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Antimycobacterial activity of selected medicinal plants extracts from Cameroon

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ABSTRACT

New drugs are highly needed to control mycobacterial infections. This study aimed at screening ethnobotanically selected plants extracted using organic solvents for their antimycobacterial activity. *In vitro* assays were performed on *Mycobacterium smegmatis*, *Mycobacterium avium*, *Mycobacterium bovis* Bacille Calmette Guerin (BCG), *Mycobacterium tuberculosis* and *Mycobacterium ulcerans* using the Resazurin Microtiter Assay. Cytotoxicity was assessed on Human lung fibroblast cells (MRC5) and bone marrow-derived macrophages (BMDM) using the MTS tetrazolium assay. The most promising extract from *Annickia chlorantha* stem bark (ACsbI) was tested for intracellular antimycobacterial activity against *M. smegmatis* using infected BMDM. Sixty crude extracts, 19 fractions, and 2 purified compounds were obtained from 19 Cameroonian medicinal plants. Results showed that crude extracts mainly inhibited BCG, while interface fractions from *A. chlorantha* stem bark (ACsbI) and stem (ACstI) displayed the strongest activity against *M. ulcerans*, with Minimal Inhibitory Concentrations (MIC) of 1.95 and 7.81 µg/ml respectively. Two compounds purified from *Sorindeia juglandifolia* fruits (SJfr 3.6 and SJfr 4.5) showed activity against BCG and *M. ulcerans* at 3.9 µg/ml and 62.5 µg/ml respectively. Finally, ACsbI showed no toxicity against MRC5 cells and BMDM and inhibited the growth of intracellular *M. smegmatis*. The results achieved in this investigation support the traditional to use of these plants and the need to investigate them in deeper details to be able to find alternatives for the existing antimycobacterial drugs.

Keywords: *Mycobacterium*, tuberculosis, Buruli ulcer, antimycobacterial activity, cytotoxicity, *Annickia chlorantha*, ethnobotanical survey.

INTRODUCTION

Mycobacteria constitute a very heterogeneous genus, comprising highly pathogenic as well as opportunistic or nonpathogenic species. Amongst the pathogenic species, *Mycobacterium*

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tuberculosis, the causative agent of tuberculosis (TB), is the leading one, responsible for an estimated one-third of the world's human population infection (WHO, 2004; TB Alliance, 2011). Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is globally the third most frequent mycobacterial disease after TB and leprosy (Asiedu et al., 2000; WHO, 2011). However, in some countries, as Benin and Côte d'Ivoire, the number of cases may exceed those of TB and leprosy (Asiedu et al., 2000). On the other hand, opportunistic or nonpathogenic mycobacteria such as *Mycobacterium avium* and *Mycobacterium smegmatis* can cause disease in imunocompromised people such as AIDS patients (Daley and Griffith, 2010; Sexton and Harrison, 2008; Cangelosi et al., 2001). Infections with these species are very hard to treat, requiring multi-drug therapy for prolonged periods (WHO, 2004; TB Alliance, 2011). Furthermore, drug-resistant strains render the treatment even more difficult, highlighting the need for a drug discovery pipeline to ensure the availability of new chemical entities with improved mechanisms of antimycobacterial action.

A large proportion of the world population relies on traditional medicine for their primary healthcare, due to cultural believes or lack of alternatives (WHO 2004; Hannan et al., 2011; Anon, 2002). Within this framework, plant products have proven interesting antimicrobial potency (Okunade et al., 2004, Vikrant, 2011). In Cameroon, there is a rich tradition of using herbal medicine for the treatment of various mycobacterial infections (Adjanohoun et al., 1996; Kimbi et al., 1996; Focho et al., 2010).

This paper reports the antimycobacterial activity of 60 crude extracts, 19 fractions, and 2 purified compounds from 19 Cameroonian medicinal plants selected based on their traditional uses against infectious diseases in general and particularly TB and related diseases.

MATERIALS AND METHODS

Collection of plant materials

Nineteen plants were selected based on their ethnobotanical use for the treatment of various ailments including cough, bronchitis, chest complaints, pneumonia, TB and other related diseases (Nishiyama et al., 2004; Ibekwe and Orok, 2010). The plants were subsequently collected between December 2009 and October 2010 at Mt Kalla (Yaoundé area-Centre region), Lolodorf (South region of Cameroon), and Dschang (West region). The ethnobotanical survey was conducted in strict respect of the current regulations of biodiversity protection and rural population's customary rights. In the preliminary step of the survey, legal authorities (villages' heads) from each study site were contacted to seek authorization to investigate within their communities. During a face-to-face interaction, the purpose and the procedure of the work, as well as the expected benefits and rights were explained to villages' heads. The traditional herbal practitioners willing to participate on the study (or their interpreters) had signed the informed consent form before further interactions. The data were collected following a questionnaire prepared for the study by Dr. Nole Tsabang, an ethnobotanist at the Institute for Medical Research and Medicinal Plants Study, Yaoundé, Cameroon. Voucher specimens of each plant were dried and deposited at the Cameroon National Herbarium, Yaoundé. The information on plant materials is summarized in Table 1 where plants are listed with their traditional uses, voucher specimen's reference numbers, the solvent and yields of extraction.

