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Original Article

GC-MS ANALYSIS, HPTLC FINGERPRINT PROFILE AND DPPH FREE RADICAL SCAVENGING ASSAY OF METHANOL EXTRACT OF *MARTYNIA ANNUA* LINN SEEDS

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ABSTRACT

Objective: To investigate the photochemical constituents present in methanol extract of *martynia annua* seeds using Gas Chromatography-Mass Spectroscopy(GC-MS), High-Performance Thin–Layer Chromatography(HPTLC) analysis and study antioxidant activity.

Methods: Methanol extract of *Martynia annua* seeds were subjected to GC-MS and HPTLC analysis. HPTLC analysis was carried out using GAMAG system with a linomate5 applicator, system mobile phase (Toluene: Chloroform: Ethanol (4:4:1 V/V/V)), two different volume of extract was applied 2 µl and 5 µl. GC-MS analysis was carried out on JEOL GC MATE II, column HP 5 MS and Quadruple double focusing mass analyzer. Antioxidant activity was determined by DPPH assay.

Results: GC-MS analysis provided 17 peaks indicating the presence of seventeen different phytochemicals in methanol extract of *martynia annua* seeds. HPTLC fingerprint showed 6 peaks at both size 2 µl and 5 µl at 254 nm whereas 4 peaks, 9 peaks were detected at 366 nm for 2 µl and 5 µl respectively. After derivatization with 10 % methanolic sulphuric acid, 8 peaks, 11 peaks were detected for 2 µl and 5 µl respectively when the derivatized plate was scanned at 540 nm. DPPH free radical scavenging result showed EC_{50} value of 44.1±1.1 µg/ml.

Conclusion: The GC-MS analysis showed the presence of fatty acids, ester, aldehydes and ketones whereas in HPTLC different peaks at different UV-lights before and after derivatization were observed. Maximum percentage inhibition using DPPH assay was found 74 at concentration of 50 µg/ml.

Keywords: *Martynia annua*, GC-MS, HPTLC fingerprint, Antioxidant activity

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INTRODUCTION

The goals of using plants as source of therapeutic agents not only to use the hole plant or part of it as a herbal remedy but also the bioactive compounds can be isolated from the plant for direct use as drugs, to use agents as pharmacological tools, to produce bioactive compounds of new or known structures as model compounds for semi-synthesis to synthesis patentable entities of higher activity and/or lower toxicity [1]. India is one of the richest in the world in the medicinal plant, has six of the system of medicine (Ayurveda, Siddha, Unani and Yoga, Naturopathy and Homoeopathy) [2]. *Martynia annua* belong to Martyniaceae family [3], it is known as Devil's Claw [4]. It is from Mexico natively [5] and found in throughout India [6]. *Martynia annua* seed is black; the most biologically active parts in this plant are leaves and seeds. Seeds are used in inflammation and considered alexiteric [7]. Due to growing condition of medicinal plants such as climate, harvest season, soil fertility, the drying process, etc, there are considerable qualitative and quantitative differences between chemical constituents of the plant and therefore in its therapeutic effect [8,9]. Nowadays the demand of herbal drugs for primary healthcare is increasing due to their safety, efficacy and rarely have side effect [10]. The pharmaceutical industries focus in investigation of higher plants as a source for novel lead structure as well as for the development of standardized phototherapeutic agents [11]. Standardization of plant material is need of the day [12] but the standardization and quality control of herbal drugs is very complicated due to variation of phytoconstituents within the same plant or from part to part of same plant [13]. Modern chromatographic techniques such as GC-MS and HPTLC fingerprint play an important role for identification, quantification of the chemical composition of plant material and may be useful for proper standardization of herbal drug formulation [14-16]. The aim of this study was to investigate the phytochemical constituents present in methanol extract of *martynia annua* seeds using GC-MS and HPTLC analysis and evaluation of antioxidant activity using DPPH assay.

MATERIALS AND METHODS

Chemicals and regents

1,1-Diphenyl-2-picrylhydrazyl radical(DPPH) was purchased from sigma-aldrish (USA), Ascorbic aid and butylated hydroxytoluene (BHT) were purchased from SDF (Mumbai). All of the other reagents were analytical grade.

