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RESEARCH ARTICLE

Bioactivity evaluation of the native Amazonian species of Ecuador: *Piper lineatum* Ruiz & Pav. essential oil

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Abstract

In the present research, the essential oil from *Piper lineatum* Ruiz & Pav. was analysed by GC/MS and GC/FID, respectively. A total of thirty-seven chemical compounds were identified, which represented 98.9% of the essential oil composition. The main compounds were apiole (21.5%), safrole (19.2%), and myristicin (13.8%), respectively. The in vitro antimicrobial activity and antifungal activity of the oil was assayed against two Gram positive bacteria, five Gram negative bacteria and two fungi. The essential oil from *P. lineatum* showed an inhibitory activity against Gram-positive bacterium *Klebsiella pneumoniae* (ATCC 9997), and against dermatophytic fungus *Trichophyton rubrum* (ATCC 28188) with a MIC of 500 µg/mL in both cases. The antioxidant activity of essential oil was explored using DPPH and ABTS radical scavenging method, by means of both assays the essential oil showed a weak antioxidant activity. To the best of our knowledge, this is the first report on the chemical composition and biological activity of essential oil from this species.

Keywords: Essential oil, *Piper lineatum*, Apiole, Safrole, Antimicrobial activity, Antioxidant activity

Introduction

The Piperaceae is a large family of flowering plants widely distributed throughout the tropical and subtropical regions (Yuncker, 1973). The Piperaceae also known as the pepper family belongs to order Piperales. The family comprises about 13 genera (The Plant List, 2013), of which the genera *Piper* and *Peperomia* contain more than 98% of the species (about 2618 species), and the other three genera contain less than 2% (about 40 species) (Missouri Botanical Garden, 2017). The genus *Piper* is the largest in the Piperaceae family and is made up of around 1457 species (Missouri Botanical Garden, 2017), many of which have been used as culinary spices and recognized by their medicinal properties in traditional medicine background. Worldwide *Piper* species are widely used for their antibacterial, antifungal, anti-inflammatory and disinfectant effects and to prevent pains, stomach-ache, and as remedies against parasites, fever, gastritis, flu, rheumatism, cough, headache, skin, prostate problems, etc. (Salehi et al., 2019). In South America, a large number of species of this genus have been reported with uses in traditional medicine (Salehi et al., 2019). As an example, *Piper umbellatum* L. is traditionally used as an anti-inflammatory in Brazil and to treat fever in Peru (Duke, Bogenschutz-Godwin, & Ottesen, 2009). *Piper marginatum* Jacq. leaves and stems are used in Brazil, especially in Paraíba State, against snake bites and as a sedative, while in north-eastern Brazil and in the northern region, especially in the Amazon, *P. marginatum* is commonly used for the treatment of inflammatory diseases, snake bites, as well as the liver and bile duct diseases (Brú & Guzman, 2016). Durant-Archibold et al. have reported on the folklore uses of *P. hispidum* in various South American countries, a leaf infusion is drunk for its antihemorrhagic and diuretic effects in Brazil while in Ecuador it is applied to kill head lice and a leaf decoction is used in Colombia to treat malaria (Durant-Archibold, Santana, & Gupta, 2018).

Piper genus is a well-known and widely distributed pantropical taxon of aromatic plants, rich in essential oils (EOs), which can be found in many tissues and organs: fruits, seeds, leaves, branches, roots and stems (da Silva et al., 2017; Naim & Mahboob, 2020). *Piper* essential oils have insecticidal, acaricidal, repellent and antifeedant effects and some are nematicidal and phytotoxic (Jaramillo-Colorado, Pino-Benitez, & González-

