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Full Length Research Paper

Cytoprotective effect of Benzyl *N'*-(indol-3-ylmethylidene)-hydrazinecarbodithioate against ethanol-induced gastric mucosal injury in rats

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Benzyl N'-(indol-3-ylmethylidene)-hydrazinecarbodithioate (BIHC) and its metal complexes have been reported to have medicinal uses (Singh and Varshney, 2006). The present study was performed to evaluate, the acute toxicity and anti-ulcer activity of this BIHC against ethanol-induced gastric ulcer. Experimental animal groups were orally pre-treated with different doses of BIHC (50, 100, 200 and 400 mg/kg) in 10% Tween 20 solution (vehicle). Normal and ulcer control groups were pre-treated with vehicle. The reference group was orally pretreated with 20 mg/kg omeprazole. After one hour, all groups received absolute ethanol to generate gastric mucosal injury except the normal control group which was administered the vehicle solution. After an additional hour, all rats were sacrificed and the ulcer areas of the gastric walls determined. Grossly, the ulcer control group exhibited severe mucosal injury, whereas pre-treatment with either derivative or omeprazole resulted in significant protection of gastric mucosal injury. Flattening of gastric mucosal folds was also observed in rats pretreated with BIHC. Histological studies of the gastric wall of ulcer control group revealed severe damage of gastric mucosa, along with edema and leucocytes infiltration of the submucosal layer compared to rats pre-treated with either BIHC or omeprazole where there were marked gastric protection along with reduction or absence of edema and leucocytes infiltration of the submucosal layer. Acute toxicity study with a higher dose of derivative (5 g/kg) did not manifest any toxicological signs in rats. In conclusions, the present finding suggests that Benzyl N'-(indol-3-ylmethylidene)-hydrazinecarbodithioate derivative promotes ulcer protection as ascertained by the comparative decreases in ulcer areas, reduction of edema and leucocytes infiltration of the submucosal layer.

Key words: Indole, S-benzyldithiocarbazone, histology, omeprazole, gastric ulcer.

INTRODUCTION

Indole derivatives constitute an important class of therapeutical agent in medicinal chemistry including antidepressive (Krishna et al., 1982), antiallergic (Unangst et al., 1989), antimicrobial (Canoira et al., 1989) and antioxidant activities (Kaneko et al., 2000; Liu and Ng, 2000). On the other hand, antimicrobial (Tarafder et al., 2002), anticancer (Ali et al., 2002), antibacterial and antifungal (Singh and Varshney, 2006) properties have been reported to be associated with the Schiff bases derived from S-alkyl- or S-aryldithiocarbazate. However, there are no data available regarding anti-ulcerogenic activity of this type of Schiff bases in rats. The present study was undertaken to evaluate the antiulcerogenic property of an indolic Schiff base of S-benzyldithiocarbazate (BIHC) against ethanol-induced gastric mucosal injury in experimental rats.

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Abbreviation: BIHC, Benzyl N'-(indol-3-ylmethylidene)-hydrazinecarbodithioate.

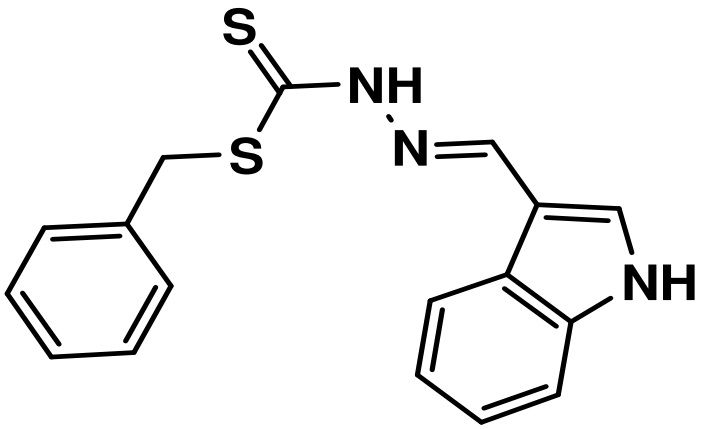

Figure 1. Benzyl *N'*-(indol-3-ylmethylidene)-hydrazinecarbodithioate.

