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Article

Antimicrobial Activity an Physicochemical Characterization of Extracts and Fractions of *Rosmarinus officinalis* and *Origanum vulgare*

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ABSTRACT

Rosmarinus officinalis L. (Lamiaceae) is a shrubby woody with a bittersweet taste, used in chips, broths, desserts, cookies, jams, fruit salads, and marmalades. *Origanum vulgare* L. (Lamiaceae) is a small plant used in meats, salads, soups, rice, potatoes, and boiled eggs, as well as perfumery and flavoring oils. Both have medicinal and aromatic properties. The aims of this study were: perform the phytochemical screening of the powder leaves and the physicochemical characterization of the crude ethanol extracts of *R. officinalis* and *O. vulgare*; determine the content of polyphenols, tannins, flavonoids; quantify rosmarinic acid; evaluate the antimicrobial activity of rosmarinic acid, extracts, fractions of *R. officinalis* and *O. vulgare* against clinical and foodborne strains. The antimicrobial activity was evaluated by the microdilution broth method. The hexane and dichloromethane fractions of *R. officinalis* and *O. vulgare* and rosmarinic acid exhibited good inhibitory activity (MIC < 100 µg/mL) against some Gram-positive bacteria and food isolates. Ethyl acetate fraction of *R. officinalis* (MIC = 62.5 µg/mL), ethanol extract (MIC = 15.6 µg/mL), hexane fraction (MIC = 62.5 µg/mL), dichloromethane fraction (MIC = 31.2 µg/mL) and aqueous fraction (MIC = 62.5 µg/mL) of *O. vulgare* and rosmarinic acid (MIC = 31.2 µg/mL) presented good inhibitory activity against *Cryptococcus*. These fractions have promising potential in the control of food pathogens and they can be used in the future as an alternative natural preservative in the industry.

Keywords: antimicrobial activity; *Origanum vulgare*; plants extracts; *Rosmarinus officinalis*.

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RESUMO

Rosmarinus officinalis L. (Lamiaceae) é um arbusto lenhoso com sabor agridoce, utilizado em batatas fritas, caldos, sobremesas, biscoitos, compotas, saladas de frutas e marmeladas. *Origanum vulgare L.* (Lamiaceae) é um pequeno arbusto utilizado em carnes, saladas, sopas, arroz, batata e ovos cozidos, bem como em óleos perfumados e aromatizantes. Ambos possuem propriedades medicinais e aromáticas. Os objetivos deste estudo foram: realizar a triagem fitoquímica do pó das folhas; a caracterização físico-química dos extratos etanólicos brutos de *R. officinalis* e *O. Vulgare*; determinar o teor de polifenóis, taninos e flavonóides; quantificar o ácido rosmarínico; avaliar a atividade antimicrobiana do ácido rosmarínico, extratos, frações de *R. officinalis* e *O. vulgare* contra cepas clínicas e de origem alimentar. A atividade antimicrobiana foi avaliada pelo método de microdiluição em caldo. As frações hexano e diclorometano de *R. officinalis* e *O. vulgare* e ácido rosmarínico exibiram boa atividade inibitória (CIM <100 µg/mL) contra algumas bactérias Gram-positivas e isolados de alimentos. A fração de acetato de etila de *R. officinalis* (MIC = 62,5 µg/mL), extrato etanólico bruto (CIM = 15,6 µg/mL), fração hexano (CIM = 62,5 µg/mL), fração diclorometano (CIM = 31,2 µg/mL) e a fração aquosa (CIM = 62,5 µg/mL) de *O. vulgare* e ácido rosmarínico (CIM = 31,2 µg/mL) apresentaram boa atividade inibitória contra *Cryptococcus*. Essas frações têm potencial promissor no controle de patógenos alimentares e podem ser utilizadas no futuro como alternativa conservante natural na indústria.

Palavras-chave: atividade antimicrobiana; *O. vulgare*; extratos de plantas; *R. officinalis*.

1. Introduction

In the last years, consumers are looking for high-quality foods, a healthier lifestyle, and environmental care (Petrescu et al. 2020). Besides that, the consumers have the knowlegment about the importance of food naturalness, preferring the food quality associated with minimally processed food, safety and free from synthetic preservatives (Román et al. 2017). The plants produce a large variety of secondary metabolites, which gives them protection against bacteria and fungi (Mendes et al. 2011). The use of substances of vegetable origin as natural food additives, especially essential oils, aromatic extracts can increase microbiological safety and extend the shelf life of products and contribute to the improvement of sensory characteristics of foods (Shan et al. 2007; Weerakkody et al. 2010; Castilho et al. 2012).

Rosmarinus officinalis L. (rosemary) is a woody, branched, shrubby plant of the Lamiaceae family (Wang et al. 2008). It has a bittersweet taste, used in chips, broths, desserts, cookies, jams, fruit salads, marmalades and, mulled wines (Loewenfeld & Back 1978). In the United States, it is used in meat, poultry, fish, and sausages (Shelef et al. 1980) and, in Morocco, it is added to butter and other foods to increase the shelf life of products (Benjamim et al. 1984; Porte & Godoi 2001). *R. officinalis* is used in folk medicine as a general stimulant, hypertensive, stomatal, pulmonary antiseptic, carminative, collagogue, choleretic, emmenagogue, anti-rheumatic and, diuretic (Carvalho & Almança 2003). The parts used of the plant are the leaves and flowers. Its composition contains active ingredients such as 1.8-cineol, α-pinene, borneol, camphor, isobornyl acetate, isonyl valerianate, citric, glycolic, glycine, rosmarinic, nicotiamide, choline, pectin, and rosmaricin. Of these, 1.8-cineol, α-pinene, borneol, and camphor have known antimicrobial activity (Gachkar et al. 2007).

Origanum vulgare L. (oregano) belongs to the Lamiaceae family. It is a small plant, subtle, with stems up to 90 cm, pubescent, hirsute or velvety, erect and sometimes reddish, with ovate, glabrous or hairy, punctate-glandular, and petiolate leaves (Cunha et al. 2009). It is an important culinary flavoring, widely used in meats, salads, soups, rice, potatoes, and boiled eggs, as well as for perfuming and flavoring oils and vinegar (Clevely & Richmond 1998; Lientaghi 2002). It is considered one of the most widely used aromatic plants in the world in the food industry and the production of cosmetics, being part of the composition of body moisturizers and perfumes (Kruppa & Russomanno 2008), which is due in particular to the presence of essential oil.

