ABSTRACT

Objective: The present study determined ameliorating effect of limonene on l-arginine induced acute pancreatitis model. Materials and methods: Acute pancreatitis was induced in five groups of rats (n = 6) by L-arginine (2 × 2.5 g/kg, intraperitoneal, 1 h apart) and 1 h later, they received a single oral dose of limonene (100 and 200 mg/kg), methylprednisolone (30 mg/kg) and vehicle (3% Tween 80). A saline (0.9% NaCl) treated group served as a normal control. The effectiveness of limonene was determined at 24 h by analyzing the level of lipase, amylase and proinflammatory cytokines including tumor necrosis factor (TNF)-α interleukin (IL)-6, C-reactive protein (CRP), lipid peroxidation (thiobarbituric acid reactive substances (TBARS), pancreatic myeloperoxidase (MPO) activity, nitrate/nitrite levels, glutathione (GSH), malonyl dialdehyde (MDA and SOD-superoxide dismutase Results: It was noted that limonene and methylprednisolone treatments significantly (P < 0.05) ameliorate the L-arginine-induced increases in pancreatic wet weight/body weight ratio and decreased the serum levels of amylase and lipase, and TNF-α, IL-6 and CRP as compared to the vehicle control. Also, pancreatic levels of MPO activity, TBARS, and nitrate/nitrite were significantly lower. Histological findings confirmed the amelioration of pancreatic injury by limonene Conclusion: limonene has the potential to heal acute pancreatitis by acting as an anti-inflammatory and antioxidant agent.

Keywords: Antioxidant, C-reactive proteins, Limonene, L-arginine, Myeloperoxidase.
INTRODUCTION
Pancreas is an organ located in the midline of the back in the abdominal cavity and inflammation of this gland is called as pancreatitis. The inflammation may be because of the use of heavy intake of alcohol, drugs like steroids, thiazides, furosemide, sulfonamides, infections like mumps and many other causes. In the previous studies the total mortality rate is reported as 20-30%. 1 Limonene is a cyclic terpene and majorly found in citrus fruits including mandarin, orange, grapefruit, lime, and lemon. Due to its pleasant fragrance, it is widely used as a pharmaceutical aid in beverages, soaps, perfumes, chewing gum and foods. 2 It was reported that limonene has toxic effect (nephrotoxic risk, carcinogenic and mutagenic) on rats and mice but subsequently it was proved to be non-toxic to humans. 3 The acute toxicity (LD50’s) is reported for d-limonene is of approximately 5000 mg/kg b.w. in the rat and 6000 mg/kg b.w. in the mouse. 4 It was reported to have good solvent for cholesterol containing gallstones, neutralization of gastric acid which supports normal peristalsis, heartburn and as a chemoprotective effect. 2 and 5 Though we have various pre-clinical experimental models are available to analyze pancreatitis but necrotizing pancreatitis has developed with administration of high dose of L-arginine by Mizunuma et al, 1984 and most used in the preclinical studies because of its reproducibility. 6 Presently we have various synthetic and semi-synthetic to treat acute pancreatitis but those have various kind of adverse reaction and they are relatively costly. Hence now we are in need of relatively reliable and readily available drug, which have fewer side effects. In the present study the use if d-limonene was because the earlier report have suggested that this active constituent have potential to produces renal tubular tumors in male rats by non-DNA reactive α2u-globulin-associated responses that were not relevant to humans. 7 Thus in this study the non-toxic dose of limonene (100 and 200 mg/kg) have been used to analyse the potential effect to ameliorate pancreatic injury induced by L-arginine.

MATERIALS AND METHODS
Animals: Thirty male Wister rats of 180-200 g weight were obtained Mahaveer Enterprises, Hyderabad was maintained at a constant room temperature (23±2 °C) with 12:12 h light-dark cycles and free access to water and standard laboratory chow. These rats were divided in five groups of six rats in each group and experiments were performed after 12 h of fasting. The experiment was conducted according to in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals and guidelines. This study was approved (320/CPCSEA dated 03-01-2001), GP college of pharmacy, Hyderabad, India
Chemicals: L-arginine, hexadecyltrimethylammonium bromide (HETAB), o-dianisidinedihydrochloride, thiobarbituric acid (TBA), Griess reagent and vanadium trichloride were procured from Sigma Aldrich Chemical Co and limonene (Ayur Shanbagh, Bangalore Karnataka). All other chemicals and reagents were of highest commercial grade available locally.

