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Original Research Article

Protective roles of *Vigna subterranea* (Bambara nut) in rats with aspirin-induced gastric mucosal injury

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ABSTRACT

Objective: *Vigna subterranea* is widely consumed as a traditional staple food in Nigeria and some West African countries. The ethanolic seed extract of *V. subterranea* (EEVS) was investigated for its gastroprotective effects on aspirin plus pylorus ligation-induced gastric ulcerated rats using an *in vivo* assay.
Methods: Gastric mucosal ulceration was induced experimentally in Groups 2 to 5 using aspirin plus pylorus ligation. Rats in Group 1 were orally pretreated with 3% Tween 80 only as normal control. Groups 2 to 5 were pretreated with 3% Tween 80 (ulcer group), 20 mg/kg of omeprazole (positive group), and 200 and 400 mg/kg of EEVS (experimental groups), respectively, once daily for 21 days before ulcer induction. Parameters including those for gastric secretions, ulcerated areas and gastric wall histology were assessed. Levels of superoxide dismutase (SOD), glutathione peroxidase (GP_X), and malondialdehyde (MDA) in the gastric tissue homogenate were also determined.
Results: Pretreatment with EEVS significantly ($P < 0.05$) reduced the ulcer index, gastric volume and total acidity in rats with aspirin plus pylorus ligation-induced ulcer. The pH and mucus of gastric content increased significantly ($P < 0.05$) while the levels of SOD and GP_X were observed to be elevated with a reduced amount of MDA. Significant severe gastric mucosal injury was exhibited in the ulcer group and EEVS or omeprazole offered significant ($P < 0.05$) protection against mucosal ulceration. Histologically, the gastric submucosal layer showed remarkable decrease in edema and leucocytes infiltration compared with ulcer group.
Conclusion: The study suggests that EEVS offered a protective action against aspirin plus pylorus ligation-induced gastric ulcers in Wistar rats. The protective effect might be mediated via antisecretory, cytoprotective and antioxidative mechanisms.

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1. Introduction

Gastric ulcers are common among gastrointestinal disorders. It is considered a serious health problem that also poses economic challenges, and thus has been a subject of many investigations both clinically and experimentally. It is the most prevalent gastrointestinal disease, affecting 3%–10% of the global population, especially in developing countries such as Nigeria [1,2]. About 10% of the world population is at risk of developing this disease at some point in their lifetime [3]. Worldwide annually, out of every 15,000 complications of gastric ulcer, an estimated 15 mortality was recorded [4,5]. Even though scientists and medical researchers cannot fully ascertain the exact pathogenesis of gastric ulcer, the likely pathways of its development have been established. Gastric mucosal ulceration occurs when the stomach secretion of aggressive factors (acid and pepsin) overwhelms that of protective factors (mucus and bicarbonate) resulting in disruption of the gastric mucosal layer [6,7].

The pathogenesis of gastric ulcer is multifactorial in nature, including high levels of acid-pepsin secretion [8], insufficient bicarbonate neutralization and reduced secretion of mucus

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[9,10]. Various factors including poor eating habits, too much consumption of nonsteroidal anti-inflammatory drugs (NSAIDs) [11,12], stress [13], smoking [14], and infection by *Helicobacter pylori* [15,16] have been implicated in gastric ulcer pathogenesis.

Numerous available orthodox drugs (such as proton pump inhibitors, histamine receptor antagonists and antibiotics) have been employed in the treatment and management of gastric ulcers. However, the major challenges of treatment continue to be the high cost, adverse effects and resistance of these drugs with prolonged use [17]. These factors triggered the quest for safer and cheaper antiulcer drugs. In view of the above challenges, it is pertinent to investigate the phytotherapy and effectiveness of folk medicinal plant formulations for treatment, management and prevention of gastric lesions. Several medicinal plants used in animal experiments have been shown to possess antiulcer effects [18,19].

Vigna subterranea, commonly known as Bambara groundnut, is an emerging plant of interest used for gastric ulcer treatment, management and prevention. It is a legume in the Fabaceae family, genus *Vigna*. It has its origin in West Africa [20,21] and exists in both wild and cultivated forms. It is a small, annual, creeping shrub, appearing in either bunched or spreading form with a height of about 0.30–0.35 m [22]. The plant is known as *Jugo* beans in South Africa, *Aboboi* in Ghana and *Nzama* in Malawi. In Nigeria, it is known locally as *Okpa* by the Igbos in Eastern Nigeria; *Gurjiya* or *kwaruru* by the Hausas in Northern Nigeria and *Epiroro* by the Yorubas in western part of Nigeria [22].

The seeds of *V. subterranea* have many therapeutic applications in folk medicine owing to their effective secondary metabolites such as alkaloids, flavonoids, saponins, resins and glycosides [23]. In Africa, infusions and decoctions of the seeds are used to treat venereal diseases, diarrhoea, cataract, beriberi and polymenorrhea [24,25]. The raw seeds are chewed to alleviate nausea and vomiting in pregnant women [21,26]. There have been documentations on its biological potencies such as antioxidative and hepatoprotective effects in animal experiments [27,28]. After literature review, experimental evidence in favour of the gastroprotective effects of the seeds of *V. subterranea* is yet to be reported. Therefore, the present study aimed to investigate the possible gastroprotective effects of the ethanolic seed extract of *V. subterranea* (EEVS) against aspirin plus pylorus ligation-induced gastric mucosal injury in rats.

