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Evaluation of hepatoprotective effect of *Withania somnifera* and *Rubia cordifolia* against carbon tetrachloride induced hepatotoxicity in albino rats

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Abstract---Liver is one of the largest gland of the body and the main site for intense metabolism and excretion. Liver is concerned with metabolism of endogenous as well as exogenous substances. "*Withania somnifera*" and "*Rubia cordifolia*" claim to have hepatoprotective action. Till now, no scientific study has been performed to evaluate such claims. This was an experiment study conducted on wistar rats, in Department of Pharmacology, Index Medical College. Healthy Albino wistar rats of either gender, weighing 150- 200g were obtained from CPCSEA approved Central Animal House. The selected rats were housed in polpropylene cages under controlled conditions of temperature (25 °C) and alternating periods of light and darkness of 12 hours each. The rats had free access to standard rat pellet diet and tap water *ad libitum*. After one week of acclimatization, the animals were rendered suitable for study. Pregnant female rats were not included in the study. ALT level in normal saline treated group was 29.5±3.35 IU/L. It was found to be significantly increased (p<0.001) with administration of CCl₄ to 433.5±48.67 IU/L. Pretreatment with known hepatoprotective preparation Liv.52 significantly (p<0.001) limited the rise in ALT

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levels after CCl₄ administration to 140.7±8.1 IU/L. ALP level in normal saline treated group was 73.9±4.63 IU/L. It was found to be significantly increased (p<0.001) with administration of CCl₄ to 600.42±52.9 IU/L. With *Rubia cordifolia* there is dose depended limitation of ALP rise after CCl₄ administration. Although the dose of 100mg/kg for 21 days showed a significant limitation (p<0.05) of ALP rise (401±45.26) when compare to CCl₄ treated group but it did not match the efficacy of Liv. 52 treated group. However, in dose of 200 mg/kg for 21 days the *Rubia cordifolia* extract had much efficacy, in limiting the ALT rise after CCl₄ administration, to 245.1±21.25 IU/L, which was significant (p<0.01). Aqueous extract *Rubia cordifolia* and *Withania somnifera* is a potent hepatoprotective agent. Further studies with its hydroalcoholic extract can be expected to provide still better results. It is suggestible that the dosage form of *Rubia cordifolia* and *Withania somnifera* aqueous extract can be changed from the current *Rubia cordifolia* and *Withania somnifera* aqueous extract form, to some other form like hydroalcoholic extract or extraction of some specific component which is involved in hepatoprotective action.

Keywords---hepatoprotective, withania somnifera, rubia cordifolia.

Introduction

Liver is one of the largest gland of the body and the main site for intense metabolism and excretion. Liver is concerned with metabolism of endogenous as well as exogenous substances. It has an important role in the maintenance, performance and regulation of homeostasis in our body as it is involved in almost all the biochemical pathways related to growth, immunity, nutrient supply, energy provision and reproduction. [1] On other hand its strategic location in the body endows liver with the capability of intercepting various harmful entities, but on other hand it also exposes the liver to myriads of insults on the other. Despite having enormous reserve capacity, continued insult to the liver results in liver diseases. [2]

Although exact epidemiology of liver diseases is unknown, but on the basis of clinical cases seen, it is hypothesized that liver diseases are a common occurrence. They generally present clinically in a few distinct patterns usually classified as hepatocellular, cholestatic (obstructive), or mixed. [3] In hepatocellular diseases (such as viral hepatitis or alcoholic liver disease), features of liver injury, inflammation and necrosis predominate. In cholestatic diseases (such as gall stones or malignant obstruction, primary biliary cirrhosis, certain drug induced liver diseases), features of inhibition of bile flow predominate. In a mixed pattern, features of both hepatocellular and cholestatic injury are present (such as in cholestatic forms of viral hepatitis and many drug-induced liver diseases). [4]

Liver disease may be primary as in the case of viral hepatitis but most often liver involvement is secondary to cardiac decompensation, alcoholism and other toxins. Histologically hepatic lesion may vary from fatty changes to hepatitis to cirrhosis and massive necrosis. Almost all kinds of liver injuries complicate into hepatocellular failure if ignored, and may ultimately lead to death. [5]

