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RESEARCH ARTICLE

# Rutaecarpine Regulates the Expression of Pro-Inflammatory Cytokines to Induce Protective Effects in the Murine Model of Acute Reflux Esophagitis

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**A B S T R A C T**

Inflammation is a hallmark of esophagitis. Rutaecarpine or rutecarpine is an alkaloid compound with both anti-inflammatory and anti-oxidative activity. Here, we sought to evaluate potential protective effects of Rutaecarpine on reflux esophagitis in a murine model of the disease, which was treated with different doses of Rutaecarpine (5, 10 and 20 mg/kg) and Omeprazole (20 mg/kg) for 6h, the results of which were compared with a non-esophagitis control group. Biochemical markers were measured based on tissue and serum samples collected from esophagitis-positive and control animals. Rutaecarpine significantly reduced macrophage cell viability, while negatively regulating nitric oxide (NO) and inducible NO synthase (iNOS), interleukin-1beta (IL-1β), cyclooxygenase-2 (COX-2) and prostaglandin E2 ($PGE_2$). Rutaecarpine treatment was associated with marked reversal of esophageal lesions, reduced gastric secretion and activity ($P<0.001$), increased gastric pH and SH groups, as well as reduced levels of $H_2O_2$, free iron, calcium ($P<0.001$). Additionally, Rutaecarpine treatment negatively regulated the expression of $H^+K^+$ATPase and histamine, while markedly altering the levels of lactoperoxidase (LPO), catalase (CAT), superoxide dismutase (SOD) glutathione (GSH) and monocyte chemoattractant protein-1 (MCP-1) ($P<0.001$). Rutaecarpine was found to confer protective effects against esophagitis in rats.

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## INTRODUCTION

Esophagitis refers to the inflammation of the gastrointestinal lining that connects the mouth to stomach (Mastracci *et al.*, 2020). An important causal factor for esophagitis is gastroesophageal reflux disease (GERD), which itself is caused by prolonged flowing of gastric secretions to esophagus (Tripathi *et al.*, 2018). The extent of esophageal damage depends on the composition on gastric secretions, as with acidity and chronicity playing major parts in its pathogenesis (Bor, 2019). While the esophageal lining is normally protected against the acidic secretions of the stomach by continuous production of mucus and bicarbonate, prolonged exposure to gastric secretions may result in esophageal lesions, nonetheless. (Bor, 2019).

While the molecular and cellular mechanisms underlying GERD are yet to be fully elucidated, several studies have proposed a role for oxidative stress (Höllwarth, 2012), which is characterized by excess generation of reactive oxygen species (ROS) that might damage the esophageal epithelium by depleting its mucosal barrier. Upregulation of ROS can, in turn, accelerate the activation of lactoperoxidase (LPO) and neutrophils, which is thought to contribute to GERD ( Singh *et al.*, 2012; Ponomarenko *et al.*, 2018). This has been partially confirmed by investigations suggesting beneficial effects for endogenous antioxidants in vulnerability to gastric ulcers (Zahid *et al.*, 2020; Lee, 2022), which are hypothesized to stem from dysregulated proinflammatory cytokines and activation of redox-sensitive transcription factors. Accordingly, administration of anti-inflammatory agents is thought to yield better clinical outcomes by

inhibiting the inflammatory response ( Dong *et al.*, 2022; Aran *et al.*, 2023).

In addition to mucin, the mucosal barrier of the esophagus also comprises nitric oxide (NO) and prostaglandins (e.g., PGE2), which serve to protect the esophageal epithelium from acidic secretions of the stomach. Being actively synthesized by both gastric epithelial and endothelial cells, NO stimulates the production and secretion of bicarbonate, while positively regulating blood flow to the mucosal lining (Korbut *et al.*, 2020) PGE2, on the other hand, is synthesized by mucosal cells (Sarosiek, 2016) and functions in a way similar to NO. Together, NO and PGE2 regulate the integrity of gastroesophageal epithelium, thus, preventing development of GERD (Sarosiek, 2016; Smith *et al.*, 2022).

Prolonged gastroesophageal is soon accompanied by esophageal injury, which is perceived as a feeling of heartburn, as well as difficult swallowing or dysphagia. Erythema, erosion and ulceration are hallmarks of esophageal injury (Bor, 2019). The primary therapeutic approach to GERS consists of proton pump inhibitors (PPIs) and type 2 histamine receptor (H2R) blockers that regulate the acidity of stomach (Bor, 2019). Omeprazole is well-known PPI, whose mechanism of action includes inhibition of proton pumps that are responsible for acidification of the stomach. Still, these medications are associated with several adverse effects that may include nausea and headache in short-term, and more serious complications such as renal failure and infections in long-term (Edinoff *et al.*, 2023; Zerr *et al.*, 2023). Ironically, PPIs themselves may result in aggravated GERD through a phenomenon known as rebound acid hypersecretion, which is the consequence of increased acid production following a period of downregulation (Thurber *et al.*, 2023). H2R blockers (e.g., famotidine), on the other hand, function by inhibiting the activity of histamine receptors, which indirectly affect acid production in a positive way. Similar to PPIs, H2R blockers may result in adverse events like ulcer relapse following long-term treatment (Meng *et al.*, 2023).

