CAPÍTULO 37

ANTIMICROBIAL EFFECT OF *Rosmarinus officinalis* LINN ESSENTIAL OIL ON PATHOGENIC BACTERIA IN VITRO

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RESUMO: O uso de plantas medicinais para doenças infecciosas é uma prática antiga. Dentre essas plantas, as aromáticas são aquelas cujas propriedades químicas vêm sido estudadas recentemente. **Objetivo:** Analisar *in vitro* o efeito antimicrobiano do óleo essencial de *Rosmarinus officinalis*, Linn, contra bactérias gram negativas e gram positivas. **Material e Métodos**: O óleo foi obtido comercialmente da Farmácia de Manipulação (Farmacotécnica). A atividade antimicrobiana do óleo foi avaliada utilizando disco-difusão em ágar (DDA) e placa-ágar com poços (MAPO). Os microrganismos utilizados foram *Staphylococcus aureus* e *Enterococcus faecalis* (gram positivos) e *Escherichia* coli e *Serratia marcescens* (Gram negativos). **Resultados:** O óleo teve ação antimicrobiana contra *S. aureus* e *S. marcescens, E. coli*, sendo que *S. aureus* foi mais sensível à ação do óleo essencial do que *E. coli*, quando testado usando técnica de disco. A amostra de óleo inibiu de forma semelhante a *S. aureus* e *S. marcescens* nos testes usando o método de placa de ágar e não teve efeito sobre *E. faecalis*. Considerando as limitações metodológicas em analisar este óleo essencial da planta em testes *in vitro*, devido às características físico-químicas do óleo e sua instabilidade na técnica de disco-difusão, os resultados obtidos são promissores para novas pesquisas. **Conclusão:** Os resultados mostraram que o óleo de *R. officinalis* Linn pode ser utilizado no tratamento de infecções por *S. aureus*, *S. marcescens* e *E. coli*.
PALAVRAS-CHAVE: alecrim, antimicrobiano, óleo essencial, fitoterapia, *S. aureus*.

ABSTRACT: The use of medicinal plants for infectious diseases is an old practice. Among these plants, the aromatic ones are those whose chemical properties have been studied.

Objective: Analyze *in vitro* the antimicrobial effect of the essential oil of *Rosmarinus officinalis,* Linn, against bacteria gram negative and gram positive. **Materials and Methods:** The oil was obtained commercially from the Pharmacy of Manipulation (Farmacotécnica). The antimicrobial activity of the oil was evaluated using Disk-diffusion in Agar (DDA) and Plate Agar with wells (MAPO). The microrganisms used were *Staphylococcus aureus*) and *Enterococcus faecalis* (gram positive) and *Escherichia coli* and *Serratia marcescens* (Gram negative). **Results:** Oil had antimicrobial action against *S. aureus* and *S. marcescens*, *E. coli,* while *S. aureus* was more sensitive to the essential oil action than *E. coli*, when tested using Disk Technology. Oil sample inhibited similarly to *S. aureus* and *S. marcescens* in the tests using well Plate Agar Method and had no effect on *E. faecalis*. Considering the methodology limitations in analysing this plant essential oil in *in vitro* investigations, due to the physico-chemical characteristics of the oil and its instability in the disc diffusion technique, the results are promising and basic for the new research, which is corroborated by the data found in this study. **Conclusion:** The results showed that *R. officinalis* Linn oil could be used in treatments of infections by *S. aureus*, *S. marcescens* and *E. coli.*
KEYWORDS: rosemary, antimicrobial, essential oil, phytotherapy, *S. aureus*.

1 I INTRODUCTION

The use of medicinal plants for relieving symptoms of colds is an old practice, which is passed on from generation to generation and is currently used as one of the main therapeutic resources for many communities (Moraes et al. et al., 2017; Mendes et al., 2017).

In the early 1990s, the World Health Organization reported that between 60 to 80% of the population in developing countries depended on medicinal plants as the only form of access to health care (Veiga Junior, Pinto, 2005). Among the medicinal plants there has been a focus on the study of the aromatic plants due to their chemical properties. The essential oils of these plants are made up of complex mixtures of volatile and usually lipophilic substances in which a pharmacologically active compound is the major component responsible for the antimicrobial activity against a variety of bacteria and yeasts, including antibiotic resistant species and antifungal agents.

Essential oils are synthesized in several plant species as a by-product of secondary metabolism, and can be extracted from stems, flowers, fruits, and roots. The antimicrobial activity of these substances is associated with the chemical constituents present in their composition, which depends on various factors indispensable to obtain a greater quantity of essential oil with a higher percentage of quality, such as: climatic factors, geographical conditions harvesting (Busatta, 2006; Bertini et al., 2005; Carvalho Filho et al., 2006).

