Ulcero-protective potential of ethyl acetate fractions of *Persea americana* seed and *Bryophyllum pinnatum* leaf binary combinations in indomethacin induced gastric ulcer

ABSTRACT

Aims: This study was aimed at investigating the protective potential of ethyl acetate fractions of *Persea americana* (PA) seed and *Bryophyllum pinnatum* (BP) leaf binary combinations in indomethacin-induced gastric ulcers.

Methodology: Fifty (50) male Wistar rats were used in this study; they were assigned into 10 groups of five animals each and respective groups received a standard rat diet and drinking water *ad libitum*. The groups were the normal control group (NC), ulcer control (UC), Omeprazole, 20mgkg^{-1} (OMEP), PA 400 mgKg^{-1} body weight, and BP 400 mgKg^{-1} body weight. Groups receiving binary combinations were (PA + BP, 1:1), (PA + BP, 1:2), (PA + BP, 1:3), (PA + BP, 2:1), (PA + BP, 3:1) each pre-treated with 400 mgKg^{-1} body weight/day of respective mixture by intubation for 21 days. On the 22nd day after overnight fasting, a gastric ulcer was induced with indomethacin (30mg/kg body weight) by intubation in a single dose. Ulcer markers, and histopathology were measured using standard methods.

Results: The present study revealed that the ethyl acetate fraction of the plants PA seeds and BP leaf possess potent gastric tissue protective effects. Analysis of gastric changes indicated a significant ($P<0.05$) reduction in ulcer index, gastric acid output, gastric mucus content and pepsin activity of ulcerated rats pretreated with PA and BP fractions. The effect of the binary combinations was significantly ($p<0.05$) higher than single plant fractions. The protective effect of the fractions was in the order OMEP > PA+BP (3:1) > PA+BP (2:1) > PA+BP (1:1), > PA+BP (1:2) > PA+BP (1:3) > PA > BP. The observed ulcero-protective effect of the binary combinations of *P. americana* seed and *B. pinnatum* leaf may be attributed to the synergy of the phytochemicals contained in the fractions.

Conclusion:

These findings suggest that *P. americana* and *B. pinnatum* ethyl acetate fraction combination favorably protect against indomethacin-induced gastric mucosal oxidative damage, reducing ulcer index, gastric acid output, gastric mucus content and pepsin activity. The ethyl acetate fractions exhibited a dose-dependent inhibition of ulcer formation in indomethacin gastric ulcer model.

Keywords: P. americana, B. pinnatum, indomethacin, gastric ulcer, ulcer index

1. INTRODUCTION

Gastric/stomach ulcers (GU) and duodenal ulcers form the two most common types of peptic ulcers. Gastric and duodenal ulcers are common digestive tract diseases highly prevalent in every part of the world. They cut across age barriers and social class. The prevalence of peptic ulcer disease and gastric cancer in Africa has been reported [1]. These disorders involve inflammation and ulceration of the digestive tract; and occupy a place only secondary to carcinoma in the field of gastroenterology [2, 3]. “However, gastric ulcer is the most prevalent gastrointestinal disorder known, affecting more than 10% of people and accounting for an estimated 15,000 mortality yearly” [4]. “The annual global incidence of GU perforation and hemorrhage are estimated at 3.8–14.0 and 19.4–57.0 per 100,000 persons, respectively” [5]. “The statistics for gastric ulcer disease in other countries are variable and are hinged primarily on the major causes of the disease, *Helicobacter pylori,* and non-steroidal anti-inflammatory drugs (NSAID)” [6].

Several factors have also been implicated in the pathogenesis of gastric ulcerations including sedentary lifestyle, alcohol intake, spicy food, NSAID, and various bacterial infections [7, 8]. Factors such as interleukin-1β (IL-1β), matrix metalloproteinases (MMP), and tumor necrosis factor-α (TNF-α) [9, 10] and reactive oxygen species (ROS) and reactive nitrogen species (RNS) are strongly linked to gastric ulcer [11, 12]. Homeostatic changes such as the generation of nitric oxide (NO) from the endothelial nitric oxide synthase (e-NOS) and growth factors are important in ulcer healing. They promote angiogenesis, regulate gastric mucosal blood flow, and stimulate gastric mucus secretion [13].

There is no single effective management therapy for gastric ulcers, therefore a combination of treatment strategies are applied depending on the severity, identified causative agent, and presenting symptoms [14]. "Current treatments have brought about remarkable changes in gastric ulcer recovery, but the efficacy is still debatable; there are incidences of relapses, adverse effects, and the danger of drug interactions during ulcer therapy. Hence, the search for an ideal anti-ulcer drug continues and has also been extended to medicinal plants/herbs in search of new and novel molecules, which may afford better protection and decrease the incidence of relapse. Plants have been identified as an important source of phytoactive compounds with verifiable medicinal properties and protective potential against toxicity" [15].

"*Persea americana* Mill. and *Bryophyllum pinnatum* are plants widely known in the tropics for their food and medicinal value. *P. americana* Mill. is an evergreen tree native to the Caribbean, commonly known as avocado and ube-beke, orewépia, and ganyen piya by Igbo, Yoruba, and Hausa tribes of Nigeria" [16]. "The reported medicinal properties of different parts of *P. americana* include anti-inflammatory, antihypertensive, anti-ulcer, hypoglycaemic and hypercholesterolaemic, and parasitic skin diseases treatment" [17, 18, 19]. "*B. pinnatum* is a member of the Crassulaceae family, indigenous to Madagascar, but largely distributed with dominance in the rainforest belt and tropical countries. They are known by names such as "air plant", "love plant", "miracle leaves", and "life plant", all attributable to their identified characteristics" [20]. "*B. pinnatum* is known as 'odaa opue', and 'ewe abamoda' or 'odundun' among the Igbos and Yorubas of South-eastern and western Nigeria respectively" [21]. "The Crassulaceae family has been widely investigated due to their xeromorphic and medicinal properties. They are ethnopharmacologically applied in the management of gastritis and bacterial infection and many diseases" [22, 23]. "Chemical composition, hepatoprotective activity, anti-inflammatory effects, anti-ulcer activity, and free radical scavenging activities have also been reported" [24-27].

