ORIGINAL ARTICLE

Evaluation of Histopathological changes in Carbon Tetrachloride induced liver injury and protective role of *Argyrolobium roseum* in animal model

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ABSTRACT

Background: Plant based hepatoprotective agents are under scientific research now days.
Aim: To provide histopathological evidence of hepatoprotective effect of *Argyrolobium roseum* (Jaub and Spach) on carbon tetra chloride induced hepatotoxicity in rabbits.
Study design: Experimental randomized controlled trial study
Place and duration: king Edward medical University and University of Veterinary Animal Sciences Lahore in 6 months duration.
Methods: thirty five adult healthy male rabbits were included in stud. Rabbits were divided into five groups containing seven animals in each group. Hepatotoxicity was induced by administering 0.4ml/kg/day of carbon tetrachloride (1:1 in olive oil). *Argyrolobium roseum* aqueous extract was administered in a dose of 200mg, 400mg, and 600 mg/kg/day daily for 14 days. Histopathological changes such as fatty change, ballooning degeneration, portal inflammation and necrosis were compared in carbon tetra chloride and Argyrolobiumroseum aqueous extract treated groups.
Results: *Argyrolobium roseum* aqueous extract treated groups showed significant changes among different parameters including weight of animal, weight of liver and histopathologicaal changes such as fatty change, ballooning degeneration, portal inflammation and necrosis (pvalue 0.0001) was observed among various groups.
Conclusion: This study provided histopathological evidence of hepatoprotective effect of *Argyrolobiumroseum aqueous* extract.
Key words: *Argyrolobiumroseum*, carbon tetra chloride ,hepatpotoxicity, rabbits, liver,

INTRODUCTION

Liver diseases are matter of great concern in developing as well as developed countries. Liver is an important target for injury by drugs, xenobiotic and infections. The incidence of predominant liver diseases such as viral hepatitis, alcoholic and non- alcoholic fatty liver disease vary from country to country. Most of the treatment modalities for liver injuries are not very effective and are also associated with severe adverse effects[1]. As a result of liver injury cellular function declines and fibrosis of the organ takes place But in spite of significant scientific development there is not even a single allopathic medicine which can protect liver from further damage and enhance its functions[2]. Herbal products are used since ancient time by mankind for the treatment of various ailments. As plant based products are associated with less adverse effects and are cost effective. Plants such as *Glycyrrhizaglabra, Phyllanthus* species, *Picrorhizakurroa, and Silybummarianum,* are effectively and widely used for the treatment of various liver diseases. These plants are effective hepatoprotective agents because of their antioxidant nature[3].So plant based hepatoprotective agents are being widely evaluated.

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CCl_4 is commonly used to induce hepatotoxicity in experimental animals. CCl_4 is metabolized by cytochrome p450 to generate trichlromethyle and peroxytrichlromethyle free radicals. CCl_4induces oxidative stress inactivating various antioxidant enzymes such as glutathione, glutathione reductase, glutathione peroxidase, super oxide dismutase[4]. CCl_4 also disturbs the functional integrity of cells as evident by elevation of serum aspartate aminotransferase, total bilirubin, alkaline phosphatase, and alanine aminotrasferase level. CCl_4 also induced various histological changes in cells such as fatty change, ballooning degeneration portal inflammation and necrosis.

*Argyrolobiumroseum*possesses antihyperglycemic as well as immune suppressant effects [5,6,7]. This plant is reported to be effective against paracetamol induced toxicity in liver[8]. Chemical induced hepatitis mimics viral hepatitis histologically, clinically and biochemically. Chemically hepatitis can be induced by various chemicals[9]. Keeping all these factors in view we decided to evaluate the hepatoproective effect of *Argyrolobiumroeum* on CCl_4 induced hepatotoxicity. CCl_4 administered for 2 weeks successfully induced hepatotoxicity in group two, three, four, and five as compared to normal control group 1 ($p < 0.001$***). Effects of *Argyrolobiumroseum* aqueous extract in doses of 200mg/kg, 400 mg/kg and 600 mg/kg on histopathological parameters were studied.

MATERIAL AND METHODS

This experimental randomized study was conducted at King Edward Medical University and University of Veterinary and Animal Sciences, Lahore. during a period of six months. Simple random sampling was done by lottery method.

Inclusion criteria: This study was carried out on thirty five adult healthy male rabbits. Weight of rabbits was between 1-1.5 kg. Animals were purchased from local market and kept housed in iron cages under natural day and night cycles. Rabbits were marked for proper identification and divided in to seven groups containing seven animal in each group. Food and water was provided ad libitum.

