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New Acetyl Triterpenoidal and Biological Activities of Euphorbia Paralias and Euophorbia Geniculata (Euphorbiaceae) from Egypt

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PHYTOCHEMICAL study of the aerial parts of *Euphorbia paralias* and *Euophorbia geniculata* belonging to family (Euophorbiaceae) grown in Egypt was carried out. This study revealed the isolation of eighteen compounds: *β-amyrin* (1), *β-sitosterol* and *Stigmasterol* mixture (2 & 3), *Cholesterol* (4), *Campesterol* (5), (*Erythradiol*, *Ovaol* and *Betulinol* mixture) (6-8), *23-acetyl-3- methyl-oleanolic acid* (9) and *β-Sitosterol–3-O-glucoside* (10) were isolated from dichloromethane fraction of *E. paralias*. While the compounds 1 to 5 and 10 were isolated from *E. geniculata*. The ethyl acetate fraction of *E. paralias* L. afforded the isolation of *Gallic acid* (11), *Ellagic acid* (12), *Querecetin-3-glucopyranoside* (13) and *Querecetin -3-arabinoside* (14), *kaempferol-3-(6"-(2"'-galloyl-glucopyranoside*) (15). While compounds 11 to 14 *in addition to Querecetin-3-rutinoside (rutin)* (16), *Querecetin-3-rhamnoside* (17), and *Querecetin* (18) were isolated from *E. geniculata* Ortega. Compound 9 (*23-acetyl-3-methyl-oleanolic acid*) was isolated for the first time from the nature. Biological activities were carried out including cytotoxic and antiviral and antimicrobial activities of the dichloromethane and ethyl acetate fractions of the aerial parts of *E. paralias* L. and *E. geniculata* Ortega revealing significant antimicrobial activity of ethyl acetate fractions of *E. paralias* L. and *E. geniculata* Ortega and strong cytotoxic activity of the ethyl acetate fractions in addition to positive antiviral activity of the total extract of *E. paralias* L.

Keywords: Triterpenes, Flavonoids, Phenolics, Cytotoxic, Antimicrobial and antiviral.

Introduction

Euphorbia is one of the largest genera of flowering plants belonging to the very diverse Family (Euphorbiaceae) with approximately 2,000 species [1]. Many of these species have been used traditionally as medicinal plants for the treatment of many diseases such as; skin diseases, migraine, gonorrhea, and intestinal parasites [2]. The triterpene alcohols found in the latex of *Euphorbia* species have been used as chemotaxonomic markers [3-4]. In addition monoterpenes, diterpenes and sesquiterpenoids, cerebrosides, glycerols, flavonoids, and steroids were also obtained [1]. The compounds isolated from genus *Euphorbia* and extracts perform many different activities, including antiproliferation, modulability of multidrug resistance, cytotoxic activity, antimicrobial, anti-inflammatory activity [1]. The phytochemical investigation of *E. paralias* L. grown in Egypt extracts has revealed the isolation of compounds such as ditepenes, triterpene and flavonoids [5 -9]. *E. geniculata* Ortega is native plant to Mexico distributed as wild plant in Delta farms of Egypt. Terpenoidal compounds such as geniculetine saponin, genicunolide A, B and C [10-11]. The present work is a comparative study on phytochemical and biological activities on the aerial parts of ***E. paralias*** and ***E. geniculata*** grown in Egypt.

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Experimental Section

General section

Evaporation of the solvents was done using a Buchi rotary evaporator. U.V. lamp for TLC visualization U.V.P., GL-58 (λ_{max} 254 and 366 nm). Circulating hot air oven, W.T-binder 7200, (Germany). Shimadzu U.V.-1700 spectrophotometer (Japan) for UV spectral analysis, using NaOMe, AlCl3, HCl, NaOAc and Boric acid as shift reagent for UV analysis of flavonoids . Infrared spectral analysis were recorded in potassium bromide disks on a Pye Unicam SP 3000 and IR spectrophotometer, Jasko, FT/IR-460 plus. EI-Ms spectra were carried out on Direct Probe Controller Inlet part to Single Quadropole mass analyzer in Thermo Scientific GCMS model ISQ LT using Thermo X-Calibur software at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Nasr City, Cairo. Polyamide 6 column was been used and Sephadex LH-20 in fractionation of ethyl acetate fractions. NMR analysis experiments were performed on a Bruker AMX 400 and 500 MH_Z for ^{1}H-NMR and with standard pulse sequences operating at 100, 125 MHz for ^{13}C-NMR. While chemical shifts are given in δ values (ppm) by using tetramethylsilane (the internal standard), DMSO-d_6 (solvent at room temperature) and solvents for NMR determination viz.: Deuterated methanol (CD_3OD), deuterated chloroform ($CDCl_3$), and DMSO-d_6 were of spectroscopic grade for spectral analysis. LC–UPLC-ESI-MS positive and negative ion acquisition mode was carried out on a XEVO TQD triple quadruple instrument. Waters Corporation, Milford, MA01757 U.S.A, mass spectrometer Column: ACQUITY UPLC - BEH C18 1.7 µm - 2.1 × 50 mm Column, Flow rate: 0.2 ml \ min, Solvent system: consisted of (A) Water containing 0.1 % formic acid, (B) Methanol containing 0.1 % formic acid and solvent systems for TLC were (solvent system 1; dichloromethane 100 %), (solvent system 2 ; 5 % Methanol in petroleum ether) , (solvent system 3; 40 % ethyl acetate in petroleum ether), (solvent system 4; 15 % Methanol in dichloromethane), (solvent system 5; 8 % Methanol in dichloromethane), (solvent system 6; HOAc15 %), (solvent system 7; BAW; n−butanol: acetic acid: water; 4:1:5; the upper layer), and (solvent system 8; EtOAc : MeOH: H_2O (30 : 5 : 4)).

