Acta Pharm. Sci. Vol 61:(3), 2023
DOI: 10.23893/1307-2080.APS6118

Chemical composition, antimicrobial and antioxidant activity of a "lipid phase" from *Zanthoxylum pistaciifolium* Griseb leaves

Yamilé HEREDIA DÍAZ[1*], Julio C. ESCALONA ARRANZ[1], Ania OCHOA PACHECO[1], Rosalia GONZÁLEZ FERNÁNDEZ[2], Pedro L. BATISTA CORBAL[2], Paul COS[3], Alexandra ESCALONA CAPARROS[1]

1 Pharmacy Department, Faculty of Natural and Exact Sciences, Oriente University, Avenue Patricio Lumumba s/n, 90500 Santiago de Cuba, Cuba

2 Medical Toxicology Centre (TOXIMED), Medical Sciences University, Highway km 1½, CP 90400. Santiago de Cuba, Cuba

3 Laboratory for Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Belgium

ABSTRACT

The present research aimed is to carry out the phytochemical analysis and to evaluate the antimicrobial and antioxidant activity, of the lipid phase from Cuban endemic plant *Zanthoxylum pistaciifolium* Griseb leaves. The total extract was fractioned by Column Chromatography, while its lipid fractions were analyzed by Nuclear Magnetic Resonance and Gas Chromatography-Mass Spectrometry. Antimicrobial activity versus a strain panel of two bacteria and six yeast was tested by microdilution method. Antioxidant activity was evaluated as the scavenging property on $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals. For the first time 31 compounds are informed, among which fatty acid derivatives and sesquiterpenes prevail. Ethyl palmitate and geranyllinalool emerge as the main compounds. Good anti-*Candida* activity as well as a moderate radical scavenging property were demonstrated. The cell viability determined reflects a slight toxicity over Vero cells (IC_{50} in 34.9 ± 0.78 µg/mL). The investigation reveals that lipid phase has an interesting potential for pharmaceutical applications.

Keywords: Antimicrobial activity, ethyl palmitate, gas chromatography-mass spectrometry, geranyllinalool, radical scavenging

*Corresponding Author: E-mail: yherediad@gmail.com
ORCIDs:
Yamilé HEREDIA DÍAZ: 0000-0003-3863-0668
Julio C. ESCALONA ARRANZ: 0000-0003-3609-4451
Ania OCHOA PACHECO: 0000-0001-9069-6897
Rosalia GONZÁLEZ FERNÁNDEZ: 0000-0001-8268-2428
Pedro L. BATISTA CORBAL: 0000-0001-7733-7638
Paul COS: 0000-0003-4361-8911
Alexandra ESCALONA CAPARROS: 0000-0003-4344-7315
(Received 02 Nov 2022, Accepted 24 March 2023)

INTRODUCTION

Zanthoxylum genus belongs to the Rutaceae family. Compiled by Linné in 1757, it comprises about 573 species distributed worldwide mainly in tropical and temperate regions[1]. Species of this genus stand out due to their economic importance as source of edible fruits, oils, wood, raw materials for industries, and for having ornamentals, culinary and medicinal applications[2]. With such applications diversity, it constitutes one of the most remarkable genera not only within its family but also the plant kingdom. From a chemical point of view, this genus is characterized by a high production of various types of alkaloids, lignans, coumarins and amides, all of which present chemotaxonomic relevance to the genre. Furthermore, other metabolite types such as flavonoids, sterols and terpenes have been isolated[3].

Zanthoxylum pistaciifolium Griseb is one of the species that grows in Cuba. Known as "palo vencedor", "pensador" or "bálsamo", it is a common shrub in arid coastal lands of Cuba, mainly in the Eastern region. Ethnobotanic reports refer that this plant has been used for aromatic baths[4], pulmonary infections and other associated diseases (particularly cold)[5], ear pain and others[6]. The species is traditionally used as an oily extract obtained from the leaf in the treatment of earaches and there are yet no reports that justify it. One of the most common infections affecting the human ear is otomycosis, also known as external fungal otitis, which occurs due to fungal growth of *Candida* spp. This fungal growth associates with bacteria and cause more complex harder to treat ear infections.

From a chemical point of view there is scarce information about the composition of this plant, only the presence of volatile compounds as α-pinene (12.35%), linalool (6.68%), 2,6-dimetil-2,4,6-octatriene (6.50%), limonene (6.19%) and phytol (6.06%) have been reported before[7]. On the other hand, no precedent of pharmacologic studies has been found, therefore, there are not enough scientific evidence to support its ethnopharmacological use as antifungal and antibacterial by the Cuban population. Considering the lack of such precedents, this work aims to investigate the chemical profile of the lipid fractions of *Z. pistaciifolium* leaves, as well as to determine their antimicrobial and antioxidant activity.

METHODOLOGY

Collection of the plant material

Leaves from *Zanthoxylum pistaciifolium* were collected in October 2017 at "El Palenque", close to Siboney neighborhood, Santiago de Cuba, Cuba. Botanical specimens were identified by Professor Félix Acosta Cantillo, and a voucher specimen (No. 21660) was deposited in the Herbarium of the Eastern Center of Ecosystems and Biodiversity.

