Phytochemical analysis, antibacterial activity and antibiotic modifying action of *Jatropha mollissima* (Pohl.) Baill. (Euphorbiaceae)

Noelly Bastos-Cavalcante[1], Cristina Rodrigues dos Santos-Barbosa[2], Raimundo Luiz Silva-Pereira[2], Débora Feitosa-Muniz[2], Henrique Douglas de Melo-Coutinho[2], Larissa Araújo-Rolim[1] & Jackson Roberto Guedes da Silva-Almeida[1]

1 Center for Studies and Research of Medicinal Plants, Federal University of San Francisco Valley, Petrolina-PE, Brazil.
2 Department of Biological Sciences, Regional University of Cariri, Crato-CE, Brazil.

Resumen

Análisis fitoquímico, actividad antibacteriana y acción moduladora de antibióticos de Jatropha mollissima *(Pohl.) Baill. (Euphorbiaceae)*

La aparición de cepas bacterianas resistentes y los efectos secundarios de sus fármacos disponibles han hecho investigar nuevos compuestos antibacterianos bioactivos. *Jatropha mollissima* es una planta endémica del bioma Caatinga, Brasil. Existen informes en la literatura de que esta especie tiene acción antioxidante, antibacteriana y antiproliferativa. El presente estudio tuvo como objetivo evaluar el potencial antibacteriano y modulador de *J. mollissima* contra cepas bacterianas multirresistentes. El perfil fitoquímico se obtuvo por cromatografía de gases junto con espectrometría de masas. *J. mollissima* mostró actividad antibacteriana moderada y moduló la actividad del antibiótico Norfloxacino, promoviendo una relación antagonista. Este fue el primer estudio de este tipo realizado con *J. mollissima*.

Palabras clave: *Jatropha mollissima;* Euphorbiaceae; Actividad Antibacteriana; Terpenos.

Abstract

The emergence of resistant bacterial strains and the significant increase in side effects of currently available antibacterial drugs have made it urgent to develop research to identify new bioactives antibacterial compounds. *Jatropha mollissima* is a plant endemic to the Caatinga biome, Brazil and this species has antioxidant, antibacterial and antiproliferative action. The present study aimed to evaluate the antibacterial and modulatory potentials of *J. mollissima* against multiresistant bacterial strains. The phytochemical profile was obtained from gas chromatography coupled to mass spectrometry. *J. mollissima* presented moderate antibacterial activity and modulated the activity of the antibiotic Norfloxacin, promoting an antagonistic relationship. This was the first study of this nature carried out with *J. mollissima*.

Key words: *Jatropha mollissima*, Euphorbiaceae, Antibacterial Activity, Terpenes.

Introduction

In recent years, increasing microbial resistance to currently available antibiotics has become a serious public health problem, and the bacterial resistance, according to the World Economic Forum Global Risks, is considered to be one of the greatest threats to human health (Blair *et al.* 2015). Currently, several studies have been developed regarding the discovery of new antimicrobial agents from vegetable extracts and other natural products, with the objective of discovering new bioactive compounds with a livability compared to the traditional drugs, but with less toxicity and greater effectiveness against the resistance of pathogenic microorganisms, besides having a lower environmental impact (Al-Tohamy *et al.* 2018, Arulmozhi *et al.* 2018, Babahmad *et al.* 2018).

In *Jatropha* L. genus some plant species are already known to have antimicrobial properties and, in recent years, several studies have been carried out in different countries, demonstrating the effectiveness of plant extracts as antimicrobial agents (Félix-Silva *et al.* 2018; Hernandez-Hernandez *et al.* 2017, Rampadarath *et al.* 2016). In addition, many plant species have also been evaluated for their potential as resistance modulating agents (Calixto-Junior *et al.* 2015). Several chemical compounds obtained from natural sources have direct activity against many species of bacteria, increasing the activity of a specific antibiotic, reversing the natural resistance of these microorganisms and causing derangements in their mechanisms of resistance (Regueira-Neto *et al.* 2017). Potentiation of antibiotic activity or reversion of resistance to antibiotics allows the classification of these compounds as modulators of antibiotic activity (Coutinho *et al.* 2010).

Jatropha mollissima (Pohl.) Baill. is a plant popularly known as "pinhão-bravo", endemic to the Caatinga biome and widely distributed in the semiarid region of Brazil (Castro & Cavalcante, 2011). There are reports in the literature that this species has antioxidant (Melo *et al.* 2010), antibacterial (Rocha & Dantas, 2009) and antiproliferative (Dias *et al.* 2019) action. In this sense, this study aims to evaluate the antibacterial effect of *J. mollissima* extracts against *Staphylococcus aureus* Rosenbach 1884 and *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900 strains by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentra-

tion (MBC) using direct antibacterial activities, as well as its modulatory effect on standard antibiotics (amicacin, ampicillin and norfloxacin) in an attempt to discover new antimicrobial agents.

