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Using Comet Assay for Genotoxic Evaluation of *Zataria multiflora* Boiss., An Iranian Endemic Plant

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HIGHLIGHTS

- No statistically significant DNA damage was observed in B lymphocytes treated with *Zataria multiflora* extract.
- The comet assay used in the current study showed that *Z. multiflora* had no genotoxic effect.
- More studies are recommended to examine the safety of plant extracts used as food preservative.

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Acronyms and abbreviations
PBS=Phosphate Buffered Saline

ABSTRACT

Background: *Zataria multiflora* Boiss. (Avishan-e Shirazi), as an Iranian endemic plant, belongs to the Lamiaceae family and may be used as a food preservative. This study aimed to detect potential genotoxic effects of *Z. multiflora* extract.

Methods: Hydro-alcoholic extract of *Z. multiflora* was prepared. Human B lymphocytes were treated with 1% extract within 3 and 24 h. Sterile Phosphate Buffered Saline (PBS) and cisplatin were used as negative and positive controls, respectively. DNA damage profiles were examined using comet assay (Single Cell Gel Electrophoresis). Data were statistically analyzed by SPSS software v. 21.

Results: No statistically significant (p=0.071) DNA damage was observed in B lymphocytes treated with either *Z. multiflora* extract or PBS after 3 and 24 h. However, there was a statistically significant difference (p=0.0001) between DNA damage in B lymphocytes that treated with cisplatin and *Z. multiflora* after 3 and 24 h.

Conclusion: The comet assay used in the current study showed that *Z. multiflora* had no genotoxic effect.

Introduction

Zataria multiflora Boiss., known as Avishan-e-Shirazi (Iranian name), belongs to the Lamiaceae family that wildly grow in the warm Mediterranean area and particularly in Iran, Afghanistan, and Pakistan. It has chemical and physiological components similar to *Thymus vulgaris* that is widely considered as medicinal plant in Iranian and western folk medicine. This aromatic plant, which reaches to 60-90 cm in height, contains essential oil in gredients and varies quantitatively and qualitatively based on different geographical growth area (Aliakbarlu and Khalili Sadaghiani, 2015; Saei-Dehkordi et al., 2010; Sajed et al., 2013).

In most developing countries, food poisoning and food-borne diseases are still known as a major concern, leading to the use of a wide variety of chemical and synthetic preservatives in food industry. There is a great deal of

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concern about safety of these preservatives, which makes consumers more interested in replacing them with safer additives based on natural herbal preservatives (Saei-Dehkordi et al., 2010).

Many herbal essential oils have antimicrobial and antifungal effects, and so they could be used as natural food preservative in commercial foods (Fazeli et al., 2007; Nakhaee Moghadam et al., 2020; Sharififar et al., 2007). Despite the benefits of herbal remedies, they may have cytotoxic and genotoxic properties (Horvathova et al., 2006; Taylor et al., 2003). So, the main objective of this study was to determine possible genotoxic properties of *Z. multiflora* extracts on human B lymphocytes using Comet assay (Single Cell Gel Electrophoresis).

Materials and methods

Ethical statement

The present study was ethically approved by Ethics Committee in Kerman Medical University (IR.KMU.RAC.2015.683). Each blood donor signed and wrote the informed consent.

Herbal extract preparation

Z. multiflora Bioss plants were collected from downtown of Shiraz (Fars Province, Iran) and dried in the shade. The plant specimens were identified by Department of Pharmacognosy Herbarium, School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. One hundred g of dried parts of both herbals were grounded using electrical miller (Moulinex, France), soaked in 100 ml of ethyl alcohol-water (80%V/V) for 48 h in dark room and then percolated (7 h, 30 drops/min). After filtration (0.24 µ filters), the solvents were evaporated in a rotating evaporator (Heidolph, Germany) under reduced pressure until dryness and finally the percentage yield of the dried extract was determined (Su et al., 2007).

Preparation of peripheral B lymphocytes

The peripheral blood was sampled from 30 healthy donors (adults without any history of carcinoma, immunodeficiency, corticosteroids, and antibiotic therapy) in an anticoagulant containing heparin tubes via venipuncture in the morning; then, the samples were transformed to the laboratory under cold chain. The blood was diluted with the equal volume of Phosphate Buffered Saline (PBS), peripheral B lymphocytes were isolated using the Ficoll solution (Labmedia, Iran) and centrifugation (Labtron, Iran) at 1 500 rpm in 20 min as described by Hanna et al. (2008). The peripheral B lymphocytes were then diluted in sterile PBS solution and cultured in RPMI-1640 (Sigma, UK) media, incubated in a CO_2 incubator (5% CO_2) at 37 ºC. The lymphocytes were then counted and adjusted to 1×10^4 cell/ml.

