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Original Article:

Safety Assessment of *Carduus pycnocephalus* hydroalcoholic Extract in Female Mice: Acute and Repeated Oral Toxicity Studies

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Abstract

Introduction: The genus Carduus belongs to Asteraceae family and is used to treat different human diseases, like cold, stomachache, and rheumatism. The current research investigated acute and repeated dose toxicity of *Carduus pycnocephalus* extract in mice.

Materials and Methods: Acute and repeated-dose toxicity experiments were performed in female mice according to OECD 423 and 407 guidelines, respectively. Physical observations were made regularly and body weight was weekly measured. The organs weight, histopathology, and blood chemistry were then analyzed. Statistical analysis was done by the GraphPad Prism 8 software, and the results were presented as mean ± SEM. P values below 0.05 were considered significant.

Results: No treatment-related mortalities were observed by oral administration of *C.pycnocephalus* extract up to the dose of 2000mg/kg. However, food consumption and water intake showed some variations in treated groups, and there were not any significant abnormalities related to treatment in treated groups in comparison to that in the control group in repeated-dose toxicity examination. Moreover, no significant alterations were noted in organ and body weight, food consumption, histopathology, and biochemical parameters in treated groups in comparison to the control group. Normal histological morphology was observed in all dose ranges and controls, except for the liver of high dose-treated samples which showed lymphocytic infiltrate and hepatocytes degeneration.

Conclusion: LD_{50} of *C.pycnocephalus* extract could be above 2000mg/kg in acute toxicity experiment. Furthermore, with sub-chronical administration of the extract at a dose of 200 mg/kg, liver tissue was slightly damaged.

Keywords: Blood chemistry, *Carduus pycnocephalus*, Herbal medicine, Histopathology, Mice, Safety

1. Introduction

Carduus pycnocephalus L. (Italian thistle) belongs to Asteraceae family which is native to Mediterranean region and some other countries [1]. It is locally known as 'Tartari-Porgol' and has long been used in Iran both for nutritional and medicinal purposes. This plant is distributed in some provinces of Iran, including Golestan, Azerbaijan, Khorasan, Isfahan, Tehran, Gilan, Semnan, and Khuzestan[2, 3]. There are a few reports on the usage of genus Carduus as traditional medicine in some parts of the world like china (for cold, rheumatism, and stomachache) [4]. Moreover, two other cases, regarding the use of this species, have been reported in Birjand (Khorasan Province, Iran). It

was used as a tonic for liver and spleen problems, constipation, and also as a herbal remedy for some diseases like headache, sores, diarrhea, stomachache, and respiratory problems [5]. Other studies [6, 7] also reported the alimentary use of *C.pycnocephalus* stems in Italy and Southern Kurdistan of Iraq. Although it is known that some Carduus species collect nitrates in toxic extents, it appears that *C.pycnocephalus* was not considered as a toxic weed [8]. New researches have shown that this plant possesses significant cytotoxic[9],antifungal[9], antibacterial[9], antiviral [10], anti-inflammatory[11], antispasmodic[11],and hypotensive[11] activities.

Phytochemical studies have demonstrated the presence of some metabolites in the plant, including some lignans[12], flavonoids, flavonoidal glycosides [9, 11, 13, 14] diosmetin, apigenin, kaempferol, triterpenes[9, 11], 3-O-acetyl-ursolic acid and lupeol, sterols[11], eta sitosterol, alkaloids [9, 15], tannins[11], coumarins[16, 17], sterols and triterpenes[18-20], and cardiac glycosides[11].

Considering that there are a large number of scholarly works that confirm the pharmacological, therapeutic, and extensive traditional usage of *C. pycnocephalus*, it is required to characterize its safety and toxicity profile for public health promotion and protection. Therefore, the present study examined acute and repeated-dose toxicities of *C.pycnocephalus* extract for predicting its safety in human application, promoting developments, and ensuring the public safety. The findings may optimize and validate its traditional use and help the developmentof future pharmaceutical products.

