RESEARCH ARTICLE

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Phytochemical screening and anti-*Helicobacter* pylori activity of *Bryophyllum pinnatum, Ocimum gratissimum* and *Vernonia amygdalina*

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ABSTRACT

Helicobacter pylori, a common gastrointestinal bacterial pathogenic isolate infects 50% and 90% of the global and developing nations population respectively. This study aimed at evaluating the bioactive components and therapeutic potential of *Bryophyllum pinnatum*, *Ocimum gratissimum* and *Vernonia amygdalina* plants extracts against *Helicobacter pylori* infection using standard physicochemical, *in-vitro* and *in-vivo* microbiological methods. Five (5) stool samples were collected from patients who presented with symptoms of gastrointestinal distress and diagnosed of ulcer at the University of Benin Teaching Hospital, for the isolation of *Helicobacter pylori*. Phytochemical screening of ethanol extract of the test plants revealed the presence of bioactive constituents such as flavonoids, tannins, cardiac glycosides, saponin, steroids, phenols, alkaloids and terpenoids. The minimum inhibitory concentration (MIC) of the plant's extracts were determined at concentrations of 125, 250, 500, and 1000 µg/ml. While the different plants extract demonstrated a better anti-*Helicobacter pylori* activity as well as MIC when compared to the commonly used antibiotic amoxicillin, and other conventional antibiotics, the anti-*Helicobacter pylori* activity and MIC of *Vernonia amygdalina* was higher, followed by *Bryophyllum pinnatum* and *Ocimum gratissimum.* The *in-vivo* study as carried out using Wistar albino rats demonstrated the promising therapeutic effect of the plants extract against *Helicobacter pylori* infection. This study therefore suggests that *Vernonia amygdalina*, *Bryophyllum pinnatum* and *Ocimum gratissimum* extracts possess anti-*Helicobacter pylori* properties, offering safe, effective, and cost-effective treatment options for the treatment of ulcer, caused by *Helicobacter pylori*.

Key words: antimicrobial, extract, *Helicobacter pylori*, phytochemical, *Vernonia amygdalina*

Introduction

Helicobacter pylori, a gram-negative bacterium has been reported as the most common disease-causing organism of the gastrointestinal tract since its uncovering (Rabia *et al*., 2020). It is an extremely diverse bacterial specie, possessing a high notch of phenotypic and genotypic heterogeneities which makes it highly acclimatised for existence in the gastric niche. The presence of *Helicobacter pylori* itself does not constitute a disease, but infections arising from their presence are sturdily connected with the cause of gastric ulcers as well as cancer of the stomach. It seems infections arising from *H. pylori* infection directly and indirectly harms the mucosal cells by increasing the discharge of gastric hydrochloric acid and decreasing the synthesis of bicarbonate in epithelial cells leading to the impairment of the gastro-duodenal lining and ulcer formation (Chatterjee *et al*., 2012). The genomic variety of *H. pylori* matches it host species, depending on colonization of the most primitive humans and co-migration out of East Africa at least 58,000 years ago (Wilkinson *et al*., 2022). The organism infects about 50% of the global population, with its infection still very predominant in developing nations, affecting about 90% of their populations while conversely, it is being reduced to below 40% in the developed climes probably due to their high living standards (Rabia *et al*., 2020). Following ingestion, *H. pylori* escape the bactericidal activity of the gastric luminal contents and go into the mucous layer leading to the degeneration of the epithelial cells (Suerbaum and Michetti, 2002; Versalovic, 2003; Mabeku *et al*., 2018; Han *et al*., 2021).

Despite the best regularly acclaimed triple *H. pylori* treatment, including proton pump inhibitor (PPI), the amalgamation of amoxicillin and clarithromycin now offer unsatisfactorily high failure rate (Fekadu *et al*., 2023). Nevertheless, the triple treatment and bismuth quadruple remedy are global managements methods utilised in the extermination of *H. pylori* and *H. pylori*-induced gastrointestinal disorders, like nausea, antibiotic resistance, recurrence, and a host of other side effects (Chatterjee *et al*., 2012). This results in relapses most often due to the patient's non-compliance with the treatment procedures, lifestyle, and food regime, but drug resistance as well as the consequences of antibiotics remain a burden (Fallone *et al*., 2019). Thus, there is obvious need to find suitable alternative for the

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treatment of diseases arising from *H. pylori* infections. The use of herbal products become handy and a better alternative for the treatment of infections of the gastrointestinal tract (Adesina *et al.*, 2013; Enerijiofi and Isola, 2019).

