



***In-vitro* Antibacterial Screening of Methanol Extracts of three Combretum species against seven strains of Methicillin Resistant *Staphylococcus Aureus* (MRSA)**

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Medicinal plants are valued as sources of natural compounds some of which are effective against several infectious diseases. Plants from the genus *Combretum* have been used traditionally in various African societies to treat variety of medical problems especially infectious diseases.

Objective: In this study, we investigated the antibacterial activity of the methanol extracts from the leaves of three *Combretum* species namely *Combretum hispidum*, *Combretum racemosum* and *Combretum platypterum* against seven strains of methicillin resistant *Staphylococcus aureus* (MRSA) *in vitro*.

Materials and methods: Methanol extract from *Combretum hispidum*, *Combretum racemosum* and *Combretum platypterum* were subjected to agar diffusion assay and broth micro-dilution test for the determination of antibacterial activity and minimum inhibitory concentration (MIC), all the extracts were tested against eight different strain of MRSA. Imipenem and ciprofloxacin were used as control.

Results: Extract from *Combretum racemosum* leaves showed significant anti-MRSA activity with zone of inhibition as high as 37 mm and MIC values ranging from 0.16 - 1.25 mg/mL on all tested strains of MRSA. Extracts from the two other species did not exhibit any activity on all tested strain.

Conclusion: Methanol extract from *C. racemosum* is highly effective against MRSA and it could be a potential source of newer antimicrobial agent against MRSA infections

Keywords: *Combretum* species, Methicillin resistance *Staphylococcus aureus*, Anti-bacteria

INTRODUCTION

The extraordinary genetic diversity in the resistance genes harbored by microbes have benefited from overuse of antibiotics by animal and man. This have resulted in increased selection pressure for resistance genes and their transmission among microbes, which in turn aided the development of multiple mechanisms of resistance against all antibiotics introduced, To evade the continuous therapeutic failure of the current antibiotics, there is need for more creative approach to the discovery of new antibiotics, thus the search for unique antimicrobial agent has re-energized scientific interest in natural products with antimicrobial

properties (Motamedi *et al.*, 2010; Chattopadhyay *et al.*, 2009).

Staphylococcus aureus has a close association with mankind as it is carried as a nasal commensal in 30% of the population, and its presence has long been linked to common skin infections such as boils (Schito, 2006). However, one of the most notorious drug resistant bacteria is Methicillin Resistant *Staphylococcus aureus* (MRSA); first recognized in the 60s as a multi-resistant strain that has shown increased resistance to different classes of antimicrobials (Enright *et al.*, 2002). Methicillin resistance *Staphylococcus aureus* is one of the leading causes of skin, soft tissue, bone, joint,

abscess, and normal heart valve infections (Barber, 1961; Ness 2010; Abouzeed *et al.*, 2013). The gross antibiotic resistance status of MRSA necessitates the search for other effective antimicrobial agents against the pathogen.

Plants as natural products provide challenging alternatives with respect to the nature of their chemical constituents, biosynthetic pathways, evolution, and biochemical mode of action (Abouzeed *et al.*, 2013; Strohl, 1997). Plant based antimicrobials represent a vast untapped source for medicines with enormous therapeutic potentials. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Borris, 1996). Phytomedicines usually have multiple effects on the body; their actions often act beyond the symptomatic treatment of disease. An example of this is *Hydrastis canadensis* (Family [Ranunculaceae](#)). *Hydrastis* not only possesses antimicrobial activity, but also increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds (Iwu, 1999).

Medicinal plants from the genus *Combretum* have been shown to demonstrate a wide range of biological activity which includes enzymatic, antiparasitic, antimalarial, larvicidal and antimicrobial activity (Olukoya *et al.*, 1993; McGaw *et al.*, 2001; Ancolio *et al.*, 2002; Odda *et al.*, 2008). They have also been indicated as recipes/or part of recipe for the traditional treatment of various diseases. (Ajaiyeoba *et al.*, 2006; Ogbole *et al.*, 2010) Several investigations into the antimicrobial activity have confirmed the broad spectrum antimicrobial activity of the genus *Combretum* (Alexander *et al.*, 1992; Martini *et al.*, 2004; Grønhaug *et al.*, 2008). A review of the antimicrobial activity of the genus indicated thirty-six species of the genus *Combretum* have been tested and confirmed for various antimicrobial activities (De Morais Lima *et al.*, 2012)

In view of the fact that *Combretum* species has demonstrated broad spectrum of activities against many pathogenic organism and in continuation of our study on the anti-MRSA activity of Nigerian medicinal plants (Ogbole *et al.*, 2011), we investigated the antimicrobial effect of three Nigerian *Combretum* species; *Combretum hispidum*. Laws *Combretum platypterum* (Welw.) Hutch. and Dalz., *Combretum racemosum* P. Beauv (family Combretaceae), on clinical isolates of Methicillin Resistant *Staphylococcus aureus*.

