SUSCEPTIBILITY OF *HELICOBACTER* AND *CAMPYLOBACTER* TO CRUDE EXTRACTS PREPARED FROM PLANTS USED IN CAMEROONIAN FOLK MEDICINE

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Summary

Crude extracts prepared from plants used in the Cameroon folk medicine, were tested for their anti microbial properties against *Helicobacter pylori* (*H. pylori*) CCUG-39500 and *Campylobacter jejuni/coli* (*C. jejuni/coli*) CPC-022004 using the well dilution and the agar diffusion techniques. The diameter of inhibition resulting from the diffusion of the extracts into the agar ranged from 8 to 42 mm, and the best acting extracts were from the barks of *Pleiocarpa* sp, *Rinorea oblongifolia*, *Drypetes gossweileri* and *Parkia biglobosa*. The lowest Minimum Active Quantity (0.63 mg) was obtained with the extract from *Drypetes gosseweileri*. Minimum Inhibitory Concentration ranged from 0.78 to 50 mg/ml and the Minimum Bactericidal Concentration ranged from 0.78 to 100 mg/ml. The ten best bactericidal extracts were further evaluated for their killing rate and the ET_{100} values (Exposure time within which the viable count (CFU/ml) drops to the lowest detectable limit after exposure to the antimicrobials) were determined. ET_{100} values obtained were all less than 10 hours; an ET_{100} value of 6 hours was obtained with five extracts, suggesting the possible presence of multiple antimicrobial components in these extracts. The extracts tested may constitute sources of new anti *Helicobacter* agents.

Keywords: *Helicobacter pylori*, *Campylobacter jejuni/coli*, medicinal plants

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Introduction

Due to its high acidity, the stomach mucosa was believed not to harbour a resident microbial flora. However, the discovery and description of *Helicobacter pylori* (*H. pylori*) in 1983 by Warren and Marshal (1), overturned the traditionally-held beliefs in the physiopathology and the clinical management of peptic ulcer disease. This gastric mucosa-adapted Gram negative bacterium has emerged as an important human pathogen, associated with gastro-duodenal ulcers and gastric malignancies (2-5). *H. pylori* was the first bacterium to be classified as a group 1 carcinogen by the International Agency for Cancer and the World Health Organisation (6, 7). Though some infected individuals may be asymptomatic, colonisation with this microaerophilic bacterium is always associated with histological gastritis and eventual progression towards active chronic gastritis, gastric carcinoma as well as Mucosa Associated Lymphoid Tissue (MALT) lymphoma is a constant threat (8). *H. pylori* eradication from the gut of infected individuals therefore constitutes a key recommendation for the management of peptic ulcer disease (9, 10). The treatment consists of a combination therapy which includes antibiotics, antacids and antisecretory agents (10-13). However, *H. pylori* strains resistant to the commonly used antibiotics have emerged and are becoming problematic world wide (2, 8, 14, 15). Moreover, side effects associated with the use of the antibiotics of choice lower patient compliance and this often leads to treatment failures. For these reasons, *H. pylori* is now viewed as one of the human pathogens in need of new therapeutic agents.

Throughout history, medicinal plant-based remedies have been used with varying degrees of success for the management of infectious diseases, and a number of compounds from plants have been shown to have potent antimicrobial properties (7, 16, 17). Thus scientific investigations in search of anti *H. pylori* medicinal plant preparations constitute an important approach to the problem of antibiotic resistant strains. The present study was therefore designed to assess the *in vitro* sensitivity of *H. pylori* and a related organism, *Campylobacter jejuni/coli,* to 21 extracts prepared from plants used in Cameroonian folk medicine for the traditional treatment of stomach disorders and complaints symptomatic of peptic ulcer disease (18).

Materials and methods

Plant materials

Details about plant materials used are provided in Table 1. The plants were identified at the National Herbarium, Yaounde. Stem barks were used for all the plants except for *Bridelia micrantha* and *Glossocalyx brevipes* (whole stem), *Garcinia kola* (Stem bark and seeds) and *Ocimum suave* (leaves). The specific plant parts were sun-dried until constant weight. The dried plant parts were ground to a fine powder which was extracted using a 1:1 solution of methanol/methylene chloride except for *Alstonia boonei, Parkia biglobosa, Picralima nitida* and *Voacanga africana* which were 1:1 ethanol/water extracts and *Ocimum suave* which was the water soluble methanol extract (19). The methanol/methylene chloride solutions were dried using a rotavapor, while the ethanol/water extracts were evaporated to dryness using a convection air oven at 40^{o}C.

Culture media, reference antibiotics and culture media supplements.

Brain Heart Infusion (CM0225), Columbia agar (CM0331), Horse Serum (SR0035), Lacked Horse Blood (SR0048), Vitox supplement (SR0090), Amoxicillin (10 µg) and Metronidazole (5 µg) disks were from Oxoid, Basingstoke, England. Amoxicillin Capsules (Maxheal Pharmaceuticals, India) were purchased from a local pharmacy.

Test organisms and culture conditions

Lyophilized *H. pylori* (strain CCUG 39500) was obtained from the Culture Collection, University of Göteborg (CCUG). The strain was revived using Brain Heart Infusion supplemented with 10% horse serum (BHI-serum). The identity of the microbe was confirmed using the rapid urease and the catalase/oxydase tests. The *Campylobacter jejuni/coli* (CPC-022004) was isolated locally from human. The microbes were maintained under microaerophilic conditions (CampyGen CN0025 in an air-tight 2.5 L anaerobic jar (Oxoid)) at 37 °C on Columbia agar supplemented with 5 % (v/v) lacked horse blood and 1% (v/v) Vitox (CA-Vitox), and were sub-cultured every 72 to 96 hours to a fresh medium. The antimicrobial diffusion test was performed on 5 to 6 mm thick CA-Vitox. Test organism inocula were prepared by suspending 72-hour colonies in 2 ml of sterile distilled water to make a turbidity standard of McFairland N° 2 and N° 1, respectively, for *H. pylori* and *C. jejuni/coli* (20). These turbidity standards of about 10^9 and 10^8 CFU/ml, respectively, for *H. pylori and C. jejuni/coli* produced semi-confluent or confluent growth. The cell suspension was evaluated using the standard plate count method (21).

Antimicrobial Well Diffusion Test

One hundred microlitres of cell suspension from the standard inoculum were plated on CA-Vitox and 6 mm wells were drilled into the agar. Fifty microlitres of extract (400 mg/ml) prepared using DMSO/H_2O (25 % v/v) were dropped into the wells. The DMSO/H_2O solution served as the negative control. Metronidazole (5 µg) and Amoxicillin (10 µg) served as reference antibiotics. The plates were incubated under microaerophilic conditions at 37 °C for 72 hours. Extracts that showed a diameter of inhibition ≥ 8 mm were considered to be active (22). The active extracts were further diluted serially using sterile DMSO/H_2O (25 % v/v) to obtain 9 serially two-fold decreasing concentrations which were re-tested in triplicate and the results expressed as a mean diameter of inhibition zone. The Minimum Active Quantity (MAQ) for each extract was determined as the minimum quantity that produced an inhibition zone ≤ 8 mm.

