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RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF EXTRACTS FROM THE STEM BARK OF *OLDFIELDIA AFRICANA* BENTH & HOOK (EUPHORBIACEAE): PRELIMINARY STUDY

F. BERINYUY JAI[1], J. NGOUPAYO[1], P.R. FOTSING KWETCHE[2,3]*, T. TABOPDA KUATE[4] J. KUOAMOUO[2,3], M.A. MAFO FOKAM [2,3], and B. NGADJUI TCHALEU[1]

[1] Faculty of Medicine and Biomedical Sciences; Yaoundé-Cameroon
[2] Université des Montagnes; Bangangté-Cameroon
[3] Cliniques Universitaires des Montagnes; Bangangté-Cameroon
[4] Faculty of Science, University of Yaoundé I; Yaoundé-Cameroon

*Corresponding author e-mail: prfotsing@udesmontagnes.org

Abstract

The present investigation focused on a semi-quantitative chemical screening of a few known secondary metabolites and the antimicrobial potentials of two extracts from the bark of *Oldfieldia africana* (*O. africana*). The targeted compounds included: alkaloids, coumarins, flavonoids, glycosides, phytosterols, quinones, saponins, terpenoids, mucilages, total phenols and tannins. Extraction was conducted with two organic solvents: methanol and ethyl acetate. The antimicrobial activity was tested on nine bacterial strains from both Gram types and *C. albicans* with the methanolic crude extract. This was done through determination of the minimal inhibitory and minimal lethal concentrations (MICs, MLCs, respectively) and the diameters of growth inhibition. All chemicals targeted were present in the plant, but not in all extracts. Extraction with methanol proved more effective than using ethyl acetate. At varying levels, the antimicrobial activity extended to all tested organisms (MIC values: 1.6 - 12.6 mg/mL). This action on both bacterial Gram-groups and fungi substantiates the use made of *O. africana* by traditional health practitioners and confirms that this plant is a good candidate for new antimicrobial drugs.

Keywords: Chemical screening, *Oldfieldia africana*, antimicrobial activity.

Introduction

The struggle against infectious diseases (IDs) caused by conventional-drug-resistant micro-organisms became a permanent challenge for all health communities worldwide short after the first antibiotic (penicillin) was discovered in the 1939s. In fact, its development from a fungal species opened ways for new investigations with the hope that IDs would entirely be controlled. With about 17 million deaths reported each year, IDs remain major causes of morbidity and mortality globally. Four major etiologic categories are incriminated in human IDs; namely protozoan, microscopic fungi, bacteria and viruses. In the early 21st century, 1407 microbial species were recognized as human pathogens, 38.2% of which were bacteria (Millar *et al*., 2007). With regards to the context, the incidence of each ID may differ from one setting to the other depending on determinants like the purchasing power of the local populations, availability and affordability of antimicrobials, general population education on drug use, the place of hygiene in local health policies, management of available facilities and human resources (Nguendo Yomsi *et al*., 2008; Nguendo Yomsi, 2011; Fotsing

Kwetche *et al.,* 2012). Some nations have provided up-to-date acceptable facilities to their populations while others are yet to. In most populations in Africa, for instance, acceptable caretaking of IDs with modern medicines is difficult because of resource limitation. Typically in those contexts, people rely on natural resources like plant derivatives in disease control.

Research initiatives on the production of available and affordable antimicrobials became paramount with phytotherapy, a promising alternative. In fact, acknowledging that about 50% of deaths are caused by IDs and that close to 80% of the population in Africa relies on herbal medicine, the WHO (2003) recommended and encouraged research and production of improved traditional medicines (ITMs). In that perspective many research groups focus on safer affordable phyto and biologically active compounds that can be used with acceptable therapeutic index in herbal medicines (Mueller *et al.,* 2000; Liu *et al.,* 2001; Li *et al.,* 2011; Dehghani *et al.,* 2012; Chougouo Kengne *et al.,* 2012; Kuate Tokam *et al.,* 2013). In general, plants are rich in large varieties of bioactive constituents like essential oils, alkaloids, terpenoids, flavonoids, tannins, coumarins, phenolic compounds and heterosides; some of which are effective in controlling microbial infections.

