PHYTOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SELECTED *CYATHEA* SPECIES FROM SOUTH INDIA

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FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BOTANY

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CERTIFICATE

This thesis entitled **"PHYTOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SELECTED *CYATHEA* SPECIES FROM SOUTH INDIA"** submitted by **Mr. N. Janakiraman** for the award of Degree of **Doctor of Philosophy in Botany** of Manonmaniam Sundaranar University is a record of bonafide research work done by him and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University / Institution.

Place : Palayamkottai

Date : 01.06.2015 **Signature of the Guide**

N. Janakiraman, M.Sc., M.Phil.,
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DECLARATION

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The PhD research that I had been through was truly a once in a lifetime experience. I realize that there are many people who deserve to be thanked for what I have been achieving and for what I have become now. It is indeed a great pleasure for me to express my profound gratitude to every distinguished one who helped me to make this humble endeavour to its fruitful completion. They will always be in my heart, in my mind and in my grave.

First and foremost, I would like to thank my greatest teacher of all: **Lord Almighty** who is always with me and showers his blessings and grace in all walks of my life.

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N. Janakiraman

ABBREVIATIONS USED

%	: Percentage
°C	: degree Celsius
µg	: microgram
µl	: microlitre
µm	: micrometre
1D	: One Dimensional
2D	: Two Dimensional
2-DE	: Two Dimensional Gel Electrophoresis
A	: Adenine
A+T	: Adenine + Thymine
ABS	: Absorbance
ACN	: Acetonitrile
ACP	: Acid Phosphatase
AD	: Anno Domini
AFLP	: Amplified Fragment Length Polymorphism
AGE	: Agarose Gel Electrophoresis
AMOVA	: Analysis of Molecular Variance
AOAC	: Association of Analytical Communities
ATP	: Adenosine triphosphate
BC	: Before Christ
BLAST	: Basic Local Alignment Search Tool
bp	: base pairs
C	: Cytosine
CBoL	: Consortium for the Barcode of Life
cm	: centimeter
Conc.	: Concentration
cpDNA	: Chloroplast DNA
CTAB	: Cetyl Trimethyl Ammonium Bromide
Da	: Dalton
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribo Nucleic Acid

dNTP	: Deoxyribo nucleotide triphosphate
DPPH	: 1,1-diphenyl-2-picrylhydrazyl
EDTA	: Ethylene Diamine Tetra Acetic acid
ESI-MS	: Electrospray Ionization - Mass Spectrometry
EST	: Esterase
ETS	: External Transcribed Spacer
eV	: electron Volt
FASTA	: FAST Algorithm
FBS	: Fetal Bovine Serum
Fig.	: Figure
FT-IR	: Fourier Transform - Infra Red
g	: gram
G	: Guanine
GA	: Gibberellic Acid
GC	: Gas Chromatography
GC-MS	: Gas Chromatography - Mass Spectrometry
GeLC-MS	: Gel Based Liquid Chromatography - Mass Spectrometry
h	: hours
HIV	: Human Immunodeficiency Virus
HPLC	: High Performance Liquid Chromatography
HPTLC	: High Performance Thin Layer Chromatography
IC_{50}	: half maximal Inhibitory Concentration
ii	: identical pairs
IR	: Infra Red
ISSR	: Inter Simple Sequence Repeat
ITS	: Internal Transcribed Spacer
kDa	: kilodalton
kV	: kilovolt
L	: Litre
LC	: Lethal Concentration
LCL	: Lower Confidence Limit
LC-MS	: Liquid Chromatography - Mass Spectrometry
$\log_{10}$	: base 10 logarithm
M	: Molarity

m/z	: mass-to-charge ratio
MALDI-TOF MS	: Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry
*mat*K	: megakaryocyte associated tyrosine Kinase
MCF 7	: Michigan Cancer Foundation 7
MEGA	: Molecular Evolutionary Genetics Analysis
mg	: milligram
min	: minutes
ml	: milliliter
mm	: millimetre
mM	: millimolar
MRSA	: Methicillin-resistant *Staphylococcus aureus*
MS	: Mass Spectrometry
MTT	: (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)
MULTALIN	: Multiple Alignment
MW	: Molecular Weight
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NAPRALERT	: Natural Products Alert
NBT	: Nitro Blue Tetrazolium
NCBI	: National Center for Biotechnology Information
NEDA	: N-(1-naphthyl) ethylene diamine dihydrochloride
ng	: nanogram
NIST	: National Institute of Standard Technology
nm	: nanometre
NMR	: Nuclear Magnetic Resonance
NTSYS	: Numerical Taxonomy System
OD	: Optical Density
PASS	: Prediction of Activity Spectra for Substances
PCR	: Polymerase Chain Reaction
Pet. ether	: Petroleum ether
pH	: power of Hydrogen ion concentration
pmoles	: picomoles
PP	: Protein Position
ppm	: parts per million

PRX	: Peroxidase
PVP	: Poly Vinyl Pyrrolidone
Py-GC-MS	: Pyrolysis - Gas Chromatography - Mass Spectrometry
QSAR	: Quantitative Structure Activity Relationship
RAPD	: Random Amplified Polymorphic DNA
*rbc*L	: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
rDNA	: Recombinant DNA
REE	: Rare Earth Element
R_f	: Retardation factor
RFLP	: Restriction Fragment Length Polymorphism
RH	: Relative Humidity
ROS	: Reactive Oxygen Species
RP-HPLC	: Reverse Phase - High Performance Liquid Chromatography
rpm	: revolutions per minute
RT	: Retention Time
SD	: Standard Deviation
SDS-PAGE	: Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
sec	: seconds
si	: transitional pairs
SNP	: Single Nucleotide Polymorphism
SPSS	: Statistical Package for the Social Sciences
sv	: transversional pairs
T	: Thymine
Taq	: *Thermus aquaticus*
TBE	: Tris Borate EDTA
TCA	: Trichloroacetic acid
TE	: Tris EDTA
TEMED	: Tetra methyl ethylene diamine
TFA	: Tri Fluoro Acetic acid
TLC	: Thin Layer Chromatography
Tris HCl	: Tris aminomethane Hydrochloride
tRNA	: transfer RNA
U	: Uracil
UCL	: Upper Confidence Limit

UPGMA	: Unweighted Pair Group Method with Arithmetic mean
USA	: United States of America
USDA	: United States Department of Agriculture
USFDA	: United States Food and Drug Administration
UV / ICP-MS	: Ultra Violet / Inductively Coupled Plasma - Mass Spectrometry
UV	: Ultra Violet
UV-Vis	: Ultra Violet - Visible
V	: Volt
v/v	: volume / volume
VOC	: Volatile Organic Compounds
w/v	: weight / volume
WHO	: World Health Organization
XCH	: St. Xavier's College Herbarium
λ_{max}	: Wavelength of maximum absorption

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1. Introduction

"Ferns in art convey the idea of solitary humility, frankness and sincerity, because they conceal their grace and beauty in forest depth"

Ferguson

"The bright coloured flowers are attracted by least intellectuals, but the beauty of form and texture of ferns require a higher degree of mental perception and a more cultivated intellect for its proper appreciation"

Abraham Stansfield

Myriads of living organisms described in terms of species and varying individuals of a species are distributed on the planet earth. Mankind is almost totally dependent on plants for their basic requirements. About 1.9 million plant species have been described so far, the estimated total number of species on earth exceeds 11 million (Chapman, 2009). Pteridophytes, the pioneer colonizers on earth, are one of the ubiquitous vegetation about 350 million years ago and they dominated the land in the Carboniferous period. It possesses simple organization and is unique in being characterized by cryptogamic mode of reproduction. They are very conspicuous and gorgeous elements of biodiversity which occurs in various kinds of habitats ranging from sea level to mountain top and tropical to subpolar regions (Dudani and Ramachandra, 2010).

Pteridophytes occupy in between the non-vascular plants and seed plants in the phylogeny of plant kingdom and represent a broad spectrum of biological types from small filmy to arborescent tree ferns and from submerged aquatics to epiphytes and xerophytes (Kumar, 1998). In the world flora, about 13,600 species of extant pteridophytes are recorded (Moran, 2008). Among which 1,300 species into 70 families and 191 genera occurs in

different biogeographical regions of India (Chandra *et al.,* 2008) with the main centers being the Himalayas, the Western Ghats and the Eastern Ghats (Chandra, 2000; Dixit, 2000).

The Indian sub-continent is bestowed with a wide range of climatic and altitudinal variations. The Western Ghats is one of the 34 global biodiversity hotspots and are one of the important centres of plant diversity and richness of fern flora of the world. It covers a distance of about 1600 km from the South of the river Tapti in Gujarat to the tip of South India, Kanyakumari in Tamilnadu. It has perennial streams, evergreen forests, grasslands and many other habitats harbouring about 320 species of ferns and fern allies with more species diversity in the southern part (Manickam, 1995). The unique physiography with mountainous terrain, narrow gorges and valleys with heavy rainfall has blessed this region with an environment most congenial to luxurient plant growth (Nampy and Madhusoodanan, 1998).

Unfortunately, ferns form a neglected group of plants in biodiversity as far as their economic value is concerned. This is not because of the misunderstood fact that they lack any economic utility, but the real fact is enough attention has not been paid towards assessing the potentialities of ferns and fern allies towards human welfare. However, with the introduction of ethnobotany by Hershberger (1896) for the study of relationship which exists between peoples of primitive societies and their plant environment, many attempts were made on the study of relationships of pteridophytes with man, particularly for their medicinal value. Theophrastus (327-287 BC) and Dioscorides (50 AD) mentioned the medicinal attributes of certain ferns. They have been successfully used in the homoeopathic, ayurvedic, unani and tribal systems of medicines (Das, 2003; Rout *et al.,* 2009). Apart from the medicinal properties, ferns have great aesthetic value for their graceful, delicate beauty and great diversity of foliage. Most of the ferns are shade loving and they are very good for interior decoration and green houses (Chandra and Kaur, 1974).

Plants synthesize a wide variety of chemical compounds which can be sorted by their chemical class, biosynthetic origin and functional groups. The medicinal value of plants lies in chemical substances or group of compounds that produce a definite physiological action in the human body (Edeoga *et al.,* 2005). The beneficial effects of plants are usually due to the secondary metabolites present in it. Medicinal plants contain active ingredients that can be used for therapeutic purposes and are precursors of chemotherapeutical semisynthesis (WHO, 1979). It also provides temporary relief to symptomatic problems, health promoting characteristics and has curative properties. With the advent of modern scientific methods, medicinal plants came under chemical scrutiny, leading to the isolation of the active principles. Soon after their isolation and characterization, these compounds either in pure state or in the form of well-characterized extracts became part of pharmacopoeias of several countries.

Chemotaxonomy had a considerable impact on plant systematics and new systems of classification were being developed which took account on the distribution of secondary metabolites (Dahlgren, 1980). Phytochemistry is one of the rapidly expanding areas of plant taxonomy (chemosystematics) which utilizes chemical information to improve the classification of plants. The origin of chemotaxonomy may date back to thousands of years i.e. from the time of using wild plants as a source of medicine (Stace, 1989). In the earlier days, only morphological characters were used to identify the drug. But now, identification of plants based on morphological parameters is very tenuous. To overcome this, molecular markers are used as an important tool to better characterize such species. Another form of accomplishing variability studies is the development of analytical techniques that can quantify chemical markers with medicinal activity in the species in study. Phytochemical characters could also be used as markers to identify and differentiate the species. These

markers can identify chemotypes and correlate them with the existent genetic variability (Silva *et al*., 2006).

According to draft guidelines stated by the USFDA, a marker compound is a chemical constituent of a botanical raw material that is used for identification or quality control purposes, especially when the active constituents are not identified. The active constituent is responsible for the intended pharmacological activity or therapeutic effects. Chemical standardization often involves chemical identification by spectroscopic or chromatographic fingerprint and chemical assay for active constituents or marker compounds if available. The analytical methods developed can be used for chemical fingerprinting and assaying of marker or active compounds (Eng and Ong, 2004). Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the chemical integrities of the herbal medicines and its products are therefore be used for authentication and identification of the plant (Farooqui *et al.,* 2014).

Plant extracts can be evaluated by various biological methods to determine pharmacological activity, potency and toxicity. Qualitative and quantitative chemical examination is designed to detect and isolate the active ingredients (AOAC, 2005). The quantitative determination of phytoconstituents has been made very easy by developments in analytical instrumentation. Recent advances in the isolation, purification and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the process of standardization. TLC, HPTLC, HPLC and GC can determine the homogeneity of a plant extract. UV-Vis and FT-IR spectrometry, MS, GC in combination with MS (GC-MS), NMR and electrophoretic techniques are powerful tools often used for standardization and to control the quality of both the raw material and the finished product. The results from these sophisticated techniques provide a chemical fingerprint as to the

nature of chemicals or impurities present in the plant extract (Bilia *et al.,* 2002; Rozylo *et al*., 2002; WHO, 2002). Information on these chemical constituents not only aid in discovering new therapeutic drugs, but such information can also help in disclosing new sources of economic materials which are precursors for the synthesis of complex chemical substances (Farnsworth, 1996).

There is a growing tendency all over the world to shift from synthetic to natural products with medicinal properties. Major classes of antimicrobial compounds from plants include phenolics, terpenoids, alkaloids, essential oils, lectins, polypeptides and polyacetylenes (Kothari *et al.,* 2010). Phytotherapy has been considered as an alternative to alleviate side effects associated with synthetic drugs (Sanchez-Lamar *et al.,* 1999). The presence of bioactive compounds in plants represents a useful area for development of natural products that can be used as substitutes for antibiotics resistant to pathogenic microorganisms. Furthermore, they provide the foundation for the development of new antimicrobials (Delahaye *et al.,* 2009).

Molecular systematic studies on plants are also essential to study the nature of distribution and variability among them and it is supported by phytochemical analysis. It is important for the identification and selection of superior genotypes for further exploitation. Molecular markers have become fundamental tools for understanding the inheritance and diversity of natural selection. Development of markers should be very useful for assessing the level of variation within the same and different groups and also for identifying the mechanisms responsible for variation. The earliest morphological markers are governed by alleles with a major phenotypic effect with little or no environmental effects (Hershey and Ocampo, 1989). The second generations of markers were biochemical which provides a useful tool for genetic fingerprinting (Pillai *et al.,* 2000). Recently, molecular markers like

proteins, isozymes, RFLP and RAPD-PCR have revealed unsuspected level of variation in a large number of species (Johnson *et al.,* 2012a; Gad *et al.,* 2013).

Electrophoresis is a versatile biochemical technique that is used as complementary strategy to traditional approaches for assessment of genetic diversity and conservation of plant genetic resources (Hamrick and Rickwood, 1990). Several different proteomic strategies were designed to characterize the proteome of a cell / tissue / organ in different states or living conditions. One of the most facile, comprehensive and unbiased strategies for protein profiling is SDS-PAGE. It enables us to identify variation in the physical and chemical properties of proteins (Rabbani *et al*., 2001). Isozyme analysis also offers a rapid and more reliable means of producing genetic profiles (fingerprints) and elucidation of genetic relationships within and different taxa. These two techniques are used as efficient tools for genetic, systematic and plant breeding studies (Mukhlesur *et al*., 2004).

Proteins can directly reflect alteration in the DNA sequence through changes in amino acid composition. If the protein occurs in variant forms, then differences in the overall ionic charges of the molecules can result. This caused variation in their electrophoretic mobilities and different forms of the protein migrate at different speeds through gel medium. The resulting banding pattern is an electrophoretic phenotype (Wendel, 1989). Bands were manually excised from the gel and to identify the cognate proteins, mass spectrometry approaches are used depending on the situation. Generally two forms of mass spectrometry are used for protein identifications, both of which employ “soft” ionization techniques (Tanaka *et al.*, 1988; Fenn, 2002). The first is MALDI-TOF MS used to perform peptide mass fingerprinting. It can rapidly measure the molecular weights of different proteins (Karas and Hillenkamp, 1988). The second is ESI-MS, which is usually coupled to HPLC sample separation and is often used in tandem mass spectrometry to undertake peptide fragmentation

(Chen, 2008). The resulting proteins are expected to be useful in describing the main metabolic pathways.

The classical morphological authentication approach was confronted with difficulties due to overly similar traits used for taxonomic characterization and an ever-decreasing number of specialists (Ching, 1978). At this multi-faceted interface, a new technology for rapid, accurate and convenient species identification termed “DNA barcoding” was recently developed (Kress *et al*., 2005; Miller, 2007). This can be potentially used to rapidly identify pteridophytes to the species level (Schneider and Schuettpelz, 2006; Ebihara *et al.,* 2009; Li *et al*., 2009), thus enabling detailed field studies linking the distribution and ecology of fern gametophytes and sporophytes. Despite these promising applications, ferns with their critical phylogenetic position as sister to seed plants have largely been neglected in choosing the standardized barcode. At present, techniques for studying the molecular phylogeny of ferns rely heavily on chloroplast genome sequence data. This is because the chloroplast genome has a simple and stable genetic structure, it is haploid, there are no (or very rare) recombination, it is generally uniparentally transmitted and universal primers can be used to amplify target sequences. Another important reason is the ease of PCR amplification and sequencing of chloroplast genes (Hurst and Jiggins, 2005).

The development of universal DNA barcoding markers for land plants is challenging and the exact choice of loci has been heavily debated (Kress *et al.*, 2005). Recently, the CBoL decided on a standard two-locus barcode for all land plants, consisting of portions of the *rbc*L and *mat*K plastid genes (Hollingsworth *et al*., 2009). It was immediately emphasized that this core barcode might have to be augmented with supplementary loci in some groups due to lack of discriminatory power and primer universality. *rbc*L has been routinely used for studies on fern phylogeny (Pryer *et al.,* 2004; Schneider *et al*., 2004a) and species discrimination (Jansen and Schneider, 2005; Schneider *et al*., 2005). The generation of *mat*K

sequences for ferns is currently problematic, because this part of the chloroplast genome underwent a strong restructuring during the evolution of the fern clade (Duffy *et al*., 2009). None of the currently existing primer sets are likely suitable for all lineages of land plants (Hollingsworth *et al.,* 2009; Li *et al*., 2009).

As far as the South Indian ferns are concerned, taxonomical and cytological studies have been carried out almost completely (Manickam and Irudayaraj, 1988; 1992 and 2003). Since then, there are limited efforts to know the bioactive compounds responsible for various biological activities and also there is a lack of knowledge to find the similarity and variation between them. In recent times, there is an increased emphasis in molecular markers for characterization of genotype, genetic fingerprinting and understanding of inter-relationship at molecular level. Using the molecular trees as a phylogenetic framework, there is an opportunity to examine and discuss similarities and dissimilarities of secondary metabolite profiles (Wink and Mohamed, 2003).

Being a vast country rich in pteridophytes, it will be more practical if detailed studies are undertaken on selected group of ferns. There exists a situation for the detailed scientific documentation and the need of time to explore the tree ferns viz., *Cyathea nilgirensis* Holttum*, Cyathea gigantea* (Wall. ex Hook.) Holttum and *Cyathea crinita* (Hook.) Copel. (Cyatheaceae) which are available in Southern part of India. Pith of *C. nilgirensis* is used against snake bite (Singh, 1999). It has central analgesic activity (Dhawan *et al.,* 1977) and anti-diabetic activity (Kumar *et al.,* 2012). Fresh rhizome of *C. gigantea* mixed with powdered black pepper seeds are taken orally with milk twice a day for one week in empty stomach against white discharges (Rout *et al.,* 2009). Rhizome is used against snake bite. Aerial parts of *C. gigantea* have anti-inflammatory properties (Asolkar *et al*., 1992). Fronds of *C. gigantea* are used for decoration by tribes. The stem is cut and used for the cultivation of epiphytic orchids (Kumar *et al.,* 2003). Rhizome and sporophyll of *C. crinita* have

antibacterial properties (Singh and Viswanathan, 1996; Singh, 1999). The fibers from the large, thick stipes are used in nurseries as potting materials to maintain moisture and for this purpose the plants are over-collected from the wild and all the species of *Cyathea* become rare and endangered.

Although there is ample reason to believe that ferns could contain astonishing bioactive compounds and variations, tree ferns are largely unexplored. In order to fulfill the lacuna, the present investigation intends to fill the gap in research for better upgradation of knowledge regarding the phytochemicals to screen the plant extracts or the pure secondary metabolites for certain types of bioactivities and to test the feasibility of species identification in the selected *Cyathea* species across a wider taxon range with a convenient DNA marker. To select the best DNA barcode, a series of tests with criteria such as DNA isolation, PCR amplification, primer efficiency, direct sequencing success rate and variation among the species were conducted.

The specific objectives of the present study are as follows:

- To carry out qualitative phytochemical screening of the selected *Cyathea* species.
- To elucidate the presence of various compounds using UV-Vis, FT-IR, HPTLC, HPLC and GC-MS analysis.
- To reveal the protein profile of selected *Cyathea* species using SDS-PAGE.
- To identify protein similarities and variations by applying MALDI-TOF-MS analysis.
- To assess the molecular variation by means of isozymic profile.
- To estimate the phylogenetic and evolutionary relationship among the selected species of *Cyathea* using *rbc*L gene.
- To evaluate the antioxidant properties of selected *Cyathea* species.
- To determine the cytotoxicity using brine shrimp lethal test and MCF 7 cell line.
- To study the larvicidal activity against the filarial vector *Culex quinquefasciatus.*

Nature has been a source of medicines for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Plants are the major component of traditional healing system in developing countries, which have also been an integral part of their history and culture. Plant-based medicinal systems continue to play an important role in primary health care, with about 80% of the world's inhabitants relies mainly on traditional medicines (Owolabi *et al*., 2007). In India, higher plants as sources of medicinal compounds have sustained to play a pivotal role in shaping the life of human beings since ancient times. Unfortunately, pteridophytes are the least exploited group of plants in India compared to other countries of the world.

Despite the richness and wide diversity of fern flora in our country, less attention has been devoted to pteridophyte research and the interest has undergone resurgence in the last decade. This renewed special attention of the researchers, plant lovers and horticulturists on ferns from diverse quarters within the scientific community.

Diversity of Pteridophytes

Many researchers have carried out significant work on South Indian fern diversity which forms the baseline for the studies even till now. The first illustrated account on the "Ferns of Peninsular India" is that of Rheede (1703) who included few ferns and fern allies from the Malabar coast. However, Beddome (1864) done the only comprehensive work on the ferns of South India. After that, Beddome (1873) in his publication entitled, "The Ferns of Southern India" has listed the various ferns from Southern India. Bir and Vasudeva (1971) recorded 118 species of ferns from the Palni hills. Nayar and Kaur (1974) in their book entitled, "Companion to Beddome's Handbook to the Ferns of British India" have listed the nomenclatural changes with regard to Beddome's (1883, 1892) names only. Chandra and

Kaur (1987, 1994) have updated the nomenclature of all the taxa illustrated in Beddome's Ferns of South India, Ferns of British India and Ferns of South and British India.

Later, Manickam and Irudayaraj (1992) mentioned 256 ferns and fern allies with suitable illustrations in "Pteridophytic Flora of the Western Ghats, South India". Chandra (1999) investigated the inventory and documentation of the Indian fern flora and compiled "The Ferns of India". Manickam and Rajkumar (1999) commented upon the polymorphism in 100 South Indian ferns. Manickam and Irudayaraj (2003) listed 144 species in "Pteridophyte Flora of Nilgiris, South India" with short description along with identification keys for genera and species. Chandra *et al*. (2008) surveyed 414 species of rare and threatened pteridophytes of political India adopting modern taxonomic concepts and nomenclature. Fraser-Jerkins (2008) gave a new picture of fern taxonomy and nomenclature in the Indian subcontinent and enumerated 300 Indian subcontinental pteridophytes.

Tree ferns

Christensen (1905) separated the genus *Cyathea* into three genera: *Cyathea, Hemitelia* and *Alsolphila* mainly on the basis of presence or absence of indusium. Holttum (1963) worked on the old world species and recognized only one genus *Cyathea*. Holttum (1965) while working on Asian species of tree ferns recognized 11 species of *Cyathea* from the Indian region of which *Cyathea nilgirensis* was described as a new species. Tryon (1970) recognized six genera of paleate Cyatheaceae viz., *Sphaeropteris, Alsophila, Nephelea, Trichopteris, Cyathea* and *Cnemideria*. Tryon (1976) revised the genus *Cyathea* and recognized 40 species. Pichi-Sermolli (1977) agrees with the view of Tryon regarding the recognition of the six genera in the family Cyatheaceae. Dixit (1984) listed 11 species under the genus *Cyathea* from India. Floristic revision studies on the family Cyatheaceae by Dixit (1998) agrees with the view of Pichi-Sermolli (1977) in recognizing only six paleate genera of Tryon (1970). Out of these six genera, he reported *Alsophila* and *Sphaeropteris* from

Indian region. Nayar and Geevarghese (1993), Rajagopal and Bhat (1998) and Pullaiah *et al*. (2003) identified certain species from South India and treated the species under the genus *Cyathea*.

Tree ferns are usually considered under a single family Cyatheaceae except Holttum (1973) who suggested a polyphyletic derivation with four families. The largest of these groups is the family Cyatheaceae which include the world's tallest tree fern *Cyathea brownii* that reaches height up to 20 m (Large and Braggins, 2004). It comprises about 500 species (Korall *et al.,* 2006) classified into four genera viz., *Cyathea, Alsophila, Cnemidaria* and *Sphaeropteris* (Lellinger, 1987). Among these, 200 species are neotropical, the majority belonging to the genus *Cyathea* (Lehnert, 2009). It is one of the most interesting families among the pteridophytes due to their striking morphology and wide geographical distribution with diversity centers in the tropics, subtropics and southern temperate regions. They are considered as primitive, though they represent different lines of evolution. These ferns display great ecological conservatism as most species are terrestrial plants of moist forests and are intolerant to longer periods of drought or frost. Furthermore, they show a greater provincialism and endemism than most fern groups (Tryon and Gastony, 1975).

In general, all these works are merely a survey of pteridophytic flora which deals with the morphology of pteridophytes. However, a well-resolved taxonomy, which would be the basis for many studies, is still unavailable. With the traditional healing system that is actively searching and expanding its pharmacopoeia in order to treat a huge number of complaints, an environment with great floral diversity is slipping away unlearned by a new generation of healers. The scientific and traditional communities need a resource where data on the phytochemical and molecular aspects of these pteridophytes are collated.

Phytochemistry in Pteridophytes - Preliminary level

Pteridophytes have long been considered as an essential group and a great deal of phytochemical work has recently been aimed at resolving relationships among the disparate groups. At the same time the utility of ferns for analysis of plant function and development has been increasingly appreciated and capitalized upon. Benerjee and Sen (1980) conducted extensive survey on antibiotic activity among ferns and reported about a hundred species having such property. Dixit and Vohra (1984) reported edible and medicinally important pteridophytic species from India. Many ferns, among many other plants, were used for medicinal purposes by the early Greeks and Romans through the middle ages. Dioscorides, a first century botanist, noted the use of spleenworts for curing maladies of the spleen (Irudayaraj and Raja, 1998).

The selection criteria of medicinal plants, which contain potentially new biological agents is based on five principle approaches viz., random, taxonomic, phytochemical, ethnomedicinal and information-managed approach. In the random approach, all the available species are collected, irrespective of prior knowledge and experience. In the taxonomic approach, plants of a specific genus or family are deemed to be of interest, and sought from diverse locations. The phytochemical (chemo-taxonomic) approach is based on a particular compound type, which is of biological interest. Taxonomic and the phytochemical approaches are closely related and cannot be clearly divided from each other. In the ethnomedicinal approach, credence is given to information on the medicinal use of the plant. Based on this information, the plant is collected and evaluated (Cordell *et al*., 1991). Information-managed plant selection collates taxonomic, biological, ethnomedicinal and phytochemical information to afford a list of plants for specific collection. The information is compiled through computerized databases such as NAPRALERT, a specialized relational database on natural products, based on systematic literature searches (Farnsworth, 1993).

Medicinal ferns from India are gaining importance in recent days due to the fact that they have been subjected to phytochemical screening. Phytochemical analysis on ferns has been done in large scale as compared to other cryptogams and studied to a lesser extent as compared to the phanerogams (angiosperms). Most of the phytochemical works on Indian ferns pertain to the primary metabolites to explain various natural phenomenons in plants. According to Irudayaraj and Raja (1998), though ferns possess less chemotaxonomic value, they are highly important from physiological, ecological and nutritional point of views.

Phytochemical analysis of the edible ferns, *Ampelopteris prolifera* and *Diplazium esculentum* shows the nutritional value (Shankar and Khare, 1985). Phytochemical analysis on rare, endangered and medicinally important spleenworts, *Asplenium* and *Psilotum* was investigated by Lal (1979), Rohtagi *et al.* (1984), Khare and Shankar (1987) and Varma (1992). Quantitative analysis of pigments (chlorophylls, carotenoids), carbohydrates (sugars, starch) and nitrogenous compounds (amino acids, proteins, nitrogen) have been done in a large number of South Indian Thelypteroid ferns (Britto *et al.,* 1991, 1993), *Hypolepis, Pteridium, Histiopteris* and *Cyathea* (Gopalakrishnan *et al.,* 1993), *Pteris* (Jesudass *et al.,* 1993) and Rajasthan ferns and fern allies (Kaur *et al.,* 1986; Vyas and Sharma, 1988; Sharma, 1989; Rathore and Sharma 1990; Harsh and Sharma, 1994; Vyas *et al.,* 1995).

The study of free amino acids in *Ophioglossum* leaves at the time of spike initiation indicates the close relationship between the genetic set-up and incorporation of amino acids into the structural and functional building blocks (Khandelwal and Goswami, 1976). Chark and Dhir (1991) analyzed the soluble proteins in *Pteris vitatta* during rhizoidal differentiation. Yadav (1995) investigated the possible role and behaviour of phytochemical compounds such as sugars, proteins and amino acids during the biorhythmic movements of leaflets in three species of *Marsilea*.

The behaviour of chlorophylls, carotenoids and phenolics in drought resistance ferns and fern allies (*Selaginella*) from Rajasthan has been studied by Bohra *et al.* (1979), Vyas *et al.* (1989), Rathore and Sharma (1991) and Sharma *et al.* (1992). A reverse trend has been observed in the content of carotenoids by Kumar (1995). Ramachandran *et al.* (1991) and Raja *et al.* (1995) have made ecophysiological studies on the ferns from Kothayar and Palni hills (South India). Ahluwalia *et al.* (2002) investigated the spectrum application of *Azolla* with regard to the effect of various metals.

Britto *et al.* (1994a) studied the phytochemicals present in *Sphaerostephanos* species of Western Ghats. Preliminary phytochemical screening of 19 species of South Indian Thelypteroid ferns showed the occurrence of steroids, alkaloids, phenolics, catechins, saponins and tannins in all the species (Britto *et al.,* 1994b, 1994c). Triterpenoids and anthraquinone are not found in any of the species investigated. Irudayaraj (1996) has reported the presence of triterpenoids in the epidermal glands of *Christella parasitica*. Jesudass *et al*. (2001) screened the phytochemicals in the members of Pteridaceae in Western Ghats, South India. Jadhav *et al.* (2011) analyzed the phytochemicals in eleven species of ferns from the Satara district of Maharashtra.

Recently, a large number of ferns and fern allies viz., *Selaginella inaequalifolia* (Irudayaraj *et al*., 2010); *Cyathea brunoniana* (Talukdar *et al.,* 2010), *Cyathea gigantea* (Talukdar *et al.,* 2010; Kiran *et al.,* 2012), *Diplazium* species (Sivaraman *et al*., 2011a), *Selaginella tenera* (Suganya *et al.,* 2011), *Azolla pinnata*, *Marsilea minuta* and *Salvinia molesta* (Mithraja *et al.,* 2011), *Acrostichum aureum* (Thomas, 2012), *Azolla microphylla* (Abraham and Aeri, 2012), *Adiantum capillus-veneris* (Ahmed *et al.,* 2012; Kumar and Nagarajan, 2012), *Hemionitis arifolia* (Bindu *et al.,* 2012), *Sphenomeris chinensis* (Aceret, 2012), *Christella* and *Adiantum* (Mithraja *et al.,* 2012) and *Asplenium aethiopicum* (Johnson

et al., 2014) have been subjected to qualitative phytochemical screening of different metabolites by various workers.

Phytochemistry in Pteridophytes - Analytical methods

In natural product drug discovery, the conventional approach of extraction, isolation, separation, identification, characterization and test for the desired biological activity suffers from problems like lower yields, de-replication, difficulty in separation and inconsistent biological activity. However, with the introduction of innovative technologies like high throughput screening and combinatorial chemistry with their promise of a seemingly inexhaustible supply of compound libraries has greatly contributed to this declining interest in the screening of natural products by the pharmaceutical industry.

In the recent decades, there has been an increasing interest in the application of chemical evidence to taxonomic problems. Biochemical markers have their own significance and importance in chemical fingerprinting. Allozymes were the best biochemical markers in plants due to various strengths. However, modern and sensitive technologies for identifying markers based on biochemical / gene expression such as UV-Vis, FT-IR, TLC, HPTLC, HPLC, GC-MS and NMR have replaced allozymes. A plant during its life span produces various phytoactive compounds as secondary metabolites for its own growth and survival. Identification and characterization of these active principles can be used in generating a species specific fingerprint (Anandjiwala *et al*., 2006, 2007). Therefore the marker based on secondary metabolites should be able to discriminate one species from another species and one accession from other accessions.

Micro FT-IR spectra of the cuticles of three Carboniferous medullosan seed-fern leaf species (*Macroneuropteris scheuchzeri, Alethopteris lesquereuxii* and *Neuropteris ovata* var. *simonii*) showed oxygenated functional groups (carboxyl and ketone) with strong bands in the aliphatic stretching region revealed the complexity of the molecular structure (Lyons *et al.,*

1995). FT-IR spectra of seed-fern *Eusphenopteris neuropteroides* and true sphenopterid fern *Oligocarpia brongniartii* were very similar except the true fern does not have aromatic bands in 700-900 cm^{-1} out-of-plane region. Py-GC-MS analysis showed more aromatic compounds for the seed fern than the sphenopterids. Comparison of FT-IR and py-GC-MS characteristics of sphenopterids and other plant groups confirmed that these two techniques have potential to identify chemotaxonomic signals from Carboniferous pteridophylls (Zodrow and Mastalerz, 2002).

Psenicka *et al.* (2005) studied the functional groups of fossil Marattialeans and chemotaxonomic implications for Pennsylvanian tree ferns and pteridophylls. The species *Pecopteris nyranensis, Pecopteris miltonii, Pecopteris aspidioides* and *Pecopteris polypodioides* are differentiable from one another by combined FT-IR characteristics and the ratio of CH_2/CH_3 is hypothesized to be a chemotaxonomic parameter for Pennsylvanian pteridophylls both in seed and true ferns. Baran and Rolleri (2010) characterized the biominerals in ferns (Marattiaceae) using IR spectroscopy. They confirmed the accumulation of biogenic silica in the tissues of *Angiopteris, Christensenia, Danaea* and *Marattia* and also showed the presence of calcium oxalate probably as weddellite.

Saito *et al.* (1989) reported the distribution of ptaquiloside and ptaquiloside-like compounds in Pteridaceae by chemical assay (TLC) and observed the widespread occurrence in a variety of ferns, including *Cheilanthes myriophylla*, *Cibotium barometz*, *Dennstaedtia scabra*, *Histiopteris incisa*, *Pityrogramma calomelanos*, *Pteris cretica*, *Pteris nipponica*, *Pteris oshimensis*, *Pteris tremula* and *Pteris wallichiana*. Sharma and Sharma (1992) identified various flavonoids in eight different ferns from Rajasthan by paper chromatography. Krishna and Dawra (1994) reported ptaquiloside in *Pteris quadriaurita* and *Onychium contiguum* for the first time in India, besides bracken fern by TLC method.

Irudayaraj and Johnson (2011a) used TLC separation to study the inter-specific relationship among the three *Asplenium* species. *Asplenium affine* and *Asplenium decrescens* showed 42% of similarity coefficient whereas *Asplenium zenkeranum* was varied from *A. affine* and *A. decrescens* with 36% variance. Pathania *et al.* (2012) analyzed the flavonoid quercetin in various ferns growing in northern India using TLC. Ferns from Himachal Pradesh viz., *Christella arida*, *Deparia japonica*, *Dryopteris cochleata*, *Dryopteris juxtaposita*, *Hypodematium crenatum*, *Polystichum squarrosum* and *Pteridium revolutum* contain a variable range of quercetin. Similarly, *P. squarrosum* and *P. cretica* from Uttarakhand contained higher concentration of quercetin.

Chikmawati *et al*. (2012) performed TLC tests in various *Selaginella* extracts to qualitatively analyze the bioactive compounds alkaloids, flavonoids and steroids. Mandal and Mondal (2012) analyzed the free amino acids present in the leaf glands of pteridophytes using TLC. DL-methionine is the common free amino acid of *Pteris vittata, Drynaria quercifolia, Ampelopteris prolifera* and *Dryopteris filix-mas*. L-tyrosine monohydrochloride are common in *D. filix-mas* and *Selaginella indica*. L-arginine monohydrochloride is also common in *D. quercifolia, Ceratopteris thallictroides* and *Marsilea quadrifolia*. Glycine is the only amino acid found in *Helminthostachys zeylanica*.

Srivastava *et al.* (2008) analyzed the HPTLC profile of *Lycopodium clavatum* stem using the mobile phase toluene: ethyl acetate: formaldehyde (6:3:1) and confirmed the presence of ferulic acid. Paul and Banerjee (2013) determined the HPTLC profile of flavonoids using the mobile phase ethyl acetate - formic acid - glacial acetic acid - water (10 : 0.5 : 0.5 : 1.3) in *Pteris vittata*. Aqueous extract showed six peaks and ethanolic extract showed twelve peaks with varied R_f values.

Smith *et al.* (1989) analyzed *Cheilanthes sieberi* from New Zealand and Australia using RP-HPLC and confirmed the presence of ptaquiloside and other potentially

carcinogenic pterosin B precursors. Lai *et al.* (2005) separated new REE-binding protein from the lamina of *Pronephrium simplex* using HPLC coupled with online UV/ICP-MS. Amino acid composition analysis by RP-HPLC indicated that the protein has relatively high contents of proline and glycine. Chen *et al.* (2007) identified phenolic antioxidant compounds from the aqueous extract of Sword Brake fern *Pteris ensiformis* by HPLC.

HPLC analysis of *Pteris biaurita* following elution from TLC plate revealed a single peak with the retention time of 8.1 min (Dalli *et al*., 2007). Ho *et al.* (2008) identified different ecdysteroids in Polynesian medicinal fern *Microsorum membranifolium* using HPLC. Paulraj *et al.* (2011) studied the presence of various kinds of terpenoids, alkaloids, tannins, saponins and flavonoids on the epidermal glands extract of the glandular morphotype *Christella parasitica* using HPLC. Zhang *et al*. (2012a) illustrated the occurrence of flavonoids in different parts of *Dryopteris erythrosora* by means of HPLC.

Mostafa and Ibrahim (2012) analyzed α-tocopherol in *Azolla caroliniana* using HPLC which showed great quantitative variations, whereas ascorbic acid and β-carotene showed marked changes in both number and area of the characterized peaks subjected to UV-B. Chikmawati *et al*. (2012) determined the highest amentoflavone (6.87 ppm) content in *Selaginella subalpina* using HPLC analysis. Pathania *et al.* (2012) quantified the carcinogen, ptaquiloside in various ferns growing in certain enzootic areas of Himachal Pradesh and Uttarakhand. *Dryopteris cochleata*, *Hypodematium crenatum*, *Pseudocyclosorus canus* and *Pteris cretica* were identified to contain ptaquiloside for the first time on HPLC and LC-MS analyses.

Flavonoids of four species of *Angiopteris* indicated that di-C-glycosyl flavones and flavone-o-glycosides might be characteristic of distinct group of eusporangiate ferns (Wallace *et al*., 1981). Yamane *et al.* (1988) isolated endogenous gibberellins from sporophytes of two tree ferns, *Cibotium glaucum* and *Dicksonia antarctica*. The total gibberellin content in *C.*

glaucum (tall) was atleast one order of magnitude greater than that of *D. antarctica* (dwarf) based on total ion current response in GC-MS and bioassay data. Oxodihydrophaseic acid was the major component of *C. glaucum* and abscisic acid was the major component present in *D. antarctica.*

Patitucci *et al.* (1995) used high resolution GC coupled with computerized MS to perform direct analysis of crude extracts and pre-fractions of Brazilian Polypodiaceae members viz., *Pleopeltis angustum, Microgramma vacciniifolia, Polypodium meniscifolium* and rhizome of *Polypodium aureum.* Wynne *et al*. (1998) confirmed new gibberellin like antheridiogen from gametophytes of the fern *Lygodium circinnatum* as the methyl ester of 9,11-didehydro-GA_{20}. Kurumatani *et al.* (2001) isolated the gibberellins A_{73} methyl ester, the most abundant antheridiogen and the methyl esters of GA_9 and several monohydroxy GA_{73} derivatives from the Schizaeaceous ferns *Lygodium microphyllum* and *Lygodium reticulatum.* Ramesh *et al.* (2001) isolated friedelin, epifriedelinol, β-amyrin, β-sitosterol, β-sitosterol 3-β-D-glucopyranoside and naringin from the dried rhizome of *Drynaria quercifolia.*

Nakane *et al.* (1999) identified six new migrated hopane triterpenoid alcohols from *Adiantum capillus-veneris* viz., pteron-14-en-7a-ol, fern-9(11)-en-3a-ol, fern-7-en-3a-ol, adian-5(10)-en-3a-ol, adian-5-en-3a-ol and fern-9(11)-en-28-ol. Alam *et al.* (2000) isolated normethyl lupine type and lanostane type triterpenes in the aerial parts of *Adiantum venustum*. On the basis of spectral data, the structures of these triterpenes have been established as 30-normethyl lupine-20-one, 30-normethyl olean-3-one-30-betol and lanost-20(22)-ene-30-ol. Bresciani *et al.* (2003) demonstrated the presence of terpenoids as predominant metabolites in *Adiantum cuneatum*. Methanolic crude extracts led to the isolation of four known triterpenoids viz., filicene, filicenal, adiantol and isoadiantone.

Melos *et al*. (2007) isolated a mixture of long-chain carboxylic acid esters as the ethyl esters of palmitic, petroselenic, oleic, (Z)-vacenic, stearic, linoleic, decosahexenoic, α-

linolenic, margaric, arachidic and behenic acids from the ethanolic extracts of *Adiantum tetraphyllum.* Further purifications led to the isolation of β-sitosterol, two triterpenes: 30-normethyl-lupan-20-one and hopan-22-ol, two diterpenes: phytol and phyten-3(20)-1,2-diol, two flavonoids: quercetin and quercetin-3-O-β-D-glucoside, a mixture of ferulic acid, caffeic acid and p-hydroxybenzaldehyde.

GC-MS characterization of *Anemia tomentosa* var. *anthriscifolia* essential oil composition detected the presence of sesquiterpenes (75%) with lower amounts of monoterpenes (15%). The sesquiterpenes were composed mainly of oxygenated components (67%) viz., α-bisabolol (51%), spathulenol (1%), caryophyllene oxide (3%), α-bisaboloxide (1%), 14-hydroxy-9-epi-(E)-caryophyllene (1%) and two unknown components (5%). The monoterpenes were dominated by neral (5%) and geranial (7%) with lower amounts of α-pinene, camphene, 6-methyl-5-hepten-2-one, 1,8-cineole and pinocarveol (Juliani *et al.,* 2004).

Santos *et al.* (2006) reported the presence of isoafricanol, a highly uncommon sesquiterpene in the essential oil of *A. tomentosa* var. *anthriscifolia.* Pinto *et al*. (2007) studied the chemical composition of the essential oil from *A. tomentosa* var. *anthriscifolia* and detected the presence of sesquiterpenes of the silfiperfolane, pre-silfiperfolane, isocumene, cariophyllene, pre-nopsane and nopsane types. They also reported the occurrence of two major components, pre-silfiperfolane-1-ol and silfiperfol-6-ene. In addition to this, Pinto *et al.* (2009a) detected thirty one substances from *A. tomentosa* var. *anthriscifolia* and Pinto *et al*. (2009b) isolated a new triquinane sesquiterpene (-)-epi-presilfiperfolan-1-ol, which was reevaluated and named as 9-epi-presilfiperfolan-1-ol (Nathan *et al.*, 2010).

Methanolic extract of *Selaginella lepidophylla* contains 3-methylenhydroxy-5-methoxy-2,4-dihydroxy tetrahydrofurane, which can act as a slight inhibitory effect on the uterus contraction (Perez *et al.*, 1994). *S. lepidophylla* is also reported to contain volatile oils

(Cetto and Heinrich, 2005). *Selaginella uncinata* has chromone glycosides, namely uncinoside A and uncinoside B which showed antiviral activities (Ma *et al*., 2002). Ethanolic extract of *S. uncinata* contains flavonoids that possess a benzoic acid substituent (Zheng *et al.,* 2008). *Selaginella sinensis* has a glucoside, namely selaginoside (Dai *et al.*, 2006), a sesquilignan, namely sinensiol A (Wang *et al*., 2007), secolignans, namely styraxlignolide D and neolloydosin (Feng *et al*., 2009). Acetone extract of *S. sinensis* contains selaginellin A, an unusual flavonoid pigment (Zhang *et al.,* 2007). *Selaginella labordei* contains 4'-methylether robustaflavone, robustaflavone, eriodictyol and amentoflavone (Tan *et al*., 2009). *Selaginella moellendorfii* contains several pyrrolidinoindoline alkaloids (Wang *et al*., 2009). Isocryptomerin isolated from *Selaginella tamariscina* showed potent antibacterial activity against Gram positive and Gram negative bacterial strains including clinical isolates of antibiotic-resistant species MRSA (Juneyoung *et al.,* 2009). *Selaginella* species also have large number of bioactive compounds, the most important being biflavonoids which constituted 13 compounds viz., amentoflavone, 2',8"-biapigenin, delicaflavone, ginkgetin, heveaflavone, hinokiflavone, taiwaniaflavone, isocryptomerin, kayaflavone, ochnaflavone, podocarpusflavone A, robustaflavone and sumaflavone (Setyawan, 2011).

