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Research paper

Inhibitory effect of medicinal plants from Cameroon on the growth and adhesion of *Helicobacter pylori*

Corinne Raïssa Ngnameko[a], Frederic Nico Njayou[a], Muinah Fowora[b], Fredy Brice Simo Nemg[a], Paul Moundipa Fewou[a,*], Stella Ifeanvi Smith[b]

[a] Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of science, University of Yaounde I, PO. Box 812 Yaounde, Cameroon
[b] Nigerian Institute of Medical Research, 6, Edmond Crescent, P.M.B.2013, Lagos, Nigeria

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ABSTRACT

Introduction: Plants in the Cameroon Pharmacopoeia are used by the population as decoctions, infusions and are macerated to relieve digestive and gastric disorders. The aim of this study was to screen for the anti-*Helicobacter pylori* and anti-adhesion activities of various plant extracts from Cameroon.

Methods: Medicinal plants used for the treatment and stomach pains and digestive disorders were identified and collected in an ethnopharmacological survey in Cameroon. Dried plant material were used to prepare crude extracts and tested against *Helicobacter pylori* strain P12 . The minimal inhibitory concentration (MIC) of plant extract was determined by broth micro dilution and agar diffusion methods. The anti-adhesion was determined by adhesion of *Fluorescein-isothiocyanate* (FITC) labelled *Helicobacter pylori* to sections of mouse gastric tissue and expression of adhesion gene in response to the plant extract treatment using RT-PCR and western blotting. Results were analyzed statistically by the Bonferroni's post hoc test.

Results: All the plant extracts demonstrated anti-*Helicobacter pylori* activity with MIC values ranging from 0.125 to 100 mg/ml. *Spathodea campanulata* and *Nicotina tabacum* showed the highest anti-*Helicobacter pylori* activity on *H. pylori* P12 strain. The lowest MIC recorded by *Spathodea campanulata* and *Nicotina tabacum* were 0.125 and 1 mg/ml respectively. Seventeen plant extracts reduced *H. pylori* adhesion to the mouse tissue stomach. In addition, plant extracts decreased the expression of blood group antigen-binding adhesion *BabA* and *H. pylori* outer membrane protein *HopZ*.

Conclusion: *Spathodea campanulata* and *Nicotina tabacum* may be useful in the development of new drugs for the treatment of *H. pylori* infection. They represent promising natural sources to be used in pharmacology.

1. Introduction

Peptic ulcers affect the stomach (gastric ulcers) and small intestines (duodenal ulcers). *Helicobacter pylori (H. pylori)* plays a predominant role in the etiology of gastric cancer and was characterized as a class I carcinogen by the World Health Organization in 1994 [1]. This disease is characterized by gastric inflammation, gastric cancer and gastric mucosa-associated lymphoid-tissue lymphoma [2,3]. *H. pylori* is a curved Gram-negative bacterium frequently present in the human stomach. It possesses two to six flagella which permit it to be mobile and to resist stomach contractions and favor penetration into the gastric mucosa. Approximately 50% of the world's population are infected with *H. pylori* and its prevalence is significantly higher in developing countries [4–6], infection reaches 80–90% of the population [7]. In Cameroon, the study conducted on 171 patients with gastric ulcer at the Yaounde University Teaching Hospital and Medical Center the Cathedral showed that 72.5% of these patients were infected by this bacterium [8].

The transmission of *H. pylori* is uncertain, but the gastro-oral, oral-oral and fecal oral routes are highly probable [9]. Once at the epithelial level, *H. pylori* adheres with the help of adhesins. There are many adhesins such as adherence-associated lipoprotein A and B (*AlpA-B*), blood group antigen-binding adhesion (*BabA*), sialic acid-binding adhesion (*SabA*), *H. pylori* outer membrane protein (*HopZ*), *H. pylori* adhesin A (*HpaA*) and Lewis^x -LPS, all of which mediate adhesion to

Abbreviations: BabA, blood group antigen binding adhesion; HopZ, *Helicobacter pylori* outer membrane protein; FITC, Fluorescein-isothiocyanate; MIC, minimal inhibitory concentration; DMSO, dimethyl sulfoxide

* Corresponding author.
E-mail addresses: corinnengnameko@yahoo.fr (C.R. Ngnameko), njayou@yahoo.com (F.N. Njayou), muinahj@yahoo.com (M. Fowora), nemgsi@yahoo.fr (F.B.S. Nemg), pmoundipa@uy1.uninet.cm, pmoundipa@hotmail.com (P. Moundipa Fewou), stellaismith@yahoo.com (S.I. Smith).

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Table 1
Primer sequences used for qualitative reverse transcriptase polymerase chain reaction (RT-PCR).

Genes	Primer Sequence (5'___3')	
	Forward	Reverse
babA	AATCCAAAAAGGAGAAAAAGTATGAAA	ACAGGATGTGGCTTTAGTGATTGT
hopZ	TTTTGAGCCGGTGGATATATTAG	CGCTAGGTGATTTCCTCGTATCCGCCGGCGTAA

gastric epithelial cells, followed by the establishment of infection [10]. *H. pylori* is sensitive to acid pH but survives in the stomach under acidic conditions due to the activity of the urease secreted by this bacterium. *BabA* plays a significant role in enhancing *H. pylori* virulence, and may represent a potential target for a curative therapy [11]. Two of the virulence factors that have been implicated in this process are cytotoxin-associated gene A (*cagA*) and vacuolating cytotoxin A (*vacA*), which are expressed by *H. pylori*. *VacA* is present in all *H. pylori* strains and contains two variable parts relevant to virulence [12], whereas *cagA* is not present in every *H. pylori* strain and is a marker for a pathogenicity island (PAI) [13] associated with more severe clinical outcomes as well as an increased risk of developing gastric cancer and peptic ulcer disease.

Current treatment still relies on a combination of antimicrobial agents, such as antibiotics, and anti-secretory agents, such as proton pump inhibitors (PPIs) [14,15]. Several studies have shown a high resistance to antibiotics namely 95.5% metronidazole, 44.7% clarithromycin and 85.6% amoxicillin [4,5,16]. The resistance of *H. pylori* to antibiotics used to treat infection makes relevant the search for new therapeutic sources with anti-*helicobacter pylori* activity [17]. Currently, plants are viewed as the main source for the discovery of new compounds [18]. Medicinal plants have always been used for the treatment of gastric diseases in Africa and specifically in Cameroon. Tan et al. [19] showed that an extract of *Pleiocarpa* sp. had inhibitory effects against this bacterium. Likewise, *Eryngium foetidum* extract presented similar effects on *H. pylori* culture [20]. In addition, the work of Tharmalingam et al. [21] has shown that piperine extract has anti-*Helicobacter pylori* and anti-adhesive activities. The anti-Helicobacter and anti-adhesive effects were investigated for a list of medicinal plants obtained from a survey held amongst herbalist in Cameroon.

