Spectroscopic Identification of New Ellagitannins and a Trigalloyl-glucosylkaempferol from an Extract of *Euphorbia cotinifolia* L. with Antitumour and Antioxidant Activity

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From an extract of leaves and small branches of *Euphorbia cotinifolia* L., 17 polyphenols were isolated including two new ellagitannins and a trigalloyl-glucosylkaempferol. Based on extensive spectral data (UV, ESI-MS, ^{1}H NMR, DEPT and 1D/2D NMR) and chemical studies, their structures were characterized as 1-*O*-galloyl-3,6-hexahydroxydiphenoyl-D-$B_{1,4}$-glucopyranose (**5**), 1-*O*-galloyl-3,6-valoneoyl-D-$B_{1,4}$-glucopyranose (**6**), and kaempferol 3-*O*-(2",3",6"-tri-*O*-galloyl)-*β*-D-glucopyranoside (**13**). Biological evaluation indicated that the 80% aqueous methanol extract (AME), chloroform extract (CE), and some pure compounds have potent scavenging activity in the DPPH assay with SC_{50} values lower than that of ascorbic acid, especially **5**, **7**–**9**, and a mixture of hyperin 6"-gallate (**11**) and isoquercitrin 6"-gallate (**12**). Moreover, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cell viability assay, **6** and **8** exhibited the highest inhibition of human hepatocellular carcinoma cells (Hep-G2), while AME, CE, **5**, **7**, **9**, and the mixture of **11** and **12** were found to be moderate growth inhibitors according to their IC_{50} values. In addition, AME, **5**, and **8** exhibited significant antiproliferative activity against colon carcinoma cells (HCT-116); however, CE and the other examined compounds displayed moderate to low antitumour activity against HCT-116 cells.

Key words: Euphorbia cotinifolia, Ellagitannins, Antitumour and Antioxidant Activity

Introduction

Euphorbia cotinifolia L. is one of about 6000 species belonging to the family Euphorbiaceae that comprises about 240 genera (Bailey, 1949). Traditionally, *Euphorbia* species have been used for the treatment of cough, hay fever, kidney stones, bronchial affection, bowel cotemplaints, and worm infestations (Hore *et al.*, 2006). Several *Euphorbia* plants were found to possess various biological effects, *e.g.* antimicrobial (Sudhakar *et al.*, 2006), antiviral (Chaabi *et al.*, 2007), antitumour (Lin *et al.*, 2006), anti-inflammatory (Singh *et al.*, 2006), antinociceptive (Ahmad *et al.*, 2005), antidiarrhoeal (Atta and Mouneir, 2005), antioxidant (Lin *et al.*, 2002), and immunosuppressive (Bani *et al.*, 2005). Different bioactive natural product classes have been reported in *Euphorbia* species, *e.g.* tannins (Yoshida *et al.*, 1994a; Amakura *et al.*, 1997; Lee *et al.*, 1991), flavonoids (Amakura *et al.*, 1997; Yoshida *et al.*, 1994b), diterpenes, and coumarins (Haba *et al.*, 2007; Fu *et al.*, 2006; Zhang *et al.*, 2006). The first report on *E. cotinifolia* leaves described the isolation of sixteen polyphenols including a quercetin analogue of **13** and isoquercitrin (**8**) from the aqueous acetone extract (Marzouk, 2008). This paper presents the second report on the polyphenols of the aerial parts of *E. cotinifolia* and the evaluation of antitumour and antioxidant activities of the aqueous methanol extract (AME) and the chloroform extract (CE) and of some of the major compounds they contain.

Material and Methods

General

The 1D and 2D NMR spectra were recorded at 270, 300, 400, 500 MHz on JEOL (Akishima, Japan) GX-270, Varian Mercury (Pleasanton, CA, USA) 300, Bruker (Madison, WI, USA) ABX-400, and JEOL GX-500 NMR spectrometers; δ values are reported in ppm relative to tetramethylsilane (TMS) as international standard. LC/ESI-MS and FAB-MS analyses were recorded on Finnigan (Bremen, Germany) LCQ deca and MAT CH_5D instruments, respectively. UV analyses of pure samples were recorded, separately, as MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu (Kyoto, Japan) UV 240 (P/N 240–58000) instrument. Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany), and polyamide S (Fluka, St. Louis, MO, USA) were used for column chromatography (CC). Also, Whatman No. 1 sheets (Whatman Ltd., Maidstone, England) were used for paper chromatography (PC). The pure compounds were visualized by spraying with freshly prepared nitrous acid reagent: a dropwise addition of glacial HOAc to an aqueous 1% $NaNO_2$ solution (1:10); and naturstoff reagent: 1% diphenyl boryloxyethanol amine complex and 5% polyethylene glycol 400 in MeOH separately sprayed. Then the dry chromatogram was heated at 120 °C for 10 min, visualized under UV light (365 nm), and sprayed with $FeCl_3$ (1% in EtOH). The solvent systems: S_1 [*n*-BuOH/HOAc/H_2O (4:1:5, v/v/v top layer)], S_2 (15% aqueous HOAc), and S_3 = BIW [*n*-BuOH/2-propanol/H_2O (4:1:5, v/v/v top layer)] were used.

Plant material

Leaves and small branches of *E. cotinifolia* L. were collected in July 2006 from plants growing in the Benha region, Egypt. The identification of the plant was performed by Dr. Wafaa M. Amer, Professor of Taxonomy, Department of Botany, Faculty of Science, Cairo University, Cairo, Egypt. A voucher specimen (No. Eu12) is deposited in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt.

