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Research Article

Development of Hepatotoxicity Model in Rats and its Application in Evaluation of Hepatoprotective Activity of Cell Wall Contents of Probiotics

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ABSTRACT

The objective of present work is Development of Hepatotoxicity model in rats and to evaluate hepatoprotective activity of cell wall contents of probiotics. Animals were divided in four groups. The groups were normal saline group, diseased control group, standard drug treated group and 4th group was CCl_4 +Cell wall contents of probiotics. In diseased control group chronic liver injury was induced by administration of Carbontetrachloride (CCl_4) via intraperitoneal route (1 ml/kg) for seventy days. For standard drug treated group 1 ml Silymarin suspension (Orally) & CCl_4 was given for seventy days. In fourth group cell wall contents (1 x 10^{12} CFU/ml/animal) and CCl_4 was given for seventy days. During disease induction & treatment period blood samples were collected and serum was separated which was used to analyse various parameters like Alanine aminotransferase (ALT), Aspartate aminotransferase, (AST), Alkaline phosphate (ALP), direct bilirubin, total protein and albumin levels to asses liver function. Along with these cholesterol, Glucose and Malondialdehyde were also measured. Liver fibrosis & cirrhosis was quantified by histopathological studies of small portion of the excised liver. Serum AST, ALT, ALP, and direct bilirubin were found to be significantly higher in CCl_4 intoxicated rats. Total protein and albumin was decreased. Manondialdehyde was found to be significantly higher in CCl_4 intoxicated rats which was main end product of Lipid Peroxidation. Whereas in cell wall contents probiotics and silymarin treated group improve the liver functions in CCl_4 toxicated rats. We conclude that protein oxidation may play a role in the pathogenesis of CCl_4 induced liver injury and that the accumulation of oxidised proteins may be an early indication of CCl_4 induced liver damage. Silymarin and cell wall contents of probiotics were effective in liver injury by inhibiting protein oxidation.

Keywords: Liver Fibrosis, Free Radicals, Lipid Peroxidation, Oxidative Stress, Carbon Tetrachloride, liver biomarkers, Cell wall content of probiotics.

INTRODUCTION

Liver is the lymphoid organ which normally regulates innate immune responses through modulation of TLR signal called **'**Liver Tolerance'[1,2]. A breakdown of tolerance may induce in-appropriate immune response leads to Acute and Chronic Liver diseases[2]**.** Many cell types present in the liver involved in Liver injury[1]. Recruitment of immune cells into the liver leads to the activation of local kupffer cells, which can further promote the fibrotic process via secretion of inflammatory and fibrogenic cytokines. Types and duration of liver injury can decide extent of fibrosis and inflammation.

CCl_4 is highly hepato-and nephrotoxicant[3] and found to be useful to study hepatotoxic effect in experimental model[4]. Dose and duration of exposure of CCl_4, covers a variety of effects. At low doses, transient effects prevail, such as loss of Ca^{2+} homeostasis[5], lipid peroxidation[6], release of noxious or beneficial cytokines[7] and apoptotic events followed by regeneration and at the with higher doses develop fatty degeneration, fibrosis, cirrhosis and even cancer[8]. Extreme doses of CCl_4 develop central nervous system depression and respiratory failure and death[9].

Probiotics are live organisms that, when ingested in adequate amounts, confer a health benefit to the host[10].The most commonly used probiotics are Lactobacilli, Bifidobacterium[11]. Ex. Of Lactobacillus species are L.acidophilus, L.casei, L.fermentum, L.jhonsonii, L.plantarum, L.rhamnosus & Bifidobacterium species are B.bifidum, B.breves, B.lactis, B.longum[12] It was found that Lactobacillus and Bifidobacterium were able to suppress transcription of IL-1β, TNF-α, NF-κB, as well as the translation of IL-1β and IL-6 in experimental colitis in Rats[13]. L.acidophilus have been reported to prevent cytokine induced intestinal

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epithelial permeability that may play significant role in intestinal epithelial cells[14]. Probiotic therapies can reduce liver aminotransferases, total-cholesterol, TNF-α and improve insulin resistance in NAFLD[15]. Probiotics also decreases intestinal permeability and endotoxemia in patients with liver disease[16]. Probiotics improve lipid peroxidation level by maintaining malondialdehyde (MDA) level - as marker of oxidative stress[17].Lipoic acid and dihydrolipoic acid serve as strong antioxidants through several mechanisms, including the scavenging of free radicals, chelation of metal ions, and regeneration of endogenous and exogenous antioxidants, such as ubiquinone, glutathione, and ascorbic acid[18].

