

Research Article

Galangin Ameliorates Inflammatory Changes in Pancreas – A Study in Rats Administered Ethanol and Cerulein

*Geetha.A¹, Fatima.CA²

¹Department of Biochemistry, Bharathi Women's College, Chennai-600 108, India.

²Research Scholar, Research and Development Centre, Bharathiar University, Coimbatore- 641 046, India.

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ABSTRACT

The study was conducted to investigate whether galangin(GA), a natural flavonoid of the rhizome *Alpinia galanga* modulates the components of NLRP3 inflammasome, a multi protein complex which mediates inflammation in rats subjected to experimental pancreatitis by ethanol(EtOH) and cerulein administration. For the study, adult male albino Wistar rats were divided into four groups. Group 1 and 2 served as control and received normal diet for 5 weeks. Group 3 and 4 received an ethanol containing isocaloric diet and cerulein (20µg/kg body wt.) intraperitoneally for the last 3 weeks of the experimental period. In addition, group 2 and 4 were administered 100µg/kg body wt. of galangin in corn oil orally by intragastric intubation for the last 3 weeks of the experimental period. The mRNA expression of the NLRP3 components apoptosis-associated speck-like protein (ASC), pyrin (PYD), and caspase-recruitment (CARD) domains, caspase-1 and the proinflammatory cytokines IL-1β and IL-18 were evaluated by real time quantitative PCR (RT-PCR) technique. Histopathological examination of pancreatic tissue was also carried out. A significant increase in the mRNA expression of ASC-CARD, ASC-PYD, caspase-1, IL-1β and IL-18 were observed in EtOH and cerulein treated rats when compared to control rats. The mRNA expression of these genes were substantially downsized in the galangin supplemented (group 4) rats. The results were supported by histological observations. The anti inflammatory effect of galangin might be due to its modulating effect on the interaction of ASC-CARD and ASC-PYD domains which activate caspase-1 to promote proinflammatory cytokines activity. Also the observations suggest that galangin downregulates the proinflammatory cytokine production thereby prevents inflammation and minimizes tissue damage.

Keywords: Caspase-1, Cytokines, Galangin, IL-1β, IL-18, Inflammasome, Pancreatitis.

INTRODUCTION

The pancreas is a secretory organ found behind the stomach and has both endocrine and exocrine role. Pancreatitis is inflammation of the pancreas associated with severe abdominal pain. Eighty percent of acute pancreatitis is linked etiologically to gallstone disease or caused by immoderate alcohol consumption[1]. Acute pancreatitis is caused by sudden and multiple episodes of inflammatory shock in pancreas associated with the release of digestive enzymes to the pancreatic interstitium and to the systemic circulation with increased cytokine production and release, which can ultimately lead to deleterious local and systemic effects[2,3]. The annual incidence of acute pancreatitis is between 5 and 80 per 100,000 people of the world population[4]. Chronic pancreatitis is characterized by progressive pancreatic damage that eventually leads to impairment of both exocrine and endocrine functions of the pancreas[5,6]. Alcohol is the single most common etiology of chronic pancreatitis[7,8,9]. Chronic pancreatitis affects 3-9 people in 100,000; 70% of cases are alcohol-induced[10].

The hallmark features of pancreatitis are abdominal pain, malabsorption, malnutrition, diabetes mellitus and

pancreatic calcification[11]. The pathology of the disease is basically an imbalance between necrosis and apoptosis and anti- and pro-inflammatory cytokines/chemokines[12]. Administration of doses of a cholecystokinin (CCK) analog cerulein beyond those that cause the maximum pancreatic secretion of amylase and lipase result in pancreatitis, which is characterized by a dysregulation of the production and secretion of digestive enzymes, particularly, the inhibition of pancreatic secretion and an elevation in their serum levels, cytoplasmic vacuolization and the death of acinar cells, edema formation, and an infiltration of inflammatory cells into the pancreas[13,14]. Chronic alcohol feeding combined with repeated cerulein treatment results in a model of pancreatitis that parallels the major aspects of the human disease[15].

