

# Cytotoxicity, acute, and sub-chronic toxicity of the *Arctium tomentosum* Mill. root extract

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## ABSTRACT

Since ancient times people around the world use the medicinal plants for their therapeutic potential. Some studies of the antioxidant, antimicrobial, anti-inflammatory, and hepatoprotective effects of the extracts from plants of the genus *Arctium* have been conducted. Basically, the extracts obtained from these plants are considered safe with low toxicities; however, little is known about toxicity of the *Arctium tomentosum* Mill. extract. Therefore, our work was aimed to study the toxic effect of a carbon dioxide-extract from the *A. tomentosum* Mill. root through MTT assay, acute, and sub-chronic toxicity studies. In MTT assay, low cytotoxic effect was observed at the concentrations between 0.078 mg/mL and 0.001 mg/mL. The IC<sub>50</sub> value – 0.091 mg/mL was determined through non-linear regression analysis. The acute toxicity test demonstrated that the LD<sub>50</sub> is >5000 mg/kg and in the sub-chronic toxicity test, a dose 200 mg/kg did not induce the toxic effect. In this way, the present study can serve as a base for the further investigation of the antimicrobial, antioxidant, and hepatoprotective effects of the *A. tomentosum* Mill. root extract.

## 1. INTRODUCTION

The use of the medicinal plants for the prophylaxis and treatment of the various diseases was distributed throughout the world since the ancient times [1]. Even ruderal plants have medical properties and are widely used in traditional medicine [2]. *Arctium lappa* L. (“greater burdock”), the well-known species from the *Asteraceae* family, along with another less-known species *Arctium tomentosum* Mill. (“woolly burdock”) were used for the treatment of the various gastrointestinal disorders and skin diseases [3].

Different parts of the plants can be used to create extracts with therapeutic effects, such as roots [4-6], leaves [7], seeds [8], fruits [9], and flowers [10]. The variety of the biologically active compounds in the content of the roots, leaves, flowers, and seeds of the greater burdock and the woolly burdock include: Hydroxybenzoic acids, hydroxycinnamic acids and their derivatives, tannins, phenolic compounds and their glycosides, flavonoids, coumarins, anthocyanins, amino acids and other nitrogen-containing compounds, polysaccharides, phenolic compounds, polyacetylenic compounds, phytosterols, triterpenoids, monounsaturated and polyunsaturated acids, and many other biologically active substances [11,12]. Arctiin

is a substance that transforms into arctigenin within the human body, exhibiting antimicrobial properties [13]. Polyunsaturated and monounsaturated fatty acids are able to destroy the cell membrane of the bacteria inducing their phagocytosis [14]. Phenolic components such as quercetin, kaempferol, and apigenin derivatives in the burdock also conduct antibacterial, antifungal, antiviral, and antioxidant effects [15,16]. Inulin is able to lower blood glucose levels and is benefit during diabetes [17]. Thus, the extracts of the *Arctium* ruderal plants have beneficial properties; however, relatively little research has been conducted on their toxicity. *In vitro* cytotoxicity tests are the preliminary stage in pharmacological studies. Test can serve as a first stage for the study of the toxic properties of the plant extracts. It will provide some background information on the potential of the active compounds against both normal and tumor cell lines. Moreover, using *in vitro* methods represents a replacement, or reduction of a number of animals used in studies, in line with the 3Rs. Subsequent acute and sub-chronic toxicity study can be included as a part of preclinical investigation. In this way, the listed stages will help to implement the toxicity testing approach for further research on the benefit properties of the plant extracts, such as antimicrobial, antioxidant, and hepatoprotective activities. [18].

In this context, the main objective of the present study was to evaluate the toxic effect of the roots of *A. tomentosum* Mill. extract and it included two stages. The first stage was the toxicity assessment on blood mononuclear cells by MTT assay. The second stage of the experiment was to study acute and sub-chronic toxicity of the extract in rats.

