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Protective effects of some Generally Recognized As Safe (GRAS) grade food preservatives against experimentally induced renal dysfunction

Barsha Dassarma[1], Saptadip Samanta[2], Dilip K Nandi[1*], Somnath Gangopadhyay[3*]

[1] Department of Physiology, Raja N.L. Khan Women's College, Midnapore, 721102, West Bengal, India.

[2] Department of Physiology, Midnapore College, Midnapore, 721101, West Bengal, India.

[3] Department of Physiology, Occupational Ergonomics Laboratory, University College of Science and Technology, University of Calcutta, 92 APC Road, Kolkata, 700009, West Bengal, India.

ABSTRACT

Drug induced nephrotoxicity is the current concern of research due to its awful worldwide occurrences. Generally recognized as safe (GRAS) grade food preservatives e.g. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), L-ascorbic acid (Vit.C) and gamma-tocopherol (Vit. E) exhibits potent antioxidant, anti-inflammatory properties against severe oxidative stress. The aim of this study was to evaluate the efficacy of food preservatives on carbon tetrachloride (CCl_4)-induced (230 mg/ kg b wt/ rat/day) nephritic damage in rats. Nephritic markers like serum urea, blood urea nitrogen, serum creatinine; antioxidant markers such as GSH, SOD, CAT, GPx, and lipid peroxidation end product, MDA were measured to establish anti-oxidant properties of said food preservatives and vitamins. The results had shown an elevated level of serum urea (387.30%), blood urea nitrogen (376%), serum creatinine (646.82%) and marked decreased activity of antioxidant markers like SOD (81.03%), CAT (72.24%), GSH (63.04%), GPx (50.34%) as well. CCl_4 induced nephrotoxicity also caused 48.14% and 59.47% increase in sodium and potassium concentration. Histological studies also confirmed that antioxidant status in renal cells was restored as BHA, BHT, L-ascorbic acid, and gamma-tocopherol successfully ameliorated certain degenerative changes caused due to CCl_4 intoxication. Therefore, it can be concluded that supplementation of certain food preservatives like BHA, BHT and like Vitamins L-ascorbic acid, gamma-tocopherol may be potentially beneficial to the community affected by severe renal dysfunction.

Keywords: butylated hydroxyanisole, butylated hydroxytoluene, L-ascorbic acid, gamma-tocopherol, CCl_4 intoxication.

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***Address for Correspondence:**

Dilip K Nandi, Department of Physiology, Raja N L K W College, Midnapore, 721102, West Bengal, India.

And

Somnath Gangopadhyay, Department of Physiology, Occupational Ergonomics Laboratory, University College of Science and Technology, University of Calcutta, 92 APC Road, Kolkata, 700009, West Bengal, India.

1. INTRODUCTION

In recent times, large-scale urbanization has effects on several risk factors for the development of several non-communicable diseases (NCD). The occurrence of non-communicable, noninfectious diseases becomes the major cause of change in mortality and morbidity worldwide. Now days, drug-induced nephrotoxicity is a widespread clinical problem which is creating a grave threat to public health in developing countries due to its high cost combating measures[1]. Therefore, the present article is able to introduce some low cost preventive measures against the said disorder.

Carbon tetrachloride (CCl_4), a colorless, odorant solvent which is uses as metal degreasing, dry cleaning, fabric-spotting, grain fumigant, to make refrigerants, propellant for aerosols, as a major component in pesticide[2]. Exposure on CCl_4 may cause Headache, dizziness, vomiting, stomach pain, lightheadedness, tiredness, weakness and blurred vision. The principal target organs of carbon tetrachloride in humans are the lungs, liver, and kidney. Carbon tetrachloride (CCl_4) is able to absorb swiftly by several route of exposure (through nasal cavity, epidermis, orally) in humans and animals and distributed among the tissues, especially those which possess a high lipid percentage in their structure, specifically PUFA. Acute exposure to CCl_4 (<6.4 mg/m^3) can be able to cause tissue necrosis through

the generation of free radicals (trichloromethyl radical). Later molecule is able to bind with membrane lipid molecules directly and thus increases the rate of lipid peroxidation[3]. Rapid absorption of CCl_4 occurs through skin which reaches peak concentrations in <1–6 µg/ hours and is metabolized mainly by the liver[3]. Antioxidants are known to diminish several ROS depended cyto-toxicity. Several antioxidative compounds which include *ginkgo* sp, black tea extracts and melatonin have been reported to ameliorate CCl_4 -induced nephrotoxicity[4,5].

Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) are lipophilic, phenol derivative synthetic antioxidants used as a food preservative for the purpose of food products last longer. This synthetic antioxidant mostly found in several food products like cooking oils, fat-containing foods, pulses, cereals, processed rice, butter, snacks, baked goods, meat, chewing gum as well as some canned and packaged foods as a preservative[6,7]. These waxy solids are reported as synthetic analog of vitamin E as well as a chain-breaking antioxidant and is able to act as a terminating agent which suppresses auto-oxidation thus prevents food from becoming rancid[8]. BHA, BHT are reported as potent molecule responsible for lowering lipid peroxidation (LPO) activity and controlling oxidative stress (OS) in an experimental model[9]. Vitamin C (Vit C), also known as ascorbic acid or L- ascorbic acid an organic, hydrophilic, antioxidant as well as micronutrient which also acts as a food preservative[10]. The acidity of ascorbic acid protects food from becoming spoilt by neutralization of oxygen through inhibition in oxidation accelerating enzyme phenolase[11]. Vitamin E is a lipophilic, organic micronutrient consist of tocopherols and tocotrienols[12]. Vitamin E is reported as an efficient antioxidant which may involve protecting cell membrane and helps to maintain cellular integrity[13]. This chain breaking antioxidant had been using as a food preservative in a broad range of products such as food and beverages, pharmaceutical drugs, biological samples, cosmetics since many years and prevents products from becoming decomposed. This chemical preservative help to reduce the risk of foodborne infections in cheese, butter, mayonnaise, sunflower oil, wheat-gram oil, chips, wine, baked goods by preventing auto-oxidation and lipid-peroxyl radical formation[14]. These can naturally be found within broccoli, spinach, cauliflower, fish, oyster, grape seed, wheat-germ, almond, peanut, cashew nut, avocado etc[14]. These synthetic antioxidants as well as vitamins are classified under generally recognized as safe (GRAS) grade of food preservatives based on rat and mice model studies in National Cancer Institute[15].

This study had been performed to compare nephro-protective activity among synthetic and natural food preservatives against CCl_4 induced nephrotoxicity in Wistar strain male albino rats.

2. MATERIAL AND METHODS

2.1. Chemicals:

Carbon tetrachloride, Butylated hydroxianisole, Butylated hydroxytoluene, Vitamin C (Ascorbic acid), Vitamin E (α-tocopherol), Methanol, Alcohol, Chloroform, Sodium chloride (NaCl), Ethylene diamine tetra acetate (EDTA), Tris buffer, Triton-X 100, Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), Potassium hydroxide (KOH), Potassium dihydrogen phosphate (KH_2PO_4), Dipotassium hydrogen phosphate (K_2HPO_4), Sodium hydroxide (NaOH), were procured from Merck Ltd., SRL Pvt. Ltd., India. Standard reduced glutathione (GSH), 5′, 5′-dithio (bis)-2-nitrobenzoic acid (DTNB) were procured from Sigma (USA).

2.2. Animal care and Selection of animals:

All the experimental animal care was provided according to the guidelines for the Care and Use of Animals[16]. Thirty six (36) healthy Wistar strain male albino rats supplied by CPSEA, Govt. of India registered firm with 100 ±15 g body weight were taken for the study and were acclimatized in laboratory condition prior to the commencement of the experiment for two weeks. They were housed (three rats/cage) in a room with temperature of 22 ±2ºC, 12–12 h dark–light cycles, 50 ±10% humidity and water *ad libitum*.

2.2.1. Experimental Design:

To carry out the study, rats were distributed into six equal groups (n= 6/gr) as follows: Group I- control group: feed normal diet + water *ad libitum*.

Group II- CCl_4 treated group: normal diet and water *ad libitum* + subcutaneous injection of CCl_4 at a dose of 230 mg/kg body wt/rat/day diluted in Olive oil[17].

Group III- pre-treated group of BHA: BHA pre-treatment with a dose of 0.5 mg/kg with normal diet + subcutaneous injection of CCl_4 at a dose of 230 mg/kg body wt/rat/day diluted in olive oil[17,18].

