

Genotoxicity evaluation of hydroalcoholic and aqueous extracts of *Dorema aucheri* by the comet assay

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Abstract

Background: *Dorema aucheri* is a plant of Apiaceae family which is used widely in some states of Iran. Different extracts and essential oil of *Dorema* species contain flavonoids and coumarin compounds which have anti-hypertensive, cholesterol- and triglycerides-lowering properties. This study was undertaken to evaluate the genotoxic properties of hydroalcoholic and aqueous extracts of *D. aucheri* on human hepatoma cells using the comet assay method for safety evaluation.

Materials and Methods: In this method, after incubation of cells with different concentrations of extracts, cell suspensions were added to pre-coated normal agarose slides. After lysis, electrophoresis and neutralization process, staining was done by ethidium bromide and comets were observed using a fluorescence microscope. Tail length, percentage of DNA in tail and tail moment parameters were measured.

Results: Statistical analysis of the results demonstrated that concentrations more than 500 µg/ml of hydroalcoholic and aqueous extract of *D. aucheri* were genotoxic.

Conclusion: It can be concluded from the results that taking the concentrations less than these dosages of extracts are safe but more studies are required to determine genotoxic mechanisms of this plant.

Key Words: Comet assay, *Dorema aucheri*, genotoxicity, HepG₂

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Received: 16.06.2014, Accepted: 11.08.2014

INTRODUCTION

In some parts of Iran, various records indicate that the percentage of some kinds of cancer is much higher than the average and many believe that this should be caused by local dietary habits.^[1,2] *Dorema aucheri*

(called Bilhar in Persian) is a member of Apiaceae family grows wild at the end of the spring in Zagros mountains in Iran. The leaf and the stem of this aborigine plant are used widely as a flavor. All parts of this plant contain secretory apparatus producing flavonoids, coumarins and essential oils.^[3] Many people believe that all plant-based therapies have less or no side effect in comparison with other therapies and use of *D. aucheri* for treatment of hypertension, high cholesterol and hypertriglyceridemia.^[4] *D. aucheri* properties are not well known, but antihyperlipidemic effect and its role on sex hormones and thyroid function have been studied in rat.^[5,6] To evaluate the safety of *D. aucheri*, we tested its genotoxicity potential on human hepatoma (HepG₂) cells. There

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.190993

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How to cite this article: Etebari M, Sajjadi SE, Jafarian-Dehkordi A, Nazmakanipour S. Genotoxicity evaluation of hydroalcoholic and aqueous extracts of *Dorema aucheri* by the comet assay. Adv Biomed Res 2016;5:199.

are several methods for evaluation of DNA damage, and among which the comet assay or single cell gel electrophoresis (SCGE) is the most well known.^[7,8] This method is based upon micro electrophoresis and can sensitively detect injurious effects on DNA.^[9] In this method, the cells mixed with low melting point (LMP) agarose and then lysed under the alkaline conditions, so they can find unwinding in the DNA helix. Under the effect of the electrical flow in electrophoresis, the DNA molecules move toward anode to form the comet shape. The comet formation pattern is determined by the size of the DNA fragments and the number of broken ends.^[10] As the percent of damage increases, the free DNA fragments move more in the tail. To perform this test, a suspension of the separated cells should be prepared. DNA damage should be assessed in the cells without giving them the opportunity of being exposed to any other genotoxic agents.^[11] Microscopic observation of DNA migration is possible using ethidium bromide staining and a fluorescent microscope.^[12] Numerous advantages offered by the comet assay method including its high sensitivity, inexpensiveness, and applicability for samples of small size have introduced it as one of the simplest and most efficient methods for the biomonitoring of genotoxic properties.^[13]

With regard to wide usage of this plant as a flavoring and the presence of active biological compounds, this study was designed to evaluate the genotoxic effects of *D. aucheri* using the comet assay for safety evaluation.

