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Hepatoprotective effect of methanolic extract of *Juniperus squamata* leaf against carbon tetrachloride induced hepatotoxicity in rats

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ABSTRACT

Liver diseases pose a major challenge to international public health. Hepatic damage is associated with distortion of metabolic functions of the liver. The present study was carried out to evaluate the hepatoprotective effect of the methanolic extract of Juniperus squamata in CCl_4-induced hepatotoxicity in albino rats. Pretreated rats were given methanolic extract at 50, 100, and 200 mg/kg dose prior to CCl_4 administration (1 mL/kg, 1:1 in olive oil). Rats pretreated with Juniperus squamata extract remarkably prevented the elevation of serum AST, ALT, LDH, and liver lipid peroxides in CCl_4-treated rats. Hepatic glutathione levels were significantly increased by the treatment with the extract in all the experimental groups. The extract at the tested doses also restored the levels of liver homogenate enzymes (glutathione peroxidase, glutathione reductase, superoxide dismutase, and glutathione-S-transferase) significantly. This study suggests that methanolic extract has a liver-protective effect against CCl_4-induced hepatotoxicity.

***Key Words*: Carbon Tetrachloride, Hepatotoxicity, Glutathione, Lipid Peroxide, Liver Homogenate.**

1.Introduction

Plants are vital sources of natural medicine. A number of modern drugs have been isolated from them. An increasing interest in the herbal remedies has been observed in several parts of the world. Many of the herbal remedies have been incorporated into orthodox medicinal plant practice [1]. Today in this modern world, even though synthetic drugs are readily available and highly effective in curing various diseases, there are people who still prefer using traditional folk medicines because of their less harmful effect [2].

Plants contain a wide range of secondary metabolites with potent antioxidant properties. Such metabolites have an immense potential in pharmaceutical and food sectors. Natural antioxidants are interesting green alternatives to artificial antioxidants mostly because of the safety concerns [3].

The liver disorders are one of the world problems. Despite its frequent occurrence, high morbidity and high mortality, its medical management is currently in adequate. Even though newly developed drugs have been used to treat chronic liver disorders, these drugs have often side effects [4]. Liver injury due to chemicals or infectious agents may lead to progressive liver fibrosis and ultimately cirrhosis and liver failure . However, no effective treatment that delays disease progression and complications has yet been found. Several recent studies suggest that traditional herbs and micronutrients such as carotenoids and selenium may be useful for this purpose [5]. Carbon tetrachloride is widely used for experimental induction of liver damage. The principle causes of carbon tetrachloride are induced hepatic damage in lipid peroxidation and decreased activities of antioxidant enzymes and generation of free radicals [6]. Various Reactive oxygen species, including superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) are generated as byproducts of normal metabolism. Cumulative oxidative damage leads to numerous diseases and disorders. The enhanced production of free radicals and oxidative stress can also be induced by a variety of factors such as radiation or exposure to heavy metals and xenobiotics [7].Carbon tetrachloride (CCl_4) intoxication in animals is an experimental model that mimics oxidative stress in many pathophysiological situations.CCl_4 intoxication in various studies has demonstrated that CCl_4 causes free radical generation in many tissues such as liver, kidney, heart, lung, brain and blood. The toxicity of CCl_4 probably depends on formation of the trichloromethyl radical ($CCl_3^{\bullet}$), which in the presence of oxygen interacts with it to form the more toxic trichloromethyl peroxyl radical ($CCl_3O_2^{\bullet}$) [8].Studies have shown that various herbal extracts could protect organs against CCl_4 induced oxidative stress by altering the levels of increased lipid peroxidation and enhancing the decreased activities of antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) as well as enhanced the decreased level of the reduced glutathione (GSH) . In the modern medicine, plants occupy a significant berth as raw materials for some important drug preparations medicinal plants have been used to treat for various diseases in all over the world [9].

2. Methods

2.1. Plant Material

J.squamata was collected from the higher altitudes of Gulmarg (Apharwat), Jammu and Kashmir state, India, in the months of September and October 2014,and identified by the Centre of Plant Taxonomy, Department of Botany, University of Kashmir.A reference specimen has been retained in the herbarium of the Department of Botany at the University of Kashmir under reference number 2211-KASH.

