Screening and characterization of potential bioactive compounds from *Selaginella wightii*

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INTRODUCTION

Biodiversity of natural resources has served not only for the primary human needs but also for health care, since time immemorial. About 80% of the world population relies on traditional systems of medicines for primary health care, where plants form the dominant component over other natural resources. The estimated no. of plant species used in health care systems worldwide is 35, 000–70, 000 (Farnsworth and Soejarto, 1991).

Figure1a. World market of herbal remedies

Figure 1b.Plants used in various Systems of medicine in India

Pteridophytes are vital component of the flora of this major region of species-diversity, next to Angiosperms in number. More than 1200 species of ferns and fern allies have been reported from India (Dixit, 1984; Chandra, 2000) but recent review of doubtful new species is showing this to be around 900 to 1000 species.

The medicinal value of the Pteridophytes have been well-known to man for more than 2,000 years, compared to the angiosperms they have found very little use in modern chemotherapy and researches on the antibiotic activity of this plant group are still in their infancy. Most of the diseases against which the ferns and fern allies are said to have curative properties, are caused by bacteria (gram-positive, gram-negative or acid fast), viruses, protozoa, and helminths (Banerjee et.al, 1980).

The overgrowth of organisms resistant to antimicrobial agents demands the discovery of new therapeutic agents. Several scientific workers have tried to find antimicrobial agents from natural products for drug discovery. Currently, natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. The interest in nature as a source of potential chemotherapeutic agents continues and natural product compounds have served as the most significant source of new leads against microbial pathogens. From this point of view, plants have formed the basis of

complicated traditional medicine systems that have been in subsistence for thousands of years and continue to provide mankind with new remedies (Lee et.al, 2008).

Selaginella

Selaginellas are primitive, seedless, vascular plants. There are about 700 species of *Selaginella*, showing a wide range of characters which are distributed mainly in warm and moist climates. Most species of *Selaginella* are known to be “resurrection plants”, because they curl up in a tight brown ball during dry times, and uncurl and turn green in the presence of moisture. They are mostly heterosporous, means that they produce different types of spores- microspores (male) and megaspores (female).

Classification

Kingdom	Plantae
Division	Lycopodiophyta
Class	Selaginellopsida
Order	Selaginellales
Family	Selaginellaceae
Genus	<i>Selaginella</i>
Species	About 700, found worldwide
Common name	Spike moss

The genus *Selaginella* is the most neglected group among Pteridophytes though it has several medicinal uses. Several *Selaginella* species are used in traditional medicine in various regions of the world to treat multiple diseases such as cancer, cardiovascular problems (Lin et.al,1994), diabetes (Darias et.al,1989),hepatitis (Lin et.al,1990), skin diseases (MacFoy et.al,1983) and urinary tract infections (Banerjee et.al,2002).

From the more than 60 species of *Selaginella* occurring in India, a few species are used medicinally (Swamy et.al, 2006) and only four *Selaginella* species, i.e., *S. tamariscina*, *S.chrysocaulos*, *S.rupestris* and *S.bryopteris* have been phytochemically investigated.

The genus *Selaginella* is a rich source of biflavonoids, some of which are cytotoxic; (Swamy et.al, 2006), other types of compounds such as alkaloidal glycosides, phenylpropanones and lignans were also reported from some *Selaginella* species.

Biflavonoids are flavonoid dimers connected with a C–C or a C–O–C bond and are known to display a variety of biological activities, such as anti-inflammatory activity (Amella et.al, 1985), inhibitory activity of mast cell histamine release (Banerjee et.al,

2002), anti-tumour activity (Chakravarthy et.al, 1981), phospholipase A2 inhibitory activity (Lee et.al, 2006), and inhibition of matrix metalloproteinase-1 production in fibroblast cells. (Kim et.al, 2007). Allocation of biflavonoids in the plant kingdom is limited to quite a few species such as *Ginkgo biloba*, *Selaginella* species, and *Garcinia kola*. (Kim et.al, 2007).

Selaginella wightii Hieron (Fig.1) was described by Hieron in 1900 stating its distribution in India (Tamil Nadu), Srilanka, Tanzania and Mauritius. It grows 5-12 cm tall and is greenish-black in appearance. It occurs on dry bare rocks or river banks: confined to sub-tropical and temperate forests. Stem is cylindrical, copiously branched from the base and there is rooting throughout; younger branches are somewhat flattened. Leaves are spirally arranged, uniform, glaucous green, linear-subulate and dentate. Sporophylls of spike are uniform; like vegetative leaves, linear-subulate and dentate. Megasporeangia and microsporeangia are found in the same strobilus; megaspores are trilete, circular, 500-600 μ in diameter and microspores are trilete, oval, 90-100 μ in diameter (Fig.2). It sporulates in the month of April-May. It is fairly abundant in the area, but absolutely rare.

Figure 2. *Selaginella wightii*; (A) Sporophyll-like uniform spikes, (B) Vegetative leaves linear-subulate and dentate, (C) Megasporeangia and microsporeangia (D) Megaspores-500-600 μ in diameter, (D1) Microspores 90-100 μ in diameter

Selaginella wightii is fairly abundant in particular area but rare among *Selaginella* species. Moreover, it has medicinal value for which it can be exploited for various drug formulations.

A chemical investigation of the whole plant of *Selaginella wightii* was undertaken and the various extracts were qualitatively and quantitatively analysed. The extracts of *Selaginella wightii* were screened for antimicrobial and antioxidant activities along with interaction-toxicity studies.

OBJECTIVE

To isolate various bioactive components.

Characterization of potential bioactive components.

To study the biological activities of extracts.

Chapter-2

Literature

Review

LITERATURE REVIEW

India represented by rich culture, traditions and natural biodiversity, offer unique opportunity for the drug discovery researchers. The country is blessed with two (Eastern Himalaya and Western Ghats) of the 18 worlds' hotspots of plant biodiversity and is 7th among the 16 Mega diverse countries where 70% of the world's species occur collectively. In India there are over 17,500 species of higher plants, 64 gymnosperms, 1200 pteridophytes, 2850 bryophytes, 2021 lichens, 15,500 fungi and 6500 algae reported. India is rich in its own flora i.e. endemic plant species (5725 angiosperms, 10 gymnosperms, 193 pteridophytes, 678 bryophytes, 260 liverworts, 466 lichens, 3500 fungi, and 1924 algae) (Sanjappa, 2005). Over 7500 plant species have been reported to be used in the Indian traditional systems including ethno medicines.

Selaginella (spike moss) is like a puzzle in the plant kingdom. Although a fascination to botanists at the turn of the 21st century, members of this genus are unexceptional in appearance, never flower, and are of no agronomic value. Selaginellas are an ancient lineage of vascular plants that arose about 400 million years ago. Lacking true leaves and roots, they are a key node of the plant evolutionary tree.

Physical Description

The stem is cutinized with an aerenchymatous cortex, and exarch, protostele (plectostele or haplostele). The leaves are microphylls with limited cutinized epidermis, mesophyll only a few cells thick and entirely spongy, and a single haplostelic vascular bundle. Typically the stem is dichotomously branched (primitive) and the spirally-arranged leaves are flattened dorso-ventrally into two morphs. At the tips of the branches strobili are found. The microphylls in the strobilus are called sporophylls. Each sporophyll has a sporangium in its axil. The sporangium consists of a stalk and a sterile jacket of cells. Inside the sterile jacket are one or more sporocytes which ultimately divide by meiosis to produce spores. *Selaginella* has both microspores and megaspores, therefore plant is called heterosporous.

Cultivation

Selaginellas are relatively easy to grow, but a successful culture requires various factors.

1. High humidity : provided by terrarium or green house.
2. Light : low levels of light intensity (filtered sunlight)
3. Temperature : 12.78° C + rise of 10-20 ° C in day
4. Soil : Mixture of osmunda fibre, sphagnum, peat and perlite or sand-peat combination provides a proper growth medium. It should be fibrous, spongy and loose and yet retain moisture all the time.
5. Container : Broad, flat container is used because of their spreading growth habit and shallow root system.

Propagation

Selaginella can be propagated by spores or cuttings. Cuttings of some species can be spread over the soil in tray in green houses covered with glass or plastic at 70°F until roots are formed. It is also likely to grow prothalia from spores as the first step in production. *Selaginella* is most often propagated by dividing mature clumps and replanting the divisions.

Altitudinal distribution of 30 species occurring in different types of forest in eastern India

The altitudinal distribution of 30 species of *Selaginella* in different forest types in eastern India is described in (Table 1). It may be well-noted that *Selaginella* species grow prolifically in tropical and subtropical forests and become completely absent from subalpine and alpine meadows of North-east frontier districts (Panigrahi et al., 1966)

Table1. Distribution patterns of different *Selaginella* species based on altitude in India

Altitude	Species distribution
Tropical forest (0-900 m)	<i>S. helferi</i> , <i>S. exigua</i> , <i>S. ciliaris</i> , <i>S. intermedia</i> , <i>S. pallida</i> , <i>S. pentagona</i> , <i>S. repanda</i> , <i>S. semicordata</i> , <i>S. bryopteris</i> , <i>S. minutifolia</i> , <i>S. radicata</i> .
Sub-tropical Forest	<i>S. chrysocaulos</i> , <i>S. decipiens</i> , <i>S. pennata</i> , <i>S. wallichii</i> , <i>S. willdenovii</i> , <i>S. bisulcata</i> , <i>S. pubescens</i> , <i>S. praetermissa</i> , <i>S. hookeri</i> , <i>S. pallidissima</i> , <i>S. involvens</i> , <i>S. argentea</i> , <i>S. semicordata</i> , <i>S. wightii</i> , <i>S. tenuifolia</i> , <i>S. miniatospora</i> , <i>S. monospora</i> , <i>S. biformis</i> , <i>S. delicatula</i> .
Temperate forest (1800-3500 m)	<i>S. argentea</i> , <i>S. chrysocaulos</i> , <i>S. pennata</i> , <i>S. bisulcata</i> , <i>S. wightii</i> .
Subalpine forest (3500-4500 m)	Total absence of <i>Selaginella</i> species.
Alpine forest (4500-5500 m)	Total absence of <i>selaginella</i> species.

