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ANTIMICROBIAL ACTIVITY OF AQUEOUS, ETHANOLIC AND METHANOLIC LEAF EXTRACTS FROM *ACACIA* SPP. AND *Eucalyptus nicholii*

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Abstract

Background: In Europe, *Acacia* and *Eucalyptus*, originate large amounts of biomass, due to their need by industries and other biological control, that can be used to extract antimicrobial substances.
Materials and Methods: Foliar aqueous, ethanolic and methanolic extracts of *Acacia baileyana* (Cootamundra wattle), *Acacia dealbata* (silver wattle), *Acacia melanoxylon* (black wattle) and *Eucalyptus nicholii* (narrow-leaved black peppermint) were assessed for antimicrobial activity against *Escherichia coli*, *Bacillus cereus*, *Candida albicans* and *Candida parapsilosis*, using the disc diffusion method.
Results: Ethanolic extracts from *A. baileyana* and *A. dealbata* showed significant ($P< 0.05$) antimicrobial activity. Concerning the microbial species tested, differences were found in *A. baileyana* ($P< 0.01$) and *E. nicholii* ($P< 0.0001$) extracts. These two extracts were effective mostly against *B. cereus*, followed by *C. parapsilosis*. According to the antimicrobial activity classification, eucalypt and Cootamundra and silver wattles extracts (both water and ethanol) presented good efficacy against *B. cereus*, a food poisoning agent, and moderate efficacy against the remaining microorganisms. *E. coli*, a Gram negative, exhibited low sensibility to all foliar extracts.
Conclusion: *A. baileyana*, *E. nicholii* and *A. dealbata* foliar biomass could be used to develop alternative substances in microbial control.

Key words: foliar extracts, *Acacia*, *Eucalyptus nicholii*, anti-microbial activity

Introduction

Plants have acquired effective defense mechanisms that ensure their survival under adverse conditions. These defenses may be physical such as thorns, but the most common protections are the chemical coumpounds. In fact, seeds, leaves, bark, and flowers contain several active ingredients that have been used medicinally for many centuries. In particular, plant extracts and/or essential oils are used in food preservation, as sources of pharmaceuticals and in alternative medicine (Deans and Titchie, 1987). The increasing resistance to the conventional antimicrobial agents is of utmost concern making the search for new biologically active metabolites in plants that are traditionally used for the microbial control, one of the most promising areas in the research of alternatives to antibiotics (Ghannoum et al., 1999; Alonso et al., 1995). The extracts of numerous traditional Australian medicinal plants, including the genera *Acacia* and *Eucalyptus*, revealed a great number of compounds with anti-microbial properties (Semple *et al*., 1998; Cock, 2008).

The majority of wattles (*Acacia* spp.) and eucalyptus (*Eucalyptus* spp.) are native from Australia, Tasmania and neighboring islands. In Europe all the *Acacia* and *Eucalyptus* species are exotic, and in Portugal these species were introduced in the 19th century. Firstly used as ornamental plants, their importance increased, as they were used for dune stabilization, furniture, tanning, pulp and paper industries, and as biomass sources for energy (Santos et al. 2006; Lourenço et al. 2008; Lorenzo et al. 2010). Many of the wattles species are considered invasive, competing with the natural flora (Expert workshop 1999; Lorenzo et al. 2010) due to their exceptionally high growth rates when planted outside of their natural habitats. Their ability to adapt to low fertile soils and resistance to wind and fire, imposed the necessity to control them in protected sites such as coastal dunes, natural parks and reserves (Lorenzo et al. 2010). The intensive use of eucalyptus trees for pulp industry and the biological control of *Acacia* spp., generate high quantities of biomass, 5 to 10 t $ha^{-1}.year^{-1}$ for *Eucalyptus* and *Acacia*, respectively (Bernhard-Reversat 1993). This biomass, documented as rich in secondary metabolites (Seigler, 2003) is mainly constituted by leaves and currently left in the fields, affecting litter decomposition and the Nitrogen cycle (Castro-Díez et al. 2012). Due the high concentration of secondary metabolites, these plant species have been used in the traditional Australian and African medicines to treat cold and cough, heal hounds and treat fungal infections (Palombo and Semple, 2001; Takahashi et al., 2004; Mutai *et al.*, 2009; Mulaudzi et al. 2011), and their extracts contain large amounts of compounds with anti-microbial activity (compounds with anti-microbial activity (Ghisalberti 1996; Semple et al. 1998; Seigler 2003; Cock 2008).

The main goal of this work is to test the effect of crude foliar extracts, obtained by maceration with three different solvents, from the wattles *Acacia baileyana* (Cootamundra wattle), *Acacia dealbata* (silver wattle) and *Acacia melanoxylon* (black wattle) and the *Eucalyptus nicholii* (narrow-leaved black peppermint) against bacteria and yeast species.

