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Original Article

INVESTIGATION OF SOME CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITY OF *ASPARAGUS SPERNGERI*

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ABSTRACT

Objective: Asparagus sprengeri (A S) is an ornamental plant cultivated in EGYPT. This study was designed to investigate some lipid and flavanoid constituents, in addition to the evaluation of antioxidant activity of different extracts of the plant.

Methods: Two phospholipid were identified by (HPTLC). The fatty acid methyl ester and unsaponifiable matter were analyzed by GC. Four flavonoids were isolated by CC and purified by PPc. Identification of isolated flavanoidal compounds was carried by spectroscopic analysis Viz, TLC,PC, UV, EL-MS and H^{-1}– NMR. The different extracts were tested for their free radical scavenging activity using DPPH and β-Carotene- linoleic acid bleaching assay.

Results: Four flavonoids, were isolated, apigenin, dihydroquercitin, naringenin, apigenin- 7-o- glucoside Nine fatty acids, Myrestic and linoliec acids were the major components. The unsaponifiable matter was found to be a mixture of hydrocarbons from (C_{13}– C_{28}) and cholesterol two classes of phospholipid were identified namely L-α-Phosphatidyl-DL-glycerol and L-α- Phosphatidylethanolamine. The alcoholic extract of the plant has moderate antioxidant activity with EC_{50} 0.114 and 0.110 mg/ml for the DppH and β-carotene methods respectively

Conclusion: Asparagus *sprengeri* can be used as the natural antioxidant. Flavonoids are suggested to be a group of key antioxidants in *Asparagus.*

Keywords: *Asparagus sprengeri*, *Liliaceae*, Lipid constituents, Flavonoids, Antioxidant activity.

INTRODUCTION

Asparagus genus, belongs to family *Liliaceae,* it represented in Egypt by three species *Asparagus sprengeri* is not growing widely in Egypt but it introduced and cultivated as an ornamental plant. It is an erect armed herb that grows up to 5ft high [1]. By reviewing the available literature, it was found a little researches concerning with chemical constituents and biological activity of *A. sprengeri*, but there are many reports about other plants of *Asparagus* genus. It was reported that, *Asparagus officinalis* has many active components and abundant nutritional value. Steroidal saponins, polysaccharides, flavonoids, tissue protein, and trace elements have been isolated from its roots and buds [2,3]. *A. racemosus* is commonly called Satavari, It's pharmacological applications, particularly from the root extracts, have recently been found to possess a phytoestrogenic effect, an effect on neurodegenerative disorders, as well as anti-diarrheal, anti-dyspepsia, adaptogenic, cardio protective, antibacterial, immuno adjuvant and antitussive effects. Its root extracts have been employed in two major forms as methanolic and aqueous extract; also it has been reported to have antioxidant activity. Methanolic root extracts were found to cause markedly increase of superoxide dismutase, catalase and ascorbic acid, while decreasing lipid peroxidase product (malondialdehyde) in rats [4-6]. In addition, it has recently been shown to contain ten steroidal saponins and racemo furan. The aqueous extracts have also been shown to exhibit an antioxidant effect in rat liver mitochondria by protecting against radiation-induced loss of protein thiols and inactivation of superoxide dismutase [7,8]. Hassan *et al.* [9] reported that *A. africanus* is used for the treatment of headache, backache, stomach pain and as an aid in child birth. The plant is also used for hematuria, hemorrhoids, malaria, lishmanians is, bilharzias, syphilis and gonorrhea [10,11]. The root extract is applied externally for the relief of pain, rheumatism and chronic gout. It is also used as a diuretic, for sore throat and otitis. Three steroidal saponins have been isolated from the roots of *A. africanus* [12,13]. The aim of the current study was to study some lipid and flavonoidal constituents in addition evaluation of antioxidant activity of different extracts of *Asparagus sprengeri*

MATERIALS AND METHODS

Plant material

The herb of *Asparagus sprengeri* was collected from the garden of National Research Centre (NRC) in January 2012. The plant was kindly identified by Dr. M. El Gibali, flora of Egypt Depart., NRC and a voucher specimen was deposited at NRC herbarium.

