In Vivo Antioxidant and Antiulcer Activity of *Parkia speciosa* Ethanolic Leaf Extract against Ethanol-Induced Gastric Ulcer in Rats

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Abstract

**Background:** The current study was carried out to examine the gastroprotective effects of *Parkia speciosa* against ethanol-induced gastric mucosa injury in rats.

**Methodology/Principal Findings:** Sprague Dawley rats were separated into 7 groups. Groups 1–2 were orally challenged with carboxymethylcellulose (CMC); group 3 received 20 mg/kg omeprazole and groups 4–7 received 50, 100, 200 and 400 mg/kg of ethanolic leaf extract, respectively. After 1 h, CMC or absolute ethanol was given orally to groups 2–7. The rats were sacrificed after 1 h. Then, the injuries to the gastric mucosa were estimated through assessment of the gastric wall mucus, the gross appearance of ulcer areas, histology, immunohistochemistry and enzymatic assays. Group 2 exhibited significant mucosal injuries, with reduced gastric wall mucus and severe damage to the gastric mucosa, whereas reductions in mucosal injury were observed for groups 4–7. Groups 3–7 demonstrated a reversal in the decrease in Periodic acid-Schiff (PAS) staining induced by ethanol. No symptoms of toxicity or death were observed during the acute toxicity tests.

**Conclusion:** Treatment with the extract led to the upregulation of heat-shock protein 70 (HSP70) and the downregulation of the pro-apoptotic protein BAX. Significant increases in the levels of the antioxidant defense enzymes glutathione (GSH) and superoxide dismutase (SOD) in the gastric mucosal homogenate were observed, whereas that of a lipid peroxidation marker (MDA) was significantly decreased. Significance was defined as p<0.05 compared to the ulcer control group (Group 2).

Introduction

Peptic ulcers, which are characterized by the presence of mucosal damage, are predominantly caused by infection with *Helicobacter pylori*, antiplatelet agents such as acetylsalicylic acid [1], non-steroidal anti-inflammatory drugs (NSAIDs) such as oral bisphosphonates, potassium chloride, immunosuppressive medications [2], serotonin reuptake inhibitors [3], alcohol consumption and cigarette smoking [4]. These factors can cause submucosal erosion and inhibit cyclooxygenase, thus disturbing the protection of the gastric mucosal layer [5]. Anatomically, peptic ulcers occur mostly in the stomach and proximal duodenum. Peptic ulcers are caused by an imbalance between the defensive (mucus secretion, mucosal barrier, blood flow, cellular regeneration and endogenous protective agents) and destructive (acid and pepsin secretion) functions of the gastric system [6]. Alcohol-induced gastric lesions impair gastric defense factors such as mucus secretion and mucosal circulation [7]. Ethanol causes necrotic lesions in the gastric mucosa through multiple pathways, directly producing necrotic lesions, which in turn reduces defensive factors, bicarbonate secretion and mucus production [8]. The gastric wall mucus is thought to play an important role as a defensive barrier against gastrointestinal damage [9]. Mucus secretion is considered to be a crucial defensive factor that protects the gastric mucosa from lesions [10]. The level of gastric wall mucus has been evaluated previously and is used as an indicator of gastric mucus secretion [11]. Researchers have reported a large number of medicinal plants with antulcer properties [12–14]. Plant-based medicines represent a vast untapped resource that has shown enormous therapeutic potential. *Parkia speciosa* is also known as stink bean or “petai.” It bears long, flat bean pods with green seeds. These beans are popular in Southeastern Asia, including Malaysia and Northeastern India. The beans are sold in the pods or as seeds that are already separated from the pods. The beans may be jarred in brine and exported. In addition, *P. speciosa* is believed by the local residents to possess medicinal properties [15] and has been reported to exhibit hypoglycemic, antibacterial, antitumor and antioxidant activity [16]. The present study was performed to...
establish the antioxidant and antiulcer activity of Parkia speciosa ethanol leaf extract against ethanol-induced gastric ulcers in rats.