Materials and methods

Bacterial samples

Investigation of antibacterial activity: The bacterial strains utilized were *Enterococcus faecalis* (Andrewes & Horder 1906) Schleifer & Kilpper-Bälz 1984 (ATCC 19433), *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 (ATCC 25922), *Klebsiella pneumoniae* (Schroeter 1886) Trevisan 1887(ATCC 13883), *Salmonella enterica* (ex Kauffmann & Edwards 1952) Le Minor & Popoff 1987 (ATCC 10708), *Serratia marcescens* Bizio 1823 (ATCC 13880), *Shigella flexneri* Castellani & Chalmers 1919 (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923). All strains were kept on slants with Müller-Hinton agar (MH, HIMEDIA). Before the assay, the cells were grown overnight at 37 °C in brain heart infusion (BHI) broth (BHI, Difco Laboratories Ltda.).

Modulation of antibiotic activity: The bacterial strains utilized were the multiresistants clinical isolates *S. aureus* (SA358) and *P. aeruginosa* (PA03) with the source and resistance profile described in table 1. All strains were maintained on slants with heart infusion agar (HIA, Difco Laboratories Ltda.). Before the assay, the cells were grown overnight at 37 °C in brain heart infusion (BHI) broth (BHI, Difco Laboratories Ltda.).

Plant material

Leaves of *J. mollissima* were collected in San Raimundo Nonato city, Piauí State, Brazil (Coordinates: 9°7'9132"S, 42°44'4716"W). The botanical identification was obtained by comparing the samples collected with a voucher specimen deposited in the Herbario of San Francisco Valley (HVASF), of the Federal University of San Francisco Valley, (Petrolina, Pernambuco State, Brazil) as No. 23193.

Preparation of *J. mollissima* extracts

For the preparation of the extracts, leaves were collected and kept submersed in ethanol 95% for 72 h. Afterwards, the extract was filtered and concentrated using a rotary vacuum evaporator

Bacteria	Origin	Resistance profile
***Staphylococcus aureus* (SA358)**	Surgical wound	Ca, Cep, Cp, Oxa, Pen, Amp, Amo, Clin, Mox, Cip, Lev, Asb, Ami, Eri, Cla, Azi.
***Pseudomonas aeruginosa* (PA03)**	Catheter tip	Com, Ctz, Imi, Cip, Ptz, Lev, Mer, Ami.

Tabla 1. Origen bacteriano y perfil de resistencia. Ca: Cefadroxila; Cep: Cefalexina; Cf: cefalotina; Oxa: Oxacilina; Pen: Penicilina; Amp: Ampicilina; Amo: Amoxicilina; Clin: Clindamicina; Mox: Moxifloxacina; Cip: Ciprofloxacina; Lev: Levofloxacina; Asb: Ampicilina + Sulbactam; Ami: Amicacina; Eri: eritromicina; Cla: Claritromicina; Azi: Azitromicina; Com: Cefepima; Ctz: Ceftazidima; Imi: Imipenem; Ptz: Piperaciclina; Mer: Meropenem.

Table 1. Bacterial origin and resistance profile. Ca: Cefadroxil; Cep: Cephalexin; Cf: Cephalothin; Oxa: Oxacillin; Pen: Penicillin; Amp: Ampicillin; Amo: Amoxicillin; Clin: Clindamycin; Mox: Moxifloxacin; Cip: Ciprofloxacin; Lev: Levofloxacin; Asb: Ampicillin + Sulbactam; Ami: Amikacin; Eri: Erythromycin; Cla: Clarithromycin; Azi: Azithromycin; Com: Cefepime; Ctz: Ceftazidime; Imi: Imipenem; Ptz: Piperacycline; Mer: Meropenem.

Phase	Yield
Jm-Hex (hexane phase)	8.54 g (10.67%)
Jm-CHCl₃ (chloroform phase)	15.45 g (19.31%)
Jm-AcOEt (ethyl acetate phase)	15.23 g (19.03%)
Jm-MeOH (methanolic phase)	31.78 g (39.72%)
Total	71.00 g (88.73%)

Tabla 2. Rendimiento de las fases obtenidas por el proceso de fraccionamiento, por cromatografía líquida al vacío, del extracto etanólico crudo de *J. mollissima* (Jm-EEC).

Table 2. Yield of the phases obtained by the fractionation process by liquid chromatography under vacuum of the *J. mollissima* crude ethanolic extract (Jm-CEE).