GC-MS analysis of antifungal component of *Pteris biaurita* revealed six major peaks in the retention time range of 7.2-10.9 min which may contain a mixture of eicosenes and heptadecanes (Dalli *et al.,* 2007). Hashemi *et al.* (2007) studied the volatile components of *Artemisia aucheri* using GC-MS which includes 1,8-cineol (22.8%), chrysanthenone (18.16%), a-pinene (8.33%) and mesitylene (7.41%). Choudhary *et al*. (2008) isolated two glycosides viz., 6'-O-(3,4-dihydroxy benzoyl)-β-d-glucopyranosyl ester and 4-O-β-d-glucopyranoside-3-hydroxy methyl benzoate along with five known compounds viz., methyl benzoate, hypogallic acid, caffeic acid, paeoniflorin and pikuroside from a fresh water fern *Salvinia molesta* showing potent antioxidant radical scavenging activity.

Peres *et al.* (2009) identified the steroid β-sitosterol, the triterpene hopan-22-ol, the flavone glycoside 6-metoxiapinenin-7-O-β-D-allopyranoside and a mixture containing the ethyl esters of hexadecanoic, oleic, 15-methyl-heptadecanoic and linoleic acids from the fronds of *Microgramma vacciniifolia*. Fons *et al.* (2010) investigated the VOC from five French ferns using GC-MS. The main volatile compound of *Adiantum capillus-veneris* was (E)-2-decenal. The volatile profiles of *Athyrium filix-femina* and *Blechnum spicant* showed similarities with small amounts of isoprenoids. The major volatile compound of *Dryopteris filix-mas* was (E)-nerolidol. Polyketides, as acylfilicinic acids, were mainly identified in this fern. *Oreopteris limbosperma* contained the highest biodiversity of VOC. 80% of the volatiles was obtained from the terpenic pathway. The most important volatiles were (E)-nerolidol, alpha-terpineol, beta-caryophyllene and other minor monoterpenes.

Nilesh *et al.* (2011) predicted the compounds 3,4-dihydroxy benzoic acid, linoleic acid, chlorogenic acid, 4-hydroxy benzoic acid and ferulic acid from the ethanolic extracts of *Polypodium decumanum.* Narasimhaiah *et al.* (2012) isolated two new glycosidic compounds viz., 2-(3,4-O-Diglucos cinnamoyl)-4-hydroxyl furan and 1-Heptaloyl, 8-hexyl, 3-(O-diglucos), 10-methyl, 9,10-dihydro naphthalene and characterized using TLC, IR, UV spectral analysis, NMR and mass spectra from the ethyl acetate extract of *Actiniopteris radiata*. Mishra and Verma (2013) revealed the presence of a flavonol glycoside, quercetin-3-O-β-glycosyl (1→2) rhamnoside from an antifungal active fraction of n-butanol soluble aqueous ethanolic extract of *Cheilanthes grisea*.

Phytochemistry - Tree ferns

The first scrutinization on flavonoid constituents in the genus *Cyathea* was carried out by Harada and Saiki (1955). They examined the leaves of *Cyathea fauriei* and *Cyathea hancockii* during a complete survey on the distribution of flavones, flavonols and flavanones in Japanese ferns. Hiraoka and Hasegawa (1975) detected flavonoid glycosides in five

Cyathea species from Tokyo. Hiraoka and Maeda (1979) isolated new acylated flavonol glycoside from the fronds of *Cyathea contaminans* and chemically characterized as kaempferol-7-(6"-succinyl)-glucoside. Yamane *et al.* (1985) identified ten gibberellins from GC-MS analysis of purified extracts of *Cyathea australis* which include the known GA_1, GA_4, GA_9, GA_{15}, GA_{24}, GA_{35}, GA_{58} and three new GAs including 12β-hydroxy GA_9 (GA_{69}), 12α-hydroxy GA_9 (GA_{70}) and 12β-hydroxy GA_4 (GA_{71}).

C. gigantea contains several active constituents viz., triterpenes, sterols, saponins, flavonoids, hentriacontane, β-sitostenone, β-sitostanone, diploterol, sitosterol, hopan-29-ol and the whole plant contains oleanolic acid (Juneja *et al.,* 1990). Gopalakrishnan *et al*. (1993) quantitatively estimated the presence of starch, total sugars, aminoacids, proteins, chlorophyll a, chlorophyll b, total chlorophylls and carotenoids on the lamina of *C. crinita, C. gigantea* and *C. nilgirensis.* They also studied the distribution of various aminoacids present in the chloroform and ethanolic extracts using the mobile phase n-butanol: acetic acid: water (12:3:5).

Cyathea has also some records of phytochemical studies related to the production of fernene, filicene and hopane triterpenes (Arai *et al.,* 1994, 1995), phenolic acids (coumaric and caffeic), protocatechuic acids and flavonoids represented mainly by kaempferol glycosides (Bringmann *et al.*, 1999). Arai *et al.* (2003) isolated dryocrassyl formate, sitostanyl formate and 12 α-hydroxyfern-9(11)-ene from the fresh fronds of *Cyathea podophylla* and their structures were elucidated by spectroscopic techniques and synthesis. Ten known triterpenoids, three derivatives of phytol, a stanol and β-tocopherol were also identified from *C. podophylla*.

Cyathea phalerata determined the presence of an active flavonoid (kaempferol-3-neohesperidopside) with antioxidant and hypoglycaemic activity (Cazarolli *et al.,* 2006). Cyathenosin A, a spiropyranosyl derivative of protocatechuic acid was isolated from the stem

pith of *C. phalerata*. Its structure was determined by MS, 1D and 2D NMR spectroscopic analyses and confirmed by single crystal X-ray analysis. Cyathenosin A is the first example of a naturally occurring compound containing a spirocyclic orthoester pyranosidic structure (Pizzolatti *et al.,* 2007). Brighente *et al.* (2007) reported the isolation of kaempferol-3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, 4-O-β-D-glucopyranosyl caffeic acid and kaempferol from the wood of *C. phalerata.* Further studies by Hort *et al*. (2008) resulted in the isolation and identification of nine substances: kaempferol-3-neohesperidoside, 4-O-β-D-glucopyranosyl caffeic acid, 4-O-β-D-glucopyranosyl p-coumaric acid, β-sitosterol, 3,4-spyglucopyranosyl protocatechuic acid, sitosterol β-D-glucoside, kaempferol, vitexin and ethyl galactoside.

Talukdar *et al.* (2010) performed TLC profiling of *C. gigantea* and *C. brunoniana* in five different solvent systems for the presence of diverse group of phytochemicals. Different R_f values of the compound in caudex and leaves extracts reflects an idea about their polarity and selection of solvents for separation of phytochemicals. Zodrow and Mastalerz (2010) applied FT-IR as an investigative technique in *Cyathea caracasana* for documenting quality spectra suitable for the interpretation of phytochemicals. Based on the absorbance spectra, five sample sets were reduced to three related-chemical groups. The reduction is principally based on aliphatic and oxygen-containing moieties.

Chemical constituents of ethanolic extracts of *Cyathea spinulosa* were isolated by chromatographic techniques and their structures were elucidated by spectral methods. The compounds include (2S,3S,4R)-2-[(2'R)-2'-hydroxytetracosanoylamino]-stigmast-3,6-dione, β-sitosterol, 1,3,4-octadecanetriol, stigmast-4-ene daucosterol, ergosterol, 3,6-dione, 1-O-beta-D-glucopyranosyl-(2S,3R,4E,8Z)-2-[(2-hydroxyoctadecanoyl)amido]-4,8-octadecadiene -1,3-diol and protocatechuic aldehyde (Jiang *et al*., 2012).

Molecular markers

Information on genetic variation within and among populations is crucial for conservation of rare and endangered species. A species may be considered as a group of individuals organized into populations that share an amalgamation of indicative characters which are not found outside the group. Survival chance of a species is indicated in genetic diversity within the population (Tsuda *et al.,* 2009). Molecular markers have been used for assessing genetic diversity and generating baseline information (Pertoldi *et al.,* 2007; Mirialili *et al.,* 2009). In order to deduct a concrete, reliable data, a marker should meet many criteria; a marker should be inheritable and have the power to discriminate between individuals. It should be easy to generate and interpret. It should be highly polymorphic in nature and frequently distributed throughout the genome. Moreover, a marker should be easy to detect and comparable with similar characters (Hillis and Moritz, 1990).

Though there are no such ideal markers existing, three classes of markers based on morphological, biochemical and molecular traits are routinely explored. Morphological markers are usually visually characterized phenotypic characters such as sorus type, shape and arrangement, indusium attachment, rhizome, type of fertile segments, vein branching, rachis scales and microphyllous leaves (Lasalita-Zapico *et al.,* 2011). Biochemical markers are differences in protein and enzymes that are detected by electrophoresis and specific staining (Pillai *et al.,* 2000). The major disadvantages of these two markers are they may be limited in number and are influenced by environmental factors or the developmental stages of the plant (Winter and Kahl, 1995).

A first map of the human genome based on molecular markers (Botstein *et al.,* 1980) fuelled the development of genomic maps in other organisms. Consequently, polymorphisms have been detected in restricted genomic DNA of plants and paved way to the development of molecular markers for plant breeding. The number of polymorphic morphological markers

is limited, especially in intra-specific crosses, and their expression is influenced by the environment. Therefore, more reliable markers such as proteins or more specifically, allelic variants of several enzymes called isozymes had to be considered (Tanksley and Orton, 1983; Weeden *et al.,* 1988).

DNA sequence-based molecular markers were introduced in 1970s, and they became the preferred method when PCR technology was invented in 1985. They are the most widely used marker predominantly due to their abundance and exhibit a much higher level of allelic variation than morphological and biochemical markers (Weising *et al.,* 2005). DNA markers arise from different classes of DNA mutations such as substitutions, rearrangements or errors in replication of tandemly repeated DNA (Paterson, 1996). They are selectively neutral because they are usually located in non-coding regions of DNA (Winter and Kahl, 1995). DNA markers may be broadly divided into three classes based on the method of their detection viz., DNA-DNA hybridization as in RFLP and AFLP, PCR technique as in RAPD, SNP, ISSR and the method of DNA sequencing (Winter and Kahl, 1995; Gupta *et al.,* 1999; Joshi *et al.,* 1999). These markers reveal genetic differences that can be visualized by using a technique called gel electrophoresis and staining with ethidium bromide, silver nitrate and detection with radioactive probes.

Proteomic analysis

Proteomic analysis started to arouse tremendous attention after the completion of sequencing human genome. One of the major goals to sequence the complete human genome is to reveal and understand the linkage between genes and their related proteins. Characterizing and understanding the proteomic composition in any biological sample can provide essential information about complex cellular regulatory networks (Domon and Aebersold, 2006). Although the concept of the "proteome" is currently receiving considerable attention, identification of specific proteins is an established technique whereby isolated

proteins are first separated by their isoelectric point in the one dimensional electrophoresis and then separated by their molecular weight in the second dimension using SDS-PAGE (Mukhlesur and Hirata, 2004).

Rodin and Rask (1990) isolated and characterized 2.2s storage protein (matteuccin) from the ostrich fern *Matteuccia struthiopteris.* Amino acid analysis revealed that the 2.2s protein is rich in arginine. Farrant *et al.* (2009) isolated total and heat stable proteins from desiccation tolerant and desiccation sensitive fronds of the fern *Mohria caffrorum.* The desiccation mechanism is seasonally regulated and it is due to the *de novo* production of the protein identified as a putative chaperonin. Deeba *et al.* (2009) carried out proteomic studies in *Selaginella bryopteris* to understand the mechanism of desiccation tolerance. About 250 putatively identified protein spots were reproducibly detected and analyzed. This indicates that the proteins involved in transport, targeting and degradation were expressed more in the desiccated frond *S. bryopteris*.

Wang *et al.* (2010) identified 138 desiccation-responsive 2-DE spots representing 103 unique proteins in resurrection fern-ally *Selaginella tamariscina*. Hierarchical clustering analyses revealed that 83% proteins were down-regulated upon dehydration. The dynamic expression changes of the desiccation-responsive proteins provide strong evidence that cell structure modification, photosynthesis reduction, antioxidant system activation and protein post-transcriptional/translational modifications are essential to the fern-ally *S. tamariscina* in response to dehydration. Bona *et al.* (2010) investigated the frond proteome of the arsenic hyperaccumulator fern *Pteris vittata* which differentially modulates expression of 130 leaf proteins with specific responses in *Glomus mosseae* or *Gigaspora margarita* colonized plants.

Revathy *et al.* (2011) determined the protein expression of *Adiantum raddianum, Arachniodes tripinnata, Dryopteris sparsa* and *Odontosoria chinensis* collected from

Kothayar, South India using SDS-PAGE. The selected ferns showed unique banding pattern of proteins in different developmental stages viz., gametophyte without sex organ, gametophyte with sex organ, gametophyte with juvenile sporophytes and matured sporophytes which represented the "protein finger print" of that particular species. Sivaraman *et al.* (2011b) studied the reproductive biology and protein expression studies on the different developmental stages of South Indian ferns viz., *Tectaria paradoxa*, *Araiostegia hymenophylloides* and *Deparia petersenii*. The gametophytes of *T. paradoxa* and *D. petersenii* are associated with algae or moss on the surface of the soil. The variation in the total number of bands in different species designates the difference in genetic diversity. The difference is expressed in mature sporophytes with minimum number of bands in *T. paradoxa* and maximum number of bands in *A. hymenophylloides*.

Balbuena *et al.* (2012) characterized the proteome of *Equisetum hyemale* rhizomes using GeLC-MS spectral counting proteomics strategy. Non-redundant proteins identified in the rhizomes apical tip and elongation zone were 1,911 and 1,860 respectively. Narayani and Johnson (2013) revealed the protein variability among ten *Selaginella* species using SDS-PAGE. A total of 190 bands with various R_f values were observed. Each region expressed different proteins with varied molecular weight which act as representative of the expression of a particular gene in the studied species of *Selaginella*.

MALDI-TOF MS analysis

Knowledge of plant development and function can be obtained by determining the distribution of proteins and metabolic processes within plant tissues. MALDI-TOF MS has recently become a popular and versatile method to analyze peptides, proteins and other biomolecules. It is used as a post separation protein identification tool and the mass patterns obtained were used for comparison with known libraries to confirm peptides. Though it is not the most rigorous approach to protein identification, it still represents an economically

convenient alternative to more complex MS systems especially when proteomic analyses are carried out on plant species whose complete genome/protein databases are complete or well annotated.

Lai *et al.* (2005) separated new REE-binding protein from the lamina of *Pronephrium simplex* and further characterization of the protein showed its molecular mass 5068.4 Da by MALDI-TOF MS and ESI-MS. Moore *et al.* (2006) purified sporopollenin from *Selaginella pallescens* and *Lycopodium clavatum* using MALDI-TOF MS. The mass spectra obtained from the spores extracted with a base, acid and solvent treatment represents pure sporopollenin. Both indicate ions at approximately m/z 8000 and 4400 to 4800 with the *L. clavatum* analysis also generating ions between m/z 6936 and 7588 (in positive ionisation mode) and at m/z 17654.

Ekman *et al.* (2008) used 2-DE and MS for comparative protein expression profiling of a cyanobacterium dwelling in leaf cavities of the water-fern *Azolla filiculoides*. Homology-based protein identification using peptide mass fingerprinting, tandem MS analyses and sequence homology searches resulted in an identification success rate of 79% of proteins analyzed in the unsequenced cyanobiont. Martinez-Cortes *et al.* (2012) purified two cationic peroxidases from *Selaginella martensii* (PRX2 and PRX3) using ammonium sulfate precipitation, adsorption chromatography and cationic exchange chromatography. The molecular mass for PRX2 and PRX3 was 36.3 kDa and 45.6 kDa according to MALDI-TOF/TOF. Both enzymes showed a typical peroxidase UV-Vis spectrum with a Soret peak at 403 nm for PRX2 and 404 nm for PRX3. The specific activities showed against several substrates and the kinetic parameters suggest that PRX2 and PRX3 have specific roles in cell wall formation and especially in lignin biosynthesis. Peptides from tryptic digestion of both peroxidases were also identified through MALDI-TOF MS/MS.

Isozymic analysis

Isozymes have been proven to be reliable genetic markers in plant systematic studies due to consistency in their expression, irrespective of environmental factors. In addition, it allows quantification of genetic homology and distance within and between species (Onus and Pickergill, 2000). These markers can correctly identify several levels of taxa, accessions and individuals since the assumption of homology can be more accurate than some genomic DNA markers (Klaas, 1998). Electrophoretic analyses of isozymes in natural populations allow exploration of levels and patterns of genetic variability. However, a subsequent investigation demonstrated that a homosporous fern with the lowest number for its genus possessed a diploid gene expression profile with Mendelian inheritance (Gastony and Gottlieb, 1982). Furthermore, it was demonstrated that the complex isozyme banding patterns of Chapman *et al*. (1979) were in part attributable to subcellularly compartmentalized isozymes (Gastony and Darrow, 1983; Wolf *et al*., 1987). Continued isozyme investigations supported the observation that homosporous ferns with base chromosome numbers for their genus have diploid expression profiles despite possessing relatively high chromosome numbers (Gastony and Gottlieb, 1985; Haufler and Soltis, 1986; Soltis, 1986; Haufler, 1987).

The use of isozymes to test hypotheses in reticulately evolved complexes has often proved valuable in fern populations, for example in the forest species (Soltis and Soltis, 1987, 1988), epiphytic species (Ranker, 1992; Hooper and Haufler, 1997; Vogel *et al.,* 1999; Wubs *et al*., 2010) and colonizing species (Wolf *et al.*, 1988). Pryer and Haufler (1993) examined the isozyme, chromosomal and morphological characters for the allotetraploid origin of *Gymnocarpium dryopteris*. Watano and Masuyama (1994) carried out isozymic variation studies on 27 natural populations of *Ceratopteris thalictroides* in Japan. Among the 15 enzyme loci examined, 8 loci were genetically polymorphic. Marked genetic differentiation was observed between populations to the south and north of Okinawa Island at six loci viz.,

Lap, Pgi-2*, Pgm*-3*, Pgm*-4*, Idh*-2 and *Skd*-2. Hauk and Haufler (1999) surveyed and compared 16 of the 24 currently recognized cryptic species of *Botrychium* subgenus *Botrychium* for isozyme variation. Inter-specific comparisons distinguished six diploid species and provided evidence of molecular differentiation between the cryptic sister species *Botrychium lunaria* and *Botrychium crenulatum.*

Herrero *et al.* (2001) studied the isozymic variation and genetic relationships among taxa in the *Asplenium obovatum* group. Electrophoretic analyses of eight enzyme systems encoded by fourteen putative loci were conducted. Alleles of the loci *Lap*-1*, Mdh*-2*, Mdh*-3*, Pgm*-1*, Pgm*-19 and 6*Pgd*-1 emerged as genetic markers for the diploids and were present in an additive pattern in most of the analyzed individuals of the tetraploid. Pajaron *et al.* (2005) evaluated the different systematic treatments of *Asplenium seelosii* by means of isozyme electrophoresis. Seventeen populations throughout the range of the complex were studied and fifteen enzymatic systems were assayed. There was no genetic identity within and between populations. Cheng *et al.* (2008) analyzed the allozyme polymorphisms of *Alsophila spinulosa* from 9 sites throughout Taiwan using 6 enzyme systems viz., esterase, isocitrate dehydrogenase, menadione reductase, 6-phosophogluconate dehydrogenase, shikimic acid dehydrogenase and malate dehydrogenase. The genetic diversity of *A*. *spinulosa* was higher than mean values of other diploid ferns and tree ferns. Highest expected heterozygosity was observed in Nanjenshan population located in South Taiwan.

Johnson *et al.* (2009) illustrated the isozymic evidence for the common origin of *Diplazium* species confined to South India and Sri Lanka. Maximum degree of diversity has been observed in *Diplazium travancorium* with the presence of fourteen bands, followed by *Diplazium polypoides* with nine bands, in contrast *Diplazium cognatum* and *Diplazium dilatum* have only four bands and two bands respectively. Stein *et al.* (2010) demonstrated the molecular profiles of tetraploids to verify their undiscovered diploid ancestor and

reconstructed *Dryopteris semicristata* using isozymes and restriction site analysis of cpDNA. Johnson *et al.* (2010a) studied the isozymic banding profiles of *Tectaria coadunata*, *Tectaria wightii* and *Tectaria paradoxa* using SDS-PAGE and confirmed that all the three species are morphologically and genetically distinct, but cytologically uniform. Johnson *et al.* (2010b) compared the isoperoxidase banding profile on selected species of *Pteris* from India. A total of thirty eight bands scored in thirty one different positions with eight active zones revealed the biochemical positions of the *Pteris* complex.

Johnson *et al.* (2010c) initiated isozymic analysis on six different species of *Adiantum* from the Western Ghats, South India. Among these, *Adiantum raddianum* and *Adiantum lunulatum* banding profile showed high percentage of similarity index compared to other species. Johnson *et al.* (2010d) studied the genetic distinction of three filmy ferns viz., *Trichomones obscurum*, *Trichomones proliferum* and *Trichomones plicatum* belonging to different morphological forms growing in different ecological niche using isozymic variation. Irudayaraj and Johnson (2011b) explored the identity and phylogenetic relationships of *Sphaerostephanos arbuscula, Sphaerostephanos unitus* and *Sphaerostephanos subtruncatus* through isozymic analysis and confirmed the distinctness of three species based on macro-micromorphology, phytochemistry, cytology and isozymic profile.

Johnson *et al*. (2012a) studied the isoperoxidase variation on three South Indian tree ferns viz., *Cyathea nilgirensis, Cyathea gigantea* and *Cyathea crinita* to reveal the inter-specific variation at molecular level. The banding pattern expressed hundred percentage genetic differentiations among the three species. Johnson *et al.* (2012b) assessed the genetic variation between different populations of *Thelypteris ciliata* collected from different localities of Tirunelveli hills using isoperoxidase. A total of six bands in six different positions with five active regions were observed in the enzyme system.

DNA barcoding

Pteridophytes have a longer evolutionary history than other vascular land plants. Therefore, it has endured greater loss of phylogenetically useful information. This factor has resulted in substantial disagreements in evaluating characters and controversy in establishing a stable classification. The first molecular systematic studies on ferns were published in the mid 1990s (Hasebe *et al*., 1994, 1995) and set the direction for modern fern systematics. The arrival of phylogenetics and molecular phylogenetics in particular, has rapidly improved our understanding of fern relationships through phylogenetic analyses of DNA sequence data (Hasebe *et al.* 1994, 1995; Pryer *et al.* 2004; Schneider *et al.* 2004a), morphological data alone (Schneider *et al.* 2009), or combined analyses of molecular and morphological evidence (Lehtonen *et al.* 2010). Since then, numerous molecular phylogenetic studies have focused on certain classically defined fern groups by sampling members from the group studied, or tested the backbone fern classification by sampling exemplar species of higher taxa. However, both kinds of studies have specific limitations to recover the complete fern tree of life.

DNA barcoding in ferns is potentially of great value when they generally lack the complexity for morphology based identification and underappreciated in ecological studies. 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 cpDNA evolves faster than mitochondria and shows considerable mutation rate, it is considered a better choice for developing a barcode (Taberlet *et al.,* 1991; Clegg, 1993).

There are many controversies existing over the value of DNA barcoding. Taxonomists consider that the traditional morphology based identification of a plant species would

diminish and result in incorrect species identification as cpDNA relies solely on genetic divergence (Kress *et al.,* 2005). Moreover, taxonomy of science is based on a detailed understanding of morphology, physiology and behavioural attributes (Ebach and Holdrege, 2005; Arvind *et al.,* 2007) and barcoding generates information, not knowledge. Species identification should be based on multigene phenotype rather than a single gene sequence (Moritz and Cicero, 2004). Though 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).

Molecular phylogenetic hypotheses for extant ferns have utilized data from several chloroplast markers (*rbc*L, *atp*A, *atp*B, *acc*D, *rps*4, 16S rDNA, ITS), one nuclear gene (18S rDNA) and three mitochondrial genes (*atp*1, *nad*2, *nad*5). A natural outgrowth of these one-gene or few-gene studies on a wide array of ferns has led to broader and increasingly robust multiple gene phylogenetic analyses.

DNA barcoding - Pteridophytes

Intergenic spacer sequences such as *trn*L-F and *rps*4-*trn*S were more effective for resolving recent divergence events in the ferns (Small *et al.,* 2005). *trn*L-F was proved to be useful in studies of Ophioglossaceae (Hauk *et al.,* 1996, 2003), Schizaeaceae (Skog *et al.,* 2002), Polypodiaceae (Haufler *et al.*, 2003; Ranker *et al*., 2004; Schneider *et al.,* 2004b), *Asplenium* (Schneider *et al.,* 2004a, 2005) and *Cyrtomium* (Lu *et al.,* 2005). Another intergenic spacer, *rps*4-*trn*S has been applied to infrageneric phylogenetic work in the ferns *Hymenophyllum* (Hennequin *et al.,* 2003), *Elaphoglossum* (Rouhan *et al.,* 2004; Skog *et al.,* 2004), *Polystichum* (Perrie *et al.,* 2003) and in the members of Thelypteridaceae (Smith and Cranfill, 2002).

Kolukisaoglu *et al.* (1995) indicated that *Selaginella* and *Equisetum* emerge earlier than *Psilotum* based on phytochrome gene. This result corresponds to chloroplast gene

(Raubeson and Jansen, 1992) and supported by *rbc*L gene (Korall *et al.,* 1999). Korall and Kenrick (2002, 2004) proved that the subgenera *Selaginella* and *Tetragonostachys* are monophyletic, *Stachygynandrum* and *Heterostachys* are polyphyletic; while the nature of *Ericetorum* is still unknown yet. Korall and Taylor (2006) indicated that the genus *Selaginella* is monophyletic based on *rbc*L map, however at subgenus level it is monophyletic or paraphyletic.

Dubuisson (1997) used *rbc*L sequences as a promising tool for 18 species of the fern genus *Trichomanes* to test the ability of this gene for resolving relationships within this taxon and to reveal the major phylogenetic tendencies. Gastony and Ungerer (1997) determined nucleotide sequences of the chloroplast-encoded *rbc*L gene for all five species of the onocleoid ferns including both varieties of *Onoclea sensibilis* and for out group member *Blechnum glandulosum.* Yatabe *et al.* (2001) crossed three sympatric *rbc*L sequence types of *Asplenium nidus* and observed that the molecularly distinct types were reproductively isolated because hybrids failed to form between atleast two pairs of *rbc*L types.

Heede *et al.* (2003) investigated the phylogenetic relationships among 20 taxa of the fern genus *Asplenium* subgenus *Ceterach* using DNA sequence data from the nuclear ribosomal internal transcribed spacers and plastid *trn*L-F intergenic spacer. In addition, a single sample per taxon was used in analysis of the plastid *rbc*L gene. Plastid *trn*L-F and *rbc*L analyses resulted in identical tree topologies. The trees produced from the separate plastid and nuclear matrices agree in the recognition of identical groups of accessions corresponding to *A. dalhousiae*, *A. ceterach*, *A. aureum*, *A. cordatum*, *A. phillipsianum* and *A. haughtonii.* Baracaldo (2004) generated robust phylogeny of the Neotropical fern genera *Jamesonia* and *Eriosorus* based on sequence data of the nuclear ETS of 18S-26S rDNA, the plastid gene *rps*4 and the intergenic spacer *rps*4-*trn*S.

Sequence data from the chloroplast *atp*B gene have proven to be useful for resolving relationships within ferns (Tsutsumi and Kato 2005; Korall *et al*., 2006), suggesting that the gene may also have phylogenetic utility for *Equisetum*, especially for resolving deep nodes. Zhang *et al.* (2005) carried out phylogenetic analysis of cryptogrammoid ferns and related taxa based on *rbc*L sequences. The resulting cladogram places *Coniogramme*, *Cryptogramma* and *Llavea* into a moderately supported clade, constituting a cryptogrammoid group distantly related to the cheilanthoid ferns. Smith *et al.* (2006) provided the most recent arrangement of Pteridaceae into five monophyletic groups based on morphological and molecular data. Many *rbc*L sequences of Pteridaceae are now available in GenBank which showed that they are variable enough to provide good resolution across the taxonomic diversity of fern taxa.

Schneider and Schuettpelz (2006) tested the principle of DNA barcoding of fern gametophytes using plastid *rbc*L sequence and successfully identified a cultivated gametophyte as *Osmunda regalis.* However, whether *rbc*L shows sufficient variation to allow general identification below genus level remains uncertain. Liu *et al.* (2007) carried out phylogenetic analysis of Dryopteridaceae family using chloroplast *rbc*L and *atp*B genes. The results indicate that Dryopteridaceae form a monophyletic group with the exception of *Didymochlaena, Hypodematium* and *Leucostegia*. They are sister to a large clade comprising Lomariopsidaceae, Tectariaceae, Polypodiaceae, Davalliaceae and Oleandraceae. Lu *et al*. (2007) studied the molecular phylogeny of the polystichoid ferns in Asia based on *rbc*L sequences. Guillon (2007) used original (*atp*B) and published (*rbc*L*, trn*L-*trn*F*, rps*4) sequence data to investigate the phylogeny of the genus *Equisetum*. Analyses of *atp*B sequences give an unusual topology with *Equisetum bogotense* branching within *Hippochaete*.

Madeira *et al*. (2008) conducted the molecular phylogeny of the genus *Lygodium* using *trn*L intron and *trn*L-F inter-genic spacer of cpDNA to determine the relationship of

Lygodium microphyllum and other *Lygodium* species. Three major clades appeared, one with *Lygodium palmatum* and *Lygodium articulatum*, second with *Lygodium reticulatum* and *L. microphyllum,* and a third comprising the other species examined. Nitta (2008) explored the utility of three plastid loci (*rbc*L*, trn*SGG and *trn*H-*psb*A) for biocoding the filmy ferns (Hymenophyllaceae) of Moorea. All the three regions were found to be potentially useful for phylogenetic studies at the appropriate taxonomic level. *trn*H-*psb*A has the greatest utility as a potential marker for DNA-based identification because of its high inter-specific variability and high degree of amplification success. *rbc*L and *trn*H-*psb*A were successfully used in combination with morphological characters to identify *Polyphlebium borbonicum*.

Song *et al.* (2009) authenticated the members of the family Polygonaceae in Chinese pharmacopoeia using DNA barcoding technique. The amplification efficiency of six DNA barcodes (*rbc*L, *trn*H-*psb*A, *ndh*J, *rpo*B, *rpo*C1, *acc*D) was 100%. Inter-specific divergence was highest for the *trn*H-*psb*A (20.05%) followed by the *nr*ITS (14.01%) across all species pairs. Schneider *et al.* (2009) used morphological dataset of 136 vegetative and reproductive characters to infer the tracheophyte phylogeny with an emphasis on early divergences of ferns (monilophytes). Independent phylogenetic analyses of morphological evidence recover the same phylogenetic relationships among tracheophytes utilizing DNA sequence data but differ within seed plants and ferns.

Ma *et al.* (2010) analyzed five DNA sequence markers (*psb*A-*trn*H intergenic region, *rbc*L, *rpo*B, *rpo*C1 and *mat*K) using six chloroplast genomic sequences from GenBank and found *psb*A-*trn*H intergenic region was a suitable DNA marker for species identification in medicinally important pteridophytes. Pryer *et al.* (2010) exposes a case of mistaken identity in the fern horticultural trade using DNA barcoding. The plastid sequences *rbc*L*, atp*A and *trn*G-R were used to demonstrate that a fern marketed as *Cheilanthes wrightii* in the horticultural trade is in fact *Cheilanthes distans.* Li *et al.* (2010) identified diminutive and

featureless stages of ferns using tissue-direct PCR combined with amplifying plant barcodes. It was very helpful for large scale ecological studies surveying distribution and population structure. Ebihara *et al*. (2010) demonstrated the effectiveness of two plastid DNA barcode regions (*rbc*L and *trn*H-*psb*A) for species identification in the Japanese pteridophyte flora.

Groot *et al.* (2011) evaluated the combination of *rbc*L with a non-coding plastid marker *trn*L-F to obtain DNA identifications for 86 fern species. All species with non-equal chloroplast genomes formed their own well supported monophyletic clade, indicating a high discriminatory power. Inter-specific distances were larger than intra-specific distances for all the tested taxa. Zhang *et al*. (2012b) used DNA sequences of four plastid loci (*rbc*L gene, *rps*4-*trn*S spacer, *trn*L intron, *trn*L-F spacer) to reconstruct the phylogeny of *Dryopteris*. The results confirmed the paraphyly of *Dryopteris* and provides the first strong molecular evidence on the monophyly of *Acrophorus, Diacalpe, Dryopsis, Nothoperanema* and *Peranema*. However, all these monophyletic groups together with the paraphyletic *Acrorumohra* are suggested to be merged into *Dryopteris* based on both molecular and morphological evidence.

Chen *et al.* (2013) identified field vittarioid gametophytes using DNA barcoding. Combinations of distance-based and tree-based approaches were performed to evaluate the discriminating power of three barcodes (*mat*K, *rbc*L and *trn*L-F) on 16 vittarioid sporophytes. Sequences of the *trn*L-F region were generated from 15 fern gametophyte populations by tissue-direct PCR and were compared against the sporophyte dataset using BLAST. *trn*L-F earns highest primer universality and discriminatory ability scores, whereas PCR success rates were very low for *mat*K and *rbc*L regions.

Molecular variation - Tree ferns

Tree ferns are a conspicuous component of tropical, subtropical and even south temperate floras, where closely related clades are especially diverse (Smith *et al.,* 2006).

They hold a critical phylogenetic position as the extant sister groups to seed plants. Understanding the organization and evolution of tree ferns can provide useful information for comparative studies across land plants. Outside the extant seed plants, arborescence is today only present in ferns, where it is mostly restricted to the "tree fern" clade (Korall *et al.,* 2006, 2007). Investigations of generation times, along with those of phenology and demography have shown that the members of primarily arborescent clade have longer generation times than closely related non-arborescent lineages (Mehltreter and Palacios-Rios, 2003; Mehltreter and Garcia-Franco, 2008).

Tree ferns are a well-established clade within leptosporangiate ferns and are strongly monophyletic. Phylogenetic relationships among the tree ferns have been controversial. Bower (1928) classified tree ferns into separate families and placed each of them near different groups, based on morphological differences in the position of sori and epidermal appendages (e.g., marginal sori and hairs in the Dicksoniaceae versus dorsal sori and scales in the Cyatheaceae). Other authors postulated that the tree ferns were closely related (Holttum and Sen, 1961; Kramer, 1990) and even included them in a single family. This was based on similarities in tree habit and oblique annuli of sporangia. Existence of intermediate genera *Metaxia* and *Lophosoria* with a combination of dorsal sori and hairs also supports monophyly of the tree ferns.

Cyatheaceae has been the focus of many taxonomic studies in the last 50 years and classifications of Cyatheaceae are still variable at the generic level. The presence of scales and the position of the sorus are the two most conspicuous characters used to separate Cyatheaceae from arborescent species in the Dicksoniaceae. The monophyly of Dicksoniaceae (excluding *Cystodium*) has been questioned in earlier studies based on DNA sequence data (Hasebe *et al*., 1994, 1995; Wolf *et al.,* 1999; Pryer *et al*., 2004) and morphology. Some previous classifications have included it either in Cyatheaceae (Holttum

and Sen, 1961; Holttum, 1963) or divided it into three separate families viz., Dicksoniaceae, Culcitaceae and Thyrsopteridaceae (Pichi-Sermolli, 1977). The most surprising addition to the tree fern clade is Hymenophyllopsidaceae. All *Hymenophyllopsis* species have scales and their sporangia resemble those found in the Cyatheaceae (Lellinger, 1984). Other affinities for *Hymenophyllopsis* have also been proposed (Lellinger, 1984) and it has even been cited as a "genus of uncertain placement" (Kubitzki, 1990).

DNA phylogenies have now greatly clarified the main subgroups of the scaly tree ferns (Holttum and Sen, 1961; Holttum, 1963; Tryon, 1970; Tryon and Tryon, 1982; Conant, 1983; Holttum and Edwards, 1983; Lellinger, 1987; Kubitzki, 1990; Conant *et al.,* 1994, 1995, 1996). In the last decade, phylogenetic studies of restriction site data and morphology led to the recognition of three evolutionary lineages within Cyatheaceae: *Sphaeropteris, Alsophila* and *Cyathea,* with *Alsophila* as sister to the other two (Conant *et al.,* 1994, 1995, 1996). Molecular studies in ferns have generally relied on a subset of the sequences used in angiosperm systematics (Pryer *et al*., 2004). The gene *rbc*L has been used extensively in studies for both higher-level and lower-level taxa (Ranker *et al.,* 2004; Schneider *et al.,* 2004a). Phylogenetic studies using nucleotide sequences of the gene encoding the large subunit of *rbc*L have been successfully revealed the relationships of ferns at both generic and familial levels (Hasebe *et al.*, 1995; Skog *et al*., 2004). Hasebe *et al.* (1994) sequenced 1206 nucleotides of the large subunit of the *rbc*L gene from 58 species representing almost all families of leptosporangiate ferns. Phylogenetic trees proved monophyletic relationship of the tree ferns with 98% of bootstrap probability in the neighbor-joining method and 73% in the parsimony method. Two morphologically distinct heterosporous water ferns viz., *Marsilea* and *Salvinia* are sister genera, the tree ferns (Cyatheaceae, Dicksoniaceae and Metaxyaceae) are monophyletic and polypodioids are distantly related to the gleichenioids in spite of the similarity of their exindusiate soral morphology.

Wolf *et al.* (1999) suggested based on *rbc*L sequence data from two *Hymenophyllopsis* species, the family was monophyletic and sister to the single Cyatheaceae species. *Lophosoria quadripinnata*, the single species in Lophosoiaceae has an affinity to other tree ferns and phylogenetic studies based on plastid DNA sequence data (Tryon, 1970; Tryon and Tryon, 1982; Wolf *et al*., 1999; Pryer *et al.,* 2004) suggest a close affinity to *Dicksonia*. Phylogenetic studies based on DNA sequence data concluded that Metaxyaceae belongs to the tree ferns, but there has been no strong support for a specific position within the clade (Hasebe *et al*., 1994, 1995; Wolf *et al*., 1999; Pryer *et al.,* 2004). Phylogenetic relationships among the major groups of tree ferns based on protein-coding plastid loci (*atp*A*, atp*B*, rbc*L and *rps*4) revealed four well-supported clades, with genera of Dicksoniaceae (Kubitzki, 1990) interspersed among them. Dicksoniaceae and Cyatheaceae are not monophyletic and new circumscriptions for these families are needed (Korall *et al.,* 2006).

Ting *et al.* (2003) sequenced chloroplast *trn*L intron and *trn*L-*trn*F intergenic spacers of *Alsophila spinulosa* and *Cyathea tsangii* belongs to Cyatheaceae. The clade showed *Sphaeropteris brunoniana*, *Sphaeropteris hainanensis* and *Cyathea contaminans* diverged from the rest of the members and the latter was further separated into two subclades corresponding to the subgenus *Alsophila* and *Gymnosphaera*. Three monophyletic terminal clades were formed separately by *Cyathea gigantea* - *Cyathea pseudogigantea* - *Cyathea tinganensis* - *Cyathea pectinata* (clade 1)*, C. contaminans* - *S. brunoniana* - *S. hainanensis* (clade 2) and *C. tsangii* - *A. spinulosa* (clade 3) suggesting that these species could be combined as three separate species: *C. gigantea*, *S. brunoniana* and *A. spinulosa*. The genus *Sphaeropteris* was placed in the basal position of Cyatheaceae, whereas the genus *Alsophila* placed as the derived sister group, which supported Tryon's hypothesis accounting for the evolutionary relationships within Cyatheaceae and the derivation of their indusium. Wang *et al.* (2004) used RAPD analysis and sequences of cpDNA *atp*B-*rbc*L intergenic spacers to

characterize the pattern of genetic variation and phylogenetic relationships of *Alsophila spinulosa*. 28 random primers generated 118 bands, out of which 26 (22.03%) were polymorphic loci, distinguishing 17 different RAPD phenotypes. AMOVA showed that 47.44% of the variance was partitioned among regions, 34.01% attributed among populations within regions, whereas only 18.55% occurred within populations. Low level of intra-specific diversity was maintained in *A. spinulosa* with Shannon diversity and gene diversity merely 0.0560 and 0.0590 respectively.

Su *et al.* (2004) sequenced cpDNA *atp*B-*rbc*L intergenic spacers of individuals of a relict tree fern *Alsophila spinulosa* collected from ten populations in Southern China. Sequence length varied from 724 to 731 bp showing length polymorphism and base composition was with high A+T content between 63.17% and 63.95%. A total of 19 haplotypes were identified based on nucleotide variation. High levels of haplotype diversity and nucleotide diversity were detected in *A. spinulosa* which allowed the accumulation of genetic variation within lineages. Su *et al*. (2005) inferred genetic differentiation and phylogeographical pattern of *A. spinulosa* in southern China using sequence variations of *trn*L-F noncoding regions of cpDNA. AMOVA analysis indicated that most of the genetic variation was partitioned among regions. Korall *et al*. (2007) investigated the phylogenetic relationships of scaly tree ferns based on DNA sequence data from five plastid regions (*rbc*L*,* *rbc*L-*acc*D IGS, *rbc*L-*atp*B IGS, *trn*G-*trn*R and *trn*L-*trn*F). A basal dichotomy resolves *Sphaeropteris* with conform scales is sister to all other taxa having marginate scales. The marginate-scaled clade consists of a basal trichotomy with the three groups viz., *Cyathea*, *Alsophila* and *Gymnosphaera*. In recent phylogenetic analyses, tree ferns were shown to be the sister group of polypods, the most diverse group of living ferns. Gao *et al*. (2009) sequenced the complete chloroplast genome of a scaly tree fern *Alsophila spinulosa.* It shares some unusual characteristics with the previously sequenced genome of the polypod fern

Adiantum capillus-veneris, including the absence of 5 tRNA genes that exist in most other chloroplast genomes. The genome shows a high degree of synteny with that of *Adiantum*, but differs considerably from two basal ferns (*Angiopteris evecta* and *Psilotum nudum*).

All these studies have given rise to growing confidence in relationships and correspondingly to the composition of taxa at familial and ordinal ranks. Nowadays it is widely accepted that any valid plant barcode will be multi-locus, preferably existing of a conservative coding region like *rbc*L, in combination with a more rapidly evolving region, which is most likely non-coding (Kress *et al*., 2009). The non-coding *trn*L intron and *trn*L-F intergenic spacer have been repeatedly suggested for this purpose (Taberlet *et al*., 2006; Hollingsworth *et al.,* 2009) and were successfully used by Li *et al.* (2009) for identification of a mysterious aquatic gametophyte. Besides the technical issues of primer universality, sequence quality and complexity, Schneider and Schuettpelz (2006) mentioned three potential difficulties for any tested marker to overcome: incomplete sampling of the online records to be used as a reference for identification (GenBank), the occurrence of mis-identified and erroneous sequences in these online databases and the potential inability of the marker to discriminate among species.

A genotypic marker in comparison to a phenotypic marker does not reveal the active principle or the chemical constituent. 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. Considerable work has been carried out to correlate DNA markers with phytochemical compositions among closely related species (Baum *et al.,* 2001; Fico *et al.,* 2003; Joshi *et al.,* 2004). Merging of these profiles will certainly help in developing a comprehensive understanding of a species.

Thus the present study area is growing rapidly on both basic (chemotaxonomy) and applied (discovery of new natural medicines) points of view with the availability of modern

techniques. The chemical taxonomy has grown in a rapid way and up to the level that today it is applied to distinguish not only species, but also individuals within a population as in the case of forensic science by applying DNA finger printing techniques (Irudayaraj and Raja, 1998). There is also a challenge for studying variation among plants despite having many advanced molecular techniques available to assess genetic variation. The level of polymorphism that different methodologies reveal is also important. If it reveals too little variation, then it may not be possible to discriminate taxa. If the variation found is too high, then the relationship between the taxa is concealed. This results in inaccurate prediction of relationships.

Biological activities

A large number of bacteria are involved in various skin infections and plants contain phytochemicals to kill bacteria involved in skin diseases (Kar and Kumar, 2010). Ethyl acetate, butanol and aqueous extracts of *Blechnum orientale* were effective against all the tested Gram positive bacteria (Lai *et al.,* 2010). Soare *et al.* (2012) demonstrated the most evident antibacterial effect in *Cystopteris fragilis* gametophytic extract which inhibited the growth of all the tested bacteria. Raja *et al.* (2012) studied the antimicrobial activity of *Cyathea nilgiriensis*, *Cyathea crinita*, *Leptochilus lanceolatus* and *Osmunda hugeliana* using paper disc diffusion assay. All the selected ferns showed inhibitory effect against *Proteus aureus, Klebsiella pneumoniae, Aspergillus niger* and *Fusarium* sp. with the maximum inhibition in the highest concentration (100 µg/ml).

Ethyl acetate fraction of *Cyathea phalerata* had marked antioxidant potential, especially as a scavenger of the hydroxyl radical and in inhibiting lipid peroxidation (Hort *et al*., 2008). Methanolic extract of *Cyathea gigantea* produced a dose dependent inhibition of *in vitro* free radical generation of superoxide anion, hydroxyl radical and DPPH radical (Madhukiran and Rao, 2011). Revathi and Sara (2014) screened *Marsilea minuta* for free

radical scavenging properties using DPPH assay and found maximum activity in frond followed by petiole and rhizome. Johnson *et al*. (2014) determined the antioxidant potential of *Asplenium aethiopicum* in different extracts using DPPH radical scavenging, phosphomolybdenum assay and scavenging of hydrogen peroxide. The best free radical scavenging activity was exerted in methanolic extract of *A. aethiopicum.*

Gleichenia linearis showed antibacterial properties in aqueous extracts (Vasudeva, 1999). Methanolic extracts of *Drynaria quercifolia* showed broad concentration dependent antimicrobial activity (Ramesh *et al.,* 2001). There are reports of *D. quercifolia* against *Neisseria gonorrhoeae* (Shokeen *et al.,* 2005). Friedelin, epifriedelirol, beta amyrin, beta sitosterol, 3-beta-D-glucopyraroside and naringin were isolated from dried rhizome of *D. quercifolia*. Flavonoids of *Drynaria fortunei* were found to protect against gentamicin ototoxicity and renal failure (Long *et al.,* 2004, 2005). Species of *Lycopodium* demonstrated anti-acetylcholinesterase activity in two separate experiments (Zhang *et al.,* 2002; Hirasawa *et al.,* 2006). *Lycopodiella cernua* was antivirally active and had been patented as a treatment for Hayfever (Cambie and Ash, 1994; Zhang *et al.,* 2002).

