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Research Article

Phytochemical Investigation and Cytotoxic Characterization of Bioactive Constituents from *Conyza dioscoridis*

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ABSTRACT

A follow-up chemical investigation to methanol extract of *Conyza dioscoridis* (Family Asteraceae) led to the isolation of seven compounds (1-7). The isolated compounds identified as 2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy-α-L-mannopyranosyl-4-(3,4-dihydroxyphenyl)-2-propenoate]-*β*-D-glucopyranoside (1), rutin (2), isoquercetin (3), *E*-caffeic acid (4), gallic acid (5), quercetin (6), *β*-Sitosterol-3-*O*-*β*-D-glucpyranoside (7). The methanol and chloroform extracts of the plant showed cytotoxic activity against brine shrimp in a preliminary assay and inhibitory activity against colon carcinoma cells (HCT-116) with LC_{50} value 20, 32 µg/ml and IC_{50} value 25, 35.3 µg/ml, respectively. Brine shrimp lethality test was conducted on the seven isolated compounds at six different concentrations 400, 200, 100, 20, 10 and 5 µg/ml; compounds 1, 2, 3, 5, 6 and 7 showed significant cytotoxicity with LC_{50} value 9, 10, 11, 22, 8 and 19 µg/ml, respectively. Compounds 1, 2, 3 and 6 exhibited inhibitory activity against colon carcinoma cells with IC_{50} value 25, 30, 34.2 and 10 µg/ml, respectively. These results indicate that; *Conyza dioscoridis* has biochemical activity as potential pharmaceuticals. The chemical structures of active constituents were unambiguously determined by analysis of ^{1}HNMR, ^{13}CNMR, ESI-MS, as well as by comparison with literature data and physical methods.

Keywords: *Conyza dioscoridis*; methanol extract; bioactive constituents; brine shrimp; HCT-116, spectroscopic data.

INTRODUCTION

Conyza dioscoridis (Family Asteraceae) are common perennial herbaceous plants grown in the Nile region (Delta, valley and Faiyum) and in the Oases of the desert. *C. dioscoridis* is a richly branched hairy shrub reputed for its folk medicinal uses. The antimicrobial effect of the ethanol extract of *C. dioscoridis* has been previously assessed against selected Gram-negative and Gram-positive bacteria, and unicellular and filamentous fungi[1]. The antinociceptive effect of the methanol extract of the aerial parts of the plant was evaluated on mice[2]. Another study revealed that oral administration of the methanol extract of the leaves (200 mg/kg) reduced the number of fecal discharge produced by castor oil in rabbits exerting a significant antidiarrheal effect[3]; this extract induced a dose-dependent relaxation of rabbit duodenal muscle, the inhibition was attributed to either a calcium-channel, or a possible ganglionic blocking effect. The insecticidal and anti-inflammatory activities of the aerial parts of the plant have been also recorded[4,5]. Chemical investigation of *C. blinii* has led to the identification of 17 new triterpenoid saponins, four new labdane diterpenoids, a new clerodane diterpenoid and a new phenolic glucoside[6-8]. Subsequent examina-tions of the ethyl acetate-soluble fraction of the plant extractive have led to the isolation of an acid belonging to a new class of diterpenoids which is named as strictic acid[9]. *Conyza* species have been reported to have expectorant, antitussive activities[10,11]. They are useful in the treatment of urinary affections, liver diseases, stomach ulcers, and to wash sores[12] as well as an antihelmintic, digestive and diuretic[13-15]. The crude methanolic extract of *Conyza bonariensis* and its subsequent solvent fractions showed antifungal and phytotoxic effects[16]. In continuation of our studies on biologically active substances from medicinal plants, we report herein the isolation and structural elucidation of an acetoside, three flavonoids, one cinnamic acid derivative and one sterol from the leaves of *Conyza dioscoridis*. In addition cytotoxicity towards brine shrimps was determined for the methanol and chloroform extracts of the leaves. The methanol, chloroform extract and major isolates (1-7) were also tested against the HCT-116 tumor cell line

MATERIALS AND METHODS

Plant Collection

Conyza dioscoridis plant were collected from various areas around Egypt during June, authenticated by the botanist Dr Wafaa M. Amer, Professor of Plant Taxonomy, Faculty of Science, Cairo University. Voucher specimens (Reg. No. C-12) of the plants were deposited at the herbarium, Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt. The plant is shade, dried and powdered by an electric mill to a fine powder and stored in airtight bottles.

