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Anticancer activity of terpenoid saponin extract of *Psidium guajava* on MCF-7 cancer cell line using DAPI and MTT assays

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***Psidium guajava* (Guava) could be an ancient remedy used for a variety of health conditions. Research suggests that guava fruits and leaves might have a variety of advantages. Guava is the tropical tree with yellowish-green skin fruits, and widely grown in Central America and Asia. Individuals use guava leaf tea as a treatment for gastric symptoms in many countries, together with India and China. In different countries, like India and Mexico, individuals have historically used the flesh of the fruit and leaves to heal wounds. Guava leaves extract had shown anticancer, antidiabetic, antispasmodic and anthelmintic effects in various research studies. In our present study, terpenoid saponin, a novel molecule isolated from the fraction of guava leaf extract studied for anticancer activity using 4′, 6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays against MCF-7 breast cancer cell line. Terpenoid saponin fraction had shown >60% apoptotic activity using DAPI staining assay against normal cell line activity and shown >99% average %inhibition activity at 400 µg/ml which is a significant result. Thus, we suggest further cell line studies of terpenoid saponin extract of guava leaf for potential anticancer effects and usage.**

Key words: Guava leaves, *Psidium guajava*, terpenoid saponin glycoside, 4′, 6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), assay.

INTRODUCTION

Psidium guajava is a small tree belonging to the Myrtaceae family popularly known as guava. It is widely available in tropical areas of Southern Mexico, Northern South America and in Asia. Many countries grow guava trees for its fruits allowing mass cultivation in suitable climatic conditions. The fruits are edible and have many medicinal properties and rich in Vitamins like A and C. The leaves are rich in flavonoids and saponins. Many folk

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medicinal preparations of leaves have abundantly been prescribed in Mexico and Nigeria as anti-diarrheal remedy. Some parts of the world used decoction of the leaves as anti-spasmodic and anthelmintic remedies. Leaves and stems of the leaves are chewed in Nigeria for oral hygiene and other dental related problems. In countries like India and China, the leaves decoction is used to treat diabetes and rheumatism. Preparations of the leaves also show antibacterial effects and are used to treat skin related problems (Lok et al, 2020; Kumar et al, 2021 and Kenneth et al, 2017).

DAPI assay

4′, 6-diamidino-2-phenylindole (DAPI) is the fluorescent stain that binds strongly to DNA at adenine thymine rich regions. It is used to study and quantify DNA in cellular systems as staining nucleic acids. Once DAPI is added to tissue culture, the cells rapidly uptake the dye and bind to cellular DNA yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence (Acharya and Hemanth, 2015).

Cell apoptosis detection using DAPI

Once cells absorb the DAPI dye the blue fluorescence will be observed by florescent microscopy. During the process of apoptosis, the dye is permeability increased and produces more fluorescence. At the same time normal cells stained uniformly and margins are clear. Apoptotic cells will have abnormity in margins of nucleus and condensed chromatin is easily stained and visible through fluorescent microscopy (Bhat et al, 2018).

Cell viability assay MTT

The MTT colorimetric assay based on reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. Principle involves conversion of MTT into formazan crystals by metabolically active cells. Viable cells containing NAD (P) H-dependent oxidoreductase enzymes reduce MTT to formazan crystals. The insoluble formazan crystals are dissolved in soluble solvent and the colored solution is quantified at absorbance of 570 nm using Elisa plate reader. The darker the solution the more metabolically viable the cells (Acharya and Hemanth, 2015).

METHODOLOGY

Guava leaves extraction and purification

Hundred grams of fresh guava leaves were collected and crushed. Methanolic extraction was carried out in 1:10 ratio in Soxhlet apparatus for 45 min. Methanolic extract was filtered and concentrated in rotary evaporator until or unless total methanol gets removed from the extract. To the concentrated extract ice cold acetone was added to get the precipitate of the saponin. The saponin fraction is filtered out and washed with ethyl acetate to remove other impurities like flavonoids and dried to light yellowish powder. The purified saponin fraction is further analyzed for its properties.

Chemical tests

Test for alkaloids

Mayer's test: A drop of Mayer's reagent was added to the test tube containing the extract and a creamy or white precipitate indicates the presence of alkaloid (Kenneth et al, 2017).

Picric acid test: To the 1% of picric acid solution in water the extract was added, and a creamy precipitate shows the presence of alkaloid (Kenneth et al, 2017).

