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Asian Journal of Plant Science and Research, 2016, 6(4):40-47

ISSN : 2249-7412
CODEN (USA): AJPSKY

Antimicrobial activities of *Combretum micranthum* extracts on *Staphylococcus aureus* strains isolated from skin infections and some reference strains

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ABSTRACT

Combretum micranthum, is an exotic medicinal plant used generally in African pharmacopoeia and particularly in Benin against several bacterial infections. In this study, the antimicrobial activity of ethanol and ethyl acetate extracts of leaves of this plant, were tested in vitro on 70 strains of Staphylococcus aureus isolated from three skin infections (pus, furuncles and abscesses) and 10 reference strains (9 bacteria and 1 yeast), by agar diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) have been determined by the macrodilution method. Phytochemical screening of the C. micranthum leaves powder revealed the presence of catechol and gallic tannins, saponins, alkaloids, flavonoids, steroids and triterpenoids. The two extracts had effect on both Gram (+) and Gram (-) bacteria. The inhibitory diameter is larger with the ethanol extract (23.5 ± 0.7 mm for S. aureus) than ethyl acetate extract (21 ± 14 mm for S. aureus) with significant variation ($p < 0.0001$). These extracts are more active on strains isolated from furuncles than those isolated from abscesses and pus ($p < 0.05$). The difference of MIC and MBC was not significant ($p > 0.05$). The C. micranthum leave is a good candidate for research into the antimicrobial active compounds in order to develop a drug for the treatment of various skin infections and particularly the furuncles.

Keywords: *Combretum micranthum*, antibacterial activities, skin infections, plant extracts, Benin.

INTRODUCTION

The infectious diseases are the world's most traded human diseases and cause as many as 50000 deaths per day [1] . However, the hope that was born with the discovery of the antibiotic has been dispelled with the emergence of resistant microorganisms to these drugs. The prevalence of resistant microorganisms to most antibiotics is growing [2]. The development of resistance to the new antibiotic molecules by pathogens responsible for most infectious diseases debilitating effects aggravated the situation [3]. These three decades, new antibiotics (3rd generation) are produced by pharmaceutical company [4]. However, these antibiotics are still unable to prevent the growth of many bacteria especially those who have the possibility of developing resistance [5].

In developing countries, the drugs used to treated most infectious diseases are imported, making their very high prices. Today, the generic drugs are out of reach of populations because the pharmaceutical firms are appropriated their production; so that people (about 90%) are moving away from modern health facilities [6] and turn to traditional medicine.
All of the above, new trails for research into new antimicrobial sources remains a challenge for pharmaceutical and research institutions [7]. One of the tracks that could be explored is the traditional medicine through its medicinal

plants. Medicinal plants are known to have a multitude of secondary metabolites [8], [9]used throughout the world for the treatment of diseases and infections[10], [11], [12].

The study we propose is within the scope of the research of new active molecules through the increase in value of plants from the traditional pharmacopeia of Benin. It aims to evaluate the antibacterial activity of *Combretum micranthum*, plant from traditional Beninese Pharmacopoeia on 10 reference strains (Gram + and Gram -) and *Staphylococcus aureus* strains from various types of skin infections.

MATERIALS AND METHODS

Plant material collection

The leaves of *C. micranthum* were collected in March 2014 in the region of Tanguieta (1°16'55''E- 10°37'88''N) northwestern Benin (Fig.1). The site is characterized by a soudano-sahelien climate with one rainy seasons (from may to November) and one dry seasons (from November to may). The Tanguieta pluviometry varied from 800 mm to 1.100 mm with maximum in August and September. The temperature varied from 15 °C to 35 °C. The harvested leaves have been authenticated at the National Herbarium of Benin (University of Abomey Calavi). The plant material was then cut and dried in the shade in the laboratory at room temperature (25 °C) for two weeks before grinded into powder.