Plant extraction

Air-dried (at room temperature) and ground (using a blender) plant materials were extracted by maceration using appropriate solvents. Annonaceae plants samples were extracted according to the method previously described by Alali et al. (1999). This method was chosen because it is designed to isolate acetogenins (or rich fractions) that were shown to exert high potency against bacteria (Vairappan and Tan, 2009; Alali et al., 1999). Plant powders (500 g) were individually macerated in 95% ethanol for 72 h. The dried ethanolic extracts obtained were further partitioned between water (H_2O) and methylene chloride (CH_2Cl_2) (1:1, v/v). The CH_2Cl_2 residues were partitioned between hexane and 90% methanol (1:1, v/v). The different fractions were evaporated to dry under reduced pressure using a Büchi R111 Rotavapor. The water layers were evaporated under ventilation at room temperature. In

addition, interface precipitates were obtained during residues partition between CH_2Cl_2/H_2O.

Samples of non Annonaceae plants that do not contain acetogenins were extracted by repeated maceration in methanol. These included *Aframomum melegueta, Chlorophytum macrophyllum, Solanum aculeastrum, Spilanthes filicaulis* and *Sorindeia juglandifolia.*

Following a Label extension strategy (Nwaka and Hudson, 2006), methanolic crude extract, fractions (SJfr 1.1, SJfr 2.1, SJfr 2.2, SJfr 2.3, SJfr 3.1, SJfr 3.2, SJfr 3.3, SJfr 3.41, SJfr 3.42, SJfr 3.5, SJfr 4.1, SJfr 4.2, SJfr 4.3, SJfr 4.4), and compounds [2,3,6-trihydroxy benzoic acid (SJfr 3.6) (formula 1) and 2,3,6-trihydroxy methyl benzoate (SJfr 4.5) (formula 2)] from the fruit of *S. juglandifolia* that were previously prepared in our laboratory and that showed good potency against infectious pathogens (Kamkumo et al., 2012) were also screened for antimycobacterial activity.

Evaluation of the biological activities

Preparation of stock solutions

Stock solutions (100 mg/ml for plants crude extracts and 2 mg/ml for fractions and purified compounds) were prepared by dissolving each extract in 2% DMSO/distilled water, and filtrating through a 0.22 µm syringe filter. For the intracellular antimycobacterial activity and cytotoxicity assays, stock solutions were prepared using 2% DMSO in complete Dulbecco's modified Eagle's medium [cDMEM, consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), 10% heat-inactivated fetal bovine serum (Sigma Aldrich)], filtrated through a 0.22 µm syringe filter. The stock solutions were serially diluted either in Middlebrook 7H9 medium supplemented with ADC (Albumin, Dextrose, Catalase) or in cDMEM for specific experiments. Positive controls were prepared at 1 mg/ml for ethambutol (EMB) and streptomycin (SM) in distilled water and sterilized through a 0.22 µm syringe filter.

Bacterial strains and growth conditions

The following mycobacterial strains were used in the experiments: *M. smegmatis* strain mc^2155 from the American Type Culture Collection (ATCC 70008); *M. avium* strain 2447 smT (smooth-transparent) isolated from an AIDS patient and obtained from Dr. F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium); *M. ulcerans* strain 1615 obtained from the University of Tennessee, USA; BCG Pasteur and *M. tuberculosis* H37 Rv, obtained from the Trudeau Institute, USA.

Mycobacteria were grown at 37 °C on solid Middlebrook 7H10 (*M. smegmatis* and *M. avium*) or Middlebrook 7H11 medium (BCG and *M. tuberculosis*) all supplemented with OADC (Oleic-Acid-Albumin-Dextrose-Catalase) and 0.5% glycerol. *M. ulcerans* was grown at 32 °C on Middlebrook 7H9 agar medium enriched with OADC.

To prepare the inocula, *M. smegmatis*, *M. avium*, BCG, and *M. tuberculosis* were freshly subcultured in Middlebrook 7H9 medium containing 0.05% Tween 80 and ADC at 37 °C. *M. ulcerans* was subcultured on solid Middlebrook 7H9 medium containing OADC enrichment for 6 weeks at 32 °C. The mycobacterial strains were used at their exponential growth phase, evaluated by their individual growth curve.

Inocula of bacteria used for *in vitro* experiments, were prepared as described by Singh et al. (2012) and Yemoa et al. (2011) with few modifications. Briefly, inocula of *M. smegmatis*, BCG, *M. ulcerans*, *M. avium*, and *M. tuberculosis* were prepared following the McFarland standards, and concentration of viable bacteria was obtained by enumerating colony forming unit (CFU) per ml on solid medium. The concentration of bacteria used in the experiments was approximately: 10^5 CFU/ml for ***M. smegmatis*****,** BCG, and *M. tuberculosis;* 10^6 CFU/ml for *M. ulcerans*, and 10^8 CFU/ml for *M. avium*. Prior to antimycobacterial assay, the absence of contamination was confirmed by culturing in the Brain and Heart Infusion (BHI) medium agar and using Ziehl-Neelsen staining.