(model Q-344B-Quimis, Brazil) and ultrathermal bath (model Q-214M2-Quimis, Brazil). The yield of crude ethanolic extract was approximately 100 g. Of the total of this material, 20 g was reserved for phytochemical analysis and biological tests. The remainder was subjected to the fractionation process using the liquid chromatography under vacuum technique. Silica gel was used as stationary phase and the solvents hexane, chloroform, ethyl acetate and methanol (in increasing polarity system) were used as mobile phase, aiming at a pre-fractionation of the substances through their polarities. For this purpose, 80 g of the crude ethanolic extract was adsorbed in a portion of the stationary phase with the aid of gral and pistil. Then, the phase separation process was carried out under vacuum (Saito *et al.* 2005). Obtaining yields of extracts presented in table 2. The solutions utilized in the tests was prepared at a concentration of 10 mg/mL, dissolved in DMSO and then diluted with distilled water to obtain a concentration of 1024 μg/mL.

Phytochemical prospecting

The phytochemical tests to detect the presence of alkaloids, anthocyanins, anthraquinones, phenolic compounds, coumarins, anthracene derivatives, lignans, mono, sesqui and diterpenes, naphthoquinones, saponins, hydrolyzable tannins, triterpenes and steroids were performed according to the method described by Oliveira *et al.* 2010. These tests were based on the visual observation of a change in color or formation of precipitate after the addition of specific reagents, and the results for the extracts studied are shown in table 3.

Chemical characterization of *J. mollissima* by gas chromatography coupled to the mass spectrometer (GC-MS)

The crude ethanolic extract was analyzed using the gas chromatography coupled to the mass spectrometer (GC-MS), in order to evaluate the chemical profile of the *J. mollissima* species. The substances present were investigated on a Shimadzu QP-2010 CG-EM apparatus. For the analyzes, the samples were resuspended in 10 mg/mL ethyl acetate (HPLC Grade) and then analyzed on a gas chromatograph coupled to a Shimadzu® mass spectrometer (QP-2010) equipped with a self-injector (AOC 20i). The following chromatographic conditions were employed: RESTEK® RTX-5MS column (30.0 mm x 0.25 mm x 0.25 mm), using helium gas (99.999%) transported with a constant flow of 1.4 mL/min, injection volume of the sample of 1.0 μL, split mode with ratio 5 (split 1:4 discard), injector temperature of 260 °C, electron impact mode at 70 eV and temperature of the ion source of 250 °C. The furnace temperature was programmed to 80 °C (isothermal for 3 min), increasing from 5°C/min to 285 °C (isothermal for 15 min) and 10°C/min to 320 °C (isothermal for 20 min). A mixture of linear hydrocarbons (C9H20-C40H82) was injected under the same conditions as the samples under analysis, and the identification of the compounds was by comparison of the mass spectra obtained with the spectra

presented by the equipment database (Wiley 7 and NIST 08 lib). The compound was considered as identified when it presented similarity index higher than or equal to 90%.

Drugs

Gentamicin, amikacin, ampicillin and norfloxacin were obtained from Sigma Chemical Corp., St. Louis, MO, USA. All drugs were dissolved in sterile water to obtain the appropriate concentrations and decrease in toxicity.

Extracts

These leaf extracts of *J. mollissima* were used to test antibacterial efficiency: crude ethanolic extract (Jm-CEE), hexanic phase (Jm-Hex), chloroform phase (Jm-CHCl$_3$), ethyl acetate phase (Jm-AcOEt) and methanolic phase (Jm-MeOH).

Antibacterial test (minimal inhibitory concentration and minimal bactericidal concentration)

The antibacterial effect was determined by the broth microdilution method as recommended by The National Committee for Clinical Laboratory Standards (NCCLS, 2008). Initially, a 25 mg/mL stock solution of the extracts was prepared using a 20% (v/v) aqueous solution of DMSO. One hundred microliters of this dilution were transferred to a microplate containing 100 μL of Müller-Hinton broth. Serial dilutions were then performed, resulting in concentrations of 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195 and 0.0975 mg/mL. The inoculums containing 5x105 CFU/mL, which corresponds to the 0.5 turbidity on the McFarland scale, were standardized by spectrophotometry, considering the interval between absorbance values of 0.08 to 0.1. Subsequently, 10 μL of these inoculums were added to each well. Wells were reserved in the microplates for control of broth sterility, bacterial growth and the actions of DMSO 20% (negative control) and reference antimicrobial (gentamicin-positive control). For gentamicin, an initial concentration of 1.6 mg/mL was used, which was diluted to concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 mg/mL. The microplates were incubated under aerobic conditions for 24 h at 37 °C and afterwards 10 μL of 2% 2,3,5-triphenyl-tetrazolium chloride (CTT) were added to each well for detection of the color change of CTT (colorless) to red, which reflects active bacterial metabolism. MIC was defined as the lowest concentration of the extract that visually inhibited bacterial growth. To determine the MBC, 10 μL aliquots was removed from each well of the previous assay and transferred to Petri dishes containing Müller-Hinton agar. Plates were incubated for 24 h at 37 °C. The appearance of bacterial colony for a given concentration indicates that it was not able to kill 99.9% or more of the bacterial inoculum used. The assays were performed in triplicate.

