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Original article

Toxicity evaluation of aromatic water of *Pinus eldarica* Medw. in acute and sub-chronic toxicity experiments

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Summary. The plants of *Pinus* genus have been traditionally used in treatment of several diseases and recent studies revealed new pharmacological and biological activities of the genus. *Pinus eldarica* (Pinaceae) is commonly known as Tehran pine and planted in many parts of Iran, Afghanistan, and Pakistan. Aromatic water of *P. eldarica* needle have been used in arthritis rheumatoid complains in Iranian folk medicine. However, there is no study regarding the composition and safety of the aromatic water administration in human, therefore in the present study components of the aromatic water were identified using gas chromatography-mass spectrometry (GC-MS) method followed by evaluation of its probable acute and sub-chronic toxicity in Wistar male rats. The aromatic water was administered with dose of 10-22.5 mL/kg in acute and with dose of 10 mL/kg in sub-chronic toxicity assay for a period of 45 days. Thymol (78.8%) and carvacrol (6.2%) were characterized as the main part of the aromatic water constituents. The results indicated no sign of toxicity and lethality after single and repetitive doses of the aromatic water of the plant with the median lethal dose (LD_{50}) higher than 22.5 mL/kg body weight for male rats. All the hematological and biochemical parameters with histological examination of liver, spleen, kidney, and lung were normal compare to normal saline. There was only significant increase in triglyceride level in the period of 23 days. Therefore, oral administration of the aromatic water of *P. eldarica* may considered as non-toxic at doses of 10-22.5 mL/kg. Thymol and carvacrol could possibly contribute to the beneficial effect of aromatic water of the plant in arthritis rheumatoid complains.

Key words: aromatic water, carvacrol, Tehran pine, toxicity, triglyceride level, thymol.

Introduction

The plant *Pinus eldarica* Medw., Tehran pine, belongs to the family Pinaceae (pine family) which is planted in many parts of Iran, Afghanistan, and Pakistan (1). Various parts of the plant have been traditionally used in treatment of several diseases like skin disorders such as irritations, wounds, allergic rashes, dermatitis, and bronchial asthma (2-4). Recent studies revealed new pharmacological and biological activities of the genus of *Pinus* like antioxidant, anti-inflammatory, antineoplastic, and immunomodulatory effects (3, 5-7), through regulation of cyclooxygenase (COX) enzymes, prostaglandin E2 (PGE2), nitric oxide, and cancer-related proteins (4, 8, 9).

Extract of *P. eldarica* fruit potently inhibited calcium oxalate stone probably by excretion of small particles from kidney and reducing the chance of stone formation (2). Different extracts of the plant bark including hydroalcoholic extract, proanthgocyanidins,

and essential oil suppressed the growth of *Pseudomonas aeruginosa*, among those the essential oil showed the highest activity against the bacteria (10). Resin of *P. eldarica* showed antibacterial activity against *Escherichia coli* strains, which were resistant to co-trimoxazole (11). Hydroalcoholic extract of the plant nut decreased blood glucose of diabetic rats without significant effect on cholesterol and triglyceride levels attributed to phytochemicals like phenolic compounds, fatty acids, and terpenoids of the plant (3). In addition, anti-depressant activity of needles extract of the plant was ascribed to the presence of flavonoids which inhibit monoamines uptake and/or monoamine oxidase (MAO) activity in the brains of rats (4). Hydroalcoholic extract of the plant bark caused mild inflammation in kidney and liver of rats and decrease in monocyte counts with dose of 500 mg/kg/day (12). Phytochemical investigation of the plant revealed presence of various components including phenolic compounds such as caffeic acid, ferulic acid, catechin, epicatechin, tyrosol, gallic acid, coumaric acid, vanillic acid, and taxifolin along with other compounds like α-pinene, β-pinene, longiflene, β-caryophyllene, α-humulene, δ-3-carene, and junipene in leaves, fruits, and bark of *P. eldarica* (1,13-15). To the best of our knowledge, there is insufficient study regarding toxicity of pine oils. However, strong somatic mutations in *D. melanogaster* was reported by oil of *Pinus sylvestris* needles with less genotoxicity for human lymphocytes *in vitro* (16). Toxicity background of aromatic water of *P. eldarica* is necessary for other preliminary experiments evaluating its efficacy in arthritis rheumatoid (AR), since it have been used to remedy AR difficulties in Iranian folk medicine. There is no document concerning the safety of the plant aromatic water, therefore, its acute and sub-chronic oral toxicity in male Wistar rats followed by composition of the aromatic water were evaluated in the present investigation.

