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Potent Gastroprotective Effect Chrysophanol and Emodin from *Rehum Emodi* via H^+K^+Atpase Inhibition and Increasing the Pge2 Level in Rats

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Abstract

The present study was aimed to determine gastro protective effect of 95% ethanol extract of *Rheum emodi* rhizomes and its active principles chrysophanol (CP) and emodin (ED) in SD rats. Ethanol extact showed significant antiulcer activity in Cold restraint induced ulcer (CRU) model (50.0%). Bioassay guided fractionation of the extract resulted in the identification of four compounds viz. chrysophanol (CP), emodin (ED), chrysophanol 8-O-β-D-glucopyranoside (CPG) and emodin 8-O-β-D-glucopyranoside (EDG) as active constituents. Chrysophenol and emodin showed significant activity against cold restraint ulcer (CRU) (50.0% and 62.5%), alcohol (AL) (70.51% and 78.48%), aspirin (AS) (37.5% and 50.0 %) and pyloric ligation (PL) (52.5% and 62.5%) induced ulcer models in Sprague dawley rats. chrysophenol and emodin significantly reduced free acids (10.72% and 25.61%), total acids (15.01% and 32.98%) and up regulated mucin secretion (32.64% and 46.64%) respectively. Further, chrysophenol and emodin inhibited H^+ K^+-ATPase activity *in vitro* with IC_{50} of 187.13 µg/ml and 110.30µg/ml respectively confirming their anti-secretory activity. Conclusively, chrysophenol and emodin were found to possess anti-ulcerogenic activity which might be due to its anti-secretory activity and subsequent strengthening of the defensive mechanism. The present study has helped in identifying a new lead that could be exploited in the treatment of gastric ulcer disease.

Keywords*: *Gastric ulcer; Proton pump; Gastric acid secretion; Rheum emodi rhizomes; Chrysophenol; Emodin

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Introduction

Gastric ulcer is a very common gastrointestinal disorder affecting a large number of people worldwide. It arises due to an imbalance between aggressive (acid, pepsin and *Helicobacter pylori* infection) and protective (mucin secretion, prostaglandin, epidermal growth factors and bicarbonate) factors in the stomach [1]. Stress, smoking, alcohol consumption, *H. pylori* infection and excessive use of non-steroidal anti-inflammatory drugs (NSAIDs) are considered as etiological factors for this disorder [2]. Major therapeutic approaches to treat gastric ulcer disease include regular feeds and adequate rest, drug therapy and averting ulcerogenic agents. Drug therapy involves reduction of gastric acid production as well as reinforcement of gastric mucosal protection [1,3]. Antacids, proton pump inhibitors, and histamine H_2 receptor antagonists are commonly used drugs [4,5]. However, beside their therapeutic efficacies, several incidences of relapse, adverse effects and drug interactions have been shown to be associated with these drugs [6]. Hence, research interest has been focused on search for new anti-ulcer molecules from medicinal plants as these molecules are more relevant to living system and already have definite biological functions and thus, may have fewer side effects. As a part of anti-ulcer drug discovery program of our lab several Indian medicinal plants including *Terminalia chebula* and *Xylocarpus granetum* have been reported to possess anti-ulcer activity [7,8]. The work on another medicinal plant *Rheum emodi* was under taken which has shown potent anti ulcer activity in the present communication.

Rheum emodi Wall. ex Meissn (family Polygonaceae) is a perennial herb which grows predominantly in the temperate and subtropical regions of Himalayas in India [9]. Rhizomes of *R. emodi* are extensively used in Ayurvedic and Asian folk medicine as a purgative, astringent, stomachic, and tonic. It is also used in traditional medicine to treat skin diseases, fevers, ulcers, bacterial infections, fungal infections, jaundice and liver disorders [10-12]. Anthraquinones are the major active constituents of this species. Compounds isolated from different parts of *R. emodi* reported to have anti-bacterial, anti-fungal, anti-viral, anti-tumor and cytostatic activities [11-13]. Keeping in consideration the above facts, we have selected this plant to determine its potential effects on gastric ulcer.

