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Anti-biofilm and Antibacterial Effect of Essential Oils and Their Major Compounds

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Abstract: Essential oils are plant secondary metabolites commonly used in traditional medicine to treat infectious diseases. Along with their compounds, oils can contribute to development of new antimicrobial/anti-biofilm products. Our study evaluated antibacterial activity of essential oils and their major compounds on *Escherichia coli* and *Staphylococcus aureus* planktonic cells and anti-biofilm activity. The effect of essential oils and their major compounds on biofilm and planktonic cells was assessed by quantifying the number of viable cells (CFU). Biomass quantification (absorbance = OD_{570nm}) was also performed to evaluate anti-biofilm activity. Planktonic cells were more susceptible to the action of agents. *Escherichia coli* was reduced by 100 % with cinnamon and palmarosa oil. The treatment showed an interesting anti-biofilm activity, whereas green tea essential oil and its major compound, terpinen-4-ol, yielded less effective results. Reduction of viable cells in biofilm biomass was significant. Although our research is one of the first experiments in anti-biofilm activity of essential oils and their compounds against *Escherichia coli* and *Staphylococcus aureus*, pharmacological data confirm that the materials used in the trial do not pose health risk. Thus, essential oils and their compounds can be safely used in research to identify new antibacterial and anti-biofilm products against pathogenic bacteria.

Key words: Natural antimicrobial, bacterial biofilms, pathogen bacteria.

Introduction

Biofilms comprise highly structured matrix-enclosed communities ⁴ and represents a mode of growth that allows bacteria to both survive in hostile environments and colonize new niches through dispersal mechanisms ^{15,22,31}. Furthermore, biofilm bacteria show coordinated behavior by building complex three-dimensional structures and functionally heterogeneous bacterial communities ^{15,37}. Biofilm cells express genes in patterns ³⁴ differently from their planktonic counterparts. Direct observation shows that biofilms are ubiquitous in

both natural and pathogenic ecosystems ^{3,5}; however, planktonic cultures in the systems should be studied as well. One of the advantages of planktonic cells forming biofilms on the surface is protection against action of antibiotics and antimicrobial agents ^{10,14,23}. Over the last two decades several publications have considered the genus *Staphylococcus* cap-able of forming biofilm bacteria, causing infections defined as “chronic infections associated with biopolymers” ¹². After biomaterial implantation, such staphylococcal infections may occur early or late ¹⁹. Due to its ability

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to adhere to inert surfaces, *S. aureus* has become the major agent of hospital-acquired infections associated with biopolymers worldwide^{17,19}.

Escherichia coli can behave as intestinal commensal, diarrheagenic and extra intestinal pathogenic agent. It is responsible for most community and hospital-acquired Gram-negative bacterial infections. *E. coli* is found in the gut flora, also colonizing the genital mucosa. Thus, bacteria may enter the urinary system and adhere to mucosal surfaces and implant devices⁹. Adherence and formation of biofilms cause infections that contribute to significant morbidity rate, although they may be short term cases⁹.

Thus, alternative strategies or effective agents to act against biofilm-producing micro-organisms are of great interest. Natural drugs could be an interesting approach to limit emergence and spread of such organisms, which are currently difficult to fight. Recently, there has been considerable interest in the study of plant materials as sources of new compounds^{18,33} for processing therapeutic agents. One approach could be the use of essential oils, which have been found to be potential and safe antibacterial agents²⁹. The functional use of natural essential oils as antibacterial agents has been increasing in medicine and dentistry. Mouthwashes containing essential oils can kill oral microorganisms by inhibiting their enzyme activity and breaking down their cell walls³². Essential oils also inhibit coaggregation between early and late colonizers, e.g. Gram-negative anaerobic periodontopathogens³². In addition, essential oils inhibit formation of bacterial biofilms on surfaces such as polystyrene¹³.

In our study, three essential oils were selected based on their use as antimicrobial agents in Brazilian traditional medicine. We also used their respective major compounds to test and compare effects on planktonic bacteria and biofilms. Thus, our study evaluated anti-biofilm and antibacterial activity of essential oils and their major compounds in planktonic cells of *E. coli* and *S. aureus*.

