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GC-MS ANALYSIS OF PHYTOCONSTITUENTS AND CONCURRENT DETERMINATION OF FLAVONOIDS BY HPLC IN ETHANOLIC LEAF EXTRACT OF BLEPHARIS MADERASPATENSIS (L) B. HEYNE EX ROTH

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ABSTRACT

Objectives: To elucidate the phytochemical constituents using GC-MS and a sensitive HPLC method has been developed for the concurrent determination of five major flavonoids in ethanolic leaves extract of *Blepharis maderaspatensis*. **Methods:** The shade-dried leaves of *B. maderaspatensis* were extracted with ethanol, the concentrated ethanolic extracts were further subjected to GC-MS and HPLC. **Results:** The GC-MS analyses determined the presence of 17 different phytochemical compounds in the ethanolic leaves extract of *B. maderaspatensis*. The phytoconsituents compounds were found in the mass spectra was matched with the National Institute of Standards and Technology (NIST) library. The major phytoconstituents are 9-Eicosyne (23.64%), Sqalene (11.51%), Phytol (8.40%), 3,4-Dihhdro-3,5,8-trimethyl-3-(4,8,12trimethyltridecyl)-(2H)-benzopyran-6-acetate (8.07%), 3,7,11,15 Trimethyl-2-hexadecen-1-ol (7.41%) and Cholestan-3-ol, 2 methylene,(3á,5à) (7.24%). Mass spectra of the compounds found in the extract were matched with the National Institute of Standards and Technology (NIST) library. The Phytochemical profile of five flavonoids contents in the ethanolic leaf extract of *B. maderaspatensis* are Gallic acid (16.0 µg/g), Caffeic acid (0.1 µg/g), Rutin (0.1 µg/g), Quercetin (0.2 µg/g) and Ferulic acid (0.01 µg/g). **Conclusions:** This is the first report of documentation of active constituents from leaves of *B. maderaspatensis*. The results of the present study reveal that the leaves of *B. maderaspatensis* indulge effective potential bioactive compounds, which may be leads to the formulation of new drugs to treat various diseases.

KEYWORDS: *Blepharis maderaspatensis* (L) B. Heyne ex Roth Phytochemicals Flavonoids.

INTRODUCTION

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. [1] Still today medicinal plants remain significant as natural alternatives to syn-thetic drugs with about 80% of the world population depending upon plants for their primary health care according to WHO estimation. [2,3] Despite the recent interest in molecular modeling, combinato-rial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products, and particularly medicinal plants, remain an impor-tant source of new drugs, new drug leads, and new chemical entities (NCEs). [4, 5, 6,] *Blepharis maderaspatensis* (L) B. Heyne ex Roth, Acanthaceae, is known as "Nethirapoondu", inTamil, is a prostrate, creeping, wiry plant, rooting at the nodes. It is seen commonly on slopes among rocks, poor gravelly soil on the hills up to 1400 m. It is used to treat the disorders such as boils, bone fracture, diarrhea and lactation. [7] Paste of leaves is mixed with limejuice and applied on cuts. [8] The leaves of *Blepharis maderaspatensis* (L.) B. Heyne ex Roth and the yellow yolks of two eggs ground into a fine paste are applied on fractured bones in traditional practice. [9] Therefore the aim of the present study was to identify the phytoconstituents using GC-MS analysis and to explore the concurrent determination of five major flavonoids in ethanolic leaves extract of *B. maderaspatensis.*

MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Material

The leaves of *Blepharis maderaspatensis* (L.) B. Heyne ex Roth were collected from Kolli Hills in Namakkal District, Tamil Nadu, India. The plant was authenticated by Plant Anatomy Research Centre, Institute of Herbal Botany, Chennai, Tamil Nadu, India. The samples were washed thoroughly in tap water, shade dried at room temperature and then ground to a fine powder in a mechanic grinder to obtain coarse powder and stored in air-tight bottles for further analysis.