2.2. Extract Preparation

The entire plant material was dried in the shade. The dried material was ground into a powder using a grinder.The dried powder (200g) obtained was successively extracted with different solvents like petroleum ether,methanol, ethanol, ethyl acetate, and water for 48 hours using a Soxhlet (60–80°C) (Figure 1).The extract was then concentrated with the help of a rotary evaporator under reduced pressure and the solid extract was stored in the refrigerator for further use.

2.3. In vivo Antioxidant Studies Animals Animals

Wistar rats of either sex, weighing 90–100 g, housed in standard conditions of temperature, humidity and light were used. They were fed with standard rodent diet and water *ad libitum* .The study was approved by IAEC(Institutional Animal Ethics Committee) department of Pharmaceutical Sciences ,University of Kashmir under registration no. 801/03/CA/CPCEA.

2.4. Dosage and treatment

Animals were divided into six groups containing six animals each. The plant extract was administered at oral doses of 50, 100, and 200 mg/kg/day. The extract was suspended in normal saline such that the final volume of extract at each dose was 1 ml which was fed to mice by gavage.

Group I – Received olive oil vehicle only at 5 ml/kg body weight for 28 days.

Group II – Received CCl_4 only.

Group III – Were administered with Vitamin (50 mg/kg body weight) for 28 days.

Group IV – Received 50 mg/kg/day extract orally for 28 days.

Group V – Received 100 mg/kg/day extract orally for 28 days**.**

Group VI – Received 200 mg/kg/day extract orally for 28 days.

On the 26th day, animals from Groups II–VI were injected intraperitoneally (i.p.) with CCl_4 in olive oil vehicle at a dosage of 1 ml/kg body weight. The mice were sacrificed 48 hours after CCl_4 administration**.**

2.5. Blood collection

Before sacrificing the experimental animals blood was collected from retro-orbital plexus without the use of anticoagulant. The blood was allowed to stand for 10 minutes before being centrifuged at 5000g for 10 minutes to obtain serum for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

3. Estimation of enzymes and proteins

3.1. ALT and AST activity

ALT and AST were estimated following the method of Kyode et al [10].Briefly 0.5ml of substrate (2mM α-ketoglutarate, 0.2M DL – alanine in phosphate buffer 0.1M pH 7.4) was incubated at 37°C for 5 minutes. 0.1 ml of freshly prepared serum was added to the aliquot and again incubated at 37°C for 30 minutes. At the end of incubation 0.5ml of 2, 4-dinitrophenylhydrazine was added and the aliquot was left for 30 minutes at room

temperature. 0.5ml of 0.4N NaOH was added and the aliquot was again left for 30 minutes. Absorbance was recorded at 505nm against water as the blank.

3.2. Preparation of liver homogenate

Liver tissue was washed in ice-cold 1.15% KCl and homogenized in a homogenizing buffer (50 mM Tris-HCl, 1.15% KCl pH 7.4) using a Teflon homogenizer. The homogenate was centrifuged at 9000g for 20 minutes to remove debris. The supernatant was further centrifuged at 15000 g for 20 minutes at 4°C to get PMS.

3.3. Protein estimation

Total protein content was estimated by the method of (Lowry *et al*., 1951)[11].Briefly 0.1ml of the tissue sample (10% w/v) was diluted to 1.0ml with distilled water and then the protein was precipitated. The samples were kept overnight at 4°C, centrifuged at 800xg for 15 min. The supernatant was decanted and discarded and the pellet was dissolved in 5ml of 1N NaOH by vortexing. Finally 0.1ml of the diluted aliquot was taken and further diluted to 1ml with distilled water before adding 2.5ml of alkaline $CuSO_4$ reagent. The alkaline $CuSO_4$ reagent was prepared by mixing Na_2CO_3 (4% w/v in 0.1N NaOH) and Na- K tartarate (2% w/v in distilled water) kept for 10 min to allow complex formation and then 0.25ml of Folin's reagent (2N, diluted to 1N with distilled water) was added. Exactly after 30min the absorbance of the blue colour was recorded at 680 nm for standard BSA (bovine serum albumin, 1mg/ml).

