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The Investigation of The Protective Effects of Achillea Wilhelmsi Aqueous and Hydroalcoholic Extracts on Oxidative Stress Caused by Arsenic

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ABSTRACT

Introduction: The release of harmful pollutants, especially heavy metals in the environment and the contact of various types of metal toxins with humans, animals and plants lead to create oxidative stress and harmful effects on human and animal health. Oxidative stress plays an important role in various diseases, especially liver diseases, in order to help people's health conditions and protect them against free radicals of heavy metals as the main factors in oxidative stress and liver damage, researchers are recommended the use of diet food including fruits and vegetables containing antioxidants as the best way to deal with oxidative stress. This research was performed with the aim of the investigation of protective effects of Achillea Wilhelmsi aqueous and hydroalcoholic extracts on oxidative damage caused by trivalent arsenic in isolated rat hepatocytes.

Materials and methods: Hepatocytes were prepared from rat liver using the perfusion method with collagenase and finally, the protective effect of Achillea Wilhelmsi aqueous and hydroalcoholic extracts on arsenic-induced cytotoxicity by measuring cytotoxicity markers, ROS production and lipid peroxidation and potential drop percentage of the mitochondrial membrane were investigated. In order to induce toxicity, arsenic trioxide with EC50,2h (Concentration 50 µM) was used.

Results: Arsenic trioxide with a concentration of 50 µM could during 3 hours of incubation lead to severe cytotoxicity, increased ROS production, increased lipid peroxidation, increased potential drop percentage of mitochondrial membrane and lysosome membrane damage compared to the control group ($P<0.05$). In all the tests, the toxicity marker induced by arsenic was inhibited by all the concentrations used of Achillea Wilhelmsi aqueous and hydroalcoholic extracts ($P<0.05$).

Conclusion: It seems that these extracts in appropriate concentrations and amounts can be effective in reducing liver toxicity caused by arsenic.

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INTRODUCTION

Life and the phenomenon of urbanization with the change of food habits have increased the consumption, expansion and distribution of metals in the living environment and the global earth and humans, animals and plants are in contact with various types of metal toxins available in their surrounding environment. Based on the conducted studies, contact with metal pollutants in the environment has led to harmful effects on human health. Some metal toxins can enter the biochemical cycle like essential metals in the body and cause activate or deactivate cellular processes in the target organs (1). Arsenic is one of these elements that in terms of frequency has dedicated 12th rank in the human body (2). Historically, this metal has had various applications in the field of medicine. For example, Flower's solution which contains 1% arsenic, was widely used in the treatment of many common disorders such as asthma from the 18th century to the middle of the 20th century (3).

KEYWORDS:
Oxidative stress,
Antioxidant,
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Also, arsenic compounds were considered the first consumed antibiotics and were widely used until the first half of the 20th century. On the other hand, compounds containing organic arsenic, especially Lewisite (dichlor and 2-chloroyl arsine), were used as chemical compounds in wars in the 20th century. This element is used not only in pharmaceutical science but also in various fields such as agriculture, livestock, electronics, industry, and metallurgy (4). Although arsenic was used in hematology as a treatment for blood cancers resistant to existing drugs, the results obtained from epidemiological studies show that inhalation contact with inorganic arsenic increases the risk of lung cancer (5). Also, there is an incidence risk of skin and bladder cancer as a result of contact with this element. Researches show that arsenic in different forms can participate in the cellular oxidation-reduction reaction and lead to excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (6). Reactive oxygen species (ROS) can participate in a complex process that its result leads to oxidative injury of the cellular major components, including amino acids, carbohydrates, lipids, proteins, and nucleic acids. Also, ROS reacts with cellular lipids and causes the production of a number of aldehydes, including malondialdehyde (MDA), which as a result of a cascade of oxidative reactions, lead to the destruction of lipids and damage to the cell membrane (7). In the following these effects, arsenic causes oxidative stress and increases lipid peroxidation and oxidative DNA damage, which can cause a variety of mutations and complications caused by it (8). Plant antioxidants, in the past and in traditional medicine, had various uses for the treatment of various diseases, and today, with the advancement of science, analysis, purification, and knowledge of the constituent compounds of each plant, they are used more as antioxidants (9). The wide use of some plants in the treatment of many liver diseases in the native medicine of the Middle East has caused a large number of researchers to study natural and artificial antioxidants and investigate their ability to prevent liver damage and the process of destruction. Major studies show that the group of plant compounds, which includes a large group of antioxidant compounds, has various antioxidant compounds such as polyphenols and flavonoids, which are cheap and available. One of the important plants that have many different groups of antioxidants is the Achilea wilhelmsi plant, which shows its antioxidant effects in the form of eliminating free radicals and increasing the level of cellular antioxidants such as glutathione (10). One of the antioxidants that can be used to reduce the oxidative effects caused by arsenic in everyday life is the Achilea wilhelmsi plant. According to the available ingredients in this plant and its antioxidant ability, this plant can be applied as a strong antioxidant and as protective against oxidative stress in the future (11). Therefore, according to the above-mentioned issues, the aim of this study is to investigate the protective effects of the Achilea wilhelmsi aqueous and alcoholic extract on the oxidative stress caused by trivalent arsenic.