Plant material collection

Martynia annua seeds were purchased from local vender-Aurangabad. It was authentication by Dr. Narayan Pandura Department of botany Dr. Babaseab Ambedkar Marathwada University-Aurangabad. Plant seeds were washed, dried in shadow at room temperature for more than 20 d, ground into powder form and kept in an airtight container in dark place until the time of use.

Preparation of extract

50 mg of dried *martynia annua* seeds powder was extracted with 95% methanol (500 ml) by using Soxhlet apparatus for 6 h. After extraction, the solvent was removed using rotary vacuum evaporator; free solvent extract was kept in amber bottle in refrigerator.

Gas chromatography-mass spectroscopy (GC-MS)

GS-MS analysis of methanol extract of *martynia annua* seeds was carried out in Indian Institute of Technology (IIT)–Madras. GC-MS analysis was done by using JEOL GC MATE II (GC model) equipped with HP 5 MS column. High pure helium as carrier gas at a constant flow rate of 1 ml/min was used for GC separation. Injector temperature was set at 220 °C and Oven temperature was set as 50 °C raised to 250 °C at 10 °C/min. Total GC running time was 30 min.

High sensitive quadruple double focusing mass analyzer was used and equipped with photon multiplier tube as the detector; mass range of 50 to 600 amu; and ionization voltage (Electron impact ionization) 70 eV was used.

High performance thin–layer chromatography (HPTLC)

HPTLC study was reformed by following Reich and Schibli guidelines [17].

Sample application

The plant extract was dissolved in chromatographic grade methanol, two and five µl of extract solution individually was loaded as 8 mm band length in the plate format 200 ml ×100 mm Merck, TLC plate's silica gel 60 F_{254} using LINOMAT 5 applicator attached to CAMAG HPTLC system which was programmed through winCAT software, version 2.5.18053.1

Spot development

After the application of sample, the chromatogram was developed in twin Trough Chamber (TTC) 20 ×10 cm which saturated with mobile phase Toluene: Chloroform: Ethanol (4:4:1 V/V/V), saturated time 20 min. After development, the plate was dried at room temperature for 5 min.

Photo-documentation

The plate In Photo-Documentation under GLP (Camag Visualiser), images were captured at white light, UV 254 nm and UV 366 nm

Scanning

The plate was fixed in CAMAG TLC Scanner 4 and scanning was done at UV 254 nm (Absorption, Lamp: Deuterium, Filter: K320) and UV 366 nm (Fluorescence, Lamp: Mercury, filter: K400).

Derivatization

The developed plate was immersed in immersion device which contains 10% methanolic sulphuric acid with dipping speed 5 mm/sec., the derivatized plate was heated at 100 °C for 3 min, the plate was photo-documented in white light and 366 nm. The scanning for a derivatized plate was done at 540 nm (Absorption, Lamp: Tungsten).

DPPH free radical scavenging assay

The antioxidant activity of the extract was determined using 1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH). DPPH scavenging activity was measured by the spectrophotometric method with minor modification (Sreejayan and Rao, 1996) [18]. 0.05 ml of the extract dissolved in DMSO were diluted to 1.0 ml using (ethanol) to attain the concentrations 10-50 µg/ml, and were added with DPPH (200 µM, prepared in 95% ethanol). An equal amount of DMSO+ethanol was added to the control. All the tests were performed using triplicate aliquots. The decrease in the absorbance of test compounds was read at 515 nm after 20 min incubation in dark using spectrophotometer (Shimadzu UV-1800) and the percentage inhibition was calculated by using the formula:

$$\textbf{\% scavenging activity} = \frac{\mathbf{A} - \mathbf{B}}{(\mathbf{A})} \times \mathbf{100}$$

Where A is a control absorbance = the measurement of DPPH solution without extract while B is a sample absorbance = the measurement of DPPH solution with the extract. Ascorbic acid and butylated hydroxytoluene (BHT) were used as standard drugs.

Statistical analysis

Antioxidant activity (DPPH assay) of methanol extract of *martynia annua* seeds were performed in triplicate (n= 3). The presented results were mean±SE (Standard Error). For calculation of EC_{50}, the data were analyzed by Non Linear Regression followed by effector v/s response analysis on Graphpad Prism 5.0.