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## 2. MATERIALS AND METHODS

### 2.1. Plant Material and Extraction Process

Samples of *A. tomentosum* Mill. were collected in the Aksai gorge of the Ile Alatau, referred to the Northern Tian Shan Mountain (43°08'52"N, 76°47'56"E). Voucher specimen is deposited in the Institute of Botany and Phytointroduction (as Herbarium AA 0002321). The roots were ground into a fine powder using an electric grinder, then sieved on the vibratory shaker and processed in the 5L capacity subcritical extraction system, detailed in Figure 1 with the following conditions:  $67 \pm 1$  bar,  $24 \pm 1$  °C. Constant liquid carbon dioxide (CO<sub>2</sub>) flow rate 10 mL/min was provided from the system pump and pressure within extraction vessel was controlled by automatic pressure regulator. Duration time of the process was approximately 4 h. Finally, the gold-colored extract was collected in a sterile container [Figure 2].

### 2.2. MTT Assay

Cytotoxicity test was conducted on mononuclear lymphocytes (BPS Bioscience, San Diego, CA). The cells were cultured in a 10% RPMI1640

culture medium containing 5 mL of fetal bovine serum, 1 mL of glutamine, and 500 µL of streptomycin. Then, 100 µL of the cell culture was placed into the 96-well microtiter plate. This microplate was then placed in a CO<sub>2</sub>-incubator at 37°C (atmosphere 5% CO<sub>2</sub> and 95% air) for 48 h. *A. tomentosum* Mill. extract was diluted in dimethyl Sulfoxide (DMSO) at the following concentrations: 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.04, 0.02, 0.01, 0.005, 0.002, and 0.001 mg/mL. These concentrations were selected by gradual double dilution of the highest concentration – 5 mg/mL in accordance with the same study on the plant extract cytotoxicity [19]. The cell culture was treated by extract solutions and incubated for 72 h at 37°C (atmosphere 5% CO<sub>2</sub> and 95%). Concentration of DMSO in the cell culture did not exceed 0.1%, thus it had no effect on the viability of the cells. The cell culture for the control test was not treated with the studied extract but was also incubated in 10% culture medium.

Investigation of the cell viability was carried out using yellow tetrazole solution (MTT), previously stored at –20°C. The medium in the cell culture was removed, then, 5 mg/mL of MTT solution was added to the cell culture 4 h before the end of the incubation. The cell viability was evaluated by observing the intensity of the purple color, which indicated the ability of living cells to restore the yellow tetrazole to purple formazan. Then, the microplate was centrifuged at 200 rpm for 5 min, after which the supernatant was removed. Then, 100 µL of DMSO was added to the microplate and it was incubated in the dark at 20°C for 20 min until the precipitate was completely dissolved.

The optical density of the test solutions was determined at 540/620 nm on the Sunrise TS universal medical microplate reader (Tecan, Austria). 0.9% of sodium chloride was used as a blank solution. The results were used for the calculation of the dead cells percentage according to the following formula [20]:

$$\text{Cell death (\%)} = 100 - \left( \frac{A_1 - A_2}{A_3 - A_2} \right) \times 100\%, \quad (1)$$

where:

A<sub>1</sub> – the optical density of the test solution (*A. tomentosum* Mill. root extract)

A<sub>2</sub> – the optical density of the blank solution (0.9% NaCl)

A<sub>3</sub> – the optical density of the control solution (culture medium)

The concentration of the *A. tomentosum* Mill. root extract which resulted in a 50% reduction of cell viability, the half maximal inhibitory concentration (IC<sub>50</sub> value), was calculated through non-linear regression analysis (Graph Pad Prism version 6.0, California).

### 2.3. Animals

Adult Wistar rats were housed in conventional standard laboratory conditions (temperature  $22 \pm 2$  °C, relative humidity  $50 \pm 10\%$ , and 12 h light/dark cycle), five animals per cage with free access to granular standard food and water. They were acclimatized for 7 days before the start of the experiments. Housing conditions and the procedures complied with the directive 2010/63/EU. The experimental protocol was approved by the Ethical Committee of JSC “Scientific Center for Anti-Infectious Drugs” No. 23/7 in accordance with the “Guide for the Care and Use of Laboratory Animals” and ARRIVE guidelines.

