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Original Article

Acute and Subchronic Toxicity and Cytotoxicity of Saffron Corm Extract

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ABSTRACT

Saffron corm is produced annually in large quantities in Iran. It contains some bioactive components like phenolic and antioxidative compounds. We extracted saffron corm by ultrasound-assisted solvent extraction method. The extraction yield and antioxidant properties, free radical scavenging ability, FRAP, and total phenolic compounds (TPC), were determined. To evaluate acute and subchronic toxicity, the extract was administered at three different doses of 1, 10, and 100 mg/kg to BALB/c male mice. Then, the rate of mortality and biochemical parameters such as LDH, ALT, AST, ALP, TG, cholesterol and creatinine were measured after 24 hours and 60 days respectively. Effect of extract on healthy (HFF) and cancerous cells (MCF-7 & HT-29) was evaluated using MTT assay. Results showed that the 40 min ultrasound-assisted extraction method with the highest extraction yield (0.98%), free radical scavenging inhibition (64.37%), and highest FRAP (643.51 µmol/feII.l) and TPC (82.23 mg/ml) could be selected as the most active saffron corm extract. According to our animal study after acute and subchronic administration of extract, no significant alteration of the levels of TG, cholesterol, ALP, ALT, AST and creatinine was observed, in mice. Cytotoxicity tests showed that saffron corm extract had no toxic effect on HFF cells, although it had toxic effects on MCF-7 & HT-29 cells. Conclusively, to our data, saffron corm extract had no significant acute and subchronic toxicity on BALB/c mice, and although it had toxic effects on cancer cells no cytotoxicity was seen on normal fibroblast cells.

INTRODUCTION

Saffron with the scientific name of *Crocus sativus* L. is one of the most valuable indigenous herbs in Iran. The most valuable part of this plant is the end of the style and the three-branched stigma, which is known as saffron and has a fragrant smell and slightly bitter taste and Iran is the world's largest producer of it with 336 tones [1]. Many studies have been conducted on saffron stigma. However, little research has been carried out on other parts of the plant, and little information is available about them. The shelf life of saffron fields for flowering varies from 5 to 10 years, depending on the initial corm density. High proliferation, scarcity of space for vegetative growth of corms and weakening of soil, greatly reduce the yield of the field. At this time, saffron onions are removed from the field. About one-eighth of the harvested saffron onions are re-cultivated, and the rest remain unused. Therefore, a large volume of saffron corm is wasted each year. Biologically active compounds, starch, protein, etc. are present in saffron corms that can be extracted and used in drugs, additives and etc. Esmaeili *et al*. (2011) determined some phenolic compounds with antioxidant activity of saffron corm in dormancy and waking stages wakefulness and identified 11 phenolic compounds in saffron corm extract. To prove the beneficial and non-toxic effects of saffron corm extract for food and medicinal use, we aimed to evaluate the acute and sub chronic toxicity of *C. sativus* corm extract in mice and its potential cytotoxicity on fibroblasts, HT-29 and MCF-7 cell

lines. Therefore, evaluation of acute and sub chronic toxicity, and cytotoxicity of this part of plant are important. In this study, we aimed to evaluate the acute and sub chronic toxicity of *C. sativus* corm extract in mice and its potential cytotoxicity on fibroblasts, HT-29 and MCF-7 cell lines [2].

MATERIALS AND METHODS

Plant Materials

Saffron corm samples, weight less than 5g, were collected at early July 2018, from Khorasan Razavi Agricultural Research Centre, Mashhad, Iran. They were dried at 50 ºC and pulverized into powdered form, using a grinder machine.

Chemicals and reagents

Folin Ciocalteu's phenol reagent, sodium carbonate, iron (II) chloride hexahydrate, TPTZ (2, 4, 6-tris (2-pyridyl)-s-triazine, gallic acid monohydrate (GA; purum), 1, 1-Diphenyl-2-picryl-hydrazyl-hydrate (DPPH), were all acquired from Sigma-Aldrich (Sternheim, Germany). All chemicals and reagents were of analytical reagent grade. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), trypsin-EDTA, and L-glutamine were supplied by Gibco, UK. MTT (3-(4, 5 dimethylthiazol-2yl) 2, 5 diphenyl tetrazolium) was provided by Sigma, USA.

