SYNTHESIS AND ANTI-ULCER ACTIVITIES OF THE SCHIFF BASE N',E,N")-N',N"-BIS(1-PYRIDI-2-YL)ETHYLIDENE)CYCLOHEXANE-1,2-DIAMINE AND ITS ZINC (II) COMPLEX

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Abstract
The Schiff base ligand (N',E,N")-N',N"-bis(1-pyridin-2-yl)ethylidene)cyclohexane-1,2-diamine and its Zinc complex was synthesized and characterized by CHN, FTIR, 1H-NMR and UV-visible analysis whereby the structure of the Schiff base was confirmed by X-ray diffraction analysis, showing that the cyclohexane ring adopts a chair conformation with the two imine groups linked at equatorial positions. The two halves of the molecule are related by a crystallographic two fold rotation axis. The dihedral angle between the pyridine rings is 75.73 (3)°. A zinc complex of this ligand was prepared through direct reaction at room temperature. All effort to produce high quality crystals was unsuccessful; however, it shows consistent spectral results with the ligand except in the position of azomethine which may be due to complexation. Both the ligand and its zinc complex were screened and evaluated for anti-ulcer activity against ethanol induced gastric ulceration. The result has shown that, administration of the ligand at the dose of 50 mg/kg markedly decreases the ulcer incidence more than both the Zn-complex and the referenced drug (Omeprazole). This is evident from the manifestation of the flattening of gastric mucosa and the complete absence of lesions in the gastric wall of the ligand treated group and appearance of some few lesions in the zinc and omeprazole groups.

Keywords: Synthesis, Characterization, Crystal structure, Anti-ulcer study

Introduction
Schiff bases are capable of binding with various metals to form complexes that have suitable theoretical and practical applications and their chelates have been extensively studied over past decades [1]. Of the various classes of Schiff bases which are prepared from the condensation of amines and carbonyl compounds, the Schiff bases of piperazine derivatives were less studied [2,3]. However, piperazine and its derivatives form very interesting ligands due to their good bridging ability which makes them useful as building blocks for the formation of coordination polymers [4] and important cyclic components in industrial field, as raw materials for epoxy resins, corrosion inhibitors, insecticides, rubber accelerators, antioxidants and in many pharmaceutical applications [5]. Herein, we report the synthesis, characterizations and anti-ulcer study of the Schiff base ligand (derived from the reaction of 1-(2-aminoethyl) piperazine and 2,4-dihydroxyphenylethanone) and its zinc complex. An exhaustive literature search shows no report preceeding the synthesis and anti-ulcer study on this ligand. This prompted the interest of this research for efficacy and possible determination of its mechanism of action. The anti-ulcer evaluation was carried out using Omeprazole as reference drug. Omeprazole is a proton pump inhibitor and is widely used as an acid inhibitor agent for the treatment of disorders related to gastric acid secretion for more than 15 years [6]. Omeprazole is a substituted benzimidazole and it inhibits acid secretion by acting on the hydrogen-potassium exchanger (H+::K+-ATPase) for the apical plasma membrane of the gastric mucosa [7]. Omeprazole is highly selective for the proton pump and undergoes catalyzed conversion into active form within the acid forming space. The active inhibitors react with SH (thiol) group of the proton pump, resulting in inhibition of acid formation [8]. The reaction scheme for the synthesis of the ligand and its zinc complex is shown below.

Materials and Methods
Cyclohexane-1,2-diamine and 1-(pyridine-2-yl)ethanone were obtained from Sigma-Aldrich, all solvents used were of reagent grade. A spectroscopic grade DMSO-d6 was also obtained from Sigma-Aldrich. All other reagents used were commercially available and used without further purification. The melting point determinations, Infra-red analysis, 1H-NMR spectroscopy, UV-Visible and Crystallographic analyses were conducted at the Department of Chemistry, University Malaya, Malaysia.

