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Original Research Article

Antitumor and cytotoxic potential of various extracts of *Gloriosa superba* L. *Centaurea behen* L., *Elaeocarpus ganitrus* Roxb and *Ficus religiosa* L. against human breast cancer (MDA-MB 231) cell lines

Satnam Singh[1]*, Amandeep Kaur[2], R. G. Saini[3]

[1]Department of Pharmacology, [2]Department of Microbiology, AIMSR, Bathinda, Punjab, India
[3]Centre for Interdisciplinary Biomedical Research, Adesh University, Bathinda, Punjab, India

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***Correspondence:**
Dr. Satnam Singh,
E-mail: sssaini76@gmail.com

ABSTRACT

Background: Medicinal plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, phenolic compounds, flavonoids etc. which have the capability to inhibit many pathways that lead to cancer. The present study was conducted with the objectives to screen the extracts of dried roots *Gloriosa superba* L., dried roots of *Centaurea behen* L. dried fruits/beads of *Elaeocarpus ganitrus* Roxb., dried leaves of *Ficus religiosa* L. and investigate their antitumor activity on human breast cancer cell lines (MDA-MB 231).
Methods: Cytotoxic activity was evaluated against non-cancerous cell lines (MCF-10A). Hexane, chloroform, methanol and water were the solvents used for extraction of phytoconstituents by Soxhlet method. Anti-proliferative potential of the plant extracts was evaluated using MTT assay. The trypan blue dye exclusion test was used to determine the number of viable cells present in a cell suspension.
Results: On MDA MB-231 cell lines, 91.94% cell death was reported with *G. superba* aqueous extract followed by *E. ganitrus* methanol extract and *F. religiosa* hexane extract with 87.93% and 81.61% cell death respectively. Moreover, none of the extracts had shown cytotoxic effect while evaluated against normal non-cancerous cell lines (MCF- 10A).
Conclusions: It is inferred from the current findings that phytoconstituents present in the plant extracts have high anticancer potential. These phytoconstituents along with some new anticancer agents present in the plant extracts reflects the high cytotoxic potential against cancer cells.

Keywords: Phytochemicals, MDA MB-231 cell lines, MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, Trypan blue dye exclusion test

INTRODUCTION

Plants have proved to be an important natural source of anti-cancer drugs for several years. About thirty plant derived compounds have been isolated so far and are currently under clinical trials. These anti-cancer compounds have been found to be clinically active against various types of cancer cells.[1] Secondary plant metabolites have proved to be an excellent reservoir of new medical compounds. Several plant species have been discovered to suppress the progression and development of tumors in cancer patients and many phytochemicals have been identified as active constituents in these plant species.[2]

Therefore, the present investigation was aimed to investigate the antitumor and cytotoxic potential of various extracts of four different plants *Gloriosa superba* L. *Centaurea behen* L., *Elaeocarpus ganitrus* Roxb. and *Ficus religiosa* L. in solvents like hexane, chloroform,

ethanol and aqueous extracts against human breast cancer cell lines (MDA-MB 231) and its possible mechanisms involved in cancer cell death.

METHODS

The direct antitumor activity of the plant extracts was tested under in-vitro conditions using cultured preparations of cancer cell lines: Human breast cancer cell lines (MDA-MB-231) which were obtained from national centre for cell science (Pune, India).

Study was conducted from January 2019 to December 2019 at department of human genetics and molecular medicine, central university of Punjab, Bathinda.

MDA MB 231 is derived from metastatic adenocarcinoma of breast epithelia derived form 51 years old female patient. The cells display aneuploidy conditions with chromosome numbers ranging between 52 to 68 and is considered excellent model for drug discovery related studies. MCF10A cell lines represent a normal breast tissue, was derived from fibrocystic disease (non-cancerous) from 36 years old female's breast epithelia, having normal karyotype and exhibits no tumorigenic potential, thus considered as a good non-cancerous model system and was used as a negative control in this study. Sixteen extracts were used for evaluating anticancer activity of different plant species.