The present investigation addressed the phytochemical screening, antibacterial and antifungal potentials of *Oldfieldia africana* (*O. africana*) a plant used by traditional practitioners in Cameroon. This work was conducted in the framework of the ongoing research program on medicinal plants put in place in research institutions throughout the world. More specifically, it focused on detection and semi-quantification of a few bioactive compounds and determination of the minimal inhibitory concentrations (MICs), the minimal lethal concentrations (MLC-minimal bactericidal-MBc and minimal fungicidal-MFc) of the crude extracts from *O. africana*. These antimicrobial tests were performed on a total of nine bacterial and a dimorphic fungal strains belonging to species commonly incriminated in IDs (either as true pathogens or as opportunistic organisms). In addition, the inhibitory zone diameters associated with the MICs and MBCs were determined for a few bacterial strains. The goal of this research was to enlarge the field of investigations and contribute to the development of new IDs treatment alternatives with potential sources for new conventional antimicrobials in the long term.

Material and Methods

1. Plant identification and work sites

Also known as "African Oak", *O. africana* (Euphorbiaceae) is an endangered plant species of about 30 m high. Identified in the National Herbarium under the Voucher number HNC 23346/$SRF_{cam,}$ its barks was harvested on December 14th, 2013 in the National Forestry School, Yaoundé.

The rest of the study was conducted in the premises of different laboratories in Cameroon. Extraction of the crude products and phytochemical screening were carried out in the Laboratories of Chemistry at the Faculty of Medicine and Biomedical Sciences and the Laboratory of Chemistry of the Higher Teacher's Training College, Yaoundé-Cameroon. The antimicrobial activity was tested in the Laboratory of Microbiology, Cliniques Universitaires des Montagnes (Université des Montagnes, Bangangté-Cameroon).

2. Crude extract Preparation

The fresh barks of *O. africana* was let to dry for three weeks, and then crushed to obtain a total raw product mass of 3 kg. Suitable organic solvents of good polarities were used for extract preparation (ethyl acetate and methanol). Two-third (≈ 66.7%) of the powdered material was macerated in 6 L of methanol for 3 days in a cool environment (≈ 15°C). The third part was macerated in 3 L of ethyl acetate for 3 days and filtered. Filtration and extract concentration were performed with a Whatman filter paper N^{o}1 and a Rotavapor (HEIDOLPH, HEIZBAB® at 45^{o}C and 200 bars), respectively. The two crude extract fractions to be screened for the presence of active secondary metabolites included then: the methanolic crude extract = 173 g; and the ethyl acetate crude extract = 72 g.

Phytochemical screening of crude extracts

The aqueous test solutions was prepared by dissolving (stirring to homogenization with a magnetic agitator) 1 g of each crude extract in 20 mL of distilled water.

Test for alkaloids (Wagner's test)

2 mL of 1% HCl was introduced into 2 mL of each test solutions. Production of a brown reddish

precipitate indicated the presence of alkaloids (Alzoreky and Nakahara, 2003).

Test for coumarins (Alkaline reagent test)

In a test tube 2 mL of NaOH was added to 2 mL of the test solutions. The development of a green or blue fluorescence indicated a positive test for coumarins (Alzoreky and Nakahara, 2003).

Test for cardiac glycosides (bromine water test)

When 2 mL of the crude aqueous extracts were mixed with 2 mL of bromine water development of a yellowish color indicated the presence of cardiac glycosides (Sofowora, 1993).

Test for total phenols (Iron Chloride test)

A few drops of iron chloride were added to 3 mL of the test solutions. If a bluish black color was observed, the test was regarded as positive, indicating the presence of total phenols (Sofowora, 1993).

Test for phytosterols (Salkowsky's test)

The test solutions were mixed with chloroform and filtered. The filtrate obtained was treated with a few drops of concentrated H_2SO_4, shaken and allowed to stand. The change into a golden yellow color was evidence of the presence of phytosterols (Alzoreky and Nakahara, 2003).