MATERIALS AND METHODS

Plant Collection

Fresh leaves of *Combretum racemosum*, *Combretum platypterum* and *Combretum hispidum*,

were collected from the Botanical garden, University of Ibadan at the onset of raining season (April/May, 2013). The plants were identified and authenticated by Mr. O. Osiyemi at the herbarium of Forestry and Research Institute of Nigeria (FRIN), Ibadan and assigned voucher specimen numbers. *Combretum racemosum* (109988), *Combretum platypterum* (109989) and *Combretum hispidum* (109991)

Preparation and extraction of Plant Material

Each plant material was shade-dried at room temperature (27-33°C) and pulverized. The dried powdered materials were extracted by maceration into methanol for 72 h and then filtered to obtain crude methanol extracts of each plant. The filtrate was concentrated with a rotary evaporator. The dry extract was kept in a refrigerator until needed.

Bacterial strains

The seven *S. aureus* strains used in this study were confirmed clinical isolates from Department of Pharmaceutical Microbiology, University of Ibadan. These *S. aureus* strains have been previously phenotypically identified as MRSA solely through resistance to ceftiofloxacin, (Ayeni *et al.*, 2014). The control MRSA strain has been completely identified by phenotypic resistance to ceftiofloxacin, growth with green colouration on chromIDTM MRSA plate (bioMérieux, France), presence of Penicillin Binding Protein-2 (PBP-2) with Slidex MRSA Detection Kit (bioMérieux, France) and detection of *mecA* gene by PCR. All the bacterial strains were grown and maintained on nutrient agar slants.

Anti-MRSA Susceptibility Assays

Agar diffusion assay

The antimicrobial activities of each of the extracts were determined using agar well diffusion methods of Stoke and Ridgway (Stoke and Ridgway, 1980). Each crude extract was diluted serially to obtain a concentration range of 200 - 12.5mg/mL. Bacterial strains were cultured overnight (24 hours) at 37°C on nutrient broth. MRSA cell suspensions adjusted to 0.5 McFarland standards (10^8 CFU/ml) were prepared. One hundred microliter (100µL) of bacteria culture was seeded into 15 mL of melted and cooled Muller Hinton agar (MHA) (Oxoid, UK) and poured into sterile Petri-dishes after mixing thoroughly; the agar was then allowed to set. Equidistant wells were then bored into the solidified agar using a sterilized cork-borer of 8 mm diameter.

For each of the extracts, 100µL of serially diluted extracts was placed in each of the wells in the inoculated agar and allowed to diffuse for 2 hrs. All plates were incubated at 37°C for 24 hours, and the resulting zones of inhibition were measured. This

experiment was carried out in triplicate and the antimicrobial activity was expressed as the mean zones of inhibition diameters (mm) produced by the plant extracts. As positive controls, discs (Oxoid) containing Ciprofloxacin 50 µg and Imipenem 10 µg were used as positive control for all bacterial strains.

Determination of Minimum inhibitory concentration

The Minimum inhibitory concentration (MIC) of the active extract, *Combretum racemosum* was determined by the agar-dilution method. Serial dilution of the extract was made to obtain a concentration range of 3.125 to 200 mg/mL. One millimeter of extract from each dilution was mixed with 19 mL of molten agar to obtain a final concentration of 0.16 to 10.52 mg/mL and poured into sterile Petri dishes allowing the agar to set. The surface of the agar was allowed to dry before streaking with overnight culture of susceptible organisms (10^8 CFU/ml). The plates were incubated in inverted position for 24 hours at 37°C and examined for the presence or absence of growth. The lowest concentration preventing visible growth of the organisms was taken as the minimum inhibitory concentration of the extract.

RESULTS AND DISCUSSION

The increasing occurrence of *Staphylococcus aureus* resistant to a wide range of antimicrobial agents, including the beta-lactams, has made antibiotic treatment more difficult. The discovery of new antimicrobial agent is essential to overcome the increasing levels of drug resistance by infectious microorganisms. There is also the problem of insufficient number of effective antibiotics against diverse bacterial species (Yu et al., 2010; Song, 2008).

This present study investigated the anti-MRSA activity of three *Combretum* species commonly used in the traditional medical practice in Nigeria. Analysis of the growth inhibitory activity by the agar diffusion method showed that only one of the three species screened, *Combretum racemosum* had inhibitory activity against all the clinical strains of MRSA, the other species; *C. hispidum* and *C. platypterum* were not active. Variations in phytochemical composition of the *Combretum* species could be responsible for this observed difference in activity. Masoko and Eloff has stated earlier that there are differences in the activities of *Combretum* species even within the same Section (Masoko and Eloff, 2006; 2007).

Combretum racemosum had a profound effect on *S. aureus* FAA001 (Control organism), the diameter of zone of inhibition against this organism at the least tested concentration in the agar diffusion assay was 28 mm (Table 1), with a minimum inhibitory concentration of 0.16 mg/mL (160 µg/mL), which make its activity comparable to the control (ciprofloxacin disc 50 µg), with a ZI of 20mm against the same organism (Tables 1 and 2). Both Isolate 2 and 3 (FAA002 and FAA003) also responded quite well to the leave extract of *Combretum racemosum*, with MIC of 0.31 mg/mL and 0.63mg /mL respectively; the extract exhibited MIC of 1.25 mg/mL against isolates FAA004, FAA005, FAA006, FAA007 and FAA008. This is an indication that all the isolates were sensitive to *C. racemosum*, though at different degrees.

