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Investigating the anti-tumor potential of *Psidiumguajava* extracts on PC-3, A549, and BT-549

Abstract

By April 2018, the number of persons diagnosed with new cancer cases in the United States stood at 1,735,350. The U.S. National Cancer Institute (2018) approximates that about 600,000 persons from this population succumbed to the disease.[1] Inferential statistics between 2015 and 2018 suggest that cancer incidence in the U.S. stands at 439.2 for every 100,000 individuals (the majority of whom are men).[2] Extracts from *Psidiumguajava*, which comprise adiverse array of polyphenols, display pharmacological properties, such as antioxidative and anti-inflammatory effects.[3] Also, the phenolic components in the plant's leaves exhibit anti proliferative properties (they deter certain cancer cell lines from replicating). The intrinsic anti tumor properties of the plant's leaves are attributed to secondary metabolites comprising specific polyphenols such as ethanolic extracts.[4] Recently, Vijaya and Manikandan also postulated that the antioxidant activity of *P.guajava* fruits could be effective for chemo therapeutic and chemo preventive uses.[5] This study investigates the antitumor potential of *P.guajava* extracts along three cancer cell lines: PC3 (prostate), A549 (lung), and BT549 (breast) cancer.

Experimental approach: *P. guajava* yielded an ethanolic extract that was prepared into varied concentrations and later exposed to PC-3, A549, and BT-549 for 24hours. A fluorescent plate, Alamar blue, and a cell viability indicator were used to conduct the enzyme-linked immunosorbent assay (ELISA). An antibody-specific, conjugated HRP (horseradish peroxidase) was used to detect the presence of caspase-3. The effect of the *Psidiumguajava* as a cellular antioxidant was evaluated via the peroxidation of lipid assays.

Outcomes: Of the seven concentrations of the *P. guajava* extract, a considerable number of concentrations of the extract indicated that the extract effectively inhibited the three types of cancer cells (PC-3, A549, and BT-549) from proliferating. By inhibiting the formation of Malondialdehyde (MDA), the plant was efficient in suppressing the lipid peroxyl radicals, especially at the highest concentrations of the extract. The tested samples results of the study also suggest that the inhibition effect of the *P. guajava* extract was dose-dependent. Furthermore, caspase-3 was detected in PC-3, A549, and BT-549 at ethanolic concentrations of *P. guajava*, ranging which ranged from 0.39 mg/mL - 3.89 mg/mL.

Conclusion: The experimental data suggest that *P. guajava* could be considered as a viable antitumor agent due to its natural cytotoxic compounds. In all, the antioxidant and anti proliferation capabilities of the extract of are exploited further for the treatment of prostate cancer, lung cancer, and breast cancer.

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Heba Alhamdi,[1] William Boadi,[2] VenkataswarupTiriveedhi,[1] Alekzander Garcia,[2] Steffani N Driggins,[3] Lewis Myles E[1]

[1]Department of Biological Sciences, Tennessee State University, USA

[2]Department of Chemistry, Tennessee State University, USA

[3]Department of Biology, Allen University, USA

Correspondence: Lewis Myles E, Professor of Biology, Department of Biological Sciences, Tennessee State University, 3500 John A Merritt Blvd, Harned Hall Room 213 Nashville, Tennessee 37209, USA, Tel +(615) 963-5789, Email emyles@tnstate.edu

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Background

Cancer has detrimental effects on the human population. Scientists are exploring new forms of therapy and studying the components of various plants to combat the disease. Plants have naturally occurring secondary metabolites that have reduced side effects and properties that can be applied to cancer therapy.[6] Herbal medicines have been used for centuries in developing countries due to their antiseptic characteristics. Numerous plant species are currently being used in the prevention and treatment of cancer. By focusing mainly on herbal medicine used in developing countries, researchers have identified different kinds of plants that exhibit anticancer properties. Some species of plants have compounds that are necessary for their survival, particularly alkaloids.[7] Alkaloids have and are still being tested for their capacity to initiate apoptosis and inhibit the growth of cancerous cells. Hence, their therapeutic effects are presently applied in cancer treatment.

