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CATECHIN: A TLR4 MEDIATED CHAIN BREAKING INHIBITOR OF SIGNALLING CASCADE IN ENDOTOXIN-INDUCED LIVER INJURY

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ABSTRACT

In view of the limitations of the existing therapy, the present study was carried out in order to evaluate the potential of catechin as a natural polyphenolic inhibitor against endotoxin-induced liver injury in a rat model. The levels of liver function enzyme markers, histological architecture, expression of signalling molecules involved in endotoxin mediated signalling pathway, levels of hepatic oxidative markers and antioxidants were analyzed in both endotoxin (10 mg/kg body weight injected intraperitoneally) challenged rats and the rats supplemented with kitchen for 15 days (50 mg/kg body weight administered orally) prior to endotoxin challenge. Oral supplementation of catechin for fifteen days followed by challenge with endotoxin decreased the levels of liver enzyme markers, nitrite and lipid peroxidation along with an increase in hepatic antioxidant activity. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis revealed the inhibition of expression of CD14, TLR4, MD2, MyD88 and IL-12B (p40) by catechin supplementation. The results of these biochemical and molecular studies correlated well with the histological alterations with once as well as earlier findings. It may be inferred from the study that catechin is able to inhibit the signalling cascade starting with the downregulation of the extrinsic molecules such as CD14, TLR4, MD2 and MyD88 and further going downstream to intrinsic molecules such as IL-12B, along with upregulation of antioxidant profile. Thus, catechin may be considered as a chain breaking inhibitor of the signalling cascade involved in endotoxin mediated liver damage and may serve as an immunomodulator to prevent endotoxin-mediated hepatotoxicity.

Key Words: Endotoxin, catechin, antioxidant, hepatotoxicity

INTRODUCTION

Liver integrity and functions are crucial for survival of patients suffering from trauma or infections (Jaeschke H, 2011). Since liver is implicated in almost all biological processes, its damage induces severe interruption of metabolism, immune response, detoxification and antimicrobial defenses. Despite current medical and surgical advances, hepatic failure is still associated with a high mortality rate. Amongst various etiological agents causing liver injury, endotoxin, also known as lipopolysaccharide (LPS), remains the predominant one. Lipopolysaccharide, a critical mediator of Gram-negative bacterial infections, is a pathogen-associated molecular pattern (PAMP) recognized by the pattern recognition receptor (PRR) i.e. Toll-like receptor4 (TLR4). TLR4, a transmembrane protein expressed mainly on the plasma membrane of monocytes and macrophages, recognizes LPS-LBP (LPS-Binding Protein) -membrane-bound CD14 complex in association with MD2 (Myeloid

Differentiation 2). The complex interaction of these molecules mediates hepatic cell activation and pro-inflammatory cytokine release (Shimazu R et al. 1999). TLR4 by itself does not function as an LPS receptor. To function as an LPS receptor, TLR4 requires MD2 as a helper molecule (Chow et al. 1999). MD2 is a 160-amino acid protein that is associated with TLR4 on the cell surface and enables TLR4 to respond to LPS (Shimazu R et al. 1999; Lien E, et al. 2000). Astronger physical association of MD2 with the extracellular domain of TLR4 makes this complex more stable which can then recognize LPS-CD14 complex. This results in the activation of nuclear factor kappa B (NF-κB), an oxidative stress sensitive transcription factor (Barnes PJ and Karin M, 1997; Nanji AA et al. 1999; Cadenas S and Cadenas AM, 2002), leading to expression of a variety of pro-inflammatory mediators including TNF-α, interleukins and inducible nitric oxide synthase (iNOS), all of which are implicated in the pathogenesis of the SIRS (Systemic Inflammatory Response Syndrome) (Christman JW et al. 1998). This in turn results in generation of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNIs) by activated cells which ultimately leads to liver damage (Bharrhan S et al. 2012). The suppression of this receptor-mediated signalling may serve as an important target for management/treatment of LPS-induced inflammatory responses. To manage oxidative stress mediated manifestations, various natural antioxidants have been put forward (Kheir-Eldin AA et al. 2001; Lynn M et al. 2003; Ozaras R, et al. 2003; Tsuji K et al. 2004; Kaur G et al. 2006; Hsu DZ et al. 2006; Kasdallah-Grissa A et al. 2007) Chemoprevention by edible phytochemicals is an inexpensive, readily applicable, and accessible approach to control and manage inflammation. For example, polyphenolic compounds have been shown to be protective against oxidative stress-induced liver injury (Hussein J, 2011). Tea polyphenols have been reported to display potent antioxidant, anti-inflammatory and antibacterial properties (Wang H et al. 2000; Higdon JV and Frei B, 2003; Sutherland BA et al. 2006). Recently, we have demonstrated that catechin modulates NF-κB (an intrinsic key molecule) mediated oxidative damage (Bharrhan S et al. 2012). In continuation, this study was designed to evaluate if catechin also inhibits extrinsic receptor mediated pathway and behaves as a chain breaking inhibitor of signalling molecules involved in endotoxin mediated liver injury.