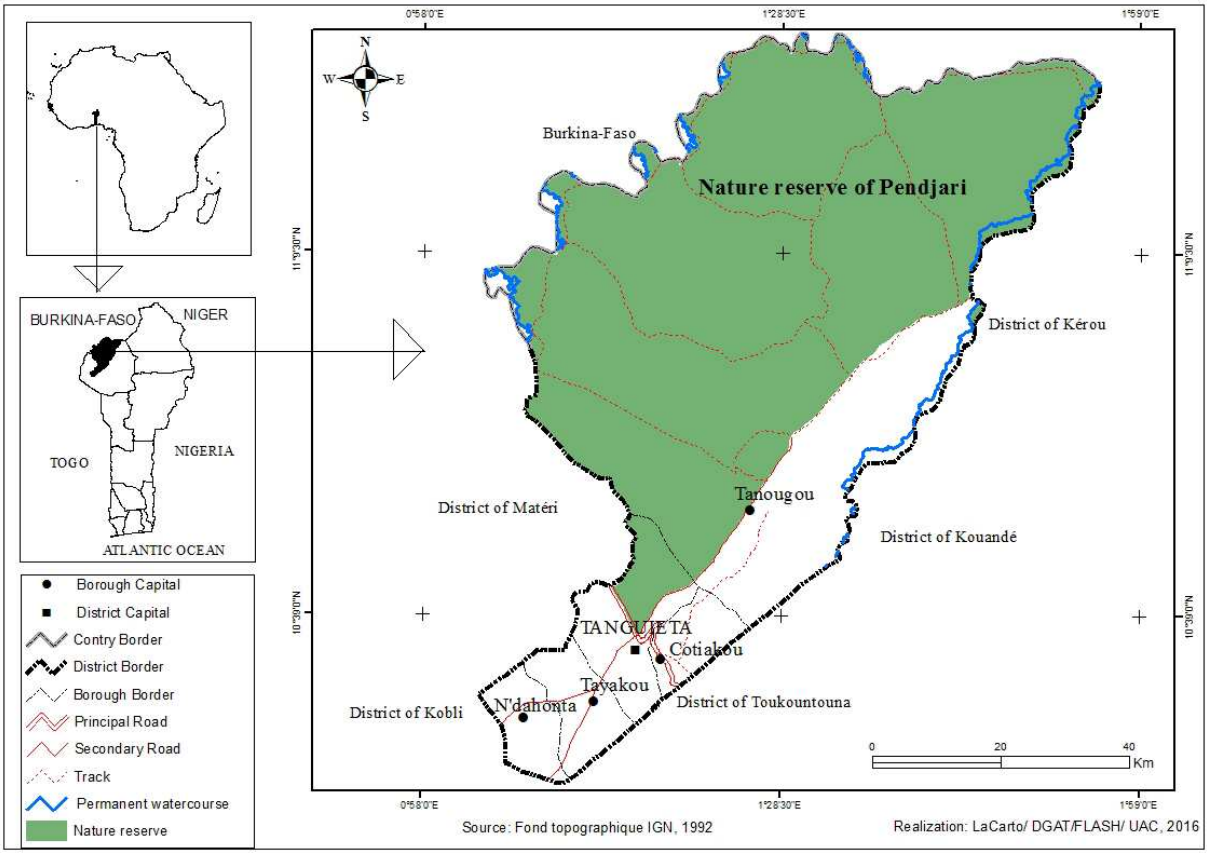

Fig 1. Geographical localization of harvested zone of *C. micranthum* leaves

Microorganisms

The used microorganisms are composed of 10 reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* T22695, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* A24974, *Micrococcus luteus*, *Proteus vulgaris* A25015, *Streptococcus oralis*, *Enterococcus faecalis* ATCC 29212, *Candida albicans* MHMR) and 70 *Staphylococcus aureus* strains isolated from various types of skin infections such as pus (31), furuncles (19) and abscesses (20). These microorganisms belonged to the microorganism collection of the Laboratory of Biology and Molecular Typing in Microbiology (University of Abomey-Calavi, Benin, West Africa).

