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HPTLC fingerprint profiles and UPLC-MS identification of potential antioxidant fractions and compounds from *Ambrosia maritima* L. and *Ammi majus* L.

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This study aimed to develop HPTLC fingerprint profile and identification of antioxidant molecules from active extract and fractions of *Ambrosia maritima* and *Ammi majus* using ultra performance liquid chromatography-mass spectroscopy (UPLC-MS). The antioxidant activity evaluated by using DPPH (1,1-diphenyl-2-picryl hydrazyl) method and HPTLC fingerprinting were carried out using CAMAG HPTLC system equipped with Linomat IV applicator, TLC scanner III, Reprostar 3, Camag twin through glass tank for development, and Wincasts1.2.3. The methanolic extract of *A. maritima* and *A. majus* shows highest antioxidant radical scavenging activity (87 and 58%, respectively). The ethyl acetate, aqueous fractions of *A. maritima* and aqueous fraction of *A. majus* shows the highest antioxidant activity (86, 82 and 81% respectively). The HPTLC profile of *A. maritima* ethyl acetate fraction indicates presence of nineteen compounds, ellagic (0.61%) and gallic phenolic acids (0.54%) content, respectively and ten compounds have been detected. The HPTLC profile of *A. majus* aqueous fraction indicate presence of twelve compounds, ellagic acid content (0.79%) and six compounds were detected. The HPTLC profile of *A. maritime* aqueous fraction indicate presence of nine compounds, ellagic acid content (2.54%) and eight compounds have been detected by UPLC-MS analysis.

Key words: *Ambrosia maritima*, *Ammi majus,* Antioxidant, HPTLC fingerprint, UPLC-MS analysis.

INTRODUCTION

Oxidative stress was initially defined as a serious imbalance between oxidation and antioxidants leading to potential damage to nucleic acid bases, lipids, and proteins that ultimately leads to cell death by necrosis or apoptosis (Halliwell and Poulsen, 2006; Mariusz and Sławomir, 2013). Cellular damage, due to free radical

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causes serious derangements such as gastric cancer (Vasavidevi et al., 2006), osteoarthritis (Yudoh et al., 2005), diabetes (Haydent and Tyagi, 2002), aortic valve stenosis (Peña-Silva et al., 2009), diabetic nephropathy (Taibur et al., 2012), high altitude pulmonary edema, high altitude cerebral edema (Bailey and Davies, 2001; Chao, 1999), neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease (Chaitanya et al., 2010; Zhang et al., 1999).

Naturally occurring phytochemical antioxidants especially polyphenols (gallic and ellagic acids) (Indira et al., 2002) act as free-radical scavengers, oxidative stress relievers, and lipoperoxidation inhibitors (Velderrain-Rodríguez et al., 2018). Over the years, research on antioxidants and medicinal plants has gained enormous popularity and emerged as a potential therapeutic (Chandra et al., 2013).

*Ambrosia maritima (*Asteraceae) is a widely available weed in the Mediterranean region and African countries, particularly Egypt and Sudan, where it is locally known as Demsissa and grows abundantly near water catchments and on the banks of the Nile River (Tarig et al., 2018). It is widely used in Sudanese traditional medicine for the treatment of urinary tract infections, gastrointestinal disturbance, kidney stones, diabetes, hypertension, asthma, rheumatic pain, bilharziasis, and cancer. It is also used as appetizer, assisting digestion and tonic (Eman et al., 2014; Tarig et al., 2018).

Ammi majus (Apiaceae) is a glabrous annual plant used traditionally for the treatment of skin disorders, psoriasis and vitiligo. It was used as emmenagogue to regulate menstruation, as diuretic, and for treatment of leprosy, kidney stones, urinary tract infections, anti-asthmatic, antihyperglycemic, antispasmodic, carminative, digestive problems, preservative and against snakebites (Boulos, 2009; Corleto, 1993; Selim, 2012).

In recent years, HPTLC has become a conventional analytical approach for the standardization of herbal drugs due to its need for minimum sample clean up (Kaul et al., 2005; Alqasoumi et al., 2011), and many samples can be run simultaneously using a little volume of mobile phase, thus reducing the time and cost per analysis (Faisal et al., 2009; Alam et al., 2011). It can be used for the identification, assay and testing for purity, stability, dissolution or content uniformity of raw materials (Biringanine et al., 2006).

This study aimed to develop HPTLC fingerprint profile of antioxidant active extract fractions of *A. martima* and *A. majus* to quantify the content of gallic and ellagic acids in active fractions.

MATERIALS AND METHODS

Plant materials

The plant materials were collected from different regions in Sudan, authenticated by a taxonomist (Dr. Yahya Suliman) and voucher specimen were kept in the Medicinal and Aromatic Plant Research Institute (MAPRI) Herbarium (Sudan).