Distribution Pattern of *Selaginella* Species

From 30 species of *Selaginella*, 12 interesting important species have been selected on the basis of its present status, distribution and physical distribution (Table2) (Panigrahi et.al, 1966)

Table2. Distribution pattern of different *Selaginella* species

Species	Geographical Distribution	Description	Reference
<i>Selaginella wightii</i> (Fairly abundant but absolutely rare)	Confined to S. India & Ceylon. New record for Eastern India.	Grows 5-12cm in height; moist soil or soil covered rocks, greenish-black in appearance	Hieron (1900)
<i>Selaginella wallichii</i> (Common)	South Burma, Malay peninsula, Sumatra. New record for India.	Grows on humid soil in forest floor, on sandy rocks, alluvial soil near ditches. Stem erect or sub-erect, drying straw colour up to 120 cm long.	Hook & Grev.(1840)
<i>Selaginella radicata</i> (Scarce)	Assam -Tipong reserve, Mountains of S. India. New record for E. India	Trailing plant on moist soil, 30cm in height	Hook & Grev. (1850); Alston (1945)

<i>Selaginella pubescens</i> (Scarce)	Burma, Siam & Indo-China. New record for India.	Grows on moist ground. spikes grow up to 1.8 cm long.	Hook & Grev. (1843)
<i>Selaginella monospora</i> (Scarce)	East India, Nepal, Bhutan, Burma, South China, Indo-China	Grows 35 cm in height, grows among grasses on the walls of roadside.	Spring (1850); Alston (1945)
<i>Selaginella minutifolia</i> (Fairly abundant)	S. Burma, Malaya, China, Cambodia, Assam-Khasi hills. New record for India	Small plants of moss-like rosette habit, grows 6 cm long on soil covered rocks.	Spring (1850); Alston (1945); Panigrahi (1966)
<i>Selaginella kurzii</i> (Scarce)	Assam to Malaya: Burma New record for Madhya Pradesh, UP	Grows 6cm in height in black mud along the road in the border of mixed forest and grassland.	Baker (1885); Alston (1945) Panigrahi (1966)
<i>Selaginella intermedia</i> (Fairly abundant)	Malay peninsula, Java, Sumatra, Borneo, Southern siam, South India. New record for Eastern India.	Grows on moist ground in forest floor, stem grows 60 cm long.	Spring (1843); Alston (1945)
<i>Selaginella ciliaris</i> (Scarce)	South India, Bihar, Orissa, Assam, Andaman Nicobar islands, South China to North Australia New record for Madhya Pradesh.	Grows 1-3 cm in height in crevices of rocks under shade in the humus containing black soil & also on brown laterite soil.	Spring (1843); Alston (1945)
<i>Selaginella rajasthanensis</i>	South Eastern Rajasthan. A new record for India	Grows 1.5-2.5 cm in height in isolated moist rock.	Gena et al. (1979)
<i>Selaginella praetermissa</i> (Scarce)	Assam, Ceylon	Grows on moist ground and stem is about 35 cm long.	Alston (1932), (1945)
<i>Selaginella willdenovii</i> (Scarce)	Nicobar islands, Burma, Indo-China. Malay peninsula, Sumatra, java, Brazil. New record for the mainland of India.	Grows on moist shady slope along the river valley, stem is about 35 cm long.	Baker (1867); Alston (1945)

Medicinal Value of *Selaginella* Species

The *Selaginella* species have potential medicinal value and has also been used in Ayurveda, Homeopathy and Siddha. In traditional Chinese medicine, *S. tamariscina* and *S. pulvinata* are used to promote blood circulation and to stimulate menstrual discharge; *S. doederleinii* is used in antibacterial and anticancer formulations, as well as for the treatment of cardiovascular diseases and snakebites. In traditional Indian medicine, *S. bryopteris* is used as a tonic for regeneration of energy and vitality, *S. rupestris* is used as a protective medicine after child birth as well as a sedative, *S. tamariscina* is used for treatment of amenorrhoea, bleeding piles and prolapse of rectum and *S. willdenovii* is used in cases of high fever and ashes are used in a liniment for backache. The Table3 illustrates interesting biological activities of various *Selaginella* species carried out by eminent authors.

Table3. Medicinal properties of *Selaginella* species

Species	Medicinal properties	Activity	Reference
<i>Selaginella bryopteris</i>	Relief from burning sensation during urination, normalcy of menstrual irregularities and given externally to pregnant women for an easy delivery& for curing jaundice	Antiplasmodial Antileishmanicidal	Kunert et.al (2008) Sah et.al (2005)
<i>Selaginella tamariscina</i>	Treatment for bloody faeces, hematuria, prolapse of the anus, for stanching & lower blood glucose levels, therapy of chronic trachitis, thrombocytopenic purpura & several forms of cancers.	Antiaging Antimetastatic Antifungal Antiproliferative	Lee et.al (2007) Yang et.al (2006) Lee et.al(2008) Gao et.al(2008)
<i>Selaginella uncinata</i>	Treatment for jaundice, dysentery, edema beriberoid diseases and tumours.	Antiviral	Ma et.al (2003)
<i>Selaginella sinensis</i>	For the treatment of chronic tracheitis	Antiviral	Ma et.al(2000)

<i>Selaginella doederleinii</i>	Treatment for cancer & cardiovascular diseases	Cytotoxic	Lee et.al (2008)
<i>Selaginella willdenowii</i>	-	Cytotoxic	Silva et.al (1994)
<i>Selaginella moellendorffii</i>	Treat acute hepatitis and bleeding	-	Zhu et.al (2008)
<i>Selaginella delicatula</i>		Cytotoxic	Chen et.al (2005)
<i>Selaginella involvens</i>	Treatment for acne	Anti-acne activity	Joo et.al (2007)

Active constituents and physical properties of different *selaginella* species

The physical properties of various compounds isolated from various species of *Selaginella* are given below. (Table 4)

Table4. Physical properties of various compounds of different Selaginella species

Species	Compound	MP (°C)	Colour	UV (MeOH) nm	IR Spectra (KBr)cm ⁻¹	Reference
<i>S. chrysocaulos</i>	Naringeninyl-(4''',O,3)-kaempferol	214	Yellow powder	270,289	3220,1653,1508	Swamy et.al (2006)
<i>S. chrysocaulos</i>	8''-Methylnaringeninyl-(4''',O,3)-kaempferol	-	Yellow amorphous solid	269,292		Swamy et.al (2006)
<i>S. chrysocaulos</i>	5'',7''-Dihydroxy-2''-phenoxychromonyl-(3''',4')-naringenin	186	Yellow powder	246,287		Swamy et.al (2006)
<i>S. chrysocaulos</i>	3',3'-Binaringenin	-	Pale yellow powder	238,268,336	3447, 1718, 1508	Swamy et.al (2006)
<i>S. chrysocaulos</i> <i>S. bryopteris</i> <i>S. tamariscina</i>	Amentoflavone	> 300	Pale yellow powder	238,268,336	-	Swamy et.al (2006) Cheng et.al (2008)
<i>S. bryopteris</i>	(2S)-2,3-Dihydroamentoflavone	-	Dark brown powder	234,276,286,330	-	Swamy et.al (2006)
<i>S. bryopteris</i>	(2''S)-2'',3''-Dihydroamentoflavone	-	Brown powder	238,268,292,338	-	Swamy et.al

						(2006)
<i>S. bryopteris</i>	(2S,2''S)-2,3,2'',3''-Tetrahydroamentoflavone		Brown powder	234,288		Swamy et.al (2006)
<i>S. bryopteris</i> <i>S. tamariscina</i>	Hinokiflavone		Dark yellow powder	236,268,338		Cheng et.al (2008)
<i>S. bryopteris</i>	(2S)-2,3-Dihydrohinokiflavone		Brown powder	242,270,292,340		Swamy et.al (2006)
<i>S. bryopteris</i>	(2''S)-2'',3''-Dihydrohinokiflavone		Dark brown powder	336,275		Swamy et.al (2006)
<i>S. bryopteris</i>	(2S,2''S)-2,3,2'',3''-Tetrahydrohinokiflavone		Dark brown powder	238,288		Swamy et.al (2006)
<i>S. bryopteris</i>	4'Methylamentoflavone (Bilobetin)		Pale yellow powder	238,268,334		Swamy et.al (2006)
<i>S. bryopteris</i>	7-Methylamentoflavone(Sequoiaflavone)		Yellow powder	238,268,334		Swamy et.al (2006)
<i>S. bryopteris</i> <i>S. tamariscina</i>	7,7'',4'''-Tri-O-methyl-amentoflavon (Heveaflavone)		Pale yellow powder	242,268,330		Cheng et.al (2008)
<i>S. bryopteris</i>	7-O-Methyl-hinokiflavone		Yellowish powder	240,270,336		Swamy et.al

	(Neocryptomerin)					(2006)
<i>S. tamariscina</i>	Selaginellin A	189-190	Red needle crystal	297,430	1595,1511, 1456	Cheng et.al (2008)
<i>S. tamariscina</i>	Selaginellin B	190-191	Red needle crystal	300,431	1597, 1512, 1488, 1449	Cheng et.al (2008)
<i>S. sinensis</i>	Sesquilignan	204-206	Colourless solid	210.1	3427, 1614	Wang et.al (2007)
<i>S. moellendorffii</i>	5-carboxymethyl-4-O-hydroxyflavone-7-O- b-D-glucopyranoside.	171–172	light yellow feather crystal	320, 258	3412, 2924, 2853, 1713, 1633, 1607, 1513, 1489,834	Zhu et.al (2008)

Active constituents and chemical properties of different *Selaginella* species

The term “flavonoid” is generally used to illustrate a broad collection of natural products that include a C6-C3-C6 carbon framework, or more specifically phenylbenzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes:

1. Flavonoids (2-phenylbenzopyrans)
2. Isoflavonoids (3-benzopyrans)
3. Neoflavonoids (4-benzopyrans)

These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related.

