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ORIGINAL ARTICLE

Anticancer Effect of Different Extracts of *Cynanchum acutum* L. Seeds on Cancer Cell Lines

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ABSTRACT

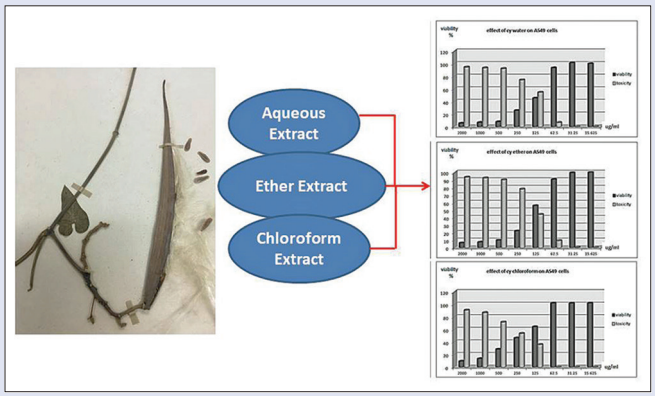
Background: *Cynanchum acutum* L. is a perennial medicinal herb, used for several traditional medicine purposes in Egypt and around the world. **Objective:** The anticancer effects of ether, chloroform subfraction, and aqueous extracts of *C. acutum* seeds were investigated. **Materials and Methods:** *C. acutum* seeds were extracted by ether, chloroform, and aqueous. Then, all extracts were subjected to gas chromatography/mass spectrometry (GC/MS) to determine alkaloids and other bioactive compounds. Furthermore, anticancer effects on malignant cell lines as well as normal cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **Results:** GC/MS analysis of the different extracts of *C. acutum* seeds revealed different compounds of alkane hydrocarbons, fatty acids, sterols, terpenoids, flavonoids, and alkaloids. The significant components of unsaturated fatty acids were linoleic acid (56.71%) in the ether extract and oleic acid (6.59%) in the chloroform extract, whereas the major saturated fatty acid was palmitic acid (3.51%) in the ether extract. Furthermore, the anticancer effects of the three extracts of the plant seeds on all tested cancer cell lines were minor. However, the higher anticancer effects were observed on lung (A549) and breast (MCF-7) cancers by water and ether extracts, respectively, as compared to positive control. Furthermore, both extracts showed low cytotoxicity toward normal cell line (WI-38) comparing to a positive control. **Conclusion:** Aqueous and ether extracts of *C. acutum* L. seeds could be considered as potential chemotherapeutic agents in lung and breast cancer treatments, respectively, with reduced systemic adverse effects.

Key words: Alkaloids, breast cancer, *Cynanchum acutum,* flavonoids, gas chromatography/mass spectrometry, lung cancer

SUMMARY

- In gas chromatography/mass spectrometry evaluation of *Cynanchum acutum* seeds, dihydrovallesiachotamine, voacristine hydroxyindolenine, pseudojervine, 6,7-dihydro-neblininane, aspidofractinine-3-methanol, phytol, cedrene, oleic acid, linoleic acid, lucenin-2, and quercetin 7,3,4-trimethoxy were identified as anticancer agents from previously reported literature.

Aqueous and ether extracts of *Cynanchum acutum* seeds possessed highly anticancer effects on lung cancer cell line (A549) and breast cancer cell lines (MCF-7), respectively, whereas chloroform extract demonstrated low anticancer activity against all studied cancer cell lines comparing to a positive control. Aqueous, ether, and chloroform extracts showed low cytotoxicities against normal cell lines (WI-38) as compared to a positive control.



Abbreviations used: MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Eth: Ether extract; Chl: Chloroform extract; Aqu: Aquatic extract of seeds.

