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ARTÍCULO DE INVESTIGACIÓN / RESEARCH ARTICLE

BIOINSECTICIDE POTENTIAL OF ETHANOL EXTRACTS FROM *Persea americana* (LAURACEAE) SEEDS ON *Aedes aegypti* MOSQUITOES

Potencial bioinsecticida de extractos etanólicos de semillas de *Persea americana* (Lauraceae) en mosquitos *Aedes aegypti*

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ABSTRACT

Mosquitoes represent the most important agent disseminating infectious diseases like yellow fever, dengue, chikungunya, and malaria, among others. An essential strategy for its control is killing them at immature stages using industrial insecticides. However, those chemicals often generate resistance and affect the environment and human health. Agricultural and plant by-products constitute a new sustainable option to obtain harmless and eco-friendly bioinsecticides to prevent mosquitoes from spreading. This study aimed to investigate the phytochemical profile of *Persea americana* Mill (Lauraceae) seed extracts and their insecticide activity against *Aedes aegypti* at larval and pupal stages. The ethanol extracts from avocado seeds were obtained by Maceration/stirring (MaE) and Soxhlet extraction (SE) methods. The main chemical profile was determined by quantitative and UPLC assays. Insecticide activity was assessed by the exposition of mosquitoes at larval and pupal stages to seed extracts. Human cell lines were used to evaluate the cytotoxicity. Soxhlet methodology was more efficient in the extraction of *P. americana* seeds metabolites (42.13±1.76 mg/mL) compared with MaE (20.46±1.66 mg/mL) ($p< 0.05$). Additionally, SE showed a higher amount of polyphenols (5.12±0.18 mg/mL). The UPLC spectra analysis revealed the presence of polyphenols, mainly catechin, and neolignan constituents. Both extracts showed larvicidal and pupicidal effects, but SE was more active at lower concentrations. Moreover, no significant toxic effects on human monocytes and fibroblast cell lines were found after treatment. In sum, avocado seed by-products can be considered an eco-friendly insecticide and its use may help to substantially decrease the vector-transmitted diseases in developing countries.

Keywords: infectious diseases, insect, natural product, phytochemicals, safety

RESUMEN

Los mosquitos representan el agente más importante en la diseminación de enfermedades como la fiebre amarilla, dengue, chikungunya, malaria, etc. Una estrategia esencial para su control es la muerte en estadios inmaduros usando insecticidas industriales.

Sin embargo, estos químicos generan resistencia y afectan el medioambiente y la salud humana. Los subproductos agrícolas y vegetales constituyen una nueva opción sostenible para obtener bioinsecticidas inocuos y eco-amigables, y prevenir la diseminación de los mosquitos. Este estudio tuvo como objetivo investigar el perfil fitoquímico de extractos de semillas de *Persea americana* Mill (Lauraceae), y su actividad insecticida contra *Aedes aegypti* en fases larval y pupa. Los extractos etanólicos fueron obtenidos mediante los métodos de maceración/agitación (MaE) y Soxhlet (SE). El perfil químico se determinó mediante ensayos cuantitativos y UPLC. La actividad insecticida se evaluó exponiendo los mosquitos a los extractos etanólicos. Se emplearon líneas celulares humanas para evaluar la citotoxicidad. La metodología Soxhlet fue más eficiente en la extracción de metabolitos de *P. americana* (42.13±1.76 mg/mL) comparado con MaE (20.46±1.66 mg/mL) ($p< 0.05$). Adicionalmente, SE mostró un contenido superior de polifenoles (5.12±0.18 mg/mL). El espectro UPLC reveló la presencia de polifenoles, principalmente catequina, y neolignanos. Ambos extractos manifestaron efectos larvicida y pupicida, pero SE resultó más activo a menores concentraciones. No se evidenciaron efectos tóxicos significativos en líneas celulares humanas de monocitos y fibroblastos. Los subproductos de semillas del aguacate pueden considerarse como insecticidas eco-amigables y su uso ayudaría a disminuir sustancialmente las enfermedades transmitidas por vectores en los países en desarrollo.

Palabras clave: enfermedades infecciosas, insecto, producto natural, fitoquímicos, seguridad

INTRODUCTION

Nowadays, special attention is paid globally to vector-borne diseases. The prevalence of infectious diseases is identified worldwide as a common cause of dysfunctional health programs, poverty, and economic burden in developing countries. Therefore, different strategies based on vector management have become an important tool for preventing vector-borne diseases. Mosquitoes represent the most important spreading agents of infectious diseases such as yellow fever, dengue, chikungunya, and malaria, among others (Elumalai et al., 2017; Wilson et al., 2020). Dengue is the most widespread mosquito-borne disease and causes more than 90 million cases and approximately 40000 deaths per year (Dahmana and Mediannikov, 2020). At the same time, *Aedes aegypti* is the mosquito species considered the main vector responsible for human health problems, often associated with high morbidity and mortality incidence in unindustrialized countries (Soonwera et al., 2017; Senthil-Nathan, 2020).