The use of medicinal plant materials has long been known to be a main basis of nature based therapeutic medications for various infectious diseases worldwide (Beverly and Sudarsanam, 2011; Enerijiofi *et al*., 2021; Martinez *et al*., 2022; Ovuru *et al*., 2023). The treatment of *H. pylori* has undergone trials with a combination of antimicrobials, but the rising resistance of this bacterium to orthodox antimicrobials remain a cause of worry to public health. Hence, the need to source for alternative anti-*H. pylori* treatments such as the use of plants extracts. Plants and their products have long been used as herbal drugs for long a time as they provide a benign and cost-effective option for the treatment of diseases with little or no side effect (Balunas and Kinghorn, 2005; Adesina and Enerijiofi, 2016; Erhabor *et al*., 2017). However, the short duration and meagre acid stability make many compounds with *in-vitro* anti-*Helicobacter* characteristics to become fruitless (Rabia *et al*., 2020). In recent years, some studies including Mabeku *et al*. (2017); Chanthaboury *et al*. (2022) and Akinduti *et al*. (2022) have advocated that infection from *H. pylori* can be curbed by using medicinal plants. It is therefore necessary to establish the *in-vitro* healing potential of plants and their herbal products by *in-vivo* test. Hence, this study aimed at evaluating the anti-*H. pylori* potential of *Bryophyllum pinnatum*, *Ocimum gratissimum* and *Vernonia amygdalina*.

Materials and Methods

Collection of stool samples and Plants materials:

Five (5) stool samples were collected from patients who presented with symptoms of gastrointestinal distress and diagnosed of ulcer at the University of Benin Teaching Hospital (UBTH), Benin City, Edo State, under sterile condition and promptly transported to the laboratory for analysis. The plants (*Bryophyllum pinnatum*, *Ocimum gratissimum* and *Vernonia amygdalina*) used in this study were obtained from the Botanic Garden, Department of Plant Biology and Biotechnology, University of Benin, Benin City and were identified accordingly.

Microbiological analysis

Determination of total heterotrophic bacterial count

Serial dilution was carried out by weighing 1g of stool sample into 10ml of sterile distilled water in a sterile test tube. Thereafter, dilution was done up to the sixth diluent and 1ml of dilution 10^3 was pipetted into sterile petri dishes containing solidified nutrient and MacConkey agars in duplicate, and then incubated in an anaerobic jar at 37^0C for 48 hours.

Identification of bacterial isolates

The distinct colonies on the Petri dishes were aseptically picked using a sterile wire loop, inoculated into plates containing freshly prepared nutrient agar and incubated at 37°C for 48 hours. The pure isolates obtained were stored in slants for further identification following the cultural, morphological and biochemical tests (Holt *et al*., 1994).

Preparation of plants extract

The plants, *Bryophyllum pinnatum*, *Ocimum gratissimum* and *Vernonia amygdalina* were dried by exposure to air for 12 days and pounded into powder using a sterile pestle and mortar. 45g of the powder was sodden in 500ml of ethanol and allowed to stand for 72h. It was thereafter filtered with Whatman filter paper. Filtrate was concentrated to paste-like form using the steam bath at 60^0C. Concentrates were dissolved with dimethyl sulfoxide and stored in sterile container and kept in the refrigerator at 4^0C until needed for use. (Amengialue *et. al.*, 2016a, 2016b; Enerijiofi *et al*., 2021).

Qualitative phytochemical analysis of extracts

Aliquot portions of the crude extract residue were weighed and used for phytochemical screening as follows:

Test for flavonoids

Five millilitres (5ml) of dilute NH_3 was added to 1ml of each plant extract filtrate. Then, 1ml of concentrated H_2SO_4 was added. The production of yellow colouration showed that flavonoid was present.

Test for tannins

One millilitre (1ml) of each plant extract was added to 2ml of water in a test tube and boiled. It was filtered and allowed to cool. This was followed by adding some drops of 0.1% ferric chloride and observed for a brownish green to a blue-black colouration which signify the presence of tannin.

