Phytochemical and Cytotoxic Studies on *Calendula Officinalis*

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Abstract: From the leaves of C*alendula officinalis* (Asteraceae) two new triterpene saponins, named sophradiol 3-*O*- α -L-$^{1}C_{4}$-rhamnopyranosyl-(1'''→4'')- *O*-β -D-$^{4}C_{1}$-galactopyranosyl (1''→6')- *O*-β-D $^{4}C_{1}$- galactopyranoside (**10**) and 23-hydroxy-3α-[(*O*-α-L-$^{1}C_{4}$-rhamnopyranosyl-(1''→4')-*O*-α-L$^{4}C_{1}$-arabinopyranosyl)-oxy]olean12-en-28-oic acid *O*-α-L-$^{1}C_{4}$-rhamnopyranosyl-(1''''→4'''')-*O*-β-D-$^{4}C_{1}$-galactopyranosyl-(1''''→6''')-*O*-β-D-$^{4}C_{1}$.galactopyranosyl ester (**11**) were isolated. In addition, nine known compounds were isolated, namely *E*- caffeic acid (**1**), quercetin (**2**), 4',5-dimethoxy quercetin (**3**), 8- sulfated apigenin (**4**) rutin (**5**), quercetin 3-*O*-β-D-$^{4}C_{1}$.neohesperidoside (**6**), 3,3',4'-trimethoxy quercetin (**7**), oleanolic acid, (**8**), oleanolic acid 3-*O*- β-D-$^{4}C_{1}$-glucopyranosyl(1''→3')- *O*-β-D-$^{4}C_{1}$-glucopyranside (**9**), All metabolites were isolated for the first time from this plant. The structures were determined mainly by spectroscopic methods (UV, ESI-MS, ^{1}H-, ^{13}C-NMR,^{1}H-^{1}H COSY, HSQC and HMBC). Cytotoxic screening of the butanol, ethyl acetate and chloroform extracts was carried out on brine shrimps. In addition the investigated butanol extract and major isolates (**10** and **11)** were also tested against the HepG2 tumor cell line
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1-Introduction

Calendula officinalis L. is a herbaceous plant belonging to the family Asteraceae which is commonly called "Marigold", *Calendula* cultivated by the Egyptians, Greeks, Hindus and Arabs, and has been used medicinally since the 12th century. It is of Mediterranean origin, although the flowers are widely appreciated for their therapeutic properties throughout the world (Chengqi, 2007). Popular knowledge and clinical studies describe numerous biological activities for this plant including antispasmodic, antiinflammatory, antibacterial, antiviral, anti-septic, wound healing, emollient, sudoriferous, dysmenorrhoea and duodenal ulcers (Yoshikawa *et al*., 2001; Lorenzi and Matos, 2002; Khalid *et al*., 2010). The triterpenoids isolated from *Calendula* species have been found to have some pharmacological activities such as hypoglycemic, gastroprotective, antiviral, antimutagenic and antiinflammatory (Elias *et al*., 1990; De Tommasi *et al*., 1991; Dellaloggia *et al*., 1994; Yoshikawa *et a*l., 2001). The main phytochemical constituents described in the flowers of this bioactive plant are essential oils (0.1-0.4%)., iononem, carotenoid pigments (Kishimoto *et al*., 2005) and sesquiterpene glycosides have been isolated from the genus *Calendula* as principle secondary metabolites (Pizza *et al*., 1987; Masterova *et al*., 1991; Ahmed *et al*., 1993). The plant has been reported to contain mainly polyphenol such *p*-hydroxybenzoic, salicylic, vanillic, caffeic, gallic acids (Gora *et al*., 1979; Gong *et al*., 2012), acylated flavonoid-*O*-glycosides and methoxylated flavonoids, amino acids (Abasova *et al*., 1995), alkaloids, carotenoids, saponins, tannins (Duke, 1992; Rodrigues *et al*., 2004), high molecular weight polysaccharides (Wagner *et al.*, 1984) and triterpenoid monoesters (Neukirch *et al.*, 2004). Alpha-cardinol (Chalchat *et al*., 1991), deltacadinol, delta-cadinine and gamma murolene (Marczal *et al*., 1987) have been identified in the essential oil. As a part of our studies on the Egyptian medicinal plants, we report herein the isolation and structure elucidation of two new triterpenoidal saponins on the basis of spectroscopic analysis including various two-dimensional (2D) NMR spectroscopic data. Also, cytotoxicity towards brine shrimps was determined for the butanol ethyl acetate and chloroform extracts of the leaves. The investigated butanol extract and major isolates (**10** and **11**) were also tested against the HepG2 tumor cell line

2. Experimental

2.1- Equipment

The NMR spectra were recorded at 300, 500 (^{1}H) and 75, 125 (^{13}C) MHz, respectively, on a Varian Mercury 300, and JEOL GX- NMR spectrometer, and δ values are reported in ppm relative to TMS in the convenient solvent. ESI-MS analyses were measured on a Finnigan LCQ deca LC/ MS and double focusing sector field MAT 90 MS spectrometer (Finnigan, Bremen, Germany). UV spectra of pure samples were recorded, separately, in MeOH using different diagnostic UV shift reagents using a Shimadzu UV 240 spectrophotometer. For column chromatography (CC), Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and microcrystalline cellulose (Merck). For paper chromatography Whatman No. 1 sheets (England)

were used, while silica gel (Sigma, 28-200 mesh) was used for saponin CC, and F_{254} for TLC (Merck, Germany).

