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Original Article

HEPATOPROTECTIVE ACTIVITY OF AQUEOUS EXTRACT OF *CAESALPINIA BONDUC* AGAINST CCL4 INDUCED CHRONIC HEPATOTOXICITY

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ABSTRACT

Objective: The leaves of *Caesalpinia bonduc* (CB) have been used against various disorders in folk medicine including the liver disorders. Earlier, we have shown the hepatoprotective effect of CB in acute hepatotoxicity model. The present study was designed to evaluate the anti-hepatotoxic and anti-fibrotic effect of the aqueous leaf extract of CB on CCl_4 (carbon tetrachloride) induced chronic hepatotoxicity/fibrosis in Wistar rats.

Methods: Animals were divided into three groups namely; preventive, curative and prophylactic, which was further subdivided into four groups each: Group I–untreated control, group II-CCl_4 control, group III-CB+CCl_4 and group IV–silymarin+CCl_4. The aqueous extract of CB/silymarin was administered orally once, daily for eight weeks in the curative group and for four weeks in preventive and prophylactic groups respectively. The chronic liver damage/fibrosis was induced by intraperitoneal injection of CCl_4 twice a week, for four weeks in preventive and prophylactic groups and for eight weeks in the curative group. Blood samples were collected for assaying serum biochemical parameters, and the livers were excised and processed for histology.

Results: The data showed that supplementation of aqueous leaf extract of CB along with CCl_4 significantly reduced the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase(ALP), total bilirubin(TB) and prothrombin time(PT) thus further restoring the total protein(TP) and albumin(ALB) in preventive, curative and prophylactic groups when compared to CCl_4 control. Significant improvement in the microscopic structure of the liver further confirmed the hepatoprotective effect of aqueous extract of CB over the liver injury and fibrosis induced by CCl_4 in rats.

Conclusion: The study, therefore, suggests that aqueous extract of CB might provide a novel and alternative approach for treating the chronic hepatotoxicity/liver fibrosis.

Keywords: *Caesalpinia bonduc,* Liver, Chronic, CCl_4, Fibrosis, Silymarin, Hepato-protection

INTRODUCTION

The liver is a major target for the chemical induced toxicity, as it participates in the metabolism, detoxification, and secretory functions. Liver disease is a worldwide problem and in most of the cases, liver damage is due to oxidative stress. Liver damage if not managed at the right time, may progress from steatosis to chronic hepatitis, fibrosis, cirrhosis and even hepatocellular carcinoma [1]. The progressive and chronic liver assault triggers the progressive wound healing response which results in the irreversible excessive production of collagen fiber and alteration of the extracellular matrix complex [2-4]. Although the precise mechanisms of the pathogenesis of liver cirrhosis are incompletely understood, the role of free radicals and lipid peroxides in inducing this has garnered considerable attention [5].

The active management of liver disease is a major concern in medical science as there are no reliable drugs available in modern medicine. Thus, there is a phenomenal increase in the search for the effective natural products worldwide to treat or to prevent liver toxicity. Keeping up with the pace, we have undertaken the screening of the hepatoprotective activity of the leaves of locally available thorny plant-*Kantakikaranja* (*Caesalpinia bonduc*) (CB). CB belongs to the family *Fabaceae*, and this plant is found in the coastal areas of South Asian countries. Different parts of this plant are used for various purposes in ethnopharmacology. The leaves of CB are used extensively in folk medicine for live ailments. However, there are no scientific evidence to prove its hepatoprotective properties. Earlier we have shown that the CB can effectively prevent the acute hepatotoxicity [6]. The hepatoprotective effect of CB in chronic hepatotoxicity is not experimented upon. Therefore, the present study was designed to understand the role of CB in chronic hepatotoxicity conditions which leads to irreversible adverse changes in the liver structure and function.

