PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES ON SELECTED SPECIES OF *SELAGINELLA* FROM SOUTH INDIA

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By

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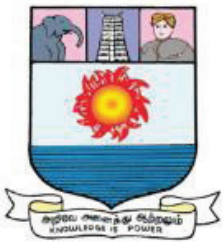

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DECLARATION

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Date:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS USED

%	: Percentage
°C	: degree Celsius
µg	: microgram
µl	: microlitre
µm	: micrometer
µM/ µmol	: micro Molar
AA	: Ascorbic Acid
A549	: adenocarcinomic human alveolar basal epithelial cells.
ABS	: Absorbance
ABTS	: 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical
ALP	: Alkaline Phosphatase
ALT	: Alanine aminotransferase
AST	: Aspartate aminotransferase
ATP	: Adenosine triphosphate
B16F-10	: Mouse Melanoma cell line
BC	: Before Christ
Bel-7402	: Apoptosis of human hepatocellular carcinoma cells
b.w	: body weight
CaCO-2	: Heterogenous human epithelial colorectal adenocarcinoma cells
CAT	: Catalase Activity Assay
cm	: centimeter
Conc.	: Concentration
Da	: Dalton
dl	: deci liter
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribo Nucleic Acid
DPPH	: 1,1-diphenyl-2-picrylhydrazyl
EC_{50}	: Half Maximal Effective Concentration

EDTA	: Ethylene Diamine Tetra Acetic acid
equi	: equivalent
eV	: electron Volt
FBS	: Fetal Bovine Serum
Fig.	: Figure
FRAP	: Ferric Reducing Ability of Plasma
FT-IR	: Fourier Transform - Infra Red
g	: gram
GAE	: Gallic Acid Equivalent
GC	: Gas Chromatography
GC-MS	: Gas Chromatography - Mass Spectrometry
GSH	: Glutathione and Sulfur Aminoacid
h	: hours
HEK293	: Human Embryonic Kidney 293 cells
HepG2	: liver hepatocellular cells
HIV	: Human Immunodeficiency Virus
HL-60	: Human promyelocytic leukemia cells
HPLC	: High Performance Liquid Chromatography
HPTLC	: High Performance Thin Layer Chromatography
HT-29	: Human colon adenocarcinoma grade II cell line
ICH	: International Conference on Harmonisation
IC_{50}	: half maximal Inhibitory Concentration
IR	: Infra Red
IRS-1	: Insulin Receptor Substrate-1
kg	: kilogram
K562	: First human immortalized myelogenous leukemia line
L	: Litre
LC_{50}	: half maximal Lethal Concentration
LPO	: Lipid Hyperoxide
M	: Molarity
MCF 7	: Michigan Cancer Foundation 7

MDA-MB-231	: Human Caucasian breast adenocarcinoma
mg	: milligram
min	: minutes
ml	: milliliter
mm	: millimetre
mM	: millimolar
MMP-2	: Matrix Metallo Proteinase-2
MS	: Mass Spectrometry
MTT	: (3-[4, 5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)
ng	: nanogram
NIST	: National Institute of Standard Technology
nm	: nanometre
N	: normality
NF-kappaB	: nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	: Nuclear Magnetic Resonance
NTSys	: Numerical Taxonomy System
OD	: Optical Density
OGTT	: Oral Glucose Tolerance Test
OVCR-3	: Human Ovarian Adeno Carcinoma cells
PASS	: Prediction of Activity Spectra for Substances
PCR	: Polymerase Chain Reaction
Pet. ether	: Petroleum ether
pH	: power of Hydrogen ion concentration
ppm	: parts per million
PPAR- γ	: Peroxisome proliferator-activated receptor gamma
QE	: Quercetin Equivalents
R_f	: Retardation factor
RH	: Relative Humidity
rpm	: revolutions per minute
RSD	: relative standard deviation
RT	: Retention Time

RT-PCR	: Reverse Transcription Polymerase Chain Reaction
SD	: Standard Deviation
sec	: seconds
SEM	: Structural equation modeling
SGOT	: Serum Glutamic Oxaloacetic Transaminase
SGPT	: Serum Glutamic Pyruvic Transaminase
SOD	: Sphincter of Oddi Dysfunction
SRB	: Sulphorhodamine-B
STZ	: Streptozotocin
TBARS	: Thio Barbituric Acid Reactive Substances
TB	: tuberculosis
TEAC	: Trolox Equivalent Antioxidant Capacity
TE	: Trolox Equivalents
TLC	: Thin Layer Chromatography
TPTZ	: 2,4,6-Tripyridyl-s-Triazine
UPGMA	: Unweighted Pair Group Method with Arithmetic mean
USA	: United States of America
UV	: Ultra Violet
UV-Vis	: Ultra Violet - Visible
V	: Volt
v/l	: volume/liter
v/v	: volume / volume
w/v	: weight / volume
WHO	: World Health Organization
XCH	: St. Xavier's College Herbarium
λ_{max}	: Wavelength of maximum absorption

1. INTRODUCTION

The ancient searched for drugs from different sources of plants to cure various diseases. The beginning of the medicinal plants usage was natural, as the case with animals. By regular practices and applications the tribals and traditional healers used specific medicinal plants for treatment of certain diseases. Approximately 5000 years old written evidence on medicinal plants usage for preparation of drugs indicated 12 recipes for drug preparation referring to over 250 various plants viz., poppy, henbane and mandrake (Petrovska, 2012). Plants are believed to be a main source of drugs in conventional and alternative systems of medicine. About 80% people of the world, particularly in the rural areas of developing countries, continue using traditional resources in healthcare.

Since time immemorial the medicinal plants are exercised as medicine and day by day their utility value has increased (Chanchal and Balasubramaniam, 2011). Medicinal plants are not only act as therapeutic agents but also serve as a big source for various chemical constituents which could serve as new leads for modern drug design (Vijyalakshmi *et al*., 2012). Gupta *et al.* (2005) enumerated more than 2000 species with medicinal properties which explain the Indian heritage. Among these only very few plants were exploited for their potential medicinal value and isolation of active principles (Sandhu and Heinrich, 2005).

Pteridophytes originated in the Silurian period (above 350 million years ago) and were abundant during the carboniferous period. Pteridophytes have been found largely in high altitude region (hill stations), moist, cool and shady places. The medicinal value of pteridophytes have been known to man for more than 2000 years. The Greek botanist Theophrastus (Ca. 372- 287

B.C.) pointed out the medicinal values of pteridophytes. Studies on food and medicinal values of pteridophytes were conducted by Nayar (1957), Hodge (1973) and Dixit (1974, 1975) clearly explained the values of pteridophytes. Recently, Ghosh *et al.* (2004) noted the medicinal and economic importance of pteridophytes viz., biofertilizers, insect repellents, food and fodder indicators.

Ferns and fern allies have little economic significance to mankind as compared to other groups, especially angiosperms (Sharpe, 2011). Due to the population growth and the requirement of the human beings has increased day by day. The utilization of plant resources has also magnified. Bad practices in the collection and utilization of plant sources have made plants rarer, more vulnerable and endangered (Chandra *et al*., 2008). The Western Ghats is one of the hotspots of the world and also one of the significant geographical regions. Indian landmass, one of the richest nations in terms of biological diversity and is counted among the 18 identified mega biodiversity countries of the world (Fraser-Jenkins, 2012). Among which the Himalayas, the Western Ghats and the Eastern Ghats are the main centres (Chandra *et al.,* 2008). In India, nearly 1300 species of ferns and fern allies distributed in different biogeographical regions (Chandra, 2000; Dixit, 2000).

Plants synthesize various secondary metabolites as natural defense mechansim to protect from various stresses and natural enemies (Bennett and Wallsgrove, 2006). Since ferns and fern allies have a long evolutionary history, they have adapted with various climatic and environmental conditions than the other primitive vascular plants (Wallace *et al*., 1991). Therefore, ferns are expected to possess various useful secondary metabolites to adapt and survive with various stress conditions. Ferns were reported to have many useful phytochemicals (secondary metabolites) such as flavonoids, steroids, alkaloids, phenolics, triterpenoids, varieties

of amino acids and fatty acids (Zeng-fu *et al*., 2008). The components responsible for the bioactivities of the ferns are mostly found to be phenolics, flavonoids, alkaloids and terpenoids (Ho *et al*., 2010). Ferns possess various biological activities, among these some of the activities are explored by various pteridologists such as antioxidant (Carcia *et al*., 2006; Chen *et al*., 2007; Ding *et al*., 2008; Shin and Lee 2010), antimicrobial (Maruzzella 1961; Banerjee and Sen 1980, Parihar and Bohra 2002; Singh *et al*., 2008a, b), antiviral (Mc Cutcheon *et al*., 1995), antidiabetic (Zheng *et al*., 2011), hepatoprotectivity (Balne *et al.,* 2013), anti-inflammatory (Punzon *et al*., 2003), anticancer (Jing *et al*., 2010) and anti-HIV (Mizushina *et al*., 1998).

The modern pharmacognosist adapt various analytical tools for the quality assessment of the crude drugs, which includes fluorescence, spectroscopic (UV-Vis, FT-IR and MS) and chromatographic (TLC, HPLC, HPTLC and GC-MS) analysis. The chromatographic techniques are accepted as a strategy for identification and evaluation of the quality of plant medicines (Farnsworth *et al*., 1985). In chemotaxonomy they are used to distinguish the medicinal sources from its adulterants, standardization of plant products and used as taxonomic tool to classify the medicinal plants. TLC and HPTLC are efficient tools for the phytochemical evaluation and widely accepted technique for its high accuracy, precision and reproducibility of results. It has many advantages because of high sample throughput at low operating cost, easy sample preparation, short analysis time and analytical assurance (Liebler *et al.,* 1996).

TLC enables reliable separation and analysis of compounds from a wide variety of classes in many types of biological samples (Sherma and Fried, 2005). HPTLC is one of the powerful analytical tools that can be utilized for linking the chemical constituents of the plant with high proficiency which in turn provides unique profiling of that particular plant (Johnson *et al*., 2012). HPLC offers advantages in identifying the isolated compounds (Zhang *et al*., 2010)

and in the quantitative determination (Douat *et al*., 2011). HPLC is highly sensitive method for detection, identification and quantification of any chemical in a particular sample using UV and visible absorbance (Hanachi and Golkho, 2009). GC-MS is a powerful technique used for many applications which has very high sensitivity and specificity. The combination of a principle separation technique (GC) with the best identification technique (MS) made GC-MS ideal for qualitative and quantitative analysis for volatile and semi-volatile compounds (Karthishwaran *et al*., 2012).

Spectroscopic (UV-Vis and FT-IR) methods together or separately can be used in this sense as well as predictable methods for detection of biomolecular composition (Socaciu *et al.,* 2005; Schultz *et al.,* 2007; Ibrahim *et al.,* 2009). FT-IR is one of the most widely used methods to identify the functional groups and pave a way to elucidate the compounds structures. It has been used as a requisite method to identify medicines in Pharmacopoeia of many countries (Liu *et al.,* 2006).

Free radicals play an important role in aerobic life and metabolism because they are the source of any biochemical reaction. To continue the normal natural life, a forceful balance between free radicals generation and antioxidants scavenging capacity is required (Shirwaikar *et al*., 2006). Due to the free radicals, a majority of the disorders are linked to oxidative stress (Gutteridgde, 1995; Celiktas *et al*., 2007). Antioxidants comprises of minerals, vitamins, enzymes, phytochemicals like phenolics and flavonoids. Antioxidants in our diet help to prevent and repair the damage caused by free radical attack. The best-known antioxidants are ascorbic acid, Vitamin-C and β-carotene found in fruits, vegetables, cereals and vegetable oils. Antioxidants like lycopene and other carotenoids, phenolic compounds, ascorbic acid and Vitamin-E lower the incidence of a number of diseases (Palmer and Pavlson, 1997). There is an

increased quest to obtain natural antioxidants with broad spectrum actions (Aquil *et al*., 2006). To reveal the antioxidant properties of ferns, a number of pteridologists and pharmacologists have carried out work in different ferns viz., *Selaginella tamariscina* (Miao *et al*., 1996), *Dryopteris crassirhizoma* (Lee *et al*., 2003), *Selaginella involvens* (Gayathri *et al*., 2005), *Pteris multifida* (Wang *et al*., 2006), *Cyathea phalerata* (Hort *et al.,* 2008), *Microgramma vacciniifolia* (Peres *et al.,* 2009), *Selaginella doederleinii* (Li *et al*., 2010) and fifteen ferns from Malaysia (Lai and Lim, 2011). In addition, *Pteris scabristipes, Microlepia rhomboidea*, *Diplazium esculentum*, *Asplenium khasianum, Microlepia hallbergii* and *Adiantum edgeworthii* (Gupta *et al*., 2014), *Asplenium aethiopicum* (Johnson *et al*., 2014), *Pteris biaurita* (Jaishee and Chakraborty, 2014), *Drynaria quercifolia* (Jinu *et al*., 2014), *Cyathea nilgirensis, Cyathea gigantea* and *Cyathea crinita* (Janakiraman, 2015) were also examined.

Mosquitoes are the vectors for the dreadful diseases of mankind. Of all the insects that transmit diseases, mosquitoes represent the greatest menace. WHO has declared the mosquito as "public enemy number one" because mosquitoes are responsible for the transmission of various terrible disease causing pathogens (WHO, 1996). Mosquitoes are the principal vectors of malaria and other vector borne diseases that contribute to major disease burden in India. Disease transmission can be interrupted by controlling the vectors using various methods. However, the extensive and unbalanced use of chemical insecticides has created problems like enhancing resistance of mosquito population to synthetic insecticides (WHO, 1992; Kumari, 1998). Plant derived products have been used traditionally by human communities in many parts of the world against mosquitoes and other species of insects. Phytochemicals derived from plant sources can act as larvicides, insect growth regulators, repellents, ovipositional attractants and have deterrent activities (Babu and Murugan, 1998). Larvicidal activity was carried out only in limited number

of ferns viz., *Amphineuron terminans*, *Chiristella parasitica*, *Cyclosorus interruptus*, *Sphaerostephanos arbuscula*, *Sphaerostephanos subtruncatus* and *Sphaerostephanos unitus* (Paulraj, 2007), *Adiantum* (Sood and Sharma, 2010), *Asplenium aethiopicum* (Johnson *et al.,* 2014) and *Cyathea* species (Janakiraman, 2015).

Brine shrimp bioassay is considered as a useful tool to detect the fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity (McLaughlin *et al*., 1991; Martinez *et al*., 1998; Barahona and Sanchez, 1999; Pelka *et al*., 2000). In cancer therapy, cell cycle arrest and apoptosis induction are important strategies (Daucas *et al*., 2006). Apoptosis or programmed cell death is a multi-step process that is important to eliminate damaged or abnormal cells (Choi and Kim, 2009). Chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, but their ultimate ability to induce apoptosis may represent unifying concept for the mechanism of chemoprevention (Taraphdar *et al*., 2001). Approximately one third of the women with breast cancer developed metastasis and ultimately died of the disease. MCF-7 cell has become a prominent model system for the study of breast cancer as it relates to the susceptibility of the cells to apoptosis. Further, it has become increasingly important in the prevention or treatment of a number of major solid tumors, particularly metastatic and drug-resistant breast cancer (Spencer *et al*., 1994). A number of reports are available on cytotoxicity of different ferns and fern allies viz., *Selaginella willdenowii* (Silva *et al*., 1995), *Selaginella involvens, S. delicatula* and *S. wightii* (Gayathri *et al.,* 2005), *Selaginella bryopteris* (Sah *et al*., 2005), *Selaginella tamariscina* (Yang *et al*., 2007), *Artocarpus heterophyllus* (Patel and Patel, 2011), *Selaginella plana* (SriHandayani *et al*., 2013) and *Cyathea* species (Janakiraman, 2015).

Diabetes is one of the most common metabolic disorders and has become an epidemic of 21st century of both genders in developed and developing countries (Rotimi *et al*., 2011). The

overall prevalence of diabetes (type I & II) worldwide was found 2.8% in the year 2000 and expected to increase up to 4.4% by the year 2030 (Wild *et al*., 2004). Hyperglycemia is an important factor in the development and progression of the complications of diabetes mellitus (Luzi, 1998). The conventional treatments of diabetes include insulin injections, oral hypoglycemic drugs, exercise, diet or combination of these (Rang *et al*., 2003). The OGTT is a test of immense value which facilitates the diagnosis of diabetes. Oral hypoglycemic drugs, besides having effective and immediate therapeutic action, have adverse side effects and also expensive one (Sinha *et al*., 2010). Ethno-botanical information indicates that more than 800 medicinal herbs and plants have been used as traditional remedies for the treatment of diabetes (Shukia *et al*., 2000). In ferns, only very few studies were carried out such as *Hemionitis arifolia* (Nair *et al*., 2006), *Selaginella tamariscina* (Zheng *et al*., 2011), *Adiantum philippense* (Paul *et al*., 2012), *Christella dentata* (Tanzin *et al.,* 2013), *Angiopteris evecta* (Rahmatullah *et al.,* 2014), *Diplazium esculentum* (Chai *et al*., 2015) and *Selaginella bryopteris* (Singh *et al*., 2014).

Liver plays a major role in detoxification and excretion of many endogenous and exogenous compounds. Injury to liver or impairment of its functions may lead to many implications on one's health (Handa and Kapoor, 2002). Liver damage is always associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels (Ramachandra *et al.,* 2007). Hepatic disorders have been recognized worldwide as an important cause of mortality in man and animals. Herbal medicines are known to play an important role in liver disorders and many traditional practioners have claimed that numerous medicinal plants can be extensively used for the alleviation of different types of liver disorders (Dash *et al.,* 2007). Previous reports on hepatoprotective activities were carried out in different ferns namely viz., *Selaginella doederleinii* and *Pteris ensiformis* (Hu *et al.,* 2004), *Selaginella labordei* (Chen *et*

al., 2005), *Lygodium flexuosum* (Wills and Asha, 2006), *Cyathea gigantea* (Kiran *et al*., 2012) and *Marsilea minuta* (Balne *et al.,* 2013).

Selaginella, a cosmopolitan genus belongs to the family *Selaginellaceae.* It comprises about 700 species distributed in subtropical and tropical areas (Tryon and Tryon 1982; Jermy 1990; Kramer and Green 1990). They grow at various climate and soil types. Some species were present in very extreme climate such as cool alpine or Arctic circle and in dry desert (Tryon and Tryon 1982; Zoller 2005), but they show highest diversity in tropical region. In South India, only 18 species of *Selaginella* has been reported. Sah (2008) reported the fern ally *Selaginella* in the famous ancient literature 'Ramayana'. *Selaginella* has sweet, spicy, bitter, cold properties and is associated with the liver, lung and stomach meridians. The main function of Selaginella is to clear heat, lessen toxicity and exhaust damp heat. *Selaginella* is used to cure coughs, sore throats, jaundice, cancer and cirrhosis of liver (Tinde van Andel *et al*., 2007). *Selaginella* is used as medicine, food, ornaments and handicrafts in Southeast Asia (Winter and Jansen, 2003). Chikmawati *et al.* (2008) reported that *Selaginella* contains a variety of secondary metabolites such as alkaloids, phenolics (flavonoids, tannins, saponins) and terpenoids (triterpene, steroid). *Selaginella* possess a large number of bioactive compounds, the most important is biflavonoid (Silva *et al*, 1995; Lin *et al*, 1999). Biflavonoid of *Selaginella* possess various bioactivities such as antioxidant, anti-inflammatory, anti-cancer, antimicrobial (anti-viral, anti-bacterial, anti-fungal, anti-protozoan), neuroprotective, vasorelaxant, anti UV-irradiation, anti-spasmodic, anti-allergy, anti-haemorrhagic and anti-nociceptive (Setyawan and Darusman, 2008). With reference to the biopotency of Indian *Selaginella* species only very few reports are available. Gayathri *et al.* (2005) studied the immunomodulatory, antioxidant and cytotoxicity of *Selaginella involvens,*

S. delicatula and *S. wightii*. Antibacterial efficacy of *Selaginella inaequalifolia* against poultry and human pathogens were studied by Duraiswamy *et al*. (2010) and Irudayaraj *et al*. (2010).

To utilize the available resources in a valuable way, the present investigation was aimed to reveal the phytochemical composition and their biopotency of *Selaginella intermedia* (Bl.) Spring*, Selaginella inaequalifolia* (Hook. & Grev.) Spring and *Selaginella tenera* (Hook. & Grev.) Spring. *S. intermedia* decoction is given for stomach-ache and is applied as poultice over the whole body for asthma (Winter and Jansen, 2003). It is also used to cure wound, postpartum and menstrual disorder (Nasution, 1993; Harada *et al*., 2002; Setyawan and Darusman, 2008). With this background, the present investigation was aimed to reveal the phytochemical constituents of *S. intermedia, S. inaequalifolia* and *S. tenera* using histochemical, preliminary phytochemical, fluorescence, chromatographic and spectroscopic analysis. In addition, the present investigation was intended to reveal the antioxidant, cytotoxic, larvicidal, anti-diabetic and hepatoprotective potentials of *S. intermedia, S. inaequalifolia* and *S. tenera*.

The specific objectives of the present study are as follows:

- To localize the secondary metabolites of selected *Selaginella* species using histochemical analysis
- To reveal the qualitative phytochemical profile of the selected *Selaginella* species using preliminary phytochemical, fluorescence, UV-Vis, FT-IR, TLC, HPTLC, HPLC and GC-MS analysis
- To evaluate the antioxidant properties of selected *Selaginella* species using DPPH, $ABTS^{+}$, FRAP, Metal chelating and Phosphomolybdenum assays
- To determine the cytotoxicity of selected *Selaginella* species using brine shrimp lethal bioassay and MCF 7 cell line

- To determine the antidiabetic potentials of selected *Selaginella* species using oral glucose test methods
- To evaluate the hepatoprotective properties of selected *Selaginella* species using SGOT, SGPT, Bilirubin and ALP test
- To know the larvicidal potentials of selected *Selaginella* species using filarial vector *Culex quinquefasciatus*

2. REVIEW OF LITERATURE

Pteridophytes including ferns and fern-allies are non-flowering, vascular and spore-bearing plants. They form a conspicuous element of the earth's vegetation and are important from evolutionary point of view as they show the evolution of vascular system and reflect the emergence of seed habit in the plants. About 250 million years ago, they formed the dominant part of earth's vegetation. But in present day flora, they have been largely replaced by the seed bearing plants. They grow luxuriantly in moist tropical and temperate forests and their occurrence in different eco-geographically threatened regions from sea level to the highest mountains are of much interest (Dixit, 2000).

India is one among eighteen megadiverse countries with significant biodiversity. The major part of India lies within the Indo-Malaya and Palearctic ecozone. With 2.5 percent of the world's land area, India accounts for 7.8% of the recorded species of the world including 45,000 species of plants and more than 1300 pteridophytes (Chandra *et al*., 2008; MoEF, 2009). Pteridophytes are distributed mostly in the high altitude mountainous regions such as Himalayas, Western Ghats and Eastern Ghats. The Indian Western Ghats harbours about 320 species of ferns and fern allies are one of the richest region in Pteridophyte diversity (Ramachandra *et al*., 2010). Ferns show various economic values towards food, fodder indicators, biofertilizers and insect repellents (Ghosh *et al.,* 2004). Ferns are used as medicines to cure diseases in various countries. In China alone, 401 kinds of pteridophytic medicines have been used for various ailments (Luo 1998). Sekar *et al.* (2011) reported the medicinal uses of 30 pteridophytes of Tamil Nadu. They were used to treat various ailments viz., wound healing, body sickness, diarrhoea, skin problems,

body pain, knee problem, cough, cold, fever, asthma, kidney problem, tonic, chronic disorders, several aches, hair growth, stomach problems, ulcer, sore throat, leprosy, ophthalmic, typhoid, urinary bladder and rheumatism.

Khare and Kumar (2007) studied ethnobotany of five pteridophytes viz., *Adiantum philippense, Diplazium esculentum, Helminthostachys zeylanica*, *Lygodium flexuosum* and *Ophiglossum reticulatum* used by the Tharu tribe of Dudhwa National Park, Lakhimpur - Kheri (U.P). The information collected by Singh *et al*. (2001) reported that 14 common Pteridophytes were used by the local people of Manipur in various forms. Kirn and Kapathi (2001) observed ethnomedical uses of 19 pteridophytes of Jammu and Kashmir.

Histochemistry

Histochemistry is the branch of histology that deals with the identification of chemical components of cell and tissues (Krishnamurthy, 1998). Histochemical methods have been developed for qualitative and quantitative analysis of all cellular components including proteins, carbohydrates, lipids, nucleic acids and the range of ionic elements occurring in cell solutions (Gahan, 1984; Conn, 1989 and Kiernan, 1989).

Lignin is an aromatic heteropolymer that is deposited most abundantly in the secondary cell walls of vascular plants. It provides structural rigidity to the plant body while enabling individual tracheary elements to withstand the tension generated during water transport; it also serves as a defensive role against herbivores and pathogens. The lignin chemical structure of some species of *Selaginella* (*Selaginella involvens, S. tamariscina*, *S. remotifolia, S. biformis, S. pennata, S. chrysorrhizos* and unidentified *Selaginella* species) are rich in syringyl nuclei. A considerable portion of syringyl nuclei of *Selaginella* lignin formed syringylglycerol-*β*-aryl ether inter monomer linkages. The major diastereomer of arylglycerol-*β*-aryl ether inter monomer

linkages of *Selaginella* lignins was the *erythro*-form exhibiting angiosperm lignin characteristics. Lignin of *Selaginella* species is typical guaiacyl-syringyl type similar to angiosperm lignin (Jin, 2007). Weng *et al*. (2008) found syringyl lignin derivative in *S. moellendorffii*. The lignin of *S. moellendorffii* possessed a high content of syringyl subunits, with a mole percentage of >70%.

Kathiresan *et al*. (2011) localized the lignin, starch grains, saponins, phenolic compounds, mucilage and suberin in the leaves, stems and roots of *Viburnum lanata, Viburnum opulus* and *Viburnum rhytidophyllum*. Regina (2014) studied the anatomical features of *Couroupita guianensis* and identified the occurrence of alkaloids, lipids, proteins, saponins and starch grains in the xylem vessels, ground tissues and also in the epidermal layers of *C. guianensis*.

Johnson *et al*. (2014) carried out histochemical analysis on *Pseudophegopteris pyrrhorhachis, Macrothelypteris torresiana, Trigonospora ciliata, Cyclosorus interruptus, Amphineuron terminans, Sphaerostephanos arbuscula, Christella parasitica, Christella dentata, Asplenium cheilosorum, Diplazium muricatum, Diplazium travancoricum, Diplazium brachylobum, Tectaria paradoxa, Arachniodes tripinnata, Arachniodes amabilis, Arachniodes aristata, Dryopteris sparsa, Bolbitis appendiculata, Blechnum orientale, Leptochilus decurrens* and *Pyrrosia porosa.* They identified the presence of lipids, polyphenols, lignin and tannins in the studied Pteridophytes.

Irudayaraj *et al*. (2014) observed the presence of lipids, polyphenols, lignin and tannins in various medicinally important primitive ferns and fern allies viz., *Lycopodiella cernua, Selaginella involvens, Selaginella inaequalifolia, Selaginella tenera, Angiopteris evecta, Marattia fraxinea, Lygodium microphyllum, Pteris argyraea, Pteris confusa, Dryopteris concolor, Cheilanthes viridis, Pellaea boivini, Heminonitis arifolia, Pityrogramma calomelanos,*

Adiantum raddianum, Pteridium aquilinum, Histiopteris incisa, Hypolepis glandulifera, Microlepia speluncae, Odontosoria chinensis, Lindsaea ensifolia, Araiostegia hymenophylloides, Nephrolepis multiflora, Trichomanes obscurum, Dicranopteris linearis and *Cyathea nilgirensis*.

Vidyarani *et al*. (2015) carried out histochemical analysis on *Adiantum hispidulum, Adiantum raddianum*, *Adiantum pervianum*, *Adiantum pubescens, Adiantum tripinnatum* and *Adiantum pacotii*. The occurrence of lignin, cutin, suberin and polyphenols were recorded with varied degree in all the studied species. Tannin showed its existence in all the studied species except *A. raddianum*. Sai Lakshmi *et al*. (2015) revealed the phytochemical constituents of *Asplenium aethiopicum, Asplenium affine*, *Asplenium decrescens* and *Asplenium viviparaum* using histochemical analysis. The analysis confirmed the existence of lignin, tannin, cutin, suberin and polyphenols with varied degree in all the studied species of *Asplenium*.

Phytochemistry

Phytochemical analysis has been done on a large number of Indian fern and fern allies. But the majority of the reports pertain to quantitative estimation of primary metabolites which are universal in occurrence and are highly variable with the environment (Khanna, 2002).

Phytochemical analysis of the edible ferns, *Ampelopteris prolifera* and *Diplazium esculentum* determined the nutritional value of the studied two ferns (Shankar and Khare, 1985; Singh *et al*., 1989). The wild or cultivated species of *Marsilea* showed more amounts of studied metabolites. A reverse trend has been observed in the content of carotenoids of *Marsilea minuta* by Kumar (1995).

Medicinal pteridophytes are gaining importance in recent days by the fact that several ferns from India have been subjected to phytochemical analysis. The rare, endangered and medicinally important Spleenworts, *Asplenium* species and *Psilotum* have been investigated

phytochemically by Lal (1979). Rohtagi *et al*. (1984), Khare and Shankar (1987) and Varma (1992) confirmed that the spore of the brackish water fern *Achrosticum aureum* seems to be a potential allergen. A potential antibiotic and anticancer chemotherapeutic agent has been extracted from various species of *Ophioglossum* by Khandelwal (1989). Rathore and Sharma (1990) studied the phytochemical composition of three species of *Isoetes* collected from Rajasthan with respect to pigments, aminoacids, proteins, carbohydrates, reducing sugar and glycosides.

Sharma and Sharma (1992) investigated the flavonoid content in common ferns from Rajasthan viz., *Actinopteris radiata, Adiantum lunulatum, A. capillus-veneris, Asplenium pumilum, Tectaria macrodonta, Cheilanthes farinosa, Hypodesmatium crenatum* and *Cyclosorus dentatus.* Patric Raja *et al*. (1992) estimated the pigments, sugars and starch content in twelve species of homosporous ferns collected from Kothayar and Palni hills.

De Britto and Manickam (1992) carried out preliminary phytochemical analysis on *Sphaerostephanos unitus, S. arbuscule* and *S. substruncatus* and confirmed the occurrence of amino acids, sugars and organic compounds. Gopalakrishnan *et al*. (1993) studied the chlorophyll, proteins, carotenoids, carbohydrates and aminoacids in *Histiopteris incisa, Hypolepis glandulifera, Microlepia speluncae* and *Pteridium aquilinum.* Joseph *et al*. (1993) studied total chlorophyll, chlorophyll a, chlorophyll b and carotenoid content in *Polystichum harpophyllum*, *Polystichum piceopaleaceum* and *Polystichum kunthianum*. Gopalakrishnan *et al*. (1993) studied the phytochemical composition of *Cyathea crinita, Cyathea nilgirensis* and *Cyathea gigantea* from Western Ghats. Jesudass and Manickam (1993) observed chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and anthocyanin in leaves and rachis of three species of *Pteris*.

Preliminary phytochemical screening on 19 species of South Indian Thelypteroid ferns showed the occurrence of steroid, alkaloid, phenol, catechin, saponin and tannin in all the studied species (De Britto *et al.,* 1994). Irudayaraj (1996) reported the presence of triterpenoid in the epidermal glands of *Christella parasitica*.

Hietz and Briones (2001) investigated the phytochemical constituents present in epiphytic ferns from Mexican cloud forest. Kale and Upadhye (2003) estimated chlorophyll contents and inorganic constituents in the dimorphic ferns *Bolbitis appendiculata, Gymnopteris contaminas*, *Ceratopteris thalictroides* and *Drynaria quercifolia* grown in different habitats of Western Ghats. Joseph *et al.* (2003) estimated carbohydrate, starch, protein, amino acids, lipids and phenol contents on sixteen species of Dryopteridaceae from Western Ghats, South India. Yang et *al.* (2004) studied the chemical constituents in rhizome of *Matteuccia struthiopteris.*

Phytochemical investigation of *Cyathea phalerata* showed the presence of an active flavonoid (kaempferol-3-neohesperidoside) with hypoglycaemic activity (Pizzolatti *et al.,* 2007). Besides the presence of cyathenosin A, a spiropyranosil derivative of protocatechuic acid was isolated from the stem pith of *C. phalerata*. Danielski *et al.* (2007) determined the occurrence of vitamins and minerals in *Equisetum giganteum*. Kale and Dongare (2007) analyzed total nitrogen, crude proteins and nitrate reductase from *Bolbitis appendiculata*, *Bolbitis virens, Osmunda regalis*, *Ceratopteris thalictroides* and *Drynaria quercifolia*. Kale (2007) studied the phytoconstituents present in *Bolbitis appendiculata* from Castle Rock and Anmode, Maharashtra.

Maridass and Raju (2010) identified the active constituents from different solvent extracts of *Elaphoglossum beddomei*. The results showed the presence of saponins, tannins, polyuronides, alkaloids, sugars, sterol and triterpenes. Sukumaran *et al*. (2012) identified the phytoconstituents present in the fronds of *Tectaria zeylanica* and confirmed the presence of

phenolics, saponins, steroids, tannins, xanthoproteins, coumarins and carbohydrates. Manonmani and Sara (2013) recorded the presence of steroids, triterpenoids, sugars, alkaloids, phenolic compounds, catechins, flavonoids, saponins, tannins, anthraquinones and amino acids in six different solvent extracts of *Actiniopteris radiata.*

Shakoor *et al.* (2013) studied the presence of different phytoconstituents in aqueous, methanolic, ethanolic and acetone extracts of 34 species of pteridophytes by qualitative screening. The analyses indicated that 34 species (100%) contained carbohydrates, proteins and free amino acids, 27 species (79.41%) with flavonoids, 26 species (76.47%) with phenolic compounds and tannins, 24 species (70.58%) with glycosides, 23 species (67.64%) with terpenoids, 22 species (64.70%) with saponins, 18 species (52.94%) with volatile oils, 15 species (44.11%) with alkaloids, 12 species (35.29%) with phlobatannins and only 3 species (8.82%) with resins.

Ruby and Sara (2014) studied the phytochemical composition of *Pyrrosia lanceolata* frond and rhizome. The fronds and rhizome were extracted with various solvents like water, ethanol, benzene, chloroform, petroleum ether and DMSO. All extracts of *P. lanceolata* frond and rhizome showed the presence of flavonoids, terpenoids, phenolics, anthraquinones, catechins, glycosides, fixed oils and fats.

Rajesh *et al*. (2014) studied the presence of phytochemicals in the aqueous, ethanolic and petroleum ether extracts of four ferns viz., *Actinopteris radiata, Drynaria quercifolia, Dryopteris cochleata* and *Pityrogramma calomelanos* by qualitative and quantitative screening methods. Ethanolic extracts of all the four ferns showed strong positivity for major phytochemicals. The phytochemicals like tannins and phenolics were well expressed in ethanolic extracts of the studied four ferns.

Spectroscopic methods have become firmly established as a key technological platform for differentiating, classifying and discriminating closely related plants (Lu *et al.,* 2004; Kim *et al.,* 2004; Biren, 2009). FT-IR 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 (Hazra *et al*., 2007; Eberhardt *et al*., 2007).

Gowtham (2013) analyzed the UV-Vis spectra of different extracts of *Asplenium aethiopicum* and identified the presence of metabolites and the functional groups. The results of FT-IR analyses confirmed the presence of alkanes, esters, primary amines alkyl halides, carboxylic acids and secondary amines in *Asplenium aethiopicum.* Janakiraman and Johnson (2014) performed UV-Vis spectroscopic analysis for different extracts of *Cyathea nilgirensis, Cyathea gigantea* and *Cyathea crinita* indicated variations in the chemical constituents. Use of macroscopical fingerprint characters not only identifies the chemical constituents of *Cyathea* species but also helped to distinguish the morphologically similar species of *Cyathea*. Janakiraman (2015) performed the FT-IR spectra to identify the functional groups of *C. nilgirensis*, *C. gigantea* and *C. crinita.*

TLC has the special ability to assay many samples at the same time on a single plate (Li *et al.,* 2004). It enables reliable separation and analysis of compounds from a wide variety of classes in many types of biological samples (Sherma and Fried, 2005). HPTLC being one of the powerful analytical tools can be utilized for linking the chemical constituents of the plant with high proficiency which in turn provides unique profiling of that particular plant (Johnson *et al*., 2012).

Dalli *et al.* (2007) studied the methanolic extract of *Pteris biaurita* using TLC bioassay. The active fraction revealed inhibition zone with R_f value 0.5 - 0.65. Silica gel from this region

was scraped, eluted in methanol and subjected to UV spectrophotometric analysis. An absorption maximum of 278 nm was recorded. HPLC analysis of TLC elute revealed a single peak with retention time of 8.1 min. GC-MS analysis revealed six major peaks in the retention time ranged from 7.2 to 10.9 min. Tsukasa and Sadamu (2011) isolated flavonoid and related compounds from six *Asplenium* species viz., *A. carruthersii*, *A. contiguum*, *A. caudatum*, *A. insiticium*, *A. laserpitiifolium* and *A. subfulexuosum*. The compounds were characterized by UV, acid hydrolysis and HPLC comparisons with authentic samples.

Irudayaraj and Johnson (2011) studied the pharmacognostical, morphological and physico-chemical characteristics of *Asplenium affine, Asplenium decrescens* and *Asplenium zenkeranum.* They revealed the presence of alkaloids, triterpenes, glycosides and flavonoids in *Asplenium* species. Based on the TLC, interspecific relationship was assessed. *A. affine* and *A. decrescens* showed 42% of similarity coefficient and *A. zenkeranum* was varied from *A. affine* and *A. decrescens* with 36% variance.

Paul Raj *et al*. (2011) studied the morphology, biochemistry and bioactivity of the epidermal glands of the glandular morphotype of *Christella parasitica.* The acetone extract of the glands showed antibacterial activities and also toxic effect against mosquito larvae and tadpoles of frog. Preliminary phytochemical analysis and HPLC studies of the gland extract showed the presence of various kinds of terpenoids, alkaloids, tannins, saponins and flavonoids. Mithraja *et al*. (2011) evaluated the phytochemical properties of *Azolla pinnata, Marsilea minuta* and *Salvinia molesta* using different solvents. They determined the alkaloids, phenolics, flavonoids, saponins, proteins, quinones, steroids, tannins, xanthoproteins, carboxylic acids, coumarins and carbohydrates presence in the studied species.

Sivaraman *et al*. (2011) carried out qualitative phytochemical screening on *Diplazium muricatum*, *Diplazium travancoricum* and *Diplazium brachylobum.* They revealed the presence of aminoacids, lipids, lignins, tannins, glycosides, flavonoids, terpenoids, steroids and saponins in the three studied species of *Diplazium*.

Mandal and Mondal (2012) studied the amino acids composition of *Pteris vittata*, *Drynaria quercifolia*, *Ampelopteris prolifera*, *Dryopteris filix* and *Selaginella indica* using TLC. Amino acid L-arginine monohydrochloride showed its common presence in *D. quercifolia*, *Ceratopteris thallictroides* and *Marsilea quadrifolia*. Glycine showed its occurrence only in *Helminthostachys zeylanica*.

Gowtham (2013) identified the presence of phenolics, tannins, flavonoids and alkaloids using HPTLC from the ethanolic extract of *Asplenium aethiopicum.* Janakiraman (2015) confirmed the presence of alkaloids, terpenoids, steroids, phenolics, flavonoids, tannins and saponins using HPTLC and find out the inter-specific variation among the three *Cyathea* species from Western Ghats, South India. Kamboj and Kalia (2014) studied the detailed pharmacognostical parameters for the histological and physico-chemical standardization of *Drynaria quercifolia.* Quantitative HPTLC and HPLC methods were successfully developed and validated as per ICH guidelines for the determination of phytochemical marker in the plant material and formulations.

Pteris vittata was evaluated for its alpha amylase inhibitory potential owing to its previously reported antihyperglycemic activity. This was also assayed for its free radical inhibition ability in ABTS decolourisation assay followed by quantification of polyphenols using HPTLC. The phenolics and flavonoids being responsible for antioxidant properties lead to quantify them which were followed by HPTLC fingerprinting of flavonoids to facilitate its

identification. Standard quercetin was used as standard along with the two extracts of *Pteris vittata* (Paul and Banerjee, 2013). Selvaraj *et al.* (2013) isolated bioactive polyphenolic compounds from methanolic extract of *Azolla microphylla*. The flavonoid compounds were isolated and characterized using TLC purified by HPTLC and identified using HPLC. Their structures and chemical bonds were analyzed using UV, FT-IR and NMR techniques. Two flavonoids were identified as rutin and quercetin.

HPLC is an important qualitative and quantitative technique, generally used for potency / purity / performance assays, pharmacokinetics / bioanalytical testing, purification, high-throughput screening, process control monitoring, quality control testing, estimation of pharmaceutical and biological samples (Guillarme *et al.,* 2012; Snyder *et al.,* 2009; Ahuja and Rasmussen, 2007).

Jaishee and Chakraborty (2015) studied the phytochemicals present in *Dicranopteris linearis* and *Pteris vittata*. Phytochemical analysis showed the presence of various bioactive compounds. Quantitatively, the phenolics, flavonoids, total sugars, proteins, vitamin C and carotenoid were found to be higher in *Dicranopteris linearis* than in *Pteris vittata.* On the other hand, tannins, total chlorophyll, chlorophyll a & b, reducing sugars and α-tocopherol were found to be higher in *P. vittata* than in *D. linearis*. Predominantly occurring phenolics identified by HPLC analysis in both the samples were catechin, caffeic, ferulic, salicylic and vanillic acid. Janakiraman (2015) revealed the HPLC fingerprint profile for ethanolic extracts of selected three *Cyathea* species from South India.

GC-MS is the best technique to identify the bioactive components of long chain hydrocarbons, alcohols and acids used in the analysis of the herbal medicines (Sridharan *et al.,* 2011). Zhongxiang *et al*. (2007) reported the presence of five new flavan-4-ol glycosides,

abacopterins E–I (5–9) and seven known flavonoid glycosides from the aerial parts of the fern *Abacopteris penangiana*. Choudhary *et al.* (2008) reported two glycosides, 62-*O*-(3,4-dihydroxy benzoyl)-â-d-glucopyranosyl ester and 4-*O*-â-d-glucopyranoside-3-hydroxy methyl benzoate along with five known compounds methyl benzoate, hypogallic acid, caffeic acid, paeoniflorin and pikuroside from a fresh water fern *Salvinia molesta*.

Melos *et al.* (2007) isolated a mixture of long-chain carboxylic acids esters from the ethanolic crude extract of *Adiantum tetraphylum*. Further purifications led to the isolation of 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*-D-glucoside, a mixture of ferulic acid, caffeic acid and *p*-hydroxybenzaldehyde. Peres *et al.* (2009) isolated and identified the steroid, sitosterol, the triterpene and hopan-22-ol; one flavone glycoside 6-metoxiapinenin-7-*O*- D-allopyranoside and a mixture containing the ethyl esters of hexadecanoic, oleic, 15-methyl-heptadecanoic and linoleic acids from the hexane and ethylacetate fractions from *Microgramma vacciniifolia* fronds ethanolic crude extract.