Test for cardiac glycosides (Keller-Killiani test)

One millilitre of each plant extract was treated with a mixture of glacial acetic acid and a drop of ferric chloride solution. This was followed by the addition of 1ml of concentrated H_2SO_4. The production of brown colour at the interface revealed that cardiac glycosides was present.

Test for saponin (Frothing test)

One millilitre (1ml) of each prepared extract was mixed with 5ml of distilled water, shaken thoroughly till the presence of persistent froth, which indicated the presence of saponin. However, further confirmation was done by adding 3 drops of olive oil, followed by thorough mixing and observed for the development of emulsion.

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Test for steroids

Two millilitres (2ml) of acetic anhydride was mixed with 0.5g of each plant extract sample followed by adding 2ml of H_2SO_4. A change in colour from violet to blue or green indicated that steroid was present.

Test for terpenoids (Salkowski test)

One millilitre (1ml) of the extract was emptied into a test tube and mixed with 2ml of chloroform and 3ml of concentrated H_2SO_4. The development of reddish brown colour at the interface indicated that terpenoids was present.

Test for phenols

About 5 drops of 10% aqueous $FeCl_3$ solution were emptied into a test tube containing 5ml of plant extract. The formation of a blue or green colour confirmed the presence of phenols.

Test for phlobatanins

Three millilitres (3ml) of the ethanol extract was added to test tube containing 2mL of 1% HCl, followed by boiling. The deposit red precipitate was indicative of phlobatanins.

Test for coumarin

Five millilitres (5ml) of plant extract was dissolved in 2ml of hot distilled water, and thereafter divided into two equal parts. For the first half volume, 0.5ml of 10% NH_4OH was added, while the second half volume served as control.

Test for alkaloids (Mayer's Test)

One millilitre (1ml) of plant extract was mixed with 3 drops of Mayer's reagent. The development of cream coloured precipitate indicated that alkaloids was present.

Test for anthraquinone

Five millilitres (5ml) of benzene was added to 1ml of each plant extract in a test tube and shaken vigorously with the addition of 2.5ml of NH_3. A pink-red colour at the bottom of the test tube indicated that anthraquinone was present.

Antibacterial Studies

In-vitro anti-Helicobacter pylori activity

The disk diffusion method was used for this assay. Blood agar plates were freshly prepared and thereafter inoculated with *H. pylori* inoculum by spread plate technique. The filter paper (6mm in diameter) was soaked in 50µl stock solution of each plant extracts and thereafter placed on the agar plates. The plates were incubated at 37ºC in a microaerophilic condition for 3-7 days. The zone of inhibition diameter was measured and recorded in millimetres (mm). Amoxicillin was used as the positive control sensitivity of isolates concentration.

Determination of minimum inhibitory concentration (MIC)

This was done by the agar dilution method. The plants extracts were reconstituted by dissolution in 10% dimethyl sulfoxide (DMSO). Sterile water was used in diluting the DMSO-based stock solution of each extract and thereafter made to the volume to 900µl in other to achieve the concentrations: 125, 250, 500 and 1000 µg/ml respectively. Thereafter, each of these final concentrations were added to 100µl of the bacteria suspension so as to align with the 0.5 McFarland's turbidity standard ($1.5x10^8$ CFU/ml). They were incubated for 1 hour and thereafter, 100µl was measured and spread on petri dish containing freshly prepared blood agar and allowed to stand, followed by boring holes using a 6mm cock borer. Thereafter, aliquots 100µL of the extract's concentrations were emptied into the labelled wells. The plates were incubated at 37ºC microaerophilically for 72 hours followed by bacterial enumeration. The lowest of all concentrations that had no bacterial growth was recorded as the MIC (Kim *et al*., 2014; Enerijiofi *et al.*, 2021). Dimethyl sulfoxide (100µl) served as the negative control while 100µl amoxicillin with a concentration of 1µg/ml, was used as the positive control. All the experiments were performed in duplicate.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was carried out on the *H. pylori* isolate using the disc diffusion method, to evaluate the sensitivity of test organism to various antibiotics. Plates were incubated at 37℃ for 24 hrs, after which zones of inhibition were recorded in millimetre. The results were interpreted as sensitive and resistant following the standard protocol (Akortha *et al*., 2010; CLSI, 2020).

