



In-vitro Cytotoxicity studies of *psidium guajava* against Ehrlich ascites carcinoma cell lines

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General Note



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ABSTRACT

The Current research explains the *in-vitro* anticancer activity of *psidium guajava* plant extract against Ehrlich ascites carcinoma cell lines. The common guava tree (*psidium guajava*) is popular in an indigenous system of folk medicines. Traditionally *Psidium guajava* is used for the treatment of various ailments. Most scientific evidence examined the clinical efficacy of guava in treating gastrointestinal disorders. Other investigations examined antiamebic, antibiotic, antidiarrheic, antihyperglycemic, antimutagenic, antispasmodic, and sedative effects, as well as anticough and narcotic-like activities of the plant species. The current research described *psidium guajava* anticancer activity against Ehrlich ascites carcinoma cell lines. Ehrlich Ascites Carcinoma cells resembles human tumors which are the most sensitive to chemotherapy due to the fact that they are undifferentiated and that they have a rapid growth rate. This study evaluated the anticancer activity of crude and fractionated extract of *psidium guajava* against Ehrlich ascites carcinoma cell lines *in-vitro* by using tryphan blue test and MTT assay.

Key words: *Psidium guajava*, Ehrlich ascites carcinoma, MTT assay

1. INTRODUCTION

India is the country of fabulous wealth and varied heritage. It is the blessed nation with the civilization of unity in diversity. On the other hand the existing diseases in the Indian society are quite awesome and threatening. Hence it is mandatory to control the intensity of the diseases and their dangerous predisposing factors in order to save India from becoming the capital of these diseases. The recent abundant facts suggesting the association of oxidative stress in the pathogenesis of various disorders and diseases. Much attention is taken by scientists and general public for the importance of antioxidants in the maintenance of human health, prevention and treatment of diseases.

The use of medicinal plants has always guided the look for new cures throughout the world for thousands of years and continues to endow with new remedies to humankind. Medicinal plants are often cheaper, locally available and easily consumable, raw or as simple medicinal preparations. Nowadays, traditional medicinal practices form vital part of complementary or alternative medicine.

Psidium guajava (Guava), belonging to the Family of Myrtaceae, which is originated in Mexico and extends all over the South America, Europe, Africa and Asia. Based on archaeological data, it has been used widely and known in Peru since pre Columbian times. *Psidium guajava* grows in all the tropical and subtropical areas of the world, adapts to different climatic conditions but prefers dry climates (Stone, 1970). The palpable reason in choosing this plant from the literature in the present study is multipurpose. *Psidium guajava* is a small tree which is 10m high with thin, smooth, patchy, peeling bark. Leaves are opposite, short-petiolate, the blade oval with prominent pinnate veins, 5–15 cm long. Flowers are somewhat showy; petals whitish up to 2cm long, stamens numerous (Stone, 1970).

Scientists have been reported that the leaves of *Psidium guajava* contain an essential oil rich in cineol, tannins and triterpenes. In addition, three flavonoids (quercetin, avicularin, and guajaverin) have been isolated from the leaves (Khadem and Mohammed, 1959). In mature leaves, the greatest concentrations of flavonoids were found in July: Quercetin > myricetin > kaempferol > luteolin (Vargas et al., 2006). Much of the medicinal properties of *Psidium guajava* are credited to these flavonoids and these bio-flavonoids are well-known for their multi-directional biological activities. Recently, Tachakittirungrod et al. (2007) also isolated quercetin, morin and quercetin–3–O–glucopyranoside from methanol crude extracts of the leaves of *Psidium guajava* and these three isolated compounds contribute importantly to the antioxidant activity and antidiabetic efficacy of *Psidium guajava* leaves. Quercetin is taught to contribute anti-diarrheal action of *Psidium guajava* leaves since it relaxes the smooth muscles of the gastrointestinal tract and also inhibits the bowel contractions (Baby Joseph and Mini Priya, 2010). Consequently, this providing a scientific basis for the use of this plant in traditional medicine.