2.2- Plant material: *Calendula officinalis* leaves collected from plants growing in El-Orman Botanical Garden, Giza, Egypt. Plant Leaves were authenticated by Professor Dr. Wafaa M. Amer, Department of Botany, Faculty of Science, Cairo University, Giza, Egypt. Voucher specimens (Reg. No.: C–1) are kept in the herbarium, Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt.

2.3- Brine shrimp lethality bioassay

Eggs of *Artemia salina* were allowed to hatch into their larvae (Fatope *et al*., 1993). The dried chloroform, ethyl acetate and *n*- butanol of *C. officinalis were* separately dissolved in distilled water to give four assay concentrations (1000, 500, 100 and 10 mg mL^{-1}). Solubility was aided by Tween 80 and each dose was examined in triplicate. Potassium dichromate was used as a reference drug and dissolved in seawater, to obtain concentrations of 1000, 100 and 10 µg ml^{-1}. Assays were performed in test tubes with ten larvae each and the final volumes were adjusted to 5 mL sea salt soln. immediately after adding the shrimps. After 24 h, the number of surviving shrimps at each dose was recorded. The LC_{50} values were calculated by the use of the Instate computer program.

2.4- Measurement of potential cytotoxicity by SRB assay

Potential cytotoxicity of the *n*-butanol extract of *C. 0ffecinalis* leaves and the isolated compounds (**10)** and (**11)** were tested at the National Cancer Institute, Egypt using the method of (Skehan *et al*., 1990). Cells were plated in a 96-well plate (104 cells/well) for 24 h before treatment to allow the attachment of cells to the wall of the plate. Different concentrations of the fractions under investigation (0, 1, 2.5, 5 and 10 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose and they were incubated for 48 h at 37 ºC in 5% CO_2. After 48 h cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer and the color intensity was measured in an ELISA reader. The survival curve of the tumor cell line was plotted for each butanol extract and compounds (**10**) and (**11**).

2.5 –Extraction and isolation

The air-dried powdered leaves of *C. officinalis* (300 g) were extracted under reflux with hot 70% MeOH (3 × 4L). After evaporation of the solvent, the obtained dry residue was defatted with $CHCl_3$ under reflux (3 × 1 L). The resulting residue (60 g) was suspended in water (500 ml) and extracted with ethyl acetate (3 × 500 ml), followed by *n*-butanol (3 × 300 ml). The *n*-butanol extract concentrated to dryness by removing the solvent in a rotary evaporator and was washed with 100 ml distilled water (X 3). 2D-PC analysis proved that the chloroform extract is free from polyphenols. Flavonoids and phenolic acid were found in the ethyl acetate fraction, whereas saponins were concentrated in the *n*-butanol extract. The ethyl acetate extract (15 g) was fractionated on a silica gel column (Ø 3.0 × 150 cm). Elution was started with chloroform, followed by ethyl acetate whose polarity was gradually increased by methanol portions. Five collective fractions were obtained (A–E). Fraction A (3.80 g) was found to be an oily, dark brown material of no phenolic character. Compound **1** (15 mg) was obtained by repeated chromatographic fractionation of fraction B (2 g) on Sephadex LH-20 using 70% EtOH as a mobile phase. Two major dark purple spots were detected in fraction C (2.90 g). Further purification of this fraction by repeated column chromatography om Sephadex LH-20 with EtOH afforded pure samples of **2** (25 mg) and **3** (20 mg). Fraction D (2 g) was rechromatographed on a Sephadex LH-20 column using 95% EtOH as an eluent to afford pure samples of compounds **4** (18 mg) and **5 (**30 mg). Column chromatography of fraction E (2.50 g) on cellulose eluted with 70 % EtOH gave subfraction (I), eluted with 60% EtOH and (II), eluted with 80% EtOH. Further purification of subfraction (I) on Sephadex with MeOH as an eluent gave a pure sample of compound **6** (20 mg), while purification of subfraction (II) gave **7** (22 mg). All separation processes were followed up by comparative PC using Whatman No. 1 paper with *n*-BuOH/HOAc/H_2O (4:1:5, upper layer) (S1) and 15% aqueous HOAc (S2) as solvent systems and specific spray reagents (*e.g.* Naturstoff, $FeCl_3$, and NH_3). The *n*-butanol extract gave negative reaction with $FeCl_3$ but pink color with sulphuric acid spray reagent on TLC when heated at 120ºC for three min. *n*-butanol extract (35 g) was subjected to column chromatography over silica gel column (Ø 5.0 x 125 cm,) using a gradient of $CHCl_3$-MeOH (9:1, 8:2, 7:3, 1:1, 3:7) and 0:1, each 75 ml) to give four main fractions (F-I) according to the differences in composition indicated by TLC analyses. Fr. F (7g) eluted with $CHCl_3$–MeOH (8:2), was purified by silica gel column, eluted with $CHCl_3$-EtOAc (8:2) to give pure **8** (18 mg). Fr. G (6.5g) eluted with $CHCl_3$–MeOH (3:7) and purified by silica gel column, eluted with $CHCl_3$-EtOH (4:7) to give pure **9** (15 mg). Crud 10 was crystallized from fr. H (9g) $CHCl_3$–MeOH (1:1) and purified by repeated crystallization from MeOH to yield pure **10** (40 mg). Fr. I (7g) (MeOH), was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH-H_2O (3:7: 0.1) to give pure **11** (55 mg). All separation processes were followed up by Co-TLC using solvent systems: $CHCl_3$- EtOAc (8:2) MeOH-$CHCl_3$ (3:7), MeOH-

