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Histological and Histomorphometric studies of ethanol-injured pylorus and duodenum of Wistar rats pre-treated with *Moringa oliefera* extract

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Abstract: *Background:* This study investigated the effects of pre-treatment with *Moringa oleifera* extract on the histomorphology and histomorphometry of the pylorus and duodenum of rats following ethanol-induced gastrointestinal injury. *Methods:* Following extract and ethanol treatment, pylorus and duodenum of the rats were processed for histological procedures, and tissue quantification of total antioxidant capacity, to access the protective abilities of the extract. *Results:* We showed that *Moringa oleifera* extract significantly reduces ($p<0.05$) total lesion area of the secretory portion of stomach following ethanol induced damage. Also the extract, to a great extent preserved the histological integrity of the pylorus and duodenum, and also significantly improved ($p<0.05$) the % of glandular mucosa of the pylorus that was intact and the ratio of villi height to crypt depth following ethanol damage. There was significant difference in pyloric and duodenal concentration of total antioxidant capacity between ethanol injured rats compared to control and extract treated groups. *Conclusion:* The present study confirms the antiulcer properties of *Moringa oleifera* extract, and demonstrates that the extract preserves the histological integrity and dimensions of the various layers of pylorus and duodenum of Wistar rats, thereby preventing a decrease gastrointestinal surface area following ethanol induced injury.
Keywords: *Moringa oleifera;* gastrointestinal injuries; ethanol; histology; histomorphometry

Introduction

Gastrointestinal disorders (such as ulcers, hyperacidity & inflammation) are very common, causing tremendous human suffering. They are mostly due to an imbalance between damaging factors within the lumen and protective mechanisms within the gastrointestinal mucosa [1].

The use of plant sources in combating these disorders may be increasingly profitable with associated fewer side effects compared to conventional drugs. *Moringa oleifera* (MO) is among the most widely cultivated species of a monogeneric family, the moringaceae and very rich in nutrient [2]. It is also referred to as "Drumstick Tree" or "Horse Radish Tree" [3]. It is a soft wood perennial tree with drooping branches and corky bark. Its leaves are feathery pale green, compound and tripinnate. All parts have been used in Ayurvedic system of medicine [4]. It has shown anti-tumor, anti-inflammatory, hepatoprotective, and antihypertensive properties [5]. All parts of the Moringa tree are edible and are eaten by humans [6]. Extracts of seeds have exhibited anti-tumor activity while its leaves have shown anti-ulcer activity [5]. Different parts of this plant contain a profile of important minerals, and are a good source of vitamins, proteins, β – carotene, and various phenolics acids [7]. The Moringa plant has been reported to contain a rich combination of zeatin, kaempferom, quercetin and many other phytochemicals [5].

Orally administered ethanol is known to produce extensive injuries to the gastrointestinal mucosa hence it is very suitable as an experimental model of gastrointestinal ulcers. It induces both petechial lesions and long ulcers in the gastrointestinal wall within a short duration [8]. In view of the foregoing, the present study has evaluated the effects of pre-treatment with *Moringa oleifera* extract on the histology and

histomorphometry of the pylorus and duodenum of rats following ethanol-induced gastrointestinal injury. The study also determined the changes in tissue total antioxidant capacity (TAC).

Material and Methods

Animal care: Twenty five male adult Wistar rats weighing between 150-200 g were obtained from the Animal Holding of the Department of Anatomy and Cell Biology and used for this research. The rats were randomly assigned into 5 groups of 5 rats each (Groups A, B, C, D, and E). Animals were housed in clean plastic cages in a clean environment of 12 hours 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 conform to the ICMR guidelines for animal research, 2001 and NIH Guidelines for care and use of laboratory animals, 2011.

