

Original article:

**PROTECTIVE EFFECTS OF CURCUMIN AND VITAMIN E ON
CARBON TETRACHLORIDE-INDUCED NEPHROTOXICITY IN RATS**

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ABSTRACT

The present investigation reports the protective effects of curcumin (CMN) and vitamin E against CCl₄ induced oxidative stress and nephrotoxicity in rats. The toxicant CCl₄ was used to induce nephrotoxicity at a dose of 1 ml/kg as 1:1(v/v) mixture with liquid paraffin twice weekly for 8 weeks. Plasma total protein, albumin, urea and creatinine concentrations were measured to assess the renal function. Antioxidant status in the kidney was estimated by determining the activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) as well as by histopathological examination. CCl₄ caused elevated levels of urea and creatinine while it decreased levels of total protein and albumin in plasma. Furthermore, CCl₄ treated rats showed marked depletion of renal endogenous antioxidant enzymes. Oral administration of curcumin (100 mg/kg, daily for 8 weeks) and in combination with vitamin E (three times weekly for 8 weeks) showed significantly attenuated renal dysfunction by increased activity of antioxidant enzymes in kidney. It also increased the concentrations of plasma total protein and albumin, while reducing the concentration of urea and creatinine. Kidney sections of CCl₄ induced rats showed deleterious alterations in micro anatomy. However, curcumin and vitamin E treatment prevented kidney damage induced by CCl₄. This study reveals that curcumin in combination with vitamin E possesses strong antioxidant and kidney protective activity compared to curcumin alone.

Keywords: carbon tetrachloride, curcumin, vitamin E, silymarin

INTRODUCTION

Various studies have demonstrated that carbon tetrachloride (CCl₄) intoxication causes free radical generation in many tissues such as liver, kidney, heart, lung, testis, brain and blood (Rechnagel et al., 1989; Kumar et al., 2005; Khan and Ahmed, 2009; Khan et al., 2009). CCl₄ intoxication in animals is an experimental model that mimics oxidative stress in many physiological situations (Ivor and Schneider, 2005). Cumulative data suggest a role for reactive oxygen metabolites as one of the postulated

mechanisms in the pathogenesis of CCl₄ nephrotoxicity (Rechnagel et al., 1989). CCl₄ is metabolically converted by cytochromes P450 to CCl₃ radicals and results in enhanced generation of trichloro methyl peroxy radicals (Cl₃COO-) and hydrogen peroxide in cultured hepatocytes as well as in mesangial cells (Knight et al., 1989). *In vitro* and *in vivo* studies indicated that CCl₄ enhances lipid peroxidation and reduced the renal reduced/oxidized glutathione ratio in kidney cortex as well as renal microsomes and mitochondria (Adewole et al., 2007; Khan et al., 2009). Antioxidants are vital

substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Shahani, 1998; Ozsoy et al., 2008). There is an increasing interest in natural antioxidants, e.g. polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage. In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin (Sundaram and Mitra, 2007). Plant and plant products are being used as a source of medicine since long. Plants used as food and in traditional medicine are more likely to yield pharmacologically active compounds. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, without side effects and of their economic viability.

Curcumin is a major yellow pigment in turmeric ground rhizome of *Curcuma longa* Linn., which is used widely as a spice and colouring agent in several foods such as curry, mustard and potato chips as well as in cosmetics and drugs (Okada et al., 2001; Joe et al., 2004). Curcumin represents a class of anti-inflammatory and antioxidants reported to be a scavenger of formed ROS (Biswas et al., 2005). Curcumin exhibited antioxidant activity in a renal cell line (Cohly et al., 1998) and ameliorated ferric nitrilotriacetic (Fe-NTA) renal oxidative stress in mice (Okada et al., 2001). However, investigations reporting the protective effects of curcumin along with vitamin E on CCl₄ induced nephropathy are scanty. Therefore, in continuation of the search for potential modulators of CCl₄-mediated renal damage, we have examined the effects of curcumin, a naturally occurring polyphenol and vitamin E on experimentally induced CCl₄ renal oxidative stress in rats.

MATERIALS AND METHODS

Chemicals and drugs

Carbon tetrachloride was purchased from MERCK India Ltd., Curcumin and α -

Tocopherol (Vitamin E) purchased from Sigma Chemicals Co. St Louis, MO/USA. Silymarin suspension purchased from Micro labs, Bangalore. Analyzing kits were obtained from Excel Diagnostics Pvt. Ltd, Hyderabad, Span Diagnostics Ltd, Surath. All other chemicals used were of technical grade.

