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Effects of Flavonoids from *Morus Alba* Leaves Extract on Experimentally Induced Gastroesophageal Reflux Disease (GERD) in Rats

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ABSTRACT

The current study investigated the effect of standardized extract of *Morus alba* leaves on gastroesophageal reflux disease (GERD) in rats. The ethyl acetate fraction of *Morus alba* leaves (MAEF) in doses of 50, 100 and 200 mg/kg were administered orally twice daily for 5 days and omeprazole in the dose of 30 mg/kg one hour prior to the induction of disease. The gastric wall mucus level was increased (from 150.4± 14.3 to 271.0± 18.5 g/g wet glandular tissue) and levels of plasma histamine (from 261.0±15.10 to 191.9±12.31 IU/milligram protein) and H^+-K^+-ATPase were significantly decreased (from 1.16±0.04 to 0.50±0.02 mmol of Pi liberated/min/mg protein) in standardized extract treated group. Treatment with standardized extract reduced the lipid peroxidation (from 0.47±0.02 to 0.41±0.02 nmol of MDA/min/mg protein) and SOD (from 166.2±13.1 to 101.1±9.8 units (U) of SOD activity/mg protein) and increased in levels of catalase (from 25.2±1.2 to 30.4±1.3 µmol of H_2O_2 consumed/min/mg protein) and GSH (from 45.5±2.8 to 59.6±3.7 nmol/g protein). *Morus alba* extract attributed to its antisecretory and antioxidant potential as that of quercetin (100 mg/kg) and proton pump blockers (30 mg/kg) to treat gastroesophageal reflux disease.

Keywords: Gastroesophageal reflux disease, histamine, mucus, lipid peroxidation.

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INTRODUCTION

Morus alba L (Moraceae) commonly known as white mulberry grown in a wide range of climatic, topographical regions and It is native of India, China and Japan. The plant is reported to contain the phytochemicals such as tannins, phytosterols, sitosterols, saponins, triterpenes, flavonoids, benzofuran derivatives, morusimic acid, anthocyanins, anthroquinones, glycosides and oleanolic acid [1-4]. It plays an imperative role in pharmaceutical industry as it contains moranoline, albafuran, albanol, morusin, kuwanol, calystegin and hydroxymoricin [5-7]. *Morus alba* has been found to act against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* as antibacterial [8], alcohol induced ulcers [9], anti-inflammatory activity and stimulate the production of nitric acid, prostaglandin E_2 and cytokines [10-11]. The flavonoids isolated from leaves of *Morus alba* showed free radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in *in vitro* studies [12]. Flavonoids are the potent antioxidant compounds and has amid with diverse pharmacological actions [13-15]. Quercetin, a member of the flavonoids family, is one of the most prominent dietary antioxidants. It has been reported to inhibit the acid production in the stomach [16-17] and prevent the oxidative stress in gastric ulcer and protect gastric lesions in glandular portion of the stomach [18]. Gastroesophageal reflux disease (GERD) is a condition in which the stomach contents (food or acid) flow upward into the esophagus. The most-common symptoms are heartburn [19], regurgitation, trouble swallowing (dysphagia) etc and less-common symptoms are sore throat (odynophagia) increased salivation (also known as water brash) nausea [20], chest pain etc. However, there are no reports on the role played by the flavonoid rich compounds of *M. alba* leaves on gastroesophageal reflux diseases induced ulcers. Therefore, the current study was undertaken to investigate the effect of standardized extract of *Morus alba* leaves and its fractionated compound quercetin on gastroesophageal reflux disease (GERD) in rats.

MATERIALS AND METHODS

Plant Material:

Plant leaves were collected in the month of June from medicinal plant garden near pilot plant of CSIR-National Botanical Research Institute, and specimen was prepared and matched with the existing live reference.

