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Original Article

An Investigation of the Cytotoxicity Effect of the *Ziziphus jujuba L., Ribes khorasanicum, Crocus sativus* Petal, and *Centella asiatica* Extracts on the NIH/3T3 Cell Line

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Abstract

Background and Aim: Medicinal plants are used for treating different diseases in humans. In the present study, we investigated the cytotoxicity effect of the hydroalcoholic extracts of *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* on the cell viability and morphology of embryonic fibroblast NIH/3T3 cell line in mice.

Materials and Methods: In this analytic cross-sectional study, we prepared the hydroalcoholic extracts of *Ziziphus jujuba L.*, *Ribes khorasanicum, Crocus sativus* petal, and *Centella asiatica* to treat NIH/3T3 cell line using various concentrations of these extracts (78.1, 156.5, 312.5, 625, 1250, 2500, and 5000 µg/ml). Subsequently, we investigated cell viability with MTT assay and morphological changes using an inverted light microscope after 24h and 48 h. One-way analysis of variance was performed to compare the groups using SPSS software.

Results: The results of this study indicated that the hydroalcoholic extracts of the *Ziziphus jujuba L.* and *Ribes khorasanicum* could induce less toxicity and morphological change in the NIH/3T3 cells ($P<0.001$). NIH/3T3 cells exposed to several concentrations of *Crocus sativus* petal and *Centella asiatica* hydroalcoholic extracts reduced cell viability and exhibited morphological changes ($P<0.01$) such as decreased number of live cells, and lost their spindle-like shape compared with the control group in a dose-dependent manner.

Conclusion: Our findings showed that none of the *Crocus sativus* petal, *Centella asiatica, Ziziphus jujuba L.* and *Ribes khorasanicum* extracts had any cytotoxicity effects on NIH/3T3 cell line due to high IC50, although *Ziziphus jujuba L.* and *Ribes khorasanicum* exhibited greater degrees of safety than *Crocus sativus* petal and *Centella asiatica*.

Keywords: Plants, Cytotoxicity, NIH 3T3 cells, Cell survival, MTT formazan

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Introduction

The use of medicinal plants in traditional cultures to develop therapeutic remedies has significantly alleviated human suffering (1). Nowadays, there is a growing worldwide interest in using these plants for curing several diseases such as gastrointestinal diseases, asthma, infections, diabetes, and cardiovascular diseases (2). Moreover, medicinal plants can be used to calm patients and prevent several types of human diseases, particularly in rural regions (3). Some of these herbs such as *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* have drawn attention because of their distinct medicinal properties (4-10).

Ziziphus species are produced in different zones such as north India, south and west Asia, southeastern Europe, and China (11). *Ziziphus jujuba L.* is a herbal plant from the Rhamnaceous family (4) that is composed of several bioactive components, including polysaccharides, terpenoids, flavonoids, pectin-A, and saponin (12, 13). *Ziziphus jujuba L.* has medicinal properties which are widely used for the treatment and improvement of diabetes, fever, bronchitis, renal nephrosis, diarrhea, some types of cancers, asthma, and digestive disorders (14).

Ribes khorasanicum is a member of the Grossulariaceae family. This plant is one of the important medicinal plants. Furthermore, its fruits are used in medicine. The common distribution of *Ribes khorasanicum* is limited to the north of Khorasan province, Iran (6). It has been used for the treatment of hypertension, gastrointestinal poisoning, constipation, and heart disease (15). Furthermore, it has pharmacologic effects such as antioxidant, antifungal, and antibacterial effects (5, 6). The fruits of this plant contain tannins, flavonoids, alkaloids, protein, ascorbic acid, and saponins (15).

Crocus sativus petal, also known as saffron, is a perennial plant of the Iridaceae family. This plant has various parts such as stamen, stigma, and petals (8, 16). Petal, as the valuable part of *Crocus sativus*, contains beneficial ingredients, including safranal, anthocyanin, thiamine and riboflavin, raw fibers, crocin, crocetin, fats, flavonoids, and proteins (16). Many studies have shown several therapeutic properties of stigma such as anti-cancer, anti-diabetic, anti-convulsant, anti-depressant, anti-Alzheimer's disease, cardioprotection, and anti-inflammatory properties (17).

