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BIOLOGICAL SCIENCES

In vitro anthelmintic activity of *Psidium guajava* hydroalcoholic extract against gastro-intestinal sheep nematodes

LUCIANA P. SILVA, RAFAEL R. DEBIAGE, JOÃO L. BRONZEL-JÚNIOR, REGILDO M.G. DA SILVA & ERIKA C.T. MELLO-PEIXOTO

Abstract: Tanniferous plants have been used for ruminants verminosis control and represent a possibility to minimize the pharmacological resistance against conventional antiparasitics. This study aimed to evaluate the antihelminthic activity of the hydroalcoholic extract of stem bark of guava tree (PgHA). It was performed the hatchability and larval migration inhibition assays to evaluate PgHA at the following concentrations 0.62, 1.25, 2.5 and 5.0 mg mL^{-1} and the control treatments. The total polyphenol, flavonoid and tannin contents were determined by phytochemical analysis, high performance liquid chromatography coupled to mass spectrometry. The antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl, ferric reducing antioxidant power and thiobarbituric acid reactive substances tests. It was also determinated total protein, intracellular H_2O_2 and antioxidant activity of enzimes: glutathione S-transferase and superoxide dismutase. PgHA was able to inhibit both hatchability and larval migration, but only hatchability inhibition presented dose-dependent pattern. The antioxidant activity was demonstrated by linear regression with IC_{50} corresponding to 534.02 µg mL^{-1}. The antiparasitic mechanism occurred through pro-oxidative activity by the increase of total proteins, intracellular H_2O_2 and the lipid peroxidation products, as well as the increase of the enzymes above related. Thus, the PgHA showed antiparasitic activity *in vitro*.

Key words: Gastrointestinal nematodes, Guava tree, medicinal plants, tannins, parasite.

INTRODUCTION

Gastrointestinal nematodes are one of the main economic barriers of sheep farming and cause losses, such as increased mortality rate and decreased weight gain, carcass yield, and production of meat, milk and wool (Roeber et al. 2013, Charlier et al. 2014). The parasitic cycle of nematodes in sheep exhibits phases that develop in the host and environment. The exogenous phase lasts from seven to 10 days from the shedding of eggs in the feces until infective larvae release into the environment. The larval stages of gastrointestinal nematodes of ruminants are highly resistant to environmental conditions, and it is extremely important to perform different parasitic control strategies to obtain effectiveness in host and environmental parasitic control (Charlier 2015, Jack et al. 2017). The control of these parasites is often performed using synthetic antiparasitics. However, the use of multiple doses of synthetic anthelmintics as well as drug underdosing favor the development of a global spread of parasite resistant has favored an increase in drug resistance, which exerts an important negative effect on the animal's natural immunity (Mcrae et al. 2015, Traversa & Samson-Himmelstjerna 2016, Keegan

et al. 2017). This resistance is observed worldwide, and the increasing use of antiparasitics opens the possibility of drug residue occurrence in the environment and animal origin products (Beynon 2012, Modi et al. 2013).

Antiparasitic management in sheep tends to restrict the use of synthetic drugs to reduce their harmful environmental effects and certify the quality of animal products (Papadopoulos et al. 2013). This tendency is directly reflected in studies that show benefits and improvements in animal management practices since the compromising use of synthetic parasiticides, even momentarily, on the certification of organic, biological and biodynamic production (European Council 2007). In view of this situation, the search for natural products promotes the discovery of new active compounds for parasitic control in sheep. These natural treatments may also result in less animal exposure to synthetic drugs, which could reduce the selection pressure on parasite resistance (Cornélius et al. 2016) and minimize environmental and food contamination.

The anthelmintic activity of some tanniferous plants are highlighted because condensed tannins are promising compounds in the control of gastrointestinal nematodes (Suleiman et al. 2014, Yoshihara et al. 2014, Raza et al. 2016). These compounds act directly on the nematodes via formation of complexes with proteins of the helminths, causing damage to the digestive tract, reproductive system and structures such as the cuticle and potentially leading to parasite death (Hoste et al. 2012). These compounds may indirectly contribute to the protein availability for absorption in the small intestine of the animal and provide the best immune response of the host and optimize the antioxidant response to the oxidative stress generated by the parasitic infection (Hoste et al. 2012, Jin et al. 2012, Lopes et al. 2016). Phenolic compounds of natural origin, such as flavonoids and tannins, also play an important role in the control of helminths (Oliveira et al. 2017).

In this context, *Psidium guajava* (L.) (Myrtaceae), known as "guava", is widespread in tropical and subtropical regions of the world, and it is highlighted for its anthelmintic potential, primarily due to its phenolic constituents (Azando et al. 2011, Klongsiriwet et al. 2015, Oliveira et al. 2017). Previous studies also demonstrated the antioxidant activity of these compounds, which is found in leaves, stem bark and fruits (Fernandes et al. 2014, Flores et al. 2015). Therefore, the present study evaluated the anthelmintic activity *in vitro* of *Psidium guajava* hydroalcoholic extract against gastrointestinal nematodes of sheep. We also evaluated the antioxidant activity and phytochemical characterization of the active compounds present in this extract.

MATERIALS AND METHODS

Ethical Aspects

The Ethics Committee on the Use of Animals of the State University of Northern Paraná certified that the present study was performed in accordance with current legislation on the ethical principles of animal experimentation (Record n° 14/2016).

Collection and processing of plant material

The plant material was collected at a property in the city of Itambaracá, Paraná, Brazil (Latitude 22° 57' 32" South and Longitude 50° 26' 20" West). After selection by the absence of macroscopic changes and packing in polyethylene bags, the plant material was sanitized in running water, weighed and dried using a forced air oven at 40 °C.

