

Research Article

Antimicrobial Activity of 23 Endemic Plants in Madagascar

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Abstract

Purpose: To screen the crude methanol extracts obtained from 23 endemic plants in Madagascar for antimicrobial activity.

Methods: In order to assess the antimicrobial properties of the extracts, their minimum inhibitory concentrations (MICs) were obtained using the broth microdilution method. The six test pathogenic species used were *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Candida albicans*. Bioautography agar overlay test and phytochemical screening were also performed on the most active extracts.

Results: From the 23 plants tested, 16 of which are used in traditional medicine, *Poivrea phaneropetala* (Combretaceae), *Koehneria madagascariensis* (Lythraceae) and *Rhopalopilium perrieri* (Opiliaceae) exhibited the broad spectrum of activity, being active against all the test organisms, while *Monoporus clusiifolius* (Myrsinaceae) showed the strongest antifungal activity against *Candida albicans* with a minimal inhibitory concentration of 0.250 mg/ml. Bioautography and phytochemical analysis of the five active extracts against bacterial strains and of one active extract against *C. albicans* indicate that the active compounds responsible for antimicrobial activity may be mainly flavonoids and/or terpenes.

Conclusion: These preliminary results are the first antimicrobial studies on these plants and lend support for the use of some of them in traditional medicine.

Keywords: Antimicrobial properties, Traditional medicine, Microdilution assay, Bioautography, Madagascar.

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INTRODUCTION

Madagascar is host to approximately 12,000 vegetable species, more than 80 % of which are endemic to the island [1]. Owing to environmental degradation, increasing deforestation, slash and burn agriculture in primary forest, this unique patrimony is threatened by extinction. Only 9 % of the original areas are currently available and the country is among 25 most critical regions for plant life protection in the world [2].

In Madagascar, herbal medicines are often used as the first line of treatment of various diseases. The practice of traditional medicine is well-embedded in the lifestyle of the eighteen indigenous tribes of Madagascar. Previous investigations on Madagascar flora mainly dealt with ethnobotanical practices of plants in folk medicine [3-5]. Screening surveys have already shown some biological activities such as antiplasmodial [6], antiviral [7], and cytotoxic activities [8]. However, very few scientific studies have been carried out on the putative antimicrobial properties of endemic plants in the island although many of them have been claimed by local traditional healers to be effective in the treatment of infectious diseases.

The aim of this study was to investigate the antimicrobial properties of 23 endemic plants obtained from different parts of the country and to identify the phytochemical class of active components.

EXPERIMENTAL

Plant materials

The plants were collected from various locations in Madagascar and were authenticated by Dr Armand Rakotozafy, the curator of the Department of Botany at the Institut Malgache de Recherches Appliquées (IMRA), Antananarivo, Madagascar. Voucher specimens were deposited at the herbarium of the Parc Botanique et Zoologique de Tsimbazaza, Antananarivo, Madagascar.

Plant names and their folkloric use are given in Table 1. Dried plant materials were ground into fine powders and preserved at the IMRA herbarium.

Table 1: List of 23 Madagascan endemic plants used in the study and their folkloric use

Family	Species name	Folkloric use
Annonaceae	<i>Xylopia buxifolia</i> Baill.	Tonic, jaundice
Apocynaceae	<i>Mascarenhasia lisianthiflora</i> A. DC.	n.i
Asteraceae	<i>Pluchea grevei</i> Humbert	Headache, tonic
Asteropeiaceae	<i>Asteropeia densiflora</i> Baker	n.i
Celastraceae	<i>Evonymopsis longipes</i> H. Perrier	Headache
Clusiaceae	<i>Symphonia clusioides</i> Baker	Hair ointment
Combretaceae	<i>Poivrea phaneropetala</i> (Baker) H. Perrier	Vermifuge
Combretaceae	<i>Poivrea grandidieri</i> (Baill.) H. Perrier	Vermifuge, icterus
Combretaceae	<i>Poivrea obscura</i> (Tul.) H. Perrier	Vermifuge, diuretic
Dilleniaceae	<i>Hibbertia coriacea</i> Baill.	Vaginitis, urethritis
Elaeocarpaceae	<i>Elaeocarpus sericeus</i> Baker	n.i
Fabaceae	<i>Piptadenia pervillei</i> Vatke	Antimalarial
Lythraceae	<i>Koehneria madagascariensis</i> (Baker) S.A.Graham, H.Tobe & P.Baas	n.i
Melastomaceae	<i>Dichaetanthera oblongifolia</i> Baker	Diarrhea, dysentery
Moraceae	<i>Pachytrophe dimepate</i> Bureau	Jaundice
Myrsinaceae	<i>Monoporus clusiifolius</i> H. Perrier	n.i
Opiliaceae	<i>Rhopalopilium perrieri</i> Cavaco & Keraudren	Antiseptic, wound healing
Rhamnaceae	<i>Bathiorhamnus louvelii</i> (H. Perrier) Capuron	Purgative
Sapindaceae	<i>Conchopetalum madagascariense</i> Radlk	n.i
Sarcolaenaceae	<i>Leptolaena pauciflora</i> Baker	Venereal disease
Sarcolaenaceae	<i>Leptolaena diospyroidea</i> (Baill.) Cavaco	Impotence to man
Sterculiaceae	<i>Rulingia madagascariensis</i> Baker	n.i
Thymelaeaceae	<i>Peddiea involucreata</i> Baker	Antimalarial

