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EVALUATION OF LEAVES´ ESSENTIAL OIL OF MYRCIA GUIANENSIS (AUBL.) DC. FROM LEGAL AMAZON: CHEMICAL, ANTIBACTERIAL ANDTOXICITY ANALYSIS

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ABSTRACT

Myrcia guianensis (Aubl.) DC., a species belonging to the Myrtaceae family, is an endemic plant in tropical areas used by communities in Legal Amazon to treat diarrhea and uterine infections**.** Although about its medicinal use, there is no description in the literature of biological activities nor determination of components of the essential oil of this species from that Cerrado biome. The present study aimed to perform chemical, biological and toxicity analysis of the essential oil (EO) of the leaves of *Myrcia guianensis* harvested from Legal Amazon. The EO was obtained by hydrodistillation process and analyzed through GC-MS. The yield calculation resulted in 1.34% and the major compounds were (E-) caryophyllene (37.43%), terpinolene (14.82%), β-bisabolene (6.07%), highlighting that the OE showed a mixture of mono and sesquiterpenes hydrocarbon and oxygenated. The evaluation of the major compounds on PASS, Osiris, PROTOX and Swiss ADME, showed great potential of antimicrobial activity and low toxicity. The essential oil was evaluated against American Type Culture Collection (ATCC) and clinical isolated bacteria, Gram-positive and Gram-negative, and it presented 2mg/mL for MIC against *Staphylococcus aureus* (ATCC 6538) and MRSA, decreasing effectively cell viability and metabolism. The inhibition of biofilm production was observed against *Escherichia coli* (ATCC 042). The essential oil of the leaves of *Myrcia guianensis* has low toxicity against *Tenebrio molitor* larvae and it promoted a great reduction on *Tenebrio molitor* larvae hemolymph bacterial load. It was concluded that essential oil of the leaves of *Myrcia guianensis* has bioprospecting potential for the development of pharmaceutical products.

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INTRODUCTION

Antimicrobial are one of the most successful class of medicine in chemotherapyhistory (Aminov, 2010). According to World Health Organization (WHO), in the report on surveillance of antibiotic consumption (2016), the antibiotic consumption has been increasing in many countries over the past two decades, proving that the overall consumption of antibiotics ranged from 4.4 to 64.4 Defined Daily Doses (DDD) per 1000 inhabitants per day. This increase on antibiotic consumption strengthens the bacterial resistance phenomenon. Beyond that, inadequate sanitary conditions and inappropriate manipulation of food may aggravate the problem (WHO, 2014; Levin-Reisman et al., 2017; CDC, 2018). Those bacteria have increased the number of mortality and morbidity what implies the need for new implementation on chemotherapy. To increase the chance of survival, bacteria use the mechanisms of antibiotic resistance such as changing the antibacterial agent's uptake and biofilm formation, for example (WHO, 2016; Frieri et al., 2017).

Researches around the world bring information about those threats as *Escherichia coli* that has showed resistance against fluoroquinilone, one of medicine wide used in urinate tract infections worldwide (Fasugba et al., 2015). Beyond that, *Staphylococcus aureus* that cause disease can range from skin infections to sepsis, pneumonia, endocarditis, osteomyelitis and necrotizing fasciitis. Its efficient pathogenicity can be attributed to a series of virulence factors that this strain has (Jenul; Horswill, 2019; Suresh; Biswas; Biswas, 2019). The medicinal plant has been used since ancient time and in the last decades has been growing the interest in validate their medicinal properties, especially for assure the safety and efficiency. The WHO says that 80% of people in developing countries use the medicinal herbs on the primary health care (Macedo, 2016). Brazil has one of the greatest biodiversity, highlighting its vegetal species accumulating 22% of the world total plants (Cerqueira et al., 2020). Although its biodiversity, less than 15% of their plants have been evaluated by some scientific study and, only, 25% of the totalregistered herbal medicine are from South America plants (Rodrigues, 2016; Cardoso et al., 2018). In this scenario, the medicinal herbs rise as the good opportunity to create new medicines

or as a source of new molecules, highlighting their essential oil that are a complex mixture present in aromatic species(Porto, 2014; Howes et al., 2020). The Myrtaceae is an important family well distributed over Brazil, endemic on tropical areas (Govaerts et al., 2008). This Family represents aromatic plants many with commercial interested like*Psidium guajava* L.(Silva et al., 2015), *Eugenia uniflora*L. (Araújo et al., 2019), Syzygiumaromaticum L. (Radünz et al., 2019), *Eucalyptus globulus* Labill. (Boukhatem et al., 2020) and *Syzygiumguineense* (Willd.) DC. *guineense* (Okhale et al., 2018). The *Myrcia* genus oil is well represented for monoterpenes and sesquiterpenes compounds, predominantly**.** Scientific researchesof the essential oil from *Myrcia* genus have showed anti-inflammatory, antinociceptive, antioxidant, antimicrobial and antifungalactivities while the extracts demonstrate hypoglycemic, anti-hemorrhagic and antioxidant action (Cascaes et al., 2015; Silva, 2019; Sampaio et al., 2020). *Myrcia guianensis* (Aubl.) DC. is a shrubthat can get up to 8 meters highand it is found in Legal Amazon, which represents 59% of Brazilian territory and concentrates most of Brazil´s biodiversity unexplored(Morais et al., 2014; Oliveira et al., 2019; Leal et al., 2020). In folk medicine, it´s leaves have been usedto treat diarrhea and uterine infections. The use in folk medicine of parts of the plant indicates the importance of a scientific investigation aiming to evaluate the effects on therapy, beyond validation of this use as medicinal herb. The aim of this study is to determine the chemical compounds andto investigate a potential antibacterial and toxicity of *Myrcia guianensis´*leavesessential oil collected from south of Maranhão state (Legal Amazon), northwest Brazil.

METHODOLOGY

Vegetal material: The plant was harvested in March 2018 in the Chapada das Mesas National Park, located inCerrado area, included in Legal Amazon, from Maranhão state, Brazil.This work is part of a project to inventory aromatic flora inCerrado area in Maranhão state authorized by Ministry of the Environment, Chico Mendes Institute for Biodiversity Conservation (ICMBio) and Biodiversity Authorization and Information System (SISBIO) for scientific purpose by number 62794-1.A plant voucher specimen was sent to the Herbarium called Museum EmílioGoeldiin Pará state and it was identified as *Myrcia guianensis* in May 2018 under registration MG230167.

Essential Oil Extraction: *Myrcia guianensis*leaves were air-dried (40 °C), ground by knife mill (DeLeo, Model Modelo EDB-5), and subjected to hydrodistillation using a Clevenger-type apparatus (Tecnal, Model TE-2765) for three hours (Brazil, 2019). The essential oils were centrifuged (4000 rpm/30 min) (Kasvi, Model K14-1215), dehydrated with anhydrous sodium sulfate (Na2SO4), packed in amber glass ampoules and kept refrigerated (5−10℃) (Coutinho et al., 2007).

