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Effects of L-arginine and L-citrulline on Indomethacin-Induced Gastric Ulceration and Gastric pH in Male Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors DU and OFS designed the study, performed the statistical analysis, and wrote the protocol. Authors OFS and ATG managed the analyses of the study. Author ATG managed the literature searches and wrote the first and subsequent drafts of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The anti-ulcerogenic activity of L-arginine and L-citrulline were evaluated in indomethacin-induced gastric ulceration. Degree of ulcerogenesis, gastric pH and microscopic histological evaluation were carried out.
Study Design: Six groups of albino rats weighing between 180-280g were pre-treated respectively with distilled water (ulcer control), omeprazole (20mg/kg, reference control 1), cimetidine (100mg/kg reference control 2), L-arginine (experimental control 1), 300mg/kg and 900mg/kg L-citrulline (experimental controls 2 and 3).
Place and Duration of Study: Department of Physiology, College of Medicine. University of Ibadan, Nigeria between April 2012 and February 2013.
Methodology: Forty-eight albino rats weighing between 180-280g were pre-treated respectively with distilled water (ulcer control), omeprazole (20mg/kg, reference control 1), cimetidine (100mg/kg reference control 2), L-arginine (experimental control 1), 300mg/kg and 900mg/kg L-citrulline (experimental controls 2 and 3) sixty minutes prior to oral administration of indomethacin to generate gastric mucosal injury.). Ulcer was induced using 40mg/kg BW Indomethacin. Four hours later, rats were sacrificed and gastric contents as well as stomach wall samples were collected. Gastric ulcer score was determined macroscopically as well as gastric pH. Tissue samples were also prepared

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and examined histologically
Results: With gross examination, ulcer control exhibited severe injury to the gastric mucosa and decreased pH of gastric contents, whereas rats pre-treated with L-arginine and L-citrulline showed significant dose-dependent reduction of gastric lesion formation accompanied by significant increase in gastric mucus production and pH of gastric fluid. Gastric protection was more prominent in L-arginine (300mg/kg) and L-citrulline (900mg/kg) groups. Histologically, the ulcer control showed the most severe and deepest gastric mucosal necrotic damage, with oedema of the submucosal layer compared to experimental and reference control groups.
Conclusion: The results suggest that a possible explanation for the protective activity of L-arginine and L-citrulline may be due its stimulation of defensive mucin secretion and a consequent increase in pH of gastric contents, which result in less mucosal injury and limited or absent oedema of submucosa.

Keywords: Arginine; citrulline; ulcer; gastric pH.

1. INTRODUCTION

Peptic ulcers are deep gastrointestinal erosions that involve the entire mucosal thickness, penetrating the muscularis mucosae [1]. They are formed in the mucosa of the stomach, duodenum, lower oesophagus and Merkel's diverticulum. For a long time it was believed that gastric and intestinal ulcerations were caused by excessive secretion of gastric acid, but many patients presenting such ulcerations had normal acid secretion rates [2]. Researchers reported that peptic ulcers were caused by an imbalance between the aggressive factors and a number of known defence mechanisms. The aggressive factors include; anti-inflammatory drugs, alcohol, psychological stress, fatty foods and *Helicobacter pylori* infection-triggered tissue necrosis [3] with their actions being carried out via mucosal ischemia, free radical generation and cessation of nutrient delivery, increased hydrochloric acid together with pepsin secretion, and pancreatic enzymes and bile decrease. The defence mechanisms of gastrointestinal mucosa are local blood flow, mucus/bicarbonate secretion and cellular growth [4,5]. Aspirin is one of the most widely used NSAIDs and it causes ulceration of gastrointestinal mucosa by irritant action, causing alteration in mucosal permeability or suppression of Prostaglandin synthesis [6]. The use of non-steroidal anti-inflammatory drugs (NSAIDs) is limited by their ulcerogenic activity as well as the ability to interfere with ulcer healing. These effects are mainly mediated through inhibition of prostaglandin synthesis [7].

NO donors have been repeatedly shown to protect gastric mucosa against damage induced by various agents [8,9,10]. However, the role of NO in regulation and maintenance of the functions of gastric mucosa has not yet been fully understood.

Considering the several side effects of modern medicine, indigenous drug therapy with fewer side effects is being looked upon as a better alternative for the treatment of peptic ulcer [11]. Peptic ulcer therapy through a combination of drugs has not been very effective due to non-compliance with drug regimen by patients and possible side effects of the drugs being taken continuously [12].

Citrulline is a non-essential amino acid first identified from the juice of watermelon, *Citrullus vulgaris* Schrad [13]. It was later obtained from tryptic digestion of casein [14]. In most

mammals, the small intestine is the major source of circulating citrulline which is utilized in the endogenous synthesis of arginine [15]. L-Citrulline given orally to children and adolescents with sickle cell disease resulted in improvement of symptoms, raised plasma arginine levels, and reduced elevated total leukocyte and segmented neutrophil counts to within normal limits [16]. Citrulline is a co-product of nitric oxide generated from the oxidation of arginine catalyzed by nitric oxide synthase. Nitric oxide (NO) functions as a cellular messenger in the cardiovascular system and is a pivotal vasoprotection molecule. However, Citrulline is also an efficient hydroxyl radical scavenger and is a strong antioxidant [17,18].

L-Arginine is a semi-essential amino acid involved in numerous areas of human physiology, including production of nitric oxide (NO) – a key messenger molecule involved in vascular regulation, immune activity, and endocrine function. Arginine is also involved in protein production, wound healing, erectile function, and fertility. Arginine is not considered essential because humans can synthesize it de novo from glutamine, glutamate, and proline, which are substrates for the synthesis of citrulline in the small intestine. Citrulline is then released into the blood circulation, where it is extracted primarily by the kidneys for conversion to arginine (intestinal-renal axis of arginine synthesis). Arginine is then released into the circulation [19]. The citrulline which exists in watermelon especially in the rind is a known stimulator of arginine and nitric oxide synthesis. Nitric oxide is thought to relax and expand blood vessels and also perform other functions as explained earlier.

