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Antioxidant Activities of Methanolic Rhizome Extract of *Podophyllum Hexandrum* Against CCl_4-Induced Kidney and Lung Injury in Rats

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Abstract: The aim of the study was to evaluate the effect of methanolic rhizome extract of *Podophyllum hexandrum* treatment on the amelioration of CCl_4 induced oxidative stress in albino rats. Exposure to CCl_4 is known to induce acute and chronic kidney and lung injuries. The antioxidant capacity of *Podophyllum hexandrum* in the CCl_4 treated rat kidney and lung tissues was monitored by assaying the activities of different antioxidant enzymes viz., Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione reductase (GR) and Glutathione S-transferase (GST). Also the effect of *Podophyllum hexandrum* on the levels of thiobarbituric acid reactive species (TBARS), reduced-glutathione (GSH) was monitored. It was found that methanolic extract of *Podophyllum hexandrum* could ameliorate the lung and kidney damage in these rats as was evident by the antioxidant status of the above mentioned parameters. Thus *Podophyllum hexandrum* has potential anti-oxidant effects, against the free radical mediated damages.

Key words: Antioxidant, CCl_4, *Podophyllum hexandrum*, SOD, GSH

INTRODUCTION

Exposure to various compounds including a number of environmental pollutants and drugs can cause cellular damages through metabolic activation of those compounds to a highly Reactive Oxygen Species (ROS). Free radical induced lipid peroxidation is believed to be one of the major causes of cell membrane damage leading to a number of pathological situations (Slater, 1984). CCl_4 was formerly used for metal degreasing and as dry-cleaning, fabric-spotting and fire extinguisher fluids, grain fumigant and reaction medium. Because of its harmful effects, these uses are now banned and it is only used in some industrial applications (De Shon, 1979). The primary routes of potential human exposure to CCl_4 are inhalation, ingestion and dermal contact. High exposure to CCl_4 can cause liver, kidney, lung and central nervous system damage and liver is especially sensitive to CCl_4 because of its role as the body's principal site of metabolism (Sakata *et al*., 1987). A number of reports clearly demonstrated that in addition to hepatic toxicity, CCl_4 also causes disorders in kidneys and lungs by generating free radicals (Ahamad *et al*., 1987; Ozturk *et al*., 2003). Findings by Perez *et al*. (1987), Ogeturk *et al*. (2005) and Churchill *et al*. (1983) suggested that exposure to this solvent causes acute and chronic kidney and lung injuries. In addition, report on various documented case studies established that CCl_4 produces renal diseases in human (Ruprah *et al*., 1985; Gossellin *et al*., 1984). A number of endogenous and exogenous

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kidney and lung risk factors generate oxygen free radicals *in vivo*. Therefore, the role of oxygen-derived free radicals and lipid peroxidation has attracted considerable attention (Gebhardt, 2002; Das *et al*., 2005). CCl_4 results in enhanced generation of trichloromethyl peroxyl radical (Cl_3COO-), hydrogen peroxide in cultured hepatocytes as well as mesangial cells (Knight *et al*., 1989). *In vitro* and *in vivo* studies indicate that CCl_4 enhances lipid peroxidation, reduces kidney, lung and liver microsomal NADPH cytochrome P_{450} and reduced/oxidized glutathione ratio (GSH/GSSG) (Rungby and Ernst, 1992). Antioxidants such as ascorbate, α-tocopherol and superoxide dismutase/catalase have been shown to ameliorate CCl_4-induced kidney and lung toxicity (Miller and Rice, 1997). Mammalian tissues are equipped with both enzymic and non-enzymic antioxidants defenses with different efficacies that protect animals against oxidative abuse caused by wide range of toxicants including CCl_4 (Karbownik *et al*., 2001).

Therefore, the present study was designed to (1) evaluate beneficial effect of methanolic rhizome extract of *Podophyllum hexandrum* in preventing acute kidney and lung damages induced by CCl_4 in rats and (2) analyze degree of kidney and lung injury via antioxidative mechanisms.

Podophyllum hexandrum belongs to family Berberidaceae and in Kashmir valley, it is locally known as Banwangon. The rhizome powder is used as a laxative or to get rid of intestinal worms and also used as poultice to treat warts and tumorous growths on the skin. Recent studies have also shown its effectiveness in the treatment of monocytoid leukemia, Hogkins disease and non-Hodgkins lymphoma brain tumors, bladder cancer, lung cancer and AIDS associated Karposis Sarcoma.

