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Inhibitory effect of β-pinene, α-pinene and eugenol on the growth of potential infectious endocarditis causing Gram-positive bacteria

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This study was led with the purpose of evaluating the effectiveness of eugenol, β-pinene and α-pinene in inhibiting the growth of potential infectious endocarditis causing gram-positive bacteria. The phytochemicals Minimum Inhibitory Concentration-MIC was determined by solid medium diffusion procedure, while the interference of the MIC values on the bacterial cell viability was performed by viable cells count. Staphylococcus aureus, S. epidermidis, Streptococcus pneumoniae *and* S. pyogenes *strains were used as test microorganisms. The assayed phytochemicals showed effectiveness in inhibiting all assayed bacteria strains presenting MIC values between 2.5 and 40 μL/mL. Eugenol showed the lowest MIC values which were between 2.5 and 5 μL/mL for the most bacteria strains. MIC values found to the phytochemicals were able to inhibit the cell viability of S. aureus providing a total elimination of the bacteria inoculum in a maximum time of 24 hours of exposure. These data showed the interesting antibacterial property of the assayed phytochemicals and support their possible and rational use in the antimicrobial therapy.*

Uniterms
- Endocarditis
- Gram positive bacteria
- Phytochemicals
- Antibacterial activity

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INTRODUCTION

Infectious endocarditis (IE) is an infectious disease attacking mainly the heart valves endocardium. Most cases of IE has been related to infections caused by *Streptococcus* and *Staphylococcus* genus, although bacteria inserted in the HACEK group (*Haemophillus parainfluenzae, H. aphrophilus, H. paraphrophilus, H. influenzae, Actinobacillus actinomycetemcomitans, Cardiobaccterium hominis, Eikenella corrodens, Kingela kingae* and *K. denitrificans*) have presented an emerging importance as IE causing agents (Baddour *et al.*, 2005; Bashore *et al.*, 2006). Fungi can act as IE etiological agents in immunosuppressed people and/or in catheter and valve prosthesis users (Niwa *et al.*, 2003; Watanabe *et al.*, 2003; Anguera *et al.*, 2005).

Even regarding the advances in the clinical therapy the IE still presents a high mortality rate (20% to 30% of the cases). It has been related to the development of antibiotic resistance by IE causing agents, mainly when regarded the *Staphylococcus* genus because the appearance of methicillin-resistant *Staphylococcus aureus* (MRSA) (Nakatani *et al.*, 2003; Ishiwada *et al.*, 2005). Some researchers have reported that the rising of MRSA strains has been responsible for an increase in the action of *Staphylococcus* genus as IE causing agent. On the other hand, the participation of *Streptococcus* genus in IE etiology has decreased (Ako *et al.*, 2003; Pigrau *et al.*, 2005; Ferreiros *et al.*, 2006).

Currently there has been an increasing interest in studying the biological properties of plant and derivatives in order to discover alternative biologically active compounds (Seidil, 2000; Araújo *et al.*, 2004; Lima *et al.*, 2005). Plant products have received a renewed interest in their use as alternative source of antimicrobial compounds because the uncontrolled use of the antimicrobial chemotherapy (Dorman, Deans, 2000). The successful history of microbial chemocontrol lies in the continuous rising of microbial strains with resistance to the classical antibiotics (Notermans, Hoogenboon-Verdegaal, 1992). Researches regarding the antimicrobial activity of phytochemicals have been yet little emphasized being the most studies addressed to evaluate the antimicrobial effect of essential oils and extracts (Nascimento *et al.*, 2000; Souza *et al.*, 2005).

This study aimed to evaluate the effectiveness of eugenol, β-pinene and α-pinene in inhibiting the growth of Gram-positive bacteria known as potential IE etiological agents.

MATERIAL AND METHODS

Phytochemicals

Eugenol, α-pinene and β-pinene were supplied by Department of Pharmaceutical Sciences, State University of Ponta Grossa, Paraná, Brazil. The phytochemicals were tested at concentrations ranging from 160 to 1.25 µL/mL, and the solutions were prepared prior to the antimicrobial assays according to Souza *et al.* (2007). Molecular structures of the phytochemicals are shown in Figure 1.

