Journal of Muhammad Medical College
Website: jmmc.mmc.edu.pk

Original Article

Histomorphometric effects of Raphanus Sativus leaf extract on Carbon Tetrachloride induced hepatotoxicity.

Shazia Parveen [1],Sajjad Ali Almani [2],Sana Kashif [3],
Muhammad Shahab Hanif [4,*], Ayesha Asad[5], Mozna Talpur [6].

1: Lecturer, Department of Pharmacology
LUMHS, Jamshoro

2: Assistant Professor, Department of Anatomy
Dow University of Health Sciences, Karachi

3: Assistant Professor, Department of Anatomy
SMBBMU, Larkana

4: Assistant Professor, Department of Anatomy
Muhammad Medical College, Mirpurkhas.

5: Assistant Professor, Department of Anatomy
Muhammad Medical College, Mirpurkhas

6: Professor, Department of Pharmacology
Isra University, Hyderabad.

***=corresponding author**
shahab_hanifhhh@yahoo.com

Abstract:

Introduction: Carbon tetrachloride (CCl4) adversely affect the structural/ and or functional properties of the hepatic tissue, leading to grave implications. Currently plant-based compounds have gained sufficient space in the treatment of liver diseases. Among such plants is Raphanus sativus, a cruciferous plant also commonly called Radish, which has since long been used in traditional medicine.

Objective: To evaluate the protective role of Raphanus sativus in CCl4-induced hepatotoxicity through assessment of liver function and oxidative stress markers in albino Wistar rats.

Methodology: This Quasi-experimental study was conducted between September 2019 to March 2020 at the Postgraduate Research Laboratory Isra University, Hyderabad. Albino Wistar selected by non-random purposive sampling were divided equally into three different groups: Group A (control group), Group B (CCl4 experimental group), and Group C (CCl4 plus Raphanus sativus group). Blood samples were collected through cardiac puncture followed by hepatic histopathological analysis using light microscope. Data was analyzed using SPSS version 24, with ANOVA and Post hoc Tukey's analysis used for comparison of different study variables.

Results: Statistically significant rise relative liver weight of group B rats compared with the group A and C rats ($P < 0.05$). Moreover, statistically significant ($P < 0.05$) rise in serum markers of hepatic functions was observed after CCl4 administration in Group B. Treatment with Raphanus Sativus administration significantly reduced serum levels of LFTs ($p<0.05$). There was a significant decline in the plasma levels of oxidative markers in group B while marked histopathological changes like necrosis, sinusoidal dilatation and congestion observed among animals of group B.

Conclusion: Raphanus Sativus exerts an anti-oxidative, and hepato-protective effect against CCl4-induced hepatic tissue damage.

Keywords: CCl4, Hepato-protective, Raphanus Sativus.

Introduction:

Liver, with its crucial role in the conservation of homeostasis and detoxifying baneful drugs, is one of the most vital organs required for survival.[1]Various elements recognized to adversely affect the structural/ and or functional properties of the hepatic tissue, leading to grave implications, have been regarded as hepato-toxic. Carbon tetrachloride (CCl4) being one of those elements, resulting in hepato-toxicity owing to its innate potential to undergo dehalogenation yielding trichloromethyl ($CCl3^-$), which in turn stimulates oxidative stress eventually leading to cell-injury and cell death.[2] In addition, actuation of Kupffer cells induces the onset of an inflammatory cascade, thereby increasing the serum levels of inflammatory markers such as necrosis factor-a (TNF-a), transforming growth factor-β 1 (TGF- β1) interleukin (IL)-1b, IL-6, etc.[3,4]

In order to overcome the side effect profile of certain drugs currently being used for the mitigation of hepatotoxicity, alternatives plant based compounds have gained tremendous interest in recent years in their potential expediency in the treatment of liver diseases.[5]Among such plants is Raphanus sativus, a cruciferous plant also commonly called Radish, which has since long been used in traditional medicine.[6]The chemicals found within the roots and leaves of R. Sativus include various nitrogenous compounds, alkaloids, and phenols, etc. which have shown to possess free radical scavenging properties that defend the body against the deleterious effects of reactive oxygen. [7]R. Sativus extract has also shown to augment the activity of various antioxidants within the body, such as catalase and glutathione peroxidase which consequently prevent lipid peroxidation.[8]

The present study is designed with an objective to evaluate the protective role of Raphanus sativus in CCl4-induced hepatotoxicity through assessment of markers of liver function and oxidative stress markers in albino Wistar rats.

