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Research Article

EXTRACTION, ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPONENTS DERIVED FROM WHOLE PLANT OF *EQUISETUM RAMOSISSIMUM* DESF

Abhilasha Jain*, Savita Shrivastava

Department of Chemistry, Govt. Maharani Laxmi Bai Girls P.G. Autonomous College, Kamla Park Rd, Professors Colony, Bhopal, Madhya Pradesh, India

**Corresponding author: aajain1012@gmail.com*

ABSTRACT

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, β-sitosterol is a phytosterol, widely distributed throughout the plant kingdom and known to be involved in the stabilization of cell membranes. The aim of the present investigation was isolation and characterization of active components derived from whole plant of *Equisetum ramosissimum (E. ramosissimum)*. The plant was extracted with petroleum ether, ethyl acetate and methanol solvent. The preliminary phytochemical results revealed that alkaloids, carbohydrates, flavonoids, triterpenoids, steroids, tannin and phenolic compound as active constituents in ethyl acetate extract of *E. ramosissimum*. The total phenolics content of whole plant of ethyl acetate extract was (213.00±0.721mg/gm), followed by flavonoids (116.33±1.154mg/gm). Ethyl acetate as bioactive fraction based on sensitivity test was subjected to TLC and column chromatography. The isolated compound was colourless powder, which was further subjected to IR, ^{1}HNMR and MASS for proper characterization and elucidation of the structure. After various spectral analysis and interpretation of same, the isolated compound was identified as β-sitosterol. Furthermore, pharmacological studies required for the isolated compounds.

Keywords: *Equisetum ramosissimum,* β-sitosterol, Phytochemical, IR, ^{1}HNMR, MASS

1. INTRODUCTION

Medicinal plants are very ancient and true natural medicines which are useful for the treatment of different diseases. They can be used directly or either as pure compounds or as standardized extracts for the management of various ailments due to the presence of various secondary metabolites [1]. According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation [2]. β-sitosterol belongs to the group of phytosterols, which particularly includes stigmasterol and campesterol. Phytosterols are crucial steroid molecules that stabilize the phospholipid bilayers of cellular membranes in plants, having similar structural and biological functions to cholesterol, and are a major group of bioactive constituents with well-proven bioactivity. Phytosterols show a variety of health benefits in vivo, in particular, protection against various chronic ailments, such as cardiovascular diseases, diabetes, cancer and hepatic injury. It is worth mentioning that phytosterols have been attracting much interest because of their well-known cholesterol-lowering property recently [3]. *E. ramosissimum* is a perennial fern from the Equisetaceae family. The plant grows best in moist soils. A black subterranean stem is usually vertical, branching horizontally. Aerial stems are hollow and lie erect above the ground, growing up to 1 meter. Thinner branches emerge in whorls at each node along the length of the stem giving it its common name of branched horsetail. Scale-like leaves form a whorled sheath above each node along the aerial stem. These leaves are narrowly lanceolate with a single vein and are often shiny-black in color [4, 5]. *E. ramosissimum* stems ointment which is used externally to treat bruises, fractures and arthritis. A

decoction is drunk as a diuretic and astringent to treat dysentery and haemorrhoids. In India it is used as diuretic and given against gonorrhoea. In Nepal juice of the roots is given to relieve fever. In South Africa, juice from the plant is used to relieve toothache and applied to the wounds after tooth extraction. In European traditional and modern alternative medicine several horsetail species were commonly used as diuretic and in baths to treat dropsy, urinary complaints and kidney affections [6]. It is used to treat hemorrhage, urethritis, jaundice, and hepatitis [7]. There have been 17 compounds that were previously isolated from the whole plant of *E. ramosissimum*: 5a,6a-expoxy-b-ionone-3-O-b-D-glucopyranoside, loliolide, cycloart-24(30)-ene-3b-ol, cycloart-22,(23)-ene-3b-ol, ergost-6,22-diene-3b,5a,8a-triol, friedelinol, apigene, genkwanin, genkwanin-5-O-b-D-glucopyranoside, apigene-5-O-b-D-glucopyranoside ,luteolin, quercentin-3-O-b-Dglucopyranoside, kaempferol-3-b-D-glucopyranoside, kaempferol-3-O-b-D-glucose-7-O-b-D-glucopyranoside, adenine, b-sitosterol and b-daucosterol [8]. The purpose of this study is to identify and characterize the bioactive principles from the whole plant of *E. ramosissimum*. In this paper, we report the isolation and characterization of known compounds from *E. ramosissimum* namely beta-sitosterol.