Preparation of the hydroalcoholic extract

To prepare the *Psidium guajava* hydroalcoholic extract (PgHA), 30 grams of stem bark was

ground using a knife mill. Vegetal material was added to 270 mL of a hydroalcoholic solution of 70% ethyl alcohol, and this solution was kept under mechanical stirring on magnetic stirrer at room temperature for 24 hours. The solution was vacuum-filtered using filter paper (WhatmanTM # 9), and the extraction procedure was repeated two more times to increase the yield. The obtained extract was concentrated in a rotary evaporator at 60 °C and vacuum at 400-500 mmHg for alcohol removal. The resulting aqueous extract was frozen and lyophilized at -50 °C and pressure of -150 mmHg to obtain the dried extract used in the assays and tests.

Hatchability test (HT)

The hatchability test was performed in quadruplicate according to the methodology of Coles et al. (1992) adapted by Bizimenyera et al. (2006). Stool samples were collected directly from the rectal ampulla of naturally parasitized animals. Egg counting test per gram of feces (ECG) was performed according to Gordon & Whitlok (1939), samples with values over 2000 were selected. Samples were homogenized in warm water (± 40 °C) and filtered through a set of sieves at 750, 250, 75 and 25 µm. The eggs were retained at the end in the 25 µm sieve.

Approximately 110 eggs were allocated in each well of a cell culture plate and subjected to a PgHA solution at the following concentrations: 625; 1250; 2500 and 5000 µg mL^{-1}. The following control treatments were also evaluated: distilled water for the negative control (NC); 300 µg mL^{-1} of albendazole + dimethyl sulfoxide (DMSO) at 0.75% for the positive control (PC), and DMSO at 0.75% for witness control (WC). DMSO was used only as a solvent because the albendazole used for the positive control is a nondissolvable oily vehicle in distilled water. Plates were homogenized manually and conditioned in a biological oxygen demand incubator (B.O.D) at 27°C for 48 hours. Hatched eggs and first stage (L1) larvae were quantified to calculate hatchability inhibition (Roberts & O'Sullivan 1950).

Larval migration inhibition test (LMIT)

Cultures for larval obtention were processed according Ueno & Gonçalves (1998), and its identification was performed using the criteria of Keith (1953).

The larval migration inhibition test (LMIT) was performed according to the methodology of Rabel et al. (1994) as adapted by Gonçalves et al. (2016). This test evaluated the same treatments assessed in the HT, except for the positive control, which was 1% levamisole hydrochloride + DMSO at 0.75%. Approximately 150 infective larvae (L3) were incubated in microtubes with 1 mL of the respective treatments in quadruplicate at 37°C for two consecutive hours. Samples were centrifuged at 6000 g for three minutes, and 200 µL of the supernatant was transferred to microtubes. A filter with 25-µm pore diameter was coupled to each well of a 24-well plate, and 1800 µL of treatments were added. Samples were incubated in B.O.D for two hours at 37 °C, and the number of L3 retained and migrated was quantified using an optical microscope at 40x magnification. The percent inhibition of migration was calculated using the formula:

% inhibition of migration = [Nr / (Nm + Nr)] x 100
"Nm": number of L3 migrated
"Nr": number of L3 retained
Tukey's test at 5% was used in Statistica® software for statistical analysis.

Total tannins determination

The tannin content was estimated according to the methodology of Fagbemi et al. (2005). The tannin content is expressed in mg of tannic acid equivalents per gram of the extract (mg TAE g^{-1}).

Total phenol determination

The Folin-Ciocalteu method was used to determine the total phenol content of the extract, using gallic acid as a comparative pattern (Stagos et al. 2012). To each 0.1-mL sample of the extract (25; 50; 75; 100; 250; 500 and 1000 µg mL^{-1}), 5 mL of distilled water, and 0.5 mL Folin-Ciocalteu reagent (molybdate, tungstate, and phosphoric acid - 1:0.25:0.50) were added. After 3 min, 1.4 mL of 25% Na_2CO_3 and 3 mL of distilled water were added, and the mixture was stocked for 1 h. The absorbance was measured at 725 nm using a UV–vis spectrophotometer (UV-M51-BEL). All measurements were performed in triplicate, and the results are expressed in mg gallic acid/g extract.

Flavonoid determination

The total flavonoid content of the extracts was determined using a UV–vis spectrophotometer, and the samples were prepared according to the methodology proposed by Yao et al. (2013) based on flavonoid complexation with $AlCl_3$, which dislocates the absorption bands to higher wavelengths. An aliquot of 1 mL of the extract at concentrations of 25, 50, 75, 100, 250, 500 and 1000 µg mL^{-1} was mixed with 4 mL of 70% ethanol and 5 mL of 5% $NaNO_2$. Six minutes later, 0.5 mL of 10% $AlCl_3$, 3 mL of a 1 M NaOH solution, and 10 mL distilled water was added. The samples were shaken in a vortex shaker, and the absorbance was measured at 510 nm. All tests were performed in triplicate, and the results are expressed in mg of rutin/g extract.

