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Original Article

Chemical and Cytotoxic activities Study of *Cuscuta campestris* Yunck

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ABSTRACT

The purpose of this study was to investigate chemical constituents and biological activity of different extracts on different human cell lines. The phytochemical investigation of whole parasitic *Cuscuta campestris* has led to the isolation and identification of β-sitosterol (1), stigmasterol (2), (1s,2s)-1,2,3-trihydroxy-1(3',4'-methylenedioxyphenyl) propane (3) isolated for first time from *Cuscuta campestris*, d-sesamin (4), oleanolic acid (5), linocinnamarin (6) isolated for first time from *Cuscuta campestris*, mixture of isomers of quercetin-3-O-β-D-glucoside and quercetin-3-O-β-D-galactoside (7,8), astragallin (9). The methylene chloride, petroleum ether and ethyl acetate fractions were tested against human lung cancer (A-549), human colorectal (cecum) cancer (LS-513), human cervical cancer (Hela), human ovarian cancer (SKOV-3), human bladder cancer (T-24) and human pancreatic cancer (PANC-1) by using SRB assay. The ethyl acetate and petroleum ether fractions showed moderate to week cytotoxic activities, but methylene chloride fraction showed strong cytotoxic activity on all tested cell lines.

Keywords: *Cuscuta campestris;* cytotoxic activities; SRB assay; phytochemical investigation

INTRODUCTION

Cuscuta campestris Yunck belong to family Convolvulaceae. It is rootles, leafless holo parasitic plant with climbing yellowish green stems and white tiny flowers [1,2]. It had common names like Golden Dodder, field dodder and Bay Parri [3]. *Cuscuta campestris* distributed in South and North America, Asia, Africa and Pacific Ocean [4]. Dodder rich with many phytochemical compounds as: coumarin, alkaloids, flavonoids, sterols, lignins, fatty acids, hydroxyl cinnamic acid derivative and glucosides [3-6]. Cuscuta campestris has many different hosts from family Brassicaceae,

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Leguminosae, Solanaceae and others [7]. It depended on its host to absorb water and nutrients by using haustoria that can penetrate and reach to the xylem of its host [7]. In folk medicine Dodder is used to strength skeletal musceles, improve vision, premature ejaculation, urinary frequency, diarrhea, back pain, dementia, prostate problem, abortion, vertigo, blurred vision and renal and hepatic diseases [8]. The biological activities of *Cuscuta campestris* were immunostimulant, antioxidant, anti-inflammatory, hepatoprotective, renal failure, antimicrobial, antifungal and anticancer [8-11]. The purpose of this study was to investigate the chemical constituents of the methanolic extract of the whole plant of *C.campestris* which led to report two compounds isolated and identified for the first time from *C.campestris*.

On the other hand, we measured the cytotoxic activities of the petroleum ether, methylene chloride and ethyl acetate fractions of the methanolic extract of *C.campestris* on six cancer cell lines: human lung cancer (A-549), human colorectal (cecum) cancer (LS-513), human cervical cancer (Hela), human ovarian cancer (SKOV-3), human bladder cancer (T-24) and human pancreatic cancer (PANC-1) by using SRB assay.

MATERIAL AND METHODS

***General*:** UV spectra was carried out on UV-visible spectrophotometer (Shimadzu 1601 PC, model TCC-240A, Japan) using spectroscopic methanol. IR spectra (cm^{-1}) was carried out on FTIR spectrometer, Rotary flash evaporator (Büchi, Switzerland) for evaporation the extracts and fractions, sonicator, all in central lab, Faculty of Pharmacy, Mansoura University. Nuclear Magnetic Resonance spectra (H^1-NMR, C^{13}-NMR, APT, HSQC, HMBC, COSY and NOESY) were obtained on a Bruker Avance III 400 spectrometer (400 and 100 MHz for ^1H and ^{13}C-NMR, respectively) in SMR unit, Faculty of Pharmacy, Mansoura University. The solvents used are CD_3OD, CD_3Cl, and DMSO-d6. Mass spectra were obtained on LC/MS/MS UPLC API 4000 (Sciex, Concord, Ontario, L4K 4V8, Canada) in Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt. Normal phase chromatography was carried out using silica gel G 60-230 mesh (Merck, Germany) packed by the dry method or the wet method in the specified solvent. Preparative reversed HPLC analysis, Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector, Column C_{18} Kromasil: 1cmx25cm, 5µm, in Nawah scientific, Cairo, Egypt. BMG LABTECH – FLUOstar OMEGA microplate reader (Ortenberg, Germany), in Nawah scientific, Cairo, Egypt. Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector. Sample was dissolved in 1 ml methanol sonicated for 15 min, filtered using 0.22 µm syringe filter then 100 µl was injected.

HPLC analysis conditions:
- Column C_{18} Kromasil: 1cmx25cm, 5µm.
- Mobile phase: Water and Methanol.
- Mode of elution: Gradient.
- Flow rate: 3ml/min.
- Temperature: Ambient.
- Wavelength: 414 nm**.**

Peaks Collected at RT (run time) 14.78, 16.4, 17.3 and 17.93 minutes.