**L-arginine powder:** prepared as a solution by dissolving in 0.9% saline to a final concentration of 500 mg/mL and the pH was adjusted to 7 with 5 N HCl. Limonene was prepared as a solution by dissolving in 3% tween 80 and 0.9% saline to a final concentration of 100 and 200 mg/mL and the pH was adjusted to 7 with 0.1 N NaOH.

**L-arginine-induced pancreatitis model:** Acute pancreatitis was induced in five groups of rats by two intraperitoneal (ip) injections of L-arginine (2.5 g/kg, 1 h apart). One hour following the last injection of L-arginine, the rats were treated orally as follows: Gr. 1 received the vehicle (3% Tween 80) of limonene and serve as disease control; Grs 2 and 3 were treated with limonene (100 and 200 mg/kg, respectively). Gr. 4 acted as positive control and received methylprednisolone (30 mg/kg), all in a volume of 10 mL/kg and Gr. 5, received saline (0.9%, NaCl, ip) in place of L-arginine and served as a normal control. After 24 h of the last injection of L-arginine or saline, a midline laparotomy was performed in rats according to the guidelines. Further the blood samples were collected from the inferior vena cava, the rats were then exsanguinated, and the whole pancreas was quickly removed and stored at -70 °C until use. The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema (mg/g).

**Macroscopic evaluation**

**Pancreas weight/body weight ratio:** The pancreas was removed immediately after the blood collection, trimmed free of fat and weighed. The pancreatic weight/body weight ratio (mg/g) was calculated for each animal, to estimate the level of pancreatic edema.

**Serum analysis:** For serum analysis, blood samples were centrifuged at 3000 g at 4 °C for 10 min. The serum amylase and lipase were determined by routine colorimetric methods using the commercial kits for amylase (Rapid diagnostics), lipase (Accurex diagnostics), C-reactive protein and interleukin-α and expressed as U/dl.
Biochemical estimations: Pancreatic total protein content was determined. Pancreatic, kidney, liver and lung (GSH, MDA, MPO, SOD, nitrate/nitrite level, TBARS level and catalase activity) and reduced GSH level were measured. The IL-6, TNF-alpha, CRP were measured according to standards kits.

Histopathological evaluation: Pancreas was removed immediately and part of it was fixed in 10% neutral buffered formalin and embedded in paraffin by standard methods. Paraffin sections of 5 µm thicknesses were cut and stained with haematoxylin and eosin, assessed under dark field microscope and examined blind by a morphologist for grading histopathological changes. Pancreatic damage was assessed and scored by grading acinar cell degeneration, interstitial inflammation, edema, and haemorrhage as described by schmidt’s standards with modification as follows: Grading for edema was scaled as 0: absent or rare; 1: edema in the interlobular space; 2: edema in the intralobular space; 3: isolated island shape of pancreatic acinus. Inflammation was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Acinar cell necrosis was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Parenchyma haemorrhage was scored as 0: absent; 1: mild; 2: moderate; 3: severe. The maximum score for acinar cell damage was 12.

Statistical analysis: Statistical analysis was performed by one way ANOVA followed by Newman Keuls as post-hoc test using Graph pad Prism 5. Values were presented as mean ± SE. The difference was considered to be statistically significant when P < 0.05.

RESULTS

Serum biochemical parameters and pancreatic edema: Induction of pancreatitis resulted in significant raise in the serum amylase, lipase and pancreatic edema. Treatment with limonene (100 and 200 mg/kg) dose dependently decreased serum amylase, lipase and pancreatic edema (Table 1). Pancreatic MPO and total protein-Induction of pancreatitis resulted in significantly increased the pancreatic MPO and decreased pancreatic total protein levels. Treatment with limonene (100 and 200 mg/kg) dose dependently reversed the change in pancreatic MPO and total protein levels (Table 1).

Pancreatic, lung, liver and kidney MDA, nitrate/nitrite, GSH and antioxidant enzymes catalase and SOD: Induction of pancreatitis resulted in a significant raise in MDA, nitrate/nitrite, catalase and SOD and decline in GSH levels. Treatment with limonene (100
and 200 mg/kg) dose dependently reversed the change in MDA, nitrate/nitrite, catalase, SOD and GSH levels (Table 1).

**Assessment of interleukins and C-reactive protein:** Induction of pancreatitis resulted in a significant raise in interleukins, TNF-α and C-reactive protein. Treatment with limonene (100 and 200 mg/kg) dose dependently decreased interleukins and C-reactive protein (Table 2).