MATERIALS AND METHODS

2.1. Agents

Lipopolysaccharide (LPS from *Escherichia coli* serotype O111:B4) and catechin hydrate were obtained from Sigma Aldrich Chemicals (St Louis, MO, USA). The preparations were made fresh every time before the commencement of the experiment. Lipopolysaccharide, dissolved in water for injection, was administered as a single intraperitoneal injection. Catechin hydrate was dissolved in warm distilled water and administered by oral gavage. All the reagents for molecular work were obtained from Fermentas Inc., Maryland, USA. All other chemicals were of analytical grade.

2.2. *Animals*

Wistar rats (200–250g) were procured from Central Animal House, Panjab University, Chandigarh, India. The animals were housed under standard laboratory conditions, maintained on a 12-h light and dark cycle and had free access to food (Ashirwad Industries Pvt Ltd, Punjab, India) and water *ad libitum*. The experimental protocols were approved by the Institutional Animals Ethics Committee of the Panjab University, Chandigarh, and conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

2.3. Experimental Design

To investigate the prophylactic potential of catechin, rats were randomized into following groups, each comprising of at least 6-8 rats. The rats in the control group were administered normal saline daily for 16 days. Rats in the LPS challenged group received a dose of LPS (10 mg/kg body weight, pre-standardized dose) intraperitoneally (i.p.). Catechin supplemented +LPS challenged rats (CT+LPS) were supplemented with catechin (50mg/kg body weight, pre-standardized dose) orally for 15 days and then challenged with endotoxin on day 16. Animals in all these groups were sacrificed 8 h post-LPS challenge by cervical dislocation. Livers were removed quickly, rinsed in cold phosphate buffer saline (0.05 M, pH 7.4) and stored at -80°C till further use.

2.3.1. Assessment of liver function enzymes

Blood was collected from the retro-orbital sinus of rats before they were sacrificed and serum was prepared. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities in serum were determined using ERBA test kits (ERBA Diagnostics, Mannheim, Germany). Alkaline phosphatase (ALP) activity was estimated using Enzopak Diagnostic kit (Reckon Diagnostics, India).

2.3.2. Histological studies

Liver tissues removed aseptically from all the groups were cut into small pieces and fixed in 10% buffered formalin. Samples were processed, stained with hematoxylin-eosin and examined under the light microscope.

2.3.3. RT-PCR analysis of liver CD14, TLR4, MD-2, MyD88, IL-12B mRNA expressions

Liver tissue of the rats subjected to the indicated treatments were harvested and immediately preserved in RNA*later* (Ambion, CA, USA) at -80°C till processing. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Equal amount of total RNA (2µg per reaction) was then, reverse transcribed by using oligo-dT primer and first strand cDNA synthesis kit (Fermentas). The cDNA obtained was then subjected to PCR using CD14, TLR4, MD-2, MyD88, IL-12B (p40) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (Sigma Aldrich Chemicals, Banglalore, India) (Table 1). GAPDH mRNA was used as an internal control.