Selaginella species showed an inhibitory effect on muscle contraction (Perez *et al.,* 1994; Rojas *et al.,* 1999). Different species of *Selaginella* showed bioactivity and exhibited anti-inflammatory, antimicrobial and antioxidant properties (Silva *et al.,* 1995; Sun *et al.,* 1997; Lee *et al.,* 1999; Lin *et al.,* 2000; Chen *et al.,* 2005). Aqueous extract of *Selaginella delicatula* has antioxidant activity and degrades blood cholesterol (Gayathri *et al*., 2005). Isocryptomerin from *Selaginella tamariscina* showed antibacterial potential against Gram positive and Gram negative bacteria (Lee *et al*., 2009). Amentoflavone from *S. tamariscina* inhibits several pathogenic fungi (Woo *et al.,* 2005; Jung *et al*., 2007).

Pteris semipinnata demonstrated anti-tumour activity in two separate investigations. *P. semipinnata* and *Pteris multifada* are both cytotoxic, but they contain diterpenes (Li *et al.,*

1998, 1999). *Pteris vittata* exhibited carcinogenic activity (Siman *et al.,* 2000). Other species of *Pteris* possessed antimutagenic, immunomodulatory and neuronal activity (Goldberg and Cooper, 1975; Lee and Lin, 1988; Wu *et al.,* 2005). Antimicrobial compounds have also been characterised from a common fern, *Pteris biaurita* (Dalli *et al.,* 2007). Fronds of *Pteris quadriaurita* showed antibacterial potential towards pathogenic multi-drug resistant strains involved in skin diseases in human beings (Thomas, 2011). *Pityrogramma calomelanos* is cytotoxic and contains flavonoids (Star and Mabry, 1971; Sukumaran and Kuttan, 1991). An oil substance which can be used as a potential antibiotic and anticancer chemotherapeutic agent has been extracted from various species of *Ophioglossum* (Khandelwal *et al.,* 1985). Aqueous extracts of *Drynaria fortunei* showed cytotoxic and antioxidant properties (Liu *et al.,* 2001). *Christella dentata* showed carcinogenic activity (Somvanshi and Sharma, 2005). Cytotoxic activity in terms of brine shrimp lethality bioassay was found to be most effective in *A. aethiopicum.* Larvicidal activity of *A. aethiopicum* against *Culex quinquefasciatus* showed highest mortality in crude acetone extracts (Johnson *et al*., 2014).

From the above review, it is clear that phytochemical analysis and molecular variation studies has been done on large number of Indian ferns and fern allies but less work has been done on the tree ferns of South India. Hence in the present investigation, an attempt has been made to study the phytochemical and molecular characterization of selected *Cyathea* species from Western Ghats, South India.

Collection of plant materials

Specimens for the present study were collected from various natural habitats of Tamil Nadu, South India (Plate I). *Cyathea nilgirensis* Holttum (Plate II A-C) were harvested in and around Kakkachi stream (1,725 m), Kothayar, Tirunelveli hills (8°44' N & 77°44' E), *Cyathea gigantea* (Wall. ex. Hook.) Holttum (Plate II D-F) from the road sides near Nadugani (2,637 m), Nilgiris hills (11°24' N & 76°44' E) and *Cyathea crinita* (Hook.) Copel. (Plate II G-I) from the Anglade Institute of Natural History, Shenbaganur, Kodaikanal (2,195 m), Palni hills (10°13' N & 77°32' E), Western Ghats, South India between December 2012 to May 2013. The plants were identified based on the "Pteridophyte Flora of the Western Ghats, South India" by Manickam and Irudayaraj (1992). Herbarium specimens were prepared using conventional methods at the collection site itself and the voucher specimens were deposited in the St. Xavier's College Herbarium (XCH), Palayamkottai, Tamil Nadu, India for further reference (*C. nilgirensis* - XCH 25423; *C. gigantea* - XCH 25422 and *C. crinita* - XCH 25424).

Morphological description

***Cyathea nilgirensis* Holttum (Fig. 1)**

Trunk cylindrical, 3 to 5 m high, with a crown of fronds at the apex. Lamina bipinnate, about 2 x 1 m, acute, pinnae about 15-18 pairs, alternate, distinctly petiolate about 15 cm apart, about 50 x 20 cm oblong lanceolate, acuminate, pinnules about 12 x 2.5 cm acuminate, margin pinnatifid upto the costa; ultimate lobes upto 21 pairs, alternate, oblong 1.3 x 0.3 cm, apex acute margin crenate, basalmost acroscopic lobe larger than the others, veins distinct well below, slightly above; pinnae pale green below, dark green above; texture

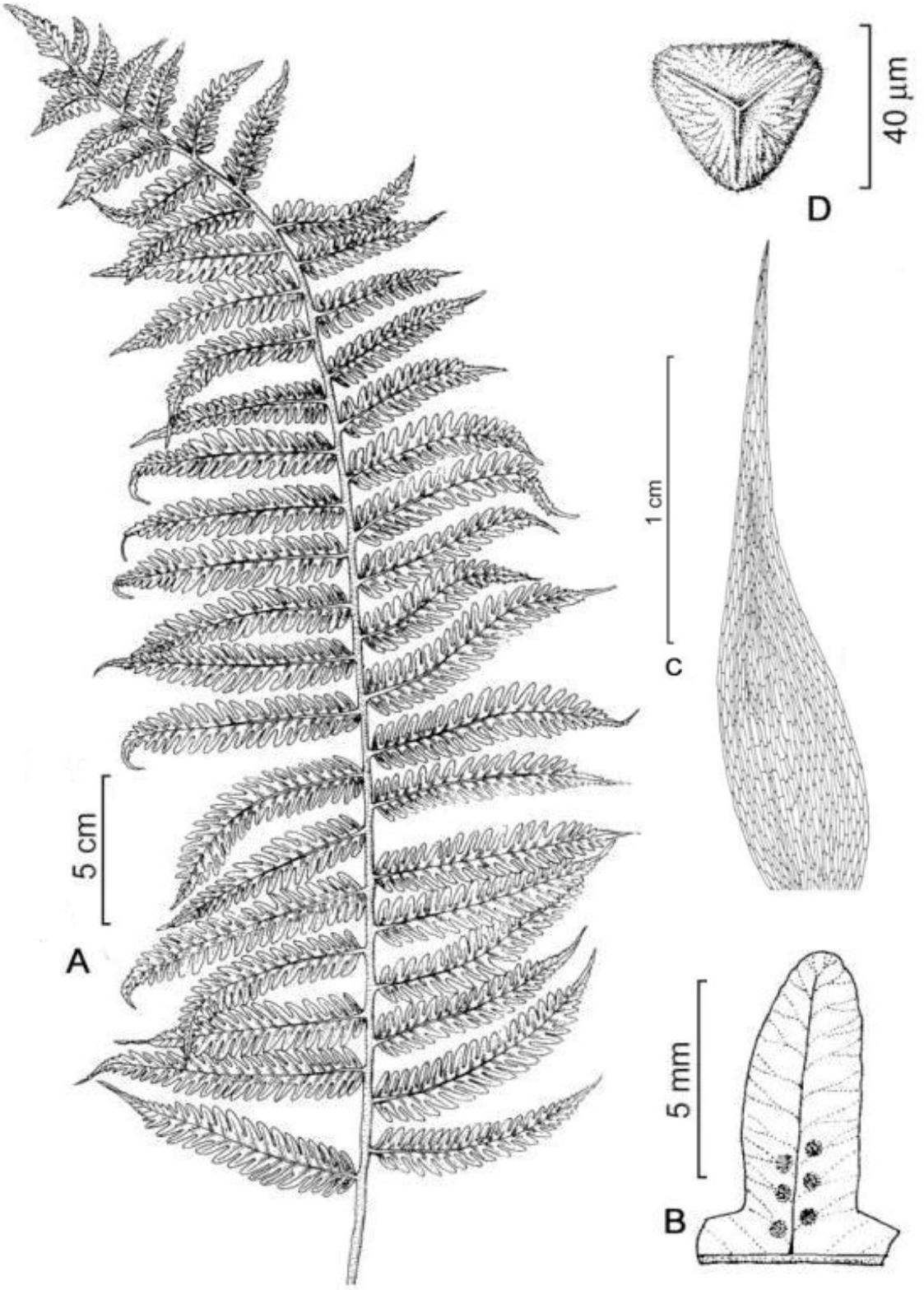

Fig. 1: ***Cyathea nilgirensis*** **Holttum (XCH 25423)**

A - Secondary pinna; **B -** Pinnule showing venation and sori; **C -** Palea; **D -** Spore.

herbaceous, pale brown. Stipes smooth hairy, costa hairy, rachis and scales shining brown. Sori seated at the vein forks of the lower half of the segments, spherical, about 1.5 mm in diameter, exindusiate, paraphysis intermingled with sporangia. Spores trilete, 35 x 40 µm with faintly reticulate exine (Manickam and Irudayaraj, 1992).

***Cyathea gigantea* (Wall. ex Hook.) Holttum (Fig. 2)**

Trunk about 2 m tall, cylindrical and 20 cm in diameter with persistent swollen base of stipes, bearing a crown of fronds at the apex; trunk densely covered, scales 5-7 x 1-1.5 mm, lanceolate, acuminate, dark brown. Stipe tufted about 70 x 2 cm, chestnut brown, glossy, abaxially rounded, adaxially grooved, densely scaly at the swollen base. Lamina bipinnate about 117 x 105 cm; primary pinna upto 15 pairs, alternate, distinctly stalked, 18 cm apart oblong lanceolate, about 50 x 20 cm, apex acuminate, base truncate, secondary pinnae about 23 pairs, alternate, shortly stalked, oblong lanceolate, 11 x 1.5 cm, apex acuminate, base subtruncate, margin usually lobed, rarely crenate, lobes upto 12 pairs, broadly deltoid about 3 x 4 mm, apex rounded. Sori median on the veins, three to five pairs, 1 mm in diameter, forming two zig-zag rows ('V' shaped) submarginally, exindusiate. Sporangia 0.3 mm in size, ovoid with oblique annulus and short stalk, paraphyses mingled with sporangia. Spores trilete, globose, 20 x 30 µm, triangular with concave margins and round corners, exine papillate (Manickam and Irudayaraj, 1992).

***Cyathea crinita* (Hook.) Copel. (Fig. 3)**

Trunk up to 8 m high, cylindrical, 15 cm thick, bearing closely set leaf bases. Stipe 125 x 3.5 cm, dark brown, rounded abaxially, grooved adaxially, swollen at the base; spines distributed densely on abaxial and lateral side of the stipes and rachis; long soft golden yellow woolly hairs densely covering the adaxial side of the primary and secondary rachis; stipes in addition to few larger scales. Lamina bipinnate, pale green to golden yellow, 208 x 104 cm oblong lanceolate, apex acute, pinnae about 23 pairs, alternate, 14 cm apart spreading

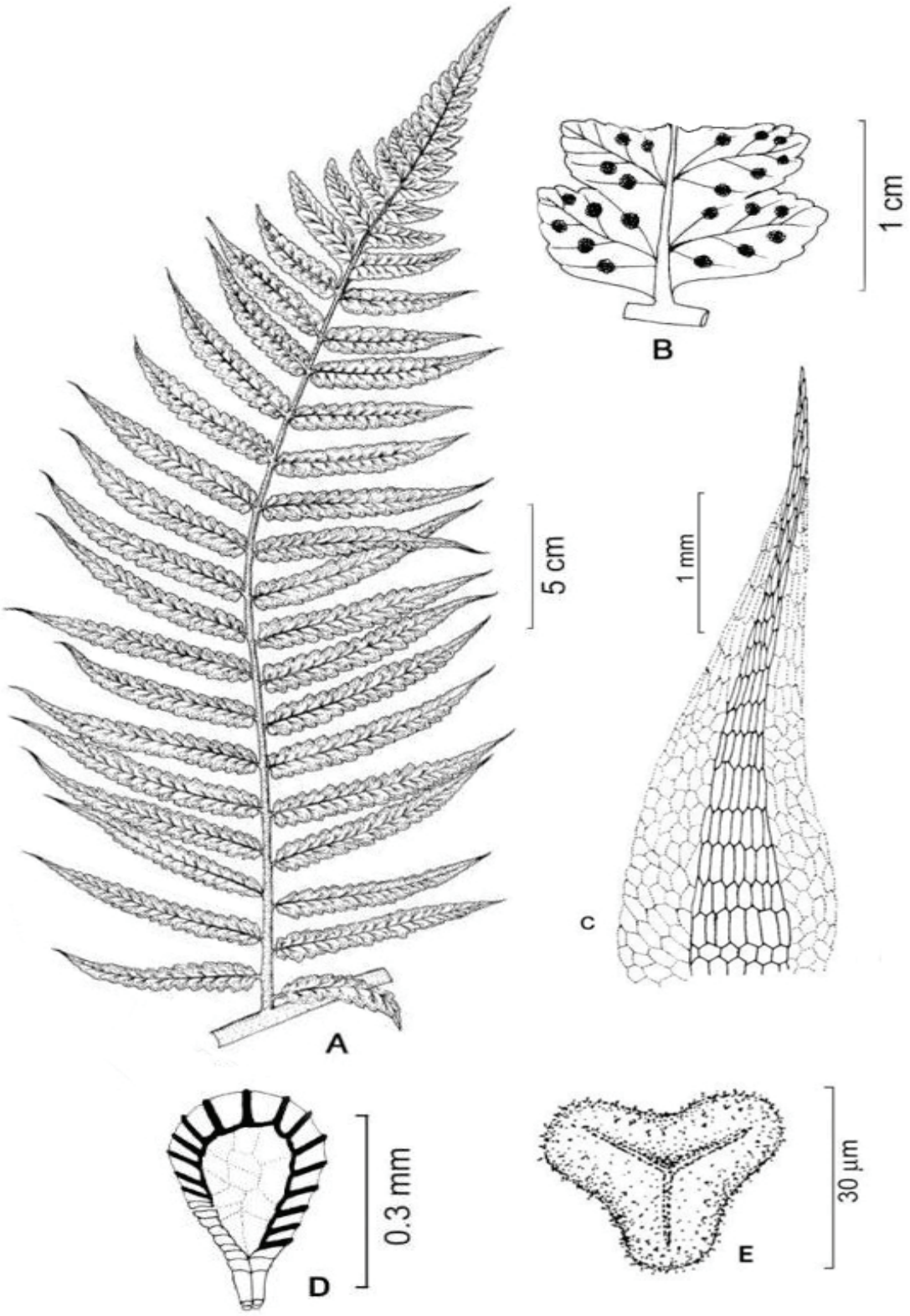

Fig. 2: ***Cyathea gigantea*** **(Wall. ex. Hook.) Holttum (XCH 25422). A -** Secondary pinna; **B -** Part of pinna showing venation and sori; **C -** Palea; **D -** Sporangia; **E -** Spore.

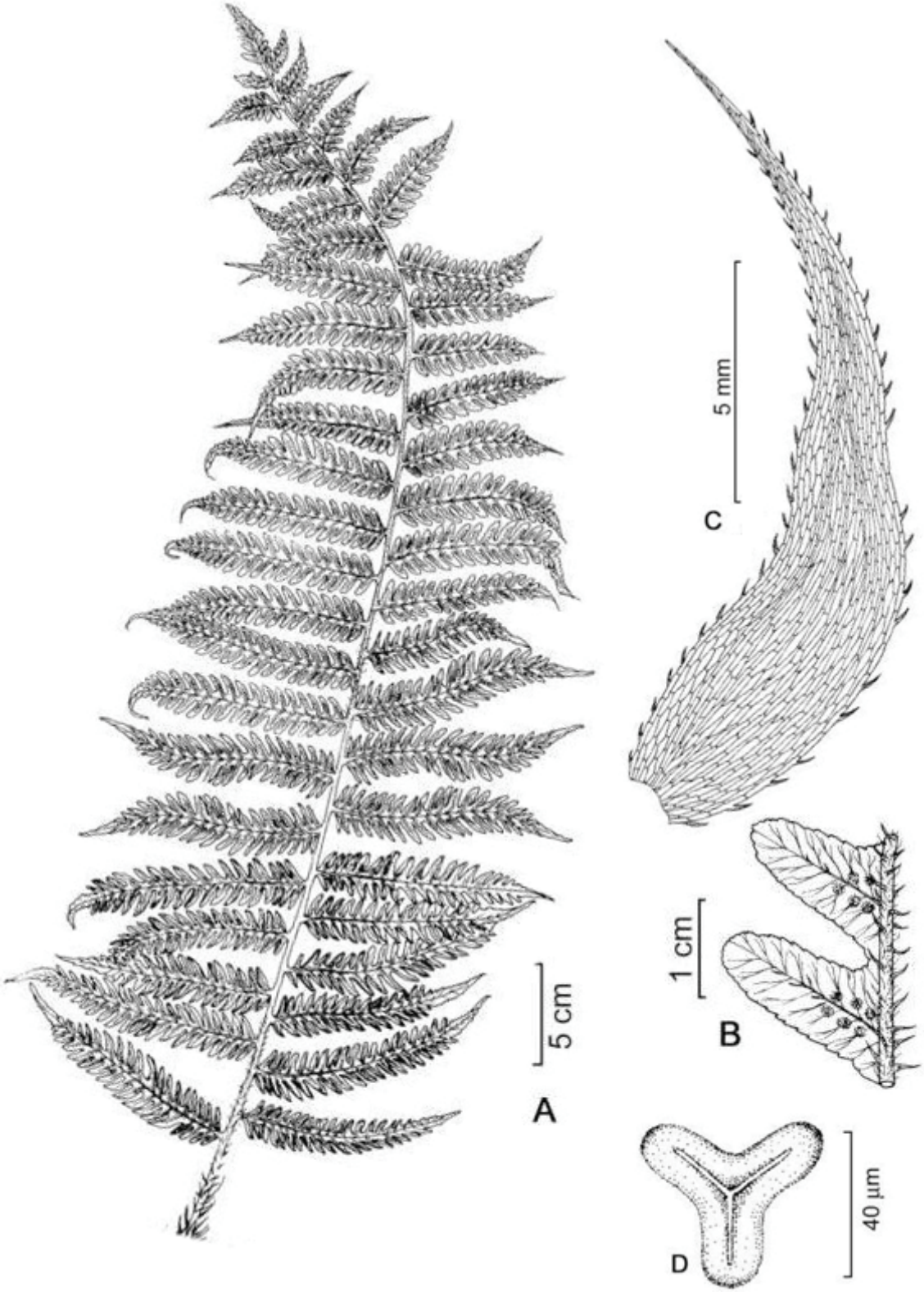

Fig. 3: ***Cyathea crinita*** **(Hook.) Copel. (XCH 25424). A -** Secondary pinna; **B -** Pinnule showing venation and sori; **C -** Palea; **D -** Spore.

distinctly stalked about 50 x 20 cm oblong lanceolate, acuminate, pinnules about 25 to 30 pairs per pinna, margin lobed upto the costa, lobes 20 to 25 pairs, alternate; small ciliated scales densely distributed on abaxial and adaxial side of the costa and costules, upper surface of the segments glabrous. Sori seated at or above the forks, spherical, 1 mm in diameter, dark brown, exindusiate, paraphyses dark brown, multicellular, uniseriate about 1 mm long. Spores trilete, 30 x 40 µm, triangular with concave margins and round corners, exine smooth (Manickam and Irudayaraj, 1992).

Phytochemical analysis

Preparation of extracts

The collected species of *Cyathea* were thoroughly washed with tap water followed by distilled water. They were blotted on the blotting paper and shade dried at room temperature under dark. The shade dried plant samples were ground to fine powder using mechanical grinder. The powdered materials were stored in refrigerator for further use.

30 g powdered samples were extracted successively with 180 ml of petroleum ether, chloroform, acetone and ethanol using Soxhlet extractor for 8-12 h at a temperature not exceeding the boiling point. The aqueous extracts were prepared directly by boiling the powder with distilled water for 3 h and filtered using Whatman No.1 filter paper. The extracts were concentrated in a vacuum at 40°C using rotary evaporator.

Qualitative phytochemical screening

The different qualitative chemical tests were performed on various extracts of selected *Cyathea* species to detect the presence of phytoconstituents viz., steroids, alkaloids, phenolic compounds, cardiac glycosides, flavonoids, saponins, tannins, anthraquinone, coumarins, catechin, terpenoids and aminoacids (Table 1).

Table 1: Preliminary phytochemical screening of metabolites (Harborne, 1998)

Test	Observation	Inference
2 ml of test solution + few ml of chloroform + 2 to 3 drops of acetic anhydride + drops of Con. H_2SO_4	Purple colour changes to blue or green colour	Steroids
2 ml of test solution + few volume of 2N HCl + few drops of Mayer's reagent	White precipitate is formed	Alkaloids
2 ml of test solution in alcohol + 1 drop of 5% $FeCl_3$ solution	Deep blue colour is formed	Phenolic compounds
2 ml of test solution + 1 drop of $FeCl_3$ + 2 ml glacial acetic acid + H_2SO_4	Yellow colour is formed	Cardiac glycosides
2 ml of test solution in alcohol + a bit of Mg + a drop of Con. HCl + heat	Red or orange colour is formed	Flavonoids
2 ml of test solution + 2 ml of H_2O and shake vigorously	Foamy layer is formed	Saponins
2 ml of test solution + H_2O + lead acetate	White precipitate is formed	Tannins
2 ml of test solution + Magnesium acetate solution	Pink colour is formed	Anthraquinone
2 ml of test solution + few drops of NaOH	Bluish green colour is formed	Coumarins
2 ml of test solution + Ehrlich reagent + drops of Con. HCl	Pink colour is formed	Catechin
2 ml of test solution + 1 ml chloroform + 1 ml acetic anhydride	Cherry red colour is formed	Terpenoids
2 ml of test solution + 1% Ninhydrin in alcohol	Blue or violet colour is formed	Aminoacids

Physico-chemical parameters

Extractive values and fluorescence analysis were determined by following the standard method (Indian Pharmacopoeia, 1996). The concentrates were transferred to pre-weighed glass vials and completely dried under a stream of air. Aqueous extracts were collected into pre-weighed glass jars and freeze-dried. The percentage yield of each dried extract in terms of the starting plant material was determined. It was then stored in the dark at 7°C until required for analysis. Fluorescent characteristics of the plant powders as such and after treating them with various chemical reagents viz., Con. H_2SO_4, Con. HCl, CH_3COOH, NaOH and 5% $FeCl_3$ were observed in visible light as well as under UV radiation at 365 nm. The changes in colour were recorded.

UV-Vis spectroscopic analysis

The UV-Vis spectra of different extracts of selected *Cyathea* species were recorded in the Shimadzu UV-Vis spectrophotometer capable of producing monochromatic light for measuring the absorbance. The extracts were centrifuged at 3000 rpm for 10 min and filtered through Whatman No. 1 filter paper by using high pressure vacuum pump. The samples were diluted to 1:10 with the same solvent. The filtered extracts were scanned in the wavelength ranging from 190-1000 nm and the characteristic peaks were observed and recorded. The analysis was repeated twice to confirm the spectrum.

FT-IR spectroscopic analysis

About 1.0 mg of crude extracts of selected *Cyathea* 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 400 to 4000 cm^{-1}. The percentage of transmissions was recorded against the wavenumber. The peak values of FT-IR were recorded and the functional groups were predicted (Mistry, 2009).

To reveal the inter-specific similarities and variations among the selected *Cyathea* species, the preliminary phytochemical profile, UV-Vis spectral profile and FT-IR spectral profile were converted into "1" and "0" matrix to indicate the presence or absence of metabolites / absorbance / functional groups respectively. Genetic similarities were estimated according to Nei and Li (1979) algorithm and a UPGMA cladogram was constructed using NTSYS version 2.0 software.

Based on the results of qualitative phytochemical screening, UV-Vis and FT-IR analysis, the ethanolic extracts of the selected *Cyathea* species were further subjected to HPTLC, HPLC and GC-MS analysis.

HPTLC analysis

Test solution preparation

25 mg of ethanolic extracts of selected *Cyathea* species were weighed accurately in an electronic balance (Shimadzu). It was dissolved in 0.5 ml of ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solution for alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and terpenoids.

Sample application

2 µl of test solutions and 2 µl of standard solution were loaded as 5 mm band length in the 5 x 10 silica gel $60F_{254}$ 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 phases (alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and terpenoids) and the plate was developed upto 90 mm (Table 2).

Table 2: Mobile phases and spraying reagents for HPTLC analysis

Metabolites	Mobile phases	Spraying reagents	Detection
Alkaloids	Ethyl acetate - Methanol - Water (10 : 1.35 : 1)	Dragendorff's reagent followed by 10% ethanolic sulphuric acid reagent	Yellow, Brownish-yellow coloured zone at visible light mode
Flavonoids	Toluene - Acetone - Formic acid (4.5 : 4.5 : 1)	1% ethanolic aluminium chloride reagent	Yellow, Yellowish blue coloured fluorescent zone at UV 366 nm
Glycosides	Ethyl acetate - Ethanol - Water (8 : 2 : 1.2)	Libermann - Burchard reagent	Brown, Brownish yellow coloured zone at visible light mode
Phenolics	Toluene - Acetone - Formic acid (4.5 : 4.5 : 1)	Folin cio-calteu reagent	Blue, Brown, Brownish-yellow coloured zone at visible light mode
Steroids	Toluene - Acetone (9 : 1)	Anisaldehyde sulphuric acid reagent	Blue, Violet coloured zone at visible light mode
Tannins	Toluene - Ethyl acetate - Formic acid - Methanol (3 : 3 : 0.8 : 0.2)	5% Ferric chloride reagent	Blue, Greenish blue, brown coloured zones at visible light mode
Terpenoids	n-Hexane - Ethyl acetate (7.2 : 2.9)	Anisaldehyde sulphuric acid reagent	Blue, bluish violet coloured zones at visible light mode

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 under visible light, UV 254 nm and UV 366 nm.

Derivatization

The developed plate was sprayed with respective spraying reagents (Table 2) and dried at 100°C in hot air oven. The plate was photo documented in visible light and UV 366 nm mode using photo documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanned at UV 254 nm and UV 366 nm. After derivatization, the plate was fixed in

scanner stage and scanned at UV 366 nm. The peak table, peak display and peak densitogram were noted. The software used was winCATS 1.3.4 version.

To reveal the inter-specific similarities among the selected *Cyathea* species, the R_f values of different tested metabolites were converted into "1" and "0" matrix to indicate the presence or absence of phytoconstituents. Phytochemical similarities were estimated according to Nei and Li (1979) algorithm and a UPGMA cladogram was constructed using NTSYS version 2.0 software.

HPLC analysis

HPLC analysis of the selected *Cyathea* species were performed on a Shimadzu system equipped with a model LC pump, UV-Vis detector, Rheodyne injector fitted with a 20 µl loop and an auto injector. A Hypersil C-18 column (4.6 × 250 mm, 5 µm size) was used. The elution was carried out with gradient solvent systems with a flow rate of 1 ml/min 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 0.45 µ membrane filter 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 (Mallikharjuna *et al*., 2007; Sharanabasappa *et al*., 2007).

GC-MS analysis

The Clarus 500 GC (Perkin Elmer) used in the analysis employed a fused silica column packed with Elite-1 and the components were separated using helium as carrier gas at a constant flow of 1 ml/min. 2 µl of ethanolic extracts of selected *Cyathea* species were employed for GC-MS analysis (Merlin *et al.,* 2009). The sample extracts were injected into the instrument and detected by the Turbo gold mass detector (Perkin Elmer) with the aid of Turbo mass 5.1 software. During the 36th min GC extraction process, the oven was maintained at a temperature of 110°C with 2 min 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: 200°C; Source temperature: 200°C). Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The MS detection was completed in 38 min.

Identification of components

The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass spectra of the respective peaks obtained in the GC-MS were compared with the mass fragmentation patterns of standards in the NIST Version 2.0 - Year 2005 library having more than 65,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The compound name, molecular weight, molecular formula and structure of the components present in selected *Cyathea* species were ascertained (Sangeetha and Vijayalakshmi, 2011). Software adopted to handle mass spectra and chromatogram was GC-MS solution version 2.53.

The biological activities of the ascertained components were predicted using PASS online and Dr. Duke's Phytochemical and Ethnobotanical Databases by Dr. Jim Duke of the Agricultural Research Service / USDA.

Molecular studies

Protein isolation and separation

Protein isolation was carried out according to the standard method described by Anbalagan (1999). The young croziers of *C. nilgirensis*, *C. gigantea* and *C. crinita* were washed in deionized water and mashed in a pre-chilled mortar with 500 µl of phosphate buffer (pH 7.0). The resultant slurry was centrifuged at 10,000 rpm for 10 min at 4°C in an Eppendorf centrifuge. The supernatant was collected and stored at 4°C before use.

Reagents for gel preparation

Solution A - 29.2 g Acrylamide and 0.8 g Bisacrylamide in 100 ml of distilled water.

Solution B - Tris HCl 1.5 M, pH 8.8

Solution C - Tris HCl 0.5 M, pH 6.8

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

10% (v/v) separation gel consisted of 3.3 ml of Solution A, 2.5 ml of Solution B, 100 µl of Solution D, 4.1 ml of distilled water and 10 µl of TEMED. The solution was mixed and poured in up to three-fourth of the gel plate mold and allowed to polymerize. A thin layer of distilled water 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 of distilled water and 6 µl of TEMED. The solution was mixed and added to 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. The chemicals required for protein separation was tabulated in Table 3.

Table 3: Chemicals preparation for SDS-PAGE

Chemicals	Preparation
Acrylamide stock 30%	29.2 g Acrylamide + 0.8 g Bisacrylamide in 100 ml water
Lower Tris	pH 8.8 for separating gel, 36.34 g Tris made up to 200 ml with distilled water + 1% SDS
Upper Tris	pH 6.8 for stacking gel, 12.1 g Tris made up to 200 ml with distilled water + 1% SDS
Ammonium per sulphate	0.05 g in 0.5 ml distilled water
Running gel buffer (5X)	15 g Tris + 5% SDS + 72 g Glycine 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 stacking gel buffer + 1 ml of glycerol + 1% SDS and bromophenol blue

Sample loading

50-60 µl of each sample was loaded directly into a well using a micropipette. Care was taken to avoid mixing of the sample with the reservoir buffer and also to avoid cross contamination of samples. SDS-PAGE was carried out at 25°C in the air conditioned room. Separation of protein was carried out at 50 V till the tracking dye reaches the separating gel and at 100 V thereafter for 3-5 h 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 using the method described by Mortz *et al*. (2001) and Sorensen *et al*. (2002).

Silver staining

After electrophoresis, the gel was removed from the cassette and placed into a tray containing appropriate volume of fixing solution for 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 sensitizing solution was added and incubated for 2 min with gentle rotation. The sensitizing solution was discarded and the gel was washed twice with deionized water (1 min each). The water was discarded. Cold silver staining solution was added and shaked for 20 min to allow the silver ions to bind to proteins. After staining, the solution was poured off and the gel was rinsed with a large volume of deionized water for 20-60 sec to remove the excess of unbound silver ions. The gel was shortly rinsed with the developing solution and 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 min. As soon as bubbling of the solution is over, the development is stopped. Moist gels were kept in 12% acetic acid at 4°C in sealed plastic bags. After staining, the gel was viewed using Vilber Lourmat gel documentation system and banding profiles of protein was determined by the migration from the origin towards the anode.

The chemicals required for silver staining was tabulated in Table 4.

Table 4: Chemicals preparation for silver staining

Solutions	Reagents
Fixation solution	50% ethanol + 12% glacial acetic acid + 0.05% formalin + 100 ml distilled water
Washing solution	20% ethanol + 100 ml distilled water
Sensitizing solution	0.02% sodium thiosulphate + 100 ml distilled water
Deionized water	Washing
Staining solution	0.2% silver nitrate + 0.076% formalin + 100 ml distilled water
Developing solution	6% sodium carbonate + 0.0004% sodium thiosulphate + 0.05% formalin + 100 ml distilled water
Terminating solution	12% acetic acid
Drying solution	20% ethanol

Molecular weight determination

The molecular weights of the dissociated polypeptides were determined by using the standard curve. The standard curve was plotted by calculating standard protein against the $\log_{10}$ of its molecular weight. Distance from the wells of the protein bands were found out. The molecular weight of the unknown protein bands and their R_f values were calculated.

Data analysis

To reveal the inter-specific relationship among the selected *Cyathea* species, every scorable protein band was considered as single allele / locus and was converted into "1" and "0" matrix to indicate the presence or absence of bands. Based on the electrophoretic bands, similarity indices were calculated. Genetic similarities were estimated according to Nei and Li (1979) algorithm and a UPGMA cladogram was constructed using the software NTSYS version 2.0.

MALDI-TOF MS analysis

MALDI spectrum of *C. nilgirensis*, *C. gigantea* and *C. crinita* were recorded using Applied Biosystems MALDI-TOF Voyager De-Pro spectrometer. The samples were prepared by mixing 1 µl of protein samples and sinapinic acid matrix solution (5 mg/ml sinapinic acid in 50% ACN / 0.1% TFA). 0.75 µl of the resulting mixture was spotted onto a freshly cleaned stainless steel MALDI target plate. The acceleration voltages applied for MS was 25 kV. After air drying, the crystallized spots were processed with a MALDI-TOF mass spectrometer. MS was recorded in the positive and negative mode within a mass range from 500 to 1,00,000 Da using a nitrogen laser (337 nm). MALDI-TOF MS was constructed using Origin 6.1. The MALDI-TOF MS spectral values were converted into "1" and "0" matrix to indicate the presence or absence of m/z ratios. Based on the MALDI-TOF MS spectral profile, similarity indices were calculated. Genetic similarities were estimated according to Nei and Li (1979) algorithm and a UPGMA cladogram was constructed.

Isozymic variation

The enzyme systems resolved in the selected *Cyathea* species includes esterase (EST), peroxidase (PRX) and acid phosphatase (ACP).

Preparation of enzyme extract

The young croziers of *C. nilgirensis*, *C. gigantea* and *C. crinita* were washed in deionized water and the crude enzyme extracts were prepared by grinding in different buffers at varied pH based on the enzymatic system described by Sadasivam and Manickam (1991).

Esterase : 0.1M phosphate buffer (pH 7.0)

Peroxidase : 1M sodium acetate buffer (pH 5.0)

Acid phosphatase : 50 mM citrate buffer (pH 5.3)

The resultant slurry was centrifuged at 10,000 rpm for 10 min at 4°C in a cooling centrifuge and the supernatant is stored at 4°C before use. The isolated enzymes were then separated using PAGE.

Separation of isozyme

PAGE was carried out at 4°C using vertical gel electrophoresis unit. Separation of isozymes were carried out at 50 V till the tracking dye reaches the separating gel and at 100 V thereafter for 3-5 h or until the tracking dye had migrated to the bottom of the gel.

Reagents for gel preparation

Solution A - 29.2 g Acrylamide and 0.8 g Bisacrylamide in 100 ml of distilled water.

Solution B - Tris HCl 1.5 M, pH 8.8

Solution C - Tris HCl 0.5 M, pH 6.8

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

10% (v/v) separation gel consisted of 3.9 ml of Solution A, 3 ml of Solution B, 4.9 ml of distilled water, 60 µl of Solution D and 10 µl of TEMED. The solution was mixed and

poured in up to three-fourth of the mold and allowed to polymerize. Disperse a thin layer of ethanol on top of the acrylamide solution to level the surface.

6% (v/v) stacking gel consisted of 0.6 ml of Solution A, 1 ml of Solution C, 1.5 ml of distilled water, 33 µl of Solution D and 6 µl of TEMED. The solution was mixed and added to the top of the separating gel after the removal of ethanol topped over the separating gel. The comb was then 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 tank buffer.

The chemicals required for isozymic separation was illustrated in Table 5.

Table 5: Chemicals preparation for isozyme analysis

Chemicals	Preparation
Acrylamide stock 30%	29.2 g Acrylamide + 0.8 g Bisacrylamide in 100 ml water
Lower Tris	pH 8.8 for separating gel, 36.34 g Tris made up to 200 ml with distilled water
Upper Tris	pH 6.8 for stacking gel, 12.1 g Tris made up to 200 ml with distilled water
Ammonium per sulphate	0.05 g in 0.5 ml distilled water
Running gel buffer (5X)	15 g Tris + 72 g Glycine 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 stacking gel buffer + 1 ml of Glycerol and bromophenol blue

Sample loading

50-60 µl of each sample was loaded directly into a well using a micropipette. Care was taken to avoid mixing of the sample with the tank buffer and also to avoid cross contamination of samples. Staining procedures were varied depending upon the specific assays.

Esterase assay

The enzymes separated in the gels were actively stained with the specific reaction mixture for isoesterase.

1M phosphate buffer	: 10 ml
Fast blue B salt	: 100 mg
α -napthyl acetate	: 80 mg
Distilled water	: 4 ml
Methanol	: 60 ml

The gel was incubated in the dark at 37°C for 20-30 min. After incubation, the gel was fixed in methanol: water: acetic acid: ethanol for 15 min.

Peroxidase assay

The enzymes separated in the gels were actively stained with the specific reaction mixture for isoperoxidase.

0.1 M phosphate buffer (pH 7.0)	: 28 ml
O-dianizidine	: 100 mg
Distilled water	: 4 ml
Hydrogen peroxide	: 6 ml
Methanol	: 60 ml

The gel was incubated in the dark at 30°C for 30 min. After incubation, the gel was fixed in 7% acetic acid for 15 min.

Acid phosphatase assay

The enzymes separated in the gels were actively stained with the specific reaction mixture for the isozyme acid phosphatase.

50 mM Tris buffer (pH 5.3)	: 100 ml
Fast blue RR salt	: 100 mg
α-napthyl phosphate	: 50 mg
Magnesium chloride	: 230 mg

The gel was incubated in the dark at 37°C for 3-4 h.

To reveal the inter-specific relationship among the selected species of *Cyathea*, every band was considered as single allele and was converted into "1" and "0" matrix to indicate the presence or absence of bands. Genetic similarities were estimated according to Nei and Li (1979) algorithm and a UPGMA cladogram was constructed using the software NTSYS version 2.0.

DNA barcoding

Isolation of DNA

DNA was extracted using the modified CTAB extraction method described by Murray and Thompson (1980); Doyle and Doyle (1987); Rogers and Benedich (1994).

Procedure

Step I

The young croziers of *C. nilgirensis*, *C. gigantea* and *C. crinita* were grounded using liquid nitrogen at -196°C to a fine powder in mortar and pestle. 1 ml of warm extraction buffer was added to 1 g of powdered sample and incubated at 65°C for 5 h in water bath.

Chloroform extraction

- Chloroform: isopropanol mixture was prepared in 24:1 ratio and it was added in equal volume to the slurry. It was centrifuged at 10,000 rpm for 10 min at 4°C.

- Supernatant was taken and 1/10th 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 and 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.
- 0.6 ml of isopropanol was added and refrigerated at -20°C for 30 min to get the DNA precipitate. It was centrifuged at 8,000 rpm for 5 min at 4°C.
- The pellets were washed with 1 ml of 80% ethanol and centrifuged at 10,000 rpm for 5 min at 4°C to remove the residual CTAB.
- The pellets were resuspended in 0.5 ml 1X TE and stored 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 and stored at -20°C.

Verification of DNA in agarose gel electrophoresis

- The sides of the gel tray were taped to hold the gel while setting and well forming combs were placed in the tray. 0.8% agarose gel was prepared by mixing 1.6 g agarose with 200 ml 1X TBE buffer. Once it was cooled, add 6 µl of ethidium bromide. The boiled agarose was poured into the tray and allowed to stand for 30 min. The combs were removed and the gel was placed into the electrophoresis tank.

- ❖ 5 µl of DNA was mixed using a pipette with the loading buffer and the mix was loaded into a well on the gel and allowed to run for about 30 min at 110 Milli Amps.
- ❖ After electrophoresis, the gel was viewed under UV transilluminator and photographed. The chemicals required for DNA isolation are as follows.

Extraction buffer (pH 8.0)

CTAB	: 2 g	Make up to 100 ml with distilled water
1M Tris HCl	: 10 ml	
0.5 M EDTA	: 4 ml	
1.4 M NaCl	: 8.2 g	
PVP	: 1.2%	

Precipitation buffer (pH 8.0)

CTAB	: 1 g	Make up to 10 ml with distilled water
50 mM Tris HCl	: 5 ml	
10 mM EDTA	: 2 ml	

Stock precipitation buffer (TE buffer)

1M Tris HCl	: 100 ml
0.5 M EDTA	: 50 ml

1X TE buffer

1M Tris HCl	: 1 ml	Make up to 100 ml with distilled water
0.5 M EDTA	: 0.2 ml	

High salt TE

1M NaCl	: 5.85 g	Make up to 100 ml with distilled water
10 mM Tris HCl (pH 8.0)	: 1 ml	
0.1 mM EDTA (pH 8.0)	: 20 µl	

10X TBE (pH 8.0)

Tris base	: 21.6 g	Make up to 200 ml with distilled water and adjust the pH with NaOH
Boric acid	: 11 g	
0.5 M EDTA (pH 8.0)	: 8 ml	

CTAB/ NaCl (10% CTAB / 0.2M NaCl)

CTAB	: 5 g	Make up to 50 ml with distilled water
NaCl	: 2.04 g	

DNA amplification

PCRs were conducted in a total reaction volume of 30 µl containing 6 µl of autoclaved ion-exchanged water, 5 µl of dNTP mixture (stock of 10 mM of each dNTP), 13 µl of 10X Taq reaction buffer (200 mM Tris pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4.7H_2O$, 1% (v/v) Triton X-100, 50% (w/v) sucrose, 0.25% (w/v) cresol red), 2 µl of *rbc*L primer (0.67 mM final concentration), 2 µl of Taq DNA polymerase and 2 µl of genomic DNA. The amplicon size of *rbc*L ranges from 654 base pairs. Primers used (5'→3') were ATGTCACCACAAACAGAGACTAAAGC and GAAACGGTCTCTCCAACGCAT (Kress and Erickson, 2007; Fazekas *et al.,* 2008). PCR samples were loaded after mixing with gel loading dye along with 10 µl of DNA ladder.

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

Components of PCR mixture

PCR vial master mix	: 20 µl
*rbc*L primer mix (5 pmoles/µl)	: 2 µl
Genomic DNA	: 2 µl
Nuclease free water	: 6 µl
Total volume	30 µl

Cycle sequencing

PCR *rbc*L amplified products were sequenced after purification and the DNA samples were sequenced according to the method described by Sanger *et al.* (1977) on an ABI Prism 3100 - Avant Genetic Analyzer. Electrophoresis was carried out at 50 V till the dye reaches three fourth distance of the gel. The gel was viewed in UV transilluminator and the banding

pattern was observed. The obtained sequence was aligned using BioEdit sequence alignment editor version 7.0.4.1.

The sequences from each DNA region were aligned using MULTALIN tool. The nucleotide sequence data of the partial *rbc*L sequences were deposited in the GenBank, NCBI. Genetic distances were calculated using MEGA 5.2 software. Phylogenetic tree was constructed using maximum likelihood and UPGMA method based on the genetic distance model described by Tamura *et al.* (2011).

Biological activities

Antioxidant activity

DPPH radical scavenging activity

The antioxidant activity of selected *Cyathea* species ethanolic extracts was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (Blios, 1958). Ethanolic extracts at various aliquots (125-1000 µg/ml) was added to 5 ml of 0.1 mM ethanolic solution of DPPH and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Ascorbic acid was used as standard and DPPH solution without extract was served as negative control. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula: % of Inhibition = [(Control OD - Sample OD) / Control OD × 100]. IC_{50} of the extracts were calculated using Microsoft Excel 2007.

Superoxide radical scavenging activity

The assay was based on the capacity of ethanolic extracts of *Cyathea* species to inhibit formazan formation by scavenging superoxide radicals generated in riboflavin-light-NBT system (Beauchamp and Fridovich, 1971). Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 6), 20 mg riboflavin, 12 mM EDTA and 0.1 mg NBT. Reaction was started by illuminating the reaction mixture with different concentrations of

sample extracts for 90 sec. After illumination, the absorbance was measured at 590 nm immediately. Ascorbic acid was used as positive control. The percentage inhibition of superoxide anion generation was calculated.

% Inhibition = [(Control OD - Sample OD) / Control OD × 100]

Metal chelating activity

The chelation of ferrous ions by *Cyathea* ethanolic extracts was estimated by the method of Dinis *et al*. (1994). 50 µl of 2 mM $FeCl_2$ was added to 1 ml of different concentration of the extracts (125-1000 µg/ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. EDTA was used as standard. All the reagents without sample extracts were used as negative control. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated.

% of Metal chelating activity = [(Control OD - Sample OD) / Control OD] × 100

Nitric oxide radical scavenging activity

The nitric oxide scavenging activity of selected *Cyathea* species ethanolic extracts on nitric oxide radical was measured according to the method described by Sreejayan and Rao (1997). Sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of ethanolic extracts (125-1000 µg/ml) and incubated at room temperature for 150 min. Griess reagent (0.5 ml), containing 1% sulphanilamide, 2% H_3PO_4 and 0.1% NEDA was added to the mixture after incubation time. The absorbance of the chromophore formed was read at 546 nm. NEDA and the same mixture of the reaction without plant extracts were employed as positive and negative control. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample. The percentage inhibition activity was calculated using the formula: [(Control OD - Sample OD) / Control OD] × 100.

Hydroxyl radical scavenging activity

The scavenging activity of *Cyathea* species ethanolic extracts on hydroxyl radical was measured according to the method of Klein *et al*. (1991). Various concentrations (125-1000 µg/ml) of ethanolic extracts were added with 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1 ml of DMSO (0.85%, v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90^{o}C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1 ml of ice cold TCA (17.5% w/v). 3 ml of Nash reagent (75 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as negative control and Vitamin C was served as positive control. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated by using the following formula: [(Control OD - Sample OD) / Control OD] × 100.

Cytotoxic activity - MTT cell proliferation assay

Cell line and culture

The cell line of MCF 7 (human breast carcinoma) was obtained from National Centre for Cell Science, Pune, India. The cells were cultured in a growth medium (DMEM, pH 7.4), supplemented with 10% FBS and antibiotics, penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml).

