fingerprints of the leaves and root of the three species exhibited distinguishable profiles, while those of the stems were undifferentiated.

Dorni *et al.* (2007) compared the quantity of plumbagin present in the hydroalcoholic extract of three *Plumbago* species viz., *P. zeylanica, P.capensis, P. rosea* was found to be 0.247%, 0.429% and 0.569% using HPTLC technique at 265 nm. Among these *P. rosea* elucidated higher plumbagin content compared to other two species. In the present work, the HPTLC analysis was carried out for different secondary metabolites using varied mobile phase viz., for Phenols: toluene - acetone - formic acid (4.5: 4.5:1), for tannins: toluene- ethyl acetate- formic acid-methanol (3:3:0.8:0.2), for Alkaloid: ethyl acetate-methanol-water (10:1.35:1), for flavanoid : ethyl acetate - butanone - formic acid - water (5: 3:1: 1), for Amino acids: Butanol-Acetic acid-water (3: 1: 1) and for plumbagin: toluene-formic acid (9.9: 0.1) in the ethanolic extract aerial parts of *Plumbago* species. The results revealed the presence of more phenolic bands (11), tannins (12), alkaloids (10), flavonoids (10) and aminoacids (10) in ethanolic extract of *P. rosea* compared to other two *Plumbago* species which was displayed in the form of varied chromatographic bands in the HPTLC profile. The presence of plumbagin was confirmed by the presence of similar RF value of 0.52 in the standard plumbagin and all the three aerial part ethnalolic extracts of *Plumbago* species with varying percentage of plumbagin (0.48%, 0.76% and 1.399%) which represents the quantity of plumbagin present in the ethanolic extract of the studied *Plumbago* species. Thus, HPTLC can be used as a chromatographic marker to distinguish variation in the phytoconstituents present in three *Plumbago* species and varied medicinal plants.

Gas Chromatography - Mass Spectrometry (GC-MS) is a method that combines the features of Gas-liquid Chromatography and Mass Spectrometry to identify different substances within a test sample. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification (Niemann *et al.,* 2005). GC-MS has been

widely heralded as a "gold standard" for forensic substance identification because it is used to perform a specific test. The results pertaining to GC-MS analysis leads to the identification and comparison of number of compounds from the GC fractions of ethanolic extracts of the three selected *Plumbago* species. They were identified through mass spectrometry attached with GC. The gas chromatogram shows the relative concentrations of various compounds getting eluted as a function of retention time. The heights of the peak indicate the relative concentrations of the components present in the three selected *Plumbago* species. The compound prediction is based on Dr. Duke's Phytochemical and Ethnobotanical Databases and PASS. The prediction of the biological activities by applying the Duke's databases was confirmed with the previous observations (Hemalatha *et al*., 2011; Kumar *et al*., 2010; Maruthupandian and Mohan, 2011). The presence of different phytochemicals in the selected *P. zeylanica, P. auriculata* and *P. rosea* have been shown to possess various activities viz., shivering, pulmonary edema, irritation, anti-inflammatory, respiratory failure, diarrhoea, hyperthermic, hepatotoxic, anemia, hematotoxic, hepatitis, antiulcerative, antitetany, antidiabetic, asthma, drowsiness, antithrombotic, antiulcerative, peptic, gastric, hypercholesterolemic, dermatologic, respiratory analeptic, thioredoxin inhibitor, visual acuity impairment, antineoplastic (breast cancer, colorectal cancer, colon cancer, pancreatic cancer), sleep disturbance etc. Paiva *et al.* (2004) isolated and quantified the individual compound plumbagin from *P. scandens* using different extraction techniques and quantified the plumbagin content using GC-MS analysis and found soxhlet extracted samples depicted higher concentration of plumbagin.

Rosy and Rosakutty (2012) compared the chemical profile present in methanolic extract of *Cissus xavierensis, C. quadrangularis* var. *rotundus* and *C. vitiginea* by GC-MS analysis. The major compound present in *C. xavierensis* was hexane (peak area. 35.09%), *C. quadrangularis*

var. *rotundus* was 1, 3, 2-oxazaborolane (peak area. 14.29%) and *C. vitigenea* was Dibutyl phthalate, 1, 2-benzenedicarboxylic acid (peak area. 16.18%). Similar to previous work, in the present study also the inter-specific variations between the *Plumbago* species were elucidated using GC-MS profile. The major compound present in *P. zeylanica* was Dihydro-4b-methyl-8-chloro-11H-isoindolo [2, 1-a] benzimidazol-11-one (RT. 26.90) in *P. auriculata* was Isoborneol (RT. 86.99) and *P. rosea* was Hexadecanoic acid (RT. 14.19). The compound hexadecanoic acid was present in all the three *Plumbago* species demonstrating varied retention time.

Ajayi *et al.* (2011) identified the phytochemical constituents present in the ethanolic root extract of *P. zeylanica* by GC-MS analysis. The results showed the presence of 24 phytochemical components among these the compound 2-Methyl-7-phenylindole (RT: 14.540 min) revealed the highest retention time. Whereas, Phenol, 2,4-bis (1,1-dimethylethyl) with RT: 6.796 min demonstrated the lowest retention time. In comparison to the work of Ajayi *et al.* (2011) the present work, was carried out to compare the general phytoconstituents present in the ethanolic aerial parts extracts of *P. zeylanica*, *P. auriculata* and *P. rosea* by GC-MS analysis. The extracts exhibited 27, 30 and 28 phytocompounds with wide range of retention time 14.04 – 45.60 min, 3.04 – 45.04 and 3.86 - 45.81 min. The major compounds present in the éthanolic aerial part extract of *P. zeylanica* was Dihydro-4b-methyl-8-chloro-11H-isoindolo [2, 1-a] benzimidazol-11-one (RT. 26.90 min), *P. auriculata* was Isoborneol (RT. 86.99 min) and *P. rosea* was Hexadecanoic acid (RT. 14.19 min). Hexadecanoic acid, methyl ester (CAS) was present in all the selected *Plumbago* species with varied retention time viz., *P. zeylanica* (26.23 min RT; peak area 8.87%), *P. auriculata* (26.21 min RT; peak area 0.73%) and *P. rosea* (26.21 min; peak area 6.24%). Similar to the present study, *P. zeylanica, P. auriculata* and *P. rosea* were observed in the ethanolic extract of *P. zeylanica* root (Ajayi *et al.,* 2011). The octadecenoic acid, methyl ester

(RT 30.50 min; peak area. 0.41%) and 9-Octadecenoic acid, methyl ester (E)- (RT 30.87 min; peak area. 11.90%) was identified in the ethanolic aerial parts extracts of *P. zeylanica*. Similarly Ajayi *et al.* (2011) also observed the Octadecenoic acid, methyl ester (RT 10.688 min; peak area. 2.783%) and 9-Octadecenoic acid, methyl ester (E) - (RT 10.528 min; peak area 1.647%) in the ethanolic root extract of *P. zeylanica* and the results of present GC-MS analysis carried out in aerial parts of *P. zeylanica* and the GC-MS analysis revealed by Ajayi *et al*. (2011) illustrated two similar compounds in different retention time. Previous works were focused on the root extracts of *Plumbago* species in the present study, emphasized and revealed the phytoconstituents in aerial parts *Plumbago* species.

Electrophoresis is a relatively simple, rapid and highly sensitive tool to study the properties of proteins and nucleic acids. It is the principal tool in analytical chemistry, biochemistry and molecular biology. Gel electrophoresis can provide information about the molecular weights and charges of proteins and nucleic acids. Protein electrophoresis is a powerful tool for population genetics (Parker *et al*., 1998). As storage proteins are not affected by environmental fluctuations, their profiling using SDS-PAGE technology is particularly considered as a reliable tool for economic characterization of germplasm (Javid *et al*., 2004; Iqbal *et al*., 2005). The protein profiling of germplasm and use of genetic markers have been widely and effectively used to determine the taxonomic and evolutionary aspects of several crops (Murphy *et al*., 1990; Khan, 2009; Javaid *et al*., 2002). The use of genetic markers and protein profiling has been successfully used to resolve the taxonomy and evolutionary problems of several crop plants including *Cajanus cajan*, *Vigna radiata*, *Vigna mungo*, *Vigna unguiculata*, *Phaseolus vulgaris*, *Pisum sativum*, *Cicer arietinum, Lens culinaris* and *Glycine max* (Javaid *et al.,* 2002).

Rout *et al.* (2010) characterized the total protein from the leaves of *P. zeylanica* L. using SDS-PAGE and visualized several protein bands of molecular weight 50.08, 41.25, 38.41, 36.21, 28.74 and 25.52 kDa and found that the protein with MW-Rf 50.08 kDa showed the thickest band when compared to other bands. Bompalli *et al.* (2013) studied the intra-specific variation among the eleven *Ocimum spp.* samples collected from northern region of India using SDS-PAGE protein profiling studies and the similarity index was studied using UPGMA analysis.

Britto *et al,* (2011) evaluated the inter genetic variation relationship between the selected species of Asclepidaceae family viz., *Calotropis gigantea, Gymnema sylvestre, Daemia extensa, Cryptolepis buchanani* and *Tylophora indica* based on SDS-PAGE protein profiling studies. The number of bands varied from 6 to 12 and the similarity index was given using UPGMA analysis. In the present study, the protein electrophoretic system was used to explore the intra-specific variation of *Plumbago zeylanica* collected from seven different localities of South India viz., Rasthakadu (Pz1), Palayamkottai (Pz2), Bangalore (Pz3), Papanasam (Pz4), Tiruppur (Pz5), Kuttichal (Pz6) and Coimbatore (Pz7).The results of SDS-PAGE protein profiling revealed the intra-specific variation and the results expressed a total of 12 bands with multiple regions of activity and with varied MW-Rf values in the range of 0.20 – 0.98. Among the proteins characterized from different localities, *P. zeylanica* collected from Bangalore (Pz3) and Coimbatore (Pz7) displayed the maximum number of bands (9). The *P. zeylanica* collected from Rasthakadu (Pz1) showed the lowest number of bands (5). The accession collected from Rasthakadu (Pz1) expressed highest variation among the studied accessions; this may be due to the ecological condition. Rasthakadu is located nearby Vattakotai - Kanyakumari seashore and other studied accessions are collected from near hill stations or plains. *P. auriculata* collected from five different accessions depicted totally 38 bands with the Rf value ranging from 0.07 - 0.85. The accession collected from Tenkasi (Pa2) expressed highest variation among the studied accessions. This may due to ecological differences because *P. auriculata* which was collected from Tenkasi hills (foot hills of Courtallam) but other *P. auriculata* species are collected from the plains.

P. rosea recognized 21 protein bands with Rf value ranging from 0.04 - 0.84. The UPGMA based cluster analysis clearly showed the variation and similarity among the various accession of *P. rosea*. The accession collected from Cheruvarankonum (Pr1) reported highest variation among the studied accession, this may due to the ecological differences because Cheruvarankonum was located in Kerala which was located in the hill station while other two accessions are located in plain surface.

In the study of the inter-specific variation among the three *Plumbago* species, *P. auriculata* recorded a distinct profile whereas *P. zeylanica* and *P. rosea* shared in the same clade of UPGMA analysis. SDS-PAGE protein profiling studies was used as a biochemical marker to analyse the variation among the plant species collected from different accessions (Gepts, 1990). These protein markers can be used as identification tools in the pharmaceutical industries.

Chuntaratin (2006) studied the plumbagin protein conjugation by glutaraldehyde reaction using SDS-PAGE. From the research it was confirmed that the molecular weight of the proteins of plumbagin-protein conjugate band was higher than the normal protein showing the molecular weight of 72817.704 and 66564.232 kDa. Norman and Russell (1988) isolated three fractions of *P. zeylanica* pollen protein (male germ unit [MGU]-rich, cytoplasmic-particulate and water-soluble proteins) using one- and two-dimensional polyacrylamide gels and identified 427 spots > 33 KDAs.

MALDI-TOF MS analysis accurately reflected the phylogenetic classification, in most cases, species identified by DNA sequence analysis clustered together by MALDI-TOF MS. The resolution of MALDI-TOF MS was performed roughly equivalent to ITS rDNA. The MALDI-TOF MS technique analyzes peptides and represents a rough equivalent to sequencing, making this method a useful adjunct for determination of species limits. It also allows simple, reliable,

and quick species identification, thus representing a valid alternative to gene sequencing for species diagnosis of bacterial and plant taxa (Respinis *et al.,* 2010). Chuntaratin (2006) studied the plumbagin protein conjugation by glutaraldehyde reaction using MALDI-TOF MS. By means of MALDI-TOF MS analysis it was confirmed that the molecular weight of the proteins of plumbagin-protein conjugate band was higher than the normal protein showing the molecular weight of 72817.704 and 66564.232. The present research showed a proximate spectra of varied ion peaks (m/z) ranged from 0 - 1, 00, 000 in correspondence to varied intensities were recorded in the peptide mass finger printing profile of individual *Plumbago* species. *P. zeylanica* collected from the accessions Bangalore (Pz3) and Kuttichal (Pz6) revealed the protein at molecular weight 75317 and 72493 which was similar to Petcharat's observation.

Respinis *et al.* (2010) investigated the peptide mass finger printing profile of 129 morphologically similar strains of *Hypocrea* and *Trichoderma* species using MALDI-TOF MS analysis and the single linkage cluster analysis was used to study the phylogenetic variation. The results were compared using ITS and tef1 sequences. Dominating peaks were observed in two different mass ranges. The most important one was situated between m/z 6,000 and 8,000. In the present study, MALDI-TOF MS analysis was carried out among the selected *Plumbago* species collected from different accessions of South India. The resulting peak lists of individual samples were submitted to NTSYs software to produce a taxonomic tree to reveal the inter-specific and intra-specific variation. Among the three *Plumbago* species, *Plumbago zeylanica* represented maximum number (94) of m/z peaks ranged from 753 - 83978 m/z values. Next to that, the *Plumbago auriculata* demonstrated nine m/z peaks ranged from 1422 – 92970. Similarly, *Plumbago rosea* depicted two distinct spectral values 822 and 4058 respectively. From the research it was reinforced that, MALDI-TOF MS analysis was used as a taxonomic tool to study

the similarity among the selected species and is a quick and reliable tool for species identification which can be a valid alternative to gene sequencing for species diagnosis. The primary advantage of MALDI-TOF MS is the speed by which identification can be made. The MALDI-TOF MS analysis can be completed in a few minutes as opposed to two or more days required for DNA sequence analysis (Druzhinina *et al.,* 2008; Hermosa *et al.,* 2004; Samuels *et al.,* 2009).

The MALDI-TOF MS analysis performed to validate the intra-specific variation in *P. zeylanica* collected from the seven accessions of South India reported 94 m/z values and more number of (27) m/z peaks ranged from 1289 to 80916 m/z values were observed in the *P. zeylanica* collected from Papanasam (Pz4) and lowest numbers of peaks (5) were observed in the accession collected from Rasthakadu (Pz1) and Tiruppur (Pz5). Among the five accessions of *P. auriculata* collected from South India, Totally 32 m/z spectral values were observed and maximum number (10) of m/z peaks ranged from 2055 to 86,132 m/z values were observed in the *P. auriculata* collected from Tenkasi (Pa2) and least m/z values were detected in the accession collected from Perunthurai (Pa3) showing five positive spectral peaks. *Plumbago rosea* collected from three accessions of South India revealed 12 m/z values and maximum number (9) of m/z peaks ranged from 1,730 to 92,000 m/z values were observed in the *Plumbago rosea* collected from Dana (Pr2) and lowest m/z values (2) was expressed in the accession collected from Bangalore (Pr3).

The inter-specific variation study among the selected *Plumbago* species based on *rbc*L sequencing and MALDI-TOF MS indicated that the species *P. rosea* was distinct from *P. zeylanica* and *P. auriculata.* The MALDI-TOF MS and *rbc*L sequencing results were directly correlated with phytochemical analysis. The dendrogram based on phytochemical and molecular

analysis showed similar pattern cluster. Neuhof *et al.* (2007) proposed the hypothesis that these proteins could be optimal biomarkers at the inter-specific and intra-specific level. The hypothesis of Neuhof *et al.* (2007) correlated with the present study demonstrating the MALDI-TOF MS analysis as a biomarker to differentiate the *Plumbago* species collected from various accessions of South India.

DNA marker technologies have greatly enhanced the ability to unravel the genetic basis of traits expressing continuous phenotype variations (Peleman *et al.,* 2003). Assessment of a significant association between trait values and markers has opened a way to use DNA markers for indirect selection of quantitative traits via marker-assisted selection. A DNA marker can distinguish between two closely related species irrespective of environment, time of collection, part of plant used, etc. Thus, it is a more reliable and robust tool for authentication of medicinal herbs at the species level. Molecular markers are highly inheritable, are available at a high number of frequencies and exhibit enough polymorphism to discriminate against closely related species (Kwon *et al.,* 2009; Feng *et al.,* 2010, Khan *et al.,* 2010, Moon *et al.,* 2010).

Nathar and Gadge (2013) isolated and quantified the DNA in *P. zeylanica, P. auriculata* and *P. rosea* to interpret the variation using CTAB method and found the DNA concentration as 4.40, 4.55 and 4.65 µg/ml in the leaf samples. But in the present study, the highly valid DNA barcoding studies were carried out in the three *Plumbago* species collected from various localities of South India using *rbc*L primer to demonstrate the inter-specific and intra-specific genetic variation among the selected *Plumbago* species. The inter-specific variation result of Nathar and Gadge (2013) represented *P. rosea* with higher DNA content. In the same way the present phylogenetic results also recognised *P. rosea* in a separate clade showing its distinct character from other two species.

Dharmar and Britto (2012) estimated the genetic diversity of *P. zeylanica* collected from five accessions of Southern Western Ghats viz., Marunthuvalmalai, Courtallam, Achankoil, Maruthamalai hills and Anchetty. Of the 12 primers screened, 6 produced highly polymorphic DNA fragments. The total number of amplified DNA fragments was 35. The P3 (Achankoil) population was found to be least diverse (0.2023) and displayed the high level of variability (0.2978) and the P2 (Courtallam) population revealed intermediate diversity (0.2671). In the present study, the highly advanced DNA barcoding studies were carried out in the three *Plumbago* species collected from various localities of South India using *rbc*L primer to analyse the inter-specific and intra-specific genetic variation study among the selected *Plumbago* species. The obtained sequences were annotated and submitted in Gen Bank GenBank accession number for *P. zeylanica* viz., KF 233546, KF 233551, KF 233552, KF 233545, KF 233544, KF 233543 and KF 233542, GenBank accession number for *P. auriculata* viz., KF 193871, KF 233550, KF 233549, KF 233548 and KF 233547 GenBank accession number for *P. rosea* viz., KF 261596, KF 261597 and KF 261598. Multiple alignments were performed for *rbc*L region using Clustal W and MULTALIN. The sequence alignment discriminated the intra-specific variation among the seven selected accessions of *P. zeylanica,* five selected accessions of *P. auriculata* and three selected accessions of *P. rosea*. Phylogenetic analysis was performed by Minimum evolution method by using MEGA 6.0 (Molecular Evolutionary Genetic analysis).

Gurudeeban *et al.* (2013) revealed congeneric relationship between *Rhizophora apiculata*, *Rhizophora mucronata*, *Ceriops decandra*, *Ceriops tagal* and *Bruguiera gymnorhiza* collected from South East coast of India, indicating a high degree of gene flow within them and the pair wise distribution of study plants among Rhizophoraceae family was calculated by mega 5 software tools. The phylogram constructed using tRNA Leu sequence clearly clustered

phylogenetic relationships of the Indian Rhizophoraceae species. The intra-specific variation of the genus *Rhizophora* was found higher than the other genera in the family. The *trn*L sequence of *Avicennia marina* was present as an outgroup in the phylogenetic tree.

Mahadani *et al.* (2013) differentiated the ethnomedicinal plants *Catharanthus roseus, Alstonia scholaris, Thevetia Peruviana, Allamanda cathartica, Tabernaemontana divaricata, Calotropis gigantea* belonging to the family Apocynaceae inhabiting in North East India by *mat*K and *trn*H-*psb*A sequences. The results showed by the NJ tree implied that the member of Rauvolfioideae formed one clade where different species clustered into different subclade. The generated sequences of *Allamanda cathartica* was found closely related to *Allamanda schottii*. Two sequences *Calotropis gigantea* and *Asclepias curassavica* formed two distinct clades at the basal position of phylogenetic tree. The present inter-specific variation among the *rbc*L sequences of the selected *Plumbago* species based on the multiple sequence alignment result of MEGA 6.0 statistical tool and the phenogram results discriminated the sequences of *P. zeylanica* and *P. auriculata* which are highly conserved and generated in a single clade whereas the sequence of *P. rosea* formed distinct clade. The overall mean distance of the three selected *Plumbago* species was 9.840. Genetic variation is essential for long term survival of species and it is a critical feature in conservation. For efficient conservation and management, the genetic composition of the species in different geographic locations needs to be assessed (Padmalatha and Prasad, 2006)

Genetic diversity at the intra-species level arises from differences in climatic conditions, geographical distribution and environmental stress. The genetic diversity may not result in morphological changes to a great extent but creates variations in the phytochemical compositions of plants (Jaalola *et al.,* 2010; Schreiner and Schuettpelz, 2005) and their medicinal properties.

Annamalai *et al*. (2012) assessed the intra-specific genetic relationship among the *Cardiospermum halicacabum* species collected from different regions of Tamil Nadu (Chennai, Coimbatore, Dharmapuri, Madurai, Triunelveli and Thanjavur) using RAPD analysis. The dendrogram constructed based on UPGMA represented two clusters, Cluster 1 included the sample from Chennai, while Cluster 2 included samples from Dharmapuri, Thanjavur, Tirunelveli, Coimbatore and Madurai. Thus the present study assists the researcher in analysing the genetic diversity by determining the phylogeny of a particular plant species and the impact of geographical locations on the plant genome of the selected *Plumbago* species. The intra-specific genomic variation of seven selected accessions of *P. zeylanica,* five selected accessions of *P. auriculata* and three selected accessions of *P. rosea* using multiple sequence alignment, phylogenetic analysis, amino acid composition and statistical parameters of the barcoded *rbc*L sequences using MEGA 6.0. The phylogenetic tree constructed using MEGA 6.0 clearly clustered the species of the same genus in individual group as follows viz., the aligned sequences of *P. zeylanica* revealed that the sequences of the accessions collected from Bangalore (Pz3) and Tiruppur (Pz5) are highly conserved and identical. Following these the accessions collected from Palayamkottai (Pz2) and Kuttichal (Pz6); Rasthakadu (Pz1) and Coimbatore (Pz7) show moderate similarity in their aligned sequences whereas the sequence of the accession Papanasam (Pz4) shows its unique presence and confirmed the variations among the seven *P. zeylanica* sequences retrieved from different accessions of South India. The overall mean distance of the selected *P. zeylanica* was 2.201.

The aligned barcoded *rbc*L sequences of *P. auriculata* revealed that the sequences of the accessions collected from Tenkasi (Pa2) and Perundurai (Pa3) are highly conserved and identical. Following these the accessions collected from Kattakadu (Pa4) and Mysore (Pa5):

Mulakumoodu (Pa1), Tenkasi (Pa2) and Perundurai (Pa3) recognised moderate similarity in their aligned sequences. The overall mean distance of the *P. auriculata* species was 1.626.

The aligned barcoded *rbc*L sequences of *P. rosea* revealed that the sequences of the accessions collected from Cheruvarankonum (Pr1) and Bangalore (Pr3) are highly conserved and identical. The accession collected from Dana (Pr2) represented an isolated clade. The overall mean distance of the *P. rosea* species was 0.002.

DNA barcoding has emerged as a powerful technique of species identification and has found wide acceptance due to its wide application in monitoring and documentation of bio-resources (Hollingworth *et al.,* 2011; Kress *et al.,* 2010; Liu *et al.,* 2010; Stech *et al.,* 2011). DNA barcodes allow the identification of species within a community associated by geography or ecology (Chase 2007; Kress and Erickson, 2007). The goal of DNA barcoding is conceptually simple. It seeks to distinguish species among the majority of the world's living organisms, and sequence DNA from these diverse sample sets to produce a large scale reference library of life on earth (Hebert *et al.,* 2003). However, most studies to date have involved an assessment of species resolution within defined taxonomic groups, highlighting areas of concordance and discordance with traditional, morphological-based taxonomic approaches (Sass *et al.,* 2007; Newmaster and Ragupathy, 2009; Seberg and Petersen, 2009; Starr *et al.,* 2009). In addition, considerable effort has been focused on the performance of barcoding regions (primers) across diverse collections of land plants (Newmaster *et al.,* 2006; Kress and Erickson, 2007; Fazekas *et al.,* 2008; CBOL Plant Working Group, 2009; Hollingsworth *et al.,* 2009).

Plant based antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials needs to take place. Antimicrobials of plant origin have enormous therapeutic potential. To promote the proper use of herbal medicine and to determine

their potential as sources for new drugs, it is essential to study medicinal plants, which are recorded in local folklore traditions in a more intensified way (Kumaraswamy *et al.,* 2008; Salau and Odeleye, 2007). Human pathogenic antibiotic resistant microorganisms have developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of such diseases (Mohanasundari *et al.,* 2007). Plants are one of the bedrocks for modern medicines to attain new principles for disease management. Modern day synthetic and chemical drugs often exhibit some negative effects or side effects but traditional herbals are safer and easier to access. Traditional healers and medicine men in India use a few medicinal plants for curing various diseases and they are more cost effective when compared to pharmaceutical drugs (Evans *et al.,* 2002).

Kumar *et al.* (2009) investigated the antibacterial activity against various bacteria by paper disc method using petroleum ether, ethanolic and aqueous extract of aerial parts of *P. zeylanica*. The maximum activity was observed in ethanolic extract against *Micrococcus luteus* (12mm) and minimum activity was observed in petroleum ether extract against *S. aureus*. In the present study, antibacterial activity was carried out by well diffusion method and the results reported maximum antibacterial activity in the aerial ethanolic extract of *P. rosea* compared to other two *Plumbago* extracts. Jeyachandran *et al.* (2009) assessed the antibacterial activity on 1 - 25 µg/ml of plumbagin, methanolic, chloroform and aqueous extracts of *P. zeylanica* root against various pathogenic bacteria using disc diffusion method. Plumbagin and chloroform extracts of *P. zeylanica* root extract showed higher antibacterial activity against *E. coli*, *S. typhi* and *S. aureus* and lower activity was observed against *P. vulgaris* and *P. aeruginosa*. In comparison to the work of Jeyachandran *et al.* (2009), in the present study, Antibacterial activity of *P. zeylanica was* screened in six different extracts with five different concentrations (20 - 100 µg/ml) against

six different pathogens viz., *S. aureus, B. subtilis, S. pyogenes* (Gram positive), *K. pneumoniae, M. morganii, P. aeroginosa* (Gram negative) using well diffusion method. Among these, 100 µg/ml of *P. zeylanica* ethanolic extract displayed higher antibacterial activity against *S. pyogenes* and *K. pneumonia* compared to the other six *P. zeylanica* extracts.

