Phytochemical investigation of medicinal plant, *Selaginella bryopteris*

A dissertation submitted in
partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE
IN
CHEMISTRY

Submitted by:
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TO WHOMSOEVER IT MAY CONCERN

It is certified that **Mr. Rushil Kapur** student of **Master of Science** in **Chemistry** at **Thapar Institute of Engineering & Technology, Patiala**, has worked in the Bio-catalysis Lab., School of Chemistry and Biochemistry, T.I.E.T, Patiala from Jan 2019 to July 2019. During the period he has worked on **"Phytochemical investigation of medicinal plant, *Selaginella bryopteris*"** and submitted his thesis in partial fulfillment of the requirements for the award of degree of **Master of Science** in **Chemistry**. The work was carried out by the candidate under my supervision and guidance.

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CANDIDATE'S DECLARATION

I hereby declare that the dissertation entitled "**Phytochemical investigation of medicinal plant, *Selaginella bryopteris***" is an authentic record of my work carried out as requirements for the award of degree of **Master of science** in **chemistry** at **Thapar Institute of Engineering and Technology, Patiala** under the supervision of **Dr. N. Tejo Prakash** (Professor, School of Energy and Environment, TIET) and **Dr. Ranjana Prakash** (Professor, School of Chemistry and Biochemistry, TIET) from January, 2019 to July, 2019. No part of the matter embodied in this report has been submitted to any other university or institute for the award of any other degree.

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It is certified that the above statement made by the student is correct and true to the best of my knowledge and belief.

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ACKNOWLEDGEMENT

I would like to express my heartfelt gratitude towards the supreme almighty for keeping me in hollow of his hands, providing me nourishment and comfort during the course of my research.

With the deep sense of gratitude I express my sincere thanks to **Dr. Amjad Ali,** (Professor and Head of School of Chemistry and Biochemistry) for giving me this opportunity to work and allowed me to use various facilities in the department.

I am highly gratified for the inspiration and guidance of **Dr. N. Tejo Prakash (Professor, School of Energy and Environment, TIET)** and **Dr. Ranjana Prakash (Professor, School of Chemistry and Biochemistry, TIET).** We had a pragmatic discussion over my interest and lab specifications after that we decided the topic for my project. I am beyond thankful for their guidance and support.

Heart-warming thanks to **Mr. Santosh Kumar Rath (Research Associate, School of Chemistry and Biochemistry)** for his diligent presence through my research. He listened to me and taught me how to work in lab in a very soothing way. I am pleased that he corrected me whenever I committed any mistake during this project work. His friendly nature helped me to complete my project smoothly. Our interaction has proved to be of great assistance**..**

I would also like to express my thanks to Ph.d scholars **Ms. Amanpreet Kaur, Ms. Anmol** and my labmate **Ms. Swati Garg** for helping me during my research work.

Most of the results described in the thesis would have not possible without the help of laboratory at **IIT Ropar**.

It is by the blessing of my mother **Mrs. Bipla Kapur** and father **Mr. Arun Kapur** and grandfather **Sh. Om Parkash Kapoor** who have showered unconditional love on me, encouraged and supported in every aspect.

Lastly I express my sincere thanks to one and all those who have contributed directly and indirectly for my investigation and help throughout my educational career.

Rushil Kapur

ABBREVIATION

Technical terms

mg	Milligram
g	Gram
ml	Milliliter
kg	Kilogram
%	Percentage
ppm	parts per million
mm	millimeter

Spectral terms

IR	Infra-red
NMR	Nuclear Magnetic Resonance
UV	Ultra violet

NMR Terms

s	singlet
bs	broad singlet
d	doublet
dd	double doublet
t	triplet

Chromatographic terms:

CC	Column Chromatography
TLC	Thin layer chromatography

Solvents

Pet. Ether	Petroleum Ether (Hexane)
DCM	Dichloromethane
MeOH	Methanol
EtOAc	Ethyl Acetate
$CHCl_3$	1Chloroform

$CDCl_3$	Deuterated chloroform
H_2O	Water
D_2O	Deuterated Water

Scientific Terms

WHO	World Health Organization
SAR	Structure activity relationship
RSV	Respiratory syncytial virus
COX – 2	Cyclooxygenase – 2
NF-κB	Nuclear Factor κB
OVCAR –3	ovarian adenocarcinoma
HIV	Human immunodeficiency virus
MMP	Matrix metalloprotease

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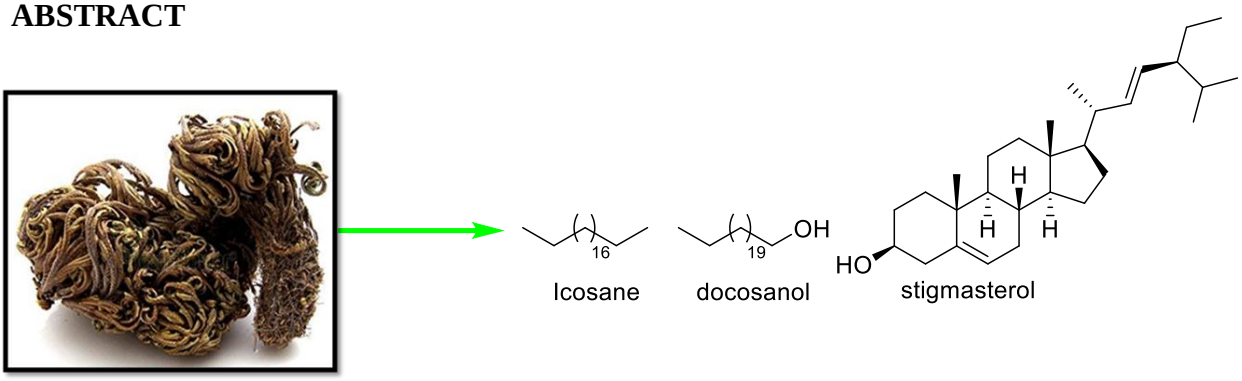
Phytochemical investigation of medicinal plant, *Selaginella bryopteris*

ABSTRACT



Selaginella bryopteris is a medicinal herb belongs to Selaginellaceae family and is considered as capable of 'resurrecting life'. It is well recognized as Sanjeevni and conventionally used for the treatment of different ailments like kidney problems, treatment of Jaundice, treatment of ulcers, delivery of pregnant women, relief from heat stroke and the burning sensation during urination, cough and cold. The present work describes the phytochemical investigation of whole plant *S. bryopteris* and resulted in the isolation of six secondary metabolites (**1-6**) of different classes, including hydrocarbon (**1**), straight chain fatty alcohol (**2**), steroids (**3**), triterpenoid (**4)**, and flavonoids (**6**). All compounds were characterised by NMR (^{1}HNMR, ^{13}C NMR).

1. INTRODUCTION

1.1. Nature and Natural Products

The word 'Natural' in broadest sense can refer to diverse variety of matrices produced by nature without the involvement of artificial or human made actions.[1] It consists of abiotic and biotic components. The biotic components are the living organisms that include humans whereas abiotic components are the non living such as water, air, soil and minerals, both being interconnected through energy flow in ecosystem. It has provided basic essentialities for existence of humans whether in terms of clothing, shelter, transportation, fragrances, flavors, food or in terms of medicines.[2]

Natural Products are the secondary metabolites derived from plants, microbes, marine and animal sources in many forms and play crucial role in the field of chemistry and medicine due to their wide range of biological activity.[3] In this connection, medicinal plants always play a vital role for the discovery of many bioactive molecules/drugs in relation to individual health care. Around 80% of the world population relies on the usage of conventional medicine which is mostly based on plant resources.[1, 3, 4]