Furthermore, the seeds of *P. americana* and leaf of *B. pinnatum* have been widely touted to be wonder cures by traditional practitioners; and applied locally in the management of gastric ulcers. It is therefore important to verify these claims to create public awareness and ensure best practices. *P. americana* seed and *B. pinnatum* leaf may have been studied for antidiabetic, hypotensive, and anti-inflammatory properties; but a comprehensive study on their ulcero-protective effect using binary combinations has not been reported. It is necessary to study the combinatorial effect of these plants which could enhance their effectiveness. The main objective of this study is to evaluate the ulcero-protective properties of ethyl acetate fractions of the binary combination of *P. americana* seed and *B. pinnatum* leaf in indomethacin-induced gastric ulcers.

2. MATERIAL AND METHODS

2.1 Chemicals/Reagents

Indomethacin (Sigma-Aldrich Mo USA), Ethanol (JHD, China), ethyl acetate (JHD, China), Sodium dihydrogen orthophosphate (JHD, China), Disodium hydrogen orthophosphate (JHD, China), Omeprazole extended-release capsules (Sanofi-aventis, Switzerland). All other chemicals and reagents used were of analytical grade.

2.2 Plant Materials

Fresh leaves of the plant *B. pinnatum* were collected from the bush around the Department of Biochemistry, Federal University of Technology Owerri, Nigeria, while fresh fruits of *P. americana* were harvested from a farm in Ugiri-ike Autonomous community, Ikeduru L.G.A. Imo State. The plant materials were authenticated by Prof. F. N. Mbagwu a plant taxonomist at the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. Plant specimens were deposited in the institution herbarium with voucher numbers IMSUH 0225 and IMSUH 0226 respectively.

2.3 Experimental animals

Fifty (50) apparently healthy male Wistar rats (*Rattus norvegicus*) weighing 80-120g (averaging 8 weeks old) were used in this study. They were purchased from the animal house of the Department of Veterinary Medicine, University of Nigeria, Nsukka, and housed in stainless steel cages under standard laboratory conditions of light, temperature (25±2^{o}C), and relative humidity (55 ± 5%). The animals were fed standard rat pellets (Vital finisher, Nigeria) and portable water *ad libitum*.

2.4 Preparation of ethyl acetate fraction

The fresh leaves of the plants were shed, sorted, and washed, while the fresh seeds were collected by carefully cutting the fruits open; the seeds were peeled and cut into cubes for easy drying. Both samples were air-dried at 30^{0}C, and reduced to a coarse powder in a mill (Kenwood BL357). Each of the plant powders (500g) was extracted with 2 L of 80% ethanol using a Soxhlet extractor. The ethanol extract was further partitioned between ethyl acetate and water to recover the ethyl acetate soluble component of the extract. The ethyl acetate fraction was recovered by distillation under reduced

pressure at 49^{o}C in a Buchi rota vapor (Switzerland), then dried to solid forms in vacuum desiccators, and stored in a freezer (≤ 4.0 ^{0}C) until when needed.

2.5 Experimental design

The ulcero-protective study of each ethyl acetate fraction was carried out in ten (10) groups of five (5) animals each grouped according to body weight (80-120mg/kgbwt.). The groups and administration regimen were organized as follows:

Group 1: Group 1 served as the normal control group (NC). They received a standard rat diet and drank water *ad libitum* for 21 days. On the 22nd day, the animals were given indomethacin vehicle distilled water.

Group 2: Group 2 served as ulcer control (UC). Ulcer control group received a standard rat diet and drank water *ad libitum* for 21 days. On the 22nd day after overnight fasting, animals in this group were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

Group 3: Omeprazole standard group (OMEP) received a standard rat diet and drinking water *ad libitum* and pre-treatment with 20mg/kg body weight of Omeprazole by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

Group 4: *P. americana* group (PA) received a standard rat diet and drinking water *ad libitum* and pre-treatment with 400mg/kg body weight of *P. americana* ethyl acetate fraction by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with Indomethacin 30mg/kg body weight by intubation in a single dose.

Group 5: *B. pinnatum* group (BP) group received a standard rat diet and drinking water *ad libitum* and pre-treatment with 400mg/kg body weight of *B. pinnatum* ethyl acetate fraction by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

Group 6: *P. americana* + *B. pinnatum* group (PA + BP, 1:1). This group received a standard rat diet and drinking water *ad libitum* and pre-treatment with 400mg/kg body weight of *P. americana* + *B. pinnatum* ethyl acetate fraction (50%: 50% (1:1)) combination by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

Group 7: *P. americana* + *B. pinnatum* group (PA + BP, 1:2). This group received a standard rat diet and drinking water *ad libitum* and pre-treatment with 400mg/kg body weight of *P. americana* + *B. pinnatum* ethyl acetate fraction (33%: 67% (1:2)) combination by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

Group 8: *P. americana* + *B. pinnatum* group (PA + BP, 1:3). This group received a standard rat diet and drinking water *ad libitum* and pre-treatment with 400mg/kg body weight of *P. americana* + *B. pinnatum* ethyl acetate fraction (25%: 75% (1:3)) combination by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

Group 9: *P. americana* + *B. pinnatum* group (PA + BP, 2:1). This group received a standard rat diet and drinking water *ad libitum* and pre-treatment with 400mg/kg body weight of *P. americana* + *B. pinnatum* ethyl acetate fraction (67%: 33% (2:1)) combination by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

Group 10: *P. americana* + *B. pinnatum* group (PA + BP, 3:1). This group received a standard rat diet and drinking water *ad libitum* and pre-treatment with 400mg/kg body weight of *P. americana* + *B. pinnatum* ethyl acetate fraction (75%: 25% (3:1)) combination by intubation for 21 days. On the 22nd day after overnight fasting, they were treated with indomethacin (30mg/kg body weight) by intubation in a single dose.