Coloma, 2019). *Piper* species EOs are rich in all classes of volatile chemical compounds, but the composition is highly variable and these differences seem to depend on polymorphism, plant part, geographical differences, environmental conditions and chemotypes (Thin et al., 2018). According to Mgbeahuruike, Yrjönen, Vuorela and Holm (2017) there are more than 270 identified compounds in Piper spp. EOs. Amongst the most important compounds found in the *Piper* EOs are monoterpene hydrocarbons, oxygenated monoterpenoids, sesquiterpene hydrocarbons and oxygenated sesquiterpenoids, also in these oils you can find the phenylpropanoids such as safrole, dillapiole, myristicin, elemicin, (*Z*)-asarone, eugenol, apiole, and sarisan (da Silva et al., 2017). Safrole is abundant in nature among diverse plant genera such as *Sassafras, Ocotea, Cinnamomum, Myristica,* and *Piper* (Kemprai et al., 2020), in EOs of some species like *Piper hispidinervum* collected in southern Brazil this compound is even found in percentages of around 80% (Andrés et al., 2017). Safrol is recognized as a weak hepatocarcinogen with demonstrated genotoxicity in rodents led to strict restrictions on its use in food by various regulatory bodies globally. Which has caused that many government authorities have enforced laws to restrict the production and harvesting of safrole-bearing plants (Kemprai et al., 2020). However, the essential oils that contain safrol show biological activity, especially repellent activity, for this reason some scientists suggest the use of these oils as a potential natural insecticide, for example for stored-product insect pests (Zapata & Smagghe, 2010).

For Ecuador, 450 species of the Piperaceae family belonging to four genera have been registered, of which the most diverse is the genus *Peperomia* with 180 native and 50 endemic species, followed by the genus *Piper* with 157 native and 61 endemic species (Jørgesen & León-Yáñez, 1999; León-Yánez et al., 2019). Ecuadorian native communities in the Amazonian region use different species of Piper. For example, the stems of *P. augustum* and *P. conejosense* are broken off and used as toothbrushes (Davis & Yost, 1983; Evans Schultes, 1985). *Piper ecuadorense*, known as *matico de monte*, is a plant used by many indigenous communities in Loja and Zamora provinces to treat hangover, as a disinfectant and in wound healing (Ramírez, Cartuche, Morocho, Aguilar, & Malagon, 2013). The Leaf and flower of *Piper aduncum* L. are used as disinfectant, gastritis, influenza, rheumatism and cough. The whole plant of *Piper barbatum* Kunth, named as Cordoncillo is used to treat headache, stomach pain, dermatitis, disinfectant, the leaf of *Piper bogotense* C. DC. called Sacha guando is used in hepatic pain, the stem and leaf of *Piper crassinervium* Kunth (Guabiduca) is used to treat diabetes, gastritis, prostate problems and the leaf of *Piper ecuadorense* Sodiro (matico del monte) is used as hangover, disinfectant, healing of wounds (Tene et al., 2007).

Piper lineatum Ruiz & Pav. is a native aromatic shrub of Ecuador and Peru. In Ecuador is widely distributed between 0-2500 m a.s.l in the Amazonian provinces of Morona Santiago, Napo, Pastaza, Pichincha, Tungurahua and Zamora Chinchipe, and in the Andean province of Pichincha (Tropicos, 2020). Ecuador is considered a biodiversity hotspot with many vegetal species per unit surface area, which locates this country in the sixth position in the world. Piper aromatic species in Ecuador have been used as a condiment or in traditional medicine, for that reason, the aim of this research was to determine the chemical composition and antifungal and antibacterial activity of the essential oil of *Piper lineatum*, as well as, to value its antioxidant activity.

Materials and Methods

Materials

Dichloromethane, sodium sulfate anhydrous, 2,2-diphenyl-1-picrylhydryl (DPPH$^\bullet$) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich. Alliphatic hydrocarbons standard was purchased from CHEM SERVICE under the name of Diesel Range Organics Mixture #2-GRO/DRO

and with the code M-TPH6X4-1ML. Helium was purchased from INDURA Ecuador. Microbiological media as Sabourad and Mueller Himton Broth were purchased from DIPCO. Dimethylsulfoxide (DMSO) was obtained from Fisher. All chemicals were of analytical grade and used without further purifications.

Plant material

The collection of the species, authorized by the Ministerio del Ambiente de Ecuador (MAE), with authorization N° 001-IC-FLO-DBAP-VS-DRLZCH-MA, was performed in Zamora canton, province of Zamora Chinchipe, Ecuadorian Amazon, at a latitude of 4°08'25" S and a longitude of 78°55'51" W. The collection was performed at an altitude of 1075 m a.s.l. The storage and transfer of the plant material was carried out in airtight plastic containers until they were hydrodistilled. Temperature collection was at room temperature (26°C) and the transfer temperature was 24 °C, the pressure was approximately 88 KPa (room pressure).