MATERIALS AND METHODS

Procurement of plant materials and chemicals

Leaves of CB were collected in the month of February from "Soans farms," Moodabidiri, Mangalore District, Karnataka, India and was authenticated by a botanist from Mahatma Gandhi Memorial College, Udupi. A voucher specimen is deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal (PP 585). CCl_4 was procured from Merck Ltd. Mumbai, silymarin and other chemicals (ascorbic acid, 2,2'-diphenyl-1-picrylhydrazyl(DPPH), Masson's trichrome staining reagents) and assay kits for assessing the (AST, ALT, ALP, total protein, Bilirubin, TriniCLOT PTEXCEL and albumin) were procured from Durga laboratory Mangalore.

Preparation of aqueous extract (Hot maceration method)

Leaves of CB were shade dried and powered. Leaf powder (200 g) was then dissolved in 1500 ml of distilled water, and a decoction was prepared at 75-80 °C. The decoction was then cooled and filtered. Finally, the filtrate was evaporated to dryness using lyophilizer.

Preparation of the test material

Carboxy Methyl Cellulose (CMC) stock solution was prepared by dissolving 250 mg of CMC in 100 ml of PBS (Phosphate Buffer Solution). Dilution of aqueous extract of CB is prepared by dissolving 500 mg of aqueous extract in 10 ml CMC of 0.25 % w/v.

α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging assay

DPPH radical scavenging activity was measured by the spectrophotometric method [7]. To measure the DPPH scavenging activity of CB, 50 µl of DPPH and 50 µl CB in methanol was taken at the concentration of 200 µg/ml and serially diluted(25, 12.5, 6.25, 3.125, 1.625 µg/ml). An equal amount of DPPH and ascorbic acid in methanol was taken as the positive control. They were further incubated for 20 min, the decrease in absorbance of the test mixture (Due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition was calculated using the formula given below [8]:

$$\text{Inhibition } \% = \frac{(\text{control} - \text{test})}{\text{control}} \times 100$$

Animals

Healthy female Albino rats (150-200 g) were maintained under standard environmental conditions at room temperature of 27 °C with 12 h day/night cycle in central animal house facility, Manipal University, Manipal. The animals were fed with a standard pellet diet and water ad libitum. Animal studies were approved by the institutional animal ethics committee (IAEC/KMC/34/2009-2010).

Toxicity study

The toxicity test of aqueous extract of CB was done as per staircase methods [9]. The rats were divided into seven groups of six animals each. The control group received saline, and the other groups received single oral dose of 100, 500, 1000, 2000, 3000, and 4000 mg/kg body weight of CB extract respectively. Then, animals were observed for various toxic signs and symptoms continuously for the first four hours and further observed for seven days. Based on the observed results, the dose of 500 mg/kg body weight was considered as optimal and safe dose for further experiments.

Treatment protocol

Animals were divided into three groups namely; preventive, curative and prophylactic based on the treatment strategy of CB. The groups were further subdivided into four groups each: Group I control, Group II-CCl_4 control, group III-CB+CCl_4 and group IV-silymarin+CCl_4.

Each subgroup comprised of eight animals (n=8). The aqueous extract of CB/silymarin was administered orally by using the intragastric tube at the dose of 500 mg/kg and 30 mg/kg body weight once daily for eight weeks in the curative group and for four weeks in preventive and prophylactic groups respectively. The chronic hepatotoxicity/liver damage was induced by intraperitoneal (i. p) injection of CCl_4 0.5 ml/kg body weight, 1:1 in olive oil, twice a week for four weeks in the preventive and prophylactic group and for eight weeks in the curative group [10].

Experimental design

A. Preventive group

Group I: control, Group II: CCl_4 control, where CCl_4 is administered twice a week, Group III: where CCl_4 and CB are administered together, Group IV: CCl_4 and silymarin are administered together. The drugs were administered for four weeks in all the treatment groups.

B. Curative group

Group I: control, Group II: CCl_4 control, where CCl_4 is administered for eight weeks, Group III: Animals were treated with CCl_4 for eight weeks, along with CB extract for the last four weeks, Group IV: Animals were treated with CCl_4 for eight weeks, along with silymarin for the last four weeks. The animals were treated for a total of eight weeks in all the treatment groups.

