

Thymol and carvacrol strongly inhibit biofilm formation and growth of carbapenemase-producing Gram negative bacilli

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Abstract: Discovery of novel drugs with new mechanisms of action and without cross-reaction with current therapeutic agents is crucial in the management of infections caused by multi-drug resistant (MDR) bacteria. The aim of the present study was to investigate effects of carvacrol and thymol on biofilm formation and antimicrobial activity against different carbapenemase-producing Gram negative bacilli. The antimicrobial and antibiofilm effect of thymol and carvacrol was investigated against strains harboring different genes related to carbapenemase resistance. Antimicrobial resistance was examined by an agar dilution method and antibiofilm effect was evaluated by microtiter plate assay and staining by crystal violet. Thymol and carvacrol had antibacterial effects ranging from 200-1600 µg/mL and 62-250 µg/mL respectively, and antibiofilm effect from 125-500 and 400-1600 µg/mL respectively. Seoul imipenemase- (SIM) producing isolates had the highest sensitivity, and NDM (New Delhi metallo-beta-lactamase) producing isolates had the lowest sensitivity to these components. Findings of the present study indicated a potential role of carvacrol and thymol in controlling carbapenemase-producing gram negative bacterial infections. These findings helped to develop herbal drugs for replacing antibiotics. In addition, their antibiofilm effects showed that carvacrol and thymol inhibit biofilm formation of carbapenemase-producing strains.

Key words: Thymol; Carvacrol; Bacterial infections; Antibiofilm; New Delhi metallo-beta-lactamase.

Introduction

Enterobacteriaceae are residents of intestinal flora and are the source of community acquired infections, hospital-acquired infections, sepsis, and urinary tract infections (1, 2). They have the propensity to spread easily among humans, mediated mostly by plasmids and transposons. The appearance of carbapenems-resistant Enterobacteriaceae is consequently a concern, and antimicrobial treatment choices are very limited (2, 3). Carbapenemase-producing Enterobacteriaceae have been increasingly reported in the past 10 years. Therefore, it is important to maintain the clinical efficacy of carbapenems (imipenem, ertapenem, meropenem, doripenem), which have become antimicrobial drugs of last recourse in treatment of infections caused by multidrug resistance Gram negative bacteria (4). These agents are critical for preventing and treating life-threatening nosocomial infections (5, 6). Carbapenemases hydrolyse most β-lactams. Enzymes having maximum carbapenemase activity are commonly imipenem-resistant phenotype carbapenemase (IMP), verona integron-encoded metallo-beta-lactamase (VIM), and New Delhi metallo-beta-lactamase (NDM) types. These enzymes show a broad spectrum of hydrolytic activity. Commercially accessible β-lactamase enzyme inhibitors (clavulanic acid, tazobactam, or sulbactam) cannot inhibit these car-

bapenemase enzyme activities. Oxacillinase 48 (OXA-48) and OXA-181 have a peculiar hydrolysis profile, sparing ceftazidime, hydrolyzing cefotaxime at a very low level, and clavulanic acid– tazobactam cannot inhibit their activity (7).

Discovery of new therapeutic agents with novel modes of action and no cross-resistance with current antibiotics is vital to counter threats created by the appearance of bacteria resistant to current therapeutic agents (8). In addition to antibiotic resistance according to producing extended spectrum beta lactamase (ESBL), bacteria growing in biofilms are commonly resistant to antimicrobial agents and are protected from the host immune response which increase chronic infections which are especially difficult to cure (9). Biofilms are organized communities of bacteria that are attached to a surface and play a major role in infectious diseases (10, 11). One mechanism of biofilm resistance to antimicrobial agents is the failure of an agent to penetrate the full depth of biofilm. A theory to describe resistance is that at least some of the cells in a biofilm experience nutrient restriction and therefore exist in a slow-growing or starved state. The latest mechanism is that at least some of the cells in a biofilm adopt a distinct and protected biofilm phenotype (12). Antibacterial effects of essential oils (EOs) and their ingredients have been examined recently (13). Spices and herbs are used globally and

Table 1. Minimum inhibition concentration of strains examined in this study.

