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Antibiotic-potentiation Activities of Cameroonian Medicinal Plants against Multidrug-resistant Phenotype *Helicobacter pylori* Clinical Isolates

Soupgui Franck Cedric[1], Kouitcheu Mabeku Laure Brigitte[2*], Eyoum Bille Bertrand[2], Hubert Leundji[3], Tamesse Joseph Lebel[1] and Francois-Xavier Etoa[4]

[1]Department of Biological Sciences, Higher Teacher's Training College, University of Yaoundé I, P.O.Box 47, Yaoundé, Cameroon.
[2]Department of Biochemistry, Microbiology and Pharmacology Laboratory, Faculty of Science, University of Dschang, P.O.Box 67, Dschang, Cameroon.
[3]Department of Gastroenterology, Laquintinie Hospital of Douala, P.O.Box 4035, Douala, Cameroon.
[4]Department of Microbiology, Faculty of Science, University of Yaoundé I, P.O.Box 812, Yaoundé, Cameroon.

Authors' contributions

This work was carried out in collaboration between all authors. Authors KMLB and FXE designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SFC and EBB managed the analyses of the study. Author TJL managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Helicobacter pylori* are the primary cause of peptic ulcer disease and an etiologic agent in the development of gastric cancer. The emergence of multi-drug resistant phenotypes is a major public health problem today in the treatment of *Helicobacter pylori* infection. The present study was designed to evaluate the anti-*Helicobacter* activities of six Cameroonian medicinal plants on ten *Helicobacter pylori* clinical isolate from dyspeptic patients and their ability to potentiate the

**Corresponding author: E-mail: laurebkouitcheu@yahoo.fr;*

effect of common antibiotics against multidrug-resistance phenotypes *Helicobacter pylori*.
Methodology: Broth microdilution assay was used for the antimicrobial evaluation of plant-extracts alone or in combination with antibiotics, while Time-kill assay was used to study the bactericidal activity.
Results: Plant-extracts showed different anti-*Helicobacter* activity with the minimum inhibitory concentration (MIC) values varying from 64 to >1024 µg/ml. The methanol extract of *E. cocaine* leaves showed the best anti-*Helicobacter* activity with MIC value of 64 µg/ml against 60% of the tested isolate. Moreover, *E. cocaine* extract at a concentration equal to 8MIC, produced from 24 to 72 h a viability decrease of 2 logs lower than those for the control against the tested clinical isolates. Synergistic concentration dependent effects were observed when combining this plant extract with erythromycin, or amoxicillin against *Helicobacter pylori* multi-drug resistant phenotypes with minimum fold inhibition of 16 and eight respectively for erythromycin and amoxicillin.
Conclusion: The overall results provide information for the possible use of *E. cocaine* extract in the control of *Helicobacter pylori* infections involving multi-drug resistant phenotypes.

Keywords: Helicobacter pylori infection; multidrug-resistance; potentiation; herbal drug.

1. INTRODUCTION

*Helicobacter pylor*us is a gram-negative rod which is responsible for a spectrum of diseases in alimentary canal including chronic superficial gastritis, chronic atrophic gastritis, gastric and duodenal ulcers, gastric cancer and mucosa-associated lymphoid tissue lymphoma [1]. A high frequency of *H. pylori* infection has been reported from resource-poor regions [2]. In general, *H. pylori* infection is curable with regimens of multiple antimicrobial agents, including amoxicillin, metronidazole, clarithromycin, tetracycline, and ciprofloxacin as well as bismuth compounds [3]. However, antibiotic resistance drastically reduced the efficiency of antibiotics, increasing the frequency of treatment failure.

For example, the worldwide resistance of *H. pylori* to metronidazole has been reported, with rates ranging from 0 to 98% [4-6]. Until recently, resistance to amoxicillin was considered to be absent or very rare; however, amoxicillin-resistant *H. pylori* strains have now been identified in different countries [4,7]. The World-wide prevalence of resistance to amoxicillin is 0-41%. [5,8,9]. The resistance of *H. pylori* to clarithromycin, a macrolide widely used in combination with a proton pump inhibitor with or without a second antibiotic has been reported, with worldwide rates ranging from 0 to 44.7% [5,6]. The overall resistance of *H. pylori* to tetracyclines, currently used for the treatment of *H. pylori* infection as part of quadruple therapy is estimated to be less than 2% [10]. However, higher resistance rates, up to 20%, have been reported in other studies from different countries [4,11]. Ciprofloxacin is a fluoroquinolone that inhibits A subunit of the DNA gyrase. Although ciprofloxacin is not the drug of choice for *H. pylori* infection, 0-20% resistance to this antibiotic has been reported in different countries [8].