Assessment of in vitro antimycobacterial activity of plant extracts

The antimycobacterial activity was assessed in 96 wells microtiter plates (Corning, Spain) using the Resazurin Microtiter Assay (REMA) as described by Singh et al. (2012). Fixed concentration of 12.5 mg /ml for 60 crude extracts, 0.25 mg /ml for 19 fractions and 2 compounds were

added to wells containing 100 µl of bacterial inocula and incubated at 37 °C for 1 day for *M. smegmatis*, 5 days for *M. avium* and BCG, and 7 days for *M. tuberculosis*. *M. ulcerans* was incubated for 15 days at 32 °C. Positive controls consisted of EMB at 1.875 µg/ml, 0.166 mg/ml and 3 µg/ml for *M. smegmatis, M. avium* and BCG, respectively, and of SM at 2 µg/ml, 18 µg/ml and 4 µg/ml respectively for *M. ulcerans, M. avium* and *M. tuberculosis*. Negative control wells contained no drugs and blank contained no inoculum and/or drug. Upon incubation periods, 30 µl of 0.01% resazurin (Acros Organic NV) were added to individual wells and the plates re-incubated for additional 1 to 3 days and checked daily for color change. Change in resazurin color from blue to pink indicated reduction of the indicator and thus bacterial growth.

The Minimal Inhibitory Concentrations (MIC) of inhibitors was assessed using the same approach. Briefly, twofold serially diluted selected extracts were incubated in the range of 12,500 - 97 µg/ml for crude extracts and 250 - 1.95 µg/ml for fractions and purified compounds with inocula in 96 wells microtiter plate (Corning, Spain). EMB and SM were used as positive controls. The MIC was defined as the lowest drug concentration that prevented visible growth of bacteria displayed by no color change.

Minimal Bactericidal Concentrations (MBC) of promising plant extracts was assessed by sub-culturing MIC test plates on solid nutrient Middlebrook 7H9, 7H10, and 7H11. The MBC was considered as the minimal concentration of plant extract needed to kill most (≥99.9%) of the initial inoculum after incubation for a fixed length of time under a given set of conditions (NCCLS, 1999). All the experiments were done in duplicate and repeated 3 times. Fractions exhibiting antimycobacterial activity with MIC ≤ 7.81 µg/ml against at least 1 mycobacterial species (ACstI, ACsbI, and SJfr 3.6) were selected and assessed for cytotoxicity against MRC5 cells.

Assessment of the cytotoxicity of promising plants extracts and fractions

Plant extracts showing antimycobacterial activity were assessed for cell cytotoxicity against MRC5 cells. Subsequently, the cytotoxicity profile of 1 fraction (ACsbI) that showed no toxic effects on MRC5 cells was assessed on BMDM prepared from mice (C57BL/6 Ly5.1) as described by Oliveira et al. (2005). The cytotoxic effect of selected plant extracts was evaluated by MTS assay ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, USA) after 48 h of incubation. Detection of cell viability was based on the reduction of the tetrazolium salt MTS to water-soluble formazan dye by metabolically active cells. Briefly, 100 µl of MRC5 cells or BMDM were cultured in 96-well plates at 1×10^5 cells/ml, 24 h before adding 100 µl of extract at 0, 125, 250, and 500 µg/ml final concentration and incubated for 48 h at 37 °C in a 5% CO_2 humidified atmosphere. Upon the incubation period, the medium was replaced with the same volume of cDMEM. MTS reagent was added to each well and incubated for 2 h at 37 °C. Untreated cells were used as negative control. The absorbance was recorded at 490 nm using an ELISA plate reader (BioteK EL800, USA). The readout was directly proportional to the number of viable cells in culture (Promega, 2011). Percent inhibition of MRC5 cells was calculated using optical density. The value of cell cytotoxicity at 50% (IC_{50}) in the MTS assay was defined as the concentration of test extract resulting in a 50% reduction of absorbance compared with untreated cells and obtained using Graph Pad Prism 5.0.

Assessment of the intracellular antimycobacterial activity of A. chlorantha stemback ACsbI fraction

To assess the intracellular activity of ACsbI that exerted no significant cytotoxicity on BMDM and MRC5 cells, BMDM were cultured in 96-well plates at 1×10^5 cells/ml for 24h and the culture medium replaced with 100 µl of *M. smegmatis* inoculum containing $5x10^5$ CFU. Upon a 4 h phagocytosis period at 37 °C under 5% CO_2 (Rastogi et al., 1987), free bacteria were washed out and 100 µl of ACsbI at MIC value (250 µg/ml in cDMEM, 20% LCCM, 2% DMSO) added and incubated at 37 ^oC under 5% CO_2 for 24 and 48 h (Rastogi et al., 1987 ; Sharbati-Tehrani et al., 2005). The infected BMDM were lysed adding 5 µl of saponin (10%), diluted and plated on the Middlebrook 7H10 medium for 3 days at 37 °C, and the CFU thereafter

enumerated. The experiment was done in triplicate and the results expressed as $\log_{10}$ CFU.

Statistical analysis

Results are expressed as Means ± SD. The comparison between control and treated samples was assessed by one-way ANOVA. The intracellular activity was evaluated by two-way ANOVA using the GraphPad Prism 5.0 software. Statistically significant differences were considered for P values < 0.05.

RESULTS

The 19 plants resulting from the ethnobotanical survey are listed in Table 1, with indication of their traditional uses, plant parts studied, extracts, fraction and yields obtained (60 crude extracts, 19 fractions, and 2 purified compounds were obtained with yields ranging from 0.019 to 25.44%) calculated in percentage (w/w) relative to the starting plant material.