Extraction and isolation

The powder of air-dried leaves and small branches (1 kg) was extracted with hot 80% aqueous MeOH (5 x 3 l) under reflux (70 °C, 2 h each). The collected extract was dried under vacuum and then preliminarily purified by $CHCl_3$ under reflux (60 °C, 1 h) to give 120 g dry residue. The residue was taken up in MeOH, then dried under vacuum to give 85 g (methanol-soluble portion). This material was dissolved in MeOH/H_2O (1:1). The MeOH was evaporated and the residual suspension fractionated on a polyamide S column (250 g, 110 x 7 cm) using a step gradient from 100% H_2O to 100% MeOH for elution to give 45 fractions (1 l each). Thereafter, the fractions were concentrated under reduced pressure and monitored by comparative paper chromatography (CoPC) using solvent systems S_1 and S_2 and UV light for collection into ten major fractions (I–X). Fractions I (7.89 g) and II (5.64 g) were isolated as sticky deep brown material of complex free sugar mixtures. Fraction III (3.73 g) was subjected to repeated CC on microcrystalline cellulose and eluted with aqueous EtOH (10–80%) to give two major subfractions. These were separately fractionated on a Sephadex LH20 column employing first EtOH to yield compounds **1** (10 mg) and **2** (15 mg) and then BIW to give **3** (20 mg) and **4** (22 mg). Fraction IV (2.59 g) was chromatographed on a Sephadex LH-20 column using *n*-BuOH saturated with H_2O as eluent to afford two subfractions that were finally purified on a Sephadex LH-20 column with EtOH to give pure **5** (28 mg) and **6** (35 mg), respectively. Pure samples of compounds **7** (30 mg) and **8** (25 mg) were obtained by repeated fractionation of fraction V (3.08 g) on a Sephadex LH-20 column using aqueous MeOH (10–80%, v/v), then 95% EtOH. Fraction VI (3.8 g) was applied onto a Sephadex LH-20 column eluted with and eluted with *n*-BuOH saturated with water followed by CC on a Sephadex LH-20 column EtOH to yield **9** (15 mg) and **10** (39 mg). Consecutive CC of fraction VII (2.29 g), three times on a Sephadex LH-20 column with BIW, then MeOH, was used for separation of two major subfractions, giving pure samples of **11** (43 mg) and **12** (31 mg). Fractionation of VIII (1.99 g) on cellulose column with *n*-BuOH saturated with water, followed by a Sephadex LH-20 column column and BIW for elution led to pure **13** (20 mg). Fraction IX (2 g) was subjected twice to CC on a Sephadex LH-20 column, the first one one was eluted with 10–90% aqueous MeOH followed by BIW in the second column. The crude major compounds were purified by preparative

paper chromatography (PPC) using S_1 as eluent to afford **14** (8 mg) and **15** (12 mg). Fraction X (890 mg) was subjected to PPC with S_1 as eluent followed by purification of each major band with MeOH on a Sephadex LH-20 column to yield **16** (10 mg) and **17** (13 mg). The homogeneity of the fractions was checked by 2D-PC and CoPC using Whatman No. 1 paper sheets, and solvent systems S_1 and S_2 for elution and different spray reagents.

1-O-Galloyl-3,6-(S)-hexahydroxydiphenoyl-D-$B_{1,4}$-glucopyranose (**5**): Off-white amorphous powder (35 mg). – R_f = 0.21 (S_1), 0.52 (S_2) on PC; deep purple spot turned to pink-red, indigo-red, and blue colour with KIO_3, HNO_2, and $FeCl_3$ spray reagents, respectively. – UV (MeOH): λ_{max} = 220, 265, 286sh nm. – Negative FAB-MS: *m/z* = 633 $[M-H]^-$, 301 [ellagic acid$-H]^-$ = $[HHDP-2H_2O-H]^-$, 169 [gallic acid$-H]^-$. – ^{1}H NMR (500 MHz, DMSO-d_6): galloyl: δ_H = 7.03 (2H, s, H-2'/6'); HHDP: 6.58 (1H, s, H-3'''), 6.51 (1H, s, H-3''); glucose: 6.22 (1H, d, *J* = 7.1 Hz, H-1), 4.62 (1H, br s, H-3), 4.37 (1H, t-like, *J* = 8.2 Hz, H-5), 4.24 (1H, dd, *J* = 11.2, 7.9 Hz, H-6a), 4.23 (1H, br s, H-4), 3.98 (1H, dd, *J* = 10.9, 8.9 Hz, H-6b), 3.89 (1H, br d, *J* = 7.1 Hz, H-2). – ^{13}C NMR (125 MHz, DMSO-d_6): galloyl: δ_C = 164.72 (C-7'), 145.49 (C-3'/5'), 138.80 (C-4'), 118.64 (C-1'), 108.93 (C-2'/6'); HHDP: 166.99, 166.64 (C-7''/7'''), 144.73, 144.64 (C-4''/4'''), 144.21, 143.89 (C-6''/6'''), 135.44, 135.31 (C-5''/5'''), 123.82, 123.01 (C-2''/2'''), 115.71, 115.42 (C-1''/1'''), 106.88, 106.01 (C-3''/3'''); glucose: 92.13 (C-1), 77.31 (C-3), 76.25 (C-5), 71.50 (C-2), 63.86 (C-6), 62.06 (C-4).

1-O-Galloyl-3,6-(S)-valoneoyl-D-$B_{1,4}$-glucopyranose (**6**): Creamy-white amorphous powder (28 mg). – R_f = 0.21 (S_1), 0.57 (S_2) on PC; deep purple spot turned to pink-red, indigo-red, and blue colour with KIO_3, HNO_2, and $FeCl_3$ spray reagents, respectively. – UV (MeOH): λ_{max} = 216, 260, 285sh; (+ MeOH + NaOMe): 210, 240sh, 325 nm. – Negative FAB-MS: *m/z* = 801 $[M-H]^-$, 469 [valoneic acid dilactone$-H]^-$, 425 [valoneic acid dilactone$-CO_2-H]^-$, 169 [gallic acid$-H]^-$. – ^{1}H NMR (500 MHz, DMSO-d_6): galloyl: δ_H = 7.04 (2H, s, H-2'/6'); valoneoyl: 6.94 (1H, s, H-3''), 6.55 (1H, s, H-3), 6.19 (1H, s, H-3'); glucose: 6.24 (1H, d, *J* = 7.9 Hz, H-1), 4.53 (1H, br s, H-3), 4.35 (1H, t-like, *J* = 8.7 Hz, H-5), 4.18 (1H, m, H-6a), 4.14 (1H, br s, H-4), 3.98 (1H, br t-like, *J* = 10.2 Hz, H-6b), 3.70 (1H, br d, *J* = 6.8 Hz, H-2). – ^{13}C NMR (125 MHz, DMSO-d_6): galloyl: δ_C = 165.22 (C-7'), 145.78 (C-3'/5'), 139.19 (C-4'), 118.99 (C-1'), 108.32 (C-2'/6'); valoneoyl: 167.44 (C-7'), 167.20 (C-7), 167.10 (C-7''), 146.00 (C-4'), 145.77 (C-6'), 144.66 (C-6), 144.50 (C-4), 142.47 (C-2''), 139.22 (C-5''), 139.20 (C-4''), 136.20 (C-5), 136.10 (C-5'), 135.73 (C-3''), 123.74 (C-2'), 122.94 (C-2), 117.89 (C-1'), 115.77 (C-1), 114.70 (C-1''), 108.99 (C-6''), 108.40 (C-3), 106.21 (C-3'); glucose: 91.80 (C-1), 79.48 (C-3), 76.11 (C-5), 71.77 (C-2), 64.06 (C-6), 63.04 (C-4).

