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Original article

Activity of *Ligustrum vulgare* L extracts against acute pancreatitis in murine models by regulation of p38 MAPK and NF-κB signaling pathways

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ABSTRACT

Pancreatitis is a fatal disease associated with significant mortality and morbidity. At present, no specific treatment is available for pancreatitis and the patients are mainly treated with supportive medication. The need for specific antipancreatitic chemotherapy is an urgent medical obligation. In the current study, protective effects of the methanolic extract of the *Ligustrum vulgare* berries were investigated in the rat model of acute pancreatitis. Acute pancreatitis (AP) was induced in the male Sprague-Dawley (SD) rats by cerulein injection. Fruit extract of *L. vulgare* L extract was prepared using the methanol. Treatment effects of *L. vulgare* were evaluated in AP rats. Serum levels of lipase, amylase, proinflamatory cytokines (TNF-α, IL-6, TL-1β), lipid peroxidase (LPO), myeloperoxidase (MPO) were determined. Histological changes in the pancreas were assessed. *L. vulgare* treatment prevented the increase in serum amylase and lipase levels, reduced the disease progression in pancreas, and reduced the serum levels of tumour necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in AP rats. Moreover, *L. vulgare* significantly suppressed pancreatic edema, inhibited oxidative damage (MPO activity and SOD activity), and inhibited the expression of NF-κB/p65 and activation (phosphorylation) of the inhibitor of NF-κB (IκBα) and p38 MAPKs. Histological examinations showed that *L. vulgare* significantly reduced the inflammatory and fibrotic changes. The results indicated the potent pancreato-protective effects of *L. vulgare* in AP.

1. Introduction

Pancreatitis, an inflammatory response of pancreatic parenchyma characterised by cell injury of pancreatic acinar cells. Acute pancreatitis (AP) is a fatal disease that often leads to significant mortality and morbidity and treatment for which is still mostly supportive. Symptoms of the disease include acute abdominal disease that can range from mild edema to severe tissue necrosis; it could be complicated by multiple organ damage (Baron and Morgan, 1999). The common cause of EP is usually attributed to factors such as biliary abnormalities, consumption of alcohol and certain drugs, infection, trauma, and autoimmune diseases (Frossard et al., 2008; Lima et al., 2013; Sah and Saluja, 2012). The underlying mechanism of EP is not fully understood. The development of its pathobiology is a complex multi-step process involving the premature ectopic activation of the key proteolytic enzyme such as conversion of inactive trypsinogen to active trypsin associated with zymogen activation and auto-digestion of the pancreas (Sah and Saluja, 2012). EP occurs due to several contributing factors characterised by loss of both intracellular and extracellular compartmentation, an obstruction of pancreatic secretary transport and an activation of pancreatic enzymes (Bhatia et al., 2005). Biliary EP is a serious complication of biliary calculous disease characterized by outflow obstruction with pancreatic duct hypertension and a toxic effect of bile salts leads to damage of pancreatic ductules, with subsequent loss of extracellular compartmentation. The pathobiology of pancreatitis implicates interplay

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between several pro-inflammatory end anti-inflammatory cytokines, such as in interleukin (IL)-1, IL-1β, IL-6, IL-8, IL-10, IL-18, IL-33 and TNF-α in injured aciner cells. In the past, a number of studies involving the EP have been performed in which the role of NF-κB inhibition has been established in the ameliorating the inflammatory response, necrosis, and other parameters of pancreatitis severity (Rakonczay et al., 2008). Additionally, the activation of MEPKs signalling pathway in the development of EP has been established in animal models (Cao et al., 2015). Therefore MEPKs pathways inhibition represents an alternative target in the treatment of EP. A number of pharmacological inhibitors have been identified which impact on the MEPKs ERK1/2, p38 end JNK/stress activated protein kinases and have been tested in different studies (Mazzon et al., 2012). *Ligustrum vulgare* L. have been used traditionally in folk medicine for disease prevention or treatment (Pieroni et al., 2000) due to its antibacterial (Jantová et al., 2000), cardio-protective (Yim et al., 2001), immunomodulatory (Barónikova et al., 1999) and anti-diabetic effects (Andrade-Cetto and Heinrich, 2005), and it has also been reported that different leaf and fruit extracts of this plant can scavenge OH and DPPH radicals. Owing to excellent pharmacological activities of *L. vulgare* L, the current study was designed to investigate the protective effects of L vulgare L fruit extract in rat models of EP.

