Hepatoprotective potential of *Indigofera tirunelvelica* Sanjappa: *in vitro* and *in vivo* studies on CCl$_4$ induced wistar albino rats

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**ABSTRACT**

The hepatoprotective efficiency of *Indigofera tirunelvelica* Sanjappa whole plant against CCl$_4$ induced hepatotoxicity was examined. Rat hepatocyte monolayer culture and wistar albino rats were exercised as *in vitro* and *in vivo* screening models of protective agent for liver. In *in vitro* analyses, the whole plant ethanolic extract of *Indigofera tirunelvelica* Sanjappa were inspected. Silymarin was chosen as a standard treatment drug. In *in vitro*, free radical scavenging property was also evaluated. In animal studies, hepatotoxicity was produced in Wistar albino rats by dispensing CCl$_4$. The degree of hepatotoxicity was examined by determining the ranges of serum enzyme. The antioxidant parameters such as superoxide dismutase, catalase, reduced glutathione, and malondialdehyde of the hepatocytes were also evaluated. In *in vitro* studies, ethanol extract of *I. tirunelvelica* whole plant was identified to be the most active than other assessed extracts. Besides, whole plant ethanol extract of *I. tirunelvelica* was noticed to be rich in phenolic and flavonoids. It exhibited expressed free radical scavenging property versus diphenylpicryl hydrazyl (DPPH) and superoxide ion radicals. In the animals studies, whole plant ethanolic extract of *I. tirunelvelica* at a ranges of doses (100, 200 and 400 mg/kg body weight) revealed considerable amount of protection against CCl$_4$ induced hepatotoxicity as evident by the protection of CCl$_4$ induced changes biochemical parameters. The results of the present study suggested that the significant hepatoprotective property of whole plant ethanol extract of *I. tirunelvelica* against CCl$_4$ induced hepatotoxicity and intimates its use as a potential medicinal drug for liver diseases.

**INTRODUCTION**

Oxidative stress has been linked in the acute and chronic development of diseases in liver injury in a variety of pathophysiological conditions such as alcoholic liver injury, intrahepatic cholestasis, hepatotoxic exposure, viral liver disease and also liver ischemia (Stehbens, 2003); (Jaeschke, 2003); (Serafini et al., 1998). Excessive synthesis of reactive oxygen species (ROS) and reactive nitrogen species (RNS), together with a substantial decline of antioxidant defence in these diseased conditions, hinders numerous cell performances throughout the...
courses of lipid peroxidation (LPO), nucleic base oxidation as well as healthy protein oxidation. Lipid peroxidation causes vicissitudes in the chemical and physical properties of cell membrane layers, thus modifying their fluidity and also permeability, resulting in impairment in membrane layer signal transduction and ion exchange, causing swelling, cytolysis, and also finally, cell death. The oxidation of healthy proteins and DNA also connects openly to cell dysfunction and fatality (Fang et al., 2002).

As necessary, results of free radical scavengers or antioxidants have been extensively evaluated for the avoidance as well as healing of immediate and persistent liver damage. In recent research studies, antioxidants have indicated beneficial impacts, especially for protection as well as therapy of persistent liver damage (Shakya et al., 2012; Rajeshkumar and Kayalvizhi, 2015; Dogan and Celik, 2012).

**Indigofera tirunelvelica** Sanjappa (Fabaceae) is an annual erect herbs, about 60 cm high, branches woody, angular, light brown pubescent when young terete, striate and glabrous at maturity. Leaves 3,5.4 cm long, pinnately trifoliolate, alternate; petioles 1-.3 cm long, slender, canaliculated above, Flowers pink, 5mm long; pedicels short, pubescent, glandular; bracts 1-1.5 mm long, lanceolate, acute, pubescent without, caducous; calyx 2mm long, 5 – lobes, lobes, 1-1.5 mm long. Flowering from November to December months and fruiting is from December to March. **Indigofera tirunelvelica** distributed in and around Tirunelveli Hills, Tamil Nadu. The literature review revealed that the pharmacognostic standardization, physicochemical analysis. Preliminary phytochemical studies and antibacterial activity of the plant were reported (Subburayalu and Asha, 2017). Our present study was destined to scrutinize the shielding potential of whole plant ethanolic extract of **Indigofera tirunelvelica** against CCl₄ induced hepatotoxicity and oxidative stress in Wistar albino rats.