n.i = no previous ethnomedical indication reported

Preparation of plant extracts

Dried and powdered plants (10 g) were macerated with agitation in 50 ml methanol (MeOH) overnight at room temperature. After filtration, the solvent was eliminated by evaporation at reduced pressure and crude extracts were dried at 45 °C using a speed-vac concentrator (Savant), and stored at the IMRA bank at 4 °C.

Microorganisms

The bacterial strains used for the investigation were *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*, and were all obtained as clinical isolates from patients at the Microbiology Department, IMRA. The yeast strain, *Candida albicans* (MUCL 31360), was obtained from the Mycothèque de l'Université Catholique de Louvain (MUCL), Belgium. Stock cultures were maintained at 4 °C on slopes of nutrient agar (Difco) for bacteria and on Sabouraud dextrose agar (SDA, Difco) for the yeast, prior to their use.

Determination of minimum inhibitory concentration (MIC)

The broth microdilution method was used to assess the MIC of the different plant extracts in a 96-well microplate using a modified method [9]. Microbial suspensions were first prepared from an overnight culture of bacterial and fungal cells grown in flasks, each containing 10 ml of Mueller-Hinton Broth (Oxoid) for bacteria and Sabouraud dextrose broth (Difco) for yeast at 37 °C and 30 °C, respectively. The turbidity of the microbial suspensions was adjusted to 0.5 McFarland using Densicheck (BioMerieux). Stock solutions of the different extracts were prepared by re-suspending the crude methanol extracts in 10 % dimethyl sulfoxide (DMSO) to produce concentrations in the range of 1.25 – 160 mg/ml. These solutions were further diluted 10-fold with water and then sterilized by filtration through 0.22 µm

membrane filters. A known volume (100 µl) of each solution was deposited in the wells. This was followed by the addition of 100 µl of the inoculum (approximately 10⁶ CFU/ml for bacteria and 10⁵ CFU/ml for *C. albicans*) was added to each well. The microplates were incubated overnight at 37 °C for bacteria and 30 °C for 48 h for *C. albicans*. After incubation, 40 µl of 0.2 mg/ml aqueous solution of methylthiazolyltetrazolium chloride (MTT) was added to each well and further incubated for 30 min at room temperature. MIC was defined as the lowest concentration in which no transformation of MTT was observed. Streptomycin sulfate and nystatin were used as positive controls and their MICs were determined using the same process. All samples were tested in triplicate and the tests were repeated twice.

Bioautography agar-overlay assay with *B. subtilis* and *C. albicans*

Plant extracts showing significant antimicrobial activity with MICs values close to 1 mg/mL against *B. subtilis* or *C. albicans* were investigated by thin layer chromatography (TLC) bioautographic agar-overlay according to the method of Rahalison et al [10] with minor modifications. Twenty microlitres of different solutions of the methanol plant extract (400 µg) were applied to pre-coated Silica gel GF254 plates (Merck KGaA, Darmstadt, Germany). TLC plates were developed with ethyl acetate/methanol 1/1 (v/v) for *B. subtilis* and ethyl acetate/methanol/water 10/10/3 (v/v/v) for *C. albicans* and dried thoroughly overnight to achieve complete removal of the solvents. The developed TLC plates were thinly overlaid with molten malt extract agar and with SDA inoculated with an overnight culture of *B. subtilis* and *C. albicans*, respectively. The plates were incubated in a dark and humid chamber at 25 °C for 24 h for *B. subtilis* and 48 h for *C. albicans*. After incubation, cultures were sprayed with MTT and further incubated for 30 min at room temperature. Microbial growth inhibition appeared as clear zones around active

compounds against a purple background. The plates were in duplicate. One set was used for bioautography experiment and the other was intended for the reference chromatogram. The experiments were repeated twice.