2. Materials and methods

2.1. Drugs, chemicals and reagents

Commercially obtained drugs, chemicals and reagents of analytical grade were used in this study. Tween 80, ethanol, carboxymethyl cellulose, diethyl ether, NaOH and HCl were procured from Sigma-Aldrich Chemical Company (St. Louis Missouri, USA). Distilled water was acquired from Ebonyi State University Biochemistry Laboratory, Abakaliki, Nigeria.

2.2. Drug preparation

2.2.1. Omeprazole

Omeprazole (Tuobin Pharmaceutical Factory, Shantou, China), purchased from Godal Pharmacy, Abakaliki, Nigeria, was used as standard antiulcer drug in this study. It was dissolved in 3% Tween 80 and orally administered to the animals at a dosage of 20 mg/kg body weight (5 mL/kg) prior to gastric ulcer induction [29].

2.2.2. Aspirin

Aspirin (May & Baker, Nigeria Plc) was purchased from Godal Pharmacy, Abakaliki, Nigeria. It was dissolved in 1% sodium carboxymethyl cellulose, and a dose of 200 mg/kg body weight (5 mL/kg) was given orally for gastric ulceration [30].

2.3. Experimental animals

Healthy adult male (8–10 weeks old) Wistar rats weighing (183.20 ± 3.67) g were used for the study. The animals were procured from the Faculty of Medicine, Preclinical Central Animal House, Ebonyi State University, Abakaliki. They were maintained on standard rat pellets (Vital Feeds Nig Ltd, Nigeria) and water *ad libitum* and were kept under controlled laboratory conditions (12 h light–dark cycle at 18–26 °C and relative humidity of 30% to 70%). The animals were allowed 1 week acclimatization before the experiment commenced.

2.4. Ethical approval

This research was carried out following the approval by the Faculty of Medicine ethics committee for animal experimentation of Ebonyi State University, Abakaliki (Ethic No. EBSU/REC/MPC/15012/07) and animal handling was according to accepted guidelines by the National Institutes of Health for care and use of laboratory animals [31].

2.5. Plant material collection and authentication

Dry seeds of *V. subterranea* were purchased from Abakpa market, Abakaliki, Ebonyi State. They were identified and authenticated by Chijioke Onyeukwu, a botanist in the herbarium section of Plant Science and Biotechnology Department, University of Nigeria, Nsukka, Enugu State. A voucher specimen (UNH 154a) was deposited for future reference.

2.6. Preparation of ethanolic extract

The dry seeds of *V. subterranea* were dehulled and ground into flour using a suitable grinder. The seed powder (1000 g) was extracted in a Soxhlet extractor at 70 °C with absolute ethanol (96%, v/v). The mixture was vacuum-filtered through Whatman No. 1 filter paper. A vacuum rotary evaporator (Eyla N-1000, Japan) maintained at 45 °C was used to concentrate the filtered extract. The resulting residue which weighed 93.4 g (recovery 9.3%) was later stored at 4 °C before use. Prior to oral administration of the extract, the required dose of 200 or 400 mg/kg body weight was obtained after the extract was reconstituted in 3% Tween 80 [32].

2.7. Qualitative phytochemical analysis

Qualitative phytochemical screening to pinpoint the presence of secondary constituents (such as alkaloids, terpenoids, saponins, anthraquinones, flavonoids, tannins, resins, glycosides, steroids and phenols) in EEVS was carried out using standard phytochemical methods described by Harborne [33].

2.8. Acute oral toxicity studies

The "fixed dose" method of Organization for Economic Cooperation and Development (2008) guideline No. 425 was employed to determine the acute oral toxicity of EEVS in adult albino rats [34]. The method was commenced with an initial dose of 2000 mg/kg body weight after overnight dietary deprivation. The animals were observed for general behavioural, autonomic and neurological behaviour during the course of the experiment.

2.9. Experimental design and treatment

In this study, gastric mucosal ulceration was induced experimentally in Groups 2 to 5 using aspirin plus pylorus ligation. The rats were allotted into five groups of five rats in each group. Group 1 (vehicle) received 3% Tween 80 only. Group 2 (ulcer control) received 3% Tween 80 before modelling. Group 3 (positive) received 20 mg/kg omeprazole while groups 4 and 5 (test groups) received 200 and 400 mg/kg body weight (5 mL/kg body weight) of EEVS respectively. All the groups were pretreated for 21 days between 8:00 a.m. and 9:00 a.m. daily by oral gavage.

2.10. Aspirin plus pylorus ligation-induced gastric ulcer

In this study, gastric mucosal ulceration was induced experimentally using aspirin plus pylorus ligation model previously described by Anupama et al. [35]. In this method, induction of gastric ulcers involved the use of both aspirin administration and pylorus ligation procedure. From the 19th to 21st day of pretreatment, groups 2 to 5 received aspirin (200 mg/kg) after 2 h fasting. All the animals (groups 1 to 5) were subjected to pylorus ligation on the 22nd day (after 18 h fast) under light ether anaesthesia. The pylorus was carefully secured and ligated with a silk thread through an abdominal midline incision below the xyphoid process. Thereafter, interrupted sutures were used to close the abdominal wall.

