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Short Communication

Origanum vulgare L. essential oil inhibits the growth of carbapenem-resistant gram-negative bacteria

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Abstract

Introduction: Plant products are sources for drug development against multidrug resistant bacteria. **Methods**: The antimicrobial activity of *Origanum vulgare* L. essential oil (OVeo) against carbapenem-resistant strains was assessed by disk-diffusion, microdilution (REMA-Resazurin Microtiter Assay), and time kill assays. **Results**: Carbapenemase production was confirmed for all strains. OVeo exhibited a minimum inhibitory concentration of 0.059% v/v for *Klebsiella pneumoniae* and *Serratia marcescens,* and of 0.015 % v/v for *Acinetobacter baumannii*. A decrease in cell count was observed after a 4 h treatment. **Conclusions**: OVeo antimicrobial effect was rapid and consistent, making it a candidate for developing alternative therapeutic options against carbapenem-resistant strains.

Keywords: Oregano. Carbapenemase. Multidrug resistance. Antimicrobial activity.

The constant selective pressure associated with clinically used antibiotics has led to a high prevalence of antibiotic-resistant microorganisms[1], prompting scientists to explore novel sources of chemicals with antimicrobial activity. Some studies have shown that *Origanum vulgare* L. (oregano) essential oil (OVeo) possesses broad-spectrum antimicrobial activity even at low concentrations, inhibiting the growth of a variety of bacteria and fungi[2]. In addition to being used as food, oregano is easily accessible, economical, and nontoxic to human cells[3].

The spread of carbapenemase genes and, consequently, of resistant strains is increasing worldwide. *Klebsiella pneumoniae*, *Serratia marcescens*, and *Acinetobacter baumannii* are characterized by their ability to develop resistance to many antimicrobials and are nosocomial pathogens often involved in hospital outbreaks[1]. Handling such infections is an ongoing clinical challenge; furthermore, the presence of carbapenemases, enzymes responsible for carbapenems resistance, is associated with higher morbidity and mortality rates[4]. For example, carbapenemase KPC (*Klebsiella pneumoniae* carbapenemase), encoded by the gene $bla_{KPC,}$ has a high dissemination potential because it is located within a highly conserved transposon, Tn*4401*, found in a plasmid transferable between species and bacterial genera[1].

Therefore, the present study aimed to assess the antimicrobial activity of OVeo against carbapenem-resistant *K. pneumoniae*, *S. marcescens*, and *A. baumannii* strains and its molecular characteristics.

Carbapenem-resistant *K. pneumoniae, S. marcescens*, and *A. baumannii* were collected and isolated from human specimens by rectal swab, urine sample, and nasal swab, respectively. Samples were taken from patients over 50 years old, hospitalized in different wards at a tertiary hospital in Brazil's Midwestern region. Selected isolates were maintained at -70 °C and later subcultured on MacConkey agar for 24 h before testing. Bacterial identification was performed using the Vitek® 2 system (bioMérieux, Hazelwood, MO) and confirmatory tests, using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), with a Microflex LT

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spectrometer (Bruker Daltonics, MA, USA)[5]. The minimal inhibitory concentrations (MICs) of the antimicrobials were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[6]. A preliminary screening for the presence of carbapenemase was performed by ertapenem hydrolysis using MALDI-TOF MS[5]. Commercial antibiotics, gentamicin (GEN), imipenem (IPM), and polymyxin B (POL), were used as controls for the assays and 0.5% Tween 80 was used as solvent for the OVeo. The study was conducted with the approval of the Research Ethics Committee from the Universidade Federal da Grande Dourados (Process No. 877.292/2014).

Polymerase Chain Reaction (PCR) was performed to determine the presence of carbapenem-resistance genes $bla_{\text{CTX-M-1-like}}$, $bla_{\text{CTX-M-2-like}}$, $bla_{\text{CTX-M-8-like}}$, $bla_{\text{CTX-M-14-like}}$, $bla_{\text{GES-like}}$, $bla_{\text{GIM-like}}$, $bla_{\text{IMP-10}}$, $bla_{\text{IMP-like}}$, $bla_{\text{KPC-2}}$, $bla_{\text{NDM-like}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48-like}}$, $bla_{\text{SHV-like}}$, $bla_{\text{SIM-like}}$, $bla_{\text{SME-like}}$, $bla_{\text{SPM-like}}$, $bla_{\text{TEM-like}}$ and $bla_{\text{VIM-like}}$ in *K. pneumoniae* and *S. marcescens* strains[1,4], and $bla_{\text{IMP-10,}}$ $bla_{\text{KPC-2,}}$ $bla_{\text{NDM-like,}}$ $bla_{\text{OXA-23,}}$ $bla_{\text{OXA-24,}}$ $bla_{\text{OXA-48,}}$ $bla_{\text{OXA-51,}}$ $bla_{\text{OXA-58,}}$ $bla_{\text{VIM-like}}$ in *A. baumannii*.

