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Research Article

Isolation, Characterization and Anticancer Activity of Seven Compounds from the Aerial Parts of *Conyza triloba*

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ABSTRACT
The increase in cancer incidence and development of chemotherapy resistant tumors demands the incessant search for novel anticancer agents. Two benzofurans; (**1**) and (**7**), two diterpenes; (**2**) and (**3**), one sesquiterepene acid; (**4**), one pyranone derivative; (**5**), and a coumarin glucoside; (**6**) were isolated for the first time from the CH_2Cl_2/MeOH extract of *Conyza triloba*. The structures of these compounds were determined by spectroscopic techniques; IR, UV, GC-MS, and NMR analyses. Most of these compounds had peroxide and superoxide scavenging activities equal to or higher than those of trolox. Compounds 4 and 5 showed a distinguished total free radical scavenging activity higher than that of tocopherol. The antiproliferative activity of these compounds was tested in 5 cell lines. Breast cells were resilient and responded only to compound 2 which had a GI50 at 6 nM. Compounds 5 and 6 were the most effective against the rest of cell lines investigated (liver, colon, prostate, and lung) and had GI50 values at 5-26 nM. Compounds 4 and 7 had similar signatures affecting only colon and prostate at comparable concentrations. Most compounds had antiproliferative activity superior to that of actinomycin D. All compounds were safe; had LC50 at high concentrations. The efficacy of these phytochemicals could be attributed to their ability to induce apoptosis since most of these compounds significantly elevated the activities of caspases 3 and 9.

Keywords: *Conyza triloba*, Asteraceae, diterpenes, coumarin glucoside, anticancer, caspases.

INTRODUCTION

According to the American Cancer Society report released in 2016, about 1.7 million new cases of cancer are expected this year in addition to about 600,000 cancer deaths. This makes cancer the second leading cause of death after heart diseases. The most common cancers based on the tissue of origin in order are breast, prostate, lung, and colon. The increase in incidence in addition to the development of chemotherapy resistant tumors demands the continuous search for novel anticancer agents. However, most of the cytotoxic and/or cytostatic agents in use are causing severe side effects and sometimes causing cancer themselves. Therefore, we need novel safe and cheap anticancer agents. Many of the available anticancer agents were first isolated from plants; taxol, silymarin, vincristine, and etoposide are just few examples[1]. Using the crude extracts of plants is not always recommended due to the failure to identify the active components and the possible potential herb/drug interactions caused by the many ingredients in the extract. The current study was undertaken to isolate and identify some active ingredients from *Conyza triloba* (family Asteraceae) in addition to investigate the effect of these active ingredients against 5 cell lines representing the most common cancers worldwide. These cell lines are human breast MCF-7, human lung A549, human prostate PC-3, human colon HT-29, and murine hepatic Hepa1C1C7. This plant could be found in tropical and temperate regions throughout the globe. In our previous study, we have found that 5 extracts of this plant with different polarities exerted significant cytotoxic activities in the previously listed 5 cell lines[1]. The most significant extracts were number II (H_2O: MeOH), III (CH_2Cl_2), and VI (CH_2Cl_2: MeOH). Therefore, and in order to fulfill our goal of discovering new safe anticancer agents, the current study aimed to investigate the anti-proliferative effects of 7 compounds isolated from the (CH_2Cl_2: MeOH) extract of *Conyza triloba*.

MATERIALS AND METHODS

Chemicals

All reagents were purchased from Sigma (St. Louis, MO, USA) except where indicated in the specified methods. The aerial parts of *Conyza triloba* were collected from Dammam, district 71 (Saudi Arabia) in March 2011, and identified by a specialized taxonomist. A voucher specimen (CO-1-11) has been deposited in the Department of Biological Sciences, College of Science, King Faisal University, Saudi Arabia. The collected plant materials were stored in a dry and dark place at room temperature

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with passive ventilation for 2 weeks. The dried plant materials were ground to powder using a plant grinder.

Chemistry

^{1}H NMR (400 MHz, $CDCl_3$), ^{13}C NMR (100 MHz, $CDCl_3$) and the 2D spectra (^{1}H-^{1}H COSY, HMQC, HMBC and NOESY) were recorded on a Bruker 400 MHz spectrometer with TMS as an internal standard. Melting points: uncorrected values were recorded on a digital melting point Gallenkamp 5A 6797 apparatus. The IR spectra (KBr) were taken on a Shimadzu FT-IR 8400S spectrometer. UV spectra were recorded on a Shimadzu UV-240 spectrophotometer. Optical rotation was determined with a Kruss P 8000 digital polarimeter. Flash Chromatography (Cartridge 40 x 150 mm, prepacked with silica gel 60, 40 – 63 □m, Pump Modules C-605, Fraction Collector C-660, Control-Unit C-620 with Sepacore Control) Column chromatography was carried out on silica gel 60 (Merck; 230 - 400 mesh) and Sephadex LH-20 (Pharmacia Co., Tokyo, Japan). TLC: precoated silica gel $60F_{254}$ plates (Merck); preparative TLC: silica gel PF_{254} (Merck, 200 x 200 x 0.25 mm); CC: silica gel type 60 (Merck). GC-MS analysis was performed on a Shimadzu GCMS –QP2010 SE equipped with a Shimadzu auto injector/auto sampler AOC-20, split/splitless injector (300 ^{o}C) and flame ionization detector (250 ^{o}C), helium was used as the carrier gas (3ml/min) [1bar] and the capillary used was DB 5 (30 m x 0.25 mm i.d., film thickness 0.32 mm), with a column temperature was held at 100 ^{o}C for 2 min and then increasing to 250 ^{o}C, the compounds were identified by comparison with their mass spectra with the NIST 11.library.

