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Elucidation of gastro-protective activity of Morin in pylorus ligation induced gastric ulcer via modulation of oxidative stress

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ABSTRACT

Peptic ulcer owes its pathological features to dysregulated oxido-nitrosative and necrotic changes in gastrointestinal mucosa. Morin possessed an array of pharmacological properties including anti-inflammatory and antioxidant activity. Hence, objective of present investigation was to unravel the antiulcer potential of morin in pylorus ligation induced gastric ulcer in laboratory animals. Gastric ulcer was induced in male Wistar rats (180-200 gm) by ligation of pylorus portion of the stomach. Morin (10, 30 and 100 mg/kg, p.o.) was provided as pretreatment 1 hr prior to pylorus ligation. Volume of gastric fluid, pH of gastric fluid, free acidity were measured whereas ulcer area, ulcer index, mucin content, superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) were determined in the gastric tissue. Histological evaluation of the stomach was also carried out. Pretreatment with Morin (30 and 100 mg/kg) significantly ($P < 0.01$ and $P < 0.05$) decreased ulcer area, ulcer index and total acidity. It also significantly increased pH of the gastric fluid. Rats treated with Morin (30 and 100 mg/kg) significantly ($P < 0.01$ and $P < 0.05$) attenuated decreased level of SOD and increased level of MDA. It also showed attenuation in histological alteration induced by pylorus ligation. In conclusion, the possible mechanism by which Morin exerts its gastro-protective action may be due to its free radical quenching ability to regulate down elevated oxidative stress.

Keywords: Antiulcer, Morin, Oxidative stress, Pylorus ligation induced ulcer

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INTRODUCTION

One of the most important diseases of the digestive system that affects thousands of people and is gaining importance due to its high rate of mortality and morbidity is Peptic Ulcer Disease (PUD) [1, 2]. If untreated, it can cause upper gastrointestinal bleeding. Peptic ulcer is a multi-etiologic chronic disease. Studies have shown that an imbalance between mucosal defensive factors (mucin, prostaglandin, bicarbonate, nitric oxide and growth factors) and injurious factors (acid, pepsin, bile and *H. pylori*) in stomach contributes to its pathophysiology [2]. Other factors include stress, smoking, nutritional deficiencies, ingestion of NSAIDS [3].

In the process of new drug development, animals model played central dogma role [4]. Peptic ulcer induced by pylorus ligation in animals mimics all the pathobiological condition that are present clinically [5]. Pylorus-ligation enhances mucosal damage through altered antioxidant levels followed by ulceration. Thus, it seems that free radical generation is associated with pylorus ligation induced ulceration in rats [6].

Therapeutic strategy for the treatment of gastric ulcer aims to attenuate the injurious factors and to sustain the defensive factors [7]. It was accomplished by the use of proton pump inhibitors, histamine receptor blockers, drugs affecting mucosal barrier and PG analogs [8]. Due to their prominent adverse effects like arrhythmia, impotence, gynecomastia, it is very important to develop an alternative therapy for the treatment of gastric ulcer. The best and safer alternative is the use of herbal drugs.

Flavonoids are the group of polyphenolic compounds possessing antioxidant, anti-inflammatory, cytotoxic and anti-nociceptive properties [9-11]. Morin (2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one; a light yellowish pigment) is one of the naturally occurring bioflavonoid, isolated from members of Moraceae family. It is mostly found in different herbs and fruits including onion, seedweeds, almond, fig and Osage orange [12]. Morin possesses an array of pharmacological properties such as antioxidant, anti-cancer, chemoprotective, anti-inflammatory [12-14]. It also showed anti-asthmatic, anti-allergic, anti-diabetic, anti-hypertensive and hepatoprotective potential [15-18]. However, there is a lack of scientific data to prove its anti-ulcer properties. Hence, the aim of the present investigation was to evaluate the effect of morin on pylorus-ligation induced ulcer in laboratory rats.

MATERIALS AND METHODS

1.1. Chemicals:

Morin (Sigma-Aldrich, USA), Ranitidine (Symed Pharmaceutical Pvt. Ltd., Hyderabad). Bovine serum albumin, Tris buffer, sucrose, copper sulphate, sodium potassium tartarate, ethylene diamine tetraacetic acid disodium salt, Folin's phenol reagent, sodium hydroxide, sodium bicarbonate, potassium chloride, hydrochloric acid and conc. Sulphuric acid was purchased from S.D. Fine Chemicals, Mumbai, India.