The botanical specimens were identified by Dr. Bolivar Merino, at the herbarium of the "Universidad Nacional de Loja". A voucher specimen is preserved in the Herbarium of the Universidad Técnica Particular de Loja under code PPN-809.

Moisture of plant material

The moisture of plant material was determined using test method AOAC 930.04-1930, Loss on drying (Moisture) in plants. For which, an analytical balance (model Mettler AC 100, ±0.0001) and an oven (Memmert Universal Oven UN260) were used. The loss on drying (LOD) as estimate of moisture content was calculated as follows: % (w/w) LOD = % (w/w) moisture = 100 x (weight loss on drying, g/weight test portion, g). All the experiments were performed in triplicated and the results are expressed as mean values.

Essential oil isolation

The material was processed fresh, immediately after arriving at the laboratory, between 2 and 4 hours after being collected. The plant material was submitted to hydrodistillation by four hours in a Clevenger-type apparatus. Subsequently, the collected essential oil was subjected to removal of moisture by addition of anhydrous sodium sulphate and finally, it was stored in amber sealed vials at 4 °C to protect it from light until being used in the subsequent analysis (Valarezo, Guamán, Paguay, & Meneses, 2019).

Determination of physical properties of essential oil

The density and refraction index of essential oils were determined according to the standard AFNOR NF T 75-111 and AFNOR NF T 75-112, respectively. For density, a pycnometer (1 mL) and an analytical balance (model Mettler AC 100, ±0.0001) were used, whereas for refraction index a refractometer (model ABBE) was used. The procedures were repeated three times and all measurements were performed at 20°C.

Gas chromatography/Flame ionization detector (GC/FID)

The analyses of the chemical composition of the essential oils were performed on an Agilent gas chromatograph (model 6890N series) equipped with a flame ionization detector (FID). A nonpolar column DB-5ms (5%-phenyl-methylpolyxilosane) 30 m x 0.25 mm, thickness 0.25 µm was used. An automatic injector (series 7683) in split mode was used. The samples, 1 µL of solution (1/100, v/v, essential oil/dichloromethane), were injected with a split ratio of 1:50. Helium was used as a carrier gas at 0.9 mL/min in constant flow mode. The initial oven temperature was held at 50°C for 3 min and then it was heated to 210 °C with a ramp of 2.5°C/min, and the temperature was maintained for 3 min until the end. The injector and detector temperatures were 210°C, and 250°C; respectively. Retention index (IR) of the compounds was

determined based on the standard of aliphatic hydrocarbons, which were injected after the oils at the same conditions.

Gas chromatography/Mass spectrometry (GC/MS)

The GC/MS analyses were performed using an Agilent chromatograph coupled to a mass spectrometer (quadrupole) detector (model Agilent series 5973 inert). The spectrometer was operated at 70 eV, electron multiplier 1600 eV, scan rate: 2 scan/s and mass range: 40-350 *m/z*. It was provided with a computerized system MSD-Chemstation D.01.00 SP1. The same columns described in GC/FID section were used. The ion source temperature was set at 250 °C. The identification of the oil components was based on a comparison of both mass spectrum data and relative retention indices with the published literature (Adams, 2007; NIST 05, 2005; NIST, 2020). The relative amounts of individual components were calculated based on the GC peak area (FID response) without using a correction factor.

Biological activity

Evaluation of antibacterial activity

Antibacterial activity was evaluated against five Gram-negative and two Gram-positive bacteria, as shown in Table 2. The bacterial strains were incubated overnight at 37°C in Mueller-Hinton (MH) broth; subsequently, the cell suspension was adjusted with a sterile physiological solution to obtain an equivalent concentration of 0.5 in the McFarland scale (1.5 x 10^8 cells/mL). Another dilution was done in MH broth to adjust the concentration of inoculum to 2 x 10^6 CFU/mL. A volume of 100 µL from the inoculum were transferred into wells containing 100 µL of MH broth with the higher concentration of the essential oil as described below, giving a final concentration of 5 x 10^5 CFU/mL.