2. Materials and methods

2.1. Survey, plant collection and extraction

The ethnopharmacological survey was carried out in Bafia (Center region Cameroon), Bazou and Foumbot (West region Cameroon). Informed consent was obtained orally from all responders prior to the interview. It was made clear to the responders that their information was purely for scientific studies and not for any commercial use. A questionnaire was developed to investigate what types of medicinal plants were used by the herbalist for the treatment of stomach pains and digestive disorders. All the traditional physicians were recommended to by neighboring populations. Each practitioner was interviewed in local language. The questionnaire contained information about different plants used to treat stomach related disease, the mode of preparation, administration of the medicinal drug, the parts of the plants used and route of administration. Every herbalist was asked to list the main diseases treated and the plants used in the treatment. Each herbalist was also asked to provide samples of the plant species from their own collection as well as from the field; and if possible plant photographs. Two voucher specimens per medicinal plant were collected in the field, pressed and then dried prior to identification at the Cameroon National Herbarium (HNC) or Society of Forest Reserves of Cameroon (SRF/Cam). All plant specimens were collected in both flowering and fruiting conditions. Samples of medicinal plants were collected for scientific identification and herbarium preparation following standard procedures.

Plants were collected in the locality of Bafia (Center region Cameroon), Bazou and Foumbot (West region Cameroon) and they were washed with distilled water and dried at room temperature for several weeks. The dried plant materials were powdered using a grinder. The powder obtained was kept at 4 °C until the preparation of extracts. Two hundred grams of powdered plant materials were soaked in 800 ml of solvent methylene chloride/methanol (1:1; v/v) or 70% ethanol for 48 h and filtered using Whatman N° 1 paper. The filtrate was dried using a rotary evaporator to obtain a residue which constituted the crude extract.

2.2. The growth condition of H.pylori P12 and determination of minimum inhibitory concentration (MICs) by the microdilution and agar dilution method

H. pylori strain P12 (*vacA* and *cagA* positive) was obtained from the Nigerian Institute of Medical Research, Lagos, Nigeria. The strain was cultured on GC agar supplemented with horse serum, vitamins and selective supplement (vancomycin 5.0 mg, trimethoprim 2.5 mg, cefsulodin 2.5 mg and amphotericin 2.5 mg). *H. pylori* strain was grown at 37 °C for 72 h under microaerophilic conditions (2.5 to 9.5% CO_2 and 6.2 to 13.2%, O_2). Except for vitamin mix obtained from Sigma, France, all reagents were purchased from Oxoid, United Kingdom.

The broth microdilution assay was performed according to the method described by Sgouras et al. [22]. To each well in the microplate 100 µl of Mueller Hinton broth, supplemented with 10% fetal bovine serum was inoculated with 6×10^8 *H. pylori* (McFarland turbidity standard 2), 100 µl of the extract, was also added to reach the final concentrations of 0.125; 0.25; 0.5; 1; 2; 10; 20; 50 and 100 mg/ml. Clarithromycin (10 mg/ml) was used as the standard drug for growth inhibition. The microplate was incubated at 37 °C under microaerophilic conditions for 3–5 days. Urease activity was determined by phenol red method with some modifications. Briefly, 5 µl of *H. pylori* cell suspension was added to 150 µl of urease reaction buffer (20% [w/v] urea and 0.012% phenol red in phosphate buffer, with the final pH adjusted to 6.5) on a microliter plate, and the plate was incubated for 1 h at 37 °C. The absorbance at 620 nm was measured with a Sunrise microliter plate reader.

The MICs of plant extract against *H. pylori* strain was evaluated by using agar dilution method. To confirm the micro-dilution, a series of concentrations of 0.125; 0.25; 0.5; 1; 2; 10; 20; 50 and 100 mg/ml of plant extract was used. The agar plates were checked for bacterial growth for 3 days. After 72 h, the MIC values were determined as the lowest concentration at which there was no strain growth judged by visual examination. Clarithromycin was used as the positive control.

2.3. Anti-adherence Assay with H. pylori

The adhesion assay was performed according to the method described by Lengsfeld et al. [23].

2.3.1. Preparation of FITC-labelled H. pylori

Briefly, 2 days after inoculation, bacteria were harvested from agar plates using a 2 µL sterile loop and resuspended in 1.0 ml of 0.15 M

Table 2
Medicinal plants used in the treatment of stomach pains and digestive disorders as identified by the ethno pharmacological survey.

