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***J. Nat. Prod. Plant Resour*., 2013, 3 (5):18-25**
(http://scholarsresearchlibrary.com/archive.html)

ISSN : 2231 – 3184
CODEN (USA): JNPPB7

Biological screening for cytotoxic potential of *Sesbania grandiflora* bark extract against human ovary epithelial teratocarcinoma using PA-1 cell lines, brine shrimp lethality bioassay and *Allium cepa* root model

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ABSTRACT

Sesbania Grandiflora (Family: Leguminosae) is a medicinal plant used as tumor suppressant agent in Ayurveda, a traditional system of medicine practiced in sub-continental India. The aim of the study was to systematically investigate and reveal the cytotoxic potential of sesbania grandiflora bark extract [petroleum ether (BP), Chloroform (BC), Methanol extract (BM) and Aqueous extract (BA)] against Brine shrimp lethality bioassay, Allium cepa root model and MTT- bioassay. We tested time and dose dependent specific cytotoxic activity of BP, BC, BM and BA on Artemia salina by brine shrimp lethality assay. The extracts were also investigated with Allum cepa root model for cytotoxic and antimitotic effect. Finally BM and BA were investigated for their cytotoxic potential using PA-1 cell lines containing human ovary epithelial teratocarcinoma cells in vitro. In Brine shrimp lethality bioassay methanolic bark extract of the plant (BM) was found to be most potent with LC_{50} values of 924.34. In Allium cepa model BM showed maximum growth retarding effect at 10mg/ml with significance of ($p<0.01$). Finally in MTT-bioassay BM was found to be active at 0.001-0.01µg/ml. Methanolic bark extract of sesbania grandiflora contains potent cytotoxic compounds with specific activity against human ovary epithelial teratocarcinoma cells. Further studies are needed to confirm the in-vivo anticancer activity and subsequent isolation and chemical characterization of the active molecules.

Key words: *Artemia salina*, Cytotoxicity, Brine shrimp, *Allium cepa,* MTT- bioassay, Indian medicinal plant.

INTRODUCTION

Sesbania grandiflora (Family: Leguminosae) is a medicinal plant used as antitumor agent in Ayurveda a traditional system that has been practiced for several millennia in the Indian subcontinent [1, 2, 3, 4, 5].

Sesbania grandiflora is quick growing, soft wood tree native of Malaysia and is grown in many parts of India such Punjab, Delhi, Bengal, Assam etc [4]. *Sesbania grandiflora* has been used for Inflammation, Gout and tumor treatment [2, 3, 5]. It has been found that the crude extract of *sesbania grandiflora* flower has anti-microbial activity [6]. The other species of sesbania such as *sesbania drummondii* seeds has found to have cancer inhibitor flavonoid sesbanimide. Ethanolic seed extract of *sesbania vesicaria, sesbania punicea* and *sesbania drummondii* showed antitumor activity [4, 7, 8, 9].

Considering the above information we investigated the in-vitro cytotoxic potential of *sesbania grandiflora* bark extract (BP, BC, BM, BA) by using brine shrimp lethality bioassay, *Allium cepa* root model and MTT-bioassay using PA-1 cell lines of Human ovary epithelial Teratocarcinoma cells. We determined the time and dose dependent cytotoxic effect of petroleum ether extract (BP), Chloroform (BC), Methanol extract (BM) and Aqueous extract (BA). We report below that *sesbania grandiflora* contains potent cytotoxic activity.

MATERIALS AND METHODS

1.1. Collection and Authentification of plant.

The bark of *sesbania grandiflora* plant was collected from Nagpur district, Maharashtra, India during their flowering season. The plant and the bark was botanically identified and confirmed from the department of botany, Rashtrasanth Tukdoji Maharaj, RTM Nagpur University, Nagpur. (A voucher specimen 9403 is retained in the departmental collection for further reference).

2.2 Chemicals and standard drugs.

Paclitaxel, Cyclophosphamide, Acetoorceine, Hydrochloric acid were of analytical grade obtained from Deepti pharmaceutical and Loba chemicals pvt.Ltd, Nagpur.