Material and methods

Plant material: The rhizomes of *Rheum emodi* were collected from Utrakhand, India and authenticated by Botany Division of CSIR-Central Drug Research Institute, Lucknow, India. A voucher specimen (Voucher No. 3755) has been preserved in the herbarium of the Institute.

Extraction, fractionation and isolation of pure compounds: The air dried, powdered rhizomes (2.0 kg) were extracted with 95% ethanol (5×3.0 lit.) at room temperature and the total combined extract was filtered and concentrated under reduced pressure below 50°C to get a dark brown mass (95% ethanol extract, 150 g). 100 g. of this extract was fractionated into hexane soluble (F1, 1.2 g), chloroform soluble (F2, 5.1 g), n-butanol soluble (F3, 40.4 g), and n-butanol insoluble fractions (F4, 53.1 g) by maceration with hexane, chloroform and butanol respectively as shown in Figure 1. All the fractions as well as crude extract were bioassayed for the antiulcer activity. Since fraction F2 has shown maximum activity, 5 g. of it was repeatedly chromatographed using a silica gel (100–200mesh) column and eluted with hexane, hexane–chloroform mixtures (4:1, 1:1 and 1:4 v:v), chloroform, and chloroform–methanol mixtures (9:1, 4:1, 7:3 and 1:1 v/v) yielding compounds K1 (120 mg) and K2 (1.6 g). Similarly fraction F3 was c hromatographed and eluted with chloroform and chloroform–methanol mixtures (9:1, 4:1, 7:3 and 1:1 v/v) yielding compounds K3 (950 mg) and K4 (220 mg). These compounds were

characterized as chrysophanol (K1) [14], emodin (K2) [14], chrysophanol 8-O-β-D- glucopyranosid (K3) [15] and emodin 8-O-β-D- glucopyranosid (K4) [16] using spectral data and comparing with those reported in literature (Figure 2). These compounds were also compared with authentic samples by thin layer chromatography.

