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ANTIBACTERIAL ACTIVITY EVALUATION OF 15 *EUCALYPTUS* SPECIES ESSENTIAL OILS AGAINST CLINICALLY RELEVANT PATHOGENIC BACTERIA

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ABSTRACT

This study aims to evaluate the potential of 15 *Eucalyptus* species essential oils as alternatives to clinical surface disinfectants with known association to antibiotic resistance. Four reference pathogenic bacteria were tested: *Pseudomonas aeruginosa* (ATCC10145), *Escherichia coli* (CECT434), *Staphylococcus aureus* (CECT976) and *Listeria monocytogenes* (ATCC15313). Gram-positive bacteria revealed higher sensitivity than Gram-negative. Essential oils from *E. bosistoana*, *E. botryoides*, *E. camaldulensis*, *E. cinerea* and *E. citriodora* showed bacterial inhibition for Gram-positive, even higher than with gentamicin and ciprofloxacin (positive controls). *L. monocytogenes* was the most sensitive and *P. aeruginosa* demonstrated resistance to all essential oils. The antimicrobial potential values were 6.25, 6.25 and 12.5% for *E. coli*, *S. aureus* and *L. monocytogenes*, respectively. This study reveals that Eucalyptus essential oils may be useful in order to control pathogenic bacteria as potential complementary treatment or as disinfectants in clinical/hospital environments.

Keywords: *Eucalyptus*, Essential Oils, Antibacterial Activity, Complementary Treatments, Disinfectants

1. INTRODUCTION

The introduction of antibiotics after World War I resulted in a dramatic decrease in death numbers due to bacterial infections. However, the increase of antibiotic resistance is the reason for this medical emergency (Schjørring and Krogfelt, 2010), which has lead inevitably to the emergence and dissemination of resistant bacteria and resistance genes. The inefficacy of the conventional antibiotics is due in part to their often excessive and inappropriate use (Saavedra *et al*., 2010). Reduced susceptibility of microorganisms to antimicrobial products may be acquired through mutation, by plasmid or transposon acquisition, or by the microorganisms' intrinsic properties conferring reduced susceptibility to antimicrobial agents (Alekshun and Levy, 2007; Simões *et al*., 2009). Therefore it becomes of high importance to take a closer look at the traditional and complementary medicine.

Plants Essential Oils (EOs) appear to be the target of intense study resulting in several studies about activity against insects (Yang *et al*., 2004; Nuchuchua *et al*., 2009;

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Senthilkumar *et al*., 2009), fungi (Hood *et al*., 2010), virus (Schnitzler *et al*., 2007) and bacteria (Cimanga *et al*., 2002; Chung *et al*., 2007).

The *Eucalyptus* genus belongs to Myrtaceae family and includes more than 700 species (Francisco *et al*., 2001). These species are distributed all over the world and Portugal is not an exception. There are many evidences that *Eucalyptus* species EOs have a strong antibacterial (Cimanga *et al*., 2002; Chung *et al*., 2007), analgesic, anti-inflammatory and antioxidant effects (Cruz *et al*., 2001; Silva *et al*., 2003).

Previous chemical analysis of *Eucalyptus* species EOs have shown that the major compounds present are 1,8-cineole (eucalyptol), (-)-α-pinene, β-eudesmol, 1-methyl-3-(1-methyl) benzene, globulol, 1-α-terpineol, α-eudesmol, (E)-pinocarveol and valenecene (Nishimura and Calvin, 1979). *Eucalyptus* has been the topic of several studies due to its extensive applications in traditional medicine, mainly in respiratory infections, proving the efficacy of its' products against upper respiratory tract infections (Ben-Arye *et al*., 2011). Globulol found in *Eucalyptus* EOs showed a relevant antimicrobial effect (Manliang *et al*., 2008). It has also been reported that macrocarpals from *E. macrocarpa* and grandinol from *E. perriniana* were effective against Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis)* (Takahashi *et al*., 2004).