2. Materials and methods

2.1. Reagents

Interleukin-6 (IL-6), interleukin-1β (IL-1β), and IL-10 ELISE kits and Mouse tumor necrosis factor-α (TNF-α), were purchased from Biolegend, Inc. (Sen Diego, CE, USE). Mouse myeloperoxidase (MPO), lipase, amylase and mouse superoxidase dismutase (SOD) kits were purchased from Uscn Life Co. (Missouri City, TX). The antibodies of NF-κB/p65, inhibitor of NF-κB (IκBα), p-JNK, JNK, p-ERK1/2, ERK1/2, p-IκBα, p-p38, p38 and glycereldehyde-3- phosphate dehydrogenase (GEPDH) were purchased from Cell Signaling Technology (Boston, ME).

2.2. Plant material

Fresh Berries of *L. vulgare* L were procured from Xiensheng Drug Store (Nenjing, Chine) and assigned a Batch number: 1403092X. Berries were washed under the running Tap water, air dried under the shade for 10 days in well ventilated rooms, grinded and then blended to a fine powder. Powder was stored in airtight containers under refrigeration for further use.

2.3. Preparation of extract

The berry powder weighing 2 kg was extracted extensively by percolation with 95% methanol using Soxhlet extractor. The extract was concentrated under vacuum at 50 °C using a rotary evaporator and then left over solvent was removed by water bath, air dried for 24 h and lyophilized until the solvent was completely removed, yielding methanolic extract weighing 250 g. Normal saline (NS), a vehicle was used as diluents for the *L. vulgare* L for treatment of animals.

2.4. Animal

Male Sprague-Dawley (SD) rats weighing 250 ± 50 g were provided by the Shanghai Jingke Industriel Co., LTD, China and used in the study. Animals were maintained in the animal holding facility of the institute under aseptic conditions. Mice were given free access to animal diet and water. Animals were kept in well ventilated rooms with controlled setting light/dark cycle of 12 h each, temperature of 23 ± 2 and humidity of 40–62%. The protocols for the animal study were approved by institutional animal ethical committee and efforts were made to minimise the animal sufferings. The animal studies were conducted in agreement with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health. Animals were kept in the animal holding facility for about a week before start of experimentation in order to allow the animals to acclimatize to the local environment.

2.5. Establishment of the acute pancreatitis animal model and treatment regimens

Forty rats were fasted for 24 h with free access to water before experimentation. Animals were randomly divided into study groups with 10 rats/group. EP was induced by intraperitoneal (i.p.) injection of cerulein at the dose of 50 μg/kg body weight for eight consecutive hours after a time span of 1 h. Cerulin was made up fresh in phosphate buffer saline (PBS). Water infusion of *L. vulgare* L fruit extract or vehicle was administered by gavage 1 h before the cerulin injection. Cerulin was injected every day until the rats were euthanized. No positive control drug was included in this EP study owing to lack of specific EP medication till date. Rats were randomized into four groups: (1) Normal control (NC) group consisted of the animals that received (0.9% NaCl, 1 mL/kg body weight) but no cerulin; (2) EP control group received the cerulin; (3) low dose L. vulgere L group that were orally administered with *L. vulgare* L (5 g/kg body weight, dry herb equivalent) fruit extract 1 h before cerulin i.p. injection and (4) high dose *L. vulgare* L group that were orally administered with L. ulgare L (10 g/kg body weight, dry herb equivalent) fruit extract 1 h before cerulin i.p. injection. At the end of the animal experimental, rats were fasted for 12 h and anaesthetized by administration via i.p injection with pentobarbital sodium (90 mg/kg body weight) to induce anaesthesia. Subsequently, animals were euthanized by cervical dislocation to obtain pancreas and blood for assays. Pancreas was rapidly removed and frozen immediately in liquid nitrogen or fixed for immune histochemical staining and ultra structural examination. Blood was collected by cardiac puncture and the serum was obtained by centrifugation. Serum obtained was stored appropriately until further analyses.

