

Antibacterial activity of the essential oil of *Origanum vulgare* L. (Lamiaceae) against bacterial multiresistant strains isolated from nosocomial patients

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RESUMO: “Atividade antibacteriana do óleo essencial de *Origanum vulgare* L. (Lamiaceae) contra bactérias multirresistentes isoladas de pacientes nosocomial”. Os antibióticos permanecem como a principal opção terapêutica para tratar infecções bacterianas, no entanto, existe a desvantagem de aumentarem a resistência bacteriana, e como alternativa, destaca-se a pesquisa de antimicrobianos de origem vegetal. Neste trabalho objetivou-se determinar *in vitro* a atividade antibacteriana do óleo essencial de *Origanum vulgare* L. (Lamiaceae) (orégano), sobre bactérias multirresistentes isoladas de materiais biológicos. Foram usadas 24 linhagens de bactérias de origem hospitalar, divididas em seis espécies distintas, que foram inibidas pelo óleo essencial no “screening” preliminar, realizado utilizando-se a técnica de difusão em ágar. A CIM foi determinada pelo método de microdiluição, partindo-se de soluções com as concentrações finais: 8 até 0,125% com os seguintes resultados: As quatro amostras (100%) de *Escherichia coli*, *Enterococcus faecalis* e MRSA foram inibidas pelo óleo essencial na concentração de 0,125%. Três amostras (75%) de *Acinetobacter baumannii* por 0,125% e uma amostra (25%) por 0,5%; *Klebsiella pneumoniae* (75%) por 0,125% e 25% por 0,25%; *Pseudomonas aeruginosa* (75%) por 0,5% e 25% por 0,25%. A CIM variou de 78 a 83%. Concluiu-se com base nos dados obtidos, que não houve diferença na concentração bactericida mínima (0,5%) do referido óleo tanto para os microrganismos Gram positivos quanto para os Gram negativos.

Unitermos: *Origanum vulgare*, infecções bacterianas, bactérias multirresistentes.

ABSTRACT: Antibiotics are considered the main therapeutic option to treat bacterial infections; however, there is the disadvantage of increasing bacterial resistance. Thus, the research of antimicrobials of plant origin has been an important alternative. This work aimed at determining the *in vitro* antibacterial activity of the essential oil of *Origanum vulgare* L. (Lamiaceae) on multiresistant bacteria isolated from biological materials. 24 strains of nosocomial bacteria were used and divided into six different species that were inhibited by the essential oil in the preliminary “screening” which was accomplished by the diffusion technique in agar. MIC was determined by the microdilution method, beginning with solutions with the final concentrations: 8 up to 0.125% with the following results: The four samples (100%) of *Escherichia coli*, *Enterococcus faecalis* and MRSA were inhibited by the essential oil at the concentration of 0.125%. Three samples (75%) of *Acinetobacter baumannii* at 0.125% and a sample (25%) at 0.5%; *Klebsiella pneumoniae* (75%) at 0.125% and 25% at 0.25%; *Pseudomonas aeruginosa* (75%) at 0.5% and 25% at 0.25%. MIC varied from 78 to 83%. It was concluded through the obtained data that there was not difference in the minimum bactericidal concentration (0.5%) of the referred oil for Gram positive as well for Gram negative microorganisms.

Keywords: *Origanum vulgare*, bacterial infections, multiresistant bacteria.

INTRODUCTION

The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases. The bacterial resistance acquired against antibiotics, becoming it difficult to fight them, mainly, when they are isolated using biological material collected from hospitalized patients. In these cases the colonization of the skin is, initially, constituted with normal microbiota, and when it is injured contributes

for the bacterial multiplication. Therefore, it is verified that, after the five first days of internment of the patient this biota is substituted by Gram negative bacteria and *Staphylococcus* strains present in the nosocomial environment, with the possibility of the presence of infections caused by some species of fungi and viruses (Martins, 2001). The most important pathogens involved in nosocomial infections, including of great burnt patients are: *Staphylococcus aureus* methicillin resistant, *Enterococcus* and multiresistant *Pseudomonas aeruginosa*, that constitute a great problem in units for

burnt treatment, increasing the etiology of cutaneous infections and eventually septicemia in patients with severe infections (Khardori & Kanchanapoom, 2002).

