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Original article

Acute, Sub-acute and Cell Toxicity of *Allium elburzense* Bulb Hydroalcoholic Extract

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Abstract

Background and objectives: *Allium elburzense* is an endemic plant in north of Iran with some nutritional and medicinal applications; however, there is no data on its safety profile. This study was aimed to investigate cytotoxicity, acute and sub-acute toxicity of hydroalcoholic extract of *A. elburzense* bulb. **Methods:** Total phenolic content of the extract was measured using Folin-Ciocalteu method. For cytotoxicity assay, human umbilical vein endothelial cells (HUVECs) were used. In acute toxicity study, single oral dose of 2000 mg/kg was administered in female and male Wistar rats and they were monitored for two weeks. In sub-acute test, 125, 250 and 500 mg/kg/day of extract were orally administered for four weeks. **Results:** Total phenolic content was estimated as 32.8 ± 2.5 mg gallic acid equivalent/g of the extract. The extract showed IC_{50} value of 366.4 µg/mL (95% CI = 246.4-566.1) in HUVECs after 24 h exposure. In acute study, there was no sign of toxicity and no mortality; however, significant increase in relative spleen weight and ALP activity and mild inflammation in kidney tissue were observed. LD_{50} > 2000 mg/kg was estimated for *A. elburzense* bulb extract. In sub-acute assay, there were significant elevations in relative spleen weight, blood urea level, AST, ALT, ALP, total WBC, lymphocyte and neutrophil count and significant decrease in blood sugar and triglyceride levels at higher doses of the extract. **Conclusion:** *Allium elburzense* bulb extract may be considered as safe at doses lower than 500 mg/kg in rats; however, assessment of liver and kidney functions is recommended during chronic uses.

Keywords: *Allium elburzense*; hematology; toxicity tests; Wistar rats

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Introduction

Herbal derived medicines are abundantly used as the basis of raw ingredients in folk medicine and also in pharmaceutical industries around the world. In most developing countries, herbal products are the main part of traditional medicine with a long history of being used for more than a thousand years [1]. According to the World Health Organization (WHO) statistics, almost 80% of people use herbal remedies in some developing countries [2].

Since medicinal plants are rich sources of bioactive components with various pharmacological properties, concerns raise about the potential toxic effects of the continuous use

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of some plants. Therefore, evaluation of the toxicity of each plant extract or herbal formulation is necessary to assure its safety for clinical use [3].

Amaryllidaceae family comprises various famous bulbous flowering herbs with almost 1600 species in 75 genera worldwide [4]. *Allium* is one of the most important genera of this family consisting of some popular plants such as garlic, onion, leek and scallion with wide spread usage as edible crops and also as valuable medications [5]. Various phytochemicals with biological and medical activities such as flavonoids, steroids, saponins, glycosides and sulfur compounds have been recognized in this genus [6].

Allium elburzense Wendelbo is an endemic plant of Elburz Mountains in north of Iran with local name of "Valak". The aerial parts of this plant have nutritional and medicinal applications [6]. In traditional medicine, *A. elburzense* has been used for treatment of some illnesses including diabetes, rheumatism, dermatitis and microbial diseases [7]. Pharmacological researches have shown antioxidant, antispasmodic, antidiabetic, immunoregulatory, fibrinolytic, and antihyperlipidemic properties of *A. elburzense* bulb extract [8-11]. However, *A. elburzense* safety has not to be elucidated yet to our knowledge; therefore, this investigation aimed to evaluate the in vitro and in vivo toxicity profile of *A. elburzense* bulb extract.

Materials and Methods

Ethical considerations

All tests were done in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23). The study was approved by the Biomedical Researches Ethics Committee of Isfahan University of Medical Sciences (ethical approval ID: IR.MUI.REC.1396.2.090, 2017).

Chemicals

Dulbecco′s modified Eagle′s medium (DMEM), fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit were purchased from Bioidea Co. (Tehran, Iran). The commercial kits for measurement of blood biochemical parameters were purchased from Pars Azmoon Co. (Tehran, Iran). Folin-Ciocalteu's phenol reagent and all other chemicals with analytical grade were purchased from Merck KGaA Co. (Germany).

Plant material and preparation of the extract

The bulbs of *A. elburzense* were collected from Damavand region in Elburz Mountains (Iran) in 2018. The plant sample was authenticated and registered (voucher No. 1145) at the Herbarium of School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. The bulbs were dried, powdered and extracted (1000 g/5000 mL) with aqueous ethanol (70%) for 48 h at room temperature using maceration method. The extract was then filtered and concentrated using a rotary evaporator under vacuum at 50 ºC and the extract (300 g) was freeze-dried and reserved in refrigerator at -20 ºC until analysis.

Determination of total phenolics content

For standardization of the plant extract, Folin-Ciocalteu method was used for determination of total phenolic content in the hydroalcoholic extract of *A. elburzense* bulb. In this colorimetric assay, the plant samples or standard solution were mixed with sodium bicarbonate (20%) and then with Folin-Ciocalteu reagent. After 2 h incubation, the absorbance was recorded at 765 nm using a spectrophotometer. A standard curve of gallic acid was prepared for estimation of total phenolic content and the results were stated in terms of mg of gallic acid equivalents (GAE)/g of extract. All experiments were done in triplicate [12].

Cytotoxicity study in vitro

The potential cytotoxicity of *A. elburzense* bulb extract was assessed on human umbilical vein endothelial cells (HUVECs; Pasteur Institute, Tehran, Iran). Endothelial cells are useful for evaluation of toxicity of substances which have entered the circulation and may interact with this cell type. HUVECs have been used in several fields of toxicology and different endpoints for viability and functionality of endothelial cells were considered in these cell line [13]. Regarding the cardiovascular effects of *Allium* plants, we used HUVECs as the normal cell line for evaluation of possible cytotoxicity of *A. elburzense* bulb extract [14].

The cells were grown in DMEM supplemented with 10% FBS and penicillin-streptomycin (100 U/mL-100 µg/mL) at 37 ºC in a humidified

atmosphere in 5% CO_2 incubator. Cytotoxicity was evaluated using MTT kit [15]. Briefly, HUVECs were seeded at 1×10^4 cells per well in 96-well plates. At the logarithmic phase of growth, the cells were exposed with various concentrations of *A. elburzense* bulb extract. Different concentrations of the extracts were prepared in distilled water. The solutions were completely dissolved in the culture medium and the final concentrations in each well were 1, 10, 100, 250, 500 and 1000 µg/mL. After 24 h exposure, the HUVECs were treated with MTT reagent for 4 h at 37 °C. Then the foramazan crystals were dissolved in dimethyl sulfoxide and the absorbance was recorded at 570 nm. Viability of the tested HUVECs was assessed by comparison of the absorbance of each sample with negative control (the cells without any exposure to the extract) and IC_{50} values were then estimated. All the experiments were done in triplicate.