Psidiumguajava, guava, is a small tree belonging to the family *Myrtaceae*.[8] The plant is native to tropical areas from southern Mexico to northern South America.[9] While most people are familiar with its fruits, medical researchers are interested in the medicinal properities of the leaves of *P. guajava*.[9] For example, the leaves of the plant are boiled and the water can be consumed to avoid bleeding in DHF, and increased platelet counts to 100.000/mm3 within a period of approximately 16 h.[10] Also, the leaves of *P. guajava* have been used to prevent the conversion of complex carbohydrates to sugars which is useful in weight loss and the prevention of diabetes.[11] Moreover, the antibacterial activity of the *P. guajava* leaves relieves gastrointestinal issues by inhibiting the growth of harmful microbes. Furthermore, the leaves can be helpful during in treating colds by suppressing microbial activity and, thus reducing mucus production.[11] The *P. guajava* leaves are also used in the regulation of blood pressure. They prevent blood thickening and ease the blood flow, which is crucial for the brain and the heart.[11] The leaves of *P. guajava* enhance the digestive process by stimulating enzyme production and they serve as great laxatives which to relieve constipation as well.[12] The leaves also inhibit histamine production which lessens the effects of allergies and makes them a useful remedy in treating insect bites.[11] The leaves are even used to treat mild tooth aches, sores, and inflamed gums.[11] The leaves of *P. Guajava* also contain copper which promotes healthy thyroid functioning, which is an important process in regulating hormone levels. Some people use the leaves of *P. guajava* to prevent and reduce the common sights of aging such as wrinkles and age spots.[11] In this regard, the extensive use and benefits of the *P. guajava* leaves have

drawn the attention of scientists, who have studied the plant in regards to its potential in the treatment of cancer.[9]

P. guajava leaves have a unique chemical composition. Extracts from the leaves contain phenolic compounds in contrast to the other plant species. Hence, they exhibit anti-inflammatory, analgesic, hepato protective, antimicrobial, and antioxidant activities. Further research suggests that the leaves contain gallic acid, catechins, epicachetin, kaempferol, naringenin, and rutin.[13] Most of these chemicals determine the leaves' inhibitive characteristics. Experts have isolated two terpenoids from the leaves, lupeol and betulinic acid, which have potential phytotoxic and antimicrobial activities.[13] The presence of flavonoids in the leaves mainly contribute to the antimicrobial actions of the leaves. Notably, all the isolated chemicals have a medicinal value.[13]

Several chemicals found in the *P. guajava* leaves have effects on human cells. The Psiguadials A, B, and guajadial present in the leaves of *P. guajava* inhibit the growth of particular cells of Prostate Cancer cell line.[13] Aqueous extracts of the plant's young leaves have an exceedingly high concentration of isoflavonoids and polyphenols.[13] The compounds are effective in suppressing the angiogenesis and cell migration processes.[13] Hence, the *P. guajava* leaves have the clinical potential to be applied as an adjuvant anticancer medication. The phenolic glycosides isolated from the leaves exhibit considerable inhibitory activity against histamine release from mast cells, in addition to the production of nitric acid from a cell line resembling murine macrophages.[13] Medical experts have established that the aqueous extracts of the guava budding leaves have chemical activities that can inhibit the development of prostate cancer in a cell line model.[13] Essentially, the leaves are a potential anti-androgen-sensitive prostate cancer agent. Thus, the therapeutic properties of the guava leaves allow for their use as an additional treatment option for cancer.

