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Research Article

EFFECT OF GALANGIN ON ETHANOL AND CERULEIN INDUCED INFLAMMATORY CHANGES IN PANCREAS: A BIOCHEMICAL STUDY IN RAT MODEL

FATIMA CYNTHIA ANTONY, ARUMUGAM GEETHA*, DEEPA C. NARAYANAN

Research and Development Centre, Bharathiar University, Coimbatore 641046, India. Email: geethav21@yahoo.co.in

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ABSTRACT

Objective: The study was conducted to examine the effects of Galangin(GA), a natural flavonoid of the rhizome *Alpinia galanga* on the levels of inflammatory markers caspase-1, IL-1β, IL-18 and antioxidant status in rats subjected to experimental pancreatitis by ethanol(EtOH) and cerulein administration.

Methods: 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 isocaloric diet , ethanol 8-12 g/ kg body weight/day and also 20µg/kg body wt. of cerulein intraperitonially for the last 3 weeks of the experimental period. In addition, group 2 and 4 were administered 100µg/kg body wt. of galangin orally by intragastric intubation for the last 3 weeks of the experimental period. Serum lipase, amylase, caspase-1, IL-1β, IL-18, thiobarbituric acid reacting substances(TBARS) and antioxidant status were assessed.

Results: A significant increase in the levels of serum lipase/amylase ratio, caspase-1 activity, IL-1β, IL-18 and TBARS were observed in ethanol-cerulein treated rats compared to those co-administered with galangin. Galangin supplementation along with ethanol-cerulein treatment significantly maintained the levels of antioxidants catalase, superoxide dismutase, glutathione peroxidase and reduced the oxidative stress in group 4 animals.

Conclusion: The caspase-1 inhibitory activity of galangin is well evidenced from the study which may be due to its modulating effect either on caspase-1 synthesis or on activation of procaspase-1 . Also the observations suggest that the anti-inflammatory activity of galangin may be attributed to its antioxidant property of scavenging the free radicals which aggravate the process of inflammation.

Keywords: Cerulein, Cytokines, Ethanol, Galangin, Inflammation, Pancreatitis.

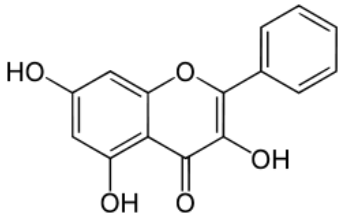
INTRODUCTION

The pancreas is an intestinal gland that synthesise and secrete the hormones insulin and glucagon, as well as digestive enzymes to help digest and absorb food. Pancreatitis is inflammation of the pancreas[1]. Pancreatitis is one of the major gastrointestinal problems that affect 70% of the alcohol consumers and is characterised by severe inflammation and abdominal pain. Acute pancreatitis is a multifactorial disease 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[2,3,4]. Chronic pancreatitis is inflammation of pancreas that does not heal or improve rather gets worse overtime and leads to permanent damage. The most common cause of chronic pancreatitis is many years of alcohol use. It can be triggered by one acute attack that damages the pancreatic duct. The damaged duct causes the pancreas to become inflamed. Scar tissue develops and the gland is slowly destroyed[5]. Chronic pancreatitis affects 3-9 people in 100,000; 70% of cases are alcohol-induced[6]. The pathology of the disease is basically an imbalance between necrosis and apoptosis and anti-inflammatory and pro-inflammatory cytokines/chemokines[7,8].

Among several animal models of experimental pancreatitis that exhibit the biochemical, morphological, and pathophysiological similarities to various aspects of human pancreatitis, cerulein pancreatitis was shown to be one of the best-characterized and widely used experimental models.[9,10] Doses of a cholecystokinin (CCK) analog cerulein beyond those that cause the maximum pancreatic secretion of amylase and lipase[11,12] 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[9,10,13].

Rise in population, prohibitive cost of treatment for common ailments, side effects of several allopathic drugs in current usage and development of resistance to currently used drugs for disease have lead to increased emphasis on the use of plant materials as source of medicines for the wide variety of human ailments including pancreatitis. Plants play a principal role in the introduction of new therapeutic agents[14,15].Recently allopathic medicines have been used to alleviate the complications associated with inflammation in pancreas[16].

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 *Alpinia galanga*, 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, stomachache and diarrhea in the Asian countries[17].