2.6. Measurement of pancreatic edema

The first histological parameter to evaluate the pancreatitis is the pancreatic edema and it was examined by measuring the gain in the water content of the parenchyma (Sersen et al., 2005). The degree of pancreatic edema assessed by calculating the ratio of pancreas to body weight (g/kg).

2.7. Serum enzymes and cytokine analysis

Blood samples were obtained from the heart of the euthanized rats by cardiac puncture to determine the levels of serum enzymes and proinflamatory cytokines. Serum was prepared by centrifugation at 10,000 rpm for 10 min. Serum levels of pencreatic lipase and amylase were determined by lipase and amylase activity kits respectively. Serum concentrations of TNF-α, IL-6, TL-1β, and IL-10 were determined using commercial ELISA kits.

2.8. Analysis of myeloperoxidase and superoxide dismutase activity in pancreas tissue

Part of the pancreatic tissues was collected for quantification of myeloperoxidase (MPO) and superoxide dismutase (SOD) activity

assays. Pancreatic tissues weighing about 100 mg were frozen in liquid nitrogen and then homogenized in normal saline. Afterwards, tissue homogenates were centrifuged (12,000 rpm for 10 min at 4 °C) to obtain the supernatant. Commercially available mouse MPO assay kit and SOD assay kit were used to measure the levels of MPO and SOD activity in the supernatant respectively. MPO activity was expressed as U/g tissue, and SOD activity was expressed as U/mg tissue.

2.9. Immunohistochemical staining analysis

Expression of NF-κB/p65 was investigated by performing the immune histochemical staining with Elivision™ detection kit (Glostrup, Denmerk) used according to the suppliers instruction. Briefly, tissue sections measuring 4–7 μm thick were cut, deparaffinised, rehydrated using xylene and graded alcohols, and then heated in 10 mM sodium citrate buffer (pH 6.0) in a microwave oven at 95 °C for 5 min for antigen retrieval. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min, and nonspecific binding was blocked with goat serum for 45 min. Subsequently, the slides were treated with the primary rabbit emit-NF-κB/p65 antibody and horseradish peroxidase (HRP)-polymer secondary antibody successively. Then, the slides were stained with dieminobenzidine (DEB) and counterstained with methyl green.

2.10. Western blot analysis

The pancreatic tissues weighing 50 mg were first digested with NP-40 lysis buffer containing 1 mmol/L phenyl methanesulfonyl fluoride (PMSF). Sample tissues were homogenised and successively centrifuged at 12,000 rpm for 10 min at 4 °C. BCA method was performed for protein quantification. Proteins were separated using SDS-PAGE and each well was loaded with 80 μg of total protein and the proteins were then transferred onto polyvinylidene fluoride membrane (Bio-Red, Hercules, CE, USE). After blocking was done for 2 h at room temperature with 5% bovine serum albumin (BSE), membrane was incubated with primary antibodies overnight at 4 °C at the following dilutions: p-p38 (1:500), p38 (1:500), p-ERK1/2 (1:500), ERK1/2 (1:500), p-JNK (1:500), JNK (1:500), NF-κB/p65 (1:500), p-IκBα (1:500), IκBα (1:500), and GEPDH (1:1000) which were diluted according to supplier's written recommendations. On next day, membranes were washed with Tris-buffered saline Tween 20 (TBST) three times, incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibody (1:5000). Protein bands were visualised by chemiluminescence using an Image J and signals were normalized to that of GEPDH.

