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Persea americana seed and *Bryophyllum pinnatum* leaf ethyl acetate binary combinations protects against non-steroidal anti-inflammatory drug-induced oxidative stress

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Abstract
The present study investigated the protective effect of binary mixtures of ethyl acetate fractions of *Persea americana* seed (PAEA) and *Bryophyllum pinnatum* leaf (BPEA) against non-steroidal anti-inflammatory drug (NSAID) induced oxidative stress in rats. The study comprised of 10 experimental units of four (4) rats each. The control groups were normal control group (NC), untreated control (UC), standard (0.02g kg^{-1} body weight Omeprazole (OMEP). Treatment groups included single and binary combinations of PAEA and BPEA, PAEA+ BPEA (1:1), PAEA+ BPEA (1:2), PAEA+ BPEA (1:3), PAEA+ BPEA (2:1), PAEA+ BPEA (3:1). The rats were initially administered 0.4gKg^{-1} b.wt. of respective mixtures orally for 3 weeks. Oxidative stress was induced by administration of 0.03 g/kg body weight indomethacin on the 22nd day after overnight fasting. Oxidative markers were determined using reference methods. The results revealed that indomethacin administration resulted in increased malondialdehyde level and significant reduction in stomach tissues GSH concentration when matched with normal control rats. Gastric tissue SOD, Catalase, GST, and GPx activity were significantly (p<0.05) reduced indicating a compromise in antioxidant protection. Administration of PAEA + BPEA (2:1) and PAEA + BPEA (3:1) resulted in significant protection against oxidative changes and restoration of GSH concentration similar to normal control. Binary combinations of the fraction had significantly (p<0.05) higher antioxidant effect than individual plant fractions. The fractions antioxidant protection was in the order PAEA + BPEA (3:1) > PAEA + BPEA (2:1) > PAEA + BPEA (1:1), > PAEA + BPEA (1:2) > PAEA + BPEA (1:3) > PAEA > BPEA.

Keywords: Oxidative stress, non-steroidal anti-inflammatory drug, *P. americana*, *B. pinnatum*, gastric ulcer, antioxidants

Introduction
Peptic Ulcer Diseases (PUD) are a group of diseases characterized by a sore or breaks on the lining of the gastrointestinal tract (Feldman *et al.*, 2016) [19]. The disease majorly affects the stomach or duodenum and are classified as gastric ulcers" and "duodenal ulcers" depending on the site of occurrence (Narayanan *et al.*, 2018) [34]. The gastro-intestinal tract are naturally protected by a network of factors such bicarbonate secretion and increased mucin production and blood flow; this functions synergistically to maintain gastric integrity (Périco *et al.*, 2020; Song *et al.*, 2023) [48]. Dyshomeostasis of internal factors such as *Helicobacter pylori* infection, gastric acid (HCl), endopeptidase pepsin, bile drain, inflammatory mediators, prolonged non-steroidal anti-inflammatory drugs (NSAIDS) use and reactive oxygen species (ROS) poses significant threat to the mucosal-defense mechanisms (Kolgazi *et al.*, 2017; Narayanan *et al.*, 2018; Sah *et al.*, 2023) [26, 34, 45]. Life style pattern such as smoking and alcoholism, genetic inheritance, and social status are implicated determinants of PUD (Räihä *et al.*, 1998; Yegen, 2018) [38, 54]. Increasing evidence implicates induction of production of ROS leading to oxidative stress (OS) as a major cause of ulcers (Sah *et al.*, 2023) [45]. Psychological, physical stress and *Helicobacter pylori* infection amount to oxidative stress in the gastric epithelium (Guo *et al.*, 2009; Suzuki *et al.*, 2012) [21, 49]. Oxidative stress arises from unchecked production of reactive oxygen species (ROS) which may induce more ROS production or deplete antioxidant defences. OS has been linked with carcinogenesis, ulcerogenesis in *H. pylori* infection and several lifestyle-related diseases (Guo *et al.*, 2009; Suzuki *et al.*, 2012; Yegen, 2018) [21, 49, 54].

Gastrointestinal disease variants such as peptic ulcer disease and gastroparesis are closely associated with antioxidant defence dysfunction (Suzuki *et al.*, 2012; Petrov *et al.*, 2022) [49, 37]. NSAIDS impair gastric protection by irreversibly blocking the function of Cyclo oxygenase 1 (COX-1) and Cyclo oxygenase 1 (COX-2) is necessary for the production of prostaglandins (Wallace, 2006; Zarghi and Arfaei, 2011) [50, 55]. The blockade increases the risk quotient of NSAID-induced gastric injury; repressing prostaglandin-mediated protection; and activating neutrophils and reactive oxygen species (ROS) infiltration (Zarghi and Arfaei, 2011; Wang *et al.*, 2021) [55,51]. Consequently, the microvasculatures are blocked by adhered neutrophils resulting in reduced mucosal blood flow, infiltration of pepsin and leukotrienes (Wang *et al.*, 2021; Morris *et al.*, 2022) [51, 32]. Integration of these factors with increased luminal acidity stimulates the release of ROS, and promotes the destruction of intestinal matrix and tissue necrosis (Whittle, 2002; Jimenez *et al.*, 2004) [52, 25].