3.4.Determination of total sulphydryl groups

The acid soluble sulphydryl groups (non protein thiols of which more than 93% is reduced glutathione (GSH) forms a yellow colored complex with DTNB that shows the absorption maximum at 412nm. The assay procedure was followed that of (Moren *et al.,* 1979) [12]. 500µl of homogenate precipitated with 100µl of 25% TCA, was subjected to centrifugation at 300xg for 10 minutes to settle the precipitate. 100µl of the supernatant obtained was added to the test tube containing the 2ml of 0.6mM DTNB and 0.9ml of 0.2mM sodium phosphate buffer (pH 7.4). The yellow color obtained was measured at 412nm against the reagent blank which contains 100µl of 25% TCA in place of the supernatant.

3.5.Lipid peroxidation assay

Lipid peroxidation in liver tissue homogenate was estimated by the formation of thiobarbituric acid reactive species (TBARS) by the method of (Nichans and Samuelson, 1968)[13]. In brief 0.1ml of tissue homogenate (PMS; Tris- HCl buffer, Ph 7.5) was treated with 2ml of (1:1:1 ratio) TBA-TCA-HCl reagent (0.37% thiobarbituric acid, 0.25N HCl, and 15% TCA), placed in boiling water bath for 15 min cooled and centrifuged at room temperature for 10min. The absorbance of the clear supernatant was measured against reference blank at 535nm.

3.6.Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assayed using the method of (Sharma *et al*., 2001)[14].The assay mixture consists of 1.49ml of sodium phosphate buffer (0.1M p.H 7.4), 0.1ml EDTA (1mM), 0.1ml sodium azide (1mM), 0.1ml 1mM GSH, 0.1ml of NADPH (0.02mM), 0.01ml of 1mM H_2O_2 and 0.1ml PMS in a total volume of 2ml.Oxidation of NADPH was recorded spectrophotometrically at 340nm and the enzyme activity was calculated a nmoles of NADPH oxidized/minute/mg of protein, using extinction coefficient of 6.22 × 103/M/cm.

3.7.Glutathione reductase activity

Glutathione reductase (GR) was assayed by the method of (Sharma *et al*., 2001).The assay mixture consisted of 1.6ml of sodium phosphate buffer (0.1 M p.H 7.4), 0.1ml EDTA (1mM), 0.1ml 1mM oxidized glutathione, 0.1ml of NADPH (0.02mM), 0.01ml of 1mM H_2O_2 and 0.1ml PMS in a total volume of 2ml.The enzyme activity measured as absorbance at 340 nm was calculated as nmoles of NADPH oxidized/minute/mg of protein using extinction coefficient of 6.22 × 103/M/cm.

3.8.Glutathione-S-transferase activity

Glutathione-S-transferase (GST) was assayed using the method of (Haque *et al*., 2003)[15].The reaction mixture consisted of 1.67ml sodium phosphate buffer (0.1 M pH 6.5), 0.2ml of 1mM GSH, 0.025ml of 1mM CDNB and 0.1ml of PMS in a total volume of 2ml. The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nmoles of CDNB conjugates formed/min/mg protein using extinction coefficient of 9.6 × 103/M/cm**.**

3.9.Superoxide dismutase activity

Superoxide dismutase (SOD) activity was estimated by the method of (Beauchamp and Fridovich, 1971)[16]. The reaction mixture consisted of 0.5ml of hepatic PMS, 1ml of 50 mM sodium carbonate, 0.4 ml of 25µM NBT and 0.2ml of 0.1mM EDTA. The reaction was initiated by addition of 0.4ml of 1mM hydroxylamine hydrochloride. The change in absorbance was recorded at 560nm. The control was simultaneously run without tissue homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT (Nitroblue tetrazolium) by 50%.

4.Statistical analysis

The values are expressed as mean ± standard deviation (SD). The results were evaluated by using the SPSS (version 12.0) and Origin 6 softwares and evaluated by one-way ANOVA.. Statistical significance was considered when value of *P* was < 0.5.