Yield Calculation: The yield was calculated for the oil volume ratio, measured from the oil volume obtained in the extraction system, divided by the moisture free base. The oil yield obtained from each sample was then calculated by the formula described below.

$$\% R = \left[\frac{V}{MFB}\right] \times 100$$

R% = Yield Calculation
V = Volume
MFB = moisture freebase: $\lceil mass - (mass - \% moisture)\rceil$

Determination of chemical composition of essential oil: The chemical composition of the essential oil of *Myrcia guianensis* leaves was analyzed by Mass Spectrometry (GC-MS) coupled gas chromatography with 1mL injection (AOC-20i Auto injector) in ultra-equipped Shimadzu QP 2010 system. Rtx-5MS (Restek, USA) silica capillary column of 30m length x 0.25mm inner diameter coated with 5% diphenyl / 95% dimethyl polysiloxane (0.25µm film thickness) GC oven was programmed from 60ºC to 240ºC (10min) at 3ºC / min, injector temperatures (Split 1:20), transfer line and ionization chamber were 250, 250, 200 ° C, respectively. Helium was used as a carrier gas at a speed of 1mL / min. Mass spectra were obtained by electron impact at 70 eV with automatic scans in the mass range 35 to 400 m / z at 0.30 scans / s. Component identification was based on time and linear retention index (C8-C20 n-alkane series), interpretation and comparison of mass spectra obtained with Adams (2006), Nist (2011) and FFNSC 2 libraries.

Evaluation of the antibacterial activity of Myrcia guianensisleaves´ essential oil: in silico analysis

Prediction of biological activities: To evaluate the biological potential of the major compounds from the *Myrcia guianensis* leaves´ essential oil, the Prediction of Activity Spectra for Substances (PASS) Online program was used. This computational tool calculates the probability of a given organic molecule to present a biological activity by comparing the molecule structure to a database composed of other organic molecules with defined biological activities(Khurana et al., 2011; Ferreira et al., 2019). Thus, the PASS online program gives the probability of a compound of being active (Pa) or inactive (Pi) on a biological target. For comparison, the biological activity of ciprofloxacin was analyzed too.

Prediction of oral bioavailability: The SwissADME program was used to predict the theoretical oral bioavailability of the major compounds from the *Myrcia guianensis* leaves´ essential oil(Daina et al., 2017). For comparison, ciprofloxacin oral bioavailability was also assessed. Information on the following properties were obtained from each compound evaluated: total polar surface area (TPSA), partition coefficient (iLogP), molecular weight, number of hydrogen acceptors (nALH) and number of hydrogen donors (nDLH). Then, an analysis based on "Rule of Five" was performed as previously described (Lipinski et al., 2001;Ferreira et al., 2019). By definition, to present a good estimated oral bioavailability, a molecule needs to meet the requirements for at least 3 of the analyzed parameters: i) total polar surface area (TPSA) <140 A^2, ii) LogP ≤ 5, iii) molecular weight <500 daltons, iv) number of acceptor hydrogen bonds (nALH) ≤ 10, and v) number of donor hydrogen bonds (nDLH) ≤ 5. If a compound violates two or more rules, it may not be orally active.

Estimation of pharmacokinetic characteristics and toxic effects : For analysis of the possible toxic effects and the theoretical pharmacokinetic parameters (absorption, distribution, metabolism and excretion) of the major compounds from the*Myrcia guianensis* leaves´ essential oil, the Osiris (Ferreira et al., 2019) and SwissADME programs were used. These parameters and the toxicity were predicted by comparison of the major compounds from the *Myrcia guianensis´* leaves essential oil chemical structure with a database containing commercially available drugs and commercially available compounds. Toxic effects were classified as mutagenic, tumorigenic, irritant and effects on the reproductive system (Ferreira et al., 2019). LD_{50} values in milligram per kilogram (mg/kg) were estimated by using the PROTOX program (Drwal et al., 2014) and those results were used to classify the major compounds of the essential oil and ciprofloxacin toxicity (Table 1). Estimations on the compounds gastrointestinal absorption, permeability through the blood brain barrier brain barrier and skin permeation (Kp) by the SwissADME program (Daina et al., 2017). Additionally, the probability of the three compounds to becoming a commercial drug ("drug-score") was calculated on the Osiris program by combining the values obtained for iLogP, drug-likeness, solubility, molar mass and toxicity in to a single value. A drug-score value of 0.1 to 1.0 was taken as an index of suitability for commercialization(Ferreira et al., 2019).

Evaluation of the antibacterial activity of Myrcia guianensisleaves´ essential oil: in vitro analysis

Bacterial Strains: The bacterial samples came from the CEUMA University Microbiology Laboratory (UNICEUMA). For the tests were used standard microorganisms (ATCC) and clinical isolates. Bacterial samples were*Staphylococcus aureus* (ATCC 6538),

Pseudomonas aeruginosa (ATCC 27853), *Escherichia coli* (ATCC 042), *Klebsiella pneumoniae* (clinical isolate), MRSA (clinical isolate 1), and MRSA (clinical isolate 2). Susceptibility to antimicrobials was determined in an automated VITEKR 2 system (BioMérieuxClinicalDiagnostics, USA) and data interpretation was performed as recommended by the Clinical Laboratory Standards Institute (CLSI, 2019). The multiple antibiotic resistance (MAR) index was calculated using the formula MAR = x/y, where "x" was the number of antibiotics to which the isolate demonstrated resistance; and "y" was the total number of antibiotics tested. The antibiotic evaluated front clinical bacteria (MRSA strain 1e MRSA strain 2) were ciprofloxacin, cotrimazole, gentamycin, oxacilin, rifampicin, teicoplamin, tetracycline, vancomycin, linezolid, cefpima, ceftriaxone, clindamicine and erythromycin. The MRSA strain 1 (clinical isolated) Multiple Antibiotic Resistance (MAR) index was 0,76 and it was resistant front oxacillin, cefpima, ceftriaxone while the MRSA strain 2 (clinical isolated) Multiple Antibiotic Resistance (MAR) index 0,38 and it was resistant front ciprofloxacin, oxacilin, rifampicin, tetracycline, cefpima, ceftriaxone, clindamicine, erythromycin.

DeterminationMinimum Inhibitory Concentration (MIC), viability and bacterial metabolic rate: The antimicrobial activity of the essential oil of the dried leaves of *Myrcia guianensis*was determined by the microdilution method (CLSI, 2019). Briefly, each bacterial strain was plated with Müeller-Hinton Agar at 37 ° C for 24h and suspended in saline (~1.5×10^8 CFU / mL), adjusted by 0.5 McFarland turbidity and then diluted 1: 10 for inoculation in wells. To determine the MIC,viability and bacterial metabolic rate 10 µL of the bacterial suspension obtained from each strain described in item 2.6.1 was inoculated into the wells of the ELISA plates containing Müeller-Hinton (MH) broth and different concentrations in the ratio 2 of the essential oil. *Myrcia guianensis* (2-0.6 mg / mL). Each well had a final volume of 100 µL. Bacterial strains were inoculated in MH broth as a positive control, whileDimethyl sulfoxide(DMSO) was used as a negative control. The plates were incubated in a bacteriological oven at 37ºC for 24 hours and the procedure was done in triplicate. MIC was the lowest concentration of essential oil of *Myrcia guianensis* in which there was no bacterial growth. In order to determine the viability was used PrestoBlue® that is a dark blue fluorescent indicator (resazurin) that is converted to a red fluorescent compound (resofurin) by metabolically active cells. Viability was determined after incubation for 90 min at 37 ° C with 10 µLPrestoBlue® according to the manufacturer's instructions. In addition, the effect of the oil on the bacterial metabolic rate was calculated through the oxidation and reduction coefficients of PrestoBlue®, according to the manufacturer's instructions.

***In vitro* evaluation of inhibition biofilm production:** The effect of *Myrcia guianensis* essential oil on the eradication of biofilm by the bacteria described in item 2.6.1was quantified according to the methodology previously described (Ferro et al., 2016). To this end, 10 µl bacterial suspension (prepared as described above) was added per well in a 96-well microplate containing different concentrations of *Myrcia guianensis* essential oil (2-0.6 mg / mL) and 100 µLMüeller Hinton broth. Vehicle-treated bacteria and medium without bacteria were used as positive and negative controlrespectively. Samples were incubated at 37 ° C and after 24h, the wells were washed three times with phosphate-saline buffer (PBS). Then, the 5% violet crystal biofilm was stained for 10 min and then immediately solubilized with methanol (200 µl / well, 100%). The reading was then performed at 550 and 630nm on ELISA reader and the result was an indication of inhibition biofilm production.

