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In vitro Antimollicute Properties of Rosmarinus officinalis Extract

Propriedades Antimollicute in vitro do Extrato de Rosmarinus officinalis

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Rosmarinus officinalis is a widely studied plant, although its activity against bacteria of the Mollicutes class is lacking. We evaluated the antibacterial potential *R. officinalis* extracts and fractions against different *Mycoplasma* species. The most active fractions were the hexane and dichloromethane ones, with MIC of 15.62 μg mL^{-1} against *M. capricolum* and the hexane fraction with MIC of 15.62 μg mL^{-1} against *M. pneumoniae*. Subfractions were obtained by silica column chromatography, with MIC of 31.25 μg mL^{-1} against *M. capricolum* and 62.5 μg mL^{-1} against *M. pneumoniae*. Purified subfractions or the isolated compound betulinic acid did not show better activity.

Keywords: Mollicutes; mycoplasma; hydroalcoholic extract; antimicrobial; natural products.

1. Introduction

Bacterial resistance to antibiotics is a matter of concern that appears to be out of control, escalating to levels where bacteria are resistant to various classes of antibiotics. Resistance genes are considered environmental pollutants and their spread is one of the most worrying public health issues in the 21st century.[1]

Antibiotics are used in the treatment and prevention of disease in animals to meet the food demand of the world population. Studies show that animal waste with an excess of antibiotics and hospital waste with untreated effluents contaminates the soil and tributaries, which in turn contaminate the food that comes from this soil and water, causing the contamination of the entire food chain.[2]

The growing need for the development of new drugs with antibacterial activity and the scant evaluation of active compounds in different plant species, whether of terrestrial or marine origin, highlights the need for the evaluation of unknown and even known plants, which did not have their potential properly explored.[3]

There is little research evaluating antimicrobial activity against mycoplasmas. Belonging to the class of Mollicutes, they are bacteria devoid of cell walls, and have the smallest known genome among microorganisms capable of self-replication; they cause a series of diseases in humans, other animals, and plants, many of them being species of great interest in the food production chain.[4]

Conventional plants which are not further exploited may be surprising in the investigation of their biological properties as much as the evaluation of new plants. *Rosmarinus officinalis* has already been studied, with antioxidant, antidiabetic, hepatoprotective, diuretic, anti-tumor, anti-inflammatory, and antibacterial activity,[5] but does not have its activity described against strains of mollicutes.

2. Experimental

The *Rosmarinus officinalis* Lamiaceae samples were collected in in the municipality of Indaial, Santa Catarina, Brazil (-26.894591, -49.209833) and independently identified by Prof. Dr. André Luís de Gasper according to standard botanical procedures; the exsiccate nº 56231 is deposited in the Dr. Roberto Miguel Klein herbarium, FURB, Blumenau, SC, Brazil.

2.1 Extraction procedures

The *R. officinalis* leaves were separated from the branches and dried in an oven at 40 °C for

three days, and then crushed and divided into two parts to be extracted by Soxhlet. For the hydroalcoholic extract (HE), 77.8 g of *R. officinalis* leaves were used with 500 mL of 70% ethanol in distilled water for three days, interrupting the process at night with an empty siphon and continuing the next day. The solvent was replaced by a new one on the second day of extraction due to excess extract in the flask to avoid reflux when emptying the siphon. In the second extraction in Soxhlet, the ethanolic extract (EE) was obtained, in 500 mL ethanol PA with 65.5 g of leaves for three days, and the solvent was renewed on the second day of the process. EE extraction was also paused at night and resumed the following morning. The extract was dried in a rotary evaporator at 50 °C until a constant weight was obtained. The partitioning of the extracts was adapted from the methodology used by Hochheim *et al.*[6] The dried HE was resuspended in 150 mL of distilled water, the insoluble part was immediately filtered, and the soluble part was subjected to liquid-liquid partitioning with hexane, dichloromethane, ethyl acetate, and butanol. The hexane (HEX.S.H), dichloromethane (DCM.S.H), ethyl acetate (AE.S.H), butanol (BUT.S.H) and aqueous (AQ.S.H) fractions were obtained from the water-soluble part. The water-insoluble residue was dissolved in dichloromethane, filtered, distilled water was added to the soluble part and the liquid-liquid partition was performed with ethyl acetate and butanol. The dichloromethane (DCM.I.H), ethyl acetate (AE.I.H), butanol (BUT.I.H), and aqueous (AQ.I.H) partitions were obtained. After the DCM.I.H partition dried, it was resuspended in hexane, obtaining the HEX.I.H partition. The same HE partitioning process was applied in the EE, obtaining the water-soluble partitions: hexane (HEX.SE), dichloromethane (DCM.SE), ethyl acetate (AE.SE), butanol (BUT.SE), and aqueous (AQ.SE), and the water-insoluble partitions: hexane (HEX.IE), dichloromethane (DCM. IE), ethyl acetate (AE.IE), butanol (BUT.IE), and aqueous (AQ.IE).