Antioxidant activity assays

DPPH (1,1-diphenyl-2-picrylhydrazyl) test

The DPPH radical scavenging activity of the extract was determined according to the technique described by Blois (1958). One milliliter of a 100 mM acetate buffer solution (pH 5.5) was mixed with 1.25 mL of absolute ethanol. A volume of 250 µL of a 500 µM DPPH solution (in ethanol), 50 µL of tested samples (25-3000 µg/mL in ethanol) or gallic acid as standard (10-60 µg/mL in ethanol) was added. Samples were incubated at room temperature for 30 min, and the absorbance was determined at 517 nm in a spectrophotometer UV-VIS (Femto, 800XI, Brazil). All determinations were performed in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation:

Antioxidant activity (%) = [(Acontrol-Asample) / Acontrol] x 100
Acontrol: absorbance of the control
Asample: absorbance of the sample

The concentration of extract that caused a 50% inhibition of DPPH (EC_{50}) was also calculated. Lower EC_{50} values correspond to a higher antioxidant activity of samples.

Ferric-reducing antioxidant power (FRAP)

The FRAP assay was performed as described by Benzie and Strain (1996). The FRAP reagent was prepared by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM in HCl solution (40 mM)), and 2.5 mL of an $FeCl_3$ solution (20 mM). Then, 90 µL of the sample (500 µg/mL) or Trolox standard (25-500 µM/L) was mixed with 270 µL of distilled water, and 2.7 mL of freshly prepared FRAP reagent. This solution was incubated at 37°C for 30 min, and the maximum absorbance values were read at 595 nm. The results are expressed as micromoles of Trolox equivalents (TE) per gram of dry extract or fraction.

Determination of antioxidant enzymes

To determine the possible anthelmintic mechanism of action against larval nematodes,

larval biomass was obtained in culture (Ueno & Gonçalves 1998). The larval solution (40 mL) was centrifuged at 1200 g for 5 minutes, and the resulting pellet (biomass) was stored at -80 °C. The pellet was resuspended in 2 mL 0.1 M phosphate buffer (pH 6.5) containing 1% (w/v) polyvinylpyrrolidone (PVP). The mixture was homogenized via vortexing for 10 seconds and centrifuged (10000 g for 10 minutes), and the supernatant was stored at -20 °C for biochemical assays.

Internal H_2O_2 levels of the larvae were determined via monitoring the formation of titanium peroxide (Jana & Choudhuri 1982). A 750-µL aliquot of the extract was mixed with 250 µL of 0.1% titanium chloride (in 20% H_2SO_4). Absorbance of the red-orange solution was measured at 410 nm. H_2O_2 concentration was calculated using its extinction coefficient (0.28l $mmol^{-1}$ cm^{-1}) and expressed as µmol g^{-1}.

The formation of malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) method (Madhava & Sresty 2000) with some modifications. To 1 mL aliquot of the extracts was added 4 mL 0.5% (w/v) TBA in 20% TCA (w/v), and the reaction mixture incubated at 95 °C for 30 minutes in a water bath. The reaction was immediately stopped via cooling in an ice bath, and the mixture was centrifuged at 10000 g for 15 minutes and vortexed. The absorbance of the mixture was read at 532 nm and corrected at 600 nm. MDA concentration (mM) was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Demiral & Türkan 2005):

$$[MAD](mM) = \frac{\text{Abs}532 - \text{Abs}600}{155{,}10} \quad (1)$$

To determine specific activity of antioxidant enzymes, total protein content was quantified. Using BSA as a protein standard, total protein concentration was measured following the procedure described by Bradford (1976). Superoxide dismutase activity was determined spectrophotometrically according to Misra & Fridovich (1972). Keeping SOD concentration below one unit (U), 3 mL of reaction mixture was used to measure the activity of the enzyme contained in 6.7 mM potassium phosphate buffer (pH 7.8), 45 µM methionine, 5.3 mM riboflavin and 84 µM nitro-blue tetrazolium chloride (NBT). One enzymatic unit was defined as the amount of enzyme required to inhibit 50% of the NBT reduction. Glutathione S-transferase activity was measured at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates (Mauch & Dudler 1993). Enzymatic reactions were initiated via the addition of 50 µL enzyme extract to 1 mL of reaction mixture (3.6 mM GSH and 1 mM CDNB in 0.1 M potassium phosphate buffer, pH 6.5). The change in absorbance at 340 nm was proportional to GST activity (nkat mg^{-1} protein).

Preparation of the extract for analysis on HPLC-DAD-MS/MS

The PgHA (10.7 mg) was dissolved in 1.0 mg of mL MeOH: H_2O 95% HPLC grade. After 5 minutes in an ultrasonic bath for complete dissolution, the PgHA was applied to an SPE cartridge (Macherey-Nagel Chromabond® C18 EC) and filtered through a 0.22-µm nylon membrane syringe filter. Two microliters were analyzed using HPLC-DAD-MS. The analysis was performed using a Shimadzu® Prominence HPLC, modular binary system, containing two LC-20AD pumps, DGU-20A3R mobile phase degasser, automatic sampler SIL-20AHT, CTO-20A column oven, and SPD-M20A diode array detector-M20A. This chromatographic system was coupled to a Bruker Amazon mass spectrometer, with an ESI-IT-MS configuration. HPLC-DAD-MS data were treated with Bruker Daltonics Data Analysis 4.1 software.

Conditions for analysis of HPLC-DAD-MS/MS of flavonoids

HPLC separation was performed on a Luna C18 (250 x 4.6 mm 5 µm) column (Phenomenex Inc., Torrance, CA, USA). The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Elution was performed at a 1 mL/min flow in linear gradient mode, beginning with 5% of B and increasing to 100% of B in 40.0 min and maintaining this composition for 10 min. Column temperature was set to 40°C, and PDA wavelength monitoring range was 200-800 nm.