Animals

Adult male albino rats Wistar strain (*Rattus norvegicus*) weighing 190 ± 30 procured from Sri Raghavendra Animal Supplier, Bangalore, Karnataka, were used for the study after the clearance from Institutional Animal Ethical Committee (CPCSEA) REGD. No. 470/01/a/ CPCSEA, DT. 24th Aug 2001. They were kept in cages under standard laboratory conditions (23 ± 2 °C, 12 h dark/light) and were fed with commercial rat feed supplied by Sai Durga Feeds and Foods, Bangalore and water *ad libitum*. Before use prior to the experimentation they were allowed to laboratory conditions for ten days.

Experimental protocol

Animals were randomly assigned into seven groups of six each. Group 1 served as normal control and received Phosphate Buffer Saline (p.o. daily for 8 weeks), Group 2 Curcumin control (100 mg/kg wt. p.o.), Group 3 Curcumin (100 mg/kg p.o. daily for 8 weeks) + Vitamin E (40 mg/kg three times weekly for 8 weeks). Group 4 received CCl₄ (1 ml/kg 1:1 in liquid paraffin p.o. twice weekly for 8 weeks), Group 5 CCl₄ + Curcumin (100 mg/kg p.o. for 8 weeks), Group 6 CCl₄ + Curcumin (100 mg/kg p.o., daily, Vitamin E 40 mg/kg weekly three times p.o. for 8 weeks), Group 7 CCl₄ + Silymarin (50 mg/kg wt. p.o. for 8 weeks).

Selection of dose

The dose of CCl₄ (1 ml/kg wt.) (Mc Lean et al., 1969; Roy et al., 2006), the dose of curcumin 100 mg/kg wt. (Sreepriya and Bali, 2006) and the dose of vitamin E

(Moawad, 2007) were selected with minor modifications.

Sacrifice of animals and collection of blood and kidney

At the end of experimentation and 24 h after the last dose of CCl₄, rats were sacrificed by cervical dislocation. Just before sacrifice, the animals were anesthetized with pentobarbital (0.6 ml/kg) and the blood collected by heart puncture and transferred into Eppendorf tubes with anticoagulant containing EDTA (1 mg/ml). Plasma was obtained by centrifugation and was used for estimation of total protein, albumin, urea and creatinine.

After sacrifice, immediately kidneys were removed and washed thoroughly with ice-cold 0.9 sodium chloride solution (saline) and cut into micro pieces fixed in 10 % formalin for histopathological examinations.

BIOCHEMICAL ANALYSIS

Plasma total protein and albumin were estimated by using kits obtained from Span Diagnostics Ltd, Surath, India. Urea and creatinine were estimated by using kits obtained from Excel Diagnostics Pvt. Ltd, Hyderabad, India.

Antioxidant enzyme assays

A ten percent homogenate of kidney was prepared by using a potter-elvehjem homogenizer at 4 °C in 0.15 M KCl. The homogenate was centrifuged (12,000 rpm for 45 min at 0-4 °C) in a Remi (C24-BL) cooling centrifuge to remove the debris and the supernatant was used for enzyme assays.

Glutathione peroxidase (GPx) assay

Glutathione peroxidase activity was estimated as described by Rotruck et al. (1973) and Ellman's (1959). Briefly, to 0.5 ml 0.4 M buffer (pH 7.0), 0.2 ml enzyme source (kidney homogenate), 0.2 ml 2 mM GSH, 0.1 ml 0.2 mM H₂O₂ were added and incubated at room temperature for 10 min along with a control tube con-

taining all reagents except enzyme source. The reaction was arrested by adding 0.5 ml of 10 % TCA, centrifuged at 4000 rpm for 5 min and the GSH content in 0.5 ml of supernatant was estimated. The activity was expressed as µg of GSH consumed/min/mg protein.

