Involvement of Inflammatory Mediators in the Gastroprotective Action of *Phaleria macrocarpa* against Ethanol-Induced Gastric Ulcer

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Abstract: This research aimed to determine the potential protective effects of *Phaleria macrocarpa* fruit extract on rat gastric mucosal injury induced by ethanol and to clarify the roles of prostaglandin E2 (PGE2), transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α). Main methods: Seven groups of rats were orally pretreated with Tween20 as vehicle control group; Tween20 as ulcer group; 20 mg/kg of omeprazole as reference drug group; and 100, 200, 500 and 1000 mg/kg of extract as experimental groups. An hour later, ulcer was induced by oral administration of 95% ethanol, except in the vehicle control group. Results showed significant ulcer protective effects through reduction of ulcer area and increase of ulcer inhibition by significant elevation of gastric pH and increase of mucus production. In addition, significant increases in levels of inflammatory mediators PGE2 and TGF-β1 and decrease in TNF-α were observed in the groups pretreated with *P. macrocarpa* compared with ulcer control group. In conclusion, our results suggested that *P. macrocarpa* pretreatment has protective effects against ethanol-induced gastric ulcers in rats by significantly stimulating inflammatory mediators PGE2, TGF-β1 and reducing TNF-α, which effect in the increase production of mucus and stomach pH to provide a protective environment against the offensive factors.

Key words: Gastroprotective • PGE2 • TGF-β1 • *Tinospora crispa* • TNF-α

INTRODUCTION

Gastric ulcer is one of the most widespread diseases in the world. Gastric ulcers are localized breaches of the gastric mucosa, with tissue destruction at least to the depth of the muscularis mucosa [1]. Gastric ulcer accounts for the highest percentage of affliction among diseases worldwide due to rising levels of stress experienced by humans, alcohol consumption, long-term anti-inflammatory drug intake (such as aspirin and indomethacin) that lead to gastric ulcer and nutritional deficiencies [2]. The disruptions between endogenous offensive factors (such as pepsin, hydrochloric acid, refluxed bile, reactive oxygen species, and leukotrienes) and endogenous defensive factors (such as bicarbonate mucus barrier, mucosal blood flow, prostaglandins, cell proliferation and enzymatic and non-enzymatic antioxidants) are important physio-pathological characteristics of gastric ulcer [3].

Numerous medications can be used to treat gastric ulcer. H2-antagonists, omeprazole, ranitidine and famotidine function as medications of gastric ulcer by inhibiting the secretion of gastric acid. However, despite 80% to 100% rate of healing through 4 to 8 weeks when proton pump inhibitors and H2-antagonists are used, the ulcer returns at a rate of 40% to 80% when therapy is stopped [4]. In addition, most of these medications have side effects with long-term use. Therefore, new therapeutic anti-ulcer agents that are more efficient and safe are necessary. In this framework, studies on the use of medicinal plants in preventing and treating diverse pathologies are underway [5]. Natural products are important in pharmaceutical manufacturing; they are also possible sources of bioactive molecules. Numerous plants and their derivatives, such as flavonoids, alkaloids and phenolic acids, have large potential for treating various diseases, including gastric ulcer. A large number of medicinal plants with gastroprotective potential have been reported in the literature [6-9].

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Phaleria macrocarpa is a traditional plant used in the treatment of inflammation, cancer, diabetes and hypercholesterolemia. P. macrocarpa fruits extract possesses anti-hyperglycemic properties; it stimulates insulin release through modulation of B cell [10]. The isolated compound from P. macrocarpa is used as an anticancer medicine [11, 12]. P. macrocarpa ethanol extract has anti-inflammatory and antioxidant activities [13, 14]. However, no data has been reported on the anti-ulcer activities of this plant. Therefore, the current study was conducted to assess the gastroprotective effects of ethanol extracts of P. macrocarpa against ethanol-induced gastric ulcers in rats and involvement of inflammatory mediators (PGE2, TGF-β1 and TNF-α) in the gastroprotective effect.

