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Original Article

Evaluation of sub-chronic adverse effects of *Dracocephalum kotschyi* hydroalcoholic extract on blood biochemical and hematological biomarkers in rats

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Abstract

Background and aims: *Dracocephalum kotschyi* is a medicinal plant native to Iran, which is known for its anti-inflammatory and spasmolytic properties. This study was conducted to investigate the sub-chronic adverse effects of *D. kotschyi* extract on biochemical and hematological biomarkers in rats, with the aim of identifying any potential adverse effects associated with long-term therapy.

Methods: A total of 50 male Wistar rats were randomly allocated into 5 separate groups, each consisting of 10 rats. The extract was administered orally for 90 days at doses of 25, 75, and 300 mg/kg in three distinct groups. A control group and a vehicle-treated group were also included in this study. Following the treatment period, blood samples were collected to assess biochemical and hematological parameters. Liver and kidney tissue samples were prepared for histological examination. Mean comparisons between groups were conducted using the one-way analysis of variance (ANOVA), with *P* values less than 0.05 considered statistically significant.

Results: The hydroalcoholic extract of *D. kotschyi* at a dose of 300 mg/kg significantly increased levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatinine (Cr). Additionally, ALT and alkaline phosphatase (ALP) levels were significantly elevated at the dose of 75 mg/kg. Other parameters, including electrolytes, glucose, lipid profiles, thyroid hormones, complete blood count (CBC), prothrombin time (PT), and international normalized ratio (INR), remained unaffected, and no tissue damage was observed in the histological examinations.

Conclusion: Long-term oral administration of *D. kotschyi* extract at anti-inflammatory doses in rats resulted in no significant changes in the measured plasma biochemical or hematological biomarkers. However, at higher doses, there was an increase in hepatic biomarkers, as well as LDH and Cr levels, indicating potential organ toxicity associated with overdose.

Keywords: *Dracocephalum kotschyi,* Liver function test, Kidney function test

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Introduction

Dracocephalum kotschyi Boiss is a perennial plant belonging to the Labiatae family, which naturally grows in the high-altitude mountainous regions of Iran (1). "Badranjboyh Denaei" is one of the local names for *D. kotschyi*; however, it is now more commonly referred to as "Zaringiah". Traditionally, remedies prepared from *D. kotschyi* have been used for the treatment of gastrointestinal disorders, joint pain, asthma, and other ailments (2). Modern pharmacological studies have confirmed the anti-inflammatory and anti-spasmodic effects of *D. kotschyi* on the bronchial and gastrointestinal systems (3-8). Pharmacological studies on the hydroalcoholic extract of *D. kotschyi* have demonstrated that this extract contains compounds with strong antispasmodic effects on smooth muscles, including those of the ileum, uterus, bladder, and tracheal tissues (9-12). These studies suggest that the anti-inflammatory and antispasmodic effects of *D. kotschyi* may be attributed to its flavonoid content, which includes apigenin, luteolin, quercetin, isokaempferide, calycopterin , and possibly other substances (10-14). Previous research has investigated the antispasmodic effects of the hydroalcoholic extract of *D. kotschyi*, along with two of its constituents, apigenin and luteolin, on rabbit tracheal smooth muscle contractions. These constituents were shown to have potent bronchodilatory properties (7). Additionally, it has been reported that these substances possess anti-inflammatory and anti-asthmatic effects in animal models of asthma (15,16). Other preclinical studies have indicated the potential anti-inflammatory and anti-fibrotic effects of the *D. kotschyi* extract (4).