In spite of the advances introduced in the attendance to the burnings victims, factors responsible now by the significant surviving and total rehabilitation, occurred in the majority of the patients, the infections remain as the main complication, with as consequence the prolongation of the nosocomial stay. However, the occurrence of multiresistant bacterial strains constitutes an additional problem by the difficulty on choosing a correct antimicrobial therapy, with effective support by the microbiology laboratory (Fernandes & Ribeiro Filho, 2000).

The microorganisms come acquiring multiresistance to antibiotics, and this fact conduct the clinical treatment using each time more the therapeutic properties of determined plants used for fighting the infections (White, 1970). In this way, and considering their pharmacological properties, different plants are studied and used for treatment of diverse types of illnesses (Thylstrup & Fejerskov, 1995). Thus, the fact of the microorganisms acquire quickly resistance to antibiotics, justify the investment, each bigger time in natural products, and the scientists are always alert in the search of new drugs, from the vegetal species, with the objective of to fight the multiresistant microorganisms (Oliveira et al., 2007; Silva et al., 2007; Coutinho et al., 2008).

Considering this premise, the study of the antibacterial action of the essential oil of *Origanum vulgare* against strains of multiresistant bacteria, from nosocomial origin and isolated from clinical materials from diverse anatomical sites in the body was motivated.

Since ancient ages essential oils (EOs) and other extracts of plants have evoked interest as sources of natural products, they are also called volatile are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, herbs, wood, fruits and roots). They can be obtained by expression, fermentation, or extraction but the method of steam distillation is most commonly used for commercial production of EOs (Van de Braak & Leijten, 1999).

It has long been recognised that some EOs have antimicrobial properties (Boyle, 1955; Nascimento et al., 2007; Costa et al., 2008) and these have been reviewed in the past like the antimicrobial properties of spices. However, the relatively recent enhancement of interest in 'green' consumerism has lead to a renewal of scientific interest in these substances (Nychas, 1995; Tuley de Silva, 1996).

Origanum vulgare is a very versatile plant and although it has been known for a long time, as a popular medicine, only now is it starting to be recognised for its potential therapeutic role such as diaphoretic, carminative, antispasmodic, antiseptic and tonic properties. Although it has a wide spectrum of antimicrobial activity, which

has been the subject of several *in vitro* and *in vivo* investigations, there is a lack of knowledge on its effectiveness against multiresistant bacteria isolated from nosocomial patients.

The aim of the present study was to evaluate the susceptibility of multiresistant bacteria isolates, obtained from patients attended at a burnt unit in an emergency hospital, to *Origanum* essential oil and its major components carvacrol and thymol.

In this way our objective was: a) to determine the bacterial etiology of infections in patients taken care of in a emergency and trauma hospital; b) to identify the incidence of multiresistant pathogens as etiologic agents of the infectious processes; and c) to observe "*in vitro*" the antibacterial activity of the EOs, on isolated bacteria from clinical samples, determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of this composition.

MATERIAL AND METHODS

Clinical samples (170) obtained from patients attended at the Hospital de Emergência e Trauma Senador Humberto Lucena (HETSHL) were used. Reference hospital for its specialty and located in the urban area of João Pessoa- State of Paraíba, Brazil and classified by the Brazilian System of Health (SUS) for attendance of urgency procedures. The biological materials was sampled from diverse anatomical small sites and processed for bacterial isolation in the Clinical Microbiology Laboratory in the Health Center Sciences at the Universidade Federal da Paraíba. The clinical samples are described on Table 1.