Extraction, separation and structural analysis of the lipid fraction compounds

A total extract in ethanol 95% was prepared with dry and milled leaves using percolation methodology. Once prepared, the extract was left to repose, filtered, and dried using a vacuum rotator evaporator (IKA-Werke, Germany) at 40°C. The obtained 60 grams were defatted using a liquid-liquid extraction with hexane to be later chemically characterized and evaluated in their biological activity. The hexane phase (lipid phase, LP) was dried and prepared for a column chromatographic separation, using gel silica 60 (0.063-0.2 mm/70-230 Mesh ASTM, Macherey-Nagel, Germany) as stationary phase. A gradient elution starting with n-hexane followed by mixtures of hexane: dichloromethane (95:5 - 5:95), pure dichloromethane, dichloromethane: ethyl acetate (95:5 - 5:95), pure ethyl acetate and ethyl acetate: methanol (95:5 - 50:50) was used.

In accordance to their behavior on the Thin Layer Chromatography (TLC), the fractions derived from previous separation were joined. Glass plates (20 x 20 cm) with internal fluorescence indicator were used, which were revealed with an UV-lamp (Biosystems, Brazil) at 254 and 365 nm and iodine vapors. The fractions that appeared as pure or non-complex were analyzed by Nuclear Magnetic Resonance (NMR), and afterwards by Gas Chromatography and Mass Spectrometry (GC-MS).

The NMR spectra was recorded in a VARIAN apparatus (USA) operated at 200 MHz (^1H). The chemical shifts were analyzed with MestRenova software version: 6.1.0-6224 of 2010. Deuterated chloroform (Cambridge Isotope Laboratories, Inc. USA) was used as solvent. The GC-MS was performed using a SHIMADZU GC/MS-QP2010 apparatus with an auto-injector AOC-20i (Japan), composed by a Mega 2 series chromatography coupled to a quadrupolar spectrometer of positive electronic impact (70e–V) as ionization mode and a mass range between 13 and 500 *m/z*. A validated program for the separation of fatty compounds was used, consisting of an Rtx-5 MS capillary column (30 m x 0.25 mm x 0.25 μm) and helium as carrier gas with a flow rate of 1 mL min^{-1}. The injection port temperature was 250°C while the ion trap and transfer-line temperatures were 250°C. The oven temperature was programmed at 60ºC (3 minutes) increasing by 40°C min^{-1} until reaching 140°C, to continue with a ramp temperature of 4°C min^{-1} until completion at 300°C.

Linear retention indexes were calculated in relation to homologous series of n-alkanes (C8-C24). Percentages of constituents were determined based on their GC-FID peak areas, using the normalization procedure without corrections for response factor (EZChrom v 6.7 software). Compounds were identified as far as possible by comparing fragmentation patterns in their mass spectra with those stored on the National Institute of Standards and Technology (NIST) library[8] and with literature data[9]. Identity was confirmed in many compounds by means of their Kovacts retention indexes. To process spectra, the software GC/MS solution version 2.70 of 2010 was used, in its "postrun analysis" option.

Microorganisms and reference drugs

The antimicrobial activity of the lipid phase was tested facing it to three bacteria and six yeast strains supplied by the Laboratory for Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Belgium. *Candida albic*ans ATCC B59630 (Azole Resistant), *Candida glabrata* ATCC B63155, *Candida kefyr* ATCC B46120, *Candida krusei* ATCC B68404, *Candida parapsilosis* ATCC J941058 and *Candida tropicalis* CDC49 were the yeast strain used while *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 were the bacteria ones. Miconazole and ampicillin (Sigma-Aldrich, USA) were used as reference drugs for fungi and bacteria, respectively.

In vitro antimicrobial activity

*In vitr*o antibacterial and antifungal activity was determined by the microdilution method with resazurin (redox indicator) in sterile 96-well microplates[10]. In each well 10 μL of the sample were added together with 190 μL bacteria inoculum ($5x10^5$ CFU/mL) and yeast inoculum ($5x10^3$ CFU/mL). Untreated control wells (100% cell growth) and medium-control wells (0% cell growth) were included in the microplates. Later on, the microplates were incubated at 37°C for 17 hours (for bacteria) and 24 hours (for yeast). Afterwards, 20 μL of resazurin (Sigma-Aldrich, USA) at 50 μgmL^{-1} per well were added and the microplates were incubated under the same temperature conditions (bacteria: 30 min and yeast: 4 hours). Microbial growth was determined by fluorimetry method (λex= 550 nm, λem= 590 nm) using a microplate reader (Tecan, Mechelen, Belgium). The product was classified as active when the bacterial growth inhibition (%) was greater than 50%. The results are expressed as percentage reduction in bacterial growth/viability compared to control wells. To accomplish this, LPs were dried and solved in dimethyl sulfoxide (DMSO) at 1 g mL^{-1}. Serial dilutions in pure water were made to obtain five levels of concentrations corresponding to: 4.0, 2.0, 1.0, 0.5, 0.25 mg mL^{-1}. Each concentration was screened by triplicate and the results were reflected as mean ± standard deviation.

Scavenging activity facing the radicals 2,2-azino-bis- (3-ethyl benzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) and 2,2 diphenyl -1-picrylhydrazyl ($DPPH^{\cdot}$)

The scavenging capacity on $ABTS^{\cdot+}$ (Merck, KGaA, Darmstadt, Germany) radical was developed according to the methodology described in the literature[11]. Different concentration of the LP (solutions of 62.5 to 1 000 μg/mL based on the extract's dry weight) were added to 3 mL of diluted $ABTS^{\cdot+}$ solution and after 90 min the absorbance was measured at 734 nm. Ascorbic acid (Fluka, 99 % pure, Germany) at a concentration of (1 mg/mL) was considered as positive control. The radical quenching activity was determined by calculating the percent inhibition of the radical. The $ABTS^{\cdot+}$ radical scavenge of *Z. pistaciifolium* extracts were estimated as a function of the extract concentration capable to quench the 50 % of the radical (IC_{50}) obtained by interpolation in the curve constructed from the five evaluated concentrations. All experiments were repeated three times.