Phospholipids kits

Avanti, Polar lipid, Inc., 10 mg, 69005P, Phospholipid from Soybean, contains: SoyPC(3.8mg), Soy PE(3.0mg), Soy PI(1.8mg), Soy PA(0.7mg) and Soy LPC(0.7mg). PH9-1 KT (sigma), contains 9 Phospholipids in quantities as indicated: P 3556 L-α-Phosphatidylcholine (100 mg), P 6386 L-α-Phosphatidylethanolamine (Approx. 98%, 25 mg), P 7769 L-α-Phosphatidyl- L-serine (Approx. 98%, 25 mg), P 0639 L-α-Phosphatidylinositol (10 mg), P 9511 L-α-Phosphatidic Acid (Approx. 98%, 50 mg), P 0514 L-α- Phosphatidyl-DL-glycerol (50 mg), L 4129 L-α-Lysophosphatidylcholine (100 mg), S 7004 Sphingomyelin (100 mg) and C 1649 Cardiolipin (Approx. 98%, 25 mg).

Instruments

1- UV-Vis spectrophotometer pc. 2401Schimadzu.

2- Bruker NMR spectrometer operating at 500 MHz for ^1H and 125 MHz for C[13] – NMR.

3- Mass spectrometer JEOL JMS-AX 500.

4- Agilant technologies 68900N series GC system for GLC

Conditions for GLC analysis of lipid fraction

Unsaponifiable matter

Apparatus: Hewlett Packard (HP6890 series GC system). **Column**: capillary Column (Hp-5),(length30m,diameter530 μm and film thickness: 0.5 μm). **Oven:** Initial temperature: 80°C (for 1.00 min),

Rate(8°C/ min),Final temperature (250°C). **Inlet temperature:** 250°c (splitless). **Flow rate:** 5 ml/min. **Detector:** FID (Flame Ionization Detector), Temperature: 300°C. **Carrier gas:** H_2 flow: 30 ml/min, N_2 flow: 30 ml/min,Air flow: 260 ml/min

Fatty acid methyl esters

Apparatus: Hewlett Packard HP 6890series GC system. **Column:** Capillary column (HP-70), (length 60m, diameter 320 µm and film thickness: 0.25 µm) Max. Temp.**:** 260ºc. **Oven:** Initial temperature: 70ºc. **Initial time:** 2 min. **Ramps:** Rate 6 º c/min. Final Temp.**:** 220º. Final time: 15 min. **Inlet temperature:** 270ºc(split 15:1). **Flow rate:** 2 ml/min. **Detector:** FID (Flame Ionization Detector), Temperature: 300ºc. **Carrier gas:** H_2 flow rate: 40 ml/min, N_2flow rate: 40 ml/min,Air flow: 45 ml/min

Phospholipids extraction

Phospholipids were extracted according to Iverson *et al*. [14]. From air dried powdered plant material (*As*, 50 gm) by sonication as Chua *et al*. [15] with $CHCl_3$: MeOH (2:1) (200 mL x 2). The extract was dried at 40°C under vacuum. The dried extract (0.9 gm) was dissolved in acetone (Hoevet*et al*. [16], Rhodesand Lee [17], filtered and left overnight at 4°C. The residue was separated using cooling centrifuge at 1°C for 10 minutes at 4500 rpm. The acetone soluble and insoluble parts were dried at 40°C under vacuum; giving a yield of (0.35 and 0.45 gm respectively).

High performance thin layer chromatography (HPTLC)

When comparing between acetone soluble fraction (total phospholipid) and the ten phospholipids references used, it appeared that the acetone soluble fraction contains L-α-Phosphatidyl-DL-glycerol and L-α- Phosphatidyl ethanol amine and neutral lipids.

Extraction of lipid constituents

About 500g of the dried powdered plant of *As* were extracted with petroleum ether (40-60 b. r.) in a soxhlet apparatus. The combined petroleum ether extract was passed through fuller's earth to remove the colored pigments, filtered, dried over anhydrous sodium sulphate and evaporated in *vacuo* at 40 ˚C till dryness to give a pale yellow residue (7.8 gm).