MATERIALS AND METHODS

Studied community

In this study, male Wistar rats were used in the weight range of 250-300 grams.

Preparation and incubation of rat hepatocytes

The rat hepatocytes were separated using the liver perfusion method with collagenase enzyme (12). To anaesthetize the animal, intraperitoneal injection of ketamine/xylazine was used, respectively 75 and 15 mg per kilogram of body weight. About 0.25 ml of heparin (lm/U5000) was injected into the inferior vena cava in order to prevent blood coagulation in the liver. After injecting heparin, using a pink angiocath (No. 18), the portal vein was cannulated near the entrance place to the liver, and the cannula was fixed in place by a suture thread. In order to wash the liver from the blood, the washing buffer with pH = 7.4, which was freshly prepared before, was perfused into the liver by a perfusion pump for 8 minutes. During the perfusion, in order to remove the liquid entered into the liver and hepatic blood, the vascular connections of the liver were disrupted. Then, using forceps and scissors, the liver was separated slowly from the viscera, gall bladder and abdominal cavity, and the rest of the operation was performed on the liver outside the animal's body. In the next step, collagenase buffer was used to digest the connective tissue. This buffer was also perfused for 10 minutes.

Finally, by separating the extra parenchymal cells with scissors immediately after the termination of the collagenase operation, the liver was slowly immersed in a petri dish containing a washing buffer. By gently shaking the liver, a part of the liver cells were separated. The next step was related to the filtration of the obtained cells in order to separate the connective tissue isolated from the parenchymal cells. For this purpose, the liver tissue was passed through a sterile gas to separate the attached cells and parts of the liver tissue. After that, the obtained cell suspension was centrifuged (500 mpr, 5 minutes, temperature 4°C) and then the sediment obtained after centrifugation was placed on the hematocytometer slide to count and prepare the appropriate cell concentration. After counting and obtaining the dilution ratio, the isolated cells were suspended with a concentration of 106 cells/ml in the incubation buffer (IV), then poured into round bottom flasks (10 ml per flask) and placed in a water bath with a constant temperature of 37°C and under a constant atmosphere (95% O2, 5% CO2) and were circulated using a bioreactor device. It is important to state that after the isolation and preparation of hepatocytes, the percentage of live cells was 90-95% at the beginning and approximately 80% in the final control under incubation conditions (atmosphere 295% O2, 5% CO2 and temperature 37 °C).

Achilia Wilhelmsii extract preparation method

A species which has been used in this research (Achilia Wilhelmsii), has been collected from Taftan foothills in Sistan and Baluchistan province-Iran, and its extract includes aqueous and hydroalcoholic extract. After collecting the plant, the stem, leaf and flower parts of the plant were dried under standard conditions, then they were made into a powder and about 15 grams of this powder was dissolved inside 150 ml of alcohol (ethanol) to prepare the hydroalcoholic extract. Then, the obtained liquid was placed on a shaker for 24 hours in a container with a wide opening with a magnet so that its extract is completely separated and the alcoholic solvent evaporates completely. After passing 24 hours of complete evaporation of the alcohol, the remaining material at the bottom of the container was scraped and collected and we transferred it to the extract storage container. To prepare the aqueous extract, about 15 grams of plant powder was completely dissolved with 150 ml of water and passed through filter paper and placed on a shaker with a magnet for 24 to 48 hours until the solvent was dried completely. Then, we collected the residual extract at

the bottom of the container and used it as a protective material for the other stages of the experiment.