In vivo model of infection induced by Staphylococcus aureus (ATCC 6538) in Tenebrio molitor larvae: The antimicrobial actions of essential oil of *Myrcia guianensis*were evaluated in an *in vivo* model of infection induced by *Staphylococcus aureus*(ATCC 6538) in *Tenebrio molitor* larvae. Briefly, *Tenebrio molitor* larvae were randomly distributed in three experimental groups (n = 10/group), and were then infected by injection of 100 µL of bacterial suspension (*Staphylococcus aureus* ATCC 6538; 1.8 x 10^9CFU/mL in Salina solution 0, 9 %). After 2h, the larvae received the essential oil of *Myrcia guianensis* dose (20 µL/mL), vehicle (Saline solution 0,9 % 100 µL) and antibiotic ciprofloxacin (0,15 µL/mL). Mortality rate was observed over 3 days post-infection.

Table 1. Toxicity Class according to LD_{50} value (mg/kg)

Class	Classificação
Class I	Fatal if swallowed $LD_{50} \leq 5$
Class II	Fatal if swallowed $5 < LD_{50} \leq 50$
Class III	Harmful if swallowed $50 < LD_{50} \leq 300$
Class IV	Harmful if swallowed $300 < LD_{50} \leq 2000$
Class V	Toxic if swallowed $2000 < LD_{50} \leq 5000$
Class VI	Non toxic $LD_{50} > 5000$

In order to assess the bacterial load in the hemolymph, in a separate set of experiments, the larvae were infected with *Staphylococcus aureus*(ATCC 6538) as described above and then received either EO of *Myrcia guianensis* (20 µL/mL; n = 5) or vehicle (Saline 0,9%; n = 5) or ciprofloxacin (0,15 µL/mL; n = 5). Larvae were incubated at 37ºC for up to one day. Five larvae of each group were culled and analyzed for bacterial load. Briefly, at each group, the larvae were cut through in acephalocaudal direction with a scalpel blade and squeezed to remove the hemolymph. Serial dilutions (10x) of the hemolymph of each larvae were made in Saline and 10µL of each dilution were incubated in MHagar and cultured for 24h at 37ºC. After this period, the plates were analyzed for CFU counting. The results are expressed as CFU/mL.

Statistical analysis: Statistical analyzes were performed using GraphPad Prism version 5.00 for Windows. The results were analysed by one-way ANOVA comparing treatments to the positive control using the Bonferroni's test. A significance level of 5% was adopted. Statistical significance was set at a level of $p < 0.05$. The survival data were analyzed using the Kaplan–Meier method, and comparisons between groups were made using the log-rank chi-square test.

RESULTS

Yield Calculation: The resulted obtained in this extraction process wascalculated in percentage resulting on a yield of 1.34%.

Determination of chemical composition of essential oil: GC-MS analysis of the essential oil from the leaves of *Myrcia guianensis*separeted 34compounds from what were identified 31, corresponding to 88,84% of the total. The compounds were, basically, monoterpenes (35,5%) and sesquiterpenes (64,5%).Only 4 components represent almost 70% of the total compounds from GC-MS analysis (Figure 1). These are the major identified components: 1 - (E-)caryophyllene (37.43%), 2 - terpinolene (14.82%), 4 - β-bisabolene (6,07%) (Table 2). The third one was a compound without identification a probably isomer of methoxyolivetol (NIST)with Molecular Weight of 194 g/mol and molecular formula of $C_{12}H_{18}O_2$(Annex I).

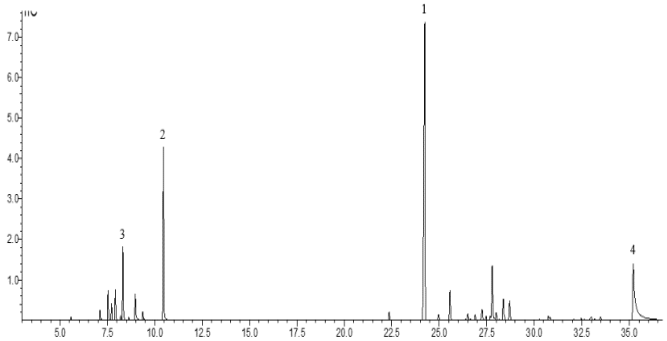


Figure 1. Chromatogram of *Myrcia guianensis* (Aubl.) DC.leavesessential oil

Table 2. Chemical composition of the essential oil of the leaves of *Myrcia guianensis* (Aubl.) DC

TR[a]	Name	%	IRC[b]	IRL[c]
5.575	α-Pinene	0,17	934	932
7.100	Myrcene	0,76	992	988
7.530	α-Phellandrene	2,2	1006	1002
7.715	δ-3-Carene	1,23	1012	1008
7.915	α-Terpinene	2,26	1017	1014
8.200	p-Cymene	0,4	1025	1020
8.320	Limonene	5,78	1029	1024
8.630	(Z)-β-Ocimene	0,22	1038	1032
8.965	(E)-β-Ocimene	2,31	1047	1044
9.365	γ-Terpinene	0,73	1059	1054
10.460	Terpinolene	14,82	1090	1086
22.370	α-Copaene	0,74	1376	1374
24.240	(E-)Caryophyllene	37,43	1422	1417
24.970	α-Guaiene	0,52	1440	1437
25.570	α-Humulene	2,97	1454	1452
26.505	γ-Muurolene	0,6	1478	1478
26.655	Germacrene D	0,09	1481	1484
26.895	β-Selinene	0,53	1487	1489
27.250	Viridiflorene	1,2	1496	1496
27.470	α-Muurolene	0,38	1501	1500
27.680	α-Bulnesene	0,34	1507	1509
27.790	β-Bisabolene	6,07	1510	1510
28.005	γ-Cadinene	0,88	1515	1513
28.375	δ-Cadinene	2,49	1525	1522
28.700	(E)-γ-Bisabolene	2,14	1533	1529
30.275	Caryolan-8-ol	0,07	1574	1571
30.750	Caryophyllene oxide	0,39	1586	1582
30.830	Viridiflorol	0,27	1588	1592
32.470	1-epi-Cubenol	0,11	1632	1627
32.995	Cubenol	0,41	1646	1645
33.480	Allohimachalol	0,33	1659	1661
35.200	NI*	11,04	1706	
IDENTIFIED TOTAL		88,84		

[a]Chromatographic column retention time
[b]Retention Index Calculated.
[c]ÍLiterature Retention Index
*No identification:isomer of methoxyolivetol (NIST); MW:194; MF: $C_{12}H_{18}O_2$
Source: Analized by Pablo L. B. Figueiredo.