RESULTS AND DISCUSSION

GC-MS: The GC-MS chromatogram is shown in fig. 1. As it is difficult to find out or isolate individual component in their purest form, the chromatogram can be used as a fingerprint for the identification of the herbal drug. The different phytochemicals corresponding to different retention time can be predicted and hence these are enlisted in table 1. The higher fatty acids and their esters are dominating. Hence this extract can be a good surfactant which helps to reduce the surface tension of the solvent. GC-MS chromatogram of methanol extract of *martynia annua* seeds (fig. 1) shows 17 peaks, indicating the presence of 17 phytoconstituents. RT value, peak area, peak area %, molecular formula and compound name of each peak was shown in table 1.

Table 1: Phytochemicals compounds identified in methanol extract of *martynia annua* seeds

	RT(min)	Peak area	Peak area %	Peak height	Molecular formula	Compound name
1	3.48	8798052	1.25	439367	C_4H_7NS	Thiazole, 4,5-dihydro-2-methyl-
2	9.47	17583168	2.5	759620	$C_6H_{12}O_2$	4-hydroxy-3-hexanone
3	10.5	14658873	2.08	769046	$C_{10}H_{14}O$	3.5-Heptadienal,2-ethylidene-6-methyl-
4	11.68	21532196	3.06	1114019	$C_{10}H_{10}N_2$	1H-Pyrazole,1-methyl-3-phenyl-
5	12.15	26220488	3.72	1674195	$C_{15}H_{32}$	Pentadecane
6	12.38	28780778	4.08	1262519	$C_{15}H_{24}O$	2,6,10-trimetyl-12-oxatricyclo[7,3,1,0(1,6)]tridec-2-ene
7	13.67	36055692	5.12	2756467	$C_{16}H_{22}O$	Cycloisolongifolene,8,9-dehydro-9-formyl-
8	13.97	40451350	5.74	1640731	$C_{15}H_{10}O_2$	Flavone
9	15.62	40906514	5.80	2632072	$C_{12}H_{14}O_3$	2-Propenoic acid,3-[4-methoxyphenyl]-, ethyl ester
10	16.77	40309114	5.72	1677498	$C_{16}H_{28}O$	2,6,10-Dodecatriene,3[E],7[E], 11-trimethyl-1-methoxy-
11	17.02	41343040	5.87	3292430	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester-
12	18.05	76337075	10.83	3251117	$C_{16}H_{32}O_2$	Palmitic acid
13	18.68	68868881	9.77	3451975	$C_{19}H_{34}O_2$	8,11-Octadecadienoic acid, methyl ester
14	19.53	80905437	11.48	3408908	$C_{19}H_{36}O$	12-Methyl-E,E-2,13-octadecadien-1-ol
15	20.67	55785668	7.92	3282854	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid[Z,Z]-
16	23.17	57618947	8.18	2430096	$C_{21}H_{38}O_2$	Isopropyl linoleate
17	25.47	46995620	6.67	2103695	$C_{21}H_{38}O_4$	9,12-Octadecadienoic acid,[Z,Z]-,2,3-dihydroxypropyl ester

GC-MS result showed that the first major percentage compound was 12-Methyl-E,E-2,13-octadecadien-1-ol (11.48%) which have been reported to have anticonvulsant, antibacterial, antihistaminic, anesthetic, antioxidant, allergenic, anagelsic, antiseptic, and anti-salmonella activity [19]. The second major percentage compound was Palmitic acid (10.83%) which possesses antimicrobial, anti-inflammatory, antiandrogenic flavor, antioxidant, 5-Alpha-reductase inhibitor, hypocholesterolemic, hemolytic, nematicide, potent mosquito larvicide, and pesticide activity [20, 21]. Third major percentage compound was 8, 11-Octadecadienoic acid, methyl ester (9.77%) which have no biological activity reported. Isopropyl linoleate was the fourth major compound with peak area percentage of 8.18%, it possesses antioxidant, antimicrobial, and anticancer activity [22]. The fifth major percentage compound was 9,12-Octadecadienoic acid[Z,Z]-(7.92%) which have been reported to have anti-inflammatory, anti-adherent vegetable, and nematicide activity [23, 24]. 9,12-Octadecadienoic acid,[Z,Z]-,2,3-dihydroxy-propyl ester possesses antieczemic, hypocholesterolemic,

nematicide, and hepatoprotective [25]. 9,12-Octadecadienoic acid,[Z,Z]-,2,3-dihydroxypropyl ester is fatty acid and have been reported to have antioxidant, antimicrobial activity [26]. Some of minor percentage compounds have been reported to have biological activities such as 2-Propenoic acid, 3-[4-methoxyphenyl]-, ethyl ester possesses antimicrobial activity [27]; Flavone possesses antioxidant, hypocholesterolemic, and androgenic activity [28]; Pentadecane has antioxidant and antibacterial activity [29]. GC-MS result showed that the *martynia annua* seeds contain bioactive compounds that have medical importance like antioxidant activity.