2.6 Sacrificing, Isolation of the stomach, and collection of gastric juice

On the twenty-second day (4 hrs post ulcer induction), the animals were humanely sacrificed under light anesthesia with dichloromethane. The animal stomach was carefully excised; thereafter opened along the greater curvature and gastric content was drained into a centrifuge tube. Ten milliliters of distilled water was added and the resultant solution was

centrifuged at 3000 rpm for 10 min. The supernatant obtained was used for biochemical analyses. The cleaned stomach tissues were preserved in ice-cold 0.1M phosphate buffer saline (1:4 (w/v), pH 7.4) before macroscopic examination.

2.7 Determination of ulcer index

The ulcer index was determined using the method described by Goyal [28]. The excised rat stomach tisseues was carefully opened along the greater curvature. Debris was cleaned with a running stream of water. The stomach tissue was then stretched out on cardboards, with the luminal surface facing up. The ulcer index was calculated from the glandular portion of the stomach, with the aid of a magnifying glass and measuring tape.

The ulcer index was calculated as:

Ulcer index = $\frac{10}{x}$

Where x= $\frac{Total\ mucosal\ surface}{Total\ ulcerated\ area}$

Measurement rules: ulcer lesions were measured along the greatest length. Five identified petechial were considered equivalent to 1 sq-mm of ulcer area. The total area of the glandular portion of the stomach and that of ulcerated mucosa were measured and used for the determination of the ulcer index [28].

2.8 Determination of free acidity, total acidity, and gastric pH

Free and total acidity were determined in the gastric content according to the method described by Kulkarni and Varghese [29]. The gastric content was collected, and made up to 10 ml with sterile deionized water. This was centrifuged and the supernatant was taken in a conical flask and titrated with 0.01N NaOH to the yellow endpoint using 100 µl Topfer's reagent as indicator. Furthermore, 100 µl phenolphthalein was added and titration continued till the phenolphthalein endpoint was reached. The amount of 0.01N NaOH required to titrate to the yellow end point was used to calculate the free acidity; the titre value for the use of both Topfer's reagent and phenolphthalein was used to determine the total acidity. The acidity was calculated by the following formula and expressed in mmol/L [29]. The pH of gastric juice was determined using a pH meter.

$$Acidity = \frac{Volume\ of\ NaOH \times Normality \times 100 mmol/L}{0.1}$$

2.9 Determination of gastric mucus content

The determination of gastric mucus content was carried out according to the method described by Corne *et al.* [30]. The excised stomach tissues were soaked in 0.05M sodium acetate buffered 0.1% alcian blue solution (pH 5.0). Excess uncomplexed dye was removed by washing with 0.025M sucrose and subsequently tissue was soaked in 0.1M $MgCl_2$. The blue solution obtained was extracted in ether and optical density was measured at 605nm. The mucin content of the sample was determined from the standard curve of alcian blue which was expressed in µg alcian blue/g tissue [30].

2.10 Determination of pepsin activity

Pepsin activity was determined according to the method of Debnath *et al.* [31] and Lowry *et al.* [32]. Briefly, about 1ml of diluted gastric juice was mixed with 0.5ml 2% hemoglobin solution in 0.06 M hydrochloric acid; and incubated for 20 mins at room temperature. The set up was precipitated with 1ml of 0.5M trichloroacetic acid, and centrifuged at 4000rpm for 10mins. Then 1.0ml of the supernatant was mixed with 1.0ml of alkaline copper sulfate solution and 0.5ml of dilute Folin-Denis reagent and incubated for 30 min at room temperature. The absorbance of the samples was determined by spectrophotometry at 610 nm. The gastric pepsin activity was expressed in µmol/L.

2.11 Determination of total nitrite contents in gastric mucosal tissues

Total nitrite content was determined in the homogenates as described by Green *et al.* [33]. A 1ml portion of the supernatant was mixed with 1ml of Griess reagent (consisting 0.5ml portion of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED) and 0.5ml of 1% sulfanilamide in 2.5% phosphoric acid). The mixture was incubated for 10 min at room temperature in the dark, and the absorbance was measured at 540 nm. The concentration of nitrite in sample was determined using a standard calibration curve and expressed in mmol/L.

2.12 Histological studies of stomach tissues

The method described by Okoro [34] was used with minor modifications.

2.13 Statistical Analysis

The study was carried out using a complete randomized design. Results of data generated were analyzed using analysis of variance with a statistical package for social sciences (version 25). Statistical significance of values was considered at $p<0.05$ using the turkey and Duncan homogeneity of variance test. Results are presented as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on gastric mucosa protection in rats

Figure 1a shows the effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on ulcer index in indomethacin-induced gastric ulcers in male albino rats. Result obtained from the study (Figure 1a) shows that indomethacin administration significantly ($p \leq 0.05$) increased the ulcer index when compared to the standard drug (omeprazole). The ethyl acetate fractions of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction and their combinations resulted in a varying degree of significant ($p<0.05$) reduction in ulcer index. The ulcer index of animals that received the ethyl acetate fraction combinations of PA+BP (1:1), PA+BP(1:2), PA+BP (1:3), PA+BP (2:1), and PA+BP (3:1) were significantly ($p<0.05$) reduced ulcer index when compared to those of groups receiving PA and BP. However, the ethyl acetate fraction combinations of PA+BP (2:1) and PA+BP (3:1) effectively reduced ulcer index but were not normalized to the same level as control or the standard drug omeprazole.