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INTRODUCTION

Recently, medicinal plants are being investigated for promising new drugs in cancer treatment. *Cynanchum acutum* L., Asclepiadaceae, is a medicinal climber herb. It is found around canal bank edges of cultivation areas of the River Nile and Mediterranean regions.[1] It is used traditionally in Egypt and around the world for the treatment of various diseases, including skin diseases, pimples,[2] skin ulcers,[3] diabetes,[4] and bacterial infections.[5] It exhibited anticancer effects against colon cancer cell line (HCT-116) and hepatocellular carcinoma cell line (HepG2).[6] Furthermore, Estakhr *et al.* (2012) reported that this plant has an anti-inflammatory effect.[7] The phytochemical investigations on *C. acutum* have revealed the presence of several natural compounds including β-sitosterol, lupeol, lupyl acetate, and α-amyrin;[8] four flavonoid glycosides[9] as well as seven flavonoids;[4] acylated flavonoid diglycosides;[10] and two simple coumarins such as scopoletin and scoparone.[11] Some species of genus *Cynanchum* which have close affiliations with *C. acutum*

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possessed bioactive compounds such as novel triterpene,[12] sinapic acid esters and phenolic glycoside,[13] alkaloids,[14-16] pregnane sapogenins,[17] carbohydrates,[18] steroidal glycosides,[19] and flavonoid compounds.[20] The medicinal importance of the seeds alone of *C. acutum* has not been investigated. Furthermore, the bioactive compounds that are in the seeds only have not been reported in the literature. We previously investigated the anticancer effects of the methanol extract of *C. acutum* seeds on different cancer cell lines.[21] The data showed potential anticancer activities against all studied cancer cell lines.[21] Furthermore, we identified several bioactive compounds in the methanol extract of the seeds alone.[21] We reported that seventeen phenolic acids and nineteen flavonoids were identified and quantified in the methanol extract of *C. acutum* seeds.[21] Kaempferol-3-glucoside-2"-P-coumaroyl was a flavonoid identified for the first time.[21] However, the different extracts of the seeds have not been investigated. Thus, this study aims to focus on the identification and quantification of new bioactive compounds of the different extracts (ether, chloroform, and aqueous) of *C. acutum* seed extracts and their anticancer effects against various cancer cell lines.

MATERIALS AND METHODS

Plant material

The seeds of *C. acutum* were collected from November to December 2017 from the Damietta Branch of the River Nile, Egypt. The plant was kindly authenticated by Plant Taxonomist, Professor Iman Al-Gohary, Department of Plant Ecology and Range, Desert Research Centre, Cairo, Egypt, and a voucher specimen was kept at the Herbarium of the Centre with code number (CAIH-0005R).

Extraction of plant seeds

Seeds were separated, washed, dried in shade, and ground to fine powder. One hundred grams of powdered *C. acutum* seeds was extracted with diethyl ether (500 ml 3× times) by cold percolation method on a shaker for 72 h. The ether extract was filtered in a Buchner funnel. After the removal of ether in a rotary evaporator at a temperature below 40°C, the residue was dried in a dissector to obtain the ether crude Ether extract (Eth). The finely powdered seeds were thoroughly moistened with 28% NH_4OH solution (pH = 9) and then were dried. The seed powder was percolated with methanol for 72 h (300 ml 3×) on a shaker. The extract filtered was neutralized with HCl (1%) and evaporated to its half volume and then extracted three times with chloroform (25 ml) in a separatory funnel. The resulting chloroform extract was dried and evaporated to obtain the alkaloid compounds in chloroform crude (Chl).[22] The resulting aqueous extract was dried and evaporated to obtain the aqueous crude (Aqu). The crudes of ether, chloroform, and aqueous extracts were subjected to GC/MS to determine the alkaloids and other bioactive compounds.

Gas chromatography/mass spectrometry

The samples of *C. acutum* L. seeds were analyzed by gas chromatography/mass spectrometry (GC-MS) (Shimadzu GC/MS-QP 5050A) instrument using a DBI column (30 mm × 0.25 mm ID × 0.25 µm, film thickness). Constant flow at 1 ml/min of carrier gas (Helium) was used for sample analysis. The injector temperature of the instrument was programmed at 280°C. Oven temperature was started from 40°C to 280°C with a ramp of 2°C/min and withholding time of 7.5 min. The injection volumes were 1 µl. The temperature of the ion source was set at 280°C. Ionization of the sample was performed in electron impact mode at an ionization voltage of 70 eV with mass range used from m/z 50-650. Interpretation of GC-MS data was performed using the database of Wiley and Nist libraries.