Materials and Methods

Materials and Reagents

All materials and reagents were obtained from Sigma (Sigma Aldrich, Germany), and the MDA, SOD and GSH activity measurement kits were purchased from Cayman Chemical Company (Cayman, USA). Omeprazole was used as a reference antiulcer drug and was obtained from the University of Malaya Medical Centre (UMMC). The drug was dissolved in 0.5% (w/v) carboxymethylcellulose (CMC) and orally administered to the rats at a dosage of 20 mg/kg body weight (5 ml/kg) according to Mahmood et al [17].

Plant Material and Preparation of the Crude Extract

Fresh Parkia speciosa leaves were obtained from Ethno Resources (Selangor, Malaysia). The identity of the plant was confirmed at the Rimba Ilmu Herbarium, Institute of Biological Sciences, University of Malaya through a comparison with the voucher specimen. Fresh leaves were hung upside down in a warm, dry place (away from direct sunlight) with good air circulation for two weeks to dry. Then, using an electrical blender, the dried leaves were converted to a fine powder. A quantity of 100 g of the powder was soaked for 3 days in a flask that contained 500 ml of 95% ethanol. The mixture was filtered using a fine muslin cloth and filter paper (Whatman No. 1). The filtered mixture was distilled using a rotary evaporator (Eyela, USA) and yielded nearly 95% ethanol. The mixture was filtered using a fine muslin cloth and tap water between 180–200 g. The animals were given standard rat pellets and tap water ad libitum.

Macroscopic Gastric Lesion Evaluation

The rat stomachs were examined under a stereomicroscope. The length and width (mm) of each individual hemorrhagic lesion was measured by a planimeter (10 × 10 mm² = ulcer area) under a dissecting microscope (1.8×). The ulcer area (UA) was calculated using the sum of the areas of all lesions for each stomach according to a previously published protocol [23]. The UA was calculated using the following formula:

\[ I\% = \left( \frac{UA_{control} - UA_{treated}}{UA_{control}} \right) \times 100 \]

Antioxidant Activity

Preparation of homogenate. Gastric tissue homogenate 10% (w/v) was prepared in ice-cold 50 mM phosphate buffer (pH 7.4) containing a mammalian protease inhibitor cocktail and then centrifuged at 4,000 rpm for 10 minutes (4°C).

Measurement of superoxide dismutase (SOD) activity. The SOD activity was measured according to the protocol of Sun et al [24]. The enzymatic activity was evaluated by measuring the enzyme’s capacity to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT). In this assay, the photochemical reduction of riboflavin generates O₂⁻; this reduces the NBT to produce a formazan salt that absorbs light at a wavelength of 560 nm. In the presence of NBT, NBT reduction is
inhibited because the enzyme converts the superoxide radical to 
peroxide. The results are expressed as the quantity of SOD 
necessary to inhibit the rate of NBT reduction by 50% in units of 
enzyme per gram of protein. The supernatants of the homogenates 
were centrifuged a second time (20 min, 12,000 rpm, 4°C), and 
the resulting supernatant was assayed. In a dark chamber, 1 ml of 
the reactant (50 mM phosphate buffer, 100 mM EDTA and 
15 mM i-methionine, pH 7.0) was mixed well with 30 μL of 
the sample, 150 μL of 75 μM NBT and 300 μL of 2 μM riboflavin. 
The solution was then exposed to fluorescent light (15 W) for 
15 min and read using a spectrophotometer at 560 nm.

**Measurement of lipid peroxidation (MDA).** Tissue mal-
ondialdehyde (MDA) (mmol/L) was determined using the double 
method of Draper and Hadley [25]. A reaction mixture 
containing 8.1% sodium dodecyl sulfate, 20% acetate buffer 
(pH 3.5) and 0.8% thiobarbituric acid (TBA) was mixed well with 
0.2 ml of stomach tissue homogenate for 3 min and then 
incubated at 95°C for 60 min. After chilling, the TBA-reactive 
substance (MDA) was extracted with 1 ml of H2O and 2.5 ml of 
an n-butanol:pyridine mixture (15:1, v/v). The upper organic 
layer containing the MDA, which was produced by lipid 
peroxidation, was measured at 532 nm.

