

Exposure to Aqueous-Alcoholic Extract of Parsley Leaves (*Petroselinum crispum*) in Lead-Treated Rats Alleviate Liver Damage

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Abstract

Lead (Pb) poisoning and the induced potential of the lesion is a global concern with harmful effects on multiple body systems, particularly the liver system. In this study, we have investigated the efficacy of aqueous-alcoholic extract of parsley leaves (PAE), which is a component of flavonoids that could play an important role in the antioxidant property, in preventing liver Pb-damages. For this, sixty adult male rats were randomly divided into six groups in a factorial arrangement: control; receiving oral gavage for 21 days with 2 mL water; 500 ppm Pb; 100 and 200 mg/kg PAE in combination form with Pb, and 200 mg/kg PAE. Liver enzymes and oxidative stress indexes were computed for liver stress, in blood serum. Apoptosis levels were assessed in the evaluation of liver gene expression. Data indicated that Pb reduced liver weight and feed intake. The results showed that Pb significantly increased the liver enzymes contents in the blood serum in the comparison vehicle group. Furthermore, the MDA contents of the Pb group rats were significantly more than that of the vehicle group. Likewise, T-AOC and the activities of CAT and SOD were significantly reduced. Meanwhile, Pb administration induced liver apoptosis-related genes by upregulating Bax and TNF- α genes and downregulating the Bcl-2 gene of animals. At the same time, administration of PAE significantly improved liver oxidative and apoptosis changes. Thus, this study provides a novel mechanistic approach concerning Pb-induced toxicity, due to PAE antioxidant activity.

Keywords: Lead, Stress, Liver, Apoptosis, Parsley leaves

Kurşun Toksikasyonu Oluşturulan Ratlarda Maydanoz Yapraklarının (*Petroselinum crispum*) Sulu-Alkollü Ekstraktı Karaciğer Hasarını Hafifletir

Öz

Kurşun (Pb) zehirlenmesi ve indüklenen lezyonun potansiyeli, başta karaciğer olmak üzere vücudun diğer birçok sistemi üzerine zararlı etkileri olan küresel bir endişedir. Bu çalışmada, antioksidan özelliğinde önemli bir rol oynayabilecek flavonoidlerin bir bileşeni içeren maydanoz yapraklarının (PAE) sulu-alkollü ekstraktının, kurşun tarafından indüklenen karaciğer hasarını önlemedeki etkinliğini araştırdık. Bunun için altmış yetişkin erkek rat, faktöriyel bir düzenle; kontrol grubu; 2 mL su ile 21 gün boyunca oral gavaj yapılan sham grubu; 500 ppm kurşun uygulanan grup; kurşun ile kombine halde 100 mg/kg ve 200 mg/kg PAE uygulanan gruplar ve 200 mg/kg PAE uygulanan grup olmak üzere rastgele altı gruba ayrıldı. Kan serumlarında karaciğer stresi için karaciğer enzimleri ve oksidatif stres indeksleri hesaplandı. Karaciğer gen ekspresyonunun değerlendirilmesinde apoptoz seviyeleri ölçüldü. Veriler, kurşun uygulamasının karaciğer ağırlığını ve yem alımını azalttığını gösterdi. Kontrol grubu ile karşılaştırıldığında kurşun uygulanan grubun kan serumundaki karaciğer enzimlerinin içeriğinin önemli ölçüde arttığı saptandı. Ayrıca, kurşun uygulanan gruba ait ratların MDA içerikleri, kontrol grubundan önemli ölçüde daha fazlaydı. Benzer şekilde, T-AOC ve CAT ve SOD aktiviteleri önemli ölçüde azalmıştı. Aynı zamanda, kurşun uygulaması, ratlarda Bax ve TNF- α gen ekspresyonlarını artırıp Bcl-2 gen ekspresyonunu azaltarak karaciğer apoptozisi ile ilgili genleri indükledi. Bununla beraber, PAE uygulaması, karaciğerin oksidatif ve apoptozis değişikliklerini önemli ölçüde iyileştirdi. Bu nedenle, PAE'nin antioksidan aktivitesiyle bu çalışma, kurşun kaynaklı toksisitede yeni bir mekanistik yaklaşım sağlamaktadır.