Glutathione reductase (GR) assay

Glutathione reductase activity was estimated by the method of Pinto and Bartley (1969). To 0.5 ml of 0.25 M potassium phosphate buffer (pH 7.4), 0.1 ml of 25 mM EDTA, 0.1 ml of 1 mM NADPH, 0.96 ml of distilled water and 0.1 ml of enzyme source (kidney homogenate). The reaction was initiated by the addition of 0.024 ml GSSG (50 mM). The change in absorbance was recorded at 1 min intervals at 340 nm for 5 min. The specific activity is expressed as µmol of NADPH oxidized/min/mg protein using an extinction coefficient for NADPH of 6.22 cm⁻¹ mmol⁻¹.

Glutathione-S-transferase (GST) assay

Glutathione-S-transferase activity was estimated as described by Habig et al. (1974). To 1.7 ml of 0.14 M buffer (pH 6.5), 0.2 ml 30 mM GSH and 0.04 ml enzyme source (kidney homogenate). The reaction was initiated by 0.06 ml 0.01 M 1-chloro-2,4-dinitrobenzene (CDNB). The activity was calculated using an extinction coefficient of CDNB-GSH conjugate as 9.6 mM⁻¹ and expressed as µmoles of CDNB-GSH conjugate formed/min/mg protein.

Catalase (CAT) assay

Catalase activity was estimated by Beers and Sizer (1952) method. The assay system contained 1.9 ml 0.05 M buffer (pH 7.0) and 1.0 ml 0.059 M H₂O₂. The reaction was initiated by addition of 0.1 ml enzyme source (kidney homogenate). The decrease in absorbance was monitored at 1 min interval for 5 min at 240 nm and activity was expressed as nmoles of H₂O₂ decomposed/min/mg protein.

Superoxide dismutase (SOD) assay

Superoxide dismutase activity was estimated by the method of Marklund and Marklund (1974) adopted as follows by Soon and Tan (2002): To 2.1 ml of 50 mM buffer, 0.02 ml of enzyme source (kidney homogenate) and 0.86 ml of distilled water. The reaction was initiated with 0.02 ml of 10 mM pyrogallol and change in absorbance was monitored at 420 nm. One unit of SOD was defined as that amount of enzyme required to inhibit the auto-oxidation of pyrogallol by 50 % in standard assay system of 3 ml. The specific activity was expressed as units/min/mg protein.

Protein concentration of the supernatant was estimated by Lowry et al. (1951) using crystalline BSA standard.

Histopathology of kidney

The histological sections of the kidney of rats were taken by adopting the procedure as described by Humason (1972). The tissues were isolated and gently rinsed with physiological saline solution (0.9 % NaCl). They were fixed in Bouin's fluid (75 ml saturated aqueous picric acid, 25 ml 40 % formaldehyde and glacial acetic acid) for 24 hours. The fixative was removed by washing through running tap water for overnight. Then the tissues were processed for dehydration. Ethyl alcohol was used as the dehydrating agent. The tissues were passed through successive series containing 30 %, 50 %, 70 %, 80 %, 90 %, 95 % and absolute alcohols. Then the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections of 5 μ thickness were cut using "sipcon" rotatory microtome. The sections were stained with Harris hematoxylin (Harris, 1900) and counter stained with eosin, dissolved in 95 % alcohol. After dehydration and cleaning, the sections were mounted in Canada balsam. Photomicrographs of the section preparations were taken using Magnus (MLX) photomicrographing equipment.

Statistical analysis

The results were expressed as mean \pm S.D. The results were analyzed using DMR test (Duncan's Multiple Range Test). $P < 0.05$ was considered as statistically significant (Duncan, 1955).

RESULTS**Effect of curcumin and vitamin E on CCl₄ induced changes in the relative kidney weight (KW)/100 g body weight (BW) and plasma profile**

Tables 1 and 2 show the relative kidney weights and plasma profile of rats in each group. It was observed that CCl₄ induced rats showed significant increase ($P < 0.05$) in relative kidney weights, urea and creatinine, whereas significant ($P < 0.05$) decrease in the concentration of total protein and albumin over normal control. Oral administration of curcumin (group V) and in combination with vitamin E (group VI) in rats treated with CCl₄ caused significantly ($P < 0.05$) reduced the relative kidney weights, urea and creatinine concentrations, while significant recovery in the concentrations of total protein and albumin.