These studies also indicate that the *D. kotschyi*

extract has the potential to cause constipation (8-11). This effect could be beneficial for patients suffering from irritable bowel syndrome, particularly those with predominant diarrhea (17). In another study, the hydroalcoholic extract of *D. kotschyi* inhibited rabbit bladder contractions (12), supporting the antispasmodic effects of the extract; however, this also suggests that the remedy could potentially lead to urinary retention. Biomarkers are measured to evaluate drug safety during toxicity studies (18,19). Both normal and abnormal cells release biomarkers into the bloodstream, making serum biomarkers often the first sign of organ toxicity (19,20). Although short-term administration of *D. kotschyi* extract appears to have no substantial adverse effects on blood biomarkers, studies assessing the safety of long-term treatment with *D. kotschyi* extract are lacking. Therefore, the purpose of this study was to investigate the long-term effects of oral administration of *D. kotschyi* extract on hematological, biochemical, and other blood biomarkers in laboratory animals to assess its general long-term safety.

Materials and Methods

Plant extraction

The aerial parts of *D. kotschyi* were collected from the farm of Kesht Pertikan Company, located in Shahan Koh, Ivanak village, Feridounshahr, during the flowering season. The cultivated plant was identified by Mr. Mohammad Asfa at the Department of Natural Resources of Isfahan as *D. kotschyi* (Boiss.). A voucher specimen of this plant was deposited in the herbarium of the Faculty of Pharmacy at Isfahan University of Medical Sciences for further reference (15,19).

Drug and solutions

The hydroalcoholic extract was prepared as a 60 mg/mL stock solution in water, emulsified with 2-4 drops of Tween 40. This stock solution was subsequently diluted as needed for oral administration.

The aerial parts of the plant were dried in the shade and ground into a fine powder using an electric grinder. Extraction was performed using the maceration technique with 70% ethanol (21). Initially, the powder was placed inside a desiccator and moistened with 70% ethanol for 2 hours. Then, it was soaked in 70% ethanol for 3 days at a ratio of 1:8 (powder/solvent), ensuring that the entire surface was covered. During this period, the extract was checked daily, and the container was shaken regularly. Afterward, the solvent was decanted and filtered through a Buchner funnel. Maceration was repeated three times to complete the extraction process. Finally, all collected extracts were concentrated using a rotary evaporator (Heidolph, Germany) at 40°C, and the yield of the extract was determined. The total phenolic content of the extract was assessed using the Folin–Ciocalteu reagent assay (22).

Pharmacological studies

Male Wistar rats weighing between 180 and 220 g were used in this study. The animals were bred in the animal house of the Faculty of Pharmacy, where they were kept at room temperature with free access to food and water. During the experimental period, the animals were housed in clean cages of appropriate size with adequate ventilation. The floors of the cages were covered with soft wood chips, and the bedding was changed daily. The researcher received training from an animal laboratory expert in animal handling and the technique of drug gavage prior to commencing the experiment.

Initially, all the animals were observed daily for symptoms such as feeding behavior, grooming, lethargy, fur color, and body weight to ensure their health. After the adaptation period, the animals were randomly allocated to five separate groups of 10 rats each. The study was conducted over a 90-day period from April 4, 2023, to July 6, 2023. The extract or vehicle was administered using the gavage technique with a rat feeding tube. Three groups of rats (A, B, and C) received daily oral administration of the extract at doses of 25 mg/kg (low dose), 75 mg/kg (medium dose), and 300 mg/kg (high dose), respectively, for 90 days. The fourth group (D) was treated with an equivalent volume of the vehicle. One group (F) received no treatment at all to serve as a comparison with both the vehicle-treated control group and the extract groups. All animals were observed daily for any abnormalities, including lethargy, aggressiveness, wounds, motor incoordination, tremors, restlessness, body weight changes, piloerection, and fur color. After three months of daily administration of the extract or vehicle, all rats were weighed again. Rats were anesthetized with isoflurane, and blood samples were collected from the corner of the eye using the capillary tube technique (23). For blood analysis, blood samples were placed in laboratory tubes containing the anticoagulant EDTA (for CBC testing) and sodium citrate 3.6% (for erythrocyte sedimentation rate and PT testing), as well as tubes without anticoagulant (for serum preparation and analysis of biochemical parameters). After blood collection, the rats were euthanized by carbon dioxide asphyxiation, and the liver and kidneys were dissected. The animal carcasses were placed in a special plastic bag and frozen for disposal. The tissues were preserved in 10% formalin before being sent for pathological examination. The tissue samples were cut using a microtome, and the staining was performed using hematoxylin-eosin stain (24). Finally, the tissue slices were examined under a microscope (Nikon YS100, with x100 and x300 magnification) by a pathologist for any abnormalities.

