Mucus secreting activity and nitric oxide concentrations of ethanol-injured pylorus and duodenum of rats pretreated with Moringa oleifera

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INTRODUCTION

Ulcerative lesions of the gastrointestinal tract are among the major adverse effects associated with ethanol consumption [1]. Oral administration of ethanol has been extensively shown to be an injurious agent to gastric mucosa in animal and clinical studies. It causes marked mucosal hyperemia, mucosal and/or submucosal hemorrhage, necrosis and edema, at concentrations greater than 40% [2, 3]. Gastrointestinal ulcers are mostly common in the stomach or in the first part of the small intestine that leads out of the stomach called the duodenum [4]. The mechanisms involved in the formation of gastrointestinal ulcers are multifaceted as several factors are responsible for the pathogenesis. A variety of factors produce damage to the gastro-duodenal mucosa including application of stress, local administration of various irritants like non-steroidal anti-inflammatory drugs (NSAIDs) that are common breakers of the mucosal barrier [5].

Ethanol-induced gastrointestinal lesion implicate a variety of mechanisms. Previous reports showed that mechanisms behind ethanol-induced gastrointestinal mucosal lesions may include; increase in oxygen-derived free radicals [6], decrease in concentrations of non-protein sulphydryl (NP-SH) contents in gastric mucosa [7], direct harm to the mucous layer or mucus synthesis [8], and activating of gastrointestinal cells’ apoptosis [9].

Mucus, which is produced by the epithelial lining of the gastrointestinal tract, forms an adhering gel to the mucosa surface and provides a self-protective barrier against injurious substance, digestive enzymes and microorganisms contained in the lumen [10]. The mucus secreted by mucous cells within gastric glands and surface mucous cells of the stomach as well as mucous glands and goblet cells of the duodenum, continuously neutralize luminal acid in the stomach and duodenum [11, 12].

Nitric oxide (NO) plays various physiologic and pathophysiologic roles in a variety of organs. NO is involved in regulation of motility and secretion in the gastrointestinal tract and in protective actions against injurious substances such as acid, ethanol, and drugs [13]. NO is normally produced from L-arginine by a variety of nitric oxide synthases (NOS) including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) [14].

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Moringa oleifera (MO) is among the most widely cultivated species of a monocogenic family, the Moringaceae [15]. It is also referred to as “Drumstick Tree” or “Horse Radish Tree” [16]. It is a perennial softwood tree with timber of low quality. However, the plant has many medicinal values as well as industrial uses [15, 16]. Many parts of the plant such as its flowers, green pods and young leaves are consumed for their nutritional benefits. It is commonly found in subtropical and tropical areas such as certain regions of India, Bangladesh, Pakistan, Philippines, South America and Arabia. It is also well distributed in many parts of Africa. MO has been used in traditional medicine for the treatment of various diseases such as ear and dental infections, skin ailments, respiratory infections, hypertension, anemia, diabetes and cancer [17, 18].

Many pharmacological effects, including diuretic, cholesterol reducing, anti-spasmodic, anti-hypertensive, anti-ulcer, hepatoprotective, antioxidant, anti-inflammatory and anti-tumor properties, have been attributed to MO [18, 19]. Extracts of its leaves have shown anti-ulcer activity while extracts of seeds displayed anti-tumor activity [19]. The Moringa plant has been reported to contain a rich and uncommon combination of zeatin, kaempferol, quercetin and many other phytochemicals [19, 20]. Also, lectins found in its seeds were observed to mediate an anti-inflammatory action via reduction in leukocyte migration, myeloperoxidase activities and also by decreasing levels of tumor necrosis factor (TNF)-α and interleukin (IL)-β [17]. Anti-tumor activities of MO have been linked to inhibition of nuclear factor kappa B (NF-κB). This regulator of intrinsic and adaptive immunity sustains proliferation and survival of many cells under most conditions via inhibition of apoptosis. By so doing, NF-κB also strengthens malignant cells in many cancerous growths. Aqueous leaf extract of MO have been shown to inhibit NF-κB signaling cascade, hence resulting in a potent anti-tumor/anti-cancer effects [21].

The objectives of the present study were to histochemically evaluate the activity of mucous secreting cells as well as determine the concentrations of NO in ethanol-injured pylorus and duodenum pretreated with MO extract, with a view to understanding some of the mechanisms behind its antiulcer activity.