Plant material and extraction process

E. paralias L. and *E. geniculata* Ortega family Euphorbiaceae were collected in the flowering stage on May 2015 and August 2015, respectively, from the North beach of Alexandria and the roadsides and beside farms in the vicinity of Benha, Qalubya, Egypt. The identification was kindly verified by Dr. Ahmed Abd El-Razik respective lecture of Plant Taxonomy, Department of Botany Faculty of Science, Benha University, Egypt. The vouchers specimens (no. S303 and S304) were deposited in National Research Center, El Doki, Egypt. The plant materials were air dried and ground by electric mill to moderately fine powder. The solvents used in this work viz.: Light petroleum (60-80 ^{o}C), dichloromethane, ethyl acetate, methanol were of the analytical grade for chromatography and crystallization. These solvents and chemicals were prepared according to the procedures described by Vogel [12]. The air-dried powdered plant materials (3 kg (*E. p*) and 2 kg (*E. g*)) were extracted by maceration with 70 % methanol until complete exhaustion. Both total extracts were separately dried under reduced pressure at 45 ^{o}C. The greenish brown viscous residues (500 g (*E. p*) and 385 g (*E. g*)) were separately dissolved in least amount of MeOH - H_2O (1:9), then subjected to fractionation by dichloromethane and ethyl acetate to give dry dichloromethane (17.5 and 12 g) and ethyl acetate (39.5 and 21 g) extracts, respectively.

Isolation of the compounds 1-10 from dichloromethane fractions

The dichloromethane soluble fractions of the aerial parts of *E. Paralias* (15 g) and *E. geniculate* (10 g) were placed separately on the top of a silica gel column. The elution started with light petroleum then the polarity was increased gradually using dichloromethane followed by ethyl acetate. Fractions were collected 1L of each, examined by TLC. The similar fractions were collected together. Fractions eluted with 40-50 % dichloromethane in petroleum ether result in the isolation of compound 1. While fractions eluted with 60 -70 % dichloromethane in petroleum ether subjected to sub-column fractionation and recrystallization affoded compounds; 2 & 3 as a mixture, 4 and 5). Compounds 6, 7 and 8(inseparable mixture) and pure 9 were isolated from fractions eluted with 30 % ethyl acetate in dichloromethane followed by PTLC (preparative thin layer chromatography with solvent system 3). Finally compound 10 was isolated from fractions eluted with 50 % ethyl acetate in dichloromethane.

Isolation of the compounds 11-18 from ethyl acetate fractions

The ethyl acetate fractions of *E. paralias* (18 g) and *E. geniculate* (10 g) were separately fractionated on Polyamide 6 CC. The column was eluted with water and then polarity decreased gradually using methanol. Ten fractions (500 ml of each) were collected, concentrated under reduced pressure and subjected to paper chromatography screening with solvents 6 and 7. The important fractions were applied on a several Sephadex LH−20 column. Eight compounds (11-18) were isolated from their fractions on the following sequence; compound 11 & 12 (20 % MeOH in H_2O), compounds 15 & 16 (40 % MeOH in H_2O), compounds 13, 14 & 17 (60 % MeOH in H_2O), and compound 18 (100 % MeOH in H_2O).

β-Amyrin (1) (18 mg); colorless needle crystals, R_f 0.38 (solvent system 1), soluble in dichloromethane and insoluble in methanol, IR spectrum $ν_{max}$ (KBr): 3426-3250, 2936, 2866, 1648, 1462 cm^{-1}. EI-MS: *m/z*: 426 [M] + corresponding to $C_{30}H_{50}O$.

β-Sitosterol and Stigmasterol (mixture 2 and 3) (40 mg): colorless needle crystals, R_f 0.43 (solvent systems 1), freely soluble in chloroform and insoluble in methanol, IR $ν_{max.}$ (KBr): 3439-3400, 2930, 2850, 1640 and 1460. EI-MS *m/z* 414 (M^+) for $C_{29}H_{50}O$ and 412 [M] + for $C_{29}H_{48}O$.

Cholesterol (4) (9 mg); colorless needle-shaped crystals, R_f 0.4 (solvent system 2), It is soluble in chloroform, and insoluble in methanol and acetone. IR $ν_{max}$ (KBr) cm^{-1}: 3430- 3400, 2936, 2867, 1665 and 1464. EI-MS: m/z 386 [M] + for $C_{27}H_{46}O$.

Campesterol (5) (10 mg); white crystals with and R_f 0.37 (solvent system 2), soluble in chloroform, insoluble in ethyl acetate and methanol, IR $ν_{max}$ (KBr) cm^{-1}: 3433- 3233, 2929, 2863, 1638 and 1456. EI-MS m/z 400 [M] + for $C_{28}H_{48}O$.

*Erythradiol, Ovaol and Betulinol (mixture of 6, 7 and 8); (*10 mg): white amorphous powder, R_f 0.52 (solvent system 3), soluble in chloroform and slightly soluble in methanol, IR $ν_{max}$ (KBr) cm^{-1}: 3427 – 3250, 2930, 2868, 1686 and 1462. EI-MS *m/z* 456 [M] + for $C_{31}H_{52}O_2$ and 442 [M] + for $C_{30}H_{50}O_2$. ^{1}HNMR and ^{13}CNMR results were collected in table 1.

3β-O-Methyl-23-acetyl-Oleanolic acid (9); (12 mg) white amorphous powder (chloroform-methanol), R_f 0.5 (solvent system 3), soluble in chloroform and slightly soluble in methanol, IR $ν_{max}$ (KBr) cm^{-1}: 3400 - 3490, 2927, 2854, 1743, 1687, 1635 and 1464. EI-MS *m/z* 528 [M] + for $C_{33}H_{52}O_5$, 513 [M- CH_3]+, 495 [M- CH_3-H_2O]+, 456 [Oleanolic acid]+, 309, 248, 207,175, 171, and 147. ^{1}HNMR and ^{13}CNMR results were collected in table 2.

β-sitosterol-3-O-β-D-glucopyranoside (10) (40 mg); white amorphous powder, and R_f 0.62 (solvent system 4), insoluble in chloroform and soluble in hot chloroform methanol mixture, IR $ν_{max}$ (KBr) cm^{-1}: 3423- 3320, 2934 -2869, 1637 and 1461. EI-MS *m/z* 576 [M] +, 414 [M - deoxy-glucosyl]+.

