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兖州卷柏水提物对四氯化碳致小鼠肝损伤的保护作用和抗氧化活性

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摘　要:研究兖州卷柏对四氯化碳(CCl_4)致小鼠肝损伤的保护效果。昆明小鼠随机分为 6 组($n=8$):正常组、CCl_4 模型组、水飞蓟素对照组、兖州卷柏水提物(高、中、低)剂量组(400、200、100 mg/kg)。各组连续灌胃给药 9 d 后,除正常组外,其余各组用 CCl_4 溶液(2 mL/kg)灌胃。结果显示兖州卷柏能显著的抑制肝损伤血清中 SGOT、SGPT、ALP、LDH、胆固醇和胆红素升高($P<0.01$)。兖州卷柏能降低损伤肝组织的脂肪含量,局灶性坏死,中心静脉充血和正弦空间拥塞。体外抗氧化实验表明兖州卷柏抗氧化活性指标 T-AOC、LPO、T-SOD 和 NO 的 IC_{50} 值明显高于维生素 C($P<0.05$)。提示兖州卷柏水提物可能通过其抗氧化活性对四氯化碳致小鼠肝损伤产生保护效果。

关键词:兖州卷柏;四氯化碳;肝损伤;抗氧化活性

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Hepatoprotective and Antioxidant Activity of *Selaginella involvens* Aqueous Extract on Carbon Tetrachloride-Induced Hepatic Damage in Mice

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Abstract: In this study, the hepatoprotective and antioxidant activity of *Selaginella involventis* aqueous extract was evaluated using carbon tetrachloride (CCl_4)-induced hepatic damaged mice model. KM mice was randomly divided into 6 groups ($n=8$), including normal, CCl_4-model, *Silymarin*-control groups and three different dosages (400, 200, 100 mg/kg) of *S. involventis* aqueous extract in experimental groups. After oral gavage for 9 days with drug, KM mice were treated with CCl_4 (2 mL/kg) by oral gavage, except for normal group. The results showed that administration of *S. involventis* aqueous extracts significantly reduced the level of SGOT, SGPT, ALP, Lactic dehydrogenase (LDH), cholesterol and bilirubin when compared to that of hepatotoxin treated mice. More importantly, histopathological data showed that *S. involventis* aqueous extracts reduced the fatty contents, focal necrosis, congestion in central vein and congestion in sinusoidal spaces in CCl_4-induced hepatic mice. In addition, the *in vitro* antioxidant assays demonstrated that the IC_{50} values of T-AOC, LPO, T-SOD and NO radical scavenging activities were increased when compared with the level of vitamin C. All these experimental results suggested that *S. involventis* aqueous extract may protect hepatic activity through antioxidant activities in CCl_4-induced mice.

Key words: *Selaginella involventis*; carbon tetrachloride; hepatic damage; antioxidant activity

Introduction

Liver is one of the few organs with highly specialized function which can undergo an astonishing degree of regeneration and drug metabolism[1]. However, a few known risk factors, such as hepatic virus, inflammation, obesity and alcohol consumption, may lead to hepatic injury, which is one of the most serious diseases affecting human health[2]. In the process of physiological liver metabolism, reactive oxygen species (ROS) includ-

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ed three states including free radicals, peroxides and excited states. When the liver underwent hepatic injury, elevated generation of ROS and decreased capacity of antioxidant could result in a state of redox imbalance, which may induce loss of cell viability and death[3]. Then, oxidative stress would trigger an irreversible situation that cannot be repaired by antioxidant and biosynthetic pathways on human pathology [4]. However, current treatment is still far from optimal to cure liver disease. Therefore, it is of great importance to develop the drugs to protect the liver with induced hepatotoxicity and antioxidant properties. In this study, we investigated the potential of traditional Chinese medicine for this purpose[5].

