

Antioxidant, Antibacterial and Antitumor Activity of Ethanolic Extract of the *Psidium guajava* Leaves

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Abstract

Psidium guajava L. has extensive use in folk medicine. The aim of this study was to quantify the levels of phenolic, flavonoids, antioxidant activity, lethality assay and antibacterial and antitumor activities of the extract of *P. guajava*. In the dry guava extract there were high levels of phenolics (766.08 ± 14.52 mg/g), flavonoids (118.90 ± 5.47 mg/g) and antioxidant activity (87.65%). The LD₅₀ was 185.15 µg/ml. The MIC value was 250 µg/ml for *Streptococcus mutans*, *Streptococcus mitis* and *Streptococcus oralis*. IC₅₀ of the extract tested in the HeLa, RKO and Wi cell lines was 15.6 ± 0.8 µg/ml, 21.2 ± 1.1 µg/ml and 68.9 ± 1.5 µg/ml, respectively. The results of all analyses allow us to conclude that the dry extract of guava leaves has promising activity to be applied topically in the oral cavity or in the development of antitumor formulation or even be used as a functional food.

Keywords

Guava, Antitumor, Medicinal Plants, Functional Foods

1. Introduction

The insertion of medicinal plants in disease treatment by the Brazilian population is rooted in regional traditions

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that are transmitted to future generations, by oral reports, family histories, rituals, or homemade preparations. In this sense, research on medicinal plants was performed based on the ethnopharmacological knowledge, as the proposal of this work, in which was evaluated the antioxidant and antibacterial action and antitumor properties of guava leaves.

Psidium guajava L. (Myrtaceae), native to tropical America is commonly known as guava or guajava or guava tree. It is a small tree, with globous or piriform fruits, yellow, 3 - 6 cm in diameter. Different parts of the plant are used in folk medicine in the treatment of diseases such as wounds, lesions, ulcers, diarrhea, cholera, hypertension, obesity and the control of *Diabetes mellitus* [1]. In India, the young leaves are used as antitussive. In China, the leaves are used as anti-inflammatory and hemostatic agent. The decoction of the leaves is used in the treatment of cholera, diarrhea and vomiting reduction [2] [3]. In Brazil, the leaves are popularly used in the treatment of gastrointestinal disorders, practice inherited originally, of the Aztec medicine of Mexico [4]. In leaves, the chemical compounds detected are tannins, flavonoids (myricetin, quercetin, luteolin and kaempferol), essential oils (caryophyllene, nerolidiol, β -bisabolene, aromadendrene, p-selinene, α -pinene and 1,8-cineol), triterpenoids (oleanic acid, ursolic acid, catecolic acid, guayavolic acid, maslinic acid, ellagic acid) and β -sitosterol [5] [6]. It has significant quantities of fatty acids and pectins [7].

Several studies have investigated the properties of natural products as antioxidants, antibacterial, anticariogenic, chemopreventive and antitumor. These are stimulated, mainly due the toxic effects produced by synthetic antioxidants used in industry [8], drug resistance, or the search for new substances that act against bacterial growth and biofilm formation [9]-[11]. In general, the antioxidants are characterized as substances present in low concentrations compared to oxidizable substrate, which significantly retard or inhibit oxidation of the substrate. Phenolic compounds have received attention, mainly by lipid peroxidation and lipoxygenase inhibition [12]. Terpenes and flavonoids are important antibacterial and anticariogenic agents. Dental caries is a common chronic disease which affects from 60% to 90% of young population. The formation of dental plaque, which plays an important role in the development of caries and periodontal disease in humans, can be initiated by several strains of oral streptococci [10] [13]. *Streptococcus mutans*, *Streptococcus mitis* and *Streptococcus oralis* are involved in the formation of dental caries. The process involves bacterial adhesion, biofilm formation, and tooth enamel demineralization by acids produced by microorganisms [14] [15]. Flavonols, mainly quercetin and kaempferol derivatives, present in guava, have been commonly found in natural products. Although their functions have been overall related with antioxidant properties, they also show an anticancer activity based in the induction of apoptosis and/or arrest of tumoral cells [16]. Although most flavonoids appear to be non toxic to humans and animals, they have been demonstrated to inhibit proliferation in many kinds of tumors cell lines. Quercetin has antiproliferative effects on squamous cell carcinoma HTB43, meningioma cells, colon cancer cells, Caco-2 and HT-29, with a dose-dependent effect. Flavonoids also have shown inhibitory effects on the growth of leukemia HL-60 cell, with an IC₅₀ value ranging from 10 to 940 ng/ml [17]. The study by Sato [18] found that the leaves of *Psidium guajava* may have anticancer activity, although it was based on limited studies. Development of new anticancer agents is vital because cancer has been the second leading cause of death in the world population. The cancer was responsible to 7.6 million deaths in 2005, will cause 9 million in 2015 and over 11 million deaths in 2030 [19].

