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Chromatographic profile from the aqueous extract of aerial parts of *Centratherum punctatum* Cass

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Abstract

Herbal medicine is as primordial as mankind and has appreciably contributed to the health care of the human society. In array to ascertain new bioactive compounds from plant sources which may possibly become new-fangled leads or new drugs, less explored plant extracts should be submitted to chemical screening hyphenated technique such as HPLC and LC-MS. In current study *Centratherum punctatum* Cass. a traditional drug belonging to the family Asteraceae which grows in arid regions is besieged for metabolite profiling. Some triterpenoids, saponins, flavonoids, isoflavonoids, sesquiterpene and flavonols are identified with the help of computer assisted evaluation of the resulting data. Some of the well-known compounds reported to have great pharmacological importance such as antioxidant and anti-inflammatory property. Such type of research opens the gate for discovery of new compounds initiate in metabolite profiling of plants. Liquid Chromatography - Mass Spectrometry (LC-MS) is proved to be a very useful technique for plant metabolite profiling and allows the identification of a large variety of common plant metabolites in a single chromatogram.

Key words : Asteraceae; bioactive compounds, *Centratherum punctatum* Cass. High Pressure Thin Layer Chromatograpy, Liquid Chromatography-Mass Spectrometry (LC-MS) and metabolite profiling.

INTRODUCTION

Plants have been in use as spices, food and folk remedies. The plant kingdom is still an unexploited reservoir of new molecules with potential therapeutic interest and only a relative small percentage of known plant species have been studied from a phytochemical or a pharmacological viewpoint[1]. Research in pharmacognosy has demonstrated that potent bioactive products can be obtained from plants. In the present study one such plant is targeted which is very less explored.

The selected plant *Centratherum punctutam* Cass. (Asteraceae), the Brazilian bachelor button, is one among 33 species of the type genus *Centratherum* and is a perennial bushy plant of 45-60cm height. It has a well branched stem with refreshing scented foliage and purple flower heads. Medicinally used as a pain killer, for snake bite, for inflammation, for sore throat, for urethritis, traumatic condition, in bleeding, cancer and in ulcer. Flower essences are used in the preparation of many herbal medicines. Flowers and leaves are used in hair oil preparations, as skin whitening and antiageing agents. In Bangladesh it is used to increase libido, in pain, bloating, snake bite and tiger bite. It is useful as an insecticidal agent because of phototoxins present in this plant [2]. High performance liquid chromatography (HPLC) and Liquid chromatography mass spectrography (LCMS) profiles were also recorded for which the plant extracts act as a fingerprint for future investigations. HPTLC allows for the separation of the chemical compounds that possess varying polarities thereby allowing for the profiling of the compounds found in the plant species and thus serving as a fingerprint for the chemical constituents of the plant species. HPTLC and LCMS analysis of plant species provide a means for the correct identification of the plant species and also to profile the chemical composition of the plants. No work on HPTLC and LCMS has been reported on this plant herb, hence the research study was taken by the authors to evaluate their biological activities.

MATERIALS AND METHODS

Procedure for material collection and extraction:

Aerial plant parts of *Centratherum punctatum* Cass. for the study were collected from Herbal garden of Srimad Andavan Arts and Science College, Trichy. The identity of the plant specimen was confirmed using Flora of Presidency of Madras [3] . The botanical identiy was confirmed by comparing with the herbarium specimen deposited at Royal botanical Garden Kew (Voucher specimen number K000373089). Aqueous extracts were prepared by boiling the powdered material with water in the ratio of 6:1. The extracts were then filtered and the filtrates were used for LCMS. HPTLC analysis was carried out as a chemical standard.

