In Vivo Study of Healing Effects of Sida acuta Leaf Extracts on Helicobacter pylori Induced Ulceration in Mice

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Abstract

Helicobacter pylori is a human pathogen that is widely distributed and known to cause peptic ulcer, gastritis and gastric cancer. Treatment is usually by combination of acid inhibitory therapy and antibiotics because of drug resistance problems. Studies now focus on the use of medicinal plants for treatment of H. pylori induced ulcers because they are inexpensive and have limited side effects. This study was undertaken to determine the effects of cold water and ethanol extracts of S. acuta leaves on H. pylori induced ulcer in mice. Oral administration with the aid of a feeding tube of 0.5 ml of 10⁷ CFU/ml of H. pylori (Accession number LT799736) in phosphate buffer saline (pH 6.8) was used to induce ulcer in mice. Effects of various concentrations of cold water and ethanol extracts of S. acuta leaves on the ulcer parameters and histology examinations of stomach tissues of mice were investigated after 2 weeks of administration of extracts into the mice. Mice treated with clarithromycin served as positive control while mice administered with H. pylori but not treated served as negative control. One-way analysis of variance (ANOVA) was used for data analysis and results were considered significant if p < 0.05. Bacterial load of stomach tissue showed significant reduction from 15.4 × 10⁶ CFU/ml to 3.5 × 10⁶ CFU/ml and 2.6 × 10⁶ CFU/ml for cold water and ethanol extracts respectively. Reduction in ulcer severity (2.00 ± 0.10 to 0.080 ± 0.05), ulcer index (8.50 ± 1.10 to 4.26 ± 0.03) and gastric volume (1.32 ± 0.12 to 0.54 ± 0.13) were observed. Histology of stomach tissues of mice treated with extracts revealed clear granulation indicating tissue repair and wound healing. S. acuta extracts were observed to enhance ulcer healing in a mice model.
Keywords

*Helicobacter pylori, Sida acuta* Leaves, Peptic Ulcer, Ethanol Extract

1. Introduction

*Helicobacter pylori* is one of the most important human pathogens, affecting more than half of the world’s population [1]. The prevalence of *H. pylori* infection varies widely according to geographical area, patient age and socioeconomic status [2]. In general, people in developing nations, residents of developed countries with low socioeconomic status and poor level of hygienic social environment have a higher prevalence of infection [3] [4].

The range of isolation is between 70% - 90% in developing countries and 25% - 50% in developed countries [5]. In various regions of sub-Saharan Africa, for example, 61% - 100% of the population may harbor the pathogen [6]. Infection with *H. pylori* is usually acquired in early childhood and persists for life [7]. While over 80% of infected individuals are asymptomatic [8], the infection can lead to peptic ulcer, gastritis, and gastric cancer [1] [9], thus, it has been recognized as the principal agent leading to gastric cancer and as a class I carcinogen by WHO. *H. pylori* uniquely colonizes the stomach where it induces inflammation and affects gastric physiology [9]. Motility has been shown to be essential for successful *in vivo* colonization by *H. pylori* and is provided by its sheathed flagella [10].

*H. pylori* eradication is very necessary and the management strategy depends on whether the patient is a first-time or a chronic non-steroidal anti-inflammatory drugs (NSAID) user [11]. Drugs that have demonstrated efficacy include amoxicillin, clarithromycin, metronidazole, tetracycline and bismuth [4]. In general, monotherapy is not recommended for the treatment of *H. pylori* infection due to poor clearance rates and problems of drug resistance [12]. Eradication is usually achieved with a combination of acid-inhibiting therapy and antibiotics. The addition of anti-secretory agents to antibiotics accelerates the ulcer healing process [13]; however, such combination therapy does not always successfully eradicate *H. pylori* and is expensive [14]. Many studies now focus on the use of medicinal plants for the treatment of *H. pylori*, because of the limited side effects on tissues and it’s inexpensive nature [15] [16] [17]. They have also been used in traditional medicine to treat a wide range of diseases including digestive disorders such as ulcers [18].

*Sida acuta* otherwise known as broom weed is a shrub belonging to Malvaceae family. The plant is widely distributed in the subtropical regions and has many traditional usages that varied from one region to another [19]. All the plant parts exert various pharmacological properties which include antiplasmodial, antimicrobial, antioxidant and cytotoxic activities [20]. In Nigeria, *S. acuta* known as Udo by the Igbos [21] and Iseketu by the Yorubas [22] has been reported to be
used in the treatment of malaria, ulcer, fever, gonorrhea, abortion, breast cancer, poisoning, inflammation, and haemorrhage [22]. This research therefore aims at determining the healing effects of cold water and ethanol extracts of *Sida acuta* leaves on *H. pylori* induced ulcer in mice.