Preparation of aqueous extract: Fresh plants *of Argyrolobiumroseum*were procured from Burkot valley Khaiberpakhtunkhawah, in the month of October. Plants were in good and healthy condition. Before use, proper identification of plants was carried out by authorized personnel from Food and Nutrition Department, PCSIR. Fresh whole plants of *Argyrolobiumroseum*(12kg) were washed thoroughly and air dried under shade for two weeks. Aqueous extract of dry plant (9kg) was prepared in PCSIR. An electric blender (Nihon blender, Robot coupe, Japan) was used to pulverize the dried plant material. For every sixty grams of powdered plant material blended in one liter of cold distilled water maintained on mechanical shaker (Mechanical shaker, Seikenshaco limited, Tokyo,Japan) and filtered using a Buchner funnel and whatman no 1 filter paper. The filtrate was quickly frozen at – 48 ºCelsius and dried for 48 hours using a freeze dryer (Eyela, Japan) .The dried extract (372g) obtained was kept in tightly closed bottle protected from light in refrigerator at 2-8ºCelsius to be used throughout the experiment[10].Total yield was about 4.1%. The aqueous extract of Argyrolobiumroseum was preferred to use for experimental process as most of the constituents of Argyrolobiumroseum were present in aqueous extract.[11]

Preparation of dose: The calculated dose for individual rabbit i.e. 200mg/kg, 400mg/kg and 600mg/kg was dissolved in 5 ml distilled water. Doses were administered through nasogastric tubes.

Induction of hepatotoxicity: CCl4 and olive oil were taken in a ratio of 1:1 and a total dose of 0.4ml/kg body weight was used to induce hepatotoxicity. CCl4 (1:1 olive oil) was administered by intraperitoneal route using 1ml disposable syringes to induce toxicity.[12]

Grouping of animals: Rabbits were divided into five experimental groups, containing seven rabbits in each group randomly

Group 1: This group was normal control group and received 0.2 ml/kg/day olive oil intraperitoneally and distilled water 5 ml orally, daily for 14 days.

Group 2: This group received 0.4 ml/kg/day carbon tetra chloride (1:1 in olive oil) intraperitoneally and distilled water 5 ml orally, daily for 14 days.

Group 3: This group received 0.4 ml/kg/day carbon tetra chloride (1:1 in olive oil) intraperitoneally and 200 mg /kg/day *Argyrolobiumroseum*aqueous extract orally, daily for 14 days.

Group 4: This group received 0.4 ml/kg/day carbon tetra chloride (1:1 in olive oil) intraperitoneally and 400 mg /kg/day *Argyrolobiumroseum*aqueous extract orally, daily for 14 days.

Group 5: This group received 0.4 ml/kg/day carbon tetra chloride (1:1 in olive oil) CCl4 intraperitoneally and 600 mg /kg/day *Argyrolobiumroseum*aqueous extract orally, daily for 14 days.

Euthanization: Twenty four hours after last dose, rabbits were sacrificed on day 15 by slaughtering.

Preparation of slides: Liver was removed, weighed and sliced. Liver slices were fixed in 10% formalin. Standard H & E staining method was used for preparation of slides and examined under microscope to observe histopathological changes such as fatty change, ballooning degeneration, necrosis and portal inflammation[13].Semiquantitave grading of histopathological changes was done as following:

___ = Absent
+ = Trace (1-25%)
++ = Weak (26-50%)
+++ = Moderate (50-75%)
++++ = Severe (75-100%)[14]

Data analysis: All the data was entered in SPSS version 16 and graph pad prism version 5 .Quantitative data was expressed as Mean ± S.D. Mean plots were used for graphical presentation to see changes in the parameters. The data was evaluated by one way analysis of variance followed by Tukey"s multiple comparison tests. Histopathological changes were expressed as frequencies and percentages; Chi-square test was used for evaluation. P-value of less than 0.05 was considered significant.

RESULTS

Mortality rate and observation of behavior during study: Total four animals died during experimental period. Two animals from group two died on day 6 six and thirteen. Two animals from group three died on day eleven and thirteen. There was no mortality in animals of group one(normal control), group four (medium dose treatment group) and group five (high dose treatment group). The apparent cause of death was hepatotoxicity. The rabbits of group one (normal control group) maintained normal appetite, health and behavior. Rabbits of group two (positive control group) were lethargic, reduced appetite. The rabbits of *Argyrolobiumroseum*aqueous extract treated groups showed decline in appetite, but normal behavior.

Effect on body weight: Changes in weight of animals of different groups are shown in (table-1,fig-1).

Gross appearance of liver: The liver of group 1 (normal control group) rabbits were light in colour with shiny and smooth surface. Texture of liver of normal control group was flexible and soft. Liver of group 2 (positive control group) rabbits were dark in colour, with tense capsule. Hemorrhagic lesions were also prominent in cut sections. The gross appearances of *Argyrolobiumroseum* treated rabbits were variable according to dose of *Argyroloiumroseum* aqueous extract. The liver of group 3 (low dose treatment group) were large with tense capsule. The liver of group 4 (medium dose treatment group) and 5 (high dose treatment group) were relatively lighter in colour and smooth capsules.

Fatty change: Total 31 liver specimens were examined for fatty change in liver. The frequency and percentage of fatty change in group 1, 2, 3, 4, and 5 were calculated. After applying Chi-square test significant difference was observed in fatty change between group 1,2,3,4, and 5 with p-value<0.001*** (table-3, fig-4).

