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Antimicrobial Properties of Velvet Bush Willow (*Combretum molle*) Crude Bark Extracts on Selected Bacteria Species

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Abstract: *Treatment and control of infectious diseases in humans and animals play a vital role in prevention of illness and death. Chemotherapy treatment usually provides effective therapy for treatment of pathogens (e.g., bacterial, fungal and protozoan infections); However, the development of resistant pathogens which do not respond to chemotherapy treatment, result in prolonged illness and in some cases ending up with death. Plant based components are used as natural antimicrobials to treat bacterial, fungal, protozoan and other infections. For cultural and economic reasons, medicinal plants constitute the major part of traditional medicine. In recent years, numerous African medicinal plants have been screened for their medicinal potential. This study was carried out to assess the antimicrobial activity of crude Combretum molle bark extracts against three selected bacteria species. Three solvents, distilled water, ethanol and acetone were used for extraction. The agar well diffusion technique was used to screen for antimicrobial activity of crude C. molle bark extracts against Streptococcus pyogenes, Pseudomonas aeruginosa and Escherichia coli; minimum inhibition concentration (MIC) of the most active extracts was determined by the broth dilution method. All the extracts tested demonstrated antimicrobial activity with zone of inhibition diameters ranging from 14 to 24 mm. Acetone was the most potent extract with its minimum inhibitory concentration (MIC) ranging from 1.25 to 2.50 mg/ml. There was no statistically significant difference (P>0.05) in the potency of the three extracts (distilled water, 95% ethanol and 95% acetone) and antibiotic (ciprofloxacin) on the different bacteria species tested. The study revealed that the crude bark extract of C. molle showed antimicrobial activity against all the test microorganisms.*

Keywords: *Combretum molle crude bark extract, Streptococcus pyogenes, Pseudomonas aeruginosa Escherichia coli; antimicrobial activity.*

List of Abbreviations Used

MIC- Minimum Inhibition Concentration

WHO- World Health Organization

MCA- McConkey Agar

BA – Blood Agar

CFU- Colony Forming Unit

1. Introduction

People all over the world have been using plants as medicine from time as far back as immemorial. It is estimated by WHO (World Health Organization), that 80% of the population, majority in developing countries, rely on plant-based medicines for primary health care (Korir *et al.*, 2012).For centuries, African have treated different disease conditions including inflammatory diseases, cancer, infertility, skin conditions, gastrointestinal parasites using medicinal plants. The truth is that African traditional medicine is usually the first contact in meeting the primary health care in Africa and is related to its affordability, accessibility, cultural and spiritual acceptance, and knowledge of its preparation and use (Mariyo 2013; Abdullah 2011).The potentials of plant-derived compounds from African medicinal plants in treatment and management of disease conditions is growing rapidly in Africa even among educated African urban dwellers. Herbal drugs are prepared from various parts of the plant such as leaves, root, stem, seeds, tubers or exudates (Mukherjee, 2002). In Africa the ethno-pharmacological and botanical knowledge on the uses of medicinal plants is often

orally passed by herbalists down from generation to generation. However, this abundance of information is in danger of disappearing since it is often kept secret until the last minutes of death of the traditional healers when they eventually call on somebody to inherit the information (PiaFyhrquist, 2007). Higher plants are currently poorly exploited as sources of new drugs (Hostettman & Terreaux, 2000). There are several ways in selecting plant materials when searching for new medicinal plants/active compounds. Ethno-pharmacological information on medicinal plants is often of substantial importance for the finding of new potential medicinal plants/new ways of using an already known plant. It has been estimated that 74 % of the pharmacologically active plant-derived components were discovered after the ethno-medical uses of the plants started to be investigated (Farnsworth & Soejarto, 1991; Wood-Sheldon *et al.,* 1997). Another important way of discovering new medicinal plants is the phylogenetic approach in which a number of highly related species of plants assumed to contain related chemical compounds (chemotaxonomy), are screened for their biological effects (Cotton, 1996; Vuorela *et al.*, 2004).