Preparation of plants extracts and fractions

Dry plant materials were powdered. 130 g of *A. majus* (seeds) and 50 g of *A. maritima* (leaves) have been taken for successive extraction two times using dichloromethane and methanol 80% as solvents by maceration for 48 h at room temperature. The extracts were filtered using cotton and Whatman filter papers; thereafter, the filtrates were concentrated under reduced pressure using Rotary evaporator and allowed to dry. The dry methanolic extracts of the plants with the higher antioxidant activity were re-dissolved in methanol (50%), and then fractionated three times using 15 ml of chloroform, ethyl acetate, and petroleum ether respectively. All fractions were concentrated under reduced pressure while the aqueous fractions were freeze dried.

Quantitative antioxidant activity using DPPH radical scavenging assay

The DPPH radical scavenging was determined according to the method of Brand et al. (1995) with some modification. In 96-wells plate, the test samples were allowed to react with 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl stable free radical (DPPH) for half an hour at 37ºC. The concentration of DPPH was kept as 300 µM. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate.

Sample preparation for chromatographic analysis

Accurately weighed 20 mg of sample was dissolved in 1 mL methanol. It was further vortexed, filtered through 0.45 µ membrane filter and used for analysis.

Selection of solvent system and HPTLC fingerprinting

After trying number of TLC in different solvent system, maximum number of spot was confirmed by TLC in a specific solvent system; thereafter that solvent system was used for analysis. Prepared samples were filtered and 8 µL of each of the solutions were separately applied on Silica gel 60 F_{254} precoated TLC plates, 5x10 cm (Merck, Germany) with the help of CamagLinomat-V (CAMAG, Switzerland) applicator and the plate was eluted to a distance of 8.5 cm at room temperature (25°C) in specific developed solvent system. The sample solution was applied to 6-mm wide band using CamagLinomat-V automated TLC applicator with the nitrogen flow providing a delivery speed of 150 nL/s from the syringe. Plates were developed in a Camag twin through glass tank pre-saturated with the mobile phase for 40 min. The plate was developed horizontally in Camag horizontal developing chamber (10 × 10 cm) at the room temperature. The scanning was carried out at 254 nm and 366 nm with a Camag TLC scanner III using the Wincats1.2.3 software.

UPLC-MS analysis of the active fraction

The 5 mg/mL solutions of each sample, filtered through 0.2 µM PTFE membrane filter as prepared previously were used for UPLC-MS analysis. In the present study, UPLC was performed on a Water's ACQUITY UPLC (TM) system (Waters Corp., MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tunable MS detector (Waters, Manchester, UK).

Table 1. List of the plant extracts and their radical scavenging activity.

Sample no.	Source	Solvent	%RSA ±SD (DPPH)
1	*Ambrosia maritima L*	Methanol	87± 0.08
2	*Ammi majus*	Methanol	58± 0.03
3	*Ambrosia maritima L*	Dichloromethane	13± 0.02
4	*Ammi majus*	Dichloromethane	Inactive
Standard	Propyl Gallate		90± 0.01

RSD = Radical scavenging activity, SD = Standard deviation.

Table 2. The antioxidant activity of the active extracts fractions.

Sample no.	Source	Solvent	%RSA ±SD (DPPH)
1	*Ambrosia maritima*	Ethyl acetate	86± 0.00
2	*Ambrosia maritima*	Aqueous	82± 0.01
3	*Ammi majus*	Aqueous	81± 0.02
4	*Ammi majus*	Ethyl acetate	43± 0.04
5	*Ambrosia maritima*	Chloroform	36± 0.04
6	*Ammi majus*	Petroleum ether	Inactive
7	*Ammi majus*	Chloroform	Inactive
Standard	Propyl Gallate		91± 0.01

The system was operated under the Empower software (Waters, USA). Data acquisition has been done in positive modes. Chromatography was performed using acetonitrile (A) and 0.5% v/v formic acid in water (B) as the mobile phases on monolithic capillary silica based C18 column (ACQUITY UPLC(R) BEH C18 1.7 µm, 2.1 x 100 mm), with the pre-column split ratio 1:5, flow rate 10 µL/min at ambient temperature. Separation was achieved by stepwise gradients from 5% B to 100% B for 20 min. The flow rate of the nebulizer gas was set to 500 L/h; for cone gas it was set to 50 L/h and the source temperature was fixed to 100°C. The capillary voltages and cone voltage were set to 3.0 and 40 KV respectively. For collision, argon was employed at a pressure of 5.3 x 10-5 Torr. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the Mass Lynx V 4.1 software incorporated in the instrument. Data obtained from UPLC-MS was processed by Mass Lynx V4.1 (Waters, USA). Separated metabolites present in different samples were tentatively identified based on their m/z ration and on literature.

RESULTS

Antioxidant activity of the extracts and fractions

The methanolic extract of *A. maritima* and *A. majus* shows variable antioxidant radical scavenging activity (87 and 58%) respectively, while dichloromethane extract of the two plants having either weak or no reactivity, is as shown in Table 1.