The scavenging capacity on $DPPH^{\cdot}$ (Merck, KGaA, Darmstadt, Germany) radical was developed according to the methodology described in the literature[12]. In short: A solution of 0.1 mM of $DPPH^{\cdot}$ was prepared using 0.00394 g dissolved in 100 mL of ethanol. A total of 0.25 mL of the LP (solutions of 62.5 to 1 000 μg/mL based on the phase dry weight) were placed in test tubes, where were added 1.5 mL of the $DPPH^{\cdot}$ solution. The mix was shaken in a vortex (Heidolph REAX 2000, Germany) and kept in the dark for 20 min. The absorbance was measured in spectrophotometer (T60 UV-Visible Spectrophotometer) at 517 nm. The positive control was an ascorbic acid solution at 1 mg/mL. The radical sequestration ability was determined by calculating the percent inhibition of the radical. The antioxidant capacity against these radicals was expressed as a function of the half inhibitory concentrations (IC_{50}) of the tested extracts obtained by interpolation in the calibration curve constructed. All the experiments were developed by triplicate.

Cell viability

Cellular proliferation and viability were assessed in Vero cells (green monkey kidney cells) purchased from ATCC (American Type Culture Collection). Cells were incubated at 37°C in 5% CO_2 atmosphere, seeded in sterile 96-well microtiter plates on a Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) and supplemented with 10% inactivated fetal calf serum (FCS) (Sigma-Aldrich, USA), 2% of L-glutamine and D-glucose (4.5 g L^{-1})[10].

Cell viability was measured as follow: 200 µL of cell inoculum ($5x10^5$ cell/well) were added in 96 well microplates and incubated by 24 hours at 37 °C in 5 % CO_2 atmosphere. The old medium was removed, and the wells were washed twice with fresh Saline Dulbecco's Phosphate Buffer, so that 100 µL of the LP at concentrations from 8 to 256 µg mL^{-1} could be added later. The microplates were incubated for another 72 hours under the same conditions. Next, a volume of 50 µL of resazurin was added to each well and the plates were incubated against for 4 hours at 37°C, 5% CO_2 to complete the assessment of cellular viability. This was performance by measuring the fluorescence at λex 550 nm, λem 590 nm with a microplate reader (Tecan, Mechelen, Belgium) and using tamoxifen (Sigma-Aldrich, USA) as a reference drug (positive control, from 3.6 to 114 µM)[13]. Untreated-control wells were used as solvent control. The results were expressed as percent reduction in cell viability as compared to untreated-control wells; the 50% cytotoxic concentration (CC_{50}) was determined.

Statistical analysis

All analyses were performed using the software SPSS v.19 by ANOVA analysis. Bonferroni test was performed to indicate significant differences between groups with $p \geq 0.05$. Results were reflected as mean ± standard deviation.

RESULTS AND DISCUSSION

Chromatographic separation and structural characterization

Eight grams of the lipid phase were fractioned using Colum Chromatography with gel-silica 60 as stationary phase. The column was eluted with an increasing polarity gradient of different mixtures of n-hexane and dichloromethane. The 56 fractions generated were grouped according to their behavior on TLC on iodine vapors and ultraviolet light at two wavelengths (254 and 365 nm) to get four main fractions (FH_1, FH_2, FH_3, FH_4).

Fraction 1 (FH_1): This fraction was obtained as a colorless semisolid and eluted with hexane and shows multiple signal from δ_H 0.77 to 0.89 ppm at NMR ^1H (200 MHz, $CDCl_3$) spectra, indicating terminal methyl groups. Additionally, signals from 1.26 to 2.05 ppm were observed, which indicate the presence of CH_2 groups from a saturated hydrocarbon chain. Signals at δ_H 2.32, 2.42, 2.99 and 3.88 ppm suggest *ortho*-CH_3 groups from a different pattern of substitution on cycled hydrocarbons exist, which are commonly present in essential oils. The double triplet at δ_H5.15 can be associated to an olefin (sp^2) proton. Those appreciations are confirmed by the results of GC/MS (see Table 1) where four non-aromatic sesquiterpenes were identified (56.96%), nine saturated hydrocarbons (29.82%) and an unsaturated triterpene squalene

(7.48%). This kind of composition has been informed before for other *Zanthoxylum* species. The hexane extract of *Z. naranjillo* was rich on sesquiterpenes including β-selinene, while the most abundant hydrocarbon was nonacosane (13.22%), compound that also appears in *Z. guilletii*[14,15].

