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Helicobacter pylori Inhibition by Medicinal Plant Extracts: An *In Vitro* Assessment

Egbe, P. A.[1*], Umeaku, C. N.[1], Iheukwumere, I. H.[1*], Iheukwumere, C. M.[2], Onwuasoanya, U. F.[3], Ezenwata, I. S.[4], Afulukwe, S. C.[5], Ikeh, V. E.[6], Ezeumeh, E. N.[5] and Egbuna, C.[7]

[1]Department of Microbiology, Faculty of Natural Sciences, Chukwuemeka Odumegwu Ojukwu University, Anambra State, Nigeria.
[2]Department of Applied Microbiology and Brewing, Faculty of Biological Sciences, Nnamdi Azikiwe University.
[3]Department of Medical Microbiology and Public Health, Faculty of Medical Laboratory Science, College of Health Sciences, Nnamdi Azikiwe University.
[4]Department of Biological Sciences, Faculty of Natural Sciences, Chukwuemeka Odumegwu Ojukwu University, Anambra State, Nigeria.
[5]Department of Medical Laboratory Science, Chukwuemeka Odumegwu Ojukwu University, Anambra State, Nigeria.
[6]Department of Biology, University of Agriculture and Environmental Sciences Umuagwo, Imo State.
[7]Africa Centre of Excellence for Public Health and Toxicological Research, University of Port Harcourt, Nigeria.

*Corresponding authors: Egbe PA; Iheukwumere, I.H: ik.iheukwumere@coou.edu.ng

Abstract

In Nigeria, the increase in antibiotic-resistant strains associated with *H. pylori*, has necessitated the search for alternative therapeutic agents from botanical source. This study focused on the assessment of inhibition of some selected medicinal plants against *H. pylori*. A total of 186 each of stool and blood samples were collected and screened for HP using Columbia agar supplemented with minor nutrients. The isolates were characterized using their morphological, biochemical and molecular properties. The phytochemical constituents of *Zingiber officinale* (ZO) rhizome, *Hunteria umbellate* (HU) leaves and *Neubouda laevis* (NL) leaves extracts were determined using gravimetric and spectrophotometric methods. The *in vitro* activities of medicinal plants against the isolates were carried out using Agar-welled diffusion method. Analysis of variance (ANOVA) and student "t" test were used to analyze the data generated from the generated from the study at 95 % confidence level. *H. pylori* strain K154 (HPK154), *H. pylori* strain BS07 (HPBS07), *H. pylori* strain K93 (HPK93) and *H. pylori* strain K115 (HPK115) were encountered in the study. Alkaloid, saponins, phenolics, flavonoids, tannins, and glycosides were the major phytochemicals significantly ($P<0.05$) detected in the plant extracts. ZO showed the highest inhibition at the optimal concentration (250 mg/ml), but the activity was statistically non-significant ($p\geq0.05$) when compared to HU and NL. NL+HU+ZO was most effective when compared to single and double combination. Therefore, ZO, HU and NL showed significant inhibition against HP, and ZO+HU+NL was most effective.

Keywords: *Helicobacter pylori, Antibiotic resistance, Medicinal plants, Phytochemicals, Zingiber officinale*

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Introduction

The era where traditional medicine practitioners are disdained has gone because everyone has discovered that there are bio-active components in medicinal plants, which are yet to be tapped (Mintah *et al.*, 2019; Riaz *et al.*, 2023). Therefore, relying on one source of antimicrobial agent, which had not yielded the desired goal, would put the health of mankind in jeopardy. As a result of this, researchers in biomedical sciences

have intensified efforts to isolating and studying pathogenic bacteria that pose a serious threat to mankind such *Helicobacter pylori* (Bi *et al*., 2014).

Helicobacter pylori is a Gram-negative, micro-aerophilic and spiral-shaped bacterium that belongs to the family *Helicobacteraceae*. The genus has several species, of which some are found in the upper gastrointestinal tract and liver of some mammals and birds. The species of the genus are motile due to presence of flagellum (Savoldi *et al*., 2018).

Research had revealed that *H. pylori* is a highly pathogenic species of the genus, which infects mostly mammals especially man (Goderska *et al*., 2018). The infection that occurs due to the presence of the bacterium in the gastrointestinal tract has been recognized globally as a threat because high level of disorderliness of the system is experienced by the infected individuals. The most debilitating aspect of the infection is that all age groups, occupation, and gender are vulnerable (Garrido-Trevino *et al*., 2022).