The inflammasome is a multiprotein oligomer consisting of caspase 1, PYCARD and NALP. It is expressed in myeloid cells and is a component of the innate immune system. The exact composition of an inflammasome depends on the activator which initiates inflammasome assembly. The inflammasome promotes the maturation of the inflammatory cytokines, interleukin 1β (IL-

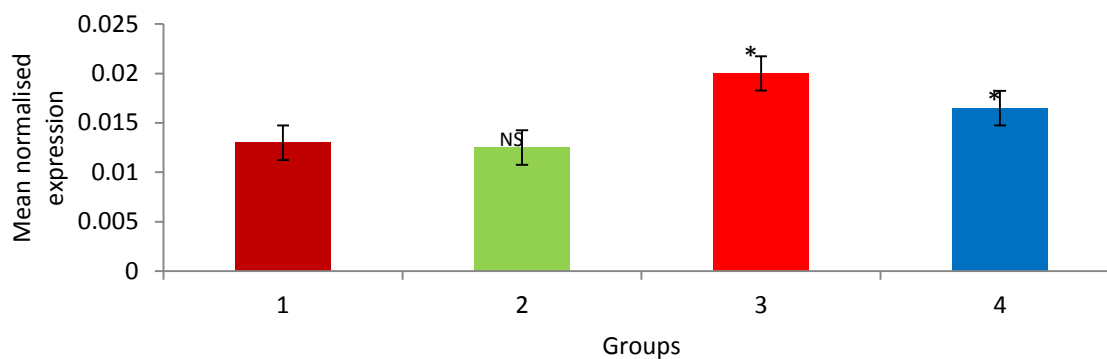


Figure 1(a) Effects of galangin on mRNA expression of ASC CARD in pancreas of experimental rats. Data were analysed by one way ANOVA followed by post hoc Bonferroni. Values are means \pm SD of six rats in each group. Statistically significant variations are expressed as * $P < 0.05$ for Group 1 Vs Group 3 and Group 3 Vs Group 4, NS - Non-Significant for Group 1 Vs Group 2.

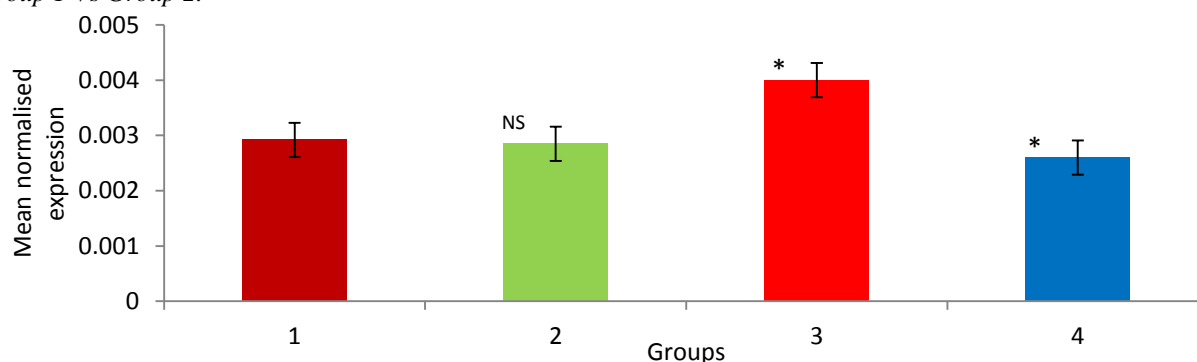


Figure 1(b) Effects of galangin on mRNA expression of ASC PYD in pancreas of experimental rats. Data were analysed by one way ANOVA followed by post hoc Bonferroni. Values are means \pm SD of six rats in each group. Statistically significant variations are expressed as * $P < 0.05$ for Group 1 Vs Group 3 and Group 3 Vs Group 4, NS - Non-Significant for Group 1 Vs Group 2.

1 β) and interleukin 18 (IL-18)[16]. The inflammasome is responsible for activation of inflammatory processes[17]. Analogous to the apoptosome, which activates apoptotic cascades, the inflammasome activates the inflammatory cascade. Once active, the inflammasome binds to pro-caspase-1 (the precursor molecule of caspase-1), either homotypically via its own caspase activation and recruitment domain (CARD) or via the CARD of the adaptor protein ASC which it binds to during inflammasome formation. In its full form, the inflammasome appositions together many p45 pro-caspase-1 molecules, inducing their autocatalytic cleavage into p20 and p10 subunits[18]. Caspase-1 then assembles into its active form consisting of two heterodimers with a p20 and p10 subunit each. Once active, it can then carry out a variety of processes in response to the initial inflammatory signal. These include the proteolytic cleavage of pro-IL-1 β at Asp116 into IL1 β , cleavage of pro-IL-18 into IL-18 to induce IFN- γ secretion, natural killer cell activation[19] and secretion of tissue-repair mediators such as pro-IL-1 α [20].

