

SUPPLEMENTARY MATERIAL

Chemical composition, antimicrobial activity and synergistic effects with conventional antibiotics under clinical isolates by essential oil of *Hymenaea rubriflora* Ducke (FABACEAE)

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Abstract:

This work aimed to investigate the chemical composition, antimicrobial activity, synergistic effect, and structure changes of the essential oil of *Hymenaea rubriflora* (EOHr). Forty-five constituents were identified in the essential oil, corresponding to 94.43% of the compounds present, being the main components E-Caryophyllene (36.72 ± 1.05 %), Germacrene D (16.13 ± 0.31 %), α -Humulene (6.06 ± 0.16 %), β -elemene (5.61 ± 0.14 %) and δ -Cadinene (3.76 ± 0.07 %). Antimicrobial activity was evaluated, presenting antibacterial and antifungal activity with MIC ranging from 0.62 to 40 μL / mL. The essential oil had a synergistic effect when combined with gentamicin and fluconazole. Structural changes were also evaluated and it was possible to observe that EOHr action was related to changes in membrane permeability. The findings obtained here suggest that the use of the essential oil of *H. rubriflora* in the treatment of infectious diseases presents a potential for the future development of pharmaceutical products.

Keywords: Essential oil, Chemical Composition, Antimicrobial Activity, Synergistic potential, Electron microscopy.

1. Methodology

1.1. Plant material

The leaves of *Hymenaea rubriflora* Ducke were collected in the fragment of the Atlantic Forest in Engenho Piedade, in the countryside of Igarassu, city of the North Coast of Pernambuco ($7^{\circ}50'03.0''\text{S}$ $34^{\circ}54'23.0''\text{W}$) in September 2016. The material used in this work was deposited in the herbarium of the Agronomic Institute of Pernambuco IPA under number 78292.

1.2. Essential oil extraction

The essential oil of leaves of *Hymenaea rubriflora* Ducke (EOHr) was obtained by hydrodistillation (HD) in a Clevenger apparatus. The fresh leaves (300g) were crushed, and the powder immersed in distilled water 1:9 (w/v) for 4h, beginning to count by the boiling point of the water. Excess water from essential oil was removed using Anhydrous Sodium Sulphate (Na_2SO_4). The yield % (v/w) of the oil was calculated based on the weight of the fresh material.

1.3. Chemical composition of essential oil

The composition of essential oil was analyzed on Agilent Technologies Gas

Chromatograph (Palo Alto, CA, USA) 5975C series, with quadrupole detection system equipped with apolar column DB-5 Agilent J&W, (60 m × 250 µm i.d. × 0.25 µm film thickness). Aliquots of 1 µL in split mode (1:50) of the essential oil in the concentration of 3000 ppm were injected in the gas chromatograph coupled to mass spectrometry (GC-MS). Subsequently, 1 µL in split (1:50) of the mixture of hydrocarbon standards: C9-C30 was injected. The essential oil mixture and the mixture of hydrocarbon standards, 1 µL (0.2 µL of alkanes and 0.8 µL of the oil) were then injected under the splitless mode. The GC temperature was maintained at 60 °C for 3 min, then increasing at 2.5 °C.min⁻¹ to 240 °C and held for 10 min at this temperature. The helium flow was maintained at a constant pressure of 100 kPa. The MS interface was set at 200 °C and injector at 250 °C and the mass spectra recorded at 70 eV (in EI mode) with a scan rate of 0.5 scan s⁻¹ of 20-350 m/z. From the analysis of the retention times (RT) of the compounds present in the essential oil sample, the hydrocarbon standards and the combination of the essential oil with the pattern mixture, the Retention Indexes (RI) for each component of the oil were calculated. Compounds were identified by comparing their mass spectra (MassFinder 4, NIST08 and Wiley Registry™ 9th Edition, integrated into the software Agilent MSD Productivity ChemStation (Agilent Technologies, Palo Alto, EUA) and Retention Index to those of authentic standards available in the Adams (2017).

The oil sample were quantified in gas chromatography with flame ionization detector (GC-FID), under the same GC-MS conditions, in triplicate for calculation of standard deviation of peak area percentage of each compound in the chromatogram. This was used to determine the proportion of the compounds in the essential oil.

