

**Comparative study of *In Vitro* and *In Vivo* antioxidant property of different *Ixora* species**

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**ABSTRACT:**

*Ixora* species (Rubiaceae) contains many important phytoconstituents in the various parts of it and they are also responsible for some important biological activities like antitumour activity, wound healing and antimicrobial activity. The antioxidant capacity of *I. coccinea* and *I. purviflora* leaves were assayed for their scavenging abilities against superoxide anion radicals, hydroxyl radical, nitric oxide radical, hydrogen peroxide, metal chelation and reducing power. All the extracts inhibited all above said free radicals in a dose-dependent manner. As antioxidant action has been reported to play a crucial role in the hepatoprotection and excellent result of these plants in *In Vitro* model of antioxidant activity, an attempt has been taken to elucidate the effect on CCl<sub>4</sub> induced hepatic damage with reference to biochemical marker enzymes & histopathology. In the present study all extracts of both the plants have been found to reduce both serum ALP and Bilirubin. Treatment with CCl<sub>4</sub> increases the levels of total lipid, total triglycerides and total cholesterol in liver. Presence of significantly high concentration of total lipid and cholesterol in the serum of CCl<sub>4</sub> treated animals and maintains of these towards near normal values in different extracts administered rats demonstrates the hepatoprotective effect of both the *Ixora* species.

**Key words:** Antioxidant, hepatoprotection, *Ixora coccinea*, *Ixora purviflora*, aspartate amino transferase, L-alanine amino transferase.

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**INTRODUCTION:**

The plants belonging to Rubiaceae family are generally a rich source of substances of phytochemical interest. Numbers of plants from this family are used in traditional system of medicine. [1] From the large list of various plants of rubiaceae family we have selected two different *Ixora* species named *Ixora coccinea* and *Ixora purviflora*. These two

plants are generally used as the ornamental plants in gardens and parks. But the review of literature showed that there are so many important phytoconstituents present in the various parts of the above said plants and they are also responsible for some important biological activities like antitumour activity, wound healing and antimicrobial activity.

Some preliminary phytochemical investigations reported that flavonoids are present in the flowers and leaves of the same. Therefore, the objectives of the present study were to investigate the *In Vitro* antioxidant activity of *I. coccinea* and *I. perviflora* leaves through the free radical scavenging, superoxide anion radical scavenging, nitric oxide scavenging, metal chelation and reducing power assay.

Liver diseases, especially viral hepatitis occurs predominantly in the developing world with an enormous impact on public health & economy. [2] Carbon tetrachloride (CCl<sub>4</sub>) is widely used in animal models to induce acute liver injury. [3-5] It is generally believed that the toxicity of CCl<sub>4</sub> results from its reductive dehalogenation by the cytochrome P450 enzyme system into the highly reactive free radical trichloromethyl radical. [6] Antioxidant action has been reported to play a crucial role in the hepatoprotection. [7]

It has been suggested that natural antioxidants are more safe and healthy than synthetic antioxidants which used in foods. [8] In the present study an attempt has been taken to elucidate the effect of the different extracts of *Ixora* species on CCl<sub>4</sub> induced hepatic damage with reference to biochemical marker enzymes & histopathology. The result of this *In Vivo* study may support these two plants to be good herbal antioxidant drugs.

## **MATERIALS AND METHODS:**

### **Plant materials**

The fresh leaves of *Ixora coccinea* Linn and *Ixora perviflora* Vahl were collected from the Medicinal Plants' Garden of Gupta College Of Technological Sciences, in the morning between 9 to 10 a.m. during the months of November and December. The herbariums of *Ixora coccinea* Linn and *Ixora perviflora* Vahl were submitted to Botanical Survey of India, Shibpur Botanical Garden, Howrah and was authenticated by the Director of the same. Reference number of the Authentication letter is CNH/ 1-1/ (201)/ 2007/ Tech. II.

### **Extraction of plant materials**

Fresh leaves of each plant were collected and shade dried at room temperature, pulverized and 100 gm (each batch) leaf powder of each plant was extracted with 50% hydro-alcohol (500 ml) and ethyl acetate separately in Soxhlet extractor. The extracts

were concentrated in a rotary flash evaporator. The residue (semisolid) was dried in a desiccator over sodium sulfite and kept in refrigerator for further study.