Cell wall Content Isolation[19]

20 gm bacteria (by weighing colonies) was suspended in 350 ml of hot water (65-68℃). In that 350 ml of 90% Phenol (65-68℃) was added & Stirred for 1hr.at 65-68℃. It was cooled in an ice bath to 2-8℃, or left overnight refrigerator. It was centrifuged at 3000 rpm for 45min. Then, upper water layer was preserved (hot phase).Residual Phenol & inter-phase was treated, if any, with another volume of hot water and proceeded as described. The Phenol layer contain lipids and the insoluble residue of cell wall proteins whereas, Aqueous phase will contain Lipoteichoic acid, Polysaccharides, Teichoic acid and Peptidoglycans. The aqueous layer was used for treatment.

HPTLC method for cell wall contents of probiotics

Probiotics (Test- Mixture of lactobacilli) were taken and cell wall contents were estimated by in house HPTLC method. The CAMAG HPTLC (CAMAG, Muttenz, Switzerland) with Win CATS Software version 1.3.4 along with sample applicator Linomat V were used for HPTLC analysis of the sample. The stock solutions (10 mg/mL) of lipoic acid (LPA) and Lipotechoic acid (LTA) was prepared by dissolving the freeze dried powders in sterile water for injection. Test compounds were prepared by Phenol-water extraction method as described above in materials and methods section. Two microlitre of different test solutions and reference solutions (LPA) and Lipotechoic Acid (LTA) were applied on precoated silica gel (10*10) of uniform thickness (0.2mm). The plate was developed in solvent system [n-Propyl alcohol: Ethyl acetate: 25% NH_3: Water (10:5:2.5:2.5) (For Lipoic Acid) and Chloroform: Acetone: Methanol: Glacial acetic acid: water (4.6: 1.7: 1.5: 1.4: 0.8) for Lipotechoic acid] to a distance of 8 cm. The distance between the tracks was kept 10 mm with bandwidth of 6 mm. The plates were developed in CAMAG flat bottom and Twin trough Chamber (20 × 10 cm). The saturation time was kept 30 min. The temperature was maintained at 25 ± 10^0C ° throughout the study. Initially, the developed plates were visualized using UV visible chamber (CAMAG, Muttenz, Switzerland) to ascertain adequate separation of the components. Finger print profile, and Rf value, were determined densitometrically at 200nm for Lipoic Acid and 540 nm for Lipotechoic Acid using CAMAG TLC scanner 3.

MATERIALS AND METHODS

Carbon Tetrachloride (CCl_4) & Arachis oil - Loba chemie Pvt Ltd, Mumbai.

Silymarin Suspension – Microlabs Ltd, Bengaluru, Karnataka

SGPT, SGOT, Bilirubin, Total Protein, Albumin, Glucose, Cholesterol, Kits- Autospan (Span Diagnostic Pvt Limited Surat)

Cell Wall Contents of Probiotics

Experimental protocol & procedure

Animals

Healthy Wistar rats of either sex, weighing 200-250 gm was approved by CPCSEA/IAEC committee before carrying out the experiments. The protocol of present study with protocol no RPCP/IAEC/2013-2014/R-32. The animals were housed in polypropylene cage at 25^0C; 12 hrs dark-light cycle, with free access to standard pellet diet (normal pellet diet) and water *ad libitum* during the course of experiment. The animals were acclimatized to surrounding for one week prior to experiment.

Experimental design

Rats were randomly divided as follows:

Group I: Received Normal Saline 1 ml/kg per day for 70 days

Group II: Received Carbon Tetrachloride 1 ml/kg for 70 days

Group III: Received Carbon Tetrachloride 1 ml/kg (i.p) & 1 ml Silymarin Suspension (p.o) for 70 days.

