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Original Research

In vivo Antioxidant Potential of *Raphanus sativus* Seeds in Rat Kidney Against CCl_4-Induced Toxicity

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Abstract

In this study, the methanolic extract of *Raphanus sativus* (RSME) seeds was evaluated for its protective effect against CCl_4-induced nephrotoxicity. The treatment of Swiss albino rats with intraperitoneal injections of CCl_4 (1 ml/kg body weight**)** on alternate days for 30 days decreased the antioxidant enzymes, while lipid peroxidation and serum toxicity markers were increased. These changes were reversed in the animals receiving an oral dose of RSME (100 and 200 mg/kg body weight) along with CCl_4, thus increasing the level of antioxidant enzymes and decreasing serum toxicity markers and thus ameliorating the toxic effect of CCl_4. These results show that seed extract of *R. sativus* can reciprocate the toxic effects of CCl_4 and can be used to make a chemopreventive drug.

Keywords: antioxidant potential, *Raphanus sativus,* seeds, rat kidney, CCl_4-induced toxicity

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Introduction

Those reactive chemical species that have only one unpaired electron in their outer shell are known as free radicals [1]. Due to the unstable configuration in their outer orbit, an energy is generated which is then released by reacting with nearby biomolecules, for example carbohydrates, nucleic acids, proteins and lipids. Oxygen-free radicals and nitrogen-free radicals, more commonly known as "reactive oxygen species" (ROS) and reactive nitrogen species (RNS), are the major free radicals causing damage to cells and tissues. Hydroxyl radical, superoxide anion and hydrogen peroxide are included in ROS and peroxynitrite and nitric oxide are included in RNS. These free radicals can start self-catalyzed reactions in such a way that substances to which they react are also transformed into free radicals in order to spread the chain of reactions causing damage. When the concentration of these reactive radicals climbs up, they cause the destruction of cell structures, including damage to membranes, lipids, nucleic acids and proteins. This type of damage caused by free radicals is called as "oxidative stress" [2].

According to some researchers, the major reductant of oxygen is present in the mitochondrial membrane and it is supposed to be "ubisemiquinone". Mitochondria is a main physiological source of superoxide radicals in living cells, as the production of this radical is more than 2–3 nmol/min per mg of protein inside mitochondria [3]. Macrophages, neutrophils, xanthine oxidase, peroxisomes, cytochrome P450, microsomes and eosinophils are some sources of free radicals inside the cell [4]. Radiations, metals, xenobiotics, ions, chlorinated compounds and various environmental agents are the exogenous sources of free radicals Valko et al. (2006).

Although an antioxidant defense system is present in the cell, oxidative injuries add up throughout the life cycle, which includes damage to lipids, proteins and DNA. Various age-dependent diseases, for example arthritis, cancer, neurodegenerative disorders and arteriosclerosis, can be caused due to the accumulation of oxidative stress [5].

For thousands of years, nature has been a source of medicinal agents and a very large number of drugs have been derived from these natural sources, among them many drugs that are based on their use in traditional medicine. Natural compounds offer a huge number of antioxidants, especially those originating from dietary sources [6].

Raphanus sativus is generally known as radish and it is commonly available in all parts of the world. Various parts of *Raphanus sativus*, such as leaves, seeds and roots, are also used in traditional medicines [7]. Leaves of radish are culturally being used as an appetizer, laxative, digestive aid, stimulant and in various stomach disorders. Old roots, leaves and seeds are also used in the treatment of chest complaints and asthma [8]. The juice extracted from fresh leaves of radish is diuretic. Its seeds are stomachic, carminative, laxative and diuretic. As the roots are antispasmodic, diuretic, antiscorbutic, digestive and astringent, it is used for the cure of acid regurgitation, indigestion, diarrhea, abdominal bloating and bronchitis [9]. The main objective of this study is to evaluate the preventive effect of *Raphanus sativus* seeds methanolic extract against CCl_4-induced nephrotoxicity as an antioxidant.

Materials and Methods

Plant Collection

The seeds of *Raphanus sativus* (L.) were collected from District Multan, Pakistan. After collection, seeds (2 kg) were washed with distilled water and dried at room temperature in shade for more than two weeks and ground using an electric grinder.

Preparation of Seed Extract

Raphanus sativus's extract was prepared by the addition of 4 liters of methanol in powdered seeds (2 kg) with occasional shaking. The extract was filtered using Whatmann filter paper No. 45 after a week. A rotary vacuum evaporator was used to evaporate the filtrate and to obtain a methanolic extract of *Raphanus sativus* (RSME).