METHODS

Animal Care

Twenty five male adult Wistar rats weighing between 150-200 g obtained from the Animal Holding of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, was used for this research. The rats were randomly assigned into 5 groups of 5 rats each. Animals were housed in clean plastic cages in a clean environment of 12 h day/light cycle at room temperature. Animals in all groups were fed on standard laboratory chow and allowed access to water ad libitum. All experimental protocols were in accordance with the guidelines for animal research as detailed in the Guidelines for the Care and Use of Laboratory Animals by the National Research Council of the National Academy of Sciences and National Institute of Health (NIH), 8th edition, 2011, and also in strict compliance with local institutional guidelines on Experimental Animal Care and Use of Laboratory Animals in Biomedical Research.

Extraction of Moringa oleifera

The leaves of Moringa oleifera were collected from Department of Botany, Obafemi Awolowo University, identified and authenticated at the same department. The leaves were air dried at room temperature for six weeks, weighed every three days to ascertain the dryness of the leaves. The air dried leaves weighing 320 g were grounded into fine powder in an electric blender and the powdered leaves were extracted in 2 l of ethanol using soxhlet extraction for 48 h. The mixture was filtered and the filtrate was allowed to evaporate at 40°C using a vacuum rotary evaporator. The wet residue was freeze-dried using a vacuum freeze-dryer for about 24 h and stored in a desiccator. An aliquot portion was prepared by dissolving 1 g of freeze dried extract in 25 ml of drinking water. The mixture was well shaken until extract was completely dissolved. This was done on the first day of administration, stored in a refrigerator, and used throughout the administration period.

Animal treatment

Animals were treated as follows:
- **Group A (control):** rats received drinking water only
- **Group B (ethanol only):** ethanol (> 99%) at 5 ml/kg body weight (BW) [22]
- **Group C (MO only):** Moringa oleifera extract only at 200 mg/kg BW
- **Group D (MO + ethanol):** MO & ethanol
- **Group E (cimetidine + ethanol):** cimetidine (CIM; 100 mg/kg BW [22]) & ethanol

MO extract and CIM (following daily preparations in drinking water) were administered orally for 5 days each. On day 6, following an overnight fast, ethanol was administered to groups D and E rats.

Surgical procedures and determination of pH of stomach content:

Animals were euthanized by cervical dislocation 1 hour after ethanol administration. A midline incision was made along the anterior abdominal wall. The stomach...
and small intestine were excised. The stomach was opened along the greater curvature, the content was drained and completely recovered by washing with 10 ml of normal saline. The content and washing was combined in a tube and centrifuged (3500 rpm, 10 min), and the pH value of the supernatant was measured using a digital pH meter [23]. The stomach was then examined macroscopically. The pylorus of stomach and duodenum of small intestine were separated. Some part of the tissues was homogenized for biochemical determination of NO and the rest was fixed in 10% formal saline for subsequent histochemical procedures. The stomach was pinned to a wooden board for macroscopic examination. Digital pictures were taken using a digital camera. Areas of gastric lesion were measured (in mm) and calculated from digital pictures of stomach by planimetry using the Java-based image processing program ImageJ developed by NIH, as described by Alvarez-Suarez et al [23]. The numbers of ulcer lesions in the glandular portion of the stomach were noted. The ulcer area (mm²) were measured and expressed as the ulcer index (UI) and Preventive Ratio (PR) was calculated as follows [3]:

\[
PR \% = \left( \frac{a - b}{a} \right) \times 100
\]

- \(a\): Ul of the ethanol only group
- \(b\): Ul of the ethanol and extract (MO) or drug (CIM) groups

**Determination of nitric oxide**

Nitric oxide was determined using a NO assay kit for the colorimetric determination of total nitrite (BioAssay Systems; Hayward, CA, USA), which is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase according to the Griess method as described in the manufacturer’s leaflet.

**Histochemical studies**

Following fixation, the tissues were processed for paraffin wax embedding. Paraffin blocked tissues were trimmed and mounted on wooden blocks for sectioning on a rotary microtome. Sections of 6 μm thickness were obtained on a rotary microtome. Periodic Acid Schiff (PAS)/hematoxylin/orange G staining [24] was used for the demonstration of mucus-secreting cells of the stomach and duodenum. Mucus is stained purple or magenta with this technique.

