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ORIGINAL ARTICLE

Identification and Characterization of Compounds from Methanolic Extracts of *Launaea procumbens* by Gas Chromatography-MS, Liquid Chromatography-Electrospray Ionization-MS/MS, and Ultra-Performance Liquid Chromatography-Electrospray Ionization-Quad Time of Flight/MS

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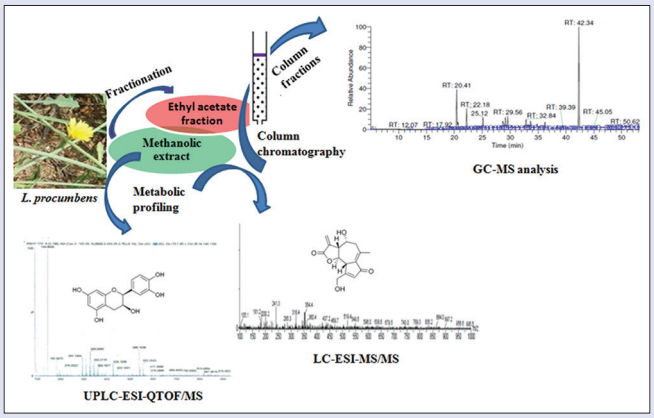
ABSTRACT

Background: *Launaea procumbens* is an important medicinal plant took its place also in the ingredient of food as preparation of sherbet, leaves in making curries, and as a goat fodder in most of the part of India. **Objectives:** The present work was aimed at investigating the phytochemicals of methanolic extract of *L. procumbens* leaves and its isolated mixtures from column fractions. **Materials and Methods:** The method was successfully developed and two new compounds (chlorogenic acid and 7-hydroxyflavanone) by liquid chromatography (LC)-electrospray ionization (ESI)-MS/MS and four new compounds (lactucin, isorhametin, 1-monopalmitin, and 1-hexacosanol) by ultra-performance liquid chromatography (UPLC)-ESI-quad time of flight (QTOF)/MS in *L. procumbens* were identified. Identification of compounds by LC-ESI-MS/MS and UPLC-ESI-QTOF/MS was identified based on the accurate mass of pseudomolecular $[M+H]^+$ ion tandem mass spectrometry (MS/MS) data and by comparing retention times, mass spectra, and molecular weights with those published in the literature. **Results:** The six new compounds, namely chlorogenic acid, 7-hydroxyflavanone, lactucin, isorhametin, 1-monopalmitin, and 1-hexacosanol were identified by in *L. procumbens*. **Conclusion:** A total of 26 compounds by gas chromatography-MS and five compounds by LC mass-ESI tandem mass spectrometry (LC-ESI-MS/MS) and seven compounds by UPLC-ESI tandem mass spectrometry/quadrupole-time-of-flight-mass spectrometry (UPLC-ESI-QTOF/MS) technique were identified.

Key words: Flavonoid, gas chromatography-MS, Launaea, liquid chromatography-electrospray ionization-MS/MS, phenolic, ultra-performance liquid chromatography-electrospray ionization-quad time of flight/MS

SUMMARY

- Total of 27 compounds was detected by gas chromatography-MS analysis
- wo new compounds (chlorogenic acid and 7-hydroxyflavanone) by liquid chromatography-electrospray ionization-MS/MS and four new compounds (lactucin, isorhamnetin, 1-monopalmitin, and 1-hexacosanol) by ultra-performance liquid chromatography-electrospray ionization-quad time of flight/MS in *Launaea procumbens* were identified.



Abbreviations used: UPLC: Ultra-performance liquid chromatography; ESI: Electrospray ionization; LCMS: Liquid chromatography-mass spectrometry; QTOF: Quad time of flight.

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INTRODUCTION

Thousands of years ago, natural products have been the source of active ingredients of medicines and used for the disease management in human health care. There are so many plant extracts or herbal formulations that find their importance in treatment in various diseases.[1] A new discipline of integrative biology with the combination of various discovery tools toward an integrative approach will surely provide a platform for achieving success in natural product drug discovery.[2] Natural product with their therapeutic active moieties has historically

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been proved a major role in human therapy in disease management.[3] Natural product-derived compounds are currently undergoing clinical trials and in preclinical development.[4] Natural products and their derivatives are shown highly biological and chemical diversity used to explore biological relevant important.[5]

