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Antimicrobial Potential of Some Plant Extracts against Multidrug Resistant Pathogenic Bacteria: Staphylococcus *Aureus* and *Klebsiella Pneumonia*

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ABSTRACT

Plants have been extensively studied as alternative agents for prevention and treatment of infectious diseases. The present work evaluates the inhibitory efficacy of different extracts prepared from seven plant species (*Erica lusitanica*, *Hypericum canariensis*, *H. inodorum*, *H. perforatum*, *Paeonia broteri*, *Quercus faginea* subsp. *broteroi* and *Sanguisorba hybrida)* against five standard strains and clinical isolates of human pathogenic bacteria (*Staphylococcus aureus* and *Klebsiella pneumonia*). *In vitro* antibacterial activity was evaluated using the serial broth microdilution method.

The polar extracts were more effective against both Gram (+) and Gram (–) strains. The MeOH and H_2O extracts of *S. hybrida* (leaves and stems), *P. broteri* (leaves) and *H. perforatum* (leaves and stems), showed high-moderate antibacterial activity against all the five multidrug resistant (MDR) bacteria tested. These plant species appear as potential leads against MDR bacteria.

KEYWORDS: plant crude extracts; antibacterial activity; microdilution method; multiresistant bacteria; staphylococcus aureus; klebsiella pneumoniae

INTRODUCTION

Plants synthesize a large diversity of compounds that perform significant biological and ecological functions, such as insect attraction and plant defense against microorganisms, insects and herbivores (Harborne, 1989). Some of these compounds have an extensive use as therapeutic agents (Kinghorn, 1992; Cragg and Newman, 2013).). It is currently acquired that natural products are an important direct source of new chemicals that may be used to treat several pathologies or may also be used as prototypes for the development of new drugs, with new chemical structures as well new mechanisms of action (Butler and Buss, 2006).

Indeed the report on plant medicinal preparations used for the treatment of ailments is very wide but there is awareness that the number of plants with unknown composition and application is even greater and a large percentage of plants is still unstudied.

Infectious diseases have always been a threat to man. The use of antibiotics has made it possible to overcome much of this threat. However, emerging antibiotic-resistant strains and mutant microorganisms are more powerful. Another problem is the formation of microbial biofilms that originate chronic infections for which it is necessary to develop effective alternatives (Olsen 2015). Plants still represent the main therapeutic tool in traditional medicine and in some societies, all plants on earth are considered as medicinal, since they can all be sources of bioactive molecules, used in therapy and disease prevention (Srinivasan *et al*., 2001). The spread of multidrug resistance among bacteria stimulated an increase in the selection of plant extracts to find compounds with a broad spectrum of antimicrobial activity (Butler, 2004; 2005). In the present work a selection of spontaneous and culture plants (Table 1), known to be used in traditional medicine, were studied. The main objective of this study was to search the antibacterial activity of plant aerial parts, mostly leaves and young stems extracts, tested against resistant bacteria strains. Extracts with strong antimicrobial activity may be good candidates for the development of new antimicrobial molecules and / or for the use of standardized herbal medicines.

Table1: Plant species identification.

Taxa	Plant Family	Voucher number	Occurrence
Erica lusitanica Rudolphi	Ericaceae	LISU 223635	endemic
Hypericum canariensis L.	Clusiaceae	LISI 771/2008	culture
*Hypericum inodorum*Mill.	Clusiaceae	LISI 773/2008	culture
Hypericum perforatum L.	Clusiaceae	LISI 365/2007	spontaneous
*Paeonia broteri*Boiss. et Reut.	Paeoniaceae	LISU 221345	endemic
Quercus faginea subsp. *broteroi* (Cout.) A.Camus	Fagaceae	LISI 503/2011	endemic
Sanguisorba hybrida(L.) Nordb.	Rosaceae	LISU 221369	endemic

MATERIAL AND METHODS

A. Plant material

Aerial parts from ten plant species were collected in Continental Portugal. The identification and voucher deposits were carried out in the Herbarium Botanical Garden of the University of Lisbon – LISU, and in the Herbarium of the Botanical Park of Tapada da Ajuda, ISA, University of Lisbon – LISI (Table 1).

B. Plant Extracts

Each fresh plant material was dried in the dark, at room temperature, powdered and weighed. Approximately 100g of every powder were extracted sequentially, with increasing polarity solvents [*n*-hexane (Hex), dichloromethane (CH_2C_{l2}), ethyl acetate (AcOEt), methanol (MeOH) and water (H_2O)], for 24 h at room temperature with occasional shaking. The extracts obtained were filtered and concentrated in a rotary evaporator under reduced pressure at a temperature of 45° C for complete solvent removal and subsequently stored at 4° C until use. The exceptions were the aqueous extracts, which have been freeze-dried.

