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Full Length Research Paper

Evaluation of the antimicrobial activity in species of a Portuguese "Montado" ecosystem against multidrug resistant pathogens

B. Lai[1], G. Teixeira[2], I. Moreira[1], A. I. Correia[3], A. Duarte[1] and A. M. Madureira[1]*

[1]Universidade de Lisboa, Faculdade de Farmácia de Lisboa, iMed-UL, Avenida Prof. Gama Pinto, 1649-003, Lisboa, Portugal.
[2]Universidade de Lisboa, Faculdade de Farmácia de Lisboa, Centro de Biologia Ambiental, Avenida Prof. Gama Pinto, 1649-003, Lisboa, Portugal.
[3]Universidade de Lisboa, Faculdade de Ciências de Lisboa, Centro de Biologia Ambiental, C2, Campo Grande, 1749-016 Lisboa, Portugal.

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Forty polar and non polar extracts from six "Montado" species (*Adenocarpus anisochilus*., *Erica lusitanica, Lavandula stoechas* subsp. *luisieri, Paeonia broteroi, Quercus faginea* subsp. *broteroi* and *Rosmarinus officinalis*) were evaluated for their antimicrobial activity against a broad panel of microorganisms that include standard and resistant strains of Gram-positive, Gram-negative bacteria, *Mycobacterium smegmatis* and the yeast *Candida albicans*. From the forty tested extracts, 87% inhibited the development of Gram-positive bacteria. Interesting results were obtained with the most polar extracts of *P. broteroi* (leaves) displaying the best MIC (3.1 to 1.9 µg/mL) when tested against *Staphylococcus aureus* standard, Vancomycin-Resistant *S. aureus* VRSA and meticillin-resistant *S. aureus* (MRSA) strains. The extracts of *E. lusitanica* and *P. broteroi* displayed the broadest antimicrobial activity spectra with the lowest minimum inhibitory concentration (MIC) values seeming very promising and are worthy for further phytochemical studies.

Key words: "Montado" flora, antibacterial activity, MIC determination, phytochemical screening.

INTRODUCTION

Infectious diseases and global antibiotic resistant pathogens are an increasing public health problem. The lack of development of new antimicrobial agents in the last decades, associated with their misuse, led to the emergence of multiresistant microorganisms. These multiresistant strains are a threat to disease management and greatly increased the treatment costs. This may result in a dark scenario where common infections become untreatable and even lethal (Norrby et al., 2005). Examples include meticillin-resistant *Staphylococcus aureus* (MRSA) which is a major cause of serious hospital-acquired infections (Appelbaum, 2006). This justifies the search for new antimicrobial drugs.

Plants used for traditional medicine are known to be effective in a wide range of diseases. The role of the natural products is particularly relevant in the infectious diseases area, where over 60% of the antimicrobial agents used in therapy are from natural origin (Newman and Cragg, 2007; Newman et al., 2003). With only 5 to 15% of the approximately 250 000 species of higher plants systematically investigated, natural products remain an important source of new molecules and scaffolds which can lead to the development of new effective drugs against multiresistant microorganisms (Newman and Cragg, 2007).

The Mediterranean region is rich in medicinal and aromatic plants. Southwestern Iberia is mainly covered by cork-oak woodland, common speaking as "Montado". This is a unique Mediterranean ecosystem, whose importance is related to its huge biodiversity, allied to a traditional and sustainable management of the ecosystem.

*Corresponding author. E-mail: afernand@ff.ul.pt. Tel: (+ 351)217946400. Fax: (+ 351)217946470.

Table 1. Botanical data of the species used in the present study.