Mass spectrometer parameters

The following ionization parameters were used: capillary voltage (+/-) 4500 V; end plate voltage (+/-) 500 V; nebulizer gas pressure at 50.0 p.s.i.; and drying gas at 10 L/min at 300°C. The acquisition range was 50 to 1500 m/z. MS/MS spectra were recorded in the auto-MS/MS mode. Fragmentation amplitude range was set to 4.0-4.0 V, and the mass spectra were acquired in (+/-) ESI-MS/MS alternate mode.

RESULTS AND DISCUSSION

PgHA inhibited the hatchability in a dose-dependent manner starting from the concentration of 5000 µg mL^{-1} (Figure 1a), as reported in previous tests with tanniferous plants (Alonso-Díaz et al. 2011, Hoste et al. 2012, Zhong et al. 2014). DMSO (0.75%) did not interfere with hatchability, which supports the use of DMSO at this concentration without causing hatchability interference.

These results show that PgHA interfered with hatchability and interrupted the beginning of the parasite's cycle, even before the release of the larvae into the environment. This fact may reduce the contamination of pastures and animal reinfection and contribute to animal parasite control (Yoshihara et al. 2013).

The maximum concentration evaluated in the present study was 5000 µg mL^{-1}, which exhibited a hatch inhibition value of 16.12%. Perhaps the problem of these low values may be solved by testing the extract in higher concentrations because of the influence of the extract in a dose-dependent manner. These results support the continuation of the present study and the

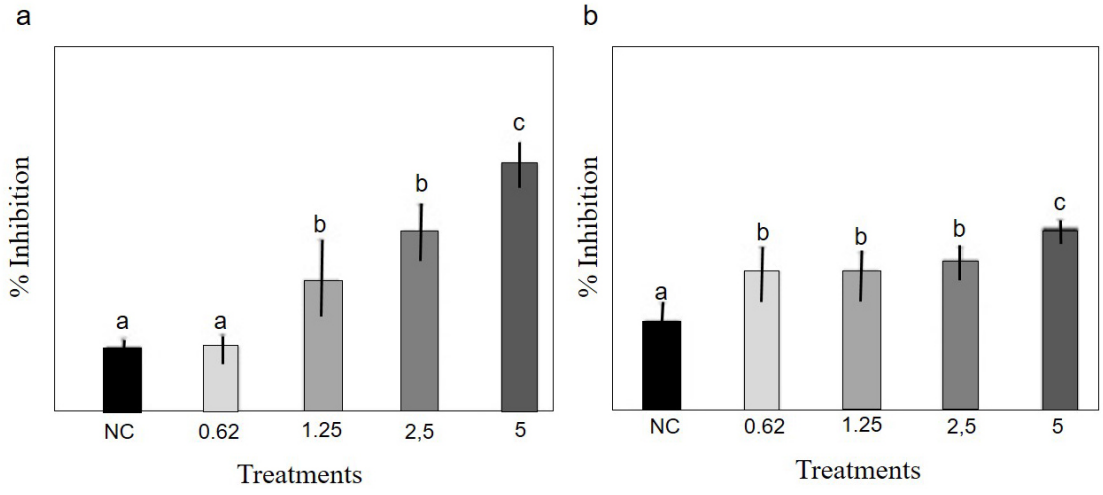

Figure 1. Percentage of hatching (a) and larval migration (b) inhibition of sheep nematodes treated with Psidium guajava hydroalcoholic extract (0.62; 1.25; 2.5 and 5.0 mg mL-1) and negative control treatment (NC). Same letters indicate no significance by Tukey test (α≤0.05).

evaluation of higher concentrations, besides toxicological tests, for application in animal parasitological control in the field.

PgHA inhibited larval migration, but it did not exhibit a dose-dependent pattern, and no significant differences were observed between the different PgHA concentrations evaluated in the present study (Figure 1b). These results are different from the inhibition of hatchability. No differences were observed between the NC and WC treatments ($p < 0.05$), which indicates that DMSO at 0.75% did not interfere with the results. The PC treatment exhibited 100% efficacy.

Alonso-Díaz et al. (2011) evaluated the anthelmintic activity of four tanniferous plants and concluded that LMIT had low sensitivity because the anthelmintic potential of some plants was not detected, which were effective in larval depletion tests. Fernex et al. (2012) also demonstrated these results in bioassays with five leguminous plants that inhibited larvae migration. However, only four of these plants promotes loss of larvae sheath, and these plants still had much lower efficacy. This result may explain the fact that the different concentrations of PgHA evaluated in the present study did not exhibit differences in larval migration inhibition.

Similar to the results presented in this study, Meenakshisundaram et al. (2016) evaluated the ethanolic extract of *Indigofera tinctoria* and demonstrated higher efficiency in the hatchability test compared to the larval migration test with values of 73.50% and 14.40% inhibition, respectively. However, the type of extraction may alter the anthelmintic properties of the plant to the larvicidal or ovicidal action (Hernández-Villegas et al. 2011).

The results of antioxidant activity, total polyphenol, flavonoids and tannins contents are shown in Table I.

Among the phenolic compounds in guava, the tannin and flavonoid contents are (Chang et al. 2013) considered promising substances in the control of helminths (Botura et al. 2013, Yoshihara et al. 2013). The content of condensed tannins found in PgHA were higher in the present study than Gonçalves et al. (2016) in buckwheat (*Fagopyrum esculentum*), which also showed

Table I. DPPH radical scavenging activity, iron ion chelating power (FRAP), inhibition of lipid peroxidation (TBARS) and determination of total phenols, flavonoids and tannins at different concentration of extract from *Psidium guajava* hydroalcoholic extract.