Kaempferol 3-O-(2'',3'',6''-tri-O-galloyl)-β-D-glucopyranoside (**13**): Yellow amorphous powder (20 mg). – R_f = 0.45 (S_1), 0.16 (S_2) on PC; deep purple spot turned to orange fluorescence with naturstoff (365 nm) and deep blue colour with $FeCl_3$ spray reagents. – UV (MeOH): λ_{max} = 220, 267, 354; (+ NaOMe) 218, 274, 326, 400; (+ NaOAc) 226, 273, 350sh, 383; (+ NaOAc/H_3BO_3) 223, 267, 353, 376; (+ $AlCl_3$) 217, 285, 306, 410; (+ $AlCl_3$/HCl) 213, 276, 300sh, 345, 405 nm. – Negative ESI-MS: *m/z* = 903 $[M-H]^-$, 751 $[M-$galloyl$]^-$, 599 $[M-2 \cdot$ galloyl$]^-$, 447 $[M-3 \cdot$ galloyl$]^-$, 285 [M–trigalloylglucoside$]^-$ = [kaempferol$-H]^-$. – ^{1}H NMR (500 MHz, DMSO-d_6): kaempferol aglycone: δ_H = 12.50 (1H, s, OH-5), 7.93 (2H, d, *J* = 9 Hz, H-2'/6'), 6.78 (2H, d, *J* = 9 Hz, H-3'/5'), 6.39 (1H, d, *J* = 2 Hz, H-8), 6.19 (1H, d, *J* = 2 Hz, H-6); galloyl: 6.98, 6.94, 6.93 (2H each, s, H-2'''/6''', H-2''''/6'''', H-2'''''/6'''''); glucose: 5.95 (1H, d, *J* = 8 Hz, H-1''), 5.42 (1H, t-like, *J* = 9.5 Hz, H-3''), 5.22 (1H, dd, *J* = 9.5, 8 Hz, H-2''), 4.34 (1H, br d, *J* = 11.5 Hz, H-6''a), 4.27 (1H, br d, *J* = 10 Hz, H-6''b), 3.87 (2H, br s, H-4''/5''). – ^{13}C NMR (125 MHz, DMSO-d_6): kaempferol aglycone: δ_C = 178.25 (C-4), 164.71 (C-7), 161.81 (C-5), 160.65 (C-4'), 157.66 (C-9), 157.42 (C-2), 133.70 (C-3), 131.13 (C-2'/6'), 121.22 (C-1'), 115.87 (C-3'/5'), 104.38 (C-10), 99.98 (C-6), 94.69 (C-8); galloyl: 165.65, 165.43, 164.91 (3 x C-7), 145.84, 145.60, 145.49 (3 x C-3/5), 139.21, 138.62, 138.40 (3 x C-4), 119.32, 118.94, 118.44 (3 x C-1), 109.32, 109.25, 108.03 (3 x C-2/6); glucose: 100.13 (C-1''), 76.11 (C-3''), 74.45 (C-5''), 72.85 (C-2''), 69.01 (C-4''), 63.23 (C-6'').

Cell culture

Two human cell lines were used in testing the anticancer activity: hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116); both lines were generous gifts from the German Cancer Research Center, Heidelberg, Germany, respectively. Hep-G2 cells were routinely cultured

in DMEM (Dulbeco's modified Eagle's medium), while HCT-116 cells were cultured in McCoy's medium. Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, and 250 ng/ml amphotericin B. The cells were maintained at subconfluence at 37 °C in humidified air containing 5% CO_2. For subculturing, monolayer cells were harvested after trypsin/EDTA treatment at 37 °C. The cells were used when the confluence had reached 75%. Test samples were dissolved in DMSO (99.9%, HPLC grade) and diluted 1000-fold in the assays. In all cellular experiments, the results were compared with DMSO-treated cells. All experiments were repeated four times, unless mentioned otherwise, and the data are represented as mean ± SD unless mentioned otherwise. All cell culture materials were obtained from Cambrex BioScience (Copenhagen, Denmark) and chemicals from Sigma/Aldrich (St. Louis, MO, USA).

Antitumour activity

Antitumour activity against Hep-G2 and HCT-116 cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cell viability assay (Hansen *et al.*, 1989). The assay is based on the ability of active mitochondrial dehydrogenase of living cells to cleave the tetrazolium rings of yellow MTT and form dark blue insoluble formazan crystals which are largely impermeable to cell membranes, resulting in their accumulation within healthy cells. Solubilization of the cells results in the liberation of the crystals, which are then solubilized. The number of viable cells is directly proportional to the intensity of the dark blue colour of the formazan solution. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm, and the relative cell viability was expressed as the mean percentage of viable cells relative to the respective DMSO-treated cells (control).

Antioxidant activity (scavenging of DPPH)

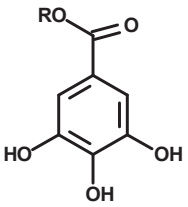
The antioxidant capacity of AME, CE, and the pure compounds was evaluated through their scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, which are deep violet because of an unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet colour decolourizes to pale yellow of the non-radical form (Amsterdam *et al.*, 1992). The change in colour and the subsequent fall in absorbance are monitored spectrophotometrically at 515 nm. The percentage of DPPH bleaching utilized for SC_{50} was calculated with reference to the absorbance (0%) and the absorbance of ascorbic acid (100%).

Results and Discussion

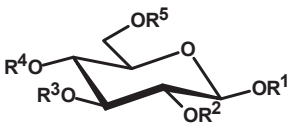
Chemistry

The compounds were identified by CoPC with authentic samples and comparison of their spectroscopic data with published data of structurally related compounds (Mabry *et al.*, 1970; Agrawal and Bansal, 1989; Okuda *et al.*, 1989). Their structures were established to be gallic acid (**1**), methyl gallate (**2**), 1,3,6-tri-*O*-galloyl-β-D-$^4C_1$-glucopyranose (**3**), 1,2,3,6-tetra-*O*-galloyl-β-D-$^4C_1$-glucopyranose (**4**), hyperin (**7**), isoquercitrin (**8**), kaempferol 3-*O*-β-D-$^4C_1$-galactopyranoside (**9**), quercetin 3-*O*-β-D-$^4C_1$-xylopyranoside (**10**), hyperin 6"-gallate (**11**), isoquercitrin 6"-gallate (**12**), ellagic acid (**14**), ellagic acid 4-*O*-methyl ether (**15**), quercetin (**16**), and kaempferol (**17**) (see Fig. 1).

