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Research paper

Assessment of anthelmintic activity and bio-guided chemical analysis of *Persea americana* seed extracts

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ABSTRACT

The aim of this study was to characterize the extracts and fractions of *Persea americana* Mill. (Avocado) seeds and to determine the composition and the *in vitro* anthelmintic activity against third-stage larvae (L3) of *Haemonchus contortus*. The fresh (F) and dried (H) avocado seeds (PA) were subjected to extraction with hot water (W-PAF, W-PAH), ethanol (E-PAF, E-PAH) or methanol 70% (v/v), and partition with solvents of increasing polarity [*n*-hexane (H-PAF, H-PAH), chloroform (C-PAF, C-PAH), ethyl acetate (Ea-PAF, Ea-PAH), and *n*-butanol (B-PAF, B-PAH)], yielding a total of 14 extracts/fractions. After considering the yield, water solubility, and the preliminary results of the larval migration test (LMT), the E-PAF, E-PAH, H-PAF, and H-PAH were selected for further experiments. E-PAH presented an efficiency concentration of 50% (EC_{50}) of 36 µg/mL on the LMT. E-PAH showed the greatest efficiency when its EC_{50} was compared to the other fractions (E-PAF = 147 µg/mL; H-PAF = 801 µg/mL; H-PAH = 77 µg/mL). After that, the E-PAH was chemically characterized, considering its quantitative polyphenolic and flavonoid contents by colorimetric and chromatographic techniques. E-PAH presented 50, 38, and 24 mg/g of dry matter of total phenol, condensed tannins (CT), and flavonoid contents, respectively. Using high performance liquid chromatography (HPLC) analysis, E-PAH had shown to have epicatechin (4.7 µg/mL), rutin (2.8 µg/mL), and chlorogenic acid (1.4 µg/mL) as its main constituents besides quercetin. These isolated compounds were evaluated using the LMT in order to relate the composition to the anthelmintic activity observed for E-PAH. Quercetin (EC_{50} = 7.8 µg/mL) and epicatechin (EC_{50} = 10 µg/mL) presented a higher efficiency than rutin (EC_{50} = 30 µg/mL). Chlorogenic acid was also tested with the LMT but did not present a significant efficiency. According to the results, the phenolic composition of E-PAH and the EC_{50} values obtained for the isolated phenols, it can be suggested that, besides the CT content, the presence of epicatechin and rutin contributed to the larvicidal activity of E-PAH. In conclusion, avocado seeds may be used as a source of polyphenols with promising anthelmintic applications.

1. Introduction

Gastrointestinal nematode infections lead to major economic losses in ruminant production across the world (Calvete et al., 2014). Among the parasites that affect small ruminants, *Haemonchus contortus*, a hematophagous parasite, is ranked as one of the most important cause for reducing animal performance and productivity, and increasing animal morbidity and mortality (Kumarasingha et al., 2016).

The use of medicinal plants as a source of bioactive compounds for the treatment of endoparasite and ectoparasite infections in livestock, has become the target of many studies and holds promise for the development of future therapeutic drugs (Githiori et al., 2006; Athanasiadou et al., 2007). One of the main reasons underlying the search for alternative drug therapy is the mitigation of problems associated with the use of semi-synthetic anthelmintic drugs. The alarming parasite resistance situation, environmental pollution, and animal by-product drug residues (Molento, 2009; Hoste et al., 2015) are some of production impeding factors.

The antiparasitic properties of plant extracts are commonly associated with the presence of secondary metabolites, such as essential oils

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or tannins (Molan et al., 2002; Hoste et al., 2006; Pavela, 2015; Pavela and Benelli, 2016). Tannins, which are polyphenol compounds, have been described as oligomeric compounds with high molecular weight (≥ 2000 Da) and are subdivided into two groups: condensed tannins (CT) and hydrolyzable tannins (Craft et al., 2012). CT, also known as procyanidins and prodelphinidins, are oligomers and polymers of flavan-3-ol, while hydrolyzable tannins are compounds that contain a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, is also called ellagitannins (Craft et al., 2012). However, it has been noted that the anthelmintic activity of plant extracts may also be attributable to other polyphenolic compounds, including phenolic acids (hydroxycinnamic and hydroxybenzoic acids) and flavonoids (flavanols, flavonols) (Barrau et al., 2005; Klongsiriwet et al., 2015, Díaz et al., 2017).

The seeds of *Persea americana* Mill. (avocado tree) are a source of bioactive compounds and the species is cultivated in various parts of the world, including Brazil. The fruit has a great commercial value because of its nutritional quality (Dabas et al., 2013). The seed represents a considerable percentage of the total fruit (12–16%, w/w) and is an under-utilized resource and a waste issue for the avocado industry (Kosińska et al., 2012). From the chemical point of view, avocado seeds contain high levels of phenolic compounds (more than 50%), such as hydroxybenzoic acids, hydroxycinnamic acids, flavanols (such as catechin, epicatechin), flavonols (such as rutin), and procyanidins (Pahua-Ramos et al., 2012).

A common approach for studying crude plant extracts and their fractions is by using bio-guided chemical analysis. The current study aimed to characterize the extracts and fractions of the avocado (*Persea americana* Mill.) seed and to relate its composition to the anthelmintic activity tested. The effect of avocado extracts and fractions, as well as, that of the isolated compounds such as chlorogenic acid, quercetin, rutin, and epicatechin, on third-stage larvae (L3) of *Haemonchus contortus* were evaluated using an *in vitro* larval migration test (LMT).