An effective way to diminish mosquito populations consists of reducing them at the first stages of their lifecycle. Most insecticides may easily act at larvae stages decreasing the mosquitos´ densities before they emerge into adults (Arun et al., 2015; Soonwera et al., 2017). In this sense, synthetic compounds have been proven as powerful insecticides able to skirmish and control the mosquitoes spreading at these immature stages. However, the uncontrolled use of chemicals frequently provokes contamination in ecosystems, affecting the biodiversity of water, air, and soil (Kumar and Kumar, 2019; Zikankuba et al., 2019). Consequently, chemical insecticides may accumulate in foods, thus leading to serious damage to humans and animals through poisoning (Siriwattanarungsee et al., 2008; Modise and Ashafa, 2016). A high number of health problems associated with insecticide exposition have been found in humans affecting specific target tissues such as the brain, lung, and gastrointestinal tract, as well as, inducing several kinds of cancers (Evangelou et al., 2016; Zikankuba et al., 2019). Furthermore, its extensive application has led to biological resistance in many vector species that have generated adaptation mechanisms, especially in mosquito populations (Prabhu et al., 2011; Modise and Ashafa, 2016).

The use of natural and eco-friendly insecticides from plants and especially their by-products seems to be a good alternative to substitute and/or diminish the excessive applications of synthetic chemicals. Additionally, the use of such biopesticides has emerged as an important approach for sustainable vector management due to less pollution in food/plant crops, less toxicity, rapid biodegradation, and feasible industrial processing with relatively low costs (Arnason et al., 2012; Senthil-Nathan, 2020).

Cuba, as a tropical country, possesses a strong epidemiological surveillance system destining substantial efforts to reduce the health impacts caused by *Aedes aegypti* vector (Peréz et al., 2018). Therefore, like the rest of underdeveloped countries, the use of new ecological methods to control the spread of the vector at immature stages has been encouraged to substantially reduce the costs associated with the control and treatment of such vector-borne diseases. In this context, plant-derived products emerge as a suitable and economically affordable resource with larvicidal and pupicidal activity against different species of mosquitoes (e.g. *Aedes aegypti*) (Zahran et al., 2010; Senthil-Nathan, 2020). It is well-accepted that secondary metabolites like essential oils, flavonoids, saponins, lignans, quinones, and others can exhibit good results when used against mosquito larvae and pupa stages (Adesina et al., 2016).

Persea americana Mill, a tree belonging to the Lauraceae family, is extensively cultivated in subtropical regions to be worldwide commercialized because of the nutritive value of their fruits. It is known as avocado or avocado pear, and the fruit value is not only associated with its nutritive attributes but also the pharmacological properties that allow the use to treat liver pathologies, inflammation, cancer, and infectious diseases (Yasir et al., 2010; Mulkay et al., 2010). After the fruit consumption, its by-products (peel and seeds) are usually discarded. In some avocado varieties, the seeds represent 50 % of the total fruit weight, having a big impact on the overall price and emerging as the main avocado

by-product. This by-product also shows some important biological effects as antimicrobial and insecticide activity against vectors (Adesina et al., 2016; Zikankuba et al., 2019). Hence, it is reasonable to explore new appropriate uses, particularly if it is accepted that avocado seeds have not been so extensively investigated from the chemical and insecticidal activity points of view.

On these bases, we investigate the phytochemical profile of *P. americana* Mill seed extracts and their insecticidal activity against *A. aegypti* at larval and pupal stages. The potentialities of avocado seed-derived products as natural insecticides may positively impact the reduction of infectious diseases in subtropical countries, thus improving people's quality of life.

MATERIALS AND METHODS

PLANT MATERIAL

The fruits from *Persea americana* Mill trees were randomly collected in Caney town, municipality of Santiago de Cuba (Latitude: 20° 03' 12.60" N and Longitude: -75° 45' 59.99" W), during the period from October-November 2019. The plantation species were taxonomically identified by a specialist from the Eastern Centre for Ecosystems and Biodiversity (BIOECO, Santiago de Cuba). Following the certified procedure, a vegetal sample was settled at the herbarium of the institution with the registration number: 21 510.

EXTRACT PREPARATION

Avocado seeds were used in fresh conditions removing the thin skin that covered them and stripping them with a grater according to Molina-Beltrán et al. (2018). A hundred grams of the striped seeds were weighed in an analytical balance to obtain two extracts: ***MaE*** by Maceration/stirring during 24 hours in a JP Selecta 3000974 (Germany), and ***SE*** by Soxhlet extraction stopping the process after six hours of the first reflux. In both cases, commercial ethanol at 94 % was used as a solvent, obtaining a final volume of 200 mL.

The extracts were filtered using a Buchner funnel and vacuum concentrated in a Kirka-Werke rotary evaporator (Germany) at 45°C. Stock solutions at a concentration (100 mg/mL) were prepared and conserved at -20 °C for analytical and biological purposes.

CHEMICAL COMPOSITION

QUANTIFICATION OF METABOLITES IN EXTRACTS

Total soluble substances

Total soluble substances were determined by a gravimetric method after drying 5 mL of the extracts in a porcelain capsule placed in an MRC oven (DNI-30, Israel) at 105 °C. The results are expressed as milligrams of extracted substances per extract milliliter.