Family	Taxa	Plant part studied	Voucher number
Leguminosae	*Adenocarpus anisochilus* Boiss.	Aerial parts and fruits	LISU 221390
Ericaceae	*Erica lusitanica* Rudolphi	Leaves	LISU 223635
Labiatae	*Lavandula stoechas* subsp. *luisieri* (Rozeira) Rozeira	Aerial parts	LISU 223640
Paeoniaceae	*Paeonia broteroi* Boiss. & Reut.	Leaves and fruits	LISU 221345
Fagaceae	*Quercus faginea* subsp. *broteroi* (Cout.) A. Camus	Leaves	LISI 503/ 2011
Labiatae	*Rosmarinus officinalis* L.	Aerial parts	LISI 2001/ 2009

Its unique characteristics were considered by the EU (Santos-Reis and Correia, 1999) as a priority habitat for conservation. Its botanical biodiversity is well adapted to the harsh meteorological conditions characteristic of Mediterranean ecosystems (inconsistent rainfall patterns, dry summers and cool winters). These "montado" species have developed various functional and structural characteristics that allow them to survive. In those extreme conditions several chemical adaptations occur, as can be exemplified by the presence of a large combination of metabolites, often present in glandular structures (Figueiredo et al., 2007).

It is known that some of those species contain a variety of biologically active compounds, such as terpenoids and tannin (Harborne, 1997), which are believed to provide protection against potential pathogenic microbes (Hammer et al., 1997; Lis-Balchin et al., 1998). The exploration of the antimicrobial properties of the Portuguese "montado" flora is very limited and restricted to a few botanical families, in particular to the Labiatae, the mint family, being their biological activities mainly attributed to the essential oils (Matos et al., 2009; Rodrigues et al., 2008, 2006).

The aim of the present study was to evaluate the antimicrobial activity of extracts of chosen indigenous species from the Portuguese "montado", against selected human pathogens, Gram-positive and Gram-negative, standard and resistant bacteria, *Mycobacterium smegmatis* and against the yeast *Candida albicans.* The preliminary phytochemical composition of each obtained extract was also evaluated by chromatographic methods.

MATERIALS AND METHODS

Plant material and preparation of the extracts

The plant materials used in this study were six different Portuguese "montado" species: *Adenocarpus anisochilus* Boiss. (Boiss.) Franco, *Erica lusitanica* Rudolphi, *Lavandula stoechas* subsp. *luisieri* (Rozeira) Rozeira, *Paeonia broteroi* Boiss. and Reut., *Quercus faginea* subsp. *broteroi* (Cout.) A. Camus and *Rosmarinus officinalis* L. (Table 1). Aerial parts were collected between June and July 2009, from wild populations growing at Herdade da Ribeira Abaixo, Grândola region, southwestern Portugal (38º 8' N - 8º 33' W). The plant material was identified in the Herbarium of the Lisbon Botanical Garden (LISU) and in the Lisbon Agronomic Institute Herbarium (LISI), where vouchers specimens are deposited.

The aerial parts were shadow dried at room temperature and in some cases different parts were separated as leaves and fruits (Table 1). The crude extracts were prepared by sequentially extracting 100 g of dried powdered plant material with 300 ml of *n*-hexane, dichloromethane, ethyl acetate, methanol and water for 24 h, at room temperature with occasional shaking. After filtration, the extracts were concentrated under reduced pressure at 40 to 45ºC, and stored at 4ºC until use. The yield of the dried extracts (as w/w percentage of the starting dried material) is available in Table 2.

Screening for antimicrobial activity

The extracts were screened for *in vitro* activity against Gram-positive bacteria: *Staphylococcus aureus* ATCC 6538, *S. aureus* standard ATCC 43866 (MRSA), ATCC 700699 (Vancomycin resistant *S. aureus*; VRSA) and ATCC 106760 (MRSA and VRSA), *Staphylococcus epidermidis* ATCC 12228 and *Enterococcus faecalis* CIP 104676; Gram-negative bacteria: *Pseudomonas aeruginosa* CIP 9027, *Klebsiella pneumoniae* ATCC 9997, *Salmonella thyphimurium* CIP 6062; *M. smegmatis* CIP 607 and the yeast *C. albicans* ATCC 10231.

Plant extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg/mL. Concentrations of plant extracts were used in a range of 500 to 0.9 µg/mL.

