Antioxidant Capacity and Antimicrobial Activity of Commercial Samples of Guava Leaves (Psidium guajava)

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ABSTRACT

***Psidium guajava* is a small tree native to South and Central America. Guava leaves have traditionally been used for treating different illnesses. These benefits can be attributed to phenolics and flavonoids produced by guava. The chemical composition of guava leaf extracts was correlated with biological activity. Total phenolics, total flavonoids, ABTS/DPPH, TZM-bl, plaque reduction, XTT, spectrophotometric and Kirby-Bauer assays were used to test phenols, flavonoids, antioxidant properties, antiviral activity, cytotoxicity, and antibacterial activity, respectively. The median cytotoxicity concentration and half-maximal effective concentration values were obtained in order to determine antiviral selectivity against human immunodeficiency virus type 1 and herpes simplex virus type 1. Antibacterial activity against *Escherichia coli* and *Bacillus subtilis* were evaluated using a spectrophotometric assay and Kirby-Bauer test. The guava leaf extracts had a high phenol (0.8 to 2.1 GAE mg/mL) and flavonoid (62.7 to 182.1 Rutin Eq mg/g DW) content that correlated with high antioxidant capacity and selective antiviral activity (therapeutic index values above 10). Results of antibacterial tests indicated that the extracts have activity against gram-negative and gram-positive bacteria.**

INTRODUCTION

Guava (*Psidium guajava*) is a small tree native to South and Central America that belongs to the family Myrtaceae. Guava can reach up to 10 m high and has thin, smooth patchy bark. The leaves are oval, between 5 and 15 cm long, the flowers are white, and fruits are fleshy and oval up to 5 cm in diameter (Kafle et al., 2008). Guava is an important food crop and medicinal plant in tropical and subtropical countries and is widely used as food and as folk medicine around the world. The leaves and bark of guava have a long history of uses in different countries (Kafle et al., 2008). Today, guava leaves are still used as laxatives, as remedies for cold and cough, and to treat diarrhea, dysentery, wounds, vomiting, gastrointestinal problems, and diabetes (Bagri et al., 2016; Gutierrez et al., 2008; Kafle et al., 2008; Wu et al., 2009). Guava leaves have exhibited beneficial effects as antioxidants (Chen and Yen, 2007; Jeong et al., 2012), antibacterial agents (Biswas et al., 2013), and as antidiabetic agents (Deguchi and Miyazaki, 2010).

Medicinal plants have been used to treat human diseases since ancient times. The health benefits of these plants are in part accompanied by low toxicity due to the potential effects of their antioxidants on reactive oxygen species in the body. In an atmosphere containing oxygen it is not surprising that aerobic organisms constantly produce a wide range of reactive oxygen species (ROS) both for useful purposes and by accidents of chemistry

(Aruoma, 1998; Seifried et al., 2007). Many physiological and biochemical processes in the human body produce oxygen free radicals and other reactive oxygen species as byproducts. Overproduction of ROS can cause oxidative damage of biomolecules leading eventually to many chronic degenerative diseases and aging (Aruoma, 1998; Betteridge, 2000; Finkel and Holbrook, 2000; Halliwell, 1994, 2012).

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Halliwell, 1994, 1996). There is considerable interest in finding antioxidants derived from plants not only for maintaining and improving human health but also for prolonging the shelf-life and maintaining quality of lipid-containing foods (Halliwell, 1996). Plants produce a wide range of natural antioxidants including phenolic compounds (flavonoids and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids, and ascorbic acid (Larson, 1988). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Paganga et al., 1999). Phenolic compounds are widely distributed in plants and provide ecological advantages such as resistance to insects and defense against other herbivores, fungi, and nematodes (Bennet and Wallgrove, 1994). Numerous protective functions have been associated with diets rich in phenolic compounds, highlighting the importance of these compounds in human health and disease. Studies indicate that long term consumption of diets rich in plant polyphenols offer protection against development of various cancers, cardiovascular diseases, diabetes, osteoporosis, and neurodegenerative diseases (Pandey and Rizvi, 2009; Shahidi and Ambigaipalan, 2015).

Plant products have also been widely studied as sources of potential antimicrobial agents (Besednova et al., 2019; Chandra et al., 2019; Cowan, 1999; Fernandez Romero et al., 2003; Leila et al., 2019). Due to the rapid rise in antimicrobial resistance, there is an urgency to renew the arsenal of antimicrobial agents to fight viral and bacterial infections. The growing concerns about resistance against conventional antimicrobial agents make the study of potential new antimicrobial agents a priority. The first step is to identify antiviral and antimicrobial activities of potential compounds using cell-based assays. In antiviral assays, as viruses are intracellular obligated parasites, they require a host cell to replicate. Therefore, such assays identify selective antiviral molecules that inhibit an invading virus without affecting the host cell viability.

