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Cytoprotective Effect of Methanol Extract of *Laportea aestuans* on Acidified Ethanol-induced Gastric Ulcer in Male Wistar Rats

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Abstract

Laportea aestuans (*Urticaceae*) plant is widely distributed in tropical rain forests and used as remedial agent for various diseases in Nigeria by herbal practitioners. Ethanol consumption is one of the common causes of gastric ulcer due to excessive generation of reactive oxygen species and erosion of gastric mucosal layer. This study investigated the cytoprotective effect of pretreatment with methanol extract of *Laportea aestuans* (MELA) for 7 days on ethanol-induced gastric ulcer in male Wistar rats using cimetidine as a standard drug. Ulcerogenic parameters, oxidative damage, inflammatory and apoptotic markers were evaluated. MELA showed a significant dose-dependent ($p < 0.01$) decrease in ulcer score (US), ulcer index (UI) and peptic activity with an increase in mucus content and percentage inhibition compared to the ethanol group. Activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), Glutathione S-transferase (GST) and level of reduced glutathione (GSH) were also increased with concomitant decrease in malondialdehyde (MDA) level in MELA treated rats. Serum levels of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and activities of cytochrome c (Cyt c), caspases-3 and -9 (CASPS-3 and -9) were also reduced by administration of MELA. Taking together, this study shows that MELA protected the gastric mucosal against the assault induced by ethanol via upregulation of the antioxidant system, and downregulation of inflammatory and apoptotic processes.

Keywords: Cytoprotective, *Laportea aestuans,* Gastric ulcer, Apoptotic markers, Antioxidant

Introduction

Gastric ulcer etiology remains multifactorial, but numerous studies have attributed the cause to an imbalance between the endogenous protective factors like mucus secretion, bicarbonate secretion blood flow and exogenous aggressive factors such as non-steroidal anti-inflammatory drugs, high gastric acid-pepsin secretion, *Helicobacter pylori* and ethanol consumption [1,2]. Ethanol solubilizes the protective mucus and exposes the mucosa to the proteolytic and hydrolytic actions of pepsin and gastric hydrochloric acid [3,4] due to its ability to penetrate the gastric mucosa, thus causing injury to the gastric cellular membrane. Ethanol also manifests its injurious effects on the gastric mucosal through generation of reactive oxygen species (ROS) [5-7] that react with most of the cell components, changing their structures and functions, thus, causing disruption of gastrointestinal tract barrier that can lead to increased gastric permeability. Stimulation of macrophages by ROS can also cause the release of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) to cause more damage to the gastric tissues [5]. Ethanol has also been reported to stimulate the process of apoptosis in the gastric cells via excessive generation of ROS [8] by activating the intrinsic apoptotic pathway or through the release of TNF-α to activates the extrinsic apoptotic pathway. Ethanol-induced gastric injury in rats is commonly used in experimental model because it shows several features that are similar to human gastric ulceration and thus provides a means for assessing agents with potential anti-ulcer capacity along with their implicated mechanisms for gastric protection [9].

The current approach for the treatment of gastric ulcers centers on the use of proton pump inhibitors, antacids, antibiotics combinations and H_2 receptor antagonists. However, the use of these drugs

has been linked to various undesirable consequences ranging from nausea, constipation, gynecomastia and impotence [10,11]. Hence, the need to search for alternative and effective agents with fewer side effects, greater availability, affordability, efficacy and safety [12]. Literatures have shown that agents with antioxidant, anti-inflammatory, and anti-apoptotic properties have displayed beneficial actions in protecting against alcohol-induced gastric ulcer [12,13].

Laportea aestuans (*Urticaceae*) is a tropical nettle weed commonly called stinging nettle or West Indian wood nettle that is widely distributed in tropical rain forests [14]. Its phytochemical constituents include saponins, tannins, flavonoids, cardiac glycosides and phenolic compounds [15]. Traditionally, *Laportea aestuans* is commonly used to treat laryngitis, burns and whitlow, urinary problems, diabetes, asthma, stroke, kidney problems and pain [14], whole plant is used as a remedy for stomach-ache [16], bronchitis and filariasi. The plant possesses antimicrobial, antioxidant activities against free radicals [17]. However, its impact on gastric ulcer has not been previously explored. Thus, the present study investigated the potential cytoprotective actions of methanol extract of *Laportea aestuans* (MELA) leaves in ethanol-induced gastric injury in Wistar rats.

Materials and methods

Plant collection and authentication

Fresh young leaves of *Laportea aestuans* were collected in June 2014 after identification of the plant from fields in Iwo, Osun State, Nigeria and were authenticated at Department of Biological Science, Bowen University, Iwo, Osun State, with voucher identification number BUH 096.

Chemicals and reagents

Cimetidine (Greenfield Pharmaceutical Company Limited, Jiangsu, China) was obtained from a reputable pharmaceutical outlet in Ibadan, Oyo State, Nigeria. Trichloroacetic acid (TCA), reduced glutathione (GSH), Folin-Ciocalteau reagent, 5 - 5`-dithiobis-(2-dinitrobenzoic acid) (DNTB), epinephrine, Bovine serum solution, methanol, sodium hydroxide, glacial acetic acid, thiobarbituric acid (TBA), sorbitol, xanthine, 1-Chloro-2,4-dinitrobenzene (CDNB), alcian blue, tris base, hydrogen peroxide, gum acacia and sulphosalicylic acid were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Others chemicals used were of analytical grade.

