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Antihepatotoxic Efficacy of Methanolic Extract of *Indigofera tinctoria* (Linn.) on Paracetamol – Induced Liver Damage in Rats

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ABSTRACT

The antihepatotoxic efficacy of aqueous extract of Indigofera tinctoria (250, 500 mg/kg body weight) and silymarin were investigated against paracetamol induced liver damage in rats. Paracetamol at the dose of 3g/kg body weight orally one day only produced liver damage in rats as manifested by the significant rise in serum levels of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), bilirubin, cholesterol, lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and decrease the protein level compared to control. Histopathological changes of liver sample were compared with respective control. Treatment of rats with I. tinctoria (250, 500 mg/kg body wt.), once daily for 10 days to paracetamol treated rats shows lowered significantly the afore mentioned clinical parameter whereas protein level increased. Further more, liver tissues were processed for histopathological observation. The extract alone treated rats did not adversely affect the serum biochemical and histopathological observation. Significant antihepatotoxic efficacies of the Indigofera tinctoria extracts were reported.

Keywords: Paracetamol; Indigofera tinctoria; transferase; liver; hepatotoxicity.

INTRODUCTION

Herbal medicines have recently attracted much attention as alternative medicines useful for treating or preventing life style related disorders and relatively very little knowledge is available about their mode of action. There has been a growing interest in the analysis of plant products which has stimulated intense research on their potential health intense research on their potential health benefits. Liver, the key organ of metabolism and excretion has an immense task of detoxification of xenobiotics, environmental pollutants and chemotherapeutic agents. Hence, this organ is subjected to variety of diseases and disorders. Several hundred plants have been examined for use in a wide variety of liver disorders. Antioxidants play an important role in inhibiting and scavenging free radicals and thus providing protection against infections and degenerative diseases.

Since, prehistoric times, man has been trying to identify plants that can be exploited as food and medicine. Traditional medicines utilizing natural products have been shown to contain bioactive compounds in vitro [**1**]. *Indigofera tinctoria* is a widely distributed small erect medicinal shrub belonging to the family of fabaceae, found throughout India. The plant has proved to be more effective against chronic myelogenous and other leukemia's [**2**]. The roots, stems and leaves are bitter, thermogenic, laxative, trichogenous, expectorant, anthelminitic, tonic and diuretic, and are useful for promoting the growth of hair and in gastropathy, asthma, ulcers and skin diseases.

Isolation of flavanoids apigenin, kaempferol, luteolin and quercetin from the plant has been reported [**3**]. *Indigofera tinctoria* was found to contained carotenoids, coumarins and flavanoids [**4**]. In traditional medicine of India and China, indigo was used in the treatment of conditions we would now call epilepsy, bronchitis, liver disease, and psychiatric illness. Based on its use for liver problems, researchers have investigated whether indigo might protect the liver against chemically induced liver injury **[5**]. In addition the plant has been used for the treatment of numerous ailments ranging from hemorrhoids to scorpion bites [**6**]. Preliminary evidence suggests that *Indigofera tinctoria* may have protective effect against carbon tetrachloride-induced hepatotoxicity. I.aspalathoides has been reported to possess anti-inflammatory activity [**7**].The present study is aimed to evaluate the hepatoprotective and antioxidant activity of methanol extract of the leaves of *Indigofera tinctoria* against paracetamol induced hepatotoxicity in rats.

MATERIALS AND METHODS

Plant material: The leaves of the plant material *Indigofera tinctoria* Linn. were collected from the Department of Agriculture, Annamalainagar Chidambaram, Cuddalore district, Tamil Nadu, India. The plant was authenticated by the Botanist, Department of Botany, Annamalai University, Annamalainagar, Chidambaram, Tamil Nadu, and India.

Preparation of methanol extract: About 500 gm of dried powder of *Indigofera tinctoria* was taken in soxhlet apparatus and extracted with methanol 72 hours. The solvent was recovered by distillation in vacuum and the residue (yield – 42.249) was stored in desiccator and used for subsequent experiments.

Preliminary phytochemical screening: Preliminary phytochemical screening [**8, 9**] revealed that, in the presence/absence of alkaloids, carbohydrates, steroids, tannins, glycosides, saponins protein, flavanoids, amino acids and terpenes.