The OVeo used was a clear greenish-yellow to brown liquid with a characteristic odor, free of impurities, extracted by leaf steam distillation, and with a density of 0.954. The sample (batch and CAS number 224 and 84012-24-8, respectively) was accompanied by a technical report, approved and certified by the responsible chemical engineer of the company from which it was acquired (Ferquima Ind. Com. LTDA, São Paulo, Brazil), indicating that carvacrol (2-methyl-5-[1-methylethyl] phenol), a phenolic monoterpenoid, was the most abundant compound (71%) in the oil, followed by γ-terpinene (4.5%), β-caryophyllene (4%), *p*-cymene (3.5%), and thymol (3%).

The antimicrobial activity of OVeo was determined using the standard agar disk diffusion method according to CLSI guidelines[6]. Filter-paper disks, 6 mm in diameter, containing 5 µL of undiluted oil (absolute concentration) were gently placed on the surface of Mueller–Hinton agar plates containing carbapenem-resistant *K. pneumoniae*, *S. marcescens*, and *A. baumannii* strains. After 30 min at room temperature (27 ± 1° C), plates were incubated at 37 ± 1 °C for 24 h and the inhibition zone's diameter was measured (mm). POL and GEN were used as positive controls. The test was performed in duplicate and the means of the values obtained were used to classify the bacteria as either sensitive (≥ 10 mm) or resistant (< 10 mm) to the oil[6].

The MIC of the OVeo was determined using broth microdilution and colorimetric assays (REMA-Resazurin Microtiter Assay)[6]. The effect of the addition of Tween 80 (0.5%) as an oil solubilizer on MIC was investigated and it was found that this concentration did not interfere with bacterial growth.

Time-kill assays were performed using the broth macrodilution technique following CLSI guidelines[6]. Both IPM and OVeo were tested using MIC values against carbapenem-resistant *K. pneumoniae*, *S. marcescens*, and *A. baumannii* strains. Time-kill assays were performed using a final inoculum concentration of approximately 1.5×10^6 colony forming units (CFU)/mL, verified spectrophotometrically using the Vitek® 2 system. Test tubes were shaken periodically and incubated at 37 °C. Samples were collected at 0, 4, 8, 12, 16, 20, and 24 h. At each sampling time, 1 µL of inoculum was collected from each tube using a sterile loop, spread onto MacConkey agar plates, and incubated for 24 h at 37 °C; colony counts were determined thereafter. For carbapenem-resistant *K. pneumoniae* and *A. baumannii,* POL was used as the positive control, whereas GEN was used as the positive control for *S. marcescens* (intrinsically resistant to POL). Brain Heart Infusion broth with one of the bacterial strains was used as a negative control and a saline solution was used as sterility control.

The sensitivity of carbapenem-resistant *K. pneumoniae*, *S. marcescens*, and *A. baumannii* strains is shown in **Table 1** and **Figure 1**. All strains were identified as carbapenemase producers using MALDI-TOF MS. PCR amplification showed

TABLE 1: Antimicrobial susceptibility patterns for carbapenem-resistant K. *pneumoniae, S. marcescens*, and *A. baumannii* (MICs in µg/mL).

	K. pneumoniae	***S. marcescens***	***A. baumannii***
Amikacin	> 32 (R)	16 (S)	16 (S)
Ampicillin	> 16 (R)	> 16 (R)	NR
Aztreonam	> 16 (R)	> 16 (R)	NR
Cefoxitin	> 16 (R)	> 16 (R)	NR
Ceftazidime	16 (R)	8 (R)	> 64 (R)
Cefepime	> 16 (R)	8 (R)	> 64 (R)
Ciprofloxacin	> 2 (R)	> 2 (R)	> 4 (R)
Polymyxin B	≤ 1 (S)	(IR)	≤ 0.5 (S)
Ertapenem	> 4 (R)	> 4 (R)	NR
Gentamicin	> 8 (R)	≤ 2 (S)	> 16 (R)
Imipenem	> 8 (R)	> 8 (R)	> 16 (R)
Levofloxacin	> 4 (R)	> 4 (R)	NR
Meropenem	> 8 (R)	> 8 (R)	> 16 (R)
Nitrofurantoin	> 64 (R)	> 64 (R)	NR
Piperacillin/Tazobactam	> 64/4 (R)	64/4 (R)	> 128 (R)
Tigecycline	2 (I)	4 (R)	2 (S)
Sulfamethoxazole/Trimethoprim	> 4/76 (R)	≤ 1/19 (S)	NR