**Determination of total glutathione (GSH).** The gastric 
mucosa was weighed, minced with scissors and homogenized at 
48°C in phosphate-buffered saline (PBS). The homogenate 
was immediately precipitated with 0.1 ml 25% trichloroacetic acid, 
and the precipitate was removed by centrifugation at 4200 rpm for 
40 min at 4°C. The supernatant was used in a 5,5′-dithiobis(2-
nitrobenzoic acid) assay to determine GSH. Absorbance 
was measured at 412 nm using a spectrophotometer [26].

**Histological Examination of the Gastric Mucosa**
The gastric wall specimens were fixed in 10% buffered formalin 
for 24 h prior to paraffin tissue processing (Leica, Germany). The 
stomach tissues were sectioned at a thickness of 5 μm and stained 
with hematoxylin and eosin to evaluate histological degeneration 
[27].

**Study of Mucosal Glycoproteins**
The sections of the glandular portion of the rat stomach were 
stained with Periodic acid-Schiff (PAS) as described by McManus 
et al [28].

**Immunohistochemical Staining**
Immunohistochemical staining for HSP70 and BAX proteins 
was conducted according to the manufacturer’s protocol (Dako-
Cytomation, USA).

**Statistical Analysis**
All values are reported as the mean ± S.E.M. and were 
analyzed by one-way ANOVA followed by Tukey’s post-hoc test 
for multiple comparisons using the Statistical Package for 
the Social Sciences software (SPSS 18). The differences between 
means were considered statistically significant when the p value 
was less than 0.05.

**Results**

**Acute Toxicity Study**
No death or significant toxicity was observed in the control or 
treatment groups (extract doses of 1, 3 or 5 g/kg for 14 days), as 
evaluated based on clinical and histopathological observations.

**Gross Evaluation of Gastric Lesions**
The antiulcer activity of the *Parkia speciosa* leaf extract in the 
ethanol-induced gastric lesion model is shown in Table 1. The 
results demonstrated that rats pretreated with omeprazole or *Parkia 
speciosa* extracts prior to treatment with absolute ethanol (groups 3– 
7) exhibited significantly smaller areas of gastric ulceration than 
did group 2 (Figure 1). Absolute ethanol produced extensive and 
visible hemorrhagic lesions in the gastric mucosa. *Parkia speciosa* 
extracts significantly inhibited the ulcer formation induced by 
absolute ethanol and obviously decreased the gastric mucosal 
damage in a dose-dependent manner, i.e., the *Parkia speciosa* 
extract significantly suppressed the formation of ulcers. Intrigu-
ingly, a flattening of the gastric mucosal folds was observed in 
group 7. The protection of the gastric mucosa was also the most 
prominent in group 7 (Table 1 and Figure 1). The inhibition of 
 gastric ulcer formation in group 6 was significant and comparable 
to that observed in the omeprazole treatment group (group 3) 
(Table 1 and Figure 1).

**The Effect of Parkia Speciosa on GWM, SOD, MDA, and 
GSH**
The effects of *P. speciosa* on the gastric wall mucus in ethanol-
induced gastric mucosal lesions in rats were examined. Group 2 
exhibited a significant decrease in the Alcian blue binding capacity 
of the gastric wall mucus, whereas groups 4–7 demonstrated a 
significant enhancement in the Alcian blue binding capacity of 
the gastric mucosa. Similarly, in group 2, ethanol reduced the SOD 
activity, while groups 4–7 exhibited a significant increase in SOD 
enzyme activity compared with group 2. The MDA activity was 
significantly higher in group 2 than in group 1, while groups 4–7 
demonstrated significantly decreased MDA activity. In addition, 
the effect of the *Parkia speciosa* extract on the total GSH in gastric 
mucosal homogenates was assessed. Ethanol treatment caused a 
significant depletion of GSH in group 2 compared to group 1. In 
contrast, groups 4–7 exhibited significantly augmented GSH 
content (Figure 2).

