Gastroprotective activity and mechanism of novel dichlorido-zinc(II)-4-(2-(5-methoxybenzylideneamino)ethyl)piperazin-1-i umphenolate complex on ethanol-induced gastric ulceration

Muhammad Saleh Salga, Hapipah Mohd Ali, Mahmood Ameen Abdullah, Siddig Ibrahim Abdelwahab

Abstract

Zinc complexes were reported to have anti-ulcer activity and used as drug for the treatment of gastrointestinal disorders. A novel compound dichlorido-zinc(II)-4-(2-(5-methoxybenzylidene amino)ethyl)piperazin-1-i umphenolate (ZnHMS) was synthesized, characterized and evaluated for its gastroprotective activity against ethanol-induced ulcer in rats. Gross and microscopic lesions, histochemical staining of glycogen storage, biochemical and immunological parameters were taken into consideration. Oral administration of ZnHMS (30 and 60 mg/kg; 14 days) dose-dependently inhibited gastric lesions. It significantly increased the mucus content and total acidity compared to the control group (P < 0.01). Serum levels of aspartate (AST), alanine (ALT) transaminases, pro-inflammatory interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and anti-inflammatory interleukin-10 (IL-10) in the rats exposed to ethanol induced ulceration have been altered. ZnHMS considerably enhances (P < 0.05) the protection of gastric epithelia by modulating the acute alterations of AST, ALT, IL-6, IL-10, TNF-α and stomach glycogen. Interestingly, ZnHMS did interfere with the natural release of nitric oxide. In addition, acute toxicity study revealed no abnormal sign to the rats treated with ZnHMS (2000 mg/kg). These findings suggest that the gastroprotective activity of ZnHMS might contribute in adjusting the inflammatory cytokine-mediated oxidative damage to the gastric mucosa.

Keywords: Schiff bases, Acute toxicity, Gastroprotectivity, Ethanol, TNF-α, IL-10

1. Introduction

Gastric mucosal erosion was reported to be associated with the imbalance between the aggressive factors (physical, chemical or psychological) in the lumen and protective mechanisms [1] in the duodenal mucosa, causing chronic inflammation that leads to a defect in the regulation of gastrin production [2]. Gastrointestinal problems have now become a global problem, and many studies were conducted towards fixing it [3]. The ability of some ulcer models to suppress the production of prostaglandins and thromboxanes, and cause irreversible inactivation of the cyclooxygenases (which is essential for the production of prostaglandins) has provided a means for intense investigations [4–6]. It was suggested that, cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) play important roles in the acute phase inflammation as well as in maintenance and regulation of the severity of gastric ulcers [7]. The ulceration bestowed by ethanol causes an intense infiltration in the submucosa, decrease mucus, depletes sulfhydryl groups and decrease blood flow, which resulted in serious damage to the gastric mucosa [8]. This ulcer model has provided a means of determining the mechanistic way by which many compounds protect gastric against ulcerations.

Zinc is an essential element which plays an imperative role in cell-mediated immune functions. It is employed as a cofactor for metalloenzymes, superoxide dismutase, collagenase, alcohol dehydrogenase, alkaline phosphatase, children’s growth and in spermato genesis [9,10]. Zinc is required for the proper functioning of mucosal cells and can arrest the advancement of gastrointestinal disease by free radical scavenging and interruption of the inflammatory process as an antioxidant and anti-inflammatory agent. Zinc deficiency can cause poor wound healing, loss of taste and smell and elevation in ROS [11,12]. Zinc complex was reported to have anti-ulcer activity and used as drug for the treatment of gastrointestinal injuries in Japan [13]. This study considers the therapeutic potentials of piperazine derivatives, and the role played by zinc in wound healing to synthesize a novel compound with combine protective activity [14,15]. The study, therefore, reported for the first time the synthesis, characterization, acute toxicity and
gastroprotective activity of Zinc(II) complex (ZnHMS) derived from the Schiff base (E)-4-methoxy-2-((2-(piperazin-1-yl)ethylimino)methyl)phenol.

2. Materials and methods

2.1. Reagents

The primary amine 2-(piperazin-1-yl)ethanamine, 5-methoxysalicylaldehyde, Zn(II)chloride dihydrate and potassium hydroxide were purchased from Merck, Kuala Lumpur, Malaysia. ELISA kits for IL-6, IL-10 and TNF-α were purchased from Ray Biotech, USA. All other chemicals and reagents were of analytical grade.