Materials and methods

Essential oils and major compounds

Essential oils of *Cinnamomum zeylanicum* (cinnamon); *Cymbopogon martini* (palmarosa) and *Melaleuca alternifolia* (green tea) extracted

from plant leaves by hydro-distillation were purchased from Ferquima Indústria e Comércio Ltda. (Vargem Grande, São Paulo, Brazil). According to the company, the major compound of cinnamon is eugenol (86 %); geraniol (86 %) in palmarosa oil; and terpine-4-ol (48 %) in green tea oil. The isolated major compounds were purchased from Sigma Aldrich.

Microorganism and inoculum standardization

We used *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 24922. Standardization of number of cells was determined by calibration curve. Cultures were grown on Trypticase Soy Agar (TSA, Merck, Portugal) added to Petri dishes and incubated at 37°C for 24 hours. Some colonies formed on TSA surface were removed and transferred to Erlenmeyer flasks containing 50 ml of Trypticase Soy Broth (TSB, Merck, Portugal), which were incubated at 37°C until reaching the number of cells necessary for the experiment, approximately 10⁸ CFU/ml.

Effect of essential oils on growth of planktonic cells

The solution was formed in accordance with the model proposed by Oliveira *et al.*³⁰ with some modification, as ethanol was replaced by dimethyl sulfoxide (DMSO). We used concentrations 0.48, 0.96 and 1.92 % of essential oils, while concentration of major compounds corresponded to oil concentration. The solution contained DMSO, 0.85 % saline water, 0.5 % Tween 80, and TSB. Aliquots of 100 µl were dispensed into 96-well micro-plates (Orange Scientific, Braine-l'Alleud, Belgium) with 100 µl TSB containing 10⁸ CFU/ml. Wells containing only TSB served as control. The plates were incubated in aerobic conditions for 24 h at 37°C in orbital shaker at 120 rpm. After incubation, planktonic cells were collected for quantification of viable cells (CFU/mL).

Effect of essential oils and compounds on bacterial biofilms

Biofilms were developed on 96-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) in solutions containing essential oils and their major compounds. Bacterial cell suspensions (100 µl of 1 x 10⁸ cells/ml in TSB), essential oils and

compounds (100 μ l of the concentrations previously described) were pipetted into each well and incubated for 24 h at 37°C in orbital shaker at 120 rpm.

Quantification of cultivable cells

Planktonic cells were quantified by plating on TSA medium. To quantify biofilm cells, the wells were washed in sterile water to remove planktonic bacteria. Viable biofilm cells were removed by sonication and serially diluted. Samples were then plated on TSA medium, and plates were incubated at 37°C in aerobic incubator for 18 h prior to enumeration. The number of cultivable bacterial cells was determined and expressed as Log CFU/mL (planktonic) and Log CFU/cm² (sessile). Assays were performed on three separate occasions.

Biomass quantification by Crystal Violet staining

Biomasses of single and mixed biofilms were quantified by adapting the crystal violet (CV) staining method by Stepanovic' *et al.*³⁶. For fixing, we added 200 μ l of 99 % methanol (Vaz Pereira, Portugal) to each well containing adhered cells or biofilms treated with essential oils, as previously described. After 15 minutes the methanol was removed, and the plates were allowed to dry at room temperature. Then we added 200 μ l of crystal violet stain (CV; 1 % v/v) (Merck, Portugal) to all wells. After 5 minutes, CV excess was removed and plates were gently washed in water. Finally, 230 μ l of acetic acid (33 % v/v) (Pronalab, Portugal) were added to the wells to dissolve CV stain, and the absorbance was measured at 570 nm. All assays were performed in triplicate on three separate occasions.

Statistical analysis

Data were analyzed with GraphPad Prism®. One-way ANOVA tests were performed and $p < 0.01$ was considered significant.

Results

Antimicrobial activity against planktonic cells

Essential oils and their major compounds showed significant antimicrobial potential ($P < 0.05$) against planktonic bacteria of *E. coli* and *S. aureus*

(Fig. 1). *Escherichia coli* was more susceptible to the action of cinnamon and palmarosa oils at concentration 0.96 %, with 100 % reduction of cell growth. The lowest concentrations were also considered effective, as reductions were greater than 5 log CFU. Eugenol and geraniol reduced more than 90 % planktonic cells of *E. coli* (Fig. 1). Essential oil of green tea was the least effective, as it reduced only 1.95 and 2.03 log CFU at concentrations 0.12 % and 0.48 % respectively. However, reduction was over 5 log CFU at concentration 0.96 % (Fig. 1) while its major compound, geraniol, reduced more than 7 log CFU for both (Fig. 1).

