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Sophora interrupta Bedd leafs aqueous extract inhibits proliferation of MCF-7 breast cancer cells by inducing apoptosis

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ABSTRACT

The identification of medicinal plants and derived natural products for cancer therapeutics was long term developing goal. Here we investigated the anti proliferative and anticancer properties of Sophora interrupta Bedd aqueous extract on MCF-7 breast cancer cells. The cytotoxicity studies were performed by MTT and LDH assays reveals that its increase in dose dependant manner form 100-1000 µg/ml for 24 hours. The microscopic studies revealed the morphological characteristics of apoptosis such as cell shrinkage, and membrane bubbling. The type of cell death was explored by fluorescence microscopy using the acridine orange/ethidium bromide (AO/EtBr) method. Significant number of treated cells because of late apoptosis was converted from green to orange color. The nucleus morphological features under gone apoptosis induced by the aqueous extract were evaluated by Hoechst 33258 staining, nucleus trails and apoptotic bodies increasing with increase concentration of aqueous extract. The dead cell count done by trypan blue method. Minimal or no oligonucleosomal DNA fragmentation was detected in untreated control cells. A clear dose response laddering pattern can be observed in treated cells. The morphological changes of cells well correlated with inter-nucleosomal DNA fragmentation. The present studies explain that aqueous extract of Sophora interrupta Bedd inducing the cell death through apoptosis and this may be potent drug target for mammary cancers. However further studies need to evaluate mode of action and molecular mechanism of Sophora interrupta Bedd inducing the cytotoxicity.

***Key words**: Sophora interrupta Bedd, MCF7 Cell lines, Brest cancer, LDH, MTT.*

INTRODUCTION

Cancer is a hyperproliferative disorder that involves transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis. The breast cancer is worlds second leading cause between all the cancers. It is anticipated that more than 1 million women are diagnosed with breast cancer every year, and it accounts for approximately 410 000 deaths per year [1]. The therapeutic options for advanced-stage breast cancers are still fairly limited [2]. In addition, these cytotoxic agents are also associated with often severe, dose-limiting, systemic toxicities. Therefore, the need for development of novel therapeutic agents active against breast cancer remains an important goal. Currently, a greater emphasis has been given towards the researches on complementary and alternative medicine that deals with cancer management. Out of that Plants have long history of use in the treatment of cancer [3-7]. One of the important plants named as *Sophora interrupta Bedd* belongs to the family; Fabaceae (Leguminaceae, Papilonaceae) is commonly called as Edwardsia maderaspatana Wight, Pili Girgoli There are approximately 219 species in this genus Sophora. Sophora interrupta Bedd which is available exclusively in Seshachalam Hill ranges of Tirumala. This plant is woody perrineal shrub with pinnate leaves, sub opposite leaflets, broadly ovate and golden yellow flowers. It has multifarious medicinal properties including antibacterial [8], anti cancer [9] and anti inflammatory [10]. It has also been proposed that, Sophora interrupta Bedd which is potentially used in cancer therapy could be due to presence of anti cancer and antioxidant bio active constituents [11]. It has been proposed that, implementation of Phyto chemicals in disease rehabilitation, provides new vistas for drug discovery system rather than synthetic drug candidates.

The current investigation taken up here to evaluate the anticancer potential of Sophora interrupta Bedd leaves (aqueous extract) on Mcf-7 Breast cancer cells using different assays.

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MATERIALS AND METHODS

Chemicals and Reagents:

Sophora interrupta bedd was collected from the Seshachalam hill ranges from Tirumala, A.P, India. The taxonomic identification of the plant by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, A.P, India and the voucher specimen number (BK-13) was deposited in the department herbarium. DMSO (dimethyl sulfoxide) was obtained from MERCK Co. RNase, Proteinase k, Triton x-100, Antibiotic solution (pen- strep), trypsin-EDTA, MTT were obtained from Hi-Media Co. RPMI- 1640 and FBS were obtained from sigma India.