GC-MS result showed the presence of seven of Fatty acids or their esters: 2-Propenoic acid,3-[4-methoxyphenyl]-,ethyl ester(5.80%), Hexadecanoic acid, methyl ester (5.87%), Palmetic acid (10.83%), 8,11-Octadecadienoic acid, methyl ester (9.77%), 9,12-Octadecadienoic acid [Z,Z]-(7.92%), Isopropyl linoleate (8.18%), 9,12-Octadecadienoic acid,[Z,Z]-, 2,3-dihydroxypropyl ester (6.67%) with retention time 15.62, 17.02, 18.05, 18.68, 20.67, 23.17 and 25.47 respectively.

HPTLC

The various HPTLC chromatograms at different sample size (2 µl and 5 µl) and at a different wavelength (254 nm and 366 nm) and after derivatization (540 nm) have been shown in fig. 2 to 9. Initially, the HPTLC developed plate was photo-documented at white light, at 254 nm and 366 nm (fig. 2) and scanned at 254 nm and 366 nm. The plates were derivatized using 10% methanolic sulphuric acid and photo-documented at white light and at 366 nm (fig. 3) and scanned at 540 nm.

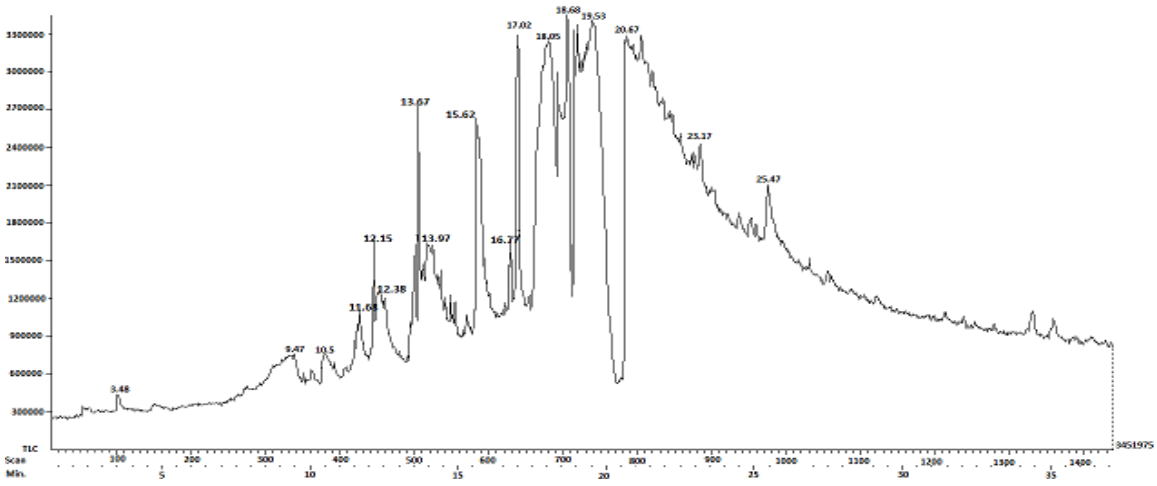

Fig. 1: GC MS chromatogram of methanol extract of *martynia annua* seeds

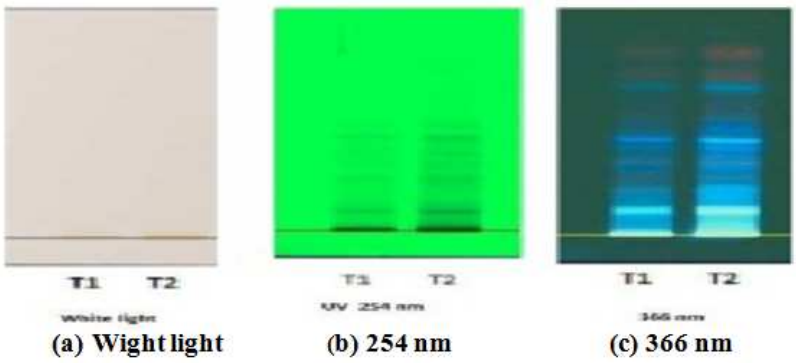

Fig. 2: HPTLC chromatogram (a) white light (b) at 254 nm (c) at 366 nm for different size T1: 2 µl, T2: 5 µl

