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Hepatoprotective and nephroprotective effects of Al-Taif Pomegranate (*Punicagranatum L*) extract against toxicity induced by Atrazine and Malathion pesticides in male albino mice

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ABSTRACT

The objective of the present study was to elucidate the hepatoprotective activity of Al-TaifPomegranate peel extract (PPE) induced by atrazine (Atra) and malathion (Mal) toxicity in male mice by using biochemical assays (liver functions and antioxidant activities in liver and kidney homogenates). Male mice were divided into seven groups and treated intraperitoneallyas following: Negative control; Mal group (27 mg/kg); Atra group (120 mg/kg); PPE group (0.4 g/Kg);Mal + PPE group (27 mg/Kg + 0.4 g/Kg, respectively); Atra + PPE group (120mg/Kg + 0.4 g/Kg, respectively); Mal + Atra + PPE combined co-administration group.Biochemical results affordeda highlysignificant increase in the liver enzymes and kidney function parameters represented by AST,ALT, LDH, Urea andcreatinine for Mal or Atra groups, alsothey elicited significant increase in lipid peroxidation end product, MDA level in both liver and kidney homogenates. In contrast, co-administration of PPEandAtra and/or Mal-treated grouprestored almost most of these liver and kidney functions to normal levels. In conclusion, the present study suggested that Atra and Mal exposure lead to hepatotoxicity and nephrotoxicityin malemice and concomitant treatment with Al-Taif Pomegranate extract restored and ameliorated the liver and kidney tissues from deleterious oxidative and toxic damage.

Key words: Malathion, Atrazine, Pomegranate peel, Hepatotoxicity, nephrotoxicity, oxidative stress.
List of abbreviations: *Pomegranate peel extract, PPE; Atrazine, Atra; Malathion, Mal.*

INTRODUCTION

Pomegranate (Punica granatum L. Punicaceae), the common name is derived from Latin words (ponus and granatus), a seeded or granular apple, is a delicious fruit consumed worldwide. Pomegranate peels are characterized by an interior network of membranes comprising almost 26–30% of total fruit weight and are characterized by substantial amounts of phenolic compounds, including flavonoids(anthocyanins, catechins and other complex flavonoids) and hydrolysable tannins(punicalin, pedunculagin, punicalagin, gallicandellagic acid). These compounds are concentrated in pomegranate peel (PoP) and juice, which account for 92% of the antioxidant activity associated with the fruit **[1]**.

Punicagranatum L. (Punicaceae), commonly known as pomegranate, is a shrub or a small tree native to the Mediterranean region**[2]**. A number of biological activities such as antitumour, antibacteria and antidiarrhoea have been reported for extracts from different parts of P. granatum**[3]**. Furthermore, antioxidant activity accompanied with radioprotective and antifibrotic properties of P. granatum peel extract have been demonstrated recently **[4]**.

Pomegranate peel extracts exhibited marked antioxidant capacity in several studies using unsafe solvents such as methanol and a mixture of methanol, acetone, ethyl acetate and water **[5]**. Knowing that no such studies were performed on Pomegranate growing in Al-Taif, we decided to carry out a comparative study to assess extracts from different Al-Taifpomegranate compartments, namely the peels, with the aim of defining an effective standardized extract having a suitable yield to be incorporated into a pharmaceutical preparation as a dietary supplement.

Liver is the main organ responsible for multitude of essential functions and plays an important role in the metabolism of foreign compounds entering the body. Human beings are exposed to these compounds through consumption of contaminated food or during exposure to chemical substances in the occupational environment. These foreign compounds produce variety of toxic manifestations particularly in the liver.In Egypt, liver diseases are one of the most prominent killers specifically fibrosis, hepatitis C virus (HCV) and cirrhosis that alter the functions of the liver **[6].**.

Reactive oxygen species (ROS) are constantly generated in vivo for physiological purposes. Their productions are often balanced by antioxidant defense system. However, excess ROS production beyond the ability of antioxidant defense system can cause oxidative damage to protein, lipid and nucleic acid **[6]**. Antioxidant defense include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), in addition to low molecular agents and dietary antioxidants. Disturbing of oxidant–antioxidant balance system is involved in development of many chronic diseases such as atherosclerosis, cancer and diabetes **[7]**.

The objective of the present study was to evaluate the hepatoprotective and nephroprotectiveeffects of PPE induced by Atra and Mal in male mice. The antioxidant activity of PPE was investigated, in addition to the antioxidant enzymes level estimation including: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and lipid peroxidation (represented by Malonaldahyde (MDA)) in the liver tissues of Atra and Mal treated mice comparing with negative control.

EXPERIMENTAL SECTION

Animals

This study was performed on 70 mature male mice, weighing about 35–45 g bw. Animals were obtained from the animal house of the King Fahd Center for Medical Research, King Abd El-Aziz University in Jeddah. They were breeding in a well-ventilated room with the temperature ranging between 22 and 25 ˚C and maintained under standardized conditions away from any stressful conditions with 12/12 light and dark cycle with free access to humidity and were fed dry balanced meals for experimental animals provided by the General Organization for Grain Silos and Flour Mills in Jeddah, with a constant source of water. All experimental procedures and animal maintenance were conducted in accordance with the accepted standards of animal care per cage (Council of Europe, European convention for the protection of vertebrate animals 2006). We have followed the European community Directive (86/609/EEC) and national rules on animal care.