Methods and Materials

Plant materials and preparation of aromatic water

The leaves of *P. eldarica* were collected from Chitgar forest park (West of Tehran, Iran) in June of 2015 and identified at the herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences with voucher number of 6566-TEH. The plants were dried at room temperature away from direct sunlight and pulverized to extract aromatic water using traditional method. The plant materials were placed in a container of water and boiled to evaporate water containing aromatic compounds. The steam was cooled using circulating cold water to liquefy and stored in a glass container at 4°C.

Gas chromatography–mass spectrometry (GC–MS)
Sample preparation

To extract aromatic compounds, aromatic water (200 mL) was saturated using sodium chloride (Merck, Germany) to salting out the aromatic compounds and extracted by equal amounts of diethyl ether (Merck, Germany) three times (17). The obtained diethyl ether portion was evaporated using rotary evaporator (Heidolph, Germany) and dried using sodium sulphate anhydrous (Merck, Germany) to analyze qualitatively.

Instrument

The aromatic compounds were analyzed using GC-MS method on Agilent 6890 with MS instrument (Agilent, U.S.) equipped with a BPX5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was raised from 50 to 300°C at a rate of 3°C/min for 75 min. The oven temperature was held for 5 min at 50°C and transfer line temperature was 290°C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min with a split ratio equal to 1/30. The quadrupole mass spectrometer was scanned over ionizing voltage of 70 eV and an ionization current of 150 μA. The Kovats index for all the components were calculated using retention times of n-alkanes (C8-C25) injected at the same temperature and conditions, after the essential oil. The compounds were identified by comparison of Kovats index with those reported in the literature together with comparison of their mass spectra with the Wiley, Adams and NIST libraries.

Acute oral toxicity study

Twenty male rats (purchased from Pasteur Institute, Tehran, Iran and weighing 200±20 g) were acclimatized 1 week prior to experiment in animal house.

They were divided into four groups and orally treated with 10, 15, and 22.5 mL/kg body weight of aromatic water of *P. eldarica* and control animals received only normal saline solution 0.9 % to observe gavage effect. The clinical and behavioral signs as well as survival of animals were observed up to 48 h. The results showed that there were no death and toxicological effects in treatment group, so it was suggested that aromatic water of the plant is relatively harmless (18) and the dose of 10 mL/kg for limit study was performed during sub-chronic toxicity test.

Sub-chronic oral toxicity study

Twenty male rats (purchased from Pasteur Institute, Tehran, Iran and weighing 107±3 g) were acclimatized 1 week prior to experiment in animal house. They were divided into two groups including treatment and control groups received aromatic water 10 mL/kg and normal saline 0.9%, respectively. Animals were gavaged 5 days of a week up to 9 weeks (18) and observed for abnormalities along with individual body weight. Food and water consumption was weighted before each daily weighing.

Hematological and biochemical analysis

The effect of aromatic water of *P. eldarica* on the hematology and blood biochemistry parameters were determined after treatments. The 12 h-fasting animals were anesthetized with ether. Cardiac blood samples were collected and centrifuged at 3000 rpm for 20 min. Several hematology and blood biochemistry variables were determined.

Histopathological examination

The organs of each rats including liver, spleen, kidney, and lung were collected for the histological studies. Buffered formalin (10%) was used to fix all organs. The tissue specimens were routinely processed into paraffin; 2 μm thick sections were stained with hematoxylin and eosin (H&E) for examination by light microscopy.

Statistical analysis

The results are reported as mean±standard deviation (SD). Data analysis was made by one-way ANOVA followed by Tukey post hoc multiple comparison tests using SPSS software. *P* values less than 0.05 were considered to be statistically significant.

Results

Chemical composition of aromatic water of *P. eldarica* was analysed using GC-MS and listed in Table 1. The amount of volatile compounds extracted from the aromatic water was scarce (3 mg in 200 mL) and eight compounds were successfully identified in the aromatic water of the plant, which comprise 95.2% of all. Thymol (78.8%), carvacrol (6.2%), and piperitenone (4.8%) were the major components of the aromatic water.