In vitro and *in vivo* studies on the inhibitory effects of guava leaves on cancer have been conducted.[14] An *in vitro* study showed that quercetin and quercetin glycosides in the extracts from *P. guajava* leaves inhibited the cell line proliferation rate.[9] The aqueous extract from it's plant's budding leaves has exhibited an anti-tumor effect against BT-549 cells.[9] The bioactivity of an enriched mixture of chemical components of guava leaves, including guajadial, psidial A, and psiguadial A and B, inhibited cell multiplication in nine human cancer lines including PC-3.[9] Further research showed that the ability of the extract from the guava leaves extracts to inhibited multiple signaling cascades involved in the proliferation of cancerous cells, which instigated an apoptotic effect on PC-3 cells.[9] Essential oils from the species' foliage have a cytotoxic impact on the PC-3 cell lines at a maximal inhibitory concentration.[9] Hence, these extracts suppress tumor genesis.

An *in vivo* study showed that the use of the extract from the guava leaves extracts resulted in apoptotic and cytotoxic effects in PC-3 cells, PC-3 cells which are androgen independent.[9] Cell death of the PC-3 cells, however, requires a high concentration of the guava plant extract.[9] In this regard, researchers learning how *Psidiumguajava* could be used for chemotherapy. Several researchers have verified the ethnomedicine applications of the guava leaves against various malignancy cell lines. The individual compounds in extracts of the leaves of *Psidiumguajava* such as catechin, guaijaverin, quercetin, and gallic acid contribute to its therapeutic efficacy. The current research study determines the effect of an extract of *P. guajava* on a prostate cancer cell line (PC-3), lung cancer cell line (A549) and a breast cancer cell line (BT549). Cell viability, detection of malondialdehyde (MDA) and the presence of caspase-3 were observed in the research study for each cancer cell line.

Methods

Cell culture

The prostate cancer cell line (PC-3), lung cancer cell line (A549) and breast cancer cell line (BT549) obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, and 1% penicillin-streptomycin, with an exception.[15] MCF-7 cells were grown in DMEM, a low glucose variant (Gibco), containing 2mM L- glutamine, non-essential amino acids, penicillin-streptomycin, 10% fetal calf Serum (Atlanta Biologicals) and also supplemented with 0.01 mg/mL insulin and 1mM sodium pyruvate.[16] Cells were grown in 5% CO_2 humidified incubator at 37°C, and passaged biweekly after reaching 80% confluency in 1:2/1:4 ratios for no more than 40 passages.[17]

Crude extraction

The crude extractions of the organic compounds from Guajava. the *P. guajava* leaves were prepared using a Soxhlet, lyophilizer and an evaporator. The dried leaves of *(Guava) P. guajava* were obtained from (the place where we got the plants). These samples were placed in a separate, plastic, chemical resistant 15-millimeter centrifuge tube and frozen for twenty-four hours in liquid nitrogen. The samples were then removed and grounded separately with a mortar and pistil. The crude extract was then infused in methanol to remove the components that were distilled for 6 six hours in a Soxhlet.[18] The methanol was removed with a rotary evaporator. All crude extracts were weighed and dissolved in dimethyl sulfoximde. The extracts were frozen in freeze vials and stored at -20 degrees Celcius.

Alamar blueTM metabolic assay

Alamar Blue®, a metabolic cell function indicator, was used as a fluorescent and as a colorimetric dye in the cell viability assay based on the metabolic activity of living cells by the reduction of the non-fluorescent reagent (resazurin) to a fluorescent compound (resaurufin).[19] The PC-3, A549, and BT-549 cancer cell lines were plated at $1X10^4$ in a 96-well plate and incubated at 37°C and 5% CO_2 for 24h. After incubation, the cells received fresh medium supplemented with treatment of the extract from the *P. guajava* leaves at concentrations ranging between 0µg/ml to 3.89µg/ml. The plates were returned to the incugator at 37C and 5% CO_2 overnight. The negative controls received fresh medium supplemented with the experimental vehicle, DMSO only.[20] 20µl of Alamar blue dye, 10%v/v, was added to each well and the plates were incubated for 25h at 37°C. The plates were read at a wavelength of 530nm to 560nm and an emission wavelength at 590nm using the Speactra Max Gemini EM microtiter plate reader.