Preparation of aqueous extract:

The leaves of Sophora interrupta bedd were washed under running tap water and rinsed with distilled water to remove the surface contaminants. Subsequently, incubated under shadow for 3 days. The dried leaves were then made into a fine powder. 10 grams of the powder was weighed and soaked in water at a concentration of 10% w/v for 8 hours and filtered using filter paper to get a clear aqueous extract. Sophora interrupta bedd aqueous extract was lyophilized and stored cold room for further analysis.

Cell culture:

Breast cancer cell line MCF–7 (Michigan cancer foundation) was obtained from NCCS, Pune. The cells were plated in 25 cm^2 tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were grown at 37° C under humidified 5% CO_2 and 95% air. The media were replaced every 2 days. At 85% confluence, cells were washed twice with phosphate-buffered saline (PBS, pH 7.3). Cells were then trypsinized and centrifuged at 1500 rpm for 4 mins at room temperature. The pellet was suspended in media and further subculture.

Effect of *Sophora interrupta Bedd* aqueous extract on cell morphology: The MCF-7 were incubated with the aqueous extract at a concentration of 100, 250, 500 and 1000 µg /mL for 24 hours. The morphology of treated and negative-control cells was observed at 3, 6 12 and 24 hours under an inverted microscope (Tokyo, Japan).The doxorubicin was used as a standard positive control for all the assays.

Cell viability assay:

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The MCF-7 ($3x10^3$ cells/well) cells were cultured in 5% CO_2at 37°C in RPMI medium (containing 10% FBS, 100 µg/mL penicillinand 100 U/streptomycin) in 96-well plates. After incubation overnight, the RPMI medium in each well was replaced with different concentrated solutions and incubated for 24 hours individually. Afterwards, 20 µL of MTT (5 mg/mL in PBS) was added to each well and the cells were incubated for another 4 h at 37°C. The supernatants were then aspirated carefully and 100µl of dimethyl sulfoxide (DMSO) was added to each well.The plates were shaken for an additional 10 min and the absorbance values were read by the Microplate Reader (Bio-Rad,Hercules, CA, USA) at 570 nm [12]. The % of inhibition of each concentration was calculated by the following formula:

$$\% \text{ of inhibition} = \frac{\text{Control O.D - Dose O.D}}{\text{Control O.D}} \times 100$$

Inhibition concentration (IC_{50}) was evaluated by plotting graph with concentration (µg) of Sophora interrupta bedd at X axis and % of inhibition at Y axis

Lactate dehydrogenase Activity (LDH) assay:

LDH assay was performed with 2×10^5 cells were seeded in a 6-well plate one day before assaying and all samples were analyzed in triplicate in culture medium of untreated confluent, by using a commercial kit (Agappe Diagnostics, India) based on the transformation of pyruvate to lactate by LDH, at pH 7.5, in the presence of NADH coenzyme. The transformation of NADH to NAD+ is accompanied by a decrease in absorbance (A) at 340 nm, which correlates with the LDH activity. The change of absorbance, in the absence or presence of different doses of extract, was recorded over a 0.5- to 4.5-min period, and the relative ΔA/min was calculated. The change in absorbance was converted to LDH international units per liter (U/l) by the following calculations: $\Delta A/\text{min} \cdot (tV \cdot 1{,}000/EMC \cdot l \cdot sV)$, where tV is the total volume, EMC is the NADH extinction micromolar coefficient (6.22 cm^2 µmol at 340 nm), l is the light pathlength (1 cm), and sV is the sample volume.

Tryphan blue dye exclusion viability cell count:

This assay measures the percentage of viable in a cell suspension. The cells were mixed with 2 ml of 1X sterile phosphate buffered saline and mixed well. The 1ml of cells was suspended in 1ml of 1X PBS and aspirated well to avoid the formation of cell clumps. 1:1 dilution of cell suspension was made using 0.4% Trypan blue solution by adding 10µl of cell suspension to 10µl of dye and 10µl of cells were loaded in the haemocytometer and left undisturbed for 2-3 minutes in room temperature. The live cells were clear and translucent where as dead cells were dark blue. The live cells were counted in WBC counting chamber [13].

