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Full Length Research Paper

In-vitro antimicrobial activity of crude acetone extract of the stem bark of *Combretum molle* against selected bacterial pathogens

Nyenje M.[1] and Ndip R. N.[1,2]*

[1]Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Fort Hare, PMB X1314, Alice 5700, South Africa.
[2]Department of Biochemistry and Microbiology, Faculty of Science, University of Buea, Box 63, Buea, Cameroon.

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Drug resistance by clinically important pathogens is now a worldwide problem with far-reaching consequences especially considering that the emergence of drug resistance is now outpacing the development of new drugs. Plants have been used for many generations for healing purposes, and screening of extracts of these plants has often yielded positive outcomes. This study was aimed at evaluating the antibacterial potential of the stem bark of *Combretum molle,* in a bid to identify potential sources of cheap starting materials for the synthesis of new drugs to address the growing antimicrobial resistance. Various solvents were used for plant extraction. The agar well diffusion technique was used to screen for antimicrobial activity of the solvent extracts against *Helicobacter pylori* PE 252C, *Streptococcus pyogenes* ATCC 49399, *Pseudomonas aeruginosa* ATCC 15442 and *Plesiomonas shigelloides* ATCC 51903. Minimum inhibitory concentration (MIC_{50}) of the most active extracts was determined by the broth dilution method. Fisher's exact test indicated a high antimicrobial activity with zones of inhibition ranging from 0 to 32 mm. Acetone was the most potent extract with its MIC_{50} ranging from 0.078 to 5.0 mg/ml. There was no statistically significant difference ($P>0.05$) in the potency of four extracts (acetone, methanol, ethanol and ethyl acetate) and antibiotic (ciprofloxacin), which served as positive control. Therefore, the acetone extract of *C. molle* contain therapeutically useful compounds, justifying the use of the plant in traditional medicine.

Key words: *Combretum molle*, medicinal plants, antimicrobial activity, minimum inhibitory concentration.

INTRODUCTION

The worldwide problem of antibiotic resistance impacts negatively on antibiotic therapy thus making successful empiric therapy much more difficult to achieve. The emergence of drug resistance is an evolutionary process that is based on selection for organisms that have an enhanced ability to survive and reproduce in the presence of a drug (Suleiman et al., 2010). Despite the existence of conventional antimicrobial agents, resistant or multi-resistant strains of pathogenic microorganisms are continuously appearing; unfortunately, the discovery and development of newer antibiotics have not kept pace with the emergence of antibiotic resistance (Gould, 2009). This therefore imposes the need to search for new effective drugs to circumvent the problem.

Streptococcus pyogenes and *Pseudomonas aeruginosa* are Gram-positive and Gram-negative organisms respectively that cause respiratory, cutaneous, and nosocomial infections, while *Helicobacter pylori* and *Plesiomonas shigelloides* which cause gastrointestinal infections have all been reported to be resistant to virtually all of the older antibiotics (Eloff et al., 2005). This leads to severe consequences ranging from failure of patients to respond to therapy and the need for expensive and/or toxic alternative drugs to the social cost of higher morbidity and mortality rates as well as the longer duration of hospitalization (Basht et al., 2009). The situation is worse in poor countries, where many of

*Corresponding author. E-mail: rndip@ufh.ac.za, ndip3@yahoo.com. Tel: +27 782696191. Fax: +27 86624759.

the second or third line therapies for drug resistant infections are not available, making the potential of resistance to first line antibiotics considerably greater. This reiterates the need to revolutionize the search for alternative treatment regimens which seem to lie in medicinal plants (Njume et al., 2009).

Traditional medicine continues to provide health coverage for over 80% of the world's population, especially in the developing world (Ndip et al., 2007). *Combretum molle* (*C. molle*), is a semi-deciduous tree of about 6 to 10 m in height, with a spreading crown (Ojowole, 2008). It is widely used in African traditional medicine for treatment of various human ailments including abdominal discomfort, body pains, respiratory disorders, cold and fever, ear and eye ailments, hookworm, schistosomiasis, dysmenorrhoea and infertility in women, snake bite and leprosy (Fyhrquist et al., 2002). Some traditional health practitioners in KwaZulu- Natal Province of South Africa have also claimed that decoctions, infusions and other extracts of *C. molle* are effective remedies for the management and/or control of an array of human ailments, including arthritis and other inflammatory conditions (Ojewole, 2008).

