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Anti-Cancer Activity of Manilkara Zapota Seed and Skin Extracts on Hela Cell Lines

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Abstract

Objective: To assess the anticancer activity of ethanolic extracts of Manilkara zapota (MZ) seeds and skin against HeLa cell lines by Methyl Thiazolyl Tetrazolium bromide (MTT) and Lactate dehydrogenase (LDH) assay. Methodology: Crude extract of seeds and skin of MZ were prepared by standard procedures using ethanol. It was filtered and evaporated on water bath. The extract was dissolved in 65% ethanol, centrifuged at -4 degrees at 4000 rpm for 10 minutes and the supernatant was used. The anticancer activity of extracts was assessed using HeLa cancer cell lines by MTT and LDH assay. For MTT Assay, 96 well plate was seeded with 5000 HeLa cells per well, incubated at 37ºC under 5% CO2. After 24 hrs, cells were treated with the extracts (0.5-12 mg/ml for both skin and seed) and Paclitaxel (0.03-1mg/ml). After 48 hrs 100µl of 0.2% MTT and later 50µl of dimethyl sulfoxide (DMSO) were added and optical density was measured at 570nm. LDH Assay was done using the supernatant cell suspension and LDH reagent and absorbance read at 490nm. Results: The extracts were found to have anticancer activity and the IC-50 (Inhibitory Concentration - 50) of seed and skin extracts and Paclitaxel were 4µg/ml, 8µg/ml and 2 µg/ml respectively. Conclusion: The ethanolic extracts of MZ seeds and skin have shown anticancer activity against HeLa cells in vitro by MTT and LDH assay.

Keywords: Manlikara zapota, Seeds, Fruit skin, Anticancer activity, MTT, LDH assay, HeLa cells

INTRODUCTION:

Cancer is the most dreadful disease known to mankind and its treatment seems to be challenging always. According to the world wide cancer data, there are around 18 million people suffering from cancer as per the latest Cancer statistics 2018.

Based on statistics published by the World Health Organization, cancer is the second leading cause of death worldwide, causing an estimated 8.8 million deaths.(1) Cancer incidences are expected to rise by approximately 70% in the next 20 years. About 70% of cancer deaths occur in low- and middle-income countries, likely because of factors such as increasing pollution levels, increased life expectancy, insufficient healthcare facilities, and expensive anticancer drugs. One way to overcome these challenges is to develop anticancer drugs from natural sources such as plants, which might lead to more affordable drugs for low- and middle-income countries.(2)

In India skin cancers constitute about 1-2% of all cancers. Though surgical procedures, radiotherapy and chemotherapy are available for the treatment of skin cancers their cost and toxicity are major limitations.

Several active components from natural sources such as leaves, fruits, seeds and skin extracts of different plants have been studied for their effect in various cancers. Among the seed extracts, grape seed(3), Indian plum(4), jack fruit seed(5), pomegranate(6) are reported to have anticancer activity. Skin peel of orange(7), lemon(7) , pomegranate(6) have also been found to possess anticancer activity. Sapota (Manilkara zapota) is one of the sweetest fruits, well consumed by most of the people and their seeds are thrown as waste unlike jack fruit and other seeds. If the seeds of sapota are evaluated for anticancer activity and found positive it can be used as a bio source for new drug development. As the anticancer activity of Sapota seeds and skin has not yet been reported we have chosen the seed and skin for evaluation.

Ethnobotanical description of Manilkara zapota:

It belongs to the family- Sapotaceae, Genus: Manilkara, Species: zapota.synonyms: Manilkara zapotilla, Manilkara achras, Mimusopus manilkara, Achras zapota. It is a large tree growing to a height of around 8 meters. It produces a dense crown and a characteristic branching system (sympodial), in which the young branches are arranged horizontally. The tree has an extensive root system. The bark is dark brown and deeply fissured. Leaves spirally arranged and clustered at the shoot tips, simple, elliptic or oblong. Flowers are hairy outside, 6-8 mm long, greenish, solitary, with a brown pubescent peduncle. Fruit is brown, fleshy, ovoid to round, 3-8 cms long, containing 5 or more shiny hard blackish brown seeds.

Phytochemical constituents:

The plant contains several phyto constituents such as alkaloids (sapotin, saponin, achrassaponin), carbohydrates (saccharose, dextrose, levulose), glycoside,tannins,triterpenes and flavonoids. It also contains protein, ascorbic acid, phenols, carotenoids and minerals like iron, copper, zinc, calcium and potassium(8-10).