Figure 3. Three different classes of flavonoids

1. Flavonoids (C6-C3-C6 Backbone)

Based on the degree of oxidation and saturation present in the heterocyclic C-ring, the flavonoids may be divided into the following groups:

Figure 4. Different types of 2-Phenylbenzopyrans

2. Isoflavonoids

Isoflavonoids possess a 3-phenylchroman skeleton that is biogenetically derived by 1, 2-aryl migration in a 2-phenyl chroman precursor. Isoflavonoids are subdivided into the following groups:

Figure 5. Different types of Isoflavonoids

3. Neoflavonoids

The Neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2*H*-1-benzopyran-2-ones), 3, 4-dihydro-4-arylcoumarins and neoflavenes.

Figure 6. Different types of Neoflavonoids

Minor Flavonoids

Chalcones and aurones are natural products containing a C6-C3-C6 backbone and are considered to be minor flavonoids. This group of compounds consists of 2'-hydroxychalcones, 2'-OH-dihydrochalcones, 2'-OH-*retro*-chalcone, aurones (2-benzylidenecoumaranone) and auronols.

Figure 7. Different types of Minor flavonoids

The chemical properties of compounds isolated from various species of *Selaginella* are given below. (Table5)

Table5. Chemical properties of various compounds of different *Selaginella* species

Species	Compound	Molecular Formula	Structure	Reference
<i>S. chrysocaulos</i>	Naringeninyl-(4''',O,3)-kaempferol	$C_{30}H_{20}O_{10}$	<p>1 R = H</p>	Swamy et.al (2006)
<i>S. chrysocaulos</i>	8''Methylnaringeninyl-(4''',O,3)-kaempferol	$C_{31}H_{22}O_{10}$	<p>2 R = Me</p>	Swamy et.al (2006)
<i>S. chrysocaulos</i>	5'',7''-Dihydroxy-2''-phenoxychromonyl-(3''',4')-naringenin	$C_{30}H_{20}O_{10}$	<p>3</p>	Swamy et.al (2006)
<i>S. chrysocaulos</i>	3',3'-Binaringenin	$C_{30}H_{20}O_{10}$	<p>4</p>	Swamy et.al (2006)

<i>S. chrysocaulos</i> <i>S. bryopteris</i> <i>S. tamariscina</i>	Amentoflavone	$C_{30}H_{18}O_{10}$	<p>5 $R^1 = R^2 = R^3 = H$</p>	Swamy et.al (2006) Cheng et.al (2008)
<i>S. bryopteris</i>	(2S)-2,3-Dihydroamentoflavone	$C_{33}H_{26}O_{10}$	<p>6</p>	Swamy et.al (2006)
<i>S. bryopteris</i>	(2''S)-2'',3''-Dihydroamentoflavone		<p>7</p>	Swamy et.al (2006)
<i>S. bryopteris</i>	(2S,2''S)-2,3,2'',3''-Tetrahydroamentoflavone		<p>8</p>	Swamy et.al (2006)
<i>S. bryopteris</i> <i>S. tamariscina</i>	Hinokiflavone	$C_{30}H_{18}O_{10}$	<p>9 $R = H$</p>	Cheng et.al (2008)

<i>S.bryopteris</i>	(2S)-2,3-Dihydrohinokifl avone		<p style="text-align: center;">10</p>	Swamy et.al (2006)
<i>S.bryopteris</i>	(2''S)-2'',3''- Dihydrohinokifl avone	$C_{30}H_{20}O_{10}$	<p style="text-align: center;">11</p>	Swamy et.al (2006)
<i>S.bryopteris</i>	(2S,2''S)- 2,3,2'',3''- Tetrahydrohinok iflavone		<p style="text-align: center;">12</p>	Swamy et.al (2006)
<i>S.bryopteris</i>	4'- Methylamentofl avone (Bilobetin)	$C_{31}H_{20}O_{10}$	<p style="text-align: center;">13 $R^2 = Me \quad R^1 = R^3 = H$</p>	Swamy et.al (2006)
<i>S.bryopteris</i>	7- Methylamentofl avone(Sequoiafl avone)	$C_{31}H_{20}O_{10}$	<p style="text-align: center;">14 $R^1 = Me \quad R^2 = R^3 = H$</p>	Swamy et.al (2006)

<i>S.bryopteris</i>	(2S,2''S)- 2,3,2'',3''- Tetrahydrohinok iflavone		<p style="text-align: center;">12</p>	Swamy et.al (2006)
<i>S.bryopteris</i>	4'- Methylameto- flavone (Bilobetin)	$C_{31}H_{20}O_{10}$	<p style="text-align: center;">13 $R^2 = \text{Me}$ $R^1 = R^3 = \text{H}$</p>	Swamy et.al (2006)
<i>S.bryopteris</i>	7- Methylameto- flavone(Sequoiafl avone)	$C_{31}H_{20}O_{10}$	<p style="text-align: center;">14 $R^1 = \text{Me}$ $R^2 = R^3 = \text{H}$</p>	Swamy et.al (2006)
<i>S.bryopteris</i> <i>S.tamariscina</i>	7,7'',4'''-Tri-O- methylameto- flavone (Heveaflavone)		<p style="text-align: center;">15 $R^1 = R^2 = R^3 = \text{Me}$</p>	Cheng et.al (2008)
<i>S.bryopteris</i>	7-O- Methylhinokifla vone(Neocrypto merin)	$C_{31}H_{20}O_{10}$	<p style="text-align: center;">16 $R = \text{Me}$</p>	Swamy et.al (2006)

<i>S.tamariscina</i>	Selaginellin A	$C_{33}H_{23}O_4$		Cheng et.al (2008)
<i>S.tamariscina</i>	Selaginellin B	$C_{34}H_{25}O_4$		Cheng et.al (2008)
<i>Selaginella sinensis</i>	sesquilignan	$C_{29}H_{32}O_8N$ a		Wang et.al (2007)
<i>Selaginella moellendorffii</i>	5-carboxymethyl-40-hydroxyflavone-7-O-b-D-glucopyranoside	$C_{23}H_{22}O_{11}$		Zhu et.al (2008)

FUTURE STUDIES

Apart from its utility as a medicinal herb, *Selaginella* species can be further explored for existence of a drought resistance gene. For example- *Selaginella bryopteris* has been reported to have the highest degrees of drought resistance. The gene responsible for it can be isolated and incorporated into the genomes of agricultural crops, such as wheat, paddy, legumes etc, with the help of biotechnology to produce transgenic agricultural crops. *S.wightii* is known to be “resurrection plant” and thus can be further investigated for the presence of drought resistance gene which would create a revolution in the field of plant genetics and molecular biology.

Chapter-3

Materials

&

Methods

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plant material collection

1. Extraction a) Solvents

Petroleum ether (PE)
Carbon tetrachloride (CCl₄)
Diethyl ether (DE)
Butanol (BuOH)
Ethanol (EtOH)
Aqueous (AQ)

b) Apparatus

Soxhlet apparatus
Rotary vacuum evaporator

2. Phytochemical tests

a) Chemical reagents

Magnesium turnings	Zinc dust
Conc. HCl	10% Tannic acid
10% NaOH	Ferric chloride
Wagner's reagent	Gelatin
Mayer's reagent	acetic anhydride
Conc. H ₂ SO ₄	

3. Quantitative estimation of Phenolic and Flavonoids

a) Phenolic compounds

Folin-Ciocalteu reagent (50%)
Sodium carbonate (20%)
Gallic acid (5mg/50ml)

b) Flavonoids

Ethanol (80%)
Potassium acetate (1M)

Aluminium nitrate (10%)

4. TLC/ HPTLC

Silica gel 60F₂₅₄

Microscopic slides

Readymade TLC plates

Solvents – Ethyl acetate

Methanol

Water

1% ethanolic Aluminium chloride reagent

Iodine chamber

5. Antimicrobial activity

a) Bacteria

Nutrient Broth

Muller Hinton Agar

b) Bacterial strains

Enterococcus faecalis (ATCC35550), *E.Coli* (ATCC13534), *Klebsiella pneumonia* (ATCC15380), *Staphylococcus aureus* (ATCC9144), *Streptococcus pneumonia* (ATCC33400), *Pseudomonas aeruginosa* (ATCC25619)

c) Fungal

Nutrient broth containing peptone and dextrose

Potato dextrose agar

d) Fungal strains

Candida albicans (MTCC227), *Candida fumata* (NCIM), *Candida rhodotrulla* (NCIM), *Neurospora carcase*, *Aspergillus flavus* and *Aspergillus fumigatus*.

6. Antioxidant activity

a) DPPH radical scavenging activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH)

Ethanol

b) β -cartenoid/Linoleic acid assay

β -carotene

Linoleic acid

Agar

7. Softwares:

Hex 4.5 (Docking)

Quantum 3.2- ADME and Toxicity studies

Origin 6.0 (Antioxidant activity)

8. Instruments:

UV-Visible Hitachi 2800 double beam Spectrophotometer.