MATERIALS AND METHODS

Plant Material Collection and Extraction

The rhizome of *Podophyllum hexandrum* was collected from higher reaches of Aharbal, JandK, India in the month of May-June, 2009 and were identified by the centre of Plant Taxonomy, Department of Botany, University of Kashmir and authenticated by a Botanist Dr. Irshad Ahmad Nawchoo (Associate Professor, Department of Botany) and Akter Hussain Malik (Curator, Centre for Plant Taxonomy, University of Kashmir). The voucher specimen has been retained in the Herbarium of Taxonomy, Department of Botany University of Kashmir for future reference under number (KASH-bot/Ku/PH-702-SAG).

The authentically identified plant material (rhizome) was shade dried under room temperature at 30±2°C. The dried rhizome material was grind into powder using mortar and pestle and sieved with a sieve of 0.3 mm aperture size. The powder obtained was successively extracted in hexane, ethyl acetate, absolute ethanol, 70% ethanol and methanol, by using Soxhlet extractor (60-80°C). The methanol extract was then concentrated with the help of rotary evaporator under reduced pressure and the solid extract was stored in refrigerator for further use.

Animals

Adult male albino rats of Wister strain weighing 200-250 g used for this study were purchased from the Indian Institute of Integrative and Medicine Jammu (IIIM). The animals were fed on a pellet diet (Hindustan Lever, Ltd., Mumbai, India) and were given access to water ad libitum. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating light-and-dark cycle. The animals used in the present study were maintained in accordance with the guidelines prescribed by

the National Institute of Nutrition, Indian Council of Medical Research and the study was approved by the Ethical Committee of the Kashmir University.

Dosage and Treatment

Rats were divided into six groups containing six rats each. The plant extract was employed at oral doses of 20, 30 and 50 mg kg^{-1} b.wt. day^{-1}. The extract was suspended in normal saline such that the final volume of extract at each dose was 1 mL and was fed to rats with gavage.

Group 1: Served as normal control and received olive oil only (vehicle) 5.0 mL kg^{-1} b.wt.
Group 2: Served as negative control and received CCl_4 1.0 mL kg^{-1} b.wt. (suspended in olive oil)
Group 3: Animals were administrated with vitamin E (α-tocopherol) 50 mg kg^{-1} b.wt. suspended in olive oil
Group 4: Animals received 20 mg kg^{-1} b.wt. of Podophyllum extract orally for all fifteen days
Group 5: Animals received 30 mg kg^{-1} b.wt. plant extract orally for all fifteen days
Group 6: Animals received 50 mg kg^{-1} b.wt. plant extract orally for all fifteen days

On the thirteenth day, animals of the groups 2-6 were injected with CCl_4 at the dose of (1 mL kg^{-1} b.wt.) intraperitonially. After 48 h of CCl_4 administration, the rats were sacrificed and the organs (kidney and lungs) isolated and PMS prepared.

Lung and kidney tissues isolated from sacrificed animals were washed in ice cold 1.15% KCl and homogenized in a homogenizing buffer (50 mM Tris-HCl, 1.15% KCl pH 7.4) using Teflon homogenizer. The homogenate was centrifuged at 9,000 g for 20 min to remove debris. The supernatant so obtained was further centrifuged at 15,000 rpm for 20 min at 4°C to get Post Mitochondrial Supernatant (PMS). This PMS is used for the estimation of lipid peroxidation by the method of Niehaus and Samuelson (1968), glutathione reduced (GSH) by Moron *et al.* (1979), Glutathione Reductase (GR) by Sharma *et al.* (2001), glutathione peroxidase (GPx) by Sharma *et al.* (2001), glutathione-S-transferase (GST) by Haque *et al.* (2003), Catalase (CAT) by Clairborne (1985) and superoxide dismutase (SOD) by Beauchamp and Fridovich (1971) and protein estimation by the method of Lowry *et al.* (1951). All the chemicals used during the experimentation were purchased from Sigma-Aldrich, New-Delhi, India.