NACHT, LRR and PYD domains-containing protein 3 (NALP3) or cryopyrin is a protein that in humans is encoded by the NLRP3 (NOD-like receptor family, pyrin domain containing 3) gene[21]. NLRP1, NLRP3 and NLRC4 are subsets of the NLR

family and thus have two common features: the first is a nucleotide-binding domain (NBD) which is bound to by ribonucleotide-phosphates (rNTP) and is important for self-oligomerization. The second is a C-terminus leucine-rich repeat (LRR), which serves as a ligand-recognition domain for other receptors (e.g. TLR). In addition to the NBD and LRR domains, NLRP3 contains a PYD domain and thus activates caspase-1 using its PYD to recruit ASC. It forms only one oligomer per cell, and its oligomer is made of seven NLRP3 molecules. It is known to be the biggest inflammasome of all[22].

Though many allopathic drugs are currently available for the treatment of pancreatitis, adverse effects are also reported in few cases. The ever increasing problem of pancreatitis demands the evaluation of more new plant based drugs which are cost effective with no side effects when compared to allopathic medicines. Medicinal plants are the source of new therapeutic agents. Flavonoids are diverse class of naturally occurring phenolic compounds that have a variety of therapeutic activities[23]. Flavonoids have been touted as anti-inflammatory, anti-oxidant, chemopreventatives with the potential to be used for prevention or treatment of such diverse diseases as arthritis [24]. Galangin(GA) chemically known as 3,5,7-trihydroxyflavone, a member of the flavonol class of flavonoids, is the active constituent of the rhizome of

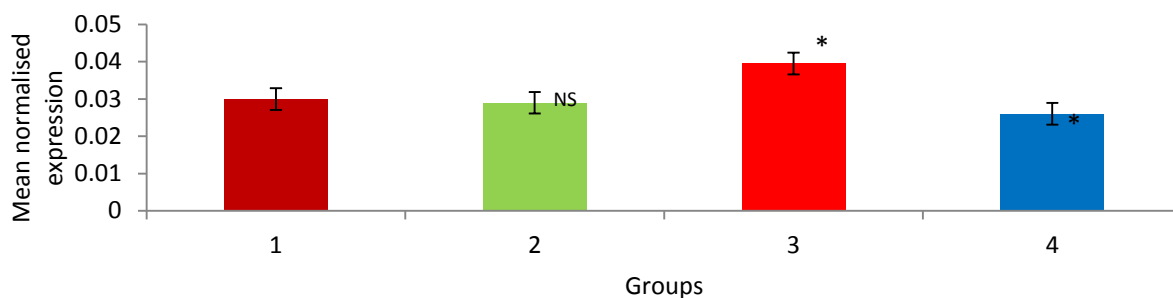


Figure 2 Effects of galangin on mRNA expression of caspase-1 in pancreas of experimental rats. Data were analysed by one way ANOVA followed by post hoc Bonferroni. Values are means \pm SD of six rats in each group. Statistically significant variations are expressed as * $P < 0.05$ for Group 1 Vs Group 3 and Group 3 Vs Group 4, NS - Non-Significant for Group 1 Vs Group 2.