MATERIALS AND METHODS

Materials

Tris, Triton X-100, H₂O₂, NaCl, EDTA, NaOH and NaH₂PO₄ (Merck Co., Germany), low-melting-point agarose (LMA), Na₂HPO₄, KCl and Ethidium bromide (Sigma Co. USA), normal melting point agarose (NMA) (Cinnagen Co., Iran), RPMI-1640, FBS and antibiotic (PAA Co., Australia) were used in this study. The aerial parts of *D. aucheri* were collected from Yasuj Mountains in the western Iran at the end of spring 2012.

Extract preparation

All extracts of aerial parts of *D. aucheri* were prepared in the faculty of pharmacy and pharmaceutical sciences, Isfahan University of Medical Sciences, Isfahan, Iran. Aqueous extract was prepared using 100 g of powdered plant and maceration method, extracted with water at room temperature. Proceeds filtrated and the residual was extracted for two more times. Finally, whole extract was collected, freeze dried and stored in a refrigerator. To prepare hydroalcoholic extract, 100 g of powdered plant enclosed in filter paper and placed in the soxhlet apparatus separately.

Ethanol was added and the reflux was performed 15 times. Extract was dried by rotary and stored in the refrigerator.^[14,15]

Cell culture

The HepG₂ cell line was obtained from Pasteur Institute (Iran) and cultured in RPMI medium (contain 10% fetal bovine serum and 250 µl of penicillin/streptomycin to avoid the growth of undesirable and pathogenic bacteria) and incubated under 5% CO₂ at 37°C in micro filter plates.

Cells were incubated with different concentrations of aqueous and hydro alcoholic extract of *D. aucheri* for 2 h. As described in our previous studies, the upper medium of wells was thrown away and washed with PBS. After trypsinization, they were neutralized by the medium and were poured in five different falcons and centrifuged. Then 1 ml of medium was added to each falcon and 300 µl of cell suspension was used for the comet assay procedure.^[14]

Comet assay

The comet assay procedure has been described in our previous studies.^[14,16,17] Briefly, incubated cell suspensions (1 × 10⁶ cells/ml) were mixed with of 1% LMP agarose (37°C) and were placed on the pre-coated slides (1% NMP agarose). The slides were respectively incubated with lysis solution (pH = 10.0) and electrophoresis buffer (pH > 13.0) for 40 min. Electrophoresis was done for 40 min (25 V, 300 mA). After this stage, the slides were rinsed with distilled water and were placed in neutralization solution (pH = 7.5) for 10 min. Slides were covered by sufficient dye solution (20 µg/ml ethidium bromide) for 5 min and washed with distilled water. Finally, comets were visualized under × 400 magnification using fluorescence microscopy with an excitation filter of 510–560 nm and barrier filter of 590 nm. All stages of comet assay were performed at room temperature and in dark conditions and all solutions were prepared freshly and used cool.

Statistical analysis

One-way analysis of variance^[18] was used to compare the results of comet assay, followed by Tukey's multiple comparison *post hoc* test. The *P* values 0.05 and less were considered as statistically significant.

RESULTS

To determine the genotoxic concentration of the aqueous and hydro alcoholic extracts of the *D. aucheri*, HepG₂ cells were separately incubated with different concentrations of these extracts for 2 h followed by the comet assay. Three factors including tail length, percentage of DNA in tail and tail moment (% DNA in

tail × tail length) were measured and compared with the negative control group.

Comet assay results of the alcoholic extract of *D. aucheri*

Three concentrations of hydro alcoholic extract of *D. aucheri* were selected and comet parameters as index of DNA damage were studied [Figure 1] The result of the one-way analysis^[18] for the tail length was significant ($P < 0.0001$). According to the results of Tukey's multiple comparison *post hoc* test, two concentrations of the hydro alcoholic extract (500 and 1000 $\mu\text{g/ml}$) had genotoxic effects on HepG₂ cells and increased the tail length significantly ($P < 0.001$) compared with the control group [Figure 1a].

The one-way analysis of the percentage of DNA in tail was significant ($P < 0.0001$). The results of Tukey's multiple comparison *post hoc* test showed the concentrations of 500 and 1000 $\mu\text{g/ml}$ had significant differences (respectively $P < 0.01$ and $P < 0.001$) compared with the control group [Figure 1b].