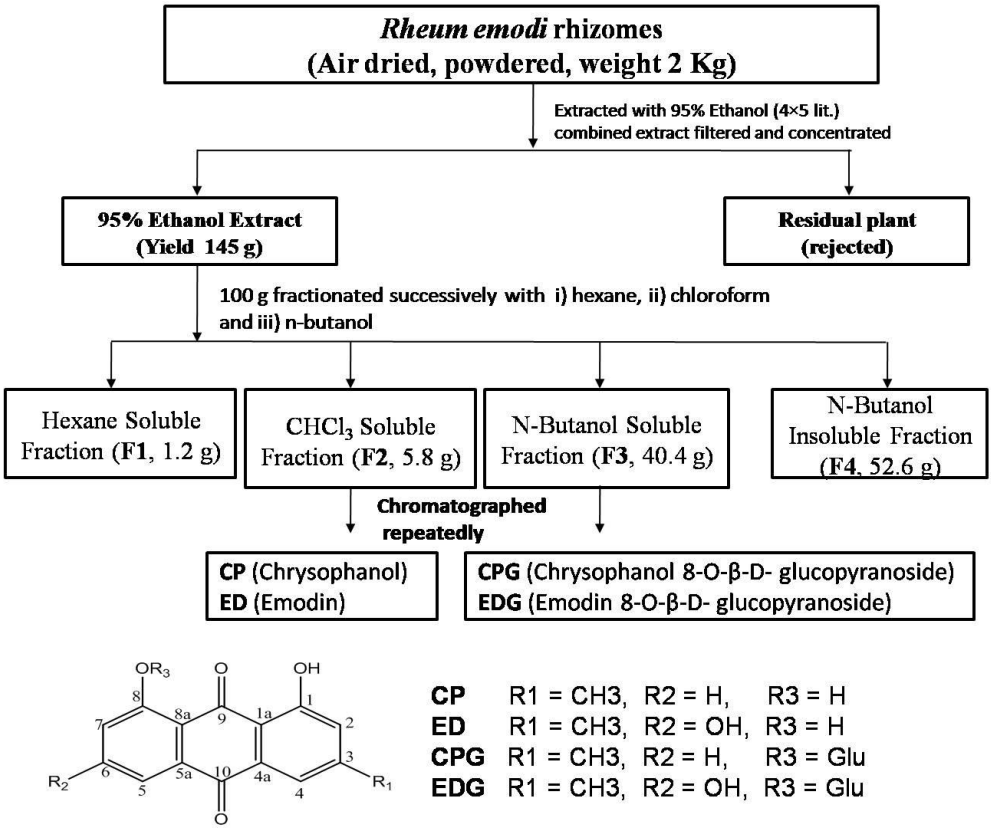


Figure 1: Procedure of extraction, fractionation and isolation of pure compounds from *Rheum emodi* rhizomes. and Chemical structure of the compounds isolated from the active fractions (F2 and F3).

Experimental animals: Adult Sprague dawley rats of either sex, weighing 180-200g were housed in raised bottom mesh cages to prevent coprophagy and were kept in environmentally controlled rooms (temperature 25 ± 2°C, humidity 60-80% and 12 hours light and dark cycle). All experimental protocols were approved by our Institutional Ethical Committee following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) which complies with International norms of INSA (Indian National Science Academy).

Drugs and chemicals: Sucralfate was obtained from Meranani Pharmaceuticals, India, whereas omeprazole and other chemicals were procured from Sigma (St. Louis, MD, USA). All other reagents used were of analytical grade.

Treatment schedule: Graded doses of extract (50, 100 and 200 mg/kg, b.w.), fractions and pure compounds (10, 20 and 40 mg/kg p.o.), reference drugs omeprazole (Omz) (10 mg/kg) and sucralfate (SUC) (500 mg/kg) were prepared in 1% carboxymethyl cellulose (CMC) as suspension and administered orally 45 min. prior to exposure of ulcerogens to animals at a volume of 1ml /200 g of body weight. Animals were fasted for 16 h before ulcerogens exposure and were divided into three groups, (n=6).

Group I (ulcer control): Animals were treated with vehicle (1% CMC) 45 min. before to the induction of ulcer.

Group II (treatment groups): Graded doses of extract (50, 100 and 200 mg/kg, b.w.) or fractions or compounds (10, 20 & 40mg/kg p.o.) were administered 45 min before the induction of ulcer.

Group III (reference groups): Animals were treated with reference anti-ulcer drugs omeprazole (Omz) (10 mg/kg, p.o.) in CRU, aspirin and pyloric ligiation model and sucralfate (500 mg/kg, p.o.) in alcohol induced ulcer model.

Anti-ulcer activity

Cold restraint induced gastric ulcer (CRU) model: Animals were subjected to cold restraint stress after 45 min. of treatment with the extract, fractions, compounds or reference drug omeprazole (Omz). All the animals were immobilized in restraint cage and kept at 4°C in an environmental chamber [17]. After two hours animals were sacrificed and stomachs were observed and scored under Magnascope for ulcers.

Alcohol induced gastric ulcers model (AL): Chilled absolute alcohol (1ml/200g, body weight) was given to animals for induction of gastric heamorrage [18]. chrysophenol (CP), emodin (ED) and sucralfate (SUC) were administered 45 minutes before alcohol treatment. After 1 hour of alcohol administration, animals were sacrificed and stomach was cut open along the greater curvature to observe the gastric lesions appearing as hemorrhagic bands along the mucosal ridges of the stomach. Lengths of the lesions were measured using Biovis image analyzer software and summated to give a total lesion score.

