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Original article

Antioxidant Potential, Hypoglycemic Effect and Safety of *Ajuga chamaecistus* Ging. ssp. *tomentella* (Boiss.) Rech. f. Aerial Parts

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Abstract

Background and objective: *Ajuga* species (Lamiaceae) are traditionally used in the treatment of jaundice, joint pain, sciatic nerve, and diabetes in different countries. The aim of this study was to investigate the antioxidant and hypoglycemic activities and safety of *Ajuga chamaecistus* ssp. *tomentella*. **Methods:** Antioxidant activity, radical scavenging effect, and total phenolics content of the aqueous and methanol extracts were assessed using ferric reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging and Folin-Ciocalteu methods. Streptozotocin (STZ) induced diabetic mice were studied in separate groups comprising aqueous and methanol extracts (200, 400, 800 mg/kg), metformin (500 mg/kg) and a negative control group. **Results:** The n-butanol fraction showed the most phenolics content (26.5 mg GAE/g of extract) and the highest antioxidant power (346.7 mmol FeII/g of extract) as well as the most considerable radical scavenging activity (IC_{50}=15.34 µg/mL). In STZ-diabetic mice, repeated oral administration of all doses of extracts showed a significant decrease in plasma glucose levels after 3, 14 and 28 days. The results of acute toxicity study showed that the ethanol extract was non-toxic up to the dose of 6000 mg/kg. Based on the sub-chronic toxicity results, a significant decrease in cholesterol and triglyceride was observed after using the extract (1000 mg/kg) for 23rd and 45th days. Histopathology of animal tissues revealed no significant differences in animal tissues between treated and control groups after 23 and 45 days. **Conclusion:** our study indicated the antioxidant potential, safety and hypoglycemic effect of *A. chamaecistus* ssp. *tomentella* extracts.

Keywords: *Ajuga chamaecistus* ssp. *tomentella*; antioxidant activity; hypoglycemic effect; safety

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Introduction

Diabetes mellitus is one of the major debilitating chronic disorders in the world and the number of people with diabetes mellitus is increasing. Currently, there are more than 220 million people with diagnosed diabetes [1]. Diabetes mellitus is caused by inefficiency of the pancreatic β-cells to

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secret insulin (type 1 diabetes) and/or the insulin function (type 2 diabetes) leading to progressive impairment of glucose tolerance and hyperglycemia [2,3]. Both types of diabetes can lead to chronic elevation of glucose levels that cause oxidative stress [4].

Clinical studies have shown the efficacy of plants in the modulation of oxidative stress associated with diabetes mellitus [5]; therefore, the use of antioxidants can protect beta cells and endothelial cells against diabetes-induced stress oxidative injury [6]. Plants with antioxidant properties would be useful in the treatment of patients with diabetes [6]. According to previous studies, more than 800 plants have been reported to have anti-diabetic activity [7]. Furthermore, recent studies demonstrated that more than 1200 plants are used in traditional medicine for their allied hypoglycemic activity [8].

The genus *Ajuga* [Kamaphytus] has been used for the treatment of jaundice, joint pain, gout, amenorrhea, sciatica and wound healing in traditional Persian medicine [9]. It has been also used as anthelmintic, against intestinal disorders [10], and as anti-diabetic in traditional medicines of different countries [11,12]. *Ajuga chamaecistus* comprises five exclusive subspecies, while *Ajuga chamaecistus* ssp. *tomentella* is endemic to Iran [13].

Several pharmacological studies on many species of this genus have been asserted in different ethnobotanical reports such as hypoglycemic [14], anti-inflammatory [15], analgesic, anabolic, antioxidant, anti-arthritis, antipyretic, hepatoprotective, cardiotonic, antibacterial and antifungal [16] properties. Some active compounds from *Ajuga* species such as flavonoids, iridoids [17], withanolides [18], phenylethyl glycosides [19] and phytoecdysteroids [20] have been identified.

The chemical composition of the essential oil from *A. chamaecistus* ssp. *tomentella* has indicated the main compounds as thymol, exo-fenchol, β-pinene, 1-octen-3-ol, α-terpineol, 2-hexanol, α-thujene, α-pinene, while the thymol has shown the highest level [21]. In 2012, Sadati et al., identified 10 natural compound in a phytochemical study of the plant including 20-hydroxyecdysone, cyasterone, ajugalactone, makisterone A, and 24-dehydroprecyasterone (phytoecdysteroids), 8-acetylharpagide (iridoid), *cis*- and *trans*-melilotoside , lavandulifolioside, leonoside B, and martynoside (phenylethanoid glycosides). They also showed that the extracts of the herb and the isolated compounds had no cytotoxic effects on cancerous or normal cells [22,23].

