Anti-ulcer activity of petroleum ether extract of leaves of Madhuca indica J. F. Gmel against pylorus ligation and naproxen-induced gastric mucosal injury in rats

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ABSTRACT

In Indian traditional system of medicine, the plant of Madhuca indica J. F. Gmel. (Sapotaceae) is recommended for the management of peptic ulcer. In light of this, the present investigation was carried out to study the antiulcer activity of various doses (100, 200 and 400 mg/kg, p.o) of petroleum ether extract of Madhuca indica J.F. Gmel, using the pylorus ligation and naproxen-induced gastric ulcer models in rats. In pylorus ligation, the extract provided significant ulcer protective effect as evinced through significant increase in gastric pH and mucin content of the stomach along with reduction in total acidity and pepsin activity. After 4 week treatment period, desired aim was achieved using petroleum ether extract of plant of Madhuca indica MI-PEE at the dose of 200 and 400 mg/kg, p.o. (P < 0.01), (P < 0.001) showed significant reduction in ulcerated area and ulcer index as compared to control group in naproxen induced ulcer model. Moreover, ulcerated area was reduced significantly in all two models. It is concluded that Petroleum ether extract of Madhuca indica leaves possesses antiulcer activity which can be attributed to its ability to increase the protective layer of mucin and decrease the damaging and or digestive effects of pepsin and acid.

Keywords: Madhuca indica J. F. Gmel, pylorus-ligation, naproxen-induced, gastric ulcer, ulcerated area, ulcer index.

INTRODUCTION

Gastric ulcer, which affects thousands of people, is becoming one of the most important diseases of the digestive system and a medical–social problem of global economic importance due to its higher and higher morbidity and mortality [1-4]. Stress, smoking, nutritional deficiencies, ingestion of nonsteroidal anti-inflammatory drugs, hereditary predisposition and infection by Helicobacter pylori are all factors that can increase the incidence of gastric ulcer [2, 5-9]. As a matter of fact, many drugs were used to treat this disease but many of them cause adverse effects and recurrent infections frequently occur within a few weeks because of difficulty in eradication of H. pylori [10-12]. Drug treatment of peptic ulcers is targeted at either counteracting aggressive factors [acid, pepsin, active oxidants, platelet aggravating factor (PAF), leukotrienes, endothelins, bile or exogenous factors including NSAIDs] or stimulating the mucosal defences [mucus, bicarbonate, normal blood flow, prostaglandins (PG), nitric oxide] [13]. This has been rationale for the development of new antiulcer drugs and search for novel molecule. Drugs of plants origin are gaining popularity and investigating for the various disorders including peptic ulcer [14-16].
Research into new medicines that enable the development of alternative therapies for the treatment of gastric ulcers is very important [15, 17]. In recent years, there has been growing interest in use of natural antioxidant, especially those derived from edible material such as fruits, spices, herbs and vegetables. Therefore, the development and use of more effective antioxidant of natural origin is desired [18]. *Madhuca indica* J. F. Gmel, a plant belonging to the family Sapotaceae. It is a large, shady deciduous tree both wild and cultivated, found in different parts of Bangladesh. It is also distributed more or less throughout India especially in the states of Jharkhand, Uttar Pradesh, Bihar, Maharashtra, Madhya Pradesh, Kerala, Gujarat and Orissa [19, 20]. The plant of *Madhuca indica* is mentioned in literature as an effective remedy for peptic ulcer. It has been traditionally used for treatment of ulcer, rheumatism, itches, bleeding, spongy gum, tonsillitis and diabetes mellitus [21].

The objective of the present study was to evaluate the effectiveness of leaves extract in preventing the formation of gastric ulcer experimentally by pylorus ligation and naproxen-induced gastric damage in rats.

**MATERIALS AND METHODS**

2.1. Plant Material:
*M. indica* (Sapotaceae) leaves were collected from areas adjoining the district of Amravati, Maharashtra, India and was authenticated at Agharkar Research Institute, Pune, India and the voucher specimen was deposited at Institute (Voucher specimen sample no – L-054).

2.2. Preparation of extract:
Weighed quantity (500 g) of air dried powder (Mesh size-16) of the leaves of *Madhuca indica* (J. F. Gmel) was macerated with petroleum ether (MI-PEE) at room temperature for 7 days and filtered. The filtrate was evaporated at room temperature. Semisolid petroleum ether extract was emulsified with 2 % Tween-80 in distilled water in order to prepare the appropriate concentration of stock suspension.

