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RESEARCH ARTICLE

Protective Effect of Silymarin on L-Arginine Induced Acute Pancreatitis in Rats

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ABSTRACT

Acute pancreatitis is an inflammatory disorder of the exocrine part of pancreas, which can lead to a systemic inflammatory response syndrome with significant morbidity and mortality in 20% of patients. Involvement of oxidative stress and inflammatory mediators are the major causative factors for the development of acute pancreatitis. Previous studies reported that treatment with α,β amyrin, Pentoxifylline , Alpha lipoic acid, N-acetyl cysteine, Eugenol, Allopurinol, Methyl prednisolone, Melatonin & Selenium have shown protective effect on L-Arginine induced acute pancreatitis by virtue of their anti oxidant & anti-inflammatory properties. Based on these reports, it is presumed that Silymarin, a potential antioxidant & anti-inflammatory agent which might exert a beneficial effect on L-Arginine induced acute pancreatitis in rats. Inflammation of pancreatic gland called pancreatitis (AP) may leads to sever complication and high mortality without treatment. The pathogenesis is not fully understood, however the leukocyte activation, microcirculatory disturbances and oxidative stress are the major constituents of AP. This is characterized by activation of widespread inflammatory cell infiltration, leukocyte and digestive proteases. Reactive oxygen, nitrogen species and various kinds of inflammatory mediators are released in inflammatory process. Previously it was reported that several factors are responsible for the AP, like alcohol, gallstones, hereditary pancreatitis, hypercalcemia, hyperlipidemia, malnutrition, abdominal trauma, penetrating ulcers, malignancy, drugs like steroids, sulfonamides, furosemide, thiazides, infections like mumps, Coxsackie virus, Mycoplasma pneumonia.

KEYWORDS

Acute pancreatitis, L-arginine, Silymarin, Oxidative stress, Inflammation

INTRODUCTION

Acute pancreatitis is a sudden inflammation of the exocrine pancreatic tissue associated with high mortality. The disease progression can be viewed as a three-phase continuum: a local inflammatory reaction that can progress to a systemic response and subsequent multi-organ failure[1].

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The pathologic spectrum of acute pancreatitis includes acute oedematous pancreatitis, necrotizing pancreatitis, haemorrhagic pancreatitis, pseudocyst pancreas, pancreatic abscess and pancreatic ascites[2].

The incidence of acute pancreatitis is increasing. In the United States, acute pancreatitis accounts for over 200,000 hospital admissions each year.

In Europe, the incidence ranges from approximately 4 to 45 per 100,000 patients a year. Almost all patients with necrotizing pancreatitis without multi organ failure survive,

where as those with multi organ failure has a median mortality of 47% [3].

Acute pancreatitis has several etiological factors, alcohol and gallstones accounts for 70-80% of cases. Moreover, other etiological factors are anatomic or functional disorders (e.g., pancreas divisum, sphincter of Oddi dysfunction), autoimmune (e.g., systemic lupus erythematosus), choledocholithiasis, congenital anomalies, certain drugs, hypertriglyceridemia, hypercalcemia, hyperparathyroidism, hypothermia, idiopathic, infections (e.g., viral, bacterial, parasitic, fungal), pancreatic or ampullary tumors, traumatic or post procedure (e.g., endoscopic retrograde cholangiopancreatography or after abdominal surgery), vascular (e.g., vasculitis) [4].

Although its pathophysiology is not fully understood, it is mainly characterized by premature activation of digestive enzymes and auto digestion with destruction of acinar cells, enzymatic colocalisation[5], Oxygen and nitrogen derived free radicals[6,] inflammatory cell recruitment[7], microcirculatory disturbances[6] and imbalance between apoptosis and necrosis[8] have been reported to play important role in determining the severity of pancreatitis. Some investigations have led to newer hypothesis, including ischemic or reperfusion injury.

The most common symptoms are severe epigastric pain radiating to the back, nausea, vomiting, diarrhea, loss of appetite, fever, chills, hemodynamic instability which include shock, tachycardia, respiratory distress, peritonitis[9].

Repeated attacks of acute pancreatitis have the potential to develop into chronic pancreatitis or pancreatic cancer characterized by fibrosis and loss of acinar cell function. There are no specific therapies for acute pancreatitis. Conflicting or inconclusive data exist regarding the efficacy of atropine, lexipafant, and low molecular weight dextran, antioxidants such as N- acetyl cystein, indomethacin, interleukin-10 and infliximab[10, 11]. Several studies and meta-analysis that evaluated the efficacy of somatostatin and octreotide suggest a slight trend toward benefit[12]. However the present treatment is mainly aimed at supportive and symptomatic relief. As a result of the limitations of conventional therapy, there is a need to develop novel and safe therapeutic agents to treat acute pancreatitis.

L-Arginine induced pancreatitis is an experimental model of severe necrotizing acute pancreatitis. Depending on the dose and duration of L-Arginine administration different phases of pancreatitis can be studied. This model has advantages like reproducibility and it causes selective, dose dependent acinar cell necrosis[13]. Administration of L-Arginine cause damage not only to pancreas but also to lungs, liver and kidney which is very similar to the human disease which may range from a local inflammatory process to a severe pancreas injury associated with extra pancreatic manifestations, such as circulatory, renal or pulmonary complications[14]. As a model of acute pancreatitis, L-Arginine induce it mainly through oxygen, nitrogen derived free radicals generation and inflammatory mediators[15-18]. So, the antioxidant and/or anti-inflammatory drugs could be useful in the management of acute pancreatitis. Previous studies reported that α,β amyrin[19], Pentoxifylline, Alpha lipoic acid[20], N-acetyl cysteine[21], Allopurinol[22], Methyl prednisolone[19], Melatonin[14,23], Eugenol[24] & Selenium[25] shown protective effect on L-Arginine induced pancreatitis by virtue of their anti oxidant & anti-inflammatory properties.

Based on these reports, it is presumed that Silymarin[26-40], a potent antioxidant & anti-inflammatory agent might exert a beneficial effect on the outcome of severity of L-Arginine induced pancreatitis. Silymarin, a naturally occurring flavonolignan compound is a major component of seeds and leaves of Milk thistle plant. Previous studies reported that silymarin possess hepatoprotective, nephroprotective, cardioprotective, antiarthritic, immunomodulating, membrane stabilizing, anticancer, antioxidant, and anti inflammatory properties.

However, no study has reported the protective effect of Silymarin on L-Arginine induced acute pancreatitis in rats.

MATERIALS AND METHOD

Chemicals

L-Arginine, Silymarin, hexadecyltrimethylammonium bromide (HETAB), o-dianisidine dihydrochloride, thiobarbituric acid, griess reagent and vanadium trichloride were purchased from Sigma-Aldrich chemical co. All other chemicals and reagents were the highest commercial grade available. All the biochemical parameters were measured by using commercially available diagnostic kits.

Drug

Silymarin, a naturally occurring flavonolignan compound is a major component of seeds and leaves of Milk thistle plant. Previous studies reported that silymarin possess hepatoprotective, nephro protective, Cardioprotective, antiarthritic, immune-modulating, membrane stabilizing, anticancer, antioxidant, and anti inflammatory properties.

