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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_4 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_4 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_4 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 perviflora*, aspartate amino transferase, L-alanine amino transferase.

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 perviflora.* 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_4) is widely used in animal models to induce acute liver injury. [3-5] It is generally believed that the toxicity of CCl_4 results from its reductive dehalogenation by the cytochrome P450 enzyme system into the highly reactive free radical trichloromethyl redical. [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_4 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 desicator 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 desicator 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 (300μM) solution, 0.75ml of NADH (936 μM) solution and 0.3ml of different concentrations of each extract. The reaction was initiated by adding 0.75ml of PMS (120 μM) 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 60μl of 1.0 mM $FeCl_3$, 90 μl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 μl of 0.17 M H_2O_2 and 1.5 ml of extract at various concentrations. Adding H_2O_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 lllosvoy 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^{2+} 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:

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^{0}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 transminase (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_{50}, 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 ± 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_{50}) of hydro-alcoholic, aqueous and ethyl acetate extracts ware 95 μg/ml, 45 μg/ml and below 10 μg/ml respectively for *I. coccinea*. Where for *I. perviflora* the half inhibition concentration (IC_{50}) of hydro-alcoholic, aqueous and ethyl acetate extracts ware 98 μg/ml, 60 μg/ml and 20 μg/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_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 120μg/ml, 55 μg/ml and 20 μg/ml respectively for *I. coccinea* and for *I. perviflora* the IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 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_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 920 μg/ml, 620 μg/ml and more then 1000 μg/ml respectively for *I. coccinea* and for *I. perviflora* the IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 930 μg/ml, 510 μg/ml and more then 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_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} 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_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 800 μg/ml, 720 μg/ml and more then 1000 μg/ml respectively for *I. coccinea* and for *I. perviflora* The IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 680 μg/ml, 540 μg/ml and more then 1000 μg/ml respectively. Now in between three extracts aqueous extract showed better result than hydro-alcoholic and ethyl acetate extracts.

Fe^{2+} chelating activity

All the three extracts of *I. coccinea* and *I. perviflora* inhibited ferrous ion in dose dependent manner. The IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 225 μg/ml, 160 μg/ml and 80 μg/ml respectively for *I. coccinea* and for *I. perviflora* the IC_{50} of hydro-alcoholic, aqueous and ethyl acetate extracts ware 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 depict 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_4 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_4 induced liver damage are similar to that of acute viral hepatitis. It has been established that CCl_4 acumulates in hepatic parenchymal cells and gets metabolically activated by CYP P-450 depended monoxygenases form trichloromethyl free radical (CCl_3). These free radical alkylates cellular proteins and other macromolecules with a simultaneous attact 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_4 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_4 treated rats reveals the severity of hepatopathy. Different extracts treated rats maintained near normally of total protein level. Treatment with CCl_4 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_4 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** (mg/dl.)	**Cholesterol** (mg/dl.)	**Total Protein** (g/dl.)	**Total Bilirubin** (mg/dl.)	**S.G.O.T** (IU/L)	**S.G.P.T** (IU/L)	**Alkaline phosphate** (KA Units)
I	Normal Control	151.72± 2.57	131.2± 1.61	7.29± 0.12	0.50± 0.02	38.26± 2.13	37.4± 2.15	7.36± 2.02
II	CCl_4	262.06± 4.09	281.2± 3.3	4.94 ± 0.27	1.85± 0.25	111.30 ±3.55	88.4± 2.55	25.26± 2.20
III	CCl_4 + Aqueous Extract	165.51± 2.60	225.0± 1.41	5.88± 1.08	1.10± 0.13	62.60± 2.44	40.8± 2.97	13.15 ± 3.57
IV	CCl_4 + Hydro-alcoholic Extract	217.93± 1.41	243.7± 2.07	6.35± 1.03	1.25± 0.03	68.11± 2.66	51.0± 1.80	15.78± 3.19
V	CCl_4 + Ethyl acetate Extract	190.21± 2.60	233.4± 1.72	6.11± 0.16	1.15± 0.03	63.10± 1.23	56.2± 1.92	14.77± 0.22
VI	CCl_4 + Silymarin	154.92± 2.43	133.8± 1.92	7.40± 0.16	0.51± 0.03	41.02± 2.60	39.41± 1.72	9.11± 0.16