Plant material: The plant material used in this study consists of whole parts of *C. campestris* (Convolvulaceae) was collected from the farm of Faculty of Agriculture, Mansoura Univeristy, Egypt during February, 2015, and identified by Prof. Dr. Ibrahim Mashaly, Professor of Ecology, Faculty of Science, Mansoura University, Egypt.

Preparation of the extracts: The plant material was air dried then powdered at room temperature (524g) after that extracted by maceration in methanol at room temperature till exhaustion. The collected methanolic extract was evaporated under reduced pressure to give (115.6 g) of viscous residue. The dried methanolic extract was dissolved in a minimum volume of methanol, diluted with 750 ml distilled water, fractionated using solvents: petroleum ether (3 x 1L), methylene chloride (2 x 1 L) and ethyl acetate (3 x 1L). The solvent, in each case, was evaporated to dryness under reduced pressure giving petroleum ether fraction (11.53 g), methylene chloride fraction (8.94 g) and ethyl acetate fraction (16.68 g).

A-1. Isolation of compounds: Petroleum ether fraction (9.53 g) was applied onto the top of a glass column (120 x 5 cm) packed with silica gel (180 g). The column was then gradiently eluted with petroleum ether-ethyl acetate (until 100% ethyl acetate) then washed with methanol. Collected fractions were subjected to purification through column chromatography and repeated crystallization to give compound 1 (3 mg) and compound 2 (3 mg) in pure form.

Methylene chloride fraction (7g) was applied onto the top of a glass column (120 x 5 cm) packed with silica gel (140 g). The column was then gradiently eluted with petroleum ether- ethyl acetate (until 100% ethyl acetate) then washed with methanol. Collected fractions were subjected to purification through column chromatography and repeated crystallization to give compound 3 (5 mg), compound 4(8 mg) and compound 5 (6 mg) in pure form.

Ethyl acetate fraction (14.68 g) was applied onto the top of a glass column (120 x 5 cm) packed with silica gel (293.6 g). The column was then gradiently eluted with methylene chloride- ethyl acetate (until 100% ethyl acetate) then washed with methanol. Collected fraction from methylene chloride – ethyl acetate (10:90) was subjected to purification through column chromatography to give compound 6 (10 mg) in pure form. The wash of ethyl acetate fraction with methanol led to isolate compound 7 (11mg) and compound 8 (10.5mg) by using preparative and reversed HPLC.

A-2. Identification of the isolated compounds: nine compounds were isolated from *Cuscuta campestris* extract. Compounds 1-9 (Figure 1) were identified by comparing their data with chemical, physical and spectroscopic published data. These compounds were β-sitosterol(1), stigmasterol (2), (1s,2s)-1,2,3-trihydroxy-1(3',4'-methylene dioxyphenyl) propane (3) isolated for first from *Cuscuta campestris*, d-sesamin (4), oleanolic acid (5), linocinnamarin (6) isolated for first time from *Cuscuta campestris*, mixture of isomers of quercetin-3-O-β-D-glucoside and quercetin-3-O-β-D-galactoside (7,8) , astragallin (9) .

Compound (3): was obtained from methylene chloride fraction as white crystal (5mg). Solvent system was pet.ether:ethyl acetat (80:20), m.p 114-118 °C , soluble in chloroform, sparingly soluble in methanol and insoluble in water. It's moleculer formula $C_{10}H_{12}O_5$, MS: *m/z* 213.076 $[M+H]^+$. according to ^{1}H-NMR using deuterated chloroform as a solvent we noted that at δ 6.84 (1 H, d, J = 1.5 Hz, H-2') ,δ 6.80 (1H, dd, J = 8.0, 1.5 Hz, H-6') ,δ 6.77 (1H, d, J = 8.0 Hz, H5') were assigned to aromatic protons,δ 5.95 (2H, s, protons of methylene dioxy),δ 4.71 (1H, d, J = 4Hz, H-l),δ 4.23 (1H, dd, J=9.5, 7 Hz, H_a-3),δ 3.87 (1H, dd, J = 9.5, 4.0 HZ, H_b-3), δ 3.07 (1H, m, H-2) . ^{13}C-NMR spectrum of compound 3 exhibited signals at 147.93 (C-3'), 147.1 (C-4'), 135.0 (C-1'), 119.35 (C-6'), 108.1 (C-5'), 106.47 (C-2'), 101.05 (OCH2), 87.6 (C-1), 72.8 (C-3), 54.61(C-2). The spectrum have one doublet-doublet at δ 6.80 (1H, dd, J = 8.0, 1.5 Hz, H-6'), two doublets at δ 6.84 (1 H, d, J = 1.5 Hz, H-2') , δ 6.77 (1H, d, J = 8.0 Hz, H5') , and singlet at δ 5.95 (2H) ,indicating the presence of methylene dioxy benzene structure. Compound(3) was (1S, 2S)-1,2,3-Trihydroxy-1-(3',4'-methylenedioxyphenyl) propane which was isolated for first time from *Cuscuta campestris,* and this reported data was compared with the published data [12].