In other hand, fresh leaves of each plant were collected and shade dried at room temperature, pulverized and 100 gm (each batch) leaf powder of each plant was extracted with distilled water (500 ml) through cold maceration. The extract was concentrated in a rotary flash evaporator. The residue (semisolid) was dried in a desiccator over sodium sulfite and kept in refrigerator for further study.

### **In Vitro Antioxidant Activity**

#### **Superoxide anion scavenging activity assay**

The scavenging activity of various extracts of *Ixora coccinea* leaves towards superoxide anion radicals was measured by colorimetric method. [9, 30] Superoxide anion was generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75ml of NBT (300 $\mu$ M) solution, 0.75ml of NADH (936  $\mu$ M) solution and 0.3ml of different concentrations of each extract. The reaction was initiated by adding 0.75ml of PMS (120  $\mu$ M) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where  $A_0$  was the absorbance of the control (blank, without extract) and  $A_1$  was the absorbance in the presence of the extract.

#### **Hydroxyl radical scavenging activity assay**

The scavenging activity for hydroxyl radicals was measured. [10, 30] Reaction mixture contained 60 $\mu$ l of 1.0 mM  $\text{FeCl}_3$ , 90  $\mu$ l of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150  $\mu$ l of 0.17 M  $\text{H}_2\text{O}_2$  and 1.5 ml of extract at various concentrations. Adding  $\text{H}_2\text{O}_2$  started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where  $A_0$  was the absorbance of the control (blank, without extract) and  $A_1$  was the absorbance in the presence of the extract.

#### **Nitric oxide scavenging activity assay**

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. [11-12, 30] 2 ml of 10 mM sodium nitroprusside in 0.5 ml of phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25 °C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml of sulfanilic acidreagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where  $A_0$  was the absorbance of the control (blank, without extract) and  $A_1$  was the absorbance in the presence of the extract.

#### **Hydrogen peroxide scavenging activity assay**

Hydrogen peroxide scavenging activity of the extract was estimated by replacement titration. [13, 30] Aliquot of 1.0 ml of 0.1mM  $H_2O_2$  and 1.0 ml of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2M  $H_2SO_4$  and 7.0 ml of 1.8 M KI. The mixed solution was titrated with 5.09 mM  $NaS_2O_3$  until yellow colour disappeared. Percentage of scavenging of hydrogen peroxide was calculated as:

$$\% \text{ inhibition} = [(V_0 - V_1) / V_0] \times 100$$

Where  $V_0$  was Volume of  $NaS_2O_3$  solution used to titrate the control sample in the presence of hydrogen peroxide (without extract),  $V_1$  was the volume of  $NaS_2O_3$  solution used in the presence of extract.

#### **Fe<sup>2+</sup> chelating activity assay**

To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of  $FeCl_2$  (2mM) was added. After 30 s, 0.1 ml ferrozine (5mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min of room temperature, the absorbance of the  $Fe^{2+}$  - Ferrozine complex was measured at 562nm. [14, 30] The chelating activity of the extract for  $Fe^{2+}$  was calculated as:

$$\text{Chelating rate (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where  $A_0$  was the absorbance of the control (blank, without extract) and  $A_1$  was the absorbance in the presence of the extract.

### **Reducing power assay**

The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate ( $K_3Fe(CN)_6$ ) (1%, w/v), followed by incubating at 50° C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 800g for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride solution (0.1%, w/v) for 10 min. The absorbance at 700nm was measured as the reducing power. [15, 30] Higher absorbance of the reaction mixture indicated greater reducing power.

### **In Vivo antioxidant activity**

#### **Experimental Animal**

Wister strain albino rats of 150-200 g body weight were used in study. Animals were procured from Institutional Animal house of Gupta College Of Technological Sciences, Asansol. All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27°C and humidity 60-65% with 12:12 light: dark cycles. Food was provided in the form of dry pellets and water *ad libitum*). All the experiments were conducted between 9.00 and 15.00 hours. The animals were allowed to get acclimatized to the laboratory conditions for 7 days before the commencement of the experiment. Food was withdrawn 18 hours prior to the commencement of the experiment. All experiments involving animals comply with the ethical standards of animal handling and approved by Institutional Animal Ethics Committee (Regd.No.- 955/a/06/ CPCSEA).