Animals

Sixty male wistar rats (190-210g) were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. They were housed individually in an environmentally controlled room with 12-h light/dark cycle and had free access to food and water. After a 7- day acclimatization period, they were randomly selected for different experimental groups. All the experimental procedures were carried out in accordance with committee for the purpose of control and supervision of experiments on animal (CPCSEA) guidelines. The study was reviewed and approved by the Institutional Animal Ethics Committee (320/CPCSEA dated 03-01-2001), G. Pulla Reddy College of Pharmacy, Hyderabad, India.

Induction of Experimental Acute Pancreatitis in Rats

Wistar rats weighing about 190-210 g were used. Acute pancreatitis was induced by non invasive L-Arginine model. L-Arginine was dissolved in 0.9% saline and the pH was adjusted to 7 with 5 N HCl and administered in a dose of 2.5 g/kg body weight [13], two intraperitoneal injections with an interval of 1 hr.

Experimental Study Design

Sixty male wistar rats were divided into five groups (n = 12) and they received following treatment

- Group I (N.C): Received saline per orally.
- Group II (D.C): Received two intraperitoneal injections of L-Arginine (2.5 g/kg, 1 hr apart) and vechicle (p.o.).
- Group III (Std): Received Methyl prednisolone 30 mg/kg/day, p.o. 1 hr after the last injection of L-Arginine.
- Group IV (S_{150}): Received Silymarin 150 mg/kg/day, p.o. 1 hr after the last injection of L- arginine.
- Group V (S_{300}): Received Silymarin 300 mg/kg/day, p.o. 1 hr after the last injection of L- arginine.

In each group half of the animals were sacrificed at 24 hours (study I) and remaining half were sacrificed at 72 hours (study II) after the last injection of L-Arginine. The treatment schedule for study I and II were shown in the figure 9. At the end of the experimental period, animals were anaesthetized with anesthetic ether and blood samples were collected from retro-orbital plexus for estimation of serum amylase and lipase and finally the animals were sacrificed and isolated the whole pancreas, lungs, liver and kidneys for the estimation of bio chemical parameters and histological examination.

Pancreas Wt/body Wt Determination

The pancreas was removed immediately after the blood collection, trimmed free of fat and weighed. The pancreatic wt/body wt ratio (mg/g) was calculated for each animal, to estimate the level of pancreatic oedema[19].

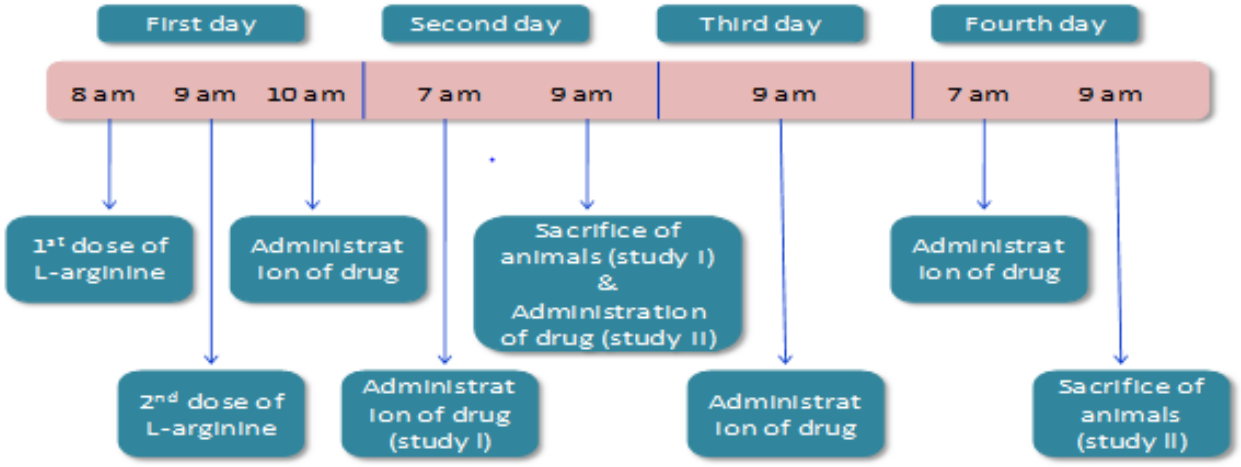


Figure 9: Flow diagram of experimental design

Serum Analysis

For serum analysis, blood samples were centrifuged at 3000 x g at 4°C for 10 minutes[19]. The serum amylase and lipase were determined by routine colorimetric methods using the commercial kits for amylase (Aspen laboratories) and lipase (Accurex diagnostics) and expressed as U/dl.

Estimation of Pancreatic Total Protein

Pancreatic total protein was determined using the commercial kit for protein (Aspen laboratories). Amount of protein in samples was expressed in g/dl[23].

Estimation of Myeloperoxidase Activity

Myeloperoxidase activity is a measure of degree of neutrophil infiltration. It was measured in pancreas, lungs, liver and kidney biopsies using the method described by Bradley et al. (1982)[41].

Estimation of Total Nitrite Content [42-43]

Nitrite estimation was done in accordance to Griess reaction.

Estimation of malondialdehyde/ Thiobarbituric Acid Reactive Substances

TBARS level in tissue is a measure of lipid peroxidation. It was measured in pancreas, lungs, liver and kidneys biopsies using the method described by Ohkawa et al[44].

Measurement of Catalase Activity

Catalase activity of pancreatic tissue was measured by the method of Aebi (1984) [45].

Measurement of SOD Activity

SOD level was measured in pancreas using the method described by Misra et al (1972)[46].

Measurement of Glutathione

Reduced glutathione (GSH) level was measured in pancreas using the method described by Ellman (1959) [47].

Histopathological assessment

Pancreas, lungs, liver and kidneys were removed immediately and part of the tissue is fixed in 10% neutral buffered formalin and embedded in paraffin by standard methods. Paraffin sections of 5 µm were cut and stained with haematoxylin and eosin and then assessed under dark field microscope and examined blind by a morphologist for grading histopathological changes.

Pancreatic damage was assessed & scored by grading acinar cell degeneration, interstitial

inflammation, oedema, and hemorrhage as described by Schmidt's standards[48].

Statistical Analysis

Results are expressed as mean ± SEM. Statistical Analysis was performed by one way ANOVA followed by Newman Keuls as post hoc test using Graph pad Prism5 for comparison of more than two groups. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Serum Amylase, Lipase and Pancreatic Oedema

As shown in Table 1 and 2, Administration of L-Arginine significantly ($p < 0.0001$) increased the serum amylase, lipase and pancreatic oedema compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) dose dependently decreased the serum amylase, lipase and pancreatic oedema compared to disease control group. Similarly treatment with Methyl prednisolone significantly decreased the serum amylase, lipase and pancreatic oedema compared to disease control group. Whereas, Silymarin (300 mg/kg) restored the L-Arginine evoked increase in serum amylase, lipase and pancreatic oedema.