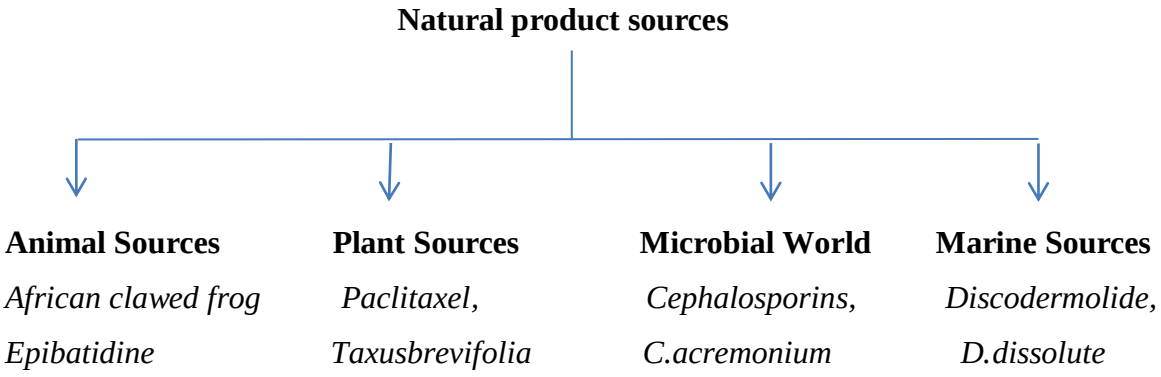
Ancient beliefs regarding medicinal practices were shared through verbal tradition or through sharing of literature from one generation to another. Herbal medicines are useful in the healing of various ailments, but these drugs have been unreasonably used and/or unscientifically exploited.[5]

The conventional medicine refers to a large range of antique natural wellbeing practices consisting folk/tribal systems such as Siddha, Ayurveda, Unani, Amchi. These therapeutic practices originated many years ago and developed progressively to a significant extent, by depending on practical experiences with no important reference to established scientific principles.[5, 6]

Almost each nation developed its own medicinal scheme, which included the traditional knowledge of people. Thus, 'Ayurveda', the Indian medical system came into existence to eradicate the major causes of diseases in concern to traditional systems.[7] Plant sources provide raw materials as crude drugs like extracts of dry herbal powder or its products for the preparation of ayurvedic medicine.[6, 7]

1.2. Sources of Natural products

The knowledge and awareness of traditional drugs has been gradually increasing, providing a disciplined, scientific explanation to natural products and their use in medicine. Researchers are engaged in developing new chemical entities from natural sources that constitute different class of compounds like glycosides, carbohydrate, lipids, tannins, alkaloids, etc. according to their chemical scaffold and biological activity as defined in modern system of medicine. Thus, it is obvious that nature will persist to be a most important source of latest structural leads. The sources of natural products can be as follows:



1.3. Role of Natural Products in drug discovery

Natural products are mainly reliable and flourishing resource for the development of drug leads. Medicinal plants are being considered as foundation for curative both bodily and divine levels, since recent times.[8] It gained augmented attractiveness in balancing resource of nutrients along with conventional medicine in ecosystem. Since olden time, uses of medicinal plants were listed in many archeological records from diverse organizations around the earth.[4, 8] For example, the primitive recognized therapeutic document of medicinal plants by Sumerians which was discovered 5000 years ago and helps in curing many illnesses.[9] Garlic (*Allium sativum*) was used by olden Egyptians for curing circulatory and cardiac disorders.[8]

Natural products are being verified to develop new leads for pharmaceutical, agrochemical and nutraceutical industries. Common sources to discover biologically active molecule are natural products which include plants, bacteria, fungus and marine. The World Health Organization (WHO) has projected that just about 65% of the world's resident's relay mostly on medicines for their good health are derived from plants. Most of the conventional drugs are plant-derived in

countries having WHO- Traditional Medicine Centers. From 122 chemical compounds, 80% were recognized for ethno medical reason which were resultant from 94 plant variety.[10]

Keeping in view the state and prospect trends of herbal drugs, the spotlight is to execute wide R&D effort on prospecting biodiversity used for its chemistry to isolate photochemical, activity guided extract, fractions and particular chemical molecules for the progress of identical and worth assured herbal drugs and formulation. Brief natural product drug developmental stages are mentioned in **(Fig 1.)** [11]

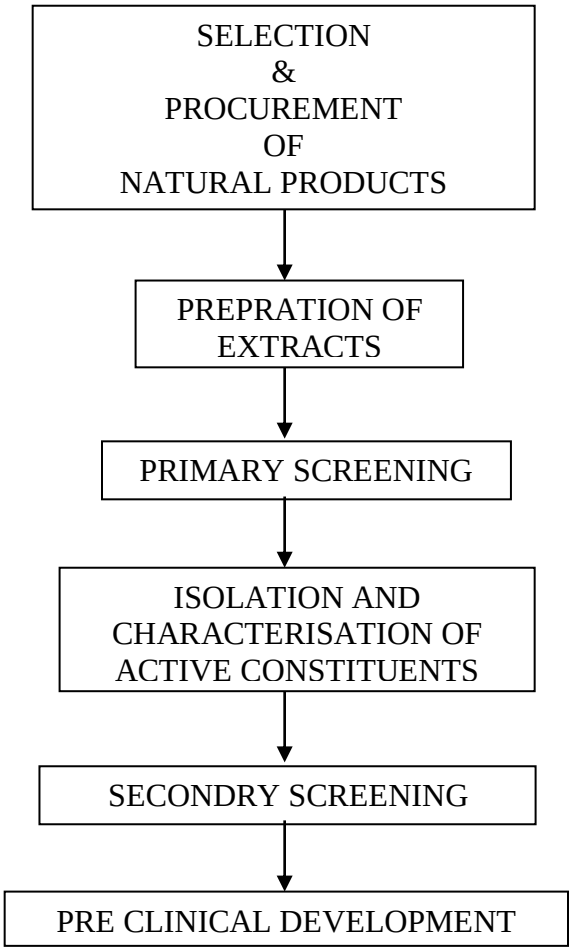


Fig 1. Discovery of preclinical drug developmental stages from natural products

1.4. Natural products as drugs

The ideal drug candidates or marketed drugs or formulations are very few in number as natural isolates in drug discovery programs. Still, many natural isolates or leads are providing basic ideas relating to structure activity relationship (SAR) studies for the development of mimics to many natural products. To minimize the severe side effects and improve the immense benefits of

natural isolates, it is thereby widely required for the synthesis of natural product analogues and their high throughout screening for specific target drug discovery. There are several natural product isolates like Taxol[12](**1**. anticancer), Papaverine[13](**2**. vasodilator), Vincristine[12](**3**.anticancer), Vinblstin[12](**4**. Anticancer), Cinchonine[13](**5**.antimaleria),Quinine[14](**6**. antimaleria), Artemisinin[15, 16](**7**. antimalarial), Lovastatin[17](**8**. Cholesterol lowering, cardiovascular) were used as drugs. **(Fig 2.)**