Centella asiatica, also known as Gotu Kola, is a member of the Apiaceae family, which is cultivated in China, India, Indonesia, and South East Asia (18, 19). It is used for the prevention and treatment of memory disorders (20), gastric ulcer (19), skin diseases (9), cellulite and striate (21), cancer, and asthma (10). The main bioactive compounds of *Centella asiatica* are triterpene acids, alkaloids, volatile and fatty oil, glycosides, flavonoids, and asiatic acids (18).

Even though some studies have been conducted about the beneficial effects of *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica*, cytotoxic effects of these plants have not been investigated. In this study, we also used mouse fibroblast cell line (NIH/3T3) to evaluate extract toxicity (22). The aim of the present study was to investigate the *in vitro* cytotoxicity effects of *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* extracts on the embryonic fibroblast NIH/3T3 cell line in mice using MTT test.

Materials and Methods

Chemicals

In this study, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium (MTT) and penicillin-streptomycin were purchased from Sigma (St Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The research project was approved by the ethical committee of the Gonabad University of Medical Sciences (ethical approval No. IR.GMU.REC.1401.099).

Preparation of Different Extracts

Fruits of *Ziziphus jujuba L.* were collected from Birjand, Iran (Aug 2021), and identified by a botanist in Herbarium of Payam Noor University of Mashhad (Voucher No: 13246). *Ribes khorasanicum* was collected from Dargaz, Iran (May 2021) and identified by botanists in the Payam Noor University of Mashhad (Herbarium number:3242). The fresh petals of *Crocus sativus* were collected from saffron farms of Ghaen,

Iran (Oct 2021) and identified by botanists in Payam Noor University of Mashhad, Iran (Herbarium No: 143-0319-1). *Centella asiatica* was collected from Bandar Anzali lagoon (Gilan, Iran) (Jun 2021) in the spring season, and this plant was identified by a botanist at the Medicinal Research Center of Gilan University of Medical Sciences (Voucher No: 13335). For preparing the hydroalcoholic extract by the macerating method, the herbs were dried, and powdered. Then, 100 g of the dried powder of each herb was added to 1800 ml of ethanol 70% and to be shaken for 72 h. Finally, the solvent was removed by an oven at 40 °C (23).

Cell Culture

Mouse embryonic fibroblast NIH/3T3 cell line was obtained from Pasteur Institute (Tehran, Iran).

The cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin antibiotic at 37 °C in a humidified atmosphere of 5% CO2.

Cytotoxicity Assay

Cytotoxicity of all the extracts on NIH/3T3 cell line was measured using MTT test. The MTT test is one of the colorimetric methods, which is used for assessing the percentage of live cells (24). Briefly, NIH/3T3 cells were seeded in a 96-well plate (7×10^{3} per well), and they were allowed to attach to the bottom of the wells for 24 h and 48 h. Then, different concentrations of *Ziziphus jujuba L.*, *Ribes* khorasanicum, *Crocus sativus* petal, and *Centella asiatica* extract (78.1, 156.5, 312.5, 625, 1250, 2500, and 5000 µg/ml) were prepared and added to each well for 24 h and 48 h. These doses were obtained from similar studies (25). Subsequently, 20 µl of freshly prepared MTT solution (5 mg/ml) dissolved in BPS was added to each well. After 4 h of incubation, the medium was removed and then 100 µL of dimethyl sulfoxide (DMSO) was added to each well to disintegrate the formazan precipitate. Finally, the absorption of each well was measured at 570 nm (with 620 nm as a reference) on a Stat FAX303 plate reader (26). All the tests were performed in triplicate. Results were represented in the IC50 value, defined as the concentration of drug which reduced 50% growth and proliferation of the cells and was used as an index in determining the toxicity effect of the extracts of these plants (27).