Preparation of ethanolic and ethyl acetate extracts of *C. micranthum*

The dried leaves were milled and the fine powder obtained was used for different extractions using various type of solvents (ethanol 96°, ethyl acetate and water). The extraction method used is an adaptation of the protocol used by [13], [14]. Briefly,leaf powder (50g) were mixed to 250 ml of ethanol96° . and macerated under stirring for 72 h before filtration with Whatman N°1 filter paper (125 mm ø, Cat No 1001 125) .

The filtrate thus obtained is separated into two fractions ethanolic 1 and ethanolic 2 extracts. The first fraction was concentrated in a rotary evaporator and the residue from this concentration was dried at 40 °C to obtain the ethanolic extract. The second fraction, was mixed with about 50 ml of distilled water and 100 ml of ethyl acetate. After homogenization the mixture was decanted and separated into two phases. The upper phase was dried to obtain the ethyl acetate extract.

Assessment of microorganism's susceptibility to Methicillin

The sensitivity was determined by disk diffusion method using Muller Hinton agar (Bio-Rad, France) as recommended by antibiotic Committee of [15]. This operation is carried out under aseptic conditions.

Evaluation of antimicrobial activity of *C. micranthum* extracts

Sensitivity test

The antimicrobial activity of the extracts was evaluated using the disk diffusion method inspired from that described [16]. Indeed, 1 ml of bacterial culture (adjusted to 0.5 McFarland standard) was used to flood a Petri dish containing Mueller-Hinton agar [15]. Two to four sterile disks (6 mm) are deposited in the Petri disk previously flooded of bacterial culture under aseptic conditions. This disk was inoculated with 30 µl of tested extract. For each extract, the experiment is duplicated and a negative control is performed with the solvent in place of the extract. The dishe are then kept at room temperature 15-30 min before being incubated at 37 °C during 24 h [17] and 48 h. The inhibition diameters were measured using a scale [18] after incubation times of 24 h and 48 h.

Determination of Minimum Inhibitory Concentration (MIC)

The method of macrodilution with visual observation previously describe by [19] was used. First, the extracts were diluted in sterilized distilled water to the highest concentration of 20 000 µg/ml and then nine dilution were performed to obtain the concentrations of 10 000 µg/ml, 5 000 µg/ml, 2 500 µg/ml, 1 250 µ/ml, 625 µ/ml, 312.5 µg/ml, 156.25 µg/ml, 78.12 µg/ml and 39.06 µg/ml in screw caped. To 1 ml of the above concentrations was added 1 ml of the bacteria inoculum (10^6 UFC/) to obtain 2 ml as a final volume. Culture medium without samples and others without microorganisms were used in the tests as control. Tubes were incubated at 37°C for 18-24 hours and growth was indicated by turbidity. The MIC is the lowest concentration of the compound at which the microorganism tested does not demonstrate visible growth (turbidity).

Determination of Minimum Bactericidal Concentration (MBC)

Referring to the results of MIC test, all tubes showing no microorganism growth were identified. Each tube is inoculated into a Petri dish containing MH agar and incubated at 37 °C for 24 h. The lowest concentration of the extract in which the microorganism did not grown on solid medium is considered to minimum Bactericidal Concentration [20].

Phytochemical screening of *C. micranthum* leaves

Phytochemical screening is based on the differential reactions (coloration and precipitation) of the main groups of chemical compounds contained in the leaves powders of *C. micranthum*. Different groups of secondary metabolites such as the terpenoids, polyphenols including flavonoids and tannins, alkaloids, saponins and quinone substances, coumarins derivatives cyanogenic mucilages, reducing compounds and anthracene derivatives have been researched according to the methods described by [21].

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SAS 9.2 software. Duncan's test was used to compare the difference of the means with 0.05 significance level.

RESULTS

Phytochemical screening of *C. micranthum* leaves

The phytochemical composition of *C. micranthum* leaves is presented in table 1. Excepted anthocyanin, several polyphenolic compounds such as tanins catechin, tanins gallic, flavonoids and leuco-anthocyans are plentiful while the alkaloid and coumarin are the secondary metabolite less abundant in the *C. micranthum* leaf. Other compounds are founds, but cardenolids, cyanogenics derivate are not founds.