Minimum inhibitory concentration (MIC) was determined by two fold serial dilution method using 96-well microtiter plates (Clinical and Laboratory Standards Institute, 2012).Twenty microliters of a solution of each essential oil [20 µL/mL of DMSO (v/v)] was dissolved in 180 µL of MH broth; subsequently, 100 µL of this solution was transferred to a next well containing 100 µL of MH broth. The procedure was done several times to achieve concentrations ranging from 1000 to 8 µg/mL. Gentamicin (1 mg/mL) was used as the positive control for Gram-positive and Gram-negative bacteria. The microplates were sealed and incubated at 37 °C for 24 hr.

Evaluation of antifungal activity

The antifungal activity of the essential oils against two fungal organisms was determined by the microdilution method (Clinical and Laboratory Standards Institute, 2008; NCCLS, 2002) as described for the antibacterial activity. A reserve solution of spore fungi was diluted with Sabouraud dextrose broth (7 mL) and stored at 4°C until analysis.

Minimum inhibitory concentration was determined using a final concentration of 5 x 10^4 spores/mL in 96-well microtitre plates. Essential oil was dissolved in Sabouraud dextrose broth with fungal inoculum to achieve the required concentrations from 1000 – 0.5 µg/mL. Itraconazole was used as the positive antimycotic control. The microplates were sealed and incubated at 28°C for 72 hr.

In the antibacterial and antifungal activity tests, a negative control with DMSO was also considered (positive growth with 5% DMSO as final concentration). MIC was the lowest concentration of essential oil that prevented visible bacterial or fungal growth, respectively. All the experiments were performed in triplicated and the results are expressed as mean values.

Antioxidant capacity

DPPH radical scavenging activity

The DPPH free radical scavenging activity of oils was measured based on the scavenging activity of the stabilized 2,2-diphenyl-1-picrylhydryl radical. This assay was performed based on the technique of Brand Williams *et al.* (Brand-Williams, Cuvelier, & Berset, 1995) with some modifications described by Thaipong *et al.* (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006). Briefly, to prepare the radical stock solution, 24 mg of $DPPH^{\bullet}$ was dissolved in 100 mL of methanol. The working solution was prepared by diluting 10 mL of the radical stock solution with 45 mL of methanol to obtain a reading of 1.1 ± 0.02 absorbance units at a wavelength of 515 nm in a UV spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific). The working solution was prepared fresh daily. Take 150 µL of the sample solution with different concentrations of *P. lineatum* essential oil (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 mg/mL) and add 2850 µL of $DPPH^{\bullet}$ working solution in an amber vial. Vigorously shake for three minutes and allowed to react for 24 hours at room temperature protected from light. The final absorbance was measured at a wavelength of 515 nm. The same amount of methanol was added instead of the sample solution as a blank control, and BHT and Trolox were used as positive controls. The percentage of free radical-scavenging capacity was calculated as follows: R (%) = [(As-Ai)/As] x 100%, where Ai is the absorbance of $DPPH^{\bullet}$ mixed with essential oil and as is the sample blank absorbance of $DPPH^{\bullet}$ in which sample has been replaced with methanol The oil concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition % against oil concentration. All measurements were performed in triplicate and reported as the average value.

ABTS radical cation scavenging activity

The ABTS assay was performed using the procedure described by Arnao *et al.* (Arnao, Cano, & Acosta, 2001) with some modifications by Thaipong *et al.* (Thaipong et al., 2006). Two stock solutions were prepared: 7.4 µM ABTS and 2.6 µM potassium persulfate. ABTS radical cation ($ABTS^{\bullet+}$) was produced after mix both solutions in equal quantities and allowed to react protected from light during 12 hours to obtain the ABTS standard solution. For the study of essential oil, 1 mL of the ABTS standard solution was diluted with 60 mL of methanol to obtain an absorbance of 1.1 ± 0.02 in a spectrophotometer UV to 734 nm. From each concentration (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 mg/mL) as well with each essential oil, 150 µL were added to 2850 µL of the $ABTS^{\bullet+}$ working solution, then was allowed to react by 2 hours protected from light, after this the absorbance was measured at 734 nm. The same amount of deionized water was added instead of the sample solution as a blank control, BHT and Trolox were used as positive controls. The sample's removal rate of ABTS radicals can be expressed as: SA (%) = (Ao-Ai)/Ao x 100%; Ao: Absorbance of $ABTS^{\bullet+}$ mixed with the sample; Ai: absorbance after reaction of $ABTS^{\bullet+}$ with the sample. The oil concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition % against oil concentration. All measurements were performed in triplicate and reported as the average value.