Family	Scientific name Voucher Specimen number	Vernacular name	Plant part	Ethnopharmacological use(s)	Administration of the drugs	FC (%)
Acanthaceae	*Acanthus montanus.*(Nees) T.Anderson. 26004/SRF/Cam	*Fozem*	Leaves	Stomach-ache, Chronic ulcers, gastritiss	Oral administration as decoction	1.22
Anacardiaceae	*Mangifera indica* Lin. 18646/HNC	*Féronneux*	Bark	Gastrointestinal disorders	Oral administration as decoction	4.29
Annonaceae	*Annona muricata* L. 32879/HNC	*Ntuè kaoh*	Leaves, Seeds	Stomach pains	Oral administration as decoction	1.84
	Enantia chloranta Oliv. 6420/HNC	*Sabasaba*	Bark	Stomach problems	Oral administration as decoction	3.68
Apocynaceae	*Voacanga africana* Stapf. 47215/HNC	*Bouleu nkan*	Bark	Stomach problems, antiulcer	Oral administration as decoction	4.9
Araliaceae	*Polyscias fulva Hiern.* 2990/HNC	*Pugue (Pûngùe)*	Bark	Venereal infections, stomach pains	Oral administration as decoction	1.84
Asteraceae	*Ageratum conyzoides* Lin. 6575/HNC	*Kéyayas*	Aerial parts	Abdominal pains, dysentery, gastrointestinal pain	Oral administration as maceration	7.36
	Elephantopus mollis Kunth. 39570/HNC	*Akiba*	Leaves	Antibacterial, stomach disorders	Oral administration as decoction	1.22
	Emilia coccinia (Sims.)g. don. 29441/HNC	*Shin mû*	Leaves	ulcer, jaundice, abdominal pains	Oral administration as decoction	1.22
	Spilanthes africana D.C. 33075/HNC	*Mièk gèp*	Flowers	Toothache, gastrointestinal disorders	Oral administration as maceration	3.68
	Vernonia guineensis Benth. 11133/SRF/Cam	*Mgbùkwet*	Rhizomes	Gastrointestinal disorders, jaundice	Oral administration as decoction	2.45
Biognoniaceae	*Spathodea companulata* P.Beaux. 50085/HNC	*Vivet*	Bark	Digestive disorders, ulcer	Oral administration as decoction	5.52
Burseraceae	*Dacryodes edulis* (g.don) h.lam. 18258/HNC	*Kéyom*	Bark	Dysentery, antimicobial	Oral administration as decoction	1.84
Caricaceae	*Carica papaya* Lin. 18647/HNC	*Tam mekal*	Leaves	Gastrointestinal disorders, stomach disorders	Oral administration as decoction	3.68
Cesalpilaceae	*Piliostigma thonningii* (schum.) m.redhead. 2689/HNC	*Pien*	Leaves	Stomach-ache, digestive disorders	Oral administration as decoction	1.84
	Senna alata (Lin.) link. 11002/HNC	*Fū bibile*	Leaves	Stomach problems	Oral administration as maceration	1.22
Clusiaceae	*Allanblackia florinbunda* Oliv. 1380/HNC	*Nsangomo*	Leaves	Digestive disorders, stomach ache	Oral administration as decoction	1.84
	Garcinia kola Heckel. 9815/SRF/Cam	*Nzoè mbeu*	Leaves	Antiulcer, gastrointestinal disorders	Oral administration as decoction	5.52
Commelinaceae	*Commelina diffusa* Burm.f. 35189/SRF/Cam	*Metit neunor*	Leaves	Antimicrobial, fever	Oral administration as decoction	1.84
Euphorbiaceae	*Alchornea laxiflora* (benth) Pax&K.H. 2093/HNC	*Meshé*	Leaves	Stomach-ache, dysentery, jaundice	Oral administration as decoction	1.22
Euphorbiaceae	*Croton macrostachyus* Hochst et Del. 17909/SRF/Cam	*Kùteshâ*	Leaves	Stomach-ache	Oral administration as decoction	1.84
	Euphorbia hirta L. 14288/HNC	*Bùmon*	Aerial parts	Gastrointestinal disorders	Oral administration as decoction	1.84
	Macaranga hurifolia Beille	*Assas*	Leaves	Gastrointestinal disorders, tubercculosis	Oral administration as decoction	3.06
Fabaceae	*Détarium microcarpum* Gill. Perr. 49834/SRF/Cam		Bark	Stomach-ache, diarrhoea, dysentery	Oral administration as decoction	0.61
	Erythrina senegalensis D.C. 35259/HNC	*Megham*	Leaves, Barks	Gastrointestinal disorders	Oral administration as decoction	0.61
	Millettia versicolor Welw. *32315/HNC*		Leaves	Digestive disorders	Oral administration as decoction	1.22
Labiaceae	*Ocimum basilicum L. 6899/SRF/Cam*	*Tsham*	Leaves	Gastrointestinal disorders	Oral administration as decoction	1.22
	Ocimum gratissimum Lin. 42852/HNC	*Messeck*	Leaves	Antiulcer, stomach pains	Oral administration as decoction	1.84
Loganiaceae	*Anthocleista schweinfurthii* Gil. 2281/HNC	*Yü'rum*	Bark	Abdominal pains	Oral administration as decoction	1.22
	Persea americana Mill. 18604/HNC	*Fou biyé*	Bark	Antiulcer	Oral administration as decoction	2.45
Loranthaceae	*Phragmentera capitata* (Spreng) Balle. 24667/HNC		Leaves	Gastrointestinl disorders, ulcer	Oral administration as decoction	1.22
Malvaceae	*Triumfetta pentandra* A. Rich. 9014/SRF/Cam	*Okon*	Whole plant	Antibacterial, induces fertility and implantation of the fetus	Oral administration as decoction	0.61
Meliaceae	*Khaya grandifoliola* D.C. 52661/HNC	*Fa' tùtù*	Bark	Stomach-ache	Oral administration as decoction	1.84
Mimosaceae	*Entada africana* Guill et Pers. 2334/HNC	*Lù*	Bark	Stomach-ache, gastrointestinal disorders	Oral administration as decoction	1.22
Moraceae	*Ficus exasperata* Vahl. 43999/HNC	*Ghùghu*	Leaves, Barks	Stomach disorders	Oral administration as decoction	1.84
Myrtaceae	*Psidium guayava* Lin. /2885/HNC	*Ntù goyave*	Leaves	Gastrointestinal disorders, ulcer	Oral administration as decoction	3.68
Piperaceae	*Piper umbellatum L.* 20934/SRF/Cam	*Boupouete*	Leaves	Antibacterial, stomach ache	Oral administration as decoction	1.22
Poaceae	*Cymbopogon ciratus* (D.C.) Stapf. 18628/HNC	*Fiba ġass*	Leaves	Stomach disorders	Oral administration as decoction	2.45
Rutaceae	*Citrus aurantifolia* Swingle.	*Lamassi*	Leaves	Stomach ache, gastrointestinal disorders	Oral administration as decoction	5.52
Solanaceae	*Nicotina tabacum* L. 1863/SRF/Cam	*Deubà*	Leaves	Toothache, digestive disorders	Oral administration as decoction	3.68
Sterculiaceae	*Cola acuminata* (P. Beauv.) Schott α Endl. 1729/SRF/Cam	*Shin pè'*	Bark	Stomach pains	Oral administration as decoction	2.45

HNC: Cameroon National Herbarium.
SRF/Cam: Society of Forest Reserves of Cameroon.

Table 3
Minimal inhibitory concentration (MIC) of plant extracts against *H. pylori*.

Scientific name	Plant part	MIC (mg/ml)
Acanthus montanus.	Leaves	50 ± 0.0
Ageratum conyzoides	Aerial parts	10 ± 0.0
Alchornea laxiflora	Leaves	20 ± 0.0
Allanblackia florinbunda	Leaves	2 ± 0.0
Annona muricata	Leaves	20 ± 0.0
Anthocleista schweinfurthii	Bark	20 ± 0.0
Carica papaya	Leaves	50 ± 0.0
Citrus aurantifolia	Leaves	50 ± 0.0
Cola acuminata	Bark	20 ± 0.0
Commelina diffusa	Leaves	50 ± 0.0
Croton macrostachyus	Leaves	100 ± 0.0
Cymbopogon ciratus	Leaves	10 ± 0.0
Dacryodes edulis	Bark	20 ± 0.0
Détarium microcarpum	Bark	50 ± 0.0
Elephantopus mollis	Leaves	50 ± 0.0
Emilia coccinia	Leaves	20 ± 0.0
Enantia chloranta	Bark	50 ± 0.0
Entada africana	Bark	50 ± 0.0
Erythrina senegalensis	Leaves	100 ± 0.0
Euphorbia hirta	Aerial parts	20 ± 0.0
Ficus exasperata	Leaves	50 ± 0.0
Garcinia kola	Leaves	20 ± 0.0
Khaya grandifoliola	Bark	20 ± 0.0
Macaranga hurifolia	Leaves	20 ± 0.0
Mangifera indica	Bark	20 ± 0.0
Millettia versicolor	Leaves	50 ± 0.0
Nicotina tabacum	Leaves	1 ± 0.0
Ocimum basilicum	Leaves	100 ± 0.0
Ocimum gratissimum	Leaves	20 ± 0.0
Persea americana	Bark	50 ± 0.0
Phragmentera capitata	Leaves	20 ± 0.0
Piper umbellatum	Leaves	20 ± 0.0
Polyscias fulva	Bark	20 ± 0.0
Psidium guayava	Leaves	50 ± 0.0
Senna alata	Leaves	50 ± 0.0
Spathodea companulata	Bark	0,125 ± 0.0
Spilanthes africana	Flowers	2 ± 0.0
Triumfetta pentandra	Whole plant	20 ± 0.0
Vernonia guineensis	Rhizomes	50 ± 0.0
Voacanga africana	Bark	10 ± 0.0

Data represent MIC values observed from three independent experiments.