C. Screening for Antimicrobial Activity

1. *Microbial Strains*

All extracts were in vitro tested against multiresistant bacterial strains: Gram-positive, *Staphylococcus aureus* ATCC 6538, ATCC 43866 (Methicillin-resistant *S. aureus*; MRSA), ATCC 700699 (Vancomycin intermediated *S. aureus*; VISA) and CIP 106760 (VISA); Gram-negative, *Klebsiella pneumonia* ATCC 9997). The test microorganisms belong to the collection of the Department of Microbiology and Immunology of the Faculty of Pharmacy, University of Lisbon. Identification and maintenance of cultures was performed using classical diagnostic microbiology procedures. Single colonies from fresh cultures were streaked in tubes containing Brain Heart Infusion Agar after growth (37°C /18–24 h) and kept at 8° C until use.

2 .Determination of the Minimal Inhibitory Concentration (MIC)

The MIC corresponds to the lowest concentration of an extract that inhibits the development of a certain microorganism. Positive values were considered when MIC < 100 µg/mL (Cos *et al*, 2006). The antimicrobial screening of the plant extracts was determined using the serial broth microdilution method (Cos *et al*., 2006; CLSI, 2008).

Tests were performed in Mueller-Hinton (MH) broth medium, in 96-well microplates, as follows: *t*o 100 µL of the medium, 100 µL of each extract solution to be tested in concentrations ranging between 500-1.75 µg/mL, were added. An inoculum of each microorganism was also added (10 µL; final concentration 10^4 cfu/mL). MH inoculated with the microorganisms without the test sample, was used as a bacterial growth control and MH alone was used as sterility control. Appropriated antibiotics were used as reference for antibacterial activities. The microplates were covered and incubated at 37°C/24h. Three independent experiments were performed in all cases. Microbial growth was evaluated by measuring the absorbance, at the wavelength of 630 nm, in a microplate reader Biotek ELX808.

3. *Phytochemical screening*

The extracts were dissolved in proper solvents, applied on silica gel TLC plates and developed with appropriate mixtures of eluents. The plates, containing an application of each extract (Hex, CH_2C_{l2}, AcOEt, MeOH e H_2O), were revealed with spray specific reagents for each class of substances, including terpenoids, phenolics, flavonoids and alkaloids (Wagner and Bladt, 1996). Results were displayed semi-quantitativein a range between absence (-) and strongly present (+++).

RESULT AND DISCUSSION

Plants produce a great diversity of substances considered responsible for several biological properties, including antimicrobial activity (Cragg and Newman, 2013; Saleem *et al*., 2010; Coates *et al*., 2002). Forty crude extracts (leaves, leaves and stems and seeds) of seven plant species belonging to six different families were assayed against MDR bacteria, namely the methicillin resistant *S. aureus* strain (MRSA 43866), Vancomycin resistant *S. aureus* (VISA 700699 and CIP 106760), a bacterial biofilm model (*S. aureus* ATCC 6538) plus a clinical strain of *K.* pneumoniae with documented ability to assemble biofilms

(Bandeira 2017; Table 2). The serial broth microdilution assays showed a differential activity of the five extracts of each plant (i.e. *n*-hex, CH_2Cl_2, AcOEt, MeOH and H_2O):

Among all the assays performed, 136 (75%) present some degree of antimicrobial activity considered as positive against more than one bacterial strain; from those, 21% inhibit medium-high the growth of *K. pneumoniae*.

Table. 2: MIC values of the tested plant extracts.
(n. t. – not tested; MIC values > 100 µg / mL were considered negative).