Examining the analgesic effects of this plant, it was found that the aqueous extract and hexane and diethyl ether fractions obtained from the methanol extract showed analgesic and inflammatory effects that suggested the use of this plant in the treatment of arthritis and sciatic nerve pain [24]. In a recent study, a chemical compound called ajugalide-E has been isolated and identified from the hexane fraction of this plant which showed larvicidal effects against *Anopheles stephensi* larvae [25].

The purpose of this study was to determine the antioxidant activity, hypoglycemic effect, acute and sub chronic toxicity of some extracts from aerial parts of *A. chamaecistus* ssp. *tomentella*, an attempt to confirm the traditional use of the plant.

Material and Methods

Ethical considerations

This study was approved by the ethics committee of Tehran University of Medical Sciences (TUMS), Tehran, Iran (IR.TUMS.PSRC.REC.1395.1166, 12/06/2016).

Plant material

Aerial parts of *A. chamaecistus Ging* ssp. *tomentella* (Boiss) Rech. f. were collected from "Sorkhe Hesar", east of Tehran, Iran, in June 2014 and verified by Prof. G Amin. A voucher specimen (THE-6697) was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Methanol extract and fractions

The air-dried and ground aerial parts of *A. chamaecistus* ssp. *tomentella* (250 g) were extracted with methanol 80% (3×1 L) at room temperature, in three days. The solvent was evaporated in a rotary evaporator and a vacuum oven to give a dark brown extract (45 g). The extract (30 g) was suspended in water and partitioned between hexane, diethyl ether (DEE), and n-butanol (NB) (Merck, Germany). After removal of solvents, DEE and NB fraction were studied for antioxidant activity.

Aqueous extract

Two hundred and fifty grams of the powdered

aerial parts were extracted with distilled water (3×1 L) at room temperature, in three days. The solvent was removed with a rotary evaporator and freeze drying process (30 g).

Ethanol extract

One kg of dried and ground plant was extracted with 70% ethanol three times with time lag of at least three days. The extract was condensed with a rotary evaporator and vacuum oven (40 °C) then refrigerated until use.

Measurement of total phenolics contents

Total phenolic contents of total aqueous and methanol 80% extracts, DEE and NB fractions were determined colorimetrically using Folin-Ciocalteu reagent as described by Velioglu et al. [26] with slight modifications. 200 µL from each sample was added to 1.5 mL of Folin-ciocalteu reagent that was 10 times diluted with distilled water and stored at 20 ºC for 5 min. Then 1.5 mL of sodium bicarbonate solution (60 g/L) was added to the mixture. After 90 min at 22 ºC, the absorbance was measured at 765 nm by spectrophotometer; the experiments were repeated three times. The calibration curves was drawn by measuring the absorption of certain concentrations (25-150 mg/L) of gallic acid as the standard and the results were stated as milligrams of gallic acid equivalents (GAE) per gram of dry matter (total extracts and fractions) as means ± SD [26].

Evaluation of antioxidant activity using TPTZ

The FRAP (ferric reducing antioxidant power assay) procedure which had been described by Benzie and Strain [27] was followed. The method was performed for water and methanol extracts, DEE and NB fractions [28]. For plotting the calibration curve, five concentrations of FeSO4 $7H_2O$ (125, 250, 500, 750, 1000 µmol /L) were used. The values were stated as the concentration of the antioxidants power as a ferric reducing ability equivalent for 1 mmol/L FeSO4. $7H_2O$

DPPH radical scavenging activity

The aqueous and methanol extracts, DEE and NB fractions were evaluated for their free radical scavenging activities using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) method according to Brand Williams et al. [29]. Half maximal inhibitory concentration (IC_{50}) value which indicates the concentration of the sample (mg/mL), required to scavenge 50% of DPPH was calculated from the plotted graph of scavenging activity versus the concentration of the extract, using linear regression analysis [30].

Animals for hypoglycemic study

Male albino mice weighing 25-30 g were obtained from Pasteur institute, Iran and maintained in groups of 7 at a constant temperature (24±1°C), with a 12 h light-dark cycle. The animals were given free access to tap water and standard laboratory mice food. They were allowed to acclimatize to the laboratory for one week before the experiments.

Animals for acute and sub-chronic toxicity

Male Wistar rats were obtained from the Pasteur Institute, Iran. One week before the tests, the animals were kept in the animal house, to be be acclimatized with animal house condition and were evaluated in terms of health. The animal house condition was as follows: temperature 24±1°C and cycle of 12 h of lighting and 12 h of darkness. Laboratory animal diets included compressed food and water for animals and animal consumption of food and water was not limited.