Boskovic et al. (2015) verified a synergistic antimicrobial activity of oregano and rosemary essential oils, using broth microdilution method, on some food-borne bacteria: *Salmonella Enteritidis* ATCC 13076 (MIC = 320 µg/mL), *Salmonella Thyphimurium* ATCC 14028 (MIC = 160 µg/mL), *Staphylococcus aureus* ATCC 25923 (MIC = 640 µg/mL), methicillin-resistant *Staphylococcus*

aureus ATCC 43300 (MIC = 320 μg/mL), *Escherichia coli* ATCC 25922 (MIC = 320 μg/mL), and *Bacillus cereus* ATCC 11778 (MIC = 160 μg/mL). Barbosa et al. (2016) observed the antimicrobial activity of the essential oils from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* (ROEO), alone or in combination at sub inhibitory concentrations (1/2 MIC OVEO + 1/2 ROEO and 1/2 MIC OVEO + 1/4 ROEO, against three pathogenic bacteria that are associated with fresh leafy vegetables: *Listeria monocytogenes* ATCC 7644 (MIC = 0,6 μg/mL for OVEO and MIC = 5 μg/mL for ROEO), *Escherichia coli* UFEPEDA 224 (MIC = 0,6 μg/mL for OVEO and MIC = 5 μg/mL for ROEO), and *Salmonella enterica* Serovar Enteritidis UFEPEDA 414 (MIC = 0,6 μg/mL for OVEO and MIC = 10 μg/mL for ROEO). Santos et al. (2020) found antimicrobial activity using the diffusion test of *O. vulgare* essential oil against all the tested strains, *E. coli* (MIC = 533,3 ± 28,87 μg/mL), *S. aureus* (MIC = 166,7 ± 28,87 μg/mL) and *Salmonella* sp. (MIC = 683,3 ± 28,87 μg/mL) and the hidroalcoolic extract presented an inhibition halo of 12,67 ± 0,577 mm against *E. coli* and of 10,67 ± 0,577 mm on *Salmonella* sp. Simirgiotis et al. (2020) verified antibacterial properties of *O. vulgare* essential oil from Chile against *Staphylococcus aureus* ATCC 29737 (MIC = 0,08 %), *Salmonella enterica* ATCC 13311 (MIC = 0,08 %), *Bacillus subtilis* ATCC 6051 (MIC = 0,08 %), *Erwinia rhapontici* MK883065 (MIC = 0,04 %), and *Xanthomonas campestris* MH885473 (MIC = 0,08 %). The essential oil presented as main components thymol (15.9%), Z-sabinene hydrate (13.4%), γ-terpinene (10.6%), p-cymene (8.6%), linalyl acetate (7.2%), sabinene (6.5%), and carvacrol methyl ether (5.6%). Janampa et al. (2021) verified the antibacterial activity of ethanol extracts of *R. officinalis* leaves 75% against *Streptococcus mutans* ATCC 25175 (inhibition halo of 12.2 ± 0.5 mm), presenting similar results on chlorhexidine 0,12% (inhibition halo of 13.0 ± 0.7 mm).

Due to an increasing necessity for controlling the growth of microorganisms in foods, the aims of this study were: to perform the phytochemical screening of the powder leaves and the physicochemical characterization of the crude ethanol extracts of *R. officinalis* and *O. vulgare*; to determine the content of polyphenols, tannins, flavonoids; to quantify rosmarinic acid; to evaluate the antimicrobial activity of rosmarinic acid, extracts, fractions of *R. officinalis* and *O. vulgare* against clinical and foodborne strains.

2. Materials and methods

2.1.Obtaining the crude ethanol extract and the fractions

The fragmented leaves of *R. officinalis* and *O. vulgare*, commercially obtained from local suppliers, were triturated in an industrial blender (Poly-Metallurgical Siemsem Ltda., Brusque, SC) and then in a processor (Model BBE 30 Electrolux, Brazil). The leaves powder was frozen (-20°C).

The volatiles content (VC), the solids content (SC), total ash content (TAC), the swelling index (SI), and the particle size distribution (PS) of the leaves powder were determined according to Brazilian Pharmacopeia (2010).

The crude ethanol extracts (CEE) of *R. officinalis* and *O. vulgare* were obtained by percolation using ethanol 80% (v/v) as extractor solvent (Moraes et al. 2011). For the extraction, 2 kg of leaves powder (HD) were placed in 10 L of extraction liquid and were left to stand for 2 h pre-swelling steps. Then, the mixture was placed in a digital mechanical stirrer (CT Tecnal 039/11) at 1000 rpm for 12 h. After this period, the mixture was placed in a percolator containing layers of cotton and filters to retain the remaining powder. The process was repeated 5 times. The extract was concentrated in a rotary evaporator (Büchi® - Model R-220 SE, Switzerland) under controlled temperature. CEE was stored in polyethylene bottles at -20°C.

For the CEE characterization, the pH, the VC, relative density (RD), alcohol content were determined according to the Brazilian Pharmacopeia (Brasil 2010).

The CEE of each plant extract (50 g) was solubilized in a methanol/water mixture (7:3 v/v) and submitted to successive partitions with increasingly polarity solvents (hexane, dichloromethane, ethyl acetate) resulting in hexane (HF), dichloromethane (DF), ethyl acetate (EAF) and aqueous fraction (AF). The fractions were concentrated on a rotary evaporator (Buchi® - Model R-3, Switzerland) at 40°C, and the AF was lyophilized (Ferri 1996). The fractions yield was calculated as follows: yield (%) = (mass fraction/weight of the crude extract) X 100.

2.2. Total phenolic content (TP), total tannins content (TT) and total flavonoids content (TF)

The Hagerman and Butler spectrophotometric method was used to determine TP and TT (Mole and Waterman 1987). A spectrophotometric method described by Rolim et al. (2005) was used for the determination of TF.

2.3. Investigation of rosmarinic acid (RA) by thin-layer chromatography (TLC)

For investigation of rosmarinic acid in CEE and powder leaves (HD) by TLC, the rosmarinic acid (RA) (Sigma Aldrich® - 97%) and samples of CEE, HD, of *R. officinalis* and *O. vulgare* was solubilized in methanol and applied in Silica plate TLC (Merck). The chromatographic analyses were performed with the mobile phase: anhydrous formic acid, acetone, and dichloromethane (5:15:80 v/v/v) (Farmacopeia Portuguesa VIII 2005). After the Silica plate was observed at 365 nm ultraviolet light and analyzed (Wagner & Bladt 2001).

2.4. Determination of RA content using High-Performance Liquid Chromatography (HPLC)

The RA content was determined by analysis on a High-Performance Liquid Chromatography (Model e2695 Waters® Milford, MA, USA). Data analysis was performed using the Empower 2.0 software.