Gallic acid (11) (17 mg) and Ellagic acid (12) (20 mg): Faint yellow needles in visible light changed to brown after exposure to ammonia vapors, spraying with $FeCl_3$ gave blue color. UPLC-MS_2 in negative mode show molecular ion peaks at (169, 125) and (301) (M-H, M-COOH), respectively. The UV spectral data of 11 exhibited an absorption band ($λ_{max}$ 217, 272 and 353, 367) characteristic for gallic and ellagic acids [16]. ^{1}H-NMR (400 MHz, DMSO–d6) (**11**): singlet signal at $δ_H$ 6.98 (2H, s, H-3/5) and broad signal at $δ_H$ 9.17 (-OH, H- bonded protons) ^{13}C-NMR (100 MHz, DMSO–d6) spectrum showed δc 145.5 (C-3/C-5), 138.1 (C-4), 120.6 (C-1), 108.8 (C-2/6) and 167.6 (C-7). ^{1}H-NMR (400 MHz, DMSO–d6) (12) δH 7.42 integrated for two aromatic protons assigned for H-5 and H-12, broad singlet at δH 10.62 integrated for four protons assigned for four hydroxyl groups at positions 3, 4, 10 and 11, ^{13}C-NMR (100 MHz, DMSO–d6) δc 148.56 (C-4 & C-11), 140.02 (C-3 and C-10), 136.84 (C-2 & C-9), 112.77 (C-6 & C-13), 110.7 (C-5 & C-12), 108.13 (C-15 & C-16), 159.6 (C-7 & C-14).

Quercetin-3-O-β-D-glucopyranoside (13) (12 mg): yellow amorphous powder gave dark purple fluorescent spot turned to yellow on PC with ammonia vapor [17], IR $ν_{max}$ cm^{-1} : 3260-3320, 2876, 1661, 1608, 1563, 1514 and 1460. UPLC-ESI-MS_2 in negative ion: 927, 463 (100 %) (M-H), 301, The ^{1}H-NMR (400 MHz, DMSO–d6) δ 7.76 (1H, dd, *J* = 8.6, 2 Hz); δ 7.53 (1H, d, *J* = 2 Hz); and 6.80 (1H, d, *J* = 8.6 Hz), two doublet at δ 6.40 and 6.20 ppm with *J* = 1.8 Hz, 5.37 (1H, d, J= 8 Hz, H-1”), 3.90-3.10 (6H, m, remaining sugar protons). ^{13}C-NMR (100 MHz, DMSO–d6): 156 (C2), 133.9 (C3), 177.7 (C4), 161.62 (C5), 99.08 (C6), 164.82 (C7), 93.93 (C8), 156.74 (C9), 104.26 (C10), 121.51 (C1’), 115.54 (C2’), 146.3 (C3’), 148.79 (C4’), 116.4 (C5’), 122.34 (C6’), 101.2 (C1”).

TABLE 1. 1H- and 13C-NMR spectral data of the 6, 7 and 8 mixture in CD_3Cl_3 .

C no	8		7		6	
	δH	δC	δH	δC	δH	δC
1	1.55,1.32, 1H, m	38.31	1.55, 1.3, 1H, m	38.7	1.55, 1.32, 1H, m	38.79
2	1.7, 1.44 m	27.38	1.67, 1.44 m	27.38	1.67, 1.44 m	27.99
3	3.2 , 2H, dd, 10, 4 Hz	79.01	3.19, 2H, dd, 12, 5 Hz	79.01	3.19, 2H, dd, 12, 5 Hz	79.02
4	-	38.87	-	38.45	-	38.71
5	1.4, 1H, t, 9.5 Hz	55.29	1.42, 1H, t, 10 Hz	55.34	1.44, 1H, t, 10 Hz	55.17
6	1.55,1.28 1H, m	17.36	1.58, 1.32 1H, m	18.31	1.59, 1.32, 1H, m	17.36
7	1.58, 1.32 1H, m	34.23	1.56, 1.32 1H, m	32.82	1.56, 1.28, 1H, m	32.5
8	-	40.8	-	39.36	-	39.43
9	1.4 1H, t , 4 Hz	50.4	1.42, t, 3.5	47.8	1.42, t, 3.5	47.66
10	-	37.31	-	37.01	-	37.08
11	1.5, 1.28, 1H, m	20.84	2.21, 1.7, 1H, m	23.39	2.26, 1.76 1H, m	23.39
12	1.52, 1.28	25.21	5.16, 1H, t, J = 8 Hz	122.62	5.31, 1H, t, J = 8 Hz	125.05
13	1.43, 1H, m	38.87	-	143.64	-	139.0
14	-	42.8	-	42.41	-	43
15	1.58, 1.32, 1H, t, J; nd	27.38	1.4, 1.12, 1H, t, J; nd	25.21	1.42, 1.16, 1H, t, J; nd	25.21
16	1.58, 1.32, 1H, t, J; nd	37.31	1.32, 1.24, 1H, t, J; nd	23	1.32, 1.24, 1H, t, J; nd	23
17	-	38.79	-	37.08	-	35.3
18	1.43, 1H, d, 3.5	47.66	2.07, 1H, d, 4	41.2	2.02, 1H, d, 4	41.2
19	2.18, 1H, m	47.8	1.46, 1.24, 1H, m	46.5	1.44, 1.23, 1H, m	39.7
20	1.64 ,1.39, 1H, m	27.99	-	30.69	-	39.43
21	1.28, 1.3, 1H, m	37.31	1.56, 1.31, 1H, m	34.23	1.54, 1.32, 1H, m	28.12
22	0.97, 3H, s	23.21	1.56, 1.31, 1H, t, 3.5	30.69	1.52, 1.28, 1H, t, 3.5	35.2
23	0.95, 3H, s	23.44	1.04, 3H, s	28.12	1.16, 3H, s	28.12
24	0.80, 3H, s	16.12	1.02, 3H, s	16.77	1.12, 3H, s	16.77
25	0.74, 3H, s	18.36	1.0, 3H, s	16.12	0.95, 3H, s	16.12
26	1.0, 3H, s	15.37	0.99, 3H, s	17.36	0.93, 3H, s	17.36
27	1.32, 3H, s	29.75	0.91, 3H, s	26	0.89, 3H, s	23.39
28	3.81, 3.83(dd,8,12 Hz)	60.56	3.20, 3.58 (2H, s)	69.95	3.55, 3.52 (2H, s)	69.95
29	-	150.63	0.81, 3H, s	33.9	0.84, 3H, s	17.36
30	4.75, 4.62, 1H, m	109.71	0.80, 3H, s	23.64	0.78, 3H, s	21.1
31	1.7, 3H, s	28.12	-	-	-	-

TABLE 2. $^{1}H^{-}$ (400 MHz) and $^{13}C^{-}$NMR (125 MHz) of compound 9 in CD_3Cl_3 .