NaCl and 0.1 M Na_2CO_3 pH 9.0, in double-distilled water. Bacterial titers were adjusted to 3.0×10^7 CFU/ml by measuring the optical density at 550 nm. Ten microliters of freshly prepared 1% fluorescein-isothiocyanate (FITC, Sigma) in dimethyl sulfoxide (DMSO) were added to the suspension, which was then incubated for 1 h at room temperature (RT) in the dark. Bacteria were recovered by centrifugation at 3000 *g*for 5 min, washed with 1 ml PBS resuspended by gently pipetting in 1 ml of PBS-Tween 20 (0.05% v/v), and pelleted by centrifugation as above. The wash cycle was repeated three times. Aliquots of labeled *H. pylori* (100 µL) were taken from the final suspension and used immediately. Gastric mice tissues were sectioned by Pathologists from Lagos University Teaching Hospital (LUTH). The sections were taken from *Helicobacter*-negative individuals, and the antrum mucosa showed no major pathologic alteration. Tissue sections were deparaffinated in xylene and 2-propanol, rinsed in water followed by PBS, and then incubated for 15 min at room temperature in blocking buffer (0.2% BSA, 0.05% Tween 20 in PBS). The FITC-labeled bacteria were diluted 20-fold in blocking buffer of which aliquots (200 µL) were placed on tissue slides. The slides were incubated for 1 h at room temperature in a humidified chamber in the dark. Slides were subsequently washed three times with PBS prior to microscopic inspection. To analyze the anti-adhesive activity of test plant extract, aliquots (100 µL) of FITC-labeled bacteria were pre-incubated with plant extract (0.1% in blocking buffer) for 2 h at room temperature in the dark. Bacteria were washed once in blocking buffer before aliquots (200 µL) were added to the gastric sections. After the sections had been carefully rinsed with PBS, FITC-labeled, untreated bacteria were added, and the assay was run as described above.

2.3.2. Validation of bacterial adherence

The validation of the bacterial adhesion was performed by fluorescence microscopy using a ranking list from 0, “no bacterial binding” to (25) for “very strong bacterial binding” (the numbers representing bacteria present in all the fields). Five microscopic fields of gastric sections were examined and the number of bacteria present counted. No bacterial binding, shows the efficacy of the extract to inhibit the binding of bacteria to mouse tissues.

2.4. RNA expression of gene of adhesion (babA and hopZ)

2.4.1. Culture and extraction of RNA

H. pylori P12 bacterial inoculum having a turbidity comparable to that of a 0.5 McFarland standard (1×10^8 cells/ml) was grown in *Brucella* broth supplemented with 10% fetal bovine serum (FBS) in the presence or absence of plant extract for three days at 37 °C on a humidity chamber in a CO_2 incubator. After this period, plant extract (10 mg/ml) was added to the bacteria incubation and vehicle control was also maintained. The plates were incubated at 37 °C under

Table 4
Effect of 4 h pretreatment with plant extract on the adhesion of FITC-labeled *H. pylori* to sections of mouse gastric tissue.

Plant extracts	Number of bacteria present	Plant extracts	Number of bacteria present
Acanthus montanus	0 ± 0	*Garcinia kola*	15 ± 2
Ageratum Conyzoide	0 ± 0	*Khaya grandifoliola*	5 ± 1.73
Alchornea laxiflora	25 ± 3	*Khaya grandifoliola Eth 70%*	5 ± 1
Allanblackia floribunda	15 ± 3.21	*Macaranga hurifolia Eth 70%*	1 ± 0.57
Annona muricata	0 ± 0.57	*Macaranga hurifolia MeOH*	1 ± 1
Anthocleista schweinfurthuii	5 ± 1	*Mangifera indica*	0 ± 0.57
Carica papaya	25 ± 3.61	*Milletia versicolor*	20 ± 2.52
Citrus aurantifolia	5 ± 1.73	*Nicotina tabacum*	0 ± 0
Cola acuminata	5 ± 0.57	*Ocimum basilicum*	5 ± 1
Commelina diffusa	25 ± 2.64	*Ocimum gratissimum*	1 ± 1
Croton macrostachyus	1 ± 1	*Persea americana*	5 ± 2.52
Cymbopogon citratus	0 ± 0.57	*Phragmantera capitata*	5 ± 0.57
Dacryodes edulis	1 ± 0.57	*Piliostigma thonnigui*	25 ± 2
Détarium microcapium	0 ± 0	*Piper umbellatum*	5 ± 2
Draceana deisteliena	6 ± 1.53	*Polyscias fulva*	5 ± 1
Euphorbia hirta	20 ± 2.08	*Psidum guayava*	25 ± 2
Elephantopus mollis	15 ± 1.52	*Senna alata*	20 ± 2.08
Emilia coccinea	1 ± 0.57	*Spathodea campanulata*	0 ± 0.0
Enantia chlorantha	0 ± 0.57	*Spilanthes africana*	15 ± 1.73
Entada africana MeOH	5 ± 2	*Triumfetta pentandra*	1 ± 0.57
Erythrina senegalensis	1 ± 0.57	*Vernonia guinensis*	5 ± 1
Ficus exasperata	25 ± 2	*Voacanga africana*	1 ± 1

Each test plant extract of a given concentration was examined at least in two or three independent experiments. Quantification of bacterial adhesion: 0, no bacterium present to 25, bacteria present in all examined field.