Extraction of Moringa oleifera: The leaves of *Moringa oleifera* (MO) was collected from Department of Botany, Obafemi Awolowo University, Ile-Ife, 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 Litre of ethanol by in a soxhlet extractor for 48 hrs. The mixture was filtered and the filtrate was allowed to evaporate at 40ºC using a vacuum Rotary evaporator. The wet residue was freeze-dried using a vacuum freeze drier 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): Control rats received drinking water only
- Group B (Ethanol only): Ethanol (>99%) at 5 ml/kg body weight [9]
- Group C (MO only): *Moringa oleifera* extract (MOE) only at 200mg/kg body weight
- Group D (MO+Ethanol): Ethanol (>99%) with MOE
- Group E (CIM+Ethanol): Ethanol (>99%) with cimetidine, 100 mg/kg body weight [9]

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

Surgical procedures and macroscopic examination of stomach: One hour after ethanol administration, animals were euthanized and a midline incision was made along the anterior abdominal wall. The stomach and small intestine were excised. Following emptying of stomach content, the stomach was cut open along the greater curvature and pinned to a wooden board. Digital pictures were taken with a Sony Cybershot DSC-W510 Digital Camera for macroscopic examination. The pylorus of stomach and duodenum of small intestine were separated. Some part of the tissues was homogenized for biochemical determination of TAC and the rest was fixed in 10% formal saline for subsequent histochemical procedures.

Areas of gastric lesion were measured (in mm) and calculated from digital pictures of stomach by planimetry using the Image Processing and Analysis in Java (ImageJ) by the National Institutes of Health (NIH, USA) as described by Alvarez-Suarez *et al.,* 2011 [10]. Also the numbers of gastric lesions were noted.

Lesion Index (LI) was calculated as follows [10]:

$$LI = \frac{Total\ lesions\ area\ (TLA)}{Total\ \ secretory\ \ area\ (TSA)}\ x\ 100\%$$

Protective Ratio (PR) was calculated as follows [11]:

$$PR(\%) = \frac{(a-b)}{a} x100$$

Where a; the lesion index of the gastric injury control group and b; the lesion index of the extract or drug treated groups.

Histology, photomicrography and histomorphometry: Tissues for histological studies were processed for routine paraffin wax embedding, sectioned on a rotary microtome at 6 µm thickness, and stained using haematoxylin and eosin (H&E) method described by Drury and Wallington, 1980 [12]. Stained sections were viewed under a Leica DM500 microscope and digital photomicrographs were taken by an attached Leica ICC50 camera.

Three stained sections each of the pylorus and duodenum were used for histomorphometric studies. Photomicrographs of haematoxylin and eosin stained sections was imported on to OpenOffice.org™ (OOo-dev 3.4.0) software for histomorphometric analysis. Thickness of four locations on the pylorus was measured for the following parameters [13];

1. Mucosa layer: from the gastric epithelial lining to the muscularis mucosa.
2. Subglandular mucosa layer: from the lamina propria to the muscularis mucosa.
3. Glandular mucosa layer: calculated by subtracting the subglandular mucosa from the mucosa layer

Also the percentage of glandular mucosa that is intact was calculated as;

$$\%Glandular\ Mucosa\ layer = \frac{Glandular\ Mucosa\ layer}{Mucosa\ layer}\ x\ 100\%$$

The small intestine is devoid of glands on the mucosa layer rather it is invested with villi and the muscularis mucosa and lamina propria are less conspicuous. Hence, five well oriented villi and corresponding crypts in the small intestine [14-15] was measured for following parameters;

1. Villi height
2. Crypt depth
3. Villi height/Crypt depth ratio was calculated.

All measurements were made in µm, at X100 magnification.

Determination of total antioxidant capacity (TAC): TAC was determined using the QuantiChrom™ Antioxidant Assay Kit (BioAssays Systems, USA). This BioAssay Systems' improved assay measures total antioxidant capacity in which Cu^{2+} is reduced by antioxidant to Cu^{+}. The resulting Cu^{+} specifically forms a colored complex with a dye reagent. The color intensity at 570nm is proportional to TAC in the sample.

Statistical analysis: One-way ANOVA was used to analyze data, followed by Student Newman-Keuls (SNK) test for multiple comparisons. GraphPad Prism 5(Version 5.03, GraphPad Inc.) was the statistical package used for data analysis. Significant difference was set at $p<0.05$.

