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Daidzein ameliorates experimental acute reflux esophagitis in rats via regulation of cytokines

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Context: Daidzein is a secondary metabolite derived from plants, has a flavonoid structure and is known for its protective activity in gastrointestinal disorders. *Objective:* The current work determines the preventive effect of daidzein against injury in the esophagus mucosa induced by esophageal reflux (RE) in an animal model. *Methods:* Adult male Wistar rats were classified into six groups: normal control, ER + different doses of daidzein and ER + omeprazole. RE was induced in all animals except controls and supplemented with daidzein and standard drugs orally for 6 hours. Serum and tissue were used for further biochemical parameters. *Results:* Daidzein as a flavonoid has antioxidant properties and shows *in vitro* antioxidant activity. The outcomes also reveal an elevation in lipid peroxidation and a decline in the levels of sulphhydryl groups and glutathione, along with the depletion in the activities of enzymatic antioxidants in the oxidative stress state. In a dose-dependent manner daidzein and omeprazole amended all macroscopic and biochemical variations and protected against the raised level of hydrogen peroxide (H_2O_2), calcium and free iron levels in esophageal tissue induced during RE. It also improved the expression and level of proinflammatory cytokines. *Conclusion*: The finding reports that daidzein has a potential to show a shielding effect against esophagus damage induced by RE in rats, at least in part *via* alteration of inflammatory cytokines.

1. Introduction

Gastroesophageal reflux disease (GERD) is defined as a situation that arises as a result of reflux of gastric juice into the esophagus in children and infant, which resultant start the damaging the mucosal membrane and boosted the oxidative stress; the problem could be asymptomatic or develop symptoms (Park and Lee 2019). The prevalence of GERD in Western countries is calculated to be 10–20 %. Literature documented that the mucosal damage occurring in GERD is caused by a handful of refluxate inducing agents to release mediators in response to stimulation of the mucosal and submucosal cells, leading to anti-inflammatory reactions, visceral hypersensitivity and other GERD symptoms (Kang and Lee 2014; Takeuchi and Nagahama 2014). The process of inflammatory cascades implies an important role in gastrointestinal problem pathogenesis involving functional activity of dyspepsia and irritable bowel syndrome (Lahiri et al. 2009; Kang and Lee 2014).

The processes causing esophageal reflux (ER) have not yet been thoroughly described. The literature suggests that oxidative stress and lipid peroxidation play important roles in gastroesophageal diseases. In this respect, several studies have shown that the elevated content of reactive oxygen species (ROS) is directly associated with esophageal lesions, leading to lipid peroxidation in the membranes through unsaturated fatty acid oxidation (Mahattanadul et al. 2006; Kang and Lee 2014; Jabri et al. 2018). Endogenous antioxidant protection mechanisms help to escape ROS toxic effects through antioxidant enzymes (Kang and Lee 2014).

Prolonged gastroesophageal reflux in humans comprises acidic gastric fluid, resulting in esophageal mucosal injury, such as bleeding, erythema, erosion and ulcers. Antacids, acid blocks, gastric motility agents and surgery are included in the medication used in the management of RE (Nam et al. 2019). Histamine type 2 (H_2) antagonists and proton pump inhibitors (PPIs) come under the categories of acid blockers and most widely used therapies in RE. Histamine type 2 (H_2) antagonists, for example ranitidine and cimetidine, decrease stomach acid production as do proton pump inhibitors (PPIs) like omeprazole and esomeprazole (Jabri et al. 2016).

PPIs are typically more effective than H_2 antagonists (Mahattanadul et al. 2006; Nam et al. 2019). Despite the significant pharmacological activity some patients are suffering from a relapse incidence and display inadequate mucosal recovery. The use of natural products controlling the redox state and inflammation for GERD treatment is increasing. By its antioxidant properties *in vivo*, *Artemisia asiatica* has been reported to prevent RE. *A. asiatica* is recommended against gastritis in South Korea. Various plant-derived natural bioactive compounds have been reported to show activity against RE (Mahattanadul et al. 2006; Lahiri et al. 2009; Kang and Lee 2014; Nam et al. 2019).

Daidzein is an isoflavonic phytoestrogen in nature with the IUPAC name 4′,7-dihydroxyisoflavone (Atkinson et al. 2005; Whitehouse-Tedd et al. 2011). This compound is mainly obtained from leguminous plants such as soybean and mung bean. It is a traditional Chinese drug and a bioactive constituent of Gegen. Its chemical structure is similar to the estrogen of mammals (Liggins et al. 2000b; Atkinson et al. 2005). Daidzein's chemical structure is similar to mammalian estrogens. It has a dual-directional function to replace/interfere with estrogen. It is commonly used in the management of fever, acute dysentery, diarrhea, diabetes, heart disease, liver injury, etc. (Liggins et al. 2000b; Cheong et al. 2014). Thus, daidzein shows defensive activity against certain diseases associated with estrogen regulation, such as breast cancer, osteoporosis, diabetes, and cardiovascular diseases. Daidzein has shown various biological activities as anti-inflammatory, or anticancer agent, prevents oxidative damage, and strengthens skin and nerve defense. Such beneficial effects are primarily due to controlling the

immune response, scavenging oxygen-free radicals, proliferation inhibition, etc (Liggins et al. 2000a; Ae Park et al. 2006; Haron et al. 2009).

In this project, we scrutinized the presumed preventive action of the bioactive compound daidzein against experimental reflux esophagitis reflux in animals and possible mechanisms.

2. Investigations and results

2.1. Cell viability and in vitro anti-inflammatory activity of daidzein

To explore the impact of daidzein on cell growth, the cells were treated with various concentrations of daidzein from 10, 20 to 40 μM up to 24 h (Fig. 1). Daidzein did not show any effect on the growth of normal cells. The activity of daidzein at various concentrations was observed during normal growth of cells. LPS stimulation caused a release of inflammatory mediators such as NO and PGE2.