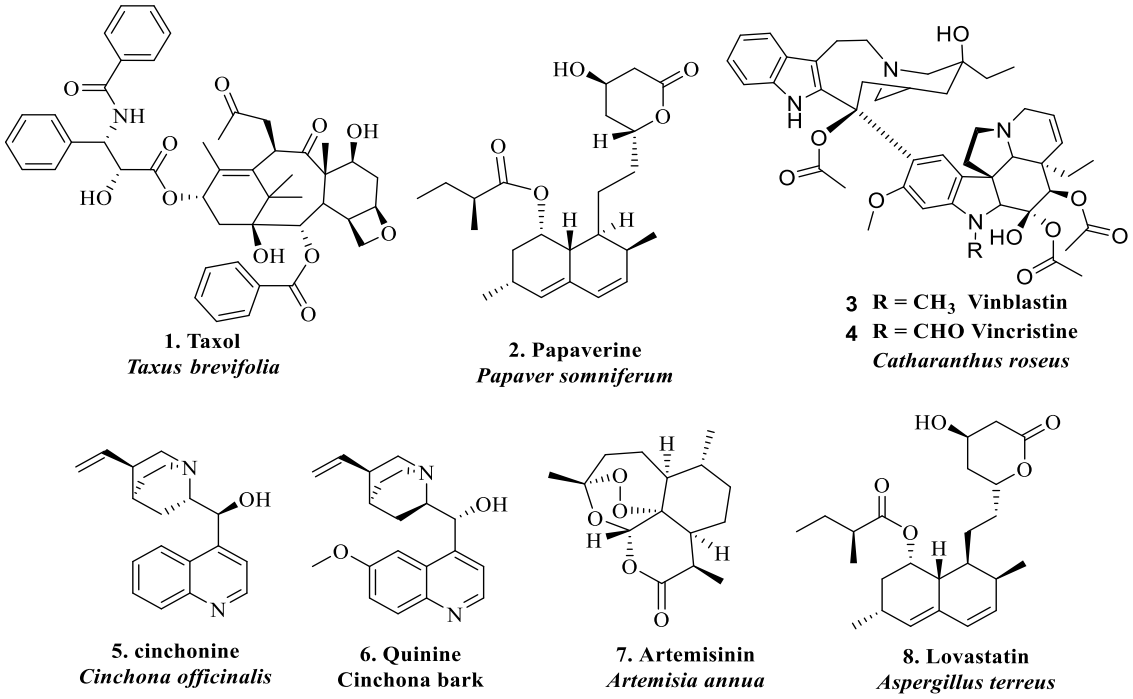


Fig 2. Examples of natural products as drugs

1.5. Semi-synthetic analogues of Natural Products as drugs

Human diseases were treated by using chemical substances resulting from plants, animals and microbes. Podophyllotoxin (**9**) is a naturally occurring antimitotic agent isolated from different plant species especially *Podophyllum peltatum*, *Podophyllum hexandrum* etc. It is used as a lead potential anti-cancer agent and many of its derivatives are found more potent. Two of its synthetic derivatives, Etoposide (**10**) and teniposide (**11**),used as drug in chemotherapeutic medication.[18] (**Fig 3.)**

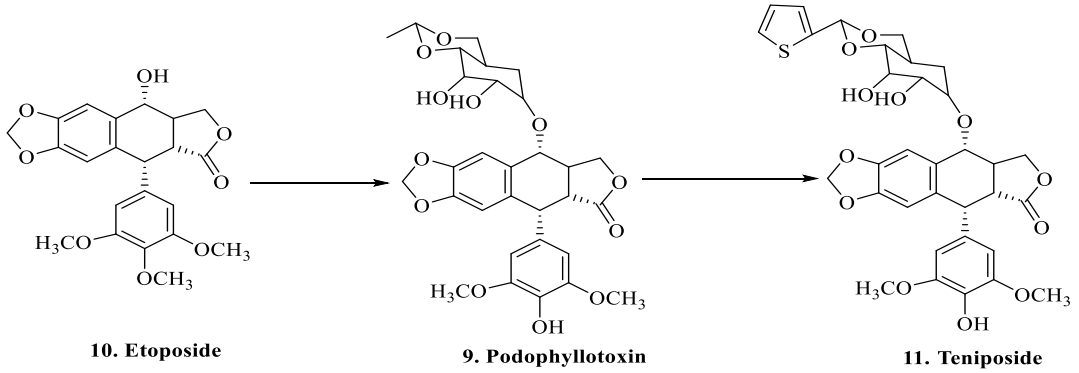


Fig 3. Podophyllotoxin analogues etoposide and teniposide

Pharmacologically active natural products

Isolated pure natural compounds have shown various pharmacological activities. Some examples are mentioned in **Table 1**.

Hepatoprotective activity[19]	Gomosin, Glycyrrhizin, Kutkoside, Picroside, Silybin
Antihyperlipidemic activity[20, 21]	Catechin, E- & Z-Guggulsterone
Anti-inflammatory activity[22]	Boswellic acids, , Curcumin, E- & Z-Guggulsterone
Antiulcer activity[23]	Glycyrrhizic acid,Curcumin, Glycyrrhetinicacid
Antiprotozoals[24, 25]	Eflornithine, Berberine,Conessine, Plumbagin, Lapachol, Gossypol
Anti-HIV activity[26]	Zidovudine, Isolicoflavonol, Glycyrrhizoflavone
Anticancer activity[12]	Vincristine, Cucurbitacin E, Combretastatin, Podophyllotoxin, wikstromol
Bronchodilators[27]	Salmeterol, Berberine, Vasicinone, Piperine, Theophylline, Formoterol
Antidiabetic activity[28]	Quercetin, Alliin, Silymarin, Rutin, Allicin, Gymnemic acid
Cardiotonic activity[29]	Lanatosides A,B,C,D , 10-Gingerol, ; Acetyldigitoxin, Arjunolic acid

Table 1. Pharmacologically active natural compounds

Keep in view the diversity, bioactivity and therapeutic value of natural products, the present study is focused on isolation and characterization of various compounds from *Selaginella bryopteris*, a well known herbal medicine mentioned in ancient literature of medicine.

2. LITERATURE REVIEW

2.1 . Pharmacognostic description of *Selaginella bryopteris*[30]

Plant Profile[30, 31]

- **Plant** : *Selaginella bryopteris*
- **Synonyms** : *Selaginella imbricata* (Roxb.) Scott
  Selaginella circinale (L.) Spring
  Lycopodium bryopteris (L.) Kuntze
  Lycopodium circinails (L.) Spring
  Lycopodium bryopteris (L.)
  Stachygynandrum bryopteris Beauv.
  Stachygynandrum circinale (L.) Beauv.
- **Botanical name** : *Selaginella bryopteris*
- **Common names** : Sanjeevani, Sanjiwani Booti, Lakshanam Booti, Mrit-sanjeevani

TaxonomicClassification[30, 31]

- **Kingdom** : Plantae
- **Superdivision** : Embryophyta
- **Division** : Lycopodiophyta
- **Class** : Isoetopsida
- **Order** : Selaginellales
- **Family** : Selaginellaceae
- **Genus** : *Selaginella*
- **Species** : *S. bryopteris*

Selaginella bryopteris belongs to Selaginellaceae family and is identified as herb which is considered as capable of 'resurrecting life'. The most mystifying and miracle herb recognized as Sanjeevni and is mentioned in the famous Indian epic "Ramayana" by the Hindi poet and literate Tulsidas**.**[31, 32] Selaginellas are one of the first vascular plants on earth which is believed to be in existence since 300 million years ago.[33] *Selaginella bryopteris* are also identified as spike moss and grow in tropical areas on rock crevices ranging from Arawali mountain terrains from east to west and the hills of South India.[33] Sanjeevani booti (*S.bryopteris*) is a lithophytes pteridophytic plant that favors shaded and moist places and feed off moss, their own dead tissue and nutrients in rain water resulting in abundant growth of this plant. These species are proved to show some special properties such as draught resistance.[34, 35] When dry Sanjeevni plants are kept in water, they unfold their fronds, changes to green leaves and become active.

Various species of *Selaginella* are being used as potential plant medicines.[36] This practice of using dry plants for making medicine for betterment of human health complications is in existence since centuries mainly by tribal people of India.[37]

Effective uses of *S.bryopteris* are: [33, 35]

- In the treatment of jaundice
- Restoring menstrual irregularities to normal
- Relief from heat stroke and the burning sensation during urination
- Helping in easy delivery of pregnant women by minimizing the labor pains

2.2. Main Active Compounds in *Selaginella*

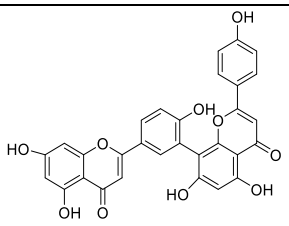
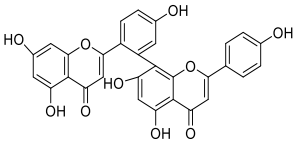
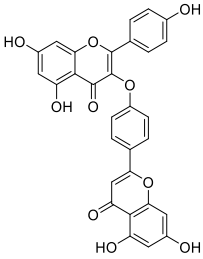
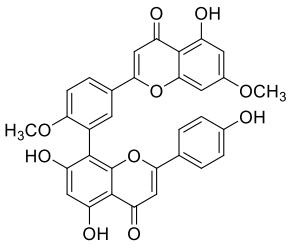
Selaginella is an effective medicinal herb, which contains different type of natural products such as phenolic (flavonoid), alkaloid and terpenoid. Biflavonoid, is a type of flavonoid which exists in dimeric form, is the most precious group of natural products in *Selaginella* species. There are 13 compounds reported from *S. bryopteris*, namely- amentoflavone **(12)**, 2',8''-biapigenin **(13)**, ginkgetin **(14)**, delicaflavone **(15)**, heveaflavone **(16)**, ochnaflavone **(17)**, hinokiflavone **(18)**, isocryptomerin **(19)**, kayaflavone **(20)** , podocarpusflavone A **(21)** , robustaflavone **(22),** taiwaniaflavone **(23)** , and sumaflavone **(24)**.[37]Earlier studies on the constituents of *Selaginella* lead to invention of several compounds, biflavonoids, the major secondary metabolite of *Selaginella*.[37, 38]

