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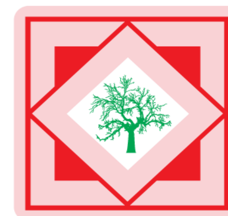

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Anti-cancer activity of *Annona squamosa* and *Manilkara zapota* flower extract against MCF-7 cell line

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ABSTRACT

Breast cancer mortality still remains the second leading cause of cancer-related deaths worldwide. Patients with breast cancer have increasingly shown resistance and high toxicity to current chemotherapeutic drugs. This has led to the identification of newer drugs/therapies for treatment of breast cancer. Plant-derived products have proved to be an important source of anti-cancer drugs. Hence the present study was directed to evaluate the anti-cancer activity of ethanolic extract of the flowers of Annona squamosa and Manilkara zapota against MCF-7 breast cancer cell lines in vitro and was compared with the normal, Vero cell lines. At 3.12 µg/ml concentration of A. squamosa and M. zapota, MTT assay showed 74.6% and 82.6% cell viability respectively and at 100 µg/ml concentration of A. squamosa and M. zapota, MTT showed 1.3% and 2.6% cell viability respectively. The IC_{50} values were 6.87 µg/ml and 12.5 µg/ml for A. squamosa and M. zapota respectively. The results indicate that M. zapota flowers showed potent cytotoxic activity as compared to A. squamosa flower extract. The study confirms the anti-cancer activity of both the plant extracts and hence can be used to develop them as a novel drug for the management of breast cancer. Their property may be due to the presence of phytochemicals such as alkaloids, phenols and flavonoids.

Keywords: Breast cancer, herbal drugs, ethanolic, MCF-7, MTT, phytochemicals.

INTRODUCTION

Cancer is a multi-step disease incorporating physical, environmental, metabolic, chemical and genetic factors [1]. Breast cancer is the most commonly occurring cancer in women, comprising almost one-third of all malignancies [2]. It accounts for approximately 25% of all female malignancies with a higher prevalence in developed countries. Breast cancer is the second leading cause of cancer-related death among females in the world [3]. Following genotoxic stress, an intact DNA damage response (DDR) is necessary to eliminate lethal and tumorigenic mutations.

The DDR is a network of molecular signaling events that control and coordinate DNA repair, cell cycle arrest and apoptosis [4]. Targeting the cell cycle to induce arrest pharmacologically is known to be effective in restricting tumor growth *in vitro* and *in vivo*, particularly in transformed cells that have an aberrant response to genotoxic and cellular damage [5]. Due to the lack of successful therapies for the treatment of cancers and other life-threatening diseases, the use of complementary and alternative therapies is increasing. Many natural products have been isolated from herbs and screened for anti-cancer activity in both cancer cell lines and in animal models of human cancer [1].
Annona squamosa (Custard apple) belonging to family Annonaceae, is commonly found in India and cultivated in Thailand and originates from the West Indies and South America. It is considered beneficial for cardiac disease,

diabetes, hyperthyroidism and cancer. The root is considered as a drastic purgative. The crushed leaves and flowers are sniffed to overcome hysteria and fainting spells, they are also applied on ulcer, wounds and cancer. The ripe fruits of this plant are applied to malignant tumors to hasten suppuration [6].

Manilkara zapota (Sapodilla) belonging to family Sapotaceae, is a long-lived, evergreen tree native to Southern Mexico, Central America and the Caribbean. It has been used in the indigenous system of medicine for the treatment of various ailments. Decoction of the bark is used for diarrhea and fever. An infusion of the young fruits and the flowers is drunk to relieve pulmonary complaints, cancer and fever. Leaf decoction is used for fever, hemorrhage, wounds and ulcers. The crushed seeds have a diuretic action and are claimed to expel bladder and kidney stones, and effective in rheumatism [7].

Hence in the present study an attempt has been made to find out the *in vitro* anti-cancer activity of ethanolic extracts of *Annona squamosa* and *Manilkara zapota* flowers in breast cancer cell line, MCF-7.

MATERIALS AND METHODS

2.1 Cell line and reagents

Cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in MEM (Minimal Essential Media) supplemented with 10% FBS (Fetal Bovine Serum), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. MEM, FBS, trypsin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMSO (Dimethyl sulfoxide) were purchased from Hi Media and Sigma-Aldrich, Mumbai.