Antibacterial activity of methanolic and chloroform extracts of *P. zeylanica* was carried out against five different pathogens viz., *S. aureus, Bacillus* spp., *P. aeruginosa, E. coli* using disc diffusion method. Methanolic extracts were more active against the entire tested organism (Devi and Thenmozhi, 2011). The antimicrobial effect of petroleum ether, chloroform and alcoholic extract of *P. zeylanica* leaves extract was evaluated against *S. aureus, B. subtilis, E. coli* and *P. aeruginosa* using agar disc diffusion method. Among these alcoholic leaf extract of *P. zeylanica* showed maximum inhibitory activity (Dhale and Markandeya, 2011). The present study also reported higher antibacterial activity in the ethanolic aerial part extract of *P. zeylanica* compared to other *P. zeylanica* extracts.

Ibrahim *et al.* (2012) studied the antibacterial activity on 500 µg/ml of *P. indica* methanolic extract against three gram positive and seven gram positive bacteria. Among these *B. subtilis* (25 mm) and *B. megaterium* (17 mm) represented lowest antibacterial activity. In association to the work of Ibrahim *et al.* (2012), the present work also recognised the antibacterial activity of *P. rosea* on six different extracts in five different concentration of 20-100 µg/ml against six selected pathogenic bacteria and the results revealed highest degree of antibacterial activity (91%) in ethanolic extract of *P. rosea* followed by acetone 64%, chloroform 56%, ethyl acetate 52%, petroleum ether 50% and aqueous extract 15%.

In conclusion, the overall antibacterial results of the three *Plumbago* species show the, highest degree of antibacterial activity (54%) in *P. rosea*. The range of antibacterial activity of

various extracts in different concentration of the selected *Plumbago* species are as follows: *P. rosea* (54%) > *P. zeylanica* (49%) > *P. auriculata* (40). Among the eighteen different extracts of three different *Plumbago* species, ethanolic extract of all the *Plumbago* species revealed superior bactericidal activity. Ethanolic extracts of *P. zeylanica, P. auriculata* and *P. rosea* showed 91%, 50% and 99% of activity against Gram positive pathogens and 66%, 26% and 89% of activity against Gram negative pathogens.

The present investigation was undertaken to study the inter-specific and intra-specific variation among the three *Plumbago* species viz., *P. zeylanica* L., *P. auriculata* Lam. and *P. rosea* L using phytochemical, biochemical and molecular analysis. To fulfil the objectives of the studies the *Plumbago* species were collected from different localities of South India. To reveal inter-specific variation among the three *Plumbago* species the phytochemical, biochemical and molecular analysis were carried out. To reveal the intra-specific variation the biochemical and molecular analysis were adopted. In pharmacognosy, the phytochemical assessment is an important and vital tool for quality assessment, which includes preliminary phytochemical screening, chemo profiling and marker compound analysis using modern analytical techniques such as fluorescence, UV-Vis, FTIR spectrum (Spectroscopic profile), TLC, HPLC, HPTLC and GC-MS (Chromatographic techniques), molecular characterization using SDS-PAGE for protein separation, MALDI-TOF MS and DNA barcoding. To calculate the similarity indices the presence and absence of the metabolites, absorption, MW-Rf and m/z were calculated as "1" and "0". The results obtained through phytochemical, biochemical and molecular analysis were documented and the similarity indices between the selected species were tabulated. The similarity indexes values were plotted in NTSys-Software and the cladograms were constructed using UPGMA. The cladograms were used to reveal the inter-specific and intra-specific variation among the selected *Plumbago* species.

The results of the preliminary phytochemical analysis showed that more number of compounds are expressed in the ethanolic extracts of *P. rosea* (77%) compared to other two *Plumbago* species (69%) in six different extracts. The UV-Vis analysis documented more number of spectral peaks in the extracts of *P. rosea* compared to other two *Plumbago* species.

The results of UV-Vis spectroscopic data directly coincides with the results of the preliminary phytochemical analysis which confirms the relationship between the presence of metabolites in preliminary phytochemical analysis and number of peaks observed in UV-Vis analysis. Based on the results of phytochemical, UV-Vis spectroscopic and TLC analysis, the ethnaolic extracts were taken for FTIR, HPTLC, HPLC and GC-MS analysis.

The FTIR spectrum was used to identify the functional group of the phytoconstituents based on the peak value in the region of infrared radiation. The results of FTIR analysis showed different peak values which confirmed the presence of various functional groups such as amides, phosphorus compounds, carbonyl compounds, phenols, carboxylic acid, alcohols, unsaturated aliphatic carbonyl compounds, sulphur compounds and halogen compounds, sulfoxides, sulfones, sulfonamides (S=O Str.), alkanes (-CH_2-, C-H DEF), intra molecular H-bounded aldehydes (C=O Str.), boron compounds and organo-phosphorus compounds (O-H Str.). The functional groups such as halides, amino acids, charged amines, alkanes, alcohols and phenols single bridge compound (O-H Str.) showed their unique presence only in the ethanolic extracts of *P. auriculata* which was absent in other two *Plumbago* species. The results revealed that *P. auriculata* represented varied functional groups compared to other two *Plumbago* species while *P. zeylanica* and *P. rosea* showed more or less similar functional groups. These spectroscopic profiles will be used as a spectroscopic marker to distinguish the *Plumbago* species from other species and its adulterant.

Similar to the results of the preliminary phytochemical and spectroscopic studies, the chromatographic studies viz., HPTLC and TLC banding profile also showed the similarity between *P. zeylanica* and *P. auriculata;* the banding profile of *P. rosea* showed the variation. Contrary to the TLC, HPTLC and phytochemical analysis the HPLC chromatographic profile of

ethanolic extracts of *Plumbago* species showed similar peak values between the ethanolic extracts of *P. auriculata* and *P. rosea* and varied peak values were observed in the ethanolic extracts of *P. zeylanica*. With reference to the standard plumbagin HPLC profiles, the ethanolic extract of *P. rosea* and *P. auriculata* showed the presence whereas the ethanolic extracts of *P. zeylanica* showed the presence of plumbagin with least peak area compared to other two species. To quantify the plumbagin in the aerial parts of the selected *Plumbago* species HPTLC analysis was analysed using the mobile phase toluene-formic acid and spraying reagent 10% methanolic acid. The presence of plumbagin was confirmed by the band existence with the Rf value of 0.52. The band with Rf value 0.52 was present in the standard plumbagin and all the three ethnalolic extracts of *Plumbago* species viz., *P. zeylanica, P. auriculata* and *P. rosea* with varied percentage of plumbagin 0.48%, 0.76% and 1.399% respectively. Highest percentage of plumbagin occurrence was observed in the ethanolic extracts of *P. rosea* (1.399%) aerial parts. The GC-MS analysis carried out on the ethanolic extract of three selected *Plumbago* species to explore the phytocontituents present in the aerial parts of *Plumbago* species and to reveal the inter-specific variation and similarity. The GC-MS analysis revealed the similarities and variation among the three studied *Plumbago* species. Out of 85 identified compounds, hexadeconoic acid methyl ester (CAS) showed its presence in all the three studied *Plumbago* species whereas, n-Hexadeconoic acid displayed its occurrence in the ethanolic extracts of *P. auriculata* and *P. rosea.* These profiles can act as pharmacognostic marker in the pharmaceutical industries to distinguish the medicinal plants from its adulterants.

SDS-PAGE protein profiling was carried out to study the inter-specific and intra-specific variation among the selected *Plumbago* species collected from South India. The protein profile of the accession collected from Rasthakadu (Pz1) represented least number of protein bands

demonstrated its variations from all the other accession of *P. zeylanica*. The accession collected from Coimbatore (Pz7), Tiruppur (Pz5), Bangalore (Pz3) and Palayamkottai (Pz2) also recognised individual banding pattern represented in separate nodes and branches in the UPGMA cladogram. The accessions collected from Papanasam (Pz4) and Kuttichal (Pz6) shared the similar protein banding pattern. The accession collected from Rasthakadu (Pz1) expressed highest variation among the studied accessions. This may be due to the ecological variations, because Rasthakadu is located nearby Kanyakumari seashore and other studied accessions are collected from near hill stations or plains.

The results of intra-specific protein electrophoretic system of *Plumbago auriculata* collected from five different accessions of South India revealed variation in the protein banding pattern. The results showed that the accession collected from Tenkasi (Pa2) showed a unique banding pattern represented in a separate clade. Similarly the accession collected from Mysore (Pa5) also recognised unique bands expressed in a distinct node. The distinctive variation among the protein banding pattern of the accession Tenkasi and Bangalore may be due to the geographical distribution; the accession collected from Tenkasi and Bangalore are situated in the foot hills of WG whereas all the other accessions are present in plains.

The results of intra-specific protein electrophoretic system of *Plumbago rosea* revealed that the accession collected from Cheruvarankonum (Pr1) showed the distinctive variation from other two accessions.

The results emphasizing the inter-specific protein electrophoretic system of the selected three *Plumbago* species collected from Karnataka illustrated that the species *P. auriculata* revealed distinct variation from other two *Plumbago* species.

The results validating the intra-specific variation based on mass spectrum values of MALDI-TOF MS analysis among the *P. zeylanica* collected from seven different localities demonstrated that the accession collected from Papanasam (Pz4) and Tiruppur (Pz5) showed its distinctive variations from other selected accessions. The samples collected from the accession Rasthakadu (Pz1) and Palayamkottai (Pz2) showed similarity in the mass spectral values. The mass spectral values of *P. zeylanica* collected from the accession Bangalore (Pz3) and Kuttichal (Pz4) showed similarity due to the cold humid climatic condition existing in the two accessions. The accession collected from Coimbatore (Pz7) showed unique m/z values. The uniqueness may be due to the moderate climatic condition existing in the Coimbatore.

The intra-specific variation among the *P. auriculata* collected from five different accessions based on the mass spectral values of MALDI-TOF MS analysis revealed that the accession collected from Mysore (Pa5) depicted its characteristic m/z values from other accessions. The accession collected from Perunthurai (Pa3) revealed the distinctive m/z values. The accession collected from Tenkasi also demonstrated a distinctive m/z values. The *P. rosea* collected from three localities of South India were subjected to MALDI-TOF analysis to know the intra-specific similarity and variation. The MALDI-TOF results emphazised that the accession collected from Dana (Pr2) showed unique m/z values whereas the accession collected from Cheruvarankonum and Bangalore revealed similar m/z values. The inter-specific variation based on MALDI-TOF MS analysis suggest that *P. rosea* was unique compared to *P. auriculata* and *P. zeylanica* which represented more or less unique m/z values.

DNA barcoding is an advanced molecular tool to assess taxonomic variation and power full tool for species identification. In the present research, DNA barcoding was used as an efficient molecular tool to differentiate the inter-specific and intra-specific variation among the

selected *Plumbago* species viz*., P. zeylanica, P. auriculata* and *P. rosea.* The results of the phylogenetic analysis using MEGA 6.0 version software acknowledged that the *rbc*L sequences of *P. zeylanica* collected from Tiruppur (Pz5) and Bangalore (Pz3) showed closely similar nucleotide sequences compared to other accessions. Similarly the accession collected from Kuttichal (Pz6) and Rasthakadu (Pz1) showed more or less similar sequence. The *P. zeylanica* accession collected from Coimbatore and Papanasam revealed similar aligned sequences. The accession collected from Palayamkottai (Pz2) demonstrated the unique sequence variation from the other accessions.

The results of phylogenetic analysis using MEGA 6.0 version software revealed the intra-specific variation among the studied accessions of *P. auriculata.* The phylogenetic analysis results documented that the *rbc*L sequences of *P. auriculata* collected from Perunthurai and Tenkasi recorded similar base paired sequences. The *P. auriculata* collected from Mysore and Kattakadu revealed similar nucleotide sequences. The *P. auriculata* accession collected from Mulakumoodu (Pa1) showed the distinctive nucleotide sequence from the other accessions.

The phylogenetic analysis discriminated the intra-specific variation among the studied accessions of *P. rosea.* The alignment showed that the *rbc*L sequences of *P. rosea* collected from Dana showed a unique base paired sequence compared to other two accessions (Cheruvarankonum and Bangalore).

The results of phylogenetic analysis using MEGA 6.0 version software showed inter-specific variation among the studied *Plumbago* species. The sequence alignment results exemplified that the sequence of *P. rosea* was distinct from *P. zeylanica* and *P. auriculata.* The molecular results showing inter-specific variation among the selected *Plumbago* species revealed

that *P. rosea* recognized distinctive molecular characteristics compared to the other two *Plumbago* species.

Thus, the results of phytochemical and molecular variation study emphasized that *P. rosea* revealed distinctive phytochemical and molecular characteristics whereas *P. zeylanica* and *P. auriculata* showed similar phytochemical and molecular features. These phytochemical and molecular analyses in particular may find application in resolving disputes of taxonomic identities, relations and authentication of the species in the pharmaceutical industries.

Finally, it can be concluded from the study that synergistic application of phytochemical, biochemical and molecular markers can be used as a proficient tool to study the taxonomic variation.

Abdelwahab SI, Abdul AB, Elhassan MM, Mohan S, Ibrahim MY, Mariod AA, AlHaj NA, Abdullah R. GC/MS determination of bioactive components and antibacterial properties of *Goniothalamus umbrosus* extracts. *African Journal of Biotechnology* 2009; **8**(14): 3336-3340.

Adams RP. Identification of Essential Oil Component by Gas Chromatography/ Quadrupole Mass spectroscopy. Allured publishing corporation Illinois, U.S.A. 2004.

Ahmad F, Slinkard AE. Genetic relationship in the genus *Cicer* L as revealed by polyacrylamide gel electrophoresis of seed storage proteins. *Theoretical and* Applied Genetics 1992; **84**: 688-692.

Ahmad I, Mehmood Z, Mohammad F, Ahmad S. Antimicrobial potency and synergistic activity of five traditionally used Indian medicinal plants. *Journal of Medicinal and Aromatic Plant Sciences.* 2000; **23:** 173–176.

Ahmad, Syed Dilnawaz, Sabir, Syed Mubashar, Saud, Halimi Mohammed Salihuddin, Yousaf. Evolutionary Relationship and Divergence Based on SDS-PAGE of *Elaeagnus umbellata*(Thunb.) Populations, a Multipurpose Plant from the Himalayas. *Turkish Journal of Biology.* 2007; **32**(1): 31-35.

Ajayi GO, Olagunju JA, Ademuyiwa O, Martins OC. Gas chromatography-mass spectrometry analysis and phytochemical screening of ethanolic root extract of *Plumbago zeylanica*, Linn. *Journal of Medicinal Plants Research* 2011; **5**(9): 1756-1761.

Ajmal Ali M, Fahad MA, Al-Hemaid, Joongku Lee, Choudhary RK, Naif A, Al-Harbi, Soo-Yong. Genetic diversity assessment of *Diplocyclos palmatus* (L.) C. Jeffrey from India using internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA. *African Journal of Biotechnology* 2011; **10**(72): 16145-16151.

Alam MZ. Herbal Medicines. APH Publishing Corporation; 2008:**32**.

Albasini A, Bianchi A, Melegari M, Vampa G, Pecorari P, Rinaldi M. Studies of *Artemisia dracunculus* L. s.l. (tarragon) *Fitoterapia*. 1983; **54**: 229–235.

Albert VASE, Williams, Chase MW. Carnivorous plants: *phylogeny and structural evolution science* 1994; **257**: 1491-1495.

Ali Azizi, Javad Hadian, Bernd Honermeier, Wolfgang Friedt. Associations between molecular markers, agro-morphological traits and chemical characteristics in a germplasm collection of the medicinal plant *Origanum vulgare* L. *Plant Systematics and Evolution* 2009; **6**: 28 - 45.

Alice Kurian, Anitha CA, Nybe EV. Variability and character association IN rose coloured Leadwort (*Plumbago rosea* Linn.). *Ancient Science of Life* 2001; **21**(2): 92–95.

Anandjiwala S, Srinivasa H, Kalola J, Rajani M. Free radical scavenging activity of *Bergia suffruticosa* (Delile) Fenzl. *Journal of Natural Medicines* 2007; **61**: 59-62.

Anbalagan K. An introduction to electrophoresis, Electrophoresis Institute Yercaud, Tamil Nadu, India. 1999; 105.

Annamalai A, Christina VL, Lakshmi PTV. Evaluation of Genetic Diversity in *Cardiospermum halicacabum* based on Geographical Variation Using RAPD Markers. *Pharmacognosy Communications* 2012; **2**(1): 62-65.

Anpin Raja RD, Jeeva S, Prakash JW, Johnson M, Irudayaraj V. Antibacterial activity of selected ethnomedicinal plants from South India. *Asian Pacific Journal of Tropical Medicine* 2011; **4**(5): 375-378.

Ansari MM, Ahmad J, Ahmad A and Ansari SH. Pharmacognostic characterization and standardization of Morus alba stem bark. *Journal of Medicinal and Aromatic Plant Sciences* 2006; **28**: 31-36**.**

Aparanji Poosarla, Rao DN, Rama Rao Athota, Venu Gopal Sunkara. Modulation of T cell proliferation and cytokine response by Plumbagin, extracted from *Plumbago zeylanica* in collagen induced arthritis. *BMC Complementary and Alternative Medicine* 2011; **11**: 114-118.

Aparicio R, Aparicio-Ruiz R. Authentication of vegetable oils by chromatographic techniques. *Journal of Chromatography* 2000; **881**: 93–104.

Aphacha J, Supachai Samappito, Karin Springob, Jürgen Schmidt, Tanja Gulder, Wanchai De Eknamkul, Gerhard Bringmann and Toni MK. *In Vitro* Plants, Callus and Root Cultures of *Plumbago indica* and Their Biosynthetic Potential for Plumbagin. *Journal of Chromatography* 2007; **2**(1): 53-65.

Arca M, Hinsinger DD, Cruaud C, Tillier A, Bousquet J. Deciduous trees and the application of universal DNA barcodes: A case study on the circumpolar *Fraxinus*. *PLOS ONE* 2012; **7**: e34089.

Ariyanathan S, Saraswathy A, Rajamanickam GV. Quality Control Standards for the Roots of Three *Plumbago* Species. *Indian Journal of Pharmaceutical Sciences* 2010; **72**(1): 86–91.

Arora DJ, Dhaliwal S, Kaur G. Antimicrobial activity of *Azardirachta indica* (Neem). *Geobios* 2005; **32**: 113-120.

Artyukova EV, Kozyrenko MM, Koren OG, Muzarok IT, Reunova GD, Zhuravlev Y.. RAPD and allozyme analysis of genetic diversity in *Panax ginseng* C.A. Meyer and *P. quinquefolius* L. *Russian Journal of Genetics* 2004; **40**(2): 178-185.

Arunachalam G, Subramanian N, Pazhani GP, Ravichandran V. Anti-inflammatory activity of methanolic extract of *Eclipta prostrata* L. (Astearaceae). *African Journal of Pharmacy and Pharmacology* 2009; **3**(3): 97-100.

Arunachalam KD, Velmurugan P, Raja RB. Anti-inflammatory and cytotoxic effects of extract from *Plumbago zeylanica*. *African Journal of Microbiology Research* 2010; **4**:1239- 45.

Arya K. Induction and evaluation of genetic variability in chethikoduveli (*Plumbago rosea*). Ph. D thesis department of plant breeding and genetics college of agriculture, Vellayani, Thiruvananthapuram; 1999.

Ashok Kumar P, Rajkumar. Kanimozhi M. Phytochemical screening and antimicobial activity from five Indian medicinal plants against human pathogens. Middle-East. *J. Sci. Res.* 2010; **5(3)**: 157-162.

Aswatha Ram HN, Shreedhara CS, Falguni P. Gajera, Sachin B. Zanwar. *In vitro* Free Radical Scavenging Potential of Methanol Extract of Entire Plant of *Phyllanthus reticulatus Poir. Pharmacology online* 2008; **2**: 440-451.

Bafeel SO, Arif IA, Bakir MA, Al Homaidan AA, Al Farhan AH, Khan HA DNA barcoding of arid wild plants using *rbcL* gene sequences. *Genetics and Molecular Research* 2012; **11**(3): 1934-1941.

Baginsky S. Plant proteomics: concepts, applications, and novel strategies for data interpretation. *Mass Spectrometry Reviews* 2009; **28**: 93–120.

Balza F, Jamieson L, Towers GHN. Chemical constituents of the aerial parts of *Artemisia dracunculus*. *J Nat Prod*. 1985; **48**: 339–340.

Baranska M, Schultz H. Application of infrared and Raman spectroscopy for analysis of selected medicinal and spice plants. *Journal of Medicinal and Spice Plants* 2006; **2**: 72–80.

Barasa M Maniafu, Lwande Wilber, Isaiah O Ndiege, Cornelius C Wanjala, Teresa Ayuko Akenga. Larvicidal activity of extracts from three *Plumbago* spp against *Anopheles gambiae. Memórias do Instituto Oswaldo Cruz Rio de Janeiro* 2009; **104**(6): 813-817.

Baum BR., Mechanda S, Livesey JF, Binns SE, Arnason JT. Predicting quantitative phytochemical markers in single *Echinacea* plants or clones from their DNA fingerprints. *Phytochemistry* 2001; **56**: 543–549.

Bennick A. Interaction of plant polyphenols with salivary proteins. *Critical Reviews in Oral Biology & Medicine* 2000; **13**(2): 184-196.

Bharat K, Kamba T, Albers M. Personalized, Interactive News On The Web. *Multimedia Systems* 1997; **6**(5):349–358.

Bhargava SK. Effects of plumbagin on reproductive function of male dog. *Indian Journal of Experimental Biology* 1984; **22**: 153–6.

Bhat TM, Kudesia R. Evaluation of genetic diversity in five different species of family solanaceae using cytological characters and protein profiling. *Genetic Engineering and Biotechnology Journal* 2011: GEBJ-20.

Bhuwan BM, Jalaj KG, Navneet K, Rakesh KS, Vyasji T, Vinod KT. An antileishmanial prenyloxy-naphthoquinone from roots of *Plumbago zeylanica. Natural Product Research* 2012; **27**: 480-485.

Biren Shah. Textbook of Pharmacognosy and Phytochemistry. *Elsevier Health*, 2009.

Birgit Ziegenhagen, Florian Scholz. Chloroplast microsatellites as markers for paternity analysis in *Abies alba*. *Can J for Res* 1998; **28**: 317-341.

Bisht R, Katiyar A, Singh R. Mittal P. Antibiotic resistance - a global issue of concern. *Asian Journal of Pharmaceutical and Clinical Research* 2009; **2**(2): 34-39.

Bizzini A, Durussel C, Bille J, Greub G, Prodhom G. Performance of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the identification of bacterial strains routinely isolated in a clinical microbiology laboratory. *Journal of Clinical Microbiology* 2010; **48**: 1549–1554.

Bodeker, G. Bhat KKS, Burley J, Vantomme P. (eds.). Medicinal plants for forest conservation and health care. Non-Wood Forest Products Series no. 11. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy. 1997: 158.

Bojaxa A, Rosy, Rosakutty PJ. GC-MS analysis of methanol wild plant and callus extracts from three *Cissus* species, Family Vitaceae. *Journal of Chemical and Pharmaceutical Research* 2012; **4**(7): 3420-3426.

Bompalli LK, Nallabilli L. Genetic diversity of *Ocimum* species through biochemical technique and UPGMA cluster analysis. *International Journal of Pharmacy and Pharmaceutical Sciences* 2013; **5**(4): 155-159.

Bose S, Frost S. An investigation on the variation of phenolic compounds in *Galeopsis* using thin-layer chromatography. *Hereditas* 1967; **58**: 145-164.

Bothiraja C, Joshi PO, Dama GY, Pawar AP. Rapid method for isolation of plumbagin, an alternative medicine from roots of *Plumbago zeylanica*. *European Journal of Integrative Medicine.* 2011; **3**: 39-42.

Briggs, David, Walters SM. Plant Variation and Evolution. London (2d ed., Cambridge, Eng. and New York; Cambridge University Press, 1984. 412; Reprinted with New York; American Book Co. 1910; 2:11.

Britto P, Benjamin Jeya Rathna Kumar. Protein profile studies of some medicinally important species of Asclepiadaceae family based on PAGE. *Life sciences leaflets* 2011; **20**: 860-865.

Buzzini P, Turchetti B, Branda E, Goretti M, Amici M, Lagneau PE, Scaccabarozzi L, Bronzo V, Moroni P. Large-scale screening of the in vitro susceptibility of *Prototheca zopfii* towards polyene antibiotics. *Medical Mycology* 2008; **46** (5): 511–514.

Cai ZM, Zhang YX, Zhang N, Gao LM, Li DZ. Testing four candidate barcoding markers in temperate woody bamboos (Poaceae: Bambusoideae). *Journal* of *Systematics and Evolution* 2012; 50: 527–539.

Calixto JB, Santos AR, Cechinel VF, Yunes RA. A review of the plants of the genus Phyllanthus: their chemistry, pharmacology, and therapeutic potential. *Medical Research Revolution* 1998; **18**(4): 225–258.

Cao H, But P, Paul. Identification of *Herba taraxaci* and its adulterants in Hong kong market by DNA fingerprinting with random primed PCR. *Zhongguo Zhang Yao Za Zhi* 1997; 22(4): 197-200.

Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: Localization of peptides and proteins using MALDI-TOF MS. *Analytical Chemistry* 1997; **69**: 4751–4760.

Carter C, Thornburg RW. Tobacco Nectarin V is a Flavin-Containing Berberine Bridge Enzyme-like Protein with Glucose Oxidase Activity. *Plant Physiology* 2004; **134:** 460-469.

CBOL Plant Working Group: Hollingsworth PM, Forrest LL, SpougeJL, Hajibabaei M, Ratnasingham S, van der Bank M, Chase MW, Cowan RS, Erickson DL, Fazekas AJ, Graham SW, James KE, Kim KJ, Kress WJ, Schneider H, van Alphen Stahl J, Barret, SCH, van den Berg C, Bogarin D, Burgess KS, Cameron KM, Carine MA DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the USA* 2009; **106**: 12794–12797.

Chan Soon Choy. Development and isolation of DNA microsatellite markers for the characterization anf identification of *Mystus nemrus* (C& V. Master's thesis, University of Puta Malaysia; 2003.

Chandra Mohan S, Dinakar S, Anand T, Elayaraja R, Sathiya Priya B Phytochemical, GC-MS analysis and Antibacterial activity of a Medicinal Plant *Acalypha indica*. *International Journal of Pharm Tech Research* 2012; **4**(3): 1050-1054.

Chandramohan A, Divya SR, Dhanarajan MS. *Matk* Gene Based Molecular Characterization of Medicinal plant – *Croton Bonplandianum* Baill. *International Journal of Bioscience Res.* 2013; **2**(3):69-72.

Chao L, Yuan L, Fang D, Qingyan M, Zewen G. Extraction, isolation and identification of plumbagin in *P. zeylanica* L. *Shizhen Guoyi Guoyao* 2006;**17**:919-22.

Charak, Drdhbala. In: Sastri R, Uppadhayaya Y, Pandeya GS, Gupta B, Mishra B. (Eds.), The Charak Samhita explained by Sastri K, Chaturvedi GN 22nd revised ed. Chaukhamba Bharti Academy, Varanasi. 1996.

Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RO, Haidar N, Savolainen V. Land plants and DNA barcodes: short-term and long-term goals. Philosophical Transactions of the Royal Society of London B, 2005; **360**: 1889–1895.

Chaurand P, Schwartz SA, Caprioli RM. Imaging mass spectrometry: A new tool to investigate the spatial organization of peptides and proteins in mammalian tissue sections. *Current Opinion in Chemical Biology* 2008; **6**: 676–681.