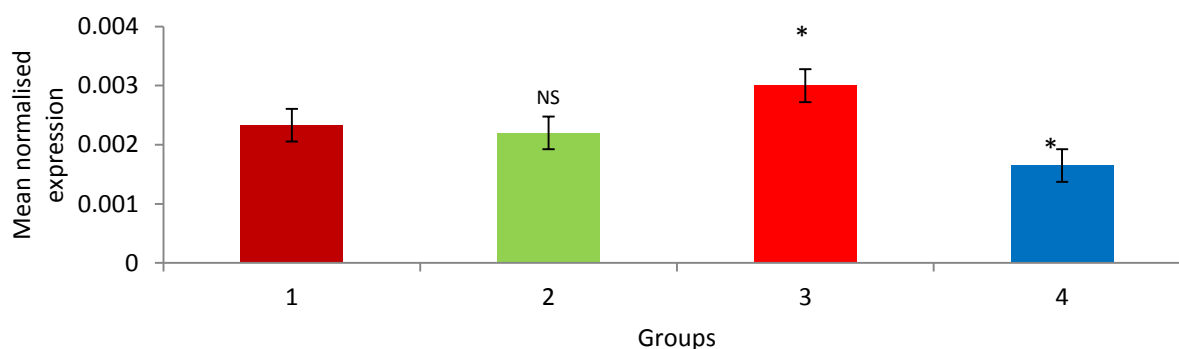


Figure 3 Effects of galangin on mRNA expression of IL-1 β in pancreas of experimental rats. Data were analysed by one way ANOVA followed by post hoc Bonferroni. Values are means \pm SD of six rats in each group. Statistically significant variations are expressed as * $P < 0.05$ for Group 1 Vs Group 3 and Group 3 Vs Group 4, NS - Non-Significant for Group 1 Vs Group 2.

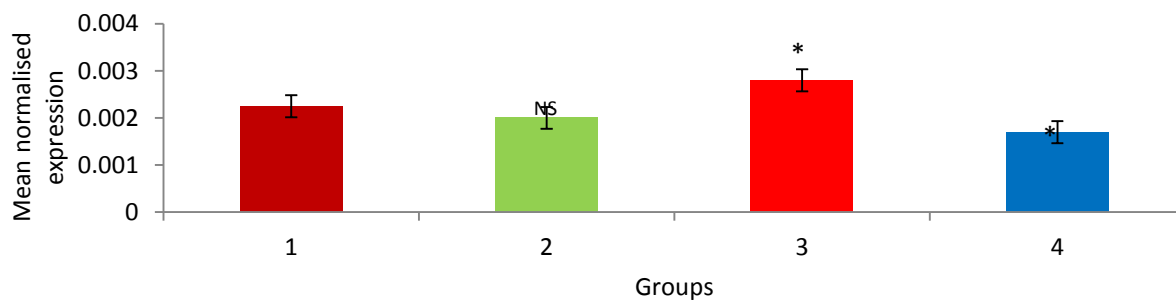


Figure 4 Effects of galangin on mRNA expression of IL-18 in pancreas of experimental rats. Data were analysed by one way ANOVA followed by post hoc Bonferroni. Values are means \pm SD of six rats in each group. Statistically significant variations are expressed as * $P < 0.05$ for Group 1 Vs Group 3 and Group 3 Vs Group 4, NS - Non-Significant for Group 1 Vs Group 2.

Alpinia galanga (known as *Chittarattai* in Tamil, *Kulanjan* in Hindi) a plant closely related to the ginger family (Zingiberaceae). The rhizome is commonly used in food preparations such as curries and soups. It is also used as traditional medicine for diabetes and for a variety of ailments like cough, cold, gastritis and diarrhea in the Asian countries[25]. The present study is proposed to validate the anti inflammatory efficacy of a flavonol galangin against ethanol and cerulein induced inflammatory changes in the pancreas of rats subjected to experimental pancreatitis in terms of molecular level alterations of ASC and caspase-1.

MATERIALS AND METHODS

RNeasy Miniprep Kit was purchased from Qiagen and cDNA reverse transcription kit was purchased from Applied Biosystems, USA. Galangin was obtained from Sigma Aldrich Chemicals co. All other chemicals and solvents used for the analysis were of analytical grade. Galangin was dissolved in corn oil and a homogenous suspension prepared was administered orally by intragastric intubation.

Animals and treatment: The work protocol was approved by the Institutional Animal Ethics Committee