Test for quinone's (Borntragers test)

When 1 mL of the test solution was mixed with equal volume of a 1N NaOH solution, quinones were revealed by the development of a red coloration **(**Trease and Evans, 1989**).**

Test for flavonoids

Alkaline test

When 2 mL of NaOH was added to 2 mL of the test solutions, an intense yellow color that turned into colorless upon addition of a few drops of diluted HCl indicated the presence of flavonoids (Alzoreky and Nakahara, 2003).

Shinoda test

A few fragments of magnesium ribbon were dispensed into the test solutions. Thereafter and drop-wise, about 2 mL of concentrated HCl were introduced into the set. A magenta color that developed indicated the presence of flavonoids (Alzoreky and Nakahara, 2003).

Test for terpenoids (Chloroform test)

2 mL of chloroform were mixed with 2 mL of the test solutions. To this mixture, 2 mL of concentrated H_2SO_4 was added and heated in a water bath (65^oC) for 120 s. A reddish brown color forming at the interface indicated the presence of terpenoids (Sofowora, 1993).

Test for saponins (foam test)

When the test solutions were mixed with 5 mL of distilled water and shaken vigorously, development of stable foam was evidence of the presence of saponins (Trease and Evans, 1989**).**

Test for mucilages

To 2 mL of the test solutions, 4 mL of absolute alcohol were added and constantly stirred. Development of air bubbles indicated the presence of mucilages (Sofowora, 1993).

Test for tannins (Iron Chloride test)

When the test solutions were mixed with a few drops of iron chloride, development of a green or blue color was evidence of the presence of tannins (Sofowora, 1993, Abba *et al.,* 2009).

Test for catechic tannins

2 mL of the test solutions were mixed with 2 mL of Stiasny's reagent (40% formol + 2/1concentrated HCl). When this preparation was incubated in a water bath (70°C) for 15 min, development of an intense red color indicated the presence of catechic tannins (Noudogbessi *et al.,* 2013).

Test for phlobotannins

In a test tube, 2 mL of the test solutions were mixed with 2 mL of 1% HCl. A red precipitate developing after 10 minutes of incubation in a water bath at 70^oC was evidence of the presence of phlobotannins in the extract (Trease and Evans, 1989). In each of the above cases, positive tests were semi-quantified based on the intensity of the test indicator.

In vitro* antimicrobial activity of the crude extracts of *O. africana

The micro-organisms used in this investigation were selected on the basis of their clinical and pharmacological significance. These included six Gram-negative bacterial strains (*E. coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* QC 76110*, Salmonella* Enteritidis*, Serratia odorifera* and *Vibrio cholerae* O1); two Gram-positive cocci (*S. aureus* QC 1625, *S. aureus* ATCC 1026^{TM}); one Gram-positive bacillus (*Enterococcus faecalis* ATCC 29212); and a dimorphic fungal specie, *Candida albicans*. In connection with the high polar nature of the solvent that theoretically allows greater absorption of active secondary metabolites, the crude extract obtained with methanol was used for antimicrobial tests.

Minimal Inhibitory Concentration (MIC)

All strains used in this experiment were seeded on Mueller Hinton agar (Liofilchem) and incubated at 37°C for 18-24 h. From the resulting overnight pure culture, a bacterial suspension equal to 0.5 McFarland (10^6-10^8 cells/mL) was prepared and adjusted to the final density required for susceptibility tests according to the ‘‘Comité de l’Antibiogramme de la Société Française de Microbiologie, CA-SFM (2014)”. A similar procedure was followed to prepare, from a fresh culture on Sabouraud agar (Liofilchem), the $2x10^6$ cells/mL suspension density used to assess the antifungal activity with conventional techniques.

The test was conducted by macro-dilution in Mueller Hinton broth (MHB) (or Sabouraud broth) as in Kuate Tokam *et al.* (2013) with slight modifications. Briefly, the original (stock) solution was prepared at 400 mg/mL. In each of a series of twelve test tubes, 2 mL of MHB were introduced. From this preparation, a serial dilution was performed to obtain extract concentrations ranging from 200 mg/mL through 0.1 mg/mL; plus two tubes with MHB without extract (positive control for growth and broth sterility control). This procedure was repeated for all microbial strains (*C. albicans* was tested in Sabouraud broth).