2.2. Extracts fractioning and compound purification

The selection of the fraction subjected to fractionation was defined considering the partitions with the lowest minimum inhibitory concentration (MIC) against mycoplasma strains, the highest yield, and the least number of spots in Thin Layer Chromatography (TLC). The partition fractionation was performed by column chromatography (40 cm high × 2.0 cm in circumference) with silica gel 60-200 mesh, using the solvents hexane, hexane/ethyl acetate, ethyl acetate, ethyl acetate/methanol, and methanol in different polarities for better separation of constituent partitions. The fractionation resulted in 136 subfractions that were combined according to their chemical similarity observed in the LTCs. The grouping resulted in 43 subfractions that had their antibacterial activity evaluated. An isolated compound of low solubility in fraction 24 was obtained, which was separated from its subfraction by successive washes with hexane: ethyl acetate (1:1).

2.3. Thin layer chromatography

Different molecular structures make it possible to qualitatively differentiate compounds using various chemical developers. The elution was carried out with hexane:ethyl acetate (9:1, 8:2, 7:3) and chloroform : methanol with acetic acid (95:5, 9:1 and 8:2) while chemical development was done with sulfuric anisaldehyde, phosphomolybdic acid, ferric chloride, and NP/PEG.[7]

2.4. Infrared spectroscopy, and ^{13}C and ^{1}H Nuclear Magnetic Resonance

The identification of organic functional groups was performed using an infrared instrument model Vertex 70 with ATR (attenuated total reflectance) from the BRUKER® brand. RMN ^{13}C and ^{1}H were performed in a BRUKER® device model Ultrashield 300 MHz for ^{1}H and 100 MHz for ^{13}C using deuterated solvent for solubilization (DMSO-d6 Sigma-Aldrich®).

2.5. Antimollicute activity

For the evaluation of the anti-Mycoplasma action of the samples, strains of *Mycoplasma capricolum* (ATCC 27343), *M. genitalium* (ATCC 33530), *M. pneumoniae* FH (ATCC 15531), *M. pneumoniae* 129 (ATCC 29342) and *M. mycoïdes* subsp. *capri* PG3 (NCTC 10137) were used. The tests were performed by microdilution in broth according to the Clinical Laboratory Standards Institute recommendations,[8] as detailed elsewhere,[9] in duplicates or triplicates until the more often common result was found. The standard antibiotic clarithromycin was used as positive control.

2.6. Statistical analysis

For statistical evaluations of the tests, we used univariate analysis (ANOVA) with a 95% confidence level, using the ezANOVA software version 0.98 (Chris Rorden®).

3. Results and Discussion

The yields of the extracts and their respective fractions are shown in Table 1. From the hydroalcoholic extract, the solid residue showed the highest yield (45.02%), followed by the aqueous fraction (24.48%), insoluble dichloromethane (10.74%) and insoluble hexane (8.06%). In the ethanolic extract, the solid residue also obtained the highest yield (28.2%), followed by the insoluble hexane fraction (22.89%), soluble aqueous (21.29%) and insoluble dichloromethane (11.79%). The high yield of the hexane and dichloromethane fractions indicates the presence of nonpolar compounds (triterpenes), while in the aqueous fraction it is likely to have more polar compounds than those of the previous fractions,

Table 1. Mass and yield of the extracts and fractions from *Rosmarinus officinalis* departing from the hydroalcoholic extract (HE) and the ethanolic extract (EE)

Extract / Fraction	EH		EE	
	Mass (g)	Yield (%)	Mass (g)	Yield (%)
Dried leaves	77.80	-	65.50	-
Original extract	18.76	28.64	20.77	26.70
Hexane from soluble part	0.01	0.03	0.01	0.04
Hexane from insoluble part	1.51	8.06	4.76	22.89
Dichloromethane from soluble part	0.07	0.37	0.06	0.29
Dichloromethane from insoluble part	2.01	10.74	2.45	11.79
Ethyl acetate from soluble part	0.74	3.92	0.71	3.43
Ethyl acetate from insoluble part	0.01	0.06	0.03	0.13
Butanol from soluble part	1.30	6.95	1.32	6.38
Butanol from insoluble part	0.01	0.06	0.07	0.34
Aqueous from soluble part	5.08	24.48	3.99	21.29
Aqueous from insoluble part	0.67	3.23	0.07	0.36
Insoluble residue	9.35	45.02	5.29	28.2

such as glycosylated compounds, which are more soluble in water due to the presence of sugars.[10, 11]

The partitions that showed the lowest inhibitory concentration against the strains of mycoplasma, the highest yield and the least number of stains present in the TLCs were chosen for fractionation, thus, the insoluble dichloromethane partitions of the two extracts were selected because they were identical in the TLCs and the antibacterial tests. By infrared analysis, the possibility of joining the dichloromethane partition of the ethanolic extract with the same fraction of the hydroalcoholic extract was verified.