Chromatographic Profiling

High performance thin layer chromatography

HPTLC was performed with a view to develop chemical standards. Flavonoids were analyzed by HPLC according to the method of [4]. In the present work, analysis of flavonoid was performed with the help of HPTLC instrument. The HPTLC system (Camag, Muttenz, Switzerland) consists of (1) TLC scanner connected with a software under MS Windows NT; (2) Linomat V Sample applicator, (3) Photo documentation system Camag, Reprostar III. About 5gm of the sample was extracted with methanol mixture kept for 24hrs with occasional shaking. Then it was filtered and concentrated to dryness. 50mg of methanolic extract was dissolved in 5ml of methanol and it was used for HPTLC analysis. The same procedure was adopted for chloroform extract. Toluene: Ethyl Acetate: Formic acid (5:4:1) was used as a mobile phase for methanol extract. Toluene: Ethyl Acetate: Diethyl Amine (70:20:10) was used as a mobile phase for chloroform extract. Camag twin trough chamber was used as a chamber used for mobile phase. Chamber Saturation was done for

18hr. TLC plate precoated with silica gel 60 F_{254} was used as stationary phase, obtained from Merck. The thickness of the plate was 0.2mm.

The Chloroform extract and methanol extract solutions were prepared. The TLC plate was activated by heating at 120°C for about 30min prior to use. Methanol extract solution and Chloroform extract solution (2 and 10µl) were applied with Linomat V applicator. The mobile phase used for chloroform extract was Toluene: Ethyl Acetate: Diethyl Amine (70:20:10) and Toluene: Ethyl Acetate: Formic acid (5:4:1) was used as mobile phase for methanol extract. No prewashing of the plate was done. Chamber (Camag twin trough) saturation time was 18h. The TLC plate was kept for development to a migration distance of 8cm. The plate was dried in hot air oven at 108°C for 10min. and scanned at 254nm. The R_f and peak area of the spots were interpreted by using software. The plate was photo documented under 254nm and 366nm light using Camag Reprostar 3, equipped with 12 bit CCD camera.

Liquid chromatography

Liquid chromatography is a fundamental separation technique used in life sciences and related fields of chemistry. Liquid chromatography (LC) combined with mass spectrometry (MS) is a powerful tool for qualitative and quantitative analytics of organic molecules from various matrices, and the use of this hyphenated technique is very common in bio analytical laboratories. In the present study, LC/MSMS methods and the required sample preparation applications were developed for detection of compounds such as flavones, terpene and sesquiterpenes lactones.

Aqueous extract was used for the analysis. For determination of secondary metabolites, micro TOF-Q II (Bruker, Germany), UV detector at 330nm and Quadrupole II for mass analysis and TOF for mass detection was utilized. The column used was UHPLC Dionex C18 RP Acclaim 120Å, 2.1 × 150mm, 3.0µm column (Dionex, USA). Solution A: ACN (1% Acetic acid) and Solution B: Water (1% Acetic acid) was used as mobile phase.