Preparation of various concentrations of plant extracts

Stock solutions of extracts were prepared by dissolving the extracts in DMSO (dimethyl sulfoxide). These solutions were then used for preparing test solutions of desired range of concentrations. Test solutions of extracts were prepared in DMSO to produce solutions of various concentrations ranging from 10 µg/100 µL to 50 µg/100 µL for final treatment purpose. Following concentrations were prepared: 10 µg/100 µL, 25 µg/100 µL and 50 µg/100 µL for anticancer evaluation study. The extracts were purified and concentrations were calculated and these extracts were then used to test their antiproliferative/anticancer activity against breast cancer (MDA MB 231) cell lines using classical MTT assay.[3]

The abbreviations used for plant extracts for graphical representation are given in Table 1.

Table 1: Abbreviations of plant extracts under study.

Name of plant	Solvent used	Name and type of extract	Abbreviation
***Centaurea behen* Linn**	Hexane	*C. behen* hexane	CBH
	Chloroform	*C. behen* chloroform	CBC
	Methanol	*C. behen* methanol	CBM
	Aqueous	*C. behen* aqueous	CBA
***Gloriosa superba* Linn.**	Hexane	*G. superba* hexane	GSH
	Chloroform	*G. superba* chloroform	GSC
	Methanol	*G. superba* methanol	GSM
	Aqueous	*G. superba* aqueous	GSA
***Elaeocarpus ganitrus* Roxb.**	Hexane	*E. ganitrus* hexane	EGH
	Chloroform	*E. ganitrus* chloroform	EGC
	Methanol	*E. ganitrus* methanol	EGM
	Aqueous	*E. ganitrus* aqueous	EGA
***Ficus religiosa* Linn.**	Hexane	*Ficus religiosa* hexane	FRH
	Chloroform	*Ficus religiosa* chloroform	FRC
	Methanol	*Ficus religiosa* methanol	FRM
	Aqueous	*Ficus religiosa* aqueous	FRA

Cell culture and treatment

The cells were grown in Dulbeco's modified Eagle's medium media (DMEM) containing 10% FBS, 100 units/ mL penicillin and 100 µg/mL streptomycin. The cells were maintained at 37°C in a 5% CO_2 humidified incubator. After the cell became confluent, the culture media was removed and washed with 1% PBS (Phosphate buffered saline) solution to inactivate the existing media in culture. After that, treatment with trypsin- EDTA (Ethylene diamine tetra acetic acid) 0.25% (w/v) solution was given for trypsinization. Subsequently, trypsin was inactivated by adding DMEM media. Cells were collected and centrifuged at 1200 rpm at 37˚C for 5 minutes. The maintenance of cultured cell line was done in 25 mL flasks. Anti-proliferative potential of the plant extracts was evaluated using MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay.[4]

Procedure of MTT assay

Cell lines in the exponential growth phase were held, trypsinized and suspended in Dulbeco's modified Eagle's Medium (DMEM). 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. They were then exposed to various concentrations (10, 25, 50 mcg/µl) of the extract for variable time points (12,

24, 36 and 48 hours). The significant changes were appeared at our interval and results were recorded accordingly. Control wells contained only maintenance medium. The plates were incubated at 37°C in an incubator with 5% CO_2 for different times of 72 hours. Cells were periodically checked for granularity, shrinkage and swelling. Afterwards, the sample solution in wells was flicked off and 50 µL of MTT dye was added to each well. The plates were gently shaken and incubated in 5% CO_2 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% (GI_{50}) was chosen as the best biological marker of cytotoxicity. The GI_{50} value represents the concentration of the tested extracts that caused 50% of cell inhibition. 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 IC_{50} value determined from these graphs.