Experimental animals

Forty male Wistar rats (150 - 170 ± 5 g) were obtained from Central Animal House, Faculty of Basic Medical Science, College of Medicine, University of Ibadan, Ibadan, Nigeria. To avoid coprophagy, rats were kept in polyethylene-walled cages in a temperature-controlled room (25 ± 2 °C) with 12 h light and 12 h dark cycle prior to the experiments and were fed with standard rat's chow (Ladokun Feeds, Nigeria) with fresh water *ad libitum*. They were acclimatized for 14 days. All procedures in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding principles in the care and use of animals [18] and as approved by the University Research Ethics Committee (No. BFSC/2019/216). The "Principle of Laboratory Animal Care" (NIH publication No. 85 - 23) guidelines and procedures were considered in this study (NIH publication revised, 1985) [19]. The rats were deprived of food for 18 h but had free access to clean water prior to the commencement of the experiment.

Preparation of extract

The leaves were washed under tap water to remove contaminants, air-dried at room temperature for 15 days, pulverized with electric blender (model MS-223; Blender/Miller III, Taiwan, China) and 500 g of the powdered leaves was extracted with 2.5 L of methanol by maceration for 48 h with intermittent shaking. The extract obtained was filtered and evaporated at 40 °C by using rotatory evaporator (Model 349/2 Corning/England). Then, the obtained semisolid mass that was lyophilized by freeze dryer. The powder extract (MELA) obtained was stored in refrigerator at −20 °C and was used in this study.

Determinations of total phenolics and total flavonoids

The total phenolic content in the plant extract was determined according to the method of Demiray *et al*. [20] Briefly, 1 mL of the extract/standard of different concentration was mixed with 2.5 mL of Folin Ciocalteau reagent (previously diluted with water 1:10 v/v) and 2 mL of Na_2CO_3 (7.6 % w/v). The tubes were vortexed and allowed to stand at 40 °C for 30 min for colour development. Absorbance was read at 765 nm using a spectrophotometer (Camspec M106, USA). Extract was estimated at a final concentration

of 1 mg/mL. Test was carried out in triplicate and the result was expressed in mg/g gallic acid equivalents from the equation of the calibration curve.

Total flavonoids were estimated using the method described by Ordon *et al.* [21]. Briefly, 1 mL of extract/standard of different concentration was mixed with 0.5 mL of 2 % aluminium chloride. After incubation for 1 h at room temperature, the absorbance of the reaction mixture was measured at 420 nm. The flavonoid content was determined from standard curve prepared using quercetin (0.012 - 0.200 mg/mL) as standard. The amount of flavonoids was expressed as quercetin mg/g dry crude extract and all tests were carried out in triplicates.

Animal grouping and ulcer induction

Gastric ulceration was induced according to the method described by Raish *et al.* [22]. Forty rats weighing 150 - 170 ± 5 g were divided randomly into 5 groups of 8 rats and were pretreated once daily orally with either MELA or cimetidine (CIM) for 7 days. The animals were fasted overnight prior to the commencement of the experiment. Rats in group 1 received 1 % gum acacia and served as the normal control group, rats in group 2 received 1 ml/kg body weight of ethanol (EtOH) (70 % v/v ethanol) and served as the ulcerated untreated group. Rats in groups 3 - 5 were pretreated for 7 days with 200 mg/kg, 400 mg/kg MELA and 50 mg/kg CIM respectively. On the 7 day, 4 h after the last dose of either MELA or CIM, ulceration was induced in rats in groups 2 - 5 with ethanol (1 mL/kg body weight EtOH). Doses of MELA used were based on our previous toxicity study.

Stomach and gastric juice collection

On the 7 day, 4 h after ulcer induction, all the rats were anesthetized using ketamine and xylazil and blood was collected via the retro-orbital vein using glass capillary tube from each rat. The blood was allowed to coagulate at room temperature and then centrifugation at 3000 rpm for 10 min to obtain serum. The sera were stored at −80 °C for biochemical assays. The stomach of each rat was opened along the greater curvature and the gastric content from each rat was collected separately into centrifuge tubes. The stomach of each rat was then excised, rinsed with 5 mL of distilled water and blotted dry. Each stomach was examined to locate the lesion using 2× hand lens. Section of the stomach of each rat was cut and preserved for histological assessment. The gastric content and the washing for each rat were then centrifuged at 1000 rpm for 10 min [23]. The supernatant obtained was used for the determination of ulcerogenic parameters.

Quantification of ulceration

Degrees of ulceration in the EtOH-treated animals were quantified using the procedure outlined by Ohara *et al*. [24] Briefly, cleaned stomachs were pinned on a corkboard and ulcers were scored using a 2× magnifying lens. Severity of the gastric mucosal damage was graded as follows; grade 0, no lesion; grade 1, pinpoint ulcer; grade 2, hemorrhagic erosion (more than 5); grade 3, many small linear ulcers (shorter than 2 m^2); grade 4, multiple linear ulcers of marked size. The ulcer index (UI) for each group was calculated by multiplying the number of rats in each grade by the number of grades divided by the number of rats in each group [25]. Percentage inhibition for each group was calculated using the formula:

Percentage protection = (UIC – UIT) / UIC×100

where UIC = ulcer index for control; UIT = ulcer index for test.

Determination of gastric peptic activity

Gastric juice (1 mL) from each rat was mixed with 1 mL albumin solution (0.5 % BSA w/v in 0.06N hydrochloric acid) in a test tube and was incubated at 37 °C for 10 min. The reaction was terminated by addition of 2 mL of 10 % TCA and the mixture was centrifuged at 1500 rpm for 20 min. Sodium carbonate 0.55 M (2.5 mL) and 1 mL of Folin's reagent were added to 1 mL of each supernatant and incubated at room temperature for 30 min. The absorbance of each sample was determined by spectrophotometer at 660 nm. The activity of pepsin was represented as mg of tyrosine/mL [26].

