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Protective effect of *Echinops galalensis* against CCl_4-induced injury on the human hepatoma cell line (Huh7)

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ABSTRACT

Phytochemical investigation of the flowering aerial parts of *Echinops galalensis* (Asteraceae) led to the isolation of a new taraxasteryl triterpene, 3β-acetoxy-taraxast-12, 20(30)-diene-11α-21α-diol (**1**), together with nine known metabolites, α-amyrin (**2**), β-sitosterol (**3**), erythrodiol (**4**), lup-20(29)-ene-1,3-diol (**5**), 1,5-dicaffeoylquinic acid (**6**), 3,5-dicaffeoylquinic acid (**7**), 3,4-dicaffeoylquinic acid (**8**), 4,5-dicaffeoylquinic acid (**9**) and apigenin-7-*O*-β-D-glucoside (**10**). The structure of the new compound was determined by comprehensive analyses of their 1D and 2D NMR, mass spectral (HR-EI) data and comparison with previously known analogs. The effect of the methanol extract of *E. galalensis*, its fractions as well as compounds (**1**–**10**) on human hepatoma cell line (Huh7) was evaluated according to aspartate aminotransferase (AST), alanine transaminase (ALT), superoxide dismutase (SOD) activities and malondialdehyde (MDA) level before and after exposure of the cells to carbon tetrachloride (CCl_4). It was found that pre-treatment of human hepatoma cell line (Huh7) with the tested samples (100 μg/ml) prior to CCl_4 challenge protected against cell injury. The protective effect of *E. galalensis* was suggested to be mediated, at least partly, by its antioxidant activity.

1. Introduction

Liver damage is one of the most serious diseases which has accompanied the adoption of modern food styles as well as the exposure to many environmental pollutants and intensive intake of medications. Regardless of the advances in modern medicine, there are no effective drugs that could stimulate liver functions and offer protection to the liver from the damage or help to regenerate hepatic cells (Chattopadhyay, 2003). In the last few decades, eastern herbal medicines have attracted the interest of modern scientific communities as alternative therapy.

The genus *Echinops* belongs to family Asteraceae and comprises over 120 species of which five are known to grow in Egypt (Takholm, 1974). Previous chemical investigation on the genus *Echinops* demonstrated the presence of thiophenes (Hymete et al., 2005; Koike et al., 1999; Nakano et al., 2011), quinoline alkaloids (Su et al., 2004), sesquiterpene hydrocarbons (Dawidar et al., 1990; Dong et al., 2008; El Sayed, 2001; Li et al., 2010) triterpenes (Metwally, 1987; Yasukawa et al., 1996), flavonoids (Ram et al., 1996; Singh et al., 2006), volatile oil (Papadopoulou et al., 2006) and lignans (Tene et al., 2004). Several members of genus *Echinops* showed hepatoprotective (Lin and Lin, 1993), anti-inflammatory (Yadava and Singh, 2006), anti-fungal (Fokialakis et al., 2006), anti-feedant, nematocidal (Chaudhuri and Tripathi, 1989), and cytotoxic activities (Jin et al., 2008).

In the present study, the possible protective effect of the methanol extract of *Echinops galalensis* as well as its major isolated compounds was assessed using a cell culture model consisting of a human hepatoma cell line that was challenged with CCl_4 to induce cell injury.

2. Results and discussion

Phytochemical investigation of the flowering aerial parts of *E. galalensis* afforded a new taraxasteryl metabolite (**1**) in addition to nine known metabolites (Fig. 1). The structures of the known compounds were established by comparing their UV, ^1H and ^{13}C NMR spectroscopic data with those in the literature and confirmed through co-chromatography with authentic samples. They were identified as, α-amyrin (**2**), β-sitosterol (**3**) (Goad and Akihisa, 1997; Mahato and Kundu, 1994), erythrodiol (**4**) (Hui-Zheng et al.,

