Research Article

PHYTOCHEMICAL SCREENING OF SECONDARY METABOLITES FROM *MYXOPYRUM SERRATULUM* A.W. HILLS BY GC-MS, HPTLC ANALYSIS

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Article Received on: 12/04/16 Revised on: 13/05/16 Approved for publication: 30/05/16

DOI: 10.7897/2230-8407.07663

ABSTRACT

Myxopyrum serratulum is a valuable medicinal plant widely distributed in Western Ghats at the altitude of 600-900m. Traditionally, the leaves of the plant used to treat asthma, cough diabetes and nerve complaints. The current study have been investigated the presence of various secondary metabolites such as saponin, flavonoids and terpenoids in the methanolic extract of the plant by Thin layer Chromatography (TLC) using appropriate solvent system and the quantification of secondary metabolites like total saponin and terpenoid content was performed by gravimetric analysis. The aqueous fraction of *M.serratulum* was characterized using GC-MS analysis and presence of iridoid glycoside in the ethyl acetate, n-butanol fractions and methanolic extract was identified by HPTLC analysis. Total terpenoid and saponin content have been observed as 6.4 ± 0.33 , 3.815 ± 0.56 respectively. Phytochemical investigation indicated that the presence of flavonoids, Phenols and higher concentration of tannin, saponin in this plant.

KEY WORDS: Saponin, Terpenoids, HPTLC, *Myxopyrum serratulum*,

INTRODUCTION

Herbal medicines remain the major source of health care for the world's population. WHO has recognized herbal medicine as an essential building block for primary health care of vast countries like India. In spite of advances in modern system of medicine, there are various areas like tropical diseases, cancer, diabetes, asthma, cough etc¹. A key factor in the widespread acceptance of natural or alternative therapies by the international community involves the modernization, standardization and quality control of these herbal plants, by use of modern science and technology. However, quality-related problems seems to be related to the lack of simple and reliable analytical techniques and methodologies for the chemical analysis of herbal materials². Modern high-performance TLC (HPTLC) is an efficient instrumental analysis, and optimised quantitative HPTLC using a densitometry evaluation can produce results analogous to those obtained with gas chromatography. Thus, HPTLC 'fingerprint analysis' may be a powerful tool for the quality control of raw plant material and may be an alternative technique, particularly in the analysis of crude plant extracts^{3,4}. The plant *Myxopyrum serratulum* is a important medicinal plant belonging to the family oleaceae, which is mainly distributed in Kerala at the altitude of about 600m. The leaves are used for cough, asthma, fever, rheumatism, neuropathy, cuts and wounds⁵. The phytochemicals were studied in this plant and reported were tannin, alkaloids, terpenoids and iridoid glycosides⁶. In the present work, we investigated the chemical composition of aqueous fraction of the methanolic extract by qualitative analysis, Thin layer Chromatography and GC-MS followed by the identification of iridoid by HPTLC fingerprinting and also quantification of phytochemicals such as saponin and terpenoids in the methanolic extract.

MATERIALS AND METHODS

Collection of plant material

The leaves of the plant *M.serratulum* were collected from western ghats, Kerala in the month of May. It was authenticated from the Central Council of Research in Ayurveda and siddha, Government Siddha Medical College, Palayamkottai, Tamilnadu, India. The voucher specimen (MS07/2013) was deposited in the Department of Pharmaceutical Technology, Anna University, Tiruchirappalli.

Plant extract preparation

The fresh leaves was washed with distilled water to remove dust particles and dried in shade. The shade dried leaves were powdered (80 mesh). 200g of the grinded coarse powder of the plant *M.serratulum* was extracted with methanol (1.0 L) using soxhlet apparatus at (55°C) for 2 days. The extract was filtered through Whatman No. 1 filter paper and then concentrated at 45°C, using a rotary vacuum evaporator. The extract was further fractionated by ethyl acetate and N- butanol successively. The obtained fractions and extract was stored at 4°C.

Phytochemical analysis

The methanolic extract of *M.serratulum* was subjected to preliminary phytochemical screening by using various chemical tests.^{7,8}