Morphological studies

For assessing the morphological changes, NIH/3T3 cell lines were treated with various concentrations of *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* extracts. The cells were observed under an inverted light microscope with a magnification of 10X. The untreated cells were used as the control groups.

Statistical Analysis

All the results were presented as mean ± SEM. One-way analysis of variance (ANOVA) was performed for normally distributed variables. If a significant difference was observed, the post-hoc Tukey test was used for comparing the results among the experimental groups. Differences were considered significant when $p < 0.05$.

Results and Discussion

Evaluation of the Cytotoxic Effects of the Extracts

In this study, we examined the cytotoxicity of various concentrations of *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* hydroalcoholic extracts using the MTT test.

After 24 h incubation with *Ziziphus jujuba L.*, MTT exhibited showed a decrease in the number of living cells compared with the control wells. A significant reduction in cell viability was only observed in 5000 µg/ml concentrations, and this reduction was about 10% in cell viability. In NIH/3T3 cells, 24h exposure to *Ribes khorasanicum* indicated decreased loss in cell viability compared with the control group. No remarkable effect in cell viability was found in NIH/3T3 cells at any of these concentrations of *Ribes khorasanicum* compared with the control well indicating the cytotoxic effect of *Crocus sativus* petal extract in NIH/3T3 cell line. This extract caused a significant decrease in cell viability from the 2500 µg/ml compared with the control group ($P < 0.001$ in 5000 µg/mL, $P < 0.01$ in 125 µg/mL).

The IC50 values of NIH/3T3 cell line treated with *Crocus sativus* petal extract were found to be 5000 µg/mL after 24 h. MTT Results revealed that the *Centella asiatica* hydroalcoholic extract could reduce cell viability of NIH/3T3 cell line compared with the control group in a dose-dependent manner, and this reduction was significant from the 2500 µg/ml concentration after 24 h ($P < 0.001$ in 5000 µg/mL, $P < 0.01$ in 125 µg/mL). The IC50 value against NIH/3T3 cells which were treated with *Centella asiatica* extract

was 2500 µg/mL. The results for 48 h were also similar to those of the 24 h (Figure 1).

Morphological Studies

Untreated NIH/3T3 cells served as the control group with a spindle-shaped and uniform distribution (figure 2) displays the morphological changes of NIH/3T3 cells which were caused by treatment with *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* hydroalcoholic extracts after 24 h. The morphology of cells after 24 h of exposure to 5000 µg/ml of *Ziziphus jujuba L.* and *Ribes khorasanicum* extracts were similar to the control group but some cells were granulated. The cells that were treated with 5000 µg/ml of *Crocus sativus* petal and *Centella asiatica* hydroalcoholic extracts exhibited morphological changes with higher severity such as having a lower number of live cells, reduction of cell density, and loss of their their spindle-like shape in comparison with the control wells.

The demand for the use of herbal medicines has attracted much attention compared with chemical medicines because of their availability, insignificant side effects, being non-narcotic, and lower costs (28, 29). Moreover, the use of these plants for treating maladies has been more beneficial than the use of chemical drugs (29). Thus, medicinal plants are utilized