Assessment and analysis of blood parameters

All analyses were performed at Al-Mahdi Laboratory (Isfahan). Complete blood cell counts were conducted using the Sysmex K-1000 Hematology Analyzer. Blood parameters, including plasma levels of glucose, lipoproteins, electrolytes (sodium and potassium), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (Cr), and thyroid hormones, were analyzed using spectrophotometry with the automatic

Hitachi 902 Chemistry Analyzer. Plasma levels of liver function markers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), as well as serum levels of bilirubin and uric acid, were also assessed using colorimetric and fluorometric assays.

Statistical analysis

The collected data for each parameter were expressed as mean ± standard error of the mean (SEM) for each group of rats (n = 10). The means of the experimental groups were compared with those of the control group using the ANOVA test, followed by Tukey's post hoc test. Statistical analyses were performed using SPSS version 27.0 (IBM Corporation, USA), and *P* values less than 0.05 were considered statistically significant.

Results

The yield of the hydroalcoholic extract was calculated to be 33.3% (w/w). The total phenolic content of the extract was found to be 248 mg of phenolic compounds per gram of the extract using the Folin-Ciocalteu method.

During the daily oral treatment of rats with the hydroalcoholic extract of *D. kotschyi* at doses up to 300 mg/kg for 3 months, no mortality or morbidity was observed. Daily subjective observations revealed no visible changes in skin or fur color, piloerection, feeding behavior, grooming, or motor movement in any group of rats. Throughout the study, no symptoms related to numbness or lethargy were noted during animal handling. Over the 90 days of the experiment, no decrease in body weight was observed; conversely, a slight increase in body weight was noted in all groups, indicating that the rats were feeding normally. However, these increases in body weight were not statistically significant when compared to the pretreatment period.

Blood analysis

Enzymes and related compounds released from tissues are also known as plasma biomarkers. For example, ALT and AST are common biomarkers of liver damage, while ALP serves as a biomarker of bone diseases (25). Based on the results of the ANOVA test, the mean levels of AST, direct bilirubin, Cr, and LDH were significantly different between the groups. Additionally, there was no significant difference in plasma levels of ALT, AST, or ALP between the normal and vehicle groups ($P = 0.6$, $P = 0.5$, and $P = 0.7$, respectively). However, in the experimental groups treated with the hydroalcoholic extract of *D. kotschyi*, the plasma concentrations of ALT and AST were significantly elevated ($P = 0.043$ and $P = 0.015$ for ALT in groups B and C, respectively, and $P = 0.005$ for AST in group C). On the other hand, a significant increase in ALP level occurred only at the medium dose ($P = 0.04$) (Table 1). Other liver

Table 1. Sub-chronic effects of oral administration of hydroalcoholic extract of *Dracocephalum kotschyi* on blood biochemical parameters in rats

