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Full Length Research Paper

In vitro anti-*Helicobacter pylori* activity of *Lycopodium cernuum* (Linn) Pic. Serm

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Helicobacter pylori*, a gram negative microaerophilic bacterium is a major etiological agent in duodenal, peptic and gastric ulcers. In this study, gastric biopsy samples were obtained from patients presenting with gastroduodenal complications. *H. pylori* was isolated from the specimens following standard microbiology procedures, and isolates subjected to pure fractions of *Lycopodium cernuum* extracts for antimicrobial assays. Extracts were fractionated by partition chromatography with solvents of increasing polarity to obtain pure fractions. 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 mm. Of the 5 fractions obtained, the hexane (Hex) fraction was the most active. The lowest MIC and MBC recorded for the hexane (Hex) fraction were 0.016 and 0.125 mg/mL, respectively. There was no statistically significant difference (P>0.05) in the potency of the fraction on the different bacterial strains tested, both for the MIC and MBC. It is concluded that this plant may contain compounds with therapeutic activity, which may be found in the Hex fraction (100%).

Key words: Antibacterial, *Lycopodium cernua*, *Helicobacter pylori,* minimum inhibitory concentration, minimum bactericidal concentration.

INTRODUCTION

Helicobacter pylori is a gram negative helical rod that colonizes the human gastric mucous layer (Marshall and Warren, 1983; Hayama et al., 2005). It chronically infects the gastric mucosa causing gastritis in more than 50% of the human population. The infection can lead to the development of peptic ulcer (Sontaq, 1997) and gastric mucosa-associated lymphoid tissue lymphoma (Du and Isakson, 2002), and an increased risk of gastric cancer in humans (Forman et al., 1994; Uemura et al., 2001). Infections have been reported to be higher in the developing than in developed countries. Studies have documented high prevalence in Africa including Cameroon (Ndip et al., 2004; Lwai-lume et al., 2005).

Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high risk populations (Sepulveda and Coelho, 2002). However, resistance of the organism to antibiotics is a growing global concern which needs public health atten-

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tion. The emerging resistance to antibiotics, especially metronidazole and amoxicillin limits their use in the treatment of infections (Smith et al., 2001; Sherif et al., 2004). This problem is encountered more in Africa (Asrat et al., 2004; Lwai-lume et al. 2005; Ndip et al., 2008). The development of safe anti-*H.pylori* compounds is therefore desirable. Studies have documented that some medicinal plant extracts have antibacterial activities, including *H. pylori* (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 *Lycopodium cernuum* exhibited potent *H. pylori* activity.

Medicinal plants constitute an effective source of both traditional and modern medicines. Herbal medicine has been shown to have profound utility, and about 80% of rural populations depend on it for their primary health care (Sofowara, 1993). Over the years, the WHO has advocated the need for interaction between modern and traditional medicines with a view to exploiting and identifying compounds that could provide safe and effective remedies for ailments of both microbial and non-microbial origins (WHO, 1978).

L. cernuum, a member of the family Lycopodiaceae is widely distributed in the tropical climates of the world including Africa (Burhill, 1935; Adjanohoun et al., 1996). It is a prostrate plant, which is rare at low altitudes and abundant at medium and high levels. The stems are stout, creeping, 30 to 100 centimeters long, and leafy throughout. The primary branches are rigidly erect, 20 to 60 centimeters long or more, much branched in the upper portion; the lower branches are divided and short, spreading or ascending, pendulous towards the tips. The leaves are inserted all around the stems and branches, crowded, narrowly linear-subulate, 2 to 3 millimeters long (Burhill, 1935).