C no	δH	δC	C no	δH	δC
1	1.32, 1.64, 2H, d, J=12 Hz	38.42	18	2.84, 1H, dd J=8, 4 Hz	40.98
2	2.02, 1.61, 2H, d, J=4 Hz	26.95	19	1.63, 1.14, J=12	45.86
3	3.24, 1H, dd, J=12, 8 Hz	79.03	20	-	30.67
4	-	51.16	21	1.32, 1.23, t, J=12	33. 7
5	0.77 1H, t, 4 Hz	55.15	22	1.88, 1.61, t=12	32.37
6	1.56, 1.29, 2H, m	18.3	23	4.31, 4.17, dd, J=12, 8	62.19
7	1.32, 1.15, 2H, t, J=4	32.42	24	0.84, 3H, S	15.65
8	-	39.28	25	0.79, 3H, S	15.32
9	1.54, m	47.63	26	0.77, 3H, S	16.9
10	-	38.74, s	27	1.14, 3H, S	25.8
11	1.96 , 1.88, 2H , t, J=12 Hz	23.16, t	28	-	183.89
12	5.29, t, J=8	122.62, d	29	0.9, 3H, S	32.9
13	-	143.64, s	30	0.94, 3H, S	23.48, c
14	-	41.63, s	31	3.9, S, 3H	55.5
15	1.3, 1.26, 2H, t, J=12	29.55, t	32	-	176.6, S
16	2.23, 1.96, 2H, t, J=8	22.85	33	2.1, 3H, S	21
17	-	46.57	-	-	-

Quercetin-3-O-β-D-arabinopyranoside (14) (13 mg): yellow amorphous powder, gave dark purple fluorescent spot turned to yellow on PC with ammonia vapor**.** IR ν_{max} cm^{-1} at 3476, 3372, 3189, 2902, 1655, 1615, 1510 and 1469. HPLC-PDA-ESI-MS in negative mode: m/z 433 [M-H]-, 301 [M-H-arabinosyl]-, [Quercetin-H]-. ^{1}H-NMR (400 MHz, DMSO–d6): δ 6.43 (1H, d, J= 1.8 Hz, H-8), 6.20 (1H, d, J= 1.8 Hz, H-6), 7.67 (1H, dd, *J* = 8.6 and 2 Hz, H-6'); δ 7.57 (1H, d, *J* = 2 Hz, 2'); and 6.83 (1H, d, *J* = 8.6 Hz, 5'), 5.38 (1H, d, J= 8 Hz, H-1"), ^{13}C-NMR (125 MHz, DMSO–d6): δ_C 177.96 (C-4), 164.77 (C-7), 161.67 (C-5), 156.75 (C-2), 156.71 (C-9), 149.07 (C-4'), 145.45 (C-3'), 134.19 (C-3), 122.53 (C-1'), 121.36 (C-6'), 116.22 (C-5'), 115.83 (C-2'), 104.34 (C-10), 101.87 (C-1''), 99.17 (C-6), 93.99 (C-8), 72.12 (C3"), 71.2 (C2"), 66.5 (C4"), 64.8 (C5").

Kaempferol3O(6"OgalloylβDglucopyranoside **(15)** (14 mg); yellow amorphous powder, R_f 0.52 (BAW), purple color under ultraviolet (UV) light turning to bright yellow when fumed with ammonia vapors**.** IR ν_{max} (KBr) cm^{-1} at 3473, 3286 and 3120, 2898, 1730, 1660 and 1568, **.** UPLC-ESI-MS_2 in negative mode: m/z 599 [M-H]-, 447 [M-H-galloyl]-, 284 [M-H-galloylglucosyl]- (100 %), and 151 [aglycone fragment of ring-B]-, Complete acid hydrolysis yielded kaempferol, gallic acid and glucose as sugar moiety in which all of them were chromatographed with authentic samples. ^{1}H-NMR (400 MHz, DMSO–d6) : δ 7.93 (*d, J* = 10.4 Hz, H2′,6′), 6.77 (*d, J* = 9.2 Hz, H3′,5′), 6.41 (d, J = 2 Hz, H-8), 6.20 (d, J = 2 Hz, H-6), δ 5.45 (1H, d, J = 7.5 Hz, H-1"), 6.93 (2H, s, 2"', 6"'), 4.27 (1H, dd, J= 12, 5.5 Hz, H-6a"), 4.17 (1H, dd, J= 12, 2.4 Hz, H-6b") and 3.90-3.10 (m, 4H, remaining sugar protons). ^{13}C-NMR (125 MHz, DMSO–d6): 157.1(C−2), 133.71(C-3), 177.7 (C-4), 161.7 (C-5), 99.39 (C-6), 164.8 (C-7), 94.3 (C-8), 156.9 (C-9), 104.45 (C-10), 121.2 (C-1'), 131.32 (C-2' and 6'), 115.62 (C-3' and 5') and 160.4 (C-4'). The glucose carbons were at δc 101.9 (C-1››), 72.79 (C-2››), 76.6 (C-3››), 70 (C-4››), 74.6 (C-5››), 63.16 (C-6››). The galloyl carbons were at δc 166.1 (C-7), 119.79 (C-1), 109.01 (C-2 and 6), 146.02 (C-3 and 5) and 138.8 (C-4**).**