Anahtar sözcükler: Kurşun, Stres, Karaciğer, Apoptozis, Maydanoz yaprağı

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INTRODUCTION

Millions of people are exposed to environmental contaminants via drinking water, which contaminates soil, air, as well as fish, and other sea organisms living in contaminated waters. Human activities, particularly mining of metallic ores, is an important reason of environmental contamination. Lead (molecular formula: Pb, molecular weight: 207 g/mol) is a one of the most important common environmental contaminants that is widely distributed all around the world. It is also known as the most abundant heavy metals in the Earth's crust^[1]. In new reports of WHO, as of August 19, 2019, there is no level of exposure to Pb that is known to be without harmful effects. Thus, its cumulative toxicant that affects multiple body systems, particularly liver organs.

The liver performs many physiological processes essential for good health. These include macronutrient metabolism, immune system support, blood volume regulation, endocrine control of growth signaling pathways, and the breakdown of xenobiotic compounds, including many current drugs^[2,3]. Some reports have pointed out that the Pb causes varying degrees of toxicities in liver system of organisms such as humans^[4-7], poultry animals^[8,9], and carp^[10].

There is an increasing interest in this topic (medicinal herbs), due to the overall indicators of health decrease in industrialized countries, but there is still much to know concerning the effect of environmental pollutants on the liver system. In folk medicine, *Petroselinum crispum*, parsley, is used to treat a wide variety of conditions^[11]. It is thought that the health promoting effect (antibacterial and antioxidant activity) of parsley may be due to its phenolic compounds^[12]. Further, Kamal et al.^[13] reported that the parsley showed a significant decrease in the blood serum activity of liver enzymes. This result indicated that parsley had able to regenerate liver system after liver cell damage in diabetes mellitus as it contains flavonoids, particularly the quercetin. In a study by Soliman et al.^[14], performed on rats, dexametasone exposure increased oxidative stress indices, while parsley extracts succeeded to modulate these observed abnormalities as indicated by the reduction of glucose, cholesterol, liver thiobarbituric acid, liver enzymes and the pronounced improvement of the investigated biochemical and antioxidant parameters. Since studies on the rat model and including Pb and liver quality data are lacking, the present study was designed to investigate the effect of antioxidant content of parsley leaves on the recovery of liver damages in Pb toxicity in a rat model.

MATERIAL AND METHODS

Chemicals and Ethics

Unless otherwise indicated, all reagents were obtained from Merck (Darmstadt, Germany). The kits to evaluate

liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALK), and various oxidative stress indices including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (T-AOC) were purchased from Nanjing Jiancheng Bioengineering Institute (China). The Animal Care and Use Committee of the Islamic Azad University (Shiraz, Iran) approved all experimental procedures of the study that were performed according to international guidelines (Permit Number: 99-02-18-44385).

Animals

Healthy adult male Wistar rats (210±25 g; 8-week-old) were purchased from the Pasteur Research Center (Karaj, Iran). Rats were kept in an air-conditioned room under 12 h light:dark cycle under standard environmental conditions (22±1°C, 52±5% humidity) with free access to tap water and commercial dry pellet diet. Rats were housed in polypropylene cages lined with pine wood husk, changed every day.

Method for Used Aqueous-Alcoholic Parsley Leaves Extraction

The method used for extraction of parsley leaves in this experiment was performed as previously described by Ozsoy-Sacan et al.^[15]. Briefly, the air-dried leaves were extracted by adding sufficient ethanol in percolator device for 24 h. The extract was then filtered, and the filtrates were evaporated, using a rotary evaporator under reduced pressure to dryness. The extract was dissolved in distilled water before the administration to normal and Pb-induced animals.

Experimental Design

Sixty adult rats were divided into six groups in a factorial arrangement: control (no treatment); receiving oral gavage for 21 days with 2 mL water; 500 ppm Pb administrated as lead acetate; 100 and 200 mg/kg aqueous-alcoholic extract of parsley leaves (PAE)^[16] in combination form with Pb, and 200 mg/kg PAE. One day after the last treatment, blood samples were processed for liver enzymes and oxidative stress parameters and liver tissue samples were processed for apoptosis-related genes expression. Body weight and feed consumption per animal were recorded weekly. Measurements were obtained in the fasting state.

Liver Enzymes Assay

Blood was obtained via the tail vein for the assay of AST, ALT, and ALK. The serum was separated by centrifugation (4000 rpm for 10 min), kept at -20°C and assessed by radioimmunoassay (MonobindInc kit), as recommended by the manufacturer.