Empirical evidence abounds on the contributions of protonophore uncouplers; aspirin and other acidic NSAIDs to mitochondrial oxidative phosphorylation uncoupling (Bjarnason *et al.*, 2018; Brandolini *et al.*, 2020) [12, 14]. This leads to shift in ATP/ADP and ADP balance in favor of the later which culminate in increased intracellular calcium concentration (Bjarnason *et al.*, 2018; Brandolini *et al.*, 2020) [12, 14]. Overall, accompanying these alterations are mitochondrial injury, proliferation of ROS, Na+/K+ balance shift culminating in mucous membrane damage and cellular necrosis (Bjarnason *et al.*, 2018; Brandolini *et al.*, 2020) [12, 14]. Current management strategies include the use of antibiotics, proton-pump inhibitors (PPIs), antacid, prostaglandins, therapeutic endoscopy and herbal remedies (Lam, 1997; Kuna *et al.*, 2019) [29, 27]. These regimens are targeted at reducing gastric acid output or neutralization of gastric HCl, gastric wound healing and surgical intervention in severe cases.

Persea americana Mill. is an evergreen, perennial tree known to grow to a height of about 15–25 m. *P. americana* is generally known as avocado pear. It is known locally by the Nigerian major tribes as Ube-beke, Ewé pia, and Ganyen piya by the Igbo, Yoruba and Hausas respectively. The avocado plant is classified as an evergreen, medium-sized tree with elliptic leaf 7-41 cm long. There are at least 500 named cultivars and generally cultivated for its delicious and highly nutritious fruit. Different sections of the plant are applied in conventional medicine practice as anti-hypertensive, antiulcer and anti-inflammatory agent. Numerous researches have investigated the biological activity and many attributed medicinal properties of the plant (Falodun *et al.*, 2013; Asiwe *et al.*, 2021a; Asiwe *et al.*, 2022) [8, 18, 7].

The plant *Bryophyllum pinnatum* is classed in the stonecrop family; it is a succulent perennial herb distributed worldwide in tropical African countries, Asia, American countries and Australia. Common names include “air plant”, “love plant”, “miracle leaves”, and “life plant” (Jain *et al.*, 2010) [24]. In South-eastern and south-western Nigeria, it is named ‘*odaa opue*’, and ‘*ewe abamoda*’ respectively (Ghasi *et al.*, 2011) [20]. They are widely applied in ethnomedicine for diverse medicinal benefits, including treatment of infection, hypertension, ulcer, inflammation, diabetes (Afzal *et al.*, 2012; Chibli *et al.*, 2014; Latif *et al.*, 2020). Previous studies have reported the phyto-active constituents, anti-inflammatory, gastroprotective, and free radical scavenging activities of the different parts (Andrade *et al.*, 2020, Emenike *et al.*, 2020; Asiwe *et al.*, 2021 a, b; Asiwe *et al.*, 2023) [8, 9].

In this work, we aim to determine the protective effect of ethyl acetate fractions of *Persea americana* seed and *Bryophyllum pinnatum* leaf binary combinations against NSAID induced oxidative stress.

Methods

Chemicals/Reagents

The following classified analytical grade chemicals and reagents were employed in the study and sourced from notable producers presented in parenthesis: Indomethacin (Sigma-Aldrich Mo USA), 2-thiobarbituric acid (Sigma-Aldrich Mo USA), Sodium dodecyl sulfate and acetic acid (Fluka-Chemie, Switzerland), trichloroacetic acid (Sigma-Aldrich Mo USA), 5’5’-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma-Aldrich Mo USA), sodium acetate, hydrochloric acid, sodium azide, reduced glutathione (Fluka Chemie, Switzerland), Sodium dihydrogen orthophosphate, Disodium hydrogen orthophosphate, Omeprazole extended-release capsules (Sanofi-aventis, Switzerland), Lactate dehydrogenase (LDH) test Kit (Randox Lab, UK).

Collection of plant sample

Matured seed of *P. americana* was collected by carefully cutting open fruits harvested from a farm in Umuodom village, Ikeduru L.G.A. Imo State. The apparently healthy fresh leaves of *B. pinnatum* were harvested from the same location in April, 2019. The samples were identified before collection using google lens imaging identification system and further authenticated at the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. The identified specimens were designated herbarium numbers IMSUH 0225 and IMSUH 0226 respectively, and deposited at the institution's herbarium by Prof. F. N. Mbagwu. The seeds and leaves were sorted, washed and the seeds sliced into irregular cubes of about 1 cm^3; both plant materials were allowed to air-dry at room temperature (30±2 °C).

Experimental animals

Experimental models used in this study were fifty (50) apparently healthy male Wistar rats (*Rattus norvegicus*) of 9-10 weeks, and weight range of 80-120g; purchased from the breeding stock at the Department of Veterinary Medicine, University of Nigeria, Nsukka. The animal handling was in strict compliance with Biochemistry department, Federal university of Technology, Owerri ethical clearance guide on the care and use of animals (BIOSC-BCH-EC-019). The models were acclimatized in aluminium cages, allowed *ad libitum* access to commercial poultry finisher pellets (Vital feeds, Nigeria) and portable water. The housing conditions were Temperature 25±2 °C, Relative humidity 55±5% and 12 hours day light.

Preparation of plant fraction

The ethyl acetate fractions preparation followed the protocol described by Asiwe *et al.*, 2021 [6, 8, 9] and Asiwe *et al.*, 2022 [7]. The dry plant materials were crushed into fine powder using a grinder (Kenwood BL357). Thereafter, 500g of each sample was extracted in 2 litres of 80% ethanol using a Soxhlet apparatus. The ethanol soluble components was recovered and evaporated to a semi-solid extract at 49 °C. The extracts were further partitioned in a column between ethyl acetate/water mixtures (2:1). The ethyl acetate soluble fraction of *P. americana* seed and *B. pinnatum* were recovered and designated PAEA and BPEA, dried under low

pressure at 49 °C in a Buchi rota vapor (Switzerland) and stored frozen at ≤ 4.0 °C.

