

Flavonoids from whole Plant of *Euphorbia hirta* and their Evaluation against Experimentally induced Gastroesophageal Reflux Disease in Rats

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ABSTRACT

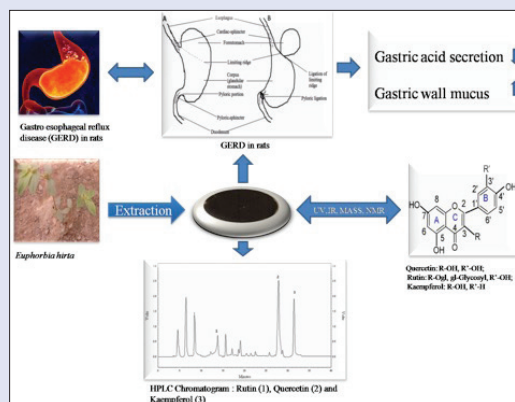
Background: *Euphorbia hirta* possesses antibacterial, anti-inflammatory, galactogenic, anti-diarrheal, antioxidant, hypoglycemic, antiasthmatic, antiamebic, antifungal, and antimalarial activities. **Objective:** The overall objective of the current study was the investigation of the whole plant extract of *E. hirta* and flavonoids from *E. hirta* on gastroesophageal reflux disease (GERD) in rats. **Materials and Methods:** The whole plant extract of *E. hirta* was characterized by analysis of flavonoids (HPLC: HPLC, UV, IR, MS and ¹HNMR). GERD model was induced surgically in Wistar rats under pentobarbitone sodium anesthesia (50 mg/kg, i.p.) and the tissue esophagus and stomach were removed. The tissues were washed with physiological saline and were examined for GERD. The whole plant extract of *E. hirta* in doses of 50, 100, and 200 mg/kg were administered orally twice daily at 10:00 and 16:00 hours, respectively, for 5 days and kaempferol (100 mg/kg) or omeprazole (OMZ) in the dose of 30 mg/kg 1 hour prior to the induction of GERD. Control groups received suspension of 1% carboxymethyl cellulose in distilled water (10 mL/kg). **Results:** The levels of gastric wall mucus increased and of plasma histamine and H⁺, K⁺ ATPase significantly decreased in groups treated by both the plant extract and flavonoids. Both the plant extract and flavonoids reduced the lipid peroxidation and superoxide dismutase and increased the levels of catalase and reduced glutathione. **Conclusions:** The whole plant extract of *E. hirta* is attributed to its antisecretory, gastroprotective, and antioxidant potential as that of quercetin, rutin, kaempferol, and proton pump blocker (omeprazole) to treat GERD.

Key words: Antisecretory, *Euphorbia hirta*, gastroesophageal reflux disease, kaempferol, quercetin, rutin

SUMMARY

- The aqueous extract of whole plant of *Euphorbia hirta* revealed the presence of kaempferol (0.0256%), quercetin (0.0557%), and rutin (0.0151%), and the ethyl acetate fraction of whole plant of *E. hirta* possesses kaempferol (0.0487%), quercetin (0.0789%), and rutin (0.0184%).
- The levels of gastric wall mucus increased and of plasma histamine and H⁺-K⁺-ATPase significantly decreased in rats groups treated by both the whole plant extract of *E. hirta* and flavonoids.

- Both the whole plant extract of *E. hirta* and flavonoids reduced the lipid peroxidation and superoxide dismutase and increased the levels of catalase and reduced glutathione in rats groups.



Abbreviation used: 1HNMR: Proton Nuclear Magnetic Resonance Spectroscopy, CAT: Catalase, EHAE: Aqueous extract of *Euphorbia hirta*, EHEF: Ethyl Acetate Fractions of *Euphorbia hirta*, GERD: Gastroesophageal reflux disease, GSH: Reduced Glutathione, HPLC: High performance liquid chromatography, IR: Infrared spectroscopy, LPO: Lipid Peroxidase, MDA: Malondialdehyde, MS: Mass Spectroscopy, OMZ: Omeprazole, ROS: Reactive Oxygen Species, SOD: Superoxide dismutase, TBHQ: tert-Butylhydroquinone, TLC: Thin Layer Chromatography, UV: Ultraviolet, UV: Ultraviolet-Visible Spectroscopy

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INTRODUCTION

Euphorbia hirta (Family: *Euphorbiaceae*, *English-Asthma herb*, *Hindi-Dudhi*) is distributed throughout the hotter parts of India and Australia, often found in waste places along the roadsides and possesses antibacterial,^[1] anti-inflammatory,^[2] galactogenic,^[3] anti-diarrheal,^[4] antioxidant, hypoglycemic,^[5] antiasthmatic,^[6] antiamebic,^[7] antifungal,^[8] and antimalarial activities.^[9] *E. hirta* contains cycloartenol, alpha-amyrin, clionasterol, phytol, linoleic acid, palmitic acid, 2-monopalmitin,^[10] quercetin, and kaempferol.^[11]

Gastroesophageal reflux disease (GERD) is a condition in which the stomach contents (food or acid) flow upward into the esophagus. However, there are no reports on the role played by whole plant of *E. hirta* on GERD. The molecules having flavonoids-like structure have

been reported for radical scavenging activity and the antioxidant activity. Quercetin, rutin, and kaempferol standardized extract may be used for treatment of GERD.^[11] In prior study, the effects of quercetin^[12] and

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rutin^[13] have been elucidated against GERD in experimental animals. However, there are no reports on the role played by kaempferol and whole plant of *E. hirta* against GERD. Therefore, the current study was undertaken to elucidate the effect of standardized whole plant extract of *E. hirta* against GERD in rats.