1.2. Animals:

Healthy adult male Wistar rats (180-200 g) were obtained from the National Institute of Biosciences, Pune (India). The animals were housed in groups of 6 in solid bottom polypropylene cages. They were maintained at 24 ^{0}C ± 1^{0}C, with a relative humidity of 45-55% and 12:12 h dark/light cycle. The animals were acclimatized for a period of two weeks and were kept under pathogen-free conditions. The animals had free access to standard pellet chow (Pranav Agro-industries Ltd., Sangli, India) throughout the experimental protocol. The animals had access to filtered water. All experiments were carried out between 09:00 and 17:00 h. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune (CPCSEA/PCL/39/2014-15) and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation.

1.3. Induction of pylorus ligation induced gastric ulcer:

The method of Shay rat ulcer was adopted [6, 19]. Rats were fasted for 24 h. and randomly selected and divided into following groups of 6 animals each as follows:

Group I: **Sham Control: (S):** Rats received a 0.5 ml of distilled water, p.o.
Group II: **Pylorus ligation control: (PLC):** Rats received a 0.5 ml of distilled water, p.o.
Group III: **Ranitidine (50 mg/kg) treated: R (50):** Rats received a 0.5 ml of Ranitidine (50 mg/kg), p.o.
Group IV: **Morin (10 mg/kg) treated: M (10):** Rats received a 0.5 ml of morin (10 mg/kg) p.o.
Group V: **Morin (30 mg/kg) treated: M (30):** Rats received a 0.5 ml of morin (30 mg/kg) p.o.
Group VI: **Morin (100 mg/kg) treated: M (100):** Rats received a 0.5 ml of morin (100 mg/kg), p.o.

The morin was freshly prepared in three different dosages (10, 30 and 100 mg/kg) and administered to the animals [15]. After the pretreatment period of 1 h, the animals were anesthetized with anesthetic ether. The abdomen was opened through a small midline incision below the xiphoid process; pylorus portion of the stomach was slightly lifted out and ligated. The 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. The animals were allowed free access to water. Nineteen hours after pylorus ligation the rats were sacrificed by etheral anasthesia, and the stomach was removed. For determination of ulcer area, each stomach was incised along the greater curvature and washed with normal saline and was scanned using CCD scanner at a magnification of 2400 dpi. The images were processed using image J software and Adobe Photoshop to determine ulcer area. The ulcer index and % inhibition was determined as per the method described previously [20-22].

1.4. Determination of gastric pH and total acidity:
The entire gastric content was transferred into centrifuge tubes. It was used for estimation of gastric pH and total acidity. The tubes were centrifuged at 1000 rpm for 10 min, and the gastric volume was directly read from the graduation on the tubes. The supernatant was then collected, and pH was determined using a digital pH meter. Total acidity was determined by titrating 1.0 ml of gastric juice against N/10 NaOH to pH 7 using phenolphthalein as the indicator and were expressed in terms of mEq/L [5].

1.5. Biochemical estimation:
500 mg tissue from the glandular portion of stomach was excised, washed, chopped and homogenized at 3000 rpm in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% w/v. The homogenates were centrifuged at 10,000 g at 0 ^{0}C for 20 min and was employed to estimate various biochemical parameters viz., total protein, superoxide dismutase (SOD) contents, glutathione (GSH) content, Lipid peroxidation [malondialdehyde (MDA)] content and mucin content according to methods described previously [10, 23-27].

1.6. Histopathological studies:
Freshly excised stomach of one animal from each group was washed with saline and preserved in 10% formaldehyde solution for histopathological studies. It processed for 12 hrs. using isopropyl alcohol, xylene and paraffin embedded for light microscopic study (Nikon E200). Paraffin embedded tissue section cut at 5µm thickness were prepared and stained by deparaffination using hematoxylin and eosin stain (H & E) to verify the morphological assessment of stomach damage. Photomicrographs were captured at a magnification of 40 X.

1.7. Statistical Analysis:
All the results were expressed as mean ± S.E.M. Statistical comparisons were made between drug-treated groups and acetic acid control groups. The data was statistically analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests using GraphPad Prism 5.0 software (GraphPad, San Diego, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

2.1. Effect of Morin on pylorus ligation induced gastric ulcer in rats
Pylorus ligation of the stomach for 19 hrs. in animals resulted in the accumulation of the gastric fluid, gastric lesions, and deep erosions thereby indicating the ulcer production (Fig.1).

