Research Article

In vitro and *in vivo* Hepatoprotective Action of L-α-phosphatidylcholine in Alcohol-induced Toxicity Model

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ABSTRACT

L-α-phosphatidylcholine, a surfactant and a constituent of the liposomal carrier system, has shown hepatoprotection in the various hepatotoxicity model. The present study was designed to assess its hepatoprotective effect against alcohol-induced toxicity in in vitro in Chang Liver cells and in in vivo in Wistar rats. For in vitro study, 7.25% v/v alcohol produced more than 50% cell death. A dose dependent increase in hepatoprotection was observed after L-α-phosphatidylcholine treatment from 7.82 to 62.5 µg/ml, while the trend was reversed in silymarin treatment. In in vivo study, Wistar rats were orally administered with 15 ml/kg (45% v/v) alcohol twice a day for 30 days. During the treatment from day 31 to day 45 with silymarin and L- α-phosphatidylcholine, alcohol was administered (25% v/v) ad libitum instead of drinking water. On 46th day, blood was withdrawn, liver was isolated after perfusion for antioxidant and histopathological examination. AST and ALT levels were significantly increased in the alcohol treated group, which was significantly reversed after silymarin treatment. A mild reversal in AST and ALT levels was observed by L-α-phosphatidylcholine treatment. Alcohol treatment showed a significant rise in the levels of TBARS and depletion of SOD, total thiols and GSH. Both drugs showed significant reversal in these oxidative stress parameters except in the levels of catalase and total thiols in L-α-phosphatidylcholine treatment. Histopathology of livers showed decrease in fatty accumulation in hepatocyte by the treatment of both drugs compared to alcohol control. The results indicated mild to moderate hepatoprotective effect for L-α-phosphatidylcholine, which were comparable to silymarin.

Keywords: L-α-phosphatidylcholine, Alcohol, Hepatoprotection, Chan Liver cells, Oxidative stress, Liver function test.

INTRODUCTION

Alcohol consumption produces a wide range of adverse illnesses, which are varying from acute to chronic conditions leading to disability, morbidity and mortality. It impacts the quality of life of the person and also affect various organs after long term consumption. The range of problems due to the chronic consumption of alcohol apart from traffic crashes includes malignant neoplasm, neuropsychiatric conditions, cardiovascular diseases and liver related diseases. Since, liver is a vital organ which detoxifies various toxins, while its location in the body immediately downstream from the intestine making it exceptionally vulnerable to toxic attack. Thus, among all the mentioned categories of disorders, liver related disorders are having higher incidences and produces chronic liver cirrhosis[1]. In year 2014, death due to chronic liver diseases were ranked 12th among top 15 leading causes of liver related deaths in the USA[2]. Alcohol as a toxicant has widely affected the population worldwide.

Alcohol produces oxidative stress in the liver, which results in activation of immune cells and Kupffer cells, leading to progression of disease to hepatitis, cirrhosis and hepatocellular carcinoma[3]. Treatment of most of the liver disorders including alcoholic liver disorders largely involves removal of toxins and usage of phytochemical agents and other supportive agents for regeneration of liver. A list of drugs is used for the regeneration of liver from plant sources viz. silymarin, phyllanthin, lecithin, catechin, glycyrrhizin, picroside, baicalein, daphnoretin, etc[4]. These plants isolates regenerate the liver and protect the liver from various toxins. Lecithin is one the major therapeutic agents, obtained from soybean as well as from egg. It is known for free radical scavenging effect against alcohol mediated free radical-induced toxicity[5]. Phosphatidylcholines are the major component of lecithin and named synonyms, sometimes[6]. They are widely being used for supporting the liver to regenerate. Phosphatidylcholine is a major component present in cell membranes and pulmonary surfactant. It protects the cells from toxic attacks and also possess antioxidant properties. Besides acting as barrier, they also act as an emulsifier of fat. Its component choline helps in the synthesis of a neurotransmitter acetylcholine, which is known to decrease inflammatory condition[7].

In earlier studies, phosphatidylcholine/lecithin has shown hepatoprotection in various animal models such as against carbon tetrachloride-induced hepatotoxicity in mice[8], D-galactosamine-induced hepatotoxicity in rats[6,9], fibrosis and cirrhosis-induced by alcohol in baboons[10] and thioacetamide-induced hepatic necrosis in rats[11]. The present study was undertaken to further study the hepatoprotective effect of L-α-phosphatidylcholine *in*

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vitro in Chang liver cells and *in vivo* in Wistar rats against alcohol-induced toxicity.