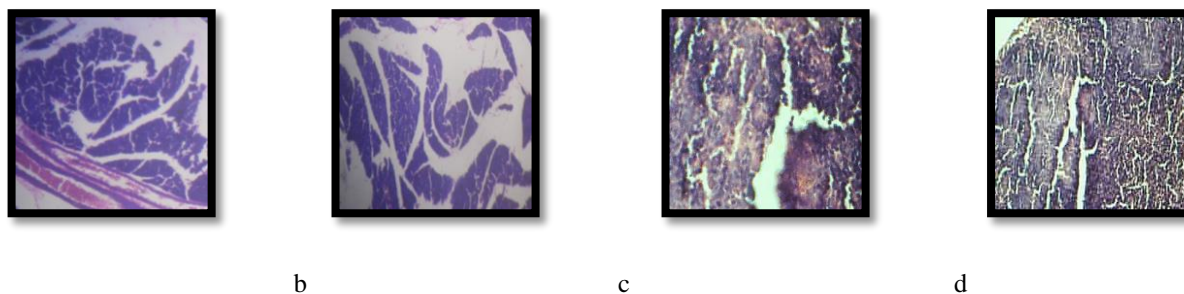


Fig.5-Histopathology of pancreas

[XIII/VELS/PCOL/23/2000/CPCSEA/IAEC/08.08.2012]. Adult male albino Wistar rats of body weight 175 to 200g [seven-eight weeks old] were used for the study. They were individually housed under hygienic conditions [22-24° C] in polypropylene cages under 12 h light/ 12 h dark cycle. The animals were allowed free access to water and standard rat chow obtained from Hindustan Lever Ltd., Bangalore, India during the acclimatization period.

Rats were randomised into four groups and were pair fed either an isocaloric control or ethanol containing diet for 2 weeks after acclimatization for a period of one week. The ethanol concentration was gradually increased from 0-36 % of the total calories during the first 9 days and the same concentration of alcohol was maintained for the rest of the period till 5 weeks. Then rats were subjected to intraperitoneal administration of cerulein at the dose of 20µg/kg and at an interval of 3,24 and 96 hours weekly for the last 3 weeks. Dose response study was conducted and 100µg/kg body weight was chosen as the optimum dose[26].

The animals were randomly divided into four groups of six animals each.

Group 1: received the normal diet (standard rat chow) for 5 weeks

Group 2: received the normal diet and GA (100µg/kg body weight/day) orally by intragastric intubation for last 3 weeks of the experimental period

Group 3: received an ethanol containing isocaloric diet and 20µg/kg body weight of cerulein for the last 3 weeks

Group 4: received an ethanol containing isocaloric diet, 20µg/kg body weight of cerulein and GA (100µg/kg body weight) for the last 3 weeks

At the end of the experimental period , rats were fasted overnight and anesthetised by intramuscular injection of ketamine hydrochloride[30mg/kg body wt.] and killed by cervical decapitation. Immediately after sacrifice, total RNA was isolated from pancreas according to the manufacturers instructions.

Gene Expression Studies: The gene expression of ASC CARD, ASC PYD, caspase-1 and pro inflammatory cytokines IL-1β and IL-18 were evaluated by Real-Time Quantitative PCR.

Quantitative RT-PCR Analysis: The RNA isolated was reverse transcribed according to manufacturer's instructions (PN. #:4374966). RT-PCR was performed in triplicate for each gene of interest using ABI Prism 7900HT Sequence Detection System (Applied

Biosystems), USA. Gene sequence was obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>) and primers were designed by Primer 3 software. GAPDH was used as endogenous control to which each gene of interest was normalized. The primer sequences of genes are shown below. RT-PCR conditions were as follows: 40 cycles of 95°C for 20 sec (denaturation), 55°C for 30 sec (annealing) and 60°C for 30 sec (extension). Ct values obtained were used to quantify mRNA expression.

Rat-GAPDH-F

CAAGGTCATCCATGACAACCTTTG

Rat-GAPDH-R GTCCACCACCCTGTTGCTGTAG

Rat-ASC PYD-F GCAATGTGCTGACTGAAGGA

Rat-ASC PYD-R TGTCCAGGTCGTGCACCAA

Rat-ASC CARD-F TGAAAACCTTGACAGCGGATG

Rat-ASC CARD-R GCTCCTGTATGCCCATGTCT

Rat-IL-18-F GAGGACTGGCTGTGACCCTA

Rat-IL-18-R ATCCCCATTTTCATCCTTCC

Rat-IL-1b-F CAGGAAGGCAGTGTCACTCA

Rat-IL-1b-R AAAGAAGGTGCTTGGGTCCT

Rat-Caspase-1-F TATGGAAAAGGCACGAGACC

Rat-Caspase-1-R CAGCTGATGGACCTGACTGA

Histopathology: For histopathology examination, the pancreatic tissues were excised and rinsed with ice-cold saline solution (0.9% sodium chloride) to remove blood and debris of adhering tissues. The tissues were then fixed in 10% formalin-saline for 24h. The fixative was removed by washing through running tap water and after dehydration through a graded series of alcohols, the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections were cut into 5µM thickness and stained with hematoxylin and eosin. After dehydration and cleaning, the sections were mounted and observed under light microscope for details.