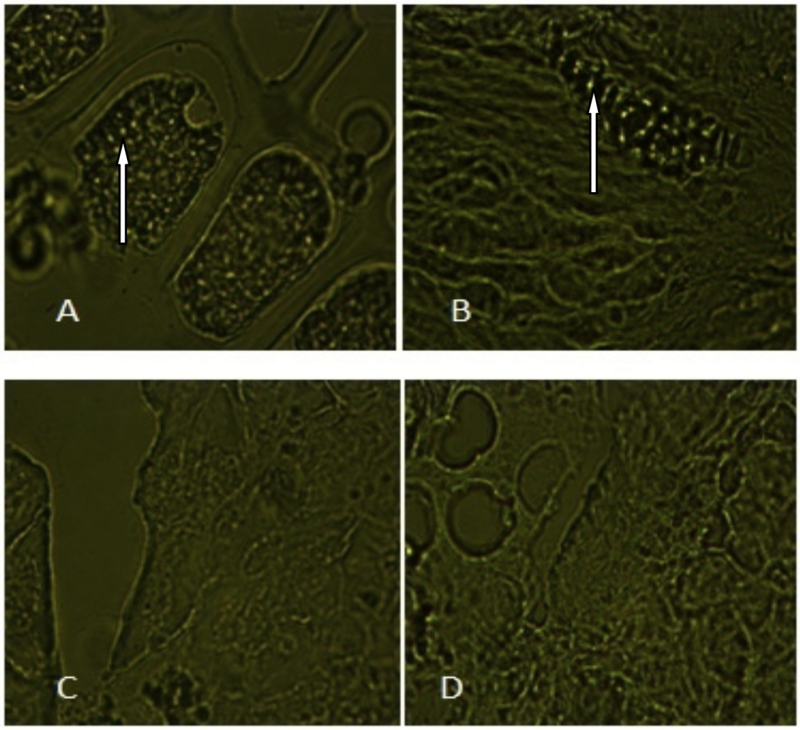

Fig. 1. Fluorescent microscopy of some representative in situ experiments with FITC-labeled *H. pylori* on mouse gastric tissue.

microaerophilic conditions for 4 h. Total RNA was extracted using silica-gel membrane adsorption for extraction of RNA (DATA SHEET, Jena Bioscience) according to the manufacturer's instruction. Expression of *H. pylori* adhesion molecules was determined by RT-PCR kit. cDNA was synthesized in a thermocycler using one step RT-PCR kit (DATA SHEET, Jena Bioscience) as directed by the manufacturer.

2.4.2. Analysis products

PCR products were analyzed by electrophoresis on a 2.0% agarose gel containing 0.5 µg/ml of ethidium bromide and gel images were captured. The primer sequences are listed in Table 1.

babA, blood group antigen binding adhesion; *hopZ*, *H. pylori* outer membrane protein

2.5. Western blot analysis

H. pylori P12 was used in this study. Three day old cultures of the *H. pylori* strain P12 were harvested into *Brucella* medium supplemented with 10% FBS to a McFarland standard of 2. 125 µl of the mixture was added to each well in a 96 well plate. One hundred and twenty five µl of plant extract at the minimum inhibitory concentration (MIC), and two concentrations below the MIC were used for this assay. The plates were

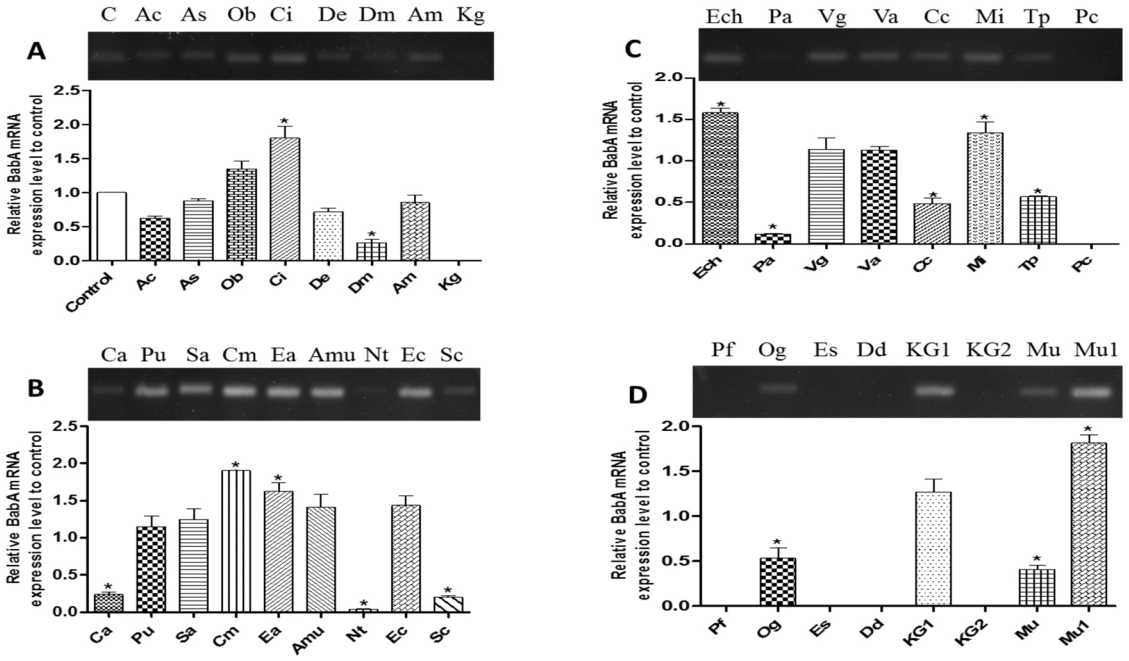

Fig. 2. Plant extract increased mRNA levels of babA gene.
H. pylori was treated with one concentration of plant extract (MIC) for 4 h and DMSO was used as loading control. RNA was extracted and relative mRNA expression level of babA was determined by reverse transcriptase-PCR. C: *H. pylori* + DMSO, the image is representative of two independent experiments. (A, B, C, D) effect of plant extract on relative *BabA* mRNA expression. Values are means ± SD of two independent experiments. ANOVA analysis: *P < 0.05 Values significantly different compared to control group. *C: control; Ac: Ageratum conyzoides; As: Anthocleista schweinfurthuii; Ob: Ocimum basilicum; De: Dacryodes edulis; Dm: Détarium microcapium; Am: Acanthus montanus; Kg: Khaya grandifoliola; Ech: Enantia chlorantha; Pa: Persea americana; Vg: Vernonia guinensis; Va: Voacanga africana; Cc: Cymbopogon citratus; Mi: Mangifera indica; Tp: Triumfetta pentandra; Pc: Phragmantera capitata; Ca: Cola acuminata; Pu: Piper umbellatum; Sa: Spilanthes africana; Cm: Croton macrostachyus; Ea: Entada africana* MeOH; *Amu: Annona muricata; Nt: Nicotina tabacum; Ec: Emilia coccinea; Sc: Spathodea campanulata; Pf: Polyscias fulva; Og: Ocimum gratissimum; Es: Erythrina senegalensis; Dd: Draceana deisteliena; KG1:Khaya grandifoliola; KG2: Khaya grandifoliola Eth 70%; Mu: Macaranga hurifolia Eth 70%; Mu1: Macaranga hurifolia MeOH.*

incubated at 37 °C under microaerophilic conditions for 4 h. After incubation, 250 µl of the mixture was transferred into a microcentrifuge tube and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was washed with phosphate buffer saline (PBS). Protein in Laemmli loading buffer was separated by 12% Sodium-Dodecyl-Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) and electro-transferred into a Polyvinylidene difluoride paper (PVDF) Blotting Membrane (GE Healthcare, Germany). The membranes were blocked with 5% w/v nonfat milk in Tris-Buffered Saline Tween-20 (TBST: 10 mM TrisHCl; 150 mM NaCl; 0.05% tween-20; pH 7.6); incubated overnight at 4 °C with primary antibodies, rinsed, and then incubated for 1 h at 25 °C with horseradish peroxidase-conjugated secondary antibodies. The blots were then developed by using developing buffer 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma, Germany). Densitometry analysis of the protein bands was performed using ImageJ Software.