2.3. Animals:
Healthy male and female wistar rats (150-200g) and male swiss albino mice (18-22 g) were obtained from National Toxicology Centre, Pune, India and housed in animal house in groups of six animals in polypropylene cages. The animals were maintained at 25 ± 2°C, relative humidity of 45 to 55% and under standard environmental conditions (12 h light 12 h dark cycle). All the animals were acclimatized for 10 days to the animal house conditions prior to the start of experimental protocol. The animals had free access to food (Amrut laboratory animal feed, Sangali, MS, India) and water ad libitum. The research protocol was approved by Institutional Animal Ethical Committee (IAEC) constituted as per the directions of the CPCSEA. All experiments were carried out between 12:00-16:00 hours.

2.4. Acute toxicity test:
Acute toxicity study was performed in healthy adult male albino mice (18-22 g) as per guideline no AOT 425 of the Organization for Economical Co-operation and Development (OECD). Petroleum ether extract of leaves of *Madhuca indica* was administered at various doses in mice were observed continuously for 2 h for behavioral and autonomic profiles and for any other sign of toxicity or mortality up to a period of seven days.

2.5. Anti-ulcer Activity:
2.5.1. Pylorus ligation induced ulcers [22]:
Rats of either sex were divided into five groups with six rats in each group. Group 1served as control group and received vehicle (2% gum acacia in distilled water, 1 ml/kg, p.o.), group 2 received reference standard ranitidine 100 mg/kg, p.o. while group 3-5 received *Madhuca indica* petroleum ether extract (MI-PEE) at doses of 100, 200 and 400 mg/kg body weight, p.o. respectively for the period of 10 days. Rats were deprived of food, but not water, for 24 h prior to the experiment. On 10th day, 1 h after the respective treatments animals was anaesthetized with ketamine (80 mg/kg, i.p). The abdomen was opened by a small midline incision below the xiphoid process; pylorus portion of stomach was slightly lifted out and ligated. Precaution was taken to avoid traction to the pylorus or damage to its blood supply. The stomach was placed carefully in the abdomen and the wound was sutured by interrupted sutures. Nineteen h after pylorus ligation the rats were sacrificed and the stomach was removed. The gastric content was collected and centrifuged. The volume, pH, total acidity of gastric fluid and mucin [23, 24] content and pepsin [25] content was determined. The stomach was then incised along the greater curvature and observed for ulcers. Ulcerated area of stomach was calculated by image processing software Image J (National Institute of Health, U. S. A.).
2.5.1.1. Estimation of mucin activity [8]:
Gastric glandular segments were removed and weighed. Each segment was immersed for 2 h in 10 ml of 0.1% v/v alcian blue dissolved in 0.16 M sucrose solution and buffered with 0.05 M sodium acetate, pH 5.8. Excess dye was removed by washing the segments twice with 0.25 M sucrose solution during a period of 15 and 45 min, respectively. Mucus-dye complex was extracted by immersing the gastric wall in 10 ml of 0.5 M MgCl₂ and shaking this solution intermittently for 1 min at 30 min intervals for 2 h. A volume of 4 ml blue extract was mixed with an equal volume of diethyl ether, shaking the mixture vigorously for 20 min. The emulsion obtained was centrifuged for 10 min at 6000 rpm and the absorbance of the aqueous layer was recorded at 580 nm using a light spectrophotometer. The free mucus in gastric content was calculated from the amount of alcian blue binding (µg/gm of wet tissue). Mucin content of the stomach was expressed as µg Alcian blue/g wet tissue.

2.5.1.2. Estimation of pepsin activity:
Aliquots of 20 µl of the gastric content were incubated with 500 µl of albumin solution (5mg/ml, 0.06 N Hydrochloric acid) at 37°C for 10 minutes. The reaction was stopped with 200 µl of 10% trichloroacetic acid and the samples were centrifuged at 1500 rpm for 20 minutes. The supernatant was alkalinized with 2.5 ml of 0.55 M sodium carbonate, 400 µl of 0.1 N Folin reagent was added to the tubes, which were then incubated for 30 minutes at room temperature. The absorbance of the sample was determined at 660 nm. A standard curve of tyrosine for the determination of the concentration of pepsin was plotted. Pepsin content of the gastric fluid was expressed as µg of tyrosine/ml.

2.5.2. Naproxen-induced ulcers [26, 27]:
Rats were divided into three sets as A, B and C with six groups in each set. Further, each group consisted of six rats. The animals were fasted for 24 for naproxen induced ulcer.
1. Normal control
2. Vehicle distilled water + Naproxen (30 mg/kg, p.o.).
3. Omeprazole (standard drug) – 30 mg/kg, p.o.
4. MI-PEE 100 mg/kg, p.o.
5. MI-PEE 200 mg/kg, p.o.
6. MI-PEE 400 mg/kg, p.o.