Treatment of B lymphocytes

One hundred µl of viable peripheral B lymphocytes (1×10^4 cell/ml) was added to each well of a sterile microtiter plate. One hundred µl of each herbal extracts (1% *Z. multiflora*) was added to each well. Cisplatin (Sobhan Oncology Co, Iran) and sterile PBS were used as positive and negative controls, respectively. The plate was incubated at 37 ºC for 3 and 24 h for detecting DNA damage using comet assay. All the tests were performed in triplicate (De Grandmont et al., 2003).

Comet assay

To determine the effects of the herbal extracts, DNA damaging was assessed using comet test according to the method explained by Pandeh et al. (2017) and Dhawan et al. (2009). Briefly, a frosted microscopic sterile slide was immersed into the normal melting point agarose gel (1%). Then, 20 µl of each treated blood cells suspensions was poured on 80 µl of low melting agar (0.7%) and mixed by vortex. After that, each mixture (30 µl of cell-agarose) was put on a laboratory slide, lidded with a cover slip, and kept in the cold box for 10 min. One hundred µl of Low Melting Agarose (LMA) was finally released on preceding layer of LMA. The slide was floated in alkaline lyses solution for 1 h and then slowly cleaned with deionized water. The slide was inserted in an electrophoresis tank, and electrophoresis was run for 25 min. After performing the test, the slides were floated in a neutralization buffer for 5 min, rinsed with deionized water and finally stained with ethidium bromide solution. The stained B lymphocytes were analyzed with a fluorescent microscope (Nikon E200, Nikon, Japan) via 400 magnifications in order to find possible DNA damage. At least 50 cells of each comet slide were looked for DNA damage, including the percentages of DNA tail, tail intensity, tail length, and tail moment. Comet software was also used as microscopic illustration analysis of DNA damage analysis (Gyori et al., 2014).

Statistical analysis

All the assessments were performed in triplicate and the data were analyzed by SPSS software version 21 using ANOVA and Students T test. Level of statistical significance was set at $p<0.05$.

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Results and discussion

The mean tail intensity, tail length, and tail moment were 18.36, 6.9, and 0.71% for B lymphocytes treated with *Z. multiflora* after 3 h, respectively closely similar to these profiles in treated B lymphocytes with PBS. There was no statistically significant differences (p=0.071) between *Z. multiflora* (tested plant extract) and PBS (negative control). Cisplatin used in present study as a genotoxic control showed a mean tail intensity, tail length, and tail moment equal to 27.12, 17.2, and 5.1%, respectively which were significantly (p=0.0001) higher versus cells treated with *Z. multiflora* extracts (Table 1).

No statistical significant difference (p=0.071) was observed between all four types of DNA damage profiles in B lymphocytes treated with *Z. multiflora* extracts and sterile PBS within 3 and 24 h (Table 2). Figures 1 and 2 shows DNA damages in stained treated B lymphocytes. As seen, there is no comet cell nucleus (tail) in B cells which treated by *Z. multiflora* extracts and PBS, while most cells treated with cisplatin showed actual comet in both incubation times.

Table 1: Levels (Mean±SD) of initial DNA damage found by comet assay in B lymphocytes treated with *Zataria multiflora* extract, Phosphate Buffered Saline (PBS), and cisplatin after 3 h

DNA Parameters	*Z. multiflora*	PBS	Cisplatin	*P* value
Tail intensity	18.36±3.15	17.15±2.95	27.12±4.60	0.004
Tail length	6.90±1.90	6.50±1.35	17.20±4.90	0.0001
Tail moment	0.71±0.31	0.63±0.20	5.10±2.69	0.0001
Tail DNA%	2889±81.26	1985.23±34.15	4101.34±2370.55	0.031

Table 2: Levels (Mean±SD) of initial DNA damage found by comet assay in B lymphocytes treated with *Zataria multiflora* extract, Phosphate Buffered Saline (PBS), and cisplatin after 24 h