Table 1. Phytochemical composition of *Combretum micranthum* leaf

Chemical groups	Sub groups	Test results
Alkaloids		+
Polyphenolic compounds	Tannins Catechin tannins Gallic tannins Flavonoids Anthocyanins Leucoanthocyanes	+++ +++ +++ +++ - +++
Quinone derivatives		+++
Saponosides		+++ (H=1,9 cm) IM=4/10
Triterpenes steroids cardenolides		++ +++ -
Cyanogénic derivate		-
Mucilages		+++
Coumarin		+
Reducting coumpound		+++
Antracens derivate	Anthracéns free combine anthracénic -O-Hétérosides -C-Hétérosides	++ - ++ +++

+++: Strong presence; ++: Mean Presence; +: Low presence; - : Absence ; H: Height of the foam; IM: Foam Index.

Susceptibility of strains isolated from skin infections to oxacillin

According to the profile of resistance to oxacillin, *S. aureus* strains have been classified into two groups: Methicillin Sensitive *S. aureus* (MSSA) and Methicillin Resistant *S. aureus* (MRSA). Of the 70 strains isolated from different skin infections, 75.71% are resistant to methicillin. All strains isolated from furuncles and abscesses are resistant to Methicillin, while 58.06% (18/31) of strains isolated from pus are sensitive to this antibiotic (Fig. 2).

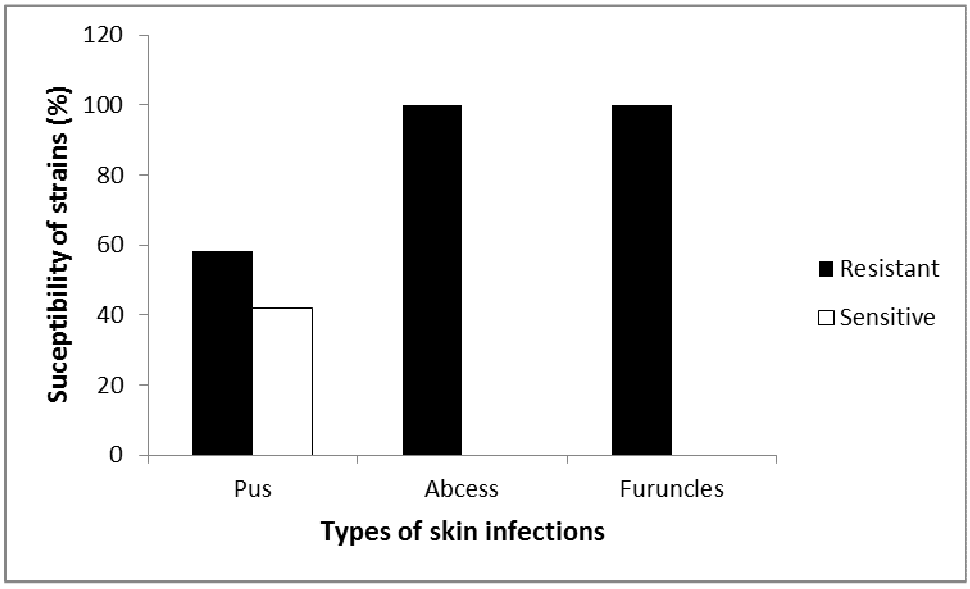

Fig 2. Susceptibility of Methicillin to *S. aureus* strains according to skin infections

Sensitivity of the reference strains to different extracts of *C. micranthum*

The figure 3 shows inhibitory activity of *C. micranthum* extracts on reference strains. For ethanolic extract, the inhibitory diameters varied from 13.5 ± 0.7 mm to 25.0 ± 0.0 mm, while the ethyl acetate extract was induced the diameters varied from 13.5 ± 0.7 mm to 21 ± 1.4 mm. The reference strains showed varying susceptibility against *C. micranthum* extract. Noted that 70% (7/10) of the strains were sensible to ethanolic extract while 50% to ethyl acetate extract. For these two extracts, the inhibition diameters obtained were very highly significant ($p < 0.0001$). The inhibition diameters varies from one species to another, but remained stable over time.