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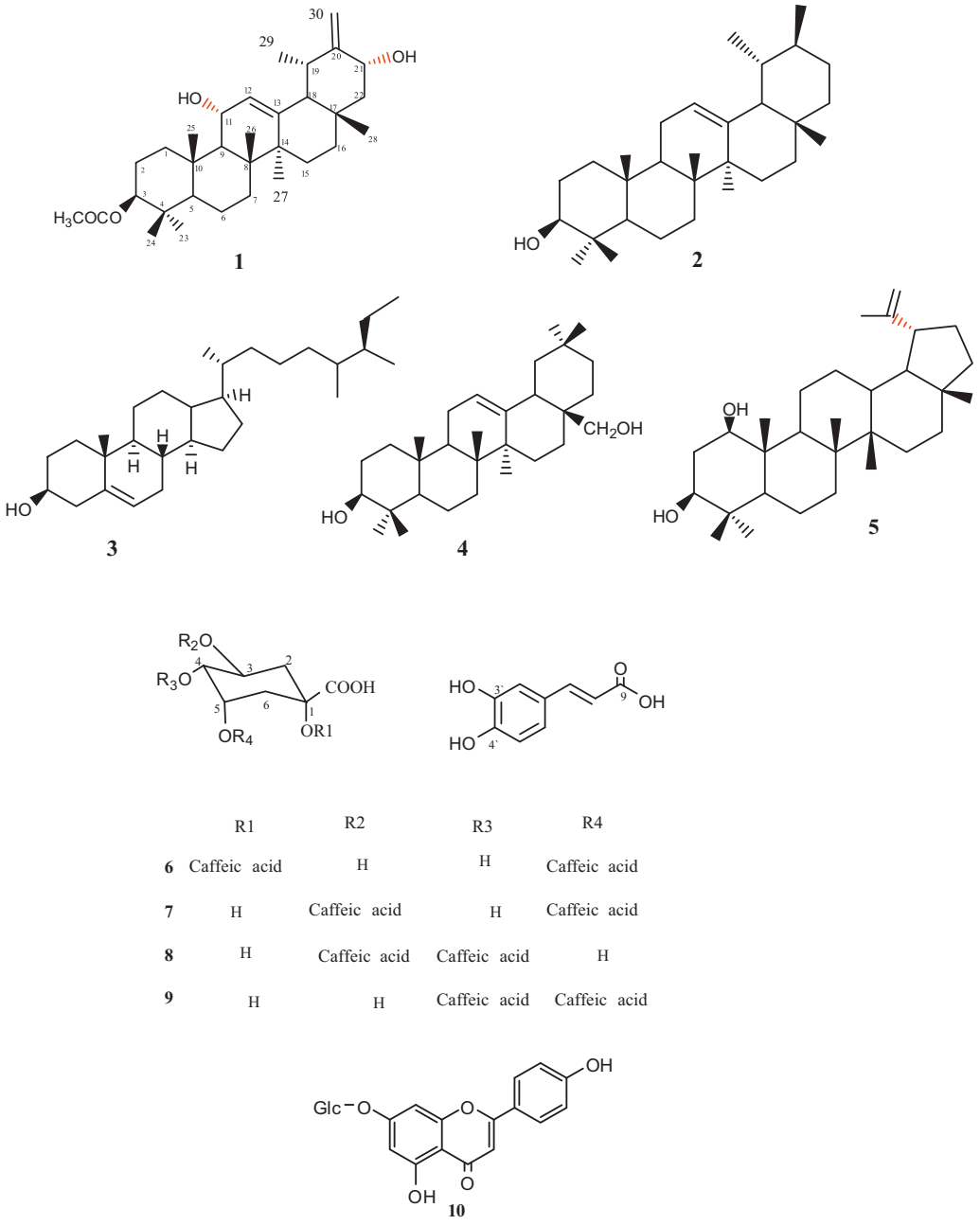

	R1	R2	R3	R4
6	Caffeic acid	H	H	Caffeic acid
7	H	Caffeic acid	H	Caffeic acid
8	H	Caffeic acid	Caffeic acid	H
9	H	H	Caffeic acid	Caffeic acid

Fig. 1. Chemical structure of the compounds isolated from *E. galalensis*.