After comparing the two spectra, the partitions were joined, resulting in the DCM.I partition with 4.422 g of sample. The fractions obtained were grouped by their chemical similarity observed in the TLCs. Subfraction 24 showed an insoluble residue when diluted with ethyl acetate. The solids were separated by successive washes with ethyl acetate and recrystallized from methanol; the crystal, Ro1, resulted in 20.0 mg.

The two extracts and their soluble and insoluble fractions had their anti-Mycoplasma activity evaluated. The inhibitory concentrations obtained are arranged in Table 2.

The MIC of the insoluble ethyl acetate partition of the hydroalcoholic extract varied between mycoplasma strains from 62.5 to 1000 µg mL^{-1}, while the same partition of the ethanolic extract showed inhibition from 62.5 to 250 µg mL^{-1}. This demonstrates that the compounds present in the ethyl acetate partition of the hydroalcoholic extract are effective against *M. capricolum*, *M. pneumoniae* FH, and *M. pneumoniae* 129, but not so much against *M. mycoïde*s and *M. genitalium*. The partition of the ethanolic extract showed inhibition of 250 µg mL^{-1} against these last two strains (Table 2).

A synergistic effect can be observed by comparing the dichloromethane fraction with its respective subfractions and its crude extracts. The dichloromethane partitions before fractionation have a lower inhibitory concentration than their respective crude extracts, but the subfractions of the DCM fraction did not show inhibition greater than their original fraction, even after separation by column chromatography, indicating that greater the separation of the compounds led to less inhibition of strains. The biggest difference obtained was the crude hydroalcoholic extract, with an inhibitory value of 125 µg mL^{-1} and its soluble hexane partition with 15.62 µg mL^{-1} inhibition of the *M. pneumoniae* FH strain (Table 2).

The subfractions had their anti-Mycoplasma activity evaluated, the minimum inhibitory concentrations obtained are shown in Table 3. The lowest inhibitory concentration obtained was 62.5 µg mL^{-1} in seven fractions: 24, 25, 28, 29, 30, 31 and 38.

To identify the compounds classes present in the fractions of the hydroalcoholic extract, which showed the best inhibitory concentrations, the chemical developer sulfuric vanillin, $FeCl_3$, NP/PEG - UV 365nm, and visualization without chemical treatment in UV light 254 nm was used In order to identify the classes of compounds and their respective colors, comparisons were made between the images obtained in this research and the images provided in the thin layer chromatography atlas by Wagner and Bladt.[7]

The development of the TLC plates in $FeCl_3$ resulted in the identification of the presence of phenolic and terpenic compounds. The development of TLC plates in NP/PEG resulted in the identification of phenolic carboxylic acids, flavonoids, flavonol, flavanone, or flavone in their glycosylated or native forms, chalcones and coumarins (Table 4).

It is possible to observe that the fractions of soluble ethyl acetate and soluble butanol are the ones that present flavonoids the most, with very intense spots. The same can

Table 2. Minimum inhibitory concentration of the hydroalcoholic extract (HE) and the ethanolic extract (EE) of *Rosmarinus officinalis* leaves and their respective partitions against different mollicute strains. The results were obtained in duplicates or triplicates until the more often common value was found. The aqueous fraction from insoluble part the ethanolic extract (EE) was not tested. Equal superscript letters indicate no statistically significant difference, considering rows or columns

