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Antioxidant Effects of Pomegranate Seed Oil Against Pentachlorophenol Toxicity in Rat Tissues*

Zeynep SOYER SARICA[1], Mürsel KARABACAK[2], Murat KANBUR[3], Gökhan ERASLAN[3], Bilal Cem LİMAN[3], Muhammet Yasin TEKELİ[3]

[1]Erciyes Üniversitesi, Deneysel Araştırmalar Uygulama ve Araştırma Merkezi, Kayseri-TÜRKİYE
[2]Erciyes Üniversitesi, Safiye Çıkrıkçıoğlu Meslek Yüksek Okulu, Hayvan Sağlığı Bölümü, Kayseri-TÜRKİYE
[3]Erciyes Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Ana Bilim Dalı, Kayseri-TÜRKİYE

Abstract: This study was conducted to evaluate the effects of pomegranate seed oil PSO on the oxidative stress induced by pentachlorophenol PCP in rats. Fourty Sprague-Dawley, 4-5 months old, 300g male rats were used in this study. Rats were assigned to four groups each containing 10 animals. First group was control group; no administration was made to animals in this group. Second, third and fourth groups were given PSO at a dose of 0.15 ml/kg bw, PCP at a dose of 40 mg/kg bw and PSO at a dose of 0.15 ml/kg bw+PCP at a dose of 40 mg/kg bw orally by gavage for 28 days, respectively. At the end of the study, brain, liver, kidney, testicle and spleen samples were collected. Malondialdehyde (MDA) level and catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities were measured in tissue samples. PCP caused an increase of MDA level in brain, kidney, liver and testicle; a decrease of CAT activity in liver and spleen; a decrease of GSH-Px activity in brain and testicle and a decrease of SOD activity in brain, liver and kidney compared to control group. When PSO+PCP group compared to PCP group, MDA level decreased in brain, liver and testicle; CAT activity increased in spleen; SOD activity increased in brain and kidney. When compared to the control group and the group given PSO+PCP, the data of PSO+PCP group were generally similar to the control group. As a result, administration of PCP at the dose of 40 mg/kg bw for 28 days caused oxidative stress and administration of PSO at the dose of 0.15 ml/kg bw alleviated severity of PCP induced oxidative stress.
Key words: Oxidative stress, pentachlorophenol, pomegranate seed oil, rat

Rat Dokularında Pentaklorofenol Toksisitesine Karşı Nar Çekirdeği Yağının Antioksidan Etkileri

Özet: Bu çalışma ratlarda pentachlorophenol PCP'nin oluşturduğu oksidatif stres üzerine nar çekirdeği yağının (NÇY) etkilerinin değerlendirilmesi için yapıldı. Çalışmada 4-5 aylık, ortalama 300 g ağırlığında 40 adet Sprague-Dawley ırkı erkek sıçan kullanıldı ve her birinde 10 hayvan olmak üzere dört grup oluşturuldu. İlk grup kontrol olarak belirlendi ve herhangi bir uygulama yapılmadı. İkinci, üçüncü ve dördüncü gruba sırasıyla 0.15 ml/kg ca dozunda NÇY, 40 mg/kg ca dozunda PCP ve 0.15 ml/kg ca dozunda NÇY ile 40 mg/kg ca dozunda PCP 28 gün boyunca gavaj yolu ile mideye uygulandı. Çalışmanın sonunda beyin, karaciğer, böbrek, testis ve dalak dokuları alındı. Doku örneklerinde malondialdehit (MDA) düzeyi ile katalaz (CAT), glutasyon peroksidaz (GSH-Px) ve superoksit dismutaz (SOD) aktiviteleri ölçüldü. Pentaklorofenolün kontrol grubuna göre, beyin, karaciğer, böbrek ve testiste MDA düzeyinde yükselme; karaciğer ve dalak CAT aktivitesinde azalma; beyin ve testis GSH-Px aktivitesinde azalma; beyin, böbrek ve karaciğer SOD aktivitesinde azalmaya neden oldu. NÇY+PCP verilen grup PCP verilen grupla karşılaştırıldığında beyin, karaciğer ve testis MDA düzeyinde azalma; dalak CAT aktivitesinde yükselme; beyin ve böbrek SOD aktivitesinde yükselmeye yol açtı. NÇY+PCP verilen grup ve kontrol grubu karşılaştırıldığında, NÇY+PCP grup verileri genel olarak kontrol grubuyla benzerdi. Sonuç olarak, ratlara 28 gün 40 mg/kg ca dozda verilen PCP'nin dokularda oksidatif strese yol açtığı; 0.15 ml/kg ca dozda uygulanan NÇY'nin, PCP kaynaklı oksidatif stresin şiddetini azalttığı belirlendi.
Anahtar kelimeler: Nar çekirdeği yağı, oksidatif stres, pentaklorofenol, rat

