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2 **ANTIBACTERIAL ACTIVITY OF THE ROSEWOOD**
3 **(ANIBA ROSAEODORA AND A. PARVIFLORA)**
4 **LINALOOL-RICH OILS FROM THE AMAZON**

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19 **ABSTRACT**
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Aims: Evaluation of antibacterial activity and essential oils composition from rosewood species (*Aniba rosaeodora* and *A. parviflora*), sampled in an experimental plantation on Lower Amazon River, Brazil, were performed between July 2014 and June 2015. Rosewood species are threatened with extinction in the Brazilian Amazon.

Methodology: GC and GC-MS analyzed the oils and the *in vitro* antibacterial potential was determined against *Escherichia coli*, *Klesbsiella pneumoniae*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, and *Streptococcus pyogenes*, using the disk-diffusion and plate microdilution assays.

Results: Showed that linalool was the principal constituent of the oils, being 88.6% and 45.0% to *A. rosaeodora* and *A. parviflora*, respectively. The oils were effective against these pathogenic bacteria, with inhibition zone values ranging from 8.8 ± 0.6 mm to 38.4 ± 1.4 mm (MIC, 1.3 to 10.0 $\mu\text{L/mL}$) for the oil of *A. rosaeodora* and 9.2 ± 0.4 mm to 15.4 ± 0.9 mm for the oil of *A. parviflora*. The bactericidal effect and the intensity have been assigned to linalool and its percentage content in the oils. Assays performed with the aqueous extracts showed no activity against the same bacteria.

Conclusion: The rosewood oils could be used in pharmaceutical formulations or to prevent food spoilage to control resistant bacteria strains, individually or in combination with traditional antibiotics.

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22 **Keywords:** *Lauraceae, pau-rosa, Amazon, GC-MS, microdilution, disc diffusion, bacteria.*
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24 **1. Introduction**
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26 The antimicrobial resistance and the lack of new antibiotic compounds have become
27 a growing health threat to the world population. The need for investment in research
28 aimed at discovering new anti-infective drugs is substantial to avoid a global crisis in
29 public health [1]. Many antimicrobial studies of herbal extracts and essential oils
30 have been carried out for the discovery of new drugs. Significant effects of various
31 essential oils and plant extracts to eliminate some pathogenic microorganisms have
32 been reported [2-5].

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34 Rosewood species are scattered in the Amazon Region of Brazil, Guyana, Peru,
35 Colombia, Venezuela, and Suriname. In Brazil, the rosewood occurs in high areas
36 of rain forest, near small rivers that flow into the basins of the Amazonas and Purus.
37 The rosewood species are trees reaching 15 to 30 m in height and 0.3 to 1.0 m in
38 diameter, with straight cylindrical trunk and brown-yellowish bark. The rosewood oil
39 market fell in the last decades, and several factors have contributed to this decline:
40 exhaustion of source supplies, logistics and production costs, Brazilian government
41 control against extinction, and the synthetic linalool trade [6].

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43 Two plant sources belonging to Lauraceae have been attributed to the species
44 producing rosewood oil in the Amazon region: *Aniba rosaeodora* Ducke (syn. *A.*
45 *rosaeodora* Ducke var. *amazonica* and *A. duckei* Kostermans) and *Aniba parviflora*
46 (Meiss.) Mez (syn. *A. fragrans* Ducke) [7]. The oil of *A. rosaeodora* is applied
47 worldwide in the perfumery and cosmetic industry due to its fragrance based on high
48 linalool content (about 85%). *Aniba parviflora* is confused with *A. rosaeodora*, the
49 real rosewood tree, by the small farmers and oil producers. It is also called,
50 "macacaporanga", and its essential oil has only 40% linalool [6,8,9]. Despite the
51 similarity, these species have very distinct scents in the oils of wood and leaves.
52 Olfactory and compositional analysis of both oils, when using enantioselective gas
53 chromatography/olfactometry and two-dimensional gas chromatography, allowed a
54 clear distinction between the two species [10,11].