1988; Mahato and Kundu, 1994), lup-20(29)-ene-1,3-diol (**5**) (Mahato and Kundu, 1994; Savona et al., 1987), four dicaffeoylquinic acid derivatives (**6–9**) (Shabana et al., 2007; Xiang et al., 2001), together with one flavone glycoside (**10**) (Mabry et al., 1970).

Compound **1** was isolated as white amorphous powder. Its HR-EIMS showed a molecular ion peak at *m/z* 498.3714 (calcd. 498.3706), corresponding to a molecular formula of $C_{32}H_{50}O_4$, which was confirmed by ^{13}C NMR and DEPT spectral data. The elucidation of compound **1** was carried out based on ^1H NMR, ^{13}C NMR, DEPT, ^1H–^1H COSY, HMQC and HMBC. The ^1H NMR spectrum (Table 1) displayed a total of seven methyl signals, i.e. one secondary methyl at δ_H 1.21 (d, J = 7.2 Hz), six tertiary methyls at δ_H 0.84 (s), 0.85 (s), 0.87 (s), 0.88 (s), 1.01 (s), 1.05 (s) ppm. Additionally, one deshielded methyl singlet was displayed at δ_H 2.05; three oxymethines at δ_H 3.35 (dd, J = 4.8, 6.6 Hz), 4.40 (dd, J = 4.8, 9 Hz) and 4.48 (dd, J = 5.4, 11.4 Hz) ppm; one trisubstituted olefinic moiety at δ_H 5.6 (d, J = 6.6 Hz) and an exocyclic olefinic moiety at δ_H 4.90 and 4.99 (br s, each) ppm.

The ^{13}C NMR spectrum displayed 32 carbon signals including seven methyls at δ_C 14.8, 15.8, 16.3, 16.4, 16.5, 27.9, 28.4 ppm, seven methines (including three oxymethines at δ_C 73.9, 71.2 and 80.9 ppm), six quaternary carbons (including one carbonyl at δ_C 170.8), and four olefinic carbons at δ_C 121.7, 145.8, 113.5 and 156.5 ppm. The ^1H and ^{13}C NMR spectroscopic data of compound **1** (Table 1) were similar to those of taraxasteryl acetate, previously isolated (Petrovic et al., 1999) except for the presence of an additional double bond, and two additional downfield protons at δ_H 3.35 and 4.40 ppm correlated in HMQC with carbon signals at δ_C 73.9 and δ_C 71.2 ppm, respectively. The substitution at C-19 by a methyl group was confirmed by the long-range correlations between the protons of this methyl group and C-18 (δ_C 48.4), 20

Table 1
^{1}H and ^{13}C NMR spectral data of compound (**1**) (600 MHz, $CDCl_3$).

Position	δ_H (J in Hz)	δ_C
CH_2(1)	1.7 and 0.94, m	38.4
CH_2(2)	1.61, m	23.7
H–C(3)	4.48, dd (5.4, 11.4)	80.9
C (4)	–	38.39
H–C(5)	0.8, m	55.0
CH_2(6)	1.51 m, 1.37 m	18.1
CH_2(7)	1.38 m	34.1
C(8)	–	41.0
H–C(9)	1.37, d (4.8)	50.2
C(10)	–	37.1
H–C(11)	3.35, dd (6.6, 4.8)	73.9
H–C(12)	5.64, d (6.6)	121.7
C(13)	–	145.8
C(14)	–	42.2
CH_2(15)	1.68 m, 0.96 m	26.4
CH_2(16)	1.31 m, 1.15 m	37.6
C(17)	–	33.8
H–C(18)	1.15, m	48.4
H–C(19)	2.15, m	38.0
C(20)	–	156.5
H–C(21)	4.40, dd (4.8, 9.0)	71.2
CH_2(22)	1.33,dd (3.6, 7.2), 1.95, dd (9.0, 13.8)	48.7
Me (23)	0.85, s	27.9
Me (24)	0.84, s	15.8
Me (25)	1.01, s	14.8
Me (26)	1.05, s	16.3
Me (27)	0.87, s	16.5
Me (28)	0.88, s	16.4
Me (29)	1.20, d (7.2)	28.4
CH_2 (30)	4.90, s, 4.99, s	113.5
C=O	–	170.8
CH_3–CO	2.05, s	21.3

Chemical investigation of *Echinops galalensis* afforded a new taraxasteryl triterpene namely 3β-acetoxy-taraxast-12, 20(30)-diene-11α,21α-diol, in addition to nine known metabolites. All isolated compounds showed a protective activity on human hepatoma cell line (Huh7).