2.11. Excision of stomach and gastric juice collection

The rats were sacrificed humanely on the 22nd day under diethyl ether anaesthetization 4 h after pylorus ligation. The stomach was excised and opened along the greater curvature following the opening of the abdomen. The gastric content of the stomach was drained into a centrifuge tube in addition with 5 mL of distilled water. The resultant solution was centrifuged at 3000 × *g* for 10 min at 4 °C and the supernatant collected was subjected to biochemical analyses thereafter [36].

2.12. Ulcer score and percentage inhibition

The gastric mucosal layer of the stomach was viewed under a magnifying lens (10×) to evaluate the gastric ulcers. The ulcerated areas were counted and scored using the method described by Kulkarni [37]. The score was graded as: 0, normal colouration; 0.5, red colouration; 1.0, spot ulcers; 1.5, haemorrhagic streaks; 2.0, deep ulcers; 3.0, perforations.

The sum of ulcer scores assigned to gastric lesions was expressed as the mean ulcer index (MUI) [38]. Percentage of ulcer inhibition was calculated according to Hojage et al. [39] using the formula: Inhibition = $(MUI_{control} - MUI_{test}) \div MUI_{control} \times 100\%$.

2.13. Determination of gastric juice volume and pH

The supernatant fluid volume was measured in millilitre using a microsyringe [40]. The gastric content samples were analysed for hydrogen ion concentration using a digital pH meter [29].

2.14. Determination of total acidity and gastric acid output

One millilitre of gastric juice was titrated with 0.01 (mEq/L) NaOH in a conical flask using phenolphthalein (two drops) as indicator until light-pink solution indicating pH 7.0 was obtained [41]. The NaOH volume added was employed in the calculation to get the total acidity using the formula below [42]: Total acidity = $Volume_{NaOH} \times normality \times 100$ (mEq/L) ÷ 0.1. Acid output was expressed as micro equivalents per hour (μEq/h) and calculated by multiplying the total acidity in mEq/L by the gastric juice volume (in litres) [43]. The result was divided by 4 to give output per hour. Acid output (μEq/h) = acidity (mEq/L) × $volume_{gastric\ juice}$ (L) ÷ 4 h.

2.15. Determination of mucus content of gastric wall

Gastric mucus study was carried out as previously described by Corne et al. [44]. The glandular portion of each rat's stomach after sacrifice was scrapped and soaked in 1% Alcian blue solution (in 10% sucrose solution, buffered with sodium acetate at pH 5.0). The glandular mucus and Alcian blue were allowed to bind for 10 min. Sucrose solution was used to rinse the excess dye which adhered to the stomach tissue. Five millilitres of 5% magnesium chloride solution were used to extract the dye which complexed with gastric wall mucus for 15 min. A 4 mL aliquot of blue extract with equal volume of diethyl ether was shaken vigorously to form emulsions which were centrifuged at 5000 × *g* for 15 min at 4 °C. The absorbance of the supernatant was recorded at 580 nm using a ultraviolet-spectrophotometer. Thereafter, the amount of extracted Alcian blue per gram of glandular tissue (net) was calculated.

2.16. Stomach homogenate preparation and assay of antioxidant and enzymatic activities

Ice cold 0.1 mol/L phosphate buffer saline (1:4 (w/v), pH 7.4) was used to homogenize the stomach and the homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C. Thereafter, the resulting supernatant was used for antioxidant status assay. Superoxide dismutase (SOD) and glutathione peroxidase (GP_X) activities in the gastric tissue homogenate were determined using the SOD and GP_X assay kits (Randox Laboratories Ltd., UK), according to instructions of the manufacturer. The gastric mucus membrane lipid peroxidation (malondialdehyde, MDA) was determined using the thiobarbituric acid reactive substance commercial kit (Randox Laboratories Ltd., UK).

2.17. Gross evaluation of gastric mucosa

Normal saline was used to rinse the mucosal layer of the stomach of each rat to remove any blood clot. The stomach was then pinned to a flat board to observe any changes in the physical appearance of the mucosa. Photographs of the gastric lesions were taken for proper observation and documentation.

2.18. Histology assay

Histopathological studies were conducted using the method described by Bancroft and Stevens [45]. After gastric content collection and scoring of gastric lesions, samples from the stomachs representing each group were fixed in 10% formalin for 24 h. The formalin-fixed specimens were embedded in paraffin wax and sections of 5 μm thick were cut in a microtome, fixed in 20% alcohol and mounted on glass slides using standard techniques. The slides were viewed under a light microscope (×40 magnifications) after staining the tissues with haematoxylin-eosin stain. Photographs of the gastric lesions were taken with a photo microscope for proper observation and documentation of histopathological lesions.

2.19. Statistical analysis

The values were expressed as mean ± standard error of mean. One-way analysis of variance was used for data comparison followed by Tukey's multiple comparison tests. Differences between

groups were considered statistically significant at $P < 0.05$ using GraphPad Prism Version 6.0 for Windows (GraphPad® Software, San Diego, CA, USA).