Nowadays, modern spectroscopic techniques have largely revolutionized compounds identification for isolated compounds, especially when compounds quantities are very small.[6] Several approaches are available for the identification of compounds, including high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), and electrochemical detection.[7] The analysis of samples must depend heavily upon chromatographic techniques as well as mass spectrometry (MS). Among these liquid chromatography-mass spectroscopy (LC-MS) techniques have gradually eliminated the need for isolating pure compound before the identification.[8] The identification by GC coupled with mass spectrometry (GC-MS) must be based primarily upon the ability of the chromatographic system to separate the *nondrug* regioisomers where different compounds exists they have potential to produce the same or nearly identical mass spectrum.[9] The combination of HPLC with the mass spectrometer has become more and more attractive for the identification of drug at a low level.[10,11]

The family *Asteraceae* (Compositae) has extremely natural taxon, consists of its unique floral and micromorphological features.[12] *Asteraceae* with 1000 genera and 20,000 species[13,14] is the largest and most distributed families of flowering plants.[15] *Launaea procumbens* is a glabrous herb, heavily branched found as a weed throughout India. Milky secretion from this plant used in constipation and leaves is useful in relieving fever in children.[16,17] It is also used in the treatment of skin itches, eczema, ulcers, cuts edema, and rheumatism. Its roots are valuable in the toothache.[18] Previous work revealed the presence of triterpenes, sesquiterpene lactones, flavonoids, coumarins, and steroids in *L. procumbens*.[19-22] In previous studies, chromatographic isolation led to the isolation of many types of compounds including a quinic acid derivative, a flavones glycoside, a pentahydroxy acetylene analog (trideca-12-ene-4,6-diyne-2,8,9,10,11-pentaol), cholistaflasid, nudicholoid, and cholistaquinate.[23]

The aim of this study was to develop an LC-electrospray ionization (ESI)-MS/MS and ultra-performance liquid chromatography (UPLC)-ESI-quad time of flight (QTOF)-MS/MS method for the identification and characterization of compounds together with GC-MS analysis in *L. procumbens* leaves.

MATERIALS AND METHODS

Chemicals

LC-MS grade solvents acetonitrile, methanol, ammonium acetate, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was produced by Milli-Q Advantage system (Millipore, Milford, MA, USA).

Plant materials

The shade dried leaves of *L. procumbens* were collected in the month of June 2018, by local area of Lucknow, and the identification of the plant was conducted in the taxonomy division of the Council of Scientific and Industrial Research, National Botanical Research Institute (NBRI), Lucknow. A voucher specimen (No. 216343) has been submitted in the herbarium of NBRI.

Extraction

The dried leaves (1 kg) of *L. procumbens* were extracted four times with methanol (3000 ml) at room temperature, and then, the filtrates were combined and concentrated to get a dark brown thick, gummy mass (45 g). It was suspended in water and was extracted with n-hexane, ethyl acetate, and saturated butanol subsequently.

Isolation by column chromatography

Column chromatography was performed using silica gel F_{254} (60–120 mesh) as the stationary phase and solvents were used as mobile phase. The ethyl acetate fraction of the *L. procumbens* was subjected to silica gel column chromatography eluting with n-hexane, n-hexane:ethyl acetate, ethyl acetate, ethyl acetate:methanol (100:00, 90:10, 80:20, 70:30, 60:40, 0:100, 95:5, 90:10, 85:15, 80:20, 0:100, respectively) in the increasing order of polarity to obtain 15 fractions. Isolated column fractions were identified by GC-MS, LC-ESI-MS/MS, and UPLC-ESI-QTOF/MS.

Gas chromatography-mass spectrometry

Samples were subjected to the silylation with 70 µL of methoxyamine hydrochloride in pyridine (20 mg/mL) for 2 h at 37°C. The resultant mixture then subjected 40 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide at 80°C for 30 min and analyzed by GC-MS.

GC-MS analysis was performed with a Thermo fisher TRACE GC ULTRA coupled with DSQ II mass spectrometer instrument using a TR 50MS column (30 m × 0.25 mm ID × 0.25 µm, film thickness). Constant flow at 1 mL/min of carrier gas (Helium) was used for the analysis. The injector temperature of the instrument was 220°C and oven temperature was started from 70°C, (hold time 5.0 min) to 290°C with the ramp of 5°C/min (hold time 5 min). The sample was injected in split mode (1:50) with an injection volume of 1µL. The ion source temperature was set at 220°C and transfer line temperature was at 300°C. The ionization of the sample was performed in electron impact mode at an ionization voltage of-70 eV. Mass range was used from *m/z* 50 to 650 amu. The chemical composition of *L. procumbens* leaves was identified by comparing their spectra with those of a NIST/Wiley library and with reference compounds.