Little is known regarding the health benefits of commercially available guava leaves. The aim of this study was to (1) determine the total phenolic and flavonoid content in five commercial samples of guava leaves; and (2) to assess the antioxidant capacity and potential antibacterial and antiviral activity of their extracts.

MATERIALS AND METHODS

Biological materials: Five newly purchased dry commercial samples of guava leaves were used in this study: (1) Tai Chi (Korea) (from Tai Chi Inc; purchased through Amazon.com), (2) Guava Leaves Tea (Korea) (from ABC Tea House; purchased through Amazon.com), (3) Sipacupa (Jamaica) (purchased from Perishables Jamaica LTD), (4) Royal King (China) (from Royal King; purchased through Amazon.com), and (5) Omura Organic Guava leaves (Puerto Rico) (from Omura Products; purchased through Amazon.com). Vero cells (ATCC, Rockville, MD) and TZM-bl cells (NIH AIDS Research and Reference Reagent Program, Germantown, MD) were grown in Dulbecco modified Eagle medium (DMEM; Thermofisher Scientific, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermofisher Scientific) and with 50U/mL of penicillin and 50µg/mL streptomycin (Thermofisher Scientific). The HIV-1_{MN} and HIV-1_{ADA-M} laboratory strain stocks were provided by Dr. Jeffrey D. Lifson of Leidos Biomedical Research, Inc. HSV-1 strain F (ATCC) was propagated in Vero cells and the viral stock titer was determined as previously described (Fernandez-Romero et al., 2012). *Escherichia coli*

HB101 K12 was obtained from Bio-Rad Laboratories (Hercules, CA). *Bacillus subtilis* (ATCC strain 19659) was obtained from Microbiologics (St. Cloud, MN). Bacterial cultures were maintained on Fisherbrand™ petri dishes (Thermofisher Scientific) by streaking to single colonies on nutrient agar (BD Difco™, Carolina Biological Supply Co., Burlington, NC) prepared according to the manufacturer. Bacterial streaks were incubated overnight at 37 °C and stored at 4 °C for up to two weeks before passaging single colonies onto freshly made nutrient agar plates. All procedures using bacteria conformed to standard sterile technique.

Guava extracts preparation: Commercial leaf samples were ground 30 seconds in a commercial coffee grinder. Twenty mg of powdered material were dissolved in 1 mL of i) Dimethyl Sulfoxide (DMSO, Sigma Aldrich, St Louis, MO), ii) 60% Methanol (Sigma Aldrich), iii) 90-50% Ethanol (Sigma Aldrich), or iv) distilled H_2O. The samples were sonicated for 5 seconds at 30% intensity using a Bransonic® Ultrasonic bath. The plant extracts were further filtered through a 0.45 µm syringe filter (Thermo Fisher Scientific, Waltham, MA) and stored at 4°C. Alternatively, one gram of powdered material was dissolved in 10 mL of 60% Ethanol/Methanol and sonicated 6 times at 10 second intervals. The extracts were centrifuged 5 minutes at 10,000 x g and 25 °C using a Sorvall Biofuge Stratos centrifuge with a Heraeus #3057 angle rotor. The extracts were filtered with a 0.45 µm syringe filter and the solvent was evaporated at 30°C. The samples were then resuspended in 1 mL of 20% DMSO. The extracts were stored at -20°C.

Total phenolic content: Total phenolic content was determined using a modified Folin-Ciocalteu's protocol (Gao et al., 2000). Absorbance was measured at 760 nm on a Biotek Synergy 4 Spectrophotometer (BioTek Instruments, Winooski, VT) using gallic acid as a standard. Total phenolic content (TPC) was expressed as mg gallic acid equivalents/mL plant extract (GAE mg/mL).

Total flavonoids content: Total flavonoids were extracted with 0.5M $NaNO_2$ (Sigma Aldrich), 0.3M $AlCl_3 \cdot 6H_2O$ (Sigma Aldrich), and 1M NaOH (Sigma Aldrich) then measured at 506 nm according to Saeed et al. (2012). The standard curve for total flavonoids was made using rutin standard solution. Results were expressed as mg of rutin equivalents/mL plant extract (RAE mg/mL).

Trolox equivalent antioxidant capacity (TEAC) assay: The TEAC assay was carried out according to Re et al., (1999) to determine the free radical scavenging capacity using the $ABTS^{\bullet+}$ radical cation. The $ABTS^{\bullet+}$ radical cation was produced by mixing a stock solution of ABTS (7 mM) and potassium persulfate (2.4 mM) at room temperature and incubating in the dark for 12-16 h before use. The cation radical solution was diluted until an absorbance at 734 nm reached 0.7± 0.05. Results are expressed as mg Trolox equivalents/mL plant extract (TEAC mg/mL).