The aim of the present study was to assess antioxidant, antibacterial and antitumor activity of ethanolic extract of the *Psidium guajava* leaves.

2. Materials and Methods

2.1. Reagents

Tannic acid, quercetin, lapachol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), butylhydroxyanisole (BHA), Folin-Ciocalteu reagent, vanillin, RPMI (Roswell Park Memorial Institute Medium), dimethyl sulfoxide (DMSO), chloramphenicol were purchased from Sigma-Aldrich (Germany/EUA). Ethanol, methanol, hydrochloric acid, Na₂CO₃, eugenol, 2,3,5-triphenyltetrazolium chloride were acquired from VETEC (Brazil). Brine shrimp *A. salina* was obtained from Artemia Salina RN[®] (Brazil). Artificial sea water was of Nature's Ocean (Brazil). Strains of streptococcus were provided by the laboratory of microorganisms reference the Instituto Nacional de Controle de Qualidade em Saúde (INCQS/FIOCRUZ), Brazil. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT or thiazolyl blue tetrazolium bromide) were used of the Invitrogen (EUA), fetal calf serum (FBS) of

Gibco (EUA). Other reagents used were analytical purity.

2.2. Extraction and *Artemia salina* Lethality Assay

Stems with leaves of guava in two distinct stages of maturation, young in early development and adult fully expanded were collected in July 2013 in Ouro Preto, Minas Gerais, Brazil. Voucher specimen was deposited in the Herbarium José Badini of Federal University of Ouro Preto, registered with number OUPR 27242.

The ethanolic extract was made according to the general methods of the Farmacopéia Brasileira V [20]. Successive re-extractions were made until the exhaustion of the plant drug. After the extraction, the filtrate was evaporated on a water bath of $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ of the solvent to dryness. The dry extract obtained (DEG) was weighted and the yield was calculated based on the initial fresh weight and the proportion of dry extract obtained (%). Standardization of organoleptic quality (color, odor and flavor) and physic-chemical analysis (pH and dry residue) were made to the extract [20].

The *Artemia salina* lethality assay procedure was adapted from Favilla [21] observed the experimental and environmental conditions. Brine shrimp eggs were obtained from a local aquarium store and hatched in glass tubes containing artificial sea water (38 g/l) in distilled water and incubated with artificial lighting (60 W lamp) at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 48 h. After this period, the newly hatched brine shrimps (nauplii) were separated and transferred to fresh sea water with a Pasteur pipette and immediately used for bioassays. Dry extract (DEG) at concentrations of 10, 100, 500 and 1000 $\mu\text{g/ml}$ were transferred into a glass tube, resuspended in 5 ml of sea water, homogenized and added to the 10 brine shrimps, previously separated. Lapachol standard was used in the same concentrations of extract as positive control. Untreated control (seawater + *Artemia*) was analyzed together with the concentrations of DEG and Lapachol. The tubes were kept under constant irradiance and average temperature of $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$, by 24 h. All tests were conducted in four replicates for all treatments (DEG, lapachol and untreated). The mortality endpoint for this bioassay was defined as the absence of forward motion of shrimps. The Probit analysis method was used for the calculate LD_{50} according to the Farmacopéia Brasileira V [20].