Modulation of antibiotic activity

Bacterial strains were maintained under ideal conditions of storage in HIA medium (Incline Heart Infusion; Difco Laboratories Ltd.). On the day before the start of the experiment, the bacterial samples were cultured and incubated under aerobic conditions for 24 hours at 37 °C in BHI medium (brain and heart infusion; Difco Laboratories Ltda). The inoculums containing 5x105 CFU/mL, corresponding to 0.5 turbidity on the McFarland scale, were standardized by spectrophotometry, considering the interval between absorbance values of 0.08 to 0.1. In order to verify the modulatory activity of the samples Jm-CEE, Jm-Hex, Jm-CHCl$_3$, Jm-AcOEt and Jm-MeOH, three antibiotics were considered standard in the treatment of infections promoted by the bacterial species tested: amikacin, ampicillin and norfloxacin. For the tests, antibiotic solutions were prepared at the initial concentration 1024 μg/mL. Initially, the Minimum Inhibitory Concentration (MIC) experiment was performed, similarly to the previous topic, with serial dilutions (1:1) of the extracts, starting at the initial concentration of 1024 μg/mL, resulting in the final concentrations of 512; 256; 128; 64; 32; 16; 8; 4; 2; 1 and 0.5 μg/mL. The experiment was carried out in microculture plates, with 96 wells, the last reserved for sterility control. DMSO 20% was used as negative control. A chromogenic indicator (Resazurin), which acts as an oxy-reduction indicator, a process that occurs in cellular respiration, in viable cells, was used to visualize whether or not there was growth of bacterial species. In the presence of these cells, the resazurin dye changes from dark blue to bright red, indicating which extracts concentrations were effective in inhibiting bacterial growth. The standard antibiotics (amikacin, ampicillin and norfloxacin) were used as controls, using the same initial concentration (1024 μg/mL) of the study samples (Coutinho *et al.* 2008).

Statistical analysis of microbiological results

The assays were performed in triplicates and results were expressed as average of replicates. The results are expressed as the geometric mean. Two-Way ANOVA was applied as Statistical hypothesis analysis using GraphPadPrism 6.0 software.

Results

Phytochemical prospecting

The preliminary phytochemical analysis carried out had the objective of detecting the main classes of secondary metabolites present in the *J. mollissima* extracts, comparing the profile found here with data present in the literature. The methodology used in this work allowed the investigation of twelve classes of secondary metabolites, suggesting the presence of important classes of these metabolites, varying in intensity depending on the sample (weak to strong). The results obtained with the preliminary study performed are described in table 3.

Chemical characterization of *J. mollissima*

The analysis of the chemical composition of the crude ethanolic extract (Jm-CEE) obtained from the leaves of *J. mollissima* was performed using gas chromatography coupled to the mass spectrometer (GC-MS). The analysis revealed the presence of 51 peaks, as can be observed in the total ion chromatogram (TIC) shown in figure 1.

From 51 compounds detected, 23 constituents were identified as shown in table 4. The Table lists the compounds, the retention time and their quantification in the crude ethanolic extract. Identification occurred by comparing the mass spectra of the compounds with the spectra of the databases present in the equipment.

Of the 23 constituents identified in the Jm-CEE, five constituents appeared in greater quantity: phytol (18.39%), γ-Sitosterol (12.12%), lupeol (9.30%), linolenic acid (6.09%) and β-amirin (6.05%). Therefore, these were classified as major compounds. The chemical structures of these compounds are illustrated in figure 2.

Antibacterial tests (MIC and MBC)

The results of the evaluation of the antibacterial effect of *J. mollissima* extracts are presented in Table 5 and are expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). According to the classification, it was possible to observe that among the results obtained for the minimum inhibitory concentration, the extracts Jm-CEE, Jm-Hex, Jm-CHCl$_3$ and Jm-AcOEt to the *S. marcescens* strain, presenting values of 0.195; 0.78; 0.78 and 0.39 mg/mL, respectively. A good inhibitory activity of the Jm-CHCl$_3$ and Jm-AcOEt phases against *E. faecalis* strain, with MIC values of 0.78 and 0.39 mg/mL respectively, was also observed. Still, these same phases obtained a good result against the

Chemical class	Jm-CEE	Jm-Hex	Jm- CHCl$_3$	Jm-AcOEt	Jm-MeOH
1	+	+	-	-	-
2	++	++	++	+	+
3	+++	+++	+	-	-
4	+++	+++	+++	+++	+++
5	+++	+	+	-	-
6	+++	-	-	++	-
7	+	-	-	+	+
8	+++	+++	++	-	-
9	-	-	-	-	-
10	+	-	+	-	-
11	+++	-	-	+++	+
12	++	++	+	+	-