MATERIALS AND METHODS

Omeprazole is a proton pump inhibitor which has been widely used as acid inhibitor agents for the treatment of disorders related to gastric acid secretion for the last 15 years (Li et al., 2004). Omeprazole is substituted benzimidazoles and it inhibits acid secretion by acting on the hydrogen-potassium exchanger (H+: K+-ATPase) for the apical plasma membrane of the gastric mucosa (Satoh et al., 1989). Omeprazole is highly selective for the proton pump and undergo 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 (Nagaya et al., 1991). In this study, omeprazole was used as the reference anti-ulcer drug and was obtained from the University Malaya Medical Centre (UMMC) Pharmacy. The drug was dissolved in 10% Tween 20 solution and administered orally to the rats in concentrations of 20 mg/kg body weight (5 ml/kg) (Pedernera et al., 2006).

Preparation of S-benzyldithiocarbazate

This compound was synthesized as reported previously (Tarafder et al., 1981). A mixture of hydrazine hydrate (10 g, 0.2 mol) and potassium hydroxide (11.4 g, 0.2 mol) in 90% ethanol (70 ml) was cooled in an ice bath. Carbon disulphide (15.2 g, 0.2 mol) was then added drop-wise with vigorous stirring. The temperature of the reaction mixture was not allowed to rise above 5°C during the period of addition of carbon disulfide. To the mixture, 40% ethanol (60 ml) was added and the solution was cooled in ice. Benzyl chloride (25.3 g, 0.2 mol) was then added slowly with vigorous stirring. The white product was separated by filtration, washed with water and dried in air. The crude product was recrystallized from absolute ethanol and the yield was 23 g (58%).

Preparation of benzyl N'-(indol-3-ylmethylidene)-hydrazinecarbodithioate

The Schiff base was synthesized as reported previously (Khaledi et al., 2008). Indole-3-carbaldehyde (4.35 g, 0.03 mol) and S-benzyl dithiocarbazate (5.94 g, 0.03 mol) were heated in methanol (300 ml) for 3 h. The solution was set aside for few hours where upon a yellow precipitate was formed. The precipitate was filtered off, washed with cold ethanol and dried over silica gel and the yield was 8.3 g (85%) (Figure 1).

Experimental animals

Adult male albino Wistar rats were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. All the animals used for study has an ethical clearance from the animal user's Committee of the Faculty of Medicine, University Malaya (Ethics No. PM 28/9/2008 MAA (R)). The rats weighed between 200 – 220 g. They were fasted for 48 h before the experiment (Garg et al., 1993), but were allowed free access drinking water until 2 h before the experiment. During the fasting period, the rats were placed individually in separate cages with wide-mesh wire bottoms. On the day of the experiment, the rats were randomly divided into 7 groups of 6 rats each. 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 (Petter and Pearson, 1971).

Acute toxicity studies

The acute toxic study was used to determine a safe dose for the BIHC. Thirty six albino Wistar rats (18 males and 18 females) were assigned equally into 3 groups labeled as vehicle (10% Tween 20); 2 and 5 g/kg of BIHC in vehicle 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, 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 animals were sacrificed on the 15th day. Hematological, serum biochemical and histological (liver and kidney) parameters were determined following standard methods (Bergmeyer, 1980; Tietz et al., 1983).

Treatment

Gastric ulcer was induced by orogastric intubation of absolute ethanol (5 ml/kg) according to the method described by De Pasquale et al. (1995) with slight modification in adult male Wistar rats. Normal and ulcer control groups were orally administered with vehicle (10% Tween 20) solution. Experimental groups were orally administered with. 50, 100, 200 and 400 mg/kg of BIHC in vehicle solution (5 ml/kg), respectively. The reference group received oral

Table 1. Observed ulcer area and inhibition percentage of Benzyl *N'*-(indol-3-ylmethylidene)-hydrazinecarbodithioate in rats.

