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Gastroprotective Mechanisms of *Entandrophragma angolense* Extract against Indomethacin-induced Gastric Ulceration in Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOO carried out all the experimental procedures designed the study. Author ATS supervised the execution of the technical aspects of the work and author FSO developed the protocol for the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The mechanisms of action of the methanolic extract of *Entandrophragma angolense* in male Wistar rats were investigated using indomethacin ulcer model.
Study Design: Laboratory experimental study.
Place and Duration of Study: Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan in 2016.
Methodology: Twenty five adult male Wistar rats weighing between 180-200 g were grouped into five groups (n=5) and treated as follows: Control (Group I), had normal rats' pellets with water given ad libitum. Groups II to IV were administered graded doses of the extract (200, 400 and 600) mg/kg for 28 days while group V received Omeprazole (positive control) for 28 days. Thirty six female mice (20-25 g) were used for acute toxicity study. Experimental ulcer was induced using indomethacin. Hematological parameters were analyzed with an automatic blood analyzer.

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Malondialdehyde (MDA), Nitric oxide (NO), Superoxide dismutase were measured by spectrophotometry method.

Results: The acute toxicity test showed LD_{50} to be 275.42 mg/kg body weight. The group pre-treated with 200 mg/kg *Entandrophragma angolense* showed a significant reduction in the Mean Ulcer Score when compared with control group showing a percentage protection of 72.41% (P=0.05), significant increase in Superoxide Dismutase activity and significant reduction MDA level when compared with the control group (P=0.05). Finally, this same group had a significant increase in Nitric Oxide and Prostaglandins E_2 concentrations when compared with the control group (*P*=0.05).

Conclusion: *Entandrophragma angolense* extract mediates its antiulcer effects via production of antioxidant enzyme {Superoxide dismutase}, stimulates the release of nitric oxide and the production of endogenous prostaglandins (PGE_2).

Keywords: Entandrophragma angolense; nitric oxide; prostaglandins (PGE2); antioxidant enzyme.

1. INTRODUCTION

The gastrointestinal tract is exposed to different kind of disorders amongst which is peptic ulceration. Peptic ulcer disease (PUD) is a group of disorders characterized by the presence of ulcers in the gastrointestinal tract [1]. Approximately 90% of PUD cases are caused by the use of Non-steroidal anti-inflammatory drugs (NSAID) or *Helicobacter pylori* infection [2,3]. Gastric "cytoprotection" refers to a reduction or prevention of chemically induced acute hemorrhagic erosions by compounds such as prostaglandins and SH-derivatives without inhibiting acid secretion in rodents [4].

Entandrophragma angolense is a popular plant commonly found on the West African coast and it belongs to the family Melicaceae. The stem bark of *Entandrophragma angolense* is widely used in ethno medical treatment of various gastrointestinal disorders including peptic ulcer in humans [5]. Another specie of the plant; *Entandrophragma utile* was shown to reduce incidence of gastric ulcer but was ineffective for duodenal ulcer in experimental ulcer models used comparable with known standard drugs. Methyl angolense; a triterpenoid isolated from this plant was reported to have an antiulcer activity while the aqueous extract is used by herbalist in treatment of heart burn and stomach pain and malaria [6]. The important advantage of medicinal plants in various treatments is their safety besides being less expensive, efficacy and availability throughout the world [7]. However, combination of Ayurvedic knowledge with modern medicine can produce better antiulcer drugs of natural origin from medicinal plants with fewer side effects [8].

A previous study has reported that the antiulcer activity of *Entandrophragma angolense* methanolic extract is due to its ability to increase in gastric mucus secretion in association with increase gastric mucus cell counts [9]. This study thus aimed at investigating the likely antiulcer mechanisms of action of the methanolic extract of *E. angolense* in indomethacin induced gastric ulceration in male Wistar rats.

2. MATERIALS AND METHODS

The materials used were; Drinkers, Feeding plates, Syringes(Sizes; 1 ml, 2 ml, 5 ml and 10 ml), Disposable hand gloves, Oral cannula, Universal and Plain bottles, Weighing scale, Sensitive weighing scale, Dissecting sets, Centrifuge, Beakers, Pipette, Homogenizer, JASCO 410 FTIR Spectrophotometer, Water bath, Micro plate reader.