Statistical Analysis

The values are expressed as Mean±SD. The results were computed statistically by using SPSS version 12.0 and graphpad prism 5 softwares and evaluated by one-way ANOVA followed by Dunnett's test. $P<0.001$, $p<0.01$ and $p<0.5$ was considered significant.

RESULTS

Protein Levels

A significant decrease in protein levels were noted in group II rat tissue homogenates after a single dose of CCl_4 administration. Pretreatment with oral doses of *Podophyllum hexandrum* methanolic extract increased the protein levels in a dose dependent manner in kidney tissue (Table 1); similar type of response was also seen in lung tissue (Table 2).

Table 1: Effect of methanolic extract of *Podophyllum hexandrum* on antioxidant enzymes and protein levels of kidney tissue in CCl_4 treated rats

Parameters	Group I (olive oil only)	Group II CCl_4 treated	Group III CCl_4+V.E	Group IV 20 mg kg^{-1} extract	Group V 30 mg kg^{-1} extract	Group VI 50 mg kg^{-1} extract
Protein (mg/100 mg tissue)	109.289±1.019	20.576±0.204	92.2400±1.041	29.576±1.17	62.439±0.885	85.145±1.715
Reduced glutathione (nm g^{-1} protein)	58.390±2.25^a	10.520±0.78^b	43.8600±1.79^{ab}	19.280±1.45^{ab}	28.480±1.40^{a,b}	35.530±1.99^{ab}
Glutathione reductase (µg GSSG utilized/minute/mg protein)	73.313±1.987^a	11.535±0.670^b	54.7530±1.808^{ab}	16.529±0.551^{a,b}	39.750±0.707^{ab}	50.715±0.414^{ab}
Glutathione peroxidase (µg GSH utilized/minute/mg protein)	72.198±2.654^a	11.112±0.128^b	51.4970±0.887^{ab}	16.369±0.265^{a,b}	39.770±0.666^{ab}	49.903±1.668^{ab}
Superoxide dismutase (units mg^{-1} protein)	34.946±1.392^a	5.576±0.328^b	33.2930±0.616*^{b}	18.743±1.199^{a,b}	24.460±0.945^{ab}	26.600±0.638^{ab}
Catalase activity (nm of H_2O_2 decomposed/min/mg protein)	3709.150±105.26^a	556.270±10.55^b	2838.170±12.74^{a,b}	778.81±25.56^{a,b}	2124.620±0.47^{a,b}	2536.810±54.46^{a,b}
Glutathione- S-transferase (nmoles of CDNB conjugated/min/mg protein)	40.138±1.268^a	19.003±0.419^b	30.228±2.745^{a,b}	20.404±2.798^{a,b}	24.687±1.220^{a,b}	25.261±2.072^{a,b}

Induced group and *: p<0.05 as compared with normal group. The data were presented as Mean±SD for six animals in each observation and evaluated by one-way ANOVA followed by Dunnett's test to detect inter group differences. Differences were considered to be statistically significant if p<0.05

Table 2: Effect of methanolic extract of *Podophyllum hexandrum* on antioxidant enzymes and protein levels of lung tissue in CCl_4 treated rats

Parameters	Group I (olive oil only)	Group II CCl_4 treated	Group III CCl_4+V.E	Group IV 20 mg kg^{-1} extract	Group V 30 mg kg^{-1} extract	Group VI 50 mg kg^{-1} extract
Protein (mg/100 mg tissue)	40.055±.964	3.752±0.927	30.636±0.924	9.139±0.584	17.839±0.736	22.30±0.443
Reduced glutathione (nm g^{-1} protein)	34.780±1.93^a	7.310±0.33^b	27.310±1.47^{a,b}	10.200±0.80^{a,b}	14.090±1.02^{a,b}	19.84±1.15^{a,b}
Glutathione reductase (µg GSSG utilized/minute/mg protein)	23.260±0.948^a	3.372±0.357^b	14.576±1.125^{a,b}	4.858±0.534^{a,b}	6.713±0.901^{a,b}	15.665±0.819^{a,b}
Glutathione peroxidase (µg GSH utilized/minute/mg protein)	19.076±1.048^a	3.234±0.359^b	11.845±0.608^{a,b}	5.446±0.491^{a,b}	7.718±1.035^{a,b}	12.479±1.429^{a,b}
Superoxide dismutase (units mg^{-1} protein)	33.259±0.602^a	4.393±0.981^b	22.445±1.392^{a,b}	10.732±0.611^{a,b}	11.241±0.587^{a,b}	15.567±0.700^{a,b}
Catalase activity (nm of H_2O_2 decomposed/min/mg protein)	179.930±30.09^a	35.470±6.89^b	116.120±22.86^{a,b}	40.730±12.08^{a,b}	67.890±11.18^{a,b}	86.070±14.72^{a,b}
Glutathione- S- transferase (nmoles of CDNB conjugated/min/mg protein)	10.114±0.413^a	5.734±0.469	9.488±0.491*^{b}	6.915±0.477^{a,b}	7.431±0.378^{a,b}	9.071±0.280^{a,b}