Among the oils with antimicrobial activity, the essential oil of *Rosmarinus officinalis* Linn has been proven to have several therapeutic properties, which fights off certain

pathogenic bacteria that affects man (Valones et al., 2016).

The rosemary is an aromatic plant that is commonly known as the common *R. officinalis* that belongs to the Labiatae family and vegetates in rocky and sandy terraces on the Mediterranean coast. It has been adapted to grow in Brazil especially in gardens that can be up to 1,500 meters high (Marchiori, 2004). The main characteristics of this plant are: linear leaves, coriaceous, green, tubular and with a strong and pleasant aroma and it reaches up to 1.50 m (Genena et al., 2008) as shown in Figure 1. It has in its composition terpenes, terpineol, sesquiterpenes, and cineol (Upadhyay, 2010; Mondello et al., 2003).

Figure 1: *R. officinalis* Linn.

The essential oil of *R. officinalis* Linn is obtained most often by the method of hydro distillation of the leaves, although it has disadvantage of undesirable substances produced due to the high temperature. Other methods may also be employed, such as: extraction with organic solvents and supercritical fluid technique. After the extraction of the oils, chromatographic techniques are implemented to analyze and identify the components found. In this case, the gas chromatography is the most used technique (Rodrigues et al., 2004).

The chemical composition of the essential oil of *R. officinalis* is regulated by an international Australian standard (ISO 4730: 2004) which specifies a minimum and/or maximum concentration of 14 components of its essential oil. According to the Australian committee, *R. officinalis* Linn oil should contain at least 30% terpinen-4-ol and at most 15% 1,8-cineole. These levels of cineol are able to irritate skin irritant, whereas terpinen-4-ol is the main contributor to its antimicrobial activity (Garcia et al., 2009; Pereira et al., 2009).

Staphylococcus aureus is a gram-positive, immobile coccus bacterium, measuring from 0.5 to 1.0 micrometers, facultative anaerobes, mesophiles, with an optimal growth temperature of 30 to 37 ºC (Bresolin, Dall'Stella, Fontoura-da-Silva, 2005).

These bacteria are part of the human microbiota that can cause diseases ranging from a simple infection, such as pimples and boils, to a more critical condition such as pneumonia, meningitis, endocarditis, toxic shock syndrome and septicemia, among others (Santos et al., 2007). *S. aureus* was one of the first bacteria to effectively be controlled with the discovery of antibiotics, however, due to its enormous capacity for adaptation and resistance, it has become one of the most infectious bacteria in hospitals and communities (Aguayo-Reyes et al., 2018).

Enterococcus sp. are classified as gram positive cocci, and are visualized as individual cells, or arranged in pairs, or in chains, with a commonly ovoid morphology. They are facultative anaerobic bacteria that grow at temperatures from 10 to 45 °C, while the optimum temperature is 35 °C (Lépesová et al., 2018).

These opportunistic pathogens are known to cause nosocomial infections, especially endocarditis and urinary tract infections and surgical wounds (Bello Gonzalez et al., 2017). Therapy for these infections has become limited because Enterococci have acquired resistance to several antimicrobials, such as ampicillin, aminoglycosides, vancomycin and teicoplanin glycopeptides (Bonten, Willems, Weinstein, 2001; Rice et al., 2003).

In the last two decades, Enterococcus sp. (Spinardi et al., 2017), and treatment difficulties due to their rapid resistance to commonly used antimicrobials such as aminoglycosides, aztreonam, cephalosporins, clindamycin and oxacillin (ANVISA, 2013).

Escherichia coli is gram-negative bacterium that belongs to the family Enterobacteriaceae, and are facultative anaerobic microorganisms. Most *E. coli* serogroups are part of the mammalian intestinal flora that exerts beneficial effects on the human organism such as suppressing the multiplication of harmful bacteria, and synthesizing a considerable amount of vitamins. However, certain serotypes are pathogenic to man and other animals, and are not part of the normal intestinal flora, called enteropathogens (Lépesová et al., 2018).

Serratia sp. belong to the genus of gram negative bacteria, facultative anaerobic, of the family Enterobacteriaceae. These enterobacteria are important contaminants of soil, water and vegetables (Akoachere, Tatsinkou, Nkengfack, 2018). These microorganisms also inhabit the intestine of vertebrate animals, including human (Kim et al., 2015).