Experimental Design

Six-week old albino rats having weight 160-210 g were obtained from the animal house of the National Institute of Health (NIH) in Islamabad. The animals were kept at the primate facility in Quaid-i-Azam University, Islamabad in regular cages. They were kept at room temperature of 25±5ºC with a 12 h light/dark cycle. Before dosing, animals were familiarized with the environment for two weeks and fed with standard laboratory food. Before any treatment initial weights were measured.

Animal Treatment

For the experiment albino rats were distributed into seven groups such that each group contains six animals. The distribution of groups was as follows:

- Group 1 (control group) without any treatment.
- Group 2 (vehicle control) was given (1 ml/kg animal body weight) corn oil only.
- Group 3 received intraperitoneally solution of 20% CCl_4 in corn oil (1 ml/kg animal body weight).
- Group 4 was given intraperitoneally 20% CCl_4 in corn oil (1 ml/kg rat body weight) and silymarin solution (100 mg/ml in DMSO).
- Group 5 was administrated intraperitoneally 20% CCl_4 in corn oil (1 ml/kg rat body weight) and was also given RSME (100 mg/kg rat body weight)

orally with the help of feeding tubes alternatively for 30 days.

- Group 6 received intraperitoneally solution of 20% CCl_4 in corn oil (1ml/kg animal body weight) and was also given RSME (200 mg/kg rat body weight) orally with the help of feeding tubes alternatively for 30 days.
- Group 7 received RSME (200 mg/kg rat body weight) in DMSO orally with the help of feeding tubes alternatively for 30 days.

Animal Dissection

After last treatment rats were unfed for 24 h and their weight was measured. Before dissection urine was collected and then chloroform was used on anesthetized animals and then dissected from the ventral side. First of all, blood was collected by piercing the heart. Blood was collected in two types of tubes, i.e., for serum analysis it was stored in small falcon tubes that were then centrifuged to obtain serum. The rest of the blood was collected in EDTA containing tubes for whole blood analysis. From the dissected animal, kidneys were removed and placed in saline solution. After drying with the help of blotting paper, kidneys were weighed. For antioxidant enzymes study, kidneys were stored in liquid nitrogen at -70ºC.

Analysis of Urine Profile

The collected urine samples were analyzed for albumin and proteins, blood, urobilinogen, leucocytes, pH and specific gravity.

Analysis of Antioxidant Enzymes and TBARS

To analyze the antioxidant enzyme activities, 80 mg of the kidney was weighed and homogenized in 10 volumes of 100 mM NaH_2PO_4 buffer containing 1 mM EDTA at pH 7.4. After homogenization, samples were centrifuged at 12,000 xg for 20 min at 4ºC and supernatant was collected. The collected supernatant was then further used to estimate the activities of various enzymes.

Peroxidase assay (POD), superoxide dismutase assay (SOD), catalase assay (CAT), glutathione reductase assay (GSR), glutathione-S-transferase assay (GST), reduced glutathione assay (GSH), glutathione peroxidase assay (GSH-Px) and lipid peroxidation assay (TBARS) were analyzed by following the protocols of [10].

Statistical Analysis

The values were expressed as means±standard deviation (SD) of six observations in each group. One-way analysis (ANOVA) of variance was carried out by SPSS 13.0 software in order to define the different treatment effects. Level of significance among the various treatments was determined by LSD at 0.05% and 0.01% levels of probability.

Results

Changes in Weights of Body and Kidney

It is clear from Table 1 that in animals without CCl_4 administration there was an increase in body weights, but in CCl_4 group oxidative stress was induced by CCl_4 administration, which decreased the body weights. Treatment of animals with RSME significantly overcomes the CCl_4 effect in body weights, and the evident increase was observed in body weights of that group. After completion of the experiment, dissection was carried out and kidneys were weighed. An increase in kidney weight was observed in the CCl_4 group as compared to control group while extract-treated groups showed a non-significant decrease in kidney weights.

Urinalysis

As compared to control group, CCl_4 treatment caused a remarkable increase ($P<0.05$) in albumin,

Table 1. Preventive effect of RSME on percentage increase in body weights and alteration in kidney weights, albumin, urobilinogen and protein.

