PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATIONS OF *ATRIPLEX SEMIBACATA* R .BR. GROWING IN EGYPT

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Abstract

The lipid content of *Atriplex semibacata* growing in Egypt was studied. The unsaponifiable fraction was identified by GLC. A series of hydrocarbons ranging from C_{14}- C_{28} in addition to cholesterol, stigmasterol and the triterpenoids α and β - amyrin were identified. GLC analysis of fatty alcohols fraction revealed the presence of six fatty alcohols in which dotriacontanol ($C_{32}H_{66}O$) was the major (14.68%). Six compounds (five coumarins and one phenolic acid) were isolated for the first time from *A. semibacata.* The coumarin constituents isolated from the chloroform and the ethyl acetate fractions of the aqueous alcoholic extract of *A. semibacata* were identified as scopoletin, umbelliferorne, coumarin, scopolin, 7-methoxy coumarin in addition to a phenolic acid *P*-coumaric acid. Also, the flavonoidal compounds isolated from the *n*-butanol fraction of the plant revealed the presence of kaempferol 3-O glucoside and acacetin. Their identity was proved by m.p., TLC, PC, UV and MS analysis. The alcohol extract showed significant antimicrobial activity against G-ve bacteria, moderate activity against G+ve bacteria. On the other hand, the pet. Ether extract showed marked activity against G+ve bacteria and fungi, also the G-ve bacteria was greatly inhibited by the chloroform extract. The different extracts of the plant exhibited no cytotoxic activity against Erlich-ascites carcinoma cells line at the tested concentrations, also showed a strong antioxidant activity using DPPH.

Key words: *Atriplex semibacata***,** unsaponifiable fraction**,** fatty alcohols**,** coumarins**,** flavonoids, antimicrobial, antifungal, antioxidant, antitumor.

Introduction

The family *chenopodiaceae* is one of the largest families of the flowering plants. It is represented in Egypt by 25 genera and 300 species. The most important genera are *Ambrosia, Atriplex, Beta, suaeda* and *Halocnemum* (Tackholm, 1974). The occurrence of flavonoids in several *chenopodiaceae* species have been reported (Rizk, 1986). Also coumarins, alkaloids and terpenes were detected in several species (Rizk, 1982).

The genus *Atriplex* is represented in Egypt by 15 species (Tackholm, 1974) and little information was represented about the constituents of these genera. *Atriplex* are characterized by their high content of sodium chloride, they are said to be useful as fodder plants (Watt and Breyer, 1962). The study of the chemical composition of several species shows that the plants have a relatively high nutritive value for ruminants and many are useful as a feed supplement (Rizk, 1986), also it was found from the literature that the ethyl alcohol extract of *A. leucolata and A. lampa* showed respectively a molluscidal and antimicrobial activities (Shoeb, *et al.*, 1987; Arellano, *et al.*, 1988; Rizk, *et al.*, 1986). Phytochemical screening of several species revealed the presence of coumarins, saponines, alkaloids and few species contain flavonoids. Alkaloids were detected in *A. littoralis* and *A. nitens* and phenolic acids in *A.astata* (Rizk, 1986). Four flavonol glycosides were isolated from *A. littoralis* (Wiestawa, 2004) and a new flavone glycoside, identified as kaempferol-3-O-[3- acetyl-α-L- arabinopyranosyl- (1-6)] -β-D- glucopyranoside has been isolated from the aerial parts of *Thalictrum Atriplex* (Guangyao, *et al.*, 2000). Previous studies revealed the isolation of protocatechuic acid, caffiec acid, *P*-coumaric acid, kaempferol and β- sitosterol from the same plant (Gao, *et al.*, 1999). It was found from literature that four triterpenoid saponines were isolated from *A. semibacata* (Shaker and Seifert, 2003).