Extract from *C. racemosum* leaves have been used in traditional medicine for treating various ailments such as inflammations, ulcers, helminthic infections, trypanosome infections, and bacterial infections of the genitourinary and gastrointestinal systems (de Moraes Lima et al., 2012; Abreu et al., 1999; Okwuosa et al., 2006; Onocha et al., 2005)

In previous studies, *C. racemosum* has demonstrated antimicrobial activity against pathogenic microorganisms such as *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and antifungal activities against *C. albicans* and *Trichophyton rubrum* with MIC of methanol extract of *C. racemosum* yielding values from 2.5-10 mg/mL (Ajibesin, et al., 2002). Another study reported that *C. racemosum* showed significant activities against the local and standard strains of *S. aureus* (MIC 25-50 mg/ml), *Shigella dysenteriae* (MIC 25-50 mg/mL), *E. coli* (MIC 25 mg/mL) and *Salmonella paratyphi* (MIC 25 mg/mL) (Sowemimo and Idika, 2007). Various authors have reported antibacterial activities and the occurrence of antibacterial compounds in different *Combretum* species (Malcolm and Sofowora, 1969; Eloff, 1998). For example, combrestatin B5, an antimicrobial stilbene have been reported to be from the leaves of *C. woodii* (Eloff et al., 2005). Antibacterial flavonoids from *C. erythrophyllum* leaves namely Genkwanin, 5-hydroxy-7,4-dimethoxyflavone, rhamnocitrin, quercetin-5,3-dimethylether showed good activity against a wide range of

Table 1. Anti-Methicillin Resistant Staphylococcus aureus activity of *Combretum racemosum*, *Combretum hispidum* and *Combretum platypterum*

Plant Extract	Concentration (mg/ml)	Mean Diameter Zone of inhibition ± S.D of Clinical Isolates (mm)							
		FAA001 (Control)	FAA002	FAA003	FAA004	FAA005	FAA006	FAA007	FAA008
<i>Combretum racemosum</i>	12.5	28 ± 0.71	18± 1.41	11± 1.41	12± 0.71	12± 0.00	12± 1.41	11± 1.33	11± 1.41
	25	29 ± 1.41	21± 1.41	12± 0.00	13± 0.00	13± 1.41	14± 0.00	13± 0.00	12± 1.41
	50	30 ± 1.41	22± 0.00	16± 1.41	15± 0.00	16± 0.00	15± 0.00	18± 0.71	14 ± 1.41
	100	33 ± 0.71	23± 1.41	17± 1.41	16± 0.00	17± 0.00	20± 0.00	20± 0.00	15± 0.00
	200	37± 0.00	27± 1.41	20± 0.00	20± 0.00	19± 0.00	25± 0.00	22± 0.00	17± 0.00
<i>Combretum hispidum</i>	12.5	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-
<i>Combretum hispidum</i>	12.5	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-
Imipenem	10 µg/ml	22 ± 0.00	25 ± 0.00	24 ± 1.41	22 ± 0.00	22 ± 0.00	25 ± 0.71	22 ± 0.00	22 ± 0.00
Ciprofloxacin	50 µg/ml	20 ± 0.71	25 ± 0.00	25 ± 0.00	17 ± 1.41	23 ± 0.71	20 ± 0.00	20 ± 0.00	17 ± 0.00

Key: - = not active

TABLE 2: Minimum inhibitory concentration (MIC) values of *Combretum racemosum* on test organisms

Test organisms	MIC value (mg/ml)
<i>S. aureus</i> FAA001	0.16
<i>S. aureus</i> FAA002	0.31
<i>S. aureus</i> FAA003	0.63
<i>S. aureus</i> FAA004	1.25
<i>S. aureus</i> FAA005	1.25
<i>S. aureus</i> FAA006	1.25
<i>S. aureus</i> FAA007	1.25
<i>S. aureus</i> FAA008	1.25

microorganisms such as *Micrococcus luteus*, *Shigella sonnei*, *Vibrio cholerae*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Martini *et al.*, 2004). These results are part of the justification of the ethnobotanical use of various *Combretum* species for diseases of bacterial origin.

The absence of Anti-MRSA activity in the other plant extracts; *Combretum platypterum* and *Combretum hispidum* in this study does not preclude the fact that they have antimicrobial activity. For instance, *C. hispidum* has been shown to have antifungal activity against *C. albicans*, and other pathogenic fungi with MIC less than 4.0 mg/mL (Baba-Moussa *et al.*, 1999)

Methicillin Resistant *Staphylococcus aureus* infection is very difficult to treat, it is therefore not surprising that despite the widely established activities of various *Combretum* species, only the crude extract of *C. racemosum* exhibited significant activity on 8 different strains of MRSA. Efforts are being made to identify the specific compound/s responsible for this anti-MRSA activity.

CONCLUSION

It can be concluded that only the crude methanol extracts of *Combretum racemosum* amongst the species tested have significant anti-MRSA activity.

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