Rutaecarpine (indolopyridoquinazolinone) or rutecarpine is an alkaloid compound that is extracted from the genus *Evodia rutaecarpa*, and empirically used in the treatment of postpartum bleeding, abdominal pain and hypertension as a component of the Asian alternative medicine due to its antihypertensive, anti-thrombotic and anti-inflammatory effects. Considering the numerous beneficial effects of Rutaecarpine, we sought to investigate whether Rutaecarpine might have any protective effects on gastrointestinal tract by developing a murine model of acute reflux esophagitis.

## MATERIALS AND METHODS

**Cell culture:** RAW 267.7 macrophages cell line (ATCC) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% P/S, which was prospectively incubated at a temperature of 37°C for a total of 7 days, during which the culture media were intermittently replaced with fresh media on a daily basis. In the end of this period, cell cultures were treated with various concentrations of Rutaecarpine and for a period of 24 h.

**Cell viability assay:** The effect of Rutaecarpine on cell viability was assessed using CCK-8 kit (Dojindo Molecular Technologies, Inc., Rockville, USA). To this end, cells were seeded into 96-well plates and treated with different concentrations of Rutaecarpine (10, 20 and 40μM) for 24h after being cultured. Spectroscopy was performed using a microplate reader set to determine absorbance at the wavelength of 450 nm.

**NO production:** To quantify NO production, RAW 264.7 macrophages were exposed to lipopolysaccharide (LPS) to induce an inflammatory response, and then treated with different concentrations of Rutaecarpine (10, 20 and 40μM) for 24h. After of the addition of Griess reagent, and cells were incubated for at room temperature for 10 min, before being evaluated by spectroscopy at 540nm.

**Cytokines and inflammatory parameters:** Quantification of IL-1β, iNOS, COX-2 and PGE2 was carried out using enzyme immunoassay kits (R&D Systems Inc., Minneapolis, MN, USA).

**Animals**: We selected a population of 12 to 14-week-old male Sprague Dawley (SD) rats weighing 250∼300g. The rats were maintained under standard laboratory conditions characterized by a mean temperature of 22±5°C and a relative humidity of 60±20%; with diurnal exposure to equal periods of dark (12h) and light (12h). The protocol of the present study was approved by the Institutional Ethics Committee of Shaanxi University of Traditional Chinese Medicine.

**Experimental protocol:** The animals were assigned to 6 groups, each containing six rats, of which 5 groups were subjected to reflux esophagitis (RE), except for the control group. To induce RE, the animals were injected with phenobarbitone for anesthetic purposes before being subjected to celotomy, which involved ligation of pylorus, fore-stomach and the corpus junction by suturing. Postoperatively, the animals were prevented from having access to food and water. Grouping of the animals was performed accordingly:

- Group I: controls
- Group II: Reflux esophagitis
- Group III-V: Reflux esophagitis + Rutaecarpine (5, 10 and 20 mg/kg)
- Group VI: Reflux esophagitis + Omeprazole (20 mg/kg)

After being treated for a period of 6 hours, animals in all groups were subjected to double ligation for autopsy. After being removed, the gastroesophageal tract was inspected for gross signs of pathology. This was followed by retrieval of tissue specimens, which were homogenized, with the resulting supernatant being collected for analytic purposes. Additionally, blood samples were taken from all groups and stored in pre-heparinized tubes.

**Gastric pH:** the excised stomach was rinsed with the 1 mL of NaCl (0.9%) using a micropipette (1000μL). The gastric pH was then measured using a pH meter device (Toledo 320, Mettler, Switzerland) (Doulami *et al.*, 2018).

**Quantification of oxidative stress markers:** the relative concentration of sulphhydryl (-SH) groups, as well as MDA, CAT, SOD and GSH were quantified based on method, which is discussed elsewhere (Wu *et al.*, 2023).

**Quantification of $H_2O_2$, iron and calcium:** Quantification of $H_2O_2$ was performed after having the compound react with 4-aminoantipyrine and p-hydroxybenzoic acid in the presence of peroxidase. The resulting product, known as quinoneimine, gives the solution a pink color, the absorbance of which can be evaluated at 505 nm to determine the concentration of $H_2O_2$.

The concentration of non-heme iron in the excised esophageal tissues was determine based on the Ferrozine method (Flores *et al.*, 2015), which involved dissociation of the transferrin-iron complex upon exposure to guanidine acetate solution. The dissociated iron was reduced by being exposed to ascorbic acid, and being subjected to reaction with ferrozine, which resulted in pink discoloration. Spectroscopy was then performed at a wavelength of 562nm.

The concentration of calcium was estimated based on the colorimetric method, in which involved the reaction of calcium with o-Cresolphthalein in an alkaline medium, resulting in generation of a colored complex, the absorbance of which was subsequently assessed at 570 nm.

