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METABOLITE ANALYSIS OF *HUGONIA MYSTAX L.* (LINACEAE) LEAVES FROM SOUTH-WEST REGION OF INDIA

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ABSTRACT

Hugonia mystax Linn., is an ethanobotanically important plant which plays a vital role in primary healthcare of tribals from Tiruvannamalai hills, Tamil Nadu, India. There is paucity in the literature regarding the active phytoconstituents of *H. mystax* contributing to the biological activities claimed by traditional healers. Therefore, the present investigation aims to analyze the metabolite profile of leaf extracts of *H. mystax*. Quantitative determination of total phenolic and flavonoid of leaves was carried out. The highest amount of phenolic and flavonoid was found in ethanol extracts of the leaves. RP-HPLC analysis of individual phenolic compounds revealed the presence of Gallic acid, Catechol, Caffeic acid, Vanillin, *p*-Coumaric acid and Ferulic acid. Catechol was found to be the highest in water extract. GC-MS of chloroform fraction and LC-MS of methanol/water fraction led to the identification of 19 and 21 compounds respectively. Chloroform fraction revealed the presence of 1-Hexanol (37.69%), Cinnamene (13.81%) and p-Xylol (9.39%), whereas, methanol/water fraction revealed the presence of p-Myrcene (19.5%), 4-Vinyphenol (12.72%) and p-Vinylguaiacol (4-VG) (7.82%) respectively as the major compounds. This is the very first report of metabolite analysis of leaves of *H. mystax* L. from south-west region of India. This investigation identified the major phytoconstituents predominantly present in leaves, which may probably be responsible for the traditional claims of its therapeutic properties.

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INTRODUCTION

Plant derived phytochemicals are a large group of substances, consumption of which protects the human system from disease conditions. They constitute the non-nutritive plant bioactive chemicals which have protective or disease preventive properties. Phytochemicals present in different fruits, vegetables and herbs work differently [1]. The functional bioactivity of a plant organic extract, depends on the presence of compounds, such as polyphenols, carotenoids, terpenoids, and chlorophyll [2-3]. This is primarily due to the antioxidant activity of the phenolic and flavonoid compounds [4-11].

Medicinal plants are widely used for the treatment and prevention of various ailments particularly in developing countries where infectious diseases are common and modern health facilities and services are insufficient [12]. There is a lot of interest in correlating the phytochemical constituents of a medicinal plant with their pharmacological activity. Screening of different active compounds from plants has lead to the discovery of new drugs, which have efficient protection and treatment roles against various diseases including cancer and Alzheimer's diseases [13].

The genus *Hugonia* L. of Linaceae family consists of about 40 species worldwide and is found in India and Srilanka. *Hugonia mystax* is locally known as Modirakanni [14]. The same species was reported by Dalzell and Gibson in Bombay Flora in 1861. Recently, it was rediscovered after a gap of 145 years in Maharashtra [15]. The roots of *Hugonia mystax* were used as anthelmintic, astringent and also used for dysentery, snake bite, fever, inflammation and rheumatism. Biological properties such as analgesic, anti-inflammatory and ulcerogenic have been associated with these plants [16-18]. The antioxidant activity of leaf was reported by Rajeswari *et al*. [19]. Antimicrobial activity of petroleum ether, chloroform, ethanol and aqueous extracts of root extracts showed significant activity against various human pathogens [20].

The literature survey and screening of scientific data revealed that although *H. mystax* leaves are traditionally used to treat various diseases for a long time, no systematic phytochemical fingerprinting data is available. Therefore, in this study an attempt was made to study metabolite profile of leaves of *Hugonia mystax* from south-west region of India and the phytoconstituents reported for the first time.