Bacteria strains

Staphylococcus aureus ATCC 13150, *S. aureus* ATCC 6538, *S. aureus* ATCC 25932, *S. aureus* ATCC LB 126, *S. epidermidis* SSI 1, *S. epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 19615, *Streptococcus*

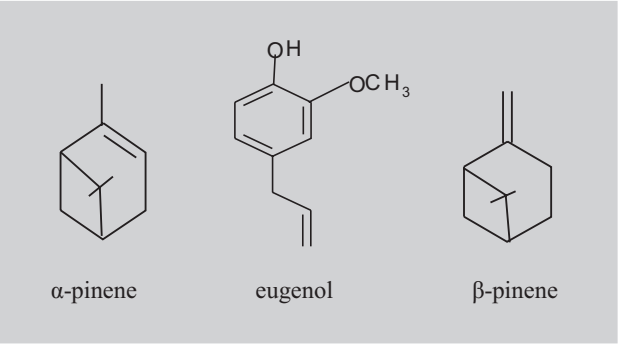

FIGURE 1 - Molecular structures of phytochemicals used in the antimicrobial assays.

pyogenes ATCC 8668 and *S. pneumoniae* ATCC 11773 were used as test microorganisms. These strains were supplied by Laboratory of Clinical Bacteriology, Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil. Stock cultures were maintained on nutrient agar slants at 4 °C. Inocula used in antimicrobial assays were obtained from overnight cultures grown on nutrient agar slants at 37 °C and diluted in sterile saline solution (0.85 % w/v) to have a final concentration of 10^6 colony forming unity (cfu)/mL (adjusted according to the turbidity of 0.5 McFarland scale tube).

Determination of the Minimum Inhibitory Concentration

Microplate bioassay was used to determine the Minimum inhibitory concentration - MIC of the assayed phytochemicals. For this was used a microplate with 96 wells (flatted bottom) and cap. The 96-well microplates were prepared dispensing into each well 100 µL of doubled strength nutrient broth inoculated with 1 mL of the bacterium inoculum prior the assay (1:9 v/v). 100 µL of the phytochemical solution with their respective concentration was transferred into seven consecutive wells. Final volume in each well was 200 µL. The solution having the highest concentration was added into the first well and the lowest concentration was added into the penultimate well. The last well contained 200 µL of nutrient broth inoculated with the bacterium inoculum was used as positive control (strain viability). The microplate was asseptically sealed, followed by mixing on plate shaker (300 rpm) for 30 s, and incubated at 28 – 30 °C/48 h (Viljoen *et al*., 2003; Sahin *et al*., 2004). Antibacterial activity was detected using a colorimetric method by adding 200 µL of resauzurin staining (0.1 %) aqueous solution in each well at the end of the incubation period. MIC was defined as the lowest phytochemical concentration able to inhibit the bacteria growth, as

indicated by resauzurin staining (bacteria died cells are not able to change the staining color by visual observation – blue to red) (Palomino *et al*., 2002; Burt, Reinders, 2003).

Kill time study

Kill time study was carried out with MIC values previously found in the microplate bioassay. For this was used the viable cells count method. 5mL of double strength nutrient broth was inoculated with 1mL of the bacterium suspension (approximately 10^6 cfu/mL). After that, 4mL of the phytochemical solution, with concentration adjusted to provide a phytochemical final concentration similar to the MIC previously determined, was added to the system and followed by shaking for 30 s using Vortex. The system was incubated at 37 °C. At different time intervals (1, 2, 4, 8, 12 and 24 hs) of exposure, 1mL of the suspension was serially diluted (10^{-1} – 10^{-5}) in sterile peptone water (0.1% w/v) and inoculated on nutrient agar Petri dishes for 24 h at 37 °C (Viljoen *et al.*, 2003; Souza *et al*., 2007). The mean number of colonies (cfu/mL) was counted and compared with that found in the control assay in which the essential oil solution was replaced by sterile distilled water. The results were expressed in log of cfu/mL.

Statistical analysis

Statistical analysis was performed to determine significant differences (P<0.05) by Tukey test in the bacteria kill time assays. For this was used Sigma stat 2.03 computer program.

RESULTS AND DISCUSSION

MIC values found for eugenol, α-pinene and β-pinene are shown in Table 1. The assayed phytochemicals presented interesting antimicrobial potential noted by low MIC values. MIC values oscillated between 2.5 (eugenol x *S. pneumoniae* ATCC 11773) to 40 µL/mL (β-pinene x *S. epidermidis* ATCC 12228). All assayed bacteria strains were sensitive to the phytochemicals. On the other hand, some strains presented resistant behavior to the standard antibiotics, mainly to gentamicin. Eugenol provided the most intense antibacterial effect showing MIC values between 2.5 to 5 µL/mL for the most bacteria strains. *S. aureus* presented as the most resistant bacterium with MIC values between 10 and 20 µL/mL for the tested phytochemicals. For the other bacteria species were found MIC values oscillating between 2.5 to 5 µL/mL for most interactions.

