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Full Length Research Paper

In-vitro anti-*Helicobacter pylori* activity of acetone, ethanol and methanol extracts of the stem bark of *Combretum molle* (Combretaceae)

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In an attempt to identify novel sources of cheap starting material for the synthesis of new anti-infective agents, the antimicrobial activity of crude acetone, ethanol and methanol extracts of the stem bark of *Combretum molle* were investigated against 32 clinical strains of *Helicobacter pylori* and a standard control strain NCTC 11638 by agar well diffusion and micro-broth dilution. Metronidazole, clarithromycin and amoxicillin were included in these experiments as positive control antibiotics. All the extracts tested exhibited anti-*H. pylori* activity with zone diameters of inhibition between 0 to 38 mm. The acetone extract showed potent anti-*H. pylori* activity, giving a percentage susceptibility of 87.5%. Minimum inhibitory concentration (MIC) values for this extract ranged from 0.078 to 2.50 mg/ml while those for amoxicillin and metronidazole ranged from 0.001 to 1.25 mg/ml and 0.004 to 5.0 mg/ml respectively. The inhibitory activity of the acetone extract was similar to amoxicillin ($P>0.05$) as opposed to metronidazole ($P<0.05$). These results demonstrate that the acetone extract may contain compounds with therapeutic activity and therefore a potential source of new anti-*H. pylori* regimen.

Key words: Antimicrobial activity, crude extracts, drug discovery, *Helicobacter pylori*, Minimum inhibitory concentration (MIC).

INTRODUCTION

Helicobacter pylori is a gram negative microaerophilic helical bacillus that affects the gastric mucosa and can be found attached to epithelial cells of the human stomach (Ndip et al., 2008). It is a risk factor and etiologic agent of chronic gastritis, peptic ulcer, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma (Adeniyi et al., 2009). The organism infects half of the world's population (Romano and Cuomo, 2004). Fortunately enough, there is significant remission from the aforementioned diseases with the eradication of *H. pylori* from the stomach (Ndip et al., 2007; Ndip et al., 2008). Eradication regimens typically involve the use of a proton pump inhibitor (PPI) or bismuth compounds in combination with two antibiotics, most commonly amoxicillin, tetracycline, clarithromycin or metronidazole with an expected success rate of 80 to 90% (Tanih et al., 2008). However, *H. pylori* is still a difficult infection to eradicate as failure rate remains at 10 to 40% (Lai et al., 2006). A major factor to this failure is the development of antibiotic resistant strains (Tanih et al., 2010). These have greatly limited the therapeutic options especially in some countries of the developing world where an alarming 100% resistance has been reported for

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metronidazole, one of the drugs used in the treatment regimen (Aboderin et al., 2007). Other factors including poor patient compliance, undesirable side effects, cost of combination therapy, the location of the organism within the stomach and the non availability of the drugs in some rural settings in Africa provide a challenge for antimicrobial therapy (Romano and Cuomo, 2004), thus necessitating the search for alternative treatment regimens against *H. pylori* infections. Alternative therapeutic agents with highly selective antibacterial activity against the organism, without the risk of resistance or other untoward effects are necessary (Njume et al., 2009).

Over the years, medicinal plants have been used to treat gastrointestinal diseases and other ailments particularly in the developing world where infectious diseases are endemic and modern health facilities and services are inadequate (Samie et al., 2005). Thus, plants would seem to be a logical source of new anti-*H. pylori* compounds. In fact, our previous studies have documented that some medicinal plant extracts have antibacterial activity against *H. pylori* (Ndip et al., 2007, 2008).

The stem bark of *Combretum molle*, a small graceful deciduous tree (3 to 13 m high) is widely used in South Africa for the treatment of stomach pains, dysentery, gastric ulcers, abdominal disorders and other illnesses (Ellof et al., 2008). It is found in the traditional medicine market where it is commercialised for medicinal purposes. Despite its traditional uses in the treatment of gastric ulcers and other stomach related morbidities, the activity of this plant has not been investigated against *H. pylori*, a major cause of gastric ulcer (Marshall and Warren, 1983).

This is surprising, particularly as the prevalence of this organism is reported to vary between 50 and 80% in South Africa (Samie et al., 2007; Dube et al., 2009), and an alarming resistance of 95.5% reported for metronidazole, one of the antibiotics used in the treatment regimen of *H. pylori* infections (Tanih et al., 2010). This study was therefore carried out to evaluate the antimicrobial activity of *C. molle* on clinical isolates of *H. pylori* in a bid to identify potential sources of cheap starting materials for the synthesis of new drugs that could be cheap and readily available to help circumvent the problem of increasing antimicrobial resistance.

