The Protective Effect of Pomegranate (*Punica granatum*) Juice against Carbon Tetrachloride-Induced Oxidative Stress in Brain Tissue of Adult Male Albino Rats

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Abstract: This study was undertaken to evaluate the effect of pomegranate (*Punica granatum, P.g.)* juice on the oxidation state of the brain tissue of adult male albino rats and whether *P.g.* juice administration improves the activity of the antioxidant enzymes after carbon tetrachloride (CCl_4) treatment as an oxidative stress agent. Rats were divided into four groups, Group I (control) was received intraperitoneal (i.p.) injection of 300µl saline solution (0.9% NaCl) for 12 weeks; Group II (CCl_4 group) was injected i.p. once per week for 10 weeks with CCl_4 (2ml/kg body weight); Group III (*P.g.* group) was administered *P.g.* juice orally for 12 weeks and Group IV (*P.g.* + CCl4) was received *P.g.* 2 weeks before CCl_4 injection and continued for 10 weeks; CCl_4 was administered as shown in group II, decapitation of all groups were carried out one week after the last dose. Determination of glutathione (GSH) content and the activity of the antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione reductase (GR), also malonaldhyde (MDA) and nitric oxide (NO) levels were carried out. The present results demonstrate that treatment with CCl_4 induced biochemical changes in brain tissue as induction of (MDA) and nitrite/nitrate level accompanied with significant decrease in GSH content, SOD, CAT, GPx, GR and GST activities. Administration of *P.g.* juice alone enhanced the activities of enzymes under investigation and decreased the level of NO. *P.g.* Juice administration before and during CCl_4 treatment attenuated the decrease in the enzymes activities and GSH content of the brain, if compared to CCl_4 group. The present study suggest that *P.g.* juice has a potential protective effect as it can elevate antioxidant defense system, clean up free radicals, lessens oxidative damages and protect the brain tissue against CCl_4-induced toxicity.
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Keywords: pomegranate (*Punica granatum*); carbon tetrachloride; oxidative stress; brain; rats.

1. Introduction

Oxidative stress results from the imbalance of reactive oxygen species (ROS) and defense mechanisms, which results in cell damage (**Hartly *et al*., 1999**). Animal tissues are constantly coping with highly (ROS), such as superoxide anion, hydroxyl radicals, hydrogen peroxide, and other radicals generated during numerous metabolic reactions **(Melin *et al*., 2000).** Oxidative stress plays a critical role in cancer, inflammatory, cardiovascular and neurodegenerative diseases as well as in aging **(Storz, 2005)**.

The enhanced production of free radicals and oxidative stress can be induced by a variety of factors such as ionizing radiation or exposure to drugs and xenobiotics (e.g., CCl_4). It is well documented **(Halim *et al*., 1997)** that the treatment with antioxidants such as vitamins C and E or S-adenosylmethionine can modulate the toxic effect of CCl_4 on liver and kidneys.

Pomegranate (*Punica granatum*) is one of the oldest edible fruit and belongs to the Punicacea family **(Fadavi *et al.*, 2006).** *P.g.* is extensively cultivated in the Mediterranean area and most Near- and Far East countries. This botanic isolation is coincident with a unique biochemistry, the seeds contain an oil of which about 80% is a rare trans 18 carbon fatty acid (punicic acid) **(Schubert *et al*., 1999).** There have been numerous reports on the *in vivo* properties of *P.g.*, namely on anti-atherosclerotic capacity **(Aviram and Dornfeld, 2001)**; anti-proliferative and pro-apoptotic activities of pomegranate extract **(Seeram *et al*., 2005)**; anti-inflammatory activity **(Adams *et al*., 2006)** as well as chemopreventive and chemotherapeutical potential towards prostate cancer **(Malik *et al.*, 2005)**.

Therefore, this study was performed in order to investigate the effect of *P. g.* juice on general oxidation status in brain tissue of adult male albino rat, and whether it protects the brain from cellular damage resulting from oxidative stress after CCl_4 intoxication.