2,2- Diphenyl-1-picrylhydrazyl (DPPH) assay: Radical scavenging activity of plant extracts against stable $DPPH^{\bullet}$ (2,2-Diphenyl-1-picrylhydrazyl hydrate) was determined spectrophotometrically (Brand-Williams et al., 1995). When $DPPH^{\bullet}$ reacts with an antioxidant compound, it is reduced. The changes in color were measured using a spectrophotometer at 517 nm. The results were expressed in percentage (%) of DPPH scavenging activity. The correlation between antioxidant capacity and total phenolic content was analyzed using a simple linear regression, and the correlation coefficient (R^2) was calculated.

High-performance liquid chromatography (HPLC): Ten mg of both gallic and ferulic acids were dissolved in methanol to prepare the stock standard solution. Five mL of the stock standard solution was transferred into a 25 mL amber flask and was brought to volume with diluent to obtain the working standard solution (20µg/mL) for both compounds. One hundred and fifty µL of each guava extracts were dried using compressed air and then reconstituted in 150 µL of 50% methanol. The samples were injected into a Phenomenex Prodigy™ 5 µm ODS-3 4.6 x 250 mm S/N H19-316755 column. The mobile phase consisted of 0.1% Trifluoroacetic acid in water (mobile phase A) and methanol (mobile phase B). The detector wavelength

was 280nm, the flow rate was 1 mL/min, and the injection volume was 15 µL at 40°C.

Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS): Liquid chromatographic separation was achieved using a Waters Acquity UPLC system and a reverse-phase BEH C18 (1.7µm, 2.1x 50 mm) column. The mobile phase solvent consisted of (A) 0.2% formic acid in water and (B) methanol, and the flow rate was maintained at 0.3 mL/min. A gradient (mobile phase 10%B to 80%B) over 5 minutes was used to elute gallic acid and catechin. This elution rate was followed by a 2 minutes column wash-out and 3 minutes equilibration to initial conditions, for a total run time of 10 minutes per sample injection. Identification of all compounds was done using multiple reaction monitoring (MRM) for protonated parent →daughter ion transitions on Xevo TQ-s mass spectrometer (Waters Corp., Milford, MA). Gallic acid and catechin identification were performed in the negative and positive ion mode, respectively. The precursor and product ion pairs for MRM were m/z 168.96 to 124.96 (gallic acid) and m/z 290.92 to 138.99 and 290.92 to 122.99 transition (catechin). The source dependent parameters and analyzer parameters were optimized in order to achieve maximum sensitivity. MassLynx software version 4.2 was used to control all parameters of UPLC and MS.

Antiviral activity: The XTT, TZM-bl and plaque reduction assays were performed to test the potential selective antiviral activity. The XTT assay was used to determine the half maximal cytotoxic concentration (CC_{50}) of the plant extracts in TZM-bl and Vero cells. The TZM-bl assay was used to determine the half maximal effective concentration (EC_{50}) against two human immunodeficiency virus type 1 (HIV-1) strains, a CCR5 virus ($HIV\text{-}1_{ADA\text{-}M}$), and a CXCR4 virus ($HIV\text{-}1_{MN}$). Additionally, the plaque-reduction assay determined the EC_{50} against HSV-1 F. Briefly, different concentrations of each extract were applied in triplicate to TZM-bl (anti-HIV activity) or Vero cells (anti-HSV activity) seeded in 96-well microplates. The non-nucleoside reverse transcriptase inhibitor MIV-150 and the polysaccharide carrageenan were used as positive controls for antiviral activity against HIV-1 and HSV-1 respectively. HIV-1 strains (TZM-bl cells) or HSV-1 strain were added to each well at a multiplicity of infection of 0.001 (including virus controls but excluding cell controls). The cytotoxicity assay was performed under the same conditions but in the absence of virus. Tween 20 (Sigma Aldrich) was used as a positive control for cytotoxicity. The plates were incubated at 37ºC, 5% CO_2 and 98% humidity for 72 h. The plates to test anti-HIV activity were stained following the MAGI procedure as previously described (Begay et al., 2011) and the number of infected cells were estimated using the ImmunoSpot analyzer (Cellular Technology Limited, Cleveland, OH). The plates to test anti-HSV activity received three cycles of freezing (-80 ºC) and thawing (room temperature) to titter the virus released in the supernatant using the HSV plaque assay (Levendosky et al., 2015). The plates for cytotoxicity were stained with XTT (Thermofisher Scientific) as previously described. The CC_{50} and EC_{50} were calculated using a dose-response-inhibition analysis on GraphPad Prism v5.0c software. The therapeutic index (TI) was calculated using the CC_{50}/EC_{50} ratio (TI values above 10 indicate potential antiviral selectivity).