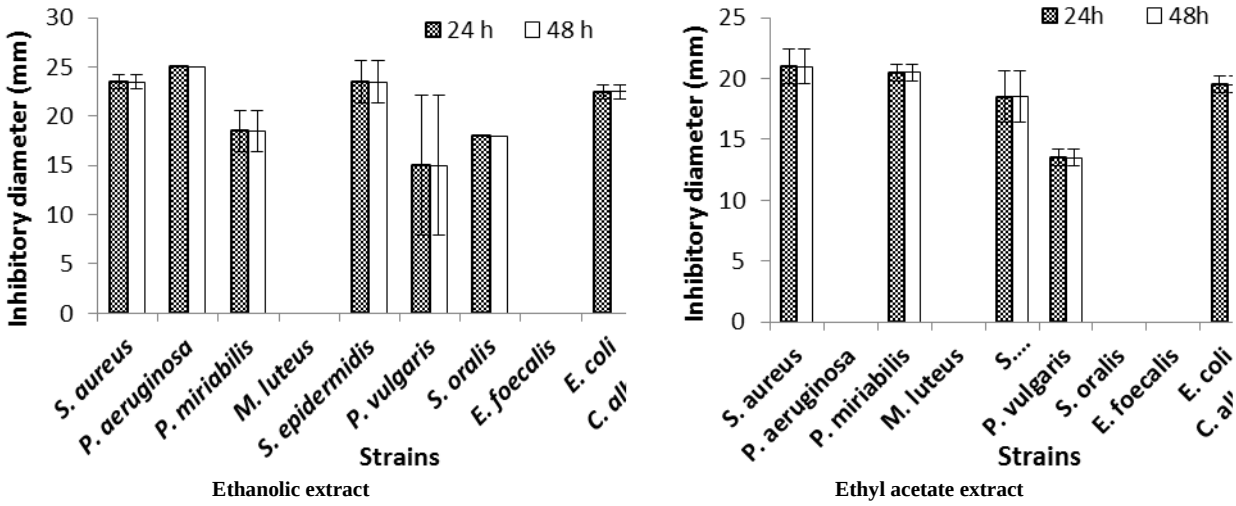

Fig 3. Inhibitory diameter zone of *C. micranthum* leaves extracts on reference strains after 24 h and 48 h

Sensitivity of *S. aureus* strains isolated from skin infections to different extracts of *C. micranthum*

Extracts were various actions on *S. aureus* strains isolated from skin infections. In the 70 *S. aureus* strains isolated, 11.43% are resistant to alcoholic extract of *C. micranthum* while 34, 29% are resistant to ethyl acetate extract (Table 2). The difference of activity is significant (p = 0.01) between alcoholic extract and ethyl acetate extract. The ethyl acetate extract (34.29% of resistance) is therefore less effective than the alcoholic extract (11.43% of resistance).

Table 2. Susceptibility (%) of *S. aureus* strains to different types of *C. micranthum* extracts according to the types of skin infections

Origin	Ethanolic extract		Ethyl acetate extract	
	Sensitive	Resistant	Sensitive	Resistant
Abscess (n=20)	90	10	65	35.00
Furuncles (n=19)	94.74	5.26	94.74	5.26
Pus (n=10)	83.87	16.13	48.39	51.61
Total (n=70)	88.57	11.43	65.71	34.29