Group	Pretreatment 5 ml/Kg	Ulcer (mm^2) X ± SEM	Inhibition %
1	10% Tween 20	922.00 ± 7.63	0
2	Omeprazol	215.0000 ± 4.54	76.68
3	50 mg/Kg	54.00 ± 3.08	94.14
4	100 mg/Kg	38.40 ± 2.400	95.84
5	200 mg/Kg	22.80 ± 2.89	97.53
6	400 mg/Kg	12.00 ± 2.400	98.70

All values are expressed as mean ± standard error mean. Means with different superscripts are significantly different. The mean difference is significant at the $p<0.05$ level. [Values are mean ± SEM of 6 animals].

doses of 20 mg/kg omeprazole in vehicle solution (5 ml/kg) as positive controls one hour after this pre-treatment; all groups of rats were gavaged with absolute ethanol (5 ml/kg) in order to induce gastric ulcers except normal control group which was only administered vehicle solution (5 ml/kg). The rats were euthanized by cervical dislocation 60 min later (Paiva et al., 1998) under an over dose of diethyl ether anesthesia and their stomachs were immediately excised.

Gross gastric lesions evaluation

Any ulcers would have be found in the gastric mucosa, appearing as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. Each 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 mm^2 = ulcer area) under a dissecting microscope (× 1.8). 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 mm^2 as described by Kauffman and Grossman (1978) with slight modification. The inhibition percentage (1%) was calculated by the following formula as described by Njar et al. (1995) with slight modification.

(1%) = [(UA control – UA treated) / UAcontrol] × 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 means + S.E.M. The statistical significance of differences between groups was assessed using one-way ANOVA. A value of $p < 0.05$ was considered significant.

RESULTS

Acute toxicity study in which the animals were treated with a dose of 2 and 5 g/kg of BIHC did not manifest any significant abnormal signs and behavioral changes. There was no mortality in these doses at the end of 14 days of observation. The serum biochemistry parameters (liver function tests) of the BIHC treated rats showed no significant change compared to the control normal rats. From these results it is concluded that this compound has no acute toxicity and the oral lethal dose for the male and female rats is in excess of 5 g/kg of BIHC.

Gross evaluation of gastric lesions

Rats pre-treated with either omeprazole or various concentrations of BIHC before being given absolute alcohol had significantly reduced areas of gastric ulcer formation compared to rats pre-treated with vehicle, 10% Tween 20 solution (ulcer group) (Table 1, Figures 2, 3 and 4). Moreover, the BIHC significantly suppressed the formation of the ulcers and it was interesting to note the flattening of gastric mucosal folds in rats pretreated with this BIHC. It was also observed that protection of gastric mucosa was more prominent in rats pre-treated with 400 mg/kg BIHC (Table 1). In addition, ethanol-induced mucosal damage was significantly and dose-dependently reduced in size and severity by pretreating the animals with BIHC.(The significant inhibition of gastric ulcer in pretreatment with this BIHC was compared with omeprazole which is a standard drug used for curing gastric ulcer).

Histological evaluation of gastric lesions

Microscopic observations of ethanol-induced gastric lesions in ulcer control rats (pre-treated with vehicle, 10% Tween 20), showed markedly extensive damage to the gastric mucosa and edema and leucocytes infiltration of the submucosal layer (Figure 5). Rats that were pre-treated with either BIHC or omeprazole each had comparatively better protection of the gastric mucosa as seen by the reduction in ulcer area, reduced submucosal edema and absence of leucocytes infiltration (Figure 6

