



SHORT COMMUNICATION

Cytotoxic and apoptosis induction potential of *Mimusops elengi* L. in human cervical cancer (SiHa) cell line



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Abstract Bullet wood tree, also called Spanish cherry (*Mimusops elengi* L.) is a medium-sized evergreen tree belonging to family Sapotaceae which is widely used in the treatment of different ailments. The present study investigated cytotoxic potential of methanolic leaf and bark extracts against human cervical cancer cell line (SiHa) by MTT (3-(4,5-dimethylthiazol-2-yl)-2; 5-diphenyl-tetrazolium bromide) assay, the apoptotic cells were quantified further by flow cytometry by FACS fluorescence activated cell sorting. The extract of *M. elengi* bark and leaf was effective towards tested cell line, with IC₅₀ values of 35.08 ± 2.92 µg/ml and 67.46 ± 4.21 µg/ml respectively. Further there is an increase in apoptotic bodies from 0.24% to 60% and 69% after treatment with extracts. These extracts exhibit significant cytotoxic effect by inducing apoptosis. The *M. elengi* has never shown anticancer activity on SiHa cells. These findings suggested that extracts and compounds from this plant could be useful for preventing and treating human gynaecologic cancer disease.

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1. Introduction

Cervical cancer constitutes the second most common cancer in women. This is due to the infection with Human Papil-

oma Virus (HPV), notably type 16 and 18 virus (Alvarez-Salas and DiPaolo, 2007). Although the mortality is high, it is preventable through screening and treatment of precancerous lesion and the use of vaccines (Berrington and Lall, 2012). Conventional cancer therapies like radiotherapy and chemotherapy will damage the cancer cells and also affect some healthy cells in the body and cause side effects like nausea, emesis, bone marrow problems, hair loss, fatigue etc., and there are no prophylactic measures available to address the side effects (Flay and Matthews, 1995), therefore natural products are being tested for the treatment but these are yet to prove their efficacy in preclinical and clinical studies.

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Recent studies have shown that antioxidants and herbal derivatives may be effective against prevailing HPV infection (Bharti et al., 2009; Su et al., 2011; Wu et al., 2006). Plants remain as an important and continuing source for treating cancers. More than 70% of the 177 anticancer drugs approved since 1950 were natural products or mimetics, and the research still continues (Brower, 2008).

Apoptosis is a process of programmed cell death and is a central event essential to maintain tissue homeostasis for all organ systems in the human body (Kumar et al., 2012). Inhibition of apoptosis results in a cell proliferation, such as cancer. Tumour cells use a variety of molecular mechanisms to suppress apoptosis (Subhadradevi et al., 2011); hence the induction of apoptosis in tumour cells is believed to be a specific therapeutic approach towards cancer chemotherapy.

Spanish cherry (*Mimusops elengi* L., Sapotaceae) is a small to large evergreen tree distributed throughout the greater parts of India. In ayurveda, the bark, flowers, fruit and seeds are having cardiogenic, alexipharmic, stomachic, astringent cooling, anthelmintic, tonic, and febrifuge properties (Mitra, 1981). The bark and fruits of this plant are used in the treatment of diarrhoea and dysentery (Jahan et al., 1995). Rinsing mouth with bark decoction is believed to strengthen the gums, reduce inflammation, prevent bleeding of gums, and to stop bad breath caused by pyorrhoea and dental caries (Baliga et al., 2011). Different parts of the plant have also been reported for anti microbial (Ali et al., 2008), anti ulcer (Payal et al., 2003), anti anxiety (Gayatri et al., 2011), anti oxidant, anti hyperglycaemic (Ganu et al., 2010), anti hyperlipidemic, anti helminthic (Gadamsetty et al., 2013), anti inflammatory and anti pyretic properties (Purnima et al., 2010). Several triterpenoids, steroids, steroidal glycosides, flavonoids and alkaloids have been identified and reported from this plant (Misra and Mitra, 1968).

Cervical cancer is a major health issue where chemotherapy is not effective due to its side effects, thus the use of pharmacological agents that are active towards HPV cancer cells with lesser side effects is strongly needed. This study is an attempt to evaluate apoptotic effects of methanolic extracts of leaves and bark of *M. elengi* on human cervical cancer cells.

2. Materials and Methods

2.1. Chemicals and reagents

Commercially available MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma (St Louis, MO). Caspase-3 apoptosis kit was purchased from BD Pharmingen, USA. All other reagents and solvents were of analytical grades.

2.2. Preparation of extracts

Fresh leaves and stem bark were collected in January 2010 in the city of Vellore (12°58' North, 79°09' East), India. The plant parts were identified and authenticated by using various taxonomic markers and other available literature by consulting a botanist in National Institute of Herbal Science, Chennai, India. The herbarium samples were prepared and stored at our

laboratory for future reference. The specimens were dried under shade, pulverized and extracted (60 g) with 400 ml hexane to remove chlorophyll and oily substances and followed by methanol at 70 °C using soxhlet extraction. The extracts were concentrated to dryness at 50 °C under reduced pressure using a rotary evaporator (Buchi, Switzerland). The extracts were kept at below 4 °C until further use (Kumar et al., 2012).