RP-HPLC

FT-IR Thermo Nicolet 330 Avatar

LC-MS

^1H and ^{13}C NMR

METHODOLOGY

3.2 Extraction

Air-dried aerial parts of herb (powdered form); 50g were taken and sequentially extracted with six solvents namely petroleum ether, carbon tetrachloride, diethyl ether, butanol, ethanol and water with the help of Soxhlet apparatus. The six extracts were then concentrated using rotary vacuum dryer.

3.2.1. Phytochemical tests

1. Flavonoids

a) Shinoda test (Harborne, 1998)

To the extract, few magnesium turnings are added and then conc.HCl is added drop wise. Scarlet or crimson red color appears after few minutes.

b) Alkaline reagent test

To the extract, few drops of 10% NaOH solution are added drop wise. An intense yellow color develops which turns colorless on adding few drops of dilute HCl.

c) Zinc Hydrochloride test

To the extract, zinc dust is added and then conc.HCl is added drop wise. It gives a red color after few minutes.

2. Alkaloids

Solvent free extract, 10 mg is stirred with few ml of dil.HCl and filtered. The filtrate is tested with various alkaloidal reagents.

a) Mayer's test (Evans,1997)

To a few ml of filtrate, 2 drops of Mayer's reagent are added by the side of the test tube. A white or creamy precipitate confirms the test as positive.

b) Wagner's test (Wagner,1993)

To a few ml of filtrate, 2 drops of Wagner's reagent are added by the side of the test tube. A reddish-brown precipitate confirms the test as positive.

c) Tannic acid test

To a few ml of filtrate, 2-3 drops of 10% Tannic acid is added. A buff color precipitate confirms the test as positive.

3. Steroids/Terpenoids (Finar, 1986)

a) Libermann-Burchard's test

The extract (10mg) is dissolved in 2ml of acetic anhydride. To this, one or two drops of conc. H_2SO_4 is added slowly along the sides of the test tubes. The solution turns red and then changes to blue and finally to green which confirms the test as positive.

b) Salkowski Reaction

To 0.5 ml of extract (dissolved in chloroform), conc. H_2SO_4 is added drop wise by the side of the test tubes. The upper layer stains red which confirms the test as positive.

4. Phenolic compounds/Tannins

a) Ferric chloride test (Mace, 1963)

The extract (10 mg) is dissolved in 1 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution are added. A dark green or blue color indicates the presence of phenolic compounds.

b) Gelatin test (Evans, 1997)

The extract (10 mg) is dissolved in 1 ml of distilled water and 2ml of 1% solution of gelatin containing 10% sodium chloride is added to it. White precipitate indicates the presence of phenolic compounds.

c) Lead acetate test

The extract (10 mg) is dissolved in 1 ml of distilled water and 3ml of 10% lead acetate solution is added. A white precipitate indicates the presence of phenolic compounds.

5. Saponins (Kokate, 1999)

The extract (10 mg) is diluted in 1 ml of distilled water and made up to 10 ml. The suspension is shaken well for 15 min. Formation of foam indicates the presence of Saponins.

3.2.2 Estimation of Phenolic compounds (Quantitative)

The phenolic contents were estimated according to the modified spectrophotometric methods of Tanner and Brunner (1979) and Kaur and Kapoor (2002). To 0.5 ml of an extract solution, 2 ml of distilled water and 0.5 ml of Folin–Ciocalteu reagent (50%) were added and mixed well. Then, 1 ml of 20% sodium carbonate was added and mixed well again.

It was then incubated at room temperature for 20 minutes. Absorbance was read at 730 nm (Karadeniz et al., 2005).

The phenolic content was calculated from the standard calibration curve obtained from gallic acid.

3.2.3 Estimation of Flavonoids (Quantitative)

A 0.1 ml of the extract was taken in a test tube and 3.8 ml of 80% ethanol and 0.1 ml of 1M potassium acetate were added.

To this solution, 0.1 ml of 10% aluminium nitrate was added. It was then incubated at room temperature for 40 minutes.

The absorbance was read at 415 nm. Flavonoid contents were then calculated using a standard calibration curve, prepared from Quercetin.

3.2.4 Thin Layer Chromatography (TLC)

a) The Silica gel G-60 PF-254 mixture (25g/50ml H₂O) is poured on small microscopic slides in a thin layer and is allowed to dry at room temperature for 1 day. It is then activated at 120°C for 30 minutes.

b) The TLC chamber is saturated with mobile phase Ethyl acetate: Methanol: Water (10:1.65:1.35) for about 30 minutes prior to placing of TLC plate inside the chamber.

c) To the activated TLC plate 4 µl of sample is loaded at a distance of 1.5 cm from the base. While spotting a few min of air drying is required in between applications.

d) The TLC plate is then placed inside the saturated chamber and allowed to run. After 10 minutes the TLC plate is removed and air-dried.

e) For visualization, the TLC plate is placed inside the Iodine chamber for 5 minutes. The spots appear as brown in color.

3.2.5 High Pressure Thin Layer Chromatography (HPTLC)

The given extract samples each 1ml were prepared by diluting with respective solvents (Ethanol and Butanol) centrifuged and made up to 5ml. These solutions were used as test solution for profile analysis.

2µl of the above test solutions were loaded in the 5cm x 10cm Silica gel 60F₂₅₄ TLC plate (0.2mm layer thickness) as 8mm band length using Hamilton syringe and CAMAG LINOMAT 5 instrument.

The sample loaded plate was then kept in TLC twin trough developing chamber with Ethyl acetate-Methanol-Water (10: 1.65: 1.35), 20min for Chamber saturation. After that, the plate was eluted with the respective mobile phase up to 80mm, as solvent front.

The eluted plate was dried by warm air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254nm and UV366nm.

The plate was sprayed with 1% ethanolic Aluminium chloride reagent and dried at 120 C in Hot air oven for 5 minutes. The plate was photo-documented at UV 366 (Flavonoid) using Photo-documentation chamber.

Finally, the plate was fixed in scanner stage and scanning was done using CAMAG TLC SCANNER 3 at UV366nm for Flavonoid.

A – Ethanol extract sample

B – Butanol extract sample

3.2.6 Column Chromatography

Here, elution chromatography is used under isocratic conditions.(constant mobile phase composition)

The extract (20 mg) was mixed with 10ml of methanol in rotary flask (1L) and to it silica gel (60-120 mesh) was added until a thick paste was obtained. It was then vaccum dried with the help of rotary vaccum dryer into a fine powder.

The mobile phase Ethyl acetate: Methanol: Water (10:1.65:1.35) up to 500 ml was prepared.

The column was rinsed with acetone to remove impurities if present any. The column was clogged with a layer of cotton at the bottom as packing material.

About 200 g of silica gel (60-120 mesh) was mixed with mobile phase to make slurry which was loaded into the column. A thin layer of absorbent cotton was put onto which the fine silica-extract powder was loaded. Then again a thin layer of absorbent cotton was put and mobile phase (10 ml) was added. The column had continuous supply of mobile phase till the final phase of elution. It was then covered with aluminum foil at the top.

The flow rate was approximately adjusted to 1ml/min and the elutes were collected in test tubes.

3.2.7 HPLC

The 24 fractions obtained from column chromatography were injected onto the chromatograph.

1mg/ml of the stock solution of amentoflavone was prepared. The samples were analysed by HPLC which consisted of freshly prepared mobile phase consisted of Ethyl acetate: Methanol: Water. (10:1.65:1.35). Ethyl acetate and methanol used were of HPLC grade. MilliQ water was used. The mobile phase prepared was then sonicated for 8 minutes. The flow rate was maintained at 1 ml/min, the column temperature at 40 °C and the detection was done at 270 and 330 nm

3.2.8 DPPH free-radical scavenging activity

The effects of herbal product extracts on DPPH radicals were studied using the modified method. [47] Briefly, 19 mg of DPPH in 500 ml of ethanol was prepared and 3.0 ml of this solution was added to the test sample. The test sample was taken in varying concentrations from 10µg/ml -500µg/ml from stock solution of 1mg/ml. The volume was made up to 500µl for different varying concentration. The reaction mixture was shaken well and incubated for 30 min at room temperature. The absorbance of the resulting solution was read at 517 nm against a blank. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

The IC₅₀ value (µg/ml) is the concentration at which the scavenging activity is 50%.

3.2.9 β-Carotene-Linoleic acid assay

1. Preparation of Agar solution

Technical agar No. 3 (0.5 g) was weighed and dissolved in 25 ml of distilled water to produce a 1.2% technical agar solution by boiling.

2. Preparation of β-carotene and linoleic acid solutions

3. β-Carotene solution. The 80 mg of β-carotene (Sigma) was weighed and dissolved in 40 ml of acetone to produce a β-carotene solution of concentration 2 mg/ml.

4. Linoleic acid solution. The 80 mg of Linoleic acid (Sigma) was weighed in a beaker and dissolved in 40 ml of ethanol to produce a linoleic acid solution of concentration 2 mg/ml.

5. Procedure

Linoleic acid solution (10 ml) and β-carotene solution (10 ml) were added to the molten agar (25 ml). This was then shaken to achieve uniform distribution of the added components. An Orange color was produced. The agar was then poured into Petri dishes. The Petri dishes were then kept in dark to exclude light and to allow the agar to set. Holes (6mm diameter) were then punched into the agar using a borer. Extracts (1mg/100µl) was transferred into the holes and the Petri dishes were then incubated at 45 °C for 6 h. A zone of color retention around the hole after incubation indicated extracts with antioxidant properties. The zone diameter was measured. Ascorbic acid (1mg/100µl) was used as a positive control. The above method used was modified method of (Graven et al., 1992).

3.3 Reducing Power

The reducing power was determined accordingly to the modified method (Shimada et.al, 1992). A concentration of different *S.wightii* extracts (600µl) was mixed with 1.5ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1.5ml of 1% potassium ferricyanide, and the resultant mixture was incubated at 50 C for 20 min.