Saponification of petroleum ether extract

About 3g of pet. Ether extract were saponified by refluxing with 100 ml 0.5 N alcoholic KOH. The alcoholic solution was concentrated to about 25 ml and diluted with cold distilled water(100 ml). The unsaponifilable matters were extracted with ether (3×100 ml). The combined ether extract was washed with distilled water, dehydrated over anhydrous sodium sulphate and evaporated in *vacuo* till dryness to give a yellowish brown semisolid residue of unsaponifiable matters (0.9g).

Extraction of the total fatty acids

The hydroalcoholic soap solution after saponification was rendered acidic (pH=2) with 5 % sulphuric acid. The liberated fatty acids were thoroughly extracted several times with ether. The combined ether extract was washed with distilled water till free from acidity and dehydrated over anhydrous sodium sulphate. The solvent was evaporated in *vacuo* at about 40˚C till dryness (1.6g).

Preparation of the fatty acid methyl esters

About 1.5 g of the total fatty acids were dissolved in 7.5 ml dry methanol,7.5 ml of Borontrifloride (12% solution(SIGMA) was added and reflexed on a boiling water bath for five minutes, the solvent was dried and the residue dissolved in 10 ml distilled water. The methyl esters of fatty acids were extracted with successive portions of ether (3×10 ml). The combined ether extract was washed with distilled water, dried over anhydrous sodium sulphate, filtered and aliquot of 2 µl was injected into GLC column.

Extraction, isolation and purification of flavonoids

About 900g of the defatted powdered *Asparagus sprengeri* herb were macerated with (80%) methanol till exhaustion. The alcoholic extract was evaporated in vacuo at about 45°C. The residue (44g) was dissolved in hot distilled water (500 ml) left overnight in refrigerator and filtered.

The aqueous filtrate was extracted with successive portion of chloroform (3X250 ml),ethyl acetate(3X250 ml) followed by butanol (3X250 ml).

The ethyl acetate fractions (5.2g) was subjected to PPC (3MM,20%acetic acid),two main bands were cut and eluted separately by 90% methanol. The flavonoidal bands were further purified on Sphadex LH_{-20} column using 90% methanol gave two pure compounds I and II which identified by PC, TLC, UV and EI/MS.

The butanol fractions (6.6g) was applied on the top of the glass column (110X6 cm) packed with polyamide in water. Elution was carried out using water followed by water/methanol mixtures and each fraction was 150 ml was collected.

The flavonoidal compounds isolated from polyamide column gave after purification on Sephadex LH_{-20} column using 90% methanol, two single flavonoidal compounds III and IV which identified by PC,TLC,UV and EI/MS.

Investigation of antioxidant activity

The antioxidant activity of the different extracts of the plant (*AS*) was determined using two methods as follow:

1- **2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method**

The free radical scavenging activity of the extract was determined according to the assay described by Lee *et al.* [18]. The reaction mixture containing various concentrations of the extract, 50, 100 and 150 µl(Conc.0. 1%) were added into 4 ml of DPPH solution (5 mg/ 500 ml MeOH) and the tubes vigorously shaken and incubated in the dark at room temperature for 30 min. After the incubation period, the absorbance of the reaction mixture was measured using UV-Visible spectrophotometer at 517 nm. The scavenging activity of DPPH free radical was calculated by the following equation:

1 ml DPPH served as a blank.

Scavenging effect (%) = 1- (absorbance of sample at 517 nm /absorbance of

control at 517 nm) X 100.

$EC_{50}$is determined from the plotted graph of scavenging activity against the concentration of the sample extract, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

2- **β-carotene linoleic acid - bleaching assay**

Determination of antioxidant activity using β-Carotene/ linoleic acid bleaching method was done as described by Velioglu *et al.* 1998[19]. Two ml of β-carotene solution (0.2 mg/ml chloroform) was pipetted into a round-bottom flask (50 ml) containing 0.02 ml of linoleic acid and 0.2 ml of 100 % Tween 20. The mixture was then evaporated at 40°C for 10 min by means of a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 ml of distilled water.