General

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The NMR spectra for all compounds were recorded at 300 (^{1}H) and 75 MHz (^{13}C) on a Varian Mercury 300 instrument, δ values are reported in ppm relative to TMS and coupling constant (*J*) by Hertz, in the convenient solvent. UV analyses of pure samples were recorded on a Shimadzu UV 240 spectrophotometer, in MeOH solution and different diagnostic UV shift reagents. ESI-MS analyses were measured on a LTQ-FT-MS spectrometer (Thermo Electron, Germany). We use for column chromatography (CC), polyamide S (Fluka) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). For paper chromatography Whatman No. 1 sheets (Whatman Ltd., England) were used, while silica gel G powder (70-230 mesh, Merck) was used for CC, 60 F_{254} for TLC (Merck, Germany) and microcrystalline cellulose (Merck, Darmstadt, Germany).

Extraction techniques and sample preparation

Extraction techniques

About 1 Kg of the dried powder leaves were exhaustively extracted with 70% MeOH (v/v 6 x 1.5 l and 3 x 2.5 l), evaporated under reduced pressure using a rotary evaporator. The aqueous methanol extract was concentrated under reduced pressure (70 °C) and defatted with petroleum ether (60 – 80 °C, 6×2 l), then extracted with CH_2Cl_2 (4×2 l) to give a brown paste form of extract which was dissolved in water, and the water-insoluble residue was removed by filtration. The water-soluble portion was desalted by precipitation with MeOH to give a dry brown residue (70 g) that was suspended in H_2O and fractionated on a polyamide column (110 × 5 cm, 350 g) using a stepwise gradient from H_2O to H_2O/MeOH mixtures up to pure MeOH. By using comparative paper chromatography (Co-PC) with the use of UV light, 1% $FeCl_3$, or Naturstoff spray reagent for detection, the individual 65 fractions were pooled into seven collective fractions (A – G). Fraction A (H_2O, 15 g) was found to be a dark brown material with no phenolic character. Fraction B (10 – 30% MeOH, 16 g) was fractionated on cellulose with MeOH as an eluent, followed by a Sephadex LH- 20 column using BIW (*n*-BuOH:2-propanol:H_2O, 4:1:5 v/v/v, organic layer) to afford pure **(1)** (45 mg). Two major dark purple spots were detected in fraction B (30-40% MeOH, 10g), which was subjected to repeated CC on Sephadex LH- 20 with 60-80% aqueous EtOH as an eluent. This fractionation resulted in pure **(2)** (43 mg) and **(3)** (55 mg). Crude **(4)** was crystallized from Fr. C (40-60% MeOH, 9 g) and purified by repeated crystallization from MeOH to yield a pure sample of **(4)** (37 mg). Fraction D (60 – 70% MeOH, 11 g) was subjected to repeated CC on cellulose and Sephadex LH-20 with 20 – 70% aqueous MeOH as an eluent, resulting in a pure samples of **(5)** (19 mg). Fr. E (70 –80% MeOH, 9 g) was rechromatographed on Sephadex LH-20 with MeOH to afford a pure sample of **(6)** (20 mg). Crude **(7**) was crystallized from Fr. F (80-90% MeOH, 9 g). Fr. G (90-100% MeOH, 8 g) was chromatographed on silica gel followed by Sephadex LH-20 with MeOH eluent to afford a pure sample of **(7)** (18 mg). All separation processes were followed by 2D-PC and CoC using Whatman No. 1 paper with 15% aqueous AcOH (S1) and *n*-BuOH:AcOH:H_2O (4:1:5, top layer) (S2) as solvent systems.

Acid hydrolysis of compounds 1, 2, 3 and 7

Each Compound (5 mg) was refluxed with 2M HCl in MeOH (5 ml) at 80 °C for 2 h in a water bath. The reaction mixture was evaporated, and the hydrolysate after dilution with H_2O (10 ml) was extracted with $CHCl_3$ (10 ml x 3). The $CHCl_3$ extract was evaporated to afford the aglycon, which was identified by Co-TLC with authentic sample using solvent system [$CHCl_3$:MeOH, 9:1]. The aqueous layer was neutralized with 2N KOH solution and concentrated to 1ml under reduced pressure. The residue was compared with authentic sugars by silica gel TLC [($CHCl_3$:MeOH:H_2O, 30:12:4), 9 ml of lower layer and 1 ml of AcOH] and by PC (iso-PrOH:*n*-BuOH:H_2O, 7:1:2); detection with aniline hydrogen phthalate, which indicated the sugars of **1** to be glucose and rhamnose. By the same method, compound **2** furnished a monosaccharide in the aqueous phase was also identified as glucose and rhamnose. Glucose was detected in hydrolysis for compounds **3** and **7**.