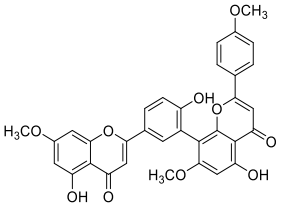
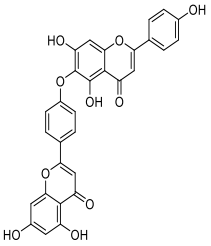
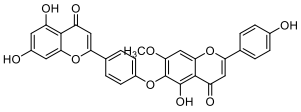
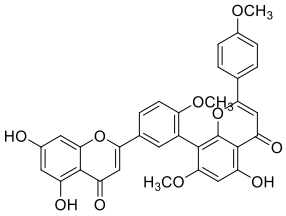
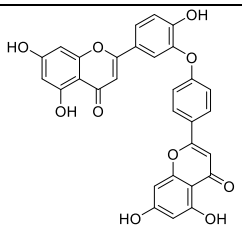
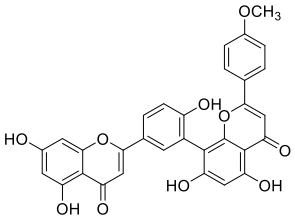
Bi et al. (2004) [39], reported that water extract of *S.tamariscina* has many natural products such as sygringaresinol, syringic acid, caffeic acid, ferulic acid, vanillic acid, arbutin, umbelliferone, tamariscinoside A, tamariscinoside B, guanosine, tamariscinoside C,tyrosine, adenosine D-mannitol and shikimic acid.[40-43] **Cheng et al. (2008)**[44] examined the whole herbs of *S.tamariscina* in EtOH extract and fractioned it in ethyl acetate and chloroform and contains selaginellin A and selaginellin B.[44] The major compounds of *S.tamariscina* are amentoflavone, hinokiflavone, isocryptomerin, bilobetin, robustaflavone and an apigenin diglucoside.[45] Sterol is found in numerous spices of *Selaginella*.[46] Three new sterols were isolated from *S.tamariscina* and reported as anticancer activity by inhibiting the growth of human leukemia Hl- 60 cells.[47] Ecdysteroid a type of steroid found in species of *S.moellendorffii*, *S.nipponcia*, *S.pachystachys*, *S.stauntoniana*, *S.remotifolia*, *S.tamariscina*, *S.uncinata*, *S.deliculata*, *S.doederleinii*.[48] **Perez et al. (1994)** examined *S.lepidophylla* methanolic extract and isolated 3-methylenhydroxy-5-methoxy-2, 4-dihydroxy tetrahydro-furane, which further observed inhibiting the uterus contraction during the bio evaluation study.[49]

Acetone extract of *S.sinensis* was found to contain selaginellin A.[50] An investigation on *Selaginella sinensis* reported a glycoside selaginoside[51], a sesquilignan sinensiol A[51], secolignans named styraxlignolide D and neolloydosin.[52]

Zheng et al. **(2008)**[53] reported that ethanolic extract of *S.uncinata* consists of flavonoids that have benzoic acid substituent.Two new chromone glycoside named uncinoside A and uncinoside B were isolated and have shownantiviral activities against PIV-3 and RSV.[54, 55]*S. doederleinii* consists of numerous phenolic compounds such as (+)-matairesinol, (-)-matairesinol, (-)-lirioresinol A, (-)-lirioresinol B, (-)-nortracheloside, (+)-nortrachelogenin, (+) - syringaresinol, (+)-wikstromol. S. doederleinii consists of a glycosidic hordenine which results in increase of hypertension.[56, 57] **Tan et al. (2009)** examined *S. labordei* and reported that it consists of 4'-methylether robustaflavone, eriodictyol, robustaflavone, and amentoflavone.[58] Various natural products, other than biflavonoid and trehalose, have various molecular properties that are of economical value and can improve human health. Natural products extracted from *Selaginella* may vary depending on location, climate, soil factor, harvesting and on procedure used for extraction. Isolated compounds from *S. bryopteris* are given in **Table 2.** from thorough literature search.

2.3. Biological Activity

S.No	Reported Flavonoids	Biological Activity	Structure
12	Amentoflavone	It acts as antioxidant,[59-61] anti cancer,[62, 63]anti-inflammatory,[64, 65]antimicrobial[66], anti depressant[67], anti-angiogenesis[68] and Antiviral activity against influenza, herpes, and respiratory syncytial virus (RSV)[69]	
13	2',8''-biapigenin	It shows anti-inflammatory and anticancer activity, reduces transactivation of iNOS gene and cyclooxigenase-2 (COX-2) by inactivating nuclear factor-κB (NF-κB).[70]	
14	Delicaflavone	Its bioactivity is not observed yet from *Selaginella*.	
15	Ginkgetin	It has properties of antiprotozoal[71], It has strong effect on cervical carcinoma (HeLa), ovarian adenocarcinoma (OVCAR-3), and foreskin fibroblast (FS-5)[72]. It inhibits cancer[73], and it can replace caffeine in food-stuff and medicines without generating addiction.[74]	

16	Heveaflavone.	It shows cytotoxic activity against murine cancer cells of L 929.[56]	
17	Hinokiflavone	It exhibits antioxidant activity.[35] It shows antiprotozoan activity against *Leishmania donovani* and *Plasmodium falciparum*.[75]	
18	Isocryptomerin.	It has cytotoxic activity against various cancer cells, including P-388 and HT-2.[38] It has antibacterial activity[76] and also has antifungal properties, which can depolarize fungal plasma membrane of *Candida albicans*.[68]	
19	Kayaflavone	It has anticancer property.	
20	Ochnaflavone	Ochnaflavone derivatives may have antioxidant activity that inhibits expression of gene COX-2 at colon cancer cell.	
21	Podocarpusflavone A	It has antioxidant properties.[61]	

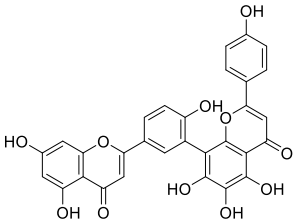
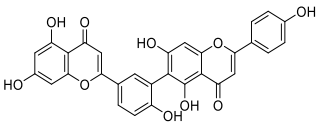
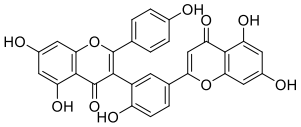
22	Robustaflavone	It has anti cancer and antivirus properties and is significantly cytotoxic to various cancer cells[62] and inhibits tumor cell of Raji and Calu-1.[63] It also has antiviral properties, which indicate high resistance topolymerase HIV-1 RTASE[77] and influenza virus (A,B), hepatitis (B), human immunodeficiency virus (HIV-1).	
23	Sumaflavone	It has anti-inflammatory property that helps to reduce production of NO, by blocking lipopolysaccharide formation that induces iNOS gene expression.[74]It inhibits the ability of UV irradiation to stimulate the activity of matrix metalloprotease-1 and -2 (MMP-1 and 2) at fibroblast of primary human skin.[38]	
24	Taiwaniaflavone	It shows anti-inflammatory[38]	

Table 2. Biological Activities of reported Flavonoids

3. AIM AND OBJECTIVES

3.1. Objectives of the present work

The aim of the present study was to carry out the phytochemical investigation of *S. bryopteris*. The objectives are:

- ❖ Extraction, Isolation and purification of natural products from the selected extracts/fractions
- ❖ Characterization of isolated molecules using various spectroscopic techniques, like NMR (^{1}HNMR, ^{13}C NMR)

3.2. Need for the study

With this entire thorough literature search, it was observed that there is different class of 13 compounds reported in *S. bryopteris*. All these compounds were found active against different diseases like cancer, bacterial/microbial infections, inflammations, HIV etc. Hence the species *S. bryopteris* is selected for further research in order to explore some new class of bioactive compounds using column chromatography technique.