Pancreatic MPO and total protein

As shown in Table 3 and 4, administration of L-arginine significantly ($p < 0.0001$) increased the pancreatic MPO and decreased the pancreatic total protein levels compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) dose dependently decreased the pancreatic MPO level and increased pancreatic total protein compared to disease control group. Similarly treatment with Methyl prednisolone significantly increased the pancreatic total protein compared to disease control group and restored the pancreatic MPO levels. Whereas, administration of Silymarin (300 mg/kg) restored the L-Arginine evoked decrease in pancreatic total protein and increase in pancreatic MPO.

Pancreatic MDA, Total Nitrite, GSH and Antioxidant Enzymes (catalase and SOD)

As shown in Table 5,6,7,8 and 9 induction of pancreatitis in rats lead to significant ($p < 0.0001$) increase in MDA, total nitrite, and decrease in GSH, catalase and SOD levels compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) dose dependently decreased the MDA, total nitrite and increased the GSH, catalase and SOD levels compared to disease control group. Similarly treatment with Methyl prednisolone significantly decreased MDA, increased the GSH and catalase compared to disease control group and restored the change in total nitrite and SOD. Whereas, administration of Silymarin (300 mg/kg) significantly decreased the L-Arginine evoked increase in pancreatic MDA, significantly increased the GSH levels compared to disease control group and restored pancreatic total nitrite, catalase and SOD levels.

Lung MDA and MPO

As shown in Table 10 and 11, induction of pancreatitis resulted in Significant ($P < 0.0001$) Rise in Lung MDA and MPO levels compared to normal control group. Administration of Silymarin (150 and 300 mg/kg) dose dependently decreased the lung MDA and MPO levels compared to disease control group. Similarly treatment with Methyl prednisolone and Silymarin (300 mg/kg) significantly decreased the lung MPO compared to disease control group and restored the L-Arginine evoked rise in lung MDA level.

Liver MDA and MPO

As shown in Table 12 and 13, induction of pancreatitis resulted in significant ($p < 0.0001$) rise in liver MDA and MPO levels compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) dose dependently decreased the liver MDA and MPO levels compared to disease control group. Similarly treatment with Methyl prednisolone significantly decreased the liver MPO compared to disease control group and restored the liver MDA. Whereas, administration of Silymarin

(300 mg/kg) restored the L-Arginine evoked rise in liver MPO and MDA levels.

Kidney MDA and MPO

As shown in Table 14 and 15, induction of pancreatitis resulted in significant ($p < 0.0001$) increase in kidney MDA and MPO levels compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) dose dependently decreased the kidney MDA and MPO levels compared to disease control group. Similarly treatment with Methyl prednisolone significantly decreased the kidney MDA compared to disease control group and restored the kidney MPO. Whereas, administration of Silymarin (300 mg/kg) restored the L-Arginine evoked rise in kidney MPO and MDA levels.

Pancreatic Histology

As shown in figure 1, histological examination of normal control group (saline treated) showed normal architecture and absence of oedema, neutrophil infiltration, hemorrhage and necrosis. Pancreatic sections of disease control group showed extensive tissue damage characterized by acinar cell degeneration, necrosis, oedema, mononuclear cell infiltration, haemorrhage. Treatment with Silymarin (150 and 300 mg/kg) dose dependently ameliorated the inflammation, oedema and more prominently acinar cell degeneration and necrosis and protected the pancreas from L-Arginine induced damage. Treatment with Methyl prednisolone (30 mg/kg) and Silymarin (300 mg/kg) significantly attenuated the L-Arginine evoked increase in oedema, neutrophil infiltration, haemorrhage and necrosis compared to disease control group.

Histology of Lungs

As shown figure 2, induction of pancreatitis resulted in significant lung injury characterized by alveolar rupture, oedema, haemorrhage and mononuclear cell infiltration when compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) dose dependently ameliorated the alveolar rupture, inflammation, oedema and haemorrhage and protected the lungs from L-Arginine induced damage. Methyl prednisolone (30 mg/kg) and Silymarin (300 mg/kg) significantly received animals showed a dramatic reduction in these features and significantly lower pulmonary injury score compared to disease control.

Histology of Liver

As shown in figure 3, histological examination of liver sections of disease control group confirmed significant liver injury characterized by central vein congestion, sinusoidal congestion, haemorrhage, mononuclear cell infiltration, necrosis and fatty changes when compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) dose dependently ameliorated central vein congestion, sinusoidal congestion, haemorrhage, mononuclear cell infiltration and necrosis and protected the liver from L-Arginine induced damage. Administration of Silymarin (300 mg/kg) and Methyl prednisolone (30 mg/kg) showed a significant reduction in histological evidence of liver injury compared to disease control group.

Histology of Kidney

As shown in figure 4, induction of pancreatitis resulted in significant kidney injury characterized by haemorrhage, mononuclear cell infiltration, tubular damage and glomerular alterations compared to normal control group. Treatment with Silymarin (150 and 300 mg/kg) and HMBA (2.5 and 5 mg/kg) dose dependently ameliorated the haemorrhage, mononuclear cell infiltration, tubular damage and glomerular alterations and protected the kidney from L-arginine induced damage. Silymarin (300 mg/kg) and Methyl prednisolone (30 mg/kg) received animals showed a dramatic reduction in these features and significantly lowered kidney injury score compared to disease control.

Table 1: Effect of Silymarin on serum amylase levels in L – Arginine induced Pancreatic Rats

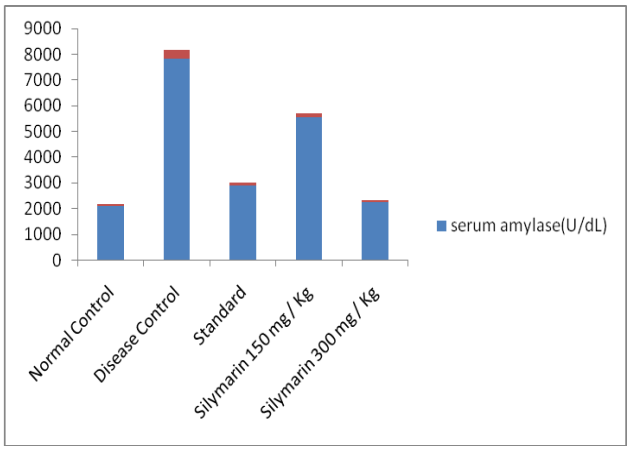


Table 2: Effect of Silymarin on serum lipase levels in L – Arginine induced Pancreatic Rats

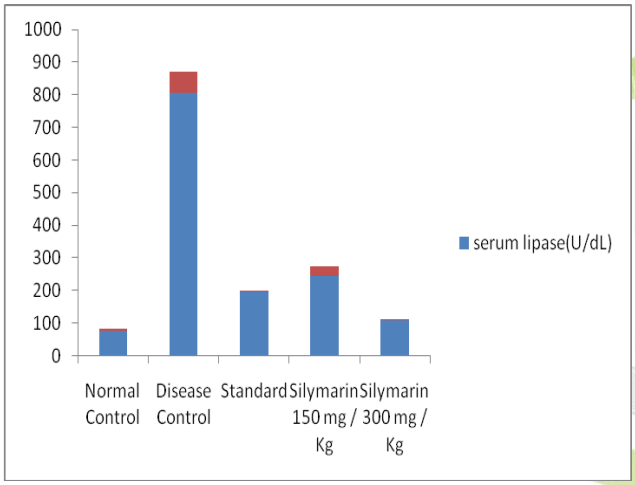


Table 3: Effect of Silymarin on pancreatic total protein levels in L – Arginine induced Pancreatic Rats