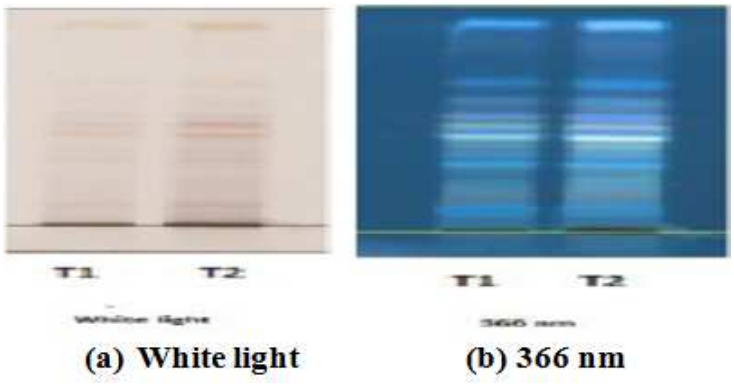

Fig. 3: HPTLC chromatogram after derivatization (a) white light (b) at 366 nm for different size T1: 2 µl, T2:5 µl

For 2 µl sample size, six peaks were detected and the highest peak area 28.10 % appears at R_f= 0.105 when the developed plate was scanned at 254 nm, but at the UV wavelength 366 nm, the number of peaks reduced to four and highest area (62.70 %) covered at peak maxima R_f= 0.123. After derivatization, the number of peaks increases to eight and highest peak get shifted to R_f= 0.426 with percentage area = 23.21 % (table 2).

When the sample size is increased to 5 µl, the highest peak area (25.01%) appears at R_f = 0.439 for 254 nm. When developed plate scanned at 366 nm, the number of peaks observed to be nine and at R_f= 0.126 highest peak area (48.33%) observed. After derivatization and derivatized plate scanned at 540 nm, the number of peaks increased to eleven with highest peak area (26.72%) at R_f= 0.473.