Group	Treatment	% increase in body weight (g)	Kidney weight (g)	Albumin (mg/dl)	Urobilinogen (mg/dl)	Protein (mg/dl)
I	Control	49.56±3.98**	1.8±0.075**	27.2±1.37**	12.1±1.27**	58.1±1.46**
II	DMSO+ Olive oil	47.45±2.45*	1.9±0.072**	28.6± 1.55**	13.9±1.21**	61.5±1.62**
III	1 ml/kg CCl_4	18.98±4.50	2.3±0.099	66.6±2.78	37.7±2.02	107.3±1.84
IV	1 ml/kg CCl_4 + silymirin	44.5±3.45**	1.9±0.083*	30.1± 1.24*	13.5± 1.19*	63.3±1.46*
V	100 mg/kg RSME +CCl_4	29.74±2.64*	2.1±0.085*	43.2±1.42*	16.4±1.50*	72.3±1.65*
VI	200 mg/kg RSME +CCl_4	37.43±3.25**	2.0±0.073**	29.4±1.71**	14.3±1.15**	62.7±1.97**
VII	200 mg/kg RSME	47.65±3.87**	1.7±0.075**	26.4±1.30*	11.7±1.08*	57.9±1.22*

Table 2. Preventive effect of RSME on urinary (pH, specific gravity, WBCs and RBCs) and serum markers (creatinine, urea).

Group	Treatment	Urinary Markers				Serum Markers			
		pH	Specific gravity	WBC/ul	RBC/ul	LDL (mg/dl)	Creatinine (mg/dl)	Urea(mg/dl)	CK-NAC
I	Control	7.1±0.11**	1.3±0.52**	17.6±1.26**	0.0±0.0**	297.24±6.67**	28.85±0.43**	35.896±1.2**	15.946±0.529**
II	DMSO +Olive oil	7.0±0.21**	1.4±0.05**	19.8±1.43**	0.04 ±0.21**	299.31±7.78**	29.56±0.39**	36.537±1.98**	17.139±0.498**
III	1ml/kg CCl_4	5.9±0.40	3.7±1.26	91.1±1.47	13.2±0.91	987.95±6.72	63.82±0.54	65.71±3.70	31.075±0.756
IV	1ml/kg CCl_4 + silymirin	7.0±0.32*	1.1 ±0.13*	20.3±1.3*	0.09±0.36*	315.28±8.36*	31.85±0.42*	37.491±2.43*	18.437±0.546*
V	100mg/kg RSME+CCl_4	6.5±0.10*	1.0±0.01*	59.2±1.00	5.2±0.95	810.42±8.67*	37.74±0.47*	48.121±1.4*	25.054±0.395*
VI	200mg/kg RSME+CCl_4	7.0±0.28**	1.0±0.08**	21.9±1.52**	1.3±0.26*	660.12±14.45	31.97±0.47*	40.23±3.71*	19.97±0.310*
VII	200mg/kg RSME	7.0±0.10**	1.0±0.01**	12.2±1.17*	0.01±0.01**	432.1±24.9*	29.55±0.92**	42.21±8.45*	16.875±0.456**

urobilinogen and urinary protein. Protective effect of RSME against CCl_4 was observed by a reduction in albumin, urobilinogen and protein levels of urine (Table 1).

As compared to control group, CCl_4 treatment causes a significant ($P<0.05$) decrease in pH of urine while a considerable ($P<0.05$) increase was found in specific gravity, WBCs and RBCs. The ameliorative effect of RSME against CCl_4 was observed on specific gravity, pH, WBCs and RBCs as pH level was increased ($P<0.05$) by the treatment of RSME in relation to CCl_4 group, and a decline of specific gravity, WBCs and RBCs count was also found (Table 2).

Assessing Kidney Serum Toxicity Markers

In serum, the important markers of renal dysfunction are CK-NAC, LDH, creatinine and urea. Significant elevation ($p<0.05$) was observed when rats were treated with CCl_4. The preventive effect was shown by RSME as it decreased its level (Table 2).

In vivo Antioxidant Enzyme Assessment

The ameliorative effect of RSME against CCl_4 was observed by the estimation of TBARS contents and determining antioxidant enzyme activities. As compared to the control group, CCl_4 treatment caused a significant ($P<0.05$) decrease in CAT, POD and SOD enzyme activity while the increase in TBARS content was found (Table 3). Treatment of RSME caused an increase in activity of these enzymes and decreased the TBARS level. As compared to the control group, CCl4 treatment caused a significant ($P<0.05$) decrease in GSR, GST, GSH and GSH-Px (Table 5), while increasing the total protein level. The treatment of RSME in relation to the CCl_4 group increased the enzyme level and decreased protein content.

