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To cite this version:

Vahinalahaja Razafintsalama, Samira Sarter, Lengo Angèle Mambu, Ranjana Randrianarivo, Thomas Petit, et al.. Antimicrobial activities of Dilobeia thouarsii Roemer and Schulte, a traditional medicinal plant from Madagascar. South African Journal of Botany, 2013, 87, pp.1–3. 10.1016/j.sajb.2013.02.171 . hal-01188116

HAL Id: hal-01188116

https://hal.science/hal-01188116v1

Submitted on 31 May 2022

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South African Journal of Botany 87 (2013) 1–3

Contents lists available at SciVerse ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Antimicrobial activities of *Dilobeia thouarsii* Roemer and Schulte, a traditional medicinal plant from Madagascar

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ARTICLE INFO

Article history:
Received 24 April 2012
Received in revised form 22 June 2012
Accepted 22 February 2013
Available online 2 April 2013

Edited by J Van Staden

Keywords:
Dilobeia thouarsii
Proteaceae
Medicinal plant
Antibacterial
Plant extract
Phenolic compound

ABSTRACT

The leaves of *Dilobeia thouarsii* (Roemer and Schulte), a tree that is endemic to Madagascar (Proteaceae), are used in traditional Malagasy medicine to treat bacterial skin infections and wounds. This study investigated the *in vitro* antibacterial activities of *D. thouarsii* leaf extracts and identified the bioactive compounds with the aim of providing a scientific basis for its use against skin diseases. Using broth microdilution method for leaf crude extract and its compounds, we investigated inhibition of the growth of *Bacillus cereus*, *Bacillus megaterium*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Vibrio harveyi*, *Vibrio fisheri*, *Salmonella* Typhimurium, *Salmonella antarctica*, *Escherichia coli*, and *Klebsiella pneumoniae*. The two purified phenolic compounds from leaf ethyl acetate extracts (**1**, **2**) were found to be more active than the crude extract itself. The structure of the two compounds was elucidated by NMR and mass spectrometry: compound **1** was identified as 4-aminophenol and compound **2** as 4-hydroxybenzaldehyde. A marked inhibitory effect (MIC < 0.1 mg/ml) was found against *S. aureus*, which is a major agent in skin infections. We observed moderate activities (MIC values of between 0.1 and 0.5 mg/ml) for *E. faecalis*, *Vibrio* spp., and *Bacillus* spp. Neither compound was active against *Salmonella* spp., *E. coli* and *K. pneumoniae* (MICs > 1 mg/ml). To conclude, the high antimicrobial activity of *D. thouarsii* leaf extracts against *S. aureus* supports its traditional use to treat skin infections.

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1. Introduction

Traditional medicine is an important component of the health care system in Madagascar and a large number of plants remain to be studied, including *Dilobeia thouarsii*, a tree that belongs to the family Proteaceae and is endemic to Madagascar (Boiteau, 1986). This species is widely distributed in the Central, Eastern, South-Eastern regions and in the high Matsiatra Fianarantsoa in Madagascar (Bosser and Rabevohitra, 1991) and is known by the common names of Vivaona, Hazontavolo and Tavolohazo (Rabesa, 1986). In southern Madagascar, decoctions of the leaves and bark of *D. thouarsii* are used for abortion, or as an anthelmintic, or a diuretic (Beaujard, 1988; Rabesa, 1986). Concerning the East coast of Madagascar (Mandraka region), our ethnobotanical investigations confirmed the use of the leaves in traditional medicine to treat bacterial skin infections and wounds (Razafintsalama, 2012).