The extracts were initially screened against *M. smegmatis*, *M avium*, BCG, *M. ulcerans*, and *M. tuberculosis* at unique concentrations of 12,500 µg/ml for crude extracts and 250 µg/ml for fractions and compounds. From the results obtained, 22 extracts and fractions and 2 purified compounds (Table 2) deriving from 10 plants species (*Aframomum melegueta*, *Spilanthes filicaulis*, *Chlorophytum macrophyllum*, *Polyalthia suaveolens*, *Sorindeia juglandifolia*, *Annickia chlorantha*, *Anonidium mannii*, *Uvaria baumannii*, *Xylopia africana* and *Polyalthia oliveri*) exhibited inhibitory actions, and were therefore selected for the MIC and MBC determination.

From the assessment of the selected extracts, MIC and MBC were determined as presented in Table 2. Crude extracts mainly acted on BCG, the most potent being UBtwMeOH (MIC= 1,562 µg/ml; MBC= 3,125 µg/ml), $XArH_2O$ (MIC= 3,125 µg/ml; MBC= 3,125 µg/ml); CMr (MIC= 3,125 µg/ml; MBC= 6,250 µg/ml), and AMntwMeOH (MIC= 3,125 µg/ml; MBC= 6,250 µg/ml). The remaining extracts presented MIC/MBC values equal or superior to 6,250 µg/ml. PSsbMeOH and $XArH_2O$ showed the broadest range of action. On the other hand, *M. ulcerans* appeared to be highly susceptible to the methanolic crude extract of *P. suaveolens* (PSsbMeOH), with MIC and MBC values equal to 3,125 µg/ml.

Concerning the susceptibility of mycobacteria to fractions, *A. chlorantha* stem bark and stem interfaces displayed the strongest activity against *M. ulcerans*, with low MIC and MBC values at 1.95 and 62.5 µg/ml respectively for ACsbI, and 7.81 and 7.81 µg/ml for ACstI. The other promising fractions acted at concentrations equal or higher than 31.25 µg/ml, and mainly against BCG.

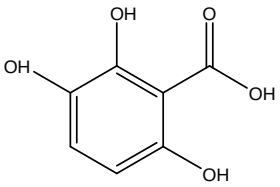
The 2 compounds purified from the fruits of *S. juglandifolia* (SJfr 3.6= 2,3,6-trihydroxy benzoic acid and SJfr 4.5= 2,3,6-trihydroxy methyl benzoate) showed antimycobacterial activity against BCG and *M. ulcerans* with respective MIC values of 3.9 µg/ml and 62.5 µg/ml.

From the MTS cytotoxicity assays using MRC5 cells, ACsbI proved to be safe at the highest concentration tested (IC_{50} > 500 µg/ml). The 2 other fractions, ACstI and SJfr 3.6, showed cytotoxicity against MRC5 (Table 3) with IC_{50} values of 50.82 µg/ml and 139.4 µg/ml respectively.

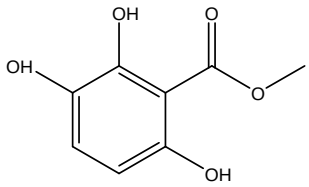
As shown in Table 3, only 1 extract from *A. chlorantha* (ACsbI) was found to be safe at 2 fold the MIC value (activity against *M. smegmatis*) and was therefore assessed against BMDM. ACsbI was also tested for cytotoxicity against BMDM (Figure 1) and showed no cytotoxic effects at MIC value and was considered for intracellular activity assessment.

For the intracellular antimycobacterial activity, BMDM infected with *M. smegmatis* were treated with the extract (ACsbI) and the result evaluated after 0, 24 and 48 h post-infection as showed in Figure 2.

As shown in Figure 2, the bacterial load in untreated macrophages nearly doubled within 48 h, while it slightly decreased or remained constant for BMDM treated with ACsbI. The time-kill kinetic showed that the activity of ACsbI is almost constant throughout the 48 h of exposition. This result suggests that drugs inhibited the intracellular mycobacterial growth.



(**1**) SJfr 3.6: 2,3,6-trihydroxybenzoic acid
Yield: 0.062%; m/z= 170; mp= 188.5-190; yellow crystals



(**2**) SJfr 4.5: 2,3,6-trihydroxymethyl benzoate.
Yield: 0.0017%; m/z= 184; mp= 139-140.5; pale yellow crystals

The synthesis of these compounds was previously described by Kreuchunas and Mosher (1956).