Unfortunately, till date, modern medicine has not been able to come up with a satisfactory answer to liver disorders. The indigenous system of Ayurvedic medicine claims to have prescriptions of therapeutic value in hepatic diseases. Various indigenous plants have been screened for hepatoprotective activity and many have been found to possess significant activity in this regard. [6] To name a few *Silybum marianum*, *Picrorhiza kurrooa*, *Boerhaavia diffusa*, *Solanum nigrum*, *Phyllanthus niruri*, are some of the mentionable plants in this context. Also, there are some formulations which have been established as hepatoprotective agents both in experimental as well as clinical studies. An outstanding example of one such formulation is Liv.52, a product of The Himalaya Drug Company. [7]

However, there are still many herbs, which have been claimed to possess therapeutic potential against liver disorders, but not been scientifically explored. Two such plants are -*Withania somnifera* and -*Rubia cordifolia* claim to have hepatoprotective action. Till now, no scientific study has been performed to evaluate such claims. Therefore, the present study is intended to evaluate the hepatoprotective activity of these plants and compare it with the already established hepatoprotective agent Liv-52 in experimentally induced hepatotoxicity in albino rats.

Aims and Objectives

To compare hepatoprotective activity of aqueous extract of *Withania somnifera* and *Rubia cordifolia* with Liv.52 in albino rats.

Materials and Methods

This was an experiment study conducted on wistar rats, in Department of Pharmacology, Index Medical College Hospital and Research Centre, Indore (M.P) from 2018 to 2021. The study was commenced after getting approval from Institutional Animal Ethical Committee of Index Medical College Hospital and Research Centre, Indore (M.P).

Healthy Albino wistar rats of either gender, weighing 150- 200g were obtained from CPCSEA approved Central Animal House of Index medical college. The selected rats were housed in polpropylene cages under controlled conditions of temperature (25 °C) and alternating periods of light and darkness of 12 hours each. The rats had free access to standard rat pellet diet and tap water *ad libitum*. After one week of acclimatization, the animals were rendered suitable for study. Pregnant female rats were not included in the study.

Test compound

1. To induce hepatotoxicity, commercially available preparation of injectable Carbon tetrachloride (Nice chemicals Pvt. Ltd. Cochin) was used.
2. Aqueous extract of *Rubia cordifolia*, to evaluate hepatoprotective activity.
3. Aqueous extract of *Withania somnifera*, to evaluate hepatoprotective activity.

Withania somnifera

Leaves of *Withania somnifera* were obtained and allowed to dry under the shade. Thereafter 50 gm of dry powdered leaves was boiled in 250ml of water for half an hour. This solution was left to stand for 24 hrs. Next day the solution was filtered and evaporated to obtain dry extract (Saman *et al.*, 2010). This dried extract was further powdered and then stored at 0-4°C. When needed the extract was suspended in water to achieve a concentration of 1%.

Rubia cordifolia

Roots of *Rubia cordifolia* were dried under shade and ground to make a fine powder in a herb grinder. One gram of finely powdered plant material was extracted with 50 ml of water. The solution was filtered using Whatman filter paper no 1. Solution was evaporated under vacuum in a rotary evaporator to make the extract dry. This dried extract was stored at 0- 4°C and dissolved in water to make a solution of 1% concentration when ever required.

The animals were randomly divided into seven groups of six animals each. The groups are described as follows:

- Group- I: control group was administered 0.9% NaCl solution in a single oral dose of 1ml/kg body wt for 21 days.
- Group -II: In addition to pellet diet and tap water ad libitum, this group was injected with toxin CCl₄ (1ml/kg) i.p only once to produce hepatotoxicity on 21st day.
- Group-III: This group was given *Rubia cordifolia* orally (100 mg/kg) (Gilani AH *et al.*,1995). As a single dose per orally every morning for 21 days followed by an injection of CCl₄ (1 ml/kg i.p.) on 21st day.
- Group -IV: This group was received *Rubia cordifolia* orally (200 mg/kg) as a single dose per orally every morning for 21 days followed by an injection of CCl₄ (1 ml/kg i.p.) on 21st day.
- Group-V: This group was given *Withania somnifera* orally (500 mg/kg) (Bhattacharya A *et al.*,2000) as a single dose per orally every morning for 21 days followed by an injection of CCl₄ (1 ml/kg i.p.) on 21st day.
- Group-VI: This group was administered *Withania somnifera* orally (1000 mg/kg) as a single dose per orally every morning for 21 days

followed by an injection of CCl₄ (1 ml/kg i.p.) on 21st day.

- Group-VII: This group received Liv.52 (1ml/kg) orally for 21 days followed by an injection CCl₄ (1 ml/kg i.p.) on 21st day.