Concentration ($\mu g.mL^{-1}$)	DPPH[a]	FRAP	TBARS	Total Phenols[b]	Total Flavonoids[c]	Total Tannins[d]
25	2.26±0.91	64.74±8.43	26.07±3.58	-	71.57	-
50	12.43±0.36	128.07±10.32	22.16±7.10	-	100.29	-
75	9.55±0.63	175.30±23.13	16.57±3.22	29.74	156.76	-
100	18.67±3.26	205.30±43.32	13.59±2.58	45.38	344.29	0.68
250	56.59±8.37	579.55±53.89	16.38±1.29	133.03	382.86	6.27
500	87.49±1.39	986.60±78.89	4.28±0.61	165.49	460.00	74.49
1000	86.81±2.23	1317.89±41.37	4.28±0.61	156.33	767.62	177.94

Values are expressed as mean ±SE. Same letters within the same column indicate no significant differences among samples by Tukey test ($\alpha \leq 0.05$). [a]Percentage values of DPPH radical scavenging activity; [b]Values of total phenol levels (mg of gallic acid equivalent/g of extract); [c]Total flavonoid levels in quercetin equivalent per mg/g of extract; [d]Tannic acid equivalent per gram of the extract (mg TAE g^{-1}).

lower anthelmintic activity than PgHA. Likewise, compared to the tannin contents found by Shad et al. (2012) in chicory (*Cichorium intybus*), a plant that also exhibits anthelmintic potential against gastrointestinal nematodes of sheep (Marley et al. 2003, Heckendorn et al. 2007), PgHA was also presented the highest concentration of these compounds.

Tannins are categorized as condensed and hydrolyzed. Condensed tannins are associated with the anthelmintic action via direct and indirect mechanisms (Hoste et al. 2012). However, hydrolyzed tannins, which are present in smaller amounts in plants, are associated with indirect effects (Ajala et al. 2014, Fortes et al. 2015). When used in feeding for ruminants, tannins improve protein and energy use by the ruminal microbiota (Hassanat & Benchaar 2013), which contributes to the physiological response of the animals if available in adequate amounts.

As for flavonoids, PgHA showed important amounts of these compounds even in the lowest concentration (Table I). Flavonoids are natural compounds of plants that exhibit a broad spectrum of biological activity and mechanisms of action that are not fully elucidated (Kerboeuf et al. 2008). The anthelmintic potential of flavonoids in gastrointestinal nematodes was reported in sheep (Bottura et al. 2013). One of the flavonoids present in the stem bark of guava is ellagic acid (Chang et al. 2013, Diaz-De-Cerio et al. 2016), which is highly valued for having several beneficial effects (Sádecká & Tóthová 2012, Girish et al. 2013), including antioxidant and immunomodulatory action (Abuelsaad et al. 2013).

As for the antioxidant activity of PgHA, it was possible to demonstrate via the increasing linear correlation (Table I) that the IC_{50} corresponded to 534.02 μg mL^{-1}, which caused a 50% inhibition of DPPH radicals. The antioxidant activity of PgHA was higher than the study of Ilha et al. (2008) in ascorbic acid (IC_{50} = 4,900 μg mL^{-1}), which is recognized as a potent antioxidant, and it was used as a reference by several authors (Kim et al. 2015, Coenraads et al. 2016). However, Fernandes et al. (2014) found IC_{50} values that varied from 4 to 10 μg mL^{-1} for extracts of guava leaves of different varieties and demonstrated that the leaf extract exhibited higher antioxidant activity compared to stem bark. Evaluating methanolic extract of the guava, Ademiluyi et al. (2016) found IC_{50} that ranged from 780 to 1,020 μg mL^{-1}. Therefore, they observed a lower antioxidant activity than the stem bark of the present study. These findings are consistent with Tachakittrungrod et al. (2007) who found greater antioxidant activity in the leaves followed by stem bark and guava fruits.

P. guajava hydroalcoholic extract was analyzed using the HPLC-DAD-MS^2 technique to accomplish an exploratory screening of its chemical constituents. The obtained (+)-ESI-MS^2 and (-)-ESI-MS^2 base peaks and UV 254 nm chromatograms are depicted in Figure 2. In this experiment, UV spectral acquired data was not sufficiently intense, and screening of the constituent substances was performed based on MS^2 spectral data, which are shown in Figure 3.

According to the evaluation of MS^2 mass spectra of figure 3, ions at m/z 303 in (+)-ESI mode (3(a), 3(b), 3(f) and 3(g) spectra) and *m/z* 301 in (-)-ESI mode (3(e) spectrum) were attributed to quercetin isomer aglycones, as described elsewhere (Saldanha et al. 2013, Wyrepkowski et al. 2014). It was possible to observe a 132 Da neutral loss in 3(g) spectrum and 162 Da neutral loss in 3(a), 3(b), and 3(e) mass spectra, which indicates the occurrence of pentose and hexose moieties in the detected substances, respectively. In the 3(c), 3(d), 3(f) and 3(h) spectra, 170 and 152 Da neutral losses are indicative of the presence of a galloyl moiety in the analyzed compound structures (Tala et al. 2013).