2. Materials and Methods

Animals:

Adult male Wistar albino rats weighing 120-150g (7-9 weeks) were obtained from the Holding Company for Biological Products and Vaccines, (VACSERA), Giza, Egypt. After acclimatization for a

period of one week, the animals were housed in cages in a room under standard conditions of illumination with a 12-hours light-dark cycle, at room temperature 25±1ºC. They were provided with water and a balanced standard pellet obtained from VACSERA used as a diet and water *ad libitum*. The animals were kept in clean and low stress environment and an enclosed door provided a substantial amount of sound proofing. All animals received care in compliance with the Egyptian rules for animal protection.

Pomegranate Juice Preparation:

Ten kg of *P.g.* were washed and manually peeled, without separating the seeds. Juice was obtained using a commercial blender (Braun, Germany), filtrated with a Buchner funnel and immediately diluted with distilled water to a volume of 1:3 and stored at 4 ºC for no longer than 2 months **(Faria *et al*., 2007).**

Experimental Protocol:

To study the protective effect of *P.g.* juice on brain tissue of CCl_4-induced toxicity in rat, twenty four adult male Wistar albino rats were randomly allocated to four groups of six rats each. Group I (Control) served as control and received i.p. injection of 300µl saline for 12 weeks. Group II (CCl_4) received weekly i.p. injection of 2ml CCl_4/kg body weight for 10 weeks as described by **Sohn *et al*. (1991)**. Group III (*P.g.)* received *P.g.* juice supplied on dark water bottles and renewed every 2-3 days **(Faria *et al*., 2007)** for 12 weeks. Their average daily intake of fluid was 5.2 to 6.5ml. The animals of Group IV (*P.g.* + CCl_4) were received *P.g.* juice as described in group III for 2 weeks before CCl_4 i.p. injection (2ml/kg body weight) for 10 weeks. *P.g.* juice supplementation to rats of this group was also continued during the whole 10 weeks. After one week of the last i.p. injection of CCl_4, the animals of all groups had cervical dislocation. Half of each rat brain was weighed and homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50mM Tris-HCl and 300mM sucrose **(Tsakiris *et al.*, 2004)**. The homogenate was centrifuged at 500g for 10 min. at 4^0C.The supernatant (10%) was used for the various biochemical determinations. The second half of the rat brain was freshly prepared to be used in flow cytometer investigation.

Chemicals:

Carbon tetrachloride (CCl_4), tris HCl buffer and 50, 50 dithiobis-2-nitrobenzoic acid (DTNB), were purchased from Sigma (St. Louis, MO, USA). Perchloric acid, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Merck. All other chemicals and reagents used in this study were of analytical grade. Double-distilled water was used as the solvent.

Determination of Nitrite/Nitrate Concentration and MDA Level:

Nitrite/nitrate (breakdown products of NO) concentration and MDA level were assayed calorimetrically in brain homogenate according to the methods of **Berkels *et al.* (2004)** and **Ohkawa *et al.* (1979)**, respectively.

Estimation of Glutathione and Anti-Oxidant Enzymes:

The brain glutathione (GSH) was determined by the method of **Ellman (1959)**. This method is based on the reduction of Ellman's reagent (5, 5` dithiobis (2-nitrobenzoic acid, "DTNB") with GSH to produce a yellow compound measured at 405nm. In addition, the level of brain antioxidant enzymes as catalase (CAT) was assayed by the method of **Aebi (1984)**.

Brain superoxide dismutase (SOD) activity was assayed by the method of **Nishikimi *et al.* (1972)**. Glutathione-S-transferase (GST) activity in the brain was assayed by the method of **Habig *et al.* (1974)**. Brain glutathione peroxidase (GPx) activity was measured by the method of **Paglia and Valentine (1967)**. Glutathione reductase (GR) activity in the brain was assayed by the method of **Factor *et al.* (1998)**.

Flow Cytometry:

Tissue samples were prepared by manual disaggregation procedure. Briefly, a few drops of RPMI media were added to tissue and then minced until complete tissue disaggregation was achieved. Suspended cells were filtered using a 50µm pore size mesh and then centrifuged at 1000 rpm for 10 min. Cells were re-suspended in protein bovine serum, counted and washed by calcium buffer then centrifuged at 1500 rpm for 5 min. The pellet was re-suspended and then cells were counted. Annexin V and propidium iodide apoptotic assay was carried out using IQP-120F Kit (IQ Products, Groningen, Netherlands). FAC scan Becton-Dickinson (BD) flow cytometer was used and data were analyzed using cell Quest software.