2. Materials and Methods

2.1. Microorganism Used

*Helicobacter pylori* (Accession number LT79936) was used for ulcer induction in mice.

2.2. Preparation of Plant Materials

The leaves were washed under running tap water, dried at 25˚C and ground into fine powder using an electric blender (Moulinex). Fifty grams of the powdered plant materials were separately soaked in 500 ml conical flasks containing 250 ml of cold water and 95% ethanol. Each flask was placed on an orbital shaker (Thermo Scientific MAXQ 400) at 100 rpm for 24 h at 25˚C. The content of the flask was filtered with Whatman No.1 filter paper and the filtrate evaporated to dryness in an oven at 40˚C. Extracts were stored in specimen bottles and stored at 4˚C for further use [23].

2.3. Experimental Design, Induction of Ulcer Using *H. pylori* and Treatment

A total of fifty four healthy swiss mice weighing 17 - 20 grams were housed in polypropylene cages under controlled conditions of temperature (25˚C) with 12 h light dark cycles [24]. They were allowed to acclimatize for 7 days and given free access to feeds and water *ad libitum*.

Mice were divided into six groups (A-F), with 6 mice in groups A-C and 12 mice in groups D-F. Mice with the exception of those in group A were orally administered with 0.5 ml of 10⁷ CFU/ml of *H. pylori* in phosphate buffer saline (pH 6.8) with the aid of a feeding tube to induce ulcer. The mice received a total of 3 doses given at 3 days interval to enable proper pathogen incubation and disease establishment [25]. Group A mice were administered with 0.5 ml of phosphate buffered saline only (healthy control) while in Group B *H. pylori* was administered without treatment (negative control). In Group C, *H. pylori* was administered and treated with 0.5 ml of 50 mg/ml clarithromycin (positive control), Group D has 12 of the mice administered with *H. pylori* but 6 of them were treated with 0.5 ml of 100 mg/ml cold water extract and the other 6 treated with 0.5 ml of 100 mg/ml ethanol extract. Group E was similarly administered with *H. pylori* but 0.5 ml of 200 mg cold water and ethanol extracts were used for treatment. In Group F as earlier stated, *H. pylori* was administered and treated with 0.5 ml of 400 mg/ml of cold water and ethanol extracts. The treatment with clarithromycin and leaf extracts were carried out twice daily for 14 days.
2.3.1. Animal Sacrifice

The mice were anesthetized by chloroform inhalation for 1min before sacrifice. Mice stomach was opened through the greater curvature and the contents used for estimation of bacterial count, evaluation of ulcer parameters and histology studies.

2.3.2. Bacterial Count

Stomach biopsies were scraped off and homogenized with 0.5 ml of phosphate buffered saline (PBS) and the homogenate serially diluted 10-fold. 0.1 ml of 10⁻⁶ dilution of the homogenate was inoculated on Columbia Agar (Oxoid) and incubated at 37°C for 5 days under microaerophilic conditions. The bacterial load was estimated by colony count and expressed as log₁₀ CFU per milliliter of homogenate [17].

2.3.3. Evaluation of Ulcer Parameters

Determination of Ulcer Index: The gastric contents were removed from the mice and blood cloths washed off with water. Mice were fixed on a board and ulcer formation examined using a 10× magnifier lens. The following scores/ratings were used to evaluate the ulcer index as well as the severity of gastric lesions [26].

0 = no lesion, 1 = mucosal edema and petechiae, 2 = one to five small lesions (1 - 2 mm), 3 = more than five small lesions or one intermediate lesion (3 - 4 mm), 4 = two to more intermediate lesions or one gross lesion (>4 mm), 5 = perforated ulcers.

\[
\text{Ulcer index (UI)} = \frac{\text{Total ulcer score}}{\text{No. of animals ulcerated}}
\]

Percentage Protective Index (PPI) was calculated as:

\[
\text{PPI} = \frac{\text{UI control} - \text{UI treated} \times 100}{\text{UI control}}
\]

Determination of Gastric Volume: Gastric content was collected and centrifuged at 300 rpm for 10 min to remove any solid mass and the volume of gastric juice determined using a graduated measuring cylinder [27].