Test for flavonoids: The extract was dissolved in desired amount of water and 5 ml of dilute ammonia solution was added followed by addition of concentrated H_2S0_4. Development of yellow coloration and disappears on standing indicates presence of flavonoids. To the powdered extract, 10 ml of ethyl acetate was added and heated over a steam bath for 3 min. To the 4 ml of filtrate, 1 ml diluted ammonia solution was added and yellow coloration indicates presence of flavonoids (Kenneth et al, 2017).

Test for tannins: To 0.5 g of the plant extract, 5 ml of distilled water was added and filtered. Ferric chloride solution was added and development of blue to dark green color precipitate indicates the presence of tannins.

Test for saponin: To 1 g of the extract 20 ml distilled water was added and boiled on water bath and was filtered. 10 ml of the filtrate was added to 5 ml of water and shaken vigorously till the froth was achieved. To the froth, olive oil was added for saponification or emulsion formation indicating the presence of saponin (Kenneth et al, 2017).

Test for terpenoids (Salkowski test): To the 5 ml of the liquid extract 2 ml chloroform and 3 ml concentrated H_2SO_4 was carefully added to form a layer and development of reddish brown coloration on the inner surface indicates the presence of terpenoid (Kenneth et al, 2017).

Test for carbohydrates

Benedict's test: 0.5 ml of Benedict's reagent was added to 0.5 ml of the filtrate and heated on boiling water bath for 2 min and development of red colored precipitate indicates the presence of carbohydrates (Kenneth et al, 2017).

Test for steroids: 2 ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2SO_4. The color changed from violet to blue or green in some samples indicating the presence of steroids (Kenneth et al, 2017).

Test for phytosterols

Libermann-Buchard's test: 2 ml of acetic anhydride was mixed

with some extract and 1 or 2 drops of concentrated sulphuric acid was added slowly along the side walls of the test tube. A change in array of colors indicates the presence of phytosterols (Kenneth et al, 2017).

Glycoside test

Antimony trichloride test: To the dry extract was added chloroform and saturated solution of antimony chloride (add chloroform in 20% acetic anhydride). Appearance of pink color on heating indicates the presence of steroids or triterpenoid glycosides (Kenneth et al, 2017).

Tetranitro methane test: To the extract tetranitro methane solution was added and formation of yellow color indicates presence of sterols or triterpenoid glycoside (Kenneth et al, 2017).

DAPI assay

The MCF-7 cells were seeded in a 24-well flat bottom micro plate containing cover slips and maintained at 37°C in CO_2 incubator for overnight. 200 µg/mL of the compounds were treated at 48 h. After the incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. 20 µL of DAPI was incubated for 5 min at room temperature in the dark, examined under fluorescent microscope. Randomly selecting the fields in the microscope and counted the number of cells undergone apoptosis. Then calculate the percentage of apoptic cells (Bhat et al, 2018).

% of apoptotic cell = (apoptotic cells + late apoptotic cells) / (total no of cells) × 100

MTT cell proliferation assay

Description of cell lines

MCF-7 cell line was purchased from National Center for Cell Sciences, Pune, with 2.0×10^4 cells/well seeding density and was stored in liquid nitrogen for further testing (Acharya and Hemanth, 2015).

Preparation of MTT Solution

MTT (5 mg/mL) was prepared in PBS and sterile filtered with 0.22 µ filter and it was used for the study as stock solution (Acharya and Hemanth, 2015).

Test system preparation

Before assay, the test system MCF-7 cells were propagated at 37 ± 1°C in a gaseous environment with 5% ± 1% carbon dioxide in humid conditions in tissue culture flasks. The flasks were supplemented with 10% fetal bovine serum and Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) and penicillin (100 units) and streptomycin (100 µg) antibiotics (Invitrogen, USA) to obtain the sub confluence of cells (70 to 90% confluent) (Acharya and Hemanth, 2015).