Number	Strains*	MIC	
		Carvacrol (µg/ml)	Thymol (µg/ml)
1	<i>K. pneumonia</i> (NDM)	250	400
2	<i>K. pneumonia</i> (VIM-1)	125	200
3	<i>K. pneumoniae</i> (OXA-48)	125	200
4	<i>K. pneumonia</i> (KPC)	125	200
5	<i>P. aeruginosa</i> (AIM)	250	800
6	<i>P. aeruginosa</i> (GIM)	250	800
7	<i>P. fluorescens</i> (BIC)	125	200
8	<i>P. aeruginosa</i> (SPM)	250	1600
9	<i>A. junii</i> (IMP)	125	200
10	<i>A. Baumannii</i> Corée (SIM)	62	200
11	<i>P. stutzeri</i> (DIM)	125	1600

* abbreviations indicates strains name (harboring gene).

their antimicrobial potential has been known since antiquity. Essential oils are natural products extracted from vegetal components, and because of their antibacterial, antifungal, antioxidant, and anti-carcinogenic properties can be used as natural additives in many foods. Purified materials derived from essential oils such as carvacrol and thymol inhibit a diversity of organisms (14, 15). The main antibacterial components of these oils are carvacrol and its isomer thymol (13).

The objective of the present study was to evaluate the antimicrobial effect of carvacrol and thymol against carbapenemase-producing Enterobacteriaceae family, and their effect on inhibition of biofilm formation of these bacteria.

Materials and Methods

Strains and substances

Eleven collection strains *Klebsiella pneumonia* (NDM), *Klebsiella pneumonia* (VIM-1), *Klebsiella pneumoniae* (OXA-48), *Klebsiella pneumoniae* (KPC), *Pseudomonas aeruginosa* (AIM), *Pseudomonas aeruginosa* (GIM), *Pseudomonas fluorescens* (BIC), *Pseudomonas aeruginosa* (SPM), *Acinetobacter junii* (IMP), *Acinetobacter baumannii* Corée (SIM) and *Pseudomonas stutzeri* (DIM) were used in the present study. All isolates were standard defined strains provided from Antwerp University, Belgium. Stock cultures were kept on TSA (Tryptone Soy Agar, Merck, KGaA, Darmstadt, Germany) at 4 °C and were subcultured bimonthly. Cultures were prepared for experiments by inoculation of a loopful of culture into BHI broth (brain heart infusion broth, Merck) and incubated overnight at 37 °C. Antimicrobial substances studied were thymol (2-isopropyl-5-methylphenol) (Carbonell, Spain), and carvacrol (5-isopropyl-2-methylphenol) (Catalogue number: 820258, Merck), with 99% and 97% purity respectively.

Antimicrobial assay

Antibacterial activity of thymol and carvacrol was determined by agar dilution method according to the CLSI M02, A11 (Clinical Laboratory Standard Institute,) guideline (16) with the following modification: a final concentration of 0.5% (v/v) Tween-20 (Sigma) was added into the agar after autoclaving to enhance oil solubility. Briefly, a series of twofold dilutions of each thymol and carvacrol oils, ranging from 2% (v/v) to 0.03% (v/v) was prepared in Mueller Hinton agar with

0.5% (v/v) Tween-20. Plates were dried at 35 °C for 30 min prior to inoculation with 1–2 µL spots containing approximately 10⁵ colony forming units (cfu)/mL of each strain. Mueller Hinton agar, with 0.5% (v/v) Tween-20 but no oil, was used as a positive growth control. Inoculated plates were incubated at 35 °C for 48 h. Minimum inhibitory concentrations (MICs) were determined after 24 h for the bacteria. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of each strain on the agar plate. The presence of one or two colonies was disregarded (17).

Biofilm assay

A Microtiter plate assay was used for determination of each strains' biofilm (18). Three to five colonies were suspended in 5mL of tryptone soy broth (TSB) and incubated for 18 h at 35±1 °C, without shaking. After incubation, the stationary phase culture was vortexed and thereafter diluted 1:100 in TSB with 0.25% glucose, and 200 µL of this solution was incubated in 96-well plates for 18 h at 35±1.0 °C. Media with suspended bacteria were then removed. The plates were carefully washed four times with water and air dried before staining with 200 µL of 0.9% crystal violet solution (Sigma, Stockholm, Sweden) for 15 min. After removing the dye solution and washing with water, the attached dye was solubilized with 95% ethanol and the optical density of the adherent biofilm was determined twice with a filter of 450/630 nm in a microtiter plate reader (OrthoReaderii, Ortho Diagnostic Systems, New Jersey, USA). In the tests, we used TSB with 0.25% glucose as a negative control (background absorbance) (18, 19). For reducing effect of vapourization, only central wells in 96 well microtiter plates were used for examination.

