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EC PHARMACOLOGY AND TOXICOLOGY
Research Article

Antibacterial Activity of Methanol Extract and Fractions from Stem Bark of *Bridelia micrantha* (Hochst.) Baill. (Phyllanthaceae)

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Received: March 25, 2019; **Published:** June 24, 2019

Abstract

The present study aims at confirming the antibacterial activity of the stem barks of *Bridelia micrantha* and to appreciate the influence of fractionation on the methanol extract antibacterial activity. The crude extract was prepared by maceration of plant powder in methanol. The dichloromethane portion was further fractionated by adsorption chromatography on silica gel. The antibacterial activity of the extract and fractions were evaluated by broth microdilution method. The phytochemical analysis of extract and fractions was done using standard phytochemical methods.

The extract showed moderate activity against most of the tested strains with MIC value of 512 µg/ml. Nine fractions designated F1 to F9 were obtained after column chromatography. F5 and F6 fractions were found to be the most active. F6 exhibited strong activity on all the tested bacteria with MIC values of 128 µg/ml on nine strains. The phytochemical analysis of the methanol bark extract of *B. micrantha* and fractions revealed the presence of triterpenes, phenols, tannins, flavonoids, saponins.

The results confirmed the antibacterial activity of methanol bark extract of *B. micrantha* and showed an increase in antibacterial activity with fractionation.

Keywords: *Bridelia micrantha; Methanol Extract; Fractionation; Antibacterial Activity*

Introduction

Plants have been used throughout history in traditional medicine for the treatment of a wide range of diseases [1]. Infectious diseases caused by bacteria, fungi and viruses are a critical challenge to health and they are believed to be one of the main causes of increasing rate of morbidity and mortality worldwide [2,3]. The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*) are responsible for the top 6 health care-associated infections (HAIs) and many have been identified as priority antibiotic-resistant bacteria [4,5]. Despite the significant progress made in the field of antibiotic therapy, medicine continues to face therapeutic failures partly due to the emergence of these resistant bacteria [6]. It is therefore necessary to ensure a therapeutic watch. Among the multitude of potential sources of antimicrobials, plants occupy a prominent place [7,8]. Previous investigations showed that many plant species were identified in Cameroon for their use in infectious diseases treatment by traditional healers [9] and some of these were proven to contain active ingredients with significant antibacterial activity [10]. *Bridelia micrantha* commonly known as coastal golden leaf is a member of the family Phyllanthaceae [11]. Ethno pharmacological knowledge revealed that its water boiled stem bark is commonly used in Cameroon to treat several ailments including amoebic dysentery, cough, diarrhoea, gastric ulcer, eye diseases, infertility and tapeworms [12,13]. Previous studies on this plant showed antibacterial [14,15], hepatoprotective [16], antioxidant [17,18], antitumor [19,20] and antiviral activities [21]. Recent studies have shown that *B. micrantha* root, stem bark and leaf contain various phytochemical compounds such as flavonoids, tannins, sterols, saponins, anthocyanins, triterpenes some of which possess potent antibacterial activity [7,22]. Therefore, the present study was undertaken to confirm the antibacterial activity of the stem barks of *Bridelia micrantha* and to appreciate the influence of fractionation on the methanol extract antibacterial activity.

Materials and Methods

Materials

Plant material

Fresh barks of *B. micrantha* used in this experiment were collected in January 2017 in the Centre Region of Cameroon. The plant was identified at the Cameroon National Herbarium where a voucher specimen N° 5714 HNC (YA) was deposited.

Chemicals

Ciprofloxacin (Sigma-Aldrich, Germany) was used as reference antibiotic. *p*-Iodonitrotetrazolium chloride [23] was used as microbial growth indicator.

Microorganisms and culture media

The antibacterial activity was carried out on two reference and eighteen routine strains. The routine strains of *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were obtained from patient suffering from gastroenteritis at the Bafang ADLUCEM hospital. Those of *Salmonella enterica serovar typhi*, *Salmonella enterica serovar paratyphi B,* and *Salmonella enterica serovar typhimurium* were obtained from the Laboratory of Bacteriology and Mycology of the "Centre Pasteur" Yaoundé-Cameroon. Methicillin-resistant *Staphylococcus aureus* strains were obtained from the culture collection of the Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Japan. Multidrug resistant *Providencia stuartii* strain was obtained from the culture collection of the University of Mediterranean, France. The bacterial features are summarized in table 1. These microorganisms were maintained at 4°C on Mueller Hinton Agar (MHA) (Liofilchem, Italy). Mueller Hinton Broth (MHB) (Liofilchem, Italy) was used for Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) determination.

