

**EVALUATION OF ANTIULCER ACTIVITY OF METHANOLIC EXTRACT OF LEAVES OF *MADHUCA INDICA* J. F. GMEL IN RATS**

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**Summary**

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 methanolic extract of *Madhuca indica* J.F. Gmel, using the pylorus ligation, ethanol-induced 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. Also, mucin content of stomach was significantly increased in ethanol induced ulcer. Moreover, ulcerated area was reduced significantly in all three models. It is concluded that methanolic 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, Ethanol-induced, naproxen-induced, gastric ulcer, ulcerated area.

### Introduction

Peptic ulcer is a lesion of gastric or duodenal mucosa occurring at a site where the mucosal epithelium is exposed to aggressive factors<sup>1</sup>. It is one of the major gastro-intestinal disorders, which mainly occur due to an imbalance between the offensive (gastric acid secretion) and defensive (gastric mucosal integrity) factors<sup>2</sup>. Potentially injurious agents such as acid, pepsin, bile acids, food ingredients, bacterial products and certain drugs have been implicated in the pathogenesis of gastric ulcer. In addition, the suppression of endogenous generation of prostaglandins, inhibition of mucosal growth and alteration of gastric mobility are other contributing factors<sup>3</sup>. Reduction of gastric acid production as well as re-enforcement of gastric mucosal production has been the major approaches for current therapy<sup>2</sup>.

Various synthetic antiulcer drugs like antacids, proton pump inhibitors, anticholinergics, H<sub>2</sub>-receptor antagonists and cytoprotective agents are being used in clinical practices, however they are unable to control high recurrence rate associated with modernized stressful life style<sup>4</sup>. The increase in financial burden of treatment produces another hurdle to the therapy and it need to be addressed<sup>5</sup>. Moreover, these drugs confer variable side effects ranging from diarrhoea, itching and dizziness to arrhythmia, impotence and gynaecomastia<sup>6-8</sup>. These conditions may worsen due to certain drug-drug interactions e.g. as in case of polypharmacy, which ultimately reduce the overall outcome of the therapy<sup>9</sup>. On the contrary, in recent years, there has been growing interest in alternative therapies and the use of natural products, especially those derived from plants since, medicinal plants are among the most attractive sources of new drugs and have been shown to produce promising results for treatment of various diseases and disorders including gastric ulcer<sup>9</sup>.

The plant *Madhuca indica* J. F. Gmel (Sapotaceae) is mentioned in literature as an effective remedy for peptic ulcer<sup>11</sup>. It has been traditionally used for treatment of ulcers, rheumatism, itches, bleeding, spongy gum, tonsillitis and diabetes mellitus<sup>12</sup>. The chemical constituents of bark of *M. indica* (J. F. Gmel) were reported to possess free radical scavenging activity<sup>13</sup>. Previous phytochemical analysis of the leaves of *Mdhuca incidica* showed presence of  $\beta$ -D-glucoside of  $\beta$ -sitosterol, oleanolic acid palmitate and a new triterpene ester identified as 3- $\beta$ -caproxy-olea-12-en-28-ol and xanthophylls<sup>29</sup>, flavonols, myricetin and quercetin<sup>30</sup>, camelliagenin A and its 22 $\alpha$ ,28-glycoaldehyde acetal, 16 $\alpha$ -O-acetyl-22 $\alpha$ -O-angeloycamelliagenin A and 16 $\alpha$ -O-acetyl-22 $\alpha$ -O-(2'-methylbutyroyl) camelliagenin A<sup>10</sup>. The methanolic extract of leaves, flowers, stem and stem bark of *Maduca indica* have been reported to possess antibacterial activity against *B. anthracis*, *B. pumilus*, *B. subtsilis*, *Sal. Paratyphi* and *Vib. Cholera*<sup>31</sup>. Earlier scientific documentation of this plant for its usefulness in generalized itching, bleeding, tonsillitis and diabetes mellitus have made it worthwhile to conduct its scientific evaluation for antiulcer potential<sup>12</sup>. Objective of the present work was to evaluate the antiulcer activity of methanolic extract of *M. indica* using pylorus ligation, ethanol-induced and naproxen-induced gastric ulcer models in rats.