Serratia sp are considered opportunistic bacteria, and are usually associated with nosocomial infections. The species most commonly isolated in clinical samples is the *S. marcescens*, which is considered the most important member of this genus. This type of species is responsible for a large percentage of hospital infections and is associated with a variety of human infections, particularly in the bloodstream, respiratory and urinary tract, and septicemia (Vahedi-Shahandashti et al., 2017). This pathogen also has a high level of resistance to antimicrobials due to a long life cycle for long periods in the hospital environment. In addition, other pathogenic species of rarer occurrence are known in the genus: *S. plymuthica, S. liquefaciens, S. rubidaea, S. odorífera* and

S. fonticola (Menezes et al., 2004).

The information from the literature are inconclusive on the antimicrobial action of *R. officinalis* Linn essential oil on *E. coli* and *Staphylococcus aureus* species, whereas some authors have stated that this oil does present bactericidal action for these species, others have observed it has no effect. These various opinions show the necessity for more in depth research to verify how this oil interacts on these species of bacteria of medical interest (Nascimento et al., 2007; Castro, Lima 2011).

The aim of this study was to evaluate *in vitro* the antimicrobial effect of the essential oil obtained from *Rosmarinus officinalis* Linn on gram positive bacteria: *Staphylococcus aureus* and *Enterococcus faecalis*, and gram-negative: *Escherichia coli* and *Serratia marcescens*.

2 I MATERIAL AND METHODS

Assays were carried out at the Laboratory of Microbiology in the Faculdade Anhanguera de Brasília (FAB).

2.1 Essential Oil

The essential oil (100%) of *R. officinalis* Linn was obtained by the Pharmacotechnical Manipulation Pharmacy Terra Flor (Yanih Cosméticos, Farmacêuticos LTDA).

2.2 Antibacterial assay

The experiments were performed using the techniques: Disk-diffusion in Agar (DDA) and Agar in Plate with Orifice (MAPO) as described in the literature (Fio Cruz / INCQS, 1992).

2.2.1 Microorganisms and inoculum

Gram-positive standard bacteria used in the experiment were: *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 51299), and gram negative bacteria were: *Escherichia coli* (ATCC 25922) and *Serratia marcescens* (BF-A 272). Bacteria were placed on Mueller-Hinton Agar plate and incubated at 36 °C for 18 to 24 h.

2.2.2 In vitro susceptibility testing using the Agar-Diffusion Technique (DDA)

The antimicrobial activity of *R. officinalis* Linn essential oil was evaluated using the disc diffusion technique according to the standards of the National Committee for Clinical Laboratory Standards (NCCLS). After the cultivated bacterial growth, bacterial colonies were selected and seeded in a culture tube containing 5 mL of Trypticasein Soy Broth (TSB) medium and incubated at 36 °C for 24 h. The turbidity acquired by the broth was measured using a spectrophotometer at a wavelength of 600 nm. Approximately $0.15x10^8$ colony forming units (CFU) were seeded in petri dishes with Mueller-Hinton Agar.

After drying the inoculum, sterile filter paper disks of approximately 6 mm in

diameter, impregnated with volumes of 1, 3, 5 and 10 µL of the pure *R. officinalis* Linn essential oil were applied without any dilution. The control test was performed using antibiotic disks in the concentration of 15 µg erythromycin (ERI) from Hyolabor Laboratory, 30µg tetracycline (TET) from Tauto Laboratory, and 30 µg amikacin (AMI) from Tauto Laboratory, available commercially. Discs of 6 mm in diameter were used as negative controls without any antibiotics. Cultures were incubated at 36 °C for 24 and 48 h. The results were expressed by measuring the diameter of the inhibition areas in millimeters. All assays were performed in triplicate.

2.2.3 In vitro susceptibility test using Well Plate Agar Technique (MAPO) Well diffusion test

The well diffusion test was performed with adaptations. It differed from the disk test, having three holes of 6 mm in diameter on the agar plates MHA in Petri dishes. The plates were inoculated on the surface by the microorganisms with a swab, and then the wells were filled with different volumes of the samples (ranging from 1 to 10 µL). The plates with Muller Hinton agar were prepared and maintained at 4 °C until the moment of use, in which were made wells with 6 mm in diameter. Using a sterile Swab, a bacterial inoculum with the turbidity of 0.5 of the MacFarland scale was evenly distributed over the surface of the agar then incubated at room temperature. After 5 min, 50 µL of essential oil of *Rosmarinus officinalis* Linn was placed in each well. At the same time plate was prepared, in a similar way as described, but in the wells placed the positive and negative controls. Negative controls used 50 µL sterile distilled water and positive antibiotic disks. Petri plates were incubated at 37 °C for 24 h. The growth inhibition hole was measured in millimeters (Laborclin, 2011).