Total proteins content

The total protein content for both extracts was estimated following Lowry´s assay (Beltrán et al., 2021). A standard

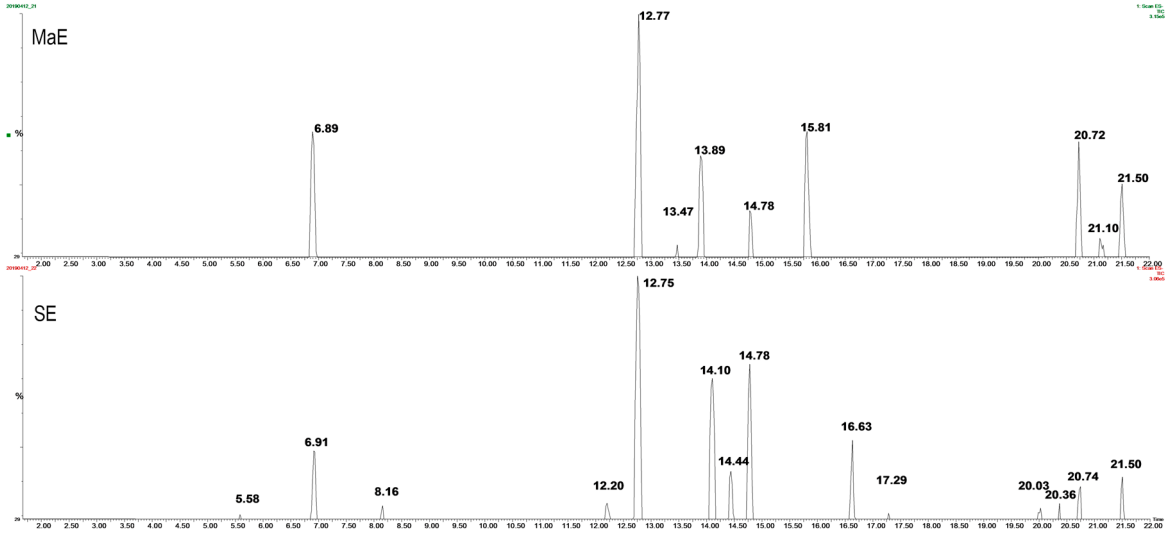

Figure 1. UPLC-DAD-MS/MS chromatogram of *Persea americana* seed ethanol extracts.

Table 1. Assigned compounds, [M-H]- and ESI negative fragment ions of the eleven peaks detected in SE and/or MaE *P. americana* seed extracts.

Peak	Rt (min)	Mass $[M-H]^-$ (*m/z*)	Molecular Formula	Error (mDa)	MS/MS ions	Tentative identification	EPP
1	6.90	441.1242	$C_{22}H_{18}O_{10}$	-3.4	289, 179, 168, 137, 125	catechin/epicatechin gallate	*SE*, *MaE*
2	12.20	327.1214	$C_{18}H_{16}O_6$	-2.7	297, 177, 149	Perseal C	*SE*
3	12.76	329.0969	$C_{18}H_{18}O_6$	-5.6	314, 299, 208, 175	3',4'-Methylenedioxy-5,7-dimethylcatechin/ epicatechin	*SE*, *MaE*
4	13.89	265.1602	$C_{19}H_{22}O$	0.7	239, 210, 189, 138, 135	-	*MaE*
5	14.10	329.2596	$C_{19}H_{38}O_4$	9.9	298, 285, 257, 241, 190	4,19-dihydroxynonadecanoic acid	*SE*
6	14.44	371.1578	$C_{21}H_{24}O_6$	0.2	356, 341, 296	-	*SE*
7	14.78	331.1248	$C_{18}H_{20}O_6$	-0.1	269, 253, 212, 165, 136	Perseal A	*SE*, *MaE*
8	15.81	325.0904	$C_{19}H_{14}O_6$	1.6	281, 221, 163, 162	p-coumaric acid glucoside	*MaE*
9	16.63	373.3218	$C_{22}H_{46}O_4$	1.7	330, 314, 313, 271, 245, 244	2-hydroxy-4-oxoicosa-dienyl acetate	*SE*
10	20.73	345.2355	$C_{18}H_{34}O_6$	-2.1	301, 285, 225, 200, 185,145	Dideoxy-hexopyranosyl-dodecanoic acid	*SE*, *MaE*
11	21.50	383.1906	$C_{20}H_{32}O_7$	3.5	365, 347, 329, 311, 225	diterpene	*SE*, *MaE*

curve of Bovine Serum Albumin (BSA, BDH-Germany) was prepared in a concentration range (5-200 µg/mL) and the mathematical equation is shown in equation 1. The results were expressed as micrograms of BSA equivalent/ mL extract.

$y = 2.7274 x + 0.016$ $R^2 = 0.9927$ (Equation 1)

Total carbohydrates content

Total carbohydrates in both extracts were determined by the Dubois phenol-sulphuric method, according to Beltrán et al. (2021). A glucose solution from 10 to 200 µg/mL (99.5 % pure, Riedel-de Haën, Germany) was used for the standard curve (equation 2) and absorbances were measured at 490 nm in a UV/VIS spectrophotometer (Genesys, Switzerland). The results were expressed as micrograms of glucose equivalent/ mL extract.