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56 The following uses are attributed to rosewood oil: analgesic, anticonvulsant,
57 antidepressant, antimicrobial, antiseptic, aphrodisiac, bactericidal, cellular stimulant,
58 cephalic, stimulant, tissue regenerator, and to reduce sleeplessness and pain
59 [12,13]. The sedative and anticonvulsant properties of linalool from *A. roseodora* oil
60 in glutamate-related seizure model, in inhibition of the compound action potential in
61 rodents, and inhibition of adenylate cyclase in chick retina, were previously reported
62 [14-18]. The linalool-rich rosewood oil induces vago-vagal bradycardia and
63 depressor reflex in rats [19]. The selective induction of apoptosis in precancerous
64 cells and cancer of the skin by rosewood oil demonstrates its potential anticancer
65 activity and linalool against other cancer cells [20,21]. The antimicrobial activity of
66 rosewood oil against several bacteria and fungi were investigated [22,23]. The *in*
67 *vitro* synergistic bacterial action of rosewood oil in combination with gentamicin was
68 reported [4]. Linalool has showed stronger antimicrobial activity against Gram-
69 positive bacteria when it was tested solely or in combination with the oil of *Ocimum*
70 *basilicum* L [24].

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72 This study aimed to investigate the antibacterial activity of the essential oils and
73 aqueous extracts of leaves from *A. rosaeodora* and *A. parviflora* against human
74 pathogenic bacteria. Also, to identify the volatile composition of these two oils, which
75 were produced from specimens sampled in an experimental plantation existing on
76 Lower Amazon River, Pará state, Brazil.

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78 **2. Material and methods**

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80 **2.1 Plant material**

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82 The samples of aerial parts (leaves and thin stems) of *A. rosaeodora* and *A.*
83 *parviflora* were collected during the crop management of these two species in the
84 experimental plantation located at the Curuá farm (Pematec Co.). It is located in the
85 Curuá-Una road, municipality of Santarém, Pará state, Brazil, with the coordinates
86 2°33'45.68 S and 54°37'00.37 W, between July and November 2009. Both species
87 were deposited in the herbarium of EMBRAPA/PA, under the numbers IAN 184,529
88 and IAN 184,897, respectively.

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90 **2.2 Plant processing and extraction of the essential oils**

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92 The aerial parts of the plants were air-dried for 48 hrs, ground and submitted to
93 hydrodistillation using a Clevenger-type apparatus (100 g, 3 hrs). The oils were
94 dried over anhydrous sodium sulfate, and their percentage contents were calculated
95 based on the plant dry weight. The moisture contents of the samples were
96 calculated using a balance with infrared moisture measurement. The oils were kept
97 in amber vials and stored at 5°C before GC and GC-MS analysis and the bioassays.
98 The procedure was performed in triplicate.

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100 **2.3 Aqueous extracts**

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102 The aerial parts of the plants were dried at 40°C under airflow in a stove and then
103 macerated in a mortar. Fifty grams of the powdered plants were extracted with
104 distilled water (1:10 w/v) at 70 ± 5°C for 3 h, under constant agitation. The aqueous
105 solution was then filtered, frozen and submitted to a lyophilization process.

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107 **2.4 Oil composition analysis**

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109 The oils were analyzed on a GC-MS Thermo Focus DSQ II, under the following
110 conditions: DB-5ms (30 m x 0.25 mm; 0.25 µm film thickness) fused-silica capillary
111 column; programmed temperature: 60–240°C (3°C/min); injector temperature:
112 250°C; carrier gas: helium, adjusted to a linear velocity of 32 cm/s (measured at
113 100°C); injection type: split (2 µl of a 1:1000 hexane solution); split flow was
114 adjusted to yield a 20:1 ratio; septum sweep was a constant 10 ml/min; EIMS:
115 electron energy, 70 eV; temperature of ion source and connection parts: 200°C. The
116 quantitative data regarding the volatile constituents were obtained by peak area
117 normalization using a GC-FID Thermo Focus operated under similar conditions to
118 the GC-MS, except for the carrier gas, which was nitrogen. The retention index was
119 calculated for all the volatiles constituents using an *n*-alkane (C8-C40,
120 Sigma/Aldrich) homologous series. Individual components were identified by
121 comparison of both mass spectrum and GC retention data with authentic
122 compounds which were previously analyzed and stored in a private library, as well
123 as with the aid of commercial libraries containing retention indices and mass spectra
124 of volatile compounds commonly found in the essential oils [25,26].