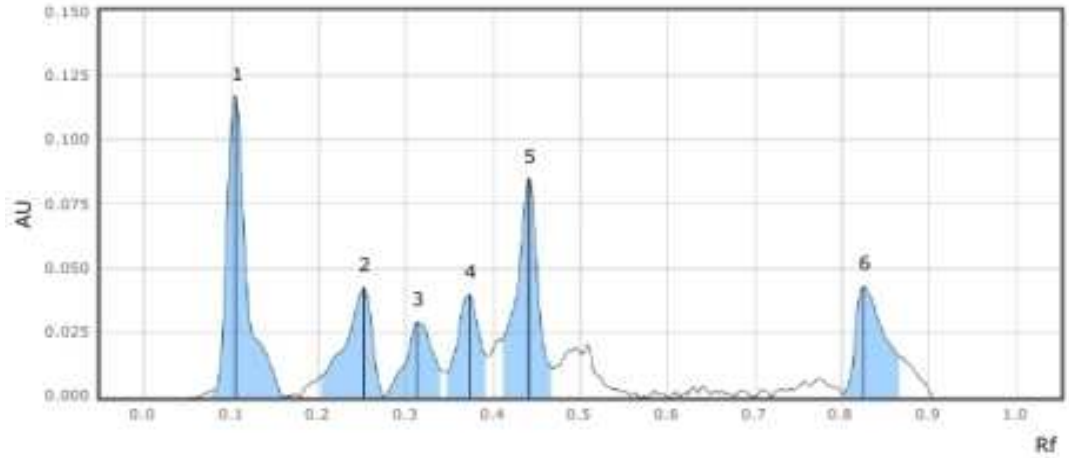

Fig. 4: HPTLC densitometric chromatogram at 254 nm, size T1: 2 µl

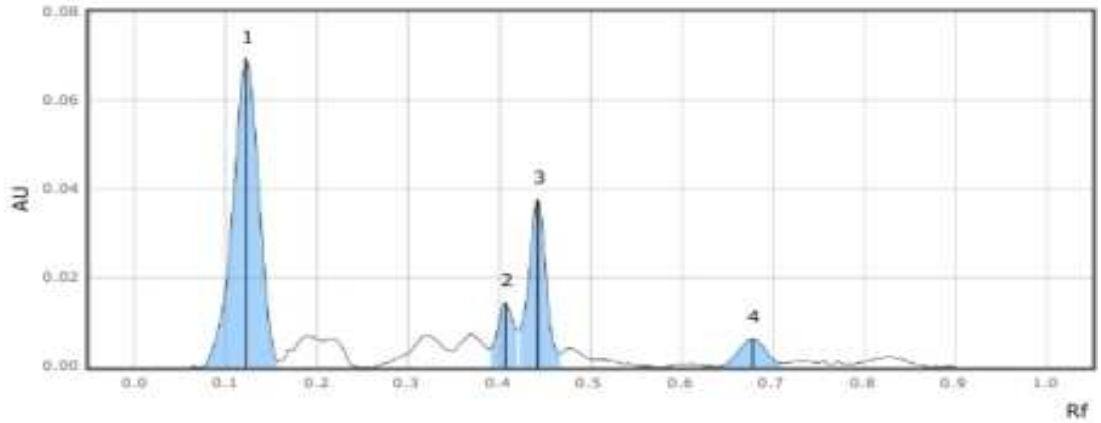

Fig. 5: HPTLC densitometric chromatogram at 366 nm, size T1: 2 µl

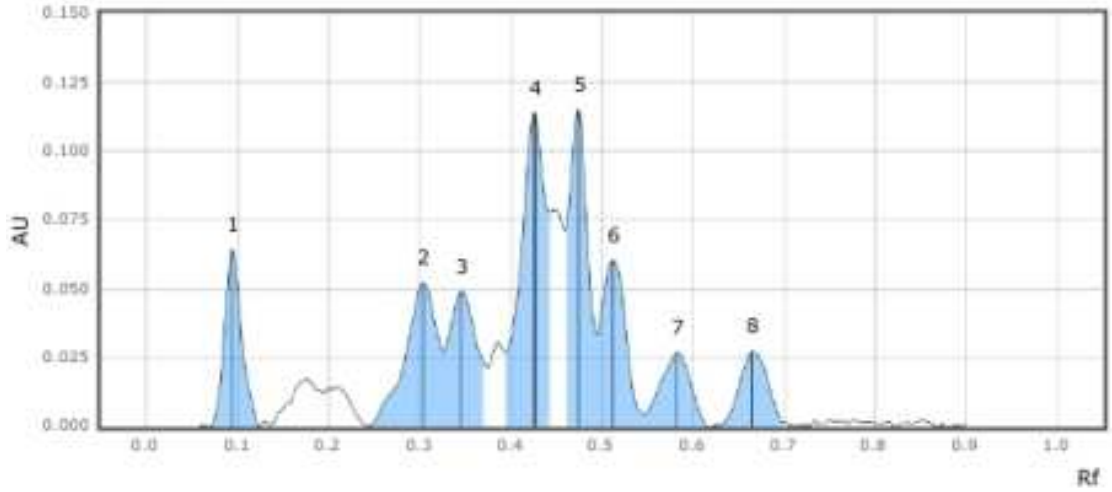

Fig. 6: HPTLC densitometric chromatogram (after derivatization) at 540 nm, size T1:2 µl

Table 2: R_f values and area % of HPTLC chromatogram of methanol extract of *martynia annua* seeds, size: 2 µl

Peak no.	254 nm		366 nm		540 nm (after derivatization)	
	(R_f) max	Area %	(R_f) max	Area %	(R_f) max	Area %
1	0.105	28.10	0.123	62.70	0.094	9.01
2	0.252	13.99	0.406	8.34	0.303	13.68
3	0.313	9.74	0.442	22.94	0.345	11.20
4	0.373	10.91	0.677	6.01	0.426	23.21
5	0.440	22.29			0.474	17.68
6	0.824	14.97			0.511	12.25
7					0.582	6.64
8					0.665	6.33