Evaluation of gastric wall mucus (GWM)

Gastric mucus content was determined according to the method of Corne *et al.* [27] Briefly, the glandular segments of each rat stomach was removed, cut and weighed. Thereafter a section was cut and immersed in 10 mL of 0.1 % w/v Alcian blue solution for 2 h. The excess dye was then washed off using 10 mL of 0.25 M of sucrose solution and the rest was washed out by 10 mL of 0.5 M of magnesium

chloride for 30 min. Then 4 mL of diethyl ether was added to the extract and shaking for 2 min, centrifuged at 4000 rpm for 10 min, the absorbance of each stomach was read at 580 nm.

Preparation of gastric tissue homogenate and assay of antioxidant status

The gastric mucosal tissue left from each rat after cutting for histological study was weighed and gastric tissue homogenate was prepared by using 0.01 M of ice-cold phosphate buffer (pH 7.4; ratio 1:10 w/v) using a Teflon Homogenizer. The resulting homogenate was centrifuged at 10,000 rpm at a temperature of 4 °C for 15 min. The supernatant fraction for each rat was collected and stored at 4 °C for biochemical estimations.

Determination of superoxide dismutase (SOD) activity

Gastric mucosa superoxide dismutase (SOD) activity was determined by its ability to inhibit the auto-oxidation of epinephrine as described by Sun and Zigma [28]. The reaction mixture (3 mL) contained 2.95 mL 0.05 M sodium carbonate buffer (pH 10.2), 0.02 mL of gastric mucosa supernatant and 0.03 mL of 0.3 mM epinephrine in 0.005 N HCl was used to initiate the reaction. The reference cuvette contained 2.95 mL buffer, 0.03 mL of epinephrine and 0.02 mL of water. The in absorbance was read at 480 nm every 30 s for 3 min with a UV spectrophotometer (UV-1650 PC, Shimadzu, Japan).

Determination of catalase (CAT) activity

Catalase (CAT) activity in the gastric mucosa tissue supernatant was assessed by the method of Kakkar and Viswanathan [29] by measuring absorbance due to the decomposition of H_2O_2 in UV recording spectrophotometer. The reaction mixture contained 0.1 mL of the supernatant in phosphate buffer (50 mM, pH 7.0) and 2.9 mL of 30 mM of H_2O_2 in the phosphate buffer pH 7.0. An extinction coefficient for H_2O_2 at 240 nm of 40.0 M^{-1} cm^{-1} was used for calculation. The specific activity of CAT was expressed as µmol H_2O_2 consumed/min/mg protein.

Determination of glutathione peroxidase (GPx) activity

The GPx activity in the gastric supernatant was measured according to the method of Moin [30] by preparing an assay mixture containing 0.8 mL of 0.1 mol/L Tris-HCl with 12 mmol/L sodium aside and 6 mmol/L ethylenediamine tetra acetic acid (EDTA), pH 8.9, 0.2 µl of gastric supernatant, 0.1 mL of 0.01 mol/L DTNB, 1 mL of 20 mmol/L t-butyl-hydroperoxide, and 0.1 mL of 4.8 mmol/L GSH. The decrease in the absorbance at 412 nm was measured spectrophotometrically (UV-1650 PC, Shimadzu, Japan) and GPx activity was expressed as unit/mg protein.

Estimation of reduced glutathione (GSH) level

Reduced glutathione (GSH) was determined by the method of Beutler *et al.* [31] by adding 2 mL of phosphate buffer to 1 mL of the gastric supernatant followed by addition of 0.5 mL of Ellman's reagent (10 mM). The yellow colour developed was read at 412 nm against blank containing 3.5 mL of phosphate buffer. Different concentrations (0 - 100 µg/mL) of the standard were also treated similarly and the amount of GSH was extrapolated from the standard curve and expressed in µmol/mg protein.

Determination of Glutathione S-transferase (GST) activity

Glutathione S-transferase activity was determined according to the method of Habig and Jakoby [32] with slight modifications using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Briefly, 20 µL of gastric supernatant was added to 270 µL of reaction mixture containing (20 mL of 0.25 M potassium phosphate buffer, pH 7.0, 10.5 mL of distilled water and 500 µL of 0.1 M GSH at 25 °C) followed by addition of 10 µL of 25 mM CDNB. The reaction was monitored for 5 min (30 s intervals) at 340 nm in a UV spectrophotometer (UV-1650 PC, Shimadzu, Japan) and GST activity expressed as unit/mg protein using the molar extinction coefficient (ε) of 9.6 mM^{-1} cm^{-1} for CDNB conjugate.

Estimation of malondialdehyde (MDA) level

Gastric tissue MDA level was determined by the method of Jiang *et al.* [33] Briefly, gastric tissue supernatant (1 mL) of each rat was deprotonated with 1 mL of 10 % TCA and centrifuged for 20 min at 2000 rpm. Then 1 mL of the centrifuged mixture was mixed with 2 mL of 0.67 % TBA and heated for 30 min at 95 °C in a water-bath. After cooling, the suspension was centrifuged at 3000 rpm for 10 min. The absorbance of pink colored reaction mixture was read at 532 nm with a UV spectrophotometer (UV-1650 PC, Shimadzu, Japan). The MDA content was expressed as mmol/g of wet tissue.