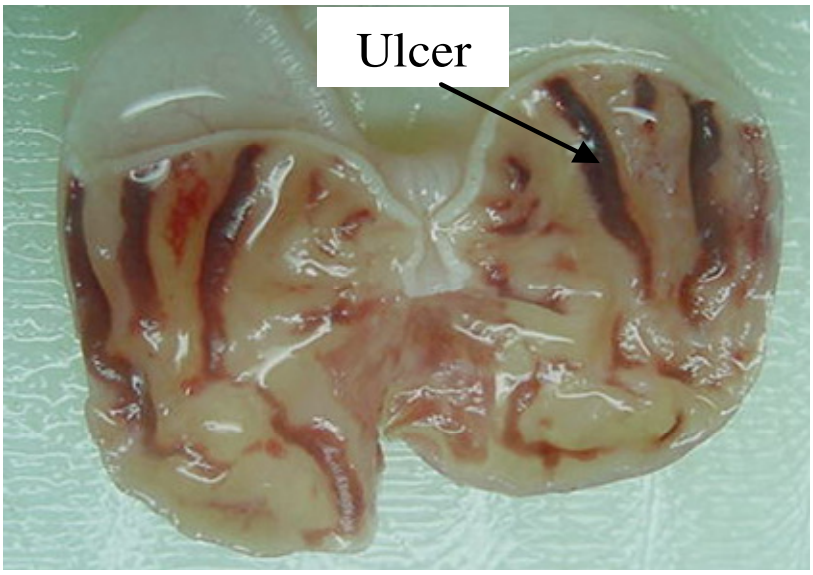

Figure 2. Gross appearance of the gastric mucosa in a rat pre-treated with 10% Tween 20 solution (ulcer control). Severe injuries are seen in the gastric mucosa.

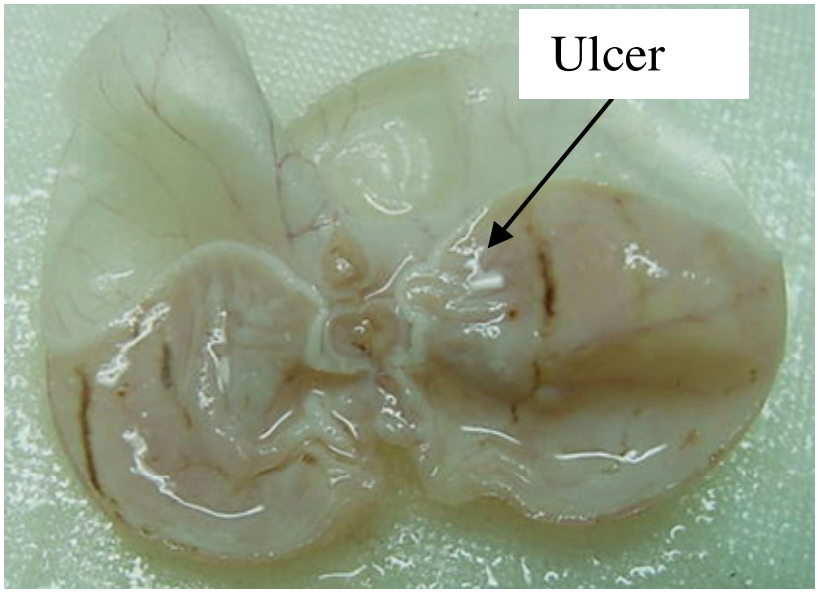

Figure 3. Gross appearance of the gastric mucosa in a rat pre-treated with 20 mg/kg of omeprazole. Injuries to the gastric mucosa are milder compared to the injuries seen in the negative control rat.

and 7). The BIHC has been shown to exert the cytoprotective effects in a dose-dependent manner.

DISCUSSION

This study investigated the effects of Benzyl N'-(indol-3-ylmethylidene)-hydrazinecarbodithioate on gastric ulcer compared to omeprazole, a drug whose ulcer healing effects have been extensively studied. The present compound (BIHC) is a hybrid molecule, obtained by condensation of two pharmacophores; Indole carbaldehyde and S-benzyldithiocarbazate. Schiff bases derived by condensation of heterocyclic aldehydes with S-benzyldithiocarbazate have been shown to exhibit significant biological activities. It has been proposed that the biological activity of these Schiff bases may come from their interaction with potential donors of biological

