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Anti-ulcer activity of *Centella asiatica* leaf extract against ethanol-induced gastric mucosal injury in rats

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The present study was performed to evaluate the anti-ulcerogenic activity of ethanol extract of *Centella asiatica* against ethanol-induced gastric mucosal injury in rats. Five groups of adult Sprague Dawley rats were orally pre-treated respectively with carboxymethyl cellulose (CMC) solution (ulcer control group), Omeprazole 20 mg/kg (reference group), and 100, 200 and 400 mg/kg *C. asiatica* leaf extract in CMC solution (experimental groups), one hour before oral administration of absolute ethanol to generate gastric mucosal injury. Rats were sacrificed and the ulcer areas of the gastric walls were determined. Grossly, the ulcer control group exhibited severe mucosal injury, whereas pre-treatment with *C. asiatica* leaf extract exhibited significant protection of gastric mucosal injury. Histological studies revealed that ulcer control group exhibited severe damage of gastric mucosa, along with edema and leucocytes infiltration of submucosal layer compared to rats pre-treated with *C. asiatica* leaf extract which showed gastric mucosal protection, reduction or absence of edema and leucocytes infiltration of submucosal layer. Acute toxicity study did not manifest any toxicological signs in rats. The present finding suggests that *C. asiatica* leaf extract promotes ulcer protection as ascertained grossly and histologically compared to the ulcer control group.

Key words: *Centella asiatica*, cytoprotection, gastric ulcer.

INTRODUCTION

*Centella asiatica* (Linn) is an ethno medical plant used in different countries by diverse ancient cultures and tribal groups. It is one of the local herbs that is claimed to possess various physiological effects and it occupies an important place in the indigenous system of medicine as a tonic in skin diseases and leprosy (Chopra et al., 1956). Different uses are claimed for the plant, the more common being its use for wound healing (Hong et al., 2005; Shetty et al., 2006), memory improvement, treatment of mental fatigue, bronchitis, asthma, dysentery, kidney trouble, urethritis, allergy, leucorrhea and toxic fever (Kan, 1986) and it is also used as a constituent of brain tonics for the mentally retarded (Kartnig et al., 1988). In addition, it has been shown to promote fibroblast proliferation and collagen synthesis (Maquart et al., 1990) and to have anti-ulcer activity (Cheng et al., 2004), antioxidant activity (Zainol et al., 2003), anti-cancer activity (Park et al., 2005), anti-bacterial activity (Zaidan et al., 2005) and anti-inflammatory activity (Guo et al., 2004). It is also commonly used as porridge for feeding pre-school children in combating nutritional deficiencies (Cox et al., 1993). Thus far, there is no data available on gastroprotective activity of *C. asiatica* leaf extracts.

In this study, Omeprazole was used as the reference anti-ulcer drug. It is a proton pump inhibitor which has been widely used as acid inhibitor agent for the treatment of disorders related to gastric acid secretion for about 15 years (Li et al., 2004). The present study was undertaken to evaluate anti-ulcerogenic properties of methanol extract of *C. asiatica* leaf in rats.

MATERIALS AND METHODS

Drugs

Omeprazole was obtained from the pharmacy of University Malaya.
Medical Centre (UMMC). The drug was dissolved in carboxymethyl cellulose (CMC) and administered orally to the rats in concentrations of 20 mg/kg body weight (5 ml/kg) (Pedernera et al., 2006). Dried leaves of *C. asiatica* leaf were obtained from Ethno Resources Sdn Bhd, Selangor Malaysia and identified by comparison with the Voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur. The leaves were then finely powdered using electrical blender. The fine powder (250 g) was soaked in 500 ml ethanol (95%) in conical flask for 6 days. After 6 days the mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No. 1) and distilled under reduced pressure in an Eyela rotary evaporator (Sigma-Aldrich, USA). The dry extract was then dissolved in carboxymethyl cellulose (CMC, 0.25% w/v) and administered orally to rats in concentrations of 100, 200 and 400 mg/kg body weight (5 ml/kg body weight) (Pasquale et al., 1995).