**MATERIALS AND METHODS**

**Chemicals and reagents**

CCl₄, Ethylene Glycol Tetraacetic Acid (EGTA), 2,2-Diphenylpicryl Hydrazyl (DPPH), Thiobarbituric Acid (TBA) and 5,5’-Dithiobis-2-Nitrobenzoic Acid (DTNB) were procured from Sigma Chemical Co. (St.Louis, MO, USA). All other chemicals and estimation kits used were of analytical grade and purchased from commercial sources.

**Plant material**

The fresh plant material of **Indigofera tirunelvelica** was gathered from Thirunelveli district of Tamilnadu, India in December month. The whole plant was identified and verified by Dr. V. Chelladurai, Research officer, Botany C. C. R. A. S. Govt. of India, (Retired). The whole plant was parched in shades, created into a abrasive powder with a mechanical grinder, distributed through 40 mesh sieves and stowed in covered vessels for further utilization.

**Table 1: Preliminary Secondary Metabolites screening of Indigofera tirunelvelica Sanjappa**

| Phytochemicals       | Terpenoids | Flavones | Steroids | Alkaloids | Phenol | Tannin | Saponin | Coumarin | Lignin | Protein | Glycosides | Quinone | Starch |
|-----------------------|------------|----------|----------|-----------|---------|--------|---------|----------|--------|---------|------------|---------|--------|
| Extraction of different solvents | ItW-He | ItW-Ch | ItW-Ea | ItW-Et | ItW-Aq |
| Terpenoids            | -         | -       | -       | +        | +       | +      | -       | -        | -      | -       | -          | -       | -      |
| Flavones              | -         | +       | +       | +        | +       | +      | -       | -        | -      | -       | -          | -       | -      |
| Steroids              | +         | +       | +       | +        | -       | -      | -       | -        | -      | -       | -          | -       | -      |
| Alkaloids             | +         | +       | +       | +        | +       | +      | -       | -        | -      | -       | -          | -       | -      |
| Phenol                | +         | +       | +       | +        | +       | +      | -       | -        | -      | -       | -          | -       | -      |
| Tannin                | -         | -       | -       | -        | -       | -      | -       | -        | -      | -       | -          | -       | -      |
| Saponin               | -         | -       | -       | -        | -       | -      | -       | -        | -      | -       | -          | -       | -      |
| Coumarin              | +         | -       | +       | +        | -       | -      | -       | -        | -      | -       | -          | -       | -      |
| Lignin                | -         | -       | -       | -        | -       | -      | -       | -        | -      | -       | -          | -       | -      |
| Protein               | -         | -       | -       | -        | -       | -      | -       | -        | -      | -       | -          | -       | -      |
| Glycosides            | -         | -       | +       | +        | +       | +      | -       | -        | -      | -       | -          | -       | -      |
| Quinone               | -         | -       | -       | -        | -       | -      | -       | -        | -      | -       | -          | -       | -      |
| Starch                | -         | -       | +       | +        | +       | +      | -       | -        | -      | -       | -          | -       | -      |

ItW-He=Hexzne extract; ItW-Ch=Chloroform extract; ItW-Ea=Ethylacetate extract; ItW-Et=Ethanol extract; ItW-Aq=Water extract; + =present; – = absent

**Table 2: Totalphenolic and flavonoid contents of various extracts of Indigofera tirunelvelica Sanjappa**

| Extracts | Total flavonoids content (mg/g) | Total phenolic content (mg/g) |
|----------|---------------------------------|-----------------------------|
| ItW-Ch   | 5.81±0.27                       | 8.24±1.51                   |
| ItW-Ea   | 12.16±0.16                      | 15.72±1.40                  |
| ItW-Et   | 26.16±0.41                      | 34.25±0.38                  |
| ItW-Aq   | 13.65±0.70                      | 21.65±0.82                  |

ItW-He=Hexzne extract; ItW-Ch=Chloroform extract; ItW-Ea=Ethylacetate extract; ItW-Et=Ethanol extract; ItW-Aq=Water extract; Values are Mean±SE (n = 3).

**Extraction procedure**

The desicated, thickly pulverized **Indigofera tirunelvelica** (500g) was extricated with ethanol [90%v/v] in soxhlet apparatus for 24h. Then the
solvent was regained entirely on the whole plant ethanol extract of *Indigofera tirunelvelica* (ItW-Et) in reduced pressure by a rotary vacuum evaporator. The condensed extract was desiccated on a water bath and stored in sealed vacuum desiccator.