## **Materials and methods**

### **Plant Material:**

*M. indica* (Sapotaceae) was 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).

### **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 99.9 % methanol (MI-ALC) (2.5 L methanol) at room temperature for 7 days and filtered. The filtrate was dried on a tray dryer maintained at 40<sup>0</sup>C to obtain the powdered form of extract. This powder was then suspended in 2 % of gum acacia in distilled water in order to prepare the stock suspension of powdered extract. Which was labeled MI-ALC.

### **Chemicals and drugs:**

Methanol (Merck, India), ethanol (96 %), petroleum ether (60:80) (Merck, India) and tween-80 (Research-Lab, India) were purchased from respective vendors. Naproxen, sucralfate, ranitidine hydrochloride and omeprazole sodium were obtained as gift sample from Symed Labs, Hyderabad.

### **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 were 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 animal were fasted for 24 for pylorus ligation and naproxen induced ulcer and overnight fasted for ethanol induced ulcer. 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.

### **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 Organisation for Economical Co-operation and Development (OECD). The 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.

**Pylorus ligation induced ulcers<sup>17</sup>:**

Rats of either sex were divided into five groups with six rats in each group. Group 1 served 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* methanolic extract (MI-ALC) 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 10<sup>th</sup> day, 1 h after the respective treatments animals were anaesthetised 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 content and pepsin 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.).

**Estimation of mucin activity<sup>14</sup>:**

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 MgCl<sub>2</sub> 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 ( $\mu\text{g}$  /wet tissue (g)). Mucin content of the stomach was expressed as  $\mu\text{g}$  Alcian blue/g wet tissue.

**Estimation of pepsin activity<sup>15</sup>:**

Aliquots of 20  $\mu\text{l}$  of the gastric content were incubated with 500  $\mu\text{l}$  of albumin solution (5mg/ml, 0.06 N Hydrochloric acid) at 37<sup>o</sup>C for 10 minutes. The reaction was stopped with 200  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{g}$  of tyrosine/ml.

**Ethanol-induced ulcers<sup>17</sup>:**

Rats of either sex were divided into five groups with six rats in each group. Group 1 served as control group and received vehicle (2% gum acacia in distilled water, 1 ml/kg, p.o.), group 2 received reference standard sucralfate 200 mg/kg, p.o. while group 3-5 received MI-ALC at doses of 100, 200 and 400 mg/kg, p.o. respectively for the period of 10 days. On the 10<sup>th</sup> day, 1 hour after the respective treatments, 96% ethanol (5ml/kg, p.o.) was administered to the overnight fasted rats of all groups. 1 h later, rats were sacrificed. The stomach was removed, inspected internally for ulcerated area and mucin content was determined by using above mentioned method. Ulcerated area was calculated by using afore mentioned method.

**Naproxen-induced ulcers<sup>24, 25</sup>:**

Rats were divided into three sets as A, B and C with five groups in each set. Further, each group consisted of six rats. Group 1 served as control and received vehicle (2% gum acacia in distilled water, 1 ml/kg, p.o.), group 2 received reference omeprazole 30 mg/kg, p.o. while group 3-5 received various doses (100, 200 and 400 mg/kg) of MI-ALC respectively. Rats in set A, B and C were treated for 10, 20 and 30 days respectively. Naproxen at the dose of 50 mg/kg, p.o. was administered for three consecutive days starting from 7<sup>th</sup> day for 10 days treatment period in set A, 17<sup>th</sup> day for 20 days treatment period in set B and on 27<sup>th</sup> 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 sacrificed on completion of 10<sup>th</sup> day, 20<sup>th</sup> day and 30<sup>th</sup> day respectively. The stomach of each rat was removed, inspected internally and ulcerated area was calculated by using aforementioned method.