Extraction

The powdered samples were mixed with 96% methanol (1: 20 w: w), at room temperature for 15 min, and subjected to ultrasonic waves (Heilscher, Germany – UP400S, kHz, 400W) for 20 and 40 min. The Solid part was separated from the blend by passing through Whatman #1 filter paper by using Buchner funnel with a vacuum. Sediment was extracted again as above. Extraction solvent was evaporated at room temperature under a vacuum. The dried samples were put in dark-glass containers and stored in a cold and dry place.

Total Extraction Yield (Y)

Total extraction yield of the optimized extract was measured using Zekovi´c *et al*. (2016) method with some modifications [3]. A volume of the extract (5.0 mL) was oven dried at 40 ˚C for 24 h and continued by 103–105˚C until a constant weight of dried matter. Three replicates of each experiment were done and results were stated as the percent of dry matter in 100 g dried corm.

DPPH Inhibition%

Antioxidant capacity was determined by the DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay according to the method reported by Ersus and Yurdagel (2007) [4]. First, 0.006% DPPH solution was prepared by dissolving DPPH in methanol. Then 1 ml DPPH solution was added to methanol extracted samples. Samples were mixed well and left to stand for 1 h in the dark. The absorbance was then measured at 512 nm. The DPPH scavenging activity was calculated as follows:

$A(\%)=(Ac-As)/Ac\times 100$. Where *A*: Inhibiton %, *Ac*: absorbance of the control, and *As*: absorbance of the sample.

Total Phenolic Compounds (TPC)

The content of phenolic was determined by using Folin-Ciocalteu's method [5]. The sample (5 mg) was extracted in 10 ml methanol and then 2.5 ml Folin-Ciocalteu's agent (Folin-Ciocalteu was dissolved in water 1:10 v/v) was added into a tube. Samples were left on the bench to start the reaction. Afterward, 5 ml of 7.5% Na_2CO_3 solution was added into the aqueous phase and after 1 min the samples were diluted to 50 ml with distilled water. Each sample was allowed to stand for 24 h in a dark place at room temperature and absorbance was measured at 765 nm. A standard curve was plotted using different concentrations of gallic acid. TPC (mg/kg) was calculated using the following equation and expressed as mg/ml. P= Y/W×100.Where, W: sample weight *Y*: the number of phenolic compounds (mg/ml).

Ferric Reducing Antioxidant Power (FRAP)

Briefly, 100 mg sample was dissolved in 10 ml ethanol. Then 30 µl of the solution was mixed into 900 µl FRAP solution and 90 µl distilled water in a test tube. Test tubes were vortexed and placed into a water bath until 37˚ ˚C. At this time absorbance was recorded at 595 nm [6]. The Fe II content was calculated using the following equation: $Y= 1782X-9.211$. Where Y: Fe II (µmol/l), X: absorbance at 595 nm

Animal toxicity

Animals

BALB/c Male mice weighing 20 to 25 g were used for testing. The mice were purchased from Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Before the tests, in order to be adapted to the environment,

they were kept in animal house of the Faculty of Medicine at appropriate temperature conditions (20 to 25 °C) and the lightness and darkness cycle of 12 hours of daylight and 12 hours of night. During the test, four mice were kept in each cage, and these animals used water and food without restriction. The diet of all groups was the same. This diet was a ready-made animal food purchased from Behparvar Company. All animal procedures were confirmed by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd (IR.SSU.MEDICINE.REC.1396.191). It was conducted based on the ethical guidelines of animal research, developed by the Ministry of Health and Medical Education Policy Planning and the Center for Ethics and Medical History of Tehran University of Medical Sciences.

Acute Toxicity Evaluation

To evaluate acute toxicity, the extract derived from saffron corm was injected intraperitoneally at three different doses of 1, 10, and 100 mg/kg to male adult mice. Forty male mice (weight: 25±5 g) were randomly divided into 4 groups (each group contained 10 animals) and the interventions were performed. The control group received only normal saline as vehicle of extract. Then, the rate of mortality, blood and biochemical parameters such as LDH, ALT, AST, ALP, TG, cholesterol and creatinine were measured after 24 hours.

Subchronic Toxicity Evaluation

To evaluate subchronic toxicity, the extract derived from saffron corm was injected intraperitoneally at three different doses of 1, 10, and 100 mg/kg to mature adult mice. Animals were randomly divided into four groups (each group contained 10 animals) and the interventions were performed for 60 days as follows, 1: the control group which received only normal saline as carrier of extract, 2: low-dose test group which received the extract at a dose of 1 mg/kg, 3: moderate-dose test group which received the extract at a dose of 10 mg/kg, 4: high-dose test group which received the extract at a dose of 100 mg/kg. Then, the rate of mortality, blood and biochemical parameters such as LDH, ALT, AST, ALP, TG, cholesterol and creatinine were measured after 60 days.