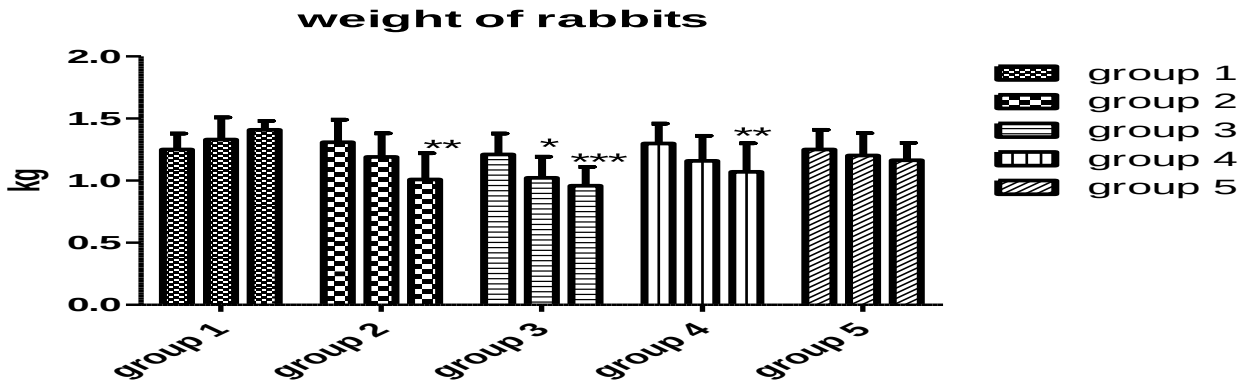
Table 1: Comparison of mean body weight (kg) between group 1, 2, 3, 4 and 5 at day 0, 7 and 15.

Body weight (kg)	Group 1	Group 2	Group 3	Group 4	Group 5	P. value
	Mean±S.D	Mean±S.D	Mean±S.D	Mean±S.D	Mean±S.D	
Day 0	1.25 ±0.13(n=7)	1.31±0.18(n=7)	1.21±0.17(n=7)	1.231±0.16(n=7)	1.25±0.16 (n=7)	0.78
Day 7	1.33±0.09(n=7)	1.19±0.19(n=6)	1.023±0.17(n=7)	1.16±0.20(n=7)	1.203±0.18(n=7)	0.04*
Day 15	1.41±0.07(n=7)	1.01±0.21(n=5)	0.96±0.15(n=5)	1.07±0.23(n=7)	1.164±0.14(n=7)	0.001***

*** P-value ≤ 0.001, ** p-value ≤ 0.01,* p-value- 0.05

Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group

Fig- 1: Mean body weight (kg) of group 1, 2, 3, 4, and 5 at day 0, 7 and 15.



Weight of liver: Mean weight of liver in g is shown in (table- 2 fig- 2).

Fig. 2: comparison of weight of liver aming group 1, 2,3,4 and five

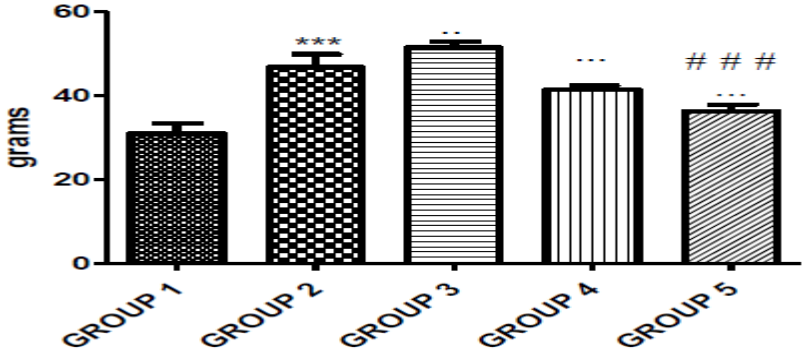


Table -2: comparison of mean weight of livers (g) in group 1,2,3,4 and 5.

Weight of liver(g)	Group 1	Group 2	Group 3	Group 4	Group 5	Value of p
Mean ± S.D	31±2.3(n=7)	46.8±6.9 n=5)	51.4±3.4(n=5)	41.4±2.4(n=7)	37.71±4.02(n=7)	0.001***

*value of p < 0.05 is considered significant

Table 3: fatty change in liver of rabbits of group 1, 2, 3, 4, and 5.

	Fatty change					Total
	Absent (-)	Trace(+)	Weak(++)	Moderate (+++)	Severe (++++)	
Group-1	7	0	0	0	0	**7**
Group-2	0	0	0	2	**3**	**5**
Group-3	0	0	0	5	**0**	**5**
Group-4	0	1	6	0	**0**	**7**
Group-5	2	4	1	0	**0**	**7**
Total	**9**	**5**	**7**	**7**	**3**	**31**

Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group.

Fig-3(a): liver of rabbit of group 1 showing soft and flexible texture, with shiny and smooth capsule.

Fig-3(b): liver of rabbit of group 2 showing darker colour, irregular surface and tense capsule.