Preparation of media for bacterial cultures: For bacterial experiments, dehydrated BD Difco™ nutrient agar and BD Difco™ Luria-Bertani (LB) broth (Carolina Biological Supply Co.) were prepared per the manufacturer. A 2x concentration of LB broth was prepared for all liquid culture experiments.

Antibacterial activity using disc-diffusion: Antibacterial activity of guava extract against *E. coli* and *B. subtilis* was tested using the Kirby-Bauer disc-diffusion method (Biemer, 1973). To prepare *E. coli* and *B. subtilis* lawns, an individual colony from a stock nutrient agar plate was used to inoculate an overnight LB broth culture. Liquid cultures were incubated overnight at 37 °C and 150 rpm in MaxQ 4450 orbital shaker (Thermofisher Scientific). Freshly grown bacteria (100-200 µL) were plated onto nutrient agar and spread using a Bel-Art™ SP Scienceware bacterial cell spreader (Thermofisher Scientific). Discs were applied and lawns were

incubated at 37 °C overnight. Zones of inhibition were measured in mm. Antibiotic control discs (Carolina Biological Supply Co.) contained tetracycline (30 µg), chloramphenicol (30 µg), or streptomycin (10 µg). LB broth was used to dilute guava extract and DMSO for disc preparation. Discs were prepared by applying 20 µL of either guava extract or DMSO to a 6 mm GE Healthcare Whatman™ Antibiotic Assay Disc (Thermofisher Scientific). Discs were air dried prior to use.

Antibacterial activity in liquid culture: For each extract, LB/guava media was prepared by mixing equal volume of guava extract and 2x LB broth. LB/DMSO control media was prepared by mixing equal volume of 10% DMSO and 2x LB broth. LB control media was prepared by mixing equal volume of sterile distilled water and 2x LB broth. Fresh overnight LB broth cultures of *E. coli* and *B. subtilis* were prepared as described above. For each bacterium, 540 µL of media was inoculated with either 6 µL of overnight culture (for a density of 10^6 cells/mL) or with 6 µL of LB broth. Each 600 µL inoculum was divided into two wells of a Corning 96-well flat-bottom tissue culture plate (Thermofisher Scientific). Microplates were incubated at 37 °C and 150 rpm in a MaxQ 4450 orbital shaker (Thermofisher Scientific). Optical density (OD) was measured at 700 nm every hour for 8 hours using a GenTek Synergy 4 spectrophotometer. At each time point, there was 10 seconds of agitation before reading the OD.

Statistical Analyses: The Friedman test was used for overall comparison between solvents for the extraction of phenolic compounds and pairwise comparisons were performed using Dunns test ($p<0.05$). Correlation between total phenolics and antioxidant activity were calculated using GraphPad Prism Software, Inc.

RESULTS AND DISCUSSION

Total Phenolic content (TPC). The Folin-Ciocalteu assay is a common and simple method to determine phenolic content in the plant samples, relying on the transfer of electrons from phenolic compounds to the Folin-Ciocalteu reagent in alkaline media. Different solvents were assessed to extract total phenolic. Total phenolic content varied among the samples and the solvent of extraction used. The highest concentration of phenolics were extracted in all the samples with DMSO, 60% methanol, 60% ethanol (Figure 1). There was a significant difference ($p<0.0001$) between DMSO and with distilled water (dH_2O) or 90% ethanol. A significant difference was also observed ($p<0.05$) between 90% ethanol and 60% ethanol. These results suggest that increasing the water in the extraction solvent enhances the amount of total phenolics content. Thus, the following experiments were conducted with DMSO, 60% ethanol, and 60% methanol (Fig 2 A).

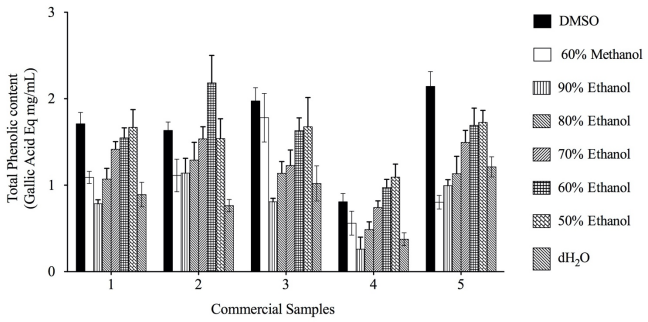

Figure 1. High variation in total phenolic content in guava commercial leaves extracts (1-Tai Chi, 2- Guava Leaves Tea, 3 Sipacupa, 4-Royal King, 5-Omura Organic Guava leaves) using eight different solvents. Bars indicate the mean value ± SE of three independent experiments.

Total flavonoid content (TFC). Flavonoids are a broad class of low molecular weight phenolics that can be found in guava leaves. High variation in total flavonoid content was observed among the samples. The highest content was observed in sample Sipacupa and the lowest in Royal King (Fig 2 B). There was a positive correlation between total phenolic content and total flavonoid content (R^2 =0.6813-0.8874). These results suggest that flavonoids are the major phenolics in these fresh samples of guava leaves.