Table 1
PCR primers for TLR4, CD14, IL-12B, MyD88, MD2 and GAPDH

Gene	Sense (5'-3')	Antisense (5'-3')	Product(bp)
TLR4	GCTTGAATCCCTGCATAGAG	TCCACAGCCACCAGATTCT	331
CD14	CTTGTTGCTGTTGCCTTTGA	CGTGTCCACACGCTTTAGAA	214
IL-12B	ACTTCTCGTAGTCCCTTTGGTTCAG	ACTTGCTCACTTTGTTGAGTCATTC	266
MyD88	TGTATGAACTGAAGGACCGC	ATGCCTCCCAGTTCCTTTG	830
MD2	TGAATCTGAGTAGGCAACAG	AACTCCATATTTGCCGAAG	221
GAPDH	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT	309

The PCR reaction mixture contained 10×Taq buffer 2.5 µl, dNTP (1mM, each) 2.5 µl, gene specific primers (Table 1 sense and antisense primers, 25 pmol/µl, each) 1 µl, Taq DNA polymerase 3.0 units and cDNA (1:10 diluted) 2 µl in a total volume of 25 µl. Amplification was performed with 35 cycles with initial incubation at 94°C for 3 min and final extension at 72°C for 7 min, each cycle of which consisted of denaturation for 45 s at 94°C, annealing for 45 s at 55°C (CD14, MyD88, GAPDH), 59.5°C (TLR4), 60°C (MD2), 54°C (IL-12B), and extension for 1 min at 72°C. Following RT-PCR, 10 µl of each amplified product was resolved by electrophoresis in 1.5% agarose gels containing ethidium bromide. The level of each PCR product was qualitatively evaluated using Gel Documentation System (Biorad Labs., Hercules, CA, USA). The sizes of the bands were determined by molecular weight standards (100 bp DNA ladder).

2.3.4. Assessment of hepatic oxidative markers and antioxidants

Samples from all the groups were tested for lipid peroxidation, nitrite levels, superoxide dismutase (SOD) activity and catalase activity. A 25% (w/v) tissue homogenate in each case was prepared in PBS (0.05M, pH 7.4) using a potter Elvehjem homogenizer. An aliquot of the liver homogenate was used for the estimation of lipid peroxidation. The remaining tissue homogenates were centrifuged at 11,269 x g for 20 min at 4°C. The supernatant thus obtained, known as post mitochondrial supernatant (PMS), was used for the estimation of nitrite levels, superoxide dismutase and catalase activities. The quantitative measurement of lipid peroxidation in liver was performed as described by us earlier (Chanana V et al. 2007) by measuring the levels of malondialdehyde (MDA). The amount of nitric oxide was determined by Griess reaction, as described by

Green et al. (1982). Superoxide dismutase (SOD) and catalase activities in the PMS preparations were estimated as described earlier (Rishi P et al. 2006).

2.4. Statistical Analysis

Results were expressed as mean±SD. The inter-group variation was measured by one way analysis of variance (ANOVA) followed by Fisher's least significant difference test. The statistical analysis was done using Jandel Sigma Stat Statistical Software v2.0. Statistical significance of the results was calculated at least at $p<0.05$.

RESULTS

3.1. Liver function tests

A significant increase in serum ALT levels (148.65±6.76 IU/L, $p<0.05$) was observed in LPS challenged group as compared to the control group (48±4.56 IU/L, $p<0.05$). The activitiy of serum ALT decreased significantly in LPS challenged group on supplementation with catechin (54.81±7.32 IU/L, $p<0.05$). The activity of AST in serum was significantly increased ($p<0.05$) in the LPS challenged group as compared with the control group (286.32±21.45 IU/L vs. 153.45±16.78). However, pretreatment with catechin in LPS challenged group significantly reduced the elevation in AST activity (146.12±17.66 IU/L, $p<0.05$). Challenge with endotoxin lead to rise in serum levels of ALP (724.32±21.22 IU/L, $p<0.05$) as compared to the levels in the control group (180±19.12 IU/L, $p<0.05$). The level of serum ALP was decreased significantly (201.67±2.41 IU/L, $p<0.05$) on supplementation with catechin (Fig. 1).

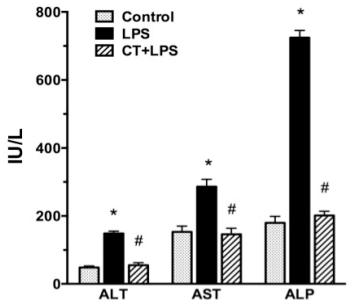

Figure 1: Effect of catechin on ALT, AST and ALP levels in the serum of LPS-challenged rats compared to control group. Values are expressed as mean ± SD of six different observations. *p< 0.05 vs. control; #p < 0.05 vs. LPS; LPS + CT: Catechin administered for 15 days prior to LPS challenge.