Chen C, Zhang H, Xiao W, Yong ZP, Bai N. High-performance liquid chromatographic fingerprint analysis for different origins of sea buckthorn berries. *Journal of Chromatography* 2007; **1154**: 250–259.

Chen CA, Chang HH, Kao CY, Tsai TH, Chen YJ. Plumbagin, isolated from *Plumbago zeylanica*, induces cell death through apoptosis in human pancreatic cancer cells. *Pancreatology* 2009; **9**(6): 797-809.

Cherkaoui A, Hibbs J, Emonet S, Tangomo M, Girard M. Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. *Journal of Clinical Microbiology* 2010; **48**: 1169–1175.

Chew FS, Rodman JE. Plant resources for chemical defense. In: Rosenthal GA, Janzen DH (Ed) Herbivores: Their Interaction with Secondary Plant Metabolites. *Acadamic Press*, New York: 1979; 271–307.

Chinese Pharmacopoeia Commission. TLC Atlas of Chinese Crude Drugs in Pharmacopoeia of the People's Republic of China. People's Medical Publishing House; 2009.

Chitravadivu C, Manian S, Kalaichelvi K. Qualitative analysis of selected medicinal plants, Tamilnadu, India. Middle - East. *Journal of science and Research* 2009; **4**(3): 144-146.

Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. Tannins and human health: A review. *Critical Reviews in Food Science and Nutrition* 1998; **38**: 421-464.

Chun-Ying Xue, De Zhu Li. Use of DNA barcode *sensu lato* to identify traditional Tibetan medicinal plant *Gentianopsis paludosa* (Gentianaceae). *Journal of Systematics and Evolution* 2011; **49**(3): 267–270.

Clauss M, Castell JC, Kienzle E, Dierenfeld ES, Flach EJ, Behlert O, Ortmann S, Streich WJ, Hummel J, Hatt JM. The influence of dietary tannin supplementation on digestive performance in captive black rhinoceros (*Diceros bicornis*). *Journal of Animal Physiology and Animal Nutrition* 2007; **91**(11-12): 449-458.

Clegg MT, Zurawki G. Chloroplast DNA and the study of plant phylogeny: Present status and future prospectus. *In* "Molecular systematics of plants". P. S. Soltis, D. E. Soltis,and J. J. Doyle, Eds Chapman and Hall, NewYork 1992: 1-13.

Clegg MT. Chloroplast gene sequences and the study of plant evolution. *Proceedings of the National Academy of Sciences* 1993; **90**: 363-367.

Cong HJ, Zhang SW, Shen Y. Guanidine alkaloids from *Plumbago zeylanica. Journal of Natural Products* 2013; **76**(7):1351-7.

Conley DJ, Schelske CL, Stoermer EF. Modification of silica biogeochemistry with eutrophication in aquatic systems. *Mar. Ecol. Prog. Ser*. 1993; **101**: 179–192.

Cook RJ. The characterization and identification of crop cultivars by electrophoresis. *Electrophoresis* 1984; **5**: 65.

Cooper RA, Halas E, Molan PC. The efficacy of honey in inhibiting strains of *Pseudomonas aeruginosa* from infected burns. *Journal of Burn Care and Rehabilitation* 2002; **23**(6): 366-70.

Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. *Journal of Natural Products* 1997; **60:** 52–60.

Cragg GM, Newman DJ. Medicinals for the millennia. *Annuals of Academic Sciences* 2001. **953**: 3-25.

Craig Costion, Andrew Ford, Hugh Cross, Darren Crayn, Mark Harrington, Andrew Lowe1. Plant DNA Barcodes Can Accurately Estimate Species Richness in Poorly Known Floras. *PLoS ONE* 2011; **6**(11): e26841

Das Talukdar A, Dutta Choudhury M, Chakraborty M, Dutta BK. Phytochemical screening and TLC profiling of plant extracts of *Cyathea gigantea* (Wall. Ex. Hook.) Haltt. and *Cyathea brunoniana*. Wall. ex. Hook. (Cl. & Bak.). *Journal of Science & Technology Biological and Environmental Sciences* 2010; **5**: 70-74.

Datte BK, Ahmed M, Banoo R, Talukder SA. Antifungal and antibacterial activity of the seed oil of *Lawsonis alba*. *Bangladesh Journal of Microbiology* 1989; **6**(2): 49-55.

Deans SG, Svoboda KP. Antibacterial activity of French tarragon (*Artemisia dracunculus* Linn.) essential oil and its constituents during ontogeny. *Journala of Horticulture and Science* 1988; **63**: 503–508.

Delazar A, Nazifi E, Movafeghi A, Nahar L, Nazemiyeh H, Moghadam SB, Asnaashari S, Sarker SD. GC-MS analysis of *Ornithogalum procerum. DARU Journal of Pharmaceutical Sciences* 2009; **17**(1): 33

Delazar A, Reid RG, Sarker SD. GC-MS analysis of essential oil of the oleoresin from *Pistacia atlantica* var *mutica*. *Chemistry of Natural Compounds* 2004; **40**: 24-27.

Demissie M, Lindtjorn B, Berhane Y. Patient and health service delay in diagnosis of pulmonary tuberculosis in Ethiopia. *BMC Public Health* 1998; **2**: 23.

Desai HP, Kapadia MD, Kharat AR. Evaluation of anthelmintic activity of *Plumbago zeylanica* Linn. *International Journal of Pharmaceutical Sciences and Research* 2012; **3**(11): 4281-4284.

Devender Rao Kodati, Shashidher Burra and Kumar Goud P. Evaluation of wound healing activity of methanolic root extract of *Plumbago zeylanica* L. in wistar albino rats. *Asian Journal of Plant Science and Research*. 2011; **1**(2): 26-34.

Devi PU. *Withania somnifera* Dunal: potential plant source of a promising drug for cancer therapy and radio sensitization. *Indian Journal of Experimental Biology* 1994; **34**: 927-932.

Devi SM, Thenmozhi M. "Antibacterial Activity of *Plumbago zeylanic*a Leaf Extract. *International Journal of Research in Biomedicine and Biotechnology* 2011; **1**(1): 1-4.

Devi VG, Vijayan C, John A, Gopakumar K. Pharmacognostic and antioxidant studies on *Clerodendrum inerme* and identification of ursolic acid as marker compound. *Journal of Pharmaceutical Sciences* 2012; **4**(2): 145-148.

Dhale DA, Markandeya SK. Antimicrobial and Phytochemical Screening of *Plumbago zeylanica* Linn. (Plumbaginaceae) Leaf. *Journal of Experimental Sciences* 2011; **2**(3): 04-06.

Dharmar K, John De Britto A. DNA fingerprinting of *Plumbago zeylanica* L. using ISSR Markers. *International Journal of Applied Bioresearch* 2012; 23-27.

Dhivya Selvaraj, Rajeev Kumar Sarma, Ramalingam Sathishkumar. Phylogenetic analysis of chloroplast *mat*K gene from Zingiberaceae for plant DNA Barcoding. *Bioinformation* 2008; **3**(1): 24-27.

Dibyajyoti Saha, Swati Paul. Pharmacognostic Studies of Aerial Part of Methanolic Extract of *Plumbagoindica* L. *Asian Jouranal of Research and Pharmaceutical Sciences* 2012; **2**(3): 88-90.

Din NUA, Mahmood GSS, Khattak I. Saeed, Hassan MF. High yielding groundnut (*Arachis hypogea* L.) Variety "Golden". *Pakistan Journal of Botany* 2009; **41**(5): 2217-2222.

Dinda B, Das SK, Hajra AK, Bhattacharya A, Chel GKD, Achari B. Chemical consitituents of *Plumbago indica* roots and reactions of *plumbagin*. *Indian Journal of Chemistry* 1997; **30**: 377-600.

Dinda B, Hajra AK, Chel G. 14 Naphthoquinonens of *Plumbago* sp. A review. *Journal of Indian Chemical Soc*iety 1997; **74**: 974-979.

Djabou N, Battesti MJ, Allali H, Desjobert JM, Varesi L, Costa J, Muselli A.. Chemical and genetic differentiation of Corsican subspecies of *Teucrium flavum* L. *Phytochemistry* 2011; **72**(11-12): 1390- 1399.

Dong LN, Wortley AH, Wang H, Li DZ, Lu L. Efficiency of DNA barcodes for species delimitation: a case in *Pterygiella* Oliv. (Orobanchaceae). *Journal of Systematics and Evolution* 2011; **49**: 189–202.

Dorni AIC, Vidyalakshmi KS, Hannah RV, Rajamanickam GV, Dubey GP. Antiinflammatory activity of *Plumbago capensis*. *Pharmacognosy magazine* 2006; **2**:239-43.

Downie SR., Katz-Downie DS, Watson MF. A phylogeny of the flowering plant family Apiaceae based on chloroplast DNA rpl16 and rpoC1 intron sequences: towards a suprageneric classification of subfamily Apioideae. *American Journal of Bot*any 2000; **87**(2): 273-292.

Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem*istry *Bulletin*. 1987; **19**:11-15.

Druzhinina IS, Komon-Zelazowska M, Kredics L, Hatvani L, Antal Z, Belayneh T, Kubicek CP.Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. *Microbiology* 2008; **154**:3447–3459.

Du ZY, Qimike A, Yang CF, Chen JM, Wang Q-F. Testing four barcoding markers for species identification of Potamogetonaceae. *Journal of Systematics and Evolution* 2011; **49**: 246–251.

Dumarey M, Van Nederkassel AM, Deconinck E, Vander Heyden Y. Exploration of linear multivariate calibration techniques to predict the total antioxidant capacity of green tea from chromatographic finger prints. *Journal of Chromatography* 2008; **1192** (1): 81-88.

Eisenhauer N, Klier M, Partsch S, Sabais ACW, Scherber C, Weisser W, Scheu S. No interactive effects of pesticides and plant diversity on soil microbial biomass and respiration. *Applied Soil Ecology* 2009; **42**: 31-36.

Ellis D, Harrigan GG, Goodacre R. Metabolic fingerprinting with Fourier-transform infrared spectroscopy. In: Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis. Kluwer Academic, Dordrecht: **111**: 2002.

Evans CE, Banso A, Samuel OA. Efficacy of some nupe medicinal plants against *Salmonella typhi*: an *in vitro* study. *Jouranl of Ethnopharmacology* 2002; **80**: 21-24.

Ezhilan, Ramasamy Neelamegam. GC-MS analysis of phytocomponents in the ethanol extract of *Polygonum chinense* L. *Pharmacognosy Research* 2012; **4**(1): 11–14.

Fabrice Guillaume A, François Guillem B, Guy Tiberghien C, Emmanuel Stip ERP. Investigation of study-test background mismatch during face recognition in Schizophrenia. *Schizophrenia Research* 2012; **134**: 101–109.

Faibbrothers FE. Chemosystematics with emphasis on systematic serology. In: Heywood V. H. (ed.*) Modern Methods in Plant Taxonomy*, London. 1968.

Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG. Multiple Multilocus DNA Barcodes from the Plastid Genome Discriminate Plant Species Equally Well. *PLoS ONE* 2008; **3**(7): e2802.

Fazekas AJ, Steeves R, Newmaster SG, Hollingsworth PM. Stopping the stutter: improvements in sequence quality from regions with mononucleotide repeats can increase the usefulness of non-coding regions for DNA barcoding. *Taxon* 2010; **59**: 694–697.

Feng T, Liu S, He XJ. Molecular authentication of the traditional Chinese medicinal plant *Angelica sinensis* based on internal transcribed spacer of nr DNA. *Electronic Journal of Biotechnology* 2010; **13**.

Ferenczi-Fodor K, Végh Z, Renger B. Thin-layer chromatography in testing the purity of pharmaceuticals. *Trends* in *Analytical Chemistry* 2006; **25**: 778-789.

Fergusan JM, Grabe DF. Identification of cultivars of perennial rye grass by SDS-PAGE of seed proteins. *Journal of Crop Science and Biotechnology* 1986; **26**: 170.

Fernanda G. Bueno, Maria A. D. Machareth, Gean P. Panizzon, Gisely C. Lopes e João C. P. Mello. Development of a UV/Vis spectrophotometric method for analysis of total polyphenols from *Caesalpinia peltophoroides* BENTH. *Química* Nova 2012; **35**(4) 822-826.

Fernie AR, Trethewey RN, Krotzky AJ, Willmitzer L. Innovation-Metabolite profiling: from diagnostics to systems biology. *Nature Reviews Molecular Cell Biology* 2004; **5**: 763-769.

Ferreira D, Gross GG, Hagerman AE, Kolodziej H, Yoshida T. Tannins and related polyphenols: Perspectives on their chemistry, biology, ecological effects and human health protection. *Phytochemistry* 2008; **69**: 3006-3008.

Ferreira L, Sanchez-Juanes F, Garcıa-Fraile P, Rivas R, Mateos PF. MALDI-TOF Mass Spectrometry Is a Fast and Reliable Platform for Identification and Ecological Studies of Species from Family Rhizobiaceae *PLoS ONE* 2011; **6**(5): e20223.

Fico A, Manganelli G, Simeone M, Guido S, Minchiotti G, Filosa S. High through put screening compatible single step protocol to differentiate embryonic stem cells in neurons. *Stem cells Development* 2008; **17**(3): 573- 584.

Frankel, Jeffrey A, Stein, Ernesto, Wei, Shangjin. Trading Blocs and the Americas: The Natural, the Unnatural, and the Supernatural. *Journal of Development Economics* 1995; **47**(1): 61–95

Fuzzati N. Analysis methods of ginsenosides. *Journal of Chromatography* 2004; **812**: 119-133.

Gairola KC, Nautiyal AR, Prasad P. Variation in Biochemical Properties in Seeds of *Jatropha curcas* Linn: A Study of Hilly Regions of Uttarakhand Bull. *Enironmental. Pharmacological Life Sciences* 2012; **2**(1): 20- 27.

Galal AM, Raman V, Avula B, Wang YH, Rumalla CS, Weerasooriya AD, Khan IA. Comparative study of three *Plumbago* L. species (Plumbaginaceae) by microscopy, UPLC-UV and HPTLC. *Journal of Natural Medicine* 2012; 59-62.

Gambhir, Nilakshi V, Bhaskar VV. HPTLC analysis of vitamin C from *Pithecellobium dulce*, Benth (Fabaceae). *Journal of Pharmacy Research* 2011; **4** (4):1197.

Ganatra SH, Durge SP, Patil SU. Preliminary Phytochemicals Investigation and TLC Analysis of *Ficus racemosa* Leaves. *Journal of Chemical and Pharmaceutical Research* 2012; **4**(5): 2380-2384.

Gangopadhyay M, Dewanjee S, Chakraborty D, Bhattacharya S. Role of exogenous phytohormones on growth and plumbagin accumulation in *Plumbago indica* hairy roots and conservation of elite root clones via synthetic seeds. *Indian Journal of Crops Production* 2011; **33**: 445-450

Gao T, Yao H, Song J, Zhu Y, Liu C. Evaluating the feasibility of using candidate DNA barcodes in discriminating species of the large Asteraceae family. *BMC evolution biology* 2010; **10**: 1–7.

Geltz Norman, Russell SD. Two-Dimensional Electrophoretic Studies of the Proteins and Polypeptides in Mature Pollen Grains and the Male Germ Unit of *Plumbago zeylanica. Plant Physiology* 1988; **88**(3): 764-9.

Gepts P, Clegg MT. Genetic diversity in pearl millet (*Pennisetum glaucum* [L.] R.Br.) at the DNA sequence level. *Journal of Heredity* 1989; **80:** 203-208.

Gepts P. Genetic diversity of seed storage proteins in plants. *Plant Population Genetics*, *Breeding and* Genetic *Resources* 1990: 64-78.

Gershenzon JME, McConkey, Croteau RB. Regulation of monoterpene accumulation in leaves of peppermint. *Plant Physiology* 2000; **122**:205-213**.**

Gershenzon, J, Croteau R. Terpenoids. In: Herbivores: Their Interactions with Secondary Plant Metabolites, Rosenthal GA, Berenbaum MR (Eds.). Vol. 1, Academic Press, San Diego, 1991: 165-219.

Ghaffoor A, M Arshad. Seed protein profiling of *Pisum sativum* L., germplasm using sodium do decyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for investigation of biodiversity. *Pakistan Journal of Botany* 2008; **40**(6): 2315-2321

Giebel R, Worden C, Rust SM, Kleinheinz GT, Robbins M. Microbial fingerprinting using matrix-assisted laser desorption ionization time of- flight mass spectrometry (MALDI-TOF MS) Applications and Challenges. *Advances in applied microbiology* 2010; **71**: 149–184.

Gopalakrishnan S, Vadivel E. GC-MS analysis of some bioactive constituent of *Mussaende frodosa* Linn. *International Journal of Pharma and Biosciences*. 2011; **2**(1): 313 - 320.

Gopinath S, Muralidharan S, Rajan S, Danaphal SP. Simultaneous Estimation of Plumbagin and Embelin by Reverse Phase-High Performance Liquid Chromatographic method. *Der Pharmacia Lettre* 2009; **1**(1): 135-142.

Grayson DH, Monoterpenoids. *Natural Product Reports* 1998; **5**: 497-521.

Greger JL, Smith SA, Johnson MA, Baier MJ. Effects of dietary tin and aluminium on selenium utilization by adult males. *Biology of Trace Elements Research* 1982; **4**: 269 – 278.

Gromova, Celis JE. Protein Detection in Gels by Silver Staining: A Procedure Compatible with Mass-Spectrometry Cell Biology: A Laboratory Handbook. 3rd Edition Elsevier. Academic Press. 2006.

Groot DGA, During HJ, Maas JW, Schneider H, Vogel JC. Use of *rbcL* and *trnL-F* as a two-locus DNA barcode for identification of NW-European ferns: an ecological perspective. *PLOS ONE* 2011; **6**: e16371.

Gunnar Samuelsson. Drugs of Natural Origin: *A Textbook of Pharamacognosy* eds. Swedish Pharmaceutic publishers 2004; **5**: 620.

Gupta AP, Gupta MM, Kumar S. High performance thin layer chromatography of asiaticoside in *Centella asiatica. Journal* of *Indian Chemical* Society 1999; **76**: 321–322.

Gupta MM, Verma RK. Combined thin layer chromatography– densitometry method for the quantitative estimation of major alkaloids in poppy straw samples. *Indian Journal of Pharmaceutical Sciences* 1996; **58**: 161–163.

Gupta S, Ahirwar D, Jhade D. Pharmacognostic standardization, physic and phytochemical evaluation of *Plumbago zeylanica* Linn root. *Drug Invention Today* 2010; **2**(9):408-410.

Gupta VK Singh J, Kumar R, Bhanot A. Pharmacognostic and preliminary phytochemical study of *Ocimum gratissimum* Linn, (Family: Lamiaceae). *Asian Journal of Plant sciences* 2011; **1**(7): 365-369.

Gurudeeban S, Satyavani K, Ramanathan T. Phylogeny of Indian Rhizophoraceae Based on the Molecular Data from Chloroplast $tRNA^{LEU}_{UAA}$ Intergenic Sequences. *Pakistan Journal of Biological Sciences,* 2013; **16**: 1130-1137.

Hai Fei Y, Gang HAO Chi Ming HU, Xue-Jun GE. DNA barcoding in closely related species: A case study of *Primula* L. sect. *Proliferae* Pax (Primulaceae) in China. *Journal of Systematics and Evolution* 2011; 49(3): 225–236.

Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends of Genet*ics 2007; **23**: 167–172.

Halinski P, Szafranek J, Szafranek BM, Goe˛biowski M, Stepnowski P. Chromatographic fractionation and analysis of the main components of eggplant (*Solanum melongena* L.) leaf cuticular waxes. *Acta Chromatographica* 2009; **21**: 127-137.

Hamrick JL, Godt MJW. Allozyme diversity in plant species. In Plant population genetics, breeding, and genetic resources (ed. A H D. Brown, M T. Clegg, AL. Kahler , BS. Weir), Sinauer, Sunderland, 1990; 43–63.

Harborne JB. Phytochemical methods, Chapman and Hall, Ltd. London. 1999. 60-66.

Haribabu Rao D, Vijaya T, Ramana Naidu BV, Subramanyam P, Jayasimha Rayalu D. Phytochemical screening and antimicrobial studies of compounds isolated from *Plumbago zeylanica*.L. *International journal of analytical, pharmaceutical and biomedical sciences* 2012; **1**(3): 82-90.

Hartmann MA. Plant sterols and the membrane environment. *Trends in Plant Science* 1998; **3**: 170-175.

Hazhar M. Muhammad, Kawkab Y. Saour, Alaadin M. Naqishbandi. Quantitative and Qualitative Analysis of Plumbagin in the Leaf and Root of *Plumbago europaea* Growing Naturally in Kurdistan by HPLC. *Iraqi Journal of Pharmaceutical Sciences* 2009; **18**: 1-6.

He SA, Sheng N. Utilization and conservation of medicinal plants in china with special reference to *Atractylodes lancea*. In; Medicinal plants for forest conservation and healthcare. G.C/Bodeker (ed) FAO, Rome, 1997.

Hebert PDN, Cywinska A, Ball SL, de Waard JR. Biological identifications through DNA barcodes. Proceedings of the Royal Society: *Biological Sciences* 2003; **270**: 313–321.

Hebert PDN, Gregory TR. The promise of DNA barcoding for taxonomy. *Systematic Biology* 2005; **54:** 855-859.

Hegnauer R, Biochemistry, distribution and taxonomic relevance of higher plant alkaloids. *Phytochemistry* 1988; **27**: 2423-2427.

Helm D, Labischinski H, Schallehn G, Naumann D. Classification and identification of bacteria by Fourier-transform infrared spectroscopy. *Journal of General Microbiology*. 1991; **137**; 69–79

Hemalatha RG, Padmini E. Studies with the latex of the milk weed- Calotropis procera (Ait) R.Br. for spermicidal effect in human. *Journal of Pharmacy Research* 2011; **4**(2): 304-307.

Hemant Kumar Singh, Iffat Parveen1, Saurabh Raghuvanshi, Shashi B Babbar. The loci recommended as universal barcodes for plants on the basis of floristic studies may not work with congeneric species as exemplified by DNA barcoding of *Dendrobium*species. *BMC Research Notes* 2012, **5**:42-46.

Hermosa MR, Keck E, Chamorro I, Rubio B, Sanz L, Vizcaino JA, Grondona I, Monte E. Genetic diversity shown in *Trichoderma* biocontrol isolates. *Mycol Res* 2004; **108**:897–906.

Hirangi DP, Gaurang B. Shah, Vandit Trivedi. Investigation of HMG Co A Reductase Inhibitory Activity of Antihyperlipidemic. Herbal Drugs Study In Vitro. *Asian Journal of Experimental Biological Sciences* **2**(1) 2011: 63-68.

Hollingsworth PM, Graham SW, Little DP. Choosing and Using a Plant DNA Barcode. *PLoS ONE* 2011; **6**(5): 19254.

Hostettmann K. Assays for bioactivity. *Methods in Plant Biochemistry*, Academic Press, San Diego. 1991; **6**; 360.

Hostettmann KA, Marston A. Saponins. Chemistry and pharmacology of natural products,Cambridge Universty Press, Cambridge, United Kingdom, 1995.

Hu Y, Zhang Q, Xin H, Qin LP, Lu BR, Rahman K, Zheng H. Association between chemical and genetic variation of Vitex rotundifolia populations from different locations in China: its implication for quality control of medicinal plants. *Biomedical Chromatography* 2007; **21**(9):967-75.

Hui Yao, Jingyuan Song, Chang Liu, Kun Luo1, Jianping Han, Ying Li, Xiaohui Pang, Hongxi Xu, Yingjie Zhu, Peigen Xiao, Shilin Chen. Use of ITS2 Region as the Universal DNA Barcode for Plants and Animals. *PLoS ONE* 2010; 5(10): e13102

Ibrahim M, Hameed AJ, Jalbout A. Molecular Spectroscopic Study of River Nile Sediment in the Greater Cairo Region. *Applied Spectroscopy* 2008; **62(**3): 306-311.

Ibrahim M, Joy Baura, Qamrul Ahasan, Torequl Islam, Zilly Homa M, Mohi Uddin Chowdhury, Aslam MH and Mohammad AR. Preliminary Phytochemical and Pharmacological Investigations of *Alpinia conchigera* Griff. and *Plumbago indica* L. *Bangladesh Pharmaceutical Journal* 2012; **15**(2): 153-157.

Ilaria Bruni, Fabrizio De Mattia, Stefano Martellos, Andrea Galimberti, Paolo Savadori, Maurizio Casiraghi, Pier Luigi Nimis, Massimo Labra. DNA Barcoding as an Effective Tool in Improving a Digital Plant Identification System: A Case Study for the Area of Mt. Valerio, Trieste (NE Italy). *PLOS ONE*, 2012; **7**(9): e43256.

Ilavenil SB, Kaleeswaran B, Ravikumar S. Evaluation of antibacterial activity and phytochemical analysis of *Crinum asiaticum. International Journal of Current Research* 2010; **1:** 035-040.

Iqbal Ahmad, Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens, *Journal of Ethnopharmacology* 2000; **74**: 113–123.

Iqbal SH, Ghafoor A, Ayub N. Relationship between SDS-PAGE markers and Ascochyta blight in chickpea. *Pakistan Journal of Botany* 2005; **37**: 87-96.

Irfan Emre, Dilek Turgut – Balik, Hasan G, Ahmet Sahn. The use of seed proteins revealed by SDS-PAGE in taxonomy of some *Lathyrus* L. species grown in Turkey. *Pakistan Journal of Biological Sciences* 2006; **9**(12): 2358 – 2361.

Ishita Ahuja, Ric CH, de Vos, Atle M. Bones, Robert D. Hall. Plant molecular stress responses face climate change. *Trends in Plant Science* 2010; **15**(12): 346-349.

Ishtiaq ChM, Mumtaz SA, Wang Y, Yiyu C, Mehmood T, Ashraf M . Proteins as Biomarkers for Taxonomic Identification of Traditional Chinese Medicines (TCMs) from Subsection *Rectae* Genus *Clematis* from China. *Journal* of *Applied* Sciences (Special Issue of *Biotechnology and Genetic Engineering* 2010; **8**: 62-70.

Itoigawa M, Takeya K, Furukawa H. Cardiotonic action of plumbagin on guineapig papillary muscle. *Planta Medica* 1991; **57**: 317–9.

Jaakola L, Suokas M, Haggman H. Novel approaches based on DNA barcoding and high-resolution melting of amplicons for authenticity analyses of berry species. *Food Chemistry* 2010; **123**: 494–500.

Jacobs DI, Gaspari M, Greef J, Heijden R, Verpoorte R. Proteome analysis of the medicinal plant *Catharanthus roseus*. *Planta*. 2005; **221**(5): 690-704.

Jain SK. Medicinal Plants. National Book Trust, India, 1968; pp.1-216

James EA, Ashburner GR. Intra specific variation in *Astelia australiana* (Liliaceae) and implications for the conservation of this Australian species. *Biological Conservation*. 1997; **82**: 253–261.

Javaid A, Ghafoor A, Anwar R. Seed storage protein electrophoresis in groundnut for evaluating genetic diversity. *Pakistan J. of Botany* 2004; **36**: 25-29.

Jetty A, Subhakar C, Rajagopal D, Jetty M, Subramanyam M, Marthanda Murthy M. Antimicrobial activities of neo- and 1- epineo-isoshinanolones from *Plumbago zeylanica* roots. *Pharmaceutical Biology* 2010; **4**8: 1007-11.