Nowadays, hospital infections are a serious concern for health authorities. In order to contain and prevent drug multi-resistant bacteria there have been intensive studies with plant extracts and EOs that show the ability to eliminate or decrease infections (Cimanga *et al*., 2002; Chung *et al*., 2007; Takahashi *et al*., 2004).

Few studies have been published about the potential effect of Eucalyptus EOs as disinfectants of surfaces in clinical and hospital environments associated to the antibiotic resistance phenomena. Thus the aim of the present work is to evaluate the potential of these compounds as alternatives to the traditional clinical disinfectants like bleach that are known to be toxic for human health. In the present study the *in vitro* antibacterial activity of the selected *Eucalyptus* EOs were tested against four clinically significant bacteria largely associated with antibiotic resistance: *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes.*

2. MATERIALS AND METHODS

2.1. Plant Material

The essential oils of *Eucalyptus bosistoana* F. Muell., *Eucalyptus botryoides* Sm., *Eucalyptus camaldulensis* Dehnh., *Eucalyptus cinerea* F. Muell., *Eucalyptus citriodora* Hook., *Eucalyptus cordieri* Trabut, *Eucalyptus dives* Schauer, *Eucalyptus globulus* Labill., *Eucalyptus pauciflora* Sieber ex Spreng., *Eucalyptus polyanthemos* Schauer, *Eucalyptus radiata* Sieber ex DC, *Eucalyptus saligna* Sm., *Eucalyptus smithii* R.T. Baker, *Eucalyptus urophylla* S.T. Blake and *Eucalyptus viminalis* Labill. were kindly provided by the Centro de Biotecnologia Vegetal, Instituto de Biotecnologia e Bioengenharia.

The essential oils were isolated by hydrodistillation for 3 h using a Clevenger-type apparatus according to the European Pharmacopoeia (CE, 2002). The *Eucalyptus* species composition in volatiles was reported previously (Faria *et al*., 2011).

2.2. Bacterial Strains

In the present study four bacterial strains were tested: Two Gram-negative (*Pseudomonas aeruginosa* ATCC10145 and *Escherichia coli* CECT434) and two Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*). These strains were obtained from American Type Culture Collection (ATCC) and from the Spanish Type Culture Collection (CECT). There are several studies on the effects of essential oils with the same bacteria but with different strains (Cimanga *et al*., 2002; Chung *et al*., 2007).

2.3. Antibacterial Assay

Antibacterial activity was tested using the disc diffusion method describe by Bauer *et al*. (1966) with some adjustments. Colonies of bacteria were picked from 24 h cultures in BHI solid medium, inoculated into 4 mL of 0.9% NaCl solution. The cultures were adjusted to 0.5 McFarland standards. A loop of bacteria from the agar-slant stock was cultured in nutrient broth overnight and spread with a sterile cotton swab into Petri dishes (90 mm of diameter) containing 20 mL of Mueller-Hinton Agar (Oxoid). Sterile filter paper discs (6 mm in diameter) (Oxoid) impregnated with 10 µL of the EO were placed on the agar plate seeded with respective bacteria and the plates were incubated in an inverted position 24 h at 37°C. The equivalent volume of solvent, dimethyl sulfoxide (DMSO), served as negative control. Gentamicin (10 µg/disc) and ciprofloxacin (5 µg/disc) (Oxoid) were used as positive control. The results were obtained by measuring the diameter in mm of the inhibitory or clear zones around the disc (Saavedra *et al*., 2010). All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition zone diameters (mm).

The antibacterial effects of the tested essential oils were classified according to the scheme referred by Aires *et al*. (2009): Non-effective (-)-inhibition halo = 0; moderate efficacy (+)-0 < inhibition halo < antibiotic inhibition halo; good efficacy (++)-antibiotic inhibition halo < inhibition halo < 2 x antibiotic inhibition halo; strong efficacy (+++) -inhibition halo >2× antibiotic inhibition halo.

The data were analyzed using the statistical program SPSS version 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Because low sample numbers contributed to uneven variation, nonparametric Wilcoxon test was used. Statistical calculations were based on confidence level equal or higher than 95% ($p<0.05$ was considered statistically significant).