MTT assay

The cytotoxicity of *C. nilgirensis*, *C. gigantea* and *C. crinita* ethanolic extracts against human breast carcinoma (MCF 7) was determined by the MTT assay (Selvakumaran *et al.,* 2003). The cells were seeded into wells of a 96 well microtitre plate at 3x10^{3} cells per well

with 100 µl of DMEM growth medium. It was then incubated for 24 h at 37°C under 5% CO_2 in a humidified atmosphere. Later, the medium was removed and fresh growth medium containing different test doses of *C. nilgirensis*, *C. gigantea* and *C. crinita* (12.5, 25, 50, 100 and 200 µg/ml) ethanolic extracts were added. 5 wells were included in each concentration. After 3 days of incubation at 37°C under 5% CO_2, the medium was removed. 20 µl of 5 mg/ml MTT (pH 4.7) was added per well and cultivated for another 4 h, the supernatant fluid was removed. 100 µl of DMSO was added per well and shaken for 15 min. The absorbance at 570 nm was measured with a UV spectrophotometer, using wells without cells as blanks. All the experiments were performed in triplicates. The absorbance of untreated cells was considered as 100%. The IC_{50} value was determined graphically. The conventional anticancer drug, adriamycin was used as a positive control. The inhibition of cell growth was calculated as a percent anticancer activity using the following formula:

% of Cell Inhibition = 100 - Sample Absorbance / Control Absorbance × 100

Brine shrimp lethality bioassay

Cytotoxic activity of different extracts of selected *Cyathea* species were evaluated using brine shrimp lethality bioassay method (Meyer *et al*., 1982). About 1 g of *Artemia salina* cysts was aerated in 1 L capacity glass jar containing filtered seawater. The air stone was placed in the bottom of the jar to ensure complete hydration of the cysts. After 24 h incubation at room temperature (25-29°C), newly hatched free-swimming nauplii were harvested from the bottom outlet. As the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. The freshly hatched free-swimming nauplii were used for the bioassay. 30 clean test tubes were taken and separated by 10 ml in each test tube. 25 tubes were used for the samples in five different concentrations ranging from 100-500 mg and 5 five tubes for control. With the help of a Pasteur pipette, 20 nauplii were transferred to each test tube containing various concentrations of petroleum ether, chloroform, acetone and

ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita*. Five replicates were made for each concentration and a control DMSO was also maintained. The standard plumbagin was used as positive control. The setup was allowed to remain for 24 h under constant illumination. After 24 h, the dead nauplii were counted with a hand lens. Using the recorded observations, LC_{50}, 95% confidence limit, LC_{90} and chi-square values were calculated using SPSS.

Larvicidal activity

Culex quinquefasciatus (4^{th} instar larvae) was collected from sewages of Tirunelveli district with the help of 'O' type brush. These larvae were brought to the laboratory and transferred to 18×13×4 cm size enamel trays containing 500 ml of water. It was maintained at 27±2°C, 75-85% RH, 14 h light and 10 h dark photoperiod cycles.

Larvicidal activity of different extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* was evaluated as per the standard method (WHO, 2005). Batches of twenty 4^{th} instar larvae of *C. quinquefasciatus* were collected separately and transferred to small disposable cups each containing 200 ml of water. The appropriate volume of dilution was added in the cups to obtain the desired target dosage (concentrations ranging from 100-500 mg) starting with the lowest concentration. Five replicates were set up for each concentration and simultaneously a control was maintained. The larval mortality in both treated and control were recorded after 24 h. The standard larvicide Temephos (Abate) was used as positive control. The control mortality was corrected by Abbott's formula (Abbott, 1925). The LC_{50} value was calculated by Probit analysis (Finney, 1979).

Qualitative phytochemical screening

Preliminary phytochemical analysis of twelve different metabolites was performed in five extracts of *C. nilgirensis, C. gigantea* and *C. crinita.* Thus out of 180 (3 x 5 x 12 = 180) tests for the presence or absence of metabolites, 71 tests conferred positive results and the remaining 109 gave negative results. All the three plant species showed significant indication about the presence of various bioactive secondary metabolites viz., steroids, alkaloids, phenolic groups, cardiac glycosides, flavonoids, saponins, tannins and terpenoids (Table 6). Coumarin was present only in *C. gigantea* and catechin demonstrated its existence only in *C. nilgirensis.* Anthraquinone and amino acids failed to show their presence in all the tested extracts of selected *Cyathea* species.

C. nilgirensis showed positive result for phenolics and cardiac glycosides in all the tested five extracts followed by flavonoids and terpenoids in four extracts. Steroids, alkaloids and saponins were present in three different extracts of *C. nilgirensis* whereas tannins and catechin showed its occurrence only in ethanolic extracts of *C. nilgirensis*. Among the five different extracts of *C. nilgirensis*, ethanolic extracts of *C. nilgirensis* illustrated the maximum number of metabolites presence (8/12) followed by acetone (6/12), petroleum ether (5/12), chloroform (4/12) and aqueous (3/12) extracts (Table 6).

Among the various solvents used to extract the phytoconstituents in *C. gigantea*, ethanolic extracts possess the maximum number of phytocompounds (8/12) followed by acetone (6/12) and aqueous (5/12) extracts. The results documented the presence of phenolics, terpenoids and cardiac glycosides in all the five tested extracts of *C. gigantea*. Saponins determined their existence in three different extracts followed by steroids, alkaloids

Table 6: Phytochemical constituents of studied *Cyathea* species

Metabolites	*C. nilgirensis*					*C. gigantea*					*C. crinita*					Total
	PE	C	A	E	Aq	PE	C	A	E	Aq	PE	C	A	E	Aq	
Steroids	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	6
Alkaloids	-	-	+	+	-	-	-	+	+	-	-	-	-	+	-	5
Phenolic compounds	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	13
Cardiac glycosides	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	11
Flavonoids	+	-	+	+	+	-	-	+	+	-	-	-	+	+	-	8
Saponins	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	8
Tannins	-	-	-	+	-	-	-	-	+	+	-	-	+	+	+	6
Anthraquinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coumarins	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
Catechine	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	1
Terpenoids	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	12
Amino acids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PE: Petroleum Ether; C: Chloroform; A: Acetone; E: Ethanol; Aq: Aqueous

and tannins in two extracts. Coumarin was present only in ethanolic extract of *C. gigantea* (Table 6).

Phytochemical studies on *C. crinita* revealed the presence of phenolics, saponins, tannins and terpenoids in three different extracts whereas steroids and flavonoids were present in two extracts. Alkaloids and cardiac glycosides confirmed their presence only in ethanolic extracts. Among the tested crude extracts, ethanolic extracts determined the presence of more number (7/12) of phytoconstituents whereas the other extracts showed minimum number of compounds (Table 6).

The cladogram was constructed based on the results of preliminary phytochemical profile of studied *Cyathea* species. The results showed two clades viz., C_1 and C_2. The clade C_1 was shared between *C. nilgirensis* and *C. gigantea* whereas clade C_2 showed the unique presence of *C. crinita* (Fig. 4).

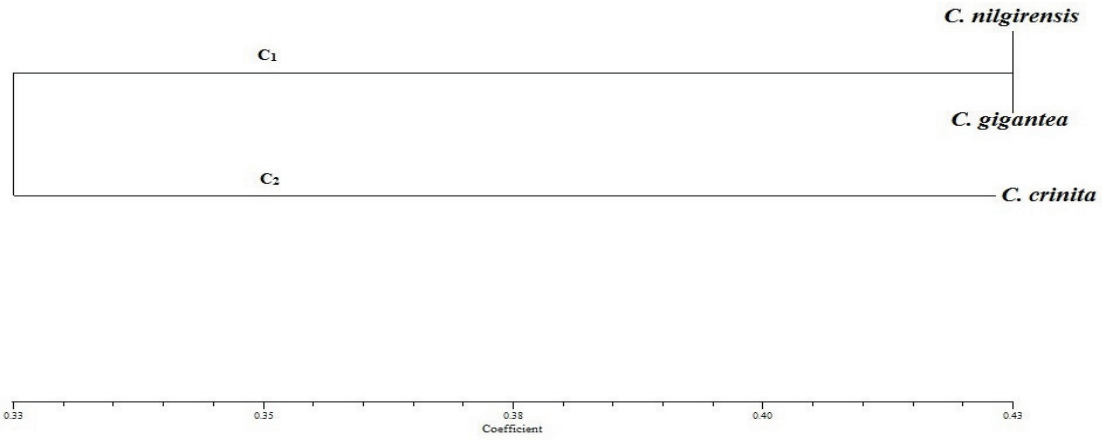

Fig. 4: Cladogram based on qualitative phytochemical profile of *Cyathea* species

Extractive values

The results of extractive values provide a basis to identify the quality and purity of the drug and help in identification and authentication of the plant material. The dry weight yield of different extracts of *C. nilgirensis, C. gigantea* and *C. crinita* were demonstrated in Table 7.

Table 7: Dry weight yield for studied species of *Cyathea*

Extracts	Extraction yield % (1 g)		
	C. nilgirensis	*C. gigantea*	*C. crinita*
Petroleum ether	0.15	0.27	0.21
Chloroform	0.28	0.31	0.58
Acetone	0.21	0.41	0.60
Ethanol	**0.57**	**0.76**	**0.85**
Aqueous	0.53	0.34	0.63

Fluorescence analysis

Fluorescence analysis of *C. nilgirensis, C. gigantea* and *C. crinita* plant powders was carried out under visible and UV light. The results showed more or less similar characters (Fig. 5). The fluorescence analysis revealed the similar and distinguished colour characteristics based on various chemical reagents and solvents employed.

Treatment	*C. nilgirensis*		*C. gigantea*		*C. crinita*	
	Ordinary light	UV light (365 nm)	Ordinary light	UV light (365 nm)	Ordinary light	UV light (365 nm)
Plant powder as such	Green	Brown	Light Green	Brown	Green	Brown
Petroleum ether extract	Dark Green	Reddish Brown	Dark Green	Brownish Green	Light Green	Brown
Chloroform extract	Light Green	Brown	Dark Green	Dark Red	Dark Green	Brownish Green
Acetone extract	Light Green	Brownish Green	Green	Brown	Light Yellow	Greenish Yellow
Ethanolic extract	Dark Green	Yellowish Green	Brown	Yellowish Green	Light Red	Dark Green
Aqueous extract	Green	Light Green	Brown	Brownish Green	Green	Brown
Powder + Con. H_2SO_4	Brown	Bluish Green	Dark Green	Brown	Brown	Brown
Powder + Con. HCl	Dark Green	Reddish Brown	Light Green	Dark Green	Brown	Brown
Powder + CH_3COOH	Yellowish Green	Pale Green	Light Green	Yellowish Green	Yellowish Green	Dark Green
Powder + NaOH	Light Yellow	Greenish Yellow	Green	Greenish Yellow	Light Green	Dark Green
Powder + 5% $FeCl_3$	Yellowish Green	Brown	Yellowish Green	Dark Green	Dark Green	Brown

Fig. 5: Fluorescent characters of studied *Cyathea* species

UV-Vis analysis

The UV-Vis spectra of *C. nilgirensis, C. gigantea* and *C. crinita* generally showed a decreased absorptivity or optical density as the wavelength increased. Although the spectra appeared to be broad and featureless showing no maxima or minima, the absorption intensity varied greatly among the selected five extracts. The presence of an absorbance band at a particular wavelength may be a good indicator for the presence of a chromophore. However, the position of the absorbance maximum is not fixed but it depends partially on the molecular environment of the chromophore and on the solvent in which the sample is dissolved.

The qualitative UV-Vis fingerprint profile of different extracts of *C. nilgirensis, C. gigantea* and *C. crinita* was selected at the wavelengths from 190 to 1000 nm due to sharpness of the peaks and proper baseline. The absorbance reveals the concentration of compound present in the expressed nanometre. These spectra are useful to identify the specific bioactive classes of molecules found in various extracts and distinguish the inter-specific variation among the *Cyathea* species.

Among the five different extracts of *C. nilgirensis*, petroleum ether and ethanolic extracts exhibited five peaks which denotes the presence of maximum number of compounds. Chloroform, acetone and aqueous extracts demonstrated only three peaks. Aqueous extract of *C. nilgirensis* showed the highest absorbance (3.373) at 204 nm and petroleum ether extract determined the lowest absorbance (0.025) at 254 and 284 nm in the UV region. In visible region, ethanolic extract represented the maximum absorbance 0.300 at 412 nm and petroleum ether extract exhibited minimum absorbance 0.014 at 668 nm (Fig. 6; Table 8).

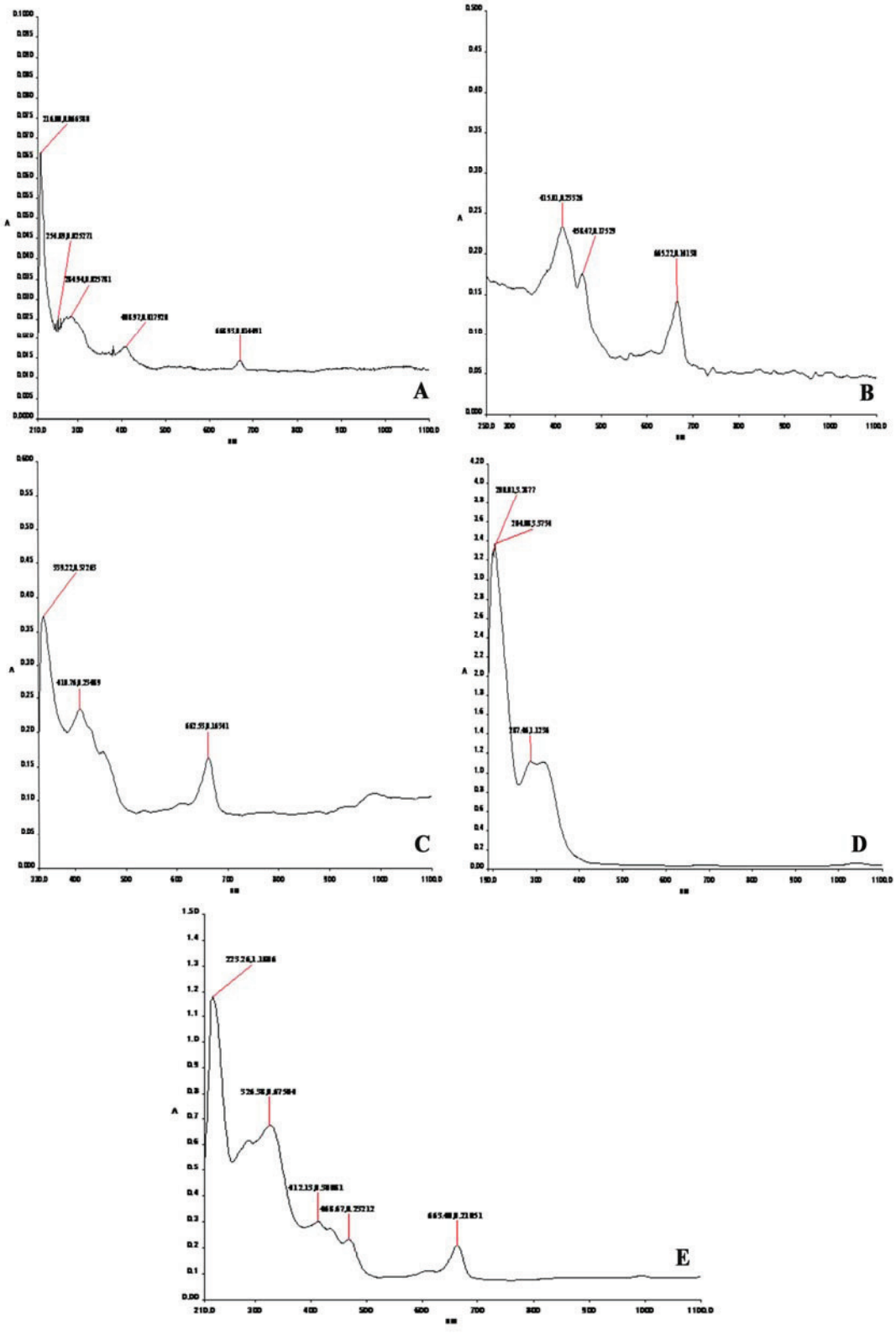

Fig. 6: UV-Vis spectra of *C. nilgirensis*

A - Petroleum ether; B - Chloroform; C - Acetone; D - Aqueous; E - Ethanol

Table 8: UV-Vis peak values of *C. nilgirensis*

Petroleum ether		Chloroform		Acetone		Ethanol		Aqueous	
λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS
216	0.066	415	0.233	339	0.372	223	1.180	200	3.287
254	0.025	458	0.175	410	0.234	326	0.675	204	3.373
284	0.025	665	0.141	662	0.163	412	0.300	287	1.123
408	0.017					468	0.232		
668	0.014					663	0.210		

In *C. gigantea,* ethanolic, chloroform and aqueous extracts expressed more number of peaks (4) followed by petroleum ether and acetone extracts (3). Aqueous extract represented highest absorbance 3.625 at 214 nm and petroleum ether extract depicted least absorbance 0.110 at 216 nm in the UV region. In visible region, acetone extract showed maximum absorbance 0.693 at 408 nm and petroleum ether extract demonstrated minimum absorbance 0.043 at 668 nm (Fig. 7; Table 9).

Table 9: UV-Vis peak values of *C. gigantea*

Petroleum ether		Chloroform		Acetone		Ethanol		Aqueous	
λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS
216	0.110	414	1.051	408	0.693	214	0.614	199	3.451
408	0.059	457	0.067	533	0.209	221	0.901	207	3.613
668	0.043	537	0.332	664	0.386	329	0.641	214	3.625
		666	0.578			663	0.149	323	1.679

UV-Vis spectra of ethanolic extract of *C. crinita* displayed more number of peaks (6) followed by chloroform and acetone extracts (4). Aqueous extract exhibited the highest absorbance 3.860 and petroleum ether extract showed the lowest absorbance 0.214 at 215 nm in the UV region. In visible region, ethanolic extract represented the maximum absorbance 1.157 at 407 nm and petroleum ether extract illustrated minimum absorbance 0.096 at 669 nm (Fig. 8; Table 10).

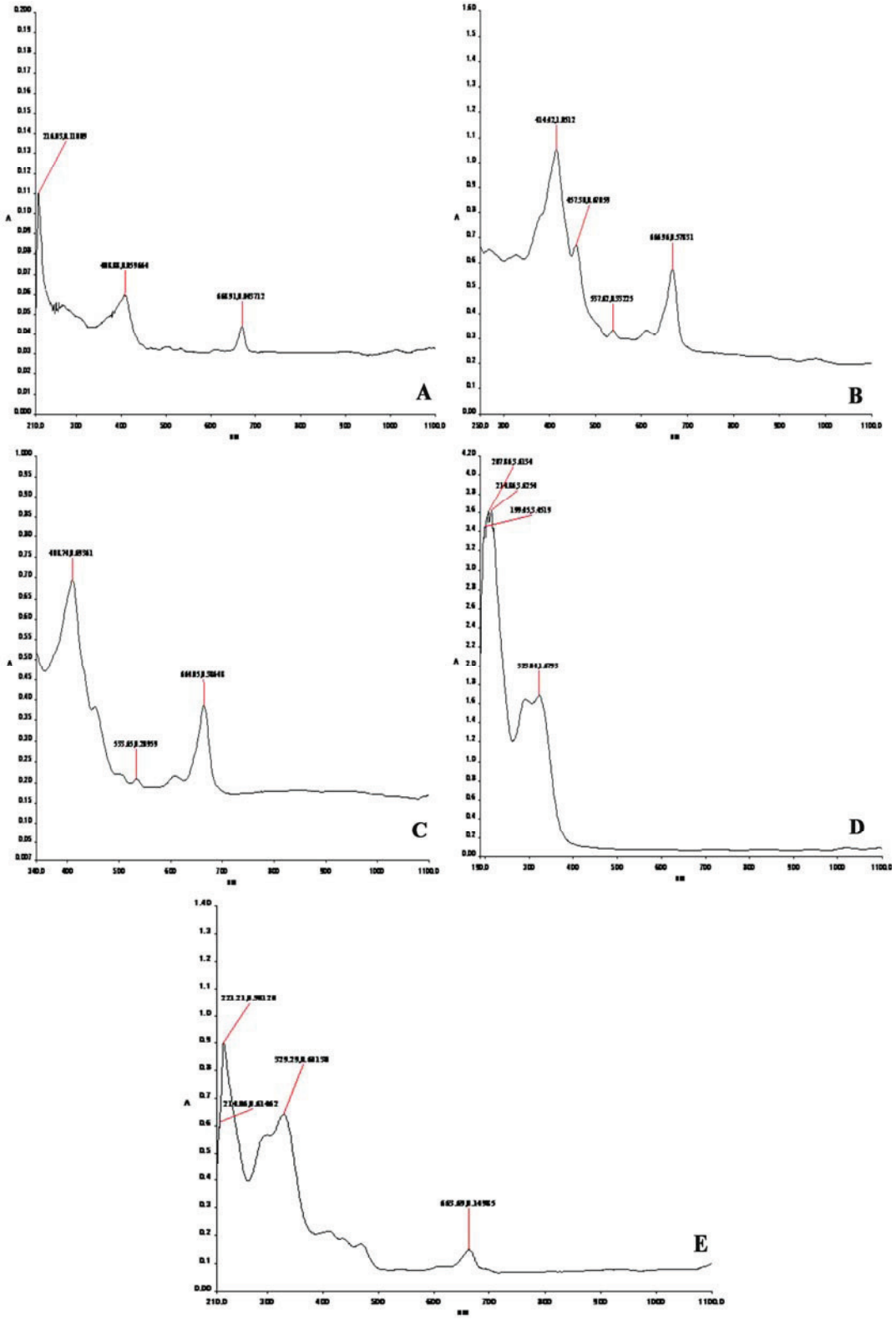

Fig. 7: UV-Vis spectra of *C. gigantea*

A - Petroleum ether; B - Chloroform; C - Acetone; D - Aqueous; E - Ethanol

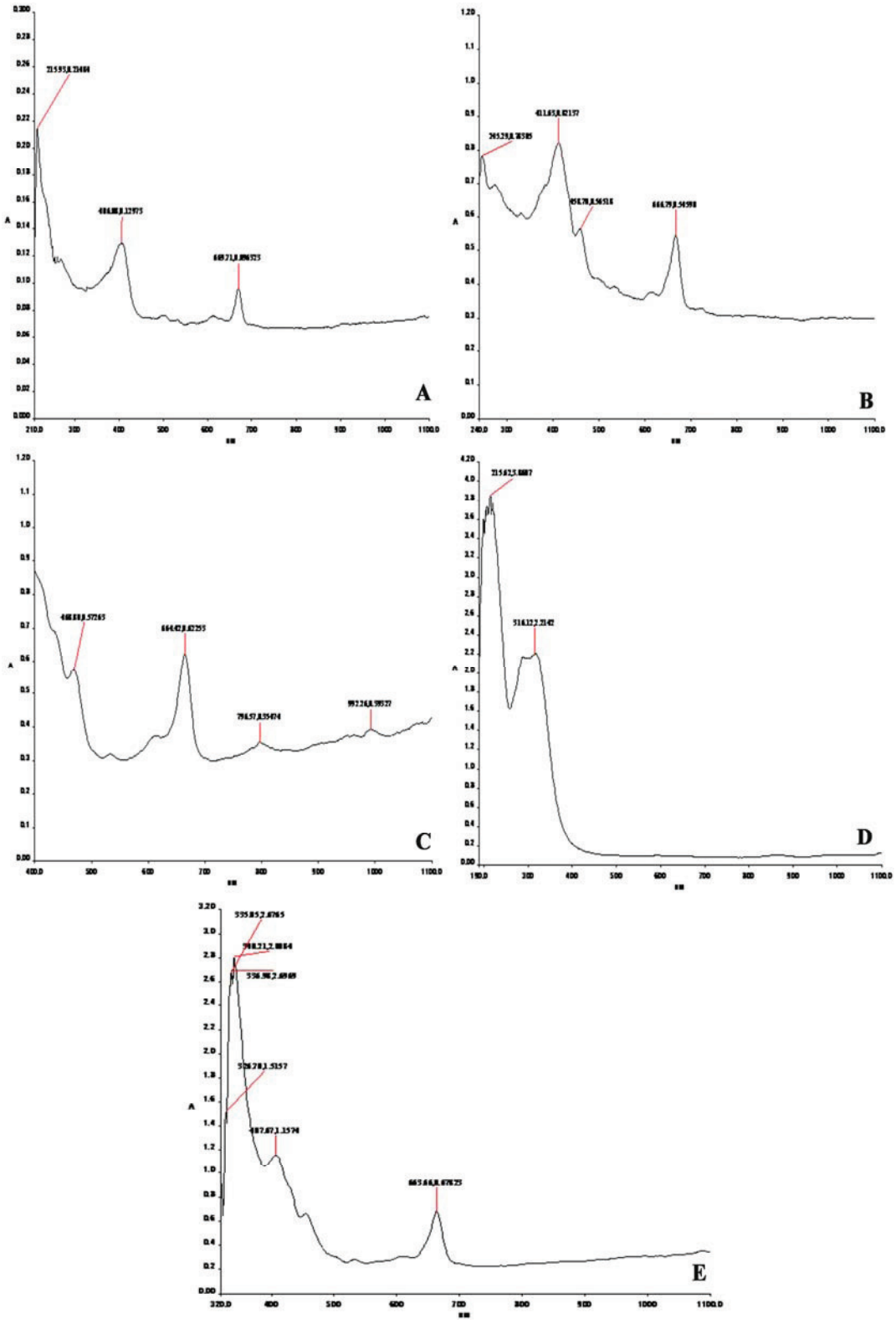

Fig. 8: UV-Vis spectra of *C. crinita*

A - Petroleum ether; B - Chloroform; C - Acetone; D - Aqueous; E - Ethanol

Table 10: UV-Vis peak values of *C. crinita*

Petroleum ether		Chloroform		Acetone		Ethanol		Aqueous	
λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS	λ_{max}	ABS
215	0.214	245	0.783	468	0.572	326	1.515	215	3.860
406	0.129	411	0.821	664	0.622	335	2.676	316	2.214
669	0.096	458	0.565	796	0.354	336	2.696		
		666	0.545	992	0.393	340	2.808		
						407	1.157		
						663	0.678		

The cladogram was constructed based on the results of UV-Vis spectroscopic profile of studied *Cyathea* species. Similar to the preliminary phytochemical analysis, the cladogram of UV-Vis profile showed two clades viz., C_1 and C_2. The clade C_1 was shared between the taxa *C. nilgirensis* and *C. gigantea* whereas clade C_2 showed the unique presence of taxon *C. crinita* (Fig. 9).

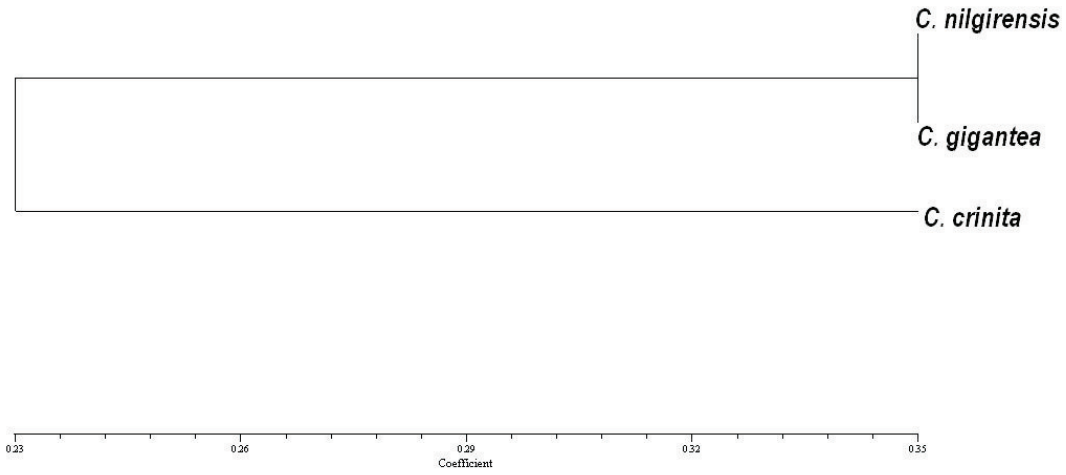

Fig. 9: Cladogram based on UV-Vis spectroscopic profile of *Cyathea* species

FT-IR analysis

The analytical evaluation of the FT-IR spectra in terms of functional groups corresponding to absorption of certain frequencies of *C. nilgirensis*, *C. gigantea* and *C. crinita* exhibits the characteristic fingerprint spectral features. It also revealed significant differences in band position and absorbance intensities. The comparative FT-IR spectrum shows that there is an apparent change in relative intensity of the bands.

The FT-IR peak values for various extracts of *C. nilgirensis* were displayed in Fig. 10-13; Table 11. The broad intensity band occurring at 3402 cm^{-1} and 1172 cm^{-1} may be due to O-H and C-O stretching vibration of alcohols. N-H stretch vibration at 3394 cm^{-1} and N-H bend at 1620 cm^{-1} and 1581 cm^{-1} determined the presence of primary amines in medium intensity. The strong band occurring at 2924 cm^{-1}, 2931 cm^{-1} and 2854 cm^{-1} related to C-H stretching vibration and the variable band at 1465 cm^{-1} and 1458 cm^{-1} indicates the occurrence of alkanes. The vibration of C=O stretch bands at 1797 cm^{-1}, 1735 cm^{-1} and 1720 cm^{-1} showed the existence of carboxylic acids, esters and aldehydes respectively. The frequency of bands occurring at 1543 cm^{-1}, 1496 cm^{-1}, 1381 cm^{-1} and 1373 cm^{-1} corresponding to N-O asymmetrical stretching revealed the presence of nitro compounds in all the four extracts of *C. nilgirensis*. Aromatics are absorbed at the region 1442 cm^{-1} only in acetone extract of *C. nilgirensis*. Strong intensity bands at 1288 cm^{-1}, 1165 cm^{-1}, 825 cm^{-1} and 725 cm^{-1} are representative for the presence of alkyl halides. The weak absorption band at 1280 cm^{-1} corresponds to aromatic amines and the characteristic absorption at 1072 cm^{-1} and 1033 cm^{-1} showed the presence of aliphatic amines. In petroleum ether extract, the strong band 956 cm^{-1} corresponding to =C-H bend indicates the presence of alkenes. O-H bend appearing at 925 cm^{-1} and 918 cm^{-1} represents the presence of carboxylic acids.

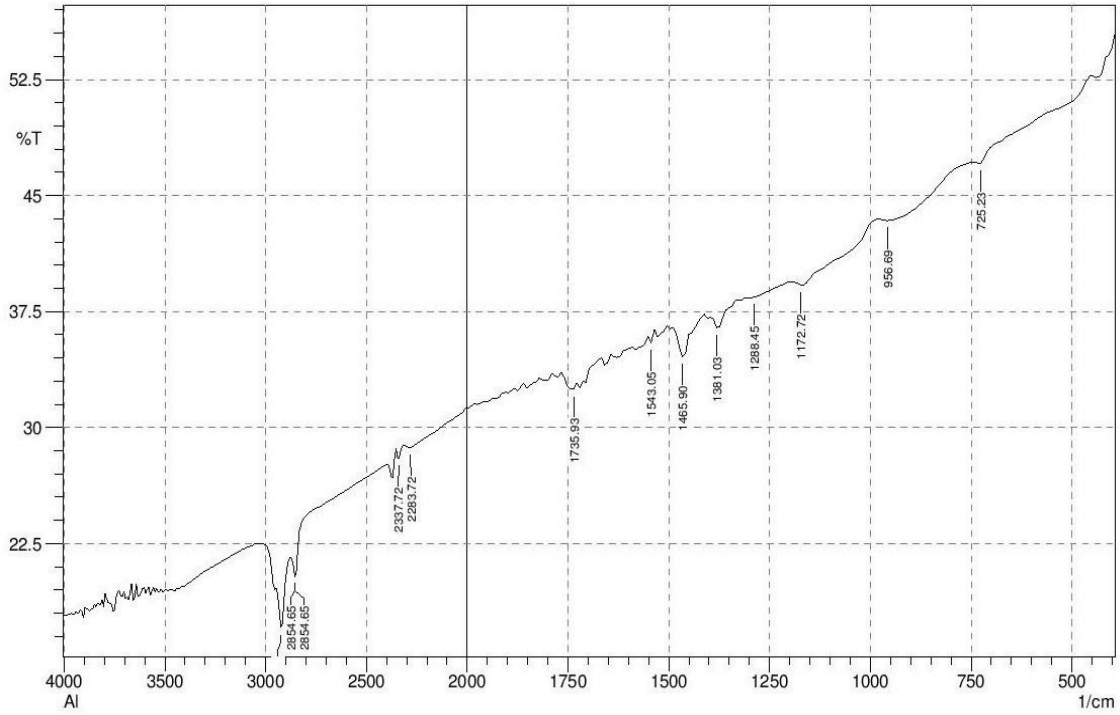

Fig. 10: FT-IR spectrum of *C. nilgirensis* petroleum ether extract

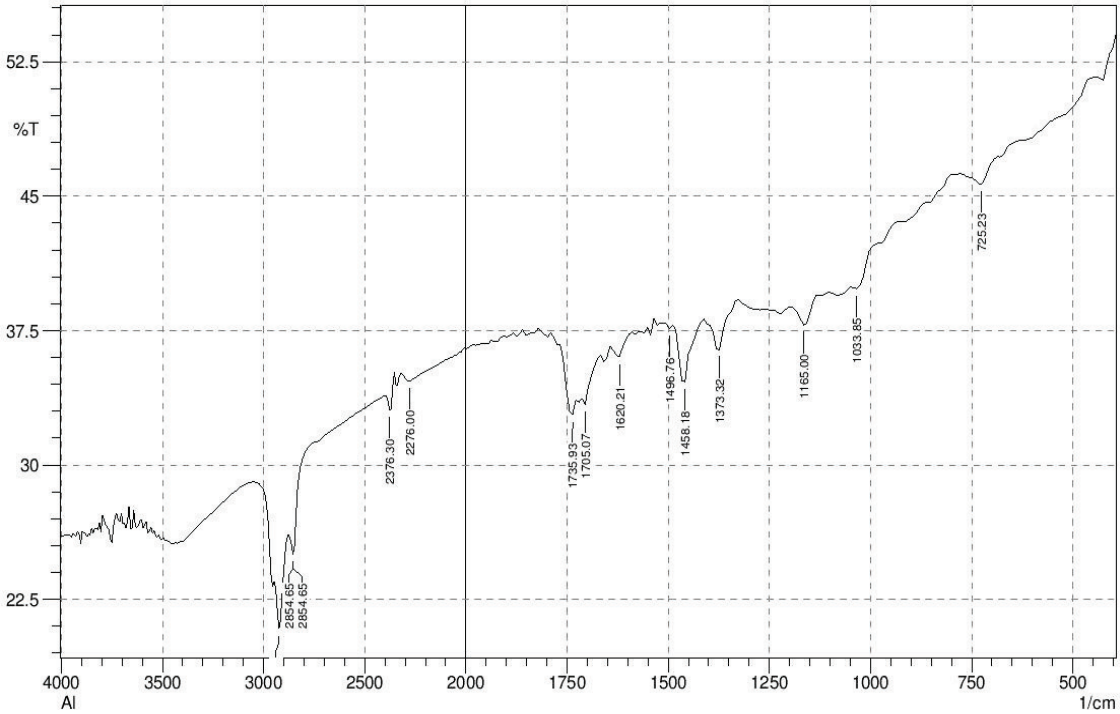

Fig. 11: FT-IR spectrum of *C. nilgirensis* chloroform extract

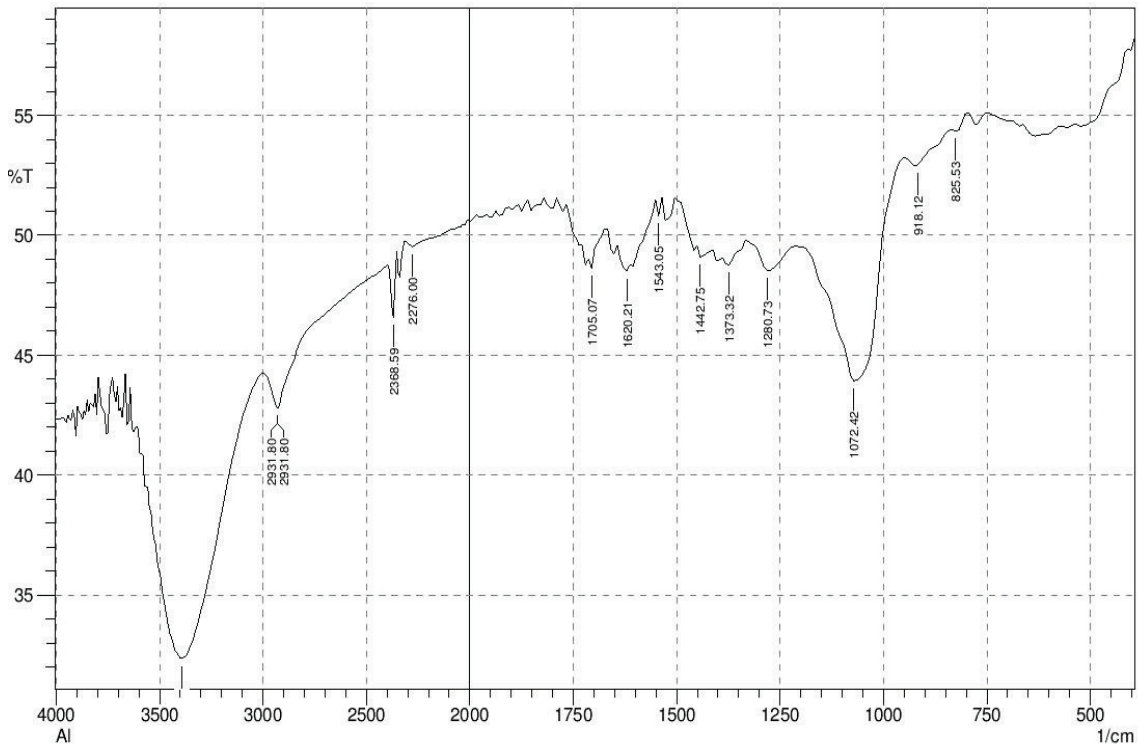

Fig. 12: FT-IR spectrum of *C. nilgirensis* acetone extract

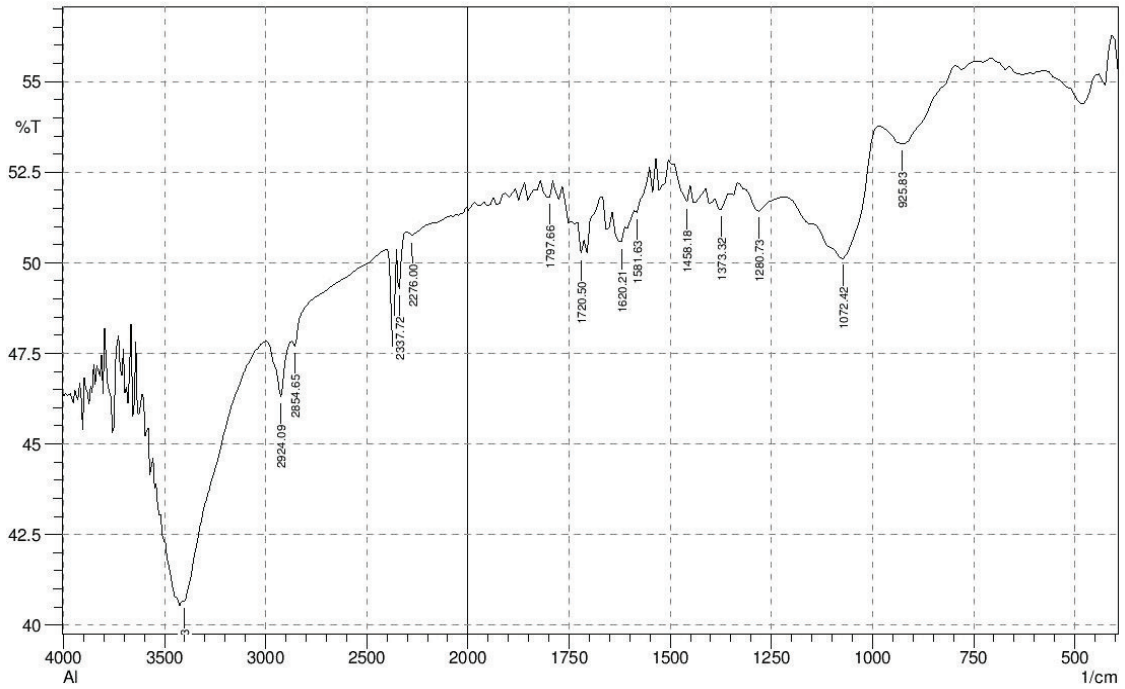

Fig. 13: FT-IR spectrum of *C. nilgirensis* ethanolic extract

Table 11: FT-IR peak values with functional groups of *C. nilgirensis*

Functional groups with bond stretching	Petroleum ether	Chloroform	Acetone	Ethanol
Alcohols (O-H stretch)	-	-	-	3402.43
Primary amines (N-H stretch)	-	-	3394.72	-
Alkanes (C-H stretch)	2924.09 2854.65	2924.09 2854.65	2931.80	2924.09 2854.65
Carboxylic acids (C=O stretch)	-	-	-	1797.66
Esters (C=O stretch)	1735.93	1735.93	-	-
Aldehydes (C=O stretch)	-	1705.07	1705.07	1720.50
Primary amines (N-H bend)	-	1620.21	1620.21	1620.21 1581.63
Nitro compounds (N-O asymmetric stretch)	1543.05 1381.03	1496.76 1373.32	1543.05 1373.32	1373.32
Alkanes (C-H bend)	1465.90	1458.18	-	1458.18
Aromatics (C=C stretch)	-	-	1442.75	-
Alkyl halides (C-F stretch)	1288.45	1165.00	-	-
Aromatic amines (C-N stretch)	-	-	1280.73	1280.73
Alcohols (C-O stretch)	1172.72	-	-	-
Aliphatic amines (C-N stretch)	-	1033.85	1072.42	1072.42
Alkenes (=C-H bend)	956.69	-	-	-
Carboxylic acids (O-H bend)	-	-	918.12	925.83
Alkyl halides (C-Cl stretch)	-	-	825.53	-
Alkyl halides (C-Br stretch)	725.23	725.23	-	-

FT-IR spectra of *C. gigantea* showed varied peak values with the presence of different functional groups (Fig. 14-17; Table 12). The broad peak intensity at 3448 cm^{-1} and 3402 cm^{-1} with O-H stretching and strong peak at 1111 cm^{-1} corresponds to C-O stretching determined the presence of alcohols. Alkanes were found to be present with C-H stretch at 2947 cm^{-1}, 2924 cm^{-1} and 2854 cm^{-1} and C-H bend at 1465 cm^{-1}, 1458 cm^{-1} and 1396 cm^{-1}. The peaks at 2839 cm^{-1}, 1797 cm^{-1}, 1751 cm^{-1}, 1735 cm^{-1}, 1720 cm^{-1}, 1705 cm^{-1}, 933 cm^{-1}, 925 cm^{-1} and 918 cm^{-1} indicates the presence of carboxylic acids with O-H stretch, C=O stretch and O-H bend type of vibrations. The wave numbers 1774 cm^{-1} and 1566 cm^{-1} present in petroleum ether extract of *C. gigantea* matching to C=O stretch and N-H bend represents the presence of anhydride and amide respectively. Alkene bands 1658 cm^{-1} and 1651 cm^{-1} with variable intensity corresponds to C=C stretching.