In vitro assays have shown that phenolic compounds are often responsible for the antimicrobial activities of different plant extracts (Shikanga et al., 2010; Tepe et al., 2005; Zampini et al., 2005). Several species belonging to the family Proteaceae, such as *Grevillea robusta*, *Toronia toru*, *Gevuina avellana*, *Kermadecia elliptica*, *Protea obtusifolia* or *Lomatia hirsuta* contain phenolic compounds (Ahmed et al., 2000; Chuang and Wu, 2007; Moure et al., 2001; Perry and Brennan, 1997; Simonsen et al., 2006; Verotta et al., 1999). In addition, species belonging to this family display antimicrobial activities against different microorganisms. *L. hirsuta*, which is used in traditional medicine in Chile, is active against the pathogenic fungus *Candida albicans* (MIC = 8 μg/ml) (Simonsen et al., 2006). A phenolic glycoside ester isolated from the New Zeland tree *T. toru* is active against *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus subtilis* (Perry and Brennan, 1997). A glycoside compound isolated from *Persoonia linearis* × *pinifolia*, a cross hybrid of *P. pinifolia* and *P. linearis*, displays antimicrobial activity against *E. coli* and *Phytophthora cinnamoni* (MacLeod et al., 1997). An extract made from leaves of *Protea simplex*, a plant used in South Africa against human dysentery and diarrhea, provides good antimicrobial

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http://dx.doi.org/10.1016/j.sajb.2013.02.171

activities against *E. coli*, *Staphylococcus aureus*, *B. subtilis* and *C. albicans* (Fawole et al., 2009).

To the best of our knowledge, no report has been published on the chemical composition and the biological activities of *D. thouarsii* (Bosser and Rabevohitra, 1991). In the present study, we investigated the antibacterial activity of *D. thouarsii* and identified bioactive compounds in order to provide a scientific basis for its traditional use, and to characterize the potential of this medicinal plant in Madagascar. Bioassay fractionation enabled isolation of two phenolic compounds that were identified on the basis of spectroscopic data including 1D NMR and mass spectrometry (MS).

2. Material and methods

2.1. Plant material

The leaves of *D. thouarsii* were harvested in Mandraka region, in the eastern part of Madagascar, 70 km from Antananarivo. Leaves were collected in April 2008. The plant was identified by Dr. Rabarison Harison from the Botany Department of Antananarivo Faculty of Sciences. Reference specimens (HERB/DBEV/4708) were deposited in the herbarium of the same department of the University of Antananarivo.

2.2. Extraction of D. thouarsii leaves

Plant materials were dried at room temperature and ground to a fine powder. The obtained powder (100 g) was extracted successively through a maceration process using 500 ml × 6 of solvents of increasing polarity (hexane, ethyl acetate and methanol). Each combined extract was evaporated under reduced pressure to yield crude hexane extract (0.7 g), EtOAc extract (5 g), and MeOH extract (10 g), respectively. Extracts were stored at room temperature until use.

2.3. Bioassay-guided extract

Part of the ethyl acetate extract (1.5 g) was subjected to flash chromatography on a silica gel 60 (10–40 μ) column (CC) eluted with 0–100% gradient of EtOAc in hexane followed by MeOH in EtOAc. Fourteen 100 ml fractions were collected: Hex–EtOAc 80:20 (1–4), Hex–EtOAc 40:60 (5–8), Hex–EtOAc 20:80 (9–12), EtOAc (13), and MeOH (14). On the basis of the analytic TLC, and according to the antimicrobial assay, similar active fractions 5–8 (0.15 g) were combined and rechromatographed on the same support using the same solvent system. Fourteen new fractions were obtained, but only two displayed antibacterial activity. Each active fraction was treated with 75% ethanol and concentrated to yield compounds **1** (100 mg) and **2** (40 mg). The antimicrobial activity of these compounds was evaluated on Gram-positive and Gram-negative bacteria.

2.4. Antimicrobial assays

2.4.1. Microorganism strains

Four Gram-positive (*Bacillus cereus* LMG 6910, *Bacillus megaterium* LMG 7127, *S. aureus* ATTC 25920, *Enterococcus faecalis* ATTC 29212) and six Gram-negative bacteria (*Vibrio harveyi* ATCC 14126, *Vibrio fisheri* ATCC 49387, *Salmonella* Typhimurium ATCC 14028, *Salmonella antarctica* LMG 3264, *E. coli* CCM 451, *Klebsiella pneumoniae* ATTC 13883) were used to study antibacterial activity. The bacteria were obtained from the collections of both the University of La Réunion (LCSNSA: *Laboratoire de Chimie des Substances Naturelles et des Sciences des aliments*, Saint Pierre) and Cirad (Montpellier, France).