MATERIALS AND METHOD

Materials

Gallic acid, quercetin, cathechol, caffeic acid, vanillin, *p*-coumaric acid and ferulic acid were purchased from Sigma Chemical Co. (USA). HPLC grade ethanol, methanol, acetic acid, Folin Ciocalteu's Phenol reagent, sodium carbonate and aluminium chloride were purchased from Merck (Germany). For LC-MS and GC-MS methanol, anhydrous sodium sulphate and chloroform (analytical reagent grade) was obtained from Merck (Germany). The Millipore water was used. All other chemicals were of analytical grade or GC grade.

Sample collection

The fresh green leaves of *Hugonia mystax* L. were collected from Mochemad–Vengurla, Sindhudurg district, Maharashtra. The geographical location of collection area was 15° 48. 040′ N, 073° 39. 283′ E. The leaves were packed in polyethylene bags and stored at 4°C until required. Plant specimen was authenticated and deposited in Blatter Herbarium, St. Xavier's College, Mumbai. Approximately 50 g of leaves were ground using a grinder. The unfermented *Hugonia mystax* leaves were kept in the oven at 40°C and put in the desiccators for at least 24 h prior to analysis.

Extraction

The air-dried leaves of *H. mystax* were made into fine powder. 5 g of powder of leaves were suspended in 50 ml of three different extracting solvent systems; distilled water, methanol and ethanol respectively for overnight extraction. Extracts were filtered using Whatman No.1 paper and were concentrated *in vacuo* using rotary vacuum evaporator. Percentage extractive values (*w*/*w*) of each extracts were calculated. For HPLC analysis, stock solutions of extracts were filtered through the syringe filter 0.45 µm pore size (Millipore).

Determination of total phenolic content

The total phenolic content (TPC) in *H. mystax* crude extracts was determined by using the Folin-Ciocalteu method [21]. Standard solutions of gallic acid of concentration 1.56 – 100 µg/ml were prepared in water. 50 µl of extracts or standard solution were added to 50 µl of distilled water. 50 µl of 10% Follin–Cicocalteu's phenol reagent and 50 µl of 1 M sodium carbonate solution were added to the mixture in a 96-well plate. Distilled water was used as blank. Reactions were incubated for 60 minutes at room temperature and protected from light. The absorbance was measured at 750 nm with a Microplate Reader (Biotek, USA.). Total phenolic contents were expressed as µg Gallic Acid Equivalents (GAE) per mg of dry plant material.

Determination of total flavonoid content

Total flavonoid content (TFC) was determined by the aluminium chloride colorimetric assay [22]. Standard solutions of quercetin of concentration 1.56 – 100 µg/ml were prepared in 80% ethanol. 50 µl of extracts (1 mg/ml) or standard solution was added to 10 µl of 10% the aluminium chloride solution and followed by 150 µl of 95% ethanol. 10 µl of 1 M sodium acetate was added to the mixture in a 96 well plate. 80% ethanol was used as reagent blank. All reagents were mixed and incubated for 40 minutes at room temperature protected from light. The absorbance was measured at 415 nm with a Microplate Reader (Biotek, USA.). Total flavonoid contents were expressed as µg Quercetin Equivalents (QE) per mg dry of plant material.

Quantitative analysis of phenolic compounds using RP-HPLC

Quantitative analysis of individual phenolic compounds present in the different parts of plant extracts was performed on a Waters HPLC system (Model 2487), using Zorbax Eclipse ODS C18 reverse phase column (150 mm x 4.6 mm ID, 5µm) (Agilent, Germany), 1% (*v*/*v*) acetic acid in water: methanol (80:20) (*v*/*v*) used as mobile phase with 1 min/ml flow rate, UV detector set at 280 nm. Phenolic compounds from each sample were identified by comparing their relative retention time with the standards of mixture chromatogram.

Statistical analysis

All the observations were taken in triplicate and the data were expressed in Mean ± Standard Error of Measurement (S.E.M).