Extraction of plant material

The air-dried aerial parts (1.2 kg) were powdered and extracted with CH_2Cl_2/MeOH (1:1) (10 L) at room temperature. The extract was concentrated *in vacuo* obtaining a residue of 90 g. The residue was pre-fractionated by CC (7 x 100 cm) on silica gel (800 g) eluted with *n*-hexane followed by CH_2Cl_2 and finally CH_2Cl_2/MeOH (85:15) (3 L each of the solvents) which yielded six fractions; fraction 1 (*n*-hexane 100 %), fraction 2 (*n*-hexane-CH_2Cl_2 3:1), fraction 3 (*n*-hexane- CH_2Cl_2 1:1), fraction 4 (*n*-hexane- CH_2Cl_2 1:3), fraction 5 (CH_2Cl_2 100 %), and fraction 6 (CH_2Cl_2-MeOH 3:1). Fraction 1 was subjected to silica gel column chromatography (400 g, 4 x 80 cm), eluted with *n*-hexane/Et_2O (9:1) and gave compounds **1 (**yellow crystals, 45 mg) and **7 (**colorless needles crystals, 50 mg)**.** Fractions 2 and 3 were collected and separated on a silica gel column (600 g, 5 x 100 cm) eluted with *n*-hexane-Et_2O (1:1) (*ca*. 1.0 L x 2) to give fractions 2-A and 2-B. Fraction 2-A was further purified on a Sephadex LH-20 column (350 g, 4 x 90 cm) eluted with *n*-hexane-CH_2Cl_2-MeOH (4 : 7 : 0.5) and gave compound **5** (colorless needles crystals, 40 mg) and impure **2** which was further purified by preparative TLC (silica gel PF_{254}), eluted with light petroleum-Et_2O (1:1) to give pure **2** (colorless oil, 15 mg). Fraction 5 was separated on a silica gel column (400 g, 5 x 100 cm) eluted with *n*-hexane-CH_2Cl_2 (5:1) (*ca*. 1.0 L x 3) to give fractions 5-A, 5-B and 5-C. Fraction 5-A was further purified on a Buchi flash chromatography (eluent: *n*-hexane-CH_2Cl_2 1:3 and flow rate: 20 ml/min) to give compound **3** (colorless needles crystals, 50 mg) and compound **4** (18 mg) which was further purified by preparative TLC (silica gel PF_{254}), eluted with light petroleum-Et_2O-MeOH (1:4:0.1). Fraction 6 was separated on a silica gel column (400 g, 5 x 100 cm) eluted with CH_2Cl_2-MeOH(3:1) (*ca*. 1.0 L x 2) to give fractions 6-A and 6-B. Fraction 6-B was further purified on a Buchi flash chromatography (eluent: CH_2Cl_2-MeOH 2:1 and flow rate: 20 ml/min) to give compound **6** (white needles 40 mg).

Characterization of the isolated compounds (1-7)

The ^{1}H NMR and ^{13}C NMR signals of all compounds **1-7** (Figure 1) could be unequivocally assigned on the basis of ^{1}H^{1}H homocorrelation experiments (COSY, NOESY), ^{1}H^{13}C heterocorrelation experiments (COSY, HMQC, and HMBC). The multiplicities of all carbon signals were assigned on the basis of DEPT-45, -90 and -135 experiments and ^{1}H-^{13}C COSY (HMQC) analysis.

***Euparin* (1)**. Yellow crystals; m.p. 118-120 ^{o}C; UV λ_{max} (nm) MeOH: 260, 289, 300 sh, 352; IR (KBr), □$_{max}$ (cm^{-1}): 3456 (OH), 1465 (aromatic), 1651 (C=COR);^{1}H NMR ($CDCl_3$, 400 MHz): □ 7.87 (1H, *s*, H-4), 6.96 (1H, *s*, H-7), 6.52 (1H, *s*, H-3), 5.74 (1H, *br s*, H-13a), 5.18 (1H, *br s*, H-13b), 2.66 (3H, *s*, CH_3-11), 2.09 (3H, *s*, CH_3-14), 12.50 (1H, *s*, 6-OH); ^{13}C NMR ($CDCl_3$, 100 MHz): □ 203.9 (C=O, C-10), 161.5 (*C*, C-8,), 159.6 (*C*, C-9), 157.8 (*C*, C-2), 132.1 (*C*, C-12), 123.4 (*CH*, C-4), 121.8 (*C*, C-6), 116.8 (*C*, C-5), 113.6 (CH_2, C-13), 102.4 (*CH*, C-3), 99.3 (*CH*, C-7), 26.7 (CH_3, C-11), 19.1 (CH_3, C-14); GC-MS (*m/z*, rel. int.): 216 (100 %) [M^+, $C_{13}H_{12}O_3$], 201 (55), 173 (10), 115 (6).

Centipedic acid (2). Colorless oil; IR (KBr), □$_{max}$ (cm^{-1}): 3450 (OH), 1680 (COOH);^{1}H NMR ($CDCl_3$, 400 MHz): □ 7.32 (1H, *br s*, H-16), 7.22 (1H, *br s*, H-17), 6.28 (1H, *br s*, H-15), 6.00 (1H, *br s*, H-7), 5.19 (1H, *br s*, H-3), 5.10 (1H, *br s*, H-11), 2.62 (2H, *m*, H-8), 2.42 (2H, *br m*, H-13), 2.15 (2H, *m*, H-12), 2.15 (2H, *m*, H-9), 2.25 (2H, *m*, H-4), 2.25 (2H, *m*, H-5), 1.65 (3H, *br s*, CH_3-1), 1.55 (3H, *br s*, CH_3-18), 1.55 (3H, *br s*, CH_3-20); ^{13}C NMR ($CDCl_3$, 100 MHz): □ 173.5 (*C=O*, C-19), 145.6 (*CH*, C-7), 142.5 (*CH*, C-16), 138.8 (*CH*, C-17), 134.9 (*C*, C-10), 132.2 (*C*, C-2), 130.6 (*C*, C-6), 124.9 (*C*, C-14), 124.5 (*CH*, C-3), 123.4 (*CH*, C-11), 111.0 (*CH*, C-15), 39.0 (CH_2, C-9), 34.5 (CH_2, C-5), 28.1 (CH_2, C-4), 28.1 (CH_2, C-8), 25.6 (CH_3, C-1), 24.7 (CH_2, C-12), 17.7 (CH_3, C-20), 15.9 (CH_3, C-18); GC-MS (*m/z*, rel. int.): 316 (95 %) [M^+, $C_{20}H_{28}O_3$], 203 (100), 185 (26), 175 (68), 160 (35), 105 (20).