**Statistical analysis:**

The results are expressed as mean  $\pm$ SEM. The statistical analysis was done by using GraphPad prism 5.0. The statistical analysis of all the results was carried out using one way ANOVA followed by Dunnett's test  $p < 0.05$  was considered as significance.

**Results****Acute toxicity test:**

In oral toxicity study administration of the extract of the graded doses 175, 550, 1750 and 2000 did not caused death of mice. MI-ALC was found to be safe upto a dose of 2000 mg/kg, p.o.

**Dose selection:**

Based upon toxicity studies and pilot studies (data not shown) three different doses of MI-ALC i.e 100, 200 and 400 mg/kg were selected for antiulcer investigation.

**Pylorus ligation induced ulcers (Modified method):**

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. All the doses of MI-ALC produced significant reduction in ulcerated area ( $p < 0.001$ ) (Table 1). At the dose of 100 mg/kg, p.o., MI-ALC produced significant ( $p < 0.01$ ) reduction in ulcerated area when compared against control group. Also at the dose of 200 mg/kg, p.o. ( $p < 0.01$ ) and 400 mg/kg ( $p < 0.001$ ) significant reduction in volume of gastric fluid and significantly reduction in acidity and pepsin content at the dose of 100 mg/kg, p.o. ( $p < 0.01$ ), 200 and 400 mg/kg, p.o. ( $p < 0.001$ ). In addition of pH of gastric fluid significantly increased at the dose 100 mg/kg, p.o. ( $p < 0.001$ ), 200 and 400 mg/kg, p.o. ( $p < 0.001$ ) and also significantly increased mucin content at the dose of 200 and 400 mg/kg, p.o. ( $p < 0.001$ ) as compared to control group. However, the doses 200 and 400 mg/kg, p.o. were found to be significant ( $p < 0.001$ ) in this regard. Ranitidine (100 mg/kg, p.o.) produced significant ( $p < 0.001$ ) reduction in ulcerated area, volume, acidity and pepsin content and also significant increases in pH and mucin content as compared to control group rats (Table 1).

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

Sr. No.	Treatment	Dose (mg/kg)	Ulcerated Area	Volume (ml)	pH	Acidity	Mucin ( $\mu\text{g}$ Alcian blue/g wet tissue)	Pepsin ( $\mu\text{g}$ tyrosine / ml)
1	Vehicle control (Pylorus ligated)	---	101.8 $\pm$ 2.77	19.82 $\pm$ 0.90	1.50 $\pm$ 0.22	113.2 $\pm$ 4.99	3.82 $\pm$ 0.44	67.96 $\pm$ 1.71
2	Ranitidine	100 mg/kg, p.o	17.25 $\pm$ 2.82***	6.55 $\pm$ 1.13***	4.66 $\pm$ 0.21***	35.33 $\pm$ 2.41***	8.75 $\pm$ 0.38***	26.58 $\pm$ 1.94***
3	MI-ALC	100 mg/kg, p.o	80.00 $\pm$ 5.18**	17.17 $\pm$ 0.75 <sup>ns</sup>	2.66 $\pm$ 0.21**	92.50 $\pm$ 6.52**	3.08 $\pm$ 0.47 <sup>ns</sup>	56.92 $\pm$ 2.44**
4	MI-ALC	200 mg/kg, p.o	44.01 $\pm$ 2.99***	14.80 $\pm$ 1.02**	3.66 $\pm$ 0.21***	71.8 $\pm$ 2.91***	6.12 $\pm$ 0.31**	44.33 $\pm$ 3.47***
5	MI-ALC	400 mg/kg, p.o	19.99 $\pm$ 3.36***	7.08 $\pm$ 0.88***	4.33 $\pm$ 0.21***	46.67 $\pm$ 1.92***	7.66 $\pm$ 0.29***	28.2 $\pm$ 11.49***

Values are expressed as mean  $\pm$  SEM, n=6, Data was analysed by one way analysis of variance (ANOVA) followed by Dunnett's test. MI-ALC: *Madhuca indica* bark alcoholic extract \* $p < 0.05$ , \*\* $p < 0.01$ ,  $p < 0.001$ .