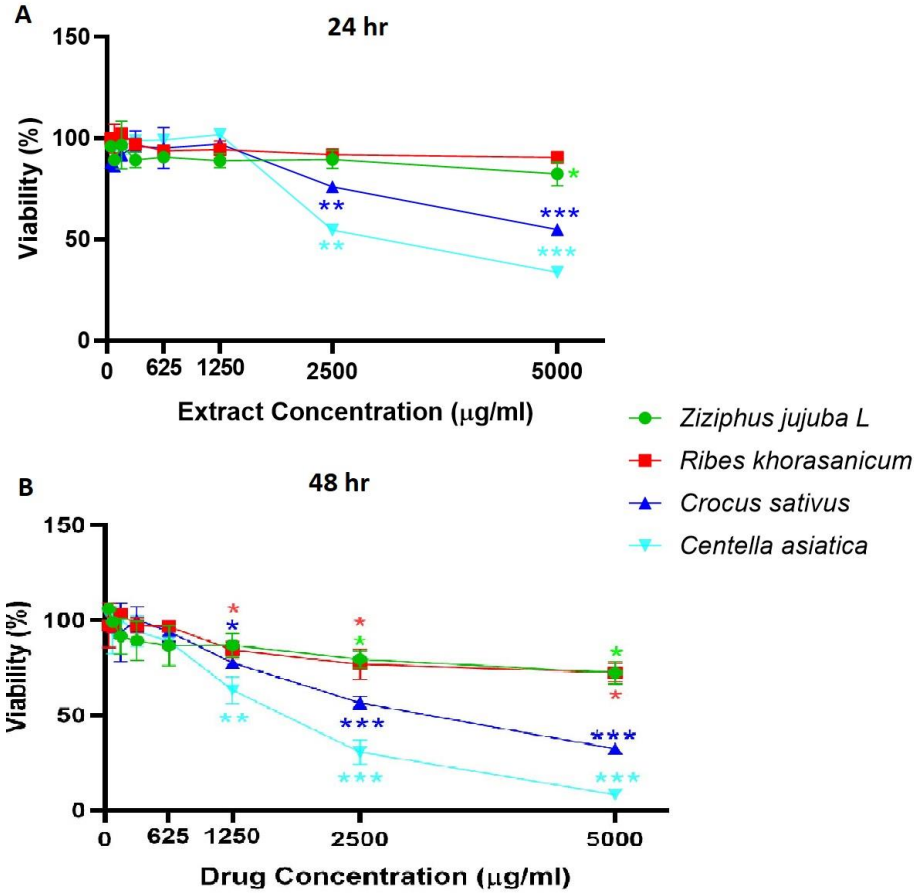

Figure 1. The comparison of the extracts on cell viability of NIH/3T3 cell line. The cells were treated with different concentrations of extract for 24 h (A) and 48 h (B). Viability was quantitated by MTT assay. The results are mean ± SEM (n = 3). *P<0.05***, *P<0.01*** and *P<0.001**** compared with the control.

Supplement table: The data of the ethanol toxicity test.

Cell line	Repeat1 (cell viability)	Repeat2 (cell viability)	Repeat3 (cell viability)
NIH/3T3 cell line	100.5±0.9	99.8±1.1	99.6±1.0