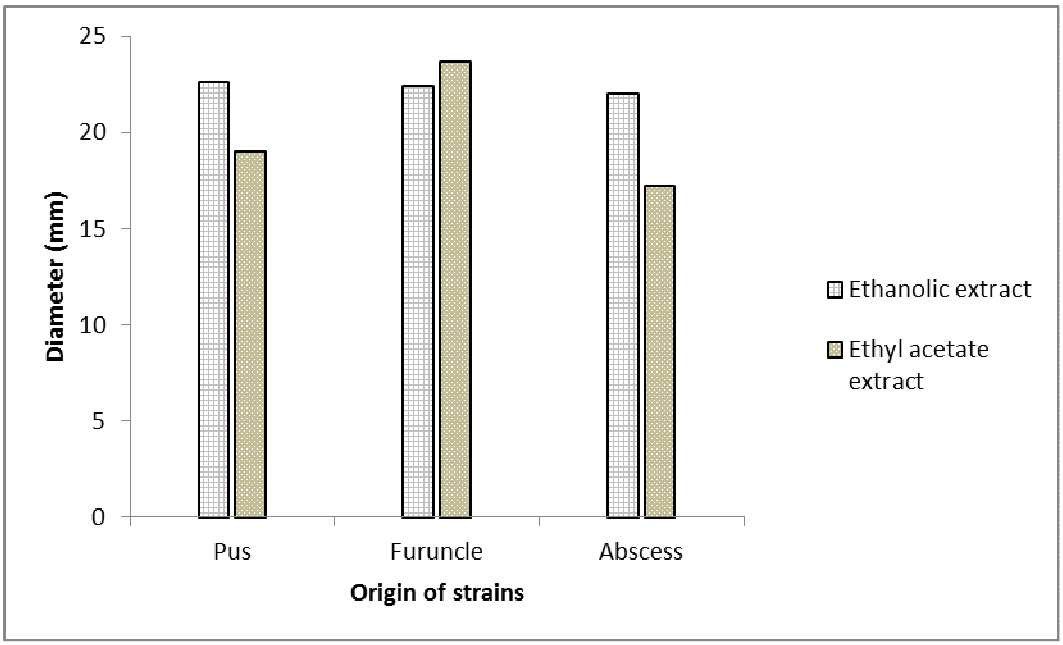

Fig 4. Average inhibitory diameter of *C. micranthum* extracts on *S. aureus* strains according to the types of skin infections after 24h of incubation

The inhibitory effect of extracts varied according to the origins of *S. aureus* (pus, furuncles and abscess). The ethyl acetate extract is more effective on strains isolated from furuncles (5.26% of resistance) than those isolated from abscess (10.00% of resistance) and pus (16.13% of resistance). This remark is similar for ethanolic extract which

more effective on strains isolated from furuncles (5.26% of resistance) than those isolated from abscess (35.0% of resistance) and pus (51.61% of resistance). According to the types of infections, the inhibitory effect is highly significant (p = 0.003) between ethanolic and ethyl acetate extracts (Fig. 4).

Sensitivity of *S. aureus* (MRSA and MSSA) strains isolated from skin infections to *C. micranthum* extracts

Among 53 MRSA studied, 88.68% were found to be sensitive to ethanol extract of *C. micranthum* while 73.58% were sensitive to ethyl acetate extract (Table 3). Among 17 MSSA, 11.76% were resistant to alcoholic extract of *C. micranthum* and 58.82% were resistant to ethyl acetate extract of *C. micranthum*. The ethanol extract is more effective than acetate extract both for MRSA (p = 0.04) or MSSA (p = 0.004). The action of the alcoholic extract was not significantly different on MRSA and MSSA while MSSA are more resistant to ethyl acetate extract than MRSA (p = 0.01).

Table 3. Susceptibility (%) of *C. micranthum* extracts with MRSA and MSSA

Type	Ethanolic extract		Acetate d'ethyl extract	
	Sensitive	**Resistant**	**Sensitive**	**Resistant**
RMSA (n = 53)	88.68	11.32	73.58	26.42
SMSA (n = 17)	88.24	11.76	41.18	58.82

RMSA : Methicillin Staphylococcus aureus resistant, SMSA : Methicillin Staphylococcus aureus sensitive

The average diameter of the inhibition of MRSA and MSSA were not statistically different (p > 0.05) whether one is in an ethyl acetate or alcoholic extract (Fig. 5).

