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Bioactive compounds from *Acokanthera oblongifolia*

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Abstract
One cardiotonic glycoside, three triterpenes and one steroidal glycoside were isolated from *Acokanthera oblongifolia* fruits (pericarp) growing in Libya. Their structures were investigated by extensive application of IR, MS, 1DNMR and 2DNMR spectroscopy. The isolated compounds have evidenced *in-vitro* cytotoxicity on selected human cell lines (A-549, H-1299) when compared to doxorubicin.
Keywords: *Cardenolide; Acokanthera oblongifolia; antitumor activity; lung carcinoma cell line (A-549, H-1299).*

1. Introduction

The most common type of cancer worldwide is lung cancer. It is accounting for 1.61 million new cases annually representing 12.7% of all new cancers. The majority of the cases now occur in the developing countries (55%) in North Africa such as Egypt about (20.6%) **(Ferlay et al., 2010).** Several studies have demonstrated that extracts from some herbal medicines or their mixtures have anticancer potential and can inhibit cancer cell proliferation *in vitro* and/or *in vivo* **(Bonham et al 2002).**

Cardiac glycosides are used for the treatment of cardiac congestion and some types of cardiac arrhythmias **(Pieter Van Der Biil 2012)**. They were also suggested to have some anticancer activity (**Inada, et al. 1993; Haux, 1999).** However, they act by a mechanism different from targeting the Na^+/K^+ ion pump (**Haux, 2002)**. Early epidemiologic studies, evidencing significantly lower mortality rates of patients with cancer receiving cardiac glycosides (**Stenkvist et al. 1979).** Numerous subsequent in *vitro* and in *vivo* studies verified these observations (**Mijatovic et al.** 2007; **Winnicka** et al. 2006; **Lopez**, 2007).

Acokanthera oblongifolia is evergreen shrub or small tree up to 6 m tall used as the source of an arrow poison **(Steyn, 1934; Verdcourt, 1969; Neuwinger, 1996; Van Wyk, 2002).** All parts of the plant are highly poisonous with the possible exception of the ripe fruits **(Kokwaro, 1993)**. *A. oppositifolia* was used medicinally to treat snake and spider bites **(Gerstner, 1939; Kokwaro, 1993; Dharani, 2002),** intestinal worms **(Pooley, 1993)** and also for aches and colds **(Neuwinger, 2000)**. In Africa, the leaves and bark are applied to treat skin disorders, and an infusion of the leaves is gargled to treat tonsillitis, fertility and gall-bladder problems. **(Bhat and Jacobs, 1995)**. Bark decoction is used menorrhagia and irregular menstruation **(Arnold and Gulumian 1984.; Maundu, 2005).** Root is used in small quantities to treat sexually transmitted diseases, an aphrodisiac and insect repellent **(Neuwinger, 1996)**.

Numerous cardenolides were detected in the wood, leaves, seeds and fruit of *A. species* **(Van Wyk, et al., 2000; Kokwaro, 1976; Omino, and Kokwaro, 1993; Hanna, 1999).** The most important being acovenosides A, B and C, which have cardio-vascular properties (**Schlegel, et al. 1955)**. Other cardenolides were isolated as spectabiline and acopieroside were showing a higher activity than digoxin as a cardiotonic. Triterpenes from various parts of *A. oblongifolia* has also been reported (**Karawya, et al. 1973**). These studies were the prime stimulus for the work presented here.

As most of these cardinolides have not been biologically investigated, more research is needed to evaluate the possible prospects of the various compounds. The current work has been carried out with the goal of exploring the anticancer property and identifying the cardiotprotective steroids principles in *Acokanthera oblongifolia*. In the present study, we are reporting the isolation of acovenoside A, lupeol, β-sitosterol-3-glucoside, ursolic acid, and oleanolic acid. Cytotoxicity of the isolates were determined using Sulphorhodamine B (SRB) assay on lung carcinoma cell lines, *the adenocarcinomic human alveolar basal epithelial cells* (A-549) and *human non-small cell lung carcinoma cell line* (H-1299).