EtOAc-$CHCl_3$-H_2O (35:32:28:2), ($CHCl_3$-MeOH-H_2O, 65:35:3) and *n*-BuOH-EtOAc- H_2O (4:1:1).

2.6- sophradiol 3-*O*- α -L-$^1C_4$-rhamnopyranosyl-(1'"→4")- *O*-β -D-$^4C_1$-galactopyranosyl (1"→6')- *O*-β-D $^4C_1$- galactopyranoside (**10**).

Creamy amorphous powder. Rf = 0.6 with solvent system $CHCl_3$-MeOH-H_2O (65:35:3) and 0.49 with solvent system, *n*-BuOH-EtOAc- H_2O (4:1:1).

Negative ESI-MS: m/z = 911.50 $[M-2H]^-$,^{1}H and ^{13}CNMR : see Table 1 and 2.

2.7- 23-hydroxy-3α-[(*O*-α-L-$^1C_4$-rhamnopyranosyl-(1"→4')-*O*-α-L$^4C_1$—arabinopyranosyl)-oxy]olean12-en-28-oic acid *O*-α-L-$^1C_4$-rhamnopyranosyl-(1""→4"")-*O*-β-D-$^4C_1$-galactopyranosyl-(1'"→6"')-*O*-β-D--$^4C_1$-galactopyranosyl ester (**11**).

Creamy amorphous powder. Rf = 0.56 with solvent system $CHCl_3$-MeOH-H_2O, (65:35:3) and 0.43 with solvent system, *n*-BuOH-EtOAc- H_2O (4:1:1). MS: *m/z* = 1219.60 $[M–H]^-$, 749.31 [M–H–deoxyrhamnosyl-2hexosyl]$^-$, 603.20 [M– H–deoxyrhamnosyldihexosyl-deoxyrhamnosyl]$^-$, 585.49 [M–H–deoxyrhamnosyldihexosyl-deoxyrhamnosyl–H_2O]$^-$, 471.40 [M–H–deoxyrhamnosyl- dihexosyl-deoxyrhamnosylpentoside]$^-$ = [aglycone– H]$^-$. ^{1}H- and ^{13}C NMR: see Table I and 2.

2.8- Alkaline hydrolysis of compounds (**11**): About 10 mg was refluxed with 10 ml 1 M NaOH for 3 hrs. The hydrolysate mixture was neutralized and the prosapogenin was extracted with *n*-butanol. The aqueous phase was then concentrated and subjected to Co-PC (solvent system: EtOAc-C_5H_5N-H_2O, 12:5:4) against authentic sugar samples, whereby D-galactose and L-rhamnose were identified.

2.9- Acid hydrolysis of compounds (**10**) and (**11**): (20 mg) were hydrolyzed with 2N HCl in EtOH on a heated water-bath for 4 hrs at 90˚C. The solvent then evaporated until most of HCl eliminated. The residue diluted with 15 mL H_2O and neutralized with $NaHCO_3$. The suspension filtered and extracted with $CHCl_3$. The sapogenin identified in $CHCl_3$ by Co-TLC MeOH-$CHCl_3$ (1.5: 9.5). The H_2O-phase was then concentrated and subjected to Co-PC (solvent system: EtOAc-C_5H_5N-H_2O, 12:5:4) against authentic sugar samples.