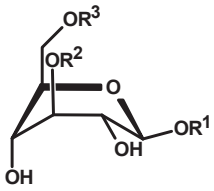
Compounds **5** and **6** showed the chromatographic behaviour (R_f values, fluorescence under UV light, and responses toward specific spray reagents) of galloyl and hexahydroxydiphenoyl (HHDP) esters (Gupta *et al.*, 1982). Characteristic UV absorption maxima at about 260 and 285 nm for gallo- and ellagitannins were recorded (Marzouk *et al.*, 2007). Following an acid hydrolysis **5** released gallic and ellagic acid, respectively, in the organic phase along with glucose in the aqueous phase while valoneoic acid dilactone was identified by CoPC also in the organic hydrolysate of **6**. Negative FAB-MS of **5** displayed a molecular ion at *m/z* 633 ($[M-H]^-$) and an intrinsic fragment of a HHDP moiety at *m/z* 301 ([ellagic acid$-H]^-$ = [HHDP$-2H_2O-H]^-$) to suggest a galloyl-hexaydroxydiphenoylglucose structure, whereas **6** showed a molecular ion peak at *m/z* 801 ($[M-H]^-$), with 168 amu more than **5** together, with characteristic fragments of a valoneoyl moity at *m/z* 469 ([valoneic acid dilactone$-H]^-$) and 425 ([valoneic acid dilactone$-CO_2-H]^-$) (Nawwar *et al.*, 1997). This evidence together with the characteristic fragment of a galloyl es-



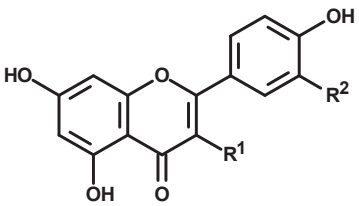
1: R = H
2: R = CH_3



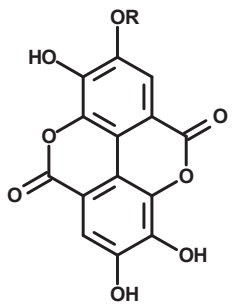
3: $R^1 = R^3 = R^5$ = galloyl, $R^2 = R^4$ = H
4: $R_1 = R^2 = R^3 = R^5$ = galloyl, R^4 = H



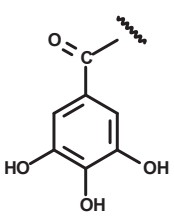
5: R^1 = galloyl, R^2--R^3 = HHDP
6: R^1 = galloyl, R^2--R^3 = valoneoyl



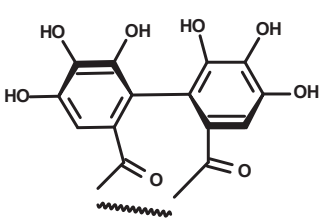
7: R^1 = *O*-β-D-glucopyranoside, R^2 = OH
8: R^1 = *O*-β-D-galactopyranoside, R^2 = OH
9: R^1 = *O*-β-D-galactopyranoside, R^2 = H
10: R^1 = *O*-β-D-xylopyranoside, R^2 = OH
11: R^1 = *O*-(6"-*O*-galloyl)-β-D-galactopyranoside, R^2 = OH
12: R^1 = *O*-(6"-*O*-galloyl)-β-D-glucopyranoside, R^2 = OH
13: R^1 = *O*-(2",3",6"-tri-*O*-galloyl)-β-D-glucopyranoside, R^2 = H
16: $R^1 = R^2$ = OH
17: R^1 = OH, R^2 = H



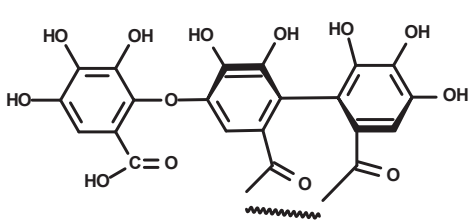
14: R = H
15: R = CH_3



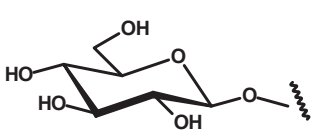
Galloyl



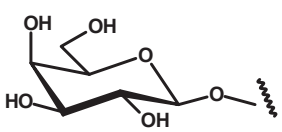
Hexahydroxydiphenoyl, (HHDP)



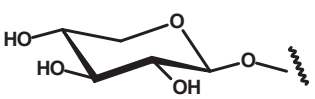
Valoneoyl



***O*-β-D-Glucopyranoside**



***O*-β-D-Galactopyranoside**



***O*-β-D-Xylopyranoside**

Fig. 1. Chemical structures of compounds **1**–**17**.