Experimental design

Induction of experimental diabetes

Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (Sigma, USA) at a dose of 150 mg/kg body weight. STZ was dissolved in 0.05 mol/L cold sodium citrate buffer, pH 4.5 immediately before use. After 72 h, hyperglycaemia was confirmed using a glucometer (Glucotrend, Germany). Only animals with non-fasting blood glucose levels higher than 2.50 g/L were selected and used in this study. Then animals were classified into 10 groups treated as described for groups 1-10, (n= 7 per group). Groups 1: received distilled water and served as the control group; Groups 2,3,4: treated with aqueous extract of *A. chamaecistus* ssp. *tomentella* at doses of 200, 400 and 800 mg/kg; Groups 5,6,7: treated with methanol extract of the plant at doses of 200, 400 and 800 mg/kg, Group 8: treated with metformin (MET) at a dose of 500 mg/kg, Group 9: non diabetic mice treated with aqueous extract of *A.*

chamaecistus ssp. *tomentella* at a dose of 800 mg/kg. All groups received All groups received the treatments by oral gavage every day for 4 weeks. The non-fasting plasma glucose levels were determined using a glucometer.

Blood sampling

Heart blood of mice was collected after anesthetized by xylazine and ketamine. Blood samples were centrifuged at 4000 rev/min for 10 min at 4 °C, and then the plasma was conserved until analysis. Plasma HBA_{1c} concentrations were defined. In addition, weight changes were also evaluated.

Acute and sub-chronic toxicity evaluation

For determining oral acute toxicity of *A. chamaecistus* ssp. *tomentella*, five male rats were selected in each group. At the beginning of the experiment, the rat weights were measured to be 120±10 g. The ethanol extract with doses of 6000 mg/kg was given to the rats in the form of gavage and after 48 h, the changes in appearance and behavior of animals, including weight, water and food intake were observed. In the study of sub-chronic toxicity, the time required for the study was 45 days. The test was in the form of oral gavage and 1000 mg/kg dose was used in this study. In addition, a control group was considered as the only group receiving normal saline of 0.9%. Animal behavior and appearance changes were measured with three degrees of low, medium and high. The number of animals was 20 and they were divided into two groups of 10. Average body weight was (156.7±1g). Samples of animals' blood and tissues were collected after 23 and 45 days [31].

Statistical analysis

All data have been presented as mean±SD. Student's t-test was used to assess statistically significant differences. Comparisons between group means were carried out using analysis of variance. Mean values were considered significantly different if $p<0.05$.

Results and Discussion

Total phenolics content (mg GAE/g of extract) of different extract and fractions were calculated using the standard curve of gallic acid (R2= 0.9912, y=0.006x-0.022). Table 1 has represented that total phenolics content of NB fraction (26.5±2.8 mg GAE/g of sample) was significantly higher than other samples ($p<0.0001$). The aqueous and methanol extracts showed similar total phenolics content, while the DEE fraction contained the lowest total phenol content.

Antioxidant effects of different extract and fractions using FRAP, was reported based on mmol FeII/g of extract. Ferric reducing antioxidant power of the extracts were calculated using the calibration curve and regression equation of ferrous sulfate (R^2= 0.9961, y= 0.0008x-0.015). According to table 1, the antioxidant power of NB fraction (346.7±31.2 mmol FeII/g of sample) was significantly higher than the aqueous ($p<0.05$) and methanol extracts and also the DEE fraction ($p<0.01$).

Table 2 has demonstrated the antioxidant power of the compounds from NB fraction. *Cis*-melilotoside showed the most antioxidant power (1373.6±59.6 mmol FeII/g of sample), while *trans*-melilotoside exhibited the lowest antioxidant power (160.2±9.4 mmol FeII/g of sample). Free radical scavenging effects of different extracts and fractions and the isolated compounds were assessed with 2, 2-diphenyl-1-picryl-hydrazyl (DPPH). IC_{50} values were exhibited in tables 1 and 2. Evaluation of the results indicated that the NB fraction (IC_{50} = 15.3 µg/mL) was the most potent radical scavenging sample which was comparable to vitamin E (14.2 µg/mL). After NB fraction, the methanol extract showed the highest radical scavenging effect (IC_{50} = 19.4 µg/mL), while the aqueous extract (IC_{50}=78.4 µg/mL) showed the least radical inhibitory power. The NB fraction showed the highest phenolic content as well as the highest antioxidant power among the samples.