It was observed that daidzein inhibited the production of NO induced by LPS dose-dependently. Following NO, the content of PGE2 liberated by the daidzein-supplemented cells was decreased relative to the LPS treated control cells. The production of IL-1β in stimulated RAW 264.7 cells was noticeably raised to that of normal control cells and was significantly diminished by daidzein concentration-dependently compared to the LPS-treated control group (Fig. 2).

2.2. Impact of daidzein on the esophageal lesion in rats

In RE induced rats, a significant elevation was observed in scores of esophageal lesions compared to normal control rats. Moreover, supplementation of daidzein in dose-dependent manner and omeprazole declined the esophageal lesion scores compared to the RE induced groups (Fig. 3a).

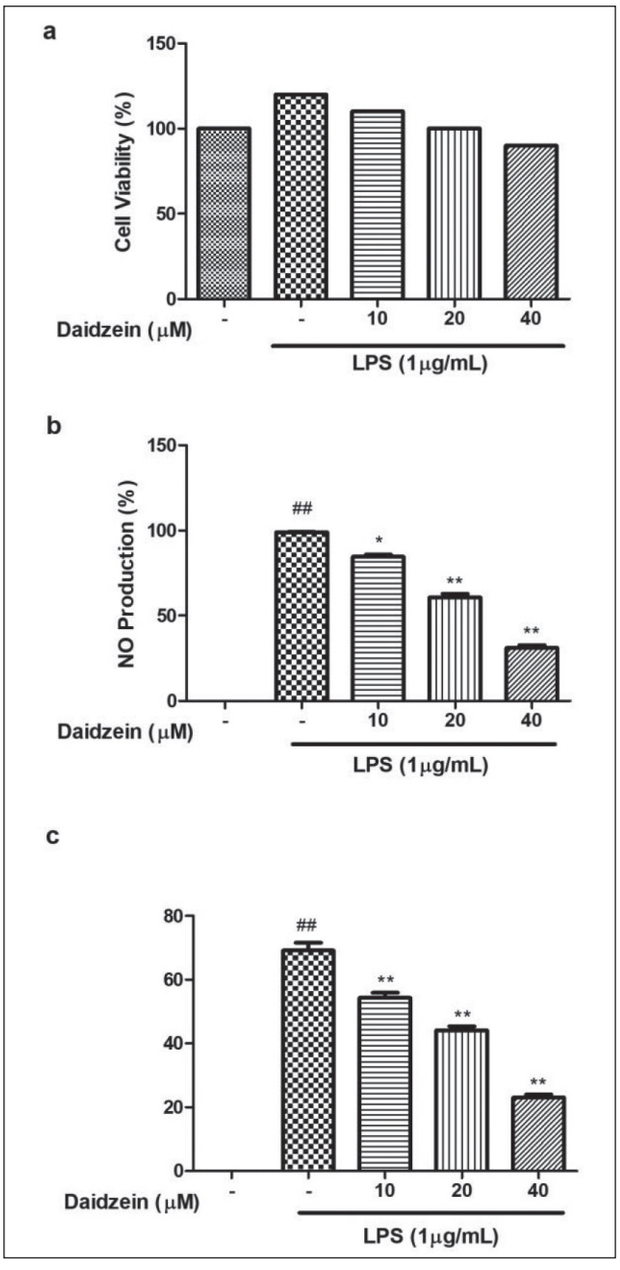

Fig. 1: Effect of daidzein on cell viability and inhibition of NO production and expression of iNOS on Lipopolysaccharide (LPS) induced in RAW 264.7 cells. A: Cell viability, B: NO production and C: iNOS expression. Data are mean±standard deviation (SD); ###p < 0.001 compared with normal control cells; **p < 0.01, and *p < 0.05 compared with LPS control cells.