Protein determination

Protein content was measured according to the method by Bradford [34]. Briefly, 1 mL of gastric supernatant from each rat was measured into a test tube and 2 mL of the biuret reagent was added to the content and mixed gently. The mixture was incubated at 37 °C for 10 min, and the absorbance was read using a UV spectrophotometer (UV-1650 PC, Shimadzu, Japan) at 540 nm. This was done in triplicate. A standard curve using bovine serum albumin (BSA) was prepared, and the protein concentration in the gastric tissue was estimated from a standard curve.

Estimations of serum levels of TNF-α and IL-1β

Protocols in the purchased Cusabio ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, China) were followed. Briefly, 100 µL of samples or standards were added into the wells already pre-coated with antibody specific for rat TNF-α or IL-1β and incubated for 2 h at 37 °C. Unbound substances were removed and 100 µL of biotin-conjugated antibody specific for rat TNF-α or IL-1β was added to the well. After washing, 100 µL of avidin conjugated Horseradish Peroxidase (HRP) was added to each well and incubated for 1 h at 37 °C, the unbound components were washed away. Then, 90 µL of tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 15 - 30 min at 37 °C to give a blue color proportional to the amount of TNF-α or IL-1β bound. The reaction was terminated by adding stop solution (50 µL) and the yellow color developed was measured at 450 nm using SpectraMax plate reader (Molecular Devices, CA, USA).

Determination of serum Cyt c, CASP-9 and CASP-3 activities

Serum cytochrome c (Cyt c), Cysteine-aspartic acid specific proteases (CASP-9 and CASP-3) concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using rat specific Cyt c, CASP-9 and CASP-3 assay kits (Cusabio Biotech Co., Ltd, Wuhan, China) according to the manufacturer's instructions. The assays rely on a quantitative sandwich enzyme immunoassay technique and the final-colored product was measured at 450 nm by SpectraMax plate reader (Molecular Devices, CA, USA). All of the experiments were performed in triplicate, and the results were presented in pg/mL.

Histological assessment

Gastric tissues histology was assessed by the method of Sun and Zigma [28]. Briefly, sections of gastric tissue fixed in 10 % formalin after sacrifice and each sample was embedded in paraffin and cut into 5 µm thick slices using a Leica RM2135 microtome (Leica, Berlin, Germany) for histological evaluation. Tissue sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Leica Microsystems, Germany).

Statistical analysis

Results were expressed as mean ± standard error of mean. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Duncan's test was used to compare values between groups. The data were analyzed by using Statistical Package for the Social Sciences (SPSS, version 17.0, Armonk, NY, USA). A $p < 0.01$ were considered to be significantly different. Ulcer protection was expressed in percentage.

Results and discussion

Total phenol and total flavonoids

The quantitative phytochemical analysis of methanol extract of *Laportea aestuans* (MELA) leaf showed the presence of phenols and flavonoids (**Table 1**).

Table 1 Total phenolic and flavonoids contents of methanol extract of *Laportea aestuans* (MELA) leaf.

Methanol extract	Total flavonoids (mg quercetin g^{-1})	Total phenol (mg gallic acid g^{-1})
Laportea aestuans	87.04 ± 0.42	61.98 ± 0.32

Values expressed per g of plant extract and as means ± standard deviation (SD) of triplicate determination.

Effects of MELA and CIM on ulcerogenic parameters in ethanol-induced gastric

The effects of MELA on ulcerogenic parameters are depicted in **Figure 1**. Administration of 1 mL/kg b.w. of EtOH caused a significant ($p < 0.01$) increase in the ulcer score (US), ulcer index (UI) and pepsin activity with concomitant decrease in gastric mucus content compared to the control rats. However, pretreatment of rats with 200 and 400 mg/kg b. w. of MELA protected the rats in a dose-dependent manner as evident in the decrease in the ulcer score, ulcer index and pepsin activity and an increase in the mucus content of the pretreated rats. The 50 mg/kg of cimetidine also decreased the US, UI, peptic activity and decreased the mucus content. The extract at 400 mg/kg b. w. offered better protection when compared with the reference drug (cimetidine). The percentage protection offered by 200 and 400 mg/kg b. w. of MELA were 78.88 and 82.40 %, respectively, while CIM gave 79.83 % to rats. These observations could be due to the presence of the phenolic and flavonoids contents in the extract as these phytochemicals have been reported to possess anti-ulcerative properties [35].

