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Antimicrobial Activity of Seed Extracts from *Albizia bernieri* E. Fourn. (Fabaceae)

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Abstract: *This work aimed at assessing the antimicrobial activity of Albizia bernieri seed extracts. Methanol extract (SME), alkaloids extracted under basic (Alk1) and acidic (Alk2) conditions, polar saponosides (Sap1) and less polar saponosides (Sap2) obtained by n-butanol fractionation were used. Their activities were tested against 9 pathogenic germs including 4 Gram (+) bacteria, 5 Gram (-) bacteria and 1 yeast using disc diffusion and microdilution methods. Regardless the method used to assess the antimicrobial activity, all extracts were efficient but their effects depended upon the germs tested. They all displayed a broad spectrum activity. Alkaloids were by far the most efficient with an excellent effect (Minimum Inhibitory Concentration or MIC<100µg/ml) against all germs tested. Streptococcus pneumoniae, Streptococcus pyogenes, Clostridium perfringens and Candida albicans were the most sensitive with MICs less than 10 µg/ml. SME and Sap 2 had moderate or low effects (MIC between 100 and 1000 µg/ml) and Sap1 was the less efficient (MIC ≥1000 µg/ml). All the extracts exerted bactericidal or fungicidal action on all sensitive germs. These preliminary results revealed antimicrobial activity of Albizia bernieri seeds that could be used to treat different infectious diseases and might lead to the development of pharmaceutical agents.*

Keywords*: Albizia bernieri, antimicrobial activity, disc diffusion method, microdilution method, minimum bactericidal concentration, minimum inhibitory concentration.*

I. Introduction

Microbial strains developing stronger resistance to various antibiotics are growing in numbers which constitutes a significant public health problem. There is an urgent need to find new disposable and affordable remedies to face this problem [1]. A systematic screening of plant extracts as a source of anti-bacterial compounds has been undertaken in different laboratories [2, 3].

Several *Albizia* species organ extracts from different countries had been reported displaying antimicrobial properties. They include the extracts of *A. zygia* leaf [4], *A. julibrissin* leaf, stem and flower [5], *A. amara* leaf, flower, pod and bark [6], *A. lebbeck* seed [7], *A. anthelmintica* root bark [8], *A. amara* and *A. saman* leaves [9], wood [10], *A. andianthifolia* bark and root [11]. Seed extracts from some endemic *Albizia* of Madagascar also exhibited antimicrobial effects [12]. The purpose of this study was to assess the antimicrobial properties of *A. bernieri* seeds, one of the 24 *Albizia* species endemic to Madagascar [13]. The effects of seed extracts were tested on pathogen germs responsible for serious infections in humans and many of which are resistant to antibiotics.

II. Materials And Methods

2.1 Plant Materials

A. bernieri is a large shrub or tree up to 25 m tall (Fig.1) growing throughout the western Part of Madagascar, from the North to the South. The plant was identified by comparison of voucher specimens, registered under 29133-SF, with herbarium samples of Department of Forest Research and Fish Farms of FOFIFA Antananarivo. Dry fruits were collected on July 2010 from Mampikony in the North West of Madagascar.