MATERIALS AND METHODS

Materials: The ripe dried seedless P. macrocarpa fruits were harvested at the Selangor housing area in the Herbarium of University Putra in Malaysia, as voucher specimens (SK1929/11). TWEEN 20 (Sigma-Aldrich, Germany), omeprazole (TROGE Medical GmbH, Germany), ethanol (Fisher Scientific, UK), xylazine and ketamine (Ilium, Australia), Alcian blue (ACROS, USA), D(+) sucrose (Fisher Scientific, UK), sodium acetate and magnesium chloride (Sigma-Aldrich, Germany), ethyl ether (Anala R, England) and PBS (Dulbecco A, Oxoid, England) were also used.

Plant Extraction: Dried fruits of P. macrocarpa were crushed. The powder (100 g) was soaked in 900 ml of 95% ethanol in a conical flask for 3 days at room temperature (30±2°C). The suspension was shaken from time to time to enable the fruits powder to completely dissolve in the ethanol and to change color to dark brown. After 3 days, the mixture was filtered using a filter paper (Whitman, 185mm) and distilled under reduced pressure in a rotary evaporator (Buchi, Switzerland). The product was kept at-20°C until use [15].

Induction of Gastric Ulcer by Ethanol: Adult healthy SD rats weighing between 200 g to 250 g were obtained from Animal House Unit, Faculty of Medicine, University of Malaya, Malaysia. They are kept in wire bottomed cages at 25±3°C temperature, 50-60% humidity and a 12 h light-dark cycle for at least a week before the experiment. They were maintained at standard housing conditions and free access to standard diet and water ad libitum during the experiment. The experimental protocol is approved by Animal Ethics Committee; with an ethic No. (PM 1 November 2011/1111/MAR (R). Throughout the experiments, all criteria of taking care of animals prepared by the National Academy of Sciences and outlined in the “guide for the care and use of laboratory animals” are applied [16]. The animals were randomly divided into six groups (six rats/group). The water was removed 1 h before the experiment. For the first group (vehicle control group), the rats were administered vehicle Tween 20 (5% v/v) (5ml/kg). For the second group (reference drug group), the rats were pretreated with 20 mg/kg omeprazole solution (5ml/kg). For others groups, the rats were pretreated with 100, 200, 500 and 1000 mg/kg P. macrocarpa fruits extracts respectively. After 1 h, all rats received 5ml/kg 95% ethanol orally to induce gastric ulcer. After 1 h, the rats were sacrificed, blood was collected and the stomach was removed and opened over the greater curvature [17].

Measurement of Ulcer Index: The ulcers were located in the gastric mucosa and appeared as hemorrhagic extended bands of lesions parallel to the long axis of the stomach. The gastric mucosa ulcer was calculated by measuring the sum of all lesion areas for each stomach, which was used in the calculation of the ulcer area (UA) equal to the sum of small squares × 4 × 1.8 = UA mm². The ulcer inhibition percentage (UI %) was calculated by [18]

\[ (UI \%) = \left[ \frac{(UA \text{ control} - UA \text{ treated})}{UA \text{ control}} \right] \times 100\% \]

Measurement of Gastric Juice pH: After the stomach was opened, gastric juice was collected and measured for acidity by using a digital pH meter [19].

Determining Gastric Wall Mucus Content: The gastric mucus contents were determined according to assay procedures previously described [7]. Adult Sprague-Dawley rats weighing between 200 g to 250 g were fasted for 24 h. The animals were randomly divided into three groups (six rats/group). The water was removed 1 h before the experiment. For the first group (vehicle control group), the rats were given vehicle Tween 20 (5% v/v) (5ml/kg). For the second group (reference drug group), the rats were treated with 20 mg/kg omeprazole solution (5ml/kg). For the third group, the rats were treated with 250 mg/kg P. macrocarpa fruits extract. After 1 h treatment, all the rats received 5ml/kg of 95% ethanol orally to induce gastric ulcer. After 1 h, all the rats were sacrificed by euthanasia and their stomachs were removed. Each glandular part was weighed and submerged into 1% Alcian blue solution (0.16 M sucrose/0.05 M sodium...
acetae, pH 5.8). After 2 h of immersion, the excess stain was rinsed with 10 ml sucrose at 0.25 M for twoconsecutive washes: the first time for 15 min and the second for 45 min. Then, the stomachs were transmitted to the tubes containing 10ml magnesium chloride at 0.5 M for 30 min. After 30 min, 4 ml of the mixture was mixed with 4 ml of ethyl ether and then shaken for 2 min. The final emulsion was centrifuged for 10 min at 3000 rpm and then the supernatant was discarded. The absorbance was read at 598 nm. The quantity of Alcian blue extracted per gram of glandular tissue was calculated.