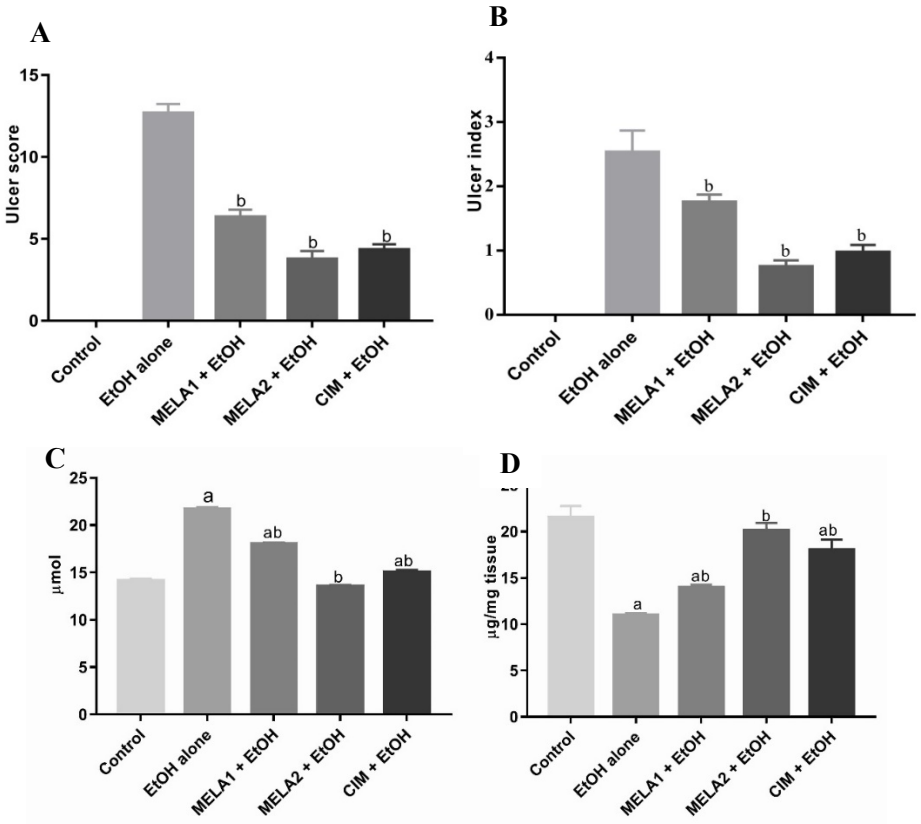

Figure 1 Effects of methanol extract of *Laportea aestuans* leaf on A) ulcer score, B) ulcer index, C) pepsin activity and D) mucus content on ethanol-induced gastric ulcer in rats; (n = 8, ± SEM). Bars with different superscripts for each parameter are significantly different ($p < 0.01$). [a] significantly different from the control group ($p < 0.01$); [b] significantly different from the EtOH group ($p < 0.01$). EtOH: Ethanol (1 mL/kg b. w.), MELA1: 200 mg/kg methanol extract of *Laportea aestuans* leaf, MELA2: 400 mg/kg methanol extract of *Laportea aestuans* leaf, CIM: 50 mg/kg cimetidine.

Biochemical analyses of gastric secretions (for pH, gastric volume, bicarbonate and pepsin activity) and mucosal integrity of stomach are usually employed to ascertain its status following exposure to pharmacological agents [36]. Reports have attributed gastrointestinal injury to erosion mucin content which is facilitated by onslaughts of both internal (pepsin and oxidants produced in the gastric lumen) and external (drugs and ethanol) aggressive agents on mucosal epithelia. Ethanol penetrates the gastric mucosa to cause the solubilization of the protective mucous and thus exposes the mucosa to the proteolytic and hydrolytic actions of pepsin and hydrochloric acid resulting in gastric epithelial cell membrane injury [37]. In the present study, following oral administration of ethanol to the rats, ulcers of various sizes were detected on the gastric mucosa of the ethanol alone group. This can be attributed to

auto digestion of the mucosal wall via the buildup of acidic gastric juice. This result is consistent with previous report [38]. Groups treated with MELA significantly decreased the rate of damage by ethanol as evident in the ulcer score and ulcer index. This observation is further supported by the histological analysis (**Figure 5**). Cimetidine also protected the gastric mucosal as seen in the ulcer score and ulcer index.

Several studies suggest that mucus gel layer provide protection against internal and external aggressive factors like ethanol and neutralize luminal acids thereby offering cytoprotection to the gastric mucosal. Ethanol disrupts the gastric mucosal layer and lowers the level of tissue proteins. Thus, compounds that have the ability to increase mucus production might possess gastroprotective activity. This present study showed decreased in mucus content in ethanol alone group [39]. However, pretreatment with MELA and cimetidine upregulated gastric mucus content against depletion by ethanol administration. Also, the increased pepsin activity coupled with decrease in mucin secretion in the ethanol-ulcerated rats indicated altered hydrophobicity and reduced protective ability of the mucosal membrane against hemorrhagic erosion. This implied decreased ability of the gastric mucosa to withstand the offensive onslaught of ethanol [40]. Pretreatments with the MELA extract in this study, arrested ulcer progression, as revealed by decreased pepsin activity and elevated mucin level in the gastric mucosa.

Effects of MELA and CIM on antioxidant status and lipid peroxidation in ethanol-induced gastric ulcer

The effects of MELA and CIM on antioxidant status and lipid peroxidation of rats administered with EtOH are shown in **Figures 2** and **3**. Effects of MELA on the activities of SOD, CAT and GPx in the gastric mucosal of ethanol-induced gastric ulcer are shown in **Figures 2A - 2C**. Activities of the antioxidant enzymes were significantly reduced ($p < 0.01$) in EtOH treated rats compared with normal control. Pretreatment with 200 and 400 mg/kg b. w. of MELA and 50 mg/kg b. w. CIM significantly ($p < 0.01$) increased the activities of the enzymes with the 400 mg/kg b. w. of MELA give a better protection than 50 mg/kg b. w. cimetidine **Figures 2A - 2C**.

Figures 3A - 3C revealed the effects of MELA leaf on the GST activity, levels of GSH and LPO of gastric mucosal of ethanol ulcerated rats. GST activity and GSH level were significantly ($p < 0.01$) decreased and the MDA level increased significantly in the EtOH alone rats compared to the normal control. But, pretreatment with 200 and 400 mg/kg b. w. of MELA and 50 mg/kg b. w. CIM significantly ($p < 0.01$) increased the GST activity and GSH level and decreased the MDA level when compared with the EtOH treated rats. Again, 400 mg/kg b. w. of MELA seem to give a better protection than 50 mg/kg b. w. cimetidine.