Determination of Oxidative Stress Indices

The method used to detect the oxidative stress indices

levels in this experiment was performed as previously described by Jiang et al.^[17]. Briefly, the levels of MDA, the enzymatic activities of SOD, and reduced CAT were measured by commercial kits, as recommended by the manufacturer. Absorbance of the supernatant was calculated at 532, 550 and 405 nm, respectively.

Firstly, 0.006 g 2,2-diphenyl-1-picrylhydrazyl (DPPH) was mixed in 15 µL methanol/water solution (1:9; 380 µL (100 mM) pH 7.4 + 20 µL Sample + 400 µL DPPH). The mixture was maintained at 20°C for 30 min and the absorption rate was measured at 530 nm.

The following protocols were used to prepare the blank (1) and the method of calculating the absorption of samples:

(1): 380 µL methanol + 400 µL DPPH + 20 µL phosphate buffer

(2): Activity [% of DPPH reduction] = $[(A-A_x)/A] \times 100\%$

A = DPPH + methanol

A_x = DPPH + sample

Quantitative Real-time PCR

Total RNA was isolated from livers weighing 25-30 mg using Trizol reagent (Life Technologies, Carlsbad, CA, USA). The 2% agarose gel electrophoresis was used to assess the integrity of total RNA and the A260/280 ratio was in the range of 1.8-2.0 evaluated by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was reverse transcribed using a PrimeScript™ RT Master Mix kit. QRT-PCR was carried out using the QuantStudio 7 Flex QRT-PCR system (Stratagene, USA) and SYBR® Premix Ex Taq™ II kit. Specific primers were designed by Invitrogen, USA (Table 1). β-actin (reference gene) was used in order to normalize the expression level of target genes. Duplicated Ct values were measured for each sample, and the comparative Ct method was used to determine the relative expression level of the target genes.

Statistical Analysis

Statistical analysis was performed using SPSS 13.0 software. The results were expressed as the means and standard

deviations (mean±SD) and performed with one-way analysis of variance (ANOVA) followed by Dunnett's new multiple range test and values of P<0.05 were considered as statistically significant.

RESULTS

Information about the phenolic compounds identified in parsley leaves are presented in Table 2.

Effects of orally administrated of Pb and PAE (single and combined) on the body and liver weights, liver enzymes and oxidative stress indexes of rats are presented in Table 3 and Fig. 1, 2, and 3, respectively.

The Table 3 shows body and liver (average) weights in each group (mean±SD). Significant difference was not observed (P>0.05) for the body weight between the treatment groups. Feed intake and liver weight was significantly lower in the Pb diet group comparing to the other groups (P<0.05). Compared with Pb-treated rats, the administration of PAE significantly (P<0.05) increased feed intake and liver weight.

Results presented in Fig. 1 showed significant increase in the liver enzymes (AST, ALT, and ALK) in Pb-treated animals. Administration of PAE (especially 200 mg/kg) in Pb given animals caused significant (P<0.01) recovery in the liver enzymes production similar to controls, however not attaining the same level in the controls.

Considering the control group, Pb-given animals exhibited significantly higher blood serum of the MDA contents. Results regarding to the oxidative stress indices showed significant (P<0.05) changes in contents in rats diet supplemented with Pb (with oral gavage) as compared to control at the end of the experiment. Concurrently, all these changes were recovered by PAE as a candidate therapy (Fig. 2, P<0.05).

Data of mRNA expression levels of apoptosis markers gene such as Bax and TNF-α and Bcl-2 were examined by QRT-PCR (Fig. 3). mRNA expression levels of apoptosis markers genes (Bax and TNF-α) were significantly up-regulated in

Table 1. Primers used for QRT-PCR, sequence, and product size

Target Gene	PCR Fragment Length (bp)	Sequences (5'-3')
BAX	111	Forward: AGGCGAATTGGCGATGAACTGG
		Reverse: AAACATGTCAGCTGCCACACGG
BCL-2	126	Forward: TGGCTCTGCCATTCTGTACG
		Reverse: GCTGCTTGCTGTTAGTTCCG
TNF-α	238	Forward: AGGCAATAGTTTTGAGGGCCAT
		Reverse: CATCAAGGATACCCCTCACACTC
β-actin	233	Forward: AGACTTCGAGCAGGAGATGG
		Reverse: GCACTGTGTTGGCATAGAGG

