Anti-ulcer potency of *Mesua ferrea* Linn.

SUMMARY

Gastric ulcers are a common global problem with increasing incident and prevalence attributing to modern life style. Gastric lesions develop due to loss of delicate balance between gastro-protective and aggressive factors. Available anti-ulcer drugs although effective at various stages of ulcer pathogenicity, pose a adverse pathological consequences. The current study unravels anti-ulcer potential of MEE against ethanol/swim induced stomach ulcer in rat model. MEE containing phenolics and flavonoids prevented ethanol/swim stress induced stomach ulcer to an extent of 85.53±0.2% (ulcer healing index). The possible mechanism involved in gastro protective activity by MEE may involve significant up regulation of gastric mucin synthesis, enhanced synthesis of antioxidant system concomitant with the inhibition of lipid and protein oxidation including inhibition of H⁺-K⁺ ATPase pump.
4.1. Introduction

Gastric ulcers are open craters or sores that develop in the inner lining (mucosa) of the stomach. Stress appears to be a major role in causing gastric ulcer (Vorder and Peura, 1990). A coating of mucus and other chemicals normally shield the stomach and duodenum; resist large variety of noxious factors. Nevertheless, when these protective mechanisms are disrupted by injurious factors such as powerful digestive acid and pepsin, thus erode the lining of these organs, cause gastric ulcers and makes the person irritating, less tolerant, violent, aggressive, etc.

Several mechanisms play a very crucial role to maintain or to protect gastric mucosal integrity. Prostaglandins play a central role in mucosal resistance. They regulate the release of mucosal bicarbonates, up-regulate gastric mucin synthesis, trigger mucosal cell proliferation, inhibit parietal cell secretion and are important in maintaining MBF (Adam and Kris, 1999; Brzozowski et al., 2004). Enhancement of aggressive factors, such as increase of acid/pepsin secretion, inhibition of prostaglandin synthesis by NSAIDs, reflux of bile and intestinal content into stomach, ROS and H. pylori infection, which tilt the balance in favor of ulcer, eventually, cause gastric ulceration (Miller, 1987; Ernö and Gold, 2000).

In developed countries, 0.5-2.5 % (per 100,000 of deaths) of the death rate is due to peptic ulcer. Whereas, in developing countries, death rate due to peptic ulcer is about 3.3 - 16.2 % per 100,000 deaths. India stands fifth rank in worldwide prevalence of peptic ulcer with 12.4 % of death rate (www.worldlifeexpectancy.com/cause-of-death/peptic-ulcer-disease). If it is untreated, it leads to Zollinger-Ellison syndrome. A modest approach to treat ulceration can be achieved via multi-step drug treatment through stimulation of gastric mucin synthesis, enhancement of antioxidant levels in the stomach, scavenging of ROS, inhibition of H⁺- K⁺-ATPase, histamine receptors and H. pylori growth (Bandyopadhyay et al., 2002). Many synthetic drugs fails to exhibit multi-step anti-ulcer effect besides adverse side effects on human homeostasis as a single entity (Waldum et al., 2005). Thus, there is an urgent need to search an indigenous drug with minimal side effects having potent antioxidant property and multi-step activity to have a better and safer alternative for the treatment of peptic ulcer from plant origin.
In the present chapter, evidence on biochemical mechanism underlying anti-ulcer activity of ethanol extract of *Mesua ferrea* L. (MEE) in ethanol and swim stress induced ulcer model has been evaluated. MEE has potent antioxidant property owing to the presence of rich polyphenols (1.005 ± 0.005 mg mg⁻¹ GAE) and flavonoids (514.82± 0.155 µg mg⁻¹). Gallic acid (412.9 mg g⁻¹) was found to be a major phenolic entity followed by ellagic acid and hydroxy benzoic acid. Among flavonoids, quercetin (753.9 mg g⁻¹) was found to be a major molecule followed by rutin and myricetin (Rajesh et al., 2013).

4.2. Materials and methods

4.2.1. Chemicals and instrumentations

Petroleum ether, chloroform, ethanol, Triton X-100, perchloric acid, acetyl acetone, Folin–Ciocalteu, trichloroacetic acid (TCA), cyclohexanone, cysteine, 2,4-Dinitrophenylhydrazine (DNPH), ethyl acetate were procured from Merck (Germany). Riboflavin, 1-chloro-2,4- dinitrobenzene (CDNB), Glutathion oxidized (GSSH), D (+) – glucosamine hydrochloride were purchased from SRL (India). Nitro blue tetrazolium disodium salt (NBT), reduced glutathione (GSH), Nicotinamide adenine dinucleotide phosphate reduced (NADPH), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Adenosine triphosphate (ATP), Coomassie brilliant blue R-250, sodium periodate, Thiobarbituric acid (TBA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). D (+) – galactose, D (+) – mannose, hemoglobin, guanidine hydrochloride were purchased from Himedia (India). Alcian blue was procured from SD fine (India). All the chemicals used were of analytical grade.

Water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA). Absorbance was measured with Systronics PC based UV-visible double beam 2202 spectrophotometer (Systronics, India). Sodium and Potassium was estimated by GBC932plus Atomic absorption spectroscopy (GBC, Australia). Cyclic voltammetry study was performed with CHI-660c electrochemical work station (CH instruments, USA), equipped with personal computer. GC-MS analysis was carried out with a Carlo Erba 5130 Mega GC linked directly to an ion trap detector (Finnigan MAT, ITD 700) operated in electron impact (EI) or chemical ionisation (CI) mode using iso-butane.
4.2.2. Preparation of plant extract

*M. ferrea* L. stem bark was collected and plant extract was prepared as mentioned in chapter 2 and stored in desiccator until further study.

4.2.3. Quantitative analysis of MEE by GC-MS

GC-MS analysis was carried out with a Carlo Erba 5130 Mega GC linked directly to an ion trap detector (Finnigan MAT, ITD 700) operated in electron impact (EI) or chemical ionisation (CI) mode using iso-butane. Fused silica capillary columns (25 m x 0.22 mm id) coated with either polar CP Wax 52 CB or non-polar CP Sil 5 CB were used with a split-split less injector (200°C) and helium as carrier gas (0.5 kg cm-2). The oven temperature was held at 50 °C for 2 min, then programmed at 6 °C min⁻¹ to 230 °C. The relative percentage amount of each component was calculated by comparing its average peak area to the total area.

Interpretation of GC-MS mass-spectrum was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components of MEE was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

4.2.4. Animals

Toxicity assessment of MEE was carried out using male Wistar albino rats weighing 150–250 g of three months old (6 per group) which were maintained under standard conditions of temperature (24±2 °C), humidity (40±2%) and light (12 h light, 12 h dark). They were provided with standard rodent pellet diet (Krish scientist shop, Bangalore, Karnataka, India) and water (Reverse osmosis water) *ad libitum* in opaque cage with stainless steel mesh tops and proper husk bedding. Animal experiments were carried out after the clearance of ethical issues from the animal ethical committee (IAEC - Reg. No. 144/1999/CPCSEA).
4.2.5. Assessment of anti-ulcer activity of MEE against ethanol and swim stress induced ulcer

Animals were grouped into two sets of five groups (n = 6) as shown in table 4.1. MEE at two doses (200 and 400 mg kg\(^{-1}\) b.w.) and ranitidine (30 mg kg\(^{-1}\)) was administered orally to animals for 14 days. At the end of 14\(^{th}\) day, first set of animals were fasted for 24 h and subjected to ethanol stress. Each animal from all the groups were administered with 100% ethanol (5 ml kg\(^{-1}\) b.w.) and allowed for 60 min. (Jainu and Devi, 2006).