**Histamine:** To measure the level of histamine, we treated the plasma samples with perchloric acid (0.2M), and centrifuged the compound at 4°C for 30 min with a velocity of 10000×g rpm. The resulting supernatant was collected and analyzed with high-performance liquid chromatography (HPLC) (Shen *et al.*, 2020).

**$H^+$-$K^+$-ATPase activity:** To determine $H^+$-$K^+$-ATPase activity, a medium containing tris-HCl buffer (70 mM) and $MgCl_2$ (5 mM) with a pH of 6.8 was mixed with 1mL of KCl (10mM) and incubated for at room temperature for a period of 60 min. The reaction was initiated with the addition of adenosine 5′-triphosphate di(tris) salt dehydrate (2mM), and terminated by adding trichloroacetic acid (10%) to the solution, which was then followed by 20 min of incubation at room temperature before being centrifugated. After the centrifugation, the supernatant was mixed with ammonium molybdate (2.5mL) and 1-amino-2-naphthal-4- sulfonic acid (0.5mL). The absorbance of the resulting compound was assessed at the wavelength of 620 nm (Shen *et al.*, 2020).

**Cytokines level:** The concentrations of TNF-α, IL-1β, IL-6 and MCP-1 were measured using relevant ELISA kits (Elabscience, Wuhan, China).

**Statistical analysis:** We used the GraphPad Prism software v9.0 (St. louis, USA) to perform statistical analysis. Based on the Tukey's multiple comparison test, the threshold of significance was considered to correspond to $p<0.05$.

## RESULTS

**NO and $PGE_2$:** We found a significance inverse dose-dependent association between Rutaecarpine and cell growth (Fig. 1a). While exposure to LPS resulted in macrophage proliferation, treatment with Rutaecarpine was found to substantially inhibit cell growth. Fig. 1b shows the upregulation of NO after exposure to LPS, which was significantly reversed after treatment with Rutaecarpine ($P<0.01$). The concentrations of IL-1β (Fig. 1c), iNOS (Fig. 1d), COX-2 (Fig. 1e) and $PGE_2$ (Fig. 1f) followed a similar trend in response to LPS and Rutaecarpine treatment.

**Esophagus lesions, gastric secretion volume** (GSV) **and activity** (GSA)**:** The negative regulatory effect of Rutaecarpine on esophageal lesions, GSV and GSA is shown in Fig. 2. Rats with RE were found to have extensive esophageal lesions, along with increased volume and acidity of gastric secretions. After Rutaecarpine treatment, we observed a marked decline in the extent of esophageal lesions (Fig. 2a), as well as GSV (Fig. 2c) and GSA (Fig. 2e). When administrated with a dose of 20 mg/kg, Rutaecarpine reduced esophageal lesions by 64.96% (Fig. 2b), with a concomitant decrease of 28.72% in GSV (Fig. 2d) and 41.84% in GSA (Fig. 2f). Treatment with Omeprazole (PPI) also led to comparable levels of alleviation with regards to esophageal lesions, GSV and GSA.

**pH:** While the acute phase of RE was associated with a pH of 2.05, we found that treatment with Rutaecarpine had a positive regulatory effect on pH in a dose-dependent manner. Administration of Rutaecarpine in doses of 5, 10 and 20 mg/kg resulted increased the pH to2.25, 3.05 and 5.25, respectively (Fig. 3). Treatment with Omeprazole increased the pH to 5.3, which was slightly higher than a comparable dose of Rutaecarpine.

**Non-enzymatic thiol:** SH- groups are required for proper activity of glutathione, which is an antioxidant. As shown in Fig. 3b, Rutaecarpine significantly upregulated SH levels in mice with RE.

**$H_2O_2$, iron and calcium concentrations:** Increased levels of $H_2O_2$ (Fig. 4a), iron (Fig. 4b) and calcium (Fig. 4c) were observed with RE. This was significantly reversed after the administration of Rutaecarpine ($P<0.001$). Treatment with Rutaecarpine at a dose of 20 mg/kg was associated with the strongest inhibitory effect on the concentrations of $H_2O_2$, iron and calcium.

**$H^+$-$K^+$-ATPase activity and histamine levels:** Induction of RE resulted in upregulation of $H^+$-$K^+$-ATPase activity (Fig. 5a) and histamine levels (Fig. 5b), which was subsequently inhibited upon treatment with Ruteacarpine and Omeprazole ($P<0.001$).

**Markers of oxidative stress:** Esophagitis was characterized by an increased level of LPO and reduced concentrations of CAT, SOD and GSH (Fig. 6). Administration of Rutaecarpine was associated with marked downregulation of LPO and upregulation of CAT, SOD and GSH.