Ethanol causes disruption of vascular endothelium of the gastric mucosal cells and triggers the release of superoxide anion and other reactive oxygen species (ROS) that produced a cascade of events leading to an increased oxidative stress [41]. The gastric tissue is endowed with antioxidant system such as SOD, CAT, GPx, GST and GSH which are the frontline endogenous antioxidant that scavenge the ROS and prevent the oxidative stress, but under pathological conditions such as gastric ulcer, this system is overwhelmed by excessive generation of ROS [42,43]. SOD converts superoxide to hydrogen peroxide and oxygen while CAT catalyzed the hydrogen peroxide to water [44]. On the other hand, GPx possesses peroxidase activity and reduces lipid hydroperoxides and free hydrogen peroxide to water [45] and GST participates in conjugation of GSH through a sulfhydryl group to the electrophilic centers of a variety of endogenous compounds such as lipid peroxides and exogenous compounds such as drugs and alcohol resulting in their detoxification [46]. Studies have shown that ROS induced peroxidation of membrane lipids constituent of the gastric cells. MDA is one of the end products of polyunsaturated fatty acid peroxidation and a marker for assessing oxidative stress [47]. In this study, exposure of rats to oral administration of ethanol diminished the activities of SOD, CAT, GPx, GST and GSH content and concomitantly increased the MDA level in the gastric tissue [48-51]. Furthermore, administration of MELA attenuated these observations, suggesting that its cytoprotective potential of may be due to its free radical scavenging activity and inhibition of lipid peroxidation in the gastric tissue. The 400 mg/kg MELA group improved the antioxidant status and prevent lipid peroxidation better than CIM group.

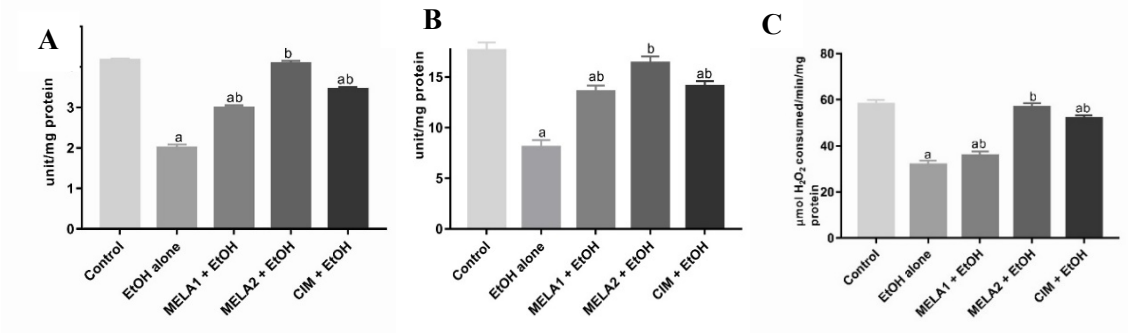

Figure 2 Effects of methanol extract of *Laportea aestuans* leaf on activities of A) superoxide dismutase, B) catalase and C) glutathione peroxidase on ethanol-induced gastric ulcer in rats; (n = 8, ± SEM). Bars with different superscripts for each parameter are significantly different ($p < 0.01$); [a]significantly different from the control group ($p < 0.01$); [b] significantly different from the EtOH group ($p < 0.01$). EtOH: Ethanol (1 mL/kg b. w.), MELA1: 200 mg/kg methanol extract of *Laportea aestuans* leaf, MELA2: 400 mg/kg methanol extract of *Laportea aestuans* leaf, CIM: 50 mg/kg cimetidine.

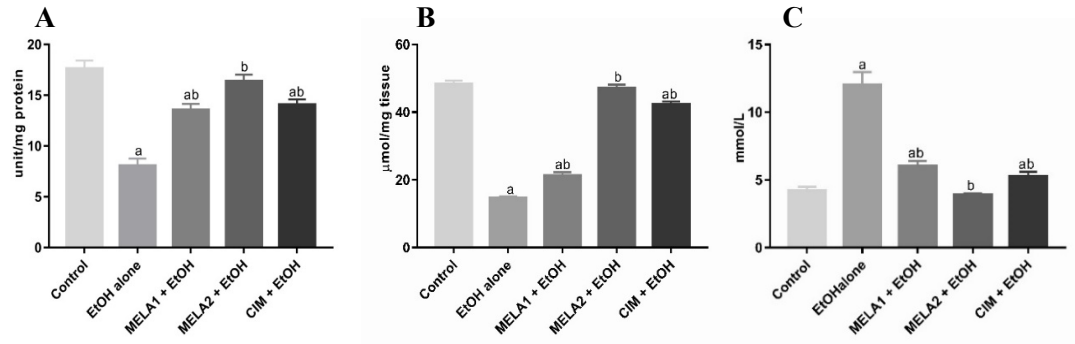

Figure 3 Effects of methanol extract of *Laportea aestuans* leaf on A) Glutathione S-transferase activity, B) reduced glutathione level and C) lipid peroxidation level on ethanol-induced gastric ulcer in rats; (n = 8, ± SEM). Bars with different superscripts for each parameter are significantly different ($p < 0.01$); [a] significantly different from the control group ($p < 0.01$); [b] significantly different from the EtOH group ($p < 0.01$). EtOH: Ethanol (1 mL/kg b. w.), MELA1: 200 mg/kg methanol extract of *Laportea aestuans* leaf, MELA2: 400 mg/kg methanol extract of *Laportea aestuans* leaf, CIM: 50 mg/kg cimetidine.