Previous works have shown that many *Combretum* species posses' antifungal, antibacterial, anti-parasitic, antioxidant, and anti-inflammatory, as well as anti-HIV type 1 reverse transcriptase activity (Asres et al., 2001; Eloff et al., 2005; Njume et al., 2011; Bessong et al., 2008; Ojewole, 2008). However, we are not aware of studies which evaluated the antimicrobial activity of the stem bark of *C. molle* against the selected pathogens which constitute potential health problems in our environment. In addition, different solvents were used in these studies as compared to the present study heralding the possibility of a novel pattern of reaction noting that different solvents have been reported to extract varying antimicrobials from plants (Eloff et al., 2005; Njume et al., 2011).

MATERIALS AND METHODS

Test bacterial strains

Bacterial strains used in the study consists of reference strains*; Streptococcus pyogenes* ATCC 49399, *Plesiomonas shigelloides* ATCC 51903, *Pseudomonas aeruginosa* ATCC 15442, *Helicobacter pylori* ATCC 43526 and *Helicobacter pylori* 252C (metronidazole-resistant clinical isolate stored in the collection of the Microbial Pathogenicity and Molecular Epidemiology Research Group laboratory) (Tanih et al., 2010). These organisms were selected based on their disease burden and increasing trend of antibiotic resistance in the developing world (Eloff et al., 2005).

Plant materials and preparation of extracts

The stem bark of *C. molle* was selected based on ethno-botanical information. The plant was harvested in the vicinity of Venda, Limpopo Province. Identification was done by botanists at the University of Venda where voucher specimens (CNU FHO 5) have been deposited. The bark was washed and dried at room temperature for 2 weeks. It was then ground to fine powder using a mechanical blender (ATO MSE mix, 702732, England).

Dried and ground *C. molle* stem bark (300 g) was serially extracted with hexane, ethyl acetate, dichloromethane, acetone, ethanol and methanol. Briefly, the plant material was macerated in three folds excess of the solvent in extraction bottles such that the solvent was above the plant material. The slurry was placed in a shaker (Edison, N.J., USA) for 48 h then centrifuged at 3000 rpm for 5 min and filtered using filter paper of pore size 60 A. The process was repeated twice for a total of three extractions (exhaustive extraction) for each solvent. The collected extracts were concentrated under reduced pressure in a rotavapor (Strike 202, Steroglass Italy) to recover the solvents. The yielded extracts were weighed and stored in a labelled tight lid container for further bioassay (Ndip et al., 2009).

Antimicrobial susceptibility testing

Agar well diffusion

Inocula were prepared from subcultures of bacteria as follows (Ndip et al., 2007): Four to five colonies of the isolate were emulsified in sterile normal saline and turbidity adjusted to 1×10^8 CFU/ml (corresponding to 0.5 McFarland standards). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the Mueller Hinton agar plates (Oxoid, Basingstoke, England). The plates were allowed to dry for 3 to 5 min. Wells about 6 mm in diameter were aseptically punched with a sterile cork borer (5 holes per plate) and filled with 50 µl extracts of different concentrations (200, 100 and 50 mg/ml). The plates (in duplicate) were left for 30 min before incubation in order for the extracts to diffuse into the agar. Thereafter, they were incubated at 37°C for 24 h and the zone of inhibition measured to the nearest millimetre. The mean zone diameter of inhibition was calculated for each solvent; 0.025 mg/ml of ciprofloxacin was used as a positive control and 80% acetone as a negative control (Boyonova et al., 2005). For *H. pylori*, it was inoculated on Columbia blood agar base (Oxoid, Basingstoke, England) supplemented with 7% sterile defibrinated sheep blood and Skirrows supplement [(amphotericin B (2.5 mg/ml); trimethoprim (2.5 mg/ml), vancomycin (5.0 mg/ml) and cefsulodin (2.5 mg/ml)]. Plates were incubated at 37°C for 3 to 5 days under micro-aerophilic conditions (5% O_2 and 10% CO_2) [Anaerocult, Basingstoke, England] (Tanih et al., 2010).

Determination of minimum inhibitory concentration (MIC_{50})

MIC_{50} was determined using the broth micro-dilution technique as previously described by Banfi et al. (2003). The extracts were dissolved in 80% acetone giving a stock concentration of 10 mg/ml. BHI broth (100 µl) was dispensed into the wells except the last row of wells which contained distilled water. A 100 µl of the stock solution was dispensed into the first well; a two-fold serial dilution was carried out up to well number 12 from which 100 µl was discarded. Twenty microlitres of standardized bacterial suspension (1×10^8 CFU/ ml) was added to the wells except the control wells (control wells contained broth only, broth+ extract, broth + isolate and distilled water only). The plates were read using an ELISA micro plate reader (Bio-Rad 680, Japan) adjusted to 620 nm before and after incubation.

