

# Antiproliferative Activity of Crude Extract and Different Fractions of *Butea monosperma* Against Lung Cancer Cell Line

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## Badgujar *et al.*: Anticancer Studies on *Butea monosperma* Leaves

The present study was conducted to study phytochemical composition, antioxidative and antiproliferative effect of methanol extract of *Butea monosperma* leaf. Superoxide scavenging assay, metal chelating assay, DPPH and MTT assay were employed. MTT assay was performed on A-549 human lung carcinoma cells and chick embryo fibroblasts were used as the control. Deoxyribonucleic acid fragmentation and real-time assay was performed to check apoptosis and gene expression level. Results obtained indicated that the methanol extract of *Butea monosperma* exhibited high level of antioxidant activity compared to standard antioxidant. Methanol extract and different fraction of the extract exhibited significant antiproliferative activity against lung cancer cell line. The chloroform fraction was found to be most active in MTT assay against A-549 cells while it was less toxic to normal cells. Cells exposed to LD<sub>50</sub> concentration of the chloroform fraction exhibited breakdown of DNA. Increased expression of p53, Bax and caspase-3 gene and reduced expression of Bcl-2 gene gave evidence that the chloroform fraction of *Butea monosperma* might induce apoptosis. These results indicated that the methanol extract and its fractions of *Butea monosperma* leaf possessed immense potential for tumour treatment. Therefore, it would be necessary to carry out further studies to isolate and identify the active principles responsible for these activities.

**Key words:** Apoptosis, A-549 cell line, *Butea monosperma* plant, cytotoxic activity, DNA fragmentation

Cancer is one of the most dreadful diseases globally and it appears to be due to extreme free radical damage, which eventually causes damage to the DNA, lipids and protein. In cell cycle, growth and division of normal cells occur in an well-ordered manner, however in cancerous cells, defective caspase-mediated cell death (apoptosis) leads to increased cell proliferation<sup>[1]</sup>. Some proteins manage the temporal order within the cell cycle highly regulated to confirm that division of cells occur only when required. Inaccuracy in this directive becomes the characteristic of cancer<sup>[2,3]</sup>. Tumour cells evolved mechanisms to resist cell death and this has motivated researchers to investigate many plants, which might selectively induce apoptosis in cancer cells<sup>[3,4]</sup>. It was reported that presence of some phytochemicals in herbs could be the main reason for antitumor activity exerted through inducing apoptosis in cancer cells<sup>[5-8]</sup>. In programmed cell death, activation of caspases (cysteinylyl, aspartate-specific proteases) leads to activation of target substrates within the

cell<sup>[6]</sup>. In chemotherapy, drugs target quickly growing cells thus these drugs might also target normal cells that proliferate quickly like those in the hair follicles, gastrointestinal tract and bone marrow along with cancer cells. Attributable to this some facet effects occur, together with hair loss, canal distress and low white corpuscle count<sup>[9-11]</sup>. For a long time, natural remedies and their preparations were shown to be effective in treating many sorts of disorders<sup>[5,11,12]</sup>. Although herbal treatments are becoming more and more popular worldwide, we all know very little concerning the active ingredients and molecular mechanisms in lots of these remedial plants<sup>[5,13]</sup>.

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Cytotoxicity studies on plant-based resources could lead on to a discovery of recent herbal-based antitumor drugs<sup>[14]</sup>.

India has distinctive plant diversities, which have been intensively studied for anticancer elements. *Butea monosperma* (subfamily- Caesalpinioideae, family- Fabaceae) grows all over the South Asian peninsula (fig. 1). In central India it is generally called as *Palash* tree<sup>[15,16]</sup>. It had been selected to study apoptotic effect of *B. monosperma* leaf extract on the lung cancer cell line, A-549 and compare these results with those obtained on chick embryo fibroblasts. Preliminary work carried out with the methanol extract established that the extract possessed antioxidant and antiinflammatory activity<sup>[17]</sup>. To date the mechanism of anticancer activity of *B. monosperma* plant extract has not been studied against lung cancer cell line. Thus, the methanol extract of *B. monosperma* and its fractions were investigated for anticancer activity on A-549 cell line along with levels of expression of p53, Bax, Bcl-2 and caspase-3 gene in comparison to chick embryo fibroblasts.