Cell seeding for cytotoxicity assessment

The cell layers were rinsed with phosphate buffer solution (PBS) and trypsinized with 0.25% trypsin in 0.2 g/l EDTA within the culture flask at 37 ± 1°C till cells get detached and gets floated then DMEM with 10% FBS was added to the flask to flush the cells. Centrifugation was carried out at 900 rpm for 5 min. DMEM is used to suspend the cells and cell count was carried out to determine the viability cell number per mL and cell number was adjusted to 2×10^5 cells/mL. Cells adjusted were taken at 0.1 mL and seeded in each well of 96 well plates. Frequent mixing was carried out while seeding was done to get uniform cell suspension for plating the cells in each well. The well designated plates were incubated 37 ± 1°C for 24 ± 1 h in gaseous environment of 5% ± 1% carbon dioxide and incubated for 24 ± 1 h. And then cells were exposed to varied concentrations of test item. Spent medium was replaced with different concentrations of test item solutions and incubated for 48 ± 1 at 37 ± 1°C in gaseous environment of 5 ± 1% carbon dioxide along with positive, negative and blank in designated wells. After 48 ± 1 h of incubation, the medium with item and positive control was removed and cells incubated for 4 h with 20 µL of MTT 5 mg/mL solution. During incubation formazan crystals were formed by mitochondrial reduction of MTT and 150 µL DMSO was added to solubilize then absorbance was read at 570 nm after 10 min of incubation and vertexing (Mosmann 1983; Cummings and Schnellmann 2004; McGarrity et al, 1980; Ligasová and Koberna 2019; Kumar et al, 2014; Manikyam et al, 2017).

Reduction in the number of living cells indicates decrease in metabolic activity leading to decrease in formazan formed which is direct correlation and can be monitored by optical density at 570 nm. Percentage viability can be calculated using the formula (Acharya and Hemanth, 2015; Kumar et al, 2014; Manikyam et al, 2017):

% Viability = 100 (O.D Test item/O.D of Control)
% Activity = 100 - %Viability

Table 1. % of apoptosis test compound.

Apoptosis	%
Control	4.25±2.90
Guava saponin	61.99±5.37

RESULTS AND DISCUSSION

Saponin content was isolated from the leaves of guava and tested through qualitative analysis which showed positive for saponin, terpenoid, carbohydrate and glycoside test and negative for the remaining tests as mentioned.

Purified terpenoid saponin glycoside was further analyzed for its anticancer activity against MCF-7 cancer cell lines using DAPI and MTT assays. Assessment of nuclear morphology by fluorescence microscopy using cell permeable nucleic acid stain, such as DAPI was commonly used for apoptosis analysis. In negative control cells, there was intact nucleus seen whereas compounds treated with cells showed nuclear condensation and nuclear fragmentation of cells was observed. Terpenoid saponin glycoside showed >60% of

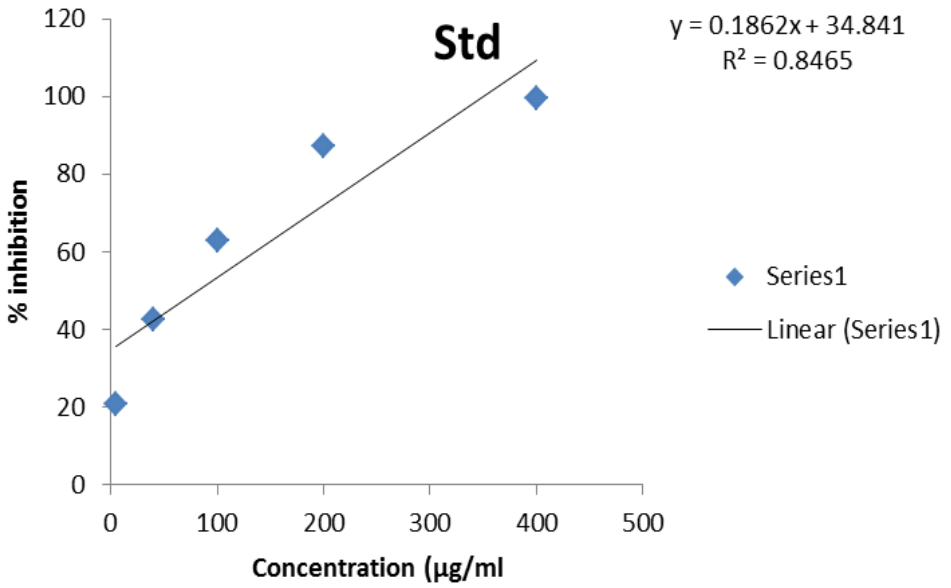


Figure 1. % inhibition of SLS.