Cell lines

Lung cancer (A549), prostate cancer (PC3), breast cancer (MCF-7), hepatocellular carcinoma (HepG-2), colon cancer (Caco-2), and normal human fetal lung fibroblast (WI-38) were obtained from tissue culture laboratory, Vacsera, Dokkey, Giza, Egypt.

Culturing of cell lines

Laminar air flow cabinet was used to maintain the sterility of the procedure. The cell culture was maintained in Roswell Park Memorial Institute medium (RPMI 1640). It contained 1% antibiotic–antimycotic mixture (10,000 µg/ml streptomycin sulfate, 25 µg/ml amphotericin B, and 10,000 U/ml potassium penicillin) and 1% L-glutamine. Fetal bovine serum (10% heat-inactivated) was used to supplement the medium.[6] Culturing and subculturing were performed according to Thabrew *et al.*[23]

In vitro cytotoxicity assay

The cytotoxicity was evaluated by the mitochondrial-dependent reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The yellow MTT undergoes a mitochondrial reduction to form a purple formazan.[24] The 96-well tissue culture microplate was inoculated at a cell concentration of 1×10^5 cells per well in 100 µl of growth medium. The microplate was incubated at 37°C (5% CO_2) for 24 h to develop a complete monolayer sheet. Growth medium was decanted from 96-well microplate after the formation of the confluent layer of cells. The extract of the seeds was dissolved in dimethyl sulfoxide (DMSO). Serial dilutions of the dissolved extract were prepared by RPMI–1640 medium to give a final concentration of 1000, 500, 250, 125, 62.5, 31.25, and 15.625 µg/ml. 0.1 ml of each concentration of the extract was added to confluent cell monolayers dispensed into 96-well microplate using a multichannel pipette. The treated cells were incubated at 37°C (5% CO_2) for 24 h. Three wells were used for each concentration of the extract. Control cells were incubated without the seeds extract. MTT powder was dissolved in phosphate-buffered solution (Bio Basic Canada Inc.,) to give a solution at the concentration of 5 mg/ml. After the end of the incubation period, 20 µl of the MTT solution was added to each well. The mixing was allowed at 150 rpm for 5 min using the shaker. Then, the 96-well microplate was incubated at 37°C (5% CO_2) for 4 h to allow the MTT metabolism. Formazan (MTT metabolic product) was resuspended in 200 µl of DMSO and placed on a shaker at 150 rpm for 5 min for a thorough mixing. The optical density was recorded using a microplate reader (Mindray-96A, China.) at 560 nm. The results were corrected using a reference wavelength of 620 nm (wells without adding MTT).[23] All experiments were carried out in triplicate.

Determination of IC_{50} values

GraphPad Prism version 5 software, Inc., California, U.S.A was used to calculate IC_{50} (the half maximal inhibitory concentration) values of different extracts of *C. acutum* seeds and doxorubicin (as a positive control) against A549, PC-3, MCF-7, HepG-2, Caco-2, and WI-38. The percentage growth inhibition was calculated using Equation:

$$\%\ \text{Growth inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \quad (1)$$

Statistical analysis

The parameters of experiments were conducted, and the results were presented as mean ± standard deviation. The statistical significance of the data obtained from *in vitro* studies was evaluated by the *t*-test at $P < 0.05$,

$P < 0.001$, and $P < 0.0001$ using Statistical software (GraphPad InStat, Version 3.10, GraphPad Software, Inc. USA).