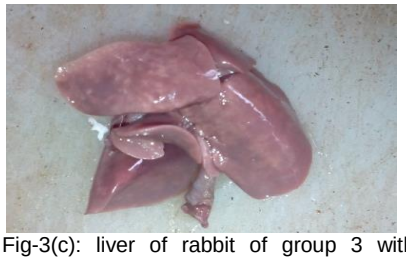


Fig-3(c): liver of rabbit of group 3 with darker colour and irregular serface.

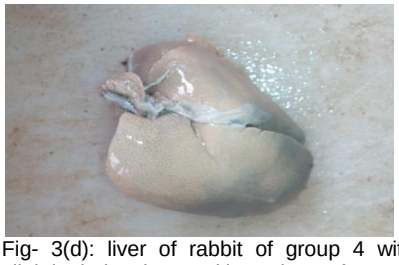
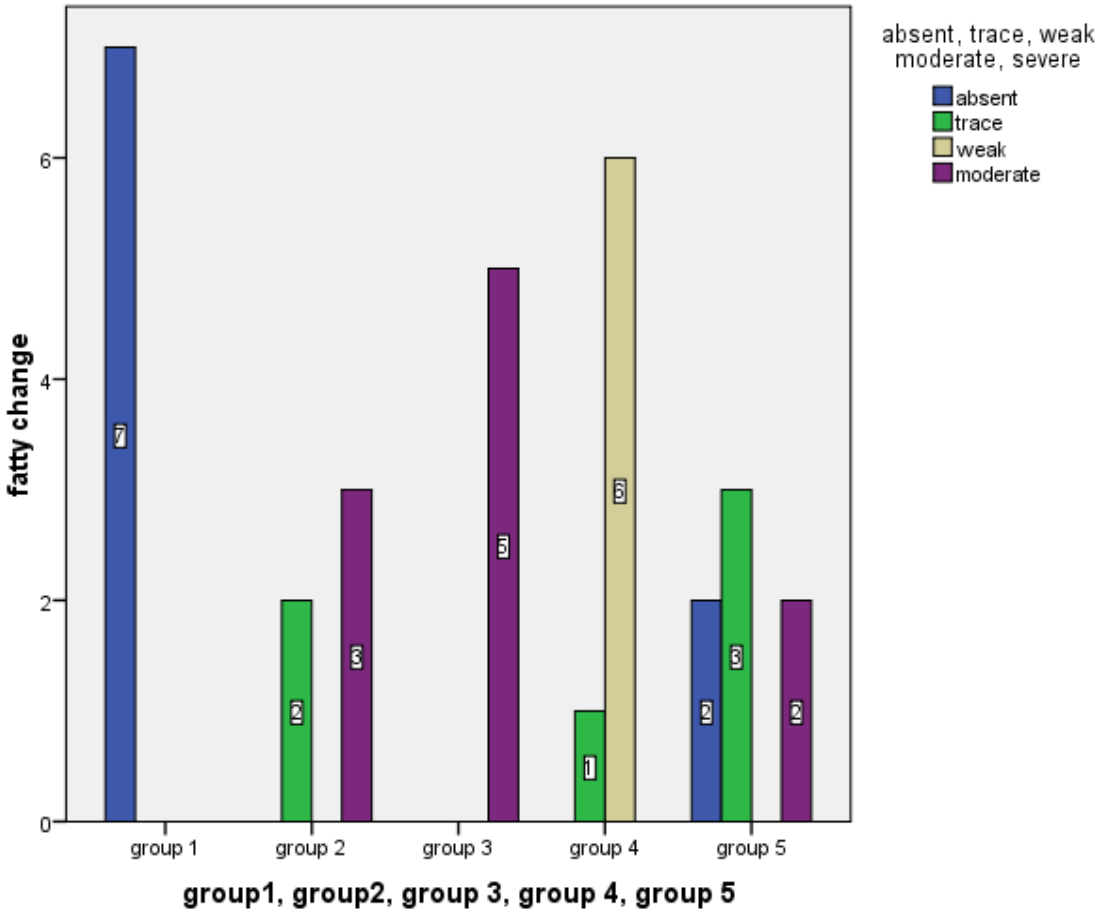


Fig- 3(d): liver of rabbit of group 4 with slightly dark colour and irregular surface

Fig-3(e): liver of rabbit of group 5 with light colour and relatively smooth surface.

Fig-4: comparison of fatty change in liver among group 1, 2, 3, 4, and 5.



Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group.