2.2. Synthesis of dichlorido-zinc(II)-4-((2-(5-methoxybenzylideneamino)ethyl)piperazin-1-iumphenolate

A weighed amount of 2-(piperazin-1-yl)ethanamine (1.29 g, 10 mmol) dissolved in an absolute ethanol 25 mL was added dropwise to an ethanolic solution 25 mL of 5-methoxysalicylaldehyde (1.52 g, 10 mmol) at room temperature and refluxed for 3 h. An orange solution was formed which after evaporation gave a red gel. The product was isolated in solid form by adding few drops of diethyl ether. Recrystallization was carried out in methanol. To a stoichiometric amount of the synthesized Schiff base (0.26 g, 1 mmol) dissolved in 25 mL methanol, an equimolar quantity of Zinc(II) chloride dihydrate (0.14 g, 1 mmol) taken in 25 mL methanol was added at room temperature. In the presence of few drops of potassium hydroxide and stirring, a pale yellow precipitate was formed. The precipitate filtered, washed with ethanol–water mixture and dried in a vacuum desiccator. Recrystallization was performed in a mixture of methanol and dichloromethane.

2.3. Acute toxicity study

2.3.1. Animals

Adult male and female Sprague Dawley rats of 7–8 weeks old weighed (165 ± 15 g) were obtained from the Animal House, Faculty of Medicine, University of Malaya, Malaysia. In this study, 24 rats were assigned into two groups of twelve rats each (six male and six female rats per group) and labeled as control and treatment groups. All animals were given human care according to approved institutional guidelines and experiments articulated by the National Academy of Sciences and published by the National Institute of Health, Malaysia.

2.3.2. Protocol for acute toxicity

The overnight fasted animals received orally a single dose of ZnHMS 2000 mg/kg/body weight, and continued fasting for 3–4 h after dosing. Daily clinical examinations of the animals did not show any significant abnormal signs of toxicity like tremor, eyes mucus, body weight changes or autonomous saliva release. No mortality was recorded at any time of observation for the period of two weeks. The study was conducted according to the “International Guidelines for Testing of Chemicals Oral Toxicity” and was approved by the Animal Ethics Committee of University of Malaya.

2.4. Gastro-protective study

2.4.1. Ulcerogenic study animals

Sprague Dawley adult male rats (220 ± 30 g) 7–8 weeks old were for ulcerogenic study. Rats were distributed into eight groups of six rats each. The animals were caged individually. They were exposed to 12 h dark–light circles and fed with a standard pellet diet and tap water under controlled temperature conditions (23 ± 2 °C). All animals were given human care according to approved institutional guidelines and experiments articulated by the National Academy of Sciences and published by the National Institute of Health, Malaysia.

2.4.2. Dose selection

A preliminary study for dose fixation was conducted using 20 mg/kg of ZnHMS to 24 h fasted rats prior to ethanol induced ulceration. It was observed that 20 mg/kg of ZnHMS did not significantly prevent the damaging effects of ethanol. Increasing the dosage of ZnHMS to 30 mg/kg was observed to give a better protection. In addition, treatment with ZnHMS for 3, 7 and 10 days showed a continuous inhibition of the ulcer area. Thus, the dose of 30 mg/kg (ZnHMS) was then considered as the low dose and the treatment extend to 14 days to further ascertain the active dosage that can give the highest protection against the damages caused by ethanol.

2.4.3. Protocol for gastroprotectivity

Animals were fasted for 24 h before ethanol-induced ulceration (Scheme 1) and orally pre-treated as follows: Groups 1 and 2 received the vehicle, carboxymethylcellulose (CMC) after 14-days treatments with food and water alone. Group 3 rats were treated with omeprazole (20 mg/kg) dissolved in CMC prior to ethanol administration. Groups 4, 7 and 8 rats were given ZnHMS dissolved in CMC for two weeks. Group 5 and Group 6 were given ZnHMS single doses of 30 and 60 mg/kg, respectively, dissolved in CMC prior to ulcer induction. One hour after this treatment, animals in Groups 2, 3, 5, 6, 7 and 8 were orally gavaged with 95% ethanol at the dose of 5 mL/kg. Group 4 rats were not given ethanol and served as normal control group. All the animals were sacrificed after 30 min by cervical decapitation under anesthesia with xylazine and ketamine. Serum was collected for biochemical analyses.

