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Original Article

ANTI-PROLIFERATIVE AND APOPTOTIC ACTIVITIES OF *SYZYGIUM SAMARANGENSE* (WAX APPLE) FRUITS EXTRACT AGAINST HUMAN A549 LUNG CANCER CELL LINES

NIVETHA THAMPI, J. VERONICA SHALINI*

Department of Biotechnology, Jeppiaar Engineering College, Rajiv Gandhi Salai, Chennai 600119, Tamil Nadu, India
Email: shalinijeyadoss@gmail.com

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ABSTRACT

Objective: Medicinal plants have been in use from time immemorial and their utility has been expanding step by step in the present world. The intention of the present study was to check the anti-proliferative and apoptotic activity of S*yzygium samarangense* fruits methanolic extract against of A549 cell lines.

Methods: The methanol extract at different concentrations were tested against A549 human lung cancer cell lines for cell viability or cytotoxicity by MTT assay and the hallmark of the apoptosis was analyzed by DNA fragmentation method. The morphological changes resulted due to apoptosis were investigated by Propidium Iodide (PI) staining technique.

Results: The results showed that the tested extracts showed strong and decreased cell viability in a concentration-dependent manner. IC_{50} value represented that the anti-proliferative activity was found with a minimum concentration of 21.86µg/ml. The presence of ladders of DNA fragments in the DNA fragmentation assay indicates a biochemical hallmark of intrinsic apoptotic cell death. Altered cell morphology after treatment with the extract demonstrated that cells experienced apoptosis.

Conclusion: The present findings encourage further for the isolation and identifying, of active components present in *S. samarangense* fruit pulps to understand the mechanism of action *in vivo* that can make an allowance for developing chemotherapeutic agent against cancer.

Keywords: S*yzygium samarangense*, Anti-proliferative activity, MTT assay, DNA Fragmentation, Apoptotic activity, PI staining.

INTRODUCTION

Cancer is a class of diseases in which a cell or a group of cells exhibit unrestrained growth, invasion and metastasis [1]. Cancer is reported as the second most common cause of death after heart disease and accounts for about 23% of the total deaths [2]. By 2020, the world population is expected to have increased to 7.5 billion; of this number, approximately 15 million new cancer cases will be diagnosed, and alarmingly 12 million cancer patients will die [3]. Cancer can be caused by carcinogenic factors, hereditary factors and also mostly by the environmental (including lifestyle) factors [4]. Induction of apoptosis in cancer cells has been developed as an indicator for the ability of naturally derived active components to treat cancer [5]. Apoptosis in cancer cells can be triggered through several tightly regulated signalling pathways, such as the receptor, mitochondrial and mitogen-activated protein kinase (MAPK) pathways. Caspases are the main promoters of apoptosis and are turned on in all pathways. The cleavage of 116 kDa PARP-1 to 89 and 24 kDa fragments induced by caspase 3 activation is an indicator of apoptosis [6].

Lung cancer is one of the five most common cancers prevalent worldwide among both men and women. Every year, more than 11 million people are diagnosed with cancer. By 2020, it is estimated that there will be 16 million new cases of lung cancers every year [7]. The occurrence and development of lung cancer are associated with a variety of factors, including immune disorders, dysfunction of lung epithelial cells, inflammation, oxidative stress, apoptosis etc [8]. Cancer treatment includes surgery, radiotherapy and chemotherapy. However, even after the surgical ablation of progressive cancer, metastasized tumour cells continue to progress and this reason makes cancer treatment problematic [9]. In order to kill tumour cells that proliferates rapidly, radioactive rays and most of the currently used anti-cancer drugs damage DNA or suppress DNA duplication and at the same time, they causes serious adverse effects, such as bone marrow function inhibition, exhibit cell toxicity, induces genotoxic, carcinogenic and teratogenic effects in non-tumor cell [10, 11].

Nature has given a phenomenal storage facility of solutions for all the ailments of mankind. Natural compounds are viewed as more secure and effectively biodegradable than synthetic compounds and the problem of drug resistance observed in synthetic drugs is additionally lessened [12]. Lignans, flavonoids and phenolic compounds that are broadly distributed in plants, have been reported to possess multiple biological effects [13]. Drugs acquired from medicinal plants comprise about 80% in developing countries and 25% of total drugs in developed countries [14].