From the medical point, it was found that *A. hortensis* and *A. patula* were used as a folk remedy for plethora, lung ailments, diuretic and emetic. Also it was found that *A. halimu* can be used for hypoglycemic treatments (Yaniv, *et al.*, 1987).

Nothing was reported about the lipid and coumarin constituents of *A. semibacata*, therefore the present work deals with the study of lipid, coumarin and non-identified flavonoidal constituents of *A. semibacata* growing in Egypt and the study of the antimicrobial and antitumor activities activity of total extracts and isolated compounds, as well as studying their antioxidant activity.

Materials and Methods

Plant material

Atriplex semibacata R.Br. was collected from Borg El-Arab near Alexandria during June 2009. The plant was

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identified by Dr. M. El-Gebaly and Dr. S. El-Kawashty, taxonomists, National Research Centre, Cairo, Egypt, to whom the authors are deeply indebted. The aerial parts of the plant were air dried and ground into fine powder. A voucher specimen was kept in the herbarium of NRC.

Antimicrobial activity: The tested organisms were obtained from Medical Microbia and Immunity Dept , Faculty of Medicine , Cairo University..

Antitumor activity: The antitumor activity of the different extracts were tested against Ehrlich-ascites of carcinoma *in vitro* in the National Institute of Cancer, Cairo.

Anti-oxidant Activity

Reagent and solvent: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich Chemical Co.), 1,1 Diphenyl-2-picrylhydrazyl (DPPH, Sigma Chemical Co.) and Methanol (HPLC grade).

Apparatus and techniques: Shimadzu UV.PC.2401 spectrophotometer. Mass spectrophotometer GC-MS Finnigan mat SSQ7000 Mass spectroscopy 70 ev. Gas liquid chromatography Hewellett HP 6890 series. Electrothermal AZ 9003 MKS 400 ^{0}C maximum far measuring the melting points.

Gas liquid chromatography (GLC) analyses of the unsap. and fatty alcohols fractions were carried out according to the following conditions:

For the unsaponifiable matter and fatty alcohol:-
Column: HP-1 (methyl siloxane) 30m length/ 0.53 x 2.65 µm, Temp. program:- Ini. Temp. 60 °C, Ini. Time 2min., program rate 10 °C/min., F. temp. 280 °C, Final time 30 min., Injection temp. 260°C, Detector (FID), T= 300°C, Flow rate of carrier gas N_2: 30 ml/min., H_2 : 35 ml/min., Air : 30 ml/min.

Isolation of Lipids: About 2 Kg of the air dried powdered plant (aerial parts) of *A. semibacata* was extracted with pet. ether (40-60). The pet. ether extract was evaporated and the residue (28g) was dissolved in boiling acetone (250ml), cooled and the formed amorphous precipitate (3.1g) was separated out. The acetone soluble fraction was saponified (N/2 alc. KOH) and the unsaponifiable matter (1.68) was separated (Radwan and Abdel-Shafeek, 2006). Results are shown in table (1,2).

Isolation of coumarins and phenolic acids: About 1.8 Kg of defatted dried powdered of *A. semibacata* was macerated with 80% ethanol. The combined alcoholic extracts were evaporated *in vacuo* at 40 ^{0}C. The residue (48g) was dissolved in hot distilled water and left over night. The aqueous filtrate was portioned with successive portions of chloroform (3 X 200 ml), followed by ethyl acetate (3 X 200 ml) and finally with *n*-butanol (3 x 200 ml). The extracts were dehydrated separately over anhydrous sodium sulfate and evaporated *in vacuo* at 45 ^{0}C. The chloroform, ethyl acetate and *n*-butanol free residues amounted to 1.9 g, 2.2 g and 2.45 g respectively.