Descriptive statistics were used to analyze the data. Statistical analysis was done by SPSS 21.0 (IBM company, USA, www.ibm.com).

Results

The MIC of carvacrol was different for strains with different carbapenemase genes (Table 1). Also, the MIC of thymol showed different effective concentrations against strains with a defined carbapenemase gene (Table 2). Figures 1 and 2 show the effect of thymol and carvacrol on biofilm formation strains of Gram negative bacilli investigated in the present study. 400-1600 µg/mL of thymol and 125-500 µg/mL of carvacrol signi-

ificantly reduced biofilm formation. In all cases, with increasing the concentrations of thymol and carvacrol, biofilm formation reduced considerably compared to the control group ($p < 0.05$) (Figures 1 & 2).

Discussion

Multidrug resistance is now emerging at an alarming rate among a diversity of bacterial species, causing both nosocomial and community-acquired infections (6). One of the most significant emerging traits is resistance to carbapenems in Gram-negative bacilli. Spread of carbapenemase-producers is by far the most important present clinical problem in antibiotic resistance in Gram-negatives and must be strictly controlled, because carbapenems are often the last line of treatment (3). Plant essential oils are a potentially suitable source of antimicrobial compounds. Potential use of essential oils as natural antimicrobial agents is less exploited than their uses as additive and antioxidant compounds. Results reported from different studies are difficult to compare, probably because of different test methods, bacterial strains, and sources of antimicrobial samples used (20). Carvacrol and thymol are known for their wide spec-

trum of antimicrobial activity, which has been the focus of some studies in vitro and in vivo (15, 21).

Our results by agar dilution method showed that the concentration of 400-1600 $\mu\text{g/mL}$ of thymol and 125-500 $\mu\text{g/mL}$ of carvacrol can be successfully used against carbapenemase-resistant bacteria. Other studies had tested a wide range of concentrations for oregano, carvacrol, and thymol, as well as for carvacrol and thymol mixtures (22). These studies suggest that mixtures of carvacrol and thymol gave an additive effect against *P. aeruginosa* and *Staphylococcus* spp. Researchers reported the inhibitory effect of thyme essential oil, thymol and carvacrol against *Shigella* spp. (22). Other researchers demonstrated antimicrobial activity of thymol, carvacrol, and other oil extracts alone or combined with eight oral bacteria by microdilution methods (23). The compounds showed an inhibitory activity on seven microorganisms, and a synergistic effect was observed with certain combinations such as thymol and carvacrol during the treatment of oral infectious diseases. In other studies researchers have indicated that essential oil and its major components thymol and carvacrol exhibited potent antimicrobial activity against *Streptococcus* as well as *Candida albicans* by using broth dilution and disk diffusion with MIC ranging from 0.625 to 10.0 mg/mL (24). Some mechanisms have been proposed to explain the antimicrobial activity of carvacrol and thymol on bacteria. Especially, carvacrol and thymol can disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane (21).

Few studies have focused on the effects of these substances on bacterial biofilm (21). Our results by microtiter plate showed that reduction of biofilm formation was seen, and began for thymol and carvacrol from concentrations of 400-1600 $\mu\text{g/mL}$ and 125-500 $\mu\text{g/mL}$ respectively. Others such as Knowles et al. (25) used carvacrol as a dried film on stainless-steel surfaces for antimicrobial purposes. Another study carried by Braga et al. (26) showed antibiofilm activity of thymol against *Candida albicans* in vitro. Soumya et al. (21) observed a different inhibitory effect on biofilm formation among the strains in the presence of concentrations (2MIC to MIC/16). Although the mechanism of action of carvacrol and thymol on biofilms remains uncertain, their amphipathic nature could account for the observed effects. Therefore, some hypothesize that the relative hydrophilicity of carvacrol and thymol may allow their diffusion over the polar polysaccharide matrix, while prevalent hydrophobic properties of these substances could lead to specific interactions with the bacterial membrane (21). This hypothesis is supported by a study by Ouhayoun et al. in which the antiplaque effects of a thymol-based mouthwash was in part attributable to rapid-killing and plaque-permeating abilities (27).