C. Prophylactic group

Group I: control, Group II: CCl_4 control, where CCl_4 is administered for four weeks, Group III: Rats were pretreated with CB for first four weeks followed by the CCl_4 for the next four weeks, Group IV: Rats were pretreated with silymarin for the first four weeks followed by the CCl_4 for the next four weeks. The animals were treated for a total of eight weeks in all the treatment groups.

At the end of the treatment period, the animals were sacrificed by ether overdose. The liver tissues and blood was collected and processed for histological and biochemical analysis respectively.

Histological analysis using Masson's trichrome stain

Each formaldehyde-fixed sample was embedded in paraffin, cut into 5 µm-thick sections and stained with Masson's trichrome according to the standard protocol [11].

Biochemical analysis

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP) levels, bilirubin, prothrombin time (PT), total serum proteins (TP) including albumin (ALB) and globulin (GLB) were estimated by using commercially available standard kits.

Statistical analysis

The results were presented as mean±standard error mean. One-way ANOVA was applied to compare between the groups, and Bonferroni's post hoc test was applied for multiple comparisons using Graph pad prism software (Microsoft, San Diego, CA, USA). $p<0.05$ was considered statistically significant.

RESULTS

DPPH radical scavenging activity

Higher the dosage of CB, greater was the inhibition of DPPH free radical activity, i.e., 25 µg/ml of CB showed maximum inhibition (46.90 %) while the lower dose (1.625 µg/ml) failed to evoke any response. This indicated that the free radical was scavenged by CB in a concentration-dependent manner (table 1).

Table 1: Effect of aqueous extract of CB on DPPH radical scavenging activity

Concentrations of CB (µg/ml)	DPPH (% inhibition)
25	46.90±2.05
12.5	32.72±2.27
6.25	15.27±0.51
3.125	6.36±0.32
1.625	3.6±1.01

Results are expressed in mean±SEM, [n=3]

Histological findings

The Masson's trichrome stain was used to assess the degree of fibrosis in the liver samples from different treated groups. Liver sections from the control group appeared normal without signs of excessive collagen deposition. In the preventive group (fig. 1), CCl_4 treated sections (fig. 1-B) revealed increased deposition of collagen fibers around the congested central vein and a remarkable increase in thickness of the capsule indicating severe fibrosis. Liver sections also revealed the inflammatory changes with the micro and macrovesicular changes around the central vein suggesting centrilobular necrosis. The tissues from silymarin-treated groups (fig. 1-D) showed minimal sinusoidal congestion with near normal architecture without visible changes in the thickness of the liver capsule. Liver tissues exposed to the CB (fig. 1-C) greatly improved the morphology with mild inflammatory changes, with minimal collagen deposition around the central vein. The liver capsule greatly reduced in thickness indicating minimal fibrosis when compared to CCl_4 treated groups. Near normal changes were observed in groups co-treated with silymarin and CCl_4 (fig. 1-D).

In the curative group (fig. 2), a liver tissue treated with CCl_4 showed severe deposition of collagen fibers and congestion in the central vein (fig. 2-B). Excessive deposition of collagen fibers was also observed in the capsule contributing to its thickness when compared to the normal control (fig. 2-A).

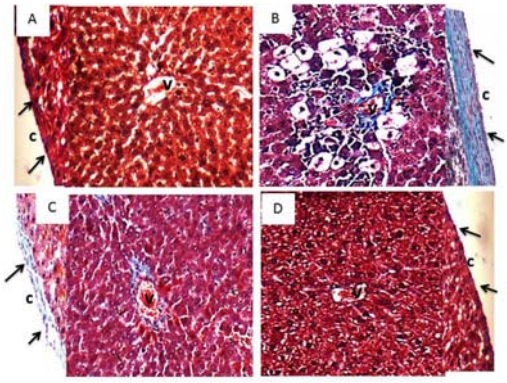

Fig. 1: Masson's trichrome staining of livers sampled from rats of the preventive group (10X), where the collagen fiber composition is (stained in green): A-control, B-+CCl_4, C-+CB, and D-+silymarin. 'c' & ↑ (arrows) = liver capsule, V= central vein

The early inflammatory changes like micro and macrovesicular although not observed; however, severe sinusoidal congestion was noticed due to the presence of the collagen fibers in the perisinusoidal spaces. Liver tissue from the rats treated with CB (fig. 2-C) extract and silymarin (fig. 2-D) showed mild collagen deposition and mild congestion around the central vein and less deposition of the fibers in the capsule when compared to the CCl_4 control.

