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EXPLORING ANTICANCER POTENTIAL OF CENTAUREA BEHEN L., ELAEOCARPUS GANITRUS ROXB, GLORIOSA SUPERBA L. AND FICUS RELIGIOSA L. AGAINST HUMAN LUNG CANCER (A-549) CELL LINES

Pharmacology

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ABSTRACT

Background: Phytochemicals form the basis of research and development of new molecules leading to the development of new novel drugs. In the oncology sector, plants have contributed more than 60% of the anti-cancer drugs, directly or indirectly. **Aims and Objectives**: The present study was conducted with the objective to explore the antitumor activity of various extracts obtained from dried roots of *Centaurea behen* L., dried fruits/beads of *Elaeocarpus ganitrus* Roxb., dried roots of *Gloriosa superba* L. and dried leaves of *Ficus religiosa* L. against human lung cancer (A 549) cell lines. **Method:** Phytoconstituents were extracted by Soxhlet method using hexane, chloroform, methanol and water as solvents. MTT assay was used to find the anti-proliferative potential of these plant extracts. The trypan blue dye exclusion test was used to determine the number of viable cells present in a cell suspension. Cytotoxic activity was also evaluated against non-cancerous cell lines (MCF-10A). **Results:** On A-549 cell lines, 93% cell death was reported with *G. superba* aqueous extract followed by *E. ganitrus* methanol extract and *F. religiosa* hexane extract and *C. behen* aqueous extract with 87%, 81.61% and 75 % cell death respectively. However, none of the extracts showed cytotoxic effect upon testing against normal non-cancerous cell lines (MCF- 10A). **Conclusions:** The results of the current findings prove that phytoconstituents present in these plant extracts have high anticancer potential. This study strongly suggests the possibility of medicinal plants as an important source of anticancer drug development.

KEYWORDS

A 549 Lung cancer cell lines, MCF-10A Cell lines, MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, Trypan blue dye exclusion test, anticancer, cytotoxic

INTRODUCTION

Lung cancer is one of the serious human health problems and many researchers have focused on identifying new therapeutic agents and effective therapies. Recently, herbal medicines have been used for the treatment of various diseases such as cancer. Despite many therapeutic treatments available for cancer, the survival rate and disease curative percentage are very low. It's become an increasing public ill health that accounts for six million cases per annum throughout the planet. Secondary plant metabolites have proved to be an excellent reservoir of many anti-cancer agents.[1] Phytochemicals exert antitumor and cytotoxic effects via distinct mechanisms. These selectively kill rapidly dividing cells, target abnormally expressed molecular factors, remove oxidative stress, modulate cell growth factors, inhibit angiogenesis of cancerous tissue and induce apoptosis.[2] Several plant species have been discovered to suppress the progression and development of tumors in cancer patients and several phytochemicals have been discovered from plants which act as dietary supplements and also suppress the viability of cancer cells. Phytochemicals exert antitumor effects via distinct mechanisms. Most phytochemicals act by interfering with several cell-signaling pathways and lead to cell cycle arrest and/or differentiation induction, apart from their apoptosis-inducing potential.[3]

The present investigation was carried out with an aim to investigate the antitumor and cytotoxic potential of various extracts obtained from four different plants namely *Centaurea behen* L., *Elaeocarpus ganitrus* Roxb., *Gloriosa superba* L. and *Ficus religiosa* L. in various solvents- hexane, chloroform, ethanol and water against human lung cancer (A 549) cell lines.

MATERIAL AND METHODS

The study was conducted at Centre for Interdisciplinary and Biomedical Research (CIBR) Adesh University, Bathinda in collaboration with Department of Human Genetics and Molecular Medicine, Central University of Punjab, Bathinda. Human lung cancer cell lines (A 549) were obtained from National Centre for Cell Science (Pune, India). The antitumor activity present in the the plant extracts was tested under in-vitro conditions using cultured preparations of cancer cell lines. A549 is an epithelial lung carcinoma cell line is derived from a 58 years old male caucasian patient. This is a composed of hypotriploid cells where chromosome number is generally 66 while frequency of cells with 67/68 chromosomes is also very high and is considered excellent model for drug discovery related studies. MCF10A cell line was used as a negative control in this study as it exhibits no tumorigenic potential. It represents a normal non cancerous breast tissue derived from 36 years old female's breast epithelia, having normal karyotype. It is thus considered as a good non-cancerous model system for various cell line studies. In the present study, sixteen extracts were used for evaluating anticancer activity of different plant species.