The methanolic extract from the *M.serratulum* was screened by thin layer chromatography (TLC), for iridoid glycosides, flavonoids, saponins, and tannin according to Stahl (1969)⁹. In order to characterize the chemical class of constituents found in this extract, the extract was dissolved in methanol (10 mg/ml)

and spotted onto a silica gel plate. Development was carried out with various solvent systems viz. n-hexane: ethyl acetate (7.2:2.9), ethyl acetate:methanol:water (8:2:1.2v/v/v), ethyl acetate: formic acid: acetic acid: water (10:1.1:1.1:2.7v/v/v), chloroform:methanol:water (6.4:5.0:1.0v/v/v). A developing system was adapted for each class of compounds. Plates were visualized under a UV detector at 254 nm and 365 nm before and after spraying with developing agents including anisaldehyde sulphuric acid (saponins, terpenoids) and. The presence of flavonoids was screened by spraying the plates with 5% aluminium chloride (AlCl₃) in ethanol. The presence of iridoid glycosides qualitatively was done by Trim & Hill colour reagent: 1 g of powdered drug was taken in a test tube and placed with 5 ml of 1% aqueous HCl, after 3–6 h 0.1 ml of the macerate is decanted into another tube containing 1 ml of the Trim–Hill reagent (made up from 10 ml acetic acid, 1 ml of 0.2% copper sulphate (CuSO₄, 5H₂O) in water and 0.5 ml conc. HCl). When the tube is heated for a short time in flame, a colour is produced if certain iridoids are present. Further, iridoid glycosides presence can be confirmed by performing TLC identity test by visualizing under UV light 254 nm and by spraying the plate with anisaldehyde sulphuric acid reagent³.

Saponin estimation

Plant Saponin was estimated according to the procedure followed by (Obadoni and Ochuko, 2001).¹⁰ About 10 gm of powder was put into a conical flask and then 50 ml of 20% aqueous ethanol was added. The sample was heated with continuous stirring at 55°C over a hot water bath for 4 hours. This mixture was filtered and the remaining residue re-extracted with another 100 ml of 20% ethanol. Both the extract combined and reduced up to 40 ml over water bath at 90°C. The concentrate obtained was transferred into a 250 ml separating funnel and 10 ml of diethyl ether was added and shaken vigorously. In separating funnel, two separate layers were observed out of which aqueous layer was recovered and the ether layer was discarded. The process of purification was repeated. To the aqueous extract 30 ml of n-butanol was added. A combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample obtained were dried in oven to the constant weight and the saponin percentage was calculated.

Estimation of total Terpenoids

500 mg of plant powder were taken separately and soaked in alcohol for 24 hours, filtered. The filtrate was extracted with petroleum ether and the ether extract was treated as total terpenoids. The residue obtained was dried and weighed.¹¹

Gas Chromatography–Mass Spectrometry (GC/MS) Analysis

GC/MS analysis of *M. serratum* fractions was performed using a Perkin Elmer GC Clauros 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with Elite-1 fused silica capillary column (30 m × 0.25 mm ID, ×1 µM, composed of 100% Dimethyl poly siloxane). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min for 50 minutes and an injection volume of 2 µl was employed (split ratio of 10:1). Injector temperature was set at 250°C with the ion-source temperature of 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 9 min. Mass spectra were detected at 70 eV with a scan interval of 0.5 seconds (45 to 450 Da). Characterisation of compounds interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library.

High Performance Thin Layer Chromatography (HPTLC)

HPTLC was performed on silica gel 60F 254, 20 × 10 cm HPTLC plate (Merk, Germany), with Chloroform: Methanol: water (60:40:4) as a mobile phase. The standard aucubin solution (concentration 1 mg/ml) and the samples includes methanolic extract, ethyl acetate fraction and n-butanol fraction of *M. serratum* were applied to the TLC plate as 10 mm bands. Sample application with CAMAG linomat IV automated spray on band applicator equipped with 100 µL syringe was used. The development of the plate has been performed with anisaldehyde sulphuric acid (0.5 ml anisaldehyde + 5 ml concentrated sulphuric acid + 10 ml glacial acetic acid + 85 ml methanol). CAMAG TLC Scanner 3 was used to quantify the bands densitometrically using WIN CATS software (Version 4 X), 20 mm/s at 254, 366 nm and 520 nm. The R_f values and finger print data were recorded by WIN CATS software.¹²

RESULTS

Phytochemical analysis

The preliminary phytochemical studies showed the presence of terpenoids, flavonoids, saponin, tannin, glycosides and iridoids in the leaves of *M. serratum*. The identification of saponin, Terpenoids and flavonoids in the methanolic extract were also done by thin layer chromatography using appropriate solvent system. Terpenoids can be observed under daylight 254 nm and 366 nm before derivatization and after derivatization, blue, bluish violet colour under visible light confirmed the presence of terpenoids in the sample and standard.

