

ANTIPROLIFERATIVE EFFECTS OF *PSIDIUM GUAJAVA* LEAF EXTRACT ON ORAL SQUAMOUS CARCINOMA CELLSSHARADA T RAJAN^{1*}, MALATHI N¹, CHAMUNDEESWARI D², ROSE C³

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ABSTRACT

Objective: Cancer is the major cause of mortality affecting population irrespective of age. Oral cancer is one among the various cancers affecting major population in India. To overcome toxicity of chemotherapy and disfiguration by surgical procedures, researchers are targeting phytochemicals for their anticancer properties. This study evaluates the antiproliferative effects of *Psidium guajava* leaf extract against OSC cells.

Methods: KB cells were purchased from NCCS, Pune. Extract from leaves of *P. guajava* was prepared with ethanol and evaluated with high-performance thin-layer chromatography (HPTLC). Antiproliferative effects of the extract were assessed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and flow cytometry.

Results: HPTLC revealed the presence of quercetin in the extract. MTT assay showed decreasing pattern in cell viability with the increasing dose of extract. Flow cytometry revealed the seizing of cycle by the extract.

Conclusion: The study results conclude the presence of antiproliferative properties in the leaf extract of *P. guajava*.

Keywords: *Psidium guajava*, Anticancer activity, Quercetin, Cytotoxicity, Flow cytometry.

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INTRODUCTION

Cancer is the most common non-communicable disease worldwide. Oral cancer is the sixth most common cancer in Indian population. It accounts for 86% of the world's oral cancer cases with a recurrence rate of 25%, according to the National Institute of Public health, February 2011. Between 2007 and 2011, 5.3% of cases were recorded in Tamil Nadu, of which 4.5% of cases were recorded from Chennai. Tobacco-related cancers account for 40–45% of all cancers in men and 15–20% of all cancers in women, in Chennai [1]. Cancer is due to sequential accumulation of genetic alterations termed as multistep oncogenesis, resulted by various endogenous and exogenous factors [2]. Histopathology of cancer structures revealed increased cell proliferation or decreased apoptosis or both [3-6]. Therapeutic targets were aimed at these various stages of oncogenesis. The surgical therapy of oral cancer is the most disfiguring, due to its wide excisional removal, while chemotherapy causes adverse cytological and systemic effects [7]. Natural products used in traditional folk medicine have been the source of many medically beneficial drugs, as many medicinal plants have been shown to present interesting biological and pharmacological activity [8]. The indigenous phytochemicals, apart from being easily available, are also less likely to produce much adverse effects that are associated with allopathic chemotherapy.

Psidium guajava is commonly called as guava and belongs to the family Myrtaceae. It is a food crop and also used in traditional folk medicine [9]. Various parts of *P. guajava* were used in folk medicine for the treatment of various human ailments such as wounds, ulcers, bowels, and systemic infections like cholera [10]. The whole plant or its roots were used in the form of infusion, decoction, and paste for analgesia in painful menstruations, miscarriages, uterine bleeding, premature labor, and wounds [11]. The bark was used as an astringent in the treatment of wounds and diarrhea in the Philippines. The root was used in West Africa, as a decoction to relieve diarrhea, toothaches, and cough. The

decoction of the leaves was used as antispasmodic and for rheumatism in India [12]. The leaves of *P. guajava* were used in the USA, as an antibiotic in the form of decoction for wounds, ulcers, and toothache [13].

Oxidative injury due to free radicals or reactive oxygen species was implicated in the pathogenesis of numerous diseases including cancer. *P. guajava* leaf extracts have been found to be potential sources of natural oxidants such as ascorbic acid, quercetin, guavin, gallic acid, and caffeic acid [12]. The aqueous extract of *P. guajava* leaves inhibited the viability of cancer cell line (DU-145) with increase of dose. Essential oil extracted from *P. guajava* was also reported to be highly effective in reducing the growth of murine leukemia P388 [14]. The most active component found in the leaves of *P. guajava* is quercetin, a polyphenolic flavonoid molecule along with quercetin-3-o-glucopyranoside and morin [15]. Quercetin (3,3',4',5,7-pentahydroxyflavone; R=OH) belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources. Quercetin was shown to possess anticancer effects in various studies [16]. With these data, our study aims to investigate the anticancer effect of the leaves of *P. guajava* on oral squamous cell carcinoma cell lines with the hypothesis that the active ingredient(s) of the leaves of *P. guajava* inhibit the growth of cancer by the inhibition of the growth signals.