The chloroform fraction was subjected to preparative paper chromatography (3MM, 15% acetic acid). Four main zones (R_f 0.74, 0.69, 0.61 and 0.41) were cut and eluted separately by 90% methanol. The eluted fractions were further purified on sephadex LH-20 column using 90% methanol as eluent. The ethyl acetate fraction was also subjected to preparative paper chromatography (3MM, 15% acetic acid) and two main zones (R_f 0.49 and 0.36) were cut and eluted separately by 80% methanol. The *n*-butanol fraction was subjected to preparative PC (3 MM, 15% acetic acid) and two main zones (F_f 0.54 and 0.09) were cut and eluted separately with 90% methanol. The eluted fractions were further purified on sephadex LH-20 column using 90% methanol.

Antimicrobial and Antifungal activities: Seven representative strains of bacteria and fungi isolates were selected viz *Staphylococcus aureus, Sarcina lutea, Bacillus subtilis, Pseudomonas aeruginosa, Proteus mirabilis, Candida albicans* and *Saccharamyces cerviceae*. The different extracts and fractions of the plant in dimethyl formamide (DMF) were tested *in vitro* against the selected strains of bacteria and fungi isolates. The Antimicrobial activities were carried out by using agar diffusion disc method (Lorian, 1980). Results of antimicrobial and antifungal activities are shown in table (3).

Antitumor activity: The plant extracts were screened *in vitro* using a single tumor (Ehrlich-ascites carcinoma cells) using a dose of 100 µg/ml, 50 µg/ml and 25 µg/ml of each extract (table 4). This screening was carried out employing the method described earlier (El-Hossary, *et al.,* 2000 ; MC-Limans, *et al.*, 1957).

Determination of scavenging effect on DPPH radicals: The effect of the plant extracts on DPPH was studying employing the modified method described by (Yamaguchi *et al.*, 1998). The decrease of the absorbance at 516 nm of the DPPH solution after addition of the sample (plant materials) was measured in a glass cuvette. An aliquot of 0.1 ml M. methanol solution of DPPH was mixed with the methanolic solution of the sample, so that the relative concentration of plant materials versus the stable radical in the cuvette was 0.13, then the solution with tested sample was shaken vigorously. The absorbance was mentioned at the start and at 30 min. after being kept in the dark against a blank of methanol without DPPH. All tests were run in duplicate and averages were calculated (Nicolaos and Maria 2002). The antioxidative of these samples were compared with Trolox. Where

$$\% \text{ RSA} = 100\% \times \frac{\text{Abs of blank}_{516\text{ nm}} - \text{Abs of sample}_{516\text{ nm}}}{\text{Abs of blank}_{516\text{ nm}}}$$

The results are expressed as radical scavenging activity (%RSA) as shown in Table 5.

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Table (1): GLC analysis of unsaponifiable fraction of *A. semibacata*

Peak No.	RT (min.)	Relative%	Constituents
1	6.42	2.3	Non identified
2	8.03	5.7	n-Tetradecane $C_{14}H_{30}$
3	9.10	3.1	n-Pentadecane $C_{15}H_{32}$
4	10.3	1.21	Non identified
5	12.36	4.81	Heptadecane $C_{17}H_{36}$
6	13.71	2.9	n-Octadecane $C_{18}H_{38}$
7	15.23	3.8	n-Eicosane $C_{20}H_{42}$
8	18.14	5.92	n-Docosane $C_{22}H_{46}$
9	19.41	0.92	n- Tricosane $C_{23}H_{48}$
10	21.22	1.98	n- Pentacosane $C_{25}H_{52}$
11	24.66	32.94	n- Octacosane $C_{28}H_{58}$
12	25.78	11.23	β - amyrin
13	37.61	1.12	Non identified
14	30.92	7.21	Cholesterol
15	33.8	4.9	Stigmasterol
16	38.8	2.72	α - amyrin