Antimicrobial Agar Dilution Test

Extract solutions (200 mg/ml) were serially diluted two-fold using sterile DMSO/H_2O (25 % v/v) to obtain 9 decreasing concentrations. The solutions were held at 70 °C for 55 to 60 minutes to decontaminate and when cooled to 40 to 45 °C, they were dispensed in duplicate 2 ml aliquots into test tubes containing 2 ml of extract-free double strength CA-Vitox in a 45 °C water bath. Concentrations of Amoxicillin ranging from 0.8 to 0.0031 mg/ml were treated in the same way. The contents were vortexed and poured into sterile petri dishes (4 cm diameter). One plate for each duplicate served as the negative control while the other was used for the test. The test plates were inoculated with 0.1 ml of the standard microbe inoculum and all the plates were incubated as described above for 72 hours. After incubation, the lowest concentration of extract that prevented visible growth was considered as the MIC

(7). The surface of each plate that showed no visible growth was washed with 100 µl of sterile distilled water and the resulting suspension was plated on an extract-free plate of CA-Vitox. The plates were incubated for 72 hours and the lowest concentration corresponding to the plate that yielded no growth was considered as the Minimum Bactericidal Concentration (MBC).

Killing rate determination

Three millilitres of BHI-serum containing 50 mg/ml of extract were inoculated with 0.3 ml of the standard cell suspension. The inoculated broth was incubated (cap loose) under microaerophilic conditions at 37°C and 0.1 ml portions removed after 0, 2, 4, 6, 8, 10, 12 and 24 hours for viable cell count. The portion removed at each time point was serially diluted 100 fold in BHI, and each dilution plated (0.1 ml) in triplicate on CA-Vitox agar plates. Colonies were counted after 72 h of incubation and the mean viable count (CFU/ml) determined (6). From the mean viable count, the killing rate curve was plotted as the Log_{10} CFU/ml as a function of time of contact with the test extract. The ET_{100} (Exposure time within which the viable count drops to the lowest detectable limit) values were then determined graphically.

Results

Antimicrobial well diffusion assay

The preliminary well diffusion test results (Table 1) show the *in vitro* anti *Helicobacter/Campylobacter* properties of the various plant extracts studied. Extracts that inhibited *H. pylori* growth also inhibited *C. jejuni/coli*. However, extracts prepared from the barks of *Sapium ellipticum*, *Aoranthe nalaensis* and the Ethanol / Water extract of the bark of *Voacanga africana* were active only against *C. jejuni/coli* (Table 1).

The inhibitory activity of all the extracts was dose-dependent (Tables 2 and 3). At the highest dose (10 mg), eight of the extracts tested on *H. pylori* showed a diameter of inhibition zone (DI) $\leq$ 24 mm, same as for Amoxicillin (10 µg). These included the *Pleiocarpa sp*, *Rinorea oblongifolia*, *Drypetes gosseweileri* (*D. gosseweileri*), *Garcinia mannii* (*G. mannii*), *Parkia biglobosa* (*P. biglobosa*), *Ocimum suave* (*O. suave*), *Markhamia lutea* (*M. lutea*) and *V. africana*. *Pleiocarpa sp* was the most active, with a DI of 42 mm (Table 2), while the least active extract was *Heisteria trillesiana* (DI= 9 mm at the dose of 10 mg). On *C. jejuni/coli*, *Alstonia boonei* (*A. boonei*), *M. lutea* as well as the extracts that were highly active against *H. pylori* (except for *O. suave*) showed DI values ≥24 mm. Extracts from *D. gosseweileri*, *Pleiocarpa sp* and *R. oblongifolia* showed DI values ≥31 mm, the DI value of Metronidazole (Table 3). The most active extract against *C. jejuni/coli* was from *D. gosseweileri* (DI= 42 mm) and the least active was from *Heisteria trillesiana* (*H. trillesiana*) (DI= 8 mm).

The MAQ values ranged from 0.63 mg to 10 mg depending on the extract. The lowest MAQ value (0.63 mg) was obtained with *D. gosseweileri* extract on both *H. pylori* and *C. jejuni/coli* and with *P. biglobosa* stem bark extract on *C. jejuni/coli* (Tables 2 and 3).

Table 1: Anti microbial activity of plant extracts against *H. pylori* and *C. jejuni/coli*

Botanical name	**Family**	**Herbarium N°**	**Antimicrobial activity**		**Yield (w/w)**	**Bioactive constituents**	**Documented Ethno-medical uses**
			Against *C. jejuni/coli*	**against *H. pylori***			
Alstonia boonei De Wild	*Apocynaceae*	HNY N° 1943	+	+	4.10%	Anti-inflammatory triterpenoids. (33)	Antipyretic, analgesic anthelminthic, Belly pains, snake bite, abscess. (18, 34)
Aoranthe nalaensis (De Wild.)	*Rubiaceae*	2496/ SRF/CAM	+	-	2.05%	Not cited in literature	none
Aulacocalyx jasminiflora	*Rubiaceae*	2677/SRFK	+	+	1.75%	Not cited in literature	none
Bridelia micrantha Baill.	*Euphorbiaceae*	3179/SRFK	+	+	1.60%	Not cited in literature	Cytotoxic, Anti diarrhoea Constipation, intestinal worms, antimalarial (35, 36)
Drypetes gossweileri S. Morre	*Euphorbiaceae*	5746/SRF/CAM	+	+	6.55%	Diterpenois (Gossweilone) (37)	Ocular irritation Respiratory problems, antioxidant properties (38)
Garcinia kola Heckel.	*Clusiaceae*	9815/SRF/CAM	Bark (+)	+	6.07%	Biflavonoids, xanthones benzophenones, kolaviron, benzophenone and flavanones (Farombi *et al.,* 2005)	Antiparasitic, antimicrobial, bronchitis, throat infections, colic, chest colds and cough, liver disorders, antiulcer anticancer (18, 39-41)
			Seed (+)	+	3.89%		
Garcinia mannii Oliv	*Clusiaceae*	21331/SRF/ CAM	+	+	3.05%	Not cited in literature	none
Glossocalyx brevipes Benth. Var	*Monimiaceae*	5862/ SRF/CAM	+	+	3.70%	Aristololactam, Liriodenine (42)	Anti malarial and antipyretic (42)
Heisteria trillesiana Pierre	*Olacaceae*	19793/SRF/ CAM	+	+	2.50%	Not cited in literature	Roots and stem bark used for abdominal pains (43)
Hypodaphnis zenkeri Stapf	*Lauraceae*	1768/SRFK/ CAM	+	+	1.95%	Not cited in literature	none
Khaya senegalensis A Juss	*Meliaceae*	HNY N° 6483	+	+	4.78%	glycosides, saponins, tannins, terpines, steroids and limonoids (44, 45).	Antiulcer, Diarrhoea, typhoid fever, dysentery, anticonvulsant, vermifuge syphilis, vaginal discharge, haemorrhoids, analgesic and anti inflammatory (43-45)
Markhamia lutea (Benth.) K.Schum	*Bignoniaceae*	1970/SRFK	+	+	4.10%	Phenylpropanoid glycosides, verbascoside and isoverbascoside, luteoside, luteoside, luteoside (46)	Antipyretic, cough and chest pain antiviral (46)
Ocimum suave	*Lamiaceae*	HNC:6077/6914(R. Letouzey)	+	+	2%	Include eugenol and mono- and sesquiterpenoids (30)	Anticathartic, febrifuge, respiratory affections, menstrual problems, stomach-ache, antiulcer analgesic (19, 47-49)
Parkia biglobosa Benth	*Mimosaceae*	HNY N°58 980	+	+	4.52%	Cardiac glycosides, steroids, alkaloids, transferulic acid, cis-ferulates gallacotechin derived compounds (51, 52)	Cough, febrifuge, toothache, wound ulcers, stomach-ache, conjunctivitis, leprosy (18, 50)

Table 1 continued.