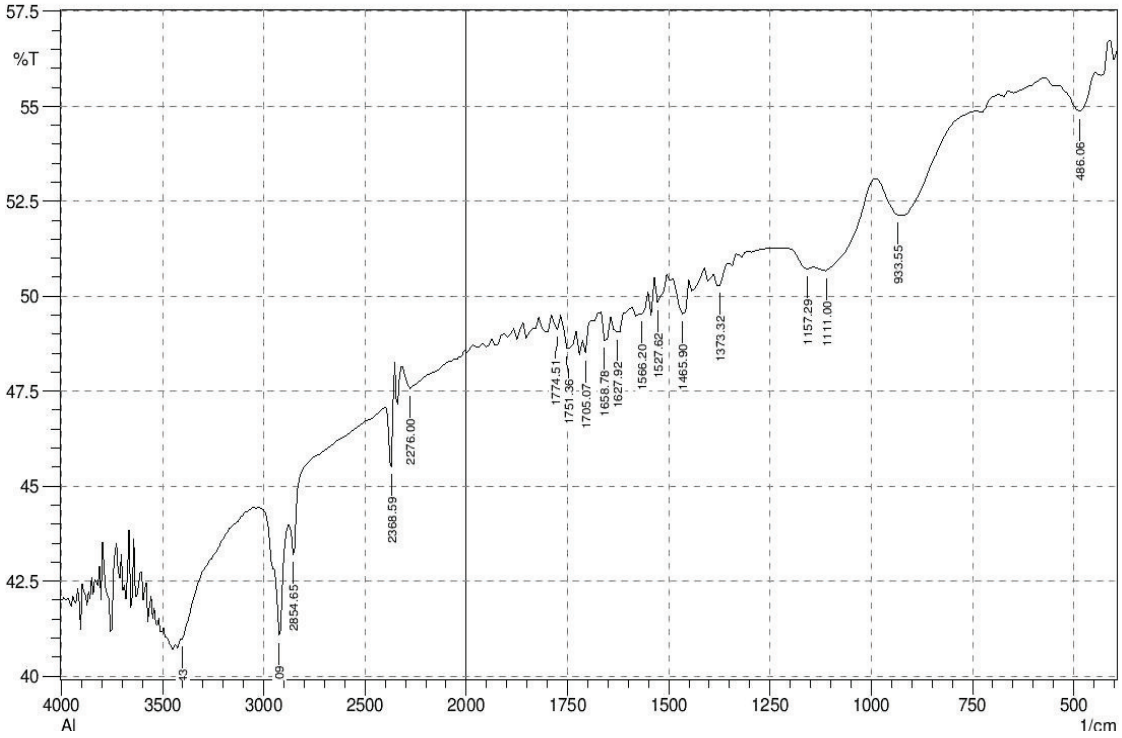

Fig. 14: FT-IR spectrum of *C. gigantea* petroleum ether extract

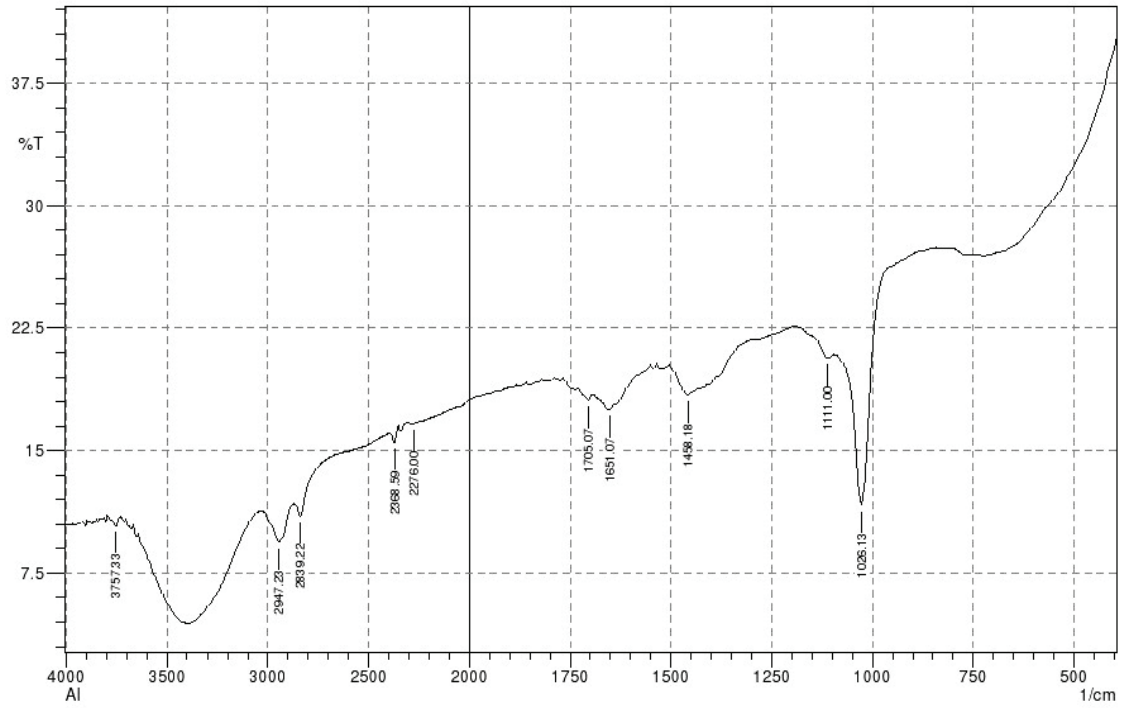

Fig. 15: FT-IR spectrum of *C. gigantea* chloroform extract

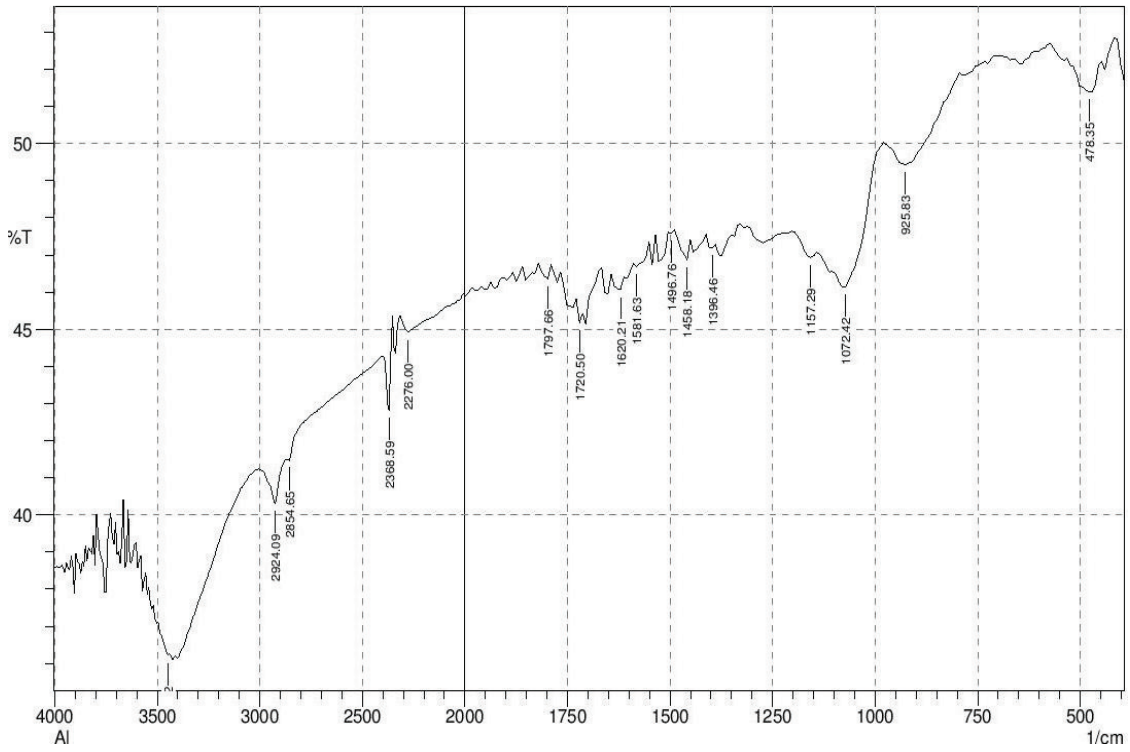

Fig. 16: FT-IR spectrum of *C. gigantea* acetone extract

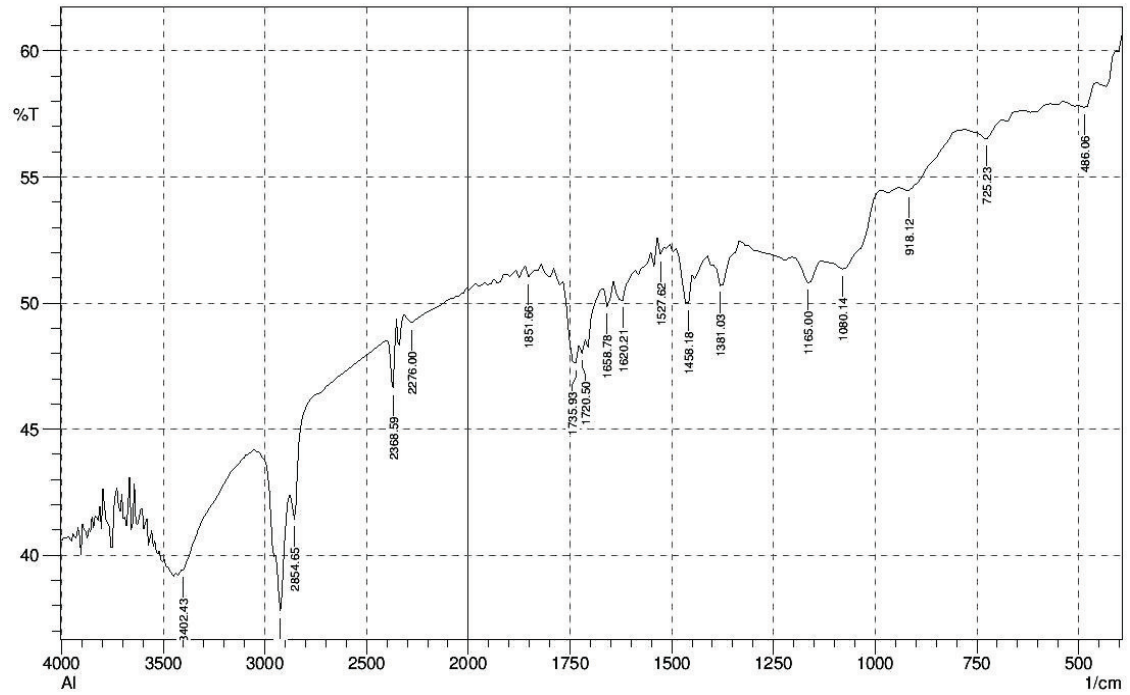

Fig. 17: FT-IR spectrum of *C. gigantea* ethanolic extract

Table 12: FT-IR peak values with functional groups of *C. gigantea*

Functional groups with bond stretching	Petroleum ether	Chloroform	Acetone	Ethanol
Alcohols (O-H stretch)	3402.43	-	3448.72	3402.43
Alkanes (C-H stretch)	2924.09 2854.65	2947.23	2924.09 2854.65	2924.09 2854.65
Carboxylic acids (O-H stretch)	-	2839.22	-	-
Anhydride (C=O stretch)	1774.51	-	-	-
Carboxylic acids (C=O stretch)	1751.36 1705.07	1705.07	1797.66 1720.50	1735.93 1720.50
Alkenes (C=C stretch)	1658.78	1651.07	-	1658.78
Primary amines (N-H bend)	1627.92	-	1620.21 1581.63	1620.21
Amide (N-H bend)	1566.20	-	-	-
Nitro compounds (N-O asymmetric stretch)	1527.62 1373.32	-	1496.76	1527.62 1381.03
Alkanes (C-H bend)	1465.90	1458.18	1458.18 1396.46	1458.18
Alkyl halides (C-F stretch)	1157.29	-	1157.29	1165.00
Alcohols (C-O stretch)	1111.00	1111.00	-	-
Aliphatic amines (C-N stretch)	-	1026.13	1072.42	1080.14
Carboxylic acids (O-H bend)	933.55	-	925.83	918.12
Alkyl halides (C-Br stretch)	-	-	-	725.23

The medium peaks obtained at 1627 cm^{-1}, 1620 cm^{-1} and 1581 cm^{-1} with N-H bending showed the presence of primary amines. Nitro compounds are absorbed at 1527 cm^{-1}, 1496 cm^{-1}, 1381 cm^{-1} and 1373 cm^{-1} with N-O asymmetrical stretching. The frequencies 1165 cm^{-1}, 1157 cm^{-1} and 725 cm^{-1} showed strong vibrations for the presence of alkyl halides. C-N stretching of aliphatic amines is located at 1080 cm^{-1}, 1072 cm^{-1} and 1026 cm^{-1} respectively.

The FT-IR analysis results of *C. crinita* different extracts was demonstrated in Fig. 18-21; Table 13. The FT-IR spectrum exhibited very broad absorption band at 3417 cm^{-1} with O-H stretching and strong intensity at 1103 cm^{-1} with C-O stretching alcohols.

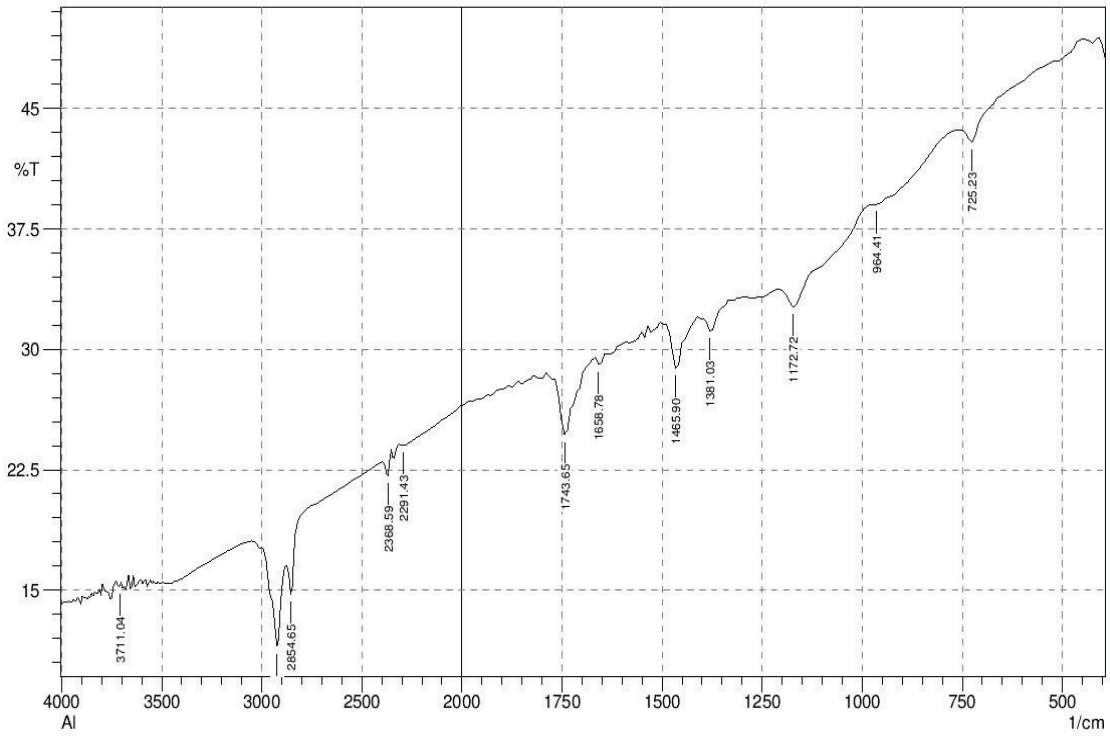

Fig. 18: FT-IR spectrum of *C. crinita* petroleum ether extract

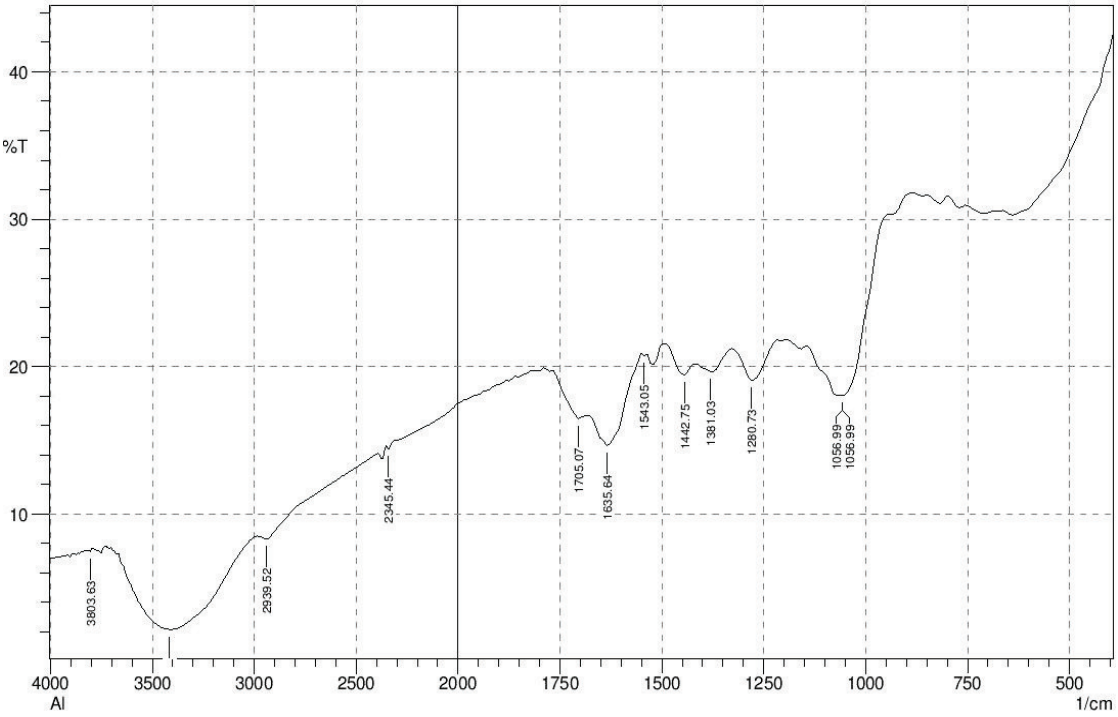

Fig. 19: FT-IR spectrum of *C. crinita* chloroform extract

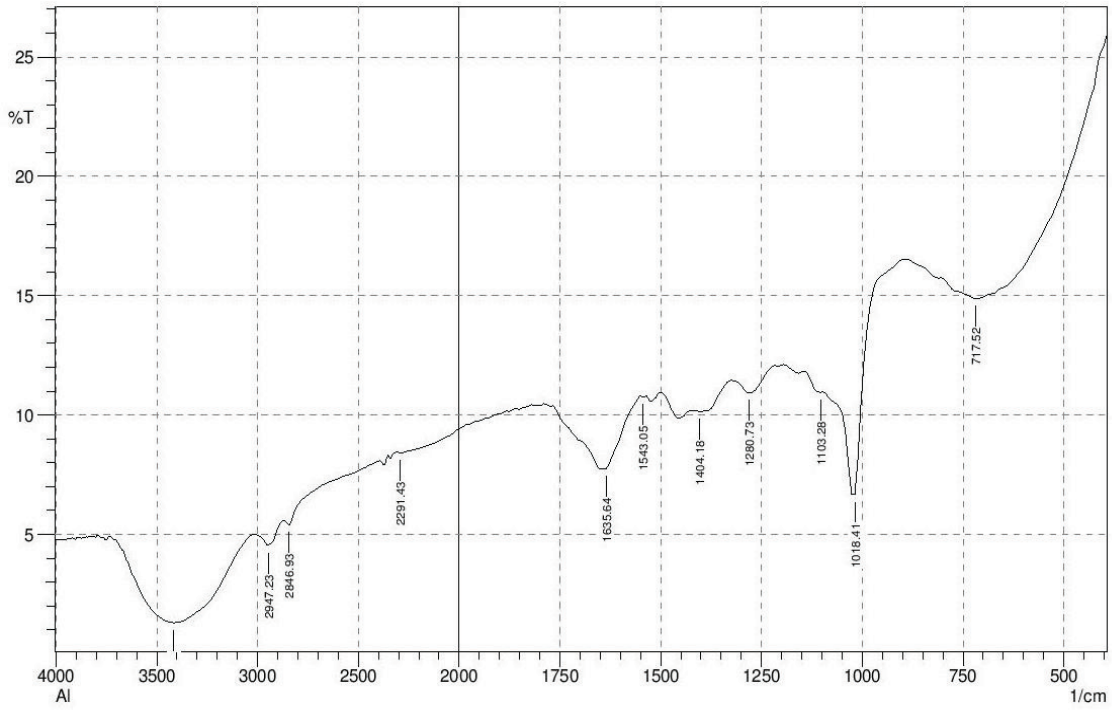

Fig. 20: FT-IR spectrum of *C. crinita* acetone extract

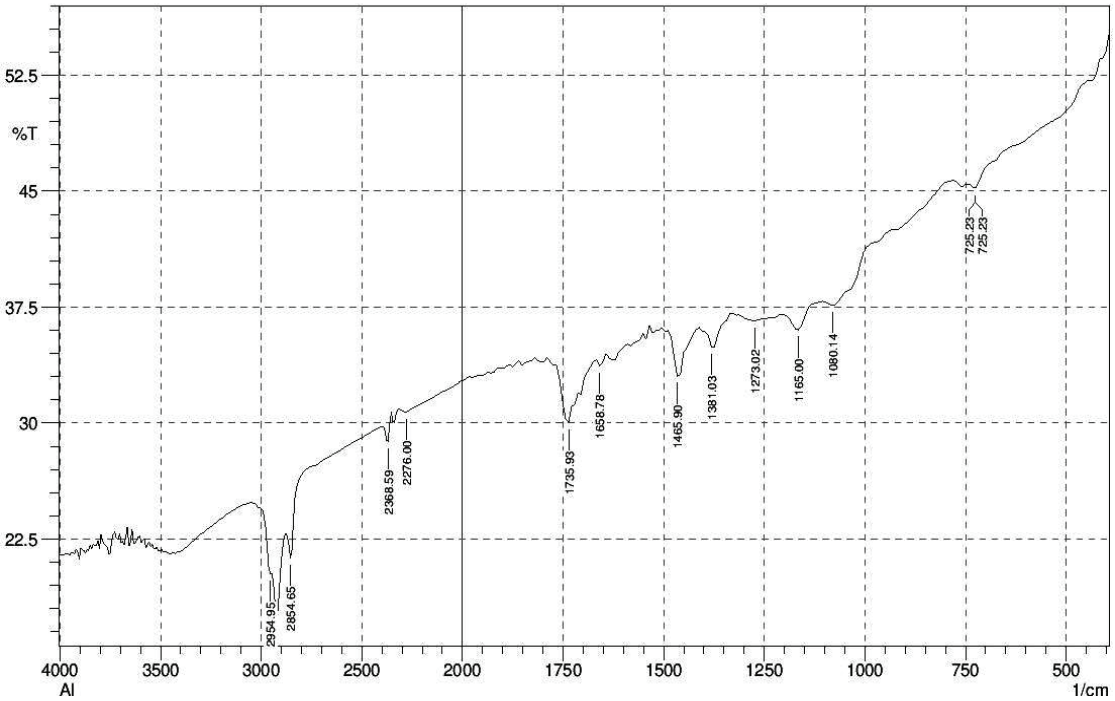

Fig. 21: FT-IR spectrum of *C. crinita* ethanolic extract

Table 13: FT-IR peak values with functional groups of *C. crinita*

Functional groups with bond stretching	Petroleum ether	Chloroform	Acetone	Ethanol
Alcohols (O-H stretch)	-	3417.86	-	-
Alkanes (C-H stretch)	2924.09 2854.65	2939.52	2947.23	2954.95 2854.65
Carboxylic acids (O-H stretch)	-	-	2846.93	-
Esters (C=O stretch)	-	-	-	1735.93
Carboxylic acids (C=O stretch)	1743.65	1705.07	-	-
Alkenes (C=C stretch)	1658.78	-	-	1658.78
Primary amines (N-H bend)	-	1635.64	1635.64	-
Nitro compounds (N-O asymmetric stretch)	1381.03	1543.05 1381.03	1543.05	1381.03
Aromatics (C=C stretch)	1465.90	1442.75	1404.18	1465.90
Aromatic amines (C-N stretch)	-	1280.73	1280.73	1273.02
Alkyl halides (C-F stretch)	1172.72	-	-	1165.00
Alcohols (C-O stretch)	-	-	1103.28	-
Aliphatic amines (C-N stretch)	-	1056.99	-	1080.14
Esters (C-O stretch)	-	-	1018.41	-
Alkenes (C-H bend)	964.41	-	-	-
Alkyl halides (C-Br stretch)	725.23	-	-	725.23
Alkyl halides (C-Cl stretch)	-	-	717.52	-

The strong C-H stretching vibration band corresponding to alkanes showed its occurrence in all the four extracts of *C. crinita* at 2954 cm^{-1}, 2947 cm^{-1}, 2939 cm^{-1}, 2924 cm^{-1} and 2854 cm^{-1} respectively. The wave numbers 2846 cm^{-1}, 1743 cm^{-1} and 1705 cm^{-1} were assigned as characteristic absorption of carboxylic acids. The fingerprint bands 1735 cm^{-1} and 1018 cm^{-1} were attributed to esters with C=O and C-O stretching respectively. The variable band 1658 cm^{-1} represents the stretching vibration of C=C and strong band 964 cm^{-1} confirmed the bending vibrations of C-H indicative of the alkenes. Medium absorption peak with N-H bending appearing in the region 1635 cm^{-1} showed the occurrence of primary amines. The strong absorption band observed around 1543 cm^{-1} and 1381 cm^{-1} was due to the N-O asymmetric stretching of nitro compounds. Medium C=C stretching vibrations were located at 1465 cm^{-1}, 1442 cm^{-1} and 1404 cm^{-1} for the presence of aromatics in *C. crinita*. The medium-weak bands in the region 1280 cm^{-1}, 1273 cm^{-1},

1080 cm^{-1} and 1056 cm^{-1} may be due to the existence of amines. The strong regions 1172 cm^{-1}, 1165 cm^{-1}, 725 cm^{-1} and 717 cm^{-1} were assigned due to stretching vibrations of alkyl halides.

The similarity indices were calculated and the cladogram was constructed based on the FT-IR spectroscopic profile of *Cyathea*. Similar to the qualitative phytochemical and UV-Vis analysis, the results of FT-IR analysis also revealed the similarities and variation among the studied *Cyathea* species (Fig. 22). The evolutionary tree constructed expressed two clusters (C_1 and C_2). The cluster C_1 includes *C. nilgirensis* and *C. gigantea* whereas cluster C_2 showed the unique presence of *C. crinita*. The preliminary phytochemical, UV-Vis and FT-IR analysis showed the similar type of cladogram.

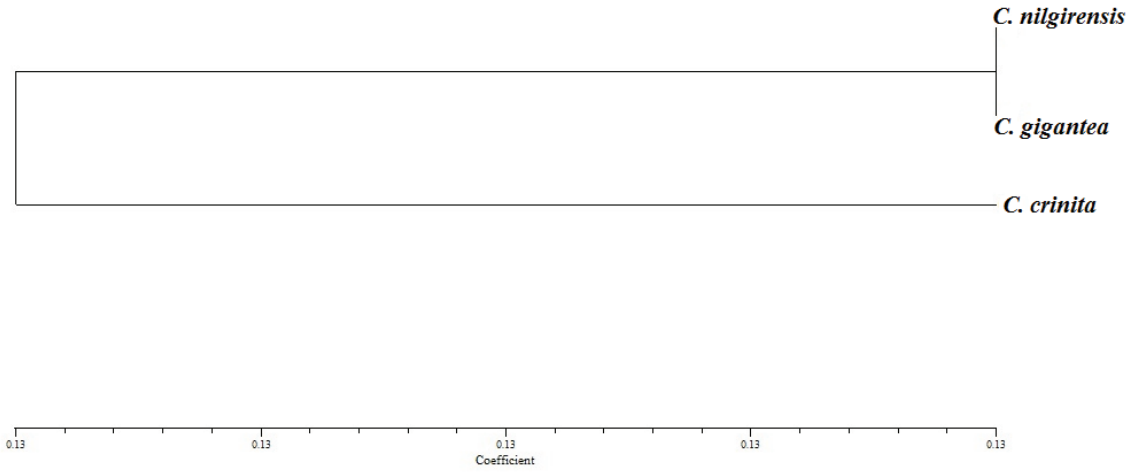

Fig. 22: Cladogram based on FT-IR spectroscopic profile of studied *Cyathea* species

HPTLC analysis

Alkaloids

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. Ethanolic extracts of studied *Cyathea* species showed 28 bands and confirmed the presence of 16 diverse types of alkaloids with varied R_f values ranged from 0.05 to 0.97 (Plate III; Table 14). In general, more degree of alkaloid diversity was observed in *C. nilgirensis* (11) when compared to the other two studied species. Among the different types of alkaloids, four alkaloids (0.05, 0.32, 0.67 and 0.97) showed their presence in all the three studied *Cyathea* species. The alkaloidal bands 0.70, 0.77 and 0.95 showed their unique presence in *C. nilgirensis* whereas the bands 0.14, 0.39 and 0.56 were present only in *C. gigantea. C. crinita* expressed two distinct bands viz., 0.23 and 0.87. Colchicine was present in *C. nilgirensis* (1.35%) and *C. gigantea* (1.10%) with the R_f value 0.53.

Table 14: HPTLC - Alkaloids profile of studied *Cyathea* species

R_f values	***C. nilgirensis***	***C. gigantea***	***C. crinita***	**Assigned substance**
0.05	+	+	+	Alkaloid 1
0.14		+		Alkaloid 2
0.16	+		+	Alkaloid 3
0.23			+	Unknown
0.26	+	+		Unknown
0.32	+	+	+	Alkaloid 4
0.39		+		Unknown
0.47	+		+	Alkaloid 5
0.53	+	+		**Colchicine**
0.56		+		Unknown
0.67	+	+	+	Alkaloid 6
0.70	+			Alkaloid 7
0.77	+			Unknown
0.87			+	Unknown
0.95	+			Unknown
0.97	+	+	+	Unknown

Glycosides

Different compositions of the mobile phase were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using ethyl acetate - ethanol - water (8 : 2 : 1.2) as the developing system. The results showed the presence of 23 bands and authenticated 12 different types of glycosides with R_f values ranged from 0.05 to 0.96 (Plate IV; Table 15). Maximum number (9) of glycosides has been observed in *C. crinita* followed by *C. nilgirensis* and *C. gigantea* (7). Among the nine different glycosides of *C. crinita*, two glycosides with R_f values 0.45 and 0.58 expressed their unique presence. The glycosidic band with R_f value 0.71 was present only in *C. nilgirensis* whereas *C. gigantea* showed the variation by the presence of glycosidic bands with R_f values 0.24 and 0.26. The glycosides with R_f value 0.05, 0.86 and 0.96 showed their common presence in all the three studied *Cyathea* species. The band with R_f value 0.62 indicates the presence of the standard glycoside swertiamarin. The glycoside swertiamarin failed to show its presence in the three studied *Cyathea* species.

Table 15: HPTLC - Glycosides profile of studied *Cyathea* species

R_f values	***C. nilgirensis***	***C. gigantea***	***C. crinita***	**Assigned substance**
0.05	+	+	+	Unknown
0.11	+	+		Glycoside 1
0.13	+		+	Glycoside 2
0.24		+		Unknown
0.26		+	+	Glycoside 3
0.28	+		+	Glycoside 4
0.45			+	Unknown
0.58			+	Unknown
0.62				**Swertiamarin**
0.71	+			Unknown
0.74		+	+	Glycoside 5
0.86	+	+	+	Glycoside 6
0.96	+	+	+	Unknown

Phenolics

HPTLC separation of phenolics determined high resolution and reproducible peaks in the mobile phase toluene - acetone - formic acid (4.5 : 4.5 : 1). Ethanolic extracts of studied *Cyathea* species determined the presence of 27 different types of phenolics bands and validated 14 different R_f values ranged from 0.05 to 0.92 (Plate V; Table 16). Maximum number (11) of phenolics has been observed in the ethanolic extract of *C. crinita*. Among the different types of phenolics, the bands with R_f value 0.05, 0.32, 0.43, 0.75 and 0.83 demonstrated their presence in all the three studied species of *Cyathea.* The phenolic band with R_f value 0.75 confirmed the presence of quercetin in the ethanolic extract of all the three studied *Cyathea* species. The percentage of quercetin presence was as follows: *C. nilgirensis* (0.19), *C. gigantea* (0.32) and *C. crinita* (0.30). The band with R_f value 0.28 validated the presence of catechin in *C. nilgirensis.* The phenolic bands with R_f value 0.28 and 0.80 showed its unique presence in *C. nilgirensis* whereas the bands 0.26 and 0.66 displayed their occurrence only in *C. gigantea*. The bands with R_f value 0.49, 0.59 and 0.72 expressed their existence only in *C. crinita.*

Table 16: HPTLC - Phenolics profile of studied *Cyathea* species

R_f values	***C. nilgirensis***	***C. gigantea***	***C. crinita***	**Assigned substance**
0.05	+	+	+	Phenolic 2
0.26		+	+	Phenolic 4
0.28	+			**Catechin**
0.32	+	+	+	Unknown
0.43	+	+	+	Phenolic 5
0.49			+	Phenolic 6
0.55	+		+	Unknown
0.59			+	Phenolic 7
0.66		+		Unknown
0.72			+	Phenolic 8
0.75	+	+	+	**Quercetin**
0.80	+			Unknown
0.83	+	+	+	Unknown
0.92		+	+	Unknown

Flavonoids

HPTLC separation of flavonoids showed high resolution and reproducible peaks in the mobile phase toluene - acetone - formic acid (4.5 : 4.5 : 1). Ethanolic extracts of studied *Cyathea* species represented the presence of 28 bands and substantiated 13 types of flavonoids with R_f values ranged from 0.05 to 0.82 (Plate VI; Table 17). Maximum number (10) of flavonoids was illustrated in ethanolic extract of *C. crinita* followed by *C. nilgirensis* and *C. gigantea* (9). Flavonoids with R_f values 0.05, 0.23, 0.30, 0.43 and 0.73 showed their presence in the studied three species of *Cyathea. C. nilgirensis* showed the presence of distinct band with R_f value 0.77. The flavonoidal bands with R_f values 0.14 and 0.71 displayed their unique presence in *C. crinita.* The flavonoid with R_f value 0.18 confirmed the presence of standard rutin which was failed to observe in the studied *Cyathea* species.

Table 17: HPTLC - Flavonoids profile of studied *Cyathea* species

R_f values	***C. nilgirensis***	***C. gigantea***	***C. crinita***	**Assigned substance**
0.05	+	+	+	Unknown
0.14			+	Flavonoid 1
0.18				**Rutin**
0.23	+	+	+	Flavonoid 2
0.30	+	+	+	Flavonoid 3
0.36	+	+		Flavonoid 4
0.43	+	+	+	Unknown
0.49		+	+	Unknown
0.60		+	+	Flavonoid 5
0.68	+	+		Flavonoid 6
0.71			+	Unknown
0.73	+	+	+	Unknown
0.77	+			Unknown
0.82	+		+	Unknown

Terpenoids

HPTLC separation of terpenoids determined high resolution and reproducible peaks in the mobile phase n-hexane - ethyl acetate (7.2 : 2.9). The results showed the presence of 23 bands and confirmed 16 types of terpenoids with R_f values ranged from 0.05 to 0.94 (Plate VII; Table 18). In general, more degree of terpenoid diversity was observed in *C. gigantea* and *C. crinita* compared to *C. nilgirensis*. *C. gigantea* and *C. crinita* revealed 9 terpenoid bands whereas *C. nilgirensis* showed only 5 terpenoid bands. Among the different terpenoids, the band with R_f value 0.05 and 0.63 was common to all the three studied species. *C. nilgirensis* confirmed the presence of one distinct band with R_f value 0.94 whereas the bands 0.14, 0.46, 0.47, 0.73 and 0.83 were present only in *C. gigantea.* The bands with R_f values 0.18, 0.33, 0.43, 0.54 and 0.75 displayed their unique presence in *C. crinita.* The terpenoid band with R_f value 0.79 depicted the presence of standard lupeol in *C. gigantea* (0.04%) and *C. crinita* (0.02%).

Table 18: HPTLC - Terpenoids profile of studied *Cyathea* species

R_f values	***C. nilgirensis***	***C. gigantea***	***C. crinita***	**Assigned substance**
0.05	+	+	+	Terpenoid 1
0.14		+		Unknown
0.18			+	Unknown
0.33			+	Unknown
0.43			+	Unknown
0.46		+		Terpenoid 2
0.47		+		Unknown
0.54			+	Terpenoid 3
0.63	+	+	+	Terpenoid 4
0.69	+		+	Unknown
0.73		+		Unknown
0.75			+	Unknown
0.79		+	+	**Lupeol**
0.83		+		Unknown
0.89	+	+		Unknown
0.94	+			Unknown

Steroids

Various solvent compositions of the developing system were examined for HPTLC analysis of steroids in order to achieve high resolution and reproducible peaks. The desired aim was achieved using the mobile phase toluene - acetone (9 : 1). The results displayed 26 bands and authenticated 15 types of steroids with R_f values ranged from 0.06 to 0.97 (Plate VIII; Table 19). In general, more degree of steroids diversity was observed in ethanolic extract of *C. gigantea* (11) followed by *C. nilgirensis* (8) and *C. crinita* (7). *C. nilgirensis* showed its unique steroidal expression with the R_f values 0.37 and 0.74. The steroid bands with R_f values 0.20, 0.52 and 0.65 were present only in *C. gigantea. C. crinita* revealed its distinct identity with the R_f values 0.15 and 0.90. The bands with R_f value 0.06, 0.23 and 0.41 were present in all the three studied species. In particular, the steroid band 0.41 confirmed the presence of standard stigmasterol with varied frequency viz., *C. nilgirensis* (0.33%), *C. gigantea* (0.29%) and *C. crinita* (0.52%).

Table 19: HPTLC - Steroids profile of studied *Cyathea* species

R_f values	***C. nilgirensis***	***C. gigantea***	***C. crinita***	**Assigned substance**
0.06	+	+	+	Unknown
0.15			+	Steroid 1
0.20		+		Unknown
0.23	+	+	+	Unknown
0.35		+	+	Steroid 2
0.37	+			Unknown
0.41	**+**	**+**	**+**	**Stigmasterol**
0.48	+	+		Steroid 3
0.52		+		Unknown
0.56	+	+		Unknown
0.65		+		Unknown
0.74	+			Unknown
0.79	+	+		Unknown
0.90			+	Unknown
0.97		+	+	Unknown

Tannins

Mobile phases of different compositions were tested in order to obtain high resolution and reproducible peaks. The desired aim was attained using toluene - ethyl acetate - formic acid - methanol (3 : 3 : 0.8 : 0.2) as the mobile phase. Ethanolic extracts of studied *Cyathea* species showed the presence of 30 bands and validated 17 diverse types of tannins with R_f values ranged from 0.05 to 0.93 (Plate IX; Table 20). Maximum number (13) of tannins was found in *C. crinita* when compared to the other studied species. *C. nilgirensis* showed 10 different tannins and *C. gigantea* demonstrated the presence of 8 different tannins. Among the different types, tannin with the R_f values 0.05, 0.09 and 0.82 showed their presence in all the studied three *Cyathea* species. The tannin bands 0.24, 0.34, 0.66 and 0.73 showed their unique presence in *C. nilgirensis*. *C. crinita* expressed three distinct bands viz., 0.49, 0.56 and 0.93. Gallic acid was present only in *C. crinita* (0.09%) with the R_f value 0.49.

Table 20: HPTLC - Tannins profile of studied *Cyathea* species

R_f values	***C. nilgirensis***	***C. gigantea***	***C. crinita***	**Assigned substance**
0.05	+	+	+	Tannin 1
0.09	+	+	+	Tannin 2
0.15		+	+	Unknown
0.22		+	+	Tannin 3
0.24	+			Unknown
0.34	+			Unknown
0.40		+	+	Unknown
0.49			+	**Gallic acid**
0.56			+	Tannin 4
0.60	+	+		Unknown
0.63		+	+	Unknown
0.66	+			Unknown
0.69	+		+	Unknown
0.73	+			Unknown
0.82	+	+	+	Unknown
0.88	+		+	Unknown
0.93			+	Unknown

Based on the HPTLC profile of the studied *Cyathea* species, the similarity indices were calculated and the cladogram was constructed (Fig. 23). The results expressed two clusters (C_1 and C_2). The cluster C_1 depicted the distinct presence of *C. nilgirensis* whereas C_2 showed the similarity between *C. gigantea* and *C. crinita*.

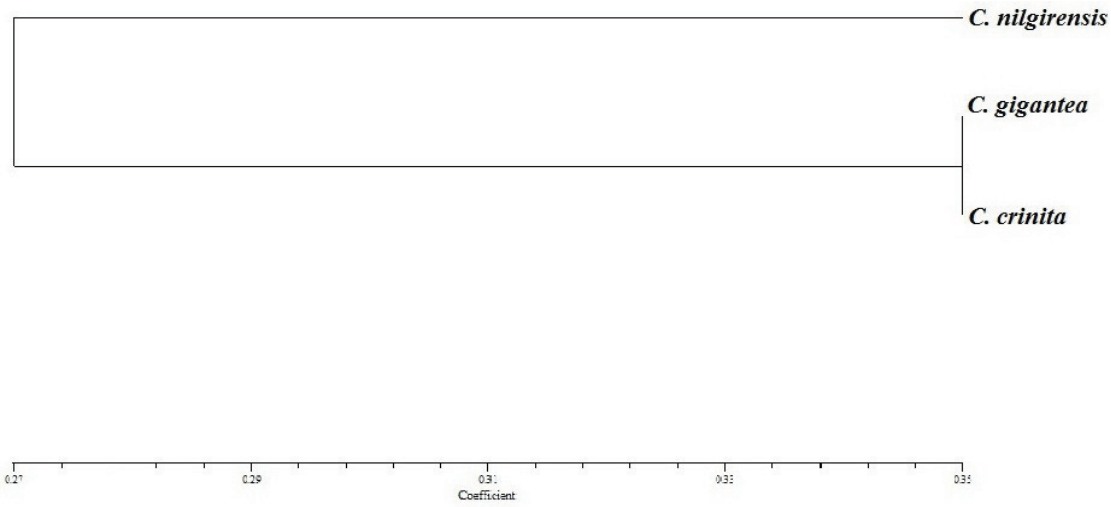

Fig. 23: Cladogram based on HPTLC profile of studied *Cyathea* species

HPLC analysis

HPLC spectral data generated by the detector was used to verify the identity of the compounds present in the chromatogram. The qualitative HPLC fingerprint profile of ethanolic extracts of selected *Cyathea* species was detected based on the sharpness of peaks and proper baseline. The results showed different peaks at various retention time with significant percent peak area (Fig 24-26; Table 21). HPLC chromatogram of *C. nilgirensis* confirmed the compound with the most abundant peak separated at a retention time of 18.09 min and peak area 99.65% (Fig. 24). The results also showed the compound separated at a retention time of 29.66 min with the peak area 0.35%. *C. gigantea* confirmed the most prominent peak with 76.77% separated at a retention time of 17.39 min (Fig. 25). The other prominent and moderate peaks reported in *C. gigantea* includes

the compound separated with a retention time of 19.24 min and 27.92 min with the percent peak area 19.50 and 3.73 respectively. The prominent peaks present in *C. crinita* were separated at a retention time of 16.06 min and 26.60 min with the percent peak area 67.75 and 32.25 correspondingly (Fig. 26).

Table 21: HPLC peak values of studied *Cyathea* species

Peaks	*C. nilgirensis*		*C. gigantea*		*C. crinita*	
	RT (min)	Area (%)	RT (min)	Area (%)	RT (min)	Area (%)
1	18.090	99.65	17.391	76.77	16.060	67.75
2	29.663	0.35	19.242	19.50	26.606	32.25
3			27.926	3.73		

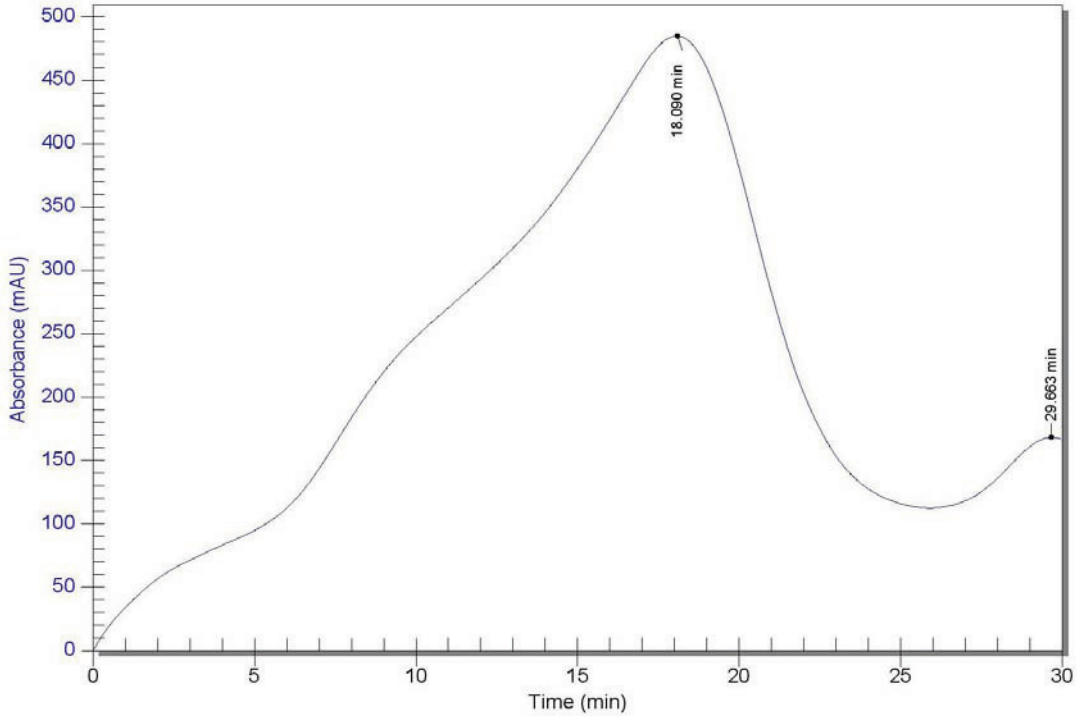

Fig. 24: HPLC chromatogram of *C. nilgirensis* ethanolic extract

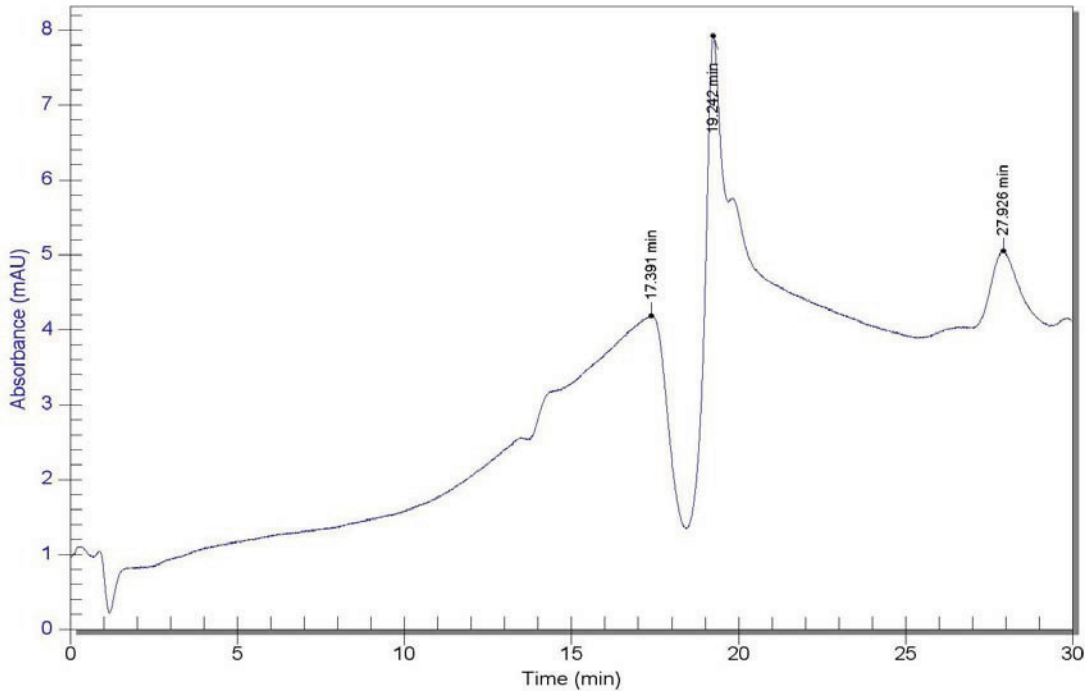

Fig. 25: HPLC chromatogram of *C. gigantea* ethanolic extract

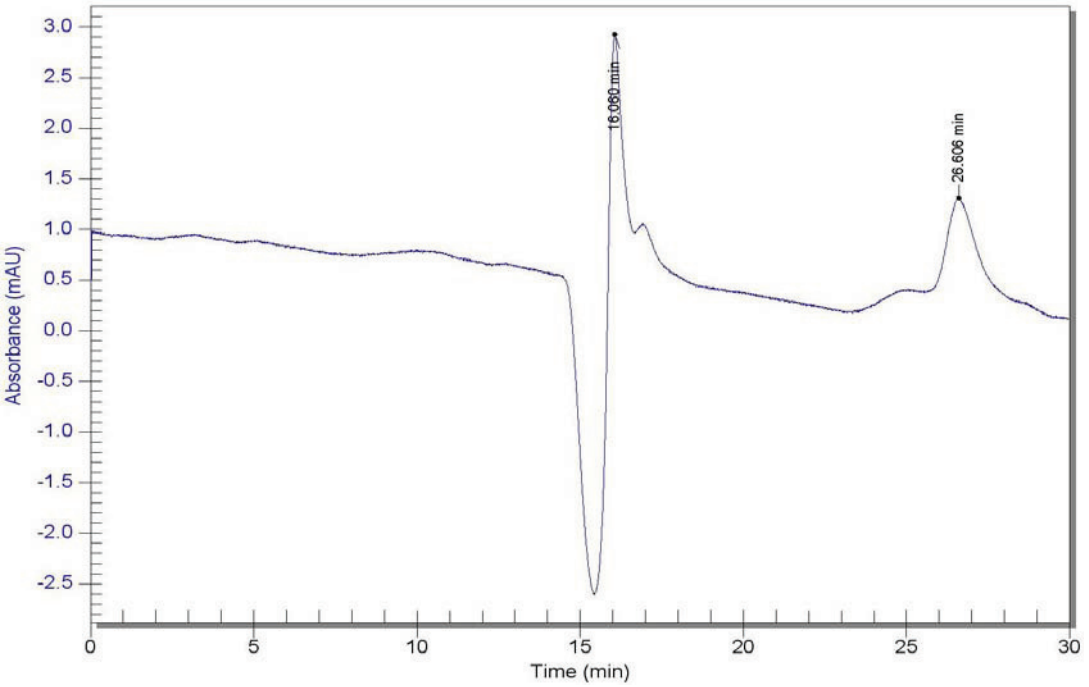

Fig. 26: HPLC chromatogram of *C. crinita* ethanolic extract

GC-MS analysis

GC-MS analysis of *Cyathea* species confirmed the presence of various phytochemical compounds in the ethanolic extracts. The active principles with their RT, molecular formula, molecular weight and concentration (%) in the ethanolic extracts of studied *Cyathea* species were presented in Table 22-24. Totally, 30 compounds were detected in each *Cyathea* species during 38.7 min measurement period. The compounds were predicted based on the mass spectrometry attached with GC and peak values of FT-IR spectrum. The major components present in *C. nilgirensis* includes Methyloctadecyl dichlorosilane (29.19%) and 2-Methylbutane-1,4-diol, 3-(1-ethoxyethoxy)- (24.48%) separated at the retention times 38.75 and 3.12 min respectively (Fig. 27; Table 22). *C. gigantea* showed the presence of 2-Methylbutane-1,4-diol, 3-(1-ethoxyethoxy)- (42.37%) and 2-Hydroxy-5-methyl Benzaldehyde (16.26%) separated at the retention times 3.13 and 16.64 min respectively (Fig. 28; Table 23). *C. crinita* confirmed the existence of the prevailing compound 2-Hydroxy-5-methyl benzaldehyde (55.45%) separated at the retention time 16.68 min (Fig. 29; Table 24).