2.1 Chemicals and Drugs

The chemicals used in this study were of analytical grade and their solutions were prepared immediately before use, where applicable. The chemicals were; 10% formalin, Sodium Phosphate monobasic anhydrous (Na_2HPO_4), Sodium Phosphate dibasic anhydrous (NaH_2PO_4), Biuret reagent, Distilled water, Dettol hand wash, Sodium thiopental, Methanol, Indomethacin (Sigma), Omeprazole (Alpha laboratories Ltd, India), Absolute ethanol.

2.2 Experimental Animals

Twenty-five (25) adult male Wistar rats weighing between 180-200 g were used for this experiment. The animals were obtained from the Preclinical Animal House, Department of Physiology, College of Medicine, University of Ibadan. The animals were marked to permit individual identification, and kept in plastic cages

with wood chips renewed every alternate day. They were acclimatized for two weeks to the standard laboratory conditions (room temperature 25°C (± 3°C), humidity 35to 60%, light and dark period 12/12 hours. All animals had regular supply of clean drinking water with standard feeds obtained at Ladokun Feeds.

2.3 Experimental Design

The experimental Wistar rats were divided into five groups with five animals in each group;

Group I: Received distilled water
Group II: Received 200 mg/kg of extract
Group III: Received 400 mg/kg of extract
Group IV: Received 600 mg.kg of extract
Group V: Received Omeprazole 20 mg/kg body weight

Group 1 animals received distilled water for 28 days, and served as the positive control, while animals in groups II, III and IV were pre- treated with different doses of 200 mg/kg, 400 mg/kg and 600 mg/kg extract respectively daily for 28 days. Omeprazole reference group was administered 20mg/kg dissolved in distilled water.

The above groupings were used for the following studies; determination of changes in weight of rats pre-treated with the extract, experimental induction of gastric ulcer in male Wistar rats pretreated with the extract determination of the gross macroscopic ulcer dimensions and the histological changes in the gastric mucosa of the rats, determination of the effect of the extract on the hematological parameters, measurement of gastric tissue level of antioxidant enzyme by assaying for proteins; Superoxide Dismutase (SOD), Malondialdehyde (MDA) levels and Nitric Oxide (NO), determining the level of Prostaglandins E_2 level in rat pretreated with the methanolic extract of *Entandrophragma angolense*

2.4 Collection and Identification of Plant

The plant material is made up of the fresh stem barks of *Entandrophragma angolense* which were collected by Mr. Odewo; a Herbarium Staff of Forestry Research Institute of Nigeria, Ibadan, Oyo State, Nigeria in December 2015. The stem barks were identified, and a Voucher specimen (No: FHI 110883) was deposited at FRIN Herbarium. The stem barks were air dried for 6 weeks and were there after ground into a powdery form.

2.5 Soxhlet Extraction

The total weight of 1800 g dried stem bark of *Entandrophragma angolense* was processed for extraction. 300 g of the sample was packed inside the chamber of the Soxhlet extractor and was moistened with methanol. Subsequently 2litres of methanol was poured into the round bottom flask and the extraction process was carried out. This process was repeated for the remaining samples. The liquid extract was later concentrated using a rotary evaporator, and the paste was later dried further using vacuum oven to remove trace of solvent. The percentage yield was calculated as 14%.

2.6 Acute Toxicity Test

Acute toxicity test was performed according to the method described by [10]. Thirty-six female mice were divided into six groups were used in this study (n=6). They were kept in separate cages and were fed with mouse pellets and water ad libitum prior to the day of the experiment. They were fasted for 24 hrs prior to the study. Different doses of the extract were administered intraperitoneally to the separate groups. A lethal dose was regarded as one which resulted in the death of the animal within 24 hrs.

2.7 Indomethacin Gastric Ulcer Inductions

Twenty four hours prior to the commencement of gastric ulcer induction, the animals were deprived of access to feeds though had access to free water. All the groups received 40mg/kg indomethacin orally as earlier described by [6]. After four hours of indomethacin administration, the animals were sacrificed with high dose of thiopental sodium (50 mg/kg, intra-peritoneal). The stomachs were surgically removed and opened along the lesser curvature. Assessment of gastric mucosal lesion was expressed in terms of the ulcer index according to previous methods [11,12].