2.3 Preparation of total bark extract.

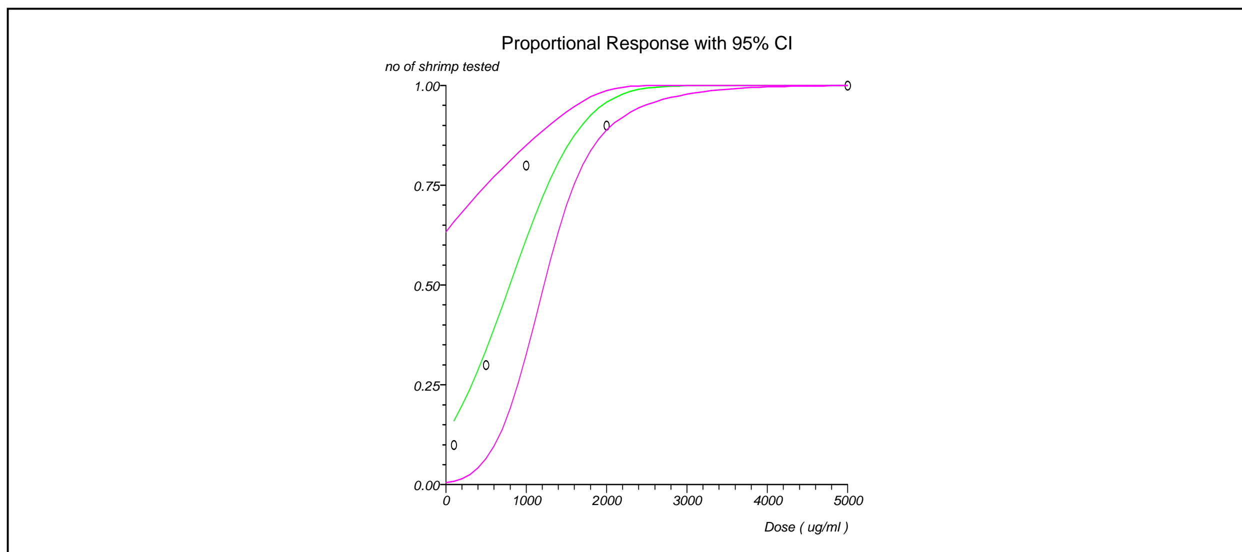
The barks were washed, air dried in the shade at room temperature (25-30^{0}C) for 6-8 days. The bark were then powdered by milling and stored at room temperature for further use. About 250g of the bark (powdered) was charged into soxhlet apparatus and extraction was carried out with following solvent. 1. Petroleum ether (60-80^{0}C), 2.Chloroform, 3.Methanol and 4. Aqueous (macerated for 48 hrs). Each time before employing the solvent of higher polarity, Marc was completely dried. The percent extracts obtained from the successive extraction with different solvent were petroleum ether extract (3.68%), Chloroform extract (7.96%), Methanol extract (12.60%) and Aqueous extract (15.90%). Each extract was then concentrated, dried and collected in glass bottles and used for screening by Brine shrimp lethality bioassay, *Allium cepa* root model and MTT- bioassay.

Experimental Models

1.2. Brine Shrimp Lethality Bioassay Model.[10,11]

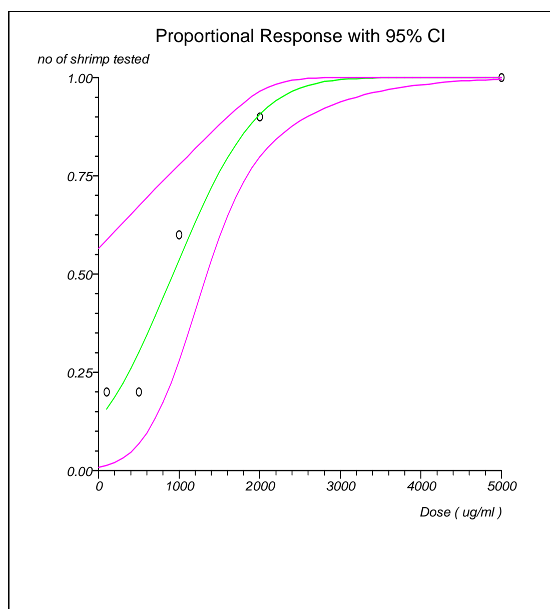
Brine Lethality bioassay was carried out to investigate the cytotoxicity of extracts of medicinal plant *sesbania grandiflora*. Brine shrimps (Artemia Salina) eggs were purchased from (Matsyakanya aquarium, Nashik) and 150 mg of the eggs were kept for hatching in a conical shaped vessel (1L), filled with sterile artificial sea water (prepared using sea salt 38g 1-1) and adjusted to pH 8.5 using 1N NaOH to avoid risk of death to the Artemia larvae by decrease of pH during incubation, under constant aeration for 72hr [12, 13, 14]**.** After 48 hours of hatching 15ml of yeast solution 0.06% was added to vessel for every litre of salt water in order to feed larvae. After 72 hours hatching takes place, active nauplii free from egg shells were collected and used for the assay [14, 15]. For the plant extract, five concentrations were tested in order to determine dose response relationship, and a control group was set with vehicle used for dilutions. Tested concentration of samples were 100, 500, 1000, 2000, 5000 µg/ml. solutions were prepared in distilled water by prior sonication and then triturated with tween-80 to achieve uniform suspension. Ten nauplii were drawn through a glass capillary and placed in test tube containing sample, filled with 5 ml total volume of artificial sea water. Experiment were conducted along with control (vehicle treated) at above mentioned concentrations of test substances in a set of three test tubes per dose. After 24 hours, live nauplii were counted and LC_{50} value was estimated using the statistical method of probit analysis [16, 17, 18, 19].