## MATERIALS AND METHODS

Fresh sample of *B. monosperma* leaves were collected from Pune, in August 2013 under the supervision of a botanist and all materials were submitted in original form to the Botany Department, Pune. Authentication of *B. monosperma* leaves was done using macroscopic, microscopic, histochemical and phytochemical characteristics of the plant.

### Preparation of plant extract:

Leaves of *B. monosperma* plant were washed, shade-dried and ground to a fine powder using a grinder mixer. Initially 50 g of the powdered plant material was extracted with 500 ml methanol at room temperature with maceration (6×24 h), after which the extract was filtered and concentrated in a rotary evaporator (at 40-50°) under reduced pressure. The percent yield was



Fig. 1: *B. monosperma* leaf

calculated from the weight of powder before and after extraction<sup>[18]</sup>. % yield = (B/A)×100, where, B is the weight of dried powder/leaves after extraction and A is the weight of dried powder/leaves before extraction. Methanol extract of dried leaves of *B. monosperma* yielded 8.16 g %. This extract was subjected to qualitative phytochemical analysis using standard protocols<sup>[19-21]</sup>.

### Estimation of total phenolic and flavonoid contents:

For determining the total phenolic content, a modified Folin-Ciocalteu method was used<sup>[22]</sup>. This assay was performed by mixing 0.5 ml of extract, 0.1 ml Folin-Ciocalteu reagents (0.5 N) and keeping it at room temperature for 15 min. Then 2.5 ml of saturated Na<sub>2</sub>CO<sub>3</sub> was added and allowed to stand for 30 min. Absorbance was measured at 760 nm using a UV/Vis spectrophotometer. Standard curve of gallic acid was created in a range of 0-28 µg/ml using the same procedure (r<sup>2</sup>=0.9454). Total phenolic content was expressed as milligram gallic acid equivalent (GAE)/g plant extract.

Total flavonoid content was studied using the modified aluminium chloride method<sup>[23]</sup>. The reaction mixture comprised of 1.0 ml of plant extracts, 0.5 ml of potassium acetate (120 mM), 0.5 ml of aluminium chloride (1.2 %) and kept for 30 min at room temperature. Absorbance was measured at 415 nm using a UV/Vis spectrophotometer. The total flavonoid content was computed from a calibration curve made with quercetin as standard (0-200 mg/ml in ethanol). The concentration of total flavonoids was then expressed as milligram quercetin equivalents/gram crude extract (r<sup>2</sup>=0.955).

### Superoxide radical scavenging activity:

The principal behind this assay was the capability to inhibit reduction of nitro blue tetrazolium (NBT) in the NBT system<sup>[24]</sup>. For determination of superoxide dismutase activity, a method developed by Martinez *et al.* was used with a slight modification<sup>[25]</sup>. Each 3 ml reaction mixture comprised of 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 2 mM riboflavin, 100 mM EDTA, NBT (75 mM) and 1 ml sample solution. The formation of blue colour formazan was followed by perceptive the rise in absorbance after 10 min lighting from a fluorescent lamp at 560 nm. The whole reaction assembly was surrounded in a box, covered with aluminium foil. Tubes with reaction mixture were kept in the dark and served as blanks. Super oxide scavenging activity

(%) =  $(A_0 - A_1) / A_0 \times 100$ , where  $A_0$  is absorption of control,  $A_1$  is absorption of tested extract solution.

#### Metal chelating activity:

Metal chelating activity was studied using the protocol as described previously by Chan *et al.*<sup>[26]</sup>. Briefly, 1 ml of plant extract was incubated together with equal volume of  $\text{FeSO}_4$  (0.1 mM) and ferrozine (0.25 mM) with vortexing for 10 min. Absorbance was measured at 562 nm. The % chelating activity was calculated using the Eqn., metal chelating ability (%) =  $(A_0 - A_1) / A_0 \times 100$ , where,  $A_0$  is the absorbance of control and  $A_1$  is the absorbance of sample.