Trypan blue exclusion test of cell viability

Trypan blue is an azo dye that is used as a dye stuff. It is a direct dye for cotton textiles. In biosciences, it is used as vital stain to selectively color dead tissues or cells blue. The dye exclusion test is used to determine the number of viable cells present in a cell suspension.[5]

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

Since, the number of variables was very high (16 extracts x three concentrations), the data was streamlined to represent extent of percentage cell death into three categories-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 result of MTT assay on breast cancer cell lines (MDA-MB 231) is shown in Table 2. *C. behen* aqueous, *G. superba* aqueous, *E. ganitrus* methanol, *E. ganitrus* hexane, *F. religiosa* hexane, *F. religiosa* chloroform extracts showed higher anticancer potential potential of 80% at 50 µg/µl dose, while extract *F. religiosa* methanol extract showed highest anticancer potential of 80% at 10 µg/µl dose (Figure 1). However, at higher concentrations of 25 and 50 µg/µl did not show any change in the anticancer potential indicating a potential issue of dose saturation. Similarly, chloroform and hexane extract also showed higher anticancer potential.

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

After confirming that these extracts show anticancer potential against various 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 have cytotoxic potential against normal cells at 24 hours-time point (Figure 2) indicating that these extracts are having a good anticancer potential and should be explored for further evaluation.

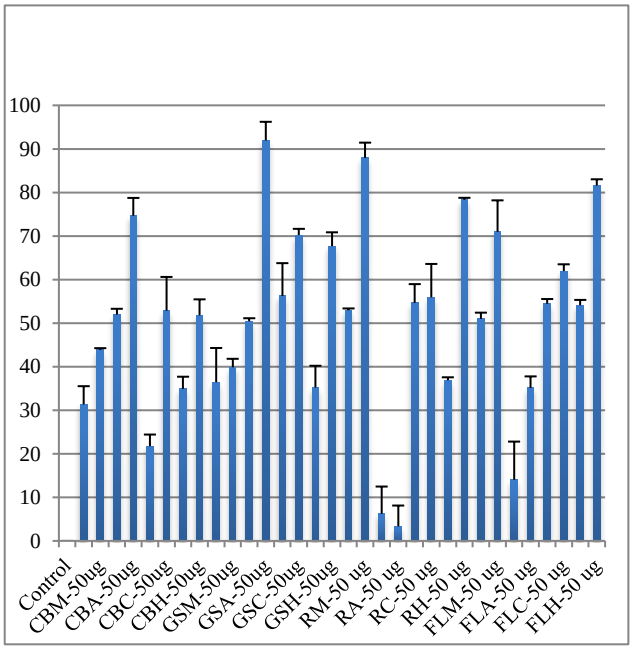

Figure 1: MTT assay of plant extracts against breast cancer cell lines (MDA- MB 231). Vertical side represents %age cell death. The experiments were performed in triplicates, data was averaged and error bars were plotted depicting SD in data.

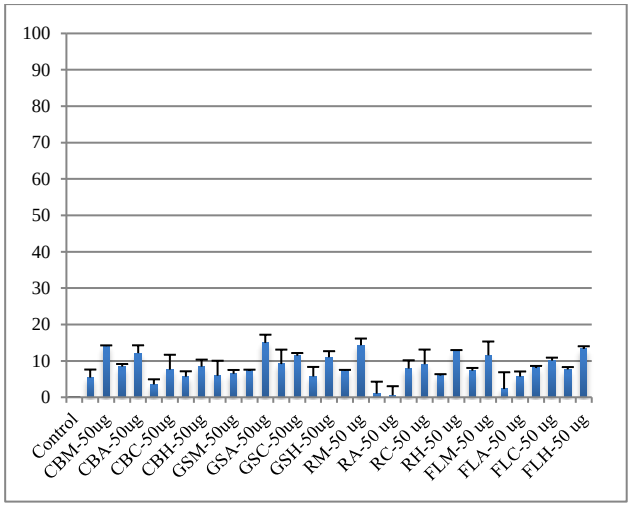

Figure 2: MTT assay of plant extracts on non-cancerous cell lines (MCF 10 A). Vertical side represents %age cell death. The experiments were performed in triplicates, data was averaged and error bars were plotted depicting SD in the data.