Methodology:

This Quasi-experimental study was conducted between September 2019 to March 2020 at the Postgraduate Research Laboratory Isra University, Hyderabad.

Thirty healthy, male Albino Wistar rats, aged 8-10 weeks and weighing between 250 to 300 grams, were procured from Agriculture University of Tando Jam, Sindh by non-random purposive sampling technique.

The sample size was calculated using the standard method of power analysis for animal studies.[9-11] This study was approved by the Ethics Review Committee of Ethics Review Committee of Isra University All the animals were handled according to the guidelines of International Research Council for laboratory animals' handling.[12]

After selecting, animals were kept for ten days of acclimatization in a well-equipped and hygienic environment at the postgraduate laboratory in Isra University, Hyderabad at the optimum temperature of 24-26 in a day-night cycle of 12/12 hours. To avoid any harm, animals were placed in plastic cages with water drinkers having stainless steel nozzles and feed containers. Rats were provided free access to chow diet and clean water ad libitum. Their bedding consisted of sawdust and was renewed daily.

Fresh leaves of Raphanus sativus were procured, taxonomically identified and authenticated from the department of Horticulture, Sindh Agriculture University, Tando Jam. Fresh leaves were splashed thoroughly to remove dirt or any potential contaminants. The leaves were dried at room temperature for 10 days. They were further dried in a hot air oven at 60ºC for six hours and then grinded in an electric grinder. The powder obtained was passed through mesh sieve and then extracted with 80% ethanol and filtered with filter paper (Whatman No. 2). The extracts were concentrated in a hot air oven at 37ºC, lyophilized by freeze drying apparatus (Christ Germany model # Alpha 1- 4LSC) and subsequently air tightly stored at -20ºC.[13]

All rats were equally (n=10) divided into Group A (control group, given a normal chow diet and clean water ad libitum), Group B (single dose of CCl4, 1.2 mg/kg in 50 mM phosphate buffer solution, subcutaneously), and Group C (single dose of CCl4, 1.2 mg/kg in 50 mM phosphate buffer solution, subcutaneously + 100 mg/Kg R. sativus extract). Treatment of R. Sativus extract orally was done by force feeding the animals for a span of 28 days through a stainless-steel feeding syringe. The level of the orally administered dosage of R. Sativus extract (100 mg/Kg) and the subcutaneous dose of CCl4 (CCl4 1.2 mg/kg) was based on previous studies.[14.15]Soon after the acclimatization period, the bodyweight of all rats was measured twice i.e., before initiation of the experiment and after completion of three weeks of the experiment using an electronic pre-

cision balance. On completion of the experiment, all rats were given anesthesia (Inj. Sodium pentobarbital at 40mg/kg, intraperitoneal) and sacrificed by cervical dislocation. For analysis of oxidative and liver function markers, blood was collected by cardiac puncture. The liver was dissected, weighed on an electronic scale after which they were fixed in 10% buffered formalin. Then, they were passed in xylene for clearing and embedded in paraffin wax. Thin tissue sections, of up to 4-µm thickness were cut manually using rotary microtome 290 and stained with hematoxylin and eosin (H&E) for examination under the light microscope (Olympus BX51, Tokyo, Japan). Histopathological analysis of hepatic tissue was done by evaluating the degree of infiltration of inflammatory cells, fibrosis, necrosis, sinusoidal dilatations, and congestion of portal vein. The changes in severity of tissue damage were observed using a graded scale adopted from a previous study as: none (0), mild (I), moderate (II), and severe (III).[11]

Statistical analysis of data was performed in SPSS version 24.0. Findings of variables like body and liver weights, oxidative, and renal function markers were expressed as mean and standard deviation while their comparison was analyzed by one-way ANOVA and Post hoc Tukey's analysis. The level of significance was considered at p= ≤ .05.