Blood Sampling

At the end of the test, the animals were anesthetized with ketamine-xylazine and blood samples were taken from their heart for about 1 to 2 ml. Then, blood samples were immediately poured into the Falcon 15 and the mice specifications were written on it. After collecting the samples, the tubes were centrifuged at 3000 rpm for 20 minutes to isolate the serum. Then, yellow serum was removed by sampler and transferred to micro tubes and stored at -80 C until determination of the considered factors.

Measurement of Biochemical Markers in Serum

The biochemical markers assessed using the kits of Zell Bio (Germany) and Pars-Azmoon (Iran) were Lactate dehydrogenase (LDH), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline phosphatase (ALP), triglycerides (TG), cholesterol (CHO), and Creatinine (CREA). The instructions on kits manual were strictly adhered to. The results of the test groups were compared with those of control group. One-way ANOVA test and Tukey post-test were used to determine the significance of differences among the groups.

Cytotoxicity evaluation

The cells

To evaluate the effect of saffron corm extract on healthy and cancer cells, healthy HFF fibroblast cells and MCF-7 and HT-29 cancer cells, purchased from Pasteur Institute, were used. The research procedure was carried out in a cell culture laboratory with a controlled environment and minimal amount of microbes. To culture MCF-7 and HT-29 cells, the culture medium contained RPMI 89%, FBS 10%, and penicillin/streptomycin 1%. To culture HFF cells, culture medium was similar to those of MCF-7 and HT-29 cells, but DMEM was used instead of RPMI for this cell line.

Preparation of different concentrations of the extract

First, a solution with a concentration of 10 mg/ml was prepared from extract in the culture medium and passed through 0.22µm filter for sterilization. Then, 100 µl of this solution was removed and diluted with 900 µl of the culture medium. Diluting was performed for several times in the same way.

Culture in 96-well plate

The cells were first cultured in 25 and 75 cell culture flasks and then transferred to 96-well plates for cytotoxic testing. Within each well, 50.000 cells were seeded. Then, they were placed in incubator for 24 hours (temperature 37 °C and CO2 5%).

After about 24 hours, we treated the cells of each well with a specific concentration of extract. For each concentration, three wells were used and their effect was investigated after 24 hours, 48 hours, and 72 hours. All tests were repeated three times.

Evaluation of cell viability (MTT test)

Following treatment, cell viability was evaluated using a MTT assay. After removal of the culture medium, cells were washed with PBS and 20 µl/well MTT solution (5 mg/ml MTT dissolved in PBS) was added to cells of each well. The plate was wrapped in a foil and placed in incubator at 37 C for 4 hours. After the incubation time, the top solution was removed and 150 µl/well of DMSO solution was added to all wells. Cells were shaken at room temperature for 10 minutes until formazan crystals were dissolved and yielded a blue-purple colour. The plate was placed in Elisa reader and the absorbance of wells was measured in 570 nm. The results were reported as percentage of the control. All experiments were conducted in triplicate wells and repeated twice.

Statistical Analysis

The data, expressed as means ± SE, were compared using one-way analysis of variance (ANOVA). Where a significant difference was detected by ANOVA, the treated groups were compared with the control one or with each other using appropriate post-tests. $P< 0.05$ was considered statistically significant.

RESULTS

Extraction Method

The effect of extraction methods on Inhibition percent, FRAP and phenolic compounds are illustrated in table 1. As can be seen, there is a significant difference between blank (without sonication), and 20 and 40 min sonication treatments. However, 40 min sonication significantly increased DPPH scavenging power (64.37 %), FRAP (643.51µmol/FeII.l) and TPC (82.23 mg/ml) ($P< 0.05$) in saffron corms extract. These results indicate that ultrasound application has a positive effect on antioxidant activities and 40 min ultrasonic was more effective than the other treatment. So we used it for our toxicological tests.