Taxon	Used plant part	Extracts %(w/w)	*Staphylococcus aureus*				*Klebsiella pneumoniae*
			(6538)	MRSA (43866)	VISA (700699)	VISA (106760)	(703)
Erica lusitanica	Leaves and stems	*n*-Hex (0,8)	> 100	n.t.	n.t.	n.t.	62,0
		CH_2Cl_2 (2,3)	> 100	n.t.	n.t.	n.t.	>100
		AcOEt (1,7)	30,0	30,0	62,0	62,0	>100
		MeOH (6,4)	15,0	15,0	7,5	3,5	30,0
		H_2O (2,3)	15,0	15,0	15,0	7,5	>100
Hypericum canariensis	Leaves and stems	*n*-Hex (3,9)	30,0	15,0	15,0	62,0	62,0
		CH_2Cl_2 (0,8)	30,0	62,0	30,0	62,0	30,0
		AcOEt (0,9)	30,0	62,0	30,0	7,5	30,0
		MeOH (4,7)	15,0	1,8	15,0	3,5	n.t.
		H_2O (0,7)	62,0	30,0	30,0	62,0	>100
Hypericum inodorum	Leaves and stems	*n*-Hex (0,3)	30,0	30,0	30,0	62,0	62,0
		CH_2Cl_2 (1,4)	62,0	62,0	62,0	30,0	30,0
		AcOEt (3,2)	62,0	30,0	30,0	62,0	30,0
		MeOH (1,5)	30,0	15,0	7,5	7,5	62,0
		H_2O (1,8)	30,0	> 100	62,0	> 100	n.t.
Hypericum perforatum	Leaves and stems	*n*-Hex (2,2)	15,0	15,0	30,0	> 100	>100
		CH_2Cl_2 (1,9)	30,0	62,0	30,0	30,0	>100
		AcOEt (0,4)	30,0	30,0	30,0	62,0	62,0
		MeOH (4,5)	15,0	7,5	15,0	15,0	62,0
		H_2O (0,6)	30,0	30,0	15,0	15,0	>100
Paeonia broteri	Leaves	*n*-Hex (1,6)	62,0	62,0	> 100	62,0	62,0
		CH_2Cl_2 (1,6)	> 100	n.t.	n.t.	n.t.	62,0
		AcOEt (1,8)	1,8	1,8	15,0	7,5	30,0
		MeOH (5,4)	15,0	3,5	3,5	1,8	15,0
		H_2O (7,2)	15,0	1,8	3,5	3,5	30,0
Paeonia broteri	Seeds	*n*-Hex (2,9)	> 100	n.t.	n.t.	n.t.	>100
		CH_2Cl_2 (1,5)	62,0	62,0	30,0	30,0	>100
		AcOEt (1,1)	62,0	> 100	30,0	15,0	62,0
		MeOH (10,9)	15,0	n.t.	3,5	7,5	>100
		H_2O (0,6)	62,0	n.t.	7,5	7,5	15,0
Quercus faginea	Leaves	*n*-Hex (1,0)	> 100	n.t.	n.t.	n.t.	>100
		CH_2Cl_2 (0,9)	62,0	> 100	62,0	15,0	>100
		AcOEt (0,9)	62,0	> 100	62,0	30,0	>100
		MeOH (0,4)	15,0	62,0	3,5	62,0	>100
		H_2O (9,3)	15,0	> 100	3,5	62,0	>100
Sanguisorba hybrida	Leaves and stems	*n*-Hex (0,8)	30,0	62,0	15,0	30,0	30,0
		CH_2Cl_2 (1,4)	30,0	> 100	30,0	15,0	62,0
		AcOEt (0,9)	15,0	> 100	30,0	62,0	62,0
		MeOH (1,9)	7,5	7,5	3,5	7,5	15,0
		H_2O (9,1)	3,5	1,8	3,5	3,5	62,0

In general, the most polar extracts (MeOH and H_2O), were more effective than the less polar extracts (*n*-hex, CH_2Cl_2, AcOEt), for both the Gram (+) and the Gram (–) strains. The MeOH and H_2O extracts of *S. hybrida* (leaves and stems), *P. broteri* (leaves) and *H. perforatum* (leaves and stems), showed antibacterial activity against all five multiresistant bacteria tested, with high activity against *S. aureus* strains, and moderate against *K. pneumoniae* (Table 2). In previous works, no activity of these plants was found against any Gram (–) bacteria (Madureira *et al*., 2014; Lai *et al*., 2012). The results obtained for methanolic and aqueous extracts of *S. hybrida* and *P. broteri*, respectively, against *K. pneumoniae* are particulary promising. This bacterium once organized within biofilms is able to increase resistance to antibiotics such as amoxicillin, gentamicin and fosfomicin 10, 257 and 1000 folds, respectively highlighting the need for alternative drugs (Bandeira 2014). Among less polar extracts, those of *H. canariensis* (leaves and stems) and *H. inodorum* (leaves and stems) were active against all the tested microbes. *n*-hex extract of *S. hybrida* (leaves and stems) was also active against all trialed bacteria. No antibacterial activity was observed for the five extracts of *Q. faginea* (leaves) against *K. pneumoniae*, what was already reported earlier by Madureira *et al*. (2014).

