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Lygodium flexuosum extract down regulates the expression of proinflammatory cytokines in CCl_4 -induced hepatotoxicity

Pallara Janardhanan Wills[1,2*], Velikkakathu Vasumathi Asha[1]

[1]*Plant Based Bioactives and Disease Biology Laboratory, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala 695014, India*
[2]*MIMS Research Foundation, Malabar Institute of Medical Sciences, Calicut-673007, Kerala, India*

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ABSTRACT

Objective: To examine the downregulation of proinflammatory cytokines in a time dependant manner on carbon tetrachloride induced toxicity in experimental animals. **Methods:** CCl_4 (150 μL/100 g) was dissolved in corn oil (1:1 v/v %) and administered orally. Group I was treated as normal control and received corn oil on 8th day. Group II was toxic control and was given a single dose of CCl_4 on 8th days. Group III was treated with *Lygodium flexuosum* (*L. flexuosum*) *n*-hexane extract (200 mg/kg) for 8 days and on 8th day a single dose of CCl_4 was received. Group IV (negative control) received *L. flexuosum n*-hexane extract (200 mg/kg) alone for 8 days. **Results:** Treatment with *n*-hexane extract prior to the administration of CCl_4 significantly prevented an increase in serum AST, ALT, LDH activity and lipid peroxidation and prevented the depletion of glutathione (GSH). Rats treated with *L. flexuosum* had reduced mRNA levels of TGF-β 1, TNF-α and IL-1 β genes in liver of CCl_4 intoxicated rats when compared to CCl_4 control as evidenced by RT-PCR. **Conclusions:** The data suggest that *L. flexuosum*, a widely available fern, significantly reduces CCl_4 induced acute hepatotoxicity by down-regulating the expression of pro-inflammatory cytokines in rats.

1. Introduction

Cytokines play a major role in the process of acute liver injury and repair. Two cytokines that appear to be candidates for a role in the process of liver injury and repair are transforming growth factor-β 1 (TGF-β 1) and tumor necrosis factor-α (TNF-α). Transforming growth factor-β 1 increases the expression of extracellular matrix proteins (ECM), thus it could be important in modulating hepatic regeneration or in promoting matrix synthesis in acute liver injury. Tumor necrosis factor-α induces neutrophil accumulation, degranulation, adhesion and phagocytosis and enhances fibroblast proliferation[1,2]. Interleukin-1 β is produced by activated Kupffer cells known to have a central role in inflammatory responses. IL-1 β induces the release of chemotactic mediators, increase the expression of adhesion molecules, activate neutrophils and endothelial cells[3,4].

Antioxidants and radical scavengers have been used to study the mechanism of CCl_4 toxicity as well as the mechanisms to protect liver cells from CCl_4 induced damage by breaking the chain reaction of lipid peroxidation[5-7]. *Lygodium flexuosum* (Lygodiaceae) (*L. flexuosum*) is a climbing fern found all over the Western Ghat region of Kerala, India. Leaf paste is used to cure jaundice by Kadar tribes of Western Ghats of India[8]. The rhizome and root is used in indigenous medicine for the treatment of jaundice by Rabha, Oraon & Mech tribes of West Bengal, India[9]. Chemical characterization revealed the presence of saponins (27.6%), bitter principles (4.6%), sterols (2.0%) and triterpene alcohols (1.7%) in the *n*-hexane extract of *L. flexuosum*[10,11]. Activity of *L. flexuosum n*-hexane extract was further studied and showed efficacy in chronic disease models[12-14]. Earlier, we studied the dose response (200 mg/kg and 100 mg/kg) effect of *L. flexuosum* extract in carbon tetrachloride induced rats[11]. The present work was carried out to study the effectiveness of *L. flexuosum* extract (200 mg/kg) in down regulating the expression of proinflammatory cytokines TGF-β 1, TNF-α and IL-1 β at different time periods (6, 24 and 48 h) after CCl_4 induced liver injury.

*Corresponding author: Pallara Janardhanan Wills, Plant Based Bioactives and Disease Biology Laboratory, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala 695014, India.
Tel: 91-471-2345899
Fax: 91-471-2348096
E-mail: willspj@yahoo.co.in; ashavv@rediffmail.com
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2. Materials and methods

2.1. Chemicals

Carbon tetrachloride from Merck, Mumbai, India. Corn oil, thiobarbituric acid, dithiobis-2-nitrobenzoic acid and LDH assay kit from Sigma Chemical Co., St. Louis, MO, USA. AST, ALT, glucose, urea, triglycerides, cholesterol and creatinine assay kits were from Dialab, Austria. Trizol reagent from Invitrogen, Carlsbad, CA, USA. AMV reverse transcriptase, oligo dT primer, RNasin Ribonuclease inhibitor, RNase free DNase, dNTPs, Taq polymerase and nuclease free water were obtained from Promega, Madison, WI, USA. 100 bp DNA ladder was from Promega and New England Biolabs, Beverly, MA, USA. All other chemicals were of high purity grade.