Figure 1b shows the percentage ulcer protective effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration in indomethacin-induced gastric ulcers in male albino rats. Result obtained from the study (Figure 1b) shows that the ethyl acetate fractions of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction and their combinations administration had a significant ($p<0.05$) protective effect against induction of gastric ulcer in rats. The percentage ulcero-protection was 2.68 ± 0.91, 89.53 ± 3.27, 25.17 ± 4.62, 12.97 ± 1.57, 47.52 ± 7.49, 45.63 ± 7.08, 35.33 ± 6.07, 65.56 ± 8.82 and 74.75 ± 5.38 % for the groups UC, OMEP, PA, BP, PA+BP (1:1), PA+BP (1:2), PA+BP (1:3), PA+BP (2:1) and PA+BP (3:1) respectively. The ulcero-protective effect of the fractions binary combinations was significantly ($p<0.05$) higher when compared to the single plant fractions, but was lower than the effect of the omeprazole standard. The protective effect of the fractions was in the order Omeprazole > PA+BP (3:1) > PA+BP (2:1) > PA+BP (1:1), > PA+BP (1:2) > PA+BP (1:3) > PA > BP.

3.2 Effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on free acidity and total acidity in indomethacin-induced gastric ulcer in rats.

Figure 1c show the effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on free acidity in indomethacin-induced gastric ulcers in the male albino rat. Results obtained from the study show that induction with indomethacin caused a significant ($p<0.05$) increase in gastric acidity when compared to normal control animals. However, the administration of the ethyl acetate fractions of PA, BP, and the combinations protected against acid reflux in the gastric mucosa, reducing free acidity. But these fractions did not normalize stomach-free acidity except for the PA+BP (3:1) group. The animals receiving PA+BP (3:1) at the dosage of 400mg/Kgb.wt were able to normalize free acidity comparable to omeprazole standard and normal control.

Figure 1d show the effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on total acidity in indomethacin-induced gastric ulcers in the male albino rat. Results obtained from the study show that indomethacin caused a significant ($p<0.05$) increase in total acidity when compared to normal control animals. The administration of *P. americana* seed and *B. pinnatum* leaf fractions resulted in a reduction of total gastric acidity except for the group that received BP (400mg/Kgb.wt). Furthermore, total acidity-reducing effects were more effective in groups PA+BP (2:1) and PA+BP (3:1) respectively. Among these groups, total acidity did not significantly ($p<0.05$) differ from the normal control and standard group.

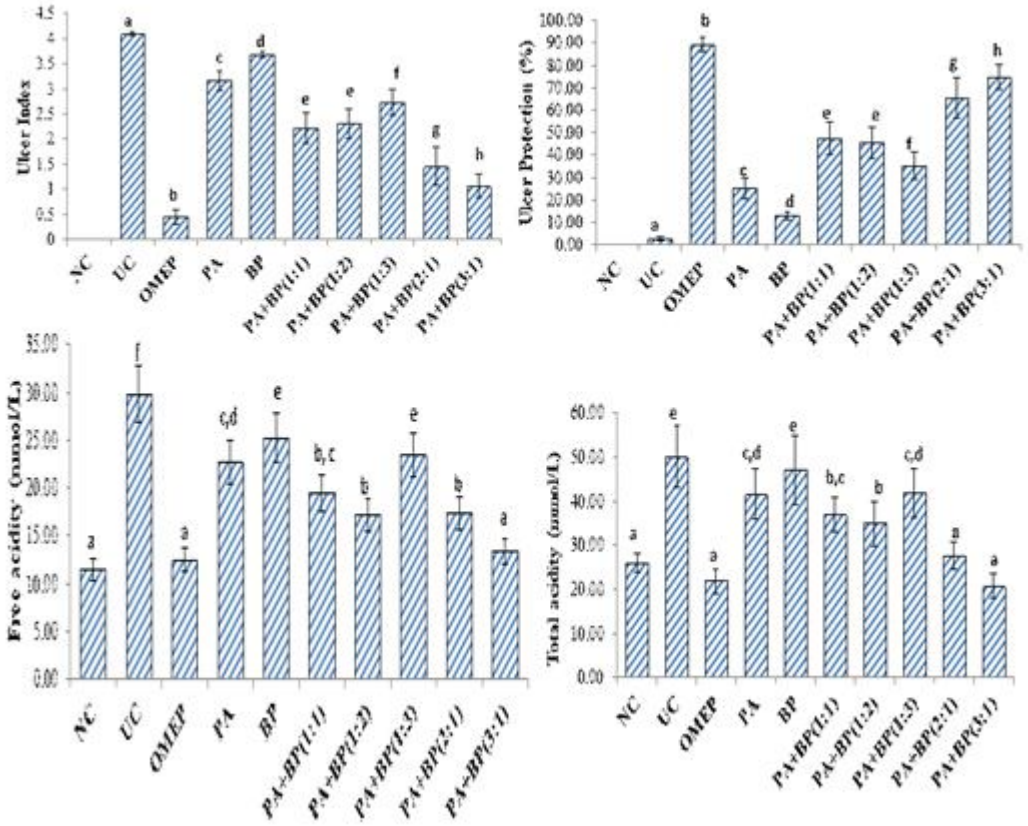

Figure 1: Effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction binary combinations administration on ulcer index, ulcer protection, free acidity, and total acidity in indomethacin-induced gastric ulcer in male albino rats. Bars are mean ± Standard of 5 determinations. Bars bearing different superscript letters across groups are significantly different ($p<0.05$).

3.3 Effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on Gastric mucus content and pepsin activity in indomethacin-induced gastric ulcer in rats.

Figure 2a show the effect of *P. americana seed and B. pinnatum* leaf ethyl acetate fraction administration on gastric mucus content in indomethacin-induced gastric ulcer in the male albino rat. The results show that indomethacin administration significantly ($p<0.05$) depleted gastric mucus content in the UC group when compared to the normal control. Administration of the ethyl acetate fractions in the combinations PA+BP (1:1), PA+BP (1:3), PA+BP (2:1), and PA+BP (3:1) showed a significant ($p<0.05$) protection of gastric mucus secretion which were comparable to OMEP and normal control.