Compound (6): Colorless needles, soluble in methanol, It's moleculer formula was $C_{16}H_{20}O_8$, MS: *m/z* 341 $[M+H]^+$. ^{1}H- and ^{13}C NMR (CD_3OD), ^{1}H NMR 400 MHz, 7.6 (d, J = 16.0 Hz, H-b), 7.45 (d, J = 8.5 Hz, H–2, H–6), 7.03 (d, J = 8.5 Hz, H–3, H–5), 6.34 (d, J = 16.0 Hz, H–a), 4.9 (d, J = 7 Hz, H–anomeric), 3.73 (s, proton of methoxy) 3.62–3.59 (2H, m H-6'a and H-6'b), 3.38–3.31 (H-of sugar), ^{13}C NMR (400 MHz,CD_3OD): 167.99 (COO), 159.5 (C-4), 144.46 (C-b), 129.4 (C-2, C-6), 128.45 (C-1), 116.57 (C-3, C-5), 115.3 (C-a), 100.4 (C-anomeric), 50.68 (C-methoxy) 76.9 (C-5'), 76.6 (C-3'), 73.5 (C-2'), 69.9 (C-4'), 61.1 (C-6'). Compound 6 was Linocinnamarin which was isolated for first time from *Cuscuta campestris*, and this reported data was compared with the published data [13].

Cell culture: human lung cancer (A-549), human colorectal (cecum) cancer (LS-513), human cervical cancer (Hela), human ovarian cancer (SKOV-3), human bladder cancer (T-24) and human pancreatic cancer (PANC-1) were obtained from Nawah Scientific, (Cairo, Egypt). Cells were maintained in DMEM (Dulbecoo's Modified Eagels Medium), media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO2 atmosphere at 37 °C.

SRB Cytotoxicity assay: Cell viability was assessed by SRB (Sulphorodamine B) assay. Aliquots of 100µL cell suspension (5x10^3 cells) were in 96-well plates and incubated in complete media for 24 h. Cells were treated with another aliquot of 100µL media containing drugs (prtroleum ether, methylene chloride, ethyl acetate fractions) at various concentrations ranging from (0.01,0.1,1,10,100 ug/ml). After 72 h of drug exposure, cells were fixed by replacing media with 150µL of 10% TCA (tri chloro acetic acid) and incubated at 4 °C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70µL SRB solution (0.4% w/v) were added and incubated in a dark place at room temperature for 10 min. Plates were washed 3 times with 1% acetic acid and allowed to air-dry overnight. Then, 150µL of Tris base (10 mM) was added to dissolve protein-bound SRB stain; the absorbance was measured at 540 nm using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany). The IC50 value was calculated from the curves generated by plotting the percentage of the viable cells versus test concentrations on a logarithmic scale using SigmaPlot 12.0 software [14-16] .

Statistical analysis: Statistical analysis of IC_{50} values were calculated from curves generated by plotting the percentage of the viable cells versus test concentrations on a logarithmic scale using SigmaPlot 12.0 software. (System Software, San Jose, CA, USA). All experiments were carried out

independently at least three times. Data were presented as mean ± SD.

RESULTS AND DISCUSSION

The investigation of this study led to the isolation of nine compounds two of them were first time isolated from *Cuscuta campestris*. The ethyl acetate, methylene chloride and petroleum ether fractions were studied for their cytotoxic activity against different cell lines as mentioned before by using SRB assay. The methylene chloride fraction showed strong ctotoxic activity on all type of cell lines compared to negative control. The petroleum ether fraction showed moderate to week cytotoxic activity on SKOV-3, PANC-1 and A-549, while LS-513, T-24 and Hela have very week cytotoxic activity. The ethyl acetate fraction heve very week cytotoxicity on all used cell lines except PANC-1 cell line produced moderate cytotoxicity. Absorbance values that are lower than the control cells absorbance values refer to a reduction in the rate of cell proliferation. All data presented in table (1) illustrate the cytotoxic activity of methylene chloride fraction on experimented cell lines and give comparison between the absorbance values of cell control and different concentration of methylene chloride fraction.

*%*cell survival= {(At-Ab)/ (Ac-Ab)} x100

Where,

At= Absorbance value of test fraction

Ab= Absorbance value of blank (0.031)

Ac=Absorbance value of control

% cell inhibition= 100-cell survival

CONCLUSION

The present study showed that the parasitic plant *Cuscuta campestris* could be potential used for developing high safety cytotoxic agent for treatment of human lung, cecum, cervical, ovarian, bladder and pancreatic cancers. These activities can be partly attributed to methylene chloride fraction which showed the highest in vitro inhibitory activities among the petroleum ether and ethyl acetate fractions on all types of tested cell lines. While ethyl acetate fraction is cytotoxic on human panceriatic cancer, while the petroleum ether fraction showed cytotoxic effect on human panceriatic, ovarian and lung cancer. The isolation of (1S, 2S)-1, 2, 3-Trihydroxy-1-(3',4'-methylenedioxyphenyl) propane compound (3) and Linocinnamarin compound (6) was the first time of isolation from *Cuscuta campestris*.