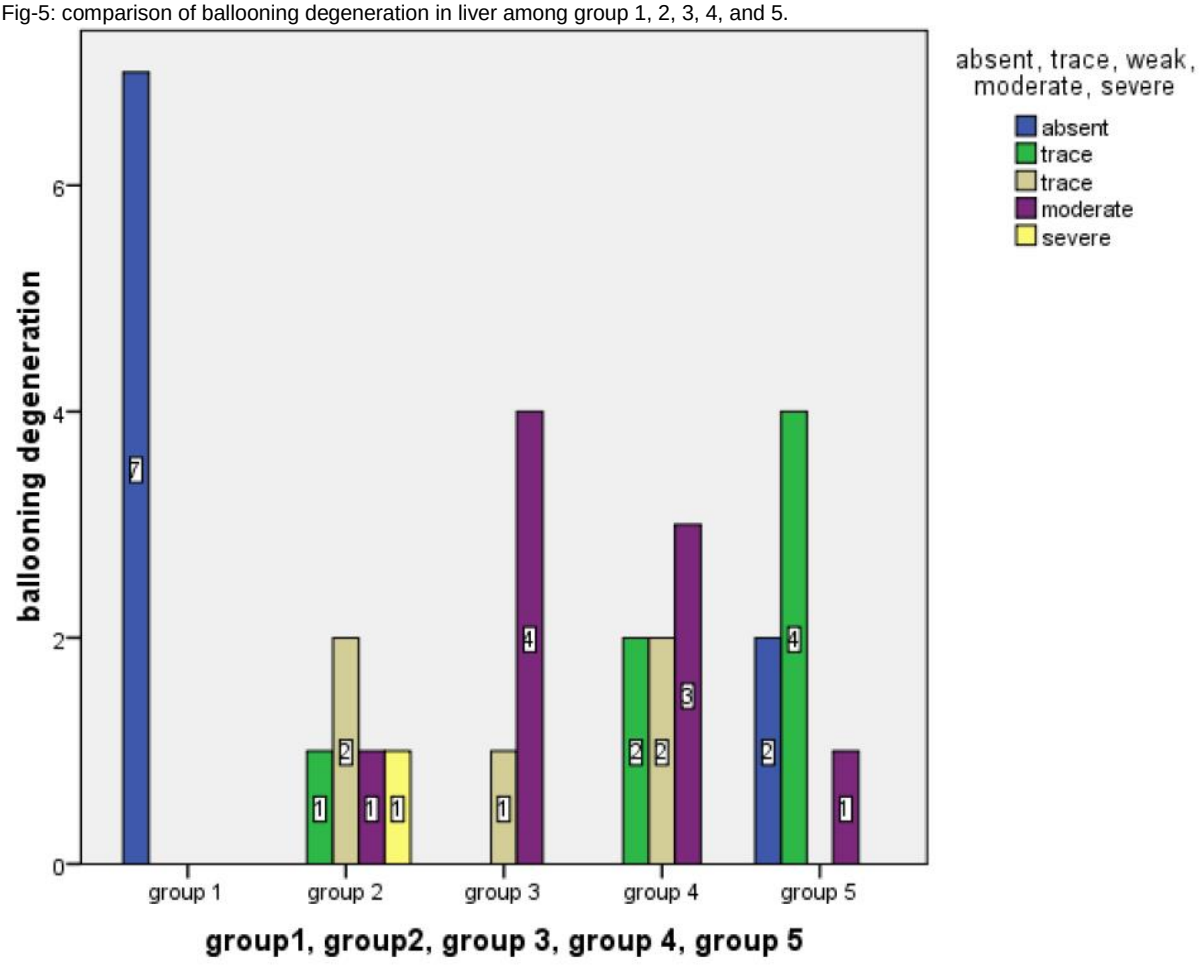
Ballooning degeneration: Total 31 liver specimens were examined for ballooning degeneration in liver. The frequency and percentage of ballooning degeneration in group 1, 2, 3, 4, and 5 were calculated.After applying Chi-square test significant difference was observed in ballooning degeneration between group 1,2,3,4, and 5 with p-value<0.001*** (Table-4,fig-5)

Table-4: comparison of ballooning degeneration in liver between group 1, 2, 3, 4, and 5

	Ballooning Degeneration					Total
	Absent (-)	**Trace (+)**	**Weak (++)**	**Moderate (+++)**	**Severe (++++)**	
Group-1	7	0	0	0	0	**7**
Group-2	0	0	1	3	1	**5**
Group-3	0	0	0	5	0	**5**
Group-4	0	5	2	0	0	**7**
Group-5	2	5	0	0	0	**7**
Total	**9**	**10**	**3**	**8**	**1**	**31**

Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group.

Fig-5: comparison of ballooning degeneration in liver among group 1, 2, 3, 4, and 5.