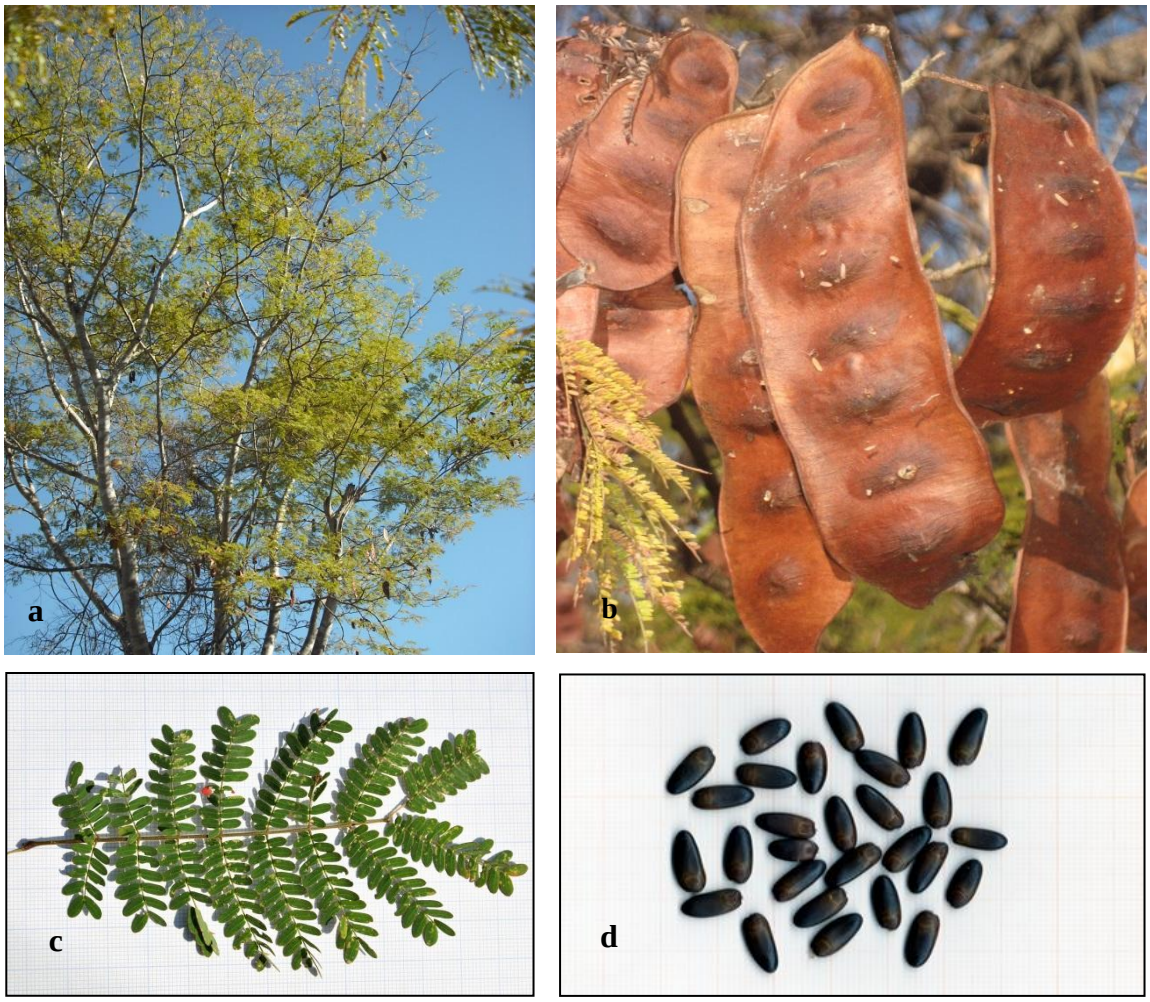

Figure 1: *Albizia bernieri*: a) the whole plant; b) fruits; c) leaves; d) seeds

Source: the authors

2.2 Microorganism Strains

The 10 microorganisms used in this study consisted of 4 Gram (-) bacteria, 5 Gram **(+)** bacteria and1 yeast (TABLE 1). They were maintained on agar slant at 4°C and cultured on a fresh appropriate agar plate 24 h prior to any antimicrobial test.

TABLE 1: List of germs used

Strains	Reference	Gram
Staphylococcus aureus	ATCC 25923	+
Bacillus cereus	ATCC 14579	+
Streptococcus pneumoniae	ATCC 6305	+
Streptococcus pyogenes	ATCC 19615	+
Enterobacter aerogenes	ATCC 13048	-
Enterobacter cloacae	ATCC 13047	-
Yersinia enterocolitica	ATCC 23715	-
Pseudomonas aeruginosa	ATCC 10145	-
Clostridium perfringens	ATCC 13124	-
Candida albicans	ATCC 10231	

2.3 Chemicals for Antimicrobial Assay

Antibiotic and antifungal used as references in this study were Neomycin 30 µg/disc and Miconazole 500 µg/disc.

2.4 Preparation of Seed Extracts

2.4.1 Seed Methanolic Extract

Ground seed powder (250 g) was delipidated with hexan (3 X 500 ml), then extracted with methanol (3 X 500 ml). After filtration using a Whatman filter paper, extract was evaporated to dryness under reduced pressure. The dry residue dissolved in sterile distilled water constituted seed methanol extract (SME).