Two different terpenoids were separated by visualising them in the R_f range of 0.57–0.76 (Tables 1). Three different types of flavonoids were observed in the methanolic extract of *M. serratum*. The R_f values determined for the flavonoids were in the range of 0.4 to 0.71. (Table 1). The suitable solvent system evaluated was hexane - ethyl acetate (7.2:2.9). Yellow or yellowish blue coloured fluorescence zone at UV 366 nm mode confirmed the presence of flavonoids in the sample and standard. Three saponin glycosides were observed in the R_f range of 0.42 to 0.74. The quantification of phytochemicals such as total saponin and total terpenoid content was evaluated and reported in the leaves of *M. serratum*, it was found to be 3.82%, 6.41% respectively.

HPTLC analysis

The presence of aucubin or aucubin derivatives type of iridoids in this plant is identified using HPTLC analysis. In the present study, HPTLC fingerprint profile of methanolic extract, ethyl acetate and n-butanol fraction of *M. serratum* was performed and recorded with reference to the standard iridoid aucubin. From the chromatogram, it was observed that the brownish violet and pale brown coloured zones on the plate after derivatization with anisaldehyde sulphuric acid showed the presence of iridoid (Figure 1). Normally the iridoids can be detected after derivatization and migrates at the R_f values in between 4.5 to 7.5 (Wagners et al. 1996). Our results confirmed the presence of iridoid in comparison with the spectrum

overlay of standard to the maximum absorption at 520nm. The chromatogram of methanolic extract showed 4 prominent peaks with different Rf values (0.57, 0.71, 0.88 and 0.93), where as 3 intense peak were detected in the n-butanol fraction with the Rf values of 0.72, 0.80 and 0.87. It is noticable that there was no prominent iridoid peak detected in ethylacetate fraction. The Rf value of n-butanol fraction and methanolic extract were observed as 0.73, 0.72 which could be coincide with the Rf value of iridoid reference standard aucubin (0.71) (Figure 1, 2 (A,B,C,D) & Table 2).

GC-MS analysis

GC/MS analysis of the Aqueous fraction of *M. serratum* led to the identification of 26 components (Table- 3, Figure 3). The major identified compounds including Hexadecanoic acid, Octane -9- enoic acid, 1,2-Benzenedicarboxylic acid, diethyl ester, Cyclooctasiloxane, Hexadecanoic acid methyl ester, 9-octadecenoic acid, methyl ester and its Mass spectrum shown in (Figure 4). Ethanone, 1-(cyclohexene-1-yl), Cyclohexasiloxane dodecamethyl, 4-phenyl-3,5-pyrazolidinedione. These compounds were abundantly found in the aqueous fraction.

Table 1: Identification of phytochemicals in the methanolic extract of *M.serratum* by thin layer chromatography

Mobile phase	Developing agent	Colour of the spot	Rf	Compound
ethylacetate:formic acid:acetic acid: water(10:1.1:1.1:2.7)	5% AlCl ₃	Yellow, brown	0.4, 0.68, 0.71	Flavonoid
n-hexane:ethylacetate(7.2:2.9)	Anisaldehyde sulfuric acid	Pink, bluishviolet	0.56, 0.75	Terpenoids
chloroform:methanol:water (6.4:5.0:1.0)	Anisaldehyde sulfuric acid	Pink, brown, violet	0.42, 0.6, 0.74	Saponin, iridoid glycoside
Ethyl acetate: methanol: water(77:15:8)	Anisaldehyde sulfuric acid	Green, blue, pink	0.76, 0.32, 0.81	Polar compounds

Table 2: Rf value of STD(Aucubin) methanolic extract and n- butanol-f, ethyl acetate-f of *M.serratum* after derivatization

S.No	Rf value				Area(AU)			
	STD	ME	EA-F	NBu-F	STD	ME	EA-F	NBu-F
1	0.73	0.57		0.72	7367.7	1007.8		1790.8
2		0.71		0.80		2043.0		688.6
3		0.88		0.87		968.6		5041.9
4		0.93				352.1		
5		0.95				298.8		

Table 3: Chemical composition of aqueous fraction of *M.serratum* by GC-MS analysis