Compound **1**: Off-white amorphous powder, R_f values: 0.73 (S1) and 0.49 (S2). Under UV light, it has a blue fluorescence colour turned to yellow with ammonia vapours and greenish-blue with Naturstoff reagent. UV: λmax = 203, 220, 231, 292 and 332 nm. Complete acid hydrolysis revealed the presence of glucose and rhamnose in the aqueous phase together with caffeic acid appears in the organic phase indicated by CoPC with authentic samples; negative ESI-MS: *m/e* = 1247.0 $[2M-H]^-$, 623.0 $[M-H]^-$, 461.0 $[M-H\text{-caffeate}]^-$, 179.0 $[caffeate]^-$, 161.0 $[caffeate-H_2O]^-$. ^{1}H NMR and ^{13}C NMR (Table 1).

Cytotoxic assay

Eggs of *Artemia salina* (Artemia Inc., California) and saline artificial sea (Instant Oceanic, Marine land Labs, USA). HCT-116 cell line was obtained from the regional center for mycology and biotechnology, Al-Azhar Univesity, Cairo, Egypt.

Brine shrimp lethality bioassay

Brine shrimp bioassay is widely used in the bioassay for the bioactive compounds[17]. The eggs of brine shrimp (Artemia salina Leach) were collected (Atremia Inc., California) and hatched in a rectangular chamber tank at a temperature around 37 °C with constant oxygen supply. Two days were enough to hatch and mature the nauplii.

Toxicity of the extracts and pure compounds were tested at 5, 10, 20, 100, 200, 400 and 600 ppm in 10 ml sea-water solutions with 1% DMSO (v/v). From each of these test solutions 100 µl were added to the prepared numbered glass test tubes containing 5 ml of sea water and 10 shrimp (larvae) nauplii. By using a Pasteur pipette take 10 living nauplii then were put to each of the vials. Three replicates were used for each concentration. A parallel series of tests with the standard potassium dichromate solution and the blank control were always conduced. After 24 hrs the vials were observed and the number of nauplii survived in each vial was counted. From this, the percentage of mortality of brine shrimp nauplii was calculated in µg/ml for each concentration of the extract and pure compounds. The data

were analyzed and LC_{50} values calculated according to Reed-Muench method[18,19].

Measurement of potential cytotoxicity by the Sulforhodamine B (SRB) assay

Potential cytotoxicity of the methanol and chloroform extracts of *C. dioscoridis* leaves and the isolated compounds **1**-**7** were tested at the mycology and biotechnology, Al-Azhar Univesity, Cairo, Egypt using the Skehan *et al*., method[20]. Cells were placed in a 96-well plate (104 cells/well) for 24 h before treatment to permit the attachment of cells to the plate wall. Different concentrations of the fractions under investigation (0, 1, 2.5, 5 and 10 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose and they were incubated for 48 h at 37 ºC in 5% CO_2. After 48 h cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer and the color intensity was measured in an ELISA reader. The survival curves of the tumor cell line were constructed by plotting for each tested sample.

RESULTS AND DISCUSSIONS

A total of 7 compounds were isolated from the desalted and defatted 70% methanol extract of *C. dioscoridis* leaves through consecutive column chromatographic separations. On the basis of chemical and hysicochemical analyses as well as their comparison with published data their structures were identified as 2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy α-L-mannopyranosyl-4-(3,4-dihydroxyphenyl)-2-propenoate]-*β*-D-glucopyranoside[21,22], rutin[23,24], isoquercetin[23,24], *E*-caffeic acid[25], gallic acid[26], quercetin[23,24] and *β*-Sitosterol-3-*O*-*β*-D-glucpyranoside[27].