RESULTS AND DISCUSSION

In the present study, we identify the compounds that were responsible for the anticancer activity of seeds of *C. acutum* extracts using GC/MS technique to help in chemical profiling and standardization of the extracts. The results of GC/MS analysis revealed that the seeds were found to contain different classes of bioactive secondary metabolites including hydrocarbons, fatty acids, sterols, flavonoids, terpenes, and alkaloids [Table 1 and Figure 1]. Sesquiterpenoids such as cedrene were detected in the ether extract. The total lipid content of *C. acutum* seeds reached a value of 12.7 g/100 g seed powder. The major components of unsaturated fatty acids were linoleic acid (RA = 56.71%) in the ether extract and oleic acid (RA = 6.59%) in the chloroform extract. On the other hand, the saturated fatty acids represented about 8.92% in the ether extract, of which palmitic acid has a relative abundance (RA = 3.51%). These fatty acids were reported to have anti-inflammatory and anticancer activity.[25-27] Furthermore, fatty acids, fatty acid ester, and aliphatic alkanes have antimicrobial and cytotoxic effects.[27] Diterpene alcohol and phytol were identified in the ether and chloroform seed extracts, whereas phytol acetate was identified in the aqueous extract (RA = 3.38%). Total phenolic compound and flavonoid contents of seeds of *C. acutum* were 39 mg gallic acid equivalents per gram dry weight and 25 mg catechin equivalents per gram dry weight, respectively.[21] Flavonoid compound, lucenine 2 (luteolin 6,8-di-C-glucoside), was identified in all seeds extracts, whereas quercetin 7,3,4-trimethoxy was identified in both aqueous and chloroform extracts. These flavonoids have antioxidant and anti-inflammatory activities.[28] The sterols, cholestan-3-ol, 2 methylene (RA = 0.30%) and corticosterone (RA = 0.16%) were identified in the chloroform extract. Furthermore, 5α-cholestan-3-one, cyclic ethylene acetate, was identified only in the aqueous extracts. These sterols were reported to have anti-inflammatory effect.[29]

The alkaloid content of seeds of *C. acutum* amounted was 5.26 ± 0.2 mg/g of dried material. Twelve alkaloids were identified in the seeds of *C. acutum*. Pleiocarpamine, colchifoline, and formyl colchicines were identified in ether extract. However, pseudojervine, 6,7 -dihydroneblininane, and dihydrocorynantheine acetate were identified in the chloroform

Table 1: Chemical constituent identification of different extracts of *Cynanchum acutum* seeds by gas chromatography/mass spectrometry