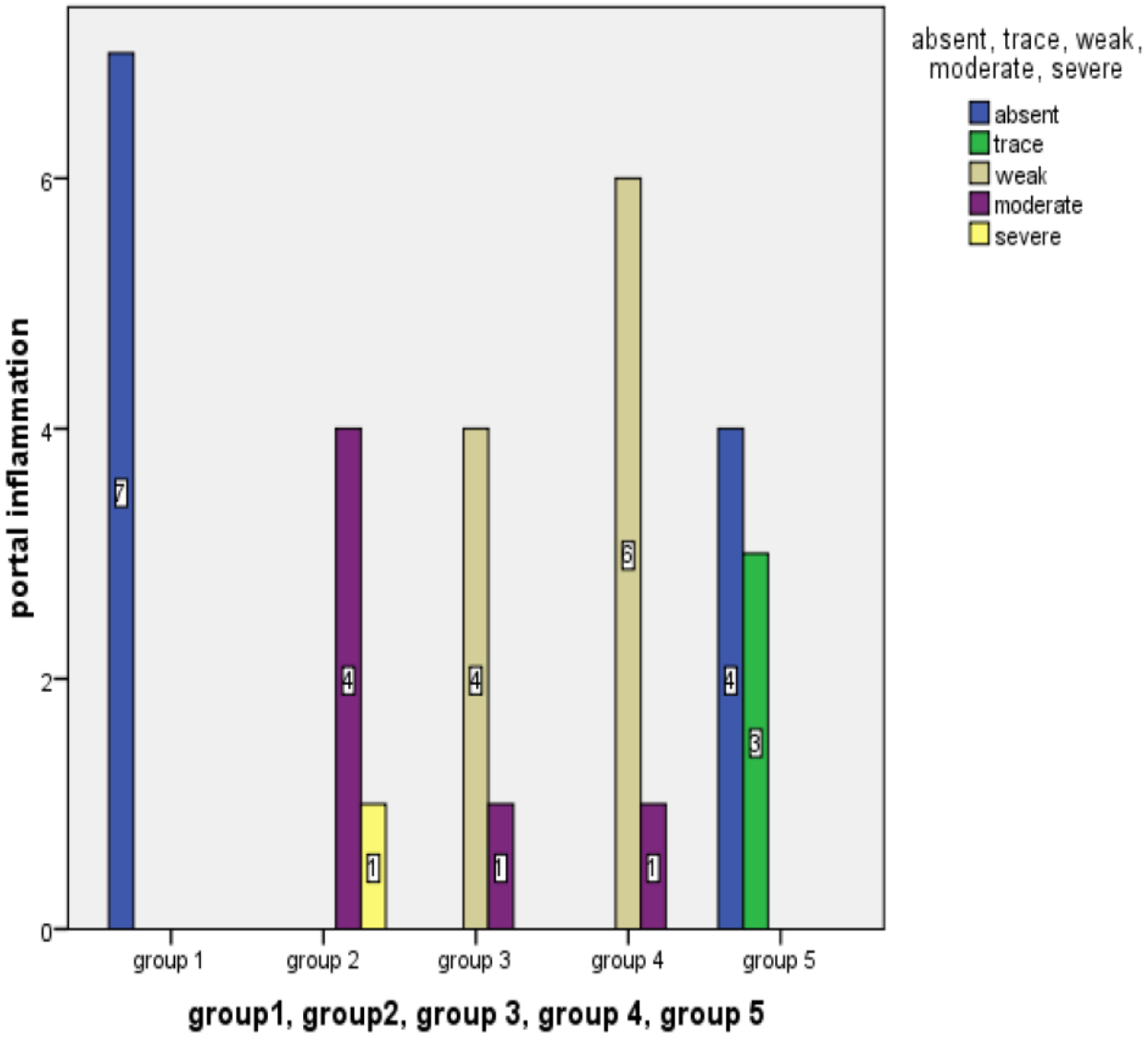
Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group.

Portal inflammation:: Total 31 liver specimens were examined for portal inflammation in liver. The frequency and percentage of portal inflammation in group 1, 2, 3, 4, and 5 were calculated. After applying Chi-square test significant difference was observed in portal inflammation between group 1,2,3,4, and 5 with p-value<0.001*** (Table-5,fig-6)

Table -5: Comparison of portal inflammation in liver between group 1, 2, 3, 4, and 5.

	Portal inflammation					Total
	Absent (-)	Trace (+)	Weak (++)	Moderate (+++)	Severe (++++)	
Group-1	7	0	0	0	0	**7**
Group-2	0	0	0	4	1	**5**
Group-3	0	0	0	2	3	**5**
Group-4	0	1	6	0	0	**7**
Group-5	4	3	0	0	0	**7**
Total	**11**	**4**	**6**	**6**	**4**	**31**

Fig-6: comparison of portal inflammation in liver among group 1, 2 3, 4 and 5.



Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group.