1.4. Antimicrobial activity

1.4.1. Microbial strains

Strains of *Klebsiella pneumoniae* ATCC 29665, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 9524, *Staphylococcus aureus* ATCC 29213 and three strains of *Staphylococcus aureus* with resistant profiles (UFPEDA, 700, 705 and 726), they were obtained of Department of Antibiotics of the Federal University of Pernambuco. Strain *K. pneumoniae* CCBH 23940 KPC⁺ (carbapenemase producer) by provider Collection of Bacterial Culture of Fiocruz Hospital. Strains of fungi *Candida albicans* URM-6543, *Candida glabrata* URM-6393, *Candida parapsilosis* URM-6557 and *Candida tropicalis* URM-6551, provided of Department of Mycology of the Federal University of

Pernambuco.

1.4.2. Antimicrobial assay

The broth microdilution susceptibility assay followed CLSI protocol M100 (2017) for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Solutions containing the essential oil were obtained according to Bessa et.al. (2016). Two-fold serial dilutions of each solution containing the essential oil (0.002- 40 $\mu\text{L}/\text{mL}$) were prepared in Mueller-Hinton broth and 10 μL of bacterial suspension (1×10^8 CFU/mL) was added. Samples were incubated at 37 °C for 24h. A resazurin solution (0.01%) was used as an indicator of growth, being taken as the lowest concentration, in which there was no color change. Subsequently, 10 μL of the cultures were seeded on plates containing Muller Hinton Agar and incubated for 24 h at 37 °C to determine the minimum bactericidal concentration (MBC). Gentamicin (Sigma Aldrich) was used as the standard drug. Each trial in this experiment was performed in triplicate. The determination of minimum inhibitory concentration and minimum fungicidal concentration was performed according CLSI protocol M27 (2008). Serial dilutions of solutions containing essential oil were performed in 96-well plates in RPM-1640 medium (buffered with MOP's) by adding 100 μL of the yeast suspension (1×10^6 UFC/mL). The samples were incubated at 30°C for 24h. The MIC was determined by inhibiting 50% growth compared to the control well (medium and inoculum only). Subsequently, 10 μL of each well were seeded on plates containing Sabouraud Dextrose Agar medium and incubated for 24h at 30 °C to determine the minimum fungicidal concentration (MFC). Fluconazole (Sigma Aldrich) was used with standard drug. Each trial in this experiment was performed in triplicate.

1.4.3. Evaluation of synergistic effect

Synergy between essential oil and antimicrobial agents (gentamicin and fluconazole) was carried out using MIC microdilution following the methodology described by Matias et al. (2016) with some modifications. For evaluation of essential oil as antibiotic resistance modulators, MIC of antibiotics was determined in the presence and absence of essential oil (MIC). Each assay was performed in triplicate.

1.4.4. Measurement of cellular permeabilization to UV-absorbing

The loss of UV absorbing was measured by 260 nm reading in a UV-VIS

spectrophotometer according to Zhou et al. (2008) with some modifications. Cells were maintained overnight in Mueller Hinton broth or RPMI-1640, for bacteria and yeast, respectively. Cells were harvested by centrifugation at $400 \times g$ for 15 min, the supernatant was discarded and the pellet was washed twice and then resuspended in PBS (pH 7.4). MIC of essential oil and control were added to the cell suspension and incubated at 37°C and/or 30 °C for 60 minutes. After treatment, the cell suspensions were centrifuged at $13,400 \times g$ for 15 min. The release of UV absorbing materials was determined by UV-VIS spectrophotometer at 260 nm. All experiments were performed in triplicate.

1.5. Scanning Electron Microscopy

The isolates that cumulatively presented antimicrobial activity, synergic effect and increasing UV absorption in the presence of essential oil were submitted to analysis by scanning electron microscopy (SEM) to analyse the structural alterations. Cells were treated as described by Veras et al. (2015). Briefly, after incubation of strains with MIC/2 of essential oil (8 hr/37 °C for bacteria and 12 hr/30 °C for yeast), they were fixed (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.2), stuck on poly-L-lysine coated coverslips for 5 minutes at room temperature, washed in 0.1 M phosphate buffer, and fixed with powders (1% osmium tetroxide for 90 minutes). Then, dehydrated in ascending series of alcohol (30% to 100%), dried at critical point, attached to metallic stubs and coated with 20 nmol/L gold for visualization of the images in the Quanta 200 F MEV (FEI company).