Fraction	*M. capricolum*	*M. pneumoniae* 129	*M. pneumoniae* FH	*M. mycoïdes*	*M. genitalium*
Original EH	62.5 ^{e}	125 ^{d}	125 ^{d}	250 ^{c}	250 ^{c}
Original EE	125 ^{d}	62.5 ^{e}	62.5 ^{e}	250 ^{c}	250 ^{c}
Hexane from insoluble part EH	31.25 ^{f}	62.5 ^{e}	62.5 ^{e}	125 ^{d}	125 ^{d}
Hexane from insoluble part EE	31.25 ^{f}	62.5 ^{e}	31.25 ^{f}	125 ^{d}	125 ^{d}
Hexane from soluble part EH	15.62 ^{g}	62.5 ^{e}	15.62 ^{g}	62.5 ^{e}	62.5 ^{e}
Hexane from soluble part EE	125 ^{d}	125 ^{d}	31.25 ^{f}	250 ^{c}	250 ^{c}
Dichloromethane from insoluble part EH	31.25 ^{f}	62.5 ^{e}	62.5 ^{e}	125 ^{d}	125 ^{d}
Dichloromethane from insoluble part EE	31.25 ^{f}	62.5 ^{e}	62.5 ^{e}	125 ^{d}	125 ^{d}
Dichloromethane from soluble part EH	15.62 ^{g}	125 ^{d}	62.5 ^{e}	125 ^{d}	125 ^{d}
Dichloromethane from soluble part EE	31.25 ^{f}	125 ^{d}	62.5 ^{e}	250 ^{c}	250 ^{c}
Ethyl acetate from insoluble part EH	62.5 ^{e}	250 ^{c}	125 ^{d}	1000 ^{a}	1000 ^{a}
Ethyl acetate from insoluble part EE	125 ^{d}	125 ^{d}	62.5 ^{e}	250 ^{c}	250 ^{c}
Ethyl acetate from soluble part EH	500 ^{b}	250 ^{c}	250 ^{c}	500 ^{b}	500 ^{b}
Ethyl acetate from soluble part EE	500 ^{b}	250 ^{c}	250 ^{c}	>1000 ^{a}	1000 ^{a}
Butanol from insoluble part EH	250 ^{c}	500 ^{b}	500 ^{b}	250 ^{c}	250 ^{c}
Butanol from insoluble part EE	500 ^{b}	250 ^{c}	500 ^{b}	1000 ^{a}	500 ^{b}
Butanol from soluble part EH	>1000 ^{a}	1000 ^{a}	1000 ^{a}	>1000 ^{a}	>1000 ^{a}
Butanol from soluble part EE	>1000 ^{a}	1000 ^{a}	1000 ^{a}	>1000 ^{a}	>1000 ^{a}
Aqueous from soluble part EH	>1000 ^{a}	1000 ^{a}	>1000 ^{a}	>1000 ^{a}	>1000 ^{a}
Aqueous from soluble part EE	>1000 ^{a}	>1000 ^{a}	>1000 ^{a}	>1000 ^{a}	>1000 ^{a}
Clarithromycin	2 ^{h}	2 ^{h}	2 ^{h}	2 ^{h}	2 ^{h}

be observed after staining in ferric chloride, which identifies phenolic compounds and terpenes. According to Barlette and Mulinacci *et al.*,[12, 13] the extraction of polyphenolic compounds is better in methanol, ethanol, or hydroalcoholic solvent, not exceeding 50% water; with the dry or fresh plant material and according to the evaluation in the TLCs, the fractions showed a high presence of phenolic compounds, as well as flavonoids and terpenes.

In the phosphomolybdic acid developer, which serves as a universal developer,[7] the presence of all classified spots and other spots that were not identified was confirmed, possibly too weak to be noticed in the other developers. With sulfuric vanillin, it was possible to identify terpenes, flavonoids, and chalcones (Table 4).

For better visualization of the analysis of the TLCs, the compiled information is displayed in Table 5 indicating the number of observed spots separated by their classes of compounds and fractions.

By chromatography on silica, the compound Ro1 was isolated, which appeared as a white, odorless solid, with a yield of 20 mg. The compound recrystallized in needle form after dissolution in methanol. When evaluating the melting point, degradation was observed at 262.5 °C, melting at 271.5 °C. The solid showed moderate solubility in methanol, requiring 4 mL to dissolve 20 mg. It showed no anti-Mycoplasma activity against *M. capricolum* or *M. pneumoniae* FH (>1000 μg mL^{-1}).

The infrared spectrum of the compound made it possible to identify at 3447 cm^{-1} the OH signal that corresponds to alcohol, and a carboxylic acid at 2938 cm^{-1}, and 2 at 868 cm^{-1} the C-H signal of Sp^3 hybridization. The C = O signal of carboxylic acid at 1684 cm^{-1}, and the signal at 1643 cm^{-1} and 886 cm^{-1} denotes the presence of an olefin exo-methylene. The existence of methyl groups in the structure is confirmed by the signal at 1375 cm^{-1}. The alcohol C-O signal is present in 1024 cm^{-1}. Based on the IR spectroscopy data, it is possible to predict that the compound presents the functional groups carboxylic acid, alcohol, methylene linked to the main chain, and the presence of a double bond.[14, 15]

The ^{13}C spectrum showed 29 carbon signals; with the data obtained by the DEPT spectrum, it was possible to classify the signals as six methyls (-CH3), eleven methylenes (-CH2-), six methines (CH), five quaternary carbons (C), one olefinic carbon and a carboxylic. The C-8 could not be identified because it was hidden from the solvent (Figure 1S, Supplementary material). The spectrum showed the signals of C-28, C-29, and C-20 at δC 178.2, 110.6, 151.3, respectively. The C-3 signal that was linked to a hydroxyl showed an e δC 77.8 signal; the other signs were between δC 56.4 and δC 15.4.

