Research Article

In Vitro and In Vivo Anti-Helicobacter Activities of Eryngium foetidum (Apiaceae), Bidens pilosa (Asteraceae), and Galinsoga ciliata (Asteraceae) against Helicobacter pylori

Laure brigitte Kouitcheu Mabeku,1 Bertrand Eyoun Bille,1 and Eveline Nguepi2

1Microbiology and Pharmacology Laboratory, Department of Biochemistry, Faculty of Science, University of Dschang, P.O. Box 67, Dschang, Cameroon
2Gastroenterology Department, Laquintinie Hospital of Douala, P.O. Box 4035, Douala, Cameroon

Correspondence should be addressed to Laure brigitte Kouitcheu Mabeku; laurebkouitcheu@yahoo.fr
Received 7 June 2016; Accepted 31 July 2016

1. Introduction

Helicobacter pylori is a Gram negative microaerophilic helical bacillus that affects the gastric mucosa and can be found attached to epithelial cells of the human stomach [1]. It is an etiologic agent of peptic ulcer disease, primary gastritis, gastric mucosa-associated lymphoid-tissue lymphoma, and gastric adenocarcinoma [2]. Approximately 20% of persons infected with H. pylori develop related gastroduodenal disorders during their lifetime [3]. Approximately 50% of persons are Helicobacter pylori positive around the world, with the developing countries having a prevalence of 80%–90% and industrialized countries like the United States having ≈35%–40% [3]. As the prevalence, the annual incidence of H. pylori infection is high in developing countries (≈4%–15%) compared with industrialized countries (0.5%) [4]. Low socioeconomic status, overcrowding, poor sanitation or hygiene, and living in a developing country are the risk factors of infection [2]. The recurrence of gastroduodenal disorders is substantially reduced by the eradication therapy of H. pylori infection. This eradication therapy entails two of the following antimicrobial agents; metronidazole, amoxicillin, tetracycline, or clarithromycin in association with a proton pump inhibitor or bismuth [5]. Quadruple regimens are used as a salvage therapy when triple therapy regimens failed [5]. The emergence of drugs resistance H. pylori strain is the most common causes of treatment failure [6]. The failure of 38% and 55% of cases, respectively, to therapy regimens containing metronidazole and clarithromycin has been documented, especially when these antimicrobial agents are used to treat infection with an organism resistant to one of the latter [7]. Consequently, there is the necessity for the development of
new antimicrobial agents from natural sources for better chemotherapeutic applications [8]. Literature clearly shows that new antimicrobial agents from natural sources for better horserewa serum and 20% glycerol and stored at 0-80°C until further use.

2.2. Animals. Swiss albino mice of 18-22 g weight obtained from Animal House, University of Dschang, Cameroon, were selected as experimental animals. These animals were fed with standard pellet diet and tap water.

2.3. Statement on Ethics Approval. Throughout the experiments, all animals received human care according to the criteria outlined in the internationally accepted principle guidelines of the European Union on Animal Care (CEE Council 86/609). Animal experiments were designated and approved by the Cameroon National Ethics Committee (Reg. number. FWA-IRB00001954).

2.4. Plant Material. The leaves of Bidens pilosa (Asteraceae) and Galinsoga ciliata (Asteraceae) were collected in Dschang (west region of Cameroon) and leaves of Eryngium foetidum (Apiaceae) in Kumba (southwest region of Cameroon). Identification of the plant was done at the National Herbarium Yaoundé (voucher specimen numbers 42254/HNC, 57409/HNC, and 42131 HNC, resp., in the listed order. HNC: Cameroon National Herbarium). The plant materials were then air-dried at room temperature and ground into a fine power.

2.5. Chemicals and Culture Media. Culture media (Columbia Agar, Brain Heart Infusion (BHI), lacked horse blood, Horse Serum, Vitox Supplement) and CampyGen gas pack were obtained from Oxoid, Basingstoke, England. Doxycycline (doxycycline 200 mg, Combitic Global Caplet, India), metronidazole (metronidazole Tablets BP 500 mg, Strides Arcolab, India), and ciprofloxacin (zoflox, ciprofloxacin 750 mg, Odypharm) were used as reference antibiotics for the determination of the MIC and MBC and for the in vivo test were obtained from local pharmacy. P-lodonitrotetrazolium chloride (INT, Sigma-Aldrich) was used to indicate microbial growth [22].