2. Experimental

Material and Methods

Plant Material

The fruits of *A. oblongifolia* were obtained from the trees growing in Benghazi, Libya, collected in November 2012, air dried and identified by Dr. Reem Samir Hamdy, Lecturer of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University, Giza, Egypt. Voucher sample of the plant is deposited at the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Material and apparatus for phytochemical study

Reference material: Material, solvent systems and spray reagents for chromatographic studies included pre-coated silica plates 60 GF_{254}, (20×20 cm) from Fluka (Sigma-Aldrich chemicals-Germany) for thin layer chromatography (TLC), silica gel 60 for normal phase column chromatography (CC), silica gel H for vacuum liquid chromatography (VLC) (Merck Darmstadt, Germany), and silica gel RP-18 (70-230 mesh) for reversed phase column chromatography were obtained from Fluka (Sigma-Aldrich chemicals-Germany). The following solvent systems were used for developing the chromatograms: S_1: *n*-hexane, S_2: *n*-hexane- chloroform (5:5)v/v, S_3: *n*-hexane-chloroform(9:1)v/v, S_4: hexane-ethylacetate (6:4)v/v, S_5: *n*-hexane -chloroform (7:3)v/v, and S_6: chloroform - methanol (9.5:0.5) v/v. Spots were visualized by spraying with *p*-anisaldehyde-sulphuric acid for steroids.

EI-MS was recorded with a Varian Mat 711 or SSQ 7000 (Finnigan mat), eV 70. IR spectra were observed as KBr discs using Jasco FT/IR-460 plus, Japan Infrared Spectrophotometer.^{1}HNMR and ^{13}CNMR spectra were recorded on a Varian Mercury VX-400NMR spectrophotometer operating at 400 (^{1}H) and 100 (^{13}C) MHz in DMSO-d_6 and $CHCl_3$-d_6 as a solvent and chemical shifts were given in δ (ppm) relative to solvent as internal standard. Ultraviolet lamp (λ max =254 and 330 nm,Shimadzu), a product of Hanovia lamps for localization of spots on chromatograms.

Extraction

The air-dried powdered fruits (pericarp) after removal of the seed of *A. oblongifolia* (225) was extracted by cold percolation with 95% ethanol (2 x1L) till exhaustion. The ethanol extract was evaporated under reduced pressure to give 62.8 g of greenish brown semi-solid residue respectively. The dried residue was separately suspended in distilled water and successively partitioned between chloroform, and *n*-butanol saturated with water. The solvent in each case was completely evaporated under reduced pressure to yield 50g, 5g of *A. oblongifolia*.

Fractionation and isolation of the components of the chloroform extractive of A. oblongifolia.

The chloroform extracts of the plant was chromatographed on a VLC column (210 g silica gel H,7x12.5cm) using *n*-hexane, *n*-hexane-chloroform and chloroform-ethyl acetate mixtures. Fractions (1-23), 200 ml each, were collected and monitored by TLC. Similar fractions were pooled together to obtain six major fractions.

Fraction **I** (4.26 g), eluted with *n*-hexane: chloroform (90:10), was purified on a silica gel column using *n*-hexane: ethyl acetate (90:10) as an eluent to obtain compound **1** (100 mg).

Fraction **II** (2.5 **g**), eluted with 20 % ethyl acetate in methylene chloride was purified on silica gel column using 0.5 % methanol in methylene chloride mixture as eluent to yield white microcrystalline powder of compound **2** (450 mg)

Fraction **III** (2.62 g**),** eluted with 35% ethyl acetate in methylene chloride, was purified on silica gel column using *n*-hexane-ethyl acetate (80:20) mixtures as an eluent revealed two spots. Further rechromatography on successive silica gel columns using *n*-hexane- chloroform mixtures yielded compound **3** (120mg).

Fraction **IV** (3.81 g), eluted with 40-45% ethyl acetate in methylene chloride was purified on silica gel column using 2 % methanol in methylene chloride mixture as eluent to yield white needle crystals of compound **4** (500 mg).

Fraction **V** (1.53 g), eluted with 50 % ethyl acetate in methylene chloride was purified on silica gel column using 4 % methanol in methylene chloride mixture as eluent to yield white microcrystalline powder of compound **5** (15 mg).

Chemicals and drugs for in vitro cytotoxic activity

Sulforhodamine-B dye was purchased from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 media, fetal bovine serum, trypsin and other cell culture materials were purchased from Gibco Invitrogen (Carlsbad, CA, USA). Other reagents were of the highest analytical grade.

Cell culture

Lung cancer cell line, A549 (Human lung adenocarcinoma epithelial cell line) H-1299 (Human non–small cell lung carcinoma cell line) derived from the lymph node maintained in RPMI-1640 supplemented with 100µg/ml streptomycin, 100µg/ml pencillin and 10% heat-inactivated fetal bovine serum in a humidified 5%(v/v) CO2 atmosphere at 37ºC. The cells were maintained as monolayer culture by serial sub culturing.