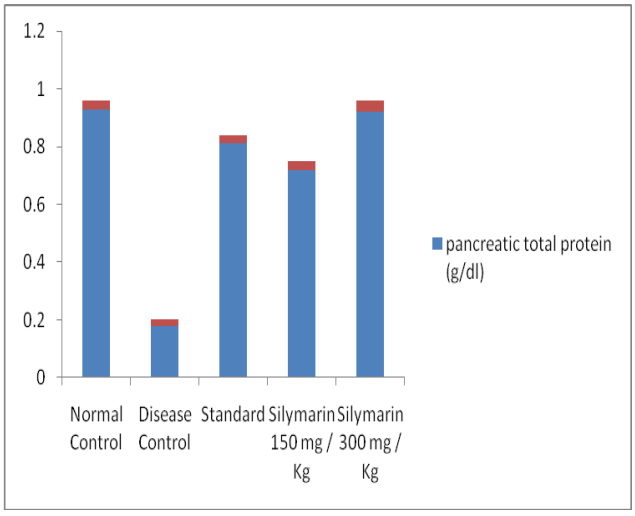


Table 4: Effect of Silymarin on pancreatic MPO in L – Arginine induced Pancreatic Rats

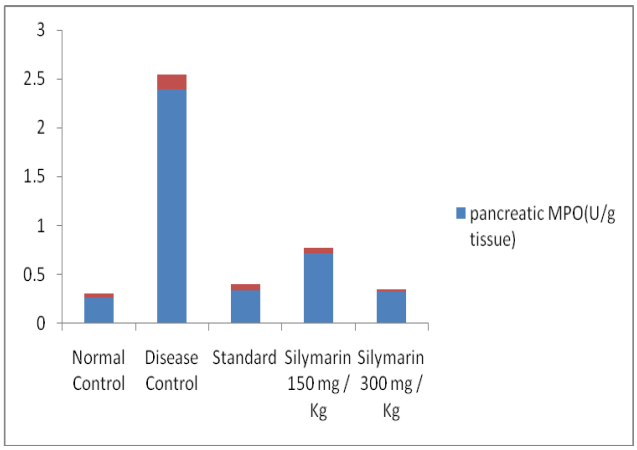


Table 5: Effect of Silymarin on pancreatic MDA in L – Arginine induced Pancreatic Rats

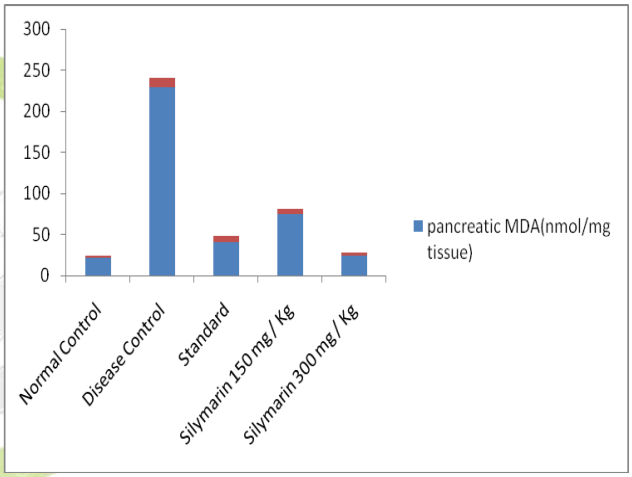


Table 6: Effect of Silymarin on pancreatic total nitrate levels in L – Arginine induced Pancreatic Rats

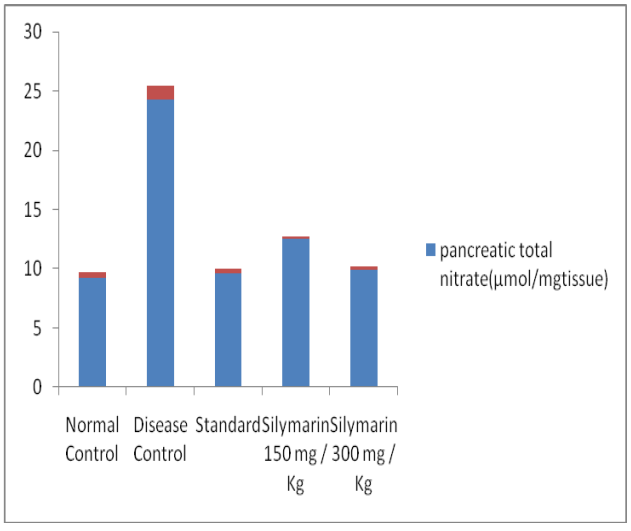


Table 7: Effect of Silymarin on pancreatic GSH levels in L – Arginine induced Pancreatic Rats

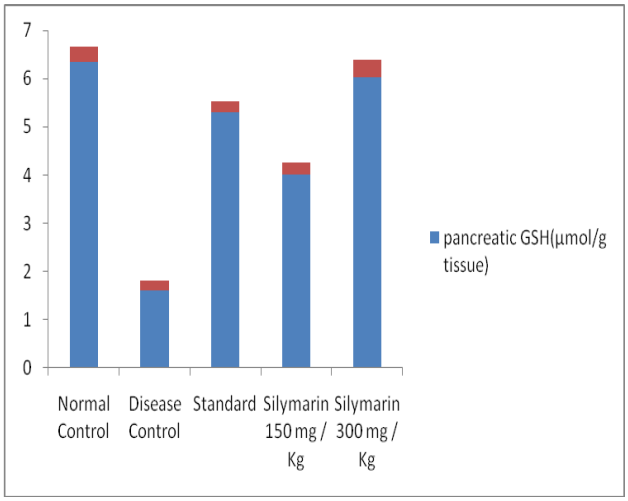


Table 8: Effect of on pancreatic catalase levels in L – Arginine induced Pancreatic Rats

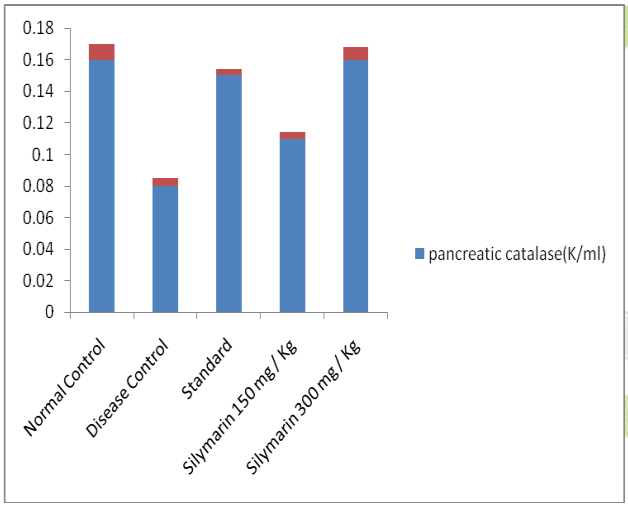


Table 9: Effect of Silymarin on pancreatic SOD levels in L – Arginine induced Pancreatic Rats