2. MATERIAL AND METHODS

2.1. Plant materials

Whole plant of *E. ramosissimum* was collected from Village Rainikheda and Tamiya, Dist. Chindwara, (M.P.) India. Herbarium of plants species were prepared graciously and submitted to Department of Botany, Saifia College of Science, Bhopal India, for authentication. Plants were authenticated by Dr. Zia-Ul-Hasan, Head, Department of Botany, Safia College of Science, Bhopal, India. Plant authentication voucher numbers obtained were 392/Bot/Saifia/16 for *E. ramosissimum*.

2.2. Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

2.3. Extraction

Collected plant material washed under running tap water and kept in shade for drying. Dried plant materials were then powdered using blender and further observed for colour, odour, and texture then placed in packed labelled air tight container for further use. Plant material was extracted by continuous hot percolation method using Soxhlet apparatus [9]. Powdered material of *E. ramosissimum* was placed in thimble of soxhlet apparatus. Soxhlation was performed at 60°C using petroleum ether as non-polar solvent. Exhausted plant material (marc) was dried and afterward re-extracted with ethyl acetate and methanol solvent. For each solvent, soxhlation was continued till no visual colour change was observed in siphon tube and completion of extraction was confirmed by absence of any residual solvent, when evaporated. Obtained extracts was evaporated using rotary vacuum evaporator (Buchi type) at 40°C. Dried extract was weighed and percentage yield for each extract was determined.

2.4. Qualitative phytochemical analysis of plant extract

The *E. ramosissimum* whole plant extract obtained was subjected to the preliminary phytochemical analysis following standard methods [10, 11]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

2.5. Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Extracts obtained from whole plant of *E. ramosissimum* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

2.5.1. Total phenolic content estimation

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentration of (20-100μg/ml) of gallic acid was prepared in methanol. Concentration of 100 μg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2 ml of a 10 fold dilute folin Ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and it was then incubated at room temperature for 30 min with intermittent shaking and the absorbance were taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve

of gallic acid and the results were expressed as gallic acid equivalent (mg/g) [12].

2.5.2. Total flavonoid content estimation

Different concentration of rutin (20 to 100μg/ml) was prepared in methanol. Test sample of near about same polarity (100μg/ml) were prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 min, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5min, and then 2 ml of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 min. Absorbance was determined at 510 nm against water as blank. Total flavonoid content was calculated by the Standard regression curve of Rutin/ Quercetin [12].

2.6. Isolation of β-sitosterol by preparative TLC

2.6.1. Preparation of stationary phase

Readymade silica gel GF 254 plates with a layer thickness of 0.25mm, dimension 20 cm×20 cm. The plates were reactivated by heating in the oven at 100°C for 15 min, left to cool, and used for application after allocation of the baseline and the solvent front.

2.6.2. Preparation of solvent system

Mobile phase for sterols (chloroform: acetone) was mixed in a conical flask and introduced in the jar. The jar was lined with a filter paper, closed tightly, and left for saturation.

2.6.3. Application of sample

About 2 gm of the sample was dissolved in absolute methanol and applied on the baseline of TLC plates.

2.7. Spectroscopic characterization

Different spectroscopic methods were used to elucidate the structure of isolated compound. Among the spectroscopic techniques IR, ^{1}H-NMR and MASS were carried out. The IR spectrum was recorded on FTIR Perkin Elmer, 1H-NMR and spectra were recorded using CDCl3 as solvent on Bruker Advance II 400 NMR spectrometer from RGPV, University, Bhopal mass spectra were recorded at high resolution on a mass spectrometer (Perkin Elmer Auto system) at Sophisticated Instrumentation centre for Indian Institute of Science Education and Research (IISER) Bhopal, Madhya Pradesh, India and the data are given in m/z values.

