Antimicrobial and antibiofilm properties of essential oils from *Piper marginatum* Jacq.

Propriedades antimicrobianas e antibiofilme de óleos essenciais de *Piper marginatum* Jacq.

Propriedades antimicrobianas y antibiofilme de los aceites esenciales de *Piper marginatum* Jacq.

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Abstract  
A low shrub growing in the Amazonian region, *Piper marginatum* Jacq, has been related to the treatment of a disease variety in folk medicine, however, still lacking scientific support. This study aimed to describe the composition of essential oils obtained from leaves (EOL) and branches (EOB) of *P. marginatum* and their antimicrobial effects on six relevant pathogenic bacteria. A combination of GC-FID and GC-MS was used to identify the phytochemical constituents. As antimicrobial assays, the oils were screened at the minimum inhibitory concentration (MIC) of 3 µg/ml for planktonic and biofilm inhibition. EOL revealed the presence of trans-muurolen, α–cymene, spathulenol, elemicin, and α–copaene, while EOB composition was mainly of myristicin, trans-nerolidol, γ–muurolen and spathulenol. The strongest inhibition of planktonic growth was achieved against *Pseudomonas aeruginosa* (EOB) and *Escherichia coli* (EOB). Overall, Gram negative bacteria were more sensitive to both EOB/EOL showing less ability of growth and biofilm formation. Our results corroborate the relevance of Piperaceae and indicate the possible use of *P. marginatum* in future developments of antimicrobials.

Keywords: *Piper marginatum*; *Piperaceae*; Essential oils; Antimicrobial; Antibiofilm.

Resumo  
Um arbusto de pequeno porte crescendo na região amazônica, *Piper marginatum* Jacq, tem sido relacionado ao tratamento de uma variedade de doenças na medicina popular, porém ainda carente de respaldo científico. Este estudo teve como objetivo descrever a composição dos óleos essenciais obtidos de folhas (EOL) e ramos (EOB) de *P. marginatum* e seus efeitos antimicrobianos sobre seis bactérias patogênicas relevantes. Uma combinação de GC-FID e GC-MS foi usada para identificar os constituintes fitoquímicos. Como ensaios antimicrobianos, os óleos foram avaliados na concentração inibitória mínima (CIM) de 3 µg/ml para inibição planctônica e de biofilme. EOL revelou a presença de trans – nerolidol, o – cimeno, espatulenol, elemicina e α – copaeno, enquanto a composição de EOB foi principalmente de miristicina, trans-cariofileno, trans-nerolidol, oxido de cariofileno, α – copaeno, γ – muurolen e espatulenol. A inibição mais forte do crescimento planctônico foi alcançada contra *Pseudomonas aeruginosa* (EOB) e *Escherichia coli* (EOB). No geral, as bactérias Gram negativas foram mais sensíveis a ambos EOB/EOL, mostrando...
menor capacidad de crecimiento e formación de biofilme. As cepas Gram-positivas parecían reagir aos óleos esenciais por adhesión masiva. Nossos resultados corroboraram a relevância de Piperaceae e indicam um possível uso de *P. marginatum* em futuros desenvolvimentos de antimicrobianos.

**Palavras-chave:** *Piper marginatum*; *Piperaceae*; Óleos essenciais; Antimicrobianos; Antibiiofilmes.

**Resumen**

Un arbusto bajo que crece en la región amazónica, *Piper marginatum* Jacq. se ha relacionado con el tratamiento de una variedad de enfermedades en la medicina popular, sin embargo, aún carece de apoyo científico. Este estudio tuvo como objetivo describir la composición de los aceites esenciales obtenidos de hojas (EOL) y ramas (EOB) de *P. marginatum* y sus efectos antimicrobianos sobre seis bacterias patógenas relevantes. Se utilizó una combinación de GC-FID y GC-MS para identificar los componentes fitoquímicos. Como ensayos antimicrobianos, los aceites se cribaron a la concentración inhibitoria mínima (MIC) de 3 µg/ml para la inhibición planctónica y de biopelículas. La EOL reveló la presencia de trans-nerolidol, α-cymene, spathulenol, elemicin y α-copaeno, mientras que la composición de EOB fue principalmente de miristicina, trans-cariofileno, trans-nerolidol, óxido de cariofileno, α-copaeno, γ-muuroleno y spathulenol. La inhibición más fuerte del crecimiento planctónico se logró contra *Pseudomonas aeruginosa* (EOB) y *Escherichia coli* (EOB). En general, las bacterias Gram negativas fueron más sensibles tanto a EOB como a EOL, mostrando una menor capacidad de crecimiento y formación de biopelículas. Las cepas Gram positivas parecían reaccionar a los aceites esenciales mediante una adhesión masiva. Nuestros resultados corroboran la relevancia de Piperaceae e indican un posible uso de *P. marginatum* en futuros desarrollos de antimicrobianos.