2.2 Plant collection and extraction

The flowers of *A. squamosa* and *M. zapota* were collected from Vellore District, Tamilnadu. The plants were identified and authenticated by a Professor from Botany Department, Voorhees College, Vellore. Collected materials were washed thoroughly with fresh running water, dried under shade at room temperature (25±1°C) for 10 - 15 days, coarsely-powdered and soaked in ethanol for 72 hours with intermittent shaking. The liquid extract so obtained was filtered and solvent was completely removed by using rotary evaporator. The resultant gummy extract of both the plants were used for subsequent assays.

2.3 Preliminary phytochemical analysis

The extracts were qualitatively tested for the presence or absence of chemical constituents like phenols, reducing sugars, flavones, glycosides, saponins, alkaloids, steroids, anthroquinones, quinones and tannins [8].

2.4 MTT Assay

Sensitivity of MCF-7 and Vero cells to ethanolic flower extract of *A. squamosa* and *M. zapota* were determined individually by the MTT colorimetric assay [9]. Cells were seeded in a flat-bottomed 96-well plate and incubated for 24 h at 37°C in 5% CO_2. Both the cell lines were exposed to two plant extracts mentioned above. The solvent DMSO-treated cells served as control. Cells were treated with MTT reagent (20 μl/well) for 4 h at 37°C and then DMSO (200 μl) was added to each well to dissolve the Formosan crystals. The optical density was recorded at 492 nm in a micro plate reader. Calculations were done and the concentration required for 50% inhibition of viability (IC_{50}) was determined graphically. Percentage of residual cell viability was determined as:

$$\text{Cell viability (\%)} = 1 - \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

2.5 DNA fragmentation assay

MCF-7 cell lines treated with the ethanolic extract of *A. squamosa* and *M. zapota* flowers was subjected to DNA fragmentation technique. A distinctive feature of apoptosis at the biochemical level is DNA fragmentation [10]. This method is used as a semi-quantitative method for measuring apoptosis [11]. The culture medium was removed and centrifuged at 3000 rpm for 5 min to collect detached cells. 2 ml of cells which was centrifuged to 3000 rpm was suspended in 200 μl of 1X TE buffer and 100 μl of 10% SDS, incubated at 60°C for 20 min. 300 μl of phenol, chloroform and isoamyl alcohol in the ration 25: 24: 1 was added and mixed well, then centrifuged at 10,000 rpm for 10 min. To the supernatant 500 μl of isopropanol was added followed by addition of 200 μl of 70% ethanol, then centrifuged at 10,000 rpm for 10 minutes. The pellet was dried at 37°C till there were no traces of solution. The

pellet was re-suspended in 20 µl of 1X TE buffer. Agarose gel electrophoresis was carried out for the extracted DNA [12]. For casting 1% agarose gel, 0.8 g of agarose was dissolved in 80 ml of diluted 1X TBE buffer. The gel was allowed to solidify without disturbing the wells. The gel was then transferred to 1X TBE buffer-filled electrophoresis tank. 2 µl of gel loading dye was added to 20 µl of sample DNA, mixed well, so that a total of 22 µl of sample was loaded to gel. The power card terminals were connected to their respective positions and the gel was run at 50 V till the gel loading dye migrated to more than half of the length of gel. The unit was switched off and the separated sample DNA was visualized with a DNA ladder marker under a UV Trans-illuminator.

RESULTS AND DISCUSSION

3.1 Extract yield

The ethanolic extract yield of *A. squamosa* and *M. zapota* flowers were 6.62% w/w and 4.02% w/w respectively (Table 1).

Table 1: Physical nature of *Annona squamosa* and *Manilkara zapota* flower extract

Plant	Part	Extract	Yield	Texture	Colour
Annona squamosa	Flowers	Ethanol	6.62%	Gummy	Dark green
Manilkara zapota	Flowers	Ethanol	4.02%	Gummy	Dark green

3.2 Phytochemical analysis

The ethanolic flower extract of *A. squamosa* and *M. zapota* contained phenols, reducing sugars, flavones, glycosides, saponins, steroids, alkaloids, proteins and tannins. Both the extracts did not have anthraquinones, quinones, amino acids and phlobotannins (Table 2).