A great variety of Chinese traditional drugs have been used in folk medicine to treat liver diseases and boost liver functions[6]. Among them, *Selaginella involven* is abundantly distributed in south China, especially in Fujian Province. This fern contains a variety of flavonoids, alkaloids, phenols, organic acid and carbohydrate compounds. It has been used as a folk herbal medicine to treat some diseases as its hemostasis, acute icteric hepatitis, hepatocirrhosis and pneumonia[7]. It is used in ethnomedical practices in certain remote villages with the belief that it had some activities, such as antitumor, antibacterial, antiviral and anti-inflammation[8]. Recent findings demonstrated that *S. involventis* aqueous extract has certain antioxidant abilities and may inhibit the growth of human esophageal carcinoma cells *in vitro*[9]. Its alkaloids may inhibit the growth of hepatocellular carcinoma cell H22 of mouse *in vitro*[10]. However, limited studies exist regarding the effect of *S. involventis* onconventional scientific studies, particularly in hepatoprotective effects area.

The purpose of this study was to evaluate the potential effect of *S. involventis* aqueousextract on carbon tetrachloride (CCl_4)-induced hepatic damage in mice model. Reactive oxygen species (ROC) were the main causes of carbon tetrachloride (CCl_4)-induced acute liver injury, which results to the mice model marked elevation of alanine aminotransferase and aspartate aminotransferase.

Materials and Methods

Preparation of *S. involventis* extract

The matured plants of *S. involventis* were collected from valleys in Sanming City, Fujian Province, China. The specimen samples were immediately sent to Prof SU Q of the Medicine Research Institute of Quanzhou for authentication. The voucher specimen (AUOCAS031) was subsequently maintained in the herbarium of Quanzhou Medical College. The herb was washed twice with distilled water to remove the contaminants, and oven-dried at 60 ℃ for 3 days, and then grinded into powder.

The powdered sample of *S. involventis* (500 g) was extracted with sterile water (5,000 mL) at 80 ℃ for 20 min. Then the solvent was precipitated in vacuum at 60 ℃ using a rotary evaporator (Eyela, Japan). The extract powder was dissolved with sterile distilled water and administered orally to mice in final concentrations of 400, 200 and 100 mg/kg body weight.

Animal model and oral treatment with *S. involventis* extract

Kunming male mice weighed at 18-20 g were obtained from Slac Laboratory Animal Ltd. (animal certification number was 2012-0002, Shanghai, China). The animals were housed in colony cages at an ambient temperature of 25 ± 2 ℃ with a 12 h light and 12 h dark circle. All the animal experiments were conducted in accordance with the guidelines of the experimental animal ethical committee of Quanzhou Medical College.

In this experiment, the animals were randomly divided into six groups, with eight mice per group, and treated as follows: Group 1, Oral gavage with ddH_2O (5 mL/kg · bw) daily for 9 days (normal control group, $n = 8$). Group 2, Oral gavage with sterile distilled water (5 mL/kg · bw) daily for 9 days + single dose of (2 mL/kg · bw, ip) CCl_4 with liquid paraffin (1∶1) for one day (positive control hepatotoxic group, $n = 8$). Group 3, Oral gavage with *silymarin* (100 mg/kg · bw) daily for 9 days + single dose of (2mL/kg · bw, ip) CCl_4 with liquid paraffin (1∶1) for one day (well established standard drug hepatoprotective group, $n = 8$). Group 4, Oral gavage with *S. involventis* aqueousex-

tract (400 mg/kg · bw) daily for 9 days + single dose of (2 mL/kg · bw, ip) CCl_4 with liquid paraffin (1∶1) for one day (treatment group, high dose, $n=8$). Group 5, Oral gavage with *S. involventis* aqueousextract (200 mg/kg · bw) daily for 9 days + single dose of (2mL/kg · bw, ip) CCl_4 with liquid paraffin (1∶1) for one day (treatment group, medium dose, $n=8$). Group 6, Oral gavage with *S. involventis* aqueousextract (100 mg/kg · bw) daily for 9 days + single dose of (2 mL/kg · bw, ip) CCl_4 with liquid paraffin (1∶1) for one day (treatment group, low dose, $n=8$).