Previous work by the authors has demonstrated that L-citrulline or L-arginine has gastro protective effects in other gastric lesion models. Limited recorded data is obtainable on research involving the isolated form of L-Citrulline and L-Arginine on indomethacin-induced ulceration; hence, the aim of this study was to evaluate and compare the possible anti-ulcerogenic activity of L-Citrulline and L-Arginine with reference drugs; Omeprazole, a proton pump inhibitor and Cimetidine, a H_2 blocker.

2. MATERIAL AND METHODS

2.1 Experimental Design

2.1.1 Animals

Thirty-six (36) male albino rats of wistar strain weighing between 180-280g were obtained from the animal house of the Department of Physiology, University of Ibadan for the gastric output, gastric juice pH, mean ulcer index and preventive ulcer index studies. They were randomly divided into six (6) groups of six (6) rats each. Female rats were not used because ulceration and gastric secretion are known to vary with phases of oestrus cycle. The animals were maintained under standard laboratory conditions and were fed with rat pellets (Ladokun feeds) and tap water *ad libitum*. The animals were allowed free access to food and water. These were withdrawn 24 hours to the commencement of ulcer induction. The groupings of the animals are as follows:

Group 1 (Normal)

These animals were pre-treated with 5ml/kg of distilled water orally an hour prior to ulcer induction.

Group 2 (Positive Control[1])

These animals were pre-treated with 20mg/kg of Omeprazole orally an hour prior to ulcer induction.

Group 3 (Positive control[2])

These animals were pre-treated with 100mg/kg of Cimetidine orally an hour prior the induction of ulcer.

Group 4

These animals were pre-treated with 300mg of L-arginine intraperitoneally an hour prior to ulcer induction.

Group 5

These animals were pre-treated with 300mg/kg of L-Citrulline intragastrically an hour prior to the induction of ulcer.

Group 6

These animals were pre-treated with 900mg/kg of L-Citrulline intragastrically an hour prior to the induction of ulcer.

2.2 Drug Preparation and Treatment

L-Citrulline (L-CIT) (Biovea, 1000 de la Gauchetiere street west, Suite 2400, Montreal, QC, Canada H3B 4W5) was dissolved in distilled water. It was prepared freshly each time and given at different doses (300 and 900mg/kg) intragastrically 60 minutes before the experiment. Control received 5ml/kg of distilled water by oral route. L-Arginine (L-ARG) (Lab Tech Chemicals, China) was dissolved in distilled water and given intraperitoneally. Omeprazole 20mg/kg (DR.REDDY's, Bachupally-500 072, A.P., India) was dissolved in distilled water and given orally. Cimetidine 100mg/kg (Jiangxi, Xier Kangtai pharmaceutical Co. Ltd, Pingxiang, Jiangxi, China) was dissolved in distilled water and given orally.

2.3 Experimental Induction of Ulceration

Ulceration was induced in rats with Indomethacin according to method proposed by Elegbe and Bamgbose. After pre-treating the animals with distilled water, Omeprazole, Cimetidine, L-Arginine and L-Citrulline, ulcer was induced with Indomethacin dissolved in 1% Sodium bicarbonate a dosage of 40mg/kg. The drug was administered orally. Indomethacin is almost insoluble in water but, totally soluble in 1% Sodium bicarbonate.

After 4 hours of administration, the rats were sacrificed and the stomachs surgically removed and opened up by an incision along the lesser curvature. Macroscopic examination of the stomach was carried out with a hand lens at X2 magnification. The method used for assessment of the degree of ulceration was that of Alphin and Ward [20] modified by Elegbe and Bamgbose [21].

Table 1. Criteria for gastric ulcer scoring (Elegbe and Bamgbose, 1976 [21])

Gastric ulcer Score	Criteria
0	Normal stomach (No Ulcer)
0.5	Punctuated haemorrhage/pin-point ulcer
1.0	Two or more small haemorrhage ulcer
2.0	Ulcer greater than 3mm in diameter

2.4 Determination of Gastric Ph

The gastric pH was determined by the use of a standard pH meter.

2.5 Determination of Gastric Acid Secretion (Gastric Acid Output)

This was performed by the method of Shay et al. [22] and Hara et al. [23], modified by Gehan et al. [24]. Four (4) hours after the induction of gastric ulcer, the abdomen was opened to remove the stomach and gastric content was collected to determine the gastric juice volume (ml).5ml of distilled water added and the resultant solution was centrifuged at 3000 rpm for 10 minutes. Gastric juice pH values were determined by the use of digital pH meter and gastric acid output in µEq/L was determined in the supernatant volume by titration with 0.0025N of sodium hydroxide.

2.6 Determination of Preventive Ulcer Index (%)

Preventive Ulcer Index (P.U.I %) was later calculated as follows:

$$\text{P.U.I \%} = \frac{\text{Ulcer index of Control} - \text{Ulcer Index of treated}}{\text{Ulcer Index of Control}} \times 100$$

2.7 Preparation of Histological Specimens

Gastric mucosal injury was assessed according to a method described previously [25]. Briefly, the tissue from the gastric mucosa of each animal was fixed in 10% formaldehyde, dehydrated in grade ethanol, and embedded in paraffin wax. Sections were cut at 5µm, mounted on clean glass slides, and dried overnight at 37ºC. The sections were cleared, hydrated, and stained with Periodic Acid Schiff for light microscopic observation. Blind analysis was performed on all samples in an Olympus BH-2 microscope for characterization of histopathological changes.

2.8 Statistical Analysis

The data were analysed using ANOVA and were expressed as Mean ± Standard error of Mean (Mean±SEM).Statistical significance was obtained at $P < 0.05$.