Lipid peroxidation assay

Lipid peroxides were analyzed by the thiobarbituric acid (TBA) assay in samples. The cells were treated with Guava leaf the *P. guajava* extract and incubated for 24hours at 37°C. The control incubations contained all reagents except the extract. After centrifugation at low-speed centrifugation, the cell pellets were saved and then sonicated by sonicator tool. The lysate was centrifuged at 3,000×g for 10 minutes

at 4°C. The resultant supernatant was used for the analysis of MDA levels as described by the manufacturer's instruction for the MDA assay kit. The following modifications were used for the MDA assay kit: 1) an aliquot (100µl) of the supernatant was obtained, 2) the R1 solution was added to each sample tube 3) 0.2N of HCl (Chromogenic reagent) was added to each tube and mixed. The samples were incubated at 50°C for an hour in the dark. After transferring the samples in 96 well plates, the absorbance was measured at 586nm in a Micro plate Reader (Bio-Tek Instruments). The MDA levels were calculated from a standard curve. [kit purchased MD Biosciencesinc, Cat. No. 437634; EMD/Calbiochem, San Diego, CA] (both are correct I don't know which one to add in the previous paragraph).

Caspase-3 activity assay

The lysates for the PC-3, A549, and BT-549 cancer cell lines were added to a 96-immunoassay plate with BSA and incubated for 24 h at 4°C. Each experimental well was washed two times with PBST. Afterward, they were incubated overnight at 4°C in a solution of caspase-3 rabbit primary antibody (Cell Signaling) at a 1:1000 a dilution in 0.1 M PBS buffer. The cells were washed with PBST (0.1 M phosphate buffer, pH 7.4, 0.1% (v/v) Tween 20) twice and incubated with biotinylated goat anti-rabbit antibody diluted at 1:500 (Secondary Ab by Cell Signaling) for 1 hr at room temperature. After two washes with PBST washes, the samples were incubated with reagent A and B for 30m in the dark. A stop solution was then added each exploratory well, and the absorbance was read in a microplate reader (FilterMax F5) at 450nm immediately.

Statistical analysis

The data were expressed as the mean±standard deviation. One-way ANOVA analysis was applied to determine the statistical differences and StatView 5.0.1. (SAS Institute Inc., Cary, NC). $P< 0.05$ was regarded as statistically significant.

Results

PC-3 (prostate), A549 (lung), and BT549 (breast) were exposed to serial concentrations (0.06mg/ml, 0.12mg/ml, 0.24mg/ml, 0.49mg/ml, 0.97mg/ml, 1.94mg/ml, and 3.89 mg/ml) of the crude extract the leaves of *P. guajava* to evaluate its the effects of the extract on the viability of each cell line. As the concentration of the leaf extract increased, the viability of the cells decreased significantly in all three cancer cell lines. Figure 1 indicates that the growth of the PC-3 cancer cells were inhibited by 80%, 90% and 97% at t 0.97mg/ml, 1.94mg/ml, and 3.89 mg/ml respectively by the leaf extract. Also, the leaf extract of *P. guajava* inhibited the growth of BT-549 by more than 80% by the following concentrations: 0.49mg/ml, 0.97mg.ml, 1.94mg/ml, and 3.89mg/ml (Figure 2). After 24hours of treatment by the plant leaf extract, the proliferation the A549 cancer cells were reduced to values close to between 70 to 90% at 0.97mg/ml and 1.94mg/ml as Figure 3.

The level of MDA as a common marker of lipid peroxidation was detected via by the lipid peroxidation assay in the PC-3, A549, and BT-549 cell lysates. Absorption for each sample was read at 586nm using a microplate reader (Filter Max, Jenway 6505, UK). Twenty- four hours after seeding of A549, BT549 and PC3 cells in 6 well plates, cells exposed with 0.45 and 0.98 mg/mL of Guava leaf extract for 24h. Then collected samples were lysed for analyzing antioxidant enzymes, Malondialdehyde (MDA).] The results of the lipid peroxidation assay indicated a very significant decrease in MDA activity levels in the PC-3 cells at 0.45 mg/ml and 0.98 mg/ml of the leaf extract in comparison to the control (Figure 4). There was also a significant decrease in the MDA activity levels in the A549 cells. The decrease was noted at occurring at 0.45mg/ml and 0.98ml as well in comparison to the control (Figure 5). On the other hand, the results indicated that there was no significant decrease in the MDA activity levels of the BT-549 cells (Figure 6). MDA activity levels in both concentrations as shown in Figure 6.