Chemicals

Mal and Atrawere produced by Misr for Agricultural Development Company, Cairo, Egypt.Atra in the commercial product as Cotrazine 80WP (an 80% wettable powder) was obtained from Alderelm limited UK.Pomegranate was supplied by local market in Al-Taif city, Saudi Arabia, the peel of pomegranate was washed and then dried and then was grinded by using electrical mixer and then was prepared for intraperitonealinjection for male mice, also we used the juice of the pomegranate fruit.

Experimental protocols

Mice were divided into seven groups, seven mice/group. Group1:Negative control group treated with 1 mg/Kg bw corn oil/day;Group2: Malgroup (27 mg/kg bw (1/50) of the LD50 for an oral dose) per day in corn oil intraperitoneally**[8]**; Group3:Atragroup treated daily with 0.24 ml vehicle suspension of 80% (w/w) Atra equivalent to 120 mg/kg body weight mg/Kg bw per day in corn oilintraperitoneally**[9]**;Group4:PPEgroup treated with PPE

(0.4 g/Kg bw/day in corn oil);Group 5 :Mal+PPEco-administration group (27 mg/Kg bw+0.4 g/Kg bw/day, respectively); Group 6:Atra +PPE co-administration group (120mg/Kg bw+0.4g/Kgbw/day, respectively); Group7: Mal + Atra + PPEcombined co-administration group treated in the same doses as previously discussed.

The dose of PPEwas chosen on the basis of previous studies **[10]**.The substances were administered in the morning (between 07.00 and 8.30 am) to non-fastedmice. Mice were injected intraperitoneally with Atra and/or Mal followed by PPE and/or PJ after 30 mins daily for successive 30 days as the treatment schedule that previously mentioned.All animals were sacrificed and dissected. The liver tissues were quickly excised for biochemical evaluation.

Biochemical estimation

Liverfunction biomarkers in the serum

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined colorimetrically by measuring the amount of pyruvate or oxaloacetate produced by forming 2,4-dinitrophenylhydrazine, the color of which was measured at 546 nm **[11]**. Lactate dehydrogenase was determined according to the method of **[12]**which depends on the oxidation of lactate to pyruvate with the simultaneous conversion of the cosubstrate NADH to NAD. The decrease in absorbance (measured at 340 nm) due to this conversion is directly proportional to LDH activity.

2.5 Kidney function parameters:

Serum urea was determined calorimetrically using Diamond kit **[13]**, according to the following reaction. Urea + $HO_2 \xrightarrow{\text{urease}}$ 2 NH_3 + CO_2. In an alkaline medium, ammonium ions react with the salicyate and hypochlorite to form a green colored indophenol (2.2 dicarboxylindophenol).

Creatinine form colored complex when react with Picrate in alkaline solution **[14]**.

Liver tissue homogenates preparation

Liver was immediately removed; weighed and washed using chilled saline solution. Tissues were minced and homogenized (10% w/v), separately, in ice-cold sodium, potassium phosphate buffer (0.01 M, pH 7.4) containing 1.15% KCl in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 10,000 Xg for 20 min at 4˚C, and the resultant supernatant was used for the determination of antioxidant enzyme and lipid peroxidation assays. All used reagents were of the highest grade commercially available.

Lipid peroxidation assay

Thiobarbituric acid reactive substances (TBARS) content was evaluated using the thiobarbituric acid (TBA) test as described by Ohkawa et al.[15]. After incubation of liver homogenate with TBA at 95 ˚C, TBARS reacts to form a colored complex. Absorbance was measured spectrophotometrically at 532 nm to determine the TBARS content. The specific activity is expressed as nmol/mg protein protein. This assay was used to estimate lipid peroxidation end product, Malonaldahyde (MDA).

Measurement of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was measured according to the method described by Marklund and Marklund[16]by assaying the auto oxidation of pyrogallol at 440 nm for 3 min. One unit of SOD activity was calculated as the amount of protein that caused 50% pyrogallol auto-oxidation inhibition. A blank without homogenate was used as a control for non-enzymatic oxidation of pyrogallol in Tris–EDTA buffer (50 Mm Tris, 10 mM EDTA, pH 8.2). The SOD activity is expressed as U/mg protein.

Measurement of catalase (CAT)

Catalase (CAT) activity was measured according to the method described by Aebi[17] by assaying the hydrolysis of H_2O_2 and the resulting decrease in absorbance at 240 nm over a 3 min period at 25˚C. Before determination of the CAT activity, samples were diluted 1:9 with 1% (v/v) Triton X-100. CAT activity is expressed as mmol/mg protein.

Measurement of glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity was measured using H_2O_2 as substrate according to the method described by Paglia and Valentine [18]. The reaction was monitored indirectly as the oxidation rate of NADPH at 240 nm for 3 min. A blank without homogenate was used as a control for non-enzymatic oxidation of NADPH upon addition of hydrogen peroxide in 0.1 M Tris buffer, pH 8.0. Enzyme activity was expressed as nmol/mg protein.

Statistical analysis

Data were collected, arranged and reported as mean ± standard error (mean±SE) for all groups (each group was considered as one experimental unit). Student t-test was computed to test the significance difference between different groups. Statistics were carried out using statistical analysis systems (SAS) program (SAS, 2005)®. $P<0.05$ was considered as the level of significance.

RESULTS

Biochemical estimation

- **Effect on Liver function parameters:**

Table 1 and Fig. 1 represented the mean values ± standard error (Mean ± SE) of liver function parameters. Different data of all experimental groups were evaluated by using student t-test.

Table (1) and Fig 1 (A,B,C) showed that treatment of normal mice with either Atra or Mal afforded highly significant increase in AST , ALT and LDH levels as compared to normal control group , Meanwhile treatment of male mice with PPE elicited non-significant changes in either AST , ALT or LDH levels with respect to normal control group.