2.2. Plant Sample Extraction

20 g of the powdered leaves of *B. maderaspatensis* were soaked in 95% ethanol for 12 h. The extracts were then filtered through Whatmann filter paper No. 41 along with 2 gm sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter

paper along with sodium sulphate was wetted with 95% ethanol. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phytocomponents of the plant material used. 2 µl of these solutions was employed for GC/ MS analysis. [10]

2.3 GC Analysis

GC-MS analysis was carried out on a GC CLARUS 500 PerkinElmer system comprising a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da. [11]

2.4. Identification of Components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained**.**

2.5 HPLC Analysis of Flavonoids

Standard Preparation

Standard stock solutions of five phenolic like Gallic acid (GA), Caffeic acid (CA), rutin (RU), Ferulic acid (FA) and Quercetin (QU) compounds were prepared in ethanol, at concentrations of 2, 4, 6, 8 and 10 µg/ml, all standard solutions were filtered through HPLC filter 0.45 mm membrane filter (Millipore)

Sample Preparation

The sample was prepared according to the procedure. The extraction was carried out using 2 ml of fermented broth with 50 mL of 95% ethanol under 80 KHz, 45°C in ultrasonic extraction device for 30 min, repeated twice. The extract was collected and filtered; the

filtrate was dried at 50°C under reduced pressure in a rotary evaporator. The dried crude extract was dissolved in the 100 ml mobile phase. After filtering through a filter paper and a 0.45 mm membrane filter (Millipore), the extract was injected into HPLC. HPLC conditions: Flavonoids were analysed using a HPLC method [12], Shimadzu Corp., Kyoto, consisting of a LC-10ATVp pump, SCL 10A system controller and a variable Shimadzu SPD-10ATVp UV VIS detector and a loop injector with a loop size of 20 µl. The peak area was calculated with a CLASSVP software. Chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250×4.6 mm i.d., particle size 5 µm, Luna 5µ C-18; phenomenex, Torrance, CA, USA) at 25°C. The gradient elution of solvent A [water-acetic acid (25:1 v/v)] and solvent B (ethanol) had a significant effect on the resolution of compounds. As a result, solvent gradients were formed, using dual pumping system, by varying the proportion of solvent A [water-acetic acid (25:1, v/v)] to solvent B (ethanol). Solvent B was increased to 50% in 4 min and subsequently increased to 80% in 10 min at a flow rate of 1.0 mL/min. Detection wavelength was 280 nm. Gallic acid (GA), caffeic acid (CA), rutin (RU), Ferulic acid (FA) and quercetin (QU) was used as internal and external standards. Phenolic acids present in each sample were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards and further confirmed by co-injection with isolated standards. The amount of each phenolic acid is expressed as micrograms per gram of fresh weight unless otherwise stated.

3. RESULTS AND DISCUSSION

The bioactive phytoconstituents present in the ethanolic extract of leaves of *B. maderaspatensis* identified by GC-MS analysis. On comparison of the mass spectra of the constituent with the NIST library, the 17 phytoconstituents were characterized and identified. The active principles with their retention time (RT), molecular formula, molecular weight and concentration of that 17 phytoconstituents present in *B. maderaspatensis* are presented in Table 1. The GC-MS analysis result reveals the presence of 17 phytoconstituents in the leaves extract of *B. maderaspatensis* were 9 – Eicosyne (23.64%), 2- Tridecen – 1-ol,(E)- (4.15%), 3,7,11,15- tetramethy-2-hexadecen- 1 –ol (7.41%), n- Hexadecanoic acid (0.62%), 4-Nonenoic acid, methylester (0.62%), n- Hexadecanoic acid (4.71%), Z-10-Pentadecen-1-ol (4.01%), Phytol (8.40%),Methoxyacetic acid, 4- tetradecyl ester (1.25%), 1-Iodo-2-methyundecane (0.89%), 3-Hexadecyloxycarbonyl-5-(2-hydroxyethyl)-4-methylimidazolium ion (4.79%), Squalene (11.51%), c- Tocopherol (4.19%), 3,4-Dihydro-3,5,8-trimethyl-3-(4,8,12- trimethyltridecyl)-(2H) 1-benzopyran-6-acetate (8.07%), Vitamin E (3.60%),