Animals

Six-week-old Wistar rats from both sexes were procured from the animal house of the School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. The laboratory conditions were preserved at a 12 h light/dark cycles under room temperature of 20-25 ºC in polypropylene cages with free access to tap water and standard chow diet. The animals were allowed to acclimatize in our laboratory conditions for 7 days before the test. For acute and subacute toxicity studies, the rats were randomly assigned to the control or treatment groups.

Acute oral toxicity study

The acute toxicity was assessed according to the guideline No. 423 of OECD [16]. This assay was done by administration of 2000 mg/kg of the hydroalcoholic extract of *A. elburzense* bulb as a single dose to the male and female rats orally using an intra-gastric tube. In the control group, the animals received equal volume of normal saline orally. Five animals were allocated in each control and experimental groups. The rats were observed during the first hour and 2, 4 and 6 h after dosing the extract and afterward daily over 14 days for any sign of toxicity or mortality. Observations were made for all possible changes in physical appearance, food and water consumption, bodyweight, behavioral, autonomic and motor activities. The animals were sacrificed at the end of the study. The critical organs were detected for gross changes and the relative organ weight (ratio of organ weight to the total body weight as percentage) was estimated for some vital organs including liver, heart, lung, kidney and spleen. The livers, kidneys and spleens were fixed and further processed for histopathological examination.

Sub-acute oral toxicity study

The sub-acute toxicity was assessed according to the guideline No. 407 of OECD [17] with a little modification. For this assay, forty rats (five per sex in each group) received the hydroalcoholic extract of *A. elburzense* bulb (125, 250 and 500 mg/kg/day) orally or equal volume of normal saline for 4 weeks. The dose range for the sub-acute study has been selected based on the previous pharmacological study [18]. The animals were weighed before the treatment and every week during the test period. After 28 days, the blood samples of 12 h-fasted rats were taken from retro-orbital sinus under anesthesia for biochemical and hematological analysis. The relative organ weight was recorded for liver, heart, lung, kidney and spleen. Histopathological evaluations were done for liver, kidney and spleen tissues.

Biochemical analysis

Various biochemical parameters were assessed after acute and sub-acute administration of *A. elburzense* bulb extract including plasma blood glucose, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, triglyceride (TG), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), total protein, urea, uric acid and creatinine. The commercial kits were used for determining the biochemical parameters based on the enzymatic colorimetric assay using a spectrophotometer.

Hematological analysis

The heparinized blood samples were evaluated for hematological parameters including red blood cells (RBC), red distribution width (RDW), hematocrit (Hct), haemoglobin (Hb), white blood cells (WBC) and differential leukocytes (neutrophil, lymphocyte, eosinophil, basophil), platelets count, mean corpuscular volume (MCV),

mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) using automated hematology analyzer.

Histopathological examination

For histopathological evaluations, the kidney, liver and spleen tissues were fixed in 10% buffered formalin. Tissues were then embedded in paraffin block, cut to 5 µm thickness sections and stained with hematoxylin and eosin (H&E) for inspection by light microscopy.

Statistical analysis

The results were reported as mean ± standard error of mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test using SPSS software version 25.0. For calculating IC_{50} value, GraphPad software (version 8; GraphPad Software, USA) was used by performing log (inhibitor) versus normalized response equation with assuming a Hill slope of 21.0 and enabling automatic outlier determination. The p value <0.05 was considered to be statistically significant.

Results and Discussion

The present study evaluated the cytotoxicity and also in vivo toxicity of oral administration of the hydroalcoholic extract of *A. elburzense* bulb in rats.

The total phenolic content was measured as 32.8 ± 2.5 mg GAE/g of dried bulbs of the plant extract using the standard curve of gallic acid ($y = 0.001x - 0.0053$, $R^2 = 0.9981$). In the study of Chen et al. assessment of various cultivars of garlic (*A. sativum*) has shown similar results from 17.16 to 42.53 mg GAE/g for total phenolics [19].

The cytotoxicity of hydroalcoholic extract of *A. elburzense* bulb was tested on HUVECs as the noncancerous cells by MTT assay after 24 h exposure in vitro. Treatment of HUVECs with different concentrations of extract revealed the IC_{50} value of 366.4 µg/mL (95% CI = 246.4-566.1) for the *A. elburzense* bulb extract (figure 1).

The results showed that *A. elburzense* bulb extract was non-toxic towards normal human cells when used at the concentrations of lower than 100 µg/mL whereas treating with high concentrations (500 µg/mL) led to a remarkable cytotoxicity. According to the obtained IC_{50} value (366.4 µg/mL), the extract appears to be cytotoxic however further in vitro experiments are also required to confirm this matter.

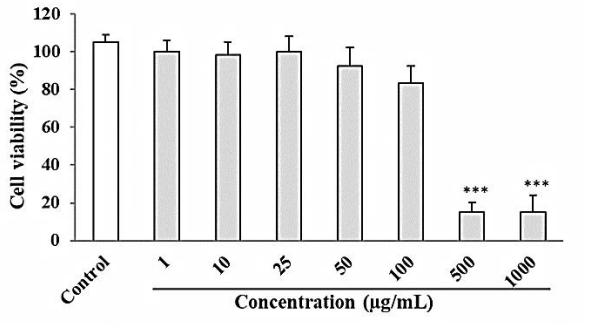

Figure 1. Cytotoxicity of hydroalcoholic extract of *Allium elburzense* bulb in HUVECs determined by MTT assay; cells were treated without (control) or with different concentrations of extracts and incubated for 24 h; values are means ± SEM from three independent experiments in triplicate; $^{***}p< 0.001$ versus control (untreated cells)

In acute oral toxicity study, administration of the single dose of 2000 mg/kg extract of *A. elburzense* bulb extract did not cause any symptoms of toxicity during 14-days test period. All animals were alive and no mortality was found representing that the median lethal dose (LD_{50}) of *A. elburzense* bulb extract was higher than 2000 mg/kg. Therefore, it could be practically safe for humans based on the criteria for classification of hazard agents [20]. No significant abnormal gross change was found in the general appearances, locomotor and behavioral properties in the males or female rats. The body weight gains were normal during the study period (figure 2; male and female body weights). In sub-acute toxicity study, no obvious sign of toxicity nor mortality was found in female and male rats during a 28-day exposure to different concentrations of *A. elburzense* bulb hydroalcoholic extract (125, 250 and 500 mg/kg). The food and water intake were normal and body weight gaining was not affected in rats with different doses of *A. elburzense* bulb extract in this sub-acute study (figure 3; male and female body weight).