2.2. Experimental animals

Male Wistar rats (150-160 g) were used in the experiments. Animals were provided standard pellet diet and water *ad libitum* and maintained on a 12 h light/dark cycle. Animal studies were approved by the CPCSEA and conducted humanely. Animals were divided into four groups. Group I was treated as Normal control, Group II was CCl_4 control, Group III was CCl_4 + *L. flexuosum* extract and Group IV was given *L. flexuosum* extract alone. Each group comprised of eighteen animals and six animals each sacrificed at 6th, 24th and 48th h.

2.3. Plant material and preparation of plant extracts

Plants were collected from its natural habitat at Thiruvananthapuram district during the month of November and authenticated. A voucher specimen (ETHNO.2) is maintained in the Institute. Plants were cleaned, dried under shade at room temperature and powdered. 10 g of dried powder of the whole plant of *L. flexuosum* was soxhlet extracted with 400 mL of *n*-hexane for 24 h. The step was repeated with fresh powder and solvent until the required quantity was achieved. The extract was concentrated in a rotary evaporator (yield 4.0%) and the concentrate was suspended in 5% Tween 80.

2.4. Study protocol

CCl_4 (150 μL/100 g) [15] was dissolved in corn oil (1:1 v/v %) and administered orally. Group I was treated as Normal control and received corn oil on 8th day. Group II was toxic control and was given a single dose of CCl_4 on 8th day. Group III was treated with *L. flexuosum n*-hexane extract (200 mg/kg) for 8 d and on 8th day a single dose of CCl_4 was received. Group IV received *L. flexuosum n*-hexane extract (200 mg/kg) alone for 8 d. Under pentothal sodium anesthesia, blood samples were collected from each rat at different time points (6, 24 and 48 h) after CCl_4 administration. Dissected liver was obtained from each rat at different time points (6, 24 and 48 h) after CCl_4 administration for hepatic glutathione and lipid peroxidation estimation and RNA isolation. ALT, AST and LDH values were estimated by using semi auto analyzer. Both glutathione and lipid peroxidation levels were estimated spectrophotometrically.

2.5. Serum AST, ALT and LDH analysis

Serum aspartate aminotransferase (AST)[16], alanine aminotransferase (ALT)[17] and lactate dehydrogenase (LDH)[18] concentrations have been used to evaluate CCl_4 induced liver injury.

2.6. Hepatic glutathione estimation

Reduced glutathione (GSH) was measured by its reaction with DTNB that gives a yellow coloured complex with absorption maximum at 412 nm[19].

2.7. Estimation of lipid peroxides

Lipid peroxidation in liver was ascertained by the formation of malondialdehyde (MDA) and measured by the thiobarbituric acid reactive substance (TBARS) method[20].

2.8. Gene expression studies

Approximately 100 mg samples of liver tissue from each group at different time intervals were homogenized and total RNA was extracted using Trizol Reagent. RNA purity and concentration were assessed by A260/A280 absorption. One microgram of total RNA from each liver sample were subjected to reverse transcription (RT) by AMV reverse transcriptase in a 30 μL reaction volume containing 1 μg Oligo dT, 40 U RNasin inhibitor and 10 mM dNTPs.

Primers for TGF-β1, TNF-α, IL-1β and G3PDH were synthesized by Sigma Genosys, Cambrigdeshire, UK based on published sequences[21,22] containing the following bases.

TGF-β1: F, 5′-TATAGCAACAATTCCTGGCG-3′
TGF-β1: R, 5′-TGCTGTCACAGGAGCAGTG-3′
TNF-α: F, 5′-GTAGCCCACGTCGTAGCAAA-3′
TNF-α: R, 5′-CCCTTCTCCAGCTGGGAGAC-3′
IL-β1: F, 5′-TGATGTTCCCATTAGACAGC-3′
IL-β1: R, 5′-GAGGTGCTGATGTACCAGTT-3′
G3PDH: F, 5′-CCCTTCATTGACCTCAACTACATGG-3′
G3PDH: R, 5′-CATGGTGGTGAAGACGCCAG-3′

All the amplification reactions were carried out in 25 mL volume that included 12.5 pmol of each primer, 1.25 U Taq polymerase and 10 mM dNTPs. Thirty cycles of denaturation at 94 ℃ for 30 s, annealing for 1 min and extension at 72 ℃ for 1 min. An initial denaturation at 94 ℃ for 4 min and a final extension at 72 ℃ for 7 min were given before and after 30 cycles respectively (iCycler, BioRad, Hercules, USA).