Effect of curcumin and vitamin E on CCl₄ induced changes in antioxidant enzyme activities in the kidney tissue (GPx, GR, GST, CAT and SOD activities)

Table 3 indicates the activities of antioxidant enzymes in the kidney of seven experimental groups. Nephrotoxicity induced by CCl₄ causes a significant decrease in antioxidant enzyme levels (catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase) as compared to the normal control group. Whereas oral administration of curcumin (group V) and its combination with vitamin E (group VI) in rats treated with CCl₄ showed recovery of the activities of antioxidant enzymes.

Table 1: Effect of curcumin and vitamin E on relative kidney weight and plasma protein and albumin of different treated groups

Name of the Experimental group	Relative Kidney (wt/100g bw)	Total protein (g/dL)	Albumin (g/dL)
Normal control	0.631 ^b ±0.122	9.565 ^a ±0.335	5.6 ^a ±0.287
CMN control	0.529 ^b ±0.0823	9.3817 ^a ±0.3	5.531 ^a ±0.09
CMN + Vit. E control	0.61 ^b ±0.0775	9.2188 ^a ±0.1974	5.2733 ^a ±0.2
CCl ₄ only	0.895 ^a ±0.02	6.09 ^d ±0.1719	3.411 ^d ±0.2724
CCl ₄ + CMN	0.752 ^b ±0.0445	8.565 ^c ±0.2843	4.37 ^c ±0.2277
CCl ₄ + CMN Vit.E	0.677 ^b ±0.033	8.948 ^b ±0.14	4.9217 ^b ±0.1259
CCl ₄ + Silymarin	0.707 ^b ±0.065	9.1 ^b ±0.1243	4.97 ^b ±0.074

Values are mean ± S.D. (n=6) (CMN - curcumin); Values with different superscripts within a column are significantly different at P<0.05 (Duncan's Multiple Range Test)

Table 2: Effect of curcumin and vitamin E on plasma urea and creatinine of different treated groups

Name of the Experimental group	Urea (mg %)	Creatinine (mg %)
Normal control	29.15 ^d ±0.2832	0.6367 ^d ±0.02
CMN control	29.1617 ^d ±0.2	0.6567 ^d ±0.02
CMN + Vit. E control	29.21 ^d ±0.17	0.6583 ^d ±0.01
CCl ₄ only	66.7 ^a ±0.6316	1.6517 ^a ±0.035
CCl ₄ + CMN	37.99 ^b ±0.695	0.8765 ^b ±0.0076
CCl ₄ + CMN Vit.E	36.548 ^c ±0.345	0.7267 ^c ±0.0075
CCl ₄ + Silymarin	36.16 ^c ±0.4479	0.7235 ^c ±0.01

Values are mean ± S.D. (n=6) (CMN - curcumin); Values with different superscripts within a column are significantly different at P<0.05 (Duncan's Multiple Range Test)

Table 3: Effect of curcumin and vitamin E on antioxidant enzymes activities (µg or µmol/min/mg protein) in the kidney of different treated groups

Name of the parameter	Group I Vehicle control	Group II CMN control	Group III CMN+ Vit. E control	Group IV CCl ₄	Group V CCl ₄ + CMN	Group VI CCl ₄ + CMN, Vit. E	Group VII CCl ₄ + Silymarin
GPx (µg GSH/min/mg protein)	8.8 ^a ±0.25	8.89 ^a ±0.38	8.875 ^a ±0.35	4.538 ^d ±0.295	6.78 ^c ±0.195	7.98 ^b ±0.356	8.665 ^a ±0.217
GR (µM/min/mg protein)	1.677 ^a ±0.025	1.653 ^a ±0.02	1.647 ^a ±0.005	0.72 ^e ±0.004	1.1007 ^d ±0.0503	1.3 ^c ±0.034	1.376 ^b ±0.019
GST (µM/min/mg protein)	0.0485 ^a ±0.0013	0.0479 ^a ±0.0013	0.0499 ^a ±0.0015	0.0187 ^d ±0.0002	0.0428 ^c ±0.002	0.0448 ^b ±0.001	0.045 ^b ±0.0008
CAT (µM H ₂ O ₂ /min/mg protein)	25.298 ^a ±0.111	25.06 ^a ±0.2349	24.913 ^a ±0.514	12.36 ^d ±0.281	20.354 ^c ±0.29	22.258 ^b ±0.4458	22.416 ^b ±0.2952
SOD (U/min/mg protein)	7.411 ^a ±0.2994	7.188 ^a ±0.16	7.21 ^a ±0.183	2.9716 ^d ±0.0587	5.6748 ^c ±0.2258	6.2552 ^b ±0.1311	6.3954 ^b ±0.3431