Dubal *et al*. (2013) investigated the bioactive compounds in the rhizome of *Tectaria coadunata*. The results revealed the presence of 16 bioactive compounds from the methanolic extract of *T. coadunata* of rhizome which may help in the protection against incurable diseases. Rukmini and Suvarnalatha (2014) studied the phytochemicals in *Nephrolepis cordifolia* using GC-MS analysis. The phytoconstituents include neophytadiene, 2,6,10-trimethyl,14,ethylene, 2 hexadecen-1-ol, 3,7,11,15 tetramethyl R-R, hexadecanoic acid, palmitic acid, 9 octadecenoic acid(z), oleic acid, octadecanoic acid, stearic acid, stigmast-4-en-3-one and 4-stigmasten-3-one. Kumar *et al*. (2014) identified thirty seven bioactive compounds which includes major compounds such as 5-7A-Isoprpenyl-4, 5-Dimethyloctahydro-1h-inden-4yl)-3-methyl-2-penta

(24.49%), n-hexadecanoic acid (18.29%), gamma-sitosterol (10.61%) and cis-vaccenic acid (9.25%) from the methanolic extracts of *Adiantum capillus-veneris.*

GC-MS analysis of the aquatic fern *Marsilea quadrifolia* showed the presence of major components hexadecanoic acid, ethyl ester (26.88%), phytol (16.97%), 9,12-octadecadienoic acid (12.46%), 1,2-benzenedicarboxylic acid, diisooctyl ester (8.62%), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (6.71%) and 2(3H)-furanone, dihydro-3-hydroxy-4,4-dimethyl (6.56%) (Sivagurunathan and Xavier, 2014). Manonmani and Sara (2015) investigated the bioactive components from the whole plant of *Actiniopteris radiata* using GC-MS. Their results revealed the existence of hexadecanoic acid, ethyl ester (20.40%), 9,12-octadecadienoic acid, methyl ester, (E, E) - (10.91%), (E)-9-octadecenoic acid ethyl ester (63.59%), docosanoic acid, ethyl ester (4.26%) and heptadecanoic acid, heptadecyl ester (0.84%).

Rukmini *et al.* (2015) determined the presence of alkaloids, flavonoids, phenolics, tannins and saponins in higher to moderate concentrations in *Hemionitis arifolia.* GC-MS analysis revealed the presence of mesityl oxide, 2- pentanone, benzene, neophytadiene, phytol, oleic acid, stearic acid and stigmast-4-en-3-one. Janakiraman (2015) carried out GC-MS analysis of *Cyathea* species and confirmed the presence of various phytochemical compounds in the ethanolic extracts.

Paul Raj (2007) studied the preliminary phytochemistry, TLC, GC-MS, larvicidal, antibacterial, insecticidal and piscicidal activity of *Amphineuron terminans*, *Christella parasitica*, *Cyclosorus interruptus*, *Sphaerostephanos arbuscula*, *Sphaerostephanos subtruncatus* and *Sphaerostephanos unitus.* Pauline Vincent (2012) determined the phytochemical profile for *A. terminans, C. parasitica* and *C. interruptus* using TLC, UV-Vis,

FT-IR, HPLC and HPTLC. In addition, antimicrobial activity of *A. terminans, C. parasitica* and *C. interruptus* were also carried out.

Babu (2013) investigated the phytochemical properties of *Adiantum latifolium, Acrostichum aureum, C. interruptus* and *Histiopteris incisa* using GC-MS and HPTLC. In addition, antibacterial, cytotoxicity, piscicidal and insecticidal properties of *A. latifolium, A. aureum, C. interruptus* and *H. incisa* were also examined.

Phytochemistry in *Selaginella*

Phytochemistry is one of the more fashionable and rapidly expanding areas of plant taxonomy 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. Preliminary pharmacological studies have been carried out on *Selaginella rupestris* by Nath *et al*. (1965). 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). New alkaloid glycosides from *Selaginella doederleinii* have been reported by Chao *et al.* (1987) and they have also been synthesized artificially (Chao *et al.,* 1990).

The relationship between carbohydrate content and life habit of four species of *Selaginella* from the Himalayas has been investigated by Loyal *et al.* (1991). 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). Certain flavonoids, notably derivatives of flavone, chrysin and apigenin, together with dimers such as amentoflavone have been found to possess anxiolytic properties without exhibiting a sedative effect (Kennedy *et al*., 1998). Large number of new biflavonoids has been

reported from different species of *Selaginella.* The extract of *Selaginella doederleinii* contained alkaloid, phytosterol and saponin compounds (Pan *et al*., 2001). Comparative study on the occurrence of amino acids in four species of *Selaginella* has been made by Paramanik *et al.* (2002).

Selaginella has various pigmentations such as blue chromatic, crimson red, variegate, yellow gold and silver. Morphological diversity and pigmentation are important characteristic in taxonomy of *Selaginella* (Dahlen 1988; Czeladzinski 2003). A set of 14 biflavonoid compounds with anti-human immunodeficiency virus activity has been studied by using quantum chemical and chemometric methodologies with the aim to calculate some molecular properties and correlate them with the biological activity (Molfetta *et al*., 2004). Two new antiviral chromone glycosides, namely unicoside A and unicoside B, have been reported from *Selaginella uncinata* (Ma *et al.,* 2003).

Further analysis in *S. uncinata* showed the presence five new biflavonoids, robustaflavone 7,4',4'"-trimethyl ether, robustaflavone 4',4'"-dimethyl ether, 2,3-dihydroamentoflavone 7,4',7"-trimethyl ether, 2,3-dihydroamentoflavone 7,4'-dimethyl ether, and 2",3"-dihydroisocryptomerin 7-methyl ether. Among the isolates, robustaflavone 4',4'"-dimethyl ether, 2,3-dihydroamentoflavone 7,4'-dimethyl ether and alpha-tocopheryl quinone exhibited cytotoxicities against P-388 and HT-29 cell lines *in vitro* (Chen *et al.,* 2005).

Four new bioflavonoids viz., robustaflavone 4'-methyl ether, robustaflavone 7,4'-dimethyl ether, 2",3"-dihydrorobustaflavone 7,4',-dimethyl ether and 2",3"-dihydrorobustaflavone 7,4', 7"-trimethyl ether, two known bioflavonoids including robustaflavone and amentoflavone, three caffeoylquinic acids viz., 3,5-di-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid have been isolated from

Selaginella delicatula (Lin *et al.,* 2000). Ginkgetin 178 isolated from *S. moellendorffii* showed selective cytotoxicity against OVCAR-3 (Kim and Park, 2002).

Amentoflavone and three other flavonoids have been isolated from the ethanolic extract of *Selaginella sinensis*. Amentoflavone have shown potent antiviral activity against respiratory syncytial virus, with an IC_{50} of 5.5 µg/ml. The contents of amentoflavone in nine species of *Selaginella* were determined by reversed-phase HPLC. *S. sinensis* showed a higher content of 1.13% (Shuang *et al.,* 2001). Amentoflavone inhibits the induction of nitric oxide synthase by inhibiting NF-kappaB activation in macrophages (Woo *et al.,* 2005).

It is important to note that structurally unique biflavonoids have been isolated from two Indian species of *Selaginella* viz., *S. chrysocaulos* and *S. bryopteris* by Swamy *et al.* (2006). Phytochemical studies on various species of *Selaginella* has not only resulted in the discovery of several new compounds of secondary metabolites, but also resulted in the discovery of new plant pigments. Two new unusual natural pigments, selaginellin A and B have been first isolated and described by Cheng *et al.* (2008) from *Selaginella tamariscina*. Like that a new steroid, 3β, 16α-dihydroxy-(5α)-cholestan-21-oic acid was isolated from the aerial parts of *Selaginella pulvinata* (Zheng *et al.,* 2007). Li *et al*. (2007) reported flavonoids from *Selaginella delicatula* and *S. willdenowii,* which are used as anticancer agent.

Selaginella lepidophylla showed the occurrence of various secondary metabolites such as alkaloids, phenolics (flavonoids, tannins, saponins) and terpenoids (triterpene, steroid). (Chikmawati and Miftahudin 2008; Chikmawati *et al*., 2008). Aguilar *et al.* (2008) isolated three different biflavonoids viz., robustaflavone, 2,3-dihydrorobustaflavone and 2,3-dihydrorobustaflavone-5-methyl ether from *Selaginella lepidophylla*. They observed that all the biflavonoids inhibited ATP production.

Preliminary phytochemical screening on different extracts of the spike-moss *Selaginella inaequalifolia* showed the presence of steroids, triterpenes, phenolic groups, tannins, sugars and catechins. Among the five different extracts, ethanolic and chloroform extracts showed the presence of maximum number of compounds (Irudayaraj *et al.,* 2010). Suganya *et al.* (2011) determined the presence of phenolic groups, steroids, triterpenoids, tannins and sugars in *Selaginella tenera.* Chikmawati *et al*. (2012) revealed the diversity of bioactive compounds and amentoflavone content of *Selaginella* from Java Island. The results showed that all the examined *Selaginella* species contained alkaloids, flavonoids, saponins, tannins and steroids. The highest amentoflavone concentration 6.87 ppm was found in *S. subalpina.*

Previous studies on some species of *Selaginella* revealed that *Selaginella* is a rich source of steroids, biflavonoids, alkaloids, secolignans, neo-lignans and caffeoyl derivatives. Other compounds such as alkaloidal glycosides, phenylpropanones and lignans were also reported in some *Selaginella* species (Sa *et al*., 2012). Rupa and Bhavani (2014) revealed the presence of alkaloids, amino acids, tannins, glycosides, steroids, flavonoids and carbohydrates in both root and stem extracts of *Selaginella bryopteris*. Terpenoids was observed only in stem extracts of *S. bryopteris*. Chikmawati *et al*. (2012) performed TLC tests in various *Selaginella* extracts to qualitatively analyze the bioactive compounds alkaloids, flavonoids and steroids.

Sun *et al.* (2006) determined the amentoflavone in *Selaginella* species which showed a good linearity in the range of 153-767 ng. The average recovery was 97.03% and RSD was 0.80% (n=5) at the detection wavelength of 330 nm. The different species of *Selaginella* showed different HPLC fingerprint characteristics. The samples of the similar species but collected in different period, different environment or different locations showed certain difference in fingerprints. However, it also generates "main fingerprint peaks", which can be used to evaluate

and distinguish the different species or intra species (Li *et al.,* 2007). Li *et al*. (2014) revealed the chromatographic profile of *S. labordei*, *S. tamariscina, S. uncinata*, *S. moellendorfii*, *S. remotifolia* and *S. pulvinata*. The results showed two major common peaks using HPLC which were identified as amentoflavone and robustaflavone.

Biological activities

Antioxidant activities

Antioxidant based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer (Devasagayam *et al*., 2004). Compounds responsible for such antioxidant activity can be isolated and used for prevention and treatment of free radical-related disorders (Middleton *et al*., 2000). Extract of *Selaginella tamariscina* contained a strong antioxidant activity that was able to reduce blood sugar levels and function as a lipid peroxide serum and increase insulin serum (Miao *et al*., 1996).

Antioxidant activity of two phloroglucinol derivatives from *Dryopteris crassirhizoma* exhibited significant antioxidant activity as assessed by DPPH radical scavenging assay *in vitro* (Lee *et al*., 2003). Antioxidant activity of aqueous extract and ethyl acetate fraction of *Equisetum telmateia* was evaluated by DPPH, TEAC and TBARS assays. Significant antioxidant activity was detected in the ethyl acetate fraction. Analysis of aqueous extract and ethyl acetate fraction by HPLC-PADESI/MS allowed the identification of the major phenolic compounds as flavan-3-ol, kaempferol and phenolic acid derivatives (Helena *et al*., 2005). Free radical scavenging activity of aqueous extract of *Pteris multifida* was evaluated using DPPH, hydroxyl radical and reducing power assay (Wang *et al*., 2006).

Aqueous extract of *S. involvens* possessed significant antioxidant effect to lipid peroxides (EC_{50} = 2 ug/ml). The aqueous extract of *S. involvens* was non toxic and degrades blood cholesterol (Gayathri *et al*., 2005). Lola Durdevic *et al.* (2007) determined total phenolics from *Ceterach officinarum*, *Asplenium trichomanes* and *Adiantum nigrum* leaves, rhizomes and rhizosphere soil. Highest amounts of phenolics were found in the leaves and rhizomes of *Ceterach officinarum*. Hort *et al.* (2008) studied the antioxidant and hepatoprotective activity of *Cyathea phalerata* hydroalcoholic extract and fractions obtained by treatment with organic solvents of increasing polarity. Ethyl acetate fraction of the crude extract displayed the best antioxidant and hepatoprotective activities. The flavonoids present in ethyl acetate fraction of *C. phalerata* could be responsible for these activities.

Mimica *et al.* (2008) evaluated antioxidant activity and phenolic composition of three different extracts of field horsetail *Equisetum arvense* by measuring the total reducing power, inhibition of lipid peroxidation, free radical scavenging capacity viz., DPPH radical and nitric oxide and total flavonoid content. In addition, they determined the phenolic constituents of each extract.

Peres *et al.* (2009) evaluated the antioxidant activity of ethanolic crude extract and fractions of *Microgramma vacciniifolia* using DPPH assay. The ethyl acetate fraction of *M. vacciniifolia* showed good activity in DPPH assay. Daonian *et al*. (2010) studied the antioxidant and hepatoprotective activity of *Arachniodes exilis* by different assays viz., reducing power, lipid peroxidation, DPPH, ABTS, superoxide anion, hydroxyl radicals and hydrogen peroxide. The aqueous extract of *Davallia solida* rhizome contains high content of phenolic compound and showed a strong DPPH scavenging activity (Chen *et al.,* 2008). Li *et al*. (2010) determined the central composite design combined with response surface methodology to optimize the

parameter of ultrasonic-assisted extraction of total flavonoids from *Selaginella doederleinii*. They observed maximum flavonoids 4.414 mg/g from 70% ethanolic extracts obtained from 50 min at 65°C extraction. The free radical scavenging activity of *Selaginella doederleinii* was ranged from 20.22 to 46.64 mg/ml.

Lai and Lim (2011) studied the antioxidant activity of selected ferns found in Malaysia. Methanolic extracts of fifteen fern species were screened and the results showed very high total phenolic content, above 2000 mg GAE/100g fresh leaves. The ferns with strong antioxidant properties were *Cyathea latebrosa*, *Cibotium barometez*, *Drynaria quercifolia*, *Blechnum orientale* and *Dicranopteris linearis*. These ferns exhibited strong DPPH radical scavenging activity (ascorbic acid equivalents 2866-3111 mg/100g), ferric ion reducing power (963-1417 mg GAE/100g) and inhibition of lipid peroxidation. Strong chelating activity was found in *Pteris vittata* and *Pteris venulosa*. Rajurkar *et al*. (2012) studied the antioxidant activity of ethanolic extract of *Adiantum capillus-veneris* and compared to the standard ascorbic acid. The results exhibit low IC_{50} values 0.3986 mg/g for DPPH assay and 0.695 mg/g for ABTS assay.

Paulsamy *et al*. (2013) reported the antioxidant activities of *Actiniopteris radiata* and *Equisetum ramosissimum* methanolic extracts. The results were found to have potent antioxidant activity against DPPH with the IC_{50} value of 93.48 and 78.58 respectively. *A. radiata* had the highest values for $ABTS^{+}$ radical (2523.11 µTE/g) and reducing power assay (0.853 absorbance at 700 µg/ml). *E. ramosissimum* exhibited higher iron chelating activity (41.18% at 5000 µg/ml).

Antesa and Grino (2013) evaluated the folkloric therapeutic claims of ten selected Philippine medicinal pteridophytes by determining the phytochemical, antioxidant and antibacterial activities. Qualitative screening of the phytochemicals showed that alkaloids, saponins, flavonoids and tannins were present in their leaves extracts. All the extracts exhibited

different extent of antioxidant activity. Among the plant samples tested, *Blechnum orientale* methanolic leaves extract showed the greatest antioxidant activity while *Lycopodium cernuum* had the least. The IC_{50} values ranged from 3.71 µg/ml to 32.37 µg/ml. Kumar *et al*. (2013) evaluated the polyphenols, vitamin E and *in vitro* antioxidant activity of methanolic and petroleum ether extract of *Adiantum capillus-veneris*. The results showed that the methanolic extracts possessed higher antioxidant potential and polyphenol contents than the petroleum ether extracts. The total phenol content in methanolic extracts of *A. capillus-veneris* was 19.05 ± 4.60 mg/g. Total flavonoid content was measured between 7.90 ± 0.90 mg/g and tannin content was 23.57±7.75 mg/g tannic acid equivalent.

Johnson *et al*. (2014) determined the antioxidant potential of *Asplenium aethiopicum*. The best free radical scavenging activity was exerted by methanolic extract of *Asplenium aethiopicum* (IC_{50} 91.4 µg/ml). Shrivastava *et al*. (2014) reported the phytochemical constituents including flavonoid, phenols, saponins, tannins and saponins in *Adiantum* and *Pteris* which indicates the antioxidant activity.

Nawaz *et al.* (2014) carried out antioxidant activities of *Azolla pinnata* and *Azolla rubra*. Extract of *A. pinnata* displayed marked radical scavenging and reducing potential when compared to *A. rubra*. The content of total phenolics and flavonoids were higher in *A. pinnata*. The higher antioxidant efficacy of *A. pinnata* could be due to the presence of high phenolic and flavonoid contents. Amoroso *et al*. (2014) determined the antioxidants, proteins and phytochemicals in *Diplazium esculentum*, *Marsilea crenata* and *Stenochlaena palustris*. The results showed quantifiable level of antioxidant activity by the DPPH assay with *D. esculentum* exhibiting the highest antioxidant activity relative to ascorbic acid (83%). The results also showed protein content as high as 4.4 mg/g for *M. crenata*. All the three fern species showed the

presence of alkaloids, saponins, phenolics and flavonoids. *D. esculentum* alone showed the occurrence of terpenes.

Gupta *et al*. (2014) evaluated *in vitro* antioxidant activity of *Pteris scabristipes, Aleuritopteris flava, Microlepia rhomboidea*, *Diplazium esculentum*, *Asplenium khasianum, Microlepia hallbergii, Adiantum edgeworthii* and *Lindsaea odorata.* A positive correlation between the antioxidant activities and phytochemical contents was observed. They observed the best antioxidant activity in the methanolic extracts of *Aleuritopteris flava* and *Lindsaea odorata* compared to other investigated ferns. Jaishee and Chakraborty (2014) determined the antioxidant activity of *Pteris biaurita* by DPPH free radical scavenging activity, hydrogen peroxide scavenging assay, nitric oxide scavenging assay, superoxide scavenging and ferric reducing antioxidant power assay. All the tested three extracts showed appreciable antioxidant activity in a dose-dependent manner.

Jinu *et al*. (2014) evaluated the polyphenolic composition and antioxidant properties of methanolic extract of *Drynaria quercifolia* rhizome. The extract yielded total phenolic content of 240 ± 0.01 mg (GAE)/100g of fresh mass and total flavonoid content of 150 ± 0.02 mg (QE)/100g of fresh mass. The extract of *D. quercifolia* rhizome exhibited remarkable scavenging capacity towards DPPH, OH, NO, H_2O_2 and $ABTS^+$. The antioxidant capacities of the extract were comparable and stronger than that of the antioxidant standard, butyl hydroxy toluene.

Ethyl acetate fraction of *Cheilanthes albomarginata* showed the strongest DPPH radical scavenging (82.54 ± 0.48%), hydrogen peroxide scavenging (3.41 ± 0.21 mg/ml) and nitrite scavenging activity (61.39%). The highest phenolic content was found in the ethyl acetate fraction followed by the butanol fraction (Lamichhane *et al*., 2014). Mathad *et al*. (2015) evaluated the *in vitro* antioxidant property and phytochemical constituents of *Actiniopteris*

radiata. The scavenging activity of DPPH, H_2O_2 and reducing power including phenolics, flavonoids, alkaloids and tannin contents were also determined. The extracts exhibited scavenging activity towards all radicals tested due to the presence of relatively high alkaloids and flavonoids content.

Janakiraman (2015) evaluated the antioxidant activity using DPPH, metal chelating, nitric oxide radical, hydroxyl radical and superoxide radical scavenging activity of *Cyathea nilgirensis, Cyathea gigantea* and *Cyathea crinita.* DPPH assay best free radical scavenging activity was exerted by *C. nilgirensis*. The highest nitric oxide radical scavenging activity was exerted by *C. nilgirensis*.

Cytotoxicity

Brine shrimp lethality assay is an important tool for preliminary assessment of cytotoxicity of herbal drugs. This method is used for the determination of LC_{50} values of herbal extracts. The brine shrimp lethality assay is used to predict the cytotoxicity and pesticidal activity (Meyer *et al*., 1982).

Priscilla *et al*. (2014) evaluated the phytochemical compositions and toxicity of *Selaginella doederleinii* extracts**.** Qualitative phytochemical analysis on ethanolic and aqueous extracts revealed the presence of saponins, tannins and cardiac glycosides in the extracts. The 50% lethal concentration (LC_{50}) in brine shrimp lethality test using ethanolic and aqueous extracts after 24 h of exposure was found to be >1000 µg/ml. Cancer-origin cell lines MDA-MB231 and HepG2 were found to be the most susceptible among other tested cell lines, with the treatments of ethanol (LC_{50}=306 µg/ml) and aqueous (LC_{50}=329 µg/ml) extracts respectively.

Janakiraman (2015) observed that the ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* were found to be more effective against brine shrimps. Chloroform and acetone extract

of selected *Cyathea* species showed moderate lethality level. Petroleum ether extract exhibited less cytotoxic effects when compared to other extracts.

Breast cancer is one of the most common and serious malignancies worldwide. Despite intensive cancer control efforts, it remains the second-leading cause of cancer death among women (Harris *et al*., 2000). While the overall response rate can be high, the duration of response is relatively short and most patients with initially responsive tumors will experience a drug-resistance phenotype. Numerous studies have centered on the field of drug resistance to improve cancer chemotherapy and management of cancers (Gottesman, 2002).

Selaginella willdenowii contains isocryptomerin, derivatives of amentoflavone and robustaflavone which is significantly cytotoxic against various cancer cells (Silva *et al*., 1995). *Selaginella tamariscina* decreased expression of MMP-2 and 9, urokinase plasminogen activator, inhibits growth of metastasis A549 cell and Lewis lung carcinoma (Yang *et al*., 2007); inhibited leukemia cancer cell of HL-60 cell (Lee *et al*., 1999); degraded leukemia cancer cell of U937 (Lee *et al*., 1996; Yang *et al*., 2007); reduced proliferation of nucleus antigen cell from stomach epithelium (Lee *et al*., 1999); chemopreventive for gastric cancer (Lee *et al*., 1999); induced apoptosis of cancer cell through DNA fragmentation and nucleus clotting (Ahn *et al*., 2006); and induced breast cancer apoptosis through blockade of fatty acid synthesis (Lee *et al*., 2009).

Selaginella tamariscina was assayed for its tumoricidal effects with 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT). Influences on expression of p53 tumor suppressor gene and induction of G1 arrest in the cell cycle were analyzed by Northern blotting and flow cytometry. Among the effective fractions, the water-extracted fraction efficiently increased p53 gene expression and induced G1 arrest (Lee *et al.* 1999).

Aqueous extract of *S. involvens* showed remarkable inhibition of lipid peroxidation. This was found to be dose dependent. At a concentration of 2 µg/ml the *in vitro* inhibition of peroxidation of lipid was almost 50%. The concentration required for 50% inhibition (EC_{50}) of *in vitro* lipid peroxidation for *S. involvens, S. delicatula* and *S. wightii* was 2.1±0.1, 38.2±1.2 and 76.6±4.4 µg/ml respectively. The aqueous extract of the three plants also showed varying levels of hydroxyl radical scavenging activity *in vitro*. *S. delicatula* was more potent in hydroxyl radical scavenging activity compared to the other two species. Concentration of aqueous extract required for 50% *in vitro* inhibition of hydroxyl radical production for *S. involvens* (Gayathri *et al.,* 2005).

Selaginella bryopteris extract may help in stress-induced complications including those due to heat shock (Sah *et al*., 2005). Guruvayoorappan and Kuttan (2007) confirmed the antimetastatic activity of amentoflavone using B16F-10 melanoma-induced experimental lung metastasis in C57BL/6 mice. Amentoflavone treatment significantly reduced tumor nodule formation accompanied by reduced lung collagen hydroxyproline, hexosamine, and uronic acid levels.

Amentoflavone extracted from *Selaginella tamariscina* was screened against five cancer cells, including HeLa (human cervical carcinoma cells), BEL-7402 (human hepatoma carcinoma cells), MCF-7 (human breast cancer cells), PANC-1 (human pancreatic cancer cells) and HL-60 (human leukemia cells). The anticancer activity was determined by means of MTT assay and Trypan Blue cytometry. The extracts of *S. tamariscina* were effective to inhibit the proliferation of HL-60, MCF-7, HeLa, BEL-7402, PANC-1 and showed reliable activity against HL-60 (Jing *et al*., 2010).

Cao *et al.* (2010) isolated three new flavones viz., 5-carboxymethyl-4',7-dihydroxyflavone, ethyl ester and butyl ester from *Selaginella moellendorffii* together with ten known compounds. Lai *et al.* (2010) studied the ethyl acetate, butanol and aqueous extract of *Blechnum orientale.* The fractions possessed strong radical scavenging activity (IC_{50} 8.6-13.0 mg/ml) and cytotoxic activity towards human colon cancer cell HT-29 (IC_{50}=27.5-42.8 mg/ml). Phytochemical analysis revealed the presence of flavonoids, terpenoids and tannins. Ethyl acetate and butanol fractions showed highest total phenolic content (675-804 mg GAE/g).

Zakaria *et al*. (2011) investigated the *in vitro* cytotoxic and antioxidant properties of aqueous, chloroform and methanolic extracts of *Dicranopteris linearis*. The cytotoxic effect was determined against the normal (3T3) and cancer cell lines (MCF-7, HeLa, HT-29, HL-60, K-562 and MDA-MB-231) using MTT assay, while the antioxidant activity was assessed using the DPPH radical and superoxide scavenging assays. Aqueous extract was not effective against any of the cancer cells studied; the chloroform extract was effective only against MCF-7 and HeLa; and the methanolic extract was effective against all the cancer cells used. All the extracts were found to exert antioxidant activity against the DPPH radical and superoxide scavenging assays; the methanolic extracts exhibiting the highest antioxidant activity in both assays. Sarker *et al*. (2011) investigated the antitumor properties of *Selaginella ciliaris*, *Marsilea minuta* and *Thelypteris prolifera*. Among the tested species, highest percentage of tumor inhibition was found in *M. minuta* (82.32%) followed by *S. ciliaris* (80%) and *T. prolifera* (75.68%) at 1000 ppm. Significant tumor inhibition was observed at 10, 100 and 1000 ppm of plant extracts on potato disc induced by *Agrobacterium tumefaciens* indicating their presence of tumor inhibitor metabolites.

Patel and Patel (2011) studied the cytotoxic activity of methanolic extracts of *Artocarpus heterophyllus* by various *in vitro* cytotoxic assays like MTT and SRB against different cell lines viz., HEK293, A549, HeLa and MCF-7. The IC_{50} values of *A. heterophyllus* methanolic extract was 35.26 µg/ml and 35.27 µg/ml against A549 cell line by MTT and SRB assay respectively. The methanolic extract of *A. heterophyllus* was found to be nontoxic to normal cells (HEK293). The methanolic extract of *A. heterophyllus* failed to show the activity against Hela and MCF-7 cell lines. Ibrahim *et al*. (2011) evaluated the cytotoxic effect of *Salvadora persica* extracts against different human cell lines. Petroleum ether extract was more potent (IC_{50} = 43.6 µg/ml) against human hepatocellular carcinoma cell line-HepG2 followed by IC_{50}=44.3 µg/ml against human breast carcinoma cell line-MCF7, 19.87 µg/ml against lung carcinoma cell line-A549 and 10.2 µg/ml against colon carcinoma cell line-HCT116. However the other extracts showed moderate activities.

Sikder *et al*. (2013) determined the methanolic extracts of leaves of *Nephelium lappaceum*, *Pandanus foetidus*, *Ludwigia repens* and the whole plant of *Adiantum philippense* for antioxidant and cytotoxic activities. The crude extract of *N. lappaccam* exhibited the highest free radical scavenging activity (IC_{50}=3.93±0.25 µg/ml). The crude methanolic extracts of *A. philippense* and *P. foetidus* demonstrated significant brine shrimp lethality with LC_{50} values 0.50±0.84 µg/ml and 0.58±0.73 µg/ml, respectively as compared to the standard anti-neoplastic drug, vincristine sulfate (LC_{50}=0.45 µg/ml).

Sri Handayani *et al*. (2013) evaluated the cytotoxic effect and apoptosis induction of *Selaginella plana* fractions against MCF-7 cells. The result showed the cytotoxic effect of hexane, methylene chloride, ethyl acetate and butanol extracts of *S. plana* against MCF-7 cells with IC_{50} value 30 µg/mL, 19 µg/ml, 24 µg/ml and 2 µg/ml respectively. The active fractions of

S. plana at its IC_{50} concentration increased apoptotic cells on the MCF-7 cells 35.33%, 20.33%, 24% and 45.67% respectively as compared to control. Butanol fraction of *S. plana* showed the highest apoptotic induction against MCF-7 cancer cells.

Li *et al*. (2014) studied the anti-proliferation effects of four fractions of *Selaginella doederleinii* (70% ethanol, petroleum ether, dichloromethane and acetic ether extracts) against five human cancer cells. The dichloromethane and acetic ether extracts showed good cytotoxicities against the studied cancer cell lines, guiding the subsequent separation.

Li *et al.* (2014) studied the cytotoxic activity of *S. labordei*, *S. tamariscina, S. uncinata*, *S. moellendorfii*, *S. remotifolia* and *S. pulvinata.* Their results showed relatively stronger activities against Bel-7402 and HeLa cells, S. *moellendorfii* demonstrated moderate antiproliferation activities, but *S. remotifolia* and *S. pulvinata* failed to show the inhibiton. The main active components were in the ethyl acetate extracts which had abundant biflavonoids. The effects of these extracts on cell proliferation and apoptosis in different cells were not the same, they were more apparent on HeLa cells than on HT-29 cells.

Kaewsuwan *et al*. (2015) isolated three coumarin derivatives, interruptins A, B and C from *Cyclosorus terminans*. Interruptins A and B inhibited the growth of MCF-7 human breast and HT-29 human colon cancer cells with IC_{50} values as low as 0.13 ng/ml yet stimulated proliferation of normal ASC stem cells with no signs of toxicity.

Chai *et al*. (2015) evaluated the glucosidase inhibitory and cytotoxic activities of *Blechnum orientale, Davallia denticulata, Diplazium esculentum, Nephrolepis biserrata* and *Pteris vittata.* The α-glucosidase inhibitory activity of *D. esculentum* (EC_{50} = 6.85 μg/ml) was considerably stronger than that of myricetin (EC_{50} = 53.21 μg/ml). *B. orientale, D. esculentum, N. biserrata* and *P. vittata* were cytotoxic to K562 cells. *P. vittata* had the strongest cytotoxicity,

although it was less potent than 5-fluorouracil. *D. denticulata* had the highest phenolic, hydroxycinnamic acid and flavonoid contents of all the extracts while *B. orientale* had the highest proanthocyanidin content.

Janakiraman (2015) studied the cytotoxic activity of ethanolic extracts of selected *Cyathea* species. *C. crinita* was found to be moderate compound to the other two studied species. The results showed that ethanolic extracts of *Cyathea* species had a moderate anticancer activity against MCF 7 cell line.

Larvicidal activity

Mosquitoes are well known group of insects, which transmit many deadful diseases causing serious health problems to human beings. The Government has taken lot of steps to control these mosquitoes (vectors) for a long time, using synthetic chemicals. But the chemicals cause pollution and result in development of resistance among mosquito species (Das and Rajagopalan, 1981; Brown, 1986; Thangam and Kathiresan, 1990). A careful and prolonged control of the vector can eliminate filariasis, but it is not an easy task due to its natural tolerance and early development of resistance to available insecticides (Brown and Pal, 1971).

Larvicidal activity of *Adiantum* ethanolic fraction showed the highest larvicidal activity at higher concentration of 70-85% at 30,000 ppm and at 5,000 ppm. The activity was found to be 30-40% between 24-120 h. *A. craccivora* showed 70-75% activity at 30,000 ppm and 50-70% activity at 5,000 ppm between 24-48 h (Sood and Sharma, 2010).

Johnson *et al.,* (2014) studied the larvicidal activity of different extracts of *Asplenium aethiopicum* against the fourth instar larvae *Culex quinquefasciatus*. Highest larval mortality was observed in acetone extract (LC_{50}=181.2 ppm) and lowest effect observed in chloroform extract (LC_{50}=288.9 ppm).

Janakiraman (2015) evaluated the larvicidal bioassay of crude petroleum ether, chloroform, acetone and ethanolic extracts of *Cyathea* species against the fourth instar mosquito larvae *C. quinquefasciatus.* The highest larval mortality was observed in ethanolic extracts of *C. crinita*.

Antidiabetic activity

Diabetes mellitus is an endocrinal disorder associated with depleted insulin secretions, damaged pancreatic β-cells with altered carbohydrate, lipid and protein metabolism and additionally increased risk of complications of various vascular diseases etc. It has been estimated that Indian people are more genetically susceptible to diabetes accounting about 40 million which further will reach to maximum of 74 million by 2025 (Porter and Barrett, 2005). The conventional treatments of diabetes include insulin injections, oral hypoglycemic drugs, exercise, diet or combination of these (Rang *et al*., 2003).

Hemionitis arifolia, a folklore fern, was evaluated for its hypoglycaemic and anti-diabetic properties using rats. Glucose lowering effect and anti-diabetic activity were studied using glucose tolerance test in normal rats and alloxan induced diabetic rats. The ethanolic extract of *H. arifolia* showed optimum activity at 200 mg/kg. The ethanolic extract of *H. arifolia* exhibited only marginal hypoglycaemic activity in overnight fasted normal rats and it was devoid of conspicuous toxic symptoms in sub-acute toxicity evaluation in mice (Nair *et al*., 2006).

Selaginella tamariscina possessed anti-diabetic activities by decreased serum levels of fast blood glucose, glycosylated hemoglobulin A1C, triglyceride, total cholesterol, free fatty acid, low density lipoprotein-cholesterol and glucagon, as well as increased serum levels of high density lipoprotein-cholesterol, insulin and C-peptide. *S. tamariscina* also improved the oral glucose tolerance test to a certain degree. Furthermore, *S. tamariscina* increased the protein

expression of PPAR-γ in adipose tissue, and increased the protein expressions of IRS-1 in hepatic and skeletal muscle tissues (Zheng *et al*., 2011).

Paul *et al*. (2012) evaluated the potential of *Adiantum philippense* in hyperglycemia induced by alloxan monohydrate, ethanolic and aqueous extracts at two dose levels of 500 mg/kg and 250 mg/kg respectively. Both the extracts exhibited significant hypoglycemic effect when compared to standard drug, glibenclamide and disease control group at an interval of 14 days is evident from plasma glucose levels and OGTT values. Antioxidant property was also evaluated in the extracts through ABTS radical scavenging assay and in the pancreatic tissue by SOD, CAT, reduced GSH and LPO assays. There was an increase in the levels of SOD, CAT, GSH whereas decrease in the LPO levels in the extracts treated groups which further support the role of *Adiantum philippense* in lowering the hyperglycemic state of experimentally induced diabetic rats.

Tanzin *et al.* (2013) determined antihyperglycemic activity of *Christella dentata* methanolic extract through oral glucose tolerance tests in glucose-loaded Swiss albino mice. The 10 mg/kg body weight of *C. dentata* methanolic extracts lowered blood sugar levels by 52.40%. The methanolic extracts of *C. dentate* exhibited favourable results than standard antihyperglycemic drug. Rahmatullah *et al.* (2014) evaluated the antihyperglycemic and analgesic potential of methanolic extract of *Angiopteris evecta* roots. The extract at doses of 50, 100, 200 and 400 mg/kg reduced blood glucose levels by 11.3, 23.0, 32.8 and 46.7% respectively.

The aqueous extract of *Selaginella bryopteris* (150 mg/kg b.w) was orally administered to alloxan induced Swiss albino mice for 26 days. It was noticed that *S. bryopteris* reduces the

level of glucose, increases the decreased body weight of mice and release the shrunken pancreas (Singh *et al*., 2014).

Hepatoprotective activity

Hepatic disorders have been recognized worldwide as an important cause of morbidity and mortality in man and animals all over the globe. Herbal medicines are known to play an important role in the treatment of various elements including liver disorders and many traditional practioners have claimed that numerous medicinal plants can be extensively used for the alleviation of different types of liver disorders (Dash *et al.,* 2007).

Hepatoprotective effects of *Selaginella doederleinii* and *Pteris ensiformis* from Taiwan have been studied by Hu *et al.* (2004). Extracts from *Selaginella labordei* down-regulated cyclooxygenase-2 gene expression, measured by real-time RT-PCR, in human colon adenocarcinoma CaCo-2 cells (Chen *et al*., 2005).

Hepatoprotective potential of *Lygodium flexuosum* was evaluated in male wistar rats against carbon tetrachloride induced liver damage in preventive and curative models. Histopathological changes induced by CCl_4 were also significantly reduced by the extract treatment in preventive and curative groups. Phytochemical studies revealed the presence of saponins, triterpenes, sterols and bitter principles in *L. flexuosum n*-hexane extract which could be responsible for the possible hepatoprotective action (Wills and Asha, 2006).

Kiran *et al*. (2012) investigated the hepatoprotective activity of *Cyathea gigantea* methanolic extract against paracetamol induced liver damage in rats. Hepatoprotective activity reduced the evaluated levels of SGOT, SGPT, ALP, TB also reversed the hepatic damage towards normal.

Balne *et al.* (2013) studied the most effective hepatoprotective fraction of methanolic extract of *Marsilea minuta* by fractionating and evaluating its fractions in three models viz., CCl_4, paracetamol and ethanol induced liver damage in rats. Pretreatment with fractions (toluene, 1-butanol, aqueous at 50, 100 mg/kg. b.w) significantly reversed the changes in serum biochemical parameters and histology of liver caused by the three hepatotoxins namely CCl_4, paracetamol and ethanol indicating their hepatoprotective activity.

Suja *et al*. (2014) studied the ethanolic extract of *Helminthostachys zeylanica* rhizome for antihepatotoxicity. *In vitro* primary rat hepatocyte culture studies with *H. zeylanica* showed significant hepatoprotection as evidenced by increased cell viability, lowering of hepatic enzyme levels ALT and AST. *In vitro* $FeCl_2$–ascorbic acid induced lipid peroxidation studies revealed the free radical scavenging effect of *H. zeylanica*. *In vivo* ethanol induced hepatotoxicity studies with *H. zeylanica* in wistar rats substantiates the above results as indicated by decreased levels of ALT, AST, ALP, total cholesterol, total lipids, triglycerides, iron and copper in serum.

Species of *Selaginella* are drought tolerant species and are usually called as resurrection plants. Numerous studies have been carried out to know the drought tolerance mechanisms, which are the basic principles of medicinal properties. *Selaginella* species are rich in the presence of variety of flavonoids, particularly bioflavonoids. *Selaginella* species also have large number of bioactive compounds viz., amentoflavone, 2',8"-biapigenin, delicaflavone, ginkgetin, heveaflavone, hinokiflavone, taiwaniaflavone, isocryptomerin, kayaflavone, ochnaflavone, podocarpusflavone A, robustaflavone and sumaflavone (Setyawan, 2011).

The above review on the distribution, phytochemistry, bioactivities and pharmacology of various species of *Selaginella* shows that this primitive *Selaginella* has a very high chemical diversity. The available literature indicated that research on *Selaginella* genus is very limited. In

Indian species of *Selaginella* only very few reprints are available in the phytochemistry and biopotentials. Hence in the present investigation, an attempt has been made to study the histochemical, phytochemical and biological activites of *S. intermedia, S. inaequalifolia* and *S. tenera* from Western Ghats, South India.

3. MATERIALS AND METHODS

To fulfill the objectives of the present study, the following *Selaginella* species viz., *Selaginella intermedia* (Bl.) Spring, *Selaginella inaequalifolia* (Hook. & Grev.) Spring and *Selaginella tenera* (Hook. & Grev.) Spring were collected from natural habitats of South India. Histochemical studies were made on the fresh sections of the stipe treated with the following reagents to identify the presence or absence of metabolites such as phenol, lignin, cutin, suberin and tannin. Lugol's iodine was used to detect tannins, cutin, suberin and lignins. Preliminary phytochemical analysis was performed by the method described by Harborne (1998). To reveal the inter-specific variation among the selected *Selaginella* species, various histochemical and phytochemical analysis were carried out viz., UV-Vis, FTIR, TLC, HPTLC, HPLC and GC-MS. To know the pharmacological potential of the selected *Selaginella* species, various biological activities viz., antioxidant, larvicidal activity, hepatoprotective, antidiabetic and cytotoxic activites using cell line culture and brine shrimp lethality test were carried out. The detailed methodologies of the investigations are as follows.

Collection of plants

Healthy, disease free *S. intermedia, S. inaequalifolia* and *S. tenera* were collected from Upper Kothayar, Tirunelveli Hills, Western Ghats, South India during the month of July 2011 (Plate I and II). The plants were identified based on the "Pteridophyte Flora of the Western Ghats, South India" by Manickam and Irudayaraj (1992). Herbarium specimens were prepared 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 (*S. intermedia* – XCH 25436; *S. inaequalifolia* – XCH 25437 and *S. tenera* – XCH 25438).

Histochemical analysis

Fresh free hand sections were made from the stem of *S. intermedia, S. inaequalifolia* and *S. tenera*. Histochemical tests were made on the fresh sections of the stem treated with the following reagents to identify the presence or absence of metabolites viz., phenol, lignin, cutin, suberin and tannin. Lugol's iodine was used to detect tannins and lignins (Haridass and Suresh Kumar, 1985; Chamberlin, 1924), 10% sodium nitrite, 20% urea and 10% acetic acid were used to detect phenolics (Reeve, 1951). The stained sections were observed under Motic trinocular microscope (Japan). They were photographed at different magnifications and at different views. The results were recorded on a photomicrograph. Based on the photographs taken, localization of tested metabolites was recorded.

Phytochemical analysis

Preparation of extracts

The collected species of *S. intermedia, S. inaequalifolia* and *S. tenera* were thoroughly washed with tap water followed by distilled water. The washed *Selaginella* species were blotted on the blotting paper and spread out at room temperature in shade to remove the excess water contents. The shade dried plant samples were ground to fine powder using mechanical grinder. The powdered samples were stored in refrigerator for further use. 30 g of powdered materials of *Selaginella* species were extracted successively with 180 ml of petroleum ether, chloroform, acetone and ethanol using Soxhlet extractor for 8 h at a temperature not exceeding the boiling point of the solvent. The extracts were filtered using Whatman filter paper (No.1) and then concentrated in vacuum at 40ºC using rotary evaporator. The aqueous extracts were prepared directly by boiling the powder with distilled water for 3 h and filtered. The residues obtained were stored in a freezer until further tests.

Qualitative phytochemical screening

Different extracts of selected *Selaginella* species were tested for the presence or absence of steroids, phenolic compounds, alkaloids, cardiac glycosides, saponins, tannins, amino acids, flavonoids and anthraquinones according to the method described by Harborne (1998). Based on preliminary phytochemical analysis, similarity indices were estimated according to Nei and Li (1979) and a cladogram was constructed by UPGMA using NTSyspc 2.0 software.