3. RESULTS AND DISCUSSION

Table 2 shows the effects of L-Arginine and L-Citrulline on Mean Ulcer Score, Percentage Inhibition, Total Acidity and pH in male albino rats

Table 2. Effects of L-Arginine and L-Citrulline on Mean Ulcer Score, Percentage Inhibition, Total Acidity and pH

Animal Groups	Treatments	Mean Ulcer Score (Mean+SEM)	Percentage Inhibition %	Total Acidity (Mean+SEM) µEq/5ml	pH (Mean+SEM) Units
1	Distilled Water (Control; 5ml/kg)	15.75±2.03	-	43.85±3.31	2.99±0.27
2	Omeprazole (20mg/kg)	12.25±2.03	22.22	25.22±4.63[a]	5.16±0.18[a]
3	Cimetidine (100mg/kg)	0.88±0.13[a]	94.41	3.68±0.85[a]	4.61±0.17[a]
4	L-Arginine (300mg/kg)	4±0.36[abc]	74.60	13.82±0.79[ab]	6.69±0.29[abc]
5	L-Citrulline (300mg/kg)	13.63±1.11[b]	13.46	18.47±2.72[ab]	5.94±0.37[ab]
6	L-Citrulline (900mg/kg)	3±1.56[ac]	80.95	8.85±1.49[abc]	2.96±0.59[bc]

n= number of animals= 6

[a]P> 0.05 implies statistically significant when compared with Control

[b]P>0.05 implies statistically significant when compared with Cimetidine (Standard drug)

[c]P>0.05 implies statistically significant when compared with Omeprazole (Standard drug)

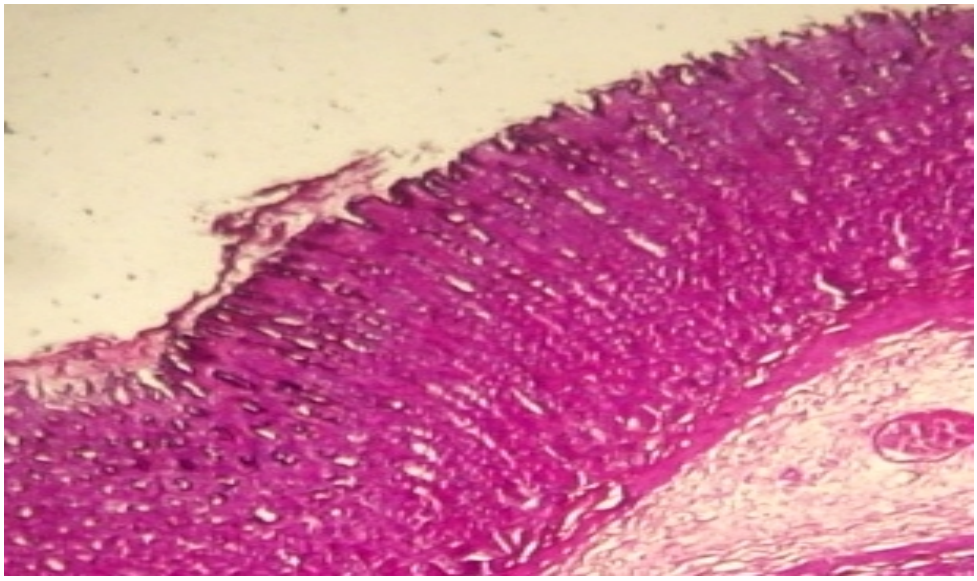

Fig. Ia.

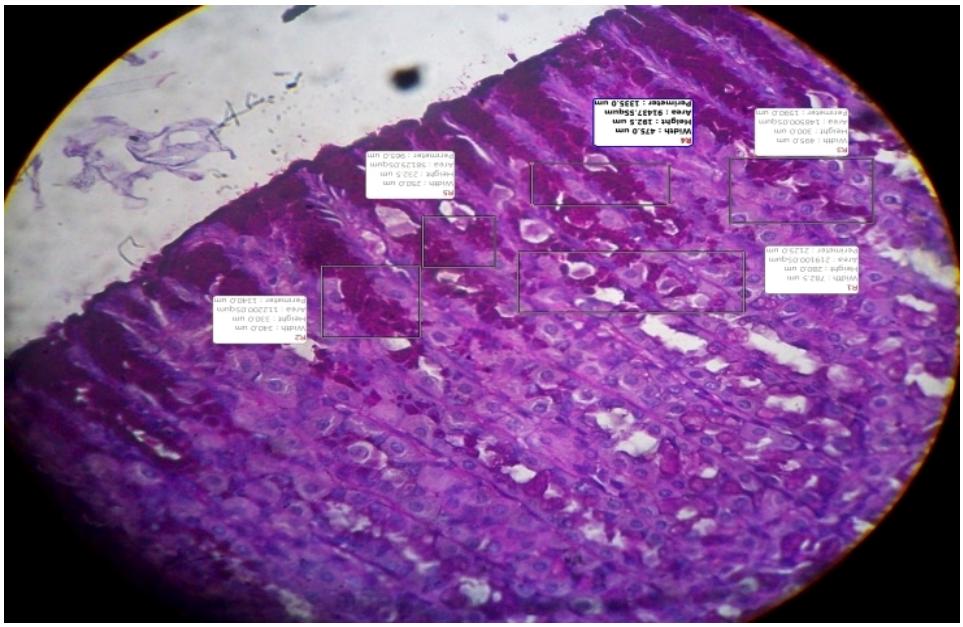

Fig. Ib.