Primer sets designed using free online software Primer3Plus (v. 0.4.0) <http://primer3plus.cgi>

Table 2. Phenolic compounds of parsley (*Petroselinum crispum*) in the aqueous-alcoholic extracts of the tested parsley leaves

Compounds	Retention Times (min)	Percentage (%)	Compounds	Retention Times (min)	Percentage (%)
Sabinene	7.79	0.211	Trans-chrysanthenyl acetate	18.88	0.422
Limonene	9.98	2.530	Carvone	19.89	4.716
Cis-b-ocimene	10.23	46.390	Unknown	20.34	9.744
d-Ocimene	10.54	6.406	Bicyclogermacrene	32.78	0.915
Dihydrotagetone	11.10	1.408	Hexadecanoic acid	53.29	3.238
Trans-β-ocimene	15.33	6.938	Eicosane	57.61	0.915
Trans-tagetone	15.95	2.393	Methyl linoleate	59.68	6.192
Verbenone	18.54	0.563	Stearic acid	60.28	0.492
Total					93.385

Table 3. Effects of dietary lead (Pb) and aqueous-alcoholic extract of parsley leaves (PAE) on body and liver weights (g) and feed intake (g) of rats (means±S.D.; n=10)

Group	Body	Liver	Feed Intake
Control	265±18.04	11.59±0.92 ^a	2.98±0.67 ^a
Sham	264±18.26	11.23±0.8 ^{ab}	2.88±0.53 ^{ab}
Pb	245±23.8	8.59±1.32 ^f	1.87±0.32 ^f
Pb+PAE 100	254.28±14.2	10.22±0.79 ^{abcde}	2.17±0.12 ^{abcd}
Pb+PAE 200	257±20.01	10.99±0.81 ^{abcd}	2.13±0.56 ^{abcde}
PAE 200	260±16.25	11.01±0.72 ^{abc}	2.24±0.42 ^{abc}

Superscripts (a-f) show significant differences in each column (P<0.05)