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	Occurrence of Steroids
2 ml of test solution + few volume of 2N HCl + few drops of Mayer's reagent	White precipitate is formed	Presence of Alkaloids
2 ml of test solution in alcohol + 1 drop of 5% $FeCl_3$solution	Deep blue colour is formed	Occurrence of Phenolic compounds
2 ml of test solution + 1 drop of $FeCl_3$ + 2 ml glacial acetic acid + H_2SO_4	Yellow colour is formed	Existence of 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	Presence of Flavonoids
2 ml of test solution + 2 ml of H_2O and shake vigorously	Foamy layer is formed	Occurrence of Saponins
2 ml of test solution + H_2O + lead acetate	White precipitate is formed	Existence of Tannins
2 ml of test solution + Magnesium acetate solution	Pink colour is formed	Presence of Anthraquinone
2 ml of test solution + 1% Ninhydrin in alcohol	Blue or violet colour is formed	Occurrence of Aminoacids

Fluorescence analysis

To reveal the fluorescence properties of selected *Selaginella* species, the crude powder and various extracts of *S. intermedia, S. inaequalifolia* and *S. tenera* were examined under visible and UV light. The crude powders were also treated with various reagents such as Con. H_2SO_4, Con. HCl, CH_3COOH, NaOH and 5% $FeCl_3$ and changes in colour were recorded (Indian Pharmacopoeia, 1996).

UV-Vis spectroscopic analysis

To find out the metabolites present in the crude extracts, the UV-Vis spectroscopic analysis was carried out using Shimadzu spectrophotometer. Different extracts of *S. intermedia, S. inaequalifolia* and *S. tenera* were centrifuged at 3000 rpm for 10 min and filtered through Whatman No. 1 filter paper. The sample was diluted to 1:10 with the same solvent. The filtered extracts were scanned in the wavelength ranged from 200 - 1100 nm using Shimadzu spectrophotometer and the characteristic peaks were observed and recorded. The UV-Vis analysis was repeated twice and the spectrum was confirmed. The peak values of UV-Vis spectra were used to distinguish the selected *Selaginella* species. To reveal the inter-specific similarities and variation among the selected three *Selaginella* species, the UV-Vis spectral profile was converted into "1" and "0" matrix, to indicate the presence or absence of the absorbance in the particular nanometer. UV-Vis spectral similarities were estimated according to Nei and Li (1979) and a cladogram was constructed by UPGMA using NTSYSpc 2.0 software.

FT-IR spectroscopic analysis

FT-IR analysis was performed using Shimadzu 8400S Spectrophotometer system. About 1.0 mg of the crude powder of *S. intermedia, S. inaequalifolia* and *S. tenera* 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 635.64 to 3425.58 cm^{-1}. The peak values of the FT-IR were recorded. Based on the Mistry (2009) guidelines, the functional groups were predicted. To reveal the inter-specific similarities and variations among the studied *Selaginella* species, the FT-IR profile was converted in to "1" and "0" matrix, to indicate the presence or absence of peak values functional compounds or groups. FT-IR similarities were estimated according to Nei and Li (1979) and a cladogram was constructed by UPGMA using NTSyspc 2.0 software.

TLC analysis

To reveal the phenolic and steroids profile of *S. intermedia, S. inaequalifolia* and *S. tenera*, TLC analysis was performed on 10 × 20 cm silica gel plates (Merck, Germany). Aliquots of various extracts of *Selaginella* were spotted on the silica gel plate with a developing solvent system of chloroform / methanol (9:1 v/v) for phenolics and benzene and methanol (9:1 v/v) for steroids. The spots were visualized under a UV detector before and after spraying the plates with spraying reagents viz., Folin-Ciocalteau reagent for phenolics and 5% alcoholic sulphuric acid for steroids (Harborne, 1999). The phenolic and steroid profiles were recorded and the R_f values of the individual spot was calculated. To reveal the inter-specific similarities among the selected *Selaginella* species, the phenolic and steroids chromatographic profile was converted into "1" and "0" matrix, to indicate the presence or absence of phenolics and steroids. Phenolic and steroids profile similarities and variations were estimated according to Nei and Li (1979). To illustrate the inter-specific relationship, a cladogram was constructed by UPGMA using NTSyspc 2.0 software.

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

HPTLC analysis

Test solution preparation

25 mg of ethanolic extracts of *S. intermedia, S. inaequalifolia* and *S. tenera* were weighed accurately in an electronic balance (Shimadzu) and dissolved in 0.5 ml of ethanol and centrifuged at 3000 rpm for 5 min. The supernatants were used as test solution for alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and terpenoids.

Sample application

2 µl of *S. intermedia, S. inaequalifolia* and *S. tenera* ethanolic extracts 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 up to 90 mm (Table 2).

Photo documentation

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

Derivatization

The developed plate was sprayed with respective spraying 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.

Table 2: Mobile phases and spraying reagents used 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

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 win CATS 1.3.4 version.

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

HPLC analysis

To know the functional compounds present in the ethanolic extracts of the selected *Selaginella* species, HPLC analysis was performed on a Shimadzu LC-10AT VP HPLC system, equipped with a model LC-10AT pump, UV-Vis detector SPD-10AT, Rheodyne injector fitted with a 20 µl loop and auto injector SIL-10AT. A Hypersil BDS C-18 column (4.6 × 250 mm, 5 µm size) with a C-18 guard column was used. The elution was carried out with gradient solvent systems with a flow rate of 1 ml min-1 at ambient temperature (25-28°C). The mobile phase consisted of 0.1% v/v methanol (solvent A) and water (solvent B). The mobile phase was prepared daily, filtered through a 0.45 µm and sonicated before use. Total running time was 15 min. The sample injection volume was 20 µl while the wavelength of the UV-Vis detector was set at 254 nm (Sharanabasappa *et al*., 2007; Mallikharjuna *et al*., 2007).

GC-MS analysis

To reveal the chemical constituents present in the selected *Selaginella* species, GC-MS analysis was performed using the Clarus 500 GC-MS (Perkin Elmer). 2 µl of selected *S. intermedia, S. inaequalifolia, S. tenera* ethanolic extracts were injected for GC-MS analysis (Merlin *et al.,* 2009). The Clarus 500 GC used in the analysis employed a fused silica column

packed with Elite-1 (100% dimethyl poly siloxane, 30 nm × 0.25 nm ID × 1 µm df) and the components were separated using helium as carrier gas at a constant flow of 1 ml/min. 2 µl sample extract injected into the instrument was detected by the Turbo gold mass detector (Perkin Elmer) with the aid of the Turbo mass 5.1 software. During the 36th 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: 2000^{0}C; source temperature: 2000^{0}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 detection employed the NIST (National Institute of Standards and Technology) Version 2.0-Year 2005 library (Sangeetha and Vijayalakshmi, 2011). Interpretation of GC-MS was conducted using the database of NIST having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components present in the test materials were ascertained. Biological activities for the identified metabolites were predicted based on PASS online.

Biological activities

Antioxidant activity

DPPH radical scavenging activity

The antioxidant activity of *S. intermedia, S. inaequalifolia* and *S. tenera* various extracts were determined in terms of hydrogen donating or radical scavenging ability, using the stable

radical DPPH (Blios, 1958). Various aliquots (50, 100, 150, 200 µg/ml) of petroleum ether, chloroform, acetone and ethanolic extracts were 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 also calculated using MS-Excel 2007.

Antioxidant activity by the $ABTS^{+}$assay

7 mM ABTS was prepared with distilled water. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and the mixture was allowed to stand in dark at room temperature for 12-16 hr before use. Prior to assay, the solution was diluted in ethanol (about 1:89 v/v) and equilibrated to 30°C to give an absorbance at 734 nm of 0.70 ± 0.02 in a 1 cm cuvette (Re *et al*., 1999). The concentration of the extracts that produced between 20-80% inhibitions of the blank absorbance was determined and adapted. After the addition of 1 ml of diluted $ABTS^{+}$ solution to 100 µl of different extracts of *S. intermedia, S. inaequalifolia, S. tenera* and Trolox standards (final concentration 0-15 µM) in ethanol, optical density (OD) was measured at 734 nm exactly 30 min after the initial mixing. The unit of total antioxidant activity (TAA) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as µM TE/g sample extracts on dry matter.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity of petroleum ether, chloroform, acetone and ethanolic extracts of *S. intermedia, S. inaequalifolia* and *S. tenera* was estimated according to the standard procedure (Pulido *et al.*, 2000). FRAP reagent (900 µl), prepared freshly and incubated at 37^{0}C,

was mixed with 90 µL of distilled water and 20 µl of test sample or methanol (for the reagent blank). The test samples and the blank reagent were incubated at 37^0C for 30 min in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 ml of 20 µM/L TPTZ solution in 40 µM/l HCl plus 2.5 ml of 20 µM/l $FeCl_3 \cdot 6H_2O$ and 25 ml of 0.3 M/l acetate buffer (pH 3.6) (Siddhuraju and Becker, 2003). At the end of incubation, the absorbance readings were taken immediately at 593 nm using a spectrophotometer. Methanolic solutions of known Fe (II) concentration, ranged from 100 to 2000 µM/l, ($FeSO_4 \cdot 7H_2O$) were used for the preparation of the calibration curve. The parameter equivalent concentration (EC_1) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 µmol/L $FeSO_4.7H_2O$. EC_1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 µM/l concentration of Fe (II) solution, determined using the corresponding regression equation.

Metal chelating activity

The chelating of ferrous ions by *S. intermedia, S. inaequalifolia* and *S. tenera* extracts were estimated by the standard method (Dinis *et al.*, 1994). The extract of selected *Selaginella* species (100 µl) were added to a solution of 2 mM/l $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 µM/l ferrozine (0.2 ml). The mixture was shaken vigorously and kept at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent/g extract.

Phosphomolybdenum assay

The antioxidant activities of the selected *Selaginella* species were evaluated by the green phosphomolybdenum complex formation according to the standard method (Prieto *et al.*, 1999). Triplicate of 100 µL of various extracts of *Selaginella* species (in 1 mM dimethyl sulfoxide, DMSO) was combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) in a 4 mL vial. The vials were capped and incubated in a water bath at 95^{o}C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The results reported (ascorbic acid equivalent) was mean values expressed as g of ascorbic acid equivalents/100 g extract.

Statistical analysis

The data obtained from the antioxidant assays were represented as means of triplicate determinations ± standard deviation (SD).

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, P^{H} 7.4), supplemented with 10% FBS and antibiotics, penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml).

MTT assay

The cytotoxicity of *S. intermedia, S. inaequalifolia* and *S. tenera* ethanolic extracts against human breast carcinoma (MCF 7) was determined by the MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) 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 *S. intermedia, S. inaequalifolia* and *S. tenera* (12.5, 25, 50, 100 and 200 µg/ml) were added. Five 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 *S. intermedia, S. inaequalifolia* and *S. tenera* were evaluated using brine shrimp lethality bioassay method (Meyer *et al*., 1982). About 1 g of *Artemia salina* cysts was aerated in 1L 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 ranged from 100-500 mg/ml and five tubes

for control. With the help of a Pasteur pipette, 20 nauplii were transferred to each tube containing various concentrations of petroleum ether, chloroform, acetone and ethanolic extracts of *S. intermedia, S. inaequalifolia* and *S. tenera*. 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.

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 *S. intermedia, S. inaequalifolia* and *S. tenera* 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 ppm) 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).

Pharmacological activities

Hepatoprotective Activity

Animals

Adult male wistar rats with 200-250g weight were obtained from Sankaralingam Bhuvaneswari College of Pharmacy, Sivakasi, Tamil Nadu. They were maintained at standard housing conditions and fed with commercial diet and provided with water ad libitum during the experiment. The institutional animal ethical committee (Reg. No. SBCP / 2015 – 16 / CPCSEA / IAEC – I / 1(m) permitted the study.

Experimental Design

Four groups of six animals were used for the study. Control group received single daily dose of 5% tween 80 (5 ml/kg; po) for 4 days and a single dose of 40% sucrose solution (1 ml/rat; po) on day 3. Paracetamol group received single daily dose of 5% tween 80 (5 ml/kg; po) for 4 days and a single dose of paracetamol suspension (2 g/kg, po) on day 3. Test groups received daily doses (400 mg/kg) of *S. intermedia, S. inaequalifolia* and *S. tenera* ethanolic extract for 4 days and single dose of paracetamol suspension on day 3. Animals were sacrified under light ether anaesthesia, 48 hour after paracetamol administration.

Biochemical Study

Animals were sacrificed by cervical dislocation. The blood samples were collected by direct cardiac puncture. The blood samples were allowed to clot and serum were separated and the serum was used for the assay of maker enzymes viz., Glutamate oxaloacetate transaminase (SGOT), Glutamate pyruvic transaminase (SGPT) (Reitman *et al*., 1957) and alkaline phosphatase (ALP) (Bessey *et al.,* 1964).

Anti-diabetic (Oral Glucose tolerance test)

All the mice were divided into 6 groups, each group containing 4 mice. The divided groups are Group I (NC - normal control), Group II (DC - Diabetic control), Group III (DS - diabetic mice receiving gibenclamide), Group IV – VI (*S. intermedia, S. inaequalifolia* and *S. tenera* ethanolic extract treated diabetic mice). Diabetes was induced to all groups except NC by intra-peritoneal injection (IP) of STZ (50 mg STZ/kg body weight for five consecutive days). The blood glucose levels were measured from tail-vein blood of all groups by glucometer. Mice with blood glucose level above 200 mg/dl were considered as diabetic and were recorded as 0 min. Without delay, glucose solution (2 gm/kg b.w) was administered to all groups by gastric tube orally. At the same time 100 mg/kg body weight each of standard drug gibenclamide, ethanolic extract of *S. intermedia, S. inaequalifolia* and *S. tenera* were administered orally to respective groups. The blood glucose level was measured after 30 min, 60 min, 90 min and 120 min (Hossain *et al.,* 2011)

Blood samples were collected from the vein of the mice and the blood glucose level was tested with glucometer according to the manufacturer's protocol. The glucose level was expressed in percentage (%) change from the initial glycaemia using the following formula: % glycaemia change = (Gx – Go) / Go X 100

G_o is the glycaemia value at time zero after overnight fasting; Gx is the glycaemia value at x minutes after vehicle or extract administration.

To reveal the phytochemical composition of three selected species viz., *Selaginella intermedia, Selaginella inaequalifolia* and *Selaginella tenera,* histochemical, preliminary phytochemical analysis, fluorescence analysis, spectroscopic and chromatographic analysis such as UV-Vis, FT-IR, TLC, HPLC, HPTLC and GC-MS were carried out. In addition, to know biological potentials of *S. intermedia, S. inaequalifolia* and *S. tenera,* antioxidant, cytotoxicity using brine shrimp lethality, anticancer using MCF cell line culture, larvicidal, antidiabetic and hepatoprotective activities were examined.

Histochemical analysis

The histochemical studies revealed the presence of phenols, tannin, cutin, suberin and lignin in the three *Selaginella* species with varied percentage (Table 3). Phenols showed their occurrence in all the three studied species. In *S. intermedia,* higher concentration of phenols was present in the endodermis and phloem (Plate III A). Lower concentration of phenols was observed in the ground tissue and xylem of *S. intermedia* (Plate III B). *S. inaequalifolia* expressed maximum concentration of phenols only in the epidermis (Plate III F) and minimum concentrations were illustrated in the all other parts (Plate III F). *S. tenera* showed high concentration of phenols in the epidermis, hypodermis and xylem (Plate III J). In ground tissue and endodermis of *S. tenera,* minimum concentrations of phenols were observed (Plate III J).

Tannins showed their occurrence in all the three studied species. *S. intermedia* expressed maximum concentration of tannin in the epidermis only (Plate III C). In *S. inaequalifolia,* high concentrations of tannins were observed in the epidermis (Plate III G), while minimum amount of tannin was illustrated in the hypodermis, xylem and phloem (Plate III G). In *S. tenera*, tannin

showed minimum concentration in epidermis and hypodermis (Plate III K). Tannin was absent in all other parts of *S. tenera* (Plate III K).

S. intermedia expressed maximum concentration of lignin, cutin and suberin in the epidermis and xylem (Plate III D). Lignin, cutin and suberin showed the minimum amount of occurrence in the hypodermis and phloem of *S. intermedia* (Plate III E). Lignin, cutin and suberin demonstrated high concentration of existence in the epidermis, hypodermis and xylem of *S. inaequalifolia* (Plate III I). Minimum concentration of lignin, cutin and suberin were present in the ground tissue and phloem of *S. inaequalifolia* (Plate III H). *S. tenera* expressed minimum concentration of lignin, cutin and suberin presence in the epidermis, hypodermis and xylem (Plate III L).

Table 3: Histochemical studies on selected species of *Selaginella*

Parts	*S. intermedia*					*S. inaequalifolia*					*S. tenera*				
	P	**T**	**L**	**S**	**C**	**P**	**T**	**L**	**S**	**C**	**P**	**T**	**L**	**S**	**C**
Epidermis	-	+	3^+	2^+	3^+	3^+	3^+	3^+	3^+	2^+	3^+	2^+	2^+	+	3^+
Hypodermis	-	-	2^+	3^+	2^+	2^+	2^+	3^+	2^+	3^+	3^+	2^+	2^+	3^+	+
Ground tissue	2^+	-	-	-	-	2^+	-	2^+	3^+	2^+	2^+	-	-	-	-
Endodermis	3^+	-	-	-	-	2^+	-	-	-	-	2^+	-	-	-	-
Xylem	2^+	-	3^+	3^+	2^+	2^+	2^+	3^+	2^+	+	3^+	-	2^+	+	3^+
Phloem	3^+	-	+	2^+	3^+	2^+	2^+	2^+	+	3^+	-	-	-	-	-

P: Phenol; T: Tannin; L: Lignin; S: Suberin; C: Cutin
[+ - Mild, 2^+ - average, 3^+- high, - data not available]

Preliminary phytochemical analysis

Preliminary phytochemical screenings of nine different metabolites were tested in four different extracts of *S. intermedia, S. inaequalifolia* and *S. tenera*. The results showed the

presence of steroids, alkaloids, phenolics, flavonoids, saponins, tannin, cardiac glycosides, aminoacids and anthraquinone in the studied three species of *Selaginella* (Table 4).

Among the four extracts of *S. intermedia*, the ethanolic extract of *S. intermedia* showed the presence of more frequency (88%) of metabolites followed by chloroform extracts (55%) and acetone extracts (44%). The least percentage of metabolites (22%) was observed in petroleum ether extracts of *S. intermedia* (Table 4; Fig. 1).

Among the tested extracts of *S. inaequalifolia,* the ethanolic and chloroform extracts of *S. inaequalifolia* showed highest percentage of metabolites (55%) existence. Subsequently, acetone extract showed the occurrence of metabolites with 44% and lowest percentage (11%) of metabolites presence was reported in the petroleum ether extracts of *S. inaequalifolia* (Table 4; Fig. 1).

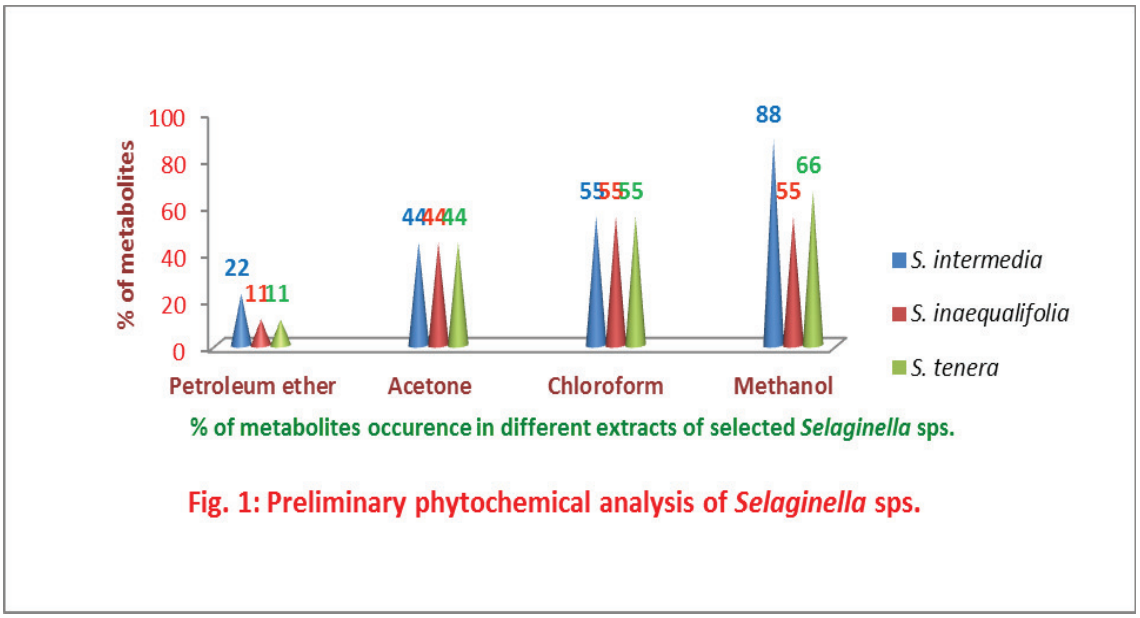

Fig. 1: Preliminary phytochemical analysis of *Selaginella* sps.

Among the four screened extracts of *S. tenera*, maximum frequencies (60%) of metabolites were observed in the ethanolic extracts of *S. tenera*. Next to that, the chloroform extracts of *S. tenera* showed 55% percentage of metabolites occurrence, followed by acetone

extracts (44%). The petroleum ether extracts of *S. tenera* illustrated only eleven percentage of metabolites presence (Table 4; Fig. 1).

Table 4: Preliminary phytochemical screening of various extracts of *S. intermedia*, *S. inaequalifolia* and *S. tenera*

Metabolites	*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
	P	C	A	E	P	C	A	E	P	C	A	E
Steroids	+	+	+	+	+	+	+	+	+	+	-	+
Alkaloids	-	-	-	+	-	-	-	-	-	-	-	+
Phenolic groups	-	+	+	+	-	-	+	+	-	+	+	+
Flavonoids	-	+	-	+	-	+	-	+	-	-	+	+
Saponin	-	-	-	+	-	-	-	+	-	+	-	+
Anthraquinone	-	-	+	+	-	+	-	-	-	+	+	-
Tannins	+	+	-	+	-	+	+	+	-	-	+	+
Aminoacids	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	-	+	+	+	-	+	+	-	-	+	-	-

P-Petroleum ether; C-Chloroform; A-Acetone; E-Ethanol

The cladogram constructed based on the existence of phytoconstituents in the *Selaginella* species showed two clusters. Cluster 1 (C_1) included *S. intermedia* only and showed 100% of divergence from other two studied species (Fig. 2). Cluster 2 (C_2) was shared by *S. inaequalifolia* and *S. tenera.*

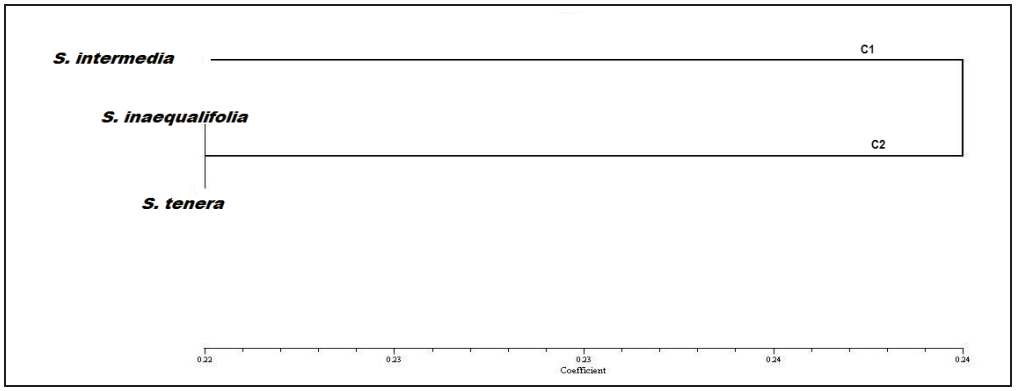

Fig. 2: UPGMA cladogram based on preliminary phytochemical analysis of studied *Selaginella* species

Fluorescence analysis

The fluorescent characteristics of the *Selaginella* species were observed under UV and day light. The results were depicted in the Fig. 3.

Treatment	*S. intermedia*		*S. inaequalifolia*		*S. tenera*	
	Visible light	UV light (365 nm)	Visible light	UV light (365 nm)	Visible light	UV light (365 nm)
Powder	Green	Brown	Green	Brown	Green	Brown
Petroleum ether extract	Light yellow	Light green	Dark green	Dark brown	Dark green	Dark red
Chloroform extract	Brown	Dark brown	Dark green	Brownish green	Dark green	Reddish brown
Acetone extract	Light green	Dark green	Light green	Green	Light green	Green
Ethanol extract	Light green	Green	Light green	Green	Light green	Green
Aqueous extract	Green	Dark green	Light green	Dark green	Green	Dark green
Powder+ H_2SO_4	Dark brown	Reddish brown	Brown	Dark brown	Dark green	Dark brown
Powder+ HCL	Brownish green	Pale green	Dark green	Reddish brown	Light green	Reddish brown
Powder+ Na OH	Yellowish green	Green	Yellowish green	Dark green	Green	Bluish green
Powder+ CH_3COOH	Light green	Dark brown	Green	Light yellow	Light green	Brown
Powder+ $FeCl_3$	Dark green	Dark green	Dark brown	Reddish brown	Light green	Dark brown

Fig. 3: Fluorescent characters of studied *Selaginella* species

UV-Vis analysis of *S. intermedia*

UV-Vis spectrum showed two diverse regions in the visible and UV region. UV region ranges from 200-340 nm where as visible region ranges from 380-900 nm. The qualitative UV-Vis fingerprint profile of different extracts of *Selaginella* species were selected at the wavelengths from 190 to 1100 nm due to sharpness of the peaks and proper baseline. The absorbance reveals the concentration of compounds present in the expressed nanometer. Among

the four different extracts of *S. intermedia* examined, petroleum ether extract of *S. intermedia* exhibited nine peaks which denotes the presence of greatest number of metabolites / compounds compared to other extracts followed by acetone (8 peaks), chloroform (4 peaks) and ethanolic extract showed least number of (3) peaks. The petroleum ether extract of *S. intermedia* showed the highest absorbance 3.311 at 404 nm (Plate IV: A) in the UV region and chloroform extract showed lowest absorbance 0.005 (Plate IV: B) in the UV region at 1021 nm (Table 5; Plate IV).

Table 5: UV-Vis analysis of *S. intermedia*

Petroleum ether		Chloroform		Acetone		Ethanol	
λ_{max}	**ABS**	**λ_{max}**	**ABS**	**λ_{max}**	**ABS**	**λ_{max}**	**ABS**
1051	0.092	1021	0.005	1071	0.054	1022	0.008
840	0.104	965	0.009	953	0.058	1004	0.009
695	1.006	653	0.306	789	0.09	658	0.082
669	1.05	600	0.152	667	1.88		
611	0.272			608	0.556		
540	0.493			539	0.76		
504	0.463			507	0.789		
404	3.311			399	4.000		
305	2.613						

UV-Vis analysis of *S. inaequalifolia*

Ethanolic extract of *S. inaequalifolia* showed highest absorbance (4.000) at 317 nm (Plate V: D) and the lowest absorbance was observed in the ethanolic extract (0.007) at 1023 nm. Among the tested extracts of *S. inaequalifolia*, petroleum ether extract of *S. inaequalifolia* exhibited 7 peaks which denotes the presence of greatest number of metabolites / compounds compared to other extracts followed by chloroform and acetone (6 peaks) and ethanolic extract showed least number (4) of peaks. The acetone extract of *S. inaequalifolia* represented highest absorbance 2.872 at 663 nm in the visible region and chloroform extract showed highest

absorbance (4.000) at 288 nm (Plate V: B) and the lowest absorbance 0.016 at 954 nm **(**Table 6; Plate V).

Table 6: UV-Vis analysis of *S. inaequalifolia*

Petroleum ether		Chloroform		Acetone		Ethanol	
λ_{max}	**ABS**	**λ_{max}**	**ABS**	**λ_{max}**	**ABS**	**λ_{max}**	**ABS**
1049	0.018	954	0.016	1022	0.01	1023	0.007
695	0.872	666	2.533	966	0.007	655	0.033
668	1.119	606	0.777	663	2.872	599	0.014
611	0.219	537	1.04	604	0.835	317	4.000
539	0.42	508	1.049	533	1.114		
504	0.412	288	4.000	505	1.219		
405	0.374						

UV-Vis analysis of *S. tenera*

Petroleum ether extract of *S. tenera* expressed more peaks (8) followed by chloroform (6) and ethanolic (5) extracts. The acetone extracts depicted least number of peaks (4). Ethanolic extract of *S. tenera* represented highest absorbance of 4.000 at 315 nm (Plate VI: D) and the lowest absorbance of 0.002 at 1021 nm. Chloroform extract of *S. tenera* depicted absorbance of 2.019 at 665 nm (Table 7; Plate VI).

Table 7: UV-Vis analysis of *S. tenera*

Petroleum ether		Chloroform		Acetone		Ethanol	
λ_{max}	**ABS**	**λ_{max}**	**ABS**	**λ_{max}**	**ABS**	**λ_{max}**	**ABS**
1021	0.002	953	0.034	1021	0.005	1021	0.008
695	0.596	665	2.019	968	0.009	965	0.011
668	1.015	605	0.653	652	0.395	656	0.082
610	0.154	537	0.88	600	0.188	603	0.047
539	0.275	508	0.871			315	4.000
504	0.296	300	4.000				
406	2.853						
291	1.679						

The cladogram constructed based on UV-Vis spectroscopic profile of *Selaginella* species showed two clusters. Cluster 1 (C_1) included only *S. intermedia* and showed 100% of divergence from other two studied species. Cluster 2 (C_2) was shared by *S. inaequalifolia* and *S. tenera* (Fig. 4).

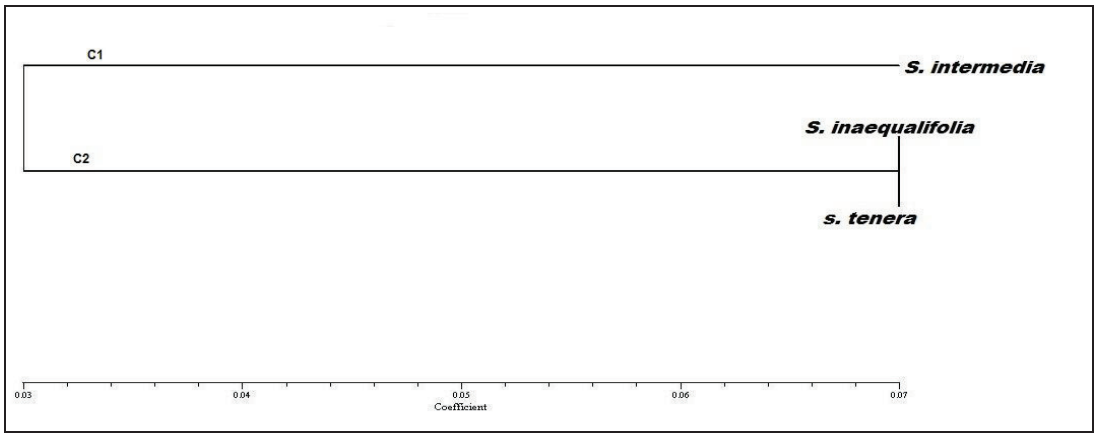

Fig. 4: UPGMA cladogram based on UV-Vis spectroscopic profile of studied *Selaginella* species

FT-IR spectroscopic profile

The FT-IR spectrum and peak values were used to identify the functional group of the active metabolites present in different extracts. The petroleum ether, chloroform, acetone and ethanolic extract of *S. intermedia, S. inaequalifolia* and *S. tenera* were passed into the FT-IR and the functional group were identified based on its peak ratio and compared with the different *Selaginella* species. The results are demonstrated in Table 8.

In the petroleum ether extract of studied *Selaginella* species, 16 different spectroscopic peak values were observed with varied range from 635.64 to 3425.58 (Table 8). The maximum number of functional groups was observed in *S. tenera* than other two studied species. The peak values 635.64, 655.80, 725.25, 736.96, 1172.72, 1373.72, 1465.90, 1658.78, 1720.50, 2276, 2345.44, 2368.50, 2854.65 and 2924.09 with the functional group alkynes, alkanes, alkyl halides,

aldehydes, saturated aliphatic, nitriles and aldehydes were present in the studied *Selaginella* species respectively. The petroleum ether extracts of *S. intermedia* and *S. inaequalifolia* displayed their similarity with the spectral peak value 1465.90 (alkanes). Similarly, *S. inaequalifolia* and *S. tenera* also showed jointly occurrence of spectroscopic peak value 2368.50 (aldehydes) and confirmed their phytochemical similarity (Fig. 5, 6 and 7; Table 8).

The chloroform extract of studied *Selaginella* species showed different spectroscopic peak values viz., 725.23, 1080.4, 1242.16, 1381.03, 1465.90, 1736.93, 2376.30 and 2854.65 (Fig 8, 9 & 10). The FT-IR analysis results confirmed the existence of various functional groups such as alkanes (C–H rock), aliphatic amines (C–N stretch), alcohols, carboxylic acids, esters, ethers (C–O stretch), alkanes (C–H bend), esters, saturated aliphatic (C=O stretch) and aldehydes (H–C=O: C–H) in the studied *Selaginella* species. The functional groups alkanes (C–H bend), esters, and saturated aliphatic (C=O stretch) showed their presence in all the three studied species of *Selaginella.* The functional groups aldehydes (H–C=O: C–H) showed their presence only in *S. inaequalifolia* (Table 8). The acetone extract of *S. intermedia, S. inaequalifolia* and *S. tenera* showed different spectroscopic peak values 635.64, 1373.32, 1435.04, 1458.18, 1635.64, 1705.07, 2345, 2368.50, 2854.65, 2924.09 and 3402 (Table 8). The spectroscopic peak values presence confirmed the existence of functional groups viz., alkynes (C≡C–H: C–H bend), alkanes (C–H rock), aromatics (C–C stretch (in–ring), alkanes (C–H bend), primary amines (N–H bend), alpha, beta–unsaturated aldehydes (C=O stretch), ketones (C=O stretch), aldehydes (H–C=O: C–H stretch), carboxylic acids (O–H stretch) and alkanes (C–H stretch) in the studied *Selaginella* species. The functional groups such as aldehydes (H–C=O: C–H stretch) and alkanes (C–H stretch) showed their jointly presence in acetone extract of *S. intermedia* and *S. inaequalifolia* (Fig. 11, 12 and 13; Table 8).