5. Results

5.1. Effect of methanolic of *J.squamata* extract on lipid peroxidation in CCl_4 treated rats

After CCl_4 administration, the MDA levels increased significantly from 0.31 to 3.8 nmol/mg protein in liver tissue homogenate. However pretreatment of extract of *J.squamata* for 28 days decreased the MDA level in a dose dependent manner .Vitamin E treated animals also showed significant decrease in the MDA levels as compared to CCl_4 treated animals. TBARS concentrations (expressed as MDA)

In order to investigate whether the antioxidant activities of *J.squamata* are mediated by an increase in antioxidant enzymes, we measured GPx, GR, SOD and GST activities in liver tissue of rats treated with *J.squamata* extract. In the present study, treatment of rats with *J.squamata* extract significantly increased SOD, GP_X, GR and GST activities in rat liver.

5.2.Effect on (GP_X activity)

Pretreatment with methanolic extract significantly increased the GP_X activity in a dose dependent manner. At higher concentrations of plant extract (200 mg/kg dose level), the activity was increased to 24 24±1.80 from CCl_4 treated group (13±0.09) in liver of rat.Vitamin E (50 mg/kg) treated animals also showed significant increase in GP_X activity in both the tested organs as shown in Table 1.

5.3. Effect on GR activity

Glutathione reductase (GR) activity was significantly decreased in CCl_4 treated animals when compared to control group. There was a significant increase in glutathione reductase activity observed in methanolic extract treated groups in liver. At the higher concentration of plant extract the activity increased many fold .Similar results were obtained with vitamin E as shown in Table 1.

5.4. Effect on SOD activity

Administration of extract proved significantly better in restoring the altered activity of antioxidant enzyme like SOD, and increased the activity in a dose dependent manner in liver. Similar results were observed in vitamin E treated group(Table 1). The SOD activity significantly decreased in CCl_4 treated group.

5.5. Effect on GSH level

CCl_4 administration markedly decreased the levels of reduced glutathione in liver demonstrating oxidative stress. Pretreatment with the *J.squamata* significantly ameliorated CCl_4-induced depletion of GSH in a dose dependent manner (Table 1)

5.6. Effect on GST activity

GST activity as measured from liver tissue homogenate of all the experimental animals have been shown in table . In homogenate decreased GST activity was observed in CCl_4 treated animals compared to the normal control group as shown in Table 1.Pretreatment with the methanolic extract for 15 days prior to CCl_4 intoxication enhanced that activity significantly in a dose dependent manner.GST activity in vitamin E and 200 mg/kg bw plant extract pretreated group was close to the normal level in lung tissue

Table 1.Effect of methanolic extract of JS on glutathione and antioxidant enzymes in CCl_4 challenged rats

Parameters	GroupI(Olive oil only)	Group II(CCl_4 treated group)	Group III CCl_4 +Vitamin C (50mg/kg)	Group IV CCl_4 treated +Extract(100mg/kg	GroupV CCl_4+ extract(200mg/kg)
Reduced Glutathione (nm/g protein	103±1.09	25±1.39	87±1.89	60±0.94	78±0.65
Glutathione Reductase (ug GSSG utilised/min/mg protein)	42±1.09	18±0.87	39±0.77	28±0.75	35±0.54
Glutathione peroxidase(ug GSSG utilised/min/mg protein)	35±1.09	13±0.09	28±0.45	20±0.79	24±1.80
Superoxide dismutase(units/mg protein)	33±1.09	11±0.99	27±0.29	19±1.07	23±0.65
Glutathione –S-transferase(nmoles of CDNB conjugated/min/mg protein)	38±1.09	10±0.79	29±0.17	21±0.89	23±0.87

Values are mean±SD, n=6 in each group. *P<0.05 when compared with respective control group (Dunnett's test).

6. Discussion

Liver plays a major role in the detoxification and excretion of many endogenous and exogenous compounds and any injury or impairment of its function may lead to several complications. Management of liver diseases is still a major challenge to modern medicine. Conventional drugs used in the treatment of liver diseases are often inadequate. It is therefore necessary to search for alternative drugs for the treatment of liver diseases that could replace the existing drug.[17].