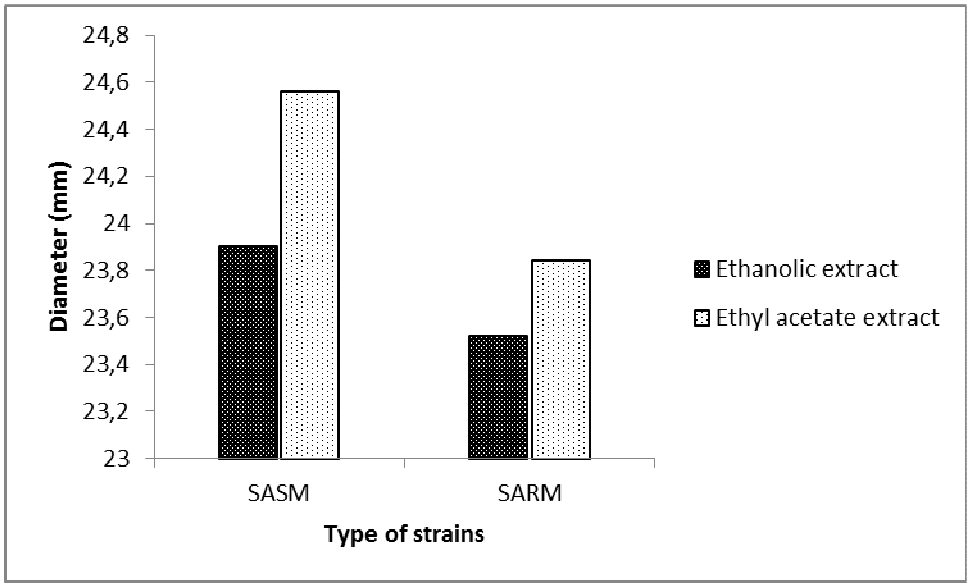

Fig 5. Average inhibitory diameters of MRSA and MSSA strains by C. micranthum extracts

Minimum Inhibitory Concentration of ethanolic and ethyl acetate extracts of *C. micranthum* on studied microorganisms.

The Minimum Inhibitory Concentrations (MIC) of studied extracts varied according to the strains and types of extracts (Table 4). These MIC variations were not statistically significant (p > 0.05). However, the MIC of ethyl acetate extract on *S. aureus* strains is greater (0.814 mg/ml). In addition, the reference strain *Streptococcus oralis* is very sensitive to both types of extracts (0.009 mg/ml). The largest MIC was observed on *S. aureus* isolated of from skin infections with values of 0.41 mg/ml (alcoholic extract) and 1.13 mg/ml (ethyl acetate extract). However, there is no statistically significant difference between these different concentrations (p > 0.05).

Micrococcus luteus, *Enterococcus foecalis* and *Candida albicans* are not sensitive to different extracts (ethanolic and ethyl acetate extract).

Table 4. Minimum Inhibitory Concentrations of ethanolic and ethyl acetate extracts of strains tested

STRAINS	Minimum Inhibitory Concentrations (mg/ml)	
	Ethanolic extract	Ethyl acetate extract
Staphylococcus aureus	0.406 ± 0.20*	1.125 ± 0.81*
Pseudomonas aeruginosa	0.312	0.625
Proteus miriabilis	0.312	0.312
Micrococcus luteus	-	-
Staphylococcus epidermidis	0.009	0.009
Proteus vulgaris	0.156	0.312
Streptococcus oralis	0.009	0.009
Enterococcus foecalis	-	-
Escherichia coli	0.312	0.625
Candida albicans	-	-

** Mean ±Standard Deviation*

Minimum Bactericidal Concentration of ethanolic and ethyl acetate extracts of *C. micranthum* on studied microorganisms.

Minimum Bactericidal Concentrations vary according to the strains and the type of extracts (Table 5). *Staphylococcus epidermidis* and *Streptococcus oralis* are more sensitive to both two extracts (MBC = 0.009 mg/ml) than others strains. The high MBC is observed with *S. aureus* which has an average of 0.406 ± 0.21 mg/ml (ethanolic extract) and 1.125 ± 0.81 mg/ml (ethyl acetate extract).