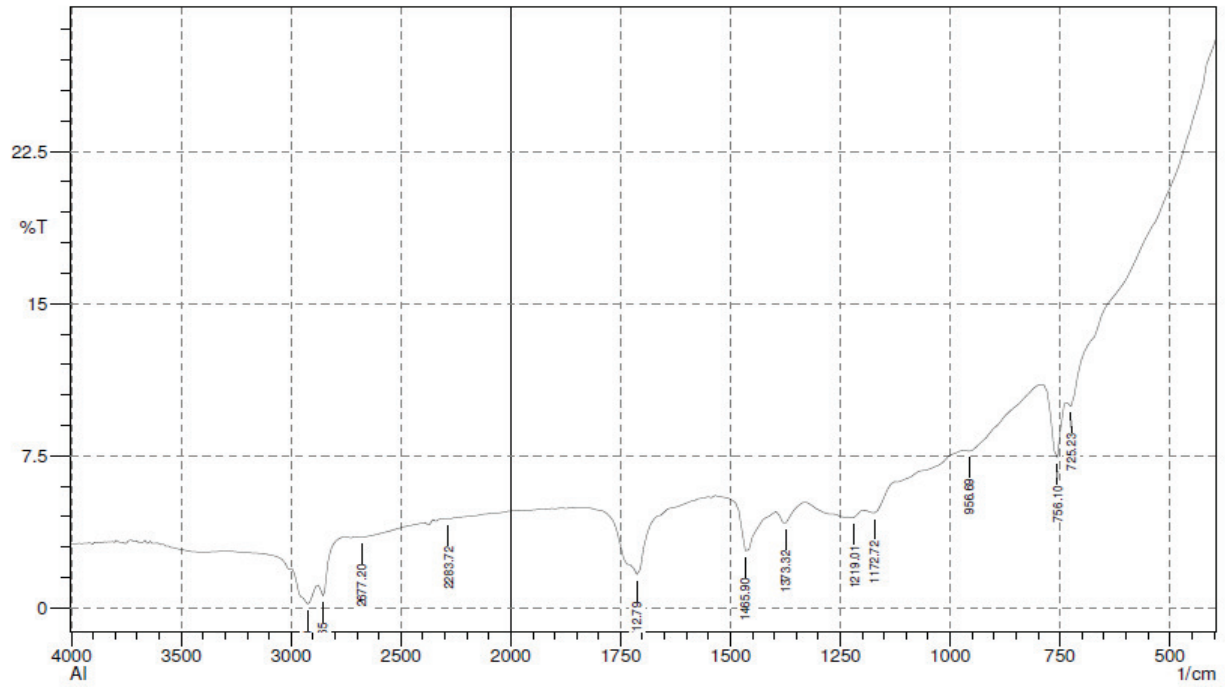

Fig. 5: FT-IR Spectrum of *Selaginella intermedia* petroleum ether extract

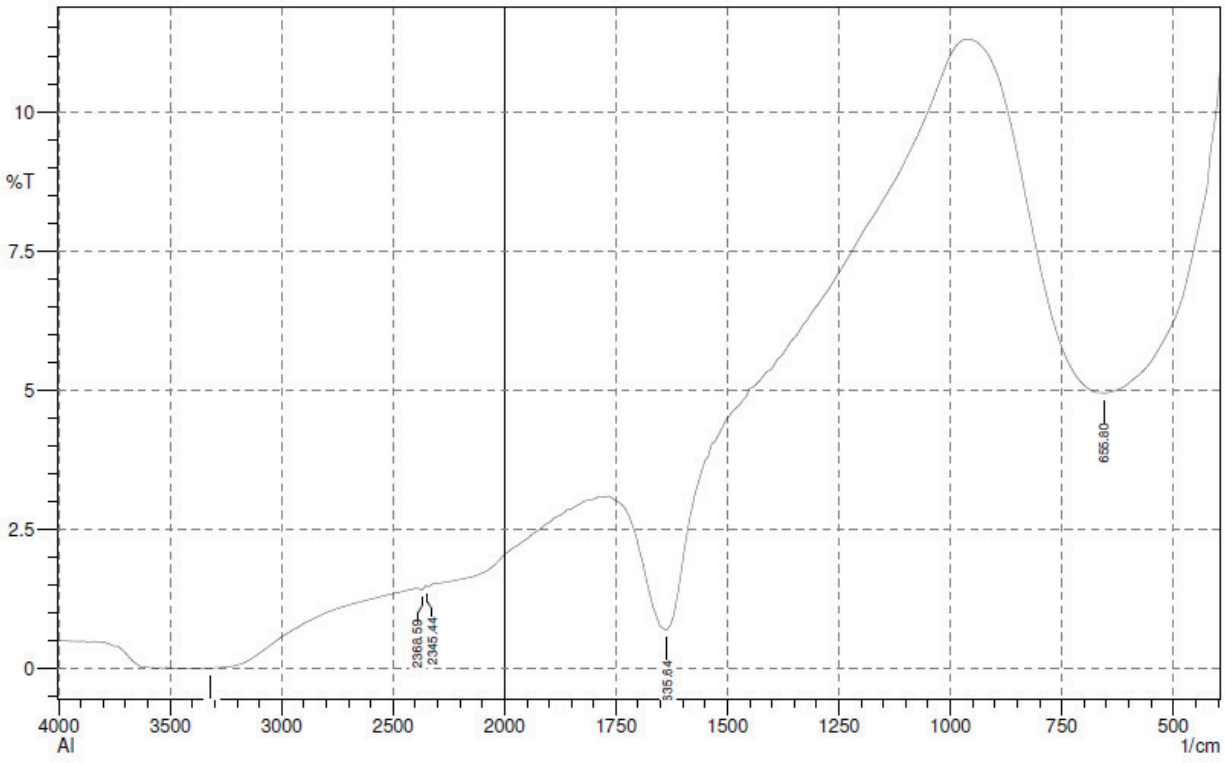

Fig. 6: FT-IR Spectrum of *Selaginella inaequalifolia* petroleum ether extract

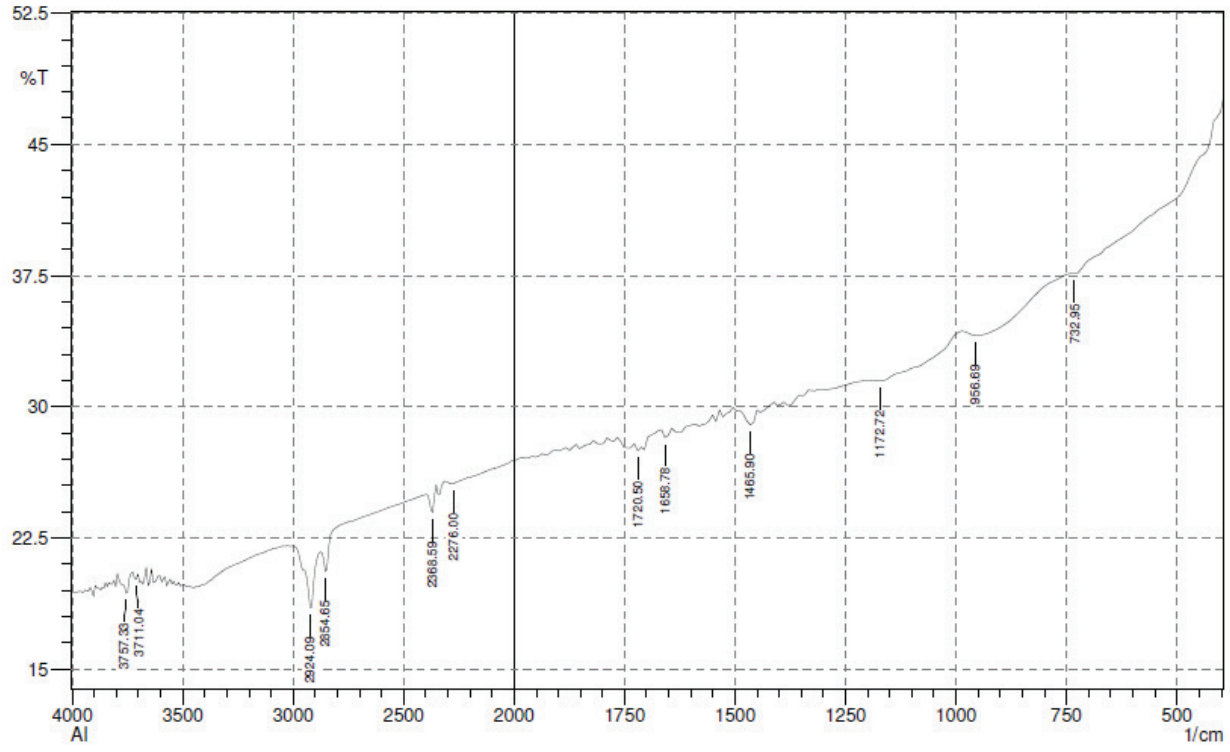

Fig. 7: FT-IR Spectrum of *Selaginella tenera* petroleum ether extract

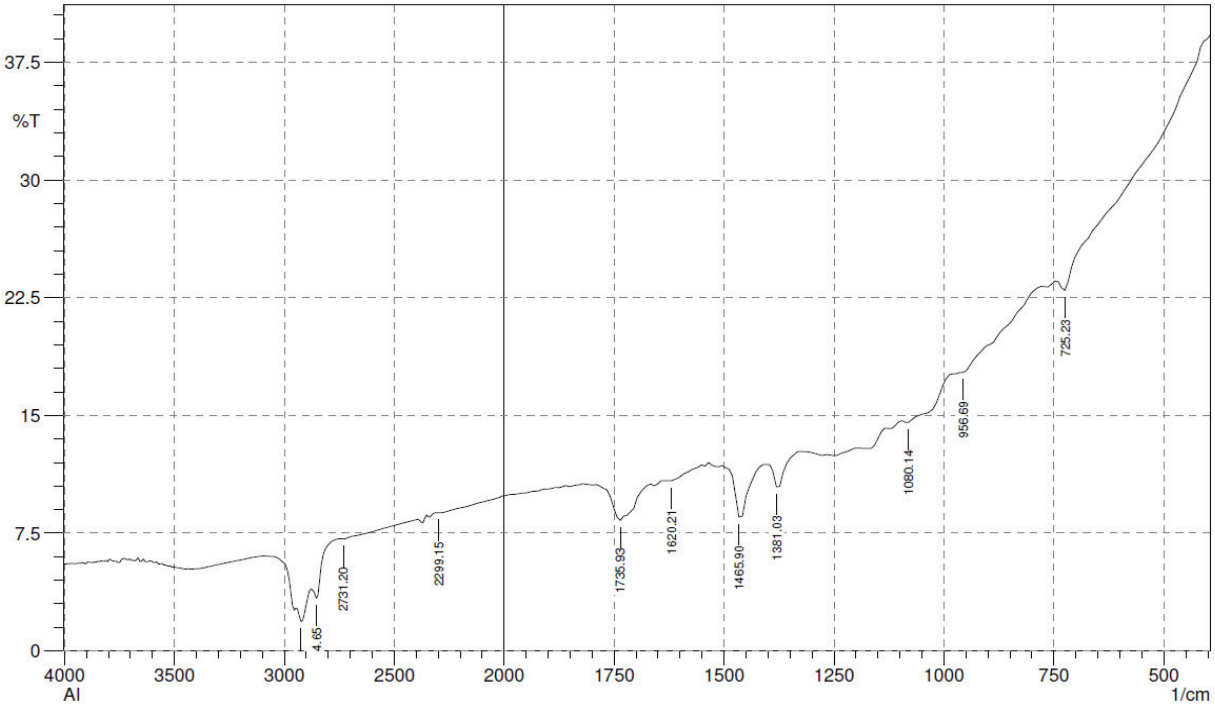

Fig. 8: FT-IR Spectrum of *Selaginella intermedia* chloroform extract

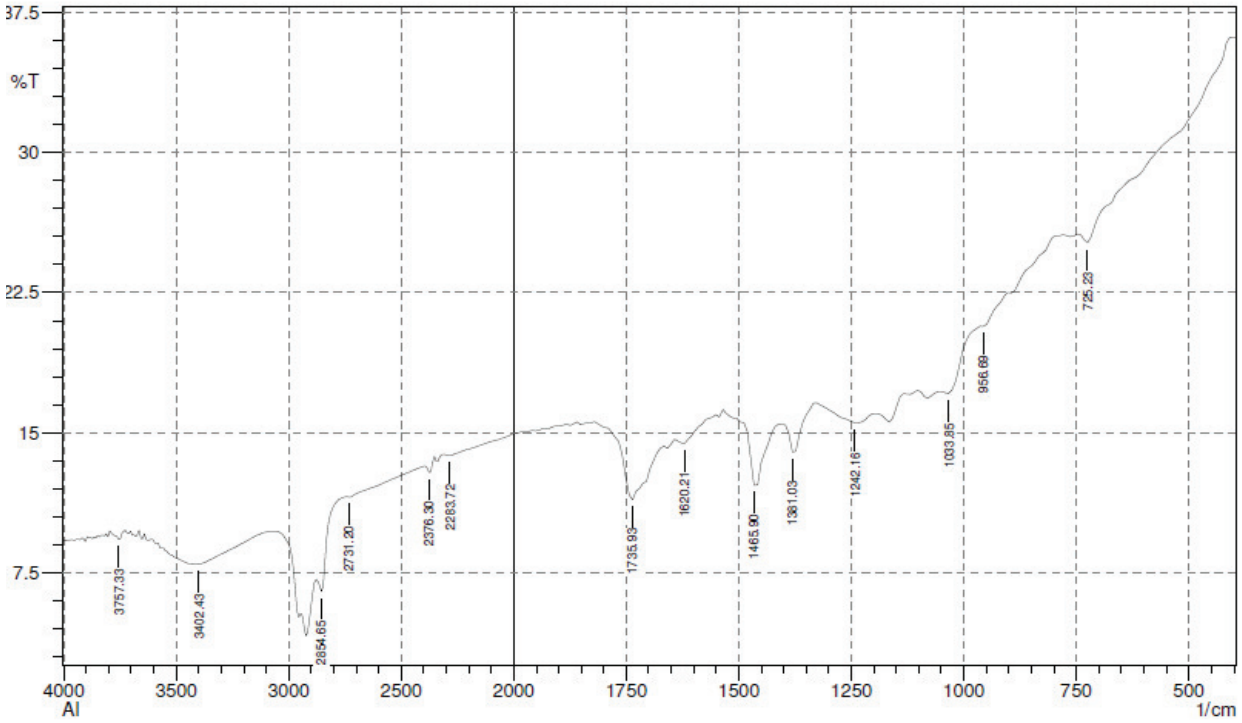

Fig. 9: FT-IR Spectrum of *Selaginella inaequalifolia* chloroform extract

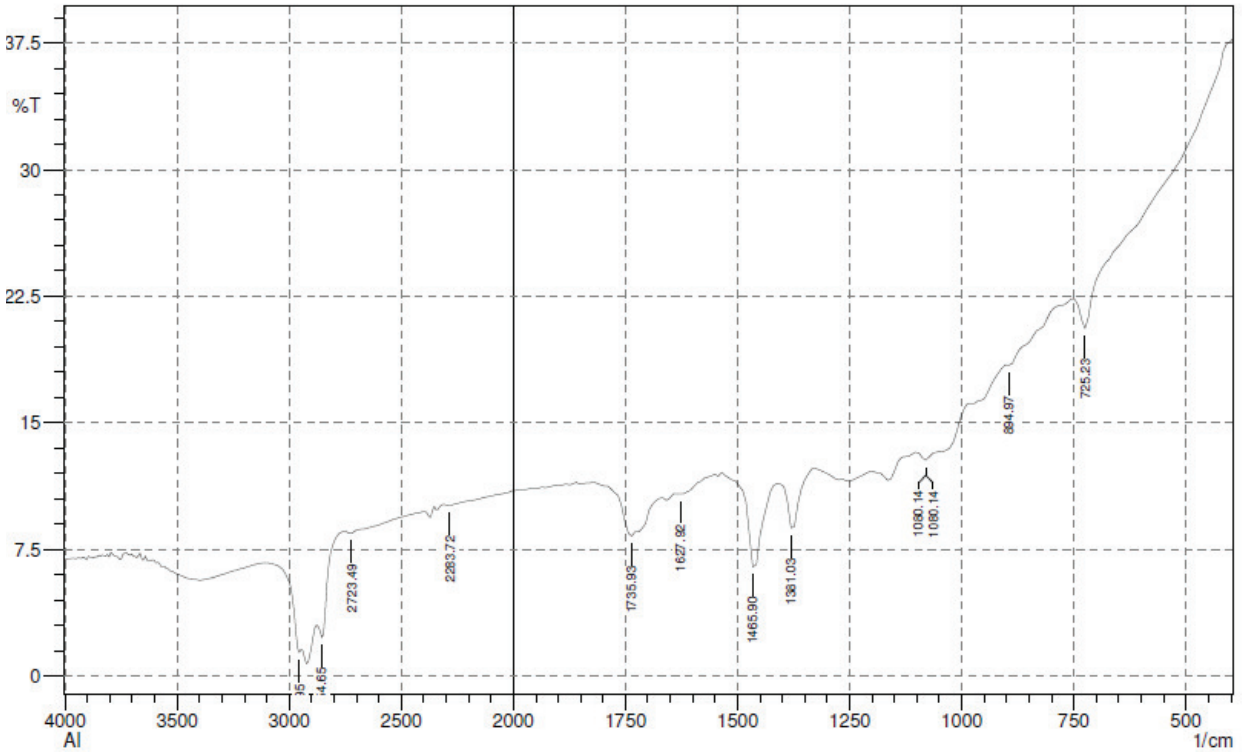

Fig. 10: FT-IR Spectrum of *Selaginella tenera* chloroform extract

In the ethanolic extract of studied *Selaginella* species, the presence of 22 different spectroscopic peak values were observed with varied range from 635.64 to 3425.58 (Table 8). The maximum number of functional groups was observed in *S. intermedia* (10) than other two studied species. The results showed different peak values viz., 671.23, 709.8, 918.12, 956.69, 1026.16, 1319.31, 1381.03, 1411.89, 1435.04, 1581.63, 1635.64, 1651.07, 1681.63, 1797.68, 2276, 2345.44, 2368.50, 2924.09, 3001.24 and 3425.58 (Fig.14, 15 and 16; Table 8) with the functional group alkynes (C≡C–H: C–H bend), alkyl halides (C–Cl stretch), carboxylic acids (O–H bend), alkenes (=C–H bend), aliphatic amines (C–N stretch), alcohols, carboxylic acids, esters, ethers (C–O stretch), alkanes (C–H rock), aromatics (C–C stretch (in–ring), primary amines (N–H bend), alkenes (–C=C– stretch), alpha, beta–unsaturated aldehydes, ketones (C=O stretch), carbonyls (general), nitriles (C(triple bond) N stretch), aldehydes (H–C=O: C–H stretch), alkanes (C–H stretch), alkenes (=C–H stretch), alcohols, phenols (O–H stretch, H–bonded) respectively (Fig. 14, 15 and 16; Table 8).

The cladogram constructed based on FT-IR peak values showed two clusters, of which Cluster 1 (C_1) was shared by *S. intermedia* and *S. inaequalifolia.* Cluster 2 (C_2) represented *S. tenera* which showed 100% of divergence from the other two studied species (Fig. 17).

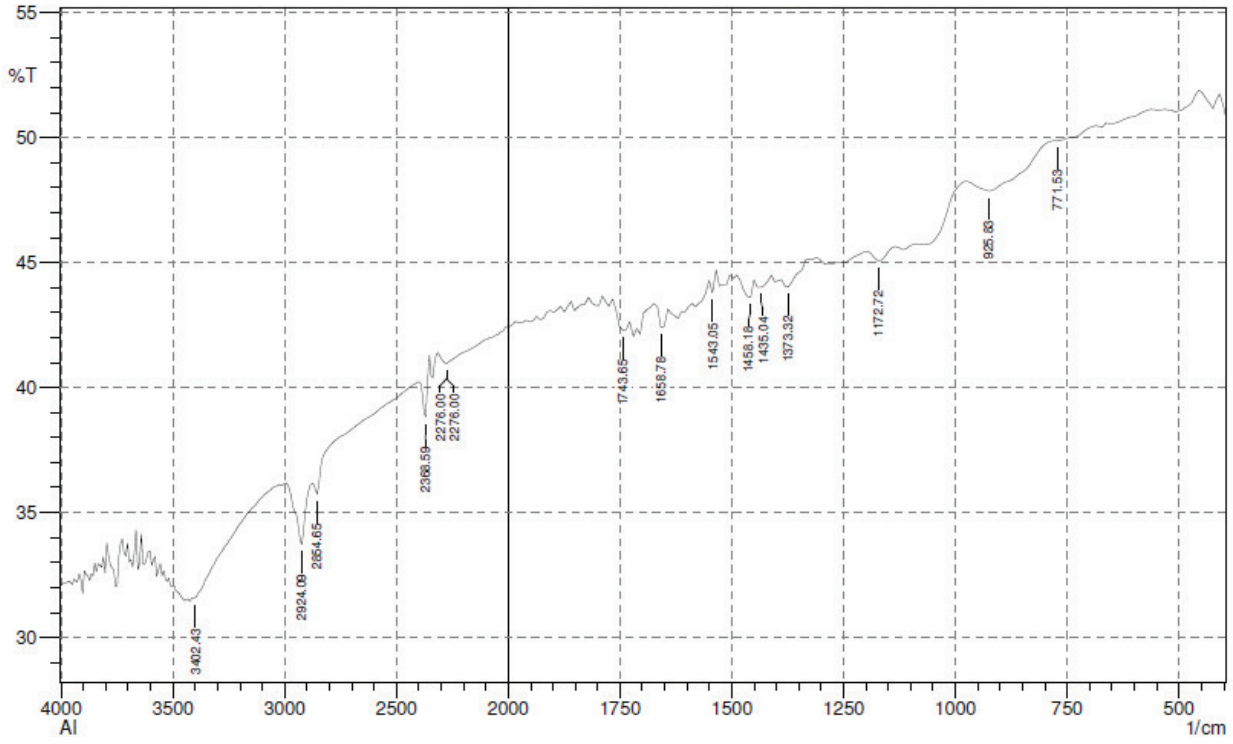

Fig. 11: FT-IR Spectrum of *Selaginella intermedia* acetone extract

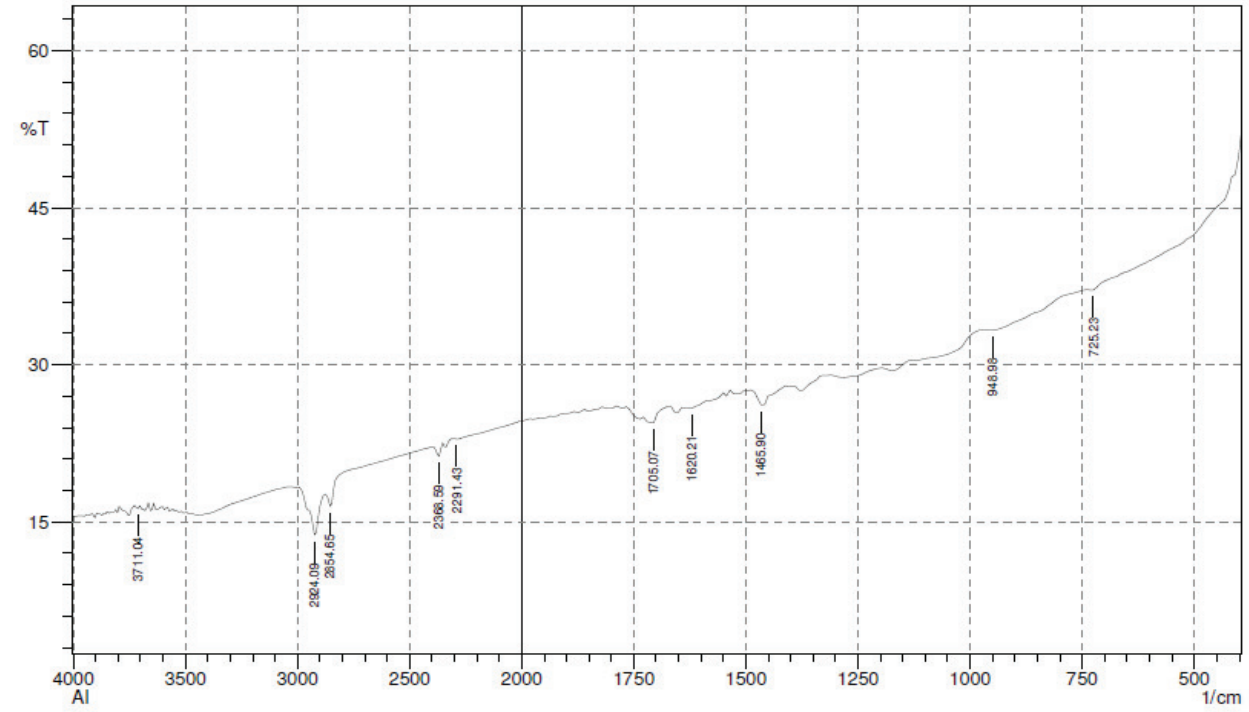

Fig. 12: FT-IR Spectrum of *Selaginella inaequalifolia* acetone extract

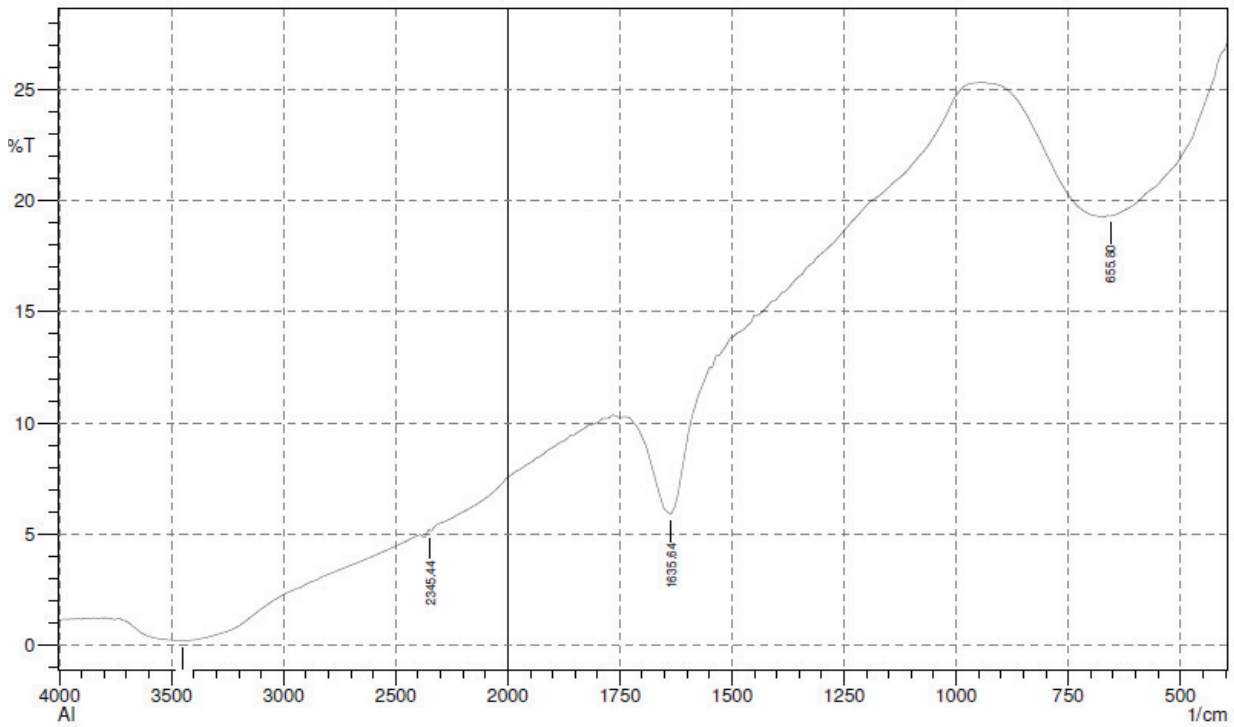

Fig. 13: FT-IR Spectrum of *Selaginella tenera* acetone extract

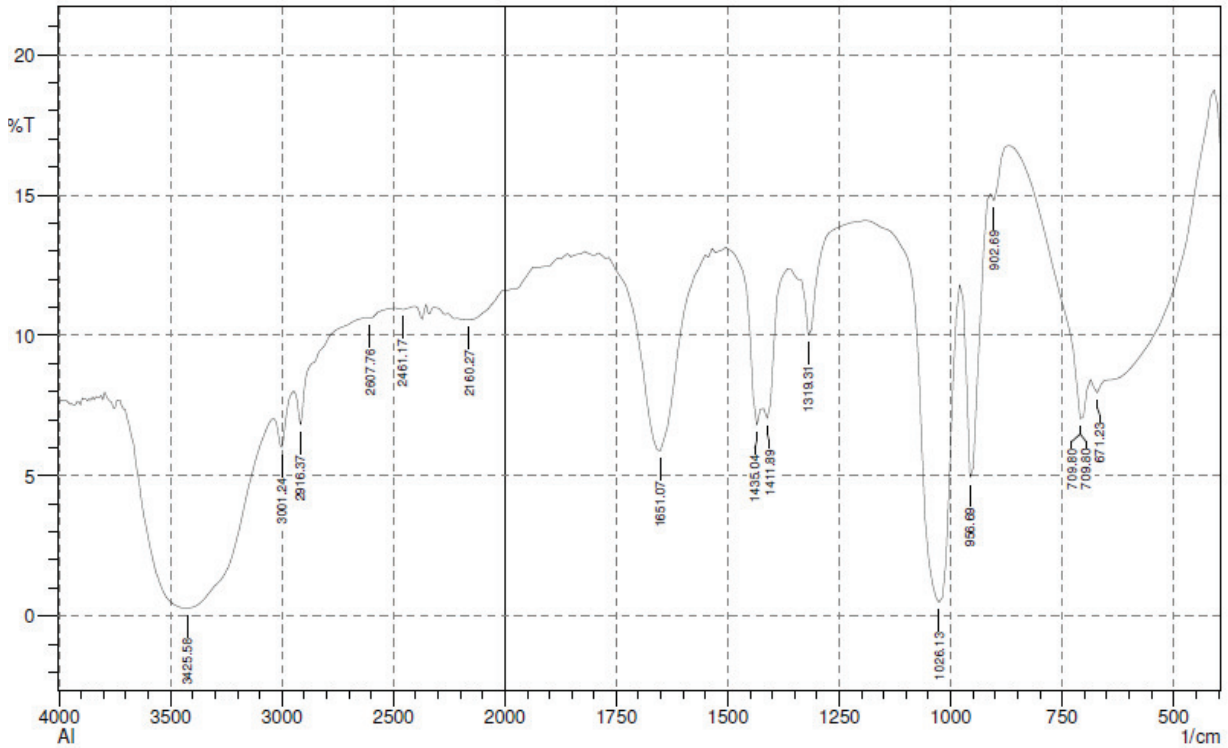

Fig. 14: FT-IR Spectrum of *Selaginella intermedia* ethanolic extract

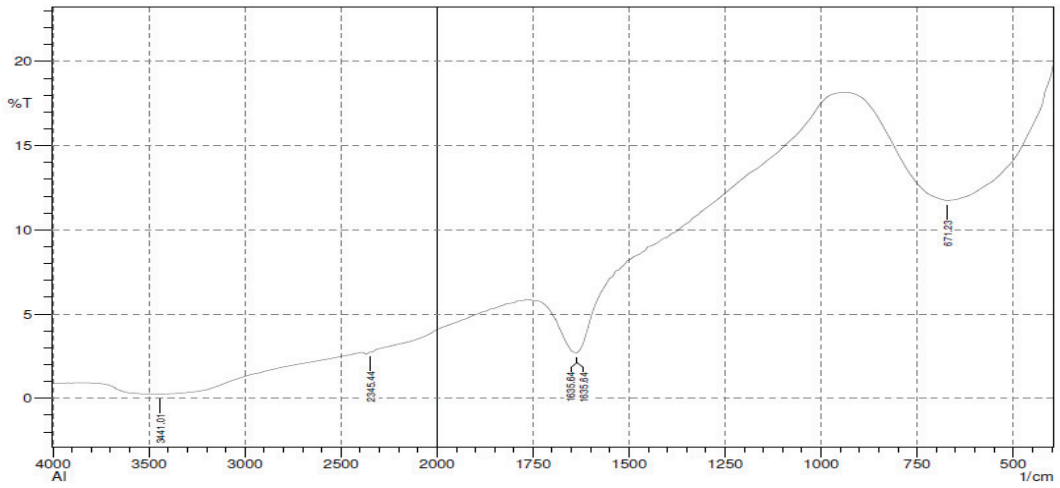

Fig. 15: FT-IR Spectrum of *Selaginella inaequalifolia* ethanolic extract

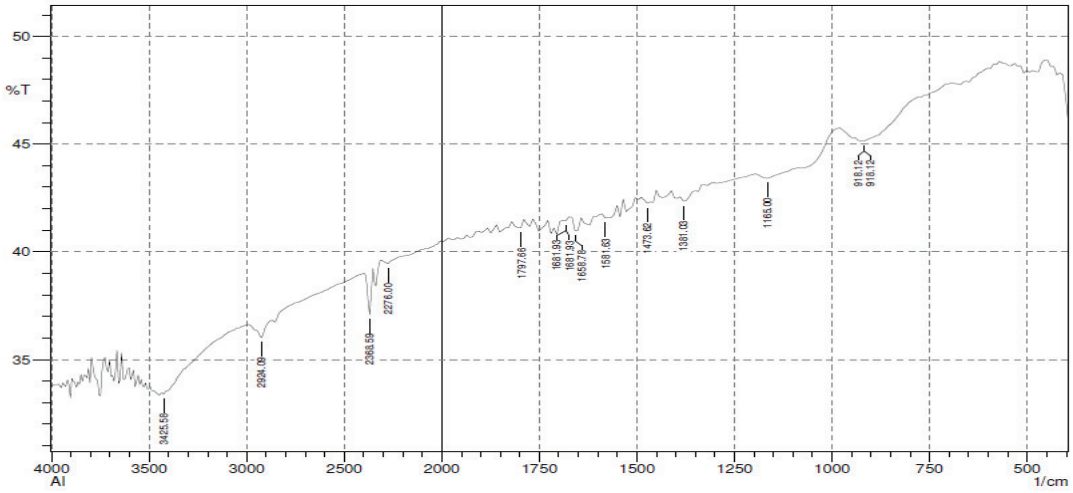

Fig. 16: FT-IR Spectrum of *Selaginella tenera* ethanolic extract

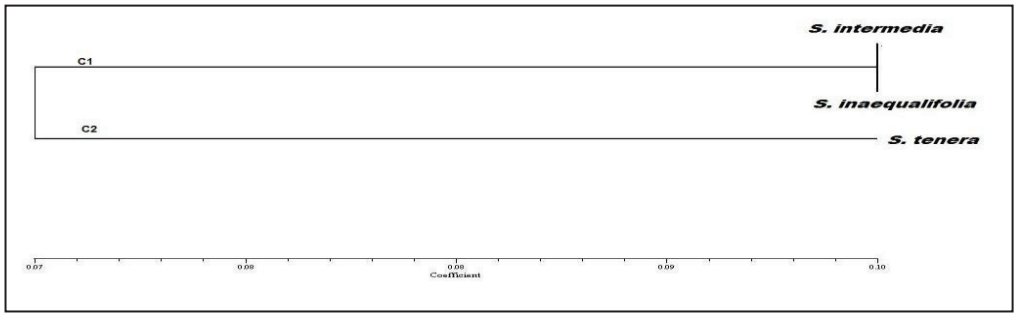

Fig. 17: UPGMA cladogram based on the FT-IR spectroscopic profile of studied *Selaginella* species

Table 8: FT-IR spectroscopic profile of studied *Selaginella* species

Peak values	Bond stretching	Functional Group	*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
			P	C	A	E	P	C	A	E	P	C	A	E
635.64	C≡C–H: C–H bend	Alkynes	-	-	-	-	+	-	-	-	-	-	+	-
655.80	–C≡C–H: C–H bend	Alkynes	-	-	-	-	+	-	-	-	-	-	-	-
671.23	–C≡C–H: C–H bend	Alkynes	-	-	-	-	-	-	-	+	-	-	-	-
709.80	C–Cl stretch	alkyl halides	-	-	-	+	-	-	-	-	-	-	-	-
725.23	C–H rock	Alkanes	+	+	-	-	-	+	-	-	-	+	-	-
732.96	C–Cl stretch	alkyl halides	-	-	-	-	-	-	-	-	+	-	-	-
756.10	C–Cl stretch	alkyl halides	+	-	-	-	-	-	-	-	-	-	-	-
918.12	O–H bend	carboxylic acids	-	-	-	-	-	-	-	-	-	-	-	+
956.69	=C–H bend	Alkenes	-	-	-	+	-	-	-	-	-	-	-	-
1026.13	C–N stretch	aliphatic amines	-	-	-	+	-	-	-	-	-	-	-	-
1080.14	C–N stretch	aliphatic amines	-	-	-	-	-	-	-	-	-	+	-	-
1172.72	C–H wag (–CH2X)	alkyl halides	+	-	-	-	-	-	-	-	-	-	-	-
1242.16	C–O stretch	alcohols, carboxylic acids, esters, ethers	-	-	-	-	-	+	-	-	-	-	-	-
1319.31	C–O stretch	alcohols, carboxylic acids, esters, ethers	-	-	-	+	-	-	-	-	-	-	-	-
1373.32	C–H rock	Alkanes	+	-	+	-	-	-	-	-	-	-	-	-
1381.03	C–H rock	Alkanes	-	+	-	-	-	+	-	-	-	+	-	+
1411.89	C–C stretch (in–ring)	Aromatics	-	-	-	+	-	-	-	-	-	-	-	-
1435	C–C stretch (in–ring)	Aromatics	-	-	-	-	-	-	-	-	-	-	-	-
1435.04	C–C stretch (in–ring)	Aromatics	-	-	+	+	-	-	-	-	-	-	-	-
1458.18	C–H bend	Alkanes	-	-	+	-	-	-	-	-	-	-	-	-
1465.90	C–H bend	Alkanes	+	+	-	-	-	+	+	-	+	+	-	-
1581.63	C–C stretch (in–ring)	Aromatics	-	-	-	-	-	-	-	-	-	-	-	+
1635.64	N–H bend	primary amines	-	-	-	-	-	-	-	+	-	-	+	-
1651.07	–C=C– stretch	alkenes	-	-	-	+	-	-	-	-	-	-	-	-

1658.78	–C=C– stretch	alkenes	-	-	-	-	-	-	-	-	+	-	-	-
1681.90	C=O stretch	Alpha,beta–unsaturated aldehydes, ketones	-	-	-	-	-	-	-	-	-	-	-	+
1705.07	C=O stretch	Alpha,beta–unsaturated aldehydes, Ketones	-	-	-	-	-	-	+	-	-	-	-	-
1720.50	C=O stretch	aldehydes, saturated aliphatic	-	-	-	-	-	-	-	-	+	-	-	-
1735.93	C=O stretch	esters, saturated aliphatic	-	+	-	-	-	-	-	-	-	-	-	-
1736.90	C=O stretch	esters, saturated aliphatic	-	-	-	-	-	-	-	-	-	+	-	-
1736.93	C=O stretch	esters, saturated aliphatic	-	-	-	-	-	+	-	-	-	-	-	-
1797.68	C=O stretch	carbonyls (general)	-	-	-	-	-	-	-	-	-	-	-	+
1916.37	C=O stretch	carbonyls (general)	-	-	-	-	-	-	-	-	-	-	-	-
2276.00	C≡N stretch	nitriles	-	-	-	-	-	-	-	-	+	-	-	+
2345.44	H–C=O: C–H stretch	aldehydes	-	-	-	-	+	-	-	+	-	-	+	-
2368.50	H–C=O: C–H stretch	aldehydes	-	-	+	-	+	-	+	-	+	-	-	+
2376.30	H–C=O: C–H stretch	aldehydes	-	-	-	-	-	+	-	-	-	-	-	-
2854.65	C–H stretch	alkanes	-	-	+	-	-	+	+	-	+	-	-	-
2924.09	C–H stretch	alkanes	-	-	+	+	-	-	+	-	+	-	-	+
3001.24	=C–H stretch	alkenes	-	-	-	+	-	-	-	-	-	-	-	-
3402	O–H stretch	carboxylic acids	-	-	+	-	-	-	-	-	-	-	-	-
3425.58	O–H stretch, H–bonded	alcohols, phenols	-	-	-	+	-	-	-	-	-	-	-	+

(P-Petroleum ether; C-Chloroform; A-Acetone; E-Ethanol)

TLC studies on *Selaginella* species

The results of TLC studies on *Selaginella* species revealed 68 phenolic bands; among the three species of *Selaginella*, maximum numbers of bands (31) were observed in *S. inaequalifolia* followed by *S. tenera* (20) and *S. intermedia* (17) with varied R_f values ranged from 0.06 to 0.97. The chromatographic profile showed the similarities and variation among the studied three species of *Selaginella*. Similar to the phenolic profile, the steroid TLC system of selected *Selaginella* species showed 64 bands. Of these, 23 bands were observed in *S. tenera*, followed by *S. inaequalifolia* with 21 bands and *S. intermedia* with 20 bands with varied R_f values ranged from 0.02 to 0.97. Among the various solvents screened, chloroform extract of *Selaginella* species showed highest number of phenolic (23) bands. The petroleum ether extract of *Selaginella* species demonstrated maximum number of steroid (25) bands. The results of phenolic and steroids profile clearly bring out the similarities and variation among the three studied *Selaginella* species.

The petroleum ether extract of *Selaginella* species expressed 16 phenolic bands. *S. intermedia* and *S. inaequalifolia* illustrated with four phenolic bands. *S. tenera* depicted highest number (8) of phenolic bands with varied R_f values ranged from 0.06 to 0.97. The phenolic band with R_f value 0.57 was shared by all the three studied *Selaginella* species. The phenolic band with R_f value 0.45 and 0.47 was shared by *S. inaequalifolia* and *S. tenera* whereas the phenolic band with R_f value 0.75 showed its jointly presence in *S. intermedia* and *S. tenera* (Table 9; Plate VII).

The chloroform extracts of three *Selaginella* species revealed 23 phenolic bands with varied R_f values. *S. inaequalifolia* showed more number (12) of bands than the other two studied species. *S. intermedia* expressed seven bands and *S. tenera* determined four bands with varied R_f

values ranged from 0.06 to 0.97. The phenolic band with R_f value 0.14 was shared by *S. intermedia* and *S. inaequalifolia* whereas the band with R_f value 0.90 displayed its jointly occurrence in *S. intermedia* and *S. tenera* (Table 9; Plate VII).

The acetone extracts of three *Selaginella* species revealed eighteen phenolic bands. *S. intermedia* showed three bands. *S. inaequalifolia* expressed 11 bands and *S. tenera* depicted four bands with varied R_f values ranged from 0.06 to 0.97. The phenolic band 0.55 was shared by *S. inaequalifolia* and *S. tenera*. The phenolic band with R_f value 0.95 was commonly present in all the three studied *Selaginella* species (Table 9; Plate VII).

The ethanolic extracts of three *Selaginella* species depicted 11 phenolic bands. *S. intermedia* showed three bands. *S. inaequalifolia* expressed four bands and *S. tenera* revealed four bands with varied R_f values ranged from 0.06 to 0.97 (Table 9; Plate VII).

The cladogram constructed based on the phenolic TLC banding profile of *Selaginella* species showed two clusters. Cluster 1 (C_1) included only *S. intermedia* and displayed 100% of divergence from other two studied species and Cluster 2 (C_2) was shared by *S. inaequalifolia* and *S. tenera* (Fig. 18).

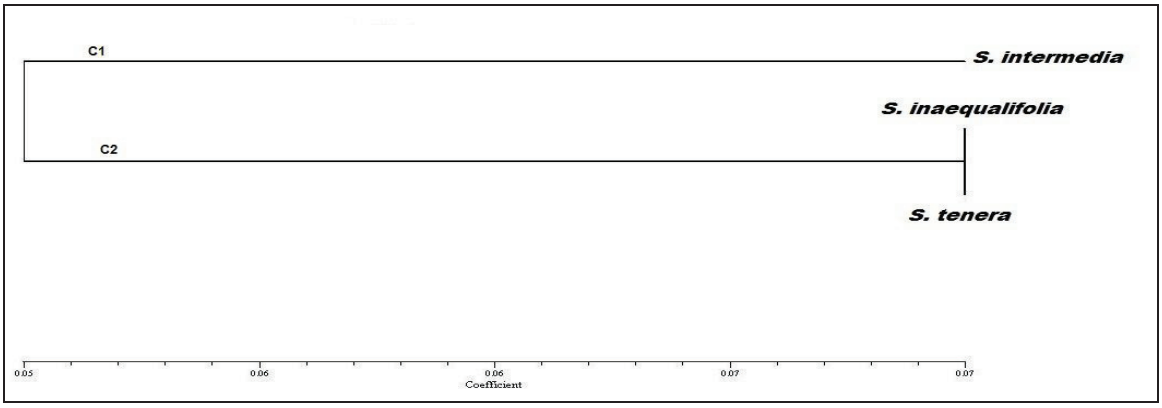

Fig. 18: UPGMA cladogram based on the phenolic TLC banding pattern of studied *Selaginella* species

Table 9: Phenolics TLC profile of studied *Selaginella* species

R_f	*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
	P	C	A	E	P	C	A	E	P	C	A	E
0.06	-	-	-	-	-	-	-	-	-	-	-	+
0.10	-	-	-	-	-	-	+	-	-	-	-	-
0.12	-	-	-	-	-	-	-	-	-	-	-	-
0.14	-	+	+	-	-	+	-	-	-	-	-	-
0.19	-	-	-	-	-	-	+	+	-	-	-	-
0.20	-	-	-	-	-	-	-	-	-	-	-	-
0.21	-	-	-	-	-	-	-	-	-	-	-	+
0.25	-	+	-	-	-	-	+	-	-	-	-	-
0.29	-	-	-	-	-	+	-	-	-	-	-	-
0.30	-	-	-	-	-	-	-	-	-	-	+	-
0.32	-	+	-	-	-	-	-	+	-	-	-	-
0.36	-	+	-	-	-	-	+	-	-	-	-	-
0.38	-	-	-	-	-	+	-	-	-	-	+	-
0.39	-	-	-	-	-	+	-	-	-	-	-	-
0.45	-	-	+	+	+	-	-	-	+	+	-	-
0.52	-	-	-	-	-	+	+	-	+	-	-	-
0.55	-	-	-	-	+	+	+	-	-	-	+	-
0.57	+	-	-	-	+	+	-	-	+	-	-	-
0.59	-	-	-	-	-	-	-	-	-	+	-	+
0.60	-	-	-	-	-	-	-	+	-	-	-	-
0.62	+	-	-	-	-	-	-	-	-	-	-	-
0.64	-	+	-	-	-	+	-	-	-	-	-	-
0.66	-	-	-	-	-	-	+	-	-	-	-	-
0.67	-	-	-	-	+	-	+	-	+	-	-	-
0.70	-	-	-	+	-	+	-	-	-	-	-	-
0.72	-	-	-	-	-	-	-	-	-	+	-	-
0.75	+	+	-	-	-	-	-	-	+	-	-	-
0.76	-	-	-	-	-	-	-	-	-	-	-	-
0.77	+	-	-	-	-	-	-	-	-	-	-	-
0.79	-	-	-	-	-	+	-	-	-	-	-	-
0.80	-	-	-	-	-	-	+	+	+	-	-	-
0.82	-	-	-	-	-	+	-	-	-	-	-	-
0.84	-	-	-	-	-	-	-	-	-	-	-	-
0.86	-	-	-	-	-	-	+	-	+	-	-	-
0.88	-	-	-	-	-	+	-	-	-	-	-	-
0.90	-	+	-	-	-	-	-	-	+	+	-	-
0.93	-	-	-	-	-	-	-	-	-	-	-	+
0.95	-	-	+	-	-	-	+	-	-	-	+	-
0.97	-	-	-	+	-	-	-	-	-	-	-	-

P-Petroleum ether; C-Chloroform; A-Acetone; E-Ethanol

A total of 64 steroid bands were observed in the steroidal TLC system of the *Selaginella* species. Petroleum ether extracts of *Selaginella* species depicted 25 steroid bands with varied R_f values ranged from 0.02 to 0.97. The steroid bands with R_f value 0.50, 0.58 and 0.70 showed their jointly presence in *S. intermedia* and *S. tenera*. The steroid band with R_f value 0.20, 0.60 and 0.90 were shared by *S. inaequalifolia* and *S. tenera.* The steroid band with R_f value 0.80 showed its presence in all the three studied *Selaginella* species.

The chloroform extracts of three *Selaginella* species revealed twenty steroid bands with different R_f values. *S. intermedia* showed eight bands; *S. inaequalifolia* expressed with three steroid bands. *S. tenera* depicted nine bands with varied R_f values ranged from 0.02 to 0.97. The steroid band with R_f value 0.40 was shared by *S. intermedia* and *S. tenera* whereas bands with R_f value 0.46 and 0.58 showed their mutual presence in *S. inaequalifolia* and *S. tenera* (Table 10; Plate VIII).

The acetone extracts of three *Selaginella* species showed the presence of fourteen steroidal bands with varied R_f values. *S. intermedia* illustrated three bands; *S. inaequalifolia* expressed more number (9) of bands and *S. tenera* depicted two bands with different R_f values. The steroid band with R_f value 0.02 showed its common presence in *S. inaequalifolia* and *S. tenera* (Table 10; Plate VIII).

Table 10: Steroids TLC profile of studied *Selaginella* species

	S. intermedia				*S. inaequalifolia*				*S. tenera*			
R_f	**P**	**C**	**A**	**E**	**P**	**C**	**A**	**E**	**P**	**C**	**A**	**E**
0.02	-	-	-	-	-	-	+	-	-	-	+	-
0.06	-	-	-	-	-	-	-	-	-	+	-	-
0.10	-	-	-	-	+	-	-	-	-	-	-	-
0.11	-	+	+	-	-	-	-	-	-	-	-	-
0.12	-	-	-	-	-	-	-	-	+	-	-	-
0.14	-	-	-	-	-	-	-	-	+	-	-	-
0.15	-	+	-	-	-	-	-	-	-	-	-	-
0.16	-	-	-	-	-	-	-	-	-	+	-	+
0.18	+	-	-	-	-	-	-	-	-	-	-	-
0.20	-	+	-	-	+	-	+	+	+	-	-	-
0.21	-	-	-	-	-	-	-	-	-	-	-	-
0.22	-	-	-	-	-	-	+	-	-	-	-	-
0.25	+	-	-	-	-	-	-	-	-	-	-	-
0.26	-	-	-	-	-	-	+	-	-	-	-	-
0.33	-	+	-	-	-	-	+	-	-	-	-	-
0.34	+	-	-	-	-	-	-	-	-	-	-	-
0.36	-	-	-	-	-	-	-	-	-	-	-	-
0.37	-	+	-	-	-	-	-	-	-	-	-	-
0.40	-	+	-	+	+	-	+	-	-	+	-	-
0.41	-	-	-	-	-	-	-	-	-	-	-	-
0.46	-	-	-	-	-	+	+	-	-	+	-	-
0.50	+	-	-	-	-	-	-	-	+	-	-	-
0.54	-	-	-	-	-	-	-	-	+	-	-	-
0.55	-	+	-	-	-	-	+	-	-	-	-	-
0.56	-	-	-	+	+	-	-	-	-	-	-	-
0.58	+	-	+	-	-	+	-	-	+	+	-	-
0.60	-	-	-	-	+	-	-	-	+	+	-	-
0.66	-	-	-	-	+	-	-	-	-	-	-	-
0.69	-	-	+	-	-	-	-	-	-	-	-	-
0.70	+	-	-	-	-	-	-	-	+	-	-	-
0.72	-	-	-	-	-	-	-	-	-	+	-	-
0.74	-	-	-	-	-	-	-	-	-	-	-	-
0.77	-	+	-	-	-	-	+	-	-	-	-	-
0.80	+	-	-	-	+	-	-	-	+	+	-	-
0.83	-	-	-	-	-	-	-	-	-	-	-	-
0.88	-	-	-	-	-	-	-	-	-	-	-	-
0.90	-	-	-	-	+	-	-	-	+	+	-	
0.92	-	-	-	-	-	-	-	-	-	-	+	+
0.97	-	-	-	-	-	+	-	-	-	-	-	-

P-Petroleum ether; C-Chloroform; A-Acetone; M-Methanol

Five steroid bands were reported in the ethanolic extract of the selected *Selaginella* species. *S. intermedia* represented with two bands, *S. inaequalifolia* showed only one band with R_f value 0.20. The steroid band with R_f value 0.16 and 0.92 showed their occurrence in *S. tenera* (Table 10; Plate VIII).