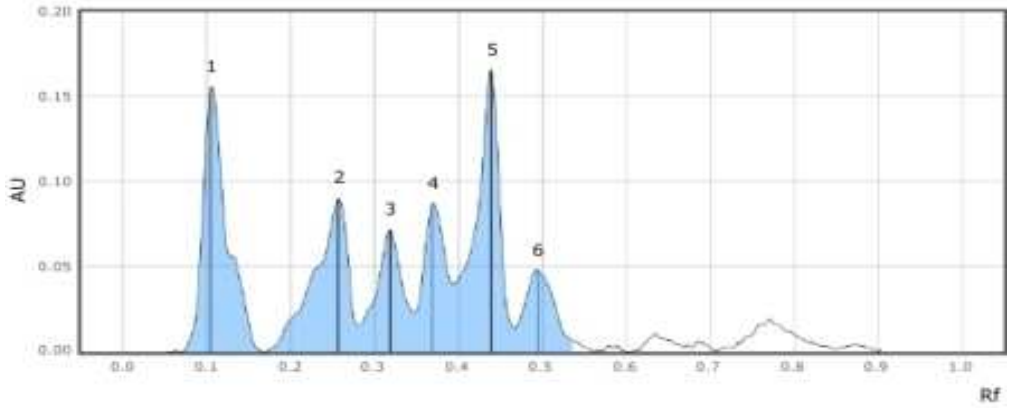

Fig. 7: HPTLC densitometric chromatogram at 254 nm, size: 5 µl

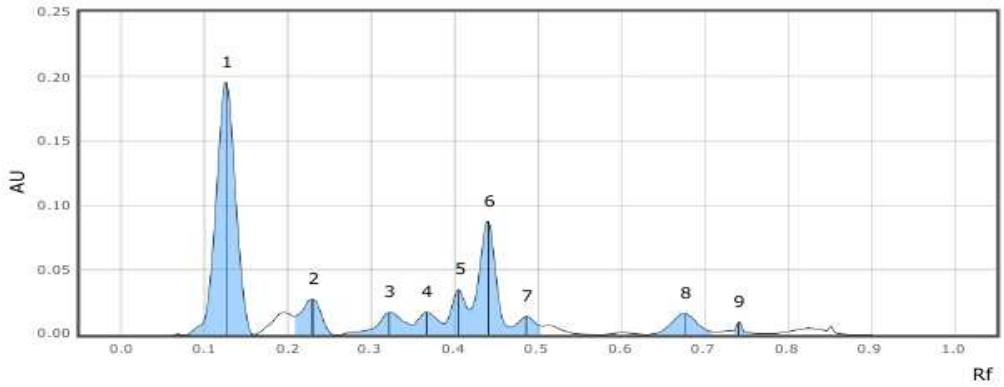

Fig. 8: HPTLC densitometric chromatogram at 366 nm, size: 5 µl

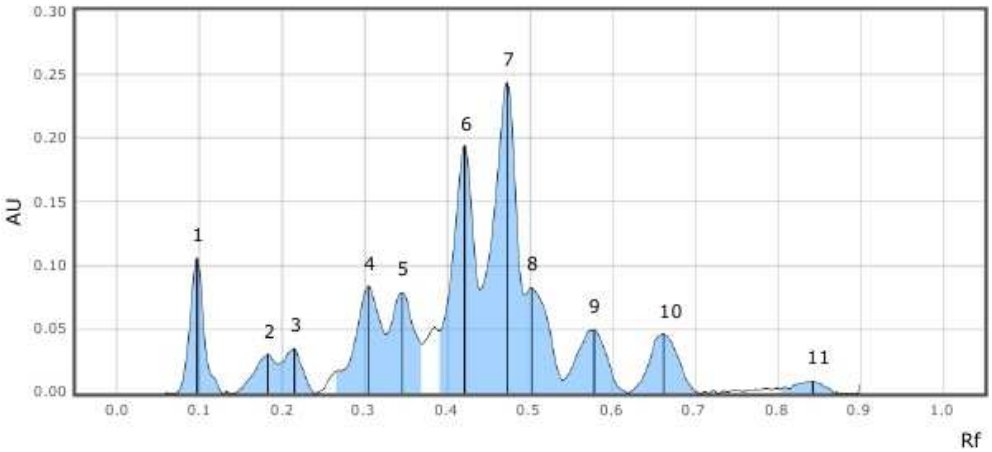

Fig. 9: HPTLC densitometric chromatogram (after derivatization) at 540 nm, size: 5 µl