Values are mean ± S.D. (n=6) (CMN - curcumin); Values with different superscripts within a column are significantly different at P<0.05 (Duncan's Multiple Range Test)

Effect of curcumin and vitamin E on CCl₄ induced changes in kidneys histoarchitecture

The histology of the kidney (Figure 1, A-F) from normal, curcumin and in combination with vitamin E control animals showed normal architecture with well developed Bowman's capsule with glomerulus and convoluted tubules enlarged, whereas rats treated with CCl₄ (Figure 2, G, H) showed degenerative changes in Bowman's capsule and glomerulus, cells with vacuolization and necrotic condition. In contrary oral administration of curcumin and in combination with vitamin E or silymarin ameliorated the renal injuries near to the normal histology of the kidneys (Figure 2, I-N).

HISTOPATHOLOGY OF KIDNEY

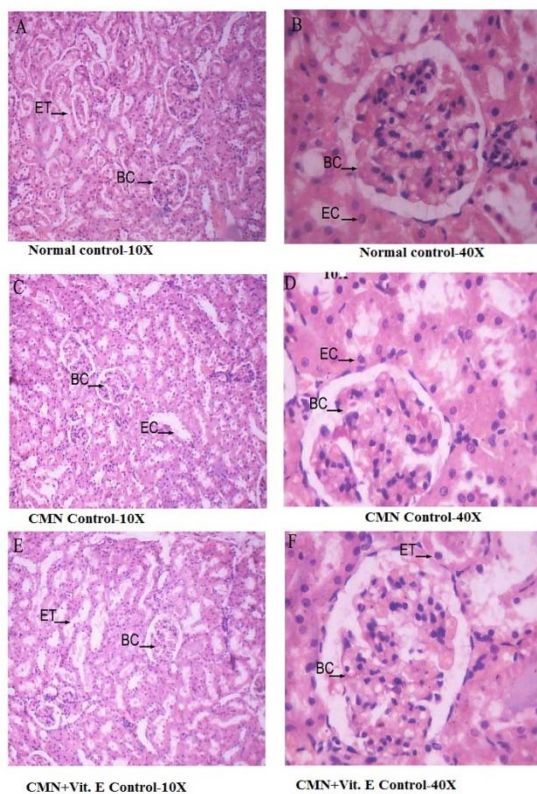


Figure 1: A (10 X) & B (40 X) Normal control (Group I), Fig C (10 X) & D (40 X) curcumin control (Group II) and Fig E (10 X) & F (40 X) curcumin and vitamin E control (Group III) showing normal architecture of kidney with prominent Bowman's capsule (BC), epithelial cells (EC), enlarged tubules (ET).