Statistical Analysis:

The obtained data were presented as means ± standard error. One-way analysis of variance (ANOVA) was carried out. The statistical comparisons among the groups were performed with **Duncan's test (1955)** using a statistical package program (SPSS version 17.0). All p-values are two-tailed and $p<0.05$ was considered as significant for all statistical analysis in this study.

3. Results

In a trial to evaluate the ability of *P.g.*juice in modulating the oxidative-antioxidative state in the brain tissue, the data obtained in the present study (Table 1) concerning treatment of adult male Wistar rats with *P.g.* juice and/or CCl_4 (2ml/kg body weight)

revealed that CCl_4 injection for 10 weeks caused a significant decrease at $p<0.05$ in GSH, GST, GPx and GR levels as compared to control group. The level of oxidative markers assessed in *P.g.* juice-treated rats group showed a significant increase at $p<0.05$ in GSH, GST levels coupled with a non significant change in GPx and GR levels. In *P.g.* juice and CCl_4-treated rats group, the data showed the toxic manifestations of CCl_4 and the recovery pattern after *P.g.* juice administration. The co-administered group exhibited an increase in GSH, GST, GPx and GR levels, if compared to CCl_4-treated group, the increase was significant at $p<0.05$ in GSH, GPx and GR.

Injection of CCl_4 induced a significant inhibition ($p<0.05$) in SOD and CAT activities in the brain tissue as compared to control group (Figure 1). The supplementation of *P.g.* juice to rats showed a significant enhancement at $p<0.05$ in CAT activity as compared to control group. In *P.g.* juice & CCl_4-treated rats, although CCl_4 effect was evident in inhibiting both SOD and CAT activities versus the control group, *P.g.* juice supplementation before and during treatment of CCl_4 indicated a significant elevation ($p<0.05$) in both enzymes activities when compared to CCl_4 group.

Table (1): The effect of *P.g.* juice and/or CCl_4 (2ml/kg body weight) on the levels of GSH, GST, GPx and GR in brain tissue of adult male albino rats.

Groups	GSH (mmol/g tissue)	GST (µmol/h/g tissue)	GPx (U/g tissue)	GR (µmol/ g tissue)
Control	32.38±0.35	0.36±0.05	1945.3±61.2	8.24±0.53
CCl_4	22.09±0.48[a]	0.21±0.03[a]	1074.1±73.1[a]	4.96±0.75[a]
P.g.	39.23±0.48[a]	0.49±0.04[a]	1891.3±55.9	10.72±1.86
***P.g.* + CCl_4**	29.16±0.42[ab]	0.29±0.04	1482.2±32.6[ab]	6.36±0.1.21[b]

Values are means ± S.E. (n=6). *a: Significant change at p<0.05 with respect to Control group.*
b: Significant change at p< 0.05 with respect to CCl_4-treated group.

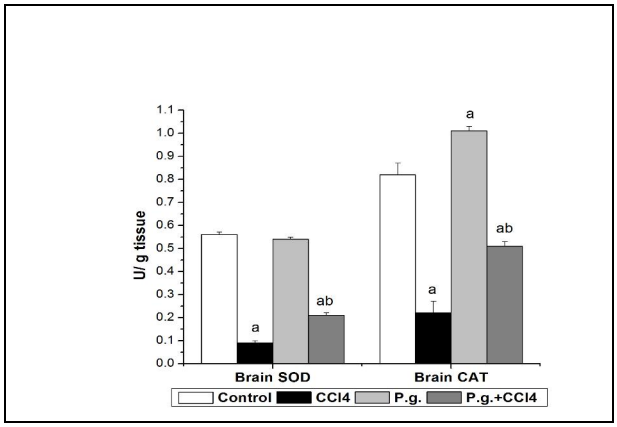

Figure (1): The effect of *P.g.* juice and/or CCl_4 (2ml/kg body weight) on SOD and CAT activities in brain tissue of adult male albino rats.