Tarapacol (3). Colorless crystals; m.p. 79-80 ^{o}C; [□$_D$] = +25^o (*c* 2.0, CH_2Cl_2); IR (KBr), □$_{max}$ (cm^{-1}): 3425 (OH), 2990 (CH);^{1}H NMR ($CDCl_3$, 400 MHz): □ 3.72 (1H, *br d*, *J*= 12.0, 3.0 Hz, H-15a), 3.61 (1H, *br dd*, *J* = 12.0, 8.5, H-15b), 3.51 (1H, *br dd*, *J* = 8.5, 3.0 Hz, H-14), 2.10 (1H, *br s*, H-12), 1.78 (2H, *m*, H-7), 1.70, 1.25 (each 1H, *m*, H-6), 1.70, 1.40 (each 1H, *m*, H-2), 1.65, 1.45 (each 1H, *m*, H-11), 1.58 (2H, *m*, H-1), 1.47 (1H, *m*, H-5), 1.40 (2H, *m*, H-3), 1.29 (3H, *s*, CH_3-17), 1.12 (3H, *s*, CH_3-16), 0.95 (1H, m, 9), 0.86 (3H, *s*, CH_3-18), 0.78 (3H, *s*, CH_3-20), 0.78 (3H, *s*, CH_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz): □ 76.0 (*CH*, C-14), 75.7 (*C*, C-8), 75.4 (*C*, C-13), 63.5 (CH_2, C-15), 56.5 (*CH*, C-9), 53.8 (*CH*, C-5), 43.7 (CH_2, C-7), 42.0

(*CH_2*, C-3), 38.9 (*CH_2*, C-1), 37.1 (C, C-10), 33.3 (*CH_3*, C-18), 33.1 (*C*, C-4), 31.5 (*CH_2*, C-12), 25.1 (*CH_3*, C-17), 25.1 (*CH_3*, C-16), 21.9 (*CH_3*, C-19), 20.0 (*CH_2*, C-6), 18.4 (*CH_2*, C-2), 15.1 (*CH_3*, C-20), 14.4 (*CH_2*, C-11); GC-MS (*m/z*, rel. int.): 309 (10 %) [(M-CH_3)$^+$, (324, $C_{20}H_{36}O_3$)-Me]$^+$, 263 (15), 245 (100), 191 (19), 137 (60), 109 (60), 95 (68), 81 (72), 55 (43).

15-Hydroxy-eudesm-4,11(13)-diene-12-oic acid (4). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz): □ 6.30 (1H, *s*, H-13a), 5.62 (1H, *s*,, H-13b), 4.12 (1H, *d*, *J* = 12.5 Hz, H-15b), 4.06 (1H, *d*, *J* = 12.5 Hz, H-15a), 2.76 (1H, dd, *J* = 6.5, 2.0 Hz, H-6a), 2.40 (1H, *m*, H-7), 2.20-1.30 (11 H, *m*, H-1, H-2, H-3, H-6b, H-8, H-9), 1.05 (3H, *s*, CH_3-14); ^{13}C NMR ($CDCl_3$, 100 MHz): □ 171.3. (*C=O*, C-12), 144.8 (*C*, C-11), 140.5 (*C*, C-5), 129.0 (*C*, C-4), 112.4 (*CH_2*, C-13); 62.9 (*CH_2*, C-15), 41.7 (*CH_2*, C-9), 41.2 (*CH*, C-7), 40.0 (*CH_2*, C-1), 35.0 (*C*, C-10), 32.0 (*CH_2*, C-6), 29.9 (*CH_2*, C-8), 27.2 (*CH_2*, C-3), 24.8 (*CH_3*, C-14), 19.0 (*CH_3*, C-2).

Pyromeconic acid (5). Off white plates, m.p. 117-119 ºC; UV λ $_{max}$ (nm) MeOH: 210, 271; IR (KBr), □$_{max}$ (cm^{-1}): 3109 (OH), 1645 (C=O);^{1}H NMR ($CDCl_3$, 400 MHz): □ 7.90 (1H, *s*, H-2), 7.80 (1H, *d*, *J* = 5.5 Hz, H-6), 6.51 (1H, *d*, *J* = 5.5 Hz, H-5); ^{13}C NMR ($CDCl_3$, 100 MHz): □ 173.7 (C=O, C-4), 155.4 (*CH*, C-6), 146.8 (*C*, C-3), 138.9 (*CH*, C-2), 113.7 (*CH*, C-5); GC-MS (*m/z*, rel. int.): 112 (100 %) [M$^+$, $C_5H_4O_3$], 64 (9), 71 (12), 69 (13), 55 (17), 42 (12).

5-methylcoumarin-4-O-□-D-glucoside (6). White needles crystals; UV λ $_{max}$ (nm) MeOH: 208, 275, 287; IR (KBr), □$_{max}$ (cm^{-1}): 3440 (OH), 1681 (C=O);^{1}H NMR (CD_3OD, 400 MHz): □ 7.42 (1H, *t*, *J* = 8.0 Hz, H-7), 7.20 (1H, *d*, *J* = 8.0 Hz, H-8), 7.10 (1H, *d*, *J* = 8.0 Hz, H-6), 5.15 (1H, *d*, *J* = 8.5 Hz, H-1'), 3.95 (1H, *m*, H-6'a), 3.82 (1H, *m*, H-6'b), 3.70 (1H, H-4'), 3.55 (2H, *m*, H-2', H-5'), 3.48 (1H, *m*, H-3'); ^{13}C NMR ($CDCl_3$, 100 MHz): □ 169.1 (*C=O*, C-2), 165.2 (*C*, C-4), 156.0 (*C*, C-8a), 139.0 (*C*, C-5), 133.2 (*CH*, C-7); 129.4 (*CH*, C-6), 116.4 (*CH*, C-8), 115.5 (*C*, C-4a), 101.3 (*CH*, C-1'), 94.3 (*CH*, C-3), 78.8 (*C*, C-2'), 78.2 (*CH*, C-3'),78.3(C-5'), 74.3 (*CH*, C-4'), 62.5 (*CH*, C-6'), 24.5 (*CH_3*, C-9). GC-MS (*m/z*, rel. int.): 338 (5 %) [M$^+$, $C_{16}H_{18}O_8$], 176 (48), 134 (100), 106 (35), 78 (20), 51 (10).