2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy-α-L-mannopyranosyl-4-(3,4-dihydroxyphenyl)-2-propenoate]-*β*-D-glucopyranoside (**1)** is predictable to be a phenylethanoid compound on the basis of its UV spectral data, chromatographic properties and acid hydrolysis products. Its negative ESI-MS spectral data indicates a dimeric adduct and molecular ion peaks at m/e, 1247.0 and 623.0 due to $[2M-H]^-$ and [M-H], respectively together with three fragment ion peaks at m/e, 461.0, 179.0 and 161.0 corresponding to $[M-H\text{-caffeate}]^-$, $[caffeate]^-$ and $[caffeate-H_2O]^-$ respectively which indicates its similarity as 3,4-dihydroxyphenethyl-alcoholcaffeoyl-rhamnosylglucoside. ^{1}H NMR spectrum (Table 1), at the aromatic region, an ABM one of H-2‴, H-5‴ and H-6‴ at *δ* 7.15, 6.85, 7.00 ppm, respectively presents together with an A^2X^2-spin coupling system of H-7‴ and H-8‴ at *δ* 7.59 and 6.29 ppm, respectively (*d*, *J* = 16.0 Hz) were indicated to an *E*-caffeoyl moiety in the compound. The ^{1}HNMR showed two anomeric proton signals at *δ* 4.46 ppm (H-1', *d*, *J* = 7.8 Hz) and at *δ* 5.27 (H-1", *brs*) together with the signal at *δ* 1.12 (3H, *d*, *J* = 6.5 Hz, CH_3-6"). Also the characteristic signals for 3, 4-dihydroxyphenethyl alcohol (aglycone moiety) were presented in the form of an ABM spin coupling system at *δ* 6.66, 6.72 and 6.55 ppm for H-2, H-5 and H-6, respectively in the aromatic ring and at the aliphatic moiety; its characteristic AX-system of two triplets responsible for H-7 and H-8 at *δ* 2.76 and 3.83, respectively. The rhamnosyl moiety guided to an *O*-α-$^1C_4$-pyranose while glucosyl moiety provides an *O*-*β*-$^4C_1$-pyranose, depending on all of their *J*- and *δ*-values in ^{1}H and ^{13}C-spectra (Table 1). The downfield position of H-4' as *t*-like at 4.91 indicated that the caffeoyl moiety on OH-4 of glucose whereas, the contact on C-2-glucoside of *O*-rhamnosyl was concluded from the characteristic downfield shift of its ^{13}C NMR signal to 79.45 ppm. Depending upon the study of all other signals of ^{1}H and ^{13}C NMR with the aid of the comparison with previously literature data, compound **1** was identified as 2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy-α-L-mannopyranosyl-4-(3,4-dihydroxyphenyl)-2-propenoate]-β-D-glucopyranoside[21,22].

Table 1: ^{1}H, ^{13}C NMR spectral data of compound **1** (300/75 MHZ, DMSO-d_6)

No.	δ_H	δ_C
1	-	131.01
2	6.66 *d* (1.8)	116.85
3	-	144.20
4	-	145.50
5	6.72 *d* (8.2)	115.90
6	6.55 *dd* (8.1, 1.8)	121.09
7	2.76 *t*-like (7.5)	36.28
8	3.83 *t*-like (7.5)	72.20
1′	4.46 *d* (7.8)	103.75
2′	4.02	79.45
3′	-	73.85
4′	4.91 *t*-like (10.5)	72.15
5′	-	76.30
6′[a]	4.10 *brd* (12.5)	62.35
6′[b]	3.36	62.39
1″	5.27 *d* (*brs*)	101.80
2″	3.88 *brs*	71.73
3″	-	70.30
4″	-	72.00
5″	3.70 *m*	69.50
6″	1.12 *d* (6.5)	18.65
1‴	-	127.45
2‴	7.15 *d* (1.5)	115.20
3‴	-	148.85
4‴	-	146.45
5‴	6.85 *d* (8.0)	116.36
6‴	7.00 *dd* (8.1, 1.8)	122.55
7‴	7.59 *d* (16.0)	147.65
8‴	6.29 *d* (16.0)	115.18
9‴	-	169.01

δ in ppm and J values (Hz)

Structure-Activity Relationship (SAR)

Brine shrimp lethality assay of methanol and chloroform extracts of C. dioscoridis show that; the methanol extract more active than chloroform extract with LC50 value 20, 32 µg/ml, respectively (Fig. 2) and compounds 1, 2, 3, 4, 5, 6 and 7 showed significant cytotoxicity with LC50 value 9, 10, 11, 90, 22, 8 and 19 µg/ml, respectively (Fig. 3).The methanol, chloroform extracts and compounds 1-**7** were