3 - Results and discussion

Repeated column chromatography of the ethyl acetate extract resulted in the isolation of six flavonoids, (**2** – **7**) and one cinnamic acid derivatives, (**1**)**.** On the basis of their chromatographic properties, acid hydrolysis products, and spectroscopic analyses (UV, ESI, 1D and 2D NMR), the structures of the isolated compounds were identified as *E*- caffeic acid (**1**), (Lu and Ly, 2002), quercetin (**2**), 4',5-dimethoxy quercetin (**3**), apigenin 8-ssulfate (**4**), rutin (**5**), quercetin 3-*O*-neohespridoside (**6**), 3, 3',4', -trimethoxy quercetin (**7**) (Harborne, 1982; Agrawal, 1989), while chromatographic separation of the *n*-butanol extract resulted in the isolation of four saponin (**8-11**) the structures of the isolated compounds were identified as oleanolic acid (**8**), oleanolic acid 3-*O*- β-D $^4C_1$-glucopyranosyl(1"→3')- *O*-β-D -$^4C_1$-glucopyranside (**9**) (Nie et al., 1984), sophradiol 3-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1'"→4")-*O*-β-D-$^4C_1$-galactopyranosyl (1"→6')- *O*-β-D $^4C_1$-galactopyranoside (**10**) and 23-hydroxy-3α-[(*O*-α-L-$^1C_4$-rhamnopyranosyl-(1"→4')-*O*-α-L$^4C_1$—arabinopyranosyl)-oxy]olean12-en-28-oic acid *O*-α-L-$^1C_4$-rhamnopyranosyl-(1""→4"")-*O*-β-D-$^4C_1$-galactopyranosyl-(1'"→6"')-*O*-β-D--$^4C_1$-galactopyranosyl ester (**11**)

Compound (**10**) gave positive Salkoweski and Molisch's reactions indicating its triterpenoid and glycosidic nature. The sugar units were assigned to be D-galactose and L-rhamnose after acid hydrolysis of (**10**) with 2 N HCl. Its negative ESI–MS showed a molecular ion peak at m/z 911.5 $[M-2H]^-$. The resonances due to eight sp^3 methyl carbons at δ C 27.7, 15.3, 16.2, 16.8, 25.1, 28.7, 32.5 and 20.5 (Table 1) and two sp^2 carbons at δ C 121.6 and 144.1 in the ^{13}C NMR spectrum of (**10**) coupled with the corresponding information from the ^{1}H NMR i.e. 8 tertiary methyl protons, hydroxymethine proton at δH 4.36 for H-3, a broad singlet vinyl proton at δ H 5.5 for H-12 and methane proton linked to oxygen bearing carbon at δH 5.21 for H-22 confirmed the aglycone moiety as sophradiol (Ndom *et al*. 2001). All assigned ^{1}H and ^{13}C-resonances of the aglycone moiety were confirmed by HSQC and HMBC correlation spectroscopy. Three anomeric proton signals were assigned at 4.60 (brd, β-galactosyl), 4.79 (d, *J* = 7.5, β-galactosyl) and 5.02 (brs, α-rhamnosyl) in the ^{1}H NMR spectrum through their direct one bond coupling in HMQC with their own anomeric carbon signals at δC 104.0, 100.0 and 99.8, respectively. The sugar moieties were deduced to adopt α -$^1C_4$-pyranose stereo-structure in case of rhamnosyl moiety and β -$^4C_1$ in case of both galactosyl moieties on the basis of *J*-values of the anomeric protons. The interglycosidic and sugar aglycone linkages were deduced from the long range three bond HMBC correlations. The HMBC exhibited correlations between H-1' (4.61) (galactosyl) and C-3 (89.2) aglycone. Similarly, correlations between H-1" (4.79) galactosyl and C-6' galactosyl (68.00), H-1'" (5.02) rhamnosyl and C-4" (77. 7) of the galactosyl were detected to establish a triglycosyl moiety at C-3 as 3-*O*- α -L-$^1C_4$-rhamnopyranosyl-(1'"→4")-*O*- β-D-$^4C_1$-galactopyranosyl (1"→6')-O- β - D-$^4C_1$-galactopyranoside. All ^{1}H and ^{13}C-resonances were assigned by the aid of HSQC and HMBC-correlation peaks and by comparison with the corresponding

published data of structural related compounds (Mimaki *et al*., 2003), and interpreted as Sophradiol 3-*O*-α-L-$^{1}C_{4}$- rhamnopyranosyl-(1'''→4'')-*O*-β-D-$^{4}C_{1}$-galactopyranosyl (1''→6')- *O*-β-D-$^{4}C_{1}$-galactopyranosy.