Group I: This group represents the histological section of the gastric mucosa in rats pre-treated with distilled water (control). Figures 1a and 1b show low and high magnification cross-sections of the surface epithelium, mucosal and submucosal layers. There is severe disruption of the surface epithelium, deep penetration of necrotic lesions into mucosa and edema of the submucosa layer with leukocyte infiltration of ulcerative tissues and fewer goblet cells (PAS.10x)

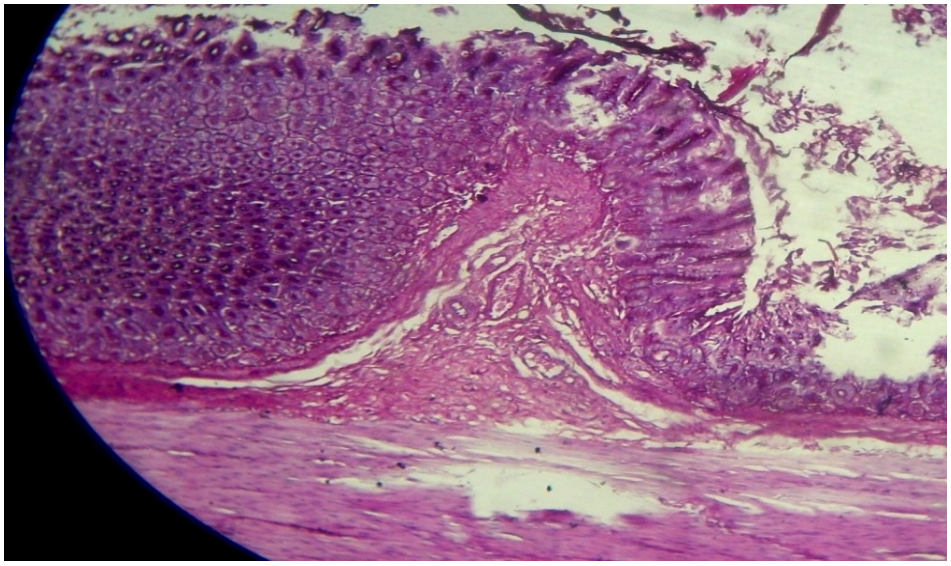

Fig. 2a.

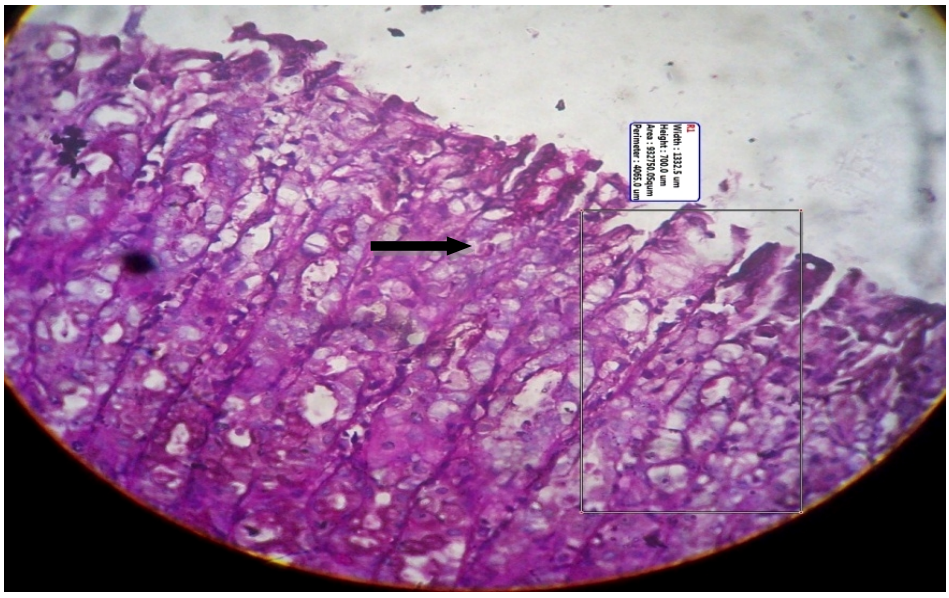

Fig. 2b.

Group 2: This group represents the histological section of the gastric mucosa in a rat pre-treated with Omeprazole (20mg/kg).Plates 2a and 2b show the magnified cross-sections of the surface epithelium, mucosa and submucosa layers of the rats' stomachs. Mild erosion of the surface epithelium and mucosal glands with mucosal debris in the lumen. The glandular cells appear enlarged and ballooned (Arrow). Goblet cells are few .This is showing an evidence of mild ulceration (PAS.10x)