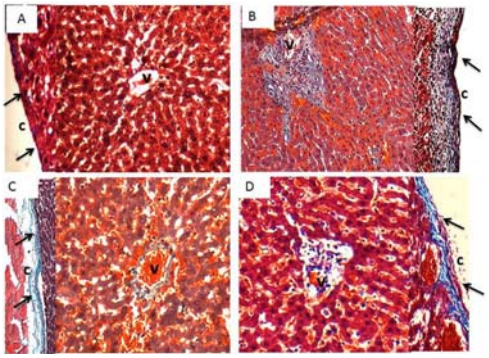

Fig. 2: Masson's trichrome staining of livers sampled from rats of curative group (10X), where the collagen fiber composition is (*stained in green*): A-control, B-+CCl_4, C-+CB, and D-+silymarin. 'c' & ↑ (arrows) = liver capsule, V= central vein

In prophylactic group (fig. 3), the liver tissue treated with CCl_4 exhibited total derangement of liver architecture with inflammatory changes (fig 3-B). Severe deposition of collagen fibers was observed around the central vein and the capsule. The increased collagen content in the capsule leads to its increased thickness when compared to the normal control (fig 3-A). The liver tissues treated with CB extract and silymarin showed the similar extent of morphological changes with fibrosis around central vein. However, no visible reduction in the thickness of the capsule in groups treated with CB and silymarin when compared to CCl_4 was observed (fig. 3-C and D). This measurement of the degree of fibrosis confirms that the treatment with CB extract might protect the animals' liver from the development of fibrosis.

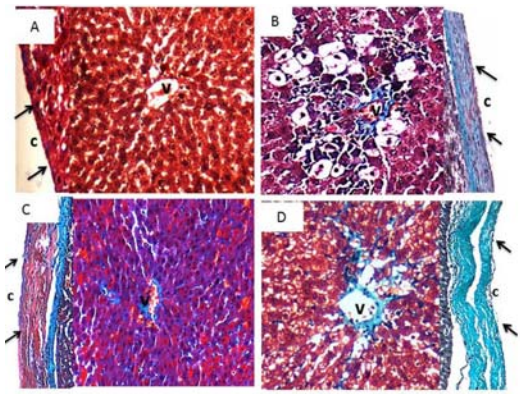

Fig. 3: Masson's trichrome staining of livers sampled from rats of prophylactic group (10X), where the collagen fiber composition is (stained in green): A-control, B-+CCl_4, C-+CB, and D-+silymarin. 'c' & ↑ (arrows) = liver capsule, V= central vein

Biochemical findings

CCl_4 intoxication leads to statistically significantly elevated levels of liver-specific enzymes (AST, ALT, and ALP), TB, PT and decreases in the levels of TP, ALB, and GLB in serum (table 2, 3 and 4). This indicates hepatocellular damage and biliary obstruction which was further endorsed by the histopathological examination of the liver sections of rats showing centrilobular necrosis, congestion of sinusoids and fibrosis (fig. 1, 2 and 3) when compared to normal control. Groups intoxicated with CCl_4, when treated with CB significantly ($p<0.05$) circumvent the toxic effects of CCl_4 by lowering the levels of serum AST, ALP,TB and PT in preventive group (table 2); serum ALT, AST and ALP in curative group (table 3) and ALP and ALB in the prophylactic group (table 4) when compared to the CCl_4 control. Other enzymes though showed improvement in its levels; however the findings were not statistically significant. Alteration in the levels of enzymes was complemented by the histopathological results.