Analysis of liver functional enzymes

To investigate the liver function, the enzymes activity was assessed through the blood samples collected from each group. On the 10th day, all animals were anesthetized with mild ether and blood sample were collected by eye bleeding method. The blood serum was separated from blood clots by centrifugation at room temperature with 4,000 rpm for 10 min. Then, liver function tests were estimated with standard kits (Amyjet Scientific Inc, Wuhan China) to detect known biochemical parameters listed as below: serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT)[11], lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total protein, albumin, sugar, cholesterol and bilirubin.

Followed by the sacrifice for all the animals, the livers were dissected using a glass rod and a ceramic potter at 4 ℃ with 0.1 M Tris-HCl (pH 7.4). Thereafter, the supernatant was collected by centrifugation at 4,000 rpm for 10 min for the spectrophotometric tests of lipid peroxide (LPO) radical scavenging assay, hydroxyl radical scavenging assay (HRSA) by using standard kits (Amyjet Scientific Inc, Wuhan china).

Various concentrations (2 μg/mL to 600 μg/mL) of *S. involventis* aqueous extract was used for the test of total antioxidant capacity (T-AOC) assay, lipid peroxide (LPO) radical scavenging assay, hydroxyl radical scavenging assay (HRSA)[12], nitric oxide radical scavenging assay and total superoxide dismutase (T-SOD) radical scavenging assay by using standard protocols with vitamin C (positive control). All the above activities were evaluated using standard kits (Amyjet Scientific Inc, Wuhan China).

Pathological examination and immunohistological analysis

For histological analysis, the livers were fixed in 10% formaldehyde for 24 h at room temperature. After rinsing with PBS twice, all the specimens were embedded in paraffin[13]. Then 5 μm thick serial sections were cut using microtome, stained with haematoxylin-eosin. The sections were scored as described using a light microscope by Jamshidzadeh *et al* [14]. The histological damage was graded on a scale of 0-3∶0 = no visible cell damage; 1 = focal hepatocytes damage on <25%-50% of the tissue; 2 = extensive, but focal hepatocytes lesion; 3 = global hepatocytes necrosis.

To further investigate whether TGFβ1 played role during the treatment, the immunohistochemistry staining was carried out in this study. Briefly, after dewaxing and dehydration, the slices were incubated with TGFβ1 primary antibody at 4 °C overnight. Followed by incubation at room temperature with a biotinylated universal secondary antibody (DAKO, CA, USA) for 15 min. Then, the antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 min. Mayer's haematoxylin (HD Scientific Pty Ltd.) was used for counter staining.

Statistical analyses

All the data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to calculate statistical significance. Least significant difference (LSD)-t test was used for multiple comparison. Statistical significance was considered as $P<0.05$ using SPSS (version 13.0).

Results

Effect of *S. involventis* extracts on liver enzymes activity

As shown in Table 1, it was noted that hepatotoxin treated mice had a significantly increased level of SGOT [(284.80 ± 14.33) IU/L], SGPT [(212.85 ± 10.56) IU/L], ALP [(649.61 ± 43.14) IU/L], LDH [(2664.17 ± 205.28) IU/L], cholesterol [(9.34 ± 2.24) mmol/L], bilurubin [(6.17 ± 1.72) umol/L] in Group 2 when compared to those of control group (P