Several researchers had reported that the ability of the bacterium to cause severe infection with acute clinical manifestations could be attributed to the presence of virulent factors such adhesins, which enable the organism to attach firmly to the mucosa of the stomach and urease, which enables it to breakdown urea, releasing ammonia and carbon dioxide (Avala *et al*., 2014; Azadi *et al*., 2019).

Some researchers had reported that the ability of the pathogen to produce ammonia from urea provides conducive environment for proliferation (Spinu *et al*., 2016; Mintah *et al*., 2019). The attachment of the organism in the mucosa enables it to destroy the epithelial cells in the tissue, thereby leading to bleeding in severe cases. Some of the infected patients had excreted the organism in faeces, which also provides relevant diagnostic information (Bouhenni *et al*., 2019). The wound caused by the organism is capable of depriving an infected person of several foods, especially when prepared using pepper, as it aggravates pain.

It is appalling that for decades, most of the drugs and antibacterial agents had not yielded the desired goal. To an extent, it was speculated that stomach ulcer caused by *H. pylori* has no cure but can only be checked. As a result of this, it has been difficult to have access to a drug that can totally cure ulcer infection, especially in developing countries, where the prevalent rate remains almost constant (Alibi *et al*., 2020).

Research had revealed that bioactive components of medicinal plants in Nigeria have extraordinary healing potentials of all kinds of diseases, though most of them have not been optimized. The researchers actually collaborated with the local herbalists, which had been using various kinds of medicinal plants for tackling diseases. According to their findings, the medicinal plants worked beyond their imagination, but scientific knowledge was needed on dosage related challenges, as local herbalists were unable to accurately determine safe dosages (Elbestawy *et al*., 2023).

Some of the plants whose bioactive potentials have been widely reported are *Ocimum gratissimum, Moringa oleifera, Aloe barbadensis, Azadirachta indica, Psidium guajava, Zingiber officinale* etc. (Ifemeje *et al*., 2014; Egbuna, 2015; Agim *et al*., 2017; Akinsanya *et al*., 2016). There are ancestral stories which had been told and passed from one generation to another concerning the potency of the aforementioned medicinal plants, and these stories have provided additional information to researchers on the potentials of each medicinal plant for further evaluation using scientific techniques. There are some religious believes that regard herbal medicine as diabolic, which had generated a lot of controversies on its acceptance in such locality, but one of the problems that biomedical researchers tend to solve is to convince people that medicinal plants are naturally endowed with bioactive agents, and it is not associated to evil forces (Akinyemi *et al*., 2017).

Materials and Methods

Sample collection: Clinical samples of blood and stool were used for the analysis. Before the collection oral consent was obtained from participants. Blood samples were collected by vein-puncture method from the anti- cubital fossa of the hand. Four milliliters (4 mL) of blood was drawn from each participant, dispensed into non-anticoagulated container and allowed to clot. Sterile plastic stool containers without preservatives were given to each subject and they were instructed to collect stool specimens following preclusive measures as described by (Cheesbrough, 2010). The collected samples were kept inside the cooler containing ice pack, and the samples were transported to the laboratory for immediate analysis.

Culture and Isolation of *H. pylori:* *H. pylori* bacteria were isolated from stool sample according to method described by Umeaku *et al*. (2022) using pre-enrichment in Columbian Agar broth (Oxoid, England), with selective antibiotic (Trimethoprim, sigma, St Louis, MO, H77883), Amphotericin B (Amresco Inc., Solar, OH, HO414), dissolved in Dimethyl-sulphoxide (DMSO) (sigma, HD5879). Stool sample was emulsified in phosphate Buffered saline and 1g of Chlorestyramine in suspension to dissolve and nullify the effect of bile in the stool as described by Ndip et al (2003). The emulsion was filtered using a sterile Muslin cloth to remove the stool debris and further filtered with membrane filter of pore size 0.45um to retain the H. *pylori* present in the stool.

Step 1 (primary culture): as recommended by (Shahamat et al 1991);
Culture broth 1; Columbia agar-based broth (oxoid–England) was prepared according to manufacture's instruction, together with the following antibiotics supplements: vencomycin (10mg), Trimethoprim (4mg), Nystatin (2.5mg). 5ml aliquot was dispensed in sterile bijou bottles. The deposite on the membrane filter was cultured on the broth and incubated at microaerophilic condition for 3-5days using anaerobic gas park (oxoid-England) at 37˚C. This was checking intermediately for the presence of visible growth (turbidity) after the first 3 days through the 12th day before discarding as no growth.