1.6. Statistical analysis

The data were analyzed using the GraphPad Prism[®] version 5.0 and expressed as mean \pm SD. All determinations were performed in triplicate. Statistically significant differences were calculated by the application of two-way (ANOVA) and Bonferroni post-hoc test. Values were considered significantly with $p < 0.05$.

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Figures

Figure S1. Synergic effect of the essential oil of *H. rubriflora* Ducke with gentamicin (A) and fluconazole (B). ***Value statistically significant ($P < 0.001$).

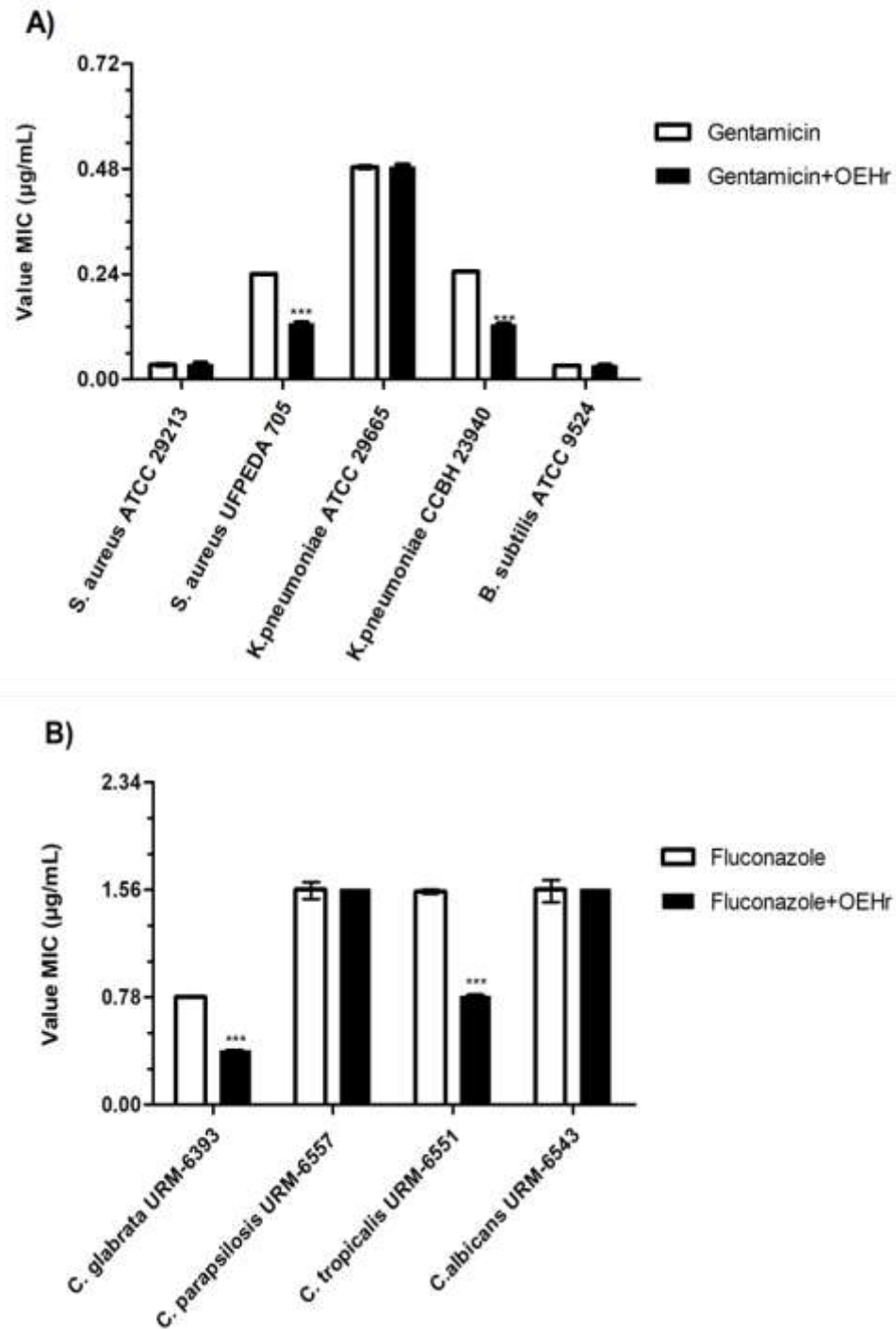


Figure S2. Presence of absorbent material at 260 nm in the strains treated with the MIC of the essential oil of *H. rubriflora* Ducke (EOHr). Values statistically significant *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$).