Table (2): GLC analysis of Fatty alcohol fraction of *A. semibacata*

PeakNo.	RT(min.)	Relative%	Constituents
1	9.507	2.11	Tetracosanol $C_{24}H_{50}O$
2	11.59	4.6	Hexacosanol $C_{26}H_{54}O$
3	14.27	3.54	Non identified
4	17.45	1.98	Non identified
5	19.74	6.44	Triacontanol $C_{30}H_{62}O$
6	21.95	14.68	Dotriacontanol $C_{32}H_{66}O$
7	25.49	9.344	Tetratriacontanol $C_{34}H_{70}O$
8	31.11	4.59	Hexatriacontanol $C_{36}H_{74}O$
9	34.51	3.48	Non identified

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Table (3): Antimicrobial and antifungal activities of *A. semibacata* extracts

Organisms	Diameter of zone of inhibition in (mm)				
	Pet. ether Extr.	**Chloroform Extr.**	**Ethyl acetate Extr.**	**Alcohol Extr.**	**n-butanol**
Gram(+) 1. *Staphylococcus aureus*	16	8	10	12	7
2. *Sarcina lutea*	12	10	14	10	9
3. Bacillus subtilis	13	9	15	12	12
Gram(-) 4. *Pseudomonas aeruginosa*	11	14	10	20	13
5. Proteus mirabilis	9	16	12	18	15
Fungi 6. *Saccharamyces cerviceae*.	14	6	6	8	8
7. *Candida albicans*	18	8	6	4	11
Solvent control	-	-	-	-	-

Table (4): Results of screening of the antitumor activity of *A. semibacata* extracts

The component	Concentration in µg/ml	% Inhibition of Cell Viability
80% ethanolic Extr.	25 50 100	0 12 36
Pet. ether extr.	25 50 100	0 0 24
Chloroform extr.	25 50 100	12 22 36
Ethyl acetate extr.	25 50 100	18 24 39
***n*-butanol extr.**	25 50 100	11 19 27

Table (5) The radical scavenging effect of samples on DPPH free radical

Tested Compounds	*Absorbance 516nm/reaction period(min.) 10 mins	30 mins	RSA%
Trolox	0.018	0.019	96.5
Unsap.	0.092	0.095	80.4
Fatty alcohol	0.20	0.179	61.4
Chloroform extract	0.057	0.059	88.62
Ethyl acetate extract	0.101	0.108	79.69
n-butanol extract	0.085	0.092	73.18

* The absorbance reading at each reaction period is the mean of two measurements.

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Results

Unsaponifiable Fraction

GLC analysis for the unsaponifiable fraction proved to be a mixture of triterpenes, sterols and a series of hydrocarbons. Identification of the compounds was carried out by comparison of their retention times with the available reference compounds Table 1.

Fatty alcohol Fraction

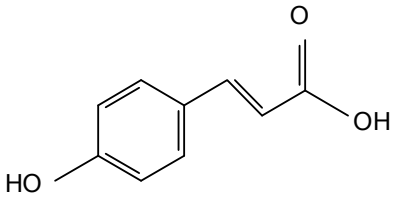
GLC analysis for the acetone precipitate fraction of *A. semibacata* revealed the presence of six fatty alcohols in which dotriacontanol ($C_{32}H_{66}O$, 14.68%) was the major component as illustrated in Table 2.

P-coumaric acid (compound A)

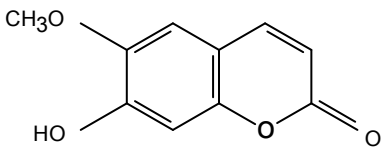
The residue obtained from the eluted band (R_f 0.74) isolated from The chloroform fraction by preparative paper chromatography (3MM, 15% acetic acid) and eluted by 90% methanol, gave after purification on sephadex LH-20 column using 90% methanol, a single compound which gave a blue color under UV light. The UV spectrum in methanol showed peaks at (317, 268 and 216 nm) that found to be identical to those of *P*-coumaric acid (Gao, *et al.*, 1999 ; Yang, *et al.*, 2002). Also the R_f of the compound was identical to that of *P*-coumaric acid in different solvents and adsorbents. Further confirmation was performed by carrying out EI-Mass spectrum which showed a molecular ion peak [M^+] 164 which corresponding to the molecular formula $C_9H_8O_3$ and fragments at m/z 147, 119 that are identical to that reported for *P*-coumaric acid (Gao, *et al.*, 1999 ; Foaad, *et al.*, 1997).