2.8 Scoring Criteria of Gastric Ulcer

0 - Normal gastric mucosa; no ulcer
0.5 - Punctuate hemorrhage / pinpoint ulcer
1.0 - Two or more hemorrhage ulcer (approximately 2 mm)
2.0 - Ulcer greater than 3 mm in diameter.

2.9 Determination of the Ulcer Index

Ulcer index was calculated as earlier described [6,13].

Ulcer Index = Mean ulcer score X Number of animals in a group / 100

2.10 Determination of Percentage Inhibition

Percentage Inhibition (%) was calculated as

PI (%) = {Ulcer index of control -Ulcer index of treated/Ulcer index of control} X 100

2.11 Histological Studies

The stomach samples were cut individually and were fixed in formalin, and then processed in an automated tissue processor; the stomach tissues were embedded, sectioned by microtome and stained with hematoxylin and eosin (H and E) stain. Each section was examined by light microscopy with magnification of x10, x40 and x100 [14]. The gastric tissue integrity (mucosa-submucosa) was then assessed for damage.

2.12 Determination of Antioxidant Activity

2.12.1 Homogenization of the stomach tissue

Each excised stomach tissue was initially blotted on the filter papers so as to remove blood and other extraneous tissues attached to it that may compromise the assays. The stomach tissue was then washed in ice cold 1.15% potassium chloride solution, weighed and cut into small pieces. 0.1 M phosphate buffer of at least four times the volume of the stomach was added and was homogenized using a Potter Elvegin Homogenizer. The homogenate was then poured into a test tube and centrifuged at 10,000 rpm at 4 degree for 10 minutes. The supernatant was carefully poured into a plain bottle for the determination of antioxidant enzyme (Superoxide Dismutase), endogenous Nitric Oxide, and determination of lipid peroxidation (Malondialdehyde) while the residue was discarded.

2.12.2 Determination of superoxide dismutase (SOD) activity

The SOD activity was determined by the method earlier described [15]. The ability of Superoxide Dismutase to inhibit the auto-oxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase.

2.13 Principle of Lipid Peroxidation Assessment

This was done by the method described by [16] in acidic conditions, the Malondialdehyde(MDA) produced from the peroxidation of fatty acid membranes react with the chromogenic reagent, 2-thiobarbituric acid (TBA) to yield a pink coloured complex with maximum absorbance at 532nm wavelength and fluorescence at 553nm. The pink chromophore is readily extractable into organic solvents such as butanol.

MDA (units/mg protein) = (Absorbance x Volume of mixture) / (E532nm x volume of sample x mg protein)

2.14 Indirect Determination of Nitric Oxide (NO)/Determination of Total Nitrite

Nitrite determination was done using the method described [17]. The assay relies on a diazotization reaction that was originally described [18]. The procedure is based on the chemical reaction which uses sulfanilamide and naphthylethylenediaminedihydrochlorate (NED) under acidic condition. Sulfanilamide and NED compete for nitrite in the Griess reaction.

2.15 Collection of Blood Samples

Blood samples were collected from the ocular vein of the animals into heparinized tubes for the determination of hematological parameters (full blood cell counts). The blood in the plain bottles was centrifuged at 3000rpm for 15min and the plasma was kept in the freezer for the assay of Prostaglandins E_2.

2.16 Determination of the Neutrophil / Lymphocyte Ratio

The full blood count was measured using an automated blood cell counter from where the Neutrophil/Lymphocyte ratio was calculated between the absolute neutrophil and absolute lymphocyte counts.

2.17 Principle of the Determination of Prostaglandins E Level

The ELISA KIT (Enzyme linked immunoabsorbent assay kit) ordered at E-lab

Science, was used for the assay of Prostaglandin E_2.The ELISA Kit utilizes Competitive-ELISA binding method. The microtiter plate provided in the kit has been pre-coated with PGE_2. During the reaction, PGE_2 in the sample or standard competes with a fixed amount of PGE_2 on the solid phase supporter for sites on the Biotinylated Detection Ab specific to PGE_2.Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme – substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measures spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of PGE_2 in the samples is then determined by comparing the OD of the samples to the standard curve.

2.18 Statistical Analysis

Data were expressed as Mean $^{\pm}$SEM (Standard Error of Means of five observations) and analyzed using ANOVA (Analysis of Variance) and student's test at P= 0.05.