Animals: Healthy adult male wistar albino rats between 2 and 3 months of age are taken and weighed about 250-300 gm were used for the study. The animals were housed in a poly propylene cages, maintained under standard conditions 12 hr light, 12 hr dark cycle.

Acute toxicity studies: Healthy adult male wistar albino rats starved overnight were divided into five groups (n=6) and were orally fed with the methanol extract in increased dose levels of 100, 250, 400 mg/kg body weight [**10**]. The rats were observed continuously for 24 hr and 72 hr for any lethality or death (25± 30°C; 35-60% humidity). They were fed with standard rat pellet diet (Hindustan lever Ltd; Mumbai, India) and water ad libitum. The institutional animal ethical committee of central animal house, RMMC and Hospital, Annamalai University (IAEC/160/ 1999/CPCSEA 2007-2010) approved for this study.

Induction of Hepatopathy: Overdose of paracetamol was reported to produce hepatotoxicity effect, which were associated with oxidative stress [**11**]. In the presence of oxidative stress, more lipids peroxidation products were formed, due to cell damage. Then the hepatotoxicity effect and protective activity was assessed by using various bio-chemical parameters.

Experimental design: The hepatotoxicity animals, divided into 5 groups (n=6) six rats each [**12**].
Group – I: *Controls received the vehicle viz. normal saline (1 ml/kg) was administered daily for 7 days.*
Group – II: *Hepatopathy control rats, paracetamol (3g/kg) was administered daily for seven days.*
Group – III: *Rats were received methanol extract of leaves of Indigofera tinctoria (250 mg/kg P.O) once daily for seven consecutive days and followed by paracetamol (3 g/kg P.O) was administered on 5th day of the extract administration [**13**].*
Group-IV: *Rats were received methanol extract of leaves of Indigofera tinctoria (500 mg/kg P.O) only for seven days.*
Group – V: *Rats were received standard drug silymarin (25 mg/kg) IP, for seven days, followed by paracetamol (3 g/kg P.O), on 5th day of silymarin administration.*

After 48 hrs of last dose of paracetamol treatment, blood samples were collected from retro-orbital plexus under mild ketamine anaesthesia. The blood samples were allowed to clot and the serum were separated by centrifugation at 2,500 rpm at 37°C and used for the assay of biochemical parameters.

Assessment of liver function: Biochemical parameters i.e, aspartate amino tansferase (AST) [**14**] alanine amino transferase (ALT) [**14**], alkaline phosphatase (ALP) [**15**], γ-glutamate transpeptidase (GGTP) [**16**], total bilirubin [**17**] and total protein [**18**] were analyzed according to the reported methods. The liver was removed, weighed and

morphological changes were observed. A 10% of liver homogenate was used for antioxidant studies such as lipid peroxidation (LPO) [**19**], superoxide dismutase (SOD) [**20**], catalase [**21**], glutathione-s-transferase (GST) [**22**]. A portion of liver was fixed in 10% formalin for histopathological studies.

Histopathological studies: To examine the extent of cellular damage caused by the paracetamol the liver of experimental and control rats were fixed in Bouin's fluid for 24 h. Following a rinse with water, the tissues were dehydrated in graded alcoholic series, cleaned in xylol and embedded in paraffin wax (58 -60C).Using a rotary microtome 6µ thick sections were cut. The sections were deparaffinized in xylene and were hydrated in graded series of alcohol from 100, 90, 70, 50, 30% and then distilled water. Then the sections were stained with Heidenhain's haematoxylin and counterstained with aqueous eosin for microscopic observations. The stained sections were mounted in DPX.

Statistical analysis: The values were expressed as mean ± SEM statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison test and data on liver weight variations were analyzed using student's 't' test, P values <0.05 were considered as significant.

