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ANTIOXIDANT ACTIVITY AND GC-MS ANALYSIS OF THE ESSENTIAL OIL OF *AMYGDALUS SPINOSISSIMA* SEEDS GROWN IN BALOCHISTAN

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Abstract

Amygdalus spinosissima also known as Prunus spinosissima belongs to the genus Prunus, its subgenus is Amygdalus. A. spinosissima is a wild thorny almond found in approximately 600 to 1500 meters above sea level. Due to its medicinal value, it is used as traditional medicine all over the world. The objective of present study was to evaluate the antioxidant activity of essential oil of A. spinosissima and other molecules present in the seed oil of this rare species through GC-MS. A. spinosissima oil showed significant antioxidant activity with IC50 value of 09.0± 0.1 µg/ml while IC50 value for ascorbic acid was 15.0± 0.5 µg/ml. These results indicate that the antioxidant activity of the oil of A. spinosissima is lower than that of ascorbic acid. A total of 17 compounds were identified in the essential oil from the seeds of A. spinosissima which were 3-hexanol (RT2.931), 2-pentanol,4-methyl(RT 2.996), Hydroperoxide,1-ethylbutyl(RT5.812), Hydroperoxide, 1-methylpentyl (RT6.045), Benzaldehyde (RT6.134), Phenol (RT 6.645), Benzyl alcohol (RT8.021), 3-Ethyl-3-methylheptane (RT8.695), Benzene, 1,3-bis(1,1-dimethylethyl)- (RT14.183), 2-Decenal, (E)- (RT14.373), 2,4-Decadienal, (E,E)- (RT15.847), Oleic Acid (RT32.912), Hexadecanamide (RT33.739), 9,12-Octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl) ethyl ester (RT37.662), 9-Octadecenamide, (Z)- (RT38.397), Diisooctyl- phthalate(RT42.179) and Gamma-tocopherol (RT38.397). It is concluded that the antioxidant activity and GC-MS analysis of the A. spinosissima seed essential oil is comparable with some other essential oils. Based on results, the seed oil may not be considered as potential sources of vegetable oil. Therefore, cannot be used as potential source in soap making industry but could be used in cosmetic industries due to vitamin E existence and antioxidant activity.

Keywords: *Ascorbic acid*, GC-MS, *IC50*, *RT*

INTRODUCTION

The family *Amygdalus* L. is one among the most imperative genera in the family Rosaceae. The family contains 29 genera and 243 species with 58 endemic taxa. Phyto-geologically, the species of *Amygdalus* are distributed in the southwest Iran, East Asia and central Asia. Iran is the core center for *Amygdalus* species in the world. Other countries surrounding Iran like, Pakistan, Turkey and Afghanistan also inhabit the different species of *Amygdalus* (1).

Prunus spinosissima also termed as *Amygdalus spinosissima*, is a wild spiny almond found in the region of 600 to 1500 m above sea level. Other species of Prunus are found in the form of a bush on sloppy hills of cold and dry stony valleys of Balochistan, Iran and central Asia that share the similar terrain (2).

Amygdalus species are deciduous bushes with mixed heights that inhabit semi-arid conditions. They also flourish on Mountains, inflexible or limestone slants at altitude around 500-5000 meters (1). The plants are usually 3 to 4 meter tall. The blooming period of the plant is April-May. The Flowers are light purple, deep pink, yellow or white having pink round petals that curve to form a "V-shaped" structure and are greenish in color. The stem is grayish white and contains long spines. The plant produces a drupe-sort fruit which ripens in the beginning of July. At the beginning, a green pericarp is produced, which after ripening becomes yellowish in color having a bluish pink cast due to a partial pubescence.

Plant seeds are a valuable source of vegetable oil having nutritional, industrial and pharmaceutical importance and have relatively high oxidative stability (3). The seed oil has powerful recuperating properties and is utilized for treatment of wounds. The gum created by this plant additionally has compelling mending properties and is utilized to treat wounds like skin burn. The capability of oil for a specific reason, however, is bowed by its attributes. No oil from any single precursor has been observed to be satisfactory for all reasons as oils from various sources by and large contrast in their unsaturated fat structure (4).

Plant seed oils are generally used to moisten the dry skin and reduce itching because of dryness. The plant seed oils are not slimy, quickly absorbed and do not irritate skin. It does not cause sensitization which may lead to allergic reactions hence have therapeutic and medicinal values.