At the same time, administration of PPE with either Mal or Atra afforded significant decrease in AST, ALT and LDH levels with respect to Mal and Atra treated groups but they showed significant increase as compared with normal control group. On the other hand treatment of male mice with combination of Atra,Mal and PPE afforded more ameliorated results as compared with other combinations.

- **Effect on antioxidant capacities in liver tissue homogenates:**

At the level of SOD, Gpx and CAT in liver tissue homogenates, Table (2) and Fig 2 (A, B,C , D)treatment with Mal or Atraled to a significant decrease of their levels in comparison with the negative control group. While co-administration of PPE with Mal or/and Atragroups led to increase in the SOD, Gpx and CAT levels but still significantly different from the negative control group.While, the level of SOD, Gpx and CAT of PPE co-administrated groupsshowed a statistically significant increase when compared with Mal or Atragroup, except at the level of SOD forAtra + PEE and CAT level of Mal +Atra + PPE groups, they showedsignificant decrease.

At the level of MDA, treatment with Mal or Atraled to a significant increase of MDA level in comparison with the negative control group. While co-administration of PPE with Mal or/and Atra groups led to decrease of MDA level but still significantly different from the negative control group. While, the level of MDA of PPE groupshoweda statistically highly significant decrease when compared with Mal or Atragroups.

Therefore, the present results elicitednoticed improvement in the antioxidant capacities of PPE, especially for the combined co-administration group (Mal + Atra + PPE), when compared with the negative control group.

- **Effect on antioxidant capacities in liver tissue homogenates:**

Table (3) and Fig 3 (A, B) showed that treatment of normal male mice with either Mal or Atra afforded highly significant increase in urea and Creatinine levels as compared with normal control group but PPE treated group afforded significant decrease in two parameters when compared with normal control group . Meanwhile, Co-administered groups treated with Mal, Atra and PPE elicited significant decrease in creatinine and urea levelsas compared with either Mal or Atra treated groups and the best combination was recorded in group treated with combination of Mal + Atra + PPE.

Table (1): Effect of Malathion (27 mg/kg),Atrazine (120 mg/kg),Al-Taif Pomegranate extract (0.4 g/Kg)and their combinations on Liver function in male mice (mean ± SE)

Groups	AST(U/ml)	ALT(U/ml)	LDH(µIU/ml)
1- Control group	13.20±0.58^{g}	12.00±0.70^{f}	371.66±47.21^{g}
2- Malathion treated group	197.80±7.39^{b}	101.00±1.18^{b}	1828.40±35.37^{a}
3- Atrazine treated group	230.80±6.02^{a}	122.80±1.82^{a}	1370.30±33.34^{b}
4- Pomegranate extract treated group	14.10±1.01^{fg}	14.20±2.10^{f}	405.32±14.06^{fg}
5- Malathion + Pomegranate extract treated group	45.00±1.67^{d}	73.20±0.86^{d}	920.00±30.37^{c}
6- Atrazine + Pomegranate extract treated group	56.40±3.12^{c}	95.40±0.50^{c}	862.66±50.63^{d}
7- Malathion + Atrazine + Pomegranate extract treated group	24.20±4.61^{e}	40.60±2.73^{e}	520.31±51.26^{e}

Means within the same column in each category carrying different litters are significant at (P ≤ 0.05) using Duncan's multiple range test, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically.

Table (2): Effect of Malathion (27 mg/kg), Atrazine (120 mg/kg), Al-Taif Pomegranate extract (0.4 g/Kg)and their combinations on oxidative stress markersin liver homogenates ofmale mice (mean ± SE)