MATERIALS AND METHODS

Bacterial strains

A total of thirty two strains of *H. pylori* were isolated from gastric biopsies of patients with gastric related morbidities undergoing endoscopy at the Livingstone Hospital, Port Elizabeth and confirmed following our previously reported scheme (Ndip et al., 2008). Ethical clearance was obtained from the Eastern Cape Department of Health and the Govan Mbeki Research and Development Centre, University of Fort Hare. Specimens were only collected from patients who had given consent and had not received antibiotics or proton pump inhibitors for at least a week. Pure cultures were suspended in eppendorf tubes containing 1 ml of Brian Heart Infusion (BHI) broth and 20% glycerol and stored at -80°C for future experiments.

Preparation of plant material

The stem bark of *C. molle* was harvested in the vicinity of the University of Venda, Limpopo Province. Identification was done by a botanist of that institution where voucher specimens have been deposited. The plant material was washed, air dried for two weeks, ground to fine powder using a blender (ATO MSE mix, England).

Preparation of plant extracts

Exactly 300 g of dried plant material was macerated separately in 600 ml of concentrated ethyl acetate, acetone, ethanol and methanol in clean glass bottles (SIMAX, Czech Republic). Aqueous extracts were also prepared by dissolving same amount of plant material in tap water. The bottles were labelled and put in an orbital shaker for 48 h. The plant extracts were centrifuged at 3000 rpm for 5 min at 4°C (Sibanda and Okoh, 2008), and filtered using a fritted filter funnel of pore size 60 Å. The procedure was repeated twice and the three extracts combined and evaporated to dryness under vacuum in a rotary evaporator (BUCHI rota vapour, Switzerland) set at temperatures depending on the solvent that was being used. The filtrate obtained from the aqueous extract was lyophilized (Castillo-juarez et al., 2009). The dried crude extracts were collected in clean universal bottles and left open in a bio-safety class ‖ □cabinet (Vivid Air, Durban, South Africa) for complete evaporation of residual solvents. Two grams of each plant extract was used for the preliminary bioassay, and where possible, another 2 g was kept in the extract bank. Stock solutions were prepared by dissolving the extracts in dimethyl sulphoxide (DMSO) or acetone, 10 and 80% respectively, concentrations we previously established to be non inhibitory to the *H. pylori* strains.

Screening of crude extracts for anti-*H. pylori* activity

This was done by the agar well diffusion method as previously reported (Manyi-Loh et al., 2010). Briefly, *H. pylori* inocula prepared at McFarlands turbidity standard 2 ($6x10^8$ CFU/ml) was plated onto Brain heart infusion (BHI) agar supplemented with 5% horse blood and Skirrows supplement (Oxoid, England). The inoculum was evenly spread on the plate and allowed to dry for about 15 min. Wells (6 mm in diameter) were punched into the agar using a sterile stainless steel borer. The wells were filled with 65 µL of the extract at 50 mg/ml. Sixty five micro litres of 0.05 µg/ml clarithromycin and 10% DMSO were included in all experiments as positive and negative controls respectively. The plates were incubated under microaerophilic conditions (Anaerocult, Oxoid, UK) at 37°C for 72 h after which the diameters of zones of inhibition were measured in millimetres. The experiment was repeated once and the mean zone diameters of inhibition recorded. *H. pylori* control strain, NCTC 11638 inoculated plate was included in all the experiments.

Determination of minimum inhibitory concentration (MIC)

Active extracts that had given a zone of inhibition of ≥13.0 mm averagely were chosen for further determination of MICs by the resazurin micro broth dilution method (Banfi et al., 2003), slightly modified by Manyi-Loh et al. (2010). The micro dilution test was performed in 96 well plates. Two-fold dilutions of each extract were prepared in the test wells in BHI broth supplemented with 5% horse

Table 1. Screening of crude extracts of *C. molle* for anti-*H. pylori* activity.