METHODS

Plant collection

Fresh leaves of *P. guajava* were collected from the medicinal herbal garden of the college and authenticated. The leaves were cleaned, dried under the shade, powdered, and stored for further processing.

Extraction and partial characterization of plant material

The powdered crude extract was further processed using Soxhlet apparatus. The extraction process was done using 100% ethanol and 70% ethanol. The extract obtained by these two procedures was

freeze-dried and stored in sterile containers. The final extract obtained with two methods, 100% ethanol and 70% ethanol were named as S1 and S2, respectively. These extracts were partially characterized and quantification done for quercetin in the extract using high-performance thin-layer chromatography (HPTLC) fingerprinting.

HPTLC analysis

Quantification of quercetin in the two extract forms of *P. guajava* was evaluated using HPTLC instrument (CAMAG, Muttenz, Switzerland). The system parameters included aluminum-coated silica gel TLC plate (Merck, Germany), mobile phase - isopropanol:water - 7:3, tungsten lamp to capture images at a wavelength of 300–600 nm. HPTLC method was performed after derivatization with 10% ammonium chloride.

About 1 mg/ml of the standard quercetin was prepared with methanol. From this, 50 µl was diluted with 950 µl of methanol, and hence, the concentration of the standard was 50 µg/ml. 10 mg/ml of extracts sample S1 and S2 were prepared with 1 ml of methanol to make the concentration of 10 mg/ml. The adsorbent was silica gel 60 F 254. The precoated TLC plate was heated in an oven for activation. 1 ml of the standard flavonoid quercetin and the ethanol leaf extract were applied dried and then kept in the developing tank. The chamber was saturated with the solvents for 20 min at room temperature. After the development of the plate, it was air-dried, then the numbers of spots were noted and retention factor (Rf) values were calculated. Average peak area of the standard was calculated. The calibration curve of the standard drug concentration (X-axis) over the average peak height/area (Y-axis) was prepared to get a regression equation by Win Cats software. The mean peak height/area of the sample was calculated and the content of quercetin was quantified using the regression equation obtained from the standard curve.

Cells and cell culture

KB cell line was purchased from National Centre for Cell Sciences, Pune. RPMI 1640 media, trypsin, ethylenediaminetetraacetic acid, sodium bicarbonate, propidium iodide (PI), RNase, triton X-100, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), penicillin, and streptomycin were purchased from Sigma-Aldrich. Fetal bovine serum and phosphate-buffered saline (×10) were purchased from Gibco, Invitrogen Life Technologies.

MTT assay

The assay was performed as described previously [17,18]. Cells (1×10⁵/well) were plated in 24-well plates and incubated in 37°C with 5% CO₂ condition. The extracts S1 and S2 were added and incubated for 24 h. 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 (5mg/ml) of 0.5% MTT was added and incubated for 4 h. After incubation, 1 ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC₅₀) was determined graphically. The percentage cell viability was calculated using the following formula:

$$\% \text{ Cell viability} = A_{570} \text{ of treated cells} / A_{570} \text{ of control cells} \times 100$$

Graphs were plotted using the percentage of cell viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control are included in each assay to compare the full cell viability assessments.

Flow cytometry assay

After 80% confluency, cells were trypsinized and seeded in 6-well plates, then, incubated overnight and treated with extracts S1 and S2, and incubated for 24 h. Then, the cells were washed with PBS, trypsinized, and centrifuged at 1200 rpm for 5 min. The collected pellets were suspended in PBS and fixed in ethanol. On the day of analysis, the cells were treated with RNase, PI, and processed in flow cytometer (BD FACSCalibur). The analysis was done with Cell Quest Pro software (Becton Dickinson, USA).

Flow cytometry offers a rapid method for measuring the DNA content of cells and provides a convenient research tool to monitor cell cycle status and regulation. An exponentially growing population of cells will have a DNA content distribution containing an initial peak of G0/G1 cells, a valley of S phase cells, and a second peak containing G2/M cells. Cells in the G2/M phase have twice the DNA content as cells in the G0/G1 phase.