3. RESULTS

3.1 Acute Toxicity Test

The animals were observed continuously for signs of toxicity and mortality at 0 min, 30 min, 60 min, 120 min and 24 hrs. There was no incidence of mortality recorded in the group I, 16.66% mortality was seen in group II, while group III with 200 mg/kg of the extract of *E. angolense* resulted in 66.66% mortality. Mice that had 600 mg/kg and 800 mg/kg body weight of the extract recorded 83.33% and 100% mortality respectively (Table 1). The LD_{50} of 275.42 mg/kg body weight intraperitoneally of the extract was obtained by extrapolation from the graph of percentage mortality against the log dose.

3.2 Effects of Methanolic Extract of *E. angolense* on Mean Ulcer Score, Ulcer Index and Percentage Inhibition

The Mean Ulcer Score was significantly reduced with 200 mg/kg *E. angolense* group (1.500± 0.333) and Omeprazole group (0.000±0.000) when compared with normal ulcer untreated group (5.833±0.333) and offers a percentage protection of 72.41% and 100.00% respectively.

The highest percentage inhibition was recorded with Omeprazole group, followed by the 200 mg/kg *E. angolense* and the 400 mg/kg *E. angolense* pre-treated groups (Table 2).

3.3 Effect of Methanolic Extract of *E. angolense* Pre-treatment on Antioxidant Enzyme Levels

3.3.1 Superoxide dismutase

Superoxide Dismutase activities were significantly elevated with all doses of *E. angolense* (200 mg/kg; 45.50±1.71, 400 mg/kg; 22.11±0.58, and 600 mg/kg; 22.76±0.98) as well as the 20 mg/kg Omeprazole group (26.06±0.88) when compared with the ulcer untreated group (19.67±0.17).

3.3.2 Malondialdehyde level (Lipid peroxidation)

Malondialdehyde (MDA) level was significantly reduced with doses of 200 mg/kg (0.59±0.03) and 400 mg/kg (0.55±0.03) extract of *E. angolense* groups, so also the 20 mg/kg Omeprazole group (0.54±0.02) when compared with ulcer untreated group (1.01±0.03). However, 600 mg/kg *E. angolense* extract showed significant increase in lipid peroxidation (1.53±0.12) compared to other treated groups.

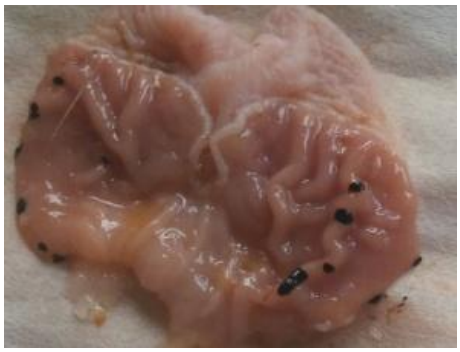
Table 1. Acute intraperitoneal toxicity study of methanolic extract of *E. angolense* in mice

Groups	Dose(mg/kg)	Log dose	No of death after 24 hour	Percentage mortality
I	50	1.70	0/6	0
II	100	2.00	1/6	16.66
III	200	2.30	2/6	33.33
IV	400	2.60	4/6	66.66
V	600	2.78	5/6	83.33
VI	800	2.90	6/6	100

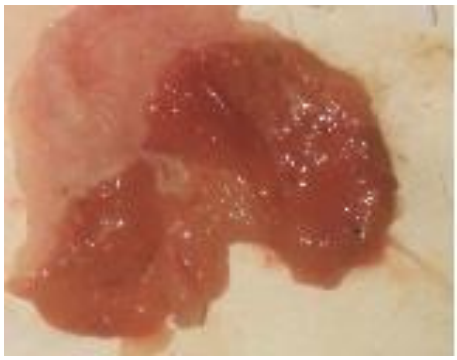
Table 2. Effect of *E. angolense* methanolic extract on mean ulcer score, ulcer index, and percentage inhibition

Groups	Mean ulcer score (MEAN ± SEM)	Ulcer index	Percentage inhibition (%)
Control	5.83±0.33	0.29	-
200 mg/kg Extract	1.50±0.33[a]	0.08	72.41
400 mg/kg Extract	4.44±1.16[b]	0.22	24.14
600 mk/kg Extract	5.67±1.01[bc]	0.28	3.45
Omeprazole	0.00±0.00[a]	0.00	100