Extract/ Control antibiotic	Mean zone diameter (mm)	Inhibition diameter range (mm)
Ethyl acetate extract (EA)	10.7±4.7	0-21
Acetone extract (A)	17.5±5.0	10-38
Ethanol extract (E)	13.0±4.7	7-35
Methanol extract (M)	13.1±5.3	7-32
Aqueous extract (H_2O)	2.8±5.5	0-20
Clarithromycin	13.7±9.1	0-32

Data are mean ± SD of 33 determinations for each extract or antibiotic.

serum and Skirrows reagent (Oxoid, England). The final extract concentrations ranged from 0.001 to 5.0 mg/ml. Twenty micro litres of an 18 h old broth culture of *H. pylori* (McFarlands turbidity standard 2) suspension was added to 180 µL of extract-containing culture medium. Control wells were prepared with culture medium and bacterial suspension and broth only respectively. Metronidazole and amoxicillin were run alongside each batch of extracts at concentration ranges of 0.005 to 5.0 mg/ml and 0.001 to 1.25 mg/ml respectively. The plates were sealed and incubated for 3 days at 37°C under microaerophillic conditions. After incubation, 5 µL of resazurin solution was added to each well, colouring them blue. Plates were incubated at 37°C for an additional period of 2 to 4 h after which they were read for colour change from blue to pink in live *H. pylori* wells. The MIC was recorded as the lowest drug or extract concentration that prevented resazurin colour change from blue to pink. Each MIC was determined twice and the mean values recorded.

Statistical analysis

Results were expressed as mean ± standard deviation using SPSS version 17.0 (Chicago Illinois, 2009) and Excel. One way ANOVA followed by Turkey's post-hoc test was used to compare the inhibitory activities of different crude extracts and antibiotics and to determine statistical differences. Differences were considered significant at $P< 0.05$.

RESULTS

Extract yield

The total amount of crude extract obtained with the different solvents showed that methanol was quantitatively the best extractant, with a crude extract yield of 5.1 g (1.7%) followed by acetone 4.6 g (1.5%), ethanol 3.2 g (1.1%), water 3.1 g (1%) and ethyl acetate 1.2 g (0.4%). Ethyl acetate, acetone and methanol extracts were dark brown in colour while ethanol and aqueous extracts appeared as brown to light brown crystals.

Antimicrobial susceptibility testing and MIC determination

All the crude extracts tested in this study demonstrated antimicrobial activity with zone diameters of inhibition ranging from 0 to 38 mm. The highest zone diameter of 38 mm was recorded for the acetone extract which also recorded the highest mean zone diameter of 17.5±5.0 mm (Table 1) and percentage susceptibility of 87.5% (Figure 1). More than 40% of the strains were resistant to clarithromycin whose mean zone diameter was 13.7±9.1 mm. It was therefore logical to consider extracts that had mean zone diameters ≥13 mm for MIC determination due to the fact that the positive control, a pure compound fell in this range. Inhibition zone diameters of the acetone extract were significantly different from those of clarithromycin and all the other extracts ($P<0.05$).

Eleven (34.4%) and 6(18.8%) of the 32 strains tested against the acetone and ethanol extracts respectively recorded susceptible zones of inhibition as opposed to the positive control which was resistant, but the differences were not statistically significant ($P>0.05$). The MIC values for the acetone, ethanol and methanol extracts ranged from 0.078 to 2.5 mg/ml, 0.156 to 5.0 mg/ml and 0.625 to 5.0 mg/ml respectively while those for amoxicillin and metronidazole ranged from 0.001 to 1.25 mg/ml and 0.004 to 5.0 mg/ml respectively (Table 2). The activity of the acetone extract was similar to amoxicillin ($P>0.05$), and significantly different from metronidazole and the other extracts ($P<0.05$).

DISCUSSION

Different solvents may be applied in the extraction of antimicrobial compounds from plants and the success in the extraction process is largely dependent on the type of solvent used (Masoko et al., 2007). The quantity of the methanol extract reported in this study is in line with many others that have documented the good extracting ability of methanol from *Combretum* species and other plants (Fyhrquist et al., 2002; Masoko et al., 2007; Ezekiel et al., 2009). However, our results also indicate that the quantity of the extract may not always relate proportionately with its activity (Figure 1).

The acetone extract of the stem back of *C. molle* has been previously reported to be active against a number of infectious organisms (Eloff, 1999; Fyhrquist et al., 2002; Asres et al., 2006). Our results are consistent with others