In each tube (accept the broth sterility control), 15 µL of the above bacterial (or fungal) preparation were dispensed. The preparations were incubated aerobically for 24 h at 37^{o}C for bacteria and 30^{o}C for *C. albicans*. When incubation was completed, the tubes were centrifuged at 5000 rpm for five minutes. For all tested micro-organisms, the MICs were read from the first tube in which no precipitate (whitish) was obtained upon centrifugation and confirmed by the broth turbidity recorded prior to centrifugation. The experiment was repeated three times for test reproducibility.

Minimal Lethal Concentration (MLC)

The tubes in which no visible microbial growth was obtained were re-homogenized. The resulting suspension was streaked (10-15 µL) on appropriate culture agars (Mueller Hinton agar for bacteria and Sabouraud agar for *C. albcans*). The cultures were allowed to incubate aerobically overnight at 37^{o}C and 30^{o}C, respectively. Upon completion of this incubation period, the MLC (MBCs for bacteria and MFC for *C. albicans*) were recorded from the first dilution in which no visible growth was obtained.

Growth inhibitory zone diameters determination

The inhibitory zone diameter was determined by the disc diffusion technique on MHA (Liofilchem) according to Hayes and Markovic (2002), with slight modifications. Briefly, the bacterial suspension prepared as mentioned above was further diluted (1/10) to the final density required for susceptibility test by standard agar diffusion technique with swabs. On the seeded agar plate, ten sterile paper discs (6 mm diameter each) were deposited for the tests. Out of these ten paper discs, 15 µL of the extract at the MIC concentration were dispensed on four (one received sterile distilled water) on one hand; 15 µL of the extract at the MIC concentration with 10% (v/v) DMSO (Merk) was dispensed on four and one was inoculated with DMSO. The same procedure was followed at the MBCs for all bacterial strains tested. These preparations were, thereafter, incubated aerobically at 37^{o}C for 18-24 h. After incubation, the inhibition zone diameter around each disc was recorded and computerized in terms of means and standard deviations.

Results

Phytochemical screening of the crude extracts

The solvents used displayed different effectiveness in the extraction process. Data obtained were summarized and presented in table 1.

Table 1: Phytochemical screening of the crude extracts

Secondary Metabolite	Tests Used	ETOAC	METH
Alkaloids	Wagner's test	+	+++
Coumarins	Alkaline reagent test	-	+
Flavonoids	Alkaline	-	+
	Shinoda test	-	+
Cardiac glycosides	Bromine water test	+	+
Phytosterols	Salkowsky's test	-	+
Quinones	Borntrager's test	+	+
Saponins	Foam test	+	+
Terpenoids	Chloroform test	++	++
Mucilages	Alcohol test	+	++
Total phenols	Iron chloride test	+	+
Tannins	Iron chloride test	-	+
	Formaldehyde test	+	+
Catechic tannins	Stiasny	++	+
Phlobotannins	Chloridric acid test	-	-

ETOAC: Ethyl acetate fraction; METH: Methanol fraction; +++: very intense; ++: intense; +: Present; -: Absent

Overall, the ETOAC and METH contained approximately 60% and 93.33% of the targeted secondary metabolites, respectively. The METH fraction contained coumarins, flavonoids phytosterols and tannins that were absent in the ETOAC's. Also, phlobotannins were absent in both crude extracts. Although at varying proportions, some secondary metabolites could be identified in either crude product. They included: catechic tannins, total phenols, mucilages, cardiac glycosides, quinines, saponosides, terpenoids and alkaloids. Catechic tannins, mucilages, terpenoids, and alkaloids were most frequently and intensely detected.

In vitro antimicrobial susceptibility tests

Microbial susceptibility to the methanol extract generated the MICs and MLCs values contained within a broad range. Related data were recorded as displayed in Table 2.