After addition of 1.5 ml of 10% Trichloroacetic acid (w/v), the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (1.5ml) was mixed with 1.5ml of deionised water and 300 µl of 0.1% ferric chloride.

The absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. The assays were carried out in duplicates and the results are expressed as means $\pm$ standard deviation. Ascorbic acid was used as standard.

Statistical analysis

All experiments were performed in duplicates. The data were recorded as means $\pm$ standard deviations and differences were considered significant at $P < 0.05$. The EC50 values were calculated using a software Microcal ORIGIN-6.0

3.3.1 Antimicrobial activity

The well diffusion method was used with certain modifications to determine the antimicrobial activity of the *S.wightii* extracts.

The medium was prepared by dissolving the specified quantity of the dehydrated medium in purified water and was dispensed into 250 ml conical flasks. The flasks were closed with cotton plugs and were sterilized by autoclaving at 121°C for 15 minutes. The contents of the flask were poured aseptically into sterile Petri plates and allowed to solidify.

Briefly, using 100 µl of a suspension containing 10^8 colony-forming units (CFU)/ml of bacteria, 10^6 CFU/ml of yeast, or 10^4 spore/ml of fungus was spread on nutrient agar (NA), potato dextrose agar (PDA), respectively with a sterile cotton swab.

The well-borer (6 mm in diameter) was used for making four wells and extracts of varied concentrations (50-500µg) were added from stock of 1 mg/ml. (dissolved in dimethylsulfoxide [DMSO]). Negative controls were prepared using DMSO.

Gentamicin (30 µg/disc) and Kanamycin (30 µg/disc) were used as the positive reference standards for bacterial strains. Bavistin (30µg/ml) was used as positive reference standard for fungal strains.

The inoculated plates were incubated at 37°C for 24 h for the bacterial strains, at 27°C for 48 h for the fungi. The antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

3.3.2 Interaction and toxicity studies

i) Molecular docking

Protein-Ligand docking is a molecular modeling technique where the goal is to predict the position and orientation of a Ligand (a small molecule) when it is bound to a protein receptor or enzyme. In the field of molecular modeling, docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Knowledge of the preferred orientation in turn may be used to calculate the strength of association or binding affinity between two molecules using for example scoring functions. Two approaches are particularly popular within the molecular docking community. One approach uses a matching technique that describes the protein and the ligand as complementary surfaces. The second approach simulates the actual docking process in which the ligand-protein pair wise interaction energies are calculated. Molecular docking may be defined as an optimization problem, which would describe the “best-fit” orientation of a ligand that binds to a particular protein of interest. During the course of the process, the ligand and the protein adjust their conformation to achieve an overall “best-fit” and this kind of conformational adjustments resulting in the overall binding is referred to as “induced-fit”. The focus of molecular docking is to computationally stimulate the molecular recognition process. The aim of molecular docking is to achieve an optimized conformation for both the protein and Ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized.

Importance of Docking: It plays a key role in rational design of drugs. The results of docking can be used to predict whether the drug can bind to the protein with good complementarities. The interaction properties can be carried out with experimental procedures but they can also be predicted in advance with bioinformatics tools so that the true positive results can be found out and false negative results can be eliminated and only the most promising experimental tools can be carried out by avoiding the negative dead ends. Furthermore, the associations between biologically relevant molecules such as proteins, nucleic acids, carbohydrates, and lipids play a central role in signal transduction. The relative orientation of the two interacting partners may affect the type of signal produced. Therefore docking is useful for predicting both the strength and type of signal produced.

Application:

A binding interaction between a small molecule ligand and a enzyme protein may result in activation inhibition of the enzyme. If the protein is a receptor, ligand binding may result in agonism or antagonism. Docking is most commonly used in the field drug design— most drugs are small organic molecules, and docking may be applied to:

- Hit identification – docking combined with a scoring function can be used to quickly screen large databases of potential drugs *in silico* to identify molecules that are likely to bind to protein target of interest.
- Lead optimization – docking can be used to predict in where and in which relative orientation a Ligand binds to a protein. This information may in turn be used to design more potent and selective analogs.
- Bioremediation – Protein- Ligand docking can also be used to predict pollutants that can be degraded by enzymes.

ii) ADME studies

ADME is an acronym in pharmacokinetics and pharmacology for absorption, distribution, metabolism, and excretion, and describes the disposition of a pharmaceutical compound within an organism. The four criteria all influence the drug levels and kinetics of drug exposure to the tissues and hence influence the performance and pharmacological activity of the compound as a drug.

Absorption/Administration

Before a compound can exert a pharmacological effect in tissues, it has to be taken into the bloodstream — usually via mucous surfaces like the digestive tract (intestinal absorption). Uptake into the target organs or cells needs to be ensured, too. This can be a serious problem at some natural barriers like the blood-brain barrier. Factors such as poor compound solubility, chemical instability in the stomach, and inability to permeate the intestinal wall can all reduce the extent to which a drug is absorbed after oral administration. Absorption critically determines the compound's bioavailability. Drugs that absorb poorly when taken orally must be administered in some less desirable way, like intravenously or by inhalation (e.g. zanamivir).

Distribution

The compound needs to be carried to its effector site, most often via the bloodstream. From there, the compound may distribute into tissues and organs, usually to differing extents.

Metabolism

Compounds begin to be broken down as soon as they enter the body. The majority of small-molecule drug metabolism is carried out in the liver by redox enzymes, termed cytochrome P450 enzymes. As metabolism occurs, the initial (parent) compound is converted to new compounds called metabolites. When metabolites are pharmacologically inert, metabolism deactivates the administered dose of parent drug and this usually reduces the effects on the body. Metabolites may also be pharmacologically active, sometimes more so than the parent drug.

Excretion/Elimination

Compounds and their metabolites need to be removed from the body via excretion, usually through the kidneys (urine) or in the feces. Unless excretion is complete, accumulation of foreign substances can adversely affect normal metabolism.

There are three sites where drug excretion occurs. The kidney is the most important site and it is where products are excreted through urine. Biliary excretion or faecal excretion is the process that initiates in the liver and passes through to the gut until the products are finally excreted along with waste products or faeces. The last method of excretion is through the lungs e.g. anaesthetic gases.

Excretion of drugs by the kidney involves 3 main mechanisms:

- Glomerular filtration of unbound drug.
- Active secretion of (free & protein-bound) drug by transporters e.g. anions such as urate, penicillin, glucuronide, sulphate conjugates) or cations such as choline, histamine.
- Filtrate 100-fold concentrated in tubules for a favourable concentration gradient so that it may be reabsorbed by passive diffusion and passed out through the urine.

iii) Toxicity studies

Computational chemists try to predict the ADME-Tox qualities of compounds through methods like QSPR or QSAR. The route of administration critically influences ADME.

Description of different types of cancer-related proteins used for Interactions-toxicity studies.

Table 6. Different types of proteins used in docking studies

Name of Proteins	Type of proteins	Disease
Ski	Oncoprotein	Responsible for all types of cancer.
Bcl-2	Apoptosis regulator	Human follicular lymphoma
E7	Viral Oncoprotein	Human papilloma virus 45
Braf-Kinase	Serine/Threonine protein	Expressed by tumours
erbB2	Signalling protein/Transferase	Brain tumour
E1a	Transcription repressor, cell cycle	Retinoblastoma
P53 wild type	Transcription/Cellular tumour antigen	a rapid senescence program in human tumour cells
Brc	Signalling protein	Breast cancer type-I
DLC2	Lipid binding protein	Liver cancer
Epha2	Transferase	Prostate and lung cancer

3.3.2.1 KEGG PLANT PATHWAY DATABASE:

KEGG PATHWAY is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction for:

- Metabolism
- Genetic Information Processing
- Environmental Information Processing
- Cellular Processes
- Human Diseases
- Drug Development

KEGG PLANT is a new interface to the KEGG resource for plant research, especially for understanding relationships between genomic and chemical information of natural products from plants. Our knowledge on biosynthetic pathways of plant natural products is largely incomplete, but the genomic information is expected to give clues to missing enzymes and reactions for biosynthesis. The genomic information may also uncover the architecture of biosynthetic pathways for generating chemical diversity of natural products. Below figure 6 shows the KEGG PATHWAY window.

KEGG PLANT
Pathway-based integration of plant genomes and natural products

KEGG2 DISEASE DRUG GLYCAN COMPOUND REACTION PLANT Organisms

KEGG PLANT
Plants are known to produce diverse chemical compounds including those with medicinal and nutritional values. **KEGG PLANT** is a new interface to the KEGG resource for plant research, especially for understanding relationships between genomic and chemical information of natural products from plants. Our knowledge on biosynthetic pathways of plant natural products is largely incomplete, but the genomic information is expected to give clues to missing enzymes and reactions for biosynthesis. The genomic information may also uncover the architecture of biosynthetic pathways for generating chemical diversity of natural products.

Plant Biosynthetic Pathways
In addition to expanding the repertoire of KEGG pathway maps for plant secondary metabolism, an overview map and several category maps have been introduced. The category maps are used to classify plant secondary metabolites.

Overview map

- [Biosynthesis of plant secondary metabolites](#)

Plant Genomes
The available complete genomes for plants are very limited in comparison to other organism groups. Thus, massive EST datasets have been processed for a number of plant species to generate the EGENES database where EST contigs are treated as genes and automatically annotated with the KO (K number) identifiers.

- [Complete genomes in KEGG](#)
- [Plant classification](#)

Phytochemical Compounds
A classification system of phytochemical compounds (plant secondary metabolites) is being developed based on the biosynthetic pathways. This system is used to categorize approved drugs and other products derived from plants.