Table 2: Preventive effects of *Caesalpinia bonduc* on liver marker enzymes

Groups/ Parameters	Control	CCl_4 control	Preventive CB	Preventive silymarin
ALT(U/l)	59.97±3.38	107.663±7.42	87.15±2.42	62.52±1.26
AST(U/l)	180.8±17.16	334.366±50.58**	153.86±1.03##	167.9±10.42##
ALP(U/l)	172.66±9.44	355.933±47.87*	118.93±52.30##	127.4±11.35##
TP(g/l)	2.546±0.05	1.401±0.66*	1.787±0.48	2.219±0.26
ALB(g/l)	1.353±0.11	1.142±0.05**	1.155±0.07	1.211±0.05
GLB(g/l)	1.193±0.22	0.259±0.81**	0.632±0.21	1.008±0.11
TB(mg/l)	4.207±0.15	5.276±0.04***	5.038±0.03#	4.997±0.19 ##
PT (s)	35.10±4.31	57.58±2.41***	45.42±3.05#	32.73±3.43###

AST-aspartate transaminase; ALT-alanine transaminase; APL-alkaline phosphatase; TP-total protein; ALB-albumin; GLB-globulin; TB-total bilirubin; PT–prothrombin time.

*, **, ***= $p<0.05$, $p<0.01$ and $p<0.001$ respectively in comparison with control; #, ## and ### = $p<0.05$, $p<0.01$ and $p<0.001$ respectively in comparison with CCl_4 treated group. Results are expressed in mean±SEM, [n=8].

Table 3: Curative effects of *Caesalpinia bonduc* on various liver enzymes

Groups/parameter	Control	CCl_4 control	Curative CB	Curative silymarin
ALT(U/l)	69.97±3.38	190.33±15.88***	104.80±8.45#	76.26±2.43##
AST(U/l)	180.8±17.16	367.7±17.16***	208.8±31.56##	229.733±2.90#
ALP(U/l)	172.66±9.44	389.26±37.31***	210.60±3.62#	186.70±11.68##
TP(g/l)	2.414±0.26	1.950±0.04	1.874±0.91	2.404±0.18
ALB(g/l)	1.353±0.11	1.068±0.04***	1.189±0.13	1.237±0.06
GLB(g/l)	1.061±0.25	0.882±0.11	0.689±0.02	1.167±0.21
TB(mg/l)	4.207±0.15	5.334±0.02***	5.061±0.20	5.012±0.20#
PT (s)	37.76±1.34	90.83±7.07***	78.98±4.19	54.10±3.42###

AST-aspartate transaminase; ALT-alanine transaminase; APL-alkaline phosphatase; TP-total protein; ALB-albumin; GLB-globulin; TB-total bilirubin; PT–prothrombin time.

*, **, ***= p<0.05, p<0.01 and p<0.001 respectively in comparison with control; #, ## and ### = p<0.05, p<0.01 and p<0.001 respectively in comparison with CCl_4 treated group. Results are expressed in mean±SEM, [n=8].

Table 4: Prophylactic effects of *Caesalpinia bonduc* on various liver enzymes

Groups/parameter	Control	CCl_4 control	Prophylactic CB	Prophylactic silymarin
ALT(U/l)	60.63±1.18	107.66±7.44	75.97±0.67	66.416±1.77
AST(U/l)	174.13±10.66	229.73±1.76	224.50±17.10	144.33±8.10#
ALP(U/l)	162.33±2.13	355.93±21.50***	143.86±6.38###	235.43±8.62##
TP(g/l)	2.546±0.05	1.401±0.66	2.588±0.14	2.484±0.97
ALB(g/l)	1.381±0.08	1.142±0.05***	1.318±0.12#	1.186±0.10
GLB(g/l)	1.165±0.03	0.259±0.13**	1.27±0.08	1.298±0.22
TB(mg/l)	4.207±0.15	5.276±0.04***	5.052±0.21	4.993±0.15
PT(s)	38.10±0.61	57.28±2.41***	52.00±3.20	46.51±2.53##

AST-aspartate transaminase; ALT-alanine transaminase; APL-alkaline phosphatase; TP-total protein; ALB-albumin; GLB-globulin; TB-total bilirubin; PT–prothrombin time.