(δ_C 156.5) and 19 (δ_C 38.0) in the HMBC spectrum (Fig. 2a), its α-orientation was deduced from the coupling constant of 7.2 Hz arising from the diaxial orientation of H-18 and H-19 (Maeda et al., 2007). The most downfield proton (H-3) was assigned to a carbon bearing an acetoxy group, and the acetyl methyl signal at δ_H at 2.05; δ_C 21.0 supported this assignment, followed by the HMBC experiment which revealed a wide range correlation between H-3 (δ_H 4.48) and the methyl of the acetoxy moiety with its carbonyl at δ_C 170.8. The other two downfield signals were assigned to protons on the carbons of two secondary hydroxyl groups, specifically to H-11 and H-21 at δ 3.35 and 4.40, respectively. Hydroxylation at C-21 was assigned from ^{1}H NMR and ^{1}H–^{1}H COSY spectra. The downfield shift of H_2-22 (δ_H 1.95, dd, J = 9, 13.8 Hz and 1.33, dd, J = 3.6, 7.2 Hz), δ_C 48.7 and its correlations with H-21 δ_H 4.40 (Fig. 2) which is correlated in the HMBC with the two olefinic carbons at δ_C 113.5 and 156.5 (assigned to the exocyclic double bond characterizing the taraxasterol moiety) (Iijima et al., 1995). Also the HMBC correlations between H_2-30 with the hydroxylated methine carbon C-21 were observed (Fig. 2). The downfield shift of C-30 (+6 ppm) (i.e. taraxasterol acetate) at δ_C 113.5 attributed mainly to the hydroxylation at C-21 (Maeda et al., 2007; Schmidt et al., 2004). Thus, as there are long range correlations between the methine proton at δ_H 4.40 and C-20 and C-30, the hydroxyl was assigned to be located at C-21. The ^{13}C NMR of the ring E was coinciding with that of the previously assigned structure as some derivatives of acyl taraxasterol moities (Maeda et al., 2007; Petrovic et al., 1999; Schmidt et al., 2004).

The lower downfield shifted proton at δ_H 3.35 gave a COSY correlation with the signal at δ_H 5.64 (d, J = 6.6 Hz) (Fig. 2) assigned for a tertiary olefinic carbon while the other olefinic carbon is quaternary indicating that the methine signal at δ_H 3.35 must be located beside the olefinic double bond. The two possibilities are either the double bond is located between C-11 and C-9 or between C-12 and C-13, the second suggestion was confirmed from the HMBC correlations between δ_H 5.64 and C-18 (δ_C 48.4) which is in turn correlated with δ_H 2.15 (H-19). On the other hand, a signal at δ_H 3.35 was correlated in the HMBC with δ_C 41.0 and δ_C 37.0 which are signals absent in the DEPT experiment indicating that they are quaternary carbons assigned to C-8 and C-10 respectively. From all the above observations, it was confirmed that the double bond exists between C-12 δ_H 5.6 (d, J = 6.6 Hz) (δ_C 121.7) and C-13 δ_C 145.8 and the hydroxylation occurs at C-11. The relative configurations of the two hydroxyl and the acetoxy groups were confirmed by NOESY (Fig. 2), in which the following correlations were found, δ_H 4.48 (Hα-3) and δ_H 0.80 (Hα-5) to δ_H 0.85(H_3-23) revealed a β-orientation for the acetoxy group at C-3. A correlation between δ_H 3.35 (H_β-11) and δ_H 1.01 (H_3-25) and δ_H 1.05 (H_3-26) demonstrated that H-11 is in a β-orientation and thus OH-11 is α-oriented. A correlation of δ_H 4.40 (H_β-21) with δ_H 0.88 (H_3-28) indicating that H-21 is a β-proton and thus OH-21 is α-oriented, this suggestion was more confirmed by the ^{3}J H,H couplings (4.8 and 9) of the axial proton at C-21. Therefore, the structure of compound **1** was assigned as 3β-acetoxy-taraxast-12, 20(30)-diene-11α,21α-diol.