Table 1. Total phenolics content, FRAP value and DPPH radical scavenging activity of *A. chamaecistus* ssp. *tomentella* extracts and fractions

Sample	TPC (mg gallic acid/g sample)	FRAP value (mmol FeII/g sample)	DPPH radical scavenging (IC_{50} µg/mL)
Aqueous ext.	18.2±2.5[1]	281.6±57.5	78.4
MeOH ext.	18.6±2.3	199.9±13	19.4
DEE	15.9±0.8	148.0±10.8	43.3
NB	26.5±2.8***	346.7±31.2*, **	15.3
Vit E	-	-	14.2
BHA	-	-	7.9

[1]Mean±SD, n=9; TPC: total phenolics content; ext: extract; MeOH: methanol; DEE: diethyl ether fraction; NB: n-butanol fraction; Vit E (Vitamin E); BHA: butylated hydroxyl anisole. *** $p<0.0001$, * $p<0.05$ compared to aqueous and MeOH ext., ** $p<0.01$ compared to DEE.

Table 2. Changes in blood hematology after repeated oral administration of the ethanol extract (1000 mg/kg) of *Ajuga chamaecistus* ssp. *tomentella* and the control groups after 23 and 45 days. Data have been expressed as mean ± SD; n=10 per group.

Hematology	23rd day		45th day	
	Control	Extract	Control	**Extract**
WBC ($10^3/\mu L$)	7.92±0.007	6.85±2.91	5.41±2.74	4.69±0.43
RBC ($10^6/\mu L$)	8.07±0.73	8.56±0.43	8.58±0.18	8.75±0.53
Hemoglobin (g/dL)	14.90±1.55	16.40±0.98	15.20±0.42	15.40±0.84
Hematocrit (vol %)	45.05±4.73	49.05±3.74	44.50±0.7	47.40 ±0.8
M.C.V (fl)	55.75±0.77	57.25±1.48	51.90±2.26	54.20±1.97
M.C.H (pg)	18.45±0.21	19.15±0.21	17.70±0.13	17.60±0.14
M.C.H.C (g/dL)	33.10±0.0	33.45±0.49	34.20±1.38	32.54±0.75
R.D.W (%)	18.00±0.9	19.40±0.84	18.55±0.35	21.45±2.89
Platelet count ($10^3/\mu L$)	875.00±28.29	1120.50±132.21	798.50±17.67	1127.00±111.72

Table 3. Blood biochemical changes after repeated oral administration of the ethanol extract (1000 mg/kg) of *A. chamaecistus* ssp. *tomentella* and the control groups after 23 and 45 days. Data have been expressed as mean ± SD; n= 10 per group.

Biochemistry	23rd day		45th day	
	Control	Extract	Control	Extract
Fasting blood sugar (mg/dL)	82.50 ± 2.12	85.50 ± 10.6	153.50 ± 17.67	130.50 ± 53.5
Blood urea (mg/dL)	51.50 ± 6.36	53.50 ± 10.6	45.00 ± 2.82	54.00 ± 5.65
Creatinine (mg/dL)	0.70 ± 0	0.80 ± 0	0.75 ± 0.07	0.75 ± 0.07
Cholesterol (mg/dL)	84.00 ± 2.82	55.50 ± 0.7*	76.50 ± 2.12	62.50 ± 13.43*
Triglyceride (mg/dL)	82.50 ± 7.5	40.50 ± 7.5*	63.00 ± 22.62	22.50 ± 7.77*
Sodium (mEq/L)	139.00 ± 0	139.00 ± 0	139.50 ± 0.5	136.00 ± 1.5
Potassium (mEq/L)	6.25 ± 1.35	5.10 ± 0.7	4.10 ± 0.1	5.00 ± 0.21
Calcium (mEq/L)	10.25 ± 0.35	10.40 ± 0.7	9.55 ± 0.07	10.70 ± 0.989
SGPT (IU/L)	51.00 ± 15.5	70.50 ± 10.6	36.00 ± 6.3	112.50 ± 21.92*
SGOT (IU/L)	149.50 ± 10.6	151.50 ± 9.19	116.50 ± 0.5	191.00 ± 22
LDH (IU/L)	1135.00 ± 261.62	748.50 ± 202.93	752.00 ± 120.20	1090.00 ± 547.59
Total protein (g/dL)	6.65 ± 0.21	6.50 ± 0.14	6.35± 0.212	6.65 ± 0.494

n= 10 per group; * $p<0.05$) compared to normal control rats.

There was a linear relationship between the antioxidant power and the phenolic contents of NB fraction, aqueous and methanol extracts ($R^2=1$). Similar to the NB fraction, the aqueous and methanol extracts showed considerable antioxidant power and radical inhibitory effect.

Figure 1 shows the plasma glucose level in diabetic mice treated with aqueous (figure 1a) and methanol extracts (figure 1b) with the doses of 200, 400 and 800 mg/kg and MET (500mg/kg), for 4 weeks. In the STZ-diabetic mice, repeated daily oral administration of all doses of aqueous extract of *A. chamaecistus* ssp. *tomentella* induced a significant fall in plasma glucose level on the 3th, 14th and 28th day

The methanol extract (400 mg/kg) showed greater improvement than MET (500 mg/kg) in decreasing glucose levels on the 14th day compared to pre-treatment level (55.24% vs 16.42%, $p<0.001$).