**Cytokines:** Our murine model of RE demonstrated increased expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (Fig. 7), which was substantially reversed

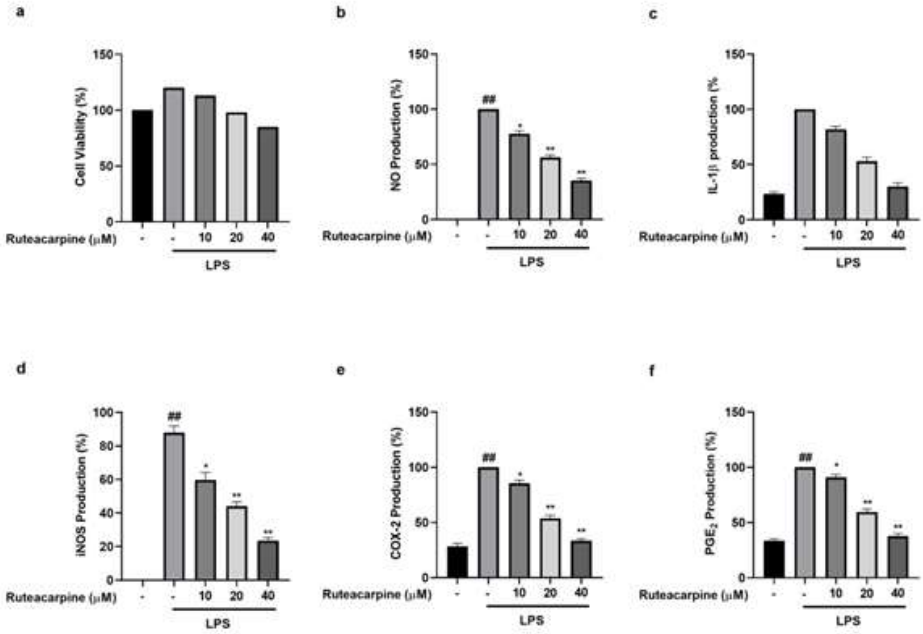



**Fig. 1:** Anti-inflammatory effects of Rutaecarpine on RAW 264.7 cells treated with LPS. a: cell viability, b: NO production, c: IL-1β, d: iNOS, e: COX-2 and f: $PGE_2$. Data are presented as mean ± standard deviation (SD); ## indicates P<0.01 compared with untreated cells, while * and ** denote P<0.05 and P<0.01, respectively.