The PCR products were size fractionated on 1.8% agarose gels, visualized by ethidium bromide staining and scanned by Multi Imager FX (BioRad, Hercules, USA). The size and density of the bands produced by the PCR products were quantified using densitometry software (Quantity One, BioRad, Hercules, USA). The transcripts were normalized against G3PDH. The identities of the PCR products were confirmed by automated DNA sequencing analysis (ABI-310 Prism, Perkin Elmer, Foster City, CA, USA).

2.9. Statistical analysis

Experiments were performed with 6 rats per group with values presented as Mean ± S.D. Statistical significance were determined by one way ANOVA followed by Tukey's post hoc analysis. *P*-values less than or equal to 0.05 were considered significant.

3. Results

3.1. Serum AST, ALT and LDH levels

Rats receiving *L. flexuosum* extract alone results no significant changes in serum AST, ALT and LDH levels compared to those of normal rats. In rats receiving CCl_4 alone, AST, ALT and LDH levels were significantly increased at 6, 24 and 48 h after CCl_4 administration. In contrast, in rats receiving CCl_4 and *L. flexuosum* extract, there were significantly less elevations of serum AST, ALT and LDH levels than in rats received CCl_4 alone (Table 1-3).

3.2. Hepatic glutathione levels

Hepatic glutathione levels (nmol/mg protein) significantly decreased in CCl_4 intoxicated rats at each time points. *L. flexuosum* treatment significantly increased the glutathione levels in hepatic tissues and almost completely restored the GSH level to the normal at 48 h after CCl_4 administration. Administration with CCl_4 resulted in a 37.1, 30.9 and 21.8% decrease in the level of hepatic glutathione (GSH) than the untreated normal control group at 6, 24 and 48 h respectively. Treatment with *L. flexuosum* treatment significantly reduced the toxicity of CCl_4 and there were 11.9, 3.3 and 1.2% decrease of GSH than that of the untreated normal control group at 6, 24 and 48 h respectively (Table 4).

3.3. Lipid peroxidation levels

Hepatic MDA levels were significantly elevated in rats receiving CCl_4 at each time point compared to normal rats. Treatment of rats with CCl_4 and *L. flexuosum* extract significantly prevented the elevation of MDA formation compared to CCl_4 alone treated rats and MDA levels of liver tissues were almost completely restored to normal levels at 48 h after CCl_4 administration. Toxicity induced by CCl_4 resulted in 72.5, 66.3 and 43.6% increase in the level of hepatic malondialdehyde (MDA) than the untreated normal control group at 6, 24 and 48 h respectively. Treatment with *L. flexuosum* treatment significantly reduced the toxicity of CCl_4 and there were 37.5, 19.0 and 6.3% increase than that of the untreated normal control group at 6, 24 and 48 h respectively (Table 5).

3.4. L. flexuosum extract reduced levels of TGF-β1 transcript in CCl_4 treated rat livers

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique is used to assess the effect of treatment with *L. flexuosum* *n*-hexane extract on the transcription of various genes associated with acute hepatotoxicity induced by CCl_4 at different time points. The level of expression of each of the transcripts was normalized with G3PDH in the same sample and expressed as a ratio.

Treatment with the *L. flexuosum* extract significantly decreased the extent of acute hepatotoxicity in the CCl_4 treated rats as determined by the levels of transforming growth factor-β1. High levels of TGF-β1 are associated with hepatic necrosis and we, therefore, investigated the effect of treatment with *L. flexuosum* extract on TGF-β1 transcript levels at 6, 24 and 48 h after CCl_4 administration. Amplified fragments specific to TGF-β1 or G3PDH transcripts yielded products of 162 and 209 bp respectively followed by sequence analysis and were confirmed to be TGF-β1 and G3PDH respectively. The mean TGF-β1: G3PDH mRNA ratio of the CCl_4 control group was 43, 40.4 and 27.7% higher than that of the untreated normal control group at 6, 24 and 48 h respectively. The mean TGF-β1: G3PDH mRNA ratio of the *L. flexuosum* treatment group was 41.1, 22.0 and 10.3% higher than that of the untreated normal control group at 6, 24 and 48 h respectively Treatment with *L. flexuosum* caused a marked reduction in the level of expression of the TGF-β1 transcript. The mean TGF-β1: G3PDH mRNA ratio of the treatment group were 3.2, 23.5 and 19.4% lower than that of the CCl_4 control group at 6, 24 and 48 h respectively. Also, the levels of TGF-β1 transcript in treatment group *L. flexuosum* alone were comparable to normal group at each time points (Figure 1).