Evaluation of the vital organs in sub-acute study showed some enlargement and significant increase in the relative weight of spleen in female rats ($p<0.01$) (table 1). There are some reports about the gender differences in severity of liver damage and sensitivity to the lethal effects of some toxic agents [21].

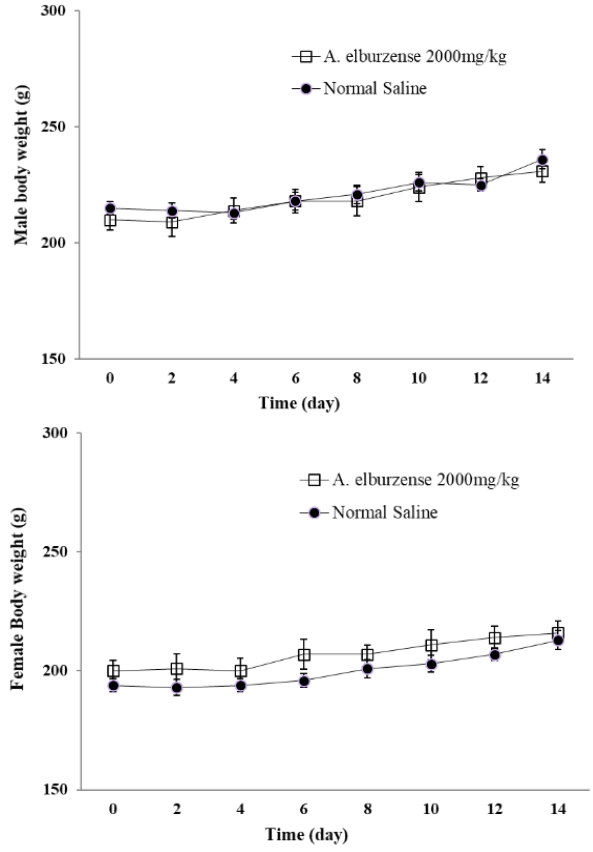

Figure 2. The effect of hydroalcoholic extract of *Allium elburzense* bulb (2000 mg/kg) on mean body weight in male and female rats in acute toxicity study; values are means ± SEM for 5 rats

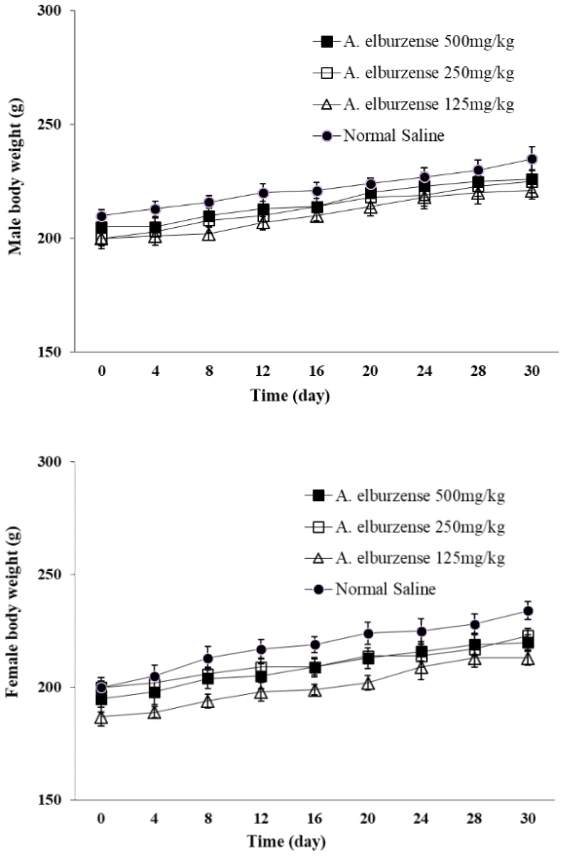

Figure 3. The effect of hydroalcoholic extract of *Allium elburzense* bulb (125, 250 and 500 mg/kg) on mean body weight in male and female rats in subacute toxicity study; values are means ± SEM for 5 rats

Significant elevation in the relative weight of spleen was also observed after administration of the highest dose of extract (500 mg/kg) for 4 weeks in both sexes of rats in sub-acute study ($p<0.05$) (table 1). Some investigations on other species of *Allium* genus have shown similar results with our findings. In the study of Kuda *et al*, administration of a diet containing 2% garlic (*Allium sativum*) for 28 days has been associated with increase in the relative weight of spleen in mice fed beef tallow [22]. Splenomegaly may be resulted from liver diseases or blood disorders described by abnormal blood cells or may be due to the spleen over-activity in removing and abolishing the blood cells. Odiase and his coworker reported that treatment with *A. sativum* bulb extract for 5 weeks in rats led to some alterations in the bone marrow and spleen histology including elevation of myeloid-erythroid cells numbers and activation of splenic sinus histiocytes and lymphoid follicles representing the immunostimulatory activities of garlic [23].

Analysis of biochemical parameters in acute toxicity revealed statistically significant increase in serum ALP activity after the administration of the high toxic dose of *A. elburzense* bulb extract in male and female rats compared to the control group ($p<0.05$) (table 2). The elevated activity of this enzyme may reflect the impairment of the integrity of plasma membranes in the liver and bile canaliculi during excretion of high dose of the extract in acute toxicity assay [24]. All other biochemical parameters including blood sugar, total protein, lipid profile, biomarkers of hepatic and renal function revealed normal levels. As shown in table 2, evaluation of biochemical markers exhibited notable decrease in serum blood sugar level at the doses of 250 and 500 mg/kg of *A. elburzense* bulb extract ($p<0.01$ and $p<0.05$, respectively) and a significant increase in blood urea at the dose of 500 mg/kg ($p<0.01$) compared to the control group in female rats in sub-acute assay.