**Tissue Homogenate Sample Preparations for Assessment of PGE2:** To assess PGE2 (Cayman PGE2 assay ELISA kit; Cat#. 400141) in gastric tissue homogenate, the gastric tissue was weighed, minced and homogenized on ice in 5 ml cold PBS buffer using a Teflon homogenizer (Polytron, Heidolph RZR 1, Germany). After each tissue stomach piece was fully homogenized, the mixture was centrifuged at 10,000xg for 15 min at 4°C. Then, the supernatant was collected in a sterile tube and kept at 80°C until use. The assay was performed as per the detailed instructions of the manufacturer. The sensitivity limit of these assays was 15.6 pg for PGE2.

**Preparations of Blood Sample for Assessment of Serum TGF-β1 and TNF-α:** After the rat blood samples were collected in tubes, they were allowed to clot for 30 min at 25°C. Then, they were centrifuged at 2000xg for 15 min at 4°C using a refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany). The serum was collected and preserved at (-80°C) until use. TGF-β1 was measured by ELISA kit (Abnova, Cat#. KA0279, version 4) and rat TNF-α by ELISA kit (Thermo Scientific, Cat#. ER3TNFA). The assays were performed as per the detailed instructions of the manufacturer. The sensitivity limit of these assays was 7.8 pg/ml for TGF-β1 and <15 pg/ml for TNF-α.

**Statistical Analysis:** The values were presented as mean ± standard division. Statistical examination of data was through one-way analysis of variance (ANOVA) and post hoc LSD test (comparing the treated groups with the control). P ≤ 0.05 was considered statistically significant compared with the control.

**RESULT**

**Gastric Lesions:** The gastroprotective effect and the enhancement of the mechanism defense of gastric ulcer by P. macrocarpa fruits or omeprazole were investigated through the induction of gastric ulcer in rats by using ethanol as necrotizing agent. This scheme resulted in mucosal injury characterized by submucosal edema, increased secretory products of the cell and disturbances in microcirculation. Pretreatment with ethanol extract of P. macrocarpa fruits at doses of 100, 200, 500 and 1000 mg/kg or omeprazole at 20 mg/kg inhibited the formation of gastric ulcer lesions in the rats. The intensity of ulcer was detected by the statistically significant ulcer area in the pretreated groups (P. macrocarpa fruits or omeprazole) (P < 0.000), which was lower than that of the ulcer control group. The ulcer inhibition percentage result reflected that results of pretreatment with all doses of the P. macrocarpa fruits were statistically and significantly (P < 0.000) higher than those of the ulcer control group (Table 1).

**Gastric pH:** The gastric pH in the experimental groups pretreated with P. macrocarpa fruits or omeprazole increased more significantly compared with that in the ulcer control group (P ≤ 0.05; Table 1).

**Mucus Barrier of the Protective Stomach:** The results of the mucus barrier contents presented in Figure 1 show statistically significant (P < 0.000) higher mucus contents in the groups pretreated with P. macrocarpa extracts compared with those in the ulcer control group.

**Evaluation of PGE2:** In the groups pretreated with P. macrocarpa fruits and omeprazole, the result showed significant (P < 0.05) increase in the level of PGE2 in the groups pretreated with P. macrocarpa or omeprazole compared with the ulcer control group (Figure 2).