Statistical Analysis: Data were analyzed by using a commercially available statistics software package (SPSS for window V.10.0). the statistical significance of mean values between different groups were determined by applying one way ANOVA with *post hoc* Bonferroni test and the P value < 0.05 was considered as significant and results were presented as mean ± S.D

RESULTS

The gene expression studies revealed the following results. Effect of galangin on mRNA expression of ASC-CARD and ASC-PYD: Figure 1(a) and 1(b) show the mean normalised expression of the ASC-CARD and ASC-PYD mRNA. The expression of the these genes were increased

in the EtOH and cerulein treated rats and significantly decreased in galangin co-administered rats.

Effect of galangin on mRNA expression of Caspase-1: Figure 2 presents the mean normalised mRNA expression of caspase-1. In EtOH and cerulein fed rats the expression was upregulated. Rats co-administered with galangin showed a significant decrease in the expression of caspase-1 gene.

Effect of galangin on mRNA expression of IL-1 β : The mean normalised expression of IL-1 β mRNA is presented in figure 3. The expression was significantly increased in the EtOH and cerulein fed rats and decreased in the galangin co-administered group 4 rats.

Effect of galangin on mRNA expression of IL-18: Figure 4 shows the mean normalised expression of cytokine IL-18 mRNA. The expression was found to be upregulated in EtOH and cerulein administered rats (group 3). The expression was significantly decreased in galangin co-administered (group 4) rats.

Effect of galangin on the histology of pancreas: The histological changes in the pancreas of control and experimental rats are shown in figure 5. The pancreas from control rats (group 1) showed normal parenchyma cells with collagen fibrils (Fig.5a). The pancreas of drug control rats (group 2) showed normal architecture with parenchymal cells (Fig. 5b). Rats received EtOH and cerulein (group 3) showed significant inflammation, mononuclear cell infiltration, fatty changes in and around acinar cell (Fig. 5c). Galangin co-administered rats (group 4) showed a significant reduction in fat accumulation, inflammation and hemorrhage in the pancreas (Fig.5d).

DISCUSSION

Alcohol abuse is a risk factor for chronic pancreatitis and is reported to be associated with chronic pancreatitis in 50-70% of patients[27]. Recurrent acute pancreatitis appears to precede the development of chronic pancreatitis [28]. Rats that are chronically fed alcohol have an increased sensitivity to pancreatitis induced by cholecystokinin. Cerulein an analogue of cholecystokinin is widely used to elicit pancreatitis by hyperstimulation of pancreas in rats. The pancreatitis induced by cerulein is characterised by edema, increased serum levels of pancreatic enzymes and inflammation(29).

Plants play a principal role in the introduction of new therapeutic agents for various common ailments[30]. A large number of plant derived natural products such as flavonoids, terpenoids and steroids have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and hepatoprotective activity[31]. Galangin, a flavonoid derivative has shown protective effects on pancreas when the organ is inflamed by EtOH- cerulein administration. Pancreatic damage is initiated by autodigestion of the tissue by acinar cell-derived proteases, followed by a massive infiltration of leukocytes such as neutrophils and macrophages[32]. The infiltrating leukocytes produce various proinflammatory cytokines, such as IL-1 and TNF- α , and release proteinases and reactive oxygen species [33,34]. Inflammasomes are a group of protein complexes

built around several proteins, including NLRP3, NLRC4, AIM2 and NLRP6. Recognition of a diverse range of microbial, stress and damage signals by inflammasomes results in direct activation of caspase-1, which subsequently induces secretion of potent proinflammatory cytokines and a form of cell death called pyroptosis[35]. I ASC is an adaptor protein composed of protein-protein interaction domains, a PYRIN domain (PYD), and a caspase-recruitment domain (CARD)[36]. Caspase-1, ASC of NLRP3 inflammasome are required for inflammation in acute pancreatitis[37]. Immunohistochemical study showed that ASC is increased in neutrophils in severe inflammatory sites of gangrenous appendicitis. The NLRP3 inflammasome has a role in the neuroinflammation occurring in Alzheimer's disease[38]. Up-regulation of ASC is closely associated with inflammation and apoptosis in neutrophils[36].