<0.05). While, the content of albumin [(18.16 ± 2.15) mmol/L] and total protein [(31.84 ± 5.230.47) mmol/L] showed a dramatically decrease ($P < 0.05$) when compared to those of control group. Interestingly, administration (400 and 200 mg/kg · bw) of *S. involventis* aqueous extracts reduced the level of SGOT, SGPT, ALP, LDH, cholesterol and bilirubin ($P < 0.05$) when compared with hepatotoxin treated group. Moreover, it was found that high dosage of *S. involventis* (400 mg/kg · bw) extracts had an optimal reduction of SGOT [(164.19 ± 15.43) IU/L], SGPT [(79.91 ± 12.83) IU/L], ALP [(256.72 ± 31.69) IU/L], LDH [(1444.02 ± 105.36) IU/L], cholesterol [(3.08 ± 0.61) mmol/L] and bilurubin [(5.18 ± 0.88) umol/L]. However, the level of albumin [(39.44 ± 4.67) mmol/L] and total protein [(64.87 ± 9.83) mmol/L] were significantly increased in the high dose group (400 mg/kg · bw) ($P < 0.05$) when compared to those of control group (Table 1).

Table 1 Effect of *S. involventis* aqueous extract on the biochemical parameters in CCl_4 induced hepatotoxicity in mice (Mean ± SD)

Parameters	Control	Hepatotoxin group (CCl_4)	*Silymarin* positive control	*S. involventis* aqueous extract (mg/kg · bw)		
				100	200	400
SGOT (IU/L)	126.27 ± 10.12	284.80 ± 14.33 *	145.73 ± 8.61 *	277.32 ± 13.36 * *	213.57 ± 17.06 * *	164.19 ± 15.43 * *
SGPT (IU/L)	52.41 ± 3.43	212.85 ± 10.56 *	68.30 ± 7.74 *	231.14 ± 16.39	139.62 ± 18.63 * *	79.91 ± 12.83 * *
ALP (IU/L)	129.51 ± 11.68	649.61 ± 43.14 *	147.05 ± 24.21	468.57 ± 34.14 * *	349.28 ± 53.17 * *	256.72 ± 31.69 * *
LDH (IU/L)	824.63 ± 114.32	2664.17 ± 205.28 *	1182.83 ± 146.25 *	2787.31 ± 247.55	1958.95 ± 181.43 *	1444.02 ± 105.36 * *
CHL (mmol/L)	2.92 ± 0.41	9.34 ± 2.24 *	2.81 ± 0.56	10.22 ± 2.84 * *	5.42 ± 0.39 * *	3.08 ± 0.61 * *
BIL (μmol/L)	3.14 ± 0.24	6.17 ± 1.72 *	5.28 ± 0.62 *	6.86 ± 1.61	5.32 ± 1.28 * *	5.18 ± 0.88 * *
ALB (mmol/L)	42.92 ± 5.16	18.68 ± 2.15 *	31.38 ± 6.41 *	20.56 ± 2.72	35.49 ± 3.39 * *	39.44 ± 4.67 * *
SUG (mmol/L)	5.29 ± 0.71	5.37 ± 0.73	6.02 ± 0.68	5.97 ± 0.83	5.55 ± 0.69	5.23 ± 0.82
TPN (mmol/L)	65.46 ± 11.07	31.84 ± 5.23 *	53.64 ± 11.42 *	48.72 ± 13.56 * *	45.61 ± 10.60 * *	64.87 ± 9.83 * *

Note: Data were mean ± standard deviation (SD) values for groups of six animals each. Values were statistically significant, * $P < 0.05$ compared with control at the corresponding time; * * $P < 0.05$ compared with CCl_4-treated group at the corresponding time.

Meanwhile, hepatotoxin itself revealed a significant ($P < 0.05$) decrease in the levels of LPO [(0.757 ± 0.028) μmol/gport)] and HRSA [(80.37 ± 7.75 U/mgport)] activities on the liver tissues when compared to that from control. On the other hand, administration of *S. involventis* aqueous extracts (400 and 200 mg/kg · bw) demonstrated a significant increase in the level of LPO and HRSA ($P < 0.05$) when compared with hepatotoxin treated mice. In addition, high dose group (400 mg/kg · bw) reached the peak reduction level for LPO [(1.337 ± 0.061) μmol/gport)] and HRSA [(154.71 ± 12.51) U/mgport)] ($P < 0.05$) when compared to that from control group (Table 2).