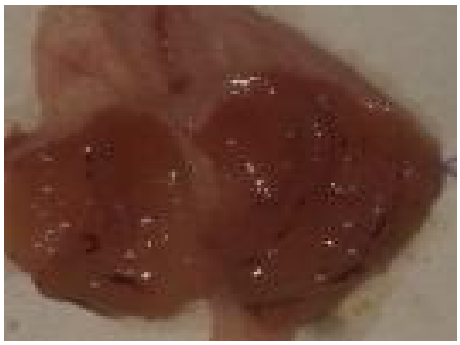
Values are expressed as Mean ± SEM. Values are significant at p=0.05 and n=5.
Keys of significance; [a] compared with normal untreated, [b]-compared with Omeprazole, [c]-compared with 200 mg/kg E. angolense,



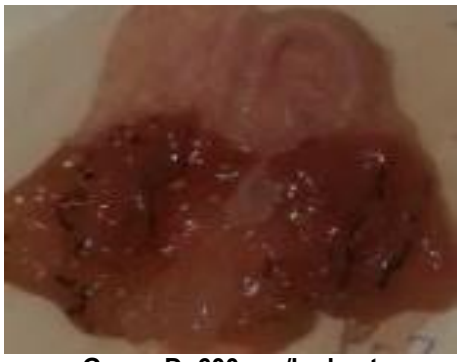
Group A: Control



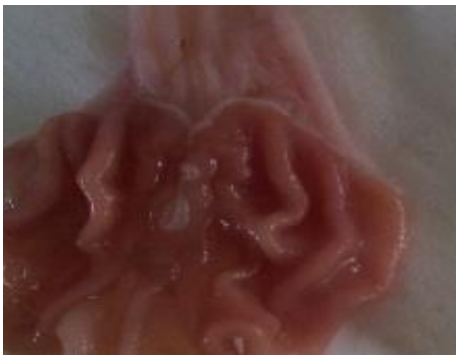
Group B: 200 mg/kg b.wt



Group C: 400 mg/kg b.wt



Group D: 600 mg/kg b.wt



Group E: 20 mg/kg Omeprazole

Plate 1. Macroscopic views of stomach samples of the various pre-treated groups

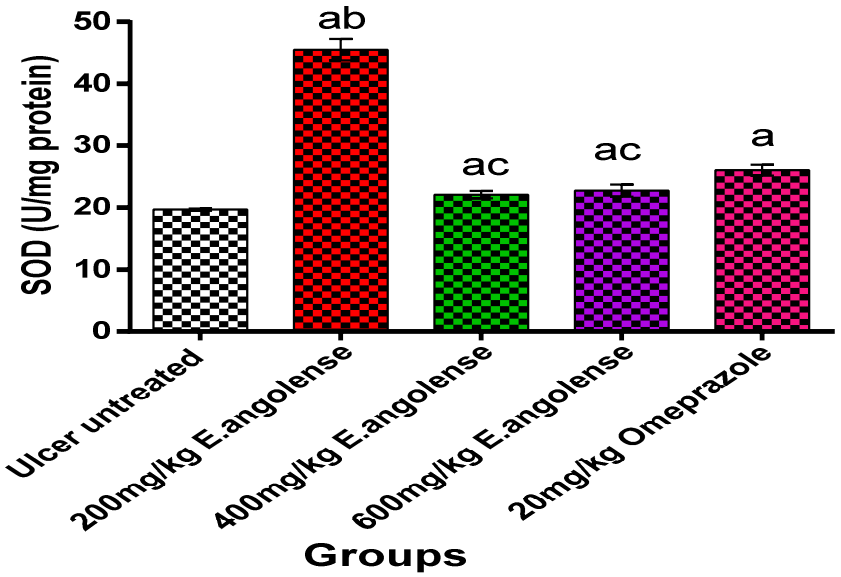


Fig. 1. Effect of *E. angolense* on superoxide dismutase
Keys of significance; [a]–compared with ulcer untreated, [b]-compared with Omeprazole [c]-compared with E. angolense 200 mg/kg, [d]-compared with E. angolense 400 mg/kg