Level of biochemical markers in serum

Determination of LDH, ALP, ALT and AST activities

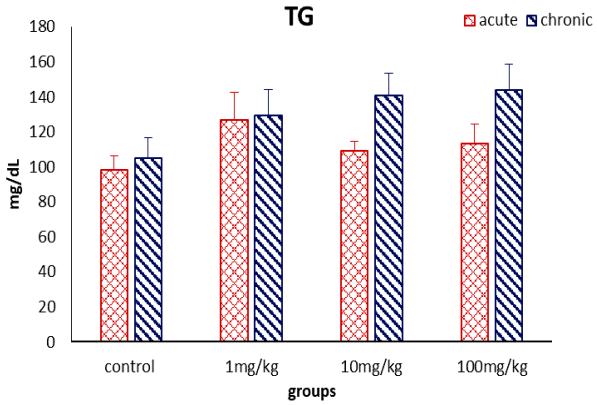

Fig. 1 Acute and Chronic Effects of extract on serum TG levels in mice. Values are expressed as mean±SE, n=6 for each group

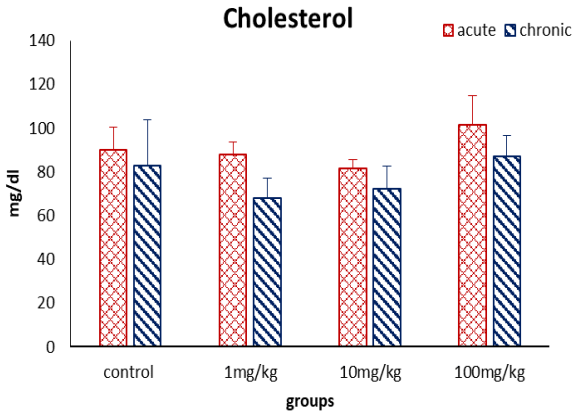

Fig. 2 Acute and Chronic Effects of extract on serum CHO levels in mice. Values are expressed as mean±SE, n=6 for each group

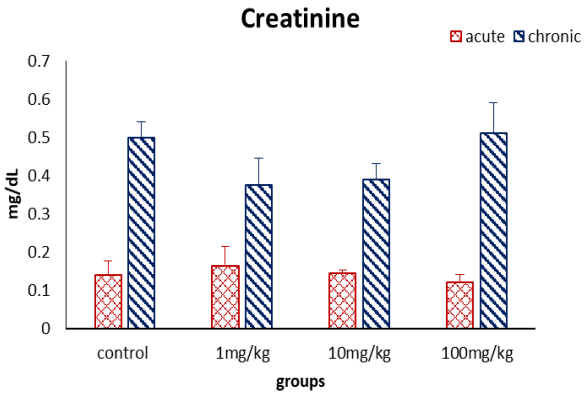

Fig. 3 Acute and Chronic Effects of extract on serum CREA levels in mice. Values are expressed as mean±SE, n=6 for each group

LDH enzyme activity in the treatment groups was higher than that of the control group, and the high LDH activity compared to control group was statistically significant. ALP, ALT, and AST enzymes activities in treatment groups in acute and chronic modes were not significantly different from the control groups (Table 2&3).

Table 1 Individual effect of extraction methods on yield, DPPH scavenging %, FRAP and TPC.

Extraction methods	Extraction Yield%	DPPH Inhibition%	FRAP (µmol/feII)	Total phenolic compounds (mg/ml)
blank	0.82±0.01 b	57.7±1.0 c	459.71±9.5 c	69.98±3.6 c
Sonication (20 min)	0.90±0.01 ab	59.53±2.4 b	527.32±10.3 b	75.23±2.4 b
Sonication (40 min)	0.98±0.01 a	64.37±2.2 a	643.51±8.4 a	82.23±3.0 a

Means ±SD within a column with the same lowercase letters are not significantly different at $P < 0.05$.

Table 2 Acute effect of extract on serum enzyme activities in mice

Groups	LDH(U/L)	ALP(U/L)	ALT(U/L)	AST(U/L)
Control	161.4±7.23	35.2±4.66	20±3.26	115±15.51
1mg/kg	564.1±34.24 **	35.7±2.86	21.9±4.08	151±11.45
10 mg/kg	935.4±38.72 **	34.3±3.27	25.5±3.67	149±8.57
100mg/kg	950.7±36.6 **	42.5±4.04	30.8±1.44	153±12.25

Values are expressed as mean±SE, n=6 for each group. **Significantly different from control group ($P < 0.01$).