R.Time	Name of the Compound	Mol.Formula	Mol. Wt	Peak area%
6.600	Bicyclo heptane, 2-methyl	C ₈ H ₁₄	110.19	0.15
7.611	Cyclotetrasiloxane, octamethyl	C ₈ H ₂₄ O ₄ Si ₄	296.62	0.14
9.178	Ethanone, 1-(cyclohexene-1-yl)	C ₈ H ₁₂ O	124.18	0.26
11.733	Naphthalene	C ₁₀ H ₈	128.17	12.54
14.004	Cyclohexasiloxane dodecamethyl	C ₁₂ H ₃₆ O ₆ Si ₆	444.9	4.05
21.832	1,2-Benzenedicarboxylic acid, diethyl ester	C ₁₂ H ₁₄ O ₄	222	10.91
18.648	4-phenyl-3,5-pyrazolidinedione	C ₁₂ H ₁₂ N ₂	216.24	1.62
28.512	Hexadecanoic acid (CAS) Palmitic acid	C ₁₆ H ₃₂ O ₂	256	5.91
38.27	1,2-Benzenedicarboxylic acid, bis(2-ethyl hexyl)	C ₂₄ H ₃₈ O ₄	390.56	10.19
25.709	Silicic acid	H ₄ O ₄ Si	96.11	0.37
30.437	9-Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296	3.33
30.945	Octadecanoic acid, methyl ester (CAS) Methyl	C ₁₉ H ₃₈ O ₂	298	0.25
26.583	Octadec-9-enoic acid	C ₁₈ H ₃₄ O ₂	282	7.10
31.008	Neophytadiene	C ₂₀ H ₃₈	278.52	0.38
31.213	2-pentadecyn-1-ol	C ₂₀ H ₄₀ O ₃ Si	356.62	8.90
31.591	Octadecanoic acid (CAS) stearic acid	C ₁₈ H ₃₆ O ₂	284	4.44
	4-p-chlorophenyl-2-dimethylamino-5-nitroso			1.83

Figure 1: Chromatogram of the sample (ME-methanolic extract ,EA-Ethylacetate fraction and n-BuF - n-Butanol fraction). a-Before derivatization at UV-254nm, b- Before derivatization at UV-366nm and c - after derivatization at 520nm. STD-Aucubin

Figure 2(A)

Figure 2 (B)

Figure 2(C)

Figure 2(D)

Figure 2: Peak destionogram of standard aucubin (A), methanolic extract of *M.serratum* (B), ethyl acetate fraction (C) n-butanol fractio(D).

Figure 3: Gas chromatogram of aqueous fraction of *Myxopyrum serratum*

Figure 4: Mass spectrum of identified compounds by GC

DISCUSSION

From the observed results of the present study, the secondary metabolites such as flavonoids, saponin glycosides, terpenoids were found in the methanolic extract of *M.serratum*. Many plant glycosides are reported to have versatile medicinal properties. The important function of glycosides in animals and humans are often eliminated poisonous substances from the body.¹³ More than 6000 different flavonoids have been

identified and reported from the medicinal plants and many of them are colorants of flowers, fruits and leaves and also many flavonoids possess important biological activities resulted in intensive research efforts to understand the impact of these compounds on human health. Chemically saponin have great variability of their structures, because of this unique property it exhibiting antitumorigenic effects by activating a variety of antitumor pathways.¹⁴ The presence of aucubin or aucubin derivatives type of iridoids in this plant was identified using

HPTLC analysis. Based on the area observed in the chromatogram, the methanolic extract and n-butanol fraction showed the comparable quantity of iridoid content. Iridoid glycoside is an important bioactive secondary metabolite found in many plant families which is used as a taxonomic marker of the plant; also it exhibit wide pharmacological activity including hepatoprotective, anti-oxidant, anti-inflammatory and neuroprotective.¹⁵

The GC-MS study showed the presence of many antioxidant compound in the aqueous fraction of *M.serratum*. The previous study of Gopalakrishnan and Rajameena, 2013¹⁶ showed the presence of 7,8 Dimethoxy 13 carbomethoxy 15 (3,4,5 trimethoxy 13,14 dihydro allobarberban, 1,6 Hexane diamine as the major components in the methanolic extract of *M.serratum*. Previous studies on secondary metabolites and bioactive phyto- constituents identification through GC/MS in different plants have been reported for many pharmacological activities including, antimicrobial, anti-inflammatory, antioxidant and anti-proliferative activities.^{17,18}

CONCLUSION

Based on the results observed in this study, it was concluded that the secondary metabolites presence such as terpenoids, flavonoids and saponins and iridoid were confirmed by various chromatographic analysis. Presence of these phytochemicals attributed the medicinal properties of the plant.

AKNOWLEDGEMENT

The authors acknowledge the Department of Science and Technology, New Delhi, sponsored National Facility for Drug Development for Academia, Pharmaceutical and Allied Industries Bharathidasan Institute of Technology, Anna University, Tiruchirappalli.

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Cite this article as:

Vijayalakshmi Maruthamuthu, Ruckmani Kandasamy. Phytochemical screening of secondary metabolites from *Myxopyrum serratum* A.W. hills by GC-MS, HPTLC analysis. Int. Res. J. Pharm. 2016;7(6):51-57 <http://dx.doi.org/10.7897/2230-8407.07663>

Source of support: Nil, Conflict of interest: None Declared

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