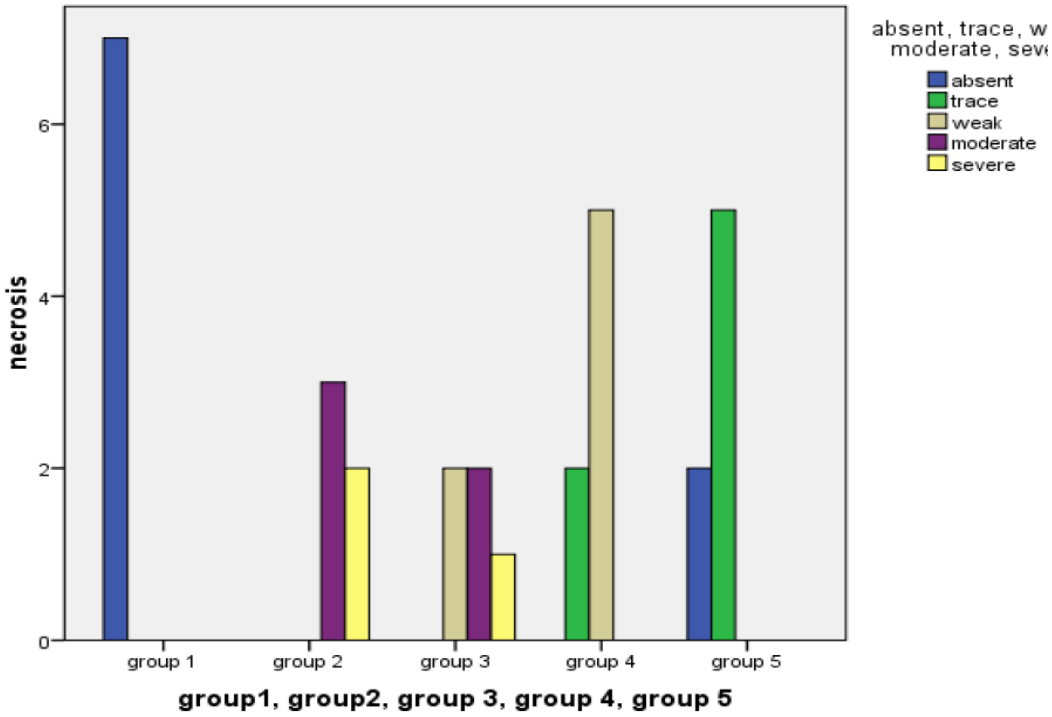
Necrosis: Total 31 liver specimens were examined for necrosis. The frequency and percentage of necrosis in group 1, 2, 3, 4, and 5 were calculated. After applying Chi-square teat significant difference in necrosis of group 1, 2, 3,4, and 5 with p-value <0.001*** (Table-6,fig-7)

Table -6: Comparison of necrosis in liver between group 1, 2, 3, 4, and 5.

	Necrosis					Total
	Absent (-)	Trace (+)	Weak (++)	Moderate (+++)	Severe (++++)	
Group-1	7	0	0	0	0	**7**
Group-2	0	0	0	3	2	**5**
Group-3	0	0	0	3	2	**5**
Group-4	0	2	5	0	0	**7**
Group-5	2	5	0	0	0	**7**
Total	**9**	**7**	**5**	**6**	**4**	**31**

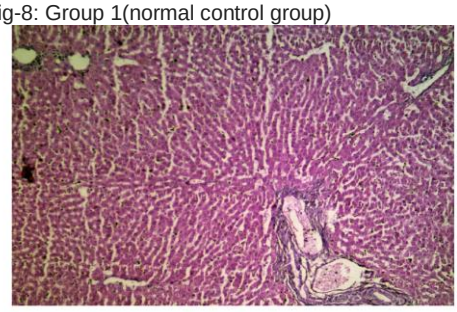
Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group.

Fig-7: comparison of necrosis in liver among group 1, 2, 3, 4 and 5.



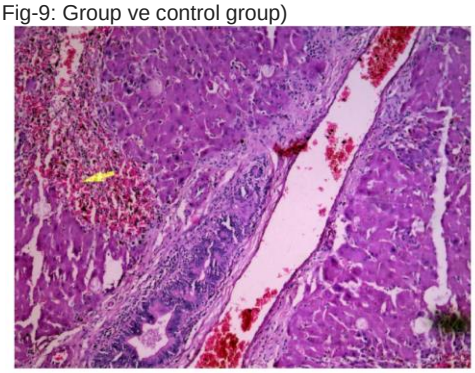
Group 1= normal control group, group 2 = positive control group, group 3 = low dose treatment group, group 4= medium dose treatment group, group 5= high dose treatment group.

Fig-8: Group 1(normal control group)



Photomicrograph revealing normal architecture of liver with central veins, hepatic cords and portal triad. (20× 10 X)

Fig-9: Group ve control group)



Photomicrograph showing hepatic vein congestion, moderate inflammatory infiltrate in portal tract and moderate necrosis of hepatocyte. (20×10 X H & E)

Fig. 10: Photomicrograph showing ballooning degeneration, fatty change, necrosis and inflammatory infiltrate. (20×10 X H& E)

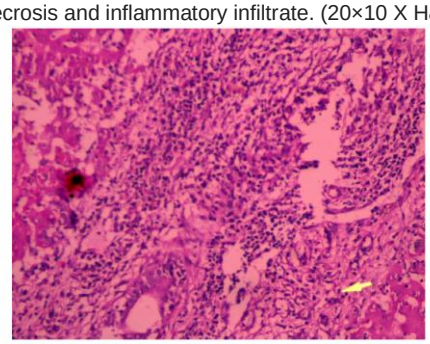
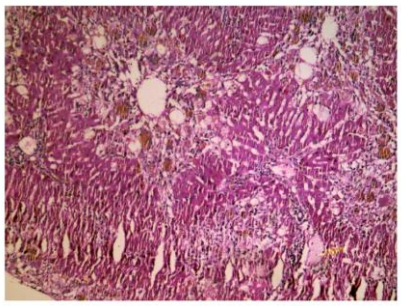
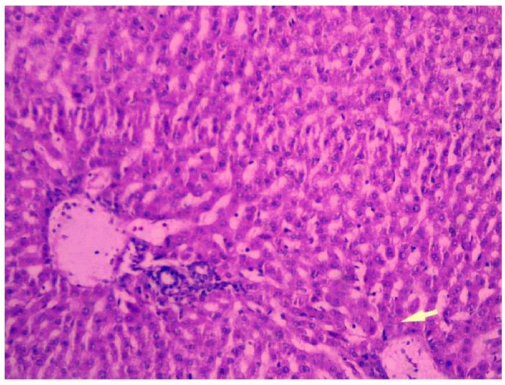