**Preliminary Secondary Metabolites screening**

Preliminary phytochemical qualitative analysis was achieved to detect the types of secondary metabolites in various extracts (Kokate, 2005)

**(A) DPPH scavenging assay**

![Graph: DPPH scavenging activity of different extracts of *Indigofera tirunelvelica* Sanjappa](image)

**Table 3: Effect of various extracts of Indigofera tirunelvelica Sanjappa and silymarin (SIL) on ALT and LDH in CCl₄ induced toxicity in rat hepatocyte monolayer culture**

| Extracts | % Restoration ALT | % Restoration LDH |
|----------|-------------------|-------------------|
| Normal   | 100               | 100               |
| CCl₄     | 0                 | 0                 |
| ItW-He   | 27.3              | 32.5              |
| ItW-Ch   | 36.9              | 41.4              |
| ItW-Ea   | 47.9              | 54.1              |
| ItW-Et   | 69.5              | 71.4              |
| ItW-Aq   | 58.6              | 63.9              |
| Silymarin| 73.1              | 79.0              |

DPPH scavenging assay: the DPPH scavenging activity was analysed according to the procedure depicted previously (Ozcelik et al., 2014). The absorbance (Abs) was taken at 517 nm. The % inhibition was computed according to the following formula:

\[
\% \text{Inhibition} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100
\]

Ascorbic acid, a well-known antioxidant, was utilized as positive control.

**Superoxide ion radical scavenging assay**: the superoxide ion radical scavenging activity was determined conferring to the procedure of Robak and Gryglewski (Zhang and Lu, 2006). The reaction mixture, comprising 3 ml of plant extract (10-500 μg/ml), 10 μl of phenazine methosulphate (60 μM) and 1 ml of NADH (468 μM) was incubated at 25°C for 5 min, and the absorbance was taken at 560 nm.

**In vitro hepatoprotective activity**

Hepatocytes were isolated from rat liver as per the reported method by Jain and Singhai (Chu et al., 2016). The isolated liver cells were suspended in William’s E medium (pH 7.4) and seeded in collagen pre-coated culture plates at a density of 2 to 3 x 10⁴ cells/well at 37°C in a humidified atmosphere of 5% CO₂ in a CO₂ incubator. After 24h of culturing, cells were exposed to CCl₄ (2.5 mM) with or without selected plant extracts (100 μg/ml) or silymarin (10 μM) and incubated for another 24h at 37°C in CO₂ incubator. After 24h incubation, the leakage of alanine transaminase (ALT) (Reitman and Frankel, 1957) and lactate dehydrogenase (LDH) Alpini (Alpini et al., 1994).

**Experimental animals**

Studies were performed using Wister albino male rats (150-200g), procured from Biogen Pvt. Ltd.,
Hosur, Tamil Nadu, India. The acquired animals were categorized and sheltered in polyacrylic cages (38 x 23 x10 cm) with 6 animals per cage and provided with 25±2°C temperature with dark and light cycle (12/12h). The experimental animals were nourished with standard pellet diet supplied by the animal suppliers and freshwater at ad libitum. All the experimental animals were adapted to animal house environment one week before the experiment. This experiments with were performed by rules of CPCSEA and Institutional Animal Ethical Committee (IAEC) of Srimad Andavan College of Arts and Science, Tiruchirappalli, Tamil Nadu. CPCSEA registration number was SAC/IAEC/BC/2016/Ph.D. - 004, and all the procedures were followed as per rules and regulation.

**Chronic oral toxicity studies**

The chronic oral toxicity studies were performed following OECD guidelines (OECD, 2000). Based on these studies, oral doses of 0.5, 1, 2, and 4g/kg b.w. were selected for in vivo studies.

**In vivo hepatoprotective activity**

The experiment was directed concurring to the method described of (Hu et al, 2014). Experimental animals were separated into 6 groups, each group have 6 animals and given doses of drug as follows: Group I (normal control), Group II administrated orally with CCl₄ (0.5 ml/150g of bw-v/v in olive oil) on 1ˢᵗ, 8ʰ and 16ʰ days, Group III-V, administrated orally with CCl₄ (0.5 ml/150 g of bw-v/v in olive oil on 1ˢᵗ, 8ʰ and 16ʰ days) and treated with W-Et (100, 200, 400mg/ Kg BW) orally for 21 days respectively. Groups VI administrated orally with CCl₄ (0.5 ml/150 g of bw-v/v in olive oil on 1ˢᵗ, 8ʰ and 16ʰ days) and treated with Silymarin (20mg/ Kg BW) orally for 21 days. On the 21ˢᵗ day, animals were sacrificed by cervical decapitation, liver and blood samples were gathered and processed for biochemical estimations.