According to Burkill (1935), the plant is used medicinally throughout Malaysia for external application; a decoction of it is used as a lotion in beriberi, coughs and uneasiness of the chest, diuretic, in rheumatism, dysentery, diarrhea and tenesmus. The leaves have been reported to be used for the treatment of vertigo (Lin, 2005). Other medicinal applications of the plant include its astringent effect, the treatment of fongoid ulcer, hay fever, abraded skin and fungal and bacterial infections (Banenjee and Sen, 1980; Combie and Ash, 1994; Adjanohoun et al., 1996; Wiart et al., 2004; Ndip et al., 2007).

Many natural occurring compounds found in medicinal plant extracts have also been shown to possess antimicrobial activities that inhibit the growth of microorganisms (Larson et al., 1996; Cowan, 1999; Seaberg et al., 2003 Lin et al., 2004). The present study evaluates the antimicrobial activity of fractionated extracts of *L. cernuum* in a bid to isolate and identify the active constituents of this plant. 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.

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, Cameroon. 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). 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^{O}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., 2007).

Preparation of crude extracts

L cernuum 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, Cameroon. Voucher specimens have been deposited at the National Herbarium, Yaounde with reference number 17385/SRF/Cam.

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 (1.6 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.

Fractionation of crude extracts

Fractionation of the crude methanolic extract was done by solvent partitioning following standard methods (Marvin and Hewitt, 2007). Forty grams of crude methanol extract was dissolved in 400 mL $MeOH/H_20$ (3/7) mixture. The resulting solution was partitioned with hexane (Hex) (3 x 400 mL), CH_2Cl_2 (3 x 400 mL) and ethyl acetate (EA) (3 x 400 mL), respectively (Figure 1).

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 extract. The test was performed according to the recommended standards of the National Committee for Clinical La-

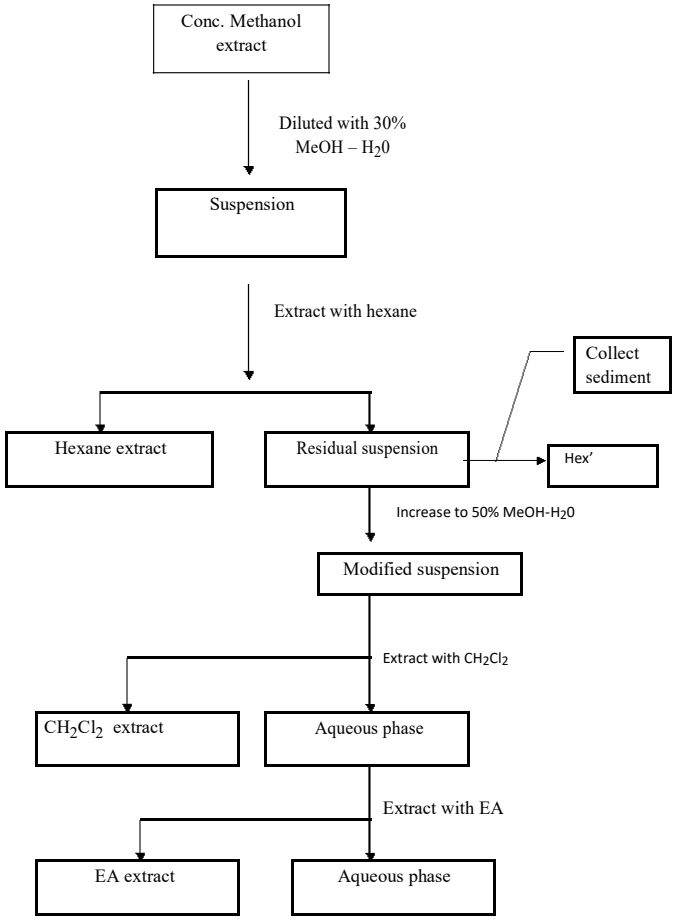

Figure 1. Extraction and Fractionation scheme for extract of *L. cernuum.*

boratory 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 used as negative control. All Petri dishes were left for 15 min at room temperature to allow diffusion of the test fractions and incubated at 37^{o}C for 48-72 h 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 fraction that showed significant activity in the primary screen was 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 tubes 1-11). The tubes were incubated at 37^{o}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^{o}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 (Chicago 2001) and EPI info (2005). The Fisher's exact test was used to statistically compare if there was any variation in MIC and MBC values of the most potent fraction against the different strains. P-values < 0.05 were considered significant.