Cell toxicity measurement

The survival percentage of the isolated liver cells was measured by trypan blue dye (0.2% w/v) (13). To avoid non-toxic or highly toxic conditions in isolated liver cells, arsenic EC50, a 2h concentration was used. To incubate water-soluble substances, about 100 µl of the concentrated stock sample 100 times the concentration was removed and then added to one of the rotating flasks containing 10 ml of the liver cell suspension to obtain the desired concentration. To measure cell toxicity, after 3 hours of incubation, the desired samples were taken from the flasks and then evaluated. The flasks were divided into three control groups, samples and types of polyphenol protective substances and Achilea wilhelmsi aqueous and alcoholic extract. The control flask contained only the suspension of liver cells and no material was incubated with it. The sample flask contained a suspension of liver cells and arsenic with a concentration of EC50, 2h(concentration of 50 µM) and the flasks of protective materials also contained a suspension of liver cells, 50 µM arsenic and the desired concentrations of protective materials. In this research, the concentration of arsenic equivalent to 50 µM and the Achilea wilhelmsi aqueous extract with the concentrations of 25 µM, 50 µM and 100 µM and the Achilea wilhelmsi hydroalcoholic extract with the concentrations of 25 µM, 50 µM and 100 µM, quercetin with the concentration of 50 µM, and gallic acid with the concentration of 10 µM were used to investigate the protective effect of these compounds on the oxidative stress caused by trivalent arsenic in isolated rat hepatocytes (14).

Active oxygen radicals (ROS) measurement

To measure the amount of ROS production in liver cells, 2, 7-dichlorofluorescein diacetate-DA (DCFH) reagent was used (15).

Lipid peroxidation investigation

To investigate lipid peroxidation, the measurement of the amount of malondialdehyde (MDA) produced in the process of lipid peroxidation was used (7). In the method used to determine the amount of lipid peroxidation, 2 molecules of barbituric acid react with one molecule of malondialdehyde in an acidic environment and the pink color produced has absorption at a wavelength of 532 nm, and the absorption intensity obtained at this wavelength is proportional to the formation of TBA-MDA complex.

Mitochondrial membrane potential drop measurement

According to the method of Anderson et al., the fluorogenic cation rhodamine 123 which has a high ability to accumulate in mitochondria, was used to investigate the mitochondrial membrane potential drop (16). This lipophilic dye enters the cytosol through diffusion, and then it accumulates specifically in the mitochondria due to its positive charge. The amount of this accumulation depends on the mitochondrial membrane potential. In this way, they are completely accumulated in healthy mitochondria with strongly negative membrane potential and as a result, they are not detected by the fluorimetric method (Quenching phenomenon). However, in case of the mitochondrial membrane potential drop and the MPT pores opening by following the contact of the cell with mitochondrial toxins, rhodamine 123 enters into the cytosol through the MPT pores. As a result, the amount of fluorescence due to its presence in the cytosol increases strongly and becomes measurable. In this way, the percentage of mitochondrial membrane potential drop is measured differently in the fluorescence intensity of rhodamine 123 in control cells and cells exposed to arsenic EC50, 2h concentration.

Lysosome membrane damage measurement

To investigate the health of the lysosome membrane, the fluorogenic dye acridine orange was used (17). In fact, Acridine orange is a weak lipophilic base with the ability to accumulate in lysosomes which chemically has fluorescence properties. Due to its high lipophilicity, Acridine orange easily passes through the cell membrane and enters into the lysosomes due to its high tendency to react with H+ (acidic hydrogen) and accumulates in them as a charged molecule (AH+) by absorbing protons. Of course, as soon as the lysosomal membrane is destroyed by active oxygen radicals (ROS) resulting from oxidative stress conditions, acridine orange enters the cytosol and loses its proton in the cytosol again and regains its fluorescence ability. Finally, the fluorescence intensity of the uncharged molecule can be measured at wavelengths of Ex: 490 nm and Em: 520 nm.