Materials and Methods

Preparation of Leaf Crude Extracts

Leaves from *Acacia baileyana* F. Muell, *Acacia dealbata* Link., *Acacia melanoxylon* R. Br. and *Eucalyptus nicholii* Maiden & Blakely were collected in March 2009, in the University *Campus*, located in Vila Real, Northeast Portugal. Plants identification was done by Eunice Bacela and confirmed by the staff of the Botanical Garden of UTAD (http://jb.utad.pt/). The collected leaves were clean, oven dried at 45 °C and milled. For extract preparation, the milled leaves were suspended in the solvent (20 % w/v): water, ethanol (95 %) and methanol (95 %). For ethanolic and methanolic extracts, the suspensions were placed at 20

ºC, under stirring for 48 h, while for the aqueous extract, the mixture was boiled for 2 h. The three suspension types were allowed to sediment and the liquid phase passed through a filter paper (WHATMAN No. 1) and dehydrated in a rotary evaporator at 80 ºC (ethanol) or 70 ºC (methanol). Stock final solutions of crude extracts for each type of solvent were prepared by thoroughly mixing the appropriate amount of dried extracts with dimethyl sulfoxide (DMSO) to obtain a final concentration of 10 mg/mL. The final solutions were filtered through a sterilized 0.22 µm syringe filter DMSO-compatible and stored at 4 °C until use.

Microbial Cultures

In testing the antimicrobial activity four species were chosen: two bacteria, *Bacillus cereus* and *Escherichia coli*, and two yeasts, *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019. The bacteria were isolated in our laboratory from soil and water samples, respectively, while the yeasts were purchased. These species are common either as food contaminants or as natural biota of mucosa. The microorganisms were incubated overnight, at 36 ºC, in fresh media: (i) for bacteria in Luria-Bertani agar (LB) $g.L^{-1}$: tryptone, 10; yeast extract, 5; NaCl, 5; and glucose, 5 and agar–agar, 15; or (ii) for yeasts, yeast malt extract agar (YMA) $g.L^{-1}$: glucose, 10; casein peptone, 5; yeast extract, 3, malt extract, 3 and agar-agar, 20.

Susceptibility Testing

The antimicrobial potential of the extracts was evaluated by disc diffusion (DD) method. For that, Petri plates (90 mm diameter) containing Mueller-Hinton (MH) agar (Difco Laboratories) were inoculated with overnight microbial cultures, previously suspended in a sterilized saline solution (0.85 %). For yeast cells, the MH agar were supplemented with 2% glucose. The microbial suspensions were spread on Petri dishes with a turbidity of 1.0 or 0.5 McFarland, respectively, for bacteria and yeast. For the susceptibility test, sterile disks in blank (6-mm diameter) embedded with 15 µL extract were allowed to dry, and placed on inoculated Petri dishes with MH. The plates were incubated at 36 ± 1˚C for 24–48 h, and the inhibitory diameter zone (DZ) measured. For all experiments three independent replicas were taken.

Negative (15 µL of DMSO) and positive (antibiotic standard) controls were included in all experiments: gentamicin (10 µg per disc) for bacteria and fluconazole (25 µg per disc) for yeasts. The interpretative criteria, according to CLSI guidelines (CLSI 2004; CLSI 2007) were: (i) for gentamicin, susceptible (S) ≥ 15 mm; intermediate (I) 14-13 mm and resistant (R) ≤ 12 mm and (ii) for fluconazole, susceptible (S) ≥ 19 mm; susceptible dose dependent (SDD) 18-15 mm and resistant (R) ≤ 14 mm. Because there are no CLSI standards for susceptibility testing against *B. cereus*, we followed the breakpoints for *Staphyloccoccus aureus*.

Antimicrobial Activity Classification

The antimicrobial effects of the tested crude foliar extracts were classified according to the inhibition halo diameter (Aires et al. 2009), as follows: non-effective (-) for inhibition halo = 0; moderate efficacy (+) for 0 < inhibition halo < antibiotic inhibition halo (AIH); good efficacy (++) for AIH < inhibition halo < two-fold AIH; strong efficacy (+++) for inhibition halo > two-fold AIH.

Statistical Analysis

To test the effect of the foliar extracts against bacteria and yeast, we tested the data for normal distribution (Kolmogorov-Smirnov test). Both the raw and the transformed data (square root, logarithm) failed to follow the normal distribution. For that reason, we performed the non-parametric Kruskal-Wallis test, followed by multiple comparisons of mean ranks groups (microorganism or solvent). All data analyses were performed using STATISTICA version 9.1 (StatSoft 2010).