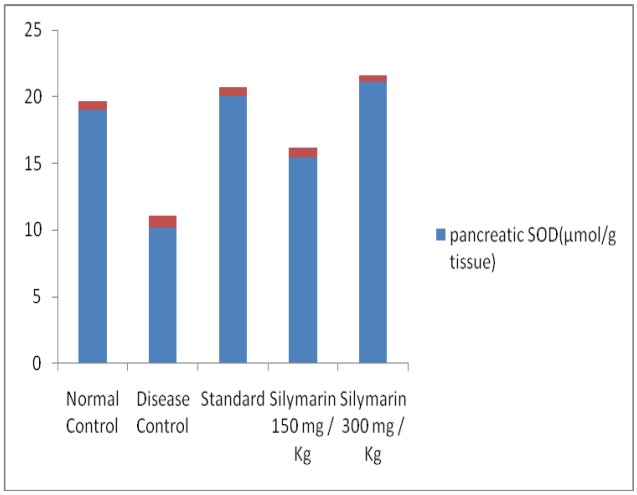


Table 10: Effect of Silymarin on Lung MDA Levels in L – Arginine induced Pancreatic Rats

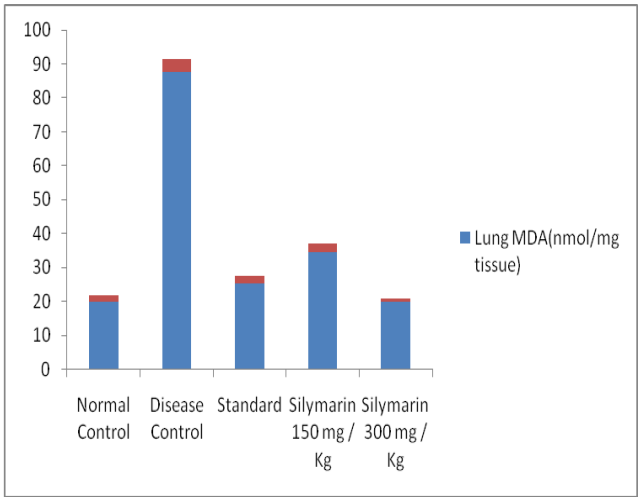


Table 11: Effect of Silymarin on Lung MPO Levels in L – Arginine induced Pancreatic Rats

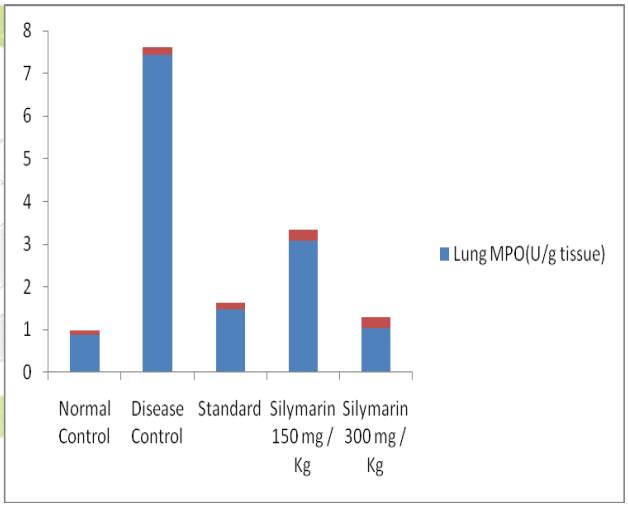


Table 12: Effect of Silymarin on Liver MDA Levels in L – Arginine induced Pancreatic Rats

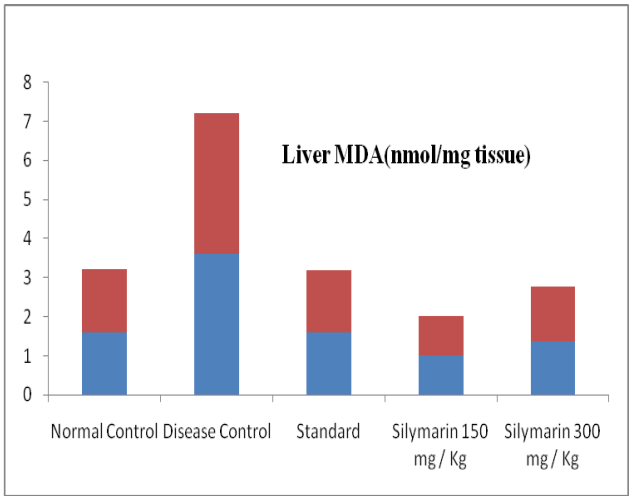


Table 13: Effect of Silymarin on Liver MPO Levels in L – Arginine induced Pancreatic Rats

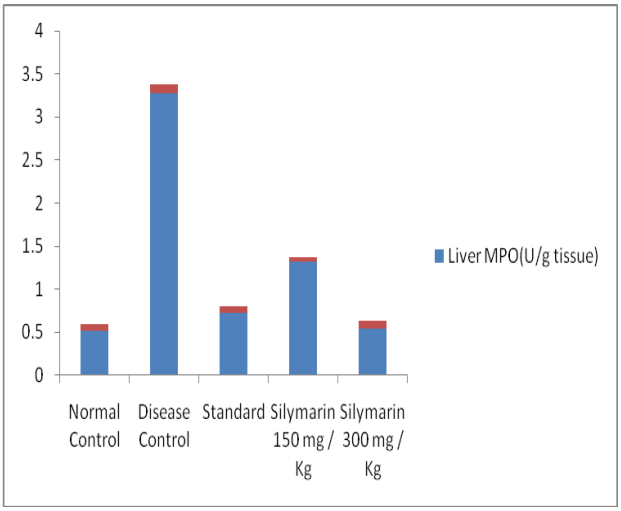


Table 14: Effect of Silymarin on kidney MDA Levels in L – Arginine induced Pancreatic Rats