Table 3. Evaluation* of the biological potential of (E-) caryophyllene, terpinolene, β-bisabolene and ciprofloxacin

COMPOUND	(E-)CARYOPHYLLENE
Total of predictable activities (Pa>0.3)	600
High probability of activity occurring (Pa>0.7)	45
Activities related to antimicrobial activity	i) antifungal (Pa=0.582 and Pi=0.020), ii) Antiprotozoal (Leishmania) (Pa=0,470 and Pi =0,029, iii) Antibacterial (Pa=0,437 and Pi= 0,023), iv) Membrane permeability enhancer (Pa=0,398 and Pi=0,059), v) DNA ligase (ATP) inhibitor (Pa= 0,335 and Pi=0.033).
COMPOUND	TERPINOLENE
Total of predictable activities (Pa>0.3)	1797
High probability of activity occurring (Pa>0.7)	123
Activities related to antimicrobial activity	i) Saccharopepsin inhibitor (Pa= 0,733 and Pi= 0,036), ii) Thioredoxin inhibitor (Pa= 0,680 and Pi= 0,014), iii) Tpr proteinase (*Porphyromonasgingivalis*) inhibitor (Pa= 0,616 and Pi= 0,011).
COMPOUND	β-BISABOLENE
Total of predictable activities (Pa>0.3)	213
High probability of activity occurring (Pa>0.7)	18
Activities related to antimicrobial activity	i) Antifungal (Pa=0.585 and Pi=0.020), ii) Antiprotozoal (Leishmania) (Pa=0,428 and Pi =0,039), iii) Antibacterial (Pa=0,413 and Pi= 0,027), iv) Membrane permeability enhancer (Pa=0,446 and Pi=0,035).
COMPOUND	CIPROFLOXACIN
Total of predictable activities (Pa>0.3)	47
High probability of activity occurring (Pa>0.7)	4
Activities related to antimicrobial activity	i) Ophthalmic antibacterial (Pa=0.940 and Pi=0,000), ii) Anti-infective (Pa=0.823 and Pi=0.005), iii) DNA synthesis inhibitor (Pa=0.786 and Pi=0.004), iv) Topoisomerase II inhibitor (Pa=0.759 and Pi=0.003), v) Antimycobacterial (Pa=0.638 and Pi=0.008), vi) Antibacterial (Pa= 0.589 and Pi=0.009), vii) Quinolone-like antibiotic (Pa=0.572 and Pi=0.001), viii) Anti-cytomegalovirus (Pa=0.448 and Pi=0.004), ix) Anti-tuberculosic (Pa=0.452 and Pi=0.019), x) DNA gyrase inhibitor (Pa=0.488 and Pi=0.001), xi) Antibiotic (Pa=0.358 and Pi=0.010), and xii) Anti-adenovirus (Pa=0.304 and Pi=0.086).

*Evaluation realized in Prediction of Activity Spectra for Substances (PASS)

In silico analysis of (E-)Caryophyllene, Terpinolene and β-Bisabolene

Identified biological activities: During the computational evaluation were found that the compounds E-)caryophyllene, terpinolene and β-bisabolenepresent many activities, which means potential activity higher than 30% of chance of happening (Pa≥0.3), being high probability of activity occurring over 70% (Pa>0.7). A close look was taken about the prediction of antimicrobial activities of the compounds and for comparison was evaluated the ciprofloxacin (Table 3).

Estimated oral bioavailability: For predicting the oral bioavailability of (E-)caryophyllene, terpinolene and β-bisabolene, their values of TPSA, iLogP, molecular weight, nALH and nDLH were analyzed (Table 4).

Table 4. Estimation of oral bioavailability of (E-) caryophyllene, terpinolene and β-bisabolene in comparison with ciprofloxacin by *in silico* test

	(E-)Caryophyllene	Terpinolene	β-Bisabolene	Ciprofloxacin	Rule of five
iLogP	3.29	2.71	3.67	2.24	<5
MW	204.35 g/mol	136.23 g/mol	204.35 g/mol	331.34	≤ 500 g/mol
TPSA	0.00 Å²	0.00 Å²	0.00 Å²	74.57 Å²	<140 A^2
nDLH	0	0	0	2	≤ 5
nALH	0	0	0	5	≤ 10

iLogP : partition coefficient water: oil – lipophilicity index; MW : molecular weight; TPSA : total polar surface area; nALH : number of acceptor hydrogen bonds; nDLH number of donor hydrogen bonds

Table 5. Estimated absorption of (E-)caryophyllene, terpinolene and β-bisabolene in comparison with ciprofloxacin by *in silico* test and using Drug-likeness score

		(E-)Caryophyllene	Terpinolene	β-Bisabolene	Ciprofloxacin
Predicted absorption	GI absorption*	Low	Low	Low	High
	BBB permeability**	No	Yes	No	No
	Log K_p***	-4.44 cm/s	-3.96 cm/s	-2.98 cm/s	-9.09 cm/s
Drug-likeness	Log S^+	-3.66	-2.34	-3.51	-3.32
	DL^{++}	-6.48	-3.02	-6.41	2.07
	DS^{+++}	0.31	0.46	0.31	0.55

*GI: gastrointestinal absorption; **BBB: blood brain barrier; ***Log Kp: skin permeation index; +Log S: solubility; ++DL: drug-likness, +++DS: drug-score

Predicted pharmacokinetic characteristics and toxic effects: This evaluation was performed in (E-) caryophyllene, terpinolene and β-bisabolene in comparison with ciprofloxacin. The prediction of pharmacokinetic characteristics shows information on the estimation of gastrointestinal absorption, permeability through the blood brain barrier (BBB) and skin permeation (log K_p in centimeters (cm)/s). No drugs were considered to be highly absorbed by the gastrointestinal tract, except the ciprofloxacin; however, only terpinolene was predicted to cross the BBB. About drug-likeness score, the terpinolene had the best result from the three compounds while (E-) caryophyllene and β-bisabolene had the same results (Table 5).

The toxicity assessment evaluated mutagenic effects, tumorigenic effects, irritant effects, hepatotoxicity, effects on reproduction, lethal dose 50% in mg/kg (LD_{50}) and toxicity class. None of the compounds, (E-)caryophyllene, terpinolene and β-bisabolene**,** had toxic effects. All the three compounds exhibited a toxicity score of 5, with the LD_{50} of all compounds between 4390mg/kg - 5000mg/kg. On the other hand, ciprofloxacin was found to have predicted mutagenic effects, with no tumorigenic actions and no effects on the liver or reproductive system. The toxicity class of ciprofloxacin was 4 with LD_{50}value of 2000mg/kg.

Evaluation of antibacterial activity of Myrcia guianensis leaves essential oil

Minimum Inhibitory Concentration (MIC): The experiment to determine the MIC showed the essential oil of *Myrcia guianensis* leaves had better results against Gram-positive bacteria, whereas front Gram-negative bacteria had little or no effect. Noteworthy was the result against *Staphylococcus aureus* (ATCC 6538), Methicillin-resistant *Staphylococcus aureus* (MRSA strain 1) and Methicillin-resistant *Staphylococcus aureus* (MRSA strain 2), which presented a minimum inhibitory concentration of 2mg/mL, while in the results front others bacteria had no significant differences were observed.