Five ml aliquots of the emulsion were transferred into different test tubes containing 50, 100 and 150 µl of the extract. Standards BHT tubes were then gently mixed and placed at 45°C in a water bath for 2 hours was used as comparison. Absorbance of the extract was measured using a UV-Visible spectrophotometer at 470 nm at the initial time (t=0) against a blank, consisting of an emulsion without β-carotene. An amount of 0.2 ml of 80% methanol in 5 ml of the above emulsion was used as the control.

The antioxidant activity (AOA) was calculated according to the following equation:

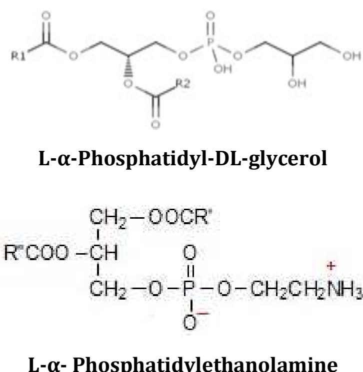
Where, AOA = [1 – (Ao – At) / (Aoo – Aot)] × 100

Ao and Aoo are the absorbance values measured at initial time of incubation for samples and control respectively, while At and Aot

are the absorbance values measured in the extract or standards and control at t = 120 min.

RESULTS AND DISCUSSION

Phospholipids content of *AS* were identified using HPTLC technique. From HPTLC, two classes of phospholipid were identified namely L-α-Phosphatidyl-DL-glycerol and L-α- Phosphatidyl ethanolamine as shown in Fig (1).



L-α-Phosphatidyl-DL-glycerol

L-α- Phosphatidylethanolamine

Fig. 1: L-α-Phosphatidyl-DL-glycerol and L-α- Phosphatidyl-ethanolamine Structures

The results of GLC analysis of the unsaponifiable fraction (Table 1) proved the presence of a mixture of saturated long chain hydrocarbons starting with n-tridecane (C13) up to n- octacosane (C28) and one steroidal compound which was identified as cholesterol (1.01%). It was found that n-eicosane (C21) and n-hexacosane (C26) constitutes the main components. (56.0% and 7.45% respectively). The GLC data of fatty acid methyl esters in (Table 2) showed that, it is a mixture of nine fatty acids, five acids are unsaturated and from about 43.79% in addition to four saturated fatty acids constitute about 56.2%. Myristic acid (C14: 0, 42.21%) was found to be the major component and followed by Linoleic acid (C18: 2, 10.93%).

This is the first report about the lipid fraction of *Asparagus sprengeri*.

The flavonoidal compounds were identified as follow

Compound -1: *Apigenin*

This compound was isolated as a pale yellow powder and appears as a yellow spot after spraying with $AlCl_3$ under the UV light. Its behavior on the chromatographic paper in different solvents proved that it is an aglycone flavone in nature. The UV spectra of this flavonoid in methanol and methanol containing five different shift additives (MeONa, $AlCl_3$, $AlCl_3$/HCl, NaOAc and NaOAc/H_3BO_3) were obtained. The UV data are as follows $\lambda_{max/nm}$): (MeOH) 263, 329; (MeONa)271, 322, 381; ($AlCl_3$) 273, 328,384; ($AlCl_3$/HCl) 271, 305, 332, 399; (NaOAc) 271, 350; (NaOAc/H_3BO_3) 263, 330. According to the method introduced by Markham [20]. The ^{1}H nmr displayed d in ppm=7.83 (2H, *d*, *J* = 8.8 Hz, H-2′and H-6′), 6.92 (2H, *d*, *J* = 8.8 Hz, H-3′and H-5′), 6.83 (1H, *d*, *J* = 2.1 Hz, H-6), 6.71 (1H, *d*, *J* = 2.1 Hz, H-8), 6.58 (1H, *s*,H-3), these data were in accordance with that reported by Markham. The EI mass spectrum gave a molecular ion peak M^+ at m/z=270 related to the molecular formula $C_{15}H_{12}O_5$, another fragments like 252[M^+ -H_2O], 242[M^+ -CO],153 and 117. All these data proved that compound -1 can be identified as Apigenin [21].