In recent years, *in vitro* systems have been used in several applications including evaluation of protective effect of plant extract on liver lesions induced by toxic compounds (Torres-Gonzalez et al., 2011). The immortalized liver cell lines (Huh7 and HepG2) are well established models and have been used for investigation of potential hepatoprotective drugs (Groneberg et al., 2002). To the best of our knowledge, this is the first report on the *in vitro* hepatoprotective effect of *E. galalensis* using a cell culture model consisting of a human hepatoma cell line that was injured with CCl_4 to induce cell damage.

This study reports the protective effect of the methanol extract of the flowering aerial parts of *E. galalensis*, its fractions and the isolated compounds (**1**–**10**) using *in vitro* assay on human hepatoma cell line (Huh7). The protective effect was evaluated

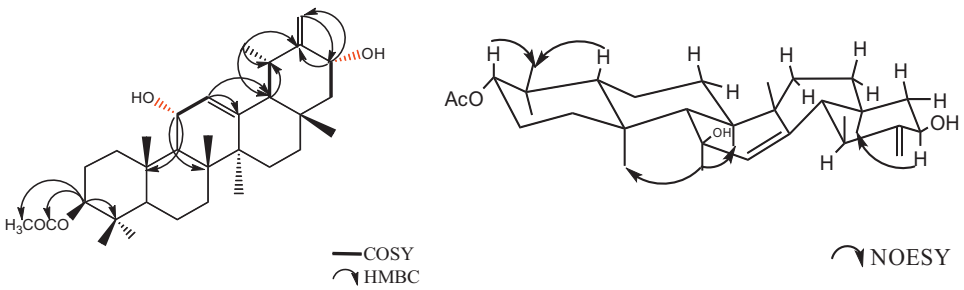

Fig. 2. Some Key HMBC, COSY and NOESY correlations of compound **1**.

based on the changes in aspartate aminotransferase (AST), alanine transaminase (ALT), superoxide dismutase (SOD) activities and malondialdehyde (MDA) level before and after exposure of the cells to carbon tetrachloride (CCl_4). Exposure of Huh7 cells to CCl_4 significantly ($p < 0.05$) increased AST, ALT and decreased SOD activities to 40.22 ± 1.45 U/ml, 40.2 ± 1.4 U/ml and 38.9 ± 5.44 U/ml, respectively and increased MDA level to 1.37 ± 0.067 nmol/ml in comparison to control untreated cells. Pretreatment with 100 μg/ml of silymarin significantly ($p < 0.05$) ameliorated CCl_4-induced damage through decreasing AST and ALT activities to 7.64 ± 0.93 U/ml and 10.21 ± 2.6 U/ml, respectively and increasing SOD activity to 406.22 ± 32.54 U/ml. At the same time silymarin decreased MDA level to 0.33 ± 0.07 nmol/ml. The same concentration of the methanol extract, chloroform, and *n*-butanol fractions as well as the isolated compounds exhibited variable promising protective effect against CCl_4-induced damage on the Huh7 (Fig. 3).

All tested compounds except **2** and **3**; significantly ($p < 0.05$) normalized elevated ALT activity, meanwhile, compounds **1**, **4** and **5** showed the highest protection to human hepatoma cell line (Huh7). They decreased ALT activity to 15.7 ± 0.9, 13.29 ± 0.87 and 15.4 ± 4.3 U/ml, respectively. On the other hand, all tested samples except methanol extract significantly ($p < 0.05$) decreased AST activity where compounds **2**, **3** and **6** were the most effective ones; they decreased its activity to 5.9 ± 3.7, 6.05 ± 1.4 and 10.59 ± 2.6 U/ml, respectively.