In spite of the recent predominance of synthetic chemistry, as a method to discover and produce drugs, the capability of bioactive plants or their concentrates to give new and novel products for disease treatment and prevention is still tremendous [15]. Among FDA approved drugs of natural origin for the treatment of cancer and infectious, it shares an account of 60% and 75% respectively [16]. Plants have an almost indefinite capacity to produce compounds that engage researchers in the quest for the discovery of new and novel entities [17]. A realistic and promising strategy for the prevention of cancer is through a continual search for new anticancer compounds in plant medicines and traditional foods [18]. As cancer cells have developed multiple resistances towards the induction of apoptosis, the re modulation of apoptosis signalling pathways by natural compounds constitutes a crucial event in these antitumor activities [19].

Wax apple is a non climacteric tropical fruit from the Myrtaceae family and botanically identified as *Syzygium samarangense* (Blume) Merr. & Perry [20]. Syzygium is derived via Latin from the Greek word 'syzygos', meaning yoked together, possibly referring to the paired leaves [21]. Wax apple produces a pink fleshy fruit which is eaten fresh and they are oblong, pear-shaped, 5 to 12 cm in length, with four fleshy calyx lobes consisting of 1 to 4 seeds. Investigators have found that aerial parts contain Ursolic acid, Jacoumaric acid and Arjunolic acid, Mearnsitrin, 2-C-Methyl-5-O-Galloylmyricetin-3-O-α-l-Rhamnopyranoside, desmethoxy-matteucinol, 4,6 Dihydroxy-2-Methoxy-3,5-Dimethylchalcone, Methyl 3-epi-betulinate, Oleanolic acid. They also contain Desmethoxy matteucinol, 5-O-Methyl-4-desmethoxymatteucinol, Oleanic acid. Quercetin glycosides are

also present in this plant which includes Reynoutrin, Hyperin, Myricitrin, Quercitrin, Quercetin, Guaijaverin. It also contains Flavanone-(S) pinocembrin, and Phenolic acids-Gallic acid and Ellagic acid [22, 23].

Based on the literature review, no reports are available regarding the study of anticancer activity against A549 cell lines from the *Syzygium samarangense* fruits. Hence, the rationale behind the present study is to evaluate the efficacy of the methanolic extract from *S. samarangense* (Wax Apple) fruits for its Anti-proliferative and Apoptotic activities against human A549 lung cancer cell lines.

MATERIALS AND METHODS

Reagents

MEM was purchased from Hi-Media Laboratories. Fetal Bovine Serum (FBS) was purchased from Cistron laboratories. Trypsin, methyl thiazolyl diphenyl-tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals, Bangalore). All of other chemicals, reagents and stains were obtained from Sigma Aldrich, Mumbai.

Collection and maintenance of A549 cell culture

A549 cell line was obtained from National center for cell sciences (NCCS), Pune. The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

Procurement of wax apple fruits

The fresh fruits of *Syzygium samarangense* (Wax Apple) (fig. 1) were collected from the areas around Western Ghats and were identified by Dr. Hari Lal, a botanist from the Department of Botany, Calicut University, Kerala, India.

Fig. 1: Image showing fresh fruits of *Syzygium samarangense* (Wax Apple)

Preparation of wax apple methanol extract

The collected fruits of *S. samarangense* were washed thoroughly in tap water to remove the dirt and surface sterilized using 70% ethanol followed by washing with sterile distilled water. The clean fruits were cut into small pieces and were dried in a hot air oven at 60 ºC overnight. The dried fruits were finely powdered using mortar and pestle. 5g of the powder was mixed with 50 ml of methanol (100 mg/ml) and was kept in a rotary shaker at 37 ºC for 48 hrs. The solution was filtered through Whatmann filter paper no.1 and was stored in air tight bottle at 4 °C [24].

Cell proliferation assay by microculture tetrazolium (MTT) method

Cytotoxic activity of *S. samarangense* methanolic fruit extract against A549 cell lines was determined by MTT assay [25]. A549 Cells (1 × 10^5/well) were plated in 24-well plate and incubated in 37 °C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100 µl/well (5 mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1 ml of DMSO was added in all the wells. Then, the absorbance was read at 570 nm in a microtitre plate reader (Bio-Rad, Richmond, CA). The % cell viability was calculated using the following formula:

% cell viability = Abs570 of treated cells/Abs570 of control cells × 100

The % cytotoxicity was calculated using the following formula:

Cytotoxicity % = 100–Viability%

The concentration required for a 50% inhibition (IC_{50}) was calculated using regression analysis in MS excel.