SRB cytotoxicity assay

Human tumor cell lines: Lung carcinoma A-549, H-1299. A549 is (Human lung adenocarcinoma epithelial cell line), H-1299 is a human non–small cell lung carcinoma cell derived from the lymph node cell lines, maintained in the laboratory of the Cancer Biology Department of National Cancer Institute, Cairo, Egypt were used. The isolated compounds at different concentrations (0-10 µg/ml) in DMSO were tested for cytotoxicity,

against the fore mentioned human tumor cell lines adopting sulforhodamine B stain (SRB) assay **(Skehan et al. 1990).** Exponentially growing cells were collected using 0.25%Trypsin-EDTA and seeded in 96-well plates at 1000-200.000 cells/well in RPMI-1640 supplemented medium after 24 hr, cells were incubated for 48h with various concentration of the tested compounds. Following 72hr treatment, the cells will be fixed with 10% trichloroacetic acid for 1hr at 4 ºC.

Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24hr and the dye was solublized withTris-HCL for 5 min on a shaker at 1600rpm. The optical density (OD) of each well was measured spectrophotometrically at 564nm with an ELISA microplate reader (ChroMate-4300, FL, USA).

The relation between surviving fractions and isolates concentration were plotted to get the survival curve of each tumor cell line after the application of the specific concentration. The results were compared to those of the standard cytotoxic drug, Doxorubicin (10 mg Adriamycin hydrochloride, in 5 ml IV injection, Pharmacia, Italy) at the same concentrations. The dose of the test solutions which reduces survival to 50% (IC_{50}) was calculated (Table1).

Data analysis for cytotoxicity

The dose- response curve of compounds was analyzed using E_{max} model.

$$\% \text{ cell viability} = 100 - R \times \frac{(1-[D]^m) + R}{K_d^m + [D]^m}$$

Where R is the residual unaffected fraction (The resistance fraction), [D] is the drug concentration used, K_d is the drug concentration that produces a 50% reduction of the maximum inhibition rate and m is a Hill-type coefficient. IC_{50} was defined as the drug concentration required to reduce fluorescence to 50% of that of the control (i.e. K_d= IC_{50}% when R=0 and E_{max} = 100-R).

On assessing the cytotoxic activity, the isolated compounds of *A.oblongifolia* exhibited specific and significant effects on the viability of the selected human cell lines, *viz.*, lung carcinoma (A-549, H-1299) cell lines. IC_{50} was (25.1, 33), (20.6, 28.2), (22.1, 22.9), (3.98, 4.73) and 15.7, 22 µg/ml for lupeol, oleanolic acid, ursolic acid, acovenoside and *β*-sitosterol-3-O- glucoside on lung carcinoma (A-549, H-1299) cell lines, respectively (Tables 2-3).

3. Results

Column chromatographic fractionation of the chloroformic fraction of the ethanol extract of the pericarp of *A. oblongifolia* allowed the isolation of five compounds **(1-5)** which were characterized through their physicochemical and spectral data.

Compound 1 (Lupeol): 50 mg, crystallize from methanol as white needle crystals. m.p. (210-212), positive test for triterpenoid skeleton. R_f values (0.77 in S_4 and 0.32 S_5); violet color with P-anisaldehyde / H_2SO_4 spray reagent**.**

IR $\sqrt{}_{Max}^{KBR}$ Spectrum: Incorporated absorption bands at 3415 cm^{-1} (OH), 2945, 2869 cm^{-1} (CH) and 1642, 880cm^{-1} for (C=CH_2).

EI Mass (70eV) m/z: showed a molecular ion peak (M^+) at 426.7 calculated for C_{30} H_{50} O with characteristic fragment ions at 411 (M^+- Me), 393 (M^+-Me-H2O), 365, 299, 297, 245, fragment ions at m/z 220, m/z 207 (allocate the hydroxyl group at C_3 position), m/z 218, m/z 205 and m/z 189 all in accordance with lupene skeleton.

^{1}HNMR (400MHz $CDCl_3$): revealed signals for seven tertiary methyl groups at δ 0.80 (3H, s, C_{28}-CH_3), 0.85 (3H, s, C_{23}-CH_3), 0.86 (3H, s, C_{24}-CH_3), 0.87 (3H, s, C_{25}-CH_3), 0.95 (3H, s, C_{27}-CH_3), 1.04 (3H, s, C_{26}-CH_3) and 1.69 (3H, s, vinylic methyl, C_{30}-CH_3), 3.15 (1H, dd, J=3Hz, 6 Hz indicative of C_3-H is α- oriented) and 4.69 (1H, s, C_{29}-H_b), 4.58 (1H, s, C_{29}-H_a).

