Full Length Research Paper

In-vitro antimicrobial activity of *Ageratum conyzoides* (Linn) on clinical isolates of *Helicobacter pylori*

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The present study evaluates the antimicrobial activity of fractionated extracts of *Ageratum conyzoides* in a bid to isolate the active constituents of the plant with *anti- Helicobacter pylori* activity. *Helicobacter pylori* was isolated from the specimens following standard microbiology procedures and isolates subjected to pure fractions of plant extracts for antimicrobial assays. Extracts of *A. conyzoides* was fractionated by silica gel and thin layer chromatography to obtain pure fractions (17). Fractions 23 - 30 and 31 - 36 were so close and had crystals; it was assumed that they had the same active components, so they were combined and considered as one (fractions 23 - 36). The disk diffusion method was used to determine the susceptibility of 15 strains of *H. pylori* to the fractions. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the most active fraction was also determined by the broth dilution method. Results were analyzed by the Fisher's exact test. All the fractions tested demonstrated antimicrobial activity with zone diameters of inhibition between 0 – 30 However, two of the 17 fractions [23 - 36(100%Hex-Hex/EA20%) and 69 - 83(Hex/EA80%)] demonstrated very potent activities. The lowest MIC and MBC recorded were 0.002 and 0.016 mg/ml respectively. However the MIC of the fractions ranged from 0.016 - 0.500 mg/ml for fractions 23 - 36 and 0.002 - 0.500 mg/ml for fractions 69 - 83. The MBC of the fractions ranged from 0.063 - 0.500 mg/ml for fractions 23 - 36; 0.016 - 1.000 mg/ml for fractions 69 - 83. There was a statistically significant difference ($P < 0.05$) in the potency of the fractions on the different bacterial strains tested, both for the MIC and MBC but comparing the activities of the most potent fractions against the different bacterial strains, they possessed no significant differences ($P > 0.05$) in their activities both for the MIC and MBC. It is concluded that this plant may contain compounds with therapeutic activity, which may be found in fractions 23 - 36 (100%Hex-Hex/EA20%) and 69 - 83(Hex/EA80%).

Key words: Antimicrobial activity, medicinal plant, minimum inhibitory concentration, minimum bactericidal concentration, *Helicobacter pylori*, antimicrobial resistance.

INTRODUCTION

Helicobacter pylori is a gram negative helical rod that colonizes the human gastric mucous layer (Shimizu et al., 1996; Hayama et al., 2005; Ndip et al., 2008). It chronicaly infects the gastric mucosa causing gastritis in more than 50% of the world's population. The infection can lead to the development of peptic ulcer (Sontaq, 1997) and gastric mucosa-associated lymphoid tissue lymphoma (Du and Isaccon, 2002); infection with the organism has also been linked with an increased risk of gastric cancer in humans (Forman et al., 1994; Uemura et al., 2001).

The development of safe anti-*Helicobacter pylori* compounds is desirable due to the problem of antibiotic-

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resistant strains that have emerged (Yoshiyuki et al., 2006). Currently, new triple therapies consisting of two antibiotics and a proton pump inhibitor demonstrate considerable eradication rates. However, with the organism rapidly acquiring resistance to some of these antibiotics coupled with the fact that some of these drugs occasionally cause side effects, as well as their high cost for treatment, there is need to search for alternative therapies (O'connor et al., 1995; Ndip et al., 2008).

Studies have documented that some medicinal plant extracts have antibacterial activities, *H. pylori* inclusive (Cowan, 1999; Isogai et al., 2000; Funtogawa et al., 2004; Ndip et al., 2007). A study recently conducted by our group (Ndip et al., 2007) indicated that crude extracts of *Ageratum conyzoides* exhibited potent *H. pylori* activity. Medicinal plants generally contain compounds which may be potential natural antibacterial agents and which may serve as an alternative effective source for the treatment of common bacterial infections. Herbal medicine has been widely used and formed an integral part of primary health care in China (Liu, 1987; Ethiopia, Desta, 1993) and Argentina (Anesini and Perez, 1993).