Group IV- pre-treated group of BHT: BHT pre-treatment with a dose of 0.8 mg/kg with normal diet + subcutaneous injection of CCl_4 at a dose of 230 mg/kg body wt/rat/day diluted in olive oil[17,18].

Group V- pre-treated group of vitamin C: Vit. C pre-treatment with a dose of 100 mg/kg with normal diet + subcutaneous injection of CCl_4 at a dose of 230 mg/kg body wt/rat/day diluted in olive oil[17, 19].

Group VI- pre-treated group of vitamin E: Vit. E pre-treatment with a dose of 50 mg/kg with normal diet + subcutaneous injection of CCl_4 at a dose of 230 mg/kg body wt/rat/day diluted in Olive oil[17, 20].

2.3. Sacrifice of animals and collection of blood and tissues:

The experimental schedule was continued for 28 days and later the animals were sacrificed afterward blood as well as organ (kidney) was collected to perform different biochemical and histological studies. The tissues were stored into -80ºC prior to the preparation of tissue homogenates. For histological examination, kidney was preserved in 10% formaldehyde solution until further process[18].

a. Separation of serum and homogenization of liver and kidney:

Kidney (1.5 g) tissue was washed with 0.9% saline prior to prepare tissue homogenates. Then immediate homogenization in the ice-cold PBS buffer (pH 7.4) was done and after centrifugation (600×g, 10 min at 4ºC) the supernatant was stored (-80˚C) for further assessments.

Serum was separated from the collected blood by centrifugation (1500×*g*, 15 min) and was preserved (-80ºC) for further use[17].

b. Biochemical determinations:

i. Biochemical markers of nephrotoxicity:

Nephrotoxicity markers like serum urea[21], serum blood urea nitrogen[21], serum creatinine[22] concentration were measured by using assay kits Sigma (USA).

ii. Electrolyte profile:

Concentrations of major electrolytes such as sodium and potassium ion in serum were measured by Electrolyte analyzer.

iii. Oxidative stress profile:

Lipid peroxidation (LPO) level by estimation of MDA content in renal tissue homogenate was measured to evaluate the degree of intracellular damage. Renal tissue homogenate was mixed with 1.34% TBA (1.5 ml) and 20% TCA (1.5 ml) allowed to boiled for 30 minute and cooled after addition of 2.5 ml butanol. Later the mixture was centrifuged (5 min in 2000×*g)*andsupernatant was collected. The optical density of the supernatant was measured at 535 nm and calculated by using the molar extinction coefficient $1.43\times10^{-3}M^{-1}Cm^{-1}$ and expressed as nmol of MDA formed/mg protein [23].

iv. Antioxidant Enzyme Profile:

Activities of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx) were measure from renal tissue homogenate for estimation of intracellular antioxidant enzyme status.

SOD activity of renal homogenate was measured through its capability to inhibit the auto-oxidation of pyrogallol by the method of Mestro and McDonald 1986[24]. SOD activity was expressed as unit/mg protein as the reaction mixtures were measured at 420 nm at 25°C for 3 min.

CAT activity of renal tissue homogenate was assessed by the method of Luck, 1963[25], using the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$for H_2O_2 and the values were expressed as unit/mg protein.

The value of GSH estimated from renal tissue homogenate was by the modified method of Ellman (1959). The tissue homogenate was mixed with 25% of TCA and then after centrifugation (2,000×g, 15 min) the supernatant was diluted to 1 mL with the help of 0.2 M sodium phosphate buffer (pH 8.0) followed by an addition of 2 mL DTNB (0.6 mM) and incubated for 10 minutes at room temperature. The optical density of the yellow-colored complex was measured at 405 nm which was formed due to the reaction of GSH and DTNB (Ellman's reagent). The values of GSH were expressed as μg of GSH/mg protein[26].