Compound 1 was suggested to be a triterpenoid since it responded positively to Liebermann- Burchard's test and has high m.p. The mass spectrum of C1 showed M^+ at m/z 426.7 calculated for molecular formula C_{30} H_{50} O suggested a triterpenoid structure. Characteristic fragmentation is represented by loss of methyl at m/z 411 (M^+-Me), m/z 393 (411-18) (M^+-Me-H_2O), 365, 299, 245 220, 207 (indicative for the hydroxyl group in C-3), 218, 205 and 189 where all consistent with skeletal pattern of lupene series **(Ogunkoya, 1981)**. The IR spectrum exhibited absorption band of hydroxyl group at 3415 cm^{-1} as well as olefinic bond absorption band at 1642, 880 cm^{-1} and 2945, 2869 cm^{-1} for (C-H).

The ^{1}HNMR spectrum (400 MHz in $CDCl_3$) exhibited seven tertiary methyl groups at δ 0.80, 0.85, 0.86, 0.87, 0.95, 1.04 ppm and the vinylic methyl at δ 1.69 ppm. Also at δ 3.15 signal with its coupling ~3, 6 Hz is indicative of C_3-H α-orientation. Two vinylic proton signals at δ 4.58 and 4.70 ppm are indicative of terminal methylene group at C-20. ^{13}CNMR (100 MHz, in $CDCl_3$) Chemical shift assignments for Compound **1**. Comparison of the reported data with the identified compound revealed no significant difference

(Sundararamaiah et al., 1976; Sholichin et al., 1980). This is the first report on isolation of lupeol from the fruits of *Akocanthera* species.

Compound 2: Oleanolic acid

^{1}H-NMR and ^{13}C-NMR resonance assignments agree with the reported literature data. (**Zhang et al, 1999**)

Compound 3: Ursolic acid

^{1}H-NMR and ^{13}C-NMR resonance assignments agree with the reported literature data. (**Sang et al., 2002**)

Compound 4: Acovenoside A

^{1}H-NMR and ^{13}C-NMR resonance assignments (Table 4) agree with the reported literature data **(Hanna et al., 1999)**.

Compound 5: β-sitosterol -3-O- β-D-glucoside

White microcrystalline powder. m.p. 290°C.Rf 0.37 in S_3. MS (EI, 70 eV): *m/z* (%) = 414 $[M]^+$ (100 %), 396 (46 %), 329 (36 %), 303 (39 %), 273 (66 %) and 255 (65 %). ^{1}H-NMR: δ (300 MHz, DMSO) 0.66 (3H, d, *J*=5.5 Hz, Me-21), 0.78 (3H, t, *J*=6.3,Me-29), 0.83 (3H, d, *J*=6.2 Hz, Me-26), 0.90 (3H, d, *J*=6.3 Hz, Me-27), 0.92 (3H, s, Me-18), 0.96 (3H, s, Me-19), 3.03 (1H, m, H-3), 4.21 (1H, d, *J*=7.5, H-1`), 5.33 (H, br.s, H-6) ppm. The ^{1}H-NMR spectrum of this compound showed the characteristic signals of β-sitosterol nucleus (**Goad and Akihisa 1997)**. In addition, an anomeric proton appeared at δ 4.20 (1H, d, J= 7.5 Hz) which indicated a β-linked sugar moiety. From acid hydrolysis, the sugar moiety was identified as D-glucose by similar PC pattern alongside with authentic D–glucose. So, compound C5 was identified as β-sitosterol -3-O- β-D-glucoside (**Goad and Akihisa 1997)**.

4. Discussion

Based on its biology, therapy, and prognosis, lung cancer is divided into two major classes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (**Ettinger, et al. 2002).** Non-small cell lung cancer accounts for about 80 % of all lung cancer cases. Small cell lung cancer (SCLC) accounts nearly for 25 % of all lung cancer. About 98 % of SCLC is attributed to cigarette smoking **(Radzikowska, et al. 2002).**

Prevention is the most cost-effective means of decreasing lung cancer development. Recently targeted therapies are designed to prevent or stop lung cancer cells from growing by targeting the new blood vessels that are needed to allow the cancer cells to survive and grow; other treatments target growth and multiplication of lung cancer cells by interfering with chemical signals required by growing or multiplying cancer cells.