Introduction

Pomegranate (*Punica granatum L., Punicaceae*) is known as a plant with valuable effects since ancient times. It can be seen from India to Iran even to south west sides of America. First usage of pomegranate is to have anti-inflammatory, antioxidant and antiparasitic effects. These effects could be originated from phenolic compounds, anthocyanins, tannins and alkaloids (6,11). Pomegranate is known to have beneficial effects for health since ancient ages;

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it is a plant whose bark of tree, peel of fruit, flower, juice of fruit and seeds are utilized. The seed of pomegranate contains active component such as monoacylglycerols, linoleic acid, linolenic acid, glycerides, sterols, campesterol, γ - tocopherol, 17-α- estradiol and macro nutrients such as protein, pectin and sugar (6,12,18,26,28,30,32). Another active ingredient which is found in high proportion in pomegranate oil is punicic acid (tricosanic acid), which is a long chain fatty acid, coming to the forefront with its anticarcinogenic effects (9,11,22,26,28). The density of this seed oil is 0.9202 to 0.9311 g/cm^3(16). Pomegranate seed oil (PSO) has hypoglycemic effects as well as anticarcinogenic effects. It is believed that this effect is related to punicic acid (6). Punicic acid contains the 74-85% of total PSO oil content (14). Herbal sterols (eg. betacytosterol, campesterol, stigma sterol) are also reported to be rich in PSO (total 4089-6205 mg/kg oil) (28). Ellagic acid which is also an anticarcinogenic and antioxidant substance is found at a highly proportion in PSO. PSO is also rich in vitamin E (26).

Pentachlorophenol (PCP) is a substance used in industrial fields like wood and wood products, paper, leather, textile, paint, glue, plastic, photography, yarn and nylon due to its insecticide, fungicide, bactericide, herbicide and molluscicide effects (48). The oral LD_{50} is 25-210 mg/kg bw in rats, 74-130 mg/kg bw in mice and 70-300 mg/kg bw in rabbits. PCP can be also toxic through respiratory tracts. This substance which is also toxic for aquatic organisms can accumulate up to 10000 times in the body of creatures like fish, algae, and invertebrates compared to water (29,38,48). The target organs in PCP exposure are liver, kidney and bone marrow (38). The most important target tissue is liver. Depending on the exposed dose, in addition to the some changes in the biochemical parameters, hepatic P450 enzyme activities, liver weight and in the liver microscopy are also observed (24,25,29,38,49). PCP has also toxic effects on kidneys. Kidney enlargement and some microscopic changes like interstitial nephritis, hyperemia, glomerular atrophy were seen in the milk cows who had PCP added to their feed (31,48). In poisoning cases, changes also occur in blood. The substance has been shown to cause anemia in cows and led to a decrease in erythrocyte count, hemoglobin concentration and hematocrit values in rats (27,38). Previous studies have shown that PCP affects especially brain (1,20), liver (46), kidney (1), testis (4) and spleen (7). Therefore, studies were carried out in these tissues. In this study, we aimed to investigate the possible antioxidant and protective effects of PSO on lipid peroxidation induced by PCP in various tissues of rats.