Sample preparation and analytical conditions for GC-MS analysis

Leaf sample of 100 mg is taken and crushed into fine powder under liquid nitrogen. To this powdered leaf sample 1 ml solution of water/methanol/chloroform (1:2.5:1) is added and extracted at 37°C for 30 min. The sample is centrifuged. For GC-MS, the lower chloroform layer (200 µl) is passed through anhydrous sodium sulphate column and this column is again eluted with 500 µl of chloroform. This 500 µl sample is filtered through 0.2 µm PTFE filter and injected directly into GC-MS.

GC– MS was performed on Varian 3800 apparatus (auto injector) coupled with Saturn 4000 MS/MS which was equipped with a split injector (split ratio is 25:1) and DB-Wax column (30 m length x 0.25 mm I.D. Agilent Technologies, USA). The DB-Wax column was operated using an injector temperature of 250°C and the following oven temperature profile: an isothermal hold at 80°C for 2 min, followed by a ramp of 15°C/min to 320°C and an isothermal hold for 10 min. All mass spectra were acquired in the EI mode (scan range of *m*/*z* 50–1000 and ionization energy of 70 eV). The carrier gas was helium (1ml/min) and the sample injected was 10 µl. The total running time for GC was 26 min.

Sample preparation and analytical conditions for LC-MS analysis

For LC-MS, the methanol/water supernatant layer (800 µl) is taken and freeze dried to remove methanol and water. To the freeze dried sample 200 µl of 0.1% formic acid and methanol (1:4) is added and shaken until the sample completely dissolves. This sample is filtered through 0.2 µm PTFE filter and injected directly into LC-MS.

LC–MS was performed on Shimadzu, Prominence UFLC series spectrometer with an IT-TOF detector. Shim-pack XR-ODS (50 mm x 2 mm L; I.D. 2.2 µm; film thickness 0.25 µm, Agilent Technologies, USA) column was used. For LC gradient system with mobile phase A containing 0.1 % formic acid aqueous solution and Mobile phase B containing Methanol was used. Gradient program (Mobile phase B: 2%-0min; 60%-10 min; 98%- 10.01 to 14 min; 2%- 14.01 min) with flow rate of 4 mL/min and column temperature of 40°C was used. All mass spectra were acquired in the ESI mode (scan range of *m*/*z* 100–1000).

Identification of volatile components

In GC-MS, the peaks were detected from the mass chromatograms and identification was conducted using the GC-MS metabolite database and the National institute Standard and Technology (NIST) mass spectrum library. In the case of LC-MS, the peaks were detected from the results of MS1 analysis, and a peak list was generated using the respective peak area values.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

Ethanol extract of leaves of *H. mystax* showed highest phenolic content (87.70 ± 5.56 µg of GAE/mg of dry plant material) in comparison to methanol and distilled water (Figure 1). Methanol extract had highest extractive value, but ethanol was observed to be the best solvent system for the extraction of phenolic compounds. The range of phenolic compound content in leaf was 33.16 ± 0.44 - 87.70 ± 5.56µg of GAE/mg of dry plant material. The range of flavonoid content in various solvents was 16.87 ± 0.43 - 102.7 ± 1.91 µg of QE/mg of dry plant material. The highest amount of flavonoid content was found in ethanol extract (102.7 ± 1.91 µg of QE/mg of dry plant material) (Figure 1). Plant phenolics are mainly classified into phenolic acids and flavonoids. These compounds are involved in growth and reproduction of plant, and also play important role in defense against ultraviolet radiation and pathogens [23, 24]. Previous findings on leaf extracts (by Soxhlet method) of *H. mystax* showed the presence of secondary metabolites such as phenolic acids, flavonoids, tannins, terpenoids, steroids; whereas qualitative determination of individual phenolic compounds of extracts revealed the presence of caffeic acid, gallic acid, *p*-coumaric acid and vanillin [25].