2.4.2 Saponosides Extraction

SME was mixed with an equal volume of n-butanol. Aqueous phase was yet two times treated with an equal volume of n-butanol. The three organic phases gathered and the aqueous phase were evaporated to dryness under reduced pressure. Seven grams (7g) of the aqueous phase residue were dissolved in 100 ml of methanol. The soluble fraction obtained was gradually added to 300 ml of acetone-diethyl ether mixture (v/v) cooled in an ice bath. After a few minutes of maceration the precipitates formed were recovered by centrifugation (1000 rpm during 5 minutes at +4°C). The supernatant was collected and treated under the same previous conditions. This operation was repeated three times. All the precipitates were gathered and solvent was removed by evaporation. The resulting residue (3.99 g) constituted the polar total saponosides named **Sap1**. Three grams (3g) of the organic phase residue were dissolved in 100 ml of methanol. The resulting solution was also submitted to the same treatment by acetone-diethyl ether mixture (v/v). After evaporation, 2.1 g of less polar total saponosides (**Sap2**) were obtained.

2.4.3 Alkaloids Extraction

Two extraction methods were used.

Alkaloid Extraction under Basic Conditions

Four grams (4g) of SME powder were moistened with 20 ml of ammoniac 20%, then suspended in 300 ml of dichloromethane. The mixture was stirred at room temperature during 24 h. After filtration, the solution was evaporated to dryness with a rotary evaporator at low pressure at 40°C. The residue obtained was dissolved in 20 ml of water and the solution was acidified with sulfuric acid (10%) until pH 2-3 and then extracted with ether to remove acidic and neutral lipophilic compounds. After adjusting its pH to 9-10 with NH_4OH (20 %), the aqueous solution was extracted with dichloromethane (3 x 20 ml). The lower organic phase was washed three times with distilled water, then dehydrated with anhydrous sodium sulphate (Na_2SO4) and evaporated to dryness under reduced pressure. The residue obtained was the crude total alkaloid **Alk1**.

Alkaloid Extraction under Acidic Conditions

SME powder (4g), moistened with HCL 1M (30 ml),was suspended in 100 ml of methanol and stirred for 24 h at room temperature. After filtration, the solution was evaporated to dryness under reduced pressure. The aqueous acidic solution was alkalinized to pH 9-10 with sodium hydroxide (NaOH 20 %), then extracted with3 x 20 ml of dichloromethane. The organic phase was dehydrated with anhydrous sodium sulphate (Na_2SO_4) and evaporated to dryness under reduced pressure to yield the crude total alkaloid **Alk2**.

The five seed extracts tested are summarized in TABLE 2.

TABLE 2: List of seed extracts tested

Abbreviations	Extracts
SME	Seed methanolic extract
Sap1	Total saponosides from aqueous butanol phase of SME
Sap2	Total saponosides from organic butanol phase of SME
Alk1	Total alkaloids under alkaline extraction
Alk2	Total alkaloids under acidic extraction

2.5 Phytochemical Screening

The reactions of chemical group detection were those developed by [14, 15].

2.6 Antimicrobial Assays

All the materials and methods used for antimicrobial assay were detailed in our previous paper [16].

2.6.1 Antimicrobial Activity Test

The *in vitro* antimicrobial activity of the extracts was determined using disc diffusion method of Pyun *et al.* [17] and Ngameni *et al.* [18]. The results were interpreted using the scale of Ponce *et al.* [19] and Celikel *et al.* [20]. Bacteria are not sensitive for an inhibition zone diameter (IZD) less than 8 mm, sensitive for IZD of 9 -14 mm, very sensitive for IZD of 15 -19 mm and extremely sensitive for IZD larger than 20 mm.

Antifungal activity was evaluated by a method described by Favel *et al*. [21].

Negative controls were prepared by using the same solvents employed to dissolve the plant extract samples while the standard antibiotics were used as positive controls. All the experiments were performed in triplicate.

2.6.2 MIC, MBC and MFC Determination

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were determined by microdilution method [22].