Table 2 Effect of *S. involventis* aqueous extract on CCl_4-induced oxidative stress in liver tissue of mice (Mean ± SD)

Liver homogenate parameters (μmol/L)	Control	Hepatotoxin group (CCl_4)	*Silymarin* positive control	*S. involventis* aqueous extract (mg/kg · bw)		
				100	200	400
LPO (μmol/gport)	0.885 ± 0.035	0.757 ± 0.028 *	1.508 ± 0.068 *	0.749 ± 0.076	0.826 ± 0.044 * * *	1.337 ± 0.061 * * *
HRSA (U/mgport)	110.56 ± 12.14	80.37 ± 7.75 *	106.18 ± 14.32	108.55 ± 9.36 * *	133.67 ± 13.29 * *	154.71 ± 12.51 * *

Note: Data were mean ± standard deviation (SD) values for groups of six animals each. Values were statistically significant; * $P < 0.05$ compared with control at the corresponding time; * * $P < 0.05$ compared with CCl_4-treated group at the corresponding time.

Effect of *S. involventis* extracts on histology of liver tissues

The representative H&E histopathological staining showed that hepatotoxin treated mice induced the maximum fatty changes, focal necrosis, congestion in central vein and congestion in sinusoidal spaces. While *S. involventis* aqueous extract at the concentration of 100 mg/kg · bw and 200 mg/kg · bw treated mice

showed the reduction in fatty changes, focal necrosis, congestion in central vein and congestion in sinusoidal spaces when compared to control group. However, no visible changes were observed with high dose of *S. involventis* extracts (400 mg/kg · bw), except for fatty changes and focal necrosis (Table 3 and Fig. 1).

Table 3 Dose dependant histopathological scores of *S. involventis* aqueous extract in CCl_4 induced hepatotoxicity in mice

Parameters	Fatty changes	Focal necrosis	Congestionin central vein	Congestionin sinusoidal spaces	Hepatocytes Deformation	Total
Hepatotoxin group (CCl_4)	3	3	2	2	0	10
Silymarin	1	1	1	0	0	3
S. involventis treated (400 mg/kg · bw)	1	1	0	0	0	2
S. involventis treated (200 mg/kg · bw)	1	1	1	0	0	3
S. involventis treated (100 mg/kg · bw)	2	2	1	1	0	6

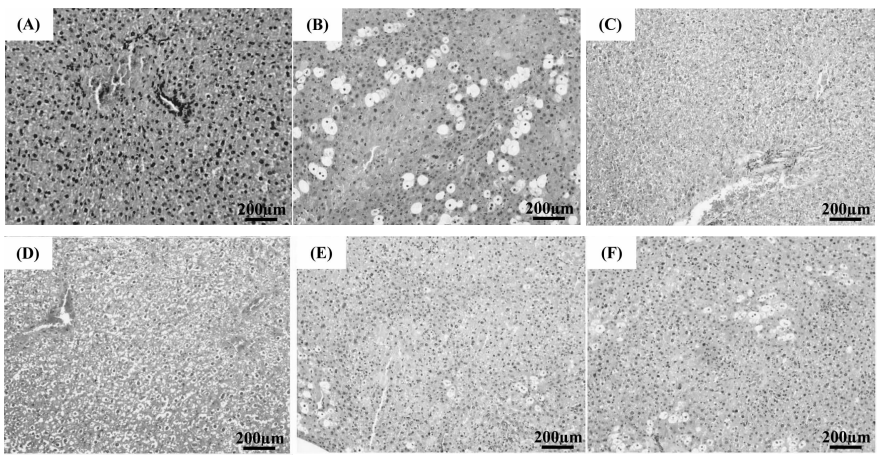

Fig. 1 Dose dependent effect of *S. involventis* aqueous extract on CCl_4 induced hepatotoxicity in mice (100 ×)

Hepatotoxin treated mice induced the maximum fatty changes, focal necrosis, congestion in central vein and congestion in sinusoidal spaces. A: Control group; B: CCl_4 treated mice; C: *Silymarin* treated mice (100 mg/kg · bw); D: 100 mg/kg · bw (Low dose) of *S. involventis* aqueous extract treated mice; E: 200 mg/kg · bw (Medium dose) of *S. involventis* aqueous extract treated mice; F: 400 mg/kg · bw (High dose) of *S. involventis* aqueous extract treated mice.