Acute toxicity test LD<sub>50</sub>

Adult male and female *Sprague Dawley* rats (6 - 8 weeks old) were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur (Ethics No. PM 07/05/2008 MAA (a) (R). The rats weighed between 150 - 180 g. The animals were given standard rat pellets and tap water and *libitum*. The acute toxic study was used to determine a safe dose for the rhizome extract. Thirty six rats (18 males and 18 females) were assigned equally each into 3 groups labeled as vehicle (CMC, 0.25% w/v, 5 ml/kg); 2 and 5 g/kg of *C. asiatica* leaf extract preparation, respectively. The animals were fasted overnight (food but not water) prior to dosing. Food was withheld for a further 3 to 4 h after dosing. The animals were observed for 30 min and 2, 4, 8, 24 and 48 h after the administration for the onset of clinical or toxicological symptoms. Mortality, if any was observed over a period of 2 weeks. The acute toxicity LD<sub>50</sub> was calculated as the geometric mean of the dose that resulted in 100% lethality and that which caused no lethality at all. The animals were sacrificed on the 15th day. Hematological and serum biochemical parameters were determined following standard methods (Bergmeyer, 1980; Tietz et al., 1983). The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Malaysia. 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.

Gross gastric lesions evaluation

Ulcers found in the gastric mucosa, appeared as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. Each specimen of gastric mucosa was thus examined for damage. The length (mm) and width (mm) of the ulcer on the gastric mucosa were measured by a planimeter (10 × 10 mm² = ulcer area) under dissecting microscope (1.8x). The area of each ulcer lesion was measured by counting the number of small squares, 2 × 2 mm, covering the length and width of each ulcer band. The sum of the areas of all lesions for each stomach was applied in the calculation of the ulcer area (UA) wherein the sum of small squares × 4 × 1.8 = UA mm² as described previously by Kauffman and Grossman (1978) with slight modification. The inhibition percentage (I %) was calculated by the following formula as described by Njar et al. (1995) with slight modification.

\[
I(\%) = \frac{[UA \text{control} - UA \text{treated}]}{UA \text{control}} \times 100\%.
\]

Histological evaluation of gastric lesions

Specimens of the gastric walls from 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 5 µ and stained with hematoxylin and eosin for histological evaluation.

Statistical analysis

All values were reported as mean ± S.E.M. The statistical significance of differences between groups was assessed using one-way ANOVA. A probability value of p < 0.05 was considered to be statistically significant.

RESULTS

Acute toxicity study

An Acute toxicity study was carried out in which the animals were treated with the rhizome extract at a dose of 2 and 5 g/kg of *C. asiatica* leaf extracts and were kept under observation for 14 days. All the animals remain alive and did not manifest any significant visible signs of toxicity at these doses. There were no abnormal signs, behavioral changes, body weight changes, or macroscopic finding at any time during the observation.
Table 1. Observed ulcer area and inhibition percentage in rats.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Pre-treatment (5 ml/kg dose)</th>
<th>Ulcer area (mm$^2$) (Mean ± S.E.M)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CMC (Ulcer control)</td>
<td>$850.00 ± 14.43^a$</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Omeprazole (20 mg/kg)</td>
<td>$145.00 ± 2.89^b$</td>
<td>82.94%</td>
</tr>
<tr>
<td>3</td>
<td>C. asiatica (100 mg/kg)</td>
<td>$81.00 ± 1.46^c$</td>
<td>90.47%</td>
</tr>
<tr>
<td>4</td>
<td>C. asiatica (200 mg/kg)</td>
<td>$55.67 ± 2.96^c$</td>
<td>93.45%</td>
</tr>
<tr>
<td>5</td>
<td>C. asiatica (400 mg/kg)</td>
<td>$7.20 ± 0.16^d$</td>
<td>99.15%</td>
</tr>
</tbody>
</table>

Figure 2. Gross appearance of the gastric mucosa in a rat 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.

period. The hematology and serum biochemistry parameters like triglycerides, creatinine, urea and hemoglobin, AST, ALT and ALP of the extract treated rats showed no significant change compared to the control normal rats. From these results it is concluded that the extract is quite safe even at these higher doses and had no acute toxicity and the oral lethal dose (LD$_{50}$) for the male and female rats was greater than 5 g/kg body weight.