Aspirin induced gastric ulcer model (AS)

Chrysophenol (CP), emodin (ED) and reference drug omeprazole (Omz) were administered 45 min. before the treatment of aspirin (150 mg/kg body weight). Animals were sacrificed after 5 hours of aspirin treatment and the stomach was dissected out, incised along the lesser curvature and the lesions were scored [19].

Pyloric ligation induced ulcer model (PL): After 45 min. of administration of chrysophenol (CP), emodin (ED) and omeprazole (Omz), ulcer was induced by pyloric ligation under chloral hydrate anesthesia (300mg/kg, i.p.). Abdomen was opened and the pyloric part of the stomach was ligated avoiding any damage to the adjacent blood vessels [20]. Stomach was replaced carefully and the animals were allowed to recover with free access to water. After 4 hours, animals were sacrificed and the stomach was dissected out. Lesions were scored and gastric fluid was collected and centrifuged at 2000 rpm for 10 min. The supernatant was collected and used for estimation of gastric secretion and mucin level.

Gastric secretion study: Free and total acidity was measured from the collected gastric juice by titrating against 0.01N NaOH, using phenolphthalein as an indicator and expressed in terms of μM equiv./ml [21]. Mucin level in gastric juice was quantified as per method reported by Crowther, et al. [22].

Ulcer Scoring: Magnascope (5X magnification) were used for ulcer scoring after induction of ulcer via different ulcerogens. Ulcer were scored According to method reported earlier [23]. The severity and intensity of the lesions were graded as following:

i) Shedding of epithelium = 10

ii) Petechial and frank hemorrhages = 20

iii) One or two ulcers = 30

iv) More than two ulcers = 40

v) Perforated ulcers = 50.

***In vitro* assay of H^+ K^+-ATPase activity**: Proton pump or *in vitro* enzymateic activity was done in microsomes isolated from stomach of normal fasted rats [24]. These microsomes were incubated with different concentrations of chrysophenol (CP), emodin (ED) and reference drug omeprazole (Omz) for 10 min at 37°C. The assay buffer containing (in mM) 150 KCl, 10 PIPES, 1 $MgSO_4$, 5 Mg ATP, 1 EGTA and 0.1 ouabain, at pH 7.2, 10µg/ml valinomycin and 2.5µg/ml oligomycin was added. The reaction was carried out at 37°C for 20 min and was stopped by adding 10% ice-cold trichloroacetic acid. After centrifugation (2000 g for 1 min), inorganic phosphate release was determined from the resulting supernatant spectrophotometrically at 310 nm wavelength [25] and expressed as µM/hr/mg protein.

PGE_2 estimation: PGE_2 was determined in gastric tissue obtained from sham, control and treatment groups. Briefly, mucosa was scrapped and rapidly rinsed with ice-cold saline. The tissue was weighed and homogenized in 10 volumes of phosphate buffer (0.1 M, pH- 7.4) containing 1 mM EDTA and 10 µ M indomethacin. The homogenate was centrifuged (10,000 rpm, 10 min, 4°C), and the supernatant was processed for PGE_2 estimation using the Biotrak enzyme immunosorbent assay kit (Cayman), following the manufacturer's instructions. Results were expressed as pg PGE_2/mg protein.

Statistical analysis: All values shown in the figures and tables represent the means ± S.E.M. IC_{50} values with 95% confidence limits were estimated using Maximum Likelihood Iterative Procedure [26]. Statistical analysis was performed with Prism version 3.0 software using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. $P<0.05$ was considered to be statistically significant.