a: p<0.001 as compared with CCl_4 induced group, b: p<0.001 as compared with normal group, d: NS as compared with CCl_4 induced group, f: p<0.01 as compared with CCl_4 induced group, g: p<0.05 as compared with CCl_4 induced group and *: p<0.05 as compared with normal group. The data were presented as Mean±SD for six animals in each observation and evaluated by one-way ANOVA followed by Dunnett's test to detect inter group differences. Differences were considered to be statistically significant if p<0.05

Reduced Glutathione Levels

The effect of Podophyllum extract on the levels of reduced glutathione in kidney tissue homogenate is shown in Table 1. A significant (p<0.001) decrease in the GSH was observed in the group II animals treated with CCl_4 (10.52±0.78 nmoles g^{-1} protein) for kidney as compared to the normal control group (58.39±2.25). Administration of methanolic extract of Podophyllum hexandrum for 15 consecutive days afforded a dose dependent protection against such depletion of GSH level. The effect of Podophyllum extract on the levels of reduced glutathione in lung tissue homogenate is shown in Table 2. A significant (p<0.001) decrease in the GSH was observed in the group II animals treated with CCl_4 (7.31±0.33 nmoles g^{-1} protein) for lung tissue, as compared to the normal control group (34.78±1.93 nmoles g^{-1} protein). Administration of methanolic extract of Podophyllum hexandrum for 15 consecutive days afforded a dose dependent protection against such depletion of GSH level. Vitamin E a known antioxidant restores the GSH content to a still larger extent in both the tissue homogenates.

Superoxide Dismutase Levels

The activity of SOD in kidney tissue homogenate was significantly decreased in CCl_4 treated animals as compared with control. Oral administration of methanolic extract at a dosage of 20, 30 and 50 mg kg^{-1} b.wt., showed significant increase in SOD activity in kidney tissue (Table 1). Similarly, the activity of SOD in lung tissue homogenate was significantly decreased in CCl_4 treated animals as compared with control. Oral administration of methanolic extract at a dosage of 20, 30 and 50 mg kg^{-1} b.wt., showed significant increase in SOD

activity in lung tissue (Table 2). The extract is thus capable of restoration of SOD level towards normalizing in both tissues studied.

Catalase Levels

CAT activity in the kidney homogenates of rats under different experimental conditions is shown in Table 1. The CAT activity observed in the kidney tissue of CCl_4 treated rats was considerably lower (556.27±10.55 nmoles/min/mg protein) as compared to normal group (3709±105.26 nmoles/min/mg protein). In the pretreatment groups, receiving methanolic extract of *Podophyllum hexandrum* for 15 days prior to CCl_4, CAT activity was significantly increased in a dose dependent manner (Table 1).

The CAT activity in the lung tissue homogenate of CCl_4 treated rats was also lower than the normal group. Oral administration of methanolic extract of *Podophyllum hexandrum* at the concentration of 20, 30 and 50 mg kg^{-1} b.wt. again restores its activity (Table 2). Vitamin E as expected restored the Catalase activity largely in both the tested organs.