The data represented in Figure (2) indicated that CCl_4 treatment induced a significant increase ($p<0.05$) in MDA and NO contents in the brain as compared to control group. The supplementation of *P.g.* juice to rats showed a significant decrease ($p<0.05$) in NO level in the brain tissue versus control group. On the other hand, *P.g.* juice supplementation before and during CCl4 injection reduced the enhanced MDA and NO levels in the brain tissue with a significant change at $p<0.05$, if compared to CCl_4-treated rats group.

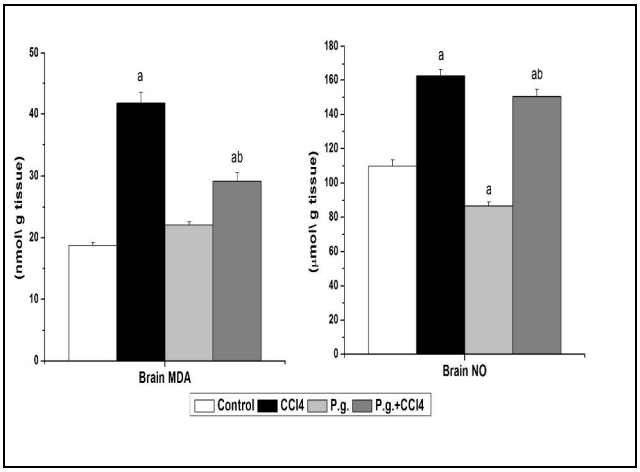

Figure (2): The effect of *P.g.* juice and/or CCl_4 (2ml/kg body weight) on MDA and NO levels in brain tissue of adult male albino rats.

Flow cytometric analysis was performed to investigate the anti-apoptotic effects of *P.g.* juice on brain cells against CCl_4-induced apoptosis and necrosis. Cells were dual-stained with Annexin V and propidium iodide (Figure 3 & Table 2). *In vitro*, treatment of brain cells with CCl_4 caused apoptosis with a percent of (62.3%). Apoptotic cells in CCl_4-treated group showed a significant ($p<0.05$) increase versus control group. The protective effect of *P.g.* juice on CCl_4-induced apoptosis in neurons were abolished by decreasing the number of apoptotic cells (44.7%), whereas, increased the number of viable cells (49.5%) when compared with CCl_4-treated group.