The antibacterial activity evaluation was performed by determination of the minimum inhibitory concentration (MIC) by the serial broth microdilution method as described by the National Committee for Clinical Laboratory Standards (CLSI; 2008). The inhibition of bacteria growth was evaluated by measuring the well´s turbidimetry on absorvance microplate reader (ELX 808, Biotek) at 630 nm. Endpoint values were established according to Cos et al. (2006a; 2006b) and samples with a MIC value ≤ 100 µg/ml were considered to have antibacterial activity.

Phytochemical screening

To characterize the major compound classes present in the extracts, preliminary phytochemical analysis was carried out through thin layer chromatography (TLC) on silica gel. After a semi-quantitative application of the extracts, the TLC plates were developed with appropriated mixtures of solvents. Spots were revealed with the following spray-reagents: Dragendorff reagent for alkaloids, anisaldehyde-sulfuric acid reagent for terpenes, Natural Products-Polyethylene Glycol (NEU) reagent for flavonoids and fast

Table 2. Plant data and MIC values (µg/mL) of plant extracts.

Species extracts (% w/w) (% w/w)	MIC (µg/mL)										
	S. aureus				*S. epidermidis*	*E. faecalis*	*M. smegmatis*	*K. pneumoniae*	*S. typhimurium*	*P. aeruginosa*	*C. albicans*
	6538	**MRSA**	***VRSA***	***MRSA/VRSA***							
***A. anisochilus* (aerial parts)**											
n-hexane (0.2)	62.0	125.0	30	15.0	125.0	125.0	125.0	250.0	250.0	250.0	250.0
CH_2Cl_2 (0.9)	62.0	125.0	125	15.0	125.0	125.0	125.0	250.0	250.0	250.0	125.0
AcOEt (0.3)	62.0	125.0	125	62.0	125.0	125.0	125.0	250.0	250.0	250.0	250.0
MeOH (7.7)	125.0	—	—	—	250.0	125.0	125.0	250.0	250.0	250.0	125.0
H_2O (2.0)	30.0	125.0	125	125.0	125.0	125.0	125.0	250.0	250.0	250.0	250.0
***A. anisochilus* (fruits)**											
n-hexane (3.0)	62.0	30.0	30.0	15.0	125.0	125.0	62.0	250.0	250.0	250.0	125.0
CH_2Cl_2 (2.3)	62.0	62.0	30.0	62.0	250.0	125.0	125.0	250.0	250.0	250.0	125.0
AcOEt (0.5)	62.0	125.0	62.0	62.0	125.0	125.0	30.0	250.0	250.0	250.0	250.0
MeOH (3.3)	250.0	—	—	—	250.0	125.0	30.0	250.0	250.0	250.0	250.0
H_2O (4.1)	30.0	125.0	125.0	62.0	250.0	125.0	30.0	250.0	250.0	250.0	250.0
***E. lusitanica* (leaves)**											
n-hexane (0.8)	125.0	125.0	—	—	250.0	125.0	125.0	250.0	250.0	250.0	250.0
CH_2Cl_2 (2.3)	125.0	125.0	—	—	62.0	125.0	125.0	250.0	250.0	250.0	62.0
AcOEt (1.7)	30.0	30.0	62.0	62.0	15.0	30.0	30.0	250.0	250.0	250.0	250.0
MeOH (6.4)	15.0	15.0	7.5	3.5	62.0	125.0	62.0	250.0	250.0	250.0	125.0
H_2O (2.3)	15.0	15.0	15.0	7.5	125.0	125.0	62.0	250.0	250.0	250.0	250.0
***L. stoechas* subsp. *luisieri* (aerial parts)**											
n-hexane (0.7)	62.0	125.0	62.0	125.0	125.0	62.0	62.0	62.0	250.0	250.0	62.0
CH_2Cl_2 (2.0)	62.0	125.0	62.0	62.0	125.0	125.0	30.0	250.0	250.0	250.0	250.0
AcOEt (1.6)	62.0	62.0	62.0	125.0	125.0	125.0	250.0	250.0	250.0	250.0	250.0
MeOH (2.5)	62.0	62.0	62.0	62.0	62.0	125.0	15.0	250.0	250.0	250.0	250.0
H_2O (4.6)	62.0	62.0	62.0	250.0	250.0	62.0	30.0	250.0	250.0	250.0	62.0
***P. broteroi* (leaves)**											
n-hexane (1.6)	62.0	62.0	125.0	62.0	250.0	250.0	125.0	250.0	250.0	250.0	250.0
CH_2Cl_2 (1.6)	250.0	—	—	—	250.0	125.0	125.0	250.0	250.0	250.0	250.0
AcOEt (1.8)	1.9	1.9	15.0	7.5	30.0	62.0	62.0	250.0	250.0	250.0	250.0