Figure 2 has presented the effect of repeated oral dose of the aqueous extract (400 and 800 mg/kg) and MET (500 mg/kg) on weight changes in diabetic mice for 4 weeks. Based on the results, the amount of body weight gain with the aqueous extract (800 mg) was statistically significant [24.2±0.54 g ($p<0.001$), 29.6±0.58 g ($p<0.01$) and 32.27±0.67 g ($p<0.001$)] in comparison with the diabetic control group; 20.85±0.34, 27.14±0.32 and 28.22±0.23 g, respectively after 3, 14 and 28 days of treatment. On the 28th day, the dose of 800 mg/kg aqueous extract produced significant (32.27±0.67 g vs 29.9±1.19 g, $p<0.05$) increase in weight gain compared to MET (500 mg/kg) as the positive control.

Figure 3 has exhibited the effect of single oral administration of the aqueous extract of (800 mg/kg) on the plasma glucose changes after 2 h in normal mice. Intake of the aqueous extracts (800 mg/kg) resulted in a slight decrease in plasma glucose levels after two hours in normal mice (154.28 ± 4.33 mg/dL at 0 h vs 125.28 ± 9.04 mg/dL after 2 h), which was not statistically significant.

Acute toxicity of ethanol extract (6000 mg/kg) showed no changes in the behavior and

appearance of animals and all of them survived after 48 h of gavage. Based on the observation of the animals, the increase in the weight, the reduction in food intake and water consumption after administration of the extract were not statistically significant ($p<0.05$).

Based on the results shown in table 2, there was no significant difference in hematologic parameters between the control and extract (1000 mg/kg) groups in the 23rd and 45th day samples.

Biochemical changes in 23rd and 45th day blood samples have been shown in table 3. According to the results, a significant decrease in cholesterol and triglyceride levels was observed after using the ethanol extract (1000 mg/kg) after 23th and 45th days and a significant increase in the plasma SGPT after the extract administration was observed compared to the control group following 45 days.

After oral administration of the ethanol extract (1000 mg/kg), no change was observed in the behavior and appearance of the animals during 45 days.