Antioxidant capacity (Trolox equivalent antioxidant capacity -TEAC). Free radicals are a major cause of the propagation stage of the oxidation process. Several methods have been developed to assess the free radical scavenging capacity of plant extracts. The most common and reliable methods involve the spectrophotometric determination of the

disappearance of free radicals ABTS$^{\bullet+}$ and DPPH$^{\bullet}$. First, the radical scavenging capacities of plant extracts from all five different commercial guava leaves were estimated using the ABTS$^{\bullet+}$ radical. This radical was generated by reaction between ABTS and potassium persulfate, which has a blue/green color. The antioxidant capacity was measured as the ability of plant extracts to decrease the color reacting directly with the ABTS$^{\bullet+}$radical. The degree of color change is proportional to the concentration of antioxidants. Antioxidant capacity of the commercial guava leaves ranged from 0.35-0.6 TEAC (mg/mL) with Sipacupa (Jamaica) and Omura (Puerto Rico) being the highest (Fig 2C). Despite the different TEAC values and TP contents among the samples, a positive linear relationship was found between TPC and TEAC values that was highest in ethanolic extracts (R^2=0.7039), moderate in DMSO extracts (R^2= 0.538), and lowest in methanolic extracts (R^2=0.4751). These results indicate that the amount of antioxidant capacity of the plant extracts depends on the solvent used for extraction and that phenolics might be an important contributor to the antioxidant capacity. Strong antioxidant capacity of guava extracts was also reported previously from plants grown in Taiwan (Chen and Yen, 2007), India (Venkatachalam et al., 2012) and Korea (Jeong et al., 2012). Positive relationship between antioxidant capacity and total phenolic content has been reported in different plants (Cai et al., 2004; Velioglu et al., 1998; Zheng and Wang, 2001). The antioxidant capacity of these phenolic compounds is due to a variety of mechanisms, including free radical-scavenging (Rice-Evans et al., 1996; Shahidi and Ambigaipalan, 2015).

The relationship between antioxidant and flavonoid content was also evaluated. In methanolic extracts there was a strong relationship between TFC and TEAC (R^2=0.7978), suggesting that flavonoids are the main contributors to the ABTS free radical scavenging capacity. However, there was a weak relationship between TFC and TEAC in ethanolic extracts (R^2=0.1312) and in DMSO extracts (R^2= 0.5948), suggesting that flavonoids could not be main compounds responsible for the scavenging capacities and that other compounds might also contribute to the antioxidant capacity of the plant extracts.

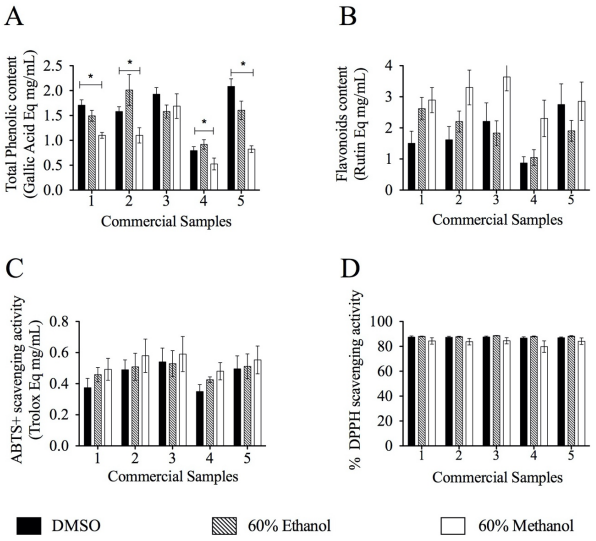

Figure 2. Variation in total phenolic content (A), flavonoids content (B), and antioxidant capacity (C and D) of guava commercial leaves extracts (20mg/mL) (1-Tai Chi, 2-Guava Leaves Tea, 3-Sipacupa, 4-Royal King, 5-Omura Organic Guava leaves). Bars indicate the mean value ± SE of three independent experiments.

In addition, the free radical scavenging capacity was evaluated using DPPH radical. DPPH radical is a stable organic free radical that has a deep purple color. The assay is based on measurement of the reducing ability of the antioxidant toward DPPH$^{\bullet}$. No significant differences in antioxidant capacity were observed among the samples or solvent extractions used in the DPPH assay (Fig 2 D). These data suggest that the ABTS assay might be more useful than the DPPH assay for detecting antioxidant capacity in a variety of foods (Floegel et al., 2011).