RESULTS

The effects of Indigofera tinctoria on serum marker enzymes are presented in Table 1. The levels of serum AST, ALT, ALP, total bilirubin, GGTP were markedly elevated and that of protein decreased in paracetamol treated animals, indicating liver damage. Administration of *Indigofera tinctoria* extract at the doses of 250 and 500 mg/kg remarkably prevented paracetamol – induced hepatotoxicity in a dose dependent manner. Analysis of LPO levels by thiobarbituric acid reaction showed a significant (P<0.001) increase in the paracetamol treated rats. Treatment with *Indigofera tinctoria* (250 mg/kg and 500 mg/kg) significantly (P<0.0001) prevented the increase in LPO level which was brought to near normal. The effect of *Indigofera tinctoria* was comparable with that of standard drug silymarin (Table 2).

Table 1: Effect of *Indigofera tinctoria* on biochemical parameters in paracetamol induced hepatotoxicty in rats

Treatment	Dose (mg/kg)	AST U/L	ALT U/L	ALP U/L	Total bilirubin mg%	Total protein mg%	GGTP U/L
Control	-	157.66±4.6	74.2±2.92	188.4±3.16	0.8±0.05	8.13±1.4	26.01±1.10
Paracetamol	600	227.5 ±6.8^{a}	176.0±4.7^{a}	578.0±8.9^{a}	1.1±0.08^{b}	6.35±0.35	62.1±2.48^{a}
Silymarin	25	151.4±6.63^{c}	89.2±3.6^{c}	228.4±5.42^{d,c}	0.72±0.03^{c}	8.12±0.56	35.3±1.78^{c}
Indigofera tinctoria extract	250	217.5±4.64^{c}	122.0±2.5^{a,c}	414.0±12.89^{a,c}	0.65±0.06^{c}	9.22±0.31	39.6±1.08^{b,c}
	500	174.25±6.73^{a}	107.0±5.42^{b,c}	299.75±11.89^{a,c}	0.7±0.04^{c}	8.62±0.96	31.5±3.61^{c}

N = 6; values are expressed as mean ± SEM; $^{a}P<0.01$; $^{b}P<0.01$; $^{d}P<0.05$ Vs control; $^{c}P<0.001$ Vs paracetamol.Data were analysed by using one way ANOVA followed by Tukey multiple comparison test.

Table 2: Effect of *Indigofera tinctoria* on antioxidants level in paracetamol induced hepatotoxicty in rats

Treatment	Dose (mg/kg)	AST U/L	ALT U/L	ALP U/L	Total bilirubin mg%	Total protein mg%
Control	-	7.85±0.92	24.61±1.68	51.29±1.67	38.75±1.96	0.38±0.05
Paracetamol	600	17.17 ±1.14^{a}	23.48±1.04	45.17±2.10^{e}	32.64±1.76^{d}	0.39±0.01^{d}
Silymarin	25	11.01±0.87^{d}	22.36±1.18	48.29±1.92^{d}	33.14±1.45^{c,d}	0.25±0.03^{c}
Indigofera tinctoria extract	250	6.54±0.51^{d}	20.72±1.39^{a}	36.24±2.10^{a}	28.52±2.45^{d}	0.48±0.004^{d}
	500	7.51±1.38^{d}	18.05±1.45^{b}	36.24±1.35^{a}	16.82±2.10^{c}	0.09±0.02^{a}

N = 6; values are expressed as mean ± SEM; $^{a}P<0.001$; $^{b}P<0.05$; $^{c}P<0.01$ Vs control; $^{d}P<0.001$; $^{e}P<0.05$ vs Paracetamol.
Data were analysed by using one way ANOVA followed by Tukey multiple comparison test.
SOD = Units/min /mg protein
CAT = µ mole of H_2O_2 consumed /min/mg protein
GPx = µ moles of GSH oxidized /min/mg protein
GST = µ moles of cDNB conjugation formed / min/mg protein
LPO = µ moles of MDA/min/mg protein.

Paracetamol treatment caused a significant (P<0.001) increase in the level of SOD, catalase, GPx and GST in liver tissue when compared with control group (Table 2). The treatment of *Indigofera tinctoria* at the doses of 200 and

500 mg/kg resulted in a significant decrease of SOD, catalase, GPx and GST when compared to paracetamol treated rats. The liver of silymarin treated rats also showed a significant increase in antioxidant enzymes levels compared to paracetamol treated rats.