Liv.52 and the test compounds (*Rubia cordifolia* and *Withania somnifera*) were administered by gavage method with animals fasted 3-4 hours prior to and 1 hour after administration of test drugs to ensure proper absorption. After administration of carbon tetrachloride, animals of all the groups were fasted for 24 hours although water remained freely available during this period thereafter animals were sacrificed under Ketamine (75 mg/kg) and Diazepam (10 mg/kg) anaesthesia given intraperitoneally.

Blood samples were collected from abdominal aorta for performing liver function tests which included total Bilirubin, Alanine Transaminase, Alkaline Phosphatase and Albumin. Also, the liver was dissected out for histopathological studies.

The liver was excised from the animals and washed with the normal saline. About one cm piece was cut and fixed in 10% neutral formalin for 12-24 hours. It was then dehydrated and cleared with ethanol and xylene respectively followed by embedding in paraffin wax from which blocks were prepared. Sections of 5m thickness were taken from the blocks using a microtome. These were processed in alcohol-xylene series and were stained with Harris haematoxylin and eosin stain and subjected to histopathological examination.

Statistical Analysis

The data thus obtained was appropriately organized and analyzed by suitable statistical methods. i.e ; ANOVA.

Results

Aqueous extract of *Rubia cordifolia* and *Withania somnifera* showed effect on biochemical parameters.

Table 1 : Effect of Liv-52, *Rubia cordifolia* and *Withania somnifera* in their respective doses on carbon tetrachloride induced changes in serum Alanine Transaminase (n=6).

TREATMENT (mg/kg)	Alanine transaminase(IU/L)(mean±SE)
Normal saline (1ml)	29.5±3.35
CCl ₄ (1)	433.5±48.67 [^]
LIV.52 (1)	140.7±8.1 [^]
<i>Rubia cordifolia</i> (100)	281±18.33 [*]
<i>Rubia codifolia</i> (200)	177.5±12.73 [*]
<i>Withania somnifera</i> (500)	304.9±36.03 [€]
<i>Withania somnifera</i> (1000)	140.2±12.1 [^]

€P < 0.05 as compare to ccl₄ treated group.

*p < 0.01 as compare to ccl₄ treated group.

[^]P < 0.001 as compare to normal saline treated group.

ALT level in normal saline treated group was 29.5 ± 3.35 IU/L. It was found to be significantly increased ($p < 0.001$) with administration of CCl_4 to 433.5 ± 48.67 IU/L. Pretreatment with known hepatoprotective preparation Liv.52 significantly ($p < 0.001$) limited the rise in ALT levels after CCl_4 administration to 140.7 ± 8.1 IU/L. With *Rubia cordifolia* there is dose depended limitation of ALT rise after CCl_4 administration. Although the dose of 100mg/kg for 21 days showed a significant limitation ($p < 0.01$) of ALT rise (281 ± 18.33) when compared to CCl_4 treated group but it did not match the efficacy of Liv. 52 treated group. However, in dose of 200 mg/kg for 21 days the *Rubia cordifolia* extract had much efficacy, in limiting the ALT rise after CCl_4 administration, to 177.5 ± 12.73 IU/L, which was significant ($p < 0.01$). With *Withania somnifera* there is dose depended limitation of ALT rise after CCl_4 administration also. Although the dose of 500mg/kg for 21 days showed a significant limitation ($p < 0.05$) of ALT rise (304.9 ± 36.03) when compared to CCl_4 treated group but it did not match the efficacy of Liv. 52 treated group. However, in dose of 1000 mg/kg for 21 days the *Withania somnifera* extract had much efficacy, in limiting the ALT rise after CCl_4 administration, to 140.2 ± 12.1 IU/L, which was highly significant ($p < 0.001$).

Table 2 : Effect of Liv-5, *Rubia cordiafolia* and *Withania somnifera* in their respective doses on carbon tetrachloride induced changes in Alkaline Phosphatase (n=6).

TREATMENT (mg/kg)	Alkaline phosphatase (IU/L)(mean \pm SE)
Normal saline (1ml)	73.9 \pm 4.63
CCl_4 (1)	600.42 \pm 52.9 [^]
LIV.52 (1)	198.9 \pm 11.6 [^]
<i>Rubia cordifolia</i> (100)	401 \pm 45.26 [£]
<i>Rubia codifolia</i> (200)	245.1 \pm 21.25 [*]
<i>Withania somnifera</i> (500)	382.1 \pm 42.62 [£]
<i>Withania somnifera</i> (1000)	204.8 \pm 24.42 [^]

£P < 0.05 as compare to ccl_4 treated group.