Gross evaluation of gastric lesions

The anti-ulcer activity of C. asiatica leaf extract in ethanol-induced gastric lesion model is reported in Table 1. Results showed that rats pre-treated with C. asiatica leaf extracts before being given absolute alcohol had significantly reduced areas of gastric ulcer formation (Figures 2 and 3), compared to rats pre-treated with only carboxymethyl cellulose (ulcer control group) (Figures 1). Moreover, the extract significantly suppressed the formation of the ulcers and it was interesting to note the flattening of gastric mucosal folds in rats pretreated with C. asiatica leaf extract (Figures 3). It was also observed that protection of gastric mucosa was more prominent in rats pre-treated with 400 mg/kg extract (Table 1).

Beside, ethanol-induced mucosal damage was significantly and dose dependently reduced in the size and severity by pretreatment of the animals with C. asiatica leaf extract. The significant inhibition of gastric ulcer in pretreatment with extract was compared with Omeprazole which is a standard drug used for curing gastric ulcer.

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.

Histological evaluation of gastric lesions

Histological observation of ethanol induced gastric lesions in ulcer control group pre-treated with only CMC, showed comparatively extensive damage to the gastric mucosa, oedema and leucocytes infiltration of the submucosal layer (Figure 4). Rats that received pre-treatment with C. asiatica leaf rhizome extract had comparatively better protection of the gastric mucosa as seen by reduction in ulcer area, reduced or absence of submucosal oedema and leucocytes infiltration (Figures 5 and 6). The C. asiatica leaf extract has been shown to exert the cytoprotective effects in a dose-dependent manner.
Figure 3. Gross appearance of the gastric mucosa in a rat pre-treated with 5 ml/kg of *C. asiatica* extract (400 mg/kg). No injuries to the gastric mucosa are seen, and showed flattening of gastric mucosa.

Figure 1. Gross appearance of the gastric mucosa in a rat pre-treated with 5 ml/kg of CMC (negative control). Severe injuries are seen in the gastric mucosa.

Figure 4. Histological section of gastric mucosa in a rat pre-treated with 5 ml/kg of CMC only. There is severe disruption to the surface epithelium, and edema of the submucosal layer with leucocytes infiltration (H&E stain, 10x).
DISCUSSION

Peptic ulcers are caused when the natural balances between the aggressive factors of acid, pepsin, defensive mechanisms of mucus, bicarbonate, mucosal turnover and blood supply (mucosal barrier) are disturbed (Piper and Stiel, 1986). Baron et al. (1980) suggested it is reported that acid and pepsin are relatively less important as causative agents and that a defect in the defensive mechanism of gastric mucosa is the first step toward ulcer formation (Marhuenda et al. 1993). Although in most cases the etiology of ulcer is unknown, it is generally accepted that it is the result of an imbalance between aggressive factors and maintenance of the mucosal integrity through the endogenous defense mechanism (Piper and Stiel, 1986). It is known that gastric lesions produced by ethanol administration appeared as multiple-hemorrhagic red bands of different sizes along the glandular stomach. Ethanol is commonly used for inducing ulcers in experimental rats and leads to intense gastric mucosal damage. Studies suggest that the ethanol damage to the gastrointestinal mucosa starts with microvascular injury, namely disruption of the vascular endothelium resulting in increased vascular permeability, edema formation and epithelial lifting (Szabo et al., 1995). Ethanol produces necrotic lesions in the gastric mucosa by its direct toxic effect, reducing the secretion of bicarbonates and production of mucus (Marhuenda et al., 1993). Exposure to ethanol increases the extension of cellular damage in a dose-dependent way (Mutoh et al., 1990).