Ethical considerations

This work was done on rats in the framework of animal studies in accordance with the principles of the World Health Organization

Statistical analysis

The results analysis was performed by one-way ANOVA test, and if the data were not normal, its equivalent non-parametric test and Dunnett's post-test, and if the data were not normal, its equivalent non-parametric test were used. To investigate the homogeneity of variance in a group, Levene's test was used and the results were reported as the average of three separate experiments (mean±SD).

RESULTS

Results related to cell toxicity measurement

Arsenic with a concentration of 50 µM during 3 hours of incubation, leads to induce 70% cell death in hepatocytes. Based on the results listed in Table 1, quercetin with a concentration of 50 µg/ml, gallic acid with a concentration of 10 µg/ml, Achilea wilhelmsi aqueous extract with concentrations of 100 and 50, 25 µg/ml and Achilea wilhelmsi hydroalcoholic extract with concentrations of 100 and 50 µg/ml. 25 were able to significantly reduce the cytotoxicity induced by arsenic during 3 hours of incubation ($P < 0.05$). Also, the Achilea wilhelmsi aqueous and hydroalcoholic extracts in different concentrations show that with the increase of Achilea wilhelmsi protective concentration, the amount of effective arsenic toxicity has a visible reduction during 3 hours of incubation.

Table 1: The investigation of the protective effect of Achilea wilhelmsi aqueous and hydroalcoholic extracts and controls (Quercetin and Gallic acid) on cytotoxicity caused by arsenic trioxide in rat isolated hepatocytes

	Cytotoxicity Percent (%)
Addition	**3h**
Control rat hepatocytes	21±2
+As^{3+} (50 µM)	70±4[a]
+Quercetin (50µM)	41±5[b]
+ Gallic Acid (10 µM)	47±3[b]
+achillea wilhelmsii aqueous extract (25µg/ml)	56±4[b]
+achillea wilhelmsii aqueous extract (50µg/ml)	42±2[b]
+achillea wilhelmsii aqueous extract (100µg/ml)	30±3[b]
+achillea wilhelmsii hydroalcoholic extract (25µg/ml)	61±3[b]
+achillea wilhelmsii hydroalcoholic extract (50µg/ml)	38±5[b]
+achillea wilhelmsii hydroalcoholic extract (100µg/ml)	23±2[b]

A** indicates a significant difference compared to the control cells (P<0.05).

B ** indicates a significant difference compared to hepatocytes exposed to arsenic trioxide (P<0.05).

Results related to amount of active oxygen radicals (ROS) measurement

Arsenic with a concentration of 50 µM caused a significant increase the amount of ROS in hepatocytes during 1 hour of incubation. According to the results listed in Table 2, quercetin with a concentration of 50 µg/ml, gallic acid with a concentration of 10 µg/ml, Achilea wilhelmsi aqueous extract with concentrations of 25, 50 and 100 µg/ml and Achilea wilhelmsi hydroalcoholic extract with concentrations of 100 and 50, 25 µg/ml were able to significantly reduce the ROS production caused by arsenic compared to the group receiving arsenic, during 1 hour of incubation (P<0.05). Gallic acid at a concentration of 10 µg/ml and at 15 and 30 minutes had a lower effect on the amount of ROS caused by arsenic. Achilea wilhelmsi aqueous extract in the mentioned concentrations increases the amount of ROS production in the first 15 minutes by increasing the concentration of the extract, but in the second and third times by increasing the concentration of the extract, its amount decreases slightly. In general, there is a significant difference in the control group ratio of rat hepatocytes in the amount of ROS produced, but the hydroalcoholic extract with increasing concentration and time had a significant effect in reducing the ROS produced compared to the group receiving arsenic.