>: more than; **≤:** less or equal to; **S:** susceptibility, **I:** intermediate, **R:** resistance, **IR:** intrinsic resistance, **NR:** not recommended.

that the bla_{KPC-2} gene was present in *K. pneumoniae*, bla_{KPC-2} and bla_{IMP-10} in *S. marcescens*, and bla_{OXA-23} and bla_{OXA-51} in *A. baumannii*. The OVeo exhibited significant inhibitory effects against tested bacterial strains, with MICs of 0.059 % v/v for *K. pneumoniae* and *S. marcescens* and of 0.015 % v/v for *A. baumannii* (**Table 2**).

Bacterial survival curves showed a linear decrease in viable cell counts over time (**Figure 2**). All three strains treated with OVeo showed decreases in cell counts of approximately 5 $\log_{10}$ CFU/mL. Considering the time of cell death, a total inhibition of carbapenem-resistant *K. pneumoniae*, *S. marcescens*, and *A. baumannii* growth was reached after 4 h of treatment. All cell counts reduced to zero, including those for *S. marcescens*, the most resistant strain from this study. In contrast, IPM showed no antibacterial activity for up to 24 h. Control antibiotics successfully inhibited these strains within 24 h, while no growth was observed in plates with saline solution.

Tested strains have intrinsic and acquired resistance to antimicrobial agents, specially KPC-2 and IMP-10-producing *S. marcescens*. Furthermore, *A. baumannii* and carbapenemase-producing Enterobacteriaceae are considered critical in the WHO list of priority for research and development of new antibiotics, and the production of carbapenemases by pathogens intrinsically resistant to polymyxins drastically reduces available therapeutic options against them[7]. In this study, OVeo was investigated as a potential novel therapeutic agent and showed encouraging and significant inhibitory effects against carbapenem-resistant bacteria. The antimicrobial action of OVeo and its time-kill curves provided evidence for its potential rapid action against *A. baumannii* (MIC, 0.015 %v/v), *K. pneumoniae* (MIC, 0.059 %v/v), and *S. marcescens* (MIC, 0.059 %v/v). OVeo inhibited tested strains within 4 h, which was as fast as the effects of POL on *A. baumannii* and faster than the effects of POL and GEN against *K. pneumoniae* and *S. marcescens*, respectively. Interestingly, this susceptibility was independent of the presence of carbapenemase-resistance genes, as *K. pneumoniae* expressed bla_{KPC-2}, *S. marcescens* expressed bla_{KPC-2} and bla_{IMP-10}, and *A. baumannii* expressed bla_{OXA-23} and bla_{OXA-51}, indicating a mode of action unrelated to the genus, species, or resistance genes. Nevertheless, its action may be related to the gram-negative cell wall structure.

The activity of this compound against methicillin-resistant *Staphylococcus aureus* (MRSA)[8], *Escherichia coli, Klebsiella pneumoniae*, and *Klebsiella oxytoca* strains insusceptible to carbapenems[9] has been reported. A multidrug sensitive *Klebsiella* strain was also reported as susceptible and moderately susceptible to OVeo with MIC values of 0.5 µg/mL[2] and close to 250 µg/mL[10], respectively. Collectively, these observations indicate that OVeo may interfere with membranes, the cell wall, or other cell structures[11]. In addition to MICs determination, our study also used the more accurate time-kill method to verify the speed at which OVeo inhibited the studied bacteria, to enhance the understanding of the underlying mechanisms of OVeo action. However, the antimicrobial action mechanism of OVeo seems complex and several pathways could be involved in its antibacterial effect. Multiple experiments would be necessary to evaluate all the possible mechanisms and provide a clearer insight into the action of OVeo.