MATERIALS AND METHODS

Plant material and preparation of extracts

The whole plant materials of *E. hirta* were collected and recorded, the accession no. 98 576 and cross identified by its vernacular names. The specimen was deposited in the Herbarium of CSIR-National Botanical Research Institute, Lucknow, India in the month of February, 2014. The plant materials were procured, dried powdered (40-mesh), and stored in polythene bags. Powdered samples (500 g) were extracted thrice with 65% methanol (v/v) (HPLC grade) containing 2 g/L TBHQ at 70°C on a water bath using Soxhlet extractor for 3 h and filtered, concentrated on Rotavapor (Buchi Analytical Inc, USA). After drying in hot air oven (40–45°C), it was stored in an air tight container in refrigerator at 5°C.

The residue was dissolved in hot distilled water, filtered and left overnight in refrigerator. The aqueous extract of *E. hirta* (EHAE, yield 2.95%) then concentrated under vacuums to ready for further analysis. Phytochemical screening of aqueous extract of whole plant of *E. hirta* (EHAE) was tested for presence of alkaloids, phenolic compounds, tannins, saponins, glycosides, flavonoids, and steroids.

The aqueous extract (EHAE) was subsequently re-extracted thrice in petroleum ether, diethyl ether, and ethyl acetate in separating funnel. 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% H₂SO₄ (10 mL/g plant material) for 2 h at 85°C for analysis of bound flavonoids. Completion of acid hydrolysis of ethyl acetate fraction was confirmed by spraying agent (i.e. 5% Fehling solution and 1% AlCl₃ solution) during TLC (Thin Layer Chromatography). Flavonoids (free flavonoids), free from sugar part, reacted with spraying agent (i.e. 5% Fehling solution and 1% AlCl₃ solution) and gave color reactions during TLC analysis. Flavonoids with sugar part (bound flavonoids) did not react with spraying agent (i.e. 5% Fehling solution and 1% AlCl₃ solution) and did not give color reaction during TLC analysis. Diethyl ether fraction gave color reaction with spraying agent and it did not need acid hydrolysis. The mixture was filtered and re-extracted thrice with ethyl acetate in a separating funnel. All EHEF (Ethyl Acetate Fractions of *Euphorbia hirta*) were pooled together separately and neutralized by adding 5% NaOH and then dried in vacuum and analyzed for flavonoids using HPLC, chromatographic separation, and elucidation of its effect against GERD.

For HPLC analysis, aqueous extract (EHAE) and ethyl acetate fraction (EHEF) from whole plant of *E. hirta* was dissolved in HPLC grade MeOH (1 mg/mL) and subjected to HPLC for the qualitative and quantitative analysis of flavonoid contents. Separation was achieved with a two pump linear gradient program for pump A (water containing 1% acetic acid) and pump B (acetonitrile). Initially started with a gradient of 18% B changing to 32% in 15.0 min and finally to 50% in 40 min followed by washing for 25 min. The flow rate was 1.0 mL/min. Results (mg/g dry wt) were obtained by comparison of peak areas (280 nm) of the samples with that of standards.

The ethyl acetate fraction of *E. hirta* plant extract (EHEF) (15 g) was chromatographed over silica gel column to obtain purified fractions

using various mobile phases in increasing polarity. Flow of mobile phase was maintained at 6 drops/min. TLC analysis of column chromatography (CC) fractions were carried out on silica gel plates using EtOAc–MeOH–H₂O (65–10–15) as a mobile phase. Flavonoid spots were visualized under UV lamp and also by staining with 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: formic acid: methanol (6: 4: 1: 0.5) which was resulted in isolation of flavonoids. The isolated flavonoid was characterized on the basis of phytochemical analysis (Shinoda test, zinc hydrochloride reduction test) and spectroscopic studies (UV, IR, MS, ¹HNMR).

Test animals

Wistar rats (100–150 g) of either sex were purchased from the animal house of the National Laboratory Animal Centre, Lucknow, India. They were put under controlled conditions of temperature 24 ± 5°C and relative humidity 40–46%, light/dark cycles of 12 h respectively for 1 week before and during the experimental study. They were given standard rodent pellet diet (Amrut, India) and the food was withdrawn 18–24 h before the experiment though water was allowed *ad libitum*. All experimental works were performed in accordance with the guide for the care and use of laboratory animals, as approved and promoted by the Institutional Animal Care Committee, CPCSEA, India (Reg. No. 1732/GO/Re/S/13/CPCSEA)

Induction of GERD and treatment

GERD model was induced in Wistar rats according to methods described by Rao *et al.*^[12] According to this method, rats were fasted for 24 h under pentobarbitone sodium anesthesia (50 mg/kg, i.p.), the abdomen of the animal was opened by a median incision of about 2 cm; then the transitional region between the fore stomach and corpus was ligated very carefully with a 2–0 silk thread, and continuously the pyloric portion was ligated. A longitudinal cardiomyotomy (1 cm length) across the cardiac sphincter was performed to enhance reflux from the stomach into the oesophagus [Figure 1]. 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 h, the animals were sacrificed by cervical decapitation and the chest was opened with a median incision and the tissue esophagus and stomach were removed. The tissue organs were opened along the greater curvature of the stomach, and the esophagus was dissected out by extending the dissection line along the major axis. The tissues were washed with physiological saline and were examined for GERD. The ethyl acetate fraction of *E. hirta* plant extract (EHEF) contains the flavonoids (quercetin, rutin and, kaempferol). So, EHEF in doses of 50, 100, and 200 mg/kg were administered orally twice daily at 10:00 and 16:00 hours, respectively, for 5 days and kaempferol (100 mg/kg) or omeprazole (OMZ) in the dose of 30 mg/kg 1 hour prior to the induction of GERD. Control groups 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 separated plasma was treated with 0.2 M perchloric acid and centrifuged at 10 000 xg for ½ h at 4°C. Then, clear supernatant was used for the determination of histamine content by the high performance liquid chromatography^[14] and expressed as IU/mg protein.

