RESEARCH ARTICLE

Cytoprotective Effects of Phloretin on Long-Term Post-Prandial Hyperglycemia-induced Esophageal Damage in Rats

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ABSTRACT

Esophageal damage arises from gastroesophageal reflux disease, where the contents of the stomach and duodenum flow back into the esophagus, causing an inflammatory lesion. Phloretin, a phytoconstituent classified as a flavonoid possessing both nutritional and medicinal properties, was the focus of this study. The experiment aimed to investigate the impact of phloretin on experimental esophagitis in albino rats. Rats in various groups underwent the induction of esophagitis through the administration of a Fructose solution (10%) and indomethacin (5mg/Kg). The resulting hyperglycemia from regular Fructose solution intake over 28 days was assessed. After sacrifice, the rats were evaluated for the esophagitis index. Additionally, esophageal tissues underwent further analysis for oxidative stress parameters, including TBARS, SOD, Catalase and GSH. Treatment with phloretin demonstrated a significant inhibition of the esophagitis index. Furthermore, phloretin contributed to the restoration of altered levels of oxidative stress parameters back to normal.

Keywords: Acid reflux, Post-prandial hyperglycemia, Esophagitis, Oxidative stress, Flavonoid.

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INTRODUCTION

The regurgitation of gastric acid and stomach contents results in epithelial damage and cell death in the esophagus, leading to an inflammatory response characterized by the infiltration of inflammatory cells and the expression of pro-inflammatory cytokines in affected tissues.[1,2] Persistent reflux of gastric acid and stomach contents into the esophagus causes various forms of esophageal damage, including inflammation, ulcers, and hemorrhaging in the epithelium.[3] Conventional treatments for gastroesophageal reflux disease (GERD) involve histamine-2 receptor antagonists, proton pump inhibitors, and antacids, yet their effectiveness is limited due to complications such as high recurrence rates, incomplete mucosal healing, and the development of esophageal strictures.[4]

Herbal remedies derived from plants, with their extensive history in clinical practice and therapeutic benefits, have gained attention. Recent studies have highlighted the protective effects of Xuanfudaizhetang in rats with reflux esophagitis, demonstrating increased esophageal pH levels and reduced inflammation.[5] Extracts from *Artemisia campestris* L. have also exhibited protective properties against esophageal mucosal injuries induced by gastric acid reflux.[2] Additionally, the positive effects of *Morinda citrifolia* fruit extract and its biomarker scopoletin on reflux esophagitis and gastric ulcers in rats have been documented.[6]

Alterations in blood glucose concentration, particularly marked hyperglycemia (≈15 mM), significantly impact gastrointestinal motor function in both normal individuals and those with diabetes mellitus. This effect extends to various digestive organs, including the esophagus, stomach, gallbladder, small intestine, colon and anorectum.[7-12] Patients with diabetes mellitus are more prone to gastroesophageal reflux, often attributed to transient lower esophageal sphincter relaxation (TLESR) as the predominant sphincter mechanism underlying reflux.[13]

L-tryptophan has been found to prevent L-tryptophan-induced esophageal mucosa (OM) and lower esophageal sphincter (LOS) damage. However, its esophagoprotective-

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promoting effect appears to be linked to endothelial metabolism stimulation rather than an amelioration of PG/COX activity.[14] In a study involving rats treated with L-Try (400 mg/kg) and indomethacin (Indo, 5 mg/kg) during post-prandial hyperglycemia (PPH),[15] the impact of these substances on esophageal damage was explored.

Phloretin, a dihydrogen chalcone flavonoid primarily extracted from apples, is known for its potent antioxidant properties. Previous research has suggested its potential therapeutic applications in cardiovascular disease, cancer, ischemia-reperfusion injury and neurodegeneration. Notably, phloretin has been shown to prevent nerve terminal toxicity induced by electrophiles such as acrolein. Despite extensive studies identifying the pharmacophore responsible for phloretin's antioxidant activity,[16,17] its ameliorative effects on esophageal damage in reflux esophagitis have not been previously reported. This study aims to investigate the potential ameliorating effects of phloretin on post-prandial hyperglycemia-induced esophagitis in rats.