**Ethanol-induced ulcers:**

Oral administration of ethanol (96 %) to the control group clearly showed hemorrhagic band like lesions along with circular lesions developed in the glandular portion of the stomach indicating ulcerated area. MI-ALC at the dose of 200 mg/kg, p.o. showed significant reduction in ulcerated area ( $p < 0.01$ ) and significant ( $p < 0.05$ ) rise in mucin content. At 400 mg/kg, p.o. dose, MI-ALC more significantly reduced ulcerated area ( $p < 0.001$ ) and increase mucin content ( $p < 0.01$ ). However, the dose of 100 mg/kg, p.o. was found insignificant in this regard. Furthermore, sucralfate (200 mg/kg, p.o.) significantly ( $p < 0.001$ ) inhibited ulcer formation as evident from reduction in ulcerated area and significantly increased mucin content ( $p < 0.001$ ) when compared against control group (Table 2).

**Table 2 Effect of MI-ALC on ulcerated area and mucin content in ethanol induced ulcer model**

Group	Treatment	Dose (mg/kg)	Ulcerated area	Mucin ( $\mu\text{g}$ Alcian blue/g wet tissue)
1	Vehicle control (Ethanol 96%)	5 ml/kg, p.o.	160.6 $\pm$ 4.96	9.05 $\pm$ 0.58
2	Sucralfate	200 mg/kg, p.o.	59.20 $\pm$ 5.38***	18.83 $\pm$ 1.96***
3	MI-ALC	100 mg/kg, p.o.	148.9 $\pm$ 3.92 <sup>ns</sup>	12.69 $\pm$ 1.78 <sup>ns</sup>
4	MI-ALC	200 mg/kg, p.o.	133.4 $\pm$ 4.27**	15.96 $\pm$ 1.59*
5	MI-ALC	400 mg/kg, p.o.	69.76 $\pm$ 5.60***	17.91 $\pm$ 2.16**

Values are expressed as mean  $\pm$  SEM, n=6, Data was analysed by one way analysis of variance (ANOVA) followed by Dunnett's test. MI-ALC: *Madhuca indica* bark alcoholic extract \* $p < 0.05$ , \*\* $p < 0.01$ ,  $p < 0.001$ .

**Naproxen -induced ulcers**

In the chronic study, time dependent reduction in ulcer area was found in control group, which is an indicative of self healing in gastric mucosa. After 10 and 20 days of pretreatment period, MI-ALC at the dose of 400 mg/kg, p.o. showed significant reduction in ulcerated area ( $p < 0.001$ ). Other two doses of MI-ALC produced insignificant reduction in ulcerated area. On the other hand, after 30 days of pretreatment period, MI-ALC produced significant reduction in ulcer index irrespective of the doses, although the level of significance was different, when compared against control groups (Table 3). Also, after 30 days of pretreatment period, all the doses of MI-ALC produced significant reduction in ulcerated area indicating its antiulcer activity. The reference standard omeprazole (30 mg/kg, p.o.) was found to be more effective in reducing ulcerated area. A time dependent reduction in the ulcerated area was observed from 20 to 30 days treatment period (Table 3).

Table 3 Effect of MI-ALC on ulcerated area in naproxen induced ulcer model

Groups	Treatment	Dose	Ulcerated Area		
			10 Days Treatment period	20 Days Treatment period	30 Days Treatment period
1	Vehicle control (Naproxen) 30 mg/kg, p.o.)	30 mg/kg, p.o.	10.96 ± 1.54	9.48 ± 1.53	7.50 ± 0.53
2	Omeprazole	30 mg/kg p.o	5.85 ± 0.40***	3.81 ± 0.39***	1.06 ± 0.27***
3	MI-ALC	100 mg/kg, p.o	8.91 ± 0.75 <sup>ns</sup>	6.62 ± 0.18*	4.42 ± 0.86**
4	MI-ALC	200mg/kg, p.o	7.67 ± 0.48**	6.07 ± 0.11**	3.43 ± 0.89***
5	MI-ALC	400mg/kg, p.o	5.87 ± 0.70***	5.05 ± 0.18***	2.93 ± 0.67***

Values are expressed as mean ± SEM, n=6, Data was analysed by one way analysis of variance (ANOVA) followed by Dunnett's test. MI-ALC: *Madhuca indica* bark alcoholic extract. UA-Ulcerated area, UI-Ulcer index, \*p<0.05, \*\*p<0.01, p<0.001.