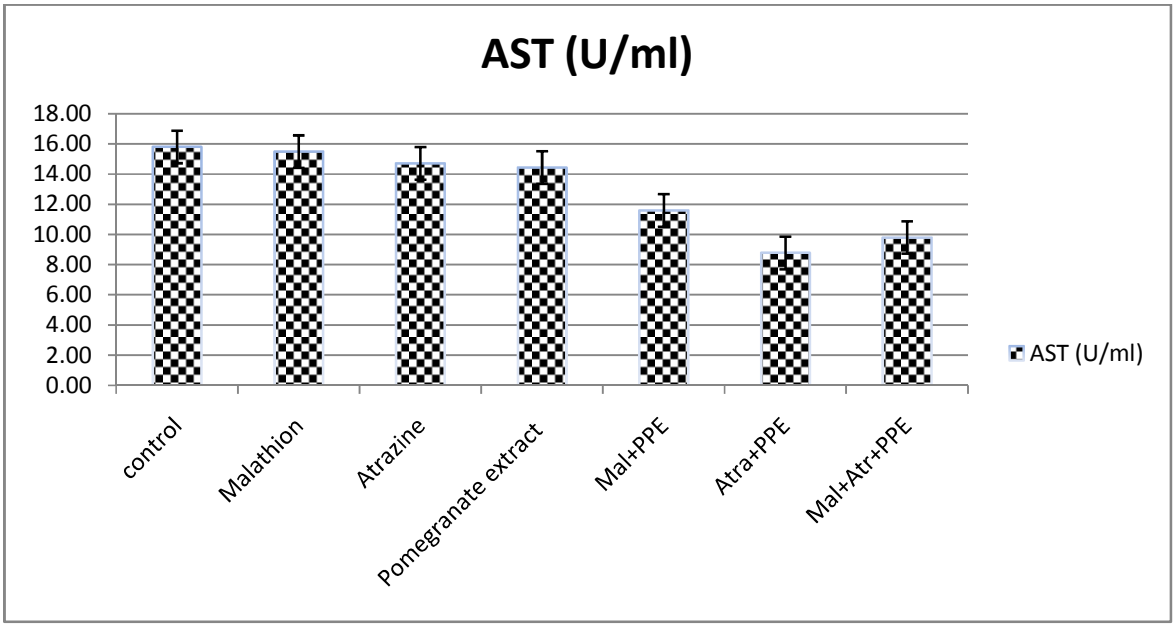
Groups	Liver Catalase (U/g)	Liver SOD (U/g)	Liver MDA (nmol/g)	Liver Glutathione Peroxidase (U/g)
1- Control group	4.29±0.01^{a}	23.32±0.87^{a}	1.74±0.10^{g}	6.91±0.75^{a}
2- Malathion treated group	1.72±0.03^{fg}	7.61±1.52^{fg}	29.35±0.26^{b}	1.12±0.48^{f}
3- Atrazine treated group	1.43±0.03^{g}	5.14±2.98^{g}	32.50±0.66^{a}	1.99±0.58^{e}
4- Pomegranate extract treated group	4.03±0.04^{b}	18.82±0.91^{b}	2.81±0.12^{f}	5.30±0.94^{b}
5- Malathion + Pomegranate extract treated group	2.57±0.04^{e}	14.94±1.66^{d}	17.30±0.25^{d}	2.01±0.82^{d}
6- Atrazine + Pomegranate extract treated group	2.72±0.05^{de}	12.92±1.59^{e}	19.38±0.38^{c}	2.03±1.56^{d}
7- Malathion + Atrazine + Pomegranate extract treated group	3.95±0.03^{c} BVGFTR5E435RTYJ,MVBNJKL;'\ \';LKJHTRE430-[';LKNBVCXZ\0.02^{d}	15.30±1.21^{c}	14.04±0.26^{e}	3.77±1.19^{c}

Means within the same column in each cat=-09q gory carrying different litters are significant at (P ≤ 0.05) using Duncan's multiple range test, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically.

Table (3): Effect of Malathion (27 mg/kg), Atrazine (120 mg/kg), Al-Taif Pomegranate extract (0.4 g/Kg)and their combinations on Urea and creatinine levels in male mice (mean ± SE)

Groups	Urea(mg/dl)	Creatinine(mg/dl)
1- Control group	22.50±1.20^{g}	0.58±0.01^{f}
2- Malathion treated group	51.00±4.08^{b}	0.97±0.02^{b}
3- Atrazine treated group	84.20±1.88^{a}	0.89±0.02^{a}
4- Pomegranate extract treated group	29.10±1.51^{fg}	0.62±0.02^{ef}
5- Malathion + Pomegranate extract treated group	42.54±0.70^{d}	0.71±0.01^{c}
6- Atrazine + Pomegranate extract treated group	49.10±2.77^{c}	0.75±0.01^{c}
7- Malathion + Atrazine + Pomegranate extract treated group	37.08±2.10^{e}	0.70±0.01^{d}

Means within the same column in each category carrying different litters are significant at (P ≤ 0.05) using Duncan's multiple range test, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically.


AST (U/ml)
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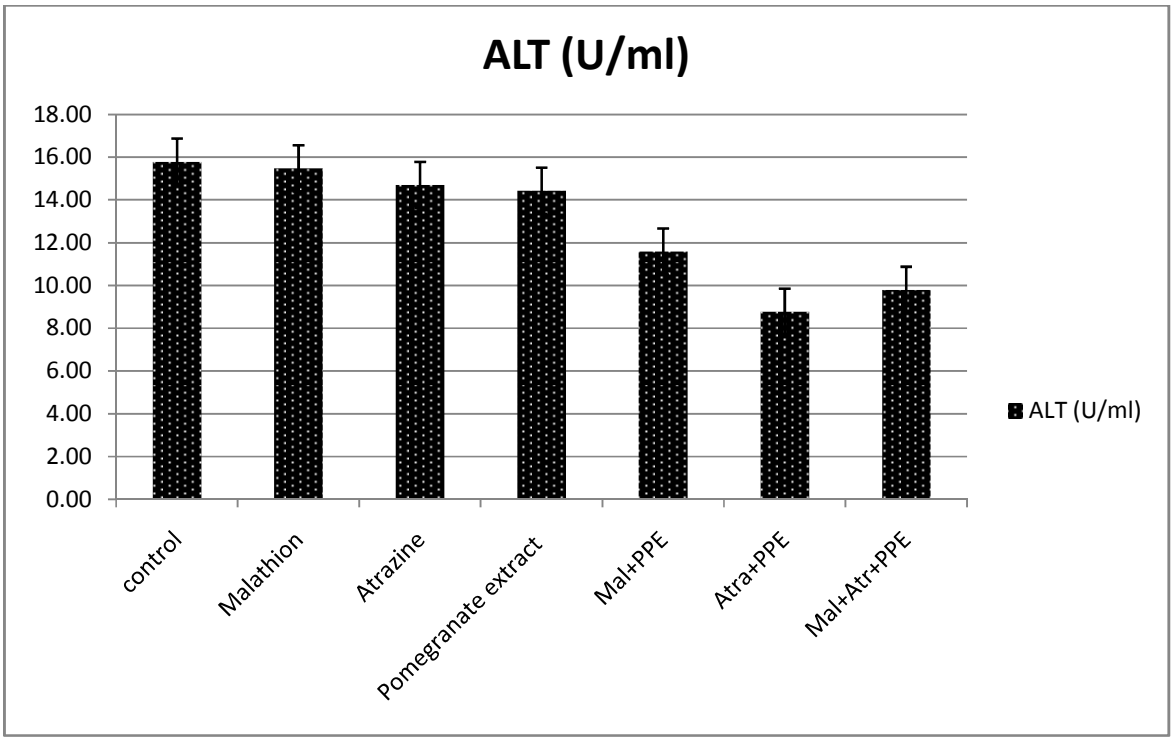
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
control
Malathion
Atrazine
Pomegranate extract
Mal+PPE
Atra+PPE
Mal+Atr+PPE
AST (U/ml)