Scopoletin (compound B)

The residue obtained from the eluted band (R_f 0.69) isolated from The chloroform fraction (21 mg) by preparative paper chromatography (3MM, 15% acetic acid) and eluted by 90% methanol, gave after purification on sephadex LH-20 column using 90% methanol, a single compound which gave a blue color under UV light and no color with p-anizaldehyde. Upon crystallization from methanol, it gave needles (m.p. 203 ^oC). The UV spectrum in methanol showed peaks at (342, 293_{sh}, 260_{sh} and 226 nm) these data are identical to those of scopoletin (Eyre and Spottis, 1965 ; Murray, *et al.*, 1982). Further confirmation was performed by carrying out EI-Mass spectrum which showed a molecular ion peak [M^+] at m/z 192 which corresponds to the molecular formula $C_8H_8O_4$ and fragments at M/z 177 (M^+- CH_3) , 164 (M^+- CO), 149, 121 that are identical to that reported for scopoletin (Murray, *et al.*, 1982).



P. Coumaric acid



Scopoletin

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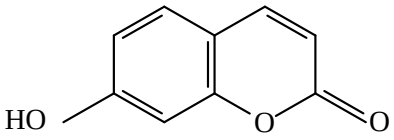
Umbelliferone (compound C)

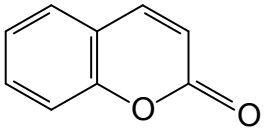
The residue obtained from the eluted band (R_f 0.61) isolated from The chloroform fraction (16 mg) by preparative paper chromatography (3MM, 15% acetic acid) and eluted by 90% methanol, gave after purification on sephadex LH-20 column using 90% methanol, a single compound which gave a blue fluorescence under UV light and no color with p-anizaldehyde. The UV spectrum in methanol showed peaks at (320, 257 and 222 nm). Upon crystallization from methanol, it gave colorless crystals (m.p. 226-227 ^{o}C). These data are identical to those of Umbelliferorne (Yang, *et al.*, 2002 ; Foaad, *et al.*, 1997). The EI-Mass spectrum showed a molecular ion peak [M^+] at m/z 162 which corresponds to the molecular formula $C_9H_6O_3$ and fragments at M/z 134 (M^+- CO), 106 that are identical to that reported for Umbelliferorne (Murray, *et al.*, 1982).



Umbelliferone

Coumarin (compound D)

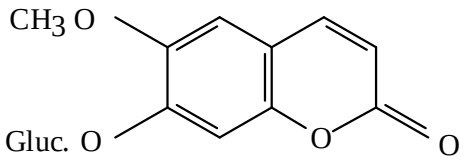
The residue obtained from the eluted band (R_f 0.41) isolated from The chloroform fraction (24 mg) by preparative paper chromatography (3MM, 15% acetic acid) and eluted by 90% methanol, gave after purification on sephadex LH-20 column using 90% methanol, a single compound which gave a blue color under UV light and no color with p-anizaldehyde. Upon crystallization from methanol, it gave faint yellow crystals (m.p. 71-72 ^{o}C), also the UV spectrum in methanol showed peaks at (310, 274 and 215 nm). These data are the same of that reported for coumarin **(Yang, *et al.*, 2002**). The EI-Mass spectrum showed a molecular ion peak [M^+] at m/z 146 which corresponds to the molecular formula $C_9H_6O_2$ and fragments ions at m/z 118, 90 that are identical to that reported for coumarin **(Murray, *et al.*, 1982**).