Oxidative stress plays an important role in the pathogenesis of various diseases including gastric ulcer, with antioxidants being reported to play a significant role in the protection of gastric mucosa against various necrotic agents (Trivedi and Rawal, 2001). Antioxidants could help to protect cells from damage caused by oxidative stress while enhancing the body’s defense.
systems against degenerative diseases. Administration of antioxidants inhibits ethanol-induced gastric injury in rat (Ligumsky et al., 1995). C. asiatica leaf extracts possess a broad spectrum of biological activities and the plant extract have been shown to contain a relatively large quantity of antioxidant compounds (Zaunol et al., 2003), and it is speculated that the gastroprotective effect exerted by C. asiatica extract could be attributed to its antioxidant property. In addition, phenolic compound are the major contributors to the antioxidant activities of C. asiatica and it could be conceivable that the anti-ulcer activity of C. asiatica extracts could be linked to the phenolic compound.

Results of the present study also revealed protection of gastric mucosa and inhibition of leucocytes infiltration of gastric wall in rats pretreated with C. asiatica extract. Similarly, Kobayashi et al. (2001) it is reported that teprenone exerts a protective effect against mucosal lesions through inhibition of neutrophils infiltration in the ulcerated gastric tissue and Shimizu et al. (2000) demonstrated that the reduction of neutrophils infiltration into ulcerated gastric tissue promotes the healing of gastric ulcers in rats. Fujita et al. (1998) observed it is also found that an increase in neutrophils infiltration into ulcerated gastric tissue delayed the healing of gastric ulcers in rats. Absolute alcohol would extensively damage the gastric mucosa leading to increased neutrophils infiltration into the gastric mucosa. Oxygen free radicals derived from infiltrated neutrophils in ulcerated gastric tissues have an inhibitory effect on gastric ulcer healing in rats (Suzuki et al., 1998). Neutrophils mediate lipid peroxidation through the production of superoxide anions (Zimmerman et al., 1997). Neutrophils are a major source of inflammatory mediators and can release potent reactive oxygen species such as superoxide, hydrogen peroxide and myeloperoxidase derived oxidants. These reactive oxygen species are highly cytotoxic and can induce tissue damage (Cheng and Koo, 2000). Suppression of neutrophil infiltration during inflammation was found to enhance gastric ulcer healing (Tsukimi et al., 1996). C. asiatica extract has been shown to contain anti-inflammatory activity (Guo et al., 2004) and it is speculated that the gastroprotective effect exerted by C. asiatica extract could be attributed to its anti-inflammatory activity. This anti-inflammatory activity could also be a key factor in the prevention of gastric ulcer as reported by Swarnakar et al. (2005).

In the present study, we also observed flattening of the mucosal folds which suggests that the gastroprotective effect of C. asiatica extract might due to a decrease in gastric motility. It is reported that the changes in the gastric motility may play a role in the development and prevention of experimental gastric lesions (Garrick et al., 1986; Takeuchi et al., 1987). Relaxation of circular muscles may protect the gastric mucosa through flattening of the folds. This will increase the mucosal area exposed to necrotizing agents and reduce the volume of Nobuhara, 1985). Ethanol produces a marked the gastric irritants on rugal crest (Takeuchi and contraction of the circular muscles of rat fundic strip. 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 (Mersereau and Hinchey, 1982). The acute toxicity profile of C. asiatica extract could be considered favorable judging from the absence of adverse clinical manifestations in experimental animals after 14 days of observation. It is concluded that the extract has no acute toxicity and that the oral lethal dose for male and female rats is in excess of 5 g/kg. In conclusion, C. asiatica extracts could significantly protect the gastric mucosa against ethanol-induced injury. Such protection was shown to be dose dependent as ascertained by the reduction of ulcer areas in the gastric wall as well as the reduction or inhibition of edema and leucocytes infiltration of submucosal layers and protection was most prominent at a dose of 400 mg/kg leaf extract. Further studies are required to determine the active ingredients responsible for the mechanism of anti-ulcer of C. asiatica extracts.

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