3. Results

3.1. Extract yield

The ethanolic extraction of 1000 g of *V. subterranea* seed powder yielded 9.3% (w/w) dark brown semisolid extract with a pleasant smell and pasty consistency.

3.2. Qualitative phytochemical analysis

The result of the preliminary qualitative phytochemical studies of EEVS showed the presence of saponins, glycosides, alkaloids, flavonoids, terpenoids and resins.

3.3. Acute oral toxicity studies

All the animals remained alive and showed no visible signs of toxicity even with the highest dose. There were no abnormal signs, changes in body weight and behaviour in the EEVS-treated animals throughout the observation period when compared to normal control animals. Thus, the median lethal dose was considered to be greater than 2000 mg/kg body weight.

3.4. Effects of EEVS on ulcer index and ulcer inhibition

The effects of EEVS on ulcer index and inhibition are reported in Table 1. Ulcer index in the ulcer control group was significantly ($P < 0.05$) increased as compared to the vehicle control group. The severity of aspirin-induced gastric ulcers was significantly ($P < 0.05$) reduced by pretreatment with EEVS or omeprazole. However, in omeprazole-pretreated group, maximum inhibition was observed, which was similar to the 400 mg/kg EEVS.

3.5. Effects of EEVS on antioxidant activities of SOD and GP_X and MDA level in gastric tissue homogenate

The effects of EEVS on activities of SOD and GP_X and MDA level in gastric tissue homogenate of aspirin plus pylorus-ligated rats are presented in Table 2. The activities of SOD and GP_X were significantly ($P < 0.05$) reduced by aspirin administration compared with the vehicle control. In contrast, SOD and GP_X activities were significantly increased ($P < 0.05$) by pretreatment with EEVS or omeprazole. MDA level in rat stomach was increased in the aspirin-induced ulcer group. In contrast, EEVS- or omeprazole-pretreated group showed significant ($P < 0.05$) reduction in MDA level in comparison to ulcer control group. The effects of EEVS on the estimated antioxidant indices were dose-dependent ($P < 0.05$).

On the other hand, pretreatment with EEVS caused an increase in activities of SOD and GPx, as well as a reduction in MDA level in comparison to omeprazole ($P > 0.05$).

3.6. Effects of EEVS on gastric juice volume and pH

The effects of EEVS on gastric juice volume and pH are reported in Fig. 1A and 1B respectively. The gastric juice volume was significantly increased ($P < 0.05$) in the ulcer control group with concomitant decrease in pH when compared to the vehicle control group. The group pretreated with 200 mg/kg EEVS produced a significant decrease ($P < 0.05$) in gastric juice volume but no significant increase in pH compared to the ulcer control group. However, a significant decrease ($P < 0.05$) in gastric volume with corresponding increase in pH was observed in groups pretreated with omeprazole (20 mg/kg) or 400 mg/kg EEVS in comparison to the ulcer control group. Meanwhile, the group administered with 200 mg/kg EEVS produced a significant ($P < 0.05$) increase in gastric juice volume with corresponding decrease in pH as compared to the omeprazole group.

3.7. Effects of EEVS on total acidity and gastric acid output

The effects of EEVS on total acidity and gastric acid output are shown in Fig. 2A and 2B, respectively. Total acidity and acid output were significantly increased ($P < 0.05$) in the ulcer control group compared with vehicle control group. Omeprazole produced a significant decrease ($P < 0.05$) in total acidity and acid output as compared with the ulcer control. A significant ($P < 0.05$) and dose-dependent ($P < 0.05$) decrease in total acidity and acid output were observed in the EEVS-pretreated groups in comparison to ulcer control group. On the other hand, the group pretreated with 200 mg/kg EEVS showed a significant ($P < 0.05$) increase in total acidity and acid output as compared with the omeprazole group.

Table 1
Effects of EEVS on ulcer index and ulcer inhibition in aspirin plus pylorus ligation-induced ulcerated rats.

Group	Treatment and dose	Ulcer index	Ulcer inhibition (%)
1 (Vehicle)	Tween 80 (2 mL/kg)	0.00 ± 0.00	100.00
2 (Ulcer control)	Tween 80 (2 mL/kg) + aspirin (200 mg/kg)	14.60 ± 1.73*	0.00
3 (Standard)	Omeprazole (20 mg/kg) + aspirin (200 mg/kg)	1.80 ± 0.95#	87.67
4 (Test)	EEVS (200 mg/kg) + aspirin (200 mg/kg)	3.50 ± 1.08#	76.03
5 (Test)	EEVS (400 mg/kg) + aspirin (200 mg/kg)	2.10 ± 0.89#	85.62

All values are expressed as mean ± standard error of mean ($n = 5$ in each group). *$P < 0.05$, *vs* vehicle control group; #$P < 0.05$, *vs* ulcer control group. EEVS: ethanolic seed extract of *Vigna subterranean*.

Table 2
Effects of EEVS on gastric tissue activities of SOD, GP_X and MDA level in aspirin plus pylorus ligation-induced ulcerated rats.