2.11. Histological examination

Pancreatic tissues fixed in 10% buffered formalin were ethanol dehydrated and then embedded in paraffin. Glass slides with tissue sections measuring 5–6 μm thickness were cut with a cryotome and mounted on glass slides and then stained with hematoxylin and eosin. Assessment of pancreatic tissue injury was performed by a trained histopathologist who was blinded to the design of experiment according to the criteria as reported previously (Zhang et al., 2017). Briefly, 10 fields from for each pancreatic tissue sample were randomly selected. Fields were cautiously observed using a light microscope at 200× magnification to investigate and score the level of damage in these sections. The level of the damage was scored at the scale of 10 to monitor the presence and degree of vacuolization, inflammatory cellular infiltration, edema, and tissue haemorrhege. The mean of the scores from each category of 10 different microscopic fields was recorded as the final damaged score for rat pancreas. Images were captured with a digital camera mounted on the microscope and the pictures were analyzed for altered appearance with imaging software from Diagnostic Instruments (Sterling Heights, MI).

2.12. Statistical analysis

All values of in vivo study were expressed as means ± S.E. for the 10 rats in each group. Statistical significance was assessed using analysis of variance, followed by Newman–Keuls post hoc test (Zar, 1984). A p-value < 0.05 were taken as statistically significant.

3. Results

3.1. L. vulgare L extract reduced cerulein-induced pancreetic edeme in rats

The ratio of pancreas to body weight (g/kg) was measured to evaluate the degree of pancreatic edema. The ratio of pancreas to body weight was increased significantly in the cerulein-treated group compared with that of the untreated group ($P < 0.05$). Ceruleen treatment induced the necrotizing pancreatitis in the EP animals. Pre-treatment with methanolic fruit extract of the plant1 h before the cerulein injection for 8 days attenuated the formation of cerulein-induced pancreatic edema (Fig. 1).

3.2. Plasma lipase activity and amylase activity

The cerulein injection induced the acute pancreatitis in the EP rats and increased the serum lipase (423 ± 30.0 U/L) and amylase (1200 ± 151.0 U/L) concentrations compared to the normal control rats (60.3 ± 11.0 U/L and 110 ± 16.0 U/L) significantly ($P < 0.05$). Treatment with *L. vulgare* L at 5 g/kg body weight did not markedly affect the plasma lipase (381 ± 36.0 U/L) and amylase (900 ± 67.0 U/L) activity in animals with cerulein-induced pancreatitis compared to the EP rats. Treatment with *L. vulgare* L at the dose 10 g/kg body weight significantly reduced cerulein-evoked increase in plasma lipase and amylase activity by approximately 36% (Fig. 2E) and 50% (Fig. 2B) compared to normal control rats.

3.3. Plasma concentrations of TNF-α, IL-6, IL-1β, end IL-10

The cerulein injection increased the plasma concentrations of pro-inflammatory cytokines such as TNF-α (Fig. 3E), IL-1β (Fig. 3B), and IL-6 (Fig. 3C) in the EP group. Treatment with the *L. vulgare* L. lowered plasma levels of these cytokines significantly ($P < 0.05$). The up-regulation IL-10 cytokine down regulates the inflammation (Fig. 3D). IL-10 levels significantly reduced in the EP group compared to that in the NC group ($P < 0.05$). However, treatment with *L. vulgare* L did not significantly increase the IL-10 level ($P > 0.05$). These findings provided strong evidence that treatment with *L. vulgare* L reduced the generation of pro-inflammatory cytokines in the rat model of EP.

3.4. Analysis myeloperoxidase of and superoxide dismutase activity in pancreas tissue

Cerulein injection significantly increased the MPO activity (Fig. 4E) and reduced the SOD activity (Fig. 4B) in EP rats compared to normal control rats ($P < 0.05$). Treatment of the EP rats with the *L. vulgare* L at 5 and 10 g/kg significantly ($P < 0.05$) reduced the MPO activity and a significant ($P < 0.05$) elevation in the SOD activity was observed compared to the rats in EP group.

