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, 53% 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 chemotherapeutic agents cause adverse cytological and systemic effects [7]. Natural products used in traditional folk medicine have been 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 extracted. Flow cytometry 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 ceasing 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.
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 WinCATS 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 (PBS) 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 WinCATS 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.

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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. 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. 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 FACS Calibur). 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.

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 depicted 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 | Rf | 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   | 225.8| 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    |          | 0.69| 3.1    | 65.2 | 466     |
| 17    |          | 0.53| 18.0   | 513.4| 466     |
| 18    | Standard quercetin | 0.40| 39.8   | 4999.1| 466     |

Rf: Retention factor
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 flavonols 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.
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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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
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