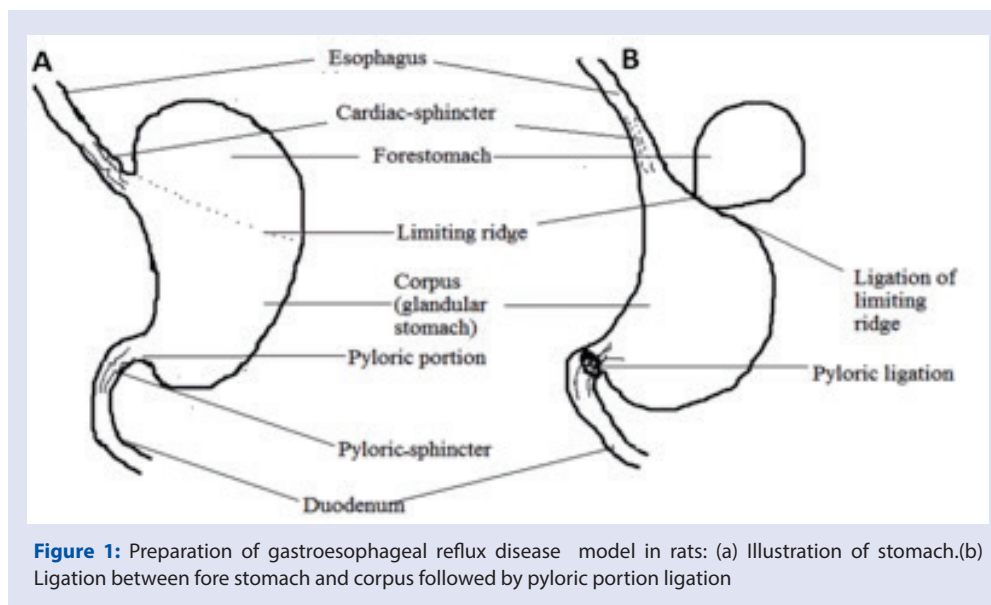


Figure 1: Preparation of gastroesophageal reflux disease model in rats: (a) Illustration of stomach.(b) Ligation between fore stomach and corpus followed by pyloric portion ligation

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 MgCl₂ 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. The reaction was terminated by adding 10% trichloroacetic acid after incubation for 20 min at 37°C. Then 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.^[15] Results were expressed as mmol of Pi liberated/min/mg protein.

Estimation of gastric wall mucus

Gastric wall mucus was measured by the modified method of Mizui and Doteuchi^[16] After washing with normal saline, the gastric mucus obtained by scraping the mucous was homogenized for 14 sec in 4 mL of distilled water. The weight of mucus (g) was obtained from the difference between the weight of homogenate and the original 4 mL of water.

Antioxidant assay

Thiobarbituric acid reactive substances, a measure of lipid peroxidation, were estimated by method of Ohkawa *et al.*^[17] and expressed as nmol malondialdehyde (MDA) eq/g protein. Superoxide dismutase (SOD) activity was estimated by the inhibition of nicotinamide adenine dinucleotide (reduced)-phenazine methosulfate-nitroblue tetrazolium reaction system as adapted by Kakkar *et al.*^[18] and the results were expressed as units (U) of SOD activity/mg protein. The decomposition of H₂O₂ in the presence of catalase (Catalase) was followed at 240 nm and one unit of CAT was defined as the amount of enzyme required to decompose 1 μmol of H₂O₂ min⁻¹, at 25°C and pH 7.0 and results were expressed units (U) of CAT activity per mg protein.^[19] Reduced glutathione (Reduced Glutathione) was determined according to the method of Ellmann^[20] and expressed as nmol/g protein.

Statistical analysis

All the data were presented as mean ± standard error of the mean for six rats and all the data were analysed by one-way analysis of variance followed by Newman-Keuls test. *P* < 0.05 were considered as significant.