**Histological Evaluation of Gastric Lesions**
Histological observations of group 1 indicated that there was no 
disruption of the surface epithelium, while the histological 
examination showed extensive damage to the gastric mucosa in 
group 2, with necrotic lesions penetrating deeply into the mucosa 
accompanied by extensive edema and leucocyte infiltration of the 
submucosal layer (Figure 3). Group 4 exhibited moderate 
disruption of the surface epithelium, with edema and leucocyte 
infiltration of the submucosal layer, and group 5 showed a mild 
disruption of the surface epithelium with edema and leucocyte 
infiltration into the submucosal layer. Group 6 showed mild 
disruption of the surface epithelium with edema and leucocyte 
infiltration of the submucosal layer. Group 7 showed mild edema 
and leucocyte infiltration of the submucosal layer, but no 
disruption of the surface epithelium. Group 3 exhibited a mild 
disruption of the surface epithelium, with submucosal edema and 
leucocyte infiltration. These results demonstrated that the plant 
extracts exerted cytoprotective effects in a dose-dependent manner 
(Figure 3).

**Periodic Acid-Schiff (PAS)**
The gastric mucosa in animals pretreated with *Parkia speciosa* 
extract or omeprazole (groups 3–7) displayed increased PAS 
staining intensity compared to the rats in group 2, indicating an 
increase in the glycoprotein content of gastric mucosa in 
pretreated rats (Figure 4).
Figure 1. The effect of *P. speciosa* on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in rats. (G1) (Normal control group); (G2) (Ulcer control group); (G3) (Omeprazole); (G4) (50 mg/kg), (G5) (100 mg/kg), (G6) (200 mg/kg), (G7) (400 mg/kg) *Parkia speciosa* extract.
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Immunohistochemistry

The expression of the HSP70 protein in the gastric mucosa was downregulated in group 2 but upregulated in group 3–7 (Figure 5). Immunohistochemical staining of the gastric mucosa of rats pretreated with *Parkia speciosa* extract or omeprazole demonstrated a downregulation of the BAX protein (Figure 6). In addition, the levels of BAX protein in group 2 rats were higher than those of groups 3–7 (Figure 6).

**Discussion**

The acute toxicity test did not suggest any toxicity or mortality in the *P. speciosa*-treated rats. This test revealed that the plant is safe and has no toxicity when administered orally up to 5 g/kg. Certain antiulcer drugs have been reported to increase the amount of gastric mucus secretion in the gastric mucosa [29]. Pretreatment with *Parkia speciosa* extract significantly increased the gastric mucus content in rats with ethanol-induced ulcers, indicating that the gastroprotective effect of *Parkia speciosa* is mediated partly by preservation of the gastric wall mucus. This mucus consists of mucin-type glycoproteins, which can be detected by Alcian blue [30]; the increase in Alcian blue staining demonstrates the protective effect of orally administered *Parkia speciosa*, which may be mediated by the formation of protective complexes between *Parkia speciosa* and the mucus that acts as a barrier against necrotizing agents introduced to the stomach [31]. Here, the *Parkia speciosa* extract prevented the decrease in the concentration of gastric wall mucus upon treatment with ethanol. Thus, one possible mechanism by which the gastric mucosa is protected by *Parkia speciosa* involves the reinforcement of the mucosal barrier resistance, generated by a protective coating. It is likely that the protective effect of *Parkia speciosa* is due at least in part to the preservation of the mucus layer in the gastric mucosa; *Parkia speciosa* was observed to prevent ethanol-induced gastric wall mucus depletion. The results of the present study demonstrated that *Parkia speciosa* extract has an effective antiulcer activity against

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>pH</th>
<th>Ulcer Area</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC (vehicle)</td>
<td>6.57 ± 0.21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CMC (control)</td>
<td>3.43 ± 0.26</td>
<td>877.45 ± 29.99</td>
<td>0</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>5.43 ± 0.21**</td>
<td>209.46 ± 12.78**</td>
<td>76</td>
</tr>
<tr>
<td>P. speciosa (50 mg/kg)</td>
<td>4.24 ± 0.20</td>
<td>373.04 ± 22.23**</td>
<td>57</td>
</tr>
<tr>
<td>P. speciosa (100 mg/kg)</td>
<td>4.75 ± 0.34*</td>
<td>215.09 ± 23.43**</td>
<td>75</td>
</tr>
<tr>
<td>P. speciosa (200 mg/kg)</td>
<td>5.36 ± 0.20**</td>
<td>102.37 ± 7.59**</td>
<td>88</td>
</tr>
<tr>
<td>P. speciosa (400 mg/kg)</td>
<td>6.04 ± 0.27**</td>
<td>39.18 ± 3.55**</td>
<td>95</td>
</tr>
</tbody>
</table>