### 2.4. Acute Toxicity Test

The acute toxicity study used five male and five female Wistar rats. Studies followed the Organization for Economic Cooperation and Development (OECD) Guidelines for the testing of chemicals, test no. 425: Acute oral

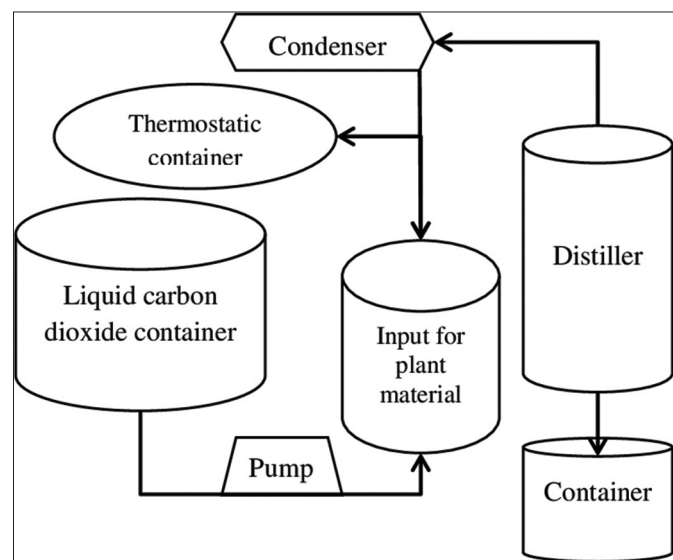


Figure 1: Schematic diagram of subcritical carbon dioxide extraction system.

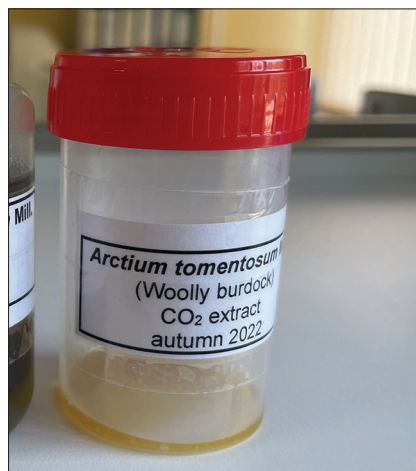


Figure 2: Carbon dioxide-extract of *Arctium tomentosum* Mill.

toxicity: Up-and-down procedure [21]. The experimental rats received doses of 2000 mg/b.w. and 5000 mg/b.w. orally; all animals survived and showed no signs of toxicity. After 14 days, the animals were euthanized and subjected to the macroscopic examination.

### 2.5. Sub-chronic Toxicity Test

The sub-chronic toxicity study was carried out in compliance with the OECD guidelines for the testing of chemicals, Test No. 407: Repeated dose 28-day oral toxicity study in rodents [22]. Two groups of female Wistar rats: The control and the test groups were formed. The extract was daily administered at a dose of 200 mg/kg to the test group for 28 days, while the control group was treated with 0.9% NaCl. During this period, the animals were observed for the physical changes, reduction of the body weight and the behavioral patterns. On the 29<sup>th</sup> day of the experiment, euthanasia was conducted, the blood samples were collected for the hematological and biochemical assay, and the main organs were subjected for the histological studies.

### 2.6. Hematology

The blood samples of the tested animals were collected under deep isoflurane narcosis from retroocular sinus on clot activator containers. Later, the animals were euthanized by anesthetic overdose. The blood samples were centrifuged at 3000 g at 4°C for 15 min and then sent to laboratory for biochemical analysis.

### 2.7. Microscopic Observation

The main organs for the histological study were the liver and kidneys. These organs were removed on the 29<sup>th</sup> day of the sub-chronic toxicity experiment. Separated organs were fixed in 10% formalin buffer solution and dehydrated in graded series of alcohol, cleared in xylol. The organ fragments were embedded in paraffin wax, cut to a thickness of 3 µm, and stained with Hematoxylin and Eosin. The microscopic investigation was conducted using the Carl Zeiss Axio Scope.A1 (ZEISS, Germany).

### 2.8. Statistical Analysis

The results are expressed as mean ± standard deviation. The IC<sub>50</sub> value was determined from non-linear regression analysis. To

assume Gaussian distribution, normality distribution was checked by Shapiro–Wilk normality test. For the determination of the difference between groups of the acute toxicity test, the one-way analysis of variance followed by Dunnett post-test was done and for the determination of the difference between groups of the sub-chronic test, the *t*-test followed by Bonferroni post-test was done (Graph Pad Prism version 6.0, California). Statistical significance was set at  $P < 0.05$ .