Results

Macroscopic examination of stomach: As shown in Figure 1, ethanol induced haemorrhagic lesions on the surface of stomach of all animals treated with ethanol. The lesions were located mostly in the corpus or body of the stomach, the portion of the stomach secreting acid and pepsin. No visible lesions were found in the non-secretory part of the stomach. These injuries were less severe in MO extract and cimetidine treated groups. No lesions were observed in Control and MO only animals.

Table 1 shows ethanol caused typical widespread gastric lesions with a Total Lesion Area (TLA) of 239.1±46.1, and covering 27.1% of the secretory areas of stomach in Ethanol only group. Pre-treatment with MO extract and cimetidine, significantly reduced ($p<0.05$) total areas of gastric lesion with TLA of 89.9±36.7 and 107.3±33.9 respectively.

MO extract and cimetidine administration reduced the percentage of gastric lesions to 12.7% and 15.7% respectively, but these were not found to be significantly different from Ethanol only group. There was no significant difference between the protective ratio of MO extract and cimetidine treated groups.

Figure-1: Stomach of Control (A), Ethanol only (B), MO only (C), MO+Ethanol (D) and CIM+Ethanol (E) rats. The stomach was cut opened along the greater curvature to show gastric lesions on stomach surface. Observe the extensive hemorrhagic lesions on Group B, treated with ethanol only compared to Groups D and E, treated with extract and cimetidine respectively. Observe that no lesions were visible in Groups A and C.

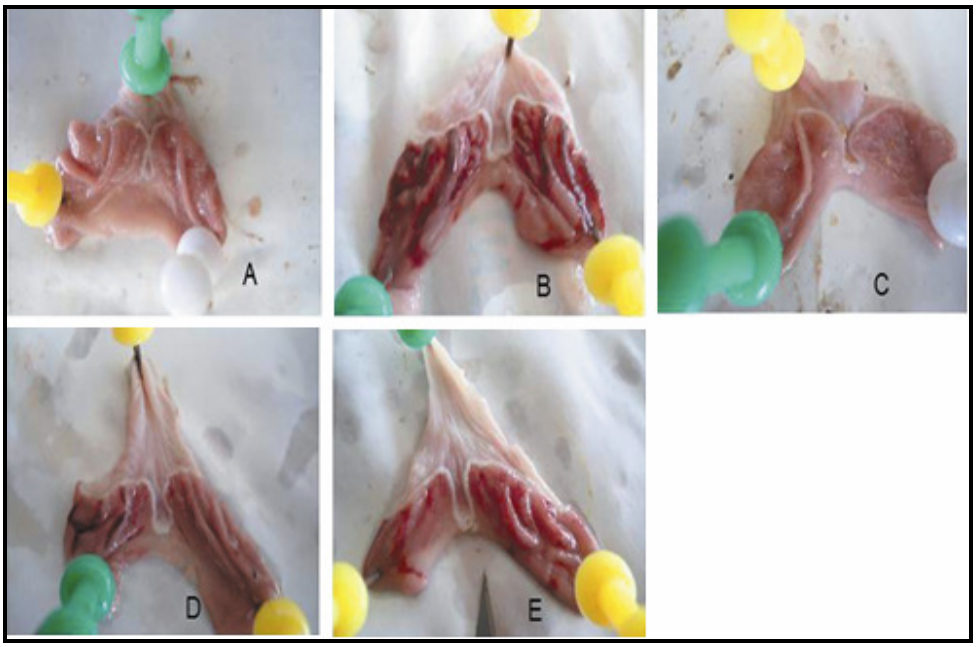


Table-1: Macroscopic measurements of areas of gastric lesions

	Lesion Numbers	Total Secretory Area (TSA) mm^2	Total Lesion Area (TLA) mm^2	Lesion Index (LI) %	Protective Ratio (PR)
Control	-	-	-	-	-
Ethanol only	13.8±1.2	861.1±46.6	239.1±46.1	27.1±3.9	-
MO only	-	-	-	-	-
MO + Ethanol	12.2±1.0	718.2±60.1	89.9±36.7β	12.7±5.0	45.1±23.9
CIM + Ethanol	10.0±1.7	670.6±41.9β	107.3±33.9β	15.7±4.6	42.6±15.6

Values are mean ± SEM of data obtained. β –$p<0.05$ compared to Ethanol only group.