### Discussion

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

Peptic ulcer is the result of hypersecretion of gastric acid and/ or decrease in gastric mucosal protection mechanism. It is generally accepted that ulcer results from an imbalance between aggressive factors and the maintenance of the mucosal integrity through the endogenous defense mechanism<sup>16</sup>. To regain the balance, different therapeutic agents including herbal preparations are used to inhibit the gastric acid secretion or to boost the mucosal defense mechanism by increasing mucus production<sup>17</sup>.



Pylorus ligation, ethanol and naproxen-induced gastric ulcer have been widely used for the experimental evaluation of anti-ulcer activity. Pylorus-ligation ulcers are caused due to accumulation of gastric acid and pepsin, which leads to auto-digestion of gastric mucosa<sup>18</sup>. 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<sup>19</sup>. The methanolic extract of *M. indica* reduced the ulcerated area and the pepsin content of gastric fluid along with an increase in pH of gastric fluid and mucin content of stomach wall.

Ethanol is widely used to induce experimental gastric ulcer in animals<sup>20</sup>. Ethanol rapidly penetrates the gastric mucosa, and it causes membrane damage, cell exfoliation, erosion, and ulcer formation. It has been suggested that ethanol induced gastric damage is mediated by the generation of free radicals<sup>21</sup>. Ethanol also induce gastric damage possibly through leukotrienes production with involvement of 5-lipoxygenase in the formation of ulcer lesion. Prostaglandins also play a role in ethanol-induced ulcer<sup>22</sup>. In our investigation, MI-ALC prevented gastric mucosal injury induced by ethanol, suggesting its possible role in enhancing cytoprotective mechanism of the gastric mucosa. Furthermore, the cytoprotective effect can also be reflected from reduced ulcer index and mucin content.

Many NSAIDs (non-steroidal anti-inflammatory drugs) have been widely used clinically as anti-inflammatory, analgesic agents. However, ulcerative lesions of the gastrointestinal tract are one of the major side effects of NSAIDs, and they are the major limitation to their use as anti-inflammatory drugs<sup>23, 25</sup>. Naproxen is a non-corticosteroid drug with anti-inflammatory, ulceration, and petechial bleeding in the mucosa of stomach as an adverse effect<sup>26, 27</sup>. Production of oxygen free radicals and lipid peroxidation play a crucial role in the development of the gastric antral ulceration induced by naproxen<sup>28</sup>. Also, naproxen causes ulceration mainly in gastric antrum and hence naproxen-induced gastric antral ulcer model can be considered suitable in the human situation<sup>23, 25</sup>. MI-ALC produced significant reduced ulcerated area in rats with naproxen induced ulcers.

The preliminary phytochemical studies revealed the the presence of flavanoids in many medicinal plants like *G. glabra*<sup>38</sup>, *Embllica officinalis*<sup>32,33</sup>, *Tinospora cordifolia*<sup>34,35</sup>, *Morus alba*<sup>36</sup> and *Cordia dichotoma*<sup>37</sup>. Various flavanoids have been reported for its anti-ulcerogenic activity with good level of gastric protection(Ref). So the possible mechanism of anti-ulcer action of methanolic extract of *Madhuca. Indica* J. F. Gmel leaves may be due ti its flavanoids content. In this study we observed that *Madhuca. Indica* J. F. Gmel provides significant anti-ulcer and cytoprotective effect against gastric ulcers in rats. However, further investigation is necessary to determine the mechanism of action.

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