3 I RESULTS

The results obtained using the pure essential oil of *R. officinalis* Linn, and the antibiotics used as positive controls for each bacterium, using the agar-diffusion technique in Agar are shown in Table 1. Inhibition zones were measured in mm per diameter total for both positive controls and treatments with pure essential oil. The diameters of inhibitory zone were formed in the positive controls using the antibiotics were: amikacin (20 mm), erythromycin (15 mm), and 24 mm of tetracycline (Table 1). Negative controls as expected did not form a zone in both diffusion disc tests and well tests.

Sample (Volume)		Inhibition zone (mm)			
		S. aureus (gram positive)	*E. faecalis* (gram positive)	*S. marcescens* (gram negative)	*E. coli* (gram negative)
Essential oil	1 µL	2.67	—	15.00	10.00
	3 µL	10.35	—	15.00	10.00
	5 µL	16.42	—	16.00	12.00
	10 µL	19.28	—	19.00	16.00
Antibiotic	Amikacin (AMI) 30 µg	20.00	—	—	—
	Erytromycin (ERI) 15 µg	—	38.00	—	—
	Tetracycline (TET) 30 µg	—	—	21.00	30.00

Table 1: Antibacterial activity of the essential oil of Rosmarinus officinalis Linn pure, using the Disk-Diffusion technique in Mueller-Hinton Agar.

The results obtained using the DDA showed that in the 1 µL of the essential oil did not inhibit growth of any of the bacteria tested. With increasing concentration of 3, 5, and 10 µL of essential oil there was inhibition of the growth of gram positive bacteria *S. aureus*, but without bactericidal activity on *E. faecalis*. The antibacterial action of the oil on the negative bacteria, only in the dilution of 10 µL there was small inhibition on *S. marcescens*, but without effect on *E. coli*. All inhibition zones were compared to the positive controls (Table 1) showed in Figure 2 (A, B, C and D) and Figure 3 (A, B, C, and D). As observed, the essential oil of *R. officinalis* Linn satisfactorily inhibited the growth of the tested microorganisms, being able to be used in the control of *S. aureus*. However, *S. marcescens* and *E. coli* were more resistant to the action of rosemary essential oil. This fact is confirmed in crop photos (Figure 2: A, B, C and D; Figure 3: A, B, C and D).