Coumarin

Scopolin (compound E)

The residue obtained from the eluted band (R_f 0.49) isolated from The ethyl acetate fraction (18 mg) by preparative paper chromatography (3MM, 15% acetic acid) and eluted by 90% methanol, gave after purification on sephadex LH-20 column using 90% methanol, a single compound which posses the same R_f values as authentic scopolin using different solvents and adsorbents. Crystallization was carried out from methanol to yield yellowish crystals (m.p. 217-218 ^{o}C) **(Yang, *et al.*, 2002**). Acid hydrolysis (2N HCl) of the compound gave scopoletin which was identified by co-chromatography using TLC, PC, UV and MS [M^+] at m/z 192 and fragments at M/z 177, 164, 149, 121 that are identical to that reported for scopoletin **(Murray, *et al.*, 1982**). The sugar moiety identified as glucose by PC. Whatman No.1, ethyl acetate – pyridine – water (12: 5: 4) and *n*-butanol-benzene-pyridine (5: 13: 3).



Scopolin

7- Methoxy coumarin (compound F)

The residue obtained from the eluted band (R_f 0.36) isolated from the ethyl acetate fraction (12 mg) by preparative paper chromatography (3MM, 15% acetic acid) and eluted by 95% methanol, gave after purification on sephadex LH-20 column using 90% methanol, a single compound which gave blue fluorescence under UV light and no color with *p*-anizaldehyde. The UV spectrum in methanol showed peaks at (349, 322, 250, 216 and 205 nm), these data are identical to those of 7-methoxy coumarin (herniarin). The EI-Mass spectrum showed a molecular ion peak [M^+] at m/z 176 which corresponds to the

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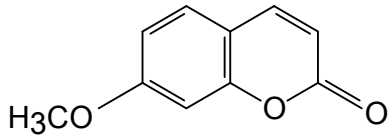
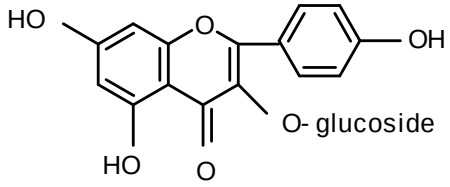
molecular formula $C_{10}H_8O_3$ and fragment ions at M/z 148 (M^+- CO), 133 (M^+- CO-CH_3), 105 and 77, that in agreement with that reported for herniarin **(Murray, *et al.*, 1982**).



Methoxy coumarin

8- Kaempferol 3-O glucoside (compound G)

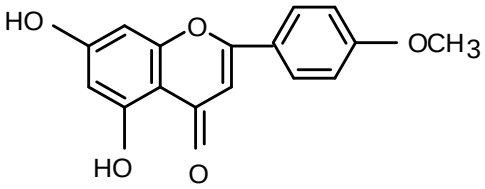
The flavonoidal band (R_f 0.54), isolated from *n*-butanol fraction by preparative PC (3 MM, 15% acetic acid) and eluted with 90% methanol, gave after purification on sephadex LH-20 column using 85% methanol, a single flavonoidal compound corresponding to that of Kaempferol 3-O glucoside (16 mg) which identified by PC and UV (Mabry, *et al*., 1970). Acid hydrolysis (2N HCl) gave Kaempferol which identified by mp (275-277°) (Eyre and Spottis, 1965), TLC, PC, UV table (6) and MS [M^+] 286 and fragments at m/z 258 (M^+- Co), 152A_1, 153 (A_1 – 1), 134 B, 124 which are characteristic for that of Kaempferol (Mabry, *et al*., 1975). The sugar moiety was identified as glucose (PC Whatmann No. 1, Ethyl acetate – Pyridine- Water (12:5:4).