The ethyl acetate, aqueous fractions of *A. maritima* and the aqueous fraction of *A. majus* shows the highest antioxidant activity (86, 82 and 81% respectively); the chloroform fraction of *A. maritima* and the ethyl acetate fraction of *A. majus* shows moderate activity (36 and 43%); while the petroleum ether and chloroform fraction of *A. majus* are inactive as shown in the Table 2. All antioxidant activity results are estimated in comparison with the result of propyl gallate as standard.

Fingerprinting of active fractions using HPTLC

The HPTLC profile of the *A. majus* aqueous fraction indicate the presence of 12 compounds with retention factors (R_f) ranges (0.01-0.87) as shown in Table 3.

The HPTLC profile of the *A. maritima* ethyl acetate fraction indicates the presence of 19 compounds having retention factors (R_f) ranges from (0.01-0.68), while the aqueous fraction of same plant indicate the presence of 9 compounds with retention factors (R_f) ranges (0.03-0.84) as shown in Table 3.

All detected compounds from active fractions of the two plants had different area under the peaks (AU) calculated from the chromatograms obtained under 254 nm and 366 nm UV wavelengths (Figure 1). The purity of the sample was confirmed by comparing the absorption spectra at start, middle and end position of the band.

Quantification of gallic and ellagic acid content in the fractions

From the HPTLC profile using gallic and ellagic acid as referencing compounds, it is clear that the ethyl acetate

Table 3. R_f values of different constituents of *A. majus* and *A. martima* active fractions.

S/N	R_f	*A. majus* aqueous		*A. maritima* aqueous		*A. maritima* ethyl acetate	
		254 nm	366 nm	254 nm	366 nm	254 nm	366 nm
1	0.01	75.2	196.7			2865.6	
2	0.03			771.7	790.6	1940.7	
3	0.04					2117.7	
4	0.06					1686.6	8441.3
5	0.08					3484.3	7533.2
6	0.10	428.5	616.9				
7	0.12					5953.9	15113.3
8	0.13	761.3	910.2	1464.8	3612.7		
9	0.15			810.6	1743.5		
10	0.18					1163.9	5555.5
11	0.23					3682.4	7580.2
12	0.25			1074.5	3237.7		
13	0.30					1467.9	4085.7
14	0.32			1290.9	4859.1		
15	0.34	1578.8	1827.5			4200.7	3482.2
16	0.38						8106.2
17	0.40					3073.9	2879.4
18	0.43	1914.8				1610.6	3438.4
19	0.49					17095.3	23145.4
20	0.50			8468.2	19850.6		
21	0.52	7873.1	19543.7			3580.7	4878.2
22	0.56					11343.3	11801.6
23	0.58	9528.9	6726				
24	0.60						725.9
25	0.62						1113.9
26	0.63	16942.8		5314.9	8118.6		
27	0.66		13669.4				
28	0.68			3647.3	5078	1301.8	1730.3
29	0.74	23579.8	21055.8				
30	0.81	5225.9					
31	0.84			4484.6	13335		
32	0.87	9006.6					

fraction of *A. maritima* contain the two acids with different percentage (0.54 and 0.61% respectively), while the aqueous fractions of *A. maritima* and *A. majus* contain only ellagic acid with different percentage (0.79 and 2.54% respectively). Those have been calculated from the AU of each peak from the fingerprint compared to AU of the standard two acids (Table 4 and Figures 2 and 3).

UPLC-MS analysis of the active plant fractions

A. maritima active ethyl acetate fraction analysis

As shown in Table 5 and Figure 4, ten compounds have been detected from ethyl acetate fraction with different R_f values ranging from (0.82 - 11.24) with different peak intensities. 3 compounds (2',5-Dimethoxyflavone, (*R*)-3-Amino-4-hydroxybutyric acid and Psilostachyin A) were found having the highest peaks indicating their presence in high concentrations.

A. maritima active aqueous extract

Six compounds have been detected from aqueous extract of *A. maritima* with different R_f values ranging from (1.34 -11.24) with different intensities (Table 6 and Figure 5).

Ammi majus aqueous fraction

Eight compounds have been detected from aqueous extract of *A. majus* with different R_f values ranging (0.08 - 6.55) with different intensities (Table 7 and Figure 6).