CCl_4 when administrated is distributed and deposited to organs such as the liver, brain, kidney, lung and heart [18]. The reactive metabolite trichloromethyl radical ($^{\bullet}$CCl3) and trichloromethyl peroxide radical ($CCl_3O_2\bullet$) has been formed from the metabolic conversion of CCl_4 by cytochrome P-450. As O_2 tension rises, a greater fraction of $^{\bullet}CCl_3$present in the system reacts very rapidly with O_2 and more reactive free radicals, like $CCl_3OO^{\bullet}$ is generated from $^{\bullet}CCl_3$. These free radicals initiate the peroxidation of membrane poly unsaturated fatty acids (PUFA), cell necrosis, GSH depletion, membrane damage and loss of antioxidant enzyme activity[19].

In this experimental study we investigated the protective effect of methanolic extract of *J.squamata* leaf. Free radicals e.g. superoxide radical, hydrogen peroxide and hydroxyl radical, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery disease, stroke, rheumatoid arthritis, diabetes and cancer [20]. High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals [21].

The level of MDA in CCl_4 treated group was significantly higher than the control group in liver. The increase in MDA level in both the tissues suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant mechanisms to prevent the production of excessive free radicals. Similar results were previously reported in kidney by Ogeturk [22], which stated that CCl_4 metabolized by cytochrome p-450 generates a highly reactive free radical, and initiates lipid peroxidation of the cell membrane of the endoplasmic reticulum and causes a chain reaction. These reactive oxygen species can cause oxidative damage in DNA, proteins and lipids. However pretreatment of *Juniperus squamata* extract in this study significantly prevent CCl_4-induced lipid peroxidation in kidney and lung tissue. GSH as we know is involved in several defense processes against oxidative damage protects cells against free radicals, peroxides and other toxic compounds [23]. Indeed, glutathione depletion increases the sensitivity of cells to various aggressions and also has several metabolic effects. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury In our study, the kidney and lung GSH level in CCl_4 treated group was significantly decreased compared with control group. Likewise Ohta et al reported a significant decrease in the GSH content in different organs of rats, when injected with CCl_4. Pretreatment however, with *J.squamata* extract increased GSH level as compared

with CCl_4 groups and thus affording protection. The antioxidant effects are likely to be mediated by the restoration of CCl_4 induced decreased SOD, GR, GPx and GST activities in various tissues of rats. Treatment of rats with *J.squamata* methanolic extract significantly increased SOD, GR, GST and GPx activities in liver. Tirkey [25] have recently conducted experiments to determine the effect of CCl_4 on the renal damages in rats and obtained similar results. All these enzymes are major free radical scavenging enzymes that have shown to be reduced in a number of pathophysiological processes and diseases such as diabetes [26]. Thus, activation of these enzymes by the administration of *J.squamata* extract clearly shows that *Juniperus* through its free radical scavenging activity could exert a beneficial action against pathophysiological alterations caused by the presence of superoxide, hydrogen peroxide and hydroxyl radicals.

7. Conclusion

The study suggests that the extract possess potential to protect liver against oxidative damages and could be used as an effective protector against CCl_4 induced kidney and lung damages. Further works are needed to fully characterize the active principles present in the plant responsible for these functions and elucidate its possible mode of action.

REFRENCES

1.Farnsworth,Akerele O, Bingel AS, Soejarto DD, and Guo ZG. Medicinal plants in therapy. *Bul. World Health Org,63(6),*1985,965-981.

2.Scalbert A, Johnson IT and Saltmarsh M. Polyphenols:Antioxidants and beyond. American. *J Clin Nut,81(1),*2005,215-217.

3.Wong SP, Leong LP and Koh JHW. Antioxidant activities of aqueous extracts of selected plants. *Food Chem,99(4),*775-83.

4.Kim HJ, Odendhal S, Bruckner JV. Effect of oral dosing vehicles on the acute hepatotoxicity of carbon tetrachloride in rats. *Toxicol Appl Pharmacol , 102(1),* 1995,34-49.

5. Mc Gregor D and Lang M. Carbon tetrachloride: genetic effects and othermodes of action. *Mutat Res , 366(2),*1996,181-195.