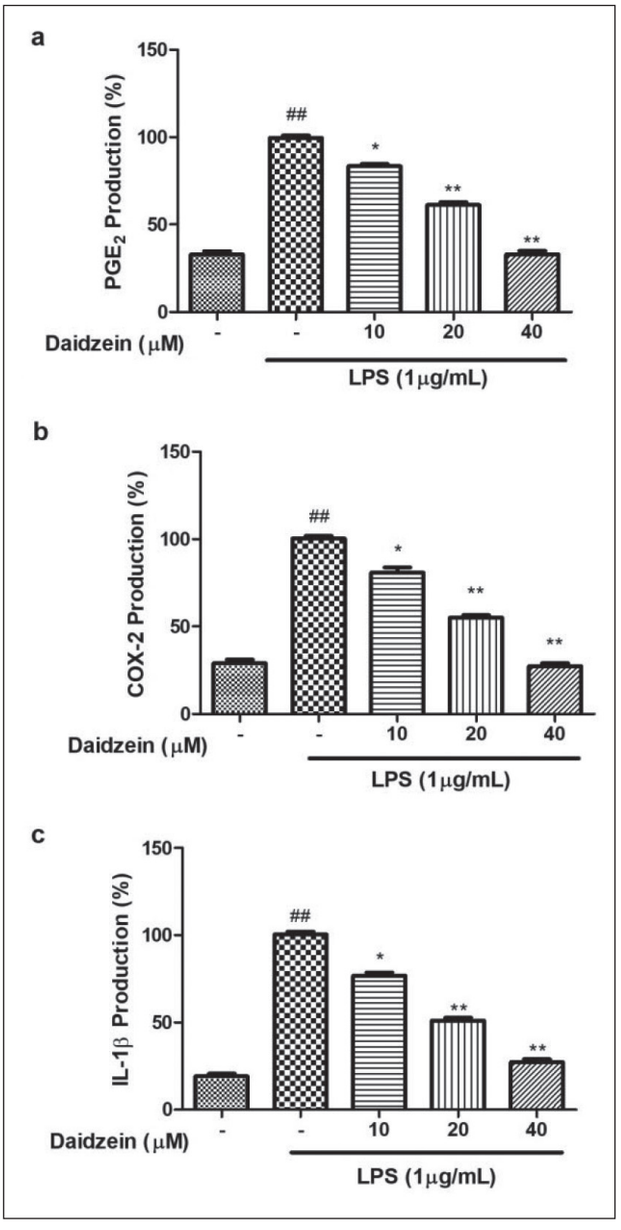

Fig. 2: Effect of daidzein on the production of inflammatory mediator on Lipopolysaccharide (LPS) induced in RAW 264.7 cells. A: PGE_2, B: COX-2 and C: IL-1β. Data are mean±standard deviation (SD); ###p < 0.001 compared with normal control cells; **p < 0.01, and *p < 0.05 compared with LPS control cells.

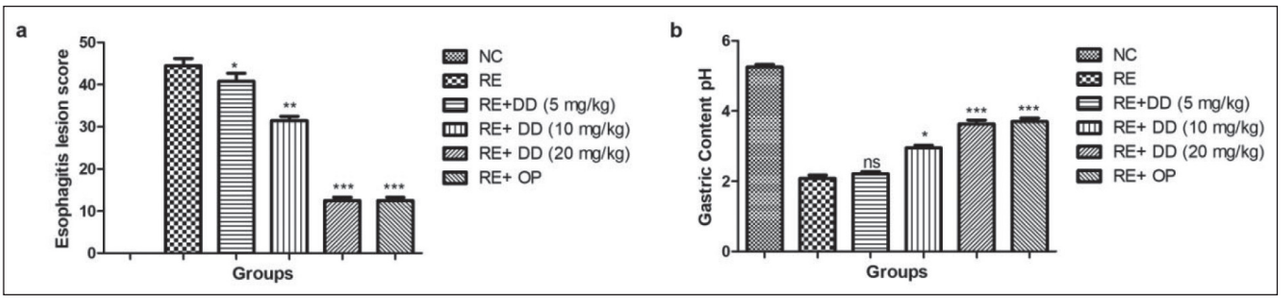

Fig. 3: Effect of daidzein on reflux esophagitis (RE) induced in animals. A: Esophagitis lesion score and B: Gastric fluid pH. Results are shown as mean±S.E.M. Treated groups compared with the RE control group rats and *.**.***Denote significant differences (p<0.05, p<0.01 and p<001).

2.3. Impact on gastric volume and pH of gastric fluid in rats

No effect was observed in the normal control group. The gastric volume in the RE induced group was considerably enhanced compared with the normal control group. Values in the RE induced group when treated with different doses of daidzein were not significantly different from those in the RE induced group, but significantly greater than in the normal control group (Fig. 3b). Supplementation of 20 mg/kg omeprazole remarkably enhanced the pH of the gastric fluid in the RE animals, whereas the values in the groups treated with all three doses of daidzein were not much different from the RE animals.

2.4. Impact of daidzein on lipid peroxide in rat tissue

To explore the impact of oxidative stress, the esophageal mucosa was used to analyze the lipid peroxidation. The levels of MDA in the esophageal tissue were considerably higher in the RE rats; however, this raised level was dose-dependently alleviated by daidzein and omeprazole (Fig. 4a).

2.5. Impact of daidzein on plasma-scavenging activity in rats

Figure 4b shows the plasma-scavenging activity (PSA). There was a significant reduction in PSA, which was accompanied by RE. The groups treated with daidzein revealed a significant and dose-dependent elevation in the concentration of PSAs relative to the RE group rats. The same was found in the omeprazole-supplemented group in RE rats used as reference.

2.6. Impact of daidzein on non-enzymatic antioxidant levels in rats

The effects of daidzein on non-enzymatic antioxidants level is illustrated in Fig. 4c. The levels of thiol groups and GSH were significantly reduced in RE group animals compared with the control rats. Administration of daidzein to RE groups dose-dependently and significantly improved the level of the GSH and thiol groups. Likewise, the administration of omeprazole enhanced the concentration of the non-enzymatic antioxidant parameters (Fig. 4d).

2.7. Impact of daidzein on depletion of enzymatic antioxidant activities in rats

Alteration in the activity of the antioxidant enzyme activity is shown in Fig. 5a-c. There was a drastical reduction in the concentration of the enzymatic antioxidants (SOA, CAT, GPx) in RE group rats. Treatment of daidzein dose-dependently and remarkably improved the depleted antioxidant enzymes. The same effects were also shown by the reference drug.

2.8. Impact of daidzein on free iron, hydrogen peroxide and ionisable calcium in rats

We also observed the impact of daidzein and RE on intracellular mediators in the esophageal tissue including hydrogen peroxide, free iron and calcium, as illustrated in Fig. 6a-c. In the RE group rats, the level of intracellular mediators was significantly upregulated. Treatment with daidzein and omeprazole reduced the RE induced intracellular mediators compared to RE rats.