For *H. pylori* isolates, BHI broth was supplemented with 7% sterile horse serum and Skirrows supplements; plates were incubated

Table 1. Extract yield of *C. molle* stem bark powder (300 g) serially extracted with six solvents.

Solvent	Mass residue extracted (g)				
	1st extraction	2nd extraction	3rd extraction	Total mass	Yield %
Hexane	0.001	0.001	-	0.002	0
Ethyl acetate	0.5	0.4	0.35	1.25	0.4
Dichloromethane	0.5	0.3	0.13	0.93	0.3
Acetone	2.5	0.99	0.48	3.97	1.3
Ethanol	0.23	0.19	0.22	0.64	0.2
Methanol	3.32	1.95	1.5	6.77	2.3

Table 2. Zone of inhibition (mm) of the extracts and ciprofloxacin against *Streptococcus*, *Pseudomonas*, *Plesiomonas* and *Helicobacter*.

Bacterial strain	Solvent extracts of the stem bark of *C. Molle* (mg/ml)										
	Acetone		Ethanol		Methanol		Ethyl acetate		Dichloromethane		Ciprofloxacin
	200	100	200	100	200	100	200	100	200	100	0.025
S. pyogenes	24±2.1	19±0.7	21±0.7	15±0.7	20±4.2	17±5.6	16	14±0.7	17±2.1	15±3.5	30±0.7
P. aeruginosa	25±1.4	18±1.4	17	15±1.4	8±2.8	3±3.5	7± 0.7	0	11±0.7	8±1.4	33±1.4
P. shigelloides	32±4.9	29±2.1	32±2.8	28±0.7	25±1.4	21±3.5	17±1.4	12	21±0.7	20	36±2.8
H. pylori 252C	15±1.4	11±2.1	11±2.8	7±0.7	13±0.7	0	7±2.1	0	0	0	16±2.8
H. pylori 43526	16±2.1	11±2.1	11±1.4	9±2.1	15±4.24	0	12±1.4	7±3.5	0	0	35

at 37ºC for 48 h under micro-aerophilic conditions (5% O_2 and 10% CO_2) (Anaerocult, Basingstoke, England). Plates were read at the same wavelength. The two absorbencies were compared and the MIC_{50} was determined as the lowest concentration (highest dilution) of extract that inhibited 50% of bacterial growth.

Statistical analysis

Analysis was performed using the SPSS version 17.0 (Illinois USA, 2009). The One way ANOVA test was used to determine if there was any statistically significant difference in the diameter of zones of inhibition of the plant extract and antibiotic and the MIC of the extracts and the antibiotics. P-values <0.05 were considered significant.

RESULTS

Extract yield

Methanol was the best solvent with a yield of 6.77 g while hexane was the least with 0.002 g (Table 1). Since the yield of hexane was small, no further tests were carried out with this extract.

Antimicrobial activity of the extracts

The zone of inhibition ranged from 0 to 32 mm, with the best activity (11 to 32 mm) demonstrated by the acetone, and least, 0 to 17 mm by dichloromethane (DCM) (Table 2). An inhibition zone diameter of ≥ 11 mm was chosen as a break-point of bacterial susceptibility of the extracts and the antibiotic. Ciprofloxacin (0.025 mg/ml) which was used as a positive control had a zone diameter of inhibition of 16 to 36 mm. *P. shigelloides* was the most susceptible organism to all extracts while *H. pylori* (clinical isolate 252C) were the most resistant with partial zones of inhibition. The zones of inhibition of the extracts and antibiotic were compared; no statistical significant difference was observed for the four extracts (acetone, methanol, ethanol and ethyl

Table 3. Mean zone of inhibition of the extracts and antibiotic against *Streptococcus*, *Pseudomonas*, *Plesiomonas* and *Helicobacter.*

Extract/ control antibiotic	Mean zone diameter (mm)	Inhibition range (mm)
Acetone	22.5 ± 7.0	8-32
Ethanol	18.8 ± 8.3	0-32
Methanol	17.8 ± 5.9	0-29
Ethyl Acetate	16.2 ± 6.5	0-25
Dichloromethane	13 ± 3.9	0-17
Ciprofloxacin	33.2± 2.3	16-36