2.3. Phenolic, Flavonols Content and Antioxidant Activity

The total phenolic content of DEG was determined according to the Folin-Ciocalteu method, adapted of Singh, Chidambara-Murthy & Jayaprakasha [22]. Four replicates of 1 mg of DEG were mixed with 2.0 ml of 10-fold-diluted Folin-Ciocalteu reagent and 1.6 ml 7.5% sodium carbonate (Na_2CO_3) solution. Blank was prepared by adding 2.0 ml of 10-fold-diluted Folin-Ciocalteu reagent and 1.6 ml 7.5% sodium carbonate (Na_2CO_3) solution. The absorbance at 765 nm was measured after 30 minutes at room temperature (21°C). The total phenolic content was calculated according to the standard curve prepared with tannic acid and the results were expressed as milligrammes of tannic acid equivalents (TAE) per gramme of solid extract.

Total flavonols contents of DEG extract was determined by the procedure adapted of Jayaprakasha, Singh & Sakariah [23]. The amount of total flavonols was assayed by the vanillin method (colorimetry). Four replicates of 40 mg of DEG were added to 2.5 ml of 4% HCl in ethanol and 2.5 ml of 1% vanillin in ethanol. Blank was prepared by adding 2.5 ml of 4% HCl in ethanol and 2.5 ml of 1% vanillin in ethanol. The absorbance at 500 nm was measured after 20 minutes in the dark at room temperature (21°C). The total flavonols content was calculated by a standard curve prepared with quercetin and the results were expressed as milligrammes of quercetin equivalents (QCT) per gramme of solid extract.

The potential antioxidant activity of the extract was assessed on the basis of the scavenging activity of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical. Four replicates of 40 mg of DEG was added to 2 ml of methanol and shaken vigorously, 1 ml of 0.1 mM methanolic solution DPPH was added to these tubes. BHA (Butylhydroxyanisole) positive control was prepared under the same conditions described for the extract. The negative control was prepared using 0.1 mM methanolic solution of DPPH. Methanol was used for the baseline correction. Absorbance was measured at 520 nm, at room temperature (23°C), in times of 0 and 30 minutes. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: $\text{AAT}\% = (\text{Abs control} - (\text{Abs sample}/\text{Abs control})) \times 100$ [22].

2.4. Antibacterial and Antitumor Activity

The microorganisms used were lyophilized strains of *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis*

(ATCC 49456) and *Streptococcus oralis* (ATCC 10557), assigned by the laboratory of microorganisms reference of the Instituto Nacional de Controle de Qualidade em Saúde (INCQS) belonging to Fundação Osvaldo Cruz (FIOCRUZ), Brazil. Microorganisms were grown on blood agar at 37°C for 24 h. The obtained cell mass was collected aseptically with sterile loop and was suspended in tubes containing 10 ml 0.85% sterile saline solution. The inoculum was prepared with approximately 10^6 colony forming units per ml (CFU/ml), equivalent to grade 0.5 McFarland and was used to evaluate the antibacterial activity by disc diffusion and minimum inhibitory concentration (MIC).

Evaluation of antibacterial activity was performed by disk diffusion method according to the Clinical and Laboratory Standart Institute protocol [24]. The inoculum was seeded in petri dishes with Mueller-Hinton agar and later the paper disks impregnated with 10 µl of the extract DEG (50 mg/ml) were applied. The negative control used was ethanol PA and the positive control was chloramphenicol (30 µg). The plates were incubated at 37°C for 24 h proceeded to read (mm) of inhibition zones of bacterial growth. The tests were performed in triplicate.