ter at 169 ([gallic acid – H]$^-$) explained that the structure of **6** contains a valoneoyl instead of the HHDP moiety in **5**. A galloyl ester was concluded at OH-1 from a 2H singlet at about δ_H 7 ppm in both ^{1}H NMR spectra. Also two 1H singlets were described at δ_H 6.58 ppm (H-3''') and 6.51 ppm (H-3'') for one HHDP moiety in **5** and three singlets at δ_H 6.94 ppm (H-3''), 6.55 ppm (H-3), and 6.19 ppm (H-3') for one valoneoyl moiety in the structure of **6** (Marzouk, 2008; Marzouk *et al.*, 2007). These data were in turn reinforced by characteristic five ^{13}C signals of each galloyl, fourteen signals of HHDP, and twenty one signals of the valoneoyl ester, respectively, especially those of carboxyl-carbonyl signals at about δ_C 165.22 ppm (galloyl), 166.99 ppm, and 166.64 ppm (HHDP), and 167.44 ppm, 167.20 ppm, and 167.10 ppm (valoneoyl). The glucose core was determined to adopt the same β-$B_{1,4}$-glucopyranose stereostructure in **5** and **6** and not β-$^1C_4$- or β-$^4C_1$-glucopyranose on the basis of δ values in ^{1}H and ^{13}C NMR spectra, splitting pattern and J values of vicinal ^{1}H-^{1}H-coupling (Okuda *et al.*, 1989; Zhang *et al.*, 2001; Amakura *et al.*, 1999). Also the β-$B_{1,4}$-pyranose structure was clearly documented because of its intrinsic $J_{1,2}$ value (> 7 Hz) and not 1C with $J_{1,2}$ of 2–3.5 Hz (Zhang *et al.*, 2001). This was clearly documented also from H-2 that appeared as br d including large $J_{ax\text{-}ax}$ ($J_{2,1}$ > 7 Hz) and small $J_{ax\text{-}eq}$ ($J_{2,3}$, not resolved). As well as H-3 was assigned as br s due to small $J_{3eq\text{-}2ax}$ and very small $J_{3eq\text{-}4eq}$ along with very narrow br s of H-4 due to dd of two very small J values ($J_{4eq\text{-}3eq}$ and $J_{4eq,5eq}$). The esterification of OH-1 with the galloyl moiety was derived from the typical downfield location of H-1 at δ_H 6.22 ppm (d). A similar downfield location of H-3 (br s), H-6a (dd), and H-6b (dd) at δ_H 4.62 ppm, 4.24 ppm, and 3.98 explained the bifunctional esterification of OH-3 and OH-6 with HHDP in the structure of **5**. Further confirmation for acylation of OH-1, OH-3, and OH-6 in both compounds was obtained from the characteristic α-downfield shift of C-1, C-3, and C-6 at δ_C 92.13 ppm, 77.31 ppm, and 63.86 ppm ($\Delta \sim +3$ ppm) and β-upfield shift of C-2 and C-4 at δ_C 71.50 ppm and 62.06 ppm (Okuda *et al.*, 1989; Zhang *et al.*, 2001). Since the corresponding δ_C and δ_H values of the glucose core are very close in both **5** and **6**, it was concluded that the valoneoyl and HHDP moieties esterify OH-3 and OH-6 in the same manner. A final proof for connectivity of the phenoyl moieties onto the glucose core was deduced from ^3J correlations between each of H-1, H-3 or H-6a and H-6b with the corresponding carbonyl carbon signal in HMBC. The assignment of all remaining ^{1}H and ^{13}C resonances was achieved by comparison with previously published data of structurally related compounds (Okuda *et al.*, 1989; Zhang *et al.*, 2001; Amakura *et al.*, 1999) and confirmed through net-correlation of ^1J, ^2J and ^3J cross peaks in ^{1}H-^{1}H-COSY, HMQC, and HMBC (Fig. 2). Thus, **5** and **6** were identified as 1-*O*-galloyl-3,6-(*S*)-hexahydroxydiphenoyl-D-$B_{1,4}$-glucopyranose and 1-*O*-galloyl-3,6-(*S*)-valoneoyl-D-$B_{1,4}$-glucopyranose (Fig. 1).

Compound **13** exhibited chromatographic properties of an acylated 3-*O*-glycosylkaempferol-like structure (Mabry *et al.*, 1970). The UV spectrum in MeOH showed a very strong broad absorption maximum at 267 nm characteristic for phenoyl masked to band II and 354 nm for band I of flavonol 3-*O*-glycoside. By adding NaOMe, a bathochromic shift of 46 nm in band I accompanied by a strong hypsochromic effect was an evidence for a free OH-4'. The remaining UV shift reagents showed characteristic effects for free OH-5 and OH-7 (Mabry *et al.*, 1970). Thus, the aglycone moiety was identified as 3-*O*-glycosylated kaempferol. Complete acid hydrolysis of **13** resulted in kaempferol and gallic acid in the organic and glucose in the aqueous phase, respectively. Moreover, negative ESI-MS gave a molecular ion peak at *m/z* 903 ([M – H]$^-$) and three consecutive fragments at *m/z* 751 ([M–galloyl]$^-$), 599 ([M–2·galloyl]$^-$), and 447 ([M–3·galloyl]$^-$) on a gradual increase of the collision-induced dissociation potential (CID) corresponding to the systematic loss of three galloyl residues. Thereafter, the highest CID led to a characteristic fragment at *m/z* 285 ([M–trigalloylglucoside]$^-$) for kaempferol by the loss of 162 mu. Accordingly, **13** was tentatively identified as 3-*O*-(trigalloylglucosyl)-kaempferol. The ^{1}H NMR spectrum showed three aromatic singlets each for 2H at δ_H 6.98 ppm, 6.94 ppm, and 6.93 ppm (3 x H-2/6), confirmative for three galloyl esters. An A_2X_2 spin coupling system was recorded at δ_H 7.93 ppm and 6.78 ppm in the form of two *ortho*-doublets (J = 9 Hz) for H-2'/6' and H-3'/5' typical for the 4'-hydroxy ring B. An AM coupling system of two *meta*-doublets (J = 2 Hz) was interpreted at δ_H 6.39 ppm and 6.19 ppm for H-8 and H-6 in the 5,7-dihydroxy