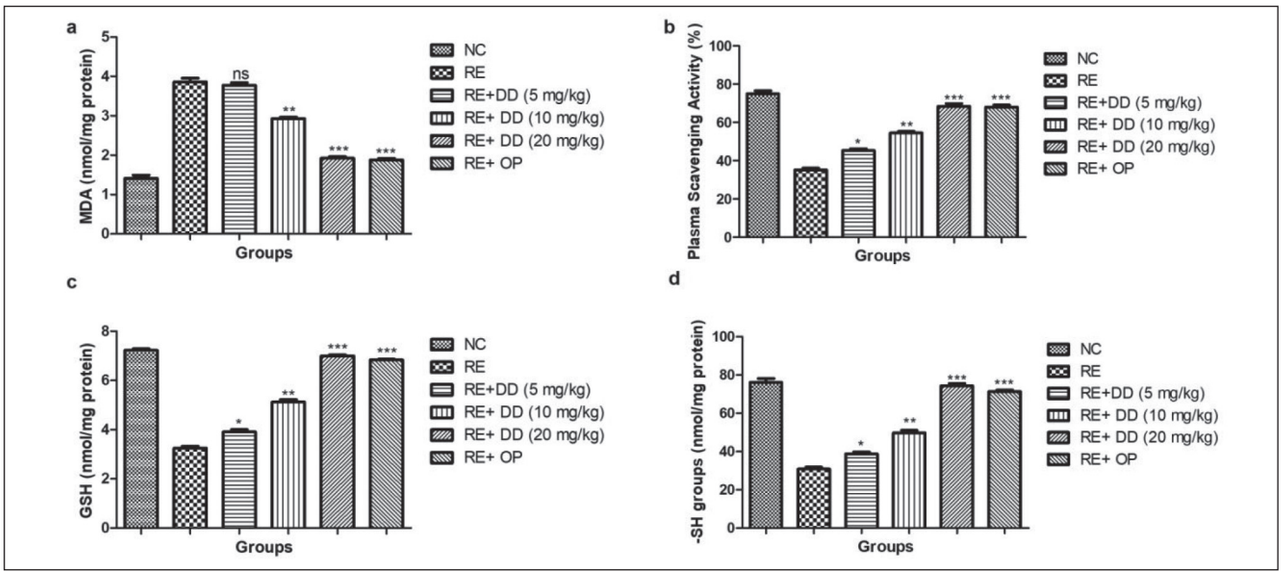

Fig. 4: Effect of daidzein on antioxidant markers of reflux esophagitis (RE) induced in animals. A: MDA, B: plasma scavenging activity, C: GSH and D: -SH groups. Results are shown as mean±S.E.M. Treated groups compared with the RE control group rats and *.**.***Denote significant differences (p<0.05, p<0.01 and p<001).

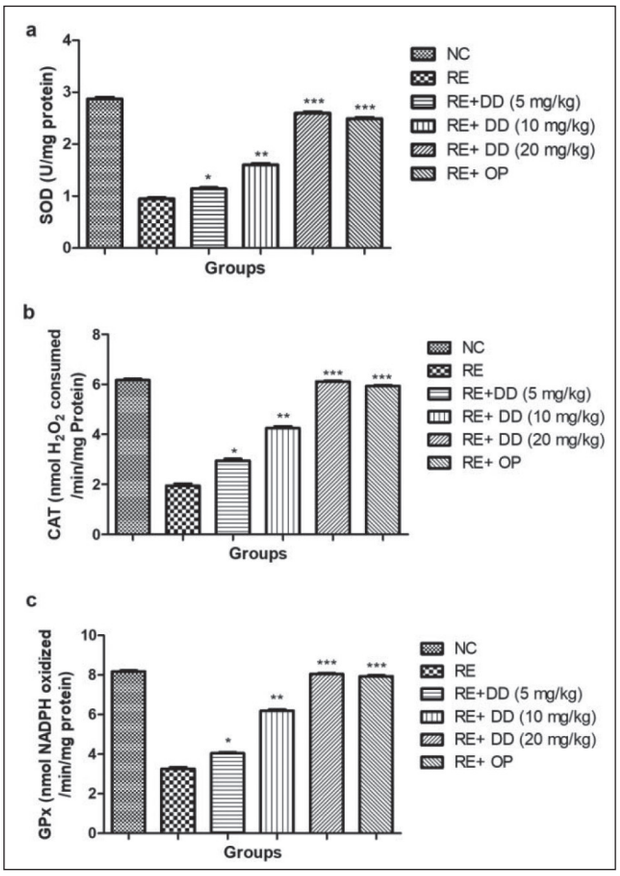

Fig. 5: Effect of daidzein on antioxidant enzymes of reflux esophagitis (RE) induced in animals. A: SOD, B: CAT and C: GPx. Results are showed as mean ± S.E.M. Treated groups compared with the RE control group rats and *.**.***Denote significant differences (p<0.05, p<0.01 and p<001).

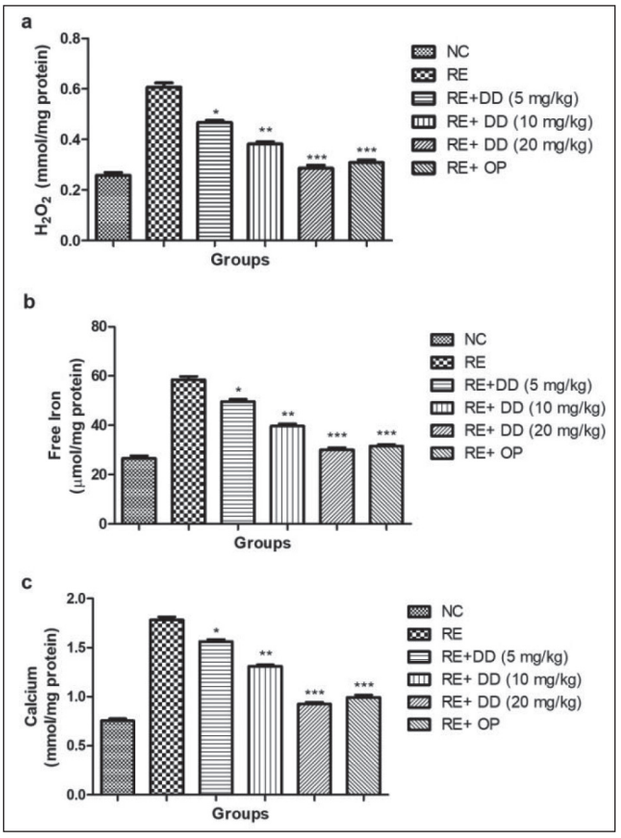

Fig. 6: Effect of daidzein on free iron, calcium and hydrogen peroxide levels of reflux esophagitis (RE) induced in animals. A: H_2O_2, B: free iron and C: Calcium. Results are shown as mean±S.E.M. Treated groups compared with the RE control group rats and *.**.***Denote significant differences (p<0.05, p<0.01 and p<001).