Jeyachandran R, Mahesh A, Cindrella, Sudhakar S, Pazhanichamy K. Antibacterial activity of plumbagin and root extracts of *Plumbago zeylanica*l. *Acta Biologica Cracoviensia series botanica* 2009; **51**(1): 17–22.

Jing Gu, Jun Xia Su, Ruo Zhu Lin, Rui- Qi Li, Pei Gen Xiao. Testing four proposed barcoding markers for the identification of species within *Ligustrum* L. (Oleaceae). *Journal of Systematics and Evolution* 2011; **49**(3): 213–224.

Joelri Michael Raj L, John Britto S, Prabhu S, Senthilkumar SR. Phylogenetic relationships of *Crotalaria* species basedon seed protein polymorphism revealed by SDS-PAGE. *International Research Journal of Plant Science* 2011; **2**(5): 119-128.

Johan D Peleman, Crispin Wye, Jan Zethof, Anker P Sørensen, Henk Verbakel, Jan van Oeveren, Tom Gerats, Jeroen Rouppe van der Voort. Quantitative trait locus (QTL) isogenic recombinant analysis: A method for high-resolution Mapping of QTL within single population. *Genetics*. 2005; **171**(3): 1341–1352.

John De Britto A, Benjamin Jeya Rathna kumar P. Protein profile studies of some medicinally important species of Asclepiadaceae family based on Poly Acrylamide Gel Electrophoresis (PAGE). *Life sciences Leaflets* 2011: **20**:860 –865.

Johnson KS, Scriber JM. Geographic variation in plant allelochemicals of significance to insect herbivores. In: Ananthakrishnan TN (Ed) Functional Dynamics of Phytophagous Insects, Oxford and IBH Publishing Co, Lebanon, 1994; 7–31

Johnson M, Usha Raja Nanthini A, Renisheya Joy Jeba Malar T. Isozyme Variation and Genetic Relationships among Three *Plumbago* Species. *Journal of Ecobiotechnology* 2010; **2**(5): 54-59.

Johnson M. Biochemical Variation Studies in *Aegle marmelos* (L.) Corr - A medicinally important plant. *Journal* of *Chemical and Pharmaceutical Research* 2010; **2**(6): 454-462.

Johnson M. Somoclonal variation studies on *Phyllanthus amarus* Schum & Thonn. *Iranian Journal of Biotechnology*. 2007; **5**(4): 240-245.

Joshi K, Chavan P, Warude D, Patwardhan B. Molecular markers in herbal drug technology. *Current Science*. 2004; **87**(2): 159-165.

Joshi R, Venkatesh K, Srinivas R, Nair S, Hasan G. Genetic dissection of itpr gene function reveals a vital requirement in aminergic cells of *Drosophila* larvae. *Genetics* 2004; **166**(1): 225-236.

Joy DA, Feng X, Mu J, Furuya F, Chotivanich K, Krettli AU, Ho M, Wang A, White NJ, Suh E, Beerli P, Su X. Early origin and recent expansion of *Plasmodium falciparum*. *Science* 2003; **300**:318–321.

Joy PP, Thomas J, Mathew S, Skarian BP. Medicinal Plants. *Tropical Horticulture* Naya Prokash, Calcutta. 2001; **Vol 2:** 449-632.

Jyoti Ranjan Rout, Satyajit Kanungo, Ritarani Das, Santi Lata Sahoo. In Vivo Protein Profiling and Catalase Activity of *Plumbago zeylanica* L. *Nature and Science* 2010; **8**(1): 87-90.

Kadry Abdel Khalik, Gamal Osman, Waeil Al-Amoudi. Genetic diversity and taxonomic relationships of some Ipomoea species based on analysis of RAPD-PCR and SDS-PAGE of seed proteins. *American Journal of Cultural Sociology* 2012; **6**(6): 1088-1093.

Kaewbumrung S, Panichayupakaranant P. Isolation of three antibacterial naphthoquinones from *Plumbago indica* roots and development of a validated quantitative HPLC analytical method. *Natural Product Research* 2012; **26**(21): 20-23.

Kaiser, Kayla Anne, Metabolic Profiling of Primary and Secondary Biosynthetic Pathways in Angiosperms: Comparative Metabolomics and Applications of Hyphenated LC-NMR and LC-MS University of California, Riverside. 2012; **406**: 3503279.

Kakjing Dadul Falang, Mary Ogonnaya Uguru, Noel Nenman Wannang, Iliya Hosea Azi, Nwoye Chiamaka. Anti-ulcer activity of *Plumbago zeylanica* linn root extract. *Journal of Natural Product and Plant Resources* 2012; **2**(5): 563-567.

Kamal AHM, Kim KH, Shin DH, Seo HS, Shin KH, Park CS, Heo HY, Woo SH. Proteomics profile of pre-harvest sprouting wheat by using MALDI-TOF Mass Spectrometry. *Plant Omics* 2009; **2**(3):110-119.

Kanchana N, Sadiq A. Hepatoprotective effect of *Plumbago zeylanica* on paracetamol induced liver toxicity in rats. *International Journal of Pharmacy and Pharmaceutical Sciences* 2011; **3**(1):151- 154.

Kantha D, Arunachalam P, Velmurugan, Balaji Raja R. Antiinflammatory and cytotoxic effects of extract from *Plumbago zeylanica*. *African Journal of Microbiology Research* 2010; **4**(12): 1239-1245.

Kapteyn J, Simon JE. The use of RAPDs for assessment of identity, diversity, and quality of *Echinacea*. *Trends in new crops and new uses.* J. Janick and A. Whipkey (eds.), In: ASHS Press, Alexandria 2002; 509–513.

Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10000 Da. *Analytical Chemistry* 1988; **60**:1199–2301.

Karlova R, Boeren S, Russinova E, Aker J Vervoort J, de Vries S. The *Arabidopsis* somatic embryogenesis receptor-like kinase1 protein complex includes brassinosteroid-insensitive1. *The Plant Cell* 2006; **18**: 626-638.

Karre Siva Venkata Subhash, Tallapudi Kiran Kumar, Chidambranathan N, Vinodth Prabhu. Phytochemical Analysis and Cardioprotective Activity of *Plumbago zeylanica* Linn in Isoproterenol Provoked Oxidative Myocardial Injury in Albino wistar Rats. *International Research Journal of Pharmacy* 2012; **3**(9): 29-32.

Kaspar S, Peukert M, Svatos A, Matros A, Mock HP. MALDI imaging mass spectrometry- An emerging technique in plant biology. *Proteomics*, 2011; **11**: 1840-1850.

Kell DB, Brown M, Davey HM, Dunn WB, Spasic I, Oliver SG. Metabolic footprinting and systems biology: The medium is the message. *Nature Reviews Microbiology* 2005; **3**: 557-565.

Kersten B, Bürkle L, Kuhn EJ, Giavalisco P, Konthur Z, Lueking A. Large-scale plant proteomics. *Plant Molecular Biology* 2002; **48**: 133–141.

Kethani Devi C, Gopala Krishna D**.** Pharmacognostic, Phytochemical And Biological study of *Plumbago zeylanica***.** *International Journal of Natural Products Research* 2012; **1**(2): 21-23.

Ketzis JK, Vercruysse J, Stromberg BE, Larsen M, Athanasiadou S, Houdijk JG. Evaluation of efficacy expectations for novel and non-chemical helminth control strategies in ruminants. *Veterinary Parasitology* 2006; **139**: 321-335.

Kevin S Burgess, Aron J Fazekas , Prasad R Kesanakurti, Sean W Graham Brian C. Husband, Steven G Newmaster, Diana M Percy, Mehrdad Hajibabaei, Spencer C Harrett. Discriminating plant species in a local temperate flora using the *rbc*L+ matK DNA barcode. *Methods in Ecology and Evolution* 2011; **2**: 333–340.

Khalid II, Elhardallou SB, Elkhalifa EA. Composition and Functional Properties of Cowpea (*Vigna ungiculata* L. Walp) Flour and Protein Isolates. *American Journal of Food Technology* 2012; **7**:113-122.

Khan MY, Aliabbas S, Kumar V, Rajkumar S. Recent advances in medicinal plant biotechnology. *Indian Journal of Biotechnology* 2009; **8**: 9-22.

Khan S, Mirza KJ, Abdin MZ. Development of RAPD markers for authentication of medicinal plant *Cuscuta reflexa*. *Journal of Biosciences* 2010; **4:** 1-7.

Khatoon S, Agnihotri AK, Singh N, Rawat AKS, Mehrotra S. Comparative pharmacognostic evaluation of *Althaea officinalis* and *Alcea rosea* root. *Hamdard Medicus* 2008; **51**(2): 56-62.

Kim SW, Ban SH, Chung H, Cho S, Chung HJ, Choi PS, Yoo OJ, Liu JR. Taxonomic discrimination of flowering plants by multivariate analysis of Fourier transform infrared spectroscopy data. *Plant Cell Reports* 2004; **23**: 246-250.

Kishore N, Mishra BB, Tiwari VK, Tripathi V. A novel naphthaquinone from *Plumbago zeylanica* L. roots. *Chemistry of Natural Compounds* 2010; **46**:517-9.

Kishore N, Mishra BB, Tiwari VK, Tripathi V. An account of phytochemicals from *Plumbago zeylanica* (Family: Plumbaginaceae): A natural gift to human being. *Chronicles of Young Scientists* 2012; **16**: 46-49.

Kokate, CK. *Pharmacognosy*. 16th Edn, Nirali Prakasham, Mumbai, India. 2001

Koleckar V, Kubikova K, Rehakova Z, Kuca K, Jun D, Jahodar L, Opletal L. Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini Reviews in Medicinal Chemistry* 2008; **8**: 436-447.

Kress JW, DL. Erickson (eds.), DNA Barcodes: Methods and Protocols, *Methods in Molecular Biology* 2007; **858:** 6-11.

Kress WJ, Erickson DL, Swenson NG, Thompson J, Uriarte M. Advances in the use of DNA barcodes to build a community phylogeny for tropical trees in a Puerto Rican forest dynamics plot. *PLoS ONE* 2010; **5**: 15409.

Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: the coding *rbc*L gene complements the non-coding trnH-psbA spacer region. *PLoSONE* 2007; **2**: 508.

Kress WJ, Erickson DL. DNA barcodes: genes, genomics, and bioinformatics. *Proceedings of the National Academy of Sciences, USA* 2008; **105**: 2761–2762.

Krishnaiah D, Devi T, Bono A, Sarbtly R. Studies on phytochemical constituents of six Malaysian Medicinal plants. *Journal of Medicinal Plant Research* 2009; **3**(2): 67-72.

Krishnaswamy M, Purushothaman KK. Plumbagin. A study of its anticancer, antibacterial and antifungal properties. *Indian Journal of Experimental Biology* 1980; **18**: 876–7.

Kshipra Dhabhai, Amla Batra. In vivo and in vitro protein profiling in Acacia nilotica (L.): A nitrogen fixing tree. *African Journal of Microbiology Research* 2011; **5**(18): 2793-2796.

Kubmarawa D, Ajoku GA, Okorie DA. Preliminary phytochemical and antimicrobial screening of 50 medicinal plants from Nigeria. *African .Journal of Biotechnology* 2008; **6**: 1690–1696.

Kubmarawa D, Ajoku GA, Okorie DA. Preliminary phytochemical and antimicrobial screening of 50 medicinal plants from Nigeria. *African .Journal of Biotechnology* 2007; **6**: 1690-1696.

Kubmarawa D, Khan ME, Punah AM, Hassan M. Phytochemical screening and antimicrobial efficiency of extracts from *Khaya senegalensis* against human pathogenic bacteria. *African Journal of Biotechnology* 2008; **7**(24): 4563-4566.

Kumar JK, Prasad AG. Identification and Comparison of Biomolecules in Medicinal Plants of *Tephrosia tinctoria* and *Atylosia albicans* By Using FTIR. *Romanian Journal of Biophysics* 2010; **21**(1): 63–71.

Kumar R, Kumar S, Patra A, Jayalakshmi S. Hepatoprotective activity of aerial parts of *Plumbago zeylanica* linn against carbon tetrachloride-induced hepatotoxicity in rats. *International Journal of Pharmaceutical Sciences* 2009; **1:** 171-175.

Kumaraswamy MV, Kavitha HU, Satish S. Antibacterial Evaluation and Phytochemical Analysis of *Betula utilis* D. Don Against Some Human Pathogenic Bacteria. *Journal of Agricultural Sciences* 2008; **4**(5): 661-664.

Kunin WE, Lawton JH. Does biodiversity matter? Evaluating the case for conserving species. In K. J. Gaston (ed.), Biodiversity: A Biology of Numbers and Difference, Oxford: Blackwell Science, 1996; 283-308.

Kwon HK, Ahn CH, Choi YE. Molecular authentication of *Panax notoginseng* by specific AFLP-derived SCAR marker. Journal of Medicinal Plant. *Research* 2009; **3**:957-966.

Lal R, Sankaranarayanan A, Mathur VS. Anti fertility and uterine activity of *Plumbago rosea* in rats. *Indian J Med res.* 1993; **78**: 287-90.

Lambert JP, Ethier M, Smith JC, Figeys D. Proteomics: from gel based to gel free. *Journal of Analytical Chemistry* 2005; **77**: 3771-3787.

Langergraber G, Fleischmann N, Van der Linden F, Wester E, Weingartner A, and Hofstaedter F.In-situ measurement of aromatic contaminants in bore holes by UV/Vis-spectrometry; In: Breh W. *et al*. (eds): *Field Screening Europe* Kluwer Academic Publishers, Dordrecht, The Netherlands, 2002: 317-320.

Lee HS, Carter RD, Barros SM, Dezman DJ, Castel WSJ. Chemical characterization by liquid chromatography of Moro blood orange juices. *Food Compos Anal*. 1990; **3**: 9-19.

Lee Y, Snell RL, Dickman RL. Dense Molecular Clouds in the Galactic Center Region,Statistical Properties of the Galactic Center Molecular Clouds. *The Astrophysical Journal* 1990; 355-536.

Lei Y, Gao H, Tsering T, Shi S, Zhong Y. Determination of genetic variation in *Rhodiola crenulata* from the Hengduan Mountain Region, China using inter-simple sequence repeats. *Genet. Mol. Biol. Sao Paulo* 2006; **29**: 2.

Lenora RDK. Dharmadasa RM, Abeysinghe DC, Arawwawala LDAM, Investigation of Plumbagin Content in *Plumbago indica* Linn. Grown under Different Growing Systems. *Pharmacologia*, 2012; **3**: 57-60.

Li H, Li H, Hao N, Xu Y, Piao Z. Study on HPLC fingerprint characteristics and chemotaxonomy of *Pulsatilla* medicinal plants. *Zhongguo Zhong Yao Za Zhi* 2011; **36**(11):1478-82.

Li HB, Jiang Y, Chen F. Separation methods used for *Scutellaria baicalensis* active components. *Journal of Chromatography* 2004; **812**: 277-290.

Li HQ, Chen JY, Wang S, Xiong SZ. Evaluation of six candidate DNA barcoding loci in *Ficus* (Moraceae) of China, *Molecular Ecology Resources* 2012; **12**: 783–790.

Li M, Zhang D, Hou Y, Jiao L, Zheng X, Wang WH. Isolation and culture of embryonic stem cells from porcine blastocysts. *Mol Reprod Dev* 2003; **65**(4): 429-34.

Liebler DC, Burr JA, Philips L, Ham AJL. Gas chromatography-mass spectrometry analysis of vitamin E and its oxidation products. *Analytical Biochemistry* 1996; **236**: 27-34.

Likhitwitayawuid K, Kaewamatawong R, Ruangrungsi N, Krungkrai J. Antimalarial naphthoquinones from Nepenthes thorelii. *Planta Medica* 1998; **64**: 237–41.

Lin Chun Shi, Jin Zhang, Jian Ping Han, Jing Yuan Song, Hui Yao, Ying Jie Zhu Jia Chun Li, Zhen Zhong Wang, Wei Xiao, Yu Lin Lin. Universality of *matK* primers for barcoding gymnosperms *Journal of Systematics and Evolution* 2011; **49**(3): 169–175.

Lin LC, Chou CJ. Metroterpenes and C- glucosyl flavonoids from the aerial parts of *Plumbago zeylanica*. *Journal of Chinese Pharmacology* 2003; **55**: 77–81.

Lin LC, Yang LL, Chou CJ. Cytotoxic naphthoquinone and plumbagic acid glycosides from *Plumbago zeylanica*. *Phytochemistry* 2003; **62**(4): 619-22.

Lindsey K, Pullen ML, Topping JF. Importance of plant sterols in pattern formation and hormone signalling. *Trends in Plant Science* 2003; **8**: 521-525.

Liu H, Sun S, Lv G, Chan KKC. Study on Angelica and its different extracts by Fourier transform infrared spectroscopy and two-dimensional correlation IR spectroscopy. *Spectrochimica Acta* 2006; **64**: 321–326.

Liu JIE, Mo¨ ller M, Gao L-M, Zhang D-Q, Li D-Z. DNA barcoding for the discrimination of Eurasian yews (Taxus L., Taxaceae) and the discovery of cryptic species. *Molecular Ecology Resources* 2010; **11**: 89–100.

Liu Y, Deng F, Liu C, Meng QY, Gao ZW. Determination of plumbagin in different parts of *Plumbago zeylanica* by RP-HPLC. *Zhongguo Zhong Yao Za Zhi* 2006; **31**: 1684-6.

Lu HF, Cheng CG, Tang X, Hu ZH. Spectrum of *Hypericum* and *Triadenum* with reference to their identification. *Acta Botanica Sinica* 2004; **46**: 401-406.

Luo K, Chen S, Chen K, Song J, Yao H. Assessment of candidate plant DNA barcodes using the Rutaceae family. *Science China Life Sciences* 2010; **53**: 701– 708.

M Ángeles Castillejo, Ana M Maldonado, Samuel Ogueta and Jesús V Jorrín Proteomic Analysis of Responses to Drought Stress in Sunflower (*Helianthus annuus*) Leaves by 2DE Gel Electrophoresis and Mass Spectrometry. *The Open Proteomics Journal* 2008; **1**: 59-71.

Mace ES, Gebhardt CG, RN Lester. AFLP analysis of genetic relationship in the tribe Datureae (Solanaceae). *Theor. Appl. Genet*. 1999; **99**: 634 - 641.

Mahadani P, Sharma GD, Ghosh SK. Identification of ethanomedical plants (Rauvolfioideae; Apocyanaceae) through DNA barcoding from north east India. *Pharmacognostic magazine* 2013; **9**: 255-63.

Mallavdhani UV, Sahu G, Muralidhar J. Screening of *Plumbago* Species for the Bioactive Marker Plumbagin. *Pharm Biol.* 2002; **40**:508-11.

Mallikharjuna PB, Rajanna LL, Seetharam YN, Sharanbasappa GK. Phytochemical studies of *Strychonos potatorum* L. f-A medicinal plant, *E. J. Cehem* 2007; **4**: 510-518.

Maniafu BM, Wilber L, Ndiege IO, Wanjala CCT, Akenga TA. Larvicidal activity of extracts from three *Plumbago* spp against *Anopheles gambiae*. *Mem Inst Oswaldo Cruz.* 2009; **104**: 813-7.

Maoela MS, Arotiba OA, Baker PGL, Mabusela WT, Jahed N, Songa EA, Iwuoha EI. Electroanalytical determination of catechin flavonoid in ethyl acetate extracts of Medicinal Plants. *International. Journal of Electrochemical Sciences* 2009; **4**: 1497-1510.

Marc L Jeanson, Jean Noel Labat, Damon P Little. DNA barcoding: a new tool for palm taxonomists? Part of a special issue on Palm Biology. *Annals of Botany* 2012: 1-7.

Marco Aurélio Caldas de Pinho Pessoa Filho, Carlos Bloch Junior, Danilo Fernandes da Silva Filho, Alexsandro Sobreira Galdino, Rodrigo Maranguape Silva da Cunha, Maria Aparecida Oliveira Alves, and Thalles Barbosa Grangeiro. Seed protein variation among pepper (*Capsicum* sp.) genotypes revealed by MALDI TOF analysis. *Protein and Peptide Letters* 2004; **11**(1): 1-6.

Mariangela Arca., Damien Daniel Hinsinger, Corinne Cruaud, Annie Tillier, Jean Bousquet, Nathalie Frascaria Lacoste. Deciduous Trees and the Application of Universal DNA Barcodes: A Case Study on the *Circumpolar Fraxinus PLoS ONE* 2012; **7(**3): e34089.

Marion Meyer JJ, Van der Kooy F, Joubert A. Identification of plumbagin epoxide as a germination inhibitory compound through a rapid bioassay on TLC. *South African Journal of Botany* 2007; **73**(4): 654–656.

Maruthupandian A, Mohan VR. GC-MS analysis of some bioactive constituents of *Pterocarpus marsupium* Roxb. *International Journal of ChemTech Research* 2011; **3**(3): 1652-1657.

Maryam Z, Farrukh Aqil, Iqbal Ahmad. The *in vitro* antioxidant activity and total phenolic content of four Indian medicinal plants. *International journal of pharmacy and pharmaceutical sciences* 2009; **1**(1): 88-95.

Masood MS, Asghar M, Anwar R Genetic diversity in wheat landraces from Pakistan based on Polymorphism for high molecular weight Glutenin subunits (HMW-GS). *Pakistan Journal of Botany* 2004; **36** (4): 835-843.

Masoumi SM, Kahrizi D, Rostami-Ahmadvandi H, Soorni J, Kiani S, Mostafaie A, Yari K. Genetic diversity study of some medicinal plant accessions belong to Apiaceae family based on seed storage proteins patterns. *Molecular Biology Reports* 2012; **9**(12): 10361-5.

Mathekaga AD, Meyer JJM. Antibacterial activity of South African *Helichrysum* species. *South African Journal of Botany* 1998; **64**: 293-295.

Mehmood Z, Ahmad I, Mohammad F, Ahmad S. Indian medicinal plants: A potential source of anti candidal drugs. *Pharmaceutical Biology* 1999; **37:** 237–242.

Menkovic, N, Savikin Fodulovi CK, Savin K, 2000. Chemical composition and seasonal variations in the amount of secondary compounds in *Gentiana lutea* leaves and flowers. *Planta Medica* **66:** 178– 180.

Mercandante AZ, Pfander II. Carotenoids from annatto: A review. *Recent Research Developments in Agriculture and Food Chemistry.* 1998; **2**: 79 – 91.

Merlin NJ, Parthasarathy V, Manavalan R, Kumaravel S. Chemical Investigation of Aerial Parts of *Gmelina asiatica* Linn by GC-MS. *Pharmacognosy Research* 2009; **1**(3): 152-156.

Minal Wani, Snehal Pande and Nitin. Determination of Plumbagin by normal phase high performance liquid chromatography *Inernational Journal of Applied Biotechnology* 2010; **2**(1): 11-14.

Mishra BB, Singh DD, Kishore N, Tiwari VK, Tripathi V. Antifungal constituents isolated from the seeds of *Aegle marmelos*. *Phytochemistry* 2010; **71**(2): 230-4.

Mistry BD. A Handbook of Spectroscopic Data Chemistry (UV, IR, PMR, ^{13}CNMR and Mass Spectroscopy). Oxford Book Company. Jaipur, India. 2009; 26-63.

Mohamed Enan, Mohammad Al-Deeb, Nael Fawzy, Khaled Amiri. DNA Barcoding of *Ricinus communis* from Different Geographical Origin by Using Chloroplast *matK* and Internal Transcribed Spacers. *American Journal of Plant Sciences* 2012; **3**: 1304-1310.

Mohammad A, Bhawani SA, Sharma S. Analysis of herbal products by thin-layer chromatography: a review. *International Journal of Pharma Bio* science *and Technology* 2010; **1**: 1-50.

Mohammed Ibrahim, Joy Baura, Qamrul Ahasan, Torequl Islam, Zilly Homa, Mohi Uddin Chowdhury, Aslam Hossain, Mohammad A. Rashid. Preliminary Phytochemical and

Pharmacological Investigations of *Alpinia conchigera* Griff. and *Plumbago indica* L. *Bangladesh Pharmaceutical Journal* 2012; **15**(2): 153-157.

Mohanasundari CD, Natarajan K. Srinivasan SA, Umamaheswari, Ramachandran A. Antibacterial properties of *Passiflora foetida* L. –a common exotic medicinal plant. *African Jornal of Biotechnology* 2007; **6**(23): 2650-2653.

Monika Olszewska, Maria Wolbis. Further flavanoids from the flowers of *Prunus spinosa* L. *Acta pilcriae phamacalica- Drug research* 2002; **59**(2): 133-137.

Moon BC, Choo BK, Cheon MS, Yoon T, Ji Y, Kim BB, Lee AY, Kim HK. Rapid molecular authentication of three medicinal plant species, *Cynanchum wilfordii*, *Cynanchum auriculatum*, and *Polygonum multiflorum* (*Fallopia multiflorum*), by the development of RAPD-derived SCAR markers and multiplex-PCR. *Plant Biotechnology Reports* 2010; **4**: 1-7.

Mordue AJ, Blackwell. A Azadirachtin: An update; *Journal of Insect Physiology*. 1993; **39**: 903–924.

Morrissey JP, Osbourn AE. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiology and Molecular Biology Reviews* 1999; **63**: 708-724.

Mortz E, Krogh TN, Vorum H, Gorg A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics* 2001; **1**(11): 1359-63.

Muhammad Ashfaq, Muhammad Asif, Zahid Iqbal Anjum and Yusuf Zafar. Evaluating the capacity of plant DNA barcodes to discriminate species of cotton (Gossypium: Malvaceae) *Molecular Ecology Resources* 2013; doi: 10.1111/1755-0998: 12089.

Muhammad Faisal Anwar Malik. Evaluation of genetic diversity in soybean (*glycine max* (l.)) Genotypes based on agronomic and biochemical traits. A Dissertation submitted to the Department of Biochemistry Doctor of Philosophy (PhD) in Biological Sciences; 2011.

Muhammad Ishtiaq PG, Xiao Q, He H, Cheng YY. Biosystematics and Plant Proteomics: Role of Proteomics in Plant Phylogenetics Analysis. *Pakistan Journal of Biological Sciences* 2009 **10**(20): 3487-3496.

Mukherjee M, Datta AK. Evaluation of genetic diversity in five species of *Ocimum* by SDS-PAGE. *Indian Journal* of *Genetics and Plant Breedin*g 2008; **68**: 212.

Mukherjee PK. Quality Control of Herbal Drugs: An approach to evaluation of botanicals. 1st ed. New Delhi: Business Horizons, 2002; 114-124.

Mukhlesur RM, Hirata Y, Alam SE. Genetic variation with in *Brassica rapa* cultivars using SDS-PAGE for seed protein and isozyme analysis. *Journal of Biological Sciences* 2004; **4**(2): 239.

Murphy RW, Sities JW, Buth DG, Haufler CH. Protein 1: Isozyme electrophoresis. In: Hillis DH, Moritz C {eds.,} 1990; Molecular systematics, Sinauer Assoc., Sunderland, MA. 45-126.

Murray HG, Thompson WF. Rapid isolation of high molecular weight DNA. *Nucleic Acids Research* 1980; **8**: 4321-4325.

Murray MG, Thomson WF. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research* 1980; 8: 4321-4325.