Group IV: Received Carbon Tetrachloride 1 ml/kg 1st for 70 days daily and 1 x 10^{12} CFU/ml/ of cell wall contents of probiotics for 70 days

Blood Sample Collection

Blood samples were collected by retro-orbital route in EDTA containing and EDTA free vials on day 0, 7, 14, 21, 28, 35, 49, 56, 63 & 70 during development & treatment of the model. After collection of blood samples, the samples were centrifuged under cooling condition at 4000 RPM for 10-15 minutes to separate plasma & serum respectively to measure all biochemical parameters such as AST, ALT, Bilirubin, albumin, total protein, Glucose, Cholesterol, Malondialdehyde except ALP and serum which was used to measure ALP activity.

Histopathological Investigation

Rats were kept on fasting and were sacrificed on day 70. Liver was collected and washed with ice cold saline. The excised liver was fixed in 10 % formalin solution and taken to the Anand Agricultural University (AAU), Department of histopathology, for histopathological study. The extent of CCl_4 induced liver injury was evaluated by assessing the morphological changes in liver sections stained with Haematoxylin & Eosin Stain (H&E Stain) and Masson's trichomes stain to observe liver fibrosis. The therapeutic effect of cell wall contents and probiotics and silymarin was also evaluated by assessing the liver section.

Statistical Analysis

All parameters were expressed as mean value ± S.D. Difference between mean value of tests and controls were evaluated statistically by Student's t-test. P value of < 0.05 was regarded as statistically significant.

Std Lipoic acid Isolated Lipoic Acid

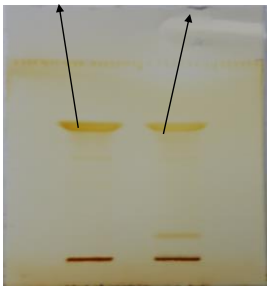

Figure 1: TLC Plate showing the spot of isolated cell wall component (Lipoic Acid) & Standard Lipoic acid.

Std Lipotechoioc Acid Isolated Lipotechoic Acid

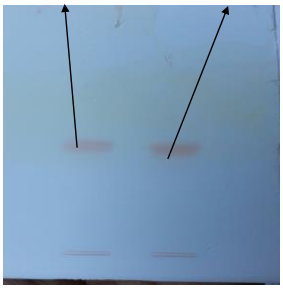

Figure 2: TLC Plate showing the spot of isolated cell wall component (Lipotechoic Acid) & Standard Lipotechoic acid.

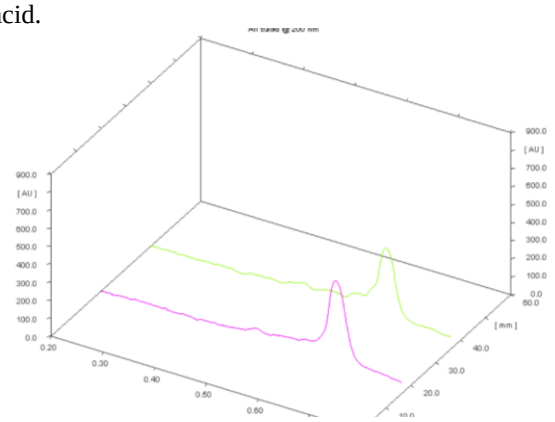

Figure 3: HPTLC for Lipoic Acid.

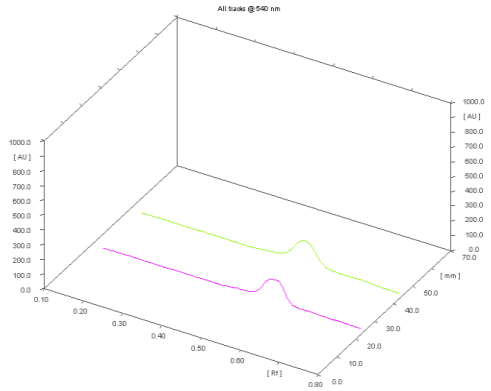

Figure 4: HPTLC for Lipotechoic Acid.

Table 1: Change in various serum parameters in rats exposed to CCl_4 daily and normal saline.