Tabla 3. Descripción de las clases de metabolitos secundarios investigados en extractos de *J. mollissima*. 1. Alcaloides 2. Antocianinas 3. Antraquinonas 4. Compuestos fenólicos 5. Cumarinas 6. Derivados antracénicos 7. Lignanos 8. Mono, sesqui y diterpenos 9. Naftoquinonas 10. Saponinas 11. Taninos y 12. Triterpenos / esteroles. Presencia de compuesto: (-) ausente, (+) débil, (++) moderado, (+++) fuerte. Jm-EEC (extracto etanólico crudo); Jm-Hex (fase hexano); Jm-CHCl$_3$ (fase cloroformo); Jm-AcOEt (fase acetato de etilo); Jm-MeOH (fase metanólica).

Table 3. Description of the classes of secondary metabolites investigated in *J. mollissima* extracts. 1. Alkaloids 2. Anthocyanins 3. Anthraquinones 4. Phenolic compounds 5. Coumarins 6. Anthracenic derivatives 7. Lignans 8. Mono, sesqui and diterpenes 9. Naftoquinones 10. Saponins 11. Tannins and 12. Triterpenes/sterols. Presence of compound: (-) absent, (+) weak, (++) moderate, (+++) strong. Jm-CEE (crude ethanolic extract); Jm-Hex (hexane phase); Jm-CHCl$_3$ (chloroform phase); Jm-AcOEt (ethyl acetate phase); Jm-MeOH (methanolic phase).
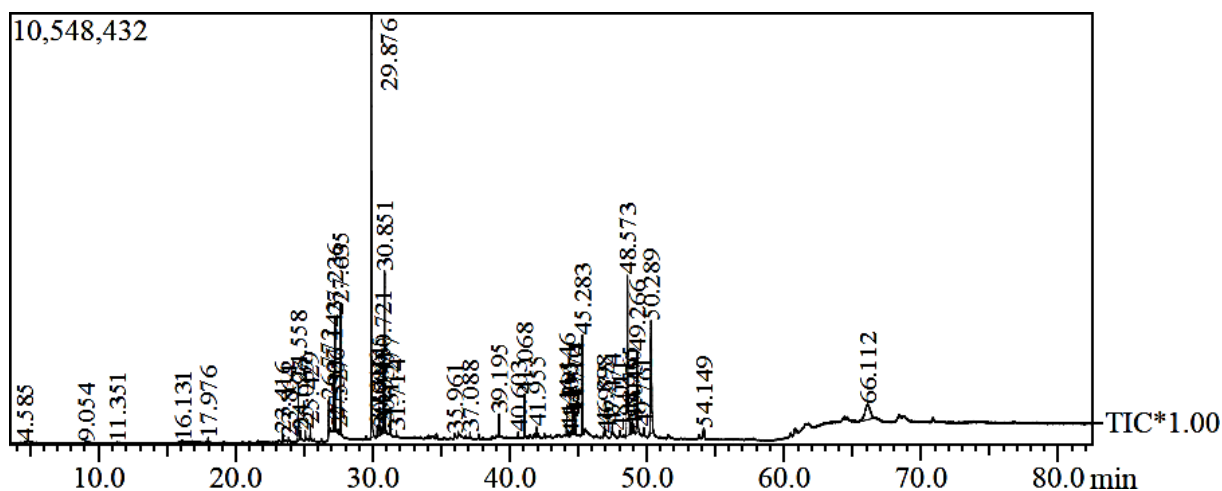
Figura 1. Cromatograma de iones totales del extracto etanólico crudo de *J. mollissima*.
Figure 1. Chromatogram of total ions of the crude ethanolic extract of *J. mollissima*.