Determination of pH: The pH of the gastric contents of both the control and the treated groups were determined using the method of [27]. An aliquot of 0.5 ml of gastric juice was diluted with 0.5 ml distilled water and the pH of the solution measured using digital pH meter.

Determination of Total Acidity: An aliquot of 0.5 ml of gastric juice was diluted with 0.5 ml of distilled water and was taken into a 50 ml conical flask. One drop of phenolphthalein indicator was added and titrated with 0.1NNaOH until a permanent pink color was observed [27]. The volume of 0.01NNaOH consumed was noted and total acidity calculated as shown below.

\[
\text{Total Acidity (Mmol/L)} = \frac{\text{No of ml of 0.1NNaOH used to achieve 2nd titre} \times 0.05 \times 1000}{\text{No of ml of gastric content used}}
\]
2.3.4. Histology Examination
This was performed according to [28]. A thin section of the stomach tissues (2 cm in diameter) was trimmed with a sharp sterile razor blade and fixed in 10% formalin for 24 h. The fixed tissues were washed under running water for 2 min and then passed through several concentrations of alcohol 70% alcohol for 24 h, 90% alcohol for 12 h and absolute alcohol for 6 h to remove water from the fixed tissues. Tissues were then, completely infiltrated with paraffin and passed through xylene for 3 h to prevent shrinkage and tissue brittleness in paraffin. Thereafter, they were embedded using the Leukhand embedded mould. The L pieces were arranged on aluminum base to form a rectangle, and molten paraffin poured into the moulds and the selected surfaces of the tissues embedded with the aid of a pair of blunt end of forceps and allowed to set. The embedded tissues were separated into different blocks and then attached to wooden blocks with the aid of an electric spatula. The blocks were thereafter trimmed using a rotary microtome and knife and arranged on ice trays in order to cut thin sections (5 μm thick). Sections were picked with the help of a camel hair brush and placed on a slide. Sections were flooded with 20% alcohol in order to spread out folds on the sections and then floated out on a water bath with a temperature of 10°C below the melting point of the wax used. The sections were picked and floated on a water bath and then picked with a pre-labeled slide. The slides were dried on a hot plate at a temperature of 10°C above the melting point of wax used for 15 min and stained with haematoxylin and eosin. They were observed under Olympus binocular microscope at ×10 and ×40 magnification lens. Photomicrographs of the tissue section were captured using Motic image plus 2.0 camera.

2.3.5. Statistical Analysis
Results of the study were expressed as mean ± standard deviation. Differences between mean on the effect of S. acuta leaf extracts on the gastric parameters were analyzed using one-way analysis of variance (ANOVA) using SPSS version 21 software. Values were considered significant if p < 0.05.

3. Results
3.1. Bacterial Load Count
All the infected mice showed stable H. pylori colonization of gastric mucosa at week 0. There was a reduction in bacterial load of gastric mucosa following the administration of the various concentrations of cold water and ethanol extracts of S. acuta and clarithromycin after weeks 1 and 2. As shown in Figure 1, bacterial load was significantly (P < 0.05) reduced after 2 weeks administration of 400 mg/ml of the extracts from 15.4 × 10^6 CFU/ml of the infected untreated mice to 3.5 × 10^6 CFU/ml for cold water extract treated mice and 2.6 × 10^6 CFU/ml for ethanol extract treated mice. The positive control (clarithromycin treated mice) showed reduction from 15.4 × 10^6 CFU/ml to 2.1 × 10^6 CFU/ml.
3.2. Evaluation of Ulcer Parameters

The results of effects of cold water extract and ethanol extract of *S. acuta* on the gastric parameters of ulcerated mice after 2 weeks of treatment are as shown in Table 1. The ethanol and cold water extracts of *S. acuta* leaves showed significant protection against *H. pylori* induced ulcer at doses 100 mg/ml, 200 mg/ml and 400 mg/ml.

Mice treated with *S. acuta* extracts and clarithromycin (Groups C-F) had their ulcer index and severity, gastric volume and total acidity considerably reduced when compared to those infected without treatment (Group B). The gastric volume was significantly (p < 0.05) increased in Group B (1.32 ± 0.12) when compared with those in Group A (0.40 ± 0.10).