Table 1.^{1}H and.^{13}CNMR of aglycone moieties in 10 and 11 (500 /125) DMSO d_6 for (10) and pyriden d_5 for (11)

	10		**11**	
No	C	H	C	H
1	39.8		38.9	
2	25.6		25.9	
3	89.2	4.36 brs	80.7	4.19 *t*-like
4	39.1		43.2	
5	55.0		46.7	
6	18.1		17.2	
7	32.5		32.9	
8	40.1		39.7	
9	47.1		47.4	
10	36.2		36.5	
11	23.1		23.5	
12	121.6	5.50 brs	122.9	5.37 brs
13	144.1		143.7	
14	41.2		41.3	
15	25.1		27.9	
16	28.7		33.5	
17	37.0		47.4	
18	44.6		41.3	3.17dd (14, 4)
19	46.1		46.7	
20	30.3		29.2	
21	41.5		34.2	
22	75.2	5.21 brs	32.9	
23a	27.7	0.94 s	63.4	3.75 d (10.5)
23b	--------	----------	--------	4.13
24	15.3	0.75 s	13.7	1.04 s
25	16.2	0.91 s	15.8	0.97 s
26	16.8	1.00 s	17.2	1.20 s
27	25.1	1.06 s	25.9	1.04s
28	28.7	0.76 s	176.1	------
29	32.5	0.97 s	32.9	0.86s
30	20.5	0.85 s	23.5	0.87s

δ in ppm and *J* values (Hz), were given in parentheses; All carbon and proton resonances were assigned on the basis of 2D (^{1}H-^{1}H COSY, HSQC and HMBC).

Compound (**11**) gave positive Salkoweski and Molisch's reactions indicating its triterpenoid glycosidic nature. Its negative ESI-MS showed a molecular ion peak at *m/z* 1219.60 $[M-H]^{-}$, corresponding to $C_{59}H_{96}O_{26}$ (MF). Fragmentation of $[M-H]^{-}$ ion gave a negative fragment at 749.32 [M-H-146-2X162]$^{-}$ (loss of a rhamnosyl and two hexosyl). The fragment ion peak at 749.32 lead to ion at 603.20 attributed to (loss of extra 146 of a second rhamnosyl), followed by 585.49 (loss of H_2O). The fragment ion peak at 603.20 gave rise to a product ion at 471.40 [genin -H]$^{-}$, corresponding to the loss of pentoside from the last fragment. These data together with the alkaline hydrolysis that afforded galactose and rhamnose and acid hydrolysis that afforded arabinose, rhamnose and galactose in the aqueous phase was compatible with a structure of hydroxyolean 28-rhamnosyl-galactosyl-galactosyl ester with an *O*-rhamnosyl-pentoside, most probably at C-3 (Mshvildadze *et al*., 2004) (Co-TLC and PC with the authentic samples). The resonances due to six sp^3 methyl carbons at δ 13.7, 15.8, 17.2, 23.5 25.9, 32.9, a primary carbinol at 63.49 and two sp^2 carbons at 122.9 and 143.7 in the ^{13}C NMR spectrum of (**11)** coupled with the corresponding information from the ^{1}H NMR [6 tertirary methyl proton singlets,, methylene protons linked to oxygen bearing carbon which constituted an AX spin system at 4.13 and 3.75 (d, *J*= 10.5Hz, H-23), hydroxylmethine proton at δ 4.19 for H-3, a proton attributed to H-18 at 3.17 (dd, *J*=14,4) and a broad singlet vinyl proton at 5.37 of H-12] confirmed the aglycone moiety as 3,23-dihydroxy-olean-12-en skeleton. The relative upfield location of C-5 at 46.7 (Δ ~ + Δ 5 ppm) was also an evidence for the γ-effect of the carbinol-OH_{23}. The resonances of C-3 at 80.7, C-28 at 176.1 together with ^{1}HNMR signal at 6.24 were characteristic of a bisdesmosidc aglycone with 3α-hydroxyl. All assigned ^{1}H and ^{13}C-resonances of the aglycone were confirmed by HSQC and HMBC correlation spectroscopy (Table1). Five anomeric proton signals were assigned at 5.08 (d, *J*= 6.5 α-arabinosyl), 5.87 (brs, α-rhamnosyl), 6.24 (d, *J*= 8.0 β-gulcosyl ester), 4.60 (d, *J*= 7.8, β-glucosyl), 6.26 (brs, α-rhamnosyl) in the ^{1}H NMR spectrum through their direct one bond coupling in HSQC with their own anomeric carbon signals at 103.90, 102.4, 95.5, 104.6 and 101.3, respectively, (Table 2). The sugar moieties were deduced to be adopt α-$^{1}C_{4}$-, α-$^{4}C_{1}$- or β-$^{4}C_{1}$-pyranose stereostructure in case of rhamnosyl, arabinosyl or galactosyl moieties, respectively on the basis of *J*-values of the anomeric protons and δ-values of their ^{13}C-resonances (Table 2). The interglycosidic and sugar-aglycone linkages were deduced from the long range three bond HMBC correlations). The HMBC exhibited correlations between H-1'(5.08) (arabinosyl) and C-3 (80.7) aglycone, H-1'' (5.87) rhamnosyl and C-4' (75.4) arabinosyl to establish a diglycoside moiety at C-3 of the aglycone as 3-*O*-α-L-$^{1}C_{4}$-rhamnopyranosyl-(1''→4')-*O*-α-L-$^{4}C_{1}$-arabinopyranoside. Similarly, correlations between H-1''' (6.24) galactosyl ester and C-28 (176.08) aglycone, H-1'''' (4.60) galactoside and C-6''' (69.4) galactosyl ester, H-1''''' (6.26) rhamnosyl and C-4'''' (78.30) of the second galactosyl were detected to establish a triglycosyl ester moiety at C-28 as 28-*O*-α-L-$^{1}C_{4}$-rhamnopyranosyl-(1''''→4'''')-*O*-β-D-$^{4}C_{1}$-galactopyranosyl-(1''''→6''')-*O*-β-D-$^{4}C_{1}$-galactopyranosyl. All ^{1}H and ^{13}C-resonances assigned by the aid of HSQC and HMBC-correlation peaks and comparison with the corresponding data of structural