Some of these phytoconstituents like tannins, phenols, etc. may be accountable for significant anthelmintic activity. It was reported that, tannins may either interfere with energy generation by uncoupling oxidative phosphorylation or they may bind to free protein of the gastrointestinal tract of the worms leading to death [26]. Polyphenols are found to be strong antioxidants that can neutralize free radicals. They also exhibit anticancer activity by regulation of signal transduction pathways of cell growth and proliferation, suppression of oncogenes and tumor formation, induction of apoptosis, modulation of enzyme activity related to detoxification, stimulation of the immune system, DNA repair and regulation of hormone metabolism [27].

RP-HPLC analysis of individual phenolic compounds

Individual phenolic compounds such as gallic acid, catechol, caffeic acid, vanillin, *p*-coumaric acid and ferulic acid in various extracts of leaves were analyzed and quantified using RP-HPLC (Figure 2). The highest amount of phenolic compound present was Catechol (1.1678 mg/ gm of dry plant material) in methanol extract of leaves (Figure 3). *p*-coumaric acid was found to be present in all solvent extracts of the leaves.

Recent in *vitro* and in *vivo* studies of leaf extracts of *H. mystax* L. with different solvent systems showed diversified biological activities such as, cytotoxic [28], anthelmintic [29], antitumor [30] , anti-inflammatory [31] and analgesic [32]. The ethanol extract from leaf of *H. mystax* showed significant anti-inflammatory activity in carrageenan induced paw edema, compared with standard drug indomethocin [31]. However, the phytoconstituents contributing to biological activity and their molecular basis for biological effect is not yet understood. Present investigation has identified vanillin, *p*-coumaric acid and ferulic acid as the major phytoconstituents present in ethanol extract from leaves of *H. mystax* L.

Rheumatoid arthritis (RA) is a chronic inflammatory disease; in which infiltration of leukocytes into synovial tissues occurs. These migrated leukocytes and synovial fibroblast generate several inflammatory factors, such as chemokines and cytokines. These factors also play important role in angiogenesis [33]. The pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α play a pivotal role in the pathogenesis of RA. On the other hand, TNF-α induce the production of vascular endothelial growth factor (VEGF) in vivo, which promotes angiogenesis. Recent in vivo studies suggested that inhibition of angiogenesis during RA might be the.

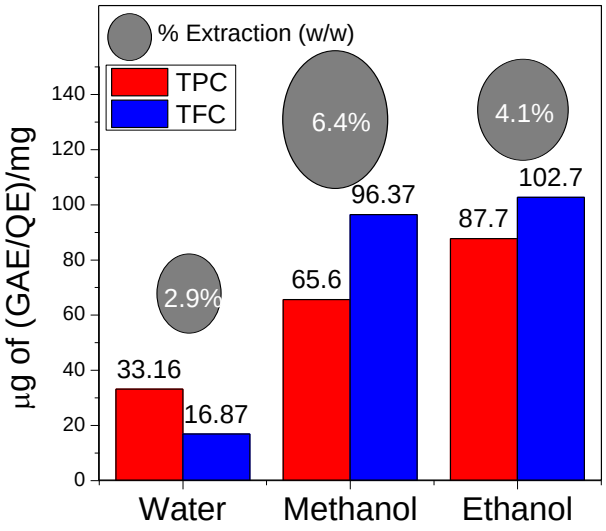


Fig. 1: Total flavonoid content, total phenolic content and percentage extraction value (w/w) in *Hugonia mystax* leaves.