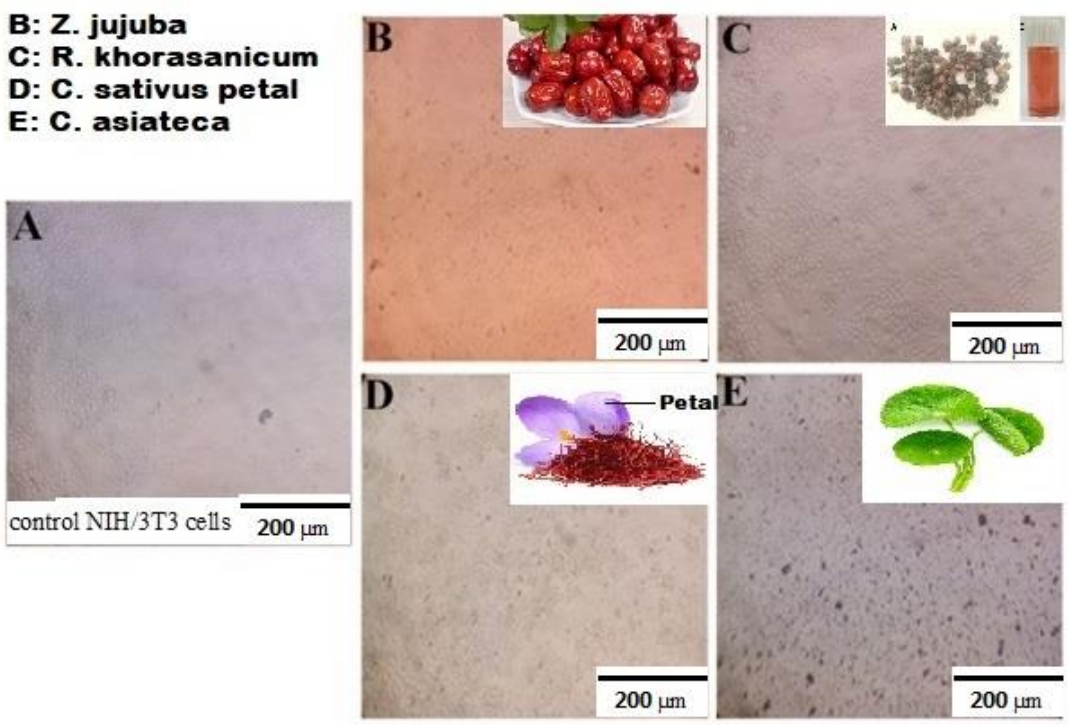

Figure 2. Morphological changes of NIH/3T3 cell line after 24h at 250 µg/ml: control NIH/3T3 cells (A), NIH/3T3 cells treated with *Ziziphus jujuba L.* extract (B), NIH/3T3 cells treated with *Ribes khorasanicum* extract (C), NIH/3T3 cells treated with *Crocus sativus* petal extract (D), NIH/3T3 cells treated with *Centella asiatica* extract (E); The images magnification is *40.

for treating different diseases such as neurologic degeneration, asthma, cancer, inflammatory disorders, and cardiovascular as well as gastrointestinal diseases (30, 31). Nevertheless, before using medicinal plants for clinical goals, various challenges that remain with these herbs must be resolved (32).

Since one of the main properties of new drugs for obtaining a certificate is to ensure their health and safety for consumers, it is necessary to know their potential for toxicity and their possible toxic effects before entering the market. Therefore, cytotoxicity experiments are usually performed on animals which are associated with emotional, ethical, and economic limitations. In recent years, efforts have been made to perform as many cytotoxicity tests as possible in *vitro*. The use of various cell lines that can be used for a long time has facilitated this goal (33). Cytotoxicity which is caused with biomaterials on cell line *in vitro* is the adverse and toxicant undesirable effect, and it is measured by multiple methods both quantitatively and qualitatively (34). In this project, we evaluated and compared the cytotoxicity effect of four significant medicinal plants which have been used in the treatment of diseases in traditional cultures on the NIH/3T3 cell line. In our work, the cytotoxicity effects of *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* hydroalcoholic extracts on the NIH / 3T3 cell line were detected by cell viability and morphological changes.

The *Ziziphus jujuba L.* belongs to the Rhamnaceous family(35). Previous studies have shown that *Ziziphus jujuba L.* has no significant cytotoxic effect in several cell lines, including C26, HTC, MCF-7, A2780, PC3, Hella, HepG2, PCL12, and DU-145 (4). Nevertheless, another report indicated the cytotoxic effect of *Ziziphus* extracts on breast cancer (35). *Ribes khorasanicum* is a shrub from the Grossulariaceae family (15). A recent report showed that anthocyanin, as the main constituent of *Ribes khorasanicum*, has been non-toxic (6). Consistent with our previous studies, the findings of this project indicated that the hydroalcoholic extracts of the *Ziziphus jujuba L.* and *Ribes khorasanicum* could induce less toxicity in the NIH/3T3 cells following 24 h exposure time. MTT results and morphological

changes also revealed that *Ziziphus jujuba L.* hydroalcoholic extracts could cause greater cytotoxic effects in NIH/3T3 cell line when compared with *Ribes khorasanicum* hydroalcoholic extracts. According to previous studies, the mechanism that caused the cytotoxicity of *Ziziphus jujuba L.* is the induction of apoptosis (36).