Results:

The mean pre-experiment body weight of group A was 213.2±3.82, group B was 215.4±3.78 and group C was 218.6±3.84. There was a significant difference in mean post experimental body weight was observed in all three groups. Animals of group A showed rise in body weight (226.3±3.76), while weight reduction was observed among animals of group B and C (189.4±2.77 and 213.2±3.54 respectively). However, in group C the weight loss in animals of group c was less when compare to group B. There was a statistically significant difference (p<0.05) between the experimental groups. The relative liver weight was significantly raised in Group B as compared with other experimental groups (p<0.05). (Table I). A statistically significant rise in serum markers of hepatic function (LFT) was observed after CCl4 administration in Group B. Treatment with Raphanus Sativus administration significantly reduced serum levels of liver enzymes (p<0.05). (Table II)

Table I. Distribution of body weight and relative liver weight among animal groups.

Groups	Body-weight (gm)	Relative liver weight
Group A	226.3±13.14 [b]	3.21±0.23 [b,c]
Group B	189.4±26.11 [a,c]	7.14±0.95 [a,c]
Group C	213.2±5.87 [b]	3.88±0.41 [a,b]

[a] p value < 0.05 as compared with Group A
[b] p value < 0.05 as compared with Group B
[c] p value <0.05 as compared Group C

Table II. Distribution of markers of liver function among animal groups.

Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total Bilirubin (mg/dL)	Direct Bilirubin (mg/dL)
A	43.21 ±8.63	45.81 ±7.4	81.6 ±15.12	0.26 ±0.07	0.19 ±0.03
B	210.4 ±17.73[a]	165.7 ±12.03[a]	261.9 ±6.73[a]	1.39.3 ±0.08[a]	1.12.2 ±0.06[a]
C	72.11 ±9.63[a, b]	71.56 ±9.02[a, b]	120.3 ±6.04[a, b]	0.51 ±0.08[a, b]	0.28 ±0.03[a, b]

[a] p value < 0.05 as compared with Group A
[b] p value < 0.05 as compared with Group B
[c] p value <0.05 as compared Group C

Statistically significant difference (p<0.05)in markers of oxidative stress was observed in all three groups i.e., in experimental group B there was a decline in the plasma levels of oxidative markers while in group C the decline on oxidative markers was less marked as seen in group B.(Table. III) Table IV shows grade wise comparison of Histopathological changes in hepatic tissues of all three groups of study animals. Marked Histopathological changes were observed in group B rats compared with group A and C.

Table III. Distribution of markers of oxidative stress among animal groups.

Groups	MDA (nmol/mg)	CAT (U/mg)	GPX (ng/dl)
Group A	2.19 ± 1.57[b]	24.31 ± 0.68[b,c]	1.39 ± 0.17[b]
Group B	4.6 ± 0.73[c,a]	18.11 ± 0.62[a,c]	0.94 ± 0.09[a,c]
Group C	2.9 ± 0.08[b]	21.14 ± 0.61[a,b]	1.28 ± 0.11[b]

[a]p value < 0.05 as compared with Group A
[b]p value < 0.05 as compared with Group B
[c] p value <0.05 as compared Group C

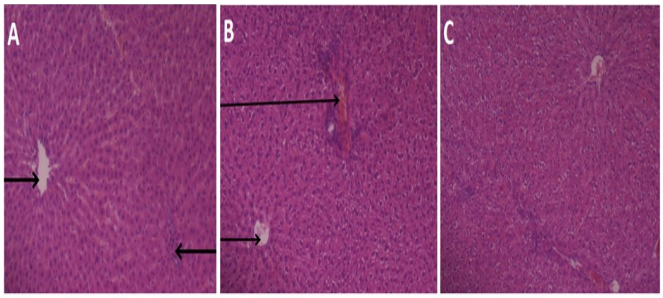
Table IV: Grading wise comparison of Histopathological changes in hepatic tissues of rats

Group	Fibrosis	Necrosis	Inflammatory cell infiltration	Sinusoidal dilatation	Congested portal vein
A	0	0	0	0	0
B	***	***	***	***	***
C	*	**	**	*	**

Grading score follows: none (0), mild (*), moderate (**) and severe (***)

On histological examination, fibrotic changes were significantly higher among experimental groups as compared to control group. Necrotic changes, hepatic inflammatory changes, sinusoidal dilatation and congestion due to inflammatory changes at intra-lobular area, were found markedly higher among animals of group B. Histomorphological changes in different groups of rats is presented in Figure1.

Figure 1. Photomicrograph showing histological section of liver of control and experimental rats. (H&E) X 400.



A) Control group rat with normal hepatic histological architecture without any infiltration.
(B) Experimental group B rat with areas of lymphocytic infiltration, marked congestion and fibrosis.
(C) Experimental group C rat with marked reduction in inflammation, necrosis and fibrosis.