## 3. RESULTS AND DISCUSSION

### 3.1. In Vitro Studies

The cytotoxic effect of the *A. tomentosum* Mill. root extract was studied on mononuclear lymphocytes by MTT-test at concentrations of: 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.04, 0.02, 0.01, 0.005, 0.002, and 0.001 mg/mL. The results of the photometric assay are presented in Table 1.

The IC<sub>50</sub> value of the *A. tomentosum* Mill. root extract was 0.091 mg/mL by MTT assay [Table 1]. Concentrations below 0.078 mg/mL showed a higher cell viability rate, up to 99%. The highest studied concentration 5 mg/mL showed the 60% decreased viability, and the lowest studied concentration 0.001 mg/mL showed the 99% cell viability [Figure 3].

Overall, this study evaluates that the *A. tomentosum* Mill. root extract has potential cytotoxic activity on the peripheral blood mononuclear cells at the higher concentrations. The greater cytotoxicity at the higher concentrations of the extract can create perspective for the hindering of cancer cell lines.

The inhibition at the higher concentrations may be due to the presence of the biologically active substances in the composition of the extract. For example, flavonoids possess hydroxyl groups and carbon bonds that are able to bind to the proteins of the human body. According to the previous study, the similar mechanism of binding flavonoid molecules to the breast cancer resistance protein causes cytotoxic aka anti-proliferative effect against cancer cells [23,24]. Presumably, this mechanism also works on the non-malignant cells, which is why the mild cytotoxic influence was manifested on lymphocytes in our study. The most common flavonoids are quercetin

**Table 1:** The cytotoxic effect of the *Arctium tomentosum* Mill. root extract on the cell culture.

Concentration, mg/mL	Absorbance	Cell viability, %	Cell death, %	IC <sub>50</sub> , mg/mL
5	0.018±0.001	39.568±2.492	60.432±2.492	-
2.5	0.014±0.001	30.935±1.246	69.065±1.246	-
1.25	0.015±0.001	32.374±2.158	67.626±2.158	-
0.625	0.018±0.001	38.130±1.246	61.870±1.246	-
0.312	0.018±0.001	38.849±2.158	61.151±2.158	-
0.156	0.021±0.022	47.482±2.158	52.518±2.158	0.091
0.078	0.036±0.002	78.417±3.296	21.583±3.296	-
0.04	0.038±0.001	82.014±2.159	17.986±2.159	-
0.02	0.039±0.001	83.453±2.492	16.547±2.492	-
0.01	0.044±0.001	94.964±2.158	5.036±2.158	-
0.005	0.045±0.001	97.122±2.159	2.878±2.159	-
0.002	0.046±0.001	99.521±0.415	0.479±0.000	-
0.001	0.047±0.001	99.760±0.415	0.240±0.000	-
Control	0.046±0.002	98.081±1.661	1.919±0.000	-

and ellagic acid. Quercetin and ellagic acid are common flavonoids. Quercetin can halt the S phase of cell division by inserting into the DNA chain, causing apoptosis; this mechanism is also observed in cancer cells [25]. Furthermore, it has been shown that quercetin causes the mitochondrial membrane depolarization, that may lead to the apoptosis too [26]. Ellagic acid can trigger the internal pathway of the apoptosis [27]. Arctigenin, the well-known representative of the lignans, is found in *Arctium* plants and it is able to enhance the cytotoxic effect of the anticancer drug doxorubicin [28]. Moreover, it is able to increase the sensitivity to another anticancer drug cisplatin in the cells of the lung cancer [29]. The mechanism relies on enhancing the apoptosis-inducing factor, which triggers apoptosis by making cell membranes more permeable, causing chromatin condensation and nuclear fragmentation [30]. Polyunsaturated fatty acids have a cytotoxic effect by generating free radicals and lipid peroxidases, which, in turn, hinders cancer cell growth [31]. In the same way, the polyunsaturated fatty acids may cause the death of the lymphocytes [32]. The phenolic compounds such as caffeic acid and chlorogenic acid are able to suppress the cell growth by enhancing the DNA methylation and interfering with the gene expression [33-35]. These compounds impact the activity of the DNA enzymes – methyltransferases that catalyze the methylation of the nucleotide residues in the DNA [36]. Steroidal saponins, the representative of the group of glycosides, along with the activation of the internal apoptosis, interact with the transmembrane proteins – aquaporins and enhances the transport of the water into the cell, what consequently disrupts the cell structure [37]. Furthermore, through interaction with the intrinsic factors of the apoptosis, saponins are able to stimulate the autophagy and phagocytosis, as well as the inhibition of metastasis [38].