**Table 4.1:** Experimental groups with sample concentration and ulcer induction models

<table>
<thead>
<tr>
<th>Set 1 (ES)</th>
<th>Set 2 (SS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Healthy</td>
<td>Healthy</td>
</tr>
<tr>
<td>2 Ulcerated</td>
<td>Ulcerated</td>
</tr>
<tr>
<td>3 Ranitidine treated</td>
<td>Ranitidine treated</td>
</tr>
<tr>
<td>4 MEE 200 mg kg(^{-1}) b. w.</td>
<td>MEE 200 mg kg(^{-1}) b. w.</td>
</tr>
<tr>
<td>5 MEE 400 mg kg(^{-1}) b. w.</td>
<td>MEE 400 mg kg(^{-1}) b. w.</td>
</tr>
</tbody>
</table>

ES: Ethanol stress, SS: Swim stress

While second set of animals were subjected to forced swim stress after 24 h of fasting (Brady et al., 1979). Briefly, rats were subjected to forced swim stress by making them swim in jar of 30 cm height and 10 cm diameter containing water up to 15 cm height for 3 h. After each stress, all groups underwent surgery according to Takeuchi et al., (1976). Animals were mildly anesthetized under anaesthetic ether and the abdomen was cut open through the midline incision. With the help of silk suture thread, ligation was done at the pylorus region with care not to damage, after which the wound was closed by suture thread and the animals were allowed to recover from anaesthesia. After pylorus ligation, feeds and drinking water was withdrawn and gastric juice was collected for 4 h. The rats were sacrificed and stomach was removed after clamping esophagus. The gastric content was collected through esophagus, centrifuged and stored in cold (-20 °C) for further studies. The stomachs were opened along the greater curvature and rinsed with phosphate
buffer saline (pH- 7.4), examined for lesions and percentage of healing index and stored in -20 °C for further studies.

4.2.6. Ulcer and percentage of healing index

The purpose of indexing is to determine the anti-ulcer effect of MEE against stress inducers. Mean ulcer index of each experimental group was calculated by using the formula \( U_I = U_N + U_S + U_P \times 10^{-1} \) (Kulkarni, 2002) where, \( U_I \) = ulcer index, \( U_N \) = average of number of ulcers per animal, \( U_S \) = average of severity score and \( U_P \) = percentage of animals with ulcer.

Severity score was recorded as follows, 0.5 – ulcerous red coloration, 1.0 – spot ulcers, 1.5 – hemorrhagic streaks of <3 mm, 2.0 - hemorrhagic streaks of 3 to 5 mm, 3.0 - hemorrhagic streaks >5 mm.

1. Ulcerous red coloration.
2. Spot ulcers.
3. Hemorrhagic streaks of <3 mm.
4. Hemorrhagic streaks of 3 to 5 mm.
5. Hemorrhagic streaks >5 mm

Percentage of ulcer healing index was calculated by using the formula

\[
\% \text{ healing index} = \left[ \frac{U_I C_U - U_I C_T}{U_I C_U} \right] \times 100
\]

where \( U_I C_U \) = ulcer index of ulcerated group, \( U_I C_T \) = ulcer index of test group (Ranitidine and MEE treated).

4.2.7. Gastric acid experiment

The collected gastric juice was measured for total volume and pH. The total acidity of gastric juice was measured by titrating against 0.01N NaOH using phenolphthalein indicator. The total protein (Bradford, 1976), hexose and hexosamine (Winzler, 1958), sialic acid (Warren, 1959) and total pepsin (Anson, 1938) content was estimated.
4.2.7.1. Estimation of hexose and hexosamine

The dissolved muco-substance in gastric juice was estimated by alcoholic precipitation of gastric juice. Briefly, to 1 ml of gastric juice, 9 ml of 90% chilled alcohol was added and incubated for 10 min. under cold condition. The mixture was centrifuged and supernatant was discarded. The obtained precipitate was dissolved in 0.5 ml of 0.1N NaOH and 6N HCl (1.8 ml) was added. The content was hydrolyzed in boiling water bath for 2 h followed by neutralization with 5N NaOH using phenolphthalein as an indicator and the volume was made up to 4.5 ml with distilled water. The obtained hydrolysate was used to estimate the total hexoses and hexosamine content.

4.2.7.2. Estimation of hexose

To 0.4 ml of hydrolysate, orcinol reagent (3.4 ml) was added. The content was incubated at 60 °C for 15 min. cooled under running tap water and absorbance was measured at 540 nm against blank. Total hexose content was determined by employing D (+) – Galactose-mannose calibration curve and expressed in μg ml⁻¹ of gastric juice.

4.2.7.3. Estimation of hexosamine

To 0.5 ml of hydrolysate, 0.5 ml of acetyl- acetone reagent was added. The content was incubated in boiling water bath for 20 min. and cooled under running tap water. 1.5 ml of 90% alcohol was added and allowed to stand for 30 min. The color intensity was measured at 540 nm against blank. Hexosamine content was determined by employing standard curve prepared by D (+) – Glucosamine hydrochloride and expressed in μg ml⁻¹ of gastric juice.

4.2.7.4. Estimation of sialic acid

To 0.5 ml of gastric juice, alcohol (4.5 ml) was added, mixed thoroughly for 10 min. and centrifuged to obtain precipitate. The precipitate was re-suspended in 0.5 ml of 0.1 N H₂SO₄ followed by hydrolysis in boiling water bath for 1 h. and volume was restored to 0.5 ml.

Total sialic acid content in hydrolysate was estimated according to Warren, (1959). Briefly, To 0.2 ml of hydrolysate, 0.1 ml of sodium periodate (0.2 M in 9 M
phosphoric acid) was added, mixed thoroughly and allowed to stand for 20 min. at room temperature. 1 ml of sodium arsenite (10%, in a solution of 0.5 M sodium sulfate-0.1 N H₂SO₄) was added and tubes were mixed thoroughly until yellow-brown color disappears followed by the addition of 3 ml of thiobarbituric acid (0.6 %, in 0.5 M sodium sulfate). The reaction mixture was incubated in boiling water bath for 15 min. and cooled to room temperature. Aliquot of reaction mixture (1 ml) was added to the test tube containing 1 ml of cyclohexanone and mixed thoroughly and centrifuged to get a clear pink layer of cyclohexanone. The absorbance of supernatant was measured at 549 nm and the amount of N-acetylneuraminic acid/sialic acid was estimated by using the formula;
μM of sialic acid = \[V \times A_{549} / 57\], where \(V\) = final volume of reaction mixture (4.3 ml).

4.2.7.5. Estimation of pepsin

Peptic activity is a major factor involved in the proteolytic activity of gastric secretion. Total pepsin activity in gastric juice was determined by measuring the amount of liberated tyrosine from hemoglobin solution (Anson, 1938). Briefly, 50 μl of gastric juice was added to test tube containing 1.25 ml of hemoglobin solution (2%) and incubated for 10 min. at 37 ºC. The 2.5 ml of TCA (4%) was added and incubated for 5 min. in boiling water bath. The mixture was centrifuged and to 0.75 ml of supernatant, 5 ml of distilled water, 0.25 ml of sodium hydroxide (3.85 N) and 0.25 ml of Folin–Ciocalteu reagent was added and incubated at room temperature for 20 min. The absorbance of the content was measured at 680 nm and the amount of liberated tyrosine was measured by employing the standard curve of tyrosine.

4.2.7.6. Estimation of Sodium (Na⁺) and Potassium (K⁺) ion concentration

The amount of sodium and potassium ion was estimated by Atomic Absorption Spectroscopy. The amount of Na⁺ and K⁺ was measured by employing standard curve of Sodium chloride and potassium chloride solution containing 0.325, 0.625, 1.250 ppm of sodium ions and 0.625, 1.250, 2.50 ppm of potassium ions respectively.
4.2.8. Estimation of antioxidant enzymes

In each stress induced model, liver and stomach homogenate (10%) was prepared with 0.15 M KCl and centrifuged at 8000 rpm for 10 min. The cell-free supernatant was used for estimation of antioxidant enzymes and total protein was estimated (Bradford, 1976).

4.2.8.1. Catalase (CAT)

Catalase activity was estimated according to the Aebi, (1984). Briefly, 10 μl of liver homogenate was added to reaction mixture consisting of 490 μl of 50 mM phosphate buffer (pH 7.0) and 500 μl of 30 mM H₂O₂. The decrease in the absorbance at 240 nm was recorded for 3 min. and CAT activity was measured by using molar extinction co-efficient of 43.6 M⁻¹ cm⁻¹.

4.2.8.2. Superoxide dismutase (SOD)

To determine the SOD activity, the riboflavin-NBT assay was adapted (Babitha et al., 2002) with slight modification. The tissue homogenate (0.1ml) was mixed with 0.1ml of 67 mM phosphate buffer (pH 7.8) containing 0.01M EDTA and 0.1 ml of 1.5 mM NBT. 3ml of 1.2 mM riboflavin was added after incubation at 37 °C for 5 min. The reaction mixture was illuminated with a 25 W light tube for 15 min.