Rats in sets A, B and C were treated with standard drug (group III) and MI-PEE (group IV to VI) with for 10, 20 and 30 days respectively. Groups of II to VI of each set of rats was administered naproxen at the dose of 30 mg/kg, p.o. for three consecutive days staring from 7th day for 10 days treatment period in set A, 17th day for 20 days treatment period in set B and on 27th day for 30 days treatment period in set C. All the animals were fasted for 24 h before administration of first dose of naproxen. The animals had free access to feed following the first dose of naproxen. Animals of set A, B and C were scarified on completion of 10th day, 20th day and 30th day respectively. The stomach of each rat was removed, inspected internally and ulcerated area was calculated by using before mentioned method.

1.6. Statistical analysis:
The results are expressed as mean ± SEM. The statistical analysis was done by using GraphPad prism 5.0. The statistical analysis of all the results was carried out using two way ANOVA followed by Bonferroni's test and one way ANOVA followed by Dunnett’s test P < 0.05 was considered as significance.

RESULTS

3.1. Acute toxicity test:
In oral toxicity study administration of the extract of the graded doses 175, 550, 1750 and 2000 did not cause death of mice. MI-PEE was found to be safe up to a dose of 2000 mg/kg, p.o.

3.2. Dose selection:
Based upon toxicity studies and pilot studies (data not shown) three different doses of MI-PEE i.e. 100, 200 and 400 mg/kg were selected for antiulcer investigation.
3.3. Pylorus ligation induced ulcers:
Pylorus ligation for 19 hours resulted in the accumulation of gastric secretions along with an increase in the total acid output of the gastric juice. Circular and linear lesions and petechiae were frequently seen in the rumenal and glandular mucosa of the stomachs of all the control animals.

Oral administration of MI-PEE produced a significant dose dependent decreased gastric mucosal damage in pylorus ligation model. At the dose of 200 and 400 mg/kg, significant (P < 0.01) and (P < 0.001) reduction in ulcer index (11.46 ± 1.09 and 6.77 ± 0.21 respectively) when compared with control group (14.35 ± 0.24). Dose of 100 mg/kg, did not show any significant changes in this regards. Ranitidine (100 mg/kg,) also exhibited significant (P < 0.001) reduction in ulcer index (1.96 ± 0.19) as compared to control group.

Discernable changes were found in the gastric parameters of MI-PEE treated group as compared with the pylorus ligation control group [Table 1]. Pylorus ligation in rats produced an increased in the volume of gastric secretion (19.82 ± 0.90), decreased in the pH (1.50 ± 0.22) and increased in acidity (113.2 ± 4.99) whereas the pretreatment of MI-PEE at dose of 200 and 400 mg/kg, show significant (P < 0.01 and P < 0.001 respectively) decreased in the volume of gastric secretion (14.58 ± 0.41 and 9.94 ± 1.31 respectively).

Table 1 Effect of MI-PEE on ulcerated area, ulcer index, volume, pH, acidity, mucin and pepsin content in pylorus ligation induced ulcer model

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment (Pylorust ligated)</th>
<th>Dose (mg/kg)</th>
<th>Ulcerated Area</th>
<th>Ulcer Index</th>
<th>Volume (ml)</th>
<th>pH</th>
<th>Acidity (µg/ml wet tissue)</th>
<th>Mucin (µg/g wet tissue)</th>
<th>Pepsin (µg tyrosine/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Vehicle control</td>
<td>---</td>
<td>101.8 ± 2.77</td>
<td>14.35 ± 0.24</td>
<td>19.82 ± 0.90</td>
<td>1.50 ± 0.22</td>
<td>113.2 ± 4.99</td>
<td>3.82 ± 0.44</td>
<td>67.96 ± 1.71</td>
</tr>
<tr>
<td>2.</td>
<td>Ranitidine</td>
<td>100 mg/kg, p.o.</td>
<td>17.25 ± 2.82***</td>
<td>1.96 ± 0.19***</td>
<td>6.55 ± 1.13***</td>
<td>4.66 ± 0.21***</td>
<td>35.33 ± 2.41***</td>
<td>8.75 ± 0.38***</td>
<td>26.58 ± 1.94***</td>
</tr>
<tr>
<td>3.</td>
<td>MI-PEE</td>
<td>100 mg/kg, p.o.</td>
<td>89.65 ± 6.78&quot;</td>
<td>13.09 ± 0.66&quot;</td>
<td>18.53 ± 1.33&quot;</td>
<td>2.66 ± 0.33&quot;</td>
<td>95.17 ± 5.63&quot;</td>
<td>4.80 ± 0.27&quot;</td>
<td>67.13 ± 4.46&quot;</td>
</tr>
<tr>
<td>4.</td>
<td>MI-PEE</td>
<td>200 mg/kg, p.o.</td>
<td>79.50 ± 5.88**</td>
<td>11.46 ± 1.09**</td>
<td>14.58 ± 0.41**</td>
<td>2.83 ± 0.30**</td>
<td>85.67 ± 6.52&quot;</td>
<td>5.06 ± 0.31&quot;</td>
<td>52.54 ± 5.26&quot;</td>
</tr>
<tr>
<td>5.</td>
<td>MI-PEE</td>
<td>400 mg/kg, p.o.</td>
<td>31.57 ± 3.62***</td>
<td>6.77 ± 0.21***</td>
<td>9.94 ± 1.31***</td>
<td>3.66 ± 0.21***</td>
<td>74.83 ± 11.54&quot;</td>
<td>6.03 ± 0.18***</td>
<td>42.08 ± 7.09&quot;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, n=6, Data was analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test. Petroleum ether extract of leaves of Madhuca indica *P < 0.05, **P < 0.01, ***P < 0.001.