Botanical name	Family	Herbarium N°	Antimicrobial activity		Yield (w/w)	Bioactive constituents	Ethno-medical uses
			Against *C. jejuni/coli*	against *H. pylori*			
			+	+	2.55%		
Pausinystalia macroceras (K Schum)	*Rubiaceae*	HNC / 49741	+	+	1.60%	Yohimbine (53)	Analgesic, sexual disorders, aphrodisiac (Data obtained from the Cameroon National Herbarium)
Picralima nitida Stapf	*Apocynaceae*	HNY N° 2138	+	+	3.91%	Akuammiline, picraline , picralicine alkaloids, Pseudo-akuammigine alkaloids (54, 55)	Fruits and bark used for fever, stomach ulcer, and belly pains, male sexual impotence, Hypoglyceamic, and for hypertention (18, 56)
Pleiocarpa sp	*Apocynaceae*	HNC / 64529	+	+	4.65%	Not cited in literature	Antipyretic antimalrial (Data obtained from the Cameroon National Herbarium)
Rinorea oblongifolia C.H Wight	*Violaceae*	5420/SRFK	+	+	1.50%	Not cited in literature	none
Sapium ellipticum (Hochst) Pax	*Euphorbiaceae*	26562/SRF/ CAM	+	-	3.55%	Not cited in literature	Bark and root used for generalised body pains, cough, constipation, dropsy and leprosy (43)
Schumanniophyton magnificum K Schum	*Rubiaceae*	2600/SRFK	+	+	2.55%	Piperidino-chromone alkaloid, Chromone alkaloids (59,60)	Anti viral, Antivenin, Anti snakebite (57-59)
Voacanga africana Stapf	*Apocynaceae*	HNC/1949	Et/H_2O (+)	-	3.08%	Ibogaine, Voacamidin Voacangine, hydroxyindolenine, Tabersonine hydrochloride (24, 61-63)	Stomach problems, Antidiarhoea, carious teeth, gonorrhoea, anti ulcer, (25,64)
			Met/Cl_2CH_2 (+)	+	8.90%		
	Amoxicillin (10 ug/ml)		+	+			
	Metronidazole (5 ug/ml)		+	+			

Antimicrobial agar dilution assay

The results of MIC and MBC determinations reported in Tables 2 and 3 indicate that 14 and 15 active extracts exhibited both inhibitory and bactericidal effects, on *H. pylori* and *C. jejuni/coli,* respectively. The MIC and MBC values of the extracts tested on *H. pylori* ranged, from 0.39 to 50 mg/ml and from 0.78 to 25 mg/ml, respectively. When the MIC and MBC values were put together, the best anti *Helicobacter* extracts were *Pleiocarpa sp* (0.39 mg/ml, 0.78 mg/ml), *P. biglobosa* (0.78 mg/ml, 0.78 mg/ml), *K. senegalensis* (0.78 mg/ml, 1.56 mg/ml) and *D. gosseweileri* (0.78mg/ml, 3.126mg/ml). *Schumanniophyton magnificum*, *Hypodaphnis zenkeri* and *H. trillesiana* extracts were poorly active and showed only bacteriostatic activity (Table 2). Extracts that showed high activity against *H. pylori* were equally the best anti *C. jejuni/coli*. The lowest MIC and MBC values against *C. jejuni/coli* were obtained with extracts *D. gosseweileri* (0.78 mg/ml, 3.13mg/ml) and *Pleiocarpa sp* (1.56 mg/ml, 3.12 mg/ml) (Table 3). The MIC and the MBC values obtained with Amoxicillin on the two test organisms were 0.0062 mg/ml and 0.0125 mg/ml, respectively (Tables 2 and 3).

Table 2: Mean (±SD) diameter of Inhibition (mm) zones, Minimum Active Quantity (mg), Minimum Inhibitory Concentration (mg/ml) and the Minimum Bactericidal Concentration (mg/ml) of crude plant extracts active against *H. pylori*

	Extract concentration							
Extract name :	0.63mg	1.25mg	2.5mg	5mg	10mg	**MAQ (mg)**	**MIC (mg/ml)**	**MBC (mg/ml)**
Alstonia boonei	0	<8	11.0±0.8	17.8±1.0	20.7±0.6	2.5	3.125	6.25
Aulaccoclalyx jasminiflora	0	0	0	9.0±1.0	17.7±0.6	5	12.5	nba
Bridelia micrantha	0	0	<8	10.0±1.0	14.8±0.8	5	3.125	nba
Drypetes gosseweileri	9.7±0.6	14.2±0.3	17.7±0.6	25.2±0.6	31.2±0.3	0.63	0.78	3.125
Gacinia kola seed	0	8.7±1.2	11.0±0.9	15.0±0.1	20.5±1.3	1.25	1.56	3.125
Garcinia kola stem bark	0	0	0	8.0±0.0	11.5±0.1	5	3.125	12.5
Garcinia mannii	0	0	8.3±0.4	17.0±0.7	29.3±1.1	2.5	6.25	nba
Glossocalyx brevipes	0	0	0	<8	12.0±0.0	10	3.125	nba
Heisteria trillesiana	0	0	0	<8	8.8±0.3	10	50	nba
Hypodaphnis zenkeri	0	0	0	<8	14.5±0.9	10	50	nba
Khaya senegalensis	0	9.2±0.8	14.8±0.8	20.3±0.3	27.3±0.3	2.5	0.78	1.56
Markhamia lutea	0	8.0±0.0	9.2±0.3	14.2±1.0	21.0±0.7	1.25	3.125	25
Ocimum suave	0	0	9.7±0.3	15.8±0.8	23.8±0.8	2.5	1.56	6.25
Parkia biglobosa	<8	8.3±0.6	11.7±1.2	25.0±00	27.8±1.0	1.25	0.78	0.78
Pauridiantha calicarpoides	0	0	0	<8	11.0±0.0	10	12.5	25
Pausinystalia macroceras	0	0	9.0±0.0	12.7±1.2	17.5±0.5	2.5	6.25	12.5
Picralima nitida	0	0	10.0±0.0	12.7±0.6	17.2±0.3	2.5	3.125	25
Pleiocarpa sp	0	8.3±0.6	15.8±1.1	30.7±0.6	42.3±1.2	1.25	0.39	0.78
Rinorea oblongifolia	0	0	11.0±00	21.2±03	32.7±0.6	2.5	3.125	25
Schumanniophyton magnificum	0	0	0	<8	11.0±0.0	10	50	nba
Voacanga africana Met/Cl_2CH_2	0	0	9.0±1.0	14.7±0.6	24±0.0	2.5	1.56	6.25
Amoxicillin disc (10µg)	23.8±0.3							
Metronidazole disc (5µg)	27.7±0.6							
						Amoxicillin	0.0062	0.0125

Et/H_2O: Ethanol/Water 50% (v/v)
Met/Cl_2CH_2: Methanol / Methylene Chloride 50% (v/v)
nba: no bactericidal activity; MAQ: Minimum Active Quantity; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration

Table 3: Mean (±SD) Diameter of Inhibition (mm) zones, Minimum Active Quantity (mg), Minimum Inhibitory Concentration (mg/ml) and the Minimum Bactericidal Concentration (mg/ml) of crude plant extracts active against *C. jejuni/coli*

	Extract concentration							
	0.63mg	1.25mg	2.5mg	5mg	10mg			
Extract name :						**MAQ (mg)**	**MIC (mg/ml)**	**MBC (mg/ml)**
Alstonia boonei	0	0	11.0±0.0	18.2±0.3	23.8±1.1	2.5	3.125	12.5
Aoranthe nalaensis	0	0	0	0	10.0±1.4	10	25	nba
Aulaccoclalyx jasminiflora	0	0	0	8.3±0.4	18.5±0.0	5	6.25	nba
Bridelia micrantha	0	0	0	0	8.2±0.3	10	25	nba
Drypetes gosseweileri	8.7±0.3	13.8±1.0	17.7±0.3	31.0±0.0	42.0±1.0	0.63	0.78	3.125
Gacinia kola seed	0	11.0±0.5	14.7±0.3	16.8±0.4	22.0±0.0	1.25	3.125	12.5
Garcinia kola stem bark	0	0	0	9.0±0.0	11.0±0.0	5	6.25	12.5
Garcinia mannii	0	0	10.5±0.5	17.7±0.8	29.0±1.0	2.5	12.5	nba
Glossocalyx brevipes	0	0	0	0	12.2±0.3	10	6.25	nba
Heisteria trillesiana	0	0	0	0	8.0±0.0	10	50	nba
Hypodaphnis zenkeri	0	0	0	9.0±0.0	15.8±0.3	5	25	nba
Khaya senegalensis	0	10.2±1.4	16.2±0.3	22.0±0.0	30.2±0.8	1.25	3.12	6.25
Markhamia lutea	0	0	9.0±0.5	14.0±0.0	24.0±0.9	2.5	6.25	25
Ocimum suave	0	0	9.7±0.9	14.2±0.3	21.5±0.9	2.5	6.25	12.5
Parkia biglobosa	8.8±0.6	10.7±0.6	18.0±0.5	24.0±0.0	28.3±0.6	0.63	1.56	1.56
Pauridiantha calicarpoides	0	0	0	0	14.7±0.8	10	12.5	25
Pausinystalia macroceras	0	0	9.0±0.0	14.8±1.0	23±0.0	2.5	12.5	12.5
Picralima nitida	0	8.8±0.3	14.0±0.5	15.8±0.3	18.8±1.0	1.25	6.25	25
Pleiocarpa sp	0	8.3±0.3	11.0±0.0	27.7±1.2	40.3±0.6	1.25	1.56	3.125
Rinorea oblongifolia	0	0	10.3±0.6	16.8±1.0	35.0±1.0	2.5	6.25	50
Sapium ellipticum	0	0	0	0	8.3±0.4	10	50	nba
Schumanniophyton magnificum	0	0	0	8.8±0.3	15.5±0.5	5	50	nba
Voacanga africana Et/H_2O	0	0	0	8.0±0.0	12.3±0.6	5	25	100
Voacanga africana Met/Cl_2CH_2	0	0	8.3±0.4	13.0±0.5	25.0±0.0	2.5	3.125	12.5
Amoxicillin disc (10µg)	23.8±0.8							
Metronidazole disc (5µg)	30.8±0.4							
						Amoxicillin	0.0062	0.0125

Et/H_2O: Ethanol/Water 50% (v/v)
Met/Cl_2CH_2: Methanol / Methylene Chloride 50% (v/v)
nba: no bactericidal activity; MAQ: Minimum Active Quantity; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration

Killing rate determination

Only extracts with MBC values lower than 25 mg/ml were selected for this step of the study. The extract concentration used (50 mg/ml) corresponded to at least 4 times the MBC. The time-kill curves of the bactericidal effects of the extracts on *H. pylori* are presented in Figures 1a and 1b. The extracts from the seed and the stem bark of *Garcinia kola* (*G. kola*), the bark extract of *P. biglobosa* and *Pausinystalia macroceras* (*P. macroceras*), and the Methylene chloride / Methanol extract of *V. africana* decreased the original bacterial load to the lowest detectable limit within 6 hours (ET_{100} = 6 hours). The extracts, prepared from *A. boonei*, *O. suave*, *K. senegalensis*, *D. gosseweileri* and *Pleiocarpa sp* could achieve this within 8 hours. The fastest killing extract was that produced from the seeds of *G. cola*. It produced a decrease of the initial cell suspension by $5Log_{10}$ of the mean viable count within 4 hours. On *C. jejuni/coli* (Figure 2a and 2b), the same observation was made except for the Methylene Chloride / Methanol extract of the stem bark of *V. africana* for which an ET_{100} of 8 hours was obtained.

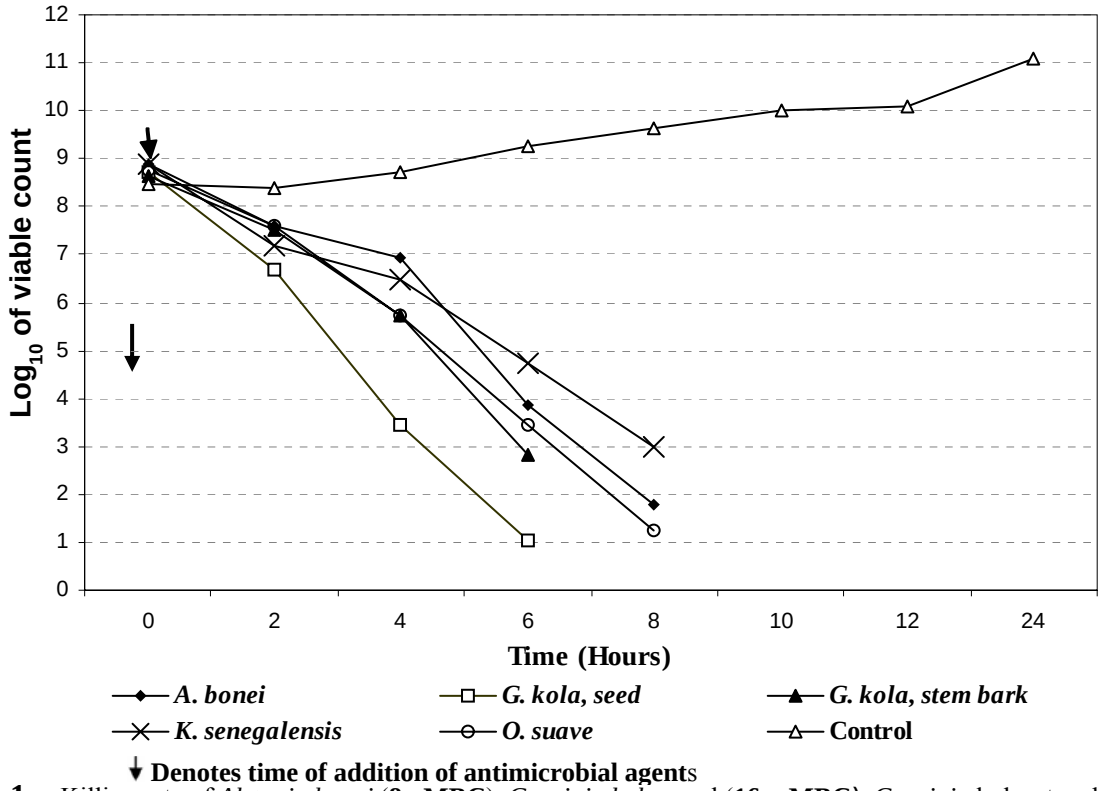

Figure 1a: Killing rate of *Alstonia bonei* (**8*x* MBC**), *Garcinia kola,* seed (**16 *x* MBC***)*, *Garcinia* kola, stem bark (*4x MBC*), *Khaya senegalensis* (**32 *x* MBC**) and *Ocimum suave* (**8 *x* MBC**) on ***Helicobacter pylori.***