The activity of GPx from renal tissue homogenate was evaluated by the method of Paglia and Valentine 1967[27] in which the absorbance of reaction mixtures were recorded at 340 nm for 5 min and values were expressed in nmol of NADPH oxidized to NADP per min/mg protein by using the extinction coefficient of $6.2\times 103M^{-1}cm^{-1}$.

c. Histological Study:

Histological analysis of liver tissue for every single experimental Group was performed by the method of Iranloye and Bolarinwa 2009[28]. Kidney tissues which were kept into formalin (10%) were proceeding for dehydration with ascending grade of alcohol (70%-100%). Then those tissues were kept in xylene overnight for the purpose of remove of alcohol and then followed by embedding block was prepared. Afterward histological sections were made with a thickness of 5 μm by using a microtome and then the sectioned tissues were placed on glass slides and stained with haematoxylin-eosin, also mounted with DPX medium. Prepared slides were then assessed for histopathological alterations under microscope (400X).

d. *Data Analysis:*

The data were calculated and statistical analyses were done by using a statistical package, Origin 6.1, Northampton, Mass, USA. The statistically calculated data were expressed as mean ± SEM, *n*= 6. Comparisons were done between the means of control and CCl_4 administered group, by one way ANOVA, *P<0.05*, level of significance.

3. RESULT

i. Biochemical markers of nephrotoxicity:

Fig.1 shows subcutaneous administration of CCl_4 caused a significant ($p<0.001$, $p<0.05$) increase in serum level of urea, blood urea nitrogen and creatinine 387.30%, 376% and 646.82% respectively in CCl_4 treated Group compared to control which was then ameliorated significantly ($p<0.001$, $p<0.05$) with the pre- treatment with BHA, BHT, Vit. C, Vit. E in serum level of urea (53.24%, 52.74 %, 50.02%, 52.74%), blood urea nitrogen (52.75%, 51.17%, 49.25%, 51.01%) and creatinine (58.01%, 57.11%, 57.28%, 57.42%) respectively compared to CCl_4 treated group.

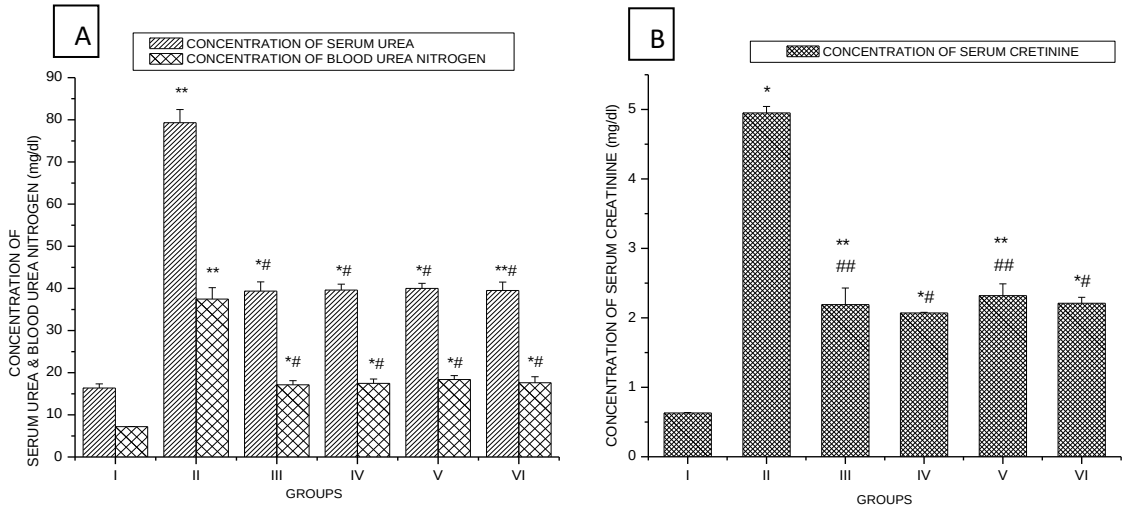

Fig. 1: Graphical representation of serum urea, blood urea nitrogen, and creatinine of different experimental groups. Values are expressed as mean±SEM, n=6. *,# and ** , ## indicates significant difference (P<0.001,P<0.05) compared to control Group and CCl4 treated group.

ii. Electrolyte profile:

Concentrations of major electrolytes such as sodium and potassium ion in serum were measured. CCl_4 induced nephrotoxicity showed significant ($p<0.001$, $p<0.05$) increase in serum sodium and potassium concentration by 48.14%, 59.47% compared to control which was significantly ($p<0.001$, $p<0.05$) reduced by the pre- treatment with BHA (19.79%, 19.637%), BHT (19.47%, 19.06%), Vit. C (19.36%, 19.59%), Vit. E (19.34%, 19.65%) respectively compared to CCl_4 treated group (Fig.2).