In-vivo anti-Helicobacter pylori activity

Male Wistar albino rats weighing between 160-210g were used for the in-vivo studies. The rats were placed in propylene cage and the environmental conditions were well maintained. These include an appropriate temperature of between 21-25ºC, relative humidity between 45-65% and light to dark cycle of 12hours.

Experimental design and dosage determination

The Wistar rats were grouped into 6, with each group comprising of 5 rats (n=5). Bacterial inoculum (1ml/mouse) was administered on all groups, except group 1 which served as normal control with 10ml/kg of distilled water administered. Group 2, the negative control was inoculated with bacterial isolate and 10ml/kg of administered sterile distilled water. Group 3, the positive control and administered with 50 mg/kg of amoxicillin. Group 4A and B were administered with *Bryophyllum pinnatum*, group 5A and B were administered with *Ocimum gratissimum*, while group 6 A and B received *Vernonia amygdalina* at varying doses of 125, 250, 500, and 1000 mg/kg respectively. One

millilitre dose (2-Mc-Farland) of *Helicobacter pylori* was inoculated intragastrically using orogastric cannula twice daily at two days interval. Concurrently, 125, 250, 500 and 1000 µg/ml of the plant's extracts were administered orally at two times daily for seven days.

Determination of active infection induced by Helicobacter pylori

After 3 hours of last administration of ether under deep anesthesia, the rats were slaughtered. A cut was made from xiphoid process on the median abdominal line. Thereafter, biopsy samples (3 × 3 cm) were harvested from the gastric mucosa, mincing, and applied to a newly prepared rapid urease test (RUT) vile, followed by incubation at 37ºC for 24h. Thereafter, examination of the urease activity was done to confirm active infection. A bright yellow colour indicated negative, thick yellow showed false (partial) positive while red or pink indicated a positive response (Hartanto, 2021).

Histopathological examination

The stomachs of the slaughtered rats were removed and half were fixated in 10 % buffered formalin. This was followed by staining with a modified Giemsa stain for *Helicobacter pylori* detection using 5µm section for histopathology block preparation. The entire tissue were studied using the microscope to determine parameters like score of *Helicobacter pylori* presence in gastric mucosa tissues (Rabia *et al*., 2020; Amengialue *et. al.*, 2023)

RESULTS AND DISCUSSION

Today, there is a renewed interest in traditional medicine and an increasing demand for more rugs from plant sources. This revival of interest in plantderived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects (Agbafor *et al.,* 2011; Amengialue *et. al.,* 2013). The in-vitro and in-vivo anti-*Helicobacter* activities of *Bryophyllum pinnatum*, *Ocimum gratissimum*, and *Vernonia amygdalina* against *Helicobacter pylori* were investigated in this study. These three plant's species were chosen due to their traditional use in herbal medicine and their potential antimicrobial properties. The phytochemical analyses revealed *Bryophyllum pinnatum* and *Ocimum gratissimum* to have high flavonoid and phenol contents, with the highest amount observed in *Vernonia amygdalina*. While phenols were present but low in *Bryophyllum pinnatum* and *Ocimum gratissimum*, they were found to be very high in *Vernonia amygdalina*. Additionally, phlabotannins, coumarin, and anthraquinone contents were not detected in the test plant extracts. The findings suggest that the high flavonoid and phenol contents observed in these plants may contribute to their potential medicinal properties. The differences recorded in the phytochemicals reported and those of earlier researchers could have resulted from the different extracting solvents and methods, nature of solvent and its absorption, and the plants parts used as well as its age (Enerijiofi and Isola, 2019). Tannins are known to hasten wound healing and swellings of mucous membrane. Saponins are known to damage cells, thereby conferring them anti-inflammatory & anticancer proficiencies as well as preventing the formation of tumour cells in animals (Odeleye *et al*., 2014). The utilization of medicinal plants as first line of treatment as well as its application in ailments and diseases continues to improve individual's health as they hold a countless prowess in the look-out for novel antimicrobial agents. This could be the reason Ere *et al*. (2014) and Erhabor *et al*., (2017) reported that 80% of the global population depend on plants associated medicines as their first aid of treatment.