**Photomicrographs and image analysis**

Stained sections were viewed under a Leica DM750 microscope and digital photomicrographs were taken by an attached Leica ICC50 camera (Solms, Germany). ImageJ software was used to analyze and quantify the PAS/hematoxylin/orange G-stained photomicrographs of tissues. This software quantifies staining intensity by measuring the pixel value of each pixel in a red-green-blue (RGB) image and converting the pixel value to a brightness or gray value, in a scale of 0 to 255 from less bright (i.e., more intensity) to more bright (i.e., less intensity). Photomicrographs of three stained sections were used for image analysis.

**Statistical analysis**

Data are expressed as mean ± SEM. One-way ANOVA was used to analyze data, followed by Student Newman-Keuls test for multiple comparisons. GraphPad Prism 5 Version 5.03 (GraphPad Inc; La Jolla, CA, USA) was the statistical package used for data analysis. Significance level was set at P < 0.05.

**RESULTS**

**Effect of Moringa oleifera extract on ulcer index**

Oral administration of ethanol induced hemorrhagic lesions on the surface of the stomach of all animals. Control and MO only groups showed no such lesions. Pretreatment with MO extract (89.9 ± 36.72 μM²) and cimetidine (107.3 ± 33.86 μM²) significantly (P < 0.001) attenuated the hemorrhagic injuries induced by ethanol (239.1 ± 46.13 μM²) (Fig. 1A). There was no significant difference among the preventive ratio of MO + ethanol (45.14 ± 23.85 μM²) and CIM + ethanol (42.64 ± 15.64 μM²) groups (Fig. 1B).

**Effects on pH of gastric contents**

Treatment with ethanol significantly (P < 0.001) reduced acidity of gastric contents in all ethanol treated groups as shown by increased pH values in all groups treated with ethanol when compared with control (4.02 ± 0.29) and MO only (4 ± 0.13) groups. Pretreatment with MO extract (5.77 ± 0.54) and CIM (6.25 ± 0.46) decreased pH value compared to ethanol only (7.03 ± 0.36), but this was not significantly different (Fig. 1C).

**Effects on nitric oxide concentration in pyloric and duodenal tissues**

There was significant reduction (P < 0.01) of NO concentrations in pyloric tissues of ethanol only (83.9 ± 12.85 μM), MO + ethanol (213.6 ± 38.65 μM), and CIM + ethanol (212.6 ± 49.49 μM) groups. Treatment with MO only (426 ± 96.29 μM) showed no significant changes in NO concentration of pyloric tissues compared to control group (624.1 ± 145.6 μM). Pretreatment with MO and CIM, attenuated the effects of ethanol on NO concentrations in pyloric tissues, though this was not significant.

Although somewhat lowered with ethanol treatment, there was no significant difference for NO concentrations of duodenal tissues among all groups (Fig. 2): control = 265.6 ± 111.3 μM; ethanol only = 175.7 ± 14.81 μM; MO only = 214.6 ± 15.5 μM; MO + ethanol = 253.9 ± 42.08 μM; CIM + ethanol = 186.9 ± 26.44 μM.
Figure 1. (A) Ulcer index of ethanol treated rats (β, P < 0.001 compared to ethanol only group); (B) Preventive ratio of ulcer injuries by extract and standard drug; (C) pH value of gastric contents of control and treated groups (α, P < 0.001 compared to control; β, P < 0.001 compared to MO only).

Result of PAS/hematoxylin/orange G staining
Intact luminal surface mucous secreting cells and numerous neck mucous secreting cells of the pyloric mucosa are observed in control and MO only treated groups (Figs.3A&C). Treatment with ethanol only reduced PAS-positively stained mucous secreting cells of the pyloric mucosa (Fig.3B). Pretreatment with MO and CIM showed slight improvement in PAS-positively stained mucous secreting cells of the pyloric mucosa (Figs.3D&E).

Numerous PAS-positively stained mucous secreting goblet cells of the duodenal villi and crypts were observed in control and MO only treated groups (Figs.4A&C). Treatment with ethanol only reduced PAS-positively stained duodenal mucous secreting goblet cells (Fig.4B). Pretreatment with MO extract and CIM was seen to have attenuated the observed effect of ethanol treatment on the mucous secreting goblet cells of the duodenal villi and crypts, as numerous positively stained cells were observed (Figs.4D&E).