**Pancreatic histology:** Histological examination of normal control group (saline treated) showed normal architecture and absence of edema, neutrophil infiltration, hemorrhage and necrosis (Fig 1). Whereas, pancreatic sections of disease control group showed extensive tissue damage characterized by acinar cell degeneration, necrosis, edema, mononuclear cell infiltration, hemorrhage and thus received significantly higher scores. Treatment with limonene (100 and 200 mg/kg) and methyl prednisolone (30 mg/kg) ameliorated the inflammation, edema and more significantly acinar cell degeneration and necrosis and protected the pancreas from L-arginine induced damage. Treatment with limonene dose dependently decreased total pathological scores compared to disease control group.

**Table 1** Effect of limonene on pancreas weight, total body weight, serum amylase, serum lipase, total nitrate, total protein, MDA, MPO and SOD after L-arginine induced acute pancreatitis.

<table>
<thead>
<tr>
<th>Parameter/Groups</th>
<th>N.C</th>
<th>D.C</th>
<th>STD</th>
<th>LI 100</th>
<th>LI200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas weight</td>
<td>870.3±15.3</td>
<td>1015±19.0*</td>
<td>911.3±18.8α</td>
<td>843.3±46.3α</td>
<td>788.3±21.3α</td>
</tr>
<tr>
<td>Total body wt</td>
<td>187.5±4.8</td>
<td>192±3.5*</td>
<td>191.2± 3.6</td>
<td>188.7 ± 5.5</td>
<td>189 ± 3.6</td>
</tr>
<tr>
<td>Pancreatic index (x10-3)</td>
<td>4.652±1.2</td>
<td>5.286±1.5</td>
<td>4.769±1.3</td>
<td>4.48 ±1.6</td>
<td>4.169 ±2.2</td>
</tr>
<tr>
<td>Serum Amylase</td>
<td>2000±85.6</td>
<td>7667±34*</td>
<td>3317±110.8α</td>
<td>2600±46a</td>
<td>2967±388.2a</td>
</tr>
<tr>
<td>Serum Lipase</td>
<td>191.7±4.0</td>
<td>566.7±30.8*</td>
<td>346.7±39.2α</td>
<td>226.7±24.2a</td>
<td>260±25.3a</td>
</tr>
<tr>
<td>Total Nitrate</td>
<td>11.87±1.3</td>
<td>16.07±1.4*</td>
<td>7.06±0.3α</td>
<td>0.53±0.8a</td>
<td>0.35±0.8a</td>
</tr>
<tr>
<td>Total Protein</td>
<td>0.73±0.0</td>
<td>0.355±0.0*</td>
<td>0.91±0.0α</td>
<td>0.68±0.07a</td>
<td>0.88±0.07a</td>
</tr>
<tr>
<td>Kidney GSH</td>
<td>0.47±0.0</td>
<td>0.284±0.0*</td>
<td>0.71±0.0α</td>
<td>0.14±0.05c</td>
<td>0.13±0.04</td>
</tr>
<tr>
<td>Liver GSH</td>
<td>0.48±0.0</td>
<td>0.284±0.0*</td>
<td>0.67±0.0α</td>
<td>0.12±0.04</td>
<td>0.13±0.0</td>
</tr>
<tr>
<td>Lung GSH</td>
<td>0.50±0.0</td>
<td>0.284±0.0*</td>
<td>0.67±0.0α</td>
<td>0.10±0.04b</td>
<td>0.09±0.0a</td>
</tr>
<tr>
<td>Pancreas GSH</td>
<td>0.48±0.0</td>
<td>0.284±0.0*</td>
<td>0.67±0.0α</td>
<td>0.12±0.04</td>
<td>0.13±0.0</td>
</tr>
<tr>
<td>Kidney MDA</td>
<td>105.6±13.6</td>
<td>170.9±10.4*</td>
<td>135.2±16.4β</td>
<td>117.3±16.6a</td>
<td>132.3±15.4b</td>
</tr>
<tr>
<td>Liver MDA</td>
<td>105.6±13.6</td>
<td>170.9±10.4*</td>
<td>135.2±16.4γ</td>
<td>119.3±12.5a</td>
<td>129.1±25.1b</td>
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<tr>
<td>Lung MDA</td>
<td>14.95±0.6</td>
<td>17.09±1.0</td>
<td>9.82±1.0β</td>
<td>15.28±1.3b</td>
<td>15±1.1a</td>
</tr>
<tr>
<td>Pancreases MDA</td>
<td>1.62±0.66</td>
<td>1.04±0.42*</td>
<td>2.39±0.9α</td>
<td>1.664±0.6a</td>
<td>1.542±0.6c</td>
</tr>
<tr>
<td>Pancreas MPO</td>
<td>4.75 ± 2.1</td>
<td>32.8 ± 4.7*</td>
<td>6.2 ± 2.2α</td>
<td>24.2 ± 3.7α</td>
<td>17.3 ± 3.7α</td>
</tr>
<tr>
<td>Lung MPO</td>
<td>7.7±0.94</td>
<td>38.92±2.2*</td>
<td>10.62±1.5α</td>
<td>20.15±2.2α</td>
<td>12.05±2.0α</td>
</tr>
<tr>
<td>Liver MPO</td>
<td>7.883±0.54</td>
<td>31.73±2.2*</td>
<td>11.6±1.9α</td>
<td>24.5±2.8ab</td>
<td>15.68±2.7aa</td>
</tr>
<tr>
<td>Kidney MPO</td>
<td>7.883±0.54</td>
<td>32.52±4.0*</td>
<td>6.953±1.0α</td>
<td>25.51±3.1aa</td>
<td>15.58±2.2αa</td>
</tr>
<tr>
<td>Pancreas Catalase</td>
<td>0.33±0.13</td>
<td>0.41±0.17*</td>
<td>0.19±0.0</td>
<td>0.2481±0.1b</td>
<td>0.1218±0.0b</td>
</tr>
<tr>
<td>kidney SOD</td>
<td>2.33 ±0.95</td>
<td>1.50±0.61</td>
<td>2.61±1.0βα</td>
<td>0.975±0.3</td>
<td>1.956±0.7g</td>
</tr>
<tr>
<td>Liver SOD</td>
<td>0.83±0.34</td>
<td>1.01±0.41</td>
<td>1.97±0.8α</td>
<td>1.835±0.7a</td>
<td>1.933±0.7b</td>
</tr>
<tr>
<td>Lung SOD</td>
<td>1.04±0.42</td>
<td>1.03±0.42*</td>
<td>3.44±1.4α</td>
<td>0.8462±0.3</td>
<td>2.152±0.8c</td>
</tr>
<tr>
<td>Pancreases SOD</td>
<td>2.09±0.85</td>
<td>1.51±0.61*</td>
<td>1.49±0.6α</td>
<td>2.757±1.1b</td>
<td>1.473±0.6</td>
</tr>
</tbody>
</table>