Table 5. Minimum Bactericidal Concentrations of ethanolic and ethyl acetate extracts of tested strains

Strains	Minimum bactericidal concentrations (mg/ml)	
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Staphylococcus aureus	0.406 ± 0.20*	1.125 ± 0.81*
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Proteus miriabilis	0.312	0.312
Micrococcus luteus	-	-
Staphylococcus epidermidis	0,009	0.009
Proteus vulgaris	0.156	0.312
Streptococcus oralis	0.009	0.009
Enterococcus foecalis	-	-
Escherichia coli	0.312	0.625
Candida albicans	-	-

** Mean ±Standard Deviation*

DISCUSSION

This study evaluates the antimicrobial activity of two extracts (ethanolic and ethyl acetate) of *C. micranthum* leaf used in beniness pharmacopoeia. About 76% of *S. aureus* strains isolated from the three skin infections (pus, furuncles and abscess) were resistant to methicillin. This proportion is higher than 36% obtained by [22] in Zou department of Benin. This difference can be explained by the fact that the work was carried out on a wider range of from other types of stem.

The ethanolic extract of *C. micranthum* leaf have a greater antimicrobial activity than ethyl acetate extract .This result corroborate those obtained by [23] who showed that the hydro- alcoholic extract of *Terminalia glaucescens* was better than other extracts to inhibit *in vitro* growth of several bacterial strains . We can be thus concluded that the ethanol better concentrate the antibacterial compounds contained in the leaves of the plant than ethyl acetate solvent. According to used extraction protocol, the ethyl acetate fraction was derivated from ethanolic extract. It is therefore possible that one finds in this extract active compounds that are not found in ethyl acetate fraction. The inhibitory activity was same both sensitive strains that resistant strains to Methicillin (Fig 4). This result differs from that obtained by [23]with *T. glaucescens* extracts on *Salmonella* typhi and *S. typhimurium* strains. Indeed, the previous authors shown that the ethyl acetate extract was the most active compared to the other extracts. Then the antimicrobial capacity of one extract varied according to types of solvent (affinity of metabolites to extract).

The average inhibition diameters are different ($p < 0.0001$) and vary from 15 ± 7.1 mm (*Proteus vulgaris*) to 25 mm (*Pseudomonas aeruginosa*) at the same concentration (20 mg/ml). The extracts of *C. micranthum* are more active on *P. aeruginosa*, we note that the extracts of this plant are active both on Gram + than Gram - bacteria. These two types of *C. micranthum* extracts have not the capacity to inhibit *Candida albicans* yeast growth. [24] showed that soothing infectious diarrhea in Africa can be explained by the antimicrobial effect of extracts *C. micranthum* on many germs found by *in vitro* studies. Similarly, [25] demonstrated that *C. micrathum* anti-inflammatory properties justifying its use in diarrheal infections by traditional practitioners.

Phytochemical screening of *C. micranthum* leaves indicates the presence of several compounds such as alkaloids, steroids, polyphenolic compounds (Tannins, Flavonoids, ...), Mucilages and Anthracene derivatives. It has been shown that the polyphenol plant compounds have antibacterial activity regarding the observation made by [26] in his study of the antioxidant and antibacterial activity of leaf *C. micranthum* extracts. The presence of these compounds that would justify the antimicrobial activity was observed. Indeed, there is evidence that flavonoids, tannins, steroids and alkaloids have antimicrobial activity [27].

CONCLUSION

This preliminary study shown the antimicrobial properties of *C. micranthum* leaves extracts (ethanolic and ethyl acetate). These results justify some ethno-pharmacological uses of these plants. This plant can be used to treat the infectious diseases. The phytochemical screening of *C. micrathum* leaves showed the presence of chemical compounds which have antimicrobial activities. It would therefore be interesting to carry out toxicity and anti-inflammation studies of purified extracts of *C. micranthum* in order to develop traditional drugs.

Acknowledgement
The authors thank the government of Benin for financial support

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