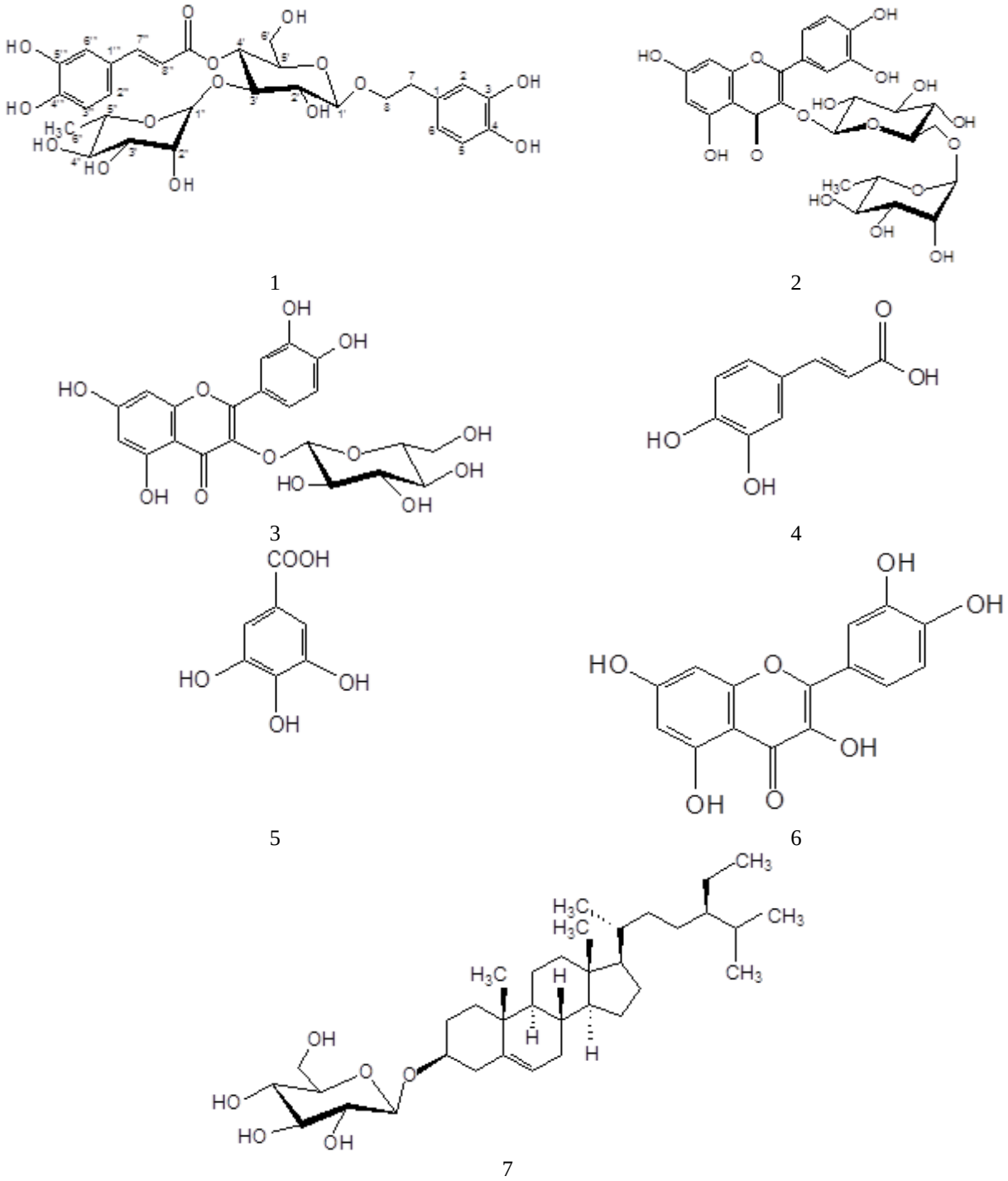

Figure 1: Structure of Compounds (1-7)

cytotoxic for HCT-116 cells and **6** was found to be the most cytotoxic agent. Methanol and chloroform extracts exhibited inhibitory effect against HCT-116 cells within IC_{50} value 25, 35.3 µg/ml, respectively (Fig.4). Compounds 1, 2, 3 and 6 exhibited inhibitory activity against colon carcinoma cells with IC_{50} value 25, 30, 34.2 and 10 µg/ml, respectively; while compounds 4, 5, 7 showed IC_{50} value 39.13, 39, 35.4 µg/ml, respectively (Fig. 5,6). Cytotoxic action of a drug is believed to be provided by disturbing the fundamental mechanisms associated with cell growth, mitotic activity, differentiation and function [28]. The obtained cytotoxic activity for these extracts and pure isolates may be due to one of these mechanisms. Phenylethanoid glycosides with caffeic side group acteoside has high biological activity that occurs in several directions on mouse cell line P-388[29], also it has significant anti-inflammatory activity in vitro and reduces the risk of atherosclerosis by inhibiting the production of the so-called ″Bad cholesterol″ (LDL), and also because of its radical-scavenging qualities[30,31]. Acteoside inhibits the human leukocyte elastase and the telomerase activity of