The results of the tail moment were completely similar to the tail length [Figure 1c].

Comet assay results of the aqueous extract of *D. aucheri*

The comet properties of aqueous extract of *D. aucheri* were studied [Figure 2]. The result of the one-way analysis^[18] for all three factors including tail length,

percentage of DNA in tail and tail moment was significant ($P < 0.0001$). According to the results of Tukey's multiple comparison *post hoc* test, the concentration of 1000 $\mu\text{g/ml}$ have increased the all factors significantly ($P < 0.001$) in comparison with the control group [Figure 2a-c].

DISCUSSION

Genotoxicity or DNA damage can lead to cancer. Research on the genotoxic properties of foreign compounds including native herbs of different regions has an important role in indicating the reason of prevalent genetic disorders of each region and improvement in safety information. There are several *in vitro* and *in vivo* tests for genotoxicity assessment and detecting DNA damage with the comet assay method is one of common methods of this assessment. For this propose, different concentrations of hydro alcoholic and aqueous extracts of *D. aucheri* were prepared and incubated with HepG₂ cells for 2 hours. All three important factors (tail length, % DNA in tail and tail moment) were compared with the control group as genotoxicity parameters. Significant increase of studied factors was seen with both extracts of *D. aucheri*. The component of these extracts has genotoxic potency on HepG₂ cells.^[19] In this study, we compare genotoxic effects of *D. aucheri* with intact cells as negative control and H₂O₂ (200 μM) was used

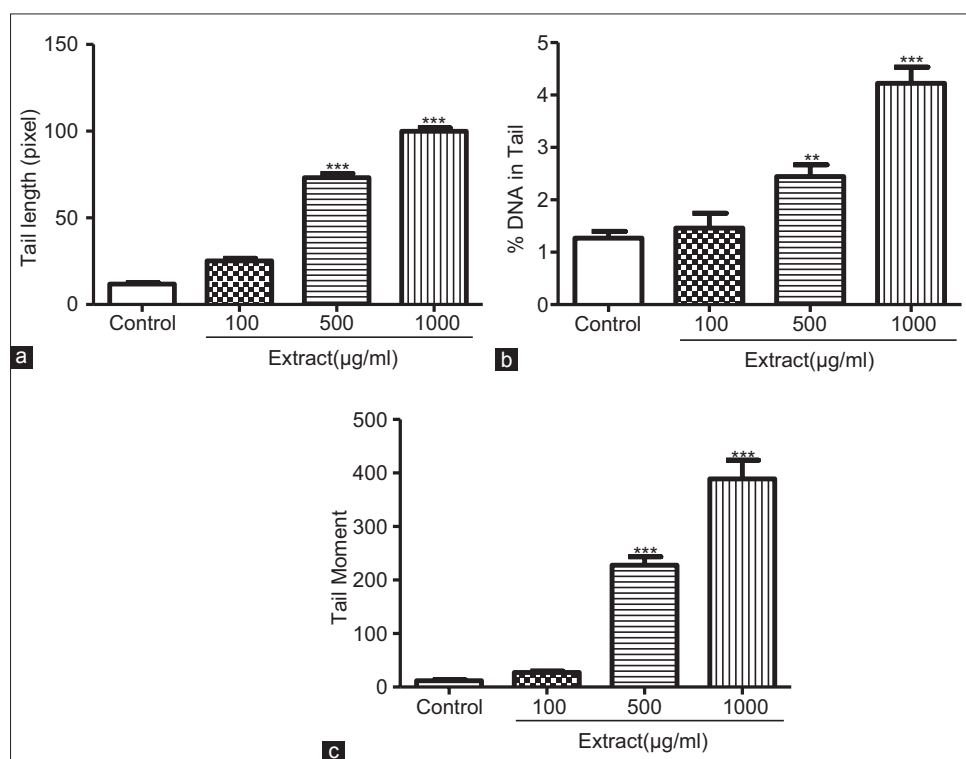


Figure 1: Comparison of (a) Tail length, (b) % DNA in tail and (c) Tail moment of different concentrations of hydroalcoholic extract of *D. aucheri*. Each graph has been represented as Mean \pm SEM. ** and *** show respectively significant results of ($P < 0.01$) and ($P < 0.001$) in compare with the control group