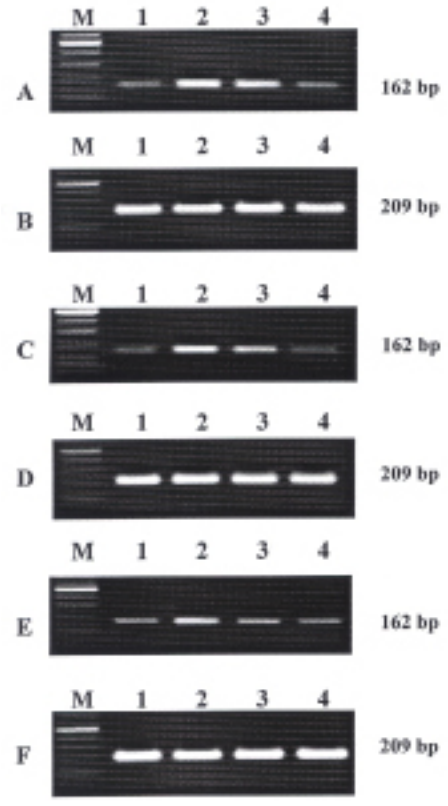

Figure 1. Treatment with *L. flexuosum* decreased the expression of transforming growth factor-β1 (TGF-β1) transcript. A, C and E are the products of the amplification of TGF-β1 (162 bp) or B, D and F are G3PDH (209 bp) at different time points, 6th h, 24th h and 48th h respectively. The sizes of fragments in 100 bp molecular weight marker (M) in base pairs. 1: Normal control, 2: CCl_4 control, 3: CCl_4 + *L. flexuosum* and 4: *L. flexuosum* alone groups.

3.5. L. flexuosum extract reduced levels of TNF-α transcript in CCl_4 treated rat livers

Fragments specific to TNF-α was amplified by RT-PCR followed by sequence analysis confirmed that the 346 bp band corresponded to TNF-α transcript. Administration with CCl_4 resulted in a 41.5, 40.6 and 16.3% increase in the level of expression of TNF-α mRNA than the untreated normal control group at 6, 24 and 48 h respectively. The mean TNF-α: G3PDH mRNA ratio of the *L. flexuosum*

treatment group was 39.5, 27.1 and 1.9% higher than that of the untreated normal control group at 6, 24 and 48 h respectively Treatment with *L. flexuosum* significantly decreased the TNF-α : G3PDH ratio to 3.3, 18.6 and 14.7% of the disease control group at 6, 24 and 48 h respectively (Figure 2).

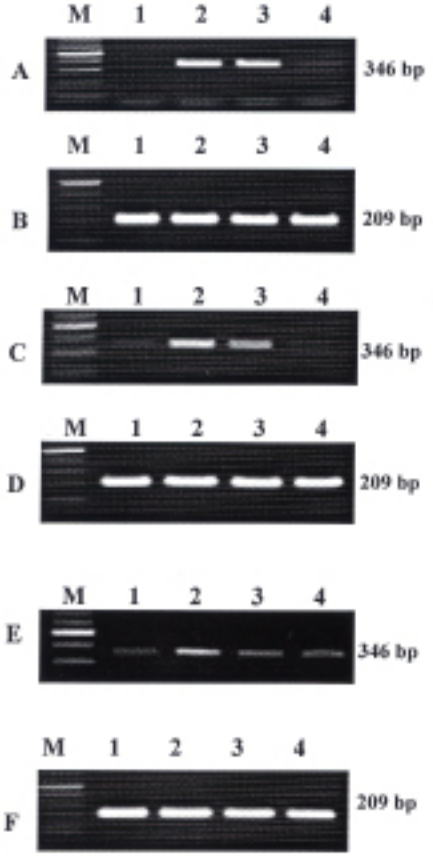

Figure 2. Treatment with *L. flexuosum* decreased the expression of tumor necrosis factor-α (TNF-α) transcript. A, C and E are the products of the amplification of TNF-α (346 bp) or B, D and F are G3PDH (209 bp) at different time points, 6th h, 24th h and 48th h respectively. The sizes of fragments in 100 bp molecular weight marker (M) in base pairs. 1: Normal control, 2: CCl_4 control, 3: CCl_4 + *L. flexuosum* and 4: *L. flexuosum* alone groups.