3. RESULTS AND DISCUSSION

Natural products have always been a preferred choice of all as it plays a great role in discovering new medicines. The crude extracts so obtained after each of the successive soxhlation extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield of extraction is very important in phytochemical extraction in order to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from the whole plants of the *E. ramosissimum* using petroleum ether, ethyl acetate and methanol as solvents are depicted in the Table 1. The petroleum ether, ethyl acetate and methanol extract of *E. ramosissimum* was subjected to screening for its phytochemical constituents. The phytochemical screening results are shown in Table 2. The ethyl acetate extracts containing alkaloids, carbohydrates, flavonoids, triterpenoids, steroids, tannin and phenolic compound. Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and TFC was then calculated with respect to rutin taken as standard. The TPC and TFC in ethyl acetate extract were found to be 213.00±0.721mg/gm and 116.33±1.154mg/gm respectively table 3 & fig. 1, 2. During extraction, solvents ethyl acetate extract of the plant was investigated by TLC which revealed the presence of β-sitosterol that appeared as spot in mobile phase (chloroform: acetone) against β-sitosterol reference standard, and the spot of extract appeared the same Rf value as that in reference standard on TLC plate as shown in Table 4, as indicated by the development of violet spots after spraying by vanillin-sulfuric acid spray reagent [13]. The IR (KBr) absorption spectrum (Fig. 3) showed absorption peaks: υ 3543, 3494, 3390 (Broad peak of OH), 3012, 2891 (alkyl C–H stretching), 2810, 1597, 1527 cm^{-1}. ^{1}HNMR (Fig.4) (500MHz, $CDCl_3$): δ (500 MHz, CDCl3) δ: 5.11 (m, 4H), 3.40 (m, 1H), 2.29 (m, 2H) 2.24 (m, 2H) 2.15 (m, 3H), 1.96 (m, 3H) 1.74 (m, 3H) 1.20 (m, 12H) 1.02 (m, 9H) 0.80 (m, 3H). HRMS m/z=414 M^+ (Fig. 5).

Table 1: Results of percentage yield of extracts

Plant Name	Percentage yield (%)		
	Pet. Ether	Ethyl acetate	Methanol
E. ramosissimum	1.26	3.24	5.31

Table 2: Phytochemical evaluation of *E. Ramosissimum* extract

S. No.	Experiment	Results (ER) Extract		
		Pet. ether	Ethyl acetate	Methanolic
1. Alkaloids				
1.1	***Mayer's reagent test***	+*ve*	+*ve*	+*ve*
1.2	Wagner's reagent test	+*ve*	+*ve*	+*ve*
1.3	*Hager's reagent test*	+ve	+ve	+ve
2. Carbohydrates				
2.1	*Molish's test*	-*ve*	+*ve*	+*ve*
2.2	*Barfoed's test*	-*ve*	+*ve*	+*ve*
3. Test for Reducing Sugar's				
3.1	*Fehling's test*	-*ve*	-*ve*	-*ve*
3.2	*Benedict's test*	-*ve*	-*ve*	-*ve*
4. Flavonoids				
4.1	*Alkaline reagent test*	-*ve*	+*ve*	+*ve*
4.2	*Shinoda test*	-*ve*	+*ve*	+*ve*
4.3	*Lead acetate test*	-*ve*	+*ve*	+ve
5. Glycoside				
5.1	*Borntrager test*	-*ve*	-*ve*	+*ve*
5.2	*Legal's test*	-*ve*	-*ve*	+*ve*
5.3	*Killer- Killiani test*	-*ve*	-*ve*	+ve
6. Tannin and Phenolic compound				
6.1	*Ferric chloride test*	-*ve*	+*ve*	+*ve*
6.2	*Lead Acetate test*	-*ve*	+*ve*	+*ve*
6.3	*Dilute Iodine solution*	-*ve*	+*ve*	+ve
7. Saponin				
7.1	*Faom Test*	-*ve*	-*ve*	-*ve*
8. Test for Proteins and amino acid				
8.1	Ninhydrin test	-*ve*	-*ve*	+*ve*
9. Test for Triterpenoids and Steroids				
9.1	*Salwonski Test*	-*ve*	+*ve*	-*ve*
9.2	*Libberman-Burchard's*	-*ve*	+*ve*	-*ve*

+***ve***: *Present;* −***ve***: *Absent*

Table 3: Total phenolic and flavonoid content of extracts

Test	Ethyl acetate extract
TPC	213.00±0.721mg/gm equivalent to Gallic acid
TFC	116.33±1.154mg/gm equivalent to Rutin