Spiro[androst-5-ene- 17,1ˈ-cyclobutan]-2ˈ-one, 3- hydroxyl-, (3à, 17á)- (2.43), Cholestan-3-ol, 2-methylene-,(3à,5á0 (7.24%) and 1-Heptatriacotanol (3.10%) respectively. The spectrum profile of GC-MS confirmed the presence of 17 major components with retention time were 11.06, 11.31, 11.50, 12.03, 12.61, 14.18, 14.23, 19.37, 2026, 22.14, 23.67, 26.86, 27.51, 27.86, 29.24, 29.67 and 30.75 were shown in Figure 1 and Table -1.

Using Dr. Duke's phytochemical and ethanobotanical database (online), the biological activity of the identified phytocomponents was ascertained. The various important phytochemicals which contributes to the medicinal activity of the plant given in Table: 2. Biological activities listed are based on Dr**.** Duke's Phytochemical the results indicated 17 phytochemical constituents have been identified from ethanolic extract of the leaves of *Blepharis maderaspatensis* by Gas Chromatogram-Mass spectrometry (GC-MS) analysis. In terms of percentage amounts squalene, phytol, 3, 7, 11, 15- tetramethy-2-hexadecen- 1 –ol, Cholestan-3-ol and Tocopherol were predominant in the extract. These five major compounds are shown to have anticancer, antioxidant, antiinflamatory, antimicrobial activity, antitumor, antiinfertility, antistroke, anticarcinogenic and anticataract activity. By interpreting these compounds, it was found that *B.maderaspatensis* leaves possess various valuable phytoconstituents which will certainly finds application in drug discovery and will serve as pharmacological tool to treat chronic diseases.

A selective and sensitive high-performance liquid chromatographic method is developed for the quantitative analysis of five naturally occurring flavonoids of ethanolic extract of leaves of *B. maderaspatensis*, namely Gallic acid (GA), caffeic acid (CA), rutin (RU), Ferulic acid (FA) and quercetin (QU). The HPLC result shows based on the Retention time (Rt), Gallic acid (Rt-5.917), Caffeic acid (Rt-9.158), Rutin (Rt-10.042), Quercetin (Rt-12.717) and Ferulic acid (Rt-24.167) content in *B. maderaspatensis* was found to be 16 µg/gm, 0.1µg/gm, 0.1 µg/gm, 0.2 µg/gm, and 0.01 µg/gm were shown in figure 2 and table 3. Simultaneous analysis of Gallic acid, Caffeic acid, Rutin, Quercetin and Ferulic acid by HPLC method has been developed. This HPLC procedure provides an excellent identification and quantification tool for these five phenolic compounds are present in the ethanolic extract of the leaves of *B. maderaspatensis* with a short analysis time of 26 minutes. The experimental results indicated that ethanolic extract of leaves of *B. maderaspatensis* contained high concentration of Gallic acid followed by Caffeic acid, Rutin, quercetin and Ferulic acid in order. Gallic acid (3, 4, 5-trihydroxybenzoic acid), found in a variety of plants, is extensively used in tanning, ink dyes,

as well as in the manufacturing of paper. [13] Regarding its biological activity, gallic acid exerts anti-bacterial, anti-viral, anti-inflammatory, antioxidant, and antimelanogenic activities via inhibition of tyrosine's activity. [14, 15] The CAPE [2-propenoic acid, 3-(3, 4-dihydroxyphenyl)-, 2-phenethyl ester] is an active component of propolis with a wide variety of biological activities at non-toxic concentrations in mammals organisms. It has shown activities as antibacterial, antiinflammatory, antioxidant, antitumor and antiproliferative.[16, 17] Rutin is a flavonol glycoside comprised of the quercetin and the disaccharide rutinose (rhamnose and glucose). It is found in many plants, fruits and vegetables. The richest source is buckwheat. Also, it is found in citrus fruits, noni, blacktea and apple peel. It has shown pharmacological benefits including antitumor; anti -allergic; anti - mutagenic; myocardial protecting; immunomodulator and hepato-protective activities. [18, 19, 20, 21, 22] Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone;) belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources. [23] Quercetin acting as free radical scavengers was shown to exert a protective effect in reperfusion ischemic tissue damage. [24, 25, 26] Ferulic acid(FA) (4-hydroxy-3 methoxycinnamic acid) is a dietary phytochemical. [27] Ferulic acid has been shown to potentially exert several beneficial effects on health. For example, it acted as a peroxyl radical scavenger and increased the resistance of LDL to oxidation and protected against some chronic diseases such as diabetes, Alzheimer's , colon and breast cancer and atherosclerosis formed during the metabolism of phenylalanine and tyrosine. [28, 29, 30]