*p < 0.01 as compare to ccl_4 treated group.

[^]P < 0.001 as compare to normal saline treated group.

ALP level in normal saline treated group was 73.9 ± 4.63 IU/L. It was found to be significantly increased ($p < 0.001$) with administration of CCl_4 to 600.42 ± 52.9 IU/L. Pretreatment with known hepatoprotective preparation Liv.52 significantly ($p < 0.001$) limited the rise in ALP levels levels after CCl_4 administration to 198.9 ± 11.6 IU/L. With *Rubia cordifolia* there is dose depended limitation of ALP rise after CCl_4 administration. Although the dose of 100mg/kg for 21 days showed a significant limitation ($p < 0.05$) of ALP rise (401 ± 45.26) when compare to CCl_4 treated group but it did not match the efficacy of Liv. 52 treated group. However, in dose of 200 mg/kg for 21 days the *Rubia cordifolia* extract had much efficacy, in limiting the ALT rise after CCl_4 administration, to 245.1 ± 21.25 IU/L, which was significant ($p < 0.01$). With *Withania somnifera* there is dose depended limitation of ALP rise after CCl_4 administration. Although the dose of 500mg/kg for 21 days showed a significant limitation ($p < 0.05$) of ALP rise (382.1 ± 42.62)

when compared to CCl₄ treated group but it did not match the efficacy of Liv. 52 treated group. However, in dose of 1000 mg/kg for 21 days the *Withania somnifera* extract had much efficacy, in limiting the ALP rise after CCl₄ administration, to 204.8±24.42 IU/L, which was highly significant (p<0.001).

Table 3: Effect of Liv-52, *Rubia cordifolia* and *Withania somnifera* in their respective doses on carbon tetrachloride induced changes in total serum bilirubin (n=6)

TREATMENT (mg/kg)	Total bilirubin (IU/L) (mean±SE)
Normal saline (1ml)	0.25±0.95
CCl ₄ (1)	2.03±0.86 [^]
LIV.52 (1)	0.46±0.42 [^]
<i>Rubia cordifolia</i> (100)	0.93±0.53 [*]
<i>Rubia codifolia</i> (200)	0.83±0.13 [*]
<i>Withania somnifera</i> (500)	0.80±0.53 [*]
<i>Withania somnifera</i> (1000)	0.52±0.64 [^]

[^]P< 0.05 as compare to ccl₄ treated group.

^{*}p< 0.01 as compare to ccl₄ treated group.

[^]P< 0.001 as compare to normal saline treated group.

Total Bilirubin level in normal saline treated group was 0.25±0.95 IU/L. it was found to be significantly increased (p<0.001) with administration of CCl₄ to 2.03±0.86 IU/L. Pretreatment with known hepatoprotective preparation Liv.52 significantly (p<0.001) limited the rise in total bilirubin levels after CCl₄ administration to 0.46±0.42IU/L. With *Rubia cordifolia* there is dose depended limitation of Total Bilirubin rise after CCl₄ administration. Although the dose of 100mg/kg for 21 days showed a significant limitation (p<0.01) of Total Bilirubin rise (0.93±0.53) when compared to CCl₄ treated group but it did not match the efficacy of Liv. 52 treated group. However, in dose of 200 mg/kg for 21 days the *Rubia cordifolia* extract had much efficacy, in limiting the Total Bilirubin rise after CCl₄ administration, to 0.83±0.13 IU/L, which was significant (p<0.01).

With *Withania somnifera* there is dose depended limitation of Total Bilirubin rise after CCl₄ administration. Although the dose of 500mg/kg for 21 days showed a significant limitation (p<0.01) of Total Bilirubin rise (0.8±0.53) when compared to CCl₄ treated group but it did not match the efficacy of Liv. 52 treated group. However, in dose of 1000 mg/kg for 21 days the *Withania somnifera* extract had much efficacy, in limiting the Total Bilirubin rise after CCl₄ administration, to 0.52±0.64 IU/L, which was highly significant (p<0.001).