The cladogram constructed based on the steroids TLC banding profile of *Selaginella* species showed two clusters. Cluster 1 (C_1) was shared by *S. intermedia* and *S. tenera*. Cluster 2 (C_2) included only *S. inaequalifolia* and demonstrated 100% of divergence from other two studied species (Fig. 19).

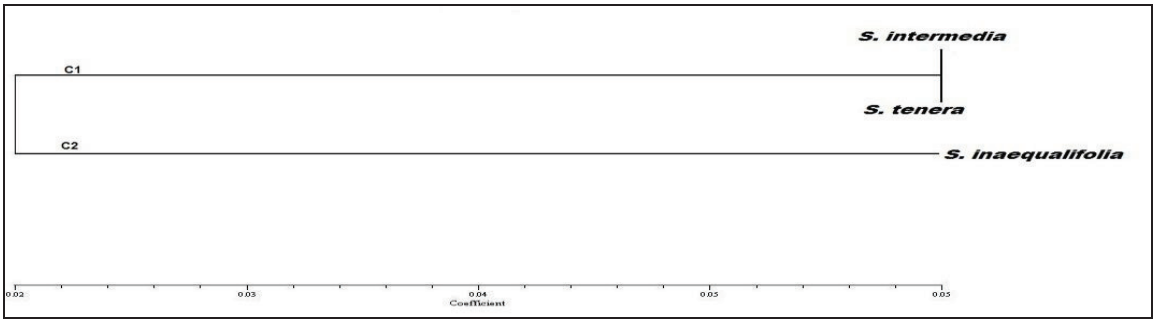

Fig. 19: UPGMA cladogram based on the steroids TLC banding pattern of studied *Selaginella* species

HPLC analysis

For the qualitative HPLC fingerprint profile of *S. intermedia, S. inaequalifolia* and *S. tenera,* ethanolic extracts were selected based on the sharpness of the peaks and proper baseline. Ethanolic extract prepared by hot extraction was subjected to HPLC for the separation and identification of constituents present in various *Selaginella* species. In *S. intermedia,* seven peaks were observed and the profile displayed one prominent peak at a retention time of 2.933 min and some moderate peaks at different retention times viz., 1.637, 2.553, 3.433, 3.793, 4.043 and 4.673 min respectively (Fig. 20). In *S. inaequalifolia,* three peaks were obtained. Of which, one prominent peak at a retention time of 3.053 min and two moderate peaks at different retention

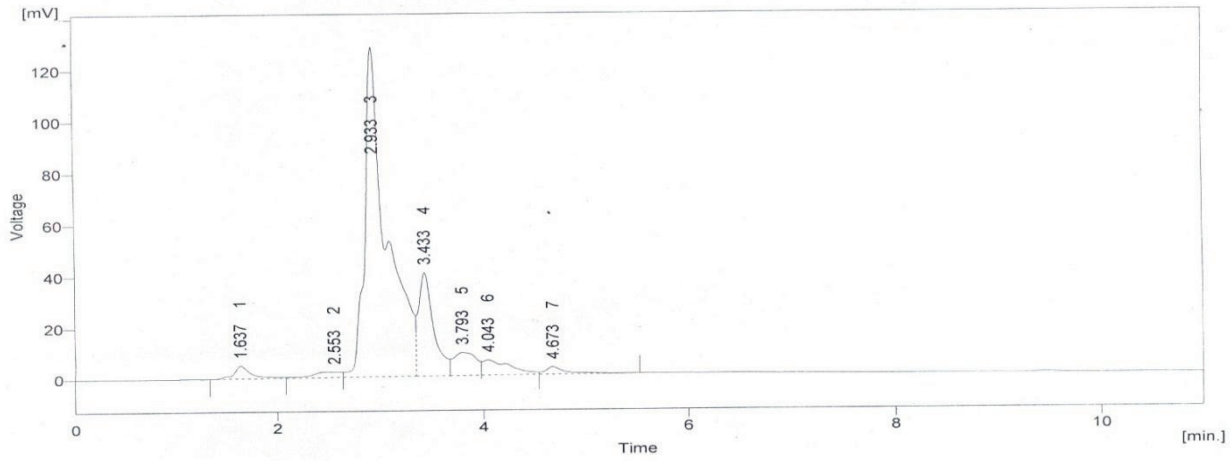

Fig. 20: HPLC chromatogram of *Selaginella intermedia* ethanolic extract

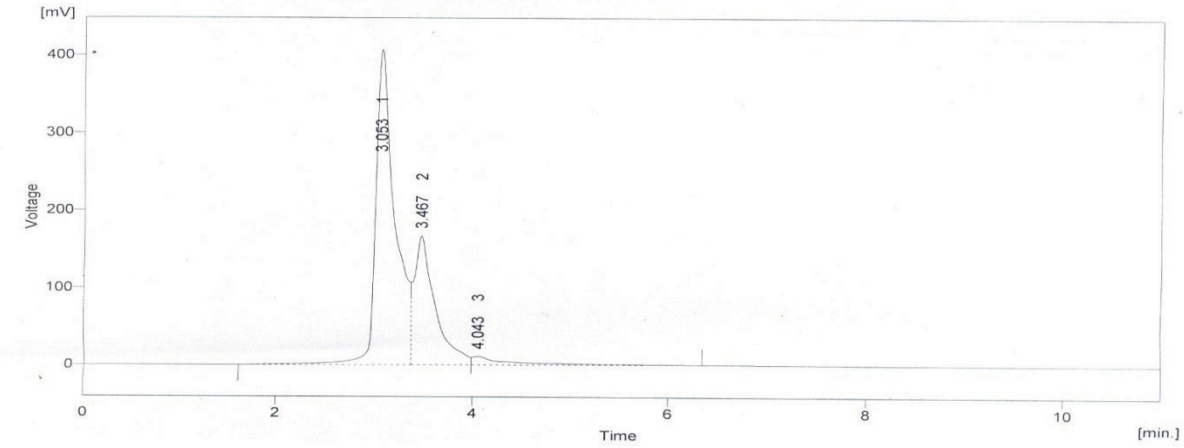

Fig. 21: HPLC chromatogram of *Selaginella inaequalifolia* ethanolic extract

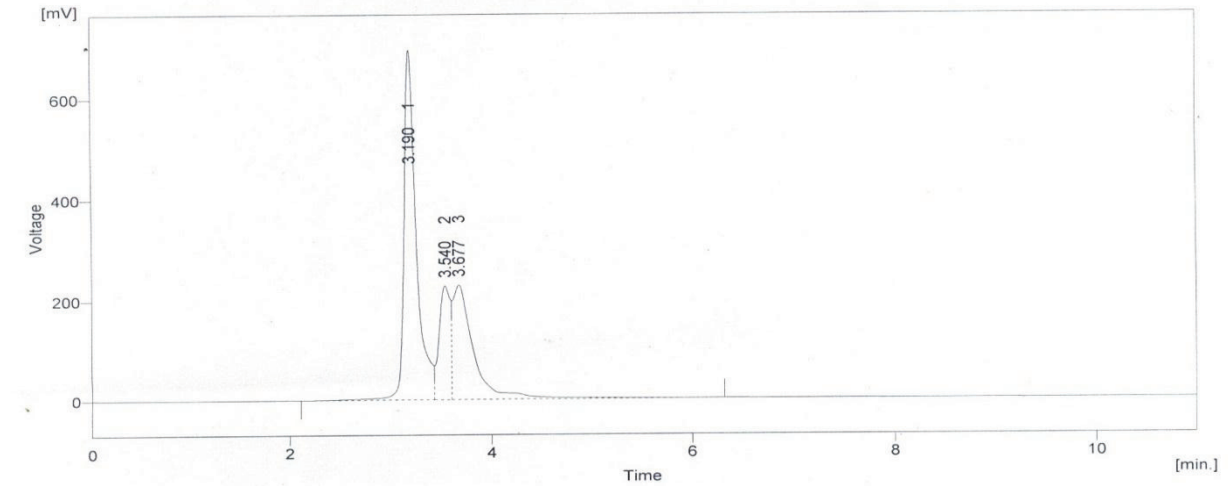

Fig. 22: HPLC chromatogram of *Selaginella tenera* ethanolic extract

times (3.467 and 4.043 min) were observed (Fig. 21). *S. tenera* illustrated the presence of three peaks with one prominent peak at a retention time of 3.190 min and two moderate peaks were also observed at a retention time 3.540 and 3.677 min respectively (Fig. 22).

HPTLC profile of *Selaginella* species

The results of the preliminary phytochemical analysis confirmed the presence of phenolics, alkaloids, glycosides, steroids, tannins, terpenoids and flavonoids in the ethanolic extracts of *S. intermedia, S. inaequalifolia* and *S. tenera*.

HPTLC phenolic profile of *Selaginella* species

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using toluene-acetone-formic acid (4.5:4.5:1) as the mobile phase. The ethanolic extract of *S. intermedia, S. inaequalifolia* and *S. tenera* showed the presence of 24 diverse type of phenolics with 22 different R_f values ranged from 0.04 to 0.94 (Plate IX). More degree of phenolics diversity was observed in ethanolic extract of *S. intermedia* (9) than other two studied species. Next to that ethanolic extract of *S. inaequalifolia* demonstrated eight phenolic bands followed by ethanolic extract of *S. tenera* (7). The phenolic with value 0.05 showed its mutual presence in the ethanolic extracts of *S. intermedia* and *S. inaequalifolia.* The phenolics with R_f value 0.14 was shared by the ethanolic extract of *S. intermedia* and *S. tenera.* The phenolic with R_f value 0.94 showed its common presence in the ethanolic extract of *S. inaequalifolia* and *S. tenera* (Table 11).

Table 11: HPTLC phenolic profile of *Selaginella* species

R_f values	*S. intermedia*	*S. inaequalifolia*	*S. tenera*	Assigned substance
0.04			+	Phenolic 1
0.05	+	+		Unknown
0.14	+		+	Phenolic 2
0.26			+	Unknown
0.27	+			Unknown
0.29			+	Unknown
0.31		+		Phenolic 3
0.32	+			Unknown
0.37	+			Unknown
0.40		+		Phenolic 4
0.45		+		Unknown
0.55		+		Phenolic 5
0.58	+			Phenolic 6
0.63	+			Unknown
0.74	+			Phenolic 7
0.75				**Quercetin**
0.76		+		Phenolic 8
0.77			+	Unknown
0.79			+	Unknown
0.83		+		Unknown
0.87	+			Unknown
0.94		+	+	Phenolic 9

HPTLC tannins profile of *Selaginella* species

In order to obtain high resolution and reproducible peaks, various compositions of the mobile phase were tested. The mobile phase toluene-ethyl acetate-formic acid-methanol (3:3:0.8:0.2) showed high resolution compared to other compositions. The ethanolic extract of *Selaginella* species showed the presence of 34 different types of tannins with 27 different R_f values ranged from 0.02 to 0.94 (Table 12; Plate X). More degree of tannins diversity was observed in ethanolic extract of *S. tenera* (12). Next to that, eleven tannins were observed in the ethanolic extract of *S. intermedia* and *S. inaequalifolia* (Table 12). Among the 34 different types of tannins, the tannin with R_f values 0.02, 0.05 and 0.65 showed their presence in all the three

examined species viz., *S. intermedia, S. inaequalifolia* and *S. tenera* (Table 12). Tannin with R_f value 0.69 showed its presence jointly in the ethanolic extracts of *S. intermedia* and *S. inaequalifolia*. The tannin with R_f value 0.88 was shared by the ethanolic extract of *S. inaequalifolia* and *S. tenera.* The tannin profile of three *Selaginella* species displayed both known and unknown tannins in the chromatographic system (Table 12; Plate X).

Table 12: HPTLC tannins profile of *Selaginella* species

R_f values	***S. intermedia***	***S. inaequalifolia***	***S. tenera***	**Assigned substance**
0.02	+	+	+	Unknown
0.05	+	+	+	Tannin 1
0.14		+		Tannin 2
0.2		+		Tannin 3
0.23			+	Unknown
0.25		+		Tannin 4
0.27		+		Unknown
0.28			+	Unknown
0.29	+			Unknown
0.33			+	Unknown
0.38		+		Unknown
0.43		+		Unknown
0.44	+			Unknown
0.45			+	Unknown
0.49				**Gallic acid**
0.56	+			Unknown
0.62	+			Tannin 5
0.65	+	+	+	Tannin 6
0.69	+	+		Tannin 7
0.71			+	Tannin 8
0.73			+	Unknown
0.75	+			Unknown
0.78			+	Unknown
0.84	+			Unknown
0.88		+	+	Unknown
0.93	+			Unknown
0.94			+	Unknown

HPTLC alkaloids profile of *Selaginella* species

Mobile phases of different compositions were tested in order to obtain high resolution and reproducible peaks. The desired aim was attained using ethyl acetate-methanol-water (10:1.35:1) as the mobile phase. The ethanolic extract of *Selaginella* species showed the presence of 20 diverse type of alkaloids with 19 different R_f values ranged from 0.04 to 0.94 (Table 13; Plate XI). High degree of alkaloid diversity was observed in ethanolic extract of *S. inaequalifolia* with ten alkaloids bands followed by *S. intermedia* with seven bands and *S. tenera* with three bands. Among the 20 different types of alkaloids, the alkaloid with R_f value 0.05 was shared by the ethanolic extract of *S. intermedia* and *S. inaequalifolia*. The alkaloid (0.42) showed its mutual occurrence in the ethanolic extracts of *S. inaequalifolia* and *S. tenera* (Table 13).

Table 13: HPTLC alkaloids profile of *Selaginella* species

R_f values	*S. intermedia*	*S. inaequalifolia*	*S. tenera*	Assigned substance
0.04			+	Alkaloid 1
0.05	+	+		Alkaloid 2
0.16		+		Unknown
0.2	+			Unknown
0.25	+			Unknown
0.27		+		Unknown
0.34		+		Alkaloid 3
0.39		+		Unknown
0.4	+			Alkaloid 4
0.42		+	+	Unknown
0.48		+		Unknown
0.53				**Colchicine**
0.67	+			Unknown
0.69		+		Alkaloid 5
0.74		+		Unknown
0.85	+			Alkaloid 6
0.92	+			Unknown
0.93		+		Unknown
0.94			+	Unknown

HPTLC flavonoids profile of *Selaginella* species

The high resolution and reproducible peaks were obtained by using the mobile phase ethyl acetate-butanone-formic acid-water (5:3:1:1). The ethanolic extract of *Selaginella* species showed the presence of 32 different types of flavonoids with 26 different R_f values ranged from 0.02 to 0.9 (Table 14). More degree of flavonoids diversity was observed in ethanolic extract of *S. tenera* with twelve flavonoids (Table 14; Plate XII) followed by ethanolic extract of *S. intermedia* (10) and *S. inaequalifolia* (10) respectively. Among the 32 different types of flavonoids, flavonoid with R_f value 0.05 showed its presence in all the three studied species of *Selaginella.* Flavonoid bands with R_f values 0.02 and 0.29 were shared by the ethanolic extract of *S. inaequalifolia* and *S. tenera* (Table 14). Similar to that, the ethanolic extracts of *S. intermedia* and *S. tenera* shared the flavonoids with R_f values 0.16, 0.23 and 0.72.

HPTLC glycosides profile of *Selaginella* species

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using n-butanol-acetic acid-water (3:1:1) as the mobile phase (Table 15). *S. intermedia, S. inaequalifolia* and *S. tenera* showed the presence of diverse type of 21 glycosides with 15 different R_f values ranged from 0.05 to 0.96 (Table 15; Plate XIII). More glycosides diversity was observed in *S. inaequalifolia* (9) followed by *S. intermedia* and *S. tenera* (7). Among the 21 different types of glycosides, two glycosides (0.05, 0.96) showed their presence in all the three studied *Selaginella* species. The glycosides with R_f value (0.09) showed its jointly presence in *S. intermedia* and *S. inaequalifolia.* The glycosides with R_f value (0.32) demonstrated its common presence in *S. intermedia* and *S. tenera*. The glycosides with R_f value 0.62 depicted the presence of standard swertiamarin in *S. inaequalifolia*.

Table 14: HPTLC flavonoids profile of *Selaginella* species

R_f values	*S. intermedia*	*S. inaequalifolia*	*S. tenera*	Assigned substance
0.02		+	+	Unknown
0.03	+			Unknown
0.05	+	+	+	Flavonoid 1
0.07		+		Unknown
0.13		+		Flavonoid 2
0.16	+		+	Unknown
0.18				**Rutin**
0.21			+	Unknown
0.23	+		+	Flavonoid 3
0.29		+	+	Flavonoid 4
0.31	+			Unknown
0.35		+		Unknown
0.36	+			Unknown
0.4		+		Flavonoid 5
0.48			+	Flavonoid 6
0.49		+		Flavonoid 7
0.53			+	Unknown
0.57		+		Flavonoid 8
0.58	+			Flavonoid 9
0.63	+			Unknown
0.64			+	Flavonoid 10
0.72	+		+	Flavonoid 11
0.73		+		Unknown
0.74			+	Unknown
0.89	+			Unknown
0.9			+	Unknown

Table 15: HPTLC glycosides profile of *Selaginella* species

R_f values	*S. intermedia*	*S. inaequalifolia*	*S. tenera*	Assigned substance
0.05	+	+	+	Glycoside 1
0.08			+	Unknown
0.09	+	+		Unknown
0.18	+			Glycoside 2
0.19		+		Unknown
0.25			+	Glycoside 3
0.28		+		Glycoside 4
0.32	+		+	Glycoside 5
0.39		+		Unknown
0.42	+			Unknown
0.55		+		Glycoside 6
0.62		+		**Swertiamarin**
0.79			+	Unknown
0.88		+		Unknown
0.96	+	+	+	Unknown

HPTLC terpenoids profile of *Selaginella* species

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using toluene-acetone-formic acid (4.5:4.5:1) as the mobile phase. The ethanolic extract of *S. intermedia, S. inaequalifolia* and *S. tenera* showed the presence of 28 diverse type of terpenoids with 26 different R_f values ranged from 0.05 to 0.95 (Table 16; Plate XIV). Maximum number of terpenoid bands (17) was observed in ethanolic extract of *S. intermedia,* next to that seven terpenoid bands were demonstrated in ethanolic extract of *S. inaequalifolia*, followed by ethanolic extract of *S. tenera* with four bands. The terpenoid with R_f values 0.63 showed its common existence in the ethanolic extracts of *S. intermedia* and *S. inaequalifolia.* The terpenoid with R_f value 0.06 was shared by the ethanolic extract of *S. intermedia* and *S. tenera.* The terpenoid band with R_f value 0.79 showed the presence of standard lupeol in *S. intermedia*.

Table 16: HPTLC terpenoids profile of *Selaginella* species

R_f values	*S. intermedia*	*S. inaequalifolia*	*S. tenera*	Assigned substance
0.05	+			Unknown
0.06		+	+	Unknown
0.1	+			Terpenoid 1
0.15	+			Unknown
0.17	+			Unknown
0.25	+			Unknown
0.27	+			Unknown
0.35	+			Unknown
0.38	+			Unknown
0.48	+			Terpenoid 2
0.51		+		Terpenoid 3
0.52	+			Unknown
0.53	+			Unknown
0.57	+			Terpenoid 4
0.63	+	+		Unknown
0.66	+			Unknown
0.67		+		Unknown
0.72			+	Terpenoid 5
0.78		+		Unknown
0.79	+			**Lupeol**
0.8			+	Unknown
0.81		+		Unknown
0.82	+			Unknown
0.88	+			Unknown
0.94		+		Unknown
0.95			+	Unknown

HPTLC steroid profile of *Selaginella* species

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using toluene-acetone-formic acid (4.5:4.5:1) as the mobile phase. The ethanolic extract of *S. intermedia, S. inaequalifolia* and *S. tenera* showed the presence of 24 diverse type of steroids with 18 different R_f values ranged from 0.06 to 0.83 (Table 17). High degree of steroids diversity was observed in

ethanolic extract of *S. intermedia* (8) followed by ethanolic extract of *S. inaequalifolia* and *S. tenera* (5). The steroid with R_f value 0.83 showed its mutual presence in the ethanolic extracts of *S. intermedia* and *S. inaequalifolia.* The steroids with R_f value 0.48 was shared by the ethanolic extract of *S. inaequalifolia* and *S. tenera.* The steroids with R_f value 0.06 showed its common presence in all the three studied *Selaginella* species (Table 17; Plate XV).

Table 17: HPTLC steroids profile of *Selaginella* species

R_f values	***S. intermedia***	***S. inaequalifolia***	***S. tenera***	**Assigned substance**
0.06	+	+	+	Unknown
0.09	+			Steroid 1
0.11		+		Unknown
0.19	+			Unknown
0.20			+	Steroid 1
0.25	+			Unknown
0.36		+		Steroid 1
0.38	+			Steroid 2
0.46				**Stigmasterol**
0.48		+	+	Unknown
0.50	+			Unknown
0.52			+	Unknown
0.58			+	Unknown
0.64	+			Steroid 3
0.83	+	+		Unknown

The cladogram based on HPTLC fingerprint profile showed two clusters, of which Cluster 1 (C_1) was shared by *S. intermedia* and *S. inaequalifolia.* Cluster 2 (C_2) included only *S. tenera* and illustrated 100% divergence from other two studied species (Fig. 23).

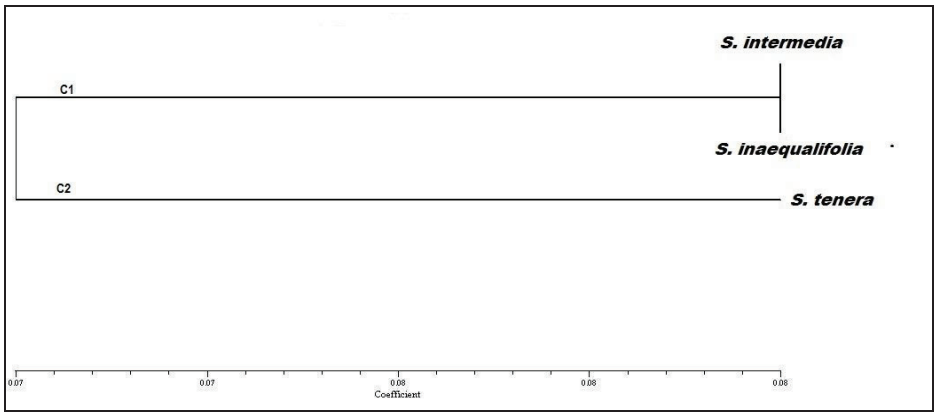

Fig. 23: UPGMA cladogram based on the HPTLC banding pattern of the studied *Selaginella* species

The amalgamated cladogram was constructed based on the UV-Vis, FT-IR, TLC and HPTLC profile. The cladogram showed two clusters, of which cluster (C_1) was shared by *S. intermedia* and *S. inaequalifolia,* cluster (C_2) included only *S. tenera* and showed 100% variation from other two studied species (Fig. 24).

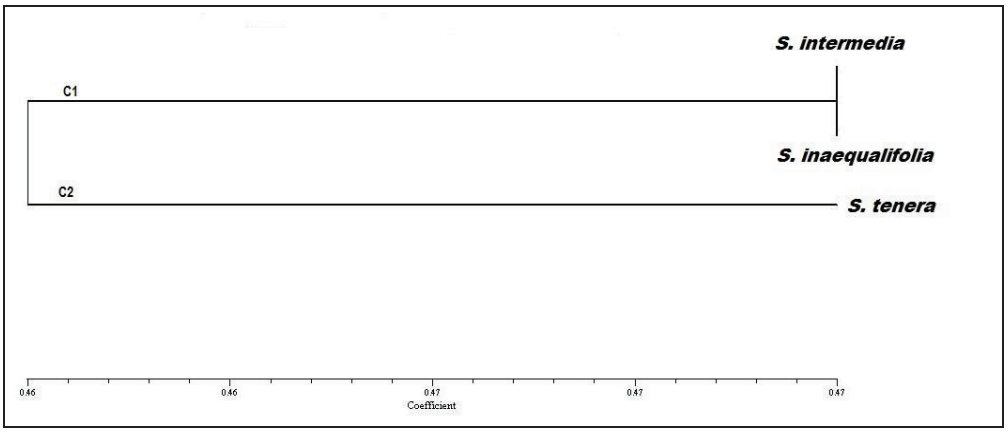

Fig. 24: UPGMA amalgamated cladogram based on the preliminary, UV-Vis, FT-IR, TLC, HPTLC profile of the studied *Selaginella* species

GC-MS analysis of *S. intermedia* ethanolic extracts

The results pertaining to GC-MS analysis on the ethanolic extracts of *S. intermedia* leads to the identification of 30 different phytocompounds with wide range of retention time (RT) ranged from 3.08 - 40.69 min and their structures were identified based on the mass spectrometry and FT-IR spectroscopic peak values. The identified compound bioactivities were predicted based on online PASS prediction. The results of *S. intermedia* ethanolic extracts GC-MS analysis revealed the presence of few prominent compounds viz., 9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans, 4-(Anisylideneamino)-cinnamic acid, Benzoic acid, 2,6-bis [(trimethylsilyl)oxy], trimethylsilyl ester, 2,6-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester, Oxazepam ditms, D-Mannopyranose, Hydroquinone, Tri chloro acetic acid, dodecyl ester, Benzenemethanol, Benzaldehyde,à-d-6,3-Furanose,methyl-á-d-glucohexodialdo-1,4-furanoside, 2-Propenoic acid, Bicyclo[3.2.2]nonane-1,5-dicarboxylic acid, 5-ethyl ester, à-D-Glucopyranose, 4-O-á-D-galactopyranosyl, Dipyridamole, Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl), Dicyclopentenyloxyethyl methacrylate, Pentadecanoic acid, 13-methyl-, methyl ester, l-(+)-Ascorbic acid 2,6-dihexadecanoate, 8,11-Octadecadienoic acid, methyl ester,9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z), Benzofuran, 3 Beta,5-epoxy-a-homo-5beta-cholest-4-en-3alpha-ol, Hexadecanoic acid and 1-(hydroxymethyl)-1,2-ethanediyl ester, The phytoconstituents of *S. intermedia* and their bio-potentials were represented in Table 18 and Fig. 25.

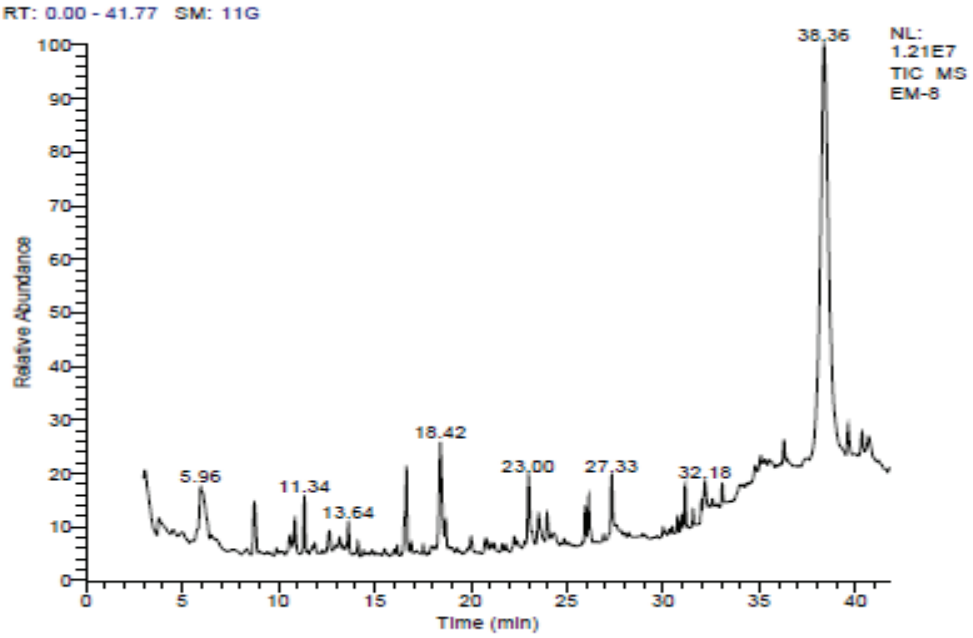

Fig. 25: Gas chromatogram of *S. intermedia* ethanolic extract

The identified compound showed biological properties such as anti-inflammatory, anti-neoplastic (colorectal cancer), astic (lung cancer), anti-fungal, anti-seborrheic, prostate disorder treatment, chlolesterol antagnosit, respiratory analeptic, anti-thrombotic and alcohol dehyrogenase inhibitor.

Table 18: Chemical constituents of *S. intermedia* ethanolic extract

Name of the compound	RT	Peak area %	Mol. Formula	Mol. Wt.	Biological activity
9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans	3.08	0.82	$C_{28}H_{44}O_4$	444	Antiinflammatory, Antineoplastic (colorectal cancer), astic (lung cancer), Acetylcholine neuromuscular blocking agent, Antifungal,
4-(Anisylideneamino)-cinnamic acid	3.78	1.04	$C_{17}H_{15}NO_3$	281	Mucomembranous protector, Vasodilator, peripheral, Antiviral (Picornavirus), Antipruritic, allergic, Cytoprotectant.
Benzoic acid, 2,6-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	5.96	4.26	$C_{16}H_{30}O_4Si_3$	370	Pterin deaminase inhibitor, Alkenylglycerophosphoethanolamine hydrolase inhibitor, Alopecia treatment, Prostate disorders treatment.
Oxazepam ditms	8.75	3.80	$C_{21}H_{27}C_lN_2O_2Si_2$	430	Cytokine release inhibitor, RNA directed DNA polymerase inhibitor, Dry eye syndrome treatment, Antibacterial, Vitamin D-like.
D-Mannopyranose	10.60	0.87	$C_6H_{12}O_6$	180	Fucosterol-epoxide lyase inhibitor, Osmotic diuretic, Anthranilate-CoA ligase inhibitor, Anaphylatoxin receptor antagonist, Beta-amylase inhibitor.
Hydroquinone	10.86	1.74	$C_6H_6O_2$	110	Antiseborrheic, Prolyl aminopeptidase inhibitor, Fucosterol-epoxide lyase inhibitor, Thioredoxin inhibitor, Vasoprotector.
1-phenylprophyl	11.34	2.78	$C_{32}H_{64}O_8Si_7$	772	Limulus clotting factor B inhibitor, Pediculicide, Cutinase inhibitor, Sclerosant, Antieczematic.
Trichloroacetic acid, dodecyl ester	11.89	1.12	$C_{14}H_{25}C_{l3}O_2$	330	All-trans-retinyl-palmitate hydrolase inhibitor, Sugar-phosphatase inhibitor, IgA-specific serine endopeptidase inhibitor, Dextranase inhibitor.
Benzenemethanol, 3-fluoro-	12.65	1.46	C_7H_7FO	126	Glucan endo-1,6-beta-glucosidase inhibitor, Antiobesity, Phobic disorders treatment, Chymosin inhibitor, Ribulose-phosphate 3-epimerase inhibitor.

2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, exo-	13.64	1.61	$C_{13}H_{20}O_2$	208	Respiratory analeptic, Acetylcholine neuromuscular blocking agent, Spasmolytic, Phosphatase inhibitor, Steroid-like, Hemostatic.
Benzaldehyde, 2-hydroxy-6-methyl-	16.65	5.76	$C_8H_8O_2$	136	Carminative, Vasoprotector, Peptidoglycan glycosyltransferase inhibitor, Alcohol dehydrogenase (acceptor) inhibitor, Adenomatous polyposis treatment.
à-d-6,3-Furanose, methyl-á-d-glucohexodialdo-1,4-furanoside	18.42	6.30	$C_7H_{12}O_6$	192	Benzoate-CoA ligase inhibitor, Aldose reductase substrate, Antioxidant, Antineoplastic (small cell lung cancer) Pullulanase inhibitor.
Bicyclo[3.2.2]nonane-1,5-dicarboxylic acid, 5-ethyl ester	18.70	0.93	$C_{13}H_{20}O_4$	240	Fragilysin inhibitor, Antiseborrheic, Centromere associated protein inhibitor, Cyclomaltodextrinase inhibitor, Sphinganine kinase inhibitor.
à-D-Glucopyranose, 4-O-á-D-galactopyranosyl-	20.01	0.86	$C_{12}H_{22}O_{11}$	342	Beta-glucosidase inhibitor, Laxative, Laminaribiose phosphorylase inhibitor, Riboflavin phosphotransferase inhibitor.
Dipyridamole	20.80	0.95	$C_{24}H_{40}N_8O_4$	504	Antihypoxic, Hydrogen dehydrogenase inhibitor, Platelet adhesion inhibitor, Leukopoiesis stimulant, Alopecia treatment.
á-D-Glucopyranose, 4-O-á-D-galactopyranosyl-	23.00	3.69	$C_{12}H_{22}O_{11}$	342	Chemosensitizer, Mucinaminylserine mucinaminidase inhibitor, Osmotic diuretic, Sucrose alpha-glucosidase inhibitor, Xylose isomerase inhibitor.
Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	23.96	1.44	$C_{27}H_{42}O_4$	430	Lipid metabolism regulator, Antiinflammatory, Antihypercholesterolemic, Prostate disorders treatment, Cytoprotectant, Gastrin inhibitor.
Dicyclopentenyloxyethyl methacrylate	25.95	1.57	$C_{16}H_{22}O_3$	262	Beta-adrenergic receptor kinase inhibitor, Antiprotozoal (Leishmania), Cholesterol antagonist, Antiulcerative.
Pentadecanoic acid, 13-methyl-, methyl ester	26.13	2.07	$C_{17}H_{34}O_2$	270	Cutinase inhibitor, Preneoplastic conditions treatment, Platelet aggregation stimulant, Fusarinine-C ornithinesterase inhibitor.
l-(+)-Ascorbic acid 2,6-dihexadecanoate	27.31	2.58	$C_{38}H_{68}O_8$	652	Respiratory analeptic, Ketol-acid reductoisomerase inhibitor, Chymosin inhibitor, Acute neurologic

					disorders treatment, Oxidoreductase inhibitor.
8,11-Octadecadienoic acid, methyl ester	31.15	2.11	$C_{19}H_{34}O_2$	294	Alcohol O-acetyltransferase inhibitor, Antithrombotic, Histone acetyltransferase inhibitor, Taurine dehydrogenase inhibitor, Fibrinogen receptor antagonist.
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	31.55	0.81	$C_{19}H_{32}O_2$	292	Mucorpepsin inhibitor, Formate-dihydrofolate ligase inhibitor, Lipotropic, Yeast ribonuclease inhibitor, Serine protease unspecified inhibitor, Sulfite dehydrogenase inhibitor.
9,12-Octadecadienoic acid (Z,Z)-	32.18	1.83	$C_{18}H_{32}O_2$	280	Thromboxane synthase stimulant, Mucositis treatment, Antihypercholesterolemic, Cutinase inhibitor, Aminoacylase inhibitor.
Benzofuran, 2,3-dihydro-2-methyl-5-phenyl-	33.06	1.03	$C_{15}H_{14}O$	210	Antidyskinetic, Cardiovascular analeptic, Antithrombotic, Kidney function stimulant, Peptidoglycan glycosyltransferase inhibitor.
As-Indacen-1(2H)-one, 3,6,7,8-tetrahydro-3,3,6,6-tetramethyl-	36.27	1.37	$C_{16}H_{20}O$	228	Ovulation inhibitor, Peptidoglycan glycosyltransferase inhibitor, Growth hormone agonist, Bile-salt sulfotransferase inhibitor.
3Beta,5-epoxy-a-homo-5beta-cholest-4-en-3alpha-ol	38.36	40.30	$C_{28}H_{46}O_2$	414	Cholesterol oxidase inhibitor, Postmenopausal disorders treatment, Prostate disorders treatment, Calcium regulator, Retinol dehydrogenase inhibitor.
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	39.62	2.04	$C_{35}H_{68}O_5$	568	Acylcarnitine hydrolase inhibitor, Sphinganine kinase inhibitor, Macrophage stimulant, Leukopoiesis stimulant.
Bis(2-ethylhexyl) phthalate	40.34	1.39	$C_{24}H_{38}O_4$	390	Antimyopathies, Histamine release stimulant, Anesthetic local, Insulin promoter, Alcohol dehydrogenase (acceptor) inhibitor.
4-Normethyl-9,19-cyclolanoststan-7-one, 3-acetoxy-	40.69	1.61	$C_{31}H_{50}O_3$	470	Hepatoprotectant, Antineoplastic, Apoptosis agonist, Sterol 24-C-methyltransferase inhibitor, Antipruritic, Chemopreventive, Antibacterial, Calcium regulator, Bilirubin oxidase inhibitor

GC-MS analysis of *S. inaequalifolia* ethanolic extracts

The results pertaining to GC-MS analysis on the ethanolic extracts of *S. inaequalifolia* leads to the identification of 29 different compounds with wide range of retention time (RT) ranged from 3.10 - 40.62 min and their structures were identified based on mass spectrometry and FT-IR peak values. The identified compound bioactivities were predicted based on online PASS prediction. The results of *S. inaequalifolia* ethanolic extracts GC-MS analysis revealed the presence of few prominent compounds viz., Octaethylene glycol monododecyl, 2,5-Dihydroxyacetophenone, bis(trimethylsilyl) ether, Cyclopentasiloxane, decamethyl-Decamethylcyclopentasiloxane, Docosanoic acid, 1,2,3-propanetriyl ester Docosanoin, 12,13-didecanoate, Hydroquinone, Cyclodecasiloxane, eicosamethyl-Icosamethylcyclodecasiloxane, 3-hydroxytetradecanoate, Tetradecanoic acid, 3-hydroxy-, methyl Ester, Cyclooctasiloxane, Salicylaldehyde hydrazone Benzaldehyde, 2-hydroxy-, hydrazone, Cyclodecasiloxane, á-D-Glucopyranose, 4-O-á-D-galactopyranosyl-Lactose, 2-Propenoic acid, 3-(4-fluorophenyl)-, ethyl ester, Cyclodecasiloxane, eicosamethyl- Icosamethylcyclodecasiloxane, î-N-Formyl-L-lysine, Pentadecanoic acid, 13-methyl-, methyl ester, Cyclodecasiloxane, l-(+)-Ascorbic acid 2,6-Dihexadecanoate, 4-Cyclohepta-2,4,6-trienyl-benzoic acid, Octadecanoic acid, 9,10-dichloro-, methyl Ester, 5,6-Dimethoxyphthalaldehydic acid, Hexadecanoic acid, 1-(2-minoethoxy)hydroxyphosphinyl]oxy]methyl]-1,2-ethanediyl ester and 4-Normethyl-9,19-cyclolanoststan-7-one. The other phytoconstituents and their bio-potentials were represented in Table 19 (Fig. 26).

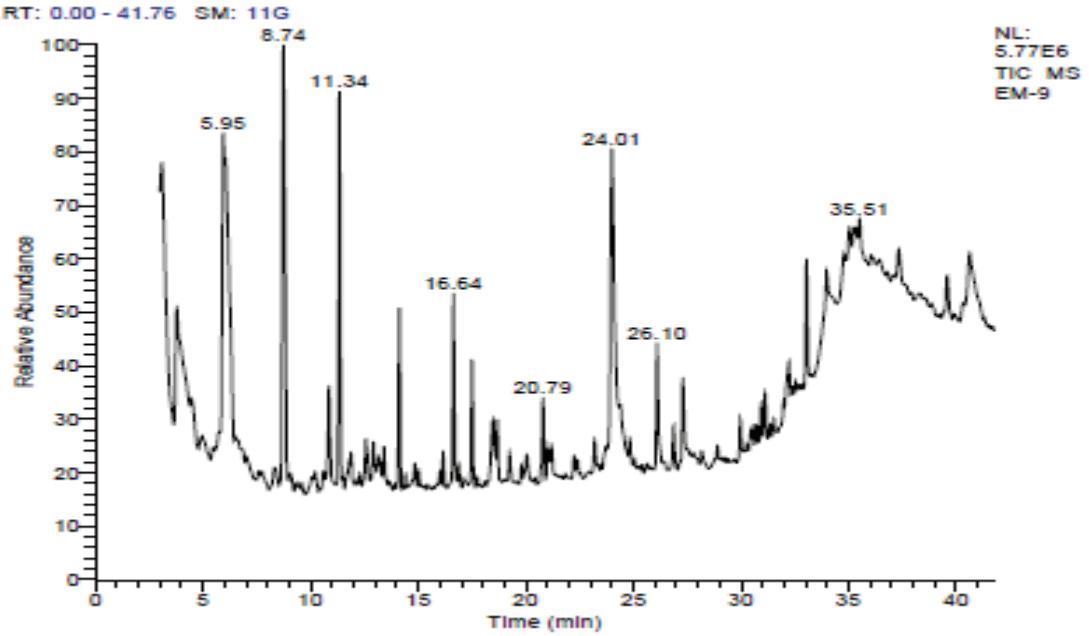

Fig. 26: Gas chromatogram of *S. inaequalifolia* ethanolic extract

The PASS prediction revealed the various biological properties for the identified compounds. They are anti-bacterial, muscular relaxant, anti-parasitic, platelet aggregation stimulant, anti-neoplastic (bone cancer), dry eye syndrome treatment, anti-viral (rhinovirus), anti-diabetic, anti-mutagenic, kidney function stimulent, neuropathy treatment, protein kinase stimulant, sweetener, osmotic and diuretic potentials.