A. conyzoides is an annual herb in the tropics and subtropics whose extracts are known to possess pharmacological and biocidal activity. It has been long known in herbal or folk medicine as a remedy for various ailments in Africa (Almagboul et al., 2001). It has a history of use in traditional medicine in various countries world wide and is commonly used to treat wounds, burns and bacterial diseases (Ming, 1999). Various extracts of the plant, including water and methanol have been shown to inhibit the growth of *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *H. pylori* (Almagboul et al., 2001; Ogbeche et al., 1997; Ndip et al., 2007).

The present study evaluates the antimicrobial activity of fractionated extracts of *A. conyzoides* in a bid to isolate and identify the active constituents of this plant subsequently. This constitutes part of an effort to identify potential sources of cheap starting materials for the synthesis of new drugs to circumvent the problem of increasing drug resistance against the pathogen. Part of this study was presented at the 21st Scientific Congress of the Austrian Pharmaceutical Society, Vienna, Austria (Ndip et al., 2009).

MATERIALS AND METHODS

Bacterial isolates

Fifteen strains of *H. pylori* isolated from gastric biopsies were used. The biopsies were obtained from the antrum and corpus of patients presenting with gastroduodenal pathologies at the Douala General Hospital. Informed consent was obtained from the patients and ethical approval from the hospital's management board (Protocol number HGD/LN158/LHN/SE/DMT/10/05). The study lasted from March 2006 to May 2008, and was conducted at the university of Buea, Cameroon. Briefly, biopsies were inoculated on to Columbia

Agar Base (CAB) (Conda Pronadisa, Spain) supplemented with 10% sterile sheep blood and *Campylobacter* select tablets [polymyxin B (2500 units/L), trimethoprim (5 mg/L), vancomycin (10 mg/L) and fungizone]. Plates were incubated at 37°C for 2 – 5 days under microaerophilic conditions (10% carbon dioxide, 5% oxygen) (Anaerocult Darmstadt, Germany). Isolates were identified following previously reported schemes (Ndip et al., 2003).

Preparation of the crude extracts

A. conyzoides was selected because we had previously demonstrated that it has potent anti- *H. pylori* activity (Ndip et al., 2007). Samples of the plant were collected from the North West Province of Cameroon; they were identified by Dr. Claire Wirmum of Medicinal Foods and Plants, Bamenda. Voucher specimens have been deposited at the National Herbarium, Yaounde with reference number 6575/SRFK.

The whole plant parts were air dried and then ground to a fine powder. The preparation of crude methanolic extracts and their subsequent chromatographic fractionation was carried out. Pure methanol (100%) was used for the extraction. Briefly, dried plants (9.5 Kg) were macerated separately in methanol in extraction pots. The mixture was left for an extended period and subsequently, the slurry was filtered and left for 48 h at room temperature, then concentrated under reduced pressure in a rotavapor (BUCHI Rotavapor R200, Switzerland) to recover the methanol. The neat crude extracts were stored under appropriate conditions for further processing. Fractionation of the extract was done following standard methods (Mohring et al., 2006) (Figure 1).

Silica gel chromatography

The neat crude extract (60 g) was adsorbed on 90 g of Celite powder. The mixture was then placed onto a silica gel column equilibrated with hexane. The column was eluted with a mixture of hexane and ethyl acetate (Hex/EA) using a stepwise gradient (100:0 to 0:100 respectively) and finally with 100% methanol. Fractions (200 ml) were collected and concentrated on a rotavapor. The fractions were further analyzed by Thin Layer Chromatography (TLC) on silica gel (Mohring et al., 2006). Fractions displaying similar constituent profile were pooled and tested against *H. pylori* using the disk diffusion assay.

Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion susceptibility test was used for primary screening of susceptibility of the isolates to the different fractions of the extracts. The test was performed according to the recommended standards of the National Committee for Clinical Laboratory standards (NCCLS) (now known as Clinical and Laboratory Standards Institute (CLSI), modified to the needs of *H. pylori* as previously described by Ndip et al. (2007). The cultures were adjusted to approximately 0.5 McFarland turbidity standards (10[8] CFU/ml) with sterile saline solution. A sterile cotton swab was used to spread the suspensions over plates containing Columbia agar base in order to get a uniform microbial growth on the test plates. The plates were allowed to dry for 3 - 5 min.

The test solutions (fractions) were prepared by weighing 0.2 g each of the different fractions into 1 ml of 10% dimethylsulfoxide (DMSO) with Tween-20 (0.5% v/v to enhance solubility of the fractions for easy diffusion). Impregnated discs were prepared by adding 25 µL each, of the different test fractions onto filter paper disks (Whatman no.2) that were previously sterilized. The disks were placed on the agar surface and gently pressed to ensure contact with the agar. Ten percent DMSO impregnated disks were
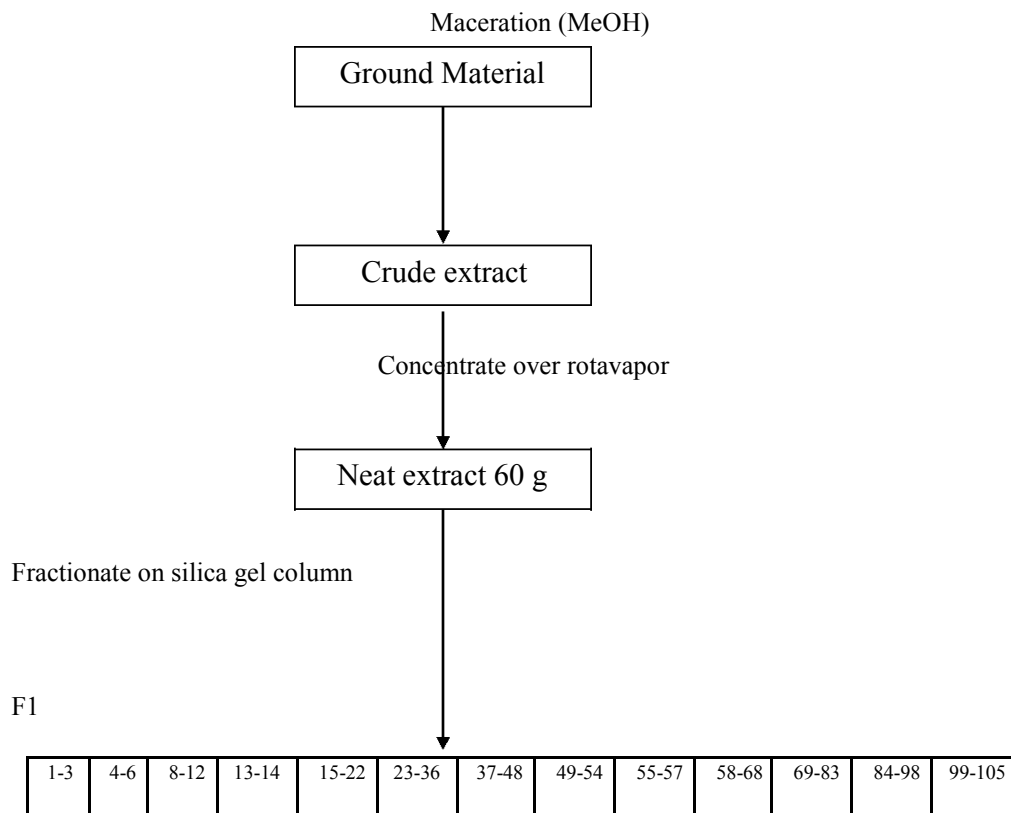
Maceration (MeOH)

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        ┌─────────────────────┐
        │   Ground Material   │
        └─────────────────────┘
                   │
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        ┌─────────────────────┐
        │    Crude extract    │
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                   │
          Concentrate over rotavapor
                   │
        ┌─────────────────────┐
        │   Neat extract 60 g │
        └─────────────────────┘
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Fractionate on silica gel column