- [Phytochemical compounds](#)
- [Drugs derived from plant natural products](#)

Herbal Medicine
The KEGG DRUG database currently contains 254 crude drugs (consisting of multiple chemical

Figure 8. KEGG PLANT Pathway window

Chapter-4

Results & Discussion

RESULTS

4. Identification of plant material

The *S.wightii* species collected from trikuti hills, Deogarh (Jharkhand) in December, India was identified by Rev. John Britto, Director, Rapinet Herbarium, Trichy.

Fig. 1 *Selaginella wightii*

4.1 Phytochemical tests

Phytochemical tests were carried out with *Selaginella wightii* extracts for qualitative estimation of phytochemicals.

The EtOH extract showed maximum strong presence of flavonoids followed by BuOH. In case of alkaloids, BuOH showed maximum strong presence followed by EtOH fraction. The DE and CCl₄ showed a weak presence of steroids/terpenoids. The EtOH and BuOH showed strong presence of phenolic compounds whereas DE showed a weak presence. Saponins were present in BuOH, EtOH and AQ extracts. The AQ extracts indicated high presence of saponins when compared to EtOH and BuOH extracts. (Table 7)

Table7. Phytochemical Tests of *S.wightii* extracts

TEST	EXTRACT					
	PE	CCl ₄	DE	BuOH	EtOH	AQ
Flavonoids						
Shinoda test	-	-	-	++	+++	-
Alkaline reagent test	-	-	-	++	+++	-
Zinc hydrochloride test	-	-	-	++	+++	-
Alkaloids						
Tannic acid test	-	-	-	++	+	-
Wagner's reagent	-	-	-	++	+	-
Mayer's reagent	-	-	-	++	+	-
Steroids/Terpenoids						
Salkowski test	-	+	+	-	-	-
Liebermann buchard test	-	+	+	-	-	-
Phenolic compounds/ Tannins						
Ferric chloride test	-	+	-	++	++	-
Gelatin test	-	+	-	++	++	-
Saponins	-	-	-	+	++	+++

- absence; + indicates weak presence; ++ indicates strong presence; +++ indicates maximum strong presence

4.1.1 Quantitative estimation for phenolic compounds

The EtOH showed high concentration of phenolic compounds (55µg/ml) when compared to BuOH extract (37.83µg/ml) and DE (30.33µg/ml). Phenolic concentration in AQ and PE extract was found to be almost similar. CCl₄ fraction (6.33 µg/ml) indicated a low concentration of phenolic compounds. (Table 8)

Table 8. Quantitative estimation of phenolic compounds for different extracts of *S. wightii*

Extracts	Concentration(µg/ml)
PE	14.38
CCl ₄	6.33
DE	30.33
BuOH	37.83
EtOH	55
AQ	15.05

4.1.2 Quantitative estimation of flavonoids

The EtOH fraction indicated highest concentration of flavonoids (4.90µg/ml) when compared to BuOH. (0.55µg/ml) CCl₄ (0.45µg/ml) and AQ extracts (0.27µg/ml) showed moderate concentration of flavonoids whereas PE (0.18µg/ml) and DE (0.09µg/ml) showed minimal concentration of flavonoids (Table 9).

Table 9. Quantitative estimation of flavonoids for different extracts of *S. wightii*

Extracts	Concentration(µg/ml)
PE	0.18
CCl ₄	0.45
DE	0.09
BuOH	0.55
EtOH	4.90
AQ	0.27

4.1.3 Thin Layer Chromatography (TLC)

The TLC was performed for ethanol and butanol extracts. Five brown spots were visible in both the extracts after keeping it for 5 minutes in iodine chamber. The mobile phase used was Ethyl acetate: Methanol: Water(10:1.65:1.35)

Figure 9. TLC analysis of S- Standard; E- Ethanol extract; B-Butanol extract

4.1.4 High Pressure Thin Layer Chromatography (HPTLC)

The mobile phase used was Ethyl acetate: Methanol: Water (10:1.65:1.35). The Peak table and densitogram were noted. Yellow fluorescence zones at UV366 nm in both the samples were observed in the chromatogram, which confirmed the presence of Flavonoid in the samples A and B. (Fig.9a) The HPTLC analysis of ethanol and butanol extracts of *S. wightii* indicated a presence of flavonoid1 of the same R_f value (Table10;Fig.9b). The spectrum comparison for both the samples of flavonoid 1 indicated similar peaks (Fig.9c).

Figure 9a. HPTLC of Flavonoids profile obtained from *S. wightii* extracts

TABLE10. HPTLC analysis of Ethanol and Butanol extracts

Track	Peak	Rf	Height	Area	Assigned substance
A	1	0.71	62.3	5253.9	Flavonoid 1
B	1	0.71	75.9	4395.2	Flavonoid 1

Track A - Baseline display (Scanned at 366nm)

Track A - Peak densitogram display

Figure 9b. HPTLC profile of ethanol extract for peak 1

Track B - Baseline display (Scanned at 366nm)

Track B - Peak densitogram display

Figure 9b. HPTLC profile of butanol extract for peak 1

Figure 9c. Spectrum comparison of “Flavonoid 1”from *S. wightii* in Track A (Ethanol) and Track B (Butanol)

4.1.5 Column Chromatography

Isocratic column chromatography was performed and 24 fractions were obtained which was further analysed by HPLC method and TLC. Out of 24 fractions, 9 fractions were analysed by TLC and rest 15 fractions by HPLC. A single peak (HPLC) or a single spot (TLC) respectively indicated the purity of the spot.

4.1.6 High Pressure Thin Layer Chromatography (HPLC)

The HPLC analysis was done to check fractions purity at two different wavelengths: 270 and 330 nm. The number of peaks obtained and the retention time of the fractions is given in the Table 11. The TLC results indicated one fraction (5A) as pure while HPLC results showed 9 fractions as pure.

Table11. HPLC Analyses of ethanolic extract of *S. wightii*, obtained from Column chromatography

Sample	No of peaks	Retention time	Purity
11 (270)	3	1.29, 1.89, 2.2	impure
11 (330)	1(broad)	1.28	impure
12 A (270)	1	1.2	pure
12 A (330)	1	1.2	pure
13A (270)	1	1.24	pure
13A (330)	1	1.238	pure
14A (270)	1	1.23	pure
14A (330)	1	1.23	pure
15A (270)	1(broad)	1.396	impure
15A (330)	2	1.184, 1.385	impure
16A (270)	2	1.224, 1.438	impure
16A (330)	2	1.232, 1.438	impure
17A-270	2	1.04, 1.3	impure
17A-330	2	1.04, 1.3	impure
18A-270	1	1.39	pure
18A-330	1	1.377	pure
19A-270	3	1.36, 2.07, 2.3	impure
19A-330	2	1.324, 1.505	impure
20A-270	1	1.398	pure
20A-330	1	1.417	pure
21A-270	many		highly impure
21A-330	many		highly impure
22A-270	many		highly impure
22A-330	many		highly impure
23A-270	1	1.363	pure
23A-330	1	1.344	pure
24A-270	2	1.289, 1.805	impure
24A-330	2	1.23, 1.58	impure
CB-270	1	1.322	pure
CB-330	1	1.318	pure
CE-270	1	1.298	pure
CE-330	1	1.298	pure
UA-270	1	1.246	pure
UA-330	1	1.24	pure

4.1.7 Spectral analysis

a) UV-Visible spectroscopy

The spectrum scan for the standard-Amentoflavone and three unknown compounds were performed. The scanning range was 200-400 nm.

Table12. UV spectra of compounds obtained from ethanolic extracts of *S.wightii*

Name of the compound/code	λ_{max} (maximum wavelength) nm
Amentoflavone	270, 336
Compound#1	270.5, 331
Compound#2	217.5
Compound#3	266.5

b) FT-IR spectra

1. Compound#1

Peak at 3438.70 indicates the presence of intermolecular hydrogen bonded hydroxyl group; 2925.20 corresponds to alkyl stretch, stretching due to methyl or methylene groups; Peak at 1628 can be either due to C=O or C=C bond. 1025.17 indicates aromatic group bending.

Figure 10a. IR spectra of Compound #1 obtained from ethanolic extracts of *S. wightii*

2. Compound #2

Peak at 3438.09 is due to dimeric associated intermolecular OH stretching (bonded); 2927.70 indicate Ar-CH₃ stretch; 2856.01 shows R-CH₃ stretch; Peak at 1636.63 can be due to C=O or C=C bond; 1023.48 is due to aromatic group bending; 791.02 shows C-H out of plane bending due to presence of 1,2,3-Trisubstituted benzene ring.

Figure 10b. IR spectra of Compound 2 obtained from ethanolic extract of *S. wightii*

3. Compound #3

Peak at 3406.13 is due to OH stretching (bonded); Peak at 1630.34 can be due to C=O or C=C bond; 1022.86 is due to aromatic group bending; 683.48 shows C-C out of plane bending due to presence of 1,3,5-Trisubstituted benzene ring.