Table 4: Effect of Liv-52, *Rubia cordifolia* and *Withania somnifera* in their respective doses on carbon tetrachloride induced changes in serum Albumin(mean± SE) (n=6)

TREATMENT (mg/kg)	Albumin (gm/dl) (mean±SE)
Normal saline (1ml)	4.6±0.82
CCl ₄ (1)	5.3±0.92
LIV.52 (1)	5.2±0.31
<i>Rubia cordifolia</i> (100)	5.4±1.14

<i>Rubia cordifolia</i> (200)	5.04±0.94
<i>Withania somnifera</i> (500)	5.14±0.88
<i>Withania somnifera</i> (1000)	5.1±0.76

There was no such difference in serum Albumin levels of any of groups and all the measurement recorded were in normal range. The Albumin levels varied from 4.2gm/dl to 5.4gm/dl showed no correlation. which was insignificant ($p>0.05$) also.

Effect on Histology

Histology of livers of normal saline treated group showed normal liver architecture. The hepatic cords and the sinusoids were well visible. (fig-1) Classical centrilobular necrosis was seen in the CCl₄ treated group. The hepatocytes around the central vein were necrosed with no distinguishable nuclei (fig-2).

Liv.52 treated group revealed vary mild signs of liver injury. Only difference from the normal saline treated group was the presence of inflammatory cells and constricted sinusoids indicating apperent hepatocyte swelling (fig-3).

Group treated with *Rubia cordifolia* with dose 100mg/kg showed feathery degeneration in the centrilobular area which is predominant histological feature, and necrosis was absent. However at dose 200mg/kg showed normal hepatic lobule architecture was seen with mild fatty changes and no necrosis was seen (fig.4-5).

Group treated with *Withania somnifera* with dose 500mg/kg showed feathery degeneration and fatty changes in the centrilobular area which is predominant histological feature, and necrosis was absent. However at dose 1000mg/kg showed almost normal hepatic lobule architecture was seen the only difference being the presence of constricted sinusoids, pointing towards mild hepatocyte swelling and no necrosis and inflammation was seen (fig.6-7).

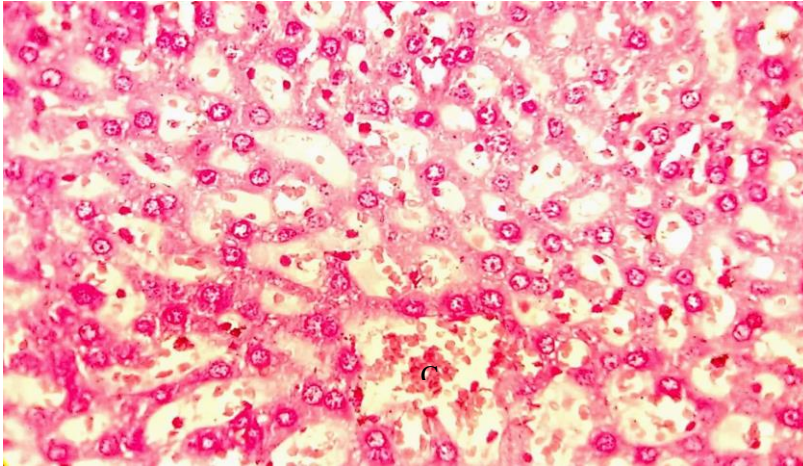


Figure 1: Microscopic feature of the liver normal saline treated group. Normal hepatic lobule architecture is seen. Hepatocytes and their nuclei are well visible. (H & E Stain x 400) CV – Central vein

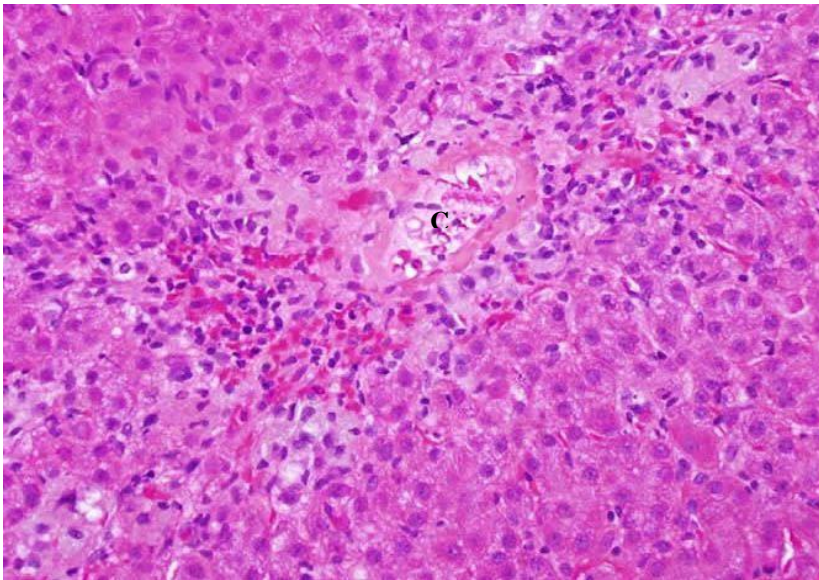


Figure 2: Microscopic feature of the liver CCl4 treated group. Extensive centrilobular necrosis is seen. Only cellular debris is seen and no hepatocytes with nuclei are discernible. (H & E Stain x 400) CV – Central vein