3.2. Hepatic histopathological changes

Histological evaluation of liver tissues did not reveal any morphological alterations in the control group (Fig. 2a). In contrast, livers of LPS-challenged rats showed marked morphological disruption such as portal triaditis, Kupffer cell hyperplasia, necrosis and lymphocytic infiltration (Fig. 2b). Supplementation with catechin resulted in significant morphological protection in terms of marked reduction in inflammation and hepatocyte damage in LPS challenged rats (Fig. 2c).

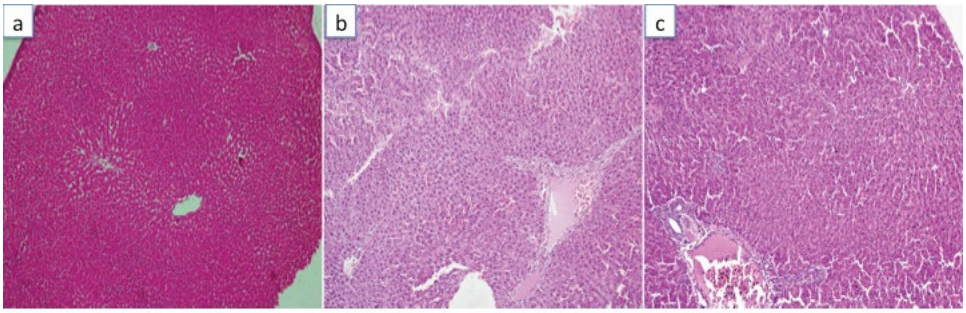

Figure 2: (a) Representative photomicrograph of the normal/control rat liver showing normal liver morphology (100x). (b) Representative photomicrograph of rat liver section 8 h post-LPS challenge (10 mg/kg body weight i.p.) showing generalized hyperplasia of Kupffer cells. There are excess of lymphocytes especially in the portal tract (middle right edge of the picture), also known as "portal triaditis". Together these changes diagnose "Reactive hepatitis" and indicate an immunological or toxic injury to the liver (100x). (c) Representative photomicrograph of liver sections of rats that were supplemented with catechin (50 mg/kg body weight) for 15 days before LPS challenge showing mild non-specific changes, but otherwise showing normal liver. The portal tracts are intact and hepatocytes are normal (100x).

3.3. Expression of signalling molecules in liver

As observed from Fig. 3, mRNA expression levels encoding GAPDH were identical in all the groups. CD14, TLR4, MD-2, MyD88 and IL-12B expressions were upregulated in LPS-challenged animals as compared to that in the control group but were substantially attenuated in rats supplemented with catechin.

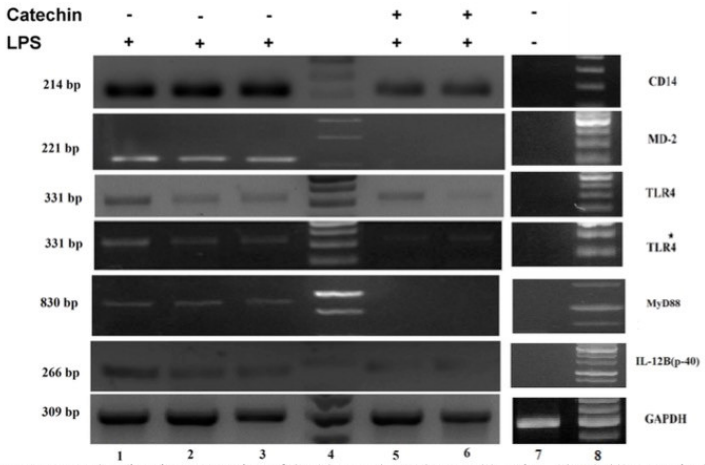

Figure 3: RT-PCR showing expression of CD14, TLR4, MD2, MyD88 and IL-12B (p40) genes in the livers of LPS-challenged rats with and without catechin supplementation. Total RNA was isolated by using the RNeasy Mini Kit (Qiagen) from liver tissues of the rats subjected to indicated treatments. RNA was then reverse transcribed and subjected to PCR using CD14, TLR4, MD2, MyD88, IL-12B and GAPDH-specific primers. GAPDH mRNA level was used as an internal control.