MATERIALS AND METHODS

Materials and chemicals

Silymarin, L-α- phosphatidylcholine, minimal essential media (MEM), fetal bovine serum (FBS) thiobarbituric acid (TBA), glutathione (GSH) and MTT reagent were procured from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. Tissue culture flasks, 96 well microculture plates were purchased from Tarsons Products Pvt. Ltd., Kolkata, India. Gentamycin, streptomycin and penicillin were procured from KMC Hospital, Manipal, Karnataka, India. Kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), Alkaline phosphatase (ALP) and total bilirubin, were purchased from Roche Diagnostic India Pvt. Ltd. BCA protein assay kit was purchased from Thermo Fisher Scientific, USA.

Test drugs preparation

All *in vitro* cytotoxic studies were performed using DMSO (0.1%) as solvent. In control samples, equimolar amount of DMSO was kept to rule out its cytotoxic effect. L-α-phosphatidylcholine and silymarin were suspended in 0.25% carboxymethyl cellulose (CMC) suspension administered orally to the animals.

Animals

Prior approval was taken from the Institutional Animal Ethics Committee for conducting this study (IAEC-KMC/07/2008-2009). Eight to ten weeks old Wistar albino rats, weighing (200 ± 20 g) were selected from an inbred colony. The animals were maintained under the controlled conditions of temperature (23±2°C), humidity (50±5% RH) and light (14 and 10 h of light and dark, respectively) at Central Animal Research Facility MAHE, Manipal-576104, India as per guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). The animals were provided with sterile food and water *ad libitum*.

Experimental method

Determination of cytotoxicity by MTT Assay[12]

The cell count was adjusted to 5000 cells/100 µL using complete medium containing 10% v/v new born calf serum. Each well of 96 well plate was added with 100 µL of this cell suspension and incubated for 24h in CO_2 incubator. Media was flicked off, and 100 µL of silymarin, L-α- phosphatidylcholine and alcohol at different concentrations were added to each well. The plate was incubated at 37°C for next 48h for drugs or 24h for alcohol in CO_2 incubator. The study was performed in triplicate at each dose level. After the treatment, drug/alcohol containing media were flicked off, washed with 200 µL of PBS, added 100 µl of 1 mg/ml MTT reagent and further incubated for 4 h at 37°C. Further, 20 µl of 5mg/ml of MTT in phosphate buffer saline (PBS) was added to each well and kept back to CO_2 incubator for next 3h. Formation of formazan crystal was observed in microscope. If the crystal formation was not proper waited for 1 more hours. Media was removed and 100 µl DMSO was added to each well. The plate was kept on shaker for 15 min. The absorbance was recorded ELISA reader at 540 nm. Calculated % cytotoxicity by the following formula:

%cytotoxicity = [(absorbance of control-absorbance of test)/absorbance of control] × 100

In vitro hepatoprotective activity against alcohol-induced toxicity

Dose levels selected for each drug sample were under the CTC_{50} value, while alcohol concentration was selected at CTC_{50}. The method for hepatoprotection were similar as used for evaluation of cytotoxicity by MTT assay for drugs and alcohol. Modifications were made in total time of incubation and pattern of addition of drugs and alcohol. Briefly, after seeding cells for 24h, the media was flicked off, and 100 µl of different drug concentrations was added to each well. The plates were then incubated at 37°C for 24h in CO_2 incubator. After 48h, 100 µl of alcohol was added to each well except control well. The plates were then incubated at 37°C for 24h in a CO_2 atmosphere. After total 72h, media was flicked off and further steps were similar as stated above for MTT assay protocol.[13]

In vivo hepatoprotection

Chronic consumption of alcohol causes alcoholic liver disease. In the present study, animals were randomly divided into six animals in four groups namely:

- Group 1 Sham
- Group 2 Control (Alcohol)
- Group 3 Silymarin (50mg/kg)
- Group 5 L-α- phosphatidylcholine (50mg/kg)

Animals were orally administered 15 ml/kg (45% v/v) two times a day for 30 days. Treatments were started on day 31st and continued until day 45 by the drugs suspended in 0.25% w/v carboxymethyl cellulose (CMC) and administered orally. The control group received only CMC. A change was made in alcohol dosing, in place of twice a day, alcohol was administered in drinking water at a concentration of 25% v/v. Total consumption of alcohol was monitored every day. On the 46th day blood was withdrawn and serum was separated from the blood by centrifuging it at 5000 RPM for 5 min. Serum was used for estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and total bilirubin using Cobas C111 autoanalyser.