Table 19: Chemical constituents of *S. inaequalifoli*a ethanolic extract

Name of the compound	RT	Peak area %	Mol. formula	Mol. Wt.	Biological activity
Octaethylene glycol monododecyl ether	3.10	2.64	$C_{28}H_{58}O_9$	538	Limulus clotting factor B inhibitor, Styrene-oxide isomerase inhibitor, Muscle relaxant, Acyl-CoA hydrolase inhibitor, Omptin inhibitor.
2,5-Dihydroxyacetophenone, bis(trimethylsilyl) ether	3.77	2.61	$C_{14}H_{24}O_3Si_2$	296	Antiparasitic, Benzoate-CoA ligase inhibitor, Acetylcholine neuromuscular blocking agent, Ornithine cyclodeaminase inhibitor.
Cyclopentasiloxane, decamethyl-Decamethylcyclopentasiloxane	5.93	8.39	$C_{10}H_{30}O_5Si_5$	370	Phobic disorders treatment, Chloride peroxidase inhibitor, Phospholipid-translocating ATPase inhibitor, Pterin deaminase inhibitor, Chymosin inhibitor.
Docosanoic acid, 1,2,3-propanetriyl ester Docosanoin, tri-	7.75	1.17	$C_{69}H_{134}O_6$	1058	Thromboxane synthase stimulant, Macrophage colony stimulating factor agonist, Leukopoiesis stimulant, Platelet aggregation stimulant, Antisecretoric.
Oxazepam ditms	8.74	15.84	$C_{21}H_{27}C_lN_2O_2Si_2$	430	Antiinflammatory, Interleukin 10 antagonist, Antineoplastic (glioblastoma multiforme), Antineoplastic (bone cancer), Catalase stimulant.
4a-Phorbol 12,13-didecanoate	10.21	1.05	$C_{40}H_{64}O_8$	672	Vanilloid agonist, Protein kinase stimulant, Antihelmintic, Retinol dehydrogenase inhibitor, Antipruritic, non-allergic, Antiviral (Rhinovirus).
Hydroquinone 1,4-Benzenediol	10.85	2.87	$C_6H_6O_2$	110	Carminative, Nicotinic alpha2beta2 receptor antagonist, Vasoprotector, Antieczematic, Erythropoiesis stimulant.
Cyclodecasiloxane, eicosamethyl-Icosamethylcyclodecasioxane	11.34	11.48	$C_{20}H_{60}O_{10}Si_{10}$	740	Aspulvinone dimethylallyltransferase inhibitor, Creatininase inhibitor, Chlordecone reductase inhibitor, Gonadotropin antagonist, Alkane 1-monooxygenase inhibitor, Cutinase inhibitor.
1-Hexadecene	11.88	1.76	$C_{16}H_{32}$	224	Mucomembranous protector, Fatty-acyl-CoA synthase inhibitor, Ubiquinol-cytochrome-c reductase inhibitor,

					Gluconate 5-dehydrogenase inhibitor, Taurine dehydrogenase inhibitor.
Methyl 3-hydroxytetradecanoate Tetradecanoic acid, 3-hydroxy-, methyl ester	12.57	1.25	$C_{15}H_{30}O_3$	258	Vasodilator, peripheral, Membrane integrity agonist, intestinal, Oxidizing agent, Preneoplastic conditions treatment.
Cyclooctasiloxane, hexadecamethyl-	14.12	5.22	$C_{16}H_{48}O_8Si_8$	592	Chenodeoxycholoyltaurine hydrolase inhibitor, Chlordecone reductase inhibitor, Thioredoxin inhibitor.
Salicylaldehyde hydrazone Benzaldehyde, 2-hydroxy-, hydrazine	16.64	5.11	$C_7H_8N_2O$	136	Cytoprotectant, Antineoplastic (melanoma), Nucleoside oxidase (H2O2-forming), Antithrombotic, Alopecia treatment.
Cyclodecasiloxane, eicosamethyl-	17.50	3.42	$C_{20}H_{60}O_{10}Si_{10}$	740	Glyoxylate reductase inhibitor, Aminobutyraldehyde dehydrogenase inhibitor, Aspartate-ammonia ligase inhibitor, Hydrogen dehydrogenase inhibitor.
á-D-Glucopyranose, 4-O-á-D-galactopyranosyl- Lactose, á-	18.47	1.82	$C_{12}H_{22}O_{11}$	342	Beta-glucosidase inhibitor, Licheninase inhibitor, Osmotic diuretic, Sucrose alpha-glucosidase inhibitor, Sweetener, Anaphylatoxin receptor antagonist, Xylose isomerase inhibitor.
2-Propenoic acid, 3-(4-fluorophenyl)-, ethyl ester	18.68	1.23	$C_{11}H_{11}FO_2$	194	Antipruritic, allergic, Antisecretoric, Antidiabetic, Dermatologic, Fibrinolytic, Antiinflammatory, Antiasthmatic, Antianginal.
Cyclodecasiloxane, eicosamethyl- Icosamethylcyclodecasiloxane	20.79	2.78	$C_{20}H_{60}O_{10}Si_{10}$	740	Osmotic diuretic, Fucosterol-epoxide lyase inhibitor, Alkenylglycerophosphoethanolamine hydrolase inhibitor, Lipotropic, Immunostimulant, Chitinase inhibitor, Alpha-amylase inhibitor.
î-N-Formyl-L-lysine	23.99	8.99	$C_7H_{14}N_2O_3$	174	Limulus clotting factor B inhibitor, Pseudolysin inhibitor, Aspartate-phenylpyruvate transaminase inhibitor, Fibrolase inhibitor.
Pentadecanoic acid, 13-methyl-, methyl ester	26.10	3.00	$C_{17}H_{34}O_2$	270	Mucositis treatment, Yeast ribonuclease inhibitor, Methylumbelliferyl-acetate deacetylase inhibitor, Vasodilator, peripheral, Antiseborrheic.

Cyclodecasiloxane, eicosamethyl-	26.88	1.24	$C_{20}H_{60}O_{10}Si_{10}$	740	Lysostaphin inhibitor, Chlordecone reductase inhibitor, Respiratory analeptic, Radioprotector, Membrane integrity antagonist.
l-(+)-Ascorbic acid 2,6-dihexadecanoate	27.31	2.25	$C_{38}H_{68}O_8$	652	Shikimate O-hydroxycinnamoyltransferase inhibitor, Kidney function stimulant, Antiseborrheic, Myeloblastin inhibitor, Histidine N-acetyltransferase inhibitor, Platelet aggregation stimulant, Carminative.
Cyclodecasiloxane, eicosamethyl-	29.97	1.02	$C_{20}H_{60}O_{10}Si_{10}$	740	Neuropathy treatment, Xanthine dehydrogenase inhibitor, Juvenile-hormone esterase inhibitor, Antiprotozoal activity enhancer, Cytokine modulator, Chorismate mutase inhibitor, Antidote, heavy metal.
4-Cyclohepta-2,4,6-trienyl-benzoic acid	30.54	0.96	$C_{14}H_{12}O_2$	212	Mucomembranous protector, Acylcarnitine hydrolase inhibitor, Electron-transferring-flavoprotein dehydrogenase inhibitor, Pterin deaminase inhibitor.
Octadecanoic acid, 9,10-dichloro-, methyl ester	31.13	1.32	$C_{19}H_{36}Cl_2O_2$	366	Antieczematic, Pediculicide, Mucositis treatment, Preneoplastic conditions treatment, Antimutagenic.
Cyclodecasiloxane, eicosamethyl-	32.22	1.66	$C_{20}H_{60}O_{10}Si_{10}$	740	Polyneuridine-aldehyde esterase inhibitor, Phobic disorders treatment, Chloride peroxidase inhibitor, Acetylesterase inhibitor, Feruloyl esterase inhibitor.
5,6-Dimethoxyphthalaldehydic acid	33.04	3.64	$C_{10}H_{10}O_5$	210	Membrane permeability enhancer, Aspartyltransferase inhibitor, Pro-opiomelanocortin converting enzyme inhibitor, Fibrinolytic, Antiseborrheic, Antineoplastic.
Hexadecanoic acid, 1-(2-aminoethoxy)hydroxyphosphinyl]oxy]methyl]-1,2-ethanediyl ester	39.59	1.40	$C_{37}H_{74}NO_8P$	691	Macrophage colony stimulating factor agonist, Choline-phosphate cytidylyltransferase inhibitor, Analeptic, Antithrombotic, Respiratory analeptic, Aspulvinone dimethylallyltransferase inhibitor.
4-Normethyl-9,19-cyclolanoststan-7-one, 3-acetoxy-	40.62	2.01	$C_{31}H_{50}O_3$	470	Antifungal, Prostate disorders treatment, Immunosuppressant, Antibacterial, Antiulcerative.

GC-MS analysis of *S. tenera* ethanolic extracts

The results pertaining to GC-MS analysis on the ethanolic extracts of *S. tenera* guides to the identification of 32 different compounds with wide range of retention time (RT) ranged from 3.12 – 40.62 min and their structures were recognized based on the mass spectrometry and FT-IR peak values. The results of *S. tenera* ethanolic extracts GC-MS analysis revealed the occurrence of Octaethylene glycol monododecyl ether, 2',6'-Dihydroxyacetophenone, bis(trimethylsilyl) ether, 2',6'-Dihydroxyacetophenone, bis(trimethylsilyl) ether, Cyclopentasiloxane, Silane,, Oxazepam ditms, Hydroquinone, Cyclodecasiloxane, Chloroacetic acid, dodecyl ester, à-D-Glucopyranoside, O-à-D-glucopyranosyl- (1.fwdarw.3)-, Cyclooctasiloxane, 1-Hexadecanol, 8-Methoxy-1,3,4,5-tetrahydro-2H- 1-benzazepin-2-one, Cyclopentanone, 2-(1-methylheptyl), à-D-Glucopyranose, 4-O-á-D-alactopyranosyl, 2-Propenoic acid, 3-(3-fluorophenyl)-, ethyl ester, Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl Ester, Androst-4-en-6-one, Pentadecanoic acid, 13-methyl-, methyl ester, Hexadecanoic acid, 1- (hydroxymethyl)-1,2-ethanediyl ester, 8-Octadecenoic acid, methyl ester, Heptadecanoic acid, 14- methyl-, methyl ester, 2,2,8,8,12,13,17,18-Octamethyl-2,3,7,8,22,24-hexahydro-porphine-5-carbonitrile, holestane, 1,4,10,13-Tetraoxa-7,16- Diazacyclooctadecane, Urs-9(11)-en-12-one-28-oic acid, 3-acetoxy-, methyl ester (14á,20á) and 4- Normethyl-9,19-cyclolanoststan-7-one. The other recognized phytoconstituents and their bio-potentials were illustrated in Table 20 (Fig. 27).

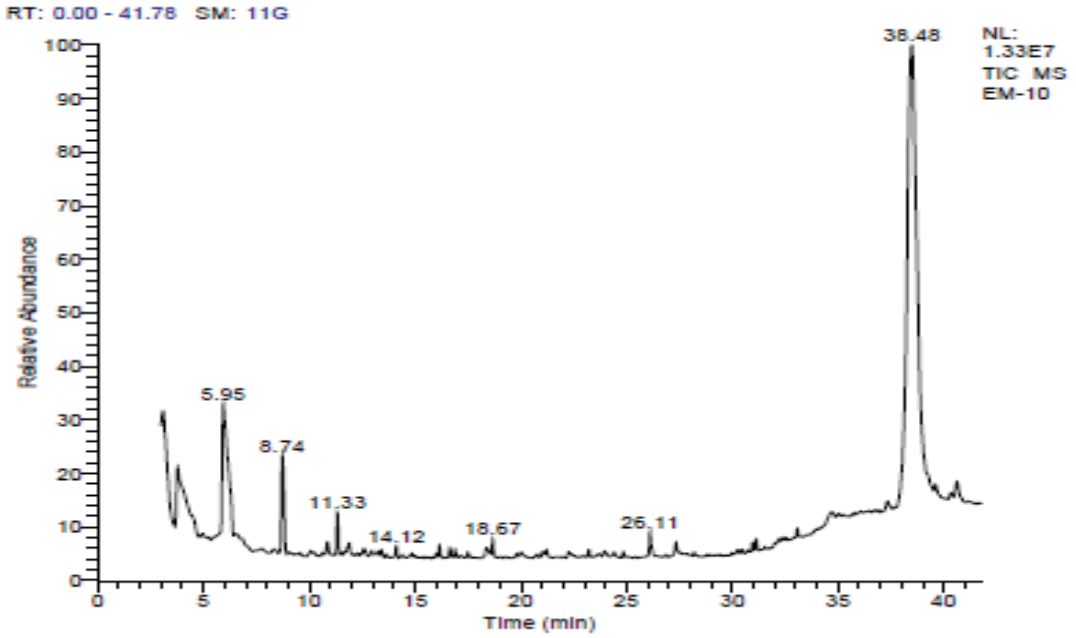

Fig. 27: Gas chromatogram of *S. tenera* ethanolic extract

The predicted compounds showed the following properties viz., anti-inflammatory, anti-diabetic, anti-oxidant, anti-bacterial activity, hepatoprotectant, anti-fungal, cholesterol antagonist, respiratory analeptic, anti-pruritic, alopecia treatment, uterine relaxant, cholesterol oxidase inhibitor, postmenopausal disorders treatment, prostate disorders treatment, calcium regulator and retinol dehydrogenase inhibitor properties.

Table 20: Chemical constituents of *S. tenera* ethanolic extract

Name of the compound	RT	Peak area %	Mol. formula	Mol. Wt.	Biological activities
Octaethylene glycol monododecyl ether	3.12	3.11	$C_{28}H_{58}O_9$	538	Fucosterol-epoxide lyase inhibitor, Eye irritation, Skin irritation, Protein-tyrosine sulfotransferase inhibitor, Oxygen scavenger.
2',6'-Dihydroxyacetophenone, bis(trimethylsilyl) ether	3.77	3.06	$C_{14}H_{24}O_3Si_2$	296	Testosterone 17 beta-dehydrogenase (NADP+) inhibitor, Erythropoiesis stimulant, Antiosteoporotic, Prostate disorders treatment, Bone diseases treatment, Carminative, Antifungal, Antineoplastic (endocrine cancer).
2',6'-Dihydroxyacetophenone, bis(trimethylsilyl) ether	4.55	0.49	$C_{14}H_{24}O_3Si_2$	296	Ubiquinol-cytochrome-c reductase inhibitor, Erythropoiesis stimulant, Ribulose-phosphate 3-epimerase inhibitor, Antiviral, Adenomatous polyposis treatment, HIV-1 reverse transcriptase inhibitor, Cyclic AMP agonist.
Cyclopentasiloxane, decamethyl-	5.93	6.18	$C_{10}H_{30}O_5Si_5$	370	Estrogen agonist, NADPH peroxidase inhibitor, Phobic disorders treatment, Shikimate O-hydroxycinnamoyltransferase inhibitor, Retinoic acid beta receptor agonist, Antiseborrheic, Platelet aggregation stimulant.
Silane, [[4-[1,2-bis[(trimethylsilyl)oxy] ethyl]-1,2-phenylene]bis(oxy)]bis [trimethyl-	6.31	0.82	$C_{20}H_{42}O_4Si_4$	458	Glucan 1,4-alpha-maltotriohydrolase inhibitor, Platelet aggregation stimulant, Antineoplastic (lung cancer), Immunosuppressant, Vasoprotector, Photosensitizer, RNA directed DNA polymerase inhibitor, Sclerosant, Pediculicide, UGT2B17 substrate.
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	8.38	0.35	$C_{26}H_{54}$	366	5 Hydroxytryptamine release stimulant, Cutinase inhibitor, Arginine 2-monooxygenase inhibitor, Leukopoiesis stimulant, Oxygen scavenger, Antipruritic, allergic.

Oxazepam ditms	8.74	6.94	$C_{21}H_{27}ClN_2O_2Si_2$	430	Gestagen antagonist, Antiinflammatory, Cytokine release inhibitor, Antiemphysemic, Uterine relaxant, Aldehyde dehydrogenase substrate, Antibacterial, Uterine relaxant.
Hydroquinone	10.86	0.76	$C_6H_6O_2$	110	Alcohol oxidase inhibitor, Antiseborrheic, Sphinganine kinase inhibitor, Fucosterol-epoxide lyase inhibitor, Alopecia treatment, Thiosulfate dehydrogenase inhibitor, Kidney function stimulant.
Cyclodecasiloxane, eicosamethyl-	11.33	2.01	$C_{20}H_{60}O_{10}Si_{10}$	740	Feruloyl esterase inhibitor, Pullulanase inhibitor, Chenodeoxycholoyltaurine hydrolase inhibitor, Ornithine cyclodeaminase inhibitor, Quinoprotein glucose dehydrogenase inhibitor, Platelet aggregation stimulant, Alopecia treatment.
Chloroacetic acid, dodecyl ester	11.89	0.70	$C_{14}H_{27}ClO_2$	262	Polyporopepsin inhibitor, Chymosin inhibitor, Saccharopepsin inhibitor, Antihypoxic, Lipid metabolism regulator, Sclerosant, Antiviral (Rhinovirus), Macrophage stimulant, Fibrinogen receptor antagonist.
à-D-Glucopyranoside, O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl	12.58	0.44	$C_{18}H_{32}O_{16}$	504	Fructan beta-fructosidase inhibitor, Respiratory analeptic, Sweetener, Osmotic diuretic, Aldose reductase substrate, Antineoplastic, Vascular dementia treatment, Wound healing agent.
Cyclooctasiloxane, hexadecamethyl-	14.12	0.58	$C_{16}H_{48}O_8Si_8$	592	Gonadotropin antagonist, Pseudolysin inhibitor, Aspartate-ammonia ligase inhibitor, Retinoic acid beta receptor agonist, Glutarate-semialdehyde dehydrogenase inhibitor, Erythropoiesis stimulant,
1-Hexadecanol	16.15	0.48	$C_{16}H_{34}O$	242	Sugar-phosphatase inhibitor, Dextranase inhibitor, Levanase inhibitor, Acrocylindropepsin inhibitor, Chymosin inhibitor, Oryzin inhibitor, Coccolysin inhibitor, Arginine 2-monooxygenase inhibitor, Urethanase inhibitor.
8-Methoxy-1,3,4,5-	16.64	0.48	$C_{11}H_{13}NO_2$	191	Nootropic, Lysase inhibitor, Neurotransmitter

tetrahydro-2H-1-benzazepin-2-one					antagonist, Prostate disorders treatment, 5 Hydroxytryptamine uptake stimulants, Alopecia treatment, Antineoplastic alkaloid.
Cyclopentanone, 2-(1-methylheptyl)-	16.88	0.45	$C_{13}H_{24}O$	196	Antieczematic, Ubiquinol-cytochrome-c reductase inhibitor, Dermatologic, Fibrinolytic, Lipoprotein lipase inhibitor, Bilirubin oxidase inhibitor, Antinociceptive.
à-D-Glucopyranose, 4-O-á-D-galactopyranosyl-	18.38	0.63	$C_{12}H_{22}O_{11}$	342	Beta-glucosidase inhibitor, Beta-mannosidase inhibitor, 4-Alpha-glucanotransferase inhibitor, Cellulose 1,4-beta-cellobiosidase inhibitor.
2-Propenoic acid, 3-(3-fluorophenyl)-, ethyl ester	18.67	0.78	$C_{11}H_{11}FO_2$	194	Antieczematic, Antidiabetic, Antianginal, Choleretic, Antiinflammatory, Fragilysin inhibitor, Vasodilator, peripheral, Feruloyl esterase inhibitor.
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	20.01	0.36	$C_{35}H_{68}O_5$	568	Leukopoiesis stimulant, Macrophage colony stimulating factor agonist, Peptide agonist, Venombin AB inhibitor, Ophthalmic drug, Antiinflammatory.
Androst-4-en-6-one, 3,17-diacetoxy-	23.22	0.38	$C_{23}H_{32}O_5$	388	Lysase inhibitor, Prostaglandin-E2 9-reductase inhibitor, Antisecretoric, Proliferative diseases treatment, Anesthetic general.
Pentadecanoic acid, 13-methyl-, methyl ester	26.11	1.34	$C_{17}H_{34}O_2$	270	Acetylesterase inhibitor, Protein-disulfide reductase (glutathione) inhibitor, Fibrinolytic, Hypolipemic, Pectin lyase inhibitor.
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	27.33	0.61	$C_{35}H_{68}O_5$	568	Xylan endo-1,3-beta-xylosidase inhibitor, Antihypoxic, Antiinfective, Feruloyl esterase inhibitor, Peptide agonist, Chitinase inhibitor, Lysostaphin inhibitor.
8-Octadecenoic acid, methyl ester	30.95	0.39	$C_{19}H_{36}O_2$	296	All-trans-retinyl-palmitate hydrolase inhibitor, Beta-adrenergic receptor kinase inhibitor, Aspulvinone dimethylallyltransferase inhibitor, Linoleate diol synthase inhibitor, Antisecretoric, Analeptic.
Heptadecanoic acid,	31.13	0.54	$C_{19}H_{38}O_2$	298	Polyporopepsin inhibitor, Chymosin inhibitor, Lipid

14-methyl-, methyl ester, (ñ)-					metabolism regulator, Carboxypeptidase Taq inhibitor, Pullulanase inhibitor, Antiulcerative.
1-Propyl-3,6-diazahomoadamantan-9-ol	33.06	0.42	$C_{12}H_{22}N_2O$	210	Cardiotonic, Kidney function stimulant, Ovulation inhibitor, Dementia treatment, Membrane permeability enhancer.
8-Ethoxy-4,5-dihydro-1-[(4-isopropylphenyl)imino]-4,4-dimethyl-1H-[1,2]dithiolo[3,4-c]quinoline	34.66	1.21	$C_{23}H_{26}N_2OS_2$	410	Antianginal, Alcohol O-acetyltransferase inhibitor, Endoglycosylceramidase inhibitor, Calcium regulator, Retinol dehydrogenase inhibitor, Lactase inhibitor, Antibiotic Tribactam-like, Prolactin release inhibitor.
2,2,8,8,12,13,17,18-Octamethyl-2,3,7,8,22,24-hexahydro-porphine-5-carbonitrile	37.33	0.89	$C_{29}H_{33}N_5$	451	T cell inhibitor, Ferrochelatase inhibitor, Cachexia treatment, Alopecia treatment, Dermatologic, Coproporphyrinogen oxidase inhibitor, Pterin deaminase inhibitor, Nucleotide metabolism regulator, Vasoprotector.
Cholestane, 1-vinyl-1-hydroxy-	38.48	63.28	$C_{29}H_{50}O$	414	Oxidoreductase inhibitor, Respiratory analeptic, Antipruritic, Peptidoglycan glycosyltransferase inhibitor, Erythropoiesis stimulant.
1,4,10,13-Tetraoxa-7,16-diazacyclooctadecane, 7,16-bis(1-oxodecyl)-	39.60	0.51	$C_{32}H_{62}N_2O_6$	570	Phobic disorders treatment, Cardiovascular analeptic, Histamine release stimulant, Simian immunodeficiency virus proteinase inhibitor, Rhodotorulapepsin inhibitor.
Urs-9(11)-en-12-one-28-oic acid, 3-acetoxy-, methyl ester (14á,20á)	40.32	0.41	$C_{33}H_{50}O_5$	526	Hepatoprotectant, Antiprotozoal (Leishmania), Antineoplastic, Transcription factor stimulant, Nitric oxide antagonist, Chemopreventive, Spasmolytic, urinary, Growth stimulant, Telomerase stimulant.
4-Normethyl-9,19-cyclolanoststan-7-one, 3-acetoxy-	40.62	1.41	$C_{31}H_{50}O_3$	470	Antifungal, Cholesterol antagonist, Acylcarnitine hydrolase inhibitor, Antiinflammatory, Antioxidant, Gestagen antagonist.

Antioxidant activity

Total Phenolics

In the present study, total phenolics obtained from various extracts of three *Selaginella* species were presented in Table 21. Maximum extractable total phenolics were recorded in ethanolic extract of *S. intermedia* (135.39 mg GAE/g) followed by *S. inaequalifolia* (115.74 mg GAE/g) and *S. tenera* (114.28 mg GAE/g). Petroleum ether extracts of *S. inaequalifolia* and *S. tenera* showed the lowest level (37.14 mg GAE/g) of total phenolics.

Table 21: Total phenolics of *Selaginella* species

Extracts	Total Phenolics in mg GAE/1g		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	38.095 ± 0.95	37.142 ± 2.51	37.142 ± 0.95
Chloroform	87.142 ± 1.98	56.0317 ± 1.67	74.285 ± 1.90
Acetone	103.57 ± 2.14	108.57 ± 0.95	89.285 ± 0.72
Ethanol	135.396 ± 0.72	115.714 ± 1.71	114.28 ± 0.95

Tannins

In the present study, tannins obtained from various extracts of *S. intermedia, S. inaequalifolia* and *S. tenera* were presented in Table 22. Highest amount of tannins were obtained in ethanolic extract of *S. inaequalifolia* (64.6 mg GAE/g) followed by *S. intermedia* (52.22 mg GAE/g) and *S. tenera* whereas other extracts showed moderate amounts of tannin occurrence. Petroleum ether extract of *S. tenera* exhibited least level of tannins (16.5 mg GAE/g).

Table 22: Total tannin of *Selaginella* species

Extracts	Total tannin in mg GAE/1g		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	19.68 ± 1.45	41.26 ± 4.88	16.5 ± 0.99
Chloroform	21.74 ± 0.72	46.66 ± 0.47	19.84 ± 1.53
Acetone	46.98 ± 0.99	51.42 ± 0.95	27.61 ± 1.90
Ethanol	52.22 ± 0.99	64.6 ± 1.53	43.17 ± 3.02

Flavonoids

Among the three studied species of *Selaginella,* the maximum extractable flavonoids was observed in ethanolic extract of *S. intermedia* (195.11 mg GAE/g) followed by *S. tenera* (183.55 mg GAE/g) and *S. inaequalifolia* (143.11 mg GAE/g). Petroleum ether extracts of *S. tenera* showed the lowest level of flavonoids (43.33 mg GAE/g).

Table 23: Total Flavonoids of *Selaginella* species

Extracts	Total Flavonoids in mg GAE/1g		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	59.77 ± 1.38	52 ± 0.66	43.33 ± 1.15
Chloroform	88 ± 1.76	70.66 ± 2.30	96.22 ± 2.14
Acetone	132.44 ± 2.03	136.88 ± 4.28	137.11 ± 2.14
Ethanol	195.11 ± 2.77	143.11 ± 2.03	183.55 ± 0.38

DPPH

The results on DPPH free radical scavenging activity of three *Selaginella* species was demonstrated in Table 24. The model of stable DPPH free radicals can be used to evaluate the antioxidative activities in a relatively short time. The absorbance decreases as a result of a colour change from purple to yellow as radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH molecule. Concentration of the sample necessary to decrease

initial concentration of DPPH by 50% (IC_{50}) under the experimental condition was determined. Therefore, lower value of IC_{50} indicates a higher antioxidant activity. The best free radical scavenging activity was exerted by ethanolic extract of *S. intermedia* (IC_{50} 67.23 µg/ml). *S. inaequalifolia* and *S. tenera* ethanolic extracts showed comparable levels of free radical scavenging activity (IC_{50} 70.08 and 77.19 µg/ml) respectively. The petroleum ether extract of *S. tenera* displayed lowest free radical scavenging activity with IC_{50} 159.54 µg/ml.

Table 24: DPPH activity of *Selaginella* species

Extracts	DPPH activity in µg/ml		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	159.48	132.8	114.7
Chloroform	115.42	90	83.41
Acetone	85.77	75.34	85.6
Ethanol	67.23	70.08	77.19

$ABTS^+$ assay

$ABTS^+$, a protonated radical, has the characteristic absorbance maximum at 734 nm which decreases the scavenging of the proton radicals. In $ABTS^+$ cation radical scavenging method, the activity of tested extracts were expressed as micromolar equivalent of Trolox solution having an antioxidant equivalent to one gram dry matter of the sample under the experimental investigation. The ABTS radical scavenging activity of various extracts of *Selaginella* species were tabulated in Table 25. Even though the samples exhibited good ABTS radical scavenging activity, ethanolic extract of *S. tenera* showed the highest activity (863.99 µM TE/g) followed by *S. inaequalifolia* (849.82 µM TE/g) whereas *S. intermedia* petroleum ether extract exhibited lower level of activity (588.59 µM TE/g) than other studied extracts of *Selaginella* species.

Table 25: ABTS^{+} activity of *Selaginella* species

Extracts	ABTS+ activity in µM TE/g		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	845.77 ± 2.33	849.82 ± 6.18	863.99 ± 2.33
Chloroform	740.47 ± 4.67	798.39 ± 9.35	760.04 ± 3.09
Acetone	673.64 ± 3.09	734.39 ± 1.16	701.99 ± 8.43
Ethanol	588.59 ± 4.67	687.82 ± 6.18	672.97 ± 2.33

FRAP assay

FRAP antioxidants, explained as reductants and inactivation of oxidants by reductants are involved in redox reactions in which one reaction species is reduced at the expense of the oxidation of another antioxidant. The antioxidant potential of various *Selaginella* species were estimated from their ability to reduce TPTZ - Fe (III) complex to TPTZ – Fe (II) complex and the results are expressed as concentration of substance having ferric-TPTZ reducing ability equivalent that of 1 µM concentration of Fe (II). The FRAP values of various extracts of *Selaginella* species were depicted in Table 26.

Table 26: FRAP activity of *Selaginella* species

Extracts	FRAP activity in µM Fe(II)/mg		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	822.22 ± 13.4	475 ± 4.40	670 ± 46.3
Chloroform	936.66 ± 32.14	608.33 ± 1.66	1071.66 ± 1.66
Acetone	1130.55 ± 12.94	825 ± 16.4	1238.33 ± 4.40
Ethanol	1220 ± 55.07	904.44 ± 4.19	1402.22 ± 5.09

Among the tested extracts of *Selaginella* species, ethanolic and acetone extracts of *S. tenera* registered highest antioxidant activity with 1402.22 and 1238.33 µM Fe (II)/mg respectively. All the samples showed reducing power but not at the same level. The order of

FRAP activity of various extracts of *Selaginella* species were as follows: *S. tenera* > *S. intermedia* > *S. inaequalifolia*.

Metal chelating assay

In the metal chelating assay, ferrozine can quantitatively form complexes with Fe^{2+}. The chelating effects on the ferrous ions of petroleum ether, chloroform, acetone and ethanolic extracts of the three *Selaginella* species were demonstrated in Table 27. All the screened extracts exhibited the ability to chelate metal ions. Among tested extracts of *S. tenera* exhibits higher metal chelating activity (5.909 mg EDTA equiv/g) followed by *S. inaequalifolia* (4.979 mg EDTA equiv/g) and *S. intermedia* showed lower activity (2.565 mg EDTA equiv/g).

Table 27: Metal chelating activity of *Selaginella* species

Extracts	Metal chelating activity in mg EDTA equiv/g		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	0.2929 ± 0.47	0.343 ± 0.21	0.606 ± 0.16
Chloroform	0.333 ± 0.07	0.353 ± 0.01	0.09 ± 0.09
Acetone	0.5959 ± 0.06	2.09 ± 0.21	2.888 ± 0.48
Ethanol	2.565 ± 0.16	4.979 ± 1.34	5.909 ± 0.51

Phosphomolybdenum assay

The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of green phosphate / Mo (V) complex with the maximal absorption at 695 nm. Among the three *Selaginella* species evaluated, ethanolic extracts of *S. tenera* showed the strongest phosphomolybdenum reduction (26.6 g AA/100 g) followed by *S. intermedia* (24.5 g AA/100 g) and *S. inaequalifolia* (22.1 AA/100 g). All the other tested extracts registered moderate phosphomolybdenum reduction (Table 28).

Table 28: Phosphomolybdenum activity of *Selaginella* species

Extracts	Phosphomolybdenum activity in g AA/100 g		
	S. intermedia	***S. inaequalifolia***	***S. tenera***
Petroleum ether	5.3 ± 0.06	4.7 ± 0.61	5 ± 0.29
Chloroform	13.9 ± 0.06	10.5 ± 0.61	12 ± 0.33
Acetone	22 ± 0.36	15.8 ± 0.17	20.2 ± 0.18
Ethanol	24.5 ± 0.25	22.1 ± 0.14	26.6 ± 024

Larvicidal activity

The larvicidal activity of petroleum ether, chloroform, acetone and ethanolic extracts of selected *Selaginella* species against the fourth instar larvae *C. quinquefasciatus* were tested at different concentration (100-500 mg/ml). All the tested extracts showed moderate larvicidal effect after 24 hrs (Table 29).

Table 29: Larvicidal activity of *Selaginella* species

Species	Extracts	LC_{50} (mg/ml)	95% confidence limits		LC_{90} (mg/ml)	Chi-Square (χ^2)
			Lower	Upper		
S. intermedia	Pet. ether	271.23	250.18	301.61	468.75	0.775
	Chloroform	209.01	171.91	251.69	406.34	4.319
	Acetone	156.36	137.50	171.74	316.78	0.125
	Ethanol	185.36	165.58	203.28	368.19	0.475
S. inaequalifolia	Pet. ether	258.03	234.86	292.15	412.58	2.599
	Chloroform	214.56	195.14	235.95	341.46	2.967
	Acetone	157.04	112.85	186.31	293..19	5.876
	Ethanol	182.11	159.34	202.44	323.34	2.717
S. tenera	Pet. ether	270.42	254.88	290.62	409.48	1.905
	Chloroform	179.65	77.82	196.27	277.94	12.991
	Acetone	227.60	214.54	242.28	366.87	3.567
	Ethanol	155.29	166.02	192.30	315.58	5.759

However the highest larval mortality in terms of lethal concentrations for 50% mortality was observed in the crude ethanolic extracts of *S. tenera* against *C. quinquefasciatus* with LC_{50} = 155.29 mg/ml followed by acetone extracts of *S. intermedia* (LC_{50} = 156.36 mg/ml) and acetone extract of *S. inaequalifolia* (LC_{50} = 157.04 mg/ml). Petroleum ether extract of *S. intermedia* (LC_{50} = 271.23 mg/ml) and *S. tenera* (LC_{50} = 270.42 mg/ml) appeared to have lowest effect against *C. quinquefasciatus*.

Brine shrimp bio-assay

The *in vivo* lethality test on a simple zoological organism such as brine shrimp nauplii was employed as a convenient tool for screening of bioactive natural products. Different extracts of *S. intermedia, S. inaequalifolia* and *S. tenera* showed different mortality rate of brine shrimp, the rate of mortality increased proportionally with the increasing concentration of the extract (Table 30).

Table 30: Cytotoxic activity of *Selaginella* species Brine shrimp

Species	Extracts	LC_{50} (mg/ml)	95% confidence limits		LC_{90} (mg/ml)	Chi-Square (χ^2)
			Lower	Upper		
S. intermedia	Pet. ether	349.45	288.18	431.57	837.34	0.684
	Chloroform	337.91	288.06	397.09	729.15	0.982
	Acetone	250.75	197.45	294.75	594.26	0.116
	Ethanol	235.66	187.28	274.93	535.33	0.786
S. inaequalifolia	Pet. Ether	328.64	275.40	390.13	743.58	2.209
	Chloroform	248.74	200.89	288.87	558.31	0.128
	Acetone	206.66	149.06	249.01	521.15	0.775
	Ethanol	274.26	239.09	307.44	520.59	2.300
S. tenera	Pet. ether	270.79	208.39	325.72	543.87	2.500
	Chloroform	243.71	94.99	331.60	665.38	3.026
	Acetone	249.80	213.81	281.67	484.93	1.511
	Ethanol	253.58	214.93	287.84	511.07	0.962

The inhibitory effect of the extract might be due to the toxic compounds present in the acetone extracts of *S. inaequalifolia* (206.66 mg/ml) which was found to be most effective at

which 50% mortality (LC_{50}) of brine shrimp nauplii occurred (Table 30). Petroleum ether extract of *S. intermedia* showed less cytotoxicity (LC_{50}=349.45 mg/ml).

Anticancer activity using cell line culture

The results of cell growth inhibition by the ethanolic extract of three *Selaginella* species against MCF-7 cell lines for various concentrations were demonstrated in Table 31 (Plate XVI). As the concentration increases there is an increase in the cell growth inhibition. Among the tested three species maximum number of cell inhibition was observed in *S. tenera* (IC_{50} = 53.89 µg/ml) followed by *S. inaequalifolia* and *S. intermedia*. The lower value of IC_{50} indicates a higher anti-cancer activity. *S. tenera* ethanolic extract showed 50% cytotoxic effect (IC_{50} = 167.0 µg/ml) followed by *S. inaequalifolia* and *S. intermedia* showed IC_{50} value > 200 µg/ml.

Table 31: Cytotoxic activity of *Selaginella* species against MCF cell line

Conc. (µg/ml)	*S. intermedia*		*S. inaequalifolia*		*S. tenera*	
	% of cell viability	% of cell inhibition	% of cell viability	% of cell inhibition	% of cell viability	% of cell inhibition
Control	100	0	100	0	100	0
12.5	0	-5.93	0	-3.000	0	-3.96
25	0	-1.674	99.15	0.848	0	-2.80
50	0	-0.135	95.18	4.815	89.63	10.37
100	97.14	2.86	89.81	10.18	63.76	36.24
200	88.84	11.16	57.50	42.49	46.11	53.89

Antidiabetic activity

400 mg/kg of *S. inaequalifolia*, *S. intermedia* and *S. tenera* ethanolic extracts and 600 µg/kg glibenclamide exerted significant hypoglycaemic activity at 0, 30, 60 and 90 min than the control and negative groups. The results indicated that the blood glucose level decrease was directly proportional to the concentraction of ethanolic extract. The ethanolic extracts of studied *S. tenera, S. intermedia* and *S. inaequalifolia* doses of extract decreased blood glucose level in

the ranges of 76.78 – 74.45, 98.69 – 86.67 and 96.27 – 82.27 respectively, from 0 to 90 min after oral glucose load (2 g/kg) as compared with control group. The hypoglycaemic activity of the studied *Selaginella* species were as follows: *S. tenera* > *S. inaequalifolia* > *S. intermedia* (Table 32).

Table 32: Oral Glucose Tolerance Test (OGTT) activity of *Selaginella* species

Drug & Treatment	**Normal Blood Glucose mg/dl**	**Blood Glucose Level in (min) mg/dl**			
		0	**30**	**60**	**90**
Normal control (saline)	1.64 ± 0.5	71.10 ± 0.81	71.21 ± 0.66	71.21 ± 0.67	71.22 ± 0.66
Diabetic control, 2 gm/kg p.o	71.90 ± 0.46	122.97 ± 2.21	151.54 ± 1.14	184.75 ± 1.79	195.81 ± 1.21
Glibenclamide 600µg/kg p.o + Diabetic 2 gm/kg p.o	74.21 ± 2.94	65.92 ± 0.54	61.33 ± 0.87	59.63 ± 0.83	53.17 ± 0.83
S. intermedia 400 mg/kg p.o + Diabetic 2gm/kg p.o	75.27 ± 2.18	101.96 ± 1.30	98.69 ± 69	91.41 ± 0.94	86.67 ± 1.77
S. inaequalifolia 400 mg/kg p.o + Diabetic 2gm/kg p.o	77.14 ± 1.44	97.91 ± 0.85	96.27 ± 0.75	88.18 ± 3.58	82.27 ± 5.91
S. tenera 400 mg/kg p.o + Diabetic 2gm/kg p.o	77.14 ± 1.44	82.26 ± 1.53	76.78 ± 1.42	75.7 ± 0.96	74.45 ± 0.43

Hepatoprotective Activity

On subcutaneous administration of paracetamol with 2.0 g / kg/b.w. dose produced acute hepatic damage in positive control (paracetamol treated) than normal control. All the groups except the normal group treated with paracetamol exhibited significant increase in level of enzymes SGOT, SGPT, ALP and serum bilirubin. Significant difference in enzyme levels (Table 33; Plate XVII) in normal, positive and test groups indicated the hepatoprotective activity of *S. inaequalifolia*, *S. intermedia* and *S. tenera* 100 mg/kg b.w. ethanolic extracts. The paracetamol induced liver damage was confirmed by estimation of elevated levels of SGOT, SGPT, ALP and serum bilirubin. This might be due to the release of these enzymes from the cytoplasm of hepatic cells, into the blood circulation rapidly after rupture of the plasma membrane and cellular damage (Datta *et al*., 2013) resulted from the paracetamol induced lipid peroxidation (Dash *et al*., 2007). Treatment with ethanolic extracts of *Selaginella* species significantly reduced the levels of these enzyme markers in paracetamol treated rats. The decrease in the levels of enzyme markers may be due to the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by paracetamol. The effects of *S. inaequalifolia*, *S. intermedia* and *S. tenera* 100 mg/kg b.w. ethanolic extracts on SGOT, SGPT, ALP and serum bilirubin levels in paracetamol-induced liver damage in rats were summarized in Table 33. Administration of paracetamol (2 g/kg; body weight), after 18 hours of intoxication resulted a significant elevation of hepatospecific serum markers SGOT, SGPT, ALP and serum bilirubin in paracetamol-treated group, in comparison with the normal control group. On administration of *S. inaequalifolia*, *S. intermedia* and *S. tenera* 100 mg/kg b.w. ethanolic extracts (Group III to VI, Table 33) and silymarin at the dose of 100 mg/kg (Group VII, Table 33) retrieved the SGOT, SGPT, ALP and serum bilirubin level. The ethanolic extracts and silymarin treated rats were found normal. The

results indicated that ethanolic extracts of *S. tenera* 100 mg/kg b.w. afforded better protection as compared to other ethanolic extracts of *S. inaequalifolia* and *S. intermedia*. It is also evident that antihepatotoxic effect of the ethanolic extracts of *Selaginella tenera* 100 mg/kg b.w. is almost close to that of the reference drug Silymarin (100 mg/kg). The hepatoprotective activity of ethanolic extracts of studied *Selaginella* species was exerted as follows: *S. tenera* > *S. inaequalifolia* > *S. intermedia*.

Table 33: Hepatoprotective Activity of *Selaginella* species

Group	Animals	SGPT (Units/ml)	SGOT (Units/ml)	Alkaline Phosphates (Units/L)	Bilirubin (Mg/dl)
Control	123.75 ± 4.8	73.11 ± 5.1	74.49 ± 2.00	15.20 ± 7.61	1.09 ± 0.035
Standard Silymarin (100mg/kg p.o)	132.5 ± 5.0	71.23 ± 5.6	81.49 ± 0.9	21.8 ± 0.64	1.2 ± 0.04
Paracetamol 2 g/kg p.o	135.0 ± 5.7	128.11 ± 1.5	129.79 ± 8.10	83.88 ± 3.77	2.49 ± 0.17
S. intermedia 100 mg/kg p.o	127.5 ± 9.5	103.29 ± 1.4	113.6 ± 5.34	72.64 ± 2.65	2.20 ± 0.10
S. inaequalifolia 100 mg/kg p.o	130.0 ± 8.1	109.66 ± 7.2	117.80 ± 3.8	71.62 ± 0.53	2.08 ± 0.09
S. tenera 100 mg/kg	135.0 ± 5.7	91.305 ± 5.1	92.20 ± 2.62	24.35 ± 0.88	1.41 ± 0.07

5. DISCUSSION

To provide protection against adverse effects of the environment, plants have the ability to produce many kinds of secondary metabolites as a defense mechanism or as natural protectants (Bennett and Wallsgrove, 2006). Since ferns and fern allies have survived from Paleozoic times, they have survived various environmental and climatic conditions (Wallace *et al.,* 1991). Therefore, they are expected to synthesize many useful secondary metabolites than other plants. Ferns and fern allies were reported to have many useful phytochemicals (secondary metabolites) such as flavonoids, steroids, alkaloids, phenolics, triterpenoids, amino acids and fatty acids (Zeng-fu *et al*., 2008). Due to the existence of these secondary metabolites the ferns and fern allies possessed various biological molecules with varied potentials viz., antioxidant, antibacterial, anti-diabetic, antitumor, antifungal, antiseptic, anti-inflammatory activities etc. (Vasudeva, 1999; Thulsi Rao *et al*., 2007; Maridass and Ravichandran, 2009; Maridass and Raju, 2009). In the present study also, the preliminary phytochemical analysis confirmed the occurrence of steroids, alkaloids, phenolics, flavonoids, saponins, tannins, cardiac glycosides, aminoacids and anthraquinone in the three studied *Selaginella* species (Table 4).