**Palabras clave:** *Piper marginatum*; *Piperaceae*; Aceites esenciales; Antimicrobianos; Inhibición de biopelículas.

1. Introduction

Upper and aromatic plants are widely used in folk medicine, since they present a wide spectrum of activity and proven inhibition against microorganisms (Duarte et al. 2005). Among many other families, *Piperaceae* has been extensively studied with special interest in *Piper* and *Potonomorph* representatives (Mesquita et al. 2004, Oliveira et al. 2013). The well known white and black culinary peppers (*Piper nigrum* L.) with high economic value are exceptions, since the majority of *Piperaceae* species are popularly used for their biological properties (Oliveira et al. 2013). Anti-parasitic activity of *Piperaceae* oils has been demonstrated against *Leishmania* spp., *Leishmania amazonensis*, *Trypanosoma cruzi* and *Plasmodium falciparum* (Marques et al. 2010; Flores et al. 2019).

Essential oils obtained from 10 species of *Piperaceae* were analyzed identifying 71 different compounds with prevalence of sesquiterpenes and monoterpenes (Santos et al. 2001). These findings were corroborated by other authors studying *P. clausenianum* (Marques et al. 2010), *P. officinarum* (Salleh et al. 2012) and *P. aduncum* (Oliveira et al. 2013).

*Piper marginatum* Jacq., called “malvarisco” or “caapeba cheirosa” in Amazonas State, Brazil, has been cited as sedative and anti-inflammatory in the treatment of snake bites (D’Angelo et al. 1997; Guimarães & Giordano, 2004). In a recent study, Pereira et al. (2020) indicated its use against erysipelas, urinary and dermatological infections, among others. The present study gives a contribution to enhance the knowledge about the composition and demonstrates evidence of antimicrobial effects of essential oils from *P. marginatum* by screening six different pathogenic bacteria growing in suspension and adhered to polystyrene microplates.

2. Methodology

2.1 Plant material - *Piper marginatum* samples were collected at the Campus-Manaus-Zona Leste (3° 4’ 38” S e 59° 55’ 46” W), Federal Institute for Education, Science and Technology of Amazonas (IFAM), located in the urban area of Manaus, Brazil, in March 2014. The exsiccate and botanic identification was provided by Professor Dr. Valdely Kinupp (IFAM) and a voucher number HUAM 9985 was deposited at the Federal University of Amazonas herbarium. To process the samples, leaves and branches were dehydrated at 40°C for 72 horas in an air circulating oven. Afterwards, all the selected plant material was stored in paper bags, at room temperature.
2.2 Obtention and analysis of essential oils - Dry samples of branches and leaves (100 g each) were processed by hydrodistillation in a Clevenger modified system for a period of 3.5 hours at constant temperature of 100°C. Subsequently, they were filtered using anhydrous sodium sulfate (Na₂SO₄) to remove water traces. The obtained oils were transferred to amber vials, sealed and kept under refrigeration to maintain the integrity of their volatile chemical constituents.