2.4. Determination of Antimicrobial Potential

The bacteria to be tested (the three strains susceptible to the EOs tested) were picked from overnight cultures in Brain Heart agar (Oxoid). The method used is one previously reported with minor modifications (Sarker *et al*., 2007). Since we are using dilutions of essential oils we chose to use the term of Antimicrobial Potential (AP instead of Minimum Inhibitory Concentration (**Table 3**). A small portion of bacteria was transferred into a bottle with 50 mL of Mueller Hinton broth (Oxoid), capped and placed in an incubator overnight at a 37°C. After 12-18 hours of incubation, the bacteria suspension was adjusted, using aseptic preparation, in order to match the optical density in the range of 0.5-1.0 that was measured at 500 nm. The resazurin solution was prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution, followed by dilution until 50% with sterile distilled water. The plates used were prepared under aseptic conditions (96 well plate, Orange Scientific). A volume of 100 µL of Mueller Hinton broth was used in each well together with 100 µL of essential oil in the first line. From the first well (belonging to the first horizontal line) were taken 100 µL, added to the next well and then this step is repeated to each of the following wells in the vertical line, allowing a serial dilution of decreasing concentration. For each essential oil were considered the percentages of the dilutions performed and therefore for the pure essential oil was considered 100% and for the other concentrations 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78%. In each of the wells were also added 20 µL of the bacteria suspension and 20 µL of resazurin solution (50%). The plates were then placed in an incubator set at 37°C for 18-24 h. All tests were performed in triplicate and the Antimicrobial Potencial (AP) was then assessed visually by the colour change of the resazurin in each well (blue to pink in the presence of bacteria growth).

3. RESULTS AND DISCUSSION

3.1. Antibacterial Screening

All 15 EOs tested had antibacterial activity at least in one of the studied bacteria (**Table 1**) with exception of *P. aeruginosa*. In general, the EOs exerted higher inhibition in Gram-positive than Gram-negative bacteria. The EOs of *E. bosistoana*, *E. botryoides*, *E. camaldulensis*, *E. cinerea* and *E. citriodora* induced total inhibition of bacterial growth in *L. monocytogenes* and *S. aureus*. On the other hand, *P. aeruginosa* demonstrated to be resistant to all EO activity, while *E. coli* did not show any sensitivity to *Eucalyptus polyanthemus* oil and *S. aureus* was only resistant to *E. saligna* oil. *L. monocytogenes* was the most sensitive bacteria tested with sensitivity to all EO. Comparing the halos obtained between the different EOs and a traditional antibiotic (gentamicin) and according to the antimicrobial efficacy ranking previously proposed (Aires *et al*., 2009) (**Table 1**), it was found that some of the tested EOs were more efficient than the antibiotic in the inhibition of bacteria growth. The EOs tested were moderately effective for all bacteria except to *P. aeruginosa*. The first five species were visibly effective against *L. monocytogenes* and *S. aureus* showing halos (mm) with more than twice of the ones found for traditional antibiotics.

3.2. Antimicrobial Potencial (AP)

The bacteria strains *E. coli*, *S. aureus* and *L. monocytogenes* that revealed sensitivity to almost all of the pure EOs (**Table 1 and 2**) were then tested against dilutions (50, 25, 12.5, 6.25, 3,13, 1.56 and 0.78% v/v) of these pure EOs in order to determine the Antimicrobial Potencial (AP) (**Table 3**). The results revealed that the most effective EO against *E. coli* was *E. radiate* (6.25%), while *E. botryoides*, *E. camaldulensis*, *E. cinerea* and *E. citriodora* were the less effective (25%), with *E. polyanthemos* showing resistant to this strain. The Eucalyptus EO species that were more effective against *S. aureus* were *E. botryoides*, *E. camaldulensis*, *E. cinerea*,

E. citriodora, E. bosistoana, E. pauciflora and *E. viminalis* (6.25%), while the less effective were *E. cordieri, E. dives* and *E. globulus* (25%). The strain *L. monocytogenes* revealed more susceptibility to the EOs of *E. bosistoana, E. botryoides, E. camaldulensis, E. cinerea, E. citriodora and E. pauciflora* (12,5%), while the other EOs were less effective (25%) with exception of *E. saligna*, to which this bacteria was resistant.