2.9. Impact of daidzein on proinflammatory cytokines TNF-α, IL-1β, IL-6 and MCP-1 levels in rats

To investigate the impact of daidzein on the content of pro-inflammatory cytokines in the serum, TNF-α, IL-1β and IL-6 and MCP-1 (a chemokine) levels in RE rodents were explored. A significant enhancement in the level of TNF-α production was observed in the RE rats. Treatment of RE rats with 20 mg/kg omeprazole and all the doses of daidzein considerably hampering the upregulation (Fig. 7a-d). Levels of IL-1β and IL-6 in the serum of the normal control group were normal. Conversely, surgically induced RE rats showed higher levels of IL-1β and IL-6 in serum, dose-dependently reversed by daidzein. Administration of omeprazole (20 mg/kg) significantly inhibited the enhanced levels of IL-1β and IL-6 following induction of RE.

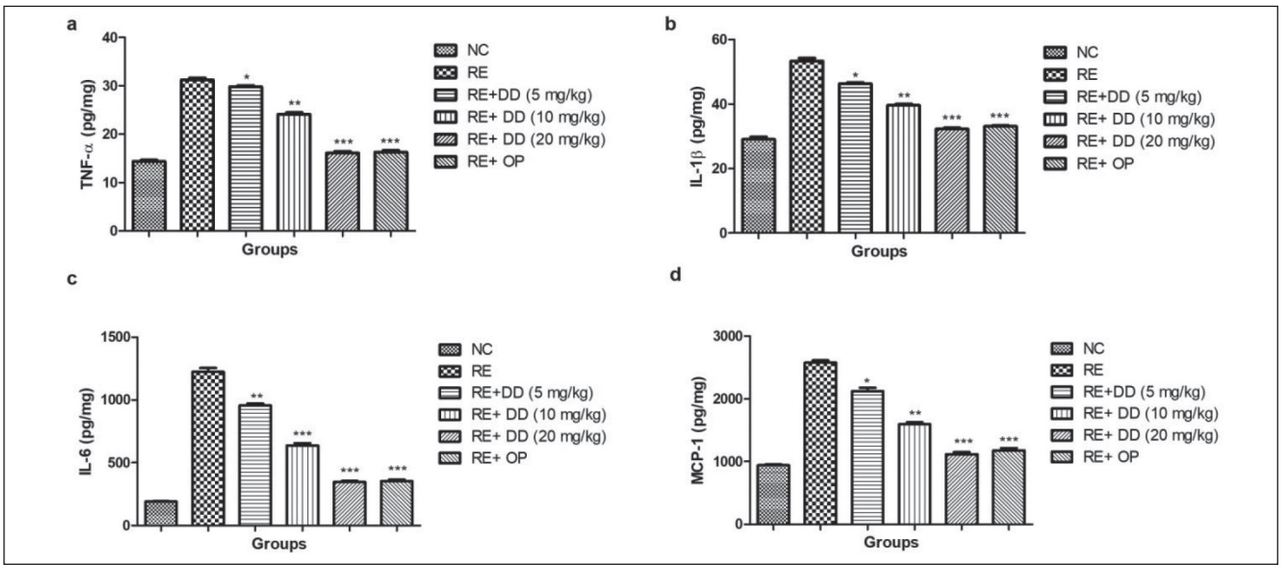

Fig. 7: Effect of daidzein on cytokines and MCP-1 level of reflux esophagitis (RE) induced in animals. A: TNF-α, B: IL-1β, C: IL-6 and D: MCP-1. Results are shown as mean±S.E.M. Treated groups compared with the RE control group rats and *.**.***Denote significant differences (p<0.05, p<0.01 and p<001).