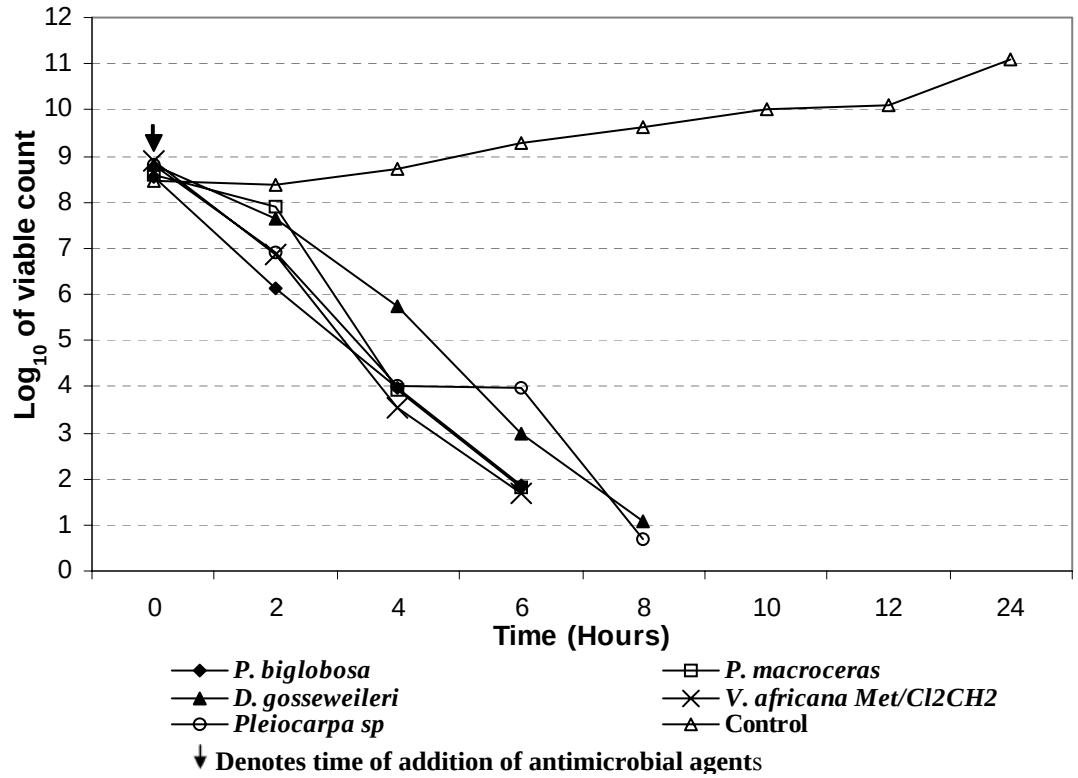

Figure 1b*: Killing rate of* *Parkia biglobosa*, ***(64* x *MBC),*** *Pausinystalia macroceras* ***(4* x *MBC),*** *Drypetes gosseweileri* stem bark ***(16* x *MBC),*** *Voacanga africana* CH_3OH/Cl_2CH_2 stem bark ***(8* x *MBC) and*** *Pleiocarpa sp* ***(64* x *MBC) on Helicobacter pylori***.

Discussion and conclusions

Table 1 shows that of the 21 plant extracts tested, only seven (*A. boonei, O. suave, P. biglobosa, G. kola, K. senegalensis, P. nitida, V. Africana*) have been cited in existing literature for their use in the traditional management of complaints symptomatic of peptic ulcer disease. We obtained supplementary information from herbalists and medicinal plant dealers in the local markets that the other 14 plants are efficiently used for gastro duodenal ulcer management. However, nowhere in the ethnobotanic literature has any antiulcer plant been cited for its specific anti H*elicobacter* properties. This is due to the fact that the involvement of *Helicobacter* in the aetiology of peptic ulcer disease is a recent discovery, and this underlines the need for a wider screening of known antiulcer plants for specific anti *Helicobacter* properties. The results of the present study are therefore novel useful findings for *Helicobacter* eradication as a component of peptic ulcer combination (triple) therapy.

H. pylori and *C. jejuni/coli,* classically known as *Campylobacter*-like organisms (CLO) (1, 2, 12), belong to the same family, the *Campylobacteriaceae* (23). Genera of this family usually express the same sensitivity or resistance profiles to antibiotics, but only the acid-stable antibiotics are selected for *H. pylori* eradication (2). For this reason, extracts that were inactive against *C. jejuni/coli,* were not tested against *H. pylori.* Three extracts (*A. nalaensis, S. ellipticum* and *V. africana*) were found to be active against *Campylobacter* but not against *Helicobacter* (Table 1). Given that the criterion for viable antimicrobial activity was a DI ≥ 8 mm, extracts with DIs < 8 mm were considered to be non active. However, inhibition zones (though < 8 mm) were observed around the wells containing these "inactive extracts", indicating that they contained low concentrations of compounds inhibitory to *H. pylori.* Thus, although the Ethanol/Water extract of *V. africana* showed neither bacteriostatic nor bactericidal activity against *H. pylori*, the Methanol/CH_2Cl_2 extract was found to be highly active against both organisms (Tables 2 and 3). This may explain the wide traditional use of the stem bark of this plant for the traditional management of peptic ulcer disease as well as the experimentally-proven antiulcer activity (24, 25). In addition, we have unpublished data which indicates that the bark extract has shown *in vitro* antibacterial activity on multiresistant nosocomial isolates of *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Alcaligenes* spp., suggesting the possible presence of broad spectrum antimicrobial principles.