n	Compounds	MF	MW	Eth		Chl		Aqu	
				RA	Rt	RA	Rt	RA	Rt
1	Dodecane	$C_{12}H_{26}$	170	0.11	19.30				
2	Tridecane	$C_{13}H_{28}$	184	0.12	22.20				
3	Phytol	$C_{20}H_{40}O$	296	0.06	24.21	2.38	41.47		
4	Phytol acetate	$C_{22}H_{42}O_2$	338					3.38	41.47
5	Tetradecane	$C_{14}H_{30}$	198	0.31	24.93				
6	Cedrene	$C_{15}H_{24}$	204	0.1	25.57				
7	5-methyl octadecane	$C_{19}H_{40}$	268	0.07	26.41				
8	Docosane	$C_{22}H_{46}$	310	0.30	26.52				
9	Pentadecane	$C_{15}H_{32}$	212	0.33	27.47				
10	Pleiocarpamine	$C_{20}H_{22}N_2O_2$	322	0.09	34.27				
11	2-Cis-9-octadecenyl oxyethanol	$C_{20}H_{40}O_2$	312	0.04	35.04				
12	7-epi-Cis-Sesquisabinene hydrate	$C_{15}H_{26}O$	222	0.84	36.89				
13	Methyl palmitate	$C_{17}H_{34}O_2$	270	0.15	36.99	1.97	44.16		
14	Methyl palmitoleate	$C_{17}H_{32}O_2$	268	1.86	38.00				
15	Palmitic acid, ethyl ester	$C_{18}H_{36}O_2$	284	3.4	38.47	0.24	46.24		
16	Palmitic acid	$C_{16}H_{32}O_2$	256	3.51	39.72	4.02	47.35		
17	Methyl linoleate	$C_{19}H_{34}O_2$	294	0.19	40.30	5.85	49.59		
18	2-monoolein	$C_{21}H_{40}O_4$	356	0.10	40.39				
19	Ethyl linoleate	$C_{20}H_{36}O_2$	308	25.07	41.88	1.18	54.73		
20	Oleic acid	$C_{18}H_{34}O_2$	282	5.85	42.25	6.59	47.43		
21	Ethyl stearate	$C_{20}H_{40}O_2$	312	1.29	42.38				
22	Linoleic acid	$C_{18}H_{32}O_2$	280	56.71	43.57	5.09	52.92		
23	Colchifoline	$C_{22}H_{25}NO_6$	415	0.08	45.68				
24	Dihydrovallesiachotamine	$C_{21}H_{24}N_2O_3$	352	0.06	47.96	0.16	62.08	0.57	53.42
25	Formyl colchicine	$C_{23}H_{25}NO_7$	427	0.04	48.68				
26	Lucenin-2	$C_{27}H_{30}O_6$	610	0.22	53.05	0.12	56.69	0.25	46.03
27	6 - Carboxypterin	$C_7H_5N_5O_3$	207					0.10	50.14
28	Eicosanoic acid	$C_{20}H_{40}O_2$	312			4.52	47.68	*0.04*	47.45
29	Linoleic acid methyl ester	$C_{19}H_{34}O_2$	294			5.85	49.59		
30	Corticosterone	$C_{21}H_{30}O_4$	346			0.16	50.16		
31	Quercetin 7,3,4-Trimethoxy	$C_{18}H_{16}O_7$	344			2.45	50.34	1.10	46.26
32	6,7- dihydroneblininane	$C_{23}H_{32}N_2O_2$	368			0.53	50.54		
33	Pseudojervine	$C_{33}H_{49}NO_8$	587			0.17	51.63		
34	Stearic acid	$C_{20}H_{36}O_2$	308			3.64	55.00		
35	2-Methylene-5α-cholestan-3β-ol	$C_{28}H_{48}O$	400			0.30	60.68		
36	Dihydrocorynantheine acetate	$C_{22}H_{28}N_2O_3$	368			0.60	60.84		
37	Dihydroerysodine	$C_{18}H_{23}NO_3$	301					1.24	42.92
38	5α-Cholestan-3-one, Cyclic ethylene acetal	$C_{29}H_{50}O_2$	430					0.14	44.69
39	2- Methyladenosine	$C_{11}H_{15}N_5O_4$	281					0.34	46.35
40	Aspidofractinine-3-methanol (Kopsinyl alcohol)	$C_{20}H_{26}N_2O$	310					4.05	62.33
41	Voacristine hydroxyindolenine	$C_{22}H_{28}N_2O_5$	400					0.33	65.62

RA: Relative abundance; MF: Molecular formula; MW: Molecular weight; Eth: Ether extract; Chl: Chloroform extract; Aqu: Aquatic extract of seeds; Rt: Retention time

extract. Finally, 6-carboxypterin, dihydroerysodine, methyladenosine, aspidofractinine-3-methanol, and voacristine hydroxyindolenine were identified in the aqueous extract. However, dihydrovallesiachotamine was identified in the three extracts. The indole alkaloid vallesiachotamine was found to have anticancer and anti-inflammatory effects.[30,31] Yubin *et al*. (2014) reported that the separation of the extracted alkaloid depends on alkaloid class, basicity, solubility differences, and the particular functional group.[22] The most detected alkaloid compounds were reported to have an antitumor effect through a different mechanism of actions. The colchicine and its derivatives were found to improve anti-inflammatory activity.[32] Colchifoline and formyl colchicine were reported for the first time in *C. acutum* and the genus of *Cynanchum*. Colchifoline was found to be toxic as same as colchicines in the menogaril-resistant mouse leukemia (P388) with a good affinity for tubulin.[33] Formyl colchicines also have anti-inflammatory activity.[34] The alkaloid, pleiocarpamine, is a potential therapeutic agent for Alzheimer's disease.[35] Dihydrocorynantheine was active against *Leishmania major*.[36] 6-carboxypterin possessed potent antioxidant and anti-inflammatory activities.[37,38] The steroidal alkaloid, pseudojervine, was reported to possess antitumor and antiplatelet activities.[39]