#### 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity:

DPPH radical scavenging activity test was performed as described by Blois<sup>[27]</sup>. To 1 ml of a 0.2 mM DPPH solution in ethanol, 1 ml of the fraction solutions at different concentration was added. The mixture was kept for 30 min at room temperature and then absorbance was measured at 517 nm. For every fraction tested the DPPH scavenging action was examined by matching its absorbance with solution having no sample. The DPPH radical scavenging activity of the plant extract was expressed as  $\text{IC}_{50}$ .  $\text{IC}_{50}$  means the amount of extract necessary to inhibit the development of DPPH radicals by 50 %. Results obtained were compared to those obtained with ascorbic acid, the standard antioxidant. Following Eqn. was used to calculate free scavenging activity; DPPH scavenging ability (%) =  $(A_0 - A_s) / A_0 \times 100$ , where  $A_0$  is absorption of control sample,  $A_s$  is the absorption of tested extract solution.

#### Partial purification of *B. monosperma* plant:

Based on the anticancer activity observed the methanol extract of *B. monosperma* was further partitioned into different fractions using an extractor funnel and solvents in increasing order of polarity. Methanol extract was dissolved in water (500 ml) and successively extracted with hexane (100 ml $\times$ 3), chloroform (100 ml $\times$ 3), ethyl acetate (100 ml $\times$ 3), butanol (100 ml $\times$ 3) and water. The hexane (1.51 g), chloroform (1.23 g), ethyl acetate (1.8 g), butanol (1.94 g) and water (5.43 g) fractions were concentrated and weighed. Stock solutions of these fractions were prepared by dissolving in 1 ml dimethyl sulfoxide (DMSO), which were further diluted with DMEM medium. Then, test solutions were filtered through a 0.22  $\mu\text{m}$  membrane filter (Axiva, SciChem Biotech) and stored at  $-20^\circ$  for more experiments.

#### Culture medium and cell lines:

A-549 (human lung cancer) cell line was acquired from NCCS, Pune. Primary culture of chick embryo fibroblast cells and cell lines were grown in DMEM containing 100 U/ml penicillin, 10 % heat-inactivated fetal bovine serum and 100  $\mu\text{g}/\text{ml}$  streptomycin solutions. Cell lines were cultured at  $37^\circ$  in a  $\text{CO}_2$  incubator. Cell culture media and reagents were purchased from Gibco Company (Germany).

#### Cytotoxic study:

MTT colorimetric assay was used to evaluate the antiproliferative activity of methanol crude extract and its fractions of *B. monosperma*. In MTT assay mitochondrial enzyme reduce soluble MTT into an insoluble colour formazan product in viable tumour cells, which may be measured spectrophotometrically<sup>[28]</sup>. Briefly, 200  $\mu\text{l}$  of cells ( $1 \times 10^4$  cells/ml) were seeded in 96 well plates and kept for 24 h ( $37^\circ$ , 5 %  $\text{CO}_2$ ). After 24 h, prepared concentrations of every sample (25-500  $\mu\text{g}/\text{ml}$ ) was added. Plant samples were dissolved in DMSO and control cells contained DMSO at the equivalent concentration (0.5 % v/v) of treated cells. After 24 h of incubation, 20  $\mu\text{l}$  of MTT solution (5 mg/ml in phosphate buffer solution) was added and kept the plate for another 4 h. To dissolve formazan crystals formed, medium containing MTT were gently replaced by DMSO. Absorbance was measured at 560 nm using an ELISA plate reader (Bio-Rad). Three independent assays were performed to calculate the results. Then 50 % cell viability of *B. monosperma* was calculated using the Eqn., cytotoxicity (%) =  $(\text{OD of control sample} - \text{OD of treated sample}) / \text{OD of control sample} \times 100$ .