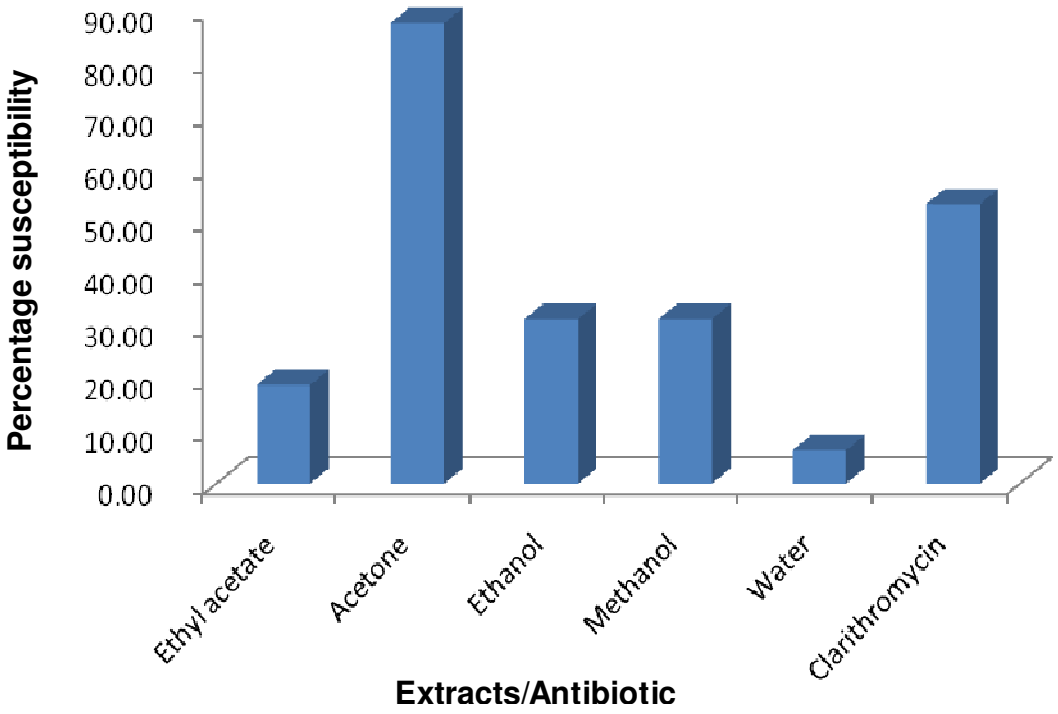

Figure 1. Anti-*H. pylori* activity of crude extracts of *C. molle* by agar well diffusion method

that point to the fact that acetone is a good solvent for the extraction of bioactive compounds from *C. molle* and other plants. Eloff (1998) in a comparison of acetone, ethanol, methanol, methylenedichloride, methanol/ chloroform/water and water observed acetone to be the best in terms of the diversity of compounds extracted. Such diverse compounds may act in synergy and produce a greater antimicrobial effect on *H. pylori*, thus resulting in high susceptibility patterns and low MIC values observed in this study. Acetone is used to extract mostly flavonoids and steroids (Eloff et al., 1998; Cowan, 1999; Afolayan and Lewu, 2009), flavonoids are known to be synthesized by plants in response to microbial infection (Hernández et al., 2000; Schinor et al., 2007), which may account for their *in-vitro* activity against a wide array of microorganisms.

MIC values for the acetone extract ranged from 0.078 to 2.5 mg/ml (Table 2) and compared favourably with amoxicillin ($P>0.05$) as opposed to metronidazole and the other extracts ($P<0.05$). *H. pylori* resistance to metronidazole is at alarming proportions in South Africa (95.5%) and many developing countries, in which resistance is up to 100% have been reported (Alarcon et al., 1999; Lwai-lume et al., 2005; Aboderine et al., 2007; Ndip et al., 2008; Tanih et al., 2010). On the other hand, resistance to amoxicillin is rare (Mégraud and Lehours, 2007; Tanih et al., 2010). It should therefore not be surprising that the activity of the acetone extract in this study was better than metronidazole.

Crude extracts of other plants have been shown to have comparable activity with amoxicillin and better activity when compared with metronidazole against *H. pylori* in other studies (Ndip et al., 2008; Castillo-juarez et al., 2009). This is quite remarkable particularly as standard antibiotics are in the purified and concentrated form whereas the extracts are crude and may harbour both pharmacologically and non-pharmacologically active compounds with the chance of some compounds having a masking effect over others. The activity of the acetone extract demonstrated herein indicates that the active components of *C. molle* are more soluble in acetone and have good antimicrobial potential, which if investigated further may lead to the isolation of potentially useful compounds for the treatment of *H. pylori* infections.

In a study to screen for antibacterial activity of 27 members of the Combretaceae against some gram positive and gram negative organisms, Ellof (1999) reported MIC values of between 0.1 to 6 mg/ml for the acetone extracts tested; with an average MIC value of 2.01 mg/ml. *C. molle* demonstrated the best activity of the 7 *Combretum* species, which were the most active. Our results seem to corroborate those of Eloff (1999), but with slightly lower MIC values. These differences may be due to the type of organisms tested, the age of the plant and its parts used as previously suggested (Ndip et al., 2007). Stem backs, used in this study are known to demonstrate a higher antimicrobial activity (Eloff et al., 2001).