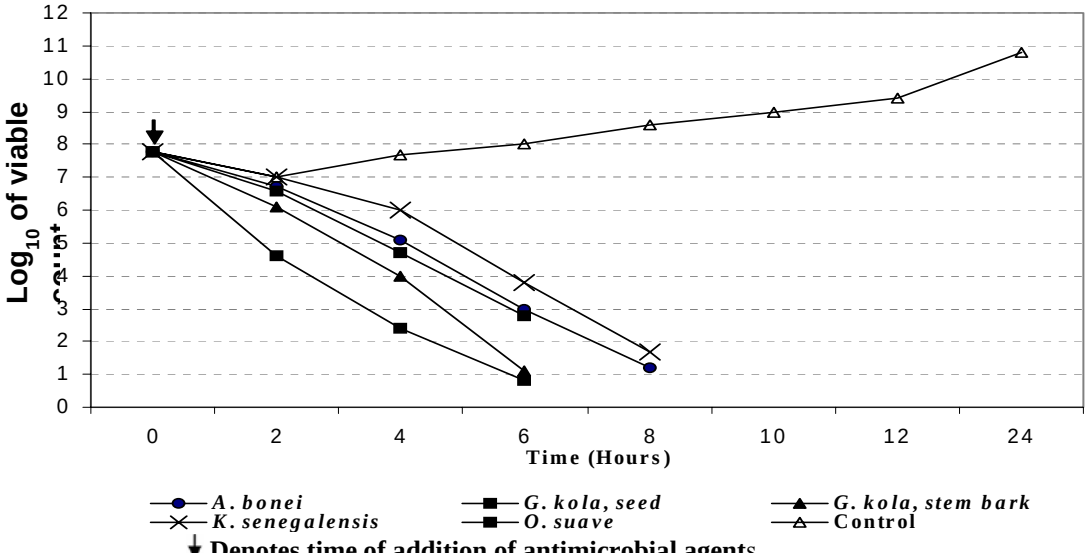

Figure 2a*: **Killing rate of** Alstonia bonei* (**4x MBC**)**,** Gacinia kola**,** seed (**4x MBC**), *Garcinia kola***,** stem bark (**4x MBC**)**,** *Khaya senegalensis* (8 x MBC) and *Ocimum suave* (**4x MBC**) on ***Campylobacter jejuni/coli***

DIs for *P. biglobosa*, *D. gosseweileri* and *Pleiocarpa sp* on both pathogens were higher compared with the reference antibiotics (Tables 2 and 3). In addition, their MIC values were lower than 1mg/ml indicating very high potency levels. Out of the 15 bactericidal extracts, the best 10 were further evaluated for their killing rates. All the 10 extracts exerted their bactericidal activity in less than 10 hours (Figures 1a, 1b, 2a, 2b). When time-kill kinetics are determined using the continuous culture technique, the experimental conditions are more reflective of the *in vivo* environment since nutrients and antimicrobials are constantly being renewed using a chemostat system in order to avoid cell death due to nutrient depletion (6). However, a number of investigators have assessed time-kill kinetics using the batch culture technique such as that employed in this study (6, 26) in which a control bactericide–free culture permits monitoring of cell growth throughout the experiment. In the present study, cell growth in the bactericide-free culture was homogeneous and continuous with time. This permitted us to conclude that the killing rates obtained were due specifically to the bactericidal effect of the extracts tested.

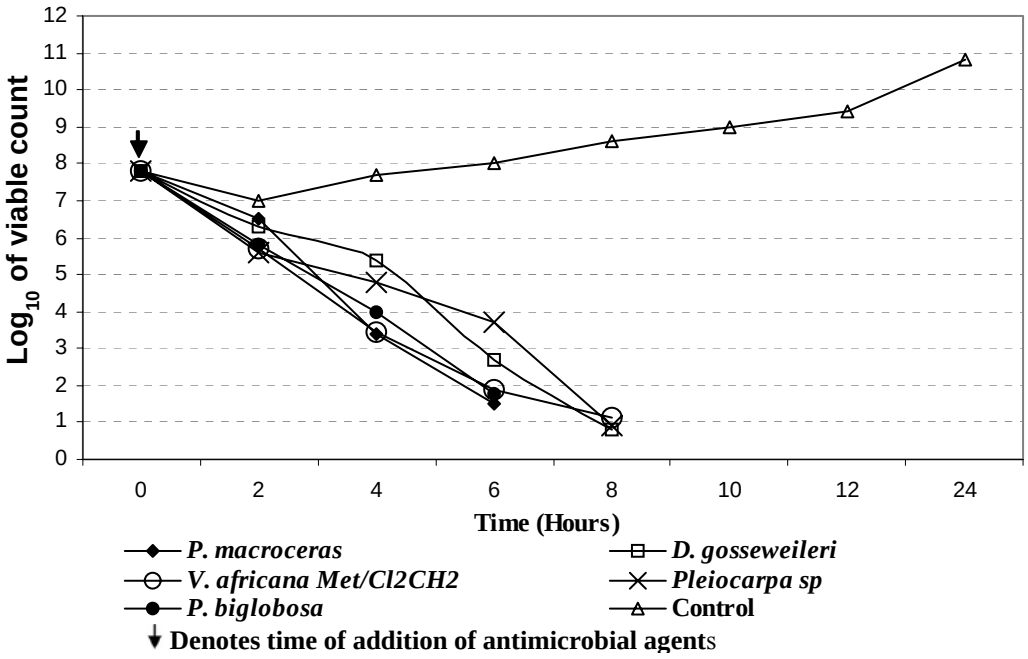

Figure 2b*: **Killing rate of** Parkia biglobosa* (**8x MBC**)**,** *Pausinystalia macroceras* (**4x MBC**)**,** *Drypetes gosseweileri* **(16x MBC),** *Voacanga africana* CH_3OH/Cl_2CH_2 (**4x MBC**) and *Pleiocarpa sp* (**16x MBC**) ***on Campylobacter jejuni/coli***

The current classical *H. pylori* eradication therapy prescribes the use of a combination of at least two antibiotics of different classes based on their mechanisms of action. The most frequently used antibiotics are Amoxicillin (an inhibitor of bacterial cell wall synthesis), Clarithromycin, Tetracycline (inhibitors of bacterial protein synthesis) and Metronidazole (inhibitor of bacterial nucleic acid synthesis). Successful *H. pylori* eradication usually occurs within 14 days when two antibiotics are combined (10-12). One of the most effective regimens with up to 96 % eradication rate is obtained with the so-called MACH 1 regimen (M, Metronidazole; A, Amoxicillin; C, Clarithromycin; H, *H. pylori*; 1, 1week) (12). The shortened period of treatment is obviously linked to the fact that the pathogen is subjected to antimicrobials with various mechanisms of action, which could therefore contribute to a reduced time-kill. In the present study, extracts from *G. kola*, *P. biglobosa*, *P. macroceras*, and *V. africana* exhibited low ET_{100} values (6 h) (Figures 1a, 1b, 2a, 2b),

suggesting that the extracts either possess active principles with different (synergistic) antimicrobial mechanisms, or that different active principles complement each other in a potentiating manner.

Many anti *H. pylori* agents are active *in vitro* but not *in vivo* due to their short stomach transit time and reduced contact with the infectious agent (27, 29), and/or due to their poor acid stability (2). Thus rapid killing activity and acid stability must govern the choice of new anti *Helicobacter* products. In addition to these virtues, acid neutralising, antisecretory or gastric cytoprotective properties of plant preparations may also be important factors. Previous studies have revealed the anti-ulcerogenic property of the bark extract of *V. africana* (25) and the cytoprotective property of the leaf extract of *O. suave* through the promotion of mucus secretion (19). Thus, some plant extracts may possess both ulcer healing and anti *Helicobacter* properties. Janssen *et al*. (1989) (29) reported that essential oils from several *Ocimum* species (including *O. suave*) possess antifungal and antibacterial properties on *Trichophyton mentagrophytes, Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. This wide spectrum antimicrobial activity of *Ocimum suave* may be attributed to eugenol and some mono and sesquiterpenoids found in this plant (30).