The inhibition of NBT reduction was determined by measuring the absorbance at 560 nm. A control (water instead of NBT), test, standard (without homogenate) and blank (water instead of NBT and homogenate) were maintained simultaneously. In addition, standards were evaluated to determine the SOD activity. Unit of SOD activity was expressed as the amount of enzyme required to inhibit the reduction of NBT using the following formula;

\[
\text{SOD activity} = \frac{\text{Decrease in OD} \times 2}{\text{S-B}} \quad \text{where Decrease in absorbance} = (\text{S-B})-(\text{T-C}). \quad \text{Where, S - Absorbance of standard, B - Absorbance of blank, T - Absorbance of test and C - Absorbance of control.}
\]
4.2.8.3. Glutathione S-transferase (GST)

Glutathione S-transferase activity was estimated according to Warholm et al. (1985) by measuring the CDNB–GSH conjugate formed using 1-chloro-2,4-dinitrobenzene as substrate. Briefly, Liver homogenate (10 μl) was mixed with reaction mixture consisting of 890 μl of 0.1 M sodium phosphate buffer (pH 6.5), 50 μl of 20 mM GSH, 50 μl of 20 mM CDNB. The change in the absorbance at 340 nm was recorded for 3 min. and GST activity was measured using molar extinction co-efficient of 0.0096 μM⁻¹ cm⁻¹.

4.2.8.4. Glutathione reductase (GR)

Glutathione reductase activity was estimated as according to Carlberg et al. (1985) by measuring the oxidation of NADPH by GSSH at 340 nm. Briefly, liver homogenate (50 μl) was added to reaction mixture containing 0.5 ml of potassium phosphate buffer (pH 7.0) containing 2mM EDTA, 50 μl of NADPH (2 mM) and 50 μl of GSSH (20 mM). Volume was make up to 1 ml by adding deionized water and change in absorbance was measured at 340 nm for 3 min. and GSR activity was measured by molar extinction co-efficient of 6.2×10³ M⁻¹ cm⁻¹.

4.2.8.5. Glutathione peroxidase (GPX)

Glutathione peroxidase activity in liver homogenate was determined as described by Flohe and Gunzler (1984) by measuring the oxidation of NADPH coupled with reduction system consisting of hydrogen peroxide and GSH. Briefly, 0.1 ml of liver homogenate was mixed with reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.0), 100 μl of glutathione reductase (2.4 U ml⁻¹), 100 μl of GSH (10 mM), 20 μl of sodium azide (1 mM), 100 μl of NADPH (1.5 mM in 1% sodium bicarbonate), 4.8 μl of EDTA (0.8 mM). The change in absorbance was recorded at 340 nm for 3 min. and GPX activity was measured by using the molar extinction co-efficient of 6.2×10³ M⁻¹ cm⁻¹.
4.2.9. Measure of level of oxidative stress

The level of oxidative stress in experimental animals was determined by measuring the amount of lipid peroxidation and total protein carbonyl content as a biomarker in liver and stomach homogenate.

4.2.9.1. Measurement of Lipid peroxidation

Level of lipid peroxidation in liver was measured as TBARS. Briefly, 0.5 ml of liver homogenate was mixed with ferric chloride (100 µl, 0.2mM w/v) and incubated at 37 °C for 30 min. Followed by the addition of 2 ml of TCA-TBA-HCl reagent (15% TCA, 0.30% TBA in 0.25 N HCl) containing 0.05% BHT and heated for 60 min. in boiling water bath. Reaction mixture was cooled to room temperature, centrifuged and absorbance of the supernatant was measured at 532 nm.

4.2.9.2. Measurement of protein carbonyl content

The fundic stomach from healthy control, ulcerated, MEE treated and ranitidine treated were homogenized in 50 mM sodium phosphate buffer (pH 7.2) to get 20% homogenate and centrifuged at low speed (600 g) for 10 min. The amount of protein carbonyl was determined by spectrophotometric DNPH method (Levine et al., 1990). Briefly, proteins from 1 ml of homogenate was precipitated and centrifuged by adding 1 ml of chilled TCA (20%, w/v). A solution of DNPH (1 ml, 10 mM) in 2 N HCl was added to protein pellet and allowed to stand for 60 min. at room temperature under dark condition with mixing for every 10 min. The content in the mixture was then precipitated with 1 ml of TCA (20 %, w/v) and centrifuged for 5 min. The protein pellet was washed one more time with TCA (20 %, w/v), and followed by three times wash with 1 ml of ethanol/ethyl acetate mixture (1:1, v/v) to remove free DNPH. Pellet was re-suspended in 1 ml of guanidine hydrochloride (6 M in 2 N HCl), allowed to stand for 15 min. at 37 °C with mixing, centrifuged at 3000 g and absorbance was measured at 362 nm. The amount of carbonyl content was determined using molar absorption co-efficient of 22,000 M⁻¹ cm⁻¹.
4.2.10. Measurement of gastric mucin

Gastric mucin content of the stomach was quantitatively determined spectrophotometrically by Alcian blue dye binding method (Ratnasooriya et al., 1995) with slight modifications. Briefly, Equal weight (0.5g) of stomach from healthy control, ulcerated, MEE treated and ranitidine treated rats were incubated with 5 ml of alcian blue (1%, w/v) for 2 h at room temperature. Later, each stomach was washed with 0.25 M sucrose and incubated with 5 ml of magnesium chloride (0.5 M) for 2 h at room temperature with shaking at 15 min intervals. After incubation, stomachs were removed and 5 ml of diethyl ether was added, mixed thoroughly and absorbance of the alcian blue solution was measured at 605 nm. The amount of bound alcian blue corresponds to amount of gastric mucin, which was estimated using calibration curve of alcian blue.

4.2.11. Histopathological examination

After measuring ulcer index, the stomach was fixed in 10% formalin until histological examination. The stomach was sectioned for histological studies. The fixed tissue samples were embedded in paraffin. The sections (5 micron) were cut using microtome and stained with hematoxylin, eosin for microscopic observation.

4.2.12. Assessment of H\(^+\)-K\(^+\)-ATPase inhibition by MEE

4.2.12.1. Preparation of H\(^+\)-K\(^+\)-ATPase enzyme

Fresh proton potassium ATPase was prepared from mucosal scraping of fresh sheep stomach obtained from local slaughterhouse (Lakkavalli, Karnataka, India) and experimental rats. Stomach was cut opened and the inner layer was scraped out for mucosal scrap (Sachs, et al., 1976). Thus obtained mucosal scrape was homogenized in 16 mM Tris buffer (pH 7.4) containing 10% Triton X-100 and centrifuged at 6000 g for 10 min. The supernatant (enzyme extract) was stored at -20 °C for further study. Protein content was determined according to Bradford's method (Bradford, 1976) using BSA as standard.
4.2.12.2. Assessment of H⁺-K⁺-ATPase inhibition

The reaction mixture containing 16 mM Tris buffer (pH 6.5) with enzyme extract (398 μg) was pre-incubated with MEE at different concentrations (0-320 μg) for 60 min. at 37 °C. The reaction was initiated by adding 200 μl of ATP (2 mM), 200 μl of MgCl₂ (2 mM) and 200 μl of KCl (10 mM). After 30 min. of incubation at 37 °C, the reaction was terminated by the addition of assay mixture containing 4.5% of ammonium molybdate and 60% of perchloric acid. Inorganic phosphate (Pi) formed was measured spectrophotometrically at 660 nm by following Fiske-Subbarow method (Fiske and Subba Row, 1925). Enzyme activity was calculated as micromoles of Pi released per hour at various doses of MEE. Results were compared with the well-known proton potassium ATPase (PPA) inhibitor Omeprazole and expressed as Mean ± SEM. Percentage of enzyme inhibition was calculated by using the formula; Percentage of inhibition = \[
\frac{\text{Activity}_{(\text{control})} - \text{Activity}_{(\text{test})}}{\text{Activity}_{(\text{control})}} \times 100
\]

4.2.13. Assessment of H⁺-K⁺-ATPase inhibition by cyclic voltammetry approach

4.2.13.1. Preparation of bare carbon paste electrode

Bare carbon paste electrode was prepared by mixing 70% of graphite powder and 30% of silicon oil manually to get a homogenous mixture (Fig. 4-1). The mixture was packed into home made PVC (3mM in diameter) and smoothened on butter paper. The electrical contact was provided by a copper wire connected to the paste at the end of the tube.

4.2.13.2. Preparation of modified carbon paste electrode

Modified carbon paste was prepared by mixing 70% of graphite powder, 30% of silicon oil and MEE/omeprazole manually in pestle and mortar to get a homogenous mixture (Fig. 4-1). The mixture was packed into home made PVC (3mM in diameter) and smoothened on butter paper. The electrical contact was provided by a copper wire connected to the paste at the end of the tube.
Step 1: Preparation of bare carbon electrode

Mix carbon powder with silicon oil in the ratio 60:40.