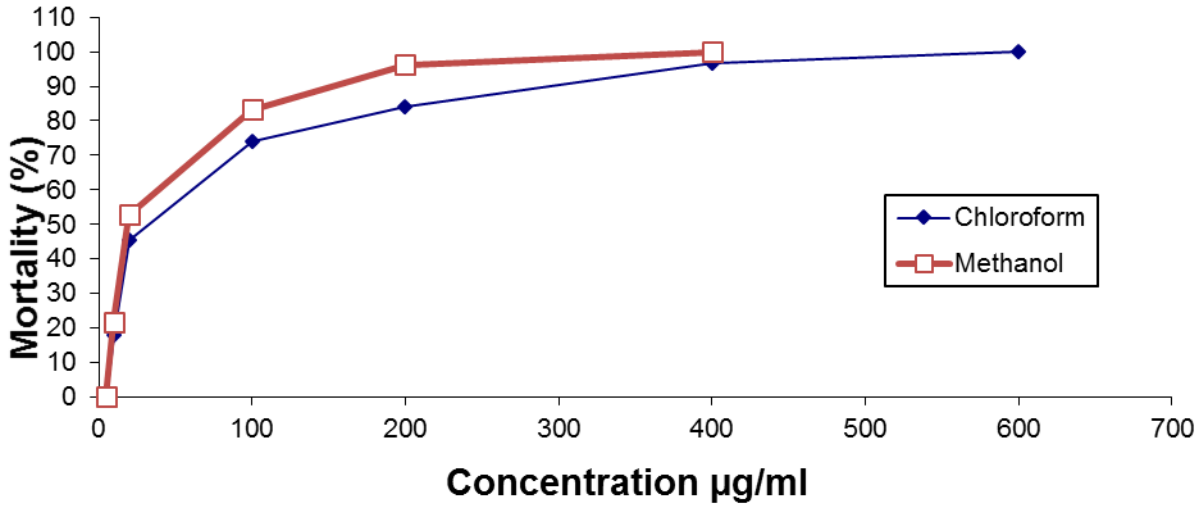

Figure 2: Brine shrimp lethality assay of methanol and chloroform extracts of *C. dioscoridis*.

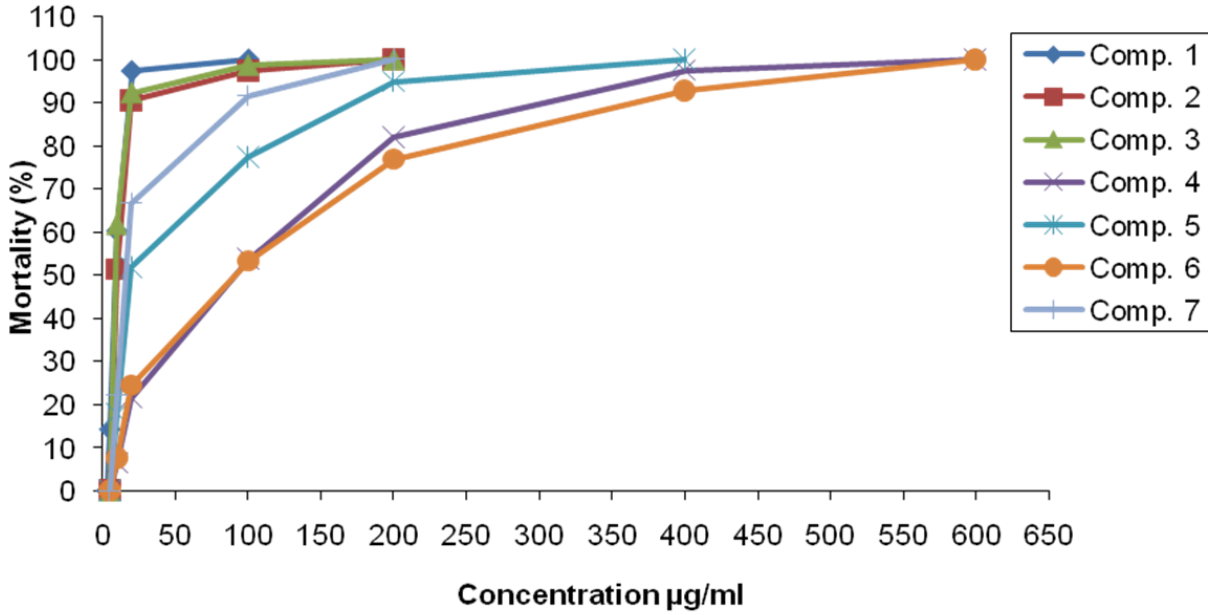

Figure 3: Brine shrimp lethality assay of compounds 1-7.