### 3.2. In Vivo Studies

At the doses of 2000 mg/kg and 5000 mg/kg of the *A. tomentosum* Mill. extract showed no negative effect on the physical state and behavioral patterns of the test group during 14-day observation period. No signs of the skin, fur and mucous membrane abnormality, as well as no mortality were indicated. Furthermore, no significant changes of the body weight and the relative weight of the organs were observed between the control and the test group [Figure 4a and b].

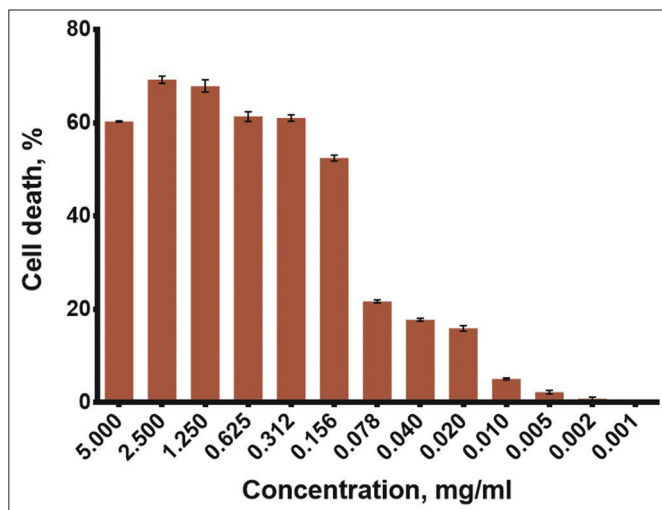
28-day oral administration of the *A. tomentosum* Mill. root extract at the dose of 200 mg/kg did not induce vigorous signs of toxicity. No mortality, as well as abnormal signs in behavior, lesions on the skin, eyes, fur, and mucous membrane were observed. Similarly, to the acute test, no significant changes in the body weight and the relative weight of the organs (heart, liver, kidneys, spleen, and thymus gland) were indicated [Figure 5a and b].

The results of the blood analysis after 28-day sub-chronic administration of the extract are presented in Table 2.

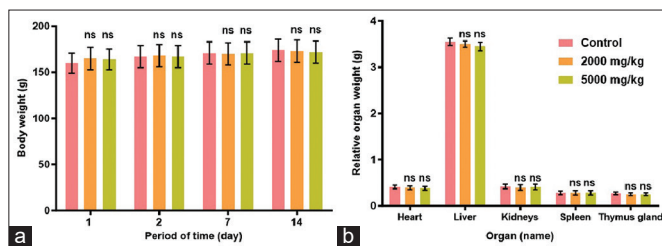
According to Table 2, a slightly decrease in WBCs count was found in the test group, compared to the control; however, it was remained within the normal range [39].

During microscopic investigation, no pathological changes in the structure of the liver [Figure 6a-c] and kidneys [Figure 7a-c] were observed.

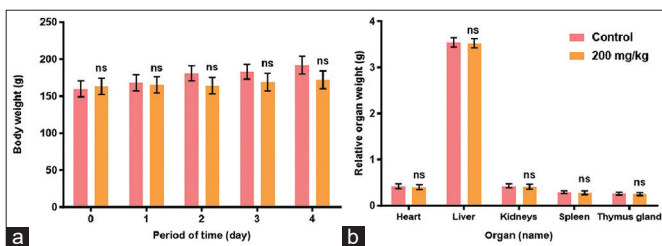
Overall, this study evaluates that the *A. tomentosum* Mill. root extract has no acute toxic effect at the doses of 2000 mg/kg and 5000 mg/kg, as well as no sub-chronic toxicity effect at the dose 200 mg/kg. It can



**Figure 3:** Concentration of *Arctium tomentosum* Mill. root extract versus the cell culture death. The left axis shows the percentage of cells died in the extract solution. The X-axis shows the concentrations of the extract solution.