Extraction, Isolation and Characterization of flavonoids:

After collection and authentication, plant materials (200 grams) were extracted thrice with 65% Methanol (HPLC grade) containing 2 g/L TBHQ at 70^0C on a water bath using soxhlet extractor for 3 hours and filtered, concentrated on rotavapour (Buchi, USA) to get an aqueous extract containing flavonoids. The resulted extract were subsequently extracted thrice in petroleum ether, diethyl ether and ethyl acetate using separating funnel by following the method of Subramanian and Nagarajan (1969)[21]. Petroleum ether fraction (Fr-I) was discarded due to presence of fatty substances. Diethyl ether fraction (Fr-II) was used for

analysis of free flavonoids and ethyl acetate fraction (Fr-III) was hydrolyzed (acid hydrolysis) to cleave glycosides by refluxing with 7% H_2SO_4 (10 ml/g plant material) for 2 hours at 85^0C for analysis of bound flavonoids. Resulting mixture was filtered and re-extracted thrice with ethyl acetate. All ethyl acetate layers were pooled together separately and neutralized with 5% NaOH. Then Diethyl ether fraction (free flavonoids) and ethyl acetate fraction (bound flavonoids) were evaporated in rotavapour to 1/10 of the initial volume [22], dried in lyophilizer, weighed and stored at -19^0C untill it was used. Completion of acid hydrolysis of ethyl acetate fraction was confirmed by spraying agent (i.e. 5% fehling solution and 1 % $AlCl_3$ solution) during TLC analysis. Flavonoids free from sugar part reacted with spraying agent (i.e. 5% fehling solution and 1% $AlCl_3$ solution) and gave colour reactions during TLC analysis. Flavonoids with sugar part did not react with spraying agent (i.e. 5% fehling solution and 1 % $AlCl_3$ solution) and did not give colour reactions during TLC analysis [23]. Diethyl ether fraction gave colour reactions with spraying agent and it did not need acid hydrolysis.

The ethyl acetate fraction of *M. alba* leaves (MAEF) contains the highest amounts of flavonoids, so 15 g of this fraction was chromatographed over silica gel column to obtain purified fractions using various mobile phases in increasing polarity : PE (Fr.1-3); PE: EtOAc 7.5:2.5 (Fr. 4-6); PE: EtOAc 1:1 (Fr. 7-9); PE: EtOAc 2.5:7.5 (Fr. 10-12); EtOAc (Fr. 13-15); EtOAc:MeOH 9.9:0.1 (Fr. 16-18); EtOAc:MeOH 9.8:0.2 (Fr. 19-21); EtOAc: MeOH 9.5:0.5 (Fr. 22-24); EtOAc: MeOH 9:1 (Fr.25-27); EtOAc: MeOH 8.5:1.5 (Fr. 28-30); EtOAc: MeOH 8: 2 (Fr. 31-33); EtOAc: MeOH 7:3 (Fr. 34-36); EtOAc: MeOH 6:4 (Fr. 37-39); EtOAc: MeOH 1:1 (Fr. 40-42); EtOAc: MeOH 2.5:7.5 (Fr. 43-45); MeOH (Fr. 46-50); MeOH: H2O 7.5: 2.5 (Fr. 51-53); MeOH: H2O 1: 1 (Fr. 54-56); MeOH: H2O 2.5:7.5 (Fr. 57-60). Flow rate of mobile phase was maintained at 6 drops/min or ml/min. TLC analysis of CC fractions were carried out on silica gel plates using EtOAC- MeOH- H2O (65-10-15) as a mobile phase. Flavonoid spots was visualised under UV lamp and also by staining with ammonia vapour or iodine vapour. Chromatographically identical fractions were combined and concentrated. Main flavonoids of each fraction group was further purified by preparative TLC on silica gel using toluene: ethyl acetate: formc acid: methanol (6:4:1:0.5) which was resulted in isolation of flavonoids. Their structures were elucidated by spectroscopic means.

Animals:

Sprague-Dawley rats (100-150 g) of either sex were purchased from the animal house of the National Laboratory Animal Centre, Lucknow, India. They were kept under controlled conditions of temperature 27 ± 2^0C and relative humidity 44-56%, light/dark cycles of 12 hours respectively for one week before and during the experiments. Animals were provided with standard rodent pellet diet (Amrut, India) and the food was withdrawn 18-24 h before the experiment though water was allowed *ad libitum*. All experiments were performed in the morning accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [24]. The protocols were approved by Institutional Committee for Ethical use of Animals and Review Board (106/IAEC/RB/7-11).