Preparation of plant extracts

DMSO (dimethyl sulfoxide) was used to prepare stock solutions of various extracts and further, the test solutions of various concentrations were prepared ranging from 10 µg/100 µL to 50 µg/100 µL (10 µg/100 µL, 25 µg/100 µL and 50 µg/100 µL). The extracts were purified and used to test their antiproliferative/anticancer activity against lung cancer (A 549) cell lines using MTT assay.[3]

Table 1: Abbreviations of plant extracts used in the study

Sr.No	Solvent used	Name of plant and type of extract	Abbreviation
1.	Hexane	C. behen Hexane	CBH
	Chloroform	C. behen Chloroform	CBC
	Methanol	C. behen Methanol	CBM
	Aqueous	C. behen Aqueous	CBA
2.	Hexane	G. superba Hexane	GSH
	Chloroform	G.superba Chloroform	GSC
	Methanol	G. superba Methanol	GSM
	Aqueous	G. superba Aqueous	GSA
3.	Hexane	E.ganitrus Hexane	EGH
	Chloroform	E. ganitrus Chloroform	EGC
	Methanol	E.ganitrus Methanol	EGM
	Aqueous	E.ganitrus Aqueous	EGA
4.	Hexane	Ficus religiosa Hexane	FRH
	Chloroform	Ficus religiosa Chloroform	FRC
	Methanol	Ficus religiosa Methanol	FRM
	Aqueous	Ficus religiosa Aqueous	FRA

Culturing of cell lines

Dulbeco's modified Eagle's medium media (DMEM) containing 10% FBS, 100 units/ mL penicillin and 100 µg/mL streptomycin was used to prepare the cell culture. The cells were maintained at 37°C in a 5% CO2 humidified incubator. When confluent growth of cells was obtained, the culture media was removed and washed with 1% PBS

(Phosphate buffered saline) solution to inactivate the existing media in culture and trypsinization was done by treatment with 0.25% (w/v) solution of trypsin- EDTA (Ethylene diamine tetra acetic acid). DMEM media was added to inactivate the trypsin and then centrifuged at 1200 rpm at 37˚C for 5 minutes. The maintenance of cultured cell line was done in 25 mL flasks. MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed to check the anti-proliferative potential of the plant extracts.[4]

MTT Cell viability assay

Cell lines were trypsinized and suspended in Dulbeco's modified Eagle's Medium (DMEM) during exponential phase of growth. Cells were seeded at 10,000 cells per well in 96 welled microtiter plates and incubated for 24 hours during which a partial monolayer was formed. The cells were synchronized by serum starvation for 12 hrs. Further, they were subjected to various concentrations (10, 25, 50 µg/µl) of the extract for different time periods (12, 24, 36 and 48 hours). Maintenance medium only was added to control wells. The plates were incubated at 37°C in an incubator with 5% CO2 for different times of 72 hours. Cells were monitored for granularity, shrinkage and swelling. The sample solution in wells was flicked off and 50 µL of MTT dye was added to each well. The plates were gently shaked and incubated in 5% CO2 incubator for 4 hours at 37°C. The supernatant was removed and 50 µL of DMSO was added. The plates were gently shaken to solubilize the formed formazan. The absorbance was measured at 570 nm. The percentage growth inhibition was calculated using the following formula:

Percentage growth inhibition=Mean optical density (OD) of individual test gp/ Mean optical density (OD) of control gp×100.