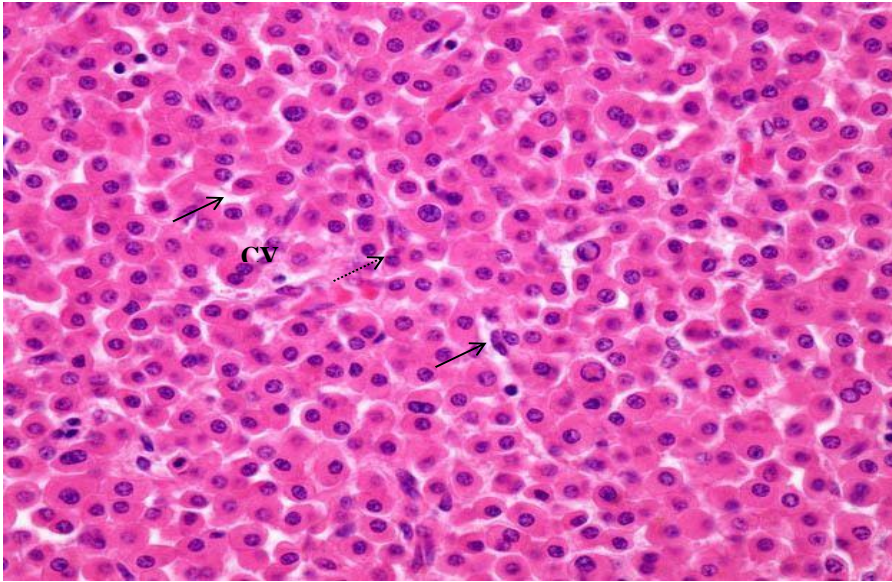


Figure 3: Microscopic feature of the liver Liv. 52 treated group.

Mild hepatocyte swelling is present as indicated by constructed sinusoids. Inflammatory cells are seen mostly around central vein.(H & E Stain x 400)

CV – Central vein

Solid Arrows : Sinusoids

Dotted Arrows : Inflammatory cells



Figure 4: Microscopic feature of the liver of animals of group treated with *Rubia Cordifolia* (100 mg/kg) . Remarkable Feathery degeneration of haepatocyte is seen around the central vein. Further away from the central vein cell with fatty changes are seen. (H & E Stain x 400)
CV – Central vein

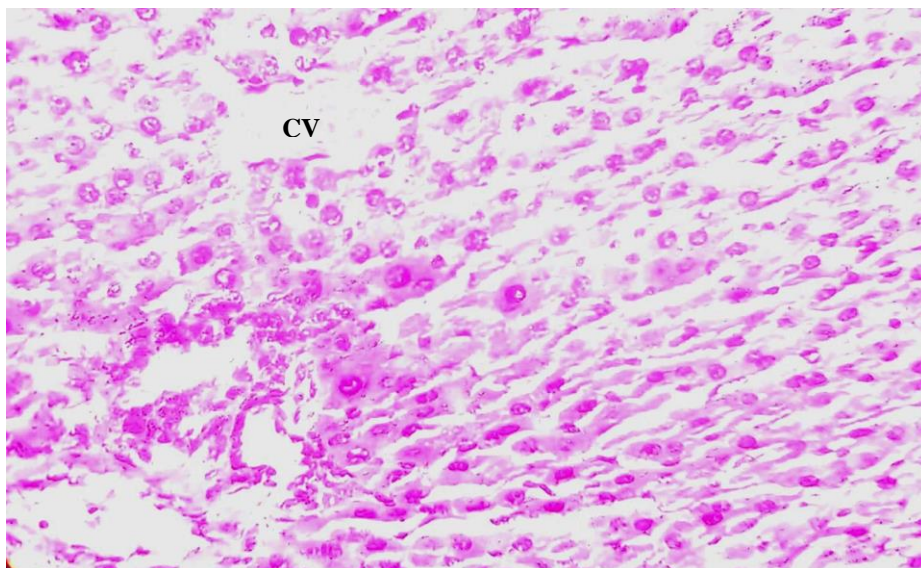


Figure 5: Microscopic feature of the liver of animals of group treated with *Rubia Cordifolia* (200 mg/kg) . Minimal feathery degeneration around central vein. Cells with fatty changes are seen. Necrosis is not seen. (H & E Stain x 400) CV – Central vein