Experimental protocol

The study followed a randomized block design as described in our earlier studies (Asiwe *et al.*, 2022) [7], and involved ten (10) groups of five (5) animals each, randomly assigned into groups based on body weight average of 90-100mgKg^{-1} b. WT. The experimental groups involved 7 test groups (Groups 3-10) pretreated with standard(OMEP), PAEA, BPEA and in fixed ratios combination of the respective ethyl acetate fraction (PAEA+ BPEA) in ratio of (50%:50% (1:1)), (33%:67% (1:2)), (25%:75% (1:3)), (67%:33% (2:1)), and (75%:25% (3:1)) by intubation for 21 days. Groups 1 and 2 served as normal and untreated control groups respectively. Induction of oxidative stress in group 2-10 was performed on the 22nd day by intubation of 0.03g/kg body weight indomethacin after overnight fasting. The animals were sacrificed after 4 hrs. The groups and administration regimen are summarized in Table 1.

Table 1: Animal grouping and experimental layout for indomethacin-induced oxidative stress.

Grouping	Treatments	Group code
1.	Finisher pellet diet and drinking water *ad libitum* for 3 weeks	Normal control (NC)
2.	0.03gKg^{-1} body weight (b. wt.) indomethacin	Untreated control (UC)
3.	Standard (0.02gKg^{-1} b. wt Omeprazole) for 3 weeks and indomethacin (0.03gKg^{-1} b. wt.)	Standard (Omeprazole (OMEP))
4.	0.4gKg^{-1} b. wt. of PAEA for 3 weeks and indomethacin (0.03 gKg^{-1} b. wt.).	PAEA
5.	0.4gKg^{-1} b.wt of BPEA for 3 weeks and indomethacin (0.03 gKg^{-1} b. wt.).	BPEA
6.	0.4gKg^{-1} b. wt. of PAEA + BPEA (50%: 50%) for 3 weeks and indomethacin (0.03gKg^{-1} b. wt.).	PAEA + BPEA (1:1)
7.	0.4gKg^{-1} b. wt. of PAEA + BPEA (33%: 67%) for 3 weeks and indomethacin (0.03gKg^{-1} b. wt.).	PAEA + BPEA (1:2)
8.	0.4gKg^{-1} b. wt. of PAEA + BPEA (25%: 75%) for 3 weeks and indomethacin (0.03gKg^{-1} b. wt.).	PAEA + BPEA (1:3)
9.	0.4gKg^{-1} b. wt. of PAEA + BPEA (67%: 33%) for 3 weeks and indomethacin (0.03gKg^{-1} b. wt.).	PAEA + BPEA (2:1)
10.	0.4gKg^{-1} b. wt. of PAEA + BPEA (75%: 25%) for 3 weeks and indomethacin (0.03gKg^{-1} b. wt.).	PAEA + BPEA (3:1)

Animal sacrificing and preparation of homogenates

The rats were anaesthesized using dichloromethane and humanely sacrificed 4 hrs post-induction. The rat stomach was carefully excised; dissected and cleaned in running portable water. The stomach tissues were weighed and homogenized in 1:4 ratio using freezing 0.1M phosphate buffered saline (pH 7.4). The resulting homogenate was spun at 4000 rpm for 15 minutes; and the clear supernatant obtained was used for biochemical analyses.

Assessment of antioxidant parameters

Determination of lipid peroxidation in gastric tissues

Malondialdehyde was measured to determine lipid peroxidation as described by Liu, *et al.* (1990) [31]. The experimental cocktail include 1.5 ml 20% acetic acid (pH 3.5), 1.5ml 1% thiobarbituric acid, 0.2ml of 8.1% sodium dodecyl sulphate, and 0.1ml of supernatant. This was thoroughly mixed and heated at 100^{0}C for 1 hr. The optical density was measured at 532nm after centrifugation for 10 minutes at 4000 rpm. Lipid peroxidation in the sample was expressed as malondialdehyde (MDA) concentration with a molar extinction coefficient of $1.56\times10^{5}M^{-1}$ cm^{-1} in µmoles MDA/mg protein.

Determination of glutathione concentration in gastric tissues

Reduced glutathione (GSH) concentration in the tissue homogenate was measured as described by Raja *et al.* (2007). The assay consisted of equal quantity of 10% trichloroacetic acid (TCA) and homogenate mixed in the ratio of 1:1 to coagulate proteins; the protein pellets was sedimented by spinning for 15 mins at 4000 rpm. The supernatant obtained (0.25ml) was reacted with 2.25ml Ellman's reagent, mixed and the optical density determined at 412 nm within 10 minutes. Glutathione content was calculated by using standard reduced glutathione (400mg/dl) of known concentration subjected to the same experimental treatment.

$$\text{GSH}\left(\frac{\mu g}{\text{mgprotein}}\right) = \frac{\text{ABS Test}}{\text{ABS STD}} \times \text{Concentration of Standard}$$

Determination of glutathione peroxidase (GPx) **activity in gastric tissues**

Determination of GPx activity in the stomach tissue homogenate was carried out in 1000μl total volume, consisting of 500μl phosphate buffer (200mM, pH 7.0), 200μl stomach homogenate, 200μl of GSH (2 mM), 100μl sodium azide (10 mM) and 100μl of H_2O_2 (0.2 mM), incubated at room temperature (25±2 °C) for 10 mins. Thereafter, 500μl of TCA (10%) was added to halt the reaction, and centrifuged at 4000 rpm for 5 min (Rotruck *et al.*, 1973). The GPx content in 500μl of the supernatant was measured spectrophotometrically as described by Ellman (1959). GPx activity was expressed as µmol GSH consumed/min/mg protein.