Table 1. Antimicrobial activity of 15 Eucalyptus EO against the tested bacteria using the disc diffusion assay and antibiotics antibacterial activity

Essential oil specie	*E. coli* CECT434	P. ATCC10145	S. aureus CECT976	L. monocytogenes ATCC15313
Eucalyptus bosistoana F. Muell.	10.3±0.6	0.0±0.0	50.0±0.0	50.0±0.0
Eucalyptus botryoides Sm.	9.3±0.6	0.0±0.0	50.0±0.0	50.0±0.0
Eucalyptus camaldulensis Dehnh	9.3±0.6	0.0±0.0	50.0±0.0	50.0±0.0
Eucalyptus cinerea F. Muell.	9.3±0.6	0.0±0.0	50.0±0.0	50.0±0.0
Eucalyptus citriodora Hook.	9.3±0.6	0.0±0.0	50.0±0.0	50.0±0.0
Eucalyptus cordieri Trabut	10.3±0.6	0.0±0.0	9.3±0.6	12.3±0.6
Eucalyptus dives Schauer	12.3±0.6	0.0±0.0	10.3±0.6	15.3±0.6
Eucalyptus globulus Labill.	9.3±0.6	0.0±0.0	12.3±0.6	13.3±0.6
Eucalyptus pauciflora Sieber ex Spreng	9.3±0.6	0.0±0.0	16.3±0.6	15.3±0.6
Eucalyptus polyanthemus Shauer	0.0±0.0	0.0±0.0	10.3±0.6	12.3±0.6
Eucalyptus radiata Sieber	16.3±0.6	0.0±0.0	11.3±0.6	10.3±0.6
Eucalyptus saligna Sm.	12.3±0.6	0.0±0.0	10.3±0.6	0.0±0.0
Eucalyptus smithii R.T. Baker	10.3±0.6	0.0±0.0	11.3±0.6	10.3±0.6
Eucalyptus urophylla S.T. Blake	10.7±0.6	0.0±0.0	12.3±0.6	10.3±0.6
Eucalyptus viminalis Labill.	10.3±0.6	0.0±0.0	15.3±0.6	12.3±0.6
Antibiotics				
CN[a]	20.7±0.6	18.3±0.6	22,3±0,6	23,3±0,6
CIP[b]	33.6±0.6	33.3±0.6	30,3±0,6	23,6±0,6

[a]Gentamicin; [b]Ciprofloxacin

The means (mm) ± SD for at least three replicates are illustrated

Table 2. Classification of the Eucalyptus EOs antibacterial activity

Essential oil specie	*E. coli* CECT434	*P. aeruginosa* ATCC10145	*S. aureus* CECT976	*L. monocytogenes* ATCC15313
Eucalyptus bosistoana F. Muell.	+	-	+++	+++
Eucalyptus botryoides Sm.	+	-	+++	+++
Eucalyptus camaldulensis Dehnh	+	-	+++	+++
Eucalyptus cinerea F. Muell.	+	-	+++	+++
Eucalyptus citriodora Hook.	+	-	+++	+++
Eucalyptus cordieri Trabut	+	-	+	+
*Eucalyptus dives*Schauer	+	-	+	+
Eucalyptus globulus Labill.	+	-	+	+
Eucalyptus pauciflora Sieber ex Spreng	+	-	+	+
Eucalyptus polyanthemus Shauer	-	-	+	+
Eucalyptus radiate Sieber	+	-	+	+
Eucalyptus saligna Sm.	+	-	+	-
Eucalyptus smithii R.T. Baker	+	-	+	+
Eucalyptus urophylla S.T. Blake	+	-	+	+
Eucalyptus viminalis Labill.	+	-	+	+