Biochemical assay	Groups					*P**
	Normal	Vehicle	25 mg/kg	75 mg/kg	300 mg/kg	
ALT (U/L)	48.6 ± 5.2	45.5 ± 1.9	60.4 ± 11.2	75.3 ± 13.5*	88 ± 15.8*	0.196
*P***	-	0.6	0.2	0.043	0.015	
AST (U/L)	210 ± 1.7	202 ± 13.8	214 ± 24.6	218 ± 27.1	273 ± 18.3**	0.003
*P***	-	0.501	0.7	0.6	0.005	
ALP (U/L)	300 ± 20.8	289 ± 19	364 ± 31.3	406 ± 13.5*	369 ± 32.7	0.055
P-value	-	0.7	0.052	0.04	0.051	
TB (mg/dL)	0.17 ± 0.016	0.19 ± 0.02	0.16 ± 0.01	0.21 ± 0.02	0.27 ± 0.05	0.302
*P***	-	0.5	0.2	0.09	0.2	
DB (mg/dL)	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.003	0.05 ± 0.004	0.1 ± 0.02	<0.001
*P***	-	0.5	0.05	0.4	0.22	
IB (mg/dL)	0.23 ± 0.03	0.17 ± 0.02	0.12 ± 0.014	0.15 ± 0.01	0.26 ± 0.07	0.196
*P***	-	0.05	0.05	0.5	0.2	
Cr (mg/dL)	0.5 ± 0.05	0.5 ± 0.04	0.6 ± 0.04	0.6 ± 0.073	0.8 ± 0.1 *	0.036
*P***	-	0.7	0.065	0.075	0.01	
BUN (mg/dL)	51.2 ± 4.9	48.9 ± 3.1	57.9 ± 3.3	59.3 ± 5.9	60 ± 4.5	0.472
*P***	-	0.7	0.06	0.14	0.06	
LDH	214 ± 18	196.6 ± 1.4	244.2 ± 39	269.8 ± 50	453 ± 31 ***	<0.001
*P***	-	0.45	0.3	0.2	0.001	

ALT: alanine transaminase; AST: aspartate transaminase; ALP: alkaline phosphatase; Cr: creatinine; BUN: blood urea nitrogen; LDH: lactate dehydrogenase; TB: total bilirubin; DB: direct bilirubin; IB: indirect bilirubin.

*Overall significant difference between all groups, **Significant difference between control and other groups.

Hydroalcoholic extract or vehicle was orally administered to rats for a duration of 90 days (n = 10 for each group). Following the three-month daily treatment, blood samples were collected for analysis. One group received no treatment at all and served as the normal control group. The collected values were expressed as mean ± standard error of the mean (SEM). Intergroup comparisons were conducted using the ANOVA, followed by Tukey's post hoc test. There was no statistically significant difference between the untreated group and the vehicle-treated group. Asterisks indicate statistically significant differences between the test group and the vehicle-treated control group (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

function parameters, including total bilirubin, direct bilirubin, and indirect bilirubin, were also investigated in this study. Based on the results, no significant differences were observed between the normal, vehicle, or treatment groups following 90 days of oral administration of *D. kotschyi* extract ($P>0.05$) (Table 1).

According to the ANOVA test, the levels of triglycerides, HDL, blood sugar, potassium, and T4 were significantly different between the groups. LDH is a routine measurement used to assess tissue damage. It was indicated that daily administration of *D. kotschyi* extract at a dose of 300 mg/kg significantly raised the serum level of LDH in the rats ($P<0.001$). However, this effect was not observed at lower doses of the extract ($P>0.05$) (Table 1).

BUN and Cr are biomarkers of kidney function (26). During the 90 days of oral administration of *D. kotschyi* extract, no significant change was observed in the plasma BUN level ($P>0.05$). However, at the highest dose of the extract (300 mg/kg), the plasma level of Cr significantly increased ($P=0.01$) (Table 1).

Other measured plasma biomarkers in this study included electrolytes, glucose, lipoproteins, and thyroid hormones. Daily administration of D. kotschyi extract for three months had no significant effect on plasma levels of potassium, sodium, fasting glucose, or lipids. Furthermore, no significant changes were observed in T_3 and T_4 levels compared to the normal group ($P>0.05$) (Table 2).

A CBC test was performed to measure red and white blood cells, platelets, and hemoglobin (Hb) levels in the blood. The administration of *D. kotschyi* extract for 3 months caused no significant changes in blood cell counts or other associated factors compared to the normal group ($P>0.05$) (Table 3). Additionally, common bleeding tests, including prothrombin time (PT) and international normalized ratio (INR), were conducted. There were no significant changes in the coagulation rate following treatment with all doses of *D. kotschyi* extract for 3 months ($P>0.05$) (Table 3).