Step 2: Preparation of modified carbon electrode

Mix carbon powder and silicon oil with MEE/Omeprazole 60:40.

Step 3: Immobilization of enzyme on modified carbon electrode

Wash to remove unbound enzymes.

$H_+ K_+\text{-ATPase}$

$\rightarrow$ Carbon paste electrode

$\rightarrow$ 20 min.

Figure 4.1: Schematic representation of cyclic voltammetric studies for $H_+ K_+\text{-ATPase}$ inhibition.
4.2.13.3. Immobilization of H⁺-K⁺-ATPase on electrode

As depicted in Fig. 4-1, H⁺-K⁺-ATPase was immobilized by adding 50 μl of enzyme preparation from sheep stomach on to the surface of carbon paste electrode and kept for 20 min. to get immobilized and unbound enzyme was washed out with buffer slowly without damaging the electrode.

4.2.13.4. Recording voltamogram

Voltamogram was recorded for all bare CPE, MEE/omeprazole modified CPE in 16 mM Tris buffer (pH 6.5) containing ATP at room temperature.

4.2.14. Assessment of acid-neutralizing capacity of MEE

MEE at three different concentration (250-1000 mg) was added to 100 ml of 0.1 N HCl and incubated for 90 min. at room temperature with shaking. Acid-neutralizing capacity was determined by titrating against standardized 0.1 N NaOH using methyl orange as an indicator. Calcium carbonate (1%, w/v) was used as a positive control.

4.2.15. Statistical analysis.

All data are expressed as mean ± SEM. A value of p < 0.05, p < 0.01 and p < 0.001 was considered as significant, very significant and highly significant respectively. Statistical analysis was performed using one way ANOVA followed by Dunnett multiple comparison test. Data was computed for statistical analysis by using IBM SPSS statistics 20.

4.3. Results and discussion

Gastric ulcer is one of the most rampant gastrointestinal disorders affecting large population in all geographical regions. ROS plays a crucial role in the etiology of ischemic injury in stomach including peptic ulcer (Perry et al., 1986; Schmassmann et al., 1997; Olaleye et al., 2007). It continues to occupy the key position in concern of both clinical practitioners and researchers in developing a multi-step anti-ulcer drug without or with minimal side effects. The effective antioxidant source from living origin is the only alternative to synthetic drugs. Therefore, in the present study MEE was examined for its mechanism of action and biochemical basis for gastro protective activity against ethanol.
and swim stress induced ulceration. Ethanol and swim stress induced gastric ulcers have been widely used for the experimental evaluation of anti-ulcer activities because of its rapid and convenient way of screening plant extracts as anti-ulcer potency.

4.3.1. Quantitative analysis of MEE by GC-MS

GC-MS chromatogram analysis of MEE showed six peaks (Fig. 4-2 and 4-3), which indicate the presence of six phytochemical constituents. On comparison of the mass spectra of the constituents with the NIST library, the six phyto-compounds were characterized and identified (Table 4.2).

4.3.2. Anti-ulcer property of MEE

Anti-ulcer property of MEE was examined by employing ethanol and swim stress induced ulcer in male albino rats. Fig. 4-4 depicts the difference between healthy and stress induced stomach ulcer. In ethanol and swim stress induced ulcers, stomach was characterized by hemorrhagic damage, inflammations and oozing of blood in gastric glandular pits (Fig. 4-4B & C). Animals pretreated with MEE (200 and 400 mg kg\(^{-1}\) b.w.) for 14 days showed significant anti-ulcer activity where ulcer index quantitatively decreased (p<0.001) compared to ranitidine pretreated group (30 mg kg\(^{-1}\) b.w.) (Table 4.3). As evident from table 4.4 and 4.5, both ethanol and swim stress showed significant increase in the volume of gastric juice, total acidity, pepsin release and total protein content, while the level of carbohydrates likes hexosamine, hexose and sialic acid were significantly decreased in ulcerated group animals. Animals pretreated with ranitidine and MEE for 14 days showed marked decrease in ulcer index (p<0.001), gastric juice (p<0.01), total acidity (p<0.001), pepsin release (p<0.01) and total protein content (p<0.001) with corresponding carbohydrates level restored.

Pepsinogen, an inactive zymogen is secreted into gastric juice from mucous cells. Once secreted, pepsinogen is activated by stomach acid into the active protease pepsin. As evidenced from table 4.4 and 4.5, significant increase in the level of pepsin activity was observed as the acidity increases. In the present study, a significant positive correlation was observed between total acidity and pepsin activity in both ethanol and swim stress induced ulcer with correlation value 0.780 and 0.892 respectively (Fig. 4-5).
Table 4.2: GC-MS analysis of MEE

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Retention time</th>
<th>Compounds Identified</th>
<th>Molecular formula</th>
<th>Relative %</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.065</td>
<td>2-Furancarboxaldehyde</td>
<td>C₅H₄O₂</td>
<td>34.375</td>
<td>Unknown</td>
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<tr>
<td>2</td>
<td>5.701</td>
<td>Benzene methanol</td>
<td>C₃H₆O</td>
<td>6.255</td>
<td>Anaesthetic activity and channel blocking activity</td>
</tr>
<tr>
<td>3</td>
<td>6.462</td>
<td>2-Furancarboxylic acid, methyl ester</td>
<td>C₆H₆O₃</td>
<td>9.375</td>
<td>Furan derivatives are reported to posses wide spectrum of biological activities like antifungal, antimicrobial, antitumor, anti-helminthic activity, anti-inflammatory, antibacterial, antimalarial, anti ulcer, anti tubercular, anticancer, antiviral. Antimicrobial, Preservative Antimicrobial, anti inflammatory</td>
</tr>
<tr>
<td>4</td>
<td>7.424</td>
<td>4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl</td>
<td>C₆H₈O₄</td>
<td>15.625</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>8.251</td>
<td>1,2-Benzenediol, 3-chloro</td>
<td>C₆H₅ClO₂</td>
<td>3.125</td>
<td>Unknown</td>
</tr>
<tr>
<td>6</td>
<td>8.684</td>
<td>2-Furancarboxaldehyde, 5-nitro-, oxime</td>
<td>C₅H₄N₂O₄</td>
<td>31.25</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Figure 4-2: GC-MS chromatogram for MEE
Figure 4-3: Mass spectra of identified compounds from MEE (A) 2-Furancarboxaldehyde, (B) Benzene methanol, (C) 2-Furancarboxylic acid, methyl ester, (D) 4H-Pyrano-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, (E) 1,2-Benzenediol, 3-chloro and (F) 2-Furancarboxaldehyde, 5-nitro-, oxime.
The increased level of total protein in gastric juice of ulcerated animals could be the reason for release of plasma protein because of gastric mucosal damage. The decrease level of carbohydrates like hexosamine, hexose and sialic acid indicates the decreased mucosal protective activity because of gastric mucosal damage. MEE pretreated animals shows the level of protein content and carbohydrates near normal level significantly when compared to ranitidine treated animals. MEE at 200 and 400 mg kg$^{-1}$ b.w. protected ethanol and swim stress induced ulcers up to 85%.

**Figure 4.4:** Macroscopic observation of ulcers in ulcer induced/protected stomachs in swim stress-/ethanol stress-induced ulcer models. Ulcer was induced in animals by either swim stress (SS) or ethanol stress (ES) in group of pre-treated/untreated animals at indicated concentrations. In (A) healthy control, (B) ethanol stress (C) swim stress induced animals, ulcers score were very high. (D and G) Ranitidine and (E, F, H and I) MEE at 200 and 400 mg kg$^{-1}$ treated animals showed dose-dependent reduction in stomach lesions.
Table 4.3: Ulcer index and healing index. Maximum ulcer index observed during stress induction was controlled in a concentration-dependent manner. Reduction in ulcer index and percent protection is depicted.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Group</th>
<th>Ulcer index</th>
<th>% of healing index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ethanol stress</td>
<td>Swim stress</td>
</tr>
<tr>
<td>1</td>
<td>Healthy</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Ulcerated</td>
<td>1.187±0.04</td>
<td>1.063±0.004</td>
</tr>
<tr>
<td>3</td>
<td>Ranitidine (30 mg kg⁻¹)</td>
<td>0.680±0.02²</td>
<td>0.357±0.004³</td>
</tr>
<tr>
<td>4</td>
<td>MEE (200 mg kg⁻¹)</td>
<td>0.343±0.007³</td>
<td>0.346±0.006³</td>
</tr>
<tr>
<td>5</td>
<td>MEE (400 mg kg⁻¹)</td>
<td>0.171±0.003³</td>
<td>0.170±0.002³</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM; n=6; a P<0.05, b P<0.01 and c P<0.001 when compare to ulcerated.