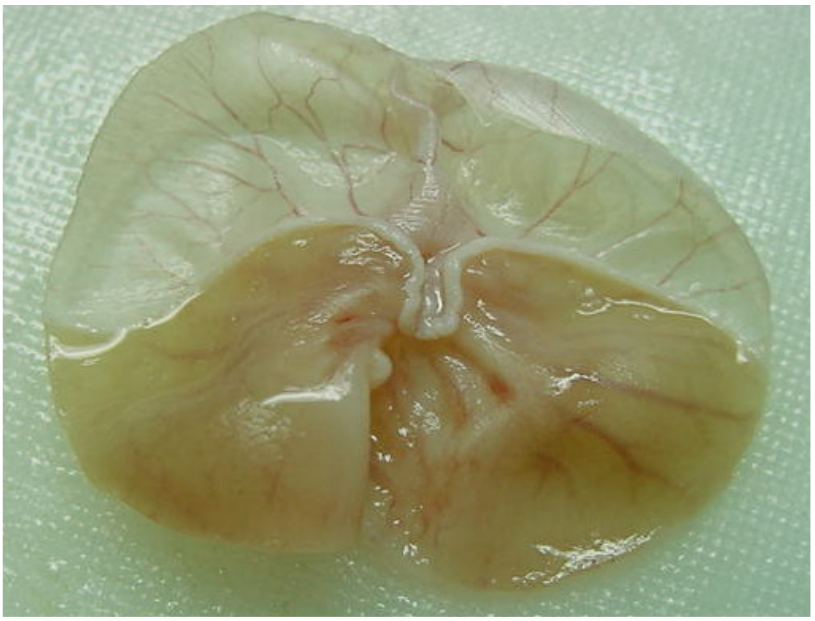

Figure 4. Gross appearance of the gastric mucosa in a rat pre-treated with 400 mg/kg of BIHC. No injuries to the gastric mucosa are seen, and showed flattening of gastric mucosa.

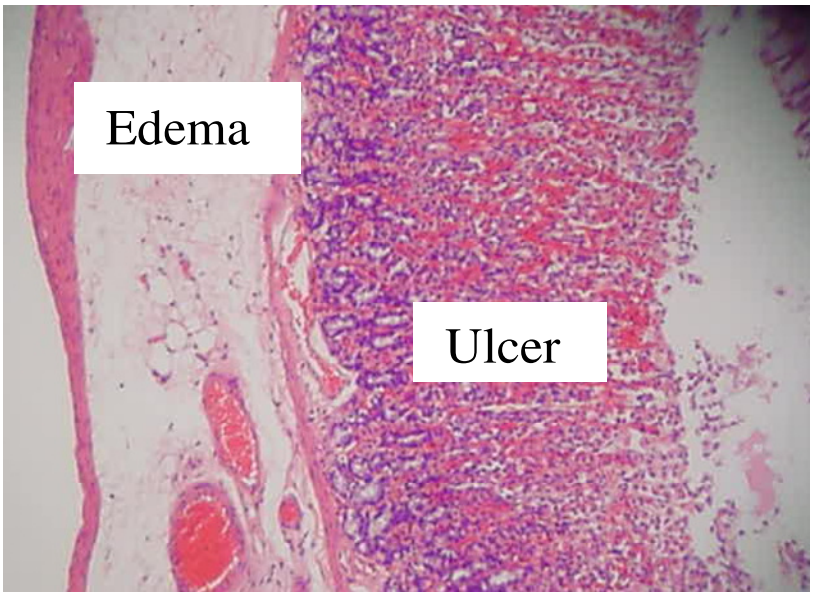

Figure 5. Histological section of gastric mucosa in a rat pre-treated with 10% Tween 20 solution (ulcer control). There is severe disruption to the surface epithelium, and edema of the submucosal layer with leucocytes infiltration (HandE stain, 10×).

heterocycles *in vivo*. In many cases, the pharmacological activity has been found to be highly dependent on the identity of the donor sequence of the ligands, as different ligands show widely different biological activities although they may vary only slightly in their molecular structure. Indole derivatives have also been shown to exhibit a wide range of biological activities including antioxidant and antiulcer activities (Bell et al., 1977).

BIHC was found to have a protective effect on the gastric mucosa similar to that of omeprazole. Omeprazole and BIHC were found to have protective effect compared to ulcer control group (10% Tween 20).