2. Material and methods

2.1. Chemicals and reagents

Water was treated in an ultra-purifier MS 2000 system from Gehaka (São Paulo, Brazil). HPLC grade acetonitrile was obtained from JT Baker (Xalostoc, Mexico), and HPLC grade formic acid was obtained from TEDIA (Darmstadt, Germany). *trans*-cinnamic acid, chlorogenic acid, gallic acid, epicatechin, quercetin, rutin, and levamisole were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ivermectin (1% w/v solution, Ivomec, Merial Limited, Duluth, GA, USA), and Folin-Ciocalteu reagent was obtained from Chromate (São Paulo, Brazil). All other used reagents were of analytical grade.

2.2. Extraction and fractionation of Persea americana seeds

Mature avocado fruits (cv *Fortuna*) were obtained from a commercial organic market. The seeds were manually removed and cut into small pieces (2 × 2 cm). Part of the seeds (315 g) was subjected to an air-drying procedure at 40 °C, until a constant weight was attained. Avocado seeds showed average moisture content of 75 ± 1.5%. The other part (193 g) was used *in natura* (fresh). The dried seeds (33 g) were submitted to hot aqueous extraction with 400 mL of water for 60 min at 70 °C (Bovo et al., 2013). The aqueous extract was vacuum filtered, concentrated in a rotary evaporator under reduced pressure at 40 °C, freeze-dried, stored at −4 °C in the dark, and designated as W-PAH. The same procedure was carried out for the fresh seeds (85 g of seeds and 500 mL of water) and the sample was designated as W-PAF, as shown in Fig. 1.

The seeds (20 g dried and 54 g fresh) were submitted to hydro-alcoholic extraction with aqueous ethanol 70% (v/v) (400 mL) or with aqueous methanol 70% (v/v) (400 mL), at 4 °C, in the dark, for 10 days. Every 2 days, 100 mL of the respective solvent was removed and 100 mL of new solvent was added, in order to optimize the extraction.

The ethanolic extracts were vacuum filtered, concentrated in a rotary evaporator under reduced pressure at 40 °C, freeze-dried, stored at – 4 °CC in the dark, and named E-PAF and E-PAH. The methanolic extracts (M-PAF and M-PAH) were also filtered, concentrated and freeze-dried under the same conditions as described before. The freeze-dried methanolic extracts were dissolved in distilled water and mixed with *n*-hexane in a 1:1 ratio (v/v). The solution was placed in a separation funnel, and after vigorous agitation, the hexane fractions (H-PAF and H-PAH) were separated. This procedure was repeated twice. Subsequently, the residual extract was fractioned using organic solvents of increasing polarity [chloroform (1:1), ethyl acetate (1:1), and *n*-butanol (1:1)] in a similar manner as described for *n*-hexane. The chloroform fractions (C-PAF and C-PAH), ethyl acetate fractions (Ea-PAF and Ea-PAH), and butanol fractions (B-PAF and B-PAH) were thus obtained.

The extraction and fractionation procedures produced 14 extracts and fractions in total, and their yields and water solubility characteristics are described in Fig. 1.

2.3. Colorimetric determination of total phenolic, flavonoid, and condensed tannin contents

The total phenolic content was determined using a microassay and the Folin-Ciocalteu reagent, which was adapted from Singleton and Rossi (1965). The calibration curve was prepared using gallic acid (GA) as a standard. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry extract. The flavonoid content was determined by the aluminum chloride method (Vennat et al., 1992), using rutin (RUT) as a standard and was expressed as milligrams of RUT equivalents per gram of dry extract. The condensed tannin (CT) content was determined by the sulfuric vanillin method (Queiroz et al., 2002). Results were expressed as milligrams of epicatechin (EPI) equivalents per gram of dry extract.

2.4. High-performance liquid chromatography (HPLC) analysis

The phenolic content of the E-PAH extract was determined using an Agilent 1200 Series high-performance liquid chromatography (HPLC) system (Agilent Co., St. Clara, CA, USA) equipped with a vacuum degasser (G1322A), quaternary pump (G1311A), manual injector (Rheodyne, 7725i), and a multi UV–vis wavelength detector (G1365D) operating at a wavelength of 235, 280, 320, 365, 375, and 525 nm. The chromatographic separation was performed using an Agilent Eclipse XDB-C-18 column (150 mm × 4.6 mm, 5 µm particle size) with 20 µL of injected sample. The mobile phase used was formic acid (1%, v/v) (A) and acetonitrile (B) set with the following gradient: 0–5 min, 5% B; 5–10 min, 10% B; 10–20 min, 30% B; 20–30 min, 50% B; and 30–35 min, 5% B, with a flow rate of 1 mL/min, at 25 °C (adaptated from Jiménez et al., 2017). The extract was diluted with methanol: water (1:4 v/v) to a concentration of 5 mg/mL and filtered through a 0.22-µm membrane filter (JetBiofil, Guangzhou, China) before injection. The standards (trans-cinnamic acid, chlorogenic acid, epicatechin, quercetin, and rutin) were prepared individually as stock solutions in methanol at a concentration of 1 mg/mL, and a diluted solution (100 µg/mL, in aqueous methanol 10%, v/v) was prepared freshly before the analysis. The phenolics were identified by comparing their retention times with those of standards. The quantification of the main compounds was determined by comparing the analytical curves of the standards (rutin, chlorogenic acid and epicatechin). To ascertain the linearity, stock solutions of the standards were prepared individually in methanol at a concentration of 1 mg/mL, and six different concentrations (2.5 to 200 µg/mL) were injected (20 µL) in triplicate to the HPLC system and the analytical curve obtained by plotting peak area versus concentration for each standard presented the square of the correlation coefficient $R^2 \geq 0.99$ as indicative of the measure of linearity [(rutin, y = 43154 × + 162195, R^2 0.9979); (chlorogenic acid,