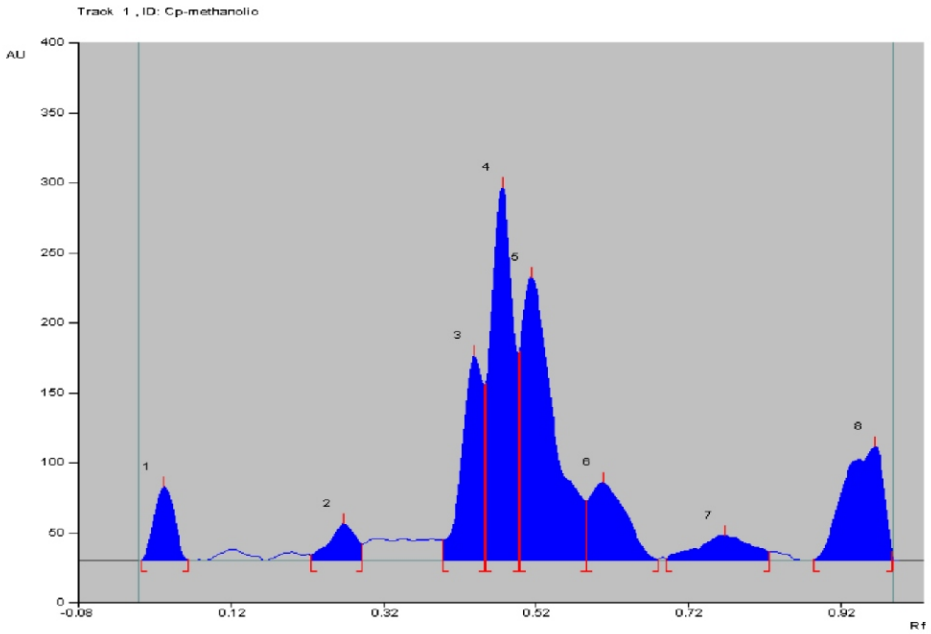


Figure 1: HPTLC chromatogram of methanol extract based on flavonoids (5l)

Table 1: HPTLC fingerprint of methanol extract based on flavonoids

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area%
1	0.00	0.2	0.03	52.5	6.18	0.07	0.5	989.5	4.43
2	0.23	4.1	0.27	26.4	3.10	0.29	12.0	660.4	2.96
3	0.40	14.6	0.44	146.3	17.20	0.45	125.0	2874.4	12.87
4	0.46	126.2	0.48	266.7	31.36	0.50	147.3	5581.9	24.98
5	0.50	149.5	0.52	202.6	23.83	0.59	42.3	6363.6	28.48
6	0.59	42.5	0.61	56.1	6.60	0.68	1.1	1907.6	8.54
7	0.69	1.3	0.77	18.0	2.12	0.83	6.4	925.0	4.14
8	0.89	0.6	0.97	81.8	9.62	0.99	6.0	3039.7	13.6

Starting with 95% B, 5% A to 5% B reached in 5min and to 25% B in another 5min, this was then brought to 45% B in 5min followed by reaching to 60% B in 5min, next 5min to reach 75% B, another 5min to reach at 90% B and finally reaching 5% B in 5min and maintained the same for 10min. 0.2mL/min flow rate 5.0µL of sample was injected. The LC/ESI-MS was conducted in positive-ion mode and operated according to defined conditions: Polarity: Negative; Auto MSn mode; ESI ionization, Nebulizer pressure: 30.5psi; Dry gas (N_2): 6.0 l/min, Capillary voltage: 4500V, Dry heater: 270°C, m/z range: 100-1500 m/z, Collision RF: 350.0Vpp; Collision energy: 15.0eV.

The spectra were obtained for each compound with varying retention time. The spectra were compared with the Mass Bank, Metlin Scripps website and the presence of nature of various compounds was evaluated. The compounds detected were confirmed by their retention time and [M+1] + peaks. The LCMS analysis was based on a method described by [5].

RESULTS

Chromatographic Profiling

High Performance Thin Layer Chromatography

HPTLC fingerprints of the methanol and chloroform extract of the plant drug also determined using single mobile phases for detecting flavonoids. Toluene:Ethyl acetate:Formic acid (5:4:1) was used as mobile phase and scanned at 254nm. The methanol extract gave 9 peaks for 10µl and gave 6 peaks for 5µl concentration [Table.1 and Figure.1; Table.2 and Figure.2] . The chloroform extract gave 7 peaks for 10µl and gave 4 peaks for 5µl concentration [Table.3 and Figure.3; Table.4 and Figure.4].