Result of H&E staining: The normal histology of the pylorus part of the stomach was observed in the control. The pylorus which is the funnel shaped part of the stomach that leads into the small intestine show a similar histological layering structure to the rest of the stomach. Three of the major layers-mucosa, submucosa and muscularis propria, were easily identified.

The tubular glands in this region are usually branched and ends as coiled secretory portions called pyloric glands. The gastric pits of these glands are deeper than that seen in other regions of the stomach, extending about half the thickness of the pyloric mucosa. The mucosal cells are intact from the luminal surface cells at the lumen of the pylorus to the chief (peptic) cells at the base. Treatment with MO extract only did not affect the histology of the pylorus, as the gastric pits were easily observable as well as the intact luminal surface cells. Following treatment with ethanol, extensive damage to the mucosa of the pylorus was observed.

The luminal surface cells were clearly eroded as well deep erosion into the glandular part of the mucosa, extending to the base of the gastric glands. Pre-treatment with MO extract and cimetidine were observed to afford some protection the mucosa of the pylorus. Though

there was erosion of the luminal surface cells in these groups, damage to the gastric glands was not as extensive as in ethanol only treated group (Figure 2). The normal histology of duodenum was seen in control rats. Three of the major layers-mucosa, submucosa and muscularis propria, are easily identified. The duodenal mucosa is intact. Numerous elongated villi are observed, with crypts between their bases. Brunner's glands were seen occupying the entire submucosa layer. Treatments with MO extract only showed normal histology and there was no observed erosion into the duodenal mucosa. Treatment with ethanol caused deep erosion into the duodenal mucosa, eroding a major part of most villi. Pre-treatment with MO extract and cimetidine were also observed to afford some protection to the duodenal mucosa as intact elongated villi were observed (Figure 3).

Figure-2: Micrographs of pylorus part of the stomach of Control (A), Ethanol only (B), MO only (C), MO+Ethanol (D) and CIM+Ethanol (E) rats. Asterisk (*) indicates the lumen. Arrows- gastric pits, dashed arrows- intact luminal surface cells. Observe the marked erosion into the mucosal layer of Group B, compared to Groups D and E. Groups A and B show intact mucosal layer with visible luminal surface cells. H&E X400