Reduction of planktonic cells of *S. aureus* was also significant ($p < 0.05$) (Fig. 1 and 2). Viable cells in biofilms were more resistant to essential oil action than planktonic bacteria (Fig. 2). Green tea essential oil showed low anti-biofilm potential while its major compound terpinene-4-ol had no significant anti-biofilm activity against *E. coli* ($P > 0.05$). The maximum reduction against *E. coli* was 1.66 log CFU at concentration 0.96 %. Palmarosa oil had the greatest anti-biofilm potential at concentration 0.96 %, reducing 3.04 log CFU. However, as well as geraniol, anti-biofilm potential was less than 50 % at lower concentrations. Cinnamon essential oil was the most effective against viable biofilm cells of *E. coli*, with all concentrations showing reduction over 3 log CFU. At concentration 0.96 %, reduction was over 75 %. Eugenol, however, did not show the same efficiency, decreasing only 1.88 log CFU (Fig. 3).

The action of green tea, palmarosa and cinnamon essential oils was significant ($P < 0.05$) in biomass reduction of *E. coli* biofilm (Fig. 3). The major compounds were also effective (Fig. 3). Formation of *E. coli* biomass was inhibited by more than 93 % in treatments with oils and their major compounds.

Reductions of viable biofilm cells of *S. aureus* were lower than in *E. coli*. At concentration 0.96 % green tea essential oil showed significant decrease ($P < 0.05$) (Fig. 3), although only reaching 1.76 log CFU. Terpinen-4-ol was not effective to prevent formation of *S. aureus* biomass ($P > 0.05$). Palmarosa essential oil was significantly effective at concentrations 0.48 % and 0.96 %, reducing 3.12 and 4.17 log CFU respectively, while

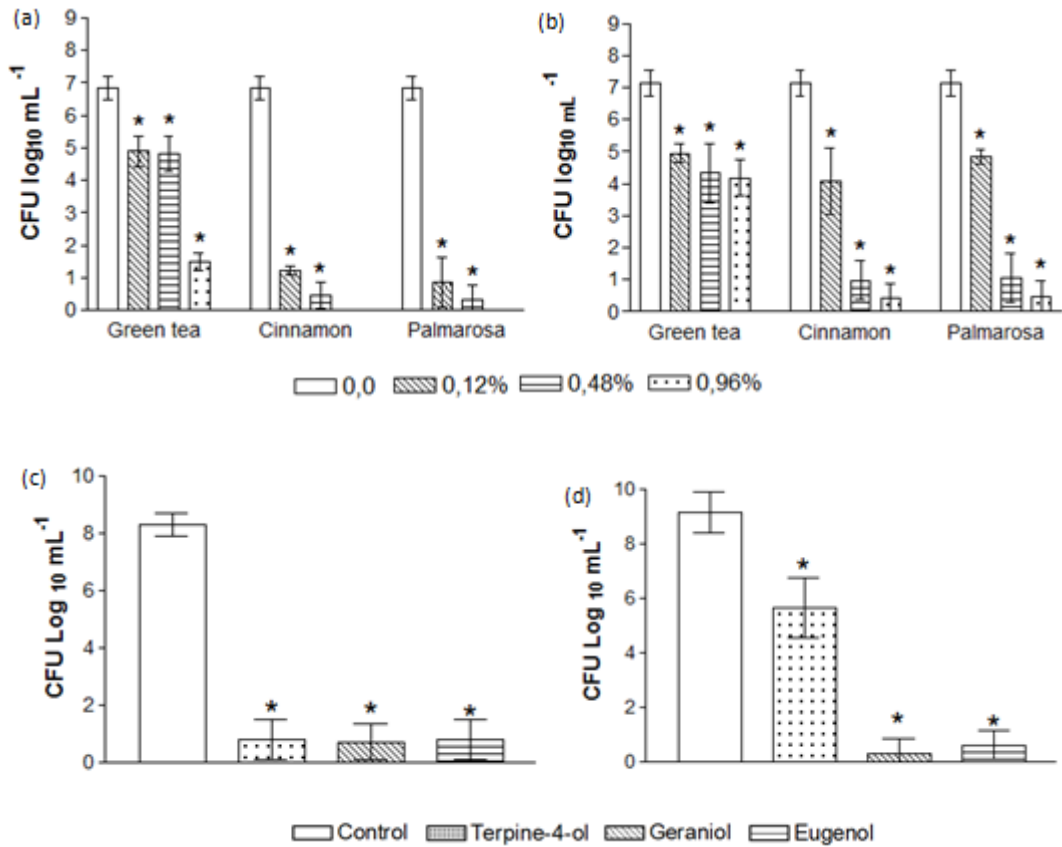


Figure 1. Inhibition *E. coli* (a) and *S. aureus* (b) viable planktonic cells for different concentrations of essential oil and *E. coli* (c) and *S. aureus* (d) for compounds majority of essential oils