Phytochemical screening

Plant extracts previously selected for TLC bioautographic assays were subjected to preliminary phytochemical screening to determine the major chemical groups corresponding to active compounds responsible for the observed inhibition zones. TLC was first developed with the same solvent systems as previously mentioned above. For the detection of alkaloid, coumarins, flavonoids and terpenes, phytochemical screening was performed using standard procedures [11]. Observations were made from two independent experiments.

RESULTS

A total of 23 methanol plant extracts of Madagascan endemic species belonging to 20 different botanical families, among which 16 are used in traditional medicine (Table 1), were tested for their antimicrobial activities against 5 bacterial species and one yeast. The results of these tests are summarized in Table 2. Eight extracts inhibited the growth of *B. subtilis* at MIC values lower or equal to 1 mg/ml. Among them, the extract of *Rhopalopilium perrieri* Cavaco & Keraudren (Opiliaceae) exhibited the lowest MIC value (0.13 mg/ml). Four of them were also found to be effective against *S. aureus* at 1 mg/ml. Five extracts were moderately active against *E. coli* and *S. typhi* with MIC values ranging from 2 to 4 mg/ml. None of the tested plant extracts displayed activity against *P. aeruginosa* (MIC > 8 mg/ml) except *Poivrea phaneropetala* (Baker) H. Perrier (Combretaceae), *Koehneria madagascariensis* (Baker) S.A.Graham, H.Tobe and P.Baas (Lythraceae) and *R. perrieri*. Of the 6 plant extracts that inhibited *C. albicans* at

MIC lower or equal to 2 mg/ml, *Monoporus clusiifolius* H. Perrier (Myrsinaceae) showed the most interesting inhibitory activity against *C. albicans* with a MIC of 0.25 mg/ml

DISCUSSION

This study showed that all but two plant extracts possessed some degree of activity against at least one tested microorganism, at the highest tested dose of 8 mg/ml. Overall, Gram positive bacteria (*B. subtilis* and *S. aureus*) were more sensitive to plant extracts than Gram negative bacteria (*E. coli*, *S. typhi* and *P. aeruginosa*). Among them, *P. phaneropetala*, *K. madagascariensis* and *R. perrieri* demonstrated a broad spectrum of activity against all the tested organisms while *M. clusiifolius* was the most active against *C. albicans*. *R. perrieri* showed the most promising antibacterial activity, confirming its use in traditional medicine as antiseptic and wound healing. The present investigation also confirmed the anti-infective property of *H. coriacea* in the treatment of vaginitis and urethritis and that of *D. oblongifolia* which is used to treat diarrhoea and dysentery in folk medicine. In contrast, the use of *Leptolaena pauciflora* Baker (Sarcolaenaceae) as anti-infective agent for the treatment of venereal diseases was not supported by our investigation. Furthermore, the oral use of this plant containing several phenolic compounds could be toxic [12].

Based on this investigation, we are unable to say categorically that extracts having the same R_f values contained the same bioactive compounds. It is possible that the observed inhibition was likely due to one or more compounds sharing the same R_f in the solvent system used, particularly for low R_f spots. Preliminary phytochemical screening revealed the presence of flavonoids, alkaloids and terpenes as the probable active compounds present in the crude extracts. It is well known that numerous members of these phytochemical groups have already demonstrated antimicrobial activity [13].

Table 2: Antimicrobial activity of 23 Madagascan endemic plants

Plant	Voucher no.	Microorganisms ^a / MIC ^b (mg/ml)					
		B.s	S.a	E.c	S.t	P.a	C.a
<i>Xylopi buxifolia</i>	AML6	-	-	-	-	-	-
<i>Mascarenhasia lisianthiflora</i>	MOR23	2	-	-	-	-	-
<i>Pluchea grevei</i>	TUL16	4	-	-	-	-	4
<i>Asteropeia densiflora</i>	TUL8	0.5	2	-	-	-	2
<i>Evonymopsis longipes</i>	MOR33	2	8	-	-	-	8
<i>Symphonia clusioides</i>	AMB34	-	-	-	-	-	-
<i>Poivrea phaneropetala</i>	MOR12	0.5	1	4	4	8	2
<i>Poivrea grandidieri</i>	TUL10	4	8	8	8	-	8
<i>Poivrea obscura</i>	MOR26	1	1	4	4	-	-
<i>Hibbertia coriacea</i>	MKR44	1	2	4	2	-	4
<i>Elaeocarpus sericeus</i>	M447	1	4	-	-	-	4
<i>Piptadenia pervillei</i>	AA17	2	8	8	8	-	-
<i>Koehneria madagascariensis</i>	TUL5	0.5	1	4	4	8	2
<i>Dichaetanthera oblongifolia</i>	BP31	1	2	-	-	-	2
<i>Pachytrophe dimepate</i>	VAM282	2	-	-	-	-	-
<i>Monoporus clusiifolius</i>	AMB43	2	8	-	-	-	0.25
<i>Rhopalopilium perrieri</i>	TUL35	0.13	1	4	4	8	2
<i>Bathiorhamnus louvelii</i>	AA68	2	-	-	-	-	-
<i>Conchopetalum madagascariense</i>	VAT652	2	-	-	-	-	-
<i>Leptolaena pauciflora</i>	MKR692	4	8	8	8	-	8
<i>Leptolaena diospyroidea</i>	MOR56	4	-	-	-	-	-
<i>Rulingia madagascariensis</i>	BP14	4	8	-	-	-	8
<i>Peddiea involucrata</i>	AA48	4	8	-	-	-	-
References (µg/ml)							
Streptomycine		3.9	3.9	31.3	31.3	-	nd
Nystatine		nd	nd	nd	nd	nd	3.13