DNA Fragmentation analysis

Based on the results obtained in the MTT assay, DNA fragmentation analysis was carried out to confirm the establishment of apoptosis or cell death [26] in the cancer cell lines treated with the methanol extract of wax apple fruits. In this protocol, 0.5 ml of the cell suspension was centrifuged and the pellet was collected and dissolved in TTE (Tris-Taps-EDTA) buffer with vigorous shaking. The mixture was centrifuged and the supernatant was transferred carefully to separate tubes. To the pellet added 0.5 ml of TTE buffer, 0.5 ml of Ice-cold 1M NaCl and 0.7 ml of ice-cold isopropanol. The mixture was incubated overnight at-20 °C to enhance precipitation. The content was centrifuged further to obtain pellet and was purified by repeated wash with 0.5 ml of 70% ice cold ethanol. The final pellet obtained was air dried and dissolved in 20-50µl of TE (Tris-EDTA) buffer. DNA samples were electrophoresed on 1% agarose gel that was visualized under UV light and photographed [27].

Morphology analysis by PI Staining

The nuclear changes in the apoptotic cells were observed by PI staining. A549 cells were seeded in 96-well plates then treated with 62.5µg/ml, 250µg/ml and 500µg/ml concentrations of wax apple methanol extracts for 24 hrs. The cells were made permeable with 50µl of acetone: methanol (1:1) mixture at – 20 °C for 10 minutes. Then 10µl of PI was added, spread by placing a cover slip over it and incubated at 37 °C for 30 minutes in the dark. The apoptotic cells with fragmented nuclei were detected using fluore scence microscope (Nikon, Japan) at 400X magnification [28].

RESULTS AND DISCUSSION

Cell proliferation assay by MTT method

In vitro cytotoxicity test is mainly performed to screen potentially toxic compounds that affect basic cellular functions. MTT assay is a rapid and high accuracy colorimetric approach that is widely used to determine cell viability and cell cytotoxicity, particularly in the development of new drug.

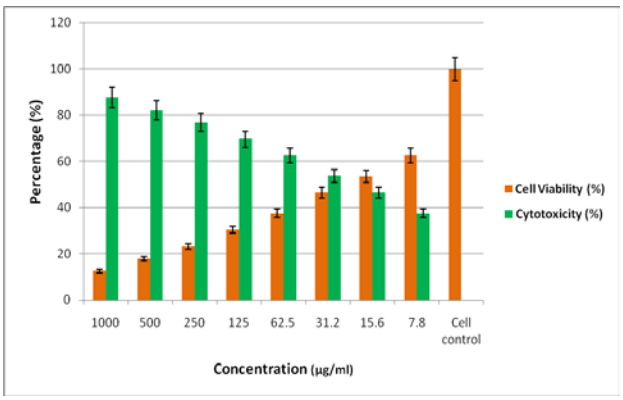

Fig. 2: % Cell viability and %Cytotoxicity in A549 cells induced by exposure to different concentration of *S. samarangense* methanolic fruit

The cytotoxic effect of *S. samarangense* fruit methanol extract was studied *in vitro* against lung cancer A549 cell lines using definite concentration ranging from 7.8 µg/ml to 1000 µg/ml. The results also show the % cytotoxicity and % cell viability of the sample have a direct dose-response relationship (fig. 2). The result showed that A549 cell proliferation was significantly inhibited by the wax apple methanol extract with an IC_{50} value of 21.86µg/ml of the concentration. The cytopathic effect of wax apple methanol extract on A549 cells was analyzed using an optical microscope. Untreated cells appeared elongated, attached smoothly on the culture surface. Following treatment with the extracts, cell shrinkage, loss of cell adhesion and reduced cell density were clearly observed with the increase in concentration of the extract (fig. 3).