Step 2. Selective plating:
As soon as turbidity was noted, it was sub cultured on *H. pylori* selective media (liophilchem, Italy) by conventional surface-streacking technique using sterile standared (0.02 ul) wire loop. Plates were incubated at 37˚C at microaerophilic condition 3 to 7days checking intermediately for growth.

Purification of the isolates: The plates that showed discrete colonies were selected and aseptically streaked each colony on sterile plates (90mm×15mm) containing nutrient agar (BIOTECH) prepared according to the manufacturer's description. The streaked plates were placed in a bacteriological incubator in inverted positions and incubated at 35 ± 2^0C for 24 h for bacteria as described in Cheesbrough (2010).

Characterization of the pure bacterial isolates: The pure isolates were characterized using the morphological, biochemical and molecular characteristics as described in the study published by Iheukwumere *et al.* (2018).

Phytochemical Analysis and In vitro Activities of the Plant Extracts
Preparation of plant materials: The fresh leaves of *Newbouldia laevis (Ogirisi plant*), seeds of *Hunteria Umbellata* and rhizomes of *Zingiber officinale* were collected from cultivated land at Uli in Ihiala L.G.A of Anambra State, Nigeria. The samples were appropriately authenticated and air dried under shade at room temperature for 14 days. The dried leaves were ground to powdered form using sterile electric grinder (LXB 242/LE Max). Twenty gram of the ground samples each were macerated with distilled water and ethanol respectively for 72 h. The mixture was filtered using Whatman No 1 filter paper. The extracts were concentrated by evaporating to dryness at room temperature in a steady air current (Iheukwumere *et al.*, 2018)

Phytochemical analysis of the plant extracts: The phytochemical components (alkaloids, glycosides, flavonoids, phenolics, tannins, steroids and saponins) of the plant extracts were determined quantitatively using the methods described by Arewande *et al.* (2018)

Preparation of test isolates: The test isolates were prepared using the method described by Iheukwumere *et al.* (2017). The isolates were aseptically sub cultured into a broth culture and incubated at $35+ 2^0$C for 24 h. The broth culture of each isolate was centrifuged using an electric centrifuge. The sediment from each culture was diluted to a turbidity that matched 0.5 McFarland standard that was prepared by mixing 0.05 mL of 1.175 % $BaCl_2$ $2H_2O$ and 9.95 mL of 1 % Conc. H_2SO_4. The prepared isolates were standardized by comparing the absorbance with that of 0.5 Macfarland standards at 640 nm using UV/visible spectrophotometer (UV1200).

***In vitro* activities of the extracts against the resistance isolates:** This was carried out by the method of Iheukwumere and Umedum (2013). Each labeled plate was uniformly inoculated with the test organism using pour plate method in H. *pylori* Muller Hinton Agar (MHA). A sterile cork borer of 5mm diameter was used to make the wells on the medium. One tenth milliliter of various concentrations of the extracts was dropped into each labeled well and then incubated at 37˚C for 24 h. Antibacterial activity was determined by measuring the diameter of the zones of inhibition (mm) produced after incubation. Diameter less than 5.50 mm was considered resistant while diameter 5.50 mm and above will be considered sensitive.

Statistical Analysis
The results of the data generated were expressed in percentage, tables and figures. The significance of the prevalence and susceptibility study were determined using Analysis of variance (ANOVA) at 95% confidence level. Pairwise comparison was carried out in an excel sheet using student "t" test (Iheukwumere *et al*., 2020).

Results

The four predominant isolates (M, N, O and P) exhibited similar cultural and morphological characteristics but differed slightly in their appearances on Columbia blood Agar and in sizes as shown in Table 1. Isolates M and P were pale grayish whereas isolates N and O were light grayish on Columbia blood Agar. The isolates were all catalase, oxidase, urease and hydrogen production positive. They fermented glucose but were negative to arabinose, lactose and maltose. They showed varied slight reactions to xylose, inositol, sorbitol and mannitol and these formed the basis of their strain variations. The sequence analysis of the bacterial isolates showed 100% identifies for all the four isolates and the identified isolates were: *Helicobacter pylori* strain K154 (HPK154, *Helicobacter pylori* strain BS07 (HPBS07) ,*Helicobacter pylori* strain K93 (HPK93) and *Helicobacter pylori* strain K115 (HPK115) as shown in Table 2.

The phytochemical constituents of *Zingiber officinale* (ZO), *Hunteria umbellate* (HU) and *Neuboudia laevis* (NL) extracts revealed the presence of saponins, flavonoids, glycosides, tannins, alkaloids, phenolics and steroids. ZO extract recorded the highest quantities of flavonoids, tannins and alkaloids; HU extract recorded the highest quantities of saponins, glycosides and phenolics whereas NL extract recorded the highest quantity of steroids as shown in Table 3. However, the amount of the phytochemicals did not portray the potency.