2.6. Extraction. Dried powdered plant material was weighed (250 g) and soaked in 3.5 L of ethyl acetate (EA) or methanol (MeOH) for 72 hours. The plant mixture was then filtered and concentrated under reduced pressure using a rotary evaporator. The extract was further concentrated by allowing it to stand overnight in an oven at 30°C.

2.7. In Vitro Study

2.7.1. Antimicrobial Assay: Determination of MIC and MBC. The clinical strains (H. pylori a1, H. pylori a2, H. pylori a3, H. pylori a4, H. pylori a5, and H. pylori a6), identified using Gram staining and enzymatic properties (catalase, oxidase, and urease), were employed to evaluate the in vitro anti-Helicobacter activity of the plant-extracts. Methanol and ethyl acetate extracts of Bidens pilosa, Galinsoga ciliata, and Eryngium foetidum were used for the determination of MICs by the INT broth microdilution method [22] using 96-well plates. Twofold dilutions of each extract were prepared in the test wells in BHI broth supplemented with 5% horse serum (BHI-serum). The final extract concentrations ranged from 0.002 to 1.024 mg/mL. One hundred microliters of inoculums prepared from 48 h colonies on supplemented Columbia Agar (Columbia Agar + 5% (v/v) lacked horse blood and 1% (v/v) Vitox) at McFarland turbidity standard 3 was added to 100 μL of the extract-containing culture medium. Control wells were prepared with culture medium and bacterial
suspension and broth, only respectively. Ciprofloxacin, doxycycline, and metronidazole at concentration ranges of 0.002 to 0.128 mg/mL were used as positive control. The plates were covered with a sterile plate sealer; the contents of the wells were mixed with a shaker and incubated for 3 days at 37°C under microaerophilic conditions. After incubation, 40 μL of 0.2 mg/mL INT was added per well and incubated at 37°C for 30 min. Living bacteria reduced the yellow dye to pink. The sample concentration that prevented the color change of the medium and exhibited complete inhibition of microbial growth is known as the MIC. The MBC was determined by adding 50 μL aliquots of the preparations which prevented the color change of the medium after incubation during MIC assays, to 150 μL of BHI-serum. These preparations were incubated at 37°C for 72 h under microaerophilic conditions. The lowest concentration of extract, which did not produce a color change after addition of INT, was considered as the MIC. The MBC was estimated by colony count and expressed as logCFU/g/mL of homogenate.

2.8.1. Inoculation of Experimental Animals. Methanol extract of *Eryngium foetidum* the most active plant-extract according to the MIC value obtained, was chosen for further *in vivo* anti-*Helicobacter* assessment. For this purpose, seventy-two mice were allowed to acclimatize for one week before initiation of experiment. After the acclimatization period, the animals were fasted for 12 h and sixty of them were infected with 0.2 mL of 10⁸ CFU/mL of *H. pylori* a2 suspension, four times in one week with a 24 h interval between each inoculation. The inoculation day was considered as day 0 and subsequent days as day 1, day 2, and day 3 up to day 21. A group of uninfected mice serving as a normal group received sterile solution of the vehicle (200 μL of 0.25% Tween 80).

2.8.2. Distribution of Animals. Inoculated mice were distributed into five groups of 12 animals and allowed to rest for 24 h after the last inoculation. Group I, a negative control group, received sterile solution of the vehicle; group II was treated with ciprofloxacin (500 mg/kg) and served as positive control; test groups III to V were treated, respectively, with 125, 250 and 500 mg/kg of *Eryngium foetidum* extract, once daily for seven consecutive days. The mice were allowed to fast for 12 h after the last day of treatment and six from each group were sacrificed with ethyl ether. The rest of animal were sacrificed after a posttreatment period of seven days. The stomach of each animal was removed and opened through the longer curvature with sterile surgical instruments. The stomach and duodena, covering all subtypes of mucosa, were used for culturing *H. pylori*.