Table 1. Effect of acute and sub-acute administration of *Allium elburzense* extract on organ weight in female and male rats

Sex	Groups	Liver (BW%)	Kidney (BW%)	Heart (BW%)	Lung (BW%)	Spleen (BW%)
Male	Control	3.85 ± 0.42	0.48 ± 0.05	0.31 ± 0.06	0.49 ± 0.05	0.39 ± 0.05
	125 mg/kg	3.92 ± 0.73	0.48 ± 0.03	0.31 ± 0.05	0.48 ± 0.09	0.41 ± 0.06
	250 mg/kg	3.79 ± 0.82	0.47 ± 0.06	0.34 ± 0.06	0.51 ± 0.09	0.45 ± 0.07
	500 mg/kg	3.98 ± 0.94	0.50 ± 0.07	0.33 ± 0.07	0.50 ± 0.07	0.49 ± 0.06*
	2000 mg/kg	3.82 ± 0.52	0.48 ± 0.04	0.34 ± 0.06	0.48 ± 0.02	0.40 ± 0.07
Female	Control	3.83 ± 0.39	0.49 ± 0.05	0.35 ± 0.07	0.59 ± 0.04	0.42 ± 0.05
	125 mg/kg	3.75 ± 0.50	0.45 ± 0.04	0.33 ± 0.06	0.58 ± 0.06	0.41 ± 0.08
	250 mg/kg	3.92 ± 0.77	0.49 ± 0.07	0.37 ± 0.08	0.57 ± 0.07	0.46 ± 0.09
	500 mg/kg	4.16 ± 0.89	0.51 ± 0.09	0.36 ± 0.06	0.61 ± 0.09	0.51 ± 0.06*
	2000 mg/kg	3.72 ± 0.57	0.59 ± 0.05	0.41 ± 0.05	0.58 ± 0.06	0.49 ± 0.08**

Values are means ± SEM (n=5); significant differences were compared with the corresponding control group which received equal volume of normal saline; *$p<0.05$ and **$p<0.01$; BW%: g % of body weight

A remarkable decline in serum concentration of triglycerides was also observed at the dose of 500 mg/kg of extract in male rats ($p<0.05$). However, there were significant rises in the activities of AST ($p<0.01$), ALT ($p<0.05$) and ALP ($p<0.05$) after the administration of the highest dose of extract in both sexes of animals. In the study of Abdel Gadir *et al*, addition of 6% *A. sativum* to rats' diet for 28 days was associated with liver and kidney degenerative alterations and elevations in AST, ALT and decrease in ALP activity [25]. They found similar nephrotic histopathological and serum enzymes activities changes with 6% onion (*A. cepa*) [25]. Oko and coworkers also reported significant raises in AST, ALT and ALP activities after 2-week administration of 400 and 600 mg/kg from ethanol extract of *A. sativum* leaves in rats [26]. In the study of Huzaifa *et al*, notable elevations were observed in the activities of these enzymes after treatment with 400 and 550 mg/kg of *A. sativum* aqueous extract for 3 weeks indicating its hepatotoxic effects at high doses in rats [24].

Our results from sub-acute study also showed significant increase in blood urea at the dose of 500 mg/kg of extract indicating its effect on kidney or liver function at high doses however further studies are required to understand the detail of *A. elburzense* bulb extract pharmacokinetics. Elevation of urea concentration has been reported by Abdel Gadir *et al* after addition of 6% garlic or onion to rats' diet for 4 weeks [25]. It is noteworthy that there are also several reports on the hepatoprotective and nephroprotective properties of some *Allium* species, albeit at low doses of administration and in pathological conditions of liver and kidney [27, 28]. Biochemical analysis in sub-acute study also exhibited significant decrease in serum blood sugar and triglyceride levels at the higher doses of extract suggesting its potential therapeutic activities in hyperglycemia and hypertriglyceridemia. Organosulfur compounds and flavonoids have been identified as phytochemical components with antidiabetic and antidyslipidemic properties in some *Allium* species [29]. In our previous study, we also found beneficial effects of *A. elburzense* bulb extract in decreasing serum concentrations of blood glucose, triglyceride, total cholesterol, LDL and increasing HDL in a model of dyslipidemia in rats [11].

In hematological analysis, all parameters showed normal levels within physiological range after the 14-day experimental period in both sexes of rats (table 3).

The effect of sub-acute dosing of *A. elburzense* bulb extract on hematological factors has been presented in table 3. Total WBC count and lymphocyte count were significantly increased at the dose of 500 mg/kg of extract ($p<0.05$) in male and female rats. The neutrophils count was also raised at the doses of 250 and 500 mg/kg ($p<0.05$) in both sexes of rats. No remarkable difference was found in other hematological indices when compared with control after the 28-day experimental period. Prominent elevations in total and differential WBC counts have been reported from some *Allium* plants such as *A. sativum* and *A. cepa* describing the immunoregulatory properties of these medicinal plants [10,30,31]. In our study, no significant changes were observed in other hematological parameters however there are controversial reports about the effects of *A. sativum* or *A. cepa* on RBC count, Hb, MCV and MCH as positive haematological effects through increasing RBC or induction of macrocytic normochromic anaemia or haemolytic anaemia in some animal species [25,30,32].

Table 2. Acute and sub-acute administration of *Allium elburzense* extract on biochemical parameters in female and male rats