Histochemical analysis results revealed the variations in the occurrence of various metabolites viz., phenolics, tannins and lipids with varied quantities in different tissues of the stem. High concentration of lignin, cutin and suberin presence was observed in epidermis, hypodermis and xylem of studied *Selaginella* species. High concentration of phenolic existence was noted in ground tissue and xylem of *Selaginella* species. High concentration of tannin presence was observed in the epidermis of studied *Selaginella*. Lignin is an aromatic heteropolymer that is deposited most abundantly in the secondary walls of vascular plants. It

provides structural rigidity to the plant body while enabling individual tracheory elements to withstand tension generated during water transport; it also serves as defensive role against pathogens and insects. Generally fern and fern allies deposit lignins that are derived primarily from guiacyl monomers together with a small portion of p-hydroxyphenyl units. Weng *et al.* (2008) reported the occurrence of syringyl monomers in the *Selaginella* species. The results of histochemical analysis also confirmed Weng *et al.* (2008) observations.

Mithraja *et al.* (2011) observed that the crude extracts of *A. pinnata*, *M. minuta* and *S. molesta* showed diverse phyto-profiles with reference to solvents of the plant extracts. The aqueous extracts of *A. pinnata* displayed maximum number of phyto-constituents (33%), followed by acetone, benzene, chloroform and ethanol (25%). The chloroform and petroleum ether extracts of *M. minuta* demonstrated maximum occurrence of phyto-constituents (58%), next to that acetone, benzene and aqueous (50%). The chloroform extracts of *S. molesta* showed maximum occurrence of phyto-constituents (58%), next to that ethanol (50%) followed by acetone, benzene, aqueous and petroleum ether (42%). Similarly in the present study also the ethanolic extract of *S. intermedia* displayed the existence of more frequency (88%) of metabolites followed by chloroform extracts (55%) and acetone extract (44%). The ethanolic and chloroform extracts of *S. inaequalifolia* showed highest percentage of metabolites (55%) existence. Maximum frequency (60%) of metabolites was observed in the ethanolic extracts of *S. tenera*. Generally the ethanolic extracts of the studied *Selaginella* species showed more number of metabolites occurrence. These may be due to the polar nature of the solvents.

Mithraja *et al.* (2012) screened the phytochemical composition of some important medicinal Pteridophytes of Western Ghats viz., *D. heterophyllum, D. linearis, B. orientale, C. thalictroides, H. arifolia, L. ensifolia, N. multiflora, P. calomelanos, P. confusa* leaves and

rhizomes of *D. quercifolia.* They obtained different phyto-profiles with reference to solvents used for the extraction. The aqueous extracts of *D. quercifolia* leaves illustrated maximum occurence of phyto-constituents (50%), next to that acetone (42%) followed by ethanol and benzene (33%). The benzene extracts of *D. heterophyllum* demonstrated maximum presence of phyto-constituents (58%), next to that ethanol and petroleum ether (50%) followed by chloroform and aqueous (42%). The ethanolic extracts of *D. linearis* showed maximum number of metabolites (50%), followed by aqueous and acetone (42%). The chloroform and petroleum ether extracts of *D. linearis* showed least percentage (33%). The benzene extracts of *B. orientale* illustrated maximum number of phyto-constituents (58%), followed by ethanol, acetone (42%) and petroleum ether (33%). The ethanolic and petroleum ether extracts of *C. thalictroides* diplayed maximum number of phyto-constituents (33%), followed by aqueous and benzene (25%). Acetone and chloroform (17%) showed least percentage of metabolites occurence.

Among the four extracts of *S. intermedia*, the ethanolic extract of *S. intermedia* showed the presence of more frequency (88%) of metabolites followed by chloroform extracts (55%) and acetone extract (44%). The least percentage of metabolites (22%) was observed in petroleum ether extracts of *S. intermedia*. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenolics, saponins, sterols etc. Thus the preliminary screening tests may be useful to detect the bioactive principles in the drug discovery process. In the present study, preliminary phytochemical analysis was carried out in *S. intermedia, S. inaequalifolia* and *S. tenera.* The results showed the presence of steroids, alkaloids, phenolics, flavonoids, saponins, tannins, cardiac glycosides, aminoacids and anthraquinones. The results suggest that these crude extracts can be used as drugs to control infectious and non-infectious diseases. The results of phytochemical analysis

further provide information regarding the activity of these tested extracts. These extracts could be used for further isolation and purification of bioactive compounds.

Irene Pearl *et al.* (2011) studied the pharmacognostical characters of *Selaginella involvens* and observed the presence of chemical compounds like steroids, triterpenes, phenolics, saponins, alkaloids, anthraquinone, tannin, sugars, amino acids and catechin. Of the five different extracts, petroleum ether extract of *S. involvens* showed the maximum number (8) of chemical compounds followed by benzene and chloroform extracts with the presence of 6 different chemical compounds each.

Secondary metabolism refers to compounds present in specialized cells that are not directly essential for basic photosynthetic or respiratory metabolism but are thought to be required for plants survival in the environment. Plants produce a large variety of secondary products (Lattanzio *et al.,* 2006). Among them, phenolic is one of the important groups of secondary metabolites due to their biological potentials (Wuyts *et al*., 2006). Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. The phenolics possesses a variety of biological properties such as anti-oxidant, anti-apoptosis, anti-carcinogenic, anti-inflammatory, anti-aging, anti-atherosclerosis, cardiovascular protection as well as inhibition of proliferation activity (Han *et al.,* 2007). Phenolic compounds also contribute to quality and nutritional value in terms of modifying colour, taste, aroma and flavour. They are also involved in plant defense mechanisms to scavenge reactive oxygen species (Vaya *et al*., 1997; Singh *et al.,* 2007; Reddy *et al.,* 2008).

Phenolics usually accumulate in the central vacuoles of guard cells and epidermal cells as well as subepidermal cells of leaves and shoots. Both monophenolic and polyphenolic compounds from a large variety of plant foods, spices and beverages have been shown to inhibit

the initiation, progression and spread of cancers in cells *in vitro* and in animals *in vivo* (Wahle *et al.,* 2010). In the present study, the histochemical analysis confirmed the occurrence of phenolics in the ground tissue, epidermis and xylem of studied three *Selaginella* species (Table 3). The results of preliminary and HPTLC analysis also supplemented the histochemical observations. The quantitative analysis determined that 135.39 mg GAE/g of phenolics occurrence in the ethanolic extracts of *S. intermedia* followed by 115.74 mg GAE/g in ethanolic extracts of *S. inaequalifolia* and 114.28 mg GAE/g in ethanolic extracts of *S. tenera*. The results of the present study clearly indicated the environmental adaptive, survival mechanism and medicinal properties of the studied *Selaginella* species.

Flavonoids are low molecular weight polyphenolic compounds which include flavones, flavonones, isoflavones, flavonols, flavon-3-ols and anthocyanins. They possess remarkable antioxidant activities and inhibit enzyme activities like lipooxygenase, cyclooxygenase and prostaglandin synthase. Flavonoids enhance the effects of vitamin C and function as antioxidants. They are also known to be biologically active against liver toxins, tumors, viruses and other microbes (Korkina *et al.*, 1997). Flavonoids are known to inhibit tumor growth and serve to protect against gastrointestinal infections (El-Mahmood *et al.*, 2008).

Flavonoids have ability to induce human protective enzyme systems. Number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers and other age-related diseases (Pandey, 2007; Kumar *et al*., 2013; Evans *et al*., 1995). The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation

ability (Kelly *et al*., 2002; Pandey *et al*., 2012). The preliminary phytochemical and HPTLC analysis confirmed the occurrences of flavonoids in the studied *Selaginella* species (Table 14). The quantitative studies estimates that 195.11 mg GAE/g flavonoids in the ethanolic extracts of *S. intermedia* followed by 183.55 mg GAE/g in ethanolic extracts of *S. tenera* and 143.11 mg GAE/g in ethanolic extracts of *S. inaequalifolia*. The results of antioxidant activity confirmed the role of flavonoids in the antioxidant defense system of the plant, medicinal and nutritional properties of the studied *Selaginella* species.

Tannins are a group of natural products widely distributed in plants. They help to reduce the risk of coronary heart diseases (Ranjithkumar, 2010). Tannins have shown potential antiviral, antibacterial and antiparasitic effects (Winter *et al*., 1993). In the present study, the histochemical analysis confirmed the occurrence of tannins in the epidemis of studied three *Selaginella* species (Table 3). The results of preliminary and HPTLC analysis also supplemented the histochemical observations. The quantitative analysis determined the presence of 64.6 mg/g of tannins in the ethanolic extracts of *S. inaequalifolia* followed by 52.22 mg GAE/g in ethanolic extracts of *S. intermedia*.

More than 36000 terpenoids compounds have been identified, making terpenoids the largest class of plant metabolites. The terpenoids show significant pharmacological activities such as anti-viral, anti-bacterial, anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities (Mahato and Sen, 1997). Terpenoids play a crucial role in plant-environment interactions including plant-plant, plant-pathogen, plant-insect and plant-animal interactions (Paschold *et al*., 2006). They are commercially employed in an immense number of industrial products such as flavouring agents, pharmaceuticals, perfumes and anti-microbial agents (Martin *et al.*, 2003). Terpenoids have been found to be useful in the prevention

and treatment of several diseases including cancer (Rabi *et al*., 2009; Wagner *et al*., 2003). In addition, terpenoids can be used as protective substances in storing agricultural products as they are known to have insecticidal properties as well (Sultana *et al*., 2008). Paulraj (2011) reported the presence of triterpenoids in the epidermal glands of six thelypteroid ferns from South India. In the present study also, the preliminary phytochemical and HPTLC analysis confirmed the occurrence of triterpenoids in the studied *Selaginella* species (Table 16). The anticancer and cytotoxic properties of the studied *Selaginella* species may be due to the occurrence of terpenoids.

Saponins act as bioactive antibacterial agents in plants and are used to treat hypercholesterolemia, hyperglycemia and obesity (Mohanta *et al*., 2007). The preliminary phytochemical analysis confirmed the occurrence of saponins in the studied *Selaginella* species (Table 4). Alkaloids are one of the largest groups of phytochemicals that have led to the invention of powerful pain killer medications (Kam and Liew, 2002). They often have pharmacological effects and are used as medications and recreational drugs (Rhoades, 1979). The preliminary phytochemical and HPTLC analysis confirmed the occurrence of alkaloids in the studied *Selaginella* species (Table 13). The result of antidiabetic analysis supplemented the previous observation. The existence of saponins in the studied *Selaginella* species may also be responsible for the antidiabetic potentials.

Some secondary metabolites of the plants such as alkaloids, terpenoids and glycosides serve either as protective agents against various pathogens (e.g. insects, fungi or bacteria) or growth regulatory molecules (e.g. hormone-like substances). As a result, secondary metabolites can serve as potential anticancer drugs, either by direct cytotoxic activity against cancer cells or by modulating the tumor development process (Kintzios, 2003). In fact, natural products are

considered as a mainstay in cancer treatment, where as 60% of worldwide anticancer drugs between 1983 and 1994 were from natural origin (Cragg *et al.,* 1997). In addition the phytochemical profile of the studied *Selaginella* species was helped to identify the phylogenetic relationship among the three species. The cladogram constructed based on the existence of phytoconstituents in the *Selaginella* species showed two clusters. Cluster 1 (C_1) included *S. intermedia* only and showed 100% of divergence from other two studied species (Fig. 2). Cluster 2 (C_2) was shared by *S. inaequalifolia* and *S. tenera.*

The physico-chemical assessment of the drug is an essential factor in identifing adulteration of drugs. Proper identification and quality of the starting material is an important prerequisite to guarantee reproducible quality of herbal medicine (Nayak and Patel, 2010). For fluorescence analysis, the powders of selected three *Selaginella* 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. Kala *et al.* (2011) employed fluorescence characters as a tool to identify the medicinal plants of South India. The phytochemical and fluorescence analysis results also distinguished the studied *Selaginella* species and these profiles may be applied to identify the *Selaginella* species from its adulterants in the pharmaceutical industries.

Chemosystematics is the attempt to classify or identify the plants according to demonstrable differences and similarities in their biochemical compositions. The coupling of morphological characters with other biochemical/phytochemical data has yielded valuable insight into underlying biochemical processes and has contributed to numerous advances in the area of chemotaxonomy. Many modern techniques have been employed to characterize and

identify metabolites present in plants and also distinguish the medicinal plants from its adulterants. Furthermore, it can provide information for unknown samples.

Phytochemical analysis requires an efficient and unbiased extraction of metabolites from plant tissues (Aparicio and Aparicio-Ruiz, 2000), identification of specific functional groups (fingerprint) of compounds / plants and other living organisms without separation and directly based on their absorption characteristics using UV-Vis and FT-IR spectroscopy (Baeten and Aparicio, 2000). UV-Vis spectrum obtained from an instrument is simply a plot of wavelength (frequency) of absorption versus the absorption intensity (Baeten and Aparicio, 2000). It is generally applied to identify molecules or inorganic complexes in solution. In the present study, UV-Vis spectra of different extracts of studied *Selaginella* species have broad features that are of limited use for identification and are very useful for quantitative measurements. However, validation studies must be performed in order to assure the ability of any analytical method to generate reliable and interpretable information. These UV-Vis spectras can be used as chemometric tool to distinguish the studied *Selaginella* species. These UV-Vis spectroscopic peak results may be used as pharmacognostic markers in the pharmaceutical industries. *S. intermedia* showed unique value absorbance 3.311 at 404 nm in petroleum ether extract and acetone extract of *S. inaequalifolia* showed their identity by the distinctive absorbance 2.872 at 663 nm. The ethanolic extract of *S. tenera* represented exceptional absorbance of 4.000 at 315 nm. In addition UV-Vis spectroscopic profile of the studied *Selaginella* species was used to identify the phylogenetic relationship among the three species. The cladogram constructed based on the UV-Vis spectroscopic profile of *Selaginella* species showed two clusters. Cluster 2 (C_2) explains the similarity of *S. inaequalifolia* and *S. tenera*. Cluster 1 (C_1) showed 100% divergence

of *S. intermedia* from the other two studied species (Fig. 4). The cladogram clearly explained the relationships among the three *Selaginella* species.

FT-IR is a rapid, non invasive, high-resolution analytical tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular fingerprint (Griffiths and de Haseth, 1986). It is one of the most widely used methods to identify the functional groups of the active principles and pave a pathway to elucidate the compound's structures. In addition it has been used as a requisite method to identify medicines in the Pharmacopoeia of many countries and used as chemotaxonomic tool to distinguish the medicinally and economically important plants (Liu *et al*., 2006). FT-IR method measures the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a biochemical or metabolic "fingerprint" of the sample. By attaining IR spectra from plant samples, it might be possible to detect the minor changes of primary and secondary metabolites (Surewicz *et al*., 1993; McCann *et al*., 1992).

Muruganantham *et al*. (2009) carried out FT-IR spectral analysis of plant parts like leaves, stem and root of *Eclipta alba* and *Eclipta prostrata* and reported the presence of characteristic functional groups such as carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, nitrates, chlorates and carbohydrates. Ragavendran *et al*. (2011) identified the functional groups carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, organic hydrocarbons and halogens in *Aerva lanata*. In the present study, FT-IR spectrum was used to confirm the functional groups presence in different extracts of studied *Selaginella* species. The results of the present study directly coincided with the previous observations in determining the presence of functional groups and generating a fingerprint for the studied *Selaginella* species. The spectroscopic peak values may be used as phytomarker for the studied species in the

pharmaceutical industries. The unique occurrence of 1026.13, 1735.93 spectroscopic peak value clearly distinguish the *S. intermedia* from other two studied *Selaginella* species. The results of the present study suggest that these spectroscopic profiles may be used as biomarkers to distinguish the medicinally important species. In addition these spectroscopic profiles are used to know the inter relationships among the three studied species. The cladogram constucted based on the FT-IR spectroscopic values clearly distinguished the studied three *Selaginella* species. The cladogram constructed based on FT-IR analysis showed two clusters, of which Cluster 2 (C_2) represented *S. tenera* which showed 100% of divergence from the other two studied species. Cluster 1 (C_1) was shared by *S. intermedia* and *S. inaequalifolia* (Fig. 10).

TLC is a quick, convenient and cost effective liquid chromatographic technique widely used for pharmaceutical and chemotaxonomical analyses. TLC has the special ability to assay many samples at the same time on a single plate (Talukdar *et al.,* 2010; Mohammad *et al.,* 2010). It is recommended as an effective method for identification of plant derivatives by Chinese, American and European Pharmacopoeias (Fuzzati, 2004). Previous reports on TLC studies have been carried out on various ferns to know the occurrence of various metabolites and to study the inter-specific relationship among the Pteridophytes and other plants. The results of the present study showed the chemical diversity of the studied *Selaginella* species and also clearly distinguished the studied three species of *Selaginella* based on the chemical profile. Irudayaraj and Johnson (2011) determined the inter-specific relationship among the three *Asplenium* species using TLC. Chikmawati *et al*. (2012) performed TLC tests in various *Selaginella* extracts to qualitatively analyze the bioactive compounds alkaloids, flavonoids and steroids. In the present study, TLC analysis on different extracts of three *Selaginella* species revealed 68 phenolic bands and 64 steroidal bands. The results showed both distinct and common banding profiles. The

results of the present study directly coincide with previous observations on the chemosystematics of plants. The cladogram constructed based on the phenolic profile showed *S. inaequalifolia* and *S. tenera* in the same clade whereas *S. intermedia* in a divergent clade. The cladogram constructed based on steroidal profile displayed *S. intermedia* and *S. tenera* in the same clade whereas *S. inaequalifolia* in a separate clade. The results of the TLC analysis indicated the chemical diversity of the *Selaginella* species and revealed the inter-specific variation among the *Selaginella* species. These profiles may be used in the pharmaceutical industry to distinguish the medicinally important *Selaginella* species.

HPTLC techniques are used for many qualitative and quantitative analytical problems in a wide range of fields including medicines, pharmaceutical, biochemistry and toxicology. In addition, finger print analysis by HPTLC has become an effective and powerful tool for linking the chemical constituents' profile of medicinal plants with botanical identity and act as biochemical markers in the chemosystematics (Sharma *et al*., 2010; Paramasivam *et al*., 2008). HPTLC has the ability to analyze several samples simultaneously using a small quantity of mobile phase. This also reduces time, cost of analysis, minimizes exposure risks and significantly reduces environment pollution through the limited usage of toxic effluents. HPTLC also facilitates repeated detection of chromatogram with same or different parameters (Kokate *et al*., 2006). HPTLC analysis was carried out to know the chemical profile of *Asplenium aethiopicum* (Gowtham, 2013); *Drynaria quercifolia* (Kamboj and Kalia, 2014) and *Cyathea* species (Janakiraman, 2015). In the present study also, HPTLC analysis has been carried out to know the alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and terpenoids profile of three *Selaginella* species. The results depicted varied degree of both known and unknown metabolites with different R_f values. The HPTLC profiles will be used as a valuable tool for

reliable identification of *Selaginella* species. In addition the results provided the chemical composition of the studied *Selaginella* species and paved a pathway to identify the active principles of studied *Selaginella* species. Further analysis may bringout some more active principles from the studied *Selaginella* species.

HPLC offers several advantages such as the possibility of automating and computer processing (Backonja *et al*., 2010), advanced reproducibility in identifying the isolated compounds (Zhang *et al*., 2010), quantitative determination (Douat *et al*., 2011) and the possibility of performing the analyses in a relatively short period of time (Reyes *et al*., 2011). It is a highly sensitive method for detection, identification and quantification of any chemical in a particular sample using UV and visible absorbance (Hanachi and Golkho, 2009). It is an important qualitative and quantitative technique used for potency / purity / performance assays, pharmacokinetics / bioanalytical testing, purification, high-throughput screening, estimation of pharmaceutical and biological samples (Guillarme *et al.,* 2012; Snyder *et al.,* 2009; Ahuja and Rasmussen, 2007). It is also the most versatile chromatographic technique for the quality control of drug components (Chen *et al.,* 2007; Ranjith *et al.,* 2008; Li *et al.,* 2011; Wang *et al.,* 2013).

In the present study, HPLC profile for *S. intermedia, S. inaequalifolia* and *S. tenera* ethanolic extracts were evolved to confirm the presence of the active constituents. Standardization is an important aspect for establishing the quality and efficacy of ayurvedic formulations or any multiple ingredient herbal formulations. Generally, two approaches are being used for standardization viz., fingerprint analysis by HPLC/HPTLC and quantification of individual chemical markers. It ensures reproducible pharmaceutical quality of herbal products. In the present study, HPLC and HPTLC fingerprint analysis were carried out for the ethanolic extract of studied *Selaginella* species to reveal the chemical diversity of studied three *Selaginella*

species. The results of HPLC and HPTLC confirmed the chemical diversity of the studied *Selaginella* species. Preliminary analysis of the ethanolic extracts of various *Selaginella* species showed varying patterns in HPLC chromatogram suggesting that there might be differences in the process of manufacturing and therefore a proper scientific validation (chromatographic fingerprinting of major constituents) is needed for quality control and medicinal utilization. These studies exhibited novel markers in standardization as useful analytical tools to check not only the quality of the powder but also the presence of adulterants in ayurvedic drugs. The generated data may be useful in suggesting chemotaxonomical inter-relationship among the studied *Selaginella* species.

GC-MS has become firmly established as a key platform for secondary metabolite profiling in medicinal plants (Robertson, 2005). It has proved to be a reliable method for the analysis of non-polar components, volatile essential oils, fatty acids, lipids and alkaloids (Bertz *et al*., 1997). It has many applications including high sensitivity and specificity. The combination of a principle separation technique (GC) with the best identification technique (MS) made GC-MS an ideal for qualitative and quantitative analysis for volatile and semi-volatile compounds (Karthishwaran *et al*., 2012). Previous studies on GC-MS analysis showed the presence of various compounds in *Abacopteris penangiana* (Zhongxiang *et al*., 2007), *Adiantum tetraphylum* (Melos *et al.,* 2007), *Salvinia molesta* (Choudhary *et al.,* 2008), *Marsilea quadrifolia* (Sivagurunathan and Xavier, 2014) and *Actiniopteris radiata* (Manonmani and Sara, 2015). The results of GC-MS analysis showed the presence of different phytoconstituents in the studied three *Selaginella* species with varied retention time. The compounds were predicted based on the compounds present in the NIST library. The biological properties of the identified compounds were predicted based on PASS online. The predicted compounds possessed the following

properties viz., anti-inflamatory, anti-diabetic, anti-oxidant, anti-bacterial, hepatoprotectant, anti-fungal, postmenopausal disorders treatment, prostate disorders treatment, calcium regulator, anti-neoplastic, anti-seborrheic, prostate disorder treatment, anti-thrombotic, muscular relaxant, anti-parasitic, platelet aggregation stimulant, anti-viral, anti-mutagenic, neuropathy treatment, protein kinase stimulant, diuretic potential etc. Previous studies on *S. tenera and S. inaequalifolia* confirmed the antibacterial potentials (Suganya *et al*., 2011; Irudayaraj *et al*., 2010). Gayathri *et al*. (2005) confirmed the anti-oxidant potentials of *S. involvens*. The results of antioxidant, anti-diabetic and hepatoprotective activity of *Selaginella* species was also confirmed in the PASS prediction. The results of PASS biological activities directly coincided with ethnobotanical and pharmacological observations of studied *Selaginella* species. The PASS prediction revealed and supplemented the biopotency of studied *Selaginella* species. Further phytochemical and pharmacological analysis on the studied *Selaginella* species may bring out some multipotent drugs.

Plant products have been traditionally used by human communities in many parts of the world against the mosquito vectors. Babu and Murugan (1998) suggested that plant derived metabolites or plant crude extracts can act as larvicides, insecticidal, anti-repellents and as anti-deterrent. Plants produce secondary metabolites that have insect growth inhibitory activity. Besides the use of such compounds as agricultural insect pest control agents, their use in mosquito larvae control is an interesting perspective. Larvicidal activites of *Goniothalamus macrophyllus* (Wattanapiromsakul *et al*., 2005), *Ricinus communis* (Mandal, 2010), *Adiantum craccivora* (Sood and Sharma, 2010), *Sida acuta* (Marimuthu, 2010), *Andrographis paniculata* (Marimuthu, 2011) and *Asplenium aethiopicum* (Johnson *et al*., 2014) confirmed the suggestions of Babu and Murugan (1998). In the present study, larvicidal efficacies of three *Selaginella*

species were studied against the filarial vector *C. quinquefasciatus.* The highest larval mortality in terms of lethal concentration 50% mortality (LC_{50}) was observed in the crude chloroform extracts of *S. tenera* against *C. quinquefasciatus* with the LC_{50} value 55.29 mg/ml. The results of the present study indicated that the studied *Selaginella* species can be used as an effective larvicidal agent to control the mosquito vectors. In the current study the ethanolic and acetone extracts of the three *Selaginella* species showed high percentage of mortallity than the other tested extracts. The ethanolic extract of *Selaginella tenera* showed the high degree of larvicidal activity against *C. quinquefasciatus* with the LC_{50} value 155.29 mg/ml.

Brine shrimp assay has good correlation with cytotoxic activity in some human solid tumors and has led to the discovery of a new class of natural active antitumor agents (Mc Laughlin *et al.*, 1998). In the present study, the inhibitory effects of studied *Selaginella* species might be due to the toxic compounds present in the extracts. Among the tested three species, *S. inaequalifolia* was found to be most effective with LC_{50} value 206.66 mg/ml. It leads to identification of novel antitumor and anticancer agents in the ethanolic extracts of *Selaginella* species. Similar to the present study, Johnson *et al.* (2014) and Babu (2013) used the brine shrimp assay to detect the cytotoxic potentials of *Asplenium aethiopicum, Adiantum latifolium, Acrostichum aureum, Chiristella interruptus* and *Histiopteris incisa* respectively. Akter *et al.* (2014) investigated the cytotoxic properties of *Diplazium esculentum* using brine shrimp lethality bioassay. They observed the LC_{50} values of chloroform and ethanolic extracts and compared to the standard anticancer drug Vincristine sulphate.

Chai *et al*. (2015) evaluated the glucosidase inhibitory and cytotoxic activities of five selected edible and medicinal ferns viz., *Blechnum orientale*, *Davallia denticulata*, *Diplazium esculentum*, *Nephrolepis biserrata* and *Pteris vittata*. The α-glucosidase inhibitory activity of *D.*

esculentum (half maximal effective concentration, EC_{50} = 6.85 µg/ml) was considerably stronger than that of myricetin (EC_{50} = 53.21 µg/ml). *B. orientale, D. esculentum, N. biserrata* and *P. vittata* were cytotoxic to K562 cells. *P. vittata* showed the strongest cytotoxicity, although it was less potent than 5-fluorouracil. *D. denticulata* displayed the highest phenolic, hydroxycinnamic acid and flavonoid contents of all the extracts while *B. orientale* had the highest proanthocyanidin content. All the fern extracts except *D. denticulata*, exhibited dose-dependent cytotoxicity against K562 cells. In the present study, the acetone extracts of *S. inaequalifolia* were found most effective.

The cytotoxic potentials of *Selaginella tamariscina* (Lee *et al.,* 1999), *Selaginella willdenowii* (Silva *et al*., 1995) and *Artocarpus heterophyllus* (Patel and Patel, 2011) were examined using MCF-7 breast cancer cell lines. Similarly in the present study also, to know the cytotoxic potentials of three *Selaginella* species ethanolic extracts were examined against MCF-7 breast cancer cell lines. The cell growth inhibition was directly proportional to concentration of the ethanolic extracts of *Selaginella* species. Maximum number of cell inhibition was observed in *S. tenera* with the IC_{50} value 53.89 µg/ml. The results showed that cytotoxicity assay using MCF-7 cell line is an appropriate method for screening new substances within a short period of time. Sarker *et al*. (2011**)** investigated the antitumor properties of *Selaginella ciliaris*, *Marsilea minuta* and *Thelypteris prolifera*. They observed highest percentage of tumor inhibition in *M. minuta* (82.32%) followed by *S. ciliaris* (80%) and *T. prolifera* (75.68%) at 1000 ppm.

Lai *et al*. (2010) evaluated antioxidant, anticancer and antibacterial activity of five solvent fractions obtained from the methanolic extract of *Blechnum orientale* leaves. The ethyl acetate, butanol and water fractions possessed strong radical scavenging activity (IC_{50} 8.6-13.0 g/ml) and cytotoxic activity towards human colon cancer cell HT-29 (IC_{50} 27.5-42.8 g/ml).

Zakaria *et al.* (2011) investigated the *in vitro* cytotoxic and antioxidant properties of the aqueous, chloroform and methanolic extracts of *Dicranopteris linearis* leaves. They observed that chloroform extract was effective only against MCF-7 and HeLa and the methanolic extract was effective against all the cancer cells used. Interestingly, all extracts failed to produce cytotoxic effect against the 3T3 cells (normal cell) indicating their safety. Similarly in the present study also the ethanolic extracts of the studied *Selaginella* species showed cytotoxic effect against the MCF-7 cell lines.

The oral glucose tolerance test is designed to evaluate the person's ability to tolerate orally administrated glucose and is well-known in diagnosis of pre-diabetic and diabetic conditions. In addition, it is also used to diagnose a malabsorption syndrome in which sugar is not properly absorbed through intestines into the blood stream (Rang *et al.,* 2003). In the present study, 400 mg/kg of *S. inaequalifolia*, *S. intermedia* and *S. tenera* ethanolic extracts and 600 µg/kg of glibenclamide were found effective in reducing blood glucose level after 0, 30 and 60 min of treatment (ethanolic extracts + glucose load) without being sedative in their respective test groups. 400 mg/kg of *S. tenera* ethanolic extracts significantly decreased blood glucose level by 76.78-74.45% at 0, 30 and 60 min after oral administration in their respective test group as compared to positive control and negative control groups. The reduction in the blood glucose level might be due to the extra-pancreatic action of extract either by inhibiting glucose absorption in intestine or by increasing glucose tolerance in mice by enhancing the glucose uptake in tissues such as muscles and liver and thereby stimulating glycolysis and hepatic glycogenesis (Qureshi *et al.*, 2009). The oral hypoglycemic agent glibenclamide (positive control) was also found significant in increasing glucose tolerance in mice as compared to control and negative control groups from 0 to 60 min. The glibenclamide is a second generation

sulfonylurea (known antidiabetic drug) which increases the release of insulin from functional β-cells of pancreas (Davis, 2006). The results of the present study identified another possible mechanism of action (*Selaginella*) in reducing the blood glucose level by acting at pancreatic level and enhancing the release of insulin which in turn decrease blood glucose level by stimulating all the anabolic effects (Nolte, 2009). Some medicinal plants were reported to enhance the release of insulin from beta-cells of pancreas such as *Christella dentata, Angiopteris evecta, Gymnema sylvestre, Ocimum sanctum* and *Phyllanthus* species (Al-Romaiyan *et al.,* 2010; Daisy *et al.,* 2004; Hannan *et al.,* 2006; Tanzin *et al.,* 2013; Rahmatullah *et al.,* 2014). Therefore, the results suggest that *S. inaequalifolia*, *S. intermedia* and *S. tenera* ethanolic extracts could be chosen for studying the long-term hypoglycemic activity in type I and II diabetic animal models which will not only help in elucidating the exact mechanism of action of the studied *Selaginella* extract in reducing blood glucose level but also pave a way to find a new bioactive compound with hypoglycemic activity.

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Tiwari, 2001). They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. Majority of the diseases / disorders are mainly linked to oxidative stress due to free radicals (Gutteridgde, 1995) which includes numerous chronic and degenerative diseases such as atherosclerosis, cardiovascular disorders, aging, diabetes mellitus, cancer, neurodegenerative diseases etc. (Metodiwa and Koska, 2000; Young and Woodside, 2001; Heinecke, 2003).

Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases (Devasagayam *et al*., 2004). Compounds responsible for such antioxidant activity can be isolated and used for prevention and treatment of free radical-related disorders

(Middleton *et al*., 2000). The scavenging effects of studied *Selaginella* species are as follows: *S. intermedia* > *S. tenera* > *S. inaequalifolia*. The results of the present study suggest that all the studied species of *Selaginella* contain powerful inhibitor compounds which may act as primary antioxidants that react with free radicals. As in selected *Selaginella* species, the crude extracts of few other pteridophytes have already been reported to exhibit strong scavenging properties (Chang *et al*., 2007; Lee *et al*., 2003).

$ABTS^{+}$, a protonated radical, has the characteristic absorbance maximum at 734 nm which decreases the scavenging of the proton radicals. Hagerman *et al*. (1998) reported the high molecular weight phenolics have more ability to quench free radicals ($ABTS^{+}$). In the present study, ethanolic extract of *S. tenera* showed the highest activity (863.99 µmol TEg^{-1}) followed by *S. inaequalifolia* (849.82 µmol TEg^{-1}). This may be due to the phenolics presence in the ethanolic extracts of studied *Selaginella* species. In contrary to Hagerman *et al*. (1998) observations, in the present study, the best free radical scavenging activity was observed in *S. tenera.* They are possessing least amount of phenolics compared to other two studied species.

The order of FRAP activity of studied *Selaginella* species are as follows: *S. tenera* > *S. intermedia* > *S. inaequalifolia*. According to Oktay *et al.* (2003), a positive relationship between total phenolics and antioxidant activity appears to be the trend in many plant species. In contrary to Oktay *et al.* (2003) observations, the results of phenolic contents of the *Selaginella* species are as follows: *S. intermedia* > *S. tenera* > *S. inaequalifolia*, but the highest FRAP activity was observed in ethanolic extracts of *S. tenera.*

The effect on the ferrous ions of studied *Selaginella* species exhibited the ability to chelate metal ions. Among the studied three species, ethanolic extracts of *S. inaequalifolia* showed higher activity. Phosphomolybdenum method is based on the reduction of Mo(VI) to

Mo(V) by the antioxidant compounds and the formation of green phosphate/Mo(V) complex with the maximal absorption at 695 nm. Among the three studied species of *Selaginella* evaluated, the ethanolic extracts of *S. tenera* displayed the strongest phosphomolybdenum reduction (26.10±0.65 g AA/100 g).

The high content of polyphenolics compounds present in the ethanolic extracts of the studied species of *Selaginella* should be able to chelate transition metal because of the high charge density of the phenoxide group generated on deprotonation (Lai *et al*., 2011). The results of the present study established that the extracts could chelate irons and the values are substantial. The transition metal ion is capable of generating free radicals from peroxides by fenton reactions and may be implicated in human cardiovascular diseases (Chung *et al*., 2002). Since Fe^{2+} has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in fenton reactions affords protection against oxidative damage. 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 results of the present study demonstrated that ethanolic extracts of studied *Selaginella* species possess significant antioxidant and free radical scavenging activities.

Previously Gayathri *et al*. (2005) studied the antioxidant properties of *S. involvens*, *S. delicatula* and *S. wightii* from the wild and tested the *in vitro* and *in vivo* lipid peroxidation, immunomodulatory property and hydroxyl radical scavenging activity. They reported that the aqueous extract of *S. involvens* has promising thymus growth stimulatory activity in adult mice and remarkable antilipid peroxidation property. In the present study, antioxidant activities of different *Selaginella* species using DPPH+, ABTS+, FRAP, metal chelating and

phosphomolybdenum reduction were studied and the results showed more scavenging properties than the previous observations.

The ability of hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Hepatocellular necrosis or membrane damage leads to high levels of serum SGOT and SGPT released from liver to circulation. Among the two, SGPT is a better index of liver injury, since SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner, thus liver GPT represents 90% of total enzyme present in the body (Achliya *et al*., 2003). Bilirubin, a metabolic product of the breakdown of heme rises in diseases of hepatocytes, obstruction to biliary excretion into duodenum or in hemolysis (Mohan, 2002).

The elevated levels of serum marker enzymes are indicative of cellular leakage and loss of functional integrity of cellular membrane in liver (Drotman and Lawhorn, 1978). ALP activities on the other hand are related to functioning of hepatocytes, its increase in serum is due to increased synthesis in the presence of increased biliary pressure (Moss and Butterworth 1974). The results of the present study confirmed the occurrence of flavonoids, saponins, tannins and phenolic compounds in the studied *Selaginella* species, which are natural antioxidants. They can scavenge off free radicals. So, the anti-oxidant principles may be involved in the hepatoprotective activity. In the experiment, paracetamol has enhanced the levels of SGPT, SGOT, Bilirubin and ALP significantly. Treatment with silymarin and 100 mg/kg of ethanolic extracts of *S. tenera* was significantly brought down the elevated levels of SGPT, SGOT, Bilirubin and ALP than *S. intermedia* and *S. inaequalifolia*. These reductions were comparable to the control and the hepatotoxin (paracetamol) used. The treatment has also demonstrated the

reduced hepatic damage or improvement in the hepatic architecture. Among the three studied extracts of *Selaginella, S. tenera* showed more significant hepatoprotective action than *S. intermedia* and *S. inaequalifolia*.

Histological examinations of the liver sections revealed the disturbance in the normal liver architecture by hepatotoxin intoxication. In the sections obtained from the rats treated with ethanolic extract of studied *Selaginella* or silymarin and intoxicated with hepatotoxin, the normal cellular architecture was retained as compared to those of the normal control rats, thereby confirming the protective effect of studied *Selaginella* ethanoilic extract or drug and thus the observation substantiates other results of the experiment.

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin and venoruton are reported for their hepatoprotective activities (Tapas *et al*., 2008). Anthocyanins have drawn increasing attention because of their preventive effect against various diseases. Zhu *et al*. (2012) demonstrated that anthocyanin cyanidin-3-O-β-glucoside (C3G) increases hepatic Gclc expression by increasing cAMP levels to activate protein kinase A (PKA), which in turn upregulates cAMP response element binding protein (CREB) phosphorylation to promote CREB-DNA binding and increase Gclc transcription. Increased Gclc expression results in a decrease in hepatic ROS levels and proapoptotic signaling. Furthermore, C3G treatment lowers hepatic lipid peroxidation, inhibits the release of proinflammatory cytokines and protects against the development of hepatic steatosis (Zhu *et al*., 2012). Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against CCl_4 induced injury in primary cultured neonatal rat hepatocytes and in rats with hepatic damage. Flavonoids at a concentration range of 1–100 μg/mL improved cell viability and inhibited cellular leakage of hepatocyte AST and alanine aminotransferase caused by CCl_4 (Wu *et al*., 2006). Similarly in an *in*

vivo experiment flavonoids at 50, 100, and 200 mg/kg oral doses significantly reduced the levels of AST, ALT, total protein, and albumin in serum and the hydroxyproline and sialic acid levels in liver. In the present study also, 100 mg/kg oral doses of ethanolic extracts of *Selaginella* species reduced the levels of AST, ALT, total protein and albumin in serum.

6. SUMMARY AND CONCLUSION

The present study was intended to investigate the phytochemical constituents present in the selected *Selaginella* species viz., *Selaginella intermedia* (Bl.) Spring, *Selaginella inaequalifolia* (Hook. & Grev.) Spring and *Selaginella tenera* (Hook. & Grev.). To know the chemical constituents of selected *Selaginella* species, the histochemical, preliminary phytochemical analysis, fluorescence analysis, spectroscopic and chromatographic analysis such as UV-Vis, FTIR, TLC, HPLC, HPTLC and GC-MS were carried out. In addition, to know the biological potentials of *S. intermedia, S. inaequalifolia* and *S. tenera,* antioxidant, cytotoxicity using brine shrimp lethality, anticancer using MCF cell line culture, larvicidal, antidiabetic and hepatoprotective activities were also carried out.

The histochemical analysis results revealed the variations in the occurrence of various metabolites viz., phenolics, tannins, cutin and suberin and lignin with varied quantities in different tissues of the studied *Selaginella* species stem. High concentration of lignin was observed in epidermis, hypodermis and xylem of the studied *Selaginella* species. High concentration of phenolic was occurred in ground tissue and xylem of the studied *Selaginella* species. High concentration of tannin presence was observed in the epidermis of studied *Selaginella* species. The results of histochemical analysis clearly confirmed the occurrence and distribution of tannin, lignin and phenolic compounds in the studied *Selaginella* species.

Qualitative phytochemical screening of studied *Selaginella* species showed the occurrence of various secondary metabolites viz., steroids, alkaloids, phenolics, flavonoids, saponins, tannin, cardiac glycosides, aminoacids and anthraquinone in the studied three *Selaginella* species. Among the four extracts of *S. intermedia*, the ethanolic extract of *S.*

intermedia showed the presence of more frequency (88%) of metabolites followed by chloroform extracts (55%) and acetone extract (44%). The least percentage of metabolites (22%) was observed in petroleum ether extracts of *S. intermedia*. Among the tested extracts of *S. inaequalifolia,* the ethanolic and chloroform extracts of *S. inaequalifolia* showed highest percentage of metabolites (55%) existence. Subsequently, acetone extract showed the occurrence of metabolites with 44% and lowest percentage (11%) of metabolites presence was reported in the petroleum ether extracts of *S. inaequalifolia.* Among the four screened extracts of *S. tenera*, maximum frequencies (60%) of metabolites were observed in the ethanolic extracts of *S. tenera*. Next to that the chloroform extracts of *S. tenera* showed 55% percentage of metabolites occurrence, followed by acetone extracts (44%). The petroleum ether extracts of *S. tenera* illustrated only eleven percentage of metabolites presence. Fluorescence analysis of *S. intermedia, S. inaequalifolia* and *S. tenera* plant powders 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.

Biological markers are a group of chemical compounds which are unique for that plant material and also correlate with biological efficacy. Chemosystematics is the attempt to classify plants based on differences and similarities in their biochemical composition. The coupling of morphological characters with other biochemical / phytochemical data has yielded valuable insight into underlying biochemical processes and has contributed to numerous advances in the area of chemotaxonomy. Many modern techniques have been employed to characterize and identify metabolites present in plants and also distinguish the medicinal plants from its adulterants. Furthermore, it can provide information for unknown samples.

UV-Vis spectra of different extracts of studied *Selaginella* species have broad features that are of limited use for identification and are very useful for quantitative measurements. *S. intermedia* showed unique value absorbance 3.311 at 404 nm in petroleum ether extract and acetone extract of *S. inaequalifolia* showed their identity by the distinctive absorbance 2.872 at 663 nm. The ethanolic extract of *S. tenera* represented exceptional absorbance 4.000 at 315 nm. These UV-Vis spectroscopic results may be used as pharmacognostic marker in the pharmaceutical industries and can be used as a chemometric tool to distinguish the studied *Selaginella* species. In addition UV-Vis spectroscopic profile of the studied *Selaginella* species was used to identify the phylogenetic relationship among the three species.

FT-IR spectrum was used to confirm the functional groups presence in different extracts of studied *Selaginella* species. The unique occurrence of 655.80, 1026.13 and 1735.93 spectroscopic peak values clearly distinguished *S. intermedia* from other two studied *Selaginella* species. The results of the present study determined the presence of functional groups and generate a fingerprint for the studied *Selaginella* species. The spectroscopic peak values may be used as phytomarker for the studied species in the pharmaceutical industries. In addition these spectroscopic profiles are used to know the inter relationships among the three studied species. The cladogram constructed based on the FT-IR spectroscopic values clearly distinguished the studied three *Selaginella* species.

Application of fingerprinting technique using modern analytical techniques like TLC, HPTLC and HPLC can give high level of quality control of the plant. Chromatographic fingerprinting should be done with emphasis on identification and quantification of specific chemical marker compound representative of specific medicinal plant. TLC analysis on different extracts of studied three *Selaginella* species revealed 68 phenolic bands and 64 steroidal bands.