Interpretation of graphs of anticancer results

Interpretation of graphs has been done to highlight the anticancer potential of various plant extracts in a simplified manner. The values of graphs are recorded in a tabulated form with a rating scale. The rating scale has been used to present the data of the graphical representation in simplified and precise manner.

Cytotoxic/ antitumorogenic potential values

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

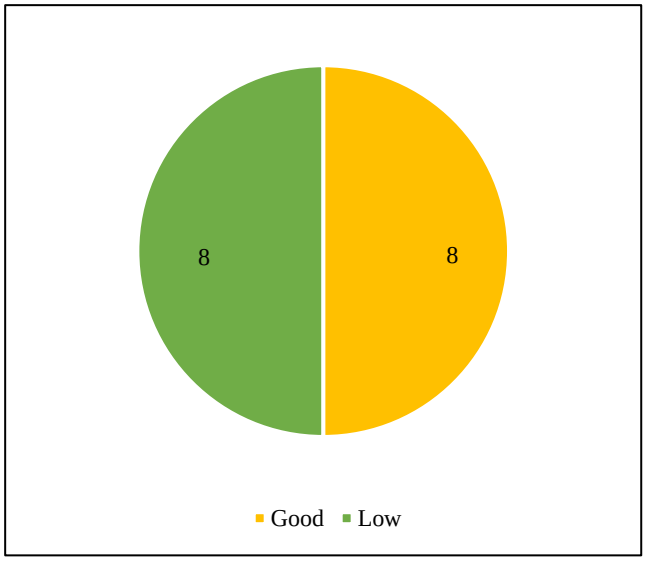

Figure 3: Anticancer potential of various plant extracts against human breast cancer cell lines (MDA MB 231) at 25 µg/µl concentration.

Table 2: Interpretation of graph of anticancer activity evaluation of plant extracts against human breast cancer cell lines (MDA MB 231).

Type of plant extract	Dose/ conc. (µg/µl)	% cell death	Effect on increased dose (On % cell death)	Anti-cancer potential
CBM	25	31.35		Low
	50	43.88	Yes	Good
CBA	25	51.98		Good
	50	74.66	Yes	Excellent
CBC	25	21.76		Low
	50	52.86	Yes	Good
CBH	25	34.90		Low
	50	51.78	Yes	Good
GSM	25	36.45		Low
	50	39.87	Yes	Low
GSA	25	50.45		Good
	50	91.94	Yes	Excellent
GSC	25	56.23		Good
	50	70.23	Yes	Excellent
GSH	25	35.23		Low
	50	67.68	Yes	Excellent
EGM	25	52.98		Good
	50	87.93	Yes	Excellent
EGA	25	6.24		Low
	50	3.35	No	Low
EGC	25	54.66		Good
	50	55.93	Yes	Good
EGH	25	36.89		Low
	50	78.33	Yes	Excellent
FRLC	25	50.98		Good
	50	70.97	Yes	Excellent
FRLA	25	14.04		Low
	50	35.14	Yes	Low
FRLM	25	54.46		Good
	50	61.96	Yes	Excellent
FRLH	25	54.11		Good
	50	81.61	Yes	Excellent

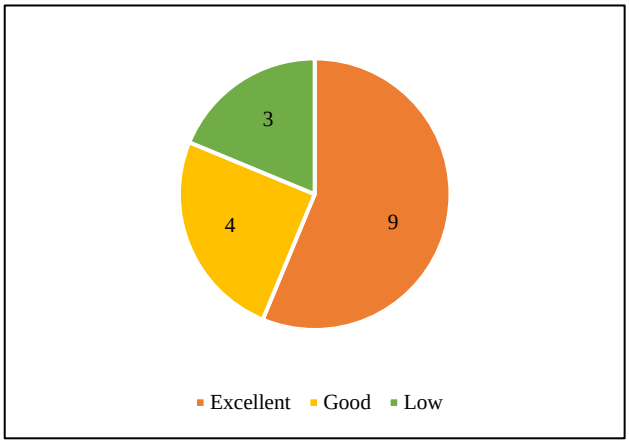

Figure 4: Anticancer potential of various plant extracts against human breast cancer cell lines (MDA MB 231) at 50 µg/µl concentration.