Discussion

CCl_4 is experimentally proved to be hepatotoxic and also an active nephrotoxin. The kidney is a multilayered organ comprised of dissimilar machinery that works in an extremely synchronized way. Various chemicals and drugs have been reported to modify the function and structure of kidney [11].

The main objective of the present study was to examine the protective effect of *Raphanus sativus* seed extract against CCl_4-induced nephrotoxicity in rats, as CCl_4 is a notorious ROS-producing chemical. So, in this study, the antioxidant potential of *Raphanus* methanolic extract was the point of emphasis. After having a look at the results obtained, it is apparent that the seed extract of *Raphanus sativus* is effective in ameliorating the damage caused by CCl_4. Due to the presence of glucosinolates and antioxidant vitamins, *Raphanus* is

Table 3. Preventive effect of RSME on protein, TBARS content and renal antioxidant enzymes.

Group	Treatment	TBARS (µg/mg protein)	CAT (U/min)	POD (units/min)	SOD (U/mg protein)	GSR (nM/min/mg protein)	GST (nM/min/mg protein)	GSH (nM/mg protein)	GSH-Px (nM/mg protein)	Protein (µg/mg tissue)
I	Control	44.35±4.14*	2.73±0.200**	5.46±0.28*	11.39±1.14*	104.31±2.67**	82.95±2.02**	32.60±2.44**	152.16±1.36**	2.62±0.14*
II	DMSO + Olive oil	46.43±3.92**	2.62±0.2610**	5.21±0.29**	10.98±1.23**	103.56±1.45**	81.34±1.65**	30.23±1.78**	151.32±1.87**	2.67±0.18*
III	1 ml/kg CCl_4	106.49±6.68	1.67±0.123	2.31±0.12	6.18±1.01	76.73±3.71	42.42±1.47	12.23±1.21	82.48±1.39	4.50±0.20
IV	1 ml/kg CCl4 + silymirin	49.52±4.32*	2.54±0.349*	4.95±0.32*	10.27±1.67*	101.28±2.45*	78.67±1.34*	31.52±1.49*	148.69±2.05**	2.58±0.21**
V	100 mg/kg RSME+CCl_4	66.58±3.86*	2.18±0.390*	3.49±0.40*	8.02±1.15*	82.38±1.34	62.60±1.98*	22.01±0.97*	124.71±2.26**	2.38±0.19*
VI	200 mg/kg RSME+CCl_4	55.54±3.31**	2.33±0.364**	4.79±0.15*	11.99±1.82**	101.39±1.37**	77.37±2.25*	32.55±2.37**	143.92±2.01**	2.57±0.22**
VII	200 mg/kg RSME	43.22±2.84**	2.48±0.300**	5.42±0.33**	12.16±1.56*	111.89±1.74*	83.20±1.61**	32.25±1.58**	154.72±2.60*	2.31±0.15*

significant in preventing chronic diseases and offering protection against oxidative damage [11, 12].

As described in the results section of this experimental study, the first thing observed after intraperitonial injection of CCl_4 was the change in body and kidney weights. As compared to untreated animals, a huge decline in body weights had been observed. This might be due to the alteration in metabolic processes inside the body or to the oxidative damage caused to tissues which had turned out to be a difficul for the body weights to increase. The loss in body weight was recovered by the co-administration of seed extract. Whereas, examining kidney weight, an increase in weight was observed in CCl_4-administrated animals, while RSME showed restorative effects by decreasing the kidney weight toward the normal group.

Analysis of the waste product produced by the kidney, i.e. urine, is the most important factor and a valuable diagnostic tool to monitor the physiological disorders of the kidney [13, 14]. The concentration of various metabolites present in urine was analyzed in this study.

Concerning the function of the liver, kidney status and acid-base balance of body, examination of urine is knowledgeable about the function of the kidney. Under normal circumstances, no protein, albumin, urobilinogen, WBCs and RBSs are excreted in urine, but a high concentration is present under extreme pathological conditions. Urobilinogen is produced by the conjugation of bilirubin and breakdown products of bacteria which, after passing through bile ducts, are processed in the intestine and finally converted to urobilinogen [15-17]. The CCl_4 treatment causes a huge increase in the concentration of creatinine, urobilinogen, WBCs and RBCs in urine, indicating severe fibrosis, renal necrosis, kidney toxicity and glomerular damage [18-21]. RSME treatment showed the protective effect by decreasing the number of WBCs and RBCs passing out in urine, and thus CCl_4 intoxication.