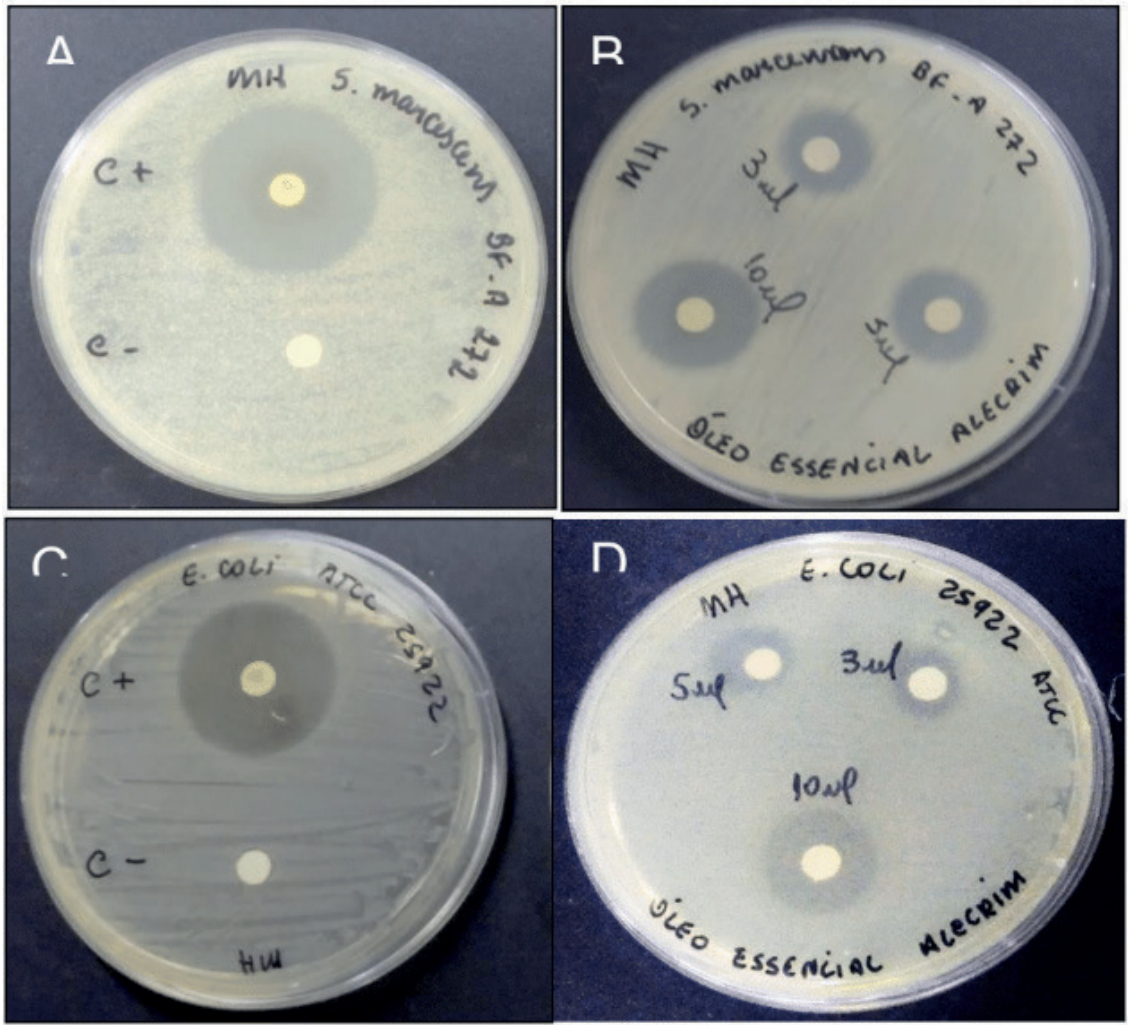

Figure 2: A and C positive and negative controls for *Serratia marcescens* and *Escherichia coli* showing inhibition with Tetracycline (A and B). In B and D treatment of bacteria *S. marcescens* and *E. coli* with essential oil of *R. officinalis*, pure Linn at the dilutions of 3, 5 and 10 µL, using the Disk-Diffusion technique in Muller-Hinton Agar.