Rats pretreated with *Parkia speciosa* (100, 200, and 400 mg/kg) extracts had significantly reduced gastric ulcer areas and gastric content pH. All values are expressed as the mean ± standard error of the mean; differences between means were considered significant at *p* < 0.05 or **p** < 0.001 levels. The data were analyzed by one-way ANOVA using the Statistical Package for the Social Sciences software (SPSS 18).

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**Figure 2.** The effects of *P. speciosa* on SOD, MDA, GSH and GWM in the gastric mucosa homogenate of ethanol-induced gastric mucosal lesions in rats. All values are expressed as the mean ± standard error of the mean. All *P. speciosa*-treated groups were significantly different from the control groups at *p* < 0.05. The data were analyzed by one-way ANOVA using the Statistical Package for the Social Sciences software (SPSS 18).

doi:10.1371/journal.pone.0064751.g002
Figure 3. The effect of *P. speciosa* on the histology of ethanol-induced gastric mucosal damage in rats. (G1) (Normal control group); (G2) (Ulcer control group); (G3) (Omeprazole); (G4) (50 mg/kg), (G5) (100 mg/kg), (G6) (200 mg/kg), (G7) (400 mg/kg) *Parkia speciosa* extract. The rats in groups 4–7 demonstrated comparatively better protection of the gastric mucosa, as shown by a reduction in or absence of the ulcer area, submucosal edema and leucocyte infiltration (H&E staining, 20×). doi:10.1371/journal.pone.0064751.g003
Figure 4. The effect of Parkia speciosa on gastric tissue glycoprotein-PAS staining in ethanol-induced gastric ulcers in rats. (G1) (Normal control group); (G2) (Ulcer control group); (G3) (Omeprazole); (G4) (50 mg/kg), (G5) (100 mg/kg), (G6) (200 mg/kg), (G7) (400 mg/kg) Parkia speciosa extract (PAS stain 20×).

doi:10.1371/journal.pone.0064751.g004
Figure 5. Immunohistochemical analysis of HSP70 expression in the gastric mucosa of rats. (G1) (Normal control group); (G2) (Ulcer control group); (G3) (Omeprazole); (G4) (50 mg/kg), (G5) (100 mg/kg), (G6) (200 mg/kg), (G7) (400 mg/kg) *Parkia speciosa* extract. HSP70 protein expression was upregulated in rats pretreated with *Parkia speciosa* (magnification 20×).

doi:10.1371/journal.pone.0064751.g005
Figure 6. Immunohistochemical analysis of the expression of the BAX protein in the gastric mucosa of rats. (G1) (Normal control group); (G2) (Ulcer control group); (G3) (Omeprazole); (G4) (50 mg/kg), (G5) (100 mg/kg), (G6) (200 mg/kg), (G7) (400 mg/kg) Parkia speciosa extract. BAX protein expression was downregulated in rats pretreated with Parkia speciosa (magnification 20×). doi:10.1371/journal.pone.0064751.g006
ethanol-induced gastric mucosal injury. The plant extract increased the mucus content of the gastric wall, which is consistent with results reported by Thirunavukkarasu et al. [32].

Our results revealed the protection of the gastric mucosa and inhibition of leukocyte infiltration into the gastric wall in rats pretreated with *Parkia speciosa* extract. The activation and infiltration of neutrophils appear to be involved in the initial processes that form these lesions. Similarly, Abdulla et al. [12] demonstrated that the reduction of neutrophil infiltration into ulcerated gastric tissues helped to prevent gastric ulcers in rats. Wasman et al. [33] showed that the oral administration of plant extract prior to ethanol administration significantly decreased neutrophil infiltration into the gastric mucosa. Ethanol causes extensive damage to the gastric mucosa and leads to increased neutrophil infiltration into this tissue.