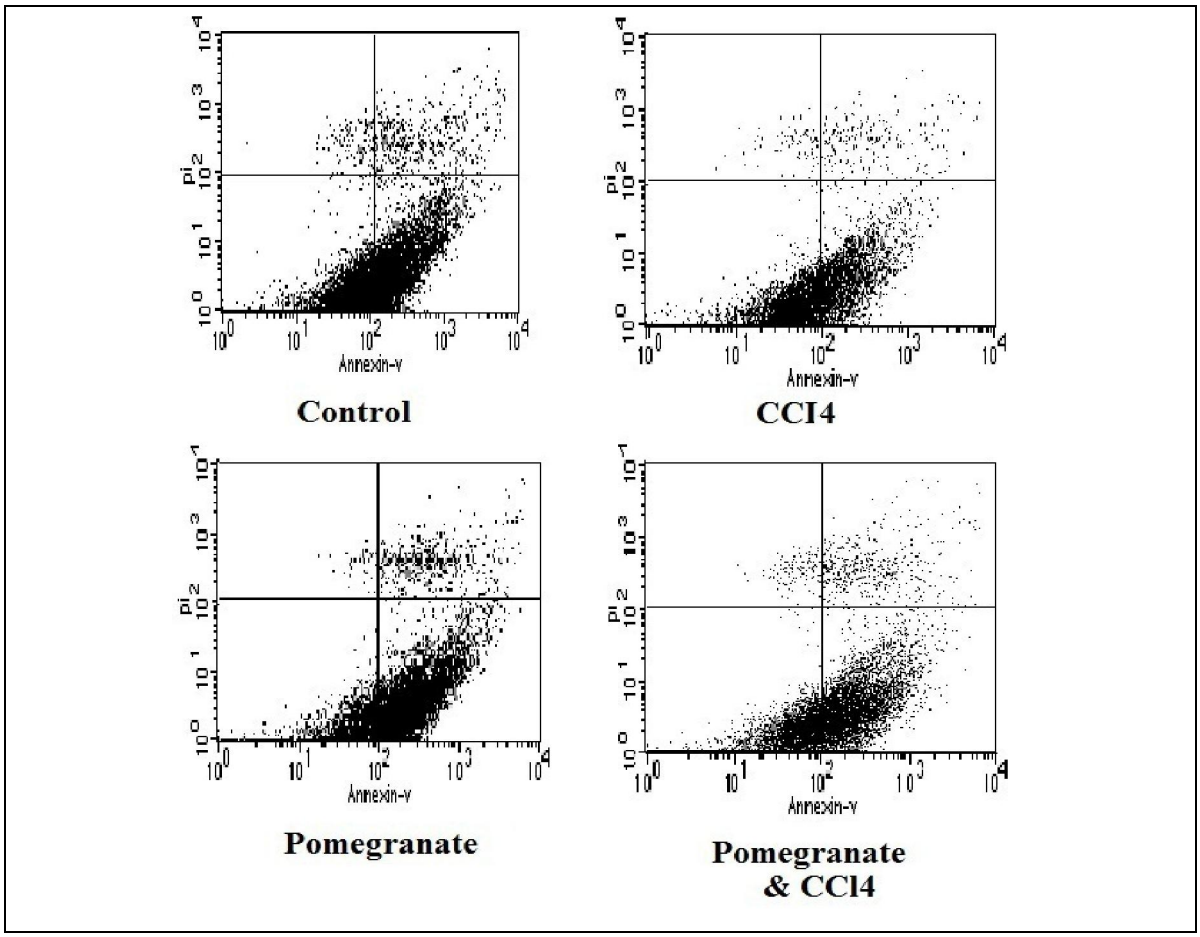

Figure (3): Assessment of apoptosis by annexin V and propidium iodide staining on brain tissue of rats treated with *P.g.* juice and/or CCl_4 (2ml/kg body weight).

Table (2): The effect of *P.g.* juice and/or CCl_4 (2ml/kg body weight) in assessing of apoptosis by annexin V and propidium iodide staining on brain tissue of adult male albino rats.

Groups	Viable Neurons	Apoptotic Neurons	Dead Neurons	Necrotic Neurons
Control	44.6±1.9	49.5±1.0	4.5±0.2	1.2±0.05
CCl_4	34.2±1.5[a]	62.3±1.8[a]	2.6±0.1[a]	0.9±0.02
P.g.	64.2±2.1[a]	30.2±1.0[a]	5.2±0.3	0.4±0.01[a]
P.g. + CCl_4	49.5±1.7[b]	44.7±0.8[b]	4.5±0.3[b]	1.3±0.02

Values are means ± S.E. (n=6). a: Significant change at $p<0.05$ with respect to Control group.
b: Significant change at $p< 0.05$ with respect to CCl_4-treated group.

4. Discussion

The present results demonstrated that treatment with CCl_4, induced biochemical signs of brain tissue injury, evidenced by decreased GSH content, increased MDA and NO levels and inhibited the enzymes activity of SOD, CAT, GPx, GST and GR which were considered as an index of lipid peroxidation.

Carbon tetrachloride, a typical hepatotoxic, exerts its toxic effects by the generation of free radicals **(Melin *et al*., 2000).** The lipid solubility of CCl_4 allows it to cross cell membranes; it is distributed and deposited to organs such as the liver, brain, kidney, and heart. The time courses of uptake and elimination of CCl_4 appeared to be governed largely by the rate of blood perfusion and lipid content of the tissue. CCl_4 is rapidly taken up by liver and brain **(Sanzgir *et al*., 1997)**.

The reactive metabolite trichloromethyl radical (CCl_3) has been formed from the metabolic conversion of CCl_4 by cytochrome P-450. These free radicals initiate the peroxidation of membrane poly-unsaturated fatty acids which results in the generation and excess production of reactive oxygen Species

(ROS) **(Hartly *et al*., 1999 and Melin *et al*., 2000)**, finally cell necrosis. However, when oxidative stress reaches a certain limit, defense mechanisms against ROS become insufficient and led to a decrease in the intracellular concentration of GSH and antioxidant enzymes **(Manna *et al.*, 2011).**