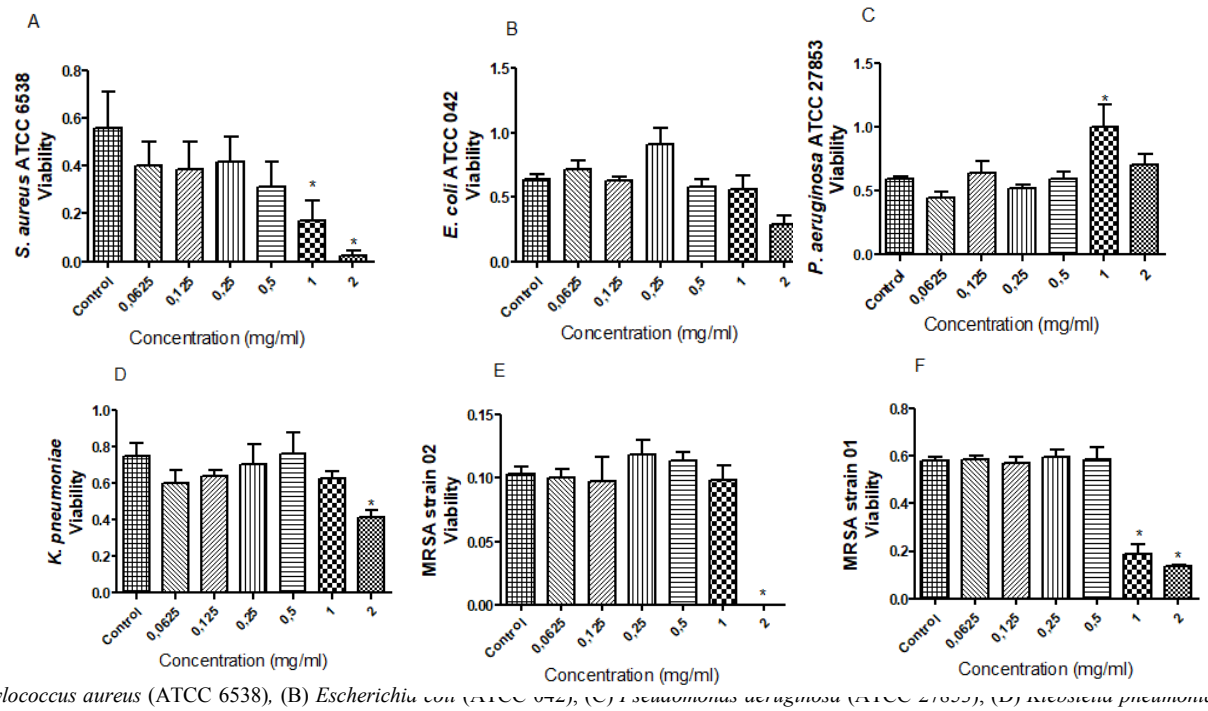
Determination of viability and bacterial metabolic rate: The essential oil of *Myrcia guianensis* leaves decreases the viability and metabolic activity of *Staphylococcus aureus* (ATCC 6538) at highest concentration tested (2mg/mL) and, statistically, it was also noted effects at 1 mg/mL.A poor oil activity was observed against *K. pneumoniae* at a concentration of 2mg/mL while was observed no effect against *Escherichia coli* ATCC 042 and *Pseudomonas aeruginosa* ATCC 27853at any concentration tested. It was seen that the best result came from Gram-positive bacteria, especially against *Staphylococcus aureus*(ATCC 6538). Thus, were tested some clinical isolated of 2 strains of MRSA, which showed that viability and metabolic were decreased by highest concentration tested (2mg/mL) (Figures 2 and 3).

***In vitro* evaluation of inhibition biofilm production:** Antibiofilm activity of the essential oil of *Myrcia guianensis* leaves was observed at the highest concentration tested (2mg/mL) against *Escherichia coli*(ATCC 042) and*Staphylococcus aureus*(ATCC 6538). However, these effects were not observed in the others strains evaluated in this test (Figure 4).

Myrcia guianensis´ leaves essential oil Reduces Bacterial Load in the Hemolymph: *Myrcia guianensis´* leaves essential oil (20mg/100mg of larvae) effects were compared to those of ciprofloxacin (1 mg/mg of larvae) and vehicle-treated larvae infected with *Staphylococcus aureus* (ATCC 6538) or saline solution 0.9%. None of the products evaluated induced any toxicity on*Tenebrio molitor*larvae (Figure 5). On those groups that received saline solution 0.9% and treatment, the essential oil and ciprofloxacin had the same result 70% of survival while the control treated with saline solution had 90% of survival. On the others groups, the essential oil analyzed had better results as the control with saline solution (80% of survival) than a ciprofloxacin group (40% of survival) after treatment.The Essential oil from *Myrcia guianensis´* leaves also reduced bacterial load in the hemolymph of *Tenebrio molitor* as the ciprofloxacin did when compared with the control (Figure 6).

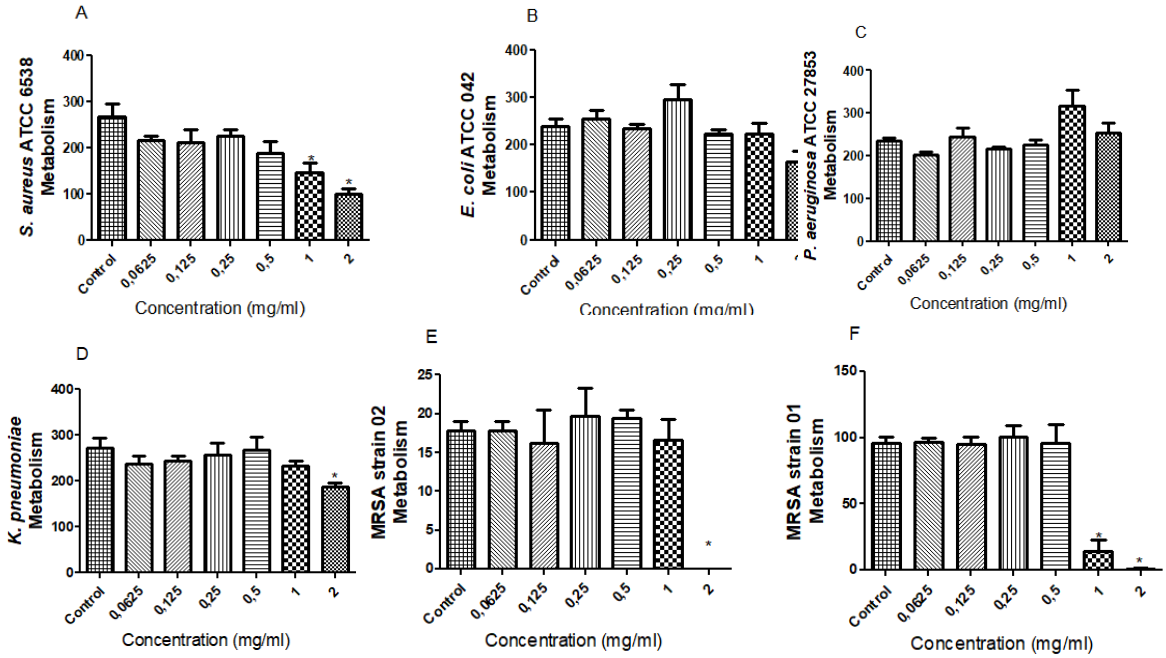
DISCUSSION

The yield calculation had a promising result of 1.34% when compared, for example, by Souza Filho et al. (2006) that had 0.11% as a result from the same species, but harvested in Para state, north of



(A) *Staphylococcus aureus* (ATCC 6538), (B) *Escherichia coli* (ATCC 042), (C) *Pseudomonas aeruginosa* (ATCC 27853), (D) *Klebsiella pneumoniae* (clinical isolate), (E) MRSA (clinical isolate 2) and (F) MRSA (clinical isolate 1). Data represent the mean ± standard error of the mean of three independent experiments performed.p*<0,05; differs from the control group.