Bacteria	Characteristics
Gram-	
Escherichia coli	
E.C 96	Routine strain: IPM^S, AUG^I, NOR^S, CFM^S, CRO^R, CIP^S, AN^I, CHL^S
E.C 99	Routine strain: IPM^S, AUG^I, NOR^S, CFM^S, CRO^S, CIP^S, AN^I, CHL^S
E.C 136	Routine strain: IPM^S, AUG^R, NOR^S, CFM^S, CRO^S, CIP^S, AN^S, CHL^S
E.C 137	Routine strain: IPM^S, AUG^S, NOR^S, CFM^S, CRO^S, CIP^S, AN^I, CHL^S
Enterococcus aerogenes	
ENT 119	Routine strain: IPM^S, AUG^I, NOR^S, CFM^S, CRO^S, CIP^S, AN^I, CHL^S
ENT 144	Routine strain: IPM^S, AUG^R, NOR^R, CFM^S, CRO^S, CIP^S, CHL^R
Klebsiella pneumoniae	
KL 111	Routine strain: IPM^S, AUG^I, NOR^S, CFM^S, CRO^I, CIP^S, AN^I, CHL^S
Salmonella enterica serovar Typhi	
SAL 9	Routine strain: IPM^S, AUG^R, NOR^S, CFM^S, CRO^R, CIP^S, AN^I, CHL^I
Salmonella enterica serovar Paratyphi B	Routine strain: AM^R, TE^R, SXT^R, NA^R, CIP^S, CHL^S
Salmonella enterica serovar Typhimurium	Routine strain: AM^R, TE^R, SXT^R, NA^R, CIP^S, CHL^S
Providencia stuartii	
PSNEA 16	Routine MDR strain, AcrAB-TolC
Pseudomonas aeruginosa	
ATCC 27853	Reference strain
PACIP 76110	Reference strain
Gram+	
Staphylococcus aureus	
ST 9	Routine strain: IPM^S, AUG^S, AM^S, DO^S, VA^S
ST 113	Routine strain: IPM^S, AUG^S, AM^S, DO^S, VA^S, E^S
ST 120	Routine strain: IPM^S, AUG^S, AM^S, DO^S, VA^S, E^S
MRSA 3	Routine multidrug strain: OFX^R, K^R, E^R, CIP^R
MRSA 9	Routine multidrug strain: OFX^R, FLX^R, K^R, E^R, CHL^R, IM/CS^R
MRSA 12	Routine multidrug strain: OFX^R, FLX^R, K^R, E^R, IM/CS^R

Table 1: *Features of bacteria strains and clinical strains used in the study.*

AN: Amikacin; AM: Ampicillin; AMC: Amoxicillin-Clavulanate; CHL: Chloramphenicol; CFM: Cefixime; CIP: Ciprofloxacin; CRO: Ceftriaxone; DO: Doxycycline; E: Erythromycin; FLX: Flomoxef; IMP: Imipenem; IM/CS: Imipenem/Cilastatin Sodium; K: Kanamycin; NA: Nalidixic acid; NOR: Norfloxacin; OFX: Ofloxacin; TE: Tetracycline; SXT: Trimethoprim-Sulfamethoxazole; VA: Vancomycin. R: Resistant; S: Sensible and I: Intermediate.

Methods

Plant extraction

B. micrantha barks were collected and dried for 21 days in an ambient environment under shade and ground into powder. The powder (500 g) was soaked in 2.5L of methanol for 3 days. The mixture was filtered using a Whatmann N°1 filter paper and the residue was re-extracted four times as previously described. The total methanol extract was concentrated using rotatory evaporator. The extract was further dried in an oven at 45°C for 24 hours.