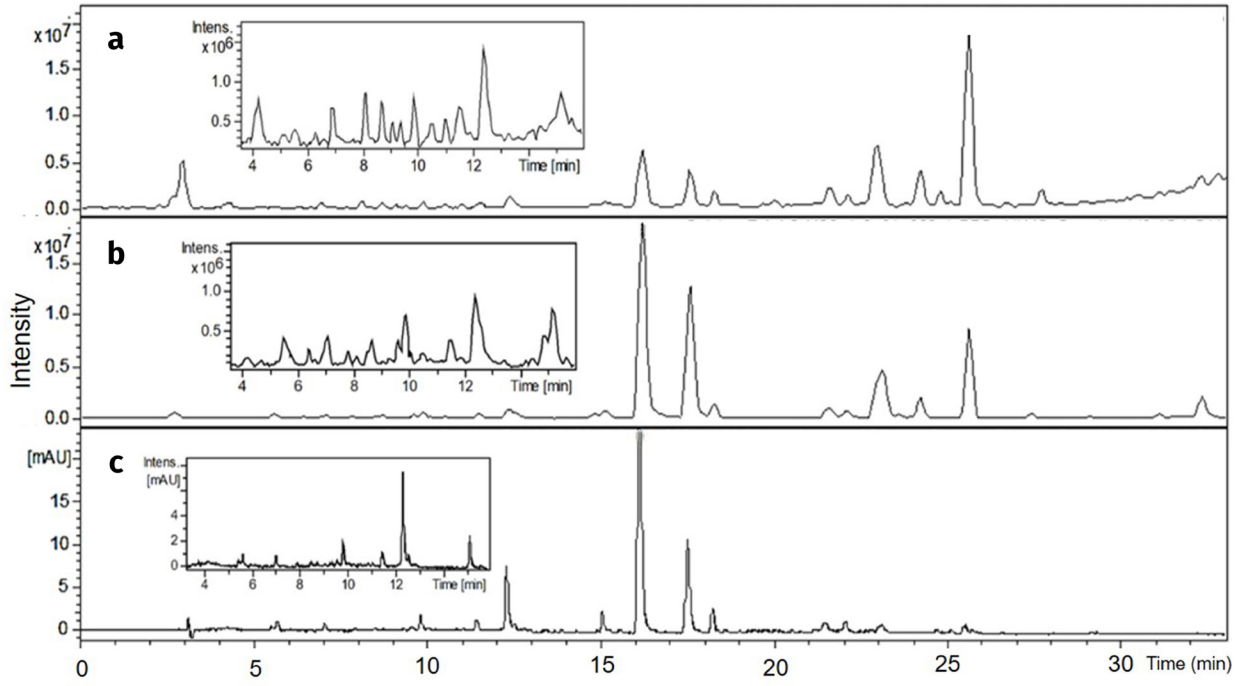

Figure 2. Chromatograms acquired by: (a) HPLC-(+)-ESI-MS2; (b), HPLC-(-)-ESI-MS2 and (c) HPLC-DAD (254nm) experiments.

Spectrum 3(d), acquired in negative mode, shows the dissociation pattern of an $[M-H]^-$ ion at *m*/*z* 577. This pattern was compatible to a type B proanthocyanidin, which consists of a (epi) catechin dimer, found in *P. guajava* leaves (Díaz-de-Cerio et al. 2016). According to the proposed dissociation mechanism shown in Figure 4, the deprotonated molecule follows one of four reaction pathways, and three of these pathways, a remote hydrogen rearrangement with H_2O neutral loss (blue arrows), a retro Diels-Alder reaction with $C_8H_8O_3$ neutral loss (red arrows), and a heterocyclic ring fission followed by a remote hydrogen rearrangement (black arrows), result in a 1,3,5-trihydroxybenzene neutral loss with 126 Da. A quinone methide cleavage reaction, shown in a separate mechanism, is the fourth reaction and provides an entire catechin/epicatechin neutral loss, producing ions with *m*/*z* 289 (Gu et al. 2003).

Spectrum 3(c) shows an $[M-H]^-$ ion at m/z 593, which exhibited the same dissociation pattern described for 3(d) spectra, indicating that this substance belongs to the same compound class, that is, a type B proanthocyanidin. From the literature data, we can infer that its structure consists of an (epi)catechin and a (epi) gallocatechin moieties, which was also reported in *P. guajava*. Although it was not possible to determine the structure of all of the detected substances, these preliminary experiments indicate the presence of flavonoids and proanthocyanidins in *P. guajava* hydroalcoholic extract (Figure 4).

Table II presents the results of the evaluation of the possible mechanism of antiparasitic action of *P. guajava* extract on ovine helminths evaluated via the determination and quantification of the total protein, enzymatic activity of the enzyme superoxide dismutase and glutathione-S-transferase, lipid peroxidation