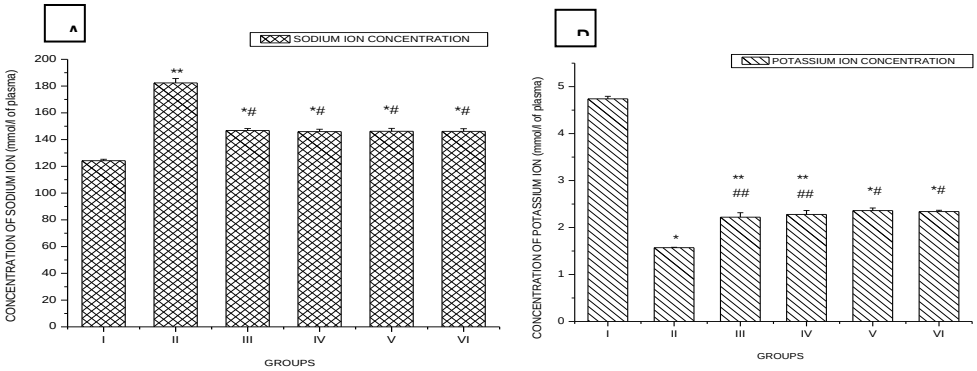

Fig. 2: Graphical representation of sodium and potassium ion concentration of different experimental groups. Values are expressed as mean±SEM, n=6. *,# and **,## indicates significant difference ($P<0.001$,$P<0.05$) compared to control Group

iii. Oxidative stress and Antioxidant enzyme profile:

In vivo production of malondyaldehyde (MDA) determines rate of lipid peroxidation. Intoxication of CCl_4 leads to an abrupt ($p<0.001$) elevation in tissue MDA level within kidney (360.54%) cells compared to control (Fig.3). However, pretreatment with BHA (18.54%), BHT (17.98%), Vit. C (17.65%) and Vit. E (18.35%) significantly ($p<0.001$, $p<0.05$) ameliorated high level of tissue MDA content in both liver and kidney cells compared to CCl_4 treated group. Subcutaneous intoxication of CCl_4 leads to decreased concentrations of intracellular antioxidant compounds (SOD, CAT, GPx, GSH)which helps to maintain normal cellular homeostasis. Concentrations of SOD, CAT, GPx, and GSHin renal tissue (81.03%, 72.24%, 50.34%, 63.04%) homogenate had been decreased significantly ($p<0.001$, $p<0.05$) due toCCl_4intoxication compared to control (Fig.3). Conversely, pretreatment with BHA, BHT, Vit C, Vit E significantly ($p<0.001$, $p<0.05$) recovered the level of intracellular antioxidant compounds like, SOD (231.65%, 233.01%, 230.14%, 230.03%), CAT (242.18%, 242.36%, 241.57%, 241.32%), GPx (35.17%, 35.62%, 34.98%, 36.01%), and GSH (62.67%, 62.21%, 60.66%, 62.23%) in renal cells compared to CCl_4 treated group.