Results and Discussion

Through hydrodistillation in a Clevenger-type apparatus, was obtained ~5 mL of essential oil from 1500 g of *Piper lineatum*, what represents a yield of 0.33 ± 0.02% (v/w) or 3.3 mL/kg, referred to fresh plant material (60 ± 1% of moisture), equivalent to 0.83% on moisture free basis, which is considered as a low yield (Molares, González, Ladio, & Agueda Castro, 2009). To our knowledge, this is the first report of the study of essential oil from *Piper lineatum*. The essential oil (EO) presented as a viscous liquid of subjective color light-yellow. The essential oil relative density was d^{20} =0.8792 ± 0.0011 and refraction index was n^{20} =1.6918 ± 0.0017.

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Essential oil compounds identification

The identification of volatile compounds present in *Piper ecuadorense* of Ecuador was carried out by means of gas chromatography equipped with a flame ionization detector (GC/FID) and gas chromatography coupled to a mass spectrometer detector (GC/MS) using capillary nonpolar column DB-5Ms. The results obtained are summarized in Table 1. Thirty-seven individual compounds were detected in the EO of *P. lineatum*, which represent a 98.87% of the total essential oil. Most essential oils are mainly composed of terpenes and sesquiterpenes, however, in this study was determined that the EO of *P. lineatum* has a higher percentage (~40%) of compounds classified as OTC (other compounds). In percentage, the compounds classified as oxygenated monoterpenes (OXM) ranked second and the aliphatic monoterpene hydrocarbons (ALM) the third. The largest number (ten) of compounds belonged to the group at ALM.

Table 1. Chemical composition of essential oil from *Piper lineatum*.

Peak #	Compound[a,b]	RI	RI[ref]	*P. lineatum*		Type	CF	MM
				%[c]	*SD*			(Da)
1	α-Pinene	932	932	1.91	0.15	ALM	$C_{10}H_{16}$	136.13
2	Camphene	947	946	tr	-	ALM	$C_{10}H_{16}$	136.13
3	β-Pinene	973	974	1.81	0.16	ALM	$C_{10}H_{16}$	136.13
4	Myrcene	986	988	0.36	0.01	ALM	$C_{10}H_{16}$	136.13
5	α-Phellandrene	1005	1002	0.39	0.04	ALM	$C_{10}H_{16}$	136.13
6	α-Terpinene	1013	1014	2.02	0.27	ALM	$C_{10}H_{16}$	136.13
7	*ρ*-Cymene	1021	1020	0.95	0.11	ARM	$C_{10}H_{14}$	134.10
8	1,8-Cineole	1029	1026	8.91	0.76	OXM	$C_{10}H_{18}O$	154.14
9	(*Z)*-β-Ocimene	1034	1032	1.17	0.12	ALM	$C_{10}H_{16}$	136.13
10	(*E*)-β-Ocimene	1045	1044	3.19	0.32	ALM	$C_{10}H_{16}$	136.13
11	γ-Terpinene	1055	1054	4.23	0.34	ALM	$C_{10}H_{16}$	136.13
12	Terpinolene	1082	1086	0.89	0.03	ALM	$C_{10}H_{16}$	136.13
13	Linalool	1098	1095	0.86	0.02	OXM	$C_{10}H_{18}O$	154.14
14	Terpinen-4-ol	1170	1174	0.07	0.00	OXM	$C_{10}H_{18}O$	154.14
15	α-Terpineol	1191	1186	0.58	0.02	OXM	$C_{10}H_{18}O$	154.13
16	Safrole	1291	1285	19.16	1.72	OXM	$C_{10}H_{10}O_2$	162.07
17	δ-Elemene	1338	1335	0.19	0.02	ALS	$C_{15}H_{24}$	204.19
18	α-Cubebene	1347	1345	tr	-	ALS	$C_{15}H_{24}$	204.19
19	α-Copaene	1376	1374	0.38	0.05	ALS	$C_{15}H_{24}$	204.19
20	(E)-Isosafrole	1375	1373	0.05	0.01	OXM	$C_{10}H_{10}O_2$	162.07
21	β-Cubebene	1388	1387	0.07	0.01	ALS	$C_{15}H_{24}$	204.19
22	β-Elemene	1390	1389	tr	-	ALS	$C_{15}H_{24}$	204.19
23	Methyl eugenol	1405	1403	3.09	0.10	OTC	$C_{11}H_{14}O_2$	178.10
24	*trans*-Caryophyllene	1415	1417	1.90	0.21	ALS	$C_{15}H_{24}$	204.19
25	β-Gurjunene	1435	1431	tr	-	ALS	$C_{15}H_{24}$	204.19
26	α-Humulene	1451	1452	0.69	0.16	ALS	$C_{15}H_{24}$	204.19
27	*trans*-Cadina-1(6),4-diene	1478	1475	1.55	0.26	ALS	$C_{15}H_{24}$	204.19
28	Germacrene D	1476	1480	1.67	0.27	ALS	$C_{15}H_{24}$	204.19
29	Viridiflorene (=Ledene)	1498	1496	0.11	0.01	ALS	$C_{15}H_{24}$	204.19
30	δ-Amorphene	1515	1511	1.96	0.02	ALS	$C_{15}H_{24}$	204.19
31	Myristicin	1519	1517	13.78	0.49	OTC	$C_{11}H_{12}O_3$	192.08