DNA Parameters	*Z. multiflora*	PBS	Cisplatin	*P* value
Tail intensity	19.96±4.45	17.25±3.86	28.65±5.40	0.003
Tail length	7.20±2.12	6.80±1.57	17.52±7.40	0.0001
Tail moment	0.77±0.33	0.61±0.26	5.60±2.99	0.0001
Tail DNA%	23485±95.96	20682.43±64.45	43632.34±2974.99	0.028

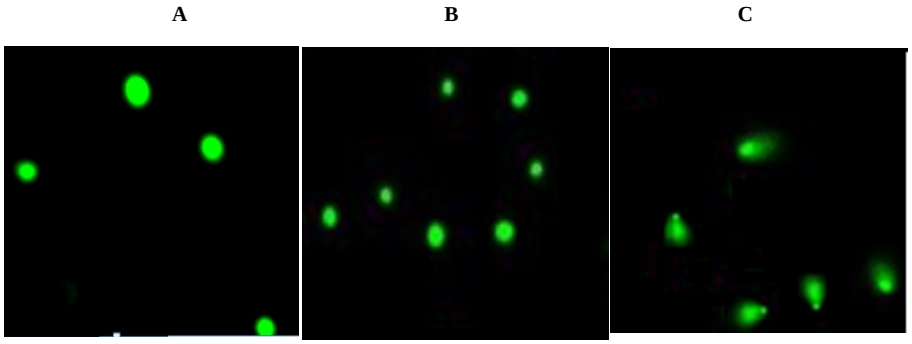

Figure 1: DNA damage of B lymphocytes treated with *Zataria multiflora* extract, Phosphate Buffered Saline (B), and cisplatin (C) after 3 h of incubation

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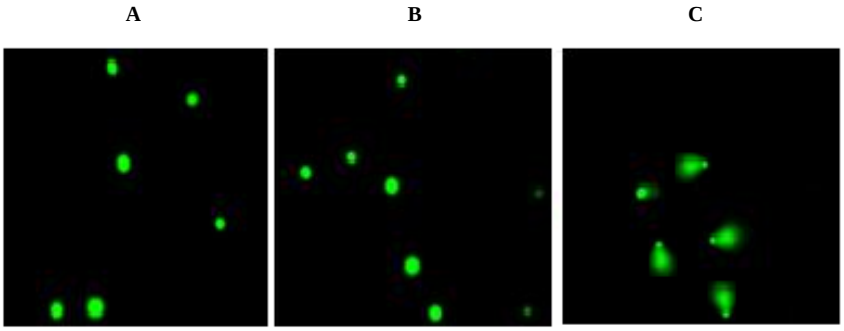

Figure 2: DNA damage of B lymphocytes treated with *Zataria multiflora* extract, Phosphate Buffered Saline (B), and cisplatin (C) after 24 h of incubation

The ongoing growth interest for application of many preservative and medicinal plants around the world has convinced scientists to investigate on the biological effects of these plants. For evaluation of their safety, preservative herbs must be analyzed based on scientific evidence-based and a well-designed tests such as comet assay (Liu et al., 2009). These tests for genotoxicity are usually revealed their feasible mutagenic and genotoxic effects (Arora et al., 2005).

The present study showed that *Z. multiflora* had no adverse genotoxic effects which is in accordance with findings of Hosseinimehr et al. (2010) who reported antioxidative activity of *Z. multiflora* for inhibiting the genotoxicity induced by cyclophosphamide in treated mice bone marrow cells. Radioprotective effect of *Z. multiflora* for the protection of human B lymphocytes exposed by γ-irradiation was also previously reported (Hosseinimehr et al., 2011) that confirms the results of the current research.

Although our study did not show any genotoxic effect of *Z. multiflora* extract, there are few studies that showed genotoxicity from alternative medicinal and preservative herbs, such as *Acacia nilotica*, *Juglans regia*, and *Terminalia chebula*, which induced different degrees of DNA damage in human B lymphocytes (Arora et al., 2005). Also, Taylor et al. (2003) examined 51 South African traditional herb extracts for their genotoxic effects using comet assay, and reported that many of them induced DNA damages conflicting the result of present study.

Conclusion

The comet assay used in the current study showed that *Z. multiflora* had no genotoxic effect. More experimental studies are recommended to examine the safety of plant extracts used as food preservative.

Author contributions

Z.A., A.A.J., and S.A.A-M. designed the study, analyzed the data and wrote the manuscript; Z.A., H.J., and M.P. conducted the experimental works. All authors read and approved the final manuscript.

Conflicts of interest

There was no conflict of interest.

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