Processing and identification

The samples had been inoculated in the plates of Agar Blood, Manitol Salt Agar and MacConkey Agar (Difco), in order for the development of different microorganisms. It was prioritized, the isolation of *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, considered as the main multiresistant pathogens. The bacterial identification was carried through standardized routine methods, with the characterization of Gram positive cocci and species of Enterobacteriaceae e Gram Negative Not Fermenters Rods (GNFR), as established in routine techniques (Koneman et al., 2001; Santos Filho, 2003).

Test of susceptibility to antimicrobials

The susceptibility tests were performed by the disc diffusion method (Bauer et al., 1966) using the drugs recommended by the NCCLS (2004): ampicillin (AMI), cefepime (CPM), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), penicillin (PEN), ampicillin

(AMP), ampicillin-sulbactam (SAM), amoxicillina-clavulanic acid (AMC), gentamicyn (GEN), cefotaxime (CFO), ceftriaxon (CFT), aztreonam (ATZ), imipenem (IMI), meropenem (MER) piperacillin (PIP), piperacillin-tazobactam (PTZ), ticarcilin-clavulanic acid (TIM), oxacillin (OXA), vancomycin (VAN)

Characterization of the samples

The bacteria that shown profile of resistance, with the following characteristics was used for the study of the antimicrobial activity of the essential oil: methicillin resistant *S. aureus* (MRSA), *E. faecalis*, *K. pneumoniae* and *E. coli* extended spectrum beta-lactamases producers (ESBL), strains *Pseudomonas aeruginosa* and *Acinetobacter baumannii* resistant to ceftazidime and Carbapenems.

Samples of *S. aureus* oxacillin-resistant (MRSA) had been confirmed using E-test® for determination of MIC and the confirmation of the resistance (Araújo et al., 2000). Samples of *K. pneumoniae* and *E. coli* producers of extended spectrum beta-lactamases (ESBL) had been confirmed using the criteria recommended by the NCCLS (2004), in which the values for the sizes of inhibition zones are established, with the test of diffusion with discs, for the presumptive selection of samples potentially producers of ESBL. Strains presenting this behavior were submitted to the confirmatory tests (Honorio et al., 2001).

Bacterial stains

From the 170 bacterial isolates, 24 bacterial strains divided into six groups of different species were selected for this study, in the following sequence: *S. aureus* (04), *E. faecalis* (04), *K. pneumoniae* (04), *E. coli* (04), *P. aeruginosa* (04) and *A. baumannii* (04). After the preliminary isolation in the identification routine scheme the strains were identified and kept in Nutrient Agar (Biobras) until its use in posterior tests.

Essential oil

The *Origanum vulgare* essential oil was supplied by the company FERQUIMA - Industry and Commerce Ltda (Vargem Grande, São Paulo, Brazil). This provider produces and commercializes essential oils and its quality parameters (appearance, color, purity, odor, density -20 °C, refraction index -20 °C) were described in an accompanying technical report. The procedure of its extraction was carried through the method of hydro distillation in industrial scale.

Screening of the evaluation of the antibacterial activity of essential oils

Initially, saline solution was prepared with bacterial strains suspensions in assay tubes with 5 mL

of sterile saline solution (NaCl 0.85% p/v). After that, such suspensions were agitated during two minutes with in a Vortex. After agitation, each suspension had its comparative turbidity adjusted to that presented by the tube 0.5 of the McFarland scale, which corresponds approximately to an inoculum of 10⁸ UFC/mL for bacteria (Chin Lu, 1971; Tavares, 1984; Drutz, 1987; Cleeland & Squires, 1991).