Figure 2b show the effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on pepsin activity in indomethacin-induced gastric ulcers in male albino rats. The results presented in Figure 2b show that indomethacin administration promoted significant ($p<0.05$) release of pepsin into the stomach. The ethyl acetate fractions did not normalize pepsin activity to levels found in control, however, showed a significant reduction of pepsin secretion in animals administered the different ethyl acetate fraction and their combinations.

3.4 Effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on nitrite concentration and Gastric pH in indomethacin-induced gastric ulcer in rats.

Figure 2c show the effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction administration on nitrite concentration of stomach homogenates in indomethacin-induced gastric ulcers in male albino rats. Stomach homogenate nitrite concentration was significantly ($p<0.05$) elevated in the ulcer control, but administration of the ethyl acetate fractions in the groups OMEP, PA+BP(1:1), PA+BP(1:2), PA+BP(1:3), PA+BP(2:1) and PA+BP(3:1) significantly normalized stomach nitrite concentration. However, PA+BP (3:1) administration was the most effective.

Figure 2d show the effect of *P. americana* seed *and B. pinnatum* leaf ethyl acetate fraction administration on gastric pH in indomethacin-induced gastric ulcers in the male albino rat. The result obtained from the study indicated an increased gastric pH value in animals receiving indomethacin. However, the increases in gastric pH were not significantly ($p<0.05$) different from that of the normal control group.

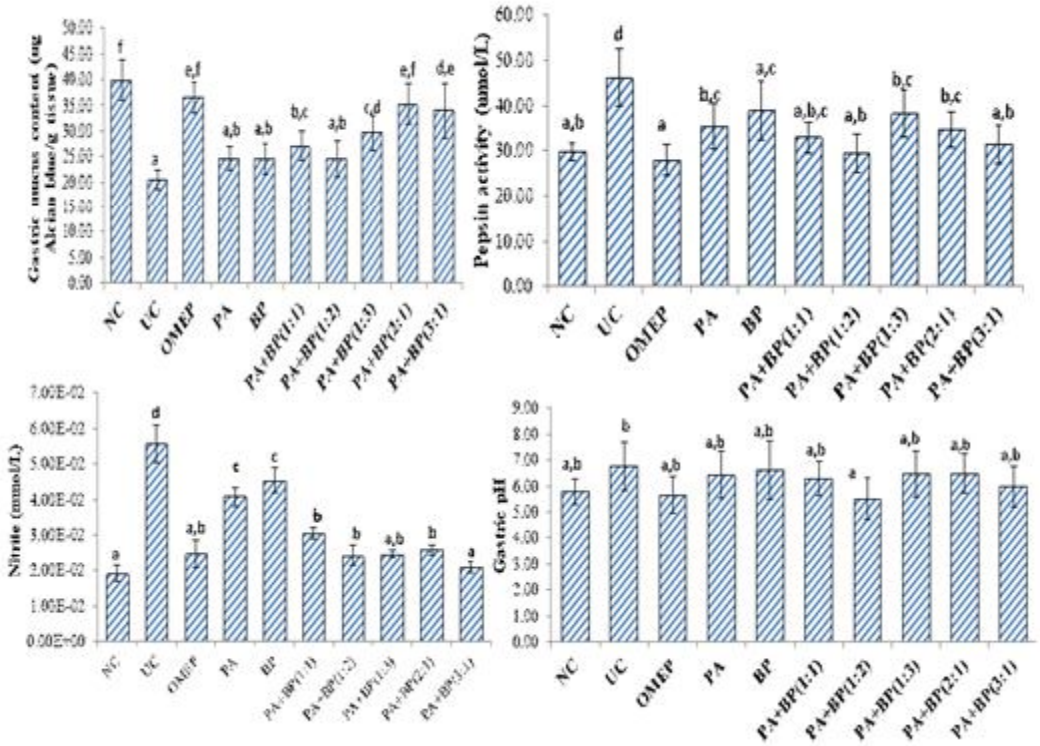

Figure 2 Effect of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction binary combinations administration on gastric mucus content, pepsin activity, nitrite concentration, and gastric pH in indomethacin-induced gastric ulcer in male albino rats. Bars are mean ± Standard of 5 determinations. Bars bearing different superscript letters across groups are significantly different ($p<0.05$).

3.5 Histological examinations of stomach sections

Histological sections (x100) of the animal stomach tissues including normal control, Ulcer control, Standard group (Omeprazole), *P. americana* (PA) 400mg/Kg bwt, *B. pinnatum* (BP) 400mg/Kg bwt, (PA + BP) (1:1) 400mg/Kg bwt, (PA + BP) (1:2) 400mg/Kg bwt, (PA + BP) (1:3) 400mg/Kg bwt., (PA + BP) (2:1) 400mg/Kg bwt and (PA + BP) (3:1) 400mg/Kg bwt. are shown in plates 1-10.

The first photomicrograph (Plate 1) shows a section of the stomach from group1 (Control) showing organized gastric epithelium or mucosa and gastric pit. Normal gastrointestinal tissue lined by both stratified squamous and columnar epithelium. The mucosa, submucosa, muscularis externa and serosa are normal.