Table 2: MICs, MLCs and MLC/MICs values

Microorganism	MIC(mg/mL)	MLC(mg/mL)	MBC/MIC
E. coli ATCC 25922	25	100	4
E. coli ATCC 35218	6.25	50	8
S. aureus QC 1625	1.6	25	15.625
P. aeruginosa QC 76110	12.5	50	4
E. faecalis ATCC 29212	3.125	100	32
S. aureus ATCC 1026™	6.25	100	16
V. cholera O1*	3.125	50	16
S. Enteritidis *	6.25	200	32
S. odorifera *	3.125	200	64
C. albicans *	3.125	200	64

**: Clinical strain*

The MIC values ranged from 1.6 mg/mL with *S. aureus* QC1625 through 25 mg/mL with *E. coli* ATCC 25922 and, 90% of the values were smaller than/ or equal to 6.25 mg/mL. In O1- *V. cholerae*, *E. faecalis* ATCC 29212, *Serratia odorifera* and *C. albicans,* growth inhibition occurred at similar concentrations, but the lethal concentrations were reached at different values. It could also be noted that *S. aureus* QC1625 was more susceptible than the *S. aureus* ATCC 1026™ strain. In *E. coli* ATCC 25922, the MIC and MBC were closer than in *E. coli* ATCC 35218. The latter strain was, however, more susceptible (MIC=6.5 mg/mL). The largest gaps between the MICs and MLCs were recorded with *S.* Enteritidis, *S. odorifera* and *C. albicans.*

Determination of the inhibitory zone diameters on MH agar generated varied results depending on bacterial strains and on whether DMSO was used in combination or not. Related data were computerized and presented in Table 3.

Table 3: Inhibitory zone diameters with and without DMSO

Microorganism	Mean IZD ± SD; DMSO -		Mean IZD ± SD; DMSO+	
	MIC	MBC	MIC	MBC
E. coli ATCC 25922	7± 0.2	14± 0.3	12± 0.5	17± 0.3
E. coli ATCC 35218	8± 0.4	13± 0.0	9± 0.4	14± 0.4
S. aureus ATCC 1026™	8± 0.2	12± 0.5	9± 0.2	13± 0.3
P. aeruginosa QC 76110	9± 0.2	13± 0.4	10± 0.0	15± 0.0
V. cholerae 01	7± 0.3	13± 0.4	9± 0.2	14± 0.0

IZD: Inhibitory zone diameter; SD: standard deviation; DMSO-: without DMSO; DNSO+: with DMSO

With a few exceptions, they indicate an increase in the inhibitory zone when DMSO was added to the extract. Overall in fact, MICs without DMSO ranged from 7± 0.2 mm with *E. coli* 25922 to 9± 0.4 mm with *E. coli* 35218. About the MBCs, the diameters were found within 13± 0.0 mm (*E. coli* 35218) and 14± 0.3 mm (*E. coli* 25922). Upon addition of DMSO at the MBCs, the largest increase was obtained with *E. coli* 25922 (from 14± 0.3 mm to 17± 0.3 mm). With or without DMSO, however, the standard deviations associated attested closeness of diameter values recorded in each case, an evidence for mean values accuracy.

Discussion

The present investigation on *Oldfieldia africana* revealed the presence of groups of bioactive chemicals that were previously identified in other plant species and known for their role in many life processes. Extraction efficiency appeared to be solvent-dependent with the highest content of tested chemicals observed with methanol. This is in accordance with earlier findings that extract richness varies with extraction solvents and techniques (Rathore *et al.,* 2012; Wendakoon *et al.,* 2012; Kuate Tokam *et al.,* 2013). In fact, the solvent polarity is higher with methanol. Also, some of these chemical compounds could not be detected by more than one of the techniques used, consistent with the necessity to use appropriate techniques in the screening of chemical compounds in raw products. More importantly the extraction products were soluble in water.