Synthesis: Ligand
To the stirred solution (0.23g, 1mmol) of cyclohexane-1,2-diamine in absolute ethanol (50ml), a stoichiometric amount (0.43g, 1mmol) of 1-(pyridine-2-yl)ethanone was added drop-wise with stirring and then refluxed for 2hrs. After concentration and cooling, orange-yellow crystals were obtained. The crystals were filtered, washed with ethanol, re-crystallized in a methanol-ethanol (60:40) mixture at room temperature and dried in a vacuum dessicator [9]. The structure is shown below (Fig. 1a).
NH₂

\[ \text{cyclohexane-1,2-diamine} \]

\[ + \]

\[ 2 \left( \text{1-(pyridin-2-yl)ethanone} \right) \]

\[ \text{NH₂} \]

\[ \text{H₃C} \]

\[ \text{Zn} \]

\[ \text{N} \]

\[ \text{N} \]

\[ \text{N} \]

\[ \text{N} \]

\[ \text{H₃C} \]

\[ \text{CH₃} \]

\[ \text{(N₁E,N₂E)-N₁,N₂-bis(1-(pyridin-2-yl)ethylidene) cyclohexane-1,2-diamine} \]

\[ \text{ZnCl₂, NaOH, Stiring} \]

\[ \text{Py, Reflux} \]

\[ \text{Dichloro-bis(1-(pyridin-2-yl)ethylidene) cyclohexane-1,2-diamine-Zn(II) Complex} \]

\[ \text{Scheme 1: Reaction pathway for the synthesis of the ligand and its zinc complex} \]

**Analysis of the ligand**

Chemical Formula: C₂₀H₂₄N₄, Mol. Wt: 320.43, Mass Spectra (m/z): 320.20 (100.0%), 321.20 (23.1%), 322.21 (2.3%); M.P. 82°C. Elemental Analysis: Anal.(calc): C, 74.78 (74.97); H, 7.45 (7.55); N, 17.32 (17.48).

Infra-red (using KBr disc): \( \nu(\text{N-H}), 3332\text{cm}^{-1}; \nu(\text{C=O}), 1619\text{cm}^{-1}; \nu(\text{C-O/phenolate}), 1296 / 1353\text{cm}^{-1}; \) phenyl ring, 622/608cm⁻¹; ¹H-NMR (in DMSO-d₆): (C=N, 7.45ppm); (N-H, 2.56ppm); (C=O, 3.6ppm). UV-Visible (in DMSO): (266nm, \( \pi-\pi^* \)) and (480nm, LMCT).

**Figure 1a: Crystal structure of the ligand (N₁E,N₂E)-N₁,N₂-bis(1-pyridin-2-yl)ethylidene)cyclohexane-1,2-diamine**

**Zn-complex**

A calculated amount (0.32g, 1mmol) of the ligand in 50ml methanol was dissolved (in 50ml methanol) and added to a stirred methanolic solution (50ml) of ZnCl₂ (0.14g, 1mmol) at room temperature and basified with 3 drops of pyridine. A white precipitate was formed. This was filtered, washed with ethanol, dried in a vacuum desiccator and dissolved in tetrachloromethane for crystallization. After 2 days, yellow needle-like crystals were obtained which remained unchanged up to 2 weeks. This eluded all our efforts to produce high quality crystals similar to that obtained for the ligand [9]. However, the target structure for the zinc complex is proposed below (Fig 1b).

**Crystallography:** Diffraction data were measured at the Department of Chemistry, University of Malaya, Malaysia using Bruker SMART Apex II CCD area-detector diffractometer (graphite-monochromated Mo-Kα radiation, \( \lambda = 0.71073 \text{ Å} \)). The orientation matrix, unit-cell refinement, and data reduction were all handled by the Apex2 software (SAINT integration, SADABS absorption correction [10].
The structure was solved using direct method in the program SHELXS-97 and was refined by the full matrix least-squares method on F2 with SHELXL-97 [10]. All the non-hydrogen atoms were refined anisotropically. The C–bound H atoms were placed at calculated positions and were treated as riding on their parent C atoms. The N– and O–bound H atoms were located in difference Fourier maps, and refined with distance restraints of O—H = 0.86 (2) Å and N—H = 0.91 (2) Å. Drawing of the molecules were produced with XSEED [11]. Crystal data and refinement are summarized in Table 1 and the structure of the complex as shown in Fig 1.