Table 3. Antimicrobial Potential (AP) a values of Eucalyptus essential oils

Essential oil specie	*E. coli* CECT434	*S. aureus* CECT976	*L. monocytogenes* ATCC15313
Eucalyptus bosistoana F. Muell.	12.5±0.0	6.25±0.0	12.5±0.0
Eucalyptus botryoides Sm.	25.0±0.0	6.25±0.0	12.5±0.0
Eucalyptus camaldulensis Dehnh	25.0±0.0	6.25±0.0	12.5±0.0
Eucalyptus cinerea F. Muell.	25.0±0.0	6.25±0.0	12.5±0.0
Eucalyptus citriodora Hook.	25.0±0.0	6.25±0.0	12.5±0.0
Eucalyptus cordieri Trabut	12.5±0.0	25.0±0.0	25.0±0.0
Eucalyptus dives Schauer	12.5±0.0	25.0±0.0	25.0±0.0
Eucalyptus globulus Labill.	12.5±0.0	25.0±0.0	25.0±0.0
Eucalyptus pauciflora Sieber ex Spreng	12.5±0.0	6.25±0.0	12.5±0.0
Eucalyptus polyanthemus Shauer	R	12.5±0.0	25.0±0.0
Eucalyptus radiate Sieber	6.25±0.0	12.5±0.0	25.0±0.0
Eucalyptus saligna Sm.	12.5±0.0	12.5±0.0	R
Eucalyptus smithii R.T. Baker	12.5±0.0	12.5±0.0	25.0±0.0
Eucalyptus urophylla S.T. Blake	12.5±0.0	12.5±0.0	25.0±0.0
Eucalyptus viminalis Labill.	12.5±0.0	6.25±0.0	25.0±0.0

[a]AP -calculated with the values of percentage of dilution of the compounds
The means (mm) ± SD for at least three replicates are illustrated
R-No inhibition; Concentrations are expressed as (v/v)

4. DISCUSSION

Plant EOs and extracts have been used in traditional medicine since remote time. Those products have also a particular interest in food preservation (Lis-Balchin and Deans, 1997), alternative medicine (Ben-Arye *et al*., 2011) and parasites control (Yang *et al*., 2004). It becomes of great importance to do extensive studies in order to determine if some EOs could be used to treat some bacterial infections or used as disinfectants in clinical environments. *Eucalyptus* species are some of the plants most described as efficient in antibacterial treatments of some infections such as those of the upper respiratory tract infections (Ben-Arye *et al*., 2011). Another main concern in modern medicine is drug-resistant bacteria and its exponential growth, so the main objective of this assay is to find some compounds that can be used in complementary medicine or as powerful disinfectants against pathogenic bacteria.

In the present *in vitro* study were tested all bacteria against antibiotics (gentamicin and ciprofloxacin) to determine their susceptibility (**Table 1**). *S. aureus* is the most sensitive bacteria followed by *E. coli*, *L. monocytogenes* and *P. aeruginosa* in the case of gentamicin. Ciprofloxacin express more activity against *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes*, respectively.