The standards used to interpret MIC results were those of Dalmarco *et al*. [2]. For crude extracts and fractions, a MIC lower than 100 µg/mL was considered as an excellent effect, from 100 to 500 µg/ml as

moderate, from 500 to 1000 µg/mL as weak, and over 1000 µg/ml as inactive. The extract action is bactericidal or fungicidal whether its ratio MBC/MIC or MFC/MIC is ≤4) and bacteriostatic and fungistatic when the ratio is>4 [23, 24, 25].

III. Results

3.1 Extraction yields

Extraction yields are shown in TABLE 3. They were calculated by reference to the starting material, *i.e.* seed powder for SME and SME for Sap1, Sap2, Alk1 and Alk2. Extraction yields ranged from 1.16% (Alk1) to SME (16.02%).

TABLE 3: Extraction yields of the different extracts

Extract	Yield
SME	16.02%
Sap1	9.97%
Sap2	5.5%
Alk1	1.16%
Alk2	2.78%

3.2 Phytochemical analysis

The major secondary metabolites identified in SME extracts can be seen in TABLE 4. Alkaloids, saponosides, tannins, polyphenols, steroids, triterpenes and cardenolids were present, while flavonoids, leucoanthocyanins, coumarins, iridoïds, steroids, unsaturated sterols and quinones were not detected. Sap1 and Sap2 were contaminated with alkaloid traces, whereas Alk1 and Alk2 were free from any contamination.

TABLE 4: Phytochemical screening of *A. bernieri* seed extracts

Chemical groups	**Tests**	**SME**	**Sap1**	**Sap2**	**Alk1**	**Alk2**
Alkaloids	Mayer	+	±	±	+++	+++
	Wagner	+	±	±	+++	+++
	Dragendorff	+	±	±	+++	+++
Saponins	Foam test	+				
	Hemolytic test	+	+++	+++	-	-
Flavonoids	Willstätter	-	-	-	-	-
Leucoanthocyanins	Bate-Smith	-	-	-	-	-
Cardenolids		+	-	-	-	-
Cyanogenetic glycosides	Grignard	-	-	-	-	-
Unsaturated lactones	Kedde	-	-	-	-	-
Coumarins		-	-	-	-	-
Tannins and Polyphenols	Gelatin 1%	+	-	-	-	-
	Gelatin-salt 10%	-	-	-	-	-
	$FeCl_3$	+	-	-	-	-
Quinones	Borntrager	-	-	-	-	-
Steroids	Liebermann-Burchard	-	-	-	-	-
Iridoïds	Hot HCl	-	-	-	-	-
Triterpenes	Liebermann-Burchard	+	-	-	-	-
Unsaturated sterols	Salkowsky	-	-	-	-	-

+: positive test; -: negative test

3.3 Antimicrobial activity

At the concentration of 1mg/disc, a concentration often used in antimicrobial activity assessment in plant extracts [26, 27, 28, 16], all the 5 extracts displayed antibacterial activity with IZD ranging from 8 to 27 mm (TABLE 5). However, activity depended on the extract and the microorganism used.

TABLE 5: Effects of *A. bernieri* seed extracts by disc diffusion method

Strains		Inhibition zone diameter (mm)						
		Extracts (1000 µg/disc)					Neomycin 30 µg/disc	Miconazole 500 µg/disc
		SME	Sap1	Sap2	Alk1	Alk2		
Gram (-)	*Enterobacter cloacae ATCC 13047*	20	18	22	26	25	27	-
	Enterobacter aerogenes ATCC 13048	7	7	9	11.5	11	19	-
	Pseudomonas aeruginosa	8	8	10	11	12	18	-
	Yersinia enterocolitica	7	6	10	13	11	20	-
Gram (+)	*Streptococcus pneumoniae*	9	9	12	14	14	23	-
	Streptococcus pyogenes	9	8	10	9	20	24	-
	Staphylococcus aureus	9	9	10	10	14	18	-
	Clostridium perfringens	9	9	10	13	11	22	-
	Bacillus cereus	9	9	10	10	14	21	-
Yeast	*Candida albicans*	6	6	6	12	10	-	25

Alk1 and Alk2 were active against all the microorganisms tested and were the most efficient. Sap2 was active against bacteria but not against *Candida albicans*. SME and Sap1 were the less active (IZD ≤ 9 mm) against all the strains tested except *Enterobacter cloacae*. *Enterobacter aerogenes, Yersinia enterocolitica* and *Candida albicans* were not sensitive to these 2 extracts.