Compound -2: *Apigenin-7-O-glucoside*

The compound was isolated as a brownish amorphous powder and it's glycosidic nature was proved through its chromatographic behavior and the acid hydrolysis as in Mabry *et al* [22] where it gave glucose as a sugar and apigenin as an aglycone. The position of the glycosylation was proved by comparison of the UV spectra of both the compound and it's aglycone in NaOAc where band –II exhibited a bathochromic shift (10 nm) relative to methanolic spectrum. the ^{1}H nmr showed signals at δ in ppm= 7.9(2H, *d*, *J* = 9.1 Hz, H-2′and H-6′), 6.81 (2H, *d*, *J* = 7.8 Hz, H-3'and H-5′), 6.83 (1H, *d*, *J* = 2.1 Hz, H-6),6.71 (1H, *d*, *J* = 2.1 Hz, H-8), 6.58 (1H, *s*, H-3), glucose moiety: 5.0 (1H, *d*, *J* = 7.6 Hz, H-1″), 3.50-3.25(other protons of the glucose)[23]. The mass spectrum for the compound after hydrolysis displayed the same data as for compound-1(apigenin), therefore compound **-2** was identified as Apigenin-7-*O*-glucoside.

Table 1: GLC data of the unsaponifiable fraction of *Asparagus sperngeri*

Peak No.	Ret. Time(min.)	compounds	Relative %
1	12.40	n- Tridecane (C13)	3.18
2	15.05	n- Hexadecane (C16)	1.36
3	15.30	n- Heptadecane (C17)	1.82
4	16.10	n- Octadecane (C18)	1.73
5	18.5	n- Nonadecane (C19)	3.91
6	20.13	n- Eicosane (C20)	56.00
7	20.60	n- Uncosane (C21)	7.31
8	22.80	n- Docosane (C22)	4.29
9	23.80	n- Tricosane (C23)	1.31
10	24.08	n- Tetracosane (C24)	1.31
11	24.58	n- Pentacosane (C25)	1.97
12	26.60	n- Hexacosane (C26)	7.45
13	27.11	n- Heptacosane (C27)	6.24
14	28.60	n- Octacosane (C28)	1.48
15	30.31	Cholesterol	1.01

Table 2: GLC data of fatty acid methyl esters fraction of *Asparagus sperngeri*

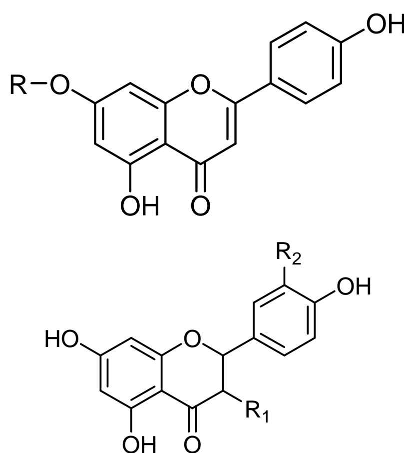
Peak No.	Ret. Time(min.)	compounds	Relative %
1	12.21	Myristic acid (C14: 0)	42.21
2	12.72	Myristoleic acid (C14:1)	11.72
3	16.02	Palmitic acid (C16: 0)	9.59
4	16.87	Palmitoleic acid(C16:1)	9.83
5	17.98	Heptadecanoic acid (C17: 0)	4.40
6	19.62	Oleic acid (C18: 1)	1.77
7	20.51	Linoleic acid (C18: 2)	10.93
8	22.34	Linolenic acid (C18: 3)	1.01
9	24.32	Arachidonic acid (C20: 4)	8.53

Compound -3: ***Dihydroquercetin***

This compound was isolated as a yellowish white powder. The UV spectra displayed λmax (MeOH) 282 nm and 322 nm (sh), which confirm the typical chromophore of flavanone [20]. In addition of NaOH, band I showed a bathochromic shift to 371 nm, indicates the presence of a free OH group at C-4`, while, the bathochromic shift of 49 nm with $AlCl_3$confirmed the existence of OH-5 group. On the other hand, the bathochromic shift (12 nm) with NaOAc indicated the presence of a free OH group at the C-7. The EI-MS showed m/z 304 [M^+]; which consistent with the molecular formula $C_{15}H_{12}O7$. The ^{1}H-nmr at δ in ppm= 6.92 (1H, s, H-6'), 6.81(2H, s, H-2', 5'), 5.85 (1H, d, J =2.1 Hz, H-8), 5.79 (1H, d, J = 2.1 Hz, H-6), 4.77 (1H, d, J = 11.2 H-3, H-2), 4.49 (1H, d, J = 10.9 H-2, H-3). these data were in agreement with that reported for Dihydroquercetin[24].