The second photomicrograph (plate 2) represent the ulcer control group. The plate show tha the mucosa, submucosa, muscularis interna and externa are not normal; there is presence of eroded gastric epithelium (EGE), necrosis (N) and atrophy (A). However, in the group that was pre-treated with 20mg/kg body weight of omeprazole and later received Indomethacin 30mg/kg, organized gastric epithelium or mucosa and gastric pit was observed (Plate 3). The normal gastrointestinal tissue lined by both stratified squamous and columnar epithelium. The mucosa, submucosa, muscularis externa and serosa are normal.
Furthermore, the group that was pre-treated with *P. americana* (PA) 400mg/Kg bwt and later received Indomethacin 30mg/kg, (Plate 4), the plate shows organized gastric epithelium or mucosa and gastric pit. Normal gastrointestinal tissues lined by both stratified squamous and columnar epithelium were observed. The mucosa, submucosa, muscularis externa and serosa are normal. Plate 5 shows the group *B. pinnatum* (BP) 400mg/Kg bwt, they was erosion of gastric epithelium (EGE) or mucosa (M); normal submucosa (SM) and muscularis interna (showing a haemorrhagic streak) is evident.

The sixth photomicrograph (plate 6) represent the group that was pre-treated with (PA + BP) (1:1) 400mg/Kg bwt and later received indomethacin 30mg/kg. The plate show organized gastric epithelium or mucosa and gastric pit as well as normal gastrointestinal tissue lined by both stratified squamous and columnar epithelium. The mucosa, submucosa, muscularis externa and serosa are normal and are better than the group pre-treated with PA or BP only and later given Indomethacin 30mg/kg. Plate 7 show the group that was pre-treated with (PA + BP) (1:2) 400mg/Kg bwt and later received Indomethacin 30mg/kg. The plate show organized gastric epithelium or mucosa and gastric pit as well as normal

gastrointestinal tissue lined by both stratified squamous and columnar epithelium. There is presence of necrosis (N) and atrophy (A) in the submucosa and muscularis layers. In the group that was pre-treated with (P.A + B.P) (1:3) 400mg/Kg bwt and later received Indomethacin 30mg/kg, (Plate 8), the plate show presence of gastric epithelium or mucosa and gastric pit with some parts eroded.

Plate 9 show the group that was pre-treated with (P.A + B.P) (2:1) 400mg/Kg bwt and later received indomethacin 30mg/kg, (Plate 9), the Plate shows presence of eroded gastric epithelium with normal submucosa and submuscularis. Also, plate 10 show the group that was pre-treated with (P.A + B.P) (3:1) 400mg/Kg bwt and later received Indomethacin 30mg/kg the plate show normal architecture of gastric epithelium or mucosa, and gastric musculature with better presentations than those in groups 6-9.

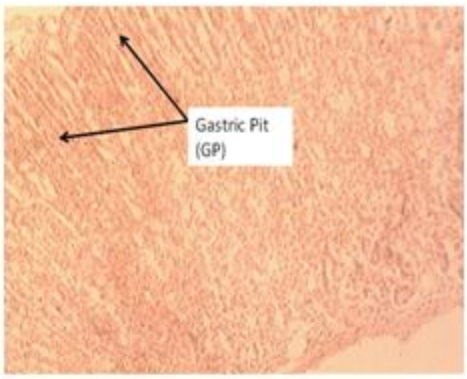

Plate 1: group 1 (Control)

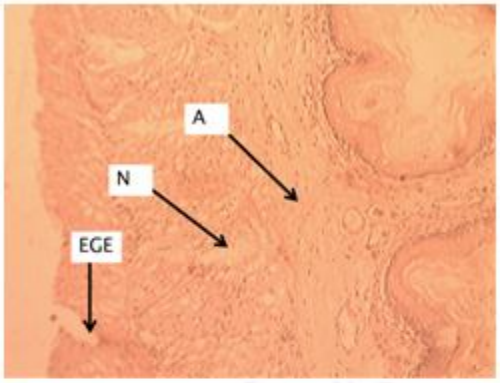

Plate 2: group 2 (Ulcer control)

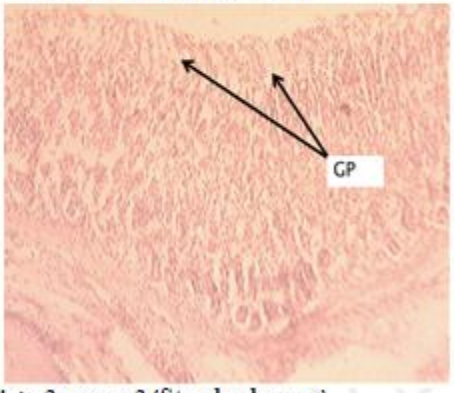

Plate 3: group 3 (Standard group)

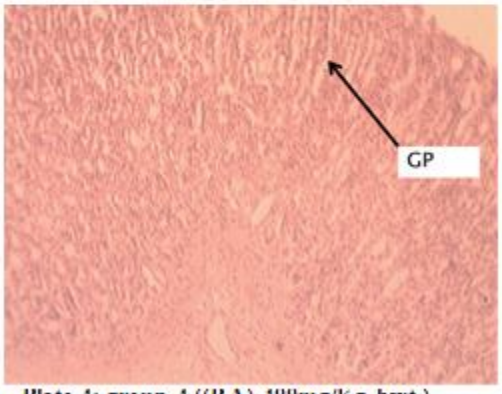

Plate 4: group 4 ((P.A) 400mg/Kg bwt)

Photomicrograph of section of the gastric tissues

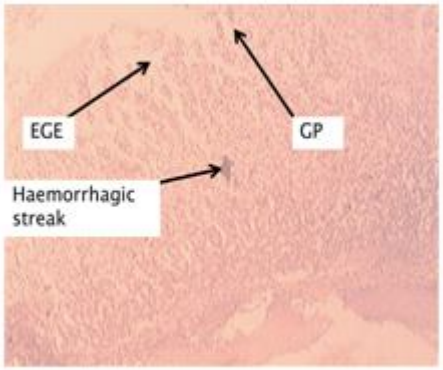

Plate 5: group 5 (BP) 400mg/Kg bwt)