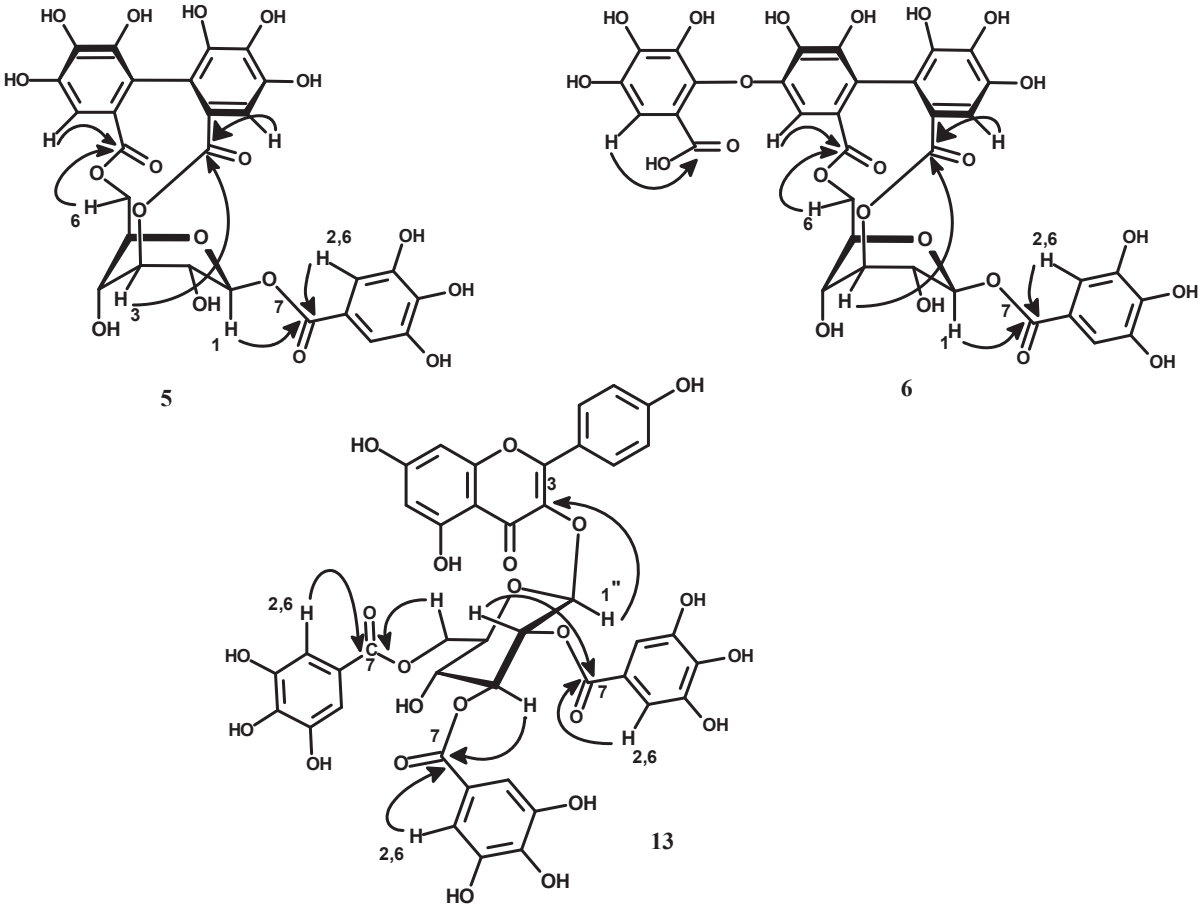


Fig. 2. Key HMBC correlations of compounds **5**, **6**, and **13**.

ring A. In the aliphatic region a β-anomeric proton doublet was located at δ_H 5.95 ppm (J = 8 Hz), whose downfield position (Δ + 0.5 ppm) is characteristic for acylation of the vicinal OH-2". Intrinsic downfield location (Δ + 1.5 ppm) of H-2" and H-3" at δ_H 5.22 ppm (dd) and 5.42 ppm (t-like) was indicative of the galloylation of OH-2" and OH-3" (Moharram *et al.*, 2006). The third galloyl ester was proven to be at OH-6" due to the characteristic downfield shift of its own diastereomeric CH_2-6" protons at δ_H 4.34 ppm and 4.27 ppm (Δ + 0.5 ppm). The stereostructure of glucose was established as β-$^4C_1$-glucopyranose depending on δ and J values of all glucose protons (Gupta *et al.*, 1982). The ^{13}C NMR spectrum showed 13 signals typical for a kaempferol aglycone including five key signals at δ_C 178.25 ppm (C-4), 133.70 ppm (C-3), 131.13 ppm (C-2'/6'), and 115.87 ppm (C-3'/5') (Agrawal and Bansal, 1989). Likewise, the characteristic five ^{13}C resonances of the galloyl moiety were repeated three times at about 165.0 ppm (3 x C-7), 145.5 ppm (3 x C-3/5), 138.5 ppm (3 x C-4), 119.0 ppm (3 x C-1), and 109.0 ppm (3 x C-2/6) (Gupta *et al.*, 1982; Moharram *et al.*, 2006). According to α/β down/upfield ^{13}C substituent additive rules of glycosidation (Agrawal and Bansal, 1989) and esterification (Okuda *et al.*, 1989), δ values of sugar signals, specially those of C-2", C-3", and C-6", were in complete accordance with a 3-*O*-glycoside and 2",3",6"-galloylation. The full assignment of all remaining ^1H and ^{13}C resonances was confirmed by comparison with previously published data (Marzouk *et al.*, 2007; Agrawal and Bansal, 1989; Moharram *et al.*, 2006) and 2D net correlation experiments (^1H-^1H, HMQC, and

HMBC, Fig. 2). Therefore, **13** was established as kaempferol 3-*O*-(2",3",6"-tri-*O*-galloyl)-*β*-D-glucopyranoside.

Antitumour activity

To our knowledge, this is the first report investigating antitumour and antioxidant activities of *E. cotinifolia* leaves and small branches. Using the MTT assay, the effect of CE, AME, some pure compounds, viz. **5**, **6**, **7**, **8**, **9**, and an equimolecular mixture of **11** and **12** on the proliferation of two tumour cell lines was studied in a 24-h treatment.

Based on their low IC_{50} values of 32.9 and 37.8 μg/ml, respectively, the gradual doses of **6** and **8** exhibited the highest inhibition of the cell growth of Hep-G2 cells as compared with untreated control cells (Figs. 3d, f). However, in case of CE, AME, **5**, **7**, **9**, and the mixture of **11** and **12**, only a moderate inhibitory effect was observed (Figs. 3a, b, c, e, g, h).

Concerning the effect on HCT-116 cells, AME and compounds **5** and **8** demonstrated high inhibition of the cell proliferation considering the low IC_{50} values of 31.3, 55.6, and 22.7 µg/ml, respectively (Figs. 4a, c, f). CE and the remaining compounds **6**, **7**, **9**, and the mixture of **11** and **12** (Figs. 4b, d, e, g, h) exhibited a moderate to low antitumour activity. The similar pattern of antioxidant, antitumour, and immunoproliferative activity of the compounds is likely due to the presence of the same phenoyl esters in their structures. It was previously found that the connection of a glucose core with galloyl and HHDP moieties in **5**, or valoneoyl in **6**, or a general glycoside with a flavonol aglycone in **7** and **8**, and again with a galloyl moiety in the mixture of **11** and **12** cooperatively produced inhibitory effects on poly(ADP-ribose) glycohydrolase purified from human placenta (Marzouk *et al*., 2007). Such polyphenols were found to act as potent inhibitors of induced histamine release from rat peritoneal mast cells (Kanoh *et al*., 2000) and as stimulators of the iodination of human peritoneal blood monocytes (Marzouk *et al*., 2007).