In order to investigate acute oral toxicity of the aromatic water, twenty male rats were divided into four groups and administered 10, 15, and 22.5 mL/kg body weight of aromatic water and normal saline. Rats did not show any clinical sign of toxicity during observation period (2 days). No abnormal gross findings as well as no significant changes in the body weight gain were detected in rats. All the animals survived with

Table 1. Identified compounds in aromatic water of *P. eldarica*.

NO.	Compound	RT (min)	Area %	KI
1	camphene	9.1	0.2	952
2	thymol	11.7	78.8	1308
3	carvacrol	12.0	6.2	1314
4	piperitenone	12.8	4.8	1349
5	β-elemene	14.5	1	1396
6	-	18.2	1.4	-
7	-	18.3	1.9	-
8	β-funebrene	18.7	1	1418
9	-	19.1	0.5	-
10	β-copaene	19.1	0.5	1432
11	-	19.9	0.4	-
12	α-amorphene	20.0	2.2	1485
13	-	20.6	0.6	-
	Known		95.2	
	Unknown		4.8	

RT: Retention time, KI: Kovats index.

no mortality indicating that the median lethal dose (LD_{50}) of aromatic water is higher than 22.5 mL/kg body weight for male rats.

Through sub-chronic evaluation, twenty male Wistar rats in two groups treated with 10 mL/kg body weight of aromatic water and normal saline as control. The animals gained weight normally in all the treatment groups without death, clinical signs, and clear differences in food or water consumption comparing to control group. Cardiac blood samples were taken to evaluate hematological and biochemical analysis. The results have been indicated in Table 2 and 3. The effect of aromatic water of *P. eldarica* demonstrated no significant change on the hematological parameters including white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, and red distribution width (RDW) (Table 2). There were no significant difference observed in biochemical parameters including blood sugar, blood urea, cholesterol, serum glutamic-pyruvic

Table 2. Hematological parameters after administration of aromatic water of *P. eldarica* and normal saline in rats, all the data represented as mean ± SD.

Hematology parameters	**23 days**		**45 days**	
	NS	AW	NS	AW
WBC (10^3/μL)	11.48±1.01	15.64±3.58	11.71±0.98	9.58±5.91
RBC (10^3/μL)	8.37±0.27	8.37±0.27	8.46±0.56	8.46±0.55
Hemoglobin (g/dL)	15.75±0.63	15.20±0.84	15.65±0.49	14.95±0.78
Hematocrit (%)	49.65±1.76	48.0±2.54	46.85±3.46	44.60±1.55
MCV (Fl)	59.35±4.03	58.60±0.28	55.30±0.42	54.95±0.91
MCH (pg)	18.80±0.14	18.55±0.07	18.55±0.63	18.4±0.58
MCHC (g/dL)	31.80±2.40	31.65±0.07	33.50±1.41	33.50±0.56
Platelet count (10^3/μL)	1015.50±187.38	950.50±0.70	672.50±102.53	846.00±21.21
RDW (%)	20.95±2.75	20.50±2.97	16.10±1.69	16.85±2.19

*WBC: White blood cell, RBC: Red blood cell, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red blood cell distribution width, NS: Normal saline, AW: Aromatic water, the level of statistical significance was p<0.05, *: There is significant differences between values.*

Table 3. Biochemistry parameters after administration of aromatic water of *P. eldarica* and normal saline in rats, all the data represented as mean ± SD.

Biochemistry parameters	**23 days**		**45 days**	
	NS	**AW**	**NS**	**AW**
Blood sugar (mg/dL)	138.5±2.12	157±11.31	184.5±2.12	147.5±2.12
Blood urea (mg/dL)	79±29.69	57.5±0.70	55.5±3.53	56±5.65
Cholesterol (mg/dL)	100±1.41	63±14.14	71±1.41	69±16.97
Triglycerides (mg/dL)	32±12.72*	110.5±19.09*	119±7.07	69.5±3.53
SGPT (IU/L)	38±2.83	48±21.21	39±14.14	53±2.83
SGOT (IU/L)	173±7.07	150±26.87	84±14.14	100±24.04