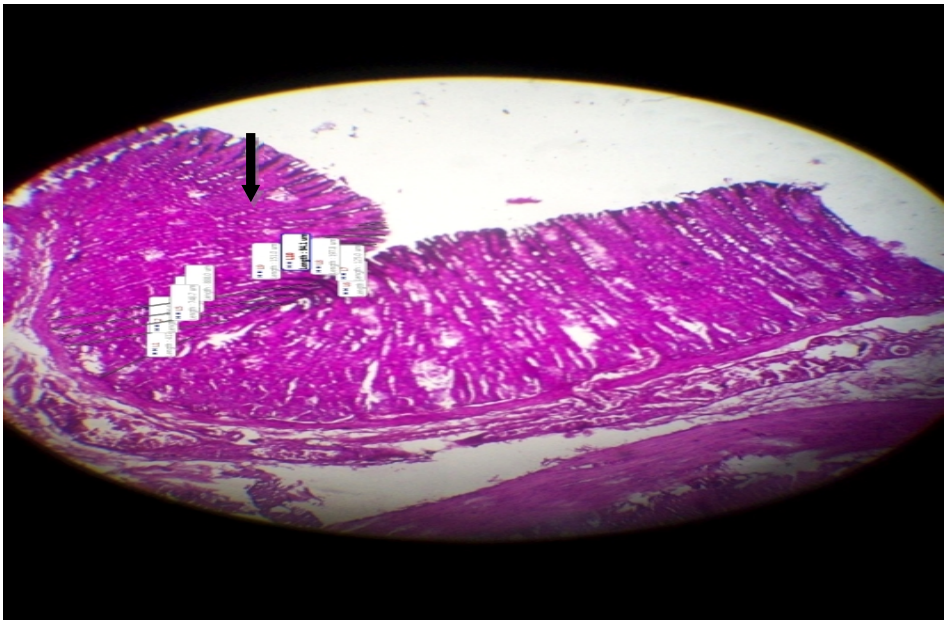

Fig. 3a

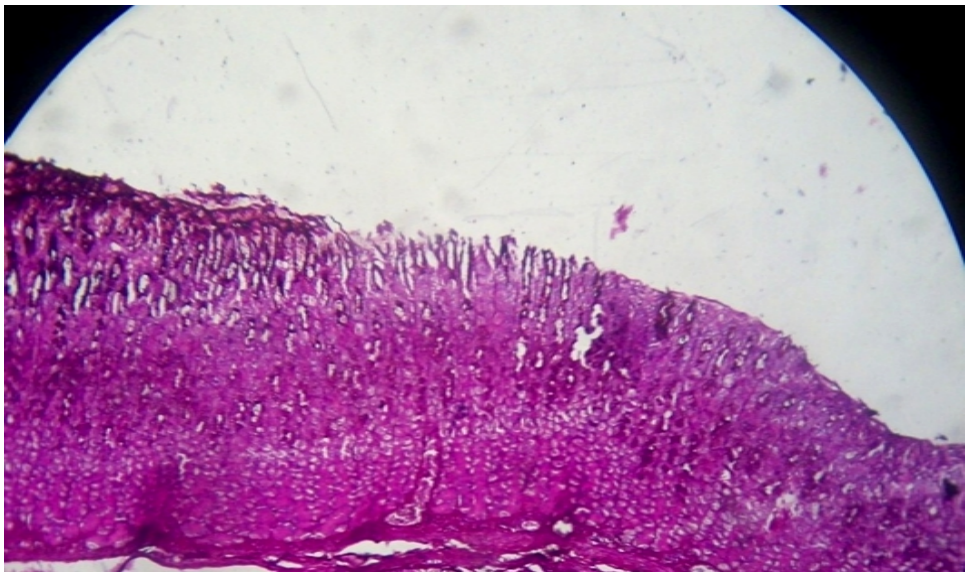

Fig. 3b.

Group 3: This group represents the histological section of the gastric mucosa in a rat pre-treated with Cimetidine (100mg/kg).Plates 3a and 3b show the magnified cross-sections of the surface epithelium, mucosa and submucosa layers of the rats' stomachs with very mild erosion (arrow) of the epithelial surface with loss of goblet cells (PAS 10x).

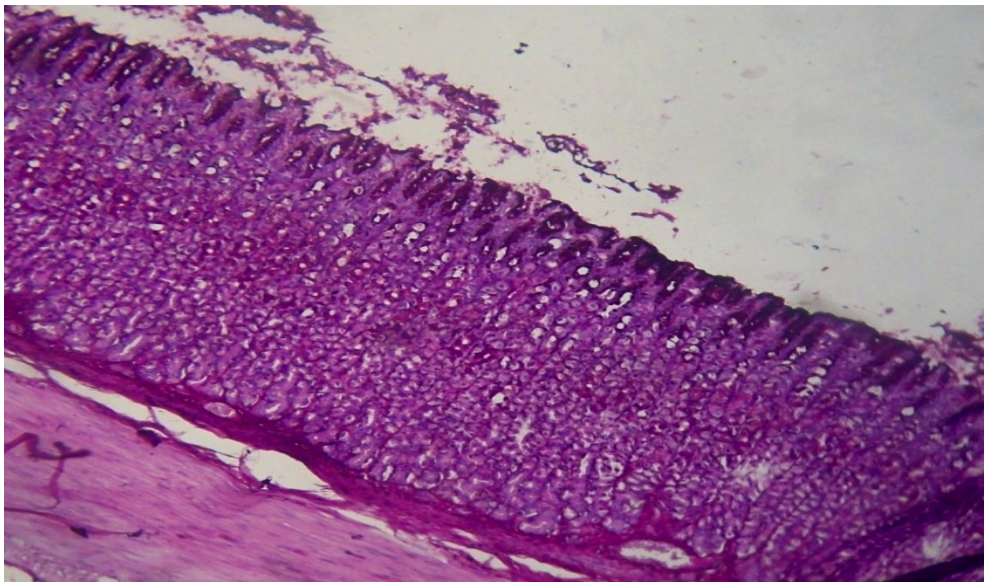

Fig. 4a.

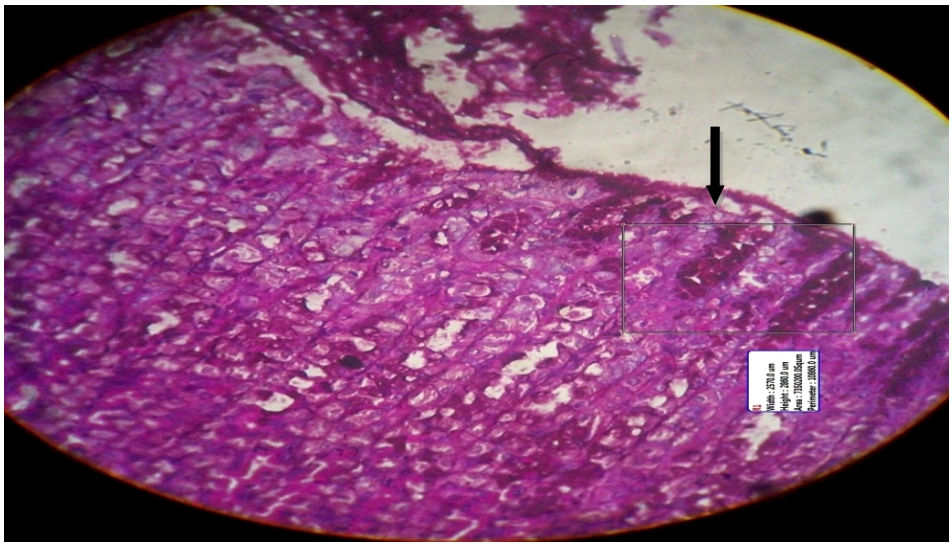

Fig. 4b

Group 4: This shows histological section of the gastric mucosa in a rat pre-treated with L-Arginine (300mg/kg).Plates 4a and 4b show the magnified cross sections of the surface epithelium, mucosa and submucosa layers of the rats' stomachs. There is mild erosion of the surface epithelium and presence of few goblet cells (arrow). The epithelium is partially broken. (PAS 10x)