Neomycin 30 µg and miconazole 500 µg were more effective than all the *A. bernieri* seed extracts.
The antimicrobial activity of the 5 extracts assessed by microdilution method is shown in TABLE 6.

Regarding MIC values, 48% were <100 µg/ml, 20% between 100 and 500 µg/ml, 26% from 500 to 1000 µg/ml and 6% >1000 µg/ml. The values ranged from 31.25 to 500 µg/ml, 62.5 to >1000 µg/ml, 15.6 to 500 µg/ml, 3.9 to 62.5 µg/ml and 1.95 to 15.6 µg/ml for SME, Sap1, Sap2, Alk1 and Alk2 respectively.

About the MBC or MFC values, 44% were <100 µg/ml, 18% between 100 and 500 µg/ml, 28% from 500 to 1000 µg/ml and 10% >1000 µg/ml. These values were 125 to >1000 µg/ml, 125 to >1000 µg/ml, 31.25 to 1000 µg/ml, 7.8 to 62.5 µg/ml, 3.9 to 62.5 µg/ml for SME, Sap1, Sap2, Alk1 and Alk2 respectively. The ratios MBC/MCI or MFC/MCI of all extracts varied from 1 to 4 which meant they had a bactericidal action against all the microorganisms tested.

IV. Discussion

A. bernieri seeds, like those of all *Albizia* species from Madagascar so far studied, mainly contained saponosides and alkaloids. The n-butanol fractionation separated the *A. bernieri* saponosides into 2 chemical groups: polar saponosides which have affinity to aqueous phase (Sap1) and less polar saponosides found in organic phase (Sap2). Alkaloids were better extracted under acidic extraction as reported by George *et al*. [29].

Regardless the method used to assess the antimicrobial activity, all the *A. bernieri* seed extracts were efficient but their effects depended upon the germs tested. They generally displayed a broad spectrum activity.

Activity levels were generally higher in liquid than in solid medium. In fact, activity considered as non-existent (IZD <8 mm) or moderate (IZD between 9 and 14 mm) in solid medium were excellent (MIC<100µg/ml) in liquid medium. As examples, against *Candida albicans* Alk1 had IZD = 7 mm and MIC = 62.5 µg/ml and against *Enterobacter aerogenes*, *Clostridium perfringens* and *Candida albicans* Alk2 had IZD = 10-11 mm and MIC = 3.9 -15.6 µg/ml. Bioactive compounds probably diffused little or not at all in solid medium. SME contained different secondary metabolites such alkaloids, polyphenols, saponins and triterpenes known for their antimicrobial properties. SME was substantially less active than saponosides and alkaloids it contained. At least, that might be partially due to negative interactions between contaminants and the active compounds. With the two methods used the alkaloids were more active than saponosides against all the strains tested. Their MIC between 1.95 and 62.5 µg/ml and their MBC between 3.9 and 62.5 µg/ml allowed to rank them among extracts with excellent effects [2]. Their activity on *Enterobacter cloacae* and *Streptococcus pyogenes*, the most sensitive bacteria tested, was comparable with that of neomycine at 30 µg/ml. It is realistic to expect that pure alkaloids could be at least as efficient as this standard antibiotic. Concerning the saponosides group, the less polar saponosides (Sap2) were more efficient that the polar saponosides (Sap1). As noted above, the standards used to interpret MIC results were those of Dalmarco *et al*. [2]. However, it should be noted that there is no consensus on the inhibition level for natural products [30]. Compared to antimicrobial effects of other *Albizia* species extracts from Madagascar, the *A. bernieri* extracts with MIC values between 1.5 and 1000 µg/ml, are by far more effective than the extracts of *A. arenicola* leaves on *Candida albicans* (MIC = 15620 µg/ml), the *A. aurisparsa* seeds on *Bacillus cereus* (MIC = 1980 µg/ml), the *A. divaricata* seeds on *Pseudomonas aeruginosa* (MIC = 3120 µg/ml), the *A. mahalao* seeds (MIC = 3750 µg/ml) and the *A. polyphylla* seeds (MIC = 2420 µg/ml) on *Staphylococcus aureus*[31]. In comparison with several plant extracts considered