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Peak #	Compound[a,b]	RI	RI[ref]	*P. lineatum*		Type	CF	MM
				%[c]	*SD*			(Da)
32	δ-Cadinene	1524	1522	1.55	0.03	ALS	$C_{15}H_{24}$	204.19
33	Elemicin	1558	1555	1.25	0.05	OTC	$C_{12}H_{16}O_3$	208.11
34	Viridiflorol	1595	1592	0.48	0.01	OXS	$C_{15}H_{26}O$	222.20
35	*epi*-α-Cadinol	1639	1638	0.17	0.01	OXS	$C_{15}H_{26}O$	222.20
36	α-Eudesmol	1647	1652	1.96	0.02	OXS	$C_{15}H_{26}O$	222.20
37	Apiole	1673	1677	21.48	1.68	OTC	$C_{12}H_{14}O_4$	222.09
	Aliphatic monoterpene hydrocarbons (ALM)			**15.98**				
	Aromatic monoterpene hydrocarbons (ARM)			**0.95**				
	Oxygenated monoterpenes (OXM)			**29.63**				
	Aliphatic sesquiterpene hydrocarbons (ALS)			**10.11**				
	Oxygenated Sesquiterpene (OXS)			**2.61**				
	Other compounds (OTC)			**39.60**				
	Total identified			**98.87**				

[a]Compounds ordered according to the elution order in the column DB5-Ms. [b]All compounds were identified by MS and RI: MS. by comparison of the mass spectrum with those of the computer mass libraries Wiley 7. Adams 2007 and NIST 05 2005; RI. by comparison of RI with those reported in literature; Adams 2007. NIST 05 2005 and NIST 2020. [c]Percentage values are means of nine determinations. tr. trace (< 0.05%); -. not detected; RI. retention indices in the a-polar column (DB5-Ms); RI[ref]. references: Adams 2007. NIST 05 2005 and NIST 2020. SD. standard deviation; CF. Chemical Formula; MM. Monoisotopic mass.

The main constituent of the essential oil was apiole (OTC, CAS 523-80-8) with 21.48 ± 1.68%, safrole (OXM, CAS 94-59-7) was the second compound with the highest percentage with the 19.16 ± 1.72%. Other main compounds were myristicin (OTC, 13.78 ± 0.49%) and 1,8-cineole (OXM, 8.91 ± 0.76%). Representative amounts of γ-terpinene 4.23 ± 0.34% and (*E*)-β-ocimene 3.19 ± 0.32% were also identified. Regarding previous studies about essential oil from species belonging to genus piper, apiol (64.24%) and safrole (64.54%) were the major constituents of the EO from Colombian species *P. holtonii* and *P. auritum* respectively (Pineda M., Vizcaíno P., García P., Gil G., & Durango R., 2012), 1,8-cineole (13.0%) and safrole (14.9%) were identified as major compounds in a study carried out on essential oil from leaves of species *P. carpunya* grown in the Peruvian Amazon (Vargas, Velasco-Negueruela, Pérez-Alonso, Palá-Paúl, & Vallejo, 2004), and apiole (28.62%) was identified as one of the main components in the *P. aduncum* essential oil (Santana et al., 2015).

