Anticancer activity of terpenoid taponin extract of tsidium guajava on MCF-7 cancer cell line using DAPI and MTT assays.

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Abstract

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. The Latin name for the common guava tree is Psidium guajava. Individuals use guava leaf tea as a treatment for Gastric symptoms in many countries, together with India and China. In different countries, like India, Mexico individuals have historically used the flesh of the fruit and leaves to heal wounds. Guava leaves extracts 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 DAPI and 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.

Keywords: Guava leaves, Novel terpenoid saponin glycoside, DAPI, MTT, Assay.

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Introduction

Psidium guajava a small tree belonging to the Myrtaceae family popularly known as Guava. It's 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. Fruits are edible and have many medicinal properties and rich in Vitamins like A and C. Leaves are rich in flavonoids and saponins. Many Folk medicinal preparations of leaves abundantly been prescribed in Mexico and Nigeria as anti-diarrheal remedy. Some parts of the world decoction of the leaves used as anti-spasmodic and anti-helminths remedies. Leaves and stems are chewed in Nigeria for oral hygiene and other dental related problems. In countries like India and China leaves decoction used to treat diabetes and Rheumatism. Preparations of leaves also shown antibacterial effects and used to treat skin related problems.

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 added to tissue culture the cells rapidly uptake the dye and binds to cellular DNA yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence.

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 increases and produce 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.

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 cells.

Materials and Methods

Guava leaf extraction and purification

100 gram of fresh Guava leaves were collected and crushed, Methanolic extraction was carried out in 1:10 ratio in Soxhlet apparatus for 45 minutes. 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 indicate the presence of alkaloid.

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.

Test for flavonoids

The extract was dissolved in desired amount of water and 5 ml of dilute ammonia solution were added followed by addition of concentrated H2S04. Development of yellow coloration and disappears on standing indicates presence of flavonoids. To the powdered extract 10 ml of ethyl acetate added and heated over a steam bath for 3 min. To the 4ml of filtrate 1 ml dilute ammonia solution was added and yellow coloration indicates presence of flavonoids.

Test for tannins

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

Test for saponin

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

Test for terpenoids (Salkowski test)

To the 5ml of the liquid extract 2ml chloroform and 3ml concentrated H2SO4 was carefully added to form a layer and development reddish brown coloration on the inner surface indicates the presence of terpenoids.

Test for carbohydrates

Benedict's test: 0.5 ml of Benedicts reagent was added to 0.5 ml of the filtrate and heated on boiling water bath for 2 minutes and development of red colored precipitate indicates presence of carbohydrates.

Test for steroids

Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H2SO4. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for phytosterols

Libermann-Buchard's test: 2ml of acetic anhydride was mixed with some extract and added 1 0r 2 drops of concentrated sulphuric acid slowly along the side walls of the test tube. A change in array of colors indicates presence of phytosterols.

Glycoside test: Antimony trichloride test: To the dry extract add chloroform and add 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.

Tetranitro methane test: To the extract add tetranitro methane solution formation of yellow color indicates presence of sterols or triterpenoid glycoside

DAPI Assay

The MCF-7 cells were seeded in a 24-well flat bottom micro plate containing cover slips and maintained at 370C in CO2 incubator for overnight. Treat the 200 µg/mL of compounds were treated at 48 hrs. 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

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

MTT cell proliferation assay

Description of cell lines: MCF-7 cell line purchased from National Center for Cell Sciences, Pune, with 2.0 × 104 cells/ well seeding density and stored in liquid nitrogen for further testing.

Preparation of MTT solution: 5 mg/mL MTT was prepared in PBS and sterile filtered with 0.22 µ filter and it was used for the study as Stock solution.

Test system preparation: Before assay the test system MCF-7 cells are propagates at 37 ± 1°C in a gaseous environment with 5% ± 1% carbon dioxide in humid conditions in tissue culture flasks. The flasks supplemented with 10% Fetal bovine serum

and DMEM (Dulbecco's Modified Eagle Medium) (Invitrogen, USA) and penicillin (100 units) and streptomycin (100 µg) antibiotics (Invitrogen, USA) to obtain the sub confluence of cells (70% to 90% confluent).