Group	Treatment and dose	SOD (U/mg protein)	MDA (μmol/g protein)	GP_X (μmol/mg protein)
1 (Vehicle)	Tween 80 (2 mL/kg)	26.50 ± 0.24	134.70 ± 3.80	1.39 ± 0.34
2 (Ulcer control)	Tween 80 (2 mL/kg) + aspirin (200 mg/kg)	13.10 ± 0.48*	218.40 ± 5.63*	0.97 ± 0.69*
3 (Standard)	Omeprazole (20 mg/kg) + aspirin (200 mg/kg)	29.30 ± 0.83#	132.50 ± 4.02#	1.43 ± 0.05#
4 (Test)	EEVS (200 mg/kg) + aspirin (200 mg/kg)	21.80 ± 1.07#	140.30 ± 3.59#	1.30 ± 0.87#
5 (Test)	EEVS (400 mg/kg) + aspirin (200 mg/kg)	27.20 ± 0.59#	135.70 ± 2.11#	1.37 ± 0.60#

All values are expressed as mean ± standard error of mean ($n = 5$ in each group). *$P < 0.05$, *vs* vehicle control group; #$P < 0.05$, *vs* ulcer control group. SOD: superoxide dismutase; GPx: glutathione peroxidase; MDA: malondialdehyde; EEVS: ethanolic seed extract of *Vigna subterranean*.

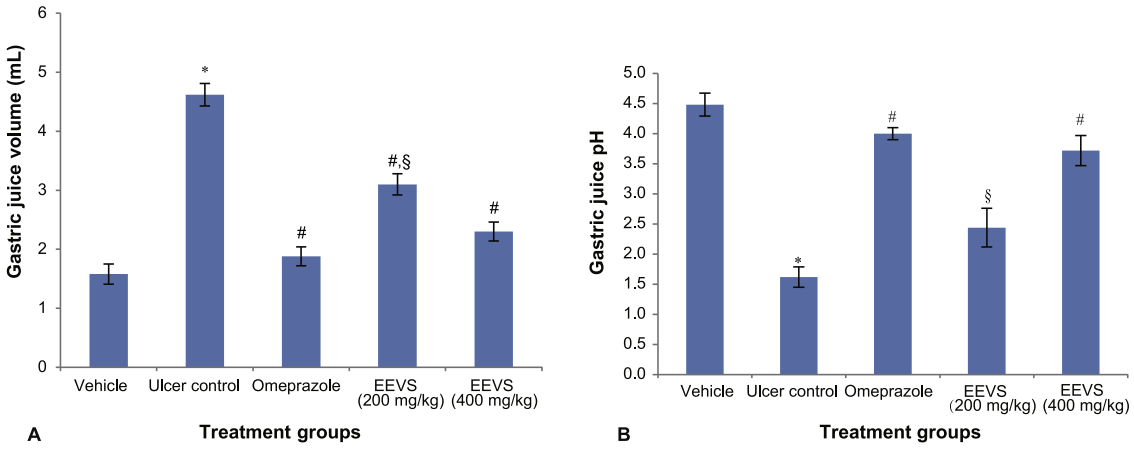

Fig. 1. Effects of EEVS on gastric juice volume (A) and pH (B) after pylorus ligation. Values are expressed as mean ± standard error of mean (n = 5 in each group). $^{*}P < 0.05$, *vs* vehicle control group; $^{\#}P < 0.05$, *vs* ulcer control group; $^{\S}P < 0.05$, *vs* omeprazole group. EEVS: ethanolic seed extract of *Vigna subterranean*.

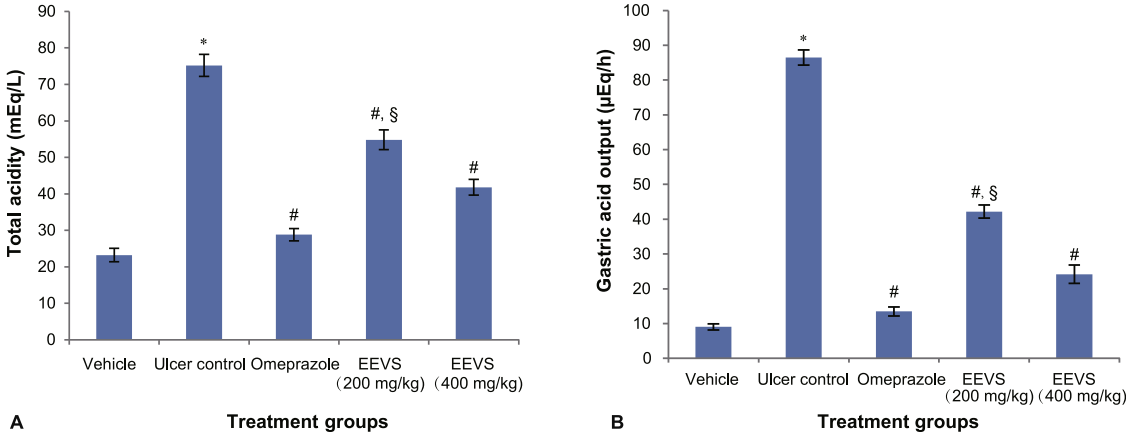

Fig. 2. Effects of EEVS on total acidity (A) and output (B) of gastric juice after pylorus ligation. Values are expressed as mean ± standard error of mean (n = 5 in each group). $^{*}P < 0.05$, *vs* vehicle control group; $^{\#}P < 0.05$, *vs* ulcer control group; $^{\S}P < 0.05$, *vs* omeprazole group. EEVS: ethanolic seed extract of *Vigna subterranean*.