Liver was dissected out after perfusion. Part of dissected was liver used for histopathological study and remaining part used for evaluation of antioxidant parameter.

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Evaluation of antioxidant parameter

A piece of liver was blot dried and homogenized in 150 mM KCl in cold condition and centrifuged in cooling centrifuge at 14000 rpm for 30 min to get the clear supernatant. The supernatant was used for estimation of following antioxidant parameter using standard protocol.

Assay for antioxidant Catalase

The catalase (CAT) activity was determined following the method of Aebi H, 1984[14]. A mixture of phosphate buffer (pH 7.0), sufficient amount of H_2O_2 (final absorbance between 0.3-0.5 at 240 nM) and tissue lysate in a final volume of 3 ml was added to cuvette and change in absorbance was recorded at 240 nm in 1 min. The catalase activity was expressed as mM/min/mg protein.

Assay for antioxidant Super Oxide Dismutase (SOD)

A mixture of 1850 µL of carbonate buffer, 50 µL of tissue homogenate and 100 µL of adrenaline was made in cuvette and a change in absorbance was recorded at 480nm in 1 min.[15]

Assay for Glutathione

The glutathione assay was performed by comparing the colour formation due to formation of derivative of DTNB. Equal volume of 5% trichloro acetic acid and the tissue homogenate was centrifuged to get a clear supernatant. The following solutions were incubated for 10 min at room temperature: 500µL of supernatant, 3mL of PBS and 500µL of DTNB. Amount of reduced glutathione calculated from the absorbance at 412 nm by extrapolating on the standard plot of glutathione. The amount was expressed in µM per mg of protein.[16]

Assay of Total thiols

A mixture of 100µl tissue homogenate, 100 µl Tris EDTA solution, 40 µl DTNB and 3.16 ml of methanol was centrifuged at 2000 rpm. The absorbance of supernatant was taken at 412 nm. Amount of total thiols was expressed in nM/mg of protein.[16]

Assay of lipid peroxidation

A mixture of 0.5 ml of tissue homogenate, 2.5 ml of thiobarbituric acid (TBA), trichloroacetic acid (TCA) and butylated hydroxyl toluene (BHT) was heated at 90°C for 10 min. The mixture was centrifuged at 2000 rpm for 5 min and absorbance was taken at 525 nm. Lipid peroxidation was expressed as nM of malondialdehyde form per mg of protein.[17]

Statistical analysis

Results were analyzed by one-way ANOVA followed by Dunnett´s post hoc method of analysis. The following were the significance values considered in the study, *p<0.05, **p<0.01 and ***p<0.001 compared to alcohol control.

RESULTS

***In vitro* cytotoxicity studies**

The drugs were tested for cytotoxicity on Chang liver cells. The cytotoxicity of silymarin was found to be very high (CTC_{50} 151.2 µg/ml) compared to L-α-phosphatidylcholine (CTC_{50} value 370.2 ± 1.39 µg/ml) (Table 1.). Four dose levels of the drugs were selected for further studies based on the cytotoxicity result of silymarin. The highest dose of the drug treatment was almost half of the CTC_{50} value, i.e., 62.5 µg/ml. Remaining concentrations were obtained by two times serial dilution *i.e,* 31.25 µg/ml, 15.6 µg/ml and 7.82 µg/ml.