Table 3 Chronic effect of extract on serum enzyme activities in mice

Groups	LDH(U/L)	ALP(U/L)	ALT(U/L)	AST(U/L)
Control	184.3±8.45	52.5±2.86	35±4.08	98.61±11.02
1mg/kg	731.14±41.02 **	46.4±4.04	24.17±5.63	123.14±9.79
10 mg/kg	820.76±38.72 **	55.5±3.21	29.11±5.71	138.32±15.92
100mg/kg	1570.76±44.04 **	58.5±5.02	44 ±6.94	134.85±13.47

Values are expressed as mean±SE, n=6 for each group. **Significantly different from control group ($P < 0.01$).

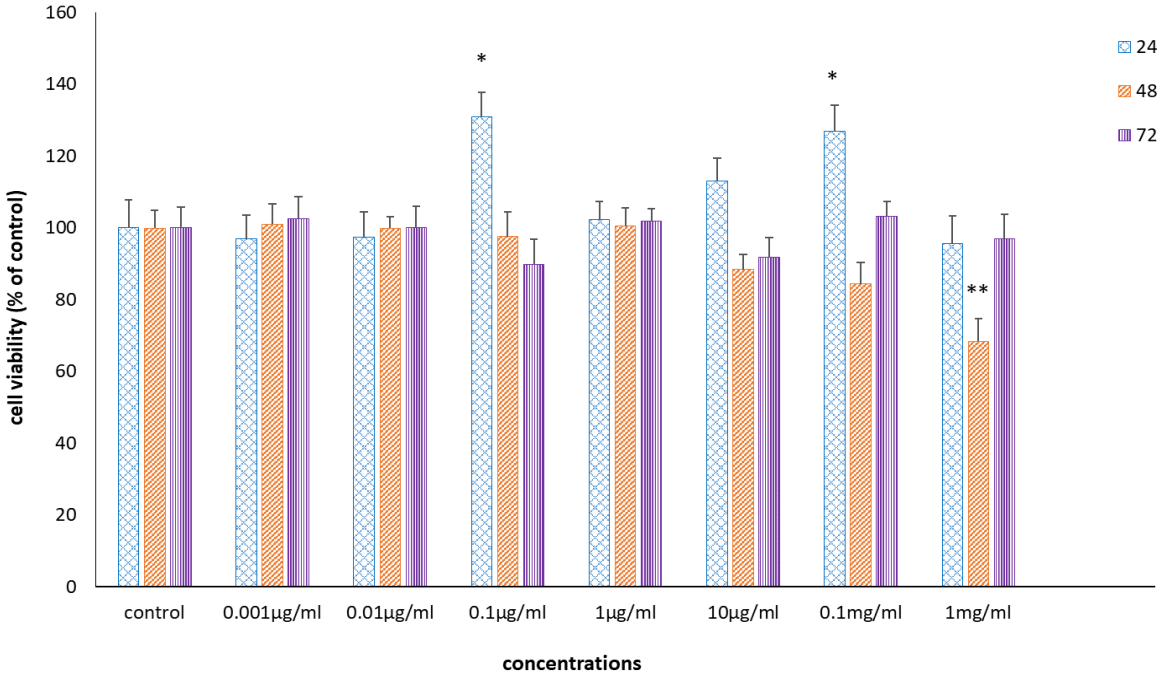

Fig. 4 The effect of saffron corm extract on the viability of HFF cells after 24, 48, and 72 hours compared to the control group. Data are shown as mean±SE. *$p < 0.05$, **$p < 0.01$