4. MATERIAL AND METHODS

4.1. Extraction, fractionation and isolation of chemical constituents

4.1.1. Extract preparation

The coarsely powdered whole plant of *Seligenella bryopteris* was extracted with Dichloromethane and methanol. The method of preparation of organic extract was followed as per the NCI protocol[78].**(Figure 4.)**

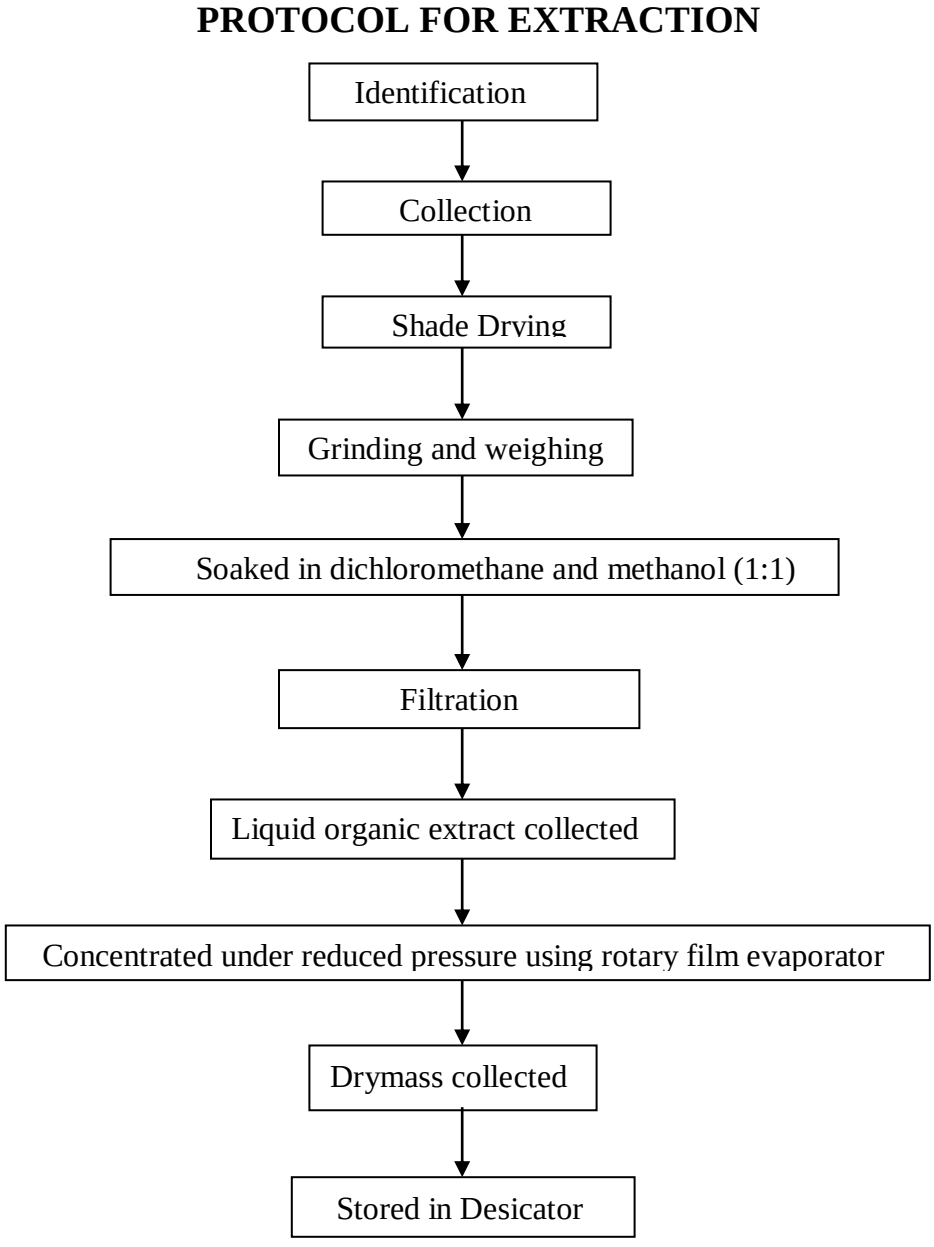


Fig 4. Extraction as per NCI protocol[78]

4.1.2. Dichloromethane and methanol extraction

Shade covered dry plant of *Selaginella bryopteris* was ground to coarse powder. The coarsely powdered plant (100g) was packed in a percolator, soaked in (1:1) dichloromethane (DCM) and methanol was kept overnight. The extract was drained, filtered and concentrated under reduced pressure using rotary film evaporator. The extraction process was repeated three times more under similar conditions. The combined extract was finally dried in vacuum desiccator and weighed.

TLC of the collected organic extract was taken initially to observe the compounds visible on the plate. Approximately more than 10 compounds were visible on the TLC plate in initial observations. With this observation, another 500 g of coarsely powdered plant was taken and above mentioned procedure was followed to isolate compounds.

Weight of plant material taken	:	600g
Weight of extract formed	:	46 g

4.2 . Column chromatography of DCM and MeOH extract

4.1.3. Isolation of Markers

Compounds were isolated from the dichloromethane and methanol extract by column chromatography and fractions were monitored on TLC.

4.1.4. Slurry Formation

Dried crude organic extract (amount 45g) was taken and dissolved in the minimum quantity of dichloromethane and then adsorbed on weighed quantity (180g) of silica gel, to get free flowing material.

4.1.5. Packing of column

A clean and dried column was taken and a cotton plug was put at the base the column. Solvent (petroleum ether) was poured into the column and packed with slurry of silica gel, (100-120 mesh) prepared by suspending it into the solvent. The adsorbed extract was then charged into the column.

4.1.6. Elution of the column and isolation of compounds

The column was first eluted with petroleum ether. Then column was eluted with the solvent by gradually increasing the percentage of ethyl acetate in petroleum ether. Four fractions of eluted

solvent were collected in 250 ml conical flask using 100% petroleum ether followed by gradual increase in polarity of mobile phase from 2% EtOAc/ petroleum ether to 60% EtOAc/ petroleum ether. Total 330 fractions were collected with gradual rise in the polarity.

4.1.7. TLC system for organic extract:

- Sample : Dichloromethane and methanol fraction
- Stationary phase : Silica Gel 100 – 200 Mesh
- Mobile phase- Ethyl acetate : Pet.Ether (4:6) and Methanol : Dichloromethane (0.5 : 9.5)
- Visualizing system: Cerric ammonium sulfate