(*R*)-6-*Hydroxytremetone (7)*. Colorless crystals, m.p. 67-68 ºC; [□$_D$] = -35º (*c* 1.0, MeOH; UV λ$_{max}$ (nm) MeOH: 219, 240, 278, 330 nm 225; IR (KBr), □$_{max}$ (cm^{-1}): 3440 (OH), 1465 (aromatic), 1651 (C=COR);^{1}H NMR ($CDCl_3$, 400 MHz): □ 7.50 (1H, *s*, H-4), 6.36 (1H, *s*, H-7), 5.23 (1H, *dd*, *J* = 8.0, 9.5Hz, H-2), 5.07 (1H, *br s*, H-13a), 4.93 (1H, *br s*, H-13b), 3.30 (1H, *dd*, *J* = 15.5, 8.0 Hz, H-3a), 2.96 (1H, *dd*, *J* = 15.5, 9.5 Hz, H-3b), 2.53 (3H, *s*, CH_3-11), 1.75 (3H, *s*, CH_3-14), 12.50 (1H, *s*, 6-OH); ^{13}C NMR ($CDCl_3$, 100 MHz): □ 201.9 (*C*, C-10), 166.6 (*C*, C-8), 165.7 (*C*, C-6), 143.1 (*C*, C-12), 126.6 (*CH*, C-4), 118.6 (*CH*, C-9), 113.7 (*CH*, C-5), 112.7 (*CH_2*, C-13), 98.0 (*CH*, C-7), 87.6 (*CH*, C-2), 33.1 (*CH_2*, C-3), 26.2 (*CH_3*, C-11), 17.0 (*CH_3*, C-14); GC-MS (*m/z*, rel. int.): 218 (95 %) [M$^+$, $C_{13}H_{14}O_3$], 203 (100), 185 (26), 175 (68), 160 (35), 105 (20).

Biology

Cell cultures

The cell lines used were hepatic mouse Hepa1C1C7, and human colon HT29, breast MCF7, lung A549, and prostate PC3. All cells, media, fetal bovine serum, DMSO, and trypsin-EDTA were obtained from ATCC (Manassas, VA, USA). Cell lines were seeded in 75-cm^2 tissue culture flasks at 37ºC in a humidified atmosphere (5% CO_2) and the medium was renewed every 2-3 days.

Peroxide scavenging activity

Peroxide scavenging activity was measured as described elsewhere [2]. Peroxide radicals were generated from mixing $FeSO_4$ and H_2O_2. The reaction mixture contained 1 ml $FeSO_4$ (1.5 mM), 0.7 ml H_2O_2 (6 mM), 0.3 ml sodium salicylate (20 mM) and either the pure compound (1-7) or trolox (100 µg/ml). The mix was incubated for 1 h at 37°C and the absorbance of the hydroxylated salicylate formed was measured at 562 nm.

The peroxide scavenging activity (%)

= [1 - (A_1 - A_2)/A_o] x 100

where A_0 is the absorbance of the control (without the investigated compound or trolox) and A_1 is the absorbance including the investigated compound or trolox, and A_2 is the absorbance without sodium salicylate.

Superoxide anion scavenging activity

Superoxide anion scavenging activity of the pure compounds was determined as described elsewhere[3]. Superoxide radicals were generated in phenazine methosulfate (PMS)-nicotinamide adenine dinucleotide (NADH) systems and assayed by the reduction of nitroblue tetrazolium (NBT). One milliliter of the investigated compound or trolox (100 µg/ml), 1.0 ml NBT solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4) and 1.0 ml NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) were mixed. The reaction was started by adding 100 µl of PMS solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The mixture was incubated at 25°C for 5 min, and the absorbance was measured at 560 nm against blank samples. The inhibition percentage of superoxide anion generation was calculated using the following formula:

Inhibition of superoxide anion (%) = [(A_0 - A_1)/A_0 x 100] where A_0 is the absorbance of control, and A_1 is the absorbance in presence of either the investigated compound or trolox.

Free radical scavenging activity

The free radical scavenging activity of *Conyza* pure compounds was evaluated using 2,2-diphenyl-1-picryhydrazyl (DPPH$^·$)[4]. A 0.5 ml of 0.1 mM ethanolic solution of DPPH$^·$ was added to the investigated compound or α-tocopherol (0-600 µg/ml). The absorbance was measured at 517 nm after incubation for 30 min at room temperature.

DPPH$^·$ scavenging effect (%) = [(A_0 -A_1)/A_0 x 100] where A_0 is the absorbance of control and A_1 is the absorbance in presence of either the investigated compound or α-tocopherol.

Determination of in vitro anticancer activity

The anticancer activity was determined as described elsewhere[1]. The cell lines were grown in the suitable medium. The cells were inoculated into 96 well plates at plating densities of ~ 5,000 cells/well, four wells were used

for each treatment. Three independent experiments were performed. The plates were then incubated at 37°C and 5% CO_2 for 24 h prior to addition of pure compounds. After 24 h, two plates of each cell line were fixed with trichloroacetic acid (TCA), to measure the cell population for each cell line at the time of addition (T0) of pure isolated compounds or actinomycin D. Aliquots of 100 µl of different compounds dilutions in DMSO vehicle (0.1%, final concentration that does not affect cell viability) were sterilized by filtration and added to the appropriate wells of fresh plates. The plates were further incubated for an additional 48 h. The assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the addition of 50 µl of cold 50 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded, and the plates were washed three times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each well, and plates were incubated for 10 minutes at room temperature. After staining, unbound dye was removed by washing three times with 1 % acetic acid and the plates were air dried. Bound stain is then solubilized with 10 mM trizma base, and the absorbance was read at a wavelength of 515 nm.

Percentage growth inhibition was calculated using the following formula:

[(T-T0)/(C-T0)] x 100 for concentrations for which T ≥ T0

[(T-T0)/T0] x 100 for concentrations for which T < T0.

T0 or time zero represents a measurement of the cell population for each cell line at the time of addition of the investigated compounds, C is the control growth, and T is the test growth at different concentrations of *Conyza* pure compounds or actinomycin D after incubation. Three dose response parameters were calculated for each experimental agent. Growth inhibition of 50 % (GI50) was calculated from [(T-T0)/(C-T0)] x 100 = 50, which is the investigated compound concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the incubation. The pure compound or actinomycin D concentration resulting in total growth inhibition (TGI) is calculated from Ti = Tz. The LC50 (concentration of *Conyza* pure compound or actinomycin D resulting in a 50% reduction in the measured protein at the end of the treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti-Tz)/Tz] x 100 = -50.

Measurement of activities of caspases 3 and 9

The activity of caspase 3 was measured using kit from Sigma (St. Louis, MO, USA) and that of caspase 9 using kit from Millipore (MA, USA) as indicated by the manufacturer. Cells were treated with the pure compounds or actinomycin D at 10 nM for 6 h.