The susceptibility study NL extract recorded the highest activity at 150mh/ml mostly against HPBS07, followed by HPK115, HPK154, and HPK93 recorded the least activity as shown in Table 4. ZO extract recorded its highest activity at 200mg/ml against the tested isolates as shown in Table 5. HU extract recorded its maximum activity at 250mg/ml against the test isolates as shown in Table 6. The combination/formation of the extracts using their optimum concentrations revealed that the mixture of NL (150mg/ml), HU (250mg/ml) and ZO (200mg/ml) in equal proportion recorded the highest activity against the test isolates, followed by HU + ZO, NL + ZO and HU + NL showed the test results as shown in Table 7.

Table 1: Morphological and biochemical characteristics of the isolates

Parameter	M	N	O	P
Appearance on Columbia blood agar	Pale greyish	Light greyish	Light greyish	Pale greyish
Size (mm)	1.00	0.80	0.90	1.10
Optical Nature	Translucent	Translucent	Translucent	Translucent
Edge	Smooth	Smooth	Smooth	Smooth
Surface	Smooth	Smooth	Smooth	Smooth
Gram reaction	-	-	-	-
Shape	Curved-spiral	Curved-spiral	Curved-spiral	Curved-spiral
Catalase	+	+	+	+
Oxidase	+	+	+	+
Urease	+	+	+	+
Hydrogen sulfide production	+	+	+	+
Glucose	+	+	+	+
Arabinose	-	-	+/-	-
Lactose	-	-	-	-
Maltose	-	-	-	-
Xylose	-	-	-	+/-
Inositol	+/-	-	+/-	-
Sorbitol	+/-	-	-	-
Mannitol	+/-	-	+/-	-

Table 2: Molecular identities of the bacterial isolates

Isolate	Maximum score	Total score	Query Cover	E-value	Identity (%)	Accession Number	Description
M	23555	23555	100	0.0	100.00	CP091771.1	*Helicobacter pylori* strain K154 (HPK154) complete genome
N	12770	12770	100	0.0	100.00	CP122947.1	*Helicobacter pylori* strain BS07 (HPBS07) complete genome
O	47493	47493	100	0.0	100.00	CP091769.1	*Helicobacter pylori* strain K93 (HPK93) complet
P	29676	29676	100	0.0	100.00	CP091770.1	*Helicobacter pylo* (HPK115) complete genome

550bp .5

Table 3: Phytochemical constituents of the medicinal plants

Parameters	*Zingiber officinale* (g/100g)	*Hunteria umbellate* (g/100g)	*Neuboudia laevis* (g/100g)
Saponins	0.78 ± 0.01	4.13 ± 0.11	0.41 ± 0.00
Flavonoids	5.43 ± 0.011	2.19 ± 0.11	1.57 ± 0.01
Glycosides	1.02 ± 0.01	1.78 ± 0.03	0.68 ± 0.01
Tannins	4.18 ± 0.14	0.91 ± 0.01	1.71 ± 0.03
Alkaloids	5.80 ± 0.11	3.18 ± 0.14	1.48 ± 0.01
Phenolics	1.28 ± 0.01	3.79 ± 0.22	0.87 ± 0.01
Steroids	0.02 ± 0.00	0.03 ± 0.00	0.42 ± 0.01

Table 4: Diameter zone of inhibition of *Neubouda laevis* (NL) extract against the test isolates

Isolate	50mg/ml	100mg/ml	150mg/ml	200mg/ml	250mg/ml
HPK154	11.00 ± 0.33	11.20 ± 0.81	11.30 ± 0.11	13.30 ± 0.17	13.40 ± 0.11
HPBS07	13.10 ± 0.11	13.40 ± 0.11	13.50 ± 0.21	13.50 ± 0.11	13.50 ± 0.14
HPK93	10.40 ± 0.14	10.70 ± 0.14	10.80 ± 0.11	10.70 ± 0.21	10.80 ± 0.33
HPK115	12.50 ± 0.14	12.90 ± 0.11	12.90 ± 0.14	12.80 ± 0.11	12.90 ± 0.11

Table 5: Diameter zone of inhibition of *Zingiber officinale* (ZO) against the test isolates