as active by the authors, the *A. bernieri* seed extracts were more efficient. As illustrations, SME was by far more efficient than other plant crude extracts (TABLE 7). All the *A. bernieri* extracts were bactericidal and fungicidal against sensitive microorganisms at the concentrations tested. This property might be due to direct action of bioactive compounds on membrane resulting in its lysis and cell death. Further chemical study will allow knowing the number of active molecules and their originality compared with molecules from other *Albizia* species or other plants.

TABLE 6: MIC, MBC, MFC (µg/ml) and MBC/MIC or MFC/MIC *A. bernieri* seed extracts

Strains		Extract	MIC	MBC or MFC	MBC/MIC or MFC/MIC
Gram (-)Bacteria	*Enterobacter cloacae*	SME	500	>1000	nd
		Sap1	1000	1000	1
		Sap2	250	250	1
		Alk1	31.25	31.25	1
		Alk2	15.6	15.6	1
	Enterobacter aerogenes	SME	500	1000	2
		Sap1	1000	>1000	nd
		Sap2	250	500	2
		Alk1	31.25	62.5	2
		Alk2	15.6	62.5	4
	Pseudomonas aeruginosa	SME	1000	1000	1
		Sap1	>1000	nd	nd
		Sap2	500	1000	2
		Alk1	62.5	62.5	1
		Alk2	15.6	15.6	1
	Yersinia enterocolitica	SME	500	500	1
		Sap1	1000	1000	1
		Sap2	250	250	1
		Alk1	31.25	31.25	1
		Alk2	15.6	15.6	1
Gram (+)Bacteria	*Streptococcus pneumoniae*	SME	31.25	125	4
		Sap1	62.5	125	2
		Sap2	15.6	31.25	2
		Alk1	7.8	31.25	4
		Alk2	1.95	7.8	4
	Streptococcus pyogenes	SME	125	125	1
		Sap1	125	125	1
		Sap2	31.25	62.5	2
		Alk1	3.9	7.8	2
		Alk2	1.95	3.9	2
	Staphylococcus aureus	SME	250	500	2
		Sap1	1000	1000	1
		Sap2	250	250	1
		Alk1	31.25	31.25	1
		Alk2	15.6	15.6	1
	Clostridium perfringens	SME	125	250	2
		Sap1	>1000	1000	nd
		Sap2	125	125	1
		Alk1	15.6	31.25	2
		Alk2	3.9	7.8	2
	Bacillus cereus	SME	500	500	1
		Sap1	>1000	>1000	nd
		Sap2	500	500	1
		Alk1	31.25	31.25	1
		Alk2	15.6	15.6	1
Yeast	*Candida albicans*	SME	500	1000	2
		Sap1	1000	>1000	nd
		Sap2	125	500	4
		Alk1	31.25	31.25	1
		Alk2	7.8	7.8	1

nd: not determined

TABLE 7: Comparison of the MIC values of SME with those of other plant crude extracts on some germs

Extracts	Germ	MIC (µg /ml)	MIC of SME (µg /ml)
aqueous trunk extract of *Harungana madagascariensis* [32]	*Pseudomonas aeruginosa*	6250	1000
hexanic leaf extract of *Crotalaria retusa* [33]	*Bacillus cereus*	1250	500
aqueous crude extract of *Allium sativum* [34]	*Streptococcus pneumoniae*	75 000	31.25
methanolic leaf extract of *Myrtusnivellei* [35]	*Candida albicans*	4500	500

V. Conclusion

The *A. bernieri* seeds contain interesting antimicrobial agents from various chemical groups but alkaloids displayed the best activity. Alkaloids are a group of major therapeutic interest in terms of number, structural diversity and range of pharmacological properties. *A. bernieri* seed extracts could be used to treat gastrointestinal infections and skin diseases caused by the germs tested. For example, they could be recommended to treat boils, wounds and injuries caused by or infected with *Pseudomonas aeruginosa* [36]. Further phytochemical research is needed to identify the active principles and to investigate other properties.

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