Compound -4: ***Naringenin***

The compound was isolated as amorphous powder and it's chromatographic behavior on paper and different solvent proved it's flavanone nature. The UV spectrum in methanol showed a band at 286.0 and 330 nm (sh), deducing its flavanone nature [20]. This compound shows 40 and 45 nm bathochromic shifts with NaOH and aluminum chloride respectively at band I and 8 nm bathochromic shift at band II with NaOAc give indication for free hydroxyl group at C-4`, C-5 and C-7. The EI-MS showed a molecular ion peak [M^+] at m/z 272 which agreed with the molecular formula $C_{15}H_{12}O_5$.



1-R= H apigenin **3**- R1=R2= OH Dihydroquercetine

2-R=glucose apigenin 7-O- glucoside **4**-R1=R2= H Naringenin

The ^{1}H nmr spectrum was consistent with a flavanone structure and it exhibited a characteristic resonance of H-2` and 6` at = 7.30, d,J= 8.5,Hz and H-3`and 5`at δ= 6.78, d,J= 8.5,HZ H-8 and H-6 (5.86, s). The presence of signals 5.42 (H-2), 3.25 (H-3a) and 2.66 (H-3b) confirm the flavanone nature of this compound, so, these spectral data were in agreement with those reported for Naringenin [25].

There are several methods for the preliminary evaluation of the antioxidant activity of a compound. In this study two methods were used. DPPH scavenging activity, and β-carotene –bleaching. The ability of a compound to donate an electron or hydrogen radical was measured by the decrease in the absorbance of the DPPH radical after forming of the DPPH stable free radical [26]. The antioxidant activity was expressed as EC_{50} (concentration of the antioxidant required to scavenge 50% of the initial DPPH radical). The lower the EC_{50}, the greater is the antioxidant activity. Table (3) shows that the total methanolic extract (TME) of *Asparagus Sprengeri*, is the highest antioxidant activity compared with that of the other fractions. The EC_{50} of (TME) is 0.114mg/ml followed by ETOAc, butanol, and chloroformic fractions 0.176, 0.207, and 0.220mg/ml respectively.

In the β-carotene –bleaching method, the antioxidant potential is measured by the power of the compound to inhibit lipid peroxidation. In the present case the model system consists of β-carotene and linoleic acid. The free linoleic acid radical (formed upon the abstraction of hydrogen atom from one of its methylene groups) attacked the β-carotene molecules, leading to the loss of its double bonds and resulting in discoloration of its orange color. The difference between the initial reading in spectral absorbance at 470 nm (zero time) and after 60 mn incubation period evaluates the rate of bleaching of the β-carotene solution. This reflects the antioxidant potential of the compound. The antioxidant activity was expressed as percent inhibition relative to control solution. The total methanolic extract (TME) showed the highest activity EC_{50} of 0.11mg/ml followed by that of EtOAc EC_{50} =0.14mg/ml. These results may be attributed to the total phenolics (flavonoids and vitamins) extracted from the plant. These results were found to be in agreement with that reported before.[26,27].

The data in table (3), fig(2)and(3) of antioxidant activity of alcohlolic extract of *Asparagus sprengeri* proved that the two methods used in this experiment gave approximately the same data but the EC_{50} are slightly different. This finding agrees with that reported by Ting *et al* [28], where they proved that, the alcoholic extract of *Asparagus* is more effective than the acetone extract of the same plant using DPPH method, also they found that total flavonoid content and the antioxidant activity of *Asparagus* had significant correlation. Therefore, flavonoids are suggested to be a group of key antioxidants in *Asparagus*. The major flavonoid antioxidant in *Asparagus* has been reported to be rutin [29].