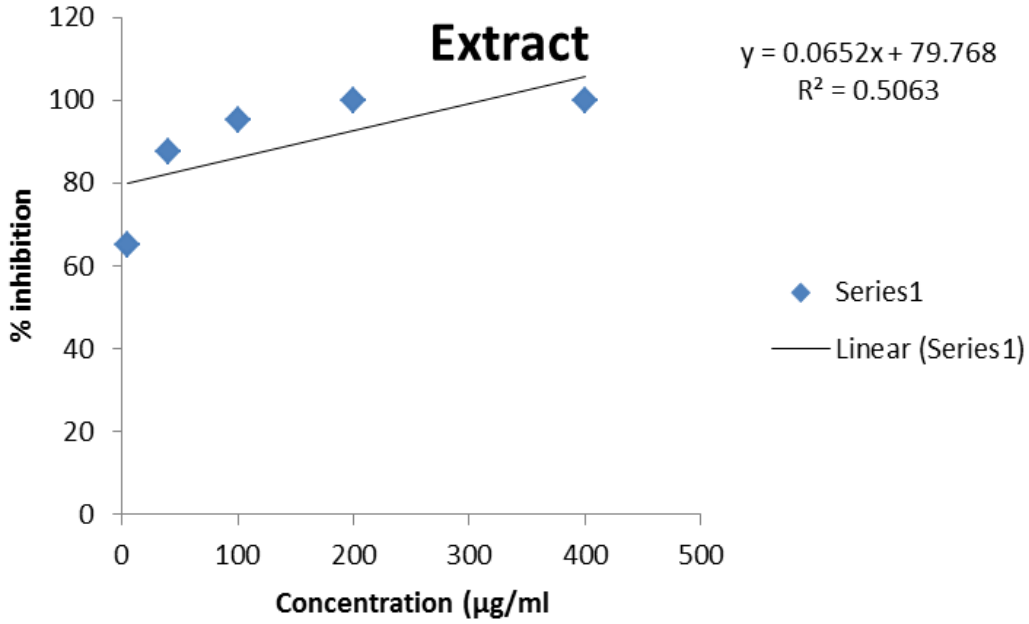


Figure 2. % Inhibition of test (terpenoid saponin-Guava leaf extract)

apoptosis activity against negative controls shown in Table 1 and Figures 1 and 2. MTT assay result showed the compound has the highest % average of anticancer activity 99.64% at 400 µg/ml concentration in correlation with positive test sample (SLS) as shown in Tables 2 and 3. Cells treated with terpenoid saponin extract of guava leaves had shown condensed chromatin with clear apoptosis when compared to Negative control using DAPI assay method as shown in Figure 3.

CONFLICT OF INTERESTS

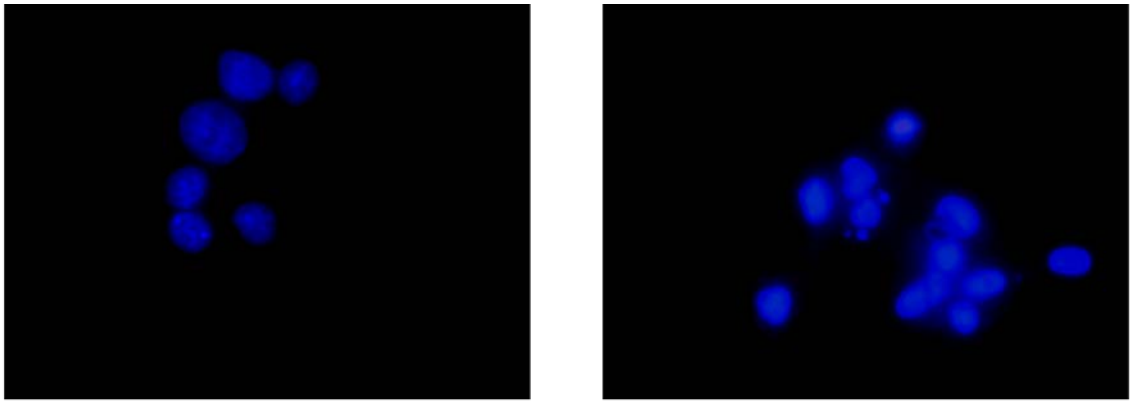
The authors have not declared any conflict of interests.

Table 2. Average % activity of test compound.

Concentration (µg/ml)	Inhibition of methanol extract of guava (%)	IC50
5	20.79	
40	42.47	
100	63.02	81.50
200	87.01	
400	99.64	

Table 3. Average % activity of positive control (SLS).

Concentration (µg/ml)	Inhibition of SLS (%)	IC50
5	65.09	
40	87.50	
100	95.20	> 400
200	99.86	
400	99.78	



DAPI assay negative control showing well defined chromatin

DAPI assay test sample showing condensed chromatin and cell apoptosis

Figure 3. Left image: DAPI assay negative control cells showing well defined chromatin and right image: DAPI assay Test sample (Guava terpenoid saponin) treated cells showing condensed chromatin and apoptosis

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