Table 2: The investigation of the amount of inhibition of ROS production caused by arsenic trioxide by Achilea wilhelmsi aqueous and hydroalcoholic extracts and controls (Quercetin and Gallic acid) on the rat isolated hepatocytes

Addition	**ROS formation**		
	15 min	**30 min**	**60 min**
Control rat hepatocytes	41±3	57±5	61±3
+As^{3+} (50 µM)	101±8[a]	104±7[a]	148±11[a]
+Quercetin (50µM)	47±5[b]	55±6[b]	62±4[b]
+ Gallic Acid (10 µM)	88±6	96±3	97±5[b]
+achillea wilhelmsii aqueous extract (25µg/ml)	46±4[b]	57±3[b]	85±4[b]
+achillea wilhelmsii aqueous extract (50µg/ml)	50±3[b]	53±6[b]	82±3[b]
+achillea wilhelmsii aqueous extract (100µg/ml)	54±1 [b]	55±4 [b]	65±5 [b]
+achillea wilhelmsii hydroalcoholic extract (25µg/ml)	85±6 [b]	91±4 [b]	102±5[b]
+achillea wilhelmsii hydroalcoholic extract (50µg/ml)	57±3[b]	59±3[b]	74±3[b]
+achillea wilhelmsii hydroalcoholic extract (100µg/ml)	49±2[b]	56±4[b]	71±5[b]

A** indicates a significant difference compared to the control cells (P<0.05).

B ** indicates a significant difference compared to hepatocytes exposed to arsenic trioxide (P<0.05).

Results related to lipid peroxidation measurement

Arsenic with a concentration of 50 µM during 1 hour of incubation, leads to induce lipid peroxidation in hepatocytes. Based on the results listed in Table 3, quercetin with a concentration of 50 µg/ml, gallic acid with a concentration of 10 µg/ml, Achilea wilhelmsi aqueous extract with concentrations of 100 and 50, 25 µg/ml and Achilea wilhelmsi hydroalcoholic extract with concentrations of 100 and 50, 25 µg/ml were able to significantly reduce the lipid peroxidation caused by arsenic during 1 hour of incubation (P < 0.05). Also, the Achilea wilhelmsi hydroalcoholic extract at a concentration of 100 µg/ml had the highest protective effect on the cells with regard to the amount of lipid peroxidation caused by arsenic compared to other concentrations.

Table 3: The investigation of the amount of inhibition of lipid peroxidation caused by arsenic trioxide by different concentrations of Achilea wilhelmsi aqueous and hydroalcoholic extracts, quercetin and gallic acid in rat isolated hepatocytes

Addition	Lipid Peroxidation (µmol/10^6 cells)		
	15 min	30 min	60 min
Control rat hepatocytes	0.162±0.03	0.177±0.04	0.196±0.08
+As^{3+} (50 µM)	0.287±0.05[a]	0.362±0.03[a]	0.408±0.13[a]
+Quercetin (50µM)	0.178±0.02[b]	0.231±0.04[b]	0.281±0.12[b]
+ Gallic Acid (10 µM)	0.266±0.06[b]	0.319±0.02[b]	0.379±0.03[b]
+achillea wilhelmsii aqueous extract (25µg/ml)	0.221±0.04[b]	0.267±0.05[b]	0.387±0.04[b]
+achillea wilhelmsii aqueous extract (50µg/ml)	0.225±0.07[b]	0.234±0.09[b]	0.284±0.08[b]
+achillea wilhelmsii aqueous extract (100µg/ml)	0.203±0.08 [b]	0.217±0.02 [b]	0.248±0.04 [b]
+achillea wilhelmsii hydroalcoholic extract (25µg/ml)	0.194±0.06[b]	0.222±0.11[b]	0.286±0.06[b]
+achillea wilhelmsii hydroalcoholic extract (50µg/ml)	0.185±0.09[b]	0.180±0.03[b]	0.238±0.06[b]
+achillea wilhelmsii hydroalcoholic extract (100µg/ml)	0.161±0.03[b]	0.173±0.05[b]	0.219±0.04[b]

A** indicates a significant difference compared to the control cells (P<0.05).

B ** indicates a significant difference compared to hepatocytes exposed to arsenic (P<0.05).