The methodology described by Canelas & Costa (2007) was validated to quantify the RA content. The chromatographic column used was an RP-18, 5 microns average diameter of the particle length and internal diameter of 250 x 4.6 mm, respectively (Waters® - X-TERRA®). The established injection volume was 20 µL. The mobile phase employed was: solvent A - 30% (v/v) acetonitrile HPLC grade (JT Baker®, Mexico) and solvent B - 70% (ultrapure water / acetonitrile / formic acid 97:2.5:0.5% v/v) under isocratic flow 0.5 mL/min. The detection wavelength was 329 nm with a scan of 192-780 nm. The temperature of the column and the sample carousel were maintained at 30°C. The analysis of the sample and of the standard time was set at 30 min.

The parameters of the system suitability: Tailing Factor (TF), Resolution (RS), Theoretical Plate Numbers (N), and standard deviation (DPR) were determined according to the Food and Drug Administration – FDA, to ensure system efficiency. The determination of RA content was evaluated according to the specifications of Resolution RE No. 899 of May 29, 2003, ANVISA (Brasil 2003). The parameters analyzed were: selectivity, linearity standard, sample linearity, the limit of quantification and detection, precision (repeatability and intermediate precision, n = 12), accuracy, and robustness. The RA content was quantified. The analysis of variance (ANOVA) was performed using Microsoft Excel 2010 software at a confidence level of 95%.

2.5.Microbial strains and determination of minimal inhibitory concentrations (MIC)

The microorganisms used on microdilution tests were presented in Table 1. To determine the antimicrobial activity, the bacteria were grown in Casey broth (Himedia, India) for 18-24 h at 35°C ± 2, transferred to Casey agar (Himedia) for 18-24 h 35°C ± 2. *Listeria innocua* and *Listeria monocytogenes* were grown in Brain Heart Infusion broth (BHI, Oxoid, England) for 18-24 h at 35°C ± 2 and then transferred to Brain Heart Infusion agar (BHI, Oxoid), for 18-24 h at 35°C ± 2. The fungi were grown on Sabouraud Dextrose agar (Himedia) at 25°C for 24-48 h (yeasts) and 48-72 h for other fungal species.

The minimum inhibitory concentration (MIC) of the CEE, fractions (HF, DF, EAF, AF) of *R. officinalis, O. vulgare*, the RA standard were determined by microdilution techniques on Mueller Hinton broth (Himedia, India), under aerobic conditions. Susceptibility tests were performed for bacteria (CLSI 2009) for yeasts (CLSI 2008a) and filamentous fungi (CLSI 2008b).

Initially, vegetal extracts were solubilized in dimethyl sulfoxide 10% (DMSO -Vetec, Brazil) and Mueller Hinton broth (Himedia, India), to obtain an initial concentration of 2000 µg/mL, for bacteria. For fungi, the samples were solubilized in DMSO 10% and Roswell Park Memorial Institute 1640 broth (RPMI) (Himedia, India), obtaining an initial concentration of 1000 µg/mL. For bacteria susceptibility tests, 200µL aliquots were distributed at the microplate wells and submitted to serial dilutions on Mueller Hinton broth, until a final concentration of 1.95 µg/mL, for each extract, and were diluted in RPMI, until a final concentration of 0.98 µg/mL, for fungi tests.

For *Listeria* strains, the cation-adjusted Mueller-Hinton broth (Fluka, Sigma-Aldrich, India) with lysed horse blood (2.5-5% v/v) was used (CLSI 2005).

Table 1 - Microorganisms used on microdilution tests.

Gram-positive bacteria	Gram-negative bacteria	Yeast and filamentous fungi
Bacillus cereus ATCC 14579	*Enterobacter aerogenes* ATCC 13048	*Candida albicans* 63U*
Bacillus subtilis ATCC 6633	*Enterobacter cloacae* HMA: FTA 502*	*Candida krusei* ATCC 34135
Listeria innocua (CT) ATCC 33090	*Escherichia coli* ATCC 8739	*Candida parapsilosis* ATCC 22019
L. innocua DF3A-MC2-LS2***	*E. coli* ATCC 25922	*C. parapsilosis* 86U*
L. innocua QMG-13****	*Klebsiella pneumoniae* ATCC 700603	*Candida tropicalis* ATCC 28707
L. innocua QMAC-11****	*Salmonella enterica* subsp. *enterica* sorotipo *Abony* NCTC 6017	*C. neoformans* var. *gatti* L3*
Listeria monocytogenes ATCC 19117	*S. enterica* subsp. *enterica* serovar *Typhi* ATCC 10749	*Criptococcus neoformans* var. *neoformans* ATCC 28957
L. monocytogenes ATCC 7644	*S. enterica* subsp. *enterica* serovar *Typhimurium* ATCC 14028	*Trichophyton mentagrophytes* ATCC 11480
L. monocytogenes 24AJ3**	*S. enterica* subsp. *enterica* serovar *Typhi (CT)* ATCC 19430	*Trichophyton rubrum* ATCC 28189
L. monocytogenes QMAC-1****	*S. enterica* subsp. *enterica* serovar *Choleraesuis* ATCC 10708	
L. monocytogenes QMAC-7****	*Serratia marcenscens* ATCC 14756	
L. monocytogenes QMG-10****	*Pseudomonas aeruginosa* ATCC 27853	
Micrococcus luteus ATCC 9341	*P. aeruginosa* ATCC 9027	
M. luteus ATCC 10240		
Staphylococcus aureus ATCC 6538		
S. aureus ATCC 25923		
S. aureus ATCC 29213		
S. aureus DF2B-10-Sa5***		
S. aureus DF2B-32-Sa3***		
S. aureus DF2B-8-Sa3***		
S. aureus DF2B-9-Sa1***		
S. aureus DF2A-MC1-Sa1***		
S. aureus DF2A-MC2-Sa1***		
S. aureus DF2A-MC3-Sa3***		
Staphylococcus epidermidis ATCC 12228		

Notes: *Donated by the Laboratory of Bacteriology and Micology from the Institute of Tropical Pathology and Public Health (IPTSP/UFG); **Donated by the Laboratory of Microbiology and Micotoxicology Food, FZEA, University of São Paulo (USP); ***Isolated from Minas frescal cheese at the Laboratory of Research on Food and Drugs Quality Control (LPCQAM/UFG) (unpublished data); ****Isolated from mozzarella cheese at LPQCAM [62] (Lima et al., 2015).