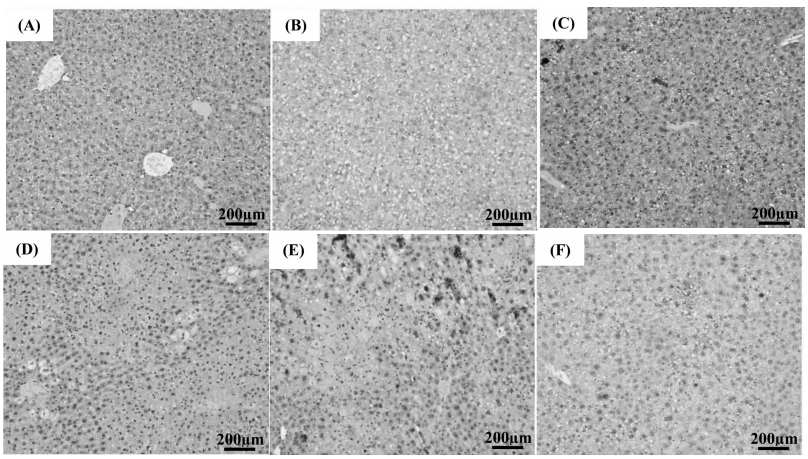

Fig. 2 Expression pattern of TGFβ1 on liver tissues. Weak expression of TGFβ1 was observed in healthy liver tissues in picture A and D.

A: Control group; B: CCl_4 treated mice; C: *Silymarin* treated mice (100 mg/kg · bw); D: 100 mg/kg · bw (Low dose) of *S. involventis* aqueous extract treated mice; E: 200 mg/kg · bw (Medium dose) of *S. involventis* aqueous extract treated mice; F: 400 mg/kg · bw (High dose) of *S. involventis* aqueous extract treated mice.

Expression pattern of TGFβ1 on liver tissues

As seen in Fig. 2, weak expression of TGFβ1 was observed in normal and healthy liver tissues, while there was strong expression of TGFβ on hepatic portal area and the interstitial collagen in CCl_4 induced-mice. When the mice was treated with *S. involventis* extracts at the dosage of 400 mg/kg · bw, it was noted that TGFβ1 was strongly expressed when compared to that from normal control group.

In vitro antioxidant activity of *S. involventis*

The *in vitro* antioxidant assays showed that the IC_{50} values were (67.44 + 2.01) μg/mL, (59.35 + 1.29) μg/mL, (102.16 + 3.65) μg/mL, (31.70 + 2.28) μg/mL and (33.63 + 1.52) μg/mL for T-AOC, LPO, T-SOD, NO and HRSA radical scavenging activities, respectively. Moreover, the results were also comparable with the positive control of vitamin C (Table 4).

Table 4 IC_{50} values of *S. involventis* aqueous extract and vitamin C with various antioxidant activities (μg/mL)

	S. involventis aqueous extract IC_{50}	Vitamin C IC_{50}
T-AOC radical scavenging	67.44 + 2.01 *	25.61 + 1.37
LPO radical scavenging	59.35 + 1.29 *	35.28 + 1.47
T-SOD radical scavenging	102.16 + 3.65 *	28.85 + 1.07
NO radical scavenging	31.70 + 2.28 *	8.33 + 1.41
HRSA radical scavenging	33.63 + 1.52	30.91 + 1.38

Note: Data were mean ± standard deviation (SD) values. Values were statistically significant: * $P < 0.05$ compared with vitamin C.