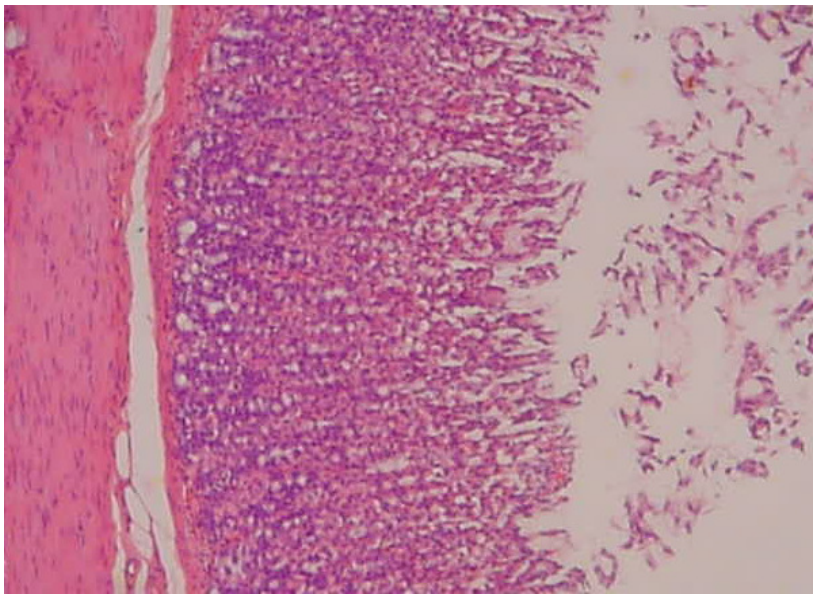

Figure 6. Histological section of gastric mucosa in a rat pre-treated with 20 mg/kg of omeprazole. There is mild disruption to the surface epithelium with no edema and no leucocytes infiltration of the submucosal layer (HandE stain 10×).

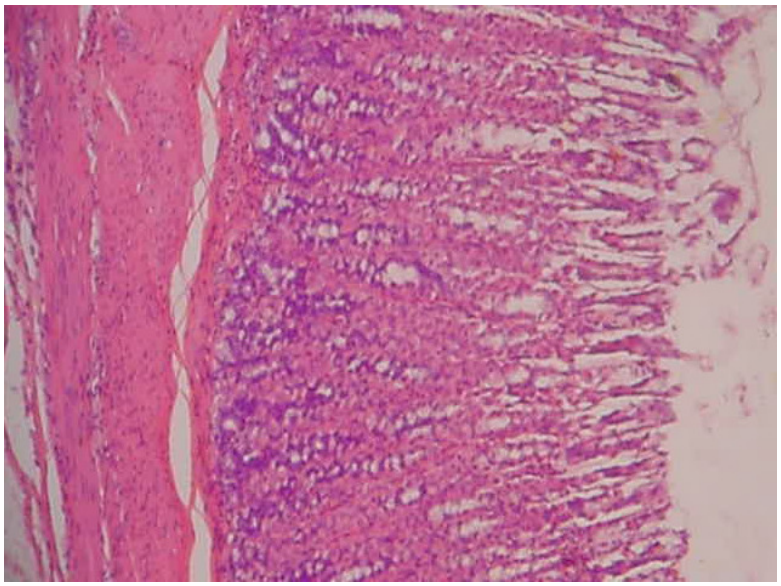

Figure 7. Histological section of gastric mucosa in a rat pre-treated with 400 mg/kg of BIHC. There is no disruption to the surface epithelium with absence of edema and leucocytes infiltration of the submucosal layer (HandE stain 10×).

This suggests that BIHC does indeed have a potential anti-ulcer effect.

BIHC has been shown to contain anti-cancer (Ali et al., 2002) and it is speculated that the gastro-protective effect exerted by BIHC could be attributed to its anti-cancer activity. This anti-cancer activity could also be a key factor in the prevention of gastric ulcer as reported by Das and Livingstone (1978).

Microbial infection of wounds delays healing (Bowler et al., 2001) and causes a more pronounced acute inflammatory reaction (Whaley and Burt, 1996) which can lead to further tissue injury and damage. It is also an

important evidence that BIHC has demonstrated an antimicrobial activity (Tarafder et al., 2002). Thus, the antimicrobial activity of BIHC may partly contribute to the wound healing effect by eliminating infection thus allowing the natural tissue repair processes to start. It also suggests that the BIHC may also play a useful role in accelerating the healing of old wounds by eradicating already established infection. The antimicrobial activity of honey and the essential oil of *Melaleuca alternifolia* is believed to underlie their usefulness as alternative therapy in wound healing (Carson et al., 1998; Molan, 1999).