Determination of superoxide dismutase (SOD) activity in stomach homogenates

SOD activity assay was determined in stomach homogenates with experimental cocktail consisting of 0.05M phosphate buffer (pH=7.4), 0.02M L-Methionine, 1% Triton X-100, 0.01M hydroxylamine hydrochloride and supernatant in volumes of 1.1ml, 0.075, 0.4ml, 0.075, and 0.1ml respectively incubated at 30 °C for 5 minutes. Subsequently, 80µl of 50µM riboflavin was pipetted into the tubes and exposed to incandescent light (200watts Lamp) for 10 minutes. Furthermore, the set-up was reacted with 2ml of Griess reagent (1ml of 1% sulphanilamide and 1ml of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED). The nitrate formed was measured spectrophotometrically at 543nm. One unit of SOD activity (1 IU) was defined as the amount of SOD that inhibited 50% of nitrate formation under the assay condition (Das *et al.*, 2000) [16].

Determination of glutathione-S-transferase activity in gastric tissues

Glutathione-S-transferase (GST) in tissue followed the method described by Habig *et al.* (1974) [22]. Exactly 1700µl of 0.14M sodium phosphate buffer (pH 6.5), 200µl GSH (30 mM), and 40µl of supernatant were mixed in a test tube. Thereafter, reaction progress was followed on addition of 60µl of 10mM 1-chloro-2, 4-dinitrobenzene (CDNB). GST activity was derived using the molar extinction coefficient of

9.6 for CDNB-GSH conjugate, and activity expressed in µmoles of CDNB-GSH conjugate formed/min/mg protein.

Determination of catalase (CAT) activity in gastric tissues

Catalase activity in stomach tissues was determined as described by Sinha (1972) [47]. The experimental cocktail consisted of stomach homogenate supernatant (0.1 mL) incubated with H_2O_2 (5mM, 2.0 mL), in the presence of 2.5ml 10mM phosphate buffer (pH 7.4). Exactly 1.0 ml portion of the reaction mixture was added to tubes containing 2 ml of dichromate/acetic acid reagent and incubated in hot water (100 °C) for 15 min. The set-up absorbance was read at 570nm at room temperature. The catalase activity was expressed in terms of µmol H_2O_2/min/mg protein.

Determination of ascorbic acid concentration in gastric tissues

Ascorbic acid in the stomach homogenates was assessed following the method of Rutkowski and Grzegorczyk, (2007) [44]. Exactly 0.5ml of stomach homogenate was deproteinized with 2.0ml of 6% trichloroacetic acid. Thereafter, 1 ml of 2, 4-dinitrophenylhydrazine (2, 4-DNPH)/thiourea solution was added to form osazone, and incubated in a thermostatic water bath at 100 ^{0}C for 15 minutes. The set-up was cooled and 5 ml of 85% H_2SO_4 was run into the tubes slanted at 45^0 and incubated at room temperature (28±2 °C) for ½ hrs. The absorbance of the separated top layer was read in a spectrophotometer at 540nm. The ascorbic acid concentration was expressed in mg/g wet tissue relative to a standard ascorbic acid solution (10 mg/ml) treated with the regimen above.

Determination of alpha-tocopherol concentration in gastric tissues

The α-tocopherol concentration in the stomach homogenate was determined as described by Baker and Frank (1968). Alpha-tocopherol in the sample was extracted by votexing (1:1 mixture of stomach homogenate: xylene; and centrifugation at 4000g for 10 minutes. Thereafter, 1ml of the xylene soluble upper layer fraction was transferred into test tubes containing 1ml α, α-dipyridyl reagents and absorbance measured at 460nm. Furthermore, 0.33 ml 1.2g/ml $FeCl_3$ solution was added to each tube and absorbance read after 15 min against the blank at 520 nm. α-tocopherol solution (1000µg/ml) was used as standard.

Calculations

α-tocopherol concentration was calculated and expressed in µg/L by the following formula-

$$= \frac{\text{Extinction of unknown at 520 nm} - \text{Extinction at 460nm x 0.29}}{\text{Extinction of standard at 520nm}} x1000$$

Determination of lactate dehydrogenase (LDH) activity in gastric tissues

LDH activity was measured according to the method of Rec (1970) [41] and Rec (1972) [41].

LDH catalyzes the reduction of pyruvate to lactate with the simultaneous release of NAD^+ group. LDH activity was determined by monitoring the rate of pyruvate reduction at 340nm.

Data Analysis

Data obtained was processed in Microsoft Excel and Statistical Package for Social Sciences (SPSS) version 25. Results are presented as mean ± standard deviation of five determinations (N=5). Means were compared using One-way-ANOVA and significant difference considered at $p<0.05$.

Results and discussion

The present study assessed the effect of ethyl acetate fractions of *Persea americana* seed (PAEA) and *Bryophyllum pinnatum* leaf (BPEA) binary mixtures against indomethacin-induced oxidative stress in rats. Empirical and clinical evidence have suggested a critical link between reactive oxygen and nitrogen species homeostasis in the etiology of gastric inflammation and ulceration. This study showed that indomethacin administration led to significant ($p<0.05$) alterations in oxidative stress markers in exposed rats. In the study, indomethacin administration resulted in increased production of malondialdehyde products; also, there was a significant reduction in stomach tissues GSH concentration when compared to the normal control. These gross changes are empirical evidence of increased lipid peroxidation and overproduction of free radicals leading to mucosal damage. Administration of PAEA + BPEA (2:1) and PAEA + BPEA (3:1) resulted in significant protection against oxidative changes significant and restoration of GSH concentration similar to those of normal control.