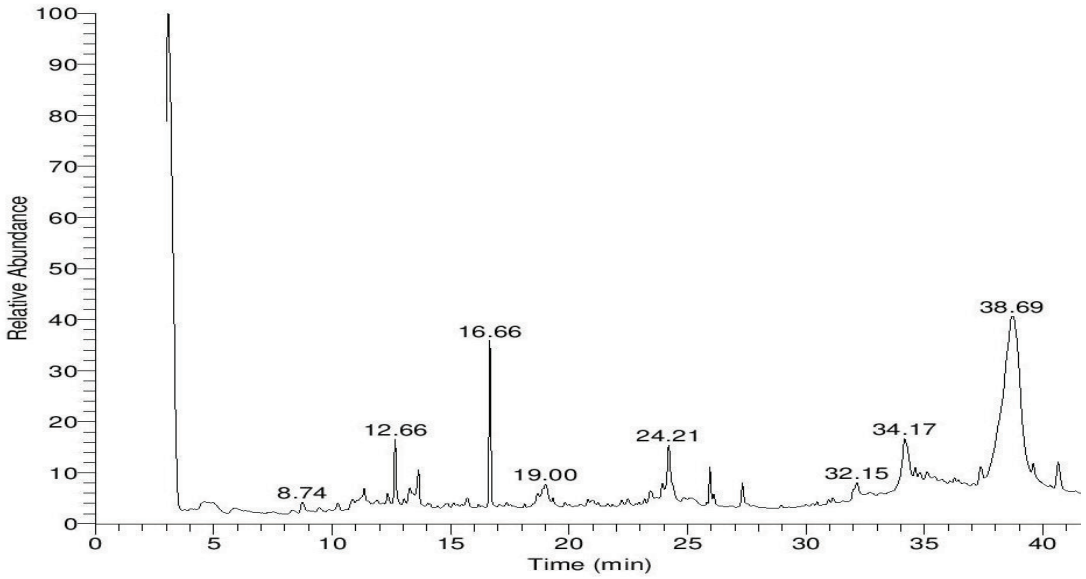

Fig. 27: Gas chromatogram of *C. nilgirensis* ethanolic extract

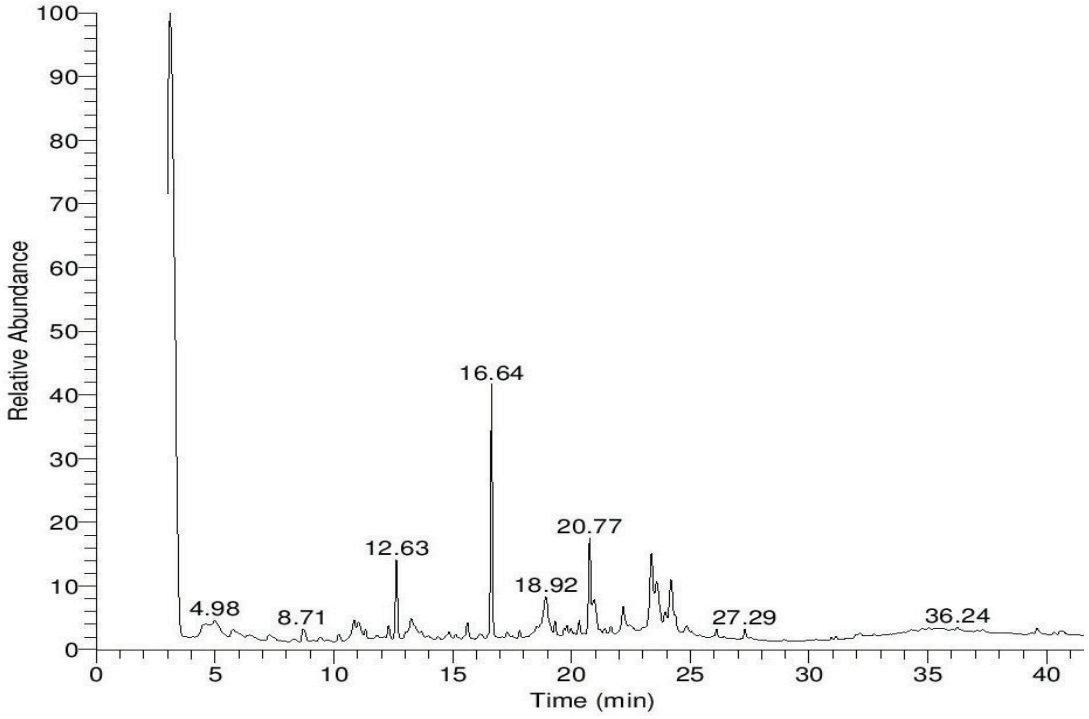

Fig. 28: Gas chromatogram of *C. gigantea* ethanolic extract

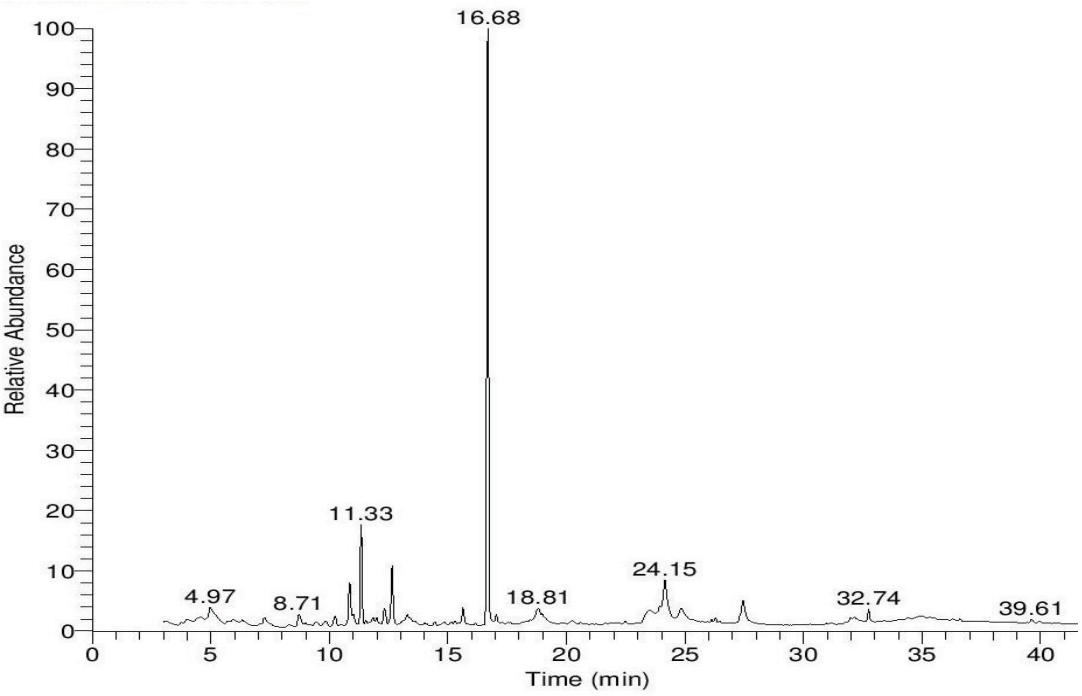

Fig. 29: Gas chromatogram of *C. crinita* ethanolic extract

Table 22: GC-MS analysis of *C. nilgirensis* ethanolic extract

Name of the compound	RT (min)	Peak area (%)	Molecular formula	MW	Biological activities
2-Methylbutane-1,4-diol, 3-(1-ethoxyethoxy)-	3.12	24.48	$C_9H_{20}O_4$	192	Sclerosant, Leukopoiesis stimulant, Phobic disorders treatment, Hemostatic, Macrophage stimulant, Antipruritic, allergic, Cholesterol antagonist, Cardioprotectant, Lipid metabolism regulator, Antipyretic, Antihypertensive, Antithrombotic, Apoptosis agonist, Antiseborrheic, Antiviral (Influenza, Herpes)
d-Glucosamine	5.84	0.76	$C_6H_{13}NO_5$	179	Anti-infective, Antiprotozoal (Leishmania), Antitoxic, Antiviral (Picornavirus), Cytostatic
Cyclohexasiloxane, dodecamethyl-	8.74	0.85	$C_{12}H_{36}O_6Si_6$	444	Antineoplastic, Estrogen agonist, Nitrate reductase inhibitor, Antiviral (Adenovirus), Vasoprotector, Oxidizing agent, Lipotropic, Contraceptive
à-D-Galactopyranose, 6-O-(trimethylsilyl)-, cyclic 1,2:3,4-bis(butylboronate)	10.24	0.51	$C_{17}H_{34}B_2O_6Si$	384	Antiviral (HIV), Antineoplastic (lung cancer), Sclerosant, Antimycoplasmal, Antiprotozoal, Lysozyme inhibitor, β-amylase inhibitor, Antiinfective, Catalase stimulant
D-Glucose, 6-O-à-D-galactopyranosyl-	10.85	0.66	$C_{12}H_{22}O_{11}$	342	β-glucosidase inhibitor, Immunostimulant, Laxative, Membrane integrity agonist, Vasoprotector Radioprotector, Antihypoxic, Hepatoprotectant, Hepatic disorders treatment
Cyclodecasiloxane, eicosamethyl	11.36	1.04	$C_{20}H_{60}O_{10}Si_{10}$	740	Estrogen agonist, Antiseborrheic, Carminative, Oxygen scavenger, Erythropoiesis stimulant, Antidyskinetic, Antihypotensive, Contraceptive female, Insecticide, Antispirochetal, Antinephritic

2H-Pyran-2-methanol, tetrahydro-	12.35	0.70	$C_6H_{12}O_2$	116	Platelet aggregation stimulant, Spasmolytic, Antiviral (Pox virus), Respiratory analeptic, Antieczematic, ATPase stimulant, Muscle relaxant, Anticonvulsant, Analgesic stimulant, Antineoplastic (renal cancer)
à-d-Mannofuranoside, isopropyl-	13.27	1.04	$C_9H_{18}O_6$	222	Vasoprotector, Wound healing agent, Antioxidant, Anticarcinogenic, Hemostatic, Anti-inflammatory, Antimetastatic, Chemosensitizer, Antihelmintic (Nematodes), Anesthetic, Antipruritic, Antitreponemal
2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1] hept-2-yl ester, exo-	13.64	2.08	$C_{13}H_{20}O_2$	208	Antieczematic, Dermatologic, Ovulation inhibitor, Antinociceptive, Antithrombotic, Antiperistaltic, Antialcoholic, Steroid synthesis inhibitor, Antimutagenic, Antisecretoric, Antirickettsial, Hair growth stimulant, Expectorant, Free radical scavenger
á-D-Galactopyranoside, methyl 2,6-bis-O-(trimethyl silyl)-, cyclic methylboronate	14.88	0.45	$C_{14}H_{31}BO_6Si_2$	362	Antileukemic, Antischistosomal, Antiparasitic, Isoamylase inhibitor, Antiprotozoal, Sweetener, Apoptosis agonist, Sclerosant, Cellulase inhibitor
Hexyl isovalerate	15.71	0.66	$C_{11}H_{22}O_2$	186	NADPH peroxidase inhibitor, Sclerosant, Antimetastatic, Antineurotic, Carminative, Peroxidase inhibitor, Catalase inhibitor, Calcium regulator
á-D-Glucopyranose, 4-O-á-D-galactopyranosyl-	18.67	0.45	$C_{12}H_{22}O_{11}$	342	Cholesterol antagonist, Lipotropic, Anti-infective, Hepatoprotectant, Antidiabetic, Mucolytic, Sclerosant, Vasodilator, Antiuremic, Chemopreventive, Antiulcerative, Wound healing agent

à-D-Glucopyranoside, O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl	19.01	1.95	$C_{18}H_{32}O_{16}$	504	Muscle relaxant, Antithyroid, Secretase stimulant, Antiviral (Influenza A), Streptomycin 6-kinase inhibitor, Antituberculosic, Antitreponemal, Antidote, Antineoplastic (solid tumours)
1,3:4,6-Dimethylene-d-glycero-d-mannoheptitol	23.43	1.04	$C_9H_{16}O_7$	236	Nucleotide metabolism regulator, Glutathione peroxidase inhibitor, Antidyskinetic, Antianginal, Carminative, Angiogenesis stimulant, Antibiotic
î-N-Formyl-L-lysine	24.21	3.04	$C_7H_{14}N_2O_3$	174	Mucositis treatment, Alopecia treatment, Venom exonuclease inhibitor, Antinaupathic, Antifibrinolytic, Insulin promoter, Ophthalmic, Antidiarrhoeal, Thrombolytic, Antiemphysemic
à-D-Mannofuranoside, 1-O-decyl-	25.21	0.55	$C_{16}H_{32}O_6$	320	Analeptic, Anti-inflammatory, Antiviral (Rhinovirus), Antinociceptive, Growth stimulant, Antinephritic, Antimycobacterial, Antitussive
2-Propenoic acid, 2-methyl-, 2-[[2,3,3a,4,7,7a(or3a,4,5,6,7,7a)-hexahydro-4,7-methano-1H-indenyl]oxy]ethyl ester	25.95	2.02	$C_{16}H_{22}O_3$	262	Hypolipemic, Skin irritation, Pediculicide, Anabolic, Mucomembranous protector, Antimetastatic, Gastric antisecretory, Vascular dementia treatment, Antipsoriatic, Antihypercholesterolemic, Skin whitener
l-(+)-Ascorbic acid 2,6-dihexadecanoate	27.32	1.47	$C_{38}H_{68}O_8$	652	Reductant, Mucomembranous protector, Spasmolytic, Immunosuppressant, Allergic, Oxidizing agent, Anesthetic, Laxative, Vasodilator, Anticataract
9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	32.15	2.21	$C_{57}H_{104}O_6$	884	Hypolipemic, Insecticide, Cytoprotectant, Antifungal, Antieczematic, Antisecretoric, Chemosensitizer, Dermatologic, Antimycobacterial, Anticarcinogenic

19-Norethandrolone tbdms	34.17	4.14	$C_{26}H_{44}O_2Si$	416	Prostate disorders treatment, Progesterone antagonist, Anti-infertility, Antiparasitic, Antiseborrheic, Antiprotozoal, Nitric oxide scavenger
Dotriacontane	34.61	0.61	$C_{32}H_{66}$	450	Leukopoiesis stimulant, Antidyskinetic, Fibrinolytic, Creatinase inhibitor, Urease inhibitor, Antimutagenic
ü-Pentamethylcyclopenta dienyl-ethylisonitril-(N,N,N',N'-tetramethylethin-1,2-diamin)-molybdaeniodid	34.81	0.44	$C_{19}H_{32}IMoN_3$	527	Antineurotic, Peroxidase inhibitor, Erythropoiesis stimulant, Superoxide dismutase inhibitor, Analeptic, Antileprosy, Antiparkinsonian,, Antiperistaltic, Photosensitizer, Muscle relaxant, Antiviral (Poxvirus)
Urs-9(11)-en-12-one-28-oic acid, 3-acetoxy-, methyl ester (14á,20á)	35.10	0.60	$C_{33}H_{50}O_5$	526	Apoptosis agonist, Septic shock treatment, Antineoplastic (ovarian cancer), Antihemorrhagic, Gynecological disorders treatment, Atherosclerosis treatment
Methyloctadecyl dichlorosilane	**38.75**	**29.19**	$C_{19}H_{40}Cl_2Si$	366	Sugar-phosphatase inhibitor, Oxygen scavenger, Pediculicide, Lactase inhibitor, Anti-infective, Skeletal muscle relaxant, Antimyopathies, Gonadotropin antagonist, Alopecia treatment, Carminative, Dementia treatment, Antifibrinolytic, Lipotropic, Antihematotoxic, Uterine stimulant, Ophthalmic, Astringent, Antiviral (Parainfluenza), Antispirochetal
Hexadecanoic acid, 1-[[[(2-aminoethoxy) hydroxyl phosphinyl]oxy]methyl]-1,2-ethanediyl ester	39.59	0.80	$C_{37}H_{74}NO_8P$	691	Antischistosomal, Anticonvulsant, Anticoagulant, Interferon agonist, Transaldolase inhibitor, Laxative, Antimycobacterial, Antidiabetic, Antipyretic, Anticataract, Antisecretoric, Lipotropic

Table 23: GC-MS analysis of *C. gigantea* ethanolic extract

Name of the compound	RT (min)	Peak area (%)	Molecular formula	Mol. Wt	Biological activities
2-Methylbutane-1,4-diol, 3-(1-ethoxyethoxy)-	**3.13**	**42.37**	$C_9H_{20}O_4$	192	Membrane integrity agonist, Antiviral (Rhinovirus), Antimyopathies, Macrophage stimulant, Antipruritic, Cholesterol antagonist, Cardioprotectant, Antidote, Vasoprotector, Antithrombotic, Antihypertensive, Antihypoxic, Antiseborrheic, Anti-inflammatory, Antifibrinolytic, Anticonvulsant, Antirickettsial
Strychane, 1-acetyl-20à-hydroxy-16-methylene-	4.49	0.61	$C_{21}H_{26}N_2O_2$	338	Antieczematic, Antiprotozoal, Antitussive, Antimetastatic, Antineoplastic, Uterine stimulant Menopausal disorders treatment
o-Acetyl-L-serine	5.00	0.57	$C_5H_9NO_4$	147	Macrophage stimulant, Radioprotector, Lipid metabolism regulator, Cytostatic, Hepatic disorders treatment, Mucomembranous protector
d-Glucosamine	5.76	0.88	$C_6H_{13}NO_5$	179	Glutamine synthetase inhibitor, Angiogenesis stimulant, Antiviral (Influenza), Nucleotide metabolism regulator, Isoamylase inhibitor, Immunomodulator, Antinociceptive, Antitoxic
Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl) methyl]cyclopropyl]methyl] cyclopropyl]methyl]-, methyl ester	7.28	0.60	$C_{25}H_{42}O_2$	374	Mucositis treatment, Antisecretoric, Vasodilator, Platelet aggregation stimulant, Fibrinolytic, Antiulcerative, Photosensitizer, Antiasthmatic, Antihelmintic (Nematodes), Calcium regulator

D-Alanine, N-propargyl oxycarbonyl-, decyl ester	8.71	1.35	$C_{17}H_{29}NO_4$	311	Skeletal muscle relaxant, Anesthetic, Antiparasitic, Anticataract, Antiglaucomic, Antiviral, Peptide agonist, Antiparkinsonian, Chemoprotective
2-Propyl-tetrahydropyran-3-ol	10.21	0.61	$C_8H_{16}O_2$	144	Antiseborrheic, Antieczematic, Antihypoxic, Sclerosant, Antipruritic, Antimetastatic, Insulin promoter, Antifungal, Antiviral (Poxvirus)
D-Glucose, 6-O-à-D-galactopyranosyl-	10.85	0.75	$C_{12}H_{22}O_{11}$	342	β-glucosidase inhibitor, Laxative, Vasoprotector, Membrane integrity agonist, Hepatoprotectant, Antihypoxic, Cholesterol antagonist
Tetrahydrofuran-5-on-2-methanol, à-[à-methoxy-(tetrahydrofuran-5-on-2-ylmethoxy)]-	12.30	0.87	$C_{11}H_{16}O_7$	260	Antiviral, Immunosuppressant, Oxygen scavenger, Antiprotozoal (Leishmania), Antioxidant, Antipsoriatic, Carminative
à-d-Mannofuranoside, isopropyl-	13.26	1.33	$C_9H_{18}O_6$	222	Anaphylatoxin receptor antagonist, Analeptic, Histamine release stimulant, Antidiabetic, Antiprotozoal (Amoeba), Anti-inflammatory, Cholesterol antagonist, Biliary tract disorders treatment
Cyclopentanecarboxylic acid, dodecyl ester	14.85	0.66	$C_{18}H_{34}O_2$	282	Antiepileptic, Antifungal, Anticoagulant, Antiviral (Hepatitis B), Antibiotic, Insulin sensitizer, Anti-ischemic, Myocardial ischemia treatment
2-Hydroxy-3-methyl succinic acid	15.62	1.03	$C_5H_8O_5$	148	Aminoacylase inhibitor, Antitoxic, Peroxidase inhibitor, Antimyopathies, Antipruritic, Antihelmintic (Nematodes), Antispirochetal, Antiinfective, Anticoagulant, Hypolipemic

2-Hydroxy-5-methyl benzaldehyde	16.64	16.26	$C_8H_8O_2$	136	Antieczematic, Antiviral, Mucolytic, Erythropoiesis stimulant, Insulin promoter, Antinociceptive, Antiparasitic, Insecticide, Hepatoprotectant, Hyaluronic acid agonist, Antiprotozoal, Antiperistaltic, Antiulcerative, Antitreponemal, Antihypotensive
L-Glucose	18.92	2.57	$C_6H_{12}O_6$	180	Lipotropic, Macrophage stimulant, Antioxidant, Dementia treatment, Alkylator, Anesthetic, Antileukemic, Antiuremic, Chemoprotective, Gout treatment, Antimycobacterial, Spasmolytic, Antiulcerative, Antineoplastic (liver cancer)
Cinnamic acid, 3-trifluoro methyl-, 6-ethyl-2-octyl ester	19.31	0.78	$C_{20}H_{27}F_3O_2$	356	Antieczematic, Dermatologic, Atherosclerosis treatment, Antisecretoric, Proliferative diseases treatment, Analgesic, Wound healing agent, Anticarcinogenic, Reductant
4,6-O-Ethylidene-à-D-glucose	19.80	0.98	$C_8H_{14}O_6$	206	Restenosis treatment, Antitussive, Laxative, Antineoplastic (gastric cancer), Antimitotic, Calcium regulator, Free radical scavenger
à-d-Glucofuranose, 1,2:5,6-bis-O-(1-methylethylidene)-3-O-à-d-ribopyranosyl-	20.33	1.03	$C_{17}H_{28}O_{10}$	392	Antipsoriatic, Anticarcinogenic, Antiarthritic, Antithrombotic, Antipruritic, Restenosis treatment, Antidiabetic, Wound healing agent, Antiasthmatic, Angiogenesis stimulant
Mannitol, 1,3,4-tri-O-methyl-, triacetate, D-	20.77	5.55	$C_{15}H_{26}O_9$	350	Cholesterol antagonist, Glucose oxidase inhibitor, Atherosclerosis treatment, Protein kinase stimulant, Antihypertensive, Antiprotozoal, Antihypoxic, Hepatoprotectant, Antieczematic, Antiglaucomic, Oxygen scavenger, Antinephritic

á-D-Glucopyranose, 4-O-á-D-galactopyranosyl-	22.18	1.50	$C_{12}H_{22}O_{11}$	342	Antiuremic, Radiosensitizer, Lipotropic, Mucolytic, Anti-infective, Antidiabetic, Hepatoprotectant, Cholesterol antagonist, Wound healing agent, Chemopreventive, Sclerosant
Desulphosinigrin	23.36	4.31	$C_{10}H_{17}NO_6S$	279	Immunosuppressant, Antimetastatic, Platelet adhesion inhibitor, Antispirochetal, Nitric oxide antagonist, Antidote, Licheninase inhibitor, Hyaluronic acid agonist, Parathyroid hormone antagonist, β-galactosidase inhibitor
Gentamicin B	24.19	3.09	$C_{19}H_{38}N_4O_{10}$	482	Protein synthesis inhibitor, Muscle relaxant, Chloride channel blocker, Cytostatic, RNA synthesis inhibitor, Mucolytic, Angiogenesis stimulant, Antituberculosic, Anticarcinogenic, Antirickettsial, Antihypotensive, Cellulase inhibitor, Antineoplastic (lung cancer)
d-Mannose	24.84	0.59	$C_6H_{12}O_6$	180	Transketolase inhibitor, Catalase inhibitor, Laxative, Galactose oxidase inhibitor, Macrophage stimulant, α-amylase inhibitor, Multiple sclerosis treatment, Leukopoiesis stimulant, Acetylesterase inhibitor
Palmitic anhydride	27.29	0.58	$C_{32}H_{62}O_3$	494	Lipid metabolism regulator, Oxidizing agent, Lipotropic, Cytoprotectant, Angiogenesis stimulant, Anti-infective, Antidyskinetic, Antithrombotic

Table 24: GC-MS analysis of *C. crinita* ethanolic extract

Name of the compound	RT (min)	Peak area (%)	Molecular formula	Mol. Wt	Biological activities
Octadec-9-enoic acid	3.98	0.44	$C_{18}H_{34}O_2$	282	Antieczematic, Phobic disorders treatment, Catalase inhibitor, Sclerosant, Antitoxic, Cytoprotectant, Fibrinolytic, Anti-inflammatory, Anti-infective
2-(2-Bromoethyl) cyclohexanone	4.46	0.64	$C_8H_{13}BrO$	204	Rheumatoid arthritis treatment, Autoimmune disorders treatment, Kidney function stimulant, Ophthalmic, Dementia treatment
2-Nitro-1-buten-3-ol	4.97	1.09	$C_4H_7NO_3$	117	Glutathione thiolesterase inhibitor, Carminative, Hepatic disorders treatment, Kidney function stimulant, Analeptic, Formaldehyde dehydrogenase inhibitor, Antifungal, Antihypoxic, Antiseptic
2-Oxiranecarboxylic acid, 3-(2,2-dimethoxyethyl)-3-methyl-, methyl ester	7.25	0.61	$C_9H_{16}O_5$	204	Antiprotozoal, Antimyopathies, Sclerosant, Free radical scavenger, Cardioprotectant, Leukopoiesis stimulant, Immunosuppressant, Antiprotozoal, Antieczematic, Antihelmintic, Antiparasitic
D-Alanine, N-propargyl oxycarbonyl-, decyl ester	8.71	1.47	$C_{17}H_{29}NO_4$	311	Muscle relaxant, Macrophage stimulant, Anesthetic, Antisecretoric, Sclerosant, Antiviral (Rhinovirus, Influenza A), Antihypertensive, Hematopoietic, Antiglaucomic, Antiparkinsonian,, Histamine agonist, Antimyopathies, Hypolipemic
4-Hexen-3-ol, 2,5-dimethyl-	9.43	0.58	$C_8H_{16}O$	128	Fibrinolytic, Carminative, Antithrombotic, Oxidizing agent, Insulin promoter, Antiulcerative, Antihypoxic, Mucositis treatment, Nitric oxide scavenger
dl-3,4-Dehydroproline methyl ester	9.84	0.60	$C_6H_9NO_2$	127	Menopausal disorders treatment, Stroke treatment, Vasodilator, Anticonvulsant, Aldosterone antagonist, Antiperistaltic, Venom exonuclease inhibitor

4,6-O-Ethylidene-à-D-glucose	10.24	1.23	$C_8H_{14}O_6$	206	Anticarcinogenic, Topoisomerase II inhibitor, Antileukemic, Apoptosis agonist, Transcription factor stimulant, Dementia treatment, Immunosuppressant, Sclerosant, Lipotropic, Antiviral (Herpes), Antirickettsial
Resorcinol	10.85	3.59	$C_6H_6O_2$	110	Antiseborrheic, Antiseptic, Oxygen scavenger, Platelet aggregation stimulant, Gonadotropin antagonist, Antihelmintic (Fasciola), Adrenaline release stimulant, Dopamine release stimulant
Benzofuran, 2,3-dihydro-	11.33	8.93	C_8H_8O	120	Phobic disorders treatment, Ovulation inhibitor, Cytoprotectant, Antineurotic, Vasoprotector, Antiseborrheic, Insulin promoter, Hematopoietic inhibitor, Antiasthmatic, Dermatologic, Antiviral (Influenza), Antineoplastic (Brain cancer)
Methyl 2,3-di-O-acetyl-4,6-di-O-methyl-à-D-mannopyranoside	11.84	0.49	$C_{13}H_{22}O_8$	306	Antineoplastic (Colon cancer), Antihelmintic, Antiviral (Rhinovirus), Antidiabetic, Antioxidant, Prostate cancer treatment, Antithrombotic
4-Methoxycarbonyl-4-butanolide	12.32	1.77	$C_6H_8O_4$	144	Chymosin inhibitor, Dextranase inhibitor, Kidney function stimulant, Antimyopathies, Malate oxidase inhibitor, Cholesterol antagonist, Neurotransmitter antagonist, Immunosuppressant, Gonadotropin antagonist, Analeptic, Hemostatic
5-Acetoxymethyl-2-furaldehyde	12.65	5.35	$C_8H_8O_4$	168	Sugar-phosphatase inhibitor, Antiseborrheic, Platelet aggregation stimulant, Pediculicide, Anticataract, Antituberculosic, Alopecia treatment, Antiprotozoal (Trichomonas), Free radical scavenger, Antipruritic, Antimyopathies, Anti-infective, Antispirochetal
à-d-Mannofuranoside, isopropyl-	13.29	0.75	$C_9H_{18}O_6$	222	Transcription factor stimulant, Anticarcinogenic, Nucleotide metabolism regulator, Apoptosis agonist, Platelet aggregation stimulant, Antiulcerative

à-D-Glucopyranoside, O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl	14.45	0.42	$C_{18}H_{32}O_{16}$	504	β-glucosidase inhibitor, Gastritis treatment, Vasoprotector, Antineoplastic, Antileukemic, Antitussive, Antihemorrhagic, Vasculitis treatment
2-Hydroxy-3-methylsuccinic acid	15.63	1.48	$C_5H_8O_5$	148	Sclerosant, Peptide agonist, Antihypoxic, Lipid metabolism regulator, Kidney function stimulant, Mucositis treatment, Anesthetic, Platelet antagonist, Expectorant, Anticoagulant, Antisecretoric
2-Hydroxy-5-methylbenzaldehyde	**16.68**	**55.45**	$C_8H_8O_2$	136	Carminative, Antieczematic, Immunosuppressant, Antiviral (Picornavirus), Erythropoiesis stimulant, Insulin promoter, Antinociceptive, Antiparasitic, Hepatoprotectant, Interferon gamma antagonist, Hyaluronic acid agonist, Mucolytic, Antiprotozoal, Gonadotropin antagonist, Oxytocic, Antiperistaltic, Cardioprotectant, Antitreponemal, Antiulcerative, Radioprotector, Antihypotensive, Insecticide
Decanoic acid, 3-hydroxy-, methyl ester	17.04	0.69	$C_{11}H_{22}O_3$	202	Vasodilator, Sclerosant, Mucositis treatment, Lipid metabolism regulator, Hypolipemic, Fibrinolytic, Antieczematic, Anti-inflammatory
d-Mannose	18.80	1.25	$C_6H_{12}O_6$	180	Peptide agonist, Glucose oxidase inhibitor, Calcium regulator, Macrophage stimulant, Chemosensitizer, Glycerol dehydrogenase inhibitor, Alcohol oxidase inhibitor, Vasoprotector, Antihypoxic, Antifungal
Desulphosinigrin	20.23	0.53	$C_{10}H_{17}NO_6S$	279	Antioxidant, Cholesterol antagonist, Anti-infective, β-amylase inhibitor, Immunosuppressant, Antinociceptive, Antimetastatic, Expectorant
à-d-Mannofuranoside, methyl	23.44	2.43	$C_7H_{14}O_6$	194	Radioprotector, Anti-inflammatory, Antithrombotic, Antiviral (Influenza), Antioxidant, Antidiabetic, Hemostatic, Wound healing agent, Cytostatic, Antidote, Antiprotozoal (Leishmania), Spasmolytic, Antihypercholesterolemic, Antibiotic

Alpha-l-rhamnopyranose	24.15	3.08	$C_6H_{12}O_5$	164	Beta-amylase inhibitor, Antineoplastic, Antioxidant, Anesthetic, Hepatoprotectant, Antidyskinetic, Immunostimulant, Antithrombotic, Antibacterial, Antimycoplasmal, Antipsoriatic, Antispirochetal, Antimycobacterial, Apoptosis antagonist
à-D-Mannofuranoside, 1-O-decyl-	24.84	1.12	$C_{16}H_{32}O_6$	320	Analeptic, Antithrombotic, Anticarcinogenic, Angiogenesis stimulant, Alpha-amylase inhibitor, Platelet adhesion inhibitor, Macrophage stimulant, Dementia treatment, Antipruritic, Antimetastatic, Antidiabetic, Antiviral, Cardioprotectant
2-Propenoic acid, 3-(2-methoxyphenyl)-, (E)-	26.28	0.37	$C_{10}H_{10}O_3$	178	Antieczematic, Carminative, Antiseborrheic, Cytoprotectant, Antihelmintic (Nematodes), Antisecretoric, Insulin promoter, Antinephritic
2-Propenoic acid, 3-(3-hydroxyphenyl)-	27.44	2.75	$C_9H_8O_3$	164	Antipsoriatic, Antimetastatic, Dermatologic, Antinociceptive, Alopecia treatment, Oxytocic, Chemopreventive, Analeptic, Nitric oxide scavenger, Antineoplastic (Pancreatic cancer), Muscle relaxant, Secretase stimulant, Gaucher disease treatment
Ergosta-5,7,22-trien-27-ol, 3-methoxymethoxy-	32.14	0.56	$C_{30}H_{48}O_3$	456	Hair growth stimulant, Testosterone agonist, Septic shock treatment, Contraceptive, Hemostatic, Anti-infertility, Antiparasitic, Antiosteoporotic
Bicyclo[4.3.0] nonan-7-one, 1-(2-methoxyvinyl)-	32.74	1.14	$C_{12}H_{18}O_2$	194	Estrogen antagonist, Antidiabetic, Ovulation inhibitor, Alopecia treatment, Antiseborrheic, Oxidizing agent, Cytoprotectant, Antitussive, Antiprotozoal, Antimetastatic, Protein synthesis stimulant, Antisecretoric, Antimyopathies
Ethyl iso-allocholate	34.91	0.46	$C_{26}H_{44}O_5$	436	Antihypercholesterolemic, Anesthetic, Hepatic disorders treatment, Anesthetic, Hepatoprotectant, Chemopreventive, Antinociceptive, Antithrombotic, Antiseborrheic, Antineoplastic (Lung cancer)

Biological properties of the ascertained phytochemical components were predicted using PASS online. They include compounds with anti-inflammatory, antieczematic, immunosuppressant, antiviral, erythropoiesis stimulant, antinociceptive, anti-infective, hepatoprotectant, antitreponemal, antipruritic, antiprotozoal, astringent, antihematotoxic, antiparasitic, cardioprotectant, antiulcerative, antimyopathies, antirickettsial, antihypoxic, antifibrinolytic, anticonvulsant, vasoprotector, antithrombotic, antihypertensive, antidote, antiseborrheic, antispirochetal activities etc.

The obtained gas chromatogram showed the relative concentrations of various compounds getting eluted as a function of retention time. The heights of the peak indicate the concentration of the components present in the studied *Cyathea* species. The mass spectrometer analyzes the compounds eluted at different retention times to identify the nature and structure of the components. The large compound fragments into small compounds giving rise to appearance of peaks at different m/z ratios. These mass spectra were served as fingerprint of that identified compound which can be identified from the data library. The obtained mass spectra were fingerprint of that particular compound which can be identified from the NIST library.

Protein profiling using SDS-PAGE

The relative positions of the protein bands revealed by SDS-PAGE showed obvious changes in the banding profiles of studied *Cyathea* species. Multiple regions of activity were observed in the protein electrophoretic system of *Cyathea* species. A total of 22 bands with various R_f values and molecular weight were observed in the SDS-PAGE gel system (Plate X; Table 25). The protein bands were appeared between the R_f values ranged from 0.14 to 0.94. The molecular weight of proteins isolated from the studied *Cyathea* species ranged from 6.16 to 89.13 kDa.

Table 25: SDS-PAGE protein profile of *Cyathea* species

R_f values	MW (kDa)	Region	Position	*C. nilgirensis*	*C. gigantea*	*C. crinita*
0.14	89.13	**2**	PP 2^1	+		
0.24	56.23	**3**	PP 3^1	+		
0.33	32.35	**4**	PP 4^1			+
0.39	31.62		PP 4^2		+	
0.40	28.18	**5**	PP 5^1			+
0.43	25.11		PP 5^2	+		
0.53	15.85	**6**	PP 6^1	+		
0.58	14.12		PP 6^2			+
0.61	12.59	**7**	PP 7^1	+		+
0.62	12.58		PP 7^2		+	
0.66	11.22		PP 7^3	+		+
0.69	10.71		PP 7^4		+	+
0.74	10.00	**8**	PP 8^1	+		
0.82	8.91	**9**	PP 9^1			+
0.83	8.31		PP 9^2		+	
0.84	8.12		PP 9^3	+		
0.92	7.76	**10**	PP 10^1			+
0.93	7.41		PP 10^2	+		
0.94	6.16		PP 10^3		+	

The highest molecular weight protein (89.13 kDa) was found in *C. nilgirensis* with lowest R_f value 0.14. The least molecular weight protein (6.16 kDa) was present in *C. gigantea* with highest R_f value 0.94. Among the three species of *Cyathea* studied, *C. nilgirensis* showed maximum number of protein bands (9) followed by *C. crinita* (8) and *C. gigantea* (5). Based on the occurrence of proteins in the *Cyathea* gel system, the protein profiles were classified into nine regions. Region 2 (PP 2^1), 3 (PP 3^1) and 8 (PP 8^1) expressed their unique presence in *C. nilgirensis* with the molecular weight 89.13, 56.23 and 10.00 kDa respectively. Region 4, 5 and 6 displayed two distinct bands in two different positions. PP 4^1 was present in *C. crinita* (R_f - 0.33; MW - 32.35 kDa) and PP 4^2 represented its individuality in *C. gigantea* (R_f - 0.39; MW - 31.62 kDa). PP 5^1 showed its unique presence in *C. crinita* and PP 5^2 demonstrated its identity in *C. nilgirensis* with the molecular weight 28.18 and 25.11 kDa respectively.

PP 6^1 was present only in *C. nilgirensis* and PP 6^2 represented its distinct presence in *C. crinita* with the molecular weight 15.85 and 14.12 kDa respectively. The protein bands with the position PP 7^1 and 7^3 were shared by *C. nilgirensis* and *C. crinita* with the molecular weight 12.59 and 11.22 kDa respectively. PP 7^2 illustrated its unique existence in *C. gigantea* (R_f - 0.62; MW - 12.58 kDa). PP 7^4 was common to *C. gigantea* and *C. crinita* with the molecular weight 10.71 kDa. Region 9 occupied three unique bands in three different positions. PP 9^1 (R_f - 0.82; MW - 8.91 kDa) depicted its presence only in *C. crinita*. PP 9^2 (R_f - 0.83; MW - 8.31 kDa) displayed its occurrence in *C. gigantea.* PP 9^3 (R_f - 0.84; MW - 8.12 kDa) represented its existence in *C. nilgirensis.* Region 10 showed three distinct bands in three different positions. The protein bands with the position PP 10^1 (R_f - 0.92; MW - 7.76 kDa) was present only in *C. crinita.* PP 10^2 (R_f - 0.93; MW - 7.41 kDa) expressed its unique presence in *C. nilgirensis* and PP 10^3 (R_f - 0.94; MW - 6.16 kDa) represented its individuality in *C. gigantea.*

NTSYS software was used to analyze the phylogenetic relationship of the *Cyathea* species based on the protein patterns. The similarity coefficient was calculated on the basis of presence and absence of bands and a UPGMA cladogram was constructed (Fig. 30).

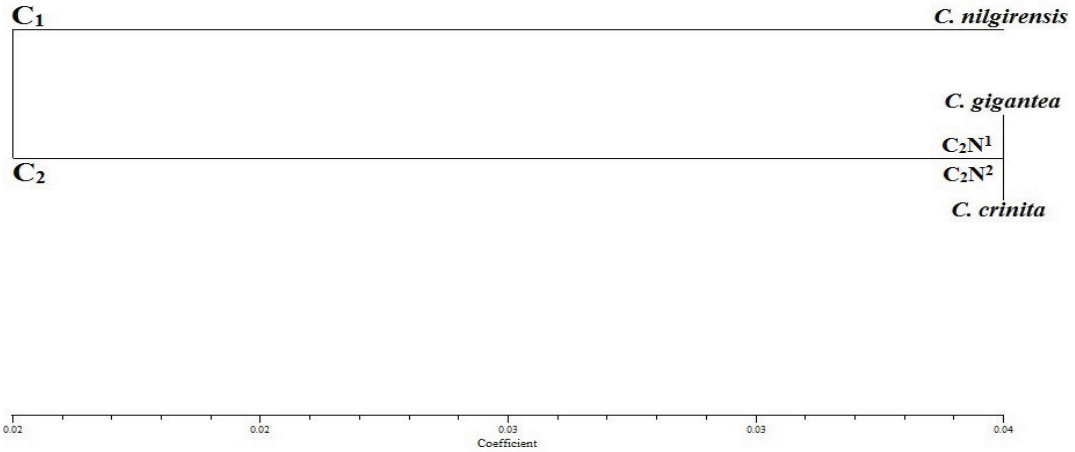

Fig. 30: Cladogram of studied *Cyathea* species based on SDS-PAGE profile

The clusters obtained from the cladogram were grouped into two main clades viz., C_1 and C_2 which clearly explained the similarity and variation among the studied *Cyathea* species. Cluster 1 (C_1) showed the unique presence of *C. nilgirensis* which showed 100% divergence from the other two studied species. Cluster C_2 is divided into two nodes viz., C_2N^1 and C_2N^2. It was shared between *C. gigantea* and *C. crinita*.

MALDI-TOF MS analysis

MALDI-TOF MS characterization of selected *Cyathea* species showed proximate spectra profile (quality and number of peaks) with varied ion peaks m/z ranged from 0 - 1,00,000 Da (Plate XI). The results showed both positive and negative peaks. To reveal the inter-specific similarity and variation among the selected *Cyathea* species, only 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. Totally 37 spectral m/z values were selected and the results were tabulated in Table 26.

Among the studied three *Cyathea* species, *C. nilgirensis* represented maximum number of m/z peaks (16) ranged from 67161.26 to 464.77 m/z values followed by *C. crinita* with 12 peaks ranged from 56684.80 to 588.45 m/z values. *C. gigantea* showed 11 peaks ranged from 98571.74 to 10298.75 m/z values. Of which, 15 specific peaks were observed in *C. nilgirensis* followed by 11 distinct peaks in *C. crinita* and 10 unique peaks in *C. gigantea.* The peak value 62973.12 m/z was shared by *C. nilgirensis* and *C. gigantea* whereas 51956.90 m/z was present in both *C. gigantea* and *C. crinita.* The unique ionic spectral peaks of different *Cyathea* species paved a way to study the variation among the species using MALDI-TOF MS analysis.

The similarity indices were calculated based on the MALDI-TOF MS analysis and the cladogram was constructed. The results revealed the inter-specific variations and similarities among the morphologically distinct *Cyathea* species.

Table 26: MALDI-TOF MS spectral values of studied *Cyathea* species

m/z values	*C. nilgirensis*	*C. gigantea*	*C. crinita*
98571.74		+	
71687.02		+	
67161.26	+		
65149.93	+		
62973.12	+	+	
58696.13	+		
56684.80			+
52807.64		+	
51956.90		+	+
48293.81			+
45544.20	+		
42413.37	+		
41956.90			+
41293.86	+		
40277.65		+	
37099.06	+		
34838.96			+
32392.27	+		
27804.30			+
21480.45		+	
20671.92			+
17410.04			+
15212.12		+	
14030.43		+	
10318.03		+	
10298.75		+	
9865.84	+		
5656.93	+		
5364.71	+		
4756.80	+		
936.84			+
911.75			+
734.15			+
713.57	+		
629.63	+		
588.45			+
464.77	+		

The cladogram displayed two clades viz., C_1 and C_2 based on the m/z peak values (Fig. 31). Clade 1 (C_1) showed the unique presence of *C. nilgirensis*. Clade 2 (C_2) was

shared by taxa *C. gigantea* and *C. crinita*. The exclusive presence of *C. nilgirensis* in a separate clade showed the presence of unique m/z peaks compared to other two species.

