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Antibacterial and efflux pump inhibitors of thymol and carvacrol against food-borne pathogens

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ABSTRACT

In this study thymol (THY) and carvacrol (CAR), two monoterpenic phenol produced by various aromatic plants, was tested for their antibacterial and efflux pump inhibitors potencies against a panel of clinical and foodborne pathogenes.

Our results demonstrated a substantial susceptibility of the tested bacteria toward THY and CAR. Especially, THY displayed a strong inhibitory activity (MIC's values ranged from 32 to 64 μg/mL) against the majority of the tested strains compared to CAR. Moreover, a significant reduction in MIC's of TET and benzalkonium chloride (QAC) were noticed when tested in combinations with THY and CAR. Their synergic effect was more significant in the case of THY which resulted a reduction of MIC's values of TET (2–8 fold) and QAC (2–8 fold).

We noted also that THY and CAR inhibited the ethidium bromide (EtBr) cell efflux in a concentration-dependent manner. The rate of EtBr accumulation in food-borne pathogen was enhanced with THY and CAR (0, 250 and 500 μg/mL). The lowest concentration causing 50% of EtBr efflux inhibition (IC 50) was noticed in *Salmonella enteritidis* (1129) at 150 μg/mL of THY and 190 μg/mL of CAR respectively.

These findings indicate that THY and CAR may serve as potential sources of efflux pump inhibitor in food-borne pathogens.

1. Introduction

Microbial pathogens in food may cause spoilage and contribute to food-borne disease incidence [1]. *Salmonella* is a facultative intracellular pathogen which constitutes a major public health problem in many countries with millions of cases of salmonellosis are noticed in the world [2]. In recent years, the emergence of multidrug-resistant (MDR) and disinfectant resistant bacteria has increased rapidly [3] causing the increase of morbidity and mortality [4]. *Staphylococcus aureus*, *Salmonella*, *Escherichia coli*, and *Pseudomonas aeruginosa*, are some of the main MDR bacteria [3]. Resistance of these organisms to the main drugs, antiseptics and disinfectants makes their environmental eradication extremely challenging [4]. The research and development of novel techniques to control the growth and survival of resistant strains is of great importance [5].

Active efflux as a mechanism for bacterial resistance to antibiotics and antiseptics, is mediated by integral membrane transporters, known as efflux pumps which extrude antibiotics from the cell allowing the bacteria to survive [6]. Efflux pump inhibitors (EPIs) are compounds that potentiate the activity of antibiotics against MDR strains [7]. Many plants extracts and natural compounds has been evaluated for their resistance modifying activities [8] especially by reversing the natural resistance of bacteria to given antibiotics [4,8]. Antimicrobial and efflux pumps inhibitor from plant sources such as rosemary [9], thymoquinone [8], tetrandrine [10], coumarins [11], and *Terminalia chebula* [12] has been reported elsewhere.

Carvacrol [2-methyl-5-(1-methylethyl)phenol] and thymol (2-isopropyl-5- methylphenol) are two monoterpenic phenol produced by various aromatic plants, including *Satureja montana* L. and *Thymus vulgaris* L. respectively [2]. They are used in low

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concentrations as a food flavoring ingredient [13] and they exert a significant antimicrobial activity against food-borne pathogens [14]. Johny et al. [15] showed that carvacrol may increase the susceptibility of MDR *S. enterica* serovar *Typhimurium* to antibiotics. Moreover, thymol and carvacrol may interact with the lipid bilayer of cytoplasmic membranes making them more permeable, which permit an increased uptake of antibiotics by the bacterial cell [14,16–19]. Meanwhile carvacrol may potentiate the effect of same antibiotics [15,20] and disinfectants [21,22]. According to Medeiros Barreto et al. [23], thymol displayed a modulatory effect of neomycin and amikacin.

In this study we reported the antibacterial activities of thymol and carvacrol alone and in combination with tetracycline (TET) and benzalkonium chloride (BC) against a panel of food-borne and spoilage bacteria. In addition, their potential effect as efflux pumps inhibitors was assessed.

2. Material and methods

2.1. Microorganisms

The tested microorganisms include Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* CIP 106510, *Bacillus cereus* ATCC 11778, and *Bacillus cereus* ATCC 14579 and Gram-negative bacteria: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC27853, *Enterococcus faecalis* ATCC 29212, *Vibrio alginolyticus* ATCC 17749, *Vibrio alginolyticus* ATCC 33787, *Salmonella typhimurium* ATCC 1408, and *Salmonella typhimurium* LT2 DT104. In addition, 12 *Salmonella enteritidis* strains responsible for collective food intoxication isolated in hospital Fattouma Bougruiba Monastir (Tunisia) in June 2000 was included. These microorganisms were kindly provided by Pr. Amel Rhim from the Regional Laboratory of Public Health of Monastir (Tunisia), and the serotyping of the strains was performed at the Pasteur Institute, Tunisia.

