



Terpenes antimicrobial activity against slime producing and non-producing staphylococci

Gallucci*, N¹; Oliva, M¹, Carezzano, E¹, Zygadlo, J²; Demo, M¹.

¹ Faculdade de Farmácia, Unidade Universitária de Ciências Exatas e Tecnológicas. Universidade Estadual de Goiás. BR 153 N° 3105 Fazenda Barreiro do Meio, Campus Henrique Santillo - CEP 75132.400 Anápolis-Goiás E-mail: (1) - Departamento de Microbiología e Inmunología. Universidad Nacional de Río Cuarto. Córdoba. Argentina. Ruta 36, Km 601. e-mail: mgallucci@exa.unrc.edu.ar . (2) -Instituto Multidisciplinario de Biología Vegetal - IMBIV - CONICET. FCEFYn - UNC .Avda. Velez Sarsfield 1600, 5000 Córdoba, Argentina. jzygadlo@efn.uncor.edu

ABSTRACT

Staphylococcus aureus, responsible of several diseases in humans and animals, produce virulence factors like an extracellular polysaccharide or “slime”. Methicillin resistant *S. aureus* (MRSA) is able to resist β -lactamic antibiotics and eventually is a slime producer. Conventional chemotherapies against MRSA are ineffective. Essential oils (EOs) are oily liquids obtained from aromatic plants chemically constituted by terpenes. Their antimicrobial activity against clinical and foodborne pathogenic bacteria has been described. The aim of this work was to study the inhibitory activity of terpenes on slime producing *Staphylococcus aureus* methicillin sensitive (MSSA) and MRSA. Terpenes were tested against MRSA and MSSA with the resazurin microdilution assay.

The terpenes active against all the microorganisms were carvacrol, thymol, geraniol and eugenol. Carvone was only active against *S. aureus* MSSA, while citronellol, menthol, menthone and myrcene did not show antimicrobial activity. The terpenes active against MRSA when slime was induced were carvacrol, eugenol, geraniol and thymol. This strain showed activity of carvacrol, carvone, eugenol, geraniol and thymol when slime was not induced. The results obtained clearly showed that carvacrol and thymol had the best antimicrobial activity. Carvacrol, eugenol and thymol could be used for therapeutical formulations in replacement of antibiotic to treat diseases caused by resistant microorganisms such as MRSA.

Keywords: terpenes, antimicrobial activity, slime, methicillin resistant *S. aureus*

Corresponding author: Mic. Nicolás Gallucci, E-mail: mgallucci@exa.unrc.edu.ar.

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INTRODUCTION

The genus *Staphylococcus* includes microorganisms widely distributed in nature. *Staphylococcus aureus*, particularly, is an important etiological agent, responsible for several diseases in humans and animals. They produce virulence factors that include exotoxins, enzymes, surface proteins and extracellular polysaccharides. (Aguilar et al. 2001; Veenstra, et al. 1996). Slime is an outer structure, formed after growth in special media and placed outside the cell wall, composed by extracellular carbohydrates forming a net where cells can reproduce themselves and adhere to the surfaces of prosthetics and plastic catheters (Aguilar et al. 2001, Salyers and Whitt. 1994). Slime producing strain loses this ability upon subculture (Rather et al. 1986). It is considered a virulence factor with the ability to inhibit chemotaxis, phagocytosis, phagocyte oxidative response and amylogen lymphocyte proliferation. It diminishes coagulation and macrophages uptake by the spleen kidney and linfatic nodule. It also constitutes a mechanical barrier against antibiotics. (Muryam et al. 1991). Slime production has been shown to play a major role in the pathogenesis of infections caused by Gram positive and Gram negative bacterias. The role of slime in infections caused by coagulase-negative staphylococci has been suggested. (Christensen et al. 1983; Christensen et al. 1985; Christensen et al. 1987, Heilmann et al. 1996, Ishak et al. 1985, Mack et al. 1992, Yamasaki, et al. 2004).

Nowadays, there are several effective chemotherapies in the treatment of *S. aureus*, but not effective against methicillin resistant *S. aureus* (MRSA). This is an emergent pathogen, which is able to resist β -lactamic antibiotics, including cephalosporines and responsible for nosocomials sepsis and infections produced on prosthetics (Moreira et al. 1997).

Essential oils are aromatic oily lipids, generally secondary metabolites, obtained from plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). They are variable mixtures of monoterpenes and sesquiterpenes. (Burt. 2004, Cowan. 1999, Dorman and Deans. 2000). Plant essential oils and their components are known to exhibit antimicrobial activities against clinical, food-borne pathogens and food spoilage bacteria. It has been reported that the antibacterial activity derives from terpenoid and phenolics compounds in the oils (Burt. 2004, Carvalho et al. 2005, Cox et al. 2000, Demo, et al. 2001, Dorman and Deans. 2000, Zunino et al. 1997).