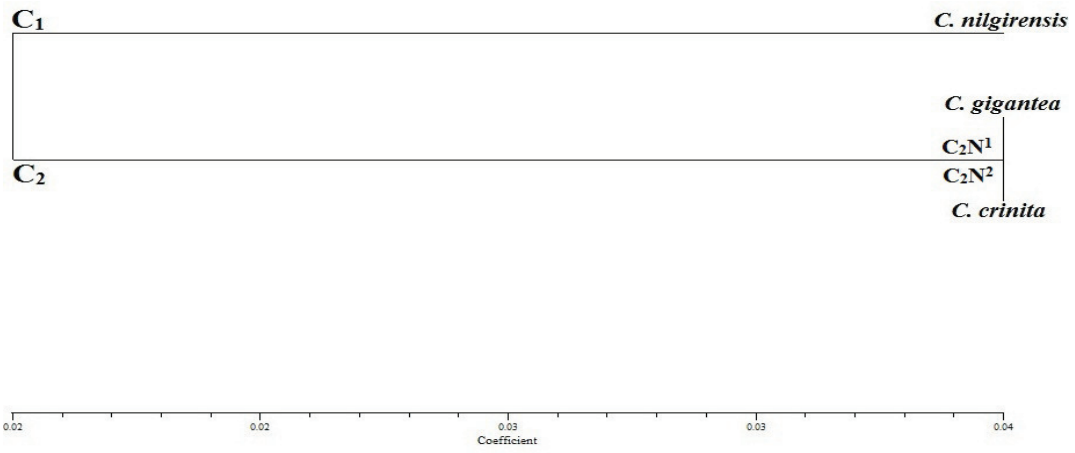

Fig. 31: Cladogram based on MALDI-TOF MS analysis of *Cyathea* species

Isozyme analysis

Isoesterase

In the isoesterase enzyme system of *Cyathea*, six regions (EST 1, 2, 6, 7, 8 and 9) of activity with seven different bands were obtained (Plate XII; Table 27). *C. nilgirensis* showed its unique banding profile only in the region EST 9^1 with R_f value 0.88. *C. gigantea* displayed its presence in four distinct zones (EST 1, 2, 7 and 8) with R_f values 0.06, 0.17, 0.63 and 0.70. The band EST 9^2 with R_f value 0.89 was expressed only in *C. crinita.* EST 6^1 (0.55) was commonly shared by *C. nilgirensis* and *C. gigantea*. All the bands showed variations among the studied *Cyathea* species except the isoform EST 6^1.

Table 27: Isoesterase enzymatic profile of studied *Cyathea* species

R_f values	Region	Position	*C. nilgirensis*	*C. gigantea*	*C. crinita*
0.06	**1**	EST 1^1		+	
0.17	**2**	EST 2^1		+	
0.55	**6**	EST 6^1	+	+	
0.63	**7**	EST 7^1		+	
0.70	**8**	EST 8^1		+	
0.88	**9**	EST 9^1	+		
0.89		EST 9^2			+

Based on the isoesterase profile of studied *Cyathea* species, the similarity indices were calculated and the cladogram was constructed (Fig. 32). The phylogenetic tree constructed based on the isoesterase profile expressed two clusters viz., C_1 and C_2. The cluster C_1 displayed the similarity and variation between the taxa *C. nilgirensis* and *C. gigantea.* The cluster C_2 showed the distinct presence of *C. crinita.*

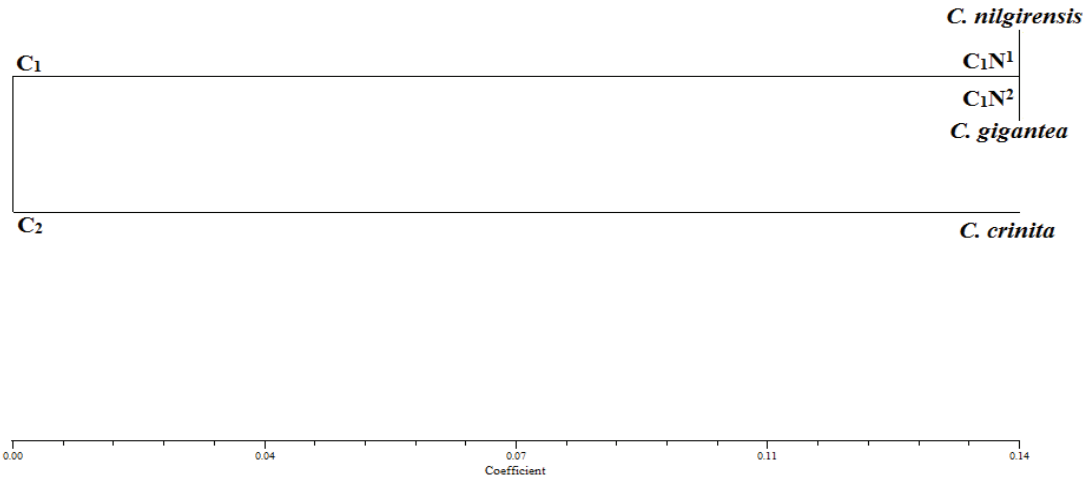

Fig. 32: Cladogram of studied *Cyathea* species based on isoesterase profile

Isoperoxidase

The isoperoxidase enzyme system of *Cyathea* showed six regions of activity (PRX 2, 3, 4, 5, 6 and 10) with nine different bands (Plate XII; Table 28). PRX 3^1 (0.22), PRX 4^1 (0.32) and PRX 10^2 (0.96) represented their unique occurrence in *C. nilgirensis.*

Table 28: Isoperoxidase profile of studied *Cyathea* species

R_f values	Region	Position	*C. nilgirensis*	*C. gigantea*	*C. crinita*
0.12	**2**	PRX 2^1			+
0.22	**3**	PRX 3^1	+		
0.23		PRX 3^2		+	
0.32	**4**	PRX 4^1	+		
0.36		PRX 4^2		+	
0.42	**5**	PRX 5^1		+	
0.57	**6**	PRX 6^1		+	
0.93	**10**	PRX 10^1			+
0.96		PRX 10^2	+		

C. gigantea showed its distinct banding profile in four different zones (PRX 3, 4, 5 and 6) with R_f values 0.23, 0.36, 0.42 and 0.57 respectively. The bands with R_f values 0.12 (PRX 2^1) and 0.93 (PRX 10^1) were present only in *C. crinita.* Peroxidase enzyme system failed to express the similarity among the studied three *Cyathea* species.

Acid phosphatase

A total of seven bands from six different positions were observed in the acid phosphatase profile of studied *Cyathea* species (Plate XII; Table 29). Multiple zones of expression were found in this enzyme system (ACP 2, 3, 4, 5, 6 and 10). In general, each band was observed in different *Cyathea* species with varied R_f values. *C. nilgirensis* showed its distinct presence in two different regions (ACP 2^1 - 0.14 and ACP 3^2 - 0.28). The bands ACP 3^1 (0.22) and ACP 4^1 (0.32) expressed their individuality in *C. gigantea. C. crinita* expressed its unique banding profile in three different zones (ACP 5, 6 and 10) with R_f values 0.47, 0.55 and 0.93 respectively. None of the band was common to all the three studied *Cyathea* species.

Table 29: Acid phosphatase profile of studied *Cyathea* species

R_f values	Region	Position	*C. nilgirensis*	*C. gigantea*	*C. crinita*
0.14	**2**	ACP 2^1	+		
0.22	**3**	ACP 3^1		+	
0.28		ACP 3^2	+		
0.32	**4**	ACP 4^1		+	
0.47	**5**	ACP 5^1			+
0.55	**6**	ACP 6^1			+
0.93	**10**	ACP 10^1			+

DNA barcoding

Among the three DNA isolation methods, the modified CTAB extraction method described by Murray and Thompson (1980) yielded a good DNA isolation. The genomic DNA isolated from the selected species of *Cyathea* was amplified using *rbc*L primer. The PCR amplification efficiency was good for the studied species (Plate XIII) and the

amplicons were sequenced (Plate XIII; Plate XIV). The sequence length was ranged from 590-593 base pairs. The obtained sequences were aligned, annotated and submitted in GenBank (*C. nilgirensis* - KF 924259, *C. gigantea* - KF 924260 and *C. crinita* - KF 924261).

>gi|575497724|gb|KF924259.1| *Cyathea nilgirensis* voucher XCH 25423 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene, partial cds; chloroplast
AAGATTATCGATTGACCTATTACACTCCCAAGTATGAGACCAAAGACACCGATATCTTGGCAGCCTTTCG
AATGACCCCGCAACCCGGAGTACCGGCTGAGGAAGCTGGAGCTGCAGTAGCTGCGGAATCTTCCACAGGT
ACATGGACCACTGTATGGACGGATGGACTTACTAGTCTCGATCGCTACAAAGGCCGATGCTATGATATCG
AACCTGTTGCTGGGGAGGATAATCAGTATATTGCATATGTAGCTTATCCTTTGGATCTATTTGAAGAAGG
TTCCGTTACCAATATGTTCACTTCCATTGTAGGTAACGTTTTTGGATTCAAGGCCTTACGCGCTCTCCGC
TTAGAAGATCTTCGAATTCCTCCTGCTTATTCTAAAACTTTCATTGGACCGCCCCATGGTATCCAGGTTG
AAAGGGATAAGCTAAACAAATATGGGCGTCCCTTATTAGGATGTACAATCAAGCCAAAATTGGGCTTATC
CGCTAAAAATTATGGGAGAGCCGTTTATGAATGTCTCCGTGGTGGACTTGACTTCACCAAGGATGATGAG
AACGTAAATTCCCAACCATTCATGCGTTGGAG

>gi|575497726|gb|KF924260.1| *Cyathea gigantea* voucher XCH 25422 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene, partial cds; chloroplast
GATTATCGATTGAACTATTACACTCCCCAGTATGAGACCAAAGACACCGATATCTTGGCAGCCTTTCGAA
TGACCCCGCAACCCGGAGTACCGCCTGAGGAAGCTGGAGCTGCAGTAGCTGCGGAATCTTCCACAGGTAC
ATGGACCACTGTATGGACGGATGGACTTACTAGTCTCGATCGCTACAAAGGCCGATGCTATGATATCGAA
CCTGTCGCTGGGGAGGATAATCAGTATATTGCATATGTAGCTTATCCTTTGGATCTATTTGAAGAAGGTT
CCGTTACCAATATGTTCACTTCCATTGTAGGTAACGTTTTTGGATTCAAGGCCTTACGCGCTCTCCGCTT
AGAAGATCTTCGAGTTCCTCCTGCTTATTCTAAGACTTTCATTGGACCGCCCCACGGTATCCAGGTTGAA
AGAGATAAGCTAAACAAATATGGGCGTCCCTTATTAGGATGTACAATCAAGCCAAAATTGGGCTTATCTG
CTAAAAATTATGGGAGAGCCGTTTATGAATGTCTCCGCGGTGGACTTGACTTCACCAAAGATGATGAGAA
CGTAAATTCCCAACCATTCATGCGTTGGAGAGA

>gi|575497728|gb|KF924261.1| *Cyathea crinita* voucher XCH 25424 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene, partial cds; chloroplast
TATCGATTGACCTATTACACTCCCAAGTATGAGACCAAAGACACCGATATCTTGGCAGCCTTTCGAATGA
CCCCGCAACCCGGAGTACCGGCTGAGGAAGCTGGAGCTGCAGTAGCTGCGGAATCTTCCACAGGTACATG
GACCACTGTATGGACGGATGGACTTACTAGTCTCGATCGCTACAAAGGCCGATGCTATGATATCGAACCT
GTTGCTGGGGAGGATAATCAGTATATTGCATATGTAGCTTATCCTTTGGATCTATTTGAAGAAGGTTCCG
TTACCAATATGTTCACTTCCATTGTAGGTAACGTTTTTGGATTCAAGGCCTTACGCGCTCTCCGCTTAGA
AGATCTTCGAATTCCTCCTGCTTATTCTAAAACTTTCATTGGACCGCCCCATGGTATCCAGGTTGAAAGG
GATAAGCTAAACAAATATGGGCGTCCCTTATTAGGATGTACAATCAAGCCAAAATTGGGCTTATCCGCTA
AAAATTATGGGAGAGCCGTTTATGAATGTCTCCGTGGTGGACTTGACTTCACCAAGGATGATGAGAACGT
AAATTCCCAACCATTCATGCGTTGGAGAGA

Multiple sequence alignment

Multiple sequence alignment performed using MULTALIN tool distinguished the inter-specific variation among the three *Cyathea* species. High consensus sequences were red in colour, low consensus sequences were blue in colour and neutral sequences were black in colour (Fig. 33).

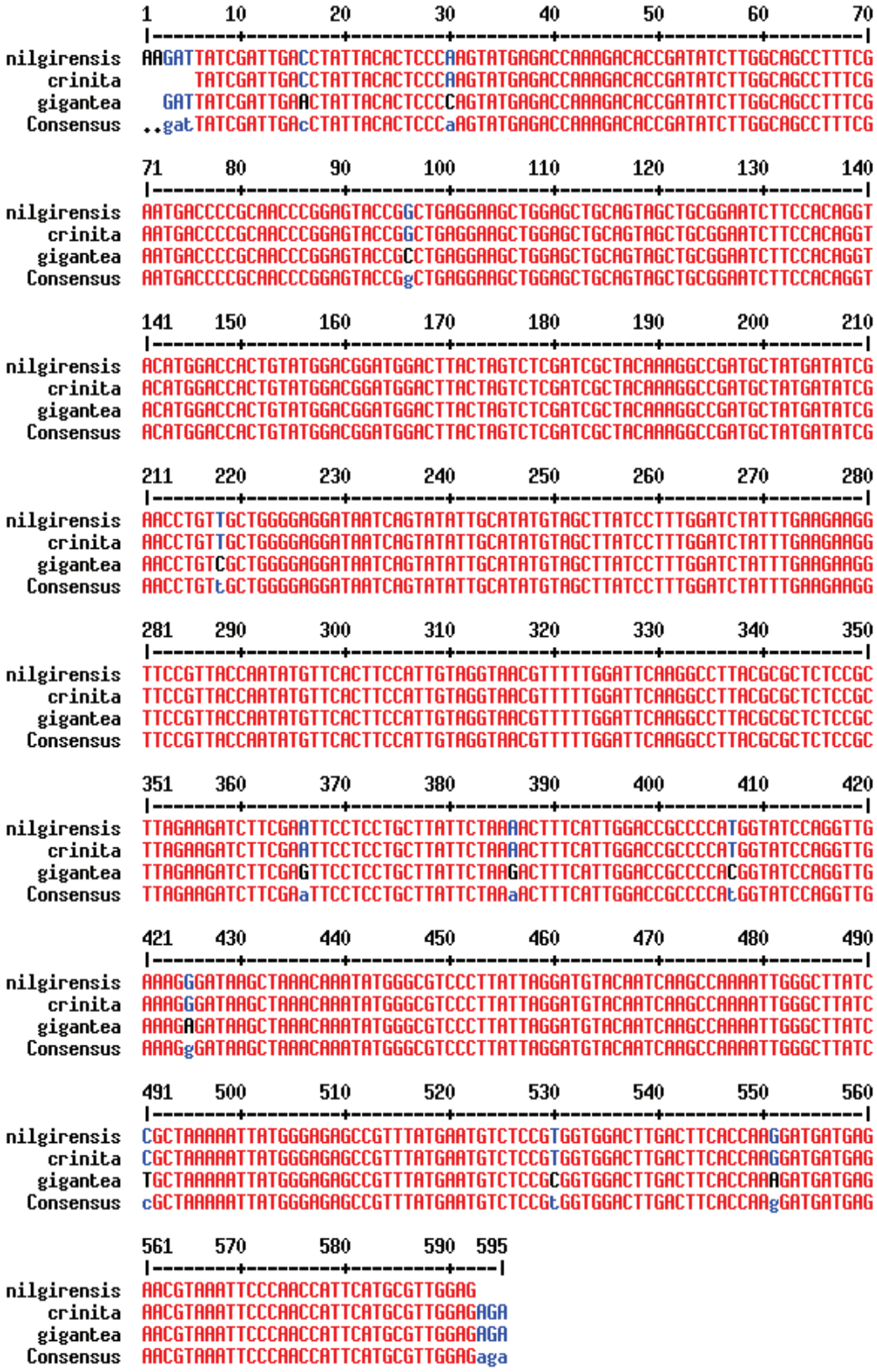

Fig. 33: Multiple sequence alignment of studied *Cyathea* species using MULTALIN

Based on the sequence alignment, phylogenetic tree was constructed using Clustal W (Fig. 34). The cladogram showed two main clades (C_1 and C_2). The clade C_1 is divided into two nodes (C_1N^1 and C_1N^2) which include the taxa *C. nilgirensis* and *C. crinita.* The clade C_2 displayed the unique presence of the taxon *C. gigantea*.

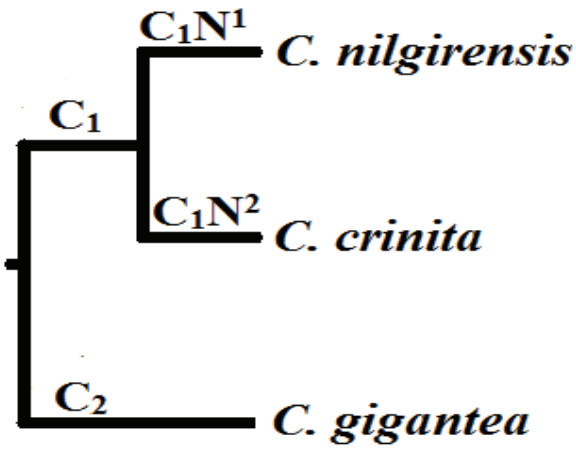

Fig. 34: Rooted phylogenetic cladogram of studied *Cyathea* species using Clustal W

Nucleotide composition

The nucleotide base frequencies of *C. nilgirensis, C. gigantea* and *C. crinita* species were represented in Table 30. The nucleotide bases were found AT rich (54.7%) in *C. nilgirensis* followed by *C. crinita* (54.6%) and *C. gigantea* (54.4%). Nucleotide bases of GC were more in *C. gigantea* (45.7%) followed by *C. crinita* (45.5%) and *C. nilgirensis* (45.3%). The average AT and GC contents were observed as 54.6% and 45.5%. Maximum frequency of thymine was found in *C. nilgirensis* and *C. gigantea* (27.5%) followed by *C. crinita* (27.5%). Cytosine content was observed to be more in *C. gigantea* (22.8%) followed by *C. crinita* and *C. nilgirensis.* Higher level of adenine was observed in *C. nilgirensis* and *C. gigantea* (27.2%) followed by *C. crinita* (27.1%). Guanine concentration was rich in *C. crinita* (23.1%) followed by *C. nilgirensis* and *C. gigantea.* The average frequencies of A, T, G and C were displayed as 27.1%, 27.5%, 23.1% and 22.4%.

Table 30: Nucleotide composition of studied *Cyathea* species

Species	T(U)	C	A	G	AT	GC	Total
C. nilgirensis	27.5	22.3	27.2	23.0	54.7	45.3	**592**
C. gigantea	27.2	22.8	27.2	22.9	54.4	45.7	**593**
C. crinita	27.5	22.4	27.1	23.1	54.6	45.5	**590**

Maximum composite likelihood estimation

The probability of nucleotide substitution from one base to another base can be estimated by maximum composite likelihood substitution pattern. Rates of different transitional substitutions of studied species of *Cyathea* were displayed in **bold** and those of transversionsal substitutions were represented in normal (Table 31). The nucleotide frequencies were 26.92% (A), 27.48% (T/U), 22.94% (C) and 22.66% (G). The transition / transversion rate ratios were k_1 = 5.47 (purines) and k_2 = 5.423 (pyrimidines). The overall transition / transversion bias was R = 2.702, where R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]. The analysis involved three nucleotide sequences of *Cyathea* species. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 587 positions in the final dataset.

Table 31: Maximum composite likelihood substitution of *Cyathea* species

Base Pairs	A	T	C	G
A	-	3.69	3.04	**16.85**
T	3.61	-	**16.50**	3.08
C	3.61	**20.02**	-	3.08
G	**19.77**	3.69	3.04	-

Pairwise distances

Among the three studied *Cyathea* species, the highest pairwise distance (13.27) was observed between *C. nilgirensis* and *C. crinita* and the lowest distance (9.25) was between *C. nilgirensis* and *C. gigantea* (Table 32).

Table 32: Pairwise distances among the studied species of *Cyathea*

Species	*C. nilgirensis*	*C. gigantea*	*C. crinita*
C. nilgirensis	1.00	-	-
C. gigantea	9.25	1.00	-
C. crinita	13.27	9.80	1.00

Nucleotide pair frequencies for directional sequences

Nucleotide pair frequencies for directional sequences estimated among the repeated 16 pair sequences found in the selected taxa groups of *Cyathea* was displayed in Table 33. The ratio of the number of transitions to the number of transversions for a pair of sequences was referred as transition / transversion bias. The estimated transition / transversion bias for studied three *Cyathea* sequences was 0.51. The nucleotide pair frequencies were high in TT (41) followed by AA (40), CC (29) and GG (28). The average identical pairs (ii) of three nucleotides were 139, transitional pairs (si) were 152 and transversional pairs (sv) were 300.

Table 33: Nucleotide pair frequencies of studied *Cyathea* species

Domain	ii	si	sv	Ratio (si/sv)	AA	TT	CC	GG	Total
Average	139	152	300	0.51	40	41	29	28	590.67
First codon	48	50	99	0.51	14	8	10	16	197.33
Second codon	48	49	100	0.49	16	16	11	5	197.00
Third codon	42	53	101	0.52	10	17	9	7	196.33

The transition / transversion bias for first codon was estimated as 0.51. The average identical pairs of first codon for three nucleotides were 48, transitional pairs were 50 and transversional pairs were 99. The transition / transversion bias for second codon was found to be 0.49. The average identical pairs of second codon for three nucleotides were 48, transitional pairs were 49 and transversional pairs were 100. The transition / transversion bias for third codon was estimated as 0.52. The average identical pairs of

third codon for three nucleotides were 42, transitional pairs were 53 and transversional pairs were 101.

Amino acid composition

The amino acid compositions of the studied *Cyathea* species analyzed using MEGA 5.2 were represented in Table 34. *C. gigantea* showed the presence of maximum number of amino acids (197) followed by *C. crinita* (196) and *C. nilgirensis* (188). Among the twenty different amino acids, leucine displayed highest percentage (15.95%) of occurrence in *C. nilgirensis* whereas cysteine expressed lowest percentage (1.06%). In *C. gigantea,* glycine and leucine showed greater percentage (8.62%) whereas histidine expressed smaller amount (0.50%). *C. crinita* represented more amounts of glycine and leucine (8.67%) and less amount of histidine (0.51%).

Table 34: Amino acids composition present in different *Cyathea* species

Amino acids	*C. nilgirensis*	*C. gigantea*	*C. crinita*	Average
Alanine	3.19	7.61	8.16	6.36
Cysteine	1.06	1.52	1.53	1.37
Asparagine	3.72	6.59	6.12	5.50
Glutamic acid	2.65	6.09	6.12	4.99
Phenyl alanine	2.65	4.06	4.08	3.61
Glycine	3.72	8.62	8.67	7.05
Histidine	1.59	0.50	0.51	0.86
Isoleucine	9.04	3.55	4.08	5.50
Lysine	5.31	5.07	5.61	5.33
Leucine	15.95	8.62	8.67	11.01
Methionine	5.31	1.52	1.53	2.75
Aspartic acid	3.72	4.06	3.57	3.78
Proline	10.10	7.10	6.63	7.91
Glutamine	2.65	2.53	2.04	2.40
Arginine	6.38	6.59	6.63	6.54
Serine	8.51	4.06	4.08	5.50
Threonine	3.72	7.61	8.16	6.54
Valine	5.85	6.09	5.61	5.85
Tryptophan	1.59	1.52	1.53	1.54
Tyrosine	3.19	6.59	6.63	5.50
Total	**188**	**197**	**196**	**193.67**

Statistical domain

The aligned nucleotide sequences of *Cyathea* species gives overall statistical domain among 590 to 593 base pairs. The statistical domain includes 31 conserved regions, 561 variable regions and 561 singleton sites. It also includes 331 zero-fold degenerate sites, 20 two-fold degenerate sites and 20 four-fold degenerate sites. The individual zero-fold, two-fold and four-fold degenerate sites were represented in Table 35.

Table 35: Statistical domain for studied *Cyathea* species

Species	Zero-fold degenerative sites	Two-fold degenerative sites	Four-fold degenerative sites
C. nilgirensis	385	119	87
C. gigantea	382	111	98
C. crinita	380	110	98

Phylogenetic analyses - Maximum likelihood method

The evolutionary history among the studied three species of *Cyathea* was inferred using the maximum likelihood method. The phylogenetic tree 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 includes *C. gigantea* and the node C_1N^2 represented *C. crinita* with both showing the similar distance value of 9.7711. The species *C. gigantea* and *C. crinita* represented the distance value of 0.6322 from the main clade. The clade 2 (C_2) consists of distinct taxon *C. nilgirensis* with the branch length of 10.4033 (Fig. 35).

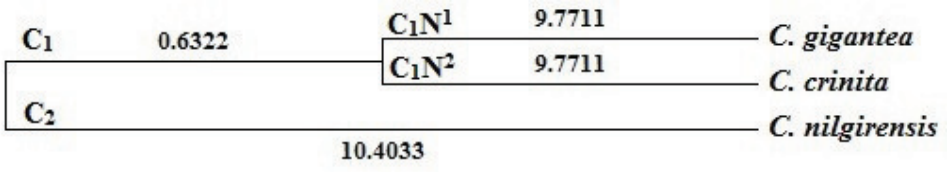

Fig. 35: Phylogenetic tree of *Cyathea* species using maximum likelihood method

UPGMA method

The phylogenetic relationship inferred using the UPGMA method represents two main clusters C_1 and C_2. The cluster C_1 was made of *C. nilgirensis* and *C. gigantea* with the distance value of 2134.7. The cluster C_2 includes *C. crinita* with the highest distance range of 4327.4. The cluster C_1 was divided into two nodes with C_1N^1 representing *C. nilgirensis* and C_1N^2 corresponds to *C. gigantea*. The nodes C_1N^1 and C_1N^2 showed the same branch length of 2192.7 (Fig. 36).

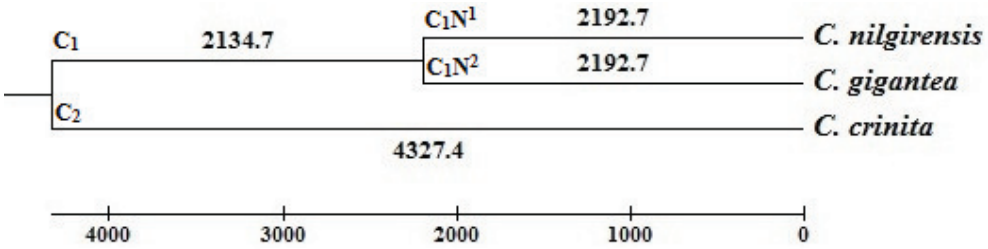

Fig. 36: UPGMA tree for studied *Cyathea* species

BLAST analysis

BLAST analysis was performed to study the sequence similarity among the three *Cyathea* species. The similar *rbc*L sequences related to *C. nilgirensis, C. gigantea* and *C. crinita* were downloaded in FASTA format. The sequences were aligned using multiple sequence alignment. UPGMA cladogram was constructed using MEGA 5.2 software. The results revealed hierarchical cluster formation among the different species of tree ferns (Fig. 37). Two major hierarchical clusters were formed. One cluster shows *Cyathea manniana* alone and another cluster comprises 39 similar species including *C. nilgirensis, C. gigantea* and *C. crinita*. The clusters were formed based on their similarity and dissimilarity of the nucleotide sequences of the individual species. It is essential to obtain an accurate phylogeny of the groups of organisms of interest for clear understanding of the evolution of characters.

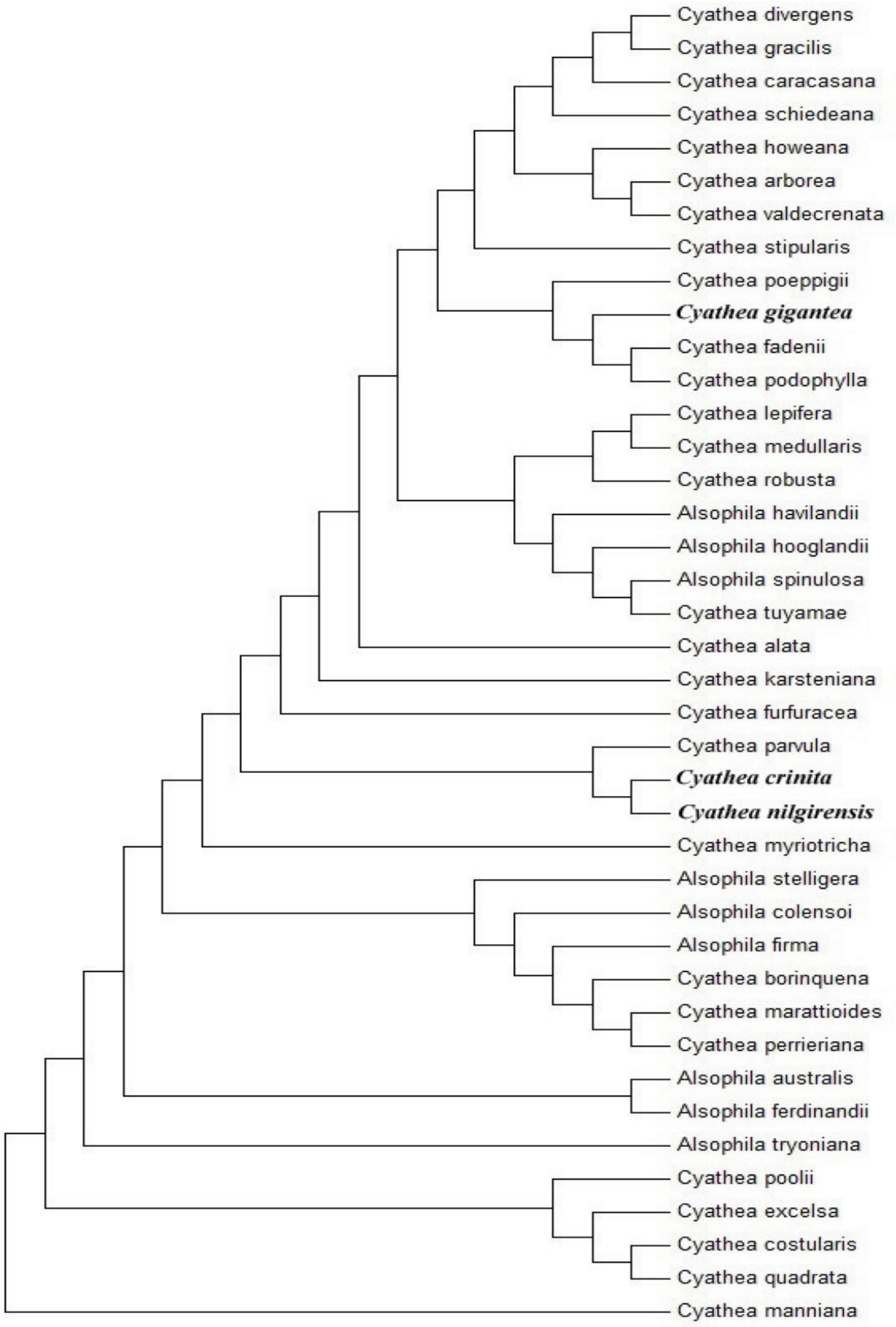

Fig. 37: UPGMA cladogram based on BLAST analysis

Biological activities

Antioxidant activity

DPPH radical scavenging activity

The results on DPPH free radical scavenging activity of tested *Cyathea* species along with the reference ascorbic acid were illustrated in Table 36. The absorbance decreases as a result of a colour change from purple to yellow as radical was scavenged by antioxidants through donation of hydrogen to form the stable DPPH molecule. Concentration of the extracts necessary to decrease initial concentration of DPPH by 50% (IC_{50}) under the experimental condition was determined. Lower value of IC_{50} indicates a higher antioxidant activity.

Table 36: DPPH radical scavenging activity of studied *Cyathea* species

Ethanolic extracts	Concentration (µg/ml)			
	125	250	500	1000
C. nilgirensis	25.91 ± 0.08	44.18 ± 0.06	67.86 ± 0.04	85.34 ± 0.03
C. gigantea	19.41 ± 0.07	36.63 ± 0.12	59.30 ± 0.06	78.89 ± 0.06
C. crinita	17.26 ± 0.04	32.22 ± 0.05	53.41 ± 0.03	75.47 ± 0.05
Ascorbic acid	46.62 ± 0.06	64.94 ± 0.03	73.56 ± 0.07	94.63 ± 0.12

Note: The values are mean ± SD of triplicates.

All the tested extracts of *Cyathea* species showed excellent DPPH radical scavenging activity. The best free radical scavenging activity was exerted by *C. nilgirensis* (IC_{50} 166.67 µg/ml). *C. gigantea* and *C. crinita* extracts showed comparable levels of free radical scavenging activity (IC_{50} 185.19 and IC_{50} 198.41 µg/ml respectively). The scavenging effect of various extracts and standards with the DPPH radical is in the following order: Ascorbic acid > *C. nilgirensis* > *C. gigantea* > *C. crinita*.

Metal chelating activity

The chelating effect on the ferrous ions by various extracts of *Cyathea* species were presented in Table 37. All the sample extracts exhibited the ability to chelate metal

ions. Among the different sample extracts tested, *C. nilgirensis* showed higher activity (80.04%) followed by *C. gigantea* (75.26) and *C. crinita* (72.20%).

Table 37: Metal chelating activity of studied *Cyathea* species

Ethanolic extracts	Concentration (µg/ml)			
	125	250	500	1000
C. nilgirensis	24.68 ± 0.03	32.63 ± 0.05	56.30 ± 0.06	80.04 ± 0.03
C. gigantea	18.49 ± 0.15	28.96 ± 0.07	52.60 ± 0.12	75.26 ± 0.04
C. crinita	18.06 ± 0.04	27.73 ± 0.02	50.21 ± 0.01	72.20 ± 0.01
EDTA	28.64 ± 0.30	51.50 ± 0.03	70.23 ± 0.02	93.96 ± 0.02

Note: The values are mean ± SD of triplicates.

Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity was estimated by using Griess reagent and the results were depicted in Table 38. In the presence of a scavenging test compound, the amount of nitrous acid will decrease and can be measured at 546 nm. The highest free radical scavenging activity was exerted by *C. nilgirensis* (76.78%) followed by *C. gigantea* (71.87%) and *C. crinita* (68.36%) ethanolic extracts.

Table 38: Nitric oxide radical scavenging activity of studied *Cyathea* species

Ethanolic extracts	Concentration (µg/ml)			
	125	250	500	1000
C. nilgirensis	18.54 ± 0.01	34.31 ± 0.01	52.26 ± 0.06	76.78 ± 0.03
C. gigantea	15.57 ± 0.02	28.43 ± 0.09	47.58 ± 0.06	71.87 ± 0.08
C. crinita	13.29 ± 0.09	22.45 ± 0.09	43.22 ± 0.01	68.36 ± 0.21
Gallic acid	29.04 ± 0.01	42.95 ± 0.04	67.05 ± 0.02	89.71 ± 0.05

Note: The values are mean ± SD of triplicates.

Hydroxyl radical scavenging activity

The scavenging abilities of *Cyathea* extracts on hydroxyl radical were investigated at the concentration ranged from 125-1000 µg/ml and were found to be dose dependent. The hydroxyl radical scavenging activity was increased with increasing concentration of extracts (Table 39). Among the tested ethanolic extracts of *Cyathea* species, *C. nilgirensis* exhibited highest radical scavenging activity (68.59%) at the concentration of 1000 µg/ml. *C. gigantea* and *C. crinita* showed comparable activity (62.78% and 58.33% respectively).

Table 39: Hydroxyl radical scavenging activity of studied *Cyathea* species

Ethanolic extracts	Concentration (µg/ml)			
	125	250	500	1000
C. nilgirensis	16.63 ± 0.06	29.42 ± 0.03	59.32 ± 0.07	68.59 ± 0.22
C. gigantea	14.38 ± 0.08	27.30 ± 0.07	52.80 ± 0.11	62.78 ± 0.06
C. crinita	12.48 ± 0.22	21.35 ± 0.09	46.28 ± 0.75	58.33 ± 0.03
Vitamin C	27.62 ± 0.09	40.69 ± 0.08	65.77 ± 0.06	87.30 ± 0.03

Note: The values are mean ± SD of triplicates.

Superoxide radical scavenging activity

The scavenging abilities of *Cyathea* species exhibited that the inhibition of formazan and the percentage inhibition were directly proportional to the concentration of the *Cyathea* ethanolic extracts (Table 40). The superoxide radical scavenging activity of different ethanolic extracts was found to be in the order of *C. nilgirensis* (78.70%), *C. gigantea* (74.63%) and *C. crinita* (66.08%). The total scavenging activity of extracts was compared with ascorbic acid which showed promising activity (91.96%).

Table 40: Superoxide radical scavenging activity of studied *Cyathea* species

Ethanolic extracts	Concentration (µg/ml)			
	125	250	500	1000
C. nilgirensis	22.45 ± 0.02	28.90 ± 0.08	56.69 ± 0.01	78.70 ± 0.06
C. gigantea	16.26 ± 0.29	30.24 ± 0.03	51.08 ± 1.11	74.63 ± 0.04
C. crinita	14.31 ± 0.07	21.26 ± 0.29	49.24 ± 0.03	66.08 ± 1.11
Ascorbic acid	35.64 ± 0.30	54.50 ± 0.03	71.23 ± 0.02	91.96 ± 0.02

Note: The values are mean ± SD of triplicates.

Cytotoxic activity

MTT cell proliferation assay

The MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. The ethanolic extracts of selected *Cyathea* species were subjected for MTT cell proliferation assay and results were presented in Table 41. The microscopic observations (Plate XV) explained the apoptosis of human breast carcinoma MCF 7 cell line. Decrease in cell viability and increase in growth inhibition was observed on MCF 7 cell line in concentration dependent manner. Maximum percentage of cell

inhibition was observed in *C. crinita* followed by *C. nilgirensis* and *C. gigantea.* As the concentration increases, there is an increase in the cell growth inhibition but it was found to be very less with only 25.26% growth inhibition in *C. crinita* at 200 µg/ml.

Table 41: Effect of ethanolic extracts of *Cyathea* species against MCF 7 cell line

Conc. (µg/ml)	*C. nilgirensis*		*C. gigantea*		*C. crinita*	
	% of cell viability	% of cell inhibition	% of cell viability	% of cell inhibition	% of cell viability	% of cell inhibition
12.5	100	0	100	0	100	0
25	99.73	0.27	100	0	100	0
50	97.77	2.23	99.24	0.76	96.38	3.62
100	95.40	4.60	94.28	5.72	84.93	15.07
200	83.95	16.05	85.42	14.58	74.74	25.26

The extracts with IC_{50} value lower than 200 µg/ml was considered to be more active and the extracts with IC_{50} value higher than 200 µg/ml were considered to be less active (Yin *et al.,* 2004). The IC_{50} value of *C. crinita* was found to be more than 400 µg/ml. Similarly, *C. nilgirensis* and *C. gigantea* showed IC_{50} value of 714.28 and 806.45 µg/ml respectively. The results showed that ethanolic extracts of *Cyathea* species had a moderate anticancer activity against MCF 7 cell line.

Brine shrimp lethality bioassay

Cytotoxic effects of petroleum ether, chloroform, acetone and ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* was found to be concentration dependent. The degree of lethality was found to be directly proportional to the concentration of the extract. The results of brine shrimp lethality bioassay were demonstrated in Table 42. The tested extracts were found to be toxic (LC_{50} < 1000 mg/ml) in the brine shrimp bioassay. Ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* were found to be more effective against brine shrimps with the LC_{50} values of 304.73, 277.45 and 287.44 mg/ml respectively. Chloroform and acetone extracts of studied *Cyathea* species showed moderate lethality level. Petroleum ether extract exhibited less cytotoxic effects when

compared to other extracts. The standard plumbagin showed 100% mortality of brine shrimp nauplii at 0.046 mg/ml.

Table 42: Cytotoxic effects of studied *Cyathea* species against *A. salina*

Species	Extracts	LC_{50} (mg/ml)	95% Confidence Limits		LC_{90} (mg/ml)	Chi-Square (χ^2)
			Lower	Upper		
C. nilgirensis	Pet. ether	563.27	501.07	668.69	958.57	0.57
	Chloroform	328.08	299.80	358.40	637.71	1.54
	Acetone	567.00	461.34	910.36	968.73	4.27
	Ethanol	304.73	273.62	336.21	642.72	0.90
C. gigantea	Pet. ether	543.59	449.60	821.95	901.17	4.67
	Chloroform	279.31	253.14	304.41	548.69	0.47
	Acetone	345.58	298.43	401.95	608.52	4.09
	Ethanol	277.45	204.62	340.50	973.77	0.50
C. crinita	Pet. ether	421.12	312.69	891.76	728.46	15.53
	Chloroform	331.08	251.65	433.57	738.91	4.78
	Acetone	340.99	311.61	373.57	663.81	0.23
	Ethanol	287.44	254.30	319.82	512.88	2.45

Larvicidal activity

The results of the larvicidal bioassay of crude petroleum ether, chloroform, acetone and ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* against the fourth instar mosquito larvae *C. quinquefasciatus* were presented in Table 43. The larvae were more sensitive to ethanolic extracts of studied three *Cyathea* species when compared to other extracts. Acetone, chloroform and petroleum ether extracts were considered to be less effective. The LC_{50} values of different extracts ranged from 320.72 to 657.03 mg/ml. Among the three different species tested, the highest larval mortality was observed in ethanolic extracts of *C. crinita* with the LC_{50} value of 320.72 mg/ml followed by *C. gigantea* (361.07 mg/ml) and *C. nilgirensis* (373.99 mg/ml). The positive control Temephos showed 100% mortality rate at 0.025 mg/ml. The 95% confidence limits LC_{50} (LCL-UCL), LC_{90} and chi-square values were also calculated.

Table 43: Larvicidal activity of different *Cyathea* species against *C. quinquefasciatus*

Species	Extracts	LC_{50} (mg/ml)	95% Confidence Limits		LC_{90} (mg/ml)	Chi-Square (χ^2)
			Lower	Upper		
C. nilgirensis	Pet. ether	607.76	512.14	894.19	893.93	3.86
	Chloroform	592.26	482.18	917.90	1078.70	2.73
	Acetone	448.88	394.96	536.88	966.31	1.04
	Ethanol	373.99	338.65	417.91	758.69	0.69
C. gigantea	Pet. ether	639.92	561.76	784.12	1013.26	1.05
	Chloroform	624.63	532.47	813.44	1151.44	0.54
	Acetone	468.00	394.65	620.38	1175.06	0.38
	Ethanol	361.07	317.84	416.12	848.87	2.29
C. crinita	Pet. ether	657.03	556.55	868.54	1178.48	0.35
	Chloroform	568.04	489.18	722.23	1103.12	0.40
	Acetone	400.36	292.74	776.28	870.41	7.37
	Ethanol	320.72	291.11	351.94	645.79	0.36

In natural systems, plants face excess of antagonist and possess myriads of multiple defense mechanisms by which they are able to cope with various kinds of biotic and abiotic stress (Ballhorn *et al*., 2009). Plants produce a high diversity of natural products or secondary metabolites which are important sources of various fine chemicals with a prominent function in protecting against predators and microbial pathogens on the basis of their toxic nature, repellence to herbivores and microbes (Schafer and Wink, 2009). Phytochemicals are bioactive substances of plants that are used directly or as intermediates for the production of pharmaceuticals and have been associated in the protection of human health against various chronic degenerative diseases.

Botanicals or phytomedicines have always been a major component of traditional systems of healing in developing countries, which have also been an integral part of their history and culture. With deep concern and relevance to Indian medicinal pteridophytes and sense of realization about its medicinal value, the present research work was undertaken to explore the phytochemical, biochemical and molecular profile of *Cyathea nilgirensis, Cyathea gigantea* and *Cyathea crinita*.

Qualitative phytochemical analysis was performed on whole plant extracts of *C. nilgirensis, C. gigantea* and *C. crinita* to reveal the presence of various active constituents which are known to exhibit medicinal as well as physiological activities. The results showed the presence of phytochemicals such as phenolics, flavonoids, tannins, cardiac glycosides, terpenoids, steroids, saponins and alkaloids. The presence or absence of the phytoconstituents in a particular species depends upon the organic solvent used for extraction and the physiological aspect of the selected species of *Cyathea*. In the present study, ethanolic extracts demonstrated the presence of maximum metabolites in *C.*

nilgirensis (8/12), *C. gigantea* (8/12) and *C. crinita* (7/12) compared to other solvents employed.

Plant phenolic compounds include flavonoids, tannins, glycosides, coumarins, anthraquinones, lignans and lignins. They may act as phytoalexins, anti-feedants and attractants for pollinators. In addition, they act as contributors to the plant pigmentation (Shahidi and Naczk, 2004). Phenolics have also been considered powerful antioxidants *in vitro* and proved to be more potent than Vitamin C, E and carotenoids (Rice-Evans *et al*., 1996). Phenolics are thought to provide a means of protection against UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage (Strack, 1997). Therefore, plants in high altitude areas which are exposed to a number of stress factors such as low air temperature, decreased partial O_2 pressure, increased UV radiation and unfavourable water regime have generally increased accumulation of antioxidants (Chanishvili *et al*., 2007). The studied three species of *Cyathea* collected from high altitude regions of Western Ghats, South India also showed high accumulation of phenolic compounds and the results of the present study coincided with Chanishvili *et al.* (2007) observations.

Flavonoids including biflavonoids, homoflavonoids, flavone glycosides and flavonol glycosides are an important group of secondary metabolites represented in pteridophytes. Amentoflavone and ginkgetin flavonoids found in ferns exhibit neuroprotective activity against cytotoxic stress. This property suggests their possible use in the treatment of neurodegenerative diseases such as stroke and Alzheimer's disease (Kang *et al.,* 2005). They also exhibit a wide range of biological activities viz., antimicrobial, anti-inflammatory, anticarcinogenic, hepatoprotective, antithrombotic, anti-allergic and vasodilatory actions. Many of these biological functions have been attributed

to free radical scavenging property of these compounds (Middleton *et al*., 2000; Williams *et al*., 2004; Soobrattee *et al*., 2005).

Tannins are good antimicrobial agents which precipitate protein thereby providing waterproof layer on the skin when used externally or protect the underlying layers of the skin and limit the loss of fluid (Buzzini *et al.,* 2008). In particular, the tannin containing remedies are in use as antihelmintics (Ketzis *et al.,* 2006), antioxidants (Koleckar *et al*., 2008), cancer treatment (Chung *et al*., 1998) and to chelate dietary iron (Clauss *et al.,* 2007). Glycosides are known to lower the blood pressure (Nyarko and Addy, 1990). Cardenolides and bufadienolides are the two basic groups of glycosides in plants which have direct effects upon cardiac function.