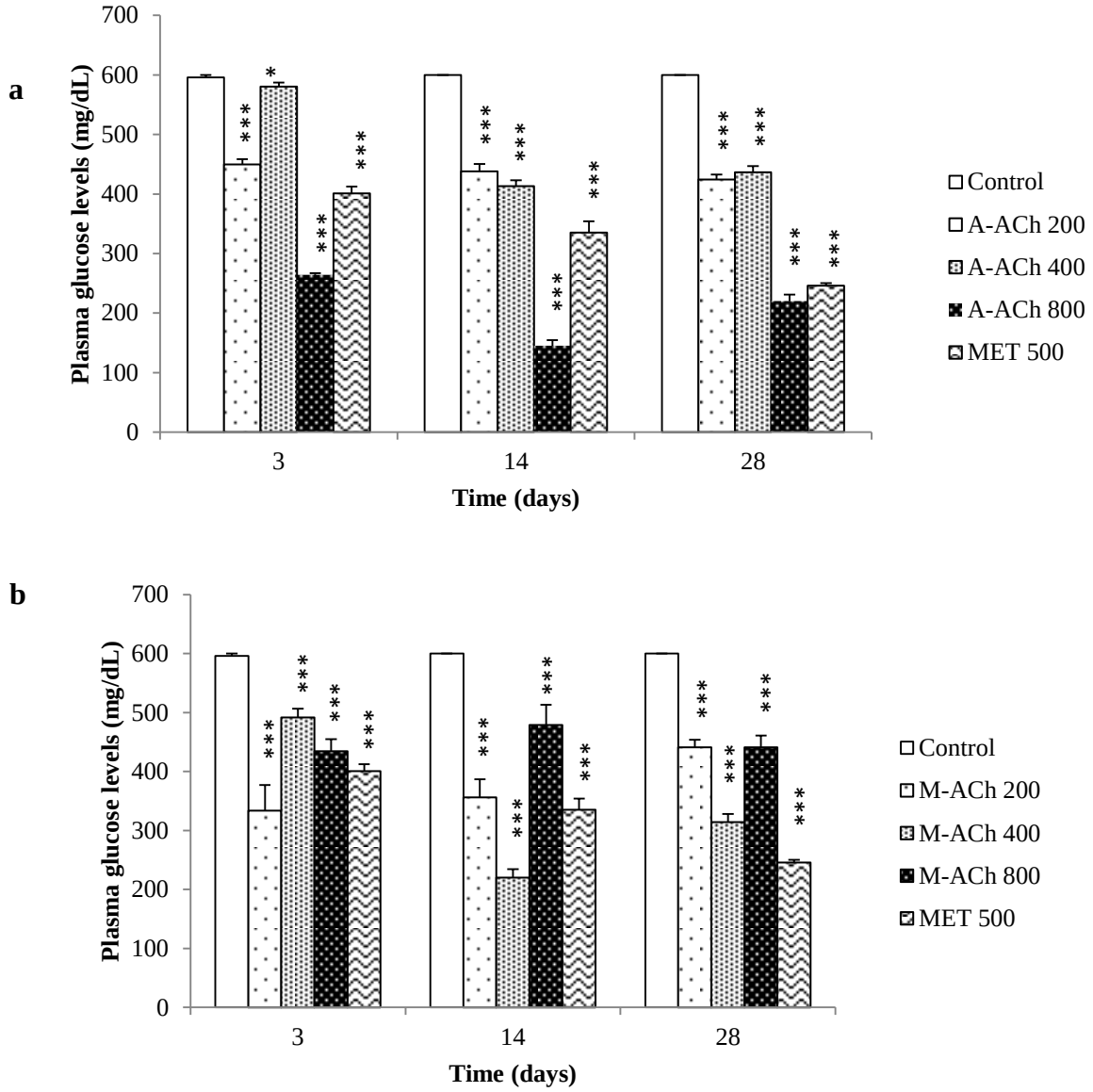

Figure 1. Plasma glucose changes after repeated oral administration of the aqueous (a) and methanol (b) extracts of *A. chamaecistus* ssp. *tomentella* and MET (500 mg/kg) within 28 days in the diabetic mice. Data have been expressed as mean ± SD; n=7 per group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to the diabetic control group. A-ACh: aqueous extract, M-ACh: methanol extract, MET: metformin

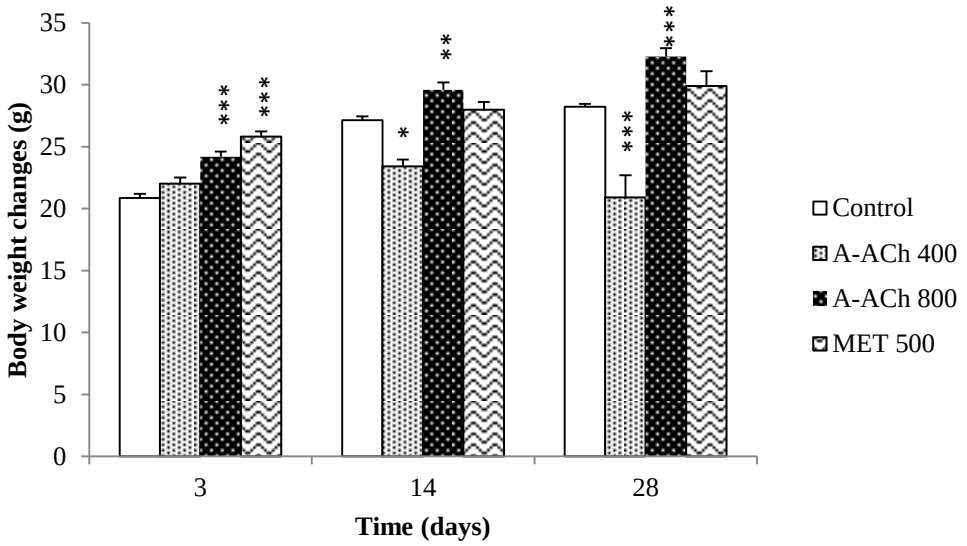

Figure 2. Weight changes after repeated oral administration of the aqueous extract of *A. chamaecistus* ssp. *tomentella* and MET (500 mg/kg) within 28 days in the diabetic mice. Data have been expressed as mean ± SD; n= 7 per group; * p<0.05; ** p<0.01; *** p<0.001 compared to the diabetic control rats. A-ACh: aqueous extract, MET: metformin.

Based on the results of histopathology of animals illustrated in figure 4, after oral administration of the ethanol extract (1000 mg/kg) for 23 and 45 days, the functioning and structure of liver, spleen, kidney and lung were normal and there were no difference with the control group.

The results of the hypoglycemic evaluation of the aqueous and methanol extracts showed that sub-chronic oral administration of the extracts caused a significant decrease in plasma glucose levels in STZ-diabetic mice after 3, 14 and 28 days of treatment. In the diabetic mice, daily dosing with MET resulted in significant hypoglycemia on the days 3, 14 and 28. A comparison of the hypoglycemic potency of test substances after daily administration (sub-chronic) showed that the aqueous extract (800 mg/kg) was more effective than MET in reducing plasma glucose levels in STZ-diabetic mice.