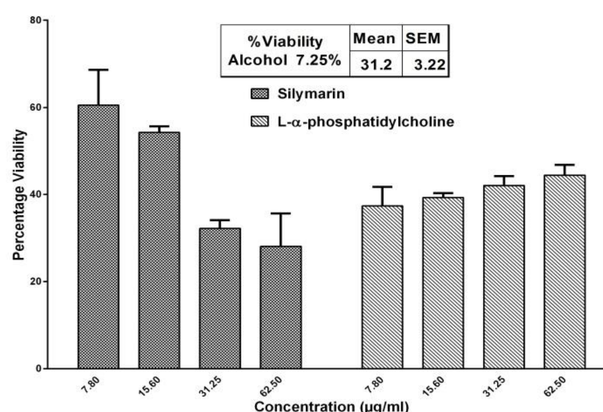
Table 1: *In vitro* cytotoxicity study on Chang liver cells by MTT assay

Drug	CTC_{50} (µg/ml)
Silymarin	151.2 ± 1.46
L-α- phosphatidylcholine	370.2 ± 1.39
Alcohol	
Concentration	% Cytotoxicity
7.25%	58.7 ± 2.14
12.30%	67.04 ± 1.97
25%	68.37 ± 1.59
50%	68.79 ± 3.22

Legend: All the values are mean±SEM of three tests in triplicate

***In vitro* hepatoprotective activity against alcohol induced toxicity**

Cells were challenged with 7.25 % v/v of alcohol. Only 31.2% cells were viable in alcohol treated cells. Silymarin showed lesser protection as concentration increased. At lowest tested concentration, silymarin (7.8µg/ml), showed maximum protection (% viability 60.53). L-α-phosphatidylcholine showed lesser protection compared to silymarin, however, a dose dependent increase in cytoprotection was observed. (Fig. 1)



Legend: All the values are mean±SEM of three readings in triplicate

Figure 1: Protective effect in Chang liver cells against alcohol-induced toxicity

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In vivo hepatoprotection study

Biochemical estimation in serum

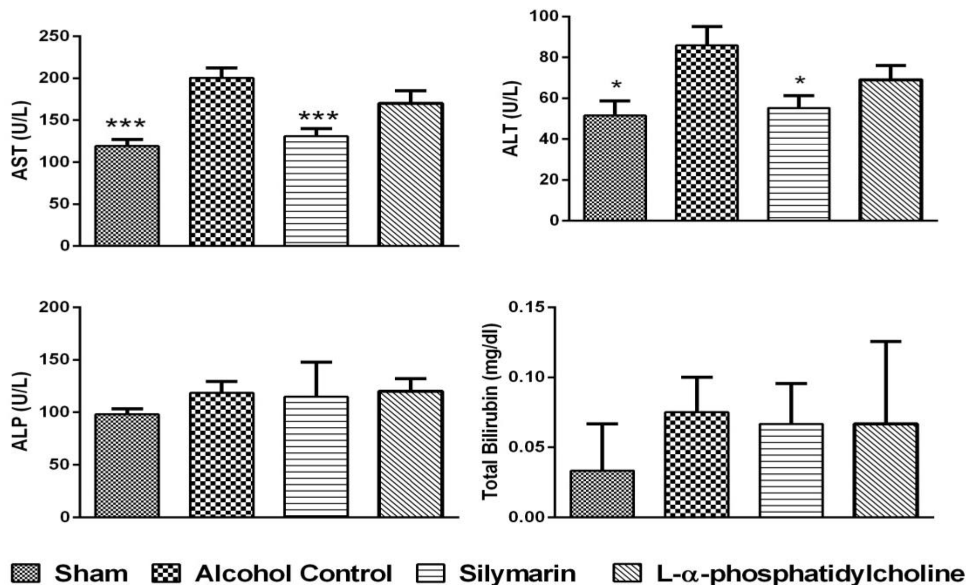
Chronic administration of alcohol showed a significant rise in AST and ALT levels compared to sham control. However, no significant change was observed in total bilirubin and ALP level by alcohol treatment compared to sham control. Silymarin significantly ($p<0.05$) prevented the rise in AST and ALT levels compared to the alcohol control group, while L-α- phosphatidylcholine was not significantly effective in controlling rises in AST and ALT levels compared to the alcohol control group. (Fig. 2)

Antioxidant parameters in liver tissue homogenate

Chronic administration of alcohol significantly ($p<0.01$) depleted the catalase, SOD, GSH and total thiols levels and significantly elevated TBARS levels [monitored in the form of malondialdehyde (MDA) formation levels] compared to sham animal in the liver tissue. Silymarin was found to be significantly effective in normalizing all tested parameters compared to the alcohol control group. L-α- phosphatidylcholine showed weaker antioxidant action, while it showed a significant improvement in SOD, GSH and MDA level compared to alcohol control group. (Fig. 3)