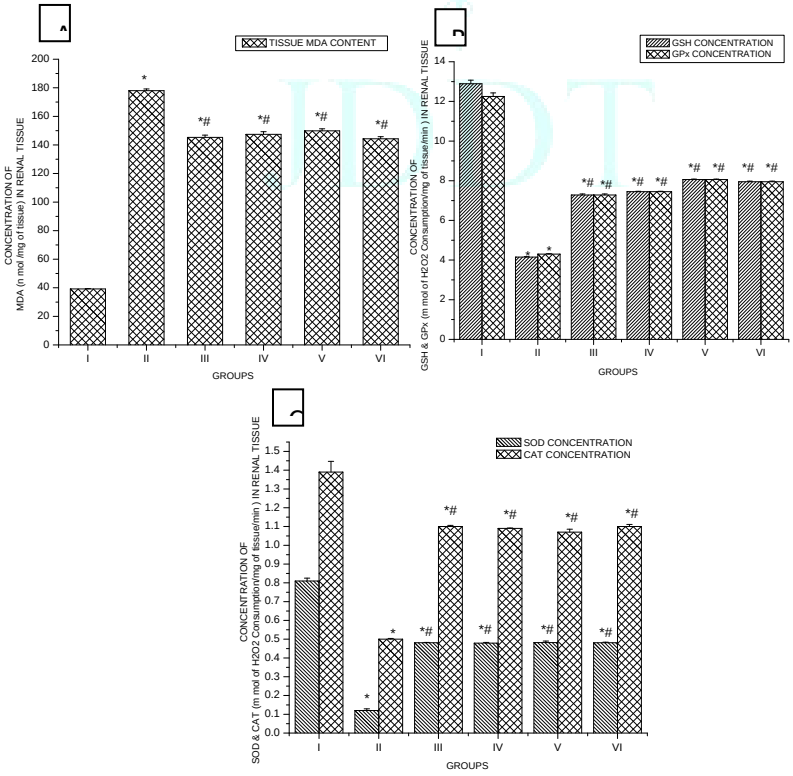

Fig. 3: Graphical representation of MDA, GSH, GPx, SOD, CAT values of different experimental groups. Values are expressed as mean±SEM, n=6. *,# and ** indicates significant difference ($P<0.001$,$P<0.05$) compared to control

iv. Histological evaluations:

The histological changes observed were ranged from none (control group) to severe (CCl4 treated group). Administration of food preservatives and vitamins to food formulations helped to ameliorate such severe damage (Fig.4; Table.1). The assessment of the structure of kidney sections of control rats showed normal architectural integrity. Administration of CCl_4 caused significant morphological damage to the renal cortex, affected glomeruli were observed with dilatation of Bowman's space and renal tubules. There was an evident infiltration of lipid molecules into the cell as a key indicator of nephro- fibrosis. Supplementation of selected antioxidants leads to no or less degenerative changes in renal glomeruli as well as less sign of accumulation of fat droplets, cellular necrosis and nephrotic vein disruption, compared to CCl_4 treated group.