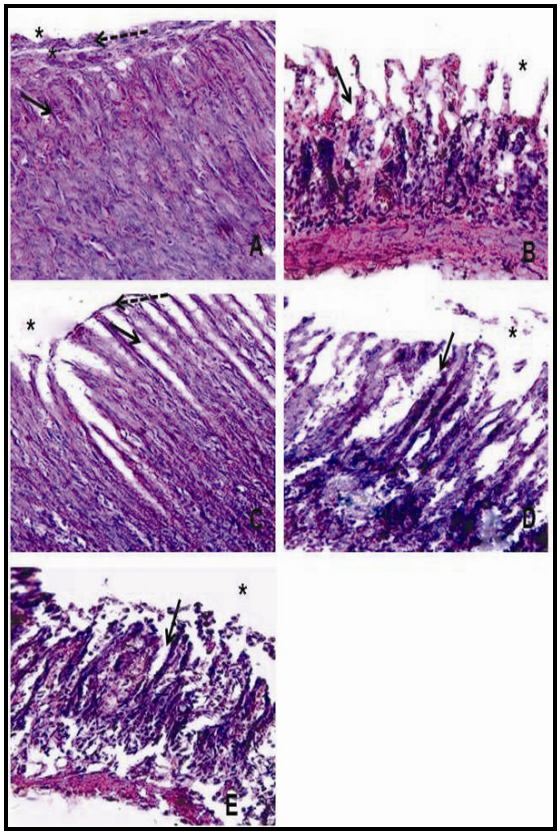
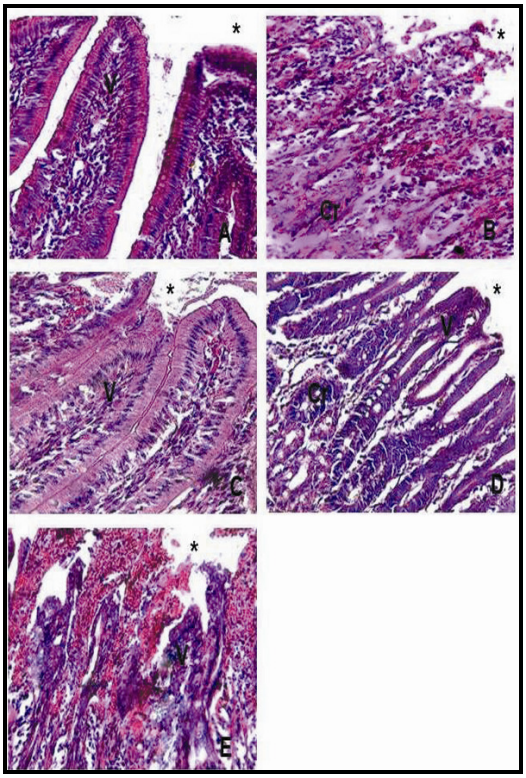


Figure-3: Micrographs of duodenum of Control (A), Ethanol only (B), MO only (C), MO+Ethanol (D) and CIM+Ethanol (E) rats. Asterisk (*) indicates the lumen. V- villi, Cr- crypts. Observe the marked erosion into villi of the mucosal layer of Group B, compared to Groups D and E. Groups A and B show intact mucosal layer with well oriented villi. H&E X400.



Results of histomorphometric measurements: Ethanol treatment significantly reduced ($p<0.001$) the thickness of mucosa layer of the pylorus, and correspondingly the glandular part of the mucosa, when compared to Control group, thus supporting histological results. Pre-treatment with MO extract and cimetidine before ethanol induced injury, significantly attenuated the effect of ethanol on the thickness of the mucosa layer and the glandular part of the mucosa. It also shows that the attenuating effect was more improved with MO extract than cimetidine pre-treatment (Table 2). Ethanol only treatment significantly reduced ($p<0.001$) the percentage of the mucosa that is glandular compared to control and other groups, and this effect was significantly attenuated with MO extract and drug treatment (Figure 4).

Villi height was significantly reduced (p<0.001) in all ethanol treated groups compared to Control (Table 3). Treatment with ethanol only significantly reduced (p<0.001) the ratio of villi height to crypt depth, thus indicating a reduction in duodenal surface mucosa area and this effect was significantly attenuated with MO extract and drug treatment (Figure 5). This also supports results of histological evaluation.

Table-2: **Histomorphometric measurements of pylorus part of stomach of control and treated rats**

	Mucosa layer (µm)	Subglandular mucosa layer (µm)	Glandular mucosa layer (µm)
Control	746.7±8.8	80.0±11.6	666.7±3.3
Ethanol only	246.7±8.8α	83.3±8.8	163.3±3.3α
MO only	700.0±25.2β	73.3±8.8	636.7±24.0β
MO + Ethanol	456.7±14.5αβδ	76.7±12.0	380.0±5.8αβδ
CIM + Ethanol	350.0±15.3αβδλ	76.7±8.8	273.3±12.0αβδλ

Values are mean ± SEM of data obtained. α - p<0.001 compared to Control. β - p<0.001 compared to Ethanol only. δ-p<0.001 compared to MO only. λ - p<0.001 between MO + Ethanol and CIM + Ethanol

Figure-4: % Glandular mucosa layer of pylorus of Control and Treated groups. α - p<0.001 compared to Control. β - p<0.001 compared to Ethanol only. δ-p<0.001 compared to MO only