A. spinosissima plant wood has been used to make coal by nomadic people for the past many years (5). The species is a threat to existence, hence numerous steps were taken to restore and preserve by seedling the *A. spinosissima*. This specific species is supposed to be a valuable source of vegetable oil because of its comparatively high stability towards oxidation is advantageous for humans in terms of health.

Eighteen wild types of almond leaf and fruit species were examined. The flowering prototype, self-incompatibility, kernel bitterness and ripening time were also studied (6). The outcome of the result shows that changes in the mean leaf dimensions among and within species were linked with average rainfall. Dry regions plants had smaller leaf size compared to semi dry and humid regions. Tiny fruit, incompatibility of pollen pistil and bitter kernel are the common hindrance to use this wild germ plasm in reproducing.

MATERIALS AND METHODS

GLASS WARE PREPARATION

All the glassware was washed thoroughly with detergent followed by tap and rinsed with distilled water. Glassware was dried in an oven at 105°C for several hours prior to use. Finally, glassware was rinsed with acetone.

SAMPLE PREPARATION

The seeds were taken out from their shell. The kernels were crushed into powder via a grinder. The 25g powder sample was placed in Clevenger apparatus and the essential oil was extracted by hydro distillation via Clevenger apparatus. The extraction was preceded for 5 hours in 1000 ml of distilled water. The 0.5 ml of oil was dissolved in hexane and desiccated over anhydrous Na2SO4. The solvent was removed by using rotary evaporator at 50°C. The oil obtained was kept in dark brown bottles and stored at 4°C in a refrigerator for further analyses.

ANTIOXIDANT ACTIVITY

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *A. spinosissima* essential oil was determined according to the (5, 6) with minor modifications. The working solutions (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml) of *spinosissima* essential oil were prepared in methanol. The standard ascorbic acid was used. 1 ml of DPPH solution and 1 ml of 0.1 milli molar methanol was mixed with 3 ml of the oil samples and standard solutions separately. The resulting mixture was stirred by vortex and kept in dark for half an hour at 25°C. The absorbance at 517 nm using UV spectrophotometer was determined with the blank 3 ml DPPH and1 ml of 0.1 mM methanol. Then DPPH radical scavenging activity was calculated by the formula:

DPPH Radical Scavenging Activity (% inhibition) = □((A0−A1)/ (A0)) × 100

Where A0 was the absorbance of the blank, and A1 was the absorbance of an oil sample mixed with DPPH. The value of IC50 was determined by plotting graph between % inhibition and concentration graph.

GC-MS Analysis

The essential oils from seeds of *A. spinosissima* were analyzed by GC-MS, GC-7000D triple Quadrupole GC gas chromatograph (Agilent technology) coupled with the 7000D MS. Identification of the compounds were compared the NIST library data of the peaks with those reported in literature, mass spectra of the peaks with literature data.

RESULTS

DPPH Scavenging Activity

The DPPH scavenging approach was adopted to determine the antioxidant activity of *A. spinosissima.* Vitamin C served as a standard, showed IC_{50} of 0.08 µM (Table I).

Table I. DPPH scavenging activity of *A. spinosissima*

Sr. No.	Sample Tested	Concentration (mg/ml)	Inhibition (%)	IC_{50} (µg/ml)
1	G1	5	90 ± 2.54	0.00099 ± 1.15[a]
2	Vitamin C[b]	0.5mM	90 ± 0.11	0.08 ± 1.14(µM)

Results are presented as Mean ± SME, (n=3); a = $p < 0.05$ versus control; [b]Standard antioxidant

Lipoxygenase Inhibition Effect

The lipoxygenase inhibition activity of *A. spinosissima* was also determined and shown in Table II.