### Biological Studies

**Experimental animals**

*Sprague Dawley* healthy adult male rats were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya. The rats were divided randomly into 3 groups of 6 rats each. Each rat that weighted between 200 - 225g was placed individually in a separate cage (one rat per cage) with wide-mesh wire bottoms to prevent coprophagia during the experiment. The animals were maintained on standard pellet diet and tap water. The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Malaysia. Throughout the experiments, all animals received human care according to the criteria outlined in the “Guide for the Care and Use of laboratory Animals” prepared by the National Academy of Sciences and published by the national Institute of health, Malaysia.

**Ligand preparation for anti-ulcer study**

Both the ligand and its zinc complex were separately dissolved in 10% Tween 20 and administered orally to the rats in concentrations of 50mg/kg body weight (5ml/kg body weight) according to the reported procedure [12].

**Gastric ulcer-induction by ethanol and tissue sample collection**

The rats were fasted for 48 hours before the commencement of the experiment, but were allowed free access to drinking water up till 2 hours before the experiment. Gastric ulcer in *Sprague Dawley* was induced by orogastric intubation of absolute ethanol (5ml/kg) according to the method described by [13]. Ulcer control groups were orally administered with vehicle (10% Tween 20, 5ml/kg). The reference group received oral doses of 20mg/kg omeprazole in 10% Tween 20 (5ml/kg) as positive controls. Experimental group was orally administered with 50 mg/kg of Ligand in 10% Tween 20 solution (5ml/kg) each, respectively. One hour after this pre-treatment; all groups of rats were gavaged with absolute ethanol (5ml/kg) for the purpose of gastric ulcer inducement. The rats were euthanized by cervical dislocation 60 minutes later [14] after being over dosed with diethyl
ether (anesthesia) and their stomachs were immediately excised.

### Table 2: Observed mucus production, pH, ulcer area and inhibition percentage in rats

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Pre-treatment with (5ml/kg)</th>
<th>Mucus weight (g)</th>
<th>pH</th>
<th>Ulcer area (mm²)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% Tween 20 (ulcer control)</td>
<td>0.34 ± 0.008a</td>
<td>3.85 ± 0.08a</td>
<td>815.00 ± 20.12a</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Omeprazole (20 mg/kg)</td>
<td>0.58 ± 0.014b</td>
<td>6.82 ± 0.01b</td>
<td>170.00 ± 8.94b</td>
<td>79.14%</td>
</tr>
<tr>
<td>3</td>
<td>Ligand 50 mg/kg</td>
<td>1.17 ± 0.002c</td>
<td>5.56 ± 0.01c</td>
<td>7.2 ± 4.56c</td>
<td>99.12%</td>
</tr>
<tr>
<td>4</td>
<td>Zn-L 50 mg/Kg</td>
<td>0.97 ± 0.002c</td>
<td>4.68 ± 0.01c</td>
<td>18.9 ± 4.56c</td>
<td>87.14%</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard error mean. Means with different superscripts are significantly different. The mean difference is significant at the 0.05 level.

**Measurements of gastric juice acid and mucus production**

The stomach was opened along the greater curvature. Samples of gastric contents were analyzed for hydrogen ion concentration by pH-meter titration with 0.1 N NaOH solutions using digital pH meter (table 2). The acid content was expressed as mEq/l. Gastric mucus production was measured in the rats that were subjected to absolute ethanol-induced gastric mucosal injury. The gastric mucosa of each rat was gently scraped using a glass slide and the mucus obtained was weighed using a precision electronic balance.