Induction of GERD and Treatment:

GERD model was induced in rats according to methods described by Rao et al. (2008) [17]. According to this method rats were fasted for 24 hours under pentobarbitone sodium anesthesia (50 mg/kg, i.p.), the abdomen of the animal was opened by a median incision about 2 cm; then the transitional region between the fore stomach and corpus was then ligated very carefully with a 2-0 silk thread, and continuously the pylorus portion was ligated. A longitudinal cardiomyotomy of about 1 cm length across the gastro-oesophageal junction was performed to enhance reflux from the stomach into the oesophagus. Immediately the incised regions were sutured and the animal were kept in recover chamber (Medi HEAT, UK) and returned to their home cages. After 6 hours, the animals were sacrificed by cervical decapitation and the chest was opened with a median incision and the tissue oesophagus and stomach were removed. The tissue organs were opened along the greater curvature of the stomach, and the oesophagus was dissected out by extending the dissection line along the major axis. The tissues were washed with physiological saline and were examined for GERD. MAEF in doses of 50, 100 and 200 mg/kg were administered orally twice daily at 10:00 and 16:00 h, respectively, for 5 days and quercetin (100 mg/kg) or omeprazole (Ome) in the dose of 30 mg/kg one hour prior to the induction of GERD disease. Control group of animals received suspension of 1% carboxymethyl cellulose in distilled water (10 ml/kg).

Estimation of Histamine*:*

The animals were sacrificed by cervical dislocation and the abdomen was opened with a median incision and blood was collected from the supraorbital plexus using the microcapillary technique and plasma was separated. The plasma was treated with 0.2 M perchloric acid and centrifuged at 10,000 xg for 30 min at 4^0C. The clear supernatant was then used for the determination of histamine content by the high performance liquid chromatography [25] and expressed as IU/milligram protein.

Assay of H^+-K^+-ATPase:

The H^+-K^+-ATPase activity was assayed in medium consisting of 70 mM Tris-HCl buffer, pH 6.8, 5 mM $MgC1_2$ and enzyme solution in the presence of 10 mM KCl in a total volume of 1 mL, and incubated for 1 hour. The reaction was initiated by adding 2 mM ATP Tris salt and further incubated for 20 min at 37^0C. The reaction was terminated by adding 10% trichloroacetic acid and after centrifugation, 2.5 ml ammonium molybdate and 0.5 ml 1-amino-2-naphthal-4-sulfonic acid were added to the supernatant and the absorbance was read at 620 nm [26]. Results were expressed as mmol of Pi liberated/min/mg protein.

Estimation of gastric wall mucus:

Gastric wall mucus was measured by the method of Corne et al. (1974) [27]. The glandular segments from stomach were removed, weighed and incubated in tubes containing 0.1% Alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH adjusted to 5.8 with

hydrochloric acid) for 2 hours. The Alcian blue binding extract was centrifuged and the absorbency of supernatant was measured at 498 nm. The quantity of Alcian blue extracted (g/g of glandular tissue) was then calculated.

Antioxidant Assay:

Lipid peroxidation (LPO) was estimated by standard method of Okhawa et al. (1979) [28] and expressed as nmol of malonialdehyde(MDA) formed/min/mg protein. Superoxide dismutase (SOD) activity was estimated by the inhibition of nicotinamide adenine dinuleotide (reduced)-phenazine methosulphate-nitrobluetetrazolium reaction system as adapted by Kakkar et al. (1984) [29] and the results were expressed as units (U) of SOD activity/mg protein. Catalase (CAT) was estimated by method of Aebi (1974)[30] and results were expressed as µmol of H_2O_2 consumed/min/mg protein. Reduced glutathione (GSH) was determined according to the method of Ellmann (1959) [31] and expressed as nmol/g protein.

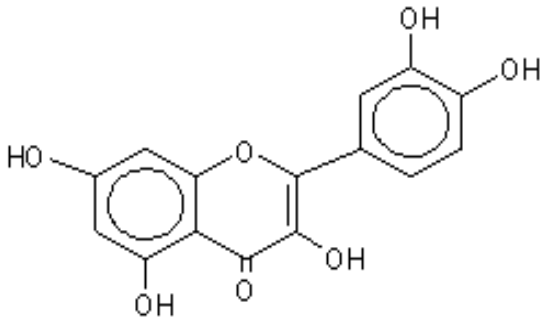
Statistical Analysis:

All the data were presented as mean ± SEM and analyzed by Wilcoxon Sum Rank Test [32] and unpaired Student's t-test for the possible significant interrelation between the various groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Elution of column with Petroleum Ether:

EtOAc 2.5:7.5 (Fr. 10-12) afforded yellowish green powder. Fr 11 & 12 on preparative TLC on silica gel using toluene : ethyl acetate : formc acid : methanol (6:4:1:0.5) gave a pure compounds which one was identified as quercetin (yield- 0.07%) on the basis of spectroscopic data. (Fig 1-3)



2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one

Molecular Formula: $C_{15}H_{10}O_7$
m.p. : 312.24^{0}C (reported 307-317^{0}C)
FW: 302.2357

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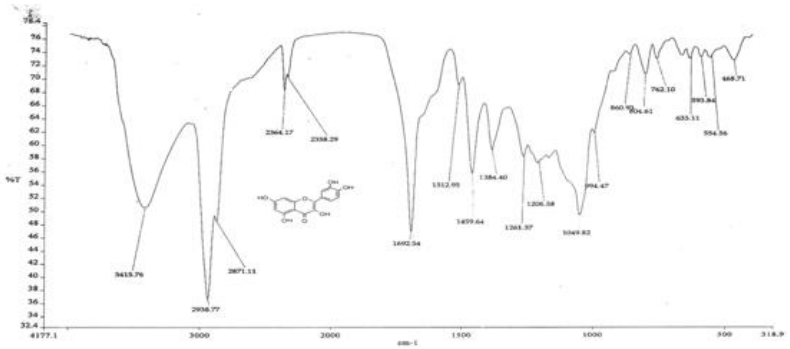

Fig 1: IR Spectrum

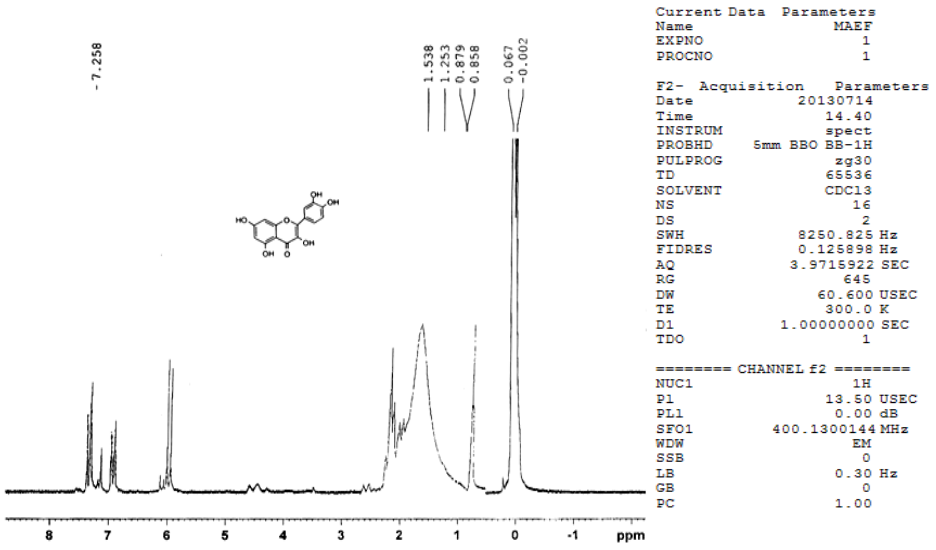

Fig 2: NMR Spectrum

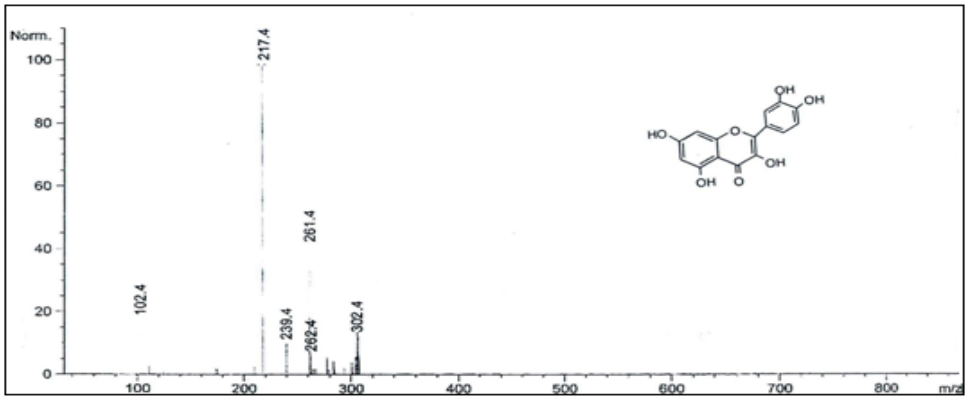

Fig 3: Mass Spectrum

Spectral data:

UV(MeOH) λmax: 255, 372 nm. IR (Kbr cm^{-1}): 3415 cm^{-1} (OH stretch) cm^{-1}, 1692 cm^{-1} (C=O), 1512 cm^{-1} (C=C), 1261 (C-O), 1049 cm^{-1} (C=C). ^{1}H NMR (400MHZ, $CDCl_3$):7.6(d 1H-2^1), 7.4(d, 2H, $5^1$and 6^1), 6.8 (d, 1H,H8), 6.2(d,1H,H6). Analysis by mass spectroscopy gave base molecular peak at 302(M+)(12%) m/z, 261(45%), 217(100%), 102(18%) analysed for $C_{15}H_{10}O_7$.

Gastroesophageal reflux disease (GERD) developed 6 h after the surgery in 100% of the animals. Effects of MAEF at dose of 50-200 mg/kg, twice a day for 5 days prevented the Gastroesophageal reflux disease (GERD) in a dose related manner. Table 1 showed that GERD group resulted in the decrease in gastric wall mucus level (141.7± 15.2) and increase in levels of plasma histamine (283.7±18.23) and H^+-K^+-ATPase (1.53±0.05). The gastric wall mucus level was increased (from 150.4±14.3 to 271.0±18.5 g/g wet glandular tissue) and levels of plasma histamine (from 261.0±15.10 to 191.9±12.31 IU/milligram protein) and H^+-K^+-ATPase were significantly decreased (from 1.16±0.04 to 0.50±0.02 mmol of Pi liberated/min/mg protein) in extract treated group (Table 1). Omeprazole showed significantly enhance in gastric wall mucus level (268.5±19.1 g/g wet glandular tissue) and decrease in levels of plasma histamine (280.5±16.12 IU/milligram protein) and H^+-K^+-ATPase (0.48±0.02 mmol of Pi liberated/min/mg protein) (Table 1).

Table 1: Effect of MAEF on Histamine, H^+-K^+-ATPase and gastric wall mucus in GERD rats

Treatment and dose (mg/kg)	Histamine (IU/milligram protein)	H^+-K^+-ATPase (mmol of Pi liberated/min/mg protein)	Gastric wall mucus (g/g wet glandular tissue)
Control	186.4±12.12	0.46±0.02	275.6± 19.1
GERD	283.7±18.23^z	1.53±0.05^z	141.7± 15.2^z
MAEF(50 mg/kg)	261.0±15.10	1.16±0.04	150.4± 14.3
MAEF (100 mg/kg)	204.7±15.10^a	0.85±0.04^a	178.7± 12.1^a
MAEF (200 mg/kg)	191.9±12.31^b	0.50±0.02^b	271.0± 18.5^b
Quercetin (100 mg/kg)	193.1±13.10^b	0.53±0.03^b	269.5±17.6^b
Omeprazole (30 mg/kg)	280.5±16.12^b	0.48±0.02^b	268.5± 19.1^b

Values are mean ± SEM for six rats. P: ^{z}<0.001 compared to respective control group, P: ^{a}<0.01 and ^{b}<0.001 compared to respective GERD group.

The lipid peroxidation is an indicator for the generation of reactive oxygen species in the oesophageal tissue in rats. Animals subjected to gastroesophageal reflux disease (GERD) showed elevation in lipid peroxidation (0.52±0.04) and SOD (186.7±15.2) and decrease in catalase (21.4±1.3) and GSH (41.1±2.6) in GERD group. Treatment with MAEF at dose of 50-200 mg/kg significantly reduced the lipid peroxidation (from 0.47±0.02 to 0.41±0.02 nmol of MDA/min/mg protein) and SOD (from 166.2±13.1 to 101.1±9.8 units (U) of SOD activity/mg protein) and increased in levels of catalase (from 25.2±1.2 to 30.4±1.3 µmol of H_2O_2 consumed/min/mg protein) and GSH (from 45.5±2.8 to 59.6±3.7 nmol/g protein). Quercetin and omeprazole showed significant inhibition in lipid peroxidation (0.40±0.02 & 0.43±0.05) and

SOD (103.1±9.5 & 110.6±8.8) and enhanced the activities of catalase (30.1±1.2 & 32.9±1.4) and GSH (55.4±3.5 & 58.5±3.3) activity as compared to MAEF GERD group (Table 2).