Table II. Lipoxygenase inhibition effect of G1

Sr. No.	Sample Tested	Concentration (mg/ml)	Inhibition (%)	IC_{50} (µg/ml)
1	G1	5	93.71±0.25	29.44±0.29
2	Baicalein[b]	0.5mM	93.79±1.27	22.41±0.30(µM)

This method was carried out according to (10). Ten µL of test solution was added in a 96-wells plate followed by the addition of 90 µL of 100 µM methanolic DPPH solution in a total volume of 100 µl. The contents were mixed and incubated at 37oC for 30 minutes. The reduction in the absorbance was measured at 517 nm using Synergy HT BioTek® USA microplate reader. Vitamin C was used as a standard antioxidant. All experiments were carried out in triplicates. For the determination of IC50 values, test solutions were assayed at various dilutions i.e., 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.015 mM. Data obtained was computed on Ez-fit software. The decrease in absorbance indicates increased radical scavenging activity which was determined by the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{Abs. of control} - \text{Abs. of test solution}) \times 100}{\text{Abs. of control}}$$

Where: Absorbance of Control = Total enzyme activity without inhibitor
Absorbance of Test = Activity in the presence of test compound

LOX Assay

LOX activity was assayed according to the method described by Sacchetti *et al.,* (2005) with slight modifications (11). A total volume of 200 µL contained 140 µl KH2PO4 buffer (100 mM, pH 8.0), 20 µl test compound and 15 µL purified LOX enzyme (600 units per well). The contents were mixed and pre-read at 234 nm and pre-incubated for 10 minutes at 25°C. The reaction was initiated by the addition of 25 µL substrate solution. The change in absorbance was observed after 6-10 min at 234 nm. Baicalein (0.5 mM per well) was used as a positive control. The reduction in the absorbance shows an elevated level of radical scavenging activity which was determined by the similar formula.

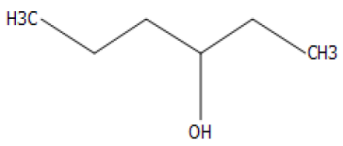
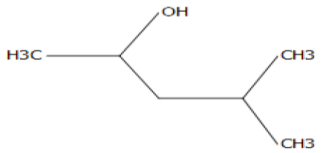
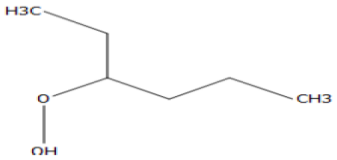
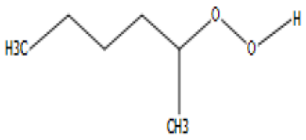
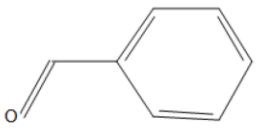
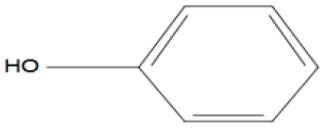
Then DPPH radical scavenging activity will be calculated by the formula:

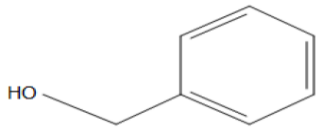
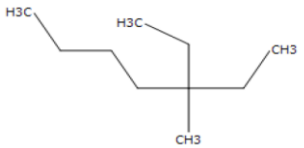
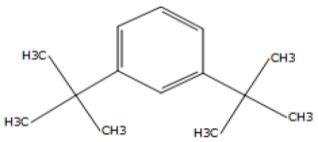
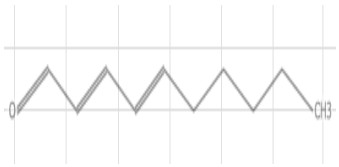
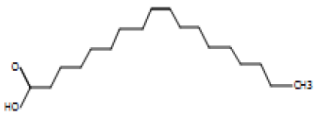
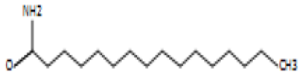
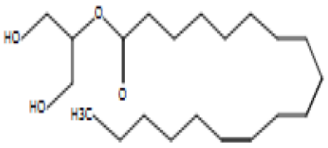
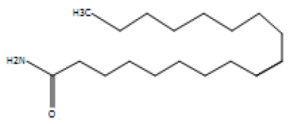
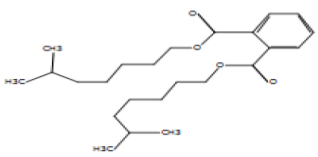
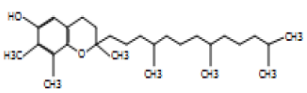
DPPH Radical Scavenging Activity (% inhibition) = $\frac{A0-A1}{A0} \times 100$