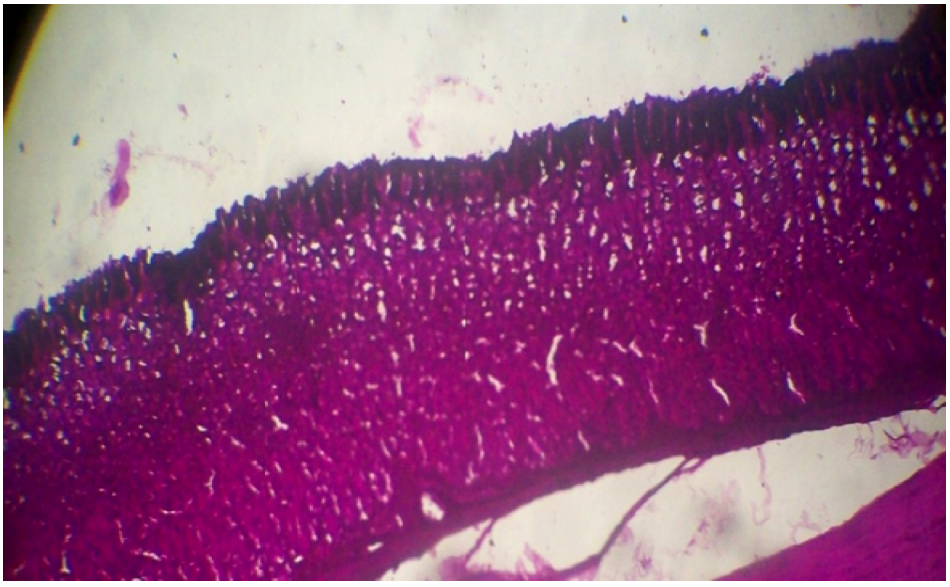

Fig. 5a.

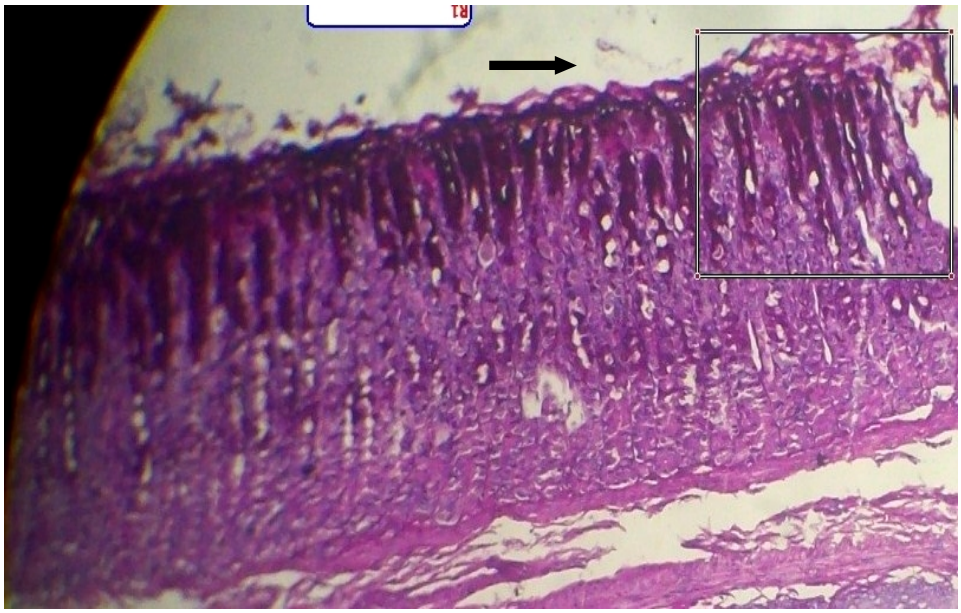

Fig. 5b

Group 5: This group represents histological section of the gastric mucosa in a rat pre-treated with L-Citrulline (300mg/kg).Plates 5a and 5b show the magnified cross-sections of the surface epithelium, mucosa and submucosa layers of the rats' stomachs. There is mild erosion of the epithelial surface and presence of globlet cells (arrow) (PAS 10x)