Also, the changes in enzyme activity involved in antioxidant homeostasis, showed a significant reduction of SOD, Catalase, GST, and GPx activity, indicating a compromise in antioxidant protection. The administration of PAEA, BPEA and their combinations showed various degrees of protection against changes in antioxidant enzyme activity compared to those of omeprazole and normal control. The groups that received the combination ratios of PAEA + BPEA (1:2) and PAEA + BPEA (1:3) showed an increase in SOD and GST activity similar to those of standard drug treated rats. Also, there was significant ($p<0.05$) increase in catalase activity for indomethacin exposed rats treated with the ethyl acetate fractions and the combinations except for groups BPEA and PAEA + BPEA (1:1). The result presented show a significant increase in GPx activity for indomethacin exposed rats treated with our ethyl acetate fractions and the combinations. GPx activity in the groups PAEA + BPEA (1:2) and PAEA + BPEA (1:3) although increased, did vary significantly from UC. The result showed a significant ($p<0.05$) increases in catalase activity for indomethacin exposed rats treated with the ethyl acetate fractions and the combinations except the groups BPEA and PAEA + BPEA (1:1) (table 1.0). The other treatments presented increases in catalase activity similar to those of animals receiving standard drug omeprazole and normal control. In addition, significant ($p<0.05$) increases in GST activity for indomethacin-exposed rats treated with the ethyl acetate fractions and their combinations was observed. The GST activity of groups PAEA + BPEA (1:2) and PAEA + BPEA (1:3) were similar to those of normal control animals and standard drug treated rats.

Furthermore, ascorbic acid and α-tocopherol concentrations in the stomach homogenates of indomethacin-exposed rats were significantly decreased. The binary combination of the ethyl acetate fractions protected against ascorbic acid depreciation but did not considerably restore vitamin E except in the group that received PAEA + BPEA (3:1).

Distortions in antioxidant enzymes activity are important indicators of oxidative toxicity. The findings of our studies align with the work of Odabasoglu *et al.* (2006) [35], which reported that indomethacin induces stress by altering antioxidant enzymes activity in rats stomach disposing them

to gastric ulceration. This is attributed to proliferation of free radicals and pro-oxidants that ultimately lead to stomach and biomolecule oxidative injury. The significant increase in antioxidant enzyme activity and reduction in MDA concentration presented in our findings is indicative of the antioxidative potential of PAEA and BPEA. Our earlier reported findings showed that *P. americana* seed and *B. pinnatum* leaf ethyl acetate fractions possess significant free radical scavenging properties (Asiwe *et al.* 2021) [6, 8, 9]. These findings closely align with previous studies, showing their antioxidative and anti-inflammatory properties against NSAIDs and other agents-induced gastric ulceration (Afzal *et al.* 2012; Sharma *et al.* 2014; Yadav, *et al.* 2016) [1, 46, 53]. Another possible mechanism of ulcer protection by the ethyl acetate fractions may be a result of their antioxidant properties.

Furthermore, in this study, LDH activity was significantly elevated by indomethacin induction when compared to normal control. Similar increases in serum LDH activity and oxidative stress markers associated with NSAID-induced ulceration have been reported in the works of Basnet *et al.* (2015) [11] and Al-Abdan and Zakia (2017) [2]. LDH is tissue specific; widespread increases in activity are associated with cellular toxicity, liver, renal, and gastric tissue disruption, and hemolysis. Administration of PAEA and BPEA to indomethacin exposed rats significantly reduced serum LDH activity when compared to the UC group except for groups PAEA + BPEA (1:1). LDH activity was significantly normalized in groups PAEA + BPEA (1:3) when compared to normal control and OMEP. Changes in LDH activity significantly corroborates observed changes in stress markers in this study.

The antioxidative effect of *P. americana* and *B. pinnatum* binary combinations against indomethacin-induced oxidative stress may be associated with the synergy of the bioactive components present in the plant fractions. Several studies have associated plant gastric ulcer protection with flavonoids and phenolic compounds action in the plant ethyl acetate fractions (Mota *et al.* 2011; Amaral *et al.* 2013; Boligon *et al.* 2014) [33, 3, 13]. Flavonoids exert their antioxidant activity through mechanisms such as metal chelation, inhibition of free radicals/ oxidant/pro-oxidant enzymes, regeneration of vitamin E from α-tocopheroxyl radicals; and inhibition of Ca^{2+} influx in glutamate induced cell death cascade (Ishige *et al.* 2001; Mota *et al.* 2011) [23, 33]. They are also promoters of tissue regeneration, gastric mucus membrane formation, and inhibit release of stomach HCl and pepsinogen. (La-Casa *et al*, 2000, Repetto and Llesuy, 2002) [28, 42]. The phytochemicals of these plants may be acting synergistically increasing antioxidant defence against indomethacin-induced stress.

Conclusions

The outcomes of this study suggest that *P. americana* and *B. pinnatum* ethyl acetate fraction binary mixtures reduced oxidative damage through reduction of stomach tissues lipid peroxidation and activation of antioxidant enzymes. The binary combination ratios of PAEA + BPEA (1:2) and PAEA + BPEA (1:3) significantly increased GSH, SOD, GPx and GST activity thereby promoting stress reduction.

Table 2: Effect of PAEA and BPEA binary mixtures on antioxidant parameters SOD, GPx, Catalase, GST, MDA, GSH, ascorbic acid, Vitamin E, and LDH activity in indomethacin-induced oxidative stress in male albino rats.