Table 2: Phytochemicals in *Annona squamosa* and *Manilkara zapota* flower extract

S. No.	Compounds	*Annona squamosa*	*Manilkara zapota*
1	Phenols	+	+
2	Reducing sugars	+	+
3	Flavones	+	+
4	Glycosides	+	+
5	Saponins	+	+
6	Steroids	+	+
7	Alkaloids	+	+
8	Anthraquinones	-	-
9	Quinones	-	-
10	Proteins	+	+
11	Amino acids	-	-
12	Tannins	+	+
13	Phlobotannins	-	-

'+' indicates presence; '-' indicates absence

Polyphenolic compounds might inhibit cancer cells by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens, while some flavonoids could also alter hormone production and inhibit aromatase to prevent the development of cancer cells. The mechanism of action of anti-cancer activity of phenols could be by disturbing the cellular division during mitosis at the telophase stage. It was also reported that phenols reduce the amount of cellular protein and mitotic index and colony formation during cell proliferation of cancer cells [13]. Extracts of the above mentioned medicinal plants contain a wide array of polyphenolic compounds which might possess cancer preventive and therapeutic properties. Our goal was to determine whether the extracts of these medicinal plants exerted an inhibitory effect on cancer cell proliferation and caused cell death. The results of our studies suggest that the ethanol extracts of *A. squamosa* and *M. zapota* possess cytotoxic effects on human breast cancer cells.

This study is correlated with a previous study [14] which indicated that the whole plant extract obtained from *Artemisia vulgaris* revealed the presence of tannins, polyphenols, flavonoids, such as eupafolin, diosmetin, rhamnetin, apigenin and their glucosides, luteolin, quercetin, rutin and vitexin which were characterized for their anti-cancer properties. In another study, the phytochemical screening of methanolic extract of leaves as well as bark

of *Holigarna grahamii* showed the presence of phenols, tannins, alkaloids and reducing sugars which are characterized for their anti-cancer properties [15].

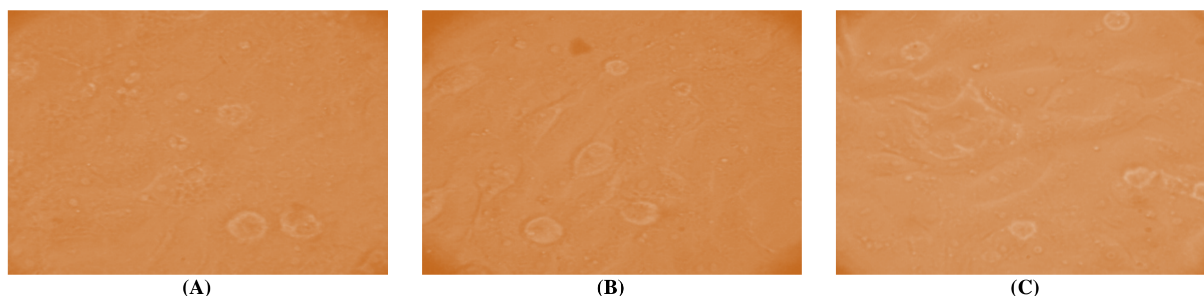
3.3 Cytotoxicity

In our study both the plant extracts showed a concentration-dependent inhibition of cell growth. The results are tabulated (Table 3). The ethanol extract of *A. squamosa* and *M. zapota* flowers on normal Vero cell lines showed that the percentage with regard to viability of cells was found to be 95.8% and 92.7% respectively at 3.12 µg/ml concentration which decreased with increase in concentration. However with the 200 µg/ml concentration of the extracts, *A. squamosa* and *M. zapota* showed 30.9% and 15.4% cell viability respectively.

Table 3: Percent cell inhibition of *A. squamosa* and *M. zapota* on Vero cell line

S. No.	Concentration (µg/ml)	Cell viability (%)	
		Annona Squamosa	*Manilkara zapota*
1	Control	100	100
2	3.12	95.8	92.7
3	6.25	91.7	86.5
4	12.5	75.2	73.1
5	25	60.8	59.7
6	50	48.4	46.3
7	100	42.2	40.2
8	200	30.9	15.4

Vero cell line, a fibroblast type, initiated from the kidney of normal adult African green monkey is employed extensively in virus replication studies and plaque assays. In the present study both the plant extracts showed a mild cytotoxic action against Vero cell line. Few cytopathological changes were observed with 200 µg/ml concentrations and photomicrographs were taken (Figure 1).