Table 1: Phytocomponents identified in the ethanolic leaves extract of *Blepharis maderaspatensis* by GC-MS.

S. No	Retention time	Name of the compound	Molecular formula	MW	Peak Area %
1.	11.06	9 – Eicosyne	$C_{20}H_{38}$	278	23.64
2.	11.31	2- Tridecen – 1-ol,(E)-	$C_{13}H_{26}O$	198	4.15
3.	11.50	3,7,11,15- Tetramethyl-2-hexadecen- 1 –ol	$C_{20}H_{40}O$	296	7.14
4.	12.03	4-Nonenoic acid, methylester	$C_{10}H_{18}O_2$	170	0.62
5.	12.61	n- Hexadecanoic acid	$C_{16}H_{32}O_2$	256	4.71
6.	14.18	Z-10-Pentadecen-1-ol	$C_{15}H_{30}O$	226	4.01
7.	14.23	Phytol	$C_{20}H_{40}O$	296	8.40
8.	19.37	Methoxyacetic acid, 4- tetradecyl ester	$C_{17}H_{34}O_3$	286	1.25
9.	20.76	1-Iodo-2-methyundecane	$C_{12}H_{25}I$	296	0.89
10.	22.14	3-Hexadecyloxycarbonyl-5-(2-hydroxyethyl)-4-methylimidazolium ion	$C_{24}H_{45}N_2O_3$	409	4.79
11.	23.67	Squalene	$C_{30}H_{50}$	410	11.51
12.	26.86	c- Tocopherol	$C_{28}H_{48}O_2$	416	4.19
13.	27.51	3,4-Dihydro-3,5,8-trimethyl-3-(4,8,12-	$C_{30}H_{50}O_3$	458	8.07

		trimethyltridecyl)-(2H) 1-benzopyran-6-acetate			
14.	27.86	Vitamin E	$C_{29}H_{50}O_2$	430	3.60
15.	29.24	Spiro[androst-5-ene- 17,1'-cyclobutan]-2'-one, 3- hydroxyl,(3à,17á)-	$C_{22}H_{32}O_2$	328	2.43
16.	29.67	Cholestan-3-ol, 2-methylene-,(3à,5á)-	$C_{28}H_{48}O$	400	7.24
17.	30.75	1-Heptatriacotanol	$C_{37}H_{76}O$	536	3.10

Table.2. Activity of phytocomponents identified in the ethanolic leaves extract of *Blepharis maderaspatensis.*

S. No	Name of the compound	Compound nature	Activity
1	n- Hexadsecanoic acid	Palmitic acid	Antioxidant, hypocholesterolemic, nematicide. pesticide, flavour, lubricant, antiandrogenic, hemolytic, 5-α – reductase inhibitor.
2.	Phytol	Diterpene	Anticancer, anti – inflammatory, antimicrobial.
3.	Tocopherol	Vitamin E	Anticancer, antitumor, antioxidant, anti-infertility, anti-stroke, anti-thrombotic, anti-carcinogenic, anti-cataract.
4.	Squalene	Triterpene	Antibacterial, Chemo preventive, immunostimulant, anti-tumor, antioxidant, anticancer, lipooxygenase- inhibitor, perfumery, pesticide, sunscreen.
5.	2- Tridecen – 1-ol,(E)	Alcoholic compound	Antifungal, Flavour and fragrance agent
6.	3,7,11,15- tetramethy-2-hexadecen- 1 –ol	Terpenene alcohol	Antimicrobial
7.	1- Hepatriacotanol	Alcoholic compound	Antimicrobial
8.	Cholestan-3-ol	Steroid compound	Antimicrobial, anticancer, diuretic, antiasthma, antiarthritic
9.	3-Hexadecyloxycarbonyl-5-(2-hydroxyethyl)-4-methylimidazolium ion	Amino compound	Antimicrobial

Table-3. HPLC Validation Data for Ethanolic Leaves Extract of Blepharis *Maderaspatensis*.