Comparison of the antioxidant capacity of extracts was also carried out at higher concentrations of plant extracts (1 g DW/mL). High variation in total phenolic and flavonoid content among the samples was observed (Fig 3A, B). Extracts from all the samples showed lower antioxidant capacity. There was poor correlation between TPC and TFC (R^2= 0.435), TPC and TEAC (R^2= 0.4298), and TFC and TEAC (R2= 0.1947). These results suggest that the ABTS scavenging capacity of the guava leaf extracts exhibited a concentration-dependent effect. At lower concentrations of plant extracts (20 mg DW/mL), the

concentration of phenolics extracted exhibited higher antioxidant capacity (Fig 2A, C). In contrast, at higher concentrations of plant extracts (1g DW/mL), higher concentrations of phenolics were extracted (Fig 3 A), but low antioxidant capacity was observed (Fig 3C). These data suggest that at higher concentrations, guava extracts behave as pro-oxidants. Phenolic compounds are known as strong antioxidants, thus when a phenolic molecule loses an electron or when it acts as a reducing agent, the molecule becomes a relatively stable radical while its oxidized intermediates can also now become pro-oxidants (Zhang and Tsao, 2016).

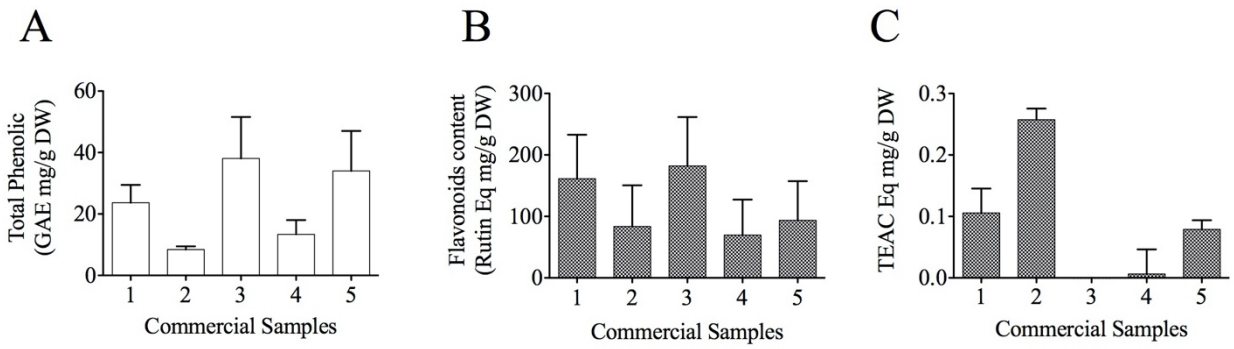

Figure 3. Variation in total phenolics content (A), total flavonoids content (B), and antioxidant capacity (C) of commercial guava leaves extracts (1g/mL) (1-Tai Chi, 2-Guava Leaves Tea, 3-Sipacupa, 4-Royal King, 5-Omura Organic Guava leaves). Bars indicate the mean value ± SE of three independent experiments.

Guava extracts were analyzed by HPLC and gallic acid was detected in all samples. The PDA scan shows that gallic acid contains 2 lambda max at 215 and 271 nm whereas ferulic acid has 3 lambda max at 218, 235 and 325 nm. Gallic acid was the predominant compound and it was present in all samples since its retention time matched the one found in the standard, furthermore the PDA scan configuration for both, samples and standards were similar. There were multiple peaks found in each sample in the region where ferulic acid is in the standard run, however none match the lambda max configuration of ferulic acid. Thus, ferulic acid was not present in any sample. Furthermore, gallic acid presence was confirmed by LC-MS-MS and with this method catechin was also identified. The guava leaves have been reported to contain different phenolic compounds, including gallic acid (Che and Yen, 2007; Goncalves et al., 2008; Jaiarj et al., 1999; Jeong et al., 2012). It has been reported that plant phenols exhibit *in vitro* antioxidant activity by acting as chain-breaking peroxyl-radical scavengers or directly scavenging reactive oxygen species. The antioxidant activity of phenolic acids depends on the number and position of the hydroxyl groups bound to the aromatic ring (Rice-Evans, et al., 1996). Gallic acid (3,4,5-trihydroxybenzoic acid) with two adjacent hydroxyl groups that can bind to transitional metal ions such as iron and copper (Miguel, 2010). Thus, the antioxidant capacity and free radical scavenging ability of guava extracts can be attributed to the presence to gallic acid, but other compounds in the extracts might also contribute to the activity.