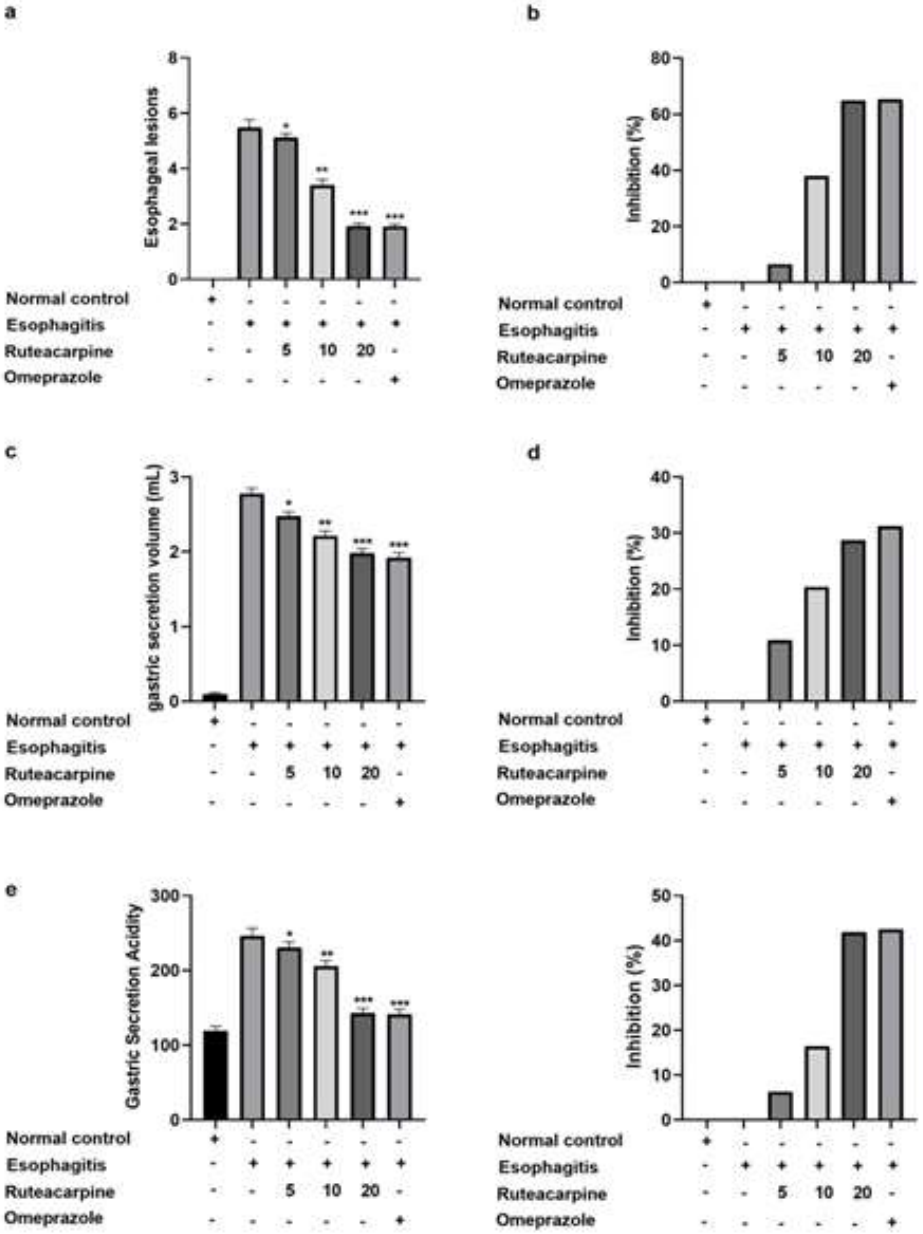



**Fig. 2:** Regulatory effects of Rutaecarpine on esophageal lesions, gastric secretion volume and acidity. a: esophageal lesions, b: esophageal lesion inhibition, c: gastric secretion volume (GSV), d: GSV inhibition, e: gastric secretion acidity (GSA) and f: GSA inhibition. Data are presented as mean ± standard deviation (SD); *, ** and *** indicate P<0.05, P<0.01 and P<0.01, respectively.

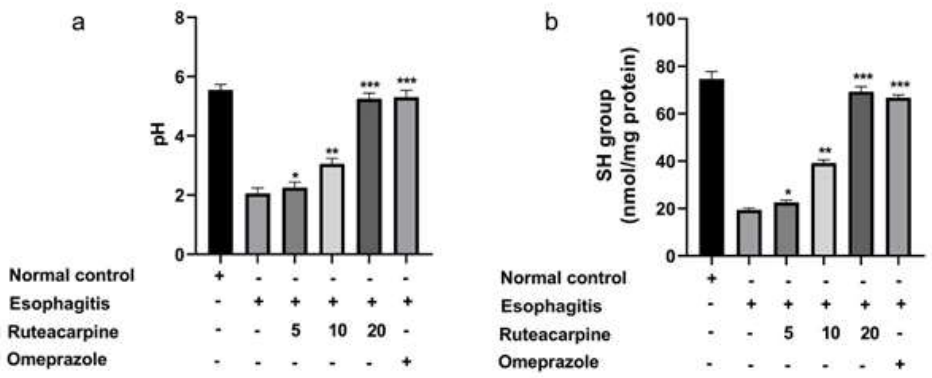
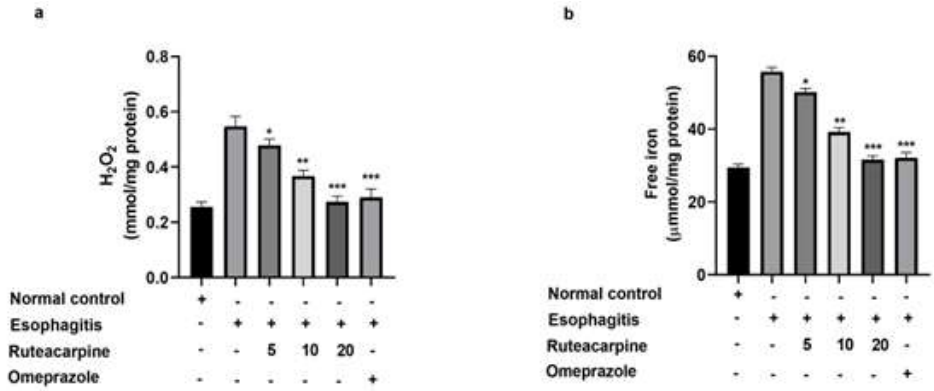



**Fig. 3:** a: Positive regulatory effect of Rutaecarpine on gastric pH.; b: Positive regulatory effect of Rutaecarpine on SH levels. Data are presented as mean ± standard deviation (SD); *, ** and *** indicate P<0.05, P<0.01 and P<0.01, respectively.



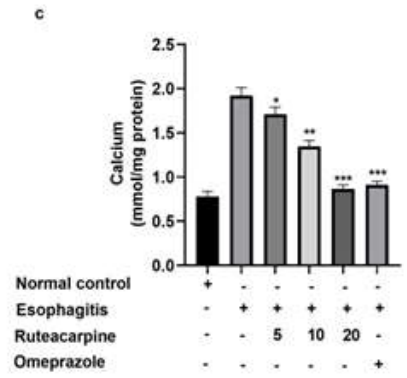




**Fig. 4:** Regulatory effects of Rutaecarpine on $H_2O_2$, free iron and calcium level. a: $H_2O_2$, b: free iron and c: calcium. Data are presented as mean ± standard deviation (SD); *, ** and *** indicate P<0.05, P<0.01 and P<0.01, respectively.