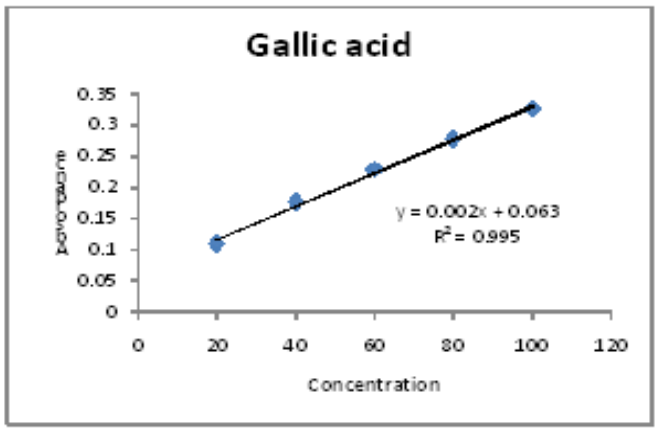

Fig. 1: Graph of estimation of total phenolic content

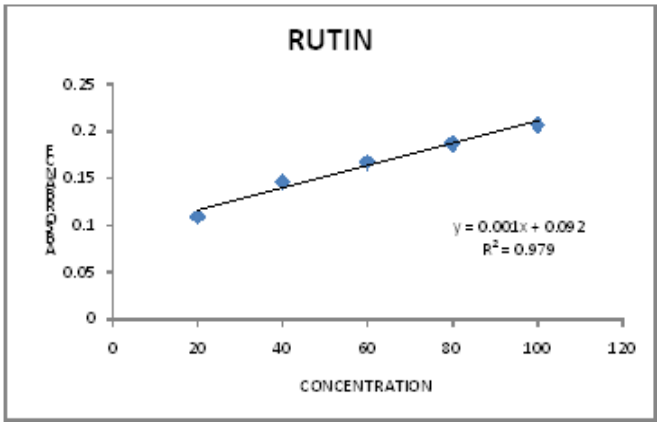

Fig. 2: Graph of estimation of total flavonoids content

Table 4: Rf value of standard and extract

Solvent system	Chloroform: Acetone
Rf value of β-sitosterol standard	0.260
Rf value of β-sitosterol in extract	0.250