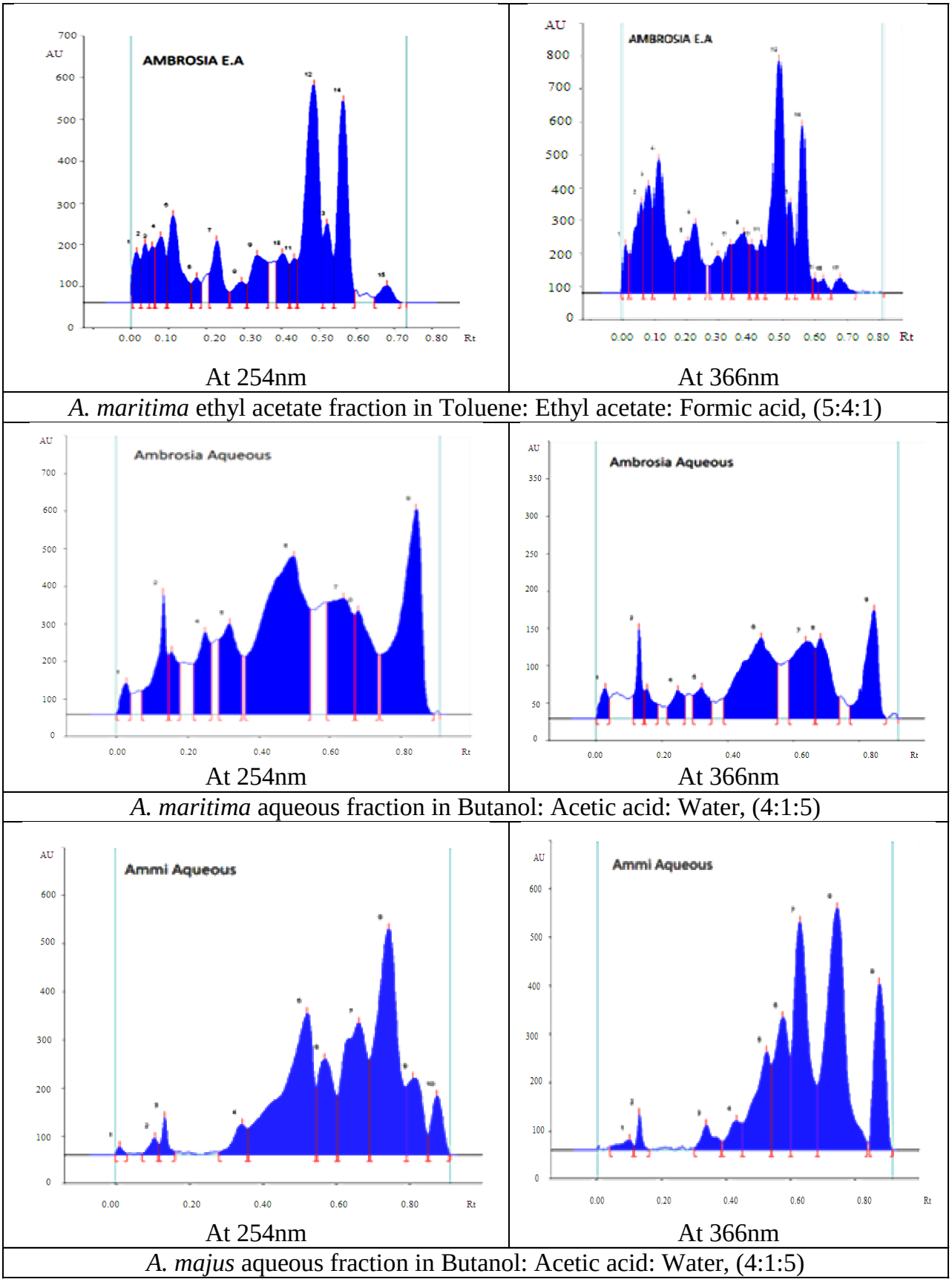

Figure 1. Fingerprint of *A. maritima* and *A. majus* active fractions at 254 and 366 nm.

Table 4. Gallic and ellagic acid content of the active fractions.

Fraction	Ellagic acid %	Gallic acid %
A. maritima ethyl acetate	0.61	0.54
A. maritima aqueous	0.79	0.00
A. majus aqueous	2.54	0.00