#### DNA fragmentation assay (apoptosis):

The induction of cell death in treated A-549 cells was determined by studying DNA fragmentation. Cells ( $2 \times 10^6$  per ml) were treated with chloroform fraction of *B. monosperma* at  $\text{LD}_{50}$  concentrations and control cells were treated with 0.5 % DMSO (v/v) for 24 h. The cells were washed twice with phosphate buffer solution after stimulation. Then cells were centrifuged for 1500 rpm for 10 min at  $14^\circ$ . The pellet was resuspended in a lysis buffer for 10 s (1 % NP-40, 50 mM Tris-HCl, 10 mM NaCl, 20 mM EDTA, pH 7.5). Supernatant was collected and extraction is repeated with lysis buffer. The supernatant was brought to 1 % SDS and kept for 60 min with RNAase-A (5  $\mu\text{g}/\text{ml}$ ) at  $56^\circ$  and then digestion with proteinase K (2.5  $\mu\text{g}/\text{ml}$ ) for at least

30 min at 56°. After addition of half of the volume of 10 M ammonium acetate, the DNA is precipitated with 2.5 volumes of ethanol. DNA samples were electrophoretically separated on 1.8 % agarose gel in 1X Tris-acetate-EDTA buffer. DNA fragmentation was observed under UV transilluminator. As a positive control, camptothecin was used in the present study.

### Real-time assay of apoptosis-related genes

The expression levels of well-known apoptosis-related genes p53, Bax, Bcl-2 and caspase were studied using two-step quantitative polymerase chain reaction (qPCR) assay as described<sup>[29,30]</sup>. Chloroform fraction at LD<sub>50</sub> concentration was used in treatment over the period of 4, 12 and 24 h. The sequence of primers used in qPCR were listed in Table 1.

## RESULTS AND DISCUSSION

Methanol extract of *B. monosperma* leaves was subjected to preliminary phytochemical analysis

**TABLE 1: THE SEQUENCES OF PRIMERS USED IN REAL-TIME PCR**

Gene	Sequence	Product size
p53 FP	TGCTCAAGACTGGCGCTAAA	145 b.p
p53 RP	CAATCCAGGGAAGCGTGTCA	145 b.p
Bax FP	CAG AGG ATG ATT GCC GCCG	113 b.p
Bax RP	AAA AGG GCG ACA ACC CGG CC	113 b.p
Bcl-2 FP	TTTGTGGAAGTGTACGGCCC	136 b.p
Bcl-2RP	GTTGACTTCACTGTGGCCC	136 b.p
Casp-3FP	TGTGAGGCGTTGTAGAAGA	158 b.p
Casp-3RP	GCACACCCACCGAAAACCAG	158 b.p
GAPDH-FP	TGGTATCGTGGAAAGGACTCA	258 b.p
GAPDH-RP	ATGCCAGTGAGCTTCCCGTT	258 b.p

that disclosed the presence of numerous secondary metabolites like tannins, flavonoids, alkaloids, carbohydrates, protein and terpenoids (active compounds) and absence of starch, saponins, glycosides and steroids (Table 2). The crude methanol extract of *B. monosperma* was further fractionated in different solvents and the fractions obtained were studied to identify the most cytotoxic fraction using the MTT assay. Results of antioxidant activity of methanol extract of *B. monosperma* were presented in Table 3. The total phenolic content was found to be 196.4±5.4 mg GAE/g for the methanol extract of *B. monosperma*. On the other hand, the total flavonoid content was 96.8±6.6 mg quercetin equivalent/g crude methanol extract of *B. monosperma*. Cytotoxicity possessed by the crude methanol extract of *B. monosperma* was evaluated against A-549 and chick embryo fibroblast cells. Results showed that the crude methanol extract inhibited the viability of A-549 cell when these cells were treated with LD<sub>50</sub> concentration for 24 h. Crude methanol extract was tested at concentrations of 25, 50, 100, 200, 300, 400 and 500 µg/ml. As shown in fig. 2, extract had considerable concentration-dependent inhibition of cell viability and proliferation of the A-549 cells and chick embryo fibroblast cells. According to these results, cytotoxic activity of the extract on A-549 cells was more than that on the chick embryo fibroblast cells. Among the fractions tested, the chloroform fraction exhibited selective cytotoxicity against cancer cells relative to normal cells (fig. 3).