Fig.1. Probit Analysis - Probit Sigmoid Curve for Standard Drug (cyclophosphamide)



Probit analysis - interpolation
Proportional response = 0.5
Value for X (Dose (µg /ml)) =794.434

Fig.2. Probit Analysis – Probit Sigmoid Curve for Methanolic extract (Bark)



Probit analysis - interpolation *Proportional response = 0.5*
Value for X (Dose (µg /ml)) = 924.348

Table 1: Lc_{50} Value of Different Extracts of Plant

SR.NO	PLANT EXTRACTS	LC_{50} µg/ml	REMARK
1	Cyclophosphamide (std.)	794.43	Active
2	Petroleum ether extract	3197.82	Not-active
3	Chloroform extract	2667.82	Not-Active
4	Aqueous extract	1426.39	Not-active
5	Methanolic extract	924.34	Active

1.3. Allium cepa root model.
The aim of this model was to evaluate the cytotoxic potential of Bark extracts of *Sesbania Grandiflora* (BC, BM, BA) in *Allum cepa* L meristem's in vitro, that affect the proliferative kinetics [20, 21].

The root Meristem of *Allium cepa* L. bulbs were grown in dark with temperature of 15^0C ±0.5^0C in beaker of about 100 ml capacities. The beaker was filled with tap water so that the bases of *Allium cepa* remained immersed in the water. The beaker was supplied with constant aeration and water was renewed every 24 hours. After 2-3 days most of the roots ranged from 2-3cm in length. Working dilutions of all the drugs were made in distill water. The different extracts of bark of *sesbania grandiflora* (BC, BM, BA) was sonicated and triturated in distill water to obtain 1mg/ml and 10mg/ml concentrations. Standard drug, Cyclophosphamide was used at a concentration of 1mg/ml and 10mg/ml. Paclitaxel was used at a concentration of 0.05mg/ml and 0.5mg/ml. The bulbs with root tips grown up to 2-3cm were placed over different extracts and incubation was carried out at ambient temperature. The length of the roots grown in the drug solution (newly appearing roots not included), root number and root length were recorded at 0hr, 48hr and 96hr and compared with that of the control bulbs [20, 22, 23].

1.3.1.Microscopic examination and determination of mitotic index.
The root tips (2-3 mms) were collected placed in 1N HCL for 5 minutes, Squashed and finally stained with 2 % acetoorceine. For Each root tip the number of Mitotic and Total Meristematic cells were counted in 5-8 field's using highly power (200 X) Motic Microscope. In all 600-900 cells were counted and cell's manifesting different stages of mitosis that is, Interphase and prophase (p), Metaphase (T) Anaphase (A) and Telophase (T) were recorded. The Mitotic Index was calculated using following formulae.

$$\textbf{Mitotic Index [MI]} = \frac{\textbf{P+M+A+T}}{\textbf{Total cells}}$$

Table 2: Observations for *Allum cepa* root length and root number attained following incubation with different extracts of *Sesbania Grandiflora* and drugs in comparison to control