Figure 2. NO concentration of pyloric and duodenal tissues (α, P < 0.01 compared to control; β, P < 0.01 compared to ethanol only group).

ImageJ analysis
Ethanol only treatment significantly (P < 0.01) reduced intensity of mucus staining in the pylorus of stomach, as represented by a higher mean gray value, compared to control and MO only group. Also percentage area of PAS positively stained mucus cells in the pylorus was significantly (P < 0.01) reduced following ethanol treatment. Pretreatment with MO extract and CIM did not significantly increase staining intensity of mucus activity. Treatment with MO extract only showed an increase in intensity of mucus staining, as indicated by a lower mean gray value compared to other groups (Table 1).

Ethanol only treatment also significantly (P < 0.01) reduced intensity of mucus staining in the duodenum, as represented by a higher mean gray value, compared to control and MO only group. Also percentage area of PAS positively stained mucus cells in the duodenum was significantly (P < 0.01) reduced following ethanol treatment. Pretreatment with MO extract and CIM significantly (P < 0.01) attenuated these effects of ethanol treatment in duodenum (Table 1).

DISCUSSION
Gastrointestinal disorders (such as ulcers and inflammation) are very common and result in great human suffering. They are mainly a result of the imbalance between damaging factors within the lumen and protective mechanisms within the gastrointestinal
Table 1. ImageJ analysis of PAS/hematoxylin/orange G photomicrographs of pylorus and duodenum tissue (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Pylorus</th>
<th>Duodenum</th>
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<tbody>
<tr>
<td></td>
<td>Mean gray value</td>
<td>Area of PAS-positive staining (%)</td>
</tr>
<tr>
<td>Control</td>
<td>39.63 ± 3.26</td>
<td>29.72 ± 4.17</td>
</tr>
<tr>
<td>Ethanol only</td>
<td>62.87 ± 4.38*</td>
<td>8 ± 2.32*</td>
</tr>
<tr>
<td>Moringa oleifera only</td>
<td>30.79 ± 3.45β</td>
<td>22.22 ± 3.10β</td>
</tr>
<tr>
<td>Moringa oleifera + Ethanol</td>
<td>55.79 ±3.13αβ</td>
<td>12.67 ± 1.24αβ</td>
</tr>
<tr>
<td>Cimetidine + Ethanol</td>
<td>60.36 ± 3.53αβ</td>
<td>10.58 ± 0.69αβ</td>
</tr>
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P < 0.01 compared to: α, control; β, ethanol only; δ, Moringa oleifera only.

Figure 3. Micrographs of pylorus part of the stomach of: (A) control, (B) ethanol only, (C) MO only, (D) MO + ethanol, and (E) CIM + ethanol rats. *, lumen; arrows, mucus secreting cells; dashed arrows, intact luminal surface mucous cells. (PAS/hematoxylin/orange G, x400)
mucosa [25]. Peptic ulcer is a breach in the gastric and duodenal epithelium associated with acute or chronic inflammation and is the most common gastrointestinal disorder in clinical practice. In spite of established antiulcer drugs, a rational therapy for peptic ulcer remains elusive and a search for safer potential drugs is being carried out [26].

Ethanol induces both long ulcers and petechial lesions within a short duration, which makes this method suitable in screening experiments for investigation of antiulcer drugs and agents [1]. The present study showed that pretreatment with MO significantly attenuated ethanol induced ulcerative lesions. This anti-ulcer effect of MO holds great clinical significance considering that NSAIDs are continually be prescribed for several ailments. The most common may be the daily intake of aspirin to reduce risk of cardiovascular disorders [27]. Regular intake of the Moringa plant as part of food may reduce the risk of ulcer in cases of continuous use of NSAIDs, sustained consumption of ethanol or repetitive exposures of the gastrointestinal tract to other injurious factors.