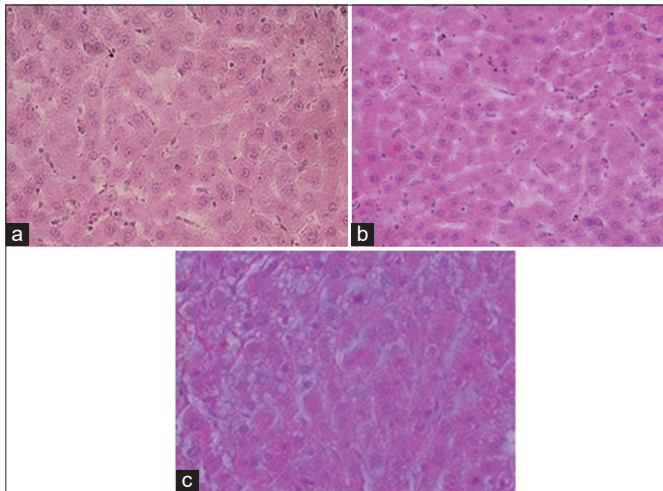
**Figure 4:** Body weight (a) and relative organ weight (b) during acute toxicity test of *Arctium tomentosum* Mill. root extract. Data are expressed as mean  $\pm$  standard deviation;  $n = 3$ ,  $*P < 0.05$ , not significant (ns) compared to the control group.



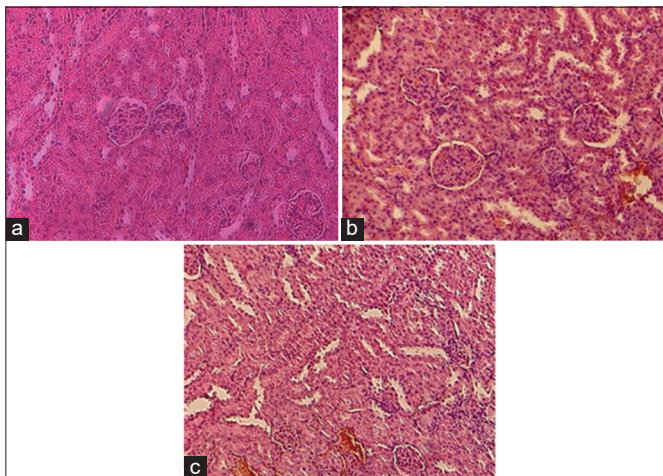
**Figure 5:** Body weight (a) and relative organ weight (b) during sub-chronic toxicity test of *Arctium tomentosum* Mill. root extract. Data are expressed as mean  $\pm$  standard deviation;  $n = 3$ ,  $*P < 0.05$ , not significant (ns) compared to the control group.

be classified as practically nontoxic according to Hodge and sterner scale [40].

The studied dose for sub-chronic toxicity was chosen based on the previous research of Yaghoobi *et al.* (2018) [41]. In the group treated with 200 mg/kg of the extract over the period of 28 days, there were no significant changes in the body weight; however, a few toxic effects on lungs were observed. Hematological profile showed no significant difference between the control group and the group received the extract at the dose of 200 mg/kg for 28 days. Thus, the present study suggests a dose  $<200$  mg/kg b.w. of the extract for the daily oral treatment. The results obtained here are in agreement with



**Figure 6:** Histologic aspect of the liver: (a) The control group, (b) the acute toxicity test group, and (c) the sub-chronic toxicity test group. There were no pathological changes in the structure and the shape of hepatocytes. Stain Hematoxylin and Eosin. Mag.  $\times 400$ .



**Figure 7:** Histologic aspect of the kidneys: (a) The control group, (b) the acute toxicity test group, and (c) the sub-chronic toxicity test group. There were no pathological changes in the structure of the cortex and medulla. Stain H&E. Mag.  $\times 400$ .