**Biochemical assay**

Existing Levels of alkaline phosphatase (ALP) (Kind and King, 1954), lactate dehydrogenase (LDH) (Lundholm et al., 1963), and alanine and aspartate transaminase (ALT and AST) (Reitman and Frankel, 1957) in serum were determined. Different ranges of lipid peroxidation (LPO) (expressed in terms of malondialdehyde, MDA) (Ohkawa et al., 1985), superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971), catalase (CAT) (Sinha, 1972) and glutathione (GSH) (Sedlak and Lindsay, 1968) were also determined by the standard procedures to examine oxidative stress.

**Statistical Analysis**

Experimental results were presented as mean ± SEM, and the statistical significance between the groups was studied through one way ANOVA followed by Tukey’s multiple comparison test. P ≤ 0.05 was considered as statistically significant.

**RESULTS AND DISCUSSION**

**Preliminary Secondary Metabolites screening**

In the preliminary Secondary Metabolites analysis, various extracts (Hexane, Chloroform, Ethylacetate, Ethanol and Water) of *I. tirunelvelica* indicated the existence of different secondary metabolites.

**Total phenolic and flavonoid contents**

Our phytochemical analysis showed the presence of moderate to high concentration of phenolics (4.56±0.28 to 21.65±0.82mg/g) and flavonoids (2.62±0.15 to 13.65±0.70mg/g) in different extracts of *I. tirunelvelica*. The rich amount of phenolic and flavonoid contents were found in W-Et.

**In vitro antioxidant activity**

In the *in vitro* evaluation, the W-Et had more powerful antioxidant capacity when we compare with other tested extracts. The IC₅₀ values of W-Et against DPPH and superoxide ion radicals were found to be 40.36 and 55.62μg/ml, respectively. Meanwhile, the ascorbic acid showed potent antioxidant activity with IC₅₀ values of 54.29 and 57.02μg/ml, respectively.
**In vitro hepatoprotective activity**

Incubation of hepatocytes with CCl₄ (2.5 mM) resulted in a significant (p < 0.05) elevation of ALT and LDH (3 and 1.5 fold, respectively) in CCl₄ control hepatocytes Table 3. Treatment with different extracts of I. tirunelvelica (100 µg/ml) or silymarin (10 µM) exhibited a medium to high hepatoprotective result as evidenced by the recom- pense of ALT and LDH levels. The high amount of restitution versus enzyme outflow was noticed with ItW-Et (69.5% and 71.4%, respectively, for ALT and LDH). In comparison, the silymarin, standard drug, exposed a shielding effect (73.1% and 79.0% regained, respectively, for ALT and LDH). The highest effective extract, ItW-Et, was chosen for in vivo hepatoprotective studies.

Normal values of ALT and LDH were 11.35±0.64 IU/L and 19.30±0.50 IU/L, respectively. In CCl₄ control group the values were found to be 33.93±2.32 and 28.06±0.76, respectively. Each value represent the mean ± SEM (n = 3). The percent of restoration was computed as 100 x (amount of CCl₄ – amount of sample) / (amount of CCl₄ control – amount of normal control). (ALT, alanine transaminase; LDH, lactate dehydrogenase; ItW-He=Hexzne extract; ItW-He=Chlorofrom extract; ItW-Ea=Ethylacetate extract; ItW-Et= Ethanol extract; ItW-Aq= Water extract)

**Chronic toxicity studies**

In chronic toxicity studies, the ItW-Et did indicate no sign and symptoms of toxicity and mortality up to 4g/kg dose, measured comparatively harmless.

**In vivo hepatoprotective activity**

The result of ItW-Et on hepatic enzymes throughout CCl₄ caused hepatotoxicity is received Table 4. The increased degrees of ALP, AST, ALT and LDH due to CCl₄ drunkenness were substantially (p < 0.05) protected against ItW-Et therapy when evaluated with CCl₄ control rats. The optimum function was noticed with the high dose. Silymarin additionally indicated considerable safety impact versus CCl₄ caused alterations. The raised LPO and also minimized level of chemical and non-enzymatic antioxidants as perceived in CCI₄ control rats Table 5, were substantially (p < 0.05) avoided in ItW-Et treated groups, showing the exceptional antioxidant result.

Each value represents the mean ± SEM (n = 6); *p < 0.05 as compared with the Group I (Normal group); §p < 0.05, respectively as compared with the Group II (treated with CCl₄ alone); AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase

Each value represents the mean ± SEM (n = 6); *p < 0.05 as compared with the Group I (Normal group); §p < 0.05, respectively as compared with the Group II (treated with CCl₄ alone); MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase.