2.4.4. Serum biochemical assays

Blood was collected through cardiac puncture. Serum samples obtained from the animals were analyzed using Hitachi Autoanalyzer at University of Malaya Medical Centre laboratory, Kuala Lumpur, Malaysia. Parameters analyzed include ALT, AST, C-reactive protein and HDL.

2.4.5. Measurement of gastric juice acidity

The stomachs removed from the experimental rats were opened along the greater curvature, and the content carefully transferred into a clean container. This was then centrifuged for 10 min at –4 °C and analyzed for hydrogen ion concentration by pH-meter titration using 0.1 N NaOH as titrant. The acid content was expressed as mEq/l [16].

2.4.6. Histological evaluation of gastric lesions

Specimens of the gastric walls removed from each rat were fixed in 10% buffered formalin and embedded in paraffin. Sections of the stomach (5 μm) were stained with hematoxylin and eosin for histological evaluation.

![Scheme 1. Experimental design.](image-url)
2.4.7. Gross gastric lesions evaluation

Ethanol induced gastric ulcers appeared as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. The ulcerated area in the gastric mucosa was measured using a planimeter ([10 × 10 mm² = ulcer area] under dissecting microscope (1.8×]) and scored by counting the number of small squares, 2 mm × 2 mm, covering the length and width of each ulcer band. Thus, UA mm² = 2 × 2 × 1.8. The inhibition percentage (%) was calculated according to the reported procedure [17] with slight modifications.

The inhibition percentage (%) = [(UAcontrol - UAtreated)/UAcontrol] × 100.

2.4.8. Measurement of mucus production

The gastric mucosa of each rat was gently scraped using a glass slide, and the mucus weight measured using electronic weighing balance.

2.4.9. Mucus histochemical staining

To investigate the protective effects of ZnHMS against ethanol induced gastric damage, gastric tissues were stained histochemically to assess the mucus content [18]. The gastric tissues were sectioned, dewaxed and stained using commercial PAS staining system kit (Sigma Aldrich, Malaysia) according to the manufacturer’s guide.

2.4.10. Role of nitric oxide on the gastro-protective effect of ZnHMS

The level of nitric oxide in the gastric of the experimental rats was evaluated as total nitrate/nitrite using Griess’s reagent [19]. The stomach homogenates in 50 mM potassium phosphate buffers (pH 7.4) were centrifuged at 4000 rpm for 30 min at 4 °C. Fifty microliters of the Griess reagent (0.1% N-(1-naphthyl) ethylenediaminedihydrochloride, 1% sulfanilamide in 5% phosphoric acid) was added to 50 μL supernatant and mixed, after 10 min; the absorbance was measured at 540 nm. The standard curves were obtained by using sodium nitrite and the results expressed as micromoles nitrate/nitrite per gram of protein.

2.4.11. Thiobarbituric acid reactive substances assay

To determine the extent at which the compound protected the gastric from lipid peroxidation caused by ethanol induced ulceration, the level of thiobarbituric acid-reactive substances (as indicators of lipid peroxidation) were examined following the reported procedure [20] with some modifications. The stomach homogenates were mixed with 0.125 mL of a solution containing 26 mM thiobarbituric acid, 0.26 M hydrochloric acid, 15% trichloric acid and many petechiae; 2: 6–10 erosions <5 mm in length; 3: 5–10 erosions >5 mm in length and many petechiae: 2: 6–10 erosions <5 mm in length; 2.5: 1–5 erosions >5 mm in length; 3: 5–10 erosions >5 mm in length; 3.5: >10 erosions >5 mm in length; 4: 1–3 erosions ≤5 mm in length and 0.5–1 mm in width; 4.5: 4–5 erosions ≤5 mm in length and 0.5–1 mm in width; 5: 1–3 erosions >5 mm in length and 0.5–1 mm in width; 6: 4 or 5 grade 5 lesions; 7: ≥6 grade 5 lesions; 8: complete lesion of the mucosa with haemorrhage.