3.3. Histological Examinations

Histopathological changes were observed in the stomach tissues of mice in groups B-F. While the micrograph of the stomach tissues of group A (Figure 2) showed full thickness of gastric body mucosa devoid of lymphoid cells, Group B revealed clear accumulation of minor fat droplets with deep ulcers (Figure 3). Meanwhile, notable repairs of the disruption of the muscularis mucosa, accumulation of inflammatory cells, hyperplasia of epithelial cells and spotty hemorrhage were observed in groups C-F with 400 mg/ml extracts (Figure 4 and Figure 5) showing healing comparable with that of clarithromycin (Figure 6).

![Figure 1](image-url) Figure 1. Bacterial load count of stomach tissues at 400 mg/ml concentration measured in Log10 CFU/ml. B: Negative control *H. pylori* induced; C: *H. pylori* induced mice treated with Clarithromycin; D: *H. pylori* induced mice treated with 400 mg/ml concentration cold water aqueous extract; E: *H. pylori* induced mice treated with 400 mg/ml concentration ethanol extract.
Table 1. Effect of cold water and ethanol extracts of *Sida acuta* leaves on gastric parameter by *H. pylori* induced ulceration in mice after 2 weeks.

<table>
<thead>
<tr>
<th>Experimental groups/Treatment</th>
<th>Ulcer severity</th>
<th>Ulcer index</th>
<th>PPI</th>
<th>Gastric Volume (ml)</th>
<th>Stomach pH</th>
<th>Total Acidity</th>
<th>% of Ulcerated Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.52 ± 0.01</td>
<td>2.84 ± 0.04</td>
<td>0.32 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>2.00 ± 0.10</td>
<td>8.50 ± 1.10</td>
<td>0</td>
<td>1.32 ± 0.12</td>
<td>2.18 ± 0.18</td>
<td>1.22 ± 0.22</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>0.6 ± 0.06</td>
<td>4.12 ± 0.05</td>
<td>39.5</td>
<td>0.54 ± 0.02</td>
<td>3.88 ± 0.04</td>
<td>0.26 ± 0.04</td>
<td>40</td>
</tr>
</tbody>
</table>

**Cold Water Extract**

| D                           | 1.2 ± 0.18     | 8.26 ± 0.5  | 20.9| 0.88 ± 0.13         | 2.82 ± 0.12| 0.68 ± 0.01   | 80                    |
| E                           | 1.00 ± 0.01    | 6.21 ± 0.50 | 40.74| 0.72 ± 0.12         | 3.14 ± 0.12| 0.48 ± 0.01   | 60                    |
| F                           | 0.90 ± 0.15    | 4.88 ± 0.10 | 41.52| 0.66 ± 0.06         | 3.38 ± 0.62| 0.27 ± 0.02   | 60                    |

**Ethanol Water Extract**

| D                           | 1.1 ± 0.15     | 6.29 ± 0.35 | 39.9| 0.88 ± 0.11         | 3.04 ± 0.04| 0.52 ± 0.04   | 60                    |
| E                           | 1.10 ± 0.15    | 6.23 ± 0.32 | 40.55| 0.86 ± 0.02         | 3.32 ± 0.35| 0.40 ± 0.02   | 60                    |
| F                           | 0.80 ± 0.05    | 4.26 ± 0.03 | 41.71| 0.54 ± 0.13         | 3.74 ± 0.15| 0.21 ± 0.02   | 50                    |

Figure 2. Photomicrograph of Group A Healthy control stomach showed (1) Intact muscularis mucosa; (2) Intact gastric glands; (3) Intact mucosa; (4) Intact gastric pits. The stomach architecture appeared normal and intact. H&E. mag. 100×.
Figure 3. Photomicrograph of Group B (Negative control). *H. pylori* induced stomach tissue showed (1) Lipid accumulation (white arrow) or lipid droplets was seen in the apical portions of epithelial cells (black arrow); (2) Deep ulcer was also observed. H&E. mag. 100×.

Figure 4. Photomicrograph of Group F treated with 400 mg/ml cold water extract *H. pylori* induced mice stomach tissue showed (1) Granulated tissue which is an evidence of tissue repair or wound healing (black arrow); (2) Hyperplasia of epithelial cells (white arrow); (3) Fibrous scar tissue. In this zone, is a mature fibrous scar tissue H&E. mag. 100×.
Figure 5. Photomicrograph of Group F treated with 400 mg/ml ethanol extract \textit{H. pylori} induced mice stomach tissue showed minor ulcer (star) and minor granulated tissue. There is evidence of hyperplasia of epithelial cells. H&E. mag. 100×.