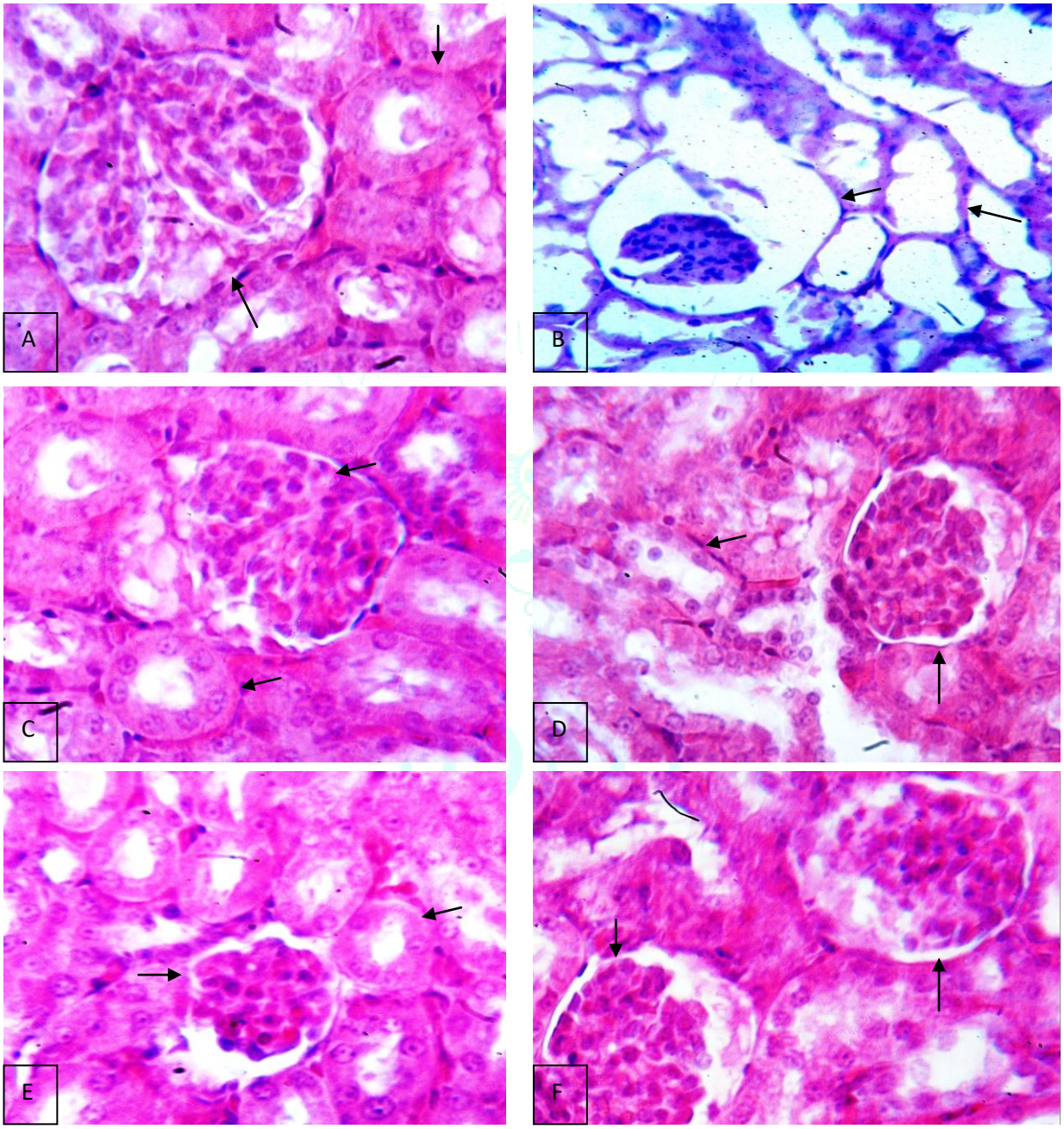

Fig.4: histological structure of kidney tissue Section A- Group I: Control, Section B- Group II: CCl_4 treated, Section C- Group III: CCl_4 + BHA, Section D- Group IV: CCl_4 + BHT, Section E- Group V: CCl_4 + Vit. C, Section F- Group VI: CCl_4 + Vit. E . (Duration- 28 Days)

Table.1. Histological changes in different experimental groups (The Ishak system (a six-point scale) was applied for scoring nephrotoxicity. Group I: Control, Group II: CCl_4 treated, Group III: CCl_4 + BHA, Group IV: CCl_4 + BHT, Group V: CCl_4+Vit C, Group VI: CCl_4+Vit E. (Duration- 28 Days).

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Lipid Accumulation	0	6	2	2	2	2
lipid infiltration	0	6	2	2	2	2
Degeneration of glomeruli	0	6	1	1	1	1
Renal cell degeneration	0	6	1	1	1	1
Cellular necrosis	0	6	1	1	1	1