Peak	RT (min)	Compound	(%)	RI	Peak	RT (min)	Compound	(%)	RI
1	4.585	*NI*	0.19	-	27	35.960	*NI*	0.07	-
2	9.055	*NI*	0.52	-	28	37.090	1,2-Benzenedicarboxylic acid (Phthalic acid)	0.20	2832
3	11.350	3-Oxo-4-fenilbutironitrile	0.29	1473					
4	16.130	*NI*	0.39	-	29	39.195	n-pentacosane	0.77	2500
5	17.975	*NI*	0.22	-	30	40.605	*NI*	0.15	-
6	23.415	-(-) Loliolide	0.56	-	31	41.070	Esqualene	1.55	2914
7	23.865	*NI*	0.08	-	32	41.955	n-tricosane	0.33	2300
8	24.560	Neofitadiene	2.59	1836	33	44.145	γ-Tocoferol	1.22	3036
9	24.695	*NI*	0.46	-	34	44.295	*NI*	0.20	-
10	25.070	*NI*	0.31	-	35	44.400	*NI*	0.77	-
11	25.430	*NI*	0.66	-	36	44.710	*NI*	0.15	-
12	26.770	9-hexadecenoic acid	1.33	1976	37	44.775	*NI*	0.91	-
13	27.145	Hexadecanoic acid	2.51	1977	38	45.285	α-Tocoferol	4.64	3149
14	27.235	Ethyl 9-hexadecenoate	4.26	1986	39	46.900	*NI*	0.70	-
15	27.305	*NI*	0.37	-	40	46.975	*NI*	0.02	-
16	27.525	*NI*	0.17	-	41	47.475	Stigmasterol	0.51	-
17	27.635	Ethyl palmitate	4.35	1993	42	48.010	*NI*	0.34	-
18	29.875	Phytol	18.39	2045	43	48.575	γ-Sitosterol	12.12	2731
19	30.315	9,12 octadecadienoic methyl ester acid	0.53	2183	44	48.765	*NI*	1.25	-
20	30.445	9,12,15 octadecatrienoic methyl ester acid	1.93	2191	45	48.930	*NI*	0.93	-
21	30.580	*NI*	0.49	-	46	49.045	*NI*	0.36	-
22	30.720	Linoleic acid	2.76	2193	47	49.265	β-amirin	6.05	-
23	30.850	Linolenic acid (omega 3)	6.09	2201	48	49.760	*NI*	0.53	-
24	30.930	*NI*	0.24	-	49	50.290	Lupeol	9.30	2848
25	31.275	Ethyl stearate	0.93	2198	50	54.150	*NI*	0.68	-
26	31.715	*NI*	0.16	-	51	66.110	*NI*	5.72	-

Tabla 4. Componentes químicos del extracto etanólico crudo de las hojas de *J. mollissima*. RT: Tiempo de retención; RI: Índice de retención, NI: No identificado.
Table 4. Chemical constituents of the crude ethanolic extract of the leaves of *J. mollissima*. RT: Retention time; RI: Retention index, NI: Not identified.
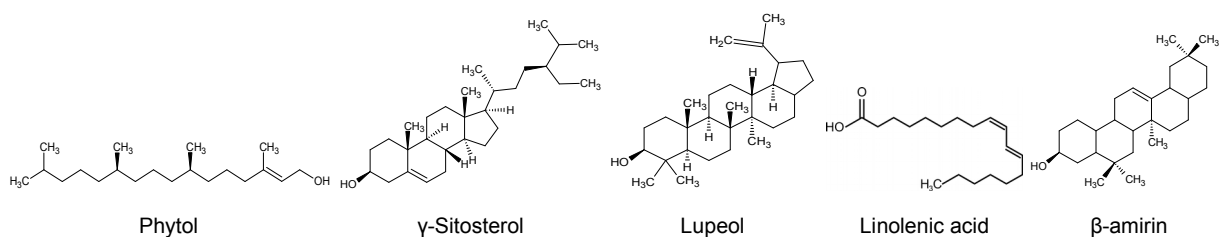

Figura 2. Estructuras químicas de los principales compuestos presentes en el extracto etanólico crudo de *J. mollissima*.
Figure 2. Chemical structures of the major compounds present in the crude ethanolic extract of *J. mollissima*.

Bacterial strain	Extract										Positive control (Gentamicin)		Negative control (DMSO)	
	Jm-EEB		Jm-Hex		Jm-CHCl₃		Jm-AcOEt		Jm-MeOH					
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. faecalis	12.5	-	12.5	-	0.78	-	0.39	-	12.5	-	0.4	0.4	-	-
E. coli	3.125	12.5	3.125	12.5	12.5	12.5	12.5	12.5	-	-	*	0.4	-	-
K. pneumoniae	6.25	-	-	-	6.25	-	-	-	-	-	0.05	0.05	-	-
S. enterica	12.5	-	-	-	6.25	-	12.5	12.5	-	-	0.05	0.05	-	-
S. marcescens	0.195	12.5	0.78	12.5	0.78	12.5	0.39	12.5	12.5	-	*	0.025	-	-
S. flexneri	3.125	-	6.25	-	3.125	-	0.195	-	12.5	12.5	*	0.025	-	-
S. aureus	12.5	-	6.25	-	6.25	-	12.5	-	-	-	0.025	0.025	-	-

Bacterial strain	Ratio MIC/MBC						
	Jm-EEB	Jm-Hex	Jm-CHCl₃	Jm-AcOEt	Jm-MeOH	Gentamicin	DMSO
E. faecalis	-	-	-	-	-	1	-
E. coli	4	4	-	-	-	*	-
K. pneumoniae	-	-	-	-	-	1	-
S. enterica	-	-	-	-	-	1	-
S. marcescens	64	16	16	32	-	*	-
S. flexneri	-	-	-	-	-	*	-
S. aureus	-	-	-	-	-	1	-
Result	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Not effective	Bactericide	Not effective

Tabla 5. Valores de concentración inhibitoria mínima (CIM) y concentración bactericida mínima (CBM) (mg/mL). Jm-CEE (extracto etanólico crudo); Jm-Hex (fase hexano); Jm-CHCl3 (fase cloroformo); Jm-AcOEt (fase acetato de etilo); Jm-MeOH (fase metanólica). (-) Ausencia de actividad en todas las concentraciones probadas; (*) Sin crecimiento en todas las concentraciones probadas.