2.4.12. Cytokines evaluations

The levels of cytokines (IL-6, IL-10 and TNF-α) in the serum were analyzed using ELISA kits (Ray Biotech, USA) for rats according to the manufacturer’s instructions. One hundred microliters of sample was added to each primary antibody coated wells and then followed with biotin conjugated secondary antibodies. To obtain color reaction, streptavidin–HRP and substrate solution were added. The absorbance was measured at 450 nm with an ELISA reader (TECAN, Mannedorf, Switzerland). Standard curves were plotted for each assay plate using recombinant IL-6, IL-10 and TNF-α in serial dilutions.

2.5. Gastric tolerability test

To assess the ulcerogenic effect of ZnHMS compound, the number of Lesions was examined under an illuminated magnifier (3×) and assessed according to a modified scoring system of Adami et al. [21]: 0: no lesions; 0.5: slight hyperaemia or ≤5 petechiae; 1: ≤5 erosions ≤5 mm in length; 1.5: ≤5 erosions ≤5 mm in length and many petechiae; 2: 6–10 erosions ≤5 mm in length; 2.5: 1–5 erosions >5 mm in length; 3: 5–10 erosions >5 mm in length; 3.5: >10 erosions >5 mm in length; 4: 1–3 erosions ≤5 mm in length and 0.5–1 mm in width; 4.5: 4–5 erosions ≤5 mm in length and 0.5–1 mm in width; 5: 1–3 erosions >5 mm in length and 0.5–1 mm in width; 6: 4 or 5 grade 5 lesions; 7: ≥6 grade 5 lesions; 8: complete lesion of the mucosa with haemorrhage.

2.6. Statistical analysis

All values were reported as mean ± SEM. The statistical significant differences between groups were assessed using one-way ANOVA. A value of P < 0.05 was considered significant.

3. Results

3.1. Spectroscopy

C₁₄H₂₁N₃O₂: IR (KBr disc cm⁻¹, 4000–400 cm⁻¹): 3270 v (O–Hs), 2941 v (C–Hs), 1638 v (C=N), 1488 v (C–O), 618 v (NH–C). ¹H NMR (400 MHz, DMSO): δ 2.1s (piperazinic N–H), δ 6.30–7.47 m (arH) δ 8.54s (iminic, HC=N), δ 11.26s (phenolic O–H), methylene δ 2.37s (CH₂), UV–VIS (λmax, mol⁻¹ L⁻¹cm⁻¹): 312 nm (2235), 268 nm (3046).

C₁₄H₂₁Cl₂N₃ZnO₂: yield, (0.2 g, 76%); anal calc. C, 54.79; H, 5.19; N, 10.51. Found: C, 53.98; H, 4.91; N, 9.54. IR: 3466 cm⁻¹, 1460 cm⁻¹ (M–N), 11.26s (phenolic O–H), methylene δ 2.37s (CH₂), UV–VIS (λmax, mol⁻¹ L⁻¹cm⁻¹): 312 nm (2235), 268 nm (3046).
any toxicity symptoms was noted with slight increase in mean body weight among the groups of rats (Fig. 2).

3.3.2. Evaluation of biochemical parameters
The ulcerated rats showed a notable increased in the enzymes AST and ALT with a decreased in high-density lipoprotein (HDL) and acute phase C-reactive protein (Table 3). These parameters remained within the normal range in the rats pre-treated with ZnHMS (30 and 60 mg/kg for 14 days). However, the rats that received single doses of ZnHMS body weight showed a moderate rise in the level of these biomarkers. The level of C-reactive protein in the rats treated with ZnHMS slightly increased compared with that of the normal rats due to inflammation. However, the ethanol induced rats manifested a significant increase in the level of this biomarker (*P<0.05) compared with treated and the normal rats (Table 3).

The serum level of TNF-α and IL-6 rises markedly in the ulcerated rats had manifested an extensive damages to the gastric mucosa as seen by reduction in ulcer area, reduced or absence of submucosal edema and leucocytes infiltration. Fourteen-day treatments with ZnHMS had shown increased gastro-protective effects when compared to a single dose of ZnHMS (Fig. 5D).