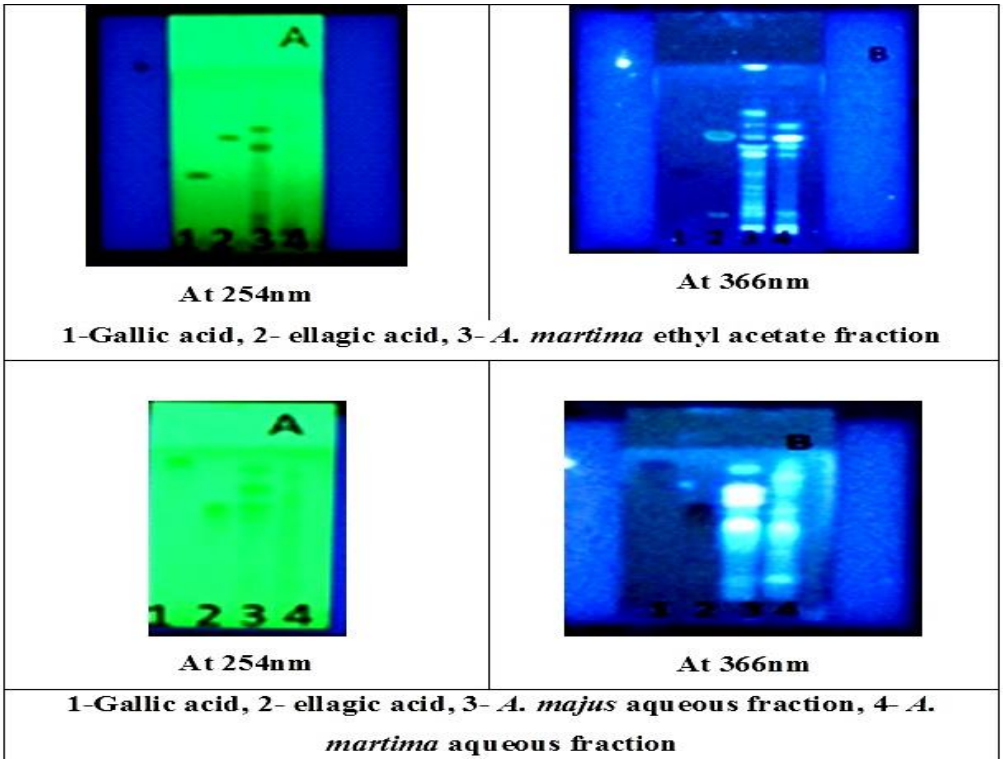

Figure 2. TLC chromatogram of *A. maritima* and *A. majus* active fractions at 254 and 366 nm.

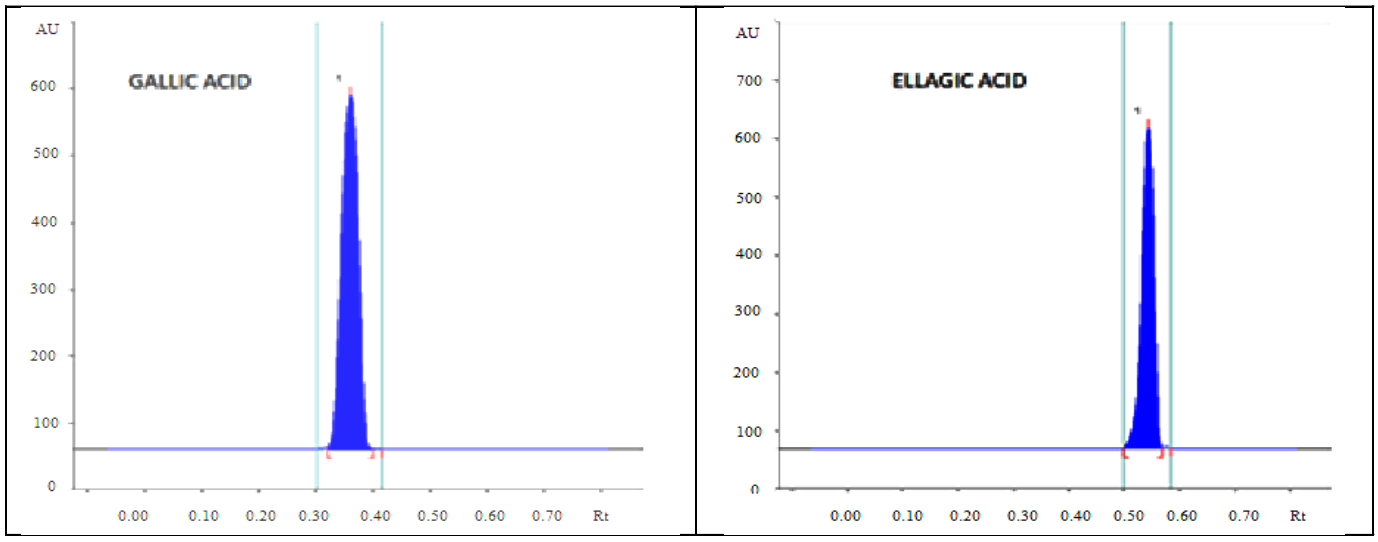

Figure 3. Chromatograms of gallic acid and ellagic acids.

Table 5. List of compounds identified from *A. maritima* ethyl acetate fraction with their R_t and molecular weight.

S/N	R_t	Mol.wt	Compound name
1	0.82	102.85	2-Aminobutyric acid
2	0.96	118.12	(R)-3-Amino-4-hydroxybutyric acid
3	2.72	227.22	Resveratrol
4	3.01	448.59	Kaempferol-3-O-glucoside
5	3.59	453.55	Scutellarein tetra-acetate
6	4.12	340.42	Esculin
7	4.59	280.19	Linoleic acid
8	5.65	111.11	Apomorphine
9	9.06	282.44	2',5-Dimethoxyflavone
10	11.24	280.32	Psilostachyin A