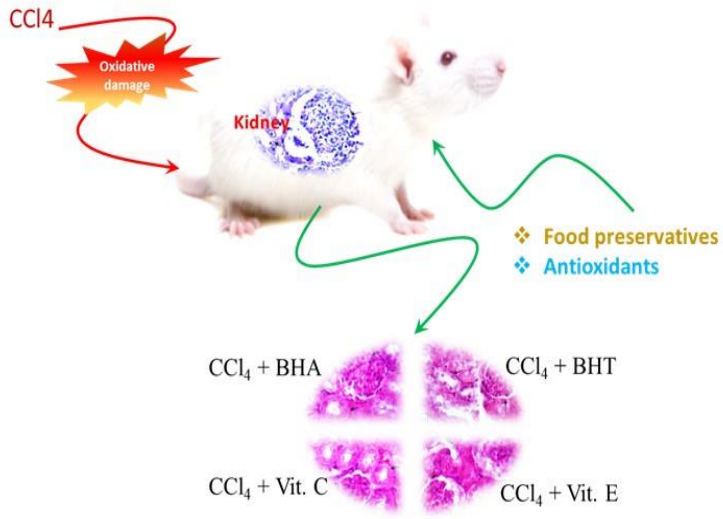

Fig. 5. Amelioration through food preservatives and vitamins in CCl4 induced

4. DISCUSSION

CCl_4 is a well known environmental pollutant specifically hepatotoxin possesses the ability to induce nephrotoxicity through the generation of huge amount of reactive oxygen species. The generation of reactive oxygen species is the prime mechanism by to induce nephrotoxicity which can be contributed by any xenobiotic component from nature. In this study, we hypothesized that proposed food preservatives would effectively protect kidneys by its antioxidant properties against CCl_4 -induced injury. To the best of our knowledge, this is the first study to evaluate the comparative antioxidative potential of food preservative against CCl_4 induced nephrotoxicity. CCl_4 lowers intracellular partial pressure of oxygen which favors reductive dehalogination of the said and produces trichloromethyl radicals ($\cdot CCl_3$) with the help of nicotinamide adenine dinucleotide phosphate (NADPH) dependent CYP450 enzyme. trichloromethyl radical in presence of oxygen converts into more toxic compound i.e. trichloro methyl peroxyl radical (Cl_3COO-) in mesangial cells of kidney[29]. Such alteration in intracellular redox status leads to development of renal ascites with increased abdominal size together with congestion, edema, hypertension and acidosis, and eventually reaches to a state of total sodium and water excess[30]. These types of toxic effects may be prevented by supplementation of antioxidants. The efficacy of these food preservatives as antioxidant was determined by its degree of amelioration against altered intracellular redox status. The serum levels of urea, creatinine, blood urea nitrogen are often considered as reliable indices for the purpose of measurement of *in vivo* renal impairment. In this study, it was observed that the experimental group treated with CCl_4 showed a significant increase in serum levels of urea and creatinine following exposure to CCl_4. A significant reduction in the plasma levels of urea is due to the decreased levels of protein in rats treated with carbon tetrachloride. Creatinine is a product of protein metabolism which is excreted in the urethra by glomerular filtration in higher concentration in comparison with its level in the blood. Therefore it can be

considered as a sign of kidney dysfunction[31, 32]. This kind of altered renal function was re-established toward normal after pretreatment with BHA, BHT, Vit C and Vit E with food formulations.

Alterations in the concentration of electrolytes are important factors in disturbances in kidney function. An abrupt increase in the concentration of sodium ion (Na^+) and a decrease in potassium ion (K^+) concentration in CCl_4 treated group successfully established failure in the renal system in experimental rats[33]. Alteration in the concentration of Na^+ and K^+ may alter the trans-epithelial transport of solutes and water in the kidney. Although the treatment with antioxidants effectively reduced such alterations within a significant level.

Our earlier works had established strong free radical scavenging activity against the same dose of CCl_4 induced toxicity in hepatic system by inhibition of lipid peroxidation by pre-treatment with synthetic antioxidants like butylated hydroxyanisole and butylated hydroxytoluene[18]. CCl_4 induces membrane-bound lipid peroxidation (specifically PUFA) thus produces trichloromethyl radical ($CCl_3^{\cdot}$). Due to alteration in intracellular partial pressure of oxygen later converts to a more toxic radical trichloromethyl peroxyl radical ($CCl_3O_2^{\cdot}$) which leads to detrimental organ damage liver, Kidney, brain[33, 34, 35]. Production of increased intracellular malondialdehyde (MDA) actually evidenced CCl_4 induced hepatonephro cellular damage due to abrupt level of intracellular lipid peroxidation (LPO) which was later replenished by administration of food preservatives[36,9,17,18]. This free radical-induced intracellular damage may be replenished by compounds with anti-oxidative properties. Superoxide dismutase (SOD) and catalase (CAT) are important detoxifying enzymes, which protect against the free radical-mediated injury[36]. CCl_4 induced oxidative stress (OS) was responsible for the decreased concentration of these above said intracellular antioxidant enzymes renal cells due to high scavenging activity[37, 38]. These free radical driven changes were recovered and intracellular SOD, as well as CAT, was restored by supplementation of BHA, BHT, Vit C, Vit E[9,18]. Subcutaneous administration of CCl_4 was also responsible for the altered concentration of intracellular thiol (-SH) defense machinery. Increased LPO drove OS lead to a decreased level of reduced glutathione (GSH) as well as glutathione peroxidase (GPx) into the cell[17] and there was the successful restoration of these above said oxidative markers by supplementation of food preservative antioxidants[9,18].

Thus, it may conclude that supplementation of synthetic as well as vitamin food preservative antioxidants mixed with food formulations able to provide protection against nephrotoxicity induced by CCl_4.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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