Bacterial suspensions were prepared in sterile sodium chloride 0.85% solution (w/v) within a range of 79.4% to 83.2% transmittance, 625 nm (spectrophotometer UV/VIS Hinotek, Model SP-2000UV, China) corresponding to a 0.5 McFarland standard. Tenfold dilution was performed to obtain a cell concentration of 10^7 CFU/mL. Each microplate received 5 µL of microbial suspensions resulting in a final bacterial concentration of 10^4 CFU/ml. The plates were incubated at 35°C for 18-24h. After the incubation period, 20 µl of triphenyl tetrazolium chloride 0.5% (TTC- Vetec, Rio de Janeiro) was added to each well. The presence of red coloration after 30 min incubation was considered positive for microbial growth and the MIC was defined as the lowest concentration able to inhibit bacterial growth. Assays were performed in duplicate, with two replicates each.

The fungi suspension was prepared in sterile sodium chloride 0.85% solution (w/v) within a range of 79.4% to 83.2% transmittance, 530 nm, equivalent to a 0.5 McFarland standard. Tenfold dilution was performed to obtain a cell concentration of 1-5 x 10^3 CFU/mL. Each well received 100 µL of microbial suspension. The concentrations of the vegetal extracts were reduced by half and a final inoculum concentration of approximately 0.5–2.5 x 10^3 UFC/mL was obtained. The plates were incubated at 25°C for 24-48h (yeasts) and 48-72h (filamentous fungi).

As control groups, they were tested separately: DMSO 10% (w / v) (as toxicity control), bacteria and fungi (as microbial growth control), extracts, and fractions of both species and rosmarinic acid (control of samples contamination).

Vancomycin (Sigma-Aldrich) (250 µg/mL), gentamicin (Sigma-Aldrich) (2000 µg/mL), ciprofloxacin (Sigma-Aldrich) (2000 µg/mL) and itraconazole (Sigma) (16 µg/mL) were used as a positive control of the technique.

The classification for antimicrobial activity criteria were: MIC<100 µg/mL (good antimicrobial activity); MIC between 100-500 µg/mL (moderate antimicrobial activity); MIC between 500-1000 µg/mL (weak antimicrobial activity), and MIC above 1000 µg/mL (inactive) (Holetz et al. 2002).

3. RESULTS

3.1. Physicochemical characterization of HD, CEE of R. officinalis, O. vulgare

The physicochemical characterization of HD, CEE of R. officinalis, O. vulgare and RA content, tannins content, and total flavonoid content of R. officinalis and O. vulgare were presented in Table 2. The yield from fractions of R. officinalis and O. vulgare were, respectively, HF (4.88%; 6.00%); DF (19.61%; 7.22%); EAF (8.39%; 18.22%) e AF (24.17%; 38.23%). The highest yield was observed in AF.

The presence of RA in samples of R. officinalis and O. vulgare by thin-layer chromatography (TLC) and the retention factor value was 0.39, corresponding to the standard RA.

Table 2 - Physicochemical characterization of powder leaves (HD), concentrated ethanol extracts (CEE) of *Rosmarinus officinalis* L. and *Origanum vulgare* L.

	Rosmarinus officinalis		***Origanum vulgare***	
Samples	HD	CEE	HD	CEE
VC (%)	9.18 ± 0.38	94.27 ± 0.00	10.75 ± 0.75	66.07 ± 0.01
SC (%)	90.82 ± 0.38	5.73 ± 0.00	89.25 ± 0.75	33.93 ± 0.01
TAC (%)	5.22 ± 0.02	NA	10.59 ± 0.15	NA
SI (mL)	2.9 ± 0.10	NA	4.37 ± 0.15	NA
pH	NA	5.83 ± 0.01	NA	4.38 ± 0.01
RD (g/mL)	NA	0.9075 ± 0.00	NA	1.096 ± 0.00
AC (v/v %)	NA	51 ± 0.00	NA	51 ± 0.00
TP (%)	9.07 ± 0.01	2.23 ± 0.01	7.45 ± 0.01	10.8 ± 0.02
TT (%)	1.10 ± 0.00	0.31 ± 0.01	1.71 ± 0.01	1.75 ± 0.01
TF (%)	3.82 ± 0.03	1.37 ± 0.03	5.03 ± 0.03	5.14 ± 0.00
RA (%)*	1.40 ± 0.03	0.49 ± 0.01	1.22 ± 0.05	6.55 ± 0.31

Notes: HD – powder leaves; CEE – concentrated ethanol extracts; VC - volatiles content; SC - solids content; TAC - total ash content; SI - swelling index; RD - relative density; AC - alcohol content; TP - total phenolic content; TT - total tannins content; TF - total flavonoids content; RA – rosmarinic acid content; NA – not applied; NR – not realized; *: ρ-value> 0,05.

3.2. System suitability, validation of analytical methodology and determination of rosmarinic acid (RA) content using High Performance Liquid Chromatography (HPLC)

The presence of RA was detected in R. officinalis and O. vulgare (Figures 1a-c). The parameters for the system suitability standard RA, RA in R. officinalis, RA in O. vulgare were, respectively: TF (1.0539; 1.1289; 1.1141), RS (2.0210; 2.0323), N (6377.58, 6508.07, 6549.34) (Table 3).

Table 3. System suitability parameters obtained for chromatograms of the rosmarinic acid standard, concentrated ethanol extracts (CEE) of *Rosmarinus officinalis* L. and *Origanum vulgare* L.

	Tailing Factor (TF)	**Resolution (RS)**	**Theoretical Plate Numbers (N)**
Rosmarinic Acid Standard	1.05389	-	6.377,58
Rosmarinic Acid – *Rosmarinus officinalis*	1.12899	2.02106	6.508,07
Rosmarinic Acid – *Origanum vulgare*	1.11410	2.03233	6.549,34
Literature specifications	≤ 2.0	> 2.0	> 2.000

Note: FT = Tailing Factor; RS = Resolution; N = Theoretical Plate Numbers.