**Estimation of TGF-β1 and TNF-α:** The results showed that the level of TGF-β1 significantly increased (P < 0.03) in the groups pretreated with P. macrocarpa extracts at 200, 500 and 1000 mg/kg doses, as well as in

### Table 1: Ulcer area, ulcer inhibition percentage and stomach pH in the pretreated groups with P. macrocarpa (P.m) and omeprazole compared with ulcer control group

<table>
<thead>
<tr>
<th>Group</th>
<th>Ulcer area (mm²)</th>
<th>Inhibition%</th>
<th>Stomach pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulcer control</td>
<td>532.8±119.2</td>
<td>0</td>
<td>2.6±0.7</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>25.2±61.7</td>
<td>95.3</td>
<td>7.5±0.5</td>
</tr>
<tr>
<td>P.m 100mg/kg</td>
<td>67±37.8</td>
<td>87.4</td>
<td>6.1±2.1</td>
</tr>
<tr>
<td>P.m 200mg/kg</td>
<td>58.3±32.9</td>
<td>89.1</td>
<td>6.4±1.1</td>
</tr>
<tr>
<td>P.m 500mg/kg</td>
<td>40.8±59.3</td>
<td>92.3</td>
<td>6.7±0.6</td>
</tr>
<tr>
<td>P.m 1000mg/kg</td>
<td>23±46.1</td>
<td>95.7</td>
<td>7.0±1.1</td>
</tr>
</tbody>
</table>

Data was expressed as mean ± SD, *significant at P<0.000.
Fig. 1: Mucus content in the pretreated groups with *P. macrocarpa*(P.m) and omeprazole (reference drug) compared with ulcer control group. Data was expressed as mean ± SD, +significant at *P* =0.000.

Fig. 2: PGE2 level in the pretreated groups with *P. macrocarpa*(P.m) and omeprazole (reference drug) compared with ulcer control group. Data was expressed as mean ± SD, +significant at *P* =0.05.

Fig. 3: TGF-β1 level in the pretreated groups with *P. macrocarpa*(P.m) and omeprazole (reference drug) compared with ulcer control group. Data was expressed as mean ± SD, +significant at *P* <0.03.

Fig. 4: TNF-α level in the pretreated groups with *P. macrocarpa*(P.m) and omeprazole (reference drug) compared with ulcer control group. Data was expressed as mean ± SD, +significant at *P* <0.01.

the omeprazole group (Figure 3). By contrast, the level of TNF-α significantly decrease (*P* < 0.01) in all doses in the groups pretreated with *P. macrocarpa* or omeprazole group compared with the ulcer control group (Figure 4).

**DISCUSSION**

This study showed that the ethanol extract of *P. macrocarpa* has gastroprotective activity as manifested by its significant inhibition of the formation of ulcers induced by ethanol. The injury of ethanol-induced ulcers is dominant in the glandular part of the stomach. Administration of absolute ethanol orally in rats has deleterious effects on the stomach, affecting the gastric mucosa topically by disabling its barrier and causing microvascular changes a few minutes after its application. A strong and rapid vasoconstriction combined with vigorous and rapid arteriolar dilation induces damage in mucosal capillaries [20, 21]. A previous study reported that ethanol stimulates the formation of leukotriene C4(LTC4)[22], mast cell secretory products and reactive oxygen species, resulting in damage of rat gastric mucosa[23]. The necrotic lesion of the gastric mucosa caused by ethanol occurs through multiple factors. It causes disruption of the mucus–bicarbonate barrier upon reaching the mucosa, as well as cell rupture in the wall of blood vessels. These effects are possibly caused by biological actions, namely, formation of free radicals, lipid peroxidation, intracellular oxidative stress and permeability and depolarization of the mitochondrial membrane before cell death [24].

Gastric ulcer defined as the damage resulting from the disturbance in the gastric mucosal defense, which means that ulcer is caused by the imbalance between offensive
and protective factors of the gastric mucosa. Pepsin and gastric acid are aggressive factors whose proteolytic effect is buffered by mucosal glycoprotein, mucin secretion, prostaglandins and cell proliferation [25]. P. macrocarpa fruits extracts decrease ulcer area and increase ulcer inhibition percentage by increasing the production of mucin from mucous cells to form the first line of defense in protecting the mucosal epithelial layer from the ulcerogenic action of ethanol. Mucus is an important factor that protects the gastric mucosa. Mucus is a transparent gel composed of water and glycoproteins that coats the gastrointestinal mucosa and preserves the gastric mucosa against agents such as ethanol and HCl [26]. Moreover, the anti-secretory activity of the P. macrocarpa fruits extract may be important in gastric mucosa protection. The gastroprotective effect has been proven by histological examination. This protection results from the inhibition of gastric acid secretion and increased stomach pH by the extracts. The obtained data revealed that the stomach pH increased in the treatment groups compared with the ulcer control group. Thus, the mechanism of mucosa protection is due to the anti-secretory activity of P. macrocarpa fruits and the enhanced production of mucin.