2.2. Effect of Morin on pylorus ligation induced alteration in ulcer area, ulcer index and percent protection
Morin treated (30 and 100 mg/kg) groups showed a significant decrease ($P < 0.01$ and $P < 0.05$) in ulcer area and ulcer index as compared to that of PL control group. Ranitidine (50 mg/kg) also showed significantly ($P < 0.001$) reduced ulcer area and ulcer index as compared to that of PL control group. Oral administration of Ranitidine (50 mg/kg) has shown 60.90% protection, whereas Morin (10, 30 and 100 mg/kg) showed 44.86%, 52.27% and 49.96% protection (Table 1).

Table 1. Effect of Morin on gastric parameters of pylorus ligation induced gastric ulcer model
Table 2.

Treatment	Ulcer Area	Ulcer Index	% Protection	Mucin content (µg/g stomach weight)	pH of gastric fluid	Total Acidity
S	-	-	-	--	-	-
PLC	13.50 ± 0.28	12.35 ± 0.21	-	68.15 ±0.57	2.26 ± 0.08	70.00 ± 7.07
R (50)	2.00 ± 0.11***	3.72 ± 0.27***	60.90	33.14 ± 0.96***	4.32 ± 0.15***	22.50 ± 7.50***
M (10)	11.25 ± 0.25	10.79 ± 1.18	44.86	59.50 ± 0.88	2.62 ± 0.30	65.00 ± 5.00
M (30)	6.00 ± 0.57**	5.80 ± 1.43**	52.27	38.39 ± 0.83**	3.97 ± 0.11**	37.50 ± 4.77**
M (100)	9.75 ± 0.25*	8.91 ± 1.18*	49.96	41.69 ± 0.34*	3.61 ± 0.22**	42.50 ± 6.29**

*Data are expressed as mean ±S.E.M. (n=5) and analyzed by one way ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to pylorus ligation control group. S: Sham control; PLC: Pylorus ligation control; R (50): Ranitidine (50 mg/kg) treated; M (10): Morin (10 mg/kg) treated; M (30): Morin (30 mg/kg) treated and M (100): Morin (100 mg/kg) treated.*