Figure 6. Photomicrograph of Group C \textit{H. pylori} induced mice treated with clarithromycin stomach tissue revealed (1) Minor disruption of the muscularis mucosa; (2) Accumulation of inflammatory cells in the superficial mucosa keeping for minor mucosal inflammation; (3) Spotty hemorrhage present. H&E. mag. 100×.
4. Discussion

Unsuccessful eradication of *H. pylori* after treatments, recurrent infections, and increasing resistance of *H. pylori* to therapeutic treatments (5% - 20% of treatments have been reported to fail due to *H. pylori* resistance to treatments) all have led to continuous research on finding new ways of treatments [29].

In this study, the research was designed to induce ulcer in mice using *H. pylori* (Accession Number LT79936) and to investigate the efficacy of the various concentrations of cold water and ethanol extracts of *S. acuta* in the ulcerated mice.

The result obtained in this study showed that all the infected mice (Groups B-F) had stable colonization of gastric mucosa by *H. pylori* which significantly reduced after 2 weeks of administration of extracts. It was observed that effects of the extracts on the studied parameters varied according to the extraction solvent, the concentration of the extract used and the length of administration of the extracts. Ethanol extracts had a significantly higher activity against the *H. pylori* than the cold water extracts. This variation may probably be due to better solubility of the bioactive components of *S. acuta* in ethanol which agrees with the reports of many researchers [30] [31] [32] [33]. They noted that components of medicinal plants are better extracted with alcoholic solvents like ethanol and methanol. The extracts inhibitory activities against *H. pylori* was also confirmed by the *H pylori* count in gastric biopsy culture, where the decrease in bacterial population was observed from samples treated with both extracts (cold water and ethanol) and clarithromycin. Several studies have also reported that extracts from several other plants, such as *Allium sativum* [15], *Centella asiatica* leaf [16], *Aframomum pruinosum* seeds [17], *Calophyllum brasiliense* Camb. [34], turmeric [35] and garlic powder and oil [36] have activities against *H. pylori*.

From the result obtained, there was increase in gastric volume after *H. pylori* was orally administered into the mice (Group B). This could be as a result of report by [37] that *H. pylori* infection leads to alteration of the motor or sensory function of the stomach, causing a delayed stomach emptying and increased gastric juice volume. Treatment with clarithromycin and varied concentrations of the extracts of *S. acuta* significantly reduced the gastric volume, ulcer severity, ulcer index and lowered the total acidity in the stomach of mice in Groups C-F in a dose dependent manner. The reduced gastric acid secretion and gastric juice volume produced by extracts of *S. acuta* in the present study may be secondary to ulcer healing, due to its anti-*H. pylori* effect or as a result of effect on auto-regulation of gastrin release. At dose 400 mg, there was no significant difference in the ulcer index of extracts and that of clarithromycin. (The ulcer index was 4.88 ± 0.10 and 4.26 ± 0.03 for cold water and ethanol extracts respectively, and 4.12 ± 0.05 for clarithromycin.) This result agrees with the work of [38] who demonstrated the anti-ulcerogenic action of *S. acuta* in a rat model induced with pylorus ligation and aspirin administration. The researchers reported the effectiveness of the ethanol extract of *S. acuta* used against different ulcer causing
agents. Several other studies have also shown the antibacterial properties of S. acuta against P. mirabilis, S. aureus and other pathogenic bacteria [14] [18] [21] [22].

The micrograph of mice stomach tissue of Group B mice revealed mucosal lipidosis and deep ulcer while that of those treated with clarithromycin (50 mg/ml), cold water and ethanol extracts (400 mg/ml each) of S. acuta, all exhibited clear granulation which represents tissue repair or wound healing characterized by fibrous scar tissues, aggregation of infiltrates, and hyperplasia of epithelial cells. In another study carried out by [39], H&E staining of the gastric mucosa of H. pylori infected mice showed extensive lesions, oedema, leucocytes infiltration and hemorrhage which showed a significant reduction after treatment with geraniol.

5. Conclusion

The findings in this study confirm positive efficacy of the ethanol and cold water extracts of S. acuta leaves against H. pylori and in controlling stomach ulcer whose efficacy is comparable to standard drug clarithromycin. Thus, S. acuta has the potential to be developed as a treatment for H. pylori associated peptic ulcer disease. Further studies to obtain bioactive compounds of S. acuta and their mechanism of action are highly recommended.

Acknowledgements

Authors wish to thank Mrs. Christy Odimegwu who provided the Sida acuta leaves used in this study and Dr. Chika Ikele of University of Nigeria Nsukka for his assistance in carrying out the Animal Tissue Histopathology.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References