Histopathological studies, showed paracetamol to produce extensive vascular degenerative changes and centrilobular necrosis in hepatocytes. Treatment with different doses of *Indigofera tinctoria* extract produced mild degenerative changes and absence of centrilobular necrosis when compared with control. All these results indicate a hepatoprotective potential of the extract (Table I & II).

DISCUSSION

Paracetamol a widely used antipyretic analgesic drug, produces acute hepatic damage on accidental overdosage. It is established that, a fraction of paracetamol is converted via the cytochrome P_{450} pathway to a highly toxic metabolite; N-acetyl-P-benzoquinamine (NAPQI) [**23**] which is normally conjugated with glutathione and excreted in urine. Overdose of paracetamol depletes glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction [**24**] and the development of acute hepatic necrosis. Several P_{450} enzymes are known to play an important role in APAP bioactivation to NAPQI. P_{450} 2E1 have been suggested to be primary enzymes for paracetamol bioactivation in liver microsomes [**25**]. Studies demonstrated that paracetamol induced hepatotoxicity can be modulated by substances that influence P_{450} activity [**26**].

In the assessment of liver damage by paracetamol the determination of enzyme levels such as AST, ALT is largely used. Necrosis or membrane damage releases the enzyme into circulation and hence it can be measured in the serum. High levels of AST indicates liver damage, such as that caused by viral hepatitis as well as cardiac infraction and muscle injury, AST catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore ALT is more specific to the liver, and is thus a better parameter for detecting liver injury.

Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver [**27**]. Serum ALP, bilirubin and total protein levels on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure [**28**].

Administration of paracetamol caused a significant ($P<0.001$) elevation of enzyme levels such as AST, ALT, ALP, GGTP, total bilirubin and decrease in total protein when compared to control. There was a significant ($P<0.001$) restoration of these enzyme levels on administration of the leaf extract in a dose dependent manner and also by silymarin at a dose of 25 mg/kg. The reversal of increased serum enzymes in paracetamol induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [**29**]. Effective control of ALP, bilirubin and total protein levels points towards an early improvement in the secretary mechanism of the hepatic cells.

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. Both silymarin and the plant extract decreased paracetamol induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cells membrane or regeneration of damage liver cell.

The increase in LPO level in liver induced by paracetamol suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Treatment with *Indigofera tinctoria* significantly reverses these changes. Hence it is likely that the mechanism of hepatoprotection of *Indigofera tinctoria* is due to its antioxidant effect.

1)