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126 **2.5 Antibacterial bioassay**

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128 The oils were assayed by the agar disk diffusion method [27,28]. The following
129 strains were used: *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa*
130 (ATCC 27853), *Klesbsiella pneumoniae* (ATCC 13883), *Staphylococcus aureus*
131 (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Streptococcus pyogenes*
132 (ATCC 19615) and *Staphylococcus epidermidis* (ATCC 12228). The
133 microorganisms were commercially obtained in lyophilized form, rehydrated in
134 nutrient broth (NB, Difco) (24 h, 36 ± 1°C) and then seeded in Petri dishes
135 containing Mueller Hinton agar (MHA, Difco) (24 h, 36 ± 1°C). The inocula (24 h)
136 diluted in sterile saline were prepared to achieve the turbidity standard of 0.5 on the
137 McFarland scale, containing the suspensions approximately 1.5 x 10⁸ CFU/ml. Filter
138 paper disks (6 mm diameter) were embedded in 10 µl of undiluted oils or 10 µl of
139 aqueous extracts, at concentrations of 60 mg/ml. The paper disks were laid upon
140 the cultures of bacteria in MHA and incubated as mentioned above. Control cultures
141 of each strain were treated with disks of gentamicin and ampicillin (10 µg/disk)
142 (Cefar, Brazil). Inhibition caused by the oils and the standards were compared (n =
143 3). Oils samples with inhibition halo ≥ 8 mm were highlighted to determine the
144 minimum inhibitory concentration (MIC) or minimum bactericidal concentration
145 (MBC).
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147 The MIC was determined by the plate microdilution method [29]. The oils were
148 serially diluted with 0.5% Tween 80 (Vetec, Brazil) resulting in concentrations from
149 0.07 µl/ml to 10 µl/ml. Each well was inoculated with the microorganisms (MH broth
150 medium, 1.5 x 10⁴ CFU/ml), followed by the addition of the oils, at different
151 concentrations. Plates were then incubated at 36°C, for 24 h. After incubation, 20 µl
152 of resazurin 0.02% (Vetec, Brazil) were added to the wells and the plates incubated
153 for another 3 h. The MIC was determined by the blue color of the resazurin dye. The
154 wells with no apparent microbial growth were selected to assess the MBC, by
155 cultures in Petri dishes (MHA medium).
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157 **2.6 Statistical analysis**

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159 Results are expressed as means ± standard deviations. The Tukey test was used at
160 the significance level of *P* = 0.05. The software Prism 3.0 program was used at the
161 confidence level of 95%.
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163 **3. Results and discussion**

164 **3.1 Analysis and characteristics of the oils**

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166 The oils of the aerial parts (leaves and thin stems) of *Aniba rosaeodora* and *A.*
167 *parviflora* yielded 1.0% and 0.5%, respectively. A yield variation of 0.7% to 1.5%
168 has been reported for the wood oil of *A. rosaeodora*, which is sold in the
169 international market and used in cosmetics [6]. Also, the wood oil of *A. parviflora* has
170 showed a yield of about 0.5%, and it is sold in the local market for cleaning products
171 and aromatic sachets. The leaf oils of both species presented pale yellow color. The
172 leaf oil of *A. rosaeodora* presented a floral and woody note while the leaf oil of *A.*
173 *parviflora* has showed the same scent, plus a peppery and citrusy note.
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176 GC and GC-MS analyzed the compositions of the oils and the identified fifty-one
 177 volatile constituents are listed in Table 1. The oil of *A. rosaedora* was dominated by
 178 linalool (88.6%) while the main components in the oil of *A. parviflora* were linalool
 179 (45.0%), β -phellandrene (17.3%), α -phellandrene (4.1%) and (*E*)-caryophyllene
 180 (3.9%).

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Table 1. Constituents of rosewood leaf oils: *Aniba rosaedora* (EOAR) and *A. parviflora* (EOAP).