3.4. Hepatic oxidative markers

LPS caused a significant increase in lipid peroxidation as compared to the control rats. However, in the presence of catechin, MDA levels were decreased significantly as shown in Table 2.

Table 2
Estimation of lipid peroxidation (MDA) levels in liver samples.

GROUPS	(nmoles of MDA/mg protein)
CONTROL	145.33±17.33
LPS	257.21±21.45
LPS+CT	158.58±35.34

*Values are expressed as mean ± S.D. of five different observations. *p< 0.05 vs. control; #p < 0.05 vs. LPS; LPS + CT: Catechin administered for 15 days prior to LPS challenge.*

Nitrite levels were also significantly higher in LPS-challenged group as compared to the control counterpart. Supplementation with catechin significantly reduced the nitrite levels as shown in Table 3.

Table 3
Estimation of Nitrite levels in liver samples.

GROUPS	(micromoles of nitrite/mg protein)
CONTROL	4±0.23
LPS	14±0.45
LPS+CT	5±0.55

*Values are expressed as mean ± S.D. of five different observations. *p< 0.05 vs. control; #p < 0.05 vs. LPS; LPS + CT: Catechin administered for 15 days prior to LPS challenge.*

*3.5. Hepatic antioxidative enzyme*s

LPS challenge significantly reduced the activity of liver SOD as compared to its activity in the control group. Supplementation with catechin significantly increased the antioxidant activity of SOD as shown in Table 4.

Table 4
Estimation of SOD levels in liver samples.

GROUPS	(U/mg protein)
CONTROL	4.98±1.33
LPS	2.01±1.45
LPS+CT	4.23±0.34

*Values are expressed as mean ± S.D. of five different observations. *p< 0.05 vs. control; #p < 0.05 vs. LPS; LPS + CT: Catechin administered for 15 days prior to LPS challenge.*

LPS challenge significantly reduced the activity of liver catalase as compared to its activity in the control group. Supplementation with catechin significantly increased the activity of catalase as shown in Table 5.

Table 5
Estimation of catalase levels in liver samples.

GROUPS	Milimoles of catalase/mg protein
CONTROL	156.46±54.39
LPS	63.21±29.76
LPS+CT	145.35±44.39

*Values are expressed as mean ± S.D. of five different observations. *p< 0.05 vs. control; #p < 0.05 vs. LPS; LPS + CT: Catechin administered for 15 days prior to LPS challenge.*