2. Materials and Methods

Plant Collection and Preparation of Extract

Carduus pycnocephalus was gathered from Misho, Marand, and East Azerbaijan in Iran. The plant was identified by Dr. KazemiVash and the voucher specimen was stored as No. 1558- AUPF at the herbarium of Faculty of Pharmacy, Islamic Azad University of Medical Sciences, Iran.

A percolation method was used for extract preparation. The aerial parts of plant (750 gram) were ground and extracted with ethanol 80° as a solvent and regularly mixed for 72 h. Then, it was filtered, dried, and kept in an appropriate bottle until being used [21].

Animals

Female Mice with a weight of 25-35 g were provided by Tehran University of Medical Sciences, Iran. The tests were conducted at the normal room temperature (22 ± 2°C) and light (12 h light/dark cycle) during the time period of 9:00 a.m. to 3:00 p.m. The steps were all done according to the guidelines proposed for laboratory animal care in the Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Iran (ethical Approval No: IR.IAU.PS.REC.1397.075).

Acute Toxicity Experiment

Guideline No. 423 of OECD was followed to evaluate acute toxicity [22]. This trial was achieved using 6 female mice (3 tests/3 controls). The target dose of the extract was specified as 2000 mg extract/kg body weight and administered orally by gavage. No mortality was observed 24 hours following the administration of the extract. The authors checked the general behavioral changes of the animals in case and control groups (including color of urine, appearance of feces, touch and sound sensitivity, aggression, hypo-activity, ataxia, ventilation disorders, and convulsions). Physical examinations were conducted, including the death, mucus membrane/skin/eye color, hair coat, respiratory rate, body temperature, lacrimation, salivation amount, body weight, and eye prominence.

Sub-Chronic Toxicity examination

Sub-chronic oral toxicity experiments were conducted according to the OECD guideline No. 407[23]. For this purpose, the female mice were assigned to three case groups and control (normal saline) with three animals in each group. The case groups were treated daily with plant extract that was orally administered at 50, 100, and 200 mg/kg doses for 28 days. Normal saline was used to treat the control group for 28 days. During the test period, behavioral parameters, water and food intake, and body weight were recorded. In order to conduct pathological examinations and biochemical tests, main organs and blood samples were gathered on the 28th day of the study.

Biochemical tests

The tested animals were fasted for 12 hours when sub-chronic toxicity investigations were completed. Then, the animals were sacrificed, followed by collecting their heart blood into dry pipes and centrifuging for 15 mins at 4°Cat 3000g. The serum samples were sent to the laboratory in order to perform biochemical analysis of creatinine, glucose, urea,SGOT, electrolytes, and SGPT.

Histopathological assay

28 days after the treatment, the researchers euthanized the animals, followed by fixing the harvested tissues (heart, kidney, liver, pancreas, ovary,

stomach, and intestine) in the 10% neutral buffered formalin (NBF, pH=7.26) for 48 hours. Then, it was processed and immersed in paraffin. After preparing sections with a thickness of 5µm, the pieces were stained using heamtoxylin and eosin (H&E). An independent reviewer evaluated the histological slides by light microscopy (Olympus BX51; Tokyo, Japan). Different samples were assessed in terms of any alterations, e.g., fatty changes, acute and chronic inflammatory responses, hemorrhage, coagulative necrosis, etc.

Statistical Analysis

Data were given as the mean ± SEM. The one-way and two-way ANOVA were employed for determining the statistical significance, and the Tukey test was used as the post-test. For this purpose, GraphpadPrism8 software was deployed. Differences at $P<0.05$ were regarded as significant.