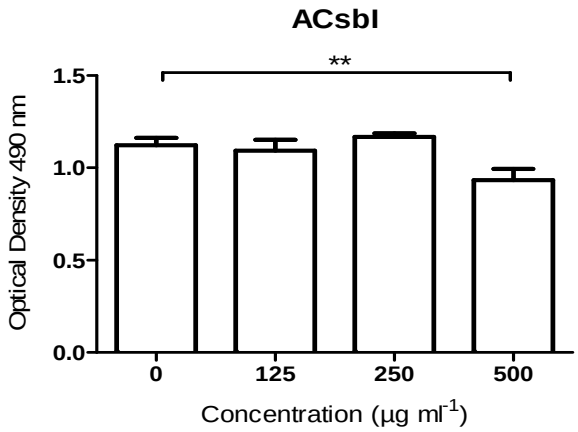


Figure 1: Cytotoxicity of *Annickia chlorantha* (ACsbI) against BMDM. The toxicity was assessed using the MTS assay. The cells were treated for 48 h with different drug concentrations. OD was measured at 490 nm. Data are reported as means ± standard deviation. Significant differences were calculated by comparing results in the presence of cells treated or not with ACSbI at different concentrations. Calculations were performed using one way ANOVA [F (3, 8)= 13.79 ; **, p = 0. 0016].

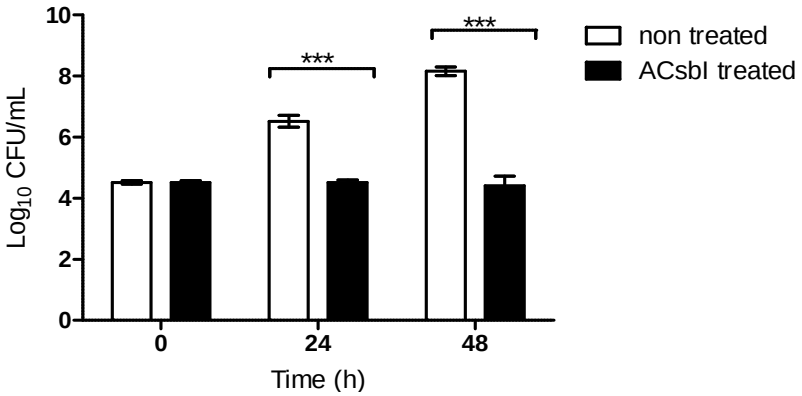


Figure 2: Intracellular antimycobacterial effect of *A. chloranta* stem bark extract (ACsbI) against *M. smegmatis* infecting BMDM. Number of intracellular surviving bacteria at 0, 24 and 48 h in untreated and treated BMDM inoculated with $5x10^5$ CFU was determined. Data are reported as means ± standard deviation. Significant differences were calculated by comparing treated and untreated cell at different time intervals. 0 h post-infection refers to 4 h after phagocytosis of *M. smegmatis*. Calculations were performed using two-way ANOVA. F (1, 12) = 569.7; ***, p <.001.

Table 1: List of plant species with their traditional uses and extraction yields.

Plant family	Species	Voucher specimen number	Traditional uses	References	Parts used	*Solvent of extraction	Extracts code	**Yield (%)
Zingiberaceae	*Aframomum melegueta* K. SChum.	43123/HNC	Dysmenorrhea, bronco-pulmonary disorders, sexual asthenia, female sterility, hay fever, migraines and wound purgative, Galactogogue, anthelmintic and haemostatic agent Diarrhea, smallpox, chickenpox, wounds, cough, anaemia, rheumatism, measles, malaria, toothache, cardiovascular diseases, diabetics and fertility control. Tuberculosis. Buruli ulcer	(Tane et al., 2006; Adeleye et al., 2008; Kadiri, 2009; Ibekwe and Orok, 2010; Yemoa et al., 2011)	Leaf	Methanol	AMl	5.40
					Fruit	Methanol	AMfr	6.78
					Root	Methanol	AMr	3.78
					Stem	Methanol	AMst	2.55
Annonaceae	*Annickia chloranta* (Oliv.) Setten and Maas	32065/SRF/Cam	Jaundice and urinary tract infections, stomach malaria, tuberculosis, hepatitis and some forms of ulcer, Sore, fevers, vomiting, fatigue, Rheumatism treatment	(Adjanohoun et al., 1996; Kimbi et al., 1996; Boyom et al., 2009; Bele et al., 2011; Tsabang et al., 2012)	Stem bark	Ethanol	ACsbMeOH	2.90
						Ethanol	ACsbI	1.98
						Ethanol	ACsbH_2O	0.31
					Stem	Ethanol	ACstMeOH	4.60
						Ethanol	ACstI	0.98
Annonaceae	*Anonidium mannii* Gaertm (D. Oliver) Engl.and Diels	45582HNC	Male infertility, complication of pregnancy and childbirth, treatment of abscess	(Noumi et al., 2011)	Stem bark	Ethanol	AMnsbMeOH	0.31
					Twig	Ethanol	AMntwMeOH	0.30
					Leaf	Ethanol	AMnlMeOH	1.65
					Leaf	Ethanol	AMnlI	2.87