Muruganantham S, Anbalagan G, Ramamurthy N. FT-IR and SEM-EDS comparative analysis of medicinal plants, *Eclipta alba* Hassk and *Eclipta prostrata* Linn. *Romanian Journal of Physics* 2009; 19(4): 285–294.

Nambiar VPK, Jayanthi A, Sabu TK. Pharmacognostical Studies on Brahmi (*Bacopa monnieri* (Linn.) Pennel) *Aryavaidyan* 2000; **13**(4): 203-221.

Nandhini Siva Kumar, David Paul Raj RS. Larvicidal antimicrobial activities of *Phyla nodiflora* and *Plumbago zeylanica* root extracts. B. Tech thesis. Dept of Biotechnology, School of Engineering, SRM University, Kattankulathur. 2008

Nathar VN, Dadge JP. Genomic DNA Isolation from *Plumbago* L. species Growing In India. *International Journal of Scientific research* 2013; **2**(7); 2277-2279.

Nayana S. Kapadia, Shalini A. Isarani, Mamta B. Shah. A simple method for isolation of plumbagin from roots of *Plumbago rosea*, *Pharmaceutical Biology* 2005; **43**(6): 551-553.

Nazeem S, Azmi AS, Harif S, Mohammad RM, Hadi SM, Kumar KS. Plumbagin induces cell death through a copper redox cycle mechanism in human cancer cells. *Mutagenesis* 2009; **24**(5): 413-418.

Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology* 2008; **7**(12): 1797-1806.

Negi RS, Sharma MK, Sharma KC, Kshetrapal S, Kothari SL, PC Trivedi Genetic Diversity and Variations in the Endangered Tree (*Tecomella undulata*) in Rajasthan. *Indian Journal of Fundamental and Applied Life* 2011 **1**(1): 50-58.

Nehete Jeetendra Y, Deshmukh VN, Shewale VV. Quantitation of Oleanolic Acid in *Achyranthes aspera* L. Roots and Leaves Extracts by High-Performance Thin-Layer Chromatography. *International J. Pharma. Res. and Develop* 2009; **5**: 234-237.

Nei M, Kumar S. Molecular Evolution and Phylogenetics. Oxford University Press, New York. 2000

Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci* USA. 1979; **76**: 5269-5273.

Neilyn P Ona, Abella C Dela Vina, Dolores A Ramirez. Genetic diversity in seed proteins among five *Solanum* Species from the Philippines. *The Philippine Journal of Crop Science* 1995; **20**(2): 122 – 128.

Neuhof T, Dieckmann R, Druzhinina IS, Kubicek CP, von Döhren H. Intact-cell MALDI-TOF mass spectrometry analysis of peptaibol formation by the genus Trichoderma/Hypocrea:

can molecular phylogeny of species predict peptaibol structures. *Microbiology* 2007; **153**:3417–3437.

Newmaster SG, Fazekas AJ, Ragupathy S. DNA barcoding in the land plants: evaluation of *rbc*L in a multigene tiered approach. *Canadian Journal of Botany* 2006; **84**: 335–341.

Newmaster SG, Subramanyam Ragupathy. Testing plant barcoding in a sister species complex of pantropical Acacia (Mimosoideae, Fabaceae). *Molecular Ecology Resources* 2009; **9**(1): 172–180.

Nguyen AT, Malonne H, Duez P, Vanhaelen-Fastre R, Vanhaelen M, Fontaine J. Cytotoxic constituents from *Plumbago zeylanica. Fitoterapia* 2004; **75**(5):500-4.

Niemann HB. The abundances of constituents of Titan'satmosphere from the GCMS instrument on the Huygens probe. *Nature* 2005; **438:** 779–784.

Nile SH, Khobragade CN. Antioxidant activity and flavonoid derivatives of *Plumbago zeylanica*. *Journal of Natural Products* 2010; **3**: 130-133.

Nisha Sharma, Vijaylata Pathania, Bikram Singh, Gupta RC. Intraspecific variability of main phytochemical compounds in *Picrorhiza kurroa* Royle ex Benth. from North Indian higher altitude Himalayas using reversed-phase high-performance liquid chromatography. *Journal of Medicinal Plants Research* 2012; **6**(16): 3181-3187.

Olagunju JA, Jobi A.A, Oyedapo OO. An investigation into the biochemical basis of the observed hyper glycaemia in rats treated with ethanol root. An update. *International Journal of Pharmacology* 1999; **7**: 316-324.

Olufunmilayo E, Adejumo, Adelodun L, Kolapo, Oladimeji P. Roleolaand Lateef, Kasim S. In vitro antisickling activities and phytochemical evaluation of *Plumbago zeylanica* and *Uvaria chamae*. *African Journal of Biotechnology* 2010; **9**(53): 9032-9036.

Omonhinmin CA, Ogunbodede OO. Genetic diversity, taxonomy and legumins implications of seed storage protein profiling in Fabaceae. *African Journal of Biotechnology* 2013; **12**(17): 2157-2163.

Onus AN, Pickergill B. A study of selected isozymes in *Capsicum baccatum, Capsicum eximium, Capsicum cardenasii,* and two inter specific F_1 hybrids in *Capsicum* species. *Turkish journal of botany* 2000; **24**: 311 – 318.

Oprean R, Tamas M, *Roman L*. Comparison of GC-MS and TLC techniques for asarone isomers determination *Journal of Pharmaceutical* and *Biomedical Analysis* 1998; **18**(1-2): 227-34.

Osbourn AE. Preformed antimicrobial compounds and plant defense againt fungal attack. *Plant Cell* 1996; **8**: 1821-1831.

Oyedapo OO. Studies on the bioactivity of the extract of *Plumbago zeylanica*. *Phytotherapy Research.* 1996; **13**: 346–348.

Padmalatha K, Prasad MNV. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India. *African journal of Biotechnolgy*. 2006; **5**: 230-234.

Paiva DSR, Fontoura LD, Figueiredo MR, Mazzei JL, Kaplan MAC. Chromatographic profile of two plumbaginaceae species: *Plumbago scandens* L. and *Plumbago auriculata* LAM. *Quimica Nova* 2003; **25**: 717–721.

Paiva SR, Marques SS, Figueiredo MR, Kaplan MAC: Plumbaginale: A pharmacology approach. *Floresta e Ambiente*. 2003; **10**: 98-105.

Pant Jagdish, Rajasekaran. HPTLC Fingerprinting Profile of Marker Compound (Berberine) in roots of *Berberis aristata* DC. *Pharmacognosy* 2011; **3**(19): 41-44.

Paramasivam M Poi R, Banerjee, H, Bandyopadhyay A. High-performance thin layer chromatographic method for quantitative determination of curcuminoids in *Curcuma longa* germplasm. *Food Chemistry* 2009; **113**: 640–644.

Parekh J, Chanda S. *In vitro* antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* Kurz. Flower (Lythaceae). *Brazilian Journal of Microbiol*ogy 2007; **38**: 204-207.

Parekh J, Chanda V. In vitro Antimicrobial activity and Phytochemical analysis of some Indian medicinal plants. *Turkish Journal of Biology* 2006; **31**: 53-58.

Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA. What molecules can tell us about populations: choosing and using molecular markers. *Ecology* 1998; **79**: 361-382.

Pascual ME, Carretero ME, Slowing. Villar KVA. Simplified Screening by TLC of plant drugs. *Plant Drugs and Pharmaceutical Biology* 2002; **40**(2): 139-143.

Patel RK, Patel MM, Patel MP, Kanzaria NR, Vaghela KR, Patel NJ. Hepatoprotective activity of *Moringa oleifera* Lam. Fruit on isolated rat hepatocytes. *Pharmacognostic magazine* 2008; **4**: 118-23.

Paterson AH, Tanksley SD, Sorreis ME. DNA markers in plant improvement. *Advances in Agronomy*. 1991; **146**: 39–90.

Patrick OE. Herbal Medicines: Challenges (Editorial). *Tropical Journal of Pharmaceutical Research* 2002; **1**(2):53-54.

Pawar BM, Wavhal VP, Pawar ND, Agarwal MR, Shinde PB, Kamble HV. Anthelmintic activity of *Gloriosa superba* Linn (Liliaceae). *International Journal of Pharmaceutical Technology Research* 2010; **2**: 1483-1487.

Pawar RK, Sharma Shivani, Singh KC, Sharma Rajeev KV. Physico-chemical standardization and development HPTLC method for the determination of Andrographonin in Kalmgh Navyas Loha. An Ayurvedic formulation. *International Journal of Research in Ayurveda and Pharmacy* 2010; **2**(1): 295-301.

Pereira VP, Weinberg HS, Singer PC. Disinfection by-product variability in a chloraminated distribution system. *Journal of American Water Works Association* 2004; **96**(11): 91 – 102.

Perumalsamy R, Ignacimuthu S. Antibacterial activity of some folklore medicinal plants used by tribals in Western Ghats of India. *Journal of Ethnopharmacology*; 2000; **69**: 63-7.

Pervaiz ZH, Rabbani MA, Shinwari ZK, Masood MS, Malik SA. Assessment of genetic variability in rice (*Oryza sativa* L.) germplasm from Pakistan using RAPD markers. *Pak. J. Bot.,* 2010; **42**(5): 3369-3376.

Petcharut Chuntaratin, Production of plumbagin by hairy root, callus and cell suspension cultures of *Plumbago indica* L. Ph. D. Thesis, Graduate school, Kasetsart University. 2006.

Peter A. Ratmond, James E Bauer. DOC cycling in a temperate estuary: A mass balance approach using natural 14C and 13C isotopes. *Limnology Oceanography* 2001; **46**(3):655-667.

Pflieger D, Bigeard J, Hirt H. Isolation and characterization of plant proteincomplexes by mass spectrometry. *Proteomics* 2011; **11**: 1824-1833.

Pharkphoom Panichayupakaranant, Supinya Tewtrakul. Plumbagin production by root cultures of *Plumbago rosea. E-Journal of Biotechnology* 2002; **5**(3): 11-12.

Pourcel L, Routaboul JM, Cheynier V. Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends in Plant Science* 2007; **12**(1): 29-36.

Pradeep Kumara S, Ranjith Kumara BD. *In vitro* and *in vivo* identification of variation in protein expression in *Artemisia vulgaria* L., *Advances in Biological Research* 2009; **3**(5-6): 237-241.

Praveen C Verma, Digvijay Singh, Laiq ur Rahman, Madan Mohan Gupta, Suchitra Banerjee. In vitro-studies in *Plumbago zeylanica*: rapid micro propagation and establishment of higher plumbagin yielding hairy root cultures. *Journal of Plant Physiology* 2002; **159**: 547–552.

Praveenkumar P, Kumaravel S, Lalitha C. Screening of antioxidant activity, total phenolics and GC-MS study of *Vitex negundo*. *African Journal of Biochemistry Research* 2010; **4**(7): 191-195.

Przybylska J, Blixt S, Hurich J. Competitive study of seed proteins in genus *Pisum* VI-Electrophoretic analysis of variation in the legumin fraction composition. *Genet. Polonica* 1986; **24**: 21-39.

Pushpangada P. Ethnobiology of India. A Status Report, GOI New Delhi, 1995.

Qian Wang, Qiu Shi Yu, Jian Quan Liu**.** Are nuclear loci ideal for barcoding plants? A case study of genetic delimitation of two sister species using multiple loci and multiple intra specific individuals. *Journal of Systematics and Evolution* 2011; **49**(3): 182–188.

Rabbani MA, Masood MS, Shinwari ZK, Shinozaki KY. Genetic analysis of basmati and non basmati rice Pakistani rice (*Oryza sativa L.*) cultivars using microsatellite markers. *Pak. J. Bot.* 2010; **42**(4): 2551-2564.

Rafi MK. Studies on genetic diversity of W*ithania somnifera* (L.) Dunal of Tamil Nadu. Thesis Submitted to Bharathidasan University for the Award of the degree of Doctor of Philosophy in Botany, P.G. and Research Department of Botany, Jamal Mohamed College, Trichy.

Raghu AV, Unnikrishnan KP, Hashim KM, Indira Balachandran, Mohanan KV. Studies on Morphological and Phytochemical Variability of Different Populations of *Tribulus terrestris*. *International Journal of Plant Breeding and Genetics* 2007; **1**: 95-100.

Rahman MS, Anwar MN. Antimicrobial activity of crude extract obtained from the root of *Plumbago zeylanica*. *Bangladesh Journal of Microbiology* 2007; **24**: 73–75.

Rahul Deo Yadav, Shashi Alok SK, Jain Amita Verma, Mahor A, Bharti JP, Jaiswal M Herbal plants used in the treatment of urolithiasis: A review. *IJPSR* 2011; **2**(6): 1412-1420.

Raj PS, Bellampalli R, Dobriyal RM, Agarwal A, Satyamoorthy K, Anantha Narayana DB. DNA barcoding of authentic and substitute samples of herb of the family Asparagaceae and Asclepiadaceae based on the ITS2 region. *Journal of integrated Ayurvedic Medicine* 2012; **3**: 136-40.

Raj, RS. Nisha. Preliminary phytochemical and in vitro antioxidant properties of *Brunfelsia americana* L. *Journal of Pharmacy Research* 2010; **3**(11): 2712-2713.

Rajendra Gyawali, Kyong Su Kim. Bioactive volatile compounds of three medicinal plants from Nepal Kathmandu University. *Journal of science, Engineering and technology* 2012; **8**(1): 51-62.

Rajendra Prasad Gujjeti and Estari Mamidala. Phytochemical Analysis and TLC Profile of *Madhuca indica* Inner Bark Plant Extract. *International Journal of Scientific & Engineering Research* 2013; **4**(10): 1507

Rajkumar T, Sinha BN. Chromatographic finger print analysis of budmunchiamines in *Albizia amara* by HPTLC technique. *International Journal of Pharmaceutical Sciences and Research* 2010; **1**(3): 313-316.

Rakesh SU, Patil PR, Salunkhe VR, Dhabale PN, Burade KB. HPTLC method for quantitative determination of quercetin in hydroalcoholic extract of dried flower of *Nymphaea stellata* willd. *International Journal of Chem. Tech. Research* 2009; **1**(4): 931-936.

Ram SG, Parthiban KT, Kumar RS. Genetic diversity among *Jatropha* species as revealed by RAPD markers. *Genetical Resource Crop Evolution* 2008; **55:** 803-809.

Ramachandran A, Viswan MB. Antibacterial potential and FTIR analysis of *Gymnema kollimalayanum: A* new record plant, India. *International Journal of Pharmaceutical Sciences Review and Research* 2011; **7**(2):167.

Ranjith A, Kumar S, Arumughan C. Simultaneous estimation of phenolic acids in sea buckthorn using RP-HPLC with DAD. *Journal of Pharmaceutical and Biomedical Analysis* 2008; **47**: 31–38.

Ranjith K, Basaveswara Rao MV, Murthy TEGK. Development and validation of assay method for meloxicam tablets by RP-HPLC. *Indian Journal of Research in Pharmacy and Biotechnology* 2013; **1**(5):679-681.

Ratnasingham S, Hebert PDN. BOLD: The Barcode of Life Data System. *Molecular Ecology Notes* 2007; **7**: 355–364.

Ravikumar VR, Sudha T. Phytochemical and Antimicrobial Studies on *Plumbago zeylanica* (L.) Plumbaginaceae *Journal of Pharmaceutical and Biomedical Analysis* 2011; **1**:185-8.

Rebuffo CA, Schmitt J, Wenning M, von Stetten F, Scherer S. Reliable and rapid identification of *Listeria monocytogenes* and *Listeria* species by artificial neural network-based Fourier transform infrared spectroscopy. *Applied and Environmental Microbiology* 2006; **72**: 994-1000.

Recio MC. A review of some antimicrobial compounds isolated from medicinal plants reported in the literature *Phytotherapy Research* 1989; **3**: 117–125.

Reda Helmy Sammour. Proteins of linseed (*Linum usitatissimum* L.) extraction and characterization by electrophoresis. *Botanical Bulletin of Academia Sinica* 1999; **40**: 121-126.

Reddy M, Chaturvedi AA. Pharmacognostical studies of *Hymenodictyon orixence* (Roxb.) Mabb. leaf. *International Journal of Ayurveda Research* 2010; **1**: 103-5.

Reynolds T. The evolution of chemosystematics. *Phytochemistry* 2007; **68**(22-24):2887-95.

Robertson DG. Metabonomics in toxicology: A review. *Journal of Toxicological Sciences* 2005; **85**: 809-822.

Rodrigues SV, Viana LM, Baumann W. UV/VIS spectra and solubility of some naphthoquinones and the extraction behaviour of plumbagin from *Plumbago scandens* roots in supercritical CO2. *Analytical and Bioanalytical Chemistry* 2006; **385**: 895-900.

Rzhetsky A, Nei M. A simple method for estimating and testing minimum evolution trees. *Molecular Biology and Evolution* 1992; **9**: 945-967.

Saarela JM, Sokoloff PC, Gillespie LJ, Consaul LL, Bull RD. DNA Barcoding the Canadian Arctic Flora: Core Plastid Barcodes (*rbcL* + *matK*) for 490 Vascular Plant Species. *PLoS ONE* 2013; **8**(10): e77982.

Sabu KK, Padmesh P, Seeni S. Estimation of active principle content and isozymes of *Andrographis paniculata* Nees (Kalmegh): An important medicinal plant of India. *Journal of Medicinal and Aromatic Plant Sciences* 2001; **23**: 637-647.

Sachin U Rakesh, Patil PR, Salunkhe VR, Dhabale PN, Burade KB. HPTLC method for quantitative determination of quercetin in hydroalcoholic extract of dried flower of *Nymphaea stellata* willd. IJCRGG *International Journal of Chem Tech Research* 2009; **1**(4): 931-936.

Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 1987; **4**: 406-425.

Salau AO, Odeleye OM, Antimicrobial activity of *Mucuna pruriens* on selected Bacteria. *African Journal of Biotechnology* 2007; **6**(18): 2091-2092.

Salim Khan A, Rajeev K Singlab, Malik Zainul Abdinc. Assessment of Phytochemical diversity in *Phyllanthus amarus* using HPTLC Fingerprints. *Indo-Global Journal of Pharmaceutical Sciences* 2011; **1**(1): 1-12.

Salim Khan, Khanda Jabeen Mirza, Malik Zainul Abdin. Development of RAPD markers for authentication of medicinal plant *Cuscuta reflexa. Eur Asian Journal of BioSciences* 2010; **4**: 1-7.

Sameera O Bafeel, Ibrahim A Arif, Mohammad A Bakir, Haseeb A Khan, Ahmad H Al Farhan, Ali A Al Homaidan, Anis Ahamed, Jacob Thomas. Comparative evaluation of PCR success with universal primers of maturase K (*matK*) and ribulose-1, 5 bisphosphate carboxylase oxygenase large subunit (*rbcL*) for barcoding of some arid plants. *Plant Omics Journal* 2011; **4**(4): 195-198.

Samuels GJ, Ismaiel A. *Trichoderma evansii* and *T. lieckfeldtiae*: two new *T. hamatum*-like species. *Mycologia* 2009; **101**: 142–156.

Sangeetha J, Vijayalakshmi K. Determination of Bioactive Components of Ethyl Acetate Fraction of *Punica granatum* Rind Extract. *International Journal of Pharmaceutical Sciences and Drug Research* 2011; **3(2):** 116-122

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the USA* 1977; **74**(12): 5463–5467.

Sangwan NS, Yadav U and Sangwan RS. Molecular analysis of genetic diversity in elite Indian cultivars of essential oil trade types of aromatic grasses (*Cymbopogon* species). *Plant Cell Report*. 2001; **20**: 437-444.

Saraswathy A, Joy Suganthan, Ariyanathan S, Hemakumar NS. Seasonal variation and storage studies in chemical constituents of *Plumbago zeylanica* Linn by HPTLC. *International Journal of Pharmacy and Technology* 2011; **3**(2): 2574-2582.

Sarkar J, Pal S, Bhattacharya S, Biswas M. Thin layer chromatographic profiling and evaluation of analgesic activity of *Psidium guajava* leaf extracts in mice. *J. Adv. Pharm. Edu. Res* 2011; **1**: 177-183.

Sasikumar, Meena A, Kavitha Srilakshmi, Sriram. HPTLC analysis of various market samples of a traditional drug source – Kodiveli (*Plumbago zeylanica* Linn). *International Journal of Pharmacy and Pharmaceutical Sciences* 2010; **2**(4): 1491.

Sass C, Little DP, Stevenson DW, Specht CD. DNA barcoding in the cycadales: testing the potential of proposed barcoding markers for species identification of cycads. *PLoSONE* 2007; **1154**.

Satheesh Kumar K, Seeni S. Production of plumbagin (5-hydroxy- 2- methyl- 1, 4-napthoquinone) in callus and cell suspension cultures of *Plumbago indica* Linn. *Indian Jounal of Biotechnology* 2002: 305 – 308.

Satyajit Kanungo, Gayatri nahak, 1santi Lata Sahoo and Rajani Kanta Sahu. Antioxidant activity and phytochemical evaluation of *plumbago zeylanica* linn. *In-vivo* and *in-vitro. International Journal of Pharmacy and Pharmaceutical Sciences* 2012; **4**(4). 522 – 526.

Savithiry S, Natarajan A, Hari B, Krishnan B, Sukla Lakshman, Wesley M, Garrett C. An efficient extraction method to enhance analysis of low abundant proteins from soybean seed. *Analytical Biochemistry* 2009; **394**: 259–268.

Savolainen V, Cowan RS, Vogler AP, Roderick GK, Lane R. Towards writing the encyclopaedia of life: an introduction to DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 2005; **360:** 1805–1811.

Saxena S, Jain DC, Gupta MM, Sharma RP. High performance thin layer chromatographic separation of hepatoprotective diterpenoids from *Andrographis paniculata. Phytochemical Analysis* 2000; **11**: 34– 36.

Schaller H. The role of sterols in plant growth and development. *Progess in Lipid Research* 2003; **42**:163-175.

Schippmann U, Leaman DJ, Cunninghan AB. Impact of cultivation and gathering of medicinal plants on Biodiversity: Global trends and issues. In (FAO). Biodiversity and ecosystem approach in agriculture, forestry and fisheries. Satellite event on the occasion of the Ninth regular session of the commission on genetic resources for food and agriculture. Rome. 2002.

Schneider H, Schuettpelz E. Identifying fern gametophytes using DNA sequences. *Molecular Ecology Notes* 2005; **6**: 989–991.

Schori M, Showalter AM. DNA barcoding as a means for identifying medicinal plants of Pakistan. *Pakistan. Jornal of Botany* 2011; **43**: 1-4.

Schultz H, Baranska M. Identification and quantification of valuable plant substances by IR and Raman spectroscopy. *Vibrational Spectroscopy* 2007; **43**: 13–25.

Schulze WX, Usadel B. Quantification in mass-spectrometry-based proteomics. *Annual Review* of *Plant Physiology* 2010**; 61**: 491-516.

Seberg O, Petersen G. How many loci does it take to DNA barcode a Crocus? *PLoSONE* 2009; **4.**

Seeni S, Sabu KK, Padmesh P. Variable-Invariably: An introduction to intra specific variations in medicinal plants. *Amruth* (FRLHT-India) 1998; **2**: 3-8.

Selma R De Paiva, Lucilene A Lima, Maria raquel figueiredo, Maria Auxiliadora C Kaplan. Plumbagin quantification in roots of *Plumbago scandens* L. obtained by different extraction techniques *Anais da Academia Brasileira de Ciências* 2004; **76**(3): 499-504.

Selma Ribeiro de Paiva, Maria Raquel Figueiredo, Tânia Verônica Aragão, Maria Auxiliadora Coelho Kaplan. Antimicrobial Activity in Vitro of Plumbagin isolated from *Plumbago* species. *Mem Inst Oswaldo Cruz, Rio de Janeiro* 2003; **98**(7): 959-961.

Selvamaleeswaran Ponnusamy, Wesely Ebenezer Gnanaraj, Johnson Marimuthu Antonisamy. Alkaloids Profile of *Clitoria ternatea* Linn by High Performance Thin Layer Chromatography (HPTLC). *Indo American Journal of Pharmaceutical Research* 2013; **3**(3): 2592-2599.

Selvaraj D, Shanmughanandhan D, Sarma RK, Joseph JC, Srinivasan RV, Ramalingam S. DNA barcode ITS effectively distinguishes the medicinal plant *Boerhavia diffusa* from its adulterants. *Genomics Proteomics Bioinformatics*. 2012; **6**: 364 -7.

Sen G, Kumar S, Ghosh S, Pal S. A novel polymeric flocculent based on polyacrylamide grafted carboxymethylstarch, *Carbohydrate Polymers.* 2009; **77**: 822-831.

Sen S, Sharma T. Asynchronous replication of constitutive heterochromatin on the two X chromosomes of female Mus dunni. *Chromosoma* (Berl) 1982; **1**:119-126.

Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases* 2009; **49**: 543–551.

Sethiya NK, Ashish Trivedi, Mayur B, Patel SH, Mishra. Comparative pharmacognostical investigation on four ethanobotanicals traditionally used as Shankhpushpi in India. *Journal of Advanced Pharmaceutical Technology Research* 2010; **1**(4): 388–395.

Shahin Aziz, Mahmudul SM, Hassan, Sudum Nandi, Shamsun Naher, Shudangshu Kumar Roy, Ram Proshad Sarkar, Hemayet Hossain. Comparative Studies on Physicochemical Properties and GC-MS Analysis of Essential Oil of the Two Varieties of Ginger (*Zingiber officinale*). *International Journal of Pharmaceutical and Phytopharmacological Research* 2012; **1**(6): 367-370.

Shalini A. Israni, Nayana S. Kapadia, Suman. K. Lahiri, Gunvat K. Yadav, Mamta B. Shah. An UV-Visible Spectrophotometric Method for the Estimation of Plumbagin. *International Journal of ChemTech Research* 2010; **2**(2): 856-859.

Shalini S, Sampathkumar P, Phytochemical screening and antimicrobial activity of plant extracts for disease management. *International Journal of Current Science* 2012; 209-218.

Shamsun Naher, Apu Ghosh, Shahin Aziz. Comparative Studies on Physicochemical Properties and GC-MS Analysis of Essential Oil of the Two Varieties of the Aniseed (*Pimpinella anisum* Linn.) in Bangladesh. *Inernational .Journal of Pharmaceutical Phytopharmacological Research* 2012; **2**(2): 92-95.

Shanley P, Luz L. The impacts of forest degradation on medicinal plant use and implication for health care in Eastern Amazonia. *Bio Science* 2003; **53** (6): 573 - 584.

Sharanabasappa GK, Santhosh MK, Shahila D, Seetharam YN, Sanjeevarao I. Phytochemical studies on *Bauhinia racemosa* Lam. *Bauchinia purpurea* Linn. and *Hardwickia binata* Roxb. *European Journal of Chemisry* 2007; **4**: 21-31.

Sharma Anubhuti, Sharma Pratibha. Genetic and Phytochemical analysis of Cluster bean (*Cyamopsis tetragonaloba* (L.) Taub) by RAPD and HPLC. *Journal of Recent Research Science* 2013; **2**(2): 1-9.

Sharma I, Gusain D, Dixit VP. Hypolipidaemic and antiatherosclerotic effects of plumbagin in rabbits. *Indian Journal of Physiology and Pharmacology.* 1991; **35**: 10–14.