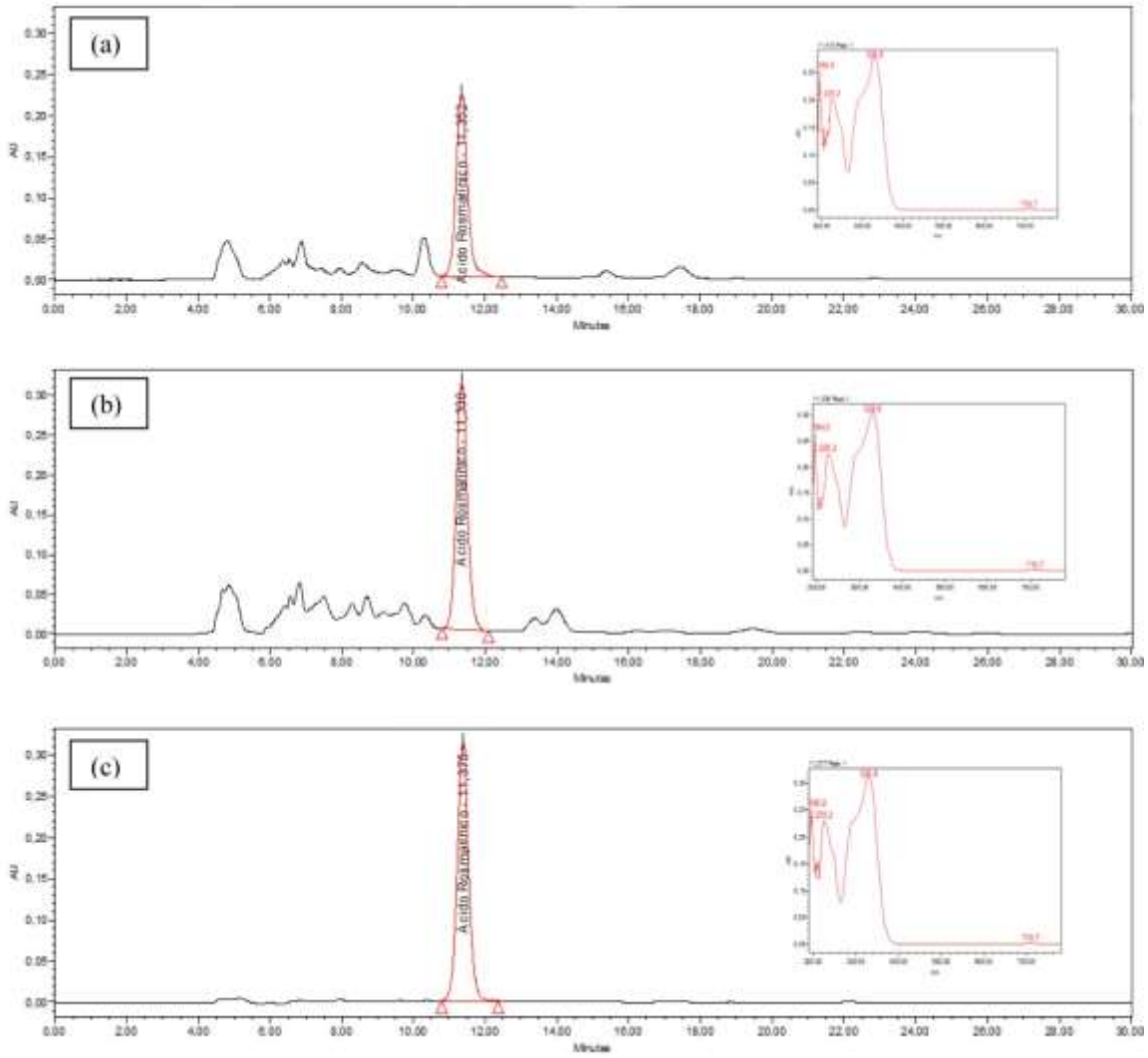

Figure 1. Chromatograms and their pattern of absorption of RA (a) and RA present in *R. officinalis* (b) and *O. vulgare* (c) obtained by High Performance Liquid Chromatography (HPLC).

Comparing the chromatograms obtained of the R. officinalis and O. vulgare CEE (Figures 1b and 1c) with RA standard (Figure 1a), the peaks were at the same retention time and same absorption spectra, both RA standard and RA present in samples were identical. Employing chromatography, it was found that there was no interference of the solvent (ethanol 80%) in the RA retention time, allowing the conclusion that the method was selective for RA.

It was verified that the proposed method is suitable and linear for RA (0.01-0.2 mg/mL); R. officinalis (4-12 mg/mL) and O. vulgare (15-40 mg/mL) (ϱ-value>0.05). The values found for the limit of detection and limit of quantification for RA were: 0.00225 mg/mL and 0.00752 mg/mL, respectively.

The method presented was precise for repeatability with an average content of 6.35 ± 0.18% (CV = 2.76%) for R. officinalis and 2.6 ± 0.04% (CV = 1.47%) for O. vulgare; and intermediate precision, with an average grade of 6.547% ± 0.312 (CV = 4.76%) for R. officinalis, and 2.626% ± 0.050 (CV = 1.92%) for O. vulgare (ϱ-value>0.05). The method proved to be accurate since the average recovery RA ranged from 100.96 to 103.69% for R. officinalis and from 104.28 to 106.46% for O. vulgare. The method showed robustness concerning the composition of the mobile phase, column temperature, and flow rate of the mobile phase (ϱ-value>0.05).

3.3. Determination of the antimicrobial activity of extracts and fractions of R. officinalis and O. vulgare

The HF of R. officinalis had good inhibitory activity against L. innocua QMAC-11 (MIC = 31.25 μg/mL), L. monocytogenes QMAC-7 (MIC = 62.50 μg/mL), and M. luteus ATCC 10240 (MIC = 62.50 μg/mL); DF against L. innocua QMG-13 (MIC=62.50 μg/mL), L. innocua QMAC-11 (MIC=31.25 μg/mL), B. cereus ATCC 14579 (MIC= 31.25 μg/mL), B. subtilis ATCC 6633 (MIC = 62.50 μg/mL), and M. luteus ATCC 10240 (MIC = 31.25 μg/mL); and EAF against C. neoformans var. neoformans ATCC 28957 (MIC = 62.50 μg/mL) (Table 4).

The CEE of O. vulgare showed good inhibitory activity against C. tropicalis ATCC 28707 (MIC = 62.50 μg/mL) and C. neoformans var. neoformans ATCC 28957 (MIC = 15.62 μg/mL). The HF had good inhibitory activity against L. innocua QMAC-11 (MIC = 7,81 μg/mL), L. monocytogenes 24AJ3 (MIC = 31.25 μg/mL), L. monocytogenes QMAC-7 (MIC = 31.25 μg/mL), S. aureus ATCC 29213 (MIC = 62.50 μg/mL), S. aureus DF2B-8-Sa3 (MIC = 62.50 μg/mL), S. aureus DF2A-MC1-Sa1 (MIC = 62.50 μg/mL), B. cereus ATCC 14579 (MIC = 62.50 μg/mL), B. subtilis ATCC 6633 (MIC = 62.50 μg/mL), L. innocua ATCC 33090 (MIC = 62.50 μg/mL); C. neoformans var. neoformans ATCC 28957 (MIC = 62.50 μg/mL). The DF had good inhibitory activity against L. innocua QMAC-11 and C. neoformans var. neoformans ATCC 28957 (MIC = 31.25 μg/mL), the EAF against C. neoformans var. neoformans ATCC 28957 (MIC = 7.81 μg/mL), and C. neoformans var. gatti L3 (MIC = 15.62 μg/mL); and the AF against C. neoformans var. neoformans ATCC 28957 (MIC = 62.50 μg/mL) (Table 4).