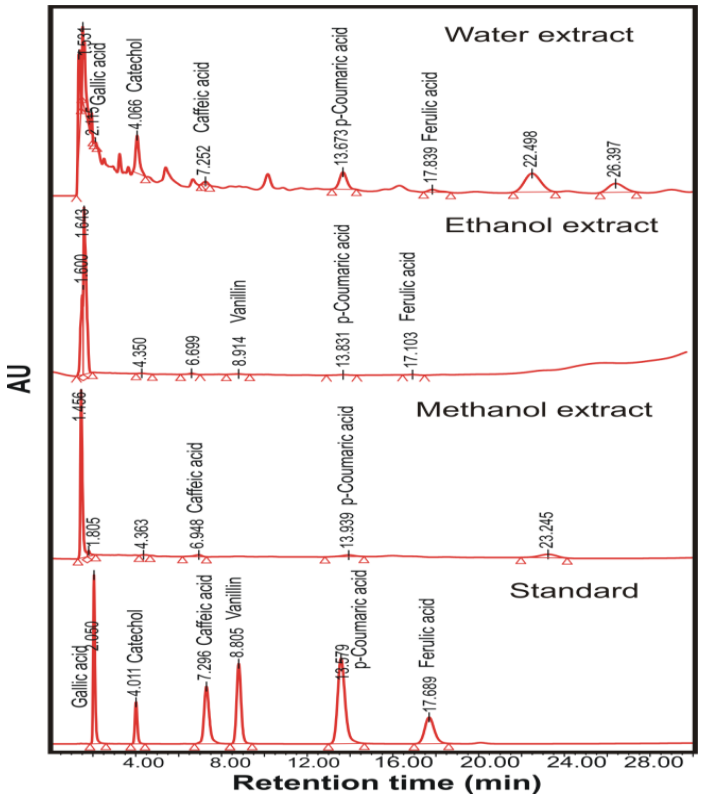


Fig. 2: RP-HPLC chromatogram of standard phenolic compounds (20 ppm each) and leaf extracts.

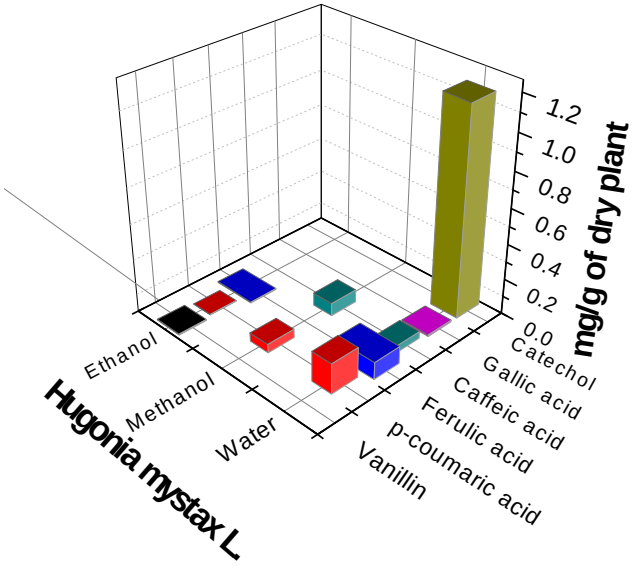


Fig. 3: Amount of individual phenolic compounds presents (mg/g of dry plant) in ethanol, methanol and water extract of *Hugonia mystax* leaves.

Therapeutic application to halt the progression of RA [34]. Wang *et al*. [35] have suggested that compounds rich in phenolic acids significantly contribute to antirheumatic activity. For example *p*-coumaric acid significantly suppresses leukocyte infiltration and pro-inflammatory cytokine expression in ankle joints of adjuvant-induced arthritic rats model [36, 37]. Hence, *p*-coumaric acid has potential of being used as immunosuppressive agent in treating autoimmune inflammatory diseases like rheumatoid arthritis. Phenolic compounds exhibit potent antioxidant activity not only by neutralizing free redicals generated during biological processes but also modulating the endogenous enzymes. Phenolic acids such as caffeic acid, gallic acid, *p*-coumaric acid and ferulic acid showed a remarkable antioxidant and antimicrobial activity. However, among these phenolic acids,caffeic acid was found to be the most potent antioxidant as compared to *p*-coumaric acid and ferulic acid [38-40].