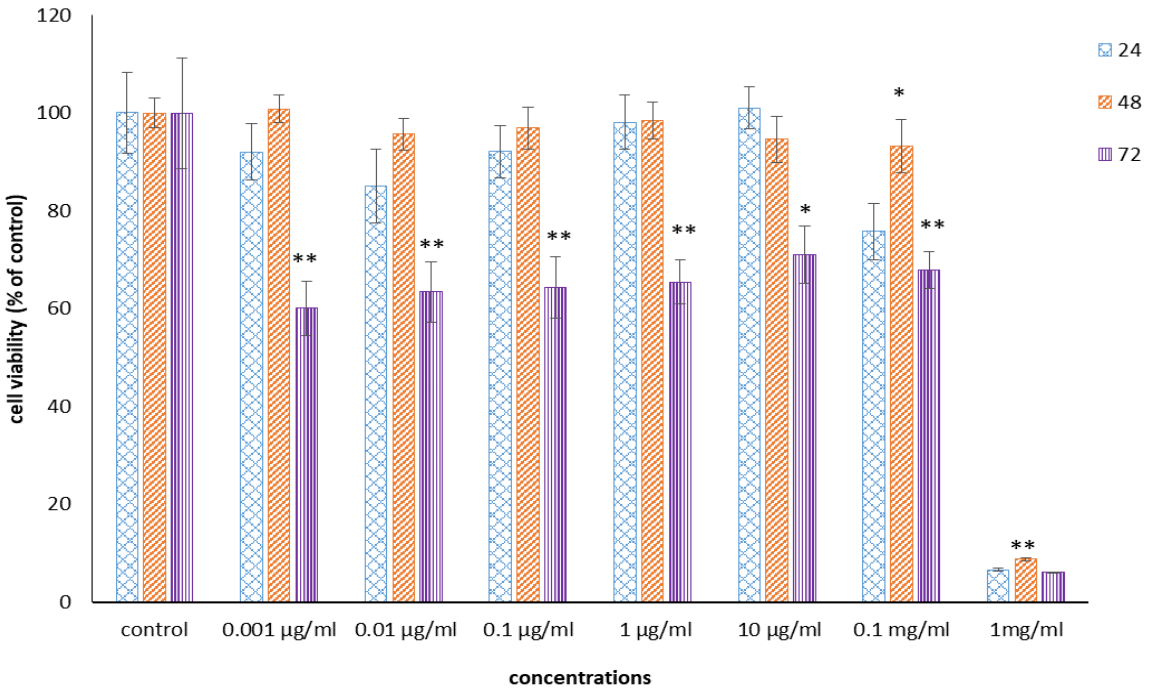

Fig. 5 The effect of saffron corm extract on the rate of viability of HT-29 cells after 24, 48, and 72 hours compared to the control group. Data are shown as SD ±mean. *p< 0.05, **p< 0.01

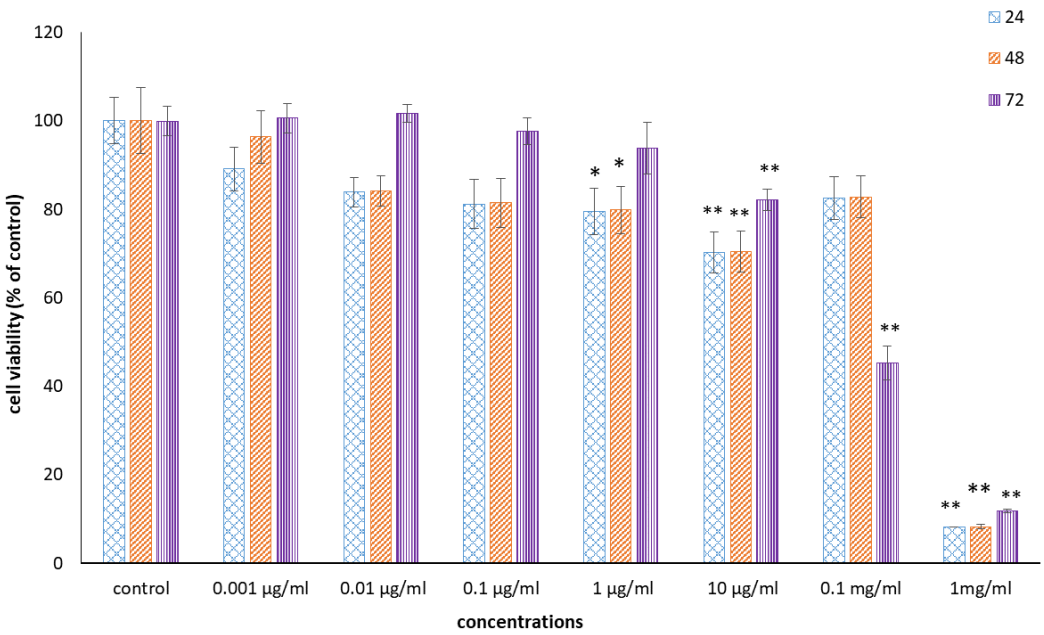

Fig. 6 The effect of saffron corm extract on the rate of viability of MCF-7 cells after 24, 48, and 72 hours compared to the control group. Data are shown as mean± SD. *p< 0.05, **p< 0.01.

Measurement of Lipid Factors and Creatinine

Serum TG, CHO, and CREA levels in treatment groups in acute and chronic modes were not significantly different from the control groups (Fig. 1-3).