Calculation:

1. Average number of cells/chamber= total no. of cells counted in all chambers/4
2. No. of cells present: No. of viable cells = average no. of cells × dilution factor $\times 10^4$/ml

Acridine orange/Ethidium bromide Staining (AO/EB):

The morphological features of apoptosis induced by the aqueous extract was evaluated by Acridine Orange-Ethidium Bromide dual (AO/EtBr) staining [14] Briefly, cells were seeded in a 6-well plate at a density of $2 \times 1\ 0^5$cells and then treated with different concentrations of the extract for 24 hours. After washing once with phosphate buffered saline (PBS), the cells were stained with 20 µL of a mixture (10 µL/mg AO and 10 µL/mg EB in distilled water) of acridine orange-ethidium bromide solutions. The cells were immediately washed with PBS and observed under fluorescence microscopy at 450–490 nm. The number of cells manifesting morphological features of apoptosis was evaluated.

Hoechst staining:

Nucleus and chromatin morphology was assessed by Hoechst staining, as described by Xia et al [15], with slight modifications. In brief, the cells were plated in dishes of 6 well plates at a density of 2X 10^5cells/well. Cells were exposed for 24 h to 100 250 500 1000 µg/ml of *S.Bedd* aqueous extract. Each sample was fixed with 4% of paraformaldehyde for 15 min in a cold room and then washed twice with 0.1% triton X-100 in PBS. The staining was carried out using Hoechst 33258 green dye (5 g mL/ml) in 0.1% of triton X-100/PBS solution, for 10 min at room temperature. The samples were visualized under a fluorescence microscope by magnification 400X, and nuclei that appeared condensed or fragmented were considered as apoptotic.

Determination of Inter Nucleosomal DNA Cleavage:

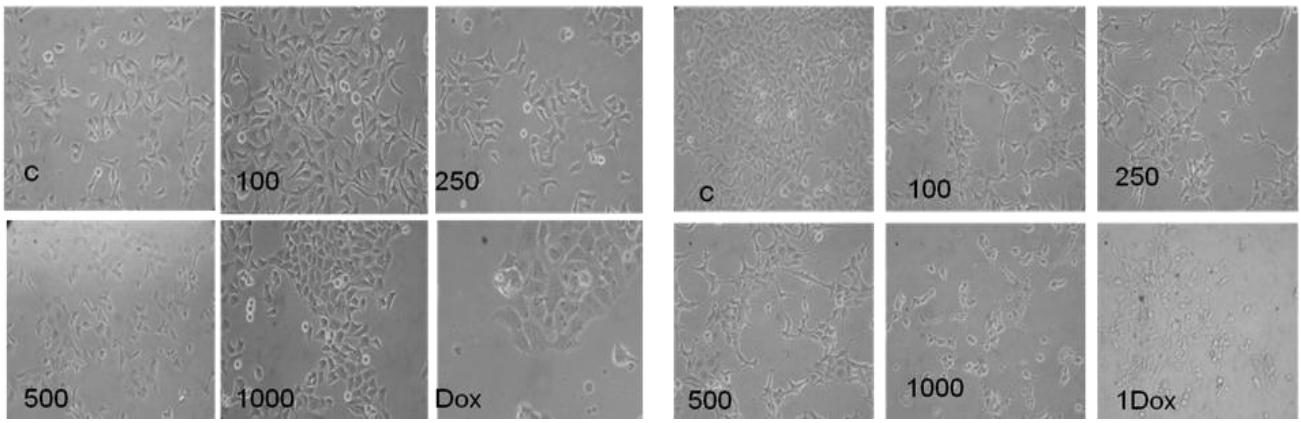
The MCF-7 cells were seeded in 6 well culture plate at a density of 3×10^5 cells/well overnight and treated with 100, 250, 500, 1000 µg/ml concentration of Sophora interrupta Bedd for 24 hr following these treatments. The cells were then lysed with lysis buffer (5 mM Tris [pH 8.0] 20 mM EDTA, 0.5% Triton X-100, Proteinase k 0.1mg/ml) on ice for 45 min. Fragmented DNA in the supernatant fraction after centrifugation at 14,000 rpm (45 min at 4°C) was extracted 2X with phenol: chloroform: isoamyl alcohol (25:24:1, v/v) and centrifuged at 14,000rpm for 10 min at 4°C.The extraction was repeated using an equal volume of chloroform and then the aqueous which was obtained was precipitated 2x with absolute ethanol and 1/10th 3 M sodium acetate overnight at 20°C and spun at maximum speed for 15 mins at 4°C. The DNA pellet was washed once with 70% ethanol, air dried and re-suspended in Tris-EDTA buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) with 100µg/ml RNase and incubated at 37°C for 2 hr.