Cell Seeding for Cytotoxicity Assessment

The cell layers were rinsed with Phosphate Buffer solution (PBS) and trypsinised with 0.25% trypsin in 0.2 g/l EDTA with in 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 carried out at 900 rpm for 5 minutes. DMEM is used to suspend the cells and cell count was carried out to determine the viability cell number per mL and cell number adjusted to 2 × 105 cells/mL. Cells adjusted were taken 0.1 mL and seeded in each well of 96 well plates. Frequent mixing carried out while seeding to get unform cell suspension for plating the cells in each well. The well designated plates were incubated 37 ± 1°C for 24 ± 1 hrs, in gaseous environment of 5% ± 1% carbon dioxide and incubated for 24 ± 1 hrs and then cells exposed to varied concentrations of test item. Spent medium is replaced with different concentrations of test item solutions and incubated for 48 ± 1 hrs at 37 ± 1°C in gaseous environment of 5 ± 1% carbon dioxide along with positive, negative and blank in designated wells. After 48 ± 1 hrs of incubation the medium with item and positive control removed and cells incubated for 4 hrs with 20 µL of MTT 5 mg/mL solution , during incubation formazan crystals formed by mitochondrial reduction of MTT and 150 µL DMSO added to solubilize then absorbance was read at 570 nm after 10 minutes of incubation and vertexing.

Reduction in 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:

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

% Activity=100-%Viability

Results and Discussion

Saponin content isolated from the leaves of Guava tested through qualitative analysis and shown positive for saponin , terpenoid, carbohydrate and glycoside test and negative for remaining tests as mentioned. Purified terpenoid saponin glycoside is 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 is commonly used for apoptosis analysis. In negative control cells there was intact nucleus was seen whereas in compounds treated cells showing nuclear condensation and nuclear fragmentation of cells was observed [Table 1-3] [Figure 1,Figure 2].

Apoptosis (%)	
Control	4.25±2.90
Guava saponin	61.99±5.37

***Table* 1 .** *% of apoptosis test compound.*

Concentration µg/ml	**percentage inhibition of methanol extract of guava**	**IC50**
5	20.79	81.5
40	42.47	
100	63.02	
200	87.01	
400	99.64	

Table 2. *Average % activity of test compound.*

Concentration µg/ml	Percentage inhibition of SLS	IC50
5	65.09	> 400
40	87.5	
100	95.2	
200	99.86	
400	99.78	

Table 3. *Average % activity of positive control SLS (Sodium Luareth sulphate).*

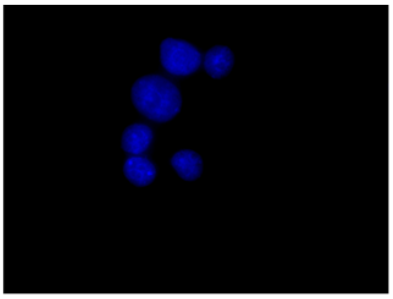

Figure 1. *DAPI assay negative control showing well defined chromatin.*

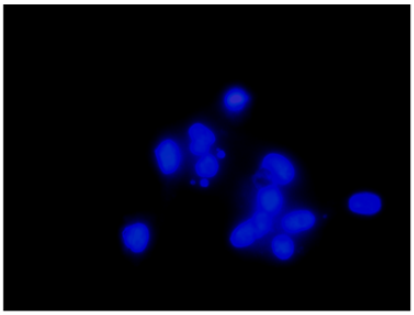

Figure 2. *DAPI assay test sample showing condensed chromatin and cell apoptosis.*

Conclusion

Terpenoid saponin glycoside had shown >60% of apoptosis activity against Negative controls as shown in table 1, figures 1 and 2. MTT assay result had shown the compound has highest % average anticancer activity 99.64% at 400 µg/ml concentration in correlation with positive test sample (SLS) as shown in tables 2, table 3.

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