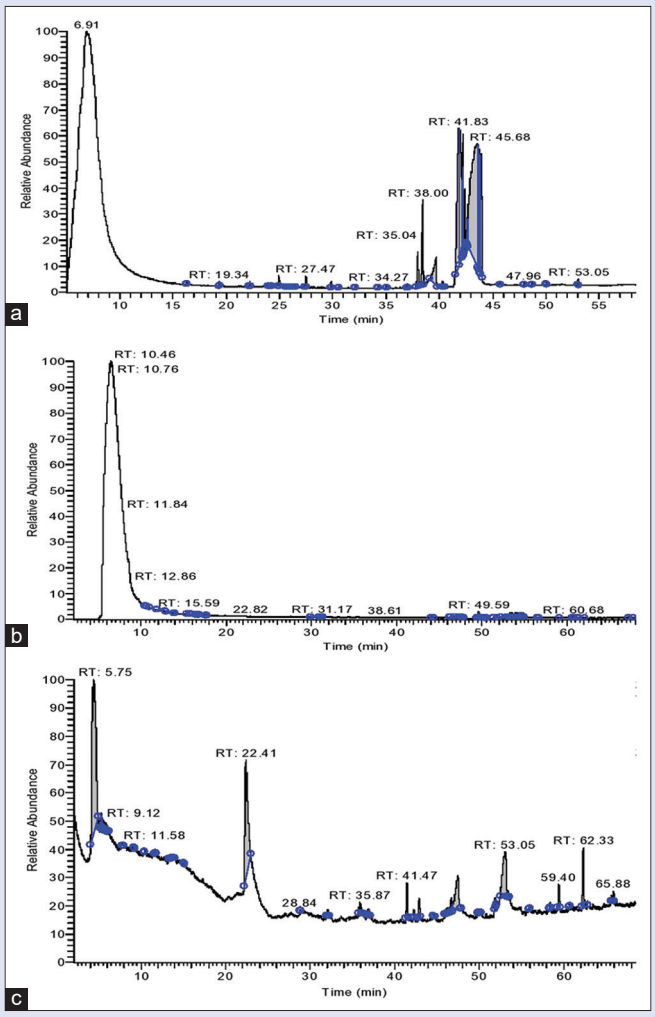

Figure 1: Gas chromatography/mass spectrometry spectra of *Cynanchum acutum* seeds – (a) ether extract, (b) chloroform extract, and (c) aqueous extract (at full page width)

Aspidofractinine-3-methanol (Kopsinyl alcohol) was reported to possess potent anti-inflammatory and antitumor activities.[40,41] Tetracyclic alkaloid of erysodine was reported to be an anti-inflammatory and antihypertensive.[42] Voacristine hydroxyindolenine was found to possess significant uterotrophic activity and prevented pregnancy when administered during the preimplantation period in Sprague–Dawley rats.[43]