*, **, ***= p<0.05, p<0.01 and p<0.001 respectively in comparison with control; #, ## and ### = p<0.05, p<0.01 and p<0.001 respectively in comparison with CCl_4 treated group. Results are expressed in mean±SEM, [n=8].

DISCUSSION

Liver is a primary organ concerned with detoxification of toxic chemicals and drugs. Chronic liver diseases are at a rise in the recent years and the treatment for these diseases have showed limited efficacy. Liver fibrosis is a wound healing response to chronic insults to the liver. The important indicator of liver fibrosis is the excessive deposition of extracellular matrix (ECM), primarily collagen fibers due to the stimulation of Hepatic stellate cells (HSCs) [12, 13]. CCl_4 toxicity is commonly employed for the study of experimental hepatic dysfunction [14]. In the present study, the liver fibrosis was induced by the intraperitoneal injection of CCl_4 for a period of 8 w.

Chronic liver disease is characterized by the excessive deposition of collagen and other ECM proteins within the liver. It is thought that activated HSCs in the perisinusoidal space are the ma in contributors to the fibrotic process [15]. The diagnosis of liver fibrosis is mainly based on the histological examination for lobular architecture, the degree of hepatocyte damage, inflammatory infiltration, and fibrous deposition. A semi-quantitative estimation of fibrosis may be performed by staining of collagen with Masson's trichrome stain [16].

Exposure of CCl_4 can rapidly lead to severe centrizonal necrosis and steatosis, and affects the activity of biochemical enzymes, breakage of DNA strands and increases telomerase activity [17]. The results of the present study revealed that the administration of CCl_4 caused hepatic injury leading to an elevation in the levels of serum marker enzymes such as ALT, AST, ALP, Bilirubin, prolonged prothrombin time and decrease in the albumin and total protein level respectively. Albumin and blood clotting factors are primarily synthesized in the liver, and in chronic liver damage, the albumin content falls and prothrombin time is prolonged considerably [12, 18]. The present study also agrees with the earlier findings.

As there is a dearth in the availability of efficacious remedies for hepatoprotection, the use of natural medicines is the need of the hour. CB is noted to be one among them. Earlier studies conducted on CB have indicated the hepatoprotective properties of the plant [19]. In our previous study, we have identified and proved the benefits of CB extracts on acute hepatotoxicity. In the study, the animals were treated for a short duration (i.e., 11 d) [6]. However, long term hepatoprotective effects of CB extract is not explored upon. This would be relevant as chronic liver diseases are on the rise. The present study is, therefore, an attempt to explore the same.

In the present study, the co-administration of CB extract and CCl_4 significantly improved the condition by decreasing the serum AST, ALT, ALP, bilirubin and total protein significantly in preventive and curative groups respectively. This indicates the effectiveness of the CB in maintaining the structural and functional integrity of the hepatocellular components. The histopathological findings also suggest that the treatment with CB extract can restore the liver architecture in CCl_4 induced liver cirrhosis in rats. The CB induced reduction of fibrosis might be due to the less activation or infiltration of the stellate cells to the injured sight. In addition, CCl_4 induced fatty degeneration; necrosis and fibrosis of liver can be prevented /repaired by administration of CB.

CONCLUSION

Aqueous extract of CB reverse the histopathology and the different biochemical indices of liver injury as observed in the present study. The long term hepatoprotective effects of CB extract is not explored upon. This would be relevant as chronic liver diseases are on the rise.

Although the underlying mechanism of the hepatoprotective activity of CB is not clearly known, its antioxidant property might be responsible for subsiding the HSCs activity thereby reducing the liver fibrosis induced by CCl_4. Therefore, CB may be considered as a potent hepatoprotective agent to treat the chronic liver toxicity. The present study is, therefore, useful in providing scientific evidence to the hepatoprotective activity of CB.

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CONFLICT OF INTERESTS

Declare none

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