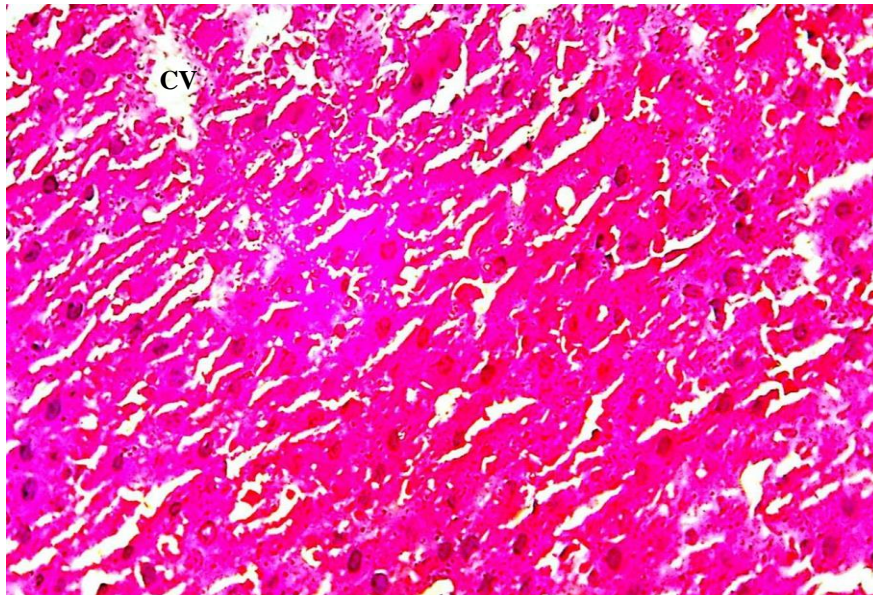


Figure 6: Microscopic feature of the liver of animals of group treated with *Withania Somnifera* (500 mg/kg) Centrilobular area is conspicuous by the presence of hepatocyte showing minimal fatty changes. Necrosis is not seen. (H & E Stain x 400) CV – Central vein.

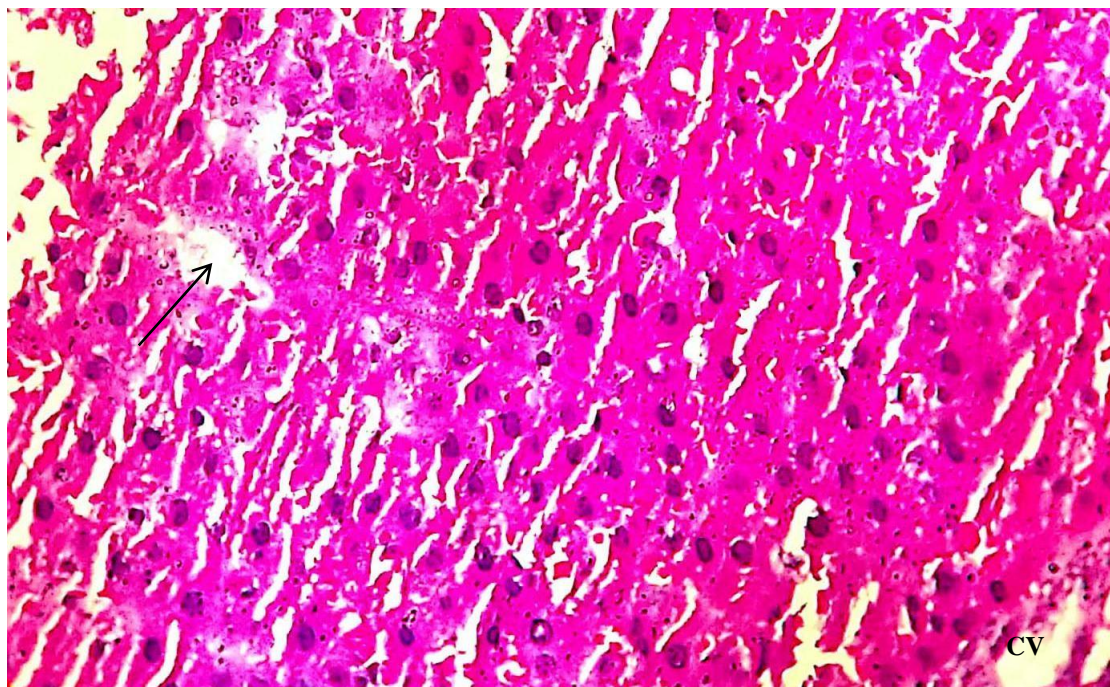


Figure 7: Microscopic feature of the liver of animals of group treated with *Withania Somnifera* (1000 mg/kg). Constricted sinusoids are seen pointing to mild hepatocyte swelling. Necrosis is not seen. (H & E Stain x 400) CV – Central vein