2.4.2. MIC and MBC determination

The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) were evaluated using the microdilution method described by Kuete et al. (2009). The samples were first dissolved in sterile distilled water. The concentration of the resulting solutions was adjusted to 7 mg/ml. This was serially diluted twofold to obtain concentration ranges of 0.027–7 mg/ml. Next, 100 μl of each concentration was added in a well (96-well microplate) containing 95 μl of Zobell medium for vibrios (1 g/l yeast extract, 4 g/l peptone, 30 g/l NaCl) or Mueller-Hinton broth for the other microorganisms and 5 μl of inoculum (standardized at 1.5×10^6 cfu/ml by adjusting the optical density to 0.125 at 600 nm). A positive control containing the bacterial culture without the extract and a negative control containing only the medium were also analyzed. The plates were covered with sterilized aluminum foil, and then incubated for 24 h at 25 °C for *Vibrio* sp. and at 37 °C for the other strains. The assay was repeated three times. The MIC of each compound was defined as the lowest concentration that inhibited the microorganism growth. Bacterial growth was visually evaluated based on the degree of turbidity (Kil et al., 2009).

For the determination of MBC, 5 μl from each well not showing turbidity was placed on Mueller-Hinton agar and incubated at 37 °C for 24 h. The lowest concentration at which no growth occurred on the agar plates after 24 h of incubation at 37 °C corresponded to the MBC.

3. Results and discussion

3.1. Active compounds identified

Compound **1** (Fig. 1) was isolated as an amorphous powder. HRESI-TOF performed in the negative mode exhibited a deprotonated molecular ion at *m/z* 108.0435 $[M - H]^-$ indicating a molecular formula of C_6H_7NO (calcd. 108.0447) requiring 4° of unsaturation. The ^{13}C NMR spectrum revealed the presence of an oxygenated quaternary carbon at δ 151.2, another quaternary carbon at δ 117.4 and a methine carbon at δ 115.8.

The ^1H NMR spectrum of this small molecule displayed an intense signal of four aromatic protons at δ 6.49. As the spectra were realized in CD_3OD, the three remaining protons not observed as suggested the molecular formula are exchangeable protons. Comparison with RMN data of the sample indicated that compound **1** was a 4-aminophenol (Sigma-Aldrich catalog).

The molecular formula of compound **2** (Fig. 1) was deduced as $C_7H_6O_2$ from the deprotonated molecular ion peak at *m/z* 122.0368 $[M - H]^-$ observed in the HRESI-MS compatible with four degrees of unsaturation. Its ^1H NMR spectrum showed the presence of an aldehyde proton at δ 9.73 and two doublets at δ 6.9 (2H, $J = 8.4$ Hz, H-3; H-5) and 7.8 (2H, $J = 8.4$ Hz, H-2; H-6). In comparison with **1**, the difference of 13 uma suggested that the amino group was replaced

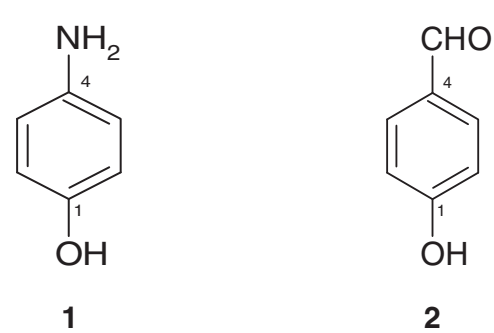

Fig. 1. Structure of the two purified compounds **1** and **2** from the leaf ethyl acetate extract of *Dilobeia thouarsii*.

by an aldehyde group (Chen et al., 1999). Consequently, compound **2** was identified as 4-hydroxybenzaldehyde.

3.2. Antibacterial activity

This is the first time that antimicrobial activity of *D. thouarsii* extracts has been reported. The two phenolic compounds determined from the leaf ethyl acetate extract (4-aminophenol and 4-hydroxybenzaldehyde) were more active against both Gram-positive and Gram-negative bacteria than the crude extract itself (Table 1). MIC and MBC values varied with the extracts and compounds tested. *S. aureus* was the most sensitive strain. According to Oussou et al. (2008), the ratio observed for MBCs and MICs (MBC:MIC < 4) indicated that the bactericidal effect of the compounds on the majority of strains tested could be expected. Globally, Gram-positive bacteria were more sensitive to these compounds than Gram-negative ones (Table 1).