RESULTS

Solvent partitioning

Fractionation of *L. cernuum* by partition chromatography yielded 5 whole neat fractions as shown in Table 1. The Hex fraction, obtained from partitioning with hexane; the Hex' fraction, which are residual sediments collected from the aqueous phase after partitioning with hexane and modified with MeOH/H_2O; the Hex'' fraction, which was a homogenized mixture of Hex and Hex' fractions in equal proportions; the CH_2Cl_2 fraction, from partitioning with methylene chloride and lastly the EA fraction, from partitioning with ethyl acetate (not used for the bioassay because its quantity was almost negligible).

Antimicrobial susceptibility testing

The susceptibility of 15 *H. pylori* strains to four fractions of *L. cernuum* using the disk diffusion tests are shown in

Table 1. Fractions of *L. cernuum* obtained through solvent partitioning.

Fraction	Solvents used for partitioning	Bioassay/consistency
Hexane (Hex)	100%Hex	Tested, sticky in nature
Hexane'(Hex')	MeOH/H_2O(3/7)	Tested, powdery
Hexane''(Hex'')	Homogenized from Hex and Hex'	Tested, powdery
Methylene chloride (CH_2Cl_2)	100% CH_2Cl_2	Tested, very sticky in nature
Ethyl acetate(EA)	100%EA	not tested

Table 2. Anti-*H. pylori* activity of fractions of *L. cernuum.*

	Zones of inhibition (mm)														
Fraction	**1***	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Hex	30	10	12	16	10	14	20	20	26	30	12	0	24	16	20
Hex'	0	0	0	0	0	0	0	6	10	10	15	0	8	8	0
Hex''	0	0	0	0	0	0	14	16	10	20	20	0	20	8	0
CH_2Cl_2	24	20	16	14	20	6	12	14	18	24	14	0	20	14	10

**H. pylori* isolates.

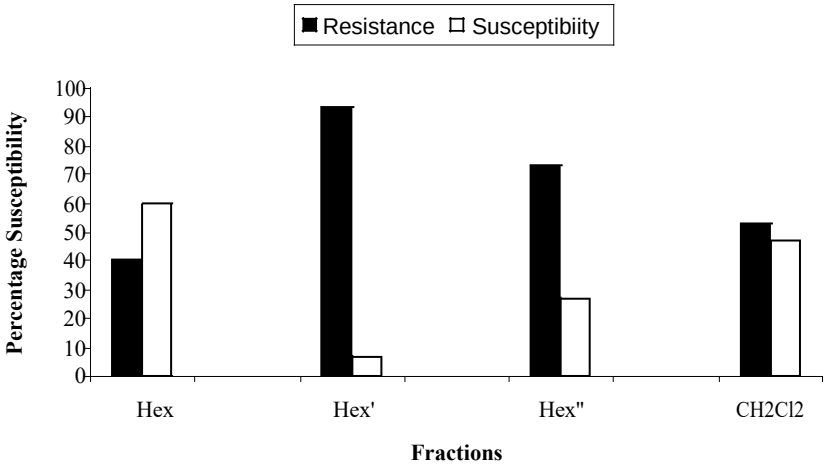

Figure 2. Percentage susceptibility of fractions of *L. cernuu* to *H. pylori* isolates.

Table 2. 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). The Hexane fraction exhibited moderate anti-bacterial activity (60%), while the other three fractions showed weak activities (Figure 2). There was no inhibition of growth with the control (10% DMSO).