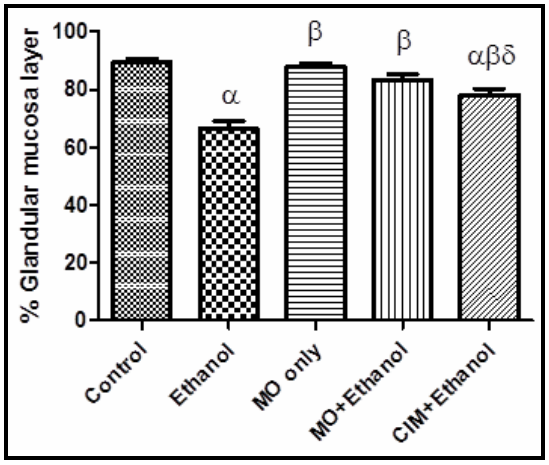


Figure-5: Villi Height/Crypt depth ratio of duodenum of control and treated rats. α - p<0.001 compared to Control. β - p<0.001 compared to Ethanol only

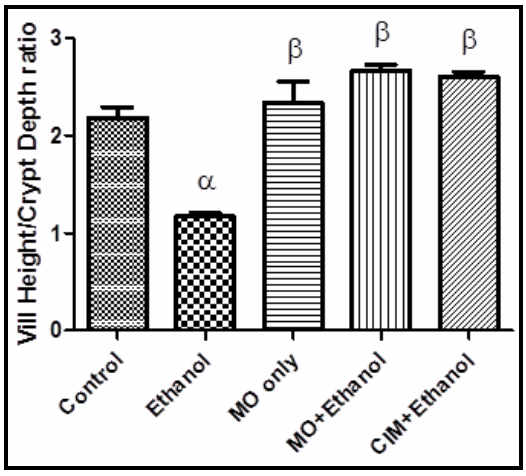


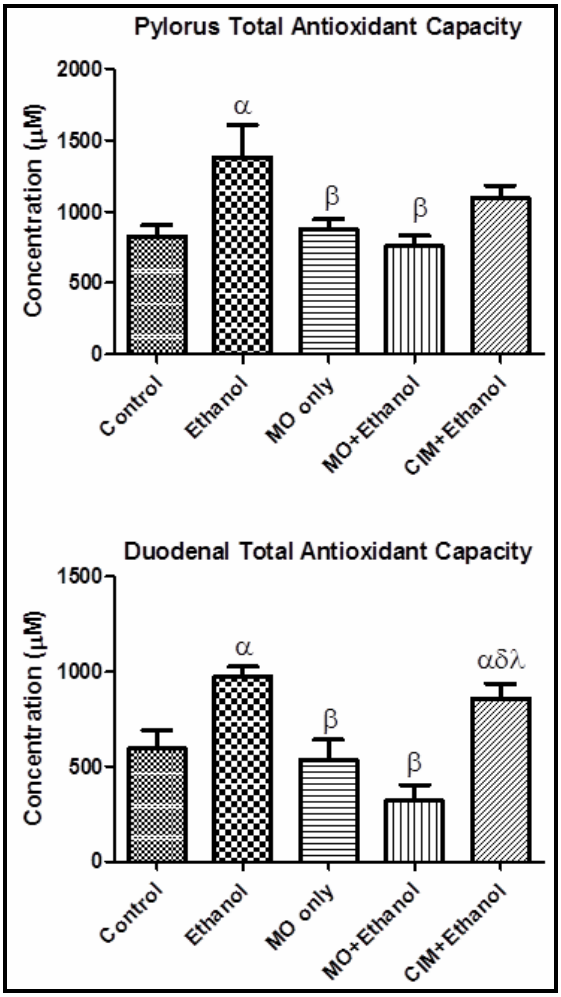
Table-3: **Histomorphometric measurements of duodenum of control and treated rats**

	Villi height (µm)	Crypt depth (µm)
Control	594.3±35.3	271.7±2.3
Ethanol only	323.3±15.1α	277.0±13.0
MO only	632.3±66.4β	270.0±4.2
MO+Ethanol	457.0±9.0αδ	171.7±6.8αβδ
CIM+Ethanol	454.7±16.5αδ	175.0±9.9αβα

Values are mean ± SEM of data obtained. α - p<0.001 compared to Control. β - p<0.001 compared to Ethanol only. δ-p<0.001 compared to MO only.