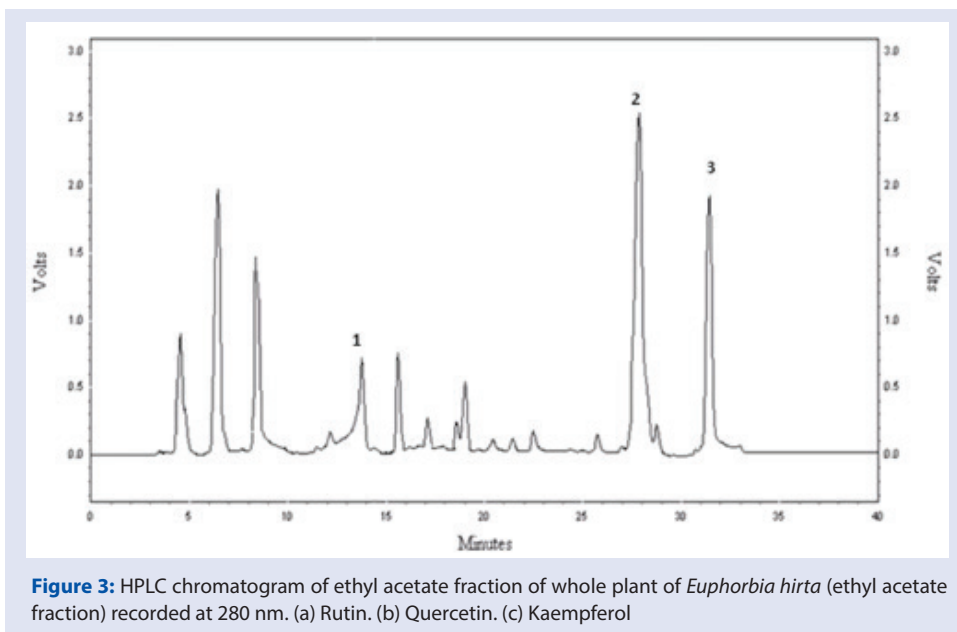
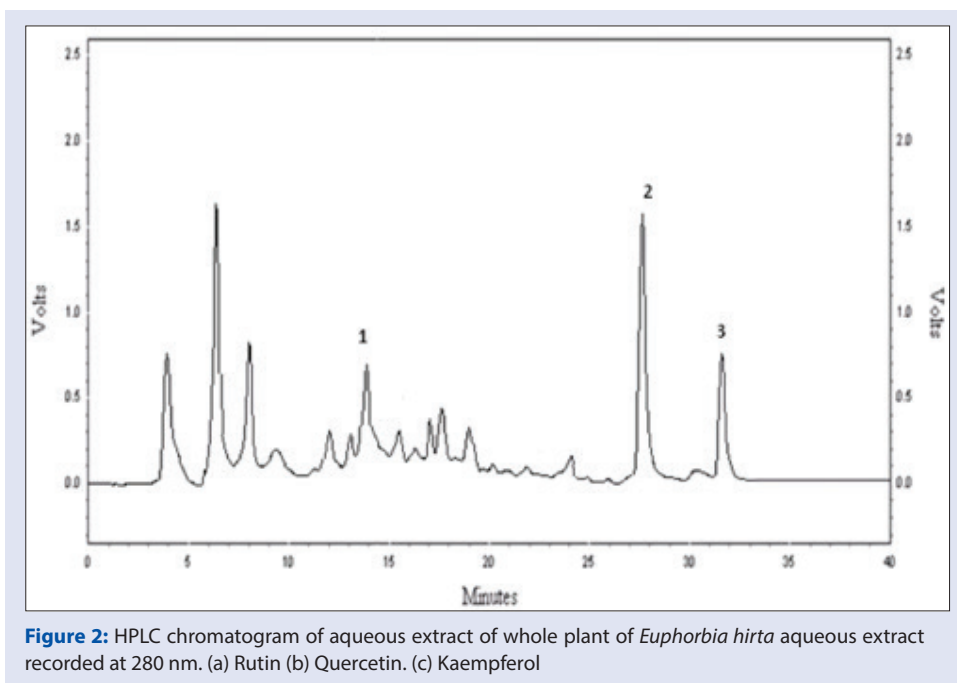
RESULTS

Phytochemical test results found the presence of alkaloids, phenolic compounds, tannins, saponins, glycosides, flavonoids, and steroids in aqueous extract of whole plant of *E. hirta* (EHAE). HPLC chromatogram of whole plant of *E. hirta* was recorded [Figure 2 and Figure 3]. EHAE revealed the presence of kaempferol (0.0256%), quercetin (0.0557%), and rutin (0.0151%). EHEF possesses kaempferol (0.0487%), quercetin (0.0789%), and rutin (0.0184%) [Figure 2 and Figure 3].

The UV spectrum of methanolic solution exhibited two major absorption bands at 372 and 255 nm (quercetin), 359 and 257 nm (rutin), 264 and 365 nm (kaempferol) which confirmed the flavonol structure. These mentioned spectral data were in close agreement with literature value of quercetin, rutin, and kaempferol. The IR, NMR, MS, melting point and the chemical test of the plant extract suggested that the isolated compounds were flavonoids (quercetin, rutin, and kaempferol).

Quercetin: ¹HNMR (CDCl₃): δ 5.36 (1H, *brs*, H-6), 3.66 (1H, *dd*, 5.5, 8.6 Hz, H-3a), 1.28 (3H, *brs*, Me-28), 1.25 (3H, *brs*, Me-19), 1.05 (3H, *brs*, Me-29), 0.91 (3H, *d*, *J* = 6.1 Hz, Me-21), 0.87(3H, *d*, *J* = 6.30 Hz, Me-26), 0.82(3H, *d*, *J* = 6.3 Hz, Me-27), 0.80 (3H, *brs*, Me-30), 0.68 (3H, *brs*, Me-18). IR ν_{max} (KBr): 3418, 2925, 2850, 1627, 1463, 1358, 1216, 1010 cm⁻¹. UV (MeOH) λ_{max}: 255, 372 nm. MS m/z (rel. int.): 428[M]⁺ (C₃₀H₅₂O) (23.58), 413 (26.1), 398 (29.2), 395 (32.5), 383 (15.2), 380 (8.7), 365 (1.1), 281(73.5), 262 (11.6), 236 (17.2), 222 (23.8), 220 (75.1), 208 (70.6), 206 (33.5), 205 (24.9), 192 (34.7), 174 (26.1), 173 (25.8), 166 (24.6), 152 (23.5), 148 (100), 144 (51.4), 143 (41.8), 134 (63.2), 133(63.3), 129 (59.2), 123 (39.8), 119 (57.2), 118 (51.0), 109 (64.8), 108 (72.6), 95 (94.76), 94 (61.3), 93 (61.1). Mol. Formula: C₁₅H₁₀O₇, m.p. 312.24°C (reported 307-317°C).