Significantly (P < 0.05, P < 0.01 and P < 0.001 respectively) increased in pH of gastric fluid at the dose of 100, 200 and 400 mg/kg, (2.66 ± 0.33, 2.83 ± 0.30 and 3.66 ± 0.21 respectively). Acidity significantly (P < 0.05 and P < 0.01 respectively) decreased at the dose of 200 and 400 mg/kg, (85.67 ± 6.52 and 74.83 ± 11.54 respectively).

Animals pretreated with MI-PEE at the dose of 200 and 400 mg/kg show significantly (P < 0.05 and P < 0.001 respectively) increased mucin content of stomach from (5.06 ± 0.31 and 6.03 ± 0.18 respectively) when compared against control group (3.82 ± 0.44).

Pepsin content of stomach significantly (P < 0.01) decreased at the dose of 400 mg/kg, (42.08 ± 7.09) as compared to pylorus ligated control rats (67.96 ± 1.71).

Ranitidine (100 mg/kg, p.o.) produced significant (P < 0.001) reduction in volume, acidity and pepsin content and also significant increases in pH and mucin content as compared to control group rats [Table 1].

3.4. Naproxen -induced ulcers:
Oral administration of naproxen (30 mg/kg, p.o.) produced small erosion throughout the glandular portion of rat stomach. However, pretreatment with MI-PEE reduced severity of naproxen-induced gastric ulcer.
MI-PEE at the dose of 400 mg/kg, p.o. showed significant ($P < 0.01$ and $P < 0.001$ respectively) reduction in ulcer index ($0.81 \pm 0.09$ and $0.53 \pm 0.09$ respectively) after 20 and 30 days of pretreatment period when compared against naproxen control group ($1.46 \pm 0.23$ and $1.28 \pm 0.27$ respectively) which did not show any significant change after 10 days pretreatment period at the dose of 400 mg/kg, p.o.

After 20 and 30 days of pretreatment period of oral administration of MI-PEE at the dose of 200 mg/kg, p.o. produced significant ($P < 0.05$ and $P < 0.01$ respectively) reduction of ulcer index ($0.94 \pm 0.04$ and $0.63 \pm 0.06$ respectively) when compared with naproxen control group. After 10 days of pretreatment period at the dose of 200 mg/kg, p.o. did not show any significant change in this regards.