A single oral dose of the aqueous extract caused a slight hypoglycemia (at two-hour post-dose) in normal mice, but not significant. The lower hypoglycemic response in normal glycemic animals compared to diabetic mice may be due to homeostasis mechanisms of glucose/carbohydrate metabolism [32].

STZ-induced diabetic models in mice showed low rate of weight gain, while daily dosing with the aqueous extract (800 mg/kg) led to a significant increase in weight gain rate in the STZ-diabetic mice ($p<0.01$).

Theoretically, the plants with hypoglycaemic activity may act with several mechanisms. Definition of the exact mechanism of the hypoglycaemic effect of *A. chamaecistus* needs more studies. Previous research on *Ajuga* species suggested that the hypoglycemic effect of *Ajuga iva* extract occurs without stimulating insulin secretion [14,32]. Possible mechanism has been proposed to be insulin mimetic effect on muscle and adipose tissues by stimulating glucose uptake and metabolism [33], insulin-like action or inhibitory effect on the hormones that increase the blood glucose levels [14,34].

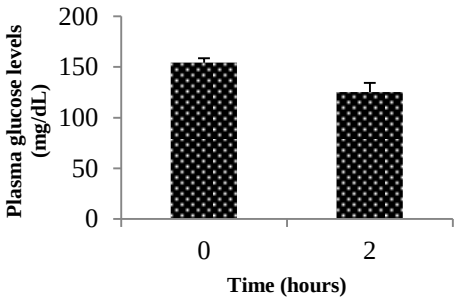

Figure 3. Plasma glucose changes after 2 h of single oral administration of the aqueous extract (800 mg/kg) of *A. chamaecistus* ssp. *tomentella* in the normal mice. Data have been expressed as mean ± SD; n= 7 per group.