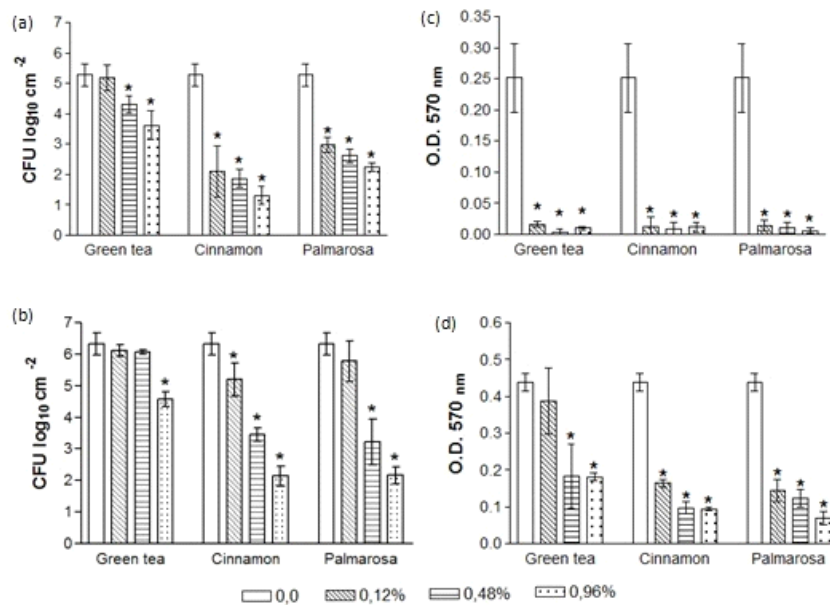


Figure 2. Activity antibiofilm of essential oils on *E. coli* (a) and *S. aureus* (b) cells cultivated in biofilms and *E. coli* (c) and *S. aureus* (d) biomass

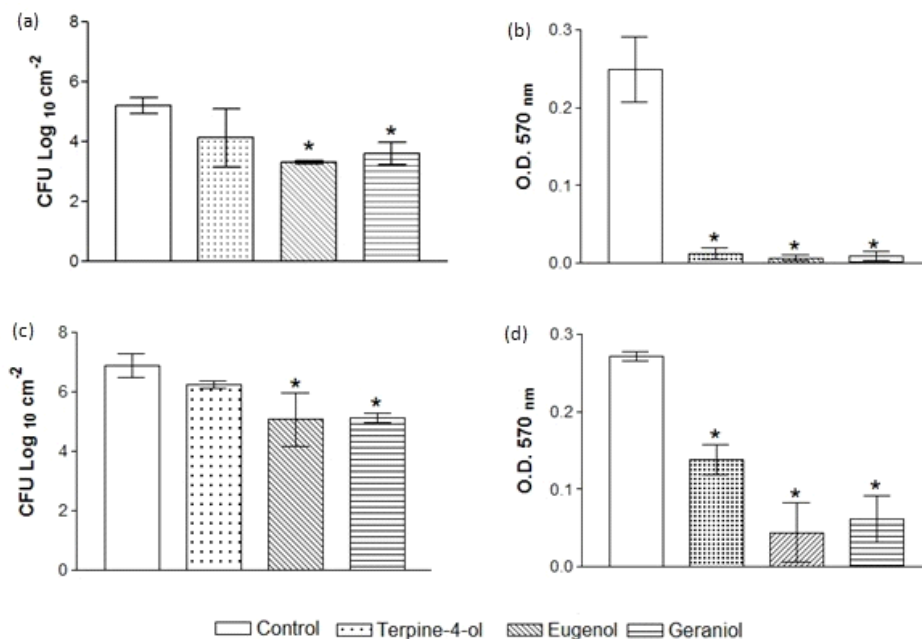


Figure 3. Activity antibiofilm diferente major compounds of essential oil of *E. coli* (a) and *S. aureus* (c) cells cultivated in simple biofilm and *E. coli* (b) and *S. aureus* (d) biomass

geraniol reduced only 25 %. As observed in *E. coli*, cinnamon essential oil also showed greater anti-biofilm capacity against *S. aureus*. Cinnamon essential oil at concentration 0.96 % reduced 66 % viable biofilm cells of *S. aureus*, equivalent to 4.19 log CFU; however, the same efficiency was not observed for eugenol, which decreased 29.22 % cells, equivalent to 2 log CFU.