Results related to mitochondrial membrane potential drop measurement

Arsenic with a concentration of 50 µM during 1 hour of incubation, leads to an increase in the percentage of mitochondrial membrane potential drop in rat hepatocytes. Based on the results listed in Table 4, quercetin with a concentration of 50 µg/ml, gallic acid with a concentration of 10 µg/ml, Achilea wilhelmsi aqueous extract with concentrations of 100 and 50, 25 µg/ml and Achilea wilhelmsi hydroalcoholic extract with concentrations of 100 and 50, 25 µg/ml were able to significantly reduce the mitochondrial membrane potential drop caused by arsenic during 1 hour of incubation (P < 0.05). Also, the Achilea wilhelmsi aqueous and hydroalcoholic extract with concentrations of 100 and 50, 25 µg/ml, respectively, leads to an increase in the concentration and time causes a significant decrease in the percentage of membrane potential drop induced by arsenic trioxide. The gallic acid in the concentration used in the second and third times had the lowest effect to prevent the mitochondrial membrane potential drop.

Table 4: The investigation of the protective effect of Achilea wilhelmsi aqueous and hydroalcoholic extracts, gallic acid against mitochondrial membrane potential drop caused by arsenic trioxide induce effect in rat isolated hepatocytes

Addition	Percent of Mitochondrial membrane potential decline		
	15 min	30 min	60 min
Control rat hepatocytes	2±1	3±1	6±2
+As^{3+} (50 µM)	12±3 [a]	14±2 [a]	43±5 [a]
+Quercetin (50µM)	3±1[b]	4±1[b]	8±2[b]
+ Gallic Acid (10 µM)	10±2[b]	11±1[b]	31±2[b]
+achillea wilhelmsii aqueous extract (25µg/ml)	6±2[b]	9±2[b]	24±3[b]
+achillea wilhelmsii aqueous extract (50µg/ml)	6±1[b]	7±3[b]	21±4[b]
+achillea wilhelmsii aqueous extract (100µg/ml)	5±2 [b]	5±1 [b]	13±2 [b]
+achillea wilhelmsii hydroalcoholic extract (25µg/ml)	7±1[b]	10±2[b]	17±3[b]
+achillea wilhelmsii hydroalcoholic extract (50µg/ml)	5±1[b]	8±3[b]	15±1[b]
+achillea wilhelmsii hydroalcoholic extract (100µg/ml)	3±1[b]	4±1[b]	9±2[b]

A** indicates a significant difference compared to the control cells (P<0.05).

B ** indicates a significant difference compared to hepatocytes exposed to trivalent arsenic (P<0.05).

DISCUSSION

Heavy metals are one of the permanent and widespread pollutants in the environment which are expanding and spreading faster with the expansion of industry and urban life and through the mechanism of creating oxidative stress in cells, affect the structure and function of body organs. Due to the impact that metallic elements have on the environment, economy, food and their quality, and the health of creatures and humans, the presence of these substances in the environment has been considered an important issue during recent decades. Arsenic metal is one of the most toxic substances available in nature that humans are exposed to it. The most important ways of contact with arsenic, are occupational contact and consumption of water and food contaminated with this metal and use of arsenic drugs (18). One of the ways of human occupational contact with arsenic is the factories producing insecticides and pesticides. In addition to industry, anthropogenic sources such as volcanoes are more important resources in the spread of arsenic pollution caused by arsenic on the surface of the global earth, so the source of one-third of atmospheric arsenic is natural sources and volcanoes. In all the mentioned cases, it has been proven that