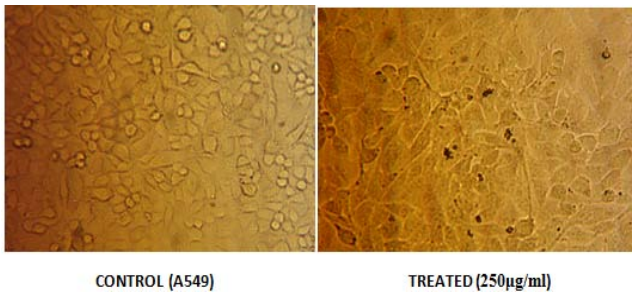

Fig. 3: Microscopic examination of morphological changes in A549 cells treated with methanolic extract of *S. samarangense* fruits

DNA Fragmentation analysis

The conventional agarose gel electrophoresis was performed on the cells treated with different concentrations (500µg/ml, 250µg/ml and 62.5µg/ml) of the plant extract for 48 hours (fig. 4). DNA fragmentation which is a typical hallmark of the apoptotic cell death was analysed in the study and found that the wax apple methanolic extract induced an anti-proliferative effect on A549 cells resulting in the degradation of chromosomal DNA into small oligo nucleosomal fragments as seen in Lanes 2, 3, and 4. In Lane 1, it shows the intact chromosomal DNA band of the untreated cells that acts as a control. Higher the concentration (500µg/ml) of the extract induced a greater laddering of DNA when compared to the lower concentration (62.5µg/ml).

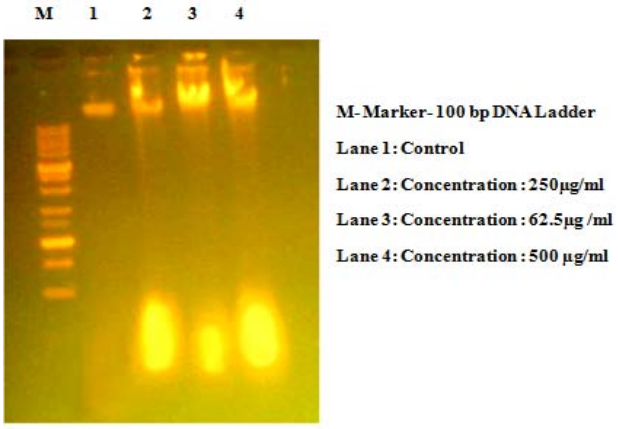

Fig. 4: DNA fragmentation pattern of extract treated and untreated A549 cells

Morphology analysis by PI staining

In order to identify whether the methanolic extract of wax apple induced inhibition of A549 cell growth via apoptosis, propidium iodide (PI) staining technique was used to observe the apoptotic morphology. The PI staining of *S. samarangense* methanolic extract treated A549 cells at different concentrations showed typical apoptotic morphology with brightly red, condensed nuclei (intact or fragmented), and formation of apoptotic bodies compared to the control A549 cells with round intact red nucleus (fig. 5). It was observed that there was a gradual increase of apoptotic cells and apoptotic bodies in a dose-dependent manner compared to the control. The maximum apoptotic cells and apoptotic bodies was found at the concentration of 500µg/ml, which indicates a significant apoptotic activity of *S. samarangense*.

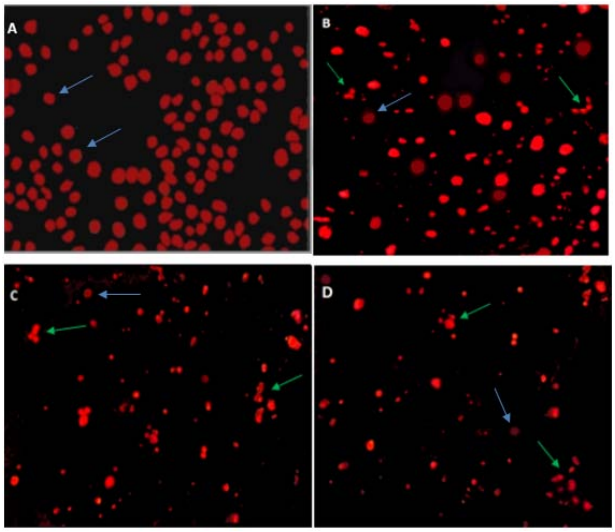

Fig. 5: PI staining to analyze the apoptotic morphology of extract treated and untreated A549 cells. (a) Untreated A549 control cells. A549 cells treated with (b) 62.5µg/ml, (c) 250µg/ml and (d) 500µg/ml. Green arrow indicates apoptotic cells while blue arrow shows normal cells.