N.C- Normal Control, D.C- Disease Control, LI 100: limonene 100 and LI 200: limonene 200 mg/kg. GSH- glutathione, MDA- malonyl dialdehyde, MPO- myeloperoxidase and SOD-superoxide dismutase. *p < 0.0001 when compared with normal control, αp< 0.0001, βp< 0.001, γp< 0.01 when compared with disease control group. LI 100: limonene 100 and LI 200: limonene 200 mg/kg. [Values are mean ± SEM from 6 animals in each group]

**Table: 2 Effect of limonene on IL-6, TNF-α and CRP after L-arginine induced acute pancreatitis.**

<table>
<thead>
<tr>
<th>Parameter/ Groups</th>
<th>NC</th>
<th>DC</th>
<th>LI1</th>
<th>LI2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>29.3±1.5</td>
<td>90.48±1.6*</td>
<td>56±2.6</td>
<td>34.2±2.0γ</td>
</tr>
<tr>
<td>TNF-α</td>
<td>19.33±1.5</td>
<td>26.32±3.0</td>
<td>21.3±4.4</td>
<td>17.03±1.5</td>
</tr>
<tr>
<td>CRP</td>
<td>415.3±7.7</td>
<td>16403±119*</td>
<td>7381±104.7</td>
<td>500±11.1γ</td>
</tr>
</tbody>
</table>

N.C- Normal Control, D.C- Disease Control, LI 100: limonene 100 and LI 200: limonene 200, IL-6-interleukin-6, TNF-α- Tumor necrosis factor-alpha and CRP-C-reactive protein. mg/kg.