Liquid Chromatography - Mass Spectrometry (lcms)

The aqueous extract was subjected to LC-ESI-MSMS analysis. Secondary metabolites present in aqueous extracts of *C.punctatum* such as triterpenoids, saponins, flavonoids,

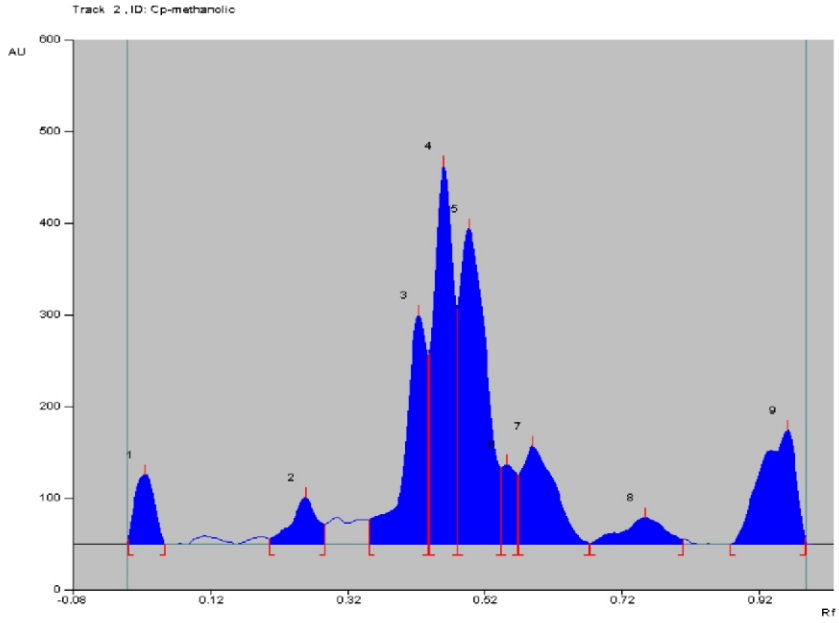


Figure 2: HPTLC chromatogram of methanol extract based on flavonoids (10l)

Table 2: HPTLC fingerprint of methanol extract based on flavonoids

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.00	6.3	0.03	76.1	5.14	0.06	0.4	1561.0	4.14
2	0.21	6.2	0.26	50.8	3.43	0.29	22.0	1365.4	3.62
3	0.35	27.0	0.43	250.2	16.89	0.44	204.4	5866.6	15.55
4	0.44	207.9	0.46	412.3	27.83	0.48	254.0	8400.7	22.27
5	0.48	256.5	0.50	344.4	23.25	0.54	83.4	9503.5	25.20
6	0.55	83.4	0.55	86.6	5.85	0.57	75.9	1323.1	3.51
7	0.57	76.1	0.59	106.8	7.21	0.67	0.7	3746.4	9.93
8	0.68	0.8	0.76	29.5	1.99	0.81	5.7	1315.3	3.49
9	0.88	0.0	0.96	124.5	8.40	0.99	6.8	4633.7	12.29

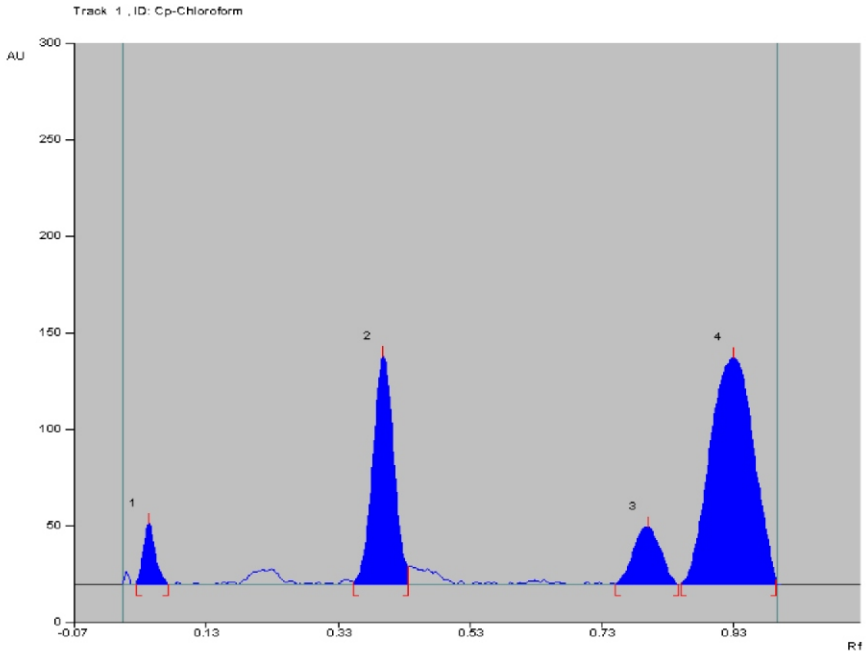


Figure 3: HPTLC chromatogram of chloroform extract based on flavonoids (5 µl)