Table 3. Antimollicute activity of the subfractions obtained from the insoluble dichloromethane partition of *Rosmarinus officinalis* leaves. The results were obtained in duplicates or triplicates until the more often common value was found. Equal superscript letters indicate no statistically significant difference, considering rows or columns

Subfraction	Minimal Inhibitory Concentration (μg mL^{-1})				
	M. capricolum	*M. pneumoniae FH*	Subfraction	*M. capricolum*	*M. pneumoniae FH*
1	>1000 [a]	>1000 [a]	23	125 [d]	62.5 [e]
2	>1000 [a]	>1000 [a]	24	62.5 [e]	62.5 [e]
3	1000 [a]	1000 [a]	25	62.5 [e]	62.5 [e]
4	1000 [a]	1000 [a]	26	250 [c]	250 [c]
5	500 [b]	500 [b]	27	500 [b]	1000 [a]
6	>1000 [a]	>1000 [a]	28	62.5 [e]	62.5 [e]
7	>1000 [a]	500 [b]	29	62.5 [e]	62.5 [e]
8	1000 [a]	500 [b]	30	62.5 [e]	62.5 [e]
9	1000 [a]	500 [b]	31	62.5 [e]	62.5 [e]
10	>1000 [a]	>1000 [a]	32	125 d	250 [c]
11	250 [c]	125 [d]	33	250 [c]	500 [b]
12	>1000 [a]	>1000 [a]	34	500 [b]	500 [b]
13	250 [c]	125 [d]	35	1000 [a]	1000 [a]
14	>1000 [a]	>1000 [a]	36	1000 [a]	1000 [a]
15	500 [b]	250 [c]	37	250 [c]	250 [c]
16	500 [b]	500 [b]	38	62.5 [e]	62.5 [e]
17	1000 [a]	500 [b]	39	1000 [a]	250 [c]
18	500 [b]	500 [b]	40	1000 [a]	500 [b]
19	500 [b]	500 [b]	41	250 [c]	250 [c]
20	500 [b]	500 [b]	42	1000 [a]	1000 [a]
21	125 [d]	250 [c]	43	250 [c]	250 [c]
22	250 [c]	250 [c]	Ro1	>1000 [a]	>1000 [a]
Clarithromycin	2 [f]	2 [f]	2 [f]	2 [f]	2 [f]

The ^{1}H NMR presented a singlet in δH 4.56 and a doublet in δH 4.69 (J = 2.47) and the presence of five tertiary methylene groups in δH 0.65, 0.77, 0.87, 0.93, and 0, 98 (Table 6S, Supplementary material). The signals of the methylene and methane groups were detected in δH 1.65 and 1.32. After comparison with the literature, it is possible to suggest that the isolated compound is betulinic acid.[16, 17] Its chemical structure and characteristics can be found at this website: https://pubchem.ncbi.nlm.nih.gov/compound/Betulinic-Acid. [18]

The major compounds present in *R. officinalis* extracts are rosmarinic acid, pirosol, carnosol, carnosic acid, caffeic acid and its derivatives, rosmadial, catechin, ferulic acid, gentisic acid, vanylic acid, and luteolin.[5] The most studied *R. officinalis* compounds are carnosic acid, carnosol, rosmarinic acid, and ursolic acid. Its essential oil is also widely studied and has in its composition the compounds 1,8-cineol, alpha-pinene, and beta-pinene as major ones.[19]

Other authors evaluated the composition of the dichloromethane:methanol (1:1) extract, in which the leaves were previously heated in a container over medium heat for five minutes and related to research data that did not use heating.[20] Derivatives of carnosic acids and carnosol were identified, two of these being novel compounds that showed better activity than carnosic acid in the control of metabolic disorders that cause type II diabetes mellitus. The researchers suggest heating plant material as an alternative in the search for active compounds.

The minimum inhibitory concentration of the hydroalcoholic extract was 62.5 μg mL^{-1} against *M. capricolum*. The ethanolic extract showed the same inhibitory concentration as the hydroalcoholic extract, however against *M. pneumoniae* FH and *M. pneumoniae* 129. Betulinic acid, $C_{30}H_{48}O_3$ (3β-hydroxi-lup-20(29)-en-28-oic acid), is a lupane skeleton pentacyclic triterpene with four six-carbon rings and a five-carbon ring. Researches attribute the anti-inflammatory, hepatoprotective, antimicrobial, anti-HIV-1, anti-hypoglycemic and cytotoxic activity against tumor cells to this compound.[21]

Regarding the fractions of the obtained extracts, those that obtained the lowest inhibitory value were the hydroalcoholic extract, the soluble hexane fraction, with inhibition at 15.62 μg mL^{-1} against *M. capricolum* and *M. pneumoniae* FH, and the soluble dichloromethane fraction against *M. capricolum*, at 15.62 μg mL^{-1}. The fractions of the ethanolic extract were also more effective