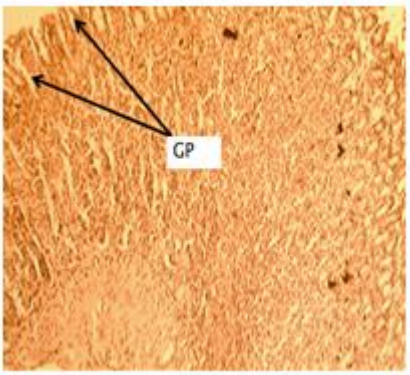

Plate 6: group 6 (PA + BP) (1:1) 400mg/Kg bwt)

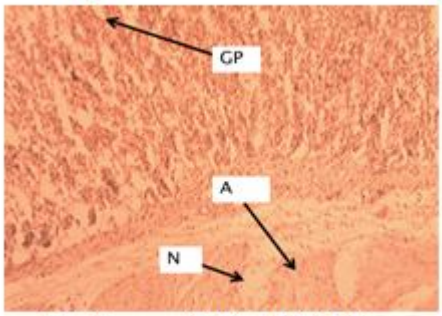

Plate 7: group 7 (PA + BP) (1:2) 400mg/Kg

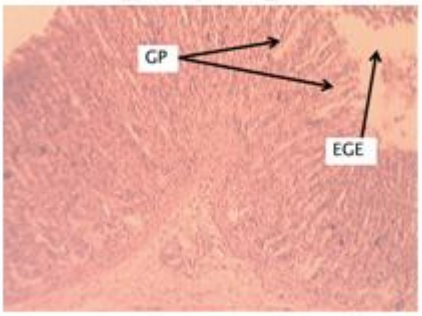

Plate 8 group 8 (PA + BP) (1:3) 400mg/Kg bwt)

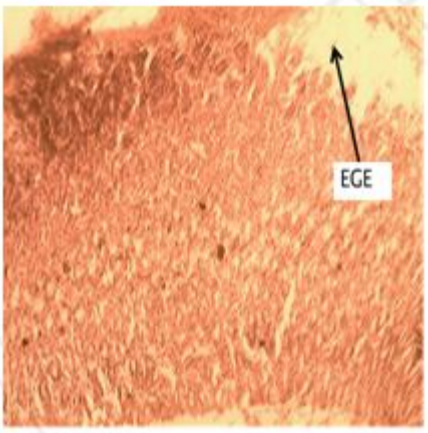

Plate 9: group 9 (P . A + B.P) (2:1) 400mg/Kg bwt).

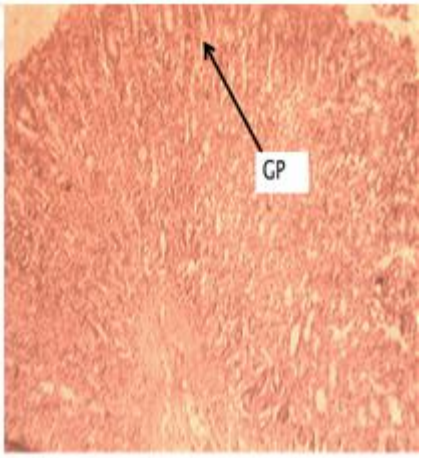

Plate 10: group 10 (P.A + B.P) (3:1) 400mg/Kg bwt)

Photomicrograph of section of the gastric tissues

3.6 Discussion

The present study assessed the ulcero-protective potential of ethyl acetate fractions of *P. americana* seed and *B. pinnatum* leaf binary combinations in indomethacin-induced gastric ulcers. Investigation of the protective effect of *P. americana* seed, *B. pinnatum* leaf, and their binary combinations against gastric ulcers showed that indomethacin administration resulted in significant changes in gastric ulcer indicators. The ethyl acetate fractions of *P. americana* seed and *B. pinnatum* leaf and their combinations provided varying degree of ulceroprotection. The ethyl acetate fraction combinations of PA+BP (1:1), PA+BP (1:2), PA+BP (1:3), PA+BP (2:1), and PA+BP (3:1) significantly reduced ulcer index when compared to groups that received PA and BP only. However, the ethyl acetate fraction combinations of PA+BP (2:1) and PA+BP (3:1) effectively reduced ulcer index but were not normalized to level found in control or the standard drug treated animals.

Analysis of the percentage ulceroprotection shows that the ethyl acetate fractions of *P. americana* seed and *B. pinnatum* leaf binary combination administration had a significant protective effect against the induction of gastric ulcers in rats. The protective effect of these fractions relative to the single plant fractions was in the order PA+BP (3:1) > PA+BP (2:1) > PA+BP (1:1) > PA+BP(1:2) > PA+BP(1:3) > PA > BP. The protective effect of the binary combinations was observed to increase with the increasing ratio of *P. americana* in the combination. The PA+BP (2:1) and PA+BP (3:1) binary combination caused a 2.5-fold and 3-fold increase in percentage protection compared to PA or BP alone. Previous studies have reported the gastroprotective properties of the ethyl acetate fractions of *P. americana* leaf and seed fractions [35-37]. Brena *et al.* [36], reported that avocado seeds may prevent gastric mucosal injury and oxidative stress induced by indomethacin. Also, the works of Sharma *et al.* [38], Amadi *et al.* [39]; De Araújo *et al.* [40] reported gastric protecting property of different solvent extracts of *B. pinnatum*. In our study, the ulceroprotective effect of the binary combinations was significantly higher when compared to the single plant fractions. The higher protective activity of the binary combinations containing higher ratios of *P. americana* seed fraction may be attributable to a the reported rich polyphenolic blue-print of this fraction [35].