Table 4.4: Effect of MEE on ethanol induced gastric ulcer in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy</th>
<th>Ulcerated</th>
<th>Ranitidine</th>
<th>MEE (200 mg kg⁻¹ b.w.)</th>
<th>MEE (400 mg kg⁻¹ b.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of gastric juice (ml)</td>
<td>1.58±0.80¹</td>
<td>6.53±0.36²</td>
<td>5.40±0.48³</td>
<td>3.13±0.85⁵, ⁶⁷</td>
<td>2.46±0.57⁷</td>
</tr>
<tr>
<td>pH of gastric juice</td>
<td>5.37±0.10⁶</td>
<td>6.97±0.32⁷</td>
<td>8.51±0.66³</td>
<td>4.88±0.48⁵</td>
<td>5.68±0.43⁸</td>
</tr>
<tr>
<td>Total acidity (mEq/l/100g)</td>
<td>80.63±1.76¹</td>
<td>9.73±2.83⁷</td>
<td>3.46±0.61⁵, ⁶⁷</td>
<td>27.39±7.08⁴</td>
<td>27.39±4.26⁴⁺</td>
</tr>
<tr>
<td>Total protein (mg ml⁻¹)</td>
<td>3.71±0.15¹</td>
<td>4.82±0.26⁷</td>
<td>4.40±0.06</td>
<td>2.05±0.63⁵</td>
<td>2.20±0.71⁸</td>
</tr>
<tr>
<td>Total peptic (µg of tyrosine)</td>
<td>509.6±28.1¹</td>
<td>183.9±77.7³</td>
<td>142.5±35.9³</td>
<td>446.1±55.5⁴, ⁶⁷, ⁸⁷</td>
<td>511.1±33.01⁵, ⁶⁷, ⁸⁷</td>
</tr>
<tr>
<td>Hexose (µg ml⁻¹)</td>
<td>185.8±43.0¹</td>
<td>72.91±3.4⁷</td>
<td>117.7±17.8</td>
<td>100.8±56.2</td>
<td>188.0±27.0</td>
</tr>
<tr>
<td>Hexosamine (µg ml⁻¹)</td>
<td>300.6±84.1¹</td>
<td>136.1±8.9²</td>
<td>295.4±26.4⁵, ⁷⁸</td>
<td>172.4±30.8</td>
<td>298.4±37.0¹⁺</td>
</tr>
<tr>
<td>Sialic acid (µg ml⁻¹)</td>
<td>0.248±0.04⁶</td>
<td>0.09±0.01³</td>
<td>0.265±0.02⁶, ⁷⁸, ⁸</td>
<td>0.258±0.06³</td>
<td>0.274±0.05⁸⁺⁺</td>
</tr>
<tr>
<td>Sodium (ppm)</td>
<td>16.3±1.1⁷</td>
<td>4.44±0.02³</td>
<td>0.97±0.04³, ⁶⁷, ⁸</td>
<td>0.65±0.05³⁺⁺, ⁶⁷, ⁸</td>
<td>0.85±0.07³⁺⁺⁺⁺⁺</td>
</tr>
<tr>
<td>Potassium (ppm)</td>
<td>4.52±0.28⁸</td>
<td>1.79±0.43³</td>
<td>4.31±0.37⁸</td>
<td>3.69±0.33⁷⁺⁺⁺⁺⁺⁺⁺⁺</td>
<td>5.64±0.08³⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM; n=6.

a<sub>H</sub> P<0.05 when compare to healthy, a<sub>U</sub> P<0.05 when compare to ulcerated,
b<sub>H</sub> P<0.01 when compare to healthy, b<sub>U</sub> P<0.01 when compare to ulcerated,
c<sub>H</sub> P<0.001 when compare to healthy, c<sub>U</sub> P<0.001 when compare to ulcerated,
Table 4.5: Effect of MEE on swim stress induced ulcer in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy</th>
<th>Ulcerated</th>
<th>Ranitidine</th>
<th>MEE (200 mg kg⁻¹ b.w.)</th>
<th>MEE (400 mg kg⁻¹ b.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of gastric juice (ml)</td>
<td>2.172±0.26ᵃU</td>
<td>6.584±1.60ᵇH</td>
<td>5.612±1.59</td>
<td>3.760±0.88</td>
<td>2.70±0.61</td>
</tr>
<tr>
<td>pH of gastric juice</td>
<td>2.048±0.16</td>
<td>2.152±0.37</td>
<td>2.234±0.56</td>
<td>3.366±0.78</td>
<td>3.583±0.22</td>
</tr>
<tr>
<td>Total acidity (mEq/l/100g)</td>
<td>79.30±4.26ᵇU,ᵇH</td>
<td>27.2±8.23ᵇH</td>
<td>94.24±6.99</td>
<td>65.77±2.29ᶜU</td>
<td>69.75±6.92ᶜU</td>
</tr>
<tr>
<td>Total protein (mg ml⁻¹)</td>
<td>3.45±0.28ᵇU</td>
<td>4.80±0.23ᵇH</td>
<td>3.48±0.29ᶜU</td>
<td>3.98±0.22</td>
<td>3.21±0.28ᵇU</td>
</tr>
<tr>
<td>Total pepsin (µg of tyrosine)</td>
<td>614.3±4.25ᶜU,ᶜH</td>
<td>320.7±3.3</td>
<td>648.7±3.5ᶜU</td>
<td>661.4±5.8ᵃU</td>
<td>552.5±3.25ᶜU,ᵃU</td>
</tr>
<tr>
<td>Hexose (µg ml⁻¹)</td>
<td>63.05±5.66ᵃU,ᵇH</td>
<td>21.37±2.49</td>
<td>32.76±5.81</td>
<td>42.98±12.5</td>
<td>57.56±13.1ᵃU</td>
</tr>
<tr>
<td>Hexosamine (µg ml⁻¹)</td>
<td>105.3±11.1</td>
<td>70.5±20.0</td>
<td>109.5±13.2</td>
<td>158.5±33.6ᵃU</td>
<td>151.6±12.8ᵃU</td>
</tr>
<tr>
<td>Sialic acid (µg ml⁻¹)</td>
<td>0.031±0.007ᵇU</td>
<td>0.066±0.008ᵃH</td>
<td>0.022±0.002ᵇU</td>
<td>0.049±0.01</td>
<td>0.047±0.01</td>
</tr>
<tr>
<td>Sodium (ppm)</td>
<td>16.2±1.8</td>
<td>20.2±1.0</td>
<td>17.6±3.5</td>
<td>18.2±3.1</td>
<td>15.0±0.19ᵇU,ᵇH</td>
</tr>
<tr>
<td>Potassium (ppm)</td>
<td>4.21±0.09ᵃU</td>
<td>2.50±0.23ᵃH</td>
<td>4.07±0.32ᵃU</td>
<td>2.34±0.86ᵃH</td>
<td>4.57±0.48ᵃU</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE, n=6.

ᵃH P<0.05 when compare to healthy,ᵃU P<0.05 when compare to ulcerated,
ᵇH P<0.01 when compare to healthy,ᵇU P<0.01 when compare to ulcerated,
ᶜH P<0.001 when compare to healthy,ᶜU P<0.001 when compare to ulcerated.

Figure 4.5: Correlation between acidity and pepsin activity in gastric juice of (A) ethanol stress induced group and (B) swim stress induced group
4.3.3. Mechanism of action

Ethanol and swim stress induced ulcers are two well accepted model for the study of oxidative stress induced ulcer. Since, ulcer is a multi-step disease, in both the cases the ulcer induction is via ROS, damage of mucosal epithelium and parietal cells H⁺-K⁺-ATPase activation (Siddaraju et al., 2009). Meantime, ulcer healing process is also a multi-step cascade involving the action of many molecules.

The generation of ROS may occur by a number of physiological and non-physiological processes. Many antioxidant enzymes like SOD, CAT, GPX, GST, GSR controls them to normal level. If any imbalance in pro-oxidants/antioxidant homeostasis, these ROS could cause tissue damage through lipid peroxidation (Fridovich, 1986; Dalle-donne et al., 2003), thus ROS involved in pathophysiology of many human diseases. Administration of MEE showed gastro-protective activity in the laboratory animals. Further, followed the protective effect of investigating the biochemical parameters such as alterations in the gastric mucin, H⁺-K⁺-ATPase and anti-oxidant enzymes level such as CAT, SOD and peroxidase in the ulcerated stomach as well in liver of all groups.