					Twig	Ethanol	AMntw CH_2CL_2	0.55
Annonaceae	*Annona muricata* Linn	32879/ HNC	Boil, cough, diarrhea, dermatosis, hypertension, rheumatism and styptic, worms and parasites. headache, fever, toothache, asthma.	(Mat Salleh and Ahmad, 1989; Adewole et al., 2009; Chan and Roslida , 2012)	Leaf	Ethanol	AnMlMeOH	4.85
					Fruit pulp	Ethanol	AnMppMeOH	2.32
					Seed	Ethanol	AnMsMeOH	3.49
					Root	Ethanol	AnMrMeOH	1.43
					Pericarp	Ethanol	AnMPMeOH	0.98
					Leaf	Ethanol	AnMlI	1.04
					Twig	Ethanol	AnMtwMeOH	1.77
Annonaceae	*Chlorophytum macrophyllum* (A. Rich.) Asch.	373445/HNC	Cough or cough with haemoptysis.	(Adjanohoun et al., 1996)	Root	Methanol	CMr	12.18
					Stem	Methanol	CMst	10.12
					Leaf	Methanol	CMl	2.50
Annonaceae	*Monodora myristica* (Gaertn.) Dunal	27690/SFR/CAM	Buruli ulcer, dysentery, diabetes, bacterial infections, fevers, pain and snake bites, hemorrhoids, stomachache, and febrile pains	(Yemoa et al., 2011; Bele et al., 2011; Igoli et al., 2011)	Stem bark	Ethanol	MMsbMeOH	0.56
					Twig	Ethanol	MMtwMeOH	0.27
					Leaf	Ethanol	MMlMeOH	0.65
Annonaceae	*Piptostigma calophylium* Mildbr. and Diels	38333HNC	Urinary tract infections	Local claims	Leaf	Ethanol	PClMeOH	1.64
Annonaceae	*Polyalthia oliveri* Engl. and Diels	19416SRF/Cam	Malaria	(Boyom et al., 2009)	Stem bark	Ethanol	POsbMeOH	1.04
					Twig	Ethanol	POtwMeOH	0.64
					Twig	Ethanol	POtwI	0.37
Annonaceae	*Polyalthia suaveolens* Engl. and Diels	1227/SRF/CAM	Malaria	(Tsabang et al., 2012)	Stem bark	Ethanol	PSsbMeOH	2.04
					Leaf	Ethanol	PSlMeOH	7.86
Solanaceae	*Solanum*	23965SRFCam	Cough and pneumonia	(Adjanohoun et	Leaf	Methanol	SAl	25.44

	aculeastrum Dunal			al., 1996)	Stem	Methanol	SAst	2.80
					Fruit	Methanol	SAfr	10.57
Anacardiaceae	*Sorindeia juglandifolia* A. Rich.	9176 SRFCam.	Liver disease and mouth sores in children	(Berhaut, 1971)	Fruit	Methanol	SJfrMeOH	12.84
						Methanol	SJfr 1.1	1.43
						Methanol	SJfr 2.1	0.09
						Methanol	SJfr 2.2	0.46
						Methanol	SJfr 2.3	1.07
						Methanol	SJfr 3.1	1.00
						Methanol	SJfr 3.2	1.57
						Methanol	SJfr 3.3	0.95
						Methanol	SJfr 3.41	0.85
						Methanol	SJfr 3.42	1.09
						Methanol	SJfr 3.5	0.12
						Methanol	SJfr 3.6	0.072
						Methanol	SJfr 4.1	0.79
						Methanol	SJfr 4.2	0.23
						Methanol	SJfr 4.3	0.19
						Methanol	SJfr 4.4	0.05
						Methanol	SJfr 4.5	0.0019
Asteraceae	*Spilanthes filicaulis* (Schumach. and Thonn.) C.D.Adams	11136/SFRCam	Cough or cough with haemoptysis	(Adjanohoun et al., 1996)	Whole plant	Methanol	SF	8.65
Annonaceae	*Uvaria baumannii* Engl. and Diels	6427/SRF/Cam	Fatigue, abscess	Local claims	Twig	Ethanol	UBtwMeOH	1.30
					Leaf	Ethanol	UBlMeOH	0.71
					Twig	Ethanol	UBtwI	1.60
Annonaceae	*Uvariodendron*	28734/SFR/CAM	Malaria	Local claims	Twig	Ethanol	UCtwI	0.50

	callophylium R.E Fries				Twig	Ethanol	UCtwMeOH	0.42
Annonaceae	*Uvaria zenkeri* Engl. and Diels	57355HNC	Gun-stock	(Bele et al., 2011)	Twig	Ethanol	UZtwMeOH	0.59
Annonaceae	*Uvariodendron molendense* (Engl. and Diels) R.E. Fries	41685 HNC	Malaria	Local claims	Twig	Ethanol	UMtwMeOH	0.20
					Leaf	Ethanol	UMlMeOH	1.44
Annonaceae	*Xylopia aethiopica* (Dunal) A Rich	28725/SFR/Cam	Buruli ulcer, Cough, carminative, and as a post-partum tonic. Stomach ache, treatment of bronchitis, biliousness and dysentery	(Iwu, 1993; Yemoa et al., 2011)	Fruit	Ethanol	XAEfrMeOH	1.98
					Leaf	Ethanol	XAElMeOH	4.27
					Fruit	Ethanol	XAEfrH_2O	0.18
					Stem	Ethanol	XAEstMeOH	0.10
					Leaf	Ethanol	XAElI	0.30
Annonaceae	*Xylopia parviflora* A Rich	38322/HNC	Bronchitis, dysentery and febrile pains, Asthma, stomach aches and rheumatism	(Bele et al., 2011)	Stem bark	Ethanol	XAsbH_2O	0.94
					Twig	Ethanol	XAtwI	0.75
					Root	Ethanol	XArI	1.45
					Leaf	Ethanol	XAlMeOH	1.83
					Stem bark	Ethanol	XAsbI	2.02
					Root	Ethanol	XArH_2O	0.31
					Root	Ethanol	XArHex	0.09
					Root	Ethanol	XArMeOH	0.49
					Leaf	Ethanol	XAlI	3.99
					Twig	Ethanol	XAtwMeOH	1.83
					Stem bark	Ethanol	XAsbMeOH	1.57
Annonaceae	*Xylopia parviflora* A Rich	42351HNC	Stomach disorders and barrenness, Headache, analgesic and antispasmodic purposes	(Nishiyama et al., 2004)	Fruit	Ethanol	XPfrMeOH	1.65
					Leaf	Ethanol	XPlMeOH	1.96
					Stem	Ethanol	XPstMeOH	2.74