3.3.3. Gastric acidity and mucus content
Oral administration of ethanol to the rats produced the lowest content of the mucus and pH of gastric acid (Table 4). ZnHMS significantly and dose dependently increased (*P<0.05) the gastric mucus content in comparison to an ulcer control group. Animals pre-treated with ZnHMS and omeprazole in combination with ethanol had significantly increased (*P<0.05) the mucus content and the pH of the gastric acid.

3.3.4. Gastric morphological assessment
The rats pre-treated with ZnHMS (Fig. 4C–D), and omeprazole (Fig. 4B) had considerably reduced areas of gastric ulcer formation compared with rats that received CMC alone (ulcer control group, Fig. 4A). In addition, flattening of gastric mucosal folds was observed in the rats treated with ZnHMS. Table 4 showed a significant (*P<0.05) reduction in an ulcer index at the doses of 30 and 60 mg/kg body weight for the period of 14 days treatment with the compound ZnHMS. The inhibition observed at these doses are statistically (*P<0.05) higher than that shown by omeprazole and the single doses of ZnHMS (30 and 60 mg/kg).

3.3.5. Histological evaluations
Histological evaluations of the gastric walls in ethanol induced ulcerated rats had manifested an extensive damages to the gastric mucosa, oedema and leucocytes infiltration of the submucosal layer (Fig. 5B). Pre-treatment with ZnHMS for two weeks (Fig. 5E–F) and omeprazole (Fig. 5C) had comparatively better protection of the gastric mucosa as seen by reduction in ulcer area, reduced or absence of submucosal edema and leucocytes infiltration. Fourteen-day treatments with ZnHMS had shown increased gastro-protective effects when compared to a single dose of ZnHMS (Fig. 5D).

3.3.6. Stomach malondialdehyde
Rats exposed to ethanol induced gastric injuries showed noticeable (*P<0.05) higher stomach’s MDA levels (Table 5). This increased MDA level decreases significantly (*P<0.05) and dose dependently in the rats that received ZnHMS for 14 days compared to the rats that received single dose of omeprazole and the compound ZnHMS.

3.3.7. Nitric oxide level
The level of nitric oxide in the gastric tissue was analyzed using Gries’s reagent and expressed as total nitrate/nitrite (Table 5). The fundus part of the gastric obtained from ethanol-ulcerated rats (Group 2) showed the lowest level of nitric oxide. Administration of ZnHMS to the animals in Groups 4, 7 and 8 for 14 days revealed significant increased (*P<0.05) in NO level compared with ethanol-induced animals. However, rats in the Group 3, 5 and 6 manifested a decrease in NO in comparison with the rats treated for 14 days.

**Table 2**

Effects of ZnHMS on liver function in rats.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Sodium (mmol/L)</th>
<th>Pottasium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>CO₂ (mmol/L)</th>
<th>Anion gap (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>144 ± 1.3a</td>
<td>4.20 ± 0.8a</td>
<td>104 ± 1.5a</td>
<td>21 ± 1.3a</td>
<td>19.8 ± 2.9a</td>
<td>6.9 ± 1.5a</td>
<td>42.5 ± 3.3a</td>
</tr>
<tr>
<td>Males (2 g/kg)</td>
<td>146 ± 1.2a</td>
<td>4.65 ± 1.9a</td>
<td>112 ± 1.8a</td>
<td>24 ± 1.8a</td>
<td>21.3 ± 2.2a</td>
<td>5.7 ± 1.8a</td>
<td>44.3 ± 2.8a</td>
</tr>
<tr>
<td>Females (2 g/kg)</td>
<td>149 ± 0.9a</td>
<td>3.85 ± 1.8a</td>
<td>109 ± 1.2a</td>
<td>26 ± 1.7a</td>
<td>18.6 ± 3.6a</td>
<td>6.3 ± 1.9a</td>
<td>46.5 ± 3.1a</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. There are no significant differences between groups. Significant value at *p<0.05.* Groups with different superscript alphabets are statistically significant. No significant difference among the groups.

**Table 4**

Effects of ZnHMS on renal function in rats.