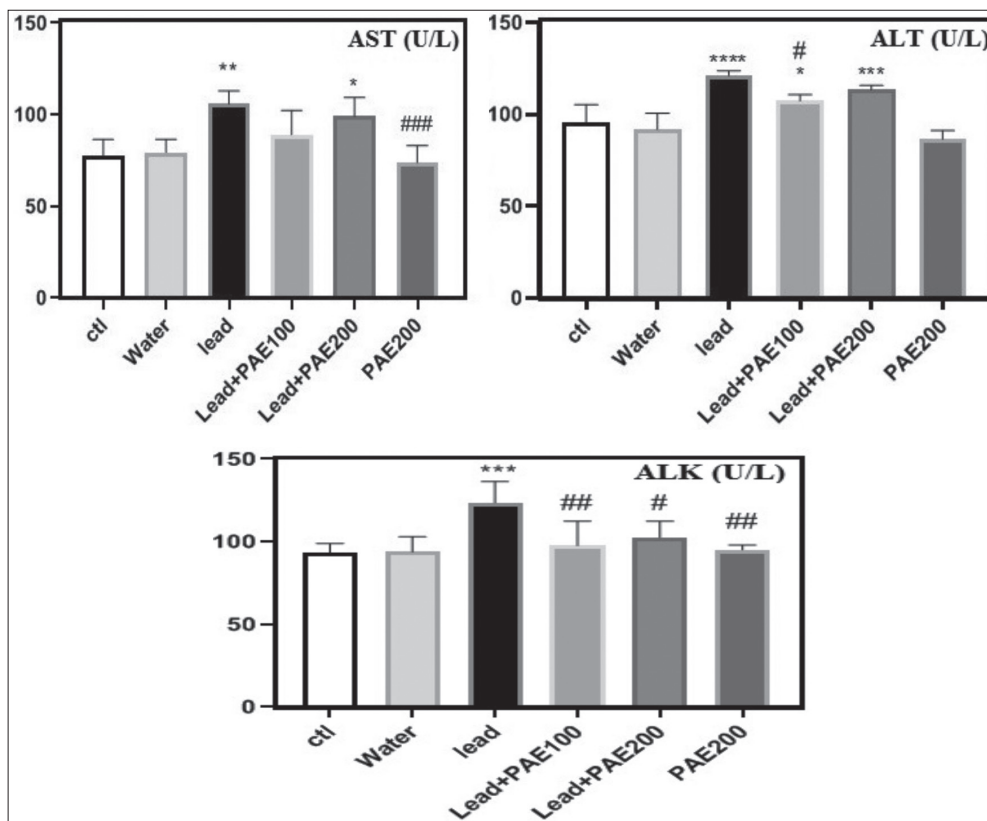


Fig 1. Effects of dietary lead and aqueous-alcoholic extract of parsley leaves (PAE) on liver enzymes level of rats. Aspartate amino transferase (AST); alanine amino transferase (ALT); alkaline phosphatase (ALK). Graphs show means±S.D. of 10 rats; Symbols indicate that treatments differ P<0.05 within each groups

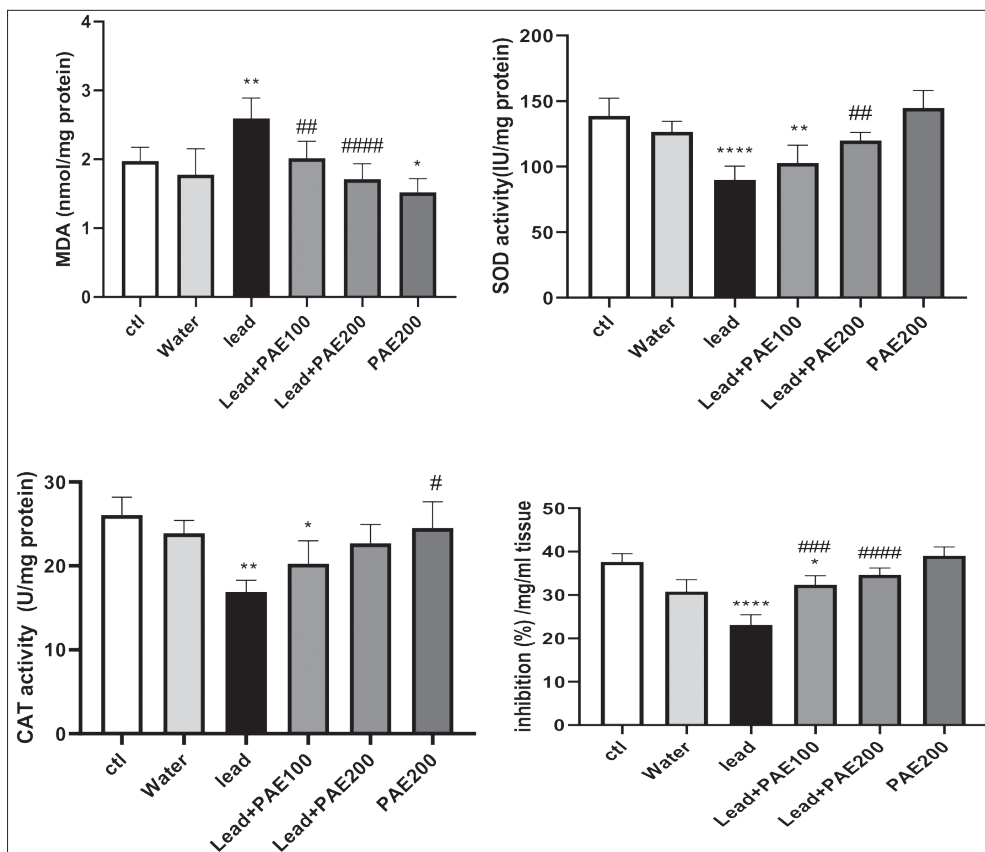


Fig 2. Effects of dietary lead and aqueous-alcoholic extract of parsley leaves (PAE) on oxidative stress indexes of rats. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity or inhibition. Graphs show means \pm S.D. of 10 rats; Symbols indicate that treatments differ P<0.05 within each groups