Detector A (280nm)				
Retention time	**Area**	**Height**	**Concentration (ppm)**	**Name**
5.917	93233	692	16.0	Gallic acid
9.158	2298	0	0.1	Caffeic acid
10.042	2531	0	0.1	Rutin
12.717	2300	3	0.2	Quercetin
24.167	341	24	0.01	Ferulic acid

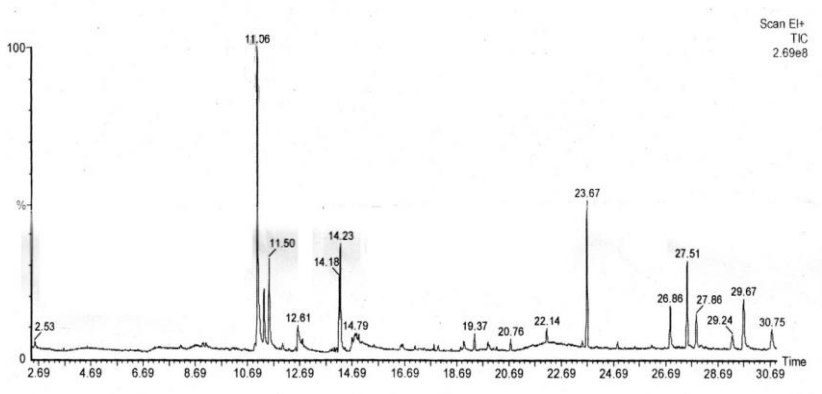

Figure 1: GC-MS Chromatogram ethanolic leaves extract of *Blepharis maderaspatensis.*

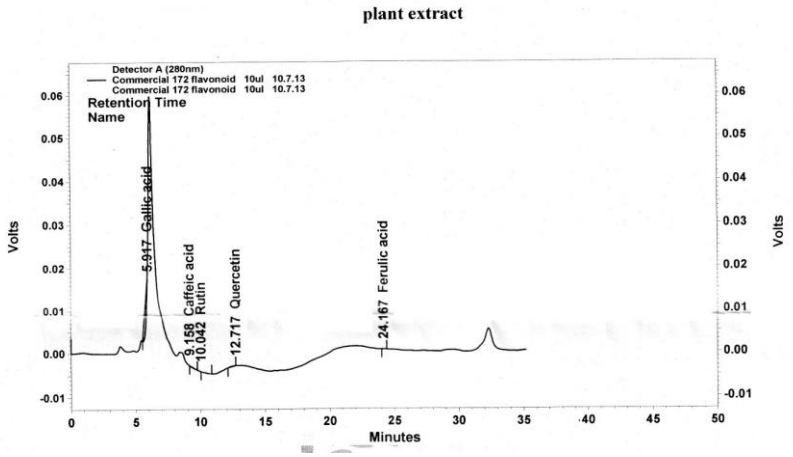

Figure 2: HPLC Chromatogram ethanolic leaves extract of *Blepharis maderaspatensis.*

CONCLUSION

The systematic investigation reveals that the characterization of *B. maderaspatensis* leaves extacts by GC-MS and HPLC separation methods shows the presence of high phenolic content and it has been found to be interesting, since phenolic compounds are known to impose of lot of health benefits, hence this analytical study signifies the presence of bioactive compounds which has been exploited in *B. maderaspatensis* leaves, whereby finds potential application in drug discovery and designing.

Conflict of Interest Statements

We declare that we have no conflict of interest.

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