Glutathione-S-transferase Activity

The effect of oxidant and antioxidants on Glutathione-S-transferase (GST) enzyme activity in kidney tissue homogenates is shown in Table 1. The GST activity was decreased in CCl_4 treated groups compared to the normal group. Only about 19.003±0.419 nmoles/min/mg protein of GST were observed in CCl_4 treated groups, as compared to the value of 40.138 in the normal controls. Pretreatment with methanolic extract largely restored the GST activity in a dose dependent manner in the kidney tissues. The effect of oxidant and levels on Glutathione-S-transferase (GST) enzyme activity in lung tissue homogenates is shown in Table 2. The GST activity was decreased in CCl_4 treated groups compared to the normal group. Only about 5.734±0.469 nmoles/min/mg protein of GST were observed in CCl_4 treated groups, as compared to the value of 10.144 in the normal controls. Pretreatment with methanolic extract largely restored the GST enzyme levels in a dose dependent manner in both the tested organs as discussed above.

Glutathione Reductase and Glutathione Peroxidase Enzyme Activities

CCl_4 administration to rats significantly decreased the activities of *Glutathione reductase and Glutathione Peroxidase* in kidney tissue homogenates, as depicted in Table 1. Oral administration with methanolic extract of *Podophyllum hexandrum* at the oral dose of 20, 30 and 50 mg kg^{-1} b.wt. significantly restored the activities of both enzymes (Table 1). Similarly, CCl_4 administration to rats significantly decreased the activities of *Glutathione reductase and Glutathione Peroxidase* in lung tissue homogenates, as depicted in Table 2. Oral administration with methanolic extract of *Podophyllum hexandrum* at the oral dose of 20, 30 and 50 mg kg^{-1} b.wt. significantly restored the activities of both enzymes (Table 2).

Lipid Peroxidation (PMS)

Effects of *Podophyllum hexandrum* rhizome extract on LPO were measured by the formation of free MDA in kidney and lung tissues of rats following short term exposure to CCl_4. The data are shown in Fig. 1. Methanolic extract significantly inhibited the formation of MDA in both the tested organs. After CCl_4 administration, the MDA levels increased significantly from 0.130±0.057 to 2.942±0.143 nm mg^{-1} protein in kidney tissue and 0.0300±0.0158 to 2.780±0.024 nm mg^{-1} protein in lung tissue respectively. However, with the oral administration of 20, 30 and 50 mg kg^{-1} b.wt. of methanolic extract of *Podophyllum*

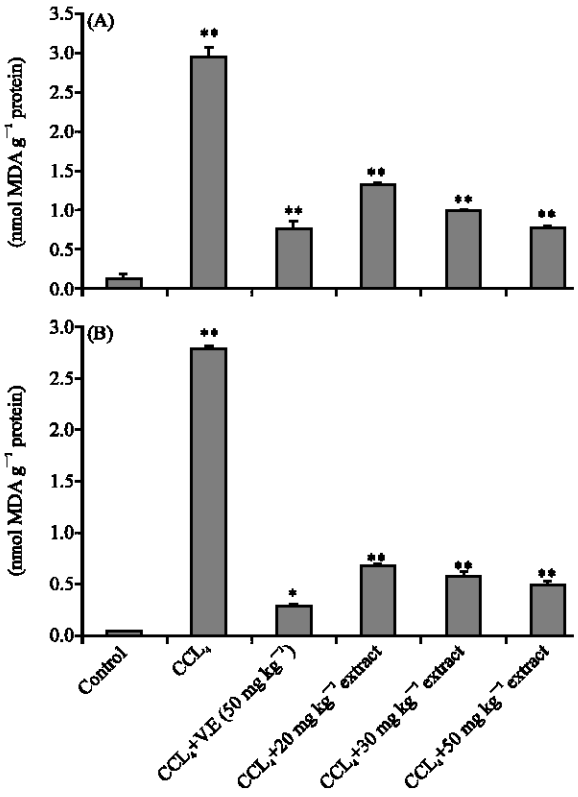

Fig. 1: Effect of Methanolic extract of Podophyllum hexandrum on lipid peroxidation in kidney and Lung tissue homogenates in CCl_4 treated rats as measured by quantifying the MDA levels. (A) The effect of increasing doses viz 20, 30 and 50 mg kg^{-1} b.wt. of *P. hexandrum* extract in kidney tissue homogenates, (B) The effects in lung tissue homogenates under similar conditions. Vitamin E (V.E) was used as a positive control. **$p<0.001$ as compared to control. The data were presented as Mean±S.D of six parallel measures and evaluated by one way ANOVA followed by the Dunnett's-test to detect inter group differences. Differences were considered to be statistically significant if *$p<0.05$

hexandrum the MDA levels decreased respectively to 1.322±0.0.034, 0.985±0.195 and 0.775±0.040 in kidney tissue (Fig. 1A). Lung tissue also exhibited LPO but to a lesser extent as compared to kidney tissue as shown in Fig. 1B. However, our test extract (methanolic) in a dose dependent manner showed inhibitory effects on these lipid peroxidations. Vitamin E (V.E) taken as positive control significantly decreased the MDA formation in both the organs tested.