The results showed both distinct and common banding profiles. The cladogram constructed based on the phenolic profile showed *S. inaequalifolia* and *S. tenera* in the same clade whereas *S. intermedia* in a divergent clade. The cladogram constructed based on steroidal profile displayed *S. intermedia* and *S. tenera* in the same clade whereas *S. inaequalifolia* in a separate clade. The results of the TLC analysis indicated the chemical diversity of the studied *Selaginella* species and revealed the inter-specific variation among the studied *Selaginella* species. These profiles may be used in the pharmaceutical industry to distinguish the medicinally important *Selaginella* species.

HPTLC analysis has been carried out to know the alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and terpenoids profile of three *Selaginella* species. The results depicted varied degree of both known and unknown metabolites with different R_f values. The HPTLC profiles will be used as a valuable tool for reliable identification of *Selaginella* species. In addition the results provide the chemical composition of the studied *Selaginella* species and pave a pathway to identify the active principles of studied *Selaginella* species. Further analysis may bringout some more active principles from the studied *Selaginella* species. HPLC analysis suggests that there might be differences in the chemical constituents of the studied *Selaginella* 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 *Selaginella* species.

GC-MS analysis showed the presence of different phytoconstituents in the studied three *Selaginella* species separated at various retention times. The compounds were identified based on the compounds present in the NIST library. The biological properties of the identified compounds were predicted based on PASS online which includes anti-inflamatory, anti-diabetic,

anti-oxidant, anti-bacterial, hepatoprotectant, anti-fungal, postmenopausal disorders treatment, prostate disorders treatment, calcium regulator, anti-neoplastic, anti-seborrheic, prostate disorder treatment, anti-thrombotic, muscular relaxant, anti-parasitic, platelet aggregation stimulant, anti-viral, anti-mutagenic, neuropathy treatment, protein kinase stimulant, diuretic potential etc. Some of the biological properties were confirmed by various pteridologists and the present study results of antidiabetic, anti-oxidant, hepatoprotective and anti-cancer studies confirmed the PASS prediction.

Larvicidal efficacies of three *Selaginella* species were studied against the filarial vector *C. quinquefasciatus.* In the current study, the ethanolic and acetone extracts of the three *Selaginella* species showed high percentage of mortality than the other tested extracts. The ethanolic extract of *Selaginella tenera* showed high degree of larvicidal activity against *C. quinquefasciatus* with LC_{50} value 155.29 mg/ml. The results of the present study indicated that the studied *Selaginella* species can be used as effective larvicidal agent to control the mosquito vectors.

Cytotoxic properties of the studied *Selaginella* species might be due to the toxic compounds present in the extracts of *S. inaequalifolia*, *S. intermedia* and *S. tenera*. Among the tested three species, *S. inaequalifolia* was found to be most effective with LC_{50} value 206.66 mg/ml. Cytotoxic potentials of three *Selaginella* species ethanolic extracts were examined against MCF-7 breast cancer cell lines. The cell growth inhibition was directly proportional to concentration of the ethanolic extracts of *Selaginella* species. Maximum number of cell inhibition was observed in *S. tenera* with the IC_{50} value 53.89 µg/ml. It leads to identification of novel antitumor and anticancer agents in the ethanolic extracts of *Selaginella* species.

Antidiabetic activity of three studied *Selaginella* species suggest that *S. inaequalifolia*, *S. intermedia* and *S. tenera* ethanolic extracts could be chosen for studying the long-term hypoglycemic activity in type I and II diabetic animal models which will not only help in elucidating the exact mechanism of action of the extract in reducing blood glucose level but also pave a way to find a new bioactive compound with hypoglycemic activity. *S. tenera* ethanolic extracts significantly decreased blood glucose level by 76.78-74.75% at 0, 30 and 60 min after oral administration in their respective test group as compared to positive control and negative control groups.

Antioxidant potentials of three studied *Selaginella* species were studied using DPPH+, ABTS+, FRAP, metal chelating and phosphomolybdenum reduction and the results showed more scavenging properties in the studied *Selaginella* species. The scavenging effects of studied *Selaginella* species are as follows: *S. intermedia* > *S. tenera* > *S. inaequalifolia*. The results of the present study suggest that all the studied species of *Selaginella* contain powerful inhibitor compounds which may act as primary antioxidants that react with free radicals.

Histological examinations of the liver sections revealed that the normal liver architecture was disturbed by hepatotoxin intoxication. Among the three studied ethanolic extracts of *Selaginella, S. tenera* showed more significant hepatoprotective action than *S. intermedia* and *S. inaequalifolia*. The cross sections of the rats treated with ethanolic extract of studied *Selaginella* and silymarin confirmed the maintenance of normal cellular architecture. The results of the present study confirmed the hepatoprotective effect of *S. intermedia, S. tenera* and *S. inaequalifolia* ethanolic extracts.

All the parameters studied in the present work would help the pharmaceutical biologist to identify and authenticate the medicinally important plants as a source to check the purity and

quality of the plant drug. The fingerprint profile using chromatographic and spectroscopic analysis would help to investigate the substitutes and adulterants. Biological properties of *Selaginella* species concluded that ethanolic extracts find use as broad spectrum antioxidant, cytotoxic, larvicidal, anti-diabetic and hepatoprotective agent in the future. This is the first step towards understanding the nature of bioactive principles and therapeutic potency of studied *Selaginella* species. These results provide a rationale for their use in traditional medicine and also form a basis for further pharmacological studies of the studied *Selaginella* species for the future benefit of mankind.

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19

Phytochemical and Histochemical Studies on Different Species of *Selaginella*

A. Sivaraman[1] and M. Johnson[1*]

ABSTRACT

The present study was aimed to reveal the phytochemical composition of various Selaginella species using qualitative tests and histochemical analysis. Different ferns viz., Selaginella involvens (Sw.) Spring, Selaginella intermedia (Bl.) Spring, Selaginella inaequalifolia (Hook. and Grev.) Spring and Selaginella tenera (Hook. and Grev.) Spring were collected from Western Ghats, South India. The powdered materials were extracted by cold extraction with various solvents viz., petroleum ether, chloroform, acetone and ethanol. Phytochemical screening of the extracts was carried out according to the method described by Harborne. Histochemical tests were made on the fresh sections of the stem treated with the following reagents to identify the presence or absence of metabolites phenol, lignin and tannin. Phytochemical analysis revealed the presence of steroids, phenolics, tannins, saponins, flavonoids, alkaloids, aminoacids and anthroquinone in various species of Selaginella. Histochemical studies revealed the presence of phenols, tannin, and lignin in the different parts of the stipes of selected four Selaginella species with varied percentage. Hence this study offers a base of using Selaginella species as herbal alternative for the synthesis of active compounds.

Keywords: Phytochemistry, Histochemistry, *Selaginella,* Pteridophytes, Stipes.

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Introduction

Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts such as Bible and the Vedas, and obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties. Medicinal plants are staging a comeback and 'renaissance' is happening all over the globe (Joy *et al.*, 2001). Ferns and their fern allies have little economic significance to humankind as compared to other groups, especially angiosperms; hence these are poorly understood, overlooked or often neglected by the society. None the less, being the major constituent of biodiversity their role in the system of nature cannot be overlooked or neglected in the global changing environmental scenario (Sharpe, 2011). Despite the large number of ferns and their allied species, a considerable percentage of them are rare and threatened. With increasing utilization of land and natural resources, it is feared that many of these threatened taxa will become yet rarer, more vulnerable and endangered, and in several cases may finally become extinct, as any disturbance or imbalance in their narrowly confined ecosystems is liable to lead to their extermination (Chandra *et al.*, 2008).

There are about 1200 species of Pteridophytes in India. Like flowering plants, pteridophytes are also used by tribals in India and other countries. *Selaginella,* with about 700 species, is world-wide in distribution, but most of the species are tropical and many of them grow in damp forests (Pichi-Sermolli, 1977). Alston (1945) enumerated 58 species of *Selaginella* from India and Sri Lanka. Of these, 45 species has been reported from India. According to Dixit (1984), 18 species are in South India (Tamil Nadu and Kerala) and 12 species have been reported from the Western Ghats, South India (Manickam and Irudayaraj, 1992). Considerable numbers of pteridophytes are used in Chinese medicines. The Chinese herb *Selaginella doederleinii* is used as an anticancer drug (Lee and Lin, 1988; Huang, 1992). The medicinal use of *Selaginella* in India goes back to the period of 'Ramayana' (Sah, 2008). According to the principles of traditional Chinese medicine, *Selaginella* has sweet, spicy, bitter and cold properties, and is associated with the liver, lung and stomach meridians. Its main functions are to clear heat, reduce toxicity and drain damp heat. More recent research has shown that *Selaginella* may be effective against both acute and chronic hepatitis. *Selaginella* may also be used externally to help stop bleeding and promote wound healing (Tinde van Andel *et al.*, 2007). Prepared *Selaginella* can be found in pill, powder and tablet forms. *Selaginella* should be taken with caution by patients diagnosed with cold deficiency. Taking large amounts of *Selaginella* may result in loss of appetite and abdominal discomfort. There are no known drug interactions associated with *Selaginella* (Lee *et al.*, 1999). *Selaginella doederleinii,* a popular anticancer herb, may contain an as yet unidentified substance that contributes to reversible bone marrow suppression (Duraiswamy *et al.*, 2010). Histochemical studies can be used to study the chemical composition of the cells resulting from growth and developments and these data can be used to make inferences about the importance of secondary metabolites in growth and development of plants. With this knowledge, the present study was aimed to reveal the phytochemical composition of various *Selaginella* species using qualitative tests and histochemical analysis.

Materials and Methods

Plant Collection

Healthy, disease free fern allies of *Selaginella involvens* (Sw.) Spring, *Selaginella intermedia* (Bl.) Spring, *Selaginella inaequalifolia* (Hook. and Grev.) Spring and *Selaginella tenera* (Hook. and Grev.) Spring were collected from Kakachi stream, Tirunelveli Hills (1000 m) in the Western Ghats. The fern allies were identified by Dr. V. Irudayaraj, St. Xavier's College, Palayamkottai.

Plant Extraction and Preliminary Phytochemical Screening

The fresh materials were washed in running tap water for 5 min and dried using blotting papers. The washed plant materials were air and shade dried for two weeks and pulverized to powder using tissue blender. The powdered materials (10 g) were extracted by cold extraction with 60 mL of various solvents *viz.*, petroleum ether, chloroform, acetone and ethanol. The samples were kept in dark for 72 h with intermittent shaking. The extracts were filtered using Whattman filter paper (No.1) and then concentrated in vacuum at 40°C using rotary evaporator. The residues obtained were stored in a freezer -70°C until further tests. Phytochemical screening of the extracts was carried out according to the method described by Harborne (1998).

Histochemical Analysis

Fresh free hand sections were made from the fronds of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera*. Histochemical tests were made on the fresh sections of the stem treated with the following reagents to identify the presence or absence of metabolites phenol, lignin and tannin. Lugol's Iodine is used to detect tannins and lignins (Haridass and Suresh Kumar, 1985; Chamberlin, 1924), 10 per cent sodium nitrite, 20 per cent urea and 10 per cent acetic acid were used to detect phenolics (Reeve, 1951). The stained sections were observed under Motic trinocular microscope (Japan). They were photographed at different magnifications and at different views. The results were registered on photomicrograph. Based on the photographs taken, anatomical description and localization of tested chemicals were done.

Results

In the present study, the phytochemical screening of petroleum ether, chloroform, acetone and ethanolic extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera* were carried out and the results were tabulated in Table 19.1. Steroids showed their presence in all the tested 16 extracts followed by phenolics and tannins in 8 extracts. Next to that, 7 extracts were illustrated their existence of saponins. Flavonoids demonstrated their occurrence in 6 different extracts. Alkaloids, aminoacids and anthraquinone showed their presence only in ethanolic extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera*.

Among the different extracts of *S. involvens,* steroids showed their presence in all the four extracts followed by flavonoids in 3 different extracts. Phenolics, tannins and saponins demonstrated their existence in 2 different extracts. In *S. intermedia,* steroids demonstrated their occurrence in all the four extracts followed by phenolics,

Table 19.1: Phytochemical analysis on different species of *Selaginella.*

Metabolites	*S. involvens*				*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
	P	*C*	*A*	*E*	*P*	*C*	*A*	*E*	*P*	*C*	*A*	*E*	*P*	*C*	*A*	*E*
Steroids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alkaloids	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+
Phenolics	–	–	+	+	–	–	+	+	–	–	+	+	–	–	+	+
Saponins	–	+	+	–	–	–	+	+	–	–	+	–	–	+	+	–
Tannins	–	–	+	+	–	–	+	+	–	–	+	+	–	–	+	+
Anthraquinone	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+
Amino acids	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+
Flavonoids	–	+	+	+	–	–	–	+	–	–	–	+	–	–	–	+

Table 19.2: Histochemical analysis on different species of *Selaginella.*

Parts	*S. involvens*			*S. intermedia*			*S. inaequalifolia*			*S. tenera*		
	P	*T*	*L*	*P*	*T*	*L*	*P*	*T*	*L*	*P*	*T*	*L*
Epidermis	–	3^+	3^+	–	+	3^+	3^+	3^+	3^+	3^+	2^+	2^+
Hypodermis	–	2^+	2^+	–	–	2^+	2^+	2^+	3^+	3^+	2^+	2^+
Ground tissue	2^+	–	–	2^+	–	–	2^+	–	2^+	2^+	–	–
Endodermis	3^+	–	2^+	3^+	–	–	2+	–	–	2^+	–	–
Xylem	2^+	–	3_+	2^+	–	3+	2^+	2^+	3^+	3^+	–	2^+
Phloem	–	–	+	3^+	–	+	2+	2^+	2^+	–	–	–

P: Phenol; T: Tannin; L: Lignin.

+: Mild, 2^+: Average, 3^+: high, –: Data not available.

saponins and tannins in two different extracts. Among various extracts of *S. inaequalifolia,* steroids illustrated their existence in all the four extracts whereas phenolics and tannins were present in 2 extracts only. In *S. tenera* also, steroids showed their presence in all the four extracts whereas phenolics, saponins and tannins were present in 2 extracts only.

The occurrence and distribution of various chemicals (phenols, lignin and tannins) in different tissues of the stipe was recorded in Table 19.2 and Figure 19.1. Based on the intensity of the resulting colour (metabolites intensity) it has been scored into three categories of concentrations such as: Low (+), Medium (2+) and High (3+).

Histochemical studies revealed the presence of phenols, tannin and lignin in the selected four *Selaginella* species with varied percentage. Phenols are mostly present in all the four selected species. *S. involvens* shows higher concentration of phenols in the endodermis (Figure 19.1A). Minimum concentration of phenols was present in the ground tissue and xylem of *S. involvens* (Figure 19.1A). In *S. intermedia,* higher concentration of phenols was present in the endodermis and phloem (Figure 19.1D). Lower concentration of phenols was present in the ground tissue and xylem of *S. intermedia* (Figure 19.1E). *S. inaequalifolia* expresses maximum concentration of phenols only in the epidermis (Figure 19.1I). Minimum concentrations of phenols were illustrated in all other parts of *S. inaequalifolia* (Figure 19.1I). *S. tenera* shows high concentration of phenols in the epidermis, hypodermis and xylem (Figure 19.1M). In ground tissue and endodermis of *S. tenera,* minimum concentrations of phenols were present (Figure 19.1I).

Tannins are mostly present in all the four selected species. *S. involvens* shows higher concentration of tannin in the epidermis (Figure 19.1B). Less concentration of tannin were present in the hypodermis of *S. involvens* (Figure 19.1B). *S. intermedia* expresses maximum concentration of tannin in the epidermis only (Figure 19.1F). Tannin is absent in the all other parts of the *S. intermedia.* In *S. inaequalifolia,* high concentrations of tannin were observed in the epidermis (Figure 19.1J). Minimum amount of tannin was present in the hypodermis, xylem and phloem (Figure 19.1J). In *S. tenera,* tannin showed minimum concentration in epidermis and hypodermis (Figure 19.1N). Tannin is absent in all other parts of the *S. tenera* (Figure 19.1N).

Lignin is present at high concentration in the epidermis and xylem of *S. involven* (Figure 19.1C). Lignin showed lesser amount in the hypodermis, endodermis and phloem of *S. involvens* (Figure 19.1C). *S. intermedia* expresses maximum concentration of lignin in the epidermis and xylem (Figure 19.1G). Lignin is minimum in the hypodermis and phloem (Figure 19.1H). Lignin is present at high concentration in the epidermis, hypodermis and xylem of *S. inaequalifolia* (Figure 19.1L). Minimum concentration of lignin is present in the ground tissue and phloem of *S. inaequalifolia* (Figure 19.1K). *S. tenera* express minimum concentration of lignin in the epidermis, hypodermis and xylem (Figure 19.1O).

Discussion

The beneficial medicinal effects of plant materials typically result from the secondary metabolites present in the plant although, it is usually not attributed to a single compound but a combination of the metabolites. The medicinal actions of

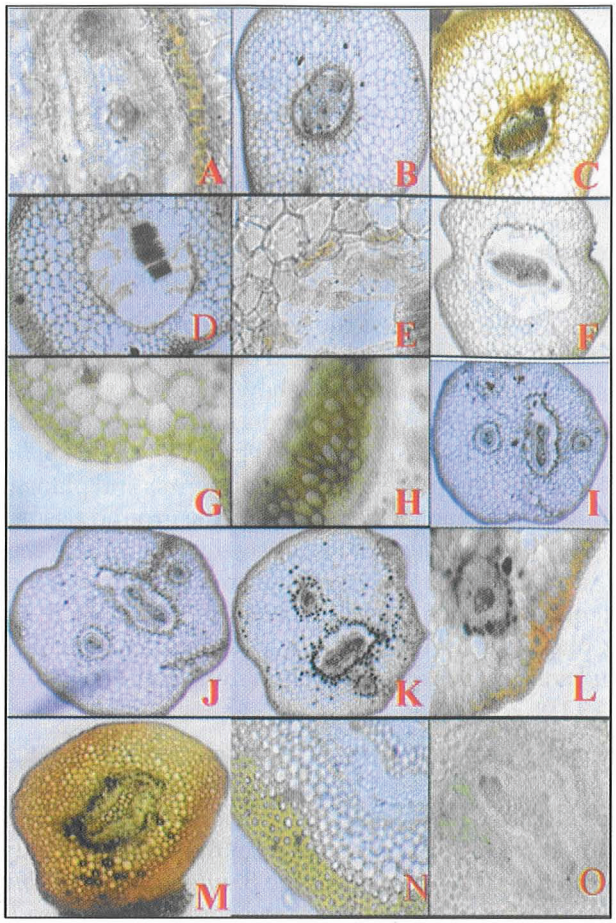

Figure 19.1

A: Phenol presence in ground tissue, endodermis and xylem of *S. involvens;* B: T.S of stipe- A portion enlarged in *S. involvens* showing the presence of tannin in epidermis and hypodermis; C: T.S of stipe – A portion enlarged in *S. involvens* showing the presence of lignin in epidermis, hypodermis, endodermis and xylem; D: T. S of stipe – A portion enlarged in *S. intermedia* showing the presence of phenol in endodermis and phloem; E: Phenol presence in ground tissue, endodermis, xylem and phloem of *S. intermedia;* F: T.S of stipe showing tannin presence in *S. intermedia* in the epidermis; G: Lignin presence in epidermis, hypodermis and of *S. intermedia;* H: Lignin presence in xylem of *S. intermedia;* I: Entire view of stipe *S. inaequalifolia* showing phenol in epidermis, hypodermis, endodermis xylem and phloem; J: Entire view of stipe *S. inaequalifolia* showing tannin in epidermis, hypodermis, xylem and phloem; K: T.S of stipe – presence of lignin in *S. inaequalifolia* in the epidermis, hypodermis, xylem and phloem; L: Lignin presence in epidermis, hypodermis, ground tissue, xylem and phloem of *S. inaequalifolia;* M: T.S of stipe – entire view of *S. tenera* showing phenol present in epidermis, hypodermis,endodermis and xylem; N: Tannin presence in epidermis and hypodermis of *S. tenera;* O: *S. tenera* showing presence of lignin in epidermis, hypodermis and xylem.

plants are distinctive to a particular plant species or group, reliable with the concept that the combination of secondary metabolites in a particular plant is taxonomically distinct (Parekh *et al.*, 2005). Steroids may serve as an intermediate for the biosynthesis of downstream secondary natural products and it is believed to be a biosynthetic precursor for cardenolides in plants (Daly, 1998). The results of the present study determined the presence of steroids in all the four extracts of studied *Selaginella* species. Tannins have been found to possess antiviral, antibacterial, antiparasitic effects, antiinflammatory, antiulcer and antioxidant property for possible therapeutic applications (Lu *et al.*, 2004; Kolodziej *et al.*, 2005). In the present study we revealed the tannins presence in acetone and ethanolic extracts of all the four species of *Selaginella.* Tannins also showed their presence in different parts of the all the studied *Selaginella* species. Saponins possess specific physical, chemical and biological activities that make them useful as drugs. Some of these biological properties include antimicrobial, anti-inflammatory, anti-feedent and hemolytic effects (George *et al.*, 2002). The results of the present study showed the presence of saponins in different extracts of *Selaginella* species except petroleum ether. Alkaloid, which is one of the largest phytochemical groups in plants, has amazing effect on humans and this has led to the development of powerful pain killer medications (Staerk *et al.*, 2002). In the present study, alkaloids presence was determined only in the presence of ethanolic extracts of all the four species. Phenolics may serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores (Vaya *et al.*, 1997). Different types of phenolic compounds have different antioxidant activities, which is dependent on their structure (Bajpai *et al.*, 2005). In the present study, we revealed the phenolics presence in acetone and ethanolic extracts and also in different parts of all the four species of *Selaginella.* Flavonoids, the major group of phenolic compounds were reported for their antimicrobial, antiviral and spasmolytic activity. They are able to scavenge hydroxyl radicals, superoxide anion radicals and lipid peroxy radicals, which highlights many of the flavonoid health-promoting functions (Havsteen, 2002). The present study results illustrated the presence of flavonoids in different extracts of *Selaginella* species except petroleum ether. Lignin is found in the cell walls of plants. Lignins impart strength to cell walls, facilitate water transport, and impede the degradation of wall polysaccharides, thus acting as a major line of defense against pathogens, insects and other herbivores (Sederoff *et al.*, 1999). In the present study, lignins illustrated their presence in all the different parts of stipes of various *Selaginella* species. Thus the present investigation on phytochemical and histochemical studies on different *Selaginella* species confirmed the presence of phenolics, tannins, alkaloids, saponins, steroids and lignin, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry. Hence this study offers a base of using *Selaginella* species as herbal alternative for the synthesis of active compounds.

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Chapter 10

Chromatographic Studies on some Selected *Selaginella* Species

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ABSTRACT

The present study was aimed to reveal the phytoprofile of four *Selaginella* species using TLC and HPLC. The TLC phenolic and steroids profile of *Selaginella involvens* (Sw.) Spring, *Selaginella intermedia* (Bl.) Spring, *Selaginella inaequalifolia* (Hook. & Grev.) Spring and *Selaginella tenera* (Hook. & Grev.) Spring was detected using the specific mobile phase and spraying agents. The appearance of blue colour and bluish green colour spot in the TLC chromatogram indicated the presence of phenolics and steroids in the crude extracts of selected *Selaginella* species. HPLC analysis was performed on the methanolic extracts of selected *Selaginella* species and the results showed various peaks at different retention times. The developed TLC and HPLC fingerprints will help the manufacturer for quality control and standardization of herbal formulations. Such finger printing is useful in differentiating the medicinally important species from its adulterant and act as a biochemical marker for this medicinally important plant in the pharmaceutical industry and plant systematic studies.

Keywords: *Selaginella, TLC, HPLC, Phytochemistry.*

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Introduction

Medicinal plants played a significant role to cure human diseases and disorders in the traditional medicine since time immemorial (Chandarana *et al.*, 2005). Based on the presence of one or more active constituents, the medicinal properties of the plants are authorised (Egwaikhide and Gimba, 2007). The ethnomedicinal plants have been used as driving force for development of novel drugs in the pharmaceutics (Robbers *et al.*, 1996). Due to importance and bioefficacy of herbal medicine, the growing demand for herbal product has increased in volume of plant materials traded within and across the countries (Suresh *et al.*, 2008). At present, the botanists focused their research on phytoconstituents of medicinal plants and examined secondary metabolites as a source of medicinal agents. The success story of chemotherapy lies in the continuous search of new drugs to counter the challenges possessed by resistant strains of microorganisms (Doughari *et al.*, 2008).

There are about 1200 species of Pteridophytes in India. Like flowering plants, pteridophytes are also used by tribals in India and other countries. *Selaginella*, with about 700 species, is world-wide in distribution, but most of the species are tropical and many of them grow in damp forests (Pichi-Sermolli, 1977). Alston (1945) enumerated 58 species of *Selaginella* from India and Sri Lanka. Of these, 45 species have been reported from India. According to Dixit (1984), 18 species are in South India (Tamil Nadu and Kerala) and 12 species have been reported from the Western Ghats, South India (Manickam and Irudayaraj, 1992). Considerable numbers of pteridophytes are used in Chinese medicines. The Chinese herb *Selaginella doederleinii* is used as an anticancer drug (Lee and Lin, 1988; Huang, 1992). The medicinal use of *Selaginella* in India goes back to the period of 'Ramayana' (Andel *et al.*, 2007). According to the principles of traditional Chinese medicine, *Selaginella* has sweet, spicy, bitter and cold properties, and is associated with the liver, lung and stomach meridians. Its main functions are to clear heat, reduce toxicity, and drain damp heat. More recent research has shown that *Selaginella* may be effective against both acute and chronic hepatitis. *Selaginella* may also be used externally to help stop bleeding and promote wound healing (Sah, 2008). Prepared *Selaginella* can be found in pill, powder and tablet forms. *Selaginella* should be taken with caution by patients diagnosed with cold deficiency. Taking large amounts of *Selaginella* may result in loss of appetite and abdominal discomfort. There are no known drug interactions associated with *Selaginella* (Lee *et al.*, 1999).

The morphological characters play a vital role in plant systematic studies and are used as a tool for the classification of a taxon. In recent times, in addition to morphological markers, anatomical, cytological, biochemical and molecular markers are also being used to classify the organisms. Correct identification and quality assurance of the starting material is important, therefore, an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy (Goldman, 2001). Choice of chemical markers is crucial step for the quality control of plant based medicines (Mukherjee *et al.*, 2006). Chemical fingerprinting has been demonstrated to be a powerful technique for the quality control of herbal medicines (Bhutani, 2000). According to regulatory guidelines and pharmacopoeias

macroscopic and microscopic evaluation and chemical profiling of the botanical materials is used for quality control and standardization (IHP, 2002; BHP, 1996). Thin layer chromatography (TLC), High Performance Liquid Chromatography and Spectrophotometric methods are frequently used for quality control and standardization of plant based drugs (WHO, 1998). The chromatogram derived using HPLC, HPTLC and GC analysis, Rf values and Retention time obtained through the chromatographic analysis are used as a phytochemical marker to characterize the medicinal plants and identify the chemical constituents present in the medicinal plants. Thin layer chromatography (TLC) is very important technique for qualitative study in both small and large scale and therefore widely-employed laboratory technique. A number of studies were reported to characterize the plants using TLC and HPLC profiles (Janakiraman *et al.*, 2012; Johnson *et al.*, 2012). Only few reports are available for pteridophytes (Talukdar *et al.*, 2010; Irudayaraj and Johnson, 2011). But there is no report on the chemical characterization of *Selaginella* using TLC and HPLC profile. With this knowledge, the present study was aimed to reveal the phytoprofile of four *Selaginella* species using TLC and HPLC.

Materials and Methods

Plant Collection

Healthy, disease free fern allies of *Selaginella involvens* (Sw.) Spring, *Selaginella intermedia* (Bl.) Spring, *Selaginella inaequalifolia* (Hook. & Grev.) Spring and *Selaginella tenera* (Hook. & Grev.) Spring were collected from Kakachi stream, Tirunelveli Hills (1000 m) in the Western Ghats. The fern allies were identified by Dr. V. Irudayaraj, St. Xavier's College, Palayamkottai, Tamil Nadu, India.

Plant Extraction

The fresh materials were washed in running tap water for 5 min and dried using blotting papers. The washed plant materials were air and shade dried for two weeks and pulverized to powder using tissue blender. The powdered materials (25 g) were successively extracted using Soxhlet extractor with 150 mL of various solvents *viz.*, petroleum ether, chloroform, acetone and methanol for 8 h at a temperature not exceeding the boiling point of the solvent. The extracts were filtered using Whattman filter paper (No.1) and then concentrated in vacuum at 40°C using rotary evaporator. The residues obtained were stored in a freezer -70°C until further tests.

TLC Analysis

The phenolic and steroids profile of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera* was detected by TLC. For phenolics, chloroform and methanol at 9: 1 ratio was used as mobile phase and benzene and methanol at 9: 1 ratio for steroids. The folin-ciocalteau reagent and 5 per cent alcoholic sulphuric acid was used as spraying agent for phenolics and steroids respectively. After spraying with specific spraying agents, the appearance of blue colour and bluish green colour spot in the TLC chromatogram indicated the presence of phenolics and steroids in the crude extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera.*

HPLC Analysis

Based on the preliminary results, methanolic extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera* were taken for the HPLC analysis. The extracts were centrifuged at 3000 rpm for 10 min and then filtered through Whatmann No.1 filter paper using high pressure vacuum pump. The sample was diluted to 1: 10 with the same solvents. HPLC method was performed on a Shimadzu LC-10AT VP HPLC system, equipped with a model LC-10AT pump, UV-Vis detector SPD-10AT, Rheodyne injector fitted with a 20 µL loop and auto injector SIL-10AT. A Hypersil BDS C-18 column (4.6 × 250 mm, 5 µm size) with a C-18 guard column was used. The elution was carried out with gradient solvent systems with a flow rate of 1 mL min-1 at ambient temperature (25-28°C). The mobile phase was consisted of 0.1 per cent v / v methanol (solvent A) and water (solvent B). The mobile phase was prepared daily, filtered through a 0.45 µm and sonicated before use. Total running time was 15 min. The sample injection volume was 20 µL while the wavelength of the UV-Vis detector was set at 254 nm.

Results

Phenolics profile present in the various crude extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera* was revealed by the TLC and the Rf values of the phenolics profile of various extracts was tabulated in Table 10.1. Of which, acetone extracts of *S. involvens* showed 8 phenolic spots, chloroform extracts of *S. intermedia* and *S. inaequalifolia* illustrated 7 and 12 phenolic bands respectively. 8 phenolic spots were detected in petroleum ether extracts of *S. tenera.* The steroids profile present in the various extracts of different *Selaginella* species was tabulated in Table 10.2. Among these, chloroform extracts of *S. involvens* showed 8 steroids spots, chloroform extracts of *S. intermedia* showed 8 steroids spots, and *S. inaequalifolia* illustrated 9 steroids spots in acetone extracts. 10 steroids spots were detected in petroleum ether extracts of *S. tenera.*

The qualitative HPLC fingerprint profile of methanolic extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera* were selected based on the sharpness of the peaks and proper baseline. Methanolic extract prepared by hot extraction was subjected to HPLC for the separation and identification of constituents present in various *Selaginella* species. In *S. involvens,* seven peaks were observed and the profile displayed one prominent peak at a retention time of 2.933 min and some moderated peaks at different retention time 1.637, 2.553, 3.433, 3.793, 4.043 and 4.673 min respectively (Figure 10.1). Methanolic extracts of *S. intermedia* showed the presence of three peaks with one prominent peak at a retention time of 3.043 min and two moderate peaks were also observed at a retention time 3.437 and 4.030 min respectively (Figure 10.2). In *S. inaequalifolia* also, three peaks were obtained, of which, one prominent peak at a retention time of 3.053 min and two moderate peaks at different retention time (3.467 and 4.043 min) was observed (Figure 10.3). *S. tenera* illustrated the presence of three peaks with one prominent peak at a retention time of 3.190 min and two moderate peaks were also observed at a retention time 3.540 and 3.677 min respectively (Figure 10.4).

Table 10.1: Phenolics TLC Profile of different *Selaginella* sps.

Rf	*S. involvens*				*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
	P	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*
0.06	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
0.09	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0.10	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
0.12	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
0.14	–	–	–	–	–	+	+	–	–	+	–	–	–	–	–	–
0.19	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–
0.20	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
0.21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
0.25	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–
0.29	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
0.30	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–
0.32	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–
0.36	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–
0.38	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–
0.39	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
0.45	–	–	–	–	–	–	+	+	+	–	–	–	+	+	–	–
0.52	–	–	+	–	–	–	–	–	–	+	+	–	+	–	–	–
0.55	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–
0.57	–	–	–	–	+	–	–	–	+	+	–	–	+	–	–	–
0.59	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
0.60	–	+	+	+	–	–	–	–	–	–	–	+	–	–	–	–
0.62	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
0.64	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–
0.66	+	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–
0.67	–	–	–	–	–	–	–	–	+	–	+	–	+	–	–	–
0.70	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
0.72	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
0.75	–	–	–	–	+	+	–	–	–	–	–	–	+	–	–	–
0.76	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
0.77	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–
0.79	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
0.80	+	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–
0.82	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
0.84	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
0.86	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–

Contd...

Table 10.1–*Contd...*

Rf	*S. involvens*				*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
	P	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*
0.88	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
0.90	+	–	+	–	–	+	–	–	–	–	–	–	+	+	–	–
0.93	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
0.95	+	–	–	–	–	–	+	–	–	–	+	–	–	–	+	–
0.97	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–

P: Petroleum ether; C: Chloroform; A: Acetone; M: Methanol.

Table 10.2: Steroids TLC Profile of different *Selaginella* sps.

Rf	*S. involvens*				*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
	P	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*
0.02	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–
0.06	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–
0.08	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0.10	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
0.11	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–
0.12	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
0.14	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–
0.15	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
0.16	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
0.18	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
0.20	–	–	–	–	–	+	–	–	+	–	+	+	+	–	–	–
0.21	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0.22	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
0.25	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
0.26	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
0.29	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0.30	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
0.33	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–
0.34	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–
0.36	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
0.37	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
0.40	–	–	+	–	–	+	–	+	+	–	+	–	–	+	–	–
0.41	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0.46	–	–	–	–	–	–	–	–	–	+	+	–	–	+	–	–

Contd...

Table 10.2–*Contd...*

Rf	*S. involvens*				*S. intermedia*				*S. inaequalifolia*				*S. tenera*			
	P	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*	*P*	*C*	*A*	*M*
0.50	–	+	+	–	+	–	–	–	–	–	–	–	+	–	–	–
0.54	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
0.55	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–
0.56	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
0.58	–	+	+	–	+	–	+	–	–	+	–	–	+	+	–	–
0.60	–	–	–	–	–	–	–	–	+	–	–	–	+	+	–	–
0.66	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
0.69	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
0.70	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
0.72	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–
0.74	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
0.77	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–
0.80	–	–	–	–	+	–	–	–	+	–	–	–	+	+	–	–
0.83	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0.88	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
0.90	–	–	–	–	–	–	–	–	+	–	–	–	+	+	–	
0.92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
0.97	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–

P: Petroleum ether; C: Chloroform; A: Acetone; M: Methanol.

Discussion

TLC studies employed the Rf values to distinguish the plants from other species and adulterant. In the present study also, we developed the phenolics and steroids TLC profile for various extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera.* This provides valuable information in authenticating the genuine mother plants along with the nature of phytoconstituents present in it. HPLC identification test are required to confirm the presence of the active constituents and potential adulterant in ayurvedic drugs. In the present study, HPLC profile for methanolic extracts of *S. involvens, S. intermedia, S. inaequalifolia* and *S. tenera* were evolved. Standardization is an important aspect for establishing the quality and efficacy of ayurvedic formulations or any multiple ingredient herbal formulations. Generally, two approaches being used for standardization are fingerprint analysis by HPLC/ HPTLC and quantification of individual chemical markers. It ensures reproducible pharmaceutical quality of herbal products. Preliminary analysis of the methanolic extracts of various *Selaginella* species showed varying patterns in HPLC chromatogram suggesting that there might be differences in the process of manufacturing and therefore a proper scientific validation (chromatographic fingerprinting of major constituents) is needed for quality control purposes. These studies exhibited novel markers in

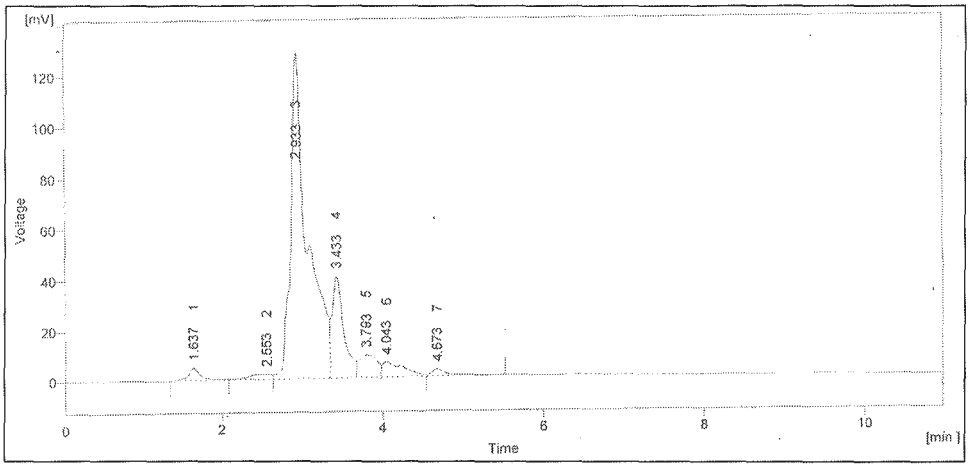

Figure 10.1: HPLC Chromatogram of *Selaginella involvens* (Sw.) Spring.

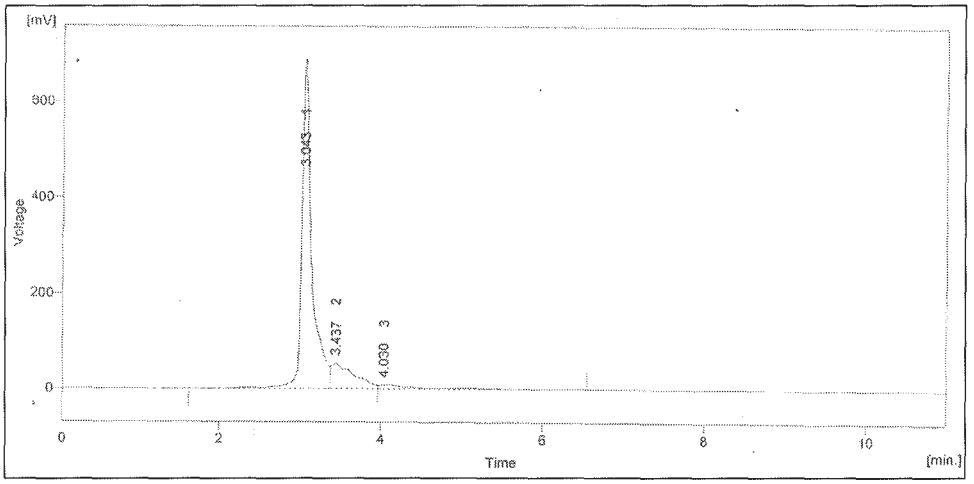

Figure 10.2: HPLC Chromatogram of *Selaginella intermedia* (Bl.) Spring.

standardization as useful analytical tools to check not only the quality of the powder but also the presence of adulterants in ayurvedic drugs.

Chemosystematics is the attempt to classify and identify organisms, according to demonstrable differences and similarities in their biochemical compositions. To date, the coupling of morphological characters with other biochemical/phytochemical data has yielded valuable insight into underlying biochemical processes and has contributed to numerous advances in the area of chemotaxonomy. Many modern techniques have been employed to characterize and identify metabolites present in plants and also distinguish the medicinal plants from its adulterants. Furthermore, it can hardly provide any information for unknown samples. All these reasons have prompted the hyphenation of high performance liquid chromatography (HPLC).

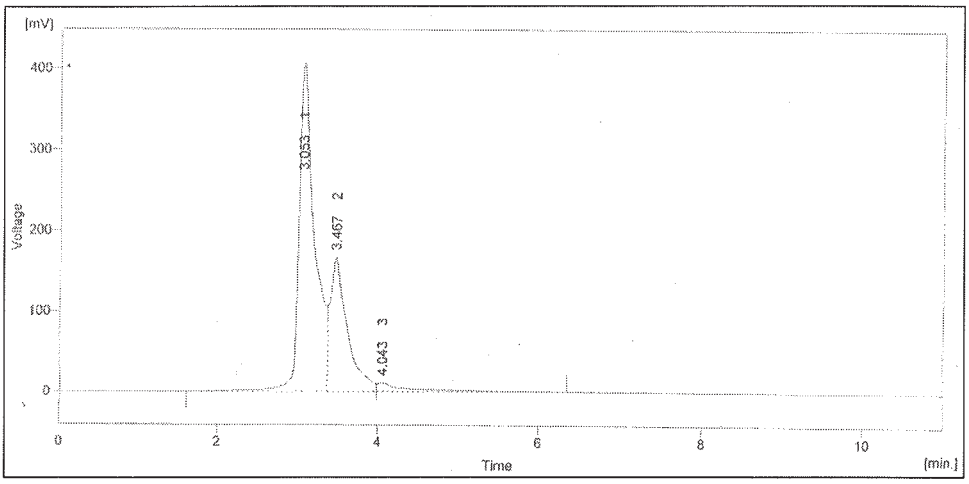

Figure 10.3: HPLC Chromatogram of *Selaginella inaequalifolia* (Hook. & Grev.) Spring.

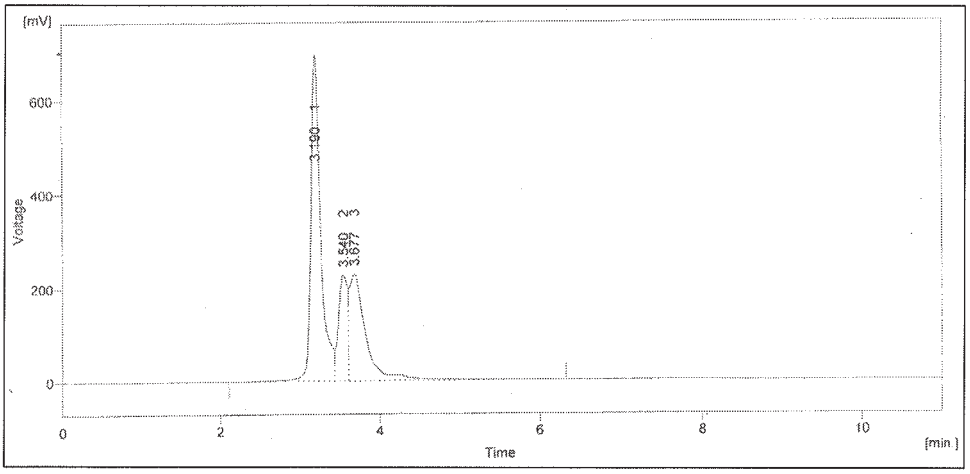

Figure 10.4: HPLC Chromatogram of *Selaginella tenera* (Hook. & Grev.) Spring.

Conclusion

The developed TLC and HPLC fingerprints will help the manufacturer for quality control and standardization of herbal formulations. Such finger printing is useful in differentiating the medicinally important species from its adulterant and act as a biochemical marker for this medicinally important plant in the pharmaceutical industry and plant systematic studies.

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