Constituents	RI	EOAR (%)	EOAP (%)
α -Thujene	921		0.2
α -Pinene	939	0.4	
β -Pinene	973	0.1	
Myrcene	983		1.6
α -Phellandrene	1002		4.1
<i>p</i> -Cymene	1019		1.6
Limonene	1024	0.2	
β -Phellandrene	1025		17.3
(<i>E</i>)- β -Ocimene	1044		1.4
γ -Terpinene	1054		0.1
<i>cis</i> -Linalool oxide (furanoid)	1064	1.5	0.1
Terpinolene	1072		0.2
<i>trans</i> -Linalool oxide (furanoid)	1081	1.4	
Linalool	1098	88.6	45.0
Borneol	1165		0.2
<i>trans</i> -Linalool oxide (pyranoid)	1168	0.1	
Terpinen-4-ol	1174		0.2
α -Terpineol	1189	0.2	0.8
Geraniol	1244		0.1
α -Cubebene	1344		0.9
α -Ylangene	1366		0.1
α -Copaene	1372	0.1	0.9
β -Elemene	1387	0.1	0.4
(<i>E</i>)-Caryophyllene	1415		3.9
γ -Elemene	1426		0.4
α -Humulene	1452		0.5
β -Chamigrene	1469	0.1	0.5
Germacrene D	1476		0.4
β -Selinene	1484	0.8	2.0
α -Selinene	1496	0.7	2.1
γ -Cadinene	1508		0.2
δ -Cadinene	1513		0.6
Hedycaryol	1543		0.1
<i>trans</i> -Dauca-4(11),7-diene (tent.)	1548	0.1	
Germacrene B	1552		0.3
(<i>E</i>)-Nerolidol	1558	0.1	0.2

Spathulenol	1576		0.9
Caryophyllene oxide	1575	0.2	1.1
Guaiol	1591	0.1	0.2
Humulene epoxide II	1603	0.1	0.2
Globulol (tent.)	1611	0.1	0.2
10- <i>epi</i> - γ -Eudesmol	1624		0.6
γ -Eudesmol	1627		2.1
Selin-3,11-dien-6- α -ol	1642	0.6	0.2
α -Eudesmol	1648		1.6
14-Hydroxy-9- <i>epi</i> -(<i>E</i>)-caryophyllene	1663	0.6	0.4
Amorpha-4,9-dien-2-ol	1701	0.5	
Nootkatol (tent.)	1704	0.1	
(<i>E</i>)- Nerolidyl acetate	1713	1.5	1.1
Eremophilone (tent.)	1731	0.1	
Benzyl benzoate	1746	0.1	0.1
Unidentified sesquiterpenes			2.6
Monoterpene hydrocarbons		0.7	26.5
Oxygenated monoterpenes		91.8	46.4
Sesquiterpene hydrocarbons		1.8	13.2
Oxygenated sesquiterpenes		4.1	8.9
Unidentified sesquiterpenes			2.6
Aromatic compounds		0.1	0.1
Total		98.5	97.7

RI = Retention index on DB-5ms capillary column.

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3.2 Antibacterial bioassay

189 *Aniba rosaeodora* oil has showed bactericidal activity against 86% of the tested
190 microorganisms. The inhibition halo to *Streptococcus pyogenes* (ATCC 19615) was
191 superior to 40 mm diameter. The oil of *A. rosaeodora* oil against the other
192 microorganism strains showed inhibition halos ranging of 8.8 to 38.4 mm diameter.
193 Although less efficient, the oil of *A. parviflora* was effective against 71% of the tested
194 microorganisms, with inhibition halos varying from 8.5 to 15.4 mm diameter. The oils
195 showed MIC values between 1.3 and >10.0 μ l/ml and MBC values between 5.0 and
196 >10.0 μ l/ml (Table 2). The aqueous extracts of *A. rosaeodora* and *A. parviflora*
197 showed no antimicrobial activity against the microorganisms tested.