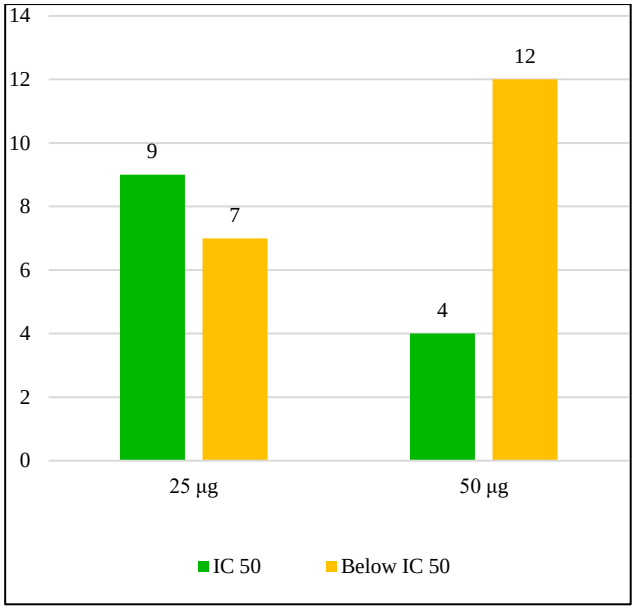

Figure 5: Number of extracts with IC 50 and below IC 50 value at 25 µg/µl and 50 µg/µl concentration. Human breast cancer cell lines (MDA MB 231).

DISCUSSION

The results in breast cancer cells indicated that aqueous extracts have a better efficiency. Chloroform and hexane extracts of all plants showed high toxicity potential.

Anticancer activity of four plants

Gloriosa superba L.

G. superba extracts showed higher anticancer activity of 60-80% at 50 ug/ul dose on MDA MB 231 cells and thus can be considered best extracts against breast cancer cells. In study done by Simon and Jayakumar (2016), anticancer activity of *G. superba* tuber extract was examined using HepG2 cell lines (Human liver cancer cells). It was reported that methanolic extract possessed maximum anticancer activity causing 50% cell death at concentration of 100 ug/ul. Death rate of Hep-G2 cells may be due to various reasons like receptor binding inhibitors, protein binding, DNA replication interaction etc.[6]

Centaurea behen L.

In the present study, all the extracts of *C. behen* showed higher anticancer potential at 50 ug/ul dose on MDA MB 231 cell lines. In a study reported by Escher et al *Centaurea* species showed low anticancer activity and pro-oxidant action without cell damage or cell death which is not in concordance with this study whereas Kubacey et al reported that methanol extract of *Centaurea* species exerted significant effect on A-549 cell line which is in line with the results of present study.[5] Csupor et al reported that different *Centaurea* species exhibited strong cytotoxic potential against HeLa cervical, A-431 epidermal and MCF-7 breast cancer cell lines.[7] Escher et al reported cytotoxic activity of *Centaurea* species against cells derived from human carcinoma of the nasopharynx with growth inhibition of 90% at concentration of10 ug/ul.[8]

Elaeocarpus ganitrus Roxb

In this study, *E. ganitrus* aqueous extracts showed least anticancer activity even at highest dose of 50 ug/ul on MDA MB 231 cell lines, *E. ganitrus* methanol and hexane extracts showed higher anticancer activity potential causing 80% cell death at 50 ug/ul dose whereas chloroform and aqueous extract showed lower anticancer activity. According to Hardainiyan et al, *E. ganitrus* has been used in traditional medicine worldwide for their reported anti-inflammatory and anti-cancer properties.[9] In a recent study conducted by Turner et al to isolate and identify potential anti-pancreatic cancer compounds from fruits of *E. ganitrus*, it was reported that crude extract was more effective in reducing pancreatic cell viability than the fractionated extracts.