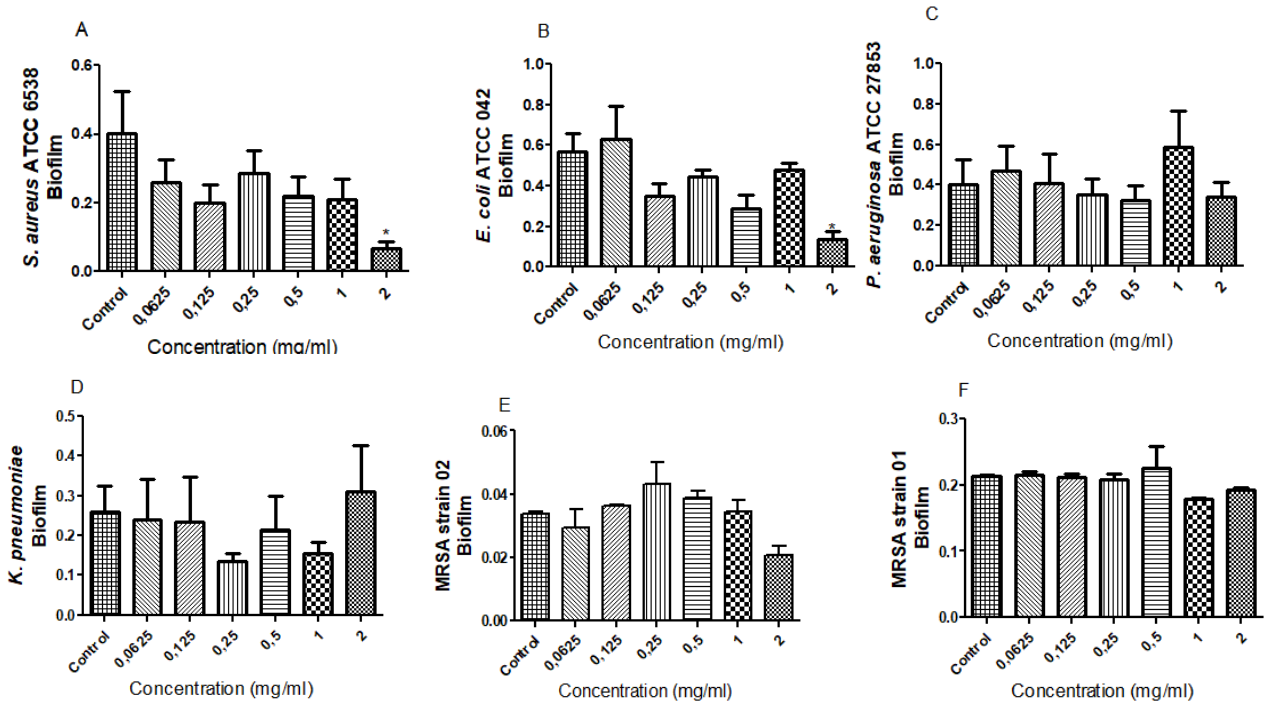
Figure 2. Effect of essential oil of leaves of Myrcia guianensis (Aubl.) DC. on bacterial growth. The viability of bacteria strains was used as an indicator of bacterial growth.



(A) *Staphylococcus aureus* (ATCC 6538), (B) *Escherichia coli* (ATCC 042), (C) *Pseudomonas aeruginosa* (ATCC 27853), (D) *Klebsiella pneumoniae* (clinical isolate), (E) MRSA (clinical isolate 2) and (F) MRSA (clinical isolate 1). Data represent the mean ± standard error of the mean of three independent experiments performed. p*<0,05; differs from the control group

Figure 3. Effect of essential oil of Myrcia guianensis (Aubl.) DC. on the metabolic activity of bacteria. The metabolic activity of bacteria strains was quantified by PrestoBlue® reagent oxidation and reduction coefficients

Brazil. Comparing with specimen that was harvested from the same plant region under analysis in this paper and it belonging to the same genus, the species *Myrcia sylvatica* (G. Mey) DC., presented the result of 0.5% (Rosa et al., 2016). It is noteworthy that the yield can be influenced by several factors such as weather conditions, time of collection, age of the plant, care in the preparation of vegetable raw material, and other factors as the time extraction process(Maia et al., 2015; Figueiredo et al., 2017). On the determination of the essential oil chemical composition, it is noted in table 2, that among the identified compounds are monoterpenes (35,5%) and sesquiterpenes (64,5%) with predominance of sesquiterpenes as occurs with other plants from the same genus. The identified major compounds, for example, are classified in the same proportion with monoterpene as terpinolene and sesquiterpenes as (E-) caryophyllene and β-bisabolene(Cascaes et al., 2015; Silva et al., 2016). The major compounds of the*Myrcia guianensis´*s leaves essential oil were evaluated by *in silico* analysis for identification of potential biological activities. The PASS program has been used for prediction of biological activity based on structural formula of a compound. It makes a qualitative evaluation as active or inactive based on



(A) *Staphylococcus aureus* (ATCC 6538), (B) *Escherichia coli* (ATCC 042), (C) *Pseudomonas aeruginosa* (ATCC 27853), (D) *Klebsiella pneumoniae* (clinical isolate), (E) MRSA (clinical isolate 2) and (F) MRSA (clinical isolate 1). Data represent the mean ± standard error of the mean of three independent experiments performed. p*<0,05; differs from the control group.

Figure 4. Effect of essential oil of *Myrcia guianensis* (Aubl.) DC. on inhibition of bacterial biofilm productionagainst different bacteria strains

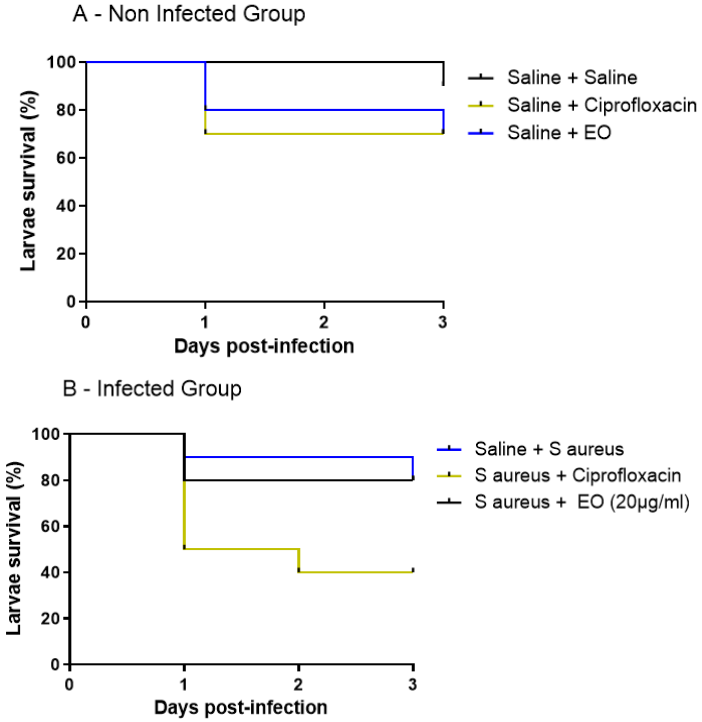


Figure 5. Effect of *Myrcia guianensis´* leaves essential oil on survival of *Tenebrio molitor* larvae infected with *Staphylococcus aureus*(ATCC 6538). *Tenebrio molitor*larvae received saline solution 0.9% (A) or *Staphylococcus aureus*(ATCC 6538) (B) and they were treated with *Myrcia guianensis´* leaves essential oil (20 mg/100 mg of larvae), Ciprofloxacin (1 mg/100mg of larvae) or vehicle (Saline 0,9 mL/100 mg),The evaluation occorred for 3-days post-infection. *n* = 10/group for survival experiments

structure-activity of compounds with known biological activity and it has been used for many researchers recently (Dmitriev et al., 2019; Ferreira et al., 2019; Sarkar et al., 2020). The results of this evaluation may be observed in table 3, where is possible to see that all major compounds have potential activity in microbiological field.

The ciprofloxacin effectiveness in microbiological activity is well known and the results from the online program showed that as well, as above-mentioned. The major organic compounds of essential oil of *Myrcia guianensis* showed prominent theoretical microbial activity what makes implication about the effectiveness the isolate compounds and it suggests that possibly of essential oil effectiveness

too. Since this showed potential for antimicrobial activity, it was realized further investigation.