There are more than 1340 plant species known to be potential source of antimicrobial compounds but only few of them have been systematically studied scientifically (Wilkins and Board, 1989). Among the plants used by most herbalists as traditional medicines is *Combretum molle* (velvet bush willow). The plant belongs to the family *combretaceae* and it is found in almost all tropical countries including Tanzania. All parts of this plant (leaves, roots, seeds or stem barks) are used to treat bacterial, parasitic and fungal infections. The parts of *C. molle* are used for variety of treatments such as for hookworm, stomach pains, snake bite, leprosy, fever, dysentery, general body swellings, abortion, wound dressing, gargling, abdominal oedema, sterility, constipation as well as in child delivery (PiaFyhrquist, 2007*).* It is a shrub or small tree up to 10m high, rarely to 16m, with a straight regular bole to 1m girth, of savannah forest and wide spread in tropical Africa. The stems are durable underground and are valuable for house posts. The wood is brownish or yellowish green, very hard and compact, strong and durable, but difficult to work. In some African countries such as Sudan a leafy and stem bark decotin is taken to treat fever, jaundice and bacterial infection. Aside of the use of the bark for tanning, its extract (stem bark), and leaves possessed significant activity against chloroquine sensitive plasmodium falciparum strain NF54 and leaf extract also totally inhibited the enzyme HIV 1 reverse transcriptase (Maroyi *et al.*, 2018)

This study was carried out to assess the antimicrobial activity of *Combretum molle* crude bark extract by using three different solvents in three selected bacteria species. These are *Streptococcus pyogenes, Pseudomonas aeruginosa* which are gram positive and *Escherichia coli* which is gram negative. *Streptococcus pyogenes* belongs to Gram-positive, non-spore-forming cocci about 0.5-1.2µm in size. The bacteria in this group often grow in pairs or chains and are oxidase and catalase negative. *S. pyogenes* colonizes the upper respiratory tract of 5-15% of normal individuals (Nyenje, 2011). The most common forms of *S. Pyogenes* disease include respiratory and skin infections.Cases of skin and soft tissue infections caused by mult- drug resistant bacteria such as methicillin resistant staphylococcus aureus are sources of ever increasing death toll (Kulher *et al*, 2006) As normal flora, *S. pyogenes* can cause infection when immunity is compromised or when the organisms are able to penetrate the constitutive immune system (Todar, 2005). *Pseudomonas aeruginosa* are oxidase-positive, Gram-negative, aerobic rod that belongs to the family Pseudomondaceae. The genus *Pseudomonas* comprises of more than 140 species, only few of these are pathogenic to man and plants. The others are essentially saprophytic and occur widely in nature (Adedeji *et al*., 2007). Although classified as aerobic organisms, *aeruginosa* is considered by many as a facultative anaerobe as it thrives not only in normal atmosphere, but also with little oxygen; and has thus colonized many natural and artificial environments. *P. aeruginosa*, a major nosocomial pathogen, is also responsible for community-acquired infections, generally associated with contaminated water and solutions (folliculitis, otitis and corneal ulcers) (Dubois *et al*., 2008).

P. aeruginosa is notorious for its resistance to the conventional antibiotics and is therefore, a dangerous and life-threatening pathogen. The bacterium is naturally resistant to many antibiotics such as aminoglycosides (gentamicin, amikacin, and tobramycin), quinolones (ciprofloxacin, levofloxacin), cephalosporins (ceftazidime, cefepime, and cefoperazone), carbapenems (imipenem, meropenem) and polmyxins (polymyxin B and colistin) due to the permeability barrier afforded by its Gram-negative outer membrane and also its tendency to colonize surfaces in a biofilm form makes the cells

impervious to therapeutic concentrations of antibiotics; also its natural habitat in the soil, living in association with the bacilli, actinomycetes and moulds (Nyenje, 2011).