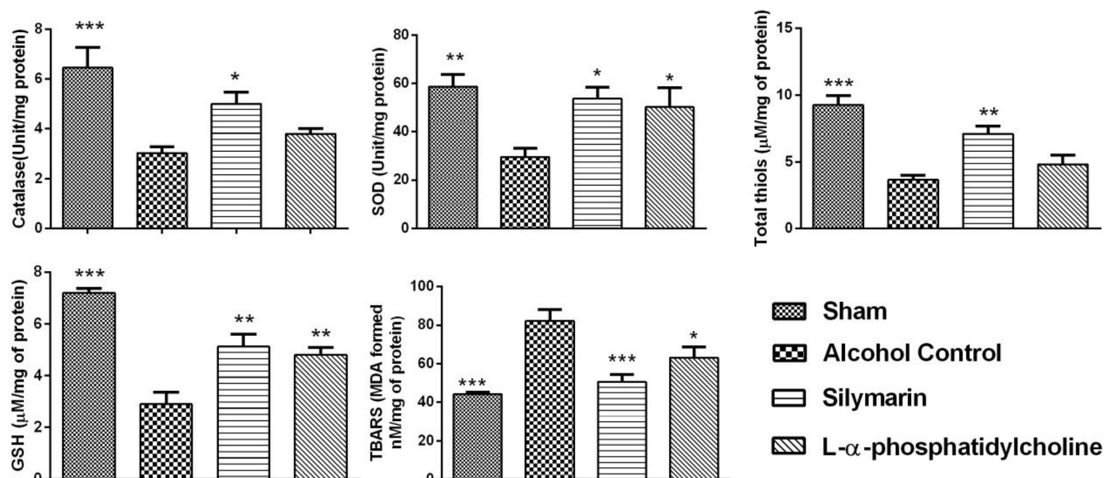
Histopathology

Chronic oral administration of alcohol to animals showed moderate damage to liver parenchyma structure. Macroscopic finding revealed liver was enlarged. Fatty accumulations were seen with mild neutrophil infiltration and spotty necrosis. Sinusoidal congestions were observed along with congestion in central vein. Treatment with both drugs showed decrease in fatty changes. Silymarin showed better improvement in the fatty changes compared to L-α- phosphatidylcholine. (Fig. 4)



All the values are mean±SEM of six animals, where ***$p<0.001$ compared to alcohol control group and *$p<0.05$ compared to alcohol control group.

Figure 2: Effect on liver function tests in alcohol-induced hepatotoxicity model in Wistar rats



All the values are mean±SEM of six animals, where ***$p<0.001$ compared to alcohol control, **<$p<0.01$ compared to alcohol control and *$p<0.05$ compared to alcohol control.

Figure 3: Effect on antioxidant parameters in liver tissue homogenate alcohol-induced hepatotoxicity model

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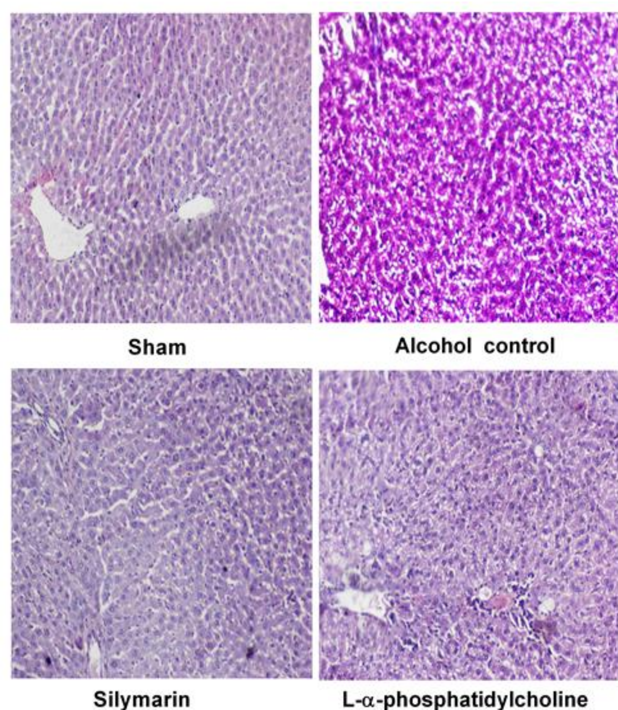