To analyse DNA fragmentation in cells, DNA is extracted from the apoptotic cells and separated on an agarose gel. Degradation of chromosomal DNA into

**TABLE 2: SECONDARY METABOLITES IN METHANOL EXTRACT OF *B. MONOSPERMA***

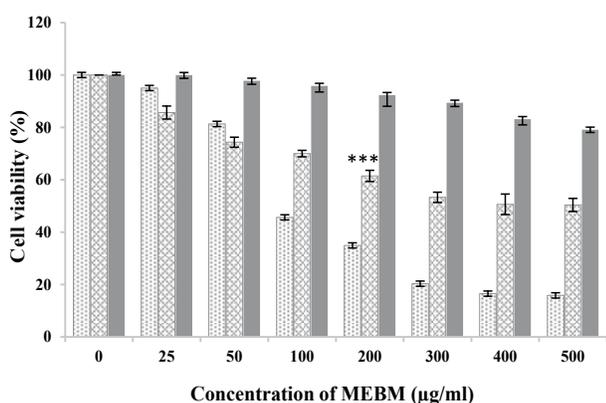
Test	Coloration	BM
Starch: weak iodine solution	Blue black	-
Flavonoids: to test solution few drops of 1 % FeCl <sub>3</sub> was added	blackish-red colour	+
Saponins: 1 ml of filtrate with 2 ml distilled water shaken vigorously and allowed to stand for 10 min	Development of foam on the surface of the mixture, lasting for 10 min	-
Glycosides: to 1 ml of the filtrate; 1 ml of FeCl <sub>3</sub> reagent (mixture of 1 vol. of 5 % FeCl <sub>3</sub> solution+99 vol. of glacial acetic acid) was added followed by a few drops of conc. H <sub>2</sub> SO <sub>4</sub>	Formation of two layers: lower layer is reddish and upper layer is bluish-green	-
Terpenoids: 1 ml of the filtrate with 2 ml CHCl <sub>3</sub> and careful addition of a few drops of conc. H <sub>2</sub> SO <sub>4</sub>	An interface with a reddish-brown coloration	+
Tannins: 1 ml of filtrate with 2 ml of FeCl <sub>3</sub>	Dark green	+
Alkaloids: 1 ml of the filtrate with 2 ml of Dragendroff's reagent	Turbid orange colour	+
Carbohydrates: 2 ml of Fehling's (A+B)+few drops of sample; heated on a burner	Formation of a red or dull violet colour at the interface of the two layers	+
Steroids: conc. H <sub>2</sub> SO <sub>4</sub> was added to ice cold extract	Colour change: violet to blue	-
Protein: few drops of 10 % NaOH was added to test solution followed by few drops of 0.1 % CuSO <sub>4</sub>	The pink or violet colour	+

BM: *B. monosperma*

**TABLE 3: ANTIOXIDANT ACTIVITY OF METHANOL EXTRACT OF *B. MONOSPERMA***

Concentration ( $\mu\text{g/ml}$ )	% Chelating effect	% Superoxide scavenging activity	DPPH activity
50	4.61 $\pm$ 1.4	5.62 $\pm$ 2.6	5.34 $\pm$ 0.9
100	12.90 $\pm$ 3.9	14.64 $\pm$ 1.9	16.97 $\pm$ 2.4
200	20.51 $\pm$ 0.6	17.85 $\pm$ 0.4	22.67 $\pm$ 1.1
300	29.83 $\pm$ 0.7	22.34 $\pm$ 2.9	35.73 $\pm$ 2.7
400	39.48 $\pm$ 1.9	35.16 $\pm$ 0.7	43.86 $\pm$ 0.5
500	42.13 $\pm$ 2.5	58.54 $\pm$ 1.8	53.13 $\pm$ 1.9
IC <sub>50</sub> of MEBM	548.44 R <sup>2</sup> =0.979	496.78 R <sup>2</sup> =0.9236	455.83 R <sup>2</sup> =0.9853
IC <sub>50</sub> of standard ( $\mu\text{g/ml}$ )	EDTA=288.37 R <sup>2</sup> =0.9914	Gallic acid=89.368 R <sup>2</sup> =0.9715	Ascorbic acid=11.59 R <sup>2</sup> =0.9769