Total amount of DNA was resolved over 1% agarose gel in 1X TBE buffer (89 mM Tris, pH 8.3, 89 mM boric acid, and 2 mM EDTA) at 50 V for 1 hr and was stained with 0.5ug/ml ethidium bromide for 20 mins and distained by single distilled water. The bands were visualized under UV transilluminator followed by digital photography [16].

RESULTS AND DISCUSSION

Morphological Observations of MCF-7:

Under phase-contrast microscope, the untreated MCF-7 cells exhibited typical growth patterns and a smooth, flattened morphology with normal nuclei (**Fig. 1**).The cells treated with *Sophora interrupta* extract, exhibited apoptotic morphological changes in dose dependent manner (**Fig. 1**), such as cytoplasmic blebbing and MCF-7 cells became enlarged, irregular-shaped, and vacuolated cytoplasm [17]. These observations provide evidence that an apoptotic pathway is triggered with the *Sophora interrupta* treatment in breast cancer cell line. The results are shown in **Fig. 1**.



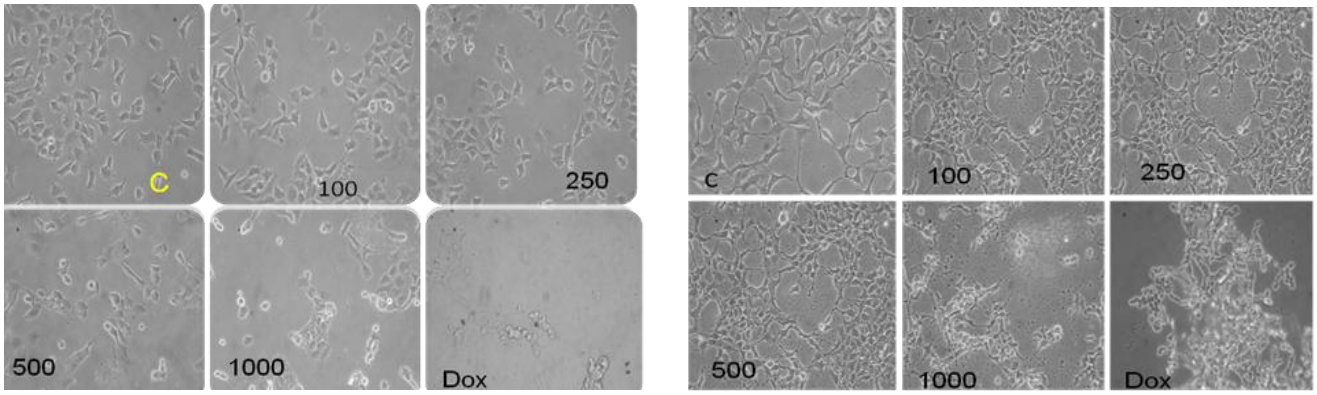


Fig. 1: Morphological observation of MCF-7 cells by phase contrast microscopy. Control cells; 100, 250,500,750, µg/ml & 1000 µg/ml of Sophora interrupta bedd treated cells A; 3hours B; 6hours C; 12hours D; 24 hours duration. Standard positive control doxorubicin.