MIC values obtained with the two compounds for *S. aureus* were lower than those of leaves and bark extracts of *P. simplex* (a Proteaceae from South Africa) which ranged between 0.147 and 0.780 mg/ml (Fawole et al., 2009). MIC values of our extracts were lower than 0.1 mg/ml for *S. aureus*, which, according to Holetz et al. (2002), can be considered as good antimicrobial activity. *S. aureus*, which is a major agent in skin infections, was sensitive to other African plant extracts of species including *Combretum vendae* (Combretaceae), *Commiphora harveyi* (Burseraceae), *Khaya anthotheca* (Meliaceae), *Kirkia wilmsii* (Kirkiaceae), *Loxostylis alata* (Anacardiaceae), *Ochna natalitia* (Ochnaceae), *Protorhus longifolia* (Anacardiaceae), *Lippia* spp., *Garcinia smeathmannii* (Clusiaceae) and *Ficus ovata* (Moraceae) (Kuete et al., 2009; Shikanga et al., 2010; Suleiman et al., 2010). Sato et al. (1997) examined the activity of three extracts from the fruiting bodies of *Terminalia chebula* RETS against methicillin-sensitive and methicillin-resistant *S. aureus* as well as 12 other Gram-negative and Gram-positive bacteria. The two compounds isolated from the Et_2O soluble part material, gallic acid and its ethyl ester derivative proved to be more effective against both types of *S. aureus* than against other species. It appears that the ability to inhibit respiratory electron transport systems plays an essential role in the antibacterial activity of alkyl gallates against Gram-positive bacteria.

The MIC values of between 0.1 and 0.5 mg/ml that we observed for *E. faecalis*, *Vibrio* spp., and *Bacillus* spp. can be considered as moderate antimicrobial activity according to Holetz et al. (2002) (Table 1). Neither purified compound (**1**, **2**) was shown to be active against *Salmonella* spp., *E. coli* and *K. pneumoniae* (MICs > 1 mg/ml).

Table 1
The minimum inhibitory concentration (MIC mg/ml) and minimum bactericidal concentration (MBC g/ml) of *Dilobeia thouarsii* leaf ethyl acetate extract against bacteria tested in microdilution assays.

Bacterial species	Leaf ethyl acetate extracts (mg/ml)				
	Crude extract	Compounds			
		1		2	
	MIC	MIC	MBC	MIC	MBC
Gram-positive					
Bacillus cereus	12.5	0.109	0.437	0.054	0.109
Bacillus megaterium	12.5	0.109	0.218	0.437	3.5
Staphylococcus aureus	12.5	0.054	0.218	0.027	0.109
Enterococcus faecalis	12.5	0.437	0.875	3.5	1.75
Gram-negative					
Vibrio harveyi	12.5	0.109	0.437	0.109	0.109
Vibrio fisheri	12.5	0.218	0.437	0.437	0.875
Salmonella Typhimurium	>100	3.5	3.5	3.5	3.5
Salmonella antarctica	>100	3.5	3.5	3.5	3.5
Escherichia coli	>100	1.75	1.75	1.75	3.5
Klebsiella pneumoniae	>100	1.75	1.75	7.0	7.0

4. Conclusion

Its antimicrobial activity against *S. aureus*, a major agent in skin infections, provides a scientific basis for the traditional Malagasy use of *D. thouarsii* (Roemer and Schulte) in the treatment of skin infections. The two purified phenolic compounds from leaf ethyl acetate extracts involved in this antimicrobial activity were 4-aminophenol and 4-hydroxybenzaldehyde. Consequently, leaf ethyl acetate extract could be used in further investigations to identify the other molecules present in this plant.

Acknowledgments

This work was financially supported by the project "*Pôle d'Excellence Régional*" (AUF/ref/no. 2708PL708) funded by AUF (*Agence Universitaire de la Francophonie*).

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