Crocus sativus is a plant belonging to the Iridaceae family (8). Ahmadi *et al*. showed that the use of *Crocus Sativus* petals extract led to significant toxicity against the HepG2 cell line (37). Moreover, previous studies have shown that *Crocus sativus* could hinder the proliferation of some types of human cancer such as lung (38) and breast cancer (39). Furthermore, *Centella asiatica* is a plant of the Apiaceae family (18). Recently, Liu *et al*. indicated that asiatic acid extracted from *Centella asiatica* significantly reduced the cell viability of cisplatin-resistant human NPC cell lines (40). Another study indicated that *Centella asiatica* ethanol extract drastically reduced the cell viability of HaCaT cells in a dose-dependent manner (10).

The results of the present study revealed that NIH/3T3 cells which were exposed to several concentrations of *Crocus sativus* petal and *Centella asiatica* hydroalcoholic extracts could reduce cell viability and exhibit morphological changes compared with the control group in a dose-dependent manner. The IC50 values of *Crocus sativus* petal and *Centella asiatica* hydroalcoholic extract in the NIH/3T3 cell line were 5000 and 2500 µg/ml, respectively. On the other hand, *Crocus sativus* petal and *Centella asiatica* hydroalcoholic extract inhibited the proliferation of the NIH/3T3 cells after 24 hr. Another notable point is that decreased cell viability caused by *Centella asiatica* hydroalcoholic extract was higher than *Crocus sativus* petal hydroalcoholic extract over the same concentrations. Our results are consistent with those of a study conducted by Wali *et al*. who reported that the decrease in cell viability was observed in MDA-MB-231 cell lines that were treated with *Crocus sativus* petal extract (8).

Moreover, according to the results, *Crocus sativus* petal extract decreased cell viability with induced apoptosis through p53-mediated apoptosis (8, 41). Furthermore, some studies suggested that the reduced viability of cells with *Centella asiatica* was probably made by cell cycle arrest and stimulation of apoptosis through increasing the Bax, caspase-3, and – 9 and decreasing the Bcl-2 (37, 42, 43). It is worthwhile to mention that in this study *Ribes khorasanicum* and *Ziziphus jujuba L.* hydroalcoholic extracts reduced cell viability and morphological changes less than *Crocus* sativus petal and *Centella asiatica* hydroalcoholic extracts, indicating that the *Ribes khorasanicum* and *Ziziphus jujuba L.* hydroalcoholic extracts are safer to be used in medical treatments. Since the aim of this study was to isolate the compounds of the extracts and not to investigate their roles, it was not possible to determine certain mechanisms for cell death of these results for each hydroalcoholic extract. However, other studies, particularly on the components of the extracts, may provide clear mechanisms that involve their toxicity effects.

Nevertheless, probably mechanism by which *Ziziphus jujuba L.*, *Ribes khorasanicum*, *Crocus sativus* petal, and *Centella asiatica* hydroalcoholic extracts caused the observed cytotoxicity effects is the induction of apoptosis. Since the *Ziziphus jujuba L.* and *Ribes khorasanicum* extracts were not cytotoxic at below 625 µg/ml concentration, it can be stated that because the cells were growing normally, the fluctuation of the number of cells could be related to different patterns of cell growth and probably was dumped in cell seeding in each well during the experiment. Thus, *Ribes khorasanicum* and *Ziziphus jujuba L.* hydroalcoholic extract could be considered as safe and promising agents to be used in medical treatments.

Conclusion

Our findings indicated that the extracts, i.e. *Crocus sativus* petal, *Centella asiatica*, *Ziziphus jujuba L.* and *Ribes khorasanicum* did not have any cytotoxicity effect on the NIH/3T3 cell line due to high IC50, although the safety of the *Ziziphus jujuba L.* and *Ribes khorasanicum* was more than the *Crocus sativus* petal and *Centella asiatica*. Further studies are required to fully recognize the mechanism involved in cell death.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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