Rutin: ¹HNMR (CDCl₃): δ 8.06 (1H, *d*, *J* = 2.1 Hz, H-6'), 7.27 (1H, *d*, *J* = 3.0 Hz, H-2'), 7.12 (3H, *s*, H-5'), 7.03 (3H, *m*, *J* = 4.5 Hz, H-6, 8), 6.89 (3H, *d*, *J* = 2.8 Hz, H-1'), 6.33(3H, *d*, *J* = 2.6 Hz, Phenolics Hs-5,7,3',4'), 5.40 (3H, *d*, *J* = 2.2 Hz, H-3'), 4.20 (3H, *d*, *J* = 2.3Hz, H-6'), 3.80 (3H, *d*, *J* = 6.7 Hz, H-5'), 3.68 (3H, *s*, H-4',6'), 3.72 (3H, *d*, *J* = 5.2 Hz, H-5'), 3.31 (3H, *s*, H-3'), 2.36 (3H, *s*, phenolics Hs-4',5', 6'), 1.25 (3H, *m*, *J* = 8.19 Hz, Me). IR ν_{max} (KBr): 3424, 2370, 1651, 1504, 1457, 1364, 1295, 1207, 1062, 803 cm⁻¹. UV (MeOH) λ_{max}: 359, 257 nm. MS m/z (rel. int.): 612[M + H]⁺ (57), 467 (45), 415 (13), 387 (19), 317 (13), 303 (100), 274 (10), 151(6),



136 (12), 115 (20), 99(82), 81 (32), 61 (42). Mol. Formula: $C_{27}H_{30}O_{16}$, m.p.: 194°C (reported 185–195°C).

Kaempferol: $^1\text{H NMR}$ (CDCl_3): δ 8.05 (2H, d, $J = 8.9$ Hz, H-2', H-6'), 6.98 (2H, d, $J = 8.7$ Hz, H-3', H-5'), 6.36 (1H, s, H-8), 6.16 (1H, d, $J = 2.1$ Hz, H-6), 4.93 (3H, s, Phenolic H-5, 7, 4'), UV (MeOH) λ_{max} : 264, 365 nm. IR ν_{max} (KBr): 3467, 2362, 2154, 1611, 1502, 1380, 1250, 1178, 1008, 883 cm^{-1} . MS m/z (rel. int.): 287.08 [$M + H$] $^+$ (100), 271.08 (21), 163.06 (15), 137.09 (13), 115.10 (12), 99.11 (20), 89.09 (12), 61.06 (23). Mol. Formula: $C_{15}H_{10}O_6$, m.p.: 278°C (reported 274–284°C).

GERD developed 6 h after the surgery in 100% of the animals. Administration of EHEF, quercetin, rutin, kaempferol, and OMZ significantly reduced esophageal index to 78.81 % ($P < 0.05$), 72.88 % ($P < 0.01$), 40.68 % ($P < 0.05$), 85.59 % ($P < 0.01$) and 98.31 % ($P < 0.001$)

respectively [Figure 4]. Effects of EHEF at a dose of 50–200 mg/kg, twice a day for 5 days, prevented the GERD in a dose-related manner. GERD group resulted in the decrement in gastric wall mucus level and increment in levels of plasma histamine and H^+ , K^+ ATPase. The gastric wall mucus level was increased (94.28%, $P < 0.001$) and levels of plasma histamine (98.61%, $P < 0.01$) and H^+ , K^+ ATPase (94.34 %, $P < 0.01$) were significantly decreased in extract treated group. Quercetin, rutin, kaempferol, and OMZ showed significantly enhancement in gastric wall mucus level 95.98% ($P < 0.05$), 69.38% ($P < 0.001$), 91.16% ($P < 0.01$), and 94.68% ($P < 0.01$) respectively and decrement in levels of plasma histamine 91.74% ($P < 0.05$), 88.06% ($P < 0.001$), 92.44% ($P < 0.01$), and 91.64% ($P < 0.01$) respectively and H^+ , K^+ ATPase (90.57%, $P < 0.01$, 78.30%, $P < 0.01$, 90.57%, $P < 0.001$, and 97.17 %, $P < 0.05$) respectively [Figure 5-7].

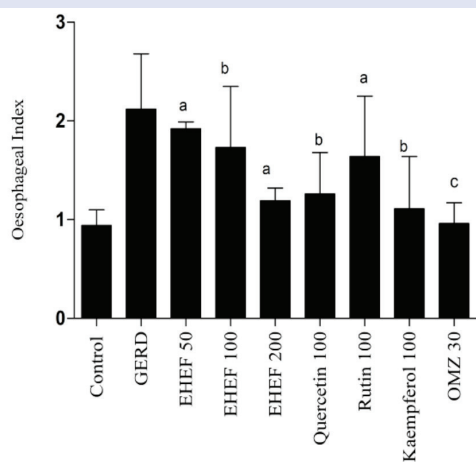


Figure 4: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on esophageal index in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

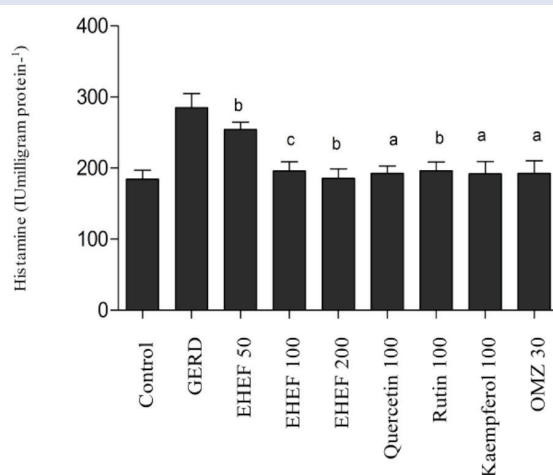


Figure 6: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on histamine in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

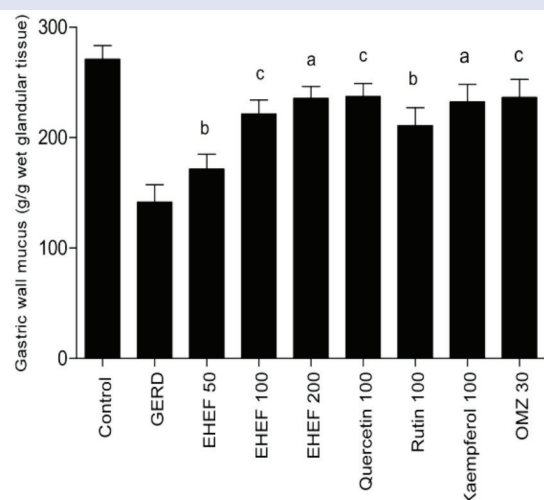


Figure 5: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on gastric wall mucus in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