$y = 12.204 x + 0.0527$ $R^2= 0.9892$ (equation 2)

Total phenols determination

The total phenolic content (TPC) was assayed by a colorimetric method using Folin-Ciocalteu's reagent (Sigma, USA) (Escalona et al., 2011). Absorbances were measured at 760 nm on a UV/VIS spectrophotometer (PG Instruments, model T60, China). A standard curve using gallic acid (GA) (Sigma, USA) ranging from 9.7 to 625 µg/mL was performed (equation 3). The results were expressed as milligrams of gallic acid equivalents/ mL of the extract.

$y = 40.973 x - 0.0204$ $R^2 = 0.9964$ (equation 3)

Total Flavonoid Content

The quantification of the total flavonoid content (TFC) was assessed through a reaction with aluminum trichloride ($AlCl_3$ 99.9 % pure, Riedel-de Haën, Germany) (Escalona et al., 2011). A calibration curve prepared with Quercetin (95 % pure, Sigma, USA) ranging from 5 to 25 µg/mL was developed measuring the absorbances at 420 nm (PG Instruments, model T60, China) (equation 4). TFC was expressed as milligrams of Quercetin equivalent /mL extract.

$y = 18.829 x + 0.0001$ $R^2 = 0.9829$ (equation 4)

QUALITATIVE SCREENING

To determine the chemical nature of the main constituents, samples were studied in a UPLC-DAD-MS/MS system using a Xevo G2-XS QTof spectrometer (Waters, Milford, MA, USA) coupled with an ACQUITY LC system equipped with MassLynx version 4.1 software.

A column BEH Shield RP18 (100 mm × 2.10 mm, 1.7 µm; Waters, Milford, MA, USA) and mobile phase consisted in H_2O + 0.1 % formic acid (A) and acetonitrile + 0.1 % formic acid (B), a gradient set (min/B %): 0.0/10, 5.0/10, 20.0/15, 30/15, 40.0/25, 45.0/25, 55.0/40, 60.0/40, 65.0/100, 70.0/100, 75.0/10, 85.0/10. Full scan data were recorded in ESI (−) and ESI (+) mode from m/z 50 to 1500 and Leucine Encephalin as lock mass. Conditions used for both methods were based on previous experiences (Berenguer-Rivas et al., 2021).

BIOLOGICAL ACTIVITY

Cytotoxicity assay

MRC-5 SV2 (human fetal lung fibroblasts) and THP-1 (human monocytes) cells were purchased from ATCC (American Type Culture Collection). Cells were cultured in MEM + Earl's salts-medium and RPMI-1640 (Gibcoâ, New York, NY, USA), respectively, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate, and 5 % inactivated fetal calf serum. Fibroblasts and monocytes (5 x

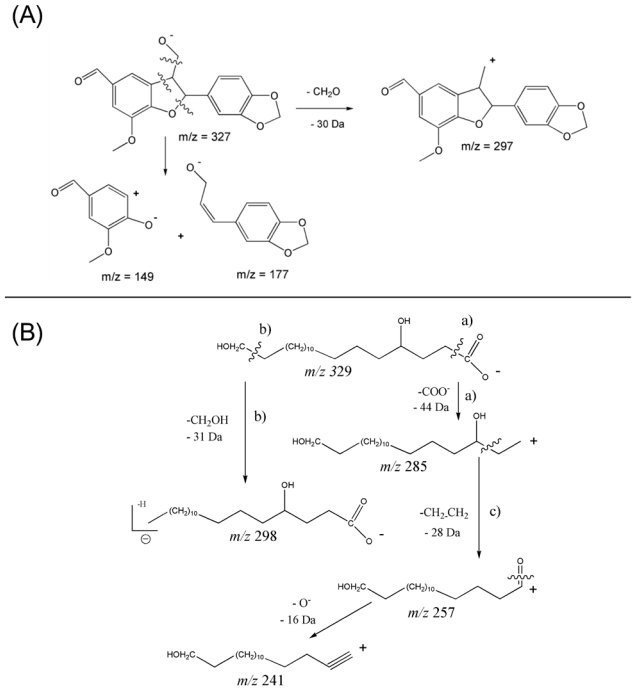

Figure 2. Fragmentation pathway proposed for (A) perseal C and (B) 4,19-dihydroxynonadecanoic acid; compounds identified in *P. Americana* Mill. seed extracts.

10^5 cells/mL) were seeded in sterile 96-well plates, containing 10 µL of the test substance at different concentrations ranging between 30 to 300 µg/mL, and incubated at 37 °C, 5 % CO_2 for 72 h. Cell growth was compared to untreated-control wells (100 % cell growth) and medium-control wells (0 % cell growth). The cell viability was assessed fluorometrically for 4 h after the addition of 50 µL/well resazurin solution (2.2 µg/mL) using a microplate reader (TECAN GENios, Germany) at λ_{ex} 550 nm, λ_{em} 590 nm. The results were expressed as percent reductions in cell growth/viability compared to control wells and IC_{50} was determined. Tamoxifen was used as a reference drug for cytotoxicity (García et al., 2019).