Antioxidants and human health

Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues, resulting in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and even lead to cell death induced by DNA fragmentation and lipid peroxidation (Beckman and Ames, 1998). With respect to this a lack of antioxidants, make possible the development of degenerative diseases, including cardiovascular diseases (CVD), cancers, neurodegenerative diseases, Alzheimer's disease and inflammatory diseases.

With respect to the previous findings, the *Psidium guajava* have antimicrobial, antioxidant and anticancer activity against oxidative stress and Cancer cell lines.

2. MATERIAL AND METHODS

In this research various materials and methods were used to study the *in-vitro* cytotoxicity of *psidium guajava* plant extract against Ehrlich Ascites carcinoma cells. List of Material and methods were followed:

Materials

- Plant leaf and body.
- Animals (Wister albino rats).
- Ehrlich ascites cell lines.
- Soxhlet apparatus.
- Haemocytometer
- Microplate reader.
- Chemicals (Tryphan blue test and MTT assay).

Animals

Wistar albino rats of 6-8 weeks age, weighing 150-180 g, were used. The albino rats were used after an acclimatization period of 7 days to the laboratory environment. The animals were fed with standard pellet diet and water was given ad libitum. This study was carried out in the animal house of Karpagam University, Coimbatore (Regd.No. 739/03/abc/CPCSEA) and this study was approved by the Institutional Ethical Committee.

Ehrlich ascites carcinoma cell line

Ehrlich Ascites Carcinoma - Maintenance of Cells (Kanematsusugiura, 1953) Ehrlich Ascites Carcinoma cells were obtained through the courtesy of Amla Cancer Research Centre, Thrissur and were maintained by weekly intraperitoneal inoculation of 1X10⁶cells/mouse.

Methodology

- Soxhlet extraction.
- Column chromatography.
- Tryphan blue test.
- MTT assay.

Soxhlet Extraction

Fresh leaves of *Psidium guajava* were collected in Coimbatore, during the months of April-May and November-December. Plant material was dried under shade at room temperature, pulverized by a mechanical grinder and sieved through 40 meshes. The coarse powder (100 g) was extracted successively with (a) Hexane, (b) Chloroform and (c) Ethanol (each 250 ml) by hot continuous percolation method in a Soxhlet apparatus for 24 hrs all the extracts were filtered through Whatman No. 41 filter paper and evaporated on a water bath and finally dried in vacuum.

In vitro Cytotoxicity - Trypan blue Test (Gothoskar and Ranadive, 1971)

Short- term in vitro cytotoxicity was assessed using Ehrlich Ascites Carcinoma cell lines cells by incubating different concentrations of the ethanolic extracts of *Psidium guajava* leaf and its isolated fraction at 37° C for 3 hours. The tumor cells were aspirated from peritoneal cavity of tumor bearing mice using an insulin syringe and transferred to a test tube containing isotonic saline. The cells were then washed in normal saline and cell number was determined using a Haemocytometer and adjusted at 10x10⁶ cells/ml. For the cytotoxicity assay, different concentrations of the extracts (100-1000 µg/ml) were added to each tubes and the final volume was adjusted to one ml with normal saline. Control tubes were kept with the saline, tumor cells and without the drugs. All the tubes were incubated at 37° C for 3 hours. After incubation 0.1ml of 0.4% tryphan blue dye in isotonic saline was added to each tube and the number of viable (unstained) and dead (stained) cells were counted using haemocytometer.

$$\% \text{ Dead cells} = \frac{\text{Total cells counted} - \text{total viable cells} \times 100}{\text{Total cells counted}}$$

Cytotoxic studies - MTT Assay (Scudiero et al., 1988)

MTT [(3-(4, 5-dimethylthiazol-2yl)-2, 5- diphenyltetrazoliumbromide] measures the metabolic activity of the viable cells. The assay is non-radioactive and can be performed entirely in a microtiterplate (MTP). The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt. This method involves culturing the cells in a 96-well microtiterplate, and then incubating them with MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number. This is applicable for adherent cells cultured in MTP.