The RA pattern showed good inhibitory activity against L. innocua ATCC 33090 (MIC = 31.25 μg/mL), C. tropicalis ATCC 28707 (MIC = 62.50 μg/mL), and C. neoformans var. neoformans ATCC 28957 (MIC = 31.25 μg/mL) (Table 4).

Table 4 - Minimum Inhibitory Concentration (MIC in µg/mL) of concentrated extracts and fractions HF, DF, EAF, AF of Rosmarinus officinalis and Origanum vulgare and RA standard against food isolated and others microorganisms

	Rosmarinus officinalis					***Origanum vulgare***					**Pd**	**GEN**	**CIP**	**VAN**	**IT**
Gram-positive bacteria	**CEE**	**HF**	**DF**	**EAF**	**AF**	**CEE**	**HF**	**DF**	**EAF**	**AF**	**RA**				
B. cereus ATCC 14579	>1000	125	31.25	>1000	>1000	500	62.50	500	>1000	>1000	>1000	<0.98	<1.95	<0.24	NR
B. subtilis ATCC 6633	>1000	125	62.50	>1000	>1000	500	62.50	500	>1000	>1000	>1000	31.25	<1.95	<0.24	NR
L. innocua ATCC 33090	>1000	125	125	250	>1000	250	62.50	500	1000	>1000	31.25	<0.98	<1.95	0.98	NR
L. innocua DF3A-MC2-LS2	>1000	250	250	>1000	>1000	1000	250	>1000	1000	1000	>1000	<0.98	<1.95	0.49	NR
L. innocua QMG-13	>1000	250	62.50	>1000	>1000	500	125	>1000	1000	>1000	>1000	<0.98	<1.95	0.49	NR
L. innocua QMAC-11	>1000	31.25	31.25	>1000	>1000	1000	7.81	15.62	500	>1000	>1000	<0.98	<1.95	0.49	NR
L. monocytogenes ATCC 19117	>1000	250	250	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	<0.98	<1.95	0.49	NR
L. monocytogenes ATCC 7644	>1000	125	125	>1000	>1000	>1000	1000	>1000	>1000	>1000	>1000	<0.98	<1.95	0.49	NR
L. monocytogenes 24AJ3	>1000	250	250	1000	>1000	250	31.25	1000	>1000	>1000	250	<0.98	<1.95	0.49	NR
L. monocytogenes QMAC-1	>1000	250	125	>1000	>1000	500	>1000	>1000	>1000	>1000	>1000	<0.98	3.9	0.49	NR
L. monocytogenes QMAC-7	>1000	62.50	125	>1000	>1000	250	31.25	>1000	>1000	>1000	>1000	<0.98	<1.95	0.98	NR
L. monocytogenes QMG-10	>1000	250	125	>1000	>1000	1000	250	>1000	>1000	>1000	>1000	<0.98	<1.95	0.49	NR
M. luteus ATCC 9341	>1000	250	250	>1000	>1000	>1000	250	>1000	>1000	>1000	>1000	250	31.25	0.98	NR
M. luteus ATCC 10240	>1000	62.50	31.25	>1000	>1000	500	125	125	>1000	>1000	>1000	250	<1.95	<0.24	NR
S. aureus ATCC 25923	>1000	250	125	1000	>1000	1000	125	1000	1000	>1000	1000	125	<1.95	0.49	NR
S. aureus ATCC 29213	>1000	>1000	125	>1000	>1000	1000	62.50	>1000	1000	>1000	1000	<1.95	<1.95	0.49	NR
S. aureus ATCC 6538	>1000	250	250	>1000	>1000	1000	250	>1000	>1000	>1000	>1000	<1.95	<1.95	<0.24	NR
S. aureus DF2B-10-Sa5	>1000	125	125	>1000	>1000	1000	125	1000	1000	>1000	>1000	250	<1.95	0.98	NR

S. aureus DF2B-32-Sa3	>1000	250	250	>1000	>1000	>1000	125	>1000	1000	>1000	>1000	500	<1.95	0.49	NR
S. aureus DF2B-8-Sa3	>1000	125	125	>1000	>1000	500	62.50	1000	>1000	>1000	>1000	500	<1.95	0.49	NR
S. aureus DF2B-9-Sa1	>1000	125	125	>1000	>1000	>1000	250	>1000	1000	>1000	>1000	250	<1.95	0.98	NR
S. aureus DF2A-MC1-Sa1	>1000	250	250	>1000	>1000	1000	62.50	1000	1000	>1000	>1000	250	<1.95	0.98	NR
S. aureus DF2A-MC2-Sa1	>1000	250	250	>1000	>1000	1000	250	1000	1000	>1000	>1000	250	<1.95	0.49	NR
S. aureus DF2A-MC3-Sa3	1000	250	125	>1000	>1000	1000	125	1000	1000	>1000	>1000	250	<1.95	0.49	NR
S. epidermidis ATCC 12228	1000	1000	500	1000	>1000	>1000	>1000	1000	>1000	>1000	1000	250	<1.95	0.98	NR
Gram-negative bacteria															
E. aerogenes ATCC 13048	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
E. cloacae HMA:FTA 502	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
E. coli ATCC 8739	>1000	>1000	>1000	>1000	>1000	>1000	1000	1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
E. coli ATCC 25922	1000	>1000	1000	>1000	>1000	>1000	1000	1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
K. pneumoniae ATCC 700603	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1000	62.5	<1.95	NR	NR
S. enterica subsp. *enterica* sor. *Abony* NCTC 6017	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	7.81	<1.95	NR	NR
S. enterica subsp. *enterica* ser. *Typhi* ATCC 10749	>1000	>1000	>1000	>1000	>1000	>1000	1000	1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
S. enterica subsp. *enterica* ser. *Typhimurium* ATCC 14028	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	500	500	NR	NR
S. enterica subsp. *enterica* ser. *Typhi* ATCC 19430	>1000	>1000	1000	>1000	>1000	>1000	1000	1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
S. enterica subsp. *enterica* ser. *Choleraesuis* ATCC 10708	>1000	>1000	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
S. marcenscens ATCC 14756	>1000	>1000	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	<1.95	<1.95	NR	NR
P. aeruginosa ATCC 27853	1000	>1000	1000	>1000	>1000	>1000	>1000	1000	1000	1000	>1000	<1.95	<1.95	NR	NR
P. aeruginosa ATCC 9027	500	500	500	1000	1000	>1000	>1000	1000	500	1000	500	<1.95	<1.95	NR	NR
Fungi															