Results

Ethanol extract and fractions of *Rheum emodi* rhizome on cold restraint induced ulcer in rats: Administration of 95% ethanol extract at a graded doses of 50, 100 and 200mg/kg, p.o. in CRU model exhibited 37.5%, 50.0% and 52.5% protection respectively which indicated antiulcer potential of this plant (Figure 2.). Subsequently it has been fractionted into Hexane soluble (F1), choloroform Soluble (F2), n-butanol soluble (F3) and n-butanol insoluble (F4) fractions. Graded doses of these fractions (10, 20 and 40mg/kg, p.o.) were evaluated in CRU model (Table1). Among these fractions only F2 showed potent activity while F3 showed moderate protection in CRU model. (Table 1)

a) b)

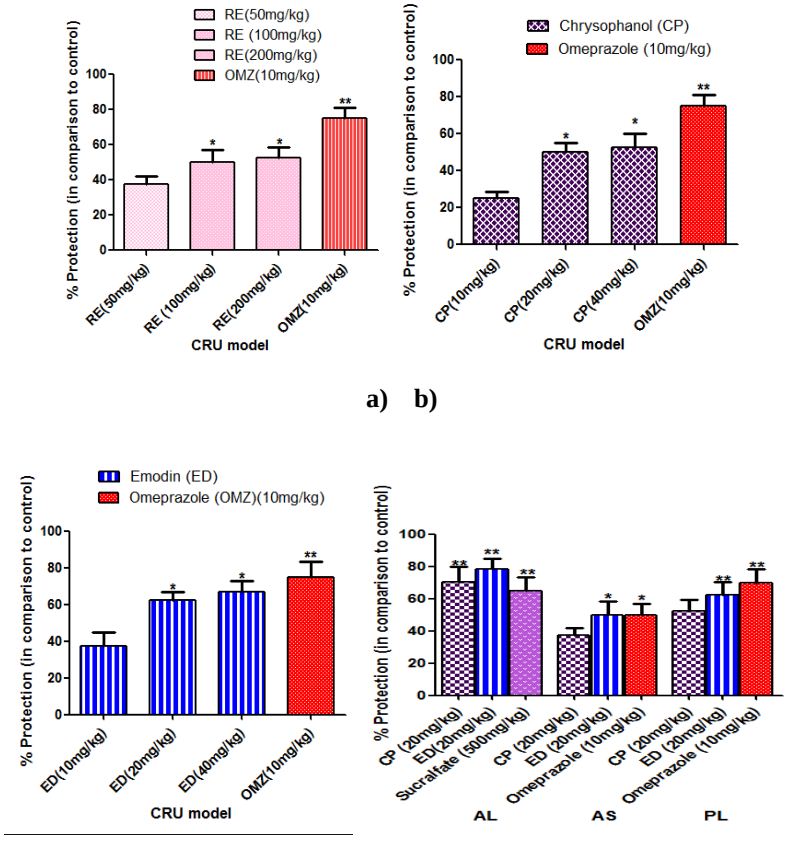


Figure 2: (a) Effect of 95% ethanol extract at graded doses 50, 100 and 200 mg/kg, b.w. **b) & c)** chrysophenol (CP) and emodin (ED) at different doses 10, 20 and 40 mg/kg p.o. compared with reference drug omeprazole (Omz) (10 mg/kg) in CRU model. **d)** Chrysophenol (CP) and Emodin (ED) against alcohol, aspirin and pyloric ligation induced gastric ulcer models in rats. Reference drugs sucralfate (SUC) (500 mg/kg) used for alcohol model and omeprazole (Omz) (10 mg/kg) used for aspirin and pyloric ligation models. Data expressed as mean % protection ± S.E.M. Statistical analysis was done by One Way ANOVA followed by Dunnett's Multiple Comparison Test. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

Fraction name isolated from *Rehum emodi* rhizome	**% Protection in cold restraint ulcer model (CRU)**		
	(10 mg/kg, p.o.)	**(20 mg/kg, p.o.)**	**(40 mg/kg, p.o.)**
Hex. fraction F1	0	0	12.5 ± 6.970
$CHCl_3$ Soluble fraction F2	25 ± 5.065	50 ± 3.891	58.5 ± 4.014
But. Soluble fraction F3	0	25 ± 2.57	37.5 ± 3.385
But. Insoluble fraction F4	0	0	0
Omeprazole (10 mg/kg, p.o)	75.0 ± 6.340		

Table 1. Graded dose analysis of various fractions of *Rehum emodi* rhizome and reference drugs omeprazole (OMZ) on percentage protection of ulcer against cold restraint induced gastric ulcer models in rats.