related compounds (Shao *et al*., 1989; Mshvildadze *et al*., 2004). Therefore, **(11)** was finally identified as 23-hydroxy-3α-[(*O*-α-**L**-$^{1}C_{4}$-rhamnopyranosyl-(1"→4')-*O*-α-**L**-$^{4}C_{1}$-arabino-pyranosyl)-oxy]olean-12-en-28-oic acid *O*-α-**L**-$^{1}C_{4}$-rhamnopyrano-syl-(1""→4"")-*O*-β-D-$^{4}C_{1}$-galactopyranosyl-(1""→6"')-*O*-β-D-$^{4}C_{1}$-galactopyranosyl ester.

In the brine shrimp lethality bioassay, the chloroform, ethyl acetate and butanol extracts were tested. The LC_{50} = 500, 60.1 and 50.2 mg/l, respectively (Figure 2). According to the standards of them National Cancer Institute (NCI), $ED_{50} \leq 20$ µg/ml for impure compounds are considered to be active (Cordell *et al.*, 1993), so we assumed the median lethal concentration (LC_{50}) as 200 ppm. According with this value, only the ethyl acetate and butanol extracts are toxic on *A. salina*. In the course of our studies, the brine shrimp lethality assay actually has proven to be a convenient system for monitoring biological activities of several *C. officinalis* extracts. Out of the several extracts screened for toxicity against the brine shrimp, the butanol and ethyl acetate extracts showed LC_{50} values less than 100 mg/l. These interesting results lend the authors for further supporting detailed phytochemical and biological studies.

Table2 ^{1}H and.^{13}CNMR of sugars moieties in 10 and 11 (500 /125) DMSO d_6 for (10) and pyridine d5 for (11)

		10			**11**	
No	C	H	HMBC	C	H	HMBC
1’	99.8	4.60 brd	C-3’, 3	103.9	5.08 d (6.5)	C-3,3’
2’	72.0	3.92 m	C-4’	69.4	4.73 dd (9, 6.5)	C-4’
3’	74.0	3.14 t-like (9.5)	C-1’, 5’	74.5	4.21 dd (8.5, 3.5)	C-1’, 5’
4’	70.6	4.29*	C-2’	75.4	4.58 m	C-2’,1’’
5’a	75.2	4.21m	C3’’	65.5	3.68 *	C-3’
5’ b	--------	----------	----------		4.17 brd	C-3’
6’ a	68.0	4.97*,	C-4’, 1’’	--------	----------	---------
6’b		3.92 dd (12.1,3)		----------	----------	--------
1’’	100.0	4.79 d (7.5)	C-3’’, 6’	102.4	5.87 brs	C-4’, 3’’
2’’	71.2	4.21 m	C-4’’	72.0	4.68*	C-4’’
3’’	75.2	3.57 t-like (9.5)	C-1’’, 5’’	72.2	4.55*	C-1’’, 5’’
4’’	77.7	4.6 brd	C1’’’, 2’’,6’’	73.5	4.30 t-like (10.5)	C-2’’. 6’’
5’’	76.2	4.42 m	C-3’’	69.4	4.33 m	C-3’’
6’’a	60.3	3.97 m	C-4’’	18.9	1.64 d(6.5)	C-4’’
6’’b		4.27m		----------	----------	C-4’’
1’’’	104.0	5.02	C-4’’, 3’’’	95.5	6.24d (8)	C-28,3’’’
2’’’	72.1	4.61*	C-4’’’, 6’’’	74.5	4.73*	C-4’’’
3’’’	72.1	4.63*	C-5’’’, 1’’’	76.2	4.42 t- like (9.5)	C-1’’’, 5’’’
4’’’	72.3	4.3m	C-2’’’, 6’’’	70.5	4.27 *	C-2’’’, 6’’’
5’’’	68.0	4.27m	C-3’’’	77.4	4.11 m	C-3’’’
6’’’a	18.2	1.08 d, (6.5)	C4’’’	69.4	4.08	C-4’’’, 1’’’’
6’’’b	-------	----------	----------		4.21	
1’’’’				104.6	4.60 brd	C-3’’’’, 6’’’
2’’’’				75.4	3.92 m	C-4’’’’
3’’’’				76.2	3.14 t-like (9.5)	C-1’’’’5, 5’’’’
4’’’’				78.3	4.29*	C-2’’’’, 6’’’’,1’’’’’
5’’’’				77.4	4.1m	C-3’’’’
6’’’’a				60.7	4.08	C-4’’’’
6’’’’b				----------	4.21	C-4’’’’
1’’’’’				101.3	6.26 brs	C-4’’’’, 3’’’’’
2’’’’’				72.2	4.66*	C-4’’’’
3’’’’’				72.4	4.79 d (7.5)	C-1’’’’’, 5’’’’’
4’’’’				73.2	4.17 m	C-2’’’’
5’’’’’				68.9	4.21)	C-3’’’’’
6’’’’’				18.2	1.60 d (6.5)	C-4’’’’