Parameters	NC	UC	OMEP	PAEA	BPEA	PAEA + BPEA (1:1)	PAEA + BPEA (1:2)	PAEA + BPEA (1:3)	PAEA + BPEA (2:1)	PAEA + BPEA (3:1)
SOD (IU/mg Protein)	122.33 ± 7.73$^{d, e,f}$	76.19 ± 13.24^{a}	130.97 ± 13.49^{f}	94.11 ± 8.58^{b,c}	88.31 ± 13.45^{a,b}	112.09 ± 6.37^{d,e}	112.83 ± 11.63^{d,e}	108.11 ± 8.28^{c,d}	127.45 ± 13.53^{f}	126.62 ± 11.17^{f}
GPx (µg GSH/min/mg protein)	15.04 ± 1.29^{c}	12.32 ± 1.44^{a}	16.67 ± 1.84^{c,d}	16.74 ± 1.46^{c,d}	14.54 ± 1.71^{a,b,c}	15.00 ± 1.88c	13.70 ± 1.01^{a,b}	13.11 ± 1.22^{a,b}	16.88 ± 1.20^{c,d}	17.39 ± 1.27^{d}
Catalase (µmol H_2O_2/ min/mg protein) x10^{-6}	14.16 ± 1.90^{a}	6.57 ± 0.64^{b}	15.49 ± 1.99^{a}	12.81 ± 1.55^{a}	9.56 ± 1.70^{c}	12.10 ± 0.58^{d}	14.38 ± 1.08^{a}	14.45 ± 1.86^{a}	12.92 ± 2.04^{a}	15.59 ± 1.97^{a}
GST (µmol GSH-CDNB /min/mgprotein)x10^{-6}	14.38 ± 1.32^{d}	8.18 ± 0.33^{a}	14.35 ± 1.55^{d}	10.41 ± 1.64^{b}	9.23 ± 0.50^{a,b}	12.36 ± 1.33^{c}	9.74 ± 0.32^{a,b}	9.56 ± 0.68^{a,b}	13.74 ± 0.60^{c,d}	14.37 ± 0.92^{d}
MDA (µmol/mg protein)	241.71 ± 26.37^{a}	380.01 ± 28.53^{e}	256.71 ± 24.00^{a}	306.19 ± 30.10^{b,c}	354.20 ± 49.67^{d,e}	320.49 ± 29.31^{b,c,d}	370.47 ± 20.76^{d,e}	332.60 ± 24.99^{c,d,e}	338.55 ± 13.42 ^{c,d,e}	283.50 ± 33.92^{a,b}
GSH (µg/mg protein)	2.76 ± 0.19^{e}	0.91 ± 0.15^{a}	2.02 ± 0.14^{d}	1.63 ± 0.07^{c}	1.17 ± 0.22^{b}	1.81 ± 0.17^{c,d}	1.77 ± 0.10^{c,d}	1.09 ± 0.13^{a,b}	1.96 ± 0.15^{d}	2.58 ± 0.25^{e}
Ascorbic acid (mg/g tissue)	5.92 ± 0.23^{f}	2.04 ± 0.20^{a}	4.54 ± 0.46^{c,d}	4.06 ± 0.27^{c}	3.42 ± 0.30^{b}	5.41 ± 0.29^{e}	4.06 ± 0.14^{c}	4.27 ± 0.16^{c,d}	5.28 ± 0.12^{e}	5.87 ± 0.40^{f}
α-Tocopherol (µg/L)	5.57 ± 0.26^{c}	4.61 ± 0.29^{a,b}	5.12 ± 0.80^{b}	4.88 ± 0.39^{b}	5.32 ± 0.25^{b}	4.64 ± 0.44^{a,b}	4.23 ± 0.24^{a}	5.28 ± 0.37^{c}	6.18 ± 0.19^{d}	10.13 ± 0.53^{e}
LDH (U/L)	31.86 ± 6.07^{b,c}	61.18 ± 7.50^{e}	17.21 ± 5.65^{a}	39.51 ± 4.41^{c,d}	38.87 ± 7.32^{c,d}	57.99 ± 8.42^{e}	40.78 ± 6.58^{c,d}	25.49 ± 4.65^{a,b}	35.69 ± 3.60^{c,d}	43.33 ± 2.08d

Values are mean ± standard deviation (N=5). Values bearing similar superscript letters across rows are not significantly different (p>0.05).

References

1. Afzal M, Gupta G, Kazmi I, Rahman M, Afzal O, Alam J, *et al*. Anti-inflammatory and analgesic potential of a novel steroidal derivative from *Bryophyllum pinnatum*. Fitoterapia. 2012;83(5):853-858.
2. Al-Abdan A, Zakia N. N-(2-mercaptopropionyl) glycine protects against gastric oxidative injury induced by indomethacin in male rats. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2017;8(2):815-821.
3. Amaral GP, Carvalho DNR, Barcelos RP, Dobrachinski F, Portella RL, Silva DMH. Protective action of ethanolic ethyl acetate fraction of *Rosmarinus officinalis* L. in gastric ulcer prevention induced by ethanol in rats. Food Chemistry and Toxicology. 2013;55:48-55.
4. Andrade AWL, Guerra GCB, Araújo DSDF, Júnior DARF, Araújo DAA, Carvalho DTG, *et al*. Anti-inflammatory and chemopreventive effects of *Bryophyllum pinnatum* (Lamarck) leaf extract in experimental colitis models in rodents. Frontiers in Pharmacology. 2020;11:1-18.
5. Asiwe ES, Ugenyi AU, Enenebeaku EU, Opara PO, Alisi CS, Ekezie J. Changes in hepatic function indices in ulcerated rats treated with *Persea americana* seed and