The emergence of antibiotic resistant *H. pylori* has necessitated the identification of alternate therapies for the treatment of this infection. In the fight against *H. pylori* infection including those due to resistant strains, investigations are being carried out to discover new effective, none or less-toxic and available anti-*Helicobacter* drugs [2,12-14]. Many scientist is also investigating synergistic compounds to potentiate the activity of the commonly used antibiotics [15]. The present work was designed to evaluate the *in vitro* anti-Helicobacter activity of some Cameroonian's medicinal plants namely *Aspilia africana* (Asteraceae), *Dichrocephala intergrifolia* (Asteraceae), *Emilia coccinea* (Asteraceae), *Erigeron floribundas* (Asteraceae) *Markham lutea* (Bignoniaceae) and *Kalanchoe crenate* (Crassulaceae) and the ability of the most active of them to potentiate the effect of common antibiotics against multidrug-resistance phenotypes *H. pylori* clinical isolates.

2. MATERIALS AND METHODS

2.1 Chemicals and Culture Media

Culture media (Columbia agar, Brain heart infusion, Lacked horse blood, Horse serum, Vitox supplement) and the CampyGen gas pack were all purchased from Oxoid, Basingstoke, England. Doxycycline (Doxycycline 200 mg, Combitic Global Caplet, India), erythromycin (Erythromycin stearate 500 mg, Cipla, India), amoxicillin (Amoxicillin trihydrate 500 mg, max

heal pharmaceutical, India), ciprofloxacin (ZOFLOX, ciprofloxacin 750 mg, Odypharm), clarithromycin (Clarithromycin 500 mg, Neurochem Laboratories, India) and metronidazole (Metronidazole 500 mg, Strides Arcolab, India) used as reference antibiotics were purchased from a local pharmacy. The P-Iodonitrotetrazolium chloride (INT, Sigma-Aldrich) was used as a microbial growth indicator [12].

2.2 Bacterial Strains

Ten strains of *H. pylori* coded as *H. pylori&1, H. pylori &2, H. pylori &3, H. pylori &4, H. pylori &5, H. pylori &6, H. pylori &7, H. pylori &8, H. pylori &9* and *H. pylori &10* were used in this study. They were isolated from gastric biopsies of patients with gastric-related morbidities undergoing endoscopy at Laquintinie Hospital in Douala-Cameroon. The study was approved by a local ethical committee of Laquintinie Hospital (Approval No. 425/AR/MINSANTE/HLD/SCM/CR). The specimens were only collected from patients who had given consent. The isolates were identified by Gram staining and enzymatic activity (catalase, oxidase, urease) [16]. Pure cultures were suspended in Brian Heart Infusion (BHI) broth supplemented with 5% horse serum and 20% glycerol and stored at -80°C until used.

2.3 Plant Material and Extraction

Plant material used in this study was collected in Baham (West Region of Cameroon) in May 2015. The plants were further identified at the National Herbarium (Yaoundé, Cameroon) where voucher specimens were deposited under a reference number. Information on plants used in this study is shown in Table 1. Air dried and powdered sample (0.1 g) of each plant was separately extracted by maceration with two solvents, methanol (MeOH, 0.3 L) and Ethyl Acetate (EA, 0.3 L) for 72 h at room temperature (25°C). After filtration using Whatman filter paper No. 1, the filtrate of each plant was concentrated under reduced pressure in a rotary evaporator, and dried at room temperature to give the crude extract. These extracts were then stored at 4°C until further use.

