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 \textit{rbc}L and \textit{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. \textit{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 \textit{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 _rbcL_ 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_.

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