The minimum inhibitory concentration (MIC) was determined according to the Clinical and Laboratory Standart Institute protocols [25]. This test was performed in sterile 96-well microplates. In each well was added 100 mL of inoculum standardized in 0.5 McFarland and 100 µl of DEG has been previously solubilized in DMSO and diluted in Mueller-Hinton broth. DEG was tested in serial dilution from 1000 µg/ml. Positive control used was Eugenol. One well with specific medium and microorganism was used as control of the growth, and one well was inoculated with the broth only to check the sterility of the media and the aseptic work conditions. The microplates were prepared in triplicate and incubated anaerobically at 37°C for 24 h. After incubation, bacterial growth was evaluated adding in each holes 20 µl 0.5% TTC (2,3,5-triphenyltetrazolium chloride) aqueous solution. The microplates were incubated again for another 3 hours at 37°C. After this final incubation, the presence of red color in the holes indicated bacterial growth. The MIC was defined as the lowest concentration of the extract (µg/ml) able to prevent bacterial growth.

The principle of this method consists of the absorption of salt MTT by the cells that is reduced within the mitochondria to formazan product. This product accumulated inside the cell, is extracted through the addition of solvent DMSO [26]. Evaluation of antitumor activity was done with human cell lines HeLa (cervical carcinoma ATCC CCL2), RKO-AS45-1 (colorectal carcinoma ATCC CRL-2579) and Wi-26VA4 (lung fibroblast ATCC CCL-95.1). The 96-well microplates was plated with 1×10^6 µl cells, homogenized in RPMI 1640 medium supplemented with fetal bovine serum (FBS) and penicillin-streptomycin antibiotics. After cell plating, microplates were incubated at least 24 h at 37°C, 5% CO₂, followed by the addition of DEG and quercetin solubilized in DMSO 1% (v/v). Negative controls were cells without treatment. Eight serial dilutions (1:10) from stock solution (10 mg/ml) using RPMI supplemented with 1% FBS were used. Cell viability was evaluated after incubation for 48 h, by removing and discarding the medium and adding 100 µl MTT 5%, followed by in additional incubation for 3 h. The supernatant was removed and discarded and the insoluble formazan product was dissolved in DMSO. Then, optical density (OD) of 96-well was measured using spectrophotometer microplate reader at 550 nm. The OD of formazan formed in untreated control cells was taken as 100% of viability. All assays were performed in triplicate [27].

2.5. Statistical Analysis

SAEG (7.0 Viçosa: Fundação Arthur Bernardes, UFV/DBG, 2013) software was used to perform analyses and standard curves. Mean results of brine shrimp mortality were plotted against the logarithms of concentrations using Probit Analysis which median lethal concentrations (LD₅₀) at 95% confidence intervals [20]. The IC₅₀ curves were elaborated using the program OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. Extraction and *Artemia salina* Lethality Assay

The procedure to obtain the ethanol extract of fresh leaves guava by re-maceration had the yield of 36.91% DEG. This percentage can be considered high when correlated to the water content found in young leaves ($\mu = 59.87\% \pm 1.84\%$) and mature leaves ($\mu = 59.52\% \pm 2.29\%$) and also when compared to yield of guava leaves extract, dried in forced ventilation oven ($\mu = 20.01\% \pm 0.12\%$) [28]. In the standardization of organoleptic and physi-

co-chemical quality DEG remained dark green color, woody odor, extremely astringent taste, pH = 6 and 2.23% desiccation residue (dry residue).

By means of probit analysis, toxicity values were calculated for leaves of *P. guajava* using *Artemia salina* test. The LD₅₀ for leaf extract was 185.15 µg/ml and lapachol was 22.74 µg/ml. Lapachol is natural product with known anticancer properties, anti-inflammatory, analgesic, antibiotic, antimalarial, anti-trypanosome, antiulcer and regarded as relatively high toxicity [29]. The assay is considered a useful tool for preliminary assessment of toxicity [30]. In toxicity evaluation of plant extracts by brine shrimp bioassay, an LD₅₀ value lower than 1000 µg/ml is considered bioactive [31]. In this study the extract had LD₅₀ value < 1000 µg/ml, therefore, the can have biological activity. The results, compared with literature data, demonstrated safety in the use of ethanol extract of guava leaves.