(A) (B) (C)

Figure 1: Cytopathological changes of Vero cell lines. Control (A), *Annona squamosa* 200 µg/ml (B), *Manilkara zapota* 200 µg/ml (C)

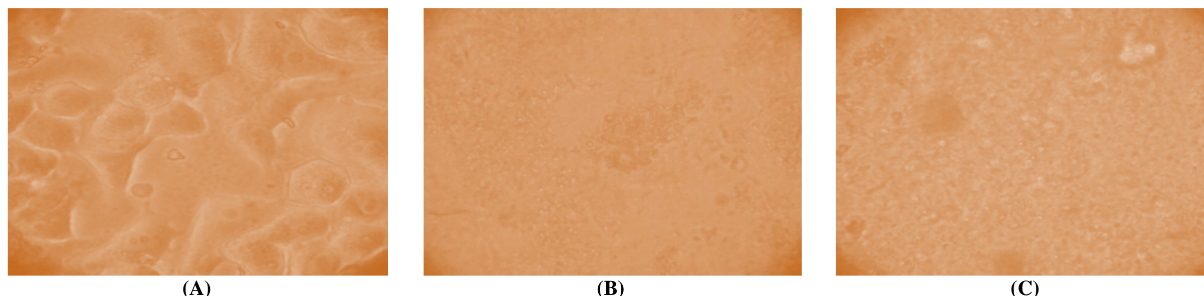
Table 4: Percent cell inhibition of *A. squamosa* and *M. zapota* on MCF-7 cell line

S. No	Concentration (µg/ml)	Cell viability (%)	
		Annona squamosa	*Manilkara zapota*
1	Control	100	100
2	3.12	74.6	82.6
3	6.25	52.0	70.6
4	12.5	36.0	50.6
5	25	20.0	28.0
6	50	4.0	6.6
7	100	1.3	2.6
8	200	0.0	0.0

Screening of ethanolic extracts of *A. squamosa* and *M. zapota* resulted in significant anti-cancer activities against MCF-7 cell lines. The treated cells were compared with the control. The percent cell viability profiles were found to be concentration dependent. The maximum concentration used in the study was 200 µg/ml and the results are summarized (Table 4). At this concentration, none of the cells survived confirming its anti-cancer property. The probable reason might be by inhibiting cell proliferation and finally killing the cells which was well implicated by the absence of Formosan crystals in the dead cells. The cell death suggesting that both the plant extract could be a good cytotoxic agent. The well-defined pathological changes such as giant cell formation, rounded and shrunken

appearance of cells, particulate and vacuolated structure, grouping and peeling of monolayer observed on both the plant extracts further confirmed the compounds potential as a cytotoxic agent against the MCR-7 cell line.

Both the plant extracts showed a well-defined pathological change on MCF-7 cell line when compared to Vero cell line (Figure 2). Morphological changes of extract-treated cells were examined using an inverted microscope and compared with the cells serving as control. These observations may be due to the presence of active biological compounds.



(A) (B) (C)

Figure 2: Cytopathological changes of MCF-7 cell lines. Control (A), ***Annona squamosa*** **200 µg/ml (B),** ***Manilkara zapota*** **200 µg/ml (C)**

The IC_{50} values of ethanolic extracts of *A. squamosa* and *M. zapota* on Vero cells and on MCF-7 cells were recorded (Table 5).

Table 5: IC_{50} values of ***Annona squamosa*** **and** ***Manilkara zapota***

S. No.	Plant extracts	Vero cell line	MCF-7 cell line
1	*Annona squamosa*	75 µg/ml	6.87 µg/ml
2	*Manilkara zapota*	42.5 µg/ml	12.5 µg/ml

This study can be correlated with the study of methanol extract of *Solanum nigrum* on HeLa cell line showing greater activity on HeLa cell line and little activity on Vero cell line, indicating *Solanum nigrum* can be used as anti-cancer agent [14]. Chloroform extract of *Epipremnum pinnatum* produced significant growth inhibition against T-47D breast carcinoma cells and analysis of cell death mechanisms indicated that the extract elicited both apoptotic and non-apoptotic programmed cell deaths [16]. A number of active compounds such as flavonoids, diterpenoids, triterpenoids and alkaloids have been shown to possess anti-cancer activity. According to the report of the American National Cancer Institute (NCI), the criterion of anti-cancer activity for the crude extracts of herbs is an $IC_{50}<30$ µg/ml [17]. The plant extracts used in this study are well below this level providing a strong confirmation of their anti-cancer effect against breast cancer cell lines.