GA is shown to be a potent scavenger of free radicals such as singlet oxygen and superoxide anion in vitro[18]. The effect of GA on the biochemical changes in alcohol cerulein induced pancreatitis has not been explored. The reported antioxidant and anti-inflammatory[19] effects of GA promoted us to investigate whether GA could prevent ethanol and cerulein induced biochemical changes in pancreas in rat model.

MATERIALS AND METHODS

Chemicals and Reagents

Galangin was obtained from Sigma Aldrich. Elisa kits for IL-Iβ and caspase-1 were purchased from Abcam and Elisa kit for IL-18 was purchased from Invitrogen. All other chemicals and solvents used for the analysis were of analytical grade.

Galangin Preparation- Galangin was dissolved in corn oil and a homogenous suspension was prepared. The suspension was administered orally by intragastric intubation.

Experimental protocol

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 [22-24º 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. The work protocol was approved by the Institutional Animal Ethics Committee [XIII/VELS/PCOL/23/2000/CPCSEA/IAEC/08.08.2012].

After acclimatization for a period of one week, rats are pair fed either an isocaloric control or ethanol containing liquid diet for 2 weeks. 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 a total period of 5 weeks. Then pancreatitis was induced by intraperitonial injection of cerulein at the dose of 20µg/kg at the interval of 3,24 and 96 hours given weekly for the last 3 weeks. Table 1 shows the composition of normal diet.

Dose response study was conducted with different doses of 50,75,100 and 200 µg/kg body weight and was observed that 100µg/kg body weight reduced the inflammation in pancreatic damage significantly and thus this dosage was chosen for the study.

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 intraperitonially for the last 3 weeks

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

Table 1: Composition of normal diet

	Normal diet%
Carbohydrate	67.3
Protein	19.3
Fat	4.3
Mineral mix	0.95
Calcium phosphate	1.23
Calcium carbonate	0.52
Potassium citrate	1.56
Vitamin mix	0.95
Choline bitartate	0.19

The animals were maintained in the respective groups for 5 weeks. At the end of the experimental period, rats were fasted overnight and anesthesied by intramuscular injection of ketamine hydrochloride[30mg/kg body wt.] and killed by cervical decapitation. Blood was collected with/without anticoagulant and plasma/ serum separated were stored until analysis.

Tissue Homogenate Preparation

Pancreas was removed carefully after sacrifice, washed and homogenized in 0.1M Tris buffer pH-7.4 and centrifuged at low speed to remove all cell debris. The supernatant was used for the determination of caspase-1, and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

Biochemical Investigations

Determination of serum lipase activity

Method of Lowry and Tinsley was used for the assay of lipase activity in serum[20]. Serum was added to 25ml olive oil/triton X-100 emulsions and lipolysis reaction was initiated. 0.3ml subsamples of reaction mixture were taken at predetermined time intervals and used for the assay of liberated free fatty acids spectrophotometrically at 715 nm. The activity of enzyme was expressed as IU/L.

Determination of serum amylase activity

Assay of amylase activity was done by the method of Gomori[21]. The enzyme activity was determined by measuring the amount of maltose liberated from starch substrate using lugol's iodine solution. The colour intensity was measured spectrophotometrically at 640nm and the enzyme activity was expressed as IU/L.

Assay procedure for IL-1β

Manufacturer's instructions (ab100767) was followed for the assay. Standards or serum samples were added to wells precoated with IL - 1β antibody and IL-1β present in a sample is bound to the well by the immobilized antibody. After wells were washed biotinylated anti-Rat IL-1β antibody was added. The unbound biotinylated antibody was removed and HRP conjugated streptavidin was added to the wells. Again the wells were washed and TMB substrate solution was added to the wells. The stop solution changes the colour from blue to yellow, and the intensity of the colour was measured at 450 nm. The intensity of the colour developed was proportional to the amount of IL-1β present in the sample. The activity of IL-1β was expressed as pg/mI.

Assay of IL-18

The assay was carried out as per the kit manual (KRC2341). The serum sample and standards were pipetted into antibody immobilized wells. After the incubation, biotinylated secondary antibody was added. After removal of excess secondary antibody, streptavidin-peroxidase was added. Then the substrate solution was added to react with the bound enzyme to produce colour. The intensity of the colour developed was measured spectrophotometrically at 450 nm. The activity of IL-18 was expressed as pg/ml.