3.2. Phenolic, Flavonols Content and Antioxidant Activity

The content of phenolic compounds in ethanol extract of guava was determined relative to the standard curve of tannic acid ($\hat{y} = 0.0342x - 0.156$; $r_2 = 0.9987$). The total amount of phenolic compounds was 766.08 ± 14.52 mg/g. It's important to remember that guavas tree have high levels of tannins, responsible for the astringent character of its fruit and leaves. Qian e Nihorimbere [32] evaluated phenolic content of aqueous and ethanol extract of guava leaves, and achieved the levels of 575.3 ± 15.5 mg/g ethanol extract and 511.6 ± 6 mg/g aqueous extract by gram of dry extract, amounts considered by the authors as too high.

In the determination of flavonols, quercetin was used as standard ($\hat{y} = 0.0039x - 0.0024$; $r_2 = 0.9998$). The content of flavonols was 118.90 ± 5.47 mg/g. According Tasca [33] the percentage of flavonoids in fruits of guava cv. Paluma represent about 13% (125.22 mg to 187.41 mg rutin/100g) and total phenolic content was 1047.55 mg/g to 1462.86 mg/g gallic acid, which allows us to infer that there are high levels flavonols and phenolic compounds in the leaves of this study. The content of bioactive compounds varies depending on biotic and abiotic factors not have been observed structural changes in chemical composition, but changes in percentage of majority compounds. The time for collecting the leaves may have promoted these higher levels, because in mature leaves, the greatest concentrations of flavonoids were found in July, myricetin (208.44 mg/kg), quercetin (2883.08 mg/kg), luteolin (51.22 mg/kg) and kaempferol (97.25 mg/kg) [6].

Qian e Nihorimbere [32] assume that is the combination of high concentrations of tannins and flavonoids responsible for the antidiarrheal action. Was identified by HPLC, the extract of guava leaves, peaks that correspond to the derivatives of quercetin (Quercetin-3-O-rhamnoside, C₁₅H₁₀O₇) which acts pharmacologically as a potential antioxidant agent. Quercetin also has anti-inflammatory, antiviral and antitumor actions in addition to inhibiting aldose reductase [1].

The potential antioxidant activity of the ethanolic extract of *P. guajava* showed a high effective free radical scavenging in the DPPH assay. This method is based on the capture of DPPH by antioxidants producing a decrease in absorbance. The increased consumption of DPPH by a sample is proportional to increased antioxidant activity [8]. The average antioxidant activity of the ethanol extract of guava leaves was $68.15\% \pm 5.34\%$ (time zero minute) and $87.65\% \pm 6.30\%$ (time 30 minutes) compared to BHA (antioxidant reference) ($\hat{y} = 0.6502x + 68,148$, $r_2 = 1$). These levels are considered high for leaves and can be correlated with the levels of flavonols and phenolic compounds. These high values, allows us to infer that the ethanol extract of leaves of guava (*Psidium guajava* L.) may have thermogenic potential or can be used as functional food. After pharmacological studies, guava extract may play a key role in preventing cardiovascular disease, cancer and degenerative diseases, due to the high antioxidant potential. Is important to remember that natural antioxidants occur in all parts of plants. These antioxidants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Several studies have revealed that the phenolic content in the plants are associated with their antioxidant activities, probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [34].

3.3. Antibacterial and Antitumor Activity

The ethanol extract of guava leaves was effective in inhibiting the growth of *S. mitis*, *S. mutans* and *S. oralis* at 50 mg/ml. The diameter of the inhibitory halo was greater than 8.2 mm (Table 1). In the evaluation of antibacterial activity by disc diffusion, the plant extract is considered active when there is formation of inhibitory halo greater than or equal to 7.0 mm [35]. The MIC value for the positive control (eugenol) was 250 µg/ml for *S.*

mutans, and 500 µg/ml for *S. mitis* and *S. oralis*. The MIC value for the extract of guava was 250 µg/ml for *S. mutans*, *S. mitis* and *S. oralis* (Table 1), proving the effectiveness of the extract used, which was higher (2×) or similar the eugenol, natural product, renowned as antiseptic, applied topically in the oral cavity and used as component in toothpastes and dental protective.