3. RESULT AND DISCUSSION

Cytotoxicity

The cytotoxicity experiments for the plant extract and its isolated fraction were monitored with Trypan blue method and MTT assay.

Trypan Blue method and MTT Assay

Short term in vitro cytotoxic effects (Trypan Blue method) of ethanolic leaf extract of *Psidium guajava* against Ehrlich Ascites Carcinoma cell lines were tabulated. Cytotoxic effect (MTT Assay) of ethanolic extract of the leaf was studied against Ehrlich Ascites Carcinoma cell lines and the results were tabulated. The *Psidium guajava* leaf extract was found to be more cytotoxic against Ehrlich Ascites Carcinoma. Concentrations of 1 µg/ml, 10 µg/ml, 15 µg/ml and 25 µg/ml showed 11.5%, 32.15%, 35.36% and 56.59% cytotoxicity was observed respectively, whereas 72.99% of cell death was found in the concentration of 50 µg/ml. Short term in vitro cytotoxic effect of the isolated fraction of *Psidium guajava* against Ehrlich Ascites Carcinoma cell lines were tabulated in Table 19. The cytotoxicity increased with increase in concentration of extract. 5 µg/ml concentrations showed 11.23 % cell death where as, in high concentration (100 µg/ml) 43.84% of cell death was noticed. Cytotoxic effect of isolated fraction of *Psidium guajava* was studied against Ehrlich Ascites Carcinoma cell lines and the results were tabulated. The isolated fraction was found to be more cytotoxic against Ehrlich Ascites Carcinoma.

Concentrations of 1 µg/ml, 5 µg/ml, 7.5 µg/ml and 10 µg/ml showed 8.19%, 17.37%, 29.51% and 81.31% cytotoxicity respectively was observed whereas 97.70% of cell death was found in the concentration of 15 µg/ml. Cell viability of the three experimental groups is displayed in Plate. The present study, reveal although the viability of treated cells (ethanolic leaf extract of *Psidium guajava* and its isolated fraction) was significantly lesser when compare with untreated control group.

Cytotoxic effect of ethanolic leaf extract of *Psidium guajava* and its isolated fraction

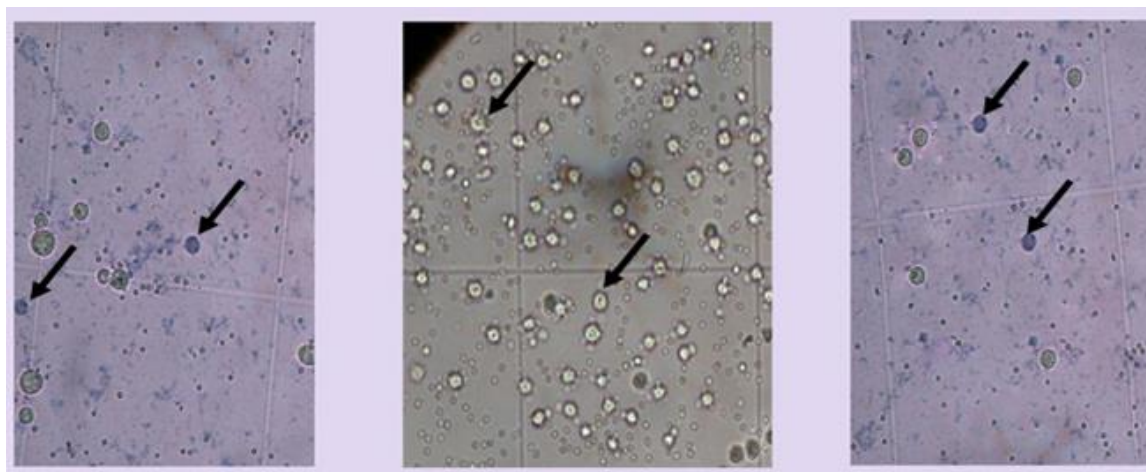
Dead Cells

(*Psidium guajava*) (1000 µg/ml)