INSECTICIDE ACTIVITY

Colonies of Mosquitoes

Larvae and pupae from *Aedes aegypti* (Rockefeller strain) were obtained from the Arthropod Laboratory collection (ARTHROLAB, Federal University of Amapá, Brazil). Adults of the F3 generation were maintained in cages of size (30 x 30 x 30 cm) and were continuously provided with 10 % sucrose solution in a jar with a cotton wick. On day 5, the adults had access to blood meal from a rat placed in resting cages. Glass Petri dishes lined with filter paper soaking with 100 mL of de-chlorinated water were kept inside the cage for oviposition. The eggs thus obtained were immersed in larval trays containing de-chlorinated tap water for the hatching and biological source of the following experiments. Mosquitoes development conditions were: temperature, 27 ± 2°C; relative humidity, 70 ± 10 % and a photoperiod of 12:12 h (light: dark).

Larvicidal bioassay

This experiment was developed according the protocol suggested by the World Health Organization (WHO, 2005). Briefly, 100 mL consistent in one milliliter of the extract diluted in 99 mL of distilled water was added to a beaker of 250 mL, forming a homogeneous solution. Then, a total of 10 four-stage larvae per group (six concentration levels for each ***MaE*** and ***SE*** extract) were immersed into the beaker and their viability was measured at 24 and 48 h by a visual inspection of the motility during at least five minutes/beaker. Larval motility was stimulated by gentle beats on the beaker borders with a pencil.

As the first biological approach, two extreme extract concentrations (5 and 500 µg/mL) as well as, two intermediate concentrations (100 and 300 µg/mL) were explored. According to those preliminary results, six new levels of concentrations were defined. Results were expressed as the mean value of lethal concentration (LC_{50}) result from the three replicates performed. A group without treatment and 99 mL of distilled water with 1 mL of commercial ethanol (96 %) was used as control.

Pupicidal bioassay

This experiment followed the procedure above-mentioned, with similar sample concentrations and control group preparation than larvicidal assay. Consequently, ten pupae by beaker were immersed in the experimental solutions (five-level concentrations of each ***MaE*** and ***SE*** extract), recording the viability/mortality responses 24 h later. In the same way, results were expressed as the mean value of lethal concentration (LC_{50}) derived from the three replicates done.

STATISTICAL ANALYSIS

GraphPad Prism 7 (Windows, V. 7.04, 2017) software package was used to process the data. The results of the secondary metabolite quantification were expressed as the arithmetic means ± standard deviation (SD) of three replicates. The statistical differences between extracts composition, as well as, their larvicidal and pupicidal activities were determined by the *t*-Student´s test. LC_{50} and IC_{50} values were estimated using the Probit correlation and Pearson correlation factor. The effects of extracts on human monocyte viability were analyzed by one-way ANOVA followed by a Tukey´s test. Differences at $p< 0.05$ were accepted as significant.