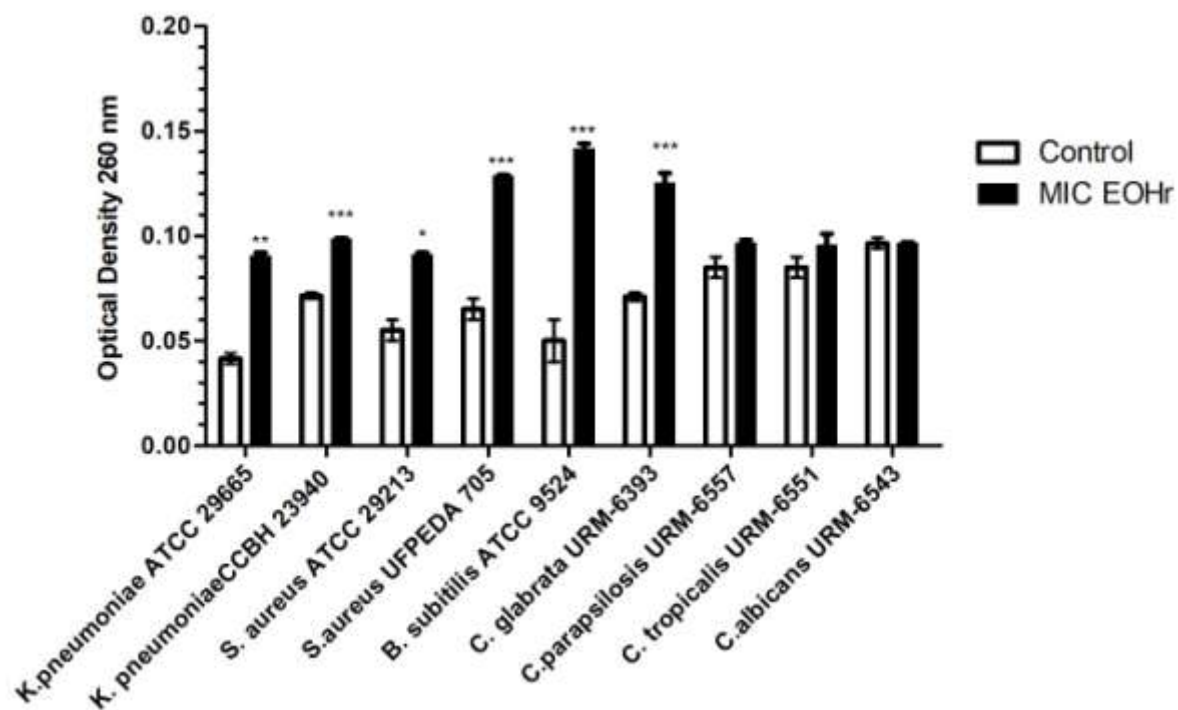