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (mg/mL). Jm-CEE (crude ethanolic extract); Jm-Hex (hexane phase); Jm-CHCl3 (chloroform phase); Jm-AcOEt (ethyl acetate phase); Jm-MeOH (methanolic phase). (-) Absence of activity at all concentrations tested; (*) No growth at all concentrations tested.

strain *S. flexneri*, with values of 3.125 and 0.39 mg/mL. Likewise, the crude ethanolic extract also obtained good inhibitory activity against *S. flexneri*, with a MIC 3.125 mg/mL.

Regarding the minimum bactericidal concentration (MBC), all the extracts were weakly active, with MBC values higher or equal to 12.5 mg/mL for all samples tested. However, as Biyiti *et al.* (2004), by means of the relationship between MIC and MBC values, a better evaluation of the antibacterial effect of bioactive compounds can be obtained. According to this author, a substance is considered to be bactericidal when the MBC/MIC ratio is ≤ 2 and bacteriostatic if the MBC/MIC ratio is 2. Based on these values, it was possible to classify the crude ethanolic extract and the hexane phase as having bacteriostatic effect against *E. coli* and *S. marcescens*, the chloroform phase as having a bacteriostatic effect against *K. pneumoniae* and *S. marcescens*, The ethyl acetate phase as having bacteriostatic effect against *S. marcescens* and the methanolic phase was classified as not effective for all bacterial strains tested. It was possible to observe that none of the extracts presented bactericidal potential against the bacterial strains tested.

Direct antibacterial test (MIC) and modulation of antibiotic activity

The Minimum Inhibitory Concentration (MIC) assay evaluated the direct *J. mollissima* extracts antimicrobial activity against *S. aureus* (SA358) and *P. aeruginosa* (PA03) strains. The MICs obtained are presented in table 6, where it is possible to observe the same MIC value obtained (≥1024 μg/mL) against the two multiresistant strains (SA538 and PA03) tested. The high MIC values obtained for the samples tested and even for the antibiotics themselves can be attributed to the mechanisms of resistance that these two bacterial strains possess. Antibiotic resistance occurs when bacteria acquire genes that allow interference with the mechanism of action of the antibiotic (Loureiro *et al.* 2016).

There was no MIC reduction of the antibiotics for any of the samples tested (there was no synergistic interaction). The samples tested, in general, did not interfere with the effect of the antibiotics, with the exception of Norfloxacin. The antibiotic activity modulation of Norfloxacin by *J. mollissima* extracts against SA358 and PA03 can be seen in figure 3.

It was possible to observe an antagonistic

Substance	Bacterial strain	
	S. aureus (SA358)	*P. aeruginosa* (PA03)
Jm-CEE	≥ 1024 µg/mL	≥ 1024 µg/mL
Jm-Hex	≥ 1024 µg/mL	≥ 1024 µg/mL
Jm-CHCl₃	≥ 1024 µg/mL	≥ 1024 µg/mL
Jm-AcOEt	≥ 1024 µg/mL	≥ 1024 µg/mL
Jm-MeOH	≥ 1024 µg/mL	≥ 1024 µg/mL
Amicacin	≥ 1024 µg/mL	≥ 1024 µg/mL
Ampicillin	≥ 1024 µg/mL	≥ 1024 µg/mL
Norfloxacin	≥ 512 µg/mL	≥ 512 µg/mL

Tabla 6. Valores de concentración inhibitoria mínima (CIM). Jm-EEC (extracto etanólico crudo); Jm-Hex (fase hexono); Jm-CHCl3 (fase cloroformo); Jm-AcOEt (fase acetato de etilo); Jm-MeOH (fase metanólica).