There is an increasing need to find new substances with the ability to inhibit resistant microorganisms and the slime producer strains. The aim of this work was to study the inhibitory activity of terpenes on slime producing methicillin resistant strains.

EXPERIMENTAL

Reagents

The terpenes used were: carvacrol, carvone, citronellol, eugenol, geraniol, menthol, menthone, myrcene, thymol. A solution of resazurin (Sigma-Aldrich) was prepared to 0,01% (w/v) in sterile distilled water. The emulsifier used was 0.15% of sterilized agar.

Tested microorganisms

The activity of the terpenes was tested against *Staphylococcus aureus* methicillin sensitive (MSSA) and *Staphylococcus aureus* methicillin resistant (MRSA)

Slime production

S. aureus MSSA and MRSA were plated on blood agar plates and incubated for 24hs, at 37°C. Colonies from blood agar plates were inoculated in tubes containing 5ml of Triptein Soy Broth (TSB), supplemented with 0.24% of glucose. They were incubated for 24h, at 37°C. After this time the liquid from the tubes was removed and they were washed with distilled water and coloured with safranin solution for 30 minutes at room temperature. They were washed and dried and "slime" production was observed like a coloured film in the botton of the tube. (Christensen et al. 1985).

Determination of the minimum inhibitory concentration (MIC)

Culture methods: Tubes containing Mueller-Hinton Broth (MHB) with 0.15% (w/v) agar were inoculated with each microorganism and incubated during 18 h, at 37°C. Firstly, it was determined, the cell concentration necessary to cause reduction of resazurin within 2 h. Serial 10 fold dilution of each culture were prepared in MHB. 170 μ l of the inoculum were dispensed into tubes containing 20 μ l of agar solution (0.15% w/v) and 10 μ l of resazurin solution and were incubated for 2 h at 37°C. The appropriate dilution to work was that one unable to reduce resazurin. Resazurin is a redox indicator that is blue in its oxidized form and pink in its reduced form.

Resazurin MIC assay: Serial twofold dilutions of each terpene were prepared by vortexing the terpene at room-temperature with sloppy agar. The assay medium (MHB) was inoculated with the tested organism to yield a final cell density \approx 1 log cycle lower than the cell density required to reduce resazurin. A sterile 96-well microtitre tray was set up with each of the test bacteria as follows: column 1-9, 170 μ l inoculum plus 20 μ l of a terpene dilution; column 10, 170 μ l inoculum plus 20 μ l terpene diluent (positive control); column 11 and 12, sterile resazurin assay medium plus 20 μ l terpene diluent (negative control). Two trays were prepared for each organism and incubated at 37°C for 3h. After incubation 10 μ l of resazurin solution was added to all wells. After a second incubation of 2 h at 37°C, wells were assessed visually for colour change, with the highest dilution remaining blue indicating the MIC. (Mann and Markham. 1998).



Determination of the Minimum Bactericidal Concentration (MBC)

To determine the MBC, 100 µl from each microwell that presented blue colour were dispersed on AMH plates and incubated 24h at 37°C. MBC was defined as the minimal concentration of terpene that inhibits growth in 99.99%. Plates that presented bacterial growth were considered bacteriostatical. (Finengold and Baron. 1992).

RESULTS

a- Slime production

Cultures of MSSA and MRSA grown in TSB supplemented with glucose 0.24% for 24h. were discarded and tubes were stained with safranin (10% v/v) to reveal the presence of the biofilm. MRSA was able to produce slime, which was observed as a viscid coloured layer of bacteria adherent to the test tube wall (Slime Induced: SI). In contrast, MSSA was a slime non-producing bacteria, demonstrated by the absence of adherence to the tubes walls.

Besides, MRSA was subcultured five times in TSB without glucose 0,24%, loosing the ability to produce slime (Slime not Induced: SNI).

b-Minimum Inhibitory Concentration of terpenes.

The terpenes active against both *Staphylococcus aureus* strains were carvacrol, eugenol, geraniol and thymol. Carvone was active against MSSA and MRSA only when slime was not induced. Citronellol, menthol, menthone and myrcene did not show antimicrobial activity (Table 1).

MSSA obtained MIC values of 0.4 mg/ml for carvacrol, 0.8 mg/ml for thymol and 0.8 mg/ml for carvone. Eugenol obtained a MIC value of 3.5 mg/ml and geraniol showed a MIC value of 23.4 mg/ml. (Table 1).

The terpenes with antimicrobial activity against MRSA when slime was induced were carvacrol, eugenol, geraniol and thymol. Carvacrol and thymol produced the lowest MIC values (MIC: 1.60 mg/ml and 3.17 mg/ml, respectively). The terpenes eugenol and geraniol obtained MIC values of 14 mg/ml and geraniol 5.8 mg/ml.