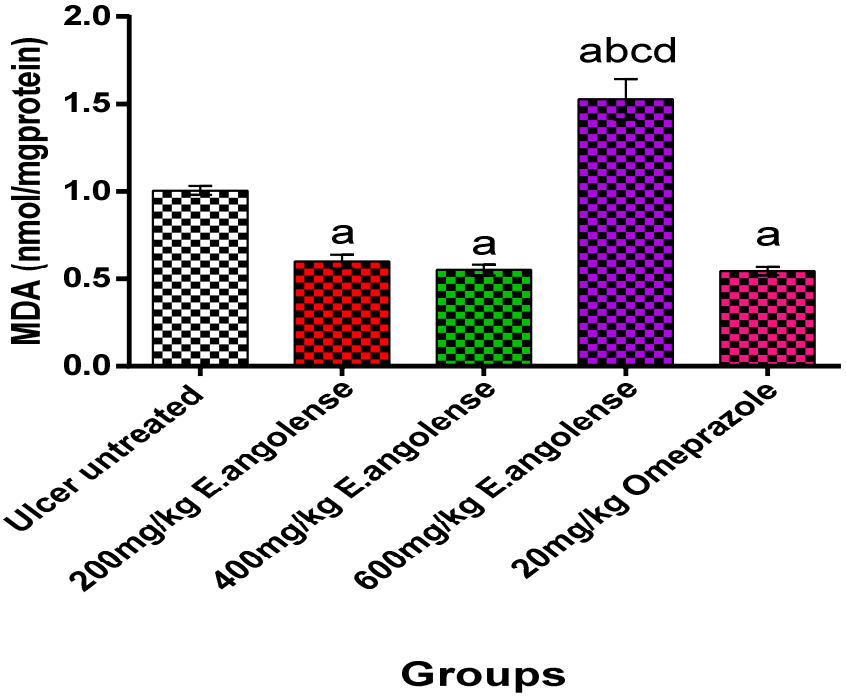


Fig. 2. Effect of *E.angolense* on malondialdehyde levels
Keys of significance; [a]– compared with ulcer untreated, [b]-compared with Omeprazole, [c]-compared with E. angolense 200 mg/kg, [d]-compared with E. angolense 400 mg/kg

3.4 Effect of Methanolic Extract of *E. angolense* Pre-treatment on Nitrite Levels (NO)

All concentrations of *E. angolense* extract produced dose-dependent increase in nitrite levels*;* 200, 400, but with out of phase drop in value at 600 mg/kg (15.89±1.11; 93.11±5.56; 45.33±2.10). These values are significantly higher than that of the control (5.06±0.28). 20 mg/kg Omeprazole (13.67±1.74) caused a slightly lower nitrite concentration to that of 200 mg/kg extract. The result is presented in Fig. 3.

3.5 Effect of Extract on Neutrophil/ Lymphocyte Ratio (NLR)

The groups pre-treated with *E. angolense* (400 mg/kg and 600 mg/kg) showed significant increase in NLR when compared with the 20 mg/kg Omeprazole group and 200 mg/kg *E. angolense.* The results are represented in Fig. 4.

3.6 Effect of Methanolic Extract of *E. angolense* Pre-Treatment on Prostaglandins E_2Concentration

Various concentrations of *E. angolense* extract 200, 400 and 600 mg/kg significantly increased Prostaglandin E_2 concentration (1396.00±60.23; 1192.00±14.13 and 1316.00±57.68) compared with control.

4. DISCUSSION

Entandrophragma angolense is considered an herbal therapy for gastrointestinal disorders and it has been shown to have significant anti-ulcer property [6].

In the study of the effect of the pre-treatment of animals with *Entandrophragma angolense* on the macroscopic assessment, the control group (ulcer untreated) showed profound gastric injury in the form of haemorrhages in pinpoints and streaks when observed macroscopically. The glandular gastric mucosa of the 200 mg/kg extract and 20 mg/kg Omeprazole (positive control) is intact (Plate 1: Groups B&E) while that of high doses extract (Groups C&D) revealed mucosa erosion of the epithelium and bleeding. This result agrees with the findings from the mean ulcer score and ulcer index. Omeprazole, a proton pump inhibitor has been found to be the most effective suppressors of gastric acid secretion via the gastric H^+/ K^+-ATPase. [19-21], and also exert antioxidant and anti-inflammatory action beyond acid suppression.These two groups exhibited significant reduction in the mean ulcer score and ulcer index (P=0.05), thus producing 74.40% and 100.00% ulcer inhibition respectively. This result is agreement