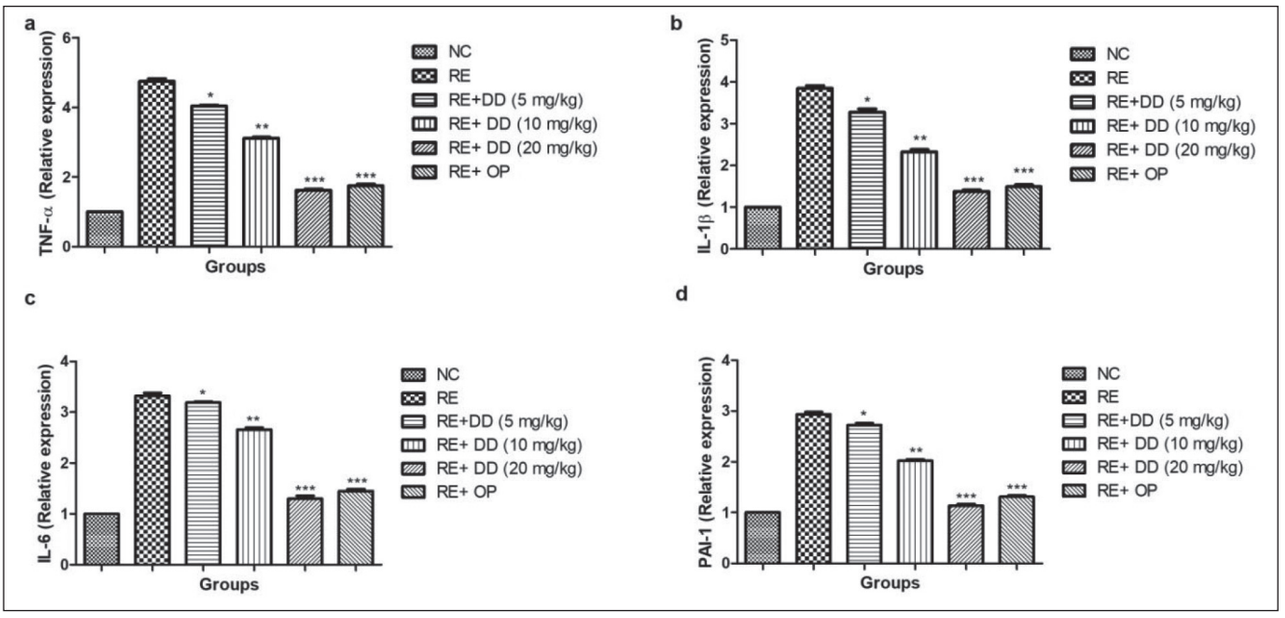

Fig. 9: Effect of daidzein on claudin-4 and claudin-5 of cytokines and PAI-1 level of reflux esophagitis (RE) induced in animals. A: claudin-4 and B: claudin-5. Results are shown as mean±S.E.M. Treated groups compared with the RE control group rats and *.**.***Denote significant differences (p<0.05, p<0.01 and p<001).

2.10. Impact of daidzein on proinflammatory cytokines TNF-α, IL-1β, IL-6 and PAI-1 mRNA expression analysed using qPCR

Figure 8a-d reveals the TNF-α, IL-1β, IL-6 and PAI-1 mRNA expression levels, analysed by PCR technique. In the normal control group, the levels of TNF-α, IL-1β, IL-6 and PAI-1 mRNA expression were low in the mucosa of the esophagus. In the RE induced rats, mRNA expression was significantly elevated because inflammatory reaction occurs in the esophagus. Supplementation of daidzein at a concentration of 20 mg/kg significantly reduced the levels of TNF-α, IL-1β, IL-6 and PAI-1 mRNA expression in the RE induced rodents.

2.11. Impact of daidzein on claudin-4 and claudin-5 protein expression in esophagus

Claudin-4 and claudin-5 expression was downregulated significantly in in RE group animals. Moreover, erosion in the esophagus tissue of RE rats declined, Claudin-4 and claudin-5 protein expression was significantly and dose-dependently elevated in the RE rats esophagus tissue (Fig. 9). We observed an enhancement to occur due to the flavonoid and omeprazole compared with RE induced rodents.

3. Discussion

Various natural therapy options have been suggested for managing RE including daidzein which is preventing esophageal injury induced by gastric acid, like other recognized herbal compounds used in the management of esophageal injury like *Morinda citrifolia* (Jabri et al. 2016). Although the effectiveness of daidzein in the management of RE has yet not been fully explored. We successfully set up an experimental model of RE in rats. In this study, stomach pariental cells released gastric acid and pepsin, whereas no alkaline content in the intestine may be a pathogenic factors in case of RE. Although bile, intestinal juice, pancreatic juice secreted from the duodenum are also concerned with an alteration of the esophageal mucosa.

The findings of this study indicate that supplementation of daidzein and omeprazole significantly and dose-dependently ameliorated RE. RE macroscopically induced alteration in the mucosa of the esophagus was also reduced. This is in agreement with earlier reports using plant-derived biomolecules such as genistein, naringin, or apigenin (Kang and Lee 2014; Takeuchi and Nagahama 2014). In the disease state of RE, various pathophysiological mechanisms are involved including an imbalance of the redox status. Enhanced MDA levels are regarded as a main indicator of LPO reduced activities of the enzymatic antioxidants SOD, CAT and GPx (Benzie and Strain 1996a, b). There was also a reduction in the sulphhydryl groups and GSH levels as well as PSA, an index of the production of free radicals in the esophagus tissue induced by RE. Moreover, it was widely agreed that lipid peroxidation and ROS in tissues causes damage to subcellular regions and are involved in the GIT diseases pathogenesis.

Scavenging free radicals protects the esophageal mucosa from injury obstructs and stimulates the antioxidant enzyme activity

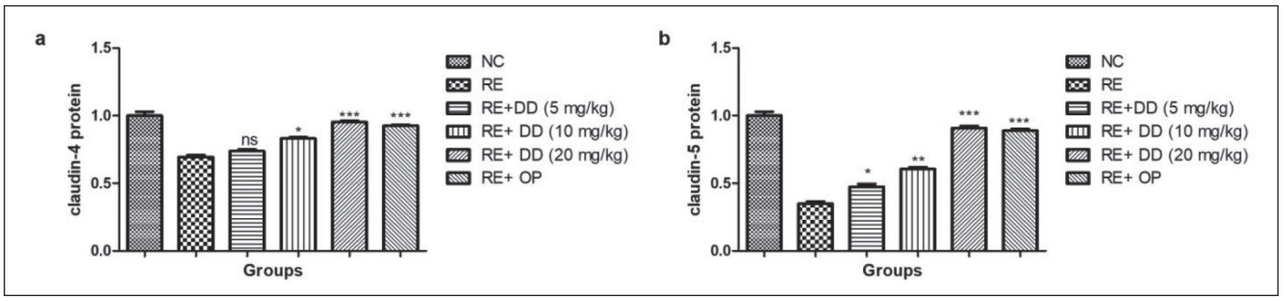

Fig. 9: Effect of daidzein on claudin-4 and claudin-5 of cytokines and PAI-1 level of reflux esophagitis (RE) induced in animals. A: claudin-4 and B: claudin-5. Results are shown as mean±S.E.M. Treated groups compared with the RE control group rats and *.**.***Denote significant differences (p<0.05, p<0.01 and p<001).