Chrysophenol and emodin, the active constituents of fraction F2, on cold restraint induced ulcer in rats: The most active fraction F2 yielded chrysophenol (CP) and emodin (ED) as major constituents. Both compounds were screened at graded doses of 10, 20 and 40 mg / kg, p.o. in CRU model and exhibited (25.0%, 50.0%, 52.5%) and (37.5%, 62.5%, 66.67%) protection respectively as compare to reference drug omeprazole (Omz) (75.00%) as shown in Figure 2. From this study, 20 mg/kg, b.w. dose was found effective and selected for further studies.

Chrysophanol 8-O-β-D-Glucopyranoside and emodin 8-O-β-D-Glucopyranoside major constituents of fraction F3 on cold restraint induced ulcer in rats: n-Butanol soluble fraction (F3) was chromatographed repeatedly over silica gel columns and purified to yield chrysophanol 8-O-β-D-glucopyranoside (CPG) and emodin 8-O-β-D-glucopyranoside (EDG). These compounds showed moderate protection in CRU model at doses of (10, 20 and 40mg/kg, p.o.) (Table 2).

Names of Compounds isolated from fraction F-3	% Protection in cold restraint ulcer model (CRU)		
	(10 mg/kg, p.o.)	(20 mg/kg, p.o.)	(40 mg/kg, p.o.)
chrysophanol -8-O-β-D glucopyranoside(CPG)	0	12.5 ± 4.150	0
emodin -8-O-β-D glucopyranoside (EDG)	0	25 ± 3.248	25 ± 6.093
omeprazole (10 mg/kg, p.o)	75.0 ± 7.024		

Table 2: Graded dose study of active constituents isolated from active fractions F-2, F-3 and reference drugs omeprazole (OMZ) on percentage protection of ulcer against cold restraint induced gastric ulcer models in rats.

Chrysophenol and emodin on alcohol induced ulcer model in rats: CP and ED showed significant anti-ulcer activity against ethanol induced ulcer showing 70.51% and 78.48% protection respectively whereas the reference drug, sucralfate (SUC), showed 65.00% protection as depicted in Figure 2 and 3.

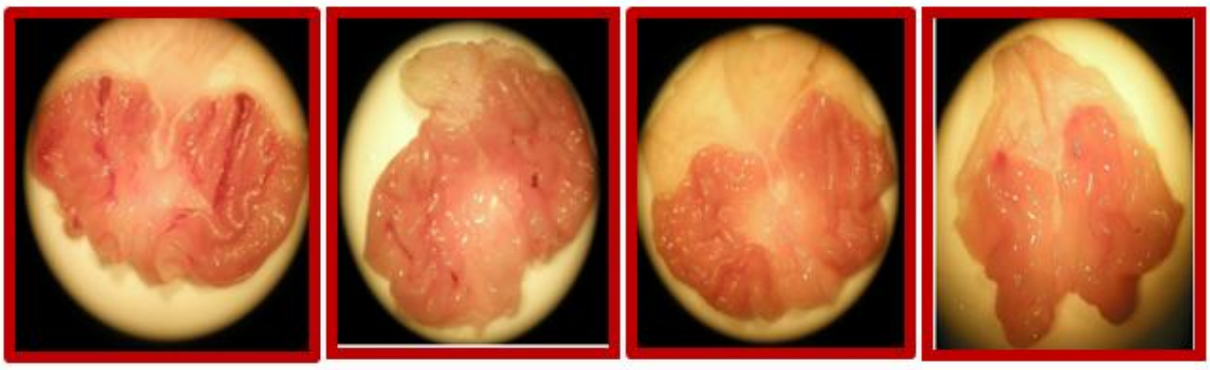


Figure 3: Photographs of ulcerated stomach obtained from rats of Control groups and treated with Chrysophenol (CP), Emodin (ED) and reference drug sucralfate (SUC) against alcohol induced gastric ulcer models in rats (n = 6 in each group).