Table (1): cytotoxic activity of methylene chloride fraction on experimented cell lines:

Number	**Cell lines**	**Cells control absorbance**	**Absorbance at different concentration of methylene chloride fraction**					**IC_{50}**
1			**0.01**	**0.1**	**1**	**10**	**100**	
2	A-549	2.0565	1.9275	1.9125	1.874	1.558	0.011	16.4
3	LS-513	2.628	2.596	2.593	2.578	2.478	0.0465	26.4
4	Hela	0.911	0.917	0.911	0.899	0.889	0.015	28.2
5	SKOV-3	0.2467	0.237	0.229	0.222	0.215	0.031	30
6	T-24	1.972	1.964	1.95	1.93	1.891	0.008	24
7	PANC-1	1.0595	1.049	1.03	0.971	0.786	0.047	18.4

Table (2): the viability % at different concentration (ug/ml) of methylene chloride fraction on the tested cell lines .

Numbers	**conc**	**A-549**	**LS-513**	**Hela**	**SKOV-3**	**T-24**	**PANC-1**
1	0.01	93.679	98.833	99.42	96.216	99.594	98.553
2	0.1	92.998	98.668	99.13	92.838	98.851	97.279
3	1	91.142	98.135	97.717	90.135	97.87	91.427
4	10	75.76	94.305	96.703	87.297	95.892	74.217
5	100	0.6484	1.7694	1.5942	12.432	0.3888	4.4675
6	**IC_{50}**	16.4	26.4	28.2	30	24	18.4

Table (3): the viability% at different concentration (ug/ml) of ethyl acetate fraction on tested cell lines .

Numbers	**conc**	**A-549**	**LS-513**	**Hela**	**SKOV-3**	**T-24**	**PANC-1**
1	0.01	95.916	95.412	93.885	99.59	88.812	99.413
2	0.1	93.377	97.312	91.187	96.516	88.481	98.9
3	1	93.24	96.193	89.045	93.443	84.963	97.912
4	10	83.007	93.92	87.247	84.153	83.805	86.131
5	100	73.853	91.665	50	71.311	82.453	42.516
6	**IC_{50}**	>100	>100	121	>100	>100	72

Table (4): the viability% at different concentration (ug/ml) of petroleum ether fraction on tested cell lines .

Numbers	conc	A-549	LS-513	Hela	SKOV-3	T-24	PANC-1
1	0.01	98.498	98.933	99.763	91.397	99.136	98.224
2	0.1	96.67	96.045	99.566	90.039	98.793	96.788
3	1	99.674	94.007	98.314	86.999	95.408	95.978
4	10	96.807	92.179	94.394	86.611	92.863	86.315
5	100	44.979	89.893	53.178	45.213	68.162	41.734
6	**IC_{50}**	87.9	>100	111	90.9	>100	70.7