In spite of the advancement in the early detection and upgraded options for cancer treatment, this disease still continues to be a major health issue in terms of morbidity and mortality. Cancer is a hostile disease, which if not detected at an early stage can metastasize to other organs of the body, potentially making even systemic chemotherapy ineffective [29]. Medicinal plants continue to play a chief role in the healthcare system and remains as an exclusive source of life saving drugs for the majority of the world's population. Flavonoids and phenolics acids are the most important groups of secondary metabolites and bioactive compounds in plants [30]. It is broadly accepted that a diet rich in plant-based foods is beneficial for cancer prevention and the polyphenols from fruits or vegetables are partly responsible for the chemo preventive effects. Dietary polyphenols may exert their anticancer effects via a variety of mechanisms such as removal of carcinogenic agents, modulation of cancer cell signalling and antioxidant enzymatic activities, and induction of apoptosis and cell cycle arrest [31, 32].

In vitro and *in vivo* studies have shown that some flavonoids modulate the metabolism and disposition of carcinogens and can contribute to cancer prevention [33]. Flavonoids are also effective in inhibiting signal transduction enzymes, protein tyrosine kinase (PTK), protein kinase C (PKC) and phosphoinositide 3-kinases (PIP3) which are involved in the regulation of cell proliferation [34]. The fruit pulp of *S. samarangense* are rich in phyto constituents that includes phenolic acids, flavonoids, glycosides and the action of these compounds could constitute for their cytotoxic activity. *In vitro* cytotoxic efficacy of the methanol extract at 1000µg/ml showed a % cell viability of 12.5%, which proves the role of wax apple fruits as a potential anti-proliferative agent. Another study reported promising cytotoxic effect of AgNPs synthesized from the leaves of *S. Samarangense* against A549 cell lines [35].

DNA fragmentation occurs in apoptotic cells that are caused by intrinsic activity which is induced by a variety of agents. This cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleosome length (180–200 bp) [36]. As a biochemical hallmark of intrinsic apoptotic cell death, DNA fragmentation was used to determine whether the antiproliferative effect of *S. samarangense* methanolic extract on cells acts through the respective apoptosis pathway [37]. The fragmented DNA was observed around 5 to 8 kbp, which was smaller than the typical fragmentation of DNA at 20 to 300 kbp when entering an early stage of apoptosis [38]. This specific DNA cleavage is due to the activation of endogenous endonuclease that cleaves at the exposed linker regions between nucleosomes. It is worthy to highlight that necrosis was not happened in this study because it associates with the random form of DNA cleavage [39].

Staining cells with fluorescent dyes are used in evaluating the nuclear morphology of apoptotic cells. The apoptotic features like chromatin condensation, nuclear shrinkage, and formation of apoptotic bodies can be seen under the fluorescence microscope after staining of nuclei with DNA-specific fluorochrome like propidium iodide (PI). PI staining is fluorescent nuclear dye that binds strongly to DNA. The red fluorescing dye propidium iodide is only permeable to dead cells and cannot enter the intact, plasma membrane of living cells [40]. Apoptosis is the process of physiologically programmed cell death in which an intrinsic pathway participates in the cell death [41]. The known molecular mechanisms of apoptosis includes activation of cysteine proteases as ICE (IL-l beta converting enzyme), calpain, Fas signaling, cell cycle interfaces, stress responses, Bcl-2 family and the tumor suppressor gene p53 [42]. Apoptosis has been demonstrated to be a major mechanism employed by many natural agents to mediate anticancer effects [19].

CONCLUSION

To the best of our knowledge, this is the first publication regarding the anti-proliferative and apoptotic effects of *S. samarangense* fruit pulp extracts against A549 lung cancer cell lines. MTT assay was carried out to measure the cytotoxicity in the cells exhibited by the wax apple extract that showed a dose-response relationship and an appreciable concentration required for 50% inhibition. The methanol extract demonstrated higher DNA damage as well as cell growth inhibition, as confirmed by DNA fragmentation analysis. Moreover, this wax apple methanol extract, through morphological analysis using PI staining procedures, showed that the extract was able to trigger cell death through apoptosis even at low concentrations. Indian traditional medicinal plants have led to several therapeutically useful preparations and compounds, generating enough encouragements among the scientists in order to explore more information about these medicinal plants. There is an emerging growth shift that hiked the markets for traditional plant-based remedies and an increased patronage strengthened by their application. Development of such modern drug from these medicinal plants should be emphasized for the treatment of cancer. A positive outcome of this study would possibly help to increase the efficacy of existing chemotherapies with reduced toxicity to normal tissues in the treatment of lung cancer. It is worthwhile to investigate the underlying molecular mechanisms leading to growth inhibition induced by single agents and combinations both *in vitro* and *in vivo*.

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CONFLICT OF INTERESTS

Declared None

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