Each essential oil was diluted in hexane and the solutions were submitted to gas chromatography (CG-FID, model CG 2010, SHIMADZU CORPORATION, Kyoto, Japan) for quantitative analysis and to determine the retention indexes. A CP-Sil 5 CB (100% dimethylpolysiloxane) column from Varian (length = 15 m, i. d.: 0.25 mm, and film thickness = 0.25 µm) was employed. A flow rate of 2.0 mL/min of Helium was applied as carrier gas. The injection was set to split mode 1:20 and performed at 250 °C. The detector was set to 240 ºC and the oven programmed to a temperature ranging from 60°C to 180°C at 3 ºC/min. Pattern linear hydrocarbons were co-injected to determine the retention indexes. To obtain the mass spectrometograms, the essential oils were analyzed by gas chromatography with mass spectrometer detector (GC-MS, model QP-2010, SHIMADZU CORPORATION, Kyoto, Japan). A column VF-1MS (Varian) was used (length = 15 m, i. d. = 0.25 mm, and film thickness = 0.25 μm). The oven conditions were the same used for GC-FID, applying the electron ionization mode at 70 eV.

The retention indices were calculated relative to the elution times of the substances and the elution times of a series of linear hydrocarbons (C₉-C₃₀) which were co-injected with the sample GC-FID. The identification of the components was obtained with a set of retention indices and mass spectra data, compared with literature data (Adams, 2009) and Wiley’s spectra library 7.0.

2.3 Target microorganisms and microbiological procedures - As microbial targets three Gram positive and three Gram negative bacteria were used as follows: Enterococcus faecalis (ATCC 29212), Streptococcus sanguinis (ATCC15300), Staphylococcus aureus (ATCC6538), Escherichia coli (ATCC8739), Salmonella enterica (ATCC13076), Pseudomonas aeruginosa (ATCC9027). All strains were obtained from INCQS, FIOCRUZ, Rio de Janeiro, Brazil. Overnight cultures in brain heart infusión (BHI) broth were used in the tests. Bacterial counts (BC) were established using a Neubauer chamber and the inoculums were standardized to a concentration of 10⁸ BC/ml.

Each 96-microtiter plate was prepared as follows: 100 µl trypticase soy broth enriched with 1% dextrose (TSB-D) was added to each well followed by 100 µl of each essential oil (EOB or EOL), and last of all, 100 µl of bacterial suspension (10⁸ BC/ml) was inoculated. Five experimental controls were used as follows: TSB-D + inoculum: as negative control, the target strain should grow without interference; chlorhexidine digluconate (CHX 2%) – as positive control of inhibition of bacterial growth; and ethylenediaminetetraacetic acid (EDTA 17%) – as positive control of biofilm inhibition and TSB-D solely as a sterility control. All plates were incubated at 35 ± 2°C for 24h. Each essential oil and control were tested in triplicate.

After the incubation period, each microplate had the results recorded by a microplate reader. The supernatant was carefully washed out (3x with sterile saline 0.9%) and the plates were left at 60°C for 1 hour in a Pasteur oven. After this, 120 µl of a crystal violet solution (0.06%) was added to each well and kept at room temperature for 5 minutes. The plates were washed gently and 40 µL of Dimetilsulfoxide (DMSO) was added to each well in order to perform the last screening by the microplate reader.

2.4 Microbiological data analysis - Three readings were performed on a microplate reader (TP READER PLUS ELx808, BIOTEK INSTRUMENTS INC., Winooski, USA) at 630nm: immediately after inoculation (T₀), after 24 hours incubation (T₂₄), and after drying with crystal violet solution (T₂₄b). For each well (essential oil or control) a difference was calculated (T₂₄
– $T_0$ for planktonic growth and $T_{24h}$-$T_0$ for biofilm formation). To relate the results of the potential inhibitors to the untreated control, the latter was considered as 100% growth/biofilm and a percentage value for each essential oil and controls was calculated (Trentin et al. 2011). Any value lower than 100% was considered inhibition of growth/biofilm formation. A comparison between means gave the significance between each essential oil and controls (EDTA, CHX and untreated control) using a Student’s $t$-test (GraphPad® Software) considering $p \leq 0.01$. (Table 2).

3. Results and Discussion

Previous studies have discussed the properties of Piperaceae oils and extracts as antibacterial, antifungal (Holetz et al. 2002; Santos et al. 2012) and anti-parasitic (Carmo et al. 2012) among other biological activities. In this study, the efficacy of the essential oils of P. marginatum was demonstrated by both planktonic and biofilm cultivation.