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. Coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination (Vogt and Dippold, 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2 and by preventing the establishment of pathogenic bacteria within the intestine (Hudault *et al.*, 2001). Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. Faecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. In rare cases, virulent strains are also responsible for haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia and Gram-negative pneumonia (Todar, K., 2007). *E. coli* is currently resistant to various conventional drugs including ampicillin, sulfonamide and tetracycline.

2. METHODOLOGY

2.1. Study Area

This study was conducted in Morogoro region. The fresh stem barks of *Combretum molle* were collected from the bushes at Doma village along the Morogoro-Iringa highway; in Mvomero district, and further processes were carried out at the department of veterinary microbiology, parasitology and biotechnology, college of veterinary medicine and biomedical sciences, Sokoine University of agriculture.

2.2. Study Design

This was a laboratory-based experimental study where by the pure strains of *Streptococcus pyogenes, Escherichia coli* and *Pseudomonas aeruginosa* were subjected to the crude extracts of *C.molle* stem barks for antimicrobial activity test.

2.3. Plant Material Preparation and Extraction

The stem barks of *Combretum molle* were harvested from bushes at Doma village in Mvomero district and transported to Sokoine University of agriculture. The barks were washed and dried at room temperature for two weeks and then ground mechanically to fine powder. Crude extracts were obtained by using two technical grade extraction solvents which are acetone and ethanol; and water as universal solvent. 50 g of the plant powder material were soaked in 150 ml of each extraction solvent (distilled water, 95% ethanol and 95% acetone) for 72 hours with intermittent shaking. The extracts were filtered by using Whatman filter paper No.1.The extracts were concentrated by evaporating the solvents using a water bath at 80^{o}C for 1-2 hours. The yielded extracts were stored in labelled tight lid containers for further bioassays.

2.4. Bacteria Strains

Pure strains of *Streptococcus pyogenes, Escherichia coli* and *Pseudomonas aeruginosa* were obtained from preserved bacterial strains in microbiology laboratory in the department of veterinary microbiology, parasitology and biotechnology, Sokoine University of agriculture. *Escherichia coli* and *Pseudomonas aeruginosa* were sub-cultured on McConkey agar (MCA) and *Streptococcus pyogenes* on blood agar (BA). They were all incubated at 37^{0}C for 24 hours. The bacterial species were identified macroscopically and microscopically (bacterial shapes and the gram stain reaction) respectively. Furthermore, biochemical tests were performed to confirm their identities.

2.5. Antibacterial Sensitivity Test

Agar well diffusion method was used for sensitivity test along with ciprofloxacin as a positive control. Inocula of pure bacterial strains were prepared from subcultures of bacteria as follows; four to five colonies of the isolates were emulsified in sterile normal saline and turbidity adjusted to $1x10^{9}$ CFU/ml (0.5 McFarland standard). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the Mueller Hinton agar plates. 6mm wells were made on the inoculated Mueller Hinton agar by using a sterile cork borer. The wells were filled with 50µl of 50mg/ml and the plates were left for 30 minutes before incubation (for the extracts to diffuse). The plates were incubated at 37^{0}C for 24 hours. The antimicrobial activity of the plant extracts were observed by measuring the inhibition zones in mm.

2.6. Determination of Minimum Inhibitory Concentration (MIC)

Nine (9) test tubes containing nutrient broth were used to determine minimum inhibitory concentration of the crude extract. A 2-fold serial dilution of the extract was made with 160mg/ml in test tube 1 and 1.25mg/ml in test tube 8 while in test tube 9 sterile 95% acetone was used as a negative control. The test tubes were inoculated with the standardized bacterial suspensions and incubated at 37^{o}C for 24 hours. MIC was determined from lowest concentration showing no bacterial growth. After 24 hours, inhibition or growth of bacteria was confirmed by transferring a loopful of bacterial suspension from each test tube and inoculating into separate solid culture media. The inoculated culture plates were incubated at 37^{0}C for 24 hours.