Oxidative stress can produce major interrelated derangements of cellular metabolism, including alteration of protein and nucleic acid structure, increase in intracellular free calcium, damage to membrane ion transport and permeability and destruction of the cells by lipid peroxidation **(Reckmage *et al.*, 1989)**. Lipid peroxidation has attracted much attention because of its association with a number of abnormal physiological processes **(Reckmage *et al.*, 1989 and Hartly *et al*., 1999).**

In the present study, a significant elevation of MDA (lipid peroxidation product) has been observed in the brain tissue of CCl_4-treated group. Evidence of lipid peroxidation by increased MDA level is one of the primary means of associated oxidative processes with an overall decrease in cellular function. The increase in MDA which indicates lipid peroxidation is an index to identify free radicals-induced injuries **(Rosenblat *et al.*, 2006)**. Moreover**, Jayakumar *et al*. (2008)** indicated that male Wistar rats exposed to CCl_4 revealed elevated levels of MDA and lowered levels of GSH, accompanied by lower activities of the antioxidant enzymes, CAT, SOD, GPx and GST in the kidneys, heart and brain.

Previous studies have demonstrated the different responses of the antioxidant enzymatic system in different tissues during oxidative stress **(Jones *et al*., 1986)**. The difference in the enzyme responses may be in part due to the differences in the amount, nature, and activities of these enzymes in particular tissues. The particular enzyme associated with the different sub-cellular fractions can exert different effects. Results obtained by **Zhang *et al*. (1989)** demonstrated a tissue- and age-specific expression of GPx, the highest activities of this enzyme being in liver and kidney but the lowest in brain and muscle. Several studies have shown that natural antioxidant defense systems have limited capacity in the brain as compared to peripheral tissues **(Del Maestro and Mc Donald, 1987 and Somani *et al*., 1995).** The brain contains small amounts of catalase and different brain regions contain different activities of antioxidant enzymes **(Somani *et al*., 1995)**.

Vohra and Hui (2001) found that, CCl_4 intoxicated cultured neurons increased lipid peroxidation product and decreased GPx activity, in a concentration dependent manner. **Szymonik-Lesiuk *et al.* (2003) f**ound that, SOD and CAT activities decreased, in the brain, after 24h of CCl_4 ingestion, but GPx activity significantly increased statistically at all time points studied. The high concentration of polyunsaturated fatty acids and aerobic metabolic activity of the brain increase the susceptibility of this organ to peroxidative damage induced by reactive oxygen species after CCl_4ingestion. It has been reported **(Del Maestro and Mc Donald, 1987 and Somani *et al*., 1995)** that the brain content of cytochrome P-450 and arachidonic acid is lower than that in the liver, demonstrating that the antioxidant defense system has limited capacity in the brain. **Szymonik-Lesiuk *et al*. (2003)** suggest that GPx is more important than catalase for detoxification of hydrogen peroxide in the brain. The brain contains small amounts of catalase, and GPx can also interact directly with lipid peroxides **(Adams *et al*., 1991). Szymonik-Lesiuk *et al.* (2003)** also suggested that brain have different coping mechanisms to deal with oxidative stress than liver.

Moreover, **Ichi *et al.* (2009)** reported that after CCl_4 (4ml/kg body weight) treatment, the enhanced activity of neutral sphingomyelinase (SMase), which was significantly increased in the liver, kidney, and brain, was accompanied by a decrease of GSH. Thus, the activation of neutral SMase via oxidative stress induced the increase of ceramide, a biologically active lipid causing apoptosis in a variety of cells during CCl_4 intoxication, in not only the liver but also other tissues. The authors found that, in the brain, the total ceramide, dramatically, increased at 36h, after CCl_4 injection. **Ichi *et al.*, (2009)** suggested that the increase in ceramide, in plasma was transferred to the kidney and the brain. This may explain the increase in apoptotic cells noticed in the brain of CCl_4-treated group when compared to control group.

Administration of CCl_4 enhanced the induction of MDA and reduced GSH levels as well GST activity in brain as reported by **Soliman and Fahmy (2011)**. The decrease in GST transcription was accompanied by a decrease in GST activity, as confirmed by **Faria *et al*. (2007).** In addition, **Moreno and Muriel (2006)** indicated that Cirrhosis produced by i.p. administration of CCl_4 did not produce a significant effect in nitrite/nitrate level but, reduced GSH and increased lipid peroxidation.