The oils from leaves and branches yielded 91.34% and 88.06% of identified phytochemicals, respectively. EOB was chemically richer, showing 25 different compounds against the 15 EOL phytochemicals. Phenylpropanoids were the main group of substances detected in EOB (32.85%) and oxygenated sesquiterpenes in OEL (48.56%). The major constituents of P. marginatum oils used in this study were myristicin (24.73%) and trans-caryophyllene (10.66%), present exclusively in the branches. Trans-nerolidol (29.20%), ocimene (27.49%), and spathulenol (10.50%) were detected in both EOL/EOB, with the highest percentage in the leaves (Table 1).

Leaf samples of P. marginatum were collected in different areas of the Brazilian Amazon and classified under seven chemotypes based on the main components of essential oils identified by GC and GC/MS. The main constituents were sesquiterpenes such as (E)-beta-ocimene, beta-caryophyllene, bicyclogermacrene, alpha-copaene and gamma-terpinene and aromatic compounds such as safrone, 3,4-(methylenedioxy) propiophenone, 2-methoxy-4,5-(methylenedioxy) propiophenone, myristicin, (E)-isosormhizole, (E)-anethole and (E)-asarone (Andrade et al. 2008). Although 4 of their samples came from the same city as those used in the present work, they did not find trans-nerolidol and ocimene which were the 2 major compounds in EOL (29.20% and 27.49%, respectively). Differences in oil components probably resulted from different environmental conditions for plant development (Simas et al. 2004). However, the data showed here could suggest the existence of another chemotype not described before.

Both essential oils were more effective in inhibiting the Gram-negative strains, being P. aeruginosa the most sensitive microorganism. E. faecalis, S. aureus and S. sanguinis responded to the presence of the essential oils by enhancing the biofilm formation. The results are summarized in Table 2.

The reduction of growth and biofilm formation in response to the essential oils was statistically significant against the Gram-negative strains, highlighting EOB. On the contrary, in contact with the Gram-positive targets, both EOB/EOL enhanced the bacterial proliferation and strongly stimulated the biofilm formation. This contradiction might be due to the differences in the cell wall structures combined with the essential oil chemical composition. EOB showed higher percentage of phenylpropanoids than EOL which were described before as inhibitors of Gram-negative species (Hyldgaard et al. 2012) including E. coli and Listeria monocytogenes (Gill & Holley, 2004) as well as S. enteritidis (Lanfranchi et al. 2010).

The inhibition of Gram-positive strains corresponded inversely to an increase in the measurements of attached cells, with the exception of E. faecalis. This behavior was also observed by all Gram-positive targets in the samples treated with EDTA. It was expected that EDTA, as a typical metal chelator would have more strongly affected the biofilm formation. This chemical property helped to attribute to EDTA the capacity of avoiding and/or removing biofilms, being one of the reasons for its application in the treatment of endodontic compromised teeth (Zehnder 2006, Dotto et al. 2020). In this particular case,
however, it promoted a higher percentage of adhesion rather than planktonic growth with exception of the Gram-negative bacteria.

Table 1 - Chemical composition of leaves (EOL) and branches (EOB) essential oils from *Piper marginatum* identified by GC-MS.