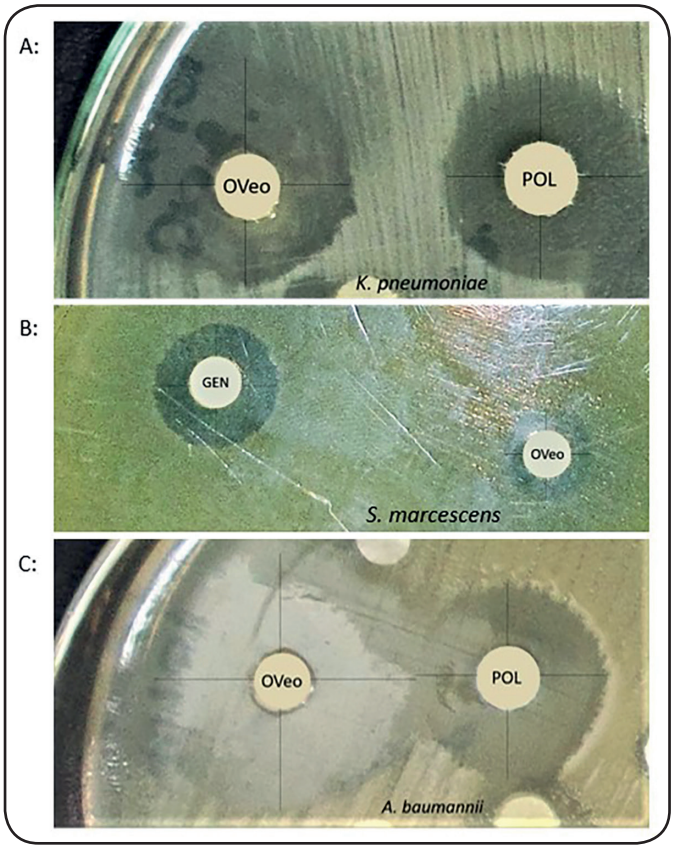

FIGURE 1: Inhibition diameter zones obtained by paper disk diffusion method for OVeo. **(A):** *K. pneumoniae*; **(B):** *S. marcescens*; **(C):** *A. baumannii*. GEN, gentamicin; OVeo, *Origanum vulgare* L. essential oil; POL, polymyxin B.

TABLE 2: Antimicrobial action of OVeo using disk diffusion and MIC methods (concentration and percentage of the oil).

	Resistance genes	Disk-diffusion	MIC (% v/v)
K. pneumoniae	bla_{KPC-2}	21 mm (S)	0.059
S. marcescens	bla_{KPC-2} and bla_{IMP-10}	12 mm (S)	0.059
A. baumannii	bla_{OXA-23} and bla_{OXA-51}	28 mm (S)	0.015

S: sensitive.