Table 4. Compound classes identified in the TLC of the hydroalcoholic extract and the ten fractions obtained from the leaves of *Rosmarinus officinalis*, with the ferric chloride, NP/PEG or sulfuric vanillin developers. RF: retention factor

Fraction	**Color (ferric chloride)**	**RF**	**Compound class**
(1) Hexane soluble	Grey	Solvent line (A)	Phenolic compound
(2) Hexane insoluble	Grey	0.59 (B); solvent line (C)	Phenolic compound
	Green, Grey after heating	0.67 (D)	Terpenoid and phenolic
	Brown, Grey after heating	0,66 (E)	Terpenoid and phenolic
(3) Dichloromethane soluble	Green, Grey after heating	0.58 (F); 0.74 (G)	Terpenoid and phenolic
	Green, Grey after heating	0.66 (H)	Terpenoid and phenolic
(4) Dichloromethane insoluble	Brown, Grey after heating	0.74 (I)	Terpenoid and phenolic
	Brown, Grey after heating	Base line (J), 0.45 (K); 0.60 (L); 0.83 (M)	Terpenoid and phenolic
(5) Ethyl acetate soluble	Green, Grey after heating	Base line to 0.38 (N); 0.53 (O)	Terpenoid and phenolic
	Grey	0.75 (P)	Phenolic compound
	Green, Grey after heating	0.83 (Q); 0.88 (R)	Terpenoid and phenolic
(6) Ethyl acetate insoluble	Brown	0,56 (S)	Terpenes
(7) Butanol soluble	Brown, Grey after heating	Linha base (T); 0.38 (U); 0.50 (V); 0.66 (X); 0.73 (Y)	Terpenoid and phenolic
	Green, Grey after heating	0.1 (W)	Terpenoid and phenolic
(8) Butanol insoluble	NV*	NV*	NV*
(9) Aqueous soluble	Grey	0.06 (Z); 0.36 (2A)	Phenolic compound
(10) Aqueous insoluble	Grey	0.06 (Z); 0.38 (2A)	Phenolic compound
Fraction	**Color** (NP/PEG)	**RF**	**Compound class**
(1) Hexane soluble	Light blue	1.0 (A)	Phenolic carboxylic acids
(2) Hexane insoluble	Light yellow	0.62 (B)	Flavonoid, flavonol, or glycosylated flavone
(3) Dichloromethane soluble	Light blue	0.41 (C); 0.98 (D)	Phenolic carboxylic acids
	Green	0.63 (E)	Glycosylated flavonoids
	Yellow with white outline	0.56 (F)	Chalcone
	Brown	0.73 (G)	Glycosylated flavanone
(4) Dichloromethane insoluble	Light yellow	0.60 (H); 0.70 (I)	Glycosylated flavonoids
	Blue	0.42 (J)	Phenolic carboxylic acids
(5) Ethyl acetate soluble	Brown	0.79 (K)	Glycosylated flavanones
	Dark green	0.0 - 0.31 (L)	Glycosylated flavanones
	Orange	0.36 (M); 0.71 (N); 0.85 (O); 0.91 (P)	Flavonol, flavone, glycosylated flavonoids
	Bright light blue	0,.1 (Q)	Cumarins
(6) Ethyl acetate insoluble	NV*	NV*	NV*
(7) Butanol soluble	Blue	0.76 (R)	Phenolic carboxylic acids
	Orange	0.69 (S)	Flavonoid
	Light yellow	0.22 (T)	Chalcone
	Yellow	Base line (U); 0.42 (V)	Flavonoid
(8) Butanol insoluble	NV	NV	NV
(9) Aqueous soluble	Yellow with white outline	Base line (X)	Chalcone
	Blue	0.10 (Y); 0.20 (W)	Phenolic carboxylic acids
(10) Aqueous insoluble	Yellow with white outline	Base line(X)	Chalcone
	Blue	0.10 (Y)	Phenolic carboxylic acids
Fraction	**Color** (sulfuric vanillin)	**RF**	**Compound class**
(1) Hexane soluble	Violet	1.0 (A)	Terpenes
	Brown	0.46 - 0.63 (B)	Flavonoids
(2) Hexane insoluble	Violet	1.0 (C)	Terpenes
	Brown	0.50 (D); 0.57 (E)	Flavonoids

Table 4. Compound classes identified in the TLC of the hydroalcoholic extract and the ten fractions obtained from the leaves of *Rosmarinus officinalis*, with the ferric chloride, NP/PEG or sulfuric vanillin developers. RF: retention factor (cont.)