2.7. Data analysis

Data analysis was done by using Microsoft excel® spread sheet for descriptive statistics, statistical significance in the mean differences of each extract based on the zone of inhibition was determined by using Chi-square test.

3. RESULTS

3.1. Antimicrobial Activity

Results obtained revealed that crude bark extracts of *C. Molle* showed antimicrobial activity against all bacterial species tested. *S. pyogenes* was the most sensitive to all extracts with mean zone of inhibition of 22.22±4.02 mm ranging from 18.00±2.00 to 26.00±2.00 mm. On the other hand, *E.coli* was the least sensitive to all extracts with mean zone of inhibition of 19.00±3.00 mm ranging from 15.67±2.52 to 20.00±2.00 mm. Acetone extract was the most effective against all the test microorganisms with mean zone of inhibition of 23.11±1.33 mm ranging from 21.33±3.10 to 26.00±4.02 mm and aqueous extract being the least effective with mean zone of inhibition of 16.56±2.52 mm ranging from 15.67±2.52 to 18.00±2.00 mm. There was no statistical significance difference between the antimicrobial activity of the extracts and the positive control antibiotics at $p< 0.05$.

3.2. Minimum Inhibitory Concentration (MIC)

All plates inoculated by bacterial suspensions from test tube 1 to 6 showed no bacterial growth for all test bacterial species. *Escherichia coli* showed growth in test tube 7, 8 and 9 while *Streptococcus pyogenes* and *Pseudomonas aeruginosa* showed growth in test tube 8 and 9 implying that the MIC of the extract was 2.5mg/ml for *E. coli* and 1.25mg/ml for both *P. aeruginosa* and *S. pyogenes*.

Table1. *Average inhibition zones (in mm) of the three extracts against the test organisms*

Test organisms	Positive control	Aqueous extract	Acetone extract	Ethanol extract
P. aeruginosa	20.33±1.53	16.00±1.00	22.00±1.00	21.00±1.15
E. coli	32.67±2.08	15.67±2.52	21.33±2.52	20.00±2.00
S. pyogenes	23.67±1.15	18.00±2.00	26.00±2.00	22.22±3.10

Table1. *Average inhibition zones (in mm) of each test organism to show mean sensitivity of each test organism to all extracts*

Test organism	Average inhibition
aeruginosa	19.78±3.29
E. coli	19.00±3.00
S. pyogenes	22.22±4.02

Table2. *Average inhibition zones (in mm) of each extract to show effectiveness of each extraction solvent*

Extraction solvents	Average inhibition zones
Distilled Water	16.56±2.52
Acetone	23.11±1.33
Ethanol	21.33±168

Table3. *Mimimum inhibittory concetration of acetone extract against the test organisms*

Test organisms	Extract concentration in mg/ml in test tubes 1-8 and 95% acetone in test tube 9								
	160 1	80 2	40 3	20 4	10 5	5 6	2.5 7	1.25 8	95% aetone 9
p. aeruginosa	-	-	-	-	-	-	-	+	+
E. coli	-	-	-	-	-	-	+	+	+
P. pyogenes	-	-	-	-	-	-	-	+	+