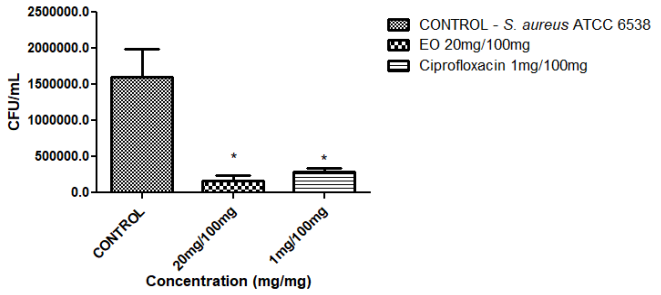


Figure 6. Effect of *Myrcia guianensis´* leaves essential oil on hemolymph bacterial load comparing with ciprofloxacin. The *Tenebrio molitor* larvae infected with *Staphylococcus aureus* (ATCC 6538) received *Myrcia guianensis´*s leaves essential oil (20mg/100mg) and were evaluated one day post-infection (n=5/group) for bacterial load quantification. Data for bacterial load is represented as mean ± SD. *$p < 0.05$, compared with control (*Staphylococcus aureus* ATCC 6538)

The estimated oral bioavailability is an important parameter on a development of bioactive molecules as therapeutic agents (Veber et al., 2002). For evaluation of new compounds is applied the "rule of 5", as shown in table 4. However, it is important to remind that there are orally active therapeutic classes outside the `rule of 5'. The exceptions are antibiotics, antifungals, vitamins and cardiac glycosides (Lipinski et al., 2001). Even so, table 4brings information showing that the three natural compounds fit the criteria to present good estimated oral bioavailability because all three compounds attended the "rule of five" meting all five criteria. Ciprofloxacin presented a TPSA of 74.57, iLogP of 2.24, molecular weight of 331.34, nDLHof 2.0 and nALH of 5.0 whichdemonstrates, as the compounds evaluated, a good predicting of oral bioavailability, assuring the test. Therefore, it implies that each compound separately, as the ciprofloxacin, had good results on estimation oral bioavailability what could lead for future oral use for isolated compound or the essential oil. Evaluating the toxic effects all the three compounds exhibited a toxicity score of class V, indicating they are classified as harmful if swallowed 2000mg/kg<LD_{50} ≤ 5000mg/kg, which means small toxicity even when compared with ciprofloxacin, drug commercially available that presented as a result a value to be classified as class IV,harmful if swallowed 300mg/ kg<LD_{50} ≤ 2000mg/kg. All natural compounds evaluated presented no mutagenic, tumorigenic, irritant effects either hepatotoxicity or effects on reproduction. Although, ciprofloxacin presented between the aspects evaluated high mutagenic effects. These toxic effects classification was expected since these essential oils from plant usually have none or small toxicity front mammal, thus getting towards the security use (Figueiredo et al., 2007). (E-)Caryophyllene, terpinolene and β-bisaboleneon the evaluation for gastrointestinal absorption presented low absorption.They did not had potential on permeability of BBB, except for terpinolene that represents an important natural substance which may be explored in terms of Central Nervous System acting drug (Table 5). As it was expected, ciprofloxacin had high potential on gastrointestinal absorption.

The skin permeability (Kp) defines the rate of a chemical penetrating across the stratum corneum. It is a linear model that relates molecular weight and lipophilicity of compounds, thus the more negative Log Kp, the less permeant on the skin will be the molecule. The exposure of the skin to naturally derived chemical is an issue due the lack of sufficient data from biological tests *in vivo* and *in vitro*. The *in silico* analysis is an alternative that is in development over the past decades (Chen et al., 2018; Guimarães, 2019). The better estimated Log K_p values were of -2.98 and -3.96 cm/s for β-bisabolene and terpinolene, respectively (Table 5). The ciprofloxacin had the lower number on Log Kp representing the lower skin permeability between the evaluated compounds. The drug-likeness estimates how close is the prediction of a structured already knew on market. This evaluation on natural products has been growing over the time and it has good resultsalready. It is noteworthy the importance of plants as source for modern drug design/discovery (Tian et al., 2015). The Log S value is related with the solubility of the compound evaluated and the lowest result is preferred (Rocha et al., 2019**)**. In this evaluation terpinolene had the best result on drug-likeness (Log S, DS and DL) being the compound with closest result from Ciprofloxacin´s (Table 5). Besides its potential, it is necessary *in vitro* and *in vivo* analyze with each compound and the essential oil as well. Once, they may present different result based on synergism or another mechanism (Azizan et al., 2017). (E-)Caryophylleneis the major components of the essential oil of*Myrcia guianensis* leaves. This compound is an important sesquiterpene and it is present in several essential oils from plants like *Copaiferalangsdorffii*Desf.(Ames-Sibin et al., 2018), *Myrcia sylvatica* (G. Mey) D.C. (Rosa et al., 2016), *Spirantheraodoratissima* A. St.-Hil. (Oliveira, 2016) e *Syzygiumaromaticum*L. (Barros Gomes et al., 2018). (E-)Caryophyllene has been demonstrating several important pharmacological activities including antioxidant, anti-inflammatory, anticancer, cardioprotective, hepatoprotective, gastroprotective, nephroprotective and antimicrobial (Machado et al., 2018). (E-)Caryophyllene also exhibits activity similar to local anesthesia, which could protect the nervous system from oxidative stress and inflammation (Gertsch et al., 2008; Tchekalarova et al., 2018).

(E-)Caryophyllene showed more pronounced antibacterial properties against Gram-positive bacteria than when compared with Gram-negative bacteria(Dahham et al., 2015)and it presented good results as an inhibitory effect on factors related to *S. mutans*biofilm formation(Yoo&Jwa, 2018). Results on inhibition of *Streptococcus* sp. by (E-)caryophyllene are highlighted because those bacteria described as the most important in the initial adhesion of dental plaque in humans. *In vitro* studies have shown natural products that are present as the main (E-)caryophyllene compound with significant antimicrobial activity, suggesting that the substance possibly participated in this activity (Pieri et al., 2016). Among the major compounds of the essential oil found in this GC analysis is highlighted the terpinolene, which is present in many plant species that have several pharmacological activities, such as analgesic and anti-inflammatory (Macedo et al., 2016).Terpinolene is one of the most monoterpenes used as a food supplement or odorant in cosmetics and pharmaceutical industry. Besides that, cell proliferation and viability significantly decreased after terpinolene exposure (Agus et al., 2018). Essential oil that had terpinolene as one of the major compounds also showed antimutagenic activity (Lima et al., 2016) and natural antioxidant potential (Coccimiglio et al., 2016). The essential oil of *Myrcia guianensis* leaves presented great amount of β-bisabolene in GC analysis. The*Copaiferamultijuga*Hayne oil, which also, presented as major compound (E-)-caryophyllene and β-bisabolene, has an interesting anti-inflammatory effect and an important effect on the CNS (Kobayashi et al., 2011). β-bisabolene, worked as an anti□cancer agent from the essential oil of opoponax that exhibits specific cytotoxicity to both human and murine mammary tumour cells *in vitro* and *in vivo* (Yeo et al., 2016). The essential oil from *Daucuscarota sativa* seeds presented 80% of β-bisabolene and it showed a great antimicrobial activity (Imamu et al., 2007) as well as the high antifungal activity presented by *Clausenalansium* essential oil with β-bisabolene being one of the major compounds (He et al., 2019).