Group	ALP	ALT	AST	Bilirubin	T.P
Normal Control	P-0.0258, 303.8 ± 30.95	P-0.0015 480.± 51.04	P-0.018, 518.3 ± 57.71	P-0.025, 0.4795 ±0.07	P-0.0106, 4.794 ±0.20
CCl_4 treated	P-0.0258 199.5 ± 31.05	P-0.0015 243.9±41.7	P-0.018, 262.0 ± 44.89	P-0.025, 0.1881 ±0.04	P-0.0106, 5.640 ± 0.23
Group	Albumin	Globulin	MDA	Glucose	Cholesterol
Normal control	P-0.0342, 2.427 ± 0.23	P-0.0122 2.538± 0.09	P-0.0058, 1.791 ± 0.11	P-0.0001, 196.2 ±10.8	P-0.0001, 214.3 ± 12.41
CCl_4 treated	P-0.0342 3.355 ± 0.34	P-0.0122 2.285± 0.20	P-0.0058, 0.3350 ±0.04	P-0.0001, 116.0 ± 12.9	P-0.0001, 111.1 ± 15.74

Data was expressed as mean±SEM (n=6) Significant difference was indicated by p<0.5 compared with normal control group.

P value between Normal control and CCl_4 treated group is <0.5, which indicates they are statistically significant.

ALP-Alkaline phosphate, ALT- Alanine Transaminase, AST-Aspartate transaminase, T.P –Total protein, MDA-Malondialdehyde

RESULTS AND DISCUSSION

Hepatotoxicity by CCl_4 depends on dose and its duration of exposure. Chronic dose of CCl_4 (1 ml /kg, i.p) serve as hepatotoxicant. Upon chronic exposure of CCl_4, rise was observed in serum enzyme levels, which is shown in Table. Along with liver enzymes, Albumin, Total protein & MDA is also affected.

Table 1 shows that when CCl_4 was given daily, there was rise in ALP, AST, malondialdehyde (MDA), Glucose, Cholesterol & Globulin. However different scenario was seen in normal controls, standard drug treated group and the group which was given cell wall contents of probiotics. Here P value between Normal control and CCl_4 treated group (table 1), CCl_4 treated group and standard drug treated group (table 2), CCl_4 treated group and cell wall contents of probiotic treated group (table 3) is <0.5, which indicates they are statistically significant. Whereas P value between standard group treated group and cell wall contents of probiotics treated group is not<0.5, which indicates no significant difference in between this group**.**

Histopathological Findings

The controlled rats were showed normal liver architecture. They showed normal hepatocytes, portal triads vasculature, and bile duct. Whereas rats which were given CCl_4 up to 70 days shows extensive liver damage. Silymarin treated rats and rats treated with cell wall contents of probiotics showed normal liver architecture.

DISCUSSION

Table 2: Change in various serum parameters in rats exposed to CCl_4 treated and standard drug treated group.

Group	ALP	ALT	AST	Bilirubin	T.P
CCl_4 treated	P-0.0083, 238.1 ± 19.52	P-0.0003 499.7±58.7	P-0.013, 550.5 ± 63.62	P-0.0001, 0.3950 ±0.05	P-0.0371, 4.827 ±0.24
Standard Drug (Silymarin) Treated	P-0.0083 138.3 ± 27.91	P-0.0003 169.3±48.5	P-0.013, 290.8 ± 71.34	P-0.0001, 0.0943 ±0.02	P-0.0371, 5.647 ±0.27
Group	Albumin	Globulin	MDA	Glucose	Cholesterol
CCl_4 treated	P-0.0124, 2.372 ± 0.32	P-0.0250 2.455± 0.01	P-0.0058, 1.791 ± 0.10	P-0.014, 188.8 ± 11.2	P-0.0001, 213.2 ± 13.43
Standard Drug (Silymarin) Treated	P-0.0124 3.658 ± 0.35	P-0.0250 1.989± 0.17	P-0.0058, 0.3350 ±0.05	P-0.014, 118.8 ± 15.2	P-0.0001, 113.8 ± 16.85

Data was expressed as mean±SEM (n=6) Significant difference was indicated by p<0.5.
P value between CCl_4 treated group and Standard drug treated group was <0.5, which indicates they are statistically significant.