The effect of essential oils and compounds in *S. aureus* biomass was good except for green tea oil at concentration 0.12 %, which did not prevent biomass formation ($P > 0.05$). Palmarosa and cinnamon essential oils as well as their major compounds showed greater ability to inhibit biomass formation over 62 %. Palmarosa oil at concentration 0.96 % showed the greatest reduction (86.04 %) (Fig.3).

Discussion

Essential oils and their compounds are known to be active against a wide variety of microorganisms, including Gram-positive and Gram-negative bacteria^{7,8,13,16} in both planktonic form^{24,26} and biofilm^{25,27,30}. Bacteria in biofilm mode of growth have been much more resistant to antibiotics than their planktonic counterparts^{21,33}. In

our study, such phenomenon also occurred in the action of essential oils and their compounds. This result confirmed the greater resistance of viable bacterial cells in biofilm rather than in planktonic mode for both *E. coli* and *S. aureus*.

According to Stoodley *et al.*,³⁷ planktonic phenotype differs profoundly from biofilm phenotype, which involves the expression of a large number of genes that allow sessile bacteria to better resist to environmental stresses and adverse conditions, as well as to the presence of antibiotics and antimicrobial agents. In a recent study, mature biofilms of *P. aeruginosa* have shown a protein profile radically different from planktonic bacteria grown in chemostats³⁴. As much as 50 % of detectable proteome (over 800 proteins) have shown a six-fold or greater difference in expression. Of these proteins, more than 300 were detected in mature biofilm samples undetectable in planktonic bacteria. The identified proteins fall into five major classes: metabolism, phospholipids and LPS-biosynthesis, membrane transport and secretion, as well as adaptation and protective mechanisms³⁴.

Several studies have addressed the use of natural compounds as antimicrobial agents^{18,29,33,38}.

However, little has been reported on the effect of cinnamon, palmarosa and green tea essential oils and their respective major compounds eugenol, geraniol and terpinene-4-ol against pathogenic bacteria. According to our knowledge, this is the first study investigating anti-biofilm action of essential oils of cinnamon and palmarosa and their major compounds against *S. aureus* and *E. coli*.

Our study showed an interesting anti-biofilm action of essential oils and their major compounds against *S. aureus* and *E. coli*. Most treatments produced good action on viable biofilm cells and biomass. Essential oils had a better performance than their individual compounds. According to Sandasi *et al.*³³, some compounds of essential oils (α -pinene, linalool, 1.8-cineole, geranyl acetate) were unable to inhibit biofilm growth in *Listeria monocytogenes*. The authors suggest that resistance to inhibition could be the fact that compounds used individually do not have the same activity as oils. Studies have shown that antimicrobial effect of essential oils is due to interaction between oils and their compounds instead of depending on individual components³⁰. Thus, our results were better against *E. coli* than against *S. aureus*, corroborating the results of experiments carried out by Budzynski *et al.*², in which Gram-negative bacteria were very susceptible to damage caused by essential oils of *Melaleuca alternifolia*, *Lavandula angustifolia* and *Melissa officinalis* and some of their major compounds (α -terpineol, terpinen-4-ol, linalool, linalyl

acetate).

Strong and fast anti-biofilm activity of TTO was noticed against *E. coli*, which was eradicated within 1 hour exposure to concentration 0.78 %². However, only planktonic cells were completely eradicated in our study. Differences in the reported data could be due to many factors including differences in the strains, concentration and chemical composition of essential oils, time of exposure to oils, growth stage of biofilm, and nature of surface to which organisms adhere^{1,11,20}.

The findings of this study highlight the promising role of compounds derived from plant secondary metabolism, especially essential oils, as antibacterial and anti-biofilm agents. Literature data on the pharmacological compounds used in this experiment suggest they pose no risk to human or animal health^{6,35}. Therefore, it may be worth further investigation.

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