F1

1-3	4-6	8-12	13-14	15-22	23-36	37-48	49-54	55-57	58-68	69-83	84-98	99-105

Figure 1. Extraction and fractionation scheme for *A. conyzoides.*

used as negative control and chlarithromycin (5 μg/ml) as positive control. All Petri dishes were left for 15 min at room temperature to allow diffusion of the test fractions and incubated at 37°C for 48 - 72h under microaerophilic conditions. The zones of inhibition were then measured following standard methods. *H. pylori* control strain NCTC 11638 was included in all the experiments.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The fractions that showed significant activities in the primary screen were chosen to assay for subsequent determination of MIC and MBC. The MIC was determined using the broth dilution method (Nariman et al., 2004). Brain heart infusion broth (BHI) was used. One millilitre of the prepared broth was dispensed into test tubes, numbered 2 - 12. A stock solution of the test fraction (2 mg/ml of 10% DMSO) was prepared and a 2-fold dilution carried out on the BHI broth as follows: 1 ml of the solution was dispensed into each of the tubes numbered 1 and 2. Subsequently, from tube 2, serial dilutions were carried out and 1 ml was transferred up to tube 11 from which 1 ml were discarded. Forty-eight-hour culture of each of the test isolates was prepared in sterile BHI broth (10^8 dilution of the broth) . From this dilution, 0.1 ml of the inoculum was transferred into each tube (from tube 1 - 11). The tubes were incubated at 37°C for 48 - 72 h under microaerophilic condition and examined for growth. Tube 12 served as control for sterility of the medium while tube 1 was used to determine the potency of the extract. The last tube in which growth failed to occur was taken as the MIC, which is the lowest concentration (highest dilution) of the extract that inhibits visible growth (no turbidity).

The MBC was determined by subculturing the MIC assay tubes onto fresh solid CAB medium (supplemented with 10% defibrinated sterile sheep blood, *Campylobacter* select tablets and Fungizone) incubated at 37°C under microaerophilic conditions. The highest dilution that yielded no single bacterial colony on the medium was taken as the MBC.

Statistical analysis

Analysis was performed using SPSS version 11.0 and EPI info 2000 software package (CDC, Atlanta, GA, USA). The Fisher's exact test was used to statistically compare if there was any variation in MIC and MBC values of the most potent fractions. P-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

The inhibitory effects of aqueous and methanolic extracts of medicinal plants have been reported (Omer et al., 1998; Olayinka et al., 1992). However these reports conflict those of Umeh et al. (2005) who documented the non inhibitory effects of methanolic extracts of various plants. It is therefore against this background of conflicting reports that we evaluated the activities of potent fractions from crude methanolic extracts of *A. conyzoides* using bioassay guided fractionation on clinical isolates of *H. pylori*.

Table 1. Fractions of *A. conyzoides* obtained through chromatographic fractionation.

Eluent	Fractions
100% Hexane	1 - 25
10% Ethyl acetate	26 - 35
20% Ethyl acetate	36 - 52
40% Ethyl acetate	53 - 66
60% Ethyl acetate	67 - 81
80% Ethyl acetate	82 - 94
100% Ethyl acetate	95 - 102
100% Methanol	103 - 105

Table 2. Fractions of *A. conyzoides* with the same TLC profile.

Fractions	Development system for TLC plate	Bioassay/consistency
1 - 3	100%Hex	Not tested
4 - 6	100%Hex	Not tested
7	100%Hex	Not tested
8 - 12	100%Hex	Not tested
13 - 14	100%Hex	Not tested
15 - 22	100%Hex	Tested-Oily
23 - 30	10%EA/Hex	Tested-crystals
31 - 36	10%EA/Hex	Tested-crystals
37 - 48	20%EA/Hex	Tested-sticky
49 - 54	20%EA/Hex	Tested-very sticky
55 - 57	40%EA/Hex	Tested
58 - 68	40%EA/Hex	Tested
69 - 83	80%EA/Hex	Tested
84 - 98	80%EA/Hex	Tested
99 - 102	100%EA	Tested
103 - 105	100%MeOH	Tested-very sticky

The crude methanolic extract of *A. conyzoides* was fractionated on a silica gel column using solvents of increaseing polarity. Eight fractions were obtained as shown in Table 1.