Table 3: HPTLC fingerprint of chloroform extract based on flavonoids

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.02	0.1	0.04	31.2	10.54	0.07	0.2	437.7	4.27
2	0.35	1.4	0.39	118.0	39.79	0.43	9.3	2875.9	28.03
3	0.75	0.2	0.80	29.6	9.97	0.84	0.0	946.4	9.22
4	0.85	0.6	0.93	117.7	36.70	0.99	2.5	6000.6	56.48

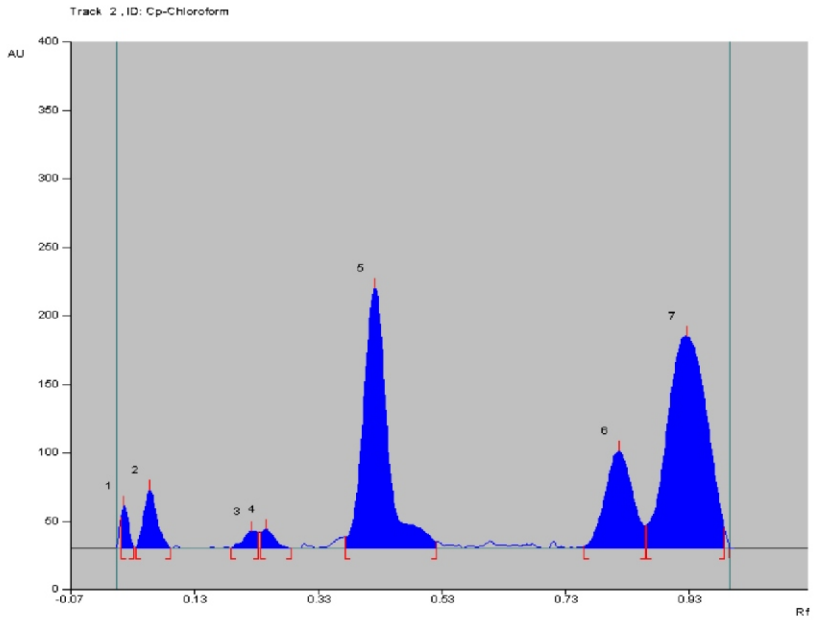


Figure 4: HPTLC chromatogram of chloroform extract based on flavonoids (10µl)

Table 4: HPTLC fingerprint of chloroform extract based on flavonoids

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.01	22.6	0.01	31.1	6.02	0.03	1.1	263.6	1.53
2	0.03	0.2	0.05	42.3	8.21	0.09	0.3	688.1	4.00
3	0.19	0.1	0.22	12.7	2.47	0.23	11.4	229.1	1.33
4	0.23	11.4	0.24	13.9	2.69	0.28	0.1	224.8	1.31
5	0.37	8.2	0.42	190.1	36.84	0.52	4.5	5711.5	33.23
6	0.76	1.3	0.81	70.9	13.75	0.86	16.2	2421.5	14.09
7	0.86	16.5	0.92	154.9	30.02	0.98	14.5	7646.6	44.49