C. albicans 63U	>1000	>1000	1000	>1000	>1000	500	1000	1000	1000	>1000	IN	NR	NR	NR	1
C. krusei ATCC 34135	250	>1000	1000	500	>1000	250	>1000	500	125	1000	500	NR	NR	NR	NR
C. parapsilosis ATCC 22019	>1000	>1000	1000	>1000	>1000	500	1000	1000	125	>1000	1000	NR	NR	NR	1
C. parapsilosis 86U	>1000	>1000	1000	>1000	>1000	125	1000	1000	250	>1000	1000	NR	NR	NR	1
C.tropicalis ATCC 28707	250	1000	>1000	125	>1000	62.50	500	125	125	125	62.50	NR	NR	NR	NR
C. neoformans var. *neoformans* ATCC 28957	>1000	125	1000	62.50	>1000	15.62	62.50	31.25	7.81	62.50	31.25	NR	NR	NR	2
C. neoformans var. *gatti* L3	>1000	250	1000	250	>1000	1000	250	250	15.62	1000	500	NR	NR	NR	2
T. mentagrophytes ATCC 11480	500	1000	1000	1000	1000	125	250	1000	500	500	500	NR	NR	NR	NR
T. rubrum ATCC 28189	>1000	>1000	>1000	1000	1000	500	>1000	>1000	1000	1000	1000	NR	NR	NR	NR

Notes: CEE – crude ethanol extract; HF- hexane fraction; DF- dichloromethane fraction; EAF- ethyl acetate fraction; AF - aqueous fraction; Pd: standard; RA: rosmarinic acid standard; - inactive; NR – not realized, GEN - gentamicin; CIP - ciprofloxacin; VAN - vancomycin; IT - itraconazol

4. Discussion

The volatile content found for the HD of *R. officinalis* and *O. vulgare* ranged from 9.18 to 10.75%. Residual water content above 14% promotes the growth and proliferation of microorganisms and enables the hydrolysis of some plant drug components (Bastos et al. 2000). Therefore, the analyzed powder leaves (HD) are within the standards.

The total ash content (TAC) of *R. officinalis* and *O. vulgare* found were, respectively, 5.22% and 10.59%. The *R. officinalis* monograph described in Portuguese Pharmacopeia (2005) shows 9% ash content maximum value, and then the levels found were within the standard. There is no data in the Brazilian Pharmacopeia about *O. vulgare*, so, the obtained results can serve as a comparison standard.

Regarding swelling index (SI), it was found that *O. vulgare* had a greater capacity to swell, which can be explained by the presence of mucilage and gums (Prathyusha et al. 2009), while in *R. officinalis*, only the presence of gums justifies its lower ability to swell (Félix-Silva et al. 2012).

The *R. officinalis* CEE presented physicochemical characteristics with close values: RD 0.964 g/ml ± 0.002, pH 5.106 ± 0.005, SC 9.66 ± 0.07% (% w/w) and AC of 38.2 ± 0.53 (% v/v) (Couto et al. 2012). No data was found in the literature on these parameters for *O. vulgare*. These parameters are important because the extracts should be standardized to generate reproducibly and to ensure their performance in biological activities, also of having adequate quality control (Gosslau et al. 2011).

Flavonoids were measured in HD (3.82 ± 0.03), and CEE (6.18 ± 0.03) of *R. officinalis* and in HD (5.03 ± 0.03), and CEE (5.22 ± 0.01) of *O. vulgare*. Almeida et al. (2010) described flavonoid content in *R. officinalis* ethanol extracts leaves from different places ranging from 0.94 mg/g to 8.66 mg/g. Kaurinovic et al. (2011) related 25.31 mg/g of flavonoid content in an aqueous extract of *O. vulgare* from the Republic of Serbia. Concentrations of flavonoids and phenols depend on the cultivation, maturation, storage, soil salinity, location, the type of the sample extract, and, therefore it is important to standardize the extracts (Almeida et al. 2010; Kaurinovic et al. 2011). The results found in this experiment were lower, possibly due to different methodologies.

The RA was detected in CEE of the *R. officinalis* and *O. vulgare*. Comparing the chromatograms obtained by analysis of the concentrated extract of *R. officinalis* and *O. vulgare* to the RA pattern, the peaks are at the same retention time. Furthermore, it can be seen that the absorption spectra of both standard and samples are identical. It was found that the developed method has adequate to detect and quantify the content of RA. In both levels analyzed coefficients of variation were within the parameters recommended by ANVISA (Brasil 2003).

The method used for the analytical parameters presented robustness regarding the mobile phase composition, the column temperature and flow rate of the mobile phase since the coefficient of variation was less than 5% for any changes to the *R. officinalis* and *O. vulgare*.

The RA content of HD of *R. officinalis* was 1.39% and the CEE was 6.54%. The rosmarinic acid values are in agreement with those described by Machado et al. (2012) to *R. officinalis* (5.99%). Other studies revealed 33 mg/100g of rosmarinic acid in fresh samples and 1286 mg/100g of rosmarinic acid from freeze-dried *R. officinalis* methanol extract (Chan et al. 2012); 8% and 22.6% of RA in methanol extract of *R. officinalis* (Erkan et al. 2018; Do et al., 2014); 1.87% and 6.42% rosmarinic acid in aqueous extract of *R. officinalis* (Afonso et al. 2013; Frescura et al. 2013); 5.74% rosmarinic acid in the dry extract of *R. officinalis* (Couto et al. 2012). The rosmarinic acid has been found in other species of Lamiaceae as the predominant polyphenol compound (Zgórka & Głowniak 2001).

The RA content of HD in *O. vulgare* was 1.22% and CEE was 2.62%. In other studies they verified RA content of 1.11 to 7.42 mg/g in leaves ethanol extract of *O. vulgare* from Lithuania (Radušienė et al. 2008); 0.1-3.5% in aqueous methanol extract of aerial parts

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of *O. vulgare* subsp. hirtum of Denmark (Grevsen et al. 2009); 0.6 mg/g 37.2 mg/g in *O. vulgare* L. subsp. *vulgare* extracts from Austria (Lukas et al. 2013).

Antimicrobial inhibitory activities were observed ranging from good to moderate of HF and DF of *R. officinalis*, CEE, HF, DF, EAF of *O. vulgare* and RA standard, against *Listeria* strains isolated cheese, with the best inhibitory activities observed for fractions: DF of *R. officinalis* and HF of *O. vulgare*. *Listeria monocytogenes* is an opportunistic pathogenic foodborne strain, known to cause high mortality (20-30%) (Carpentier & Cerf 2011; Almeida et al. 2013) commonly found in raw food or food ready for consumption (APC), especially milk and dairy products (medium-high moisture cheeses) and other meat products (Almeida et al. 2013). The invasive form affects a group of people who have a higher susceptibility to the micro-organism, usually immunocompromised patients (severe infections of the central nervous system) and pregnant women, may cause miscarriage, premature birth, meningitis, sepsis, and even death (Carpentier & Cerf 2011; Barancelli et al. 2011).