Sheeja E, Joshi SB, Jain DC. Bioassay guided isolation of anti-inflammatory and antinoceptive compound from *Plumbago zeylanica* leaf. *Pharmaceutical Biology* 2010; **48**(4): 381-387.

Sher AK, Habib A, Shah MK, Ayub K, Sardar A, Muhammad S. Confirmation of sunflower F1 hybrids using SDS-PAGE analysis. *African Journal of Biotechnology* 2010; **9**(29): 4516-4520.

Sherif M Sharawy. Taxonomic Relationships of Some Taxa of Subfamily Asclepiadoideae (Apocynaceae) as Reflected by Morphological Variations and Polymorphism in Seed Protein and RAPD Electrophoretic Profile. *International Journal of Botany* 2013; **9**: 18-29.

Sherma J, Fried B. Thin Layer Chromatographic analysis of biological samples - A Review. *Journal of Liquid Chromatography and Related Technologies* 2005; **28**: 2297-2314.

Shevchenko A, Loboda A, Shevchenko A, Ens W, Standing KG. MALDI quadrupole time-of-flight mass spectrometry: A powerful tool for proteomic research. *Analytical Chemistry* 2000; **72**(4): 2132–2141.

Shilin Chen, Hui Yao, Jianping Han, Chang Liu, Jingyuan Song, Linchun Shi, Yingjie Zhu, Xinye Ma, Ting Gao, Xiaohui Pang, Kun Luo, Ying Li, Xiwen Li, Xiaocheng Jia, Yulin Lin, Christine Leon.Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS ONE* 2010; **5** (1): e8613.

Shin DH, Kamal AHM, Tatsuro Suzuki, Young-Ho Yun, Moon-Soon Lee, Keun-Yook Chung, Heon-Sang Jeong, Cheol-Ho Park, Jong-Soon Choi, Sun-Hee Woo. Reference proteome map of buckwheat (*Fagopyrum esculentum* and *Fagopyrum tataricum*) leaf and stem cultured under light or dark. *American Journal of Cultural Sociology* 2010; **4**(8):633-641.

Shivraj H. Nile, Khobragade CN. Antioxidant activity and flavonoid derivatives of *Plumbago zeylanica. Journal of Natural Products* 2010; **3**:130-133.

Siddig Ibrahim Abdelwahab, Ahmad Bustamam Abdul, Manal Mohamed Elhassan, Syam Mohan, Mohamed Yousif Ibrahim, Abdelbasit Adam Mariod, Nagi AA and Rasedee Abdullah. GC/MS determination of bioactive components and antibacterial properties of *Goniothalamus umbrosus* extracts. *African Journal of Biotechnology* 2009; **8** (14): 3336-3340.

Siddiqui MF, Naz N. Protein landmarks for diversity assessment in wheat genotypes. *African Journal of Biotechnology* 2009; **8**(9): 1855.

Siddiqui MJ. Guggul. An excellent herbal panacea. *Asian Journal of Pharmaceutical Health Sciences* 2011; **1**:35-9.

Siddiqui S, Verma A, Rather AA, Jabeen F, Meghvansi MK. Preliminary phytochemicals analysis of some important medicinal and aromatic plants. *Advances in Biological Research* 2009; **3**(5): 188-195.

Sikorska M, Matawska I, Gowniak K, Zgorka G. Qualitative and quantitative analysis of phenolic acids in *Asclepias syriaca* L. *Acta Pol Pharmaceuticals* 2001; **57**: 69-72.

Silver LL, Bostian KA. Discovery and development of new antibiotics: the problem of antibiotic resistance. *Antimicrobial Agents and Chemotherapy* 1993; **37**: 377–383.

Simon Creer, Anita Malhotra, Thorpe RS, Reto S, Favreau P, Chou WH. Genetic and Ecological Correlates of Intra specific Variation in Pit viper Venom Composition Detected Using Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) and Isoelectric Focusing. *Journal of Molecular Evolution* 2003; **56**:317–329.

Simonsen HT, Nordskjold JB, Smitt UW, Nyman U, Palpu P, Joshi P, Varughese G. *In vitro* screening of Indian medicinal plants for antiplasmodial activity. *Journal of Ethnopharmacology.* 2001; **74:** 195–204.

Simpson, MG. Plant Systematics. *Elsevier* 2006; 163-166.

Sinha S, Murugesan T, Muriti K, Gayen JR, Pal M, Saha BP. Evaluation of anti-inflammatory potential of B. 7iliate Sternb. Rhizome extract in rats. *J. Pharma Pharmacol*ogy 2001; **53**: 193 – 196.

Siva R, Krishnamurthy KV. Isozyme diversity in *Cassia auriculata* L. *African Journal of Biotechnology* 2005; **4**(8): 772-775.

Skaar, Irene, Jordheim, Monica. New anthocyanidin and anthocyanin pigments from blue *Plumbago*. *Journal of Agricultural and Food Chemistry* 2012; **60**(6): 1510-1515.

Smila H, Johnson M, Rajasekarapandian M. Studies on varietal difference, tissue specificity and developmental variation of esterase and peroxidase isozymes in pearl millet (*Pennisetum glacum* (L.) R. Br.). *Indian Journal of Biotechnology* 2007; **6**: 91 – 99.

Smith JSC, Smith OS. Environmental effects on Zein Chromatograms of maize inbred lines revealed by reversed-phase high performance liquid chromatography. *Theoretical and Applied Genetics* 1986; **71**: 607.

Sneath PHA, Sokal RR. *Numerical Taxonomy*. Freeman, San Francisco. **1973**

Snyder LR, Kirkland JJ, Dolan JW. Introduction to Modern Liquid Chromatography. *John Wiley and Sons, Inc.* Hoboken 2009; 1 – 7.

Socaciu C, Ranga F, Diehl H. UV-Vis spectrometry applied for the quality and authenticity evaluation of edible oils from Romania. *Bulletin of USAMV-CN* 2005; **61**: 295–300.

Solomon MJ, Harper JW, Shuttleworth J. CAK, the p34cdc2 activating kinase, contains a protein identical or closely related to p40MO15. *EMBO Journal* 1993; **12**: 3133-3142.

Soltis DE, Soltis PS. Phylogenetic relationships in Saxifragaceae Sensu lato: a comparison of topologies based on 18S rDNA and rbcL sequences. *American Journal of Botany* 1995; **84**(4): 504–522.

Sophie De Respinis, Guido Vogel, Cinzia Benagli, Mauro Tonolla, Orlando Petrini, Samuels GJ. MALDI-TOF MS of *Trichoderma*: a model system for the identification of micro fungi. *Mycol Progress* 2010; **9**:79-100.

Sorensen BK, Hojrup P, Ostergard E, Jorgensen CS, Enghild J, Ryder LR, Houen G. Silver staining of proteins on electroblotting membranes and intensification of silver staining of proteins separated by polyacrylamide gel electrophoresis. *Analytical Biochemistry* 2002; **304**(1): 33-41.

Spengler B, Hubert M, Kaufmann R. MALDI ion imaging and biological ion imaging with a new scanning UV-laser microprobe. 1994; In: Proceedings of the 42nd annual conference on mass spectrometry and allied topics. Chicago, Illinois.

Sreelatha T, Hymavathi AJ. Madhusudhana Murthy PU, Rani J Madhusudana Rao, Suresh Babu K. Bioactivity-guided isolation of mosquitocidal constituents from the rhizomes of *Plumbago capensis* Thunb. *Bioorganic & Medicinal Chemistry Letters* 2010; **20**(9): 2974-2977.

Srivastava S, Gupta MM, Verma RK, Kumar S. Quantitaion of 1,3 benzodioxanes derivatives from *Piper mullesua. American Organization of Analytical Chemists International (AOAC International)* 2000; **83**: 1484–1488.

Stace, CA. Plant taxonomy and Biosystematics. Edward Arnold Publishers Ltd., London, 1980.

Starr J, Naczi R, Chouinard B. Plant DNA barcodes and species resolution in sedges (Carex, Cyperaceae). *Molecular Ecology Resources* 2009; **9**: 151–163

Stebbins N, Holland MA, Cianzio SR, Polacco JC. Genetic tests of the roles of the embryonic ureases of soybean. *Plant Physiology* 1991; **l97**: 1004-1010.

Stech M, Kolvoort E, Loonen MJJE, Vrieling K, Kruijer JD BryophyteDNA sequences from faeces of an arctic herbivore, barnacle goose (*Branta leucopsis*). *Molecular Ecology Resources* 2011; **11**: 404–408.

Steven G. Newmaster and Subramanyam Ragupathy Testing plant barcoding in a sister species complex of pantropical Acacia (Mimosoideae, Fabaceae). *Molecular Ecology Resources* 2009; 9(1): 172–180.

Subramanyam Ragupathy, Steven G Newmaster, Maruthakkutti Murugesan, Velusamy Balasubramaniam. DNA barcoding discriminates a new cryptic grass species revealed in an ethnobotany study by the hill tribes of the Western Ghats in southern India. *Molecular Ecology Resources* 2009; 9(1): 164–171.

Sugie S, Okamoto K, Rahman KM, Tanaka T, Kawai K, Yamahara J, Mori H. Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats. *Cancer Lett* 1998; **127**: 177–83.

Sugimoto T, Amano M, Tokumoto T, Ishikawa K. Unusual phosphatase activity resistant to SDS and pronase treatments in Xenopus ovary Biochemical and *Biophysical Research Communications*1999; **26**(4): 1-4.

Sule WF, Okonko IO, Joseph TA, Ojezele MO, Nwanze JC, Alli JA, Adewale OG, Ojezele OJ. *In vitro* Antifungal Activity of *Senna alata* Linn. Crude Leaf Extract. *Journal of Biological Sciences* 2010; **5**(3):275-284.

Sun K, Chen X, Ma R, Li C, Wang Q, Ge S. Molecular phylogenetics of *Hippophae* L. (Elaeagnaceae) based on the internal transcribed spacer (ITS) sequences of nrDNA. *Plant Sysematics and Evolution*. 2002; **235**: 121–134.

Sunil H. Ganatra, Shweta P Durge, Patil SU. Preliminary Phytochemicals Investigation and TLC Analysis of *Ficus racemosa* Leaves. *Journal of Chemical and Pharmaceutical Research* 2012; **4**(5): 2380-2384.

Suresh Lalitharani, Veerabahu Ramasamy Mohan and Gnanasingh Selial Regini GC-MS analysis of ethanolic extract of *Zanthoxylum rhetsa* (Roxb.) DC spines *Journal of Herbal Medicine and Toxicology* 2010; **4**(1): 191-192.

Sweta T, Upendra B, Laxmi S, Anand G, Shailendra S. Quantitative analysis of glycyrrhizic acid by HPTLC in herbal formulation. *Asian Journal of Pharmacy & Life Science* 2011; **1**(2): 124-127.

Syamasundar KV, Ramesh S, Chandrasekhara RS. Volatile Constituents of *Alpinia galanga* Flower oil. *Journal of Medicinal and Aromatic Plant Sciences* 1999; **21**: 46.

Syed Mehar Alishah, Hidayat-ur-Rahman, Fida Muhammad Abbasi, Malik Ashiq Rabbani, Ijaz Ahmad Khan, Zabta Khan Shinwari, Zahir Shah. Interspecific variation of total seed protein in wild rice germplasm using SDS-PAGE. *Pakistan Journal of Botany* 2011; **43**(4): 2147-2152.

Szmidt AE, Wang XR. Genetic markers in forest genetics and breeding - The tunnel remains dark. In: Forest Genetics and Sustainability, C. Matyas (ed) 2000; **63**: 31-48.

Taha Shahat El Alfy MA , Hamida Mohamed A El Gohary, Nadia Mohamed Sokkar, Sahar Abd El Tawab, Dalia Adel Mohamed Al Mahdy Botanical and genetic characteristics

of *Celtis australis* L. and *Celtis occidentalis* L. grown in Egypt. *Bulletin of Faculty of Pharmacy,* Cairo University 2011; **49**(1): 37-57.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 2007; **24**:1596-1599.

Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbour joining method. *Proceedings of the National Academy of Sciences* (USA) 2004; 101: 11030-11035.

Tamura K., Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 2013; 10: 197.

Taylor JLS, Rabe T, McGraw LJ, Jager AK, van Staden J. Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulators*. 2001; **34**: 23-37.

Teshome K, Mariam TG, Asres K, Perry F, Engidawork E. Toxicity studies on dermal application of plant extract of *Plumbago zeylanica* used in Ethiopian traditional medicine. *Journal of Ethnopharmacology* 2008; 117: 236-248.

Thenmozhi M, Bhavya PK, Rajeshwari Sivaraj. Compounds identification using HPLC and FTIR in *Eclipta alba* and *Emilia sonchifolia*. *International Journal of Engineering Science and Technology* 2011; **3**(1): 292-298.

Thoria R. Mohamed, Sayed F. Khalifa and Reem M. Salah El-din. Leaf Protein Electrophoretic Profiles and Chromosome Numbers of Some Araceae. *International Journal of Agriculture & Biology* 2006; **8**(2): 231–234.

Tilak JC, Adhikari S, Devasagayam TP. Antioxidant properties of *Plumbago zeylanica,* an Indian medicinal plant and its active ingredient, plumbagin. *Redox Report* 2004; **9**(4): 219-27.

Timmins EM. Howell SA, Alsberg BK, Noble, WC, Goodacre R. Rapid differentiation of closely related Candida species and strains by pyrolysis-mass spectroscopy and Fourier transform– infrared spectroscopy. *Journal of Clinical Microbiology* 1998; **36**: 367–374.

Trease GE, Evans WC. Pharmacognosy. 15th Ed. London: Saunders Publishers; 2002. pp. 42–44. 221–229, 246–249, 304–306, 331–332, 391–393.

Tuna Uysal, Emine Arslan, Osman Tugay, Kuddisi Ertuğrul Determination of the relationship between some *Centaurea* species based on SDS-PAGE. *Turkish Journal of Biology* 2010; **34**: 125-131.

Uma B, Prabhakar K, Rajendran S, Lakshmi, Sarayu Y. Studies on GC/MS Spectroscopic Analysis of some Bioactive Antimicrobial Compounds from *Cinnamomum zeylanicum. Journal of Medicinal Plants* 2009; **8**(31): 125 – 131.

Umamaheswari A, Nuni A, Shreevidya R. Evaluation of antibacterial activity of *Boerhaavia diffusa* L.leaves. *Interantional Journal of Green Pharm*acy 2010; **4**:75-78.

Uniyal SK, Singh KN, Jamwal P, Lal B. Traditional use of medicinal plants among the tribal communities of Chhota Bhangal, Western Himalaya. *Journal of Ethnobiology and Ethnomedicine* 2006; **2**: 14.

Unnikrishnan KP, Raja SS, Balachandran I. A reverse phase HPLC-UV and HPTLC methods for determination of plumbagin in *Plumbago indica* and *Plumbago zeylanica*. *Indian Journal of Pharmaceutical Sciences* 2008; **70**: 844-7.

Uzer A, Ercag E, Apak R. Selective spectrophotometric determination of TNT in soil and water with dicyclohexylamine extraction. *Analytica Chimica Acta* 2005; **534**: 307-317.

Vaughan JG. The use of seed protein in taxonomy and phylogeny. In: *Seed Proteins*. L: Daussant J, Mosse J, Vaughan J. Academic Press, London New York. 1983; **198**: 135-150.

Vere N, Rich TCG, Ford CR, Trinder SA, Long C. DNA Barcoding the Native Flowering Plants and Conifers of Wales. *PLoS ONE* 2012; **7**(6): e37945.

Vijaya kumar R, Senthilvelan M, Ravindran R, Devi RS. *Plumbago zeylanica* action on blood coagulation profile with and without blood volume reduction. *Vascular Pharmacol* 2006; **45**(2):86-90.

Vijayakumari B, Yadav RH, Nithya SV. Pharmacognostic aspect of *Acalypha indica, vitex negundo* and *Coriandrum sativum. Biotechnology Research Asia* 2008; **5**(1): 269-276.

Vijayalakshmi R, Ravindhran R, HPTLC method for quantitative determination of gallic acid in ethanolic root extract of *Diospyrus ferrea* (Willd.) Bakh and *Aerva Lanata* (L.) Juss. Ex schultes – A potent Indian medicinal plants. *Asian Journal of Pharmaceutical and Clinical Research* 2012; **5**(4): 66-69.

Vinatoru, Maricela Toma M, Otilia Radu PI. Filip D, Lazurca TJ. Mason The use of ultrasound for the extraction of bioactive principles from plant materials. *Ultrasonics Sonochemistry* 1997; **3**: 242-246.

Vishnukanta S, Rana AC. Evaluation of anticonvulasant activity *Plumbago zeylanica* Linn leaf extract. *Asian Journal of Pharmaceutical Clinical Research* 2011; **3**: 76-8.

Wang GL, Wei M, Wang J, Lu Y, Mahady GB, Liu D High-performance liquid chromatography with Photodiode Array (HPLC-PAD) quality control of menoprogen, a traditional Chinese Medicine (TCM) formula used for the management of menopause. *International Journal of Medicinal Plants Research* 2013; **2** (1): 146-151.

Wang Q, Yu QS, Liu JQ. Are nuclear loci ideal for barcoding plants? A case study of genetic delimitation of two sister species using multiple loci and multiple intraspecific individuals. *Journal of Systematics and Evolution* 2012; **49**:182–188.

Wang Y, Taylor DE. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. *Biotechniques* 1993; **14**: 748-750.

Wang YC, Huang TL. Anti-*Helicobacter pylori* activity of *Plumbago zeylanica* L. *J. Chromatograph A* 2005; **1094**: 99-104.

Warburton D, Wuenschell C, Flores Delgado G, Anderson K .Commitment and differentiation of lung cell lineages. *International Journal of Biochemistry & Cell Biology* 1998; **76:**971-995.

Waterman JM. Mating tactics of male Cape ground squirrels (Xerus inauris): consequences of year-round breeding. *Animal Behaviour*. 1998; **56**:459-466.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*. 1990; **18**: 6531–6535.

Wolf HTI, Zundorf T, Winckler R Bauer, Dingermann T. Characterization of *Echinacea* species and detection of possible adulterations by RAPD analysis. *Planta Medica* 1999; **65**:773–774.

Wu W, Liang Z, Zhao Z, Cai Z. Direct analysis of alkaloid profiling in plant tissue by using matrix-assisted laser desorption/ionization mass spectrometry. *Journal of Mass Spectrometry* 2007; **42**(1): 58–69.

Wuyts N, Lognay G, Swennen R Waele DD. Nematode infection and reproduction in transgenic and mutant *Arabidopsis* and tobacco with an altered Phenyl propanoid metabolism. *Journal of Experimental Botany* 2006; **57** (11): 2825–2835**,**

Xiang B, Du GH, Wang XC, Zhang SX, Qin XY, Kong JQ, Cheng KD, Li YJ, Wang W. Elucidating the structure of two cyclotides of Viola *tianshanica maxim* by MALDI TOF/TOF MS analysis. *Yao Xue Xue Bao* 2010; **45**(11): 1402-9.

Xing Guo, Mark P. Simmons, Paul Pui Hay But, Pang Chui Shaw, Rui Jiang Wang. Application of DNA barcodes in *Hedyotis* L. (Spermacoceae, Rubiaceae) *Journal of Systematics and Evolution* 2011; **49**(3): 203–212.

Xiufeng Zhao, Chengqiang Ding, Lin Chen, Shaohua Wang, Qiangsheng Wang and Yanfeng Ding. Comparative proteomic analysis of the effects of nitric oxide on alleviating Cd-induced toxicity in rice (*Oryza sativa*L.). *Plant Omics Journal* 2012; **5**(6): 604-614.

Yadav RK. Seed protein electrophoresis studies in cucurbits-a review. *Agricultural Reviews* 2008; **29**(1): 21-30.

Yan Li, Lian-Ming GAO, Ram C, De-Zhu Li, Alan Forrest. High universality of *matK* primers for barcoding gymnosperms. *Journal of Systematics and Evolution* 2011; **49**(3): 169-175.

Yang JB, Wang YP, Möller M, Gao LM, Wu D. Applying plant DNA barcodes to identify species of *Parnassia* (Parnassiaceae). *Molecular Ecology Resources* 2012; **12**: 267–275.

Yang JH, Lin HC, Mau JL. Antioxidant properties of several commercial mushrooms. *food chemistry* 2001; **77**: 229-235.

Yanli Wang, Qing Zhou, Bin Li, Baoping Liu, Guoxing Wu, Muhammad Ibrahim, Guanlin Xie, Hongye Li, Guochang Sun. Differentiation in MALDI-TOF MS and FTIR spectra between two closely related species *Acidovorax oryzae* and *Acidovorax citrulli*. *BMC Microbiology* 2012; **12**: 182-185.

Yogananth N, Jothi Basu M. TLC method for the determination of plumbagin in hairy root culture of *Plumbago rosea* L. *Global Journal of Biotechnology & Biochemistry* 2009; **4**(1): 66-69.

Yogesh V Ushir, Krishnakant N Patel, Navin R Shet**.** HPTLC Fingerprint Profile For Quantitative Determination Of Various Phytoconstituents In *Anisomeles* Species. *American Journal of Pharm Tech Research* 2005; **1** (3) 283 – 290.

Yong Li, Ping Liu, Yonghua Li. Intraspecific variation of *Achyranthes bidentata* (Amaranthaceae) in the geo-authentic product area based on internal transcribed spacer sequences of ribosomal DNA. *American Journal of Cultural Sociology* 2012; **6**(12):1655-1660.

Yong Ming, Jing Wang, Jin Yang, Wei Liu. Chemical Constituents of *Plumbago Zeylanica L. Advanced Materials Research* 2011; **308**(310): 1662-1664.

Yousaf Z, Masood S, Shinwari ZK, Khan MA, Rabani A. Evaluation of taxonomic status of medicinal Species of the genus *Hyoscyamous, Withania, Atropa* and *Datura* based on poly acrylamide gel electrophoresis. *Pakistan Journal of Botany* 2008: **40**(6): 2293-2297.

Yousaf Z, Shahid Masood, Zabta Khan Shinwari, Mir Ajab Khan, Ashiq Rabani. Evaluation of taxonomic status of medicinal species of the genus *Solanum* and *Capsicum* based on Poly Acrylamide Gel Electrophoresis. *Pakistan Jouranl of Botany* 2011; **38**(1): 99-106.

Youxiong Que, Liping Xu, Jianwei Lin, Miaohong Ruan, Muqing Zhang, and Rukai Chen. Differential Protein Expression in Sugarcane during Sugarcane-*Sporisorium scitamineum* Interaction Revealed by 2-DE and MALDI-TOF-TOF/MS. *Comparative and Functional Genomics* 2011.

Yu J, Xue JH, Zhou SL. New universal *matK* primers for DNA Barcoding angiosperms. *Journal of Systematics and Evolution* 2011; **49**: 176-181.

Yu Jiang, Chun-bang Ding, Li Zhang, Ruiwu Yang, Yonghong Zhou, Li Tang. Identification of the genus *Epimedium* with DNA barcodes. *Journal of Medicinal Plants Research* 2011; **5**(28): 6413-6417.

Yuan L, Fanq D. Chao L. Qing-Yan M. Ze-Wen G. Determination of plumbagin in different parts of *Plumbago zeylanica* by RP-HPLC, *Zhongguo Zhong Yaoza Zhi* 2006; **31**(20): 1684-1686.

Yuan-Miao Fu, Wei-Mei Jiang, Cheng-Xin Fu. Identification of species within *Tetrastigma* (Miq.) Planch.(Vitaceae) based on DNA barcoding techniques. *Journal of Systematics and Evolution* 2011; **49** (3): 237-245.

Zahid Ali, Showkat Hussain Ganiea, Alka Narula, Maheshwar Prasad Sharma, Prem Shankar Srivastava. Intra-specific genetic diversity and chemical profiling of different accessions of *Clitoria ternatea* L. *Industrial Crops and Products* 2013; **43**:768– 773

Zahin M, Aqil F, Ahmad I. The in vitro antioxidant activity and total phenolic content of four Indian medicinal plants. *International Journal of Pharmaceutical Sciences* 2009; **1**: 89-95.

Zeidler M. Electrophoretic analysis of plant isozymes. *Acta Universitatis Palackianae Olomucensis, Facultas Rerum Naturalium, Mathematica* 2000; 38: 7 – 16.

Zhang A, Sun H, Wu G, Sun W, Yuan Y, Wang X. Proteomics analysis of hepatoprotective effects for scoparone using MALDI-TOF/TOF mass spectrometry with bioinformatics. *OMICS* 2013; **17**(4): 224-9.

Zhang JS, Guan J, Yang FQ, Liu HG, Cheng XJ, Li SP. Qualitative and quantitative analysis of four species of *Curcuma rhizomes* using twice development thin layer chromatography. *Journal of Pharmaceutical and Biomedical Analysis* 2008; **48**: 1024-102.

Zhang Y, Bailey A, Matthies HJG, Rende RB, Smith MA, Speese SD, Rubin GM, Broadie K. Drosophila fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell* 2001; **107**(5): 591-603.

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STUDIES ON INTER-SPECIFIC VARIATION IN THE GENUS *PLUMBAGO* (PLUMBAGINACEAE) FROM SOUTH INDIA USING PHYTOCHEMICAL ANALYSIS

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ABSTRACT

The objective of the present investigation was aimed to reveal the inte-specific variation among the three *Plumbago* species *viz., Plumbago zeylanica* Linn, *Plumbago auriculata* Lam, *Plumbago rosea* Linn using preliminary phytochemical screening and TLC profiles. To identify the various phytoconstituents and functional groups present in the *Plumbago* species, the preliminary phytochemical analysis was carried out using Harborne *et al.,* method. TLC chromatographic technique was used to predict the secondary metabolites presence in the screened extracts of *Plumbago* species based on the band formation. The preliminary phytochemical assay reported the presence and absence of thirteen different compounds in varied degree among these ethanolic extract of all the three *Plumbago species* showed maximum number of eight compounds. TLC profiling of the extracts of *Plumbago* depicted the presence phenol and steroid in different range of band formation. Among all the plant extracts chloroform and ethanolic extract reported maximum number of spots compared to all the other extracts. These techniques paved a way to predict and compare the phytoconstituents present in all the three selected *Plumbago* species which can be used for further medicinal properties.

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INTRODUCTION

Plants are the important source of secondary metabolites with interesting biological activities [1]. The World Health Organization (WHO) has reported that approximately 80% of the world's population currently exercises herbal medicines as teas, decocts or extracts with easily available solvents such as water or alcohol [2, 3]. Due to less side effect and availability for practice, the application of herbalism became popular throughout the world during the last century. In spite of the great advances achieved in contemporary medicine, plants still make a significant contribution to health care. This is due to the recognition of the value of traditional medicinal systems [4, 5]. In pharmacognostical analysis, the qualtiative and quantitative determination of phytoconstituents are largely estaimated by chromatographic and spectroscopic analysis [6].

The roots of *Plumbago* species are the main source of plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) which is commercially important for its broad range of pharmacological activities, e.g. anti *Helicobacter pylori* [7], anti-tumor [8], antiparasitic [9], insect antifeedant [10], anti-hepatoma, anti-intestinal carcinogenesis [11], antioxidant [12] and antimalarial [13] activities. This plant also contains alkaloids, glycosides, tannins, saponins and steroids [14]. Jetty *et al.* [15] identified the number of active compounds presence in the roots of *P. zeylanica* viz., naphthoquinones (plumbagin, chitranone, maritinone, elliptinone and isoshinanolone), coumarins (seselin, 5-methoxyseselin, suberosin, xanthyletin and xanthoxyletin), plumbagic acid glucosides (3′-*O*-β- glucopyranosyl plumbagic acid and 3′-*O*-β- glucopyranosyl plumbagic acid methyl ester) [8], neoisoshinanolone and 1-epineo-isoshinanolone.