Importantly, environmental changes could have a different impact on populations of *Myrcia guianensis*, and these changes are responsible for changes in yield and chemical composition of essential oils. Examples of these environmental factors include rainfall, seasonality, altitude, atmospheric composition, temperature, UV radiation, age, pathogen attack, water, macronutrients and micronutrients (Martins et al., 2006; Gobbo-Neto& Lopes, 2007). The results seen in figures 2 and 3 show that the best results of *Myrcia guianensis* oilwere in Gram-positive bacteria, specifically front *Staphylococcus aureus* ATCC 6538 and clinical isolated MRSA. No other work on *Myrcia guianensis*was verified with this evaluation. This result also

corroborates the results above-mentioned as (E-) caryophyllene play. Other studies with other species of the genus Myrcia, such as *Myrcia oblangata*DC., showed inhibition of the activity of Gram-positive bacteria (*Enterococcus faecalis, Staphylococcus aureus, Bacillus subtilis* and *Staphylococcus epidermidis*), while it was recorded with no activity front Gram-negative bacteria tested (Santana et al., 2018). The work of Cerqueira et al. (2007) corroborates the results found in this study, since evaluating the species *Myrcia myrtifolia* DC.from Salvador, Bahia, Brazilpresented better results against *Staphylococcus aureus* and MRSA. Some Myrcia species presented good antimicrobial activity being showed better results front *Staphylococcus aureus* in which some species (*Myrcia citrifolia, Myrcia minutiflora, Myrcia paivae* and *Myrcia magnoliifolia*) presented moderate to high antibacterial activity and others (*Myrcia fallax, Myrcia sylvatica, Myrcia paivae*and *C. spruceana*) showed moderate actions (Pereira Junior, 2018). The essential oil exhibited potential bactericidal effects against the tested pathogenic bacteria, even the clinical isolated. Although, the mode of action is still not fully understood. Moreover, the antibacterial activity may also be influenced by the susceptibility of the bacterial cells, especially *Staphylococcus aureus*, towards the chemical composition of the essential oil. It is noteworthy that others compound may also contribute the antibacterial properties by synergism and/or additive effects due to a single and/or several different mechanism(s) of action of one or several compounds. Mode of actions of the antibacterial activity may differ considerably depending on their interactive functions (Azizan et al., 2017).

Essential oils are also known for inhibit and eradicate of biofilm against bacteria decreasing this virulence factor and the chance of bacterial survival (Kim, ES et al., 2015; Kim, BS et al., 2015; Rossi et al., 2018). It is possible to observe in figure 4 the statistically significant result of inhibition of factors of biofilm formation against*Escherichia coli* (ATCC 042) and *Staphylococcus aureus* (ATCC 6538). However, as above-mentioned there were no observation on biofilm from *Staphylococcus aureus* (ATCC 6538) probably because of the decrease of bacteria population. On the other hand, the population of *Escherichia coli* (ATCC 042) did not suffer interference of the essential oil while there was an inhibition of factors of biofilm formation in the highest concentration tested what corroborates for a potential action on biofilm. The results of the essential oil from plants of genus *Myrcia*, as *Myrcia ovata,* was effective agent against *E. faecalis*biofilm (Cândido et al., 2010). However, it also was observed essential oil of *Orthosiphonstamineus* Benth and *Ficusdeltoidea,* which had as a major compound (E-)caryophyllene, were found no biofilm effect, which may happen because of synergism or effect of others compounds of the essential oil together (Azizan et al., 2017). As *Myrcia guianensis´* leaves essential oil exhibited stronger actions on *Staphylococcus aureus* (ATCC 6538), it was performed an *in vivo* infection assay using *Tenebrio molitor* larvae. This larvae has been used as experimental model on many activity evaluation as antimicrobial, insecticide and cytotoxicity (Rezende, 2018).

Revealing a larva survival rate of 80%, the *Myrcia guianensis* essential oil and the vehicle-treatedof considered being of low toxicity (Figure 5) as said on the baseline of Firmo´s work(2018). It is important highlight that, there were no difference statically about the survival test between any of the groups compered. The results on ciprofloxacin groups may be explained by the current understanding that says the bacterial cells may reach persistence by diverse pathways even from the antibiotic what can increase virulence in some cases (Chen et al., 2011; Kubistova et al., 2018). On the other hand, by analyzing the bacterial load, it was noted that the *Myrcia guianensis´* leaves essential oil significantly reduces the number of *Staphylococcus aureus*(ATCC 6538) in *Tenebrio molitor* hemolymph samples in comparison with control, as indicated by CFU counting (Figure 6). It is noteworthy that the bacterial load had slight better results when comparing with ciprofloxacin, which had both great results comparing with the control. This effect was noted from 24h post-infection. The average of CFU in bacterial load quantification in control group was 100 while in Ciprofloxacin group was 18 (SD = 5) and in essential oil group was 10 (SD = 9)**.** It is important to note that to estimate pathogen load is vital to determinate how the infection is especially within immune cells (Brazil, 2019). Overall, the *Myrcia guianensis´* leaves essential oil had promising results, especially, against *Staphylococcus aureus* andoninhibition of biofilm formation against *Escherichia coli* (ATCC 042). The association of essential oil with antibiotics it is also an opportunity of improvement what may create different mechanism of action, thus providing new choices in overcoming the unsolved crisis of bacterial.

Conclusion

This paper brings relevant scientific information about *Myrcia guianensis* present in Legal Amazon in Brazil. The results showed that the essential oil from *Myrcia guianensis* leaves is a promising bactericidal agent, based on the characteristics of the constituents identified, together with the absence of toxicity identified in the tests performed. Thus, promoting the bioprospecting potential of the species, as well as identifying it as a promising agent for the development of herbal medicines.

Credit author statement

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Jorge Fernando Viegas Pessoa: Investigation.

Pablo Luis Baia Figueiredo: Methodology, Investigation.

Odair dos Santos Monteiro: Methodology, Resourses.

Saulo José Figueiredo Mendes: Methodology, Writing - Original draft preparation, Software, Resourses.

Flavia Maria Mendonça do Amaral: Writing – Review & Editing.

Thiago Azevedo Feitosa Ferro: Conceptualization, Methodology, Validation, Formal Analysis, Resourses, Writing- Review & Editing.

Denise Fernandes Coutinho: Conceptualization, Resourses, Writing – Review Editing, Project administration

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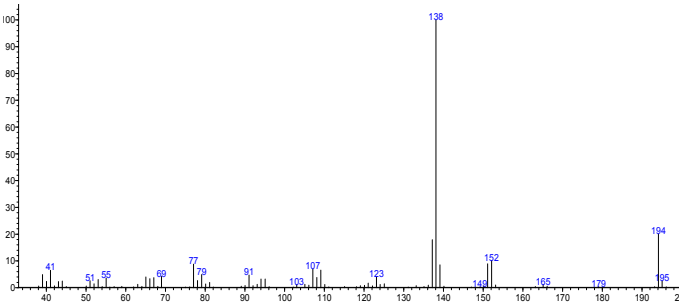
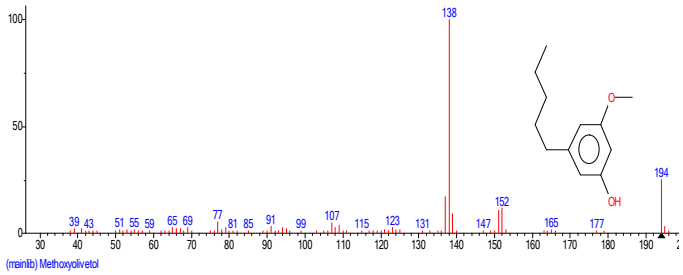
Supplementary file - Annex I

MASS SPECTROMETRY OF COMPOUND IR1706 (PM 194, FM, $C_{12}H_{18}O_2$)



NIST METOXIOLIVETOL (IR 1601- Estimated)



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