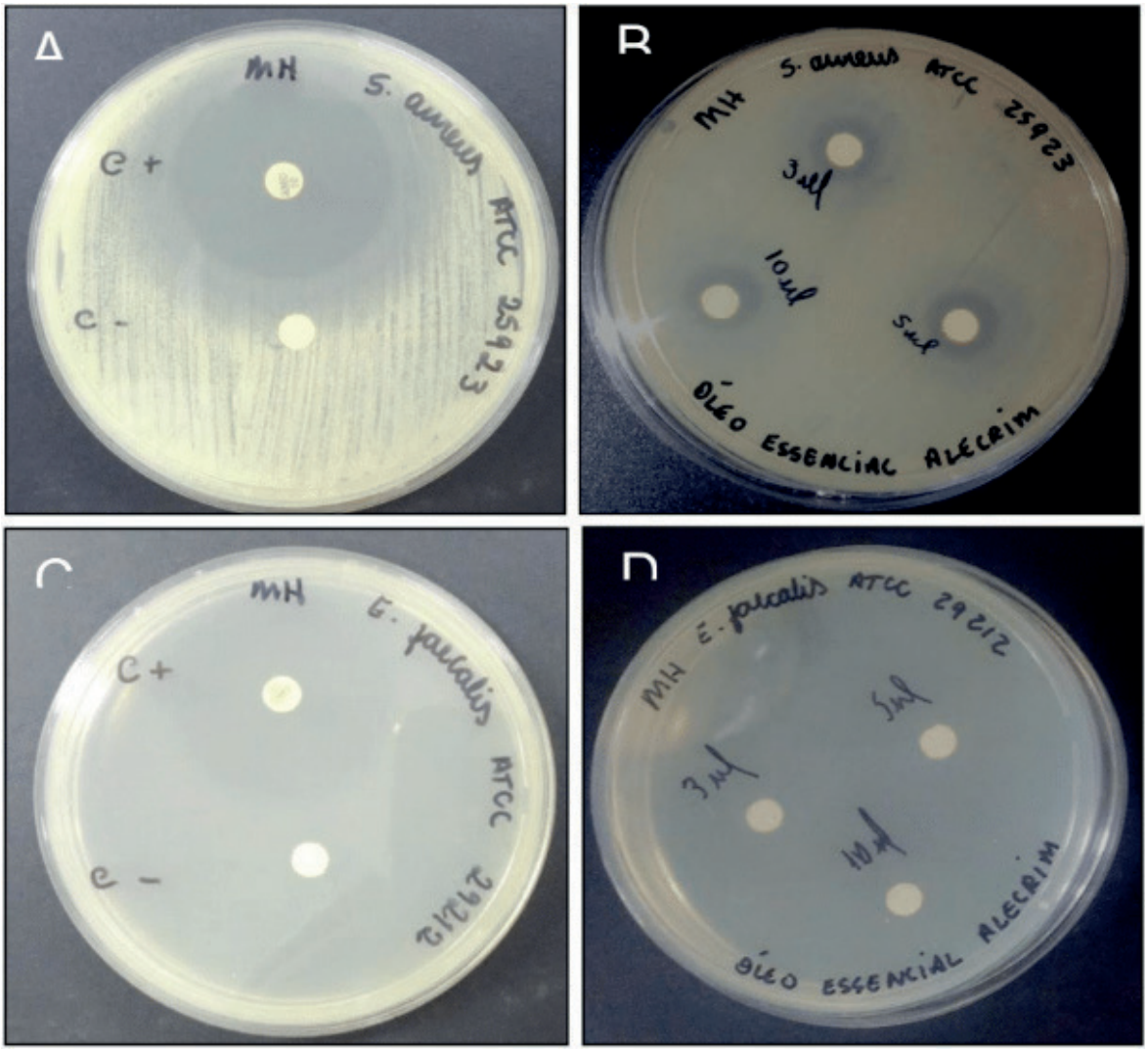

Figure 3: A and C positive and negative controls for *Staphylococcus aureus* and *Enterococcus faecalis* showing inhibition with the antibiotic Amicacin (A) and Erythromycin (C). In B and D treatment of bacteria *S. aureus* and *E. faecalis* with essential oil of *R. officinalis*, pure Linn at the dilutions of 3, 5 and 10 µL, using the Mueller-Hinton Agar-Diffusion technique.

In Table 2 the data determined using the MAPO method confirmed the susceptibility of *S. aureus* and *S. marcescens* on growing when treated with essential oil of *R. officinalis* Linn, comparing to the positive controls (Figure 4: A, B, C, and D; Figure 5: A, B, C, and D).

Sample (Volume)		Inhibition zone (mm)			
		S. aureus	*E. faecalis*	*S. marcescens*	*E. coli*
	50 µL	14.50	__	15.00	__
Essential oil	50 µL	15.00	__	15.00	__
(Volume)	50 µL	15.00	__	17.00	__

Antibiotics	Amikacin (AMI) 30 µg	20.00	—	—	—
	Erytromycin (ERI) 15 µg	—	38.00	—	—
	Tetracycline (TET) 30 µg	—	—	21.00	30.00

Table 2: Antibacterial activity of *Rosmarinus officinalis* Linn essential oil using the Orifice Plate Agar Method (MAPO) using Mueller-Hinton Agar.

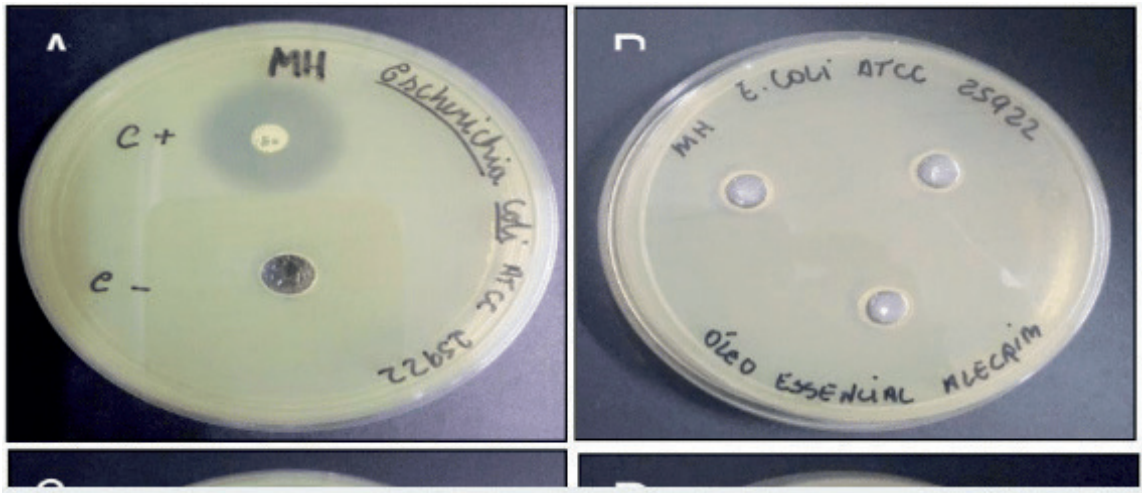

Figure 4: A and C positive and negative controls for Staphylococcus aureus and Enterococcus faecalis showing inhibition with Amikacin (A) and Erythromycin (C). In B and D treatment of bacteria S. aureus and E. faecalis with essential oil of R. officinalis, pure Linn at the dilutions of 3, 5 and 10 µL, using the Platelet Agar Method (MAPO) with Mueller- Hinton.