*NS: Normal saline, AW: Aromatic water, SGPT: Serum glutamic-pyruvic transaminase, SGOT: Serum glutamic-oxaloacetic transaminase, the level of statistical significance was p<0.05, *: There is significant differences between values.*

transaminase (SGPT), and serum glutamic-oxaloacetic transaminase (SGOT) after 23 and 45 days of treatment (Table 3) except for triglycerides levels. Although, the level of triglycerides in treatment group (110.5±19.09) was significantly higher than control group (32±12.72) after 23 days, there was a decrease in triglycerides level in treatment group (69.5±3.53) compare to control group (119±7.07) after 45 days. No macroscopic and microscopic changes were observed in organs specimens of rats including liver, spleen, kidney, and lung after 23 and 45 days of administration of aromatic water of *P. eldarica* (Fig. 1-4).

Discussion

The main constituents of the aromatic water of *P. eldarica* were thymol and carvacrol in the present study. Previous experiments revealed anti-inflammatory effect of thymol and carvacrol in the animal models (19-

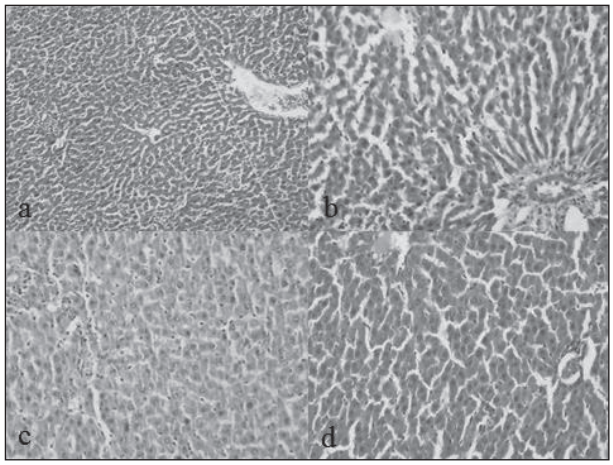

Figure 1. Histologic appearance of rat liver (magnification, x40), a: 23 days after administration of aromatic water of *P. eldarica*, b: 23 days after administration of normal saline, c: 45 days after administration of aromatic water *P. eldarica*, d: 45 days after administration of normal saline.

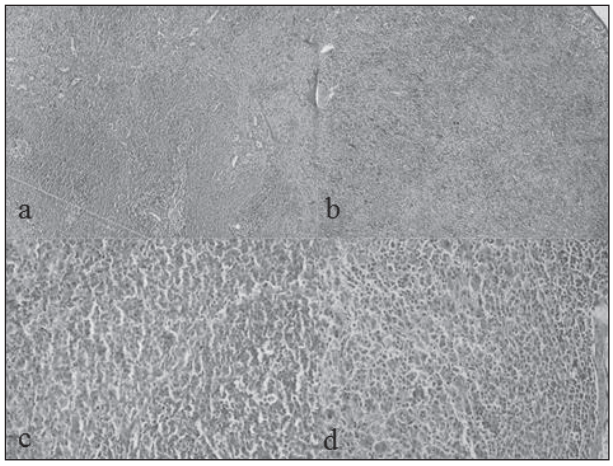

Figure 2. Histologic appearance of rat spleen (magnification, x40), a: 23 days after administration of aromatic water of *P. eldarica*, b: 23 days after administration of normal saline, c: 45 days after administration of aromatic water *P. eldarica*, d: 45 days after administration of normal saline.

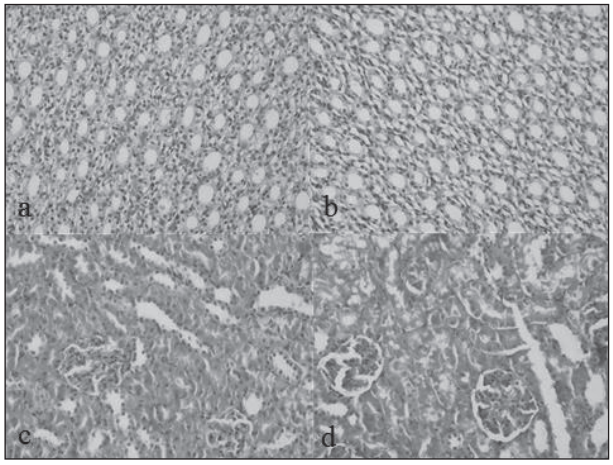

Figure 3. Histologic appearance of rat kidney (magnification, x40), a: 23 days after administration of aromatic water of *P. eldarica*, b: 23 days after administration of normal saline, c: 45 days after administration of aromatic water *P. eldarica*, d: 45 days after administration of normal saline.