4.3.3.1. Histological analysis of gastric mucosa

Histology of haematoxylin and eosin (H & E) stained ulcer control stomach tissue showed (Fig. 4-6B & c) completely damaged gastric mucosa with inflammatory exudates. MEE treatment showed evidence of restoration of mucosal epithelium and reduced inflammatory exudates after 14 d treatment in both ethanol (Fig. 4-6E & F) and swim induced (Fig. 4-6H & I) stress models. In addition to these changes a complete healing with reorganized glandular structures was observed.

Stress induces mucosal necrosis and leads to gastric ulcers. It also induces gastric ulcer indirectly by stimulating several mediators of mast or blood cells (Szabo et al., 1985). The histopathological changes induced by stress can be prevented or reduced by hampering the release of mediators (Harvey et al., 1985). The present study demonstrated that, pre-treatment with MEE protected the gastric mucosa from stress inducers and mucosal normal architecture was preserved.
Figure 4.6: Histopathology of stomach tissue. Haematoxylin and eosin stained tissue samples of (A) Healthy control, (B) ethanol stress induced, (C) swim stress induced, (D and G) Ranitidine treated, (E and F) MEE at 200 and 400 mg kg\(^{-1}\) treated animals in ethanol stress induced, (H and I) MEE at 200 and 400 mg kg\(^{-1}\) treated animals in swim stress induced animals.

4.3.3.2. Normalization of anti-oxidant system

Increase in free radicals and oxidative processes contribute to gastric ulcer. It is hypothesized that the ability of a plant extract to scavenge free radicals and elicit antioxidant properties could be one of the mechanism in which extract exerts protective effect. Previous reports suggest that, antioxidants play a pivotal role in order to protect gastric mucosal damage and repair in oxidative stress induced ulcer (Trivedi and Rawal, 2001). Table 4.6 shows the effect of MEE on level of anti-oxidant enzymes in rat liver.
and stomach when animals were exposed to stress inducers. SOD, CAT, GPX, GST and GSR contributes to maintain antioxidant status during oxidative stress condition such as ulcer. Previous studies have demonstrated the low-level expression of SOD, CAT and increased level of peroxidases in the gastric mucosa of stress induced gastric inflammation (Sangiovanni et al., 2013; Das and Banarjee, 1993). Elevation in the level of GPX, GST is to scavenge peroxyl radicals generated during ulceration. Depletion of SOD and CAT indicates the utilization of these components towards the neutralization of free radicals generated during ulcer. In the present study, administration of MEE resulted in a significant increase in the level of SOD, CAT, GSR and reduced GPX, GST, suggesting the efficacy of MEE in preventing the free radical induced gastric damage.

4.3.3.3. Normalization of oxidative damage

Generally, oxidative stress leads to hypoxia and near or actual “ischemia” in stomach through sympathetic and parasympathetic stimulation. In ischemic condition, hydrogen peroxide conjugates with oxygen to form hydroxyl radicals through Haber-Weiss reaction (Das et al., 1997). Thus, a generated hydroxyl radical oxidizes life-sustaining molecules like lipids, proteins and nucleic acid etc., causes tissue damage through lipid peroxidation and it can be evidenced by accumulation of malondialdehyde (Das and Banerjee, 1993).
Table 4.6: Anti-oxidant enzymes and MDA levels in ethanol and swim stress-induced ulcer model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (U mg⁻¹)</th>
<th>CAT (M mg⁻¹)</th>
<th>GPX (n M mg⁻¹)</th>
<th>GST (n M mg⁻¹)</th>
<th>GSR (m M mg⁻¹)</th>
<th>MDA (n M mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>44.0±1.56ᵇ</td>
<td>0.60±0.02ᶜ</td>
<td>28.9±5.76ᶜ</td>
<td>1.39±0.29ᶜ</td>
<td>22.0±1.53ᵇ</td>
<td>0.98±0.05ᵇ</td>
</tr>
<tr>
<td>Ulcerated</td>
<td>22.6±4.57ᵇ</td>
<td>0.36±0.03ᶜ</td>
<td>98.4±0.74ᶜ</td>
<td>3.29±0.19ᶜ</td>
<td>9.06±0.61ᵇ</td>
<td>2.66±0.41ᵇ</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>35.2±1.55</td>
<td>0.55±0.03ᵇ</td>
<td>46.8±8.67ᶜ</td>
<td>1.88±0.11ᵇ</td>
<td>16.4±2.20ᵈ</td>
<td>1.18±0.34ᵇ</td>
</tr>
<tr>
<td>MEE 200 mg kg⁻¹</td>
<td>42.2±4.94ᵃ</td>
<td>0.54±0.02ᵇ</td>
<td>61.0±13.0ᵃ</td>
<td>1.85±0.02ᶜ</td>
<td>13.5±1.58ᵈ</td>
<td>1.35±0.13ᵃ</td>
</tr>
<tr>
<td>MEE 400 mg kg⁻¹</td>
<td>41.8±4.99ᵃ</td>
<td>0.57±0.01ᶜ</td>
<td>33.4±2.08ᶜ</td>
<td>1.71±0.18ᶜ</td>
<td>20.1±3.62ᵇ</td>
<td>0.90±0.02ᵇ</td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>0.093±0.007ᶜ</td>
<td>0.08±0.004ᵇ</td>
<td>0.231±0.02ᶜ</td>
<td>0.15±0.003ᵇ</td>
<td>0.67±0.04ᵇ</td>
<td></td>
</tr>
<tr>
<td>Ulcerated</td>
<td>0.022±0.003⁴</td>
<td>0.02±0.002⁴</td>
<td>0.36±0.02 (1.5ᵃ)</td>
<td>0.37±0.03 (2.4ᵃ)</td>
<td>1.95±0.24 (2.9ᵃ)</td>
<td></td>
</tr>
<tr>
<td>Ranitidine</td>
<td>0.015±0.004ᵈ</td>
<td>0.06±0.01ᵃ</td>
<td>0.262±0.01ᵇ</td>
<td>0.21±0.02ᵃ</td>
<td>1.32±0.08ᵈ</td>
<td></td>
</tr>
<tr>
<td>MEE 200 mg kg⁻¹</td>
<td>0.023±0.001ᵃ</td>
<td>0.06±0.01ᵃ</td>
<td>0.236±0.01ᶜ</td>
<td>0.18±0.005ᵃ</td>
<td>0.85±0.11ᵇ</td>
<td></td>
</tr>
<tr>
<td>MEE 400 mg kg⁻¹</td>
<td>0.028±0.008ᵈ</td>
<td>0.07±0.001ᵃ</td>
<td>0.223±0.01ᶜ</td>
<td>0.18±0.04ᵃ</td>
<td>0.68±0.08ᵇ</td>
<td></td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>92.3±5.7ᵇ</td>
<td>0.46±0.02ᵇ</td>
<td>24.2±1.51ᵇ</td>
<td>1.98±0.20ᵈ</td>
<td>16.9±0.24ᵇ</td>
<td>1.95±0.17ᵃ</td>
</tr>
<tr>
<td>Ulcerated</td>
<td>78.6±4.09 (1.4𝑉)</td>
<td>0.28±0.03 (1.6𝑉)</td>
<td>40.9±2.71 (1.7ᵇ)</td>
<td>2.49±0.25 (1.3ᵃ)</td>
<td>0.56±0.09 (3.0ᵇ)</td>
<td>3.29±0.56 (1.7ᵃ)</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>85.9±9.72</td>
<td>0.53±0.02ᶜ</td>
<td>28.6±2.37ᵃ</td>
<td>1.80±0.06ᵃ</td>
<td>0.98±0.05ᵈ</td>
<td>1.40±0.34ᵇ</td>
</tr>
<tr>
<td>MEE 200 mg kg⁻¹</td>
<td>86.5±2.59</td>
<td>0.45±0.01ᵇ</td>
<td>34.0±4.00ᵈ</td>
<td>1.78±0.08ᵃ</td>
<td>0.59±0.16ᵈ</td>
<td>1.30±0.20ᵇ</td>
</tr>
<tr>
<td>MEE 400 mg kg⁻¹</td>
<td>79.4±0.62</td>
<td>0.51±0.03ᶜ</td>
<td>30.5±2.01ᵃ</td>
<td>1.86±0.06ᵈ</td>
<td>14.2±0.22ᵃ</td>
<td>1.75±0.10ᵃ</td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>0.026±0.001ᵃ</td>
<td>0.06±0.004ᵇ</td>
<td>0.249±0.03</td>
<td>0.15±0.03ᵈ</td>
<td></td>
<td>0.58±0.04ᵃ</td>
</tr>
<tr>
<td>Ulcerated</td>
<td>0.018±0.003 (1.4𝑉)</td>
<td>0.03±0.001 (2.0𝑉)</td>
<td>0.27±0.03 (1.1ᵇ)</td>
<td>0.23±0.002 (1.5ᵃ)</td>
<td></td>
<td>2.15±0.03 (3.7ᵃ)</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>0.015±0.001</td>
<td>0.06±0.007ᵃ</td>
<td>0.218±0.01</td>
<td>0.17±0.01ᵈ</td>
<td></td>
<td>0.88±0.39ᵃ</td>
</tr>
<tr>
<td>MEE 200 mg kg⁻¹</td>
<td>0.031±0.002ᵇ</td>
<td>0.05±0.007ᵈ</td>
<td>0.202±0.04</td>
<td>0.12±0.004ᵃ</td>
<td></td>
<td>0.78±0.07ᵈ</td>
</tr>
<tr>
<td>MEE 400 mg kg⁻¹</td>
<td>0.027±0.001ᵃ</td>
<td>0.05±0.002ᵃ</td>
<td>0.202±0.03</td>
<td>0.09±0.03ᵃ</td>
<td></td>
<td>0.57±0.03ᵃ</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. n=6; a P<0.05, b P<0.01 and c P<0.001 when compare to ulcerated