Table 1. Relative abundance in the lipid phase of *Z. pistaciifolium* identified by GC-MS

Nº	Compound	RIc[a]	PIr[b]	A[c] (%)
Fraction 1 (FH_1)				
FH_{1-1}	α–Himachalene	1449	1449	3.63
FH_{1-2}	Selina-4,11-diene	1474	1476	5.99
FH_{1-3}	β-selinene	1492	1492	23.92
FH_{1-4}	α-selinene	1500	1501	23.42
FH_{1-5}	Octadecane	1799	1800	2.49
FH_{1-6}	Nonadecane	1898	1900	2.20
FH_{1-7}	Eicosane	1999	2000	2.30
FH_{1-8}	Heneicosane	2099	2100	2.35
FH_{1-9}	Docosane	2199	2200	2.43
FH_{1-10}	Tricosane	2296	2300	2.67
FH_{1-11}	Unknown	2354		5.74
FH_{1-12}	Tetracosane	2397	2400	2.16
FH_{1-13}	Squalene	2837	2836	7.48
FH_{1-14}	Nonacosane	2898	2900	13.22
Total identified (%)				92.9
Hydrocarbon compounds				94.26
Non terpene				29.82
Sesquiterpenes				56.96
Triterpenes				7.48
Fraction 2 (FH_2)				
FH_{2-1}	Ethyl tetradecanoate	1795	1794	1.82
FH_{2-2}	Ethyl palmitate	1999	1993	86.38
FH_{2-3}	Ethyl heptadecanoate	2095	2097	1.90
FH_{2-4}	Oleic acid	2171	2171	3.25
FH_{2-5}	Ethyl stearate	2196	2197	6.65
Total identified (%)				100
Oxygenated compounds				100
Fatty acid and it derivatives				100
Fraction 3 (FH_3)				
FH_{3-1}	Pentadecane	1497	1500	1.16
FH_{3-2}	Ethyl 9-oxononanoate	1508	1507	2.49

FH_{3-3}	Hexadecane	1597	1600	2.31
FH_{3-4}	Heptadecane	1696	1700	1.96
FH_{3-5}	Benzyl Benzoate	1785	1789	5.40
FH_{3-6}	Benzyl salicilate	1889	1886	2.33
FH_{3-7}	Ethyl palmitate	1992	1993	24.57
FH_{3-8}	Methyl 10-octadecenoate	2100	2100	1.16
FH_{3-9}	Ethyl-9,12-octadecadienoate	2165	2166	23.73
FH_{3-10}	Oleic acid	2172	2171	29.96
FH_{3-11}	Ethyl stearate	2191	2197	2.14
FH_{3-12}	Phytol acetate	2216	2212	2.79
Total identified (%)				100
Hydrocarbon compounds				5.43
Non terpene				5.43
Oxygenated compounds				86.84
Fatty acid and it derivatives				81.56
Terpenes				2.79
Others				2.49
Aromatic compounds				7.73
Fraction 4 (FH_4)				
FH_{4-1}	(5Z)-6,10-Dimethyl-5,9-undecadien-2-one	1453	1450	1.73
FH_{4-2}	3-Cyclohexene-1-carboxaldehyde, 4-(4-methyl-3-pentenyl)	1535	1534	1.91
FH_{4-3}	Selin-6-en-4α-ol	1639	1636	10.94
FH_{4-4}	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-	1922	1923	3.23
FH_{4-5}	Geranyllinalool	2036	2034	65.99
FH_{4-6}	Hexadecane, 5-octyl	2273	2270	2.60
FH_{4-7}	Bis(2-ethylhexyl) phthalate	2555	2552	9.45
FH_{4-8}	Hexacosane, 8 methyl	2630	2634	1.99
FH_{4-9}	Nonacosane	2903	2900	2.16
Total identified (%)				100
Hydrocarbon compounds				9.98
Non terpene				6.75
Terpene				3.23
Oxygenated compounds				90.02
Sesquiterpenes				10.94
Diterpenes				65.99
Others				13.09
Total compounds				36

[a]Calculated retention index, [b]Reported retention index, [c]Relative area

Fraction 2 (FH_2): This fraction was obtained as an intense yellow semisolid and eluted with hexane: dichloromethane (45:55 v/v), it shows a singlet at δ_H 0.82 and 0.86 ppm, which is characteristic of terminal CH_3; as well as one at δ_H1.23 which characterizes CH_2 from aliphatic hydrocarbons. Nevertheless, a singlet at 2.01 and a triplet at 2.26 ppm indicate unsaturation and/or α-carbonyl CH_2 group. The multiplet at 4.10 ppm can be associated to substitutions type O-CH_2, while the doublet at δ_H 5.23 ppm is related to olefinic proton (CH). (Figure 1). Altogether, this fraction looks like a mixture of free or esterified saturated or un-saturated fatty acids. The GC-MS analysis (Table 1) confirmed a 96.75% of esterified fatty acid plus the oleic acid (3.25%) in agreement with the suggestions derived from NMR experiment. Fragments *m/z* 88 and 101 are the most abundant in most of them corresponding to alpha and beta carbonyl bond-breaking (Figure 2). Molecular ions were identified at *m/z* 228,