Table 2. Contd.

MeOH (5.4)	15.0	3.8	3.8	1.9	15.0	62.0	62.0	250.0	250.0	250.0	250.0
H_2O (1.8)	15.0	1.9	3.8	3.8	15.0	62.0	30.0	250.0	250.0	250.0	125.0
***P. broteroi* (fruits)**											
n-hexane (2.9)	125.0	—	—	—	250.0	250.0	250.0	250.0	250.0	250.0	250.0
CH_2Cl_2 (1.5)	62.0	62.0	30.0	30.0	250.0	125.0	62.0	250.0	250.0	250.0	250.0
AcOEt (1.1)	62.0	125.0	30	15.0	30.0	250.0	62.0	250.0	250.0	250.0	250.0
MeOH (10.9)	15.0	3.8	3.8	7.5	15.0	62.0	125.0	250.0	250.0	250.0	250.0
H_2O (0.6)	62.0	1.9	7.5	7.5	15.0	125.0	15.0	250.0	250.0	250.0	125.0
***Q. faginea* subsp. *broteroi* (leaves)**											
n-hexane (1.0)	250.0	—	—	—	250.0	125.0	62.0	250.0	250.0	250.0	250.0
CH_2Cl_2 (0.9)	62.0	125.0	62.0	15.0	250.0	125.0	125.0	250.0	250.0	250.0	250.0
AcOEt (0.9)	62.0	125.0	62.0	30.0	62.0	125.0	62.0	250.0	250.0	250.0	125.0
MeOH (0.4)	15.0	62.0	3.8	62.0	30.0	125.0	62.0	250.0	250.0	250.0	125.0
H_2O (9.4)	15.0	125.0	3.8	62.0	30.0	125.0	62.0	250.0	250.0	250.0	125.0
***R. officinalis* (aerial parts)**											
n-hexane (2.4)	125.0	—	—	—	125.0	125.0	125.0	250.0	250.0	250.0	250.0
CH_2Cl_2 (3.1)	62.0	125.0	30.0	62.0	125.0	125.0	30.0	250.0	250.0	250.0	250.0
AcOEt (2.5)	30.0	62.0	15.0	62.0	30.0	30.0	30.0	250.0	250.0	250.0	250.0
MeOH (4.7)	62.0	125.0	30.0	62.0	125.0	125.0	62.0	250.0	250.0	250.0	250.0
H_2O (6.9)	62.0	125.0	250.0	250.0	125.0	125.0	30.0	250.0	250.0	250.0	250.0

blue salt reagent for phenolic compounds, prepared according to Wagner and Blader (1996). Results were displayed semi-quantitatively in a range between absence (–) and strongly present (+++).