The reduction in AST and ALT activities by the extracts and the isolated compounds is in agreement with the commonly accepted

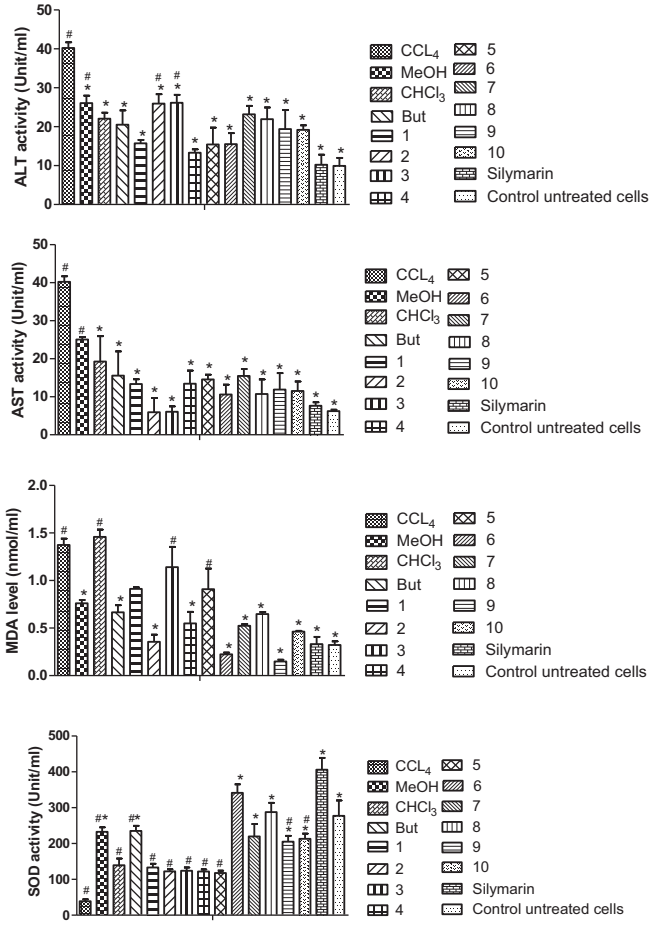

Fig. 3. Protective effect of methanol extract of *E. galalensis*, its fractions as well as its isolated compounds (**1**–**10**) (100 μg/ml) relative to CCl_4 on the activity of AST, ALT and SOD and level of MDA using *in vitro* assay on human hepatoma cell line (Huh7). *Significantly different from CCl_4 at $p < 0.05$. #Significantly different from silymarin at $p < 0.05$.

view that serum levels of transaminases return to normal due to the treatment of the hepatic damage caused by $CC1_4$ through stabilization of the plasma membrane of hepatocytes (Thabrew et al., 1987).

The lipid peroxidation is accelerated when free radicals are formed as the results of losing a hydrogen atom from the double bond in the structure of unsaturated fatty acids. Scavenging of free radicals is one of the major antioxidation mechanisms to inhibit the chain reaction of lipid peroxidation. Reduced lipid peroxidation was revealed in all tested compounds by a significant ($p < 0.05$) decrease in MDA level except for compounds **3** and **5.** Compounds **6** and **9** displayed the highest activity by decreasing MDA level to 0.224 ± 0.01 and 0.14 ± 0.01 nmol/ml, respectively. Moreover, compounds **6**–**10** significantly ($p < 0.05$) reduced the oxidative stress through increasing SOD activity. Compounds **6** and **8** proved the highest activity through increasing SOD activity to 341.65 ± 23.55 and 287.7 ± 25.7 U/ml, respectively.

In conclusion, the protective effect of *E. galalensis* methanolic extract, its fractions as well as the isolated compounds is at least partly due to their antioxidant activities as evidenced by the reduction in MDA level and the increase in SOD activity. The isolated triterpenes exhibited their protective effects by different grades based on the diversity of their skeleton which include lupene (**5**), ursane (**2**), oleanane (**4**) and taraxastane (**1**), in addition to stigmastane (**3**) skeleton. The activity of compound (**2**) on AST and MDA could be due to its similarity to the skeleton of ursolic acid which has been reported to exhibit a potent protective effect on damaged liver cells (Liu et al., 1994; Wu et al., 2011). On the other hand, compound **3** is known for its liver protecting activity (Adnyana et al., 2001). Moreover the activities of compounds **4** and **5** that belong to oleanane and lupane nucleuses were in accordance with the previously reported effects of oleanolic and betulinic acids which showed a protective effect on the liver through affecting its oxidative stress status (Marzouk, 2009; Miura et al., 1999).