Fractionation

The extract (128g) was dissolved in a mixture of methanol (500 mL), distilled water (250 mL) and dichloromethane (500 mL). The dichloromethane phase and the methanol/water phase were separated. Each phase was concentrated using a rotatory evaporator. The dichloromethane phase (31.2g) was chromatographed through a silica gel (250 - 300 Mesh) column (5 cm of internal diameter and 40 cm of height) using n-hexane-EtOAc (90:10; 80:20; 70:30; 50:50; 70:30; 0:100) and EtOAc-MeOH (100:0; 95:5; 90:10; 80:20; 70:30; 50:50; 0:100) as eluent. Seventy-two fractions of 250 mL each were collected and combined on the basis of their thin layer chromatography (TLC) profiles into nine major fractions labelled F1 to F9 (F1: 1-3; F2: 4-10; F3: 11-23; F4: 24-44; F5: 45-49; F6: 50-57; F7: 58-66; F8: 67-69; F9: 70-72).

Antibacterial activity assay

The *in vitro* antibacterial activity of the crude extract and fractions were evaluated by determining the Minimum Inhibitory Concentrations using broth microdilution method [24]. Briefly, the stock solution of the extract was prepared with 5% DMSO in broth culture medium. Bacterial suspension of about 1.5 x 10^8 CFU/ ml following N° 0.5 Mc Farland turbidity standards was prepared from 18 hours old bacterial culture. These suspensions were further diluted in Mueller Hinton broth to give 1.5 x 10^6 CFU/ml. The antibacterial susceptibility tests were performed in 96-well microtiter plates. A serial two-fold dilution of the plant extract and fractions was performed to obtain final concentrations ranging from 1024 to 8 µg/ml in a total volume of 100 µl/well. These wells were finally inoculated with 100 µl inoculum. The plates were incubated at 37°C for 18h. Following incubation, bacterial growth was monitored colorimetrically using *p*-iodonitrotetrazolium chloride (INT). Viable bacteria change the yellow dye of *p*-iodonitrotetrazolium violet to a pink colour. Minimum inhibitory concentration (MIC) value was recorded as the lowest concentration of the test substance that completely inhibited bacterial growth [23]. The antibacterial activity was classified as strong if the extract displayed a MIC values less than 500 µg/ml, moderate from 500 to 1500 µg/ml and weak when over 1500 µg/ml [25].

The Minimum Bactericidal Concentrations (MBC) were determined by adding 50 µl aliquots of the preparations which did not show any growth after incubation during MIC assays to 150 µL broth culture medium. These preparations were incubated at 37°C for 24h. The MBC values were regarded as the lowest concentration of extracts which did not produce any color change after addition of INT as mentioned above [26].

The experiments were performed in duplicate and repeated thrice. Ciprofloxacin was used as positive control while 5% dimethylsulfoxide (DMSO) was used as negative control.

Phytochemical analysis

Qualitative phytochemical tests were conducted on the methanol extract and fractions using standard methods [27].

Results

Antibacterial activity

The methanol extract and fractions from stem bark of *B. micrantha* were evaluated for their antibacterial activities on a panel of bacteria strains including two reference and eighteen routine strains (Table 2). These results showed moderate activity of the methanol extract against most of the tested pathogenic bacteria strains (MIC = 512 µg/ml). Strong antibacterial activity of this extract was obtained on three bacteria strains with MIC value of 256 µg/ml.

Nine fractions were obtained from *B. micrantha* methanol extract. Among them, F1 was found to be inactive while F2 to F9 exhibited antibacterial activity with MIC values ranging from 128 µg/ml to 1024 µg/ml. F5 and F6 fractions were the most active. F5 showed strong activity on ten of the twenty tested bacteria strains with MIC value of 256 µg/ml while F6 exhibited strong activity on all the tested bacteria with MIC values of 128 µg/ml on nine strains, including *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, *Enterobacter aerogenes and Salmonella sp* strains (Table 3).