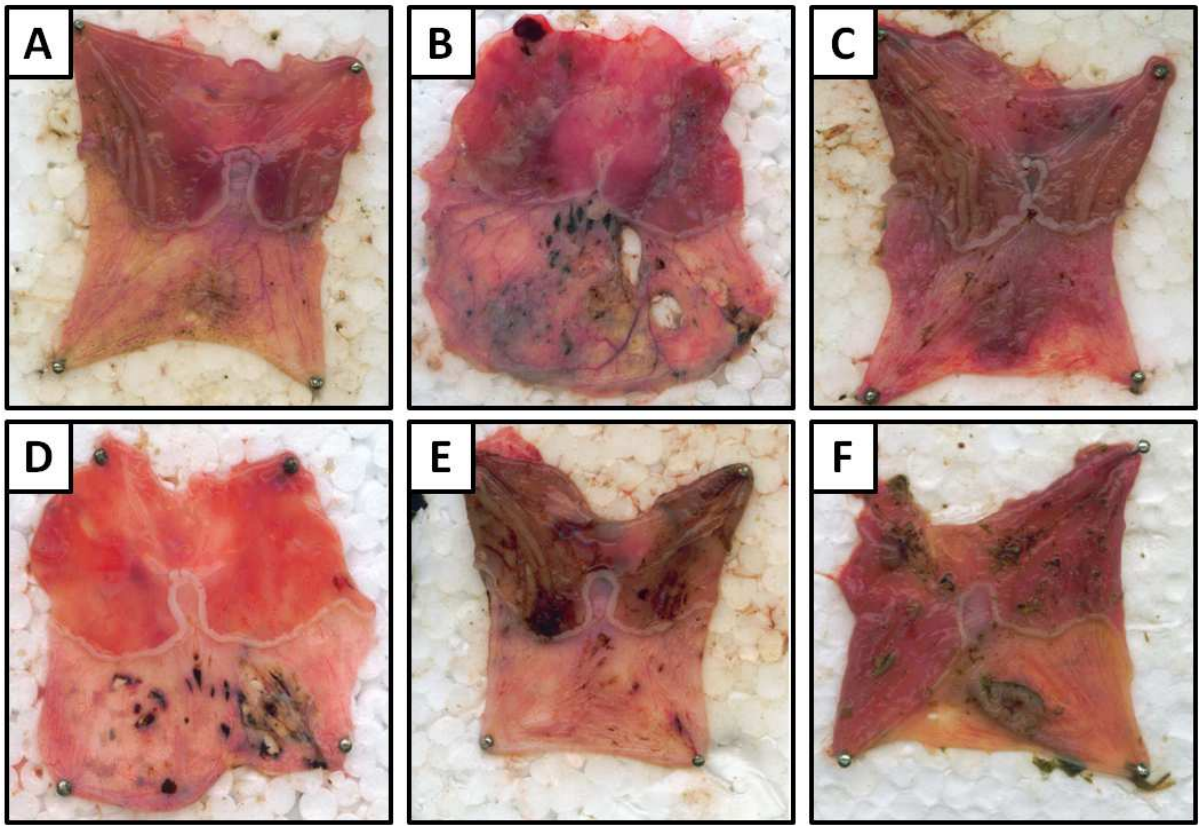

Fig. 1 Representative stomachs images of rats after pylorus ligation induced gastric ulcer. (A) Sham control rat, (B) Pylorus ligation induced ulcer rat, (C) Ranitidine (50 mg/kg) treated rat, (D) Morin (10 mg/kg) treated rat, (E) Morin (30 mg/kg) treated rat and (F) Morin (100 mg/kg) treated rat.

2.3. Effect of Morin on pylorus ligation induced alteration in pH of gastric juice and total acidity:

Compared to that of PL control group, Morin (30 and 100 mg/kg) treated groups showed an increase ($P < 0.01$) in gastric pH. Rats treated with Ranitidine (50 mg/kg) showed significantly ($P < 0.001$) increased gastric pH as compared to that of PL control group (Table 1).

There was a significant ($P < 0.001$) decrease in total acidity of Ranitidine (50 mg/kg) treated group as compared to that of PL control group. Whereas Morin treated groups (30 and 100 mg/kg) also showed a significant ($P < 0.01$) decrease in total acidity as compared to PL control rats (Table 1).

2.4. Effect of Morin on pylorus ligation induced alteration in Mucin content:

Pylorus ligation resulted in significant ($P < 0.001$) decreased in mucin content in Ranitidine (50 mg/kg) treated group as compared to that of PL control group. Whereas treatment with Morin (30 and 100 mg/kg) showed a significant ($P < 0.01$ and $P < 0.05$) decrease in mucin content as compared to that of PL control group. (Table 1)

2.5. Effect of Morin on pylorus ligation induced alteration in SOD and GSH:

SOD and GSH level was decreased significantly ($P < 0.001$) in PL control group as compared to sham control rats. However, Ranitidine (50 mg/kg) treated group showed significant increased ($P < 0.001$) in SOD level as compared to that of PL control group. Rats treated with Morin (30 and 100 mg/kg) showed significant ($P < 0.01$ and $P < 0.05$) increase in SOD level as compared to that of PL control group (Fig. 2B). Administration of Morin (30 and 100 mg/kg) failed to produced any significant increase in GSH level as compared to that of PL control group (Fig. 2C).