MATERIAL AND METHODS

Seven-week-old sprague Dawley rats, weighing between 150 to 200 g, were accommodated in conventional rat cages during the experimental period. The rats were provided continuous access to ample food and water, housed in an environment with a 12-hour light/dark cycle, at a temperature maintained between 21 to 25˚C and sustained at a humidity level ranging from 35-60%. Following a one-week adaptation period, the rats were randomly allocated into five groups of six each. These groups included group I (Control group), receiving tap water for 28 days. Group II (Toxic group) received fructose solution (10%) and indomethacin (5 mg/Kg) for 28 days, group III (Standard group) received omeprazole (30 mg/kg) along with Fructose solution (10%) plus indomethacin (5 mg/Kg) for 28 days, group IV (Test I) receiving phloretin (25 mg/kg, p.o.) along with fructose solution (10%) plus indomethacin (5 mg/Kg) for 28 days and group V (Test II) receiving phloretin (50 mg/kg, p.o.) along with Fructose solution (10%) plus indomethacin (5 mg/Kg) for 28 days. The rats were fasted 18 hours before the sacrifice but maintained water supply. Rats were euthanized and the esophagus samples along with the stomach of each rat were immediately removed and, washed with saline and photographed for examination of esophageal damage (Figure 1 and 2). A small portion of tissue was immediately fixed in 10% formalin and sent to pathology for histopathological examination (Figure 3).[18] Remaining tissue was kept at -20˚C for further studies. A blood sample was also taken for estimation of blood glucose levels. TBARS, SOD, catalase and GSH levels were estimated using tissue homogenate of the esophagus. The research received approval from the Institutional Animal Ethics Committee of the Faculty of Pharmacy, Integral University, Lucknow, India, with registration number 1213/PO/Re/S/08/CPCSEA. All presented data were expressed as mean ± SD and subjected to statistical analysis using one-way ANOVA, followed by Dennett's t-test to identify potential significance among different groups. Statistical significance was denoted by $p < 0.05$, $p < 0.01$ and $p < 0.001$. GraphPad software (3.2), located in San Diego, CA, was utilized for the statistical analysis.

RESULTS

Results are depicted in Table 1.

Estimation of Blood Glucose Level

The blood sample was tested for estimation of glucose level using the glucometer "My Lyf" and recorded for statistical analysis. The treated group IV and group V both showed a significant decrease in blood glucose levels as compared to toxic group II (Figure 4).

Estimation of Esophageal Erosion Index[19]

After sacrificing the animals, the extraction of the esophagus and the stomach was done. The esophagus was opened along the greater curvature and meticulously dissected by extending the dissection line along its major axis. Subsequently, the excised tissue underwent a thorough washing with normal saline and was examined for any lesions. The extent of erosions was assessed using a scoring system detailed in a table. The severity score was then determined by dividing the total score by ten, resulting in the calculation of the esophagitis index (Table 2, Figure 5).

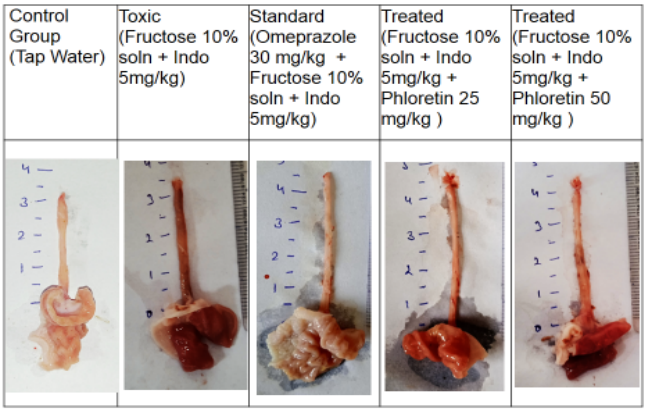


Figure 1: Effect of phloretin on macroscopic appearance of rat esophagus (external)