3. Results

Acute and sub-chronic toxicities of C.pycnocephalus extract

Body weight changes, behavioral patterns, and physical changes

At the 2000 mg/kg dose, no adverse effects were noted for *C.pycnocephalus* extract on the behavioral responses of the study mice during 14 days. No indications of changes in the fur, eye, skin, mucous membrane, tremors, behavior patterns, diarrhea, and salivation were observed in the physical observations done on the mice. Also, there was not any mortality at the studied dose and after the oral administration of the extract (2000 mg/kg) in the acute examination. Body weight changes were not significant statistically ($P=0.5231$). Nevertheless, an increase was observed in body weight in the case group receiving the 2000mg/kg doses in comparison to that of the control group (Fig.1A). Also, no obvious toxicity symptoms were induced in mice by the daily oral administration of *C. pycnocephalus* extract at the 50, 100, and 200 mg/kg doses for 28 days. Additionally, there were no deaths or clinical indications in the case group during the test period. There were not any signs of toxicity in the physical observation of the treated animals during the research period. Similar to the acute examination, there were not any changes in the mice body weight following the oral exposure of the extract (50, 100, and 200 mg/kg) in sub-chronic test (Fig.1B).

Changes in water intake, relative organ weight, and food consumption

Single dose oral exposure of *C. pycnocephalus* extract (2000 mg/kg) showed an increase in the food consumption pattern until the 7th day similar to the that in the control group, yet significantly ($P<0.05$) lower. (Fig. 2A).

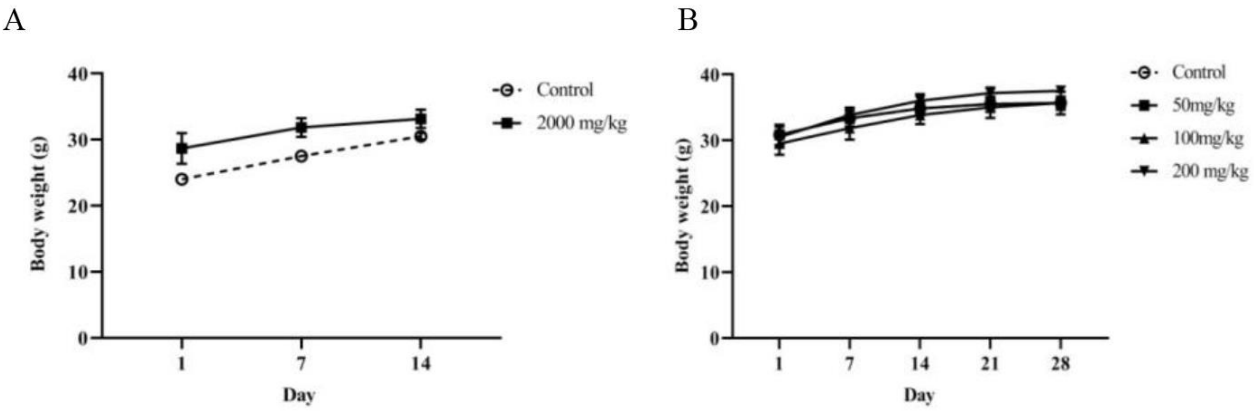

Figure 1. Impact of *C. pycnocephalus* extract on body weight as function of time (in days) following administering a single dose (2000mg/kg) (A) and repeated dose (50, 100 and 200 mg/kg) (B) in case groups in comparison with the control group