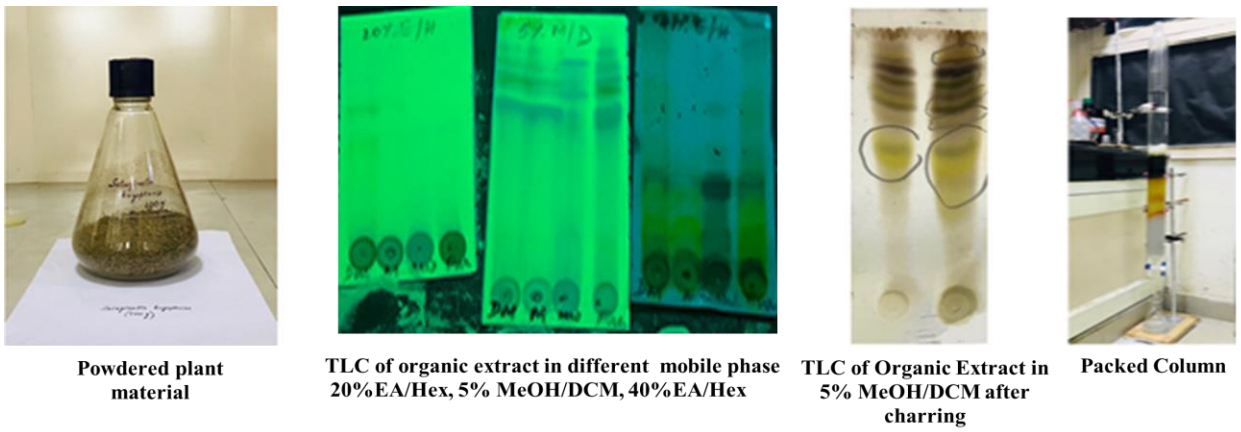


Fig 5. Packing of column and TLC optimization of *S. bryopteris*

4.1.8. Characterization techniques

All the solvents were obtained from Rankhem and were distilled prior to use to perform the present work. The purified compounds were monitored by TLC on 0.25 mm silica gel 60 F254 plates (E. Merck) visualized using UV illumination and 2% ceric ammonium sulfate solution as spraying reagents for detection of the spots on the TLC. Confirmation of compounds was initially done by column chromatography using Silica gel 100-200 mesh stationary phase. ^{1}H NMR and ^{13}C NMR spectra were recorded on JEOL (400 MHz; JEOL JNM-ECS 400) instrument using $CDCl_3$ as the solvent with TMS as internal standard. The chemical shifts were represented in δ ppm and coupling constants in Hertz. The abbreviations used are as follows: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; m,multiplet. The spectral data are consistent with the assigned structures. A high-resolution mass spectrum (HRMS) and MS was recorded on Agilent Technologies 6540 instrument.

5. RESULT AND DISCUSSION

5.1 . Isolation and visualization of natural products

The plant *S. bryopteris* was collected from costal region of Odisha (India). The whole plant was shade dried, powdered and extracted with CH_2Cl_2: MeOH. The concentrated CH_2Cl_2: MeOH (1:1) extract was subjected to column chromatography over silica gel. With the gradual increase in the ethyl acetate (%) in petroleum ether while running the column, resulted in seven pure compounds and with rest being mixtures of compounds. Total of 330 fractions were collected and seven pure compounds were identified which were observed to belong to different class of compound such as long straight chain hydrocarbons, steroids, terpenoids and coumarins. The details of isolated mixture and pure compounds were mentioned in **Table 3**.

S.No.	No. of fractions eluted	Column solvent system	Compounds isolated	TLC of Compounds
1	1 - 4	Petroleum ether	PURE 1 **Icosane/Eicosane**	
2	5 - 29	2% EtOAc/ Petroleum ether	Mixture	
3	30 - 40	5% EtOAc/ Petroleum ether	Mixture	
4	41 - 50	5% EtOAc/ Petroleum ether	Mixture	
5	51 - 80	5% EtOAc/ Petroleum ether	PURE 2 **Docosanol**	

6	80 - 103	5 % EtOAc/ Petroleum ether	PURE 3 **Stigmasterol**	
7	104 - 106	12% EtOAc/ Petroleum ether	Mixture	
8	107 - 130	15% EtOAc/ Petroleum ether	Mixture	
9	130 - 150	15% EtOAc/ Petroleum ether	Mixture	
10	150 - 189	15% EtOAc/ Petroleum ether	Mixture	
11	190 - 202	25% EtOAc/ Petroleum ether	Mixture	
12	203 - 250	40% EtOAc/ Petroleum ether	PURE 4 Unknown	
13	251 - 275	40% EtOAc/ Petroleum ether	PURE 5 Flavonoid	
14	276 - 300	50% EtOAc/ Petroleum ether	Mixture	
15	301 - 330	60% EtOAc/ Petroleum ether	PURE 6	

Table 3. Collection of fractions from dichloromethane and methanol extract

5.2. Isolation of compounds:

By using repeated column chromatography, 6 compounds have been isolated and ^{1}H NMR was performed for 3 of these compounds.

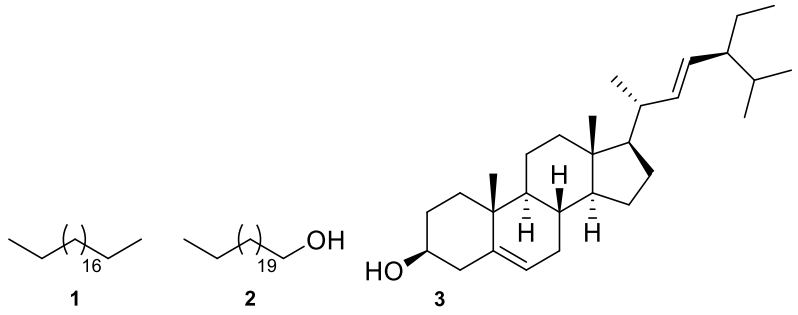
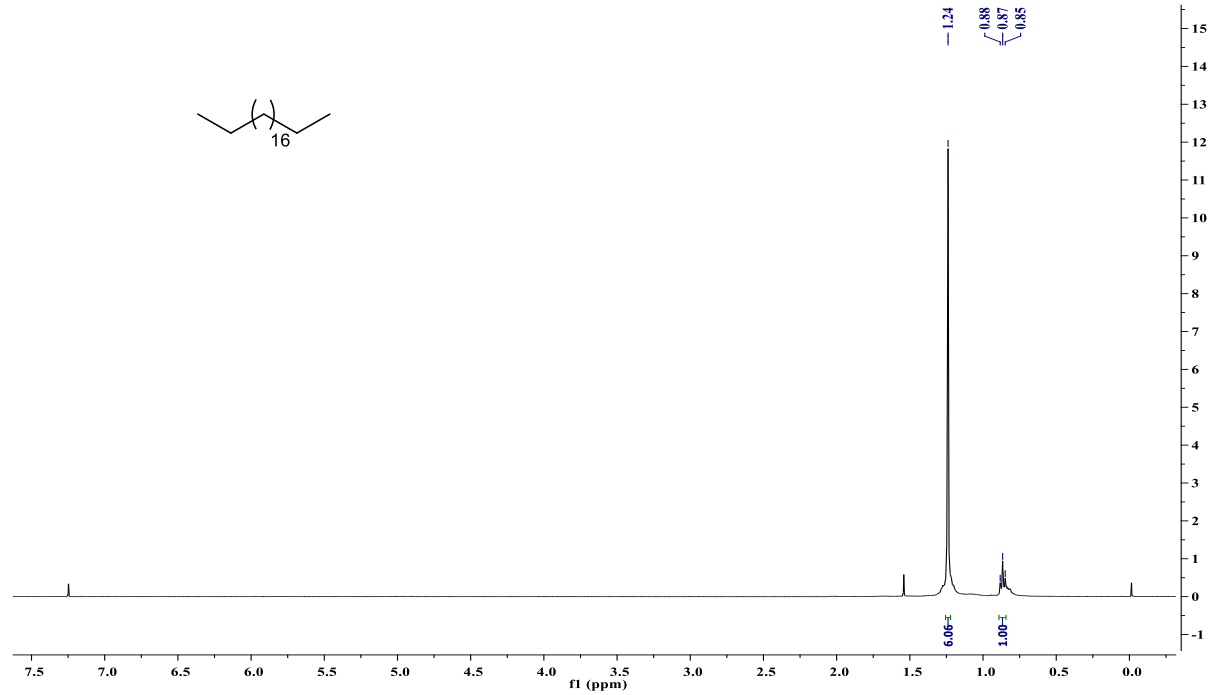
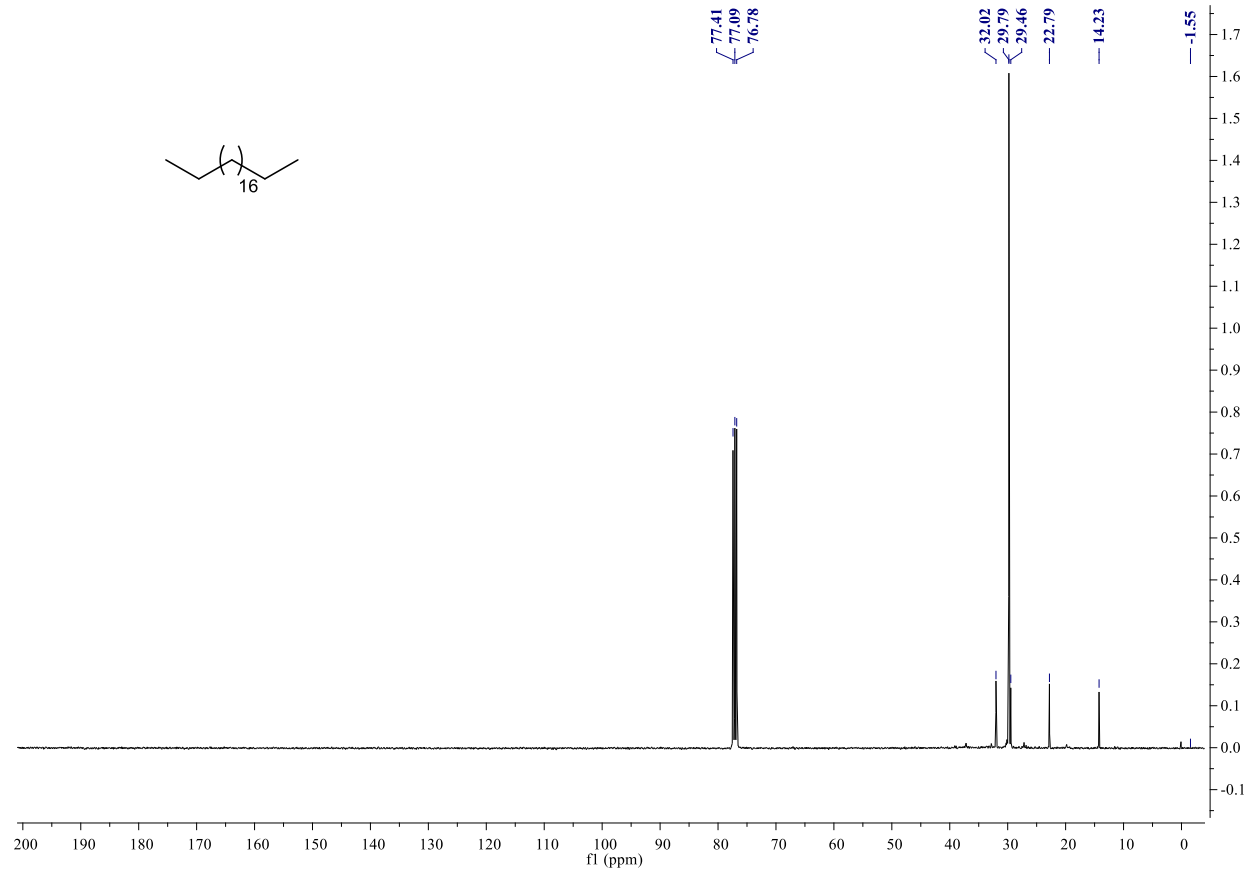