Fraction	Color (ferric chloride)	RF	Compound class
(3) Dichloromethane soluble	Violet	0.48 (F)	Terpenes
(4) Dichloromethane insoluble	Violet	0.67 (G)	Terpenes
	Brown	0.6 (H)	Flavonoids
(5) Ethyl acetate soluble	Violet	0 - 0.29 (I)	Terpenes
(6) Ethyl acetate insoluble	NV*	NV*	NV*
(7) Butanol soluble	Yellow	0.29 (J); 0.42 (K); 0.77 (L)	Glycosylated chalcones or flavanones
	Violet	0.1 (M)	Terpenes
	Blue	0.59 (N)	Terpenes
	Brown	Base line (O)	Flavonoids
(8) Butanol insoluble	NV*	NV*	NV*
(9) Aqueous soluble	Yellow	0.25 (P)	Flavonoids
	Blue	0.64 (Q)	Terpenes
	Brown	Base line (R)	Flavonoids
(10) Aqueous insoluble	Yellow	0.33 (P)	Flavonoids
	Blue	0.64 (Q)	Terpenes
	Brown	Base line (R)	Flavonoids

*not observed.

Table 5. Classes of compounds identified by TLC in extracts and fractions of *Rosmarinus officinalis* leaves, according to the developer used

Fraction	NP/PEG				Ferric chloride		Anisaldehyde
	Flavonoid and glycosylated flavonoid	Glycosylated flavanone	Glycosylated flavonol and / or flavone	Phenolic Carboxylic Acid	Phenolics	Terpenes	Terpenes
(1) Hexane soluble	-	-	-	+	+	-	+
(2) Hexane insoluble	-	-	+	-	++	+	++
(3) Dichloromethane soluble	++	+	+	++	++	+	+
(4) Dichloromethane insoluble	-	-	+	+	++	+	++
(5) Ethyl acetate soluble	++	+	+	-	+++	+++	++
(6) Ethyl acetate insoluble	-	-	-	-	+	+	-
(7) Butanol soluble	+	-	++	+	+++	+++	++
(8) Butanol insoluble	-	-	-	-	+	-	-
(9) Aqueous soluble	-	-	-	+	++	+	+
(10) Aqueous insoluble	-	-	-	+	+	+	-

against *M. capricolum* and *M. pneumoniae* FH, with the best inhibitory values, respectively, the insoluble hexane fraction (MIC = 31.25 μg mL^{-1} and 31.25 μg mL^{-1}), the insoluble and the soluble dichloromethane (31.25 μg mL^{-1} and 62.5 μg mL^{-1}) fractions, and the soluble hexane fraction (125 μg mL^{-1} and 31.25 μg mL^{-1}), against these strains. For natural products, extracts with MIC lower than 10 μg mL^{-1} are considered to have an excellent antibacterial activity; extracts with MIC values between 10 and 100 μg mL^{-1} are considered to have a good activity; extracts with MIC values between 100 and 500 μg mL^{-1} are considered to have moderate activity; extracts with MIC values between 500 and 1000 μg mL^{-1} are considered to have low activity, and extracts with MIC above 1000 μg mL^{-1} are considered inactive. For pure compounds, only samples with MIC lower than 100 μg mL^{-1} are considered active.[22]

The insoluble dichloromethane fraction initially presented the lowest MIC, of 31.25 μg mL^{-1}. After its fractionation seven subfractions had a MIC of 62.5 μg mL^{-1}, showing that the more fractionated the sample the weaker will be the interaction between its compounds, decreasing their antibacterial activity. This may suggest a synergistic effect on this fraction.

Previous studies with hydroalcoholic extract of *R. officinalis* and its fractions also showed an inhibitory and bactericidal effect against strains of *E. faecalis* and *P. aeruginosa.* Inhibition also occurred with strains of *C. albicans*, *S. aureus*, *Sctinomyces* spp, *Streptococcus* spp, *E. coli*, *Lactobacillus acidophilus*, and *Veillonella* spp.[23]

In another study, the *R. officinalis* O ethanolic extract

and the dichloromethane partition showed better inhibitory concentrations than the ethyl acetate and butanol fractions against *S. epidermidis* (MICs of 16, 16, 32 and 512 μg mL^{-1} respectively for the extract, dichloromethane, ethyl acetate, and butanol fractions), *P. aeruginosa* (128, 128, 512, and 512 μg mL^{-1} respectively), *B. cereus* (32, 32, and 256 μg mL^{-1} for the extract, dichloromethane, and ethyl acetate fractions) and *Staphylococcus aureus* (128, 64, 256 μg mL^{-1} respectively).[24] The authors found that rosmarinic acid was the only compound present in the dichloromethane partition that was in a higher concentration than in the original extract (21.5 times more); and that only carnosic acid was in a higher concentration in the original extract when compared to the ethyl acetate partition (9.36 times more). They suggest that the antibacterial activity of the dichloromethane partition is due to the presence of rosmarinic acid; whereas part of the bacterial inhibition of the ethyl acetate partition is influenced by the presence of carnosic acid; and that the synergy between rosmarinic, carnosic and chlorogenic acids in equivalent concentrations would denote the inhibitory concentration in the butanol partition. Indeed, in our study the dichloromethane fraction was one with the best activities, just behind the hexane fraction, which is not commonly studied. The hexane fraction was demonstrated to be especially rich in phenols and terpenes, while the dichloromethane fraction presented also especially phenolic carboxylic acids and flavonoids and glycosylated flavonoids.