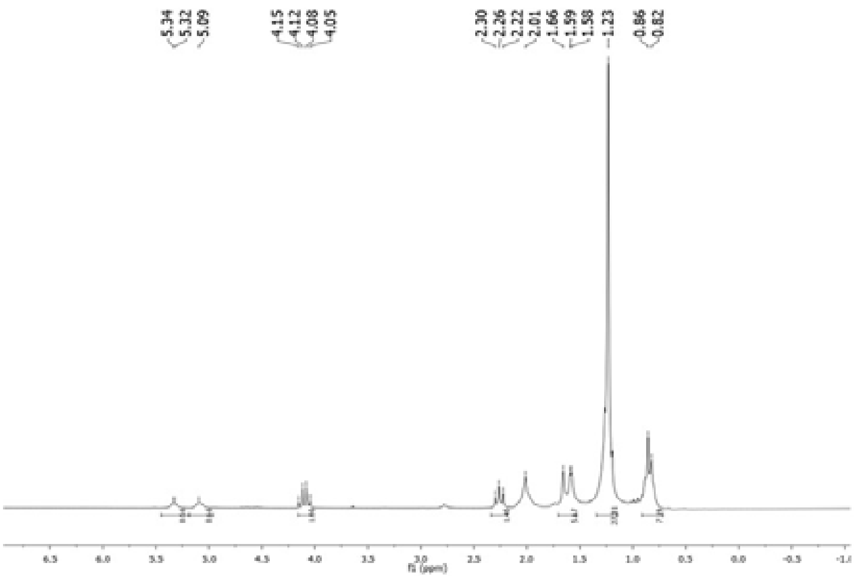

Figure 1. NMR 1H spectrum ($CDCl_3$, 500 MHz) for FH_2 fraction

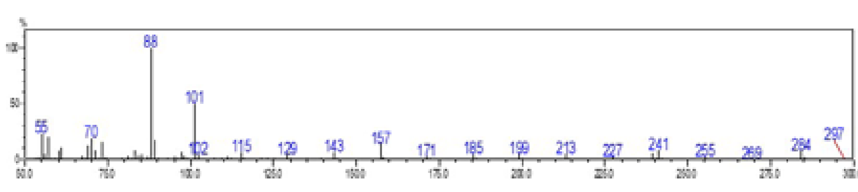

Figure 2. Mass spectra for the ethyl palmitate (FH_{2-2}) (positive electronic impact)

Fraction 3 (FH_3): This fraction was obtained as an intense yellow semisolid and eluted with hexane: dichloromethane (35:65 v/v). With a similar NMR pattern that the previous sample (FH_2) it shows few differences as double at 8.05; 7.10 ppm and the singlet at 7.41 ppm, as well as small signals at 3.46 ppm characteristic of methoxyl moiety. GC/MS shows that oxygenated compounds are quite abundant (Table 1), and account for up to 86.84% but mostly with fatty acids (81.56%). Oleic acid was classified as the main compound with a relative abundance of 29.26%, followed by aromatic compounds (7.73%) and non-terpene hydrocarbons (5.43%).

Fraction 4 (FH_4): This fraction was obtained as a tenuous yellow oil and eluted with pure dichloromethane showing the classical hydrocarbon signals at δ_H 0.86, δ_H 1.23 and δ_H 1.65 ppm. The singlet at 2.02 ppm looks like protons at α-carbonyl group are present, while the signal at δ_H 4.06 ppm can be associated to O-CH_2 moiety. At last, evidence at δ_H 5.10 ppm and a multiplet signal at 5.42 ppm indicate olefin protons belonging to conjugated or non-conjugated systems (Figure 3). GC/MS analysis confirms those observations (Table 1) with geranyllinalool as the most abundant compound with 23.368 minutes as retention index. This compound appears in the middle of the chromatogram along other eight compounds with retention indexes between 10 and 42 minutes (Figure 4). Considering the relative abundance, once again compounds with oxygen moiety constitute the majority. The fragment *m/z*= 69 is characteristic to the bond-breaking of the last double-bond of geranyllinalool (Figure 5).