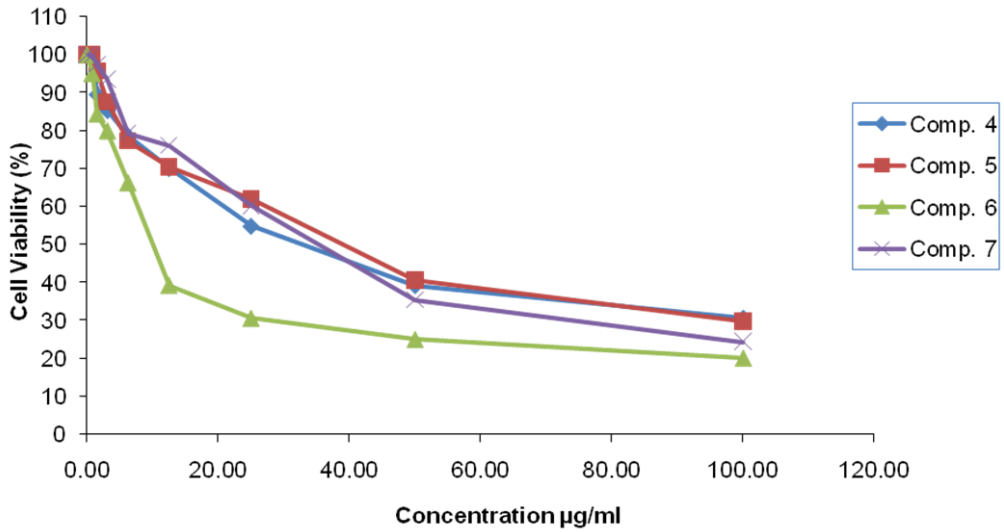

Figure 4: The cytotoxic activity of *C. dioscoridis* chloroform and methanol extracts against HCT-116 colon cancer.

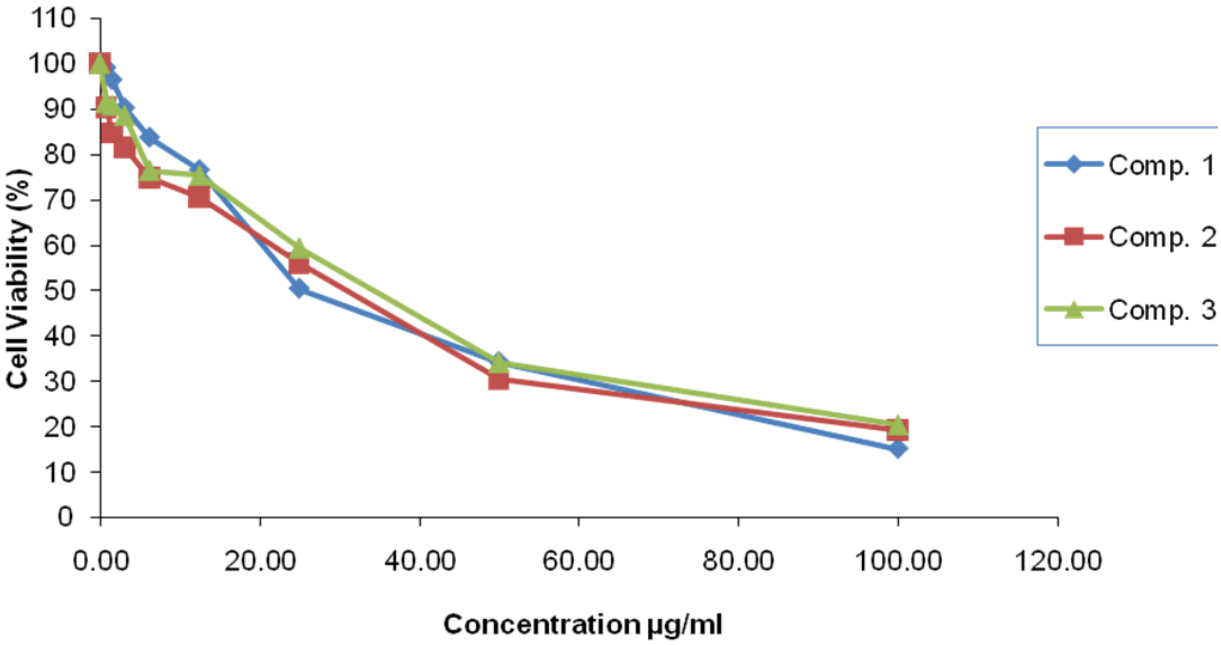

Figure 5: The cytotoxic activity of compounds 1,2 and 3 against HCT-116 colon cancer.