GC-MS analysis

The phytoconstituents present in chloroform fraction of leaves were analyzed by GC-MS (Figure 4). The active principals with their retention time (RT), formula, molecular weight (MW) and concentration (%) the extract are presented in Table 1. Nineteen compounds were detected in the extract of *H. mystax* leaves. The results revealed that 1-Hexanol (37.69%), Cinnamene (13.81%), p-Xylol (9.39%), were found to be major components followed by beta-Ionone (3.58%), alpha-Ionone (2.8%), m-Xylol (2.23%), 1-Heptanol (1.98%), Hemimellitene (1.97%), Benzofuranone (1.93%), p-Cymene (1.86%), 3-Ethylcyclopentanone (1.82%), n-Tetradecane (1.66%), Hexahydrofarnesyl acetone (1.26%), Azulene (1.2%), Durol (1.17%), 1-Nonanol (1%), Ethyl palmitate (0.97%), Phytol (0.86 %) and Geranyl acetone (0.74%).

Previous GC-MS profile of ethanolic extract of *H. mystax* leaves was done by Rajeshwari *et al*. [41]. Thirteen compounds were identified from the analysis. 1,2-Benzenedicarboxylic acid, diisooctyl ester (48.75%) was found to be major component followed by n-Hexadecanoic acid (13.52%), Phytol (9.25%), Squalene (6.41%), Vitamin E (4.09%). From the previous and present analysis it was observed that, there is difference in phytochemical content identified in the leaves of *H. mystax*, except phytol, which showed its presence in both the analysis.

Among the identified phytochemicals in the present study, 1-Hexanol is an anesthetic and nicotinic antagonist [42]. Since these compounds inhibit the action of acetylcholine (ACh) at nicotinic acetylcholine receptor they are mainly used for peripheral muscle paralysis in surgery. Phytol detected in *H. mystax* leaves is effective at different stages of the arthritis. *H. mystax* has been traditionally used to cure rheumatoid arthritis. The results show that, reactive oxygen species promoting substances such as phytol constitute a promising novel class of pharmaceuticals for the treatment of rheumatic arthritis and possibly other chronic inflammatory diseases [43].

LC-MS analysis

In the present study, LC-MS analysis of methanol: water extract of *H. mystax* leaf resulted in the identification of 21 medicinally valuable compounds (Figure 5 and Table 2). The results revealed that p-Myrcene (19.5%), 4-Vinylphenol (4-VP) (12.72%), p-Vinylguaiacol (4-VG) (7.82%) and 1, 2-.

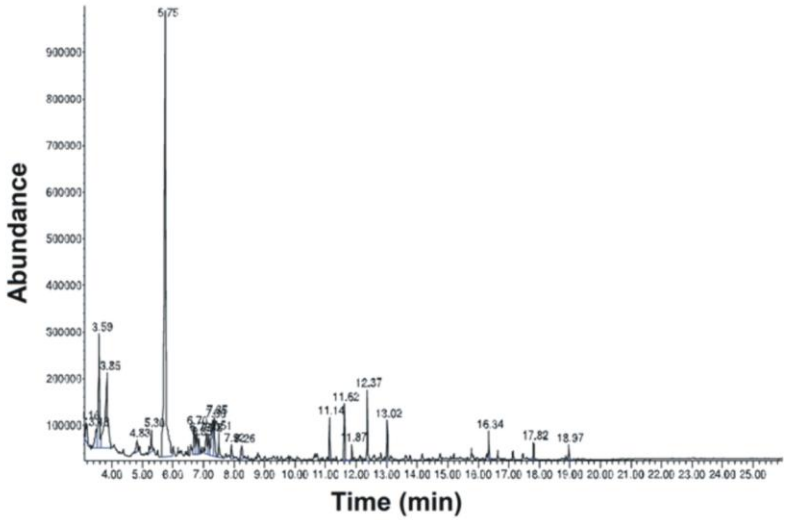


Fig. 4: GC-MS chromatogram of non-polar fraction of *Hugonia mystax* L. (Linaceae) Leaves.

Table 1: Identified metabolites by GC-MS from the chloroform extract of *H. mystax* leaves.