Rutin (16) **(**15 mg); yellow powder, R_f 0.37 (solvent system 7), gave dark purple fluorescent spot turned to yellow on PC with ammonia

vapor. UPLC-ESI-MS_2 in negative mode: m/z 609 [M-H]-, 463 [M-H-rhamnosyl]-, 301 [M-H-rutinosyl]-, (100 %), and 151 [aglycone peak of ring-B]. ^{1}H-NMR (400 MHz, DMSO–d6): δ_H 7.6 (IH, d, *J*=2.1Hz, H-2`), 7.55 (1H, dd, dd, *J* =8, 2 H-6`), 6.85 (1H, d, 8Hz, H-5`), 6.39 (1H, d, J=2 H-8), 6.20 (1H, d, J=2.1, H-6), 5.34 (1H, d, 7.8 Hz, H-1``), and 4.39 (1H,d, J= 2.0 Hz,H-1```), while the rest of protons in the sugar moieties resonated between 3.08 - 3.73 ppm and 1.1 (CH3-6''' of rhamnose , 3H, d, J = 6 Hz). ^{13}C-NMR (125 MHz, DMSO–d6) spectral data displayed 27 carbons, 15 carbons of aglycone and 12 carbon resonances of the two sugar moieties. The carbon signals appeared at δ 104.8 and 102.4 which assignable of the anomeric carbons of glucose and hamnose. Anther signals δC 177.9 (C-4), 164.76 (C-7), 161.66 (C-5), 156.74 (C-2), 156.71 (C-9), 149.1 (C-4›), 145.45 (C-3›), 134.19 (C-3), 122.53 (C-1›), 121.36 (C-6›), 116.22 (C-5›), 115.83 (C-2›), 104.34 (C-10), 102.4 (C-1››), 99.17 (C-6), 93.99 (C-8) and sugars moieties at 74.41 (C-5``),73.65 (C-3››), 71.38 (C-2›››), 60.75 (C-6``), 73.1 (C-2``), 71.63 (C-3```), 73.9 (C-4```), 68.38 (5```), and 17.73 (C-6```).

Quercetin 3-O-α-L-rhamnoside (17) (4 mg); yellow powder, R_f 0.84 (solvent system 8), gave dark purple fluorescent spot turned to yellow on PC with ammonia vapor. UPLC-ESI-MS_2 in negative mode: m/z 447 [M-H]-, 301 [M-H-rhamnosyl]-, (100 %), and 151 [aglycone peak of ring-B]. The ^{1}H-NMR (400 MHz, DMSO–d6): δ_H 7.76 (IH, br s, H-2`), 7.27 (1H, d, 8 Hz, H-6`), 6.88 (1H, d, 8.5 Hz, H-5`), 6.39 (1H, br s, H-8), 6.21 (1H, br s, H-6), 5.39 (1H, br s, H-1``), 1.03 (3H, d, J = 6 Hz, CH3-6''); while the rest of protons in the sugar moiety resonated between 3.15 - 3.98. ^{13}C-NMR (100 MHz, DMSO–d6): δ ppm 177.8 (C–4), 165.43 (C–7), 161.8 (C–5), 158.01 (C–2), 156.4 (C–9), 148.51 (C–4`), 145.56 (C–3`), 134.27 (C–3), 122.24 (C–6`), 121.40 (C–1`), 115.8 (C–5`), 115.62 (C–2`), 104.10 (C–10), 101.86 (C–1''), 98.98 (C–6), 94.45 (C–8), 71.46 (C–4''), 74.15 (C-4''), 70.55 (C- 2''), 70.62 (C-3''), 17.54 (C-6'')

Quercetin (18) (6 mg); yellow amorphous yellow powder, R_f value is 0.32 (solvent system 6). UPLC-ESI-MS_2 in negative mode shows molecular ion peaks at: 301 (M-H) and 151. The ^{1}H-NMR (400 MHz, CD_3OD) showed δ ppm 7.669 (*1H, br d, J* = 8.8 Hz, H-6′), 7.53 (1H, br s, H-2′), 6.92 (*J* = 9.2 Hz, H-5′), 6.43 (1H, d, J= 2 Hz, H-8), 6.22 (1H, d, J= 2 Hz, H-6), ^{13}C-NMR (100 MHz, CD_3OD) displayed 176.29 (C-4), 164.33 (C-7), 161.18 (C-5), 158.99 (C-9), 156.58 (C-2), 148.15 (C-4′),145.51(C-3′),136.49 (C-3),122.41, (C-1′), 120.49 (C-6′),116.18 (C-5′),115.44 (C-2′), 103.46 (C-10), 98.63 (C-6), 93.80 (C-8).

Evaluation of antimicrobial activity

The investigation was carried out in Regional Center for Mycology and Biotechnology, Al-Azhar University. The agar diffusion technique [18 & 19] was used for screening the antimicrobial activity of different plant fractions including dichloromethane and ethyl acetate fractions of the two plants. The zones of inhibition were measured (mm) and recorded in table 3.

Evaluation of the cytotoxic activity

Cytotoxicity evaluation was carried out using viability assay [20 & 21] for the ethyl acetate and dichloromethane fractions. The percentage of inhibition and IC_{50} values with their standard deviations of different fractions were reported in tables 4 and figures 3 and 4.

Evaluation of in Vitro Antiviral Activity

The study was carried out on the activities of total extract from *E. paralias* and *E. geniculata* against hepatitis A virus for the first time using CPE inhibition Assay [22]. This assay was selected to show specific inhibition of a biologic function, i.e., cytopathic effect (CPE) in susceptible mammalian cells [22 & 23]. Antiviral activity was determined by the inhibition of cytopathic effect compared to control, *i.e.*, the protection offered by the tested extracts to the cells was scored [23]. Ribavirin was used as a positive control under this assay system. Viral inhibition rate was calculated as follows: [(ODtv-ODcv)/(ODcd-ODcv)] × 100% Where ODtv, ODcv and ODcd indicates the absorbance of the tested extracts with virus infected cells, the absorbance of the virus control and the absorbance of the cell control, respectively.

Results and Discussion

Chemistry

Compounds 1- 5 and 10

Direct comparison their data (IR, MS, and co-TLC) with published data of authentic samples, they proved to be 1 (*β*-amyrin), 2 and 3 (mixture of *β*-sitosterol and Stigmasterol), 4 (cholesterol) [24], 5 (Campesterol), and 10 (*β*-sitosterol-*3-O-β*-D-*glucopyranoside*) [25-27].