*P < 0.0001 when compared with normal control, γP< 0.01 when compared with disease control group.
**Histopathology:** Fig. 1-Effect of d-limonene on pancreatic histopathological changes after L-arginine induced acute pancreatitis [(A) normal control, (B) standard control, (C) disease control 24 h hemorrhage (D) disease control 24 h edema and necrosis, (E) limonene 100 mg/kg, ip, 24, (F) limonene 200 mg/kg, ip, 24 h] (H&E ×200)

A. Normal Control

B. Standard Control

C. Disease control 24 hrs hemorrhage

D. Disease control 24 hrs Oedema & necrosis

E. Limonene 100 mg/ kg, ip, 24 hrs

F. Limonene 200 mg/ kg, ip, 24 hrs

**DISCUSSION**

By the present experimental model it was noticed that d-limonene (200 mg/kg) significantly attenuated pancreatitis in L-arginine induced acute pancreatitis model. In consistent with previous reports d-limonene has significantly reduced the raised level of acinar cell necrosis,
serum amylase and lipase. 1, 18 and 19. Amylase and lipase are called as important marker in diagnosis of acute pancreatitis, in the present study it was noticed that the raised level to peak at 24 hours were significantly reduced by treatment with limonene (200 mg/kg). From the previous report it is well know that the markers like MPO, MDA, nitrite, catalase and SOD, which are increased at the time of acute pancreatitis. 5 Staying on the same conclusion of previous reports of d-limonene decreased level of neutrophil infiltration, SOD, nitrite, catalase, MPO and MDA. 19 Staying on the same conclusion of previous reports induction of pancreatitis with L-Arginine increased the pancreatic MPO levels. 20, 21 It was noted that the neutrophil infiltration can attenuate the pancreatic injury. 22 It was noted that therapy with d-limonene significantly lowers pancreatic MPO levels probably due to its anti-inflammatory action. The earlier reports have stated that the lipid peroxidation marker is elevated in L-arginine treated rats. It was stated that the lipid peroxidation is a process mediated by free radicals and this may results in impairment of the membrane functional and structural integrity resulting in oxidative deterioration of polyunsaturated fatty acids of cell membrane. 23,24 It could be attributed to the accumulation of free radicals proposed to be generated by L-arginine. It was stated that the catalase and SOD level being changed and the story remains controversial. The same was reported as fall in the enzyme level at 24 hours. The change in levels of catalase and SOD remains controversial. Czako and Takacs reported the fall in these enzyme levels at 24 h. 25 But one of the earlier report has suggested that raised levels of these enzymes. 26 Earlier reports suggested by Robbins, the present study have showed significant increase in catalase and SOD level was observed. 1 The present study suggested that oxidative stress caused by L-arginine may up-regulate the activity of antioxidant enzymes to facilitate rapid removal of accumulated reactive oxygen and nitrogen species. 27 The studies have been reported that GSH is found to be decreased in L-arginine treated rats indicating enhanced oxidative stress as the disease progresses. 28 For the earlier studies it has been noticed that nitric oxide role and progression of acute pancreatitis remains unclear. Nitric oxide increases the pancreatic blood flow and/or secretion in response to endothelium derived nitric oxide and ameliorates the pancreatic dysfunction, others suggested that NO aggravates pancreatic oxidative stress and damage. 29, 30,31 Consistent with previous report, the present study has showed significant increase in nitric oxide level and pancreatic edema in in L-arginine received rats. 31 In Takacs et al. has showed that administration of excess L-arginine could induce iNOS activity and increase the NO levels in pancreas. 32 The present study have suggested that increased levels of nitric oxide can increase vascular/micro capillary permeability and may contribute to the pancreatic edema and acinar cell damage. Limonene
has significantly restored the pancreatic GSH, MDA, edema, nitrite, catalase and SOD in L-arginine received rats. In the study of Passaglia, it was suggested that acinar cells are the protein factory of the body. In acute pancreatitis, catabolism of proteins could increase up to 80 present. In the present study it was noticed that sharp decline in protein content was observed in pancreas. Earlier study Sidhu et al, have suggested that pancreatic total protein content, a marker of the tissue damage was found to decrease in L-arginine received rats. Therapy with d-limonene has significantly increased the total protein content. According to previous reports its well knows that the extent of pancreatic tissue damage in acute pancreatitis correlates with the levels of inflammatory mediators and free radicals. In consistent with previous report in the present study the histopathological assessments revealed that induction of pancreatitis resulted in pancreatic damage characterized by haemorrhage, mono nuclear cell infiltration, acinar cell necrosis and edema. Therapy with d-limonene ameliorates the pancreas from L-arginine induced injury. Further it was concluded that, the present study have suggested that d-limonene significantly ameliorated the severity of L-arginine induced pancreatitis by reducing the oxidative stress markers and neutrophil infiltration. In present study this was suggested that this effect is due to anti-inflammatory and antioxidant property.

Conflicts of interest statement
We declare that we have no conflict of interest.

REFERENCE