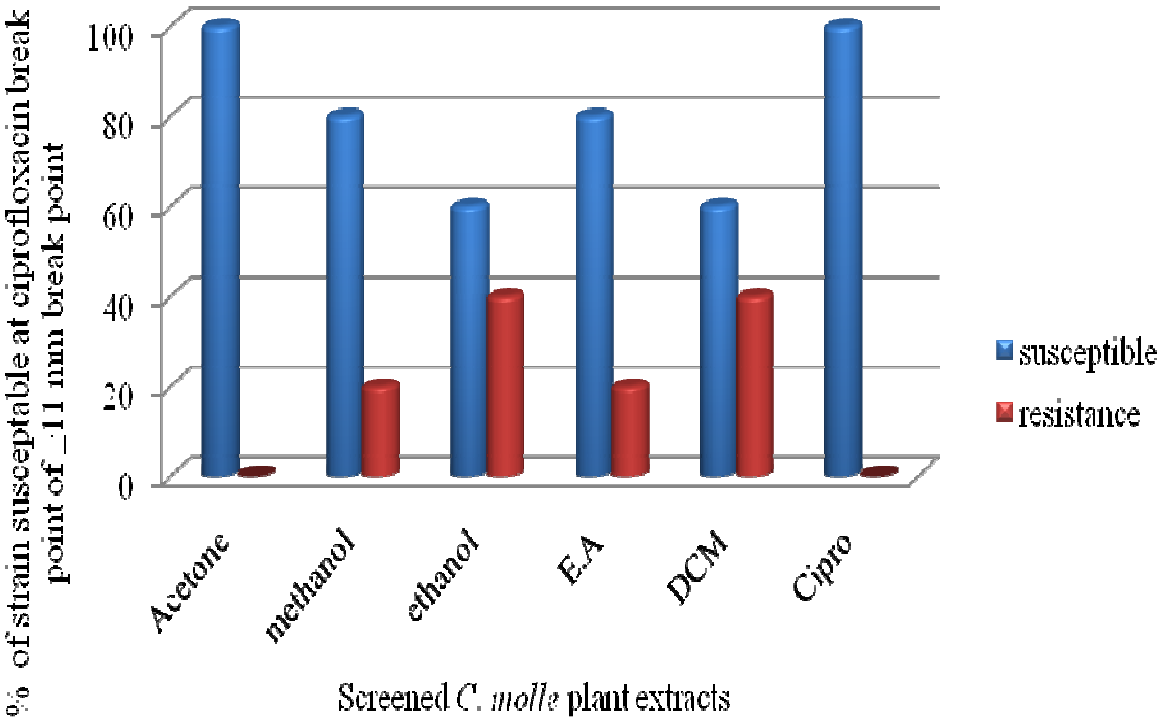

Figure 1. Susceptibility of the extracts against the test organisms at 200 mg/ml. E.A., ethyl aceate; DCM., dichloromethane.

acetate).

Table 3 shows the mean zone diameter of the individual extracts against the organisms; acetone had potent activity (22.5 ±7.0 mm) followed by ethanol (18.8 ± 8.3mm) and the least was DCM extract (12.8 ± 9.6 mm).

Of the five organisms subjected to the plant extracts and antibiotic, 100% susceptibility was recorded for the acetone extract and antibiotic. Susceptibility of methanol and ethyl acetate was 80% while ethanol and DCM was 60% (Figure 1).

MIC_{50} determinations

The MIC results also showed that acetone extract was the most active with a MIC_{50} ranging from 0.078 to 2.5 mg/ml followed by ethanol, 0.625 to 5 mg/ml while ethyl acetate was the least (2.5 mg/ml). On the other hand, the MIC_{50} of ciprofloxacin ranged from 0.00312 to 0.0156 mg/ml (Table 4). There was no statistically significance difference between the MIC of the extracts and control antibiotic ($P > 0.05$).

DISCUSSION

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.,

Table 4. Antibacterial activity of *C. molle* and ciprofloxacin at MIC $_{50}$ against Streptococcus, *Pseudomonas*, *Plesiomonas* and *Helicobacter.*

Organism	MIC$_{50}$ of the extracts/antibiotic (mg/ml)					
	Acetone	Ethanol	Methanol	DCM	E.A	Cipro
S. pyogenes;	0.156	0.625	2.5	0.625	2.5	0.00312
P. shigelloides	0.078	0.625	0.156	0.312	2.5	0.00312
P. aeruginosa	0.625	1.25	2.5	2.5	2.5	0.0156
H. pylori 252C	2.5	1.25	ND	ND	ND	0.0125
H. pylori 43526	1.25	5	ND	ND	ND	0.0125

DCM, Dichloromethane; E.A., ethyl acetate; Cipro, ciprofloxcin; ND, not determined.