Antiviral activity. Selective antiviral activity was observed in all samples (Figure 4 and Table 1). TI values were above 10 in most of the samples and EC_{50} values were between 0.05 and 3 mg/mL for HIV-1 strains and below 0.2 mg/mL for HSV-1. Interestingly, there was a more potent antiviral activity against the CXCR4 virus (HIV-1_{MN}) than the CCR5 virus (HIV-1_{ADA-M}). There are reports showing that some compounds like polyanions, have a higher affinity for the gp120 glycoprotein on the surface of HIV-1 CXCR4 phenotypes versus HIV-1 CCR5 phenotypes (Moulard et al., 2000). This may explain our results and further investigation should look into the presence of similar compounds in our extracts. Our results also showed a good correlation

between the antiviral activity and the high phenol and flavonoids content detected in each extract. This corroborate findings by others suggesting that phenols and flavonoids have a wide-spectrum antiviral activity against naked and envelop viruses (Besednova et al., 2019; Daglia, 2012; Leila et al., 2019). While the antiviral activity against HIV-1 may not translate into a feasible compound(s) to treat or prevent HIV infections, the activity against HSV-1 could prompt the further development of topical formulations to treat or prevent HSV oral infections.

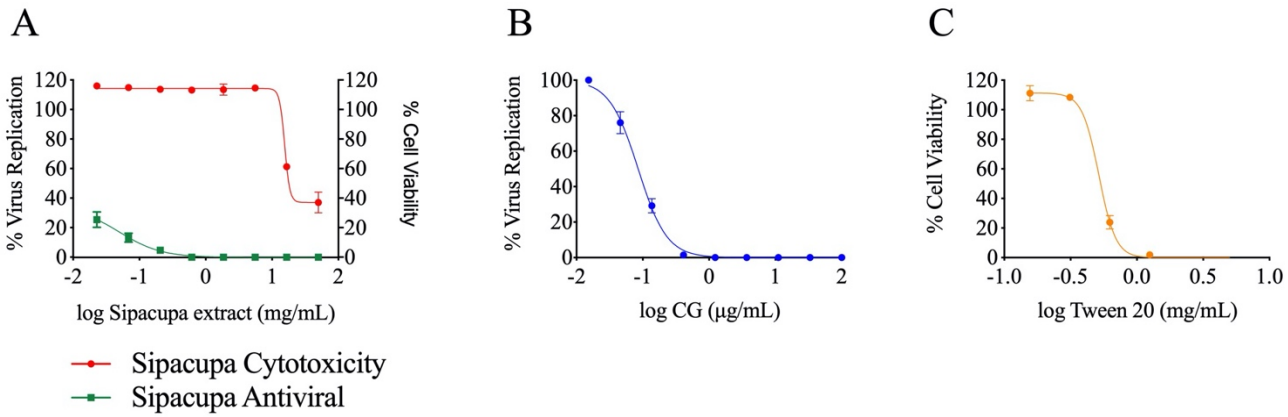

Figure 4. The Sipacupa guava leaf extract has a selective anti-HSV-1 activity. The cytotoxicity was estimated using the XTT assay in Vero cells; CC_{50}~15 mg/mL. The antiviral activity was tested using the plaque-reduction assay; EC_{50}<20 µg/mL and TI>750 A). Carrageenan control showed the expected selective antiviral activity with EC_{50}= 85.7 ng/mL (78 to 92.9) B). Tween 20 control showed the expected dose-response in the cytotoxicity experiment C). The graphs show the combined result of two independent experiments.

Table 1: Guava leaf extracts show selective antiviral activity against HIV-1.

Sample	**CC_{50} (mg/mL)**	**HIV-1_{ADA-M}**		**HIV-1_{MN}**	
		EC_{50} (mg/mL)	**TI***	**EC_{50} (mg/mL)**	**TI***
Tai Chi	10.1	0.34	29.5	0.07	148.5
Guava Leave Tea	10.3	0.56	18.3	0.08	125.6
Sipacupa	10.9	0.26	41.6	0.05	231.9
Royal King	23.4	2.96	7.9	1.24	18.9
Omura Organic	11.3	0.94	12.03	0.23	48.7

*TI>10 means selective antiviral activity; data from two independent experiments.

Antibacterial activity. The commercial guava extracts were tested for antibacterial activity during bacterial growth in liquid culture. *E. coli* and *B. subtilis* were independently grown in microplate wells in either the presence or absence of each guava leaf extract. Bacterial growth was monitored hourly by spectrophotometry for a total of eight hours. Figure 5 shows that at their highest concentrations, there was no detectable growth of either bacteria. Control experiments confirmed that DMSO present in the samples did not contribute to the inhibition. It was therefore concluded that one or more components in each guava extract was responsible for inhibition.

In a disc-diffusion assay using Tai Chi extract (#1), inhibition of bacterial growth was observed on *B. subtilis* lawns. Table 2 shows inhibition zones measured for four dilutions of this extract, with minor but visible zones seen with as little as 250 mg/mL (5 mg) of extract. Alternatively, there was no zone inhibition observed on *E. coli* lawns. Results of the disc-diffusion, however, is not consistent with the

growth kinetics assay in which inhibition was observed for both bacteria. Sipacupa extract (#3) was also tested with disc-diffusion, but did not exhibit inhibition of either bacteria even with concentrated extract (data not shown), whereas the extract completely inhibited both bacteria in liquid culture (Figure 6). The results imply that phytochemicals in guava leaves that are active against bacterial growth in culture behave differently in agar media. It should also be considered that whereas growth in culture was monitored for only eight hours, the extracts might lose activity with extended time of incubation in agar lawns.