6. Dashti H, Jeppsson B, Hagerstrand I, Hultberg B and Srinivas U.Thioacetamide and carbon tetrachloride-induced liver cirrhosis. *Eur Surg Res 21(1),*1982,83-91.

7. Behar-Cohen FF, Heydolph S, Faure V and Droy-Lefaix MT.Peroxynitrite cytotoxicity on bovine retinal pigmented epithelial cells in culture. *Biochem Biophys Res Commun , 226(3),*1996,842-849.

8. Rajesh MG and Latha MS.Protective activity of Glycyrrhiza glabra Linn on Carbon tetrachloride-induced peroxidative damage. *Indian J Pharmacol ,36(1),*2004,284-287.

9.Halliwell B, Gutteridge JMC and Cross CE. Free radicals, antioxidants and human disease: where are we now? *Journal of Laboratory and Clinical Medicine, 119(1),*1992,598-620.

10.Kayode AA, Kayode OT, Aroyeun OA anf Stephen MC. Hematologic and Hepatic Enzyme Alterations Associated with Acute Administration of Antiretroviral Drugs, *Journal of Pharmacology and Toxicology, 6(3),*2006,293-302

11. Lowry OH, Rosenbrough NJ, Farr AI, and Randall RJ. Protein estimation with the Folin phenol reagent. *J Biol Chem, 193(2),*1951,265-275.

12. Moren MA, Depierre JW and Mannervick B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochem Biophys Acta , 582(1*),1979,67-78

13. Nichans WG and Samuelson D. Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur J Biochem , 691(3),*1968,126-130.

14. Sharma N, Trikha P, Athar M, and Raisuddin S. Inhibition of benzo[a]pyreneand cyclophosphamide-induced mutagenicity by cinnamomum cassia. *Mutat Res , 480 (2),*2001,179-188.

15. Haque R, Bin-Hafeez B, Parvez S, Pandey S, Sayeed I and Ali M, Raisuddin S. Aqueous extract of walnut (Juglans regia L.) protects mice against cyclophosphamide induced biochemical toxicity. *Hum Exp Toxicol , 22(3),*2003,473-480.

16. Beauchamp C and Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gel. *Anal Biochem , 44(2),*1971,276-287.

17.Pihan G, Regillo C and Szabo S.Free radicals and lipid peroxidation in ethanol aspirin induced gastric mucosa injury. *Dig. Dis. Sci, 32 (12),* 1978, 1395-1401.

18.Recknage R.O, Glender E.A and Walter R.L.Mechanism of Carbon tetrachloride toxicity. *Pharmal ther, 43(3),*1981,139-154.

19.Tamayo R.P.Is Cirrhosis of the Liver Experimentally Produced by CC14 an Adequate Model of Human Cirrhosis?.*Hepatology, 3 (1)*1983, 112-120.

20.Wiseman H and Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression *to cancer Biochem. 31(3),* 1996, 17-29.

21.Zhao R, Xiang Z.J, Ye T.X, Yaun J.Y and Guo X. Z.Antioxidant activities of Salvia miltiorrhiza and Panax notoginseng. *Food Chemistry, 99(1),*1996 767-774.

22.Ogeturk M, Kus I, Colakoglu N, Zararsiz I, Ilhan N and Sarsilmaz M.Caffeic acid phenethyl ester protects kidneys against carbon tetrachloride toxicity in rats. *J Ethnopharmacol ,97(1),*2005,273-280.

23. Sies H. Glutathione and its role in cellular functions. *Free Radic Biol Med ,27(3),*1991,916-921.

24. Ohta Y, Kongo M and Sasaki E.Therapeutic effect of melatonin on carbon tetrachloride-induced acute liver injury in rats. *J Pineal Res , 28(1),*2000,119-26.

25. Tirkey P, Pilkwal S, Kuhad A and Chopra K. Hesperidin, a citrus bioflavonoid, decrease the oxidative stress produced by CCl_4 in rat liver and kidney.*BMC Pharmacol, 5(2),*2005,193-198.

26.Cohen G and Heikkila RE. The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. *J Biol Chem 249(2),*1974,2447-2452.