Statistical analysis of data

Statistical analyses were performed using ANOVA, followed by Fisher's protected least significant difference multiple range test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Consumption of vegetables and fruits is known to inversely correlate with cancer incidence. This is due to the many antioxidant and anticancer agents in these foods. However, it is somewhat different when it comes to antitumor therapy where we need higher concentrations and controlled administration of specific pure anticancer agents.

In the present work, our interest in the search for natural safe anticancer compounds led us to isolate 7 compounds for the first time through repetitive chromatographic separation of the CH_2Cl_2/MeOH (1:1) extract of the aerial parts of *C. triloba*. These compounds were identified based on spectroscopic techniques including, IR, UV, GC-MS, and extensive 400 MHz 1D- and 2D- NMR analyses (^{1}H, ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments) as euparin (**C1**)[5], centipedic acid (C**2**)[6], tarapacol (**C3**)[7], 15-hydroxy-eudesm-4,11(13)-diene-12-oic acid **(C4)**[8], pyromeconic acid **(C5)**[9], and 5-methylcoumarin-4-*O*-□-D-glucoside **(C6)**[10,11], and 6-hydroxytremetone (**C7**)[12]. These pure compounds isolated from *Conyza triloba* could be classified according to their ability of peroxide and superoxide scavenging activities into three categories. The first category, compounds with scavenging activity similar to that of trolox, these were compounds 1 and 7 for peroxide, and 1 for superoxide scavenging activities (Table 1). The second category includes those with scavenging activities lower than trolox. These include compound 7 only for the superoxide radical. The rest of pure compounds (2-6) showed a remarkable scavenging activity against both radicals investigated and largely higher than those of trolox (Table 1). Compounds 4 and 5 showed a distinguished total free radical scavenging activity profile higher than that of tocopherol at all concentrations investigated (Figure 2). Compound 2 was almost similar to tocopherol while compound 1 was at the bottom and had the lowest activity (Figure 2). Since many genotoxic and epigenetic (non-genotoxic) carcinogens cause cancer and growth of neoplasms through elevation of oxidative stress and challenging the antioxidant milieu of cells, antioxidants and free radical scavenging phytochemicals could be of value in fighting against cancer[13]. Noteworthy, the correlation between antioxidant/ scavenging activities and antiproliferative activity where compounds 1 and 7 were the least effective antioxidant and anticancer agents.

As for the antiproliferative activity of the pure compounds isolated from *Conyza* against the 5 cell lines investigated (colon; HT29, lung; A549, prostate; PC3, breast; MCF7, and liver; Hepa1C1C7), the compounds investigated were highly efficient and caused a 50% growth inhibition (GI50) at low concentrations (nM). Breast cells were resilient and needed higher concentration (µM). Compound 2 was the most effective against MCF7 cells with GI50 of ~ 6nM. It was more potent than actinomycin D which had GI50 at 600 nM. The rest of compounds had their GI50 against breast cells at concentration ranging from ~ 6 to > 100 µM. The weakest activity against the breast cells was shown by compounds 5 and 6 (Table 2). Taxol which is the most used drug against breast cancer is derived from a diterpene[14]; the same class compound 2 belongs to. With the exception

Table 1: Peroxide and superoxide scavenging activities of seven pure compounds isolated from *Conyza triloba*.

Compound	Peroxide scavenging activity (%)[a,b]	Superoxide scavenging activity (%)[a,b]
1	35.7 ± 4.2	58.1 ± 4.6
2	100.1 ± 6.4	90.3 ± 6.8
3	78.1 ± 5.5	96.7 ± 6.0
4	94.3 ± 6.0	99.3 ± 7.2
5	69.3 ± 4.8	86.7 ± 4.3
6	51.7 ± 2.9	93.1 ± 7.1
7	37.3 ± 3.9	11.3 ± 1.9
Trolox	31.3 ± 2.5	58.0 ± 4.9

[a] The data are expressed as means ± SEM, n = 3.
[b] The activity is expressed as equivalent of trolox (µg trolox)

of their effects on breast cells, compounds 5 and 6 were the most effective against all cell lines investigated having GI50 at low concentrations (5-6 nM) against other human cells, and 10-26 nM against murine liver cells. In comparison, actinomycin D which had GI50 against all human cell lines at double that concentration (~10-12 nM), and at ~ 88 nM against murine cells. Away from this broad anticancer activity of compounds 5 and 6, the other compounds were selective in their inhibitory effects, for example, compound 2 was most effective against colon, liver, and breast cells at ~ 6-8 nM and compounds 4 and 7 against colon and prostate (GI50 at ~ 6-7 nM). A synthetic compound very similar to compound 7 had similar results in colon and prostate cells through inhibiting aurora B kinase[15]. The activity of compound 3 was moderate while the activity of compound 1 was the weakest among all isolated compounds against all cell lines (Table 2).

Similar results were obtained for the total growth inhibition (TGI) of the cancer cells over the course of the assay (Table 3). MCF7 cells were very resilient to all compounds except compound 2 which had a TGI at 60 nM. Compound 1 was weaker than the other compounds and needed higher concentrations to achieve the TGI (Table 3). TGI values for compounds (2-7) were 12-92 nM for colon cells (82 nM for actinomycin D); 34-1880 nM (57 nM for actinomycin D) for lung cells but compound 2 had a high value of 10 µM; 10-862 nM for prostate cells (71 nM for actinomycin D) and 62-1200 nM (921 nM for actinomycin D) for murine hepatic cells. Similarly, compounds 5 and 6 were the most effective achieving their TGI activity at low concentrations of 12, 34, and 10 nM (for compound 5), and 28, 40, and 10 nM (for compound 6) against human colon, lung, and prostate cells, respectively (Table 3). Interestingly, the behavior of these compounds was different against the murine hepatic cells against which the most effective were compounds 2 and 3 with TGI values of 62 and 64 nM, respectively. The possibility of metabolic activation of these compounds by hepatic enzymes expressed by Hepa1C1C7 cells cannot be ruled out. Compound 5 was also as effective with a TGI at a concentration of 76 nM (Table 3).