Compounds 6-8 mixture

The physical and chemical data of mixture 6- 8 suggested a triterpenoidal skeleton [13-15]. The IR

spectrum revealed the presence of broad hydroxyl absorption peak at 3427 - 3250 cm^{-1}, peaks at 2930, 2868 (C-H stretching), peaks at 1686 cm^{-1} (C=C), 1462 (- CH_2 bending) suggesting an unsaturated steroid or triterpenoid alcohol. [24, 27, 29]. The EI-MS showed a molecular ion peak at *m/z* 456 [M]+ for $C_{31}H_{52}O_2$, in addition to 442 [M]+for ($C_{30}H_{50}O_2$), and 411 (base peak 100%), 423, 393, 302, 273, 248, 207, and 189 are characteristics for triterpenes of lupan nucleus [26]. Other peaks like 248, 203, 175, 133, 95 and 55 indicate the presence of oleanane skeleton triterpene and the peaks of 438, 300, 248, 235, 203, 147, 133, 119 and 105 are characteristics for ursane skeleton group of triterpene. Accordingly, these spectra revealed the presence a mixture of three types of triterpenoidal compounds with lupane, oleanane and ursane skeletons [26-28], and with direct comparison of (IR, MS, H^1-NMR. attached proton of ^{13}C-NMR, HSQC and HMQC) with published data of compounds of the mixture 6- 8 was proved to be mixture of Erythradiol, Ovaol and Betulinol [30 & 31]. According to the available literature, this is the first report about the isolation of this mixture from genus Euphobia. Only Ovaol was isolated from *E. paralias* grown in Egypt [8].

Compound 9

The physical and chemical data suggested a steroidal or triterpenoidal skeleton [13-15]. The IR spectrum revealed the presence of a broad hydroxyl absorption peak at 3400 - 3490 cm^{-1}, peaks at 2927, 2854 cm^{-1} (C-H stretching), peaks at 1743, and 1687 corresponding to two (C=O) groups, peaks at 1635 cm^{-1} (C=C), 1462 (- CH_2 bending) suggesting an unsaturated steroid or triterpenoid acid ester [24, 26-27]. The ^{1}H-NMR (400 MHz, CDCl3) spectrum of 9 (table 2) showed a triplet at δ 5.29 ascribable to CH=C proton of C12 (1H, t, J = 8), 3.9 (3H, S, CH3 of C-31), 3.24 (1H, dd, J = 12, 8 Hz, H-3). Anther protons appear as two signals at δ 4.31 and 4.17 (2H, dd, dd, J=12, 8 HZ, CH_2-23), 0.74 (1H, t, H-5), 2.1 (3H, S, methyl ester C-33), 1.61 and 2.02 (2H, dd, Ha, Hb of C-2), 1.56 and 1.29 (2H, m, Ha, Hb, C-6), 1.48 and 1.29 (2H, t, Ha, Hb, C-7), 1.54 (1H, m, C-9), 1.88 and 1.94 (2H, t, Ha, Hb, C-11), 1.3 and 1.26 (2H, t, Ha, Hb, C-15), 2.23 and 1.96 (2H, m, m, Ha, Hb, C-16), 2.84 (1H, H-18), 1.63 and 1.14 (2H, d, Ha, Hb, C-19), 1.23 and 1.32 (2H, t, Ha, Hb, H -21), 1.74 and 1.56 (2H, t, Ha, Hb, C-22), 0.84 (3H, s, C-24), 0.79 (3H, s, C-25), 0.79 (3H, s, C-26), 1.14 (3H, s, C-27), 0.9 (3H, s, C-29), 0.94 (3H, s, C-30) [32, 34 & 35]. Attached proton of ^{13}C- NMR (CDCl3, 125 MHz) (table 2) 183.89 (s, carbonyl group C-28), 176.6(S, carbonyl group C-32), 143.55 (C-13), 122.6 (C-12), 79.03 (C-3), 62.19 (S, C-23), 55.5 (C-31), 51.16 (C-4), 52.15 (C-5), 32.9 (C-29), 23.48 (C-30), 21(C-33) [34-35]. HSQC spectrum showed correlations between C-12 (122.6) and H-12 (5.29), C-23 (62.19) and 2H-23 (4.31& 4.17), C-3 (79.12) and H-3 (3.23), C-31 (55.2) and 3H-31 (3.9), C-33 (21) 3H-33, C-19 (45.85) and 2H-19 (1.14 Ha and 1.63 Hb), C-18 (41.53) / H-18 (2.84), C-5 and H-5, C-9 and H-9, C-16 H-16. Based on these spectral data, the compound was identified as a triterpenoid acid ester [34 & 35]. The HMBC showed correlation of H-19 and H-21 with C-29; H-25 and H-11 with C-9, H-9 with C-11. The position of the double bond at C-12, 13 was also evident from the HSQC correlation of the olefinic proton H-11 at 5.29 ppm with its own carbon at δc 122.6 (C-12). The connection of an acetyl group to OH-23 was documented from the long rang correlation of CH3-33 and 2H-23 with the carbonyl carbon C-32 [32]. According to the comparison of its properties and spectral data with the corresponding of structurally related compounds, it was identified as 23-acetyl-3β-O-methyloleanolic acid that is a new natural product [30-33].

Compounds 11 and 12

The physical and chemical properties of compound 11 and 12 suggested a phenolic acid like structures [17]. UPLC-MS_2 spectra in negative mode show molecular ion peaks at: 169 and 301 [M-H]- corresponding to the MFs of $C_7H_6O_5$, and $C_{14}H_6O_9$,, respectively. The UV spectral data of 1 and 2 exhibited an absorption band (λ_{max} 217, 272 and 253, 367) characteristic for gallic and ellagic acids [16-34]. Depending on the comparison with their previously published spectral 1H and 13C-NMR data and Co-chromatography with the authentic samples, 11 and 12 were identified as gallic and ellagic acid, respectively [35 & 36].

Compounds 13, 1 4 and 16-18

Based on their physical and chemical characters, compounds 13, 14, 16 and 17 were suggested to have a flavonol 3-O-glycoside structure [17]. Their spectral data i.e. HPLC-PDA-ESI-MS, 1H-and 13C-NMR spectral data were in accordance with the published data of quercetin 3-O-β-D-glucopyranoside, (Isoquercitrin, 13) [37-38], quercetin 3-O-β-D-arabinopyranoside (14), rutin (16) [38-40], quercetin 3-O-α-L-rhamnoside (17)

[41]. Moreover, 18 was identified as quercetin depending on its chromatographic behaviour and comparison of its spectral data with the that published before [42].