Fig 6. Structure of isolated compounds from *S. bryopteris*

Compound 1 (Icosane/Eicosane)

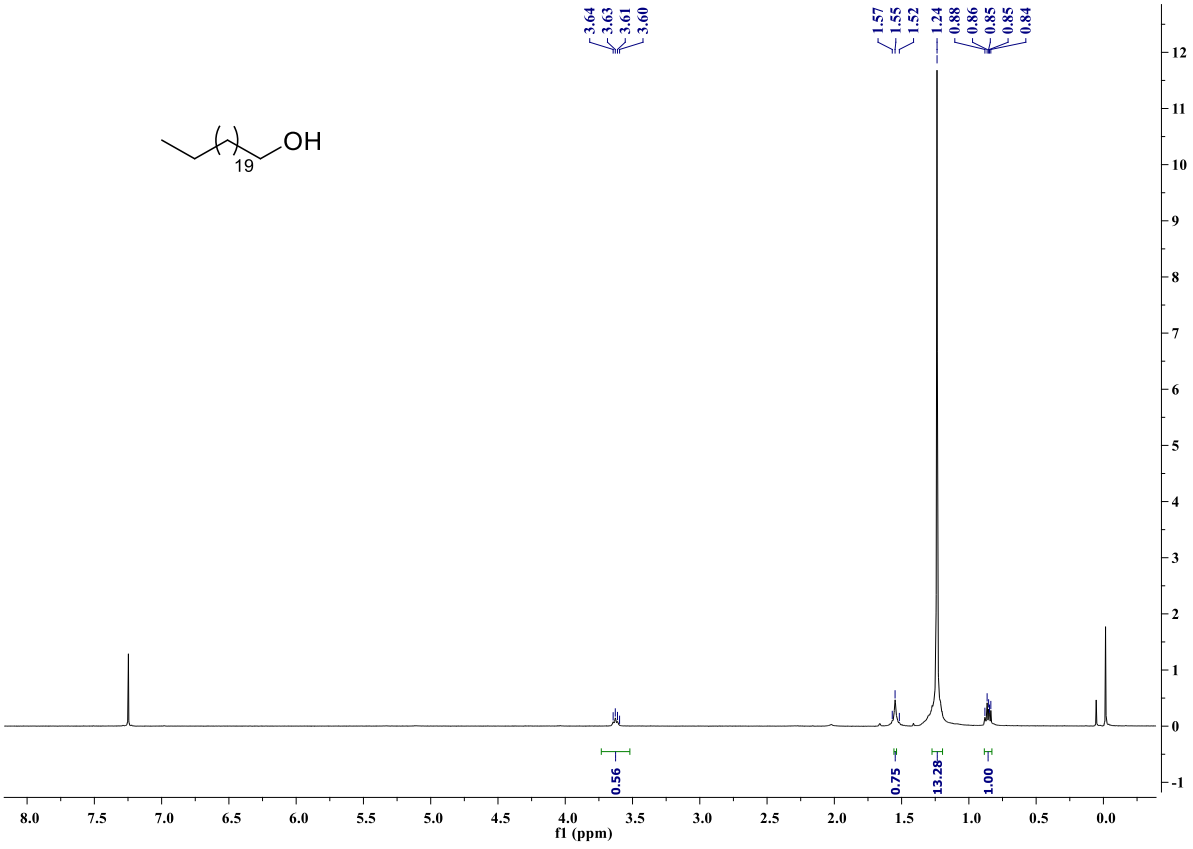
Compound **1** was identified as a long chain hydrocarbon from the ^{1}H NMR analysis. Compound **1** is white crystalline solid found to be icosane also called as eicosane ($C_{20}H_{42}$), and further final confirmation was done by ^{13}C NMR result. From the ^{1}H NMR analysis, the multiplet was found at 0.87 which signifies two CH_3 (ie. 6 protons) at the terminal end of the compound and the singlet at 1.24 signifies 36 protons ie. 18 numbers of CH_2 observed within the compound.^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (s, 36H), 0.87 (m, 6H). The ^{13}C NMR (100 MHz, CHLOROFORM-D) δ 14.12 ($2CH_3$), 22.79 (CH_2), 29.40 (CH_2), 29.79 (CH_2), 32.02 (CH_2) confirmed the compound as icosane. Icosane is a bioactive compound was identified from *Streptomyces* stain KX852460 and found as antifungal compound when screened against the *R. solani* AG-3 which is causative agent of target spot disease in tobacco.[79]