It is important for the pure compounds to exert their anticancer / antiproliferative activity in a concentration away from toxic concentration. Measuring the lethal concentration 50 (LC50; concentration of the compound that killed 50% of the cells present at the beginning of the experiment is then of great value (Table 4)). In general, all compounds were safe causing the LC50 of the cells at high concentrations. Apparently only compounds 5 and 6 and only against colon cells, had low LC50 (0.8-1.0 µM) versus 2.1 for actinomycin D, and compound 6 against liver cells (LC50 1.0 µM) versus 2.2 for actinomycin D. However, deep analyses of the numbers would show that compound 5 and 6 are still safe and exerted their anticancer activity (TGI) at concentrations far away from their LC50 against colon cells. The LC50 values for compounds 5 and 6 were 83x and 29x concentration required to achieve TGI against HT29 (Tables 3 and 4). On contrast, the LC50 for actinomycin D was ~26x the concentration required to achieve TGI against these cells. Out of seven pure isolated compounds from *Conyza*, only compound 6 was slightly toxic and only to the murine hepatic cells Hepa1C1C7 but still safer than actinomycin D (Table 4). These compounds belong to chemicals classes that were shown to have significant anticancer activities; diterpenes[16], sesquiterepene acid[17], pyranone derivatives[18], coumarin glucoside[19], and benzofuran derivatives[20].

Examining the apoptotic pathways is one of the crucial mechanisms through which almost all anticancer drugs should be piped onto. The aspartic acid protease caspase-9 has been linked to the intrinsic (mitochondrial) death

Table 2: The 50% growth inhibition (GI50) data of seven isolated pure compounds from *Conyza triloba* in cells lines.

Cpd	Potency of isolated compounds (nM) in cell lines (mean ± SEM), n = 5				
	HT29	A549	PC3	MCF7*	Hepa1C1C7
Actinomycin D	10.1 ± 1.2	11.0 ± 0.8	12.2 ± 1.0	0.6 ± 0.1	87.7 ± 6.9
1	9800 ± 780	281.8 ± 24.1	63.6 ± 5.5	10.0 ± 1.1	273.2 ± 30.4
2	7.2 ± 0.3	186.4 ± 13.9	63.6 ± 7.1	0.006 ± 0.0	8.4 ± 1.0
3	22.2 ± 1.1	49.0 ± 3.5	22.8 ± 2.3	6.2 ± 0.5	18.6 ± 1.2
4	5.8 ± 0.2	69.6 ± 7.1	5.8 ± 0.2	6.8 ± 0.4	90.6 ± 6.7
5	5.6 ± 0.6	6.0 ± 0.3	5.6 ± 0.4	> 100 [a]	26.4 ± 1.6
6	6.2 ± 0.9	6.0 ± 0.4	5.2 ± 0.5	> 100 [a]	10.0 ± 1.5
7	7.0 ± 0.9	61.8 ± 5.1	7.2 ± 0.8	70.6 ± 8.7	61.8 ± 7.7

[a] extrapolated from dose-response curve. HT29 (human colon), A549 (human lung), PC3 (human prostate), * MCF7 (human breast, concentration is expressed in µM), Hepa1C1C7 (murine liver). GI50 is the concentration of a compound that causes 50% growth inhibition.

Table 3: The total growth inhibition (TGI) data of seven isolated pure compounds from *Conyza triloba* in cells lines.

Cpd	Potency of isolated compounds (µM) in cell lines (mean ± SEM), n = 5				
	HT29	A549	PC3	MCF7	Hepa1C1C7
Actinomycin D	0.082 ± 0.01	0.057 ± 0.01	0.071 ± 0.01	11.211 ± 0.12	0.921 ± 0.11
1	> 100 [a]	7.980 ± 0.90	0.966 ± 0.11	> 100 [a]	4.880 ± 0.20
2	0.058 ± 0.01	10.000 ± 0.80	0.862 ± 0.10	0.060 ± 0.01	0.062 ± 0.01
3	0.082 ± 0.01	1.880 ± 0.20	0.078 ± 0.05	> 100 [a]	0.064 ± 0.01
4	0.092 ± 0.01	0.758 ± 0.11	0.036 ± 0.01	> 100 [a]	0.810 ± 0.10
5	0.012 ± 0.01	0.034 ± 0.01	0.010 ± 0.00	> 100 [a]	0.076 ± 0.01
6	0.028 ± 0.01	0.040 ± 0.01	0.010 ± 0.00	> 100 [a]	0.248 ± 0.01
7	0.054 ± 0.01	1.020 ± 0.20	0.054 ± 0.01	> 100 [a]	1.202 ± 0.12

[a] extrapolated from dose-response curve. HT29 (human colon), A549 (human lung), PC3 (human prostate), MCF7 (human breast), Hepa1C1C7 (murine liver). TGI is the concentration of a compound (µM) that yields no net growth over the course of the assay

Table 4: The 50% lethality (LC50) data of seven isolated pure compounds from *Conyza triloba* in cells lines.

Cpd	Potency of isolated compounds (µM) in cell lines (mean ± SEM), n = 5				
	HT29	A549	PC3	MCF7	Hepa1C1C7
Actinomycin D	2.1 ± 0.3	3.5 ± 0.5	6.7 ± 0.8	14.3 ± 2.1	2.2 ± 0.4
1	> 100 [a]	> 100 [a]	> 100 [a]	> 100 [a]	> 100 [a]
2	27.4 ± 1.8	> 100 [a]	> 100 [a]	25.6 ± 2.9	10.0 ± 1.2
3	15.2 ± 1.6	> 100 [a]	55.0 ± 4.8	> 100 [a]	10.0 ± 0.5
4	10.2 ± 0.8	> 100 [a]	9.8 ± 1.5	> 100 [a]	> 100 [a]
5	1.0 ± 0.2	10.0 ± 0.8	44.6 ± 5.1	> 100 [a]	10.2 ± 0.7
6	0.8 ± 0.1	10.0 ± 1.1	65.4 ± 5.7	> 100 [a]	1.0 ± 0.1
7	9.8 ± 1.4	> 100 [a]	30.8 ± 4.0	> 100 [a]	> 100 [a]

[a] extrapolated from dose-response curve. HT29 (human colon), A549 (human lung), PC3 (human prostate), MCF7 (human breast), Hepa1C1C7 (murine liver). LC50 is the concentration of a compound (µM) that kills 50% of the cells that were present at the time of the addition of the compound.

Table 5: Effect of seven pure compounds isolated from *Conyza* on the activity of caspase 9 (µmolpNA/min/ml) in cell lines after 6 h incubation.