Key: - = no bacterial growth, + = bacterial growth

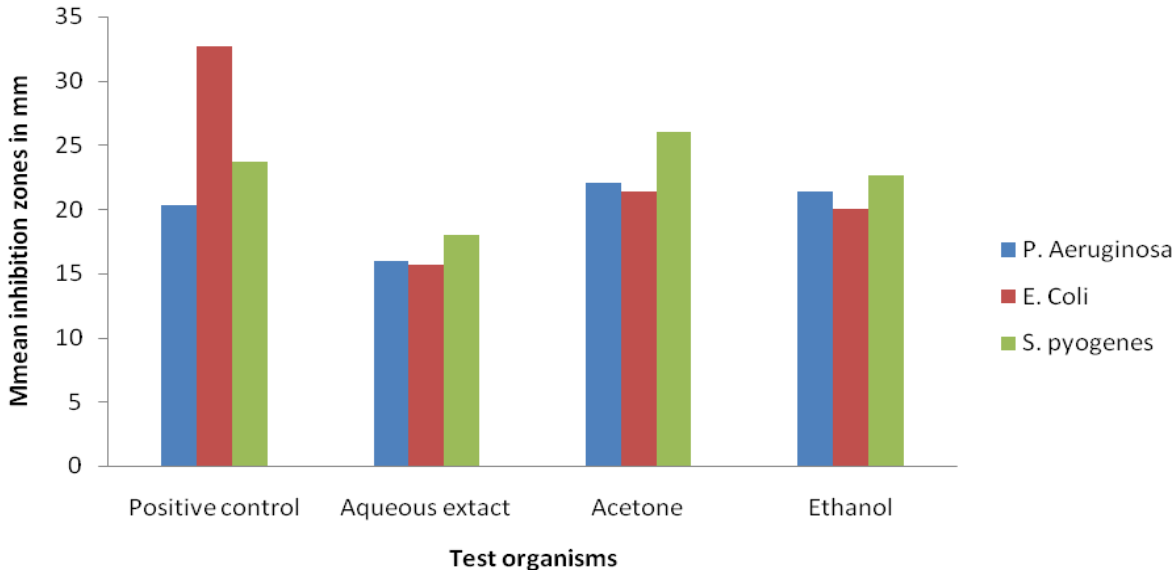

Figure1. *Average inhibition zone of the three extracts against the test organisms*

4. DISCUSION

The increasing resistance to conventional antibiotics by microorganisms has necessitated the search for new, efficient and cost effective ways for the control of infectious diseases (Samie *et al*., 2005; Ndip *et al*., 2007). Previous studies have reported the antimicrobial activity of *C. molle* against bacteria, fungi and helminths (Asres *et.al.* 2001; Eloff *et al*., 2005; Ojewole, 2008). Although the phytochemical constituents of the stem bark of *C. Molle* are known, the exact bioactivity against these test organisms has not been established. This study assessed the antimicrobial activity of crude bark extracts of *C. Molle* against *P. aeruginosa*, *E. coli* and *S. pyogenes*. It was observed that acetone was good extractant. The findings correlate with previous studies by (Asres *et al.*, 2001; Eloff *et al.*, 2005; Masoko *et al.*, 2006) who also found that acetone has potential to extract more compounds of *Combretum* species. All the three extracts tested showed varying degree of antibacterial activity against the test bacterial species. The antibacterial activities of acetone and ethanol extracts were comparable to that of standard antibiotic (ciprofloxacin) at $p < 0.05$. Acetone extract showed minimum inhibitory concentration (MIC) against the test organisms ranging from 1.25 to 2.5mg/ml. This finding are comparable to the earlier study of Nyenje (2011); who found that acetone extract had minimum inhibitory concentration (MIC) ranging from 0.078 to 2.5mg/ml.

The weak activities demonstrated by some of these extracts *in vitro*, does not necessarily imply that they would demonstrate weak activities *in vivo* because of immuno-modulation of chemical compounds from medicinal plants which has been proven to be inactive or weakly active *in vitro*. Also, as with some drugs, some of these plant extracts may be more potent *in vivo* due to metabolic transformation of their components into highly active intermediates (Ngemenya *et al*., 2006; Ndip *et al*., 2009).

5. CONCLUSION

From this study, therefore it can be concluded that *C. molle* bark extracts have antimicrobial activity against the bacterial species tested. Also, acetone crude extract of the stem bark of *C. molle* demonstrated good antimicrobial activity against (*aeruginosa, S. Pyogenes* and *E. coli*). Furthermore, the active compounds of *C. molle* are non-volatile.

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