Antioxidant activity

AME proved to be a strong scavenger of DPPH radicals as indicated by the relatively low SC_{50} value (16.2 μg/ml, Fig. 5), which is almost 3-fold stronger than that of CE (47.5 μg/ml). This could be attributed to the high polyphenolic content (tannins and flavonoids) in AME relative to the trace amount detected in CE. All tested compounds were highly efficient in bleaching DPPH radicals as was obviously from their low SC_{50} values compared to that of the well-known antioxidant ascorbic acid (6.8 μM) (Marzouk *et al*., 2006, 2007), except for **9** which was a less potent scavenger (SC_{50} 22.5 μg/ml). Compounds **5**, **6**, the mixture of **11** and **12**, **7**, and **8** gave effective SC_{50} values as low as 7.4, 8.4, 9.2, 10.6, and 11.5 μg/ml, respectively (Fig. 5). In our findings, the strong antioxidant activity of tannins in pure (*i.e.* in **5**, **6**) or extract form (*i.e.* in AME) could be explained mainly by the presence of a large number of phenolic hydroxy groups in a large extended π-electron conjugation system in the galloyl, HHDP, or valoneoyl moiety, respectively. This property is the factor responsible for stabilization of phenoxide radicals and hence increases the scavenging capacity in the oxidation reaction (Marzouk *et al*., 2007). Based on this fact, **6** showed higher antioxidant capacity than **5** because of the presence of an extra galloyl moiety that extended the conjugation from HHDP into the valoneoyl moiety. Also, the higher antioxidant activity of the mixture of **11** and **12** as compared to **7** or **8** can be explained by the presence of a galloyl moiety in the structures of **11** and **12**. In the case of flavonoids, the strong antioxidant activity depends on their intrinsic structural features, *i.e*. again a large π-electron conjugation system, which extends over rings A and B through an α,β-unsaturated ketone group in ring C. The drop of antioxidant capacity of **9** relative to that of **7** or **8** could be attributed to its lacking 3'-OH and consequently the 3',4'-*ortho*-dihydroxy substituents. This function was reported to play an essential role in the stabilization of the aryloxy radical after H-donation by the +R-effect of hydroxy groups at the 3-, 3'-, and 4'-positions (Moharram *et al*., 2006). However, the mixture of **11** and **12** showed highest activity among all flavonoids because of the extra galloyl moiety with three *ortho*-hydroxy groups.

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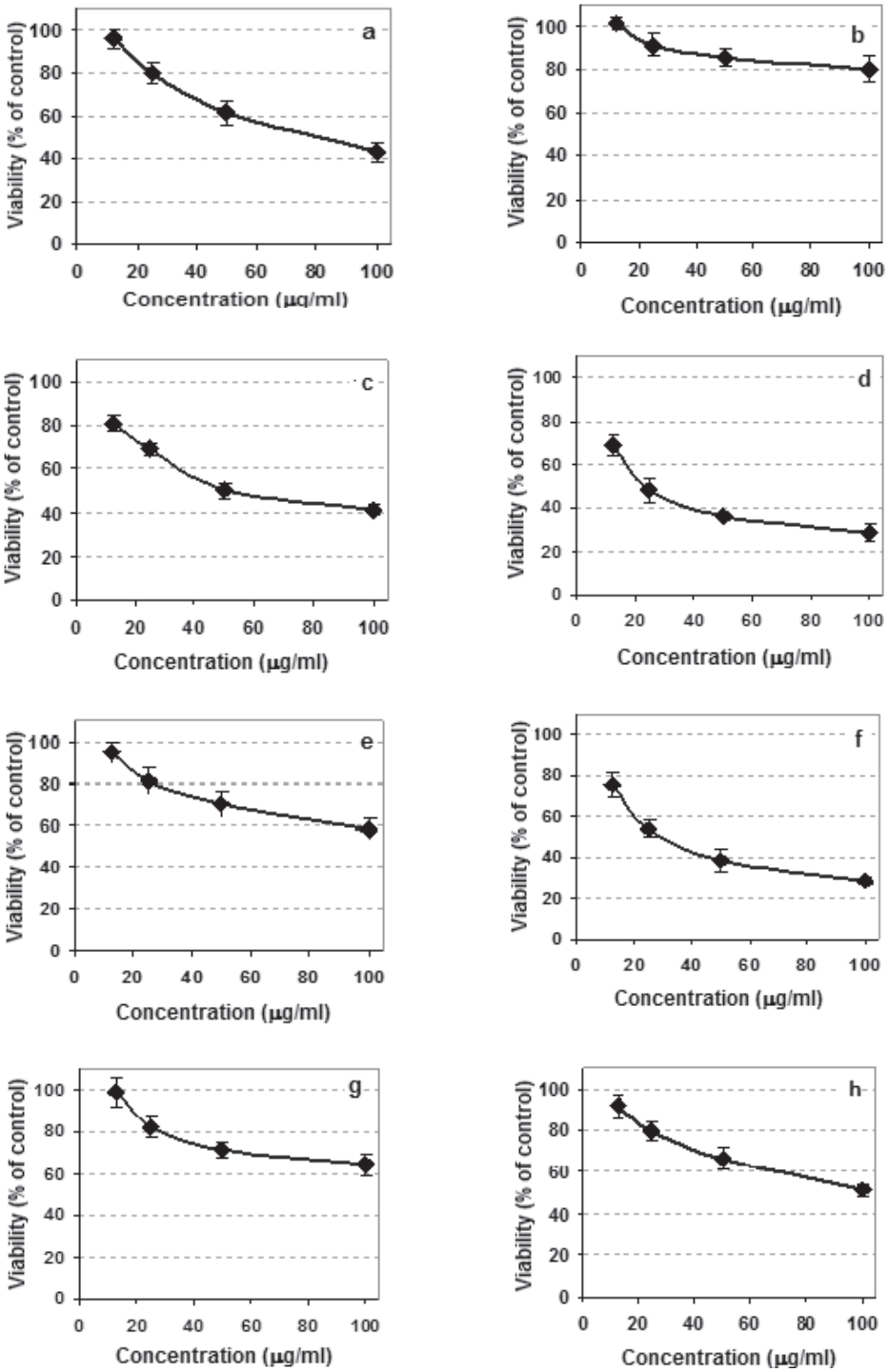

Fig. 3. Antitumour activity of AME, CE, and pure compounds from *E. cotinifolia* against hepatocellular carcinoma cells (Hep-G2): (a) AME (IC_{50} 81.52 μg/ml); (b) CE (IC_{50} 234.08 μg/ml); (c) **5** (IC_{50} 71.36 μg/ml); (d) **6** (IC_{50} 32.84 μg/ml); (e) **7** (IC_{50} 115.35 μg/ml); (f) **8** (IC_{50} 37.68 μg/ml); (g) **9** (IC_{50} 130.76 μg/ml); (h) mixture of **11** and **12** (IC_{50} 97.62 μg/ml).