Histological studies

Microscopic examination of the liver and kidney tissues was conducted after 90 days in the groups receiving the extract, as well as in the vehicle and control groups. At the microscopic level, no evidence of damage was observed in the liver and kidney tissues (Figure 1).

Discussion

Testing new drugs for adverse effects begins with toxicity tests in animals. Measurement of biomarkers is an important tool for assessing organ toxicity. Biomarkers provide significant information about any pathophysiological changes that may occur during systemic drug therapy. The application of high doses, along with long-term administration of the drug in laboratory

Table 2. Subchronic effects of oral administration of hydroalcoholic extract of *Dracocephalum kotschyi* on plasma lipid and sugar profiles, electrolytes, and thyroid hormones

Other plasma assays	Groups					*P**
	No treatment	Vehicle	25 mg/kg	75 mg/kg	300 mg/kg	
Cholesterol (mg/dL)	150±3.5	163±5.8	168±8.7	160±4.6	160±7.2	0.067
*P***	-	0.08	0.63	0.7	0.7	
TG (mg/dL)	108±4.7	118±5.1	147±12.3	84±10.6	122±12.4	0.004
*P***	-	0.2	0.08	0.01	0.7	
LDL (mg/dL)	107±5.2	111±3.4	116±7.9	121±3.8	105±6.9	0.268
*P***	-	0.552	0.6	0.06	0.45	
HDL (mg/dL)	25.1±1.1	24.9±1.2	21.7±0.75	21.8±1.13	27.5±1.2	0.025
*P***	-	0.9	0.06	0.08	0.165	
FBS (mg/dL)	96.9±19.7	81.3±8.04	89.6±11.3	61.9±4.6	54.8±10.1	0.004
*P***	-	0.5	0.56	0.051	0.053	
K^+(mM)	3.8±0.2	4.2±0.1	3.7±0.11	4.32±0.15	3.5±0.34	0.005
*P***	-	0.45	0.1	0.4	0.065	
Na^+(mM)	139±0.8	139±0.8	137±1.9	140±0.7	142±1.1	0.063
*P***	-	0.8	0.4	0.2	0.06	
T_3 (ng/dL)	1.51±0.16	1.88±0.2	1.4±0.13	1.55±0.1	1.3±0.07	0.347
*P***	-	0.2	0.4	0.24	0.06	
T_4 (µg/dL)	5.63±0.45	5.2±0.5	6.2±0.2	5.8±0.4	6.11±0.4	0.036
*P***	-	0.5	0.07	0.345	0.153	

TG: triglycerides; LDL: low-density lipoprotein; HDL: high-density Lipoprotein) FBS: fasting blood sugar; T_3: triiodothyronine; T_4: thyroxine.
*Overall significant difference between all groups, **Significant difference between control and other groups.
The hydroalcoholic extract of *D. kotschyi* or a vehicle was orally administered to rats for 90 days (n=10 for each group). Following the 3-month daily treatment, blood samples were collected for analysis. One group received no treatment at all (normal group). The collected values were expressed as mean±standard error of the mean (SEM). Intergroup comparisons of the data were conducted using the ANOVA, followed by Tukey's post hoc test. There was no statistically significant difference between the normal group, the vehicle-treated group, and the rats treated with the extract (ANOVA).

Table 3. Subchronic effects of oral administration of hydroalcoholic extract of *Dracocephalum kotschyi* on hematological parameters (CBC) in rats