Figure 10c. IR spectra of Compound 3 obtained from ethanolic extract of *S. wightii*

d) LC-MS spectra

1. Compound #1

Figure 10d. LC-MS spectra of Compound 1 obtained from ethanolic extract of *S. wightii*

2. Ethanol extract

D:\2009\JISC\IPC\SRK\APR\SAMPLE_1

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3. Butanol extract

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Figure 10e. LC-MS spectra of ethanolic and butanolic extract of *S. wightii*

e) NMR spectra

1 a. Compound #1(¹³C)

1 b. Compound #1 (¹H)

Figure 10f. NMR spectra of Compound 1 obtained from ethanolic extract of *S. wightii*

2 a. Compound#2(¹³C)

2 b. Compound #2 (¹H)

Figure 10g. NMR spectra of Compound #2 obtained from ethanolic extract of *S. wightii*

4.1.8 Antioxidant activity

4.1.8.1 DPPH-Free radical scavenging activity

The DPPH free radical scavenging activities of *S.wightii* extracts and BHT are presented in Table13. A solution of each extract at a concentration of 1.0 mg/ml was prepared. The activities of sample extracts were between 5.879 and 248.199 μ g/ml at 1.0 mg/ml. Most of the samples showed high antioxidant activity using DPPH in *S. wightii*. With regard to IC₅₀ values (the concentration of antioxidant required to achieve absorbance equal to 50% that of a control containing no antioxidants), Compound#1 (IC₅₀ = 5.879 $\pm$ 0.0) EtOH (IC₅₀= 8.813 $\pm$ 0.050), DE (IC₅₀= 25.483 $\pm$ 0.017) and Aqueous (IC₅₀=28.550 $\pm$ 0.055) had the highest radical-scavenging abilities, whereas CCl₄ (IC₅₀ = 248.199 $\pm$ 0.064), BuOH (IC₅₀= 98.299 $\pm$ 0.039) had the lowest radical-scavenging abilities. PE did not show any antioxidant activity (IC₅₀= undetermined). BHT showed IC₅₀ value as 33.085 $\pm$ 0.006 (Fig.11).

Figure11. DPPH-Free radical scavenging activity of *S. wightii* extracts

Table 13 DPPH- Free radical scavenging activity of *S.wightii* extracts

Extracts	IC ₅₀ (µg/ml)
PE	Undetermined
CCl ₄	25.483 ± 0.017
DE	248.199 ± 0.064
BuOH	98.299 ± 0.039
EtOH	8.813 ± 0.050
AQ	28.550 ± 0.055
Compound#1	5.879 ±0.000
BHT(Standard)	33.085 ±0.006

IC₅₀ (µg/ml): Amount required for 50% reduction of DPPH after 30 min.

Each value is mean ± standard deviation of duplicate replicate tests.

4.1.9. β- -Carotene-Linoleic acid assay

The extracts of *S. wightii* exhibited significant scavenging properties. It showed antioxidant activity of 12 mm mean zone of color retention when compared to positive control ascorbic acid. (18 mm) A PE did not exhibit any antioxidant activity compared to other extracts. The antioxidant activity of *S. wightii* extracts may be described due to the presence of flavonoids and other phenolic compounds.(Table 13)

Table 14 β- -Carotene-Linoleic acid assay of *S.wightii* extracts

Extracts	Zone of color retention (mm)
PE	-
CCl ₄	12
DE	10
BuOH	13
EtOH	13
AQ	12
Compound#1	11
Ascorbic acid(Standard)	18

Fig.12 β -Carotene-Linoleic acid assay of different extracts of *S. wightii*.

4.2. Reducing Power

Fig.13 shows the reducing power of *S.wightii* extracts as a function of their concentration. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. The presence of reducers in the test solution results in reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. The reducing power of diethyl ether extract (0.759) was excellent; at 1 mg/ml the reducing power was higher than Ascorbic acid. The reducing power of BuOH extract was (0.355) followed by compound#1(0.292), ethanol extract (0.234) and AQ (0.238).The reducing power of petroleum ether extract was (0.227) and that of CCl_4 was (0.229) which was comparatively very low. The reducing power of ascorbic acid at 1 mg/ml was 0.742.

Figure 13. Reducing power assay of different extracts of *S. wightii*

4.3 Antimicrobial activity

The antimicrobial activities in various fractions of the *Selaginella wightii* extracts were assessed by a well-diffusion assay. The results indicated variation in the antimicrobial properties of the plant fractions (Table 15). In general, the EtOH, DE and AQ extracts were more effective in inhibiting the growth of bacterial strains while in case of fungal strains BuOH was also found to be effective along with DE fraction. Surprisingly, all the extracts of *S.wightii* showed highly potential anticandidal activity.

Table15. Antimicrobial activity of *S.wightii* extracts

a) Bacterial strains

Extracts	Concentration (µg/ml)	Zone of inhibition (mm)					
		Ef	Sa	Sp	Pa	Ec	Kp
PE	50	24.5	-	-	13.4	-	-
	100	25	-	-	15	-	-
	250	28.5	-	-	14	-	-
	500	29.2	-	-	16	-	-
CCl ₄	50	-	15.5	8.75	14.5	-	-
	100	-	14.75	7.05	17	-	-
	250	-	15.50	-	16.25	-	-
	500	-	16.25	-	15.75	-	-
DE	50	32.5	-	8.5	16.25	-	13.5
	100	33.5	-	9.0	15	-	17.5
	250	26.5	-	8.75	14.5	-	15
	500	25.0	-	9.30	14.75	-	15.0
BuOH	50	12.75	-	-	16	-	10.5
	100	9.1	-	-	17.5	-	14.0
	250	9.9	-	-	14.5	-	15.0
	500	10.5	-	-	14.75	-	11.5

EtOH	50	32	-	-	15.50	11.5	-
	100	37	-	10.5	14.45	13.5	-
	250	-	-	-	15.00	13.0	-
	500	-	-	-	17.0	12.0	-
AQ	50	-	-	-	10.52	16.5	13.5
	100	-	-	-	15.50	16.0	12.75
	250	>40	-	-	15.75	16.5	13.5
	500	>42	-	-	16.00	17.5	12.0
Kanamycin	30 µg/disc	17.5	-	18	22	21	14
Gentamycin	30 µg/disc	17.5	18	-	19	14	15

Ef- *Enterococcus fecalis*; **Sa-** *Staphylococcus aureus*; **Sp-** *Streptococcus pneumonia*; **Pa-** *Pseudomonas aeruginosa*; **Ec-** *E.Coli*; **Kp-** *Klebsiella pneumonia*.

b) Fungal Strains

Extracts	Concentration (µg/ml)	Zone of inhibition (mm)					
		Ca	Cf	Cr	Nc	Afl	Afu
PE	50	23	-	-	8.5	-	-
	100	30	-	-	-	-	-
	250	30.5	-	-	-	-	-
	500	31	-	-	11.5	-	-
CCl ₄	50	29.5	-	11.5	-	-	-
	100	28.5	-	13.5	-	-	-
	250	28.75	-	-	-	-	-
	500	30	-	-	-	-	-
DE	50	26	11.5	13.5	-	-	-
	100	31	9.5	14.5	8.75	-	-
	250	29	13	14.25	12	-	-
	500	30.5	12.5	13.5	15.1	-	-

BuOH	50	26	-	13	16	-	-
	100	25	-	12.5	17.5	-	-
	250	32.5	-	11.5	14.5	-	-
	500	33.5	-	12.5	14.75	-	-
EtOH	50	30	-	-	-	-	-
	100	31	-	-	-	-	-
	250	31.5	-	-	12.6	-	-
	500	33.25	-	-	15.05	-	-
AQ	50	18.5	-	-	-	-	-
	100	20.5	-	-	10	-	-
	250	22.5	-	-	10.5	-	-
	500	24	-	-	12.1	-	-
Bavistin	30 µg/ml	22	18	20	19	40	40

Ca- *Candida albicans*; **Cr-** *Candida rhodotrula*; **Cf-** *Candida fumata*;

Nc- *Neurospora carcassae*; **Afl-** *Aspergillus flavus*; **Afu-** *Aspergillus fumigatus*.

Staphylococcus aureus (CCl₄)

Enterococcus faecalis (EtOH)

Streptococcus pneumonia (DE)

Streptococcus pneumonia (EtOH)

Pseudomonas aeruginosa (BuOH)

Pseudomonas aeruginosa (H₂O)

Figure 14a. Antibacterial activity (p-u) of various extracts of *Selaginella wightii*

Candida rhodotrula (BuOH)

Candida rhodotrula (DE)

Candida rhodotrula (Control)

Neurospora carcassae (H₂O)

Candida fumata (DE)

Candida fumata (Control)

Figure 14b. Antifungal activity (a-o) of various extracts of *Selaginella wightii*

4.4 Interactions and toxicity studies of selected Flavonoids

Hex4.5 reads protein or DNA molecules in PDB file format. In order to run a docking calculation in Hex, receptor and ligand PDB structure are loaded into the software from file pull down menu. Then the following procedure is carried out:

Ligand-Robustflavone

Step 1: This is the initial step where the protein and ligand are to be selected from file menu to load into the 3D window.

Step 2: The receptor is loaded from file menu which are saved as PDB file format.

Figure shows the scene obtained after loading the protein.

Step 3: Figure below shows the scene obtained after loading the ligand (from file menu) with the receptor and docking process.

DOCKING RESULTS

a) Ski protein

b) Bcl-2 protein

c) E7 protein

d) Braf protein

e) Erbb2 protein

f) E1a protein

g) P53 wild type protein

h) Brc protein

i) Liver cancer protein

j) EphA2 protein

Ligand- Hinokiflavone

a) Ski protein

b) Bcl-2

c) E7

d) B-raf

e) Erbb2

f) E1a

g) P53 wild type

h) Brc

i) Liver cancer

j) Epha2

Ligand- 2, 3-Dihydro-2-(4-hydroxyphenyl)-5, 6, 7, 8-tetramethoxy-4H-1-benzopyran-4-one

a) Ski

b) Bcl-2

c) E7

d) Braf

e) Erbb2

f) E1a

g) P53 wild type

h) Brc

i) Liver cancer

j) Epha2

Ligand- Amentoflavone

a) Ski protein

b) Bcl-2 protein

c) E7 Protein

d) Braf protein

e) Erbb2 protein

f) E1a protein

g) P53 wild type protein

h) Brc protein

i) **Liver cancer protein**

j) **Epha2 protein**

Ligand- Naringeninyl-(4''',O,3)-kaempferol

a) Ski

b) Bcl-2

c) E7

d) Braf

e) Erbb2

f) E1a

g) P53 wild type

h) Brc

i) Liver cancer

j) EphA2

Ligand -8''-Methylnaringeninyl-(4''', O, 3)-kaempferol

a) Ski

b) Bcl-2

c) E7

d) Braf

e) Erbb2

f) E1a

g) P53 wild type

h) Brc

i) Liver cancer

j) EphA2

Ligand- 5'', 7''-Dihydroxy-2''-phenoxychromonyl-(3''', 4')-naringenin

a) Ski

b) Bcl-2

c) E7

d) Braf

e) Erbb2

f) E1a

g) P53 wild type

h) Brc

i) Liver cancer

j) EphA2

Protein-ligand docking

Molecular docking was carried out with seven ligands belonging to flavonoids class and ten cancer related proteins. The energy minimization score is presented in Table 16. The lower energy minimization score indicates high stability of the protein –ligand complex. Considering the above into account, compounds 5, 6 and 7 have the lowest energy minimization scores.