δ in ppm and *J* values (Hz), were given in parentheses; All carbon and proton resonances were assigned on the basis of 2D (^{1}H-^{1}H COSY, HSQC and HMBC).

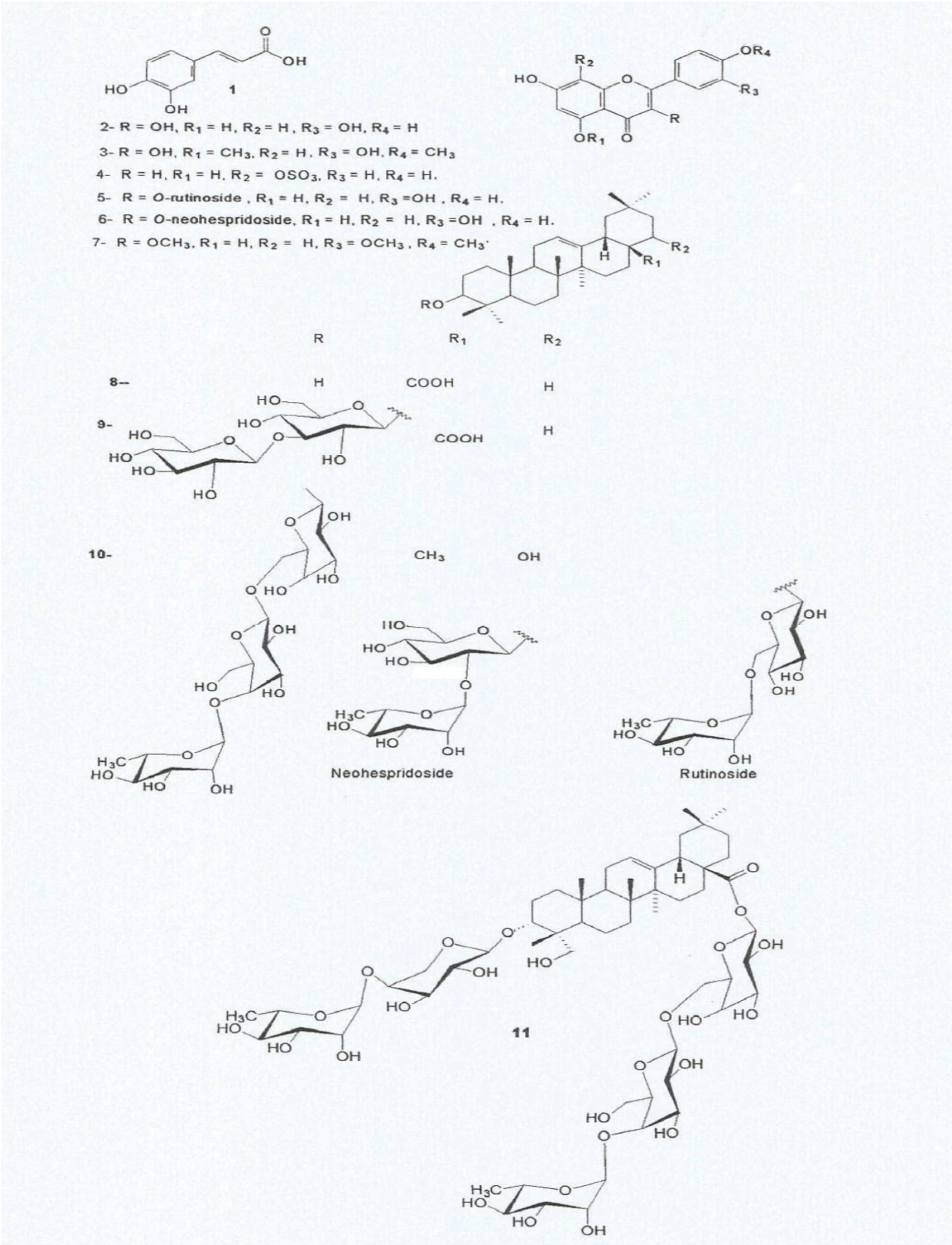

Fig. 1-Structures of isolated compounds.