Sr. No.	RT	Chemical name	Mol. formula	Mol. Wt.	Peak area (%)
1.	3.17	3-Ethylcyclopentanone	$C_7H_{12}O$	112.17	1.82
2.	3.48	m-Xylol	C_8H_{10}	106.16	2.23
3.	3.59	p-Xylol	C_8H_{10}	106.16	9.39
4.	3.85	Cinnamene	C_8H_8	104.15	13.81
5.	5.31	Hemimellitene	C_9H_{12}	120.19	1.97
6.	5.75	1-Hexanol	$C_6H_{14}O$	102.17	37.69
7.	6.7	p-Cymene	$C_{10}H_{14}$	134.21	1.86
8.	6.86	1-Heptanol	$C_7H_{16}O$	116.20	1.98
9.	7.19	Durol	$C_{10}H_{14}$	134.21	1.17
10.	7.93	1-Nonanol	$C_9H_{20}O$	144.25	1
11.	8.25	Azulene	$C_{10}H_8$	128.17	1.2
12.	11.13	n-Tetradecane	$C_{14}H_{30}$	198.38	1.66
13.	11.61	alpha-Ionone	$C_{13}H_{20}O$	192.29	2.8
14.	11.86	Geranyl acetone	$C1_4H_{24}O$	208.33	0.74
15.	12.37	beta-Ionone	$C_{13}H_{20}O$	192.29	3.58
16.	13.01	Benzofuranone	$C_8H_6O_2$	134.13	1.93
17.	16.35	Hexahydrofarnesyl acetone	$C_{18}H_{36}O$	268.47	1.26
18.	17.82	Ethyl palmitate	$C_{18}H_{36}O_2$	284.47	0.97
19.	18.97	Phytol	$C_{20}H_{40}O$	296.53	0.86

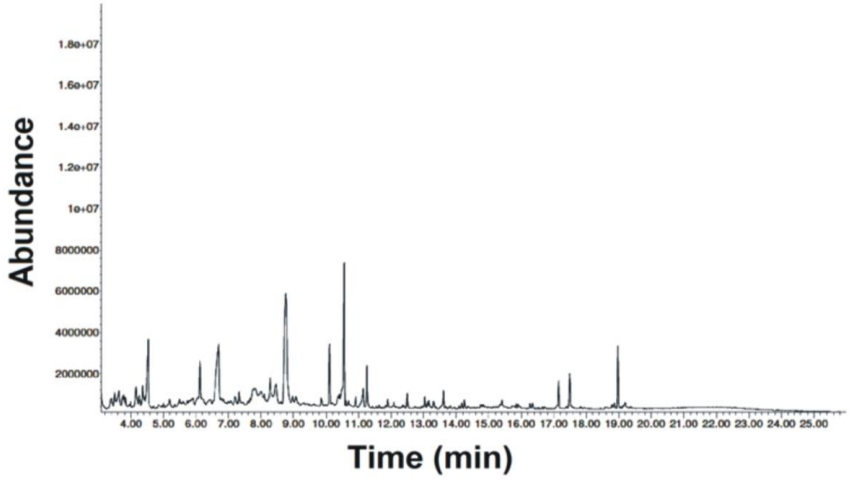


Fig. 5: LC-MS spectra of polar fraction of *Hugonia mystax* L. (Linaceae) Leaves.

Table 2: Identified metabolites by LC-MS from the hydroalcoholic extract of *H. mystax* leaves.