Parameters	Male					Female				
	Treatment (mg/kg)									
	Control	125	250	500	2000	Control	125	250	500	2000
Blood sugar (mg/dL)	129.3 ± 10.4	141.2 ± 15.3	144.1 ± 10.8	122.7 ± 18.2	122.1 ± 12.5	134.4 ± 12.8	131.6 ± 12.7	85.8** ± 12.5	108.4* ± 18.4	116.5 ± 18.7
Urea (mg/dL)	59.1 ± 4.5	56.8 ± 5.1	50.2 ± 6.3	54.4 ± 4.3	58.8 ± 4.9	45.2 ± 3.7	51.4 ± 7.3	52.8 ± 4.9	72.5** ± 4.8	47.2 ± 4.9
Creatinine (mg/dL)	0.51 ± 0.05	0.51 ± 0.07	0.54 ± 0.03	0.53 ± 0.06	0.51 ± 0.03	0.58 0.04	0.52 ± 0.05	0.55 ± 0.1	0.51 ± 0.03	0.51 ± 0.04
Uric acid (mg/dL)	0.8 ± 0.2	1.1 ± 0.1	0.9 ± 0.3	0.8 ± 0.2	1.1 ± 0.5	1.4 ± 0.1	1.4 ± 0.2	1.1 ± 0.4	1.2 ± 0.2	1.4 ± 0.4
Cholesterol (mg/dL)	70.8 ± 4.3	91.3 ± 3.8	83.2 ± 6.5	81.5 ± 9.7	68.5 ± 9.1	88.2 ± 3.4	86.3 ± 5.8	77.2 ± 5.9	102.1 ± 9.8	75.4 ± 8.8
TG (mg/dL)	111.2 ± 17.2	95.2 ± 20.6	108.1 ± 21.7	70.3* ± 28.1	94.4 ± 15.2	118.4 ± 19.1	94.5 ± 21.6	90.6 ± 21.8	98.2 ± 21.3	112.5 ± 8.7
HDL (mg/dL)	40.3 ± 1.4	48.1 ± 4. 2	41.5 ± 2.4	39.4 ± 2.6	38.4 ± 4.2	38.2 ± 1.6	40.1 ± 2.2	37.6 ± 4.7	41.2 ± 2.7	41.5 ± 4.4
LDL (mg/dL)	42.5 ± 8.2	48.3 ± 7.6	36.7 ± 8.9	46.1 ± 3.2	39.9 ± 8.9	36.1 ± 7.9	35.5 ± 6.7	36.2 ± 7.5	35.2 ± 5.9	38.1 ± 8.5
AST (IU/L)	112.6 ±6.8	115.2 ± 4.0	122.2 ± 6.1	132.1** ± 5.2	126.1 ± 16.7	104.2 ± 7.4	106.6 ± 7.2	108.4 ± 6.5	123.6** ± 9.3	101.5 ± 9.2
ALT (IU/L)	51.8 ± 8.4	59.2 ± 6.0	61.0 ± 9.1	73.8* ± 8.2	52.8 ± 5.3	43.8 ± 5.6	51.1 ± 6.2	53.2 ± 8.9	64.4* ± 5.9	42.8 ± 5.4
ALP (IU/L)	535 ± 24.3	538 ± 21.9	551 ± 19.2	634* ± 28.4	693* ± 49.7	323 ± 19.3	329 ± 22.1	325 ± 25.1	399* ± 26.5	386* ± 46.5
Protein (g/dL)	7.2 ± 0.2	7.5 ± 0.1	7.2 ± 0.2	7.1 ± 0.3	7.1 ± 0.5	7.0 ± 0.1	7.4 ± 0.1	7.4 ± 0.2	7.5 ± 0.4	7.4 ± 0.4

Values are means ± SEM (n=5); significant differences were compared with the corresponding control group which received equal volume of normal saline; * $p<0.05$ and ** $p<0.01$

Table 3. Acute and sub-acute administration of *Allium elburzense* extract on hematological parameters in female and male rats

Parameters	Male					Female				
	Treatment (mg/kg)									
	Control	125	250	500	2000	Control	125	250	500	2000
WBC (10^3/µL)	7.53 ± 0.36	8.23 ± 0.66	8.14 ± 1.42	9.04* ± 0.35	6.93 ± 0.59	7.36 ± 0.34	7.64 ± 0.31	8.44 ± 0.95	9.20* ± 1.63	7.64 ± 0.31
Neutrophils (%)	12.9 ± 3.6	14.3 ± 7.2	24.2* ± 6.7	28.4* ± 6.4	11.3 ± 8.2	10.6 ± 2.5	15.4 ± 4.6	24.8* ± 7.3	23.8* ± 5.7	11.8 ± 4.7
Lymphocyte (%)	65.4 ± 3.9	68.2 ± 9.8	61.0 ± 8.3	70.2* ± 5.9	69.2 ± 9.9	63.6 ± 4.5	68.7 ± 6.6	55.6 ± 9.2	71.4* ± 5.5	67.7 ± 8.9
Eosinophil (%)	1.0 ± 0.3	0.8 ± 0.5	0.8 ± 0.5	1.4 ± 0.6	0.9 ± 0.5	0.9 ± 0.6	1.2 ± 0.5	1.0 ± 0.3	1.1 ± 0.4	0.8 ± 0.7
Monocyte (%)	3.9 ±0.8	4.1 ± 0.5	4.5 ± 0.4	5.8 ± 0.9	4.4 ± 0.9	4.2 ± 0.6	3.6 ± 0.8	4.5 ± 0.3	5.2 ± 0.4	5.6 ± 0.5
RBC (10^3/µL)	7.47 ± 0.15	7.55 ± 0.31	7.44 ± 0.35	6.95 ± 0.41	7.82 ± 0.41	7.05 ± 0.18	6.94 ± 0.21	6.85 ± 0.35	6.79 ± 0.34	7.41 ± 0.30
Hb (g/dL)	13.44 ± 0.19	13.60 ± 0.46	12.92 ± 0.79	12.87 ± 0.68	13.70 ± 0.52	12.52 ± 0.53	12.72 ± 0.18	12.52 ± 0.35	12.98 ± 0.24	13.43 ± 0.64
HCT (%)	37.91 ± 0.56	37.73 ± 0.69	36.98 ± 1.75	38.12 ± 1.95	38.23 ± 0.83	37.23 ± 0.64	36.69 ± 0.72	36.13 ± 0.98	37.06 ± 0.59	38.29 ± 0.85
MCV (Fl)	50.16 ± 0.33	54.32 ± 0.87	53.82 ± 0.65	55.75 ± 0.53	49.97 ± 0.62	53.75 ± 0.46	50.12 ± 0.75	52.80 ± 0.69	52.76 ± 0.63	53.11 ± 0.63
MCH (pg)	17.53 ± 0.40	18.23 ± 0.75	18.81 ± 0.84	18.89 ± 0.87	17.58 ± 0.84	18.62 ± 0.51	18.42 ± 0.36	18.91 ± 0.49	18.33 ± 0.54	17.36 ± 0.45
MCHC (g/dL)	33.4 ± 0.52	34.1 ± 0.47	34.4 ± 0.62	34.9 ± 0.71	32.9 ± 0.52	34.6 ± 0.43	33.9 ± 0.59	35.8 ± 0.94	34.6 ± 0.54	35.5 ± 0.64
Platelets (10^3/µL)	715 ± 35.6	748 ± 52.1	796 ± 67.5	735 ± 56.4	770 ± 51.8	635 ± 65.5	712 ± 72.4	641 ± 59.3	723 ± 82.5	658 ± 78.3
RDW (%)	14.3 ± 0.45	14.6 ± 0.51	14.6 ± 0.62	14.8 ± 0.53	15.1 ± 0.72	14.2 ± 0.47	14.5 ± 0.68	14.8 ± 0.42	14.6 ± 0.61	13.8 ± 0.84

Values are means ± SEM (n=5); significant differences were compared with the corresponding control group which received equal volume of normal saline, * $p<0.05$.

The presence of several bioactive components including flavonoids, sulfuric compounds, sapogenins and saponins such as elburzensoides may be responsible for various effects of *A. elburzense* bulb on different tissues and organs [8,9].