(A)


ALT (U/ml)
18.00
16.00
14.00
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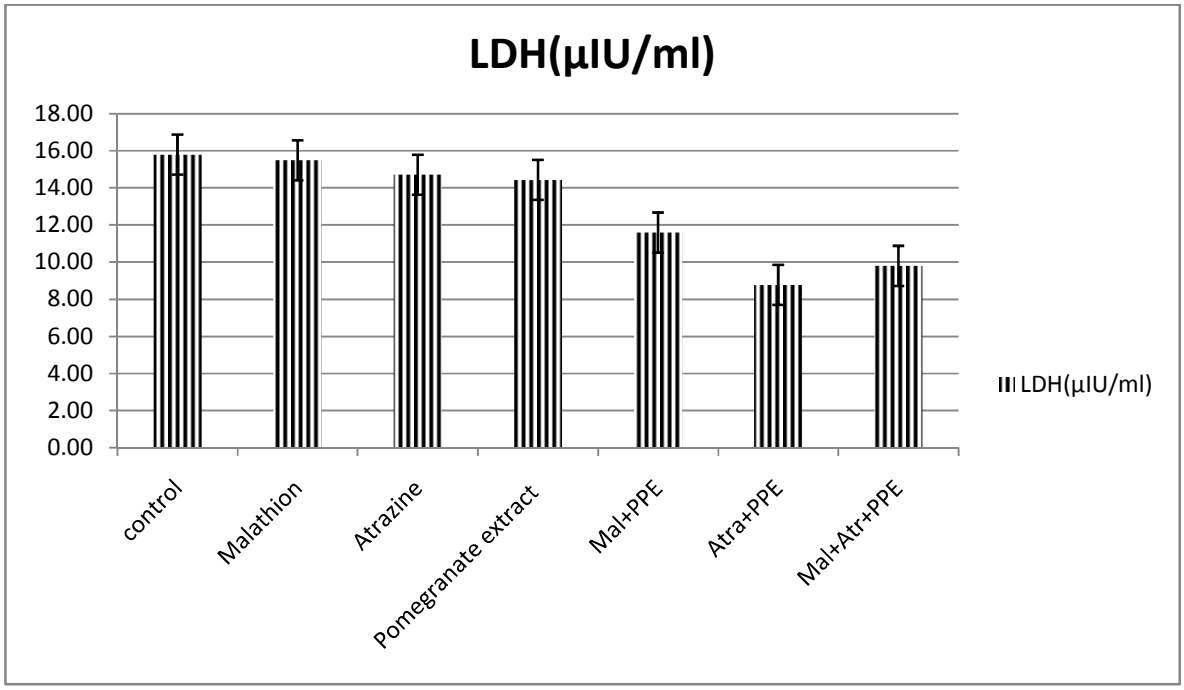
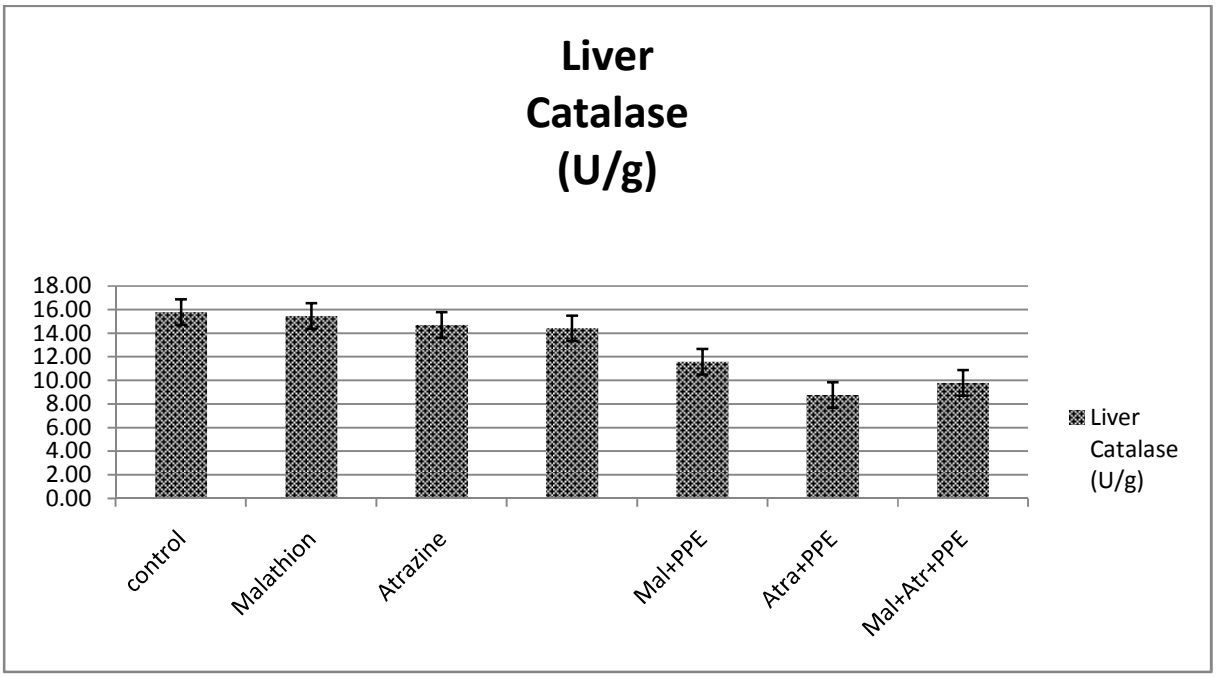
8.00
6.00
4.00
2.00
0.00
control
Malathion
Atrazine
Pomegranate extract
Mal+PPE
Atra+PPE
Mal+Atr+PPE
ALT (U/ml)