In many reports, ethanol or methanol is used for alkaloid extraction (Eloff, 1998; Kumaraswamy et al., 2008; Ezekiel et al., 2009). The lower activity of ethanol

Table 2. Minimum inhibitory concentrations of acetone, ethanol and methanol crude extracts of *C. molle* and antibiotics (mg/ml).

	Crude extracts/ Antibiotics				
***H. pylori* strains**	**Acetone**	**Ethanol**	**Methanol**	**Metronidazole**	**Amoxicillin**
PE2A	0.312	1.25	–	0.625	0.011
PE5A	0.625	–	–	–	1.250
PE9C	1.25	5.0	5.0	–	0.011
PE11A	0.156	0.312	0.625	2.50	0.312
PE11C	0.156	5.0	–	–	0.312
PE14C	0.156	1.25	1.25	–	0.011
PE26A	0.312	1.25	2.50	2.50	0.005
PE70A	0.625	2.50	5.0	2.50	0.011
PE76A	1.25	0.625	1.25	0.625	0.005
PE84C	0.625	1.25	5.0	–	0.005
PE93A	0.078	0.156	2.50	1.25	0.005
PE93C	1.25	0.625	2.50	5.0	0.005
PE102C	0.156	0.625	1.25	2.50	0.005
PE115A	0.312	1.25	0.625	1.25	0.005
PE155A	2.50	1.25	2.50	0.156	0.005
PE162A	1.25	–	–	1.25	0.039
PE219A	0.312	1.25	2.50	0.625	0.005
PE252C	0.312	1.25	1.25	0.625	0.011
PE258C	0.312	5.0	1.25	0.625	0.005
PE265C	0.625	1.25	–	–	0.079
PE296C	0.312	–	–	1.25	0.011
PE308C	1.25	–	–	1.25	0.011
PE369A	0.625	0.625	1.25	2.50	0.005
PE369C	1.25	0.625	1.25	–	0.312
PE397C	0.312	1.25	0.625	1.25	0.005
PE402A	0.625	–	–	1.25	0.011
PE411A	0.625	1.25	2.5	–	0.078
PE411C	1.25	–	–	–	0.001
PE430A	0.312	–	–	–	0.001
PE430C	0.312	5.0	1.25	–	0.001
PE435A	0.312	5.0	1.25	–	0.625
PE466C	0.156	5.0	–	0.156	0.001
NCTC 11638	2.50	-	-	0.004	0.001
Mean ±SD	0.68±0.62	2.15±1.91	2.05±1.39	1.41±1.17	0.09±0.25

–, Value not within susceptible range; SD, Standard deviation. The results shown are representative of duplicate determinations for each strain and 33 determinations for each extract or control antibiotic.

and methanol crude extracts observed in this study may imply that the components extracted by these two solvents may not be very effective as antimicrobials against *H. pylori* infections. However, their demonstrated activity gives an indication of their potential as useful bioactive substances.

A number of authors have reported on the good antimicrobial activity of the ethyl acetate fraction of *Combretum* species (Onocha et al., 2005; Eloff et al., 2005), but *H. pylori* was not among the organisms tested. Our findings provide preliminary evidence that the ethyl acetate extract of *C. molle* has very little activity against *H. pylori*. Ethyl acetate is used to extract mostly esters and non polar compounds, substances that may not easily diffuse through an aqueous agar matrix (Valgas et al., 2007; Eloff et al., 2008). In order to detect the antimicrobial activity of such extracts by agar diffusion methods therefore, the inoculated system may have to be kept at lower temperatures for a few hours before incubation to favour compound diffusion over microbial growth, which may not be suitable when working with *H. pylori*, a very fastidious organism.

The aqueous extracts of *C. molle* showed very little activity (6.3%) against *H. pylori*, other studies have also reported very low antimicrobial activity of the aqueous extract of other members of the Combretaceae (Eloff et al., 2005, 2008). Generally, in this study, zone diameters of inhibition and percentage susceptibilities decreased with increase in polarity of the extractant from acetone to water (Figure 1) which may imply that the strains were not very sensitive to the polar compounds of this plant or at least, not many anti- *H pylori* compounds were extracted by polar solvents.

In conclusion, the results of this study provide preliminary scientific validation of the traditional medicinal use of this plant in the treatment of infections symptomatic of *H. pylori*. This plant may contain compounds, mostly in the acetone crude extract that could be used as lead molecules for the synthesis of novel drugs against *H. pylori* infections. Bioassay-guided fractionation of the biologically active components of this plant and a detailed assessment of their *in-vivo* potencies is already receiving attention in our group.

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