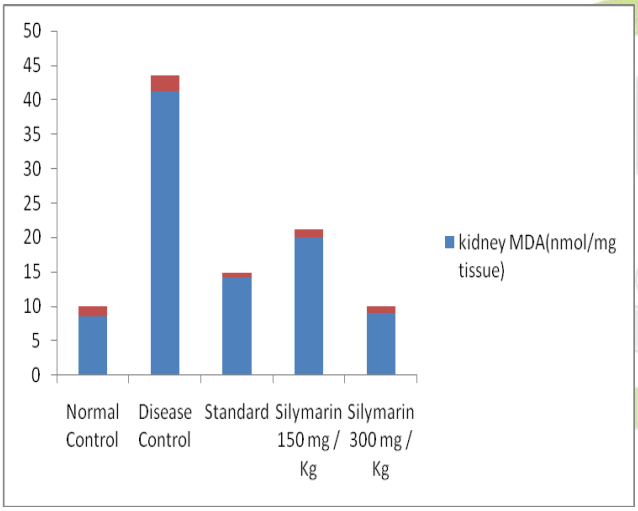
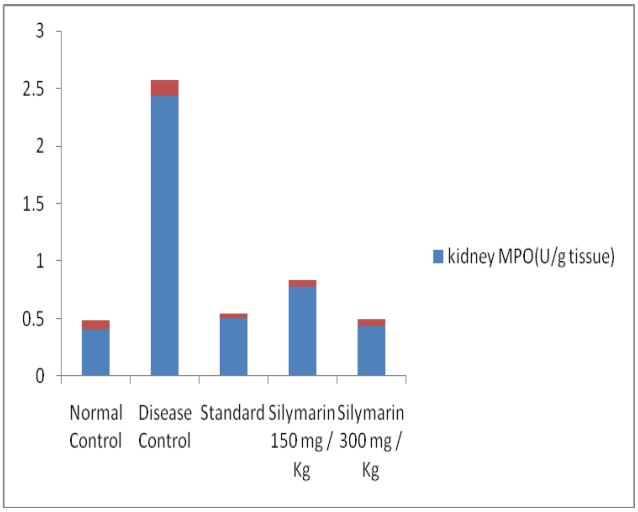
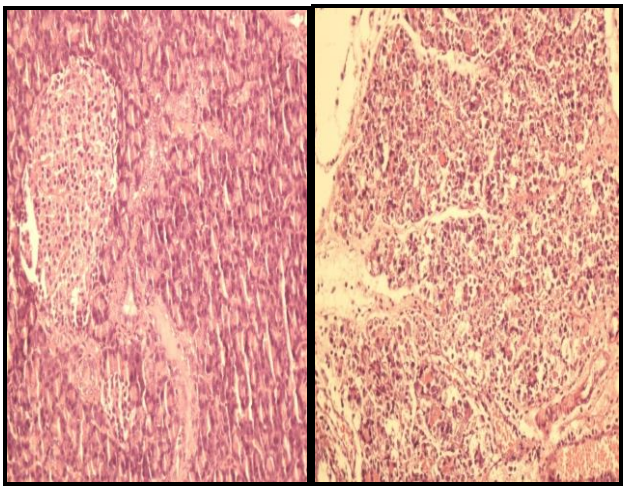
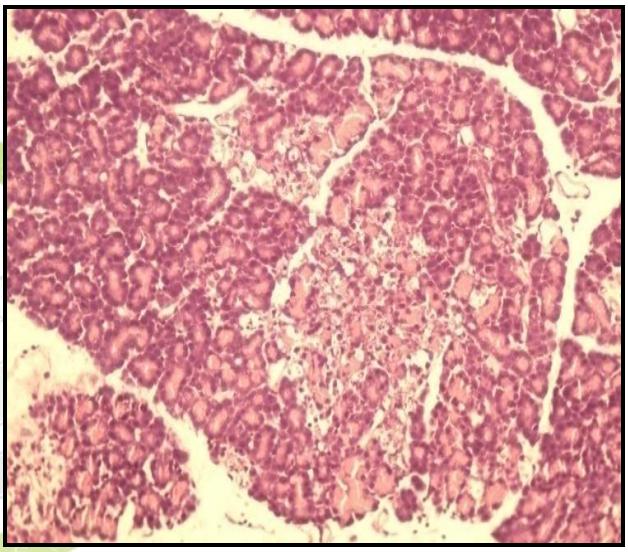


Table 15: Effect of Silymarin on kidney MPO Levels in L – Arginine induced Pancreatic Rats

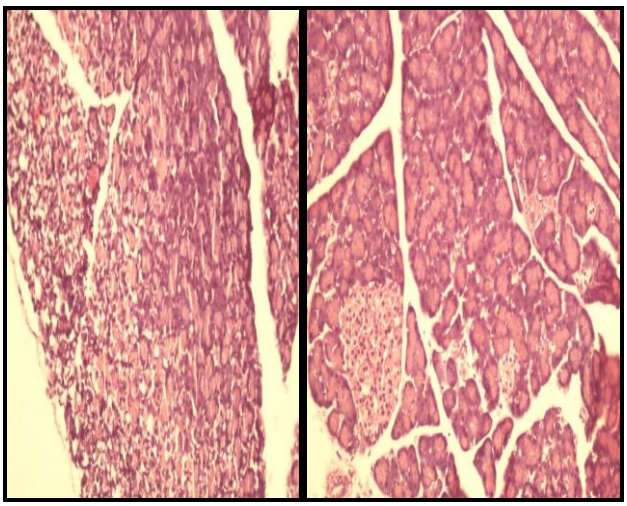




Normal Control Disease control



Methyl prednisolone (30 mg/kg)



Silymarin (150 mg/kg) Silymarin (300 mg/kg)

Figure 1: Representative photo micrographs of pancreatic sections (200x) of Study I

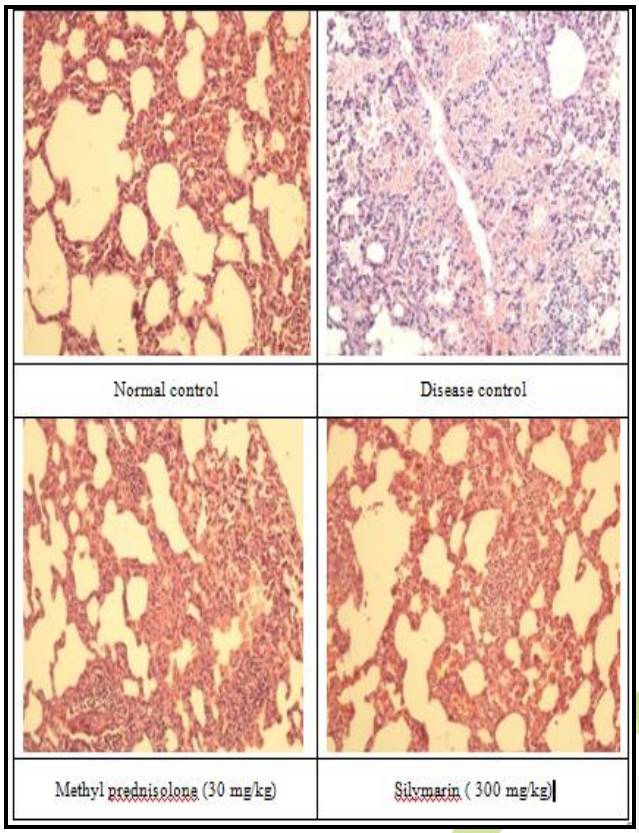


Figure 2: Representative photo micrographs of lung sections (200x) of study I.

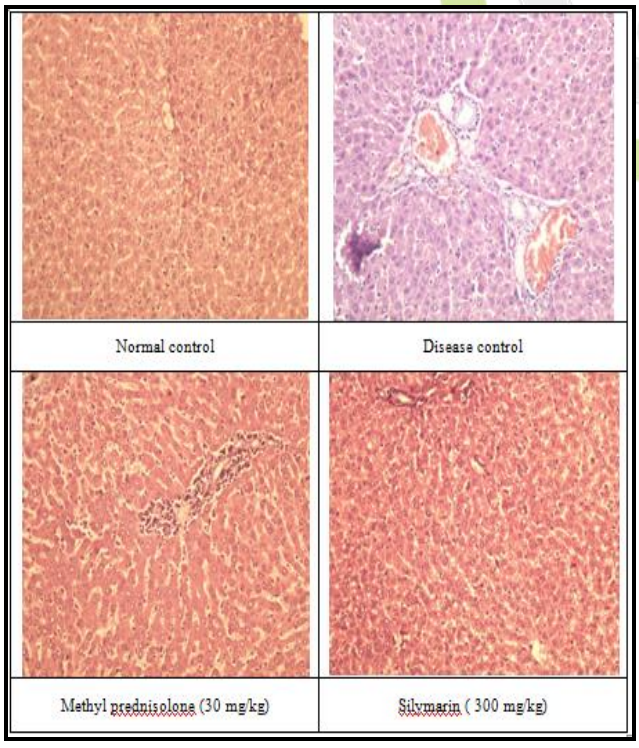


Figure 3: Representative photo micrographs of liver sections (200x) of study I.