(B)

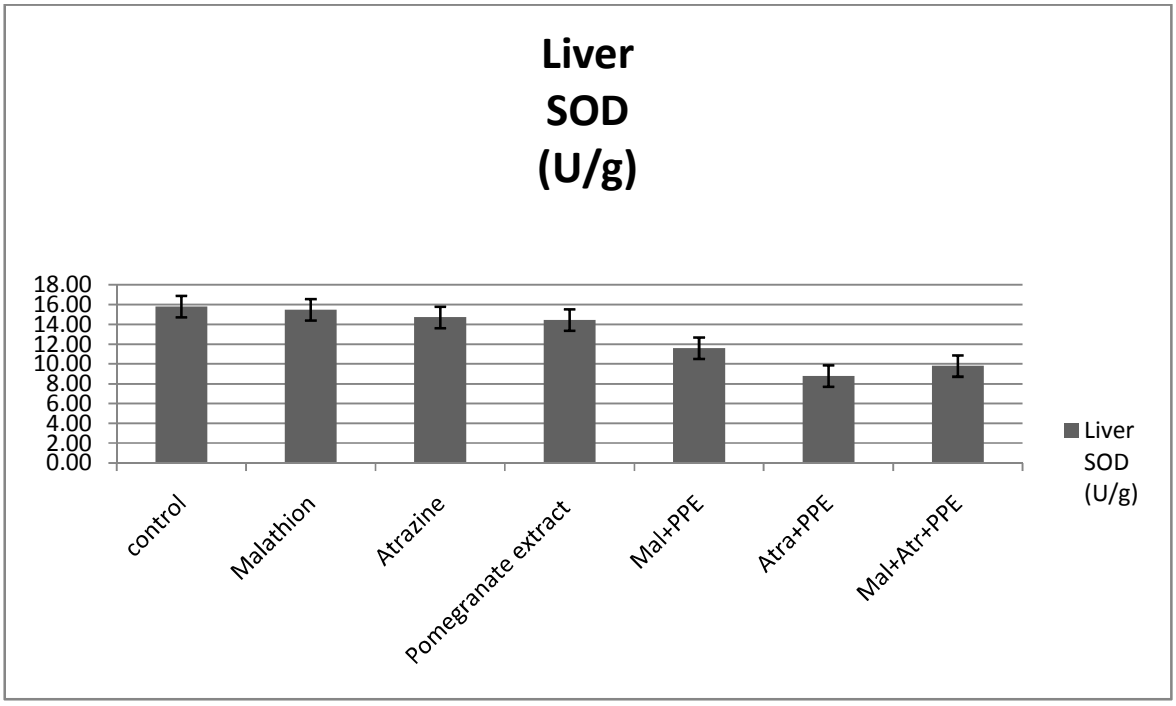


(C)

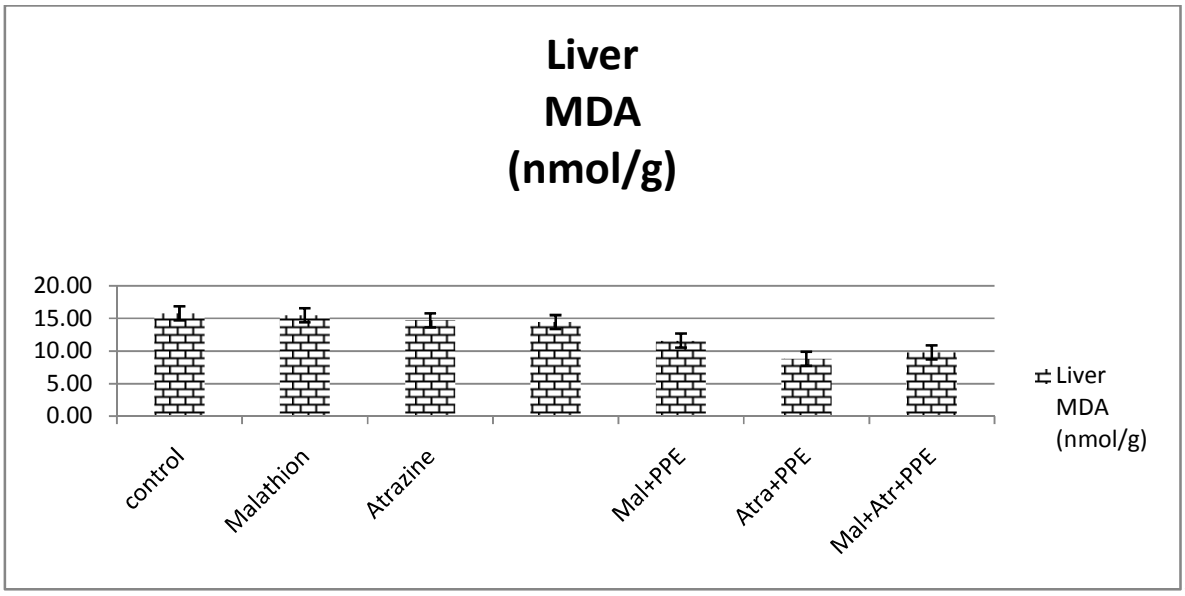
Fig (1): Effect of Malathion (27 mg/kg), Atrazine (120 mg/kg), Al-Taif Pomegranate extract (0.4 g/Kg)and their combinations on Liver function in male mice (mean ± SE)