With regard to toxicity of the apiol (CF: $C_{12}H_{14}O_4$, MM: 222.09), it has been reported that parsley apiole has acute toxicity to humans, the lowest total dose of parsley apiole causing death is 4.2 g (2.1 g/day for two days) the lowest fatal daily dose is 770 mg, which was taken for 14 days; the lowest single fatal dose is 8 g. At least 19 g has been survived. Parsley apiole is hepatotoxic and nephrotoxic (Tisserand & Young, 2014). In scientific reports, safrole (CF: $C_{10}H_{10}O_2$, MM: 162.07) is considered a human carcinogen based on sufficient evidence of hepatocarcinogenic in rats and mice. The EPA has evaluated the carcinogenicity of safrole and classifies it as possibly carcinogenic to humans (Group B2: the agent (mixture) is possibly carcinogenic to humans). Safrole cannot be used as fragrance ingredient, the International Fragrance Association (IFRA) recommend that the essential oils containing safrole should not be used at levels in which the total concentration exceeds 0.01% in consumer products (Clarke, 2008; Tisserand & Young, 2014). However, *P. lineatum* essential oil also contains 1,8-cineole (eucalyptol, CF: $C_{10}H_{18}O$, MM: 154.14) which is used in the treatment of respiratory tract diseases due to its antimicrobial, mucolytic, broncholytic, and antiinflammatory properties (Aprotosoaie, Luca, Trifan, & Miron, 2019), Santos in the 2004 (Santos et al., 2004) stated that 1,8-cineole reduce colon inflammation in rats.

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Biological activity

Essential oils are complex mixtures of chemical compounds, which may have biological synergism or antagonistic properties. When two or more carcinogenic constituents are present in an essential oil or in a mixture of essential oils, it has been have assumed that such actions are additive. When an essential oil or a mixture of oils contains a minority of carcinogenic, and a majority of anticarcinogenic constituents, it is assumed that the principle of antagonism applies (Tisserand & Young, 2014). To estimate the net activities (including synergism and antagonism) of the compounds present in the essential oil, the biological activity was determined based on their antibacterial, antifungal and antioxidant activities. Essential oils obtained from aerial parts of *P. lineatum* were assessed by microdilution broth method and the values of minimum inhibitory concentration (MIC, expressed in µg/ml) are shown in Table 2.

Table 2. Antibacterial and antifungal activity of essential oil from *Piper lineatum,* given as minimal inhibitory concentration (MIC, µg/mL).

Microorganism	***Piper lineatum***	**Positive control[b]**
	MIC (µg/mL)[a]	
Gram-negative bacteria		
*Pseudomonas aeruginosa (*ATCC 27853)	>1000	0.39
Klebsiella pneumoniae (ATCC 9997)	500	0.39
*Proteus vulgaris (*ATCC 8427)	>1000	0.39
*Escherichia coli (*ATCC 25922)	>1000	0.39
Salmonella typhimurium (LT2)	>1000	1.95
Gram-positive bacteria		
*Enterococcus faecalis (*ATCC 29212)	>1000	1.95
*Staphylococcus aureus (*ATCC 25923)	>1000	0.39
Dermatophytes Fungi		
*Trichophyton rubrum (*ATCC 28188)	500	0.39
Trichophyton mentagrophytes (ATCC 28185)	>1000	0.39

[a] Mean of nine determinations. [b] Gentamicine for Gram-negative and Gram-positive bacteria and Itraconazole for fungi.