2.8.3. Culture and Bacterial Load. Stomach biopsies were scraped off and homogenized with 0.5 mL of PBS in a 1.5 mL Eppendorf tube. The homogenized sample was serially diluted 10-fold. Each 0.1 mL of homogenate was plated on supplemented Columbia Agar and incubated at 37°C for 2 to 3 days under microaerophilic conditions. *H. pylori* was identified through macroscopic characteristics (small translucent colonies), microscopic characteristics and Gram staining (Gram negative curved rods), and enzymatic properties (urease, catalase, and oxidase positive). The bacterial load was estimated by colony count and expressed as log₁₀ CFU per milliliter of homogenate.

2.9. Statistical Analysis. The results were expressed as means ± standard deviation. Before analysis, bacterial densities were expressed in log₁₀. The analysis of variance followed by the paired Student’s *t*-test was used for the statistical evaluation of data. The differences between groups were considered significant at *p* < 0.05.

3. Results

3.1. MIC and MBC Determination of Plant-Preparations and Antibiotics. Plant species showed different anti-*Helicobacter* activity with each other with MIC values ranging from 64 to >1024 μg/mL. The methanol extract of *E. foetidum* shows the best activity for MIC values between 64 and 512 μg/mL against 3/6 (50%) evaluated strains. Methanol extract from *E. foetidum* also displayed the best spectrum of bactericidal effect with a ratio of MBC/MIC 1 obtained on two tested *H. pylori* strains. MIC values from 128 to 512 μg/mL were also recorded with *E. foetidum* (AE), *Bidens pilosa* (MeOH), *Bidens pilosa* (AE), *Galinsoga ciliata* (MeOH), and *Galinsoga ciliata* (AE) extracts, respectively against 2/6 (33.33%), 4/6 (66.66%), 1/6 (16.66%), 4/6 (66.66%), and 4/6 (66.66%) of the evaluated bacteria. The best MIC value (64 μg/mL) was obtained with the methanol extract from *E. foetidum* against *H. pylori* a1 and *H. pylori* a2.

3.2. Phytochemical Screening. According to Table 2, alkaloids, phenoloids, flavonoids, anthraquinones, and sterol are the chemical compounds present in the methanol extract of *E. foetidum*.

3.3. Culture. After the cessation of treatment, the number of *H. pylori* positive animals in treated groups (plant-extract and ciprofloxacin) was lower than the number recorded in the untreated infected group (control group) (Table 3). None of the animals treated with the highest dose of plant-extract (500 mg/kg) or ciprofloxacin was *H. pylori* positive. However, only 83.33% of animals treated with 125 and 250 mg/kg of the
Table 1: MIC/MBC of crude plant-extracts and antibiotics (µg/mL).

| Crude extracts/antibiotics | H. pylori α1 | H. pylori α2 | H. pylori α3 | H. pylori α4 | H. pylori α5 | H. pylori α6 |
|---------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| E. foetidum (MeOH)        | 64/64       | 64/64       | 512/512     | >1024       | >1024       | >1024       |
| E. foetidum (AE)          | 256/256     | 512/512     | >1024       | >1024       | >1024       | >1024       |
| Bidens pilosa (MeOH)      | 1024/1024   | 512/1024    | >1024       | 256/1024    | 512/1024    | 256/1024    |
| Bidens pilosa (AE)        | 1024/1024   | >1024       | >1024       | 256/1024    | >1024       | 1024/1024   |
| Galinsoga ciliata (MeOH)  | 512/1024    | 256/512     | >1024       | >1024       | 128/512     | 256/512     |
| Galinsoga ciliata (AE)    | 256/512     | >1024       | 256/512     | 256/512     | >1024       | 128/512     |
| Doxycycline               | 64/64       | 32/32       | 32/32       | 32/32       | 4/16        | 32/32       |
| Ciprofloxacin             | 8/8         | 32/32       | 2/2         | <2          | 16/16       | 8/8         |
| Metronidazole             | >128        | >128        | >128        | >128        | >128        | >128        |

All the values given in the table are means of three determinations. MeOH: methanol; AE: ethyl acetate.