2007). Several 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, to our knowledge, the bioactivity against our selected test organisms has not been established. Therefore our study evaluated the antimicrobial activity of extracts of the stem bark of *C. molle* against the selected test organisms.

The current study, observed that methanol and acetone were good solvents for extraction with yield of 6.77 g (2.3%) and 3.97 g (1.3%) respectively while hexane was the least 0.002 g (0%). The findings concurs with the work of others (Asres et al., 2001; Eloff et al., 2005; Masoko and Eloff, 2006) who also found that acetone and methanol extract more compounds of *C molle, Combretum woodii* and *Combretum hereroense*. Angeh et al. (2007) worked on different species of *Combretum* and found that the yield differed with species but methanol yield was high in all species. Saponins and tannins which are reported to be in abundance could be extracted by methanol and acetone (Masoko and Eloff, 2006). This might explain why methanol and acetone yielded more than other solvents. Non- polar solvents yield more lipophilic components, while alcoholic solvents give a larger spectrum of polar material. Acetone is usually preferred because it extracts polar and non polar components (Masoko and Eloff, 2006).

The antibacterial activity of acetone, ethanol, methanol and ethyl acetate extracts compared favourably with that of the standard antibiotic (ciprofloxacin) ($p > 0.05$) and appeared to be broad spectrum as its activity was independent of the organism being Gram-positive or negative. The results imply that these extracts may contain compounds with therapeutic potential comparable to the antibiotic. Previous works have demonstrated potent antimicrobial activity of this plant against Gram-positive bacteria (Kelmanson et al., 2000; Kumar et al., 2010). However, in this study it was realized that *P. shigelloides,* a Gram-negative bacterium was the most susceptible organism with a MIC$_{50}$ value ranging from 0.078 to 0.625 mg/mL. Most plant extracts have been reported to be more active against Gram-positive than Gram-negative bacteria; this has been attributed to the fact that Gram-negative bacteria contains an outer membrane with lipopolysacharide layer which render them impermeable to certain antibiotics and bactericidal compounds (Nikaido, 1996; Fennell et al., 2004).

The weak activities shown by some of these extracts *in vitro* may not imply that they would demonstrate weak activities *in vivo* because of immuno-modulation of chemical. 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).

The acetone extract was the most potent among the five extracts with a zone diameter of inhibition ranging from 11 to 32 mm. The findings corroborate previous works (Asres et al., 2001; Krugger 2004; Masoko and Eloff, 2007; Mishra et al., 2009). In their study, Asres et al. (2001) demonstrated good activity of the acetone extract against *Mycobacterium tuberculosis, Trypanosoma brucei rhodesiense* STIB900 and *Plasmodium falciparum* 3D7. The activity was attributed to high amount of hydrolysable tannins present in the stem bark of *C. molle*. It is generally believed that tannins are non-selective enzyme inhibitors due to their poly-phenolic groups. However, it has been shown that some hydrolysable tannins display selective cytotoxicity. Therefore, the potential of this group of compounds for drug development should not be undermined, especially when they are proved to be the active ingredients of plants used in traditional medical practices (Asres et al., 2001; Funatogawa et al., 2004).

Krugger (2004) worked on *Terminalia sericea* and also reported marked activity with the acetone extract against *Staphylococcus aureus* compared to other solvents. Similarly, the acetone extracts of *Carex nelsonii, Clarias albopuctactum*, *and Combretum imberbe* and *Terminalia sericea* possessed growth inhibitory activities against fungal pathogens *in vitro* (Masoko and Eloff, 2007).

Mishra et al. (2009) screened the antifungal activity of *Cinnamomun zeylanicum* (CZ) against the growth of two moulds (*Alternaria solani* and *Curvulana lunta*) and found that acetone extract was the most potent exhibiting 100% inhibition of spore germination. Therefore these findings

suggest that an organic solvent, in particular, acetone is a good solvent as it extracts more active compounds from plant material. Flavonoids and steroids have been reported to be extracted using acetone (Eloff, 1998); plants produce flavonoids in response to microbial infection (Schinor et al., 2007) which may account for the *in vitro* activity of *C. molle* observed in this study.

Conclusion

The findings of this study indicate the *in vitro* activity of the crude extracts of *C. molle* and therefore further supports its use in traditional medicine. The plant may provide novel or lead compounds, which could become starting materials for the synthesis of new drugs. Therefore, further studies to isolate and characterize the active compounds are essential.

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