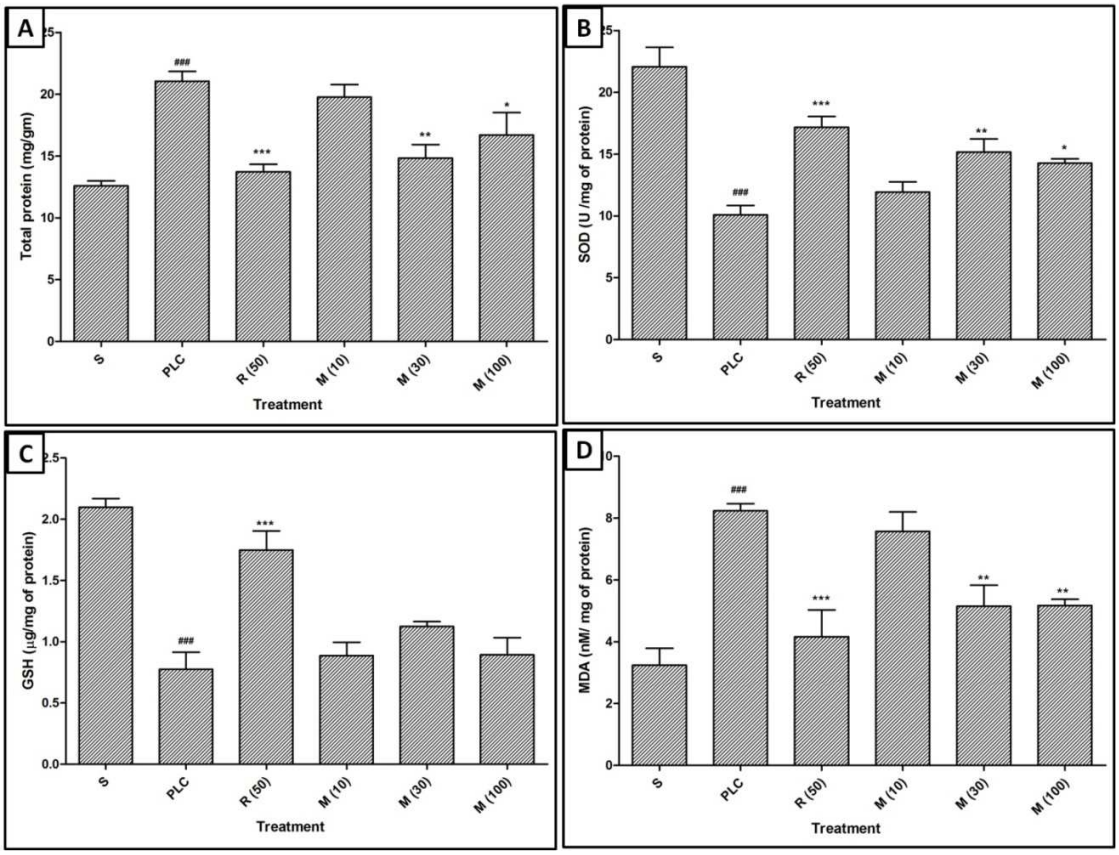

Fig. 2 Effect of Morin on pylorus ligation induced alteration in (A) Total protein level, (B) SOD level, (C) GSH level and (D) MDA level in gastric tissue.

*Data are expressed as mean ±S.E.M (n=5) and analyzed by one way ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to acetic acid control group. S: Sham control; PLC: Pylorus Ligation control; R (50): Ranitidine (50 mg/kg) treated; M (10): Morin (10 mg/kg) treated; M (30): Morin (30 mg/kg) treated and M (100): Morin (100 mg/kg) treated*

2.6. Effect of Morin on pylorus ligation induced alteration in MDA and total protein:

Pylorus ligation resulted in a significant increased ($P < 0.001$) in MDA and total protein level in PL control rats as compared to sham control rats. There was significant ($P < 0.001$) decrease in MDA and total protein level in Ranitidine (50 mg/kg) treated group as compared to that of PL control group. Morin (30 and 100 mg/kg) treated groups also showed significant ($P < 0.05$ and $P < 0.01$, resp.) decrease in MDA and total protein as compared to the PL control group (Fig. 2D and 2A, resp.).

2.7. Effect of Morin on pylorus ligation induced alteration in histology of stomach:

Figure 3A depicted the normal architecture of stomach from sham control rats devoid of inflammatory infiltration, necrosis, oedema, haemorrhage and congestion. Epithelium remained intact in the stomach of sham control rats. Pylorus ligation of the stomach in animals resulted in the loss of mucosal integrity, epithelial necrosis, inflammatory infiltration and hemorrhage (Fig. 3B). However, Ranitidine (50 mg/kg) treated groups showed decreased hemorrhage, necrosis and oedema induced by pylorus ligation (Fig. 3C). Whereas morin (30 and 100 mg/kg) treated rats also showed decreased hemorrhage, necrosis and oedema formation (Fig. 3E and 3F, resp.). Stomach tissue from Morin (10 mg/kg) treated rats showed the presence of inflammatory inflammation, necrosis, oedema and loss of mucosal integrity (Fig. 3D) (Table 2).