The crude extract activity observed on micro-organisms in the present investigation was likely associated with its chemical composition in secondary metabolites. These included alkaloids, tannins, terpenoids, phytosterols, total phenols, quinones, and glycosides, reported in previous investigations (Bios *et al*., 1988; Audu *et al.,* 2007; Mute, 2009; Kumar *et al*., 2010; Sutar *et al*., 2010; Dandjesso *et al.*, 2012) and known for their role in some biological processes**.** For instance, wide ranges of biological activities such as emetic, antiseptic, antitumor, diuretic, antiviral, antitussive, anti-inflammatory and antimicrobial have been associated with alkaloids (Bruneton, 1999; Aiello *et al*., 2011; Gonzales and Tolentino, 2014). Alkaloids could also act on haemostasis and vasoconstriction. Like alkaloids, administration of tannins can help control heamostasis and abnormal cell proliferation that might eventually result in tumors and cancers (Ayoola *et al.,* 2008; Savithramma *et al.,* 2011).

Also detected in larger amount in the crude extract of *O. africana,* terpenoids are known to possess other pharmacological properties like antiviral, antibacterial, anti-malarial, anti-inflammatory, can be used to regulate blood cholesterol and blood sugar, and like tannins, process an anticancer potential (Mahato and Sen, 1997).

On test micro-organisms in particular, the crude extract presented a certain degree of activity on Gram-positive bacteria. Strains of *S. aureus* are causative agents of human conditions like suppurative infections, superficial lesions (boils, impetigo, for instance), throat sore, pneumonia, mastistis, phlebitis, meningitis, food poisoning, urinary tract infections and others in both clinical and community settings (Wendakoon *et al.,* 2012; Sen and Amla, 2012; Daniel *et al*., 2012; Djeussi *et al.,* 2013). Representative strains were susceptible to the extract at relatively low concentrations. This activity of the crude extract extended to ubiquitous opportunistic Gram-negative bacteria (*P. aeruginosa, E. coli*, *S. odorifera*) identified as major causes of infections in surgical wards and intensive care units of healthcare facilities with low hygiene standard; and professional pathogens such as O1-positive *Vibrio cholerae* (most frequent cause of epidemic cholera worldwide) and *S.* Enteritidis (incriminated in gastro-intestinal disorders). These Gram-negative bacteria can also be incriminated in diffused pneumonia, osteomyelitis, dermatitis, cystic fibrosis, and diarrhea with abdominal cramps in immune-depressed individuals.

At a relatively reduced concentration, the crude extract was active against *C. albicans.* This opportunistic fungal species is known for its role in oral and genital infections, onchomycosis and other types of mycosis. Daniel *et al*. (2012) reported that saponins could inhibit *C. albicans* growth**.** Accordingly, the susceptibility of *C. albicans* could be justified by the presence of saponins. Based on current knowledge on phytochemicals and microbes, similar conclusions could be drawn as far as other identified chemicals are concerned. From these findings, however, it cannot be asserted that one component or the other is actually responsible for the antimicrobial potential observed for at least three major reasons: 1- only a few known bioactive elements have been tested; 2- interactions among the chemical identified have not been investigated through (neutral, additional, synergetic or antagonistic); 3- interactions between those identified and the ones that cannot be detected with the techniques used cannot also be assessed. Further concentrating the crude extract would generate more satisfactory results.

Activity on fungi, Gram-positive and Gram-negative bacteria reported in the course of the present survey highlighted the wide spectrum of activity regardless of the chemical composition of the microbial cell envelope. This activity was enhanced with addition of DMSO known to facilitate cell permeability and substance diffusion as reported earlier (Chougouo Kengne *et al.,* 2012; Kuate Tokam *et al.,* 2013).

Improved traditional drugs in general, and those from *O. africana* would be very important in developing communities with respect to the large arrays of potentials they can have on various groups of diseases, unlike conventional medicines, made up of a single or limited concentrated bioactive compounds commonly referred to as active principles, then more focus. But typically, to achieve the expected goal, in connection with the relatively low content per mass unit of raw material, adequate bioavailability (at the infection site, for instance) should be ensured via administration of large amounts of the product per time unit. This demands that specific strategies should be put in place and monitored by health coordinators in all settings.

Conclusion

Oldfieldia africana appears as a reliable alternative in many human diseases, consistent with its use by traditional health practitioners throughout generations and with the advocacy of the WHO to capitalize local natural resources for health. It is, therefore, important to find out proper strategies to use optimally the virtues of this plant in the needy populations.

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