2.4 Antimicrobial Susceptibility Tests

MICs were determined by the P-Iodonitrotetrazolium chloride (INT) broth microdilution method [17] using 96-well plates. Two-fold dilutions of each selected antibiotics or plant-extract were prepared in the test wells in BHI broth supplemented with 5% horse serum (BHI-serum). The final concentrations ranged from 0.125 to 512 µg/ml for antibiotic and from 1024 to 8 µg/ml for plant-extract. One hundred microliters of inoculums prepared from 48 h colonies of each isolate on supplemented Columbia Agar (Columbia Agar + 5% (v/v) lacked horse blood and 1% (v/v) Vitox) at McFarland turbidity standard three was added to 100 μl of the antibiotic or plant-extract containing culture medium. Control wells were prepared with culture medium and bacterial suspension, and broth only.

The plates were covered with a sterile plate sealer; the contents of the wells were mixed with a shaker and incubated for three 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 antibiotic or plant-extract concentration that prevented the color change of the medium, exhibited complete inhibition of microbial growth was known as the MIC. Each MIC was determined in triplicate, and the mean values were recorded. MIC value for each antibiotic was compared to the break- point MIC value recommended by the European Committee on Antimicrobial Susceptibility Testing 2015 on *H. pylori* and the isolate were classified as susceptible or resistant.

2.5 Time-kill Bactericidal Activity

Time–killing studies are known as a useful method for evaluating the clinical potential of anti-*H. pylori* agents rather than susceptibility testing alone. So, bactericidal activity of the best anti-*Helicobacter* extract tested judging from the obtained MIC value (64 µg/ml); methanol extract of *Emilia coccinea* was studied using a modified time-kill assay [18] by evaluating the decrease in viable cells during exposure to this plant-extract. The assay was performed in Brain Heart Infusion broth containing 5% horse serum (BHI-serum) incubated in a microaerophilic environment. Plant-extract at concentrations of 0.5, 1, 2 and four-time MIC against *H. pylori* & 8 were prepared in broth medium in a 100-ml baffled flask. A 48-h culture of *H. pylori* & 8 isolates was diluted with fresh broth and then inoculated to each plant-extract solution at a final concentration of 10^6 CFU/ml. The bacterial suspension was incubated with circular shaking,

Table 1. Information on plants used in this study

Plants samples and herbarium voucher number	Parts used	Popular names	Traditional used	Known pharmacological activities of plants
Aspilia africana (Asteraceae) 50749 HNC	Leaves	Cia-Samsung	Properties of stopping bleeding, to draw up exudations; it is used in the treatment of gonorrhea and stomach -troubles; to relieve a feverish headache; to assist in childbirth and also to increase milk flow [30].	Antimicrobial [26], anti-fertility [27] activity. The wound healing and anti-ulcer activity [28, 29].
Dichrocephala intergrifolia (Asteraceae) 37065 HNC	Leaves	Mbac-fack	Hepatic disorders [31].	Anticancer, antimicrobial, anti-inflammatory, anti- oxidant and anti- helmintic [32, 33].
Emilia coccinea (Asteraceae) 59675 HNC	Leaves	Mhei-lapin	Treatment of fever, convulsions, epilepsy in children [34]. Treatment of ulcers, body rashes and abscesses [34]. Treatment of wounds, sores and sinusitis ulcer, ringworm jaundice, abdominal pains and gastritis [35].	Antibacterial, antioxidant and anti-inflammatory activities [36].
Erigeron floribundus (Asteraceae) 15603 HNC	Whole plant	Mhei-gam	Used in HIV/AIDS therapy [37], skin disorders [38].	Analgesic and anti-inflammatory activities [39]. Antidermatophyte [38]. Immunomodulatory Effect [37].
Kalanchoe creneta (Crassulacées) 35196 HNC	Whole plant	Ntan-keyouc	Antihelmintic, antiemetic, antiseptic, antiinflammatory, anti-malarial, anticancer, cicatrizant, sedative and tonic [40].	Chemical investigations of this plant revealed that it is rich in terpenes, sterols and alkaloids [41].
Markhamea lutea (Bignoniaceae) 48359 HNC	Leaves	Wagne	Used to reduce symptoms of watery and bloodless diarrhea [42], root bark is used in the treatment of anemia and diarrhea [43], to cure various parasitic and microbial diseases [44].	antiplasmodial activity [45]. The cytotoxic potential against cervical carcinoma, colon adenocarcinoma, and skin carcinoma [46]. Anti-Alzheimer's disease drug lead due to its high phenolic content [47].

and samples (0.1 ml) taken at 0, 4, 8, 24, 48 and 72 h after drug exposure were tenfold serially diluted with saline and inoculated in duplicate onto drug-free supplemented Columbia agar plates for colony counts. The CFU/ml was calculated from the number of colonies that appeared after incubation at 37°C for 72 h under microaerophilic atmosphere.