Liquid chromatography-mass spectroscopy

Sample analysis was performed with a Waters ACQUITY UPLC™ system (Waters; Milford, MA, USA) attached to a hybrid triple quadrupole-ESI source. Chromatographic separation was performed with a SUNFIRE C_{18} column (250 mm × 4.6 mm, 5µm) and positive mode LC-ESI-MS/MS. For the separation of individual compounds, the mobile phase used (A) acetonitrile (B) 5mM ammonium acetate in 1.5% methanol, under a gradient system: 95% B in 0–1 min; 70% B in 1–10 min; 40% B in 10–14 min; 40% B in 14–16 min; 20% B in 16–24 min; 20% B in 24–32 min; 95% B in 32–35 min; 95% B in 35–40 min. Nitrogen gas was used as the nebulizing and drying gas at flow rates of 30 and 950 L/h respectively. The capillary voltage of ESI source potential was 3.5 kV; cone potential at 30 V for every experiment. Source and desolvation temperature were at 125 and 350°C respectively. Electrospray mass spectra data were recorded on a positive ionization mode for a mass range *m/z* 100 to *m/z* 1000. Data acquisition and processing were performed by using MassLynx V4.1 SCN 714 software.

Ultra performance liquid chromatography/ mass spectrometry (ultra performance liquid chromatography-electrospray ionization-quad time of flight/MS)

The high accuracy mass spectrometric data were performed on a Waters Acquity UPLC chromatographic system (Waters Corp., Milford, USA) coupled with a Waters Q-TOF premier instrument. The separation was carried out a Thermo Betasil C_8 column (250 mm × 4.5 mm, 5µm). The column temperature was maintained at 25°C. The mobile phase consisted of (A) acetonitrile and (B) methanol containing 0.1% formic acid using a gradient elution of 40%–95%, 0–8 min, 95%–95%, 8–25 min, 95%–40%, 25–35 min and initial condition was maintained for 5 min. The injection volume was 10µL. The mass spectrometer was operated in positive ESI mode and spectra were recorded by scanning the mass range from *m/z* 100 to 1500 in MS mode. The accurate mass data of the molecular ions were performed using the Mass Hunter Workstation software.

RESULTS

Characterization of compounds by gas chromatography-MS

The identification of compounds by GC-MS of fractions was based on computer searches by NIST98 and Wiley MS data library 11 edition. The reference compounds of samples such as butanoic acid, azelaic acid, myristic acid, vanillic acid, pentadecanoic acid, palmitic acid, phthalic acid, dibutyl phthalate, octadecanoic acid, maleic acid, glutaric acid, hexanedioic acid, 1-monopalmitin, 1-hexacosanol, β-amyrin acetate, stigmasterol, β-amyrin, α-amyrin and lupeol mentioned under study were also run in GC-MS. The results of the GC-MS analysis of the isolated column fractions of *L. procumbens* leaves are shown in Table 1. A total of 26 compounds were identified from the column fractions obtained from the methanolic extract of leaves of *L. procumbens*. The principle constituent dioctyl phthalate detected in our GC-MS analysis has not been reported in this plant, however, reported in another genus of Launaea, *L. residifolia*.[24] In previous studies, the chemical composition of *L. procumbens* possessed triterpenes such as α-amyrin, β-amyrin, lupeol, taraxasterol, ψ-taraxasterol, 3β-taraxerol, nudicauline A and nudicauline B, 3-keto-13 (28)-epoxy-urs-11-ene, olean-11,13 (18)-diene, 3β-hydroxy-13 (28)-epoxy-urs-11-ene[25,26] and fatty acids like tetradecanoic acid, pentadecanoic acid, hexadecanoic acid.[12] Other compounds salicylic acid, vanillic acid, gallic acid, β-sitosterol acetate have also been reported.[27] The present GC-MS results resemble the chemical composition of previously reported compounds.