Yaghoubi *et al.* (2018) study of the plant of the same genus *A. lappa* L. Overall, in the presented *in vivo* adult Wistar rats appeared to tolerate *A. tomentosum* Mill. root extract at the acute dose of 5000 mg/kg b.w. well. The phytochemical composition of *A. tomentosum* Mill. root has not been widely analyzed so far, however, arctiin and arctigenin were found in its fruit extract [42]. Antioxidant compounds such as campesterol, squalene, lupeol, and tocopherols were found in the flowers and leaves [43,44]. Tannins are the most common in the root of the burdock. Since they exert inhibitory and cytotoxic activity on the tumor growth, they can cause some toxic effect on the liver and kidney in large doses [45].

#### 4. CONCLUSION

The present study was conducted to evaluate the cytotoxic and toxic potential of the *A. tomentosum* Mill. root extract. The first objective was to study the cytotoxicity of the extract by MTT test.

**Table 2:** Blood cells and biochemical parameters of rats after 28-day oral administration of *Arctium tomentosum* Mill. root extract.

Parameter	Control	200 mg/kg
RBC, $10^6$ cells/ $\mu$ L	6.10 $\pm$ 0.6	5.30 $\pm$ 0.7
HGB, g/dL	16.10 $\pm$ 0.7	15.30 $\pm$ 0.8
HCT, %	32.0 $\pm$ 0.9	30.50 $\pm$ 1.0
MCV, fl	60.0 $\pm$ 1.0	59.20 $\pm$ 1.5
MCH, pg/cell	30.3 $\pm$ 1.1	28.9 $\pm$ 1.6
MCHC, g/dL	51.20 $\pm$ 1.0	49.70 $\pm$ 1.3
WBC, cells/ $\mu$ L	5850 $\pm$ 775	5600 $\pm$ 850*
PLT, $10^3$ cells/ $\mu$ L	815 $\pm$ 115	857 $\pm$ 125
SGOT, IU/L	145.0 $\pm$ 2.5	157.0 $\pm$ 3.7
SGPT, IU/L	35.1 $\pm$ 1.7	38.5 $\pm$ 2.0
Creatinine, mg/dL	0.5 $\pm$ 0.0	0.6 $\pm$ 0.0
Total plasma protein, g/L	6.5 $\pm$ 0.0	6.1 $\pm$ 0.0
Urea, mg/dL	46.8 $\pm$ 2.0	48.2 $\pm$ 2.2

Data are expressed as mean $\pm$ SD,  $n=3$ , \* $P<0.05$ , compared to the control group, RBC: Red blood cells, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular hemoglobin, MCHC: Mean corpuscular concentration, WBC: White blood cells, PLT: Platelets, SGOT: Serum Glutamic-oxaloacetic Transaminase, or Aspartate aminotransferase, SGPT: Serum Glutamic pyruvic Transaminase, or Alanine aminotransferase

As a result, the 78–99% cell viability indicating the low cytotoxic effect was observed at the concentrations between 0.078 mg/mL and 0.001 mg/mL. The  $IC_{50}$  of the *A. tomentosum* Mill. root extract on the peripheral blood mononuclear cells was 0.091 mg/mL. The second objective was to study the acute and sub-chronic toxicity of the *A. tomentosum* Mill. extract in the adult Wistar rats. As a result, the extract showed no signs of toxicity at the doses of 2000 mg/kg and 5000 mg/kg b.w. and no death was observed in the first 24 h after oral administration of the extract. There were no significant changes in the body weight, physical state, and behavioral patterns between the control group and groups received the extract. In conclusion, the present work demonstrated that the *A. tomentosum* Mill. root extract is practically non-toxic and its  $LD_{50}$  is  $>5000$  mg/kg. Sub-chronic administration of the extract at the dose of 200 mg/kg did not cause any obvious pathology in the organs of rats. Thus, the extract can be considered as safe in the limited doses.

#### 5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

#### 6. FUNDING

This research received no external funding.

#### 7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 8. ETHICAL APPROVALS

The animal studies were approved by the Ethical Committee of JSC “Scientific Center for Anti-Infectious Drugs” No. 23/7 in accordance with the “Guide for the Care and Use of Laboratory Animals” and ARRIVE guidelines.

## 9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

## 10. PUBLISHER’S NOTE

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