Cell viability assay:

The cytotoxicty effects of Sophora interrupta aqueous extract on the growth of MCF-7 cells were examined by the MTT assay. The enzyme activity (mitochondrial dehydrogenase) directly proportional to amount of formazan formed. This indicates percentage of viability of cells in the well. The shape of dose-response curves indicates a significant inhibition of cell growth in dose-dependent manner form 1-1000 µg/ml concentration in 24 hrs period of treatments (**Fig. 2**). Cell growth was significantly lower ($p < 0.05$) if extract-treated cells were compared to control cells. The IC_{50} was graphically obtained from the dose response curve. The extract at the dose above of 500 µg/ml induced marked cytotoxicity. This results well correlates with previous reports [18]. The doxorubicin used at 0.01, 0. 05 and 1µg/ml concentrations as positive control (**Fig. 2**)

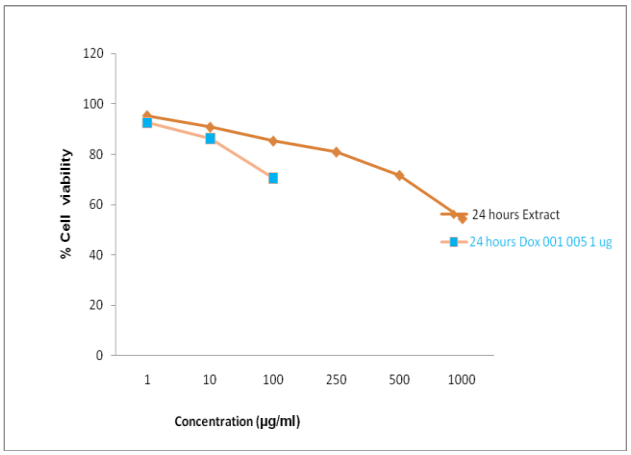


Fig. 2: Effect of *Sophora interrupta Bedd* on cell viability.MCF 7 cells were treated with different concentration of extract for 24 duration. Standard positive control doxorubicin at 0.01, 0.05, and 1 µg/ml concentration for 24 hours duration.

Lactate dehydrogenase Activity (LDH) assay:

Quantification of LDH activity released from damaged cells was measured using the LDH assay. The LDH release from MCF-7 lines by aqueous extract was investigated. Cells were incubated with 100, 250, 500 1000 µg/mL for 3, 6, 12, 24 hours. Data analysis of results revealed that the release of LDH from cells into the culture medium increased significantly after 12 h in MCF-7-treated cancer cells compared with untreated cells. This suggested that treatment aqueous of S.bedd caused plasma membrane rupture and cellular damage in cancer cells increase in dose dependent manner (**Fig. 3**). However, in normal cells, there was no significant difference in LDH release. (**Fig. 3**)

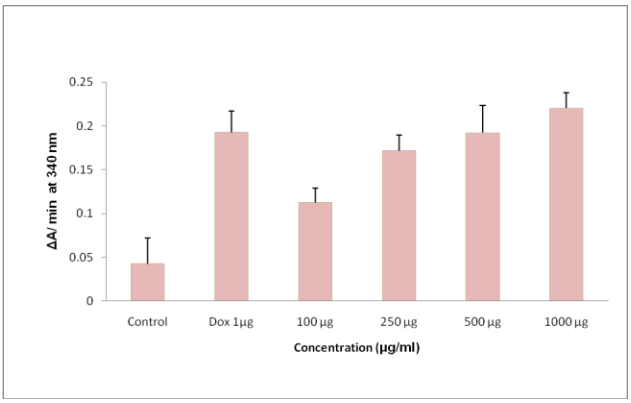


Fig. 3: LDH levels after 24 hours exposure of MCF cells to Sophora interrupta bedd leaf aqueous extract

Tryphan blue dye exclusion viability cell count:

The Percentage cell viability of cell lines was carried out by using Trypan blue dye exclusion technique [13]. **Table. 1** shows that *S.bedd* was reduced cell viability of tumor cells in dose dependent manner. There was a remarkable growth rate decrease to 50% in the number of treated cells when compared to control cells after 24 hrs while significant decrease was observed at 500 µg/ml concentration. The results obtained indicate the possibility that *Sophora interrupta Bedd* may have been involved in cell cycle arrest may be in G0/G1 phase, which led to reduction in MCF-7 tumor cell numbers in the cell culture.