Materials and Methods

Animals

All the procedures with animals in this study were approved by from Erciyes University Experimental Research and Application Center (Approval date and number: 17.12.2008-08/74). Fourty, sixteen weeks old Sprague-Dawley male rats weighing of 300 g in the same condition were selected (Erciyes University Experimental Research Center). They were provided with adequate commercial feed (Produced by Purina, Düzce ,Turkey) and tap water. The rats were arranged in four groups and each group were arranged four cages (25 cm width by 40 cm length by 20 height). Each of the cages contained two or three rats and provided coarse sawdust bedding (Kayseri, Turkey). Rats were housed under conventional experimental animal housing conditions with controlled temperature (23±2 °C), humidity (50±5%), air change (12 air change per hour), room had an equal 12 h of light and darkness and ad libitum feed. General health status of the rats was monitored prior, during and at the end of the study. In this study, cold pressed PSO containing meristic acid (0.09%), palmitic acid (3.29%), palmitoleic acid (0.12%), margaric acid (0.05%), stearic acid (2.13%), oleic acid (8.66%), linoleic acid (5.81%), linolenic acid (0.06%), arachidic acid (0.42%), ecosanoic acid (0.70%) and punicic acid (78.67%) indicated on the product was used (Obtained From Bükaş ®, İzmir, Turkey; 50 ml cold press oil). PCP sodium salt (Fluka 76480) was used and this compound was dissolved in distilled water.

Experimental Protocol

Rats were randomly divided into four groups and each group contains 10 rats. Group I is control group and no administration was made. Group II received PSO (0.15 ml/kg bw), group III received PCP (40 mg/kg bw) and group IV was administrated PSO+PCP (0.15 ml/kg bw+40 mg/kg bw) intragastrically using a 22 G ball tip needle for 28 days, respectively. Applied PSO and PCP doses are determined from previous studies (1,8,24,38,44,48). Twenty four

hours after the last administration, tissue samples from brain, liver, kidney, testis and spleen of the rats were collected under light ether anaesthesia. The removed organs were washed with cold distilled water. Afterwards the fat tissue on the organs was removed. These tissue samples were homogenized with phosphate buffer, at pH 7.4 (Heidolph, Silent Crusher M). The homogenized tissues were centrifuged at 20000 rpm for 1 hours at + 4 °C and the supernatants were transferred to Eppendorf tubes. All samples were stored at -80 °C till analysis. Protein analysis was employed in accordance with the method modified by Miller (37) and based on the method of Lowry (33). The measurement of MDA levels of all tissues are determined by the method reported by Ohkawa (39); the estimation of SOD activities by Sun's method (45), the estimation of CAT activities by Luck's method (34) and the estimation of GSH-Px activities by Paglia's method (40) was performed. All analysis of samples was assayed as spectrophotometrically (Helios Alpha UV-Vis spectrophotometer). The units of MDA levels, the activities of SOD, CAT and GSH-Px were expressed as nmol/mg protein, U/mg protein, k/g protein and U/g protein, respectively.

Statistical analysis

The statistical analysis was made with SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA). Differences of groups were compared using One-way ANOVA and Duncan's test. The data were given as mean±standart deviations (SD) and values of $p<0.05$ were considered as statistically significant.

Results

MDA: When compared control group, there is no significant statistical changes on MDA levels of all tissues in PSO group ($P>0.05$). When PCP group compared to the control group, it was seen an increase of brain, liver, kidney and testis MDA levels at PCP group ($P<0.05$). When control and PCP+PSO group was compared, no statistical change in brain and spleen MDA levels was determined ($P>0.05$) but statistical decrease in liver, kidney and testis MDA levels of PCP+PSO was observed ($P<0.05$). Compared with the PCP and PCP+PSO groups, brain, liver and testis MDA levels were statistically significant ($P<0.05$) (Table 1).

Table 1. MDA levels in rat tissue of control and experimental groups (nmol/mg protein).