**Histological evaluations of gastric lesions**

Specimens of the gastric walls of each rat were fixed in 10% buffered formalin and processed in a paraffin tissue processing machine. Sections of the stomach were made at a thickness of 5μm and stained with hematoxylin and eosin for histological evaluation. The assessment criteria of the histological score were carried out according to the method described by [14].

**pH of gastric content and Mucus production**

The acidity of gastric content in experimental animals pretreated with the Ligand and its Zn complex were decreased significantly compared to that of the ulcer control group. The mucus production of gastric mucosa also increases significantly in animals pretreated with Ligand and its Zn complex compared to ulcer group.

**Gross evaluation of gastric lesions**

The results of anti-ulcer activity of the Ligand and its zinc complex in ethanol-induced gastric lesion model is shown in table 2. The results showed that rats pretreated with Ligand and/or its Zn complex before being given absolute alcohol had a significantly reduced areas of gastric ulcer formation compared to rats pre-treated with 10% Tween 20 (ulcer control group) (Fig 2). Moreover, the Ligand significantly suppressed the formation of ulcers and more interestingly had manifested the flattening of gastric mucosal folds in all the rats that are pretreated with this compound. The extent of inhibition of gastric ulcer in the rats pretreated with Ligand and its zinc complex was compared with that of omeprazole (the referenced drug used).

**Figure 2.** Gross appearance of the gastric mucosa in rats. (1a) Pre-treated with 5 ml/kg of 10% Tween 20 (ulcer control). Severe injuries are seen in the gastric mucosa. (1b) Pre-treated with 5 ml/kg of omeprazole (20 mg/kg). Injuries to the gastric mucosa are milder compared to the injuries seen in the negative control rat. (1c) Pre-treated with 5 ml/kg of Ligand (50 mg/kg). No injuries to
the gastric mucosa are seen, and showed flattening of gastric mucosa. (1d) Pre-treated with 5 ml/kg of Zinc complex (50 mg/kg). No injuries to the gastric mucosa are seen, and showed flattening of gastric mucosa.

Figure 3. Histological section of gastric mucosa in a rat. (2a) Pre-treated with 5 ml/kg of 10% Tween 20 (ulcer control). There is severe disruption to the surface epithelium, and edema of the submucosal layer with leucocyte infiltration. (2b) Pre-treated with 5 ml/kg of omeprazole (20 mg/kg). There is mild disruption to the surface epithelium with no edema and leucocytes infiltration of the submucosal layer. (2c) Pre-treated with 5 ml/kg of Ligand (50 mg/kg). There is no disruption to the surface epithelium with no edema and no leucocytes infiltration of the submucosal layer. (2d) Pre-treated with 5 ml/kg of Zinc complex (50 mg/kg). There is no disruption to the surface epithelium with no edema and no leucocytes infiltration of the submucosal layer (H&E stain 10x).

Histological evaluation of gastric lesions
Histological observation of ethanol induced gastric lesions in ulcer control group pre-treated with 10% Tween 20, showed comparatively extensive damage to the gastric mucosa, and oedema and leucocytes infiltration of the submucosal layer (Fig 3). Rats that received pre-treatment with Ligand and its Zn complex had a comparatively better protection of the gastric mucosa as seen by reduction in ulcer area, reduced or absence of submucosal edema and leucocytes infiltration (Fig. 3).

Discussion
The ligand was prepared by reflux condensation of cyclohexane-1,2-diamine and 1-(pyridine-2-yl)ethanone in ethanol. The yellow crystals obtained were treated with the solution of zinc chloride to afford the zinc complex (Scheme 1). The FT-IR spectra of both the ligand and the complex showed couple of prominent band regions appearing at 3332cm⁻¹ assignable to ν(N-H) and at 1619cm⁻¹ which is attributable to the azomethine ν(C=N) expected to originate in the ligand spectra respectively. The shifts observed in the position of these band regions in the spectra of the complex have confirmed the complexation. This is supported by other spectral information obtained from NMR where δ (C=N) appeared at 7.45ppm. The chemical shift observed at 2.56ppm could be assigned to the δ (N-H) vibration. Henceforth, the Uv-visible analysis displayed absorptions at 266nm and 480nm which were ascribed to the bathochromic shifts due to (π-π*) and (LMCT) transitions respectively.