*Plant materials were extracted by maceration at room temperature; **extraction yields were

Table 2: MIC and MBC from the antimycobacterial screening of plant extracts.

Extracts code	MIC (µg/ml)					MBC (µg/ml)				
	MS	**MA**	**BCG**	**MU**	**MTB**	**MS**	**MA**	**BCG**	**MU**	**MTB**
Plants fractions and purified compounds at 250 µg/ml										
ACstI	250	NA	NA	7.81	NA	>250	NA	NA	7.81	NA
ACsbI	250	NA	125	1.95	NA	≥250	NA	>250	62.50	NA
SJfr 3.2	NA	NA	15.62	62.50	NA	NA	NA	62.50	≥250	NA
SJfr 3.3	NA	NA	31.25	NA	250	NA	NA	31.25	NA	250
SJfr 3.41	NA	NA	NA	62.50	NA	NA	NA	NA	250	NA
SJfr 3.42	NA	NA	31.25	NA	NA	NA	NA	31.25	NA	NA
SJfr 3.6	NA	NA	3.90	NA	NA	NA	NA	31.25	NA	NA
SJfr 4.2	NA	NA	125	NA	250	NA	NA	125	NA	250
SJfr 4.3	NA	NA	250	NA	NA	NA	NA	250	NA	NA
SJfr 4.4	NA	≥250	NA	NA	NA	NA	>250	NA	NA	NA
SJfr 4.5	NA	NA	NA	62.50	NA	NA	NA	NA	125	NA
Plants crude extracts at 12,500 µg/ml										
PSsbMeOH	6,250	>12,500	12,500	3,125	6,250	12,500	>12,500	12,500	3,125	12,500
XArH_2O	12,500	NA	3,125	NA	6,250	12,500	NA	3,125	NA	12,500
POsbMeOH	12,500	NA	6,250	NA	NA	≥12,500	NA	12,500	NA	NA
AMl	NA	NA	12,500	NA	NA	NA	NA	12,500	NA	NA
AMfr	NA	NA	6,250	NA	NA	NA	NA	6,250	NA	NA
SF	NA	NA	12,500	NA	NA	NA	NA	12,500	NA	NA
CMst	NA	NA	12,500	NA	NA	NA	NA	12,500	NA	NA
CMr	NA	NA	3,125	NA	12,500	NA	NA	6,250	NA	12,500
AMnsbMeOH	NA	NA	6,250	NA	12,500	NA	NA	6,250	NA	12,500
XAsbH_2O	NA	NA	12,500	NA	NA	NA	NA	12,500	NA	NA
XAsbI	NA	NA	12,500	NA	NA	NA	NA	12,500	NA	NA
UBtwMeOH	NA	NA	1,562	NA	NA	NA	NA	3,125	NA	NA
AMntwMeOH	NA	NA	3,125	NA	6,250	NA	NA	6,250	NA	6,250
Positive controls (µg/ml)										
SM	NT	>18.00	NT	0.12	0.50	NT	NT	NT	0.25	0.5
EMB	0.46	>160	1.50	NT	NT	>1.87	>160	>3.00	NT	NT

Abbreviations used in the table: MS: *M. smegmatis*; MA: *M. avium*; ; MU: *M. ulcerans*; MTB: *M. tuberculosis*.NA= no activity; NT= not tested; SJfr 3.6: 2,3,6-trihydroxy benzoic acid; SJfr 4.5: 2,3,6-trihydroxy methyl benzoate; SF: *S. filicaulis*; XArH_2O: *X. africana* root water fraction CMr: *C. macrophyllum* root, AMntwMeOH: *A. mannii* twig methanolic fraction; PSsbMeOH: *P. suaveolens* stem bark; ACsbI: *A. chlorantha* stem bark interface; ACstI: *A. chlorantha* stem interface.

Table 3: Cytotoxicity of ACsbI, ACstI, and SJfr 3.6 against MRC5 cells.

Plant fraction	*IC_{50} ± SD (µg/ml)
ACstI	50.82 ± 1.04
ACsbI	> 500
SJfr 3.6	139.4 ± 1.03

*Plant fractions were tested in triplicate; IC_{50} = extract concentration that inhibits 50% of cell growth.

DISCUSSION

Amongst the 19 plants collected in this study, 9 were reported to be used to treat respiratory tract infections, including cough, bronchitis, asthma, pneumonia, TB, cough with haemoptysis and other broncho-pulmonary disorders. In addition, 3 plants were reported as used to treat ulcers (ex. BU) and 7 to control other general symptoms like fevers, headache, and pain (Nishiyama et al., 2004; Ibekwe and Orok, 2010).