3.6. L. flexuosum extract reduced levels of IL-1β transcript in CCl_4 treated rat liver

Treatment with the *L. flexuosum* extract significantly decreased the extent of acute hepatotoxicity in the CCl_4 treated rats as determined by the levels of IL-1β. Fragments specific to IL-1β was subjected to sequence analysis confirmed that the 378 bp band corresponded to IL-1β transcript. Intoxication with CCl_4 resulted in a 48.1%, 32.6% and 27.0% increase in the level of expression of IL-1β mRNA than the untreated normal control group at 6, 24 and 48 h respectively. The mean IL-1β : G3PDH mRNA ratio of the *L. flexuosum* treatment group was 33.9%, 23.0% and 10.2% higher than that of the untreated normal control group at 6, 24 and 48 h respectively. Treatment with *L. flexuosum* significantly decreased the IL-1β : G3PDH ratio to 21.4%, 9.6% and 12.3% of the CCl_4 control group at 6, 24 and 48 h respectively (Figure 3).

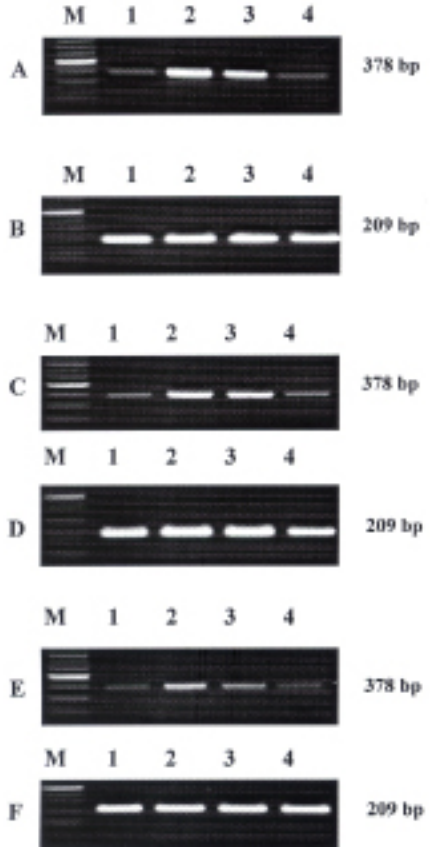

Figure 3. Treatment with *L. flexuosum* decreased the expression of interleukin-1β (IL-1β) transcript. A, C and E are the products of the amplification of IL-1β (378 bp) or B, D and F are G3PDH (209 bp) at different time points, 6th h, 24th h and 48th h respectively. The sizes of fragments in 100 bp molecular weight marker (M) in base pairs. 1: Normal control, 2: CCl_4 control, 3: CCl_4 + *L. flexuosum* and 4: *L. flexuosum* alone groups.

Table 1

Serum aspartate aminotransferase (AST) levels during the treatment with *L. flexuosum* at different time periods, 6th h, 24th h and 48th h in rats during carbon tetrachloride exposure (n=6).

Treatment Groups	AST (IU/L) 6th h	AST (IU/L) 24th h	AST (IU/L) 48th h
Normal control	150.6 ± 5.9	151.4 ± 5.2	151.1 ± 4.5
CCl_4 control (150 μL/100 g; oral)	337.6 ± 10.2*	762.2 ± 30.1*	1 151.2 ± 43.8*
L. flexuosum extract (200 mg/kg) + CCl_4	186.0 ± 8.8†	261.5 ± 10.6†	334.4 ± 14.8†
L. flexuosum extract alone (200 mg/kg)	154.8 ± 5.7†	150.5 ± 5.6†	151.0 ± 6.5†

*$P<0.05$ *versus* normal control, †$P<0.05$ *versus* CCl_4 control, Values are Mean ± S.D.

Table 2

Serum alanine aminotransferase (ALT) levels during the treatment with *L. flexuosum* at different time periods, 6th h, 24th h and 48th h in rats during carbon tetrachloride exposure (n=6).