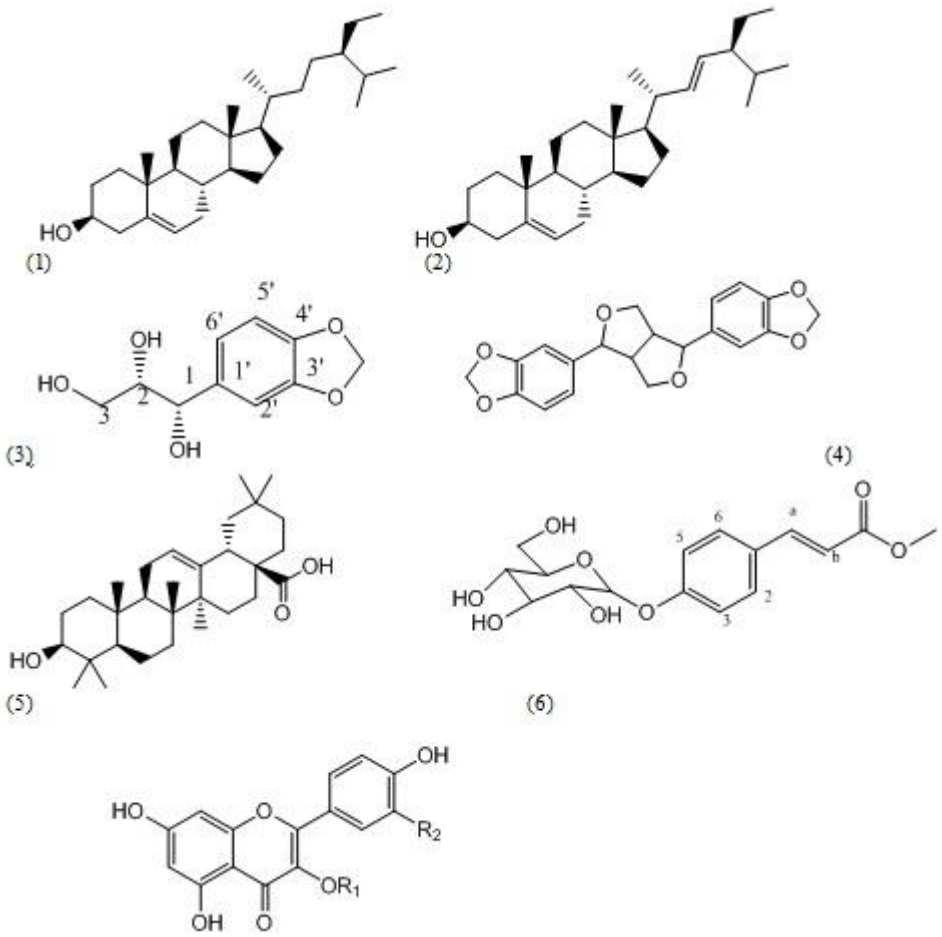

Figure (1): natural compound isolated from *C.campestris*.

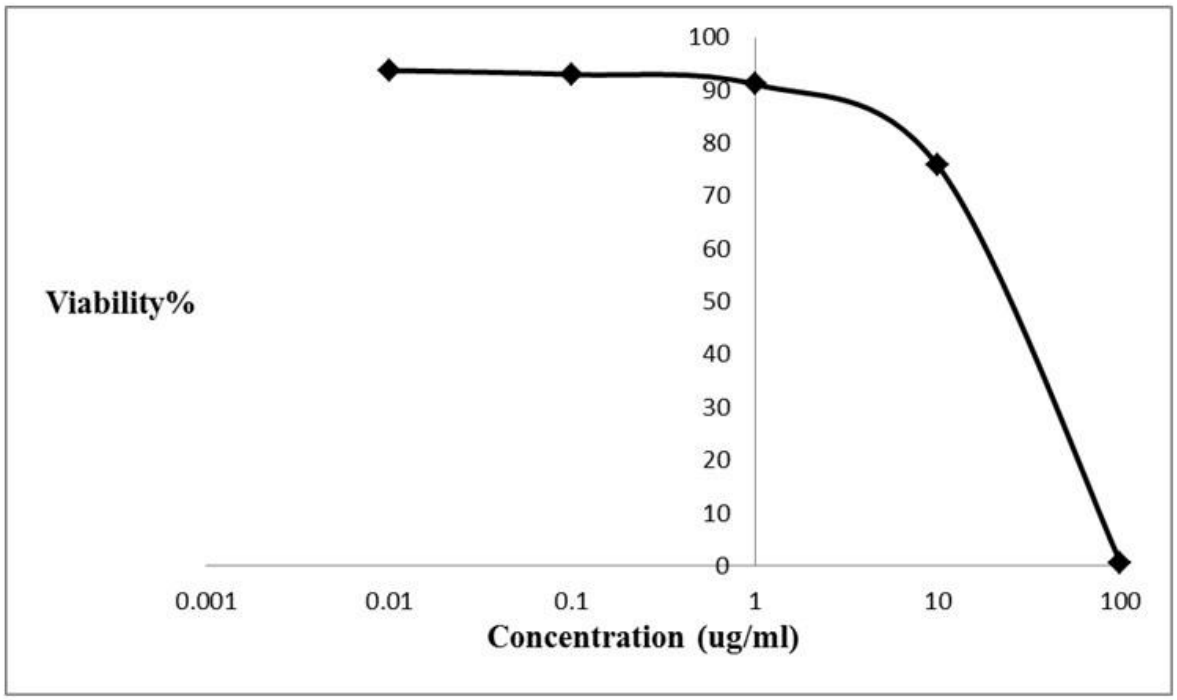

Figure (2): cytotoxic activity of methylene chloride fraction against human lung cancer A-549 cell line using SRB assay .The cell inhibition at10 ug/ml was 24.24% and at 100ug/ml was 99.352% which indicate very strong cytotoxicity with IC_{50} 16.4 ug/ml.