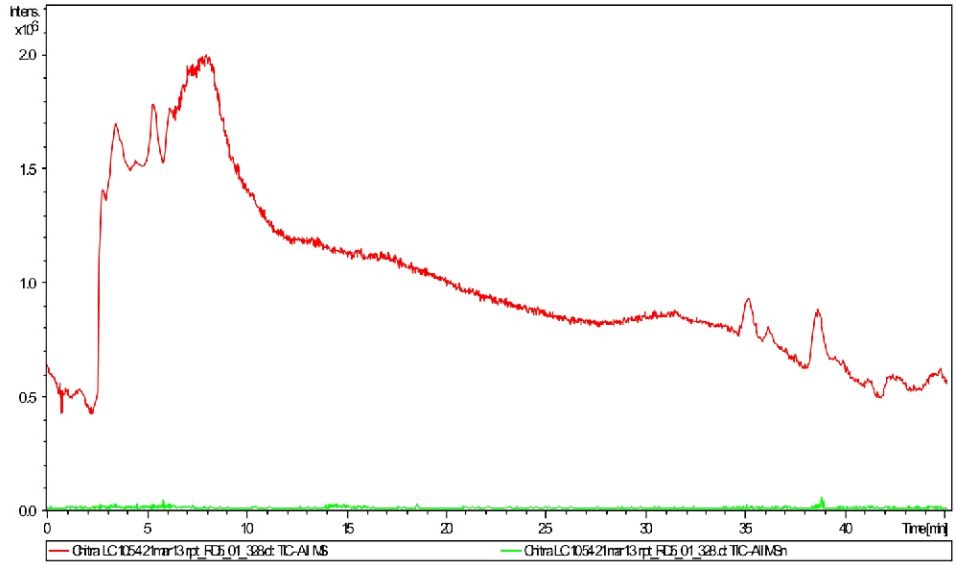


Figure 5.1: Ion Chromatogram of aqueous extract of *C.punctatum* obtained from LCMS