2.6. Statistical analysis

Data analysis was carried out using the GraphPad Prism 5.03 software. Excel was used to cited all the plants of the survey. For the MIC and bacteria adhesion, the results were expressed as mean ± SEM of three independent experiments. Regarding gene and protein expression, data were presented as mean ± SEM and analyzed by one-way ANOVA using GraphPad Prism 5.0 followed by the Bonferroni's post hoc test whenever significant differences were observed between the variances. Comparisons were made between untreated group and DMSO control groups. Differences between compared groups were considered significant for $p < 0.05$.

3. Results

3.1. Ethnopharmacological survey

A total of 28 traditional physicians (15 males and 13 females) was interviewed in this study. Sixteen were from Bafia (10 males and 6 females), 7 from Bazou (2 males and 5 females) and 5 from Foumbot (3 males and 2 females). The age of the traditional physicians ranged 30 to 65 years. Table 2 below presented the botanical name, family, local name, plants parts, administration route and frequency of citation of all collected plants. Forty-one plant species belonging to 27 families were collected from the study area. The largest number of species was noted from family *Asteraceae* (5 species), followed by *Euphorbiaceae* (4 species).

3.2. Effect of plant extracts on growth inhibition

The anti-*H. pylori* activities of plant extract, represented by the minimum inhibitory concentration (MIC) are shown in (Table 3**)**. The MIC values of plant extracts ranged from 0.125 to 100 mg/ml. *Spathodea campanulata* and *Nicotina tabacum* have high potential because the MICs were 0.125 and 1 mg/ml respectively.

3.3. Adherence of H. pylori to sections of mouse stomach tissue

Seventeen plant extracts completely and strongly reduced the adhesion of *H. pylori* **(**Table 4**)**. Fig. 1 displays characteristic binding with pretreated and none treated *H. pylori* on sections of mouse gastric tissue.

adhesion of non-treated bacteria, (B) adhesion of pretreated bacteria with DMSO, (C, D) adhesion of pretreated bacteria with plant

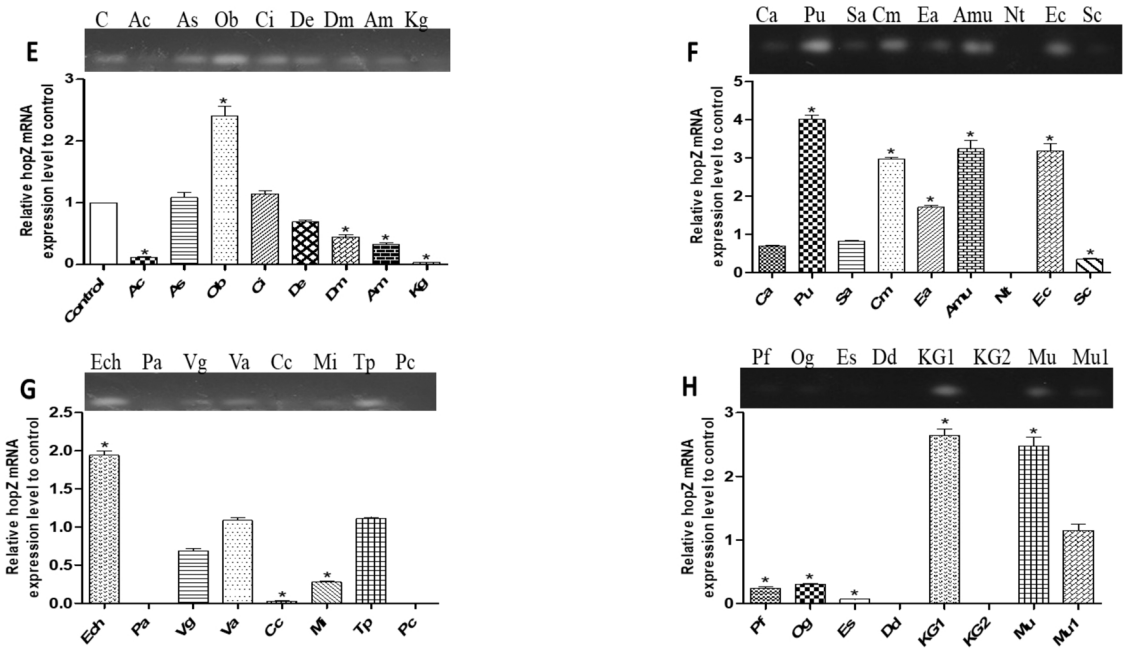

Fig. 3. Plant extract increased mRNA levels of hopZ gene.
H. pylori was treated with one concentration of plant extract (MIC) for 4 h and DMSO was used as loading control. RNA was extracted and relative mRNA expression level of *hopZ* was determined by reverse transcriptase-PCR. C: *H. pylori* + DMSO, the image is representative of two independent experiments. (E, F; G, H) effect of plant extract on relative *hopZ* mRNA expression. Values are means ± SD of two independent experiments. ANOVA analysis: *P < 0.05 Values significantly different compared to control group. *C: control; Ac: Ageratum conyzoides; As: Anthocleista schweinfurthuii; Ob: Ocimum basilicum; De: Dacryodes edulis; Dm: Détarium microcapium; Am: Acanthus montanus; Kg: Khaya grandifoliola; Ech: Enantia chlorantha; Pa: Persea americana; Vg: Vernonia guinensis; Va: Voacanga Africana; Cc: Cymbopogon citratus; Mi: Mangifera indica; Tp: Triumfetta pentandra; Pc: Phragmantera capitata; Ca: Cola acuminata; Pu: Piper umbellatum; Sa: Spilanthes africana; Cm: Croton macrostachyus; Ea: Entada africana* MeOH; *Amu: Annona muricata; Nt: Nicotina tabacum; Ec: Emilia coccinea; Sc: Spathodea campanulata; Pf: Polyscias fulva; Og: Ocimum gratissimum; Es: Erythrina senegalensis; Dd: Draceana deisteliena; KG1:Khaya grandifoliola; KG2: Khaya grandifoliola Eth 70%; Mu: Macaranga hurifolia Eth 70%; Mu1: Macaranga hurifolia MeOH.*

extract. The image is representative of three independent experiments.