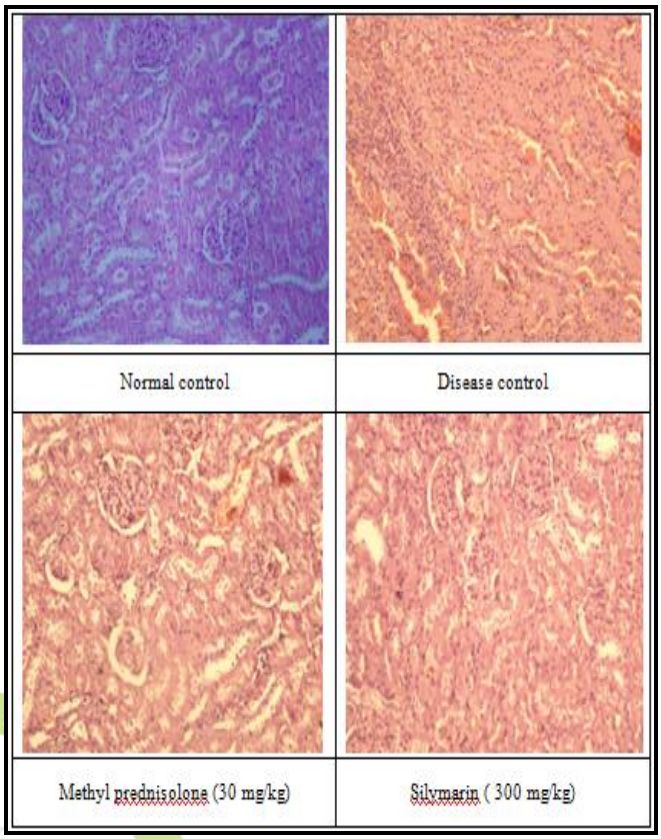
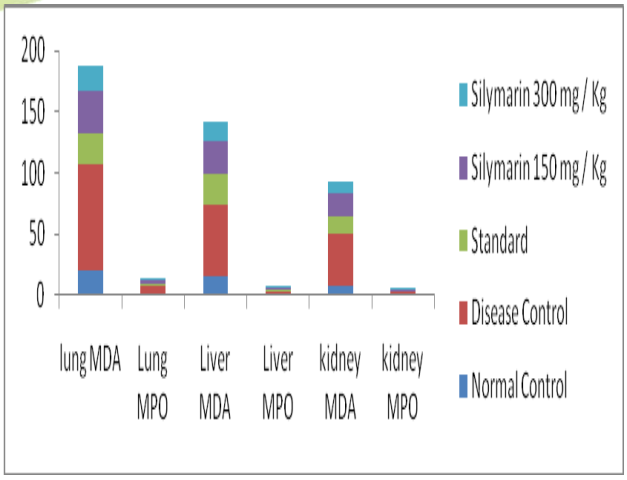


Figure 4: Representative photo micrographs of kidney sections (200x) of study I.

Comparative Study

Table 14: Comparative effect of Silymarin on lung, liver, and kidney MDA and MPO levels in L-arginine induced pancreatic rats

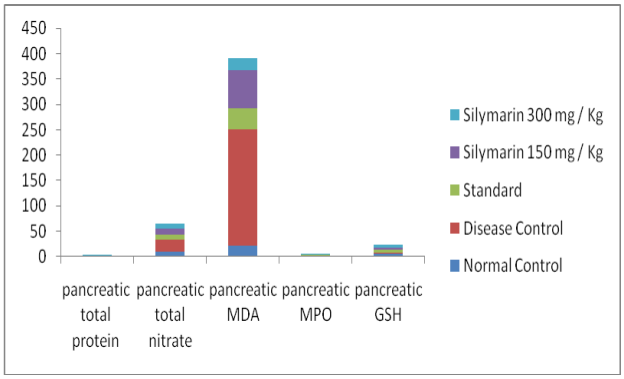


Data presented as mean^{+}-SEM (n=6)

$^{\alpha}p<0.0001$,$^{\beta}p<0.001$,$^{\gamma}p<0.001$ when compared to normal control

$^{a}p<0.0001$when compared to disease control

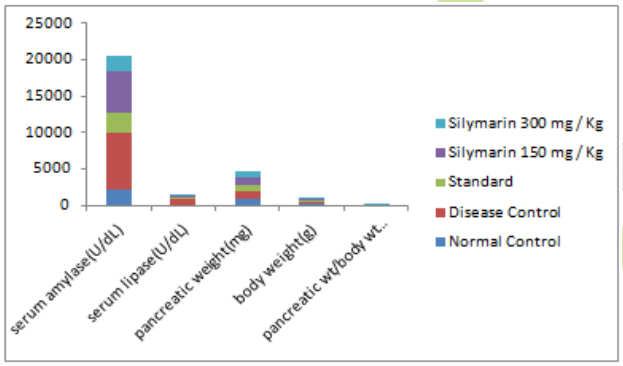
Table 15: Comparative effect of Silymarin on pancreatic total protein, total nitrate, MDA, MPO and GSH levels in L-arginine induced pancreatic rats



$^{\alpha}p<0.0001, ^{\beta}p<0.001, ^{\gamma}p<0.001$ when compared to normal control

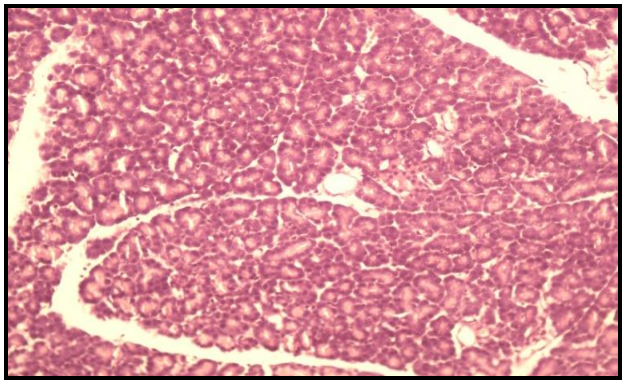
$^{a}p<0.0001$, $^{b}p<0.001$when compared to disease control

Table 16: Comparative effect of Silymarin on serum amylase, lipase, pancreatic weight levels in L-arginine induced pancreatic Rats