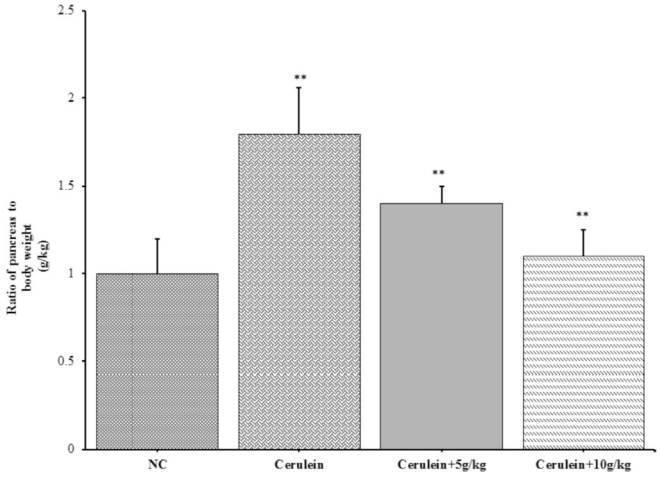

Fig. 1. *L. vulgare* L extract reduced cerulein-induced pancreetic edeme in rats.

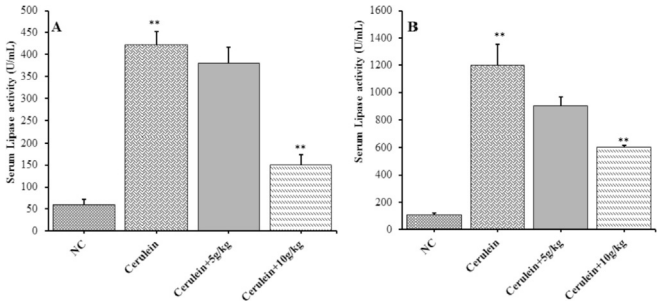

Fig. 2. Plasma lipase activity and amylase activity.

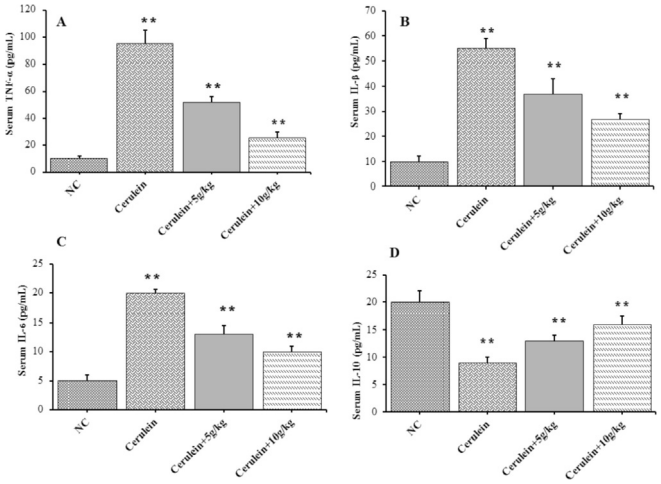

Fig. 3. Plasma concentrations of TNF-α, IL-6, IL-1β, end IL-10.

3.5. L. vulgare L extract treatment inhibited the expression of NF-κB/p65 and phosphorylation of IκBα in mice with cerulein-induced EP

We evaluated NF-κB/p65 expression and phosphorylation of IκBα in the pancreas to further assess the effect of *L. vulgare* L on EP by immunohistochemistry and western blot (Fig. 5). NF-κB/p65 expression and IκBα phosphorylation was up-regulated after cerulein injection in EP rats. Contrary to this the expression and phosphoryletion of this proteins was significantly $P < 0.01$) reduced in the EP rats treated with *L. vulgare* L at 5 or 10 g/kg body weight.

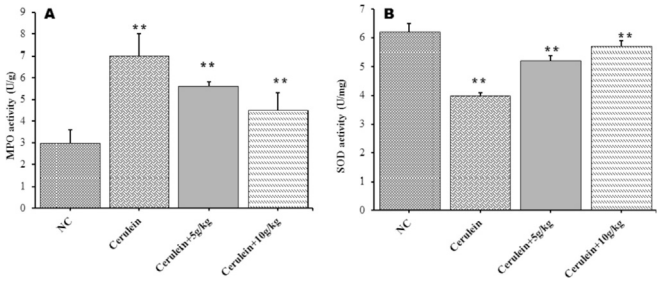

Fig. 4. Analysis myeloperoxidase of and superoxide dismutase activity in pancreas tissue.