humans are exposed to chronic arsenic toxicity. Contamination of groundwater with arsenic in many regions of the world that use groundwater as drinking water has become a widespread problem and a challenge for scientists around the world. This contamination has been reported from different parts of the world, including the United States of America, China, Chile, Bangladesh, Taiwan, Mexico, Argentina, Poland, Canada, Hungary, Japan and India. As a result, in all of the mentioned cases, exposure to arsenic is inevitable and thus measures should be considered to prevent its toxicity increase in the body. One of the most important and least complicated strategies to reduce the toxicity of various poisons is the use of effective compounds available in food, just as food can cause enter these substances into the body, as a result, it also causes to neutralize and remove from the body (19). The liver is one of the target and sensitive tissues of toxicity, and it is one of the accumulation tissues of heavy metals such as arsenic, chromium, lead, and cadmium. One of the pathogenic mechanisms of arsenic is the creation of oxidative stress due to the production of ROS and RNS, which induces DNA destruction, chromosomal abnormalities, liver and neurotoxicity, and various cancers. In other words, it is determined that oxidative stress caused by arsenic leads to DNA damage, an increase of free radicals, lipid peroxidation, activation of oxidative enzymes and reduction of antioxidant defence power. The damage and reduction of the antioxidant defense system involve several mechanisms, including a decrease in the activity of SOD, CAT and a change in the expression of GPX (20). GSH is a critical factor against the harmful effects of arsenic in the cell. Methylated metabolites of arsenic, such as MMA, are strong inhibitors of GSH reductase and thioredoxin reductase, which is probably due to the high tendency and interaction of arsenic with thiol groups available in these molecules. Inhibition of cell antioxidant enzymes may change the cellular oxidation-reduction system and decrease the ability of the cell to protect against oxidative damage to the body. The reduction of the power of the natural antioxidant system of the cell eventually leads to cytotoxicity. Arsenic causes changes in the activity of enzymes involved in the mitochondrial respiration system, the opening of the mitochondrial MPT pores and the release of cytochrome c from the mitochondria, the mitochondrial membrane potential drop, defect in the electron transport chain, the production of ROS, and the occurrence of morphological changes in the mitochondria. Arsenic also by affecting on the lysosome by destroying and damaging the wall and membrane of lysosomes, causes the release of enzymes inside the lysosome, so that these events will in turn lead to cell death (21). The results obtained from the present study on hepatocytes also confirmed the cytotoxicity of arsenic with a significant increase in cell death in hepatocytes exposed to arsenic compared to the control group. The first ROS species produced in arsenic-induced oxidative stress is the superoxide radical. By inducing NADPH oxidase and increasing NADPH, arsenic causes to produce of superoxide radicals which its production activates the secondary cascade of production of other active oxygen species such as H2O2 and OH. The reaction of these active oxygen species with some cellular targets leads to DNA damage, lipid peroxidation and cell death (22). In a study which is conducted, the results obtained examining all five factors of cell death rate, ROS production, lipid peroxidation, mitochondrial membrane potential drop, and lysosome membrane damage also showed that arsenic has caused a significant increase in ROS production, lipid peroxides production, lysosome damage, the percentage of mitochondrial membrane potential drop and induction of cell death in hepatocytes compared to control cells. The Achilea wilhelmsi extract neutralizes free radicals by using available thiol groups in the structure of its compounds. Another antioxidant mechanism that some studies imply to it, is the inhibition of oxidative enzymes such as xanthine oxidase, lipoxygenase, and NADPH oxidase by the available compounds in Achilea wilhelmsi extract. These enzymes play a key role in the production of free radicals and the occurrence of cell damage caused by free radicals (23). The Achilea wilhelmsi extract also stabilizes the mitochondrial membrane of hepatocytes by inhibiting free radicals. The results obtained from a study which was of conducted on the protective effect of Achilea wilhelmsi extract over acetaminophen-induced oxidative stress on rat hepatocytes show that Achilea wilhelmsi extract increases the level of glutathione (GSH), decreases the activity of superoxide dismutase (SOD) and decreases of lipid peroxidation in rat hepatocytes (24). Also, it was shown in this study that Achilea wilhelmsi aqueous and hydroalcoholic extract has significantly decreased the amount of ROS production, the amount of lipid peroxidation, the percentage of mitochondrial membrane potential drop and finally the amount of cell death caused by arsenic compared to the control group. A research that was conducted for the first time to show the protective and antioxidant effect of Achilea wilhelmsi extract against oxidative stress caused by trivalent arsenic on rat hepatocytes, indicated that Achilea wilhelmsi extracts in the concentrations of 50, 25 and 100 µg/ml based on increasing concentration and also with the passage of time, leads to decrease the amount of ROS produced, was reduced the amount of lipid peroxidation, decrease the percentage of mitochondrial membrane potential drop, and in general significantly decreased the amount of cell death caused by arsenic. The Achilea wilhelmsi hydroalcoholic extract also was used with the concentrations 25, 50 and 100 µg/ml, the results obtained of it showed that with the increase of the protective concentration and incubation time, the amount of (ROS) produced, the amount of lipid peroxidation, the percentage of mitochondrial membrane potential drop and cell death caused by arsenic significantly decreased. From the comparison and examination of the results obtained from the Achilea wilhelmsi aqueous and hydroalcoholic extracts, we conclude that both types of extracts cause a protective effect and decrease cell death and are consistent with the results of studies that have been done previously. Another point is the amount of release and removal of antioxidant compounds in the Achilea wilhelmsi hydroalcoholic extract is a little greater, considering that the rate of cell death in the Achilea wilhelmsi hydroalcoholic extract is lower than the Achilea wilhelmsi aqueous extract. These results and the results of previous studies show that Achilea wilhelmsi plant extract can be used as a drug or product that can reduce the oxidative effect of trivalent arsenic which has caused chronic poisoning of people exposed to the poison (25). Since the Achilea wilhelmsi plant has high amounts of flavonoid and polyphenolic compounds, including quercetin and gallic acid, some of the protective effects of the Achilea wilhelmsi plant can be attributed to these compounds. The studies which have been conducted on the percentage and type of components of the Achilea wilhelmsi plant show that this plant contains polyphenolic, flavonoid and monoterpene compounds, which are smaller subunits including tricycle, alpha thogen, alpha pinene, quercetin, and gallic acid. , sabinene, and camphor that most of them have antioxidant effects (17). In this study, in addition to the protective effect of the Achilea wilhelmsi aqueous and hydroalcoholic extracts the protective and antioxidant effects of quercetin and gallic acid polyphenols existing in the Achilea wilhelmsi plant were investigated as a control group. These polyphenols whose antioxidant properties have been measured and proven many times, are among the main components of the Achilea wilhelmsi plant. These compounds can also be obtained from other food