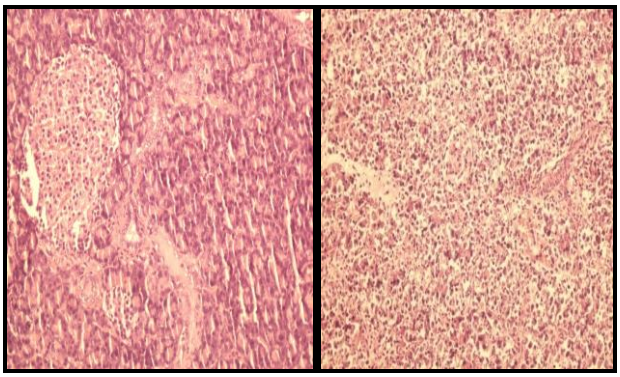


$^{\alpha}p<0.0001, ^{\beta}p<0.001, ^{\gamma}p<0.001$ when compared to normal control

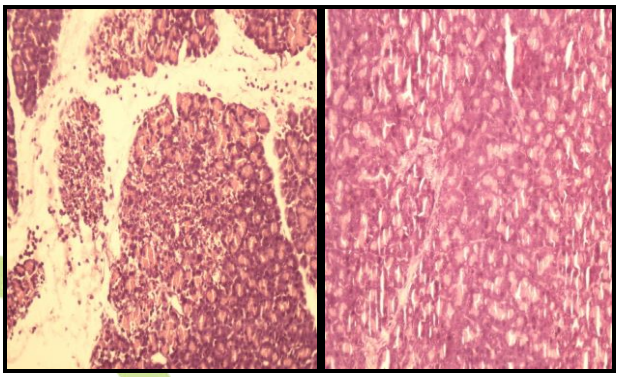
$^{a}p<0.0001$ when compared to disease control



Methyl prednisolone (30 mg/kg)



Normal control Disease control



Silymarin (150 mg/kg) Silymarin (300 mg/kg)

Figure 5: Representative photomicrographs of pancreatic sections (200x) of study II.

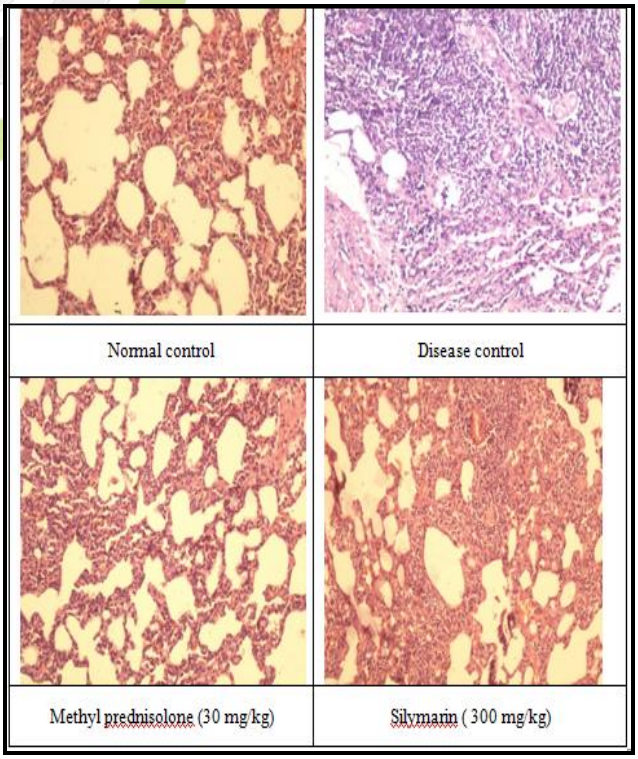


Figure 6: Representative photomicrographs of lung sections (200x) of study II

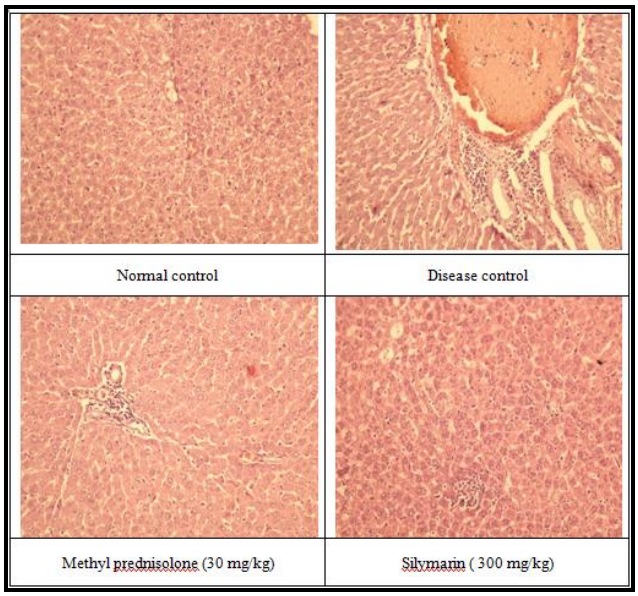


Figure 7: Representative photomicrographs of liver sections (200x) of study II.

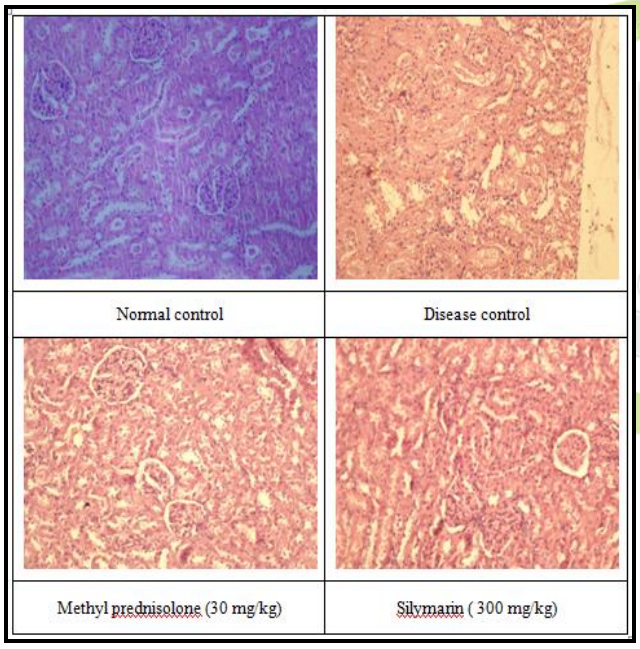


Figure 8: Representative photomicrographs of kidney sections (200x) of study II.

SUMMARY AND CONCLUSION

Summary

In the present study, administration of L-Arginine developed acute pancreatitis characterized by:

- Elevated levels of serum amylase and lipase at 24 hours.
- Increase in pancreatic oedema.
- Increased levels of ROS and altered antioxidant status in pancreas, lung, liver and kidneys.
- Fall in pancreatic total protein level.
- Histoarchitectural changes of pancreas, lungs, liver and kidney.

Except serum amylase and lipase all other parameters were more pronounced at 72 hours when compared to 24 hours after induction of pancreatitis. Treatment with Silymarin (150 and 300 mg/kg/day) dose dependently restored all the parameters at both 24 and 72 hours.

Conclusion

In conclusion the present study suggest that treatment with Silymarin significantly ameliorated the L-Arginine induced acute pancreatitis and systemic complications associated with acute pancreatitis probably due to its antioxidant and anti-inflammatory properties.

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