Viable Cells

Dead Cells

(*Psidium guajava*) (Isolated fraction) (100 µg/ml)



Psidium guajava plant leaf extract without fraction

Crude extract of anticancer activity of *psidium guajava* (Cell counting using tryphan blue staining test)

Concentration of Plant extract (µg/ml)	Number of Viable cells	Viable cells (%)	Number of Dead cells	Dead cells (%)
Control	110	94.82	6	5.18
100	121	76.58	37	23.42
250	97	60.62	63	39.38
500	75	45.45	90	54.55
1000	37	24.66	113	75.34

Cytotoxic effect of ethanolic leaf extract of *Psidium guajava* against Ehrlich Ascites Carcinoma cell lines (MTT assay)

Concentration µg/ml	OD-1	OD-2	OD-3	Average	% Cytotoxicity
Control	0.311	0.317	0.305	0.311	--
1	0.281	0.275	0.270	0.275	11.5
10	0.211	0.218	0.204	0.211	32.15
15	0.197	0.191	0.186	0.191	35.36
25	0.141	0.135	0.130	0.135	56.59
50	0.087	0.084	0.083	0.084	72.99

***Psidium guajava* Extract of Isolated fractionation:**

Isolated fraction of *Psidium guajava* against Ehrlich Ascites Carcinoma cell lines (Trypan Blue Method)

Concentration of Plant extract ($\mu\text{g/ml}$)	Number of Viable cells	Viable cells (%)	Number of Dead cells	Dead cells (%)
5	87	88.77	11	11.23
10	96	76.19	30	23.81
25	82	70.08	35	29.92
50	71	61.20	45	38.80
100	82	56.16	64	43.84

Cytotoxic effect of isolated fraction of *Psidium guajava* against Ehrlich Ascites Carcinoma cell lines (MTT assay)

Concentration $\mu\text{g/ml}$	OD-1	OD-2	OD-3	Average	% Cytotoxicity
Control	0.307	0.312	0.297	0.305	-
1	0.287	0.284	0.271	0.280	8.19
5	0.261	0.254	0.243	0.252	17.37
7.5	0.211	0.210	0.226	0.215	29.51
10	0.076	0.054	0.043	0.057	81.31
15	0.003	0.008	0.011	0.007	97.70

DNA Fragmentation analysis

The DNA fragmentation effect of two concentration of ethanolic extract of the *Psidium guajava* leaf and one concentration of isolated fraction of *Psidium guajava* against the Ehrlich Ascites Carcinoma cells were studied and reported in gel. Both *Psidium guajava* leaf extract and it fractioned compound fragmented the DNA of Ehrlich Ascites Carcinoma cells effectively. Fragmentation of DNA clearly suggests that the *Psidium guajava* leaf extract and it fractioned compounds might activate the apoptosis in cancer cells.

DNA Ladder Analysis of ethanolic leaf extract of *Psidium guajava* and its isolated fraction



Lane 1: DNA of EAC control

Lane 2: DNA of EAC + 25 $\mu\text{g/ml}$ of ethanolic leaf extract of *Psidium guajava*

Lane 3: DNA of EAC + 50 $\mu\text{g/ml}$ of ethanolic leaf extract of *Psidium guajava*