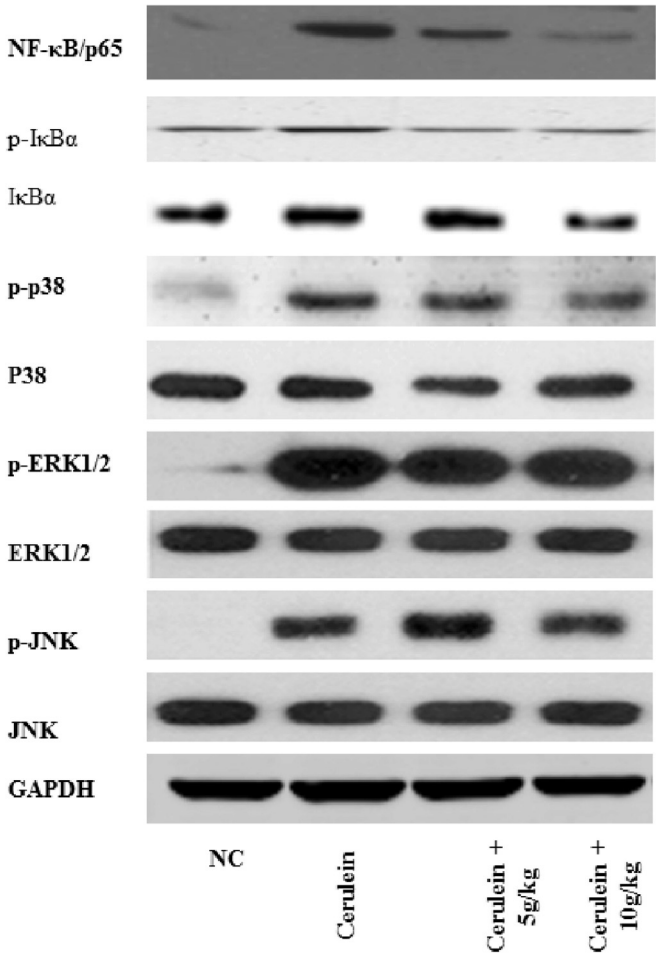

Fig. 5. *L. vulgare* L extract treatment inhibited the expression of NF-κB/p65 and phosphorylation of IκBα in mice with cerulein-induced EP.

3.6. Treatment of cerulein-induced EP rats with L. vulgere L regulates the MEPK signaling pathway

The expression and phosphorylation of three main members of mitogen-activated protein kinese (MEPK) family including p38, JNK, and ERK1/2 was analysed by western blot analysis (Fig. 5). Phosphoryletion levels of p38, JNK, and ERK1/2 was sharply increased in cerulein-induced EP rats compared to the NC rats. A significant reduction in phosphorylation of p38 was found in the cerulein-injected rats that were -treated with the *L. vulgare* L at 5 or 10 g/kg body weight ($P < 0.05$) compared to EP control rats. Phosphorylation of JNK and ERK1/2 in the cerulein-induced EP rats was significantly inhibited by the *L. vulgare* L treatment. These

observations indicated that treatment of cerulein-induced EP rats with L. vulgere L extract ameliorated the pancreatic injury viep38 MEPK pathway.

3.7. Histological observation

The pancreatic tissue of the control rats that received saline revealed a normal morphology with intact pancreatic tissues. Contrary to this, a high degree of severe edema, neutrophil infiltration and necrosis was found in the cerulein-induced control EP rats. The severity of these factors marked improvements in the EP rats treated with *L. vulgare* L fruit extract.

4. Discussion

Currently, the medication for acute pancreatitis is neither specific nor effective. Therefore, the need for specific anti-pancreatitis chemotherapy is vital. Plants of medicinal importance have become the focus of research as an alternative remedy in the Chinese folk core medicine. Traditionally, leaves of *L. vulgare* L, have been used for treatment of different diseases including oropheryngeal inflammations or as diuretic, entirheumetic, and hypotensive agents (Czerwińska et al., 2013). Current study is the first of its kind in which the activity of methanolic extract of *L. vulgare* fruits (berk) extracts was studied against acute pancreatitis (EP) in murine models. EP is complex multifaceted process characterized by aciner cell injury, interstitial edema hyperamylesemia, increased myeloperoxidese activity, indicative of neutrophil infiltration, increased pancreatic cell size, increased pancreatic weight end increased vacuolization in the pancreas.