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Lipid peroxidation causes loss of membrane fluidity, integrity, impaired ion transport and finally cellular death. Therefore, oxidative stress is considered as one of the major reason in causing ulceration. Malondialdehyde (MDA) in liver and stomach was determined as marker of lipid peroxidation (Fornai et al., 2005). The level of MDA in stress induced group was significantly more (2–3 fold increase) when compare to healthy and treated groups. The ethanol administration and swim-induced stress significantly elevated the level of hepatic and gastric mucosal MDA (ES: liver; 2.66±0.41, stomach; 1.95±0.24 nm mg⁻¹, SS: liver; 3.29±0.56, stomach; 2.15±0.03 nm mg⁻¹), since it significantly decreased the level of SOD and CAT (Table 4.6). However, pretreatment with MEE significantly lowered the level of hepatic and gastric mucosal MDA almost to the level of normal with consequent increase in SOD and CAT (Table 4.6). SOD and CAT plays a pivotal role as first line of defense against oxidative stress. Lowering level of these enzymes can results in the accumulation of highly reactive free radicals that cause deleterious effects ultimately leads to lipid peroxidation. SOD catalyses the reactions of scavenging superoxide anion radicals, dismutation of hydrogen peroxide (H₂O₂), while CAT degrades H₂O₂ into a molecule of oxygen and water (Saravanan et al., 2003). In the present study, a significant negative correlation was observed between the level of MDA with SOD and CAT level respectively (Fig. 4-7). This indicates that, treatment with MEE resulted in significant increase in the activities of SOD and CAT and a decrease in MDA formation, reflecting their antioxidant potential.

Cellular proteins are believed to be the first target inflicting the deleterious effect of oxidative stress. ROS including radicals: superoxide, hydroxyl, alkoxyl, hydroperoxyl, peroxy; non-radicals: hydrogen peroxide, hypochlorous acid, singlet oxygen, ozone, peroxynitrite (Dean et al., 1997; Berlett et al., 1997) leads to protein oxidation beyond the stoichiometry and produce stable carbonyl group in the protein side chains. So, protein carbonyls was estimated to measure the level of protein oxidation and oxidative stress. The level of protein carbonyl was significantly high (2.6 fold increase) in stress induced groups when compare to healthy animals (Fig. 4-8). The level of protein carbonyl was less in MEE treated animals significantly (P<0.01).
Figure 4.7: Correlation between (A) SOD and MDA in liver, (B) SOD and MDA in stomach, (C) CAT and MDA in liver and (D) CAT and MDA in stomach of ethanol induced ulcer. (E) SOD and MDA in liver, (F) SOD and MDA in stomach, (G) CAT and MDA in liver and (H) CAT and MDA in stomach of swim stress induced ulcer.
The accumulation of lipid peroxidation and protein oxidation product slowdown the rate of ulcer healing (Srikanta et al., 2010). Administration of antioxidants prior to stress causes significant decrease in ulcer healing index, lipid peroxidation, protein oxidation suggesting the involvement of reactive oxygen species in stress induced gastric ulceration. In the current study, there was a remarkable decrease in the level of oxidation (P<0.01) in MEE treated animals when compare to stress induced group in both the stress condition. The antioxidant and anti-radical property of MEE may rapidize the ulcer healing proves through suppressing the oxidative stress.

4.3.3.4. Modulation of gastric mucin content

The mechanism of gastric mucosal defense systems includes several protective factors, which allow the mucosa to resist against aggressive factors. Prostaglandins play a crucial role in maintaining mucosal integrity and protect from injurious factors (Halter et al., 2001; Brzozowski et al., 2005a). The determination of gastric mucin represents a source of continues prostaglandin production. Gastric mucin in healthy, ulcerated, ranitidine and MEE treated rats stomach was determined quantitatively by Alcian blue dye binding assay. Since Alcian blue is specific dye, which binds to intact gastric mucin.
Any damage or reduction in gastric mucin will result in reduction of dye binding to stomach tissue. Interestingly, in the present study animals treated with MEE showed normalized mucin level (Fig. 4-9) when compared to stress-induced ulcer (P<0.01). In ulcerated animals, ~4.46 folds decrease in the level of gastric mucin content was observed whereas it has been appreciated that, the mucin/mucosal damage was recovered and modulated by MEE in treated animals.

![Figure 4-9: Analysis of gastric mucin by Alcian blue dye binding method. Values are expressed as mean±SEM; n=6. * P<0.05, ** P<0.01, *** P<0.001 and **** Non significant when compared to ulcerated group.](chart)

**4.3.3.5. Inhibition of H⁺-K⁺-ATPase by MEE**

The inner surface of the stomach is formed into numerous gastric pits and the enzyme complex proton pumps (H⁺-K⁺-ATPase pump) present in lumenocyte of the parietal cells lining the gastric pits are responsible for acid secretion. The proton pump actively transport proton into the stomach lumen with the exchange of potassium to the parietal cell against their concentration gradient with the hydrolysis of ATP. The proton pump inhibitors (Clinically used proton pump inhibitors Omeprazole, Lansoprazole, Dextansoprazole, Esomeprazole, Pantoprazole and Rabeprazole) are the drugs which reduce acid secretion in the stomach. Drug binds irreversibly to the proton pump and prevents the active transport of protons, this dramatically decreases the acid secretion of the stomach (Sachs et al., 1978). Over expression of H⁺-K⁺-ATPase leads to acidity and
ulcer. Since, this regulatory enzyme has been found to be a pharmacological target to treat ulcer. Presently ulcer is treating by providing allopathic PPI, antioxidants, H₂-receptor antagonist etc. but these are associated with side effects particularly under conditions of pregnancy/lactation and alcohol or any other drug consumption (Waldum et al., 2005; Ryan and Madanick, 2011). Therefore, there is a vital need of PPI of plant origin. We studied the ability of MEE in inhibiting H⁺-K⁺-ATPase isolated from sheep stomach (Fig. 4-10) as well as from the experimental rats. The 2.6 and 2.9 fold increase in the level of H⁺-K⁺-ATPase in ethanol and swim stress induced ulcer was observed respectively and was normalized upon pre-treatment with MEE. Oral pre-treatment of MEE inhibited the H⁺-K⁺-ATPase activity by 1.8 and 2.4 fold reduction at 200 and 400 mg kg⁻¹ b. w. respectively in ethanol stress induced animals. In swim stress induced experimental animals, 1.7 and 2.5 fold reduction in H⁺-K⁺-ATPase activity was observed at 200 and 400 mg kg⁻¹ b.w. MEE pretreated animals respectively (Fig. 4-11).