P. zeylanica roots were reported to possess antioxidant, hypolipidemic, anti-artherosclerotic, central nervous system stimulant and anti-fertility properties [16]. *Plumbago rosea* L. (Plumbaginaceae), commonly known as Rakta Chitrak, grows in the wild and in abundance in India. The *P. rosea* is used to treat inflammatory disorders, skin diseases [17], gastric acidity, constipation, abdominal pain [18] and as an abortifacient [19] by the traditional medical practioner. The roots of *P. rosea* have been reported to possess antitumor [20] and antiatherogenic [21] activities. The following active constituents are reported from *P. rosea* plumbagin, hydroxy-1,4-napthaquinone, sitosterol glycoside, fatty alcohol and tannins [22]. Johnson et al. [23] studied the *inter*-specific variation among the *Plumbago* speceis using isozyme and RFLP profiles. For efficient conservation and management of the selected medicinal plants diversity, the genetic phytochemical composition of species needs to be assessed. Based on morphology, it is very difficult to identify the medicinal plants in the form of crude drug and juvenile stage. Phytochemical marker is very useful in pharmacognosy. In addition, previous studies on the *Plumbago* species were focused on the roots only, very few reports are availbale on the aerial parts the selected *Plumbago* species. To supplement the previous observations as an attempt was made to reveal the phytochemical properties of three *Plumbago* species aerial parts and find out the *inter*-specific variation among the selected three speceis of *Plumbago* using preliminary phytochemical screening and TLC analysis. These phytochemical analyses of intraspecific variation in particular may find application in resolving disputes of taxonomic identities, relations and authentication of the species in the pharmaceutical industires.

MATERIALS AND METHODS

The aerial parts of selected three *Plumbago* species viz., *Plumbago zeylanica* Linn., *Plumbago auriculata* Lam. and *Plumbago rosea* L. were collected from natural habitats at Tenkasi, Coimbatore and Mysore were air and shade dried for fifteen days and pulverized to powder using tissue blender. The dried and powdered leaves (50 g) were extracted successively with 300 mL of petroleum ether, chloroform, acetone, ethyl acetate, ethanolic and aqueous by using Soxhlet extractor for 8 h at a temperature not exceeding the boiling point of the solvent. The extracts were filtered using Whatmann filter paper (No. 1) and then concentrated in vacuum at 40°C using rotary evaporator.

Preliminary phytochemical screening of various secondary metabolites from different extracts of *P. zeylanica, P. auriculata, P. rosea* (steroids, alkaloids, phenolic groups, cardiac glycosides, flavanoids, saponins, tannins, aminoacids, anthroquinones, coumarin glycosides, carbohydrates, terpenoids, catechins) were carried out according to the standard method described by Harborne [24].

To know the qualitative profile of phenol and steroid present in the selected *Plumbago* species, the specific TLC method described by Harborne method was adopted. TLC was carried out on 10 × 20 cm silica gel plates (Merck, Germany). To detect the phenolic presence in the extracts of *Plumbago* specceis, the chloroform and methanol with 9: 1 ratio and folin ciocalteau reagent was served as mobile phase and spraying agent respectively. To detect steroid bands in the selected *Plumbago* species benzene and methanol with 9:1 ratio and 5% alcoholic sulphuric acid was exercised as the mobile phase and spraying agent respectively. Each separation was repeated twice and confirmed the banding pattern and their RF values were recorded.

For the inter-specific relationship studies, the TLC chromatogram was converted into a "1" and "0" matrix, to indicate the presence or absence of the Rf Values, respectively. Genetic similarities (GS) were estimated according to Nei and Li [25]. To demonstrate the *inter*-specific relationship, a dendrogram was constructed by UPGMA using NTSYSpc-2.0 software.

RESULTS

The prelimnary phytochemical analysis revealed various degree of phytoconstituents presence with refernce to solvents used for extraction. Among the tested extracts, ethanolic extracts of all the three *Plumbago* species showed maximum number of metabolites (8) out of thirteen different tests screened. The results of preliminary phytochemical analysis were demonstrated in the Table 1.

Table 1: Preliminary phytochemical screening of *Plumbago* species

Metabolites	P			C			A			EA			E			A			*T*
	Pz	*Pa*	*Pr*	*Pz*	*Pa*	*Pr*	*P.z*	*Pa*	*Pr*	*Pz*	*Pa*	*Pr*	*Pz*	*Pa*	*Pr*	*Pz*	*Pa*	*Pr*	
Steroids	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	**13**
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	**1**
Phenolic groups	-	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	-	-	**9**
Cardiac glycosides	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+	**14**
Flavonoids	-	+	-	+	+	-	-	-	+	-	+	-	-	+	+	+	-	-	**8**
Saponins	+	+	-	-	+	-		-	+	-	+	-	-	-	+	-	-	-	**6**
Tannins	+	+	-	+	+	-	-	-	+	-	-	-	+	+	+	+	+	+	**11**
Coumarin glycosides	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	**3**
Carbohydrates	-	-	+	+	-	+	-	+	-	+	+	+	+	+	+	-	-	-	**10**
Terpenoids	-	+	-	+	+	+		+	-	+	-	-	+	+	-	-	-	-	**8**

***Pz- Plumbago zeylanica; Pa- Plumbago auriculata; Pr- Plumbago rosea;* P- Petroleum ether extract; C-Chloroform extract; A- Acetone extract; EA- Ethyl acetate extract; E- Ethanolic extract and Aq- Aqueous extract; T – Total**

The results of TLC analysis revealed the phenolic and steroid profile of the studied *Plumbago* species (Table 2 – 7). The chloroform, ethyl acetate and ethanolic extract of *P. zeylanica* showed maximum numebr (5) of phenolic bands with different Rf values ranging from 0.35 - 0.53 (Table 2).

Table 2: Phenolic Profile of *Plumbago zeylanica*

RF value	P	C	A	E	E	Aq
0.22	-	-	-	+	-	-
0.25	-	-	+	-	-	-
0.29	-	-	+	-	-	-
0.33	-	+	-	-	+	-
0.35	-	+	-	-	-	-
0.36	+	-	-	+	-	-
0.38	-	-	+	-	+	-
0.40	-	+	-	+	+	-
0.44	+	-	-	+	+	-
0.49	-	+	+	+	-	-
0.50	+	-	-	-	-	-
0.53	-	-	-	-	+	-
0.58	-	+	-	-	-	-
0.62	+	-	-	-	-	-
Total	4	5	4	5	5	-

P-Petroleum ether extract, C- Chloroform extract, A- Acetone extract, EA- Ethyl acetate extract, E- Ethanolic extract, Aq- Aqueous extract

The petroleum ether extracts of *P. zeylanica* showed maximum numebr (7) of steroidal bands compared to other tested extracts of *Plumbago zeylanica* (Table 3). Where as chloroform and acetone extract of *P. auriculata* illustrated seven phenolic bands with varied Rf values (Table - 4).

Table 3: Steroid Profile of *Plumbago zeylanica*

RF value	P	C	A	EA	E	Aq
0.08	-	-	-	-	+	-
0.09	-	-	+	+	-	-
0.10	+	-	-	-	-	-
0.15	+	-	+	-	+	-
0.18	-	+	+	-	-	-
0.2	-	-	-	+	-	-
0.25	+	-	-	-	-	-
0.32	-	-	-	+	-	-
0.38	-	-	-	+	-	-
0.41	+	-	-	-	-	-
0.5	+	-	-	+	-	-
0.55	+	-	-	-	-	-
0.58	+	-	-	-	-	-
0.6	-	-	+	-	-	-
0.65	-	+	-	-	-	-
0.68	-	-	-	-	+	-
0.78	-	+	-	-	-	-
Total	7	3	4	5	3	Nil

Table 4: Phenolic profile of *Plumbago auriculata*

RF value	P	C	A	EA	E	Aq
0.1	-	+	-	-	-	-
0.3	-	-	+	-	-	-
0.35	-	+	+	+	-	-
0.38	-	+	+	+	+	-
0.41	-	+	-	-	-	-
0.43	-	-	+	+	+	-
0.48	-	+	-	+	-	-
0.5	+	-	+	-	-	-
0.53	-	+	-	-	-	-
0.56	-	-	+	-	-	-
0.58	-	+	-	-	-	-
0.6	+	-	+	-	+	-
0.65	+	-	-	+	-	-
0.72	+	-	-	-	-	-
Total	4	7	7	5	3	Nil

P-Petroleum ether extract, C- Chloroform extract, A- Acetone extract, EA- Ethyl acetate extract, E- Ethanolic extract, Aq- Aqueous extract

Similar to *P. zeylanica*, the petroleum ether and chloroform extract showed maximum number (8) of steroidal bands. The aqueous extracts of *P. auriculata* failed to express the phenolic and steroid presence (Table 5).

Table 5: Steroid profile of *Plumbago auriculata*

RF value	P	C	A	EA	E	Aq
0.03	+	-	-	-	-	-
0.07	-	+	-	-	-	-
0.14	-	+	-	-	-	-
0.16	+	-	-	-	-	-
0.20	-	-	+	-	-	-
0.23	-	+	-	-	-	-
0.26	+	-	-	-	-	-
0.30	-	+	+	-	-	-
0.32	-	-	+	-	-	-
0.44	-	+	-	-	-	-
0.5	+	-	-	-	-	-
0.52	-	-	-	+	-	-
0.57	-	+	-		-	-
0.58	-	-	-	+	-	-
0.60	+	-	-		-	-
0.65	-	+	-	+	-	-
0.67	+	-	-	-	+	-
0.68	-	-	+	-	-	-
0.75	-	+	-	-	-	-
0.81	+	-	-	-	-	-
0.90	+	+	-	-	-	-
Total	8	8	4	3	1	Nil

P-Petroleum ether extract, C- Chloroform extract, A- Acetone extract, EA- Ethyl acetate extract, E- Ethanolic extract, Aq- Aqueous extract

The phenolic profile of *P.rosea* depicted in the table 6. The phenolic TLC profile of *P. rosea* ethanolic extracts showed seven bands with different Rf values ranges from 0.20- 0.57. Similar to that, ethanolic extracts of *P. rosea* illustrated maximum number (8) of steroidal bands with varied Rf value ranged from 0.08 to 0.68 (Table - 7). Cluster analysis was performed using preliminary phytochemical and chromatographic data and it produced

stable and consistent patterns (Figure 1- 4). The cladogram based on the phytochemical analysis and phenolic profile shown that two clusters, of which cluster 2 includes only one species viz., *P. rosea* showed 100% of divergence with other two species. Cluster 1 (C_1) showed two nodal (N) branches (C_1N^1 and C_2N^2). C_1N^1 was *P. zeylanica* and C_2N^2 was *P. auriculata* (Fig. 1 and 2).

Table 6: Phenolic profile of *Plumbago rosea*

RF value	P	C	A	EA	E	Aq
0.20	-	-	-	-	+	-
0.27	-	-	+	-	-	-
0.30	-	+	+	-	+	-
0.33	-	-	-	-	+	-
0.38	-	+	+	-	+	-
0.40	+	+	-	-	-	-
0.42	-	-	+	+	-	-
0.45	-	+	+	-	+	-
0.50	+	-	-	+	-	-
0.54	+	-	-	-	+	-
0.57	-	+	-	-	+	-
0.60	-	-	-	+	-	-
0.70	-	-	-	+	-	-
Total	3	5	5	4	7	Nil

Table 7: Steroid profile of *Plumbago rosea*

RF value	P	C	A	EA	E	Aq
0.05	+	-	+	-	-	-
0.08	-	-	-	-	+	-
0.11	-	-	+	-	+	-
0.13	+	-	-	+	-	-
0.15	-	+	-	-	+	-
0.19	-	-	-	+	-	-
0.20	-	-	-	-	+	-
0.22	-	-	+	-	+	-
0.25	+	+	+	-	-	-
0.28	-	-	-	-	-	-
0.30	-	-	+	+	+	-
0.41	-	+	-	-	-	-
0.44	-	-	-	-	+	-
0.46	+	-	-	-	-	-
0.5	-	-	+	+	-	-
0.55	-	-	-	-	-	-
0.63	+	-	-	-	-	-
0.68	-	+	-	-	+	-
0.7	-	-	-	+	-	-
0.8	-	-	-	+	-	-
0.9	-	-	-	+	-	-
Total	5	4	6	7	8	Nil

P-Petroleum ether extract, C- Chloroform extract, A- Acetone extract, EA- Ethyl acetate extract, E- Ethanolic extract, Aq- Aqueous extract

Fig. 1. Cladogram of the selected *Plumbago* species based on Phytoconstituents

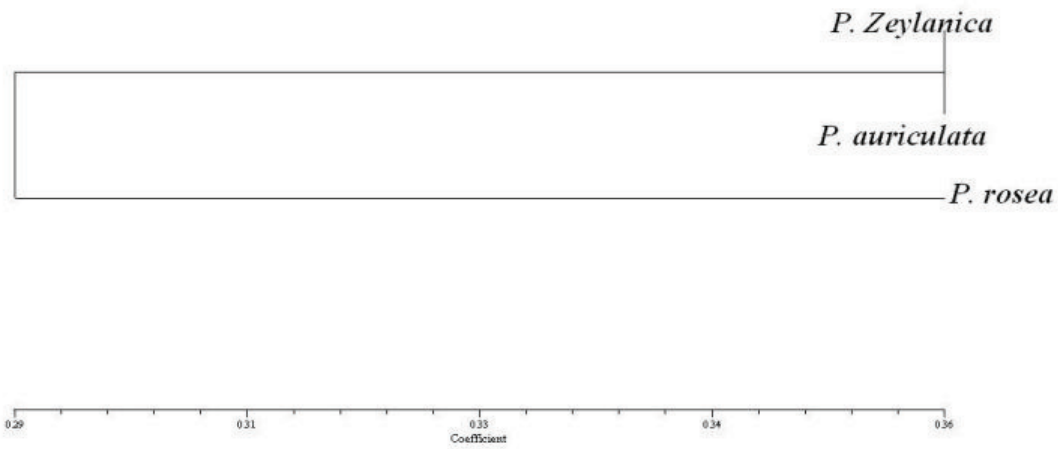
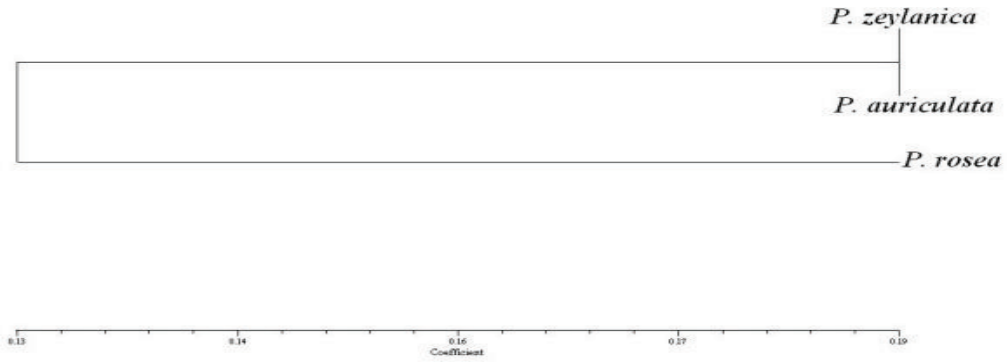


Fig. 2. Cladogram of the selected *Plumbago* species based on Phenolic profile



Contrary to that the cladogram constructed based on the steroidal profile and amalgamated cladogram based on the phenolic and steroidal profile showed two clusters, of which cluster 2 includes only one species viz., *P. auriculata* showed 100% of divergence with other two species. Cluster 1 (C_1) showed two nodal (N) branches (C_1N^1 and C_2N^2). C_1N^1 was *P. zeylanica* and C_2N^2 was *P. rosea* (Fig. 3 and 4).

Fig. 3. Cladogram of the selected *Plumbago* species based on Steroid profile

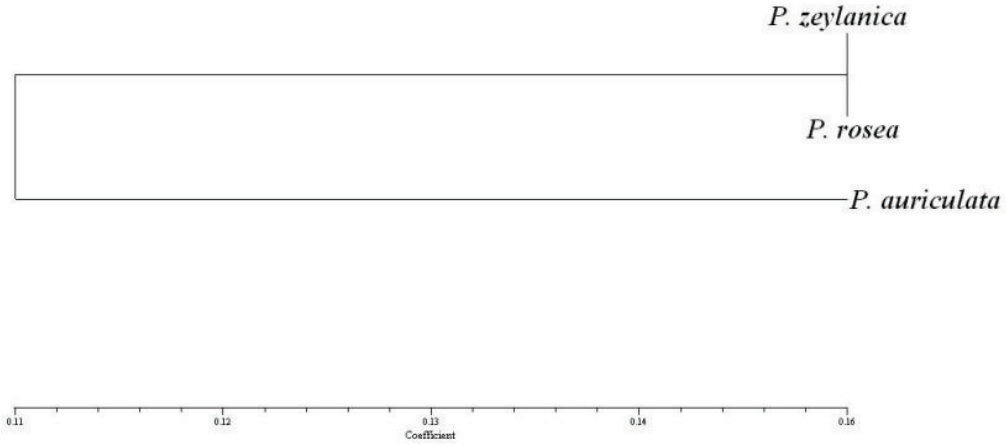
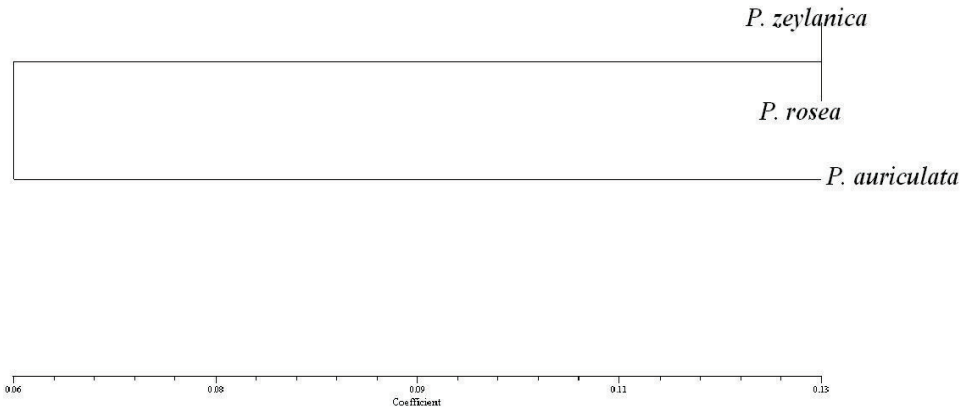


Fig. 4. Cladogram of the selected Plumbago species based on Phenolic and Steroidal Profile



DISCUSSION

A number of phytochemist reported the presence of potentially rich secondary metabolites such as alkaloids, flavonoids, tannins and terpenoids in the medicianl plants as a defence emchanism [26]. The metabolites phenolic and flavonoids compounds reported as antioxidant agents, which perform as free radical terminators and have revealed medicinal properties [27]. Ajayi *et al.* [28] identified the presence of anthraquinones, alkaloids, carbohydrates, cardiac glycosides, flavonoids, saponins, steroids and tannins in the ethanolic root extract of *P. zeylanica* from Nigeria. Similar to the Ajayi *et al.,* observations, in the present study also ethanolic extract of *P. zeylanica* aerial parts collected from Tenkasi showed the presence of steroids, alkaloids, phenol, cardiac glycosides, tannins, coumarin glycosides, sterols, carbohydrates, terpenoids. Sheeja *et al.* [11] indicated the presence of tannins, flavanoids, triterpenoids, and napthoquinones in the acetone extract of *P. zeylanica* from Mandsaur, Madhya Pradesh. The ethanolic extract of *P. zeylanica* collected from Mandsaur, Madhya Pradesh showed the presence of carbohydrates, glycosides, tannins, flavonoids and saponins. Contrary to Sheeja *et al.,* observations in the present study, acetone extract of *P. zeylanica* aerial parts showed the presence of steroids only. Kantha *et al.* [29] revealed the phytochemical screening of dichloromethane extract of *P. zeylanica* root and confirmed the presence of terpenoids, flavonoids and absence of steroids, carbohydrate, alkaloids and tannins. Kethani Dev and Gopala Krishna [30] revealed the presence of alkaloids and glycosides in the root, stem and leaf of methanolic extract of *P. zeylanica*. With reference to Kantha *et al.,* and Kethani *et al.,* observations, in the present study, ethanolic extracts of *P. zeylanica* aerial parts showed maximum number of compounds like steroids, flavonoids, terpenoids, phenol, carbohydrates, cardiac glycosides and alkaloids compared to other tested extracts of *P. zeylanica*.

Mohammed Ibrahim *et al.* [31] reported the preliminary phytochemical analysis on methanolic extract of *P. rosea* and showed the presence of alkaloids, glycosides, saponins, tannins, steroids and reducing sugars. Simlar to Ibrahim et al obsevatin in the present study also we observed the presence of steroids, cardiac glycosides, saponins and tannins, in the ethanolic extracts of *P. rosea* collected from Mysore, India except alkaloids. In addition, we observed the phenols, flavanoids, coumarin glycosides, and carbohydrates presence in the ethanolic extracts of *P. rosea* collected from Mysore, India. Kantha *et al.* [29] subjected TLC profiling of dichloromethane extract of *P. zeylanica* using Hexane: Ethyl acetate (7:3) and Methanol: Ethyl acetate (2:8) as mobile phase. Similar to the work of Kantha *et al.,* in the present study also the phenolic and steroid profile of six different extracts of *Plumbago* species were carried out using chloroform and methanol as a mobile phase for phenol and benzene and methanol for steroid with 9:1 ratio.

Pawar *et al.* [32] revealed the plumbagin presence in the chloroform extract of *P. zeylanica* root collected from Ghaziabad which resolved as a dark grey colour band with the Rf. 0.84. Similar to that in the

present study also, the plumbagin presence in the selected Plumbagao species was identified with the standard plumabagin using the TLC plate. The results revealed that the plubagin was identifed in the selected palnt species of Plumbago. The acetone, ethanolic and aqueous extracts of standard plubagin was showed a band with the Rf value 0.62; the ethyal acetate with 0.65, choroform with 0.67 and petroleum ether with 0.68. Among the tested extracts, the petroleum ether extract of *P. zeylanica* confirmed the plumbagin presence with Rf value 0.62. The other extracts were showed with varied profiles.

Ariyanathan *et al.* [33] reported the TLC profile of *P. auriculata* using with toluene: ethyl acetate as solvent system, they observed fluorescent yellow spots with Rf value 0.4. But the results of the present study showed varied profile with different Rf values. We observed the plubagin presence in the petroleum ether and ethyl acetate extract of *P. auriculata* with the Rf value of 0.65. The results were directly coincided with the standard plumbagin. The chloroform and acetone extracts of *P. auriculata* showed maximum number of phenolic bands (7) with different Rf values. Chloroform and petroleum ether extracts of *P. auriculata* showed maximum of steroidal (8) bands with varied Rf values. The ethanolic extracts of *P. rosea* showed maximum of phenolic bands (7). Similar to that ethyl acetate extracts of *P. rosea* displayed 8 steroidal bands. These banding profiles will be used as pharmacognostical markers in the pahrmaceutical industires and it will help the phytochemist to distingusih the medicinal plants from its adulteratnts using the phenolic and steroid TLC profile of studied *Plumbago* species. Previous studies on the *Plumbago* species were focused on the roots only, to supplement the previous observations as an attempt was made to reveal the phytochemical properties of three *Plumbago* species aerial parts, the results of the present study confirmed the medicinal properties presence in the aerial parts of the selected three species of *Plumbago*. The cladogram constructed based on the steroidal profile and amalgamated cladogram of the steroid and phenolic profile of the *Plumbago* speceis were similar to the cladogram constructed by Johnson *et al*. [24] using isozymes and RFLP profiles.

CONCLUSION

The present preliminary and chromatographic studies results suggests that the aerial parts of three *Plumbago* species viz., *P. zeylanica, P. auriculata, P. rosea* also posses the medicinal properties in equalence with the roots of the selected species. In addition the results of the present study revealed the *inter*-specifc variation and similarty among the selected species. These profiles can be used as a phytochemical marker to distinguish and characterize the medicinally important plants.

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Conflict of interest:

We declare that we have no conflict of interest.

REFERENCES

1. De-Fatima A, Modolo LV, Conegero L S, Pilli R A, Ferreira C V, Kohn L K, De-Carvalho JE. Lactones and their derivatives: biological activities, mechanisms of action and potential leads for drug design. *Curr. Med. Chem*, 2006, 13, 3371-3384.