On further purification on thin layer plates, the fractions were differentiated into sixteen fractions based on the similarity of their TLC profile as shown in Table 2. It is also interesting to note that fractions 23 - 30 and 31 - 36 had crystals, an indication of closeness and also their TLC profiles were almost similar hence equal proportions were combined, homogenized and considered as a single fraction (23 - 36). Plant extracts are complex mixtures which contain many constituents and the biological activity of a given extract probably reflects contributions from a number of the constituents. Consequently, the initial observation of biological activity in a plant extract is typically followed by bioassay-guided fractionation which is designed to isolate and identify the bioactive constituents. This study then is a logical extension of our previous study (Ndip et al., 2007), which reports the anti-*Helicobacter pylori* activity of medicinal plants from Cameroon.

The amount of active components in crude extracts from medicinal plants maybe small or diluted and when fractionated, these components become concentrated and therefore exhibit activity. Thus, fractions from crude medicinal plant extracts have great potential as anti-microbial compounds against microorganisms and can be used as potential sources for antibacterial agents in the treatment of infectious diseases caused by microbes.

The susceptibility of 15 *H. pylori* strains to eleven fractions of *A. conyzoides* using the disk diffusion test is shown in Table 3. The zones of inhibition ranged from 0 – 30 mm. An inhibition zone of 15 mm was chosen as a cut-off point for bacterial susceptibility to plant fractions (Ndip et al., 2007). Some of these fractions were bacteriostatic but not bactericidal while others were neither bacteriostatic nor bactericidal. Their non bacteriostatic or

Table 3. Anti- *H. pylori activity* of fractions of *A. conyzoides.*

|	Zones of inhibition (mm)														
	Isolates														
Fractions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
15 - 22	18	0	0	0	10	10	0	0	0	0	0	0	14	10	16
23 - 30	14	20	10	20	10	20	20	14	18	10	14	14	16	18	14
31 - 36	20	30	0	0	18	14	18	18	0	18	18	0	20	16	10
37 - 48	30	0	0	0	14	0	0	24	10	18	0	24	0	0	6
49 - 54	0	25	0	0	0	0	0	0	0	10	0	12	14	10	10
55 - 57	8	0	8	0	8	10	10	6	18	6	8	0	6	8	6
58 - 68	20	0	0	8	8	10	26	10	8	22	8	0	0	0	0
69 - 83	21	30	20	20	24	24	26	18	0	20	20	24	20	23	0
84 - 98	18	10	18	10	18	10	10	10	0	10	10	16	10	18	18
99 - 102	10	0	0	0	12	18	12	16	6	0	0	10	0	0	0
103 - 105	24	0	0	0	0	0	0	0	20	0	6	16	0	0	0
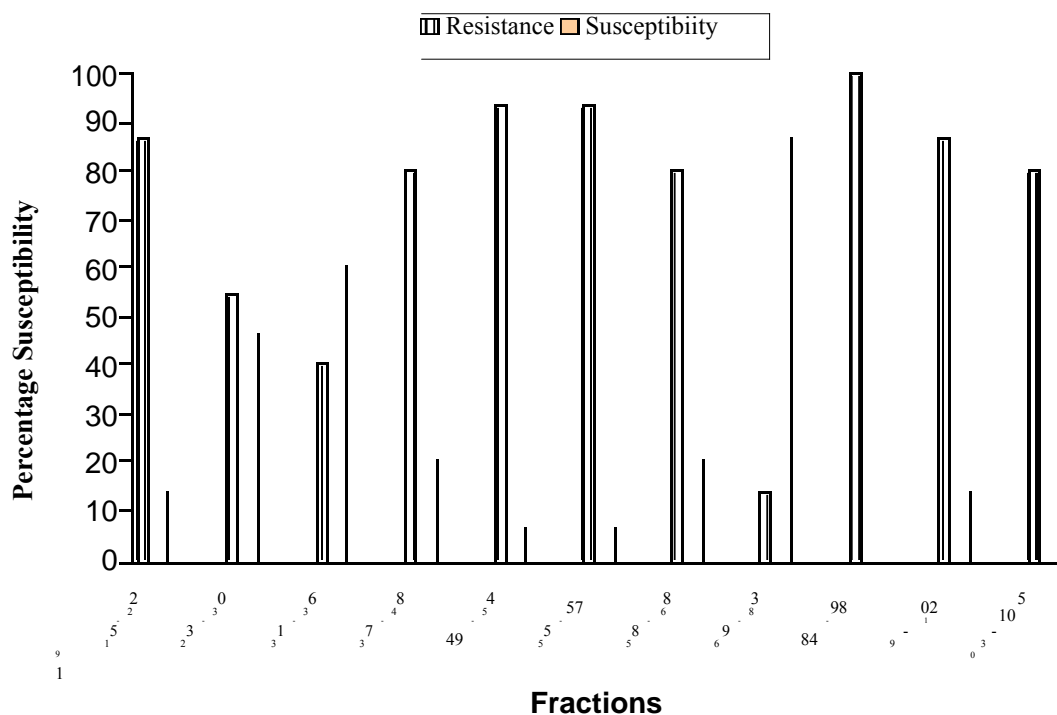
Figure 2. Percentage susceptibility of fractions of *A. conyzoides* to *H. pylori* isolates.