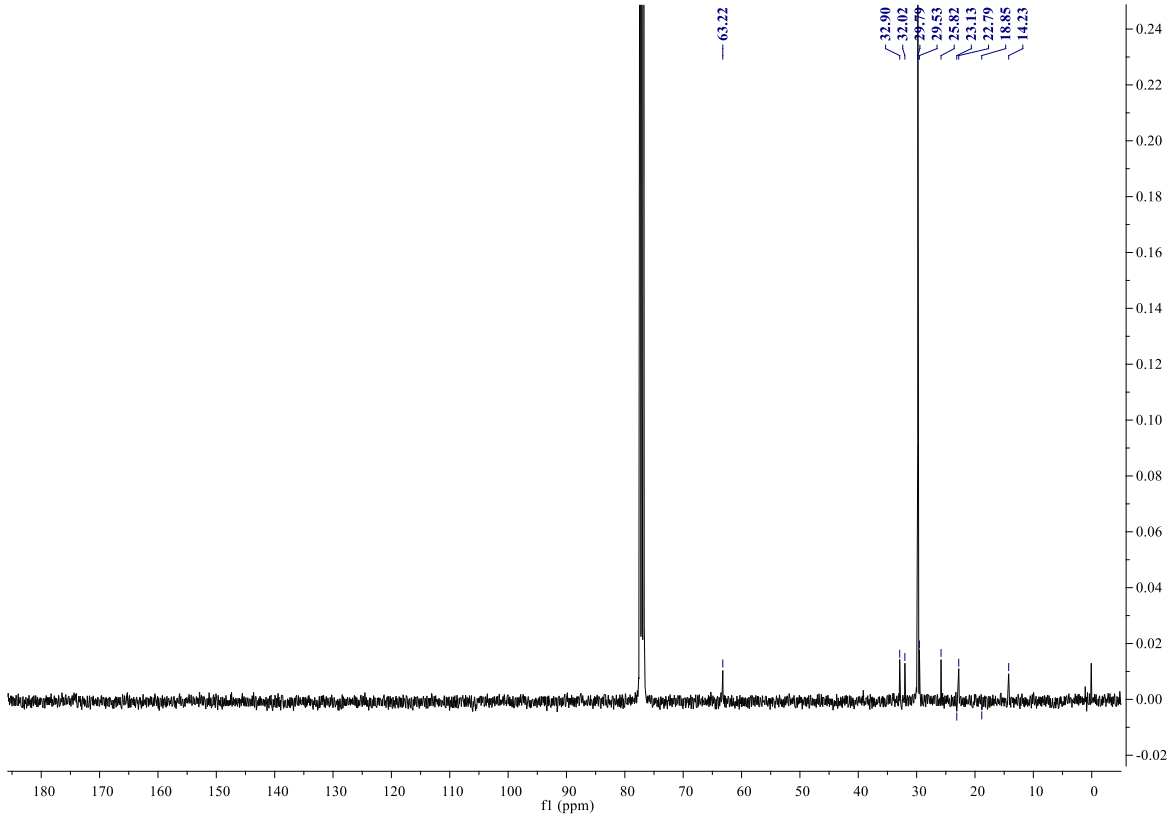

16
1.24
0.88
0.87
0.85
6.06
1.00
f1 (ppm)


16
77.41
77.09
76.78
32.02
29.79
29.46
22.79
14.23
-1.55
f1 (ppm)

Compound 2 (Docosanol/ behenyl alcohol)

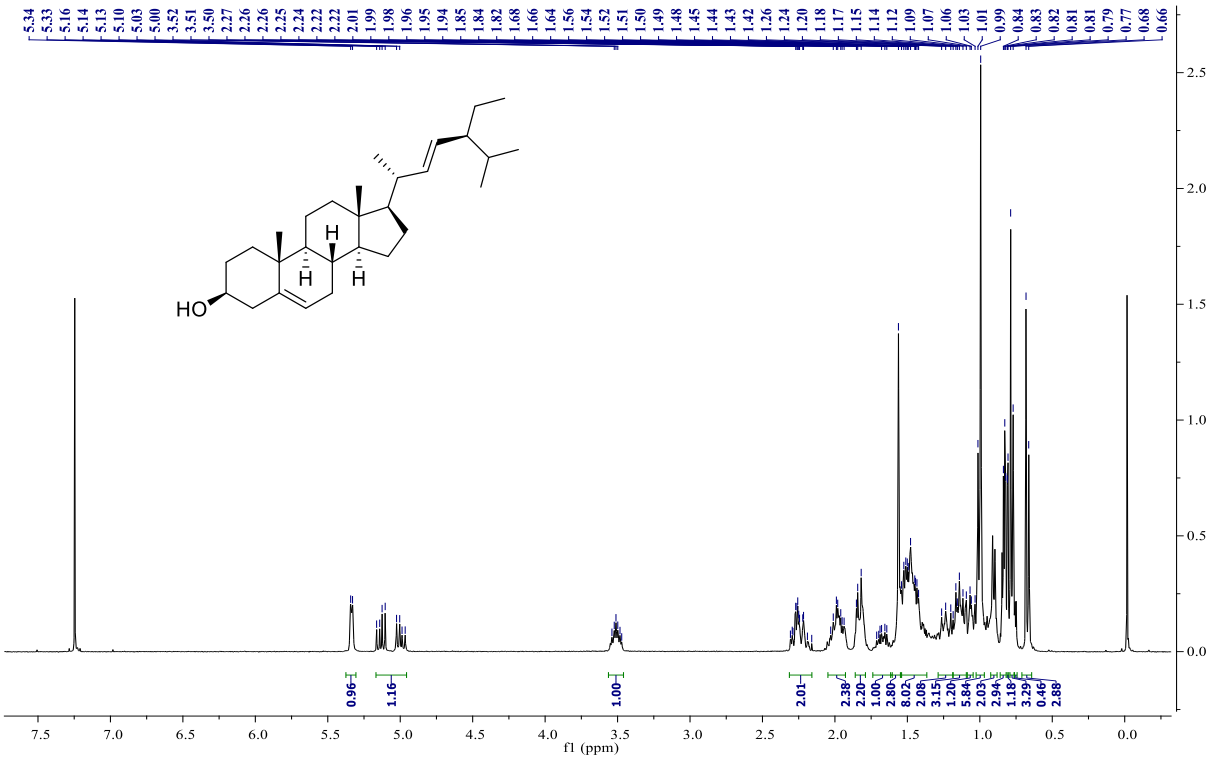
Compound **2** was identified as a long chain hydrocarbon from the ^{1}H NMR analysis. Compound **2** is white crystalline solid found to be docosanol ($C_{22}H_{44}O_2$), and later final confirmation was done by ^{13}C NMR result. From the ^{1}H NMR analysis, the multiplet was found at 0.85 which signifies one CH_3 (ie. 3 protons) at the terminal end of the compound and the singlet at 1.24 signifies 40 protons ie. 20 numbers of CH_2 observed within the compound, while one CH_2 (ie. 2 protons) at 3.63 signifies the CH_2 adjacent to the terminal alcohol group and its next to methylene proton observed at 1.55.^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62 (m, 1H), 1.56 (m, 2H), 1.24 (s, 40H), 0.85 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 63.22, 32.90, 32.02, 29.79, 29.53, 25.82, 23.13, 22.79, 18.85, 14.23.This long chain saturated fatty acid was reported from ethyl acetate extract of stem bark of *A. adianthifolia* and leaves of *Loropetalum chinense*.[80] This compound is being used as ingredient in hair conditioners, lubricating oil and anti-foaming agent in detergents.[80]





Compound 3 (Stigmasterol)

Compound **3** was identified as a steroid from the ^{1}H NMR analysis. Compound **3** is white needle type crystal and the ^{13}C NMR result is being awaited for further confirmation of the compound. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.34 (d, *J* = 5.1 Hz, 1H), 5.06 (ddd, *J* = 55.1, 15.2, 8.6 Hz, 1H), 3.50 (m, 1H), 2.27 (m, 2H), 2.01(m, 2H), 1.98 (m, 2H), 1.70 (m, 1H), 1.56 (s, 3H), 1.54 (m, 2H), 1.52 (m, 2H), 1.49 (m, 2H), 1.44 (m, 2H), 1.24 (m, 2H), 1.17 (m, 3H), 1.07 (m, 1H), 0.99 (d, *J* = 7.6 Hz, 6H), 0.90 (d, *J* = 6.4 Hz, 2H), 0.83 (m, 3H), 0.81 (d, *J* = 1.7 Hz, 1H), 0.79 (d, *J* = 6.9 Hz, 3H), 0.75 (d, *J* = 2.8 Hz, 1H), 0.67 (d, *J* = 7.4 Hz, 3H).


HO
H
H
H
f1 (ppm)

6. CONCLUSION

Phytochemical investigation of *Selaginella bryopteris* resulted in the isolation of six pure compounds belonging to hydrocarbon, fattyacid, terpenoid and flavonoid class of secondary metabolites. The pure compounds were initially confirmed by TLC analysis and further identified and characterizedby^{1}H NMR spectroscopic technique. The three characterized pure compounds are eicosane and docosanol and Stigmasterol. The structures of these two compounds were extensively analyzed by their literature NMR spectral results matching with ^{1}H NMR ande ^{13}C NMR spectra.

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