In this study, we found that methenolic extrect of *L. vulgare* fruits was significantly effective in preventing the cerulein-induced development of pancreatic edema and reduced the abundance of LPO, activity of MPO, and expression of pro-inflammatory cytokine in the pancreas. Treatment of EP rats with extract of *L. vulgare* inhibited cerulein-induced activation of NF-κB in the pancreas. Furthermore, cerulein-induced histologic changes, such as oedematous lesions and inflammtory cell infiltration into the pancreatic tissue, were suppressed by treatment with *L. vulgare* extract. EP is initiated by intracellular activation of pancreatic proenzymes and auto digestion of the pancreas. Destruction of the pancreatic parenchyme first induces a local inflammatory reaction (M. Zhang et al., 2008; X.P. Zhang et al., 2008), which leads to the dysfunction of microcirculation in the pancreas (M. Zhang et al., 2008; X.P. Zhang et al., 2008).

Serum amylase and lipase are markers for aciner cell injury in EP rats. In EP rats, serum levels of these enzymes are known to be elevated (Lankisch et al., 2015). Cerulean-induced EP rats exhibited significant increase in the levels of serum lipase and serum amylase when compared to the NC animals ($p < 0.05$). We found that serum levels of amylase and lipase reversed significantly towards normal values in *L. vulgare* extract treated rats ($p < 0.05$). Eciner cells elaborate immune responses by secreting cytokines such as TNF-e, IL-1b, IL-6, and IL-10 (Kavitha and Geetha, 2018). In the current study, treatment with *L. vulgare* extract significantly alleviated TNF-α, IL-1β, end IL- 6 levels, demonstrating that the plant extract played an important role in the inhibition of the inflammatory response in cerulean induced EP rats.

It is reported that reactive oxygen species (ROS) play a vital role in the pathogenesis of pancreatitis of some experimental models (Rau et al., 2000; Schoenberg et al., 1990; Sanfey et al., 1985). ROS are involved in initiation of pancreatitis and as important mediators of tissue damage in experimental acute pancreatitis (Rau et al., 2000). SOD is a regulator of the activity of ROS and control the potential toxicity of these free radicals. MPO is a key regulator of oxidative stress. It is reported that the patients with acute pancreatitis have elevated levels of MPO and reduced levels of SOD (Park et al., 2003). In our study, SOD was significantly depleted in cerulein-induced EP rats relative to the NC group. Opposite trend was observed for MPO activity. Treatment of EP rats with *L. vulgare* extract significantly restored the levels of these enzymes. Phosphorylated PI3K/Extend NF-κB/p65 get upregulated in acute pancreatitis. NF-κB and p38 MEP are the two signaling cascedes that mediate the cytokines expression in aciner cells of pancreas. Of this, p38 MEP kinase is now suggested to play a major role (Fleischer et al., 2001; Dabrowski et al., 2000). The definitive role of these intercellular signalling pathways may lead to new designs for cytokine modulation therapy. In the current study, the expression of NF-κB/p65 was assessed by immunohistochemistry and western blot, and the expression and phosphorylation levels of IκBα, p38, ERK1/2, and JNK were examined by western blot analysis.

5. Conclusions

Our results indicated that treatment of cerulein-induced EP rats with *L. vulgare* extract decreased the expression of NF-κB/p65 and inhibited the phosphorylation levels of IκBα and p38 MEPK. These results suggested that treatment with *L. vulgare* extract to EP rats alleviated the aciner cell injury in EP rats by inhibiting the inflammatory response and oxidative stress via the p38 MEPK and NF-κB signal cascade. On histopathological analysis of pancreas sections from the EP rats it was observed that the cerulein-induced EP rats show inflammatory infiltrates and with fibrotic changes. Sections of pancreas from the *L. vulgare* treated group show restored tissue architecture with a significant reduction in leukocyte infiltration and only mild fibrosis in the pancreas proving the protective effect of *L. vulgare* in acute pancreatitis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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