The compound of essential oil of herbs and spices can vary significantly depending upon the geographical region, variety, age of the plant, procedure of drying, and the method of oil extraction. This makes standardization and comparison of outcomes in the application of essential oils difficult. It is recognized that diffusion procedure is only useful as a qualitative screening method (28). The diameter of the zone of inhibition will depend on the capacity of the test substance to diffuse uniform-

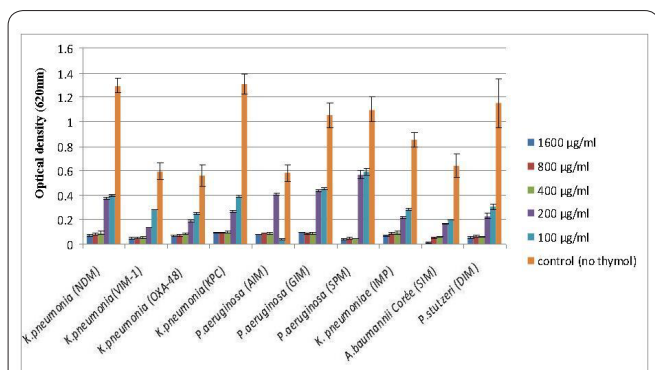


Figure 1. Biofilm formation by *Klebsiella pneumoniae* (NDM), *Klebsiella pneumoniae* (VIM-1), *Klebsiella pneumoniae* (OXA-48), *Klebsiella pneumoniae* (KPC), *Pseudomonas aeruginosa* (AIM), *Pseudomonas aeruginosa* (GIM), *Pseudomonas fluorescens* (BIC), *Pseudomonas aeruginosa* (SPM), *Acinetobacter junii* (IMP), *Acinetobacter baumannii* Corée (SIM), *Pseudomonas stutzeri* (DIM) in the range of thymol concentration (from 1600 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$).

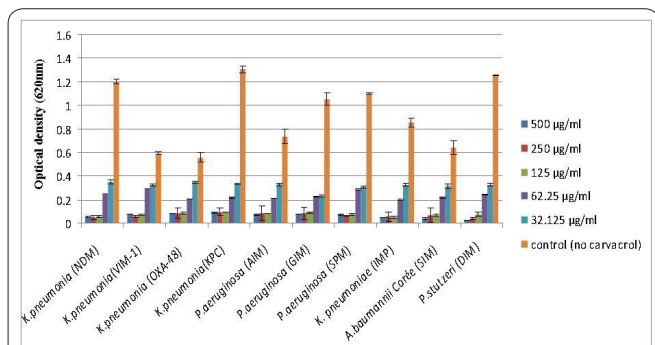


Figure 2. Biofilm formation by *Klebsiella pneumoniae* (NDM), *Klebsiella pneumoniae* (VIM-1), *Klebsiella pneumoniae* (OXA-48), *Klebsiella pneumoniae* (KPC), *Pseudomonas aeruginosa* (AIM), *Pseudomonas aeruginosa* (GIM), *Pseudomonas fluorescens* (BIC), *Pseudomonas aeruginosa* (SPM), *Acinetobacter junii* (IMP), *Acinetobacter baumannii* Corée (SIM), *Pseudomonas stutzeri* (DIM) in the range of carvacrol concentration (from 500 $\mu\text{g/ml}$ to 32.125 $\mu\text{g/ml}$).

mly over an agar medium. Most essential oils and their active substances are very volatile and show poor solubility in the aqueous stage (29, 30). Dilution methods confer more quantitative results (23). It is essential to standardize test methods. MICs depend on many factors such as temperature and time of incubation, and the size of the test inoculum (30, 31)

Due to widespread use of antibiotics, carbapenemase-producing bacteria have increased significantly. For this reason, the main purpose of this series of studies is to find drugs for hospital infection control; so the following hypothesis was formed: if thymol and carvacrol can reduce biofilm formation, they can be used as a drug for treatment of infectious disease. Our data showed that the essential oil tested and its major phenolic compounds (thymol and carvacrol) had antimicrobial activity against the main group of carbapenemase-producers and their biofilm formation. In conclusion, findings of the present study indicate the potential role of these extracts in controlling carbapenemase-producing Gram negative bacterial infections. These findings suggest that herbal drugs may replace antibiotics under certain circumstances. In addition, they can be used for inhibiting biofilm producer isolates because of their antibiofilm effects. This work represents an advance in biomedical science because it shows the importance of thymol and carvacrol on treatment and control of biofilm of recently emerged carbapenemase-resistance isolates.

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