Effects of MELA and CIM on TNF-α and IL-1β in Ethanol-induced gastric ulcer

The effects of MELA on pro-inflammatory cytokines are depicted in **Figure 4**. Administration of 1 mL/kg b.w. of EtOH significantly ($p < 0.01$) increased the TNF-α and IL-1β levels in the rats compared to the control rats. But, pretreatment of rats with 200 and 400 mg/kg b. w. of MELA decreased the TNF-α and IL-1β levels in a dose-dependent manner. Cimetidine also decreased the levels of these cytokines. The extract at 400 mg/kg b. w. offered better protection when compared with the reference drug (cimetidine).

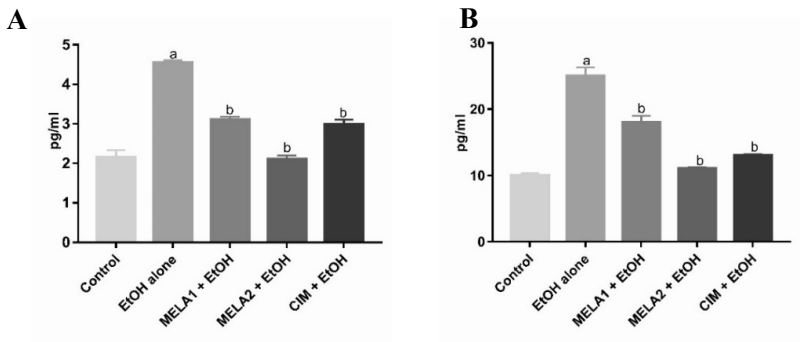

Figure 4 Effects of methanol extract of *Laportea aestuans* leaf on A) TNF-α level and B) IL-1β level on ethanol-induced gastric ulcer in rats; (n = 8, ± SEM). Bars with different superscripts for each parameter are significantly different ($p < 0.01$); [a] significantly different from the control group ($p < 0.01$); [b] significantly different from the EtOH group ($p < 0.01$). EtOH: ethanol (1 mL/kg b. w.), MELA1: 200 mg/kg methanol extract of *Laportea aestuans* leaf, MELA2: 400 mg/kg methanol extract of *Laportea aestuans* leaf, CIM: 50 mg/kg cimetidine.

Inflammation has been associated with ethanol-induced gastric ulceration [52,53]. Ethanol produces inflammatory response by initiating the recruitment and infiltration of neutrophil to the site of inflammation and cause the release of pro-inflammatory cytokine especially TNF-α and IL-1β. Continuous release of these cytokines and generation of ROS promote gastric ulceration. TNF-α particular triggers the expression of the nuclear transcription factor kappa-B (NF- κB) which in turn causes the release of other cytokines and subsequent exacerbation of gastric mucosal damage [54]. Consistent with other studies, our results showed that rats administered with ethanol alone had elevated levels of these cytokines [8]. MELA down-regulate the levels of these pro-inflammatory cytokines, an implication of the anti-inflammatory property of MELA. Furthermore, 400 mg/kg of MELA decreased these cytokines better than cimetidine.

Effects of MELA and CIM on activities of Cyt c, CASP-9 and CASP-3 in ethanol-induced gastric ulcer

Figure 5 showed the makers of intrinsic apoptosis in the rats. Administration of 1 mL/kg b.w. of EtOH caused a significant ($p < 0.01$) increase in CYT-C, CASP-9 and CASP-3 activities in the rats compared to the control rats. But, pretreatment of rats with 200 and 400 mg/kg b. w. of MELA decreased the activities of these markers in a dose-dependent manner. Cimetidine also decreased the levels of these apoptotic markers. The extract at 400 mg/kg b. w. seems to offered better protection when compared with cimetidine.

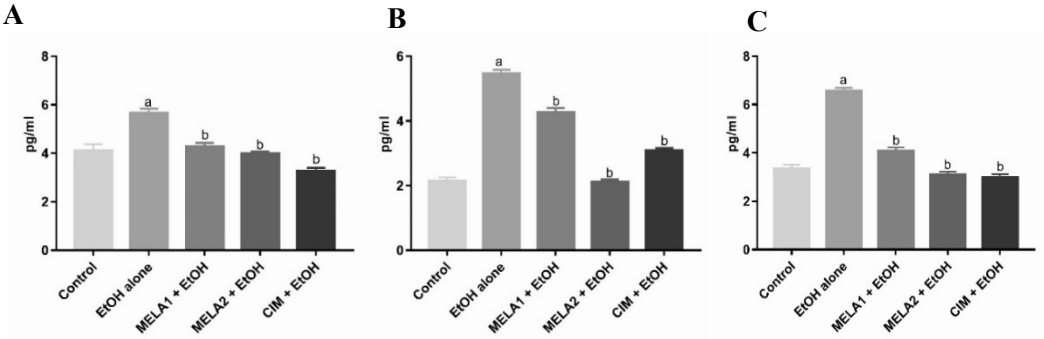

Figure 5 Effects of methanol extract of *Laportea aestuans* leaf on activities of A): CYT-C, B) CASP-9 and C) CASP-3 on ethanol-induced gastric ulcer in rats; (n = 8, ± SEM). Bars with different superscripts for each parameter are significantly different ($p < 0.01$); [a] significantly different from the control group ($p < 0.01$); [b] significantly different from the EtOH group ($p < 0.01$). EtOH: Ethanol (1 mL/kg b. w.), MELA1: 200 mg/kg methanol extract of *Laportea aestuans* leaf, MELA2: 400 mg/kg methanol extract of *Laportea aestuans* leaf, CIM: 50 mg/kg cimetidine.