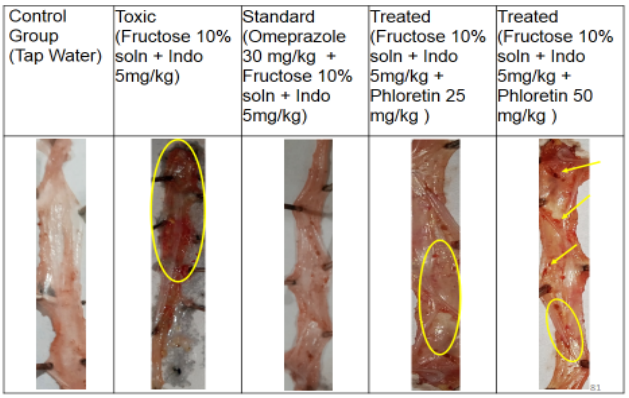


Figure 2: Effect of phloretin on macroscopic appearance of rat esophagus (internal)

Table 1: Effect of phloretin on assessed parameters

Parameter	*Control (Tap Water)*	*Toxic (Fructose 10% soln + Indo 5 mg/kg)*	*Standard (Omeprazole 30 mg/kg + Fructose 10% soln + Indo 5 mg/kg)*	*Treated (Fructose 10% soln + Indo 5 mg/kg + Phloretin 25 mg/kg)*	*Treated (Fructose 10% soln + Indo 5 mg/kg + Phloretin 50 mg/kg)*
EL Index	119.5 ± 5.1961	192.75 ± 3.3040#	132.5 ± 3.4156**	142.5 ± 3.8729**	157.25 ± 2.2173**
Glucose Level	0.025 ± 0.050	3.1075 ± 0.0788#	0.6075 ± 0.1043**	1.14 ± 0.0365**	1.465 ± 0.1379**
TBARS	0.0829 ± 0.0027	0.1505 ± 0.0243#	0.0965 ± 0.0031**	0.1046 ± 0.0112**	0.1188 ± 0.0167*
SOD	0.6104 ± 0.0458	0.3932 ± 0.0069#	0.3889 ± 0.2198**	0.47 ± 0.0220**	0.3974 ± 0.0187*
CAT	0.1765 ± 0.0106	0.0498 ± 0.0280#	0.1366 ± 0.0814**	0.1353 ± 0.0355ns	0.0490 ± 0.0276*
GSH	18.842 ± 0.7168	6.132 ± 3.6258#	16.972 ± 1.8204**	14.786 ± 1.0022**	9.204 ± 5.1606ns

All the values were expressed as Mean ± SD, N = 5

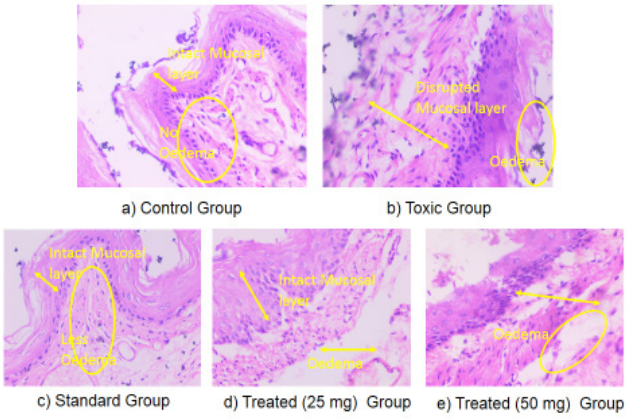


Figure 3: Histological section of rat esophagus showing the effect of Phloretin Low dose (25mg/kg) and High dose (50mg/kg) (40X magnification, H&E stain; Scalar bar: 100 µm)