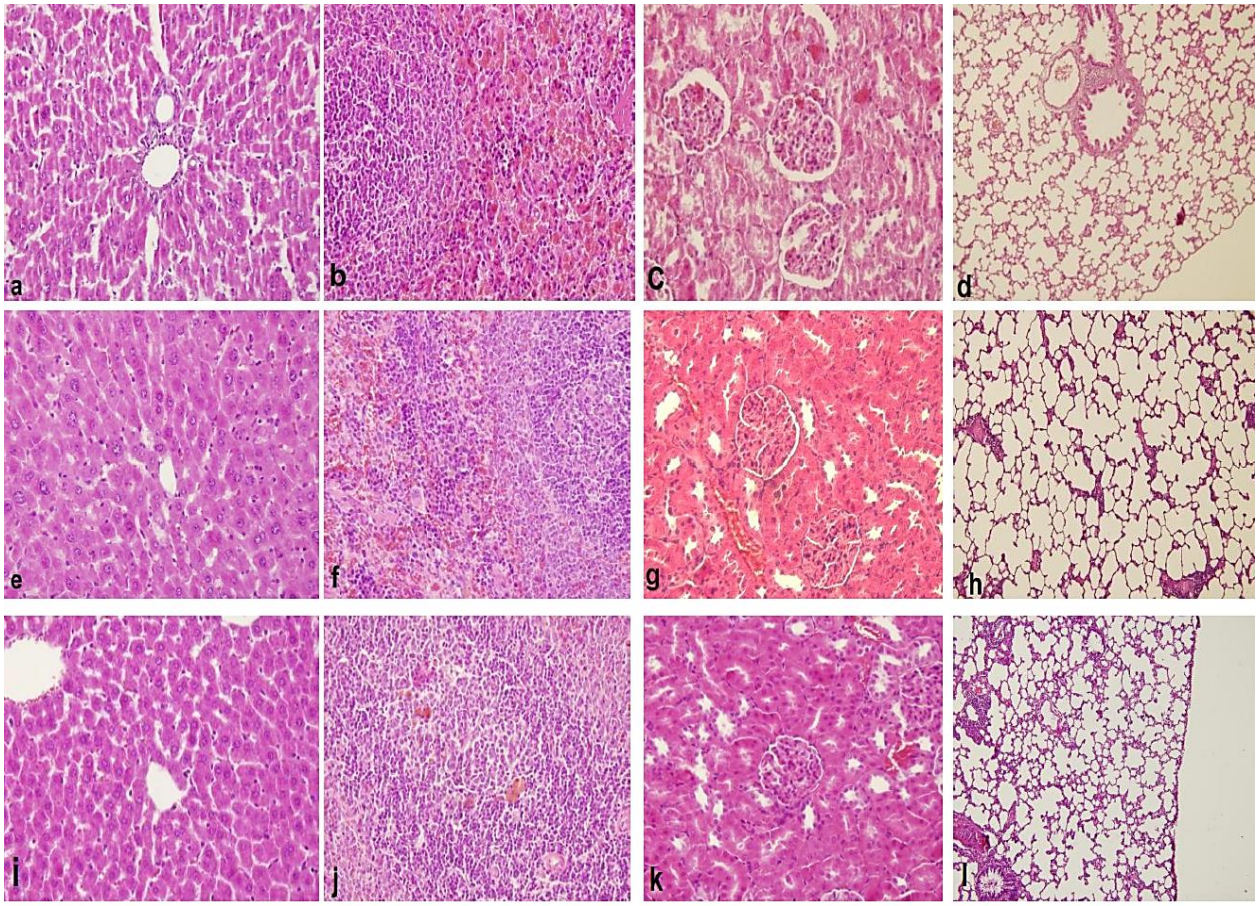

Figure 4. Photomicrograph of some organs after 23 and 45 days of repeated oral administration of the ethanol extract (1000 mg/kg) of *A. chamaecistus* ssp. *tomentella* in normal rat. a) liver; b) spleen; c) kidney; d) lung after 23 days; e) liver; f) spleen; g) kidney, h) lung after 45 days; i) liver; j) spleen; k) kidney; l) lung in control rat after 45 days

Chemical studies on *Ajuga* species have revealed the presence of several flavonoids, tannins, terpenes, steroids and phytoecdysteroids [35]. Flavonoids act as free radical scavengers [36] and like tannins and triterpenes have antioxidant effects [37]. Previous reports have also indicated that phytoecdysteroids have good antioxidant function against free radicals and inhibit galvinoxyl radicals [38]. Moreover, in the plants of the genus *Ajuga*, a variety of phytoecdysteroids and flavonoids have shown anti-diabetic and hypoglycemimc effects [39-41]. In a recent study, the amount of 20-hydroxyecdysone (20E) in the methanol extract of *A. chamaecistus* ssp. *tomentella* was analyzed to be 2.58% (w/w) (0.46% in dry plant) that was higher than other plants containing phytoecdysteroids [42].

According to the previous studies, hyperglycemia, hyperinsulinemia and insulin resistance in type 2 diabetes increase free radical production and oxidative stress [43]. In addition, changes in the antioxidant parameters status have been reported in various tissues in diabetes [44,45]. Clinical studies have shown the efficacy of natural antioxidants in the modulation of oxidative stress associated with diabetes mellitus [5]. Therefore, the use of antioxidants can prevent the diabetes-induced oxidative injury and protect beta cells and endothelial cells against oxidative stress [6]. This result suggests that *A. chamaecistus* ssp. *tomentella* might be effective by preventing oxidative damage and decreasing complications of diabetes due to its antioxidant and anti-diabetic properties.

In conclusion, our study indicated that both acute and repeated oral administration of the extract caused hypoglycemic effect, while this effect was significant in repeated treatment groups. This implies that *A. chamaecistus* ssp. *tomentella* treatment can prevent or be helpful in reducing the complications of diabetes. These results

confirmed the traditional use of some *Ajuga* species for the treatment of diabetes, so it can be used as a therapeutic supplement in diabetic problems. Based on the results of toxicity study, there were no acute and sub-chronic deaths, side effects and behavioral changes with oral administration of the extract of *A. chamaecistus* subsp *tomentella* in rats and the plant could be classified as none-toxic. The effect of reducing cholesterol and triglyceride in sub-chronic consumption of the extract can be an idea for future studies in hyperlipidemia models. Our result suggested that comprehensive chemical and pharmacological research is required to reveal the mechanism of the hypoglycaemic effect and identify the active constituents of *A. chamaecistus* that are responsible for this effect. Also, further clinical investigations considering the non-toxic nature of this plant are recommended.

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Author contributions

Seyede Nargess Sadati Lamardi was involved in conception and design of study, analysis of data, critical revision; Zahra Majidi did acquisition and analysis of data and drafting of article; Chamaan Alipour performed the hypoglycemic study and data analysis; Sajjad Farajzadeh performed the toxicity study and data analysis; Ameneh Gohari performed the antioxidant study and data analysis; Hamed Shafaroodi designed the hypoglycemic study, acquisition and analysis of data; Mahdi Vazirian designed the antioxidant study, acquisition and analysis of data; Seyed Nasser Ostad designed the toxicity studies, acquisition and analysis of data, and critical revision.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

FRAP: ferric reducing antioxidant power; DPPH: 2, 2-diphenyl-1-picryl-hydrazyl; GAE: gallic acid equivalents; STZ: streptozotocin; TPTZ: 2,4,6-Tri(2-pyridyl)-*s*-triazine; DEE: diethyl ether; NB: n-butanol