Kaempferol 3-O glucoside

9- Acacetin (compound H)

The flavonoidal band (R_f 0.09), isolated from *n*-butanol fraction by preparative PC (3 MM, 15% acetic acid) and eluted with 90% methanol, gave after purification on
sephadex LH-20 column using 95% methanol, a single flavonoidal compound corresponding to that of acacetin (18 mg) which identified by TLC, PC, UV (**Mabry, *et al*., 1970**), **table (6)** and MS [M^+] 284 in addition to fragment ions at m/z 269 (M^+- CH_3), 256 (M^+- Co), 253 (M^+- OCH_3), 153, 118 which are characteristic for that of acacetin (**Mabry, *et al*., 1975**).



Acacetin

Table (6): UV spectral data (nm) of the isolated flavonoids

Flavonoids	UV.abs in MeOH	NaOMe	$AlCl_3$	$AlCl_3$ + HCl	$NaOA_C$	$NaOA_C$+ H_3BO_3
Compound G	256, 315, 826	368,271h 219	396, 308 276,213	397,313 277,225	366,318 277	363, 315, 269
Compound H	328, 304, 268	261, 291sh, 276	378, 340, 290 sh, 273, 257 sh	382, 340, 264 sh	358, 301sh, 280	331, 307 sh, 272

Sh = Shoulder
Compound G = Kaempferol 3-O glucoside
Compound H = Acacetin

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Discussion

Fractionation of the constituents of pet. ether extract of *Atriplex semibacata* was carried out and the components of various fractions were identified by GLC. The unsaponifiable fraction was found to contain a series of hydrocarbons ranging from C_{14}- C_{28}, in which C_{28} (32.94%) was the main component. Cholesterol and stigmasterol were represented by 7.21% and 4.9% respectively and the triterpenoids α and β - amyrin were represented by 11.23% and 2.72%. GLC analysis of fatty alcohols fraction revealed the presence of a mixture of six fatty alcohols in which dotriacontanol ($C_{32}H_{66}O$) represented the major alcohol (14.68%). The study of the coumarin fraction revealed the isolation and identification of scopoletin, umbelliferorne, coumarin, scopolin, 7-methoxy coumarin in addition to a phenolic acid *P*-coumaric acid. Also, the study of the flavonpidal compounds isolated from the *n*-butanol fraction of the plant revealed the isolation and identification of kaempferol 3-O glucoside and acacetin. Their identity were proved by m.p., TLC, PC, UV and MS analysis, in addition to acid hydrolysis of the glycoside compounds. This the first record of the coumarins in *Atriplex semibacata*. The results of antimicrobial activities Table 3 showed that the alcohol extract exhibited higher antimicrobial activity against G-ve bacteria, *Pseudomonas aeruginosa* and *Proteus mirabilis*,moderate antimicrobial activity against G+ve bacteria, *Staphylococcus aureus, Bacillus subtilis*. On the other hand the pet.ether extract showed a significant antimicrobial activity against G+ve bacteria, *Staphylococcus aureus* and fungi, *Candida albicans*, also it is clear from table (3) that G-ve bacteria was greatly inhibited by the chloroform extract. The results of the antitumor activity Table 4 of the different extracts of the plant showed no cytotoxic activity against Erlich-ascites carcinoma cells line at the tested concentrations (100 µg/ml, 50 µg/ml and 25 µg/ml). Also, the radical scavenging effect of the tested extracts and isolated compounds using DPPH free radical were represented in Table 5. The chloroform, ethyl acetate and *n*-butanol extracts showed a strong antioxidant activity due to the presence of the main coumarin and flavonoidal compounds. Also, unsaponifiable fraction, posses a moderate antioxidant activity followed by fatty alcohol fraction compared to Trolox (standard antioxidant compound).

Conclusion

The significant antimicrobial and antifungi activities of the petroleum ether and chloroform extracts may be attributed to the presence of the coumarin compound and unsaponifiable fraction. The biological activities of different extracts were available, but the antimicrobial and antifungi activities of the different isolated compounds were not available due to their small quantities. Therefore, we will complete the research in a large scale to isolate enough quantities to measure the biological activity of the isolated compound in the near future in another research.

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