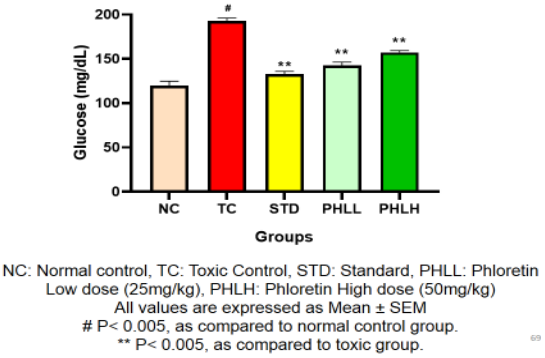


Figure 4: Effect of phloretin on blood glucose level

The treated group IV and V both showed a very significant decrease in esophageal erosion index as compared to toxic group II (Figure 2).

Estimation of thiobarbituric acid reactive substance (TBARS)

Required reagents for TBARS estimation were prepared and the tissue sample of the esophagus was homogenized. Out of whole tissue homogenate, 1-mL was taken out for TBARS activity and the remaining was kept for further studies. Estimation was performed as studied by Tahir and co-workers.[20] The calculation of TBARS content was determined using the following formula:

Table 2: Scoring for assessment of erosion and severity

Erosion (millimeter)	1 or less	1–2	2–3	>3
Score	1	2	3	4

$$nM \text{ of } MDA = A \times 540 \times V/0.156$$

Where A is the absorbance at 540 nm and V is the volume of the test solution.

The treated group IV showed a more significant decrease in TBARS levels in comparison to group V but both showed a significant decrease as compared to toxic group II (Figure 6).

Estimation of Superoxide Dismutase

Buffers and other reagents were prepared and estimation was performed as studied by Khushtar and co-workers.[21] The supernatant was assayed for superoxide dismutase (SOD) activity based on the inhibition of pyrogallol autoxidation. SOD levels were computed using the formula:

$$\text{Unit of SOD per mL of sample} = [(A - B) \times 100/A \times 50]$$

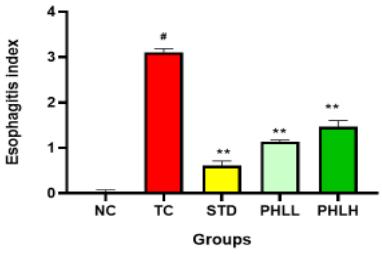
Where, 'A' is the difference of absorbance in 1-minutes in control and 'B' is the difference of absorbance in 1 min in the test sample

The results showed a significant increase in SOD levels in treated group IV but a non-significant effect in treated group V as compared to toxic group II (Figure 7).

Estimation of Catalase

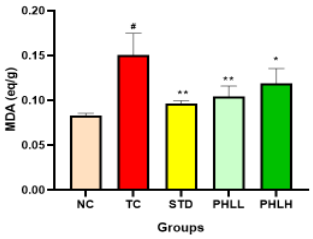
The supernatant of the tissue homogenate, buffers and other reagents were prepared and estimation is done by the method of Khushtar and co-workers.[21] Catalase activity expressed as η moles of H_2O_2 consumed/minute/mg protein and calculated with the formula:

The results showed a significant increase in catalase levels in treated group IV but non-significant effect in treated group V as compared to toxic group II. (Figure 8)



NC: Normal control, TC: Toxic Control, STD: Standard, PHLL: Phloretin Low dose (25mg/kg), PHLH: Phloretin High dose (50mg/kg)
All values are expressed as Mean ± SEM
# P< 0.005, as compared to normal control group.
** P< 0.005, as compared to toxic group.