The increasing resistance of strains of *Helicobacter* and *Campylobacter* species to some of the most commonly used antibiotics has been cited as a major cause of treatment failure (2, 8, 14, 31). Moreover, the side effects associated with the use of a good number of the antibiotics of choice significantly limit their usefulness (13, 32). The anti *Helicobacter/Campylobacter* properties exhibited by the extracts tested in this study provide scientific support for their use in the traditional management of peptic ulcer disease. Toxicity studies as well as *in vivo* antimicrobial tests are necessary in order to provide further scientific validity for their traditional use.

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References

1. Schillio Y. *Helicobacter pylori* et ulcère gastro–duodénal. Medecine Digest 1996; 22(5), 12–17.
2. Skirrow MB. *Campylobacter* and *Helicobacter enteritis*; gastritis in Greenwood *et al.* (eds) Medical Microbiology 14th edition ELBS Churchill Livingstone, Low price edition 1992: 353–361.
3. Korwin JD. Management of *Helicobacter pylori*-associated gastrointestinal disease. Laborama 1999; 6: 10–13.
4. Sizemore CF, Quispe JD, Amsler KM, *et al*. Effects of Metronidazole, Tetracycline, and Bismuth-Metronidazole-Tetracycline Triple Therapy in the Helicobacter pylori SS1 Mouse Model after 1 Day of Dosing: Development of a H. pylori Lead Selection Model. Antimicrobial Agents and Chemotherapy 2002; 46 (5), 1435–1440.
5. Gisbert JP, Pajares JM. *Helicobacter pylori* infection and perforated peptic ulcer prevalence of the infection and role of antimicrobial treatment. Helicobacter 2003; 8(3), 159–167.
6. Hassan JI, Stark MR, Greenman J, Millar RM Activity of β-Lactams and Macrolides against *Helicobacter pylori*. Antimicrobial Agents and Chemotherapy 1999; 43(6): 1387–1392.

7. Mahady GB, Pendland SL, Yun SG, Zhi-Zhen L, Stoia A. Ginger (Zingiber officinale Rsocoe) and the Ginerol Inhibit the growth of cag A+ Strains of Helicobacter pylori. Anticancer Research 2003; 23: 3699–3702.

8. Fauchere JL. Bacteriological characteristics and diagnosis of *Helicobacter pylori*. Laborama 1999; 6: 10–14.

9. Walsh JH, Peterson WL. The treatment of Helicobacter pylori infection in the management of peptic ulcer disease. New England Journal of Medicine 1995; 333: 984–991.

10. Beales, ILP. Efficacy of Helicobacter pylori eradication therapies: a single center observational study. BMC Gastroenterology 2001; 1: 1–9.

11. Hentschel EG, Brandstatter B, Dragosics AM, *et al.* Effect of ranitidine and Amoxicillin plus Metronidazole on the eradication of *H. pylori* and recurrence of duodenal ulcer. New England Journal of Medicine 1993; 328: 308–312.

12. Dunn BE, Cohen, H, Blaser, MJ. *Helicobacter pylori*. Clinical Microbiology Reviews 1997; 10(4): 720–741.

13. Kanamaru T, Nakano Y, Toyoda Y, *et al. In Vitro* and *In Vivo* Antibacterial Activities of TAK-083, an Agent for Treatment of *Helicobacter pylori* Infection. Antimicrobial Agents and Chemotherapy 2001; 45 (9): 2455–2459.

14. Kato M, Yamaoka Y, Jae JK, Reddy R, Asaka M, *et al.* Regional Differences in Metronidazole Resistance and Increasing Clarithromycin Resistance among *Helicobacter pylori* Isolates from Japan. Antimicrobial Agents and Chemotherapy 2000; 44(8): 2214–2216.

15. Okamoto T, Yoshiyama H, Nakazawa T, In-Dal P, Myung-Woong C, Yanai H, Okita K, Shirai M. A change in PBP1 is involved in amoxicillin resistance of clinical isolates of *Helicobacter pylori*. Antimicrobial Agents and Chemotherapy 2002; 50: 849–856.

16. Cowan MM. Plant products as antimicrobial agents. Clinical Microbiology Reviews 1999; 12(4): 564–582.

17. Mahady GB, Pendleand SL, Stoia A, Chadwick LR. *In vitro* susceptibility of *Helicobacter pylori* to Isoquinoline Alkaloids from *Sanguinaria canadensis* and *Hydrastis canadensis*. Phytotherapy Research 2003; 17: 217–221.

18. Adjanohoun JE, Aboubakar N, Dramane K, *et al.* Traditional Medicine and Pharmacopoeia: Contribution to Ethnobotanical and Floristic Studies in Cameroon. Organization of African Unity Scientific, Technical and Research Commission. Centre Nationale de Production des Manuels Scolaires, Porto-Novo. Benin 1996.

19. Tan VP, Nyasse B, Dimo T, Mezui C. Gastric cytoprotective anti-ulcer effects of the leaf extract of Ocimum suave (Lamiaceae) in rats. Journal of Ethnopharmacology 2002; 82: 69–74.

20. NCCLS (National Committee for Clinical Laboratory Standards). Performance standards for antimicrobial susceptibility testing- Ninth Informational supplement: Document M100-S9. NCCLS, Villanova, 1999.

21. Cheesbrough M. Microbiological tests. In Cheesbrough M. (editor) District Laboratory Practice in Tropical Countries Part 2. Cambridge Low Price Edition. 2000: 45–157.

22. Sgouras D, Maragkoudakis P, Petraki K, *et al. In Vitro* and *In Vivo* Inhibition of H*elicobacter pylori* by *Lactobacillus casei* Strain Shirota Applied and Environmental. Microbiology. 2004; 70 (1): 518-526.

23. Vandame P, De Ley J. Proposal for a new family, Campylobacteraceae. International Journal of Systematic Bacteriology 1991; 41: 451–455.

24. Tan VP, Nyasse B. Anti Ulcer Compound from Voacanga africana with Possible Histamine H2-receptor Blocking activity. Phytomedicine 2000; 7(6): 509–515.

25. Tan VP, Penlap VB, Nyasse B, Nguemo JDB. Anti-Ulcer Action of the Bark Methanol Extract of *Voacanga africana* in Different Experimental Ulcer Model in Rats. Journal of Ethnopharmacology 2000; 73: 423–428.

26. Fung-Tomc JC, Gradelski E, Valera L, Kolek B, Bonner DP. Comparative Killing Rates of Fluoroquinolones and Cell Wall-Active Agents. Antimicrobial Agents and Chemotherapy 2000; 44(5):1377 – 1380.

27. Haas CE, Nix DE, Schentag JJ. *In vitro* selection of resistant *Helicobacter pylori*. Antimicrobial Agents and Chemotherapy 1990: 34: 1637–1640.

28. Krakowka S, Eaton KA, Leunk D.R. Antimicrobial therapies for *Helicobacter pylori* Infection in Gnotobiotic piglets. Antimicrobial Agents and Chemotherapy 1998; 42(7): 1549–1554.

29. Janssen AM, Scheffer JJ, Ntezurubanza, Baerheim L, Svendsen A. Antimicrobial activities of some *Ocimum* species grown in Rwanda. Journal of Ethnopharmacology 1989; 26(1): 57-63.

30. Chogo JB, Crank G. Chemical composition and biological activity of the Tanzanian plant Ocimum suave. Journal of Natural Products 1981; 44(3): 308-11.

31. Luber P, Wagner J, Hahn H, Bartelt E. Antimicrobial Resistance in Campylobacter jejuni and Campylobacter coli Strains isolated in 1991 and 2001-2002 from Poultry and Humans in Berlin, Germany. Antimicrobial Agents and Chemotherapy 2003; 47(12): 3825–3830.