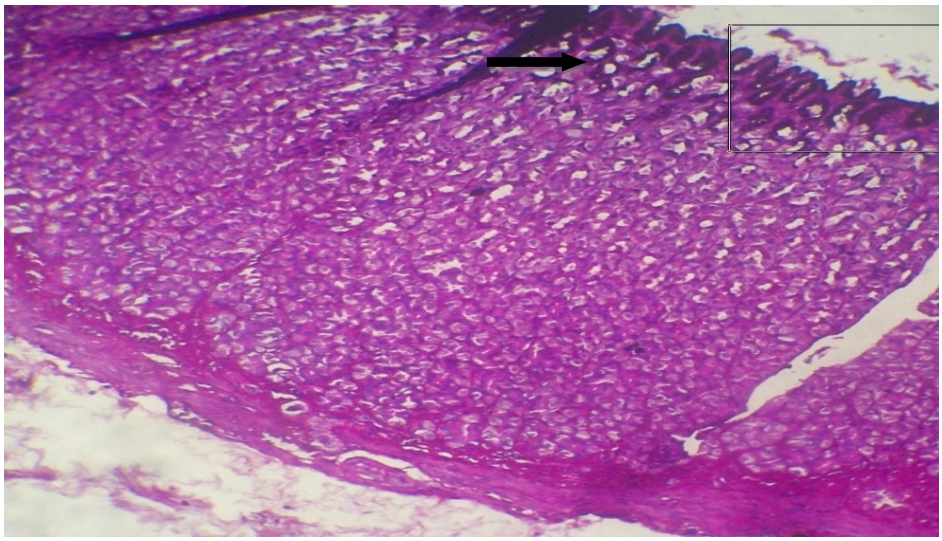

Fig. 6a

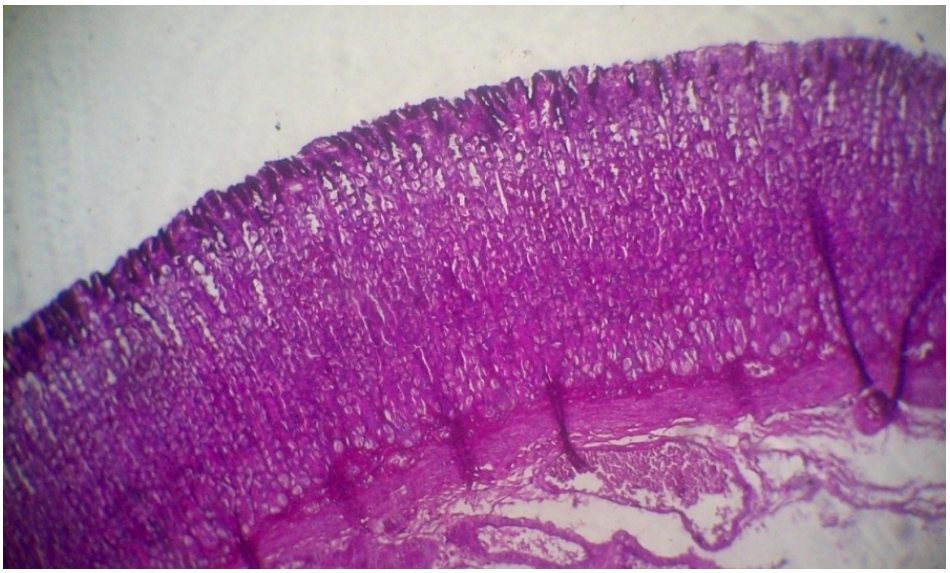

Fig. 6b.

Group 6: This group represents the histological section of the gastric mucosa in a rat pre-treated with L-Citrulline (900mg/kg).Plates 6a and 6b show the magnified cross-sections of the surface epithelium, mucosa and submucosa layers of the rats' stomachs. There is partial erosion of the surface epithelium and is very mild, and there is no submucosal edema and no leucocytes infiltration and presence of numerous goblet cells (arrow) (PAS.10x)

4. DISCUSSION

Gastrointestinal injury is induced by various chemical agents [26]. The induction of gastric lesions by Indomethacin is a popular method of screening plant extracts for anti-ulcer potency in experimental animal models. It has been reported that Indomethacin administration produced ulceration after four (4) hours and that dosages between 30 and 40mg/kg body weight of indomethacin were capable of producing gastric ulceration [27,28]. The cytoprotective properties of a plant are evaluated by assessing the sizes of macroscopically and microscopically visible lesions that are induced in a gastric mucosa. The pathological effect of Indomethacin, according to Menguy and Desbaillets [29], was its exertion of an ulcerogenic effect by decreasing the rate of secretion of gastrointestinal mucus and the lowering of the concentration of the carbohydrate component of the gastric mucous substance. Reduction in the mucous secretion will expose mucous lining of the GIT to the action of acid-pepsin secretion resulting in lesions.

Omeprazole is a proton pump inhibitor used for the treatment of peptic ulcers. In Table 1, the group II animals that were pre-treated with only Omeprazole had significantly reduced Mean Ulcer Score, Mean Total Acidity and Percentage Inhibition of 22.22 while the mean gastric pH was significantly increased compared with the control ($P<0.05$). Plates IIa and IIb showing histological appearance of rats' stomach in group II confirmed partial erosion as the epithelial cells were partially aligned. However, the histological appearance of rats' stomach

in the normal control group showed numerous haemorrhagic ulcers. It also showed split open blood vessels, mucosal layer damage and broken epithelium.

Cimetidine is a H_2-receptor antagonist which works by decreasing the amount of acid produced in the stomach. Animals pre-treated with Cimetidine in group III had the lowest significantly reduced Mean ulcer Score, Mean total acidity and the highest Percentage inhibition. The mean gastric pH was also significantly increased when compared with the control ($P<0.05$). Plates IIIa and IIIb representing stomachs of animals in group III show that there is mild disruption of the surface epithelia; an evidence of mild ulceration. This is however very slight when compared with the mucosal disruption in the control.

In group IV, animals were pre-treated with L-arginine Percentage Inhibition was found to be significantly increased while Mean gastric pH was also significantly increased when compared with the control ($P<0.05$). Also, from the histological appearance of rats' stomachs in group IV, there was disintegration of the epithelial cells but the effect was not as pronounced as that in the control group. The disintegration further supports the fairly low Mean Ulcer Score value when compared with the normal control group I rats.

Animals pre-treated with L-Citrulline (300mg/kg) had significantly reduced Mean Ulcer Score and Mean Total, while percentage Inhibition was 13.46. The Mean gastric pH was significantly increased when compared with the control ($P<0.05$). Plates Va and Vb representing stomachs of animals in group V show mild erosion of the epithelial surface, an evidence of very mild ulceration.