Values of absorbance were converted into percentage of residual viability. Growth inhibition concentration of 50% (GI50) was chosen as the best biological marker of cytotoxicity. The GI50 value represents the concentration of the tested extracts that caused 50% of cell inhibition.[5]

Appropriate controls were used in the study. Result was established on comparing data in triplicate. Percentage cell death was plotted in form of graphs on Microsoft excel software to assess anticancer potential of all extracts and IC50 value determined from these graphs.

Trypan blue exclusion test of cell viability

Trypan blue is an azo dye used as vital stain to selectively color dead tissues or cells as blue. The dye exclusion test is used to determine the number of viable cells present in a cell suspension.[6]

Procedure

Cell suspension was mixed with a drop of trypan blue dye and then visually examined to determine whether cells take up or exclude dye. The viable cells appeared colorless whereas non-viable cells appeared blue due to retention of dye in dead cells.

Criteria for anticancer potential

The data was represented in three categories according to the extent of percentage cell death -Low (upto 40%); good (41-60%) and excellent (greater than 60%).

Statistical analysis

The MTT assay results were obtained and plotted in Microsoft Excel to calculate average cell death at each concentration of respective plant extract. Statistical analysis was done using analysis of variance (ANOVA) and student t-test to analyse the results.

RESULTS

The results of MTT assay on lung cancer cell lines (A 549) showed that there is significant dose dependent increase in anticancer potential when the cells were treated with various extracts. Figure 1 shows that almost all the extracts were showing more than 50% cell death upon treatment at 50µg/100µLdose. Amongst these, *G. superba* L. aqueous extract showed maximum anticancer potential of 60% at 25µg/100µLdose while 90% cells were dead at higher dose of 50 µg/100µL. On the other hand, *G. superba* L. methanol extract showed very low anticancer potential (40% at 50 µg/100µLdose) while *E. ganitrus* Roxb. aqueous and *F. religiosa* L. aqueous extracts showed least anticancer potential (less than 20%) even at highest dose of 50 µg/100µL. Overall trends indicated that chloroform and hexane extracts showed higher anticancer potential followed by methanol while aqueous extracts were least toxic with exception of *G. superba* L. aqueous extract.

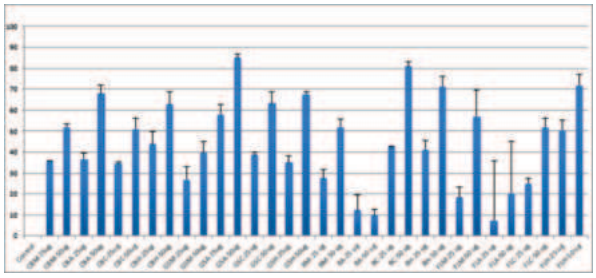


Figure 1 : Graph showing MTT assay of plant extracts against lung cancer cell lines (A 549). Vertical side represents % age cell death. The experiments were performed in triplicates, data was averaged and error bars were plotted depicting standard deviation (SD) in the data.

MTT assay on non-cancerous/ normal (MCF 10A) cell lines

These extracts show high anticancer potential against human cancer cell lines, their cytotoxic potential was also tested against non-cancerous cell line (MCF 10A**)** which represents a normal breast tissue.

The results showed that none of the extracts showed cytotoxic potential against normal cells at 24 hours-time point (Figure 2).

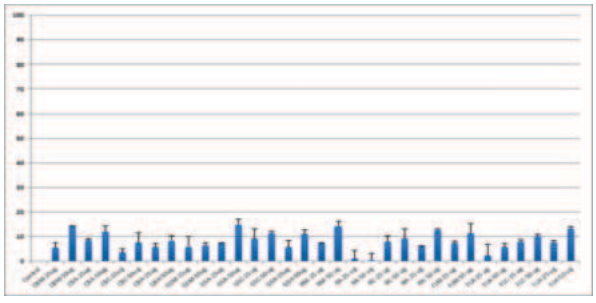


Figure 2: Graph showing MTT assay of plant extracts on non-cancerous cell lines (MCF 10A). Vertical side represents %age cell death. The experiments were performed in triplicates, data was averaged and error bars were plotted depicting standard deviation (SD) in the data.