3.4. Effect of plant extracts on adhesion gene expression

H. pylori adhesion to the gastric epithelium is mediated by the adhesin molecules. As demonstrated in the adhesion assay, *H. pylori* adhesion to mouse gastric tissue sections was inhibited by the action of some plant extracts.

Therefore, RT-PCR was performed to determine whether expressions of the adhesin genes was influenced by the plant extract. The levels of the expression of adhesins genes *babA and hopZ* (Figs. 2 and 3) on bacterium treated with plant extract were evaluated. Ten plant extracts inhibited *hopZ* expression and five plant extracts inhibited *babA* expression.

3.5. Plant extract regulation of BabA and HopZ protein expression

Western blot results revealed that expression of *BabA* and *HopZ* was reduced by some plant extracts treatment. To confirm this binding of bacteria on the mouse gastric tissue sections, we performed western blot to determine whether the protein level of both protein molecules was diminished. As shown in Fig. 4 and 5, exposure of *H. pylori* to the plant extract for 4 h resulted in a marked decrease of *BabA* and *HopZ*-protein level. The molecular weights of the *H. pylori BabA* and *HopZ* are 78KDa and 74KDa. Based on the predicted molecular weight, we observed the suppression of both *BabA* and *HopZ* protein synthesis in plant extract treated cells.

4. Discussion

In Cameroon, various studies have shown that plant extracts have *H. pylori* inhibitory effects [19,20,24,25]. These suggest that plants from Cameroonian pharmacopoeia could be potential sources of new bioactive molecules against *H. pylori*. The purpose of this work was to determine the anti *H. pylori* and anti-adhesion effects of some plant extracts from Cameroon. We demonstrated that the extracts of plants of Cameroonian pharmacopoeia exhibited anti-*H. pylori* activity at minimum inhibitory concentrations ranging from 0.125 to 100 mg/ml (Table 3). The screening of the best plant extracts with anti-*H. pylori* and anti-adhesion activities was determined respectively by broth micro-dilution, agar dilution and FITC-label. From this methodology, we found that extracts of *Spathodea campanulata* and *Nicotina tabacum* exhibited higher anti-*H. pylori* activity *in vitro* with MIC values of 0.125 and 1 mg/ml respectively. We also found that seventeen plant extracts totally inhibited *H. pylori* adhesion to the mouse stomach tissue. Similar results were obtained by Zheng et al. [26] who showed ethanolic extract of *Centella asiatica* inhibited *H. pylori* growth at effective concentration of 2 mg/mL.

Similar work was carried out by Ndip et al. [24] on inhibitory activity of plants extracts from *Ageratum conyzoïdes, Acanthus montanus, Emilia coccinea* and *Euphorbia hirta* on *H. pylori*. From the results obtained by these authors, the activity of the plant extract *Ageratum conyzoïdes* was confirmed in the present study. Moreover, this plant extract showed inhibitory activity of gene expression experiments carried out in this study.

The bactericidal activity could be related to the presence of secondary metabolites polyphenolic compounds, flavonoids and tannins in plants [27–29]. Indeed, these groups of metabolites could perform their bactericidal activities by inhibiting proton pumps, protein biosynthesis, peptidoglycan disruption or inhibition of nucleic acid synthesis or function. In addition, the anti-adhesive activity could be due to the presence of polysaccharides, polyphenols, tannins and flavonoids which

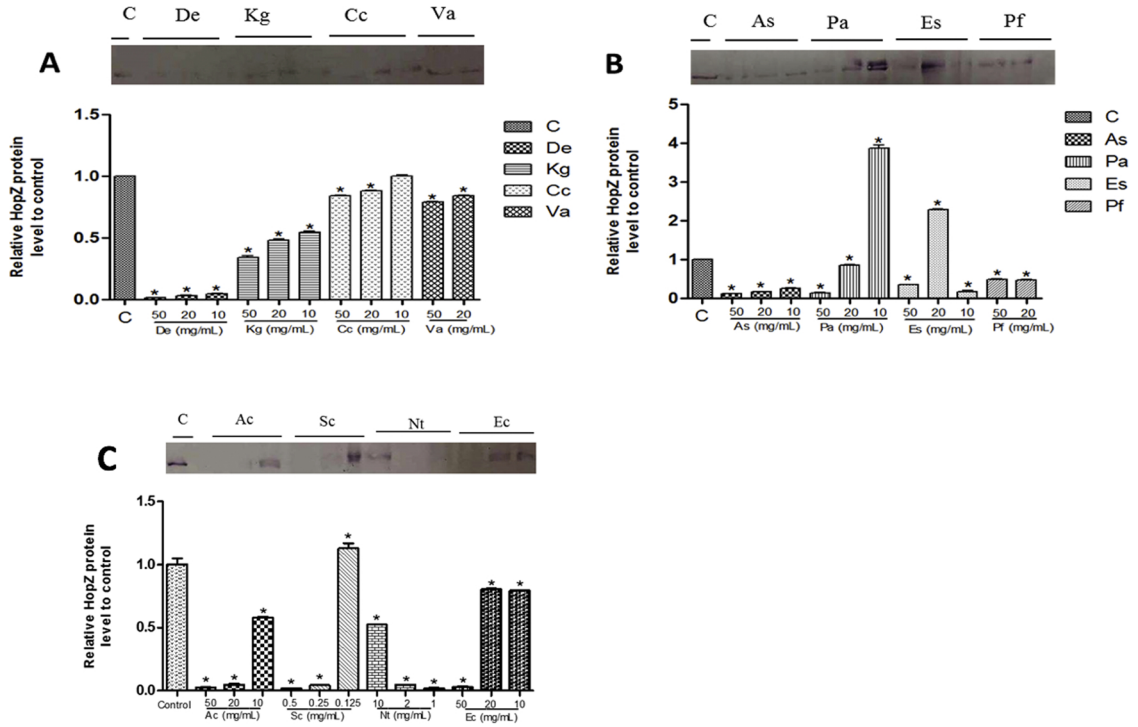

Fig. 4. Effect of plant extract on *H. pylori* P12 adhesin molecule HopZ.
H. pylori was treated with various concentrations of plant extract for 4 h and protein levels of adhesin molecule HopZ were determined. After treatment, total proteins were extracted and expression were determined by western blot. DMSO was used as loading control. Each blot represents one of two independent experiments. (A, B, C) effect of plant on HopZ expression. Densitometry analysis of blot for plant extracts. Values are means ± SD of two independent experiments in triplicate. ANOVA analysis: Values significantly different compared to control group ($P < 0.05$). Values significantly different compared to control group. *De: Dacryodes edulis; Kg: Khaya grandifoliola; Cc: Cymbopogon citratus; Va: Voacanga Africana; As: Anthocleista schweinfurthuii; Pa: Persea Americana; Es: Erythrina senegalensis; Pf: Polyscias fulva; Ac: Ageratum conyzoides; Sc: Spathodea campanulata; Nt: Nicotina tabacum; Ec: Emilia coccinea*

have the capacity to mimic the specific receptor form of these adhesions [23,30,31].

