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 O₂ 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 (cocaïne), 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 infrared 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 C₁ includes *C. nilgirensis* and *C. gigantea* whereas cluster C₂ 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 therapeutics. 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
phytotherapeutics. 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, antiseborrhic, 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, Araiosegia 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), *Trichnomones* (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 *rbcL* 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 *rbcL* 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 *rbcL* to test the performance of the barcode. The applicability of *rbcL* 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 *rbcL* sequences useful for phylogenetic analysis even at the interspecific 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 rbcL sequence data. The results obtained in the present study have shown that rbcL 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 C1 included C. gigantea and C. crinita and C2 showed C. nilgirensis only.

Su et al. (2004) sequenced cpDNA atpB-rbcL 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\textsubscript{50} of \textit{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 \textit{C. nilgirensis} was higher compared to \textit{C. gigantea} and \textit{C. crinita}. The findings of the present study established that the studied \textit{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\textsubscript{2} to produce its stable products such as nitrate and nitrite through intermediates NO\textsubscript{2}, N\textsubscript{2}O\textsubscript{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.