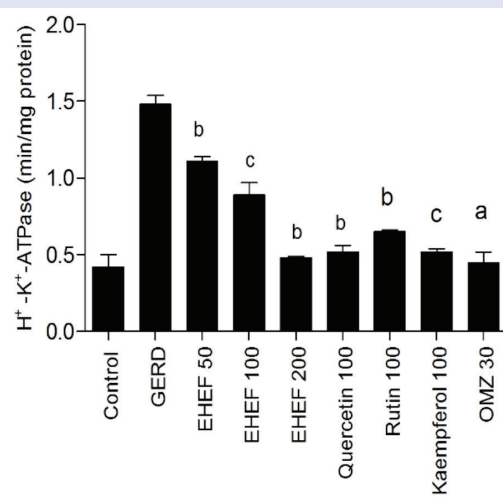


Figure 7: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on H^+K^+ -ATPase in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

The lipid peroxidation is an indicator for the generation of reactive oxygen species (ROS) in the esophageal tissue in rats. GERD-induced animals showed elevation in lipid peroxidation (0.55 ± 0.02 nmol MDA eq/g protein) [Figure 8] and SOD (201.2 ± 13.5 units of SOD activity/mg protein) [Figure 9] and reduction in CAT (22.7 ± 1.2 units of CAT activity per mg protein) [Figure 10] and GSH (45.2 ± 3.2 nmol/g protein) [Figure 11]. The ethyl acetate fraction of whole plant of *Euphorbia hirta* EHEF at dose of 50–200 mg/kg significantly reduced the lipid peroxidation (23.08–84.62%, $P < 0.05$ – $P < 0.001$) [Figure 8] and activity of SOD (43.32–95.73%, $P < 0.05$ – $P < 0.001$) [Figure 9] and increased the activity of CAT (43.55–82.26%, $P < 0.05$ – $P < 0.001$) [Figure 10] and level of GSH (35.20–87.15 %, $P < 0.05$ – $P < 0.001$) [Figure 11].

Quercetin, rutin, kaempferol, and OMZ showed significant inhibition in lipid peroxidation 92.31% ($P < 0.001$), 84.62% ($P < 0.01$), 92.31% ($P < 0.01$)

and 92.31% ($P < 0.05$) respectively [Figure 8] and SOD 90.52% ($P < 0.001$), 62.27% ($P < 0.01$), 87.20% ($P < 0.01$) and 92.32% ($P < 0.001$) respectively [Figure 9] and improved the activity of CAT 90.32% ($P < 0.001$), 79.03% ($P < 0.01$), 84.68% ($P < 0.01$) and 91.13% ($P < 0.001$) respectively [Figure 10] and GSH (88.83% ($P < 0.001$), 83.24% ($P < 0.001$), 89.94% ($P < 0.01$), and 98.84% ($P < 0.05$) respectively [Figure 11].

DISCUSSION

Quercetin, is a bioflavonoid possessing 3, 5, 7-trihydroxy in ring A, 3', 4'-dihydroxy in the ring B and C2-C3 double bond conjugated with a 4-keto group in ring C. Rutin, also called quercetin - 3 - O - rutinose, is a bioflavonoid comprised of quercetin and the disaccharide rutinose [a - L - rhamnopyranosyl - (1 \rightarrow 6) - β - D - glucopyranose]. Rutin possesses 5, 7-dihydroxy in ring A, 3', 4'-dihydroxy in the ring B and C2-C3

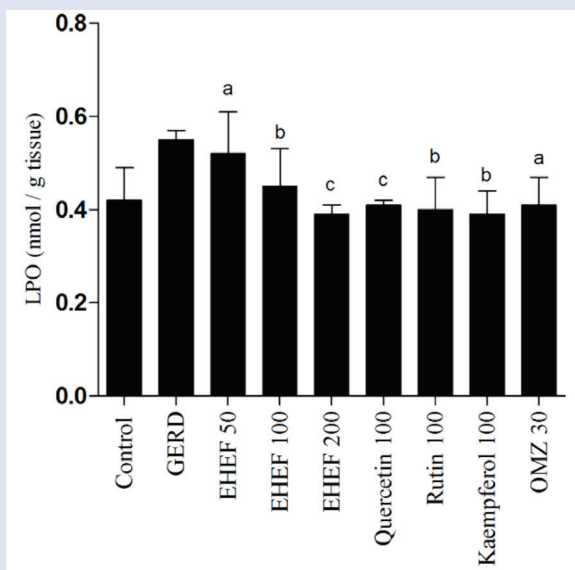


Figure 8: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on lipid peroxidation in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

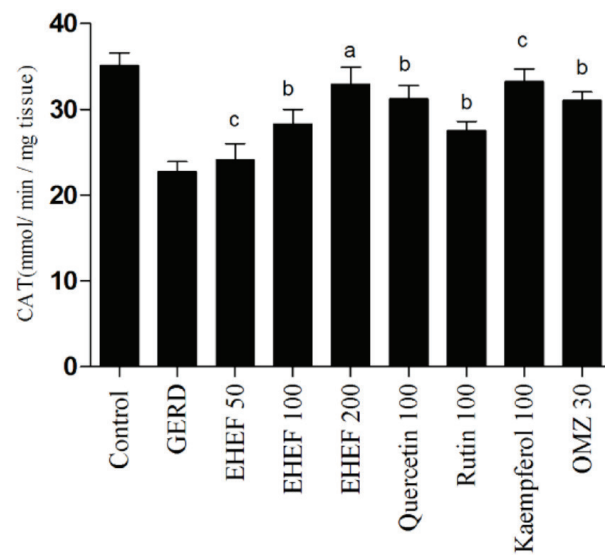


Figure 10: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on CAT in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