<table>
<thead>
<tr>
<th>Animals</th>
<th>T. protein (g/L)</th>
<th>Albumin (g/L)</th>
<th>Globulin (g/L)</th>
<th>TB (µmol/L)</th>
<th>CB (µmol/L)</th>
<th>AP (IU/L)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>72 ± 1.4a</td>
<td>36.2 ± 1.3a</td>
<td>29.6 ± 2.3a</td>
<td>5.9 ± 1.2a</td>
<td>1.0</td>
<td>120 ± 2.9a</td>
<td>56 ± 3.9a</td>
<td>247 ± 4.3a</td>
</tr>
<tr>
<td>Males (2 g/kg)</td>
<td>76 ± 1.7a</td>
<td>37.3 ± 1.8a</td>
<td>31.3 ± 2.5a</td>
<td>6.6 ± 1.3a</td>
<td>1.0</td>
<td>123 ± 2.6a</td>
<td>57 ± 3.4a</td>
<td>249 ± 4.2a</td>
</tr>
<tr>
<td>Females (2 g/kg)</td>
<td>77 ± 2.1a</td>
<td>38.8 ± 2.2a</td>
<td>33.5 ± 2.5a</td>
<td>6.8 ± 1.8a</td>
<td>1.0</td>
<td>125 ± 2.8a</td>
<td>59 ± 4.6a</td>
<td>248 ± 4.5a</td>
</tr>
</tbody>
</table>

TB, total bilirubin; CB, conjugated bilirubin; AP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

* Groups with different alphabets are statistically significant. No significant difference among the groups.
This suggests that, 14 days treatment with ZnHMS had enhanced the production of NO and thus provides more protection than the single dose regime of this zinc complex (Table 5).

### 3.3.8. Mucus staining

The Periodic Acid Schiff (PAS) staining was used to assess the level of glycogen in experimental animals as presented in Fig. 6. Rats pretreated with ZnHMS had the highest ability to restore the mucin in the glandular cells when compared with ethanol-induced ulcerated animals. This high tendency is evidenced by the accumulation of the magenta staining of PAS was not observed abundantly in the stomach of the animals induced with ethanol ulcer. This suggests that, 14 days treatment with ZnHMS had enhanced the production of NO and thus provides more protection than the single dose regime of this zinc complex (Table 5).

### 3.4. Gastric tolerability

ZnHMS when administered to the animals did not produce any significant gastric lesions. The changes were observed in range of 0–1 according to the Adami’s scoring scale. Namely, only slight hyperaemia or few petechiae were registered in rat stomach regardless of given dose.

### 4. Discussion

The IR spectral analysis showed absorption at 1638 cm$^{-1}$ which can be ascribed to azomethine, confirming the formation of Schiff base and the absence of aldehydeic group [22–25]. This band shifted to 1629 cm$^{-1}$ in the spectra of the complex which indicates chelate formation [26–28]. This coordination was confirmed by the appearance of new bands at 592 and 416 cm$^{-1}$ and metal-nitrogen (M-N) bands, respectively [29–34]. Furthermore, the proton NMR spectra of the Schiff base indicated the presence of phenolic hydrogen at the chemical shift $\delta$ 11.26, this band was absent in the spectra of the complex [29,35,36] supporting the coordination between the Schiff base and the metal ion. The spectral data obtained from UV–visible was in agreement with the data obtained from IR and NMR. It showed an absorption at 312 nm and 268 nm that can be attributable to charge transfer in the imine area C=N (before complexation) and $\pi$–$\pi^*$ electronic transition (of the phenyl ring), respectively. These absorptions shifted to 342 nm and 267 nm affordable to ligand–metal charge transfer [37,38] and resonance effects of $\pi$-electrons in the phenyl ring due to complexation [39].

The 14 days treatment with ZnHMS showed increased in the body weight of the experimental animals (Fig. 2). The abnormal signs of toxicity were noticeably absent throughout the study period. The biochemical evaluations of liver and kidney functions did not show significant differences between the normal and the treated rats in the acute toxicity experiment. The liver enzymes AST, ALT did not alter significantly in the rats treated with the compound ZnHMS. The level of albumin, globulin, alkaline phosphatase, total and conjugated bilirubin are all within normal range in the rats that received ZnHMS (Table 1). The level of sodium, potassium, chloride, urea and creatinine in the kidneys is also comparable with the normal rats (Table 2). The histological evaluations of liver and kidney manifested minor damages in the liver cells of female rats (Fig. 3E) when compared with that of the male rats (Fig. 3C) and the normal group (Fig. 3A). This showed that female rats are more susceptible to ZnHMS effects than their male counterparts.