Figure 1: Effects of different treatment on the recovery of H. pylori from infected mice. Each circle represents the bacterial count for one animal. Inf: infected + vehicle; Inf + Ef 125: infected + E. foetidum pinnatum 125 mg/kg; Inf + Ef 250: infected + E. foetidum 250 mg/kg; Inf + Ef 500: infected + E. foetidum 500 mg/kg; Inf + Cp 500: infected + ciprofloxacin 500 mg/kg; Uninf: normal group. Data column of the different treatment with superscript * are significantly different compared with infected control group (∗ p < 0.05).

plant-extract were H. pylori negative. There were no differences among the groups treated with three doses of plant-extract and ciprofloxacin at the second posttreatment period of time. Almost all the infected treated animals were H. pylori negative at the second posttreatment period, indicating the lasting action of the plant-extract or ciprofloxacin.

3.4. Bacterial Load. Infected mice showed stable H. pylori colonization of gastric mucosa as shown in the number of bacterial counts (Figure 1). The bacterial load of gastric mucosa in infected mice was significantly reduced following the administration of 500 mg/kg of E. foetidum (381.9 ± 239.5 CFU) and ciprofloxacin (248 ± 153.2 CFU) compared to that recorded with untreated infected mice (14350 ± 690 CFU).

Table 2: Phytochemical screening of methanol extract of E. foetidum.

| Compounds        | Methanol extract of E. foetidum |
|------------------|--------------------------------|
| Alkaloids        | +                              |
| Flavonoids       | +                              |
| Phenolics        | +                              |
| Saponins         | –                              |
| Tannins          | –                              |
| Triterpenes      | –                              |
| Anthraquinones   | +                              |
| Anthocyanins     | –                              |
| Steroid          | +                              |

*: present; –: absent.

4. Discussion

In this study, the antimicrobial activities of the ethyl acetate and methanol extract of three medicinal Cameroonian plants were evaluated against six H. pylori strains. In vitro, the tested plants displayed selective antibacterial activities and this activity was different within the same plant species from one extraction solvent to another. In fact, the type of solvent used largely affects the effectiveness of the extracts. The differences observed in the antibacterial activities of the plants species could be due to the differences in their chemical composition and in the mechanism of action of their bioactive constituents [30]. It is known that the antibacterial activity of a plant-extract is considered significant when MIC values are less than 100 µg/mL, moderate when being between 100 and 625 µg/mL, and weak when being greater than 625 µg/mL [31]. Thus, the MIC value of 64 µg/mL obtained with E. foetidum methanol extract against H. pylori strains. 

The preliminary phytochemical analysis of the tested plant-extract revealed the presence of alkaloids, phenols, flavonoids, anthraquinones, and steroid (Table 2). The result of the antimicrobial activity of E. foetidum obtained herein correlates with that of Lingaraju et al. [22] showing that the
ethyl acetate extracts of this plant have antimicrobial activity against Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and fungus Candida albicans using agar well diffusion method. The activity of the methanol extract of E. foetidum against H. pylori a1 and H. pylori a2 was similar to that of doxycycline, a standard drug against H. pylori a1 (Table 1). This is quite remarkable particularly because the standard antibiotics are in the purified form whereas the extracts are mixtures of both pharmacological and nonpharmacological substances. Anti-Helicobacter activity of the methanol extract of E. foetidum was better than that of metronidazole, another antibiotic used in the treatment of H. pylori infection. Metronidazole-containing regimens have recently been shown to have limited effectiveness because of the increasing prevalence of resistance to this drug [32]. Its prevalence varies from 10 to 90% in different countries [33]. Studies of Ndip et al. [34] and Tanih et al. [32] carried out, respectively, in Cameroon and South Africa, documented a very high resistance rate of 95.5% to metronidazole.