Results and Discussion

The DZ obtained by the DD method for the four plant crude extracts are shown in Fig. 1. Among the bacteria, *E. coli* was less susceptible than *B. cereus*. Cock (2008) tested the methanolic extracts of 25 Australian native species against four bacteria (two Gram-positive and two Gram-negative) by the DD technique and observed that *B. cereus* was the most susceptible bacteria for 54 % of the tested extracts. Also, *Acacia aulacocarpa* and *Eucalyptus major* leaf extracts were among the extracts with good anti-bacterial activity. By contrast, others (Egwaikhide et al. 2008) have tested the methanolic extract from *Eucalyptus globulus* leaves against several bacterial species and reported that *E. coli* (DZ = 17 mm) was more susceptible to the extract than *B. cereus* (DZ = 14 mm). Voravuthikunchai et al. (2004) tested 38 medicinal plant species commonly used in Thailand, both its aqueous and ethanolic extracts, against different strains of *E. coli*. Among the plant extracts tested was *Acacia catechu*, with DZ ranged from 9 to 11 mm, results very similar to ours (6 to 10). Other works have also reported high susceptibility of the Gram-positive bacteria to plant extracts, when compared to Gram-negative bacteria (Palombo and Semple, 2001; Taguri et al. 2006).

The susceptibility differences between these two bacterial groups may be due to cell wall structural differences, with the outer membrane of the Gram-negative cell wall, acting as a barrier to many compounds, including antibiotics (Russel 1995). Nevertheless, *C. albicans*, whose cell wall displays a parallel structure to the Gram-positive bacteria, had a similar susceptibility to *E. coli*. The growth of these organisms has not been inhibited by any of *A. melanoxylon* extracts and was weakly exhibited by the ethanolic extract of *A. dealbata*. Also, *C. albicans* was less susceptible to foliar extracts than *C. parapsilosis*, a trend also found by others. In a study conducted by Hamza and colleagues on the effect of extracts of 56 plant species, among them *Acacia nilotica* and *Acacia robusta*, against yeasts species, *C. parapsilosis* was more susceptible than *C. albicans* (Hamza et al. 2006). Also, *C. parapsilosis* ATCC 22019 was, in general, more susceptible to essential oils, than the isolates of *C. albicans* (Carvalhinho et al. 2012).

The extracts belonging to *A. baileyana* and *E. nicholii* were the most efficient in inhibiting all the tested microorganisms, with the exception of eucalyptus against *E. coli* (DZ = 6 mm). The other two *Acacia* species tested were less effective in the

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inhibition of microbial control, with the exception of ethanolic extract from leaves of *A. dealbata*, against *B. cereus*. By contrast, Taguri and collaborators related that *A. dealbata* extracts had weak potency against *B. cereus* and *B. subtilis* (Taguri et al. 2006). Nevertheless, the extract was obtained from fruits using water as the solvent. The aqueous extract from this species also had a weak effect against the organims tested in this work.

The non-parametric test Kruskal-Wallis showed differences among the plant species ($H_{(3;\ 144)}$ = 49.79; P< 0.0001) and among microbial species ($H_{(3;\ 144)}$ = 34.01; P< 0.0001). Multiple comparisons revealed that plant extracts efficacy, in decreasing order of importance, was (*A. baileyana* = *E. nicholii*) > (*A. dealbata* = *A. melanoxylon*). The effect of solvent and microbial species was analyzed within each plant species (Table 1). Regarding the solvent, ethanol was superior to water only in *A. baileyana* (P<0.05) and *A. dealbata* (P<0.0001). Despite the high DZ values obtained with *E. nicholii* ethanolic extracts, the differences between extracts were not significant. On average, the extraction with ethanol seems to be the most effective, except for *A. melonoxylon*, against *C.*

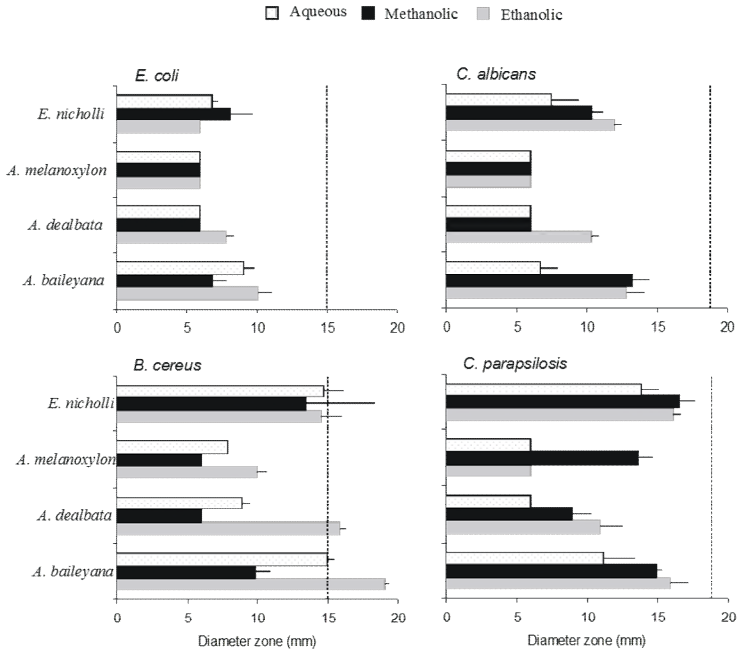

Figure 1: Antimicrobial activity, expressed by the average DZ (mm) and standard deviation (SD), of four crude foliar extracts against *E. coli*, *B. cereus*, *C. albicans* and *C. parapsilosis*. Doted lines indicate the susceptible (S) antibiotic breakpoint for gentamicin (bacteria) or fluconazole (yeasts).