2.3. Preliminary phytochemical screening

Preliminary phytochemical screening of methanolic extracts of *M. elengi* leaves and bark was done for the presence of alkaloids, anthraquinones, amino acids, carbohydrates, flavonoids, saponins, steroids, tannins, and phenols by employing standard screening tests (Harbone, 1998).

2.4. Evaluation of cytotoxic activity

2.4.1. Cell culture

The HPV16 positive human cervical cancer cell line (SiHa) was obtained from the American Type Culture Collection (ATCC No. HTB-35), USA and checked for PCR positivity and contaminations. SiHa cells were cultured in a sterile T75 flask containing DMEM medium supplemented with fetal bovine serum (10% v/v) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ incubator.

2.4.2. Cytotoxicity assay

The Cytotoxicity effects of methanolic extracts of leaf and bark against SiHa were determined by MTT dye uptake method (Mahata et al., 2012). The SiHa cells (1×10^3) seeded and grown overnight in 96-well plate, were treated with plant extracts at a concentration ranging from 1 to 100 µg/ml for 24 h at 37 °C in a CO₂ incubator. Two hours prior to the completion of treatment 0.025 ml of MTT solution (5 mg/ml in PBS solution) was added to microplates and incubated at 37 °C. After incubation lysis buffer (20% SDS and 50% dimethyl formamide) was added to each well and then incubated overnight at 37 °C for solubilization of formazan crystals. Absorbance was measured at 570 nm with the aid of a 96-well multi-scanner autoreader (Biotek, Winooski, Vermont) with the lysis buffer serving as blank. The percentage of cell viability was calculated using the formula:

$$\text{Percentage cell viability} = \left\{ \frac{\text{OD of the experiment samples}}{\text{OD of the control}} \right\} \times 100.$$

2.5. Flow cytometric analysis of apoptotic cell death by quantitation of Caspase-3

The activity of caspase 3 was measured using the active caspase 3 apoptosis kit (BD Pharmingen, USA) following the manufacturer's protocol. Briefly, 50 µg of the extract was incubated with SiHa cells for 12 h and harvested. The attached and detached cells were pooled and pelleted with centrifugation at 200xg for 5 min at 4 °C (Mahata et al., 2011). The cells were permeabilized, fixed and stained for active caspase 3 (PE-conjugated). The cells were then analysed by fluorescence activated cell sorting (FACS). The number of 10,000 events was acquired and the cells were properly gated for analysis using FACS Aria instrument equipped with Flowjo software (Bravo-Cuellar et al., 2010).

2.6. Data analysis

Results of triplicate experiments were analysed using Microsoft Excel 2007 and presented as mean \pm SD ($n = 3$).

3. Results

3.1. Preliminary phytochemical analysis

The preliminary phytochemical screening of methanol extracts showed abundant amount of saponins and flavonoids and trace amounts of carbohydrates and anthraquinones in both leaf and bark extracts. Amino acids and tannins were absent in both the extracts.

3.2. Cytotoxic activity

In SiHa cell lines after treatment with appropriate concentrations of plant extracts, the cell viability was tested after 24 h by MTT assay. The results of both the extracts are summarised in Fig 1, which showed a dose dependant activity towards the SiHa cell line. The results are demonstrated as percentage viability and represented as mean \pm standard deviation ($n = 3$).

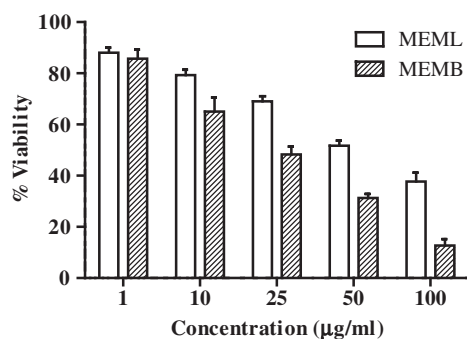


Figure 1 Cytotoxic activity of methanolic extract of *M. elengi* leaves (MEML) and methanolic extract of *M. elengi* bark (MEMB) against Cervical cancer cell lines (SiHa) Values are Mean \pm SD ($n = 3$).

From the results, the methanolic extract of *M. elengi* bark and leaf showed promising cytotoxic activity towards SiHa cells with IC_{50} values of $35.08 \pm 2.92 \mu\text{g/ml}$ and $67.46 \pm 4.21 \mu\text{g/ml}$ respectively.

3.3. Quantitation of caspase-3 activity by flow cytometric analysis

In order to confirm the apoptotic effect of the extract the activation of caspase-3 by flow cytometric analysis was done. Treatment of SiHa cell lines with methanolic extract of *M. elengi* at a concentration of $50 \mu\text{g/ml}$ resulted in the expression of caspase-3. The expressed caspase-3 cells were quantified flow cytometrically and the results are summarised in Fig. 2. The result showed a significant increase of caspase-3 levels after treatment of test extracts.

4. Discussion

Cervical cancer is a major problem worldwide, reporting about 15% of neoplasms (Um et al., 2002). Current treatments include chemotherapy, radiotherapy and surgical ablation which show less satisfactory results, thus there is a need for safer and effective alternative treatment for different kinds of cancers.