The Effect of Extract on HT-29 Cell Line

HT-29 and MCF-7 cell lines are cancer cell lines which related to breast cancer and colon cancer, respectively. Cancer cells have faster growth than fibroblast and healthy cells, and if a substance can reduce their growth or increase their deaths, it can potentially be considered as an anticancer drug. The results show that saffron corm extract at all concentrations significantly reduced the viability of HT-29 cells after 72 hours and it significantly increased cell death at the highest concentration at all times (Fig. 5).

The Effect of Extract on MCF-7 Cell Lline

The results show that saffron corm extract at concentration of 10 µg/ml and 1 µg/ml increased cell death significantly at all times. It also led to significant reduction in viability of MCF-7 cells at concentration of 1 µg/ml after 24 hours and 48 hours and at concentration of 0.1 µg/ml after 72 hours (Fig. 6).

DISCUSSION

Large quantities of Saffron (*C. sativus*) corm, produced annually in Iran, are wasted or consumed by livestock. We subjected this part of the plant to a series of toxicological tests to evaluate its safety and to find out if it can be used as a valuable ingredient, too. In this study, the ultrasound-assisted extraction with comparison of blank was applied for the extraction of saffron corm extract and acute and subchronic toxicity and also cytotoxicity of it were investigated.

Results demonstrated that the sonication significantly improved the yield of extraction that is probably due to sheer force and the high energy content of these waves breaks up and collapses the cell walls, increases the mass transfer and probability releases their contents to the extraction environment, reduces particle size and increases the contact surface and, as a result, increases the solvent emission [7]. so improves the extraction yield of bioactive material.

Ultrasonic treatment and 20 and 40 minutes sonication extracted more significantly TPC than blank ($P< 0.05$) (Table1). Ultrasonic treatment extracted more phenolic compounds than blank because of cavitation's bubbles destruction near cell wall cause more effective contact between solvent and materials, in addition to it produced the rapid flow of waves acted as a micro pump enforcing solvent into cells and dissolved related compounds [8]. In fact, ultrasound propagation in liquid-solid phase creating an expansion, contraction cycles (causing to produce growing destructed bubbles), the vibration of liquid-solid particles, speeding up under ultrasound wave action, as well as emulsification, diffusion and tissue damage that lead to more extraction.

Heidari Majd *et al*. (2012) argued that the ultrasonic method extracted more phenolic compounds than the maceration method, which was due to the shear stress obtained by ultrasound on phenolic compounds [9]. In the research on coconut skin the optimization of phenolic compounds extraction by ultrasonic, the same result was observed [10].

Increasing the ultrasonic exposure time significantly ($P< 0.05$) increased the DPPH free-radical scavenging rate in saffron corm extracts at the time of 20 and 40 min (Table 1). According to statistical analyses, the 40 min extraction time was appropriate for nearly complete leaching for high rates of DPPH free-radical scavenging. In general, the increase in time increases the solubility of the desired substance; however, some phenolic compounds that are sensitive are subject to degradation or adverse reactions such as enzymatic oxidation due to time [11]. In addition, according to the second law of penetration, after a certain time, the concentration of the soluble matter will be balanced between the solid and solvent matrices. Therefore, a longer time is not suitable for the extraction of compounds with antioxidant effect and extraction time optimization is very important [12].

FRAP capacity was increased with increasing extraction time. The highest antioxidant capacity was observed at higher extraction time. Probably, time to allow a bubble to grow to a size sufficient to cause disruption and resulting increase in extraction yield. Therefore, the bubbles may need time during rarefaction to collapse through processing. For that reason, 256 the high frequencies will not have the ability to cause enough cavitation in the extracts [13]. Teh and Birchin (2014) described that phenomenon to be providing more time to release bioactive compounds from plants tissue as well as enhancing the diversity of the extracted compounds. According to statistical analyses, the interaction effects of treatment time, temperature, power, and frequency were significant on ferric reducing antioxidant power [14].