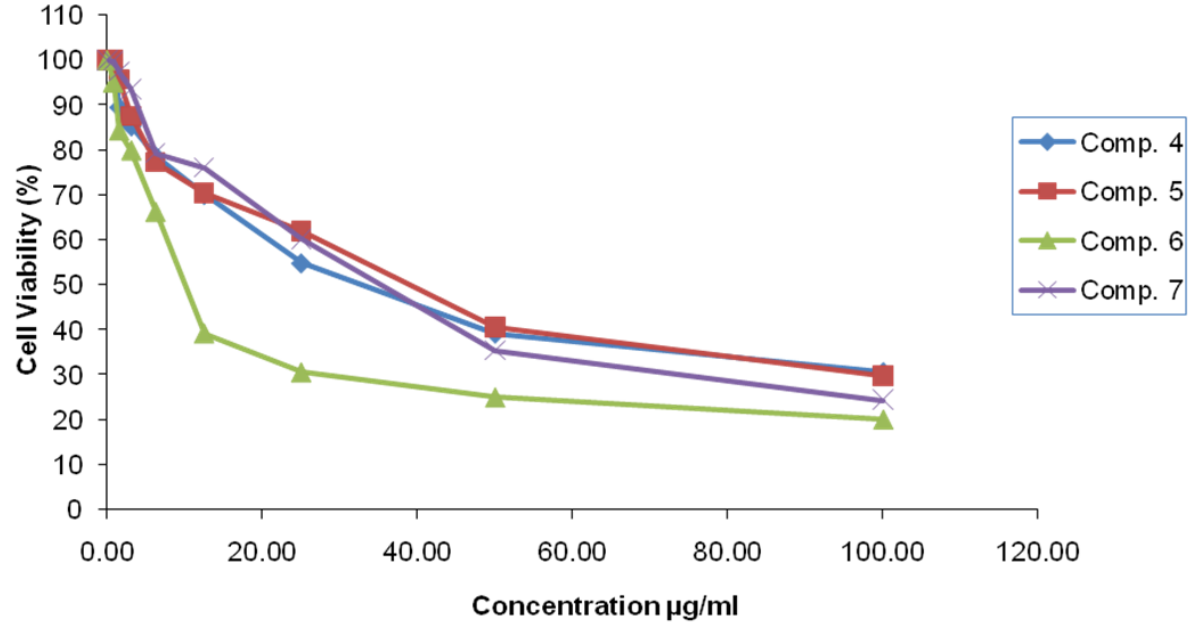

Figure 6: The cytotoxic activity of compounds 4,5,6 and 7 against HCT-116 colon cancer.

human gastric adenocarcinoma cells MKN45, and show antiproliferative effect against B16F10 cells [32,33]. The analysis shows that the 3,4-dihydrophenethyl alcohol radicals of the acteoside is to a greater extent responsible for these effects than the caffeoyl groups. The results showed that phenyl groups with caffeic acid esters as acteoside were more potent than those with p-coumaric acid esters indicating that the type of phenylpropanoid acid affects the activity[34].

Quercetin and rutin should be considered as potential pharmaceutical molecules that might be used as cancer inhibitors. The phenolic acids are characterized by the presence of a benzene ring, a carboxylic acid moiety and one or more hydroxyl or methoxy moieties in the molecule, which confer antioxidant properties to it[35-37]. The phenylpropanoids act as antioxidants by eliminating oxygen free radicals[38,39]. Inoue *et al*., (1995), elucidated that the carboxyl group of gallic acid is presumably implicated in distinguishing between normal and cancer cells and the three adjacent phenolic hydroxyl groups should be essential to the cytotoxicity[40].

This study is the first trial to investigate the cytotoxicity of the methanol, chloroform extracts of *C. dioscoridis* leaves against HCT-116 solid tumor cell lines. The strong anti-

cancer activity of *C. dioscoridis* leaves methanol extract may be attributed to the corresponding anti-tumor activities of its constituents.

CONCLUSION

In conclusion this is a study reporting cytotoxic activity of isolated metabolites from *Conyza dioscoridis* (Asteraceae) methanol extract within chloroform extract against brine shrimp and colon carcinoma cells (HCT-116). All isolated compounds exhibited anti-cancer activity especially quercetin was found to remarkably inhibit colon cancer activity, so *C. dioscoridis* can be used in many pharmacological as well as biological actions. However, further studies are required to know the mechanistic action of these compounds using suitable animal models to confirm this attribution.

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