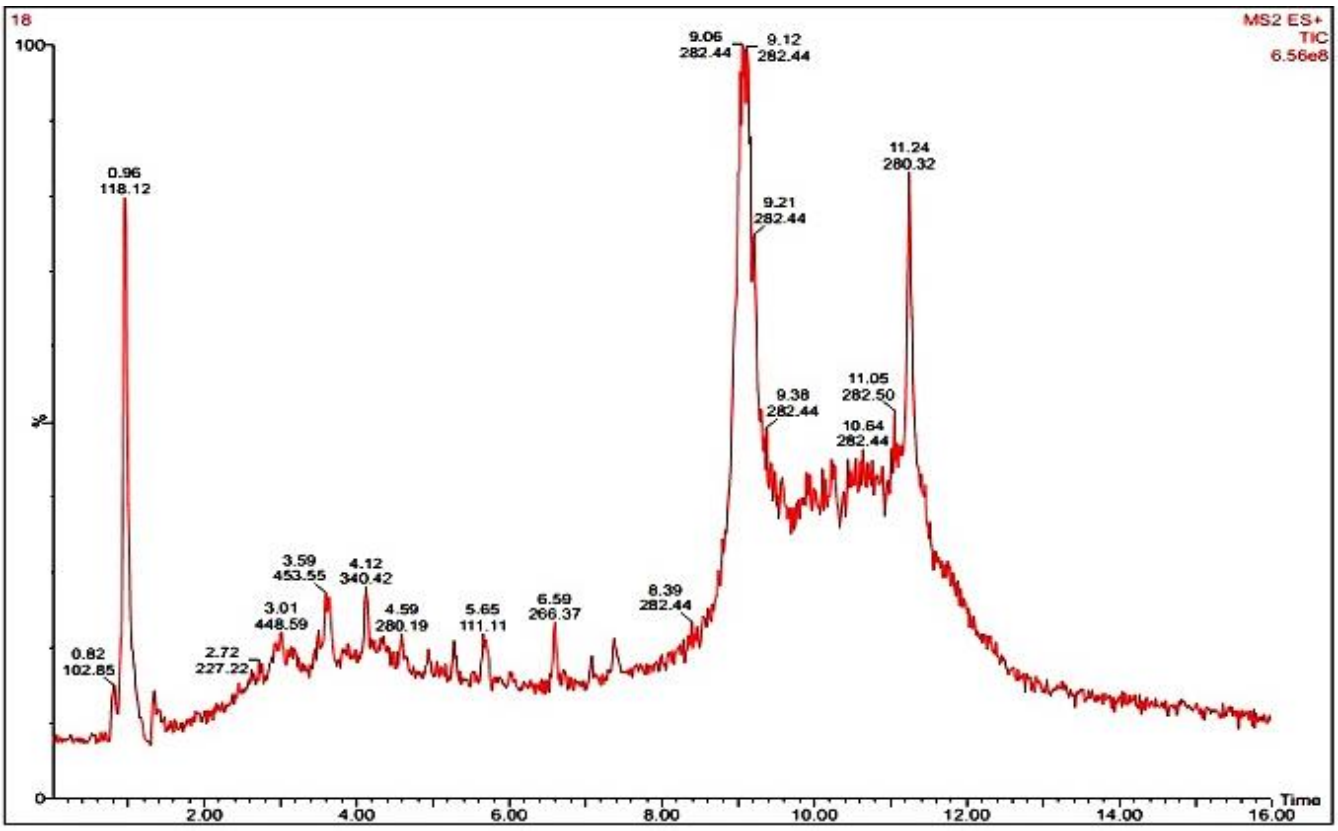

Figure 4. UPLC spectrum of *A. martima* ethyl acetate fraction.

Table 6. List of compounds identified from *A. maritima* aqueous fraction with their R_t and molecular weight.

S/N	R_t	Mol.wt	Compound Name
1	1.34	130.16	Isoleucine
2	2.99	448.40	Kaempferol-3-O-glucoside
3	3.63	453.55	Scutellareintetraacetate
4	4.13	340.49	Esculin
5	5.68	247.19	Matrine
6	11.24	280.52	Psilostachyin A