The increased potassium level (Table 4.3 & 4.4) in gastric juice significantly reflects the inhibition of H⁺-K⁺-ATPase and prevented the active transport of proton and potassium. This was further substantiated by using in vitro sheep H⁺-K⁺-ATPase inhibition by MEE at different concentrations (0-320 μg). At a maximum concentration (320 μg) of MEE and omeprazole showed 71.88±1.08 and 63.69±0.7 % of inhibition respectively. MEE inhibited H⁺-K⁺-ATPase activity in a concentration-dependent manner with an IC₅₀ of 45.45±0.4 μg mL⁻¹ when compare to standard omeprazole (IC₅₀= 46.89±0.4 μg mL⁻¹) under similar experimental conditions. The results suggests that, MEE has higher inhibition property against H⁺-K⁺-ATPase when compare to existing proton pump inhibitors.
Figure 4-10: *In vitro* effect of MEE and omeprazole on H⁺-K⁺-ATPase activity: H⁺-K⁺-ATPase activity was measured without (control) and with 20–320 μg of MEE and omeprazole. Values are expressed as mean±SEM; n=3.

Figure 4-11: *In vivo* effect of MEE on H⁺-K⁺-ATPase activity in healthy, ulcerated, ranitidine and MEE treated rats. Values are expressed as mean±SEM; n=6. *P<0.05,  †P<0.01,  ‡P<0.001 and  §Non significant when compare to ulcerated group.
In order to have further evidence to show the inhibition of $\text{H}^+\text{K}^+$-ATPase by MEE, an electrochemical method cyclic voltammetry was conducted. Fig. 4-12, shows a cyclic voltamogram for ATP in absence and presence of $\text{H}^+\text{K}^+$-ATPase enzyme in 16 mM Tris buffer (pH 6.5) at a scan rate of 100 mV/S at room temperature. In the presence of ATP alone, little anodic peak current was obtained at a potential of 1.25 V (Black color). When the enzyme immobilized electrode was dipped and after 30 min. of incubation, ATP peak diminished. Since, ATP readily undergoes hydrolysis to form ADP and inorganic phosphate as shown below.

\[
\text{Adenosine triphosphate (ATP)} \xrightarrow{\text{H}^+\text{K}^+\text{-ATPase}} \text{Adenosine diphosphate (ADP)} + \text{Inorganic phosphate (Pi)}
\]

$\text{H}^+\text{K}^+$-ATPase inhibition was done by using MEE and standard PPI, Omeprazole. As stated above (section: 2.6.3.3), enzyme was immobilized and incubated for 30 min. on MEE and omeprazole modified electrode and immersed in the solution containing ATP. The cyclic voltamogram was generated under similar experimental conditions as mentioned above. The voltamogram shows the presence of stable ATP even in the presence of immobilized enzyme. Indicating the inhibition of $\text{H}^+\text{K}^+$-ATPase by MEE and omeprazole as this evidenced by reduction in current response.

4.3.3.6. Effect of MEE on acid neutralization

Antacids are effective in accelerating healing of duodenal and gastric ulcers. The ulcer healing action of antacids was thought to be due to the neutralization of gastric luminal acid (Tarnawski et al., 1995). In the present study, the titer value did not portray any significant antacid activity (Table 4.7).
Figure 4-12: *In vitro* electrochemical detection of H⁺–K⁺–ATPase inhibition by MEE and Omeprazole. (a) cyclic voltamogram for ATP in presence of H⁺–K⁺–ATPase. (b) cyclic voltamogram for ATP in presence of H⁺–K⁺–ATPase and MEE. (c) cyclic voltamogram for ATP in presence of H⁺–K⁺–ATPase and omeprazole.

Table 4.7: Acid-neutralizing capacity of MEE

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Test</th>
<th>Volume of NaOH consumed (ml)</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>107.2±0.20</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>CaCO₃</td>
<td>57.85±0.05</td>
<td>46.04±0.147</td>
</tr>
<tr>
<td>3</td>
<td>MEE (250 mg)</td>
<td>105.3±0.40</td>
<td>1.305±0.277</td>
</tr>
<tr>
<td>4</td>
<td>MEE (500 mg)</td>
<td>105.6±0.60</td>
<td>0.746±0.001</td>
</tr>
<tr>
<td>5</td>
<td>MEE (1000 mg)</td>
<td>105.7±0.30</td>
<td>1.490±0.002</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM
The effect of MEE is more encouraging exhibiting gastro-protective activity through the re-establishment of the continuous surface epithelial layer, increased synthesis of gastric mucin, normalization of antioxidant system, inhibition of oxidative stress and inhibition of H⁺-K⁺-ATPase. Similar observation has been made by many investigators and the present findings are in consistent with earlier reports on anti-gastric ulcer property of various phenolic rich plant extracts in animal models (Mahendran et al., 2002; Naik et al., 2007; Siddaraju et al., 2009; Morais et al., 2010; Karthikeyan and Gobianand, 2010; Zakaria et al., 2011; Choudhary et al., 2013; Palacios-Espinosa et al., 2014). General class of phytochemicals with anti-ulcer property includes; flavonoids, xanthones; saponins, tannins, phenolic acids and alkaloids (Lewis and Hanson, 1991; Borrelli and Izzo, 2000; Falcão et al., 2008). Qualitative analysis of MEE showed the presence of tannins, flavonoids, phenolics and xanthones and earlier literature clearly reveals the presence of quercetin, myricetin, rutin as major flavonoid molecules (Rajesh et al., 2013).

Xanthones are the major class of compounds, which are present in Mesua species. Some of major xanthones present in M. ferrea are mesuaxanthone, ferraxanthone 1,7-dihydroxyxanthone, 1,5-dihydroxy-3-methoxyxanthone, 1, X6-trihydroxyxanthone, 1,5-dihydroxyxanthone, 1-hydroxy-7-methoxyxanthone, mesuabixanthone etc. Presence of xanthone derivative has also been reported from various parts of the plant (Sharma et al., 2002; Chow et al., 1968; Govindchari et al., 1967). Most of the authors investigated and showed significant gastroprotective activity of flavonoids, xanthones, phenolic acids, saponins and tannins (Yamamoto et al., 1992; Ezaki Barnaulov et al., 1982; Martin et al., 1998; Barnaulov et al., 1985; Guerrero et al., 1994; Ateufack et al., 2014; Jesus et al., 2012; Souza et al., 2007). Additionally, natural products are safe with less associated toxicity and exerts a wide rage of biochemical and pharmacological properties. Therefore, it can be deduced that the gastro-protective activity exhibited by MEE could be due to the presence of significant antioxidant class of molecules like phenolics, flavonoids, tannins, xanthones and other chemical constituents.

GCMS profiling of MEE also showed the presence of phytocompounds. Among the identified compounds, 2-Furancarboxylic acid, methyl ester and 4H-Pyran-4-one, 2,3-
dihydro-3,5-dihydroxy-6-methyl have inherent property of anti-ulcer and anti-inflammatory activity. This also justifies the anti-ulcer potency of MEE and traditional use of *M. ferrea* to treat gastrointestinal disorders.

### 4.4. Conclusion

The findings from the present study showed that MEE exerts gastro-protective effects via various mechanisms (Fig. 4-13) such as normalization of antioxidant system, modulation of oxidative damage, H⁺-K⁺-ATPase inhibition, increased cytoprotection and gastric mucin synthesis. The ability of MEE to reduce total acidity and gastric secretion with increased gastric mucin suggests the balanced protection of MEE against the aggressive and defensive factors of gastric ulcer. Presence of high phenolic, flavonoid, tannins and saponin clearly explain the effectiveness of MEE in protection against gastric ulcer.

![Diagram](image)

**Figure 4-1:** Ulcer healing mechanism by MEE. (A) Reason for the cause of ulcer, (B) factors which delays ulcer healing and (C) MEE accelerated the ulcer healing by multi-step mechanism
References


CHAPTER OUTLINE

Objective: To isolate, screen free radical scavenging and H⁺-K⁺-ATPase inhibition property of phyto-constituents of Mesua ferrea Linn.

Work flow

Collection of bark

MPE

MCE

MPE

Isolation of phytoconstituents through column chromatography

Free radical scavenging activity

Structure elucidation of phytoconstituents

H⁺-K⁺-ATPase inhibition study

Molecular docking

Results: (a) Potent free radical scavenging phytoconstituents were isolated and characterized (b) Isolated phytoconstituents showed remarkable H⁺-K⁺-ATPase inhibition (c) In vitro H⁺-K⁺-ATPase inhibition was substantiated by molecular docking studies. All the phytoconstituents exhibited minimum binding energy.

Conclusion: Overall result of the investigation accentuates that, isolated phytoconstituents are potent antioxidant molecules and may poses gastro protective activity against induced oxidative stress.