bactericidal activities are represented as zero on the table. Three of the eleven fractions screened exhibited potent antibacterial activity (Figure 2) and these activities were graded accordingly: Fractions 69 - 83 (86.7%) showed high activities, followed by fractions 31 – 36 (60.0 %) with moderate activities and lastly fractions 23 - 30 (46.7%) which showed lower activities.

Although fractions 84 – 98 were eluted with similar solvents to fractions 69 – 83, they demonstrated a weaker activity (40%). We may not be certain as to the reason for this discrepancy but speculate that it could have emanated from differences in evaporation of the solvents from the fractions thus generating fractions with slight differences in potency. The other seven fractions showed weak activities. There was no inhibition of growth with the control (10% DMSO).

The presence of bioactive substances has been reported to confer resistance to plants against bacteria

Table 4. MIC's and MBC's of fractions of *A. conyzoides* against *H. pylori* isolates.

Isolates	MIC (mg/ml) Fractions 23 - 36	MIC (mg/ml) Fractions 69 - 83	MBC (mg/ml) Fractions 23 - 36	MBC (mg/ml) Fractions 69 - 83
1	0.125	0.002	0.250	0.016
2	0.016	0.063	0.063	0.500
3	0.500	0.063	0	1.000
4	0.063	0.125	0.500	0.500
5	0.125	0.063	0.250	0.250
6	0.125	0.031	0.250	0.250
7	0.250	0.002	0.500	0.063
8	0.063	0.125	0.250	1.000
9	0.500	0	0	0
10	0.031	0.250	0.125	0
11	0.031	0.125	0.250	1.000
12	0.063	0.031	0.250	0
13	0.125	0.250	0.250	0
14	0.031	0.500	0.125	0
	0.063	0	0.250	0

(Scinivasan et al., 2001) and may explain the demonstration of antibacterial activity by the different fractions of the plant extracts used in this study. Many pharmacological active compounds have been found in *A. conyzoides* which could be responsible for their antimicrobial effect. They include alkaloids, almarins, essential oils, tannins, agertochromone, 2, 6-dimefloyageratochromone and eugenol. Also reported are flavonoids such as conyzoigun and dotriaconthene (Oliver-Bever, 1980). Phenol and phenolic esters, which are known disinfectants as well as other antimicrobials and insecticidals have also been reported (Okwori et al., 2007; Moreira et al., 2007). Plants have also been reported to have different constituents depending on the climatic conditions in which they are growing (Ngemenya et al., 2006).