Isolate	50mg/ml	100mg/ml	150mg/ml	200mg/ml	250mg/ml
HPK154	11.50 ± 0.41	13.10 ± 0.14	13.40 ± 0.11	13.60 ± 0.14	13.60 ± 0.11
HPBS07	13.40 ± 0.21	16.40 ± 0.31	16.80 ± 0.21	16.80 ± 0.11	16.80 ± 0.21
HPK93	10.90 ± 0.11	12.60 ± 0.14	12.70 ± 0.17	12.90 ± 0.11	12.90 ± 0.14
HPK115	13.10 ± 0.82	15.80 ± 0.14	16.50 ± 0.21	16.60 ± 0.14	16.60 ± 0.11

Table 6: Diameter zone of inhibition *Hunteria umbellate* (HU) extract against the test isolates

Isolate	50mg/ml	100mg/ml	150mg/ml	200mg/ml	250mg/ml
HPK154	10.30 ± o.42	11.70 ± 0.11	11.80 ± 0.17	11.80 ± 0.11	11.90 ± 0.14
HPBS07	12.70 ± 0.11	13.80 ± 0.14	14.10 ± 0.11	14.20 ± 0.21	14.30 ± 0.33
HPK93	9.20 ± 0.14	10.40 ± 0.33	10.80 ± 0.14	11.00 ± 0.21	11.10 ± 0.11
HPK115	12.10 ± 0.17	13.30 ± 0.33	13.60 ± 0.21	13.80 ± 0.14	13.80 ± 0.11

Table 7: Diameter zone of inhibition of the mixture of the plant extracts using their maximum inhibitory concentrations

Isolate	HU + NL	HU + ZO	NL + ZO	NL + HU + ZO
HPK154	11.80 ± 0.11	18.40 ± 0.11	15.10 ± 0.22	19.40 ± 0.33
HPBS07	15.10 ± 0.21	19.70 ± 0.42	17.70 ± 0.11	21.00 ± 0.33
HPK93	11.10 ± 0.11	17.40 ± 0.33	14.60 ± 0.21	18.90 ± 0.21
HPK115	14.90 ± 0.14	18.80 ± 0.31	15.90 ± 0.14	19.90 ± 0.11

Discussion

The characteristics and identities of different strains of *H. pylori* encountered in both stool and blood samples are in line with the reports of many researchers (Egwu and Chukwubike, 2014; Lopes *et al*., 2014; El-Shabrawi *et al*., 2018; Nwachukwu *et al*., 2020; Hussein *et al*., 2021). *H. pylori* strain K154 (HPK154), *H. pylori* strain BS07 (HPBS07), *H. pylori* strain K93 (HPK93) and *H. pylori* strain K115 (HPK115) were encountered in the studied samples. Man researchers (El-Shabrawi *et al*., 2018; Nwachukwu *et al*., 2020; Hussein *et al*., 2021) encountered *H. pylori* from their studied samples but with varied strains.

The study revealed the presence of alkaloids, saponins, flavonoids, phenolics, tannins, glycosides and steroids in the rhizome of *Zingiber officinale* (ZO), leaves of *Hunteria umbellata* (HU) and *Neuboudia laevis* extracts and these phytochemicals were also detected by Ajayi *et al*. (2017), Arawande *et al*. (2018), Lawal *et al*. (2018), Virshette *et al*. (2019) and Iheukwumere *et al*. (2020). These phytochemicals could be responsible for the plant extracts' activities as Ujah *et al*. (2021) and Iheukwumere et al. (2018) reported. The variations in the phytochemical constituents of similar plant extracts could be traced from the source of the plants and soil nutrients. A similar deduction was drawn by Iheukwumere *et al*. (2020). The pronounced activities of the plant extracts against *H. pylori* could be attributed to the activities of the phytochemical constituents as reported by Owoyale *et al*. (2019) and Iheukwumere *et al*. (2020). The study further revealed that the mixture of the extracts mainly HU + NL + ZO showed the most pronounced activity, and these could be attributed to the synergistic activities of phytochemical constituents.

Conclusion

The study revealed that *Helicobacter pylori* strain K154 (HPK154), *H. pylori* strain BS07 (HPBS07), *H. pylori* strain K93 (HPK93) and *H. pylori* strain k115 (HPK115) were encountered in the studied stool and blood samples.The study further revealed that the mixture of the rhizomes of *Zingiber officinale* (ZO), leaves of *Hunteria umbellata* (HU) and *Neuboudia laevis* extracts contained potent phytochemicals, and exhibited significant level of inhibition against *H. pylori*, and the combination of the three extracts at their optimal activities recorded the highest inhibition.

Competing interest

There is no competing interest.

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