The anticancer effects of ether, chloroform subfraction, and aqueous extracts of *C. acutum* seeds were evaluated against lung cancer (A549), PC3, breast cancer (MCF-7), hepatocellular carcinoma (HepG-2), colon cancer (Caco-2), and normal human fetal lung fibroblast (WI-38) cell lines. The seed extracts revealed various anticancer effects against cancer cell lines and cytotoxic effect toward normal cell line [Table 2 and Figure 2]. The anticancer effects of aqueous and ether extracts on lung cancer (A549) and breast cancer (MCF-7), respectively, were higher than other cell lines. However, the cytotoxic effects of the three extracts toward normal cell lines were reduced. All the IC_{50} values of the extracts were compared to the IC_{50} values of a positive control, doxorubicin against the malignant and nonmalignant cell lines. For example, the highest anticancer effect on lung cancer cell lines (A549) was revealed by aqueous extract (IC_{50} of 115 ± 6 µg/ml), whereas ether extract (IC_{50} of 1130 ± 7 µg/ml) and chloroform extract (IC_{50} of 172 ± 22 µg/ml) showed decreased anticancer activities against lung cancer cell lines (A549) comparing to a positive control (IC_{50} of 100 ± 2 µg/ml). Furthermore, aqueous extract showed low cytotoxic effect (IC_{50} of 217 ± 21 µg/ml) toward normal cell lines (WI-38) comparing to a positive control (IC_{50} of 137 ± 10 µg/ml) [Table 2 and Figure 2]. The highest anticancer effect on breast cancer cell lines (MCF-7) was demonstrated by the ether extract (IC_{50} of 146 ± 11 µg/ml), whereas the anticancer effects of water extract (IC_{50} of 152 ± 5 µg/ml) and chloroform extract (IC_{50} of 192 ± 6 µg/ml) were reduced on breast cancer cell lines (MCF-7) as compared to a positive control (IC_{50} of 115 ± 7 µg/ml) [Table 2 and Figure 2]. The cytotoxic effect of ether extract against normal cell lines (WI-38) (IC_{50} of 209 ± 17 µg/ml) was low compared to a positive control (IC_{50} of 137 ± 10 µg/ml) [Table 2 and Figure 2]. Therefore, the major compounds including phytol acetate (RA = 3.38) and aspidofractinine-3-methanol (Kopsinyl alcohol) (RA = 4.05) found in the aqueous extract may possess the potentially highest anticancer effect on lung cancer. Whereas, palmitic acid (RA = 3.51), ethyl linoleate (RA = 25.07), oleic acid (RA = 5.85), and linoleic acid (RA = 56.71) found in the ether extract may be responsible for the highest anticancer effect on lung cancer. However, the other

Table 2: Cytotoxic effects of different extract fractions of *Cynanchum acutum* seeds on cancer cell lines

	IC_{50} (µg/ml)					
Cell Lines	**A549[a]**	**PC3[b]**	**MCF-7[c]**	**HepG-2[d]**	**Caco-2[e]**	**WI38[f]**
Water Extract	115±6*	151±20**	152±5**	185±21***	288±6***	217±21**
Ether Extract	130±7**	181±19***	146±11*	128±5***	285±34**	209±17**
Chloroform Extract	172±22**	222±19***	192±6***	230±13***	307±16***	592±24***
Doxorubicin	100±1	57±6	115±7	67±11	106±12	137±10

IC_{50}: The half maximal inhibitory concentration; [a]Human lung cancer (A549), [b]Prostate cancer (PC3), [c]Human breast cancer (MCF-7), [d]Human hepatocellular carcinoma (HepG-2), [e]Human colon cancer (Caco-2) and [f]Normal human fetal lung fibroblast (WI-38). Each value represents mean±SD from three independent experiments. *Significant difference from doxorubicin $P<0.05$; **High significant difference from doxorubicin $P<0.001$, ***Very high significant from doxorubicin $P<0.0001$