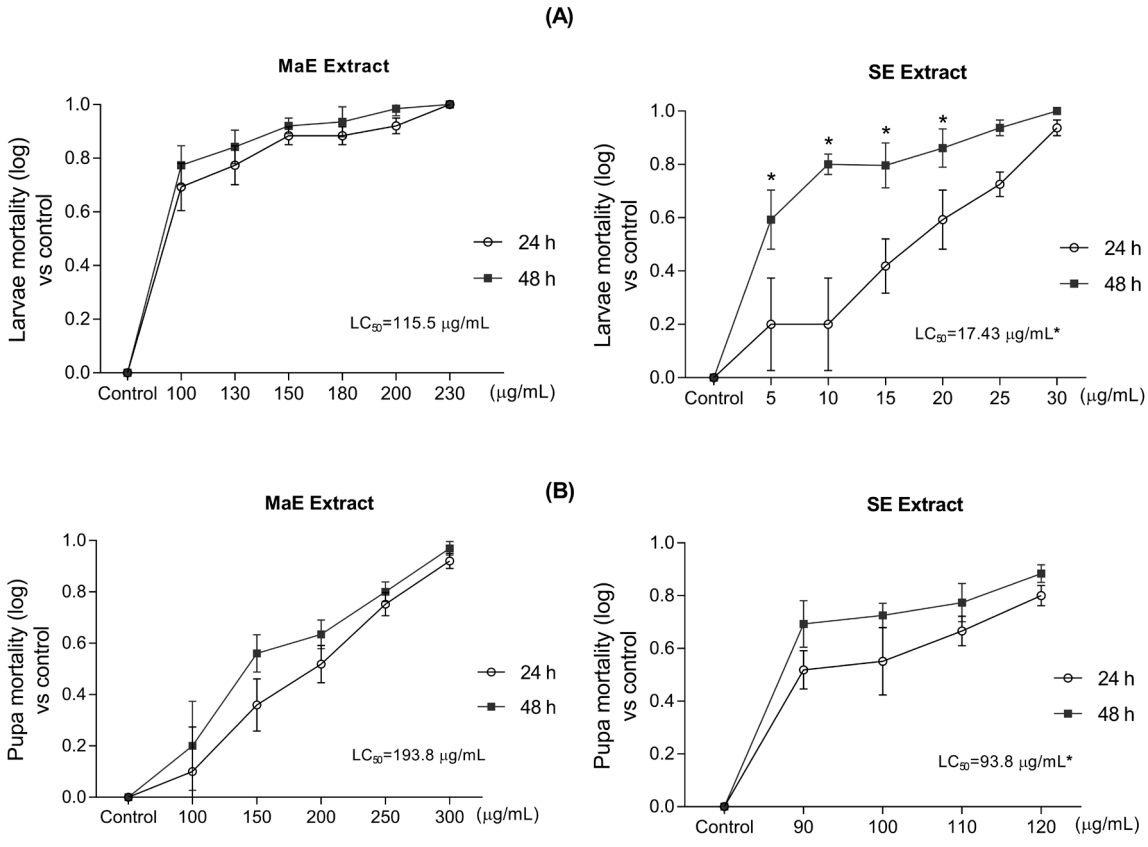

Figure 3. Larvicidal (A) and pupicidal (B) activity of *Persea americana* seed ethanol extracts (MaE and SE) against *Aedes aegypti*.

All values are expressed as the arithmetic mean ± SD of three replicates. (*) Express significant differences for each extract and distinct letters indicate statistical differences between LC_{50} ($p< 0.05$ in the *t*-Student´s test).

RESULTS

CHEMICAL COMPOSITION

QUANTIFICATION OF METABOLITES IN EXTRACTS

Secondary metabolites are commonly associated with insecticide activity; therefore, their quantification allows correlating their presence with the biological activity evaluated. Total extractable substances express the amount of substance that the solvent/extracting method combination can extract from the vegetal material. Considering that the solvent was the same for both extracts, differences in this parameter can be directly associated with the methodology (MaE or SE). Results demonstrated that the Soxhlet methodology was more efficient in the extraction of *P. americana* seeds metabolites with 42.13 ± 1.76 mg/mL, a value statistically different from the 20.46 ± 1.66 mg/mL obtained by maceration/shaking method ($p<0.05$). Statistical differences for these extractive methods were also found for total phenols, in which the 5.12 ± 0.18 mg/mL in SE were significantly different from the 3.57 ± 0.06 mg/mL obtained in MaE. On the other hand, the maceration/stirring method favored the extraction of carbohydrates contained in *P. americana* seeds, rendering values of 0.88 ± 0.08 mg/mL, higher than the 0.40 ± 0.14 mg/mL obtained when Soxhlet extraction was used. The other two metabolites quantified in this study did not evidence differences between extraction procedures: 0.49 ± 0.04 *vs* 0.43 ± 0.02 mg/mL for total proteins, and 2.28 ± 0.06 *vs* 1.88 ± 0.33 mg/mL for total flavonoids when Soxhlet and maceration/shaking methods were respectively used. In general, the sum of the quantified metabolites represents 19.68 and 33.04 % of the total extractable substances in SE and MaE, respectively, suggesting that for Soxhlet extraction other types of metabolites were extracted in higher amounts and the influence that heat can exert in the extraction of the metabolites from avocado seeds.

QUALITATIVE SCREENING

The screening indicated that there are some coincidences in the main peaks between both extracts with the following

retention times: Rt= 6.90, 12.75, 14.78, 20.73, and 21.50 minutes (Fig. 1, peak intensity over 25 % intensity) (Table 1).

Fig. 1 and Table 1 also show the higher complexity of SE extract, but the nature of the compounds remains analogous with a high incidence of polyphenol compounds as catechins and neolignanes derivatives, as well as some fatty acids/alcohols. Nine of the eleven peaks were assigned according to their pattern fragmentation.

Peak one was assigned to catechin or epicatechin gallate. Because of the nature of this spectroscopic method, it was impossible to define which of the isomers was present. Nevertheless, both substances (catechin and epicatechin) have been previously isolated from avocado seeds (Figueroa et al., 2018). Similar happened with peak 3 in which isomers were impossible to define. Compound two was identified as perseal C and its fractioning pathway proposed is illustrated in Fig. 2A. The other neolignan identified in this study was perseal A, corresponding to peak seven. These compounds have been previously informed in the species *Persea obovatifolia* (Tsai et al., 1996). Peak four was impossible to identify, but its molecular formula and fragmentation pattern suggest an unsaturated fatty alcohol. Peaks five and ten are free fatty acids, while peak nine was an acetate derivative. Pattern fragmentation allows defining the hydroxyl/ketone substituent but not the double bond positions in the case of compound nine. A plausible fragmentation pathway for a representative compound of this group (compound five) is presented in Fig. 2B. Peak six was the second one declared as unknown; Peak four was also impossible to identify, but its molecular formula and fragmentation pattern suggest some kind of lignin. Compound eight was assigned to a p-coumaric acid glucoside, a ubiquitous substance that has been also isolated from *P. americana*. At last, peak 11 was classified as a diterpene; several diterpenes have been informed for avocado seeds that match with the loss of four water molecules (MS/MS peaks with m/z = 365, 347, 329, 311) as perseanol and cinnzeylanone (Bhuyan et al., 2019).