PLATE-2

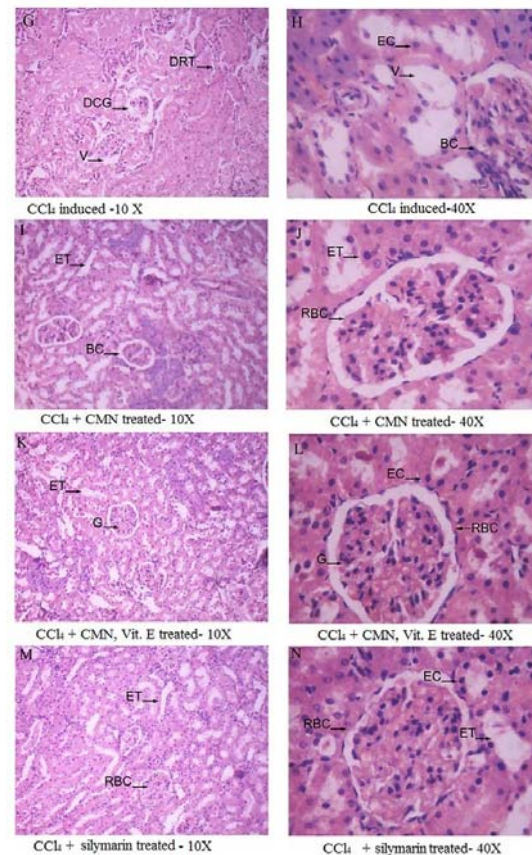


Figure 2: G (10 X) & H (40 X) CCl₄ treated (Group IV) showing degenerative changes in renal tubules kidney tissue (DRT/DCG). Structural changes in glomeruli with vacuolization (V). Fig I (10 X) & J (40 X) CCl₄ + curcumin (Group V) showing regenerative Bowman's capsule (RBC) and vacuolization. Fig K (10 X) & L (40 X) CCl₄ + curcumin along with vitamin E (Group VI) showing regenerative Bowman's capsule (RBC) and reduced vacuolization. Fig M (10 X) & N (40 X) CCl₄ + standard drug (silymarin) (Group VII) showing regenerative Bowman's capsule (RBC) and reduced vacuolization

DISCUSSION

The present study revealed ameliorative effect of curcumin and vitamin E on CCl₄ induced renal toxicity in rats. The pathogenesis of kidneys is a crucial public health problem. It is well known that the kidneys play a pivotal role in the regulation of various chemicals. Administration of CCl₄ causes nephrotoxicity as indicated by elevation in urine and serum level of urea, creatinine and urobilinogen while it decreased

the creatinine clearance. These pathological changes signify the potential damage to liver and kidney cells induced with CCl₄ treatment (Ogeturk et al., 2005). From the present study it is evident that elevation in plasma urea and creatinine levels can be attributed to the damage of nephron structural integrity (Khan and Siddique, 2012). In addition, decrease in the plasma total proteins and albumin concentrations in CCl₄-treated rats might have resulted from remarkable leakage due to hyper cellularity of both glomeruli and tubules (Adewole et al., 2007). Histopathological examination also revealed the degenerative changes in glomerulus, renal tubules and vacuolization of cells.

Curcumin with or without vitamin E treatment significantly improved the concentrations of total proteins and albumin in plasma, while significant recovery was noticed in the levels of urea and creatinine. Earlier studies have also shown that curcumin treatment decreased serum creatinine and urea concentrations in cyclosporine induced renal injury in rats in dose dependent (Tirkey et al., 2005). This effect may be related to the antioxidant properties of curcumin since it has been found that ROS may be involved in the impairment of glomerular filtration rate (Hughes et al., 1996; Farombi and Ekor, 2006).

Of the antioxidant enzymes, superoxide dismutase and catalase are extremely effective antioxidant enzymes responsible for catalytic dismutation of highly reactive toxic superoxide radicals to H₂O₂ and for the catalytic decomposition of H₂O₂ to oxygen and water, respectively (Reiter et al., 2000). CCl₄ induced oxidative stress in renal tissues led to accumulation of superoxides and hydrogen peroxides. In this study it is evidenced decline in the activities of GPx, GR, GST, CAT and SOD in the kidney tissue. These results were in agreement with earlier findings (Khan and Siddique, 2012). Curcumin and vitamin E treatment significantly improved the antioxidant enzyme activity in the kidney tissue. This suggests that antioxidant potential of curcumin. More and more

studies now established the ability of curcumin to mainly eliminate the hydroxyl radical (Reddy and Lokesh, 1994), superoxide radical (Sreejayan and Rao, 1996), singlet oxygen (Rao et al., 1995), nitrogen dioxide (Unnikrishnan and Rao, 1995) and NO (Sreejayan and Rao, 1997). It has also been demonstrated that curcumin inhibits the generation of the superoxide radical (Ruby et al., 1995).

In our studies simultaneous supplementation of vitamin E and curcumin to CCl₄-treated rats was found to ameliorate the renal toxicity. Therefore, it is assumed that administration of both vitamin E and curcumin co-operatively act on ROS induced by CCl₄, curcumin alone also shown positive effect, however, it is less effective compared with curcumin combination with vitamin E. In support of our results earlier findings also have suggested that curcumin and vitamin E effectively ameliorates the L-Thyroxine (T4) induced oxidative stress in renal cortex of male rats, whereas curcumin alone unable to do so (Jena and Chainy, 2011). Histopathological examinations are in agreement with biochemical analysis. Regenerative changes in glomerulus and convoluted tubules observed in curcumin and vitamin E supplemented rats. Comparatively curcumin in combination with vitamin E showed almost normal architecture of kidney than curcumin treatment alone.

CONCLUSION

Our study suggests that curcumin combination with vitamin E may be considered as potentially combating oxidative stress and nephrotoxicity induced by CCl₄.

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