Hematological assay	Groups					*P**
	Normal	Vehicle	25 mg/kg	75 mg/kg	300 mg/kg	
WBC ($\times 10^3$ n/μL)	5.3±0.4	5.6±0.4	7.1±0.53	6.9±0.6	5.9±0.4	0.090
*P***	-	0.52	0.051	0.13	0.72	
RBC ($\times 10^6$ n/µL)	7.3±0.23	7.4±0.3	7.9±0.2	6.7±0.24	6.42±0.4	0.003
*P***	-	0.97	0.115	0.07	0.07	
HB (g/dL)	15.02±0.32	13.9±0.4	14.4±0.15	12.4±0.9	11.8±1	0.221
*P***	-	0.056	0.32	0.115	0.052	
HCT (%)	40±0.8	38.5±0.73	41.9±1.63	36.1±1.16	35.2±1.4	0.013
*P***	-	0.09	0.07	0.1	0.055	
MCV (µm3)	55.5±1	54.1±0.9	54.5±0.4	56±0.4	56.2±0.7	0.077
*P***	-	0.3	0.7	0.084	0.083	
MCH (pg)	19.6±0.4	19.1±0.5	17.9±0.7	18.6±0.43	18.9±0.6	0.666
*P***	-	0.51	0.16	0.4	0.7	
MCHC (g/dL)	34.9± 0.7	33.7±0.8	32.4±0.7	32.7±0.8	33±1.1	0.570
*P***	-	0.25	0.26	0.41	0.62	
PLT ($\times 10^3$ n/µL)	589±51.5	572±60.11	611±35.5	554±49.6	504±51.4	0.645
*P***	-	0.8	0.6	0.8	0.4	
RDW (%)	15±0.6	14.6±0.44	13.3±0.5	14.3±0.3	14.7±0.3	0.120
*P***	-	0.6	0.086	0.6	0.8	
INR	1.4±0.1	1.5±0.1	1.8±0.2	1.5±0.07	1.5±0.06	0.749
*P***	-	0.6	0.3	0.9	0.6	
PT	16.1±0.4	15.9±0.5	18.5±1.15	17±0.5	16.4±0.4	0.072
*P***	-	0.8	0.08	0.1	0.5	
ESR	2.1±0.4	2.1±0.04	3.8±0.7	2.1±0.5	2.5±0.6	0.052
*P***	-	1.0	0.07	1.0	0.6	

CBC: complete blood count; WBC: white blood cells; RBC: red blood cells; HB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet; RDW: red cell distribution width. INR: international normal ratio; PT: prothrombin; ESR: erythrocyte sedimentation rate.

*Overall significant difference between all groups, **Significant difference between control and other groups

The hydroalcoholic extract of *D. kotschyi* or a vehicle was orally administered to rats for 90 days (n=10 for each group). Following the 3-month daily treatment, blood samples were collected for analysis. One group received no treatment at all (normal group). The collected values were expressed as mean±standard error of the mean (SEM). Intergroup comparisons of the data were conducted using the ANOVA, followed by Tukey's post hoc test. There was no statistically significant difference between the normal group, the vehicle-treated group, and the rats treated with the extract (ANOVA).