3.8. Effects of EEVS on gastric mucus secretion

The effects of EEVS on gastric mucus content are shown in Fig. 3. Aspirin treatment decreased the gastric wall mucus significantly ($P < 0.05$) in the ulcer control group compared to the vehicle control group. Pretreatment with EEVS replenished the depleted gastric mucus significantly ($P < 0.05$). Also it was found that in the 200 and 400 mg/kg EEVS-pretreated groups, the amount of gastric mucus in the aspirin plus pylorus ligation-induced rats increased significantly ($P < 0.05$) in a dose-dependent manner ($P < 0.05$). Similarly, gastric mucus content in the omeprazole group was significantly increased ($P < 0.05$) as compared to the ulcer control group. Interestingly, pretreatment with 400 mg/kg EEVS produced a significant increase ($P < 0.05$) in gastric mucus secretion in comparison to the omeprazole group.

3.9. Effects of EEVS on macroscopic appearance of the gastric mucosa

In Fig. 4, the vehicle control group showed normal gastric mucosal architecture. Ulcer control group showed severe gastric mucosal injuries with extensive haemorrhagic necrosis of the gastric mucosa (white arrow). EEVS-pretreated groups (200 and 400 mg/kg) exhibited a fairly protected mucosa in aspirin plus

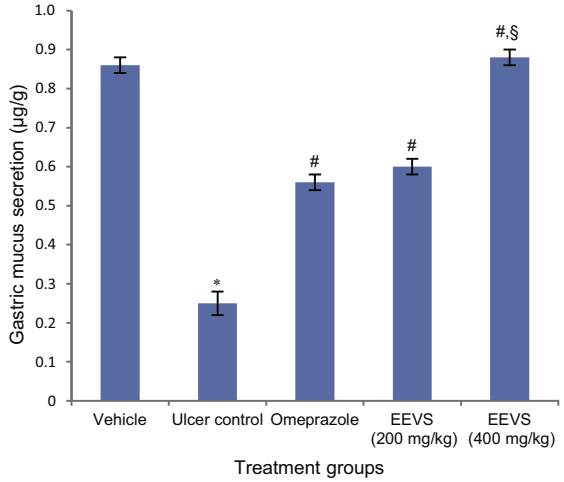

Fig. 3. Effects of EEVS on gastric mucus secretion in aspirin plus pylorus ligation-induced ulcerated rats. Values are expressed as mean ± standard error of mean (n = 5 in each group). $^{*}P < 0.05$, *vs* vehicle control group; $^{\#}P < 0.05$, *vs* ulcer control group; $^{\S}P < 0.05$, *vs* omeprazole group. EEVS: ethanolic seed extract of *Vigna subterranean*.

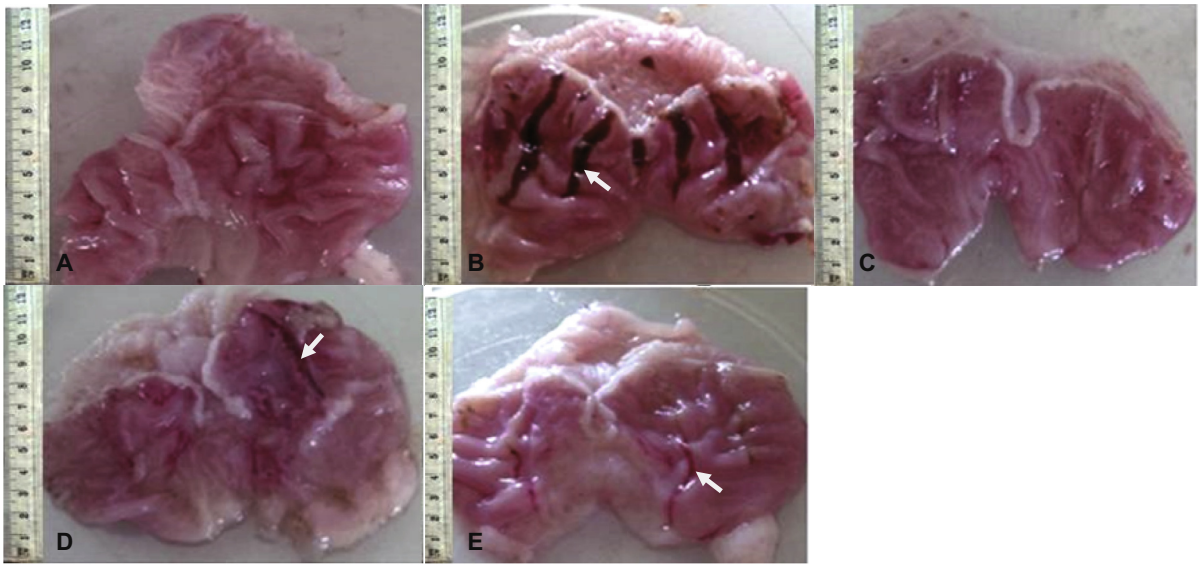

Fig. 4. Gross appearance of the gastric mucosa in aspirin plus pylorus ligation-induced ulcerated rats. A: Vehicle control group; B: Ulcer control group; C: Omeprazole group; D: EEVS (200 mg/kg) group; E: EEVS (400 mg/kg) group. White arrows point to the gastric mucosal injuries. EEVS: ethanolic seed extract of *Vigna subterranean*.

pylorus ligation model compared to the injuries seen in the ulcer control rats.