There are hundreds of species of *Eucalyptus*, but in this study 15 were tested that are previously described in literature as the most economic and world distributed species (Cimanga *et al*., 2002; Chung *et al*., 2007; Francisco *et al*., 2001; Evtuguin *et al*., 2003; Freitas *et al*., 2008). Its importance at several industries, such as pharmaceutical, cosmetic and paper, justifies the necessity to develop more studies with *Eucalyptus*. There are several studies on the antimicrobial activity of *Eucalyptus* EOs (Cimanga *et al*., 2002; Chung *et al*., 2007; Lis-Balchin and Deans, 1997; Ghalem and Mohamed, 2008). The results of the present work show that Gram-positive bacteria are more sensitive to the EOs in general than Gram-negative bacteria. This fact can be related to the different cell wall structure of Gram-positive and Gram-negative bacteria (Gootz, 2010). The present *in vitro* study also showed that the *Eucalyptus* EOs inhibited bacterial growth but their effectiveness varied. The most active EOs were *E. bosistoana*, *E. botryoides*, *E. camaldulensis*, *E. cinerea* and *E. citriodora*, which induced total inhibition of bacterial growth in *L. monocytogenes* and *S. aureus*. A previous study (Lis-Balchin and Deans, 1997) with *L. monocytogenes* revealed that *E. citriodora* and *E. radiata* caused a total inhibition of every strains tested. The EO of *E. camaldulensis* and *E. globulus* have a stronger activity in *S. aureus* than *E. coli* from clinical isolates (Ghalem and Mohamed, 2008). The chemical composition of this EO is very heterogeneous because *E. camaldulensis* is rich in 1,8-cineole and *E. citriodora* only has 1.2% of this molecule. On the other hand, *E. citriodora* is rich in citronellal (72.7%) and the EO of *E. camaldulensis* does not possess this compound (Cimanga *et al*., 2002). So it is probable that this bactericide effect is due to the presence of some minor compound of the *Eucalyptus* EO. Previously authors indicated that globulol (an EO minor component) extracted from *E. globulus* leaf has a strong activity against fungus such as *Alternaria solani*, *Fusarium graminearum*, *Rhizoctonia solani*, *Venturia pirina* and

some bacteria like *Xanthomonas vesicatoria* and *Bacillus subtilis* (Manliang *et al*., 2008). Other authors also relate antimicrobial activity of globulol from *E. globulus* L. fruits (Tan *et al*., 2008). Other cause can be the fact that the composition of each essential oil depends on the age of the plant, climate region, season of the year, EOs extraction method, (Cimanga *et al*., 2002).

P. aeruginosa is one of the most difficult bacteria to treat in hospital infections. The results found in **Table 1** demonstrate that this bacteria, in particular this strain have a strong resistance to all EOs tested. But there are some works in which *P. aeruginosa* is sensitive to peppermint, orange and *Eucalyptus* EO (Cimanga *et al*., 2002).

Previous studies reported an inhibition zone (7-20 mm) of ten *Eucaliptus* species EOs (Cimanga *et al*., 2002) against *E. coli* with higher values, while against *S. aureus* the values found (8-25 mm) were within the range of the values presented in our study. This previous report, when comparing the strains *S. aureus* and *E.* coli, revealed the same tendency shown in the present study with higher inhibition zone attributed to *S. aureus*. Other authors reported values of minimum inhibitory concentration for *E. globulus* EO of 125 µg mL^{-1} against *S. aureus* and 250 µg mL^{-1} for *E. coli* (Dessi *et al*., 2001). When comparing the values obtained for these two bacteria, this last study reveals the lowest MIC for *S. aureus*, while in the present study (Table 3) the lowest AP was found for *E. coli* (*E. globulus* EO). Lis-Balchin and Deans (1997) previously reported the effect of EOs from *E. radiata* and *E. citriodora* showing antimicrobial effect against several strains of *L. monocytogenes*.

5. CONCLUSION

The 15 *Eucalyptus* EOs revealed a strong activity against Gram-positive bacteria which may indicate that in their constituents there are some compounds that can be used as a pharmacological active ingredient or as a main component of a powerful antiseptic disinfectant for clinical surfaces. In general, our results reveal a high susceptibility of the pathogenic bacteria tested to the 15 *Eucalyptus* EOs, with exception of *P. aeruginosa*. The development of clinical surfaces disinfectants from the *Eucalyptus* EOs may be of great importance, as alternatives to other chemical products that are currently being used, that are known for their toxicity for the human health. This is a preliminary study and therefore it is necessary in future to perform more studies to evaluate the level of toxicity of these EOs regarding its antibacterial action since the current applications are more focused on topical use as an antiseptic, as well as the chemical composition of the EOs tested and also evaluate their potential application as disinfectants or even antibacterial agents.

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6.1. Author's Contributions

All authors equally contributed in this work.

6.2. Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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