Furthermore, it is well established that taraxasterol acetate (nucleus of compound **1**) was proved to have a significant antihepatitis effect due to the presence of two essential pharmacophores, the acetoxy group and the exocyclic double bond at ring E. The ability of compound **1** to significantly ($p < 0.05$) decrease the activity of ALT (15.71 ± 0.88 U/ml) and AST (13.34 ± 1.27 U/ml) indicates its ability to inhibit the degeneration and necrosis of liver cells (Iijima et al., 1995). Based on this fact, the activity of compound **1** could be due to the presence of similar moieties between the two compounds. Also it is obvious theoretically that, the hydroxylation at C-21 and C-11 as well as the unsaturation at C-12 in **1** did not significantly alter the activity.

The effect of phenolic acids (**6**–**9**) is in agreement with the previously published data. Caffeoyl group might be the active pharmacophore for the hepatoprotective effect (Xiang et al., 2001). Also, dicaffeoylquinic acid derivatives were reported to have a potent radical scavenging effect to which their inhibitory effects on the lipid peroxidation of hepatic mitochondria and microsomes might be related. Therefore, the radical scavenging activity was considered to play an important role in the hepatocyte protective effect of the isolated dicaffeoylquinic acid derivatives (Xiang et al., 2001).

3. Experimental

3.1. General

Optical rotation was measured with a DIP-360 automatic polarimeter (Jasco,Tokyo, Japan). UV spectra were recorded in MeOH solutions before and after addition of different diagnostic shift reagents (Mabry et al., 1970) on a UV IKON 940 spectrophotometer. HR-EIMS was measured with a JEOL JMX-AX 505HAD

mass spectrometer at an ionization voltage of 70 eV. NMR analyses were run on AVANCE AV-600 MHz BRUKER spectrometers relative to TMS in DMSO or $CDCl_3$. For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck) and polyamide 6S (Riedel de Hean AG, Seelze, Hannover, Germany), Silica gel 60 H for VLC (E-Merck, Darmstadt, Germany), silica gel 60 for column Chromatography (Fluka, 70–230 mesh, Germany), silica gel $100C_{18}$–Reversed phase (70–230 mesh, Fluka) were used. Pre-coated silica gel 60 F_{254} plates (Merck, Germany) were used for TLC.

3.2. Chemicals for biological study

Silymarin, carbon tetrachloride (CCl_4) and dimethyl sulfoxide were purchased from Sigma–Aldrich chemicals Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin 0.25%, penicillin G, streptomycin and phosphate-buffered saline were obtained from Gibco Invitrogen (Carlsbad, CA, USA). Kits for measurement of AST, ALT, SOD activities and MDA level were purchased from Biodiagnostic for diagnostic reagents (Dokki, Giza, Egypt).

3.3. Cell culture

Human hepatoma cancer cells (Huh-7) obtained from VACSERA (Dokki, Giza, Egypt). Cells were maintained in DMEM medium supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% CO_2 atmosphere at 37 °C.

3.4. Assessment of hepatoprotective effect

To assess the hepatoprotective effect of the tested samples (methanol extract, its fractions as well as all isolated compounds); CCl_4 induced hepatic cell injury was induced as previously described (Torres-Gonzalez et al., 2011). Briefly, exponentially growing cells were collected using 0.25% trypsin-EDTA and plated in 6-well plates at 10^5 cells/well. Cells were exposed to a sub-cytotoxic concentration of test samples in PBS (100 μg/ml) for one hour and subsequently challenged with 40 mM CCl_4 and further incubated for two hours. The supernatant was collected and assayed for AST, ALT, SOD activities and MDA level (Marklund and Marklund, 1974; Ohkawa et al., 1979; Reitman and Frankel, 1957).