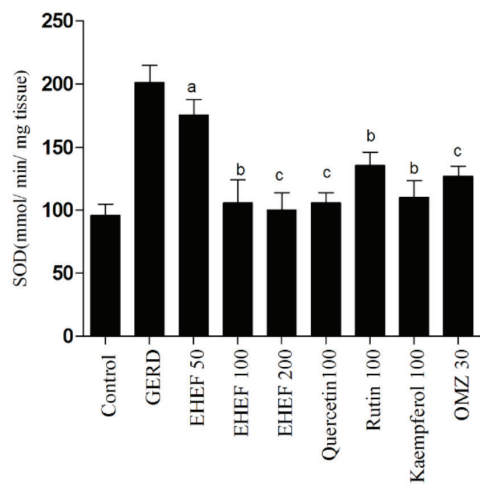


Figure 9: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on SOD in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

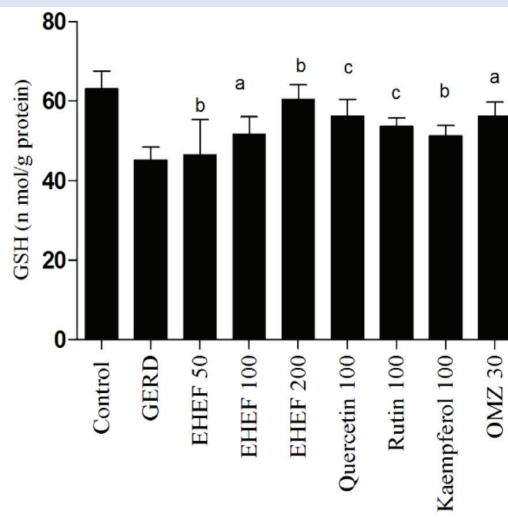


Figure 11: Effect of ethyl acetate fraction of whole plant of *Euphorbia hirta* on GSH in gastroesophageal reflux disease rats. Values are mean \pm standard error of the mean for six rats. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the respective gastroesophageal reflux disease group

double bond conjugated with a 4-keto group in ring C. Kaempferol, a natural flavonol, a type of flavonoids, is found in a variety of plants and plant-derived foods. Kaempferol possesses 3, 5, 7-trihydroxy in ring A, 4'-hydroxy in the ring B and C2-C3 double bond conjugated with a 4-keto group in ring C [Figure 12]. The presence of 5-OH group in ring A, 3',4'-dihydroxy in the ring B and C2-C3 double bond conjugated with a 4-keto group in ring C^[21,22] have been reported for free radical scavenging activity in flavonoids. Therefore, having these types of structure, quercetin, rutin, and kaempferol standardized extract may be used for treatment of GERD.

The free radical scavenging of flavonoids is due to its phenolic group and it depends on its molecular structure and the substitution pattern

of hydroxyl groups on rings A and B. The presence of a 3', 4'-dihydroxy in the ring B and possessing electron donating properties is essential for effective radical scavenging in flavonoids. The C₂-C₃ double bond conjugated with a 4-keto group in ring C, which is responsible for electron delocalization from the ring B, increases the radical scavenging capacity.^[21,23] The presence of 5-OH group in ring A also enhances the radical scavenging property of flavonoids.^[21]

The investigative study on the structure activity relationship of the inhibition of lipid peroxidation by flavonols was started by characterizing the influence of substituents on the activity of phenol. It was revealed that the nature of the substituents as well as its position determine the activity of flavonols. These findings can be explained by the different

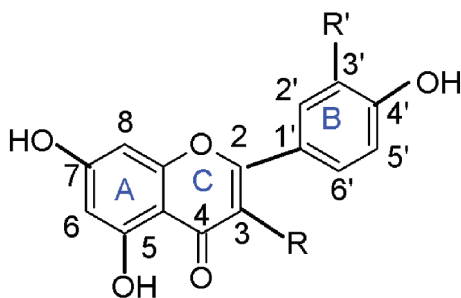


Figure 12: Structural features of flavonoids isolated from ethyl acetate fraction of whole plant of *Euphorbia hirta* (Quercetin: R-OH, R'-OH; Rutin: R-Ogl, gl-Glycosyl, R'-OH; Kaempferol: R-OH, R'-H)

electron-donating effect of the various substituents at different positions in flavonols.^[24] Structure activity analysis of flavonols (flavonoids) molecule suggests that the polyhydroxylated substitutions on rings A and B and C₂-C₃ double bond conjugated with a 4-keto group in ring C would confer its antiperoxidative properties.^[25,26] The scavenging activity increases with the number of hydroxyl groups substituted in ring B. It is suggested that the overall antioxidant activity of flavonoids on lipid peroxidation may be due to their hydroxyl radical (OH) and superoxide radical (O⁻²) scavenging properties and the reaction with peroxy radicals (RO₂).^[27,28]

Quercetin is one of the most prominent dietary antioxidants. It has been reported to inhibit the acid production in the stomach^[12] and prevent the oxidative stress in gastric ulcer and protect gastric lesions in glandular portion of the stomach.^[29] Rutin is one of the most effective inhibitors of superoxide anions. Flavonoids inhibited the MDA formation in rat liver microsomes. It is concluded that antioxidant properties of flavonoids are due to its scavenging of superoxide anions.^[30] The antioxidant potential of the kaempferol on lipid peroxidation due to the metal chelation, proton radical and hydroxyl radical scavenging has been demonstrated by Nagaya *et al.*^[15] and Singh *et al.*^[31] Therefore, quercetin, rutin and kaempferol standardized extract may be used for treatment of GERD.