The results obtained on this strain when slime was not induced showed the same MIC values for carvacrol, eugenol, geraniol and thymol.

It is worth to note that when the ability to produce slime was absent in *S. aureus* MRSA it became sensitive to carvone action, while this microorganism was resistant to this terpene when slime was present. The MIC value of carvone was 0.8 mg/ml for MRSA without slime and for MSSA (not slime producer) (Table 1).

Carvacrol and thymol showed the best antimicrobial activity for *S. aureus*, while it was needed 4 times more concentration of eugenol to inhibit MRSA compared to MSSA and 4 times more concentration of geraniol to inhibit MSSA compared to MRSA.

The MBC was performed with terpenes that showed inhibitory activity against the strains. Carvacrol and thymol showed bactericidal activity while eugenol and geraniol were bacteriostatic.

MO	<i>S. aureus</i>	<i>S. aureus</i> MRSA	
Terpenes	MSSA NSP		
		SI	SI
Carvacrol	0.40	1.60	1.60
Carvone	0.8	NI	NI
Citronellol	NI	NI	NI
Eugenol	3.5	14.0	14.0
Geraniol	23.4	5.8	5.8
Menthol	NI	NI	NI
Menthone	NI	NI	NI
Myrcene	NI	NI	NI
Thymol	0.8	3.17	3.17

Ref: NSP: Not slime producer; SI: Slime induced; SNI: Slime not induced; NI: No inhibition

DISCUSSION

The results obtained with terpenes clearly showed that carvacrol and thymol had the best antimicrobial activity against the microorganisms used in these experiences. Geraniol and eugenol were active but in a minor concentration compared to carvacrol and thymol.

Carvacrol, eugenol and thymol are monocyclic, oxygenated, aromatic monoterpenes like menthol and menthone. The formers were highly active while the last ones did not show antimicrobial activity. Geraniol and citronellol are acyclic, oxygenated, aromatic monoterpenes. Geraniol showed the least inhibitory activity and citronellol was not active. Myrcene, the only hydrocarbonated monoterpene tested was not able to inhibit any of the microorganisms. Several reports on the antimicrobial activity of some monoterpenes showed that the number of double bonds in the structure and acyclic, monocyclic and/or bicyclic structure has no significant influence on its activity but in this experience aromatic monoterpenes (carvacrol, eugenol and thymol) showed the best inhibitory activity. (Zygadlo et al. 1996, Zygadlo and Juliani. 2000). The relative inactivity of



hydrocarbons like myrcene is associated with its low aqueous solubility, while the formation of hydrogen bonding is found to be associated with high antimicrobial activity. However, oxygenated terpenoids show characteristics and distinct activity pattern towards microorganisms. Moreover, the essential oils constituents that contain alcohols possess higher activity than the corresponding carbonyl compounds. (Zygadlo and Juliani. 2000)

The gram positive bacteria *S. aureus* MSSA and MRSA showed similar behaviours against the terpenes. It is worth to note that carvacrol, eugenol, geraniol and thymol were able to inhibit MRSA with slime at the same MIC, what means that these terpenes could be able to pass through the exopolisaccharide layer and maintain the inhibitory and bactericidal effects. In contrast, carvone was able to inhibit MSSA and MRSA when slime was not present; but it was unable to inhibit MRSA when slime was present which could be caused by the fact that this terpene is unable to pass through the slime. The slime is an exopolisaccharide structure located around the cell wall of bacteria, with some differences from the capsule of some gram positive bacteria, that could be observed with electronic microscopy. The slime is associated to the colonization of microorganisms; its formation depends on the genotype and the environment, increasing its production when hydrocarbons and amino acid concentration is high. The ability to form slime could be lost when microorganisms are successively cultivated in artificial media, depending on the culture composition. (Baselga et al. 1994, Rather et al. 1986). It also functions as a mechanical barrier for antibiotics due to its polianionic composition and it helps in the cells replication. (Muryam et al. 1991). Terpenes with activity on slime producing strains could be used to inhibit and/or kill them. What is more, essential oils, with major terpenic components as carvacrol and thymol could have similar effects as the individual terpenes over slime producing strains.

In the future carvacrol, eugenol and thymol could be used for terapeutical formulations in replacement of antibiotic to treat diseases caused by resistant microorganisms such as *S. aureus* MRSA and slime producig strains. Citotoxic and genotoxic experiences in-vivo are needed in order to use these compounds for human and animal illnesses and to preserve food.

Note: Part of this study was presented at the 'II Reunión de Biotecnología aplicada a plantas medicinales y aromáticas' (Second Biotechnology Meeting on Medicinal and Aromatic Plants), Córdoba, Argentina, 2009.

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