Medicinal uses reported:

Seeds have been proved to have diuretic (11),antibacterial (12),anthelminthic activity(13)and hypoglycemic activity(14).Seed kernel oil is used as skin ointment. It is also traditionally used for the treatment of fever and pain. The leaves of the plant possess analgesic and anti inflammatory(15), antioxidant, antihyperglycemic and hypo cholesterolemic activities (16).Roots are found to have hypoglycemic activity (17). Fruit, leaves and bark extracts have been evaluated for antitumor activity (18-20).

OBJECTIVES:

1. To evaluate the anticancer activity of ethanolic extracts of Manilkara zapota seeds and skin against HeLa cell lines in vitro using MTT and LDH assay.

2. To compare the in vitro anticancer activity of these extracts with standard anticancer drug Paclitaxel.

MATERIALS AND METHODS:

Preparation of seed extract:

The fresh fruits of Manilkara zapota were obtained from Kanchipuram district of Tamilnadu and washed thoroughly. The skin and seeds were carefully separated from the fruit. 500 gm of the skin was shade and air dried for 7 days. After complete drying, the skin was powdered and subjected to cold extraction using ethanol as solvent for 72 hours.

The seeds were washed with distilled water thoroughly to remove traces of contaminants and shade dried for one month. After complete drying, 500 gm of seeds were powdered and extracted with ethanol similarly.

Both the extracts were separately filtered and the filtrate was allowed for complete evaporation of the solvent on water bath. After extraction the yield obtained was 32 gm (6.4%) and 25 gm (5%) from skin and seed extracts.

Figure 1: Extract Preparation

The extracts were dissolved in 65% ethanol, centrifuged at -4 degrees at 4000 rpm for 10 minutes and the supernatant was used.(14)

Cell Lines:

HeLa Cell lines were procured from the National Centre for Cell Sciences in Pune. Minimal Essential Media (MEM) was used to maintain the cell lines. 10% Fetal bovine serum (FBS), Penicillin 100U/ml and Streptomycin 100µg/ml served as supplements. The cells were maintained in a humidified atmosphere of 5%CO2 at 37° C.

Chemical reagents:

MEM, FBS, MTT, DMSO, LDH reagent, Penicillin, Streptomycin, Paclitaxel, trypsin and Phosphate buffered saline (PBS) were purchased from Sigma Aldrich and Hi-media, Mumbai.

Invitro assays:

The invitro assays were carried out using HeLa cancer cell lines by MTT and LDH assays.

MTT assay: 96 well plate was seeded with 5000 HeLa cells per well, incubated at 37 ºC under 5% CO2. After 24 hrs, cells were treated with different concentrations of Paclitaxel and the extracts (0.5,1,2,4,8,12 µg/ml). After 48 hrs 100µl of 0.2% MTT and 3 hrs later 50µl of DMSO were added and optical density measured at 570nm. The results were expressed as percentage death of cancer cells calculated using the formula:

% Viability = Mean absorbance of sample × 100 / Mean absorbance of control

LDH assay was performed and absorbance read at 490nm. 90µl cell suspension was seeded in a 96 well plate at optimized cell density. All the reagents were thawed before use. 10 µl lysis solution was added to LDH control wells.10 µl test compound was added to experimental wells. 10 µl PBS was added to untreated control wells. Plate was incubated at 37◦C and 5% CO2 for 30-45 minutes. Cell lysis was observed in maximum LDH control cells microscopically. Aseptically transfer 50µl supernatant from each well to a new 96 well plate. Add 50µl LDH reagent to each well and incubate the plate at room temperature for 15-30 minutes. For end point assay 50µl of stop solution was added to each well and the absorbance was read at 490nm. The results were expressed as percentage of LDH release and calculated using the formula:

(Optical density of test/ Optical density of control) x 100

RESULTS:

Both the seed and skin extracts exhibited dose dependent inhibition of cell growth. 50 % inhibition of cellular growth was observed at 4µg/ml for seed extract and 8µg/ml for skin extract in MTT assay. Paclitaxel inhibited 50 % of cell growth at 2µg/ml.

Table 1: Cell viability with Skin, Seed extracts and Paclitaxel:

	Cell viability in %					
	Skin		Seed		Paclitaxel	
DOSE	Mean	SE	Mean	SE	Mean	SE
0.5 µg	89.51	0.05	83.59	0.01	75.6	0.04
1 µg	75.78	0.01	70.64	0.01	66.73	0.02
2 µg	61.79	0.01	58.43	0.01	50.34	0.02
4 µg	57.33	0.00	50.4	0.02	41.54	0.01
8 µg	50.45	0.01	42.45	0.01	37.68	0.02
12 µg	41.65	0.00	30.36	0.00	26.69	0.01

Figure 2: MTT Assay: Cell Viability with Skin, Seed Extracts and Paclitaxel:

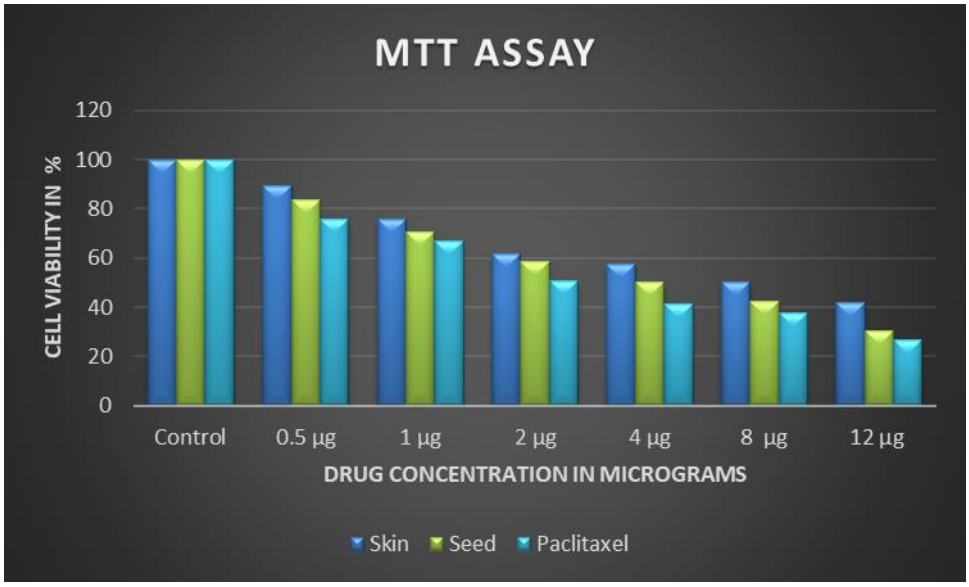
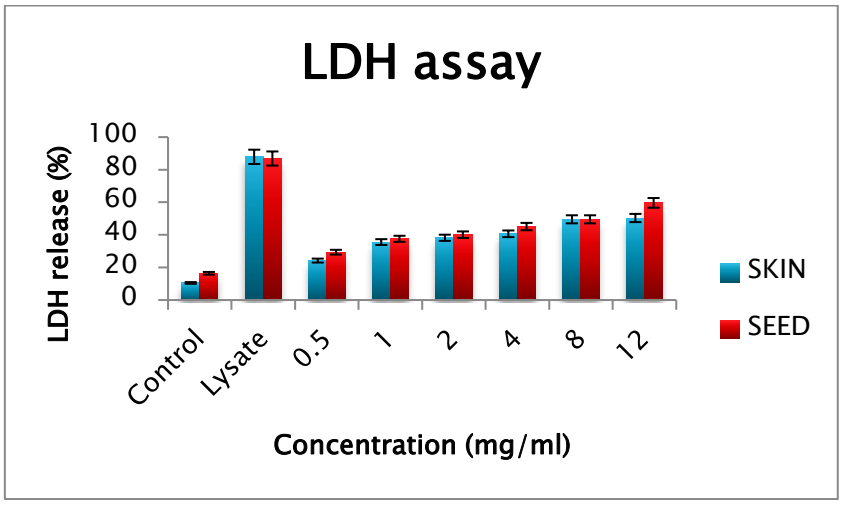


Figure 3: Cytotoxic Effect of Seed and Skin extracts on HeLa Cell lines by LDH assay:



DISCUSSION:

The in vitro anticancer activity assays have shown that the seed and skin extracts have cytotoxic effect on HeLa cell lines in a concentration dependent manner in MTT and LDH assays. The IC 50 value of seed extract was 4 µg/ml and skin extract was 8 µg/ml. Seed had potent cytotoxic effect than skin against HeLa cell lines.

In an earlier invitro study done on Human hepatocellular carcinoma cells, it was observed that Manilkara zapota leaf extracts significantly reduced the cell viability in comparison with the untreated cells similar to our study.(22)

Mohanapriya et al have observed that acetone extracts of Manilkara zapota seeds have free radical scavenging activity and can be used as natural antioxidants. (23)

The phytochemicals present in the seed are alkaloids, steroids, flavanoids, saponins, tannins, phenols and glycosides.(23) Studies on the phytochemical analysis of skin are limited. However, Abdi Dharma et al have identified only alkaloids in the skin extract. The presence of additional phytochemicals, flavanoids, saponins, tannins and sterols in the seed would have contributed to the potent anticancer activity of the seed extract as these phytochemicals have been already reported to have anticancer activity.

CONCLUSION:

The present invitro study has confirmed the anticancer activity of both the seed and skin extract of Manilkara Zapota against HeLa cell lines and the seed extract is more potent than the skin extract.

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