Determination of MIC and MBC

Table 3 shows the results of MIC and MBC of Hex fraction of *L. cernuum* on the test isolates. The MIC and MBC values ranged from 0.016-1.000 and 0.125-1.000 mg/mL, respectively. The MIC and MBC values of the Hex fraction were statistically compared to determine any variation in their efficacy against the isolates. There was no statistically significant difference ($p>0.05$) in the potency of the fraction on the different strains tested, both for the MIC and MBC. Some of these fractions were bacteriostatic but not bactericidal while others were neither bacteriostatic nor bactericidal. Their non bacterio-static or bactericidal activities are represented as zero on the table.

DISCUSSION

Plant extracts are complex mixtures which contain many constituents and the biological activity of a given plant

Table 3. MIC and MBC of Hex fraction of *L. cernuum* against *H. pylori* isolates.

Isolate	MIC (mg/mL)	MBC (mg/mL)
1	0.063	0.500
2	1.000	1.000
3	1.000	0
4	0.250	0
5	1.000	0
6	0.500	0
7	0.031	0.250
8	0.125	1.000
9	0.031	0.125
10	0.125	0
11	1.000	0
12	0	0
13	0.016	0.125
14	0.500	0
15	0.125	0

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 the previous study (Ndip et al., 2007), which reports the anti-*H. pylori* activity of *L.cernuum.*

The inhibitory effects of aqueous and methanolic extracts of medicinal plants have been reported (Tignokpa et al., 1986; Olayinka et al*.,* 1992; Omer et al*.,* 1998). However these reports conflict those of Elegami et al. (2001). and 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 *L. cernuum* using bioassay guided fractionation on clinical isolates of *H. pylori*.

Results obtained in the present study revealed the antimicrobial efficacy of fractions of *L. cernuum*. The presence of bioactive substances has been reported to confer resistance to plants against bacteria (Scinivasan et al., 2001) and may explain the demonstration of antibacterial activity by the different fractions of the plant extracts used in this study. The Hex fraction was the most potent demonstrating moderate activities of about 60%. The Hex' fraction had the lowest activity and when combined with the Hex fraction (Hex'' fraction), its activity reduced drastically suggesting that the Hex' fraction had components which were antagonist to or interfering with the activities of the components in the Hex fraction.

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 (Adeniyi and Anyiam, 2004). Thus, fractions from crude plant extracts have great potential as antimicrobial compounds against microorganisms, and can be used as potential sources for antibacterial agents in the treatment of infectious diseases caused by microbes. Pharmacological active compounds such as alkaloids and serratene triterpenes have been found in *L. cernuum* which may be responsible for their antimicrobial effect (Combie and Ash, 1994; Zhang et al., 2002).

Hexane is a non-polar solvent and since the hexane fraction showed the highest activities, we could suggest that some of the principal anti-*H. pylori* components of this plant were lipid soluble. In addition, the CH_2Cl_2 fractions showed considerable activity, which were lower to those exhibited by the hexane fraction suggesting that partitioning with hexane was not done exhaustively (only 3 times). It is likely that some of the excess active components must have been dissolved by CH_2Cl_2 since its polarity is intermediate. This corroborates previous findings (Desta, 1993; Eloff, 1998).

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 MIC and MBC of the Hex fraction ranged from 0.016-1.000mg/mL and 0.125-1.000mg/mL, respectively. However, there was no statistically significant difference ($p<0.05$) in the potency of the fraction on the different strains tested, both for the MIC and MBC. The low MIC values observed for these fractions are a good indication of high efficacy against the organism at low concentrations. The MBC results varied considerably from the MIC. These variations may suggest that the MBC values obtained from plate cultures with dilutions of the fraction is more reliable and accurate compared to MIC results obtained visually using turbidity as an index.

Conclusions

Based on our results, solvent partitioning of the extract yielded 5 fractions with the hexane fraction (100% hexane) exhibiting the highest activity. These results confirm the rationale behind the use of this plant in traditional medicine and also support the traditional application of the plant extracts. 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, which is already receiving attention in our group, should be conducted.

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