Plants and plant products both as extracts and derived compounds are known to be effective and versatile chemopreventive agents against various types of cancers **(Aruna and SivaramaKrishnan, 1990).** A remarkable surge of interest in chemoprevention research has thus led to the identification of many phytochemicals as effective chemopreventive agents **(Cordell et al., 2002).**

Several studies have demonstrated that extracts from some herbal medicines or their mixtures have anticancer potential and can inhibit cancer cell proliferation *in vitro* and/or *in vivo* **(Bonham, et al 2002).**

Cardiac glycosides are used for the treatment of cardiac congestion and some types of cardiac arrhythmias **(Pieter van der Bijl 2012)**.

The action of cardiac glycosides are explained by inhibition of Na+/K+ ATPase leading to an increase of intracellular Ca2+, which leads to a better interaction between actin and myosin filaments in cardiac myocytes. They were also suggested to have some anticancer activity (**Inada, et al. 1993; Haux,1999)**, however, by a mechanism different from targeting the Na+/K+ ion pump (**Haux, 2002)**. Early epidemiologic studies, evidencing significantly lower mortality rates of patients with cancer receiving cardiac glycosides (**Stenkvist, et al. 1979).**

Numerous subsequent in *vitro* and in *vivo* studies verified these observations (**Mijatovic, et al.** 2007; **Winnicka**,et al. 2006; **Lopez**, 2007). Anticancer treatments such as anthracyclines are effective; however, they induce cardiotoxicity by releasing radical oxygen species (ROS). *Acokanthera oblongifolia* isolates were evidenced cytotoxicity on lung carcinoma cell line (A-549, H-1299) as well as a cardio protection effect against cardiotoxicity of doxorubicin .We evaluated cardiodynamics, as well as biochemical and pathological parameters, to emphasize the effectiveness of the treatment with *Acokanthera* isolates.

5. Conclusion

Cytotoxic effects of acovenoside A and lupeol are reported for the first time from *Acokanthera* species. Cytotoxicity of ursolic, oleanolic and *β*-sitosterol-3-β-O-glucoside was previously reported **(Vechia, et al., 2009)**. *β*-sitosterol-3-β-O-glucoside can affect the structure of cell membranes and alters the signaling pathways that regulate tumor growth and apoptosis **(Holtz et al., 1998).** Moreover, *β*-sitosterol-3-β-O-glucoside has shown a decrease in proliferative changes and tumor yields when added to diets of mice and rats treated with colon carcinogens.

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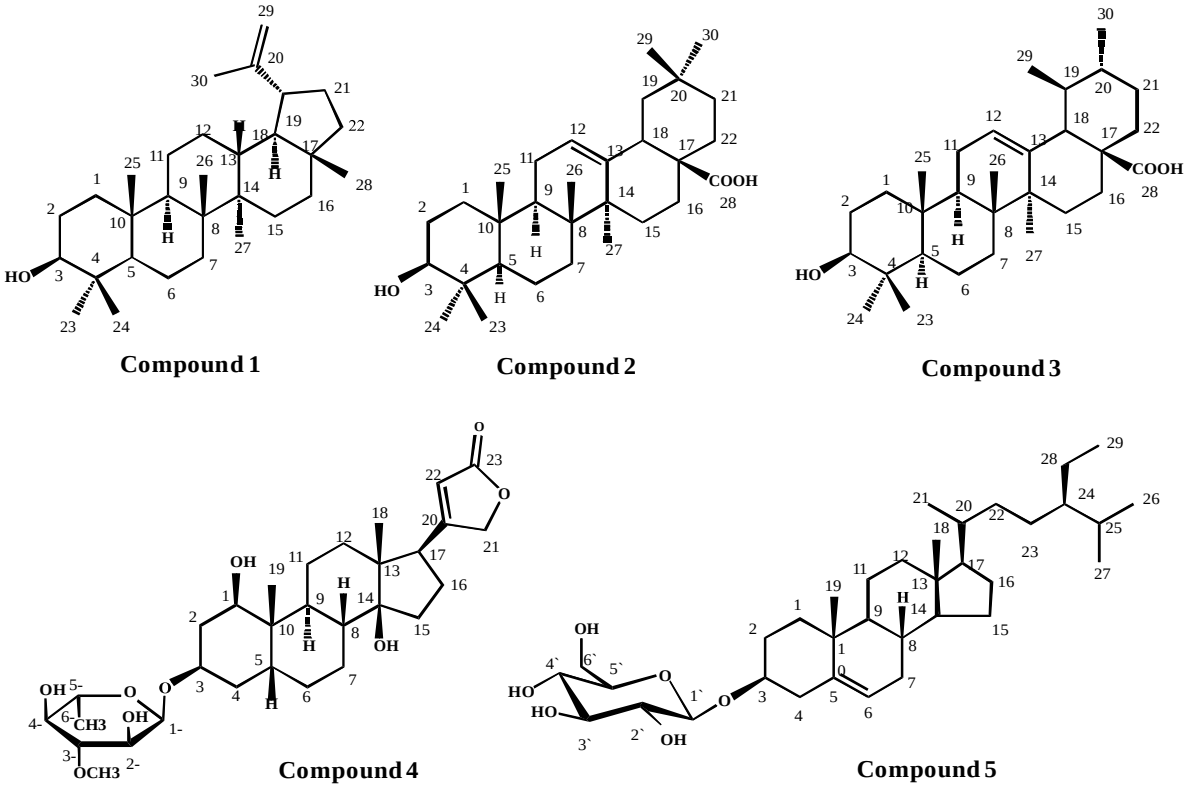