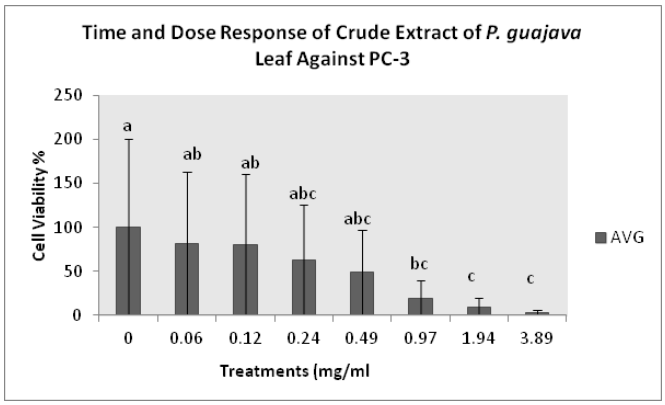

Figure 1 Time and dose response of crude extract of *P. guajava* leaf on cell proliferation of PC-3 cancer cell line. Cells were plated at 1X10^4 cells per well in a 96-well plate. Values are presented as means (n=3)±S.D.*Statistical difference ($P< 0.05$).

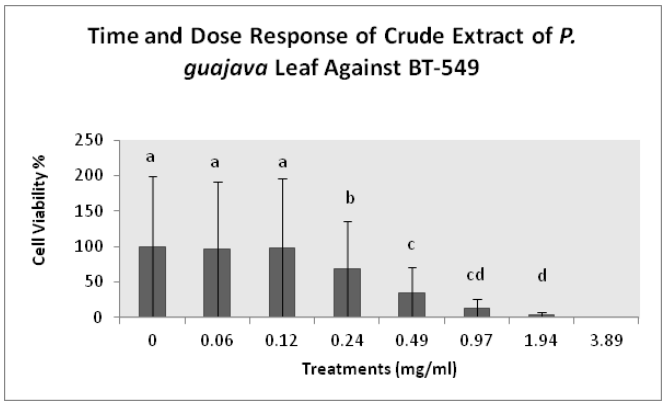

Figure 2 Time and dose response of crude extract of *P. guajava* leaf on cell proliferation of BT-549 cancer cell line. Cells were plated at 1X10^4 cells per well in a 96-well plate. Values are presented as means (n=3) ± S.D.*Statistical difference ($P < 0.05$).

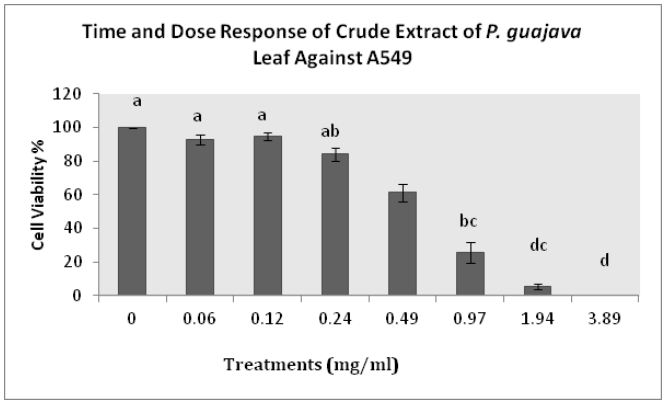

Figure 3 Time and dose response of crude extract of *P. guajava* leaf on cell proliferation of A549 cancer cell line. Cells were plated at 1X10^4 cells per well in a 96-well plate. Values are presented as means (n=3)±S.D.*Statistical difference ($P < 0.05$).