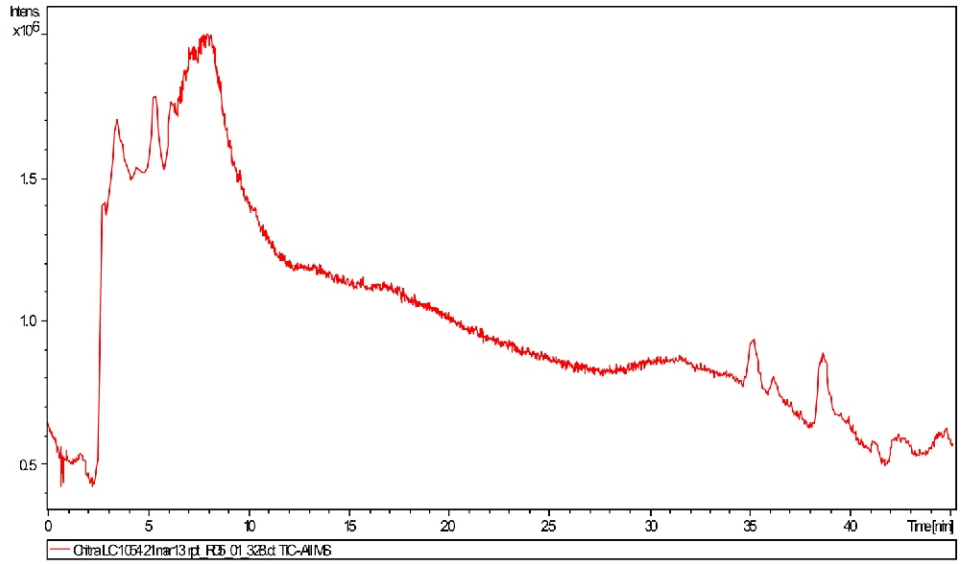


Figure 5.2: MS of aqueous extract of *C.punctatum* obtained from LCMS

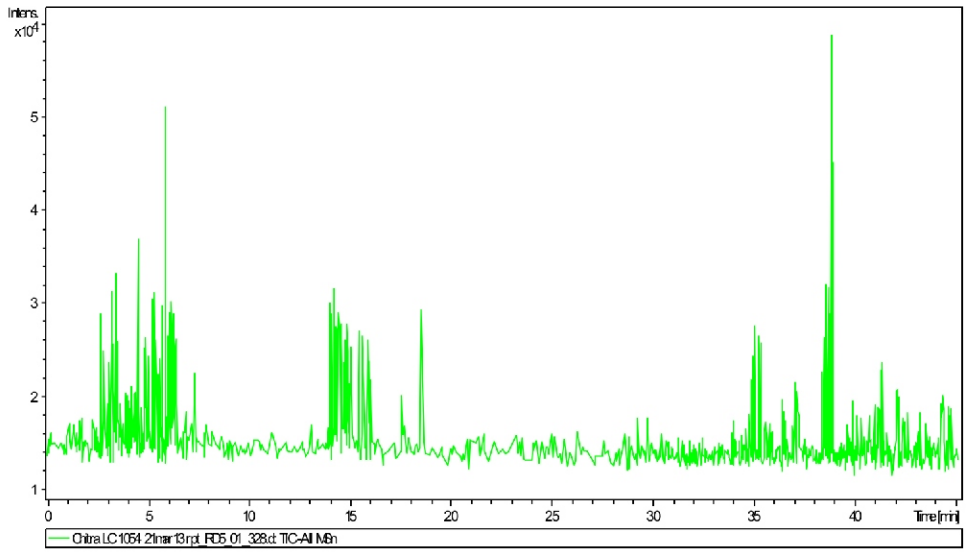
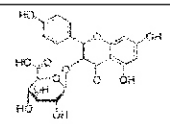
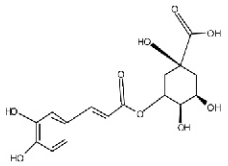
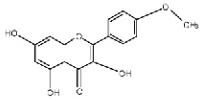
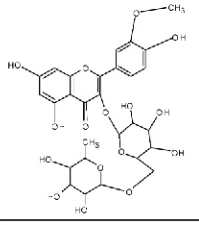
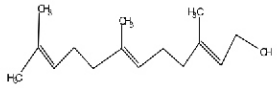
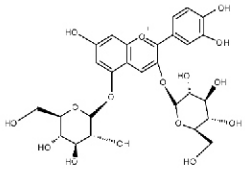
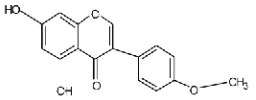
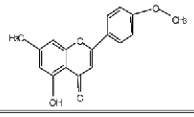
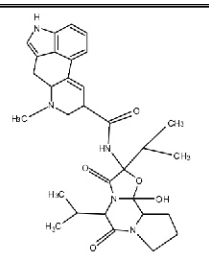
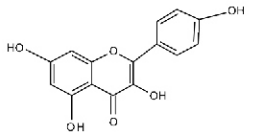
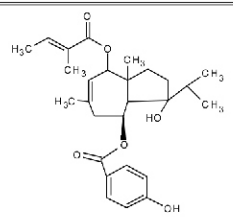
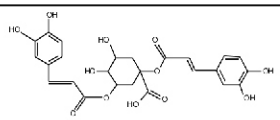
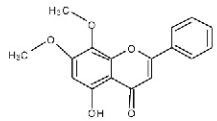
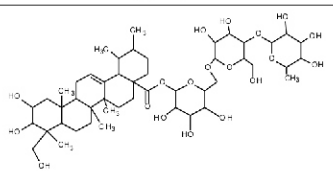
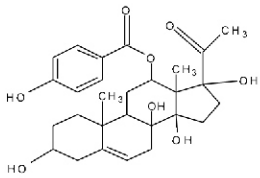
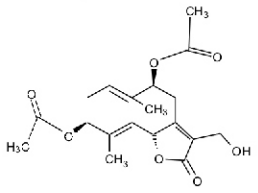
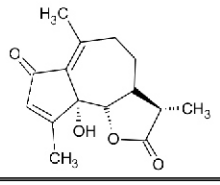