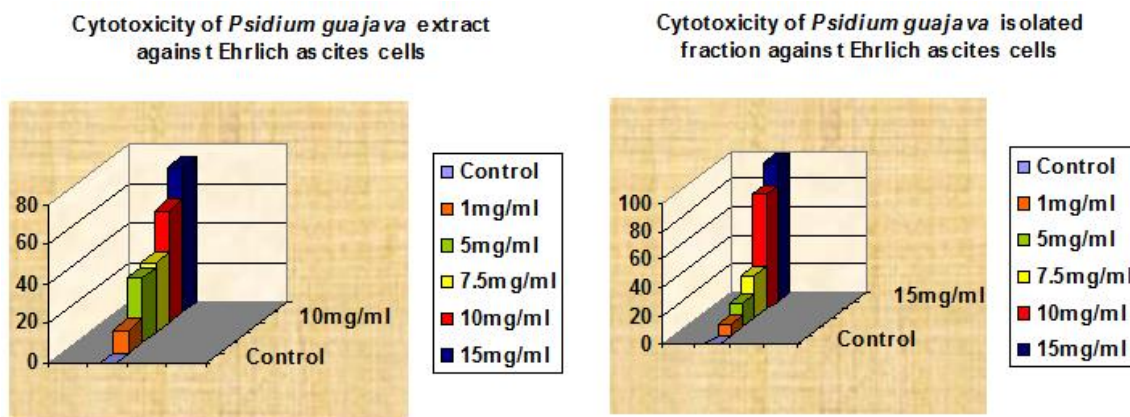
Lane 4: DNA of EAC + 10 $\mu\text{g/ml}$ isolated fraction of *Psidium guajava*

4. DISCUSSION

Experimental data of the present study showed that the extracts found to be cytotoxic against Ehrlich Ascites Carcinoma. The cytotoxicity increased with increase in concentration of extract. 100 $\mu\text{g/ml}$ concentrations showed 23.42% cell death where as, in

high concentration (1000 µg/ml) 75.34% of cell death was noticed. The isolated fraction found to be cytotoxic against Ehrlich Ascites Carcinoma. At low concentration 5 µg/ml leaf extract showed 11.23% cytotoxicity whereas at high concentration 100 µg/ml showed 43.84% of cytotoxicity.

As a part of the apoptosis precede the loss of membrane integrity there by the cells were permeable to Trypan Blue. The extracts were found to have considerable cytotoxic effects and it may be found that it activates the apoptotic pathway inside the cancer cells. Further in depth cytotoxic activity of the plant extract and its isolated fraction under study were evaluated against Ehrlich Ascites Carcinoma cell lines (MTT assay). 24hrs treatment with plant extracts showed growth inhibition of Ehrlich Ascites Carcinoma cells.



In the present investigation, leaf extract showed 72.99% of cytotoxicity with IC₅₀ Value 30 µg/ml and isolated fraction showed 97.70% of cytotoxicity with IC₅₀ Value 7.5 µg/ml. The death of the cells caused by the extract under study was due to the loss of mitochondria which is one of the hallmark of the apoptosis pathway. From the results it is clearly evident that at minimum concentration the extract activates the apoptotic pathways and results in death of Ehrlich Ascites Carcinoma cell lines.

DNA Fragmentation of Ehrlich ascites carcinoma cells

In the current investigation, the electrophoretic run of DNA of Ehrlich Ascites Carcinoma cells treated with two different concentrations of the ethanolic extract of the *Psidium guajava* exhibited extensive double strand breaks there by yielding a ladder appearance. The degradation of DNA may be due to activation of endonucleases. The presence of DNA fragmentation has been extensively used as a marker for apoptotic cell death. In the present study DNA fragmentation caused by the plant extract and isolated fraction clearly indicated that the extract activates the apoptotic pathway of cancer cells.

The isolated fraction quercetin of this study has the ability to interfere with different targets identified as "hallmarks of cancer" makes this molecule, together with several other phytochemicals, a multi-target inhibitor with pleiotropic and synergistic effects in tumor cells (Lee et al., 2011). Quercetin inhibits the growth and proliferation of cancer cell lines of different origins (prostate, cervical, lung, breast, and colon) in vitro (Russo et al., 2012).

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Conflict of Interest: The authors declare that there are no conflicts of interests.

Peer-review: External peer-review was done through double-blind method.

Data and materials availability: All data associated with this study are present in the paper.

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