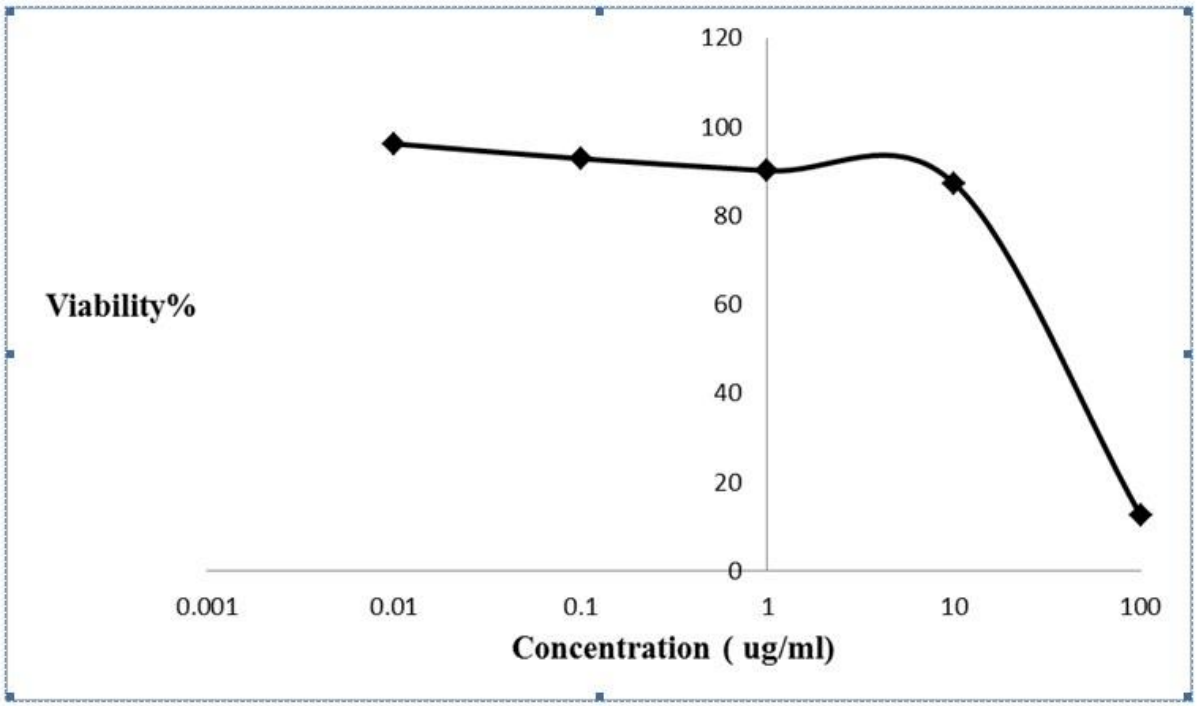

Figure (3): cytotoxic activity of methylene chloride fraction against humanovarian SKOV-3 cancer cell line using SRB assay .The cell inhibition at 100ug/ml was 87.568% which indicate strong cytotoxicity with IC_{50} 30ug/ml.

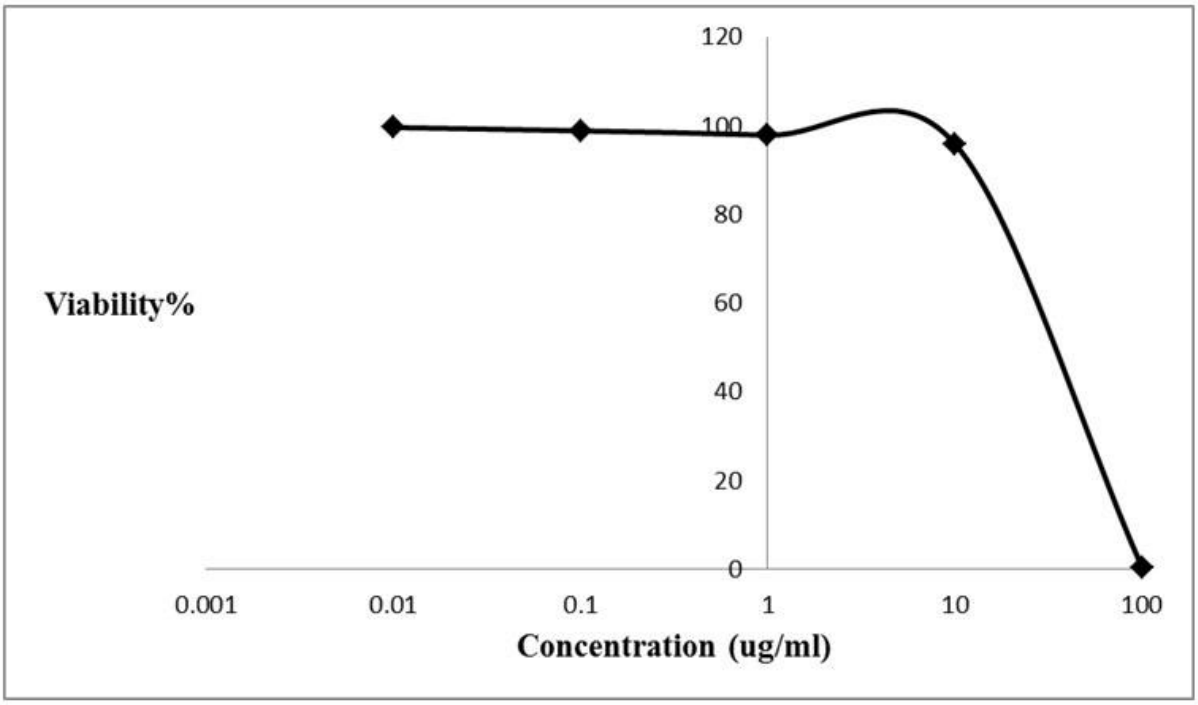

Figure (4): cytotoxic activity of methylene chloride fraction against human bladder T-24 cancer cell line using SRB assay .The cell inhibition at 100ug/ml was. 99.6112 % which indicate very strong cytotoxicity with IC_{50} 24 ug/ml.