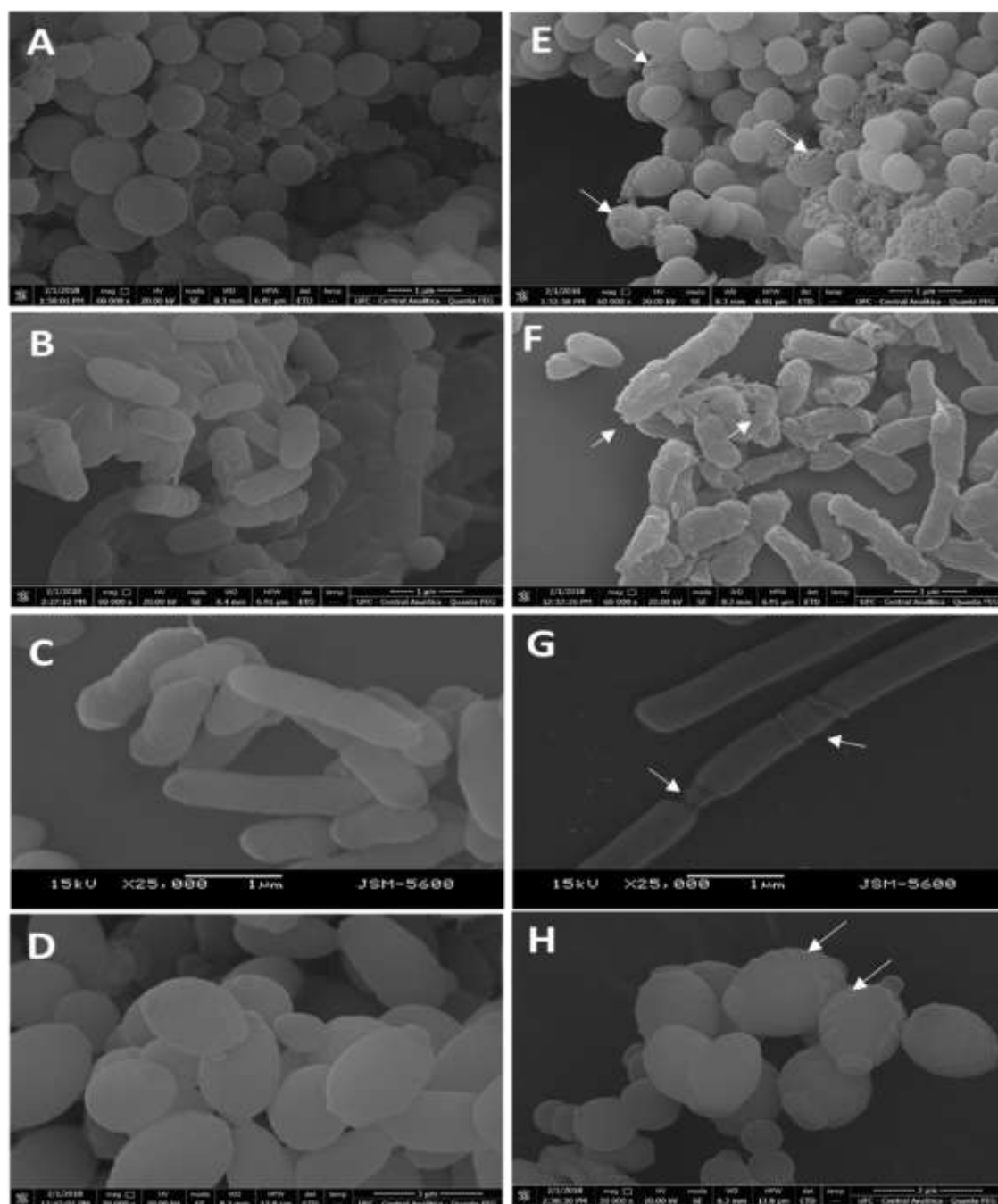