The HF, DF of *R. officinalis*, the CEE, and HF of *O. vulgare* showed good or moderate inhibitory activity against food isolates of *S. aureus*. This strain is associated with food poisoning due to their ability to produce enterotoxins, causing gastroenteritis or inflammation of the intestinal mucosa (Kadariya et al. 2014).

Studies revealed antimicrobial activity of essential oils from the leaves of *R. officinalis* (MIC = 320-1280 μg/cm^3) against strains isolated from milk and cheese, as *Geobacillus stearotermophillus*, *B. cereus*, *Bacillus subtilis* var. *niger*, *Enterococcus faecium*, *Salmonella enteritidis* e *Escherichia coli* (Žižovic et al. 2009); *E. coli* multidrug-resistant strain isolated from curd cheese (Ribeiro et al. 2013). Bubonja-Sonje et al. (2011) observed good activity (MIC = 0.083 mg/mL) of the polyphenol extract of *R. officinalis* obtained commercially against *L. monocytogenes* strain EGD (serotype 1/2a), four invasive clinical isolates of *L. monocytogenes,* and the virulent *L. innocua.*

The HF, DF of *R. officinalis*, HF of *O. vulgare,* and RA showed good inhibitory activity (MIC 31.25-62.5 μg/mL) against Gram-positive strain ATCC, including the *B. cereus* and *B. subtilis*. The *B. cereus* is often associated with food poisoning, causing serious and fatal gastrointestinal infection, by the production of exoenzymes (Bottone 2010). Despite *B. subtilis* is a "generally recognized as safe" (GRAS) bacteria (Yeo et al. 2012) some of these bacteria are capable of producing a toxic peptide, amylosin associated with outbreaks of food poisoning (Apetroaie-Constantin et al. 2009).

The CEE, HF, DF of *R. officinalis*, the EAF of *O. vulgare*, and the RA pattern showed moderate activity against *P. aeruginosa* ATCC 9027, a microorganism associated with severe infections such as otitis external, endocarditis, meningitis, pneumonia, and septicemia (Bodey et al. 1983).

Bernardes et al. (2010) verified the antibacterial activity of the crude ethanol extract of leaves of *R. officinalis* (MIC 350 to >400 μg/mL), and the mixture of hexane and ethyl acetate fractions of leaves (MIC 70–10 μg/mL) against oral pathogens. Abdel-Massih et al. (2010) described the activity of the crude extract, aqueous fraction and ethyl acetate fraction of *R. officinalis* leaves against *E. coli* and *Klebsiella pneumoniae*. Bayoub et al. (2010) found the antimicrobial activity of ethanol extracts of leaves of *R. officinalis* against *L. monocytogenes*. Silva et al. (2013) observed antimicrobial activity of essential oils from *R. officinalis* leaves against *Staphylococcus epidermidis* ATCC 12228 and several food pathogens such as *Bacillus cereus* ATCC 1247; *Clostridium perfringens* ATCC 1324; *Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 25923.

Scientific studies have shown antimicrobial activity of essential oil from leaves *O. vulgare* against food pathogens when added to fresh sausages (Busatta et al. 2007) and against Gram-positive pathogens such as *B. subtilis* and *B. cereus* (Falco et al 2013). Licina et al. (2013) found antimicrobial activity of aqueous extract of aerial parts of *O. vulgare* against *Bacillus pumilis* NCTC824 and *S. aureus* PMFKGB12 and Martins et al. (2014) verified the activity of the hydroalcoholic extract of aerial parts of *O. vulgare* against *E. coli.*

EAF of *R. officinalis*, CEE, HF, DF, EAF, and AF of *O. vulgare* and RA standard showed good inhibitory activity against *C. neoformans* var. *neoformans* ATCC 28957. In addition, the CEE of *O. vulgare* and the RA pattern showed good inhibitory activity against *Candida tropicalis* ATCC 28707. Cryptococcosis is a disease that usually affects the lungs and the central nervous system and can cause cryptococcal meningitis and usually is associated with people with weakened immune systems, is rare in immunocompetent persons (CDC, 2015). *Candida tropicalis* cause candidiasis and are quite common in tropical countries, very virulent in patients with low levels of

neutrophils (Chai et al. 2010). This may cause candidemia in immunocompromised patients or patients having malignant diseases such as leukemia or even patients involved in organ transplantation (Kontoyiannis et al. 2001).

The RA pattern showed good inhibitory activity against strains of *L. innocua* ATCC 33090 and *C. tropicalis* ATCC 28707. Therefore, a moderate inhibitory activity of the CEE of *R. officinalis* can be explained by their higher RA content (6.5%). Abedini et al. (2013) also found that as well as an extract from the leaves and stems of *Hyptis atrorubens* Poit. (Lamiaceae), RA showed antimicrobial activity, both bactericidal and inhibitory, against eight pathogenic bacteria. They found that RA showed moderate activity (MIC = 300 µg/mL) against *S. epidermidis* 5001, *Stenotrophomonas maltophilia,* and *Enterococcus faecalis* C159-6. Stanojkovic et al. (2013) found that rosmarinic acid showed good inhibitory activity against *L. monocytogenes* NCTC 7973 (MIC = 25 µg/mL) and against clinical isolates of fungi: *C. albicans* (MIC = 50 µg/mL) and *C. krusei* (MIC = 12.5 µg/mL). Gohari et al. (2009) found that the RA isolated from *Hymenocrater calycinus* showed antifungal properties. In a review, Nieto et al (2018) reported the antibacterial activity of *R. officinalis* oil against *E. coli, Bacillus cereus, Staphylococcus aureus*, *Staphylococcus aureus, Clostridium perfringens, Aeromonas hydrophila, Bacillus cereus, and Salmonella choleraesuis*. The inhibitory effect of rosemary is the result of the synergy of various secondary metabolites, like rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol, and isorosmanol. They act at the cellular membrane level, changing the genetic material and nutrients and altering the function of electron transporters; and interaction of membrane proteins, resulting in loss of functionality membrane.

The DF and HF of *R. officinalis* and HF of *O. vulgare* showed equal or better inhibitory activity against some Gram-positive bacteria and food isolates concerning the antibiotic gentamicin and has potential as an antimicrobial agent in the food industry. These fractions have promising potential in the control of food pathogens and they can be used in the future as an alternative natural preservative in the industry.

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