It is not surprising that there are differences in the antibacterial activity of the tested species as they belong to

different botanical families, whose phytochemical disparities are already known (Trease and Evans, 2009). Data on phytochemical screening are in Table 3 and in general: i) terpenoids are moderately distributed across all extracts of all plants, although present in higher concentration in the less polar extracts; ii) phenolic compounds appear in all extracts with rare exceptions; iii) flavonoids appear in higher concentration in the more polar extracts; iv) none of the species under study present alkaloids in their composition.

Table 3 – Phytochemical screening of taxa extracts: semi-quantitative and qualitative evaluation.
(ls leaves and stems: l, leaves; s, seeds; A/SA –anisaldehyde/ Sulfuric Acid; FBS – Fast Blue Salt; NEU – Dual NP / PEG developer.

	Screening of plant extracts chemical composition			
Taxa Extractss	**Terpenoids (A/SA)**	**Phenolics (FBS)**	**Flavonoids (NEU)**	**Alkaloids (Dragendorff)**
***E. lusitanica*(ls)**				
n-hex	+++	++	++	-
CH_2Cl_2	++	+++	-	-
AcOEt	++	+	+++	-
MeOH	+	+	+++	-
H_2O	-	-	++	-
***H. canariensis*(ls)**				
n-hex	+	+	-	-
CH_2Cl_2	++	+++	-	-
AcOEt	+	+++	++	-
MeOH	-	++	+	-
H_2O	-	+++	+	-
***H. inodorum*(ls)**				
n-hex	+	+	++	-
CH_2Cl_2	++	+	-	-
AcOEt	+	++	+	-
MeOH	-	+	-	-
H_2O	-	-	-	-
***H. perforatum*(ls)**				
n-hex	+++	+	-	-
CH_2Cl_2	++	-	-	-
AcOEt	+	+	+	-
MeOH	+	++	+	-
H_2O	-	+	+	-
***P. broteri* (l)**				
n-hex	++	++	+++	-
CH_2Cl_2	++	++	+++	-
AcOEt	++	+	++	-
MeOH	++	+++	++	-
H_2O	++	+	++	-
***P. broteri* (s)**				
n-hex	+++	+	++	-
CH_2Cl_2	++	-	-	-
AcOEt	+++	+++	+	-
MeOH	+	+	+	-
H_2O	-	+	+	-
***Q. faginea*(l)**				
n-hex	++	+	-	-
CH_2Cl_2	++	+	-	-
AcOEt	++	+++	+++	-
MeOH	++	+++	++	-
H_2O	+	+	+	-
***S. hybrida*(ls)**				
n-hex	+	++	+++	-
CH_2Cl_2	++	-	+++	-
AcOEt	++	+	++	-
MeOH	+	+	++	-
H_2O	++	+	-	-

The selective antibacterial effect of the most polar extracts of the tested plants over the less polar extracts can be attributed to the presence of different secondary metabolites. The concentration of phenolic and flavonoids compounds is higher in most polar extracts. The relationship between the antimicrobial activity and the phenolic and flavonoid compounds is known (Ghasemzadeh and Ghasemzadeh, 2011; Cazarolli *et al.*, 2008; Tsuchiya *et al.*, 1996), but in some plant extracts the high content of terpenoids should not be neglected and might also play an important role in the inhibition of the bacterial development (Rodrigues *et al.*, 2012; Cowan, 1999; Scortichini and Rossi, 1991). The bacterial cell envelop composition and structure also interferes, as it is less complex in Gram (+) than in Gram (-) bacteria (Slama, 2008; Cos *et al.*, 2006). Gram (-) bacteria have a highly hydrophobic outer membrane, functioning as a natural barrier with low permeability. This partially explains the absence of significant antibacterial activity when exposed to antibiotics (Stavri *et al*., 2007) and to the extracts of some plants tested here. Some secondary metabolites probably owe their activity to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al.*, 1996). The mechanisms by which essential oils can inhibit microorganisms may be related with their hydrophobicity. Some of the components of the essential oils act as membrane permeabilizers (Nazzaro*et al.*, 2013), making it more permeable to the uptake of the antimicrobial agents (Helander et al., 1998).

CONCLUSIONS

The present work has shown that most of the chosen species are potentially good sources of antimicrobial agents as they displayed activity against *S. aureus* MDR strains and *K. pneumoniae*. It appears to be a correlation between lipophilic and the antibacterial activity, being the more polar extracts related with the higher activity. Those are preliminary results and further studies are needed to evaluate the extracts general cytotoxicity and isolate the bioactive compounds.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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