The crystal structure of the ligand presented in Fig 1 showed that the cyclohexane ring adopts a chair conformation with the two imines linked at equatorial positions. The two halves of the molecule are related by a two-fold rotation. The dihedral angle between the two pyridine rings is 75.73 (3)°. The crystal structure is devoid of any inter- or intra- molecular interactions. The bond distances and angles in the title molecule are in agreement with the corresponding bond distances and angles reported in some related structures [15,16,17]. The weighted R-factor wR and goodness of fit S are based on F², conventional R-factors R are based on F, with F set to zero for negative F². The threshold expression of F² > σ(F²) is used only for calculating R-factors (gt) etc. and is not relevant to the choice of reflections for refinement. R-factors based on F² are statistically about twice as large as those based on F, and R-factors based on ALL data will be even larger (Table 1)[9,10].

The results of the biological studies showed that both the Ligand and its zinc complex are capable of providing prophylactic anti-ulcer effects against irritant substances. The ligand is capable of completely inhibiting the lesion formed by the ethanol more than the complex and the complex more than the referenced drug as shown in Table 2. The significant increase in the mucus production suggests that the gastric mucosal strengthening mechanism contributes to the anti-irritant potentials of the Ligand and its zinc complex in descending order. It is evident from the result that an increase in the mucus production (1.17 ± 0.002g) must have largely contributed towards the
preventive effect of the Ligand when compared with the mucus produced (0.97 ± 0.002g) in the group treated with the zinc complex and that (0.58 ± 0.014g) of omeprazole (Table 2). Similar finding exist in the literature [14,18] where plant extracts prevent gastric ulceration in rats. The gastric wall mucus is playing an important role as a defensive factor against gastrointestinal damage [19]. Pretreatment with the ligand and its zinc complex had significantly increased the gastric mucus content (Table 2) suggesting that the gastroprotective effect of these compounds is mediated partly by preservation of gastric mucus secretion. Similarly, the result of this study has manifested the protection of gastric mucosa and inhibition of leucocytes infiltration of gastric wall in rats pretreated with the Ligand and its Zn complex, Fig 3. Similarly, [12,20] reported that plant extract exerts protective effect against mucosal lesions through inhibition of neutrophil infiltration in the ulcerated gastric tissue while [21], explained that the reduction of neutrophil infiltration into ulcerated gastric tissue promotes the healing of gastric ulcers in rats. It was narrated [22] that an oral administration of plant extracts before ethanol administration significantly decreased neutrophil infiltration of gastric mucosa. This is supported by [23] where he observed that an increase in neutrophil infiltration into ulcerated gastric tissue delayed the healing of gastric ulcers in rats. It is well known that an absolute alcohol would extensively damage the gastric mucosa leading to increased neutrophil infiltration into the gastric mucosa. However, in this study, the flattening of the mucosal folds has confirmed the gastroprotective effect of the compounds (Ligand and its Zn complex) used which might be attributed to the decrease in gastric motility (Fig 2 & 3). It was reported that the changes in the gastric motility may play a role in the development and prevention of experimental gastric lesions [24]. Moreover, relaxation of circular muscles may protect the gastric mucosa from damage through flattening of the folds which will subsequently increases the mucosal area on exposition to the necrotizing agents and reduce the volume of the gastric irritants on rugal crest [12,14,24]. In addition, ethanol produces a markedly contraction of the muscles of rat gastric wall. Such a contraction can lead to mucosal compression at the site of the greatest mechanical stress, at the crests of mucosal folds leading to necrosis and ulceration [22,24]. In conclusion, the ligand and it Zinc complex could possibly protect the gastric mucosa against ethanol-induced injury. This was ascertained by the reduction of ulcer areas in the gastric wall and edema as well as due to the inhibition of leucocytes infiltration of submucosal layers.

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