3.10. Histological evaluation of gastric lesions

In Fig. 5, the vehicle control group showed normal mucosal epithelium (yellow arrow) and submucosal layer (black arrow). Histological evaluation of aspirin plus pylorus ligation-induced rats showed severe disruption of the epithelial surface with deep penetration of necrotic lesions into the mucosa (yellow arrow) and presence of edema of the submucosa with leucocytes infiltration (black arrow). In the omeprazole group, disruption of epithelial surface was mild but there was no deep mucosal damage, edema of the submucosa and leucocytes infiltration (Fig. 5). Animals pretreated with EEVS (200 and 400 mg/kg) had better gastric mucosal protection which is manifested through mild disruption to the epithelial surface with reduced or absence of submucosal edema and leucocytes infiltration.

4. Discussion

There is increasing attention on the prevention and management of gastrointestinal diseases including gastric ulcer through identification and evaluation of drugs from natural products as an alternative therapeutic approach. The current study employed aspirin plus pylorus ligation-induced ulcer model to investigate the possible gastroprotective effects of EEVS. This model represents some of the most common causes of gastric ulcer in humans, and demonstrated that EEVS possesses significant gastroprotective activity against aspirin-induced gastric lesion in rats. To the best

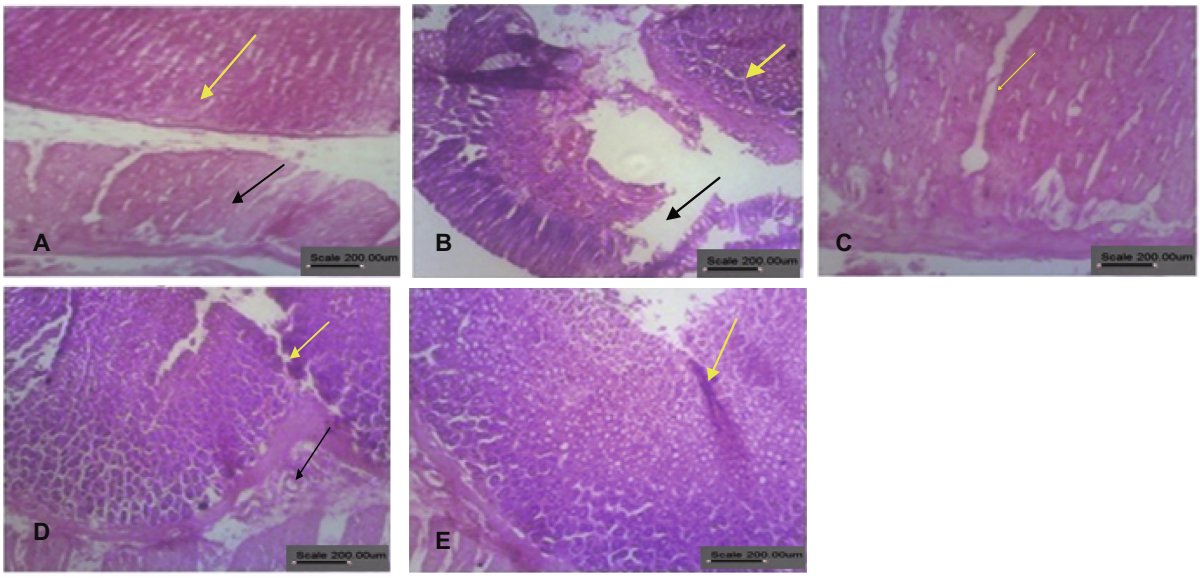

Fig. 5. Histological evaluation of aspirin plus pylorus ligation-induced gastric mucosal damage in rats (H&E stain, ×40 magnifications). A: Vehicle control group; B: Ulcer control group; C: Omeprazole group; D: EEVS (200 mg/kg) group; E: EEVS (400 mg/kg) group. Yellow and black arrows point to the gastric mucosal epithelium and submucosal layer respectively. EEVS: ethanolic seed extract of *Vigna subterranean*.

of our knowledge, this is the first report to show that EEVS is capable of ameliorating aspirin plus pylorus ligation-induced gastric mucosal ulceration in rats. At low and high doses, the extract offered 76.03% and 85.62% mucosal protection respectively against aspirin plus pylorus ligation-induced ulcerations. Interestingly, both doses of extract showed an increase in gastric mucus secretion over omeprazole. This is an indication that EEVS has better therapeutic activity than omeprazole. However, the mechanisms behind these gastroprotective effects of EEVS in rats have not yet been ascertained.