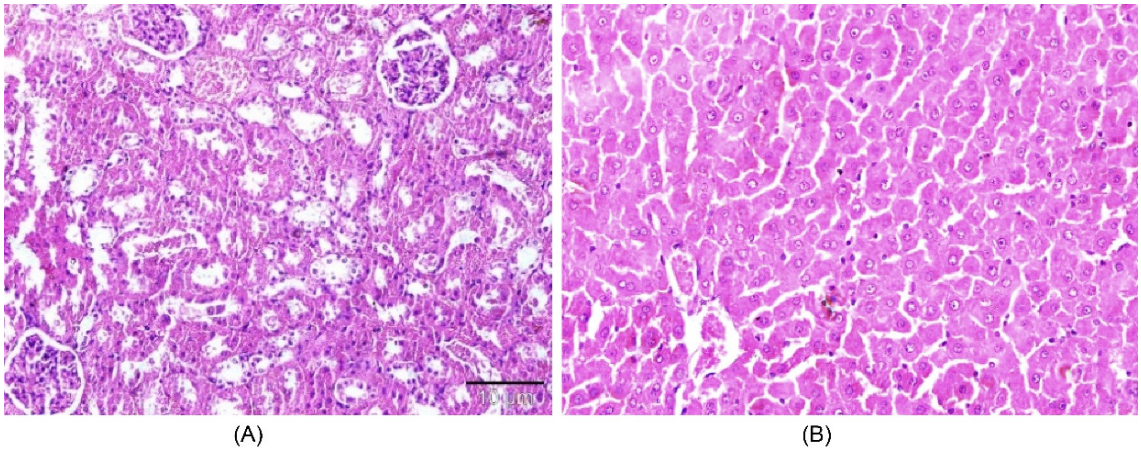

Figure 1. Microscopic features of rat kidney (A) and liver (B) tissues following subchronic treatment with *D. kotschyi* extract (hematoxylin-eosin staining, 200×). The hydroalcoholic extract of *D. kotschyi* (300 mg/kg) was administered orally for 90 consecutive days. Tissue samples were collected from rats exhibiting elevated plasma levels of LDH, ALT, AST, and Cr. Histopathological examination revealed a normal appearance of the tissues

animals, can reveal any hidden potential adverse effects that may arise during drug therapy (27). There is a wide range of biomarkers that are often measured during both preclinical and clinical investigations. Blood parameters are considered safety biomarkers and have been validated in both animal and human studies. Plasma biomarkers are

easily accessible and provide vital information about liver and kidney functions, as well as other tissues; therefore, they are routinely used for detecting drug toxicity (20).

In this study, three different doses of *D. kotschyi* extract (low, medium, and high) were administered daily to rats for a period of three months. The low and medium doses (25 and 75 mg/kg) were selected because these doses exhibited significant anti-inflammatory properties in the rats (4-6). To further investigate even small potential adverse effects, one group of rats was treated daily at a high dose of the extract (300 mg/kg).

Blood analysis has traditionally been used for drug toxicity assessment. Biochemical parameters, hematological factors, electrolytes, hormones, and other plasma parameters have been utilized as specific biomarkers for assessing normal physiological functions (28). For example, the assessment of thyroid hormones can indicate changes in energy levels. There was no change in thyroid plasma levels, suggesting that *D. kotschyi* extract is unlikely to affect the basal metabolic rate or thyroid-related conditions.

Measurement of Cr levels is considered a key parameter for assessing kidney toxicity, as an increase in Cr indicates impaired kidney function. In the examination of serum Cr concentration, no significant difference was observed compared to the control group; however, long-term administration of a high dose of the extract was associated with a significant rise in Cr levels (Table 1). Cr and BUN are waste products produced during protein metabolism. Cr is a byproduct of creatine phosphate in muscle tissue. An elevated BUN level usually reflects a reduction in glomerular filtration rate (GFR), while a rise in Cr levels typically indicates renal failure (26). Although renal failure is often associated with electrolyte imbalances, this study found no significant changes in sodium or potassium plasma levels, suggesting that GFR was likely not affected. Some drugs, including aspirin, corticosteroids, cimetidine, trimethoprim, pyrimethamine, and vitamin D derivatives, have also been reported to increase plasma Cr levels without affecting GFR (29).

The high dose of *D. kotschyi* extract also elevated plasma levels of ALP, ALT, and AST. An elevated blood ALP level is indicative of potential bone or liver damage and may also suggest a blockage in the bile ducts (30). To differentiate the source of the elevation, a specific ALP isoenzyme test is required. Elevated ALT levels are more specific biomarkers of liver damage, as these enzymes are released into the bloodstream when the liver is under stress (30). However, it is important to note that AST is also present in skeletal and cardiac muscle, as well as in erythrocytes (30).

LDH is another enzyme released into the plasma during tissue damage. LDH is found in various tissues, including skeletal muscle, kidneys, liver, lungs, and red blood cells (RBCs) (30). Therefore, the observed increase in total LDH levels induced by the high dose of *D. kotschyi* extract does not specify which tissue has been damaged; rather, it serves as a warning sign of potential tissue injury that requires further investigation. Although lysis of RBCs during blood collection and handling could contribute to increased LDH levels, no changes were observed in the control groups. Furthermore, hemolysis is typically accompanied by hyperkalemia, making it unlikely that erythrocyte lysis is the source of the elevated enzyme levels.