RESULTS

A total of forty extracts prepared from six different “montado” species were screened for their antibacterial activity. The results are showed in Table 2. The best results were obtained against standard and multiresistant *S. aureus* strains. 80% of the crude extracts inhibit the development of the *S. aureus* standard and from those, 30% displayed low MIC values (<30 µg/mL). Ethyl acetate extract of *P. broteroi* leaves displayed the lowest MIC value (1.9 µg/mL). Very interesting results were also obtained (MIC 15 µg/mL) with methanol and water extracts of *P. broteroi* leaves, *P. broteroi* fruits methanol extract, *E. lusitana* and*Q. faginea* subsp. *broteroi* methanol and water extracts. All the active extracts were then assayed against the *S. aureus* multiresistant strains (MRSA, VRSA and MRSA/VRSA). *P. broteroi* leaves and fruits and *E. lusitana* polar extracts (methanol and water) displayed MIC values of 1.8 to 15 µg/mL, being very interesting for further phytochemical investigation.Thirty three percent of the tested extracts inhibited the *S. epidermidis* growth with MIC values between 15 to 62 µg/mL,

exhibiting the polar extracts of *P. broteroi* leaves and fruits the lowest MIC (15 µg/mL). Similar results were obtained when the *E. faecalis* was tested, being 20% of the extracts actives with MIC values ranging from 30 to 62 µg/mL. The development *M. smegmatis* was inhibited by 62% of the tested extracts (MIC 15 to 62 µg/mL), being *P. broteroi* fruits water extracts the most active. With exception of the *L. stoechas* subsp. *luisieri n*-hexane extract (Table 2), no activity was found when Gram-negative bacteria were assayed. The *E. lusitana* ethyl acetate and the *L. stoechas* subsp. *luisieri n*-hexane and water extracts were the only ones displaying moderate activity against *C. albicans* growth.

P. broteroi leaves extracts (ethyl acetate, methanol and water), *P. broteroi* fruits water and methanol and *E. lusitanica* and *R. officinalis* ethyl acetate extracts displayed the broadest antibacterial activity being active against all the Gram-positive standard and multiresistant tested bacteria and against *M. smegmatis*.

The phytochemical data are displayed in Table 3. The analyzed plants are not rich in alkaloids, displaying *A. anisochilus* vestigial amounts. *P. broteroi* (leaves), *L. stoechas* subsp. *luisieri*, *E. lusitanica*, *Q. faginea* subsp. *broteroi* and *R. officinalis* extracts have a high content in flavonoids/phenolics. Terpenes were also detected in some of the screened extracts.

DISCUSSION

The present study showed the potential use of “montado” flora as a source of antimicrobial agents. Different extracts of chosen “montado” species exhibited antimicrobial activity against standard and resistant microbial strains. Our results revealed that all plant extracts exhibited stronger activities against Gram-positive bacteria and *M. smegmatis*. The Gram-negative antibacterial activity was not significant. These bacteria possess outer membrane that is highly hydrophobic, providing these microorganisms with a permeability barrier. This partially explains the greater resistance observed by Gram-negative bacteria when exposed to antibacterial drugs (Stavri et al., 2007) and to the plant extracts tested herein. The antifungal activity against the yeast *C. albicans* was also not significant, with the exception of *E. lusitanica* ethyl acetate extract and *L. stoechas* subsp. *luisieri n*-hexane and aqueous extracts, which showed a moderate activity.

The relationship between the antimicrobial activity and the phenolic and flavonoid compounds is well known and this might be in part, responsible for the results showed by the plants extracts (Cazarolli et al., 2008). Only the *A. anisochilus* (aerial parts and fruits) ethyl acetate and methanol extracts displays positive results for alkaloid screening, which is in agreement with the data of the Leguminosae family and of other *Adenocarpus* species (Veen et al., 1992).

In some plant extracts, the high content of terpenes should not be neglected and might also play an important role in the inhibition of the bacterial development (Cowan, 1999). The two Labiatae species, *L. stoechas* subsp. *luisieri* and *R. officinalis* are the richest in terpenes, phenolics and flavonoids and so almost all their extracts displayed moderated activity against the tested Gram-positive bacteria. In *Lavandula* genus, the essential oils have different chemiotypes, being that *L. stoechas* subsp. *luisieri* essential oils are very peculiar, possessing rare necrodane derivatives (necrodane type) (González-Coloma et al., 2006) to who are attributed some of its biological activities. 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 permeabilitzers (Nicolson et al., 1999), making it more permeable to the uptake of the antimicrobial agents, including antibiotics (Helander et al., 1998).