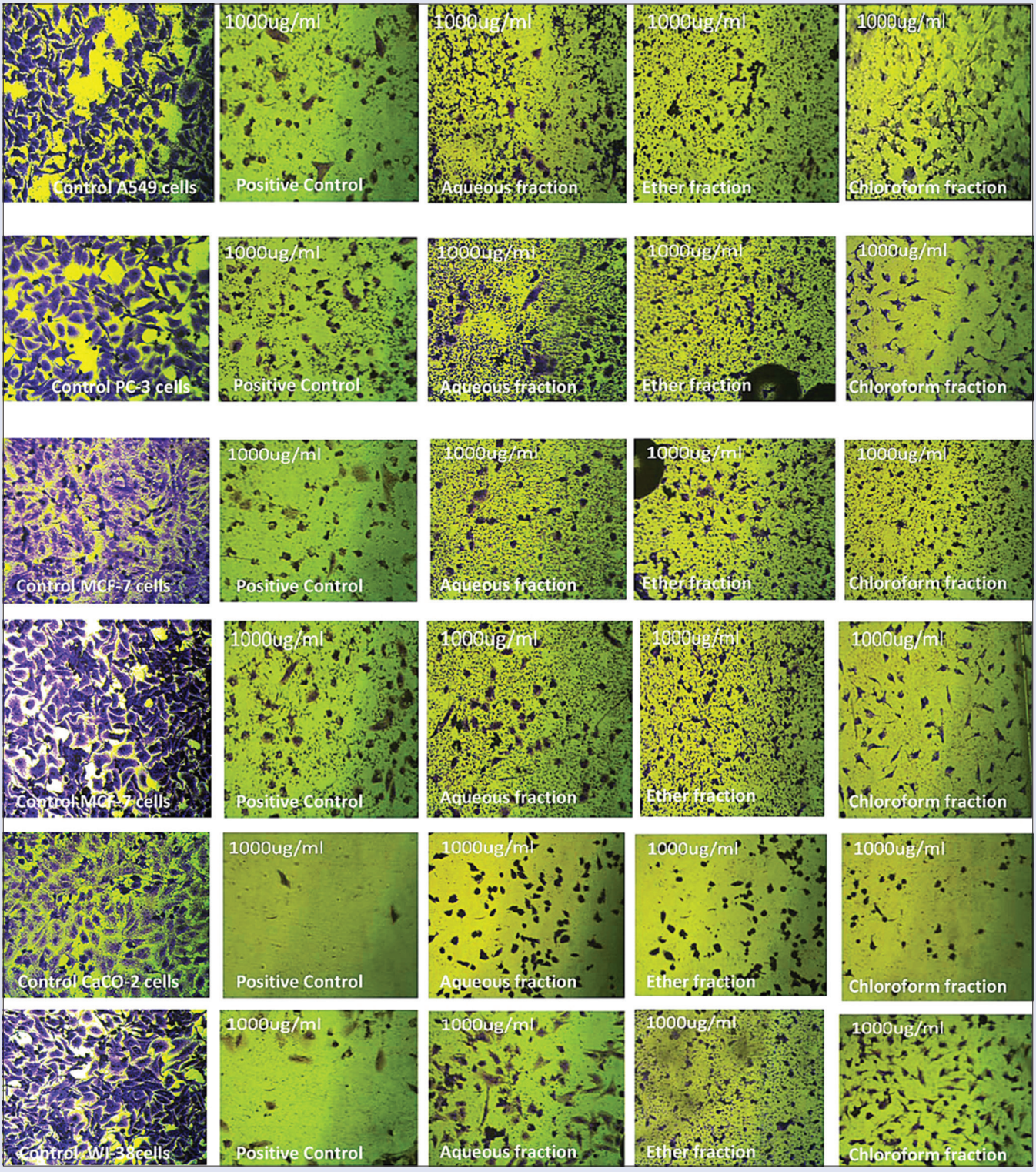

Figure 2: Anticancer effects of different extracts of *Cynanchum acutum* seeds on cancer cell lines (at full page width)

identified compounds in the aqueous and ether extracts showed low RA percentage [Table 1]. Therefore, isolation of these compounds from ether and aqueous extracts of *C. acutum* seeds and studying their anticancer effects on lung and breast cancers are strongly recommended. Thus, we can verify their potential effect for lung and breast cancer treatments with possibly reduced systemically adverse effects.

CONCLUSION

From our results, it can be concluded that *C. acutum* seeds are abundant sources of several pharmaceutically essential compounds which can be used as sources of new and useful anticancer chemical entities. The aqueous and ether extracts of seeds exhibited anticancer activities against lung cancer and breast cancer, respectively, with a potential safety

toward normal cell lines. These anticancer activities may be attributed to the presence of different classes of bioactive compounds such as fatty acids, sterols, flavonoids, and alkaloids. This indicates that aqueous and ether extracts may be considered as potential drugs for lung and breast cancer treatments with reduced their systemic adverse effects. Further isolation and structure clarification of pure compounds which may be responsible for the anticancer activities of *C. acutum* are needed, and further experiments are warranted to explore their action mechanism.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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