In study of animal models of esophagitis as well as those on human esophageal tissue, ROS that are generated in the process of reflux esophagitis were found to be responsible for the esophageal tissue damage, and these findings were further supported by the studies viewing that tissue damage could be prohibited with the use of antioxidants.^[32] Free oxygen radicals in general and superoxide radical (O⁻²) in particular were revealed to rise in animals with esophagitis and it was claimed that free radical scavengers like SOD could stop the tissue damage.^[33] Studies performed in adults with reflux esophagitis are in support of the experimental esophagitis models showing that free oxygen radicals do take part in the pathogenesis of reflux esophagitis.^[34]

In general, the balance of aggressive and defensive factors plays a pivotal role in integrity of gastrointestinal wall.^[35] The aggressive factors encompass the rise in acid output and subsequent lipid peroxidation, which is due to the reaction between oxy radicals and the polyunsaturated fatty acids. The defensive factors are gastroprotective in nature and involve the antioxidative enzymes; superoxide dismutase (SOD, superoxide-scavenging enzyme) which catalyses the dismutation of superoxide radical (O⁻²) into less noxious hydrogen peroxide (H₂O₂), and CAT or GSH peroxidase that inactivate hydrogen peroxide (H₂O₂) to water (H₂O) and oxygen (O₂).^[15]

It has been found that oxygen-derived free radicals are drawn in the mechanism of acute and chronic ulceration in the gastric mucosa^[36]

and scavenging-free radicals can play an appreciable role in healing ulcers. Histamine is widely distributed in the gastrointestinal tract in different cells and involves in the pathogenesis of gastroduodenal ulceration, gastric inflammation, and gastric acid secretion,^[17] whereas a significant increase in plasma histamine concentration was observed after development of GERD. The 1950s studies revealed that flavonoids could stop the secretion of histamine.^[37] Antigen binding to the mast cell-attached immunoglobulin E (IgE) then triggers the mast cell to take action and this response results in histamine secretion.^[12] The flavonols significantly inhibited IgE, able to mediate histamine release in RBL-2H3 cells.^[26] Flavonoids are known to hinder the enzyme activity of histidine decarboxylase and lessen the formation of histamine in the gastric mucosa has reported that kaempferol significantly inhibited histamine release.^[31]

Flavonoids-rich extracts have been screened for free radical scavenging and H⁺, K⁺ ATPase inhibitory activity in different *in vitro* models.^[38] The inhibitory potency of the flavonoids on the H⁺, K⁺ ATPase is attributed due to the presence and position of hydroxyl groups in flavonoids. H⁺, K⁺ ATPase inhibitory activity of flavonoids is due to action on the ATPase by competing with ATP binding.^[14]

The stomach has mucus to line and defend the gastric wall from the acid. Without this gastric wall mucus, the stomach wall would be likely to such things as ulcers. Flavonoids show cytoprotective effects by stimulating the mucosal content of prostaglandins and mucus in gastric mucosa. It also treats gastric mucosal lesions produced by various models of experimental ulcer and protects the gastric mucosa against different necrotic agents.^[22]

Lipid peroxidation is a natural process in small amount in the body system, primarily by the cause of numerous ROS (hydroxyl radical, hydrogen peroxide, etc.). These ROS readily attack the polyunsaturated fatty acids of the fatty acid membrane, starting a self-propagating chain reaction and change membrane lipid composition further aggravates gastric damage. The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are especially dangerous for the viability of cells, even tissues. Enzymatic (CAT, superoxide dismutase) and nonenzymatic (vitamins A and E) natural antioxidant defence mechanisms exist.^[39] Dismutation of superoxide anions by SOD interrupts the free radical chain reaction at the very beginning of the reaction and prevents reflux in oesophagus of rats.^[40] In a recent study, we evaluated the implication of oxygen-derived-free radicals in reflux esophagitis of human.^[34]

CAT is active in the cells and tissues throughout the body, where it breaks down hydrogen peroxide (H₂O₂) molecules into oxygen (O₂) and water (H₂O). At low level, hydrogen peroxide is involved in chemical signalling pathways, but at high level it produces toxicity in body cells. CAT breaks down hydrogen peroxide and stops production of ROS that can damage DNA, proteins, and cell membranes. GSH, a tripeptide (glutamyl-cysteinyl-glycine), is an extremely important cell protectant against damage by ROS. The cysteine provides an exposed free and very reactive sulphhydryl group (SH), an abundant target for radical attack. This radical attack oxidizes GSH; however, its reduced form is regenerated in a redox cycle involving GSH reductase and the electron acceptor NADPH. The role of oxygen derived free radicals in GERD has been reported in the induction of GERD in recent animal studies.^[40,41]

The present study demonstrates that ethyl acetate fraction of *E. hirta* plant extract (EHEF) has suppressive effect on gastric acid secretion by stimulation of gastric mucus secretion, blocking of H⁺, K⁺ ATPase and opposition to the action of histamine due presence of flavonoids. Our observations indicate that ethyl acetate fraction of *E. hirta* have beneficial effect in the GERD treatment.

CONCLUSION

It is concluded that molecular structure of flavonoids has been reported for radical scavenging activity, antioxidant activity, stimulation of gastric mucus secretion, blocking of H⁺, K⁺ ATPase, and opposition to the action of histamine. Quercetin, rutin, and kaempferol standardized *E. hirta* plant extract played a crucial role in gastric mucus secretion and suppression of gastric acid secretion. The results of the study prove that *E. hirta* plant extract is effective against GERD in rats. That's why, it can be recommended that positive effect of the *E. hirta* plants may be attributed to its antisecretory and antioxidant potential, justifies the use of these seeds to treat GERD.

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Conflicts of interest

There are no conflicts of interest.

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