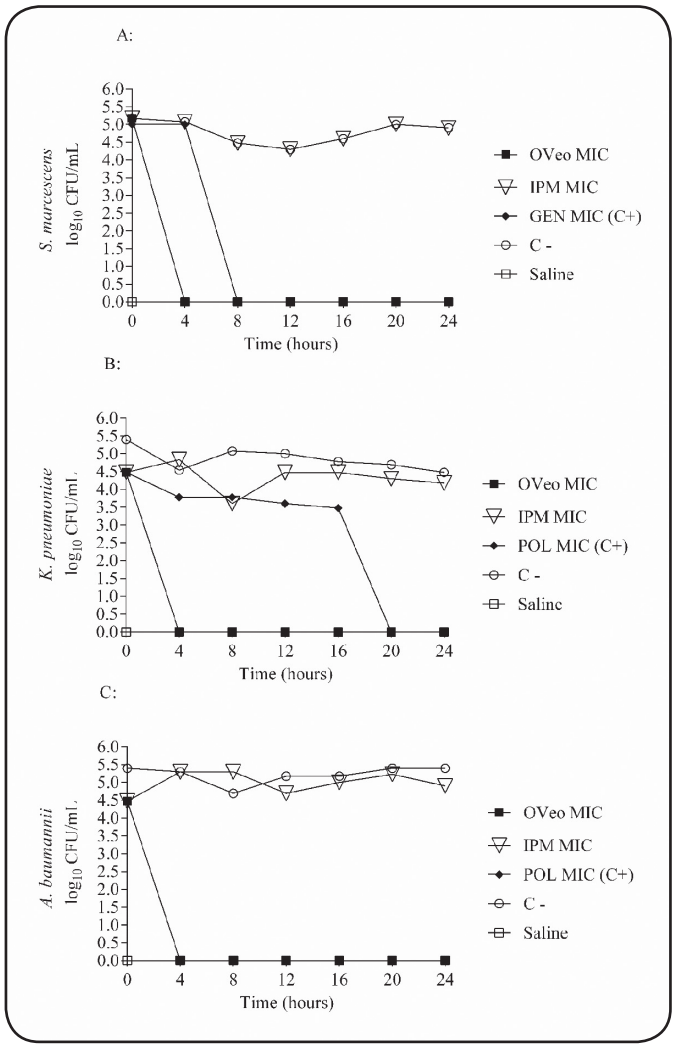

FIGURE 2: Time-kill curves with OVeo against carbapenem-resistant strains. **(A):** *S. marcescens*; OVeo MIC of 0.059 % v/v; IPM MIC of 8 µg/mL; GEN MIC of 4 µg/mL; **(B):** *K. pneumoniae*; OVeo MIC of 0.059 % v/v; IPM MIC of 8 µg/mL; POL MIC of 1 µg/mL; **(C):** *A. baumannii*; OVeo MIC of 0.015 % v/v; IPM MIC of 16 µg/mL; POL MIC of 0.5 µg/mL. GEN, gentamicin; IPM, imipenem; MIC, Minimal Inhibitory Concentration; OVeo, *Origanum vulgare* L. essential oil; POL, polymyxin B; C+, positive control; C- negative control.

The antimicrobial activity of essential oils can involve single or multiple targets, and therefore, their mechanisms of action could not be attributed to a unique site of action but to a cascade of reactions within the bacterial cell. Several mechanisms for the antibacterial activity of oregano have been proposed, including the impairment of a variety of enzyme systems —such as those involved in energy production and structural component synthesis[11]. *K. pneumoniae, S. marcescens*, and *A. baumannii* can produce beta-lactamases and carbapenemases, which hydrolyze and destroy antibiotics. Essential oils are naturally hydrophobic, a characteristic responsible for the disruption of the bacterial cytoplasmic membrane, increasing cell permeability, leading to leakage of cell contents, and reducing ATPase activity[3].

Carvacrol, abundant in OVeo, is largely responsible for its antimicrobial activity[11]. Two monoterpenes, γ-terpinene and *p*-cymene (the carvacrol precursor), are also constituents of OVeo, but no antimicrobial activity has been reported for them[12]. This lack of antimicrobial activity is probably due to the absence of phenolic hydroxyl groups, since phenols are known for their membrane-disturbing activities[13].

The three studied bacteria can cause infections at multiple sites in the human body, therefore, other potential activities of OVeo, apart from its antimicrobial activity, could be very useful; the oil in the natural state could play an important therapeutic role with additional antioxidant or antispasmodic activities because of its compounds[8]. Gram-negative bacteria are known to be more resistant to antibiotics than gram-positive bacteria because of their more complex outer membrane; and terpenes are minor components of plant essential oils that, owed to their lipidic nature, can enhance the ability of the oil to penetrate the lipid-rich gram-negative cell membrane[11].

In this study, one multidrug resistant strain of each species was subjected to antibacterial analysis to investigate the potential of OVeo to suppress the growth of these hard-to-kill bacteria. Testing only one isolate of each species might be considered a limitation of the study; nevertheless, if OVeo can act on these multidrug resistant strains that have a variety of resistances and carbapenemase enzymes, then OVeo can potentially have antibacterial action.

In addition, to our knowledge, toxic effects of *O. vulgare* L. have not been reported thus far. Previous studies have shown that *O. vulgare* L. extract protects human lymphocytes against genotoxicity induced by internal irradiation, suggesting its effectiveness as a free-radical scavenger and its likely provision of concentration-dependent radioprotection, and protection against DNA damage[14]. Furthermore, carvacrol, the major compound of OVeo, is classified as a generally recognized as safe (GRAS) compound, approved for use in food items[3]. Data on the acute and short-term *in vivo* effects in different animal species are available and they suggest that carvacrol may not pose a risk to human health[6]. The oral median lethal dose (LD_{50}) values in rats are also available for carvacrol (810 mg/kg body weight)[15].

This study demonstrates the efficacy of OVeo in growth inhibition of carbapenem-resistant gram-negative bacteria associated with nosocomial infections. The antimicrobial effect was rapid and consistent, making OVeo a favorable candidate for the development of alternative treatments. This finding is significant, considering the critical need for novel sources of antibiotics to address the increasing incidence of drug- and multidrug-resistant human pathogens such as the clinical strains used in this study. Microorganisms are excellent survivors with a remarkable ability to adapt to hostile situations such as the host environment. Thus, future studies on OVeo activity and its antibacterial mechanisms in animal models are necessary to enhance our understanding of its action and establish its efficacy.

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Conflict of Interest

All authors declare that they have no conflict of interest to disclose.

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