The protective activity of the compound was ascertained by comparing the gastric damage caused by ethanol to the rats in the ulcer control group (Fig. 4B) with the stomachs of the rats in the normal (Fig. 4A) and the treatment groups (Fig. 4C–F). It is apparent that the compound significantly and dose-dependently protects the mucosal membrane against ethanol-induced injury (Fig. 4C–F). This protection was high in rats that received ZnHMS for two weeks (Fig. 4E–F) than those given a single dose of the compound (Fig. 4D) and omeprazole (Fig. 4C). The ability of a mucosa membrane to protect the gastric walls depended solely on equilibrium between the aggressive and the protective factors [40–42]. The compound ZnHMS has strengthened the protection of mucosal membrane from ethanol damaging effects. This was evidenced by the inhibition of leucocytes infiltration of gastric wall (Fig. 5C–F) when compared with the ulcerated rats (Fig. 5B). These rats showed a severe disruption to the surface epithelium, necrotic lesions which penetrate deeply into mucosa, extensive edema of submucosal layer and leucocytes’ infiltration.
Two-week treatment with ZnHMS in ulcer induced groups have notably enhanced the mucus secretion (Fig. 6C–D) when compared with the normal rats (Fig. 6A). Ethanol served as the ulcer model that is used for evaluating the protective and healing activity of many drugs [7,43]. It effectively reduced the level of NO in the gastric mucosa, affects the flow of gastric blood and

### Table 5
Effects of ZnHMS on lipid peroxidation, nitric oxide and cytokines on ethanol induced ulcer.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatments*</th>
<th>Malondialdehyde (μmol/g Tissue)</th>
<th>Nitric oxide (μmol)</th>
<th>Tumor necrosis factor alpha (pg/mg of stomach tissue)</th>
<th>Interleukin-6</th>
<th>Interleukin-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Normal</td>
<td></td>
<td>9.98 ± 2.67a</td>
<td>9.4 ± 1.81a</td>
<td>9.10 ± 0.2a</td>
<td>6.5 ± 0.3a</td>
<td>453 ± 18a</td>
</tr>
<tr>
<td>2 Ethanol ulcer group</td>
<td></td>
<td>29 ± 1.40b</td>
<td>4.2 ± 0.3b</td>
<td>610 ± 18b</td>
<td>78 ± 4.1b</td>
<td>84 ± 5.2b</td>
</tr>
<tr>
<td>3 Omeprazole (20 mg/kg)</td>
<td></td>
<td>16.5 ± 0.9a</td>
<td>8.3 ± 1.4a</td>
<td>104 ± 2.8a</td>
<td>8.2 ± 0.43a</td>
<td>249 ± 5.7a</td>
</tr>
<tr>
<td>4 ZnHMS-alone 60 mg/kg</td>
<td></td>
<td>10.5 ± 2.57c</td>
<td>9.3 ± 1.20a</td>
<td>10.8 ± 0.9a</td>
<td>7.5 ± 0.83c</td>
<td>427 ± 26a</td>
</tr>
<tr>
<td>5 ZnHMS (single dose; 30 mg/kg) + ethanol</td>
<td></td>
<td>19.6 ± 2.1d</td>
<td>5.8 ± 1.4c</td>
<td>132 ± 2.7a</td>
<td>14.2 ± 2.3c</td>
<td>158 ± 4.8a</td>
</tr>
<tr>
<td>6 ZnHMS (single dose; 60 mg/kg) + ethanol</td>
<td></td>
<td>18.2 ± 1.8f</td>
<td>6.2 ± 1.5c</td>
<td>105 ± 1.4c</td>
<td>13.6 ± 2.8c</td>
<td>189 ± 5.4f</td>
</tr>
<tr>
<td>7 ZnHMS (14 days, 30 mg/kg) + ethanol</td>
<td></td>
<td>10.0 ± 1.10e</td>
<td>9.2 ± 0.9a</td>
<td>75 ± 4.5c</td>
<td>9.6 ± 1.2c</td>
<td>305 ± 4.9f</td>
</tr>
<tr>
<td>8 ZnHMS (14 days 60 mg/kg) + ethanol</td>
<td></td>
<td>9.5 ± 0.56d</td>
<td>9.9 ± 0.85a</td>
<td>11.2 ± 0.4c</td>
<td>7.2 ± 3.6c</td>
<td>401 ± 21f</td>
</tr>
</tbody>
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* Groups with different alphabets are statistically significant.