BIOLOGICAL ACTIVITY

LARVICIDAL ACTIVITY

The larvicidal effects of avocado seed extracts against mosquito larvae were tested (Fig. 3A). Soxhlet extract exhibited statistically significant differences in larvae mortality of *A. aegypti* (LC_{50} 17.4 µg/mL) in the first 24 h at lower concentrations (20-30 µg/mL), augmenting the larvicidal effect at 48 h ($p< 0.05$). It is highlighted that an increase in the concentration of the SE extract raised the death at this vector stage, following a dose-response pattern (R^2= 0.9677). On the opposite, MaE extract similarly inhibited the larvae growth but at high concentrations either 24 h or 48 h with an LC_{50} of 115. 5 µg/mL.

PUPICIDAL ACTIVITY

Both ethanol extracts exerted pupicidal activity against *Aedes aegypti* mosquitoes after 24 and 48 h of exposition (Fig. 3B). In general, the ES extract showed the highest pupicidal mortality (LC_{50} 93.8 µg/mL) when compared with MaE

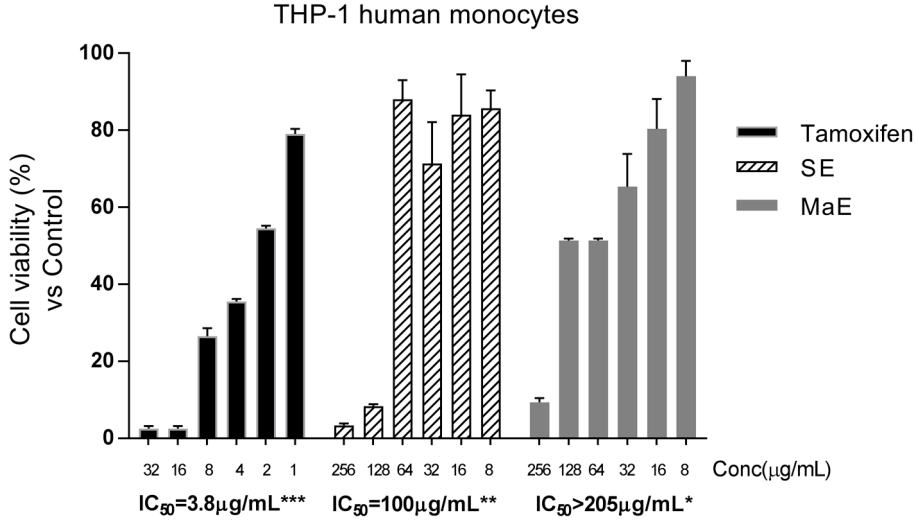

Figure 4. Effect of *Persea americana* seed ethanol extracts on THP-1 human monocyte cells viability.

The effect of ethanol extracts on the viability of human monocyte cells was determined by the Resazurin dye reduction test. Tamoxifen was used as positive control of cytotoxicity. All values are expressed as the arithmetic mean ± SD of six replicates. Distinct letters express significant differences at $p<0.05$ for IC_{50} (one-way ANOVA followed by a Tukey´s test).

(LC_{50} 193.8 µg/mL), following also a dose-response pattern (R^2=0.9235).

CYTOTOXICITY ACTIVITY

The avocado seed extracts were not cytotoxic to human monocyte cells at the doses used for insecticidal assays (MaE: IC_{50}>205 µg/mL and SE: IC_{50}=100 µg/mL), as judged by the results obtained from Resazurin cell viability assay (Fig. 4). Similar pattern was found for human fetal lung fibroblast cells (MRC-5 SV2) with an IC_{50}>64 µg/mL for SE extract, while MaE extracts showed an IC_{50}>256 µg/mL (data not shown). Tamoxifen treatment resulted in cell damage in both human cell lines.

DISCUSSION

The use of botanical products to control the incidence of different vectors has gained in attention, mainly because of the negative effect of chemical insecticides, which are continuously used despite their enormous mammalian toxicity. Natural resources like plants represent potential biofactories to obtain active bioproducts lacking hazardous effects.

Various strategies have been performed to "mosquito's control" using herb extracts and in preference plant by-products; most specifically during the life period of greatest vulnerability: larvae and pupae stages. The results obtained in this paper highlight that: a) *Persea americana* seed extracts exhibit an important matrix rich in different phytochemical active metabolites; b) the plant extracts act as an insecticide against mosquitoes at larvae and pupa stages; c) the ethanol extracts from avocado seed were not cytotoxic to human cell lines.

Overall, plant extracts showed some qualitative and quantitative differences in their chemical composition; therefore, some differences in the biological profile can also be expected. Several studies have shown the relationship between the chemical composition of avocado seeds and their biological activity (Dabas et al., 2013). A summarized revision of the scientific literature reveals that aqueous, methanol, and ethanol extracts from avocado seeds exhibited different *in vitro* and *in vivo* biological activities such as antimicrobial, anti-inflammatory, anti-hypertensive, anti-cancer, etc., probably due to the low-molecular-weight compounds identified (Dabas et al., 2013; Egbuonu et al., 2018). However, only hexane and methanol extracts were evaluated as insecticide (larvicidal) showing a noxious effect in *Aedes aegypti* mosquito species, but at very high concentrations (16.7 and 8.9 mg/mL), respectively (Leite et al., 2009).