32. Feldman M, Brurton ME. Histamine H2-receptor antagonists: standards therapy for acid peptic diseases. New England. Journal of Medicine 1990; 323(24): 1672-1680.

33. Rajic A, Kweifio-Okai G, Macrides T, Sandeman RM, Chandler DS, Polya GM. Inhibition of Serine proteases by Anti-inflammatory triterpenoïds. Planta Medica. 2000; 66 (3): 206-210.

34. Olajide AO, Awe SO, Makinde JM, *et al.* Studies on the anti-inflammatory, antipyretic and analgesic properties of *Alstonia boonei* stem bark. Journal of Ethnopharmacology 2000; 71(1-2): 179-186.

35. Lin J, Puckree T, Mvelase TP. Anti-diarrhoeal evaluation of some medicinal plants used by Zulu traditional healers. Journal of Ethnopharmacology 2002; 79(1): 53-56.

36. Ajaiyeoba EO, Abiodun OO, Falade MO, *et al.* In vitro cytotoxicity studies of 20 plants used in Nigerian antimalarial ethnomedicine. Phytomedicine 2006; 13(4): 295-298.

37. Ngouela S, Ngoupayo J, Noungoue DT, Tsamo E, Connolly JD. Gossweilone: A new podocarpane derivative from the stem bark of Drypetes gossweileri (Euphorbiaceae) Bulletin of the Chemical Society of Ethiopia 2003; 17(2): 181-184.

38. Ngangu Z, Foussereau J. Tropical woods and contact eczema. Derm Beruf Umwelt 1982; 30(6): 193-5.

39. Iwu M. Handbook of African medicinal plants. CRC Press, Boca Raton, FL.1993.

40. Akpantah AO, Oremosu AA, Ajala MO, Noronha CC, Okanlawon AO. The Effect of Crude Extract of *Garcinia Kola* Seed on the Histology and Hormonal Milieu of Male Sprague-Dawley Rats' Reproductive Organs. Nigerian Journal of Health and Biomedical Sciences 2003; 2(1): 40–46.

41. Farombi EO, Adepoju BF, Ola-Davies OE, Emerole GO. Chemoprevention of aflatoxin B1-induced genotoxicity and hepatic oxidative damage in rats by kolaviron, a natural biflavonoid of *Garcinia kola* seeds. European Journal of Cancer Prevention 2005; 14(3): 207-214.

42.Mbah JA, Tane P, Ngadjui BT, *et al.* Antiplasmodial agents from the leaves of Glossocalyx brevipes. Planta Medica 2004; 70(5): 437-440.

43. Mshana NR, Abbiw DK, Addae-Mensah I, *et al*. Traditional Medicine and Pharmacopoeia Contribution to the revision of Ethnobotanical and floristic study in Ghana. Organization of African Unity Scientific, Technical and Research Commission (OAU/STRC) 2000.

44. Adesina SK. Examination of *Khaya senegalensis* and *Khaya ivorinsis*. Fitoterapia 1983; 3:141-143

45. Adebayo JO, Yakubu MT, Egwim EC, Owoyele VB, Enaibe BU. Effect of ethanolic extract of Khaya senegalensis on some biochemical parameters of rat kidney. Journal of Ethnopharmacology 2003; 88(1): 69-72.

46. Kernan MR, Amarquaye A, Chen JL, *et al*. Antiviral phenylpropanoid glycosides from the medicinal plant Markhamia lutea. Jounal of natural Products 1998; 61(5): 564–670.

47. Watt JM, Gerdinat M, Breyer-Brandwij K. Medicinal and poisonous plants of southern and Eastern Africa. 2nd edition. London 1962.

48. Seyoum A, Kabiru EW, Lwande W, Killeen GF, Hassanali A, Knols BG. Repellency of live potted plants against Anopheles gambiae from human baits in semi-field experimental huts. American Journal of Tropical Medicine and Hygiene 2002; 67(2): 191-195.

49. Makonnen E, Debella A, Zerihun L, Abebe D, Teka F. Antipyretic properties of the aqueous and ethanol extracts of the leaves of Ocimum suave and Ocimum lamiifolium in mice. Journal of Ethnopharmacology 2003; 88(1): 85 – 91.

50. Ajaiyeoba EO. Phytochemical and antibacterial properties of *Parkia biglobosa* and *Parkia bicolor* leaf extracts. African Journal of Biomedical Research 2002; 5(3): 125–129.

51. Kouadio F, Kanko C, Juge M, Grimaud N, Jean A, N'guessan YT, Petit JY. Analgesic and anti-inflammatory activities of an extract from Parkia biglobosa. Phytotherapy Research. 2000; 14(8): 635-637.

52- Tringali C, Spatafora C, Longo OD. Bioactive constituents of the bark of *Parkia biglobosa.* Fitoterapia 2000; 71(2): 118-125.

53. Kreutz S. Yohimbe and erectile dysfunction. Cockatoo health publications 2001, Website: http://www.asiatour.com/health/yohimbe/index.htm. Last date visited: 24th July 2006

54. Duwiejua M, Woode E, Obiri DD. Pseudo-akuammigine, an alkaloid from Picralima nitida seeds, has anti-inflammatory and analgesic actions in rats. Journal of Ethnopharmacology 2002; 81(1): 73–79.

55. Carme CL, Joule JA. Synthetic studies related to the akuammiline alkaloids. Arkivoc x 2004; 20–38.

56. Nkere CK, Iroegbu CU Antibacterial screening of the root, seed and stem bark extracts of Picralima nitida. African. Journal of Biotechnology 2005; 4(6): 522–526.

57. Martz W. Plants with a reputation against snakebite. Toxicon 1992; 30(10): 1131-1142.

58. Houghton PJ, Skari KP The effect on blood clotting of some West African plants used against snakebite. Journal of Ethnopharmacology 1994; 44(2): 99-108.

59. Houghton PJ, Woldemariam TZ, Khan AI, Burke A, Mahmood N. Antiviral activity of natural and semi-synthetic chromone alkaloids. Antiviral Research 1994; 25(3-4): 235-244.

60. Houghton PJ, Osibogun IM, Woldemariam TZ, Jones K. Heteronuclear NMR studies of the chromone alkaloids and revision of the structure of some piperidino-chromone alkaloids. Planta Medica 1995; 61(2): 154-157.

61. Thomas DW, Biemann K. The hydroxyindolenine derivative of voacangine, a new indole alkaloid from *Voacanga africana*. Tetrahedron 1968; 24(11): 4223-4231.

62. Kombian S.B, Saleh TM, Fiagbe NI, Chen X, Akabutu JJ, Buolamwini JK, Pittman QJ. Ibogaine and a total alkaloidal extract of Voacanga africana modulate neuronal excitability and synaptic transmission in the rat parabrachial nucleus in vitro. Brain Research Bulletin 1997; 44(5): 603-10.

63. Jenks CW. Extraction studies of Tabernanthe iboga and Voacanga africana. Natural Products Letters 2002;16(1): 71-6.

64. Tona L, Kambu K, Mesia K *et al.* Biological screening of traditional preparations from some medicinal plants used as antidiarrhoeal in Kinshasa, Congo. Phytomedicine 1999; 6(1): 59-66.