Fig-11: Group 4 (medium dose treatment group)



Photomicrograph of liver showing weak ballooning degeneration, fatty change and inflammatory infiltrate (20×10 X H & E)

Fig-12: Group 5 (high dose treatment group)



Photomicrograph of liver revealing normal lobular architecture, hepatocytes arranged in cords with mild inflammation in portal tracts. (20×10 X H & E)

DISCUSSION

Carbon tetra chlorideis a commonly used hepatotoxin to induce hepatotoxicity in experimental animals.Rabbits of group 1 gained weight during experimental period. Whereas body weight of CCl_4 exposed rabbits (group 2) was significantly reduced (p.value<0.001***). *Argyrolobiumroseum* aqueous extract helped to restore body weight in experimental animals. Weight loss may be due to anorexia and disturbance of protein, carbohydrate and lipid metabolism. As the constituents of *Argyrolobiumroseum* possesses the ability to restore deranged activities of enzymes involved in carbohydrate metabolism thus help to maintain body weight. Sivakumar et al demonstrated the effect of d-pinitol on the impaired activities of hepatic key enzymes in carbohydrate metabolism of streptozotocin-induced diabetic rats[15]

Weight of liver was high in positive control group (group 2) as compared to *Argyrolobiumroseum* treated groups (p<0.001***). This effect was mainly due to fatty change in liver as evident by histopathological examination. The weight of liver in group 3 was slightly high then group 2. This effect was due to the presence of more fibrotic changes in group 2 as compared to group 3 which caused decrease in weight of liver. However in group 4 and 5 weight of liver was less as compared to positive control group. This effect is in consistence with previous study done by Chen, Yieng-How, et al who also explained that carbon tetrachloride results in increase in weight of liver due to fatty change. Damage to structural and functional integrity of hepatocytes causes inhibition of apolipoprotein synthesis required for transport of lipids resulting in accumulation of lipids within the cells[16] However in Argyrolobiumroseum aqueous extract treated group weight of liver was less as compared to carbon tetrachloride treated group. This effect may be due to anti-hyperlipidemic effect of pinitol[17]

The present study showed that carbon tetra chloride induced significant fatty change in liver of rabbits. *Argrolobiumroseum* aqueous extract significantly reduced fatty change. This effect may be due to the anti-hyperlipidemic effect of d.pinitol. D.pinitol possesses inhibitory effect on various enzymes involved in lipid metabolism such as HMG-CO A reductase, acetyl co A transferase which are main enzymes involved in synthesis of various lipids in cells. This result is in consistence with previous study which showed that d.pinitol decreases the level of total cholesterol, free fatty acids, phospholipids and triglycerides in liver, kidney, heart, brain and serum.[18]

The present study has shown that the incidence of ballooning degeneration was significantly reduced in *Argyrolobiumroseum*aquous extract treated groups. This effect may be due to membrane stabilizing effect of *Argyrolobium roseum*.[19]Thus antioxidant nature of d.pinitol may be responsible for prevention of ballooning degeneration by *Argyrolobiumroseum.*

Carbon tetra chloride induces significant portal inflammation. The present study has shown that portal inflammation is significantly reduced in *Argyrolobumroseum*aquous extract treated groups. Hepatotoxins cause activation of kupffer cells which in turn release chemokine such as interleukin 1 beta, interferon gamma, tumor necrosis factor alpha and interleukin 6. These cytokine increase expression of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 in portal and sinusoidal endothelial cells. Leukocytes mainly enter in liver through portal tract, resulting in portal inflammation[20]**.** This effect may be due to the ability of d.pinitol to inhibit production of proinflammatory cytokines such as TNF-alpha, NF-KB, IL-6, and IL-1b [21].

The present study has shown that *Argrolobiumroseum*aqouous extract has significantly reduced the incidence of necrosis in liver of rabbits (p. value 0.001***). This effect may be due to the antioxidant

nature of d.pinitol. It is already known that d.pinitol increases the level of cellular enzymatic and nonenzymatic antioxidants such as vitamin E, vitamin C and reduced glutathione, superoxide dismutase, glutathione peroxidase and glutathione reductase, glutathione s-transferase and catalase.[22,23]

CONCLUSION

The present study confirms the hepatoprotective effect of *Argyrolobiumroseum*aqueous extract. This study may be helpful for development of economical safe and efficacious hepatoprotecive agent. Further researches are required to explore the active constituents responsible for hepatoprotective effect.

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