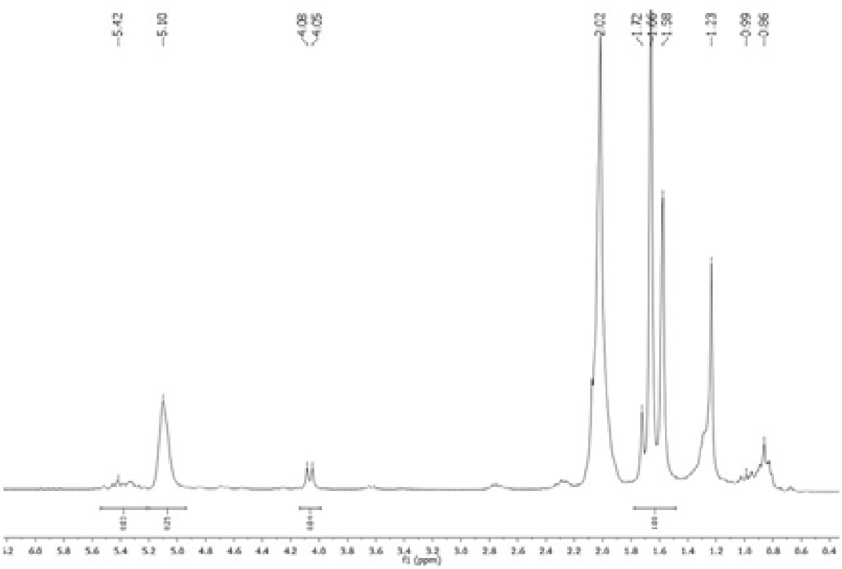

Figure 3. NMR ^1H spectrum ($CDCl_3$, 500 MHz) for FH_4 fraction

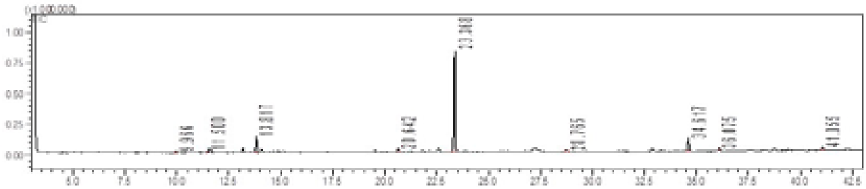

Figure 4. GC-MS chromatogram obtained for FH_4 fraction

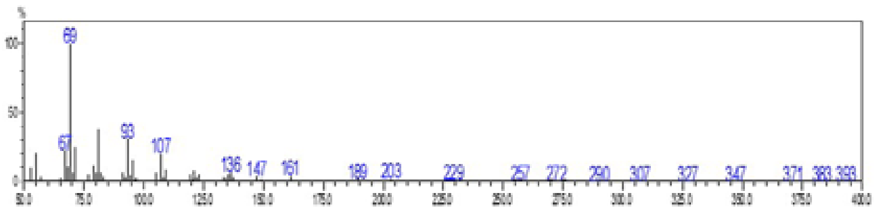

Figure 5. Mass spectra for geranyllinalool (FH_{4-5}) (positive electronic impact)

In general, this phytochemical procedure allowed to identify 35 compounds that represent 97.22% of all the peaks. In the fraction 1, thirteen compounds were identified, of which β-selinene and α-selinene were the most abundant. Five compounds were identified for fraction 2, ethyl palmitate was the most abundant. On the other hand, for fraction 3 the oleic acid was it the majorities of the twelve total compounds. Of the nine metabolites recognized in fraction 4, geranyllinalool was the most abundant. Four substances were identified in more than one fraction: ethyl palmitate, ethyl stearate, oleic acid (Fractions 2 and 3) and nonacosane (Fractions 1 and 4).

Ethyl palmitate looks like the most abundant one of all compounds. Many chemically different types of compounds were identified: 14 non-terpene hydrocarbons, eight fatty acid derivatives, five sesquiterpenes, two diterpenes, two aromatic compounds and others. A low level of free fatty acid was observed as well as the absence of steroids. This chemical composition matches reports of hexane extracts prepared from other species for the genre as *Z. armatum, Z. dipetalum*, *Z. kauaense* and *Z. hawaiiense* in which the terpene substances are common, as well as saturated or unsaturated hydrocarbons[16,17]. Nevertheless, the relative abundance and diversity of fatty acids is higher than the one previously informed for the *Zanthoxylum* genre[18].

Sesquiterpenes were the third most abundant metabolite type, which matches previous reports of chemical composition for *Z. naranjillo* in which β-selinene was also identified[14]. Essential oil studies of other *Zanthoxylum* species refer to an abundance of this kind of compounds, such is the case of *Z. schinifolium* in which a 46.45% was informed with β-selinene as main compound[19]. The species in study (*Z. pistaciifolium*) is not an exception, as our research group once again reports β-selinene in its composition, together with selin-4,11-diene, and α-selinene (main compounds)[7].

These results enrich the knowledge about *Z. pistaciifolium* leaves' chemical composition, considering that 31 of the identified compounds are reported for the first time in this plant.

In vitro antibacterial and antifungal activity

Table 2 shows the concentration in which 50% or more of the microbial growth is inhibited. A good of activity against some yeast as *Candida albicans*, *Candida glabrata*, *Candida kefyr*, *Candida krusei*, *Candida parapsilosis* and *Staphylococcus aureus* bacteria can be observed. The antifungal activity of *Zanthoxylum* spp. extracts reports are quite common, but not those of lipoid nature. The activity of petroleum ether extracts from *Z. acanthopodium* against *C. albicans* and *C. krusei* as well as the hexane extract of *Z. armatum* facing *T. longifusus* and *M. canis* can be considered as the exception[20,21].

Table 2. Antimicrobial activity of the lipid phase from *Z. pistaciifolium* extract

Microorganism	Concentration (mg mL^{-1})	Inhibition ± SD[a] (%)
Yeast		
C. albicans (azole resistant)	< 0.25	93.78 ± 0.01
C. glabrata	< 0.25	90.96 ± 0.71
C. kefyr	< 0.25	96.12 ± 1.50
C. krusei	< 0.25	95.03 ± 0.08
C. parapsilosis	< 0.25	94.45 ± 0.30
C. tropicalis	< 1.0	77.61 ± 1.30
Bacteria		
E. coli	NA[b]	NA
P. aeruginosa	NA	NA
S. aureus	< 0.25	54.98 ± 2.95

[a] Standard deviation, [b] Non-activity

It is a general consent that the antimicrobial potential of *Zanthoxylum* species is related to the presence of terpene compounds[22-25]. Due to this, the antimicrobial activity observed for this lipid phase of *Z. pistaciifolium* leaves can be associated with the presence of those kinds of compounds, specifically with the presence of geranyllinalool, an oxygenated diterpene which has been proven to have a high antimicrobial and insecticide activity[26,27]. Nevertheless, other metabolites isolated in this extract of *Z. pistaciifolium* leaves as ethyl palmitate and other fatty acid derivatives, can also contribute to the measured activity[28].

DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ scavenging activity

Results associated to scavenging activity are displayed in Figure 6. The IC_{50} observed for DPPH$^{\cdot}$ radical was moderate, considering the statistic difference ($p < 0.05$) between the IC_{50} values of lipid phase (0.31 ± 0.009 mg/mL) and ascorbic acid (0.042 ± 0.003 mg/mL). On the contrary, for the ABTS$^{\cdot+}$ radical the activity can be considered as good, even when statistic difference was present, but in this case, closer IC_{50} values for the lipid phase (0.42 ± 0.03 mg/mL) regarding the standard antioxidant compound (0.35 ± 0.01 mg/mL) can be observed.