In another study, the treatment of the early plaque settlers with 1 mg/ml aqueous extract leaf of *Psidium guajava* reduced the cell-surface hydrophobicity of *Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces* sp., by 54.1%, 49.9% and 40.6%, respectively [36]. These results provide some scientific rationale for its use in the treatment of dental diseases and suggested that guava leaf extracts may inhibit the caries-inducing properties of *Streptococcus* and thus may be beneficial for the dental care [6].

The use of medicinal plants and functional foods in chemoprevention seeks to prevent, reduce the incidence or assist in controlling the proliferation of cancer cells. The antitumor activities have been reported in ethnopharmacological studies as one of the numerous nutritional benefits of consuming the fruits and juice of *P. guajava* [37]. The effect of the extract *P. guajava* and quercetin on the growth of cervical carcinoma (HeLa), colorectal carcinoma (RKO-AS45-1) and lung fibroblast (Wi-26VA4) cell lines investigated by MTT assay are shown in Table 2.

According to the National Cancer Institute (USA), crude extracts are cytotoxic when their IC₅₀ values are less than 30 µg/ml [38]. The extract of *P. guajava* showed significant antitumor activity on HeLa and RKO-AS45-1. However, Wi-26VA4 cells were the least affected by the cytotoxic effects of the extracts (Table 2). These could be because the extract were not cytotoxic to the lung fibroblast but affected cervical carcinoma and colorectal carcinoma cell.

The use of quercetin was based on the studies as Seufi [39] on the preventive effect of quercetin on hepatocellular carcinomas *in vivo* by RAPD-PCR methodology, where quercetin exerted preventive effect via reduction of oxidative stress and antioxidant activity. Phenolic compounds are potential chemopreventive agents especially flavonoids present in leaves of guava. Flavonoids can act through their ability to inhibit various PKs (protein kinase) inhibiting events in the signal transduction of cell proliferation by stopping the cell cycle via CDKs (cyclin-dependent kinases) by modulating the activity of MAPKs (mitogen-activated PKs) with possible anti-neurodegenerative and immoestimulante actions. Flavonoids have been found to arrest cell-cycle progression at either G1/S or G2/M boundaries. However, the molecular mechanisms involved in cell-cycle arrest by flavonoids remain largely unclear, although the modulation of multiple cell-cycle regulatory proteins seems to be involved. Flavonoids may exert antioxidant activity and anti-inflammatory action through direct inhibition of metalloenzymes pro-oxidants (LOXs: lipoxygenases; COXs: cyclooxygenases; and XO: xanthine oxidase) and inhibition of ornithine decarboxylase (induced by tumor promoters) [17] [40].

Table 1. Antibacterial activity (50 mg/ml) and minimum inhibitory concentration (MIC) of extracts of *Psidium guajava* L. and eugenol.

Strain	Guava extract		Eugenol
	Inhibition zone (mm)	MIC (µg/ml)	MIC (µg/ml)
<i>Streptococcus mutans</i> (ATCC 25175)	8.6 ± 0.57	250	250
<i>Streptococcus mitis</i> (ATCC 49456)	8.2 ± 0.28	250	500
<i>Streptococcus oralis</i> (ATCC 10557)	9.3 ± 0.76	250	500

Table 2. Antitumor activity of alcoholic extract of leaves *P. guajava* on three human cell lines.

Cell line	<i>Psidium guajava</i> L.	Quercetin
HeLa	15.6 ± 0.8	160.1 ± 0.7
RKO-AS45-1	21.2 ± 1.1	139.4 ± 1.2
Wi-26VA4	68.9 ± 1.5	92.3 ± 0.5

The data indicate the mean of concentration producing 50% growth inhibition (IC₅₀) µg/ml ± SD.

4. Conclusion

The results of all analyzes allow us to conclude that the dry extract of guava leaves has promising activity for use in the oral cavity or in the development of antitumor formulation or even be used as a functional food.

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