Another group of serum markers comprising of gamma-GT, total bilirubin, creatinine and urea also highly increase due to oxidative atmosphere. Creatinine is a waste product formed by slow degradation of creatine phosphate. It is the creatine kinase that catalyzes the formation of creatine phosphate through the transfer of phosphate bond from a high-energy ATP molecule. Under CCl_4-induced oxidative stress, ATP declines occur due to the blockage of TCA cycle, so increasing the amount of free creatine, whose degradation results in creatinine formation and therefor increasing its serum level [22] – a condition specific to nephritis [23].

In this study, because of the production of free radicals by the metabolism of CCl_4 and tissue damage, urine of the CCl_4-treated rats exhibited a minor reduction in pH and a rise in specific gravity. Co-administration of *R. sativus* seed extract increases the pH and decreases specific gravity toward normal, as shown by similar results reported by Khan, Rizvi [24] and Khan, Khan [25].

A brownish yellow pigment called bilirubin is found in bile and is produced during the breakdown of waste RBC; usually found in conjugation with albumin. The bilirubin tests are used for identification of liver damage. Increased levels of total or direct bilirubin may be due to jaundice, liver tube blockage or bile ducts, cirrhosis. Our results of this study show a marked increase in these tested parameters. RSME prevented renal injuries with a subsequent restoration of all these parameters discussed above. This study is in relation to the studies of Khan, Khan [26] and Venkatanarayana, Sudhakara [27] who investigated how the administration of CCl_4 caused marked oxidative stress in kidneys. Serum creatinine, total bilirubin, gamma-GT and urea concentrations were significantly higher in the CCl_4-treated rats.

LDL and albumin are some other parameters that were checked in this experiment. Lipid peroxidation products have been shown to affect a variety of cellular processes. During early stages of oxidation, the antioxidants act to prevent the formation of oxidation products. Once these antioxidants are used up, the formation of lipid hydroperoxides of polyunsaturated fatty acids (PUFAs) appear, which are rapidly reduced to their hydroxide form [28]. As is evident from previous studies, lipid abnormalities are often linked with renal function deterioration [29, 30], and together with this there exists an independent risk factor for renal injury known as hypercholesterolemia [31, 32]. The probable cause for hypercholesterolemia is an increase in oxidative stress, increased formation of oxidized low-density lipoprotein (Ox-LDL), and renal inflammation [33]. Serum albumin is the predominant serum protein, which reflects the synthetic function of the liver. In the present study an increase in all the parameters of lipid abnormalities occurred by CCl_4 administration for 18 days on alternative basis, except HDL and albumin, which decreased considerably by CCl_4 toxicity as compared to rats of the control group. A decrease in albumin is known as hypoalbuminemia. It can be proposed that high cholesterol levels in serum are associated with low HDL and high LDL. *R. sativus* methanolic extract blunted the elevated levels of these parameters when given along with CCl_4.

A relationship between oxidative stress and renal damage is reported in various studies. In order to protect the body from oxidative stress and damage caused by radicals, a family of antioxidant enzymes exists, but oxidative stress reaches a certain level and the antioxidant defense system present in the body becomes useless and inadequate for recovering the damaging effects of reactive oxygen species [34-36].

In this experimental work, the protecting effects of *R. sativus* seed extract on damaged antioxidant enzymes were observed, such as POD, CAT, SOD, GSH, GSR, GSH-Px, and GST, and TBARS level was also examined.

Many *in vivo* and *in vitro* studies have supported the fact that CCl_4 enhances peroxidation of lipids in tissues by the production of CCl_3 radical produced by the metabolic conversion carried out by CYP-450 2E1Adewole, Salako [37] and Khan and Siddique [19]. In this study, after the administration of CCl_4 in rats, the TBARS contents increased significantly. By keeping in mind the results of previous experiments it can be supposed that lipid peroxidation has occurred, showing tissue damage [20, 38]. Various antioxidant constituents such as vitamins and glucosinolates are present in the methanolic seed extract of *R. sativus*, so it counters the effect of CCl_4-produced toxicity. Similar results regarding the level of TBARS content in CCl_4-intoxicated rat tissues after treatment with black tea was observed [39].

A similar study was carried out by Khan, Khan [40] in which methanolic extract of *Launaea procumbens* avoided the renal injuries by stabilizing the membranes, scavenging of free radicals, and ameliorating the toxic effects of CCl_4.

Conclusion

Results obtained in this study show that RSME can cure the damage caused in renal tissue of rats with CCl_4. So it can be concluded that in future *Raphanus sativus* can be used to cure toxicity, as no abnormal signs were observed in animals treated with RSME only.

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Conflict of Interest

The authors declare no conflict of interest.

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