Forward angle scatter (FSC) is a measure of cell size. Side scatter (SSC) is a measure of cellular components or granularity. A dot plot showing FSC versus SSC (cell size and granularity) was drawn and fluorescence voltage channel was adjusted to allow the system to trigger on fluorescent particles. In the FSC versus SSC plot, cells were acquired between channels 200 and 800. The lower level discriminator was adjusted to partially eliminate sample debris below the cell population and diploid G0-G1 peak at channel 200. A gate (R1) was drawn to show the total events. The emitted fluorescent light of the DNA dye (PI emits fluorescence in FL2 channel) generates an electronic signal that can be recorded as high (FL2H) for the intensity of the staining as well as measured as pulse-area (FL2A) and pulse-width (FL2W) of the samples. By plotting the FL2W versus FL2A in a dot plot graph, G1 doublet discrimination was made. In the FL2-width versus FL2-area dot plot, a gate (G0/G1/S/G2/M) was set around the single population. The instrument computes the data and presents various cell cycle phases in the form of an integrated histogram from which percentage of DNA content in each phase of cell cycle was calculated [19].

RESULTS

HPTLC fingerprinting of S1 and S2

The HPTLC analysis revealed the presence of quercetin in the two extract forms at proportion of 0.23% in S1 and 0.48% in S2. Quercetin detected by brown-yellow color zone. The Rf values of extract and standard were shown in Table 1 and chromatograms of S1, S2, and standard quercetin showing the area and peak values are depicted in Fig. 1.

Antiproliferative activity of *P. guajava*

MTT assay

MTT cytotoxicity assay revealed decreased cell viability of KB cells with increase in concentration of S1 and S2. The IC₅₀ value for S1 was 12.5 µg/ml and for S2 was 6.25 µg/ml. S2 extract showed effective cell proliferation inhibition at a lower dose as shown in Fig. 2.

Flow cytometric analysis

The effect of extracts S1 and S2 on cell cycle progression of KB cells is shown in Fig. 3. After the treatment period, there was decrease in

Table 1: Chromatogram peak table with Rf values, area and height of extract S1 and S2, and quercetin

S. No.	Compound	R.F	Height	Area	λ max NM
1	S1	0.11	28.5	260.8	466
2		0.28	7.2	193.7	466
3		0.42	2.7	59.8	466
4		0.45	2.7	16.2	466
5		0.46	2.3	11.8	466
6		0.50	4.2	28.0	466
7		0.61	2.7	90.6	466
8		0.69	1.6	24.7	466
9		0.74	20.3	441.4	466
10	S2	0.11	20.6	186.7	466
11		0.14	12.2	223.8	466
12		0.27	7.4	201.7	466
13		0.38	5.6	124.7	466
14		0.48	2.8	16.0	466
15		0.57	2.0	69.7	466
16		0.68	3.1	65.2	466
17		0.53	18.0	513.4	466
18		Standard quercetin	0.40	39.8	4999.1