TABLE I - MIC of some phytochemicals on potential infectious endocarditis causing gram-positive bacteria

Bacteria	Phytochemicals (µL/mL)			Penicillin	Gentamicin
	Eugenol	β-pinene	α-pinene	(10 µg/mL)	(10 µg/mL)
S. aureus ATCC 13150	10	20	20	S	R
S. aureus ATCC 6538	10	20	20	S	S
S. aureus ATCC 25923	10	20	20	S	S
S. aureus LB 126	10	20	10	S	S
S. epidermidis SSI 1	5	20	5	S	S
S. epidermidis ATCC 12228	5	40	5	S	S
S. pyogenes ATCC 19615	5	20	5	S	R
S. pyogenes ATCC 8668	5	20	10	S	R
S. pneumoniae ATCC 11773	2.5	20	5	R	S

S: sensitive; R: resistant

Figure 2, 3 and 4 show the effect of eugenol, β-pinene and α-pinene MIC values on the *S. aureus* ATCC 6538 cell viability. *S. aureus* was chosen to be included in the kill time assays because it is known as the main etiological agent of IE acquired at the community or in nosocomial environment (Niwa *et al.*, 2003; Ishiwada *et al.*, 2005). Phytochemicals MIC values were able to cause a significant ($p<0.05$) inhibition of *S. aureus* cell viability providing a bactericidal effect in a maximum time of 24 hours of exposure. Most intense inhibitory effect was showed by β-pinene and eugenol causing a total elimination of the initial bacterial inoculum after 8 hours of exposure. β-Pinene and eugenol presented a bacteriostatic effect until 2 hours of exposure and from this time on it was established a progressive decrease in the bacteria cell count. α-pinene provided a bacteriostatic effect until 4 hours of exposure being established its cidal effect after 24 hours of exposure.

Some researches have found an antimicrobial effectiveness in phytochemicals, although these data still are considered as incipient when compared to the availability of researches regarding the antimicrobial properties of plant extracts and essential oils (Vasquez *et al*., 2001; Nostro *et al*., 2004). Gayoso *et al*. (2004) and Lima *et al*. (2005) found antimicrobial effectiveness of pinenes against moulds and pathogen yeasts providing a cidal effect between 2 and 5 hours of exposure. Hao *et al*. (1998) reported antimicrobial activity of eugenol on Gram positive bacteria, including *S. aureus* strains.

Phytochemicals are small organic biomolecules generally hydrophobic and designated as naturally occurring antibiotics. Cytoplasm membrane coagulation, breakdown of protons motive force, breakdown of electron flux and active transport unbalance are some events responsible for providing the antimicrobial property of phytochemicals (Sikkema, 1995; Carlson *et al*., 2002). These biological events have been believed for no occurring separately being some of them activated as consequence of other ones (Sikkema, 1995; Burt, 2004).

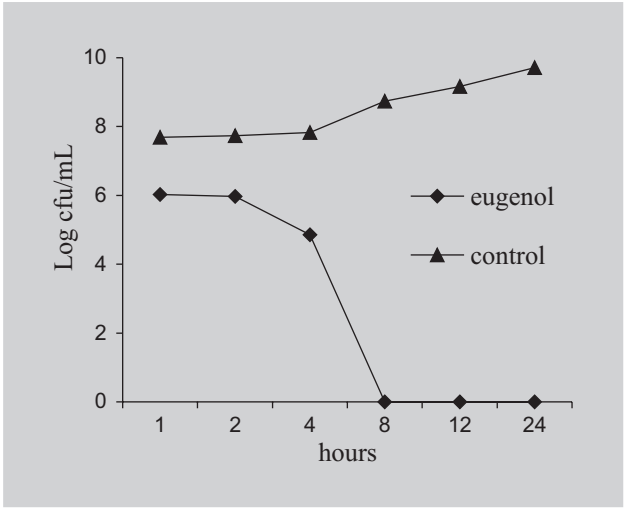

FIGURE 2 - Effect of eugenol (10 µL/mL) on the *S. aureus* ATCC 6538 viable cells number.