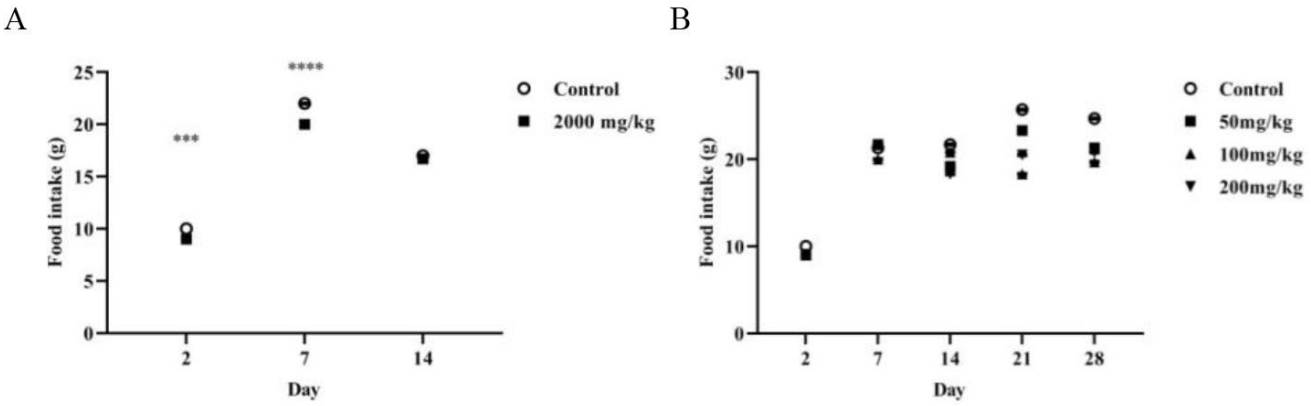

Figure 2. Effect of *C.pycnocephalus* extract on food consumption as function of time (in days) following single dose administration(2000mg/kg) (A) and repeated dose administration(50, 100, and 200 mg/kg) (B) in case groups in comparison to the control group; ***p<0.001; ****p<0.0001

However, the repeated dose test showed an increased consumption pattern in all groups until the 7th day and then a constant pattern until the last day of the experiment. The treated and control groups did not show any significant differences (Fig 2B).

Significant variations were observed in the water intake of both the control and case groups treated with a single dose of the extract (p-value=0.0001). However, no statistically significant variation was seen in the water intake of both control and case groups in repeated doses (p-value = 0.6942 and p-value =0.3518, respectively). (Fig 3A -B).

There were not any significant differences in organ weight to body weight index (heart, kidney, pancreas, intestine, ovary, and stomach) in case groups in comparison to the control group (Fig 4). Nevertheless, significant differences were observed in liver weight to body weight index treated with the highest dose of extract (200mg/kg) in comparison with that of the control (p-value = 0.0093) (Fig 4).

Blood biochemistry analysis

As for the impact of sub-chronic administration of *C. pycnocephalus* extraction, some biochemical parameters are presented in Table 1. Biochemical parameters (glucose, creatinine, SGOT, SGPT, urea, and electrolytes) did not show any changes in the mice following oral administration of the extract (50, 100, and 200 mg/kg) in sub-chronic test in comparison with those in the control group.

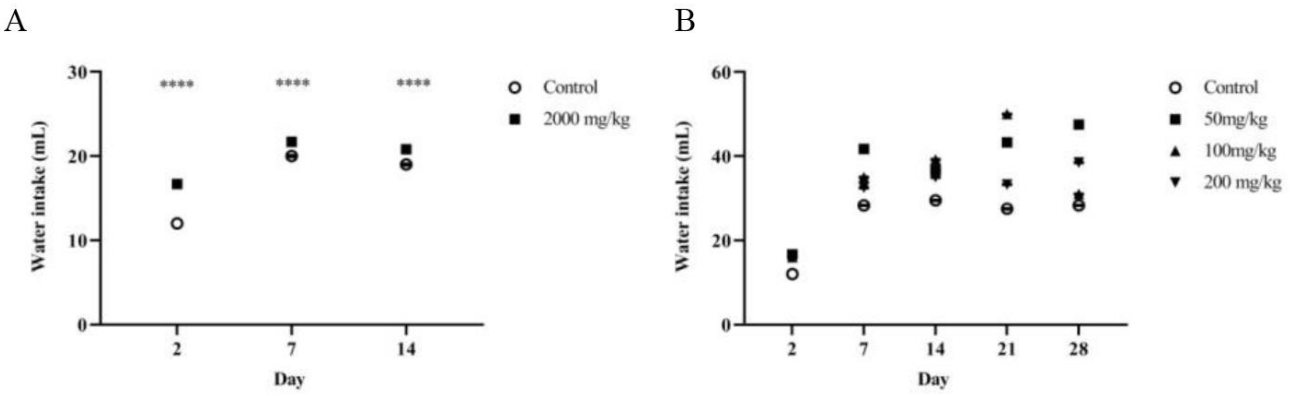

Figure 3. Effect of *C.pycnocephalus* extract on water consumption as function of time (in days) following single dose administration(2000mg/kg) (A) and repeated dose administration(50, 100, and 200 mg/kg) (B) in case groups in comparison to the control group; ****p<0.0001