Bryophyllum pinnatum leaf ethyl acetate fraction. Tropical Journal of Natural Product Research. 2023;7(9):4084-40891.
6. Asiwe ES, Igwe CU, Iheanacho KME, Onyeocha IO, Onwuliri VA. Antioxidative and free radical scavenging properties of ethyl acetate fractions of *Persea americana* seed and *Bryophyllum pinnatum* leaf. Tropical Journal of Natural Product Research. 2021;5(8):1486-1492.
7. Asiwe ES, Igwe CU, Iheanacho KME, Onwuliri VA, Alisi CS, Chigbu ENG, *et al*. Ulcero-protective potential of ethyl acetate fractions of *Persea americana* seed and *Bryophyllum pinnatum* leaf binary combinations in indomethacin-induced gastric ulcer. Asian Journal of Biochemistry, Genetics and Molecular Biology. 2022;12(4):139-153.
8. Asiwe ES, Igwe CU, Iheanacho KME, Onyeocha IO, Onwuliri VA. Antioxidative and free radical scavenging properties of ethyl acetate fractions of *Persea americana* seed and *Bryophyllum pinnatum* leaf. Tropical Journal of Natural Product Research. 2021;5(8):1486-1492.
9. Asiwe ES, Igwe CU, Onwuliri VA, Iheanacho KME, Iheanacho JN. Characterization of chemical composition of *Bryophyllum pinnatum* leaf ethyl acetate fraction. Asian Journal of Advanced Research and Reports. 2021;15(4):15-24.
10. Baker H, Frank O. Tocopherol. In: Clinical vitaminology: methods and interpretation. New York: Interscience Publishers, John Wiley and Sons, Inc.; 1968. p. 172-3.
11. Basnet S, Adhikari A, Kumar V, Shivanandappa BT, Veerapur V. Protective effect of *Blumea lacera* DC aerial parts in indomethacin-induced enterocolitis in rats. Inflammopharmacology. 2015;23(6):355-363.
12. Bjarnason I, Scarpignato C, Holmgren E, Olszewski M, Rainsford KD, Lanas A. Mechanisms of damage to the gastrointestinal tract from nonsteroidal anti-inflammatory drugs. Gastroenterology. 2018;154(3):500-514. https://doi.org/10.1053/j.gastro.2017.10.049
13. Boligon AA, Freitas DRB, Brum DTF, Waczuk EP, Klimaczewski CV, de Ávila DS, *et al*. Antiulcerogenic activity of Scutia buxifolia on gastric ulcers induced by ethanol in rats. Acta Pharmaceutica Sinica B. 2014;4(5):358-367.
14. Brandolini L, Antonosante A, Giorgio C, Bagnasco M, d'Angelo M, Castelli V, *et al*. NSAIDs-dependent adaptation of the mitochondria-proteasome system in immortalized human cardiomyocytes. Scientific Reports. 2020;10(1):18337. https://doi.org/10.1038/s41598-020-75394-x
15. Chibli LA, Rodrigues KC, Gasparetto CM, Pinto NC, Fabri RL, Scio E, *et al*. Anti-inflammatory effects of *Bryophyllum pinnatum* (Lam.) Oken ethanol extract in acute and chronic cutaneous inflammation. Journal of Ethnopharmacology. 2014;154(2):330-338. https://doi.org/10.1016/j.jep.2014.03.035
16. Das K, Samanta L, Chainy G. A modified spectrophotometric assay of superoxide dismutase using nitrite formation by superoxide radicals. Indian Journal of Biochemistry and Biophysics. 2000;37:201-204.
17. Emenike BA, Emmanuel CO, Obiora CU, Valentine ON, Chioma AA, Petra ON, *et al*. Ulcer-protective property of *Bryophyllum pinnatum* leaf extract and their phytosomal formulations. Tropical Journal of Natural Product Research. 2020;4(12):1201-1207.
18. Falodun A, Engel N, Kragl U, Nebe B, Langer P. Novel anticancer alkene lactone from *Persea americana*. Pharmacology and Pharmacy. 2013;51(6):700-6.
19. Feldman M, Friedman LS, Brandt LJ. Sleisenger and Fordtran's gastrointestinal and liver disease: pathophysiology/diagnosis/management. 10th Ed. Philadelphia, PA: Saunders/Elsevier. 2016;2(31):2369-2389.
20. Ghasi SE, Achukwu PU, Onyeanusi J. Assessment of the medical benefit in the folkloric use of *Bryophyllum pinnatum* leaf among the Igbos of Nigeria for the treatment of hypertension. African Journal of Pharmacy and Pharmacology. 2011;5(1):83-92.
21. Guo G, Jia KR, Shi Y, Liu XF, Liu KY, Qi W, *et al*. Psychological stress enhances the colonization of the stomach by Helicobacter pylori in the BALB/c mouse. Stress. 2009;12(6):478-85. https://doi.org/10.3109/10253890802642188
22. Habig WH, Pabst MJ, Jacoby WB. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry. 1974;249:7130-7139.
23. Ishige K, Schubert D, Sagara Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Radical Biology and Medicine. 2001;30(4):433-66.
24. Jain VC, Patel NM, Shah DP, Patel PK. Antioxidant and antimicrobial activities of *Bryophyllum calycinum* salisb leaf. Pharmacologyonline. 2010;1:393-405.
25. Jimenez MD, Martín MJ, Lastra ADLC, Bruseghini L, Esteras A, Herrerías JM, *et al*. Role of L-arginine in ibuprofen-induced oxidative stress and neutrophil infiltration in gastric mucosa. Free Radical Research. 2004;38(9):903-911.
26. Kolgazi M, Kumral OZN, Ozturk CC, Demirci EK, Yuksel M, Sirvanci S, *et al*. Anti-inflammatory effects of nesfatin-1 on acetic acid-induced gastric ulcer in rats: Involvement of cyclo-oxygenase pathway. Journal of Physiology and Pharmacology. 2017;68:765-777.
27. Kuna L, Jakab J, Smolic R, Raguz-Lucic N, Vcev A, Smolic M. Peptic ulcer disease: A brief review of conventional therapy and herbal treatment options. Journal of Clinical Medicine. 2019;8(2):179. https://doi.org/10.3390/jcm8020179
28. La Casa C, Villegas I, Alarcón LC, Motilva V, Martín CMJ. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol-induced gastric lesions. Journal of Ethnopharmacology. 2000;71(1-2):45-53.
29. Lam SK. Current strategies in ulcer management with special reference to the use of antibiotics. The Yale Journal of Biology and Medicine. 1997;70(1):27-31.
30. Latif A, Ashiq K, Ashiq S, Ali E, Anwer I, Qamar S. Phytochemical analysis and *in vitro* investigation of anti-inflammatory and xanthine oxidase inhibition potential of root extracts of *Bryophyllum pinnatum*. Journal of Animal and Plant Sciences. 2020;30:219-228.
31. Liu J, Edamatsu R, Kabuto H, Mori A. Antioxidant action of Guilingji in the brain of rats with FeCl3 induced epilepsy. Free Radic Biol Med. 1990;9(5):451-454.
32. Morris G, Gevezova M, Sarafian V, Maes M. Redox regulation of the immune response. Cell Mol Immunol. 2022;19(10):1079-1101. https://doi.org/10.1038/s41423-022-00902-0