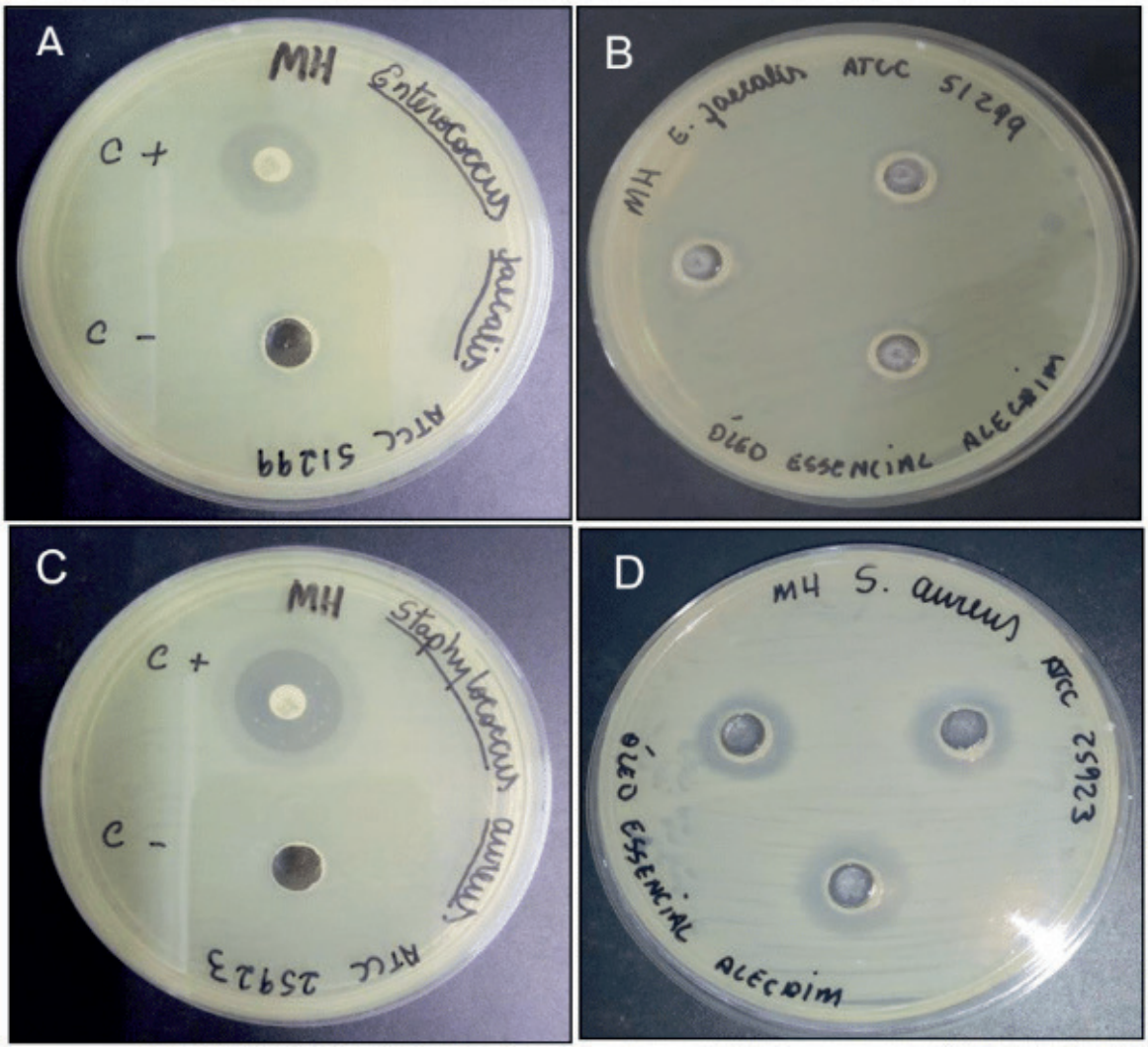

Figure 5: A and C positive and negative controls for *Staphylococcus aureus* and *Enterococcus faecalis* showing inhibition with the antibiotic Amikacin (A) and Erythromycin (C). In B and D treatment of bacteria *S. aureus* and *E. faecalis* with essential oil of *R. officinalis*, pure Linn at dilutions of 3, 5 and 10 µL, using the Platelet.

The difference determined in this investigation, using the two methodologies DDA and MAPO, is due to the different concentrations of the essential oil used (Table 1 and 2). Possibly these findings are related to the physical and chemical properties of the essential oil, mainly due to the solubility and diffusibility in the disc diffusion tests.