2.2. Chemicals used

All the media used in this study were purchased from Biorad (France). Thymol (THY), Carvacol (CAR), and tetracycline (TET) from Sigma-Aldrich (Switzerland). Benzalkonium chloride (QAC) from Acros organics (USA).

2.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) value determination assay

The minimum inhibitory concentration (MIC) of THY and CAR was determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute [24]. Bacterial strains suspensions were adjusted to 0.5 McFarland standard turbidity with a densimat. Cell suspensions (200 μL) were inoculated into the wells of 96-well microplates. THY and CAR was dissolved in ethanol and then in Muller Hinton Broth (MHB) as required and transferred to microplate well in order to obtain a twofold serial dilution ranging from 1 to 1024 μg/mL. Tetracycline and BC were dissolved in water. Then wells containing MHB only and MHB with bacteria were used as negative and positive controls, respectively. Plates were incubated at 37 °C for 24 h and bacterial growth was evaluated by the presence of turbidity and a pellet on the bottom. MIC value was defined as the lowest concentration of the tested compound that had no macroscopically visible growth. All MICs tests were repeated twice in separate times.

To determine the minimum bactericidal concentration (MBC) values, 10 μL of each well medium with no visible growth was inoculated in MH plates. After 24 h of incubation at 37 °C, the number of surviving organisms was determined. MBC was defined as the lowest concentration of compounds (THY, CAR, TET and BC) at which 99% of the bacteria were killed. Each experiment was repeated twice [8,25].

2.4. Resistance modifying assay

For the evaluation of THY and CAR as a potential modulator of antibiotic and disinfectant resistance, the TET and BC MICs (ranging from 1 to 1024 μg/mL) were determined alone and in combination with THY and CAR (1/2 MIC) respectively [26]. All experiments were carried out three times.

2.5. Ethidium bromide efflux and accumulation assays

Ethidium bromide (EtBr) efflux assay was carried out as described previously [27]. Briefly, cells were grown to an OD_{600} of 0.6 than cells were washed with 20 mM potassium phosphate buffer containing 1 mM $MgCl_2$. EtBr was added at a final concentration of 5 μg/mL.

Bacterial cultures were incubated at 20 °C with shaking for 60 min. After centrifugation for 5 min at 2500 g at 20 °C, the supernatant was decanted and the pellet was re-suspended in 20 mM potassium phosphate buffer with 1 mM $MgCl_2$ and 5% glucose to energize the cells. Following, fluorescence was measured over 15 min at excitation and emission wavelengths of 530 and 600 nm respectively with a spectrofluorophotometer, model RF-5301PC (Shimadzu). The fluorescence intensity of EtBr is higher when EtBr binds to DNA molecules. Thus, the efflux of EtBr from the cell can be monitored by the detection of a decrease in the level of fluorescence unite over time.

To evaluate the effects of CAR and THY on the efflux of EtBr, cell suspensions were prepared in the same way as described above, pre-incubated for 5 min at 37 °C with different concentrations of each compound separately (0, 250 and 500 μg/mL) prior to the addition of glucose (5%) than fluorescence were measured.

3. Results and discussion

3.1. Antibacterial activity of THY and CAR

As presented in Table 1, the THY and CAR were active *in vitro* against the majority of the tested strains. We noted also that THY was more effective than CAR, with a lowest MIC values of 32 μg/mL against six isolated strains. THY exhibited a significant antibacterial activity with a MICs values ranged from 32 to 128 μg/mL and 32 to 64 μg/mL respectively. Moreover, the lowest MICs values of CAR were 64 μg/mL against *S. epidermidis* CIP 106510 and *S. entiritidis* (1129). Our result was agree with Lambert et al. [14] who found that THY possess a stronger antibacterial activity than CAR against *P. aeruginosa* and *S. aureus*. Similar result was found by Rivas et al. [28] against verocytotoxigenic *E. coli* and *E. coli* O157:H7 suggesting the antibacterial activity of THY and CAR at 500 μg/mL. Similarly, Olasupo et al. [29] found that THY was very effective compound against *S. Typhimurium* and *E. coli* (MIC values ranged from 1 to 1.2 mmol/L respectively) followed by carvacrol.

Our result revealed also that CAR was less effective than THY against *Vibrio* strains (Table 1). This result was contradictory with Rattanachaikunsopon and Phumkhachorn [30] who found that CAR exhibited a dose dependent inhibitory effect on *Vibrio cholerae* ATCC 14033, VC1, and VC7 with a lowest concentrations of required to eliminate viable cells about 7.5, 5 and 5 ppm respectively.