Table 3: Change in various serum parameters in rats exposed to CCl_4 treated and cell wall contents of probiotics treated group

Group	ALP	ALT	AST	Bilirubin	T.P
CCl_4 treated	P-0.0258, 303.8 ± 30.95	P-0.0015 480.± 51.04	P-0.018, 518.3 ± 57.71	P-0.025, 0.4795 ±0.07	P-0.0106, 4.794 ±0.20
Cell Wall contents of probiotics treated	P-0.0258 199.5 ± 31.05	P-0.0015 243.9±41.7	P-0.018, 262.0 ± 44.89	P-0.025, 0.1881 ±0.04	P-0.0106, 5.640 ± 0.23
Group	Albumin	Globulin	MDA	Glucose	Cholesterol
CCl_4 treated	P-0.0342, 2.427 ± 0.23	P-0.0122 2.538± 0.09	P-0.0058, 1.791 ± 0.11	P-0.0001, 196.2 ±10.8	P-0.0001, 214.3 ± 12.41
Cell Wall contents of probiotics treated	P-0.0342 3.355 ± 0.34	P-0.0122 2.285± 0.20	P-0.0058, 0.3350 ±0.04	P-0.0001, 116.0 ± 12.9	P-0.0001, 111.1 ± 15.74

Data was expressed as mean±SEM (n=6) Significant difference was indicated by p<0.5
P value between CCl_4 treated group and cell wall contents of probiotics treated group was <0.5, which indicates they are statistically significant.

The haematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status for both animals and humans[19]. Hematotoxicology is the study of adverse effects of drugs, non-therapeutic chemicals and other agents in our environment on blood and blood-forming tissues[20]. This subspecialty draws on the discipline of hematology and the principles of toxicology. The vital functions that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication, makes the hematopoietic system unique as a target organ. Accordingly, it ranks with liver and kidney as one of the most important considerations in the risk assessment of individual patient populations exposed to potential toxicants in the environment, workplace, and medicine cabinet.

Serum aminotransferase enzymes

Biochemical parameters are an important marker to evaluate the organs and cellular functions. Among the evaluated parameters, such as AST, ALT, and ALP, total bilirubin, unconjugated bilirubin and conjugated bilirubin, total protein, albumin, globulin, and A/G ratio are considered as liver function markers[21]. The analysis of these parameters is important because several reports of liver toxicity are related to the use of phytotherapeutic products[22].

The most specific test for hepatocellular damage is the ALT level. The AST may also be elevated but is not as specific as the ALT. The ALT is the gold test in hepatocellular damage. AST and ALT are enzymes that are present in all the cells, however, they are found in highest concentration in the hepatocytes: ALT in the cytoplasm and AST in the cytoplasm as well as the mitochondria[23]. Cholestatic injury is best diagnosed by an elevated ALP level. Bile acids stimulate ALP production, but duct obstruction or damage prevents bile acid secretion into the duodenum. Therefore, the ALP level in serum rises dramatically. Because ALP can be derived from other body tissues (e.g. bone, intestine), a concurrent elevation of GGT helps to support a cholestatic mechanism.

In the present study serum AST, ALT, ALP, Bilirubin activities were greatly increased ($p < 0.05$) in rats exposed to CCl_4 as compare to normal control. The increased serum levels of hepatic markers have been attributed to the liver injury, because these enzymes are