In acute investigation, histopathological evaluation of kidney tissues showed mild inflammation with infiltration of inflammatory cells and mild cloudy swelling of tubules in a few areas after administration of 2000 mg/kg of *A. elburzense* bulb extract (figure 4E). Liver tissues showed no pathological change in acute toxicity study (figure 5E); however, mild histiocytosis was observed in spleen tissues (figure 6E). Histopathological examination of kidney and liver tissues of all animals treated with different doses of *A. elburzense* bulb in sub-acute assay showed normal architectures without any pathological changes (figures 4B-4D and 5B-5D).

Our biochemical analysis showed mild elevations in AST, ALT and ALP activities. Although high levels of these enzymes can reflect the liver damage, histopathological alterations usually occur at serum levels 3 times greater than the upper limit of the normal range [33]. Assessment of spleen tissues showed mild lymphoid follicular activation and mild histiocytosis in animals receiving 500 mg/kg of *A. elburzense* bulb extract (figure 6D).

In conclusion, the toxicity information acquired in this study revealed that the hydroalcoholic extract of *A. elburzense* bulb may be considered as relatively non-toxic at low doses; however, assessment of liver and kidney functions is recommended during chronic usage. Concerning the presence of various bioactive components, more surveys are required to explore the precise safety profile of this plant.

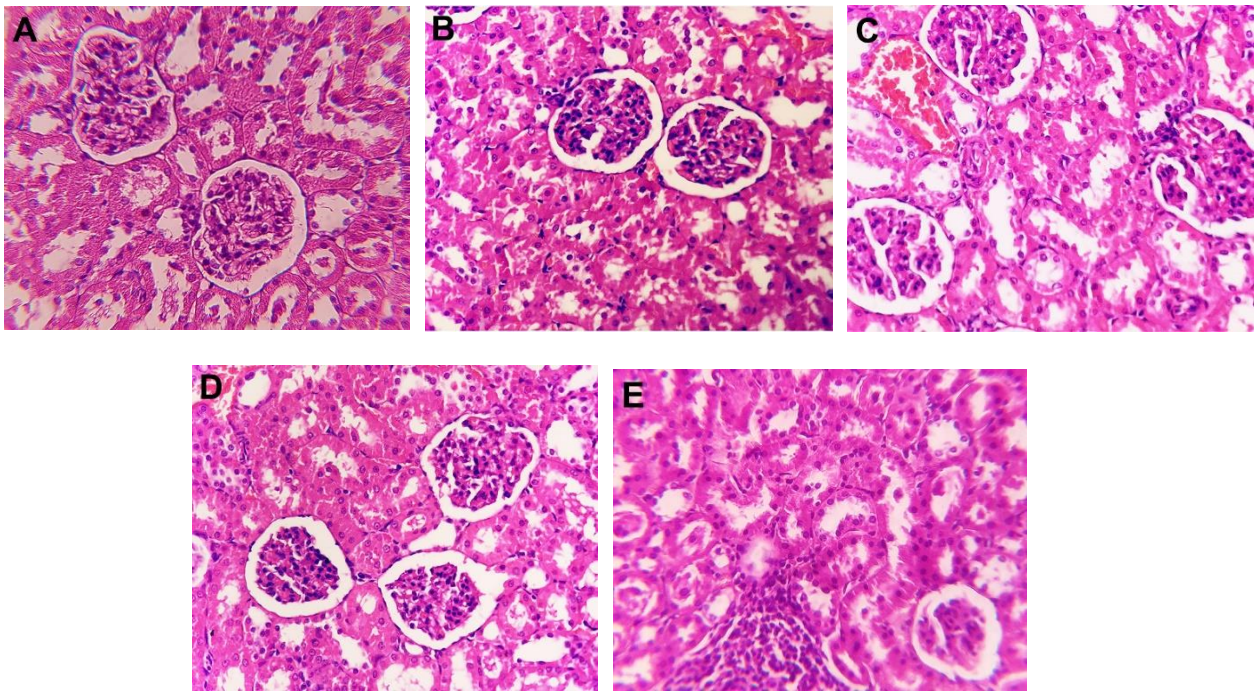

Figure 4. Hematoxylin and eosin histologic sections of kidney tissues of control (A) and treated rats with *Allium elburzense* bulb extract at the doses of 125 mg/kg (B), 250 mg/kg (C), 500 mg/kg (D) and 2000 mg/kg (E) in sub-acute and acute toxicity studies (magnification ×40); mild inflammation with infiltration of inflammatory cells and mild cloudy swelling of tubules could be observed in rats treated with single dose of 2000 mg/kg.