Table 6. Minimum inhibitory concentration (MIC) values. Jm-CEE (crude ethanolic extract); Jm-Hex (hexane phase); Jm-CHCl3 (chloroform phase); Jm-AcOEt (ethyl acetate phase); Jm-MeOH (methanolic phase).
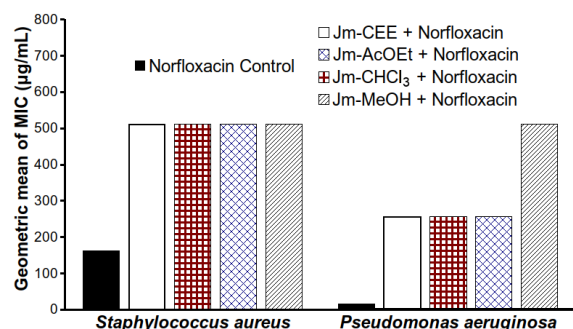

Figura 3. Efecto modulador de los extractos de *J. mollissima* (Jm-CEE, Jm-Hex, Jm-CHCl₃, Jm-AcOEt y Jm-MeOH) en la actividad antibiótica del norfloxacino contra cepas resistentes a múltiples fármacos de *S. aureus* (SA358) y *P. aeruginosa* (PA03). Todos los extractos causaron efectos significativos en comparación con el control (p <0.0001).

Figure 3. Modulatory effect of extracts from *J. mollissima* (Jm-CEE, Jm-Hex, Jm-CHCl₃, Jm-AcOEt and Jm-MeOH) in the antibiotic activity of Norfloxacin against multidrug resistant strains of *S. aureus* (SA358) and *P. aeruginosa* (PA03). There were significant effects of all extracts with respect to the control (p<0.0001).

interaction (increase of antibiotic MIC) between the antibiotic Norfloxacin and all the samples tested. The crude ethanolic extract increased the MIC of the antibiotic from 16 µg/mL to 256 µg/mL compared to the *P. aeruginosa* species and from 161 µg/mL to 512 µg/mL for *S. aureus*. The hexane phase, in turn, increased the MIC of the antibiotic from 16 µg/mL to 512 µg/mL compared to the *P. aeruginosa* species and from 161 µg/mL to 512 µg/mL for *S. aureus*. Chloroform and methanolic phases, as well as the crude ethanolic extract, increased the MIC of the antibiotic from 16 µg/mL to 256 µg/mL compared to the *P. aeruginosa* species and from 161 µg/mL to 512 µg/mL for *S. aureus*. Finally, the ethyl acetate

phase increased the MIC of the antibiotic from 16 µg/mL to 645 µg/mL compared to the *P. aeruginosa* species and from 161 µg/mL to 512 µg/mL to *S. aureus*.

Discussion

Currently, several studies have been developed regarding the discovery of new antimicrobial agents from vegetable extracts and other natural products, with the objective of discovering new bioactive compounds with a hability compared to the traditional drugs, but with less toxicity and greater effectiveness against the resistance of pathogenic microorganisms, besides having a lower environmental impact. In the evaluation of the antimicrobial activity of plant extracts, different methods can be used, being the best known the method of diffusion in agar, disc-diffusion and methods of macrodilution and microdilution in broth (De Bona *et al.* 2014).

In the present study, it was possible to observe that *J. mollissima* acted as a moderate inhibitor against the species *S. marcescens*, *E. faecalis* and *S. flexneri*, probably due to the presence of phenolic compounds and terpenes in the chemical composition of the extracts (as was suggested in preliminary phytochemical screening). Thee substances present antimicrobial potential, as already reported in the literature. By means of the chromatographic analyzes by CG-MS it was possible to observe that the extracts analyzed present in their compositions different compounds of the class of terpenes. By observing the preliminary phytochemical profile, it was also possible to perceive that the crude ethanolic extract and its phases have in their composition these same constituents. Our work resembles a study by Rahman *et al.* (2014) with the *Jatropha curcas* L. species, in which metabolites such as 10-octadecenoic acid methyl ester, octadecanoic acid, 9,12-octadecadienoic acid methyl ester and n-hexadecenoic acid with antimicrobial potential were found.

Terpenes have aroused great interest due to the various biological activities already reported in the literature attributed to these compounds, such as anti-inflammatory, antibacterial, antifungal, antiviral, antitumor, antidiabetic, antiulcerogenic, hepatoprotective, neuroprotective, antiparasitic, analgesic and antioxidant (Coloma *et al.* 2011). In this sense, these metabolites may be associated with the antimicrobial activity presented by *J.*

mollissima, because, according to studies, these have the ability to inhibit microbial growth. The mechanism of action responsible for its activity is not fully understood, but is probably due to disruption of the plasma membrane by its lipophilic compounds (Cowan 1999).

The use of plant extracts as antimicrobial agents minimizes the possibility of microorganisms becoming resistant to their action, since they are complex mixtures, making the microbial adaptability difficult. These natural compounds, when associated with certain antibiotics, may promote direct activity against various bacterial species, decreasing or increasing the activity of a specific antibiotic. The potentiation of antibiotic activity or the reversion of resistance to antibiotics allows the classification of these compounds as modulators of antibiotic activity (Coutinho *et al.* 2009).

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