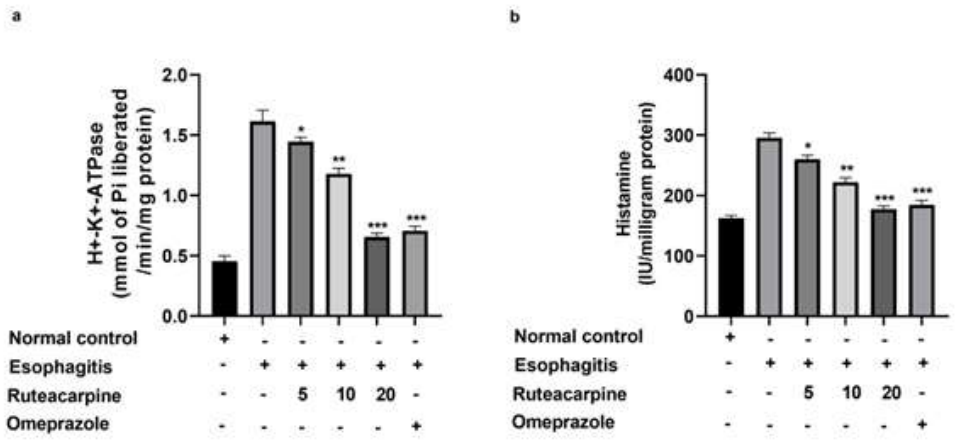



**Fig. 5:** Inhibitory effects of Ruteacarpine on $H^+K^+$ATPase and histamine expression. A: $H^+K^+$ATPase and b: histamine. Data are presented as mean ± standard deviation (SD); *, ** and *** indicate P<0.05, P<0.01 and P<0.01, respectively.

by Rutaecarpine treatment (P<0.001). Additonally, MCP-1 was found to be negatively modulated upon treatment with Rutaecarpine (Fig. 7d).

## DISCUSSION

A highly prevalent gastroesophageal condition, GERD is associated with several important complications such as esopheageal strictures or narrowing and Barrett's esophagus, with the latter being a known risk factor for esophageal carcinoma (Di Capua *et al.*, 2023). At its lowermost portion, the esophagus is surrounded by a muscular sphincter known as the lower esophageal sphincter (LES), which serves to prevent the reverse flow of gastric content into the esophagus. LES is a highly important structure, as the esophagus cannot withstand the

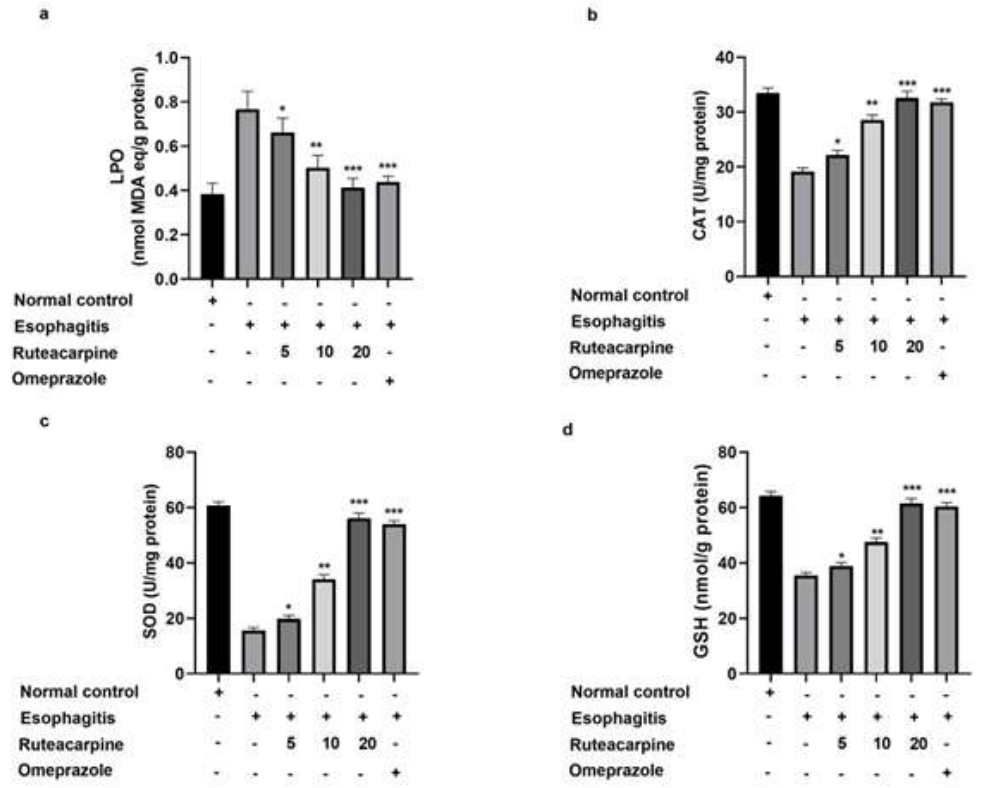



**Fig. 6:** Anti-oxidative effects of Rutaecarpine on markers of oxidative stress. a: lactoperoxidase (LPO), b: catalase (CAT), c: superoxide dismutase (SOD) and d: glutathione (GSH). Data are presented as mean ± standard deviation (SD); *, ** and *** indicate P<0.05, P<0.01 and P<0.01, respectively.