(Jang et al. 2012; Kang and Lee 2014; Jabri et al. 2018). Several endogenous and exogenous compounds are used to obstruct free radicals derived from oxygen. (Konturek et al. 2013; Kang and Lee 2014). Various reports show that daidzein exerts significant antioxidant activity (Lee et al. 2005; Rüfer and Kulling 2006).

Our results showed that the reflux of gastric material into the esophagus compartment greatly increased the levels of ionisable calcium, hydrogen peroxide and free iron (Mahattanadul et al. 2006; Kang and Lee 2014). Deficiency in iron and excess iron direct to cellular dysfunction, and thus it is crucial to maintain a proper iron homeostasis. In fact, by engaging in the Fenton process, the accumulation of iron catalyzed hydroxyl radical-induced oxidative harm. It has to be mentioned that daidzein is able to chelate free iron and scavenge H_2O_2.

Similarly, the state of oxidative stress may lead to the enhancement of the levels of calcium the cytoplasm of different cells, due to the secretion of calcium from organelles present in a cell, or the import from extracellular spaces through the plasma membrane (Mahattanadul et al. 2006; Kang and Lee 2014; Jabri et al. 2018). In this study, we have shown that supplementation of daidzein prevents from deregulation in RE induced rats and re-establishes homeostasis. Thus it is suggested that daidzein has beneficial properties in part by chelation of free iron and quenching of H_2O_2 resulting in homeostasis of calcium.

Oxidative stress, inflammation and apoptosis are correlated with the reflux esophagitis pathogenesis. Various researches have been performed to explain the repressive activity of daidzein on inflammation (Liggins et al. 2000b; Hämäläinen et al. 2007). TNF-α, IL-1β and IL-6, pro-inflammatory cytokines, are a response to any kind of pathogenic infection or damage to any body tissue (Kumar et al. 2015, 2016; Verma et al. 2018). Supplementation of daidzein revels a significant reduction in pro-inflammatory gene expression The principal cytokines are TNF α and IL-1 in which TNF-α has many physiological functions like inducing septic shock, inflammation, and cytotoxicity (Gu et al. 2018; Kim et al. 2020). TNF α is also termed as cachectin because it arbitrates fever and cachexia and is accountable for various effects of bacterial sepsis, rheumatoid arthritis and Crohn's disease. Following several stimuli such as bacterial LPS, monocytes and macrophages release TNF α, as a key mediator of endotoxin deleterious effects (Jia et al. 2012). IL-1 is also an inflammatory cytokine that is released by innate immune cells such as macrophages in response to infection or cell injury (Jeong et al. 2009).

IL-6 is generated in various cells including fibroblasts, monocytes, T-cells, B-cells, microglia, endothelial cells, neurons, and astrocytes. In the host reaction to inflammation, IL-6 is a B-cell differentiation factor and is prepared in response to IL 1β, which plays an important role in provoking the acute inflammatory proteins synthesis (Yoon et al. 2012; Lee et al. 2013). IL-1β and IL-6 participate in the phase of the acute immune response (Yoon et al. 2012). IL-6 is a central regulator of cell development, survival, and differentiation and as such engaged in a number of biological responses including immune response, inflammation, hematopoiesis, and oncogenesis. MCP-1 belongs to the family of C-C chemokines and has anti-inflammatory activity. It plays a crucial role in employment and stimulates the leukocytes in the acute inflammation phase. Upregulation of MCP-1 is connected with the employment of macrophages, survival of cancer cells and angiogenesis (Yoon et al. 2012; Lee et al. 2013; Cho et al. 2015). Daidzein has anti-inflammatory properties against cytokines like TNF-α, IL-1β and IL-6. In this study, RE rats developed high gastric volumes and gastric fluid pH and following administration of daidzein, there was not too much alteration in the data. It also improved the level of all proinflammatory cytokines.

Claudin is present in the epithelium and plays role as barrier of tight junctions (Camire et al. 2015). The normal control group had the highest expression of claudin-4 and claudin-5. Daidzein alleviated the esophagus damage in RE rats in a dose-dependent manner. The regulation of claudins in the destructed cells and tissues is important in determining the gastroesophageal reflux disease in our experimental model. Daidzein modulated this by controlling the immune system during disease state.

In summary, the protective effect of daidzein against RE damage may be accredited to the antioxidant and anti-inflammatory activities due to its flavonoid nature. It will be worth mentioning that significant anti-inflammatory activities of daidzein have been reported, since most of the anti-inflammatory drugs used in modern medicine are ulcerogenic. Studies are nonetheless necessary to further explore daidzein's usability in RE treatment.

4. Experimental

4.1. Animals and treatment

Healthy Wistar albino rats with an average wt of 150±20 g were used. They are used according to the ethics committee of the university and animal care was done according to the recommendations of NIH. All rats were fed with standard food and water *ad libitum*. All rats were kept in the experimental cage under maintained temperature and a 12 h light/dark cycle. All the animals were categorized into 6 groups of 8 rats each. RE in each group, except the normal control group, was induced. After 1 h, under light anesthesia (phenobarbitone), celotomy was performed to induce the RE by ligation of pylorus and fore stomach and corpus junction using silk sutures (2–0) in rats according to the procedure explained by Renu et al. All the rats were kept away from food and water.

Group 1 served as the normal control group and orally received a physiological solution; Group 2 were RE induced; Groups 3, 4, and 5 received daidzein (5, 10 and 20 mg/kg, b.w., p.o.) orally, whereas Groups 6 was treated with omeprazole at 20 mg/kg, b.w., p.o. After 6 h, double ligation was performed for the autopsy of animals. A part of the gastroesophageal digestive tract was rapidly excised and inspected to evaluate its appearance and PBS was used for homogenization (Mahattanadul et al. 2006; Kang and Lee 2014). After homogenization, the supernatant was collected to examine the various biochemical parameters. Blood was obtained and stored in heparinized tubes. For estimating the PSA, plasma was treated after centrifugation at 3000 g for 15 min.