Rf: Retention factor

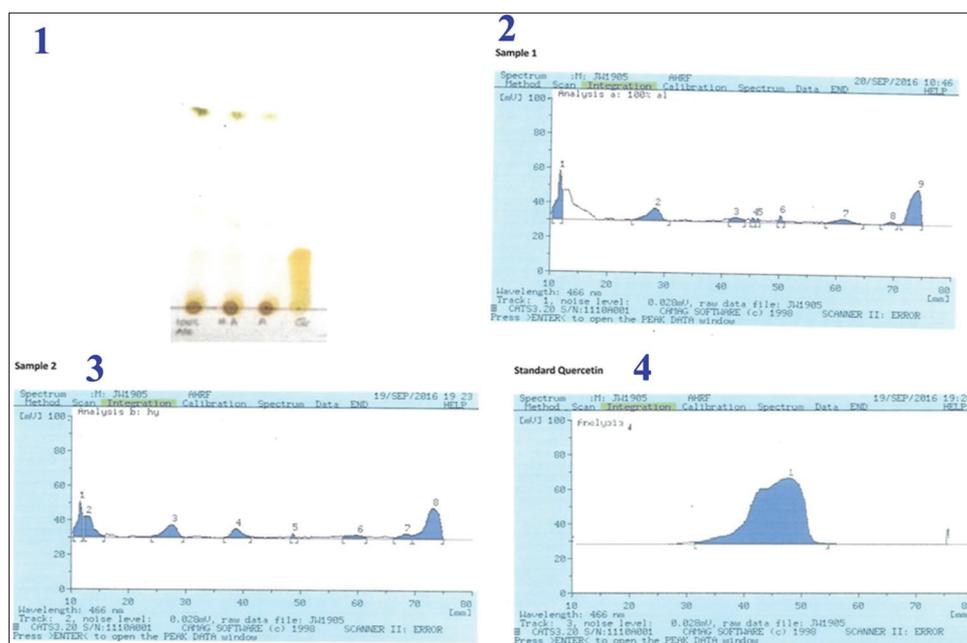


Fig. 1: High-performance thin-layer chromatography chromatogram of ethanolic extract at 466 nm showing different peaks (bands) of phytoconstituents in *Psidium guajava* and standard quercetin. Concentration of extracts 10 mg/ml. 1: Chromatograms of extract in HPTLC analysis after derivatization with color zones, 2: Chromatogram of ethanolic extract S1, 3: Chromatogram of ethanolic extract S2, 4: Chromatogram of standard quercetin

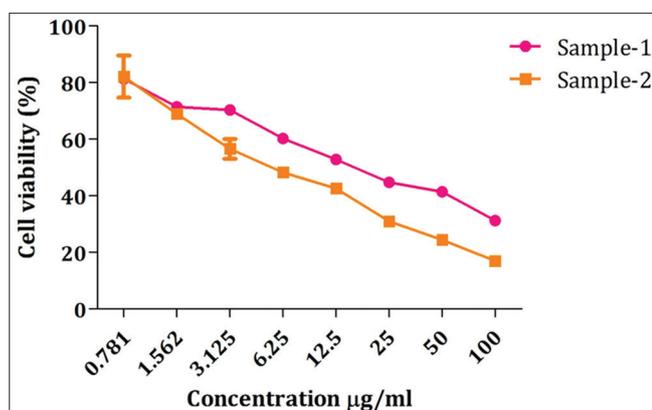


Fig. 2: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay, 50% inhibition (IC_{50}) value of the *Psidium guajava* leaf extracts S1 and S2. IC_{50} of S1 - 12.5 µg/ml and S2 - 6.25 µg/ml

G0/G1 phase cells from 87% (control/untreated) to 54% with S1 treatment and 41% with S2 treatment, decrease in S phase cells from 1.9% (control/untreated) to 0.19% with S1 treatment and 0.09% with S2 treatment, decrease in G2/M phase cells from 2.92% to 0.14% with S1 treatment and 0.08% with S2 treatment. The decreased G2/M phase cells signify arrested cell growth or cycle. Flow cytometric scatterplots showed increased apoptosis with both extracts S1 and S2 compared to control/untreated cells.

DISCUSSION

Cancer remains one of the leading causes of death worldwide. Many cancer therapies are in research which includes the use of natural products from plants [20]. There is a need to find out novel, effective and scientifically reliable phytochemicals are urgent. Natural products provide a fertile ground for seeking out treatments with fewer side effects and equal or better efficacy. The pharmacological effects of plant products were due to their constituent phytochemicals that include carbohydrates [21], carotenoids, polyphenols, alkaloids, and

sulfur-/nitrogen-containing compounds [22]. Phytochemicals extracted from plants are excellent pharmacological agents and they are easily available, non-toxic, inexpensive, and well tolerated [23].

The aim of the present study was to assess the antiproliferative effects of the leaf extracts S1 and S2 of *P. guajava* on oral squamous cell carcinoma cells and to propose the possible mechanism of action of the anticancer effect of the same. KB cells were reported to have high proliferative activity and they are undifferentiated in nature [24]. Some plant antioxidants have been suggested to contribute to their anticarcinogenic effects and their flavanols have been reported to inhibit cancer cell proliferation *in vitro* [25]. S2 showed more proportion of quercetin composition compared to S1. Commercially available quercetin was found to downregulate expression of mutant p53 protein, thereby causing an arrest of cells in the G2-M phase of the cell cycle. Mutations of p53 are among the most common genetic abnormalities in human cancers. The G1 checkpoint controlled by the p53 gene is a major site for the control of cellular proliferation. Quercetin has been found to arrest human leukemic T cells in the late G1 phase of the cell cycle. Quercetin has also been found to inhibit production of heat shock proteins in colon cancer. Heat shock proteins along with mutant p53 form a complex which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses and are associated with shorter disease-free survival and chemotherapy drug resistance. Quercetin has also been found to inhibit the expression of p21 ras oncogene in colon cancer cell lines. This mutation which causes continuous activation of the signal for DNA replication has also been implicated in a number of human cancers. Quercetin was also found to inhibit the expression of tyrosine kinase involved in the transduction of growth factor signals to the nucleus. It also binds to type II estrogen receptor, thereby offering a greater growth inhibition of ER-negative cells [16]. Both the extracts arrested cells of G2/M phase and G1 phase as revealed by above-described studies with other antiproliferative agents and cancer cells. In comparison, S2 extract was more effective than S1. S2 decreased G1 phase cells to 41% and G2/M phase cells to 0.08% which may be correlated to composition of quercetin in the extract. Both the extracts S1 and S2 have shown antiproliferative effects in the KB cells.