Discussion and Conclusion

Increasing evidences demonstrated that lots of Chinese traditional herbal medicines could relieve patients' pains against some liver diseases; however, there are still little known whether these herbal extracts possess this capability. Among of them, *S. involventis* is widely used in some different therapeutic applications in folk medicine. In this study, hepatoprotective potential of this plant was investigated via *in vivo* model with hepatocellular damage induced by CCl_4 in mice, which has been established by published protocols with significant elevations of SGOT and SGPT activities[15,16]. In the early of liver injury, SGPT was found with in a high concentration in cytosolic, while SGOT was localized in cytosol and mitochondria and released into the circulation. Continuous hepatic damage would result in an elevation ALP, LDH and bilirubin activity in the serum and further lead to severe hepatic dysfunction. In the present work, oral administration of *S. involventis* aqueous extract can reduce all the elevated biochemical parameters such as SGOT, SGPT, ALP, LDH and bilirubin levels.

In hepatotoxin intoxicated mice, the reduction in the level of total protein and albumin might be due to the damage produced and localized in the endoplasmic reticulum which leads to its functional failure with decrease in protein synthesis and accumulation of triglycerides. Intoxication of CCl_4 also results in inhibition of the synthesis of the bile acids from the cholesterol and leads to elevated level of cholesterol [17]. Suppression of cholesterol level in the serum parameters suggests the inhibition of synthesis of the bile acids from cholesterol, and is reversed by the pre-administration of the *S. involventis* aqueous extracts. Reduction in the level of SGOT, SGPT towards the normal value is an indication of the stabilization of the plasma membrane as well as repair of hepatic tissue damage caused by CCl_4[18]. Reduction in the level of ALP and bilirubin suggested the stabilization of the biliary function and the increased level of protein as well as albumin suggested the stabilization of the endoplasmic reticulum leading to the protein synthesis.

Oxidative stress is the state of imbalance between the level of antioxidant defense system and production of the oxygen derived species[19]. The increased level of oxygen and oxygen derived species such as superoxide radicals, hydroxyl radicals and peroxide radicals causes the oxidative stress[20]. The *in vitro* assays such as T-AOC radical scavenging assay, LPO radical scavenging assay, T-SOD radical scavenging assay, NO radical scavenging assay and HRSA radical scavenging assay

suggested the ability of *S. involventis* aqueous extract to reduce the biological oxidative stress. We observed significant increases in the liver levels of LPO radical scavenging and HRSA radical scavenging in *S. involventis* aqueous extract treated mice compared with CCl_4-treated mice. Hence, the hepatoprotective effect of the aqueous extract may be achieved by the scavenging free radical activity of the oxidative stress [21,22].

Moreover, the histopathological analysis showed that, the normal liver architecture was disturbed by the hepatotoxin treated mice. But, the liver sections obtained from the mice treated with the aqueous extract and intoxicated with hepatotoxin, the normal cellular architecture retained as compared with *silymarin* treated mice, thereby further confirming the protective effect of the extract.

TGFβ1 has a prominent role in the disease process to stimulate hepatic stellate cell activation and transformation into fibroblast and then affecting liver fibrosis positively. TGFβ1 could increase the expression of platelet derived growth factor (PDGF) and its receptor in platelets, and then promote hepatic stellate cell proliferation. In this study, immunohistochemistry data showed increased expression of TGFβ1 in CCl_4 induced chronic liver injury. *S. involventis* inhibited TGFβ1 protein expression in liver tissues, indicating that *S. involventis* extracts may possess the capability of anti-liver injury through TGFβ1 protein expression levels.

The results of the present study suggested that *S. involventis* aqueous extract might possess hepatoprotective properties through its antioxidant activity *in vivo*. However, more in-depth studies will be required to quantitatively and structurally determine the phytochemical principles during these activities.

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