Sr. no	Groups	Concentration	Root length in cm		
			0 hrs	48 hrs	96 hrs
1	Control	1mg/ml	2.76±0.12 (n =14)	2.98±0.24 (n =26)	3.26±0.14 (n =35)
3	Methanolic extract (bark)	1mg/ml	2.52±0.09 (n =16)	2.19±0.10 (n =18)	1.92±0.05 * (n =19)
4	Aqueous extract	1mg/ml	2.45±0.22 (n =19)	2.70±0.15 (n =34)	2.81±0.18 (n =42)
5	Cyclophosphamide	1mg/ml	2.49±0.08 (n =17)	2.14±0.12 (n =19)	1.82±0.07 * (n =23)

Table 3: Observations for *Allum cepa* root length and root number attained following incubation with different extracts of *Sesbania Grandiflora* and drugs in comparison to control

Sr. no	Groups	Concentration	Root length in cm		
			0 hrs	48 hrs	96 hrs
1	Control	10mg/ml	3.54±0.18 (n =16)	3.76±0.13 (n =31)	4.11±0.11 (n =41)
2	Methanolic extract (bark)	10mg/ml	2.55±0.10 (n =18)	1.22±0.08 (n =16)	0.92±0.14 ** (n =15)
3	Aqueous extract	10mg/ml	2.49±0.19 (n =18)	2.73±0.16 (n =23)	2.86±0.11 (n =29)
4	Cyclophosphamide	10mg/ml	2.51±0.11 (n =19)	1.84±0.14 (n =18)	1.40±0.17 * (n =18)
5	Paclitaxel	0.5mg/ml	2.58±0.14 (n =11)	1.34±0.13 (n = 9)	0.82±0.06 ** (n = 5)

*Statistical significance is given for comparison of root length attained at 48 and 96 hr with respect to 0 hr control * p< 0.05; ** p<0.01.*

Table 4. Mitotic index in *Allium cepa* Meristem following incubation with various drugs

Sr.No	Groups	Concentration (mg/ml)	Mitotic Index		
			0 hr	48 hr	96 hr
1	Control		51.71± 0.7	53.26±0.3	53.66±0.2
2	Paclitaxel	0.05mg/ml	53.16± 0.4	42.50± 0.5	30.11± 0.3*
3	Methanolic extract	1mg/ml	57.13± .2	49.34± 0.4	40.18± 0.2
4	Cyclophosphamide	1mg/ml	62.36± 0.1	54.36± 0.4	44.31± 0.2
5	Aqueous extract	1mg/ml	61.17± 0.1	57.05± .5	50.57± 0.5
6	Chloroform extract	1mg/ml	54.77±0.3	51.66±0.5	48.48±0.3

*Statistical significance is given for comparison of mitotic index obtained at 48 and 96 hr with respect to 0 hr control * p< 0.05; ** p<0.01*

Table 5: Mitotic index in *Allium cepa* Meristem following incubation with various drugs

Sr.No	Groups	Concentration (mg/ml)	Mitotic Index		
			0 hr	**48 hr**	**96 hr**
1	Control		64.06± 0.6	66.0.±0.2	68.83±0.4
2	Paclitaxel	0.5mg/ml	63.93± 0.4	47.02.± 0.1	30.91± 0.5**
3	Methanolic extract	10mg/ml	61.13± 0.6	47.0.1± 0.3	30..85± 0.1*
4	Cyclophosphamide	10mg/ml	65.08± 0.3	53.94± 0.6	39.82± 0.1*
5	Aqueous extract	10mg/ml	57.98± 0.7	53.86± 0.4	48.73± 0.5
6	Chloroform extract	10mg/ml	62.49±0.1	57.38±0.2	51.24±0.4

*Statistical significance is given for comparison of mitotic index obtained at 48 and 96 hr with respect to 0 hr control * $p < 0.05$; ** $p < 0.01$*

1.4. MTT- Bioassay

In study of MTT Assay Cell Proliferation activity of various extracts of *Sesbania grandiflora* bark had been evaluated, which estimated the effect of various extracts on the growth of cell in vitro. Measurement of cell viability and proliferation forms the basis for this in vitro assay. For evaluation of cytotoxic potential of *Sesbania Grandiflora* plant through MTT Assay, Methanolic extract (bark) and Aqueous extract had been send to "Deshpande Laboratories", Bhopal. Further screening and evaluation had been carried out at Deshpande Laboratories and the results obtained were provided in table no 6.