(A)

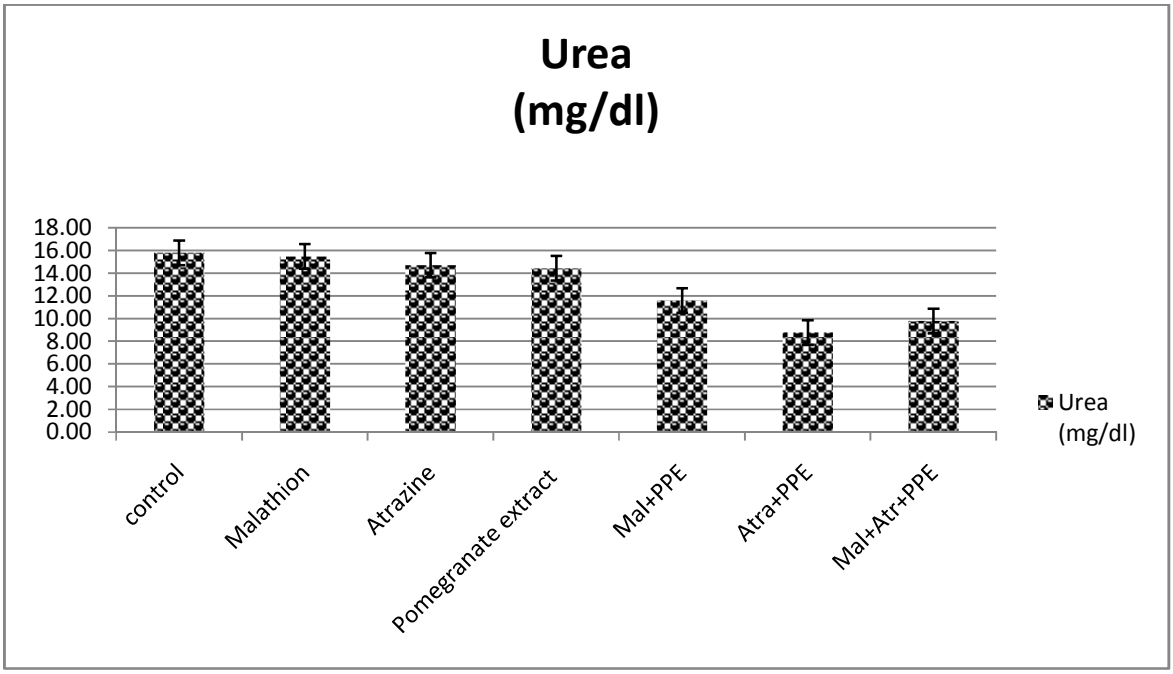


(B)

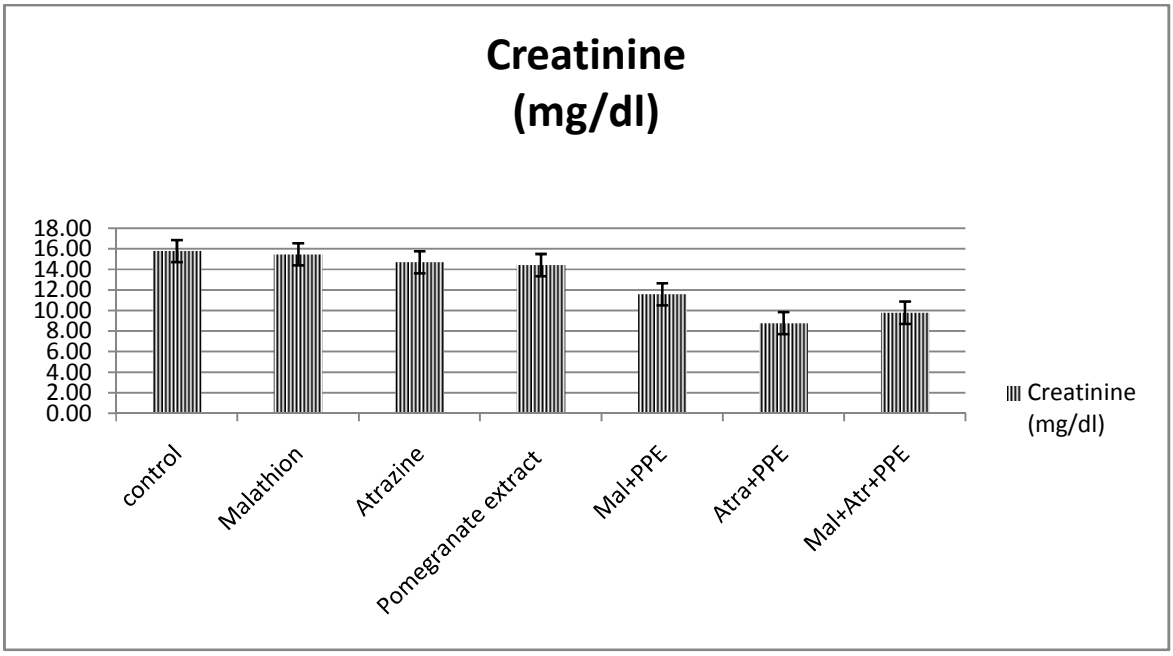


(C)