After determining the anti-adhesive activity of the plant extracts, the type of adhesin responsible for this activity was investigated. For this purpose, we incubated the plants in the presence of the bacterium, and then the total proteins as well as the total RNAs were extracted to evaluate the gene expression responsible for the biosynthesis of *babA* and *hopZ* adhesins by RT-PCR as well as the expression of *BaBA* and *HopZ* proteins by western blot. Two adhesins were investigated *BabA* and *HopZ*, we found that some plant extracts used at their MIC displayed strong inhibition of the synthesis of the two adhesins: *Anthocleista schweinfurthuii; Ageratum conyzoides, Spathadea Campunalata* and *Nicotina tabacum*. The strongest inhibition was observed with extracts from *Spathadea Campunalata* and *Nicotina tabacum* which were effective at concentrations of 0.125 mg/ml and 1 mg/ml respectively for *babA* and *hopZ* (Fig. 3) when compared to control. It is important to notice that the results on the inhibition of *H. pylori* growth and anti-adhesion effect of extract match each other regarding *S. campanulata* and *N. tabacum*. These plant extract exhibited low MICs values (0.125 mg/ml and 1 mg/ml, respectively) and strongly inhibited adhesins expressions at the RNA and protein levels. This work provides information on inhibition of *H. pylori* adhesins and further studies need to be done to better understand the action of plant extracts in inhibiting *H. pylori* adhesion to epithelial cells.

In this study, we showed that plant extracts inhibited the growth of *H. pylori* and adherence to mouse stomach sections by inhibiting the expression of RNA and protein adhesion molecules *BabA* and *HopZ*. Our study suggests that these plant extracts could be good candidates for overcoming tolerance of antibiotics for the treatment of *H. pylori* mediated gastric disease.

A drawback of the study is that only organic extracts were used to screening the efficacy of the plant extracts. Hence, it is possible that more potent phytochemicals present in the plants tested may have been obtained in different solvents used during the extraction process.

5. Conclusion

Cameroon plants inhibited *H. pylori* growth and adhesion. Our work has given additional scientific support to the traditional use of these plants in Cameroon to treat *H. pylori* related disorders. *S. campunalata* and *N. tabacum* were the plants presenting the strongest inhibitory effect. To the best of our knowledge, this is the first report showing inhibition of adhesion to gastric mouse tissue by plant extracts from Cameroon.

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Author contributions

CRN, FNN, SIS and PMF defined the research subject and its aims, conceived and designed the experiments. PMF and SIS provided facilities to perform the work. CRN, MF and FBS prepared the plant extracts and performed the experiments. CRN, FBS and PMF analyzed the data and wrote the paper. All the authors read and approved the final version of this manuscript.

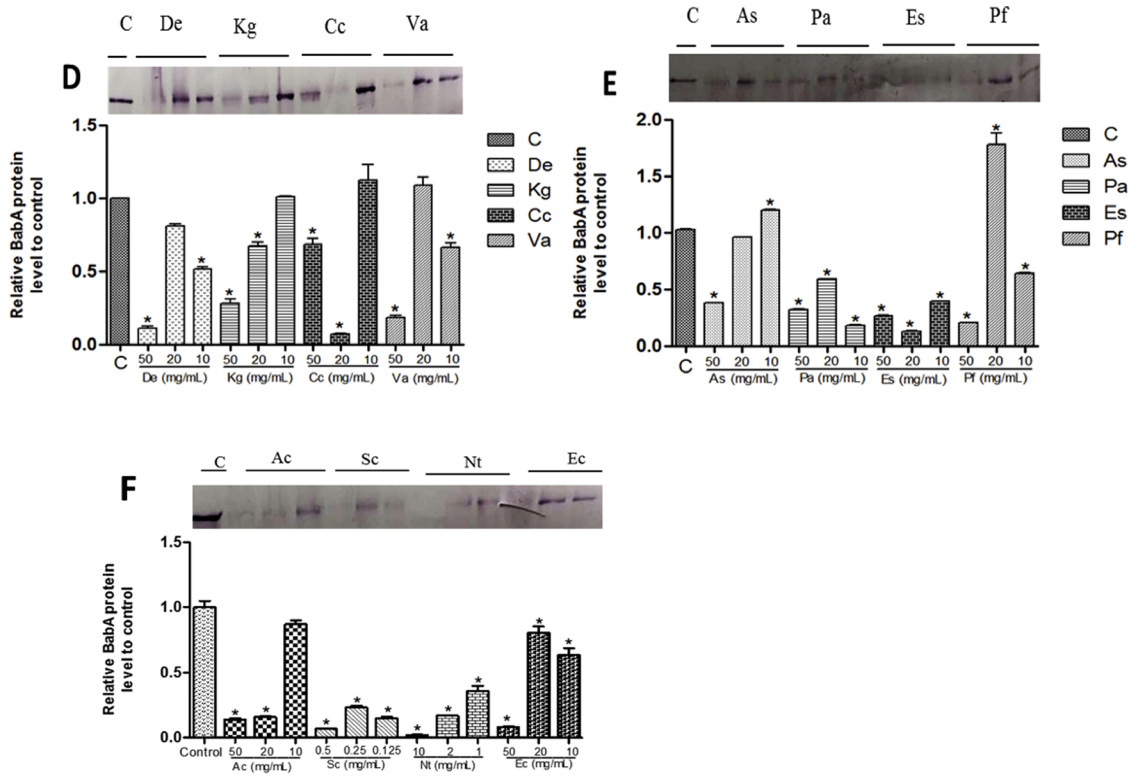

Fig. 5. Effect of plant extract on *H. pylori* P12 adhesin molecules BabA.
H. pylori was treated with various concentrations of plant extract for 4 h and protein levels of adhesin molecules BabA were determined. After treatment, total proteins were extracted and BabA expression were determined by western blot. DMSO was used as loading control. Each blot reprents one of two independent experiments. (D, E, F) effect of plant on *BabA* expression. Densitometry analysis of blot for plant extracts. Values are means ± SD of two independent experiments in triplicate. ANOVA analysis: Values significantly different compared to control group ($P < 0.05$). Values significantly different compared to control group. *De: Dacryodes edulis; Kg: Khaya grandifoliola; Cc: Cymbopogon citratus; Va: Voacanga Africana; As: Anthocleista schweinfurthuii; Pa: Persea Americana; Es: Erythrina senegalensis; Pf: Polyscias fulva; Ac: Ageratum conyzoides; Sc: Spathodea campanulata; Nt: Nicotina tabacum; Ec: Emilia coccinea*

Declaration of Competing Interest

The authors declare no conflicts of interest

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