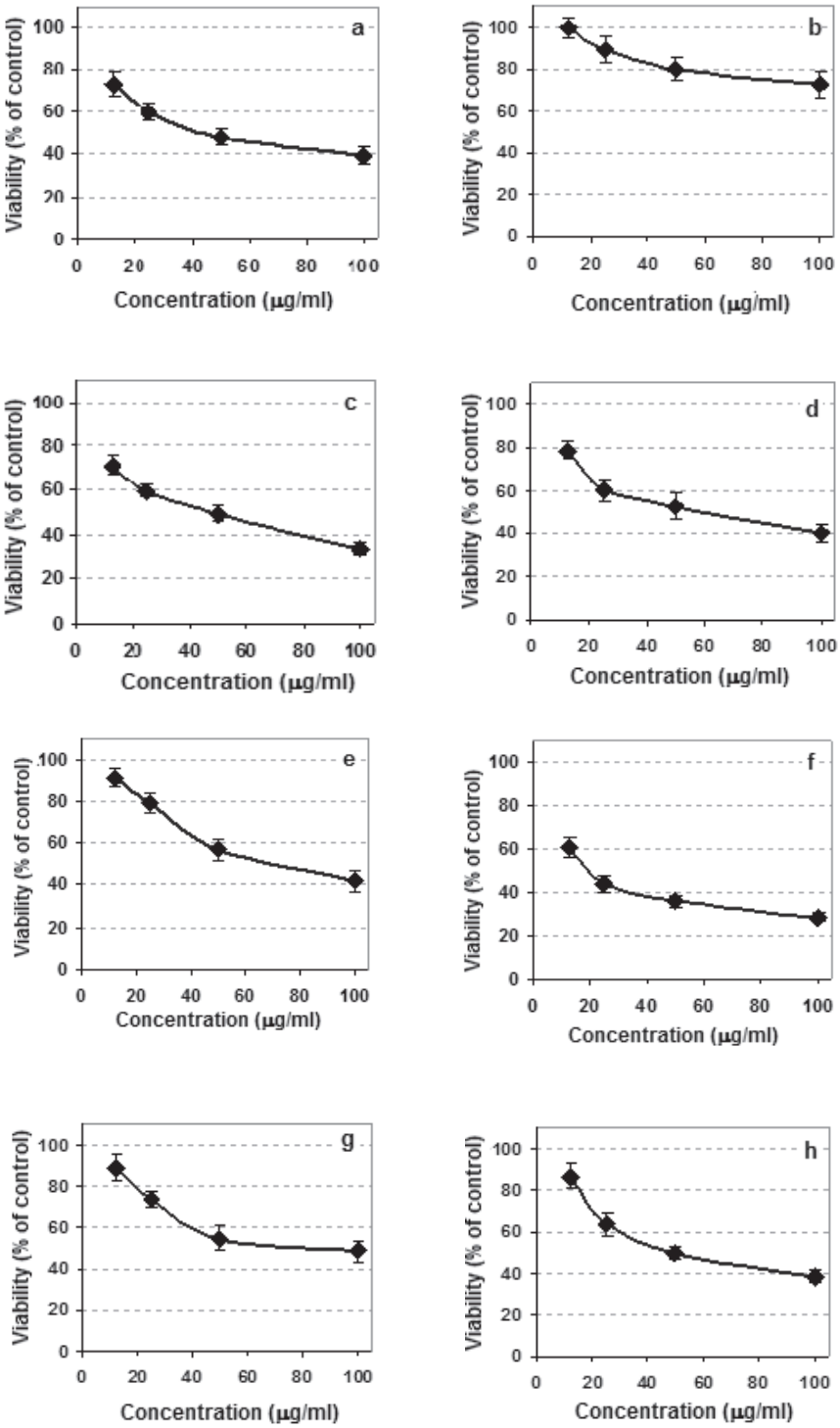

Fig. 4. Antitumour activity of AME, CE, and pure compounds from *E. cotinifolia* against colon carcinoma cells (HCT-116): (a) AME (IC_{50} 31.34 µg/ml); (b) CE (IC_{50} 169.32 µg/ml); (c) **5** (IC_{50} 55.56 µg/ml); (d) **6** (IC_{50} 68.1 µg/ml); (e) **7** (IC_{50} 77.97 µg/ml); (f) **8** (IC_{50} 22.65 µg/ml); (g) **9** (IC_{50} 85.27 µg/ml); (h) mixture of **11** and **12** (IC_{50} 66.65 µg/ml).

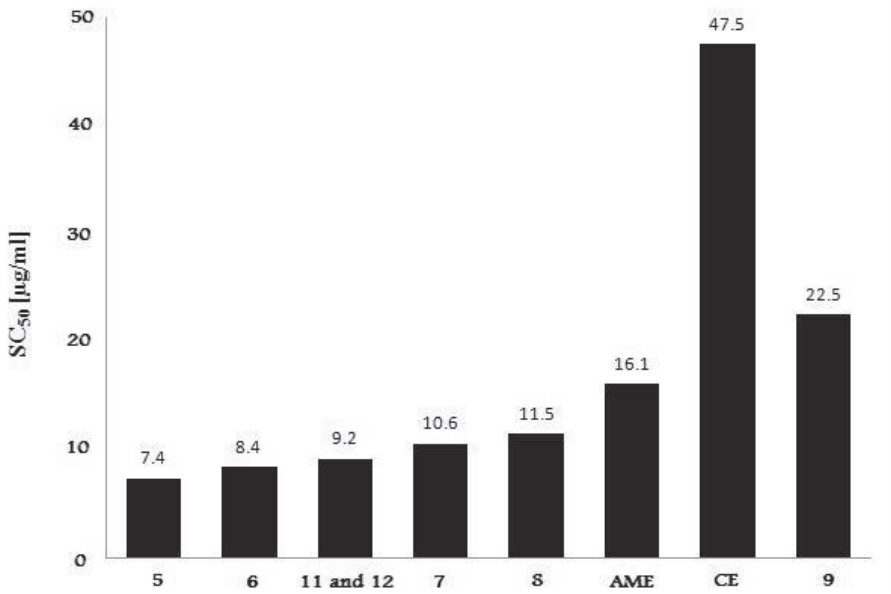

Fig. 5. Radical scavenging activities of AME, CE, and pure compounds from *E. cotinifolia* against DPPH radicals.

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