CCl₄, a well-known hepatotoxin, is traditionally used to monitor new hepatoprotective agents (Al-Snafi et al., 2019); (Ali et al., 2016). This xenobiotic is swiftly altered by cytochrome P450 2E1 to a CCl₃ extreme which is changed into a peroxyl major in the inclusion of oxygen. These radicals may relate with macromolecules of cell and instigate the peroxida-

In the present study, experimental rats treated with CCl₄ alone, developed significant hepatic injury and oxidative damage, as evident by the high increase activities of AST, ALT, ALP and LDH when compared with normal rats. This is an manifestation of cellular leakage and damage of functional reliability of liver cell membrane (Hu et al., 2014). Lessening enzyme functions to the respective Group I animals by ItW-Et at various dose levels (100, 200 and 400 mg/kg) is a symptom of stadyiness of plasma membranes and healing of liver injury.

### Table 5: Effect of whole plant ethanol extract of Indigofera tirunelvelica Sanjappa and Silymarin (SIL) on hepatic MDA, GSH, SOD, and CAT in CCI₄-induced hepatotoxicity in rats

| Groups       | LPO (µmol/g protein) | GSH (µg/g tissue) | SOD (µU/mg protein) | CAT (µU/mg protein) |
|--------------|-----------------------|-------------------|---------------------|---------------------|
| Group I      | 47.71±0.35            | 10.36±0.12        | 12.00±0.90          | 23.01±0.42          |
| Group II     | 121.82±1.42*          | 5.67±0.18*        | 1.35±0.06*          | 6.02±0.67*          |
| Group III    | 91.09±1.55§           | 7.08±0.25§        | 4.78±0.35§          | 11.33±0.36§         |
| Group IV     | 70.45±1.36§           | 8.95±0.46§        | 6.59±0.55§          | 13.84±0.24§         |
| Group V      | 49.51±0.87§           | 9.94±0.24§        | 9.97±0.27§          | 20.25±0.48§         |
Elimination of free radicals and prevention of LPO is essential in the treatment of CCl₄ mediated liver damage (Narwal et al., 2011). Inactivation, detoxification, elimination of ROS and other free radicals be figured out by enzymatic as well as non-enzymatic antioxidants. The crucial chemical antioxidants in the tissue are SOD, CAT and also glutathione peroxidase (GPx). These antioxidants along with GSH perform to prevent the advancement of toxic substances as well as therefore prevent oxidative anxiety (Khan and Sultana, 2011).

In our experimental conditions, CCl₄ induced a severe depletion in hepatic GSH, SOD and CAT concerning normal control group. Moreover, this effect was accompanied by a high level of LPO. This would in turn mean that LPO is a significant factor in the pathogenesis of CCl₄ induced liver damage. Rats administered with ItW-Et revealed a considerable rise in the levels of GSH, SOD and CAT along with a marked reduction in MDA when compared with CCl₄ control rats, indicating remarkable antioxidant effects.

Previous phytochemical investigations on I. tirunelvelica reported the existence of flavonoids and phenolics (Jaeschke, 2003); (Serafini et al., 1998). Consistent with previous results, we also found the abundant amount of phenolics and flavonoids in I. tirunelvelica. Taking into account the fact that such constituents have demonstrated significant hepatoprotective and antioxidant activity (Bhadauria et al., 2012); (Arora et al., 2012); (Patel et al., 2014), their presence in the ethanol extract could explain the biological effects here reported. Our experimental findings also revealed a positive association among phenolic content and antioxidant activity, signifying that phenolics and flavonoids might be the active secondary metabolites in I. tirunelvelica.

CONCLUSIONS

In conclusion, the hepatoprotective activity of Indigofera tirunelvelica Sanjappa whole plant extract against CCl₄ induced hepatic toxicity was evaluated. Our results demonstrated that whole plant ethanol extract of Indigofera tirunelvelica possessed significant (p < 0.05) protection against CCl₄ induced hepatotoxicity, which might be associated with its antioxidant activities through scavenging free radicals to reorganize oxidative stress and protect lipid peroxidation. The secondary metabolites analysis exposed the high amount of phenolics and flavonoids in ItW-Et, which might be accountable for its more potent biological activities. These preliminary results on antioxidant and hepatoprotective activities here reported lend support to the use of I. tirunelvelica as a hepatoprotective agent. Further studies on identify and characterize the active principle(s) and the working mechanism will throw more light on its possible use of whole plant extract of I. tirunelvelica in hepatoprotective drug formulation.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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