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Treatment (Naproxen)</th>
<th>Dose</th>
<th>Ulcer area (mm$^2$) 10 Day</th>
<th>Ulcer index 10 Day</th>
<th>Ulcer area (mm$^2$) 20 Day</th>
<th>Ulcer index 20 Day</th>
<th>Ulcer area (mm$^2$) 30 day</th>
<th>Ulcer index 30 Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Vehicle (Naproxen)</td>
<td>30 mg/kg, p.o.</td>
<td>10.96 ±1.54</td>
<td>1.67 ± 0.21</td>
<td>9.81 ± 1.50</td>
<td>1.46 ± 0.23</td>
<td>8.73 ± 1.79</td>
<td>1.28 ± 0.27</td>
</tr>
<tr>
<td>2.</td>
<td>Standard (Omeprazole)</td>
<td>30 mg/kg, p.o.</td>
<td>5.85 ± 0.40***</td>
<td>0.83 ± 0.05***</td>
<td>3.81 ± 0.39***</td>
<td>0.54 ± 0.05***</td>
<td>1.06 ± 0.27***</td>
<td>0.15 ± 0.03***</td>
</tr>
<tr>
<td>3.</td>
<td>MI-PEE 100 mg/kg, p.o.</td>
<td>9.91 ± 1.18*</td>
<td>1.52 ± 0.18*</td>
<td>7.45 ± 0.86***</td>
<td>1.12 ± 0.14***</td>
<td>5.14 ± 0.79*</td>
<td>0.74 ± 0.11*</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>MI-PEE 200 mg/kg, p.o.</td>
<td>8.96 ± 0.50**</td>
<td>1.37 ± 0.07**</td>
<td>5.93 ± 1.13*</td>
<td>0.94 ± 0.04*</td>
<td>4.09 ± 0.76**</td>
<td>0.63 ± 0.06**</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>MI-PEE 400 mg/kg, p.o.</td>
<td>7.79 ± 0.29**</td>
<td>1.30 ± 0.03**</td>
<td>4.98 ± 1.06**</td>
<td>0.81 ± 0.09**</td>
<td>3.27 ± 0.87***</td>
<td>0.53 ± 0.09***</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, n=6. Data was analyzed by two way analysis of variance (ANOVA) followed by Bonferroni’s post hoc. MI-PEE Madhuca indica leaves petroleum ether extract *P < 0.05, **P < 0.01, ***P < 0.001.

On the other hand, after 30 days of pretreatment period, MI-PEE produced significant ($P < 0.05$) reduction in ulcer index at the dose of 100 mg/kg, p.o. ($0.74 \pm 0.11$) when compared with naproxen control group. The dose of 100 mg/kg did not show any significance result from 10 and 20 days treatment period.

The reference standard omeprazole (30 mg/kg, p.o.) after 10, 20 and 30 days pretreatment was found to be more significantly ($P < 0.001$) reduction in ulcer ($0.83 \pm 0.05, 0.54 \pm 0.05$ and $0.15 \pm 0.03$ respectively) when compared with naproxen control group [Table 2].

**DISCUSSION**

Peptic ulcers are resulted due to overproduction of gastric acid and/or decrease in gastric mucosal protection mechanisms [2, 6, 8, 11, 24]. That is why, the potential anti-ulcerogenic and ulcer-healing drugs are known to possess the property of decreasing offensive factors or of increasing the defensive factors.

The present investigation revealed significant antiulcer effect of petroleum ether extract of *Madhuca indica* leaves (MI-PEE) in experimental models of gastric ulcers induced by pylorus ligation and naproxen.

Pylorus-ligation and naproxen-induced gastric ulcers have been widely used for the experimental evaluation of anti-ulcer activity [8, 16, 24]. Disturbances in gastric secretion, damage to gastric mucosa, alteration in permeability, gastric mucus depletion and generation of free–radical production are reported to be the pathogenic effects of pylorus ligation and naproxen-induced ulcer [28].

Pylorus-ligation ulcers are caused due to accumulation of gastric acid and pepsin, which leads to auto-digestion of gastric mucosa and breakdown of the mucosal barrier. In addition to gastric acid secretion, reflex or neurogenic effect has also been suggested to play an important role in the formation of gastric ulcer in this model [29].

The petroleum ether extract of *M. indica* reduction in the ulcer index, volume, total acidity and pepsin content of gastric fluid along with an increase in its pH of gastric fluid and mucin content of stomach wall suggests an inhibition of aggressive factors. In the present study, we examined the effects of NSAIDs in model that attempt to mimic relevant clinical scenarios of NSAID use [30-32].

NSAIDs represent one of the most widely used classes of drugs and are used primarily to alleviate the symptoms (e.g. pain and swelling) of osteoarthritis, rheumatoid arthritis and other inflammatory disorders; however, the use of...
NSAIDs is significantly limited by their ability to induce the formation of erosions and ulcers in the gastrointestinal (GI) tract [9, 33-36].

The effectiveness of NSAIDs in reducing pain and swelling lies in their ability to inhibit PG synthesis. COX-1 and COX-2 are the key enzymes for the synthesis of PGs, which have hyperalgesic effects and can augment edema formation. PGs also mediate many components of the GI mucosal defense, including mucus and bicarbonate secretion, mucosal blood flow, epithelial cell turnover, and mucosal immunocyte function [31, 32].

By suppressing mucosal PG synthesis (as well as through direct topical irritant effects on the epithelium), NSAIDs can impair mucosal defense and render the stomach more susceptible to injury. The oral administration of MI-PEE showed a significant protection against naproxen-induced gastric antral ulcer.

In this study we observed that *Madhuca. Indica* J. F. Gmel provides significant antiulcer and cytoprotective effect against gastric ulcers in rats. However, further investigation is necessary to determine the mechanism of action.

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