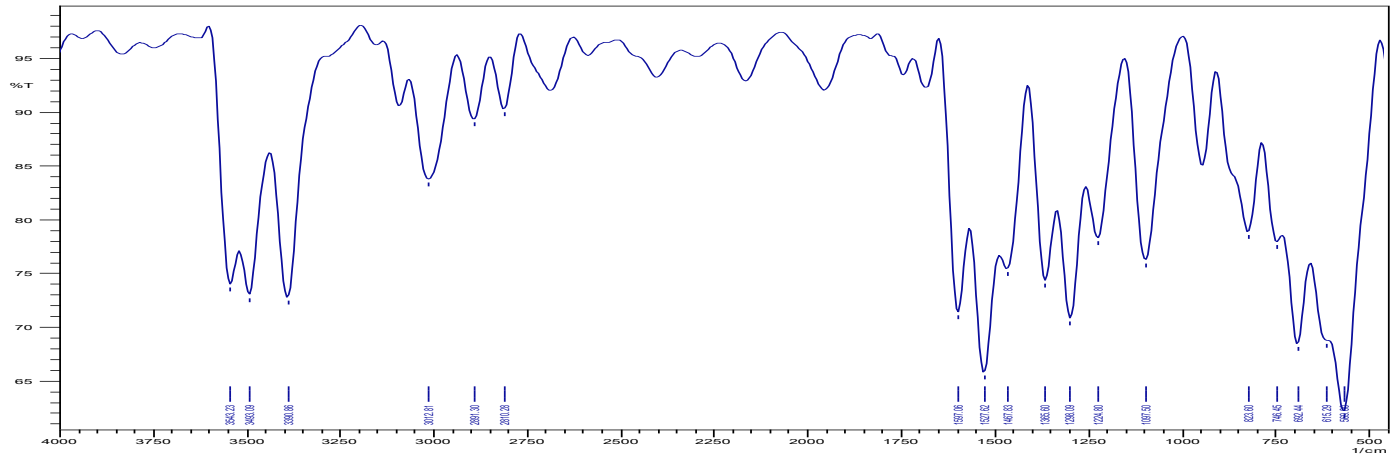

Fig. 3: The Fourier transforms infrared spectra of the β-sitosterol

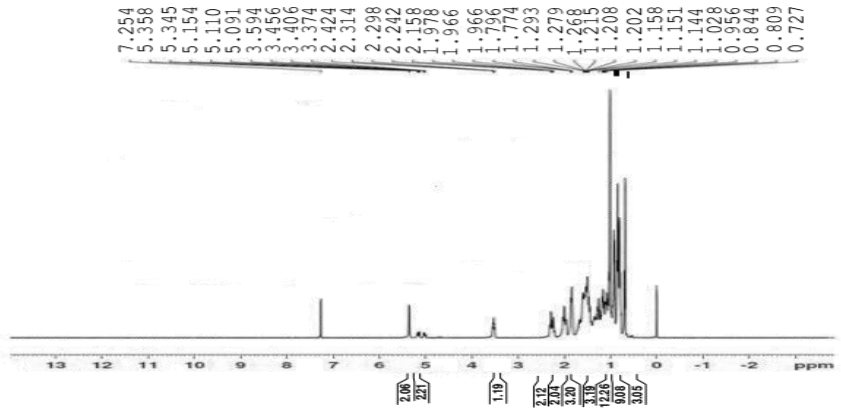

Fig. 4: [1]HNMR Spectrum of the β-sitosterol

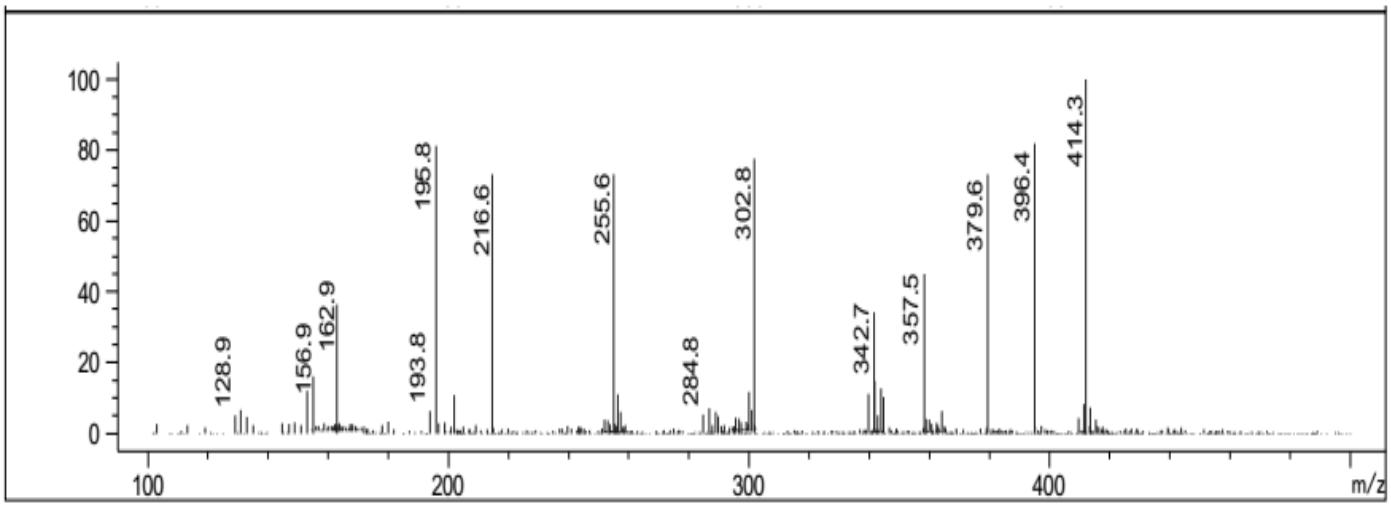

Fig. 5: Mass spectrum of the β-sitosterol

4. CONCLUSION

The therapeutic effects of *E. ramosissimum* have not been attributed to a specific compound however biological investigations have shown that β-sitosterol like phytosterol acts to attenuate heart disease and high cholesterol. It is also used for boosting the immune system and for preventing colon cancer, as well as for gallstones, hair loss, bronchitis, migraine and headache. Some men use β-sitosterol for enlarged prostate (benign prostatic hyperplasia or BPH). Some women use it for symptoms of menopause. β-sitosterol isolated from *E. ramosissimum* could have the good future scope of this cheap medicinal plant.

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