GC-MS

The seed essential oil *A. spinosissima* was separated, identified and quantified via GC-MS. Seed essential oil sample was then analyzed by using a temperature control program with a DB-5 GC-MS capillary column. Seventeen different compounds from the essential oil *A. spinosissima* were identified, that contains of alcohol, phenols, aldehydes, ketones, ether, esters, epoxides and terpenic hydrocarbons. The abundance of hydrocarbons, aromatic molecules and alcohol were high compared to others. The results of the components were listed according to elution on a DB-5 column with their retention time, molecular formula, molecular mass, structure and mass/charge ratio (Table III and IV). All the samples of essential oil predominantly demonstrated compounds of the cyclic series. A total of 17 different molecules were identified in the essential oil from the seeds of *A. spinosissima* where there were 3-hexanol (RT2.931), 2-pentanol,4-methyl (RT 2.996) , Hydroperoxide,1-ethylbutyl (RT5.812), Hydroperoxide, 1-methylpentyl (RT6.045), Benzaldehyde (RT6.134), Phenol (RT 6.645), Benzyl alcohol (RT8.021), 3-Ethyl-3-methylheptane (RT8.695), Benzene, 1,3-bis(1,1-dimethylethyl) (RT14.183), 2-Decenal, (E)- (RT14.373), 2,4-Decadienal, (E,E)- (RT15.847), Oleic Acid(RT32.912), Hexadecanamide (RT33.739), 9,12 Octadecadienoic acid (Z, Z), 2-hydroxy-1-(hydroxyl methyl) ethyl ester (RT37.662), 9-Octadecenamide, (Z)- (RT38.397), Diisooctyl-phthalate(RT42.179) and Gamma -tocopherol (RT38.397).

Table III. Molecules identified in the hexane oil extract of *A. spinosissima* seed

Compound Name	Molecular formula	Molecular mass	Structure	RT
3-hexanol	$C_6H_{14}O$	102		2.931
2-pentanol,4-methyl	$C_6H_{14}O$	102		2.996
Hydroperoxide,1-ethylbutyl	$C_6H_{14}O_2$	118		5.812
Hydroperoxide, 1-methylpentyl	$C_6H_{14}O_2$	118		6.045
Benzaldehyde	C_7H_6O	106		6.134
Phenol	C_6H_6O	94		6.645

Benzyl alcohol	C_7H_8O	108		8.021
3-Ethyl-3-methylheptane	$C_{10}H_{22}$	92		8.695
Benzene, 1,3-bis(1,1-dimethylethyl)-	$C_{14}H_{22}$	190		14.183
2-Decenal, (E)-	$C_{10}H_{18}O$	154		14.373
2,4-Decadienal, (E, E)-	$C_{10}H_{16}O$	152		15.847
Oleic Acid	$C_{18}H_{34}O_2$	282		32.912
Hexadecanamide	$C_{16}H_{33}NO$	255		33.739
9,12-Octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{21}H_{38}O_4$	354		37.662
9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281		38.397
Diisooctyl- phthalate	$C_{24}H_{38}O_4$	390		42.179
Gamma ,-tocopherol	$C_{28}H_{48}O_2$	416		56.117

INTEGRATION PEAK LIST

Table IV. Retention time and percentage area of oil extract of *A. spinosissima* seed

Peak	Start	RT	End	Height	Area	Area %
1	2.971	2.996	3.039	78689.25	140969.43	0.26
2	6.605	6.645	6.685	59711.61	115238.09	0.21
3	32.728	32.912	33.427	426305.66	6202617.51	11.51
4	33.656	33.739	33.914	1841482.83	6999139.84	12.99
5	34.594	34.665	34.72	57878.8	202175.11	0.38
6	37.573	37.662	37.711	535764.41	2252590.57	4.18
7	37.711	37.836	37.987	11084431.01	53885218.11	100
8	38.302	38.397	38.676	334629.63	2793163.43	5.18
9	42.062	42.176	42.415	271142.83	1719796.85	3.19
10	55.921	56.12	56.475	105877.38	1335818.39	2.48