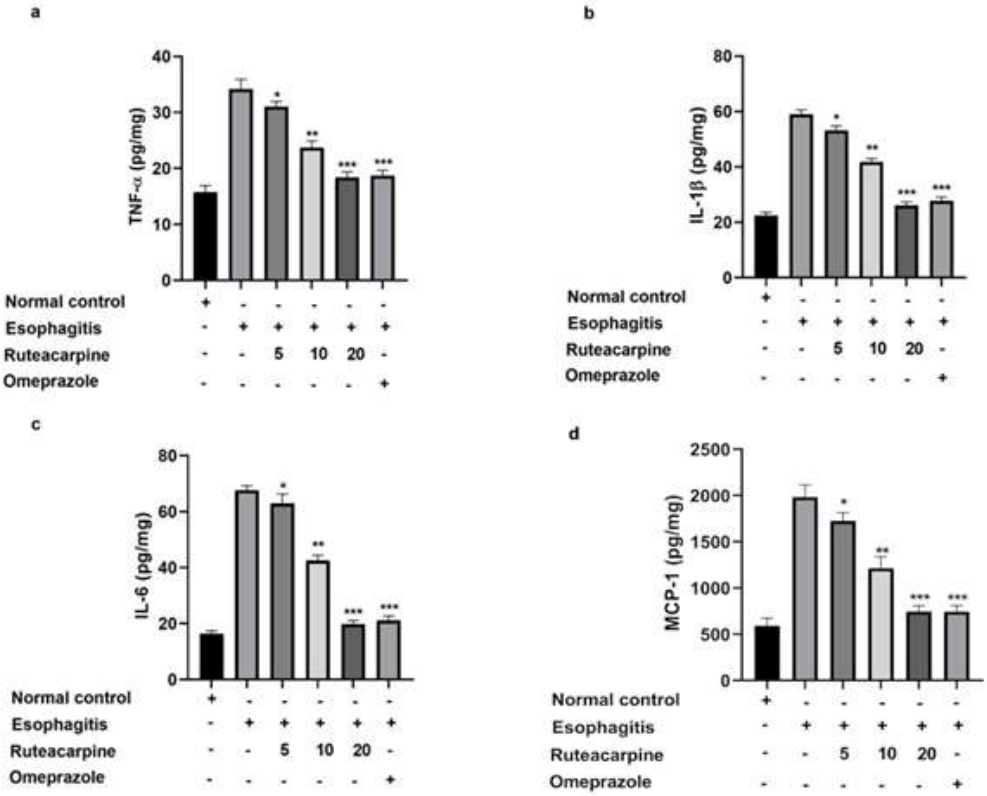



**Fig. 7:** Inhibitory effect of Rutaecarpine on pro-inflammatory cytokines. a: TNF-α, b: IL-1β, c: IL-6 and d: MCP-1. Data are presented as mean ± standard deviation (SD); *, ** and *** indicate P<0.05, P<0.01 and P<0.01, respectively.

acidic contents of stomach beyond the lower one-eighth of its length. Based on this logic, LES is an evolutionarily developed structure which functions as a valve to prevent esophageal epithelium from being exposed to gastric secretions (Manzo *et al.,* 2021). LES is subject to regulation by peristaltic movements of the esophagus, which are a series of rhythmic longitudinal contractions, followed by subsequent relaxations in a retrospective

manner. These movements ensure continued flow of the ingested food towards the stomach, while preventing reverse flow. When peristaltic movements reach the LES, it becomes relaxed, allowing the passage of food to the stomach (Elisha *et al.*, 2023).

Gastric acid is an important driver of esophageal injury and lesions, as the epithelial layer lining the inner esophageal wall is not specialized to withstand the exceedingly low pH of the gastric acid. While the gastric epithelium consists of columnar cells, the esophageal epithelium is made up of stratified squamous cells. This type of epithelium is adept at directing ingested material and delivers sufficient resistance against food passage, albeit being highly vulnerable to acidic environments (Tripathi *et al.*, 2018). The extent of mucosal damage depends on acidity and duration of exposure. Prolonged exposure in the absence of proper treatment can lead to serious complications including ulceration and metaplasia. As such, it is highly recommended to address gastroesophageal issues in a timely manner (Yuan *et al.*, 2017). Symptoms often include heartburn sensation, which can be perceived as chest pain, and dysphagia or difficult swallowing (Bor, 2019; Mastracci *et al.*, 2020). Recurrent exposure to highly acidic secretions is highly anticipated to accelerate the natural history of esophagitis (Bor, 2019). Various investigations have indicated that reflux esophagitis is associated with increased volume of gastric secretions with a lower pH, which can aggravate esophageal inflammation (Mastracci *et al.*, 2020).