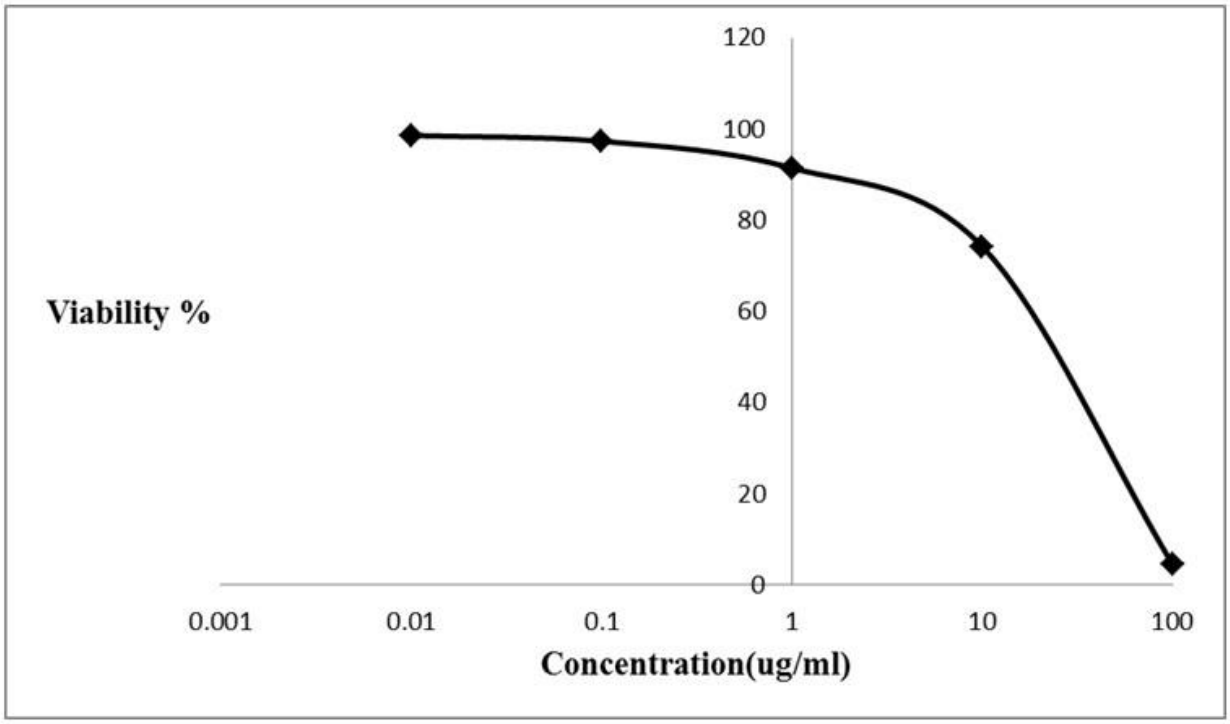

Figure (5): cytotoxic activity of methylene chloride fraction against human pancreatic PANC-1 cancer cell line using SRB assay .The cell inhibition at 100ug/ml was. 95.533 % which indicate very strong cytotoxicity with IC_{50} 18.4 ug/ml.