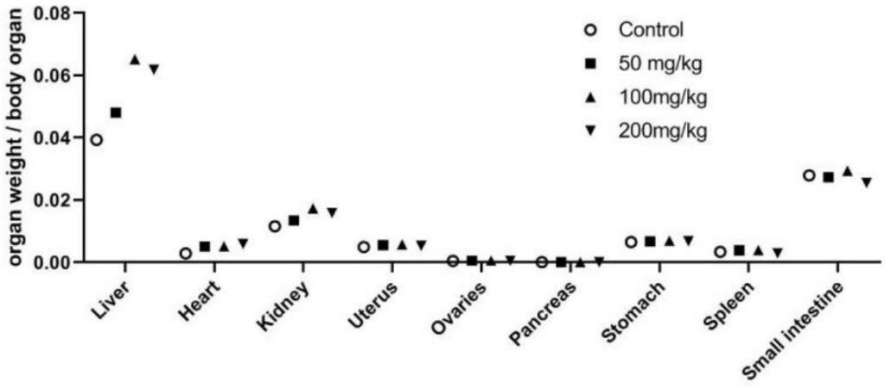

Figure 4. Effects of sub-chronic oral toxicity of *C. pycnocephalus* extract on theorgan weight to body weight ratio

Table 1. Effects of *C.pycnocephalus* extract on some serum biochemical parameters in the sub-chronic toxicity in female mice

	Control	50 mg/kg	100 mg/kg	200 mg/kg
Blood sugar (mg/dL)	147.3 ± 16.6	194.0 ± 6.8	232.3 ± 10.7	167.7 ± 40.1
SGOT (U/L)	305.7 ± 62.5	204.0 ± 42.1	257.0 ± 15.7	227.7 ± 16.8
SGPT (U/L)	137.0 ± 29.5	72.7 ± 20.1	86.0 ± 12.1	76.0 ± 1.7
Urea (mg/dL)	83.0 ± 9.1	67.7 ± 5.3	78.3 ± 0.3	87.3 ± 2.9
Creatinine (mg/dL)	0.51 ± 0.02	0.52 ± 0.00	0.51 ± 0.00	0.46 ± 0.01
Ca^{2+} (mM/L)	10.23 ± 0.3	10.7 ± 0.3	10.6 ± 0.0	10.8 ± 0.2
Na^{+} (mM/L)	148.0 ± 0.0	146.3 ± 1.4	147.3 ± 0.3	149.0 ± 2.1
K^{+} (mM/L)	9.9 ± 0.8	9.0 ± 1.3	11.6 ± 0.5	10.4 ± 1.1

The extract of *C.pycnocephalus* was exposed in sub-chronic oral doses (50, 100, and 200 mg/kg/day) during 28 days (n=3). The measurement of the serum biochemical parameters was performed at the end of the test period. Data were presented as mean±SEM; n=3.

Histopathological studies

H&E-stained liver pieces of test groups were histologically evaluated. Normal tissues (control group) are illustrated in figure 5 (Ctrl). The histopathological evaluation of the stomach, intestine, liver, kidney, heart, ovary, and pancreas showed intact tissues in all treatment doses 28 days after treatment (Fig.5). The histopathological assessment of liver in samples treated with 50 and 100 mg/kg revealed a normal tissue, while in 200 mg/kg it showed severe fatty change (hepatocytes degeneration) and infiltration of inflammatory cells (Fig. 5).