Following with aid of a sterile swab absorbed with the prepared bacterial suspension, all surface of a Mueller Hinton (Difco) agar plate was inoculated and paper filter discs absorbed with 0.02 mL of the products submitted to analysis were used. Finally, the plate was incubated 35-37 °C during 18 the 24 hours (Bauer et al., 1966; Chin Lu, 1971; McGinnis, 1980; Vincent & Vincent, 1994). It was considered as positive for antibacterial activity the cases where the product applied on the plates inoculated with the bacterial suspension made formation of inhibition area equal or superior to 10 mm of diameter around the disc (Naqui et al., 1991; Lima et al., 1993; Cole, 1994; Alves et al., 2000; Souza et al., 2005).

Determination of the minimum inhibitory concentration (MIC) using the microdilution technique

The MIC of the essential oil was determined against 24 bacterial strains isolated from clinical samples. It was established varied concentrations of the essential oil in the following scheme: in a sterile assay tube 3.2 mL of the essential oil were placed, added with 0.04 mL of TWEEN 80 and q.s.p. 5 mL with distilled water, and agitated during two minutes in a Vortex. Following a serial dilution, each sterile tube with 2.5 mL of sterile distilled water was added of 2.5 mL of previous dilution and agitated again during two minutes (Allegrini & Simeon, 1972). With this sequence of procedures, the final obtained concentrations correspond to 8, 4, 2, 1, 0.5, 0.25 and 0.125 dilutions.

The determination of the MIC of the *O. vulgare* EOs was carried through the microdilution technique using sterilized microplates with 96 (12 x 08) wells with deep flat and proper cover, where was placed 100µL of the essential oil solution. After that, 100 µL of nutrient broth doubly concentrated were added and inoculated with the assayed microorganism. In the eighth line of the microdilution plate, 200 µL of the broth inoculated with the microbial suspension (positive control of the assayed strain viability) were placed, in this way that the first sequence of wells presented the highest concentration of the essential oil, and the seventh line, presents it lesser concentration of the studied oil. The system was incubated at 35-37 °C during 24 hours (Viljoen et al., 2003; Sahin et al., 2004).

For the reading of the microdilution assay the colorimetric method was used, which consists of the

Table 1. Clinical samples used for isolating the microorganisms.

Material	Number	%
Urine	08	33.33
Blood	04	16.67
Brochoalveolar wash	04	16.67
Tracheal secretion	02	8.33
Secretion of righ leg	02	8.33
Others	04	16.67
Total	24	100.00

Table 2. Determination of the Minimal Inibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the *Origanum vulgare* essential oil against different bacteria in broth medium.

Microorganismos	Essential oil (%) (CIM)							Essential oil (%) (CBM)		
	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.5%	0.25%	0.125%
<i>A. baumannii</i> 01	-	-	-	-	-	-	-	-	+	+
<i>A. baumannii</i> 02	-	-	-	-	-	-	-	-	+	+
<i>A. baumannii</i> 03	-	-	-	-	-	-	-	-	+	+
<i>A. baumannii</i> 04	-	-	-	-	-	+	+	-	+	+
<i>E. coli</i> 01	-	-	-	-	-	-	-	-	+	+
<i>E. coli</i> 02	-	-	-	-	-	-	-	-	+	+
<i>E. coli</i> 03	-	-	-	-	-	-	-	-	+	+
<i>E. coli</i> 04	-	-	-	-	-	-	-	-	+	+
<i>E. faecalis</i> 01	-	-	-	-	-	-	-	-	+	+
<i>E. faecalis</i> 02	-	-	-	-	-	-	-	-	+	+
<i>E. faecalis</i> 03	-	-	-	-	-	-	-	-	+	+
<i>E. faecalis</i> 04	-	-	-	-	-	-	-	-	+	+
<i>K. pneumoniae</i> 01	-	-	-	-	-	-	-	-	+	+
<i>K. pneumoniae</i> 02	-	-	-	-	-	-	-	-	+	+
<i>K. pneumoniae</i> 03	-	-	-	-	-	-	+	-	+	+
<i>K. pneumoniae</i> 04	-	-	-	-	-	-	-	-	+	+
<i>P. aeruginosa</i> 01	-	-	-	-	-	+	+	-	+	+
<i>P. aeruginosa</i> 02	-	-	-	-	-	+	+	-	+	+
<i>P. aeruginosa</i> 03	-	-	-	-	-	+	+	-	+	+
<i>P. aeruginosa</i> 04	-	-	-	-	-	-	+	-	+	+
MRSA 01	-	-	-	-	-	-	-	-	+	+
MRSA 02	-	-	-	-	-	-	-	-	+	+
MRSA 03	-	-	-	-	-	-	-	-	+	+
MRSA 04	-	-	-	-	-	-	-	-	+	+