N=3. All the values are expressed as mean $\pm$ SD; MEBM: methanol extract of *B. monosperma*

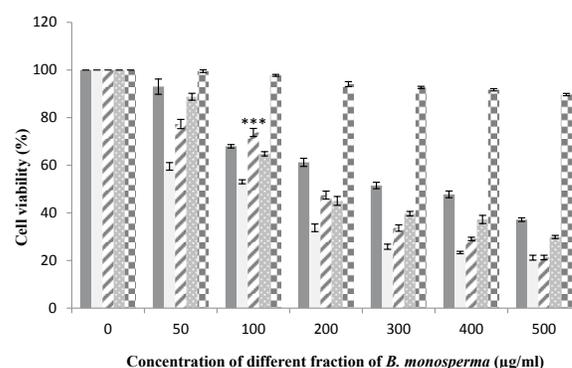


**Fig. 2: Cytotoxicity of methanol extract of *B. monosperma* leaves against A-549 and chick embryo fibroblast cells**

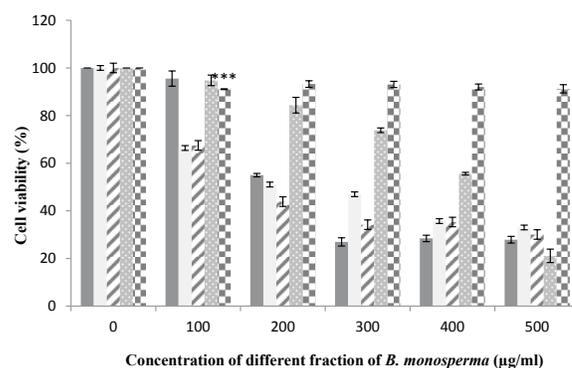
Cytotoxic effect of methanol extract of *B. monosperma* leaves (MEBM) was measured against A-549 (lung cancer cell line) and chick embryo fibroblast cells using the MTT assay. Data presented as mean $\pm$ SD (n=3). \*\*\*p<0.001, as compared with control; ▨ A-549, ▩ chick embryo fibroblast cells, ■ DMSO

small inter nucleosomal fragments occurred when cells treated with chloroform fraction and methanol extract, which was regarded as a biochemical hallmark of cells undergoing apoptosis, thus fragmented DNA confirmed antiproliferative effect of the extract (fig. 4A). Cells without any treatment did not show any fragmentation (fig. 4B). A-549 cells exhibited morphological changes after treatment like chromatin condensation, cell shrinkage and sphere-shaped form (fig. 4C), which were descriptive of apoptotic cells.

The expression levels of some apoptosis-related genes such as p53, Bax, Bcl-2 and caspase-3 were examined to understand the molecular mechanism behind chloroform fraction-induced programmed cell death in A-549 cells. The relative quantification of p53, Bax, Bcl-2 and caspase-3 mRNA levels were performed. In A-549 cells treated with chloroform fraction of *B. monosperma* for 4, 12 and 24 h and expression levels of these genes were compared to untreated control cells. Changes in expression level of p53, Bax,



A.