The findings of the present study indicated that CCl_4 enhances NO generation in the brain tissue of rat. This elevation in NO production may inhibit key enzymes of energy metabolism, damage DNA, deplete intracellular glutathione and react with superoxide to form the much more powerful oxidant peroxynitrite **(Bashkatova *et al.*, 2004).** Peroxynitrite is a key element in resolving the contrasting roles of NO in physiology and pathology. The generation of peroxynitrite over long periods of time will result in substantial oxidation and potential destruction of host

cellular constituents, leading to the dysfunction of critical cellular processes, disruption of cell signaling pathways, and the induction of cell death through both apoptosis and necrosis **(Pacher *et al.*, 2007).**

There is an intimate relationship between nutrition and the antioxidant defense system, as some exogenous low molecular weight antioxidants may be supplied by the diet. These two main systems of the antioxidant defense act in coordination, their levels being regulated by each other, to avoid oxidative stress events **(Masella *et al.*, 2005)**. In the past few years, a considerably large group of molecules widespread in plants has come into focus.

Herbal medicine has been used for more than 5000 years. The interest in polyphenols has grown considerably because of their high capacity to trap free radicals associated with different diseases. Phenols and flavonoids are very important plant constituents because of their antioxidant activity. The plant phenolics are commonly present in fruits, vegetables, leaves, nuts, seeds, barks, roots and in other plant parts **(Lansky and Newman, 2007)**. The antioxidant activity of phenolic compounds is mainly due to their redox properties which play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metals **(Mustafa *et al.*, 2010).**

The present study showed that *P.g.* juice supplementation before and during treatment of CCl_4 exhibited an increase in GSH, GST, GPx, GR levels, SOD and CAT activities, while reduced the enhanced MDA and NO levels, if compared to CCl_4-treated rats group.

As depicted from the present results, treatment of *P.g.* juice with CCl_4 resulted in a reduction in oxidative stress clearly indicating the antioxidant ability of *P.g.* which was attributed to diverse phenolic compounds **(Kaur *et al.*, 2006)** present in *P.g.* juice, including punicalagin isomers, ellagic acid derivatives and anthocyanins **(Seeram *et al.*, 2005)**. These compounds are known for their properties in scavenging free radicals and inhibiting lipid oxidation.

Another reliable oxidative stress indicator is the measure of GSH level. Glutathione is an antioxidant marker which plays a protective role in the metabolism of a large number of toxic agents. It acts as a free radical trapping agent and preserves cytochrome P-450 by blocking lipid peroxidation **(Yuan *et al.*, 2008)**. In the present study, *P.g.* juice markedly increased the brain GST activity and maintained GSH level, when compared to CCl_4-treated rats group. The mechanism of protection by *P.g.* juice against CCl_4 toxicity might be due to restoration of the GSH level.

From the present results the *P.j.* juice was active and possessing very potent and novel therapeutic agents for scavenging of NO. *P.g* juice may also exert its effects on the regulation of pathological conditions caused by excessive generation of NO and its oxidation product-peroxynitrite. The protective effect of *P.g.* juice treatment agreed with **Aviram *et al*. (2000)** who reported that *P.g.* reduced the oxidative stress.

However, polyphenols are known to modulate the transcription and expression of proteins related to the endogenous antioxidant defense by interacting with antioxidant response elements in gene promoter regions of genes encoding proteins related to oxidative injury management **(Myhrstad *et al*., 2002 and Moskaug *et al*., 2005).** In addition, in the present study, *P.g.*juice supplementation considerably increased the formation of antioxidant products which may be regarded to the phenolic constituents and its antioxidant activity. Flavonoids present in *P.g.* juice have been shown to alleviate the oxidative stress by increasing the endogenous antioxidant status, protecting cells against free-radical damage by increasing resistance to oxidative stress **(Li *et al.*, 2006 and Bachoual *et al.*, 2011).**

It could be concluded that, *P.g.* juice reversed the deteriorated antioxidant enzymes activity in the brain tissue of adult male albino rats intoxicated with CCl_4 through scavenging free radicals, decreasing lipid peroxidation, attenuating the brain susceptibility to oxidative stress thus, improving cellular membrane and organ functioning more profoundly and brought variables towards control values.

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