Fig.3. Dose response curves of compounds against PA-1

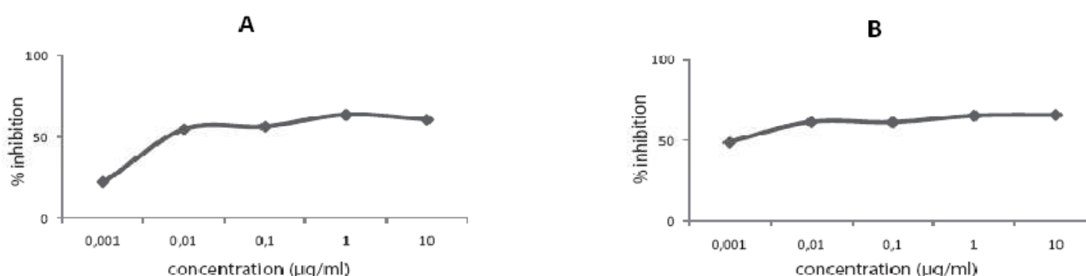


Table. 6 Cytotoxicity analysis of *sesbania grandiflora* bark extracts against Teratocarcinoma

Assay	MTT
Time of incubation	96h
Cell Line	PA-1
Organism	*Homo sapiens* (human)
Organ	Ovary
Tissue	Epithelial
Disease	Teratocarcinoma
Derived from metastatic site	Ascites
Antigen expression	HLA A28, B12
Oncogene	N-ras + (activated)
DNA Profile (STR):	Amelogenin: X CSF1PO: 9,12 D13S317: 9,10 D16S539: 9,12 D5S818: 11 D7S820: 9 THO1: 7,9 TPOX: 11 vWA: 15,17
Cytogenetic Analysis:	NA
Isoenzymes:	G6PD, B
Age:	12 years
Gender:	Female
Comments:	The line was established from cells taken from ascitic fluid and cells form tightly knit colonies, and differentiate to form embryoid bodies when cultured in low serum concentration, or at low plating densities or when treated with 5-bromo-2'-deoxyuridine. The embryonic antigen PCC4 is expressed, but F9 is not detectable.
Assay used :	MTT

Indicates the cytotoxic activity of methanolic extract (B) and Aqueous extract (A) against human ovary epithelial teratocarcinoma cell using PA-1 cell lines. IC50 value of A is 0.01µg and B is 0.001-0.01µg.

Table 7. IC_{50} Values of Methanolic and Aqueous Extract of Plant

Compound	IC_{50}	Status
A (Aqueous extract)	0.01 µg	Active
B (Methanolic bark extract)	0.001-0.01 µg	Active
Standard doxorubicin	500nM	Active

1.5. Statistical analysis

All the experiments were conducted in duplicate and repeated at least twice. For brine shrimp lethality bioassay, five different dilution of the test samples were tested along with control and Lc50 values was estimated using statistical method of probit analysis. For *Allum cepa* root model the values are given as mean ± SEM and the data was analyzed by student t-test.

RESULTS AND DISCUSSION

Brine shrimp is a crustacean whose larvae are sensitive to a variety of substances. This test is used particularly in the developing countries, were 85% of the population use medicinal plants in traditional therapies [24, 25]. In cytotoxicity evaluation of plant extracts by brine shrimp lethality bioassay, An Lc_{50} value lowers than 1000 µg/ml is considered bioactive [15,19].

In brine shrimp lethality bioassay the Lc_{50} values for petroleum ether extract, Chloroform extract, Methanol extract and Aqueous extract of *sesbania grandiflora* bark was calculated with that of a positive control Cyclophosphamide which is given in table no.1. The degree of lethality was found to be directly proportional to the concentration of extract. In the evaluation of cytotoxic potential using brine shrimp Lc_{50} values of the standard drug Cyclophosphamide was found to be 794.43. In comparison with the standard drug maximum mortalities took place in methanolic bark extract (BM) of *sesbania grandiflora* plant with Lc_{50} values of 917.37. The statistical comparison for standard drug Cyclophosphamide and methanolic bark extract (BM) is given in Fig.1 and Fig 2.