Liquid chromatography-electrospray ionization-MS/MS analysis of compounds

Two new compounds in *L. procumbens*, namely chlorogenic acid, 7-hydroxyflavanone and three known compounds methyl gallate, lupenone and β-amyrin acetate were identified by LC-ESI-MS/

Table 1: Chemical composition analysis of column fractions of methanolic extract of *Launaea procumbens* leaves

Compounds	Area %															
	RT	Fr 1	Fr 2	Fr 3	Fr 4	Fr 5	Fr 6	Fr 7	Fr 8	Fr 9	Fr 10	Fr 11	Fr 12	Fr 13	Fr 14	Fr 15
Butanoic acid	7.81	-	-	-	-	-	-	-	7.86	27.78	-	-	-	-	-	-
3-Hydroxybutyric acid	9.28	-	-	-	-	-	-	-	1.58	1.39	-	3.81	-	-	-	-
Butanedioic acid	15.40	-	-	-	-	-	-	-	0.08	-	-	2	-	-	-	-
2,6-Bis (tert-butyl) phenol	20.39	-	2.45	14.93	-	7.27	-	-	0.35	1.68	-	-	5.62	5.41	6.33	-
2,4-Di-tet-butyl phenol	22.20	3.78	3.96	6.50	-	-	0.23	-	0.48	0.95	-	1.01	10.19	11.78	4.05	1.32
Azelaic acid	26.73	-	-	-	-	0.21	-	-	-	-	-	-	-	-	-	1.13
Myristic acid	26.83	-	-	-	-	1.51	-	1.07	-	-	-	0.06	-	1.46	-	1.20
Vanillic acid	27.03	-	-	-	-	-	-	-	-	-	-	1.06	-	-	-	-
Pentadecanoic acid	28.77	-	-	-	1.87	9.49	-	1.97	-	-	-	-	-	-	-	-
Palmitic acid	30.68	3.87	-	3.75	41.51	12.77	0.56	0.01	5.23	4.88	6.66	6.28	11.14	8.50	9.92	1.96
Phthalic acid	31.50	1.87	1.35	1.03	0.18	1.63	0.21	41.28	1.31	2.28	8.30	8.44	1.88	1.58	1.74	1.46
7,9-Di-butyl -1-oxaspiro (4,5) deca-6,9,-diene-2,8-dione	32.31	1.64	4.08	3.49	1.31	0.48	0.37	6.68	1.24	2.00	2.88	11.63	6.77	9.19	4.68	8.48
Dibutyl phthalate	33.66	6.72	8.17	2.73	0.73	0.81	0.76	0.15	0.21	0.26	1.86	3.52	10.98	8.75	9.37	2.07
Octadecanoic acid	34.13	1.72	-	-	1.25	5.23	-	19.82	3.88	2.52	2.96	14.99	1.27	4.77	1.77	-
Maleic acid	34.84	-	-	1.97	-	-	0.11	-	0.14	-	-	-	-	-	-	-
Glutaric acid	36.72	1.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexanedioic acid	38.51	3.17	-	1.24	-	-	0.11	0.78	0.61	1.30	1.36	1.96	2.16	-	6.26	-
1-Monopalmitin	39.29	-	-	-	-	-	-	1.05	5.89	0.86	-	1.10	-	-	0.52	12.48
Diisooctyl phthalate	42.32	11.96	1.83	34.24	0.87	4.18	1.23	3.38	2.77	1.31	18.38	5.25	14.42	9.51	13.00	22.90
1-Hexacosanol	43.85	-	1.98	-	-	0.70	0.46	-	-	0.44	2.59	1.80	-	-	4.07	1.19
β-Amyrin acetate	45.30	1	-	-	2.08	-	20.38	-	-	-	-	-	-	-	1.20	-
Stigmasterol	48.95	0.74	0.62	-	7.37	-	14.50	1.52	13.60	1.74	4.76	2.01	-	-	0.57	8.32
Tris (2,4-di-tert -butylphenyl) phosphate	50.64	36.02	-	-	2.55	5.08	4.08	0.13	-	11.85	-	0.28	-	9.24	4.07	2.32
β-amyrin	51.47	0.52	30.23	-	-	1.75	-	0.37	3.43	0.89	-	-	-	-	-	6.63
α-amyrin	52.51	0.03	11.24	-	-	0.58	-	0.15	-	-	-	-	-	-	-	0.75
Lupeol	52.88	0.15	6.39	-	-	0.43	24.51	0.12	-	-	-	-	-	-	-	1.57