Discussion

Liver damage induced by CCl_4 is a commonly used model for the screening of hepatoprotective drug. [11] The rise in serum levels of Alanine Transaminases, Alkaline Phosphatase, Total Bilirubin and Albumin has been attributed to the damaged structural integrity of the liver, because they are cytoplasmic in location and released into circulation after cellular damages. [8] When rats were treated with Carbon tetrachloride it induces hepatotoxicity by metabolic activation, therefore it selectively causes toxicity in liver cells and causes poorly altered metabolic function. Carbon tetrachloride is metabolically activated by the cytochrome P-450 dependent mixed oxidase in the endoplasmic reticulum to form trichloromethyl free radical (CCl_3) which combined with cellular lipids and proteins in the presence of oxygen to induce lipid peroxidation. [13] This results in changes of structure of the endoplasmic reticulum and other membrane, loss of metabolic enzymes activation, leading to liver injury and elevated levels of Alanine Transaminases, Alkaline Phosphatase, Total Bilirubin and Albumin etc. [9]

In the present study it was observed, that Liv.52, a well known hepatoprotective agent, significantly suppressed the rise of ALT and ALP after CCl_4 challenge. It also normalized the bilirubin levels. This biochemical protection was also reflected in

the histology. The aqueous extract of *Rubia cordifolia* and *Withania somnifera* exhibited dose dependant hepatoprotection, both biochemically and histologically. The aqueous extract of *Withania somnifera* at dose of 1000mg/kg provided better results with ALT as compare to Liv.52 and histologically almost similar as it showed mild hepatocellular swelling without any inflammation. Same dose for same duration also gave good protection from increasing ALP as compared to liv.52 and slightly less protection from increasing Total Bilirubin as compare to Liv.52. Nosignificant change was seen with albumin levels.

The possible hepatoprotective mechanism of aqueous extract of *Rubia cordifolia* and *Withania somnifera* have been not clearly known. It is assumed that the effect of these extract on liver protection may be due to several reasons.

Hepatocytes have a practically unlimited capacity for proliferation, with full regeneration. Possible mechanism for regeneration of liver are changes in gene expression, an array of transcription factors (NF-kB, STAT3, fos and jun) and various hepatic mitogenes which promote hepatocyte regeneration. ^[10] Like silymarin, a proved hepatoprotective agent (acts through entry inside the nucleus and stimulate protein synthesis), may enhance *Rubia cordifolia* and *Withania somnifera* the activity of one or more of the above factors to stimulate the hepatic regeneration.

The hepatoprotective activity of aqueous extract of *Withania somnifera* was comparable in the dose of 1000mg/kg for 21days to that with Liv.52 syrup (1 ml/kg) for 21 days. Liv.52 is a mixture of several compound and usually not used in healthy individuals for prophylactic purposes, but in contrary to that the extract may *Rubia cordifolia* and *Withania somnifera* be given to healthy persons prophylactically so that chances of occurrence liver disease can be reduced. Further the pharmacokinetic studies of *Rubia cordifolia* and *Withania somnifera* are largely unknown. An elaborate investigation to explore the pharmacokinetic profile may lead to better efficacy and potency of *Rubia cordifolia* and *Withania somnifera* at smaller doses.

Conclusion

Aqueous extract *Rubia cordifolia* and *Withania somnifera* is a potent hepatoprotective agent. Further studies with its hydroalcoholic extract can be expected to provide still better results. It is suggestible that the dosage form of *Rubiocordifolia* and *Withania somnifera* aqueous extract can be changed from the current *Rubia cordifolia* and *Withania somnifera* aqueous extract form, to some other form like hydroalcoholic extract or extraction of some specific component which is involved in hepatoprotective action. Also the safety profile of *Rubia cordifolia* and *Withania somnifera* aqueous extract has been very encouraging in this study.

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