Table 2: Tai Chi guava leaf extract resulted in a zone of inhibition on *B. subtilis* lawns. Zones of inhibition were measured in mm for different concentrations (125 mg/mL - 1 g/mL) of Tai Chi extract.

Plant Extract Concentration	Mean Diameter (mm) ± SE
1 g/mL	8.33 ± 0.11*
500 mg/mL	7.13 ± 0.48
250 mg/mL	7.42 ± 0.22
125 mg/mL	6.33 ± 0.17

* Results show the average of three independent trials ± standard error (SE).

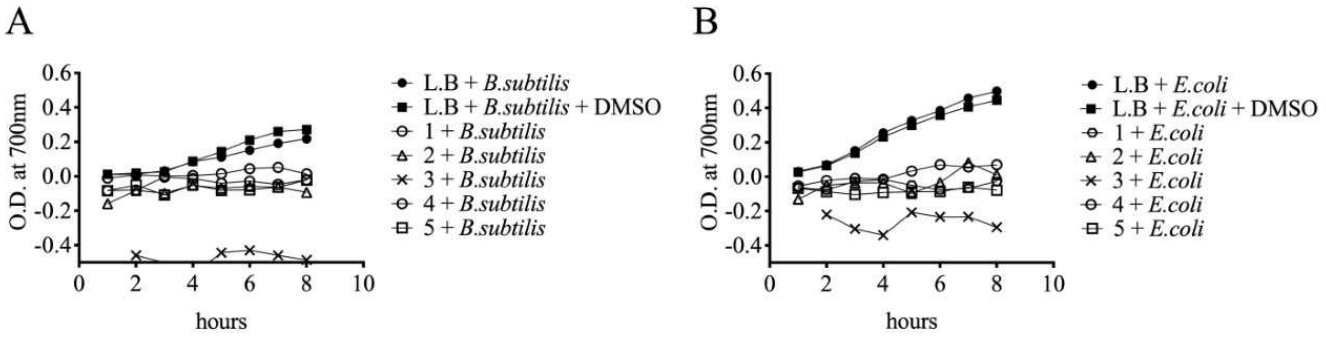

Figure 5. Commercial guava leaf extracts inhibited growth in culture of *E. coli* and *B. subtilis*. Bacteria were grown in 10 mL LB broth ± extract at 37°C and 150 rpm. OD readings were measured against appropriate blanks (LB; LB/10% DMSO; LB/extract). OD values ≤ zero indicate no growth. 1-Tai Chi, 2-Guava Leaves Tea, 3-Sipacupa, 4-Royal King, 5-Omura Organic Guava Leaves. Results show average of 2 readings per sample at each time point.

The disc diffusion results are consistent with those of Biswas et al. (2013), who previously reported that methanol and ethanol extracts of guava leaves using the well diffusion method showed inhibitory activity against gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus*, but no activity against gram-negative *E. coli* and *Salmonella enteritidis.* In contrast, and consistent with the liquid culture results shown here, there have been reports of guava leaf extract activity against *E. coli* and other gram-negative bacteria using well diffusion (Chandra and Kaneria, 2011; Vieira et al., 2001).

The antioxidant capacity, antimicrobial, and antiviral activity of guava extracts could be attributed to the accumulation of phenolic compounds in the leaves, mainly gallic acid. It has been reported that gallic acid has shown both antioxidant activity (Brewer, 2011; Miguel, 2010) and antimicrobial activity (Daglia, 2012). Furthermore, the synergistic effect of polyphenols in combination with conventional antimicrobial agents against clinical multidrug-resistant microorganisms has been proposed (Daglia, 2012; Nascimiento et al., 2000). Guava extracts at lower concentrations exhibited high antioxidant capacity with high total phenol and flavonoid content. Guava extracts at higher concentrations showed prooxidant action with high total phenol and flavonoid content, and strong antiviral and antibacterial activity. Variable results were observed among the different commercial samples in terms of phenol content, antioxidant

properties, as well as antimicrobial activity. These variable results could be attributed to variable raw material composition as well as different ecological conditions in the areas where the guava leaves were collected and/or ways in which they were harvested, dried and stored. The biological activities of these guava extracts could help to understand the traditional uses of guava leaves; however further evaluation of guava leaf extracts should be performed to validate all their uses in traditional medicine. Additional preclinical studies should be performed to evaluate the potential of these natural extracts as antimicrobial and/or antioxidant agents.

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