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The *A. rosaeodora* and *A. parviflora* leaves essential oils showed significant antibacterial activity. As the aqueous extracts were inactive, the antibacterial activity observed could be attributed to the volatile components of the oils, which presented a lipophilic character. The composition of the leaf oil of *A. rosaeodora* was not much different from those previously described, with a predominance of linalool in the range 81-96% [6,30,31]. Linalool occurs as a racemic mixture of the oil of *A. rosaeodora*, with the enantiomers (-)-linalool and (+)-linalool in almost equal proportions and a slight predominance of the levorotatory isomer [18]. The literature related to the oil chemical composition of *A. parviflora* is very scarce. Two papers have mentioned only its main constituents and are related to *Aniba fragrans* Ducke,

209 its botany synonymy [8,32]. A more recent paper has utilized the *A. parviflora* oil to
 210 highlight the chromatographic method in comprehensive two-dimensional gas phase
 211 for identification of its constituents, but without informing their percentage values
 212 [11]. Therefore, the total chemical composition analysis of the *A. parviflora* oil is
 213 being reported for the first time (Table 1), whose main component is also linalool,
 214 with a percentage (45.0%), which is half of that found for the *A. rosaeodora* oil
 215 (88.6%). Also, the monoterpene hydrocarbons α - and β -phellandrene contributed
 216 more than 20% for the composition of *A. parviflora* oil, resulting in the peppery-
 217 citrusy note presented for this oil.

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Table 2. Antibacterial potential of leaf oils and aqueous extracts of rosewood: *Aniba rosaeodora* (EOAR) and *A. parviflora* (EOAP).

Microorganisms	Standard Antibiotics		Essential Oils					
	AMP ^a	GEN ^b	EOAR			EOAP		
		DDM	DDM	MIC	MBC	DDM	MIC	MBC
(-) <i>E. coli</i>	> 10	21.9 ± 0.2	13.2 ± 0.5 ^{ab}	> 10	> 10	na	nd	nd
(-) <i>P. aeruginosa</i>	> 10	21.4 ± 0.1	na	nd	nd	na	nd	nd
(-) <i>K. pneumoniae</i>	> 10	20.7 ± 0.1	11.6 ± 0.1 ^{ab}	> 10	nd	9.20 ± 0.4 ^{ab}	> 10	> 10
(+) <i>S. aureus</i>	34.9 ± 0.3	22.3 ± 0.1	26.7 ± 1.9 ^{ab}	1.3	10	15.4 ± 0.9 ^{ab}	> 10	> 10
(+) <i>E. faecalis</i>	23.4 ± 0.6	14.2 ± 0.4	8.80 ± 0.6 ^{ab}	5	> 10	11.2 ± 0.9 ^{ab}	> 10	> 10
(+) <i>S. epidermidis</i>	20.7 ± 0.1	25.4 ± 0.1	38.4 ± 1.4 ^{ab}	5	> 10	13.3 ± 0.8 ^{ab}	> 10	> 10
(+) <i>S. pyogenes</i>	39.1 ± 0.5	27.1 ± 0.4	> 40 ^b	1.3	5	13.3 ± 1.0 ^{ab}	1.3	5

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^{a,b} P = .05 for comparison of means; AMP = ampicillin, GEN = gentamicin; DDM = Disk Diffusion Method (Mean inhibition zone diameter ± SD, mm); MIC = Minimum Inhibitory Concentration (µl/ml); MBC = Minimum Bactericidal Concentration (µl/ml); na = not active; nd = not determined.

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As mentioned earlier, the rosewood oil producers do not distinguish these two species in the field easily. Another important aspect in rosewood oils is that the older the leaves, the greater the percentage of components of the oxidation process of the linalool, such as α -terpineol, terpinen-4-ol and linalool oxides, which leads to changes in the bouquet of the oils. In this respect, the different scents of the rosewood oils have helped the producers in the collection of plants, propagation and cultivation, given that this task becomes more difficult without a previous analyzing the composition of the oils.

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Essential oils are natural compounds that protect plants against microbial attack, and it is suggested that their constituents, alone or synergistically, are more efficient than those found in aqueous plant extracts [3]. Under the experimental conditions, the aqueous extracts of *A. rosaeodora* and *A. parviflora* showed no antimicrobial activity against the microorganisms tested, suggesting absence or insufficient concentration of active compounds. The antimicrobial efficiency exhibited against Gram-positive and Gram-negative bacteria might be due to the presence of linalool in both species of *Aniba*. Accordingly, the different antibacterial actions observed for these oils can be due to the different percentages found for linalool. However, the possibility of a synergistic interaction amongst linalool and others minor constituents of the oils cannot be discarded. For example, beta-phellandrene showed content of 17.3% in the oil of *A. parviflora* and essential oils with a significant percentage of this constituent has displayed bactericidal activity [33,34].