Treatment	Caspase 9 activity (fold induction) in cell lines (mean ± SEM), n = 5				
	HT29	A549	PC3	MCF7	Hepa1C1C7
Actinomycin D	2.51 ± 0.29[a]	2.29 ± 0.25[a]	2.22 ± 0.31[a]	2.46 ± 0.26[a]	2.37 ± 0.22[a]
1	1.40 ± 0.13	1.03 ± 0.11	1.11 ± 0.08	0.91 ± 0.07	1.12 ± 0.12
2	2.09 ± 0.18[a]	1.28 ± 0.15	1.09 ± 0.11	1.87 ± 0.09[a]	1.23 ± 0.09
3	1.46 ± 0.17	1.61 ± 0.10	1.21 ± 0.09	1.03 ± 0.09	1.34 ± 0.10
4	2.73 ± 0.20[a]	1.38 ± 0.12	2.13 ± 0.13[a]	1.06 ± 0.10	1.26 ± 0.13
5	2.85 ± 0.14[a]	2.09 ± 0.18[a]	1.71 ± 0.09[a]	0.98 ± 0.11	1.81 ± 0.14[a]
6	2.79 ± 0.16[a]	1.98 ± 0.12[a]	1.86 ± 0.10[a]	0.94 ± 0.10	1.86 ± 0.12[a]
7	2.57 ± 0.11[a]	1.16 ± 0.12	2.01 ± 0.14[a]	1.05 ± 0.08	1.23 ± 0.09

[a] significant difference ($p < 0.05$) as compared to untreated cells. HT29 (human colon), A549 (human lung), PC3 (human prostate), MCF7 (human breast), Hepa1C1C7 (murine liver).

pathway. Induction of stress signaling pathways JNK/SAPK causes the release of mitochondrial of cytochrome c and activation of apaf-1, which in turn cleaves the pro-enzyme of caspase-9 into the active form. Caspase-9 activates procaspase-3 which could also be activated by caspases 8 and 10[21]. Caspase 3 plays a central role in the execution phase of apoptosis[22]. Numerous anticancer drugs and novel agents from plant origin were found to act through stimulation of apoptosis via inducing the activities of caspases 9 and 8[23,24]. We have examined the activity of two caspases; one initiator (caspase 9) which is the most studied of intrinsic apoptotic pathway and one effector (caspase 3). Data in tables (5 and 6) show that compounds 5 and 6 achieved their significant anticancer activity through inducing caspases 3 and 9 and these compounds elevated both caspases by ~ 2-3 folds in all cell lines investigated except breast cells where these compounds along with actinomycin D had no effect on caspase 3 in MCF7 cells. Compounds 1 and 3 were devoid of any significant effect on caspase 9. Compound 2, the only effective compound against MCF7 cells, was also the unique compound able to inducing caspase 9 in these cells but without any significant effect on the activity of caspase 3. It is now well established that MCF7 cells do not express caspase 3[25]. Compound 2 along with compounds 4, and 7 resulted also in a ~ 2-3 fold induction in caspase 9 in colon cells. Compounds 4 and 7 had a similar signature on prostate and colon cells inducing the activities of caspases

Table 6: Effect of seven pure compounds isolated from *Conyza* on the activity of caspase 3 (µmolpNA/min/ml) in cell lines after 6 h incubation.

Treatment	Caspase 3 activity (fold induction) in cell lines (mean ± SEM), n = 5				
	HT29	A549	PC3	MCF7	Hepa1C1C7
Actinomycin D	3.20 ± 0.25[a]	3.12 ± 0.28[a]	3.42 ± 0.19[a]	1.11 ± 0.27	3.10 ± 0.21[a]
1	0.88 ± 0.10	1.02 ± 0.09	1.14 ± 0.10	0.97 ± 0.10	1.01 ± 0.11
2	1.13 ± 0.12	1.07 ± 0.11	1.28 ± 0.12	1.26 ± 0.14	1.24 ± 0.12
3	1.03 ± 0.09	2.00 ± 0.13[a]	1.22 ± 0.10	1.15 ± 0.09	1.81 ± 0.12
4	1.94 ± 0.09[a]	1.11 ± 0.09	2.00 ± 0.10[a]	1.16 ± 0.08	1.59 ± 0.11
5	2.34 ± 0.18[a]	2.23 ± 0.15[a]	2.29 ± 0.14[a]	1.11 ± 0.08	2.11 ± 0.12[a]
6	2.51 ± 0.18[a]	2.33 ± 0.19[a]	2.08 ± 0.15[a]	1.09 ± 0.07	2.34 ± 0.21[a]
7	1.97 ± 0.17[a]	1.20 ± 0.09	2.08 ± 0.12[a]	0.98 ± 0.08	1.16 ± 0.09

[a] significant difference ($p < 0.05$) as compared to untreated cells. HT29 (human colon), A549 (human lung), PC3 (human prostate), MCF7 (human breast), Hepa1C1C7 (murine liver).