In Allum cepa root model different extracts of *sesbania grandiflora* ((BC, BM, BA) was evaluated on the growth (cytotoxic activity) of Allum cepa root meristem's and the effect was compared with standard anticancer drug Cyclophosphamide and Paclitaxel. Cytotoxic effect of *sesbania grandiflora* is evident in the form of shortening and decaying of roots which has been quantified as decrease in the root length and root number in the extract treated group as compare to control. A progressive increase in root number and root length was observed in control group at 0hr, 48hr and 96hr (Table no 2 and 3.). Incubation of bulbs in different concentration of bark extracts produced a growth retarding effect that was associated with a decrease in root number. The cyclophosphamide and methanolic bark extract (BM) arrest the root growth. The root number did not increases any further at 10mg/ml concentration. Paclitaxel at 0.05mg/ml and 0.5mg/ml produce root decay and decrease the root length and root number significantly at 48 h and 96 h as compare to that of 0 h with significant of ($P<0.01$) given in table no.2 and 3.

However among the different extract of *sesbania grandiflora* which was tested, Methanolic extract (BM) was shown the maximum growth retarding effect at 10mg/ml when compared to standard drug paclitaxel and Cyclophosphamide after 0h, 48h and 96h with significance of ($P<0.01$) given in table no.3.

The mitotic cells were counted in the root meristems in the above groups at 0, 48 and 96 h of incubation with each drug. The mitotic index ranged between 51.71± 0.7 to 53.66±0.2 (Table 4) and 64.06± 0.6 to 68.83±0.4 (Table 5) in the control group over a period of 96 h. Methanolic extract produced a significant decrease in mitotic index that was dose and time dependent. The mitotic index at 10mg/ml concentration of methanolic extract was 47.01± 0.3 at 48 h and 30.85± 0.1 at 96 h ($p<0.05$). Treatment with 0.5mg/ml of paclitaxel significantly reduces the mitotic index to 30.91± 0.5 at 96 h ($p<0.0$). Treatment with cyclophosphamide at 10mg/ml concentration brought down the mitotic index to 39.82 ± 0.1 at 96 h ($p<0.05$). *Allium cepa* root tip meristems have been widely used for the evaluation of cytotoxic and anti-mitotic activity of various compounds [26]. In the present study we have tested the cytotoxic and anti- mitotic effect of methanolic extract, aqueous extract, standard anticancer drugs, paclitaxel and cyclophosphamide in *Allium cepa* root tip meristem model. Paclitaxel, an inhibitor of microtubule assembly was the most effective in inhibiting mitosis in *Allium cepa* root tip meristems. In this model, its cytotoxic effect was evident

in the form of shortening and decaying of roots. Anti-tumor drugs that interact with microtubules and tubulin are known to block mitosis and induced cell death by apoptosis. On the other hand, cyclophosphamide alkylates DNA and protein after it has been metabolized by cytochrome P450 to yield phosphoramide mustard and acrolein. It induces plasma membrane blebbing, DNA fragmentation and cleavage of poly (ADP-ribose) polymerase (PARP) and produces cell death by apoptosis [27]. The cytotoxic effect of methanolic extract was significantly comparable to that of the paclitaxel and cyclophosphamide. Thus our study demonstrates that methanolic bark extract of *sesbania grandiflora* exhibits cytotoxic properties like standard anticancer drugs paclitaxel and cyclophosphamide. However, the mechanism for such an effect needs further evaluation.

In study of MTT Assay Cell Proliferation activity of various extracts of *Sesbania grandiflora* bark was evaluated, which estimated the effect of various extracts on the growth of cell in vitro. Measurement of cell viability and proliferation forms the basis for this in vitro assay. The Methanolic extract and aqueous extract of *Sesbania grandiflora* L had been evaluated at Deshpande Laboratories, Bhopal.

Analogous to the results obtained in previous models, Methanolic extract (bark) was found to be active with IC 50 value of 0.001-0.01 µg/ml and also aqueous extract was found to be active with IC 50 value of 0.01µg/ml.

CONCLUSION

Methanolic bark extract of *sesbania grandiflora* contains potent cytotoxic compounds with specific activity against human ovary epithelial teratocarcinoma cells. Further studies are needed to confirm the in-vivo anticancer activity and subsequent isolation and chemical characterization of the active molecules.

Acknowledgements
The authors are very much thankful to Mr. Manoj V. Balpande and Dr. U.N. Mahajan Principal of Dadasaheb Balpande College of Pharmacy, Besa, Nagpur for providing necessary facilities to carry out the research work. The authors are also grateful to Department of Botany, R.T.M. Nagpur University, Nagpur. We also acknowledge the support of Deshpande laboratories Bhopal.

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