3.5. Plant material

The aerial flowering parts of *E. galalensis*, were collected in April 2010 from Wadi Hagol, Cairo, Egypt. The identity of the plant was authenticated by Dr. Ibrahim El-Garf, Plant Taxonomy Department, Faculty of Science, Cairo University. A voucher specimen (No. EG-5) is kept in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

3.6. Extraction, isolation and identification

The air dried powdered flowering aerial parts of *E. galalensis* (2 kg) were extracted with methanol (4 × 5 L) at room temperature, till exhaustion. The combined extracts were filtered and concentrated to give a brown residue of 378 g. Part of the residue (350 g) was suspended in distilled water (500 ml) and partitioned successively with chloroform (4 × 500 ml), and *n*-butanol (4 × 500 ml) to yield 50, and 40 g, respectively. The chloroform fraction was chromatographed over silica gel 60 H for vacuum liquid chromatography VLC (5 × 7.5 cm, 30 g) with *n*-hexane, *n*-hexane–chloroform mixtures, chloroform-ethyl acetate mixtures with a gradual increase in polarity up to 100% ethyl acetate. Fractions (200 ml each) were collected and monitored by TLC. The spots were visualized after spraying with *p*-anisaldehyde followed by heating. Similar fractions were pooled to give four main fractions (A–D). Fraction B (40–50% chloroform–*n*-hexane, 3.5 g) was rechromatographed over silica gel column (25 cm × 2 cm, 50 g), using *n*-hexane as an eluent to afford compound **2** (20 mg) and **3** (40 mg). Fraction C (55% chloroform–*n*-hexane, 2.5 g) was further subjected to silica gel column (25 cm × 2 cm, 50 g) and eluted with a *n*-hexane–ethyl acetate mixture (9.5:0.5, v/v) to yield compound **1** (55 mg). Fraction D (60% chloroform–*n*-hexane, 2.9 g) was rechromatographed over silica gel 60 column (25 cm × 2 cm, 50 g), using *n*-hexane-ethyl acetate (9.5:0.5, v/v) as an eluent to give two subfractions. The two subfractions were purified over sephadex LH-20 column using $CHCl_3$–MeOH (1:1, v/v) as an eluent to yield two compounds **4** (65 mg) and **5** (46 mg).

The *n*-butanol fraction was suspended in water, precipitated with excess EtOH, filtered and dried (10 g). The dried residue was suspended in water, applied on a polyamide column (5 cm × 100 cm, 250 g) and eluted with H_2O, then H_2O–MeOH mixtures to give four fractions (I–IV). Fraction I (100% H_2O, 2 g) was examined by paper chromatography and was found to be free from polyphenol character. Fraction II (80% H_2O, 1 g), was rechromatographed over silica gel $100C_{18}$–Reversed phase using acetonitrile–water, 0.5:9.5 (v/v) as an eluent to afford compounds **6** (17 mg), and **7** (22 mg). Fraction III (60% H_2O, 1 g), was treated as fraction II eluted with acetonitrile:water (1:9, v/v) to afford compounds **8** (20 mg), and **9** (23 mg). Fraction IV (40% H_2O, 1 g) was rechromatographed on Sephadex LH-20 column using (*n*-BuOH–isoprOH–H_2O, (4:1:5, v/v/v), upper layer) as an eluent to afford compound **10** (19 mg).

Compound **1**: 3β-acetoxy-taraxast-12, 20(30)-diene-11α,21α-diol was obtained as white amorphous powder; $[\alpha]_D^{24}$ +55 (c 0.001, MeOH); HR-EIMS: 498.3714 (calcd. 498.3706). For ^1H (600 MHz, $CDCl_3$), ^{13}C NMR (150 MHz, $CDCl_3$) (see Table 1).

3.7. Statistical analysis

All variables were measured in triplicates and the experiment was repeated three times. Results were expressed as mean ± standard error of the mean (S.E.M). Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey–Kramer's post hoc test. Statistical significance was accepted at a level of $p < 0.05$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2012.10.012.

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