From the results presented (Table 2), extracts were more active on slow growing strains such as *M. tuberculosis*, *M. ulcerans*, and BCG than on faster growing strains such as *M. smegmatis* and *M. avium*.

Overall, apart from *M. avium*, all the mycobacterial strains showed susceptibility to at least 1 extract. *M. avium* is basically resistant to many drugs used to treat other mycobacterial infections. Of note, this bacterial species showed low susceptibility to the positive controls (SM, MIC> 18 µg/ml; EMB, MIC > 160 µg/ml). This resistance is usually ascribed to the impermeability of its lipid-rich cell wall, but it may also occur through emergence of highly resistant strains, patient intolerance to treatment, or erratic compliance (Portillo-Gomez et al., 1995; Ramirez et al., 2007). Furthermore most *M. avium* isolates segregate into smooth-transparent-, smooth-opaque- and rough-colony-type variants on agar plates. *M. avium* strain 2447 is a smooth transparent (SmT) colony isolated from AIDS patients (Cangelosi et al., 2001) and for unknown reasons, transparent variants tend to be more drug-resistant than opaque variants (Florido et al.,1999). The low susceptibility of *M. avium* strain 2447 might also be due to the bacterial load used in this study.

Two fractions from *A. chlorantha* stem and stemback (ACstI and ACsbI) showed activity against *M. smegmatis* at MIC value of 250 µg/ml for both and against *M. ulcerans* at MIC values of 1.95 and 7.81 µg/ml respectively. ACstI and ACsbI showed bactericidal activity on *M. ulcerans* with MBC values of 7.81 and 62.50 µg /ml respectively. In a similar approach, previous studies were conducted on *M. ulcerans.* The MIC value obtained by Coulibaly et al. (2011) with an aqueous extract from *Phyllanthus amarus* was 32 mg/ml, while results obtained by Yemoa et al. (2011) from the screening of 49 methanolic extracts from different plants showed MIC values ranging from 125-250 µg/ml.

Overall, investigation of plants products is so far scarce for the management and control of BU depite the urgent need for effective drugs. *M. ulcerans* has shown high susceptibility to *A. chlorantha* stem and stem bark extracts (ACsbI and ACstI), *S. juglandifolia* compound (SJfr 4.5). *A. chlorantha* also known as the African yellow wood, is a dense forest tree found in Cameroon, Nigeria and Gabon where Buruli ulcer is endemic (WHO, 2011). In the southern forest zone of Cameroon, it is used traditionally to treat tuberculosis and some forms of ulcer (Adjanohoun et al., 1996; Kimbi et al., 1996). This plant should be further investigated for the treatment of BU.

BCG appeared to be the most sensitive strain to much of extracts and fractions. Extracts from *S. juglandifolia* were found to be the most active on this strain. These results suggest that, the fruits of *S.*

juglandifolia should be further investigated for the treatment of bovine tuberculosis.

Results from the cytotoxicity studies suggest a critical management of doses of traditional potions that are given to patients attending traditional herbal practitioners. Extract from *A. chlorantha* (ACsbI) was found to be safe at 2 fold the MIC value (activity against *M. smegmatis*). A previous work assessed the cytotoxicity of the water extract of *A. chlorantha* and showed to be safe on Vero cells up to 0.025 mg/ml (Fasola et al., 2011), corroborating our findings. This result suggests that the activity of this fraction might be directed towards microorganisms rather than eukaryotic cells. The result obtained with ACsbI against the intracellular bacteria supports the local use of *A. chlorantha* stem bark in the treatment of a wide range of infections (Adjanohoun et al., 1996; Kimbi et al., 1996; Boyom et al., 2009; Bele et al., 2011; Tsabang et al., 2012). Moreover, ACsbI fraction, which is obtained from an Annonaceae plant, might contain acetogenins derivatives. This group of compounds was reported to be the most powerful of the known inhibitors of complex I (NADH: ubiquinone oxidoreductase) in mammalian and insect mitochondrial electron transport systems (Lewis et al., 1993). In addition, they were shown to be potent inhibitors of NADH oxidase of plasma membranes (Morré et al., 1995); leading to ATP deprivation and apoptosis (programmed cell death). Moreover, they are potently cytotoxic with bactericidal activities (Rupprecht et al., 1990; Guadaño et al., 2000; Rakotomanga et al., 2004; Kamkumo et al., 2012) that could explain the exerted antimycobacterial activity.

Conclusion

The study designed to assess the antimycobacterial activity of extracts from 19 Cameroonian medicinal plants highlighted the potency of natural products against 4 species of mycobacteria, *M. smegmatis*, BCG, *M. tuberculosis*, and *M. ulcerans*. Moreover, ACsbI significantly inhibited the growth of intracellular *M. smegmatis* and was also shown not to be cytotoxic for MRC5 cell line and BMDM. Considering the anti-TB drug resistance and the increasing burden of the emerging BU, the results achieved are promising, and indicate that new drug candidates could be discovered through this approach. Of note, very few reports are available on investigation of plant products against *M. ulcerans*. We anticipate that further detailed studies within this focus will enable us to uncover the hidden potency of particular natural products from the Cameroonian biodiversity against TB and BU.

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