Silica gel column fractionation showed that fractions eluted using 100%Hex-Hex/EA 20% and Hex/EA 80% contained active anti-*H. pylori* compounds. These results correlate to those of Iqbal et al. (2007) who documented the fungicidal activity of the *n*-hexane fractions of *A. conyzoides.* Hexane: ethyl acetate (20:80) fractions, (69 - 83) were the most potent of all the fractions with 86.7% activity. This was followed by fractions 31 - 36 with moderate activities (60.0%) and 23 - 30 with low activities (46.7%). Since fractions 31 - 36 and 23 - 30 were so close with both having crystals and showing almost similar TLC profile, it was assumed that they had the same active component; hence it could have been a smear of activity through the column. So these two fractions were combined and considered as one (Fractions 23 - 36).

Also, from the results, we could suggest that some of the principal active substances of the plant must be lipid soluble since fractions 23 - 36 were obtained between elution with 100%hexane and hexane (80): ethyl acetate (20). Hexane is a non- polar solvent which must have easily extracted the lipid soluble phytochemicals such as essential oils and coumarins and diffusion rates of these phytochemicals within the agar matrix may explain the wider zone of inhibition observed (Cowan, 1999). In addition, the hexane (20): ethyl acetate (80) fractions (fractions 69 - 83) showed high activities, suggesting that some of the active components had intermediary polarity since Hex/EA80% is an intermediate polar solvent.

Eloff (1998) had earlier indicated that crude extracts or mixtures of compound-rich residues are used for initial screening of plants for anti- microbial activities followed by TLC with several solvent systems for the elution of enormous water and organic solvent soluble anti-microbial compounds, which is in line with our study. It is also important to note that the polarity of solvents will affect the quantity and types of bio-molecules eluted from extracts; more polar solvents generally elute more active molecules (Eloff, 1998).

Table 4 shows the results of MIC and MBC determinations of fractions on the test isolates. The MIC and MBC values for fractions 23 - 36 ranged from 0.016 - 0.500 mg/ml and 0.063 - 0.500 mg/ml respectively; while those for fractions 69 - 83 ranged from 0.002 - 0.500 mg/ml (MIC) and 0.016 - 1.000 mg/ml for MBC. The lowest MIC values of 0.002 and 0.016 mg/ml were obtained from fractions 69 - 83 and 23 - 36 respectively; while the lowest MBC of 0.016 and 0.063 mg/ml were exhibited by fractions 69 - 83 and 23 - 36 respectively. The MIC and MBC values of the most potent fractions were statistically compared to determine any variation in

their efficacy against the isolates. No statistically significant difference (p > 0.05) was observed between the MIC and MBC values of these fractions. However, there was a statistically significant difference (p < 0.05) in the potency of the different fractions on the different strains tested, both for the MIC and MBC.

The MIC values were found to be lower than the MBC values suggesting that the fractions were bacteriostatic at lower concentrations and bactericidal at higher concentrations. The lowest MIC and MBC recorded were 0.002 and 0.016 mg/ml respectively. The low MIC values observed for these fractions are a good indication of high efficacy against the organism at low concen-trations. The MBC results varied considerably from the MIC. These variations may suggest that the MBC values obtained from plate cultures with dilutions of the fractions is more reliable and accurate compared to MIC results obtained visually using turbidity as an index.

Conclusion

The results of this study confirm the rationale behind the use of this plant in traditional medicine and also support the traditional application of the plant extracts. Fractions 69 - 83 obtained by eluting with hexane: ethyl acetate (20:80) exhibited high activities followed by fractions 23 - 36 obtained between elution with 100% hexane and hexane (80): ethyl acetate (20) with moderate activities. The plant is therefore a potential source for antibacterial agents for the treatment of *H. pylori.* Further investigation using bioassay guided fractionation to isolate and characterize the active constituents should be conducted.

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