The authors reported that acetone extract of the plant was found to significantly decrease the viability of four pancreatic cell lines and induced apoptosis in BXPC-3 and HPDE (Human pancreatic duct epithelial) cells. Triterpenoidcucurbitacin-I was identified by high performance liquid chromatography as a likely component of the extract which significantly reduced the viability of HPDE and BXPC-3 cells.[10]

Ficus religiosa L.

In the present study, *F. religiosa* aqueous extracts showed lesser anticancer activity at highest dose of 50 ug/ul. In case of MDA MB 231 cell lines, *F. religiosa* hexane and chloroform extracts showed higher anticancer activity at 50 ug/ul dose, while methanol and aqueous extracts showed lesser anticancer activity of at 50 ug/ul dose.

In a study conducted by Khulood et al to evaluate cytotoxic effect of *F. religiosa* leaves on human prostate cancer cell lines, leukemic cancer and breast cancer cell lines, it was reported that serotonin and tannic acid or their isomers were detected as active compounds in chloroform extract of *F. religiosa* plant which showed anticancer activity to cancer cells.[11] These compounds perform several important functions such as free radical scavengers which may account for their health promoting properties. Antioxidant properties of the plant may reduce certain types of anticancer activity associated with chemotherapy.[12]

Different studies have elucidated that *F. religiosa* acetone extract exhibited significant anticancer activity on cervical cell lines and human breast cancer cell lines-MCF7 with low anticancer activity to non-tumorigenic mammary epithelial cells.[13,14]

CONCLUSION

The overall results indicate that most of the extracts have excellent anticancer potential against breast cancer cell lines (MDA MB-231) pertaining to human breast cancers. These extracts should be explored further for cancer avenues. It is inferred from the current findings that phytoconstituents along with some new anticancer agents present in the plant extracts reflects the high cytotoxic potential against cancer cells. The various cytotoxic compounds from plants may inhibit growth of cancerous cells by different mechanisms than the currently used anticancer agents and may have potential clinical value in the treatment of breast cancer which is posing very serious threat to the health care system and become a challenge for the clinicians nowadays. This study strongly suggests the possibility of medicinal plants as an important source of anticancer drug development.

The keystone in cancer combat has been conventional chemotherapy but it is associated with normal cell toxicities. Due to lack of specificity, conventional cancer treatments often cause severe side effects and toxicities. Generally, natural agents are considered safe while treating or prevention diseases; however, some compounds as flavonoids, alkaloids, phenolic compounds, terpenoids, triterpenoids, phytosteroids and similar other secondary plant metabolites have shown great potential in the combat against cancer. Plant-derived compounds have a high impact as cancer therapeutic agents both alone or in combination with conventional drugs. As cancer chemoprevention and treatment using natural phytochemicals have been such an attractive approach, further efforts are required to thoroughly understand their potencies, pharmacokinetic performances, pharmacodynamic responses, metabolisms, toxicities, drug-drug interactions, polymorphisms formulations, stabilities, degradations and dosage regimens. The plant based new medicines will be helpful for the patient as they will be more efficacious and freer from side effects as compared to traditional synthetic therapeutics in producing many irreversible side effects in the patients and development of multiple drug resistances in the pathogens. Such natural drugs will be helpful to improve the health care system and reduce the disease burden too. It is very pertinent to mention here that the availability of novel plant-based medicine/pharmacotherapeutics is also the need of the hour as the mortality rate due to the burden of above-mentioned diseases is increasing day by day. Further, research and discovery of mechanism of action are needed to study their anticancer potential *in vivo* which will greatly contribute to the development of novel lead molecules to reduce burden deadly diseases like cancer.

Limitations

The present study was conducted *in vitro*. Further research is required to explore the scientific evidences to support the anticancer activity of extracts of *Gloriosa superba* L. *Centaurea behen* L., *Elaeocarpus ganitrus* Roxb and *Ficus religiosa* L. by performing *in vivo* studies. Further research and discovery of mechanism of action are needed to study their anticancer potential *in vivo* which will greatly contribute to the development of novel lead molecules to reduce the burden deadly diseases like cancer.

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