resources. Previous studies have shown that quercetin prevents kidney damage caused by lead by avoiding the production of free radicals and by eliminating them (26). In the present study, the protective effects of quercetin on hepatotoxicity caused by trivalent arsenic were investigated in rat isolated hepatocytes. The concentration of quercetin used significantly reduced the amount of ROS production, the amount of lipid peroxidation, the percentage of mitochondrial membrane potential drop and finally the amount of cell death caused by arsenic compared to the group that was exposed to arsenic alone. Another polyphenol compound which is found in the Achilea wilhelmsi plant, is gallic acid. In previous studies, the hepatoprotective effects of gallic acid against hepatotoxicity caused by carbon tetrachloride and acetaminophen have been reported. Gallic acid increases cell survival and increases GSH in the cell and decreases the production of malondialdehyde, and in different studies, it has shown good cell protective effects even more than α-tocopherol. Gallic acid has the ability to inhibit the production of ROS and RNS and is known as a strong antioxidant and has shown potential ability to inhibit lipid peroxidation in various studies (27). In the present study, gallic acid in the concentration used, has decreased significantly the amount of ROS production, the amount of lipid peroxidation, the percentage of mitochondrial membrane potential drop and finally the amount of cell death caused by arsenic compared to the group that was only exposed to arsenic. These findings are consistent with other studies that have proven the protective effects of gallic acid against oxidant toxins. By considering that these compounds are present with significant amounts in the Achilea wilhelmsi plant, a percentage of the Achilea wilhelmsi plant antioxidant property can be attributed to these compounds. According to this fact that the safety of the Achilea wilhelmsi plant has been proven in the past and with experience, we can use its aqueous and hydroalcoholic extracts of this plant to protect against oxidative damage caused by heavy metals (19).

CONCLUSION

According to all the results obtained of this study and other studies that have proven the antioxidant effects of polyphenols, flavonoids, tannins and the Achilea wilhelmsi extract, and considering that the research shows that different compounds available in the Achilea wilhelmsi extract can prevent the metals accumulation in the blood and tissue and reduce poisoning with them, and despite the high content of antioxidant and useful compounds in the Achilea wilhelmsi plant and various vegetables, the use of these food sources can be a promising therapeutic strategy to prevent and reduce the effects arsenic poison in the body.

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