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

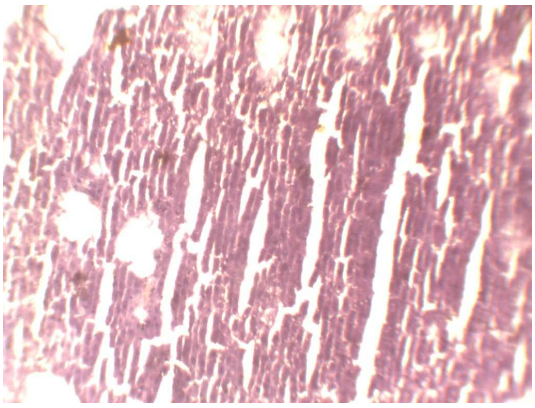
Groups	Treatment	Triglycerides (mg/dl.)	Cholesterol (mg/dl.)	Total Protein (g/dl.)	Total Bilirubin (mg/dl.)	S.G.O.T (IU/L)	S.G.P.T (IU/L)	Alkaline phosphate (KA Units)
I	Normal Control	152.32± 1.15	130.5± 1.01	7.52± 0.26	0.60± 0.03	40.11± 1.33	35.3± 2.22	7.00± 1.03
II	CCl_4	282.16± 3.11	285.1± 2.3	4.00 ± 0.33	2.00± 0.05	121.2± 3.01	85.5± 1.05	30.02± 1.10
III	CCl_4 + Aqueous Extract	160.01± 2.01	235.0± 1.04	6.02± 1.05	1.15± 0.10	71.30± 2.02	47.3± 2.07	21.01 ± 3.27
IV	CCl_4 + Hydro-alcoholic Extract	220.00± 1.04	244.2± 2.21	5.85± 0.03	1.35± 1.03	78.11± 2.01	52.0± 1.20	25.54± 2.11
V	CCl_4 + Ethyl acetate Extract	190.01± 1.00	232.2± 1.02	6.10± 0.15	1.25± 1.03	60.01± 0.06	51.1± 1.02	22.21± 0.54
VI	CCl_4 + Silymarin	154.92± 2.43	133.8± 1.92	7.40± 0.16	0.51± 0.03	41.02± 2.60	39.41± 1.72	9.11± 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_4 treated group. Centrilobular necrosis accompanied by fatty changes and ballooning degeneration were observed in group treated with CCl_4 (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.



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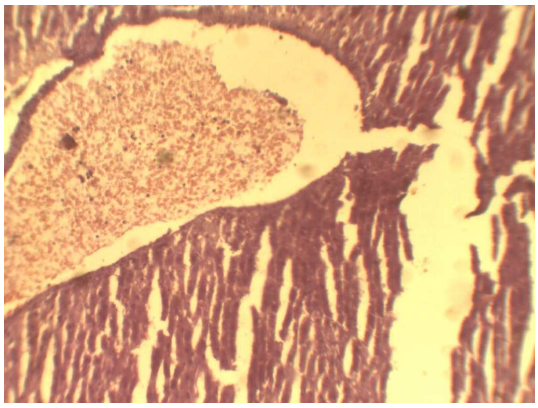

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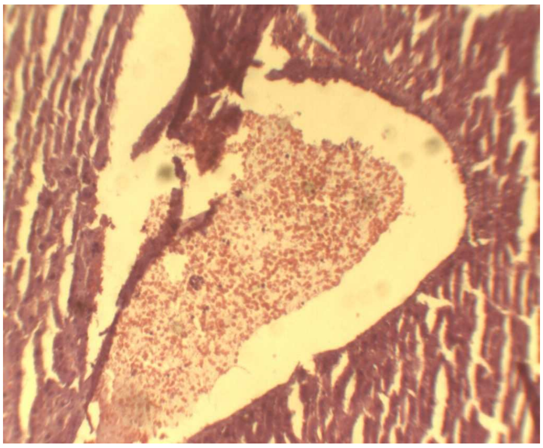

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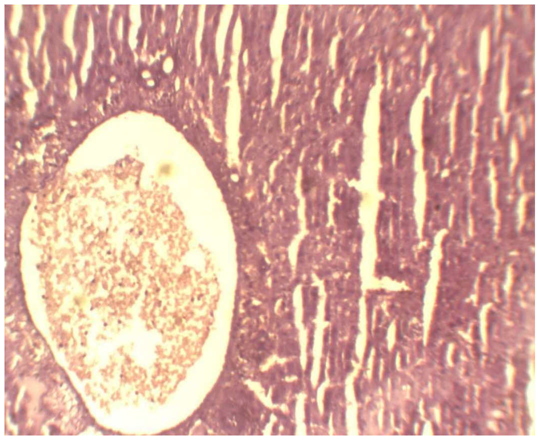

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Fig. 1: Histpathological Section of A) CCl_4 treated Liver , B) *I. coccinea* Extract Treated Liver, C) *I. perviflora* Extract Treated Liver , D) Silymarin Treated Liver

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