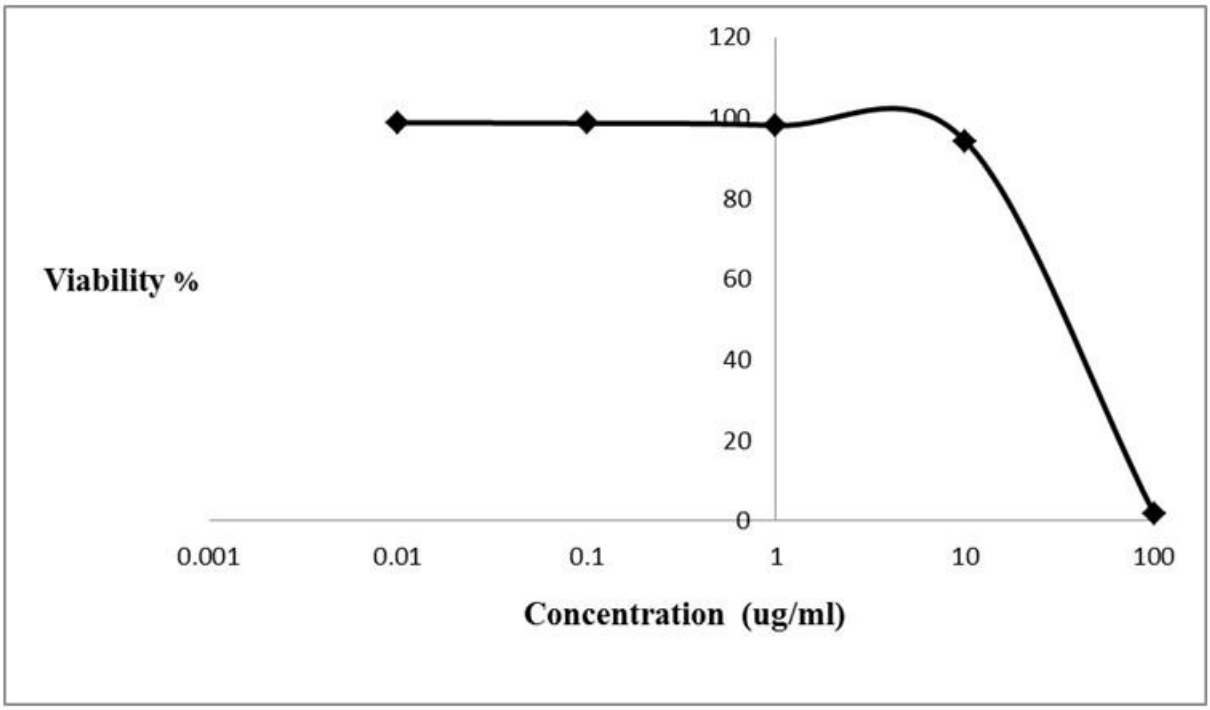

Figure (6): cytotoxic activity of methylene chloride fraction against human colorectal (cecum) LS-513 cancer cell line using SRB assay .The cell inhibition at 100 ug/ml was 98.231 % which indicate very strong cytotoxicity with IC_{50} 26.4 ug/ml.

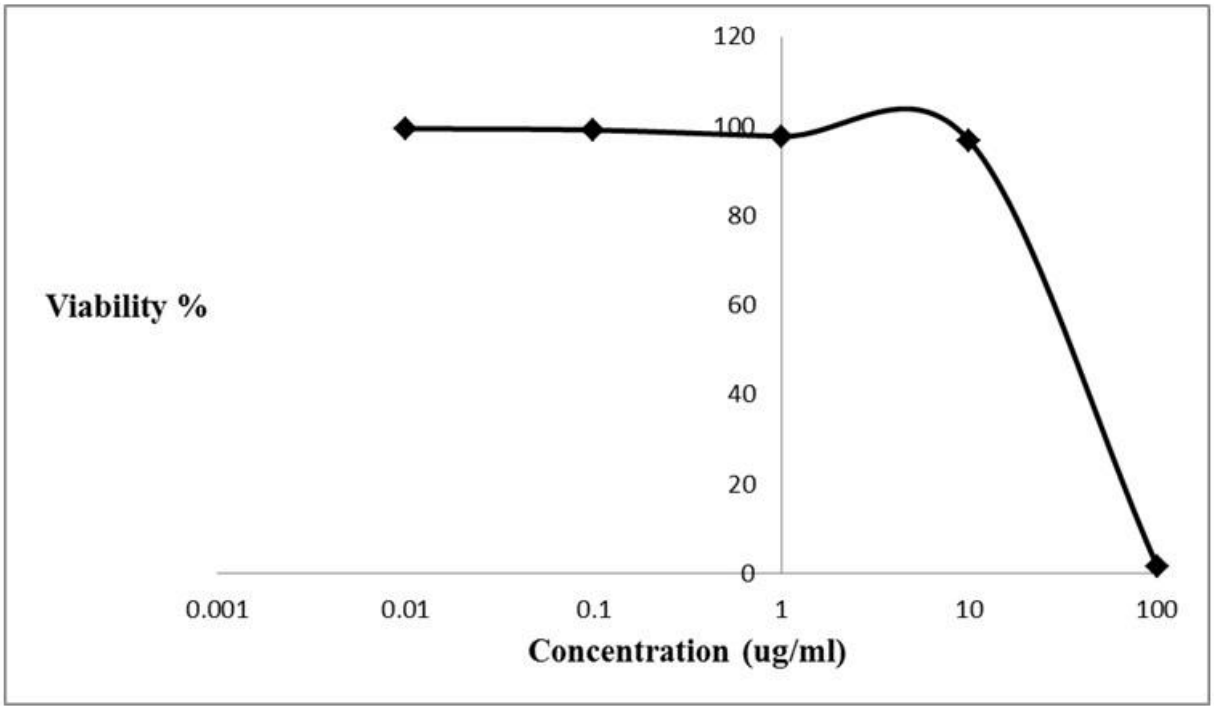

Figure (7): cytotoxic activity of methylene chloride fraction against human cervical (Hela) cancer cell line using SRB assay .The cell inhibition at 100 ug/ml was 98.406 % which indicate very strong cytotoxicity with IC_{50} 28.2 ug/ml.

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