#### **Experimental Procedure**

Hepatopathy was induced in animals by administration of  $CCl_4$  interperitoneally (i.p) at the dose of 1.25 ml/kg,i.p, in liquid paraffin for 14 days .The rats were equally divided into Six groups, each group contains six animals. [18,23]

Group-I was considered as control, Group-II was considered as  $CCl_4$  (1.25 ml/kg,i.p,/14 days) treated animals, Group-III was considered as  $CCl_4$  and aqueous extract (250mg/kg,p.o/14 days) treated animals, where Group-IV was considered as  $CCl_4$  and hydro-alcoholic extract (250mg/kg,p.o/14 days) treated animals, Group-V was treated as  $CCl_4$  and ethyl acetate extract (250mg/kg,p.o/14 days) treated animals and at last Group-VI was considered as  $CCl_4$  and silymarin (100 mg/kg, p.o/14 days) treated animals.

At the end of the treatment, rats were sacrificed by cervical dislocation, blood samples were collected by direct cardiac puncture and serum was used for the assay of marker

enzymes and other parameters, Liver was dissected out and immediately preserved in 10% formaldehyde solution for histopathological study.

### **Serum biochemical estimations**

Triglycerides, Cholesterol, Total Protein, Total Bilirubin, Serum glutamate- oxaloacetate transaminase (S.G.O.T) or Aspartate amino transferase (AST), Serum glutamate-pyruvate transaminase (S.G.P.T) or L-alanine amino transferase (ALT), Alkaline phosphate (ALP) were estimated using standard commercial kits from SPAN India Ltd. Surat, India. [16-19,21-29]

### **Statistical Analysis**

In *in-vitro* activity tests were carried out in triplicate. The amount of extract needed to inhibit free radicals concentration by 50%, IC<sub>50</sub>, was graphically determined and means, standard deviation & correlation were computed by using Microsoft Excel.

In *in-vivo* activity the values are expressed in terms of Mean  $\pm$  S.E.M. of (n=6).

Prism 3.0 software was used to perform all statistical analysis. It was carried out by one-way analysis of variance (ANOVA), followed by Tukey Kramer multiple comparison post test. P values < 0.05 were considered as significant.

## **RESULTS AND DISCUSSION:**

### ***In Vitro* activity**

#### **Superoxide anion scavenging activity**

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system. The Superoxide anion scavenging activity of the extracts from *Ixora coccinea* and *Ixora perviflora* assayed by the PMS-NADH system is shown. The Superoxide anion scavenging activities of all the extracts of *Ixora coccinea* were increased markedly with the concentrations. Now in between three extracts ethyl acetate and aqueous extracts showed better result than hydro-alcoholic extract. The half inhibition concentration (IC<sub>50</sub>) of hydro-alcoholic, aqueous and ethyl acetate extracts were 95  $\mu$ g/ml, 45  $\mu$ g/ml and below 10  $\mu$ g/ml respectively for *I. coccinea*. Where for *I. perviflora* the half inhibition concentration (IC<sub>50</sub>) of hydro-alcoholic, aqueous and ethyl acetate extracts were 98  $\mu$ g/ml, 60  $\mu$ g/ml and 20  $\mu$ g/ml respectively. These results suggested that all the extracts of *Ixora coccinea* and *Ixora perviflora* leaves had important superoxide radical scavenging effect. But the aqueous

extract and ethyl acetate extract of *I. coccinea* showed better superoxide anion scavenging activity than any other extracts of *I. perviflora*.

#### **Hydroxyl radical scavenging activity**

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. The result showed the above three extracts of *I.coccinea* and *Ixora perviflora* leaves exhibited concentration dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC<sub>50</sub> of hydro-alcoholic, aqueous and ethyl acetate extracts were 120µg/ml, 55 µg/ml and 20 µg/ml respectively for *I. coccinea* and for *I. perviflora* the IC<sub>50</sub> of hydro-alcoholic, aqueous and ethyl acetate extracts were 150µg/ml, 78 µg/ml and 15 µg/ml respectively. The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups.