Assay of caspase-1

Method of Thornberry[22], was adopted for the colorimetric assay of Caspase-1 activity by using serum or pancreatic extract, as the enzyme source. Briefly, the pancreas was homogeniseed in a lysis buffer(25mM HEPES pH 7.5], 1mM EDTA, 10µg of aprotinin/mL, 10 µg of leupeptin/mL , 2mM dithiothreitol) at 5mL/100 mg of pancreas tissue. Extracts were centrifuged at 15,000g for 30 mins at 4º C, and the supernatant was centrifuged again at 200,000g for 1 h at 4º C. The cytosol was used for measurement of capase-1 activity. The assay in undiluted serum or pancreas extract was done as per the kit manufacturer instruction. Reactions with enzyme preparation alone, enzyme mixed with caspase-1 substrate(Ac-YVAD-pNA) or inhibitor (Ac-YVAD-CHO), and with substrate alone were also run as controls. The activity was measured by proteolytic cleavage of Ac-YVAD-pNA for 4 h at 37º C. A recombinant caspase-1 enzyme was used as a positive control. The plates were read at 405nm.

Estimation of lipid peroxides

Thiobarbituric acid-reacting substances (TBARS) was measured to determine level of lipid peroxides in plasma[23]. The value was expressed as nmol/ml plasma.

Estimation of antioxidant enzymes

Glutathione peroxidase (GPx) was assayed according to the method of Flohe and Gunzler[24]. nM of glutathione oxidized / min / mg protein expresses the activity of GPx. Superoxide dismutase (SOD) activity was measured according to method the of Kakker et al,[25]. The inhibition of reduction of nitroblue tetrazolium to blue coloured formazan in the presence of phenazine methosulfate and NADH was measured at 560 nm using n- butanol as blank. The enzyme activity was expressed as units/mg protein. Catalase (CAT) activity was kinetically measured at 240nm[26]. CAT activity was defined as the amount of enzyme required to decompose hydrogen peroxide. The enzyme activity was expressed as µM of H_20_2 consumed/ min/ mg protein.

Estimation of protein

Bradford method was used to determine the protein concentration in the tissue homogenate[27]. The protein level was used to calculate the enzyme activity in tissue homogenate.

Statistical Analyses

Commercially available statistics software package (SPSS for window V.10) was used for the data analysis. The statistical significance of mean values between different groups was determined by applying one way ANOVA with *post hoc* Bonferroni test and the P value <0.05 was considered as significant.

RESULTS

The biochemical estimations revealed the following results:

Effect of Galangin on serum lipase and amylase

The serum lipase and amylase ratio, the marker enzymes of pancreatic functions are presented in table 2. Serum amylase and lipase activities were increased significantly in EtOH and cerulein received animals (group 3) when compared to group1 rats. The rats (group 4) co-administrated with galangin showed decrease in the level of serum lipase and amylase.

Table 2: Levels of serum lipase and amylase in experimental animals

Groups	Lipase /Amylase ratio(IU/L)
Control	1.85 ± 0.33
Galangin Control	1.29 ± 0.18^{NS}
EtOH+Cerulein	4.37 ± 0.57*
EtOH+Cerulein+Galangin	3.14 ± 0.47*

Values are expressed as mean ± SD for 6 animals in each group. Control Vs Galangin, control Vs EtOH+Cerulein, EtOH+Cerulein Vs EtOH+Cerulein+Galangin

* P < 0.001, NS- Non-significant

Table 3: Levels of serum IL-1β and IL-18 in experimental animals

Groups	IL-1β (pg/ml)	IL-18(pg/ml)
Control	12.63 ± 2.46	238.85 ± 57.57
Galangin Control	14.13 ±0.76 ^{NS}	199.68 ±11.34 ^{NS}
EtOH+Cerulein	21.92 ± 2.31*	315.38 ± 22.48$^{@}$
EtOH+Cerulein+Galangin	15.80 ± 3.82$^{@}$	212.28 ± 14.86*

Values are expressed as mean ± SD for 6 animals in each group. Control Vs Galangin, control Vs EtOH+Cerulein, EtOH+Cerulein Vs EtOH+Cerulein+Galangin

* P < 0.001, @ P < 0.002, NS- Non-significant

Table 4: Levels of caspase-1 in serum and pancreas of experimental animals

Groups	Serum Pg/ml	Pancreas p mole/min/mg ptn
Control	9.9 ± 1.76	10.6 ± 0.95
Galangin Control	8.7 ± 1.16 ^{NS}	12.53 ± 1.38 ^{NS}
EtOH+Cerulein	15.58± 2.76*	14.13 ±1.18*
EtOH+Cerulein+Galangin	10.55 ±1.09$^{@}$	11.21 ± 0.98$^{@}$