Organisms	Extract	F1	F2	F3	F4	F5	F6	F7	F8	F9	Ciprofloxacin
K. pneumoniae											
KL111	512/512	-/-	1024/1024	1024/1024	1024/1024	512/512	256/256	512/512	512/1024	512/1024	1/16
E. aerogenes											
ENT167	512/512	-/-	512/512	1024/1024	1024/-	256/256	128/512	512/512	512/512	512/512	8/128
ENT144	256/512	-/-	512/-	512/1024	1024/-	256/256	256/256	512/512	512/1024	512/1024	1/128
ENT119	512/512	1024/-	512/-	512/1024	1024/-	512/512	256/512	512/1024	512/1024	512/1024	1/-
S. aureus											
ST9	512/512	-/-	512/-	1024/1024	1024/-	256/512	128/512	512/512	512/512	512/512	1/64
ST113	256/256	1024/-	512/1024	512/-	1024/1024	256/1024	128/512	512/1024	512/512	512/512	1/64
ST120	256/256	-/-	512/-	1024/1024	512/-	256/256	256/512	512/512	512/512	512/512	8/128
MRSA9	512/512	-/-	512/-	1024/1024	512/-	512/512	256/256	512/1024	512/-	1024/-	2/4
MRSA3	512/512	-/-	512/-	1024/1024	512/1024	256/512	128/256	512/512	512/512	512/1024	32/-
MRSA12	512/512	-/-	512/-	1024/1024	1024/1024	512/512	256/512	512/512	512/-	512/-	2/16
E. coli											
EC96	512/1024	-/-	1024/-	512/1024	512/-	256/1024	128/512	512/512	512/512	512/1024	1/128
EC99	512/512	-/-	512/-	1024/-	1024/1024	512/512	256/-	512/512	512/-	512/-	4/16
EC136	512/512	-/-	1024/1024	1024/1024	1024/1024	512/512	256/256	512/512	512/512	1024/1024	1/8
EC137	512/512	-/-	512/-	1024/1024	1024/1024	256/512	128/256	512/512	512/1024	512/512	16/128
P. stuartii											
PSNEA16	512/1024	-/-	512/-	1024/1024	1024/-	512/-	256/-	512/512	512/-	512/-	1/16
S. enterica serovar Typhi											
SAL 9	512/-	-/-	1024/-	1024/1024	1024/1024	512/512	256/-	512/1024	256/-	512/1024	1/16
S. enterica serovar Typhimurium	512/512	-/-	512/512	1024/1024	1024/-	256/256	128/512	512/512	1024/1024	512/512	32/64
S. enterica serovar paratyphi B	512/512	1024/-	512/512	1024/1024	1024/-	512/512	128/512	512/512	1024/1024	512/512	1/128
P. aeruginosa											
ATCC 27853	512/512	-/-	512/512	1024/1024	1024/-	512/512	256/512	512/512	512/512	512/512	1/64
CIP 76110	512/512	-/-	512/1024	1024/1024	512/1024	256/256	128/512	512/512	512/-	512/512	1/64

Table 2: *Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of B. micrantha methanol extract and its fractions.*

	Extract	Fractions								
		F1	F2	F3	F4	F5	F6	F7	F8	F9
Saponins	++	-	-	+ +	+	+	+	+	++	++
Polyphenols	+++	+	+	+	++	+++	+++	+++	+++	+++
Anthraquinones	+	++	++	+	+	-	-	+	+	+
Anthocyanes	++	- 6 -	-	+ +	+	+	+++	++	+	+
Triterpenes	++	+	-	+	+	+++	+	+++	+	+
Sterols	-	- -	- + +	-	-	-	-	-	-	-
Flavonoids	+	+	++	+	+	++	++	-	+	+
Alkaloids	++	+	++	++	+	++	+	+	++	++
Coumarins	++	++	++	+	+	++	+++	+++	+	+
Tannins	+++	+	+	+	++	+++	+++	-	+++	+++
Glucosides	++	-	-	-	-	-	++	+	++	+

Table 3: *Phytochemical composition of B. micrantha methanol barks extract and fractions.*

(–): Absence; (+): Presence of chemical compound; (+) < (++) <(+++): Base of the intensity of characteristic colour.

Phytochemical screening

The qualitative phytochemical composition showed that polyphenols, tannins, anthocyanins, triterpenes, glucosides, coumarins, alkaloids and saponins are the major compounds in the methanol extract of *B. micrantha* bark.

Phytochemical composition of sub-fractions revealed that except triterpenes, F1 and F2 fractions have the same composition as well as F3 and F4 had the same compositions. Moreover, saponins, polyphenols, anthocyanins, triterpenes and alkaloids were found in all fractions while flavonoids, anthraquinones, tannins and glucosides were selectively present.