#### **Nitric oxide scavenging activity**

Nitric oxide (NO) is a potent pleiotropic mediator of physiological process such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities. All the three extracts of *I.coccinea* and *I. perviflora* moderately inhibited nitric oxide in dose dependent manner. The IC<sub>50</sub> of hydro-alcoholic, aqueous and ethyl acetate extracts were 920 µg/ml, 620 µg/ml and more than 1000 µg/ml respectively for *I. coccinea* and for *I. perviflora* the IC<sub>50</sub> of hydro-alcoholic, aqueous and ethyl acetate extracts were 930 µg/ml, 510 µg/ml and more than 1000 µg/ml respectively. Now in between three extracts aqueous extract showed better result than hydro-alcoholic and ethyl acetate extracts.

#### **Hydrogen peroxide scavenging activity**

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H<sub>2</sub>O<sub>2</sub> can probably react with Fe<sup>2+</sup>, and possibly Cu<sup>2+</sup> ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. All the three extracts of *I.coccinea* moderately inhibited hydrogen peroxide in dose dependent manner. The IC<sub>50</sub> of hydro-

alcoholic, aqueous and ethyl acetate extracts were 800 µg/ml, 720 µg/ml and more than 1000 µg/ml respectively for *I. coccinea* and for *I. perviflora*. The IC<sub>50</sub> of hydro-alcoholic, aqueous and ethyl acetate extracts were 680 µg/ml, 540 µg/ml and more than 1000 µg/ml respectively. Now in between three extracts aqueous extract showed better result than hydro-alcoholic and ethyl acetate extracts.

#### **Fe<sup>2+</sup> chelating activity**

All the three extracts of *I. coccinea* and *I. perviflora* inhibited ferrous ion in dose dependent manner. The IC<sub>50</sub> of hydro-alcoholic, aqueous and ethyl acetate extracts were 225 µg/ml, 160 µg/ml and 80 µg/ml respectively for *I. coccinea* and for *I. perviflora* the IC<sub>50</sub> of hydro-alcoholic, aqueous and ethyl acetate extracts were 210 µg/ml, 200 µg/ml and 90 µg/ml respectively. In between that ethyl acetate extract showed more effective scavenging of ferrous ion than hydro-alcoholic and aqueous extracts.

#### **Reducing power activity**

It depicts the reductive effect of *Ixora coccinea* and *Ixora perviflora* similar to the antioxidant activity, the reducing power of both *I. coccinea* and *I. perviflora* increased with increasing dosage. High absorbance at 700nm indicates high reducing power.

#### **In-vivo activity**

Liver injury induced by CCl<sub>4</sub> is the best characterized system of xenobiotic-induced hepatotoxicity and is commonly used models for the screening of anti hepatotoxic and/or hepatoprotective activities of drugs. The changes associated with CCl<sub>4</sub> induced liver damage are similar to that of acute viral hepatitis. It has been established that CCl<sub>4</sub> accumulates in hepatic parenchymal cells and gets metabolically activated by CYP P-450 dependent monooxygenases form trichloromethyl free radical (CCl<sub>3</sub>). These free radical alkylates cellular proteins and other macromolecules with a simultaneous attack on poly unsaturated fatty acids in the presence of oxygen to produce lipid peroxides. Leading to liver damage, Lipid peroxidation will initiate pathological changes such as depression of protein synthesis, elevation of serum marker enzymes such as AST, ALT and ALP. SGPT is more specific to the liver and a better parameter for detecting liver damage.

All the extracts at the dose of 250 mg/kg decreased the level of both AST and ALT significantly in CCl<sub>4</sub> treated rats indicating maintenance of functional integrity of hepatic cell membrane. Serum ALP and bilirubin levels are also related to the status and function of hepatic cells. Increase in serum ALP is due to increased synthesis, in presence of increasing biliary pressure.

In the present study all extracts of both the plants have been found to reduce both serum ALP and Bilirubin in the treated groups compared with the untreated ones. The site specific oxidative damage of some of the susceptible amino acids of proteins is regarded as the major cause of metabolic dysfunction during pathogenesis. Hypoalbuminaemia is most frequent in the presence of advanced chronic diseases. Hence decline in total protein content can be deemed as a useful index of the severity of cellular dysfunction in chronic liver diseases. The lowered level of total proteins recorded in the serum as well as lever of CCl<sub>4</sub> treated rats reveals the severity of hepatopathy. Different extracts treated rats maintained near normally of total protein level. Treatment with CCl<sub>4</sub> increases the levels of total lipid, total triglycerides and total cholesterol in liver. Presence of significantly high concentration of total lipid and cholesterol in the serum of CCl<sub>4</sub> treated animals and maintains of these towards near normal values in different extracts administered rats demonstrates the hepatoprotective effect of both the *Ixora* species. In between all the extracts, the aqueous extract has shown better result than other ethyl acetate and hydro-alcoholic extracts (Table 1 and Table 2).