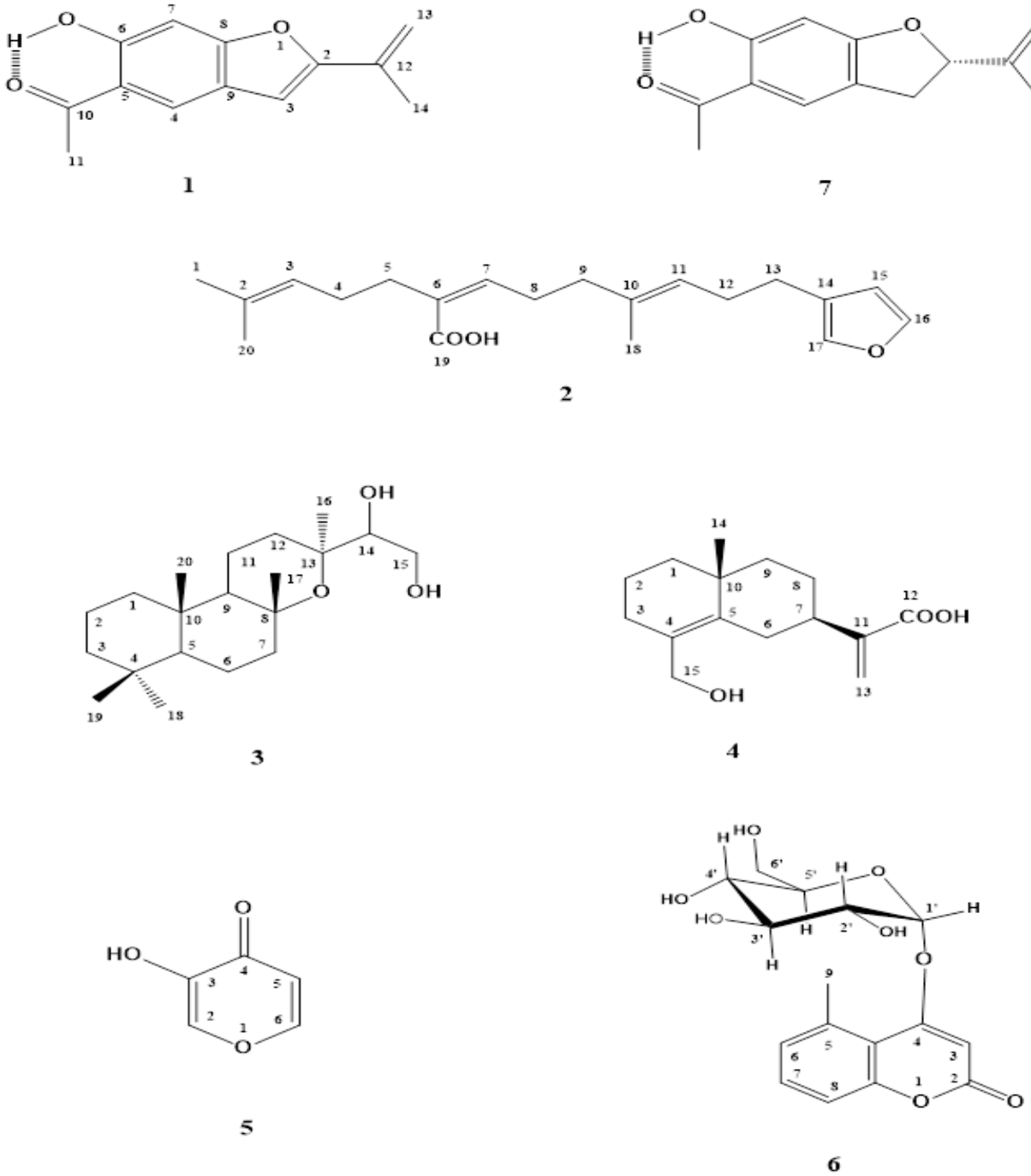

Figure 1: compounds isolated from *Conyza triloba*

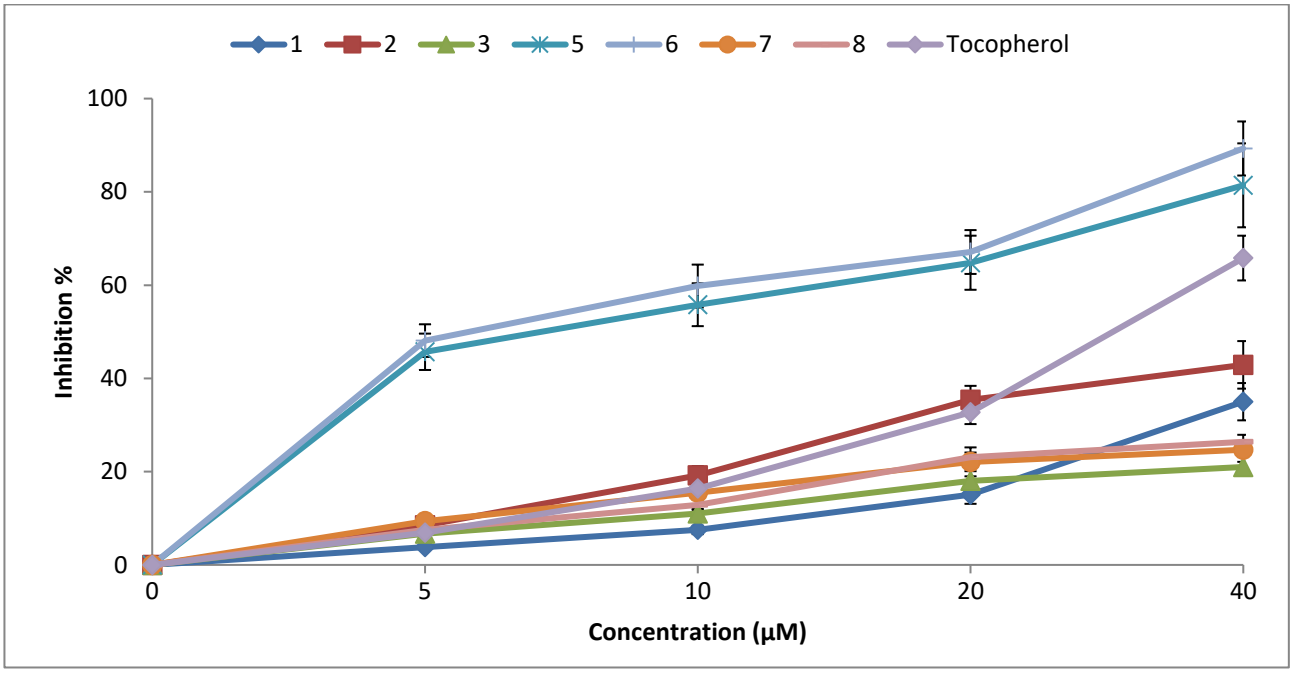

Figure 2: Free radical scavenging activity of seven isolated compounds from *Conyza triloba*

3 and 9 in these cells (Tables 5 and 6). Compounds 1 and 2 had no significant effect on caspase 3. Interestingly, compound 3 was able to elevate the activity of caspase 3 without affecting caspase 9 in lung cells (Table 6). A similar approach was previously reported in lung cells, where caspase 3 was activated by caspase 8 not 9[26]. This study firmly proved that some of these pure compounds have cytotoxic/cytostatic activities in a concentration range of nano-molar away from toxic concentration, which means they are safe. The antiproliferative activity of these pure phytochemicals were better than that of actinomycin D and at concentrations much less toxic. The antioxidant/scavenging properties, especially against peroxide radical, of these compounds correlate well with their antiproliferative activity. These compounds exerted their anticancer activity through the intrinsic apoptotic pathway by inducing the initiator caspase 9 and the execution caspase 3. Compounds 5 and 6 (pyromeconic acid and 5-methylcoumarin-4-*O*-□-D-glucoside) had a broad anticancer activity against all cell lines except breast cells where Compound 2 (centipedic acid) was the only effective agent against the breast cells. We also understand that in vitro studies have some limitations and lacking some key advantages of animal studies. Nevertheless, this study will be used to launch an educated in vivo study investigating both compounds 5 and 6 against dimethyl hydrazine-induced colorectal cancer.

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CONFLICT OF INTERESTS

None declared.

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