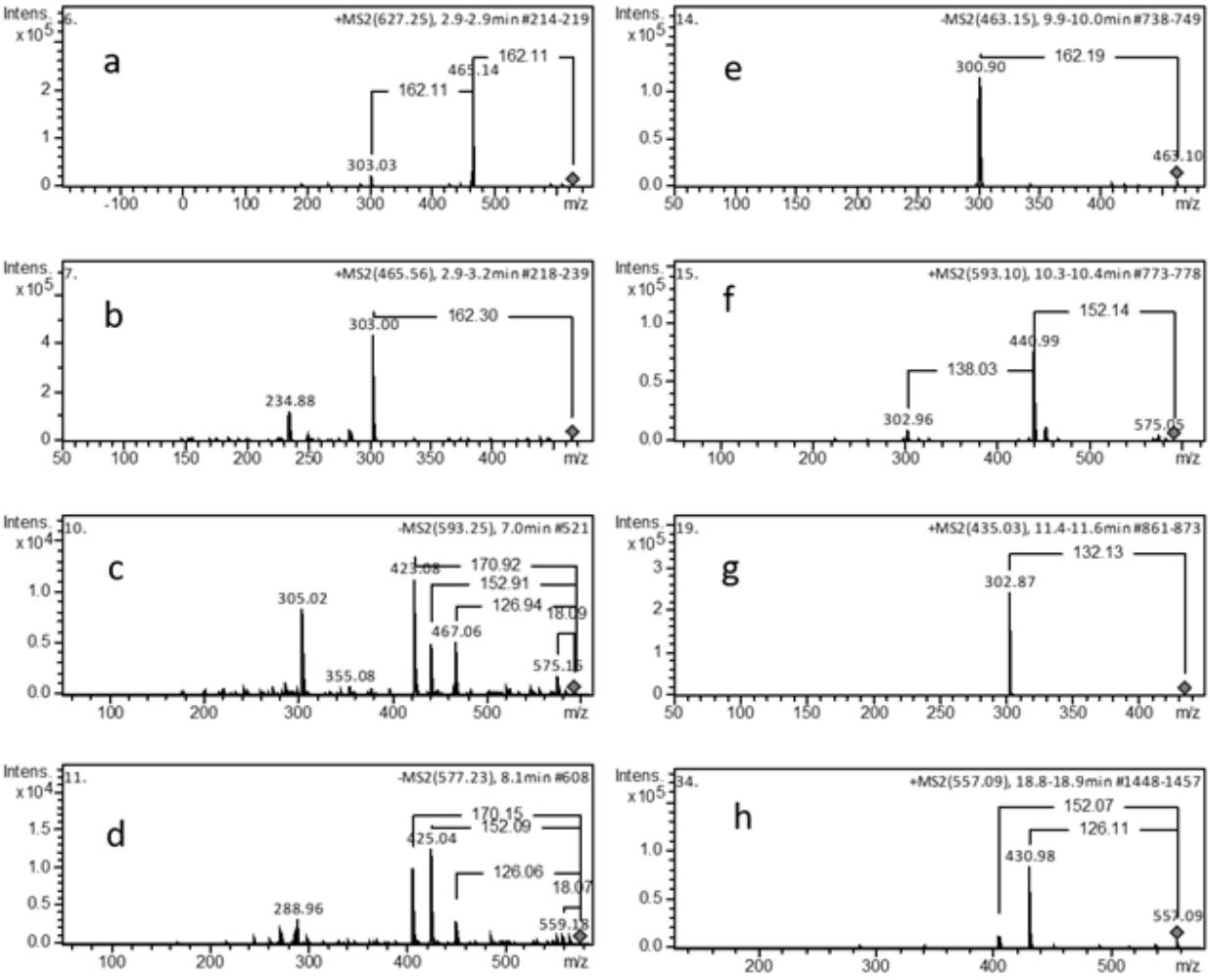

Figure 3. Tandem MS2 mass spectra acquired by HPLC-DAD-MS/MS experiment. Letters indicate retention time order.

and intracellular hydrogen peroxide in larvae of helminths obtained from sheep and exposed to the extract of *P. guajava*.

Exposure to *P. guajava* extracts produced an increase (dose-dependent) in the internal intracellular production of hydrogen peroxide, concentration of lipid peroxidation products, glutathione S-transferase and superoxide dismutase activity. All of these alterations are related to the antioxidant defense metabolism of helminths. Therefore, these effects may be correlated with the increase in total proteins observed in the helminth larvae exposed to the extract.

The increases observed in the presence of the extract were significantly different than the control. These results were observed for the first time in larvae of helminths to vegetal extracts because previous scientific studies expressed the antioxidant enzymatic activities produced in the hosts of the parasites in Callahan et al. (1988), Batra et al. (1990), Kosik-Bogacka et al. (2011) and Cuesta-Astroz et al. (2017).