**Table 1: Serum Biochemical Estimations of *Ixora coccinea* Extracts**

Groups	Treatment	Triglycerides	Cholesterol	Protein	Total Bilirubin	S.G.O.T	S.G.P.T	Alkaline phosphate
		(mg/dl.)	(mg/dl.)	Total				
<b>I</b>	Normal	151.72±	131.2±	7.29±	0.50±	38.26±	37.4±	7.36± 2.02
	Control	2.57	1.61	0.12	0.02	2.13	2.15	
<b>II</b>	CCl <sub>4</sub>	262.06±	281.2±	4.94 ±	1.85±	111.30	88.4±	25.26±
		4.09	3.3	0.27	0.25	±3.55	2.55	2.20
<b>III</b>	CCl <sub>4</sub> +	165.51±	225.0±	5.88±	1.10±	62.60±	40.8±	13.15 ±
	Aqueous Extract	2.60	1.41	1.08	0.13	2.44	2.97	3.57
<b>IV</b>	CCl <sub>4</sub> + Hydro-alcoholic Extract	217.93±	243.7±	6.35±	1.25±	68.11±	51.0±	15.78±
		1.41	2.07	1.03	0.03	2.66	1.80	3.19
<b>V</b>	CCl <sub>4</sub> + Ethyl acetate Extract	190.21±	233.4±	6.11±	1.15±	63.10±	56.2±	14.77±
		2.60	1.72	0.16	0.03	1.23	1.92	0.22
<b>VI</b>	CCl <sub>4</sub> +	154.92±	133.8±	7.40±	0.51±	41.02±	39.41±	9.11±
	Silymarin	2.43	1.92	0.16	0.03	2.60	1.72	0.16