2. Julsing KM, Quax JW & Kayser O. The Engineering of Medicinal Plants: Prospects and Limitations of Medicinal Plant Biotechnology. In Oliver Kayser and Wim J Quax, ed. Medicinal Plant Biotechnology. WILEY-VCH Verlag GmbH and Co. KGaA, Weinheim, 2007, 527- 31443-0.
3. Kalidass C, Daniel A, Mohan V R. Rapid propagation of *Plumbago zeylanica* L. An important medicinal plant. *J. Am. Sci.,* 2010, 6 , 1027-1031.
4. Mohammed Ibrahim, Joy Baura, Qamrul Ahasan, Torequl Islam, Zilly Homa, M. Mohi Uddin Chowdhury Md, Aslam Hossain and Mohammad A Rashid, Preliminary Phytochemical and Pharmacological Investigations of *Alpinia conchigera* Griff. and *Plumbago indica* L, *Bangladesh Pharmaceutical Journal* 2012,15(2),153-157.
5. Pour BM, Sasidharan S. *In vivo* toxicity study of *Lantana camara*. *Asian Pacific Journal of Tropical Biomedicine* 2011, 1(3), 189-191.
6. Eisenhauer N, Klier M, Partsch S, Sabais ACW, Scherber C, Weisser W , Scheu S. No interactive effects of pesticides and plant diversity on soil microbial biomass and respiration. Appl. Soil Ecol, 42**,** 2009, 31-36.
7. Wang, Y.C. and Huang, T.L. 2005. High-performance liquid chromatography for quantification of plumbagin, an antiHelicobacter pyloricompound of *Plumbago zeylanica* L. J Chromatogr A.1094: 99-104
8. Lin L C, Yang L L ,Chou C J, Cytotoxic naphthoquinones and plumbagic acid glucosides from *Plumbago zeylanica*. *Phytochemistry,* 2003,62 , 619-622.
9. Chan-Bacab M J, Peña-Rodríguez L M. Plant natural products with leishmanicidal activity. *Nat Prod Rep* 2001, 18, 674-688.
10. Villavicencio M A & Perez-Escandon B E, Plumbagin activity (from *Plumbago pulchella* Boiss. Plumbaginaceae) as a feeding deterrent for three species of Orthoptera. *Folia Entomol Mex,* 1992, 86, 191-198.
11. Sheeja E, Joshi S B, Jain D C, Antiovulatory and estrogenic activity of *Plumbago rosea* leaves in female albino rats, *Indian journal of Pharmacol,* 2011,41, 273-277.
12. Nahak G and Sahu RK. Antioxidant activity of *Plumbago zeylanica* and *Plumbago rosea* belonging to family Plumbaginaceae. *Natural Product: An Indian Journal*, 2011, **7,** 51-56.
13. Likhitwitayawuid K, Kaewamatawong R, Ruangrungsi N, Krungkrai J, Antimalarial naphthoquinones from *Nepenthes thorelii*. Planta Med, 1998, 64, 237-241.
14. Zaheer A, Ahsana D. Advances in Natural products Importance in health and Economy, 2008
15. Jetty A, Subhakar C, Rajagopal D, Jetty M, Subramanyam M , Murthy M M, Antimicrobial activities of neo- and 1-epineoisoshinanolones from *Plumbago zeylanica* roots. *Pharm. Biol,* 2010, 48, 1007-1011.
16. Mallikadevi T, Paulsamy S, *Plumbago zeylanica*– a potential plant for antimicrobial activity. *Plant Arch,* 2010,10, 547-550.
17. Dorni AIC, Vidyalakshmi KS, Hannah RV, Rajamanickam GV, Dubey GP. Antiinflammatory activity of *Plumbago capensis*. *Pharmacognosy Mag,* 2006, 2, 239–43.
18. Misra MK, Behera SK, Panda A, Behera SK. Medicinal plants used by the Kandhas of Kandhamal district of Orissa. *Ind J Trad Knowledge* 2006, 5, 519–28.
19. Nath SC, Purkayastha J. Biological activity of ethnomedical claim of some plant species of Assam.*Ind J Trad Knowledge,* 2004, 5, 229–36.
20. Devi P U, Solomon F E, Sharada A C. *In vivo* tumor inhibitory and radiosensitizing effects of an Indian medicinal plant, *Plumbago rosea* on experimental mouse tumors. Indian *J Exp Biol,* 1994,32, 523-528.
21. Mary NK, Babu BH, Padikkala J. Antiatherogenic effect of Caps HT2, a herbal Ayurvedic medicine formulation. *Phytomedicine*,2003, 10, 474–82. (PubMed)

22. Lal R, Sankaranarayanan A, Mathur VS. Antifertility and uterine activity of *Plumbago rosea* in rats. *Indian J Med Res,* 1993, 78, 287–90. (PubMed).
23. Johnson M, Usha Raja Nanthini A, Renisheya Joy Jeba Malar T, Isozyme Variation and Genetic Relationships among Three *Plumbago* Species. Journal of Ecobiotechnology 2010, 2 (5), 54-59.
24. Harborne J.B. Phytochemical methods. London, Chapman and Hall Ltd. 1998; 49-188.
25. Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci.,* 1979, 76, 5269-5273.
26. Olagunju JA, Fagbohunka BS, Oyedapo OO, Abdul AIA . Effects of an ethanolic root extract of *Plumbago zeylanica* Linn. on some serum parameters of the rats. RPMP-Drug Dev. *Mol*, 2006. **11,** 268-276.
27. Omale J, Okafor PN, Comparative antioxidant capacity, membrane stabilization, polyphenol composition and cytotoxicity of the leaf and stem of *Cissus multistriata*. *Afr. J. Biotechnol.,* 2007, 7 (17), 3129-3133.
28. Ajayi GO, Olagunju JA, Ademuyiwa O and Martins CO. Gas chromatography-mass spectrometry analysis and phytochemical screening of ethanolic root extract of *Plumbago zeylanica* Linn. Journal of Medicinal Plants Research 2011, 5(9), 1756-1761
29. Kantha D. Arunachalam P, Velmurugan and Balaji Raja R, , Anti-inflammatory and cytotoxic effects of extract from *Plumbago zeylanica*, *African Journal of Microbiology Research* 2010, 4(12) , 1239-1245.
30. Kethani Devi, Gopala Krishna. Pharmacognostic, phytochemical and biological study of *Plumbago zeylanica, International Journal of Natural Products Research.* (2012), 1(2), 21-23.
31. Mohammed Ibrahim, Joy Baura, Qamrul Ahasan, Torequl Islam, Zilly Homa, M. Mohi Uddin Chowdhury Md, Aslam Hossain and Mohammad A Rashid, Preliminary Phytochemical and Pharmacological Investigations of *Alpinia conchigera* Griff. and *Plumbago indica* L, *Bangladesh Pharmaceutical Journal* 2012,15(2),153-157.
32. Pawar RK., Sharma shivani , Singh KC. and Sharma Rajeev K. HPTLC method for the determination of plumbagin from *Plumbago zeylanica* Linn. (root) *International Journal of Pharmacy and Pharmaceutical Sciences* 2010, 2, 26-28.
33. Ariyanathan A, Saraswathy G, Victor Rajamanickkam. Phytochemical Investigation of *Plumbago capensis* Thunb, *International journal of pharmacy & life sciences* 2011, 2(4), 670 - 673.

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Chapter 10

Phytochemical and Bio-efficacy Studies on *Plumbago rosea* L.

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1. Introduction

In fact, plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times (Farombi, 2003). Secondary metabolites produced by plants constitute a source of bioactive substances and nowadays the scientific interest has increased due to the search for new drugs from plant origin (Gothandam *et al.*, 2010). *Plumbago rosea* L. (Family: Plumbaginaceae), a perennial evergreen shrub with about 2 to 4 feet in height is traditionally used in skin disease, anaemia, irregular menstruation and leucorrhoea in the southeast area of Bangladesh (Yusuf *et al.*, 2007). The roots contain an alkaloid called plumbagin, a natural napthaquinone (5-hydroxy-2-methyl-1,4-naphthoquinone), possessing various pharmacological activities such as antimalarial (Didry *et al.*, 1994), antioxidant activity, anticancer, cardiotonic, antifertility action, antibiotic and antineoplastic (Nahak and Sahu, 2011). *Plumbago* species are reported in the literature for its biological activities such as: antiparasitic (Chan-Bacab and Peña-Rodríguez, 2001), insect anti-feedant (Villavicencio and Perez-Escandon, 1992), antitumoral (Devi *et al.*, 1994) and others, some of them attributed to the presence of special chemical compounds, such as naphthoquinones. All parts of *P. rosea* were used, but the roots have fascinated the chemists and biologists due to tremendous pharmacological properties. The pulped roots or aerial parts are reported abortifacient, while powdered bark, root or leaves are used to treat gonorrhoea, syphilis, tuberculosis, rheumatic pain, swellings, and wound healing (Thakur *et al.*, 1989). Root decoction with boiled milk is swallowed to

treat inflammation in the mouth, throat and chest. A paste of the root in vinegar, milk, and water is considered significant against influenza and black water fever, while root infusion is taken orally to treat shortness of breath (Teshome *et al.*, 2008). *Plumbago* species has been described for its significant anticancer (Xu *et al.*, 2010), antitumor (Yang *et al.*, 2010), anti-inflammatory (Sivakumar *et al.*, 2005), antimycobacterial (Patil *et al.*, 2011) and antimicrobial activities (Ravikumar *et al.*, 2011). The plant is also effective against rheumatic pain, sprains, scabies, skin diseases, and wounds. The roots of the plant and its constituents are credited with potential therapeutic properties including antiatherogenic, cardiotonic, hepatoprotective, neuroprotective, and central nervous system stimulating properties (Bagla *et al.*, 2011; Siddique *et al.*, 2011). With this background the present study was aimed to reveal the phytochemical and antibacterial activities of *P. rosea* against the selected human pathogens *viz.*, *P. aeroginosa*, *S. aureus*, *P. vulgaris*, *K. pneumoniae* and *B. subtilis*.

2. Materials and Methods

2.1. Collection of Plant Material

The aerial parts of *Plumbago rosea* were collected from the natural habitats at Chankanachari, Kerala, India. The plant materials were washed under running tap water to remove the surface contamimants and the leaves and stem were separated mechanically. The separated parts were air dried under shade. The dried sample was powdered using mechanical grinder and used for further extraction.

2.2. Extraction of Plant Material

25 g of air dried powder of the sample was extracted successively with organic solvents with 150 ml of solvents *viz.*, petroleum ether, chloroform, methanol and water with the increasing order of polarity using soxhlet apparatus. The extraction was carried out for 8 hours and the extract was concentrated by evaporation in a rota-vaccum. The extract obtained was used for the further assessment of phytochemical and antibacterial activity.

2.3. Preliminary Phytochemical Screening

To reveal the presence of steroids, terpenoids, cardiac glycosides, saponins, tannins, phenolics, amino acids, alkaloids, the preliminary phytochemical screening of various extracts of *Plumbago rosea* was carried out according to the method described by Harborne (1998).

2.4. Antibacterial Activity

Screening of antibacterial activity was performed by disc diffusion technique (Mukherjee, 2004). The methanolic extracts with various concentrations (50, 100, 150, 200, 250 µg/ml) were screened for antibacterial studies against selected human pathogens *viz.*, *P. aeroginosa*, *S. aureus*, *P. vulgaris*, *K. pneumoniae* and *B. subtilis* (Hardi *et al.*, 2004). Commercially available antibiotic disc Amikacin was implanted along with the crude extract disc on the surface of the Muller-Hinton agar plates, which is used as a positive control against Gram positive and Gram negative microbe respectively. The inoculated plates were incubated at 37°C for 18-24 hours and the zone of inhibition was measured and the results were tabulated.

3. Results and Discussion

3.1. Preliminary Phytochemical Screening

The secondary metabolites are responsible for the therapeutic properties of plants and the composition of these secondary metabolites varies from plant species to species. The composition of these compounds with the same species of plant can vary with the nutrient composition of the soil, climatic season, development stage of the plant and natural association with other plants (Renisheya Joy Jeba Malar *et al.*, 2012; Arunkumar and Muthuselvam, 2009). In the present study also preliminary phytochemical screening of nine different chemical compounds (steroids, saponins, phenolics, tannin, alkaloids, anthroquinone, cardiac glycosides, amino acids and terpenoids) were tested in four different extracts. Thus out of 36 (9 x 4 = 36) tests for the presence or absence of the above compounds, only 17 gave positive results and the remaining 19 gave negative results. The 17 positive results show the presence of steroids, saponins, tannin, anthroquinone, amino acid, terpenoids, phenolics, cardiac glycosides and alkaloids. Among the four different extracts, methanolic extract of *P. rosea* showed the maximum (7/9) presence *viz.*, steroids, tannin, alkaloids, phenolics, saponin, cardiac glycosides, and terpenoids. Chloroform extract of *P. rosea* showed the presence only five (5/9) compounds *viz.*, tannin, alkaloid, phenolics, cardiac glycosides and steroid. Petroleum ether extract showed the presence of only minimum (3/9) compounds (phenolics, saponin and steroid). Aqueous extract showed the presence of only two (2/9) compounds such as tannin and phenol (Table 10.1). Sheeja *et al.*, 2011 revealed the presence of phytoconstituents with vaired degree in *P. rosea* petroleum ether, chloroform, acetone, ethanol and aqueous extracts. They observed that ethanolic extract of *P. rosea* showed the presence of maximum number of (5) compounds *viz.*, naphthoquinone, carbohydrates, glycosides, tannins, flavanoids and saponins. But in the present study we observed seven metabolites present in the methanolic extracts of *P. rosea viz.*, steroids, tannin, alkaloids, phenolics, saponin, cardiac glycosides, and terpenoids. The results of preliminary phytochemical analysis confirmed the presence of tannin, saponin and cardiac glycosides; in addition

Table 10.1: Phytochemical screening of *P. rosea* various extracts.

Test	*Plumbago rosea*			
	Methanol	*Chloroform*	*Pet ether*	*Aqueous*
Steroid	+	+	+	–
Alkaloid	+	+	–	–
Phenol	+	+	+	+
Saponin	+	–	+	–
Tannin	+	+	–	+
Anthroquinone	–	–	–	–
Aminoacid	–	–	–	–
Terpenoid	+	–	–	–
Cardiac glycosides	+	+	–	–

we observed the presence of steroids, alkaloids, phenolics and terpenoids in the methanolic extracts of *P. rosea*. Sheeja *et al.*, observed five compounds *viz.*, cardiac glycosides, glycosides, tannins, flavanoids, proteins and saponins presence in the aqueous extracts of *P. rosea*. Contrary to Sheeja *et al.*, observation, in the present study aqueous extract showed the presence of only two (2/9) compounds such as tannin and phenol.

3.2. Antibacterial Activity

The *in vitro* antibacterial activity of methanolic extract of *P. rosea* was assessed by the agar disc diffusion method. The results were compared with the standard antibiotic Amikacin. 250 µg/ml methanolic extract of *P. rosea* showed good activity against *K. pneumoniae* (20 mm), *B. subtilis* (19 mm), *S. aureus* (17 mm) and moderate activity was observed against *P. vulgaris* and *S. aureus* (16 mm) and lowest activity was observed in 50 µg/ml of methanolic extract of *P. rosea* against *P. vulgaris* (6 mm). The antibiotic Amikacin showed highest zone of inhibition against *K. pneumoniae* (12 mm) and lowest activity was observed against *P. vulgaris* with 9 mm (Table 10.2). Haribabu Rao *et al.*, 2012 revealed that the aqueous extracts of *P. zeylanica* showed no inhibitory zones where as butanol extracts of *P. zeylanica* showed best inhibitory activity than benzene extracts. In the present study we observed the best inhibitory activity in the methanolic extracts of *P. rosea*. Shafiqur Rahman and Nural Anwar (2007) reported the antimicrobial activity of the crude ethanolic extract (250 µg/disc and 500 µg/disc) of *P. zeylanica* against the pathogenic bacteria *viz.*, *B. subtilis*, *B. cereus*, *B. megaterium*, *S. aureus*, *E. coli*, *V. cholerae*, *S. sonnei*, *S. typhi*, *S. paratyphi*, *P. mutabilis*) using the disc diffusion method. The ethanolic extract exhibited zone of inhibitions ranged from 8 to 18 mm in diameter with 250 µg/disc and 16-30 mm in diameter with 500 µg/disc concentration against the test bacteria. In the present study also we tested various concentrations of methanolic extracts using disc diffucion method. We also observed highest zone of inhibition 250 µg/ml methanolic extract of *P. rosea* against *K. pneumoniae* (20 mm), *B. subtilis* (19 mm), *S. aureus* (17 mm) and moderate activity was observed against *P. vulgaris* and *S. aureus* (16 mm). The present study results clearly showed that the methanolic extracts of *P. rosea* had significant and considerable antibacterial activity against various pathogens and further evaluation is necessary to find out the active principle compound responsible for bioactivity.

Table 10.2: Antibacterial activity of *Plumbago rosea* Methanolic extracts.

Pathogens	*Zone of Inhibition (mm)*					
	Concentration of Extracts in µg/ml					
	Amikacin	*50*	*100*	*150*	*200*	*250*
P. aeroginosa	10	9	11	13	14	16
S. aureus	11	7	10	12	16	17
P. vulgaris	9	6	8	10	14	16
K. pneumoniae	12	11	13	15	17	20
B. subtilis	11	7	9	12	14	19

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References

Arunkumar, S. and Muthuselvam, M., 2009. Analysis of Phytochemical Constituents and AntimicrobialActivities of *Aloe vera* L. against clinical pathogens. *World Journal of Agricultural Sciences*, 5(5): 572–576.

Bagla, V.P., McGaw, L.J. and Eloff, J.N., 2011. The antiviral activity of six South African plants traditionally used against infections in ethnoveterinary medicine. *Vet. Microbiol.*, doidx.doi.org/10.1016/j.vetmic. 09.015.

Chan-Bacab, M.J. and Peña-Rodríguez, L.M., 2001. Plant natural products with leishmanicidal activity. *Nat. Prod. Rep.*, 18: 674–688.

Devi, P.U., Solomon, F.E. and Sharada, A.C., 1994. *In vivo* tumor inhibitory and radiosensitizing effects of an Indian medicinal plant, *Plumbago rosea* on experimental mouse tumors. *Indian J. Exp. Biol.*, 32: 523–528.

Didry, N., Dubrevil, L. and Pinkas, M., 1994. Activity of anthraquinonic and naphthoquinonic compounds on oral bacteria. *Die Pharmazie*, 49: 681–683.

Farombi, E.O., 2003. African indigenous plants with chemotherapeutic potentials and biotechnological approach to the production of bioactive prophylactic agents. *African. J. Biotech.*, 2: 662–671.

Gothandam, K.M., Aishwarya, R. and Karthikeyan, S., 2010. Preliminary screening of antimicrobial properties of few medicinal plants. *Journal of Phytology*, 2: 1–6.

Harborne, J.B., 1998. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, 3rd edn. Chapman and Hall, New York, pp. 1–150.

Hardi, A.L. and Uddin, M.I., 2004. Seasonal variation in the intestinal bacterial flora of hybrid Tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) culture in earthen pond in Saudi Arabia. *Aquaculture*, 229(124): 37–44.

Haribabu Rao, D., Vijaya, T., Ramana Naidu, B.V., Subramanyam, P. and Jayasimha Rayalu, D., 2012. Phytochemical Screening and antimicrobial studies of compounds isolated from *Plumbago zeylanica*. L *IJAPBS*, pp. 82–90.

Mukherjee, K.L., 2004. *Medical Laboratory Technology*. Tata McGraw Hill Publishing Company Ltd., New Delhi.

Nahak, G. and Sahu, R.K., 2011. Antioxidant activity of *Plumbago zeylanica* and *Plumbago rosea* belonging to family plumbaginaceae. *Natural Product: An Indian Journal*, 7(2): 51–56.

Patil, C.D., Patil S.V., Salunke, B.K. and Salunkhe, R.B., 2011. Bioefficacy of *Plumbago zeylanica* (Plumbaginaceae) and *Cestrum nocturnum* (Solanaceae) plant extracts

against *Aedes aegypti* (Diptera: Culicide) and nontarget fish *Poecilia reticulata*. *Parasitol. Res.*, 108: 1253–1263.

Ravikumar, V.R. and Sudha, T., 2011. Phytochemical and Antimicrobial Studies on *Plumbago zeylanica* (L) Plumbaginaceae. *IJRPC*, 1: 185–188.

Renisheya Joy Jeba Malar, T., Johnson M., Nancy Beaulah, S., Laju, R, S., Anupriya, G. and Renola Joy Jeba Ethal, T., 2012, Anti-bacterial and antifungal activity of *aloe vera* gel Extract. *IJBAR*, 3(3).

Shafiqur Rahman and Nural Anwar, 2007. Antimicrobial activity of crude extract obtained from the root of *Plumbago zeylanica*. *Bangladesh J. Microbiol.*, 24(1): 73–75.

Sheeja, M., Joshi, S.B. and Jain, D.C., 2011. Antiovulatory and estrogenic activity of *Plumbago rosea* leaves leaves in female albino rats. *Indian J. Pharmacol.*, 41(6): 273–277.

Siddique, Y.H., Ara, G., Faisa, M. and Afzal, M., 2011. Protective role of *Plumbago zeylanica* extract against the toxic effects of ethinylestradiol in the third instar larvae of transgenic *Drosophila melanogaster* (hsp70–lacZ)Bg9 and cultured human peripheral blood lymphocytes. *Alternative Medicine Studies*, 1: 726–729.

Sivakumar, V., Prakash, R., Murali, M.R., Devaraj, H. and Devaraj, S.N., 2005. *In vivo* micronucleus assay and GST activity in assessing genotoxicity of plumbagin in Swiss albino mice. *Drug. Chem. Toxicol.*, 28: 499–507.

Teshome, K., Gebre-Mariam, T., Asres, K., Perry, F. and Engidawork, E., 2008. Toxicity studies on dermal application of plant extract of *Plumbago zeylanica* used in Ethiopian traditional medicine. *J. Ethnopharmacology*, 117: 236–248.

Thakur, R.S., Puri, H.S. and Husain, A., 1989. *Major Medicinal Plants of India*. Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

Villavicencio, M.A. and Perez-Escandon, B.E., 1992. Plumbagin activity (from *Plumbago pulchella* Boiss. Plumbaginaceae) as a feeding deterrent for three species of Orthoptera. *Folia Entomol Mex.*, 86: 191–198.

Xu, K.H. and Lu, D.P., 2010. Plumbagin induces ROS–mediated apoptosis in human *Promyelocytic leukemia* cells *in vivo*. *Leuk Res.*, 34: 658–65.

Yang, S.J., Chang, S.C., Wen, H.C., Chen, C.Y., Liao, J.F. and Chang, C.H., 2010. Plumbagin activates ERK1/2 and Akt via superoxide, Src and PI3–kinase in 3T3–L1 Cells. *Eur. J. Pharmacol.*, 638: 21–28.

Yusuf, M.A., Wahab, M.Y., Chowdhury, J.U. and Begum, J., 2007. Some tribal medicinal plants of Chittagong Hill Tracts, Bangladesh *Bangladesh J. Plant Taxon.*, 14: 117–128.

Nucleotide

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Plumbago auriculata voucher XCH 28090 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233547.1

FASTA Graphics

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  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 600)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
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Nucleotide

Display Settings: GenBank

Plumbago auriculata voucher XCH 28091 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233548.1

FASTA Graphics

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  TITLE     Studies on inter-specific and intra-specific variation in the genus
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  JOURNAL   Unpublished
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  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
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Plumbago auriculata voucher XCH 28092 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233549.1

FASTA Graphics

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  TITLE     Studies on inter-specific and intra-specific variation in the genus
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REFERENCE   2  (bases 1 to 593)
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  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
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Plumbago auriculata voucher XCH 28093 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233550.1

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  TITLE     Studies on inter-specific and intra-specific variation in the genus
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  JOURNAL   Unpublished
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  JOURNAL   Submitted (13-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
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  TITLE     Studies on inter-specific and intra-specific variation in the genus
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GenBank: KF261597.1

FASTA Graphics

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      421 aaattgaaca aatatgggcg tcccctattg ggatgtacta ttaaacctaa attggggttg
      481 tccgctaaga actacggtcg agctgtttat gaatgtcttc gcggcggact tgattttacc
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Nucleotide

Display Settings: GenBank

Plumbago indica voucher XCH 28102 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF261596.1

FASTA Graphics

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LOCUS       KF261596                586 bp    DNA     linear   PLN 10-AUG-2013
DEFINITION  Plumbago indica voucher XCH 28102 ribulose-1,5-bisphosphate
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ACCESSION   KF261596
VERSION     KF261596.1  GI:528917479
KEYWORDS    .
SOURCE      chloroplast Plumbago indica
  ORGANISM  Plumbago indica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 586)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            Plumbago (Plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 586)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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Nucleotide

Display Settings: GenBank

Plumbago indica voucher XCH 28103 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF261598.1

FASTA Graphics

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LOCUS       KF261598                591 bp    DNA     linear   PLN 10-AUG-2013
DEFINITION  Plumbago indica voucher XCH 28103 ribulose-1,5-bisphosphate
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            chloroplast.
ACCESSION   KF261598
VERSION     KF261598.1  GI:528917485
KEYWORDS    .
SOURCE      chloroplast Plumbago indica
  ORGANISM  Plumbago indica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
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            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 591)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            Plumbago (Plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 591)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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Nucleotide

Display Settings: GenBank

Plumbago zeylanica voucher XCH 28089 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233545.1

FASTA Graphics

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LOCUS       KF233545                591 bp    DNA     linear   PLN 10-AUG-2013
DEFINITION  Plumbago zeylanica voucher XCH 28089 ribulose-1,5-bisphosphate
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            chloroplast.
ACCESSION   KF233545
VERSION     KF233545.1  GI:528917454
KEYWORDS    .
SOURCE      chloroplast Plumbago zeylanica
  ORGANISM  Plumbago zeylanica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 591)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            plumbago (plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 591)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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Nucleotide

Display Settings: GenBank

Plumbago zeylanica voucher XCH 28095 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233546.1

FASTA Graphics

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ACCESSION   KF233546
VERSION     KF233546.1  GI:528917457
KEYWORDS    .
SOURCE      chloroplast Plumbago zeylanica
  ORGANISM  Plumbago zeylanica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 593)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            plumbago (plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 593)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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Nucleotide

Display Settings: GenBank

Plumbago zeylanica voucher XCH 28096 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233544.1

FASTA Graphics

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VERSION     KF233544.1  GI:528917450
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            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 593)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            plumbago (plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 593)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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Nucleotide

Display Settings: GenBank

Plumbago zeylanica voucher XCH 28097 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233543.1

FASTA Graphics

Go to:

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VERSION     KF233543.1  GI:528917446
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            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 404)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            plumbago (plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 404)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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Nucleotide

Display Settings: GenBank

Plumbago zeylanica voucher XCH 28098 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233552.1

FASTA Graphics

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LOCUS       KF233552                587 bp    DNA     linear   PLN 10-AUG-2013
DEFINITION  Plumbago zeylanica voucher XCH 28098 ribulose-1,5-bisphosphate
            carboxylase/oxygenase large subunit (rbcL) gene, partial cds;
            chloroplast.
ACCESSION   KF233552
VERSION     KF233552.1  GI:528917476
KEYWORDS    .
SOURCE      chloroplast Plumbago zeylanica
  ORGANISM  Plumbago zeylanica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 587)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            plumbago (plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 587)
  AUTHORS   Renisheya Joy Jeba Malar,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     SAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRWR"
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      121 tcttctactg gtacatggac aactgtgtgg accgatggac ttaccagcct tgatcgttac
      181 aaaggacgat gctaccacat cgagcctgtt cctggagaag aaagtcaatt tattgcttat
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      361 cctcctgctt attcgaaaac tttccaaggc ccgcctcacg gtatccaagt tgaaagagat
      421 aaattgaaca aatatgggcg tcccctattg ggatgtacta ttaaacctaa attggggttg
      481 tccgctaaga actacggtcg agctgtttat gaatgtcttc gcggcggact tgattttacc
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Nucleotide

Display Settings: GenBank

Plumbago zeylanica voucher XCH 28099 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233551.1

FASTA Graphics

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LOCUS       KF233551                586 bp    DNA     linear   PLN 10-AUG-2013
DEFINITION  Plumbago zeylanica voucher XCH 28099 ribulose-1,5-bisphosphate
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            chloroplast.
ACCESSION   KF233551
VERSION     KF233551.1  GI:528917473
KEYWORDS    .
SOURCE      chloroplast Plumbago zeylanica
  ORGANISM  Plumbago zeylanica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 586)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            Plumbago (Plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 586)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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            Sequencing Technology :: Sanger dideoxy sequencing
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                     /country="India: Palayamkottai, Tirunelveli district,
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                     /identified_by="Dr. M. Johnson"
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      361 cctcctgctt attcgaaaac tttccaaggc ccgcctcacg gtatccaagt tgaaagagat
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Nucleotide

Display Settings: GenBank

Plumbago zeylanica voucher XCH 28100 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: KF233542.1

FASTA Graphics

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            chloroplast.
ACCESSION   KF233542
VERSION     KF233542.1  GI:528917443
KEYWORDS    .
SOURCE      chloroplast Plumbago zeylanica
  ORGANISM  Plumbago zeylanica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            Caryophyllales; Plumbaginaceae; Plumbago.
REFERENCE   1  (bases 1 to 363)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Studies on inter-specific and intra-specific variation in the genus
            plumbago (plumbaginaceae) from south India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 363)
  AUTHORS   Renisheya Joy Jeba Malar,T. and Johnson,M.A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2013) Centre for Plant Biotechnology, Department
            of Botany, St. Xaviers College (Autonomous), North High Ground
            Road, Palayamkottai, Tirunelveli, Tamilnadu 627002, India
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                     KPKLGLSAKNYGRAVYECL"
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