**Fig. 3.** Body weight changes among the experimental rats.
resulted in the development of hemorrhagic necrosis. [44–45]. This causes an increased flow of Na+ and K+, amplified pepsin secretion, and enhances loss of H+ ions and histamine in the lumen [46].

The chemo-preventive activity of ZnHMS can be presumed considering the changes noticeable in the acute phase C-reactive proteins and HDL. The serum level of AST and ALT elevated significantly in the ulcerated rats indicating the level of inflammation in the liver. In contrast, the level of these enzymes has decreased significantly in the rats pre-treated with ZnHMS and omeprazole (Table 3).

Pre-treatment with omeprazole and ZnHMS had led to the rise in gastric pH, improved the production of mucus and caused flattening of mucosal folds (Table 4). The single doses of ZnHMS showed a decreased activity compared to omeprazole. However, two-week treatments with ZnHMS give better protection of 90.8% and 95.8% at low and high doses, respectively. These percentages are higher than 79.0% shown by omeprazole. This apparently established the protective effect of ZnHMS.

The possible changes in the level of cytokines IL-6, TNF-α and IL-10 were investigated (Table 5). The observed increased in the production of IL-6 and TNF-α in plasma can be afforded to the necrotizing effects of ethanol [47]. This effect was mild in the animals treated with omeprazole and ZnHMS at all doses. However, a slight increased was noticed in the single doses of ZnHMS, this rise was not significant when compared to the ethanol induced ulcerated rats.

The increase in the level of NO, IL-10 (Table 5) and inhibition of acid secretion to fix the inflammation and mucosal erosion caused by ethanol [48] can be afforded to the continuous treatments with ZnHMS for the period of 14 days. Nitric oxide (NO) was reported to have an ability of inhibiting the neutrophil infiltration and provides a protective barrier to the gastric mucosa against ethanol attack [49]. In addition, the potential anti-ulcer

![Gross appearance of the gastric mucosa in the experimental rats](image-url)
drugs can exert their protective effect against mucosal lesions through inhibition of neutrophil infiltration in the ulcerated gastric tissue [50]. Furthermore, neutrophil accumulation in a gastric mucosa has been shown to induce microcirculatory abnormalities. However, the results of this study demonstrated the ability of ZnHMS to inhibit the level of pro-inflammatory mediator (TNF-α and IL-6) and neutrophil infiltration. This is similar to the reported activity of plant extract against indomethacin induced ulceration [1,51]. This anti-inflammatory activity could also be a key factor in the prevention of gastric ulcer as reported [52]. To further substantiate the mechanism of action of this compound, MDA level was evaluated (Table 5). The current study also showed an appreciable reduction of MDA level in the treatment groups compared with the ulcerated rats. This also suggests the facility of ZnHMS to prevent the formation of reactive oxygen species (ROS) and consequently, stopped the lipid peroxidation process in the gastric mucosa [52].

In conclusion, ZnHMS has notably and dose-dependently protect the gastric mucosa against ethanol-induced injury. The compound activity is considered as a synergistic effect between zinc and the Schiff base as it was evident that zinc has protective potentials. Interestingly, ZnHMS enhanced the release of NO. The current findings revealed that ethanol induced ulceration affects the enzymes AST and ALT causing a serious inflammation in the liver. This had led to the response of acute phase C-reactive proteins due to increased inflammation and was evident by the rise in the level of interleukin IL-6 and TNF-α. In contrast, the rats that received ZnHMS had enhanced immune system, which raises the secretion of interleukin IL-10 and HDL to reverse cholesterol transport, increase the level of NO and inhibit lipid peroxidation [52]. However,
this study suggests further research on the compound dichlorido-zinc-Il-4(2, 5-methoxy benzylideneamino)ethyl)piperazin-1-iun-phenolate (ZnHMS) to exactly ascertain its possible therapeutic activity against gastric ulcer caused by different etiologies.

Conflict of interest statement

None declared.

Acknowledgements

The authors acknowledge the Grants PS358/2009C and ER009-2011A provided by University of Malaya for the conduct of this study.

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