**Table 2: Serum Biochemical Estimations of *Ixora perviflora* Extracts**

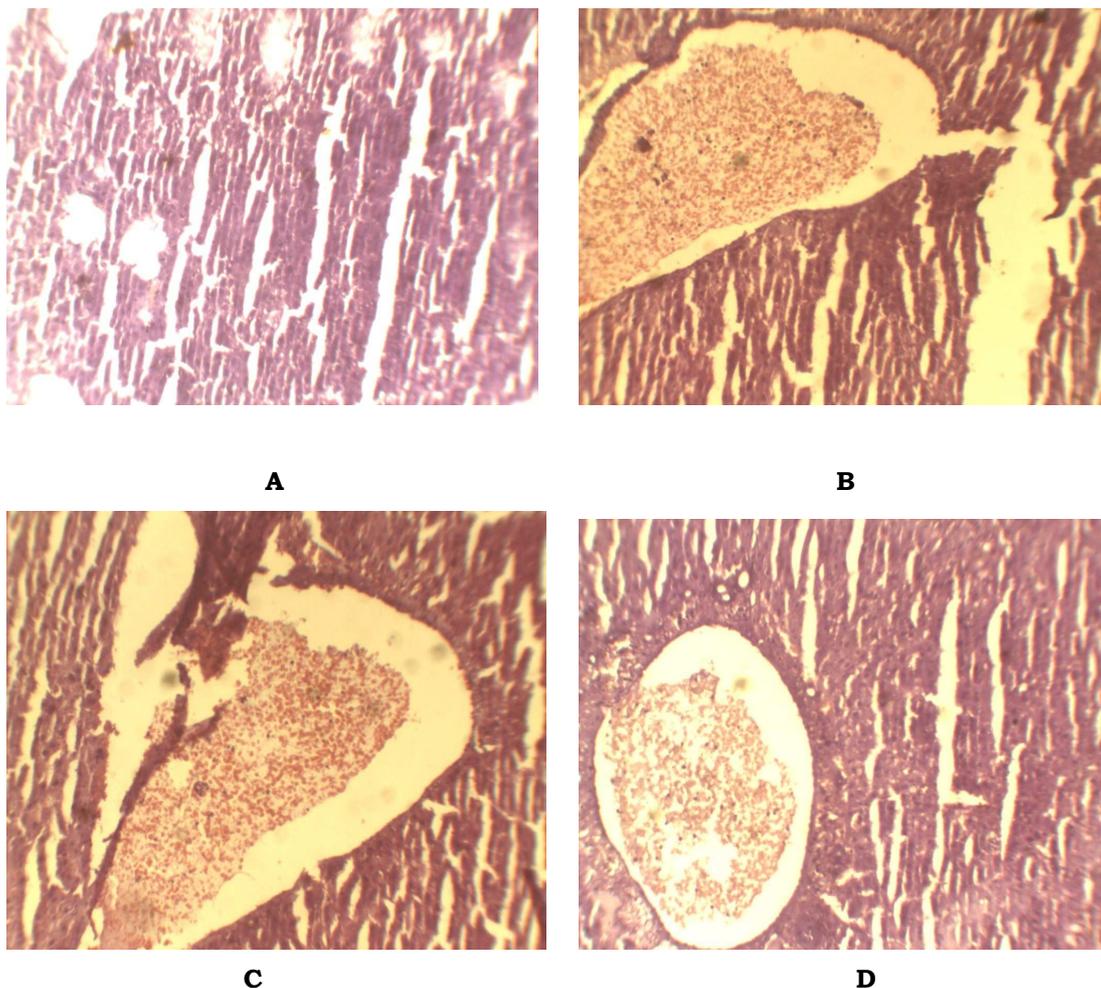
Groups	Treatment			Protein				Alkaline phosphate (KA Units)	
		Triglycerides (mg/dl.)	Cholesterol (mg/dl.)	Total (g/dl.)	Total Bilirubin (mg/dl.)	S.G.O.T (IU/L)	S.G.P.T (IU/L)		
<b>I</b>	Normal	152.32±	130.5±	7.52±	0.60±	40.11±	35.3±	7.00± 1.03	
	Control	1.15	1.01	0.26	0.03	1.33	2.22		
<b>II</b>	CCl <sub>4</sub>	282.16±	285.1±	4.00 ±	2.00±	121.2±	85.5±	30.02±	
		3.11	2.3	0.33	0.05	3.01	1.05	1.10	
<b>III</b>	CCl <sub>4</sub> + Aqueous Extract	160.01±	235.0±	6.02±	1.15±	71.30±	47.3±	21.01 ±	
		2.01	1.04	1.05	0.10	2.02	2.07	3.27	
<b>IV</b>	CCl <sub>4</sub> + Hydro- alcoholic Extract	220.00±	244.2±	5.85±	1.35±	78.11±	52.0±	25.54±	
		1.04	2.21	0.03	1.03	2.01	1.20	2.11	
<b>V</b>	CCl <sub>4</sub> + Ethyl acetate Extract	190.01±	232.2±	6.10±	1.25±	60.01±	51.1±	22.21±	
		1.00	1.02	0.15	1.03	0.06	1.02	0.54	
<b>VI</b>	CCl <sub>4</sub> + Silymarin	154.92±	133.8±	7.40±	0.51±	41.02±	39.41±	9.11±	
		2.43	1.92	0.16	0.03	2.60	1.72	0.16	

### Histopathological Study

Histopathological studies also provided supportive evidence for the biochemical analysis. The different extracts treated groups and Silymarin treated group showed the normal parenchymal architecture without noticeable alteration compared to CCl<sub>4</sub> treated group. Centrilobular necrosis accompanied by fatty changes and ballooning degeneration were observed in group treated with CCl<sub>4</sub> (Fig. 1).

### CONCLUSION:

Both *I. coccinea* and *I. perviflora* showed a promising result in *In Vitro* and *In Vivo* antioxidant activity. So the conclusion of this research work is to find out two different medicinal plants which have positive antioxidant property.



**Fig. 1: Histopathological Section of A) CCl<sub>4</sub> treated Liver , B) *I. coccinea* Extract Treated Liver, C) *I. perviflora* Extract Treated Liver , D) Silymarin Treated Liver**

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