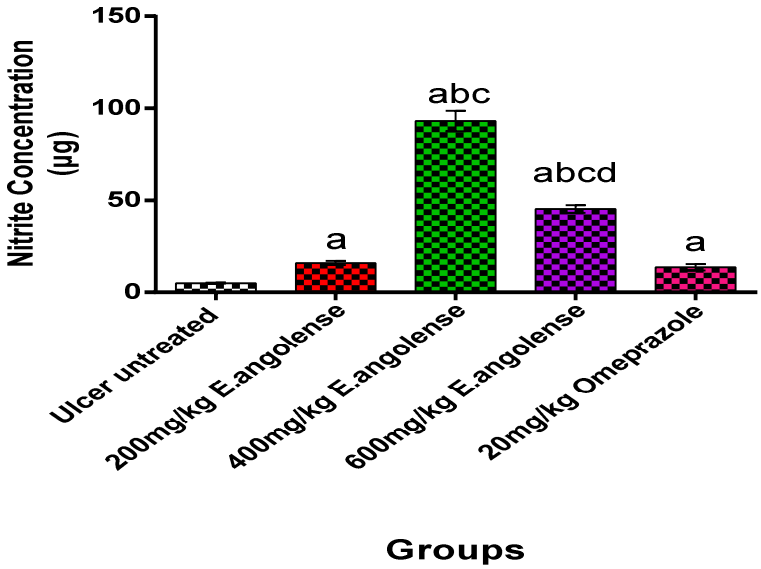


Fig. 3. Effect of *E. angolense* extract on nitrite concentration

Keys of significance; [a]– compared with ulcer untreated, [b]-compared with Omeprazole, [c]-compared with E. angolense 200 mg/kg, [d]-compared with E.angolense400 mg/kg