| Constituents                  | *IK* | EOL   | EOB   | EOL     | EOB     |
|-------------------------------|------|-------|-------|---------|---------|
|                               | IK₁  | SI    | IK₂  | SI      | Percental composition (%) |
| sabinene                      | 975  | 1007  | 93%  | -       | 0.15    |
| β-pinene                      | 979  | 1010  | 95%  | -       | 0.64    |
| β-myrcene                     | 991  | 1018  | 92%  | -       | 0.30    |
| α-cymene                      | 1026 | 1043  | 97%  | 1042    | 27.49   |
| limonene                      | 1029 | 1046  | 94%  | -       | 0.66    |
| cis-β-cymene                  | 1037 | -     | -    | 1052    | 93%     | 0.26    |
| trans-β-cymene                | 1050 | -     | -    | 1060    | 91%     | 0.31    |
| linalool                      | 1097 | 1105  | 93%  | -       | 1.29    |
| bornyl acetate                | 1289 | -     | -    | 1286    | 94%     | 0.81    |
| tridecane                     | 1300 | -     | -    | 1295    | 97%     | 1.20    |
| ciclostratene                 | 1371 | -     | -    | 1374    | 95%     | 0.99    |
| α-copaene                     | 1377 | 1375  | 93%  | 1375    | 92%     | 3.64    |
| β-bourbonene                  | 1380 | 1384  | 91%  | 1383    | 92%     | 0.97    |
| cis-methyl isoeugenol         | 1454 | 1405  | 94%  | 1404    | 96%     | 1.59    |
| β-selinene                    | 1490 | 1486  | 93%  | 1484    | 92%     | 1.58    |
| trans-caryophyllene           | 1419 | -     | -    | 1418    | 96%     | 10.66   |
| germacrene D                  | 1485 | -     | -    | 1428    | 94%     | 1.17    |
| trans-nerolidol               | 1434 | 1434  | 87%  | 1431    | 90%     | 29.20   |
| myristicin                    | 1519 | -     | -    | 1461    | 90%     | 24.73   |
| cis-eudesma 6,11–diene        | 1490 | -     | -    | 1484    | 89%     | 0.48    |
| γ-murolene                    | 1480 | -     | -    | 1499    | 92%     | 4.75    |
| δ-cadinene                    | 1523 | -     | -    | 1522    | 92%     | 1.15    |
| elemicin                      | 1557 | 1558  | 89%  | 1557    | 90%     | 4.47    |
| γ-asarone                     | 1574 | -     | -    | 1577    | 89%     | 2.21    |
| spathulenol                   | 1578 | 1578  | 95%  | 1581    | 95%     | 10.50   |
| caryophyllene oxide           | 1583 | 1582  | 98%  | 1581    | 95%     | 7.80    |
| α-muurolol                    | 1646 | -     | -    | 1645    | 86%     | 0.46    |
| elemol acetate                | 1681 | -     | -    | 1648    | 91%     | 0.79    |
| viridiflorol                  | 1593 | 1658  | 87%  | -       | 1.06    |
| α-cadinene                    | 1639 | -     | -    | 1657    | 81%     | 0.97    |

Monoterpene hydrocarbons       | 29.24| 2.1   |
Oxygenated monoterpenes         | 1.29 | 0.81  |
Sesquiterpenes hydrocarbons     | 6.19 | 27.81 |
Oxygenated sesquiterpenes       | 48.56| 23.29 |
Phenylpropanoids                | 6.06 | 32.85 |
Others                          | -    | 1.20  |
Total identified compounds      | -    | 91.34 | 88.06 |

*IKovats index determined on the DB-5 capillary column referring to n-alkanes (Adams, 2009); Kovats index determined on the CP-Sil 5 CB capillary column referring to n-alkanes IK₁(leaves) IK₂(branches); SI – similarity index. Source: Authors.*

Comparing the results obtained for CHX, EOB achieved the best performance, inhibiting growth and biofilm formation of all Gram-negative strains. EOL was not able to avoid growth at a significant level but was very effective in preventing biofilm formation by *E. coli* and *P. aeruginosa*. Two probable explanations would be, first, an interaction of small molecules present in EOL with some bacterial wall structures or, second, an interference with the exoenzymes involved in quorum sensing mechanisms which are essential to build biofilms (Nazzaro et al. 2013).
Table 2 - Percentual values* of planktonic growth (G%) and biofilm (B%) formation of pathogenic bacteria in contact with essential oils of *Piper marginatum* Jacq.

|                          | EOB   | EOL   | EDTA  | C (+)  | C (-)  |
|--------------------------|-------|-------|-------|--------|--------|
| *E. faecalis*            |       |       |       |        |        |
| G%                       | 133.7±0.0 | 125.5±0.1 | 86.1±0.0 | 51.5±0.0 | 0.546 ± 0.1 |
| B%                       | 767.3±0.1 | 994.4±0.4 | 687.0±0.1 | 48.8±0.0 | 0.149 ± 0.0 |

| *S. aureus*              |       |       |       |        |        |
| G%                       | 50.7±0.1 | 53.3±0.1 | 32.4±0.0 | 25.7±0.0 | 1.147 ± 0.0 |
| B%                       | 1666.9±0.6 | 1542.4±0.6 | 851.1±0.3 | 72.8±0.0 | 0.061 ± 0.0 |