3.4 DNA fragmentation Assay

DNA fragmentation analysis was performed by agarose gel electrophoresis of extract-treated MCF-7 breast cancer cell lines (Figure 3). The DNA migrated as discrete bands which were compared to DNA markers, giving a ladder of approximately 200 base pairs. Such DNA ladders are considered to be a hall mark of apoptosis. Continuous smears may also indicate DNA fragmentation due to apoptosis. The ladder from DNA fragmentation is catalyzed by an endogenous endonuclease that cleaves inter nucleosomal DNA to form ladder like bands of oligo nucleosome fragments. Results revealed that the DNA fragment shows that the ethanolic extract of *A. squamosa* and *M. zapota* has anti-cancer activity in the MCF-7 breast cancer cell lines.

DNA fragmentation assay confirmed the anti-cancer effect of extracts. A cancer cell is a mutant human cell that differs from normal cell only in the rapid growth of cell. The DNA of the rapidly multiplying cancerous cells is more exposed as compared to normal cells. Thus, the cell DNA is one of the targets for the treatment of cancer. Breakdown of DNA molecule is one of the sign of inhibition of DNA replication. Furthermore, DNA fragmentation assay clearly indicates potential of both the plant extracts in cancer management.

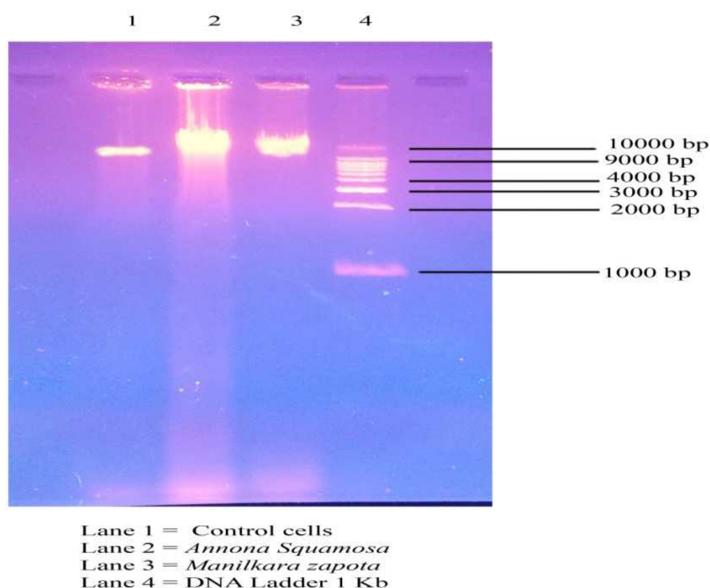


Figure 3: DNA Fragmentation by the plant extracts

These results support the previously reported apoptosis-inducing activity of resveratrol against tumor cell lines [18]. Willow extracts also caused DNA fragmentation [19]. Luffin from the seeds of *Luffa aegyptiaca* was found to induce an increase in cytosolic oligonucleosome-bound DNA in both melanoma and Ehrlich ascites tumour cells, the level of DNA fragmentation in the former cell line being higher than in the latter [20].

CONCLUSION

Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around the world. Approximately, 60% of the anti-cancer drugs currently used have been isolated from natural products from the plants. At this time, more than 3000 plants worldwide have been reported to possess anti-cancer properties. The plants, *A. squamosa* and *M. zapota* have been commonly used in traditional Indian medicine for the treatment of various human ailments for many years. Results of anti-cancer activity of the flower extracts of *Annona squamosa* and *Manilkara zapota* showed a potent cytotoxic activity against MCF-7 breast cancer cell line and a very little activity against the non-cancerous Vero cell line. Thus, we can assume that the possible mechanism of its anti-cancer activity may be apoptosis induced by DNA fragmentation and this is contributed by active phytochemicals such as alkaloids, phenols and flavonoids found in the extracts. Our findings support the reported therapeutic use of these plants as an anti-cancer agent in the Indian system of medicine. Further experiments are however needed, both *in vitro* and *in vivo* to obtain more details on its mechanism of action.

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