33. Mota CS, Freitas RB, Athayde ML, Boligon AA, Augusti P, Somacal S. Effect of Vernonia cognata on oxidative damage induced by ethanol in rats. Hum Exp Toxicol. 2011;30(7):675-684.
34. Narayanan M, Reddy KM, Marsicano E. Peptic Ulcer Disease and Helicobacter pylori infection. Mo Med. 2018;115(3):219-224.
35. Odabasoglu F, Cakir A, Suleyman H, Aslam A, Bayir Y, Halici M. Gastroprotective and antioxidant effects of usnic acid on indomethacin-induced gastric ulcer in rats. J Ethnopharmacol. 2006;103(1):59-65.
36. Perico LL, Emílio-Silva MT, Ohara R, Rodrigues VP, Bueno G, Barbosa-Filho JM, *et al*. Systematic analysis of monoterpenes: advances and challenges in the treatment of peptic ulcer diseases. Biomolecules. 2020;10(2):265. https://doi.org/10.3390/biom10020265
37. Petrov L, Kachaunov M, Alexandrova A, Tsvetanova E, Georgieva A, Dolashki A, *et al*. Snail mucus protective effect on ethanol-induced gastric ulcers in mice. Life (Basel). 2022;12(8):1106. https://doi.org/10.3390/life12081106
38. Raiha IK, Kemppainen H, Kaprio J, Koskenvuo M, Sourander L. Lifestyle, stress, and genes in peptic ulcer disease: A nationwide twin cohort study. Arch Intern Med. 1998;158(7):698-704. doi:10.1001/archinte.158.7.698
39. Raja S, Ahmed N, Kumar V, Mukherjee K, Bandyopadhyan A, Mukherjee KP. Antioxidant effect of *Cytisus scoparius* against carbon tetrachloride treated liver injury in rats. J Ethnopharmacol. 2007;109(1):41-47.
40. Rec. GSC. Determination of lactate dehydrogenase activity using UV method. J Clin Chem Clin Biochem. 1970;8(6):658.
41. Rec. GSC. Optimised standard colorimetric methods. Serum alkaline phosphatase (DGKC). J Clin Chem Clin Biochem. 1972;10(3):182.
42. Repetto MG, Llesuy SF. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. Braz J Med Biol Res. 2002;35(5):547-558.
43. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. Science. 1973;179(4073):588-590.
44. Rutkowski M, Grzegorczyk K. Modifications of spectrophotometric methods for antioxidative vitamins determination convenient in analytic practice. Acta Sci Pol Technol Aliment. 2007;6(3):17-28.
45. Sah DK, Arjunan A, Lee B, Jung YD. Reactive oxygen species and H. pylori infection: A comprehensive review of their roles in gastric cancer development. Antioxidants. 2023;12(9):1712. https://doi.org/10.3390/antiox12091712
46. Sharma A, Bhot M, Chandra N. *In vitro* antibacterial and antioxidant activity of *Bryophyllum pinnatum* (Lam.) Kurz. Int J Pharm Pharm Sci. 2014;6(4):558-560.
47. Sinha AK. Colorimetric assay of catalase. Anal Biochem. 1972;47(2):389-394.
48. Song C, Chai Z, Chen S, Zhang H, Zhang X, Zhou Y. Intestinal mucus components and secretion mechanisms: what we do and do not know. Exp Mol Med. 2023;55(4):681-961. DOI: 10.1038/s12276-023-00960-y
49. Suzuki H, Nishizawa T, Tsugawa H, Mogami S, Hibi T. Roles of oxidative stress in stomach disorders. J Clin Biochem Nutr. 2012;50(1):35-39. https://doi.org/10.3164/jcbn.11-115SR
50. Wallace JL. COX-2: A pivotal enzyme in mucosal protection and resolution of inflammation. Sci World J. 2006;6:577-588.
51. Wang B, Wu L, Chen J, Dong L, Chen C, Wen Z, *et al*. Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. Signal Transduct Target Ther. 2021;6(1):94. https://doi.org/10.1038/s41392-020-00443-w
52. Whittle BJ. Gastrointestinal effects of nonsteroidal anti-inflammatory drugs. Fundam Clin Pharmacol. 2002;17(3):301-313.
53. Yadav R, Angolkar T, Kaur G, Buttar HS. Antibacterial and anti-inflammatory properties of bovine colostrum. Recent Pat Endocr Metab Immune Drug Discov. 2016;10(1):1-5.
54. Yegen BC. Lifestyle and peptic ulcer disease. Curr Pharm Des. 2018;24(18):2034-2040. https://doi.org/10.2174/1381612824666180510092303
55. Zarghi A, Arfaei S. Selective COX-2 inhibitors: A review of their structure-activity relationships. Iran J Pharm Res. 2011;10(4):655-683.