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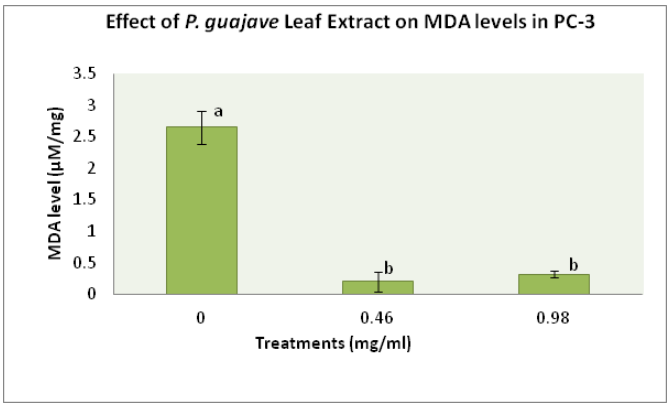

Figure 4 Effect of *P. guajava* leaf extract on MDA level activites in PC-3. Cells were plated at 1X10^4 cells per well in a 96-well plate. Values are mean ± SEM (n = 3), ($p < 0.05$).

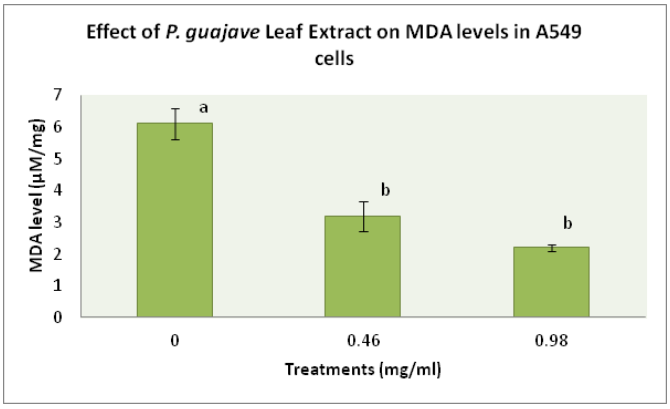

Figure 5 Effect of *P. guajava* leaf extract on MDA level activities in A549. Cells were plated at 1X10^4 cells per well in a 96-well plate. Values are mean±SEM (n = 3), ($p < 0.05$).

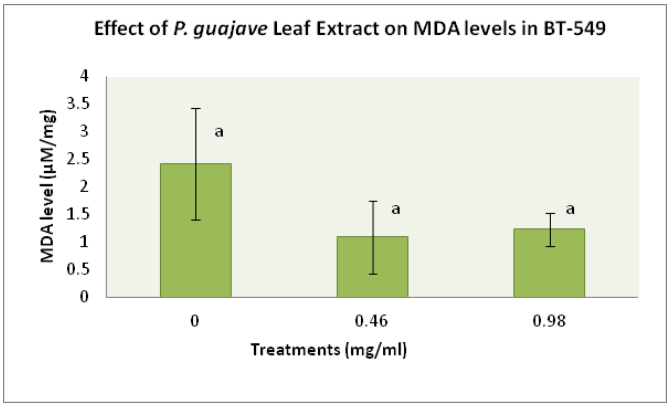

Figure 6 Effect of *P. guajava* leaf extract on MDA level activites in BT-549. Cells were plated at 1X10^4 cells per well in a 96-well plate. Values are mean ± SEM (n = 3), ($p < 0.05$).

Caspase 3 is the most generally studied of the effector caspases. Cleaved caspase-3 is considered a biomarker of apoptosis.[21] After A549, BT549 and PC3 cancer cells were treated with the serial dilutions of the *P. guajava* leaf extract there was a marked increase in the level of caspase-3 in the cells ($P < 0.05$) in comparison with the control (DMSO). As demonstrated in Figure 7, 8 & 9. The increase in cleaved caspase-3, however, was measured by Elisa assay using anti-caspase-3. These findings indicate that Guava leaf extract -induced cell death is mediated by Caspase-3 pathway activation through enzymatic activity.