2.6 The Anti-helicobacter Activity of Extract and Antibiotic Combination

The anti-*H. pylori* activity of antibiotics to which all tested isolates were resistant was carried out in combination with methanol extract of *Emilia coccinea* to check for any potentiation effect of this plant-extract. Different combinations were made between each non-active antibiotics (amoxicillin, metronidazole, and erythromycin) judging by the MIC value obtained and *Emilia coccinea* extract, and each of them was then tested to resistant clinical isolates.

The broth microdilution method as described above was used with BHI broth supplemented with 5% horse serum (BHI-serum) as culture media. A two-fold serial dilution of each selected antibiotic ranging from MIC to MIC/64 was mixed together with two fixed concentration of *Emilia coccinea* extract corresponding to its MIC and MIC/2 value. So, for the selected antibiotics, the concentrations tested ranged from 512 to 2 µg/ ml, from 256 to 2 µg/ ml and from 128 to 2 µg/ ml respectively for amoxicillin, metronidazole, and erythromycin; and from 0.0625 to 0.125 µg/ ml for the tested plant-extract.

Antibiotics were serially diluted in BHI-serum into a 96-well round bottom sterile plates and the plant-extract solution separately prepared in test tubes were added. Then, 100 µl of the multi-resistant isolate *H. pylori* & 8 suspensions prepared from 48 h colonies on supplemented Columbia Agar at McFarland turbidity standard three were distributed into wells containing various concentrations of the different compounds. The inoculated 96-well round bottom was incubated for three 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. The minimum inhibitory concentrations of the combination were determined as described above. Each MIC was determined in triplicate and the mean values recorded. The fractional inhibitory concentration (FIC) index value was then calculated, and the interactions were considered synergistic if the FIC index was < 0.5, additive if the FIC index was equal to 1 and antagonistic if the FIC index exceeded 4.

3. RESULTS

3.1 Antimicrobial Susceptibility Tests

The MIC value of the 12 tested plant-extracts (methanol and ethyl acetate extract of the six selected plants) against *H. pylori* clinical isolate using the broth microdilution method is given in Table 2. Plant-extract showed different anti-*Helicobacter* activity each other with MIC values ranging from 64 to >1024 µg/ml. The lowest MIC value, 64 µg/ml was obtained with Methanol extract of *E. cocaine* leaves. This lowest MIC values (64 µg/ml) was obtained against isolate *H. pylori* & 1, & 4, & 6, & 8, & 9 and & 10. Each tested isolate showed different susceptibility to the plant-extracts tested. Judging from the obtained MIC value (0.125 µg/ml), doxycycline, clarithromycin, ciprofloxacin were the most active among the six antimicrobial agents tested. A 100% resistance was obtained with erythromycin, amoxicillin, and metronidazole against the tested clinical isolates, indicating their multidrug resistant phenotypes.

3.2 Time-kill Bactericidal Activity

The bactericidal studies of *Emilia coccinea* (Fig. 1) showed that at concentrations of MIC/2 and MIC, *Emilia coccinea* had a very little effect on the growth of *H. pylori* isolate tested throughout the experimental period. However, at 72 h, a bactericidal time-dependent effect of 1 log lower than those for the control was observed at 2MIC and 4MIC concentration. At a concentration equal to 8MIC, bacterial numbers were approximately 2 logs lower than those for the control from 24 to 72 h. So, *Emilia coccinea* produced a time-dependent viability decrease against the tested clinical isolates at a concentration equal to 8MIC (512 µg/ml).