Chrysophenol and emodin on aspirin induced ulcer model in rats: CP and ED showed potential anti-ulcer activity (37.5% and 50.0% protection respectively) in aspirin induced ulcer model, whereas omeprazole showed 50.0% protection in comparison to control as shown in Figure 2.

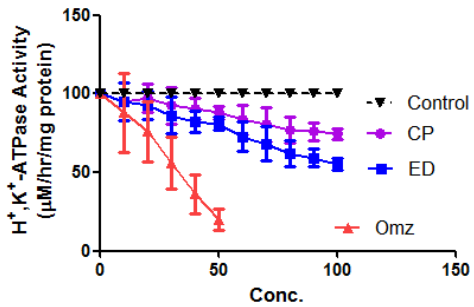
Chrysophenol and emodin on pyloric ligation induced ulcer model in rats*:* Anti-ulcer activity of CP and ED was also observed against pyloric ligation induced ulcer model in rats where it showed protection of 52.5% and 62.5% respectively whereas reference drug omeprazole (Omz) showed 70.0% protection (Figure 2).

Effect of chrysophenol and emodin on gastric secretion: As shown in Table 3, treatment with CP and ED at a dose of 20 mg/kg body weight has significantly reduced the free acidity by 10.72% and 25.61% and total acidity by 15.01% and 32.98%, respectively (Table 3). On the other side, CP and ED at a dose of 20 mg/kg body weight increased the mucin secretion by 32.64% and 46.64% respectively in comparison to control (Table 3).

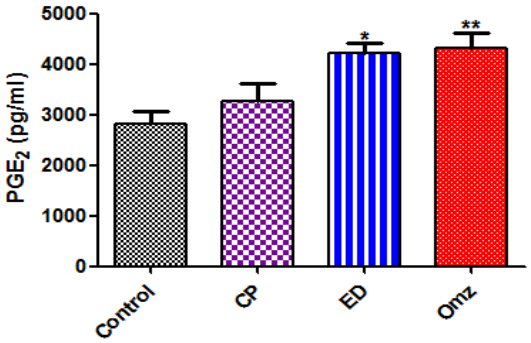
Treatment	Free acid	Total acid	Mucin
	µequiv./ml	µequiv./ml	µg/ml
Control	75.26 ± 3.242	136.90 ± 3.570	544.03 ± 47.39
CP (20mg/kg)	67.19 ± 8.027	116.35 ± 8.310	807.69 ± 35.06*
ED (20mg/kg)	55.98 ± 5.634*	91.74 ± 4.096*	1019.57 ± 92.48**
Omz(10mg/kg)	49.07 ± 6.801**	76.59 ± 6.124**	990.41 ± 49.50*

Table 3: Effect of chrysophenol (CP), emodin (ED) and omeprazole (OMZ) on free acidity, total acidity and mucin contents in pyloric ligation model (n= 6 in each group).

Effect of chrysophenol and emodin on H^+ K^+-ATPase activity: To establish the gastro protective activity of the compounds CP and ED, we investigated the effect of these compounds on H^+ K^+-ATPase inhibitory activity in gastric microsomes isolated from rat stomach. CP and ED inhibited the proton pump activity with an IC_{50} 187.1319µg/ml and 110.3046µg/ml respectively comparable to reference drug omeprazole with an IC_{50} 30.24 µg/ml. it is significant anti-secretary activity of the CP and ED (Figure 4).



a)



b)

Figure 4: a) Effect of chrysophenol (CP), emodin (ED) and reference drug omeprazole (Omz) on H+ K+-ATPase activity in the rat gastric microsomes. Dots and lines are mean ± S.E.M. of experiments performed in triplicates (n=3). **b)** Effect of chrysophenol (CP), emodin (ED) and Omz on gastric PGE_2 level in comparison to ulcer control group. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

Effect of Chrysophenol and Emodin on PGE_2 level: PGE2 generation in the ulcer control group was 2814 ± 255.9 pg/mg tissue protein. The PGE2 value of CP, ED and OMZ treated group was found to be 3273 ± 355.3, 4214 ± 206.1, 4315 ± 304.9 respectively (Figure 4).