Tissue	Group (n:10) (Control)	Group II (n:10) (PSO)	Group III (n:10) (PCP)	Group IV (n:10) (PSO+PCP)	P
Brain	11.39±1.2^{a}	12.40±2.1^{a}	18.79±2.7^{b}	14.68±3.5^{a}	P<0.05
Liver	1.77±0.3^{a}	1.95±0.4^{a}	3.78±0.7^{c}	2.77±0.2^{b}	P<0.05
Kidney	3.20±0.6^{a}	3.24±0.5^{a}	5.67±0.5^{c}	4.61±1.3^{bc}	P<0.05
Testis	3.81±0.7^{a}	4.21±0.6^{ab}	7.77±1.6^{c}	5.53±0.8^{b}	P<0.05
Spleen	6.32±1.4	7.03±1.2	8.11±0.3	7.15±1.1	P>0.05

(*) Different letters of the groups in the same line are statistically significant (P<0.05).
PSO: Pomegranate seed oil, PCP: Pentachlorophenol, PSO+PCP: Pomegranate seed oil and pentachlorophenol.

CAT: When compared to the control group, no significant changes were observed in the CAT activities of the PSO group on all tissues ($P>0.05$). When compared to between control and PCP group, CAT activities of liver and spleen decreased at PCP group ($P<0.05$). When compared to control and PCP+PSO group, there were no significant differences ($P>0.05$). When compared to PCP and PCP+PSO groups, CAT activities decreased in

Table 2. CAT activities in rat tissue of control and experimental groups (k/g protein).

Tissue	Group I (n:10) (Control)	Group II (n:10) (PSO)	Group III (n:10) (PCP)	Group IV (n:10) (PSO+PCP)	P
Brain	44.97±18.8	46.45±19.8	57.05±11.3	52.50±6.5	P>0.05
Liver	210.67±42.5^{b}	199.35±14.6^{b}	146.5±28.1^{a}	177.62±20.5^{ab}	P<0.05
Kidney	177.68±53.1	191.61±54.5	180.10±28.5	186.96±43.1	P>0.05
Testis	44.54±14.8	56.40±9.9	40.18±15.2	42.52±8.51	P>0.05
Spleen	43.02±21.8^{b}	42.44±14.5^{b}	17.02±3.0^{a}	39.9±11.4^{b}	P<0.05

(*) Different letters of the groups in the same line are statistically significant (P<0.05).

spleen tissue of PCP group (P<0.05) (Table 2).

GSH-Px: When the control group was compared with the PSO group, there was no significant change in brain, liver and kidney of GSH-Px activities (P>0.05) but there were statistically increase in testis and spleen tissues of PSO group (P<0.05). When control and PCP group was compared, it was seen significant decrease on GSH-Px activities in brain and testis of PCP group (P<0.05). There was no significant difference in liver, kidney, testis and spleen GSH-Px activities between control and PCP+PSO groups but significant difference has brain tissue (P<0.05). When PCP and PSO+PCP group are compared, there was no significant difference on GSH-Px activities of PSO+PCP group (P>0.05) (Table 3).

SOD: When control and PSO group were compared, there were no significant changes in all tissues SOD activities (P>0.05). Comparing control and PCP group, there was significant decrease in SOD activities of brain, liver and kidney of PCP group (P<0.05). When PCP and PSO+PCP group were compared, it was observed that brain and kidney SOD activities increased at PCP+PSO group (P<0.05) (Table 4).

Table 3. GSH-Px activities in rat tissue of control and experimental groups (U/g protein).

Tissue	Group I (n:10) (Control)	Group II (n:10) (PSO)	Group III (n:10) (PCP)	Group IV (n:10) (PSO+PCP)	P
Brain	226.48±43.3^{b}	299.34±53.5^{b}	122.9±24.3^{a}	126.65±11.5^{a}	P<0.05
Liver	139.04±28.3^{ab}	150.95±33.6^{b}	113.61±21.7^{a}	136.52±10.7^{ab}	P<0.05
Kidney	126.66±30.8^{ab}	159.73±47.6^{b}	92.92±19.1^{a}	107.79±12.3^{a}	P<0.05
Testis	111.70±15.1^{b}	185.09±15.1^{c}	89.04±11.4^{a}	97.30±9.4^{ab}	P<0.05
Spleen	205.88±26.5^{a}	280.58±36.5^{b}	181.31±78.0^{a}	200.23±30.74^{a}	P<0.05

(*) Different letters of the groups in the same line are statistically significant (P<0.05).