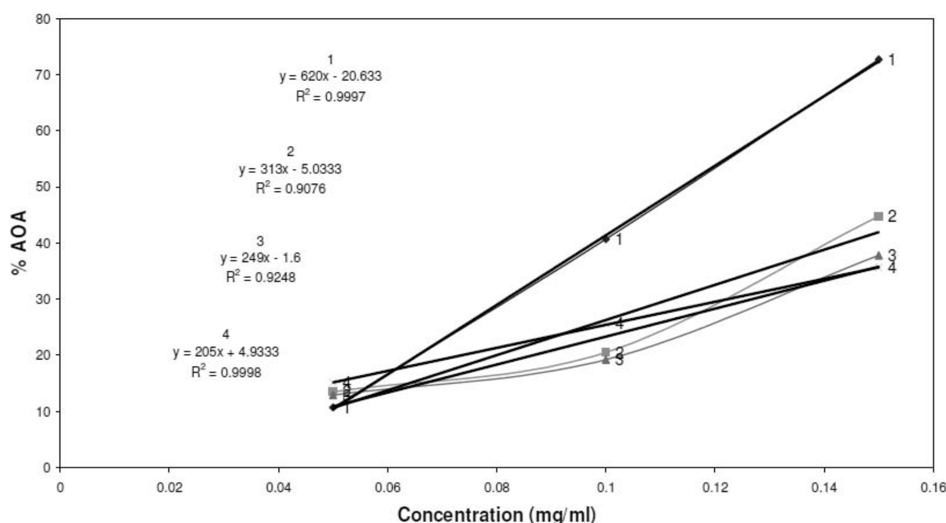


Fig. 2: Antioxidant oxidative activity percentage (AOA%) using DPPH

Table 3: Antioxidant activity of tested extracts (n=3) ±S. D.

Extract number	DPPH	β-carotene-linoleic
	EC 50 (mg/ml)	
Total methanol extract of As	0.114±0.06	0.110±0.005
EtOAc fraction	0.176±0.09	0.140±0.005
Butanol fraction	0.207±0.03	0.163±0.007
Chloroform fraction	0.220±0.04	0.165±0.002

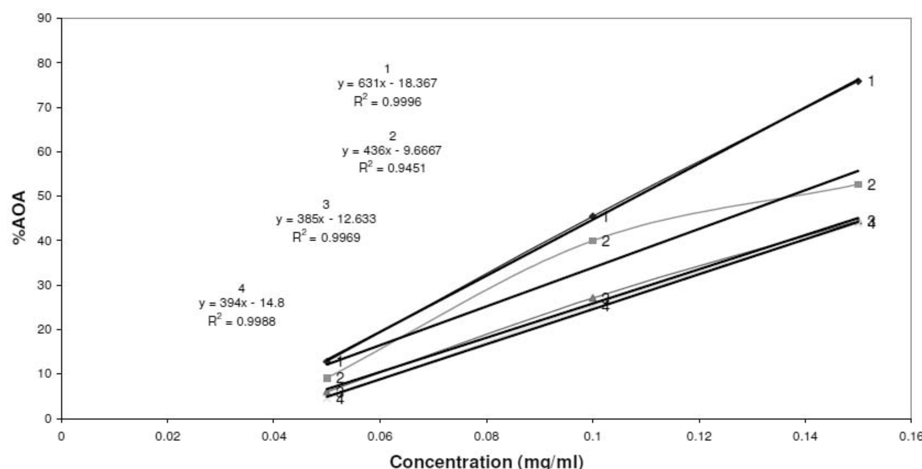


Fig. 3: Antioxidant oxidative activity percentage (AOA%) using Caroten Bleaching Methods

CONCLUSION

The phytochemical investigation of *Asparagus sprengeri* grown in Egypt, led to identification of 9 fatty acids in which myristic and linoleic acids are the main constituents. Also the phospholipid fraction was found to contain L-α-Phosphatidyl-DL-glycerol and L-α-Phosphatidylethanolamine. The investigation of the flavonoidal fraction led to the isolation and identification of four compounds, apigenin, dihydroquercitin, naringenin, apigenin- 7-*o*- glucoside, in which suggested to be a group of key antioxidants in *Asparagus*.

CONFLICT OF INTERESTS

Declared None**.**

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