On the molecular level, it is presumed that esophagitis can, in part, be the result of increased generation of free radicals, which are known to disrupt cellular activity and accelerate oxidative stress, thereby, instigating a vicious cycle that involves further production of free radicals (Tripathi *et al.*, 2018). Normally, the defense mechanisms of the esophageal lining neutralize the threat associated with these insults. However, should the balance be disrupted by events such as GERD, oxidative stress can very well aggravate the process of injury and ulceration (Anwar *et al.*, 2021). Lipid peroxidation is another type of molecular insult which occurs once reactive oxygen species (ROS) react with polyunsaturated fatty acids present within cell membranes. Generations of hazardous byproducts ensuing these sorts of reactions may jeopardize cellular integrity and result in tissue damage (Tripathi *et al.*, 2018).

Mucus is a protective barrier in the gastrointestinal tract, and while it is present throughout the alimentary tube, this barrier is strongest within the confinements of the stomach and tends to become weaker either proximally and distally. $H^+$-$K^+$-ATPase is an enzyme responsible for acidification of the stomach. Therapeutic inhibition of $H^+$-$K^+$-ATPase is a widely practiced approach to the treatment of esophagitis (Oshima and Miwa, 2018; Choi, 2022). Other mediators, such as histamine, can facilitate acidification. As such, inhibition of histamine receptors may confer protective effects against GERD and esophagitis. In the present work, we observed that Rutaecarpine treatment downregulated $H^+$-$K^+$-ATPase and histamine, which suggests a gastroprotective effect for this compound.

The pathogenesis of RE is highly intertwined with inflammatory response, oxidative stress and apoptotic cell death. Rutaecarpine has already been shown to have anti-inflammatory effects in certain conditions such as cognitive impairment (Gong and Jia, 2023), diabetic cardiomyopathy (Lv *et al.*, 2023), pulmonary arterial hypertension (Gong *et al.*, 2023), cancer (Yu *et al.*, 2023) and even coronavirus disease 2019 (COVID-19) (Lin *et al.*, 2023). Certain pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are known to be upregulated in the esophagus in response to GERD (Tripathi *et al.*, 2018). Secretions of such pro-inflammatory cytokines is a means by which our tissues recruit inflammatory cells to elicit an inflammatory response (Tripathi *et al.*, 2018). In addition to inflammation, cytokines can also alter the normal function of LES (Manzo *et al.*, 2021). TNF-α is strongly implicated in inflammation, and is known to be substantially upregulated in cancer, acquired immunodeficiency syndrome (AIDS), and septicemia (Zhao *et al.*, 2022). A central orchestrator of inflammatory response, TNF-α is released in response to stimuli such as lipopolysaccharides (LPS), which was used in this study to induce immune response in macrophages (Kopplin and Shifera, 2019). Excess production of TNF-α in the context of esophagitis is thought to underlie esophageal injury (Zhao *et al.*, 2022). According to a number of studies, TNF-α is upregulated in epithelial cells of the esophagus upon exposure to gastric acid. This has further been supported by studies reporting potential protective effects for TNF-α refluxing the treatment of esophagitis (Clayton *et al.*, 2019). IL-6 is another comparably active pro-inflammatory cytokine that prompts mucosal injury. Several lineages of immune cells including monocytes and granulocytes, as well as T lymphocytes, produce IL-6 in response to pro-inflammatory stimuli. Additionally, IL-6 is substantially overexpressed esophagitis, as compared to normal esophagus (Clayton *et al.*, 2019), which is speculated to stem from a hyperactive immune state (Clayton *et al.*, 2019). IL-1β is similarly involved in orchestration of acute reflux esophagitis Here, we found that IL-1β was markedly upregulated in rats with acute reflux esophagitis. A direct response to exposure to the acidic contents of the stomach (Zhao *et al.*, 2022), IL-1β has been suggested to mediate esophageal mucosal injury by accelerating pro-inflammatory processes and oxidative stress, which is evident by the increased production of ROS. (Clayton *et al.*, 2019). Collectively, pro-inflammatory cytokines are highly suspected to be involved in the progression of reflux esophagitis. As such, therapeutic inhibition of these cytokines, by means of Rutaecarpine, could be a promising strategy in the treatment of reflux esophagitis.

Nuclear factor kappa B (NF-κB) is a transcription factor, which is frequently implicated inflammation and immune response. Esophageal synthesis of NF-κB is stimulated in response to gastric acidic, which is speculated to underlie esophageal injury, particularly in the earlier stages of the disease by stimulating the expression of adhesion molecules and furthering the recruitment of immune cells. Inhibition of NF-κB is suggested to ameliorate inflammation in animal models of reflux esophagitis (Nan *et al.*, 2018).

In this study, Rutaecarpine was found to reduce the production of NO, IL-1β, iNOS, COX-2 and $PGE_2$, while negatively regulating esophageal lesion, GSV and GSA. Additionally, Ruteacarpine also resulted in substantial

repression of $H_2O_2$, free iron, calcium, as well as H+K+ATPase and histamine receptors. Lastly, Rutaecarpine was found to confer protective effects against reflux esophagitis through negative modulation of oxidative stress.

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**Authors contribution:** WC, LL, XD, conceived the idea and designed the research project. QY, CZ and YL were involved in data analysis. All authors were actively involved in the execution of the research project. All authors approved the final version of the manuscript.

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