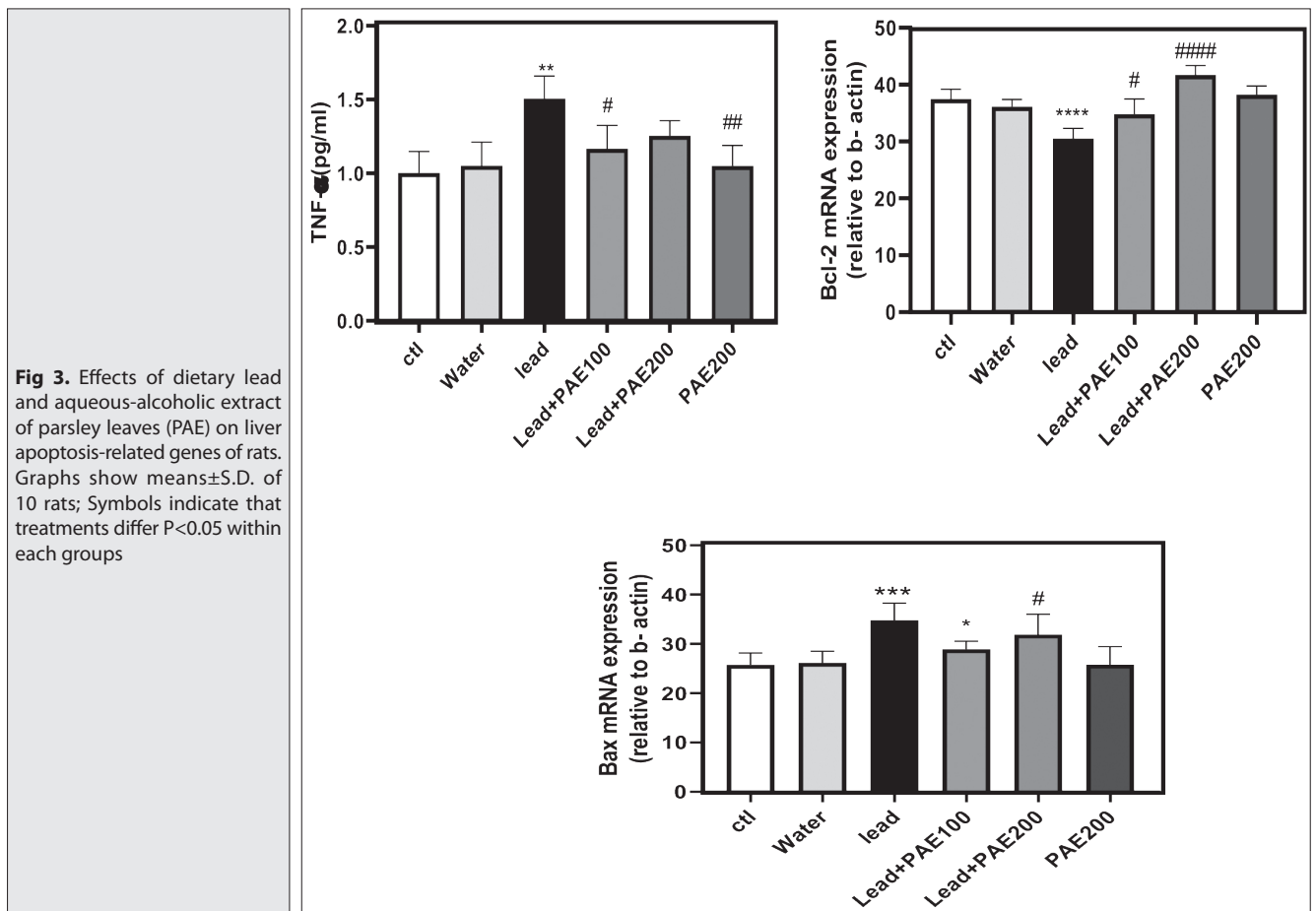


Fig 3. Effects of dietary lead and aqueous-alcoholic extract of parsley leaves (PAE) on liver apoptosis-related genes of rats. Graphs show means \pm S.D. of 10 rats; Symbols indicate that treatments differ P<0.05 within each groups

Pb exposed groups compared to control group ($P < 0.05$). However, Bcl-2 was down-regulated by Pb administration. Meanwhile, exposure to PAE in this study, led to a significant decrease in apoptosis related genes ($P < 0.05$). All these changes proportionally were abolished by treatment with PAE (especially 200 mg/kg).

DISCUSSION

In the era of rising environmental contaminant, scientists need to be more aware of the detrimental effects of these agents on multiple body systems, particularly liver system. Nowadays, many environmental pollutants affect the liver system, and common therapeutic methods often results in unsatisfactory results for management of disorders of the liver, including liver disease, hepatitis, cirrhosis and liver cancer.

Supplementing medicinal herbs in animal model for the promotion of health is an increasingly more common management tool. Likewise, this experiment reported the potential beneficial health effect of PAE on Pb-induced liver dysfunction in rats and explored the possible mechanisms.