Treatment groups	ALT (IU/L) 6th h	ALT (IU/L) 24th h	ALT (IU/L) 48th h
Normal control	52.6 ± 3.7	53.5 ± 3.2	51.9 ± 3.4
CCl_4 control (150 μL/100 g; oral)	184.1 ± 9.3*	328.6 ± 18.6*	748.1 ± 23.9*
L. flexuosum extract (200 mg/kg) + CCl_4	96.0 ± 5.8†	163.3 ± 10.2†	238.7 ± 11.5†
L. flexuosum extract alone (200 mg/kg)	54.0 ± 3.9†	56.1 ± 3.9†	52.7 ± 3.3†

*P<0.05 *versus* normal control, †P<0.05 *versus* CCl_4 control, Values are Mean ± S.D.

Table 3

Serum lactate dehydrogenase (LDH) levels during the treatment with *L. flexuosum* at different time periods, 6th h, 24th h and 48th h in rats during carbon tetrachloride exposure (n=6).

Treatment groups	LDH (IU/L)6th h	LDH (IU/L)24th h	LDH (IU/L)48th h
Normal control	156.9 ± 4.2	156.3 ± 4.7	156.6 ± 4.7
CCl4 control(150 μL/100 g; oral)	1 344.4 ± 38.5*	1 816.3 ± 80.6*	2 178.8 ± 111.7*
L. flexuosum extract(200 mg/kg) + CCl_4	458.6 ± 20.9†	535.8 ± 34.6†	626.4 ± 39.0†
L. flexuosum extract alone (200 mg/kg)	150.5 ± 6.3†	155.4 ± 6.0†	159.0 ± 4.0†

*P<0.05 *versus* normal control, †P<0.05 *versus* CCl_4 control, Values are Mean ± S.D.

Table 4

Hepatic glutathione (GSH) levels during the treatment with *L. flexuosum* at different time periods, 6th h, 24th h and 48th h in rats during carbon tetrachloride exposure (n=6).

Treatment groups	GSH (nmol/mg protein)6th h	GSH (nmol/mg protein)24th h	GSH (nmol/mg protein)48th
Normal control	0.468 ± 0.004	0.475 ± 0.004	0.472 ± 0.003
CCl_4 control(150 μL/100 g; oral)	0.294 ± 0.007*	0.328 ± 0.006*	0.369 ± 0.005*
L. flexuosum extract(200 mg/kg) + CCl_4	0.412 ± 0.005†	0.459 ± 0.005†	0.466 ± 0.004†
L. flexuosum extract alone (200 mg/kg)	0.479 ± 0.004†	0.480 ± 0.004†	0.478 ± 0.004†

*P<0.05 *versus* normal control. †P<0.05 *versus* CCl_4 control. Values are Mean ± S.D.

Table 5

Hepatic malondialdehyde (MDA) levels during the treatment with *L. flexuosum* at different time periods, 6th h, 24th h and 48th h in rats during carbon tetrachloride exposure (n=6).

Treatment groups	MDA (nmol/mg tissue)6th h	MDA (nmol/mg tissue)24th h	MDA (nmol/mg tissue)48th h
Normal control	0.585 ± 0.004	0.587 ± 0.004	0.586 ± 0.004
CCl_4 control (150 μL/100 g; oral)	2.140 ± 0.030*	1.740 ± 0.010*	1.040 ± 0.010*
L. flexuosum extract (200 mg/kg) + CCl_4	0.944 ± 0.008†	0.725 ± 0.007†	0.626 ± 0.006†
L. flexuosum extract alone (200 mg/kg)	0.585 ± 0.004†	0.586 ± 0.004†	0.585 ± 0.004†

*P<0.05 *versus* normal control. †P<0.05 *versus* CCl_4 control. Values are Mean ± S.D.

4. Discussion

Lipid peroxidation in liver associated with CCl_4 exposure occurs early in the injury and it has been associated as a critical event because it is associated with reductions of enzyme activity in the endoplasmic reticulum, fatty acid transport and protein synthesis. CCl_4 treatment of hepatocytes causes a sustained elevation of intracellular Ca^{2+} by affecting Ca^{2+} regulation at the level of the endoplasmic reticulum, plasma membrane and mitochondria[23]. This elevation in cytosolic Ca^{2+} precedes evidence of necrosis as determined by leakage of aminotransferases like aspartate aminotransferase (AST) and alanine aminotransferase (ALT) into the extracellular medium. It is also associated with covalent binding of CCl_4-derived free radicals to cellular lipids and lipid peroxidation[24]. Necrotizing agents like CCl_4 produce significant injury to hepatic parenchyma to cause increases in AST, ALT and LDH levels at different time intervals in the serum. Since *L. flexuosum* prevented the effect of CCl_4 on serum AST, ALT and LDH levels, it is reasonable to suggest that *L. flexuosum* prevented the severity of acute liver injury. *L. flexuosum* showed ability to prevent CCl_4 induced levels of MDA content suggesting that *L. flexuosum* inhibit lipid peroxidation in the liver. Increased levels of MDA at 6-24 h after CCl_4 administration was normalized at 48 h after CCl_4 administration. In our experiment, it was also found that the hepatic glutathione level was higher in rats receiving CCl_4 and *L. flexuosum* extract than in rats exposed to CCl_4 alone at 6-48 h and the GSH level was replenished to a greater extent at 48 h after CCl_4 administration, which was comparable to normal values. Therefore, *L. flexuosum* appears to exert its protective effect against CCl_4 induced hepatic injury by protecting the integrity of the cell membrane and the free radical scavenging activity of the extract.