In the present study, the observed downregulation of ASC PYD and ASC CARD mRNA could possibly be due to the anti inflammatory effect of galangin. Galangin might have interacted with CARD and PYD domains of ASC to down size the expression of these genes in the galangin treated group 4 rats.

Caspases are a class of cysteine-proteases involved in the regulation of inflammation and apoptosis[39,40]. Caspase-1 has emerged as the main intracellular processing enzyme responsible for maturation of active IL-1 β and IL-18, which are then released into the extracellular space[41,42]. Caspase-1, also known as interleukin-1 β converting enzyme, is activated by inflammasomes, formed by caspase-1, several members of the NOD-like receptors family and the adaptor protein ASC. In addition to its well-established proinflammatory role, caspase-1 can also execute a program of cell death, termed pyroptosis, to kill infected macrophages[43]. However, caspase-1 retains a direct role in non-infectious cell death processes[44]. Caspase-1, indeed, also acts as a tumor suppressor regulating proliferation and apoptosis of epithelial cells[45,46]. Moreover, in human cancers, caspase-1 is frequently downregulated, especially in prostate cancer[47,48]. In this study, overexpression of caspase-1 gene has been observed in group 3 animals. Galangin supplementation seems to have reduced the caspase-1 gene expression in group 4 rats. Galangin might have interrupted the activation of procaspase to caspase-1 by modulating the ASC-PYD-CARD domains and thus responsible for the significant decrease in the caspase-1 gene expression observed in galangin administered group 4 rats.

Inflammatory mechanisms are central in modulating the severity of AP and development of CP. In both human and experimental CP models, a series of inflammatory mediators have been identified that play key role in the progressive injury to pancreatic cells [49,50,51]. Inflammasomes are key signalling platforms that detect pathogenic microorganisms and sterile stressors, and that activate the highly pro-inflammatory cytokines interleukin-1 β (IL-1 β) and IL-18[52]. Cytokines are regulators of host responses to infection, inflammation, and trauma. Some cytokines act to make disease worse

(proinflammatory), whereas others serve to reduce inflammation and promote healing (anti-inflammatory)[53]. In present study, the mRNA expression of proinflammatory cytokines IL-1 β and IL-18 were over expressed in group 3 EtOH and cerulein administered rats. The expression of IL-1 β and IL-18 was downsized in galangin treated group 4 rats. These observed results indicate that galangin might have shown an anti inflammatory effect by regulating the activation of cytokines. This is in accordance with the earlier study reports which show that any anti inflammatory drug should reduce the level of cytokine formation which is the first level prevention of inflammation to reduce tissue injury. Galangin shows anti inflammatory effect by affecting the gene level expression of cytokines and the activating factor caspase-1. This could be attributed to the fact that flavones and hydroxyflavones can inhibit the phosphorylation of proteins involved in the signal transduction[54]. The honey bee propolis, the bee hive extract, has been reported to possess anti inflammatory effect by altering the activation of NF- κ B[55]. Galangin being one of the active constituents of propolis has been shown responsible for the anti inflammatory activity of propolis[56]. This property of galangin disables the inflammatory reactions within the cells. Earlier findings indicated that flavonols have a prebiotic-like effect on the anti-inflammatory activity of certain gut microbiota[57] and that hydroxyflavones could modulate the IL-1 β gene expression in activated macrophages via inhibiting gene transcription[58]. Galangin supplementation significantly reduced the level of inflammation, fatty steatosis and hemorrhage in pancreas. The anti inflammatory effect of galangin is supported by the histopathological observations.

CONCLUSION

The present study has demonstrated the mechanism of pancreato protective nature of galangin effected by its anti-inflammatory action . Galangin ameliorates the inflammatory changes by minimizing the production of activated IL-1 β and IL-18. Galangin exerts anti inflammatory effect probably by modulating the mRNA expression of ASC-CARD and ASC- PYD domains in NLRP3 inflammasome. Thus the data reinforces the anti inflammatory effects of galangin and its potential application for inflammation related ailments. However it demands further study on the evaluation of protein expression of the various domains of NLRP3 inflammasome.

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