Acridine orange/Ethidium bromide Staining (AO/EB):

The results obtained with the acridine orange/ethidium bromide (AO/EB) staining of MCF-7cells exposed 24 h at various concentration of *S.Bedd* extraction from 100 to 1000 µg/mL concentrations are shown (**Fig. 4**).The untreated, control MCF-7 cells were characterized by bright green nucleus with uniform intensity and the absence of ethidium bromide uptake, while apoptotic cells appeared orange [19] (**Fig. 4**).Compared with the spontaneous apoptosis observed in the control cells (early apoptotic 3.20%, 0% late apoptotic and 0% necrotic cells), MCF-7 cells treated with extracts of S.Bedd showed increased percentages of early apoptotic cells for 24 h treatment, increased percentages of early apoptotic (12.22%), late apoptotic (51.11%) and necrotic cells (17.78%) after 24 h at higher concentration of *Sophora interrupta Bedd* aqueous extract. The results were compared with positive control dexorurucin as shown in **Fig. 4**.

Table No. 1: Viable cell count of Sophora interrupta bedd leaf aqueous extract treated with MCF-7 Cell line after 24 Hours

Sophora interrupta Bedd leaf Aqueous extract concentration	24 hour	
	Viable cells (10^4 Cells)	Dead cells (10^4 Cells)
Control	40	12
Dox	54	32
100	24	10
250	38	19
500	42	2
1000	44	31

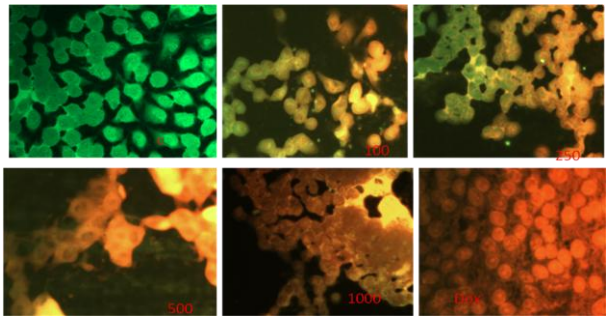


Fig. 4: typical morphological changes of MCF-7 cells induced by 100, 250, 500, 100µg/mL aqueous extracts of *S.bedd*, stained with AO/EB. The images were taken using fluorescence microscopy at 40×. C: control.

Hoechst staining:

It has been shown that the mechanism of action of many anticancer drugs is based on their ability to induce apoptosis [20, 21]. Based on this it was desirable that cancer cells treated with Sophora interrupta bedd undergo apoptosis as their mode of cell death. Analysis of nuclear chromatin condensation and apoptotic index: MCF-7 cells were treated with Sophora interrupta bedd leaf extracts. At the end of the incubation period the cells were stained with Hoechst 33342 dye and the cells were observed under the microscope for chromatin condensation. One of the characteristics of cells undergoing apoptosis is nuclear chromatin condensation. The DNA in condensed chromatin stained strongly with fluorescent dyes allowing non apoptotic cells to be discriminated from apoptotic ones. The results for MCF-7 are shown in Figure 5. Apoptotic cells can be differentiated from non-apoptotic cells as the former are bright and their nuclei condensed. The nuclear condensation can be clearly seen from 100ug/ml to 1000ug/ml concentration. Typical morphology of apoptotic nuclei stained with the DNA-binding fluorophore Hoechst 33258. The treated cell nuclei appear slightly smaller than normal nuclei (**Fig. 5**), probably because apoptotic cells have a tails nuclei with apoptotic bodies (**Fig. 5**).The nucleus apoptotic were found in dose dependent manner, the shape of nucleolus completely vanished in at higher concentration and dexorurucin.