Figure 4: Effect on histology in alcohol induced hepatotoxicity model

DISCUSSION

L-α-phosphatidylcholine is widely used for nanocarrier development, such as liposome and obtained from soybean and egg yolk. For the present study, L-α-phosphatidylcholine from soybean source were selected for hepatoprotective effect. Its efficacy against alcohol-induced toxicity in *in vitro* on Chang liver cell line and *in vivo* in Wistar rats were compared with standard silymarin. Although the hepatoprotection of these drugs are well established, the present study compared the *in vitro* and *in vivo* hepatoprotective effect of L-α-phosphatidylcholine in alcohol-induced toxicity model. The highest dose selected for screening of *in vitro* hepatoprotective action was 62.5µg/ml, which was less than half of the CTC_{50} value of the silymarin (CTC_{50} 151.2 ± 1.46). The other three dose levels were 7.82, 15.6, and 31.25µg/ml for silymarin and L-α-phosphatidylcholine. The dose level for alcohol was 7.25%v/v, which produced toxicity more than 50 % cytotoxicity. From the present study, we found that silymarin showed better protection than L-α-phosphatidylcholine against alcohol-induced toxicity. However, the trend of hepatoprotection was opposite for both drug, a dose dependent decrease for silymarin, while a dose dependent increase for L-α-phosphatidylcholine. This dose dependent changes in these two drugs might be due to the fact that CTC_{50} value of L-α- phosphatidylcholine was more than two times of silymarin. In our previous study also, silymarin was found to be a better hepatoprotective agent compared to L-α-phosphatidylcholine in D-galactosamine-induced acute hepatotoxicity models.[6]

Chronic alcohol administration is reported to produce liver damage in Wistar rats, marked by raised levels of liver enzymes parameters (Liver function test, LFT)[18]. In the present study, 45 days of oral administration of ethanol showed a significant ($p<0.05$) elevation in AST, ALT levels compared to sham group, while no significant increase in ALP and total bilirubin levels were observed in alcohol treated group. These changes indicated a mild to moderate liver damage. Elevated AST and ALT levels were significantly lowered by silymarin treatment compared to alcohol treated control group, while L-α-phosphatidylcholine showed a mild reversal.

Elevated oxidative stress in the liver is one of the important markers of hepatotoxicity in alcoholic liver disease, which initiate a series of reactions resulting in liver dysfunction[18]. Oxidative stress starts by inducing a hypoxia condition to hepatocyte followed by depletion in GSH level and S-adenosine methionine level[18]. The similar finding has been observed in our study. The depletion in antioxidant levels, namely GSH, total thiols, catalase, and SOD levels was observed in alcohol-intoxicated group. Alcohol intoxication mediated depletion in antioxidant system is also accompanied by increased reactive oxygen species (ROS) formation by cytochrome P450-2E1 (CYP2E1) and leads to the initiation and propagation of lipid peroxidation[18]. Finding in this study indicated a similar condition; alcohol intoxication increased the TBARS level (measured by malondialdehyde levels). Treatment of silymarin significantly ($p<0.05$) prevented the rise in MDA level. Silymarin was able to normalize the oxidative stress significantly, reflected by changes in oxidative stress markers in the liver homogenate. L-α-phosphatidylcholine also showed significant reversal in oxidative stress parameters except total thiols and catalase levels.

The product of lipid peroxidation i.e., malondialdehyde forms immunogenic adduct with transport protein, which in turn initiate humoral and cellular immune response[18] leading to immune cell infiltration and progresses liver disease from fatty liver to hepatitis. Similar effects were in seen in liver histology of alcohol intoxicated livers. The liver histology showed microvascular fatty accumulation and infiltration of immune cells. These changes reflected initial stage of alcohol induced liver damage, *i.e.,* alcoholic fatty liver disease (AFLD)[19]. These changes were minimized by the treatment of silymarin and L-α-phosphatidylcholine.

CONCLUSION

The present study reflected mild to moderate hepatoprotective potential of L-α- phosphatidylcholine against alcohol-induced hepatotoxicity.

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