The antibiotic susceptibility test of *H. pylori* isolates from stool samples as shown in table 2 revealed high resistance rate against cefotaxime, gentamycin, cefuroxime, imipenem, and ciprofloxacin. This resistance level corroborates earlier studies of Bello *et al*. (2019) that reported a high resistance level to cefuroxime and ciprofloxacin. The sensitivity zone diameter (mm) of plant extracts against *Helicobacter pylori* isolates investigated in this study suggest the plants to have more potential as a natural alternative to amoxicillin for treating *Helicobacter pylori* infections. Furthermore, *Vernonia amygdalina* demonstrated the greatest *in-vitro* anti-*Helicobacter pylori* efficacy, completely halting the bacteria growth at concentrations of 125, 250, 500, and 1000 µg/ml, ranging from 12 to 18mm. From the results obtained, *Vernonia amygdalina* may become a good option for the research and development of fresh anti-*Helicobacter pylori* medications. However, Amengialue *et. al.,* 2013 had earlier reported the ethanol extract of *Ocimum gratissimum* to have antibacterial activity against some selected enteric bacteria pathogens. There is need for more research that will lead to the determination of the specific compounds that are responsible for their inhibitory effects and to explore their potential use in combination therapies.

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Table 1. Qualitative phytochemical analysis of ethanol extracts

Active ingredients	*Bryophyllum pinnatum*	*Ocimum gratissimum*	*Vernonia amygdalina*
Flavonoids	++	++	+++
Tannins	++	+	++
Cardiac glycosides	+++	+++	-
Saponin	-	-	+++
Steroids	++	++	++
Phenols	++	++	+++
Phlabotannins	-	-	-
Coumarin	-	-	-
Alkaloids	+	-	-
Anthraquinone	-	-	-
Terpenoids	+	++	++

KEY: - Negative (Absent); + Positive (Present) but low; ++ (High); +++(Very high)

Table 2. Antibiotic susceptibility test of *H. pylori* isolates from stool samples

Isolates	CRO (45µg)	CTX (25µg)	GN (10µg)	CXM (30µg)	IMP (10µg)	CIP (5µg)	LBC (5µg)	ZEM (5µg)
AMA1	R	R	R	R	R	R	S	S
BMA2	S	R	R	R	R	S	S	S
CMA1	R	R	R	S	S	S	R	R
CNA2	S	R	R	R	R	R	S	S

Key: S= Sensitive; R= Resistance; CXM=cefuroxime; GN= gentamycin; IMP= imipenem; LBC=levofloxacin; ZEM=cefexime; CTX= cefotaxime; CRO= ceftriaxone, CIP= Ciprofloxacin

Table 3. Sensitivity zone diameter (mm) of plant extracts against *Helicobacter pylori isolates.*

Isolates	*Vernonia amygdalina*	*Ocimum gratissimum*	*Bryophyllum pinnatum*	Amoxicillin
AMA1	16	12	13	6
BMA2	15	12	14	8
CMA1	13	10	12	6
CNA2	15	12	14	4

The *Helicobacter pylori* did not show resistance against the extracts tested in this study. However, resistance was reported in the studies carried out by Bluemel *et al*. (2020) and Rabia *et al*., (2020) for Amoxicillin. The extracts showed improved results at 125µg/ml as against previous work carried out on garlic powder that prevented growth at 250µg/ml (Loolaie *et al*., 2017). Moreover, it is likely that the MICs reported in this study can be reduced to lower values through the application of specific sequestered compounds identified and found by compound isolation of plants.

In this study, the histological section of the group 2a and b stomach revealed the presence of mucosa, which is made up of epithelium, lamina propria, and muscularis mucosae. The mucoid layer plays a crucial role in protecting the underlying mucosa from mechanical and chemical damage. It acts as a barrier against harmful substances and aids in

Table 4. *In-vitro* MIC of ethanol extracts of test plants against *Helicobacter pylori* isolates

Isolates	Dose (µg/ml)	VA (mm)	OG (mm)	BP (mm)	AM (mm)
CNA2	125	10	7	7	4
	250	12	10	8	4
	500	15	12	12	5
	1000	18	14	14	7
AMA1	125	10	8	10	6
	250	10	8	11	7
	500	12	12	12	7
	1000	15	12	13	8
CMA1	125	12	10	8	4
	250	13	11	9	4
	500	15	12	12	6
	1000	16	13	13	8
BMA2	125	12	8	10	5
	250	14	12	12	7
	500	15	14	15	8
	1000	18	16	16	9