Discussion and conclusion

Pomegranate (*Punica granatum L.*) which is thought to have no significant adverse effects is a widely used plant in many cultures (35). According to this positive effect, in recent studies it is proposed that PSO is good alternative for food supplement or dietary supplements (6). Faria et al. (19) reported that, the levels/ activities of GSH and GSH-Px, SOD, and CAT in liver were decreased in rats that were fed with pomegranate juice ad libitum for 4 weeks compared to the control group, whereas lipid peroxidation did not cause a significant change in hepatic MDA levels which is considered as the most important oxidative stress marker (19). According to a study, because of oxidative stress, antioxidant enzyme activities such as SOD, CAT and GSH-Px significantly decreased compared to control group and also the administration of α-eleostearic acid and punicic acid was observed to support the cellular antioxidant defence mechanism by modulating the activity of these antioxidant enzyme against the oxidative stress (42). According to another study, pomegranate juice extract (200 µg gallic acid equivalent/mouse/day) was administrated to mice for 2 weeks and positively affected on cel-

Table 4. SOD activities in rat tissue of control and experimental groups (U/mg protein).

Tissue	Group I (n:10) (Control)	Group II (n:10) (PSO)	Group III (n:10) (PCP)	GroupIV (n:10) (PSO+PCP)	P
Brain	5.21±0.3^{a}	5.09±0.7^{a}	3.41±0.5^{b}	4.99±0.6^{a}	P<0.05
Liver	2.97±0.7^{b}	3.25±0.3^{b}	1.97±0.4^{a}	2.57±0.6^{ab}	P<0.05
Kidney	3.84±0.6^{b}	3.65±0.4^{b}	2.19±0.5^{a}	3.07±0.6^{b}	P<0.05
Testis	6.82±2.1^{ab}	7.14±1.5^{b}	4.90±0.7^{a}	6.22±0.5^{ab}	P<0.05
Spleen	2.59±0.8	2.50±0.5	2.24±0.2	2.45±0.5	P>0.05

(*) Different letters of the groups in the same line are statistically significant (P<0.05).

lular oxidative stress (2). Antioxidant activities of pomegranate juice is evaluated and compared to green tea and red wine, and it was reported that pomegranate juice which is presented to public consumption, has rich aryl content and antioxidant activities are 3 times higher than the green tea and red wine. This potent antioxidant activity attributed to tannins which can be hydrolysable of pomegranate juice main antioxidant compound, and also to anthocyanins and ellagic acid derivatives (21). In this presented study, it was observed that the administration of PSO at a dose of 0.15 ml/kg bw for 28 days to rats increased GSH-Px activities in the testis and spleen without any significant negative effect on lipid peroxidation. Elevation of testis and spleen GSH-Px activities may be related to the accumulation of PSO at more high levels in these tissues. As mentioned in earlier studies this effect can be associated with especially punicic acid which is conjugated linoleic acid presence in PSO compound (51). Different parts of pomegranate have different antioxidant properties, 12 -20% of the total seed weight can be oil, and 80% of these oils contain conjugated fatty acid. Apart from these compounds, such as sterols, steroids are also found in minor compounds which form the main components of the nervous system. Pomegranate products have antibacterial, antiviral, antidiabetic and anticancer effects (32). Pomegranate extracts have effects like scavenging free radicals, reducing oxidative stress. This oil increases plasma antioxidant capacity and reduces cellular oxidative stress products and increases the levels of decreased GSH (5,26,32,41). In the studies regarding the protective effects of pomegranate products on experimental models of toxicity and disease, administration of pomegranate extract in liver-damaged rats caused elevation in CAT, SOD and GSH-Px enzyme activities and reduction in the formation of lipid peroxidation products in the liver (13). It was reported that administration of pomegranate extract to streptozotocin-induced diabetic rats, significantly reduced pancreatic MDA levels and brought the levels of GSH and the activities of antioxidant enzymes (SOD, CAT and GSH-Px) similar to the control group (3). Another study stated that pomegranate tea was administered to rats and the MDA levels in liver, kidney, brain and heart tissues were shown to be elevated in the trichloroacetic acid exposed group and that the stated parameters were approached towards the control values in the PSO+TCA group. They noted that the antioxidant and protective effects of PSO may be associated with the fatty acids present in its content (15). Previous study Soyer-Sarıca and Liman (44) noted that was decreased serum MDA and NO levels and erythrocyte SOD activities compared to the PCP group and PCP+PSO group, whereas erythrocyte CAT and GSH-Px activities was elevated. It has also shown that PSO administration reduced renal MDA levels in studies previously performed on kidney damage induced by different compounds in experimental animals (8-11).