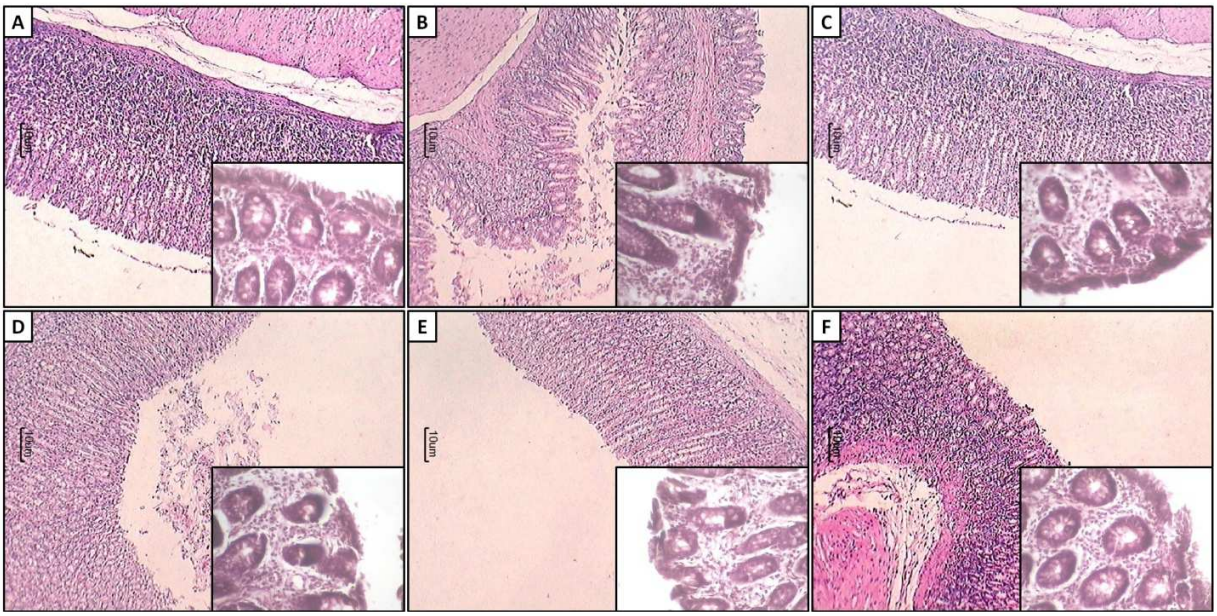

Fig. 3 Photomicrographs of stomach sections from pylorus ligated rats stained with H & E. Stomach microscopic image of (A) Sham control rat, (B) Pylorus ligation induced ulcer rat, (C) Ranitidine (50 mg/kg) treated rat, (D) Morin (10 mg/kg) treated rat, (E) Morin (30 mg/kg) treated rat and (F) Morin (100 mg/kg) treated rat. Images (at 100X magnification) and respective inset (at 400X magnification) are typical and representative of each study group.

Table 3. Effect of Morin on pathological changes of rat stomach in pylorus ligation induced ulcer

Treatment	Necrosis	Haemorrhage	Inflammation	Congestion	Oedema	Cellular in filtration
S	-	-	-	-	+	-
PLC	+++	++++	+++	+++	+++	+++
R (50)	+	+	-	+	+	-
M (10)	+++	+++	+++	+++	+++	++
M (30)	++	+	++	+	+	+
M (100)	++	++	+	+	+	++

S: Sham control; PLC: Pylorus ligation control; R (50): Ranitidine (50 mg/kg) treated; M (10): Morin (10 mg/kg) treated; M (30): Morin (30 mg/kg) treated and M (100): Morin (100 mg/kg) treated.

Note:

-: no abnormality detected, +: damage/ active changes up to less than 25 %

++: damage/ active changes up to less than 50 %, +++: damage/ active changes up to less 75 %

++++: damage/ active changes up to more than 75 %

DISCUSSION

Peptic ulcer is a chronic and most prevalent modern age epidemics affecting almost 10% of world population [8]. It has been well documented that the imbalance between acid and pepsin as well decrease in strengthening of mucosal defensive barrier leading to peptic ulcer disorder. The scientific literature is evident that active enrollment of reactive oxygen species and free radical plays a vital role in etiology and pathophysiology of peptic ulcer [28]. Moreover, excess consumption of alcoholic beverages, overuse of anti-inflammatory agents, stress as well as *Helicobacter pylori* infection also leads to gastric ulcers [29].