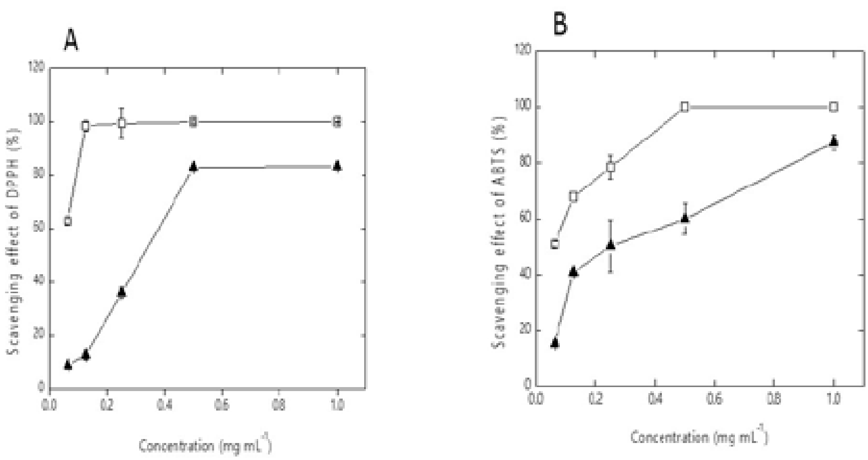

Figure 6. Scavenging activity of the lipid phase (□) from *Z. pistaciifolium* extract and the reference ascorbic acid (▲). 6A) DPPH· scavenging, 6B) ABTS·+ scavenging

Acceptable scavenging activity demonstrated for the lipid phase of *Z. pistaciifolium* leaves extract can also be related to the presence of terpenoids, which have been reported in the literature as good scavengers, mainly monoterpene and sesquiterpene types[29]. Once again, fatty acid and its derivatives can also contribute to this determined activity.

Cell viability

The cell viability results demonstrated that even when the IC_{50} is not too high which is expected for a natural extract, the value of 34.9 ± 0.78 μg/mL is higher that of tamoxifen reference (3.59 μg/mL). Other studies refer higher IC_{50} values when lipid fractions are faced to Vero cells, as is the case of *Z. rhoifo-*

lium[30,31]. Nevertheless, the scarce number of reports using this cell line makes a deeper analysis hard. Because of this, further *in vivo* and/or *in vitro* experiments will be necessary.

Antimicrobial and scavenging activity was demonstrated for the lipid phase from *Z. pistaciifolium* leaves extract, most importantly the activity against *Candida* spp. and ABTS$^{\cdot+}$ radical. Both activities were associated to the presence of several types of terpene substances as well as fatty acid and its derivatives. This phytochemical study allows us to report 35 compounds of different chemical profile. These findings justify for the first time that *Z. pistaciifolium* species could be a potential candidate for the treatment of ear diseases in ethno-botanical practices.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided here in.

FUNDING SOURCES

The authors declare that there is no funding sources of interest regarding the publication.

REFERENCES

1. The Plant List. [Internet]. 2013. Available from: http://www.theplantlist.org/ [Accessed 2020/10/24].

2. Yang G, Chen D. Alkaloids from the roots of *Zanthoxylum nitidum* and their antiviral and antifungal effects. Chem Biodivers. 2008;5(9):1718-1722. https://doi.org/10.1002/cbdv.200890160.

3. Patiño LOJ, Prieto RJA, Cuca SLE. *Zanthoxylum* Genus as potential source of bioactive compounds. In: Rasooli I, editor. *Bioactive compounds in phytomedicine* Rijeka, Croatia: InTech, 2012,184-218.

4. Roig JT. *Plantas medicinales, aromáticas o venenosas de Cuba*. La Habana: Editorial Científico-Técnica; 2012. p. 736.

5. Heredia YD, Tuenter E, Garcia JD, Ochoa AP, Cos P, Pieters L, et al. Novel flavonol-3-*O*-methylethers from *Zanthoxylum pistaciifolium* Griseb. (Rutaceae). Nat Prod Res. 2022;36(19):4869-4878. https://doi.org/10.1080/14786419.2021.1906240

6. Martínez J. Yerberos de Cuba. La Habana: Fundación Fernando Ortiz; 2013. p. 32

7. Heredia Y, González R, Escalona J, García J, de la Vega J. Influencia del medio de extracción en la composición de sustancias volátiles de las hojas de *Zanthoxylum pistacifolium* Griseb. Rev Cub Quím. 2016;28(1):490-506.

8. National Institute of Standards and Technology. [Internet]. 2020. Available from: https://www.nist.gov/srd/nist1a.cfm [Accessed 2020/12/22].

9. Adams RP. Identification of essential oil components by Gas Chromatography/Mass Spectrometry. Illinois: Allured Publishing Corporation; 2007. p.15.

10. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. J Ethnopharmacol. 2006;106(3):290-302. doi:10.1016/j.jep.2006.04.003

11. Choi Y, Lee SM, Chun J, Lee HB, Lee J. Influence of the heat treatment on the antioxidant activities and polyphenolic compounds of Shiitake (*Lentinus edodes*) mushroom. Food Chem. 2006;99(2):381-387. doi:10.1016/j.foodchem.2005.08.004

12. Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem. 1992;40(6): 945-948. https://doi.org/10.1021/jf00018a005

13. McMillian MK, Li L, Parker JB, Patel L, Zhong Z, Gunnett JW, et al. An improved resazurin-based cytotoxicity assay for hepatic cells. Cell Biol Toxicol. 2002;18:157-173. https://doi.org/10.1023/A:1015559603643

14. Bastos JK, Gottlieb OR, Sartia SJ, Filho DS. Isolation of lignans and sesquiterpenoids from leaves of *Zanthoxylum naranjillo*. Nat Prod Lett. 1996;9(1):65-70. https://doi.org/10.1080/10575639608043580

15. Ombito OJ, Matasyoh CJ, Vulule MJ. Chemical composition and larvicidal activity of *Zanthoxylum gilletii* essential oils against *Anopheles gambiae*. Afr J Biotechnol. 2014;13(21):75-80. https://doi.org/10.5897/AJB2014.13711

16. Kumar V, Reddy SGE, Chauhan U, Kumar N, Singh B. Chemical composition and larvicidal activity of *Zanthoxylum armatum* against diamondback moth, *Plutella xylostella*. Nat Prod Res. 2015;30(6):689-692. https://doi.org/10.1080/14786419.2015.1036270

17. Marr KL, Tangs CS. Volatile insecticidal compounds and chemical variability of hawai-

ian *Zanthoxylum* (Rutaceae) species. Biochem Syst Ecol. 1992;20(3):209-217. https://doi.org/10.1016/0305-1978(92)90055-I

18. Xia L, You J, Li G, Sun Z, Suo Y. Compositional and antioxidant activity analysis of *Zanthoxylum bungeanum* seed oil obtained by supercritical CO_2 fluid extraction. J Am Oil Chem Soc. 2010;88(1):23-32. https://doi.org/10.1007/s11746-010-1644-4

19. Wang CF, Yang K, Zhang HM, Cao J, Fang R, Liu ZL, et al. Components and insecticidal activity against the maize weevils of *Zanthoxylum schinifolium* fruits and leaves. Molecules. 2011;16(4):3077-3088. https://doi.org/10.3390/molecules16043077

20. Devi OZ, Rao KS, Bidalia A, Wangkheirakpam R, Mukherjee OS. GC-MS Analysis of phytocomponents and antifungal activities of *Zanthoxylum acanthopodium* DC. Collected from Manipur, India. European J Med Plants. 2015;10(1):1-9. https://doi.org/10.9734/EJMP/2015/19353

21. Alam F, Saqib QN. Evaluation of *Zanthoxylum armatum* Roxb for *in vitro* biological activities. J Tradit Complement Med. 2017;7(4):515-518. https://doi.org/10.1016/j.jtcme.2017.01.006

22. Vuuren SF, Viljoen AM. Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. Flavour Fragr J. 2007;22(6):540-544. https://doi.org/10.1002/ffj.1843

23. Park SN, Lim YK, Freire MO, Cho E, Jin D, Kook JK. Antimicrobial effect of linalool and a-terpineol against periodontopathic and cariogenic bacteria. Anaerobe. 2012;18(3):369-372. https://doi.org/10.1016/j.anaerobe.2012.04.001

24. Dai J, Zhu L, Yang L, Qiu J. Chemical composition, antioxidant and antimicrobial activities of essential oil from *Wedelia prostrata*. Excli J. 2013;12:479-490. https://doi.org/https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4669986/

25. Pejin B, Savic A, Sokovic M, Glamoclija J, Ciric A, Nikolic M, et al. Further *in vitro* evaluation of antiradical and antimicrobial activities of phytol. Nat Prod Res. 2014;28(6)372-376. https://doi.org/10.1080/14786419.2013.869692

26. Jirovetz L, Buchbauer G, Schmidt E, Stoyanova AS, Denkova Z, Nikolova R, et al. Purity, antimicrobial activities and ol factoric evaluations of geraniol/nerol and various of their derivatives. J Essent Oil Res. 2007;19(3):288-291. https://doi.org/10.1080/10412905.2007.9699283

27. Zatla AT, Dib MEA, Djabou N, Tabti B, Meliani N, Costa J, et al. Chemical variability of essential oil of *Daucus carota* subsp. *sativus* from Algeria. J Herbs Spices Med Plant. 2017;23(3):216-230. https://doi.org/10.1080/10496475.2017.1296053

28. Kujumgiev A, Bankova V, Ignatova A, Popov, S. Antibacterial activity of propolis, some of its components and their analogs. Pharmazie 1993; 48(10):785-786.

29. Chandra M, Prakash O, Kumar R, Kumar RB, Bhushan B, Kumar M, et al. β-selinene-rich essential oils from the parts of *Callicarpa macrophylla* and their antioxidant and pharmacological activities. Medicines. 2017;4(3):52. https://doi.org/10.3390/medicines4030052

30. Melo NB, Leitão JM, Oliveira LG, Santos SE, Carneiro SM, Rodrigues KA, et al. Inhibitory effects of *Zanthoxylum rhoifolium* Lam. (Rutaceae) against the infection and infectivity of macrophages by *Leishmania amazonensis*. An Acad Bras Cienc. 2016;88(3):1851-1861. https://doi.org/10.1590/0001-3765201620150131

31. Moura-Costa GF. Nocchi SR, Ceole LF, de Mello JC, Nakamura CV, Dias FB et al. Antimicrobial activity of plants used as medicinals on an indigenous reserve in Rio das Cobras, Parana. Brazil J Ethnopharmacol. 2012;143(2):631-638. https://doi.org/10.1016/j.jep.2012.07.016