Table 2: Effect of MAEF on LPO, CAT, SOD and GSH activities in GERD rats

Treatment and dose (mg/kg)	LPO	CAT	SOD	GSH
Control	0.40±0.03	33.6±1.8	94.2±9.6	60.1±3.4
GERD	0.52±0.04^{x}	21.4±1.3^{x}	186.7±15.2^{y}	41.1±2.6^{y}
MAEF (50 mg/kg)	0.47±0.02	25.2±1.2	166.2±13.1^{a}	45.5±2.8
MAEF (100 mg/kg)	0.45 ±0.02^{b}	29.1±1.2^{a}	125.1±10.2^{b}	50.9±3.1^{b}
MAEF (200 mg/kg)	0.41±0.02^{c}	30.4±1.3^{b}	101.1±9.8^{c}	59.6±3.7^{c}
Quercetin (100 mg/kg)	0.40±0.02^{c}	30.1±1.2^{b}	103.1±9.5^{c}	55.4±3.5^{c}
Omeprazole (30mg/kg	0.43±0.05^{c}	32.9±1.4^{c}	110.6±8.8^{c}	58.5±3.3^{c}

Values are mean ± SEM for six rats, P: ^{x}<0.05 and ^{y}<0.001 compared to respective control group, P: ^{a}<0.05, ^{b}<0.01 and ^{c}<0.001 compared to respective GERD group.

DISCUSSION

The UV spectrum of methanolic solution of quercetin exhibited two major absorption bands at 372 nm and 255 nm, which confirmed the flavonol structure. The above mentioned spectral data were in close agreement with literature value of quercetin. The IR, NMR, melting point and the chemical test of MAEF suggests that the isolated compound is flavonoid, quercetin.

The effect of the extract is then evaluated on gastric acid secretion. The present study demonstrates that ethyl acetate fraction of *M. alba* leaves extract (MAEF) have suppressive effect on gastric acid secretion by opposition to the action of histamine, stimulation of gastric mucus secretion and blocking of H^{+}-K^{+}-ATPase. Histamine is widely distributed in the gastrointestinal tract in various cells. It is involved in the pathogenesis of gastroduodenal ulceration, gastric inflammation and gastric acid secretion [33]. Whereas, a significant increase in plasma histamine concentration was observed after development of GERD. Our observation indicated that treatment with MAEF caused the reduction in histamine concentration in GERD models, indicating the gastric defensive effect. The effect MAEF against is due to simultaneous inhibition of gastric acid secretion and stimulation by mucus production. In general, the balance of aggressive and defensive factors plays a pivotal role in GERD. The aggressive factors may include gastric acid back diffusion and oxy radical generation [34] while defensive factors are mucus production. In the disease state, oxidative stress of the stomach may occur, resulting in an elevation of mucosal lipid peroxides that are generated from the reaction of oxy radicals and cellular polyunsaturated fatty acids. It has been found that oxygen-derived free radicals are implicated in the mechanism of acute and chronic ulceration in the gastric mucosa [35] and scavenging these free radicals can play an appreciable role in healing these ulcers. The extract inhibits membrane lipid peroxidation that plays pivotal role membrane damage. The role of free radicals is reported in the induction of GERD [17]. Oxygen derived free radicals cause lipid peroxidation, which leads to membrane fluidity and increases the influx of Ca^{2+} ions, resulting in

reduced membrane integrity of surface epithelial cells, thereby causing GERD. Antioxidant activities of the extract had beneficial effect on the use of MAEF in the GERD treatment.

CONCLUSIONS

The results of our study prove that *M. alba* extract is effective against experimentally induced GERD models. Hence, it can be suggested that positive effect of the *M. alba* may be attributed to its antisecretory and antioxidant potential, justifies the use of this herb to treat gastroesophageal reflux disease (GERD).

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