LDH levels also increase during intense exercise, indicating that muscle damage is associated with the release of LDH (31). However, muscle damage also leads to an increase in serum levels of creatine phosphokinase (CK), which is a more specific indicator of muscle injury (32). If the skeletal muscle is the source of the elevated LDH, it is likely that excessive doses of *D. kotschyi* extract cause muscle fatigue or possibly rhabdomyolysis. Both LDH and CK enter the bloodstream when muscle tissue is damaged; therefore, blood tests assessing CK levels, as well as specific cardiac biomarkers, are recommended to determine if there is any risk of muscle fiber damage.

According to the ANOVA test, RBC and hematocrit (HCT) levels were significantly different between groups. Factors such as Hb, HCT, and blood cell counts provide a comprehensive assessment of how the drug affects the blood profile. A CBC can evaluate the possible myelosuppressive effects of the extract. The primary blood cells, including red and white blood cells, as well as platelets, are produced in the bone marrow. Drug-induced bone marrow toxicity may result in low white blood cell count (WBC) (neutropenia), low RBC counts (anemia), or low platelet levels (thrombocytopenia) (30). However, three months of oral treatment with *D. kotschyi* extract had no significant effect on blood factors, including WBC, RBC count, HCT, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), platelet count (PLT), Hb, and red cell distribution width (RDW) (Table 3). Therefore, it is very unlikely that the extract affects the immune system or causes anemia. Furthermore, it had no effect on blood clotting tests, indicating that no anticoagulant activity is anticipated.

In summary, excessive doses of *D. kotschyi* extract increased the levels of biomarkers associated with liver stress, indicating a potential for hepatotoxicity. This toxicity may be attributed to the active ingredients metabolized in the liver or their metabolites. However, it has been reported that anti-inflammatory doses of *D. kotschyi* extract are very effective in preventing induced liver fibrosis in rats (33). Therefore, at therapeutic doses, *D. kotschyi* extract appears to have a protective role against liver injury rather than causing liver toxicity. Furthermore, tissue examinations in this study and previous research (18,33) did not reveal any signs of pathology or abnormalities. Therefore, the elevated plasma levels of liver biomarkers should be regarded as side effects rather than toxic effects, as they did not result in tissue damage. Additionally, the elevated Cr levels observed at excessive doses of the extract are indicative of potential renal adverse effects. The hydroalcoholic extract of *D. kotschyi* contains

many constituents, and the observed adverse effects may be due to compounds other than those with anti-inflammatory or anti-spasmodic properties. Therefore, it is recommended to separate and identify the beneficial ingredients from the ineffective compounds.

All drugs and herbal medicines can have adverse effects and may be toxic at excessive doses (34,35). The margin of safety depends on the therapeutic window of the drug. While there is a clear safety margin between the anti-inflammatory doses of *D. kotschyi* extract and the doses that increase blood biomarkers in rats, it is recommended that liver and renal biomarker screening be conducted during clinical trials to ensure safety in humans. Given that *D. kotschyi* extract is pharmacologically regarded as a potent agent, toxic effects at excessive doses are to be expected. Therefore, *D. kotschyi* extract should be considered a therapeutic medicine rather than a complementary agent, and unrestricted consumption as an herbal remedy must be avoided.

Conclusion

Although oral administration of *D. kotschyi* extract at anti-inflammatory doses did not affect the blood biomarkers of liver and kidney functions, this study concludes that high doses of *D. kotschyi* extract may have contraindications in cases of renal or liver dysfunction.

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Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

Animal care and experiments were conducted in accordance with the guidelines for the care and use of laboratory animals established by Isfahan University of Medical Sciences. The project was approved by the Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.RESEARCH.REC.1400.009).

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