Apoptosis, a programmed cell death, is vital for normal cell survival. Abnormal buildup of ROS has been linked to induction of cellular apoptosis through peroxidation of phospholipid constituents of the membrane [55] leading to the release of cytochrome c and subsequently the intrinsic apoptotic pathway [56]. Additionally, inflammatory process through TNF-α has also been implicated in the extrinsic apoptotic pathway [57]. Apoptosis in mammalian cell has been associated with caspases which play a critical role in the destruction of gastric mucosal structure in ethanol-induced ulcer [58,59]. In our present study, administration of ethanol to rats induced the release of CYT-C, CASP-9 and CASP-3; the executioner caspase as well as elevation of TNF-α level, suggesting that the induction of apoptosis in ethanol-induced gastric ulcer is linked to both ROS generation and inflammation. Our result is consistent with other studies reported [60]. Furthermore, MELA ameliorated these observations, indicating that MELA has the potential to protect the gastric mucosal against ethanol damage by regulating apoptosis.

Histological assessment of gastric lesions

Histological observation of the gastric tissues of the rats showed an intact gastric tissue in the normal control group (**Figure 5A**); the group treated with 1 mL/kg bwt of EtOH alone showed disruption of the epithelia cells, marked congestion in the mucosal, infiltration of the mucosa by inflammatory cells, lacerations and necrosis (**Figure 5B**). The rats pretreated with 200 mg/kg MELA before EtOH administration (**Figure 5C**) showed mild disruption of the surface epithelium with leucocyte infiltration and fissure of the submucosal; the rats pretreated with 400 mg/kg MELA before EtOH administration (**Figure 5D**) showed restoration of the surface epithelium, intact mucosal muscularis and no leucocyte infiltration and the group pretreated with 50 mg/kg CIM prior to EtOH administration (**Figure 5E**) revealed mild disruption of the surface epithelium, inflammatory cells infiltration and mild splitting of the submucosal (**Figure 5**).

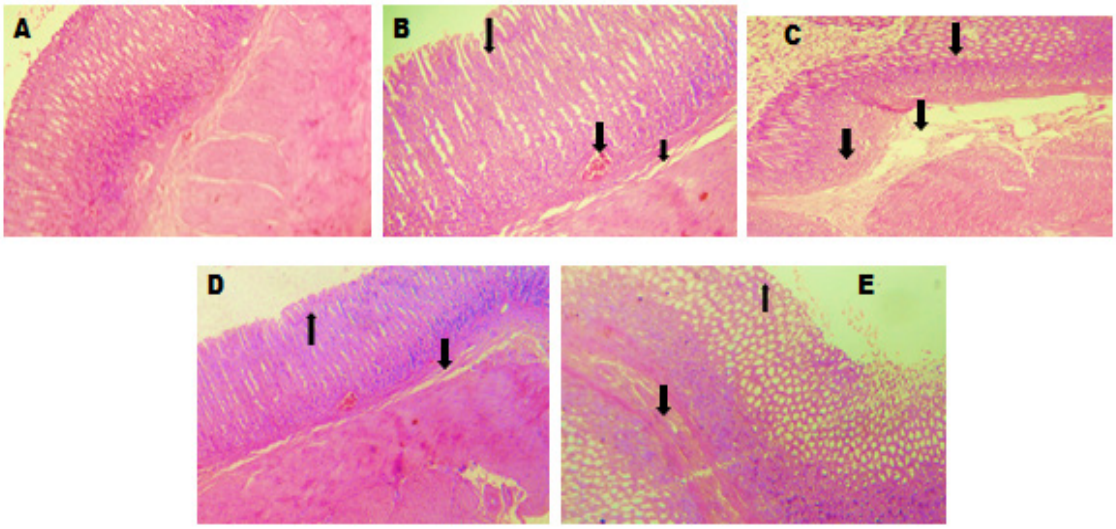

Figure 5 Representative histological effects of methanol extract of *Laportea aestuans* leaf on ethanol-induced gastric mucosa damage (ulcer) in Wistar Rats. A) control stomach architecture appears intact, B) treatment with 1 mL/kg bwt of EtOH showing damage of the mucosa with disruption of the epithelia cells, marked congestion in the mucosa and infiltration of the mucosa by inflammatory cells, C) MELA1 (200 mg/kg bwt) + 1 mL/kg bwt of EtOH showing mild disruption of the surface epithelium with necrosis, infiltration of the mucosa by inflammatory cells, D) MELA2 (400 mg/kg bwt) +1 mL/kg bwt of EtOH showing restoration of the mucosa cells and increased muscularis thickness, E) CIM (50 mg/kg bwt) + 1 mLkili/kg bwt of EtOH showing partial restoration of the surface epithelia cells. (H & E, X 100).

Conclusions

Overall, our results highlight the evidences for the cyto- and gastroprotective potentials of methanol extract of *Laportea aestuans* leaf in ethanol-induced gastric ulcer model in rat. Our results also revealed that MELA at 400 mg/kg significantly reduced gastric injury by protecting the mucosal barrier and enhance microcirculation via antioxidant and anti-inflammatory processes and also inhibit apoptosis by modulating expression of apoptotic factors in the gastric tissue. Our results thus show the promising potential of *Laportea aestuans* leaf as a natural adjuvant therapeutic agent for alleviating gastric mucosal injury. Further studies are ongoing to explore the mechanisms of action of MELA that can aid its use as an alternative in clinical setting for the management of gastric ulcers.

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