Table 3: R_f values and area % of HPTLC chromatogram of methanol extract of *martynia annua* seeds, size: 5 µl

Peak No.	254 nm		366 nm		540 nm (after derivatization)	
	R_f max	Area %	R_f max	Area %	R_f max	Area %
1	0.105	23.38	0.126	48.33	0.097	6.85
2	0.256	18.64	0.229	6.97	0.182	2.87
3	0.318	11.87	0.321	5.67	0.215	3.21
4	0.369	12.17	0.366	4.50	0.305	9.64
5	0.439	25.01	0.405	6.23	0.345	8.62
6	0.494	8.93	0.440	18.64	0.421	19.30
7			0.485	3.44	0.473	26.72
8			0.676	5.31	0.502	8.59
9			0.740	0.90	0.577	6.85
10					0.661	6.10
11					0.842	1.25

DPPH free radical scavenging assay

Fig. 10 showed the DPPH scavenging activity of various concentrations methanol extract of *martynia annua* seeds. Increase extract concentration leads to increase antioxidant activity (DPPH scavenging activity) and maximum percentage inhibition was found in the extract has 50 µg/ml concentration. EC_{50} is a concentration of drug or extract required to obtain a 50% antioxidant effect. Table 4 showed EC_{50} values of methanol extract of *martynia annua* seeds and two standard drugs: ascorbic acid and BHT (butylated hydroxytoluene). Lowest EC_{50} value means highest antioxidant activity.

Sample which have EC_{50} lower than 50 µg/ml, is a very strong antioxidant, and 50-100 µg/ml is a strong antioxidant, and 101-150 µg/ml is a medium antioxidant while a weak antioxidant with EC_{50}>150µg/ml [30]. EC_{50} value of extract was found 44.1±1.1µg/ml while EC_{50} of ascorbic acid and BHT were found 11.5±0.6 µg/ml and 12.7±0.4 µg/ml respectively.

Table 4: EC_{50} value of DPPH free radical scavenging of extract

EC_{50} µg/ml	
Ascorbic acid(standard)	11.5±0.6
BHT(standard)	12.7±0.4
Martynia annua extract	44.1±1.1

Values were obtained as mean±SE (Standard Error), n = 3.

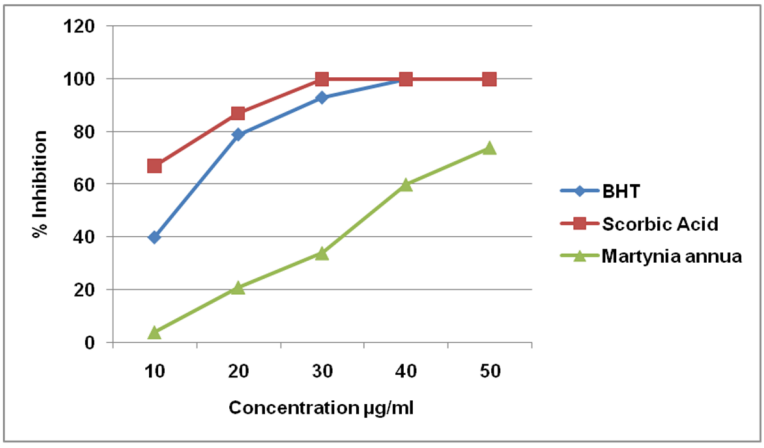

Fig. 10: DPPH radical scavenging activity of methanol extract of *martynia annua* seeds

CONCLUSION

The GC-MS analysis showed the presence of saturated and unsaturated free fatty acids,esters of fatty acids, flavones, alkaloid,aldehydes and ketones whereas in HPTLC different peaks at different UV-lights before and after derivatization were observed. Methanol extract of *martynia annua* seeds exhibited very good inhibition percentage (74%) of DPPH scavenging activity at concentration 50 µg/ml.

AUTHORS CONTRIBUTIONS

Ali Alrabie, Ola Basa□ar, and Inas al-qadsy carried out the experiment and wrote the manuscript. Dr. Mazahar Farooqui, the research supervisor conceived the original idea, supervised the project and corrected the scientific content of the manuscript.

CONFLICT OF INTERESTS

The authors have no conflict of interest

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