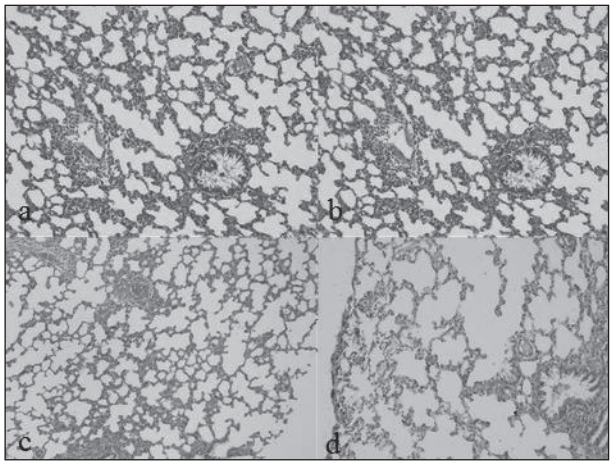

Figure 4. Histologic appearance of rat lung (magnification, x40), a: 23 days after administration of aromatic water of *P. eldarica*, b: 23 days after administration of normal saline, c: 45 days after administration of aromatic water *P. eldarica*, d: 45 days after administration of normal saline.

22). Anti-inflammatory property of carvacrol explicated by inhibition of leukocyte migration along with its anti-edematogenic and anti-chemotactic effects *in vivo*, while thymol caused irritation probably through release of hisatamine and prostanoid (22). However, thymol inhibits COX-1 and -2, key enzymes in inflammation, *in vitro* (23). Aromatic water of *P. eldarica* has been used in Iranian folk medicine for AR difficulties. Although, additional pharmacological and clinical trials are necessary, thymol and carvacrol with other constituents of the aromatic water may contribute to its beneficial effect in AR.

The results of the present study suggested that aromatic water of the plant is relatively non-toxic. There are few pertinent researches to toxicity of pine oil except a report of genotoxicity of *P. sylvestris* oil *in vitro* (16). Toxicity of oral administration of hydroalcoholic extract of *P. eldarica* bark in both male and female rats were evaluated by Ghadirkhomi et al. Up to dose of 2000 mg/kg of the extract of *P. eldarica* bark, no abnormal gross alteration or mortality were reported suggesting that LD_{50} of the extract is higher than 2000 mg/kg. In addition, no sign of toxicity was provided after repeated administration of *P. eldarica* for a period of 28 days (12). Although, all of the hematological and biochemical biomarkers showed normal levels in administration of the aromatic water of the plant in the present study, triglycerides level significantly increased for a period of 23 days. However, the level of triglycerides in treatment group was decreased statistically significant after 45 days. Cholesterol levels were decreased in both period of 23 and 45 days comparing to control group. There were some controversial reports regarding the efficacy of *P. eldarica* nut on cholesterol and triglyceride levels in animal models (3,12,24). Although, extract of *P. eldarica* nut showed no effect on the levels of cholesterol and triglyceride in hypercholesterolemic diabetic rats (3), blood cholesterol level in hypercholesterolemic rabbits decreased followed by administration of the nut extract (24). Interestingly, the results of another investigation revealed that extract of *P. eldarica* bark has hypotriglyceridemic effect only in male rats (12). The discrepancy of *P. eldarica* effects on the cholesterol and triglyceride levels could be described by differences in the composition of the plant extracts, administration doses, and duration of treatments. However, comprehensive evaluation is needed to confirm the effect of the plant extract on the lipid profile.

In conclusion, identification of aromatic components of *P. eldarica* and toxicity data obtained in the present study revealed that the aromatic water of the plant is relatively non-toxic at doses of 10-22.5 mL/kg in Wistar male rats. The finding of our study would be particularly helpful for further detailed investigations to examine efficacy of the aromatic water of *P. eldarica* in AR.

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