The pathogenic effects of aspirin plus pylorus ligation-induced lesions have been established to include increased secretion of gastric acid, which resulted in increased gastric volume, reduced pH, and increased total acidity, acid output and ulcer index [46]. In pylorus ligation, gastric blood flow is altered by stasis of gastric contents [47]. The autodigestion of the mucosal wall by accumulated gastric juice causes mucosal barrier breakdown leading to gastric ulcer formation [48]. Findings from this study showed that in aspirin plus pylorus ligation-induced rats, the EEVS-pretreated groups (200 and 400 mg/kg) produced a significant decrease in acid output, total acidity, and volume of gastric content with a corresponding increase in pH compared to the ulcer control group. This decrease in gastric juice volume in the extract-pretreated groups may be due to a decrease in acid production as evidenced from the gastric juice total acidity. This is an indication that EEVS contains some biological compounds that reduce the acidity of gastric secretions which had been increased by aspirin plus pylorus ligation.

Aspirin, which is an example of NSAID, produces gastric mucosal ulceration by irreversibly inhibiting the cyclooxygenase enzyme that synthesizes endogenous prostaglandins, which protect the gastric mucosa. Prostaglandins, especially prostaglandin E2 and prostaglandin I2, enhance secretion of bicarbonate and mucus, sustain blood flow of the gastric mucosa, and regulate turnover and repair of mucosal epithelial cells. Therefore, inhibition of the synthesis of prostaglandin will lead to susceptibility of the gastric wall to injury and ulceration [49]. Agents that inhibit the effects of aspirin will exhibit cytoprotection. From this study, the results showed that EEVS can significantly prevent the gastric damaging effects of aspirin by protecting the mucosa with a significant decrease in ulcer index, and an increase in mucus production in comparison to the ulcer control group. This observed cytoprotection offered by the extract may be mediated by strengthening of the mucosal barrier (gastric mucus restoration) through increase in prostaglandin synthesis, enabling it to resist the toxic effects of aspirin.

Previous studies have implicated oxidative stress in pathogenesis of gastric mucosal ulceration [50,51]. It was observed in the present study that gastric mucosal ulceration via oxidative stress was induced by oral administration of aspirin, since it evokes lipid peroxidation and affects antioxidative enzyme activities in the gastric tissue homogenates. This is signified by the increase in the level of total antioxidant and activities of SOD, GP_X, and markedly attenuated the increase in MDA level following treatment with EEVS which was overwhelmed by aspirin-induced oxidative stress. This is consistent with several reports both *in vitro* [52] and *in vivo* [53], which have shown that the antioxidant effect of *V. subterranea* seed extracts is potent. The antioxidant activity of EEVS is due to its strong scavenging effect on reactive oxygen species and free radicals [27]. Antioxidants have been reported to protect the gastric mucosa against different ulcerogens [51]. Antioxidants protect cells from oxidative damage while improving the body's immune system against degenerative diseases. Large amounts of antioxidant compounds have been reported to be present in the seeds of *V. subterranea* [27]. The active compounds flavonoids and glycosides which are present in the seeds have been reported by Mota et al. [52] and Gill and Bali [53] as antioxidant materials. Hence, it is speculated that the antioxidant potential of this extract, which offers a first line of defence against any ulcerogenic agent by bolstering the mucosal defence system, may be attributed to its gastroprotective activity.

Histological evaluation revealed that the gastric mucosa is protected and infiltration of leucocytes into the submucosa is inhibited or reduced in rats pretreated with EEVS. The gastric mucosa is otherwise extensively damaged by aspirin, leading to increased infiltration of neutrophils into the submucosa. Inflammatory mediators are mainly formed by neutrophils that can release free radicals, which are harmful to cells and tissues [54]. According to Hajrezaie et al. [55], a decrease in infiltration of neutrophils into ulcerated gastric tissue enhances gastric ulcer healing in rats. Gastric ulcer healing in rats is inhibited by oxygen-free radicals released by neutrophils that infiltrated into ulcerated gastric tissues [56]. This inhibition of leucocyte infiltration by EEVS into the submucosa suggests that the extract may possess anti-inflammatory properties, which could also play a role as reported by Bhattacharyya et al. [57] in gastric ulcer prevention.

EEVS has been shown to contain phytochemicals such as flavonoids, glycosides and terpenoids which are among the cytoprotective materials known to possess antiulcer [46,52], antioxidant [53] and anti-inflammatory [58] activities, respectively. It is suggested that these secondary metabolites can enhance bicarbonate, mucus and prostaglandin secretion and attenuate the damaging effects of free radicals in the gastrointestinal lumen [59]. Therefore, it is pertinent that in this study, the EEVS decreased gastric lesions as well as volume and acidity of gastric fluid, and increased gastric mucus secretion, all of which could be due to the presence of these biological compounds or some other mechanisms yet to be unravelled. Hence, further studies are required to establish the exact mechanism of action and isolate the active ingredients in the seeds responsible for the observed gastroprotective effects so as to provide new alternatives for the clinical management of gastric ulcers.

In conclusion, the study suggests that EEVS possesses significant gastroprotective effects against aspirin plus pylorus ligation-induced gastric lesion in rats. The observed gastroprotective effect might be possibly due to its antisecretory, cytoprotective, and antioxidative properties. Our findings may have beneficial application in the management of gastric mucosal lesions associated with aspirin plus pylorus ligation-induced gastric ulceration.

Competing interests

The authors declare no conflict of interest.

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