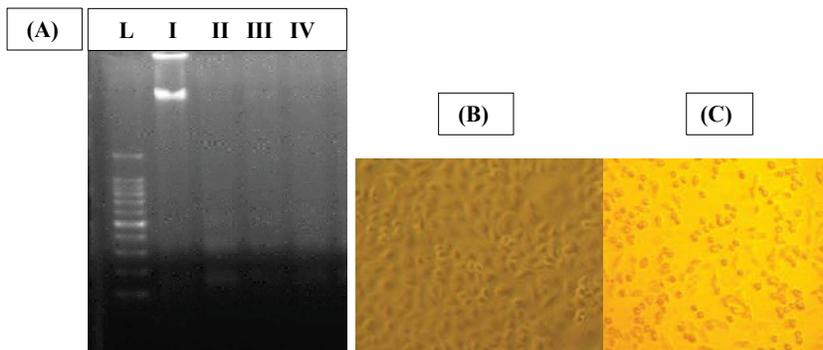


B.

**Fig. 3: Cytotoxicity of different fractions of *B. monosperma* against A-549 and chick embryo fibroblast cells**

Cytotoxic effect of different fractions of *B. monosperma* was measured against (A) A-549 cells and (B) chick embryo fibroblast cells using the MTT assay. Data presented as mean $\pm$ SD (n=3), ■ hexane extract, ▨ chloroform extract, ▩ ethylacetate extract, ▧ butanol extract and ▩ water extract of *B. monosperma* leaves. \*\*\*p<0.001 as compared with control

Bcl-2 and caspase-3 genes were summarised in fig. 5. After treatment, several-fold increase was observed in the number of transcripts of p53, Bax and caspase-3. A decrease in Bcl-2 expression was observed with chloroform fraction-treated cells and Bax expression was significantly increased (fig. 5), which showed that the treatment induced programmed cell death by changing the Bax and Bcl-2 ratio in favour of apoptosis. Chemotherapy and chemoprevention used

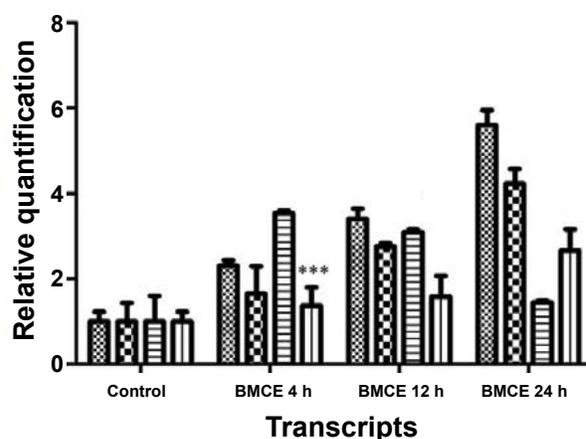


**Fig. 4:** Agarose gel electrophoresis of the chromosomal DNA extracted from A-549 cells and antiproliferative effects of *B. monosperma* extract

(A) Agarose gel electrophoresis (1.8 %) of the chromosomal DNA extracted from A-549 cells. L- 1 kb DNA marker, I- control cells treated with 0.5 % DMSO, II and III- cells treated with chloroform fraction, IV- cells treated with methanol extract at  $IC_{50}$  concentration for 24 h. Antiproliferative effects of extracts of *B. monosperma* (B). A-549 cells without treatment at 20X and (C) cells treated with chloroform fraction of *B. monosperma* extract for 24 h at 20X

to treat cancer by causing caspase-mediated cell death could represent an appropriate methodology to restrain the proliferation of cancerous cells. Many anticancer agents are developed to treat cancer but critical side effects and resistance are the severe issues. Therefore, there is a need for development of more effective anticancer agents. Over the last few years, screening of traditional plants and their parts for cytotoxic properties has gained interest in drug industry. Therapeutically important bioactive components generally occur as secondary metabolites in all plants but their concentration varies consistent with the plant parts, season, climate, and growth phase. Cold percolation method used in the present study has advantages over the Soxhlet extraction that refutes the chances of loss of heat labile components during the extraction. Moreover, the bioactivity of various parts of *B. monosperma* in terms of antiinflammatory, antioxidant, antimicrobial, antiviral, anticonvulsant, anticonceptive and hepatoprotective properties have been reported. According to Sahare *et al.* *B. monosperma* leaves and roots significantly inhibited the motility of microfilariae in a concentration-dependant manner ( $IC_{50}$ - 83 ng/ml)<sup>[31]</sup>. *B. monosperma* leaves showed considerable hypoglycaemic and antioxidant effects<sup>[32,33]</sup>. Borkar *et al.* reported antiinflammatory activities of petroleum ether, hexane, ethanol, ethyl acetate and chloroform extracts of *B. monosperma* leaves<sup>[17]</sup>.