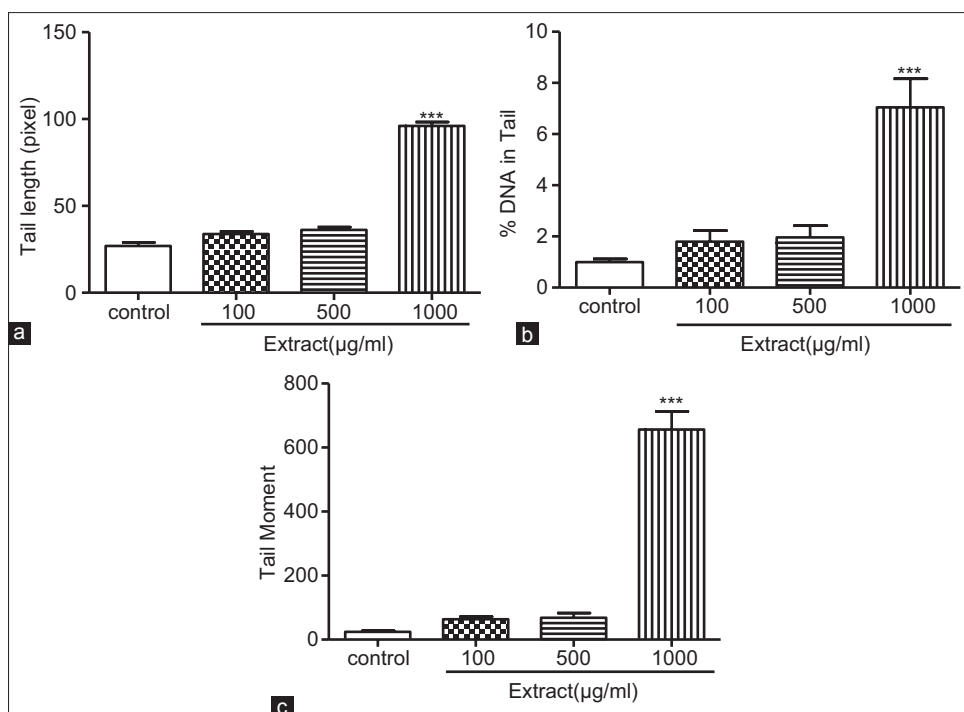


Figure 2: Comparison of (a) Tail length, (b) % DNA in tail and (c) Tail moment of different concentrations of aqueous extract of *D. aucheri*. Each graph has been represented as Mean \pm SEM. ***shows significant results of ($P < 0.001$) in compare with the control group

as positive control of method. The concentrations of 500 and 1000 µg/ml of hydro alcoholic extract and the concentrations of 500 µg/ml of aqueous extract showed significant results. Separated study of these extracts showed that the potency of hydro alcoholic extract genotoxicity is more than aqueous extract. Then we can assume that these extracts are safe in lower concentrations. Therefore, other genotoxicity tests need to be performed on this plant. *D. aucheri* samples which are sourced from the other parts of the country should also be checked by similar tests.

CONCLUSION

It can be concluded from the discussion that *Dorema aucheri* has genotoxic effect on HepG₂ cells. With regard to this finding lower concentration of this plant could be suggested to have no genotoxic effects.

ACKNOWLEDGEMENT

This paper was derived from a pharmacy thesis in Isfahan University of Medical Sciences, Isfahan, Iran. We would like to acknowledge the research department of Isfahan University of Medical Sciences, Isfahan, I.R. Iran, for their co-operation and financial supports.

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Source of Support: Nil, **Conflict of Interest:** The authors declare that they have no conflict of interest.