The relation between acid output and formation of acute gastric mucosal lesions (AGML) has been previously reported [28]. Thus, the pylorus-ligation induced gastric ulcer is an important animal model to evaluate the possible changes that are associated with gastric content [5, 6]. Increase in the formation of acid and pepsin in the stomach resulted in the formation of ulcer which is the basis of pylorus ligation induced gastric ulcer. Moreover, quality and quantity of gastric mucus secretion determine the mucosal defense barrier against the unpleasant attack of acid-pepsin in the stomach [7]. There are number of mechanisms that has played a role in the prevention of gastric mucosa from vicious ulceration process. It includes inhibition of release of hydrogen ions during peristalsis as well as increase in mucus secretion from the stomach [30]. Moreover, it has been reported that histamine plays an intermediate role in the formation of ulcers in pylorus ligated animals via increased gastric secretion stimulated by

gastrin, vagal stimulation, and cholinergic agents [31]. However, enhanced gastric wall mucus secretion played a defensive role in inhibition of pylorus-ligation induced gastric mucosal damage. In the present study, administration of Morin significantly inhibits the formation of gastric ulcer that may be due to its mucosecretary property.

A study carried out by researcher reported that in the presence of pepsin accumulated acid caused the formation of gastric ulcer in pylorus ligated rats [32]. However, the activation of pepsinogen to pepsin is only occurred at about pH 2. Moreover, inactivation of pepsin is occurred at about pH 6. However, in between pH 3 and 6 the pepsin is stable but remain inactive [6]. Thus, in the present study the gastric pH of pylorus ligated control rats is about 2-3 that is favorable for pepsin to induce gastric ulcers. However, administration of morin caused increase in gastric pH which may inhibit the activation of pepsin thus decreasing the formation of gastric ulcer.

Use of antioxidant supplements or free radical scavengers for a variety of diseases has been well recognized as an alternative treatment for oxidative stress related diseases [33-39]. The formation of vicious by-products of a normal cellular redox process i.e. generation of Reactive oxygen species (ROS) caused lipid peroxidation and cellular damage of cell membrane [40, 41]. However, endorsement of endogenous enzymatic and non-enzymatic antioxidants like superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and total glutathione (GSH) played an important role in neutralizing free radicals and maintained normal redox balance in body [42-44]. Any disturbance in this process leads to the generation of excess free radical [45]. Thus elevated oxidative stress caused diminished membrane integrity, enzyme and receptors activities, and activation of cells that resulted in gastric ulceration. Hence, the therapeutic moiety with free radical scavenging ability has been proven as effective strategies for prevention of gastric damage.

Superoxide dismutase played an important role in the reduction of superoxide radicals to hydrogen peroxide that further converted to nontoxic water molecule by glutathione peroxidase [46-48]. Whereas, a non-enzymatic component i.e. GSH has a pivotal role in the maintenance of gastric mucosal cell integrity and has a central role in the antioxidant network [49]. In a variety of disease states elevated free radical generation caused lipid peroxidation that is the hallmark of ROS generation reflected by increased level of MDA [50]. During lipid peroxidation, formation and propagation of lipid radicals occurred that leads to uptake of oxygen and rearrangement of double bonds in unsaturated lipids which eventually results in the destruction of membrane lipids [51, 52]. As biological membranes are enriched source of unsaturated fatty acids and bathed in oxygen-rich metal containing fluid thus, this lipid membrane is more prone to peroxidative attack [53]. In pyloric ligation induced gastric ulcer alters the level of endogenous SOD, and GSH caused elevated free radical generation resulted in induction of tissue injury and formation peptic ulcer. Treatment with morin resulted in the increased SOD level that may decrease of lipid peroxidation that reflected by decreased MDA level in gastric tissue.

It has been reported that infiltration of inflammatory cells resulted in alteration of mucosal integrity and neutrophils are one of the major inflammatory cells amongst them [6]. The activation of neutrophils causes enhanced ROS formation that has potential to damage mucosal microvasculature [5]. In the present investigation, histopathological examination of stomach tissue from pylorus ligated control rats showed elevated inflammatory infiltration, however, administration of morin showed inhibition in this inflammatory infiltration thus shown to have protective effect against gastric ulcers induced by pylorus ligation.

In conclusion, the result of the present investigation showed that morin exerts its gastro-protective effect through its antisecretory and antioxidant potential to attenuate pylorus ligation induced ulcer in laboratory rats.

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