Currently, there are no accepted standard criteria for defining the *in vitro* antimicrobial activity of natural products. However, from an extensive review about this issue, Van Vuuren and Holl (2017) suggested a more detailed classification scale for extracts and essential oils that resulted more relevant to characterize the activity of our samples. For an essential oil, a moderate activity is reported when a MIC value lies between 500 to 1000 µg/mL, from 101 to 500 µg/mL the activity is strong, meanwhile, an activity ≤ 100 µg/mL is considered to be very strong. On basis, the essential oil from *P. lineatum* presented a strong activity against *Klebsiella pneumoniae* (ATCC 9997) and *Trichophyton rubrum (*ATCC 28188) with a MIC of 500 µg/mL in both cases. The antibacterial capacity of the EO of *P. carpunya* may be due to its high concentration in 1,8-cineole, which has been reported as a compound with antibacterial capacity (Hendry, Worthington, Conway, & Lambert, 2009). Further, 1,8-cineole showed significant levels of synergistic interaction combined with antimicrobial compounds like chlorhexidine gluconate (CHG) against *Staphylococcus aureus*, *S. aureus, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis* and *Candida albicans* (Şimşek & Duman, 2017). According to Stefano *et al.* apiol and myristicin resulted inactive against bacterial strains *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* and *Pseudomonas aeruginosa,* and antifungal strains *Candida albicans* and *Candida tropicalis*, at the maximum tested concentration of 200 µg/mL (Stefano, Pitonzo, & Schillaci, 2011). However, the essential oil extracted from the seeds of dill (*Anethum graveolens* L.), which contains 16.8% of apiole, showed antifungal capacity against *Aspergillus flavus*, the antifungal

activity of dill oil results from its ability to disrupt the permeability barrier of the plasma membrane and from the mitochondrial dysfunction-induced reactive oxygen species accumulation (Tian et al., 2012). Additionally, the essential oil of *P. lineatum* contains safrole which is considered a hepatotoxin. Using *Escherichia coli*-expressed human P450, recombinant cytochrome P450 (CYP, P450) and human liver microsomes, was demonstrated that safrole is a potent inhibitor of human CYP1A2, CYP2A6, and CYP2E1, also inhibits with relatively less potency CYP2D6 and CYP3A4 (Ueng, Hsieh, & Don, 2005).

Antioxidant capacity

The essential oil of *P. lineatum* was explored for antioxidant activity using DPPH radical and ABTS radical cation scavenging activity, IC_{50} was used as a measure value of inhibition concentration of 50% of the activity, and BTH and Trolox as positive control. Through $DPPH^{\bullet}$ method, the essential oils studied showed little antioxidant activity (~25% of inhibition to 2500 ppm), but did not provide IC_{50} values (>2500 μg/mL) in the concentration ranges tested. Among standards tested Trolox (IC_{50} = 580 ± 40 μg/mL) was the most efficient. Employing the ABTS technique, with 2500 ppm an inhibition of 21% was obtained, but did not provide IC_{50} values in the concentration ranges tested (Table 3).

Table 3. Antioxidant activity of essential oils of *Piper lineatum*

Sample	$DPPH^{\bullet}$	$ABTS^{\bullet+}$
	IC_{50}* (μg/mL)	
Piper lineatum EO	>2500	>2500
BHT	980 ± 50	290 ± 20
Trolox	580 ± 40	380 ± 30

* IC50 = Inhibition Concentration of 50%

The absence of antioxidant activity, for the essential oil of this study, in the DPPH assay can be explained by the fact that terpene compounds are not capable of donating a hydrogen atom and the low solubility provided by them in the reaction medium of the assay, because of this test utilizes methanol as solvent (Mata et al., 2007). However, the ABTS method is especially useful for investigations of lipophilic antioxidants and is appropriate for the investigation of antioxidant activity of essential oils (Andrade et al., 2013), the absence of antioxidant activity in essential oils of *P. lineatum* mainly consisting of monoterpene and sesquiterpene with antioxidant activity may be due to antagonism with carcinogenic compounds like safrole. In this regard, it should be considered that the apiole, the main compound in the EO of *P. lineatum*, has been shown to inhibit human colon cancer cell (COLO 205 cells) growth through induction of G0/G1 cell cycle arrest and apoptotic cell death (Wu et al., 2019). Therefore, this compound could be isolated from the essential oil and used as an active principle or as a raw material to obtain derivatives, since apiol derivatives have also shown induced anti-proliferative effects on colon cancer (Wu et al., 2019).

Conclusion

In Ecuador, many species belonging to the genus piper are used as culinary spices or in traditional medicine especially in the Amazon, without making an exact distinction by species, which causes that medicinal properties are attributed to most species of the genus *Piper*, however many of these species possess compounds that can be harmful to human health. In this research a complete characterization of the chemical composition, physical properties, and antimicrobial and antioxidant activity was conducted over the essential oil of *Piper lineatum.* A total thirty-seven individual compounds were detected in the essential oil, being the apiole and safrole the main compounds. The essential oil showed to be active against *Klebsiella pneumoniae* and *Trichophyton rubrum.*

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