- Absence of growth and + Presence of growth.

addition of 20 µL of the water solution of the resazurin die (Sigma-Aldrich) in the 0.01% concentration, which is recognized as a colorimetric marker of oxide-reduction reaction (Mann & Markan, 1998; Salvat et al., 2001; Burt & Reinders, 2003). The change of coloration of the die (blue for rose) indicates microbial growth. The lesser concentration of the oil essential capable to inhibit the growth of strain assayed, verified for the blue coloration of the die is considered as a measure of the MIC (Espinell-Ingrof et al., 1992).

Determination of minimum bactericidal concentration (MBC)

For the determination of the MBC, 50 µL of the four last concentrations of the oil were removed, in the microdilution plate with growth absence, and were inoculated in a plate contend Nutrient Agar (Biobras). The same ones were incubated at 35 ± 1 °C during 24-48 hours and, after this, the reading was made. The minimum bactericidal concentration (MBC) was considered starting at the point that did not allow to the reactivation of the microorganism in the medium without antimicrobials (Benoudia et al., 1988; Smith Palmer et al. 1998; Salvat et al., 2001; Sahin et al., 2004).

RESULTS AND DISCUSSION

Of the carried through microbiological analyses in 170 clinical samples of some biological materials, 24 bacterial strains (sixteen Gram negative and eight Gram positive) were selected considering that they presented the profile for the considered study.

The results presented on Table 2 demonstrate that of the four strains of *A. baumannii*, three (01, 02 and 03) corresponding to 75% were inhibited by the essential oil of *O. vulgare* at the 0.125% concentration. The strain 04 (25%) was inhibited by the 0.5% concentration. The four strains (100%) of *E. coli*, *S. faecalis* and *S. aureus* were inhibited with the 0.125% concentration. From the four strains of *K. pneumoniae*, three (75%), strains 01, 02 and 04 were inhibited at the 0.125% concentration, on the other hand, the strain 03 was inhibited in the 0.25% concentration. Considering the strains of *P. aeruginosa*, three of them (01, 02 and 03) corresponding to 75% were inhibited at the 0.5% concentration, however, the strain 04 (25%) was inhibited in a lesser concentration (0.25%). Emphasize that, good results with these normally multiresistant bacteria were obtained with the referred oil, while Hammer et al. (1997) had determined the MIC of different oils using extracts at $\leq 2\%$ concentration. Moreover, Souza et al. (2006), although testing the action of the *O. vulgare* oil against other bacterial species, had also obtained good results. The MBC corresponded to 100% when was used the 0.5% concentration in the twenty four tested strains showing the effectiveness of the oil. Santurio et al. (2007) testing the action of *O.*

vulgare against serum variant of *Salmonella enterica* also had determined a very good MBC.

CONCLUSION

From the obtained data we can concluded that: the essential oil of *O. vulgare* in low concentration was capable to prevent the growth of the strains: *A. baumannii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *E. faecalis* and *S. aureus* methicillin resistant (MRSA) and the majority of these bacteria had the minimum inhibitory concentration (MIC) equal to the minimum bactericidal concentration (MBC). And a significant number of these microorganisms were also inhibited by a concentration slightly lower.

It was observed that there was no difference in the minimum bactericidal concentration (MBC) of the *Origanum vulgare* essential oil as much to the Gram positive as well as for the Gram negative microorganisms.

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