Fig (2): Effect of Malathion (27 mg/kg), Atrazine (120 mg/kg), Al-Taif Pomegranate extract (0.4 g/Kg)and their combinations on oxidative stress markers in male mice (mean ± SE)



(A)



(B)

Fig (3): Effect of Malathion (27 mg/kg), Atrazine (120 mg/kg), Al-Taif Pomegranate extract (0.4 g/Kg)and their combinations on Urea and creatinine levelsin male mice (mean ± SE)

DISCUSSION

The ubiquitous occurrence of contaminantsin the environment has become an ever-present concern. Such these compound s cause toxicity and sever damage even at low exposure levels.In the present study, mice treated with Mal or Atra showed increase in liver and kidney function parameters markers.

Mal and Atra induced a decrease in the antioxidant enzymes level of SOD, Gpx and CAT, while it showed an increase of the lipid peroxidation end product, MDA. Our results reported the toxicity of Atraand Mal treatment.Reactive oxygen species (ROS) are constantly generated in vivofor physiological purposes. Their productions are often balanced by antioxidant defense system. However, excess ROS production beyond the ability of antioxidant defense system can cause oxidative damage to protein, lipid and nucleic acid [19]. Antioxidant defense include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

The present results are in agreement with previous results, in which Akunnaet al.[20]and Durak et al. [21]reported the toxic potential ofAtrain rat testicular tissue and Mal in human erythrocytes treatment, respectively.They estimated a significant decrease in the activity level of GPx, SOD and CAT when compared to control group. Further, a significant increase in lipid peroxidation was observed also in Atraand Mal treated group when compared to control group. It has been largely reported that Atraand Mal induced organ toxicity could be as a result of their metabolites which may be involved in electron transfer, reactive oxygen species formation, and oxidative stress [22].

Co-administration of PPE and Atraor/and Mal treated groups restored almost most of these antioxidant defense capacities to normal levels and improved the hepatic damage and alleviates the toxic potential of Atra and Mal. This might be explained by their antioxidant potential against pesticides toxicity.

The conception of antioxidant action of phenolic compounds is not novel [23].According to Verstraeten et al. [24], in addition to known protein-binding capacity of flavanols and procyanidins, they can interact with membrane phospholipids through hydrogen bonding to the polar head groups of phospholipids. As a consequence, these compounds can be accumulated at the membranes' surface, both outside and inside the cells.

The kidneys are easily susceptible to damage from drugsbecause of larger perfusion and accumulation of excreted compounds that occur in renal tubular cells during absorption and secretion.

Pomegranate has become more popular because of the attribution of important physiological properties, such as anticancer, cholesterol-lowering, and cardioprotective[25].our results are in accordance with Cekmen et al. [25] and this study demonstrated ameliorative effects of PEE, a phenolic antioxidant, on GEN-induced nephrotoxicity, in line with the consideration that oxygen-free radicals are important mediators of GEN-induced acute renal failure.

Our results recorded an increment in Urea and creatinine levels in eith Mal or Atra treated groups while these parameters are highly ameliorated in groups treated with combination of PPE with Mal and Atra and these results are greatly agreed with the obtained results of Cekmen et al. [25]who reported increased serum urea and creatinine levels in GEN-treated rats reflect the renal damage .Meanwhile,Administration of PPE protects the kidney function from GEN as indicated by preventing an increase in serum urea and creatinine levels. PPE restores the renal function by preserving the structural integrity of renal cells against GEN challenge, evidenced by significantly preventing an increase in the levels of serum creatinineand urea.

The structural/functional integrity was assessed by the status of their respective biomarker enzymes. So, here, we measured the MDA, CAT, GSH, and SOD, as a means of oxidative stress. Our findings corroborate those of the earlier studies demonstrating that an enhanced endogenous oxidative stress has a major role in the severity of Mal or Atra-induced acute renal failure [26].

MDA, a stable lipid hydroperoxide, provides an index of the LPO in biological tissues.42 In this study, we found increased MDA levels in either Atra or Mal treated groups and this parameter was significantly decreased in PPE treated group and also in combined treated groups of Mal or Atra with PPE and these results are greatly reinforced by Cekmen et al. [25] who reported also increase in MDA level in the GEN-treated group, and as a protective effect of PEE, lower MDA levels were found in the group determined by GEN+ PPE.

GSH antioxidant systemis considered as the most notable cellular protective mechanism. GSH has a very important role in protecting against oxygen-free radical damage by providing reducing equivalents for several mechanism, as well as scavenging hydroxyl radicals and singlet oxygen. Its depletion is a common consequence of increased formation of ROS[27]likeMal or Atra-induced nephrotoxicity and this depletion is attenuated by co-administration of PPE. Our results go hand in hand with Cekmen et al. [25]who reportedthat group treated with GEN+ PE, we found increased GSH levels. So, PPE may enhance LPO by a different way. These findings strongly indicate that PE is important in protecting the kidney fromMal and Atra-induced injury through improvement in oxidant status.

At the end, the current results showed pesticides toxicity represented by Atra and Mal on the liver and kidney function parametersand antioxidant markers as a result of oxidative stress. In addition, we evaluate Al-Taif Pomegranate peel extract (PPE) antioxidant potential in ameliorating pesticides toxicity. Also we recommend further studies to assess Al-Taif Pomegranate potentials.

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