Cytotoxic/ antitumorogenic potential

Rating scale to interpret the cytotoxic potential value of graphs: Low up to 40%: 41% to 60%: Good and above 60%: excellent.

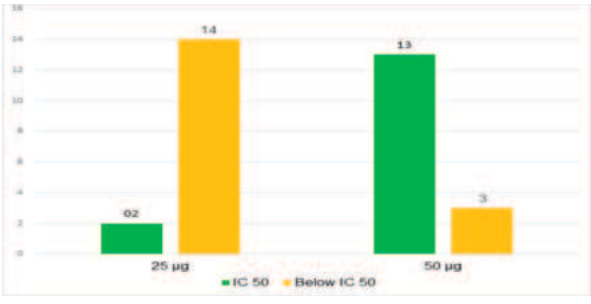
Table 2: Summary and interpretation of graph of anticancer activity evaluation of plant extracts against human lung cancer (A-549) cell lines

Sr.No.	Type of the plant extract	Dose/Cocn.(µg/ µl)	% Cell death	Effect on increased dose (On % Cell Death)	Anticancer Potential
1.	CBM	25 µg	35.74		Low
		50 µg	51.99	Yes	Good
2.	CBA	25 µg	36.66		Low
		50 µg	67.78	Yes	Excellent
3.	CBC	25 µg	34.45		Low
		50 µg	50.98	Yes	Good
4.	CBH	25 µg	43.77		Good
		50 µg	62.77	Yes	Excellent
5.	GSM	25 µg	26.89		Low
		50 µg	39.87	Yes	Low
6.	GSA	25 µg	57.88		Good
		50 µg	84.95	Yes	Excellent
7.	GSC	25 µg	38.66		Low
		50 µg	63.25	Yes	Excellent
8.	GSH	25 µg	34.98		Low
		50 µg	67.68	Yes	Excellent
9.	EGM	25 µg	27.65		Low
		50 µg	51.95	Yes	Good

10.	EGA	25 µg	12.19		Low
		50 µg	9.94	No	Low
11.	EGC	25 µg	42.34		Good
		50 µg	80.94	Yes	Excellent
12.	EGH	25 µg	41.12		Good
		50 µg	71.35	Yes	Excellent
13.	FLM	25 µg	18.33		Low
		50 µg	56.89	Yes	Good
14.	FLA	25 µg	7.41		Low
		50 µg	20.46	Yes	Low
15.	FLC	25 µg	25.03		Low
		50 µg	51.94	Yes	Good
16.	FLH	25 µg	50.39		Good
		50 µg	71.84	Yes	Excellent

Figure 3: Anticancer potential of various plant extracts against Human lung cancer cell lines (A-549) at 25µg/µl concentration

Figure 4: Anticancer potential of various plant extracts against Human lung cancer cell lines (A-549) at 50µg/ µl concentration



Ic50 Value

Figure 5: Number of extracts with IC 50 and below IC 50 value at 25 µg/µl and 50 µg/µl concentration. Human lung cancer cell lines (A-549)

MTT assay results of different extracts of all four medicinal plants obtained with two concentrations (ignoring the results obtained at concentration of 10µg/µl) namely, 25µg/µl and 50 µg/µl against Human lung cancer cell lines (A-549) becomes equal to 32 value (16 extracts x 1 cell line x 2 concentrations = 32) . Out of 32 total extracts at two different concentrations tested against Human lung cancer cell lines (A-549) at concentration of 25 µg/µl (out of 16 extracts), 02 extracts (12.5%) showed IC 50 value and 14 (87.5%) showed below IC 50 values. At 50 µg/µl concentration (out of 16 extracts), IC 50 value was shown in case of 13 (81.25%)) extracts and 3 (18.75%) showed below IC 50 values.