Figure 5: Effect of phloretin on esophagitis index



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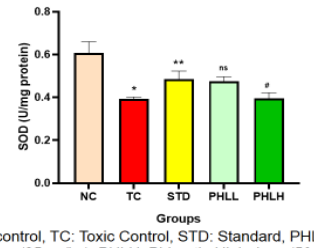
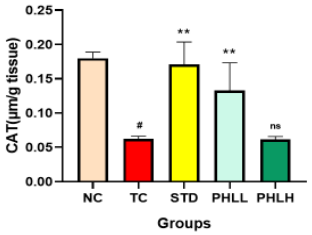
All values are expressed as Mean ± SEM
# P< 0.005, as compared to normal control group.
** P< 0.005, as compared to toxic group.
* P<0.05, as compared to toxic group.

Figure 6: Effect of phloretin on TBARS level



NC: Normal control, TC: Toxic Control, STD: Standard, PHLL: Phloretin Low dose (25mg/kg), PHLH: Phloretin High dose (50mg/kg)
All values are expressed as Mean ± SEM
# P< 0.005, as compared to normal control group.
** P< 0.005, as compared to toxic group.
ns P>0.5, as compared to toxic group.
# P< 0.05, as compared to toxic group.

Figure 7: Effect of phloretin on SOD level



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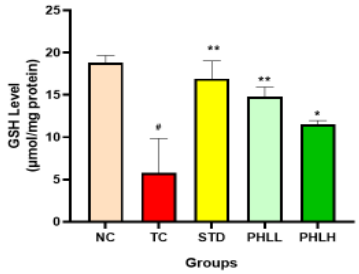
All values are expressed as Mean ± SEM
# P< 0.005, as compared to normal control group.
** P< 0.005, as compared to toxic group.
ns P>0.5, as compared to toxic group.

Figure 8: Effect of phloretin on catalase level



NC: Normal control, TC: Toxic Control, STD: Standard, PHLL: Phloretin Low dose (25mg/kg), PHLH: Phloretin High dose (50mg/kg)
All values are expressed as Mean ± SEM
# P< 0.005, as compared to normal control group.
** P< 0.005, as compared to toxic group.
* P<0.05, as compared to toxic group.

Figure 9: Effect of phloretin on GSH level

Estimation of Reduced Glutathione

EDTA, DTNB, TCA and buffers are prepared along with the homogenate of tissue samples of the esophagus for estimation of reduced glutathione level.[20] GSH level expressed as µg/mg of protein using the equation:

Treated groups IV and V both showed a significant increase in GSH levels as compared to toxic group II. (Figure 9)

DISCUSSION

Phloretin shows significant cytoprotective effects on low dose (25 mg/kg) with a marked decrease in Esophageal Lesion Index and TBARS level, in comparison to its high dose (50 mg/kg) of test drug. Further, in the dose (25 mg/kg), phloretin is able to improve endogenous antioxidant levels (CAT, SOD) and GSH levels as similar to the standard drug (Omeprazole), while in high dose (50 mg/kg) it shows no significant changes in antioxidant parameters, as compared to toxic groups. These results justify the outcomes of the toxicological study of the drug and also resemble the work of other researchers[22] as well. Moreover, it was recorded that the test drug phloretin significantly decreased blood glucose levels to produce a cytoprotective effect.

CONCLUSION

Two doses of phloretin significantly ameliorate the esophageal damage in rats. Hyperglycemia caused tissue damage, which was elevated by the cytotoxic actions of indomethacin. The selected model post-prandial hyperglycemia successfully induced damage in esophageal tissues. The low dose (25 mg/kg) of phloretin displayed a marked decrease in the esophageal lesion index and TBARS level, in contrast to high dose (50 mg/kg). The endogenous antioxidant parameters were found to be significantly near to the standard drug (Omeprazole) treated group in a dose 25 mg/kg of phloretin. Whereas, in high dose of 50 mg/kg no significant changes were recorded. Findings revealed that the higher dose of phloretin has lesser or no effect as compared to a lower dose, which indicates the prospective efficacy at low doses and may involve the ceiling effects of the drug. Phloretin positively expressed cytoprotection and amelioration of antioxidant parameters, but more in-depth study is required in this context at the molecular

level to understand the reasoning.

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