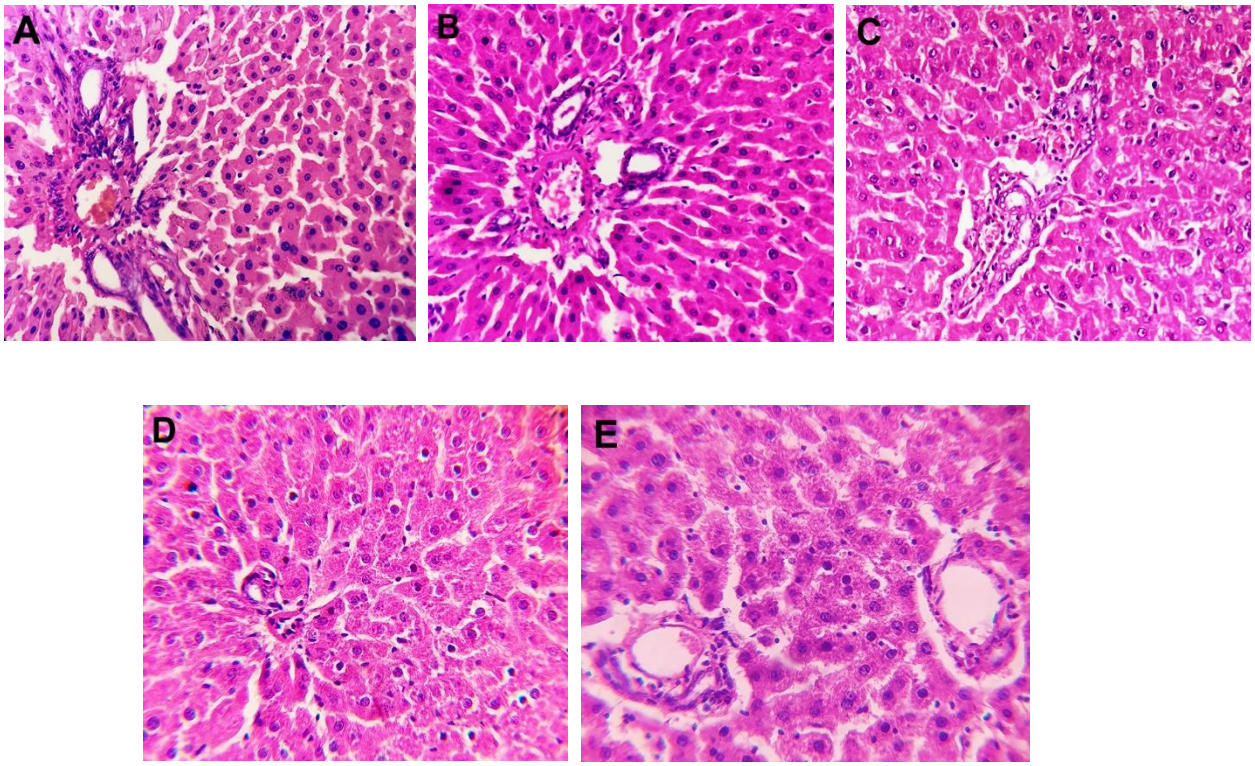

Figure 5. Hematoxylin and eosin histologic sections of liver tissues of control (A) and treated rats with *Allium elburzense* bulb extract at the doses of 125 mg/kg (B), 250 mg/kg (C), 500 mg/kg (D) and 2000 mg/kg (E) in sub-acute and acute toxicity studies (magnification ×40); Normal tissues have been presented at all doses.

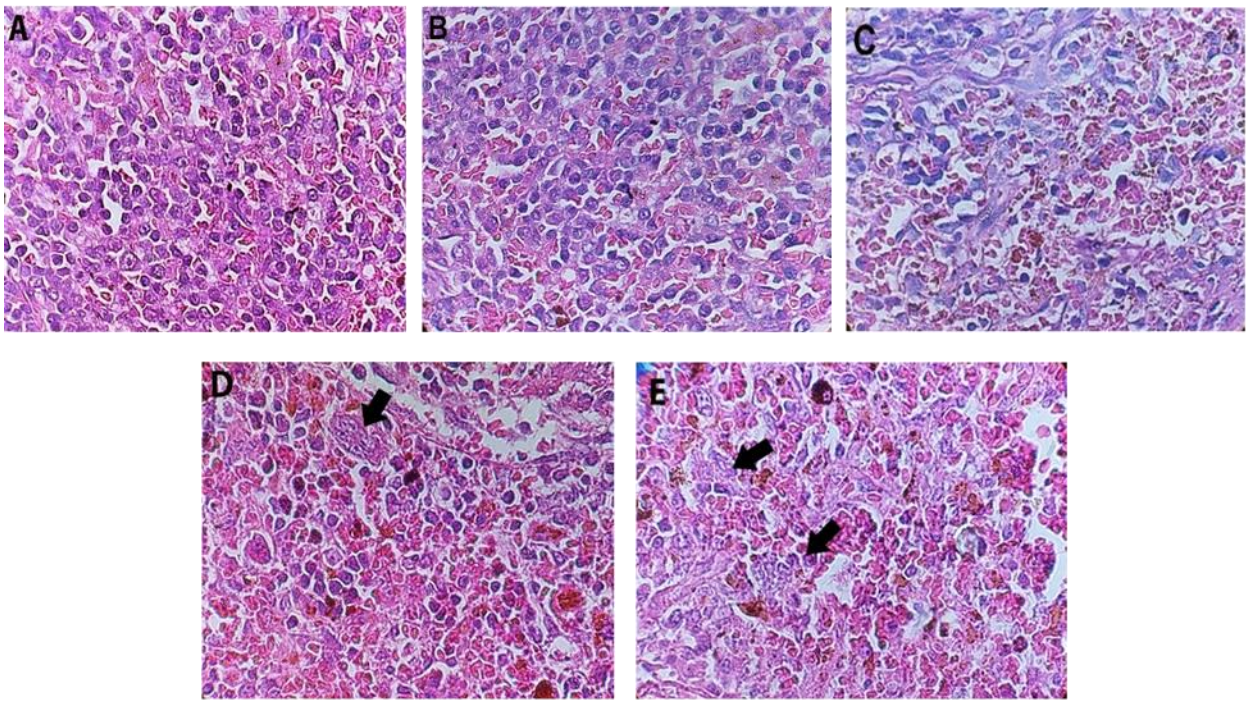

Figure 6. Hematoxylin and eosin histologic sections of spleen tissues of control (A) and treated rats with *Allium elburzense* bulb extract at the doses of 125 mg/kg (B), 250mg/kg (C), 500 mg/kg (D) and 2000 mg/kg (E) in sub-acute and acute toxicity studies (magnification ×100); mild lymphoid follicular activation and mild histiocytosis are seen in rats receiving 500 and 2000 mg/kg of extract (figure 6D).

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Author contributions
Bahareh Yavarian analyzed the data and wrote the manuscript; Leila Safaeian performed the stereological plan, designed the animal studies and edited the manuscript; Behzad Zolfaghari designed the herbal studies; Mahmoud Etebari designed the cellular study; Hamidreza Sharifi was involved in animal handling and treatments.