Values are expressed as mean ± SD for 6 animals in each group. Control Vs Galangin, control Vs EtOH+Cerulein, EtOH+Cerulein Vs EtOH+Cerulein+Galangin

* P < 0.001, @ P < 0.002, NS- Non-significant

Effect of galangin on caspase-1 and cytokine activities

Table 3 and 4 shows the level of serum IL-1β, IL-18 and caspase-1 in serum and pancreas of experimental animals. In EtOH and Cerulein fed rats (group 3) the level of IL-1β, IL-18 was found to be increased in serum. Also the levels of serum and pancreatic caspase-1 was increased in EtOH and Cerulein fed rats Rats co-administered with galangin(group 4) showed a significant decrease in the levels of IL-1β and IL-18 and a well pronounced decrease in the levels of caspase-1 in serum and pancreas was observed in rats that received galangin.

Table 5: Levels of TBARS in plasma of experimental animals

Groups	TBARS in plasma nM/ml
Control	0.22 ± 0.03
Galangin Control	0.22 ± 0.03 ^{NS}
EtOH+Cerulein	0.49 ± 0.06*
EtOH+Cerulein+Galangin	0.40 ± 0.05$^{@}$

Values are expressed as mean ± SD for 6 animals in each group. Control Vs Galangin, control Vs EtOH+Cerulein, EtOH+Cerulein Vs EtOH+Cerulein+Galangin

* P < 0.001, @ P < 0.05, NS- Non-significant

Effect of galangin on TBARS in plasma

Table 5 shows the levels of TBARS in plasma of experimental animals. In EtOH and Cerulein fed rats (group 3) the level of TBARS was found to be increased in plasma. Rats co-administered with galangin (group 4) showed a significant decrease in the level of TBARS in plasma.

Table 6: Levels of antioxidant enzymes in experimental animals

Groups	GPx (nM GSH oxidized min/mg ptn)	SOD (Units/mg ptn)	CAT(µmol H_2O_2 consumed /min/mg ptn
Control	3.23 ± 0.74	15.76± 0.90	137.7 ± 9.66
Galangin Control	4.11 ± 0.87 ^{NS}	15.76± 1.36 ^{NS}	132.18± 11.96 ^{NS}
EtOH+Cerulein	1.85 ± 0.45$^{\$}$	9.00 ± 0.93*	99.68 ± 11.53*
EtOH+Cerulein+Galangin	2.99 ±0.60$^{\$}$	11.51 ± 0.57$^{@}$	121.96 ±7.13$^{\#}$

Values are expressed as mean ± SD for 6 animals in each group. Control Vs Galangin, control Vs EtOH+Cerulein, EtOH+Cerulein Vs EtOH+Cerulein+Galangin

* P < 0.001, @ P = 0.002, # P < 0.01, $ P < 0.05 , NS- Non-significant

Effect of galangin on antioxidants in pancreas

The activities of SOD, CAT and GPx in the pancreas of control and EtOH-Cerulein received rats with/without galangin administration are presented in table 6. EtOH and Cerulein received rats showed significantly low level of enzymatic antioxidants in pancreas. Galangin co-administration in group 4 rats was found to maintain the level of antioxidants

DISCUSSION

Medicinal plants have been consumed for its rich polyflavonoid content. Most of the beneficial health effects of flavonoids are mainly due to its antioxidant properties. Galangin is a flavonol found in honey, *Alpinia officinarum, Helichrysum aureonitens* and in propolis. It showed anti-mutagenic, anti-clastogenic, anti-oxidative, radical scavenging, metabolic enzyme modulating activity and effective against certain types of cancers[28].

Flavonoids are polyphenolic compounds that are present in any medicinal plant accounting for their therapeutic potential. 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[29]. Galangin, a flavonoid derivative has shown protective effects on pancreas when the organ is inflammed by EtOH- Cerulein administration.

In the present study, galangin has shown to reduce the serum lipase and amylase activity. EtOH- Cerulein group of animals have shown increased level of lipase and amylase due to cell damage in pancreas. Galangin administration is found to reduce the cell damage as well as the release of enzymes into blood circulation. The functional efficiency of pancreas is determined by measuring the Serum amylase and lipase . These enzymes are synthesized by pancreatic cells which when subjected to injury release the enzymes to blood circulation and elevating the enzyme level in serum[30,31]. Galangin supplemented rats showed reduced activity of the enzymes in serum showing the protective activity on the pancreas.