Compound 15

The physical and chemical data of compound 15 suggested a flavonol 3-glycoside like structure [17]. UPLC-ESI MS_2 spectrum negative mode shows molecular ion peaks at: 599 [M-H]- corresponding to a MF of C28H23O15, two characteristic fragment ions at 447 [M- H-152]- and 284 [M-H-152-162]-, (100%) corresponding to the loss of galloyl then galloylglucoside, respectively. The last fragment is confirmed to be oxidized kaempferol to indicate the nature of the aglycone [17]. Complete acid hydrolysis yielded kaempferol, gallic acid and glucose as sugar moiety using co-chromatogrphy with authentic samples. ^{1}H NMR spectrum showed two ortho doublets at δ 7.93 (*d, J* = 10.4 Hz, H2′, 6′) and 6.77 (*d, J* = 9.2 Hz, H3′, 5′) characteristic for ring B, also two signals appeared at δ 6.41 (*d, J* = 2 Hz, H8), and 6.2 (*d, J* = 2 Hz, H6) for the ring A, which were confirmative data for kaempferol 3-O-substituted aglycone. In the aliphatic region a doublet signal of the anomeric proton appeared at δ 5.45 (*d, J* = 7.5 Hz) for the sugar moiety whose βconfiguration. A singlet signal appeared at δ 6.93 was assignable to the two equivalent protons of the galloyl ester moiety (2''' and 6'''). The 6″ (CH2) position of the sugar moiety showing downfield shift for 2H-6″ at δ 4.27 and 4.17 indicating the position of attachment to gallic acid moiety [39]. The galloylation of OH-6" was evidenced from the downfield location of CH2-6" diastereomeric protons at 4.27 and 4.17 ppm [39]. The ^{13}C-NMR spectrum showed twenty four signals, six of them attributed to the sugar moiety, thirteen to the kaempferol aglycone and the remaining five signals for gallic acid. The downfield shift of of the C6″ to δc 63.16 (+2.5-3 ppm) was an evidence for the site of attachment of gallic acid moiety to C-6''. The other aromatic carbons were in accordance with the published data of *kaempferol 3- O (6″OgalloylβDglucopyranoside*), which were identical with those reported in literature [39].

Antimicrobial Activity

Drug-resistant pathogens resulted rottenly from prolonged use of broad-spectrum antibiotics in both agriculture and in medicine. The need for novel and efficacious antimicrobial agents has increased due to new threats such as biological warfare [43]. Generally, it is considered that compounds produced naturally, more than synthetically, will be biodegraded more easily and so will be more environmentally acceptable [18 & 19]. Both the dichloromethane and ethyl acetate fractions were effective against *Bacillus subtilis* and *Escherichia coli* bacteria with significant zone of inhibition, but lacking activity against *Candida albicans, Aspergillus flavus,* and *S. typhimurium* except the effect of dichloromethane fraction of *E. p*aralias on *C. albicans* (10 ± 0.5 mm) and ethyl acetate fraction of *E. P*aralias on *S. aureus* (8 ± 0.43 mm). The best antibacterial effect was obtained on *E. coli* (15 ± 0.34 mm) which is nearly equal to that of the standard antibiotic Gentamicin (GM) 15 ± 0.1 mm and was exerted by the ethyl acetate of *E. g*eniculata and also on *B. subtilis* (12 ± 0.35 mm) which is equal to that of the standard antibiotic Gentamicin (12 ± 0.31 mm) and was exerted by the ethyl acetate of *E. paralias*. Zones of inhibition were measured (mm) and recorded in table (3).

In Vitro Cytotoxic Activity

***Fractions of E. paralias* L.:** Evaluation the effect of DCM fraction of *E. paralias* on HEP-G2 (hepatic carcinoma cell line) revealed by weak inhibition in the cell proliferation and high IC_{50} values 244 ± 8.6 µg / well, While ethyl acetate fraction has a high anti-tumor activity represented by high inhibition in the cell proliferation as concluded by the low IC_{50} values 26.4 ± 1.2 µg /well (tab. 4). *Fractions of E. geniculate:* Evaluation of dichloromethane fraction of *E. geniculata* on $HEPG_2$ revealed weak anti-tumor activity with IC_{50} values > 500 µg /well. While EA fraction revealed a moderate anti-tumor activity as concluded by the moderate IC_{50} values 48.8 ± 1.4 µg / well.

In Vitro Antiviral Activity

Recent antiviral screenings have demonstrated that some *Euphorbiaceae* species, *e.g. E. pekinensis, E. peplus, Phyllanthus nanus* and *P. amarus*, are effective against virus infections [44]. With the aim of finding plants containing promising antiretroviral compounds, we have studied the activities of total extract from *E. paralias and E. geniculat*a against hepatitis A virus for the first time using CPE inhibition Assay. The antiviral study on the total extracts of the two plants revealed significant antiviral activity of *E. paralias* and no activity of *E. geniculata* against hepatitis-A virus (HAV-10). The results were summarized in the following table.

13, R = glucose
14, R = arabinase
16, R = rutinosde
17, R = rhamnoside
18, R = H

Fig. 1. Chemical structures of the isolated compounds .

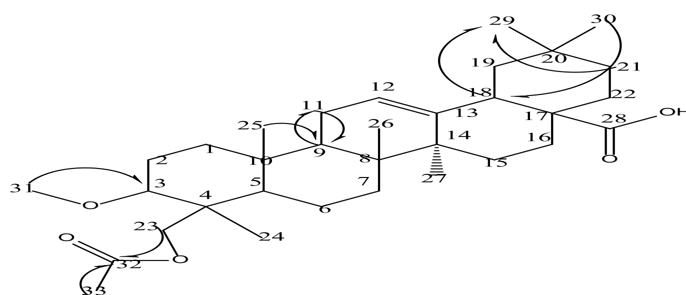

Fig. 2. Important HMBC correlations of compound 9.

TABLE 3. Antimicrobial activity of E. geniculata and E. paralias fractional extracts in terms of inhibition zones (mm).

Tested microorganisms	*E. geniculata*		*E. paralias*		Control
	DCM	EA	DCM	EA	
Gram Positive Bacteria:					*GM*
Staphylococcus aureus (RCMB010010)	NA	10 ± 0.36	NA	8 ± 0.43	16 ± 0.21
Bacillus subtilis (RCMB 015 (1) NRRL B-543	8 ± 0.53	9± 0.45	11 ± 0.4	12 ± 0.35	12 ± 0.31
Gram Negatvie Bacteria:					GM
Salmonella typhimurium RCMB 006 (1) ATCC 14028	NA	NA	NA	NA	17±0.25
Escherichia coli (RCMB 010052) ATCC 25955	10 ± 0.54	15 ± 0.34	11 ± 0.56	14 ± 0.52	15 ± 0.1
Fungs					KC
Aspergillus flavus (RCMB 002002)	NA	NA	NA	NA	16 ± 0.2
Candida albicans (RCMB 005003 (1) ATCC 10231)	NA	NA	10 ± 0.5	NA	11± 0.12

NA = no activity, GM = Gentamycin and KC = Kitoconazol.