The findings in this study also showed that pretreatment with P. macrocarpa fruits extract induced a remarkable decrease in the level of serums gastrin and pepsin and caused an increase in PGE2 level compared with the ulcer control group, indicating the mechanism of P. macrocarpa fruits in more than one anti-ulcer mechanism. Gastric secretion decreased after P. macrocarpa fruits extract pretreatment. The foregoing may be a negative feedback mechanism that is, increase in mucus and PGE2 caused a reduction in gastrin and pepsin release in rats treated with P. macrocarpa fruits extract.

The protective mechanism depends on mucus and bicarbonate secretion and to a large degree, PGE2 secretion; the activity of the cyclooxygenase enzyme also has an important role in this function [27]. Our results proved that pretreatment of rats with P. macrocarpa fruits extract induced increase in PGE2 levels compared with rats in the ulcer control group. These results give a clear indication of the involvement of PGE2 in the gastroprotective mechanism of P. macrocarpa fruits extract. In the stomach, prostaglandins cause elevation of the mucus and bicarbonate secretions, keeping the normal response of the gastric environment to intrinsic factors and inhibiting inflammatory mediator release from mast cells and free radical production. A previous study [28] examined the increase in blood flow in the ulcer region and suggested that it may be caused by higher increase in PGE2 concentration in ulcerative regions compared with other parts of the gastric mucosa because PGE2 causes vasodilatation. The supply of blood reveals the active re-epithelization, which requires an abundant supply of oxygen and glucose [29]. Therefore, increasing the production of PGE2 gained in the treatment with P. macrocarpa fruits extracts clearly indicated the excitement of cytoprotective factors that accelerate healing of gastric ulcer.

One of the important findings in this study was that the level of pro-inflammatory factor TNF-α significantly decreased in the groups pretreated with P. macrocarpa fruits extract, which increased the level of anti-inflammatory cytokine TGF-β1. These results indicated that the immunomodulatory effect of P. macrocarpa might be effected through the inflammatory mediator. Gastrointestinal mucosa integrity depends on the balance between defensive and offensive factors. Many of the components of mucosal defense have proven to be influenced by inflammatory mediators, such as prostaglandins, leukotrienes and thromboxanes [30]. Prostaglandins can also have anti-inflammatory effects through inhibition of leukocyte recruitment, which can contribute to their beneficial effects during situations such as inflamed gastrointestinal mucosa [31]. TNF-α is a key mediator of intestinal injury caused by endotoxins. One of the mechanisms through which TNF-α can develop inflammatory responses and tissue injury is via its regulation of the expression of receptors for other inflammatory mediators, including leukotriene B4 and platelet-activating factor [32].

Prostaglandins are powerful inhibitors of TNF-α released from both the macrophage [33] and the mast cell [30], which may cause elevation of PGE2 level during pretreatment with P. macrocarpa fruits extract. In turn, they are caused by the inhibition of the production of TNF-α. Another immunomodulatory effect of P. macrocarpa is inciting the production of TGF-β1, an important cytokine that accelerates gastric ulcer healing. The healing of gastric ulcer is a complex process controlled by numerous factors, hormones and cytokines. TGF-β1 is one of the multifunctional peptide growth factors that exhibit positive, regulatory healing of gastric ulcer by inducing cell migration and angiogenesis and improving extracellular matrix production. TGF-β1 exerts its action by binding to its transmembrane serine/threonine kinase receptor, which in turn triggers activation of various intracellular signaling pathways [34]. A previous study has reported that the immunoreactive effect of TGF-β1 protein is focuses on epithelial cells and thus, the proliferation of gastric glands [35].
In conclusion, our results indicate that *P. macrocarpa* pretreatment has protective effects in ethanol-induced gastric ulcers in rats. Moreover, these results provide evidence that these protective effects of *P. macrocarpa* are carried out by stimulation of some inflammatory mediators, such as PGE2, TGF-β, and TNF-α. Moreover, important increase in the mucus production and elevated stomach pH can prevent gastric injury.

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