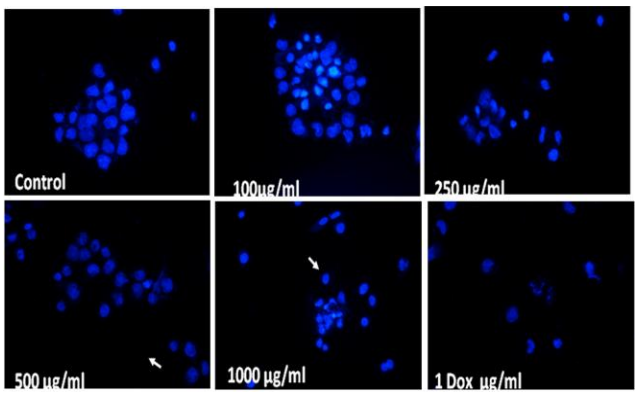


Fig. 5: Hoechst 33258 staining nucleus staining for 24 hours of treatment of S.bedd different concentrations aqueous extract. Fluorescence microscopy at 40×. C: control

DNA fragmentation:

In order to further investigate the cell death mechanism induced by Sophora interrupta, DNA of the cells treated with 100-1000µg/ml concentrations induced the inter-nucleosomal breakdown of chromatin DNA, resulting in ladder-like agarose electrophoretic patterns of degraded DNA products, typical of classical apoptosis [22, 23]. As shown in **Fig. 6**, the *Sophora interrupta Bedd* Minimal or no oligonucleosomal DNA fragmentation was detected in untreated control cells. A clear dose response effect can be observed in **Fig. 2** (lanes 3 to 7). This data suggest that cell death induced by Sophora interrupta Bedd is apoptosis. It is likely that DNA fragmentation seen in the current study does not solely represent apoptotic cell death but also primary or secondary necrosis. The morphological observations and DNA cleavage are well correlated interns of cell death was accompanied by cell shrinkage, membrane blebbing, partial chromatin condensation pattern.

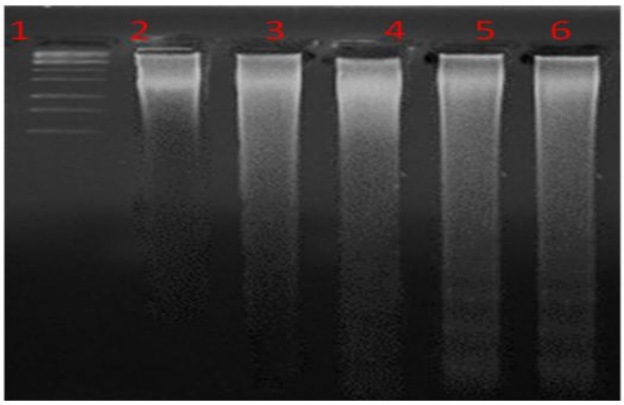


Fig. 6: DNA Fragmentation of Sophora interrupta Bedd aqueous extract treated with MCF-7 cells after 24 hours Lane: 1 kb ladder Lane2 : control Lane3:100 µg/ml Lane4:250 µg /ml Lane5:500µg/ml, Lane6:1000µg/ml/ml

CONCLUSION

Thus the study concludes that *Sophora interrupta Bedd* leaves (aqueous) inhibits MCF-7 cell proliferation by inducing the apoptosis and suppression of the survival pathways. The induction of apoptosis was evident when the morphological changes of MCF-7 cells treated with leaves aqueous extract resulted in morphological apoptotic changes after 24 hr, along with DNA fragmentation and cells and nucleus stainings. However, further research should be carried out to elucidate the relationship between *Sophora interrupta* (aqueous extract) induced cell death in breast cancer cells.

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