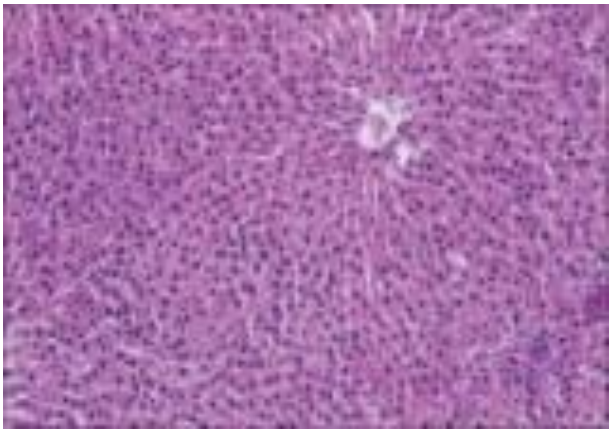

Figure 4a. Normal Histology of liver

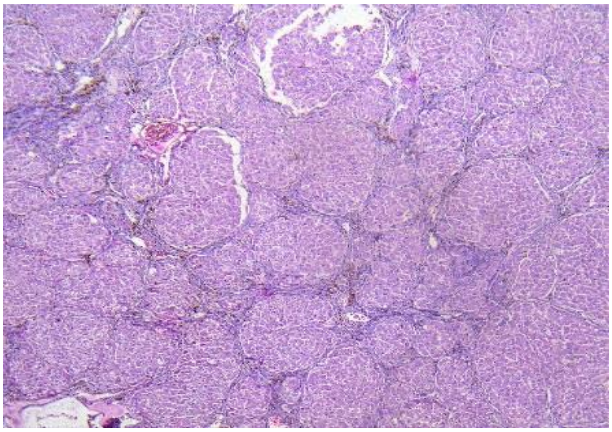

Figure 4b: Proliferation of fibrous were Evident in hepatic lobule resulting into pseudolobulation (Day 70)

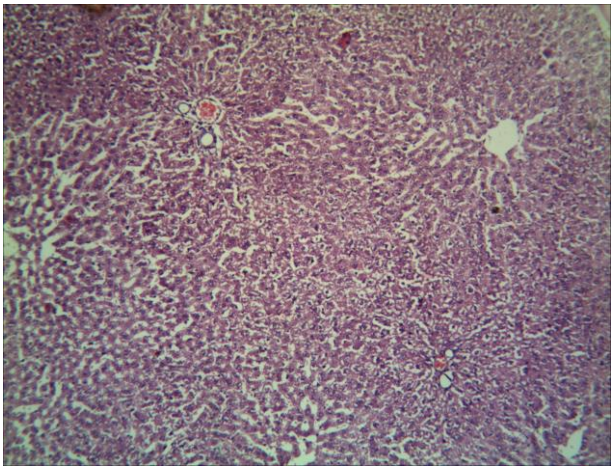

Figure 4c: Did not reveal any specific changes (CCl_4 + Silymarin)

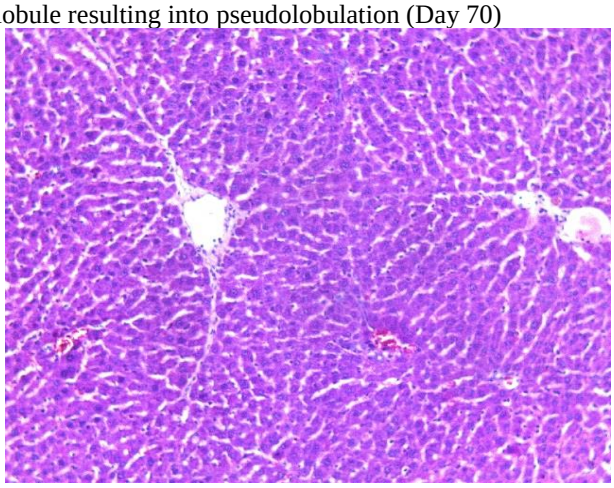

Figure 4d: Did not reveal any specific lesion (CCl_4 + cell wall contents of probiotics 1 x 10 ^{12} CFU/ml/animal)

place in cytoplasmic area of the cell and are released into circulation in case of cellular damage**.**

L.acidophilus have been reported to prevent cytokine induced intestinal epithelial permeability that may play significant role in Intestinal epithelial cells[24]. It has been reported that probiotic therapies can reduce liver aminotransferases, total-cholesterol, TNF-α and improve insulin resistance in NAFLD[25]. Lactobacillus GG (LGG) could prevent endotoxemia which was due to its ability to improve barrier function[26]. It was studied that lipoic acid and Lipotechoic acid can serve as antioxidant through scavenging of free radicals, chelation of metal ions and regeneration of endogenous and exogenous antioxidant[27].

CONCLUSION

From the results and discussion it was concluded that Carbon Tetrachloride has potential to cause chronic liver injury (Fibrosis & Cirrhosis). The proposed animal model of chronic liver injury might be suitable for screening of drugs used to treat liver injury and associated disorders. Here Silymarin as a standard drug which shows hepatoprotective effect in developed model.

In current study, cell wall contents of (lactobacilli bacteria) probiotics were highly effective in treating chronic liver disease. The cell wall components of probiotics (Lipoic acid & Lipotechoic acid) are highly effective in treatment of liver disease by inhibiting lipid peroxidation and normalizing the liver enzymes level.

CONFLICT OF INTEREST

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