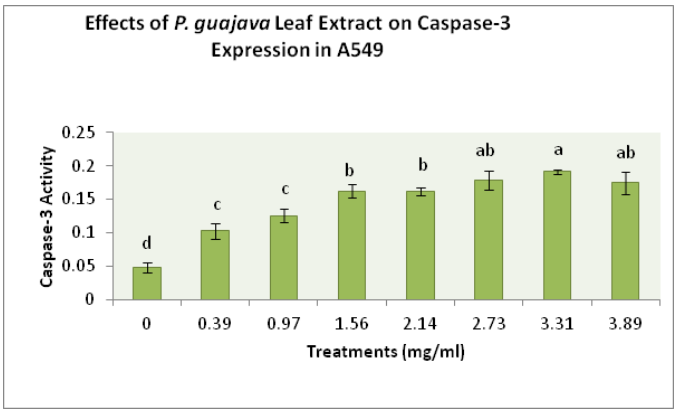

Figure 7 Effect of *P. guajava* leaf extract on caspase-3 expression in A549. Cells were plated at 1X104 cells per well in a 96-well plate. Values are mean ± SEM (In = 6), ($p < 0.05$).

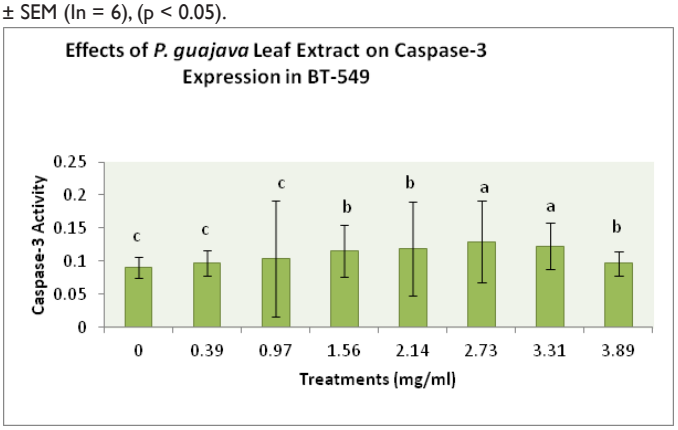

Figure 8 Effect of *P. guajava* leaf extract on caspase-3 expression in BT-549. Cells were plated at 1X104 cells per well in a 96-well plate. Values are mean ± SEM (In = 7), ($p < 0.05$).

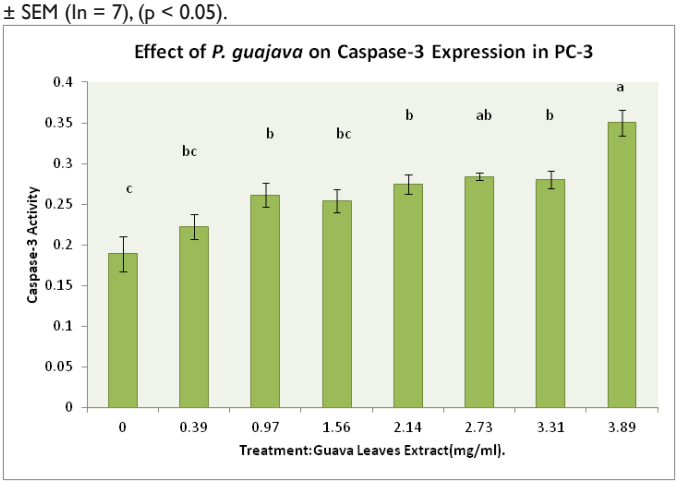

Figure 9 Effect of *P. guajava* leaf extract on caspase-3 expression in PC-3. Cells were plated at 1X104 cells per well in a 96-well plate. Values are mean ± SEM (In = 3), ($p < 0.05$).