DISCUSSION

ANTIOXIDANT ACTIVITY

Decrease in the absorbance of DPPH in the presence of antioxidants correlates with the free radical scavenging potential of the antioxidant. The scavenging activity might be due to the presence of different compounds *A. spinosissima* oil showed significant antioxidant activity with IC50 value of 09.0± 0.1 µg/ml while IC50 value for ascorbic acid was 15.0± 0.5 µg/ml. The results indicate that the antioxidant activity of the oil of *A. spinosissima* is lower than that of ascorbic acid. The essential oils were screened for antioxidant activity. The use of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) as a reagent for screening the antioxidant activity of small molecules has been reported (7). The inhibition percentage of the radical-scavengers activity in the essential oil was 9.91% (2270 µg/ml-1) at the flowering stage, 7.47% (2390 µg/ml-1) at the fruit-bearing stage, and 6.89% (2320 µg/ml-1) at the vegetative stage. The reference compound quercetin showed a scavenging effect of 90% (20 µg/ml-1), 91% (40 µg/ml-1), 93% (80 µg/ml-1), 94% (160 µg/ml-1) and 97% (320 µg/ml-1). In this test, the scavenging of the DPPH radical is followed by monitoring of the decrease in absorbance at 517 nm, which occurs due to the antioxidant reduction, and has been used to assess the ability of phenolic compounds to transfer labile H atoms to radicals (8). The lower antioxidant activity has been attributed to the absence and/or lower amount of the donor groups of the electron in ortho position in relation to phenolic hydroxyl, and the presence of larger amounts of hydrocarbons terpenes. This result agrees with the other studies of essential oils with similar patterns (9).

GC-MS

Gas chromatography-mass spectrometry (GC-MS) has been used in the separation, identification and quantification of complex mixtures, such as essential oils. As a rule, the identification of these compounds is not precise, because the mass spectra of these compounds were very similar and determination with the standard MS library was very difficult. For this reason, the retention index –IR was

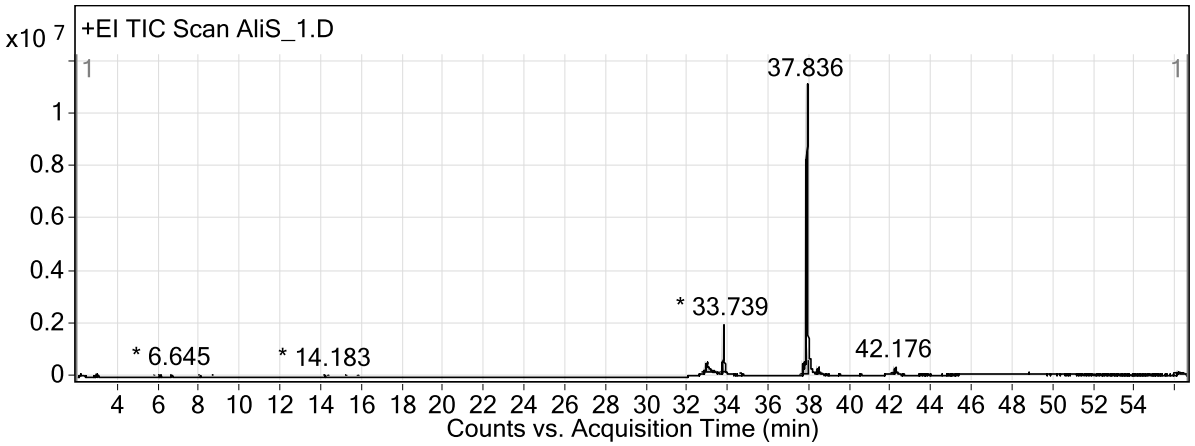


Fig. 1. Complete GC-MS spectrum and individual spectrums of molecules

used as a parameter for the GC qualitative analysis of the complex mixtures of isomers. These oil samples were then analyzed by GC-MS using a temperature program with a DB-5 capillary column. A total of 17 different compounds from the essential oil *A. spinosissima* were identified, including the presence of terpenic hydrocarbons, ether, alcohol, aldehydes, ketones, esters, phenols and epoxides. Alcohol and hydrocarbons were the predominant class. Due to the complexity of the results the components were listed in order of elimination on a DB-5 column and their retention index and percentage composition are described in Table III, V and Fig. 1. The abundance of hydrocarbons, aromatic molecules and alcohol were high compared to other essential oils.

CONCLUSION

The GC-MS analysis and antioxidant activity of the *A. spinosissima* seed essential oil is comparable with some other essential oils. Based on the result, the seed oil may not be considered as potential sources of vegetable oil. Therefore, cannot be used as a potential source in the soap making industry but could be used in cosmetic industries due to vitamin E existence and antioxidant activity. Further, extensive studies are required to find out more possible anti-nutritional components.

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