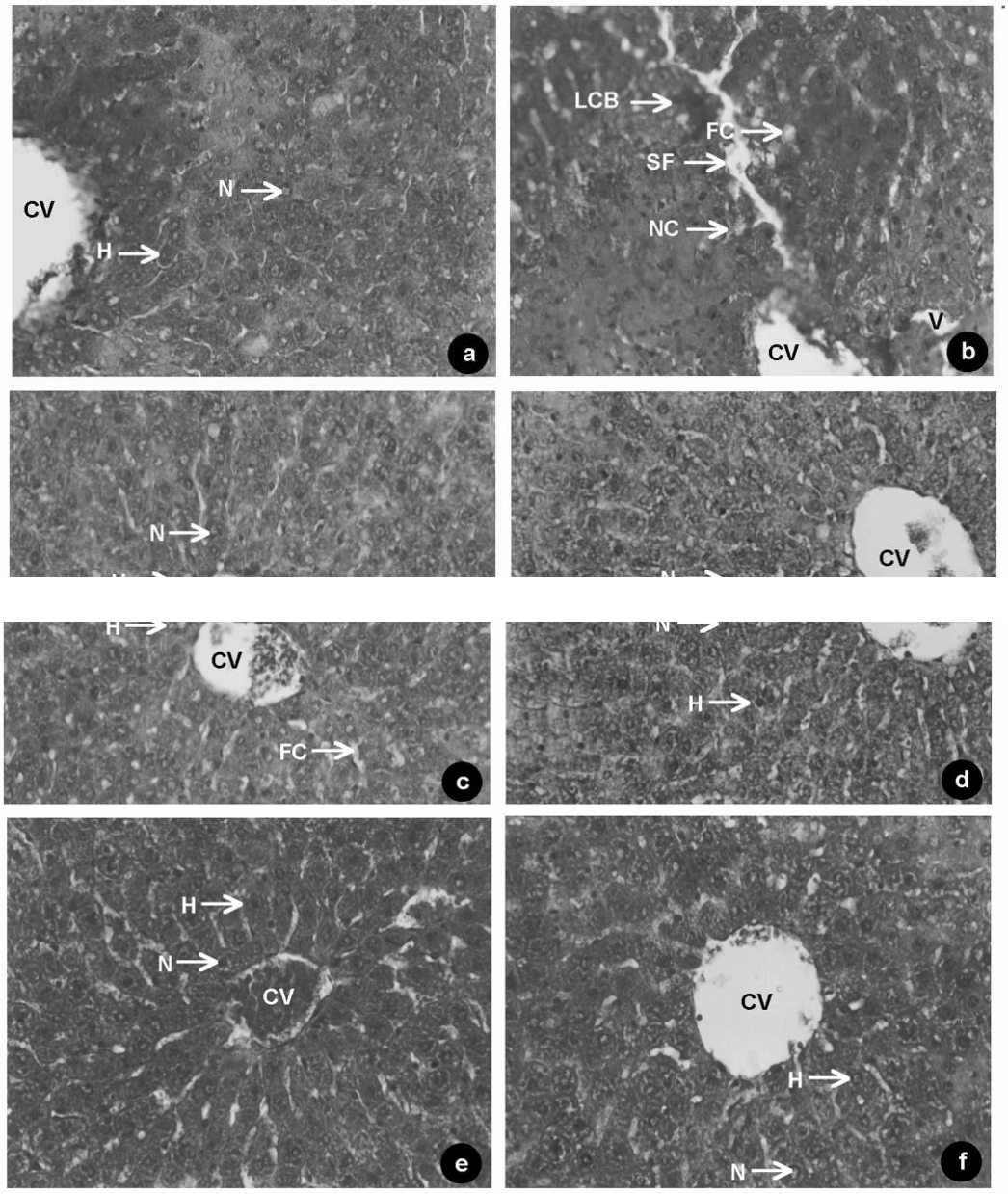

Fig.1. Liver section taken from (a) control rats showing normal histoarchitectural pattern; (b) paracetamol treated rats; (c) Paracetamol + methanol extractof *Indigofera tinctoria* (250 mg/kg body weight); (d) Paracetamol + methanolic extract of *Indigofera tinctoria* (500 mg/kg body weight); (e) Paracetamol +silymarin (25 mg/kg body weight); (f) methanolic extract of *Indigofera tinctoria* (500 mg/kg body weight) alone. H & E x 200.

CV-Central vein, H-Hepatocyte, N-Nucleus, FC-Fatty changes, NC-necrosis, V-Vacuole, SF-Space formation, LCB-Loss of cell boundaries.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepato cellular damage and is the most sensitive enzymatic index in liver injury [**30**] SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical inducing oxidative damage to liver Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [**31**]. Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of

superoxide radical and hydrogen peroxide. A higher dose (500 mg/kg) decreases the level of CAT as produced by silymarin, the standard hepatoprotective drug.
Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is substrate for glutathione peroxidase (GPx) [**32**]. Decreased level of GSH is associated with an enhanced lipid peroxidation in paracetamol treated rats.

Extensive vascular degenerative changes and centrilobular necrosis in hepatocytes was produced by paracetamol. Treatment with different doses of methanolic extract of leaves of *Indigofera tinctoria* produced only mild degenerative changes and absence of centrilobular necrosis, indicating its hepatoprotective efficiency.

Free radical mediated process has been implicated in pathogenesis of most of the diseases. The protective effect of *Indigofera tinctoria* on paracetamol induced hepatotoxicity in rats appears to be related to inhibition of lipid peroxidation and enhancement of antioxidant enzyme levels in addition to free radicals scavenging action. A preliminary phytochemical study reveals the presence of flavanoids in methanolic extract of *Indigofera tinctoria*. Flavanoids are hepato protectives [**33**, **34**]. The observed antioxidant and hepatoprotective activity of *Indigofera tinctoria* may be due to the presence of flavanoids. Further studies to characterize the active principles and to elucidate the mechanism are in progress.

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