Another study evaluated avocado seed extracts as a botanical pesticide to control the *Anopheles gambiae* mosquito at the larvae phase, one of the vectors responsible for malarial transmission. Ethyl acetate, chloroform, and acetone extracts from avocado seeds displayed good larvicidal activities against this mosquito species. Nevertheless, the authors did not correlate the biological activity with the proximal avocado seed composition (Adesina et al., 2016). These studies highlight the need to link the potential active compounds with the observed effects.

We were previously informed by a qualitative phytochemical analysis of the presence of low-molecular-weight metabolites such as alkaloids, coumarins, tannins, flavonoids, sugars, and amino acids in similar ethanol seed extracts (Molina-Beltrán et al., 2018). Here, we additionally quantified the phenols and flavonoid constituents extracted by maceration- and Soxhlet methods, which were statistically higher in the SE extract. Moreover, the UPLC-DAD-MS/MS analysis revealed the presence of phenolic compounds type catechin and neolignans, as well as, fatty alcohols/acids. The determination of these UPLC profiles can be also used as a fingerprint for the tested extracts monitoring the quality control of those potential herb insecticides. Taking into account the distribution of this plant worldwide, mainly in tropical countries, and its growth in different soil conditions, this powerful tool would allow better control of the chemical composition of extracts.

Both ethanol extracts were effective against *Aedes aegypti* mosquito. Nevertheless, in this work, the best larvicidal and pupicidal activities were observed in Soxhlet-derived extract. It is noted that SE extract was more lethal at lower concentrations showing not significant *in vitro* toxic effect to mammalian cells at those concentrations. SE extract was also more active against mosquito larvae at 48 h of treatment; however, the bioactivity evidenced at 24 h was even better compared with MaE extract.

The increment of vector mortality evidenced in this study could be associated with the presence of low-molecular-weight compounds in the ethanol extracts, which potentially inhibit the developmental larvae phase of *Aedes aegypti*. Soxhlet-derived ethanol extract showed a higher polyphenols and flavonoid composition that can support the chemical correlation with the insecticidal action. It has been suggested that the action may be triggered by the interaction of these bioactive compounds with cuticle membrane or larvae, ultimately disordering the membrane, and provoking the larval death (Adesina et al., 2016).

A study explored the larvicidal activity of four flavonoids identified in *Milletia pinnata* (L) seed extract against different mosquito species. The flavonoids karanjachromene, pongamol, and pongarotene, strongly inhibited mosquito larval, whereas karanjin exhibited less marked effect (Perumalsamy et al., 2015). Likewise, Gautam et al. (2013) stated that two flavonoid-enriched extracts from *Vitex negundo* (L) and *Andrographispa niculata* (Nees) exerted larvicidal activity against instar larvae of *Aedes aegypti* and *Anopheles stephensi*. The authors summarized that extracts could be useful to develop a flavonoid-based eco-friendly pesticide as an alternative to chemical insecticides.

In this study, flavonoid-type catechin derivates are found in both SE and MaE avocado seed extracts, and these compounds have been extensively recognized as good insecticides (Muema et al., 2016). In this context, Elumalai et al. (2016) reported the larvicidal activity of catechin isolated from *Leucas aspera* against *Aedes aegypti*, *Anopheles stephensi,* and *Culex quinquefasciatus*. Neolignans also have good activity as insecticides (Chauret et al., 1996; Narciso et al., 2014). These compounds are present in both tested extracts. Therefore, there is evidence that the insecticidal activity observed in this work could be related to the presence of catechins and neolignans in those prepared extracts.

In previous work, we presented that an ethanolic extract from *P. americana* seed obtained by the Soxhlet method largely diminished the viability of larvae and the neolarva period to adults of *Musca domestica*. The extract, rich in different types of secondary metabolites, also affected the post-embryonic development of the vector (Molina-Bertrán et al., 2018).

In addition to low-molecular-weight compounds, primary metabolites like proteins and carbohydrates are known to exert a wide spectrum of biological activities (Hikal et al., 2017). The main biochemical composition of dried and powder avocado seeds have been also examined by Ejiofor et al. (2018) but with the objective of determining the nutritional value. They found higher carbohydrate (49.03 ± 0.02 g/ 100 g) and protein (15.55 ± 0.36 g/ 100 g) contents compared with the values reported in the current study. However, to the best of our knowledge, there are no further evidences that correlate these primary metabolites with the biological activity focused to decrease the vector spreading.

CONCLUSIONS

In sum, *Persea americana* seed-derived extracts contain different bioactive phytochemicals, which would be useful as natural larvicides to combat mosquito populations. Ethanol extracts rich in polyphenols, mainly catechin and neolignans constituents, inhibited the larval and pupal immature stages of *Aedes aegypti* mosquitos. Soxhlet-derived extract showed a higher vector mortality rate at low concentrations. The study demonstrated that *P. americana* was non-toxic to mammalian cells. Finally, plant by-products like avocado seed can be considered an eco-friendly insecticide and its use may help to substantially decrease the vector-transmitted diseases in developing countries.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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