^a MIC: minimal inhibitory concentration (mg/ml)

^b Microorganisms: B.s = *Bacillus subtilis*; S.a = *Staphylococcus aureus*; E.c = *Escherichia coli*; S.t = *Salmonella typhi*; P.s = *Pseudomonas aeruginosa*; C.a = *Candida albicans* (-): MIC > 8 mg/ml for plant extracts and MIC > 64 µg/ml for streptomycin; nd = not determined

Overall, five plant extracts with MICs lower than 1 mg/ml against either *B. subtilis* or *C. albicans* were subjected to TLC bioautographic agar overlay test. Rf value of the inhibition zones and the probable chemical group of compound responsible for the inhibition are listed in Table 3. The tests based on colour development suggested that the active compounds belong mainly to flavonoid or terpene groups. Furthermore, a positive test with Dragendorf reagent suggests that *Asteropeia densiflora* Baker (*Asteropeiaceae*) and *R. perrieri* may contain alkaloids at Rf of 0.02 (at base line).

Table 3: Phytochemical screening of active compounds responsible for microbial inhibition based on bioautography assays

Plant	Rf value	Phytocompound ^a			
		A	C	F	Te
<i>Monoporus clusiifolius</i> ^b	0.68	-	-	+	+
<i>Poivrea phaneropetala</i> ^c	0.1	-	-	+	+
<i>Koehneria madagascariensis</i> ^c	0.1	-	-	+	+
<i>Asteropeia densiflora</i> ^c	0.02	+	-	+	+
	0.75	-	-	+	+
<i>Rhopalopilium perrieri</i> ^c	0.02	+	-	+	+

^a A= Alkaloids; C= Coumarins; F= Flavonoids; Te= Terpenes

^b Test performed on TLC bioautographic agar-overlay with solvent system EtOAc/MeOH/H₂O (10/10/3) using *C. albicans* as organism test

^c Test performed on TLC bioautographic agar-overlay with solvent system EtOAc/MeOH (1/1) using *B. subtilis* as organism test

In many cases, these substances serve as plant defense mechanisms against aggression by microorganisms, insects, and herbivores either synthesized during the plant normal development (constitutive resistance factors) or induced only after contact with the pathogen (induced resistance factor) [14].

To the best of our knowledge, no previous antimicrobial survey has been reported on these plants. However, some of them were previously reported for other biological activities. Literature searches indicate that *Evonymopsis longipes* (H. Perrier) H. Perrier (Celastraceae) was particularly potent and could completely inactivate the Herpes Simplex Virus at a concentration lower than 25 µg/ml [7]. *Peddiea involucreata* Baker (Thymelaeaceae) and *Piptadenia pervillei* Vatke (Fabaceae) were said to possess antimalarial activity [15-16]. From this latter was isolated (+)-catechin 5-gallate and (+)-catechin 3-gallate which demonstrated, *in vitro*, high activity against the chloroquine-resistant strain FcB1 of *Plasmodium falciparum* as well as (+)-catechinant ethylgallate which were less effective [16]. With regard to antibacterial activity, however, these plants were less effective, being active only against *B. subtilis* with MIC ranging from 2 to 4 mg/ml.

While no ethnomedicinal practices were reported to date for 7 of the 23 plants, the study revealed that *A. densiflora* and *K. madagascariensis* were found to possess effective antibacterial activity; in addition, the extract from *M. clusiifolius* may be a good candidate in the search for potential antifungal compounds. Further studies on these plants are being undertaken in our laboratory to isolate and elucidate the compounds responsible for the observed anti-candidiasis and antibacterial activities.

CONCLUSION

This survey contributes new data to the existing knowledge of antimicrobial activity of the endemic flora of Madagascar and may

serve as a basis for further pharmaceutical investigation. The findings also lend support for the use of some of these plants in traditional medicine in Madagascar.

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