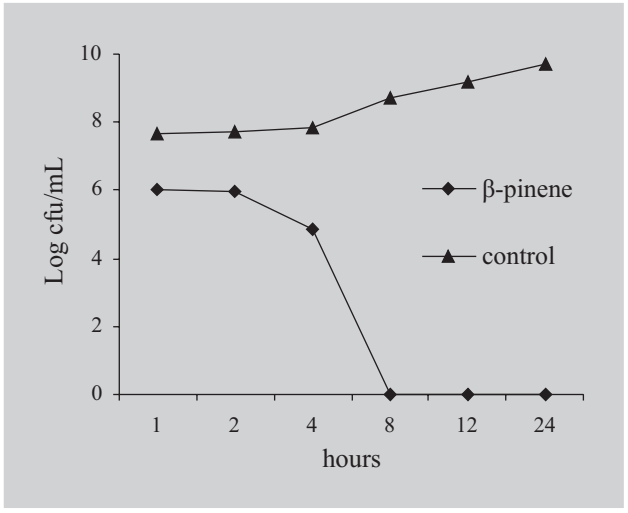

FIGURE 3 - Effect of β-pinene (20 µL/mL) on the *S. aureus* ATCC 6538 viable cells number.

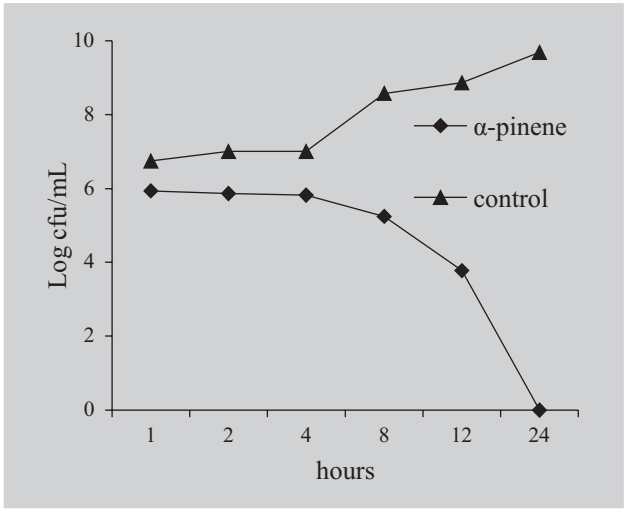

FIGURE 4 - Effect of α-pinene (20 µL/mL) on the *S. aureus* ATCC 6538 viable cells number.

Our data show the intense antimicrobial potential of eugenol, β-pinene and α-pinene which were able to inhibit significantly the growth and cell viability of potential infectious endocarditis causing gram-positive bacteria. These results support the recognizing of phytochemicals as alternative antimicrobial compounds to be used in pharmaceutical formulations used in the antibacterial therapy. Further researches are needed to

evaluate their antimicrobial effectiveness against pathogen microorganisms able to act as etiological agents of different infections diseases, as well as regarding their toxicological and pharmacological aspects.

RESUMO

Efeito inibitório de eugenol, β-pineno e α-pineno sobre o crescimento de bactérias Gram-positivas potencialmente causadoras de endocardite infecciosa

Este estudo foi conduzido com a proposta de avaliar a efetividade de eugenol, β-pineno e α-pineno em inibir o crescimento de cepas de bactérias Gram-positivas potencialmente causadoras de endocardite infecciosa. A Concentração Inibitória Mínima-CIM dos fitoconstituintes foi determinada através do método de difusão em meio sólido, enquanto a interferência da CIM sobre a viabilidade celular bacteriana foi avaliada através da contagem de células viáveis. Cepas de Staphylococcus aureus, S. epidermidis, Streptococcus pneumoniae *e* S. pyogenes *foram utilizadas como microrganismos teste nos ensaios antimicrobianos. Os fitoconstituintes ensaiados mostraram efetividade em inibir todas as cepas bacterianas utilizadas como microrganismos testes apresentando valores de CIM entre 2.5 e 40 μL/mL. Eugenol apresentou os menores valores de CIM, os quais estiveram entre 2,5 e 5 μL/mL para a maioria das cepas bacterianas. Os valores de CIM encontrados para os fitoconstituintes foram capazes de inibir a viabilidade celular de* S. aureus *causando uma total eliminação do inóculo bacteriano em um tempo máximo de 24 horas de exposição. Estes dados mostram o intenso potencial antibacteriano dos fitoconstituintes ensaiados e suportam sua possível e racional aplicação na terapia antimicrobiana.*

UNITERMOS: Endocardite. Bactérias Gram-positivas. Fitoconstituintes. Atividade antibacteriana.

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