parapsilosis and *E. nicholii* against *E. coli*, where the methanolic extract clearly had the highest performance (Fig.1). Our findings are supported by others. Three solvents (water, hexane and ethanol) were used to prepare extracts from 82 plant species, which were tested against five bacteria. The results indicated that the ethanolic extracts showed a superior activity to the extracts obtained with the other two solvents (Ahmad et al. 1998).

Table 1: Non parametric Kruskal-Wallis test, followed by multiple comparisons of mean ranks. d.f. - degree of freedom; n - number of observations; n.s. – not significant (P> 0.05).

Plant species	Group variable	H (d.f.; n)	P-value	
A. baileyana	Solvent	6.86 (2; 36)	<0.05	Ethanol > water
	Microbial species	15.59 (3; 36)	<0.01	*B. cereus* > *E. coli*
				C. parapsilosis> *E. coli*
E. nicholii	Solvent extract	0.87 (2; 36)	n.s.	
	Microbial species	26.56 (3; 36)	<0.0001	*B. cereus* > *E. coli*
				C. parapsilosis > *E. coli*
				C. parapsilosis > *C. albicans*
A. dealbata	Solvent extract	20.76 (2; 36)	<0.0001	Ethanol > (methanol ≡ water)
	Microbial species	5.46 (3; 36)	n.s	
A. melanoxylon	Solvent extract	0.21 (2; 36)	n.s	
	Microbial species	12.48 (3; 36)	n.s	

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The differences among foliar extracts may be due to the distinct chemistry of plant species, namely in the phenolic fraction and in the polarity of the solvents used to obtain the extracts. In a work on four extract-types (ethanolic, hydro-alcholic, methanolic and acetone) from *A. melanoxylon* and *A. dealbata* aerial parts (Luís et al. 2012), regardless of the solvent used, the extracts of *A. dealbata* had higher antioxidant activity than that of *A. melanoxylon*. The methanolic extracts of *A. melanoxylon* and *A. dealbata* differed in the content of phenolics (syringic, *p*-coumaric, ferulic and ellagic acids), which may explain the distinct activities against the tested micro-organisms obtained in the present work. Phenolic acids may inhibit ergosterol biosynthesis and compromise the integrity of fungal cytoplasmic membrane (Li et al., 2015). Also, gallic and ferulic acids led to irreversible changes in bacterial membranes (Borges et al., 2013).

When we compared the antimicrobial efficacy of leaf extracts with those obtained with gentamicin and fluconazole (Table 2), it was clear that the most efficient extracts were the ethanolic and aqueous extracts of *A. baileyana* and *E. nicholii*, followed by the ethanolic extracts of *A. dealbata*. Contrary, the extracts of *A. melanoxylon* were the least effective

Table 2: Classification of crude foliar extracts for their antimicrobial activity, relative to gentamicin (bacteria) and fluconazole (yeasts). Non-effective (-) for inhibition halo = 0; moderate efficacy (+) for 0 < inhibition halo <AIH and good efficacy (++) for AIH < inhibition halo < two-fold AIH.

		Foliar Extracts			
Microorganism	Solvent	*A. baileyana*	*A. dealbata*	*A. melanoxylon*	*E. nicholli*
B. cereus	Water	++	+	+	++
	Ethanol	++	++	+	++
	Methanol	+	-	-	+
E. coli	Water	+	-	-	+
	Ethanol	+	+	-	-
	Methanol	+	-	-	+
C. albicans	Water	+	-	-	+
	Ethanol	+	+	-	+
	Methanol	+	-	-	+
C. parapsilosis	Water	+	-	-	+
	Ethanol	+	+	-	+
	Methanol	+	+	+	+

e. *B. cereus* was the most susceptible microorganism followed by *C. parapsilosis* and *C. albicans*. All the foliar extracts were ineffective, or moderately effective, against *E. coli* and *C. albicans*.

In conclusion, our results may prove useful to forest producers or those involved in plant invasive control programs, in using the leaf biomasses of *A. baileyana*, *E. nicholii* and *A. dealbata*, to obtain alternative substances to conventional antimicrobials.

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Conflict of interest

The authors declare that they have no conflict of interest.

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