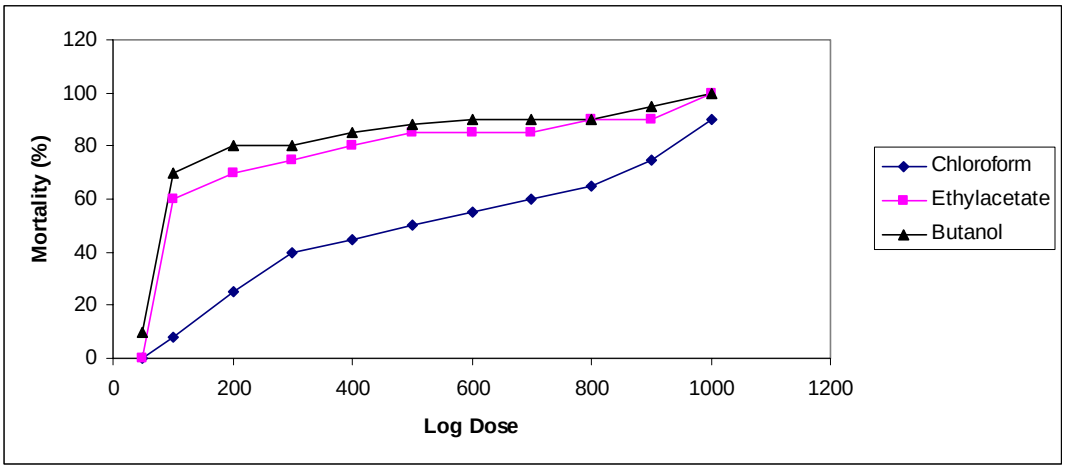

Figure 2. The cytotoxic activity of *C. officinalis* $CHCl_3$, EtOAc and MeOH extracts against brine shrimp (*A. salina*).

The butanol extract and compounds (**10**), (**11**) were cytotoxic for HepG2 cells and (**11**) was the most cytotoxic agent (IC_{50} = 14.3, 7.35 and 3.73 ug/ml), respectively, Figure 3.

Although different extracts of *C. officinalis* have been investigated for their different biological activates including anti-inflammatory, antispasmodic, and anti-septic (Yoshikawa *et al*., 2001; Lorenzi & Matos, 2002) to our knowledge, this work is the first trial to investigate the cytotoxicity of the butanol extract and some isolates of *C. officinalis* leaves against HepG2 solid tumor cell lines. The anti-cancer activity of *C. officinalis* butanol extract may be attributed to the corresponding activities of the extract constituent.

Many bidesmosidic oleanane type triterpene saponins were reported to have a cytotoxic activity (Lee *et al*., 2000). The tumor specificity of the cytotoxic action seems to be influenced by the structure of the sugar portion of the saponins (Kuroda *et al*., 2001). It was reported that the aglycone with five sugar units exhibited greater available cytotoxicity than that, which possessed three sugars (Huang *et al*., 2008). The results suggest that the presence of an additional sugar moiety of the oleanane-type saponins plays a role in mediating cytotoxicity.

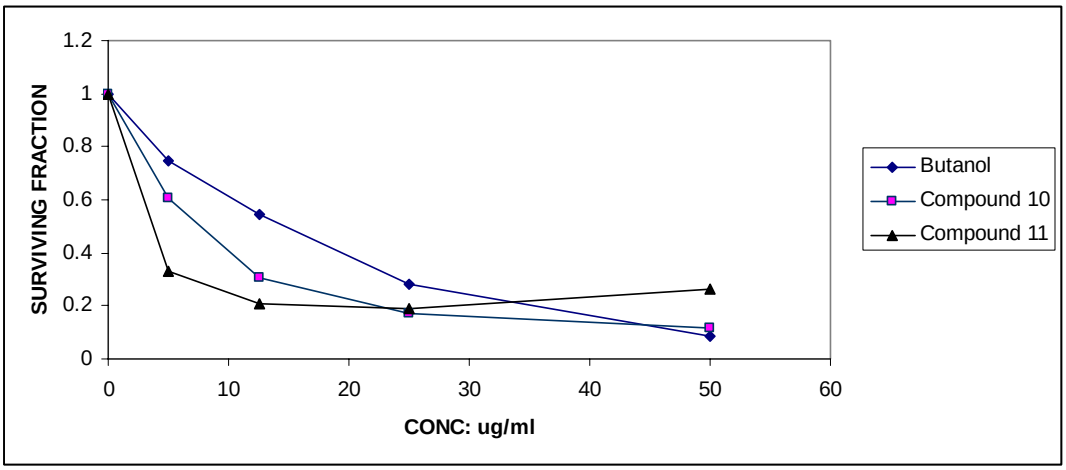

Figure 3. The cytotoxic activity of *C. officinalis* BuOH extract, 10 and 11 against HepG2 cell line.

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