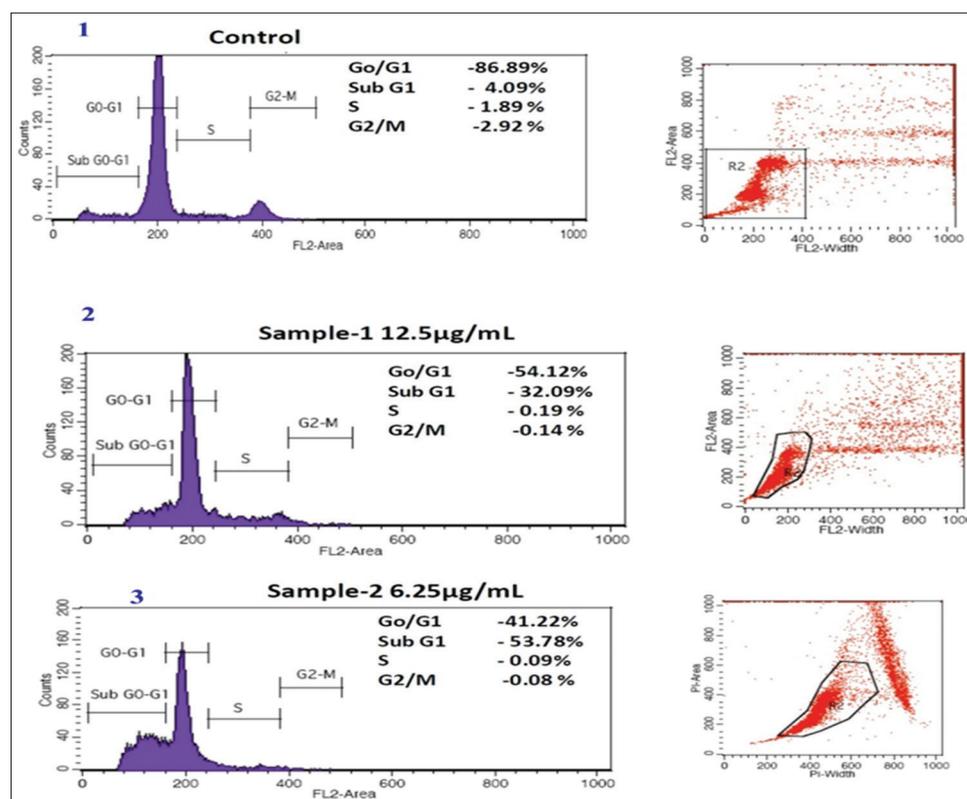


Fig. 3: Flow cytometric analysis of apoptosis in KB cells. KB cells were treated with the corresponding IC₅₀ concentrations of S1 and S2 and stained with propidium iodide (PI) dye and analyzed by flow cytometry. Flow cytometric patterns of 1: Untreated cells, 2: S1, and 3: S2

CONCLUSION

The results of the present study conclude the antiproliferative effect of *P. guajava* leaf extract. Active chromatographic fractions induced apoptosis and also arrested cell cycle. Further studies are required to identify the active ingredient, which can be developed as a chemotherapeutic neutraceutical with further clinical trials.

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AUTHORS' CONTRIBUTIONS

Dr. Sharada, the principal investigator of the project, performed the sample collection and prepared the manuscript. Dr. Malathi, Guide, Prof., and Head of the Department, is the coinvestigator of the project and helped in conceptual designing of the study and supervised and edited the manuscript preparation. Dr. Chamundeeswari and Dr. C. Rose, research advisors, guided in analysis of the results and helped in reviewing the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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