Discussion

Psidium Guajava is a medicinal plant that belongs to the *Myrtaceae* family and is used as a source of food. Its trees grow in the tropical

regions such as Florida, the Caribbean, Central, and South America and they have a great healing capability, which can be traced, back to its use for many centuries by the native people.[22] The guava leaves contain antioxidants, antibacterial and anti-inflammatory agents, tannins and pain relievers.[23] The antioxidants in guava leaves include Vitamin C and quercet in which helps to slowdown the aging process and prevent devitalizing diseases such as cardiovascular ailment, arthritis, cancer, diabetes, stroke and muscle degeneration through the for aging away off reeradicals in the body and enhancement of the immune system.[24] Various studies have identified that plants contain various phyto chemicals that can be isolated from different plant tissues and employed in the treatment of numerous illnesses.[25] The role of phyto chemicals in the treatment of cancer has particularly been aprospective area in the pharmacological industry.[26] Currently, there is no cure for cancer.[27] However, recent studies have identified that various compounds, sourced from plants, can induce cytotoxicity against different cancer cell lines in humans.[28] This discovery has prompted further investigation into the medical benefits of plant compounds against cancer. This investigation, in turn, has led to the illumination of the mechanisms of action of known plant bioactive compounds as well as the discovery of new phytochemicals that can induce apoptosis and cytotoxicity in various human cell lines.[29] So far, there are different literatures that provide scientific evidence of natural plant-induced cytotoxicity in human cells.

The inhibition of cell proliferation of A549(Lung), BT549(Breast) and PC3(Prostate) cancercells was measured at six different concentrations after 24h of treatments Figure 1). This procedure was conducted 3 times and readings were averaged for statistical significance. The profile of results obtained by the cell viability assays for Guava Leaf extracts was similar. From the graph, it appears that the highest concentrations of *Psidiumguajava* 3.89mg/ml) have the biggest effect on cancer cells growth which is cell viability close to zero comparing to the control. It becomes clear that *Psidiumguajava* is effective plant in term of anti proliferation. The conclusion is that cancer cells respond to variations in psidium concentrations in the same way.

In the present data, we evaluated the capacity of *Guava Leaf* extract to induce apoptosis and inhibit oxidative stress and cancer cell proliferation. MD Aassay showed that incubation of A549(Lung) and PC3(Prostate) cancer cells with Guava Leaf extract significantly reduced the lipid per oxidation from oxidative stress.[30] The protection against oxidative stress was mediated by the inhibition of MDA activity and which is known to be end product of lipid per oxidation.[31] Oxidative stress markers levels such as MDA may also reveal the effectiveness of cancer growth treatment or surgical procedures.[32] Postoperatively in lung cancer patients following removal of cancer-associated parenchyma, Reduced MDA levels have been demonstrated.[33] Another finding (significantly reduced MDA levels) has also been showed after surgical treatment in colorectal cancer patients.[34] Thus, these results suggest that, by comparing the levels of lipid peroxidation products before and after therapy may be used to utilize the effectiveness of therapy or medical procedure in patients with cancers. Apoptosis is regulated by multiple signaling pathways of a family of cysteine proteases (caspases), which are extrinsic pathway, and intrinsic pathway. Caspase 3 is the most generally concentrated of the caspase cascade, cleaved caspase-3 induces apoptosis. Our results show that guava Leaf extract activates caspase-3 in apoptotic cells. By Using ELISA assay and anti-caspase 3 we measured active caspase 3 in cancer cells.

Conclusion

In summary, based on the results of this study the extract of Guava Leaves may protects human A549(Lung), BT549(Breast) and PC3(Prostate) cancer cells from oxidative stress-induced cell death, which is associated with the suppression of MDA level. Our results show that Guava Leaves Extract has a significant induction of apoptosis in a dose caspase-3 dependent mechanism that could also serve as a diagnostic monitor the progress or effectiveness of therapy in cancer patients. Therefore, Guava Leaves extract has a potential natural antioxidant that can be beneficial in cancer management and natural agent with inhibition and apoptotic effects against cancer cells.

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Conflicts of interest

The authors declare that there is no conflicts of interest.

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