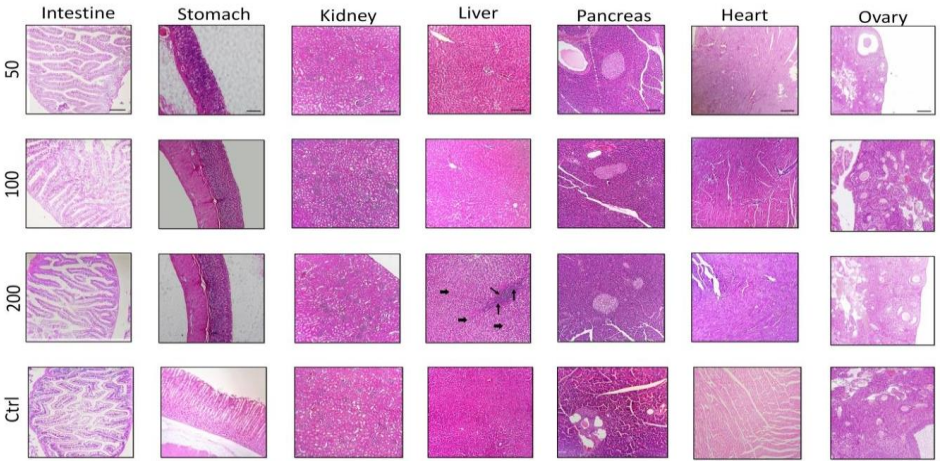

Figure 5. Histopathology sections of the intestine, stomach, kidney, liver, pancreas, heart, and ovary in experimental groups, Ctrl: Control, H&E stain, Thin arrows: mononuclear inflammatory cells. Thick arrows: degeneration of hepatocytes (fatty change)

4. Discussion

This research showed the relative safety of *Carduus pycnocephalus* extract in a mouse model. Acute toxicity and repeated 28-day sub-chronic toxicity examinations indicated that there were not any significant physiological changes. Single oral dose of *C. pycnocephalus* extract up to 2000 mg/kg did not cause any significant morbidity related to treatment in female mice. It is possible to consider LD_{50} of *C. pycnocephalus* extract as being above 2000 mg/kg, since we did not observe any deaths at this dosage. Therefore, the extract of *C.pycnocephalus* could be considered as Slightly Toxic according to Hodge and Sterner scale [24]. The observed changes in organ weight, body weight, food consumption, clinical chemistry parameters, histopathology, and water intake were not significant in 28-day repeated dose experiments. Nevertheless, relative water intake and food consumption in acute treated groups showed significant variations in comparison to those of the control group. No significant changes were seen in the relative body weight in most studied organs, except for the liver weight/body weight ratio in the highest dose-treated groups in comparison to the control group in a concentration-dependent toxicity way. In terms of the obtained clinical chemistry values, different treatment groups did not reflect significant changes. The histopathological evaluation of intestine, stomach, kidney, liver, pancreas, heart, and ovary showed intact tissues in all treatment doses after 28 days post treatment; however, at the dose of 200 mg/kg, it showed severe fatty changes (hepatocytes degeneration) and infiltration of inflammatory cells in liver tissue.

A daily oral exposure of below 200 mg/kg was suggested in this work for the long-term administration of *C.pycnocephalus* extract. Therefore, establishing a scientific foundation for the therapeutic measures of this folk medicine is required since it can be used as a source to develop more efficient therapies.

5. Conclusion

According to the findings of this research, *C.pycnocephalus* extract showed to be slightly toxic with the LD_{50} above 2000 mg/kg. Nevertheless, the repeated exposure of *C.pycnocephalus* extract for a period of 28 days and at a moderately lower dose caused some organ damages in the mice's livers. There were not any significant alterations in biochemical parameters at the 200 mg/kg dose, while histological analysis revealed slight microscopic tissue damage in liver. Hence, it is better to use *C. pycnocephalus* extract at a less than 200 mg/kg so that its adverse effects would be mitigated.

Further evidence is conferred by these findings in the sense that the administration of the extract at non-recommended doses may induce functional damage to such critical organs as liver in animals and probably in humans. Overall, the current research showed that female rats seemed to well endure *C.pycnocephalus* extract at the acute dose of 2000 mg/kg of body weight.

Conflict of interest

None declared**.**

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