3.3 The Anti-helicobacter Activity of Extract and Antibiotic Combination

A 100% resistance was obtained with erythromycin, amoxicillin, and metronidazole against the tested clinical isolates. Thus, the susceptibility of the resistant isolate to these antibiotics was evaluated in combination with the most active plant extract. Two plant-extract concentration were selected, a sub-inhibitory concentration (MIC/2) and minimal inhibitory

Table 2. MIC (µg/ ml) of plant-extracts and antibiotics tested

Plant extract / antibiotics (Extraction's solvent)	MIC value of plant-extract and antibiotics (µg/ ml)									
	H. pylori &1	***H. pylori &2***	***H. pylori &3***	***H. pylori &4***	***H. pylori &5***	***H. pylori &6***	***H. pylori &7***	***H. pylori &8***	***H. pylori &9***	***H. pylori &10***
Aspilia africana (MeOH)	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Aspilia africana (EA)	512	512	>1024	>1024	512	512	>1024	256	>1024	>1024
Dichrocephala intergrifolia (MeOH)	512	1024	1024	512	1024	1024	>1024	1024	512	1024
Dichrocephala intergrifolia (EA)	>1024	1024	>1024	>1024	>1024	>1024	512	>1024	>1024	>1024
Emilia coccinae (MeOH)	64	256	512	64	256	64	128	64	64	64
Emilia coccinae (EA)	1024	1024	1024	1024	256	1024	512	1024	512	512
Erigeron floribundus (MeOH)	>1024	512	>1024	>1024	512	512	1024	256	512	>1024
Erigeron floribundus (EA)	512	1024	512	1024	1024	1024	1024	1024	>1024	1024
Kalanchoe creneta (MeOH)	>1024	>1024	>1024	>1024	1024	>1024	>1024	>1024	>1024	>1024
Kalanchoe creneta (EA)	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Markhamea lutea (MeOH)	>1024	512	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Markhamea lutea (EA)	512	1024	512	512	512	1024	1024	512	512	>1024
Doxycyclin	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125
Erythromycin	128	128	128	128	128	128	128	128	128	128
Amoxicillin	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
Ciprofloxacin	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125
Clarithromycin	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125
Metronidazole	256	256	256	256	256	256	256	256	256	256

MIC: minimum inhibitory concentrations, H. pylori: Helicobacter pylori, (EA): Ethyl acetate, (MeOH): Methanol

concentration (MIC) and then tested on *H. pylori* &8, resistant isolate to both non-active antibiotics mentioned above.

The results are summarized in Tables 3. Synergistic effects were observed with the association between erythromycin, amoxicillin and *E cocaine.* These synergistic effects were concentration dependent since the highest minimum fold inhibition was obtained with the higher concentration of the plant-extract. No case of antagonism was observed, but at high concentration of the plant extract, an additive effect was noticed with metronidazole. The best combination with the highest minimum fold inhibition (16) was obtained with erythromycin at high concentration of plant extract, followed by amoxicillin with a minimum fold inhibition of 8.

4. DISCUSSION

The antimicrobial activity of 12 plant-extracts against *H. pylori* clinical isolate were examined in the present study and their potency were quantitatively assessed by the determination of MIC values. It is considered that if the extract displays a MIC less than 100 µg/ml, then the antimicrobial activity is good; from 100 to 500 µg/ml the antimicrobial activity is moderate; from 500 to 1000 µg/ml the antimicrobial activity is weak; over 1000 µg/ml, the extract is considered inactive [19]. Given the above classification, the activity recorded with most of the extracts used against the pathogenic bacteria tested can be considered moderate or weak. However, the methanol extract of *E. cocaine* leaves showed a good and the best anti-*Helicobacter* activity with MIC values of 64 µg/ml against 60% (6/10) of the tested isolate. This differences in susceptibility within the clinical isolate against antimicrobial substances in *E. cocaine* methanol extract may be explained by the differences in cell wall composition and inheritance genes on plasmids that can easily be transferred among bacterial strains [20]. Previous phytochemical studies on *E. coccinea* have reported the presence of alkaloids, tannin, saponin, steroid, terpenoid, flavonoid and cardiac glycoside [21]. The presence of these chemical compounds in this extract may explain some of their antimicrobial actions since antimicrobial actions of most of these phytochemical substances have been documented [22].