TABLE 4. Cytotoxic activity of E. geniculate and E. paralias fractional extracts.

conc. (µg/ml)	Fractions of E. paralias						Fractions of E. geniculata					
	DCM			EA			DCM			EA		
	Inh %	SD (±)	IC_{50}	Inh%	SD (±)	IC_{50}	Inh %	S.D (±)	IC_{50}	Inh %	S.D (±)	IC_{50}
500	68.4	0.92	244 ± 8.6 µg / well	94	0.32	26.4± 1.2 µg / well	17.1	0.75	>500 µg / well	90.1	0.19	48.8± 1.4 µg / well
250	51.1	1.75		88	0.14		3.57	0.09		80.5	0.75	
125	29.6	2.37		79	0.32		0.24	0.12		68.3	0.96	
62.5	12.7	0.84		71	0.59		0	-		57.1	1.37	
31.25	4.5	0.18		58	3.46		0	-		40.8	1.52	
15.6	0.2	0.06		31	2.35		0	-		23.8	0.91	
7.8	0	-		11	0.82		0	-		7.42	0.22	
3.9	0	-		2.9	0.21		0	-		0.53	0.15	

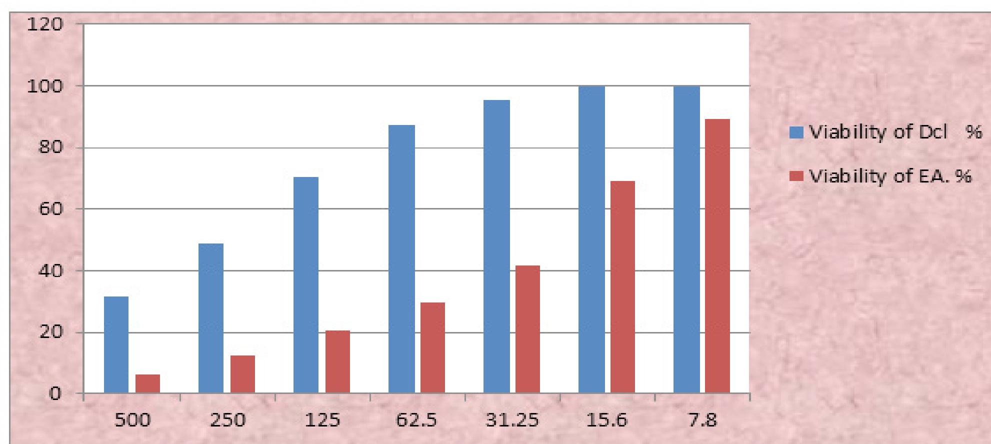

Fig. 3. Cytotoxic activity of E. paralias fractions.

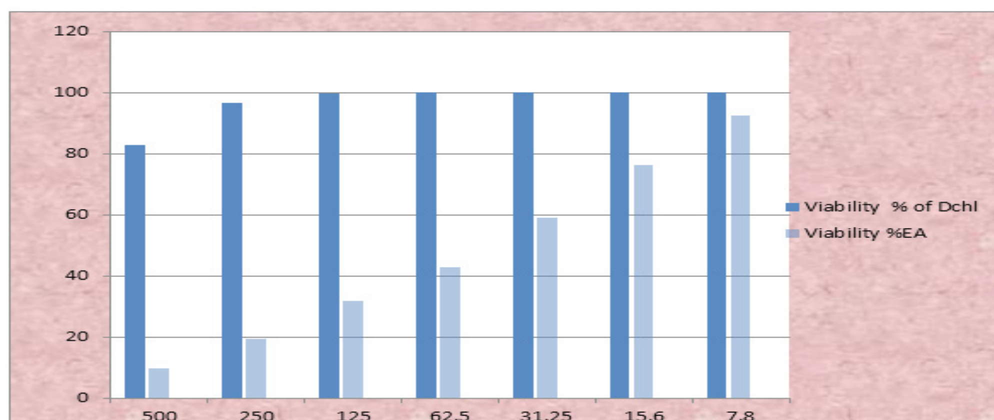

Fig.4. Cytotoxic activity of E. geniculata fractions.

TABLE 5. Antiviral activity of E. geniculata and paralias .

Total extract	HAV-10	
E. paralias	++	Tested at maximum non-cytotoxic conc. (200 µg/ml)
*E. geniculat*a	-	Tested up to 2000 µg/ml

++ Significant activity, - no activity.

Conclusion

The study of chemical compositions of dichloromethane fractions of the aerial parts of *E. paralias* L. and *E. geniculata* Ortiga revealed the presence of several phytosterols, triterpenes alcohols and a new acetyl-methoxyoleanolic acid ester. As known plant sterols have very important role in human health as Cholesterol-lowering effects, Immunomodulatory effects, Anticancer and Prostatic hyperplasia. Phytochemical study on the ethyl acetate fractions of the two plants revealed the isolation of several compounds phenolic acids and flavonoids. Flavonoids also play very important role in human health because of their high pharmacological activities as antioxidant activity, in coronary heart disease prevention, anticancer activity and some flavonoids exhibit an effect as potential for anti-immunodeficiency virus in human. The antimicrobial study revealed strong antibacterial effect was exerted by the ethyl acetate and dichloromethane of *E. paralias* and *E.* geniculata in comparison with standard antibiotic Gentamicin and Kitoconazol (standard antifungal). The significant anti-tumor activity of ethyl acetate fractions which may be due to the presence phenolic and flavonoidal compounds [20]. The significant antiviral activity of *E. paralias* L. on HAV-10 virus may be due to the presence of bioactive compounds such as di, triterpene, phenolic and flavonoidal compounds. So it is recommended to more flow up in vivo studies on biological activities of *E. paralias* and *E. geniculata* extract.

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