The mRNA levels of proinflammatory cytokines involved in acute hepatotoxicity namely, TGF-β1, TNF-α and IL-1β were analyzed. Kupffer cells, the resident macrophages of liver following activation of CCl_4 release TGF-β1, TNF-α and IL-1β. TGF-β1 is an indicator of pathologic cell status; it is induced in Kupffer cells after CCl_4 intoxication. CCl_4 causes oxidative stress to mitochondria that via intricate signaling cascades involving the activation of caspases, results in apoptosis, or cirrhosis and necrosis during TNF-α activation. IL-1β is also increased following CCl_4 induced TNF-α release. This inflammatory reaction is predominantly carried out by Kupffer cells[25].

The expression levels of proinflammatory cytokines was increased in CCl_4 induced liver injury. The treatment with *L. flexuosum* extract decreased this effect to a great extent within 6-48 h of the time period. A sharp decline was noticed in the expression levels of TGF-β1 and TNF-α in the *L. flexuosum* treated rats 24th h after CCl_4 exposure. In CCl_4 treated rats, TGF-β1 and TNF-α were equally expressed at the 6th and 24th h level, even though TNF-α expression level declined at 48 h. This is probably due to the stimulation of TNF-α gene expression within the confines of the first 24 h when inflammation and necrosis predominate. The subsequent rise in TGF-β1 gene expression relative to TNF-α occurred when the liver was halting its phase of regeneration and was engaged in matrix repair and remodeling[1]. In *L. flexuosum* treated rats, TNF-α level was strongly reduced at 6th, 24th and 48th h levels and was comparable to normal values indicating the protection by the extract against the inflammation caused by the toxin. In *L. flexuosum* treated rats, interleukin-1β showed a marked reduction in their expression levels at the 6th h after CCl_4 induced hepatic injury and subsequent decline in the levels during the 24-48th h indicates the anti-inflammatory role of *L. flexuosum*. Moreover, the expression of TGF-β1, TNF-α and IL-1β mRNA levels in group IV (*L. flexuosum* extract alone) was comparable with normal control show the non-toxic effect of *L. flexuosum*.