DISCUSSION

Plants have been used for treatment of various human cancers without causing anticancer activity to host cells.[7] A great number of *in vitro* and *in vivo* methods have been developed to measure the efficiency of natural anticancer compounds either as pure compounds or as plant extracts. In vitro methods include – Trypan blue dye exclusion assay, MTT assay, XTT assay etc. Among all, MTT assay is the most common method for estimating anticancer activity and has been used in present study.[8]

In the present study, all the extracts of *C. behen* showed more than 50% cell death upon treatment at 50 µg/100µLdose on A549 cell lines. Chloroform and hexane extracts showed higher anticancer activity followed by methanol extract while aqueous extracts showed least anticancer activity on A549 cell lines. *E. ganitrus* aqueous extracts showed least anticancer activity (less than 20%) even at highest dose of 50 ug/ul. In the present study, *F. religiosa* chloroform and hexane extracts showed higher anticancer activity followed by methanol extract while aqueous extracts showed least anticancer activity on A549 cell lines.

In another study, the *Pedalium murex* leaves methanolic extract showed the anticancer activity against A549 cell line in a dose dependant manner. The *Pedalium murex* leaves methanolic extract showed a maximum inhibition of 68% at 500 µg/ml against A549 lung cancer cells.[9] The cytotoxicity of the compounds were tested in A549, lung cancer cell line and compared with L132, normal embryonic lung cell line by MTT assay. The cell viability test showed that the compounds had toxic effect against A549 cell line and negligible toxicity was found against L132 cell line.[10] The ethanolic extract of *Nepeta paulsenii* Briq. showed considerable cytotoxic effects against cancerous cell line A549. In vitro cytotoxicity of the aqueous extract of the flowers, stem, and leaves was also determined against the A549 cell line based on an MTT assay, and all the aqueous extracts of the flowers, stem, and leaves showed activity against lung cancer (A549) cell lines. However, none of the extracts were found to be cytotoxic in normal cell lines.[11] In an another study, the *Anacyclus pyrethrum* plant extract was found to significantly inhibit lung cancer cell (A549) proliferation.[12] Ashraf et al. reported that the ethanolic extract of the *F. religiosa* exhibits strong anticancer activity compared to other extracts analyzed.[13] A study quoted that methanolic extract of *F. religiosa* bark displayed a significant antioxidant activity with the IC_{50} value of 48 µg/mL.[14]

The mechanism of action involves upregulating the expression of p53, p21, and pRb proteins. This was followed by downregulation of the phospho Rb (ppRb) protein expression, that subsequently terminates the cell cycle progression at the G1/S phase. On the other hand, the plant extracts induce apoptosis by increasing the intracellular Ca^{2+} level, which results in the loss of mitochondrial membrane potential. It also promotes the release of Cytochrome C and upregulated the Caspase-3 expression.[15] Overall, the mechanism of action involves the downregulation of MMP-2 and Her-2, as well as viral oncoproteins E6 and E7 expression.[16,17] The pharmaceutical industry develops the drugs with the consideration of important characteristics such as solubility, membrane permeability, metabolic stability and systemic pharmacokinetics and pharmacodynamics.[18]

CONCLUSION

Plant parts have attracted much attention for their various pharmacological potentials contributed by the phytochemicals present in the plant matrix. Isolation and characterization of the chemical constituents present in the plant extracts and exploration of their molecular targets in cancer cells will be the broad goals of this work that may be investigated in future studies. New strategies or compounds need to be discovered because most known cancer treatments have adverse effects and all tumors do not react in the same way to treatment. Plant-based medicines have good potential as a primary source for chemotherapeutic drugs.

In addition, adverse effects and drug interactions are major restrictions in synthetic anticancer drugs; therefore, plants have been investigated across the world to exploit novel and potential sources of anticancer agents. Further studies will help to identify the level of chemical components of these extracts responsible for anticancer activity and elucidate more detailed molecular mechanisms of cell death. Therefore, more research is required to correlate its pharmacological activity with chemistry based evidences by strengthening linkage

between researches being carried out by different groups across the world so that it can be developed as potential drugs.

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Ethical approval: *The study was approved by the Institutional Ethics Committee*

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