A good anticancer agent should destroy or incapacitate cancer cells without causing unnecessary damages and side effects to normal cells. This perfect condition is attainable by causing apoptosis in malignant cells<sup>[34]</sup>. Generally cells undergoing this condition exhibit some



**Fig. 5:** Expression of mRNA of apoptosis-related genes over 24 h

Real-time polymerase chain reaction analysis of apoptosis-related genes showed that p53, caspase-3 and Bax were up-regulated in cells treated with all three extracts, but Bcl-2 was down regulated. Data represented are means $\pm$ SD of three independent experiments. BMCE=chloroform extract of *B. monosperma*. \*\*\* $p < 0.001$ , as compared with control, P53, Bax, Bcl-2, caspase-3

morphological changes like cell shrinkage, blebbing, DNA fragmentation, loss of cell-membrane attachment and chromatin condensation<sup>[35]</sup>. Cells treated with plant extract would change their morphology from adherent epithelial like shape and also lose their attachment which is characteristics of apoptotic cells (fig. 4C). Lastly, the plant extract elicited programmed cell death in treated cells, not necrosis. Treatment with the plant extract resulted in breakdown of chromosomal DNA into smaller fragments (fig. 4A), which was a biochemical trademark of cells undergoing programmed cell death<sup>[5]</sup>. Electrophoresis of cells DNA resulted in a smudge, but not a ladder and this confirmed that cells were killed due to apoptosis and not necrosis.

In the present study, to know the possible anticancer mechanisms of chloroform fraction, the expression of apoptotic genes was analysed. After treatment with plant extract, expression of caspase-3 was increased and decrease in Bcl-2 expression level (fig. 5) was observed. Thus expression of Bcl-2 mRNA was decreased but the expression of p53, Bax and caspase-3 mRNA was increased. Therefore, the Bax/Bcl-2 ratio was also elevated. The results obtained in this study indicated that chloroform fraction induced programmed cell death through the mitochondrial apoptotic pathways, which was confirmed by the observed enhanced expression levels of caspase-3 after treatment. These pro- and antiapoptotic genes have been the main regulators of the intrinsic pathway of cell death. According to earlier reports, ratio of Bax to Bcl-2 decided the vulnerability of cells undergoing apoptosis<sup>[36]</sup>. Bcl-2 expression was significantly inhibited whereas the expression of p53, Bax, and caspase-3 increased after the treatments in a time-dependant manner. Chloroform fraction might encourage programmed cell death through p53 balancing the ratio of Bax to Bcl-2. Bcl-2 family is one of major classes of regulators in the intrinsic pathway<sup>[37]</sup>. The possible method by which p53 control the apoptosis by increasing gene expression of pro-apoptosis genes and inhibiting antiapoptotic genes<sup>[38,39]</sup>. Apoptosis occurred due to extrinsic or intrinsic or mitochondrial pathway<sup>[40]</sup>. Based on the above activity exhibited by the chloroform fraction, identification of active substances would play a significant role in the safe, effective use of chloroform fraction therapeutically. In the end, the current study confirmed that the chloroform fraction killed A-549 cells by induction of apoptosis.

This investigation established for the first time that the chloroform fraction of *B. monosperma* up regulated p53, Bax, caspase-3 gene and down regulated Bcl-2 gene as. Therefore, the chloroform fraction of *B. monosperma* could serve as a potential source to find new molecules for cancer treatment. Since the above results demonstrated the cytotoxic potential of the chloroform fraction of *B. monosperma in vitro*, additional studies are required to investigate possible *in vivo* cytotoxic activity of the chloroform extract and to find out the molecular mechanisms of such activity.

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### Conflict of interest:

We declare that we have no conflict of interest.

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