The main cause of gastric ulcer is destruction of gastric mucosal barrier consisting of the surface epithelium and mucosal coat. This destruction may be due to increase in gastric acid secretion, decrease in mucus production or decrease in mucosal blood flow (Abd El-Dayem and El-Againy, 1993). Ethanol is a simple method used for inducing gastric ulcer in experimental rats and lead to severe gastric mucosal injury. Ethanol shows its harmful effects either through direct generation of reactive metabolites, including free radical species that react with most of the cell components, changing their structures and functions, or promote enhanced oxidative damage (Kato et al., 1990; Nordmann, 1994). Ethanol damage to the gastrointestinal mucosa with microvascular injury, disruption of the vascular endothelium resulting in increased vascular permeability, edema formation and epithelial lifting (Szabo et al., 1995). It 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). Ethanol is metabolized in the body releasing superoxide anion and hydroperoxyl free radicals which are involved in the mechanism of acute and chronic ulceration in the gastric mucosa. Salim (1990) observed that administration of ethanol caused disturbances in gastric secretion, damage to the gastric mucosa, alterations in permeability, gastric mucus depletion and free-radical production.

Oxidative stress plays a major role in the pathogenesis of various diseases including gastric ulcer. Indolic compounds have shown to possess antioxidant activity (Kaneko et al., 2000; Liu and Ng, 2000) which may mediate the protection of gastric mucosa. Antioxidants are important inhibitors of lipid peroxidation, not only for food protection but also as a defense mechanism of living cells against oxidative damage (Vimala and Adenan, 1999). Antioxidants have been reported to play a significant role in preventing gastric ulcers. It appears that antioxidant may be an important contributory factor in the protection of gastric mucosa (Shukla et al., 1999). Studies have shown that antioxidant significantly strengthen gastric walls and protect tissues from oxidative damage (Martin, 1996).

The result of the present study also revealed protection of gastric mucosa and inhibition of leucocytes infiltration of gastric wall in rats pretreated with BIHC. Similarly, Kobayashi et al. (2001) reported that teprenone exerts a protective effect against mucosal lesions through inhibition of neutrophil infiltration in the ulcerated gastric tissue and Shimizu et al. (2000) demonstrated that the reduction of neutrophil infiltration into ulcerated gastric tissue promotes the healing of gastric ulcers in rats. Cheng and Koo (2000) showed that oral administration of plant extract before ethanol administration significantly decreased neutrophil infiltration of gastric mucosa. Absolute alcohol would extensively damage the gastric mucosa leading to increased neutrophil infiltration into the gastric mucosa. Oxygen free radicals derived from infiltrated neutrophils in ulcerated gastric tissues have inhibitory effect on gastric ulcers 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). Furthermore, neutrophil accumulation in gastric mucosa has been shown to induce microcirculatory abnormalities (Bou-Abboud et al., 1988). Suppression of neutrophil infiltration during inflammation was found to enhance gastric ulcer healing (Tsukimi et al., 1996).

In the current study, we observed flattening of the mucosal folds which suggests that gastro-protective effect of BIHC might be 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 the gastric irritants on regal crest (Takeuchi and Nobuhara, 1985). Ethanol produces a marked contraction of the circular muscles of rat fundic strip and 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 BIHC could be considered favorable judging for the absence of adverse clinical manifestations in experimental animals after two week of observation. It is concluded that acute toxicity study with a higher dose of BIHC (5 g/kg) did not manifest any toxicological signs in rats and that the oral lethal dose for male and female albinos Wistar rats is in excess of 5 g/kg. Based on acute toxicity test, BIHC was found safe.

In conclusion, BIHC could significantly protect the gastric mucosa against ethanol-induced injury. Such protection was shown to be dose dependent as ascertained by the reduction or inhibition 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 BIHC.

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