The biological activity of *R. officinalis* is attributed both to its volatile fraction and to the phenolic constituents, subdivided into three classes: rosmarinic acid, flavonoid fraction, and diterpene fraction.[12]

In our tests, the minimum inhibitory concentration increases as the polarity of the partitions increases, indicating that the more lipophilic fractions are more effective in inhibiting bacteria. Nonpolar compounds may cross the cell wall of Gram-positive and Gram-negative bacteria easier; the lipophilic area of lipoteicoic acid present in the cell wall facilitates the passage of compounds of less polarity through the wall and cytoplasmic membrane.[25]

The fact that the inhibitory concentration of the fractions was higher than in the partition originating shows the synergistic effect, which has already been observed in other studies with *R. officinalis*.[26] Hence, our study corroborates that this synergistic mechanism may be important to the antibacterial activity of *R. officinalis* compounds.

There is extensive research that addresses the antibacterial activity of *R. officinalis* against strains of common bacteria, and a survey of research that evaluates which compounds confer the biological activity of *R. officinalis*,[27] among these, 30% attribute the biological activity to carnosic acid, followed by 17% attributed to carnosol, 12% to rosmarinic acid and 6% to ursolic acid. The synergistic effect between carnosic acid and gentamicin against methicillin-resistant *Staphylococcus aureus* was found both in type strains and in isolated clinical strains.[28] Gentamicin at 0.1 μg mL^{-1} inhibited 22% of a clinical isolate, carnosic acid inhibited 40% the same strain at a concentration of 0.8 μg mL^{-1}, while a mixture of carnosic acid (0.8 μg mL^{-1}) and gentamicin (0.1 μg mL^{-1}) inhibited 100% of the samples. It is possible to propose a synergy between these compounds in the dichloromethane fraction, considering that the fraction presented a MIC of 31.25 μg mL^{-1}, while fractions 24, 25, 28, 29, 30, 31, and 38 showed MICs of 62.5 μg mL^{-1}.

The compound isolated in our work did not show antimollicute activity. On the other hand, the major activity of betulinic acid seems to be the antitumor one, having an inhibiting effect on the formation of breast cancer colonies of the strains MCF-7 and MDA-MB-231, suppression of aerobic pathways (RCAR e OCR) and reduced expression of LDH-A, c-Myc, and p-PDK1/PDK1. Against MMTV-PyVT+/- cells (breast tumor women are prone to) betulinic acid inhibits cancer growth and decreases tumor burden.[29] The antitumor activity of betulinic acid is considered to have a broad spectrum, being evaluated in tumor cells that afflict the lung, pancreas, prostate, stomach, ovary, colorectal region, and in chronic myeloid leukemia, melanoma, glioblastoma, and cervical carcinoma.[3]

In short, it is evident that the potential biological properties of natural products, even the relatively better-known ones, remains an open and vast field to be studied. The main limitation of our work is that, although *R. officinalis* is a well-known plant, we did not perform gas or liquid chromatography coupled to mass spectrometry to characterize the obtained extracts, in addition to the TLC. It is possible for a plant grown in a different environment to present a distinct chemical profile, especially regarding secondary metabolites.

4. Conclusion

In conclusion, we evaluated the antibacterial potential of the ethanol extract (EE) and the hydroalcoholic extract (HE) of *R. officinalis* and their fractions against *Mycoplasma mycoïdes*, *M. genitalium*, *M. capricolum*, and *M. pneumoniae*. The most active fractions were hexane and dichloromethane from HE, both with MIC of 15.62 μg mL^{-1} against *M. capricolum* and the hexane fraction with MIC of 15.62 μg mL^{-1} against *M. pneumoniae*. Fractions were obtained with MIC of 31.25 μg mL^{-1} against *M. capricolum* and 62.5 μg mL^{-1} against *M. pneumoniae*. Neither the more purified subfractions nor the isolated compound betulinic acid did showed better activity compared to their original subfractions, suggesting the synergistic effect of its components.

Supplementary Information

Supplementary Information (NMR data of the isolated compound Ro1) is available free of charge at https://rvq.sbq.org.br/

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