In response to cell injury, the inflammatory cytokines namely interleukins are produced to alleviate the cellular damage, but when they are produced in excess they can even damage the normal cell to promote tissue injury. In a variety of diseases including cancer, infectious diseases and myocarditis, interleukin 1β known as pro-inflammatory cytokines is expressed at the site of chronic inflammation[32,33]. IL-18 plays an important role in the pathogenesis of inflammatory diseases such as atopic dermatitis, rheumatoid arthritis (RA), adult-onset Still's disease, Sjogren's syndrome, and inflammatory bowel diseases including Crohn's disease[34]. We could find significant increase in the serum level of IL- 1β and IL-18 in EtOH - Cerulein fed rats when compared to control rats. Galangin supplementation is found to reduce the level of interleukins significantly in group 4 rats. A significant reduction in the serum concentration of IL- 1β and IL-18 shows potent anti-inflammatory nature of Galangin.

Caspase-1/interleukin-1 converting enzyme is an enzyme that proteolytically cleaves other proteins, such as the precursor forms of the inflammatory cytokines interleukin 1-β and interleukin 18, into active mature peptides[35,36]. It belongs to a family of cysteine proteases known as caspases that always cleave proteins following an aspartic acid residue[37]. Caspase-1 is produced as a zymogen that is cleaved into 20 kDa (p20) and 10 kDa (p10) subunits that become part of the active enzyme. Active caspase 1 contains two heterodimers of p20 and p10. It interacts with another CARD domain containing protein called PYCARD (or ASC) and is involved in inflammasome formation and activation of inflammatory processes[38].

Caspase 1 has been shown to induce cell necrosis or pyroptosis and may function in various developmental stages. Studies of a similar protein in mouse suggest a role in the pathogenesis of Huntington's disease[39]. The relationship between active IL-1β and caspase-1 suggests that caspase-1 promotes osteoarthritic tissues progression by activating this proinflammatory cytokine[40]. Caspase-1 activity was found to be significantly increased both in serum and pancreas of group 3 rats indicating the role of caspase-1 in inflammatory changes. An observed decrease in the activity of caspase-1 in galangin administered rats could be due to the effect of galangin on an oligomeric protein inflammasome analogous with apoptosome.

Galangin was found to minimize the level of TBARS formation and peroxides both in serum and pancreas. Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and peroxynitrite are involved in promoting inflammation in cells[41].The deleterious effect of these radical acts as stimuli for pro inflammatory cytokines production[42]. Enzymatic antioxidants and non-enzymatic antioxidants scavenge free radicals produced by ROS and protect organ from ROS induced oxidative stress[43]. EtOH- cerulein feeding was found to enhance the free radicals formation which might be accounted for the inflammation and the high level of IL-1β and IL-18 in blood. This effect shows that galangin ameliorates inflammation probably by reducing the free radical formation.

Galangin exhibits a strong inhibitory effect on the cvclo-oxvgenase family of enzymes. This provides a strong cardioprotective effect by inhibiting the aggregation of platelets, and providing a consistent systemic anti-inflammatory effect. Galangin can prevent heart disease and has anti-oxidative effect on endothelial tissues. Therefore, it helps preserve other protective antioxidants such as vitamin E, vitamin C, and other flavonoids and also can prevent lipid peroxidation[44].

Results from in vitro and in vivo studies indicate that galangin with anti-oxidative and free radical scavenging activities is capable of modulating enzyme activities and suppressing the genotoxicity of chemicals[45]. Our results are in accordance with the other reports which showed that galangin scavenge free radicals and also play an important role in maintaining endogenous antioxidant status invivo.

The present study showed that galangin supplementation along with EtOH-cerulein markedly reduces the pancreatic damage and provides protection to the pancreas.

CONCLUSION

The beneficial pancreato protective nature of galangin has been demonstrated experimentally in the present study. The abnormal changes induced by simultaneous administration of EtOH- cerulein in rat pancreas is modulated by galangin through its action, by minimizing amylase and lipase release from pancreas into circulation, cytokine production and also by maintaining the antioxidant status in the glandular organ. However, to understand and to validate the mechanism of action further study on the effect of galangin on the expression of cytokines and caspase -1 and their regulation by inflammasomes is warranted.

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