In group VI, animals pre-treated with L-Citrulline (900mg/kg) showed Mean Ulcer Score of 3±1.56, Mean Total Acidity of 8.85±1.49, Percentage Inhibition of 80.95 and Mean gastric pH of was significantly reduced when compared with the normal control group ($P<0.05$). Also, the histological appearance of rats' stomach in group VI showed mild and slight disruption of the surface epithelial cells. This disruption further supports the fairly low value of Mean Ulcer Score and Mean Total Acidity when compared to group I rats.

The animals in the control group had the highest Mean Ulcer Score and Mean Total Acidity but lowest Percentage Inhibition and Mean pH when compared individually with the other groups. The differences between the control group and the other groups were not only seen in the Mean Ulcer Score, Mean total Acidity, Percentage Inhibition and Mean pH but also in their histological appearances. Plates Ia and Ib taken from animals in the control group pre-treated with distilled water show blood stained haemorrhagic ulcers while the histological appearances of the other groups (Groups II, III, IV, V and VI) with the mucosal layers slightly intact showing little or no ulceration.

Consumers around the world take watermelon because of its colourful appearance and its sweet taste while most people avoid taking the rind due to its unappealing flavour but unknowingly miss out on the many hidden advantages of its intake.

Watermelon is the number two fresh vegetable crop in the world in terms of area harvested and total production [30]. Recently, it has been identified as a healthy food because of its high content of the carotenoid, lycopene. Apart from watermelon being the leading fruit and vegetable source of lycopene, it also contains other antioxidants and amino acids that have health promoting activities. Amino acids have well established individual roles in disease prevention. Arginine, an essential amino acid, functions as one of the 20 building blocks of protein and in free form as a physiological amino acid. L-Citrulline is a physiological amino

acid, endogenous to most living systems. These amino acids are directly involved in clearing excess metabolic ammonia from the human body and indirectly involved in cardiovascular function, immune stimulation, and protein metabolism [31]. Ingested arginine is cleared by hepatic cells, but L-citrulline is not and can serve as an arginine source in other parts of the body.

Watermelon is rich in citrulline [32] but differences among cultivars have not been adequately studied nor have effects of production environments. The citrulline which exists in the rind is a precursor of L-arginine. L- Arginine can be obtained from diet and intracellular protein degradation [33] or synthesized from citrulline by successive actions of arginnosuccinate synthase (AS) and arginnosuccinate lyase (AL), the third and fourth enzymes of the urea cycle (Ornithine cycle) [34]. Moreover, the highest rates of L-Arginine synthesis occurs within the hepatic urea cycle [35]. Citrulline which is co-produced with Nitric Oxide can be recycled to L-Arginine via a pathway that has been termed the Citrulline-NO cycle [36].

Besides being the starting point for urea and ornithine synthesis, L-Arginine serves as substrate for the synthesis of nitric oxide (NO), a radical involved in such divergent actions as smooth muscle relaxation and host defense [37]. Nitric oxide may be important for the integrity of the gastric mucosa in health and disease both through its antimicrobial action and by influencing mucous production by the gastrointestinal mucosa. Nitric oxide has also been suggested to activate cyclooxygenase (COX) enzymes directly. However, it is unlikely that NO activates COX by binding directly to the heme-prosthetic group [38], and other mechanisms have been suggested. Both COX-1 and COX-2 contribute to gastric defence via the action of prostaglandins E and F2α. These prostaglandins acts by stimulating epithelial cells to release more bicarbonate and mucus inhibit gastric secretion, increase mucosal blood flow, downregulate the release of a number of the inflammatory mediators that have been suggested to contribute to the generation of mucosal injury in certain circumstances, and accelerate ulcer healing [39].

Humans can efficiently absorb L-Citrulline from watermelon, which also increases plasma arginine levels [40,41]. Recently, subjects consuming watermelon or synthetic citrulline as a drink, combined with exercise, had reduced arterial blood pressure compared with a placebo [42]. The heightened importance of watermelon as a source of bioactive compounds such as L-Citrulline highlights the need for a better understanding of the genetic control of this amino acid and other phyto-nutrients in watermelon.

In this present study, pre-administration of L-Citrlline and L-Arginine significantly prevented gastric mucosa injury accompanied by increased mucus production and pH of gastric content, inhibition of oedema as well as neutrophil infiltration of submucosal layer. There is growing evidence that L-citrulline can be readily converted to L-arginine in the kidney, vascular endothelium, and other tissues, thus raising plasma and tissue levels of L-Arginine. Moreover, L-arginine could inhibit the increase of inducible nitric oxide synthase (iNOS) expression induced by myocardial ischemia-reperfusion [43]. Therefore, the inhibitory effect of L-citrulline on inducible nitric oxide synthase (iNOS) expression might be mediated via conversion into L-arginine in vivo. The increased mucus production contributes to the preventive effect of the amino acids. These effects may be due to activation of defensive factors involved in protection of gastric mucosa and also inhibition of offensive factors. Shimizu et al. [44] demonstrated that the reduction of neutrophil infiltration into ulcerated gastric tissue promotes the healing of gastric ulcers in rats, and Fujita *et al*. [45] described that an increase in neutrophil infiltration into ulcerated gastric tissue delayed the healing of

gastric ulcers in rats. Neutrophils mediate lipid peroxidation through the production of superoxide anions [46]. Oxygen free radicals derived from infiltrated neutrophils in ulcerated gastric tissues have inhibitory effect on gastric ulcers in rats [47].

5. CONCLUSION

The findings suggest that L-Arginine and L-Citrulline displayed gastroprotective activity in indomethacin-induced gastric ulceration. Grossly, they were observed to significantly suppress the extent of ulceration and also increase gastric mucus production and pH of gastric content. Histology showed comparatively decreased gastric mucosal injury and inhibited oedema and leukocytes infiltration in submucosal layer of stomach.

It is therefore safe to conclude that L-Arginine and L-Citrulline have the capability of preventing ulceration. Patients suffering from gastric ulcer should be advised to take L-Arginine and L-Citrulline as these will reduce the ulcer severity.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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