Discussion

The use of medicinal plants in the treatment of diseases is an ancient tradition that has co-existed with human habitation [28]. Nowadays, with the increase of resistance of numerous microorganisms to conventional drugs [29], these medicinal plants could be used as an alternative as the antibacterial activity of some of them have been well established in most bacteria including drug resistant bacteria [10,30,31]. The Cameroonian flora is very rich and varied, and many plants have not yet been explored for their pharmacological properties including antibacterial activity.

The methanol extract of *B. micrantha* stem bark revealed moderate antibacterial activity on most microorganisms tested. This result is in line with previous authors who reported the antibacterial activity of this plant [32-34]. However, the activity of this extract, although moderate, appears relevant compared to the results mentioned by these authors. Indeed, Steenkamp *et al*. [32] using broth micro-dilution method found that the extract showed no activity against *E. coli* and *P. aeruginosa* while MIC value of 4000 µg/mL was obtained against *S. aureus*. The very weak activity obtained by these authors might be due to the impact of geographical and therefore climatic differences since these factors have been reported to affect plant secondary metabolite composition [35,36]. Adefuye., *et al*. [34] revealed MIC_{50} values of ethyl acetate and acetone stem bark extract of *B. micrantha* ranged from 0.078 to 1.25 mg/ml and 0.078 to 0.625 respectively on *Staphylococcus aureus, Shigella sonnei, Salmonella Typhimurium* and *Helicobacter pylori* strains. These values, which inhibit 50% of the bacterial growth, are clearly greater than those reported in the present work and which, contrary to them, represent concentration that inhibit any bacterial growth. Some phytochemicals; polyphenols, tannins, anthocyanins, triterpenes, glucosides, coumarins, alkaloids and saponins, found in this extract may explain the antibacterial capacity. These secondary metabolites serve a defense mechanism against invasion by many microorganisms, insects and other herbivores [37]. Significant inhibitory properties against different pathogens have been reported. For example, flavonoids are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection [38]. Antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell [39]. Tannins bind to proline rich proteins and interfere with the protein synthesis [40]. The obtained results may justify the use of the plant for ethnomedicine [41].

The antibacterial activity of the methanol extract of the stem bark of *B. micrantha* was distinguished from their fractions. F5 and F6 fractions were found to be more active than the crude methanol extract on most of the tested bacteria. Increase antibacterial activity with fractionation reveals that the active principles of the stem barks of this plant are concentrated during fractionation in some fractions and highlights the fractionation as alternative to ameliorate plant extracts antimicrobial activity. Similar approach was reported by several authors [15,42,43].

The fraction F6 was found to be the most active with this activity extended to methicillin-resistant *Staphylococcus aureus* (MRSA strains). Infections caused by MRSA are generally severe with the highest mortality rate [44]. Actually, very few antibiotics as Lysocin E are efficient against MRSA [45].

The activity of the fraction F6 was extended to ESKAPE pathogens, particularly *S. aureus*, *K. pneumonia*, *P. aeruginosa*, *E. aerogenes* strains. The ESKAPE pathogens are differentiated from other pathogens due to their increased resistance to commonly used antibiotics. This increased resistance, combined with their clinical significance in the medical field, results in a necessity to combat them with novel antibiotics [2]. Therefore, the fraction F6 could be used directly as antibacterial or could provide molecules which could be useful as antibacterial or substrates for the synthesis of new broad spectrum antibiotics which could overcome gastrointestinal tract infection bacteria.

Regarding the MBC values of the extract and fractions, it was seen that MBC/MIC ratio was less than 4 in many cases meaning that they all exerted a bactericidal activity on many of the tested organisms [46].

Conclusion

The study aimed at confirming the presence of antibacterial substances in *B. micrantha* stem barks and to appreciate the influence of fractionation on the methanol extract antibacterial activity. The results confirmed the antibacterial activity of the stem barks methanol extract of *B. micrantha* and revealed an increase activity with fractionation with the F6 fraction obtained with AE/MeOH (20%) being the most active.

Acknowledgement

Authors are grateful to Pr Victor Kuete for microorganisms supply.

Funding

The authors received no direct funding for this research.

Competing Interests

The authors declare no competing interest.

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