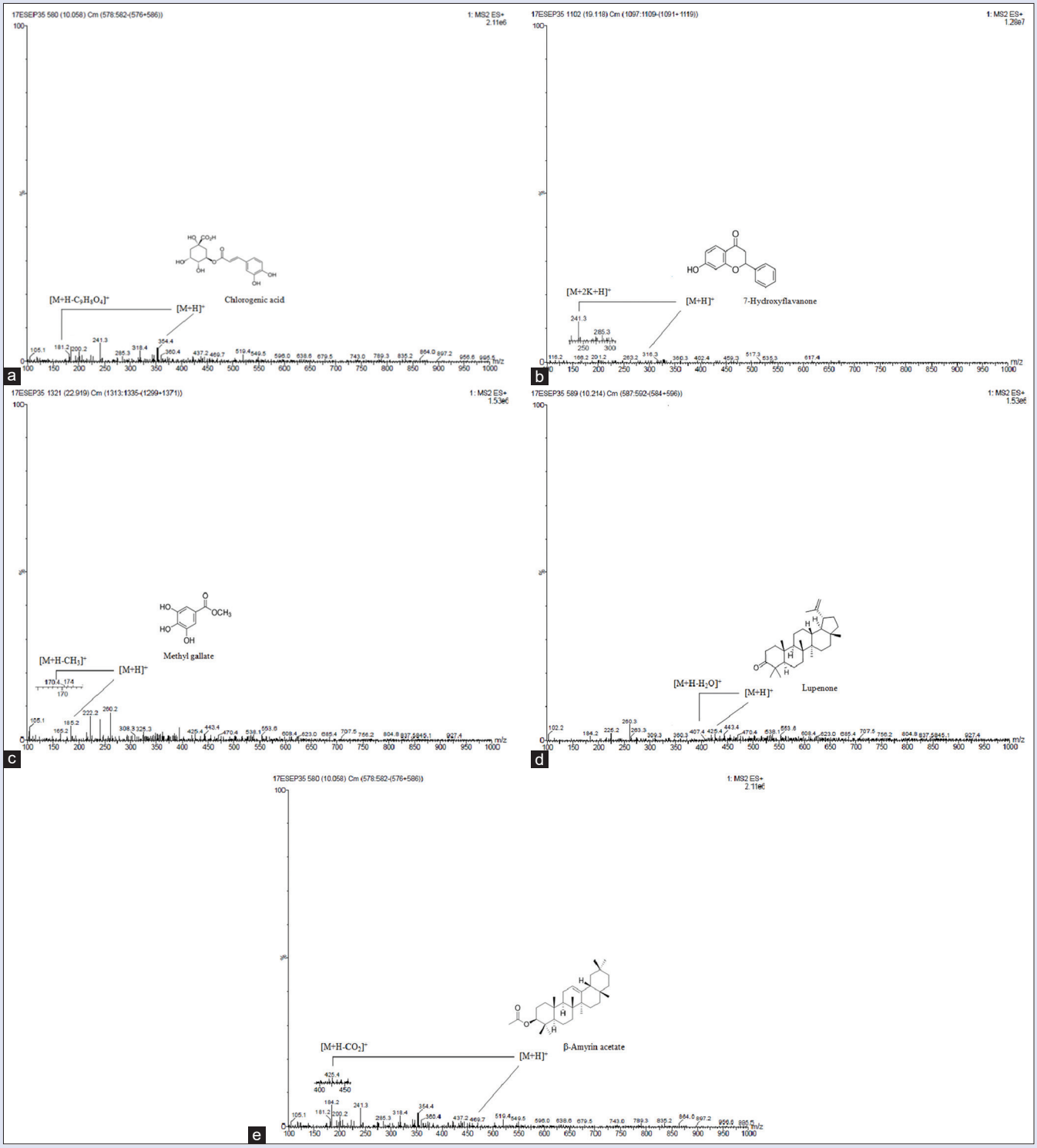

Figure 1: (a-e) MS/MS spectra of identified compounds in *Launaea procumbens* leaves

MS analysis. The identifications were based solely on accurate mass measurements and MS/MS spectra [Figure 1]. The compounds detected in this study were tentatively characterized by means of MS data, together with the mass fragmentation pattern of the observed LC-ESI-MS/MS spectra in comparison with those already reported in the literature.[28]