Sr. No.	RT	Chemical name	Mol. formula	Mol. Wt.	Peak area (%)
1.	3.378	2H-Pyran-2-one	$C_5H_4O_2$	96.08	2.34
2.	4.412	Angelica lactone	$C_5H_6O_2$	98.09	2.93
3.	4.53	1,2-Cyclopentanedione	$C_5H_6O_2$	98.09	7.82
4.	5	Furancarboxyaldehyde, 5-methyl	$C_6H_6O_2$	110.11	1.17
5.	6.789	3-Hexenoic acid	$C_6H_{10}O_2$	114.14	1.56
6.	6.845	Guaiacol	$C_7H_8O_2$	124.13	1.56
7.	7.209	4,4 - Dimethylcyclohex-2-en-1-ol	$C_8H_{14}O$	126.19	1.95
8.	7.326	3-Mercaptohexanol	$C_6H_{14}OS$	134.23	2.54
9.	8.76	4-Vinylphenol	C_8H_8O	120.14	12.72
10.	10.111	p-Vinylguaiacol	$C_9H_{10}O_2$	150.17	7.82
11.	10.546	p-Myrcene	$C_{10}H_{16}$	136.23	19.5
12.	10.605	Phenol, 2, 6-dimethoxy-, acetate	$C_{10}H_{12}O_4$	196.19	1.56
13.	11.133	Tetradecane	$C_{14}H_{30}$	198.38	2.93
14.	11.627	alpha-Methyl Ionone	$C_{14}H_{22}O$	206.32	1.17
15.	11.897	Eugenol acetate	$C_{12}H_{14}O_3$	206.23	1.95
16.	12.085	4-Acetoxycinnamic acid	$C_{11}H_{10}O_4$	206.19	1.56
17.	12.379	beta-Methyl Ionone	$C_{14}H_{22}O$	206.32	1.56
18.	12.496	1-Dodecanamine	$C_{12}H_{27}N$	185.34	2.54
19.	13.037	2-Coumaranone	$C_8H_6O_2$	134.13	1.95
20.	14.094	Megastigmatrienone	$C_{13}H_{18}O$	190.28	1.27
21.	14.176	Drimenol	$C_{15}H_{26}O$	222.36	1.27

Cyclopentanedione (7.82%) were found to be major components. The compound Myrcene has an analgesic effect and is likely to be responsible for the medicinal properties of extract. Myrcene showed significant cytotoxic effects in crown gall tumors, MCF-7 breast carcinoma, HT-29 colon adenocarcinoma, and other cell lines [44]. Silva *et al*. [45] investigated the cytotoxicity of myrcene against HeLa (human cervical carcinoma), A-549 (human lung carcinoma), HT-29 (human colon adenocarcinoma), and Vero (monkey kidney) cell lines as well as mouse macrophages. Myrcene exhibited significant anti-inflammatory and anti-catabolic effect in human chondrocytes, which indicates the therapeutic potential of myrcene to halt process of cartilage destruction and osteoarthritis progression [46].

The enzymatic decarboxylation of phenolic acids such as ferulic acid and *p*-coumaric acid leads to the production of corresponding volatile phenols viz. 4-vinylguaiacol and 4-vinylphenol respectively [47]. Therefore, 4-VP and 4-VG identified from MeOH: Water fraction of *H. mystax* leaves might be converted from *p*-coumaric acid and ferulic acid respectively. The anti-angiogenic and anti-inflammatory activity of *p*-coumaric acid and 4-VP has recently been elucidated. 4-VP may possess stronger in vivo anti-angiogenic activities than *p*-coumaric acid because 4-VP could significantly inhibit angiogenesis in breast tumors [48]. It is found that the angiogenesis and inflammation plays a critical role in the pathogenesis and progression of rheumatoid arthritis. Hence, the presence of *p*-Coumaric acid, p-Myrcene and 4-VP in leaves of *H. mystax* justifies its use in rheumatism by traditional healers.

CONCLUSION

From the present investigation, it can be concluded that the plant *Hugonia mystax* L. is very valuable in medicinal usage with highly bioactive phytochemicals present in it. This is the very first study to report metabolites from leaf extract of *H. mystax* from south-west part of India using RP – HPLC, GC-MS and LC-MS techniques. The presence of various bioactive metabolites justifies the use of leaves of *H. mystax* by traditional healers for various ailments. However, in depth in vitro and in vivo study of *Hugonia mystax* leaf extracts will provide a good concrete base for all the biological functions mentioned above. The compounds need further investigation on toxicological aspects to develop safe drugs.

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