Also, analysis of other gastroprotection markers showed that administration of indomethacin caused a significant rise in gastric free and total acidity. However, the administration of *P. americana* seed, *B. pinnatum* leaf, and binary combinations significantly protected against gastric acid reflux. The potency of the protective effect was found to be similar to the trends seen for ulceration. Results demonstrated a dose-dependent effect on gastric acidity which progressively increased with the ratio of *P. americana* seed ethyl acetate fraction in the binary combinations of the two plants. Administration of the combinations PA+BP (1:1), PA+BP (1:2), PA+BP (2:1), and PA+BP (3:1) to indomethacin-exposed animals effectively resulted in a reduction of gastric acidity, but only PA+BP (2:1) and PA+BP (3:1) produced a normalization of gastric acidity similar to those seen for animals receiving Omeprazole and normal control animals. The result obtained for gastric pH indicated a slight elevation of gastric pH values in animals receiving indomethacin. However, the changes were not significantly different from the normal control group. Administration of *P. americana* seed and *B. pinnatum* leaf ethyl acetate fraction and the different combinations in this study maintained gastric acidity preventing gastric acid concentration increase. In addition, indomethacin administration significantly depleted gastric mucus content in the UC group when compared to the normal control. Administration of the ethyl acetate fractions in the combinations PA+BP (1:1), PA+BP (1:3), PA+BP (2:1), and PA+BP (3:1) showed significant protection on gastric mucus secretion which was comparable to OMEP and normal control. Also, stomach homogenates nitrite concentration was significantly elevated in the ulcer control, but administration of the ethyl acetate fractions in the groups receiving binary combinations, significantly normalized stomach nitrite concentration, with the group PA+BP (3:1) being more effective.

Furthermore, indomethacin induction promoted the significant release of pepsin into the stomach. The ethyl acetate fractions did not normalize pepsin activity to the concentration found in control; they showed a significant effect on pepsin secretion in animals administered the different ethyl acetate fractions and their combinations. These observations are confirmed by microscopic evidence obtained in our histopathological analysis. In histological observation, the stomach of control animals showed no damage (Plate 1). However, in the ulcer control group (Plate 2), exposure to indomethacin presented damage to gastric tissue at a microscopic level. Histopathological injury caused by indomethacin administration is characterized by erosion of gastric epithelium, necrosis, and cell atrophy (Plate 2). Also, the mucosa, submucosa, muscularis interna and externa were not normal.

However, administration of the *P. americana* and *B. pinnatum* ethyl acetate fractions combinations was able to protect against damage caused by indomethacin to a varying degree. The ethyl acetate fraction combination PA + BP (3:1) 400mg/Kg bwt, was able to effectively protect the histological structure of the gastric mucosa, preventing distortion and mucosal damage. This was similar to that observed for normal animals and those treated with omeprazole standard. The findings of the histopathological analysis corroborate our biochemical findings on the gastro protective effect and the reduction in gastric acid secretion by the combinations of ethyl acetate fraction of *P. americana* and *B. pinnatum* at the different binary ratios.

Non-steroidal anti-inflammatory drugs such as indomethacin produce anti-inflammatory effects by inhibiting cyclooxygenase enzymes, this in turn, suppresses the formation of prostaglandins and thromboxane from arachidonic acid [41]. This suppressive effect leads to gastric lesions due to the reduction of cytoprotection of prostaglandins in the gastric mucosa [42] or via the production of oxygen radicals [43]. The gastroprotective mechanism of these fractions may be attributed to several possible mechanisms which though speculative may bring insight into their effect. The homeostasis of mucosal integrity is achieved by a number of mucosal protective factors among which are secretion and action of mucus and bicarbonate [44, 45]. Prostaglandins (E2 and I2) are known to stimulate secretion of mucus and bicarbonate, maintain mucosal integrity as well promote mucosal regeneration [44, 46]; as well promote mucosal regeneration. From the result of our study, it can be seen that the fractions and their combinations inhibited indomethacin-induced ulceration of the mucosa. The ethyl acetate fractions of the extracts may be acting by preventing the inhibitory effect of indomethacin on prostaglandin synthesis.

Furthermore, the gastro-protective effect and the reduction in the gastric acid secretion of the ethyl acetate fraction may also be attributed to the active component of the ethyl acetate fraction polyphenols, alkaloids, and flavonoids which have been proven to be useful for digestive disorders and disturbances of the gastrointestinal tract [47, 48]. NSAIDs such as indomethacin produce anti-inflammatory effects by suppressing of formation of prostaglandins and several other mechanisms which result in the production of oxygen radicals. Therefore, oxidative stress has been strongly suggested to be a dominant presence in the progression of indomethacin-induced gastric ulcers [49, 50].

4. CONCLUSION

The outcome of this study revealed that oral pre-administration of these ethyl acetate fractions before the exposure of the gastric mucosa to indomethacin resulted in significant protection from ulceration. These findings suggest that *P. americana* and *B. pinnatum* ethyl acetate fraction combination favorably protect against indomethacin-induced gastric mucosal oxidative damage, reducing ulcer index, gastric acid output, gastric mucus content and pepsin activity. The ethyl acetate fractions exhibited a dose-dependent inhibition of ulcer formation in indomethacin gastric ulcer model.

ETHICAL APPROVAL

The animal rights and ethical committee of the Federal University of Technology Owerri approved the study before the commencement of the study. All treatment of the animals was in accordance with the Principles of Laboratory Animal Care (NIH Publication, 1985 to 1993; revised, 1985).

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

There was a total collaboration between the authors in the execution of this study. CUI, ESA, VAO, KMEI, and CSA designed the study and wrote the protocol, while CUI, ESA, COU, and NGE performed the statistical analysis and interpretation of study data. Authors ESA and NGE did the literature searches, while ESA wrote the first draft of the manuscript and incorporated all corrections from co-authors. CUI, VAO KMEI, and CSA critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.
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LIST OF ABBREVIATIONS

GU: Gastric ulcer; **PA**: *Persea americana;* **BP**: *Bryophyllum pinnatum;***NC**: Normal control; **UC**: Ulcer control; **OMEP**: Omeprazole; **NSAIDS**: Non-steroidal anti-inflammatory drugs.