| *S. sanguinis*           |       |       |       |        |        |
| G%                       | 96.1±0.1 | 77.0±0.0 | 52.8±0.1 | 49.1±0.0 | 0.540 ± 0.1 |
| B%                       | 1144.9±0.1 | 1104.9±0.1 | 997.6±0.3 | 121.2±0.0 | 0.117 ± 0.1 |

| *E. coli*                |       |       |       |        |        |
| G%                       | 9.6±0.0  | 8.4±0.2  | 30.6±0.2  | 21.4±0.0  | 0.627 ± 0.1 |
| B%                       | 26.4± 0.0 | 31.5± 0.0 | 44.1±0.0 | 38.4±0.0 | 0.145 ± 0.0 |

| *P. aeruginosa*          |       |       |       |        |        |
| G%                       | 8.9±0.0  | 54.3±0.1 | 12.7±0.0  | 44.4±0.0  | 1.379 ± 0.1 |
| B%                       | 16.8±0.0  | 21.5±0.0  | 15.7±0.0  | 35.6±0.0  | 0.253 ± 0.0 |

| *S. enterica*            |       |       |       |        |        |
| G%                       | 27.5±0.0 | 83.9±0.0 | 67.9±0.0 | 100.0±0.0 | 1.064 ± 0.0 |
| B%                       | 40.7±0.0 | 49.3±0.0 | 68.4±0.0 | 36.0±0.0 | 0.066 ± 0.0 |

*The percentage values in the lines G% (growth) and B% (biofilm) were calculated considering the optical density (nm) mean values of the untreated samples (negative control) as 100%. EOB: essential oil from branches; EOL: essential oil from leaves; EDTA: ethylenediaminetetraacetic acid (5.7%); C (+): positive control as chlorhexidine digluconate (0.7%); C (-): negative control considering each strain cultivated in TSB-D 1% with no inhibitors. Source: Authors.

*P. aeruginosa* was the most sensitive microorganism, followed by *E. coli* and *S. enterica*, showing that EOB probably pursues a unique combination of chemical compounds which interferes more effectively with specific structures present in the Gram-negative cell wall and/or penetrates through the porin channels to act inside the bacteria. Even though the majority of essential oil molecules are small, they are also hydrophobic and do not enter the Gram-negative cells as easily as they do the Gram positive (Burt & Reinders, 2003). Tariq et al. (2019) reviews a variety of essential oils tested for antimicrobial effects and indicates multiple mechanisms of action depending on the microbial strain. Many of the compounds described in this work were also found by other authors to be effective against the same pathogens.

A mechanism of action proposed for myristicin (EOB main compound) on the mitochondrial membrane of leukemia cells would suggest a parallel with bacterial inhibition, considering the similarities between prokaryotes and mitochondria (Martins et al. 2014). Trans-noridol was found to be effective against *S. aureus* and *E. coli*, but not *P. aeruginosa*, in an agar diffusion assay (Skatsa et al. 2000). However, other molecules even in small amounts would act synergistically to affect bacterial cells, so that faced with the complexity of essential oil composition it is difficult to explain exactly why or which component exerts the main bactericidal or bacteriostatic effect (Nazzaro et al. 2013). A mechanism of reversible fluidity enhancement of bacterial membrane was recently described as a possible mode of action for isoeugenol. This molecule would facilitate the permeability of bacteria, thus collaborating with the entrance of other components present in the essential oils (Hyldgaard et al. 2015). The samples collected for this study indicated the presence of isoeugenol in both EOB (2.98%) and EOL (1.59%).
4. Conclusion

In this work, the essential oils obtained from leaves and branches of *Piper marginatum* Jacq. were described concerning their chemical composition and antimicrobial effect against six pathogenic bacteria. Myristicin and trans-nerolidol were the main components detected in branches and leaves essential oils, respectively. Gram negative bacteria were the most susceptible targets, growing planktonic or in biofilms, highlighting the inhibition of *Pseudomonas aeruginosa* by the branches oil. These results indicate *P. marginatum* as a promising *Piperaceae* representative in further developments of phytochemical based antimicrobial products.

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