R. officinalis, the other Labiatae included in our study, is a very popular herb in the Mediterranean region. Interesting results were obtained with the ethyl acetate extract of this species, such as the antimicrobial activity against *S. aureus* resistant to vancomycine. The antibacterial activity of the *R. officinalis* leaves extracts against highly drug-resistant Gram negative Bacilli was previously mentioned (Abdel-Massih et al., 2010). Oluwatuyi et al. (2004) report on the isolation of abietane diterpenes on its aerial parts chloroform extract which seemed to be responsible for the antibacterial activity against strains of *S. aureus* possessing efflux mechanisms of resistance.

Q. faginea subsp. *broteroi* leaves ethyl acetate and methanolic extracts also displayed interesting results on the antimicrobial assays. The phytochemical screening revealed that those extracts have a high content in phenolics and flavonoids. The presence of those compounds is well known in *Quercus* genus and the antibacterial activity has been reported in some species (Andrensek et al., 2004), and could explain the MIC value obtained in this work.

The most important results were obtained with the *P. broteroi* (both leaves and fruits) and *E. lusitanica* more polar extracts (ethyl acetate, methanol and water). Almost all of those extracts displayed very low MIC values against all the *Staphylococcus* strains. A synergic effect between the terpenes, phenolics and flavonoids is probably related with the antibacterial activity of these extracts.

In conclusion, our work revealed that several extracts of the chosen “montado” species displayed very low MIC values against a large panel of Gram-positive bacteria. The activity on different microorganism seems to depend on the extract chemical profile but in general the activity increases with the polarity of the extracts. Further

Table 3. Qualitative evaluation of extracts chemical composition.

Plants extracts	Terpenes	Phenolics	Flavonoids	Alkaloids
A. anisochilus **(aerial parts)**				
n-hexane	+++	+	++	-
CH_2Cl_2	+	+	-	-
AcOEt	+	+	+	+
MeOH	+	-	-	+
H_2O	-	+	-	-
A. anisochilus **(fruits)**				
n-hexane	+++	+	++	-
CH_2Cl_2	+	+	-	-
AcOEt	+	+	+	+
MeOH	++	+	-	+
H_2O	-	+	-	-
E. lusitanica **(leaves)**				
n-hexane	+++	++	++	-
CH_2Cl_2	++	+++	-	-
AcOEt	++	+	+++	-
MeOH	+	+	+++	-
H_2O	-	-	++	-
L. stoechas **subsp.** ***luisieri*** **(aerial parts)**				
n-hexane	+++	++	++	-
CH_2Cl_2	+++	++	++	-
AcOEt	+++	++	+	-
MeOH	++	++	+++	-
H_2O	++	++	+	-
P. broteroi **(leaves)**				
n-hexane	++	++	+++	-
CH_2Cl_2	++	++	+++	-
AcOEt	++	+	++	-
MeOH	++	+++	++	-
H_2O	++	+	++	-
P. broteroi **(fruits)**				
n-hexane	+++	+	++	-
CH_2Cl_2	++	-	-	-
AcOEt	+++	+++	+	-
MeOH	+	+	+	-
H_2O	-	+	+	-
Q. faginea **subsp.** ***broteroi*** **(leaves)**				
n-hexane	++	+	-	-
CH_2Cl_2	++	+	-	-
AcOEt	++	+++	+++	-
MeOH	++	+++	++	-
H_2O	+	+	+	-
R. officinalis **(aerial parts)**				
n-hexane	+	+	+	-
CH_2Cl_2	++	++	+++	-

Table 3. Contd.

AcOEt	++	++	++	-
MeOH	+	+	+	-
H_2O	+	++	-	-

experiments will be necessary to assess the potential of some species, including *E. lusitanica* and *P. broteroi*, which seems to be quite interesting species being worthy of a detailed phytochemical characterization.

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