Key: VA= *Vernonia amygdalina;* OG = *Ocimum gratissimum;* BP = *Bryophyllum pinnatum*; AM =Amoxicillin;

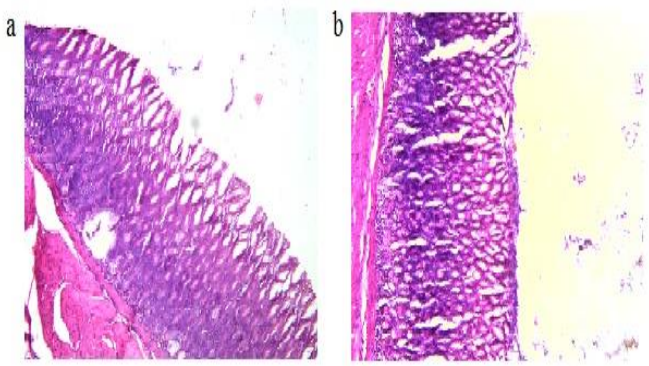

Figure 1. *Section of the group 2a and b stomach showing mucosa consisting of epithelium, lamina propria and muscularis mucosae. Above the mucosa layer is mucoid layer.* ***Magnification****: X100*

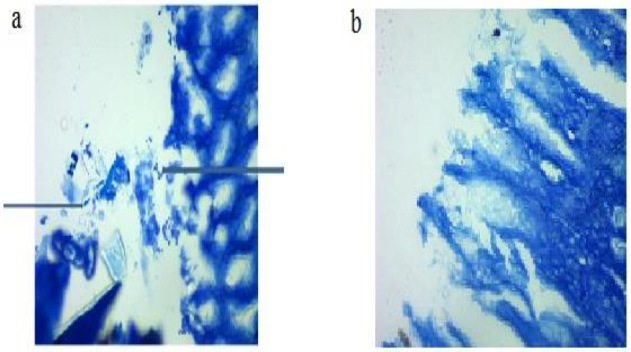

Figure 3. (a) *Giemsa special stain shows presence of some H. pylori organisms (arrows) within the mucus layer in negative control. (b) No H. pylori organism was identified in positive control group.* ***Magnification****: X400.*

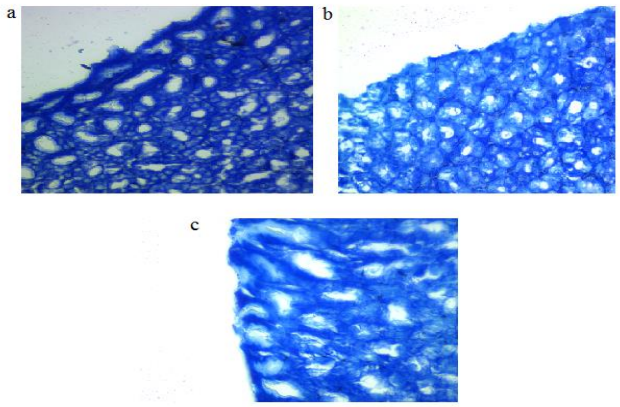

Figure 2. *No H. pylori organism was identified.*
Magnification*: X100*

lubrication of the stomach lining. The absence of *H. pylori* reported in this study as in figure 2 is significant as it suggests that the mucoid layer is effective in protecting the underlying mucosa from potential infection. This finding supports the belief that the mucus layer plays an important function in maintaining the overall health and integrity of the stomach lining. In the negative control group as in figure 3, the presence of some *H. pylori* organisms within the mucus layer suggests reduced significant threat to the stomach lining. However, further investigation is needed to determine if there are any potential long-term effects or complications associated with this low level of *H. pylori* presence.

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Conclusion

Findings from this study has revealed the extracts of *Bryophyllum pinnatum*, *Ocimum gratissimum* and *Vernonia amygdalina* to have anti-Helicobacter pylori property. This offers a new, safe, effective, and cost-effective approach to treating the pathogen. This could reduce the need for conventional antibiotics, which often have negative side effects and lead to antibiotics resistance. Further research into the medicinal potential of the studied plant extracts could lead to new, long-lasting treatments for Helicobacter pylori infections.

Conflict of interest

The authors declare that no conflict of interest exist between them as a result of the paper

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