DISCUSSION

In the recent past, we have reported that catechin downregulates NF-κB signalling and prevents endotoxin-induced liver injury (Bharrhan S et al. 2012). Although NF-κB is the key intrinsic molecule playing a pivotal role in LPS induced inflammatory response, its activation requires binding of LPS to host cells via receptor mediated signalling. Therefore, the first step in the extrinsic signalling pathway leading to NF-κB activation and subsequent pro-inflammatory gene transcription is the recognition of the LPS molecule by specific cells of the immune system. LPS is first captured by LPS-binding protein (LBP), then LPS-LBP complex interacts with a membrane form of CD14 (mCD14) on the surface (Schumann RR et al. 1990; Tobias PS et al. 1992; Ulevitch RJ and Tobias PS, 1995; Yu B and Wright SD, 1996; Su GL et al. 2002). mCD14 is anchored into the cell surface only through a glycosylphosphatidylinositol (GPI) -linkage. It lacks a transmembrane component and thus is devoid of the ability to transduce cytoplasmic signals (Akashi S et al. 2000), unlike TLR4, a transmembrane protein, which is specific for LPS. TLR4, in association with MD2, recognizes the LPS-CD14 complex. Thus, CD14, TLR4 and MD2 are the major extrinsic components of receptor complex, which play an important role in signal transduction of LPS. The TLR4/MD2 complex activates the signalling pathway via the recruitment of adapter proteins, such as MyD88 and Toll-IL-1R domain-containing adapter-inducing IFN-b (TRIF), to its intracellular Toll-IL-1R domain (Takeda K and Akira S, 2004). In the present study, we found that LPS challenge enhances the expression of CD14, TLR4, MD2 and MyD88. It was reported earlier that CD14 transgenic mice were susceptible to LPS-induced liver damage while CD14 knockout mice were resistant to LPS Fenton MJ and Golenbock DT, 1998). Similarly, TLR4 deficient/TLR4 mutant mice have been shown to be hyporesponsive to LPS, demonstrating that TLR4 is a critical receptor for LPS signalling. The expressions of these molecules were found to be downregulated upon catechin supplementation. Earlier, it has been reported that bicyclol, a synthetic anti-hepatic drug suppresses transcription of CD14 and TLR4 (Zhao JI et al. 2008). To the best of our knowledge, it is the first report on catechin showing modulation of these important molecules of the LPS-receptor complex. It may be recalled here that suppression of these molecule might have downregulated the expression of NF-κB and further going downstream to TNF-α and iNOS mediated inflammatory response to LPS, as observed in our previous study (Bharrhan S et al. 2012). Specific blockade of a single inflammatory mediator such as TNF-α is now not being perceived to be a worthwhile strategy in human diseases because numerous other cytokines would replace its function. Recent attention has turned to the role of interleukin (IL-12) family of cytokines in the pathogenesis and potential treatment of inflammatory diseases. IL-12 family of cytokines includes IL-12 and IL-23, both of which share IL-12p40 subunit. Thus, anti-IL-12p40 subunit can inhibit either one of the cytokines (Barrie AM and Plevy SE, 2005). Therefore, in the present study expression of IL-12p40 was also studied. Catechin downregulated the expression of IL-12p40 which, remains elevated in the non-supplemented endotoxin challenged rats, thereby indicating its potential to suppress IL-12B levels in addition to TNF-α.

Further, it has been established that cellular sensitivity or resistance to TNF-α is also correlated with decreased or increased levels of SOD, respectively (Rishi P et al. 2008). In the present study also, increased levels of TNF-α after LPS injection correlated with the increased level of lipid peroxidation and nitrite along with the decreased activities of SOD and catalase. However, catechin attenuated the rise of liver malondialdehyde levels, increased the levels of hepatic antioxidants after LPS-challenge and the liver injury was ameliorated. These effects might have been due to the antioxidative property of catechin in the supplemented rats. It may be attributed to the ability of catechin to localize near the membrane surface, trapping directly any radical generated in the lipid environment of the membranes as well as ROS generated in the aqueous phase. This might block the superoxide radicals in the tissues and decrease SOD consumption thus enhancing SOD activity. Endotoxin challenge has been reported to disrupt the permeability of plasma membrane causing the leakage of enzymes from hepatocytes that leads to

alleviation in the levels of serum transaminases such as ALT, AST and ALP (Singh A et al. 2011). Downregulation of the extrinsic and intrinsic molecules and upregulation of antioxidant enzymes might have reduced the levels of serum transaminases responsible for functional activity in the liver. Further, amelioration of the functional activity of these enzymes upon catechin supplementation correlated well with the clinical manifestations of the disease, i.e. histopathological alterations in the hepatic tissue following LPS insult as observed in the present study. Taken together, the findings of our previous and present study, catechin may be perceived as a chain breaking inhibitor of signalling cascade initiated by LPS receptor complex.

CONCLUSION

It is concluded from the study that catechin has the potential to attenuate endotoxin-induced liver injury by downregulating the expression of its receptors, CD14, TLR4, MD2 and MyD88 and further going downstream the signaling cascade including IL-12B and by enhancing the hepatic antioxidant system. Our data provides a possible mechanism for catechin to act as a chain breaking inhibitor of endotoxin-induced signalling cascade, which provides the basis to develop immunomodulators like catechin for prevention of endotoxin-mediated hepatotoxicity. It may also be inferred that catechin, if not alone, may at-least be used in conjunction with the conventional drugs for the treatment of liver diseases. This may lower the effective dose of drugs used for the treatment, thereby reducing the associated side effects besides conferring health benefits to the individuals.

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