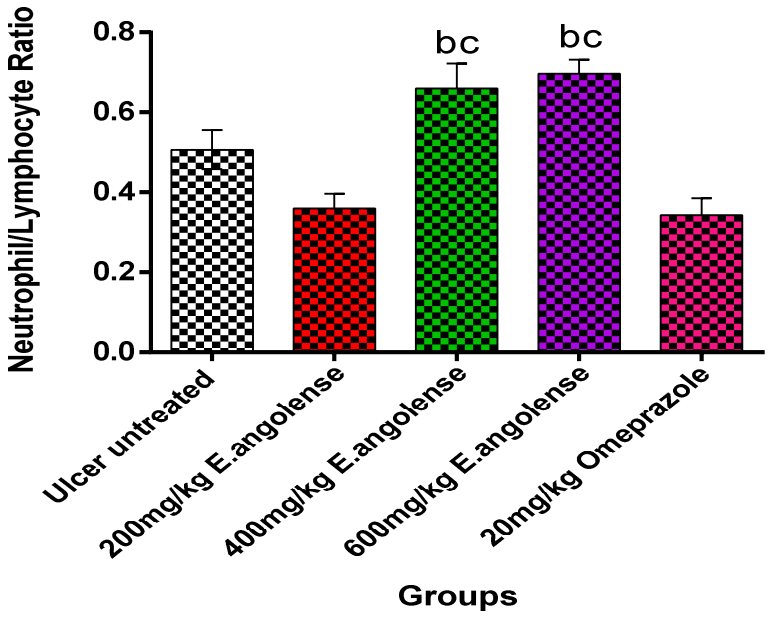


Fig. 4. Effect of *E. angolense* extract on Neutrophil/ lymphocyte ratio

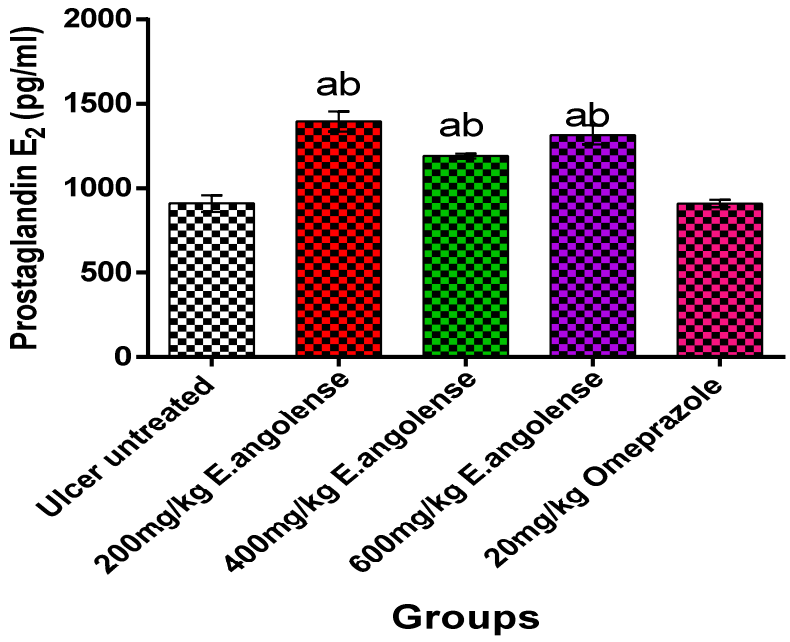


Fig. 5. Effect of *E. angolense* on Prostaglandin E_2 level

Keys of significance; [a]– compared with ulcer untreated, [b]-compared with Omeprazole, [c]-compared with 200 mg/kg E. angolense

with earlier findings [6] that methanolic extract of the stem bark of *E. angolense* has antiulcer activity. However, there was significant increase in the mean ulcer score of groups pre-treated with 400 mg/kg and 600 mg/kg *E. angolense* when compared to the control group. It should be noted that these doses are exceedingly higher than the LD_{50} of 275.42 mg/kg body weight

recorded in mice in this study. The highest preventive ulcer index (percentage inhibition) was recorded with the Omeprazole group, followed by the *E. angolense* 200 mg/kg.

Gastroprotection of the gastric mucosa is said to be controlled by the gastric microcirculation. Prostaglandin of the E series (PGE_2) is a potent vasodilator, producing its effect in the stomach through EP2/EP4 receptors [22,23]. It increases mucosal blood flow, thereby increasing the resistance of the gastric mucosa to injury [24]. In this study, the PGE_2 concentration was significantly increased across all groups pretreated with the varying doses of *E. angolense* when compared with the control group and the Omeprazole group. This implies that the extract of *E. angolense* mediates its gastroprotective ability via the production of PGE_2. This is further justified by the modulation of prostaglandin biosynthesis by nitric oxide as there was equally a significant increase in the nitric oxide concentration across all the groups pretreated with *E. angolense* (Fig. 5).

Studies have shown that neutrophils infiltrate in gastric damage and have been found to play a role in the development of injury and inflammation in tissues of the body [25]. In this study, there was no significant difference in neutrophil counts, and neutrophil lymphocyte ratio (NLR) in the groups pretreated with 200 mg/kg *E. angolense* when compared with the control group. Similar observation is seen with Omeprazole. Neutrophil lymphocyte ratio (NLR) is a potent marker for inflammation, and is associated with prevalent chronic conditions. This follows that the above dose of *E. angolense* is protective to the gastric mucosa as against the high doses, 400 and 600 mg/kg *E. angolense* which had significantly higher values compared to the control (P=0.05).

There was a significant increase in SOD level with 200 mg/kg *E. angolense* and omeprazole (P=0.05) when compared with the control group. SOD dismutates the reactive superoxide ion into less reactive hydrogen peroxide [26], and it also alongside with catalase and glutathione subsequently scavenge the hydrogen peroxide and break it into water and oxygen [27]. Similarly, in the study on lipid peroxidation using Malondialdehyde (MDA) as its indicator, the other two groups (200 and 400 mg/kg) pretreated with *E. angolense* as well as the 20 mg/kg Omeprazole group had significantly low values of MDA when compared with the control group. The reduced values in the groups is probably due to the ability of the extract to develop resistance to lipid peroxidation of the gastric tissues, while the increased value in the group pretreated with 600 mg/kg *E. angolense* can be justified from the toxic effect of this dose. This is observed in the macroscopic assessment of this group (Plate 1).

5. CONCLUSION

These findings suggest that the anti-ulcer mechanism of actions of the methanolic extract of the stem bark of *Entandrophragma angolense* might be mediated via the following; scavenging of free radical species by the production of antioxidant enzymes which include Superoxide Dismutase, generation of nitric oxide and increase production of endogenous Prostaglandins (PGE_2).

Furthermore, the gastroprotective potency of the extract is dose specific in that the group pretreated with 200 mg/kg had the most potent gastroprotective ability.

CONSENT

It is not applicable.

ETHICAL APPROVAL

We hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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