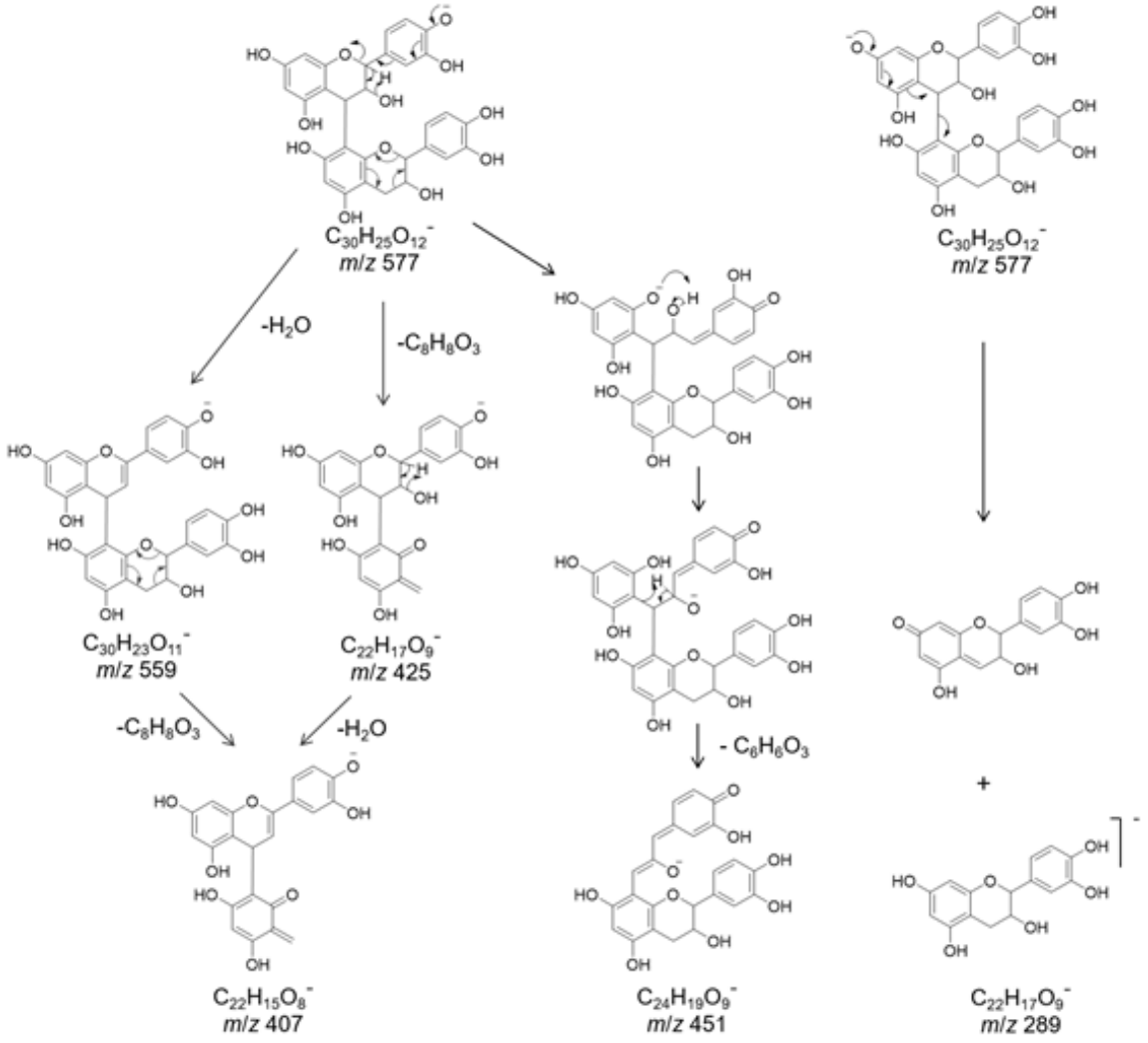

Figure 4. Proposed dissociation mechanism for m/z 577 [M-H]- ion.

Table II. Evaluation of total proteins (TP), intracellular concentrations H_2O_2 (PH); lipid peroxidation products concentration (LP); glutathione S-transferase (GST) and superoxide dismutase (SOD) of larval of sheep nematodes submitted to *Psidium guajava* hydroalcoholic extract (0.62; 1.25; 2.5 and 5.0 mg mL^{-1}) and negative control treatment.

Treatments $mg.m^{-1}$	TP ($mg.mL^{-1}$)	PH ($\mu M.g^{-1}$)	LP ($mM.L^{-1}$)	GST ($nm.mL^{-1}.min^{-1}$)	SOD $U.mL^{-1}$)
0	38.04±2.32[a]	0,19±0.02a	0,37±0.09a	1.64±0.23a	0.73±0.06a
0.62	42.31±1.98[a]	0.27±0.09b	0,45±0.12a	1.86±0.21a	0.75±0.09a
1.25	53.23±2.33b	0.35±0.02b	0,69±0.18b	2.17±0.14b	0.89±0.24a
2.50	89.54±2.65c	0.61±0.06c	1,18±0.23c	2.69±0.16c	1.23±0.11b
5.00	197.67±3.42d	1.47±0.11d	2,65±0.63d	3.89±0.14d	3.49±0.23c

Values are expressed as mean±SE. Same letters within the same column indicate no significant by Tukey test (α≤0.05).

CONCLUSION

The results obtained in the present study verified that PgHA showed an anthelmintic potential *in vitro* as well as antioxidant activity. These results justify the continuation of the present study to evaluate higher concentrations, besides toxicological tests, and apply the animal parasitological control in the field. Notably, these results are particularly important for organic, agroecological and biodynamic systems of animal production.

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LUCIANA P. SILVA[1]
https://orcid.org/0000-0002-0914-2094

RAFAEL R. DEBIAGE[1]
https://orcid.org/0000-0001-8892-9348

JOÃO L. BRONZEL-JÚNIOR[2]
https://orcid.org/0000-0001-5645-2485

REGILDO M.G. DA SILVA[3]
https://orcid.org/0000-0002-0300-0247

ERIKA C.T. MELLO -PEIXOTO[1]
https://orcid.org/0000-0002-9608-4282

[1]Universidade Estadual do Norte do Paraná (UENP), Departamento de Produção Animal, Laboratório de Controle Parasitológico Animal em Agroecológico e Sistemas Orgânicos de Produção, s / n, Rodovia BR - 369, 86360-000 Bandeirantes, PR, Brazil

[2]Universidade Estadual Paulista (UNESP), Instituto de Química, Av. Prof. Francisco Degni, 55, Jardim Quitandinha, 14800-900 Araraquara, São Paulo, Brazil

[3]Universidade Estadual Paulista (UNESP), Faculdade de Ciências e Línguas de Assis, Departamento de Biotecnologia, Laboratório de Fitoterapia e Produtos Naturais, Av. Dom Antônio, 2100, 19806-900 Assis, SP, Brazil

Correspondence to: **Regildo M.G. da Silva**
E-mail: regildos@yahoo.com.br

Authors contribution

Luciana P. Silva: Concept, design and experimental studies - Anthelmintic and Oxidative Activity; Rafael R. Debiage: Experimental studies - Anthelmintic Activity; João L. Bronzel-Júnior: Phytochemical analysis. Regildo M.G. Da Silva: Experimental studies, data acquisition, statistical analysis and manuscript review; Erika C.T. Mello-Peixoto: Concept, design, experimental studies data acquisition, statistical analysis and manuscript review.