Our results of animal study indicate that acute and sub chronic administration of extract up to 100 mg/kg did not induce any effects on survival or behavioral changes, in mice. No significant alteration of the levels of triglycerides, cholesterol, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine was observed too. The only marker that significantly increased after acute and chronic administration of corm extract was lactate dehydrogenase (LDH). LDH is an enzyme involved in energy production that is found in almost all of the body's cells, with the highest levels found in the

cells of the heart, liver, muscles, kidneys, lungs, and in blood cells. In a study, Keyhani and Sattarahmady (2002) detected three L-lactate dehydrogenase isoenzymes in saffron corms. All three dehydrogenases were substrate-inhibited by ferricyanide, but at different concentrations; maximum enzymatic activity was observed for 250, 100 and 600 μM ferricyanide, at pH 5.5, 7.5 and 9.5, respectively [15]. As we used photometric method to detect LDH, maybe high level of LDH in treatment groups compare to control group is because of LDH content of saffron corm, itself.

The results of our cytotoxicity tests showed that saffron corm extract not only did not increase the death of normal cells (HFF cell line), but also increased the growth of cells at some concentrations after 24 hours. On the other hand, saffron corm extract significantly reduced the viability of HT-29 cells at all concentrations after 72 hours and significantly increased cell death at the highest concentration at all times. It also led to significant reduction in viability of MCF-7 cells at concentration of 1 μg/ml and 10 μg/ml after 24 and 48 hours and at concentration of 0.1 mg/ml after 72 hours.

According to Escribano et al. study, corms of *C. sativus* L. (Iridaceae) contain a glycoconjugate that shows cytotoxic activity on tumoral cells in culture. Studies of intracellular calcium fluctuations, and release of lactate dehydrogenase in human cervical epitheloid carcinoma cells, showed that this compound caused plasma membrane damage, allowing movements of both calcium and macromolecules, and leading to cell lysis. Analysis of DNA fragmentation showed that cell death was not mediated by apoptosis. This molecule is active against human tumoral cells derived from fibrosarcoma, cervical epithelioid carcinoma and breast carcinoma, with IC_{50} values of 7, 9 and 22 μg/ml, respectively. The proteoglycan is about 8 times more cytotoxic for malignant cells than for their normal counterparts. In addition, 100 μg/ml of proteoglycan produced 50 % *in vitro* lysis of normal human erythrocytes, whereas 320 μg/ml induced about 60 % cell death on cultured human hair follicles [16,17]. Altogether, these results suggest a distinctive cytotoxic activity of this molecule on different human cell types. This is somewhat consistent with our findings about anti-tumor effect of saffron corm extract.

Although saffron is a well-known plant, very little research has been done on the biochemical composition of saffron corm and methods for extracting its bioactive compounds in Iran and in the world. Esmaeili *et al*. (2013) determined the total phenolic content in *Crocus sativus* L. corms in dormancy and waking stages by the Folin-Ciocalteu method. Numerous compounds were detected and 11 compounds were identified. The highest phenolic content in waking corms was observed for gentisic acid (5.693 ± 0.057 μg/g) and the lowest for Gallic acid (0.416 ± 0.006 μg/g); also these two phenolic compounds are the highest (0.929 ± 0.015 μg/g) and lowest (0.017 ± 0.001 μg/g) phenolic in dormant corms, respectively. The results from quantization and GC-MS analysis showed a high concentration of phenolic compounds in waking corms than the dormant stage [18]. Furthermore, the radical scavenging activities of saffron corms were studied by 1, 1-diphenyl-2-pycrylhydrazyl (DPPH) test and EC_{50} values were determined about 2055 ppm and 8274 ppm for waking and dormant corms, respectively [2,18].

CONCLUSION

To date bountiful studies have been done to evaluate the effects of the usable part of *C. sativus* (the end of the style and the three-branched stigma), but little information is existing about saffron corm. We suggest the ultrasonic-assisted, solvent extraction for saffron corm. Ultrasound application resulted in a positive effect on antioxidant activities of the extract. We used this extract for our toxicological tests and taken together, our findings indicate that although LDH significantly increased after acute and sub chronic administration of corm extract, no significant alteration of the levels of TG, cholesterol, ALP, ALT, AST and creatinine was observed, in mice. According to our cytotoxicity tests results, saffron corm extract has no toxic effect on normal fibroblast cells, although it had toxic effects on cancer cells.

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Authors' Contribution

Fatemeh Tavakoli and Soodabeh Einafshar desighned the research . Soodabeh Einafshar Collected the plant and performed saffron corm preparation for extraction and analyzed of data. All authors Performed experimental tests of this research. Fatemeh Tavakoli and Soodabeh Einafshar analyzed of data and prepared the article . Azadeh Emami helped in manuscript preparation.

Conflict of Interest

The authors declare that there is no conflict of interest.

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