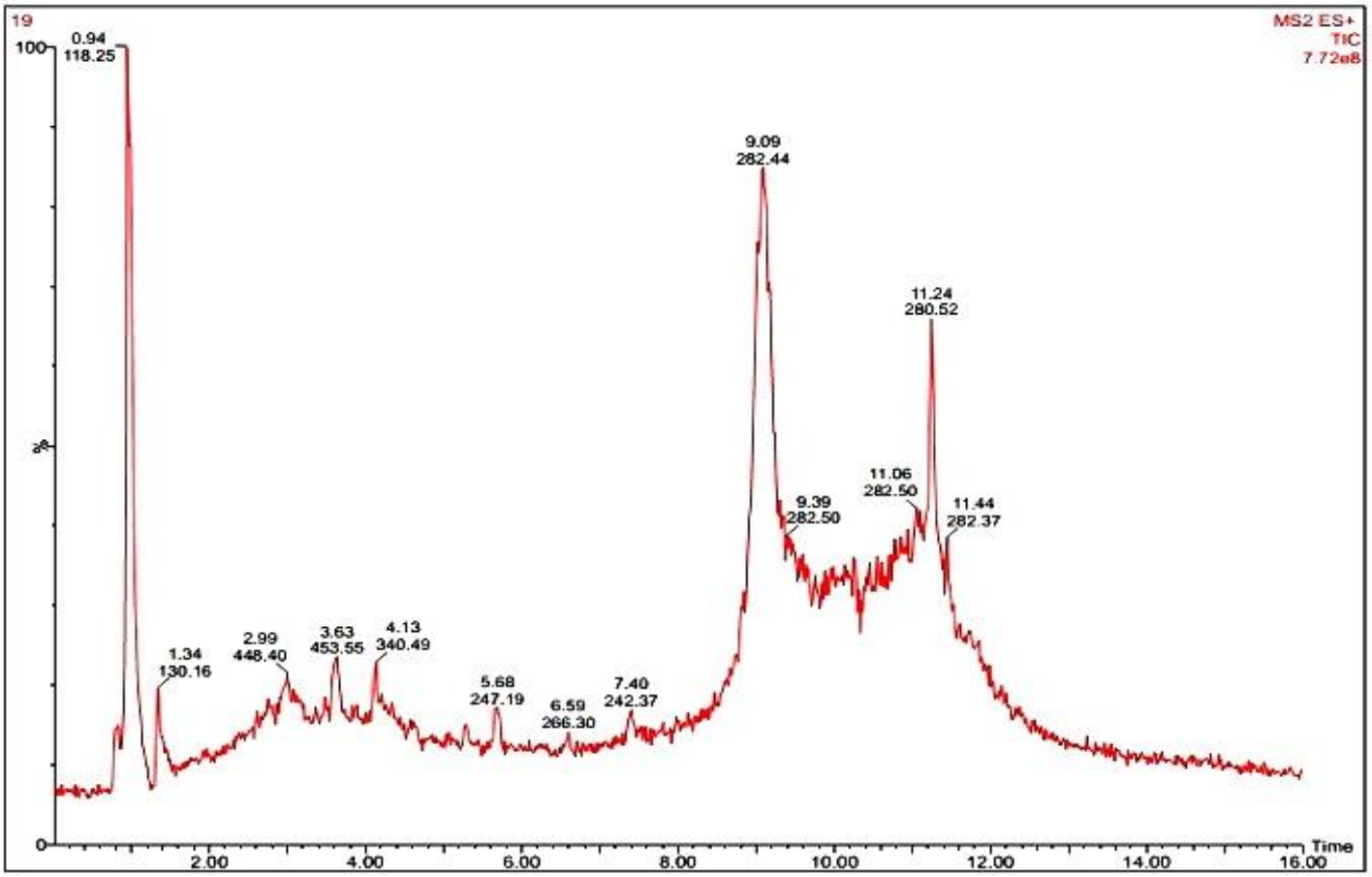

Figure 5. UPLC spectrum of *A. martima* aqueous fraction.

Table 7. List of compounds identified from *A. majus* aqueous fraction with their R_t and molecular weight.

S/N	Rt	Mol.wt	Compound name
1	0.81	128.04	3-Thiophenecarboxylic acid
2	0.99	116.14	Betaine
3	1.34	130.22	4-Hydroxy-L-proline
4	2.60	163.09	2-Coumaric acid
5	3.49	409.19	Mangostin
6	4.04	407.40	Nodakenin
7	5.61	261.21	Tryptophylglycine
8	6.55	275.36	Eserine

DISCUSSION

Herbal medicines have a long therapeutic history. However, the quality control and quality assurance of herbal drugs, singularly and in combinations, still remains a challenge because of the high variability of chemical compounds. This creates a challenge in establishing quality control standards for raw materials and standardization of finished herbal drugs (Chandrakar, 2018). Fingerprint analysis approach using HPTLC has become the most potent technique not only an alternative analytical tool for authentication, but also for quality control of complex herbal medicines (Lalhriatpuii, 2020), as well as qualitative and quantitative estimation of chemicals and bio-chemical markers (Chandrakar, 2018).