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Previously, the essential oils of wood and leaf of *A. rosaeodora* inhibited the growth of *E. coli*, *K. pneumoniae*, *S. aureus* and *E. faecalis* [22,23]. The results are very

251 close to this work, including linalool, which was tested separately. A synergistic
252 interaction was observed against some pathogenic strains using a combination of *A.*
253 *rosaedora* oil and gentamicin, with improvement in the antimicrobial effectiveness,
254 particularly against Gram-negative bacteria [4]. Those authors concluded that there
255 was a synergistic effect between the oil and the antibiotic, mainly due to the
256 presence of linalool. In another paper, the antifungal activity of the wood oil of *A.*
257 *rosaedora* showed MIC and MFC values between 0.5 to 20 $\mu\text{L}/\text{mL}$ for more
258 resistant fungi, such as *Aspergillus flavus* (ATCC 9170), *A. terreus* (ATCC 16792)
259 and *Trichoderma viride* (IAM 5061) [30]. In said oil, linalool was the main
260 component, with the percent of 81.3%. Also, it was observed that the oil of *Ocimum*
261 *basilicum* (Lamiaceae) and linalool, its main constituent (50-60%), showed greater
262 activity against bacterial strains than against fungal strains. Also, the oil of *O.*
263 *basilicum* and linalool have showed stronger antimicrobial activity against Gram-
264 positive bacteria than against Gram-negative bacteria [24].

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266 In some experiments, (-)-linalool has showed greater efficacy in physiological
267 parameters stress than the racemic mixture (\pm)-linalool that occurs in the oils of *A.*
268 *rosaedora* and *A. parviflora* [35]. In this work, the antibacterial assay was tested
269 also with a commercial sample of (-)-linalool and the bactericidal efficiency was
270 slightly higher than the (\pm)-linalool existing in the *A. rosaedora* oil. However, these
271 results should also be compared with the separate enantiomers of *A. rosaedora* oil,
272 which will be done in future studies.

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274 The antimicrobial action mechanism of the oils of *A. roseadora* and *A. parviflora* can
275 be explained by the lipophilic character of the mono- and sesquiterpenoid
276 compounds contained in these oils. These compounds act by disrupting the
277 cytoplasmic membrane or perturbation of the lipidic fraction of the plasmid
278 membrane. This action results in alteration of the membrane properties, such as
279 dissipation of the proton motive force, inhibition of several enzymes due to leakage
280 of essential ions, and the active transport and the coagulation of cell contents [36-
281 39]. The synergistic effect between chemical components present in some essential
282 oils has been evaluated [40,41]. Those authors have concluded that essential oils
283 showed higher antibacterial activity than the isolated mixture of its main
284 components. Thus, components present in low concentrations can be essential to
285 enhance the biological effect given by these oils.

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287 **4. Conclusion**

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289 GC and GC-MS analyzed the compositions of the oils and identified fifty-one volatile
290 constituents. The oil of *A. rosaedora* was dominated by linalool while the main
291 components in the oil of *A. parviflora* were linalool, β -phellandrene, α -phellandrene
292 and (*E*)-caryophyllene. *Aniba rosaedora* oil has showed bactericidal activity against
293 86% of the tested microorganisms. Although less efficient, the oil of *A. parviflora*
294 was effective against 71% of the tested microorganisms. Based on the results, it is
295 assumed that the rosewood oils could be used in pharmaceutical formulations or to
296 prevent food spoilage to control resistant bacteria strains.

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305 **Competing interests**

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307 Authors have declared that no competing interests exist.

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309 **Authors' contributions**

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311 This work was carried out in collaboration between all authors. All authors read and
312 approved the final manuscript.

313

314 **Consent**

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316 It is not applicable.

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318 **Ethical approval**

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320 It is not applicable.

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