DISCUSSION

It is well established that free radicals are implicated in a large number of diseases/ metabolic alterations. The toxic effects of CCl_4 *in vivo* are well known to be mediated through radical reactions (Recknagel *et al*., 1989). The CCl_3O-and/or CCl_3OO-radicals produced as a

result of metabolic conversion of CCl_4 is reported to induce organ damage through lipid peroxidation and decreased activities of antioxidant enzymes (Aleynik *et al*., 1997). CCl_4 is a widely used toxicant to experimentally induce animal models of acute kidney and lung tissue damages. CCl_4 and its metabolites are capable of initiating a chain of lipid peroxidation reactions by abstracting hydrogen from polyunsaturated fatty acids (PUFA). Peroxidation of lipids, particularly those containing PUFA, can dramatically change the properties of biological membranes, resulting in severe cell damage and play a significant role in pathogenesis of some diseases (Aleynik *et al*., 1997). Enhanced lipid peroxidation (LPO) expressed in terms of Thiobarbituric Acid Reacting Substance (TBARS) is a measure of membrane damage as well as alteration in structure and function of cellular membrans (Halliwell *et al*., 1995). The level of LPO increased after the CCl_4 treatment in both kidney and ling tissues indicating membrane damage, however pretreatment with methanolic extract of Podophyllum hexandrum decreased the LPO levels, which may be due to the free radical scavenging activity of the extract.

Reduced GSH levels are important for maintaining the structural and functional integrity of different organs. The maintenance of cellular GSH levels dependent upon the activities of Glutathione Reductase (GR) and NADH (Meister and Anderson, 1983). *In vivo* studies indicate that CCl_4 reduces kidney and lung ratio of GSH/GSSG (reduced/oxidized glutathione) (Rungby and Ernst, 1992). The present study also shows that CCl_4 induces a significant reduction in the GSH/GSSG ratio and a decrease in the GR activity and that pretreatment with *Podophyllum hexandrum* was capable of restoring the GSH/GSSG ratio and GR activity to that of normal. Similar results were reported by Ali *et al*. (2000), were traditional medicinal plant Rhazya stricta Decne at 4 g kg^{-1} b.wt. was able to restore the GSH levels in rats.

Glutathione Peroxidase (GSH-Px), Glutathione Reductase (GR), Glutathione-S-Transferase (GST), Superoxide Dismutase (SOD), Catalase (CAT) constitute a mutually supportive team of defense against ROS (Bandhopadhy *et al*., 1999). In the present study it was observed that CCl_4 induced significant decrease in SOD, CAT, GSH-Px and GST activities and enhanced lipid peroxidation in kidney and lung tissues in rats. The decreased activity of SOD in lung and kidney tissues in CCl_4 treated rats may be due to the enhanced lipid peroxidation or inactivation of the antioxidative enzymes. Pretreatment with *Podophyllum hexandrum* was able to restore the activities of SOD, CAT, GSH-Px and GST to that of normal. Similar to our observations, Gupta and colleagues have reported that methanolic extract of Chamomile recutita Capitula exhibited significant antioxidant activity by showing increased levels of GSH-Px, GST, GR, SOD, CAT in CCl_4 induced liver injury (Gupta *et al*., 2006), also reported same observations with ethanolic extract of Indigofera trita Linn against CCl_4 induced tissue injury (Kumar *et al*., 2008).

In conclusion, present study demonstrates that methanolic extract of *Podophyllum hexandrum* has definite antioxidant effect. The mode of action of methanolic extract in affording the protective activity against CCl_4 may be due to the cell membrane stabilization, cell regeneration and activation of antioxidant enzymes such as SOD, CAT, GSH-Px and GST.

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