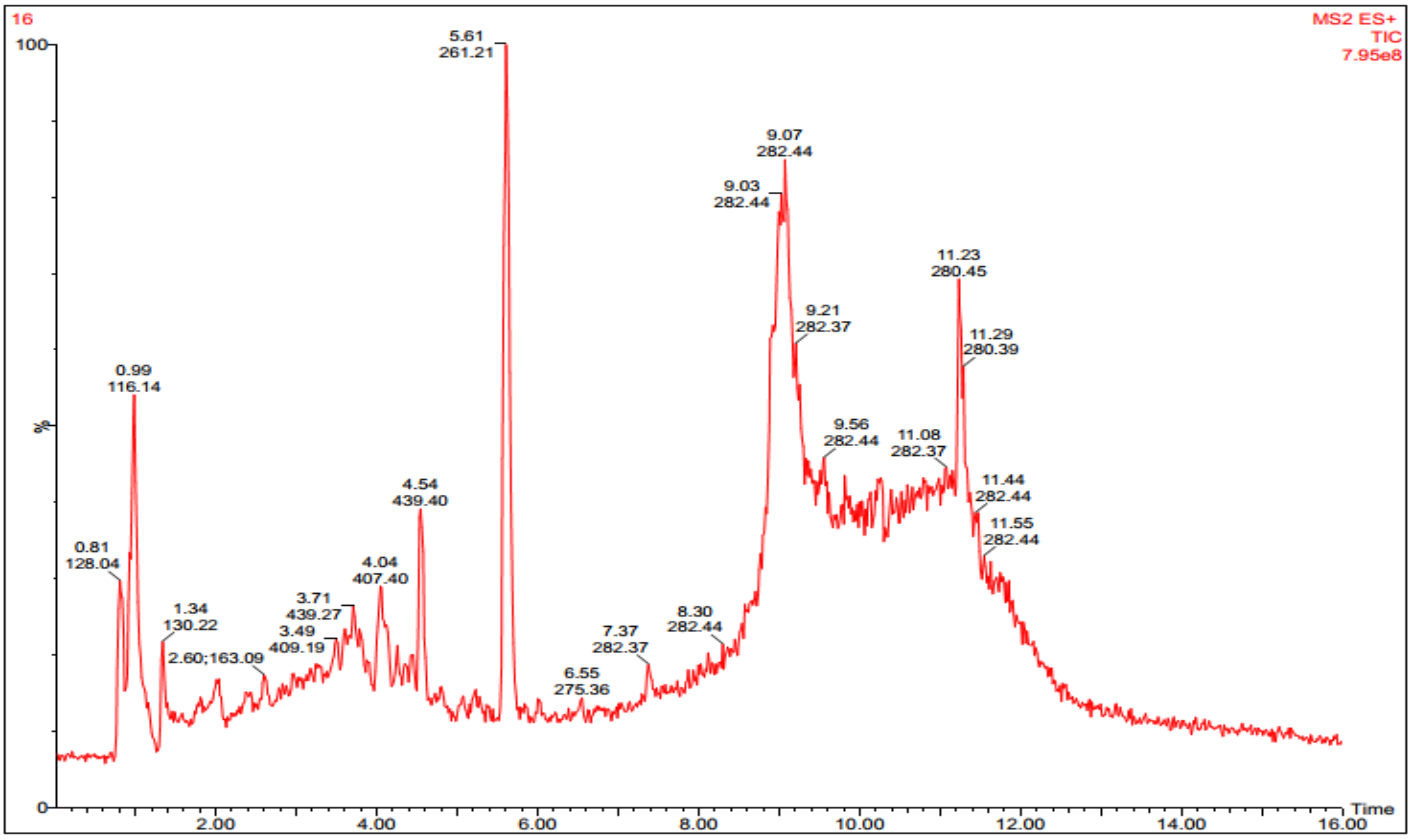

Figure 6. UPLC-MS spectrum of *A. majus* aqueous fraction.

Methanolic extract of *A. maritima* and *A. majus* showed acceptable antioxidant radical scavenging activity (87 and 58%) respectively compared with standard antioxidant agent Propyl Gallate (90%). The ethyl acetate, aqueous fractions of *A. maritima* and the aqueous fraction of *A. majus* showed the highest antioxidant activity (86, 82 and 81% respectively) compared to other fractions (Tables 1 and 2).

The HPTLC profile of the active fractions indicated different number of compounds (twelve for *A. majus* aqueous, nineteen for *A. maritima* ethyl acetate and nine for *A. maritima* aqueous) with different R_t values ranging from 0.01-0.87 (Table 3 and Figure 1). From HPTLC profile, the ethyl acetate fraction of *A. maritima* contain gallic and ellagic acid with different percentage (0.54 and 0.61% respectively) while the aqueous fractions of *A. maritima* and *A. majus* contain only ellagic acid with different percentage (0.79 and 2.54% respectively) (Table 4 and Figures 2 and 3).

UPLC-MS analysis of the active plant fractions indicated presence of ten compounds from *A. maritima* ethyl acetate with R_f values ranging from 0.82 - 11.24 (Table 5 and Figure 3), six compounds from aqueous extract of *A. maritima* with different R_f values ranging from 1.34 -11.24 (Table 6 and Figure 5) and eight compounds from aqueous extract of *A. majus* with R_f values ranging from 0.08 -6.55 (Table 7 and Figure 6).

Conclusion

HPTLC fingerprints and UPLC-MS analysis of the active fractions from *A. maritima* and *A. majus* have been done for the first time, and can be used as rapid and reliable methods in the quality control of the target plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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