Cytotoxicity test is mainly carried out to screen the cytotoxic potentiality of the compounds present in the extracts which affect basic cellular functions. Cytotoxic potential of the sample was measured by MTT dye uptake method, where active cells convert the water soluble MTT to an insoluble purple formazan, further solubilised and its concentration was determined (Liu et al., 1997). We found that the extracts of *M. elengi* bark and leaf were significantly toxic to SiHa cells with IC_{50} values 35.08 ± 2.92 and $67.46 \pm 4.21 \mu\text{g/ml}$ respectively, when compared to the control. The bark extract was more toxic than leaf towards SiHa cell line. Cytotoxic activity of *M. elengi* is reported earlier as well, where Bhujbal et al. (2011) reported *in vitro* cytotoxic activity of methanolic bark extracts of *M. elengi* by *A. cepa* root tip assay. Bark extract was found to significantly decrease the percentage mitotic index and root length. In another study, Kar et al. (2012) reported the anti tumour activity of leaves of *M. elengi* against

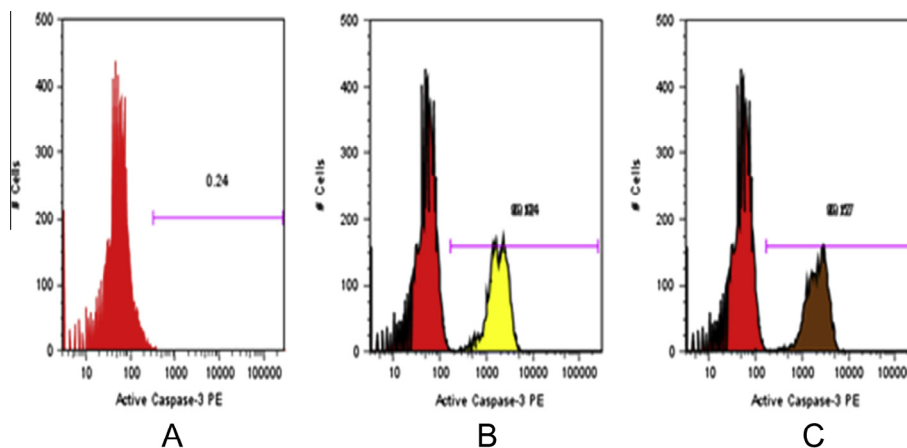


Figure 2 Flow Cytometric analysis of activation of Caspase-3 in cervical cancer cell lines (SiHa). (A), control untreated cells. (B), cells treated with MEML. (C), cells treated with MEMB.

Ehrlich's ascites carcinoma treated mice. Hence, the results imply that the methanolic extracts of leaf and bark were toxic towards tumour cells.

Apoptosis plays a major role in the regulation of tissue homeostasis and elimination of abnormal cells. Apoptosis is believed to be deregulated in cancer. An ideal therapeutic goal for cancer is to trigger apoptotic death selectively in tumour cells. Most of the antitumour drugs kill the cancer cells by stimulating the apoptotic pathway (Evan and Vousden, 2001). The key biochemical event involved in the induction of apoptosis is the activation of caspase-3 which is mediated through proteolytic cleavage of procaspase-3 via upstream caspases (caspase 7/9 or caspase 8). Activity of the caspase-3 which is an effect of caspases involved in both mitochondrial (extrinsic) as well as death receptor (intrinsic) pathways of apoptosis was expected in drug induced cytotoxicity of cancer cells. Therefore, drugs that restore the apoptotic pathways have the potential for effective treatment of tumours (Gao et al., 2011). To provide further evidence supporting the involvement of apoptosis, the caspase-3 was quantified by flow cytometry. Treatment of SiHa cell lines with methanolic extracts of leaves and bark of *M. elengi* at a concentration 50 µg/ml, for 12 h resulted in an increase of expression of caspase-3 from 0.24% to 69% and 60% for bark and leaf extracts, respectively. Thus it proves that the cytotoxic cell death is due to apoptosis. So the extract possibly induces caspase-3 and promotes apoptosis.

A number of bioactive molecules including flavonoids, phenolic compounds, alkaloids, and terpenoids previously reported for their cancer properties were identified from this plant in several studies (Gami et al., 2012). Among those isolated bioactive molecules, Lupeol, betulinic acid, gallic acid, and taraxerol have been shown in several reports to possess anti-cancer activity (Shoeb, 2006; Swain et al., 2012). In addition, many number of bioactive molecules were isolated and identified from the leaf and bark of this plant (Misra and Mitra, 1968). Therefore, the anti-cancer activity observed in crude leaf and bark extracts may be specifically due to any single chemical compound or it may due to phytochemical constituents. According to the results obtained, *M. elengi* leaf and bark extracts appeared to be potent anti-cancer agent and to the best of our knowledge this is the first report on anti-cancer activity of *M. elengi* against SiHa cell line.

In conclusion, the present study demonstrates the cytotoxic properties of methanolic extracts of leaf and bark of *M. elengi* L. Further studies to characterise the active principles and to elucidate the mechanism of action are in progress.

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