Figure 1 Structure of Compounds isolated from *Acokanthera oblongifolia* Pericarp

Table 1: Results of cytotoxic activity of isolates of *Akocanthera oblongifolia* on lung carcinoma cell lines (A549, H-1299).

Tested Human cell line	IC_{50} µg/ml				
	Lupeol (1)	Oleanolic acid (2)	Ursolic acid (3)	Acovenoside (4)	*Beta*-sitosterol-3-O-glucoside (5)
A 549	25.1	20.6	22.1	3.98	15.7
H-1299	33	28.2	22.9	4.73	22

Table2: Cytotoxicity of the isolates of *Akocanthera oblongifolia* on lung carcinoma cell line (A-549).

Conc. (µg/ml)	Mean of surviving fraction				
	C1	C2	C3	C4	C5
	Mean± SD				
0.000	1.000±0.044	1.000±0.044	1.000±0.044	1.000±0.044	1.000±0.044
5.000	0.989±0.040	0.904±0.037	0.944±0.041	0.377±0.030	0.709±0.060
12.500	0.832±0.046	0.732±0.042	0.853±0.043	0.379±0.029	0.548±0.044
25.000	0.501±0.034	0.377±0.037	0.387±0.021	0.239±0.103	0.347±0.022
50.000	0.250±0.053	0.192±0.020	0.212±0.027	0.271±0.040	0.201±0.034

Conc = concentration

Table 3: Cytotoxicity of the isolates of *Acokanthera oblongifolia* on lung carcinoma cell line (H-1299).

Conc. (µg/ml)	Mean of surviving fraction				
	C1	C2	C3	C4	C5
	Mean± SD				
0.000	1.000±0.026	1.000±0.026	1.000±0.026	1.000±0.026	1.000±0.026
5.000	0.980±0.021	0.946±0.045	0.931±0.067	0.459±0.033	0.889±0.048
12.500	0.850±0.024	0.798±0.040	0.849±0.042	0.497±0.052	0.712±0.085
25.000	0.560±0.036	0.534±0.029	0.434±0.023	0.441±0.031	0.433±0.032
50.000	0.379±0.043	0.301±0.046	0.263±0.020	0.352±0.045	0.301±0.026

Conc = concentration

Table 4: ^{1}HNMR and ^{13}CNMR (400MHz, 100MHz) of compound C4

Position	δH ppm	δC ppm
1	3.7	72.4
2	1.89, 1.95	31.7
3	**4.26**	**72.3**
4	1.83, 1.62	28.2
5	1.9	30.4
6	1.81, 1.36	25.9
7	1.72, 1.25	20.9
8	1.62	41.9
9	1.49	37.6
10	--	40.2
11	1.35, 1.36	21.1
12	1.30, 1.53	39.9
13	--	49.4
14	--	85.3
15	2.1, 1.73	33.1
16	2.14, 1.89	26.9
17	2.79	50.8
18	0.91	15.8
19	1.12	18.8
20	--	174.3
21	4.8, 5.0	73.4
22	5.9	117.8
23	--	174.1
1'	**5.0**	**97.6**
2'	3.88	68.4
3'	3.37	75
4'	3.88	69.7
5'	3.98	66.7
6'	1.36	16.5
3'-OCH_3	3.51	55.6