Figure 5.3: MSMS of aqueous extract of *C.punctatum* obtained from LCMS

Figure 5: Molecular Compounds identified by LC-MS in the aqueous extract of *C.punctatum*

S. No.	Compound	MW	RT (min)	Structure
1.	Kaempferol-3-glucuronide	462	2.7-2.1	
2.	Chlorogenic acid	354	2.7-2.9	
3.	Kaempferide	300	3.6-3.7	
4.	Isorhamnetin-3-0-rutinoside	624	18.5	
5.	Farnesol	222	34.134.2	
6.	Cyanidin-3,5-di-0-glucoside	611	36.9-37	
7.	Biochanin-1	284	40.1-40.5	
8.	5-Hydroxy-4'-methoxy-7-methylflavones	282	42.2-42.7	

9.	Ergocornine	561	8.9	
10.	Kaempferol	286	20.2	
11.	Feritidin	456	38.3-39.9	
12.	3,4-dicaffeoylquinic acid	516	2.7-3.1	
13.	7-0-methylwogonin	298	4.1	
14.	Asiaticoside	959	40.1-40.6	
15.	Qingyangshengenin	500	38.5-39.6	
16.	Germacranolide derivative	364	9.1	
17.	Germacranolide derivative	262	9.6	

isoflavonoids, sesquiterpene and flavonols were identified with the help of this technique. The active principles with their molecular weight, retention time and structure are presented in Table 5. The chromatogram and the double mass spectrum of the aqueous extract of the test drug are shown in Figure 5.

The aqueous extract was subjected to LCMS analysis to understand the major molecules present in the selected plant. In the aqueous extracts of *C.punctatum* molecules such as Kaempferol-3-glucuronide, Chlorogenic acid, Kaempferide, Isorhamnetin-3-0-rutinoside, Farnesol, Cyanidin-3,5-di-0-glucoside, Biochanin-1, 5-Hydroxy-4'-methoxy-7-

methylflavones, Ergocornine, Kaempferol, Feritidin, 3,4-dicaffeoylquinic acid, 7-0-methylwogonin, Asiaticoside, Qingyangshengenin and Germacranolide derivative were identified.

DISCUSSION

HPTLC profiles was carried out as it could be used as a chemical standard to detect the genuineness of the selected plant drug. The pharmaceutical and nutraceutical industries are nowadays confronted with adulteration and cheating [6]. Determination of salient standards for herbal drug is inevitable in this field to check adulteration and substitution. HPTLC finger printing observed in the present study could serve as a chemical standard to check the quality and genuineness of the selected plant drug.

Liquid Chromatography - Mass Spectrometry (LC-MS) is proved to be a very useful technique for plant metabolite profiling and allows the identification of a large variety of common plant metabolites in a single chromatogram. The enhanced separation of analytes and better throughput available in this technique for detecting various constituents, lead the bio analytical laboratories to shift from traditional high-performance liquid chromatography (HPLC) to UPLC [7&8].

The research reveals the potential of *C.punctatum* leaves as a good source of bioactive compounds such as Kaempferol-3-glucuronide, Chlorogenic acid, Kaempferide, Isorhamnetin-3-0-rutinoside, Farnesol, Cyanidin-3,5-di-0-glucoside, Biochanin-1, 5-Hydroxy-4'-methoxy-7-methylflavones, Ergocornine, Kaempferol, Feritidin, 3,4-dicaffeoylquinic acid, 7-0-methylwogonin, Asiaticoside, Qingyangshengenin and Germacranolide derivatives that justify the use of this plant for its various ailments by traditional practitioners

CONCLUSION

The HPTLC profile could serve as chemical standard. The chromatographic assay proposed, involving detection by MS, allowed for rapid identification of the main components present in the aqueous extracts and provides an option for the characterization of plant material. In conclusion, the present study gave useful information of standardization parameters for authentication and carry out the further investigation for its use in different ailment.

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