**Figure 4.** Micrographs of duodenum of: (A) control, (B) ethanol only, (C) MO only, (D) MO + ethanol, and (E) CIM + ethanol rats. *, lumen; arrows, mucus secreting goblet cells of the villi and crypts. (PAS/hematoxylin/orange G, x400)
There has been a great debate as to the effects of ethanol on gastric acid secretion from being a mild stimulant to being a powerful inhibitor. It is generally agreed that alcoholic beverages stimulate gastric acid secretion. Various researches have observed a rise in histamine, gastrin and carbachol levels following wine and beer ingestion [28-30]. Unexpectedly, this increase in secretagogue concentrations did not occur after consumption of equivalent amount of pure ethanol. The stimulatory effect of the beverages was therefore attributed to other ingredients than ethanol [29]. This has instigated researches to determine whether pure ethanol influences gastric acid secretion. In summary, the results of this effort proved to be very contentious. Chacin et al [28] postulated that low dose (2-20%) ethanol stimulates gastric acid secretion, whereas high concentrations (>20%) were shown to have an inhibitory effect. In contrast to these findings, other investigations concluded that acid secretion is reduced under low-dose conditions [29].

Del Valle et al [30], not only demonstrated cessation of acid secretion following low-dose (1-20%) ethanol exposure, but also decreased ATP and cAMP concentrations in the affected parietal cells. Also, Kopić et al [31] were able to demonstrate that ethanol at doses as low as 1% decreased acid secretion significantly both on the single-cell/gland and on the whole-stomach level. In the present study, treatment with ethanol significantly reduced the acidity of the gastric contents in all ethanol treated groups indicating a decrease in acid secretion, as shown by increase in pH values compared with control and MO only group. Although not significantly different, the pH values increased more in ethanol only group compared to pretreatment with cimetidine and MO. It has been hypothesized that the inhibitory effect of ethanol on gastric acid secretion may be mediated by the activation of AMP-activated protein kinase (AMPK), due to immediate rise in AMP concentrations in liver cells following acute ethanol exposures [32].

Previous reports suggest that ethanol-induced gastrointestinal mucosal lesions are due to various possible mechanisms including increased oxygen-derived free radicals [1] and direct damage to the mucus layer or mucus synthesis [8]. Mucus helps to prevent injury to the stomach and duodenum from luminal acid, noxious agents and microorganisms [31]. Mucus has been suggested to be involved in neutralizing the effects of oxygen-derived free radicals [33]. Histochromic evaluation of mucus secreting cells in the present study shows that ethanol depletes the mucus secreting cells of the stomach and duodenal tissue and pretreatment with MO significantly prevented this depletion of the gastroduodenal mucus cell population and subsequent mucus production.

NO has been shown to stimulate mucus and bicarbonate secretion, maintain gastric blood flow, and promote angiogenesis i.e. the process of growth of new blood vessels from pre-existing blood vessels. Inhibition of NOS has been reported to significantly delay ulcer healing, reduce gastric blood flow around the ulcer, and impair angiogenesis in the granulation tissue. On the other hand, administration of NO donor or L-arginine, the substrate of NOS, significantly enhanced healing and reversed NOS induced delay in healing [14]. In view of the foregoing, endogenous NO may play an important role in the protection of gastric mucosa from injuries. In the present study, ethanol administration significantly lowered NO concentration in pyloric tissues and, though not significantly, NO concentration was also lowered in duodenal tissues. Reduction of endogenous NO concentration in pyloric and duodenal tissues may be a contributing factor in the mucosa damaging effects of ethanol in the gastrointestinal tracts. Also NO has been reported to play a critical role in proper functioning of goblet cells which are mucus secreting cells in the gastrointestinal tracts. NO donors could increase mucus release from gastric mucosal cells and enhance mucus gel thickness in rats [34]. Hence reduction in endogenous NO may affect normal production of mucus, which may worsen gastro-duodenal mucosa damage in ulcerative injuries. In the present study, pretreatment with both MO and cimetidine, improved lowered NO concentration in pyloric and duodenal tissues. Also treatment with MO only showed no significant changes in NO concentrations compared to control. These suggests that MO may maintain endogenous NO concentrations in gastrointestinal lesions, hence contribute to its ability to protect gastrointestinal mucosa.

In conclusion, the present study reaffirmed that Moringa oleifera possesses antiulcer properties against ethanol-induced gastrointestinal damage. The antiulcer activity of MO seem to be attributed to the preservation of mucus secreting cells and maintaining production of endogenous NO concentrations in ethanol-injured gastro-duodenal tissues.

COMPETING INTERESTS

The authors declare that they have no conflict of interests.
REFERENCES


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