4 I DISCUSSION

The use of solvents, detergents or emulsifying agents to facilitate the dispersion of essential oils using disks or impregnated wells has been increasingly used to obtain essential oils. However, these solvents are removed by evaporation after extraction and do not impair the antimicrobial activity (Nascimento et al., 2007). According to the quality control of the Laborclin Manual for disk diffusion antibiogram following the technique of Kirby and Bauer, the values obtained in this study (Laborclin, 2011) of the area of inhibition exerted by the antibiotics used as positive control are within the normal

standard (Castro, Lima 2011). Other studies using the essential oil of *R. officinalis* Linn determined the inhibition of growth of different species of bacteria and fungi (Lima, Souza, Lima, 2006).

Packer, Luz (2007) investigating the effect of *R. officinalis* Linn essencial oil against strains of *S. aureus* and *E. coli* determined inhibition zones of 10 to 60 mm of diameter around the bacterial growth. These data showed that, in spite of the differences between the techniques, the antimicrobial activity was higher for *S. aureus*, demonstrating agreement with the present study. These data showed that despite the differences between the techniques, the antimicrobial activity was higher for *S. aureus*, demonstrating agreement with the present study. The results obtained in our experiments suggest that gram-negative bacteria have less sensitivity than gram-positive to this medicinal oil. However, experiments with *R. officinalis* essential oils had a significant effect on the control of gram-positive bacteria such as *S. aureus* (Haida et al., 2007).

Other reports about using essential oil of *R. officinalis* as antibiotic test observed that action was more effective against bacteria of the genus gram positive. Gram-negative bacteria are present in the cell structure of an outer membrane that protects the cell wall, which hinders the action and diffusion of hydrophobic compounds from the external wall of bacteria. The mechanism in which essential oils exert an inhibitory effect on microorganisms is not yet clear, however, it is proposed that liposoluble compounds play a large role in the mechanism of bacterial cell rupture. Thus, their constituents probably interact with cell structures that exhibit lipophilic affinities (Nascimento et al., 2007).

Each essential oil has different chemical components, so they have specific interactions and abilities to break or penetrate the bacterial wall structure, preventing its replication and growth. Therefore, it is very important that new studies should be carried out in order to isolate the components of the oils and to test them separately, to verify which ones have inhibitory effects of gram positive and negative bacteria (Ribeiro et al., 2012).

The antifungal activity of the essential oil of *R. officinalis* Linn was observed from the 0.25% concentrations of the essential oil, being able to alter the permeability and the fluidity of the membrane of *Candia albicans*. This effect would be responsible for causing an electrolytic imbalance and consequently death of the fungus (Lima, Souza, Lima 2006; Hammer, Carson, Riley 2004).

Phongpaichit et al. (2004) observed a collapse and denaturation of the fungus when studying fungi exposed to medicinal plants extracts, analyzed using electron microscopy. These findings suggest that similar mechanisms might occur with other plant substances, such as oils, on other pathogenic microorganisms such as bacteria.

Antimicrobial activity of *R. officinalis* Linn essential oil was evaluated in rats infected with strains of *S. aureus* sensitive and resistant to the antibiotic penicillin. The results showed that the essential oil presented antimicrobial activity *in vitro* and *in vivo*

on penicillin-sensitive strains and inhibited the growth of penicillin-resistant strains in vitro (Simoes et al., 2002). These results corroborate with those found in our research, showing the effect of the essential oil of *R. officinalis* Linn in the control of *S. aureus* infections.

5 I CONCLUSIONS

The essential oil of *R. officinalis* Linn has antimicrobial activity for gram positive *S. aureus* bacteria, and for gram negative bacteria: *S. marcescens*, *E. coli*, *S. aureus* being more sensitive to the action of essential oil than to *E. coli*, when tested using the Disk-Diffusion Technique, although with no effect against *E. faecalis* growth. The oil inhibited similarly to *S. aureus* and *S. marcescens* when tests were performed using Orifice Plate Agar Method, and had no effect on *E. faecalis* and *E. coli*. It was also observed that the results, regarding to the sensitivity of the antimicrobial effect, differed in the tests evaluated. Therefore, the sensitivity response of the antimicrobial action depends on the test adopted. One of the difficulties in antimicrobial tests *in vitro*, using the essential as medicinal plant, is due to the physicochemical characteristics of the oil. In spite of instability of the disc-diffusion technique, the results are promising and fundamental for further research. Thus, it is suggested that this substance could be used in treatments of human infections caused by *S. aureus*, *S. marcescens* and *E. coli*.

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