Discussion

In present times, Natural products have gained powerful attention due to its effective roles in chemo-therapeutic agents. The anti-ulcer activity of chrysophenol (CP) and emodin (ED) isolated from *R. emodi* has been studied against various models of experimentally induced gastric ulcer in order to evaluate its mechanism of action involved in prevention of ulcer formation.

Further a dose dependent anti-ulcer study of chrysophenol (CP) and emodin (ED) in CRU model. CRU is a well-accepted model for the induction of gastric ulcers, in which peripheral sympathetic activation and increased acid secretion play important roles [27]. In addition, chrysophenol (CP) and emodin (ED) exerted a protective effect against ethanol-induced gastric lesions in contrast to reference drug, sucralfate. Ethanol damages the superficial epithelial layers and inhibits the release of mucosal prostaglandins and depresses the gastric defensive mechanisms [28]. chrysophenol (CP) and emodin (ED) appear to augment the gastric mucosal defense indicating the cyto protective potentials.

Furthermore, gastric acid is an important factor for the genesis of ulceration in pyloric-ligated model [20]. In this model, auto-digestion of mucosa by gastric acid results in the development of ulcers [29]. chrysophenol (CP) and emodin (ED) significantly reduced free and total acidity in this model, which suggests its anti-secretary potency.

In an attempt to clarify the mode of action of chrysophenol (CP) and emodin (ED), through the anti-secretory pathway, its influence on gastric secretion was studied using inhibition of H^+ K^+-ATPase (Proton pump). Proton pump is a membrane bound enzyme that catalyses H^+ transport at the expense of ATP hydrolysis. Thus the inhibition or the blockade of H^+ K^+-ATPase may account for suppressed acid secretion observed in the *in vivo* studies. The results obtained with gastric microsomes isolated from rat stomach showed that chrysophenol (CP) and emodin (ED) potently inhibited the H^+ K^+-ATPase activity comparable to the positive control. Omeprazole, thus suggesting that chrysophenol (CP) and emodin (ED) might be imparting anti-ulcer activity through decrease in acid secretion via proton pump inhibition.

The cytoprotective ability of chrysophenol (CP) and emodin (ED) was evident with increase in mucin content in pyloric ligation model and protection against ethanol induced ulcer model in comparison with the reference drugs. To further substantiate the cytoprotective potency of chrysophenol (CP) and emodin (ED), its effect against NSAIDs induced ulcer model was explored. Studies suggest that NSAIDs induces ulcers due to their effect on cyclooxygenase enzyme leading to reduced prostaglandin production and increase in acid secretion [29,30]. Chrysophenol (CP) and emodin (ED) significantly reduced ulcer incidence, which may be mediated by prostaglandins. The level of prostaglandins was also measured and was found that it also significantly increased the level of prostaglandins.

Though different biological activities of *R. emodi* has been reported earlier, anti-ulcer mechanism of this plant and its active constituents has not been reported till date. Our study is the first of its kind to show anti-ulcer effect of *R. emodi* rhizomes and its active constituents chrysophenol (CP) and emodin (ED).

Conclusion

Thus, the present study demonstrated that the chrysophenol (CP) and emodin (ED) impart gastro protective effects through the inhibition of H^+ K^+-ATPase (proton pump) activity and increasing the PGE2 level. Thus, chrysophenol (CP) and emodin (ED) may emerge as a more potent therapeutic agent in treating gastric ulcer disease because these compounds possess both anti-secretary and cyto protective potentials.

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