The bactericidal studies of *Emilia coccinea* (Fig. 1) showed at 72 h, a relatively similar bactericidal time-dependent effect of 1 log lower than those for the control at 2MIC and 4MIC concentration. This relatively similar bactericidal effect observed at 2MIC and 4MIC of *Emilia coccinea* extract, suggests a possible break-point concentration which was reached at 8MIC concentration. In fact, at a concentration equal to 8MIC, bacterial numbers were approximately 2 logs lower than those for the control from 24 to 72 h. So, *Emilia coccinea* produced a time-dependent viability decrease against the tested clinical isolates at a concentration equal to 8MIC (512 µg/ml). Could this high concentration of *Emilia coccinea* attain the gastric mucosal; the site of *H. pylori* infection without severe side effects? Because the plant part tested are commonly eaten as a vegetable for soups or salads in many countries in Africa [23,24], one may expect that this

Table 3. MIC (µg/ml) of antibiotics in the absence and presence of the sub-inhibitory and inhibitory concentrations of *Emilia coccinea* against multidrugs resitant clinical isolate *Helicobacter pylori*

Antibiotics	*E. coccinea* concentration (µg/ml)	Combination MIC value (µg/ml)	FIC index value	Interaction's type
ERY	0	128		
	MIC/2	128	1	Additive
	MIC	8	0.0625	Synergy
MET	0	256		
	MIC/2	> 256	> 1	/
	MIC	256	1	additive
AMO	0	> 512		
	MIC/2	128	0.25	synergy
	MIC	64	0.125	synergy

MIC: minimum inhibitory concentrations, H. pylori: Helicobacter pylori, AMO: amoxicillin, MET: metronidazole, ERY: erythromycin

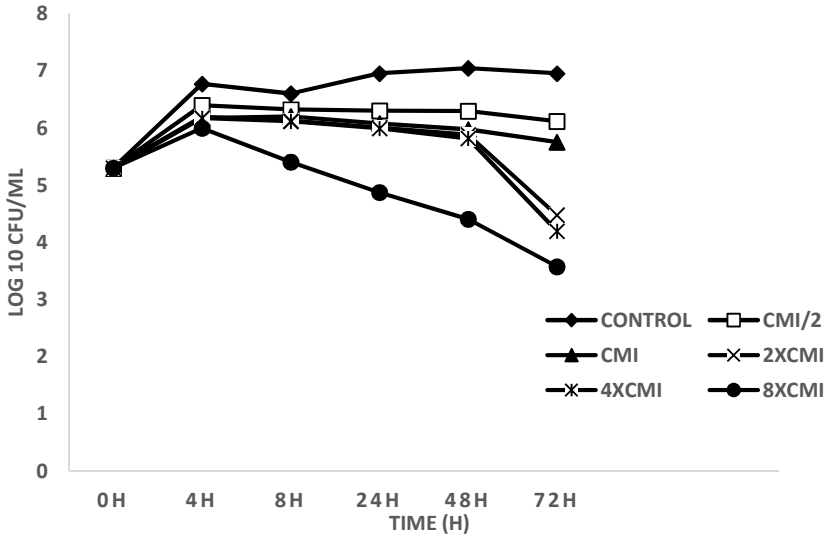

Fig. 1. Bactericidal effect of methanol extract of *Emilia coccinea* against clinical isolate *Helicobacter pylori* &8 at MIC/2, MIC, 2 MIC, 4 MIC and 8 MIC

concentration could be considered with limited toxicity. However, this hypothesis needs to be evaluated.

Judging from the obtained MIC value, the overall tested clinical isolates were resistant to erythromycin, amoxicillin, and metronidazole. Thus, the susceptibility of the resistant isolate to these antibiotics was evaluated in combination with the most active plant sample to find a possible synergistic effects. Significant synergistic effects were noted with erythromycin, amoxicillin and *E. cocaine* extract. The association of natural products such as plant extracts and antibiotics constitutes an alternative in the fight against multidrug resistant bacteria. Such effects might be due either to the action of the active compounds or possible inhibition of the resistance mechanism exhibited by the strain by other compounds of the extract. However, the synergistic effects observed indicate that active compounds of the extract could also present different mode(s) of action from those of the studied antibiotics. The lowest synergistic effects were observed with metronidazole, obviously because their actions via activation within the bacteria, leading to the production of toxic metabolites [25].

5. CONCLUSION

The overall results of the present work provide baseline information for the possible use of *Emilia cocaine* methanol extract in the treatment of *Helicobacter pylori* infections. In addition to this anti-*Helicobacter* activity, the data reported herein indicate that the association of this plant-extract with amoxicillin or erythromycin could be used in the control of *Helicobacter pylori* infections involving multidrug resistant phenotypes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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