Hydroxycinnamic acid derivative

To the best of our knowledge, chlorogenic acid was not reported in this plant, however, reported in the other genus of Launaea.[29,30] The MS/MS of the chlorogenic acid showed the pseudomolecular ion $[M+H]^+$ at *m/z* 354 gave the dominant product ions at *m/z* 181

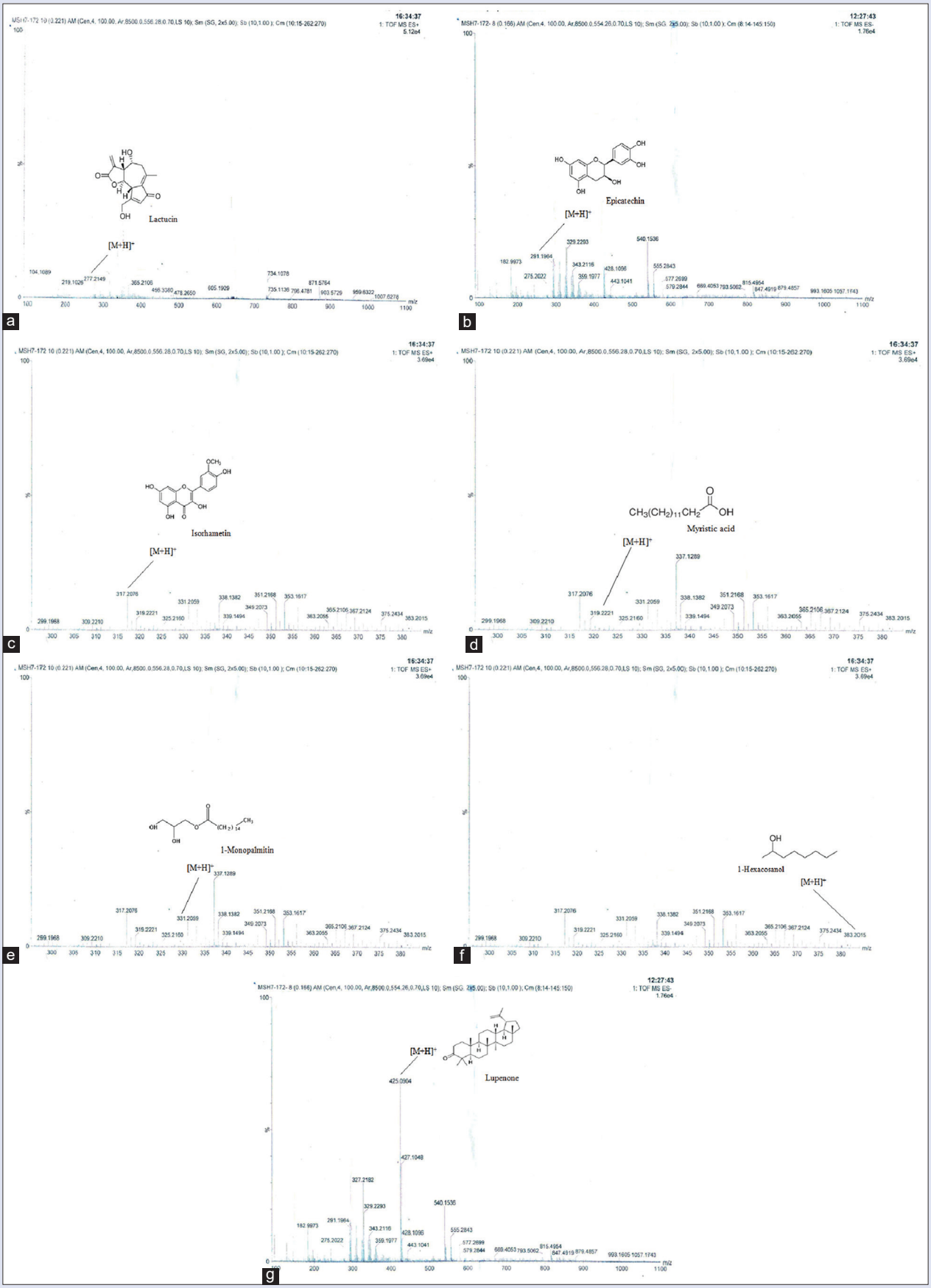

Figure 2: (a-g) Ultra-performance liquid chromatography- electrospray ionization/MS spectra of identified compounds in *Launaea procumbens* leaves

and 193 corresponding to caffeic acid and quinic acid respectively revealed the constitution of chlorogenic acid.[31] Considering the chlorogenic acids are a series of esters formed by quinic acid and certain cinnamic acid, the fragmentation patterns should be similar with those of caffeoylquinic acid. Thus, the cinnamic acid moiety, quinic acid moiety, H_2O and CO should be common chemical groups to be easily eliminated from chlorogenic acid to afford their respective diagnostic product ions.[32]