In the present study, the difference in feed intake, liver weight, liver enzymes, oxidative biomarkers and apoptosis-related genes between control and treated Pb animals is likely attributed to differences in daily content of diet with oral gavage of Pb and PAE. In Pb given groups feed intake was reduced, thus during the study maybe the animals compensate their need to energy through lipolysis. It can be concluded that Pb increases lipolysis through increasing the production of free radicals (*Fig. 2*) and formation of reactive oxygen species and decreasing feed intake (*Table 3*). Our study proved that Pb promotes the excessive generation of free radicals, which could disrupt the balance between oxidant and antioxidant enzyme systems in liver tissues. Thus, the activities of antioxidant enzymes fail to inhibit the excessive generation of lipid peroxide, especially MDA contents (the end product of lipid peroxidation). In Pb-given groups, liver damage is associated with enhanced MDA levels. And, most of the recent literature have reported that excess of reactive oxygen species (ROS) will break homeostasis, such as the increase of MDA content, the reduction of oxidative damages, and the ability to resist OH% and inhibition of many anti-oxidative enzyme activities^[18,19]. The low levels of MDA in PAE groups might be responsible for the withdrawal of the inhibitory effect of Pb toxicity on liver antioxidant system as well as the diminution in free radical generation. All these changes in oxidative stress indices proportionally were abolished by PAE as a candidate therapy.

Organism's exposure to environmental pollutants are usually analyzed through the evaluation of blood. Results of blood analysis show that a significant increase in liver

enzymes and the amount of oxidative stress indices reported post Pb exposure, partly due to increased oxidative stress. This phenomenon is in line with the earlier reports in humans^[5-7], poultry^[8,9], and carp^[10] models, which have been reported that change in liver status may be due to tissue-specific toxicity of Pb on it.

Concretely, the liver health including liver enzymes and the amount of oxidative stress indices improved in rats with PAE administration. However, results of blood analysis show that the recovery with medicinal herbs therapy (100 or 200 mg/kg PAE; 21 days continuously) to stimulate liver function is rapid. This rapid improvement could be associated with antioxidant activity of PAE by preventing lipid peroxidation and hepatic cells from oxidative damage by scavenging of free radicals. In agreement with the results of current study, previous studies have reported that PAE demonstrates a significant hepatoprotective effect since significantly cause lower levels of blood glucose, ALT and ALK in diabetic rats^[20], and reduction of cholesterol, liver thiobarbituric acid, and the pronounced improvement of the investigated biochemical and antioxidant parameters^[14], due to its flavones components^[12]. Further understanding of natural antioxidant drugs and their effects on the liver system could help improving health treatments in patients.

By analyzing the expression level of several genes related to cell apoptosis (Bax and TNF- α) and survival (Bcl-2) apoptosis was shown to occur as a possible side effect of Pb use. Our data proved that Pb as an extracellular stimuli, could cause apoptosis-mediated pathway in the liver system of rat model, which might ultimately act either as a death safeguard effects. Pb administered in male rat generates an inflammatory-oxidative microenvironment that induces apoptosis of liver tissue by activation of Bax, and TNF- α increases together with Bax/Bcl-2 imbalance. The results of mRNA expression levels of apoptosis markers exhibited that Pb could induce mitochondrial impairment in liver cells or tissues of rat model, which maybe one of the potential mechanisms of Pb hepatic toxicity. Thus, study on apoptosis maker expressions in the liver tissues of laboratory animal may be advantageous to understand whether Pb induce apoptosis response against hepatic toxicity. Collectively, our findings indicated that PAE exposure could change expression of genes related to cell apoptosis. Our results exhibited a marked up-regulation of the expressions of Bcl-2. Overexpression of Bcl-2 increases cell viability and prevents apoptosis in adverse tissue circumstances^[21].

Taken together, these results demonstrated that PAE exerted a vital protective effect on liver dysfunction in a rat model, which could be attributed to its direct protective effect on damaged liver via dual inhibiting oxidative changes and regulating the expressions of apoptosis-related genes.

AVAILABILITY OF DATA AND MATERIALS

This manuscript contains original data and it is not under editorial consideration elsewhere. We have adhered to the ethical guidelines of your journal.

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COMPETING INTERESTS

None.

AUTHORS' CONTRIBUTIONS

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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