Table 16. Energy minimization of protein-ligand docking

LIG AND S	PROTEINS									
	ENERGY MINIMIZATION SCORE (KJ/Mole)									
	Ski	Bcl-2	E7	Braf	ErbB2	E1a	P53(w)	Brc	Liver Cancer	Epha2
1	-36.28	-43.75	-40.41	-31.63	0.00	-48.75	-45.54	-34.61	-47.68	0.00
2	-36.28	-43.75	-40.41	-31.63	0.00	-48.75	-45.54	-34.61	-47.68	0.00
3	-36.28	-43.75	-40.41	-31.63	0.00	-48.75	-45.54	-34.61	-47.68	0.00
4	-36.28	-43.75	-40.41	-31.63	0.00	-48.75	-45.54	-34.61	-47.68	0.00
5	-248.28	-262.51	-253.58	-291.17	-14.35	-271.33	-248.93	-181.82	-251.76	-13.27
6	-241.58	-266.02	-249.63	-284.69	-22.27	-267.09	-245.24	-199.0	-263.57	-20.64
7	-248	-266.16	-267.03	-264.59	-15.20	-278.03	-258.5	-242.42	-255.13	-15.28

1: Robustaflavone

2: Hinokiflavone

3: 2, 3-Dihydro-2-(4-hydroxyphenyl)-5, 6, 7, 8-tetramethoxy-4H-1-benzopyran-4-one

4: Amentoflavone

5: Naringeninyl-(4'', O, 3)-kaempferol

6: 8''Methylnaringeninyl-(4'', O, 3)-kaempferol

7: 5'', 7''-Dihydroxy-2''-phenoxychromonyl-(3'', 4')-naringenin

ADMET STUDIES

The Log P values for the seven compounds were less than equal to 5. The molecular weight of all these compounds were greater than 500 except 2, 3-Dihydro-2-(4-hydroxyphenyl)-5, 6, 7, 8-tetramethoxy-4H-1-benzopyran-4-one (360.4). The number of rotatable bonds for all the compounds was in the range 9 -10. The LD₅₀ values of seven the compounds were in the range 0.5g/kg - 2.1g/kg. The fraction absorbed was 93% and Caco-2 permeability was 3×10^{-5} cm/s; volume distribution range for compounds was 7.3 -544.3.(Table 18)

Table17. ADMET studies of various compounds

Compounds	Vol Distr (L)	FA %	Caco-2 Permeability (cm/s)	LogP	LD ₅₀ (g/kg)	Mol. weight	Rotable bonds
Amentoflavone	77.8	93	3E-05	3.5	2.0	538.5	9
2'',3''-dihydrohinokiflavone	7.3	93	3E-05	4.6	2.1	540.5	9
5'',7''-Dihydroxy2''-phenoxychromonyl-(3'',4')-naringenin	179.6	93	3E-05	4.0	1.3	540.5	9
8''Methylnaringeninyl-(4'',O,3)-kaempferol	16.5	93	3E-05	5.0	0.7	554.5	10
2,3-Dihydro-2-(4-hydroxyphenyl)-5,6,7,8-tetramethoxy-4H-1-benzopyran-4-one	11.9	93	3E-05	2.8	0.5	360.4	10
Naringeninyl-(4'',O,3)-kaempferol	544.3	93	3E-05	4.6	0.5	540.5	9
Robustaflavone	-	-	-	-	-	-	-
Hinokiflavone	-	-	-	-	-	-	-

DISCUSSION

The pteridophyte *Selaginella wightii* exhibited significant antioxidant and antimicrobial activities. These activities may be due to the presence of flavonoids and phenolic compounds as its presence was validated by performing qualitative and quantitative tests.

Flavonoids are large compounds found ubiquitously in food plants and also distributed in lower non-agronomic pteridophytic plants like *Selaginella*. They occur as glycosides and contain several phenolic hydroxyl groups on their ring structure. Many flavonoids are found to be strong antioxidants capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups (Cao et.al, 1997). In present study, the flavonoids showed a significant positive correlation with the antioxidant activity of the *S. wightii* extracts. Phenols are secondary metabolites in plants and are known to possess a wide range of therapeutic uses, such as antioxidant, antimutagenic, anticarcinogenic, free radical-scavenging activities and also decrease cardiovascular complications (Yen et.al, 1993). The scavenging ability of the phenols is mainly due to the presence of hydroxyl groups. From the results obtained, it is inferred that total phenols (Table 8) and flavonoids (Table 9) were present in reasonable amounts in the whole plant of *Selaginella wightii*. Moreover, significant correlation was observed between the total phenol content and the antioxidant activity of the extracts

The β -cartenoid-Linoleic acid assay was done as preliminary step to detect the presence of antioxidant capacity of *S. wightii* extracts. It showed positive for antioxidant giving a vague idea about the % inhibition in different extracts of the whole-herb. (Table 14) That's why two alternative methods were carried out for obtaining confirmatory results. *Sesuvium portulacastrum* showed antioxidant activity of 15.9 mm mean zone of color retention (Magwa et.al, 2006).

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et.al., 1995). In this assay, the yellow color of the test solution changes to green depending on the reducing power of test specimen. The presence of reductants in the solution causes the reduction of the Fe³⁺/ferricyanide complex to the ferrous form. Therefore, Fe²⁺ can be monitored by the measurement

of the absorbance (OD value) at 700 nm (Zou et al., 2004). The reducing power of DE and BuOH extracts was significantly high among other extracts (Fig.13). This was also demonstrated (Chua et al, 1998) in case of *Cinnamomum osmophloeum* for BuOH fraction.

In case of DPPH assay, the IC₅₀ value for each extract, defined as the concentration of extract causing 50 per cent inhibition of absorbance, was determined from the curves plotted using a software (ORIGIN 6.0) and tabulated (Table 13). Since IC₅₀ is a measure of inhibitory concentration, a lower IC₅₀ value would reflect greater antioxidant activity of the sample. The present investigation revealed lower IC₅₀ value (8.8 µg/ml) in EtOH extract of *S.wightii* suggesting that the herb can be used as potential natural antioxidant. A comparison of IC₅₀ values (Table 13) indicates that extracts of herb are more potent than BHT. The methanolic extract of sorghum showed an average IC₅₀ value (5.75 µg/ml) (Kil et al, 2009). It can be well-inferred from the above three antioxidant assays that DPPH method is the most suitable method for this particular herb *S. wightii*.

In the antimicrobial experiments the MIC was found to be 50 µg/ml for *S.wightii* extracts and EtOH, DE, AQ fractions were more effective in inhibiting the growth of bacterial strains while in case of fungal strains BuOH was also found to be effective along with DE fraction. Surprisingly, all the extracts of *S.wightii* showed highly potential anticandidal activity (Table 15). In Sorghum, methanol extract only showed significant antimicrobial activity; MIC was 250 and 500 µg/ml (Kil et al, 2009) and in *Adiantum* species also methanol extract only showed potential antimicrobial activity (Singh et.al,2008) suggesting that *S.wightii* extracts can be used to target wide number of microorganisms.

Finally, interaction-toxicity studies were carried out by docking various Oncoproteins with the selected ligands-flavonoids. Out of 10 ligands chosen, only a few ligands showed good binding flexibility (Table 16) i.e. lower the energy minimization score; stable is the protein –ligand complex and all the compounds were found to be moderately toxic i.e. the LD₅₀ values of the compounds were in range 0.5 – 5 g/kg.

The experimental results indicated the presence of Phenolic compounds and flavonoids which can significantly prevent the microbial and free-radical induced diseases. The observations may be used to validate the scientific reasoning that free radical-scavenging and microbial inhibition is indeed the mode of operation of these plants in the treatment or prevention of the onset of deadly disorders like arthritis, breast cancer, atherosclerosis, urinary-tract infections, pneumonia and other microbial and fungal diseases etc. The present investigations if emphasized in-depth on biological systems can open up new avenues in the search for natural antioxidants and herbal drugs that can be employed successfully in further clinical trials.

In this present study, *S. wightii* extracts showed potential antioxidant and antimicrobial activities and bioinformatics studies highlighted a few potential flavonoids which may be used as anticancer drug. So considering this into knowledge, *Selaginella wightii* can be one of the potential medicinal herb for future research work.

CONCLUSION

- The extracts of *Selaginella wightii* were obtained by sequential extraction with six solvents and ethanol extract was subjected to column chromatography for isolation of 3 pure compounds.
- The ethanol, diethyl ether and aqueous extract possessed high antioxidant and antimicrobial activity compared to other extracts.
- All the extracts of *S.wightii* showed an excellent anticandidal activity.
- With docking and ADMET studies, a few compounds showed good binding flexibility and LD₅₀ values with different cancer-related proteins.

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