Effects on TAC: Treatment with ethanol only significantly increased (P<0.05) TAC in pyloric tissues compared to control, MO only and MO+Ethanol groups but no significant difference was observed compared to CIM+Ethanol group. Correspondingly, ethanol only as well as CIM+Ethanol groups significantly increased (P<0.05) TAC in duodenal tissues compared to control, MO only and MO+Ethanol (Figure 6).

Figure-6: Total antioxidant capacity of pyloric and duodenal tissues; α - p<0.05 compared to Control, β - p<0.05 compared to Ethanol only. δ-p<0.05 compared to MO only. λ - p<0.05 between MO + Ethanol and CIM + Ethanol.



Discussion

The present study shows that ethanol induced haemorrhagic lesions on the surface of stomach of all animals treated with ethanol. Narcotizing agents such as ethanol, when given by mouth to rats produce serious gastric hemorrhagic erosions. Ethanol induced both long ulcers and petechial lesions within a small duration, which makes this method appropriate for screening experiments for investigation of antiulcer drugs and agents [8]. 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. The use of natural drugs in gastric ulcer has been reported [16]. Ulcerative lesions of the gastrointestinal tract are one of the major side effects associated with the use of NSAIDs, alcohol, stress, and ischemic reperfusion [8]. In this study, the lesions were located mostly in the corpus or body of the stomach, the portion of the stomach secreting acid and pepsin. No visible lesions were found in the non-secretory part of the stomach. These injuries were less severe in MO extract and cimetidine treated groups. No lesions were observed in Control and MO only animals.

Ethanol administration induced severe lesions characterized by coagulative necrosis of the glands with diffuse haemorrhages of the mucosa [17]. Histological results of the present study confirm the serious microscopic damages to mucosal layer of the pylorus and duodenum of treated rats caused by ethanol. Pre-treatment with MO and cimetidine afforded protection to the mucosal layer of these tissues. Also histomorphometric analysis showed that ethanol treatment significantly reduced the thickness of mucosa layer of the pylorus, the glandular part of the mucosa and correspondingly the percentage of intact glandular mucosa, when compared to Control group.

Pre-treatment with MO extract and cimetidine before ethanol induced injury, significantly attenuated the effect of ethanol on the thickness of the mucosa layer and the glandular part of the mucosa. It also shows that the attenuating effect was more improved with MO extract than cimetidine pre-treatment. There was significant reduction in the villi height of the duodenal tissue of all ethanol treated group when compared with control and reduction in the ratio of the villi height to villi crypt of the ethanol only group when compared with the other groups in this present study. Also pre-treatment with extract and cimetidine, improve the damaging effects of ethanol in the duodenum.

Increase in oxygen-derived free radicals has been implicated amongst the possible mechanisms by which ethanol induces gastrointestinal lesions [8, 18]. Considering these reports, the increased TAC in the pyloric and duodenal tissues following ethanol induced lesions may be attributed to an increase in oxidative stress, thus necessitating

an increase in antioxidants in these tissues. Hence, our results show that MO extract inhibited oxidative stress in these tissues following ethanol injury, and this effect was not demonstrated by the reference drug, cimetidine. These results confirms the antiulcerogenic properties of *Moringa oleifera*, and that MO extract preserve the microanatomical integrity and dimensions of the various layers of pylorus and duodenum of Wistar rats. Therefore the extract prevents a decrease in gastrointestinal surface area following ethanol induced injury, via inhibition of oxidative damages that accompanies ethanol-induced gastrointestinal injuries.

Abbreviations

MO – Moringa oleifera
CIM – Cimetidine
NSAIDs –Nonsteroidal Anti-inflammatory Drugs

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