Alkaloids rank among the most efficient and therapeutically significant plant metabolites (Okwu, 2005). They are one of the largest groups of phytochemicals in plants having significant effects on humans which have led to the development of powerful pain killer medications (Kam and Liew, 2002). Plant alkaloids are used as basic medicinal agents for analgesic and antispasmodic activities (Stray, 1998). They are also used as antidepressant (morphine), stimulants (caffeine), anaesthetic (cocaine), anti-tumour (vinblastine) and antimalarial (quinine) agents (Cowan, 1999; Heinrich *et al.,* 2004; Gurib-Fakim, 2006).

Terpenoids are the main component of many plant essential oils (Kretovich, 1966). They are a diverse group among the pteridophytes which includes triterpenoids, diterpenoids, hemiterpene glycosides and clerodane diterpene glycosides. Terpenoids are medicinally significant for a wide range of treatments viz., cytotoxic against human cancer cell lines and anti-inflammatory activity (Loggia *et al*., 1994). Steroids and saponins are the derivatives of terpenoids. Steroids may serve as an intermediate for the biosynthesis of downstream secondary products and it is believed to be a biosynthetic precursor for

cardenolides in plants. The presence of steroids in every organism suggests that they have a powerful role in chemosystematics (Herl *et al*., 2006; Gavidia *et al*., 2007). Saponins have a diverse range of medicinal properties viz., haemolytic (Oda *et al.,* 2000), anti-inflammatory (Sun *et al.,* 2010), anti-cancer (Musende *et al.,* 2009), molluscicidal, insecticidal and antimicrobial (Sparg *et al.,* 2004). Saponins are also of great interest as valuable adjuvants (Sun *et al.,* 2009).

The results of qualitative phytochemical analysis confirmed the presence of phenolics, flavonoids, tannins, cardiac glycosides, terpenoids, steroids, saponins and alkaloids in the studied *Cyathea* species. The present study results suggest that the studied *Cyathea* species may be used as antioxidant, anticancer, antimicrobial, anti-inflammatory, insecticidal and haemolytic agents. Hepatoprotective activity of *C. gigantea* was confirmed by Kiran *et al.* (2012). The other pharmacological properties of *Cyathea* species were unexplored. The results of the present study paved a way to find the chemical constituents of the studied *Cyathea* species which may lead to quantitative estimation and also in locating the source of bioactive principles for various pharmacological properties.

The physico-chemical evaluation of the drug is an important parameter in detecting adulteration or improper handling of drugs. Extractive values are useful for the determination of exhausted drugs and help in estimation of specific constituents soluble in a particular solvent (Ozarkar, 2005). Correct identification and quality assurance of the starting material is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy (Nayak and Patel, 2010). For fluorescence analysis, the powders of selected *Cyathea* species were treated with various chemical reagents. The fluorescence colour is unique for each compound. A non-fluorescent compound may fluoresce if mixed with impurities that are fluorescent. Similar to the present study, Kala *et al.* (2011) previously applied fluorescence characters as a tool

to characterize the different medicinal plants of South India. The results of the fluorescence analysis of studied *Cyathea* species may be applied to identify the purity of the drug in the pharmaceutical industries.

Authentication of medicinal plants as chemical and genetic level is a critical step in the use of botanical materials for academic and commercial purposes. For any living organism, identity is very important in order to distinguish itself from other organisms within the population and other populations. In general, morphological characters play a vital role in plant systematic studies and are used as an efficient tool for the classification of a taxon. In addition to morphological characters, anatomical, cytological, biochemical, phytochemical and molecular markers are also used to classify the organisms (Rouhan *et al*., 2004; Irudayaraj and Johnson, 2011a; Narayani and Johnson, 2013).

Molecular absorption spectrophotometry in UV-Vis light 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 were transferred to the molecules of the medium under analysis to cause electron transitions associated with vibrational and rotational transitions (Leal *et al*., 2008). The changes that occur in the UV spectrum due to complex formation are generally identified by widening of peak areas. The displacement at maximum UV absorption by the effect of complex formation can be explained by the partial protection of excitable electrons and chromophores present in the sample. The development of less onerous methods, easier to execute and normally using less complex apparatus, requires validation studies of these techniques for each plant species, in order to assure the reliability of the results (Silva-Corazza *et al*., 2010).

The UV-Vis analysis results showed that ethanolic extracts of studied *Cyathea* species displayed more number of peaks when compared to other extracts. The cladogram

constructed based on the UV-Vis spectroscopic profile showed two clades. C_1 was shared between *C. nilgirensis* and *C. gigantea* whereas clade C_2 showed the unique presence of *C. crinita.* UV-Vis spectra generally show only a few broad absorbance peaks. It provides a limited amount of qualitative information compared with FT-IR which produces many narrow peaks. Although UV-Vis spectra do not enable absolute identification of an unknown substance, they are frequently used to confirm the identity of a substance through comparison of the measured spectrum with a reference spectrum. The number of peaks increases with higher orders of derivatives. This increase in complexity of the derivative spectra can be useful in qualitative analysis, either for characterizing materials or for identification purposes.

FT-IR spectroscopy has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plant extracts. It records the interaction of infra red radiation with experimental samples, measuring the frequencies at which the sample absorbs the radiation and the intensities of the absorptions. Determining these frequencies allows identification of the sample's chemical makeup since chemical functional groups are known to absorb light at specific frequencies (Chen *et al*., 1998; Coimbra *et al*., 1998).

Studies based on FT-IR spectral bands analysis in conjunction with plant taxonomic classification have yielded fruitful data. Kim *et al*. (2004) proposed that FT-IR fingerprinting was an excellent method for the determination of phylogenetic relationships between different groups of plants. Lu *et al*. (2004) employed FT-IR spectroscopy for identifying the species *Hypericum* and *Triadenum.* Kumar and Murugan (2012) used FT-IR approach in taxonomic identification of various accessions of *Solanum capsicoides.* Renisheya and Johnson (2014) used UV-Vis and FT-IR spectroscopic profile as pharmacognostic criteria to distinguish the medicinally important *Plumbago* species.

Previous reports on FT-IR analysis of ferns viz., *Macroneuropteris scheuchzeri, Alethopteris lesquereuxii, Neuropteris ovata* var. *simonii, Eusphenopteris neuropteroides, Oligocarpia brongniartii, Pecopteris nyranensis, Pecopteris miltonii, Pecopteris aspidioides* and *Pecopteris polypodioides* showed the presence of various functional groups with strong bands in different regions. They employed FT-IR characteristics to distinguish the ferns and used as a chemotaxonomic parameter for identifying ferns (Lyons *et al.,* 1995; Zodrow and Mastalerz, 2002; Psenicka *et al.,* 2005).

Similarly, FT-IR analysis was carried out to identify the similarity and variation of functional groups present in *C. nilgirensis, C. gigantea* and *C. crinita.* The data for infrared spectra of *Cyathea* were shown based on the most common and characteristic group frequencies. The common functional groups present in studied *Cyathea* species include alkanes, nitro compounds, carboxylic acids and aromatics whereas alcohols, primary amines, alkyl halides, amide, anhydride, alkenes and esters were fingerprint peaks present only in a particular extract. The range of the peaks in the FT-IR spectrum is a direct indication of the amount of compounds present in the extracts. The evolutionary tree constructed expressed two clusters. The cluster C_1 includes *C. nilgirensis* and *C. gigantea* whereas cluster C_2 showed the unique presence of *C. crinita*. In addition, FT-IR spectra of pure compounds provide unique chemical "fingerprint" with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material.

Chromatography is the lynchpin of phytochemistry and is the key to obtain pure compounds for development into therapeuticals. Separation, identification and structure elucidation of biologically active compounds has been facilitated by continual development of chromatographic and spectroscopic methods. It also plays a fundamental role as an analytical technique for quality control and standardization of

phytotherapeuticals. Generally, two approaches being used for standardization are fingerprint analysis by HPTLC/HPLC and quantification of individual chemical markers (Patel and Mishra, 2012). It ensures reproducible pharmaceutical quality of herbal products. Characteristic HPTLC fingerprinting of particular plant species will not only help in identification of that species but also provide basic information useful for the isolation, purification and characterization of marker chemical compounds of the species (Yadav *et al*., 2011). It is useful as a phytochemical marker and also a good estimator of genetic variability in plant populations. The presence or absence of chemical constituent has been found useful in the placement of the plant in taxonomic categories. HPTLC profile differentiation is an important procedure (Kpoviessia *et al.,* 2008) which produces visible chromatograms and complex information about the entire sample. It also provides visualization of the separated constituents and online identification of the analyte by *in situ* spectrum scanning and post chromatographic derivatization, along with R_f comparison with the standard (Faiyazuddin *et al*., 2011). HPTLC method can be used for phytochemical profiling and quantification of compounds present in plant samples.

With the increasing demand for natural products as medicines, there is an urgent need for standardization of plant products. Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to traditional system of medicine throughout the world (Halinski *et al*., 2009). The optimized chromatographic fingerprint is not only an alternative analytical tool for authentication, but also an approach to express the various patterns of chemical ingredients distributed in the plant material and to preserve such "database" for further sustainable studies (Sweta *et al*., 2011). HPTLC results on ethanolic extracts of *C. nilgirensis, C. gigantea* and *C. crinita* provided an impressive result that directing towards the presence of diverse type of phytochemicals (alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and

terpenoids). The selection of appropriate solvent system for a particular plant extract can be achieved only by analyzing the R_f values of compounds in different solvent system. The variation in R_f values of the phytochemicals provides an important clue in selection of appropriate solvent system for separation of pure compounds by column chromatography. Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extracts. The developed HPTLC method will provide sufficient information about therapeutic efficacy of the drug and also in the identification, standardization and quality control of studied *Cyathea* species. The cladogram constructed based on the HPTLC profile expressed two clusters. The cluster C_1 depicted the distinct presence of *C. nilgirensis* whereas C_2 showed the similarity between *C. gigantea* and *C. crinita*. This revealed a better separation of individual secondary metabolites and further facilitates their quantitative estimation and qualitative separation of pharmacologically active chemical compounds.

HPLC identification tests are required to confirm the presence of the active constituents and potential adulterant in ayurvedic drugs. Standardization is an important aspect for establishing the quality and efficacy of any multiple ingredient herbal formulations (Mallikharjuna *et al*., 2007; Sharanabasappa *et al*., 2007). HPLC analysis of *C. nilgirensis, C. gigantea* and *C. crinita* showed varying patterns in the chromatogram. The results showed various peaks separated at different retention times. The chromatogram also confirmed the presence of most abundant peak separated at a retention time of 18.09 min with the peak area 99.65% in *C. nilgirensis*, *C. gigantea* with 76.77% peak area separated at a retention time of 17.39 min and *C. crinita* with the peak area 67.75% at a retention time of 16.06 min respectively. The results of the HPLC analysis suggests that there might be differences in the chemical constituents of the studied *Cyathea* species and therefore a proper scientific validation of active principles is needed

for the medicinal utilization. The generated data may be useful in suggesting chemotaxonomical inter-relationship among the studied *Cyathea* species.

Metabolomics techniques are used to screen the potential "biomarkers" in plants (Kooy *et al.,* 2009). These techniques play an important role in many aspects of biomedical and phytochemical research including biomarker screening, chemotaxonomy, quality control, bioactivity and toxicity prediction (Liu *et al*., 2010). The most widely used and powerful methods to identify the profile of low molecular weight chemicals are based on chromatographic separation followed by detection and validation by mass spectroscopy (Tolstikov and Fiehn, 2002). GC in combination with MS are able to detect several chemicals including sugars, sugar alcohols, organic acids, amino acids, fatty acids and a wide range of diverse secondary metabolites (Fiehn *et al*., 2000; Roessner *et al*., 2001).

GC-MS analysis is an efficient way to analyze metabolic fingerprinting of phytomedicine and to evaluate the global chemical difference in medicinal plants which provides high separation efficiencies (Hall *et al*., 2002; Sumner *et al*., 2003). It is also a valuable tool for reliable identification of phytocompounds present in plant extracts. Chemical compounds found in plants, including secondary metabolites have various functions ranging from defense against herbivores and microorganisms to ecological adaptations. Previous studies have reported a direct correlation between plant secondary metabolites and their biological activities (Fernie *et al.,* 2004; Saito *et al*., 2006).

In the present study, GC-MS analysis was carried out to identify some of the potent chemical constituents present in *Cyathea* species. The most prevailing compounds identified in *C. nilgirensis* are Methyloctadecyl dichlorosilane (29.19%) and 2-Methylbutane-1,4-diol,3-(1-ethoxyethoxy)- (24.48%) separated at the retention time of 38.75 and 3.12 min respectively. *C. gigantea* showed the presence of 2-Methylbutane-1,4-diol, 3-(1-ethoxyethoxy)- (42.37%) and 2-Hydroxy-5-methyl benzaldehyde (16.26%)

separated at the retention time of 3.13 min and 16.64 min respectively. *C. crinita* confirmed the existence of major constituent 2-Hydroxy-5-methyl benzaldehyde (55.45%) separated at the retention time 16.68 min. In general, all the predicted compounds showed various pharmacological properties viz., antieczematic, antinociceptive, hepatoprotectant, antirickettsial, antiviral, anti-infective, antiprotozoal, antihematotoxic, cardioprotectant, antitreponemal, antiulcerative, astringent, antimyopathies, antipruritic, anti-inflammatory, antihypoxic, antifibrinolytic, vasoprotector, antiparasitic, antithrombotic, antihypertensive, antiseborrheic, anticonvulsant, antidote, antispirochetal activities etc. The presence of various bioactive compounds with biological properties confirmed the application of *Cyathea* species for various ailments.

Most of the currently available molecular modeling methods such as docking are designed to study the ligand-receptor interaction for one specific biological macromolecule at a time while QSAR analysis is generally applicable to the optimization of lead compounds' properties within the same chemical series (Waterbeemd, 1996). In contrast to both these techniques, the computer program PASS (Gloriozova *et al.,* 1998; Poroikov and Filimonov, 2001) is able to predict biological activities for compounds from different chemical series on the basis of their 2D structural formulas in a very rapid manner. The set of pharmacological effects, molecular mechanisms of action and specific toxic compounds with side effects that might be exhibited by a particular compound in its interaction with biological entities which is predicted by PASS is termed as "biological activity spectrum" of the compound. It is the "intrinsic" property of a compound which depends only on its structure and physico-chemical characteristics. Further hyphenated spectroscopic studies are required for structural elucidation and identification of compounds detected in the studied *Cyathea* species. However, isolation of individual phytochemical constituents may proceed to find a novel drug formulation.

The breeding system of a species is thought to be a major determinant of patterns of genetic variation between same groups of plants (Hamrick and Godt, 1989). The outcrossing mating system is thought to be predominant in terrestrial ferns (Soltis and Soltis, 1990a; 1990b). Although most diploid homosporous fern species exhibit high outcrossing (Soltis and Soltis, 1992), a gametophyte of a homosporous fern may have the potential for self-fertilization because of hermaphroditism, an important mechanism to construct a population from a single spore after long-distance dispersal (Baker, 1955; Klekowski, 1979). Reported as an outcrossing fern species, tree ferns produces sporophytes primarily by inter-gametophytic mating, especially inter-gametophytic crossing (Chiou *et al*., 2003). The reasons that *Cyathea* species maintains relatively higher genetic variation might be closely related to its mating system. The outcrossing habit of *Cyathea* may play a key role in the maintenance of its genetic diversity.

Electrophoretic separation using SDS-PAGE plays an important role in genetic diversity analysis and conservation of plant genetic resources. It has been successfully used to resolve the taxonomy and evolutionary problems of several ferns and fern allies viz., *Adiantum raddianum, Arachniodes tripinnata, Dryopteris sparsa, Odontosoria chinensis, Tectaria paradoxa*, *Araiostegia hymenophylloides*, *Deparia petersenii* and *Selaginella* species by many researchers (Wang *et al.,* 2010; Revathy *et al.,* 2011; Sivaraman *et al.*, 2011b; Narayani and Johnson, 2013). These ferns demonstrated unique banding pattern of proteins to distinguish one species from another. In the present study also, SDS-PAGE protein profiling is used to differentiate the studied *Cyathea* species based on the protein bands. The distinct protein bands with respective molecular weight present in the *Cyathea* species represented the “protein fingerprint” of that particular species. The present study results showed that SDS-PAGE is used as a biochemical tool for identification of genotypes based on proteins.

MALDI has the potential to provide new insights into the molecular analyses of plants by providing high resolution information on the spatial arrangement of peptides and proteins. The most important advantages of the MALDI mass spectrometric approach are: (1) only small amount of biological material is required (i.e. less than 100 ng) and (2) both measurement and data interpretation processes are very fast and relatively easy. MALDI-TOF MS offers relatively high tolerance against sample impurities (salts and detergents), as well as fast and accurate molecular mass determination and the possibility of automation, which makes it a powerful alternative to classical biological methods (Lewis *et al.,* 2000). The utility of MALDI for protein and peptide analyses lies in its ability to provide highly accurate molecular weight information on intact molecules. The ability to generate such accurate information can be extremely useful for protein identification and characterization (Dickinson *et al.,* 2004). For example, a protein can often be unambiguously identified by the accurate mass analysis of its constituent peptides produced by either chemical or enzymatic treatment of the sample. Protein identification can also be facilitated by analysis of the protein's proteolytic peptide fragments in the gas phase. Previous studies on MALDI-TOF MS analysis of pteridophytes viz., *Pronephrium simplex*, *Selaginella martensii, Selaginella pallescens, Lycopodium clavatum* and *Azolla filiculoides* has been used for the characterization and measurement of accurate molecular weight of proteins (Lai *et al*., 2005; Moore *et al*., 2006; Ekman *et al*., 2008; Martinez-Cortes *et al*., 2012). The MALDI-TOF mass spectra obtained from the present study sufficiently provided relative differences among the studied *Cyathea* species. Among the studied three *Cyathea* species, 15 specific peaks were observed in *C. nilgirensis* followed by 11 distinct peaks in *C. crinita* and 10 unique peaks in *C. gigantea.* These unique ionic spectral peaks of different *Cyathea* species act as a spectroscopic tool and paved a way to study the variation among the species using MALDI-TOF MS analysis.

Isoenzymes are widely used for their relative efficacy and cost-effectiveness, particularly in studies of intra and inter-specific variability (Siva and Krishnamurthy, 2005). They are useful as genetic and biochemical markers and also as good estimators of genetic variability in plant populations. In plants, most enzymes routinely assayed have several isozyme forms often with specific sub-cellular locations and the majority of isozymes have different allozymic variants (Harris, 1966; Tanksley and Orton, 1983).

The remarkable inter-specific difference in isozymic pattern of studied three species of *Cyathea* clearly showed the difference in their phylogenetic relationship. Each and every species of *Cyathea* of the present study belongs to different intra-generic taxonomical groups. The species *C. nilgirensis* and *C. gigantea* belong to the same subgenus *Cyathea* and section *Alsophila.* The species *C. crinita* belongs to the different subgenus *Sphaeropteris*. The isozymic variation in studied *Cyathea* species may be due to the morphological and genetical variation between the species.

The changing pattern of isozymes during development may be interpreted as evidence for differential timing of gene expression correlated with the physiological changes (Presley and Fowden, 1965; Johnson *et al.,* 1973; Mehta and Ali, 1996). Isozymic variation studies using esterase, peroxidase and acid phosphatase has been chosen to reveal the diversity existing at molecular level in different *Cyathea* species. The present study revealed the genetic differentiation and banding profiles among the three species expressed with diversified banding pattern. The active regions occupied by a particular isozyme in the form of bands are the representatives of the expression of a particular gene locus coding for that isozyme. In certain regions, more than one distinct bands are resolved which represents allelic isozymes, coded by different alleles of the same gene at a locus and thus occupy that particular zone on the system. In the present study also, similar

kind of banding profiles were obtained in the studied *Cyathea* species expressing the presence of multiple alleles.

The genetic variation of medicinal ferns using isozymes has already been carried out previously in *Diplazium* (Johnson *et al*., 2009), *Tectaria* (Johnson *et al*., 2010a), *Pteris* (Johnson *et al*., 2010b), *Adiantum* (Johnson *et al.* 2010c), *Trichomones* (Johnson *et al.* 2010d), *Sphaerostephanos* (Irudayaraj and Johnson, 2011b) and *Thelypteris ciliata* (Johnson *et al*., 2012b). Similar to the previous observations, the unique banding pattern of isoenzymes (isoesterase, isoperoxidase and acid phosphatase) has been observed in each species of *Cyathea* which represents the fingerprint of that particular species. This confirms the real distinctness of *Cyathea* species which will be useful in differentiating *Cyathea* species from other ferns and can act as biochemical markers.

Until now, most studies of pteridophyte DNA sequences have focused on phylogenetic placement (Schuettpelz and Pryer, 2007). Results derived from pteridophyte samples with small coverage may not be readily extendable to other pteridophytes (Fazekas *et al*., 2008). Furthermore, some of the studies suggesting the possibility of species identification using standard DNA sequences were carried out within a narrow pteridophyte taxon (Nitta, 2008). DNA phylogenies have now greatly clarified the main subgroups of the scaly tree ferns (Lellinger, 1987; Conant *et al.,* 1994, 1995, 1996).

The identification of tree ferns is especially difficult when the country of origin is not known (Pryer *et al.,* 2010). The scaly tree ferns (Cyatheaceae) are distinguished from all other members of the tree fern clade by an indument composed of scales and hairs, by their sori being in a dorsal position with a capitate receptacle, exindusiate or with small scale-like to globular indusia completely covering the sorus. They usually develop an erect trunk of large size. The unique appearance of a particular character has almost certainly led to identify it as the correct species. Upon reexamining the identity of *Cyathea* species,

the traditional use of stipe scales for tree fern identification suggested that this identity was incorrect. The identification of juvenile *Cyathea* sporophytes is very difficult with the morphological characters in the natural habitats. To overcome this lacuna, the more recently developed approach of using cpDNA sequences as barcodes for species identification (Kress *et al.,* 2005; Chase *et al.,* 2005; CBoL, 2009) has been shown to complement traditional analyses based on morphological characters. In the present study, DNA was isolated, amplified and sequenced from the young sporophytes (croziers) without any reproductive characters (sori and spores). They were used to distinguish the *Cyathea* species. The *rbc*L gene provided sequence variation with its strong resolving power.

Proper sequence alignment is essential for any phylogenetical analysis method (DeSalle *et al.*, 2005). Phylogenetic studies using nucleotide sequences of the gene encoding the large subunit of *rbc*L have been successfully revealed the relationships of ferns at both generic and familial levels (Hasebe *et al.*, 1995; Skog *et al*., 2004). In the present study, a new chloroplast-based barcode was developed for three *Cyathea* species consisting of the coding locus *rbc*L to test the performance of the barcode. The applicability of *rbc*L genes at lower hierarchic levels is a consequence of the antiquity of the group and the consequent accumulation of nucleotide substitutions over the several hundred million years of pteridophyte evolution. The most extant fern groups are not substantially older than angiosperms; nucleotide substitution rates are probably faster in ferns, which make *rbc*L sequences useful for phylogenetic analysis even at the inter-specific level.

The phylogenetic relationships among the studied *Cyathea* species were congruent with those from recently published studies (Korall *et al*., 2007; Janssen *et al*., 2008). Hasebe *et al.* (1994) proved the monophyletic relationship of tree ferns with 98% of

bootstrap probability in the neighbor joining method and 73% in the parsimony method. Wolf *et al.* (1999) suggested that the family Cyatheaceae was monophyletic based on *rbc*L sequence data. The results obtained in the present study have shown that *rbc*L sequences were variable enough to provide good resolution across the taxonomic diversity of tree fern taxa. The sequential based cladogram showed two clades. The clade C_1 included *C. gigantea* and *C. crinita* and C_2 showed *C. nilgirensis* only.

Su *et al.* (2004) sequenced cpDNA *atp*B-*rbc*L inter-genic spacers of individuals of a relict tree fern *Alsophila spinulosa* collected from ten populations in Southern China. Sequence length varied from 724 to 731 bp and base composition was with high A+T content between 63.17% and 63.95%. In the present study, sequence length of selected *Cyathea* species varied from 590 to 593 bp showing length polymorphism and the nucleotide base frequencies were found AT rich (54.7%) in *C. nilgirensis*.

Cyatheaceae occur from southern temperate to tropical vegetation zones and from coastal to high mountain habitats. In the Western Ghats region, tree ferns are found in dense evergreen rainforests over the entire altitudinal range, they are quite frequent in cloud and crest forests, and also occur on forest margins and open habitats. Despite their high species diversity, differentiation of their respective ecological niches is relatively weak in tree ferns. However, the species occupy a well-defined altitudinal belt. Cyatheaceae are not homogenously distributed in the Indian forests, but frequently occur in patches, i.e. individuals of one or different species appear. The sequential difference in the three species of *Cyathea* may also be attributed to the difference in the habitat ecology of these three species. In the present study, *C. nilgirensis* were collected from shaded stream banks and on the roadsides of Tirunelveli hills (Kothayar). *C. gigantea* were collected from the road sides of Nilgiris (Nadugani). *C. crinita* were collected from Palni hills (Anglade Institute of Natural History, Shenbaganur, Kodaikanal). The selected three

species showed the variation in the distribution. As per the literature, *C. nilgirensis* was distributed in Tirunelveli hills, Palni hills and Nilgiris (Manickam and Irudayaraj, 1992). But the other two studied species showed limited distribution. The results of maximum likelihood method also revealed the similarity between the high altitude tree fern species *C. gigantea* and *C. crinita* by representing in the same clade whereas *C. nilgirensis* expressed in separate clade showed more variation. This may be due to the distributional differences of the species.

Genetic differentiation among populations is primarily a function of gene flow among populations via pollen and seed dispersal in seed plants (Loveless and Hamrick, 1984). However, in ferns, gene flow is principally carried out by spore dispersal (Tryon, 1986). In general, widespread species should have higher levels of gene flow than species with restricted or isolated populations. Similar to the previous observations, the results of the present study was directly coincided by showing the commonly distributed *C. nilgirensis* in a separate clade. The species *C. gigantea* and *C. crinita* with limited distribution showed the similarity by representing monophyletic nature in maximum likelihood method. This confirmed the higher level of gene flow in *C. nilgirensis*.

Pryer *et al*. (2010) mentioned that DNA barcoding exposes a case of mistaken identity in the fern horticultural trade in advanced countries like USA. They have strongly advocated the barcoding approach as a valuable new technology available to the horticulture industry to help correct plant identification errors in the international trade. Since tree ferns, in general, are rare and endangered in India, there is a restriction to collect the species from the wild. The molecular marker based on DNA barcoding will be helpful for conservation of tree ferns and it can also be used as a taxonomical tool to characterize the tree ferns.

The antioxidant activities of plant extracts are mainly contributed by the active compounds existence. It is important to characterize the extracts by a variety of antioxidant assays. The DPPH radical scavenging activity is related to the nature of phenolics contributing to their electron transfer / hydrogen donating ability (Brand-Williams *et al.,* 1995). Presence of flavonoids generally possesses higher antioxidant activity because of double bonds existing in C-ring. Generally the radical scavenging activity of flavonoids depends on their structure and hydroxyl group arrangement. The highest radical scavenging activity is exhibited by compounds that have an ortho 3',4'-dihydroxy structure at B ring. The obtained results revealed that IC_{50} of *C. nilgirensis* (166.67 µg/ml) was lower which indicate powerful inhibitor compounds and may act as primary antioxidants that react with free radicals.

Reactive oxygen species are formed as a natural byproduct of the normal metabolism of oxygen and various target structures such as lipids, proteins and carbohydrates can be affected (Halliwell, 1997). Chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Gulcin *et al.,* 2007). The high contents of phenolic compounds present in the extracts should be able to chelate transition metals because of the high charge density of the phenoxide group generated on deprotonation (Hyder *et al.,* 2001). The chelating effect on the ferrous ions by *C. nilgirensis* was higher compared to *C. gigantea* and *C. crinita.* The findings of the present study established that the studied *Cyathea* species has the ability to chelate metal ions and the values are substantial.

Nitric oxide is a very unstable species and under aerobic condition it reacts with O_2 to produce its stable products such as nitrate and nitrite through intermediates NO_2, N_2O_4. Nitric oxide is believed to participate in the regulation of the oxidation / reduction potential of various cells and may be involved in protection or the induction of oxidative

stress within various tissues depending upon its concentration (Bland, 1995). Previous evidence suggests that diseases are related to either an inadequate or excessive production of nitric oxide (Moncada and Higgs, 1993). Nitric oxide has also implicated for inflammation, cancer and other pathological conditions (Moncada *et al*., 1991). As a natural antioxidant provider, the inhibition shown by the ethanolic extracts of studied *Cyathea* species can have a significant role in scavenging nitric oxide radical.

Hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage *in vivo*. It has been implicated as a damaging species in free radical pathology, capable of damaging almost every molecule found in living cells such as sugars, amino acids, lipids and nucleotides (Wang *et al.,* 2008). This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. It is formed by the oxidation reaction with DMSO to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent (Singh *et al.,* 2002). Removing hydroxyl radical is very important for the protection of living systems. The results obtained in the present study indicate that *C. nilgirensis* with highest hydroxyl radical scavenging activity (68.59%) at the concentration of 1000 µg/ml can act as a good scavenger of such harmful radicals.

Superoxide anions are the most common free radicals *in vivo* and the concentration of superoxide anions increases under conditions of oxidative stress (Lee *et al*., 2002). NBT assay was carried out to test whether extracts of *Cyathea* species scavenge superoxide anions. Superoxide radical is known to be a very harmful species to cellular components as a precursor of more ROS (Halliwell and Gutteridge, 1985). The percentage inhibitions obtained in the present study clearly demonstrated the efficiency of *Cyathea* extracts in scavenging the ROS.

In vitro cytotoxicity test using cell lines was performed to screen potentially toxic compounds that affect basic cellular functions. MCF 7 cell line had a cobblestone-like phenotype with strong cell-cell adhesion. However, when the cells were exposed to cytotoxic components, two distinct modes of cell death were recognized viz., apoptosis and necrosis. Apoptosis or programmed cell death involves a sequential cascade of cellular event, resulting from chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing and cell shrinkage (Boe *et al.* 1991). The observed results showed that ethanolic extracts of *Cyathea* species caused marked cell growth inhibition in the human breast carcinoma MCF 7 cell line. Most of the MCF 7 membranes blebbed during shrinkage and the apoptotic bodies were formed around cells treated with ethanolic extracts of studied *Cyathea* species. Manosroi *et al.* (2006) suggested that sample with IC_{50} value between 200 and 5000 µg/ml was considered to have moderate potential to be developed into a cancer therapeutic agent. Similar to the previous observations, ethanolic extracts of *Cyathea* species with IC_{50} value ranging from 400-806.45 µg/ml showed moderate cytotoxic effects. Literature data proved that terpenoids and flavonoids are biologically active against many human cancer cell lines (Min *et al.,* 2000; Havsteen, 2002). In the present study, qualitative phytochemical screening and HPTLC analysis confirmed the presence of terpenoids and flavonoids in the ethanolic extracts of *C. nilgirensis, C. gigantea* and *C. crinita.* Moderate cytotoxic activity shown by *Cyathea* species may be attributed mainly due to the presence of terpenoids and flavonoids in the extracts. However, a wide range of phytocompounds are capable of exhibiting non-specific cytotoxicity, extracts of *Cyathea* species with significant cytotoxic activity should be further assayed using animal models to confirm anti-tumour activity.

The evaluation of the toxic action of plant extracts is indispensable in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and

the effects of acute overdose (Padmaja *et al*., 2002). The brine shrimp lethality bioassay has been used routinely in the primary screening of the crude extracts as well as isolated compounds to assess the toxicity. It could also provide an indication of possible cytotoxic properties of the tested plant extracts. It is frequently used as a model system to measure cytotoxic effects of variety of toxic substances and plant extracts against brine shrimps nauplii (Morshed *et al*., 2011). It is also considered as a reliable indicator for the preliminary assessment of toxicity and it can be extrapolated for cell line toxicity and anti-tumour activity (Mc Laughlin *et al*., 1991). A number of novel anti-tumour and pesticidal natural products have been isolated using this bioassay (Meyer *et al.,* 1982). In the present study, ethanolic extracts of *C. nilgirensis, C. gigantea* and *C. crinita* were found to be more effective against brine shrimps with the LC_{50} values 304.73, 277.45 and 287.44 mg/ml respectively compared to chloroform, acetone and petroleum ether extracts. The results obtained from the brine shrimp lethality bioassay can be used as a guide for the isolation of cytotoxic compounds from the ethanolic extracts.

The control of mosquito larvae by chemical substances is not safe at present because of environmental imbalance and insecticide resistance by vectors which leads to deleterious effects. The major drawback with the use of chemical insecticides is that they are non-selective and could be harmful to other organisms in the environment. Hence, an alternative mosquito control method is needed (Pavela, 2008). The extracts which are obtained from plant parts have been used as conventional larvicide (Das *et al*., 2007; Rana and Rana, 2012). The observed results were also comparable with earlier reports. The fruit extract of *Croton caudatus*, flower extract of *Tiliacora acuminata* (Singha *et al*., 2011), leaf extract of *Typhonium trilobatum* (Haldar *et al*., 2011) and flower extract of *Tagetes erecta* (Nikkon *et al.,* 2011) were found to cause larval mortality against *Culex quinquefasciatus*. In the present study, the different extracts of *C. nilgirensis*, *C. gigantea*

and *C. crinita* exhibited a dose dependent activity. The results observed were similar to previous studies which have also reported dose dependency of plant extracts against mosquito larvae (Kaushik and Saini, 2008; Govindarajan, 2010). The larvae were more sensitive to ethanolic extracts of studied three *Cyathea* species when compared to other extracts. The mechanism of action exhibited by the studied *Cyathea* species may therefore possibly be due to its toxic effects on the larvae.

The present study intended to investigate the phytochemical constituents present in various extracts of *C. nilgirensis, C. gigantea* and *C. crinita* using various qualitative and quantitative analysis viz., preliminary, fluorescence, UV-Vis, FT-IR, HPTLC, HPLC and GC-MS. In addition to phytochemical analyses, protein profiling using SDS-PAGE, MALDI-TOF MS analysis for protein identification, PAGE for isoenzyme separation was carried out to know the biochemical and molecular relationships among the selected species of *Cyathea.* To select the best DNA barcode using *rbc*L gene, a series of tests with criteria such as DNA isolation, PCR amplification, primer efficiency, direct sequencing success rate and variation among *Cyathea* species was conducted. Biological properties of the selected *Cyathea* species were also studied using antioxidant, cytotoxic and larvicidal activities.

Qualitative phytochemical screening of studied *Cyathea* species showed the significant indication of various secondary metabolites viz., steroids, alkaloids, phenolic groups, cardiac glycosides, flavonoids, saponins, tannins and terpenoids. Among the tested five extracts (petroleum ether, chloroform, acetone, ethanol and aqueous), ethanolic extracts illustrated more number of phytoconstituents in all the three studied species of *Cyathea*. The extractive values showed the maximum percentage of dry weight yield in ethanolic extracts. Fluorescence analysis of plant powders of *C. nilgirensis, C. gigantea* and *C. crinita* under visible and UV light showed more or less similar characters. This may provide a basis to identify the quality and purity of the drug and also helps in identification and authentication of the plant material.

The UV-Vis spectra of *Cyathea* species showed generally a decreased absorptivity or optical density as the wavelength increased. The presence of an absorbance band at a particular wavelength may indicate the presence of a chromophore. Among the five different extracts tested, ethanolic extracts exhibited more number of peaks in all the three studied *Cyathea* species which denotes the presence of maximum number of compounds. These spectra are useful to identify the specific bioactive classes of molecules found in various extracts. FT-IR spectra exhibits the characteristic fingerprint peak features. It also revealed significant differences in peak position and absorbance intensities. The comparative FT-IR spectra of *C. nilgirensis, C. gigantea* and *C. crinita* showed that there is an apparent change in relative intensity of the peaks. This has proven to be a valuable tool for the characterization and identification of functional groups (chemical bonds) present in an unknown mixture of *Cyathea* extracts and is used as a chemotaxonomic parameter to distinguish *Cyathea* species. The UPGMA cladogram based on the qualitative phytochemical, UV-Vis and FT-IR analysis revealed the similarities and variation among the studied *Cyathea* species. The phylogenetic tree constructed expressed two clusters. The cluster C_1 includes *C. nilgirensis* and *C. gigantea* whereas cluster C_2 showed the unique presence of *C. crinita*.

Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to traditional system of medicine. The optimized fingerprint using HPTLC is not only an alternative analytical tool for authentication, but also an approach to express the various patterns of chemical ingredients distributed in studied *Cyathea* species and to preserve such "database" for further sustainable studies. HPTLC results on ethanolic extracts of *C. nilgirensis, C. gigantea* and *C. crinita* indicate the presence of alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and terpenoids with distinct R_f values. This variation in R_f values of the

phytochemicals provides an important clue in selection of appropriate solvent system for separation of pure compounds by column chromatography.

HPLC spectral data of studied *Cyathea* species generated by the detector was used to verify the identity of compounds present in the chromatogram. The most abundant peak was found in *C. nilgirensis* (99.65%) followed by *C. gigantea* (76.77%) and *C. crinita* (67.75%). The results also showed varying patterns in the HPLC chromatogram which suggests that there might be differences in the process of manufacturing and therefore a proper scientific validation is needed for quality control purposes. These identification tests are used to confirm the presence of the active constituents and potential adulterant in ayurvedic drugs.

GC-MS analysis was carried out to identify the potent chemical constituents present in the studied *Cyathea* species. It is an efficient way to analyze metabolic fingerprinting of phytomedicine and to evaluate the global chemical difference in plants. It is also used as a valuable tool for reliable identification of phytocompounds which include Methyloctadecyl dichlorosilane and 2-Methylbutane-1,4-diol,3-(1-ethoxyethoxy) in *C. nilgirensis*, 2-Methylbutane-1,4-diol, 3-(1-ethoxyethoxy) and 2-Hydroxy-5-methyl benzaldehyde in *C. gigantea* and 2-Hydroxy-5-methyl benzaldehyde in *C. crinita*. PASS was used to predict the biological activities for the predicted compounds of studied *Cyathea* species on the basis of their 2D structural formulas in a very rapid manner. PASS analysis predicted the following biological properties viz., immunosuppressant, anti-inflammatory, antieczematic, antiviral, erythropoiesis stimulant, antinociceptive, anti-infective, hepatoprotectant, antitreponemal, antifibrinolytic, antiprotozoal, astringent, antihematotoxic, antiparasitic, antidote, antihypoxic, cardioprotectant, antiulcerative, antimyopathies, antirickettsial, antiseborrheic, antipruritic, anticonvulsant, antithrombotic, antihypertensive and antispirochetal activities from the identified compounds of *Cyathea*.

The biological activity spectrum is the "intrinsic" property of a compound which depends on its structure and physico-chemical characteristics.

Spectroscopic studies gives direct preliminary information on the fingerprint of functional groups present in the studied *Cyathea* species while chromatography gives qualitative and quantitative information related to individual molecules which characterize the *Cyathea* species or specific extract. The results of the present study revealed the spectroscopic and chromatographic features of three studied *Cyathea* species. These chemical profiles of studied *Cyathea* species may be used either in metabolomics research or in laboratories for food and phytopharmaceuticals in order to evaluate their quality. Secondary metabolite profiles of the studied *Cyathea* species may be used as markers for biological or geographical authenticity, as well for adulteration and traceability studies, representing the metabolic profile or fingerprint.

SDS-PAGE was carried out to study the inter-specific relationship among the *Cyathea* species based on protein profile. A total of 22 bands with various R_f values and molecular weight were observed in the SDS-PAGE gel system of studied *Cyathea* species. The protein bands were appeared with varied R_f values ranged from 0.14 to 0.94. The molecular weight of proteins separated from the studied *Cyathea* species ranged from 6.16 to 89.13 kDa. The distinct protein patterns with respective molecular weight could be used to differentiate the studied *Cyathea* species which represent the “protein finger print” of that particular species. The present study results showed that SDS-PAGE derived protein profiles may be used as a biochemical tool for identification of genotypes.

There is still a big gap in linking MALDI-TOF MS peptide / protein profiles with proteomic identification of individual biomarker molecules which need to be expanded. MALDI based identification and classification of *Cyathea* species utilize peptide / protein profiles containing characteristic biomarker peaks in the m/z region of 0 - 1,00,000 Da

range. The unique ionic spectral peaks obtained in the present study can act as a spectroscopic tool and will definitely involve a continuous updating of current commercial databases together with the instrumentation as well as building up new databases for specialized research purposes. UPGMA cladogram based on SDS-PAGE protein profiling and MALDI-TOF MS of studied three *Cyathea* species showed the similarities and variation with two clades. Clade 1 displayed the unique presence of *C. nilgirensis* and clade 2 was shared between *C. gigantea* and *C. crinita.*

Isozymic variation studies using isoesterase, isoperoxidase and acid phosphatase have been employed to reveal the diversity existing at molecular level among morphologically distinct *Cyathea* species. The peroxidase and acid phosphatase gel system showed 100% divergence whereas esterase gel system demonstrated the similarity and variation among the studied *Cyathea* species. The distinct banding profiles revealed the genetic differentiation among the studied *Cyathea* species. The active regions occupied by a particular isozyme in the form of bands are the representatives of the expression of a particular gene locus coding for that isozyme. In certain regions, more than one distinct bands are resolved which represents allelic isozymes, coded by different alleles of the same gene at a locus and thus occupy that particular zone on the gel system.

DNA barcoding using *rbc*L gene was employed to study the inter-specific variation among the three *Cyathea* species from Western Ghats, South India. The PCR amplification efficiency was good for the studied *Cyathea* species. The amplicons were sequenced and sequence length was ranged from 590-593 bp. The *rbc*L gene provided sequence variation with its strong resolving power. The obtained sequences were aligned, annotated and submitted in GenBank (*C. nilgirensis* - KF 924259, *C. gigantea* - KF 924260 and *C. crinita* - KF 924261). Multiple sequence alignment performed using MULTALIN tool distinguished the inter-specific variation among the three *Cyathea*

species. The sequential difference in the three species of *Cyathea* may also be attributed to the difference in the habitat ecology of studied *Cyathea* species. The studied three species also showed the variation in the distribution. *C. nilgirensis* was distributed in Tirunelveli hills, Palni hills and Nilgiris. But the other two studied species showed limited distribution compared to *C. nilgirensis*. The results of maximum likelihood method also revealed the similarity between the high altitude tree fern species *C. gigantea* and *C. crinita* by representing in the monophyletic clade whereas *C. nilgirensis* expressed in paraphyletic clade showed more variation. This may be due to the distributional differences of the species. Since tree ferns, in general, are rare and endangered in India, there is a restriction to collect the species from the wild. The molecular marker based on DNA barcoding will be helpful for conservation of tree ferns and it can also be used as a taxonomical tool to characterize the tree ferns.

Damage to cells caused by free radicals played an important role in the aging process and disease progression. Antioxidants are first line of defense against free radical damage. They are capable of stabilizing or deactivating free radicals before they attack cells and are absolutely critical for maintaining optimal cellular and systemic health and wellbeing. The reduction ability shown by the active ethanolic extracts of studied *Cyathea* species confirmed that the extracts defend against harmful free radicals and can be considered as potential sources of natural antioxidants for food and nutraceutical products.

In vitro cytotoxicity test using MCF 7 cell line was performed to screen potentially toxic compounds that affect basic cellular functions. The observed results showed that ethanolic extracts of *Cyathea* species caused marked cell growth inhibition in the human breast carcinoma cell line. Most of the MCF 7 membranes blebbed during shrinkage and the apoptotic bodies were formed around cells treated with ethanolic extracts of *Cyathea*

species. The ethanolic extracts with significant cytotoxic potential should be further assayed using various cell lines to confirm anti-tumour activity.

The brine shrimp lethality bioassay has been used routinely in the primary screening of crude extracts as well as isolated compounds to assess the toxicity. Ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* were found to be more potent against *A. salina* with the LC_{50} values of 304.73, 277.45 and 287.44 mg/ml respectively. The obtained results can be used as a guide for the isolation of cytotoxic compounds from the ethanolic extracts of studied *Cyathea* species. The control of mosquito larvae by chemical substances is not safe at present because of environmental imbalance and insecticide resistance by vectors which leads to deleterious effects. In the present study, *C. quinquefasciatus* was more sensitive to ethanolic extracts of studied three *Cyathea* species when compared to other extracts. The LC_{50} values of different extracts ranged from 320.72 to 373.99 mg/ml. The results of larvicidal activity suggest that the ethanolic extracts of *Cyathea* species may be used as conventional larvicide in the near future.

With the recent advances in molecular biotechnology and analytical approaches to phylogenetic research, systematists have made unprecedented progress towards reconstructing 'tree of life'. The results of the present study suggested that *C. nilgirensis*, *C. gigantea* and *C. crinita* may have rich sources of phytoconstituents and produced pharmacognostical and phytochemical markers to identify the presence of adulterants in ayurvedic drugs. However, isolation of individual phytochemical constituents may proceed to find a novel drug formulation. Therefore, using newer analytical techniques as markers can be generated for the use of common man to evaluate the quality of herbal drug incorporated in pharmacopoeias. Quantitative analyses of these phytochemicals may also be done to guide the researchers on which particular bioactive class of compounds may be subjected to subsequent target isolation.

The results on molecular characterization of studied *Cyathea* species inferred the phylogenetic relationship among them. UPGMA cladogram showed monophyletic and paraphyletic expression which was a reflection of genetic as well as geographical variation. The results also revealed distinctive characteristics which may be helpful in resolving disputes of taxonomic identities, relations and authentication of the species in the pharmaceutical industries. It can also be concluded from the present study that synergistic application of phytochemical, biochemical and molecular markers can be used as a prominent tool to characterize the species specific taxonomic variation among the tree ferns.

Biological properties of *Cyathea* species concluded that ethanolic extracts find use as broad spectrum antioxidant, cytotoxic and larvicidal agent after extensive investigation. This is the first step towards understanding the nature of bioactive principles in the studied *Cyathea* species and this type of study will be helpful for further research into the pharmacological activites which may add new knowledge to the information in the traditional medical systems. The search for novel bioactive compounds in plants is a promising endeavour. 80% of the world's population relies on plants for their medical care and with a booming world population, there is no doubt that the studied *Cyathea* species are effective and need to be used more efficiently in future.

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