In summary, the protective effects of *L. flexuosum* extract may possibly be attributed to the combination of more than one biological compound that are strong antioxidants in down regulating the proinflammatory cytokines in carbon tetrachloride induced hepatotoxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Czaja MJ, Flanders KC, Biempica L, Klein C, Zern MA, Weiner FR. Expression of trumor necrosis factor-α and transforming growth factor-β1 in acute liver injury. *Growth Factors* 1989; **1**: 219-226.
[2] Montes AS, Rizo VD, Borunda JA. Differential gene expression of proinflammatory and anti-inflammatory cytokines in acute and chronic liver injury. *Hepatol Res* 2000; **16**: 181-194.
[3] Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J. Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J Clin Invest* 2001; **107**: 1529-1536.
[4] Bassiouny AR, Zaky A, Kandeel KM. Alteration of AP-endonuclease1 expression in curcumin-treated fibrotic rats. *Ann Hepatol* 2011; **10**: 516-530.
[5] Chao J, Lee MS, Amagaya S, Liao JW, Wu JB, Ho LK, et al. Hepatoprotective effect of shidagonglao on acute liver injury induced by carbon tetrachloride. *Am J Chin Med* 2009; **37**: 1085-1097.
[6] Kim SH, Cheon HJ, Yun N, Oh ST, Shin E, Shim KS, et al. Protective effect of a mixture of *Aloe vera* and *Silybum marianum* against carbon tetrachloride-induced acute hepatotoxicity and liver fibrosis. *J Pharmacol Sci* 2009; **109**: 119-127.
[7] Shah VN, Shah MB, Bhatt PA. Hepatoprotective activity of punarnavashtak kwath, an Ayurvedic formulation, against CCl_4-induced hepatotoxicity in rats and on the HepG2 cell line. *Pharm Biol* 2011; **49**: 408-415.
[8] Henry AN, Hosagoudar VB, Ravikumar K. Ethno-medico-botany of the Southern Western Ghats of India. In: Jain SK. (ed.). *Ethnobiology in Human Welfare*. Proceedings of IV International Congress of Ethnobiology held at Lucknow, India, 17-21 November 1994, New Delhi: Deep publications; 1996, p. 173-180.
[9] Kumar K. Notable pertinence of *Lygodium flexuosum* (L.) Sw. in Tribal medicine of India: an ethnopharmacognostical investigation. In: Govil JN, Singh VK. (eds.) *Recent progress in medicinal plants*: *Ethnomedicine and pharmacognosy*. Vol. 1. Texas: SCI Tech Publishing LLC; 2002, p. 315-323.
[10] Wills PJ, Asha VV. Protective effect of *L. flexuosum* (L.) Sw. (Lygodiaceae) against D-galactosamine-induced liver injury in rats. *J Ethnopharmacol* 2006a; **108**: 116-123.
[11] Wills PJ, Asha VV. Protective effect of *L. flexuosum* (L.) Sw. extract against carbon tetrachloride-induced acute liver injury in rats. *J Ethnopharmacol* 2006b; **108**: 320-326.
[12] Wills PJ, Suresh V, Arun M, Asha VV. Antiangiogenic effect of *L. flexuosum* against N-nitrosodiethylamine-induced hepatotoxicity in rats. *Chem-Biol Interact* 2006; **164**: 25-38.
[13] Wills PJ, Asha VV. Protective mechanism of *L. flexuosum* extract in treating and preventing carbon tetrachloride induced hepatic fibrosis in rats. *Chem-Biol Interact* 2007; **165**: 76-85.
[14] Wills PJ, Asha VV. Chemopreventive action of *Lygodium flexuosum* extract in human hepatoma PLC/PRF/5 and Hep 3B cells. *J Ethnopharmacol* 2009; **122**: 294-303.
[15] Simile MM, Banni S, Angioni E, Carta G, De Miglio MR, Muroni MR, et al. 5′-Methylthioadenosine administration prevents lipid peroxidation and fibrogenesis induced in rat liver by carbon-tetrachloride intoxication. *J Hepatol* 2001; **34**: 386-394.
[16] Bergmeyer HU, Bowes GN, Horder M, Moss DW. Provisional recommendations on IFCC methods for the measurement of catalytic concentrations of enzymes: part 2. IFCC method for aspartate aminotransferase. *Clin Chim Acta* 1976; **70**: F19-F42.
[17] Bergmeyer HU. IFCC methods for the measurement of catalytic concentrations of enzymes: part 3. IFCC method for alanine aminotransferase (l-alanine: 2-oxoglutarate aminotransferase). *Clin Chim Acta* 1980: **105**; 147-154.
[18] Amador E, Dorfman LE, Wacker WEC. Serum lactic dehydrogenase: An analytical assessment of current assays. *Clin Chem* 1963; **9**: 391-401.
[19] Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. *Biochem Biophys Acta* 1979; **582**: 67-78.
[20] Ohkawa H, Ohishi W, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351-358.
[21] Wasser S, Ho JM, Ang HK, Tan CE. *Salvia miltiorrhiza* reduces experimentally induced hepatic fibrosis in rats. *J Hepatol* 1998; **29**: 760-771.
[22] Masson S, Scotte M, Francois A, Coeffier M, Provot F, Hiron M, et al. Changes in growth factor and cytokine mRNA levels after hepatectomy in rat with CCl_4-induced cirrhosis. *Am J Physiol* 1999; **277**: G838-846.
[23] Dahm JL, Jones PD. Mechanisms of chemically induced liver disease. In: Zakim D, Boyer TD. (eds) *Hepatology: a textbook of liver disease*. Vol 1. Philadelphia: WB Saunders; 1996, p. 875-890.
[24] Gitlin N. Clinical aspects of liver disease caused by industrial and environmental toxins. In: Zakim D, Boyer TD. (eds.) *Hepatology-A Textbook of Liver Disease*. Vol 2. WB Saunders: Philadelphia; 1996, p. 1018-1050.
[25] Weber LWD, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of halaoalkanes: Carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; **33**: 105-136.