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

References
[1] Aschwanden C. Herbs for health, but how safe are they? *Bull World Health Organ*. 2001; 79(7): 691-692.
[2] Willcox ML, Bodeker G. Traditional herbal medicines for malaria. *Br Med J*. 2004; 329(7475): 1156-1159.
[3] Sharwan G, Jain P, Pandey R, Shukla SS. Toxicity profile of traditional herbal medicine. *J Ayu Herb Med*. 2015; 1(3): 81-90.
[4] Christenhusz MJM, Byng JW. The number of known plants species in the world and its annual increase. *Phytotoxa*. 2016; 261(3): 201-217.
[5] Neshati F, Fritsch RM. Seed characters and testa sculptures of some Iranian *Allium* L. species (Alliaceae). *Feddes Repert*. 2009; 120(5-6): 322-332.
[6] Zolfaghari B, Shokoohinia Y, Ramezanlou P, Sadeghi A, Mahmoudzadeh M, Minaiyan M. Effects of methanolic and butanolic fractions of *Allium elburzense* Wendelbo bulbs on blood glucose level of normal and STZ-induced diabetic rats. *Res Pharm Sci*. 2012; 7(4): 201-207.
[7] Fritsch RM, Maroofi H. New species and new records of *Allium* L. (Alliaceae) from Iran. *Phyton*. 2010; 50(1): 1-26.
[8] Barile E, Capasso R, Izzo A, Lanzotti V, Sajjadi SE, Zolfaghari B. Structure-activity relationships for saponins from *Allium hirtifolium* and *Allium elburzense* and their antispasmodic activity. *Planta Med*. 2005; 71(11): 1010-1018.
[9] Barile E, Zolfaghari B, Sajjadi SE, Lanzotti V. Saponins of *Allium elburzense*. *J Nat Prod*. 2004; 67(12): 2037-2042.
[10] Radjabian T, Hosseinpur Yektaei Z, Ghazanfari T, Nasiri Z, Fotovvat M. The immunoregulatory effects of four *Allium* species on macrophages and lymphocytes viability. *Immunoregulation*. 2018; 1(3): 143-152.
[11] Safaeian L, Zolfaghari B, Karimi S, Talebi A, Aghaye Ghazvini M. The effects of hydroalcoholic extract of *Allium elburzense* Wendelbo bulb on dexamethasone-induced dyslipidemia, hyperglycemia, and oxidative stress in rats. *Res Pharm Sci*. 2018; 13(1): 22-29.
[12] Yegdaneh A, Ghannadi A, Dayani L. Chemical constituents and biological activities of two Iranian *Cystoseira* species. *Res Pharm Sci*. 2016; 11(4): 311-317.
[13] Schleger C, Platz SJ, Deschl U. Development of an in vitro model for vascular injury with human endothelial cells. *ALTEX*. 2004; 21(3): 12-19.
[14] Bahadoran Z, Mirmiran P, Momenan AA, Azizi F. *Allium* vegetable intakes and the incidence of cardiovascular disease, hypertension, chronic kidney disease, and type 2 diabetes in adults: a longitudinal follow-up study. *J Hypertens*. 2017; 35(9): 1909-1916.
[15] Akbari V, Sadeghi HM, Jafarian-Dehkordi A, Abedi D, Chou CP. Improved biological activity of a single chain antibody fragment against human epidermal growth factor receptor 2 (HER2) expressed in the periplasm of *Escherichia coli*. *Protein Expr Purif*. 2015; 116: 66-74.
[16] Organization for Economic Co-operation and Development. Test No. 423: acute oral toxicity-acute toxic class method. OECD Publishing [Accessed 2020]. Available from: https://read.oecdilibrary.org/environment/test-no-423-acuteoral-toxicity-acute-toxic-classmethod_9789264071001-en#page1.
[17] Organization for Economic Co-operation and Development. Test No. 407: repeated dose 28-day oral toxicity study in rodents. OECD Publishing [Accessed 2020]. Available from: https://read.oecdilibrary.org/environment/test

-no-407-repeated-dose-28-day-oral-toxicity-study-inrodents_9789264070684-en#page1.
[18] Safaeian L, Zolfaghari B, Aghaye-Ghazvini M, Behnampour M. Evaluation of fibrinolytic and antioxidant effects of *Allium elburzense* bulb extracts. *Avicenna J Phytomed*. 2017; 7(3): 223-231.
[19] Chen S, Shen X, Cheng S, Li P, Du J, Chang Y, Meng H. Evaluation of garlic cultivars for polyphenolic content and antioxidant properties. *PLoS One*. 2013; 8(11): 1-12.
[20] United Nations. The globally harmonized system (GHS) of classification and labelling of chemicals. [Accessed 2020]. Available from: http://www.unece.org/trans/danger/publi/ghs/ghs_rev07/07files_e0.html."
[21] Pohjanvirta R, Miettinen H, Sankari S, Hegde N, Lindén J. Unexpected gender difference in sensitivity to the acute toxicity of dioxin in mice. *Toxicol Appl Pharmacol*. 2012; 262(2): 167-176.
[22] Kuda T, Iwai A, Yano T. Effect of red pepper *Capsicum annuum* var. *conoides* and garlic *Allium sativum* on plasma lipid levels and cecal microflora in mice fed beef tallow. *Food Chem Toxicol*. 2004; 42(10): 1695-1700.
[23] Odiase DE, Osazee LO. Histological effects aqueous extract of *Allium sativum* (Alliaceae) bulb on bone and spleen of adult Wistar rats. *J Appl Sci Environ Manage*. 2017; 21(3): 538-544.
[24] Huzaifa U, Labaran I, Bello AB. Effect of oral administration of aqueous garlic (*Allium sativum*) extract on liver function on rats. *Tech Sci Afr J*. 2013; 8(2): 113-115.
[25] Abdel Gadir EH, Abdel Gadir WS, Adam SEI. Response of Wistar rats to low levels of dietary *Allium cepa*, *Allium sativum* and sodium selenite. *J Pharmacol Toxicol*. 2006; 1(3): 284-288.
[26] Oko MO, Anyim C, Nworie O, Agah MV, Okoli CS. The effects of ethanol extract of *Allium sativum* leaves on aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase in albino rats. *J Chem Biol Phy Sci Sec B*. 2012; 3(1): 256-263.
[27] Ilyas N, Sadiq M, Jehangir A. Hepatoprotective effect of garlic (*Allium sativum*) and milk thistle (silymarin) in isoniazid induced hepatotoxicity in rats. *Biomedica*. 2011; 27(2): 166-170.
[28] Anusuya N, Durgadevi P, Dhinek A, Mythily S. Nephroprotective effect of ethanolic extract of garlic (*Allium sativum* L.) on cisplatin induced nephrotoxicity in male Wistar rats. *Asian J Pharm Clin Res*. 2013; 6(8): 97-100.
[29] Zeng Y, Li Y, Yang J, Pu X, Du J, Yang X, Yang T, Yang S. Therapeutic role of functional components in *Alliums* for preventive chronic disease in human being. *Evid-Based Compl Altern Med*. 2017; Article ID 9402849.
[30] Olaniyan OT, Meraiyebu AB, Arogbonlo A, Dare JB, Shekins O, Shafe MO. Effects of aqueous extract of garlic (*Allium sativum*) on blood parameters in adult Wistar rats (*Rattus novergicus*). *Int J Pharm Sci Invent*. 2013; 2(3): 42-45.
[31] Mirabeau TY, Samson ES. Effect of *Allium cepa* and *Allium sativum* on some immunological cells in rats. *Afr J Tradit Complement Altern Med*. 2012; 9(3): 374-379.
[32] Banerjee SK, Maulik SK. Effect of garlic on cardiovascular disorders: a review. *Nutr J*. 2002: 1(1): 1-14.
[33] Aulbach AD, Amuzie CJ. Biomarkers in nonclinical drug development. In: Faqi AS, Ed. A comprehensive guide to toxicology in nonclinical drug development. London: Academic Press, 2017.

Abbreviations

HUVECs: human umbilical vein endothelial cells;
LD_{50}: median lethal dose