4.2. Cell culture

The ATCC raw 267.7 macrophage cell line was used for the current study. The cells were produced in DMEM medium nourished with FBS (10%) and P/S (1%) in an incubator at 37 °C. Cells were cultured for 7 days and medium was replaced every second day. It was supplemented with various concentrations of daidzein and incubated with LPS (1 μg/mL) for 24 h.

4.3. Cell viability assay

The effect of BB on the viability of the cells was estimated using the Cell Counting kit (CCK)-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA), in accordance with the manufacturer's instructions. Cells were seeded in a 96-well plate and then incubated with various concentrations (10, 20 and 40 μM) of BB for 24 h. Absorbance was measured with a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm.

4.4. NO production

RAW 264.7 cells ($2.5x10^4$ cells/ml in a 96-well plate) were treated with lipopolysaccharide (LPS; 1 μg/ml) alone or with BB (10, 20 or 40 μM) for 24 h. The culture supernatants were mixed with an equal volume of Griess reagent (Promega Corporation, Madison, WI, USA) and incubated at room temperature for 10 min. Absorbance was measured at 540 nm using a microplate reader. Nitrite levels in the samples were determined by comparisons against a sodium nitrite curve.

4.5. PGE_2 and IL-1β production

RAW 264.7 cells were seeded in a 96-well plate at a density of $2.5x10^5$ cells/ml and then treated with LPS (1 μg/ml) alone or with BB (10, 20 or 40 μM) for 24 h. The PGE2 concentration in the culture supernatants was quantified using a competitive enzyme immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA) in accordance with the manufacturer's instructions. IL-1β levels were determined using a commercially available ELISA kit (R&D Systems Inc.), in accordance with the manufacturer's instructions. The ELISA was performed in 96-well polystyrene microplates with a specific monoclonal antibody coating. Absorbance was measured at 540 nm in a microplate reader.

4.6. Biochemical parameters

4.6.1. Determination of pH, titrable acidity and volume of gastric juice

The stomachs were opened six hours after the drug administration along the greater curvature and the gastric contents were collected and centrifuged at 2000 g for 10 min. The overall gastric content volume was expressed as mL/100 g body weight (Dai and Ogle 1973). The supernatant (1 mL) was diluted with distilled water to 10 mL and the total acid content was calculated by phenolphthalein titration with sodium hydroxide (0.01 N) as an indicator and is expressed as mEq/L/100 g (Xin et al. 2012). A pH meter was used to determine the pH values.

4.6.2. Lipid peroxidation measurement

Malondialdehyde (MDA) measurements were used to assess esophageal lipid peroxidation according to the double heating process (Draper and Hadley 1990).

4.6.3. Plasma scavenging activities

Using the DPPH radical process, the free radical scavenging activities of plasma were calculated according to a literature method with minor modification (Brand-Williams et al. 1995; Alimi et al. 2012).

4.6.4. Sulphhydryl groups (–SH) determination

According to the Ellman process, the complete concentration of thiol groups (-SH) was determined (Ellman 1959).

4.6.5. Reduced GSH determination

Using the process described by Sedlak et al. GSH was estimated in esophageal tissue (Sedlak and Lindsay 1968).

4.7. Antioxidant enzyme activities assays

4.7.1. Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was calculated using modified assays of epinephrine (Misra and Fridovich 1972).

4.7.2. CAT activity assay

By calculating the initial rate of H_2O_2 disappearance at 240 nm, CAT activity was assessed (Jabri et al. 2016).

4.7.3. GPx activity assay

Glutathione peroxidase (GPx) was estimated according to the procedure described by Flohé and Günzler (1984) with minor modification.

4.7.4. H_2O_2 determination

According to Dingeon et al. (1975), the amount of esophageal H_2O_2 was calculated.

4.7.5. Iron measurement

As proposed by Leardi et al. (1998), esophageal tissue non-heme iron was colourimetrically calculated using ferrozine.

4.7.6. Calcium determination

The calcium level of the esophageal tissue was determined using a Stren and Lewis colourimetric system (Stern and Lewis 1957).

4.7.7. Protein determination

As a minor change in the Lowry process, the protein concentration was calculated according to Hartree (1972). As a norm, serum albumin was used.

4.8. Proinflammatory cytokines

Pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) were estimated using the ELISA kit following the manufacture protocol.

4.9. qPCR estimation

TRIzol reagent was used for the isolation of the total RNA (Invitrogen Life Technologies, Inc., Grand Island, NY, USA), following the manufacturers protocol. For the estimation of RNA content, spectrophotometry was used. qPCR estimation was done via using the SYBR Green PCR kit. The primers are presented in the Table.

4.10. Statistical analysis

The data were analyzed by the t-test of Student unpaired and expressed as mean±-standard error of the mean (SEM). 10 independent experiments are representative of the data. All statistical tests were two-tailed and considered to be significant with a p value of 0.05 or less.

Conflicts of interest: None reported.

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Table: List of primers

S. No.	Gene	Primer sequence	
		Forwarded	Reverse
1	**TNF-α**	5'-CCA GGA GAA AGT CAG CCT CCT-3'	5'-TCA TAC CAG GGC TTG AGC TCA-3'
2	**IL-1β**	5'-CAC CTC TCA AGC AGA GCA CAG-3'	5'-GGG TTC CAT GGT GAA GTC AAC-3'
3	**IL-6**	5'-CGAAAGTCAACTCCATCTGCC-3'	5'-GGCAACTGGCTGGAAGTCTCT-'3
4	**PAI-1**	5'-CCGATGGGCTCGAGTATGA-3'	5'-TTGTCTGATGAGTTCAGCATCCA-3'
5	**GAPDH**	5'-ATGGCACAGTCAAGGCTGAGA-3'	5'-CGCTCCTGGAAGATGGTGAT-3'

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