Figure S3. Electron microscopy images of the cells treated with and without essential oil. The images A, B, C and D control cells, present conserved morphology and regular lengths in *S. aureus* 705, *K. pneumoniae* 23940, *B. subtilis* ATCC 9524 and *C. glabrata* URM-6393, respectively. The images E, F, G and H cells treated with MIC/2 of EOHr, have irregular shapes, surface depressions and evidence of incomplete divisions.



Tables

Table S1. Chemical composition of the essential oil of leaves of *H. rubriflora* Ducke (EOHr), highlighting in bold the major components.

No.	Compound	RI Lit.	RI Cal.	Percentage (%)	S.D.
1	Geijerene	1138	1141	0.09	0.01
2	δ - elemene	1335	1337	0.43	0.02
3	α -Cubebene	1348	1350	0.52	0.05
4	α -Ylangene	1373	1371	0.08	0.01
5	α -Copaene	1374	1376	2.24	0.07
6	β -Bourbonene	1387	1385	0.47	0.03
7	β-elemene	1389	1392	5.61	0.14
8	Cyperene	1398	1399	0.25	0.01
9	Z-Caryophyllene	1408	1406	0.01	0.00
10	Cis- α -Bergamotene	1411	1415	0.08	0.01
11	E-Caryophyllene	1417	1421	36.72	1.05
12	β -Copaene	1430	1429	0.57	0.01
13	α -trans-Bergamotene	1432	1436	0.51	0.02
14	Aromadendrene	1439	1439	0.31	0.02
15	Trans-muurolo-3,5-diene	1451	1451	0.12	0.01
16	α-Humulene	1452	1454	6.06	0.16
17	allo-aromandendrene	1458	1461	0.61	0.03
18	4,5-di-epi-aristolochene	1471	1470	0.06	0.01
19	γ -Muurolene	1478	1478	2.09	0.04
20	Germacrene D	1480	1483	16.13	0.31
21	β -Selinene	1490	1487	3.07	0.06
22	trans-Muurolo-4(14),5-diene	1493	1493	0.01	0.00
23	α -selinene	1498	1496	3.16	0.10
24	β -alaskene	1498	1497	3.63	0.10
25	α -muurolene	1500	1501	0.28	0.01
26	Germacrene A	1508	1506	0.53	0.04
27	γ -Cadinene	1513	1515	0.55	0.58
28	δ-Cadinene	1522	1524	3.76	0.07
29	Trans-Cadina-1.4-diene	1533	1533	0.11	0.01
30	α -Cadinene	1537	1538	0.15	0.00
31	α -Calacorene	1544	1544	0.06	0.01
32	Germacrene B	1559	1558	0.27	0.06
33	β -Calacorene	1564	1565	0.11	0.00
34	Spathulenol	1577	1579	0.74	0.03
35	Caryophyllene oxide	1582	1584	1.51	0.02

36	Viridiflorol	1592	1593	0.15	0.01
37	Ledol	1602	1604	0.16	0.03
38	Humulene Epoxide II	1608	1610	0.15	0.06
39	Caryophylla-4(12),8(13)-dien-5 β -ol	1639	1638	0.10	0.00
40	tau.-Muurolol	1644	1643	1.13	0.05
41	Cubenol	1645	1647	0.01	0.00
42	α -Cadinol	1652	1656	1.44	0.05
43	santalol<(Z)- α >	1674	1673	0.40	0.08
44	Eudesma-4(15),7-dien-1 β -ol	1687	1688	0.01	0.00
45	Mintsulphide	1740	1739	0.01	0.00
Total (%)				94.43	
Hydrocarbon monoterpenes (MH)				0.09	
Oxygenated monoterpenes (MO)				0.01	
Hydrocarbon sesquiterpenes (SH)				89.03	
Oxygenated sesquiterpenes (SO)				5.03	
Total (%)				94.43	

RICal-Experimental retention indices relative and RILit- Literature retention indices;
S.D. Standard Deviation.

Table S2. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) Minimum and Fungicidal Concentration (MFC) of the essential oil of *H. rubriflora* Ducke.

Strains	Essential oil of <i>H. rubriflora</i>			Gentamicin			Fluconazole		
	MIC	MBC-MFC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MFC	MFC/MIC
	(μ L/mL)	(μ L/mL)	MFC/MIC	(μ g/mL)	(μ g/mL)		(μ g/mL)	(μ g/mL)	
<i>K. pneumoniae</i> ATCC 29665	20	40	2	0.48	0.48	1	-	-	-
<i>K. pneumoniae</i> CCBH 23940	20	40	2	0.24	0.48	2	-	-	-
<i>P. aeruginosa</i> ATCC 27853	40	-	-	0.24	0.96	4	-	-	-
<i>B. subtilis</i> ATCC 9524	5	10	2	0.03	0.06	2	-	-	-
<i>S. aureus</i> ATCC 29213	5	10	2	0.03	0.061	2	-	-	-
<i>S. aureus</i> UFPEDA 700	10	40	4	0.12	0.96	8	-	-	-
<i>S. aureus</i> UFPEDA 705	5	10	2	0.24	0.96	4	-	-	-
<i>S. aureus</i> UFPEDA 726	40	-	-	0.03	0.06	2	-	-	-
<i>C. albicans</i> URM 6543	1.25	2.50	2	-	-	-	1.56	3.12	2
<i>C. glabrata</i> URM 6393	0.62	2.50	4	-	-	-	0.78	1.56	2
<i>C. parapsilosis</i> URM 6557	1.25	2.50	2	-	-	-	1.56	3.12	2
<i>C. tropicalis</i> URM 6741	0.62	2.50	4	-	-	-	1.56	3.12	2