The present study, when PSO+PCP group was compared to PCP group, it was observed that the MDA levels in brain, liver, and testis significantly decreased; CAT activity in spleen and SOD activity in brain and kidney significantly increased. When the control group was compared to the PSO+PCP group, the parameters examined were found to be similar to the control group. These results show that PSO reduces the severity of PCP-induced oxidative stress. These beneficial effects may be related to active substances, which are found at high levels in PSO, known to be protective against free radical damage. On the other hand the antioxidant effects of PSO can be attributed to active ingredients such as conjugated linoleic and linolenic fatty acids, flavonoids and vitamin E as mentioned in previous studies (6,12,18). In addition, fatty acids such as gallic acid, ellagic acid and punicic acid; flavonols and flavonol glycosides like quercetin, kaempferol, luteolin and apigenin; anthocyanins like delphinidin, cyanidin and pelargonidin; amino acids like methionine, valine and proline has protective features (3,9,13,15,32).

PCP is a compound which has a wide spectrum of biocidal activity, which is widely used as antifungal, especially in the wood industry, and which continues to have a residue problem in the nutrients due to its extremely slow degradation in the environment (5,20). PCP is carcinogenic and genotoxic in various experimental animals and that these effects may be mediated by free oxygen radicals resulting from its biotransformation in the liver (43,50). Chlorophenols studies as a metabolism poison. ATP production is reduced without effecting the electron transport chain in mitochondria. Thus, the oxidative phosphorylation is used in the body heat

instead of the energy metabolism that breaks the chain. With increasing body heat, metabolism increases and the cycle continues with acceleration. (27,17,29). PCP increases hepatic CYP-P450 enzyme levels, thus leading to the formation of free radicals (47). In present study, the MDA levels in brain, kidney, liver and testis were increased in the PCP group compared to the control group whereas the CAT activity in liver and spleen; GSH-Px activity in brain and testes; SOD activity in brain, liver and kidney were decreased. The results of the study indicate that PCP causes oxidative stress in the brain, liver, kidney and testes. As mentioned in previous study, the lipid peroxidative effects of PCP in tissues can be attributed to the production of free radicals due to increased CYP-P450 enzyme activity (47). Various enzymes such as SOD, CAT and GSH-Px are involved in the inactivation of free radicals. SOD reduces the superoxide radical to a less oxidizing agent H_2O_2, whereas GSH-Px and CAT inactivate H_2O_2 formed by SOD (23,36). In this study, decrease in antioxidant enzyme activities can be associated with excessive availability of free radicals produced by PCP exposure in the biological system.

In conclusion, it was determined that administration of PSO at the dose of 0.15 ml/kg bw dose for 28 days had no adverse effects on oxidant/antioxidant balance in rats, administration of PCP at the dose of 40 mg/kg bw for 28 days caused oxidative stress in brain, kidney, liver and testicular tissues in rats and administration of PSO at the dose of 0.15 ml/kg bw had a alleviating effect on the severity of PCP induced oxidative stress. In this context, pomegranate can be used as a supportive care in combination with other medical treatment options for PCP poisoning. As well as other preventive approaches, when there is a risk of exposure to PCP poisoning, it may be given as food supplement to minimize the toxic effects.

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Sorumlu Yazar:
Dr. Zeynep SOYER SARICA
Erciyes Üniversitesi,
Deneysel Araştırmalar Uygulama ve
Araştırma Merkezi,
Kayseri-TÜRKİYE
Tel:0352 20076666/24414
E-posta:zeynepsoyer_94@hotmail.com