The present study established that pretreatment with *Parkia speciosa* reduced neutrophil infiltration into ulcerated tissue. We also observed a flattening of the mucosal folds, which suggests that the gastroprotective effect of the *Parkia speciosa* leaf extract might be attributed to a decrease in gastric motility. Changes in gastric motility have been implicated in the development and prevention of experimental gastric lesions. The relaxation of the circular muscles may protect the gastric mucosa through a flattening of the folds. This flattening increases the mucosal area exposed to necrotizing agents and reduces the volume of the gastric irritants that come into contact with the rugal crest [12,33].

Gastric tissue homogenate prepared from the groups that were pretreated with plant extract exhibited significant antioxidant activity, with decreased levels of MDA and elevated levels of GSH and SOD, in response to oxidative stress due to ethanol treatment. SOD converts superoxide to hydrogen peroxide (H₂O₂), which is transformed into water by catalase in the lysosomes or by glutathione peroxidase in the mitochondria [34]. MDA is the final product of lipid peroxidation and is used to determine lipid peroxidation levels [35]. Lipid peroxidation causes a loss of membrane fluidity, impaired ion transport and membrane integrity and ultimately a loss of cellular function.

Our experimental results indicate that *Parkia speciosa* ethanolic extract significantly inhibited the negative effects of ethanol on gastric GSH levels at all doses used. The gastric GSH level was highest for the 400 mg/kg dose and lowest for the 50 mg/kg dose, while the GSH level was decreased in the ulcer control group. The current results and previously published articles indicate that there is an important relationship between gastric GSH levels and ulcer severity. GSH and GSH-related enzymes are known as important tissue protective agents due to their antioxidant properties [36,37].

The periodic acid-Schiff (PAS) histochemical method produces a characteristic carmine staining in stomach regions that secrete mucopolysaccharides. Tissue sections from the group of rats that was treated with 400 mg/kg plant extract showed intense staining reflecting mucus secretion in the gastric glands. Mucus production is one of the major mechanisms of local gastric mucosal defense [38].

BAX promotes apoptosis [39], while BCL-2 inhibits this process. Apoptosis may be caused by an imbalance in the expression of BCL-2 family antiapoptotic proteins and apoptotic BAX proteins in stress ulcers [40]. HSP70 is a 70 kDa protein from the HSP family that is expressed in mammalian cells. These proteins are responsible for protecting cellular homeostatic processes from environmental and physiologic injuries by preserving the structure of normal proteins and repairing or removing damaged proteins [41], and the study of this protein can thus provide interesting data for the elucidation of possible mechanisms of action. HSP70 defends cells from oxidative stress or heat shock. Ethanol-generated reactive oxygen species (ROS) normally act to inhibit the expression of HSP70 and increase the expression of BAX. HSP70 prevents these partially denatured proteins from aggregating and allows them to refold. The upregulation of HSP70 that was observed in this study suggests that the *Parkia speciosa* extract protected the gastric tissues through the upregulation of HSP70. Additionally, HSP70 has been suggested to exert its cytoprotective activity by protecting mitochondria and interfering with the stress-induced apoptotic program. Immunohistochemical staining showed that BAX protein expression was downregulated in rats pretreated with *Parkia speciosa* extract.

**Conclusion**

In conclusion, *P. speciosa* extract was clearly demonstrated to function as an antiulcer agent. The results suggest that the *P. speciosa* extract may act by enhancing the gastric mucosal defense and/or by inhibiting leukotriene synthesis. The plant extract protected the gastric tissues through the upregulation of the HSP70 and downregulation of the BAX protein. *Parkia speciosa* reversed the decrease in PAS staining induced by ethanol, significantly increased the GSH and SOD activities, and decreased the level of lipid peroxidation (MDA) in the *P. speciosa*-pretreated groups.

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**Author Contributions**

Conceived and designed the experiments: MAA. Performed the experiments: RAB. Analyzed the data: AMA RAB. Contributed reagents/materials/analysis tools: MMJ. Wrote the paper: FAR. Literature search: HMA HAH.

**References**