Since the in vitro anti-Helicobacter properties of a compound can become ineffective in vivo, the most active plant-extract in vitro was also evaluated in vivo on the mouse model. As indicated by the confirmation tests for the presence of H. pylori and bacterial load, both methanol extract of E. foetidum (500 mg/kg) and ciprofloxacin significantly reduced H. pylori colonization of gastric mucosa in infected mice as compared to infected untreated mice. Thus, our data showed that the anti-Helicobacter activity of the methanol extract of E. foetidum was effective in vitro and in vivo. The activity of the plant-extract demonstrated herein is very important when considering that the tested plant-extracts are from edible plant parts and also when considering the medical importance of the tested bacteria. In fact, H. pylori is a virulent pathogen as evidenced by its low infective dose and high prevalence in human populations and the principal cause of type B gastritis, peptic ulcer disease, gastric adenocarcinoma, and MALT-lymphoma [35]. It has also been classified as a Class I carcinogen by the World Health Organization [35]. Furthermore, the continuous emergence of drugs resistant to H. pylori drastically reduced the limited range of antibiotics that have efficacy in the treatment of this infection leading globally to an increase in the frequency of therapeutic failure [36].

In conclusion, the present data provided evidence that methanol extract of E. foetidum could be a rich source of

### Table 3: Effects of different doses of E. foetidum and ciprofloxacin on H. pylori gastric colonization of infected and normal mice.

| Treatment group (mg/kg) | Tests | 1 day after cessation of treatment | 7 days after cessation of treatment |
|-------------------------|-------|-----------------------------------|-----------------------------------|
|                         |       | Animals number                     | Animals number                     |
|                         |       | 1 2 3 4 5 6 Negative (%)           | 1 2 3 4 5 6 Negative (%)           |
| Normal                  |       |                                   |                                   |
|                         | Urease| − − − − − − 100                   | − − − − − − 100                   |
|                         | Catalase| − − − − − − 100                   | − − − − − − 100                   |
|                         | Oxidase| − − − − − − 100                   | − − − − − − 100                   |
|                         | Gram st| − − − − − − 100                   | − − − − − − 100                   |
| H. pylori + vehicle     |       |                                   |                                   |
|                         | Urease| + + + + + + 0                     | + + + + + + 0                     |
|                         | Catalase| + + + + + + 0                     | + + + + + + 0                     |
|                         | Oxidase| + + + + + + 0                     | + + + + + + 0                     |
|                         | Gram st| + + + + + + 0                     | + + + + + + 0                     |
| H. pylori + E. foetidum, 125 | | | |
|                         | Urease| − − − + + + 50                    | − − − + + + 67                    |
|                         | Catalase| − − − + + + 83                   | − − − + + + 100                   |
|                         | Oxidase| − − − + + + 100                   | − − − + + + 100                   |
|                         | Gram st| − − − + + + 67                    | − − − + + + 83                    |
| H. pylori + E. foetidum, 250 | | | |
|                         | Urease| − + + − − − 67                    | − + + − − − 83                    |
|                         | Catalase| − + + − − − 100                   | − + + − − − 100                   |
|                         | Oxidase| − + + − − − 100                   | − + + − − − 100                   |
|                         | Gram st| − + + − − − 83                    | − + + − − − 100                   |
| H. pylori + E. foetidum, 500 | | | |
|                         | Urease| − − − + − + 100                   | − − − + − + 100                   |
|                         | Catalase| − − − + − + 100                   | − − − + − + 100                   |
|                         | Oxidase| − − − + − + 100                   | − − − + − + 100                   |
|                         | Gram st| − − − + − + 100                   | − − − + − + 100                   |
| H. pylori + ciprofloxacin, 500 | | | |
|                         | Urease| − − − + − − 100                   | − − − + − − 100                   |
|                         | Catalase| − − − + − − 100                   | − − − + − − 100                   |
|                         | Oxidase| − − − + − − 100                   | − − − + − − 100                   |
|                         | Gram st| − − − + − − 100                   | − − − + − − 100                   |

All the percentages given in the table are means of the six animals (n = 6) that tested negative per group. (+): positive test; (−): negative test.
metabolites with antimicrobial activity to fight *Helicobacter pylori* infections. Further investigations on purification and structure elucidation of the compounds are in progress.

**Competing Interests**

The authors declare that they have no competing interests.

**Acknowledgments**

The authors are grateful to the Cameroon National Herbarium for the identification of plants.

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