Discussion:

The present study sought to investigate the hepatoprotective effects of Raphanus Sativus exerted on CCl4-induced hepatotoxicity in a rat model by examining different biochemical and Histopathological parameters associated with hepatic function of intoxicated and treated rats. Significant alteration in liver functions and Histopathological status were observed in experimental rats intoxicated with CCl4. However, significant improvement was observed in body weight and all biochemical parameters related to liver function causing significant reduction in LFTs, and oxidative markers levels as well as improving the hepatic histological architecture. These results show that Raphanus Sativus therapy can ameliorate CCl4-induced hepatotoxicity by improving levels of endogenous antioxidants. CCl4, a common solvent for various refrigerants and dry-cleaning agents, is known to have an adverse effect on the liver and kidneys.[16]In our experiment, administration of CCl4 was followed by a reduction in the weight of the experimental animals whereas the animals receiving Raphanus Sativus showed a much less reduction in their weights, which is consistent with the findings of Anwar et al. who observed that Raphanus Sativus ameliorates weight loss secondary to hepatotoxicity.[15] In this study, there was an increase in the LFTs of all the animals receiving CCl4 as compared with the controls. These findings are similar to the results reported by Baek et al. and Jeongtae et al.[16, 17] However, LFT levels in the rats receiving adjunct therapy of Raphanus Sativus were far less than those who re-

ceived CCl4 alone which is also consistent with the findings of Baek et al. and Jeongtae et al.[17,18]

Rahman et al. also reported a significant rise in LFT levels in experimental animals which received CCl4 but the experimental animals receiving concomitant Raphanus Sativus therapy showed near normal levels, which is consistent with the current study.[14]

In this experiment, there was an increase in the MDA levels and decrease in the serum GPX and CAT levels of CCl4-treated rats denoting oxidative stress. However, Raphanus Sativus therapy prevented the change in the MDA, CAT, and GPX levels. These findings are in agreement with published research[6,14,19,20] that showed the consumption of Raphanus Sativus was inversely associated with biomarkers of oxidative stress.

In our study, histological examination of the liver showed clear evidence of CCl4-induced hepatotoxic injury manifested as marked infiltration of inflammatory cells, fibrosis, necrosis, sinusoidal dilatations, and congestion of portal vein, which is consistent with previous findings.[19]

However, these changes were much less evident in the Raphanus Sativus treated animals. These findings are consistent with the findings of Shariq et al. and Rahman et al[14,19], who noted that histopathological changes were reversed in the nephrotoxic animals who received Raphanus Sativus as well.

The main limitation of our study was lack of funds to assess further parameters of liver function, such as plasma albumin levels, serum levels of inflammatory markers such as CRP, TNF-a, TGF – β1 for further evaluating the hepatic damage and related complications due to induction of CCl4.

Conclusion

The present study concluded that Raphanus Sativus exerts an anti-oxidative, and hepatoprotective effect against CCl4-induced hepatic tissue damage. However, further studies should be carried out for a more detailed evaluation of the protective effects of Raphanus Sativus on other organs as well, to give more insight of the subject.

References:

1. Singh H, Sidhu S, Chopra K, Khan M. Hepatoprotective effect of trans-Chalcone on experimentally induced hepatic injury in rats: inhibition of hepatic inflammation and fibrosis. Canadian journal of physiology and pharmacology. 2016;94(08):879-87.
2. Choi H-S, Kang J-W, Lee S-M. Melatonin attenuates carbon tetrachloride–induced liver fibrosis via inhibition of necroptosis. Translational Research. 2015;166(3):292-303.
3. Ma J-Q, Ding J, Zhang L, Liu C-M. Hepatoprotective properties of sesamin against CCl4 induced oxidative stress-mediated apoptosis in mice via JNK pathway. Food and Chemical Toxicology. 2014;64:41-8.
4. Huang W, Li L, Tian X, Yan J, Yang X, Wang X, et al. Astragalus and Paeoniae radix rubra extract inhibits liver fibrosis by modulating the transforming growth factor□β/Smad pathway in rats. Molecular medicine reports. 2015;11(2):805-14.
5. Rajaratnam M, Prystupa A, Lachowska-Kotowska P, Załuska W, Filip R. Herbal medicine for treatment and prevention of liver diseases. Journal of Pre-Clinical and Clinical Research. 2014;8(2):55-60.
6. Ahn M, Kim J, Hong S, Kim J, Ko H, Lee N-H, et al. Black radish (raphanus sativus L. Var. Niger) extract mediates its hepatoprotective effect on carbon tetrachloride-induced hepatic injury by attenuating oxidative stress. Journal of medicinal food. 2018;21 (9):866-75.
7. Jaafar NA, Ahmed AS, Al-Sandooq DL. Detection of active compounds in radish Raphanus Sativus L.and their various biological effects. Plant Archives. 2020;20(2):1647-50.
8. Kamble S, Ahmed MZ, Ramabhimaiaha S, Patil P. Anti-inflammatory activity of Raphanus sativus L in acute and chronic experimental models in albino rats. Biomedical and Pharmacology Journal. 2015;6 (2):173-7.
9. Hanif MS, Baloch MS, Meghji KA, Abbas A, Kashif S, Qureshi R. Histopathological changes in the gastric mucosa induced by carbaryl toxicity: an experimental rat model. Khyber Medical University Journal. 2020;12 (2).
10. Charan J, Kantharia N. How to calculate sample size in animal studies? Journal of pharmacology & pharmacotherapeutics. 2013;4(4):303.
11. Meghji KA, Memon TF, Ahmed I, Memon SG, Noor N, Abbas A. Nephro protective Effects of L-Arginine

against Chemotherapy Induced Acute Kidney Injury in Wistar Rats. Journal of Islamabad Medical & Dental College. 2020;9(4):249-55.

12. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. 8th edition. Washington (DC): National Academies Press (US); 2011. Available from: https://www.ncbi.nlm.nih.gov/books/NBK54050/ doi: 10.17226/12910.
13. Kim WK, Kim JH, Da Hee Jeong YHC, Kim SH, Cho KJ, Chang M-J. Radish (Raphanus sativus L. leaf) ethanol extract inhibits protein and mRNA expression of ErbB2 and ErbB3 in MDA-MB-231 human breast cancer cells. Nutrition research and practice. 2011;5(4):288.
14. Rahman HS, Bayz KA, Hussein RH, Abdalla AI, Othman HH, Amin KM, et al. Phytochemical analysis and hepatoprotective activity of Raphanus sativus var. sativus in Sprague-Dawley rats. Tropical Journal of Pharmaceutical Research. 2020;19(8):1745-52.
15. Anwar O, Iqbal M, Khan AM, Tariq S, Ambreen A. Effect of Raphanus sativus (Radish) Leaf Extract and High Doses of Atorvastatin on Body Weight, Liver Weight and Liver/Body Weight Ratio. Annals of Punjab Medical College (APMC). 2020;14(4):313-7.
16. Mughal TA, Saleem MZ, Ali S, Anwar KK, Bashir MM, Babar M, et al. Evaluation of Hepatotoxicity of Carbon Tetrachloride and Pharmacological Intervention by Vitamin E in Balb C Mice. Pakistan Journal of Zoology. 2019;51(2).
17. Baek S-H, Park M, Suh J-H, Choi H-S. Protective effects of an extract of young radish (Raphanus sativus L) cultivated with sulfur (sulfur-radish extract) and of sulforaphane on carbon tetrachloride-induced hepatotoxicity. Bioscience, biotechnology, and biochemistry. 2008;72(5):1176-82.
18. Kim J, Ahn M, Kim S-E, Lee HS, Kim HK, Kim GO, et al. Hepatoprotective effect of fermented black radish (Raphanus sativus L. var niger) in CCl4 induced liver injury in rats. Journal of the Preventive Veterinary Medicine. 2017;41(4):143-9.
19. Syed SN, Rizvi W, Kumar A, Khan AA, Moin S, Ahsan A. In Vitro Antioxidant and In Vivo Hepatoprotective Activity of Leave Extract of Raphanus sativus in Rats Using CCL 4 Model. African Journal of Traditional, Complementary and Alternative Medicines. 2014;11(3):102-6.
20. Rahmouni F, Badraoui R, Amri N, Elleuch A, El-Feki A, Rebai T, et al. Hepatotoxicity and nephrotoxicity in rats induced by carbon tetrachloride and the protective effects of Teucrium polium and vitamin C. Toxicology mechanisms and methods. 2019;29 (5):313-21.