Flavonoid

The pseudomolecular ion $[M+H]^+$at *m/z* 241 was identified as 7-hydroxyflavanone with its potassium adduct at *m/z* 316 [M+2K+H] often form during storage of the sample in glass solution and detected during flavonoid analysis in ESI (+ve mode).[33] Launaea the genus contains various types of flavonoids and flavonoids glycosides such as flavanone, 7-hydroxyflavanone have been reported in the literature.[34] This paper describes, for the first time, this compound in *L. procumbens*.

Triterpenoid

Two triterpenoids were identified. The pseudomolecular ion $[M+H]^+$ at *m/z* 425 was identified as lupenone corresponding to its dehydrated protonated molecule $[M+H-H_2O]^+$ at *m/z* 407 and pseudomolecular ion $[M+H]^+$ at *m/z* 469 was identified as β-amyrin acetate corresponding to its fragment ion at *m/z* 425 originate from the loss of CO_2 from the carboxylic acid group.[35] Lupenone and β-amyrin acetate were also reported in the other genus of Launaea. These types of pentacyclic triterpenoid are chemio-characteristic of Asteraceae family, including the Launaea genus and reported to have many important activities like anti-inflammatory, hepatoprotection, antioxidant and certain forms of cancer.[36]

Phenolic

The psuedomolecular ion $[M+H]^+$ at *m/z* 185 was identified as a gallate of gallic acid, methyl gallate which gave characteristic fragment ion at *m/z* 170 due to loss of CH_3.[32] This compound has been reported in the *L. procumbens*. Gallic acid is an important polyphenolic compound. It acts as anti-inflammatory and antioxidant agent.[37]

Ultra performance liquid chromatography-electrospray ionization-quad time of flight/MS analysis of compounds

In order to acquire more metabolism information, the extract of *L. procumbens* leaves, it is necessary to develop effective analytical methods for analysis. The compounds, lactucin, isorhametin, 1-monopalmitin and 1-hexacosanol detected first time in this plant by UPLC-ESI-QTOF/MS. It is also shown their confirmation in GC-MS analysis. The peaks of the identified compounds are shown in Figure 2.

In the positive ion mode, seven phytochemicals yielded with their protonated molecule $[M+H]^+$ were tentatively identified based on their mass. Peaks corresponding to the molecular species of lactucin (*m/z* 277), epicatechin (*m/z* 291), isorhametin (*m/z* 317), myristic (*m/z* 319), 1-monopalmitin (*m/z* 331), 1-hexacosanol (*m/z* 383) and lupenone (*m/z* 425) were identified. β-amyrin and α-amyrin cannot be distinguished based on UPLC-ESI-QTOF/MS due to the same molecular formula and exact mass. Lactucin, 1-monopalmitin and 1-hexacosanol and isorhamnetin were not detected in this plant, however, were identified in other Launaea species according to the literature reported. The spectral data showed complete agreement with those reported in the literature and also close similarities reported from the same genus, based on literature report and our findings some of the expected phytochemicals compounds in *L. procumbens*.

CONCLUSION

This study investigated a GC-MS, LC-ESI-MS/MS and UPLC-ESI-QTOF/MS metabolomics-based strategy for the quality assessment of *L. procumbens*. The GC-MS analysis of column fractions revealed that *L. procumbens* was predominantly composed of phthalate, triterpenoids and fatty acids. Phthalates are great importance in the enteric coating of pharmaceutical pills, and as nutritional supplements such as lubricants and emulsifying agents, triterpenoids in anticancer and rich in fatty acid content gives it nutritional value, used as fodder for goats, in the preparation of cooling sherbet and leaves are used locally in curries.[38] Results from phytochemicals study stay in agreement with the literature to the Launaea genus, being the first-ever report of the presence of these compounds (chlorogenic acid, 7-hydroxyflavanone, lactucin, isorhametin, 1-monopalmitin and 1-hexacosanol) in this *L. procumbens*, enhancing the chemical knowledge of this species.

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Conflicts of interest

There are no conflicts of interest.

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