ORIGINAL ARTICLE

Evaluation of DNA damage of hydro-alcoholic and aqueous extract of *Echium amoenum* and Nardostachys jatamansi

Mahmoud Etebari, Behzad Zolfaghari[1], Abbas Jafarian-Dehkordi, Roya Rakian
Departments of Pharmacology, [1]Pharmacognosy, Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Background: Today most of herbal medicines are marketing without any standard safety profiles. Although common assumption is that these products are nontoxic but this assumption may be incorrect and dangerous, so toxicological studies should be done for herbal drugs. According to the frequent use of *Echium amoenum* as immunostimulant and useful in conditions including pain, cough, sore throat and arthritis, and *Nardostachys jatamansi* as tranquilizer and sleep inducer and evidences of some toxicities, we assessed the probable effect of their extracts on DNA of $hepG_2$ cells using the comet assay. **Materials and Methods:** Different concentrations of above extracts of the plants are incubated with $hepG_2$ cells for 24 h. A mixture of cell suspension and agarose gel were put on slides, then slides were embedded in a lysing solution and were put in electrophoresis buffer (pH = 13). Then the electrophoresis procedure took place in an alkaline solution and after neutralization stage, colorization was done by ethidium bromide and comets were observed using a fluorescence microscope. At least 100 cells of each sample were evaluated and three parameters including comet length, percent of DNA in tail, and tail moment were assessed. **Results:** Both Aqueous and hydro-alcoholic extract of *E. amoenum* were genotoxic in the concentrations of 25 mg/ml and aqueous and hydro-alcoholic extract of *N. jatamansi* were genotoxic in the concentrations 5 and 10 mg/ml, respectively. **Conclusions:** Although *E. amoenum* and *N. jatamansi* are highly used in medicine, these herbs have genotoxic effects in determined concentrations and they should be used cautiously.

Key words: Comet assay, DNA damage, *Echium amoenum*, *Nardostachys jatamansi*

INTRODUCTION

Today most of herbal medicines are marketing without any standard safety and toxicological trials. Although common assumption is that these products are nontoxic but the true is that, this assumption is incorrect and dangerous, so toxicological studies should be done for herbal drugs. According to the frequent use of *Echium amoenum* and *Nardostachys jatamansi* and evidences of their active toxic compounds, we made an effort to assess probable effect of aqueous and hydro-alcoholic extract of *E. amoenum* and *N. jatamansi* on DNA of $hepG_2$ cells using the comet assay.[1]

In many countries *N. jatamansi* (belongs to the family Valerianaceae) is traditionally used in mental disorders, insomnia, and disorders of blood and circulatory system.[2]

Access this article online	
Quick Response Code:	Website: www.journals.mui.ac.ir/jrms
	DOI: ***

N. Jatamansi contains numerous chemical compounds such as, β-eudesmol, elemol, β-sitosterol, angelicin, jatamansinol, valrenone, valtrate, valerenic acid, and didrovaltrate. From them the most important is valeranon which is responsible for the sedative effect of *N. Jatamansi*.[3,4]

E. amoenum (belongs to the family Boraginaceae) is one of the most important medicinal plants in Iranian traditional medicine.[5] Petals of *E. amoenum* have rosmarinic acid, alkaloids, flavonoid aglycons, volatile oils, and anthocyanidine.[6]

This herb is used in Iranian traditional medicine as tranquilizer, anti-inflammatory, analgesic, heart tonic, anti-tussive diuretic, anxiolytic, and sedative.[5,7,8]

In the recent two decades, cytotoxicity and genotoxicity of these herbs drew much attention. *E. amoenum* has been thought to have potential toxicity because of the presence of pyrrolizidine alkaloids.[9,10] There are some evidence suggesting that *N. jatamansi* is cytotoxic and probably genotoxic due to its valtrate and didrovaltrate components.[4]

Two types of variations in the genetic material of somatic cells which might lead to aging are DNA damage and

Address for correspondence: Prof. Mahmoud Etebari, Department of Pharmacology, Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: etebari@pharm.mui.ac.ir
Received: 09-11-2011; **Revised:** 22-05-2012; **Accepted:** 03-07-2012

mutations. DNA damages consist of a variety of chemical disorders in polynucleotide structure of the double helix, such as pyrimidine, apurinic sites, crosslinks, and both large and small chemical additions, named adducts.[11]

DNA damage has a basic role in most of human diseases including cancer.[12] An accurate, fast and sensitive method is required to evaluate DNA damage in clinical studies, this method should be able to monitor DNA repair properly.[13]

Several different tests such as MN (Micronucleus test), UDS assay (unscheduled DNA synthesis), intracellular ROS assay, 8-OHdG assay, Ames assay, and comet assay have been used to assess the rate of DNA damage or genotoxicity. Comet assay or single cell gel (SCGE) electrophoresis was used in the current study to determine DNA damage.[14-18]

SCGE has been established as a simple, rapid, cheap, flexible and most importantly sensitive method to detect DNA damage in individual cells.[19]

This assay is particularly notable since it requires little equipments. The SCGE can estimate single and double-strand breaks in DNA of any eukaryotic cells caused by a variety of physical, chemical, and biological agents. The alkaline comet assay was described in 1998 by Singh *et al*. and since 1998, some modifications have increased its sensitivity.[13]

The comet assay is built on the foundation that DNA damage decreases the size of DNA fragments. This effect could be evaluated by utilization of electrophoretic field to lysed cells. The spotted DNA fragments create a typical comet-shaped migration pattern.[19]

Many studies about the efficacy of *E. amoenum* and *N. jatamansi*, their chemical compounds and effective substance have been published that introduce these two herbs much beneficial and harmless, without any notification to their adverse effects and toxicity. A few assessments have been done about their toxicity but, the important question is that, is this point of view correct or are these two herb really without harmfulness and nontoxic in any concentration therefore the necessity of study about their harmfulness or harmlessness are feeling.

In this study, we sought to determine the effects of hydro-alcoholic and aqueous extract of *N. jatamansi* and *E. amoenum* on DNA of $hepG_2$ using the comet assay method. Also the relationship between amount of damage in DNA and concentration of the extract of these two herbs was studied.

MATERIALS AND METHODS

Material

Desiccated petals of *E. amoenum* and rhizomes of *N. jatamansi* were purchased from a local medicine market in Isfahan, Iran and were identified by the experts. Ethidium bromide, Tris, Triton X-100, H_2O_2, NaCl, EDTA, NaOH, and NaH_2PO_4 were purchased from Merck Chemical Co. LMA (low melting agarose), NMA (normal melting agarose), and Na_2HPO_4 and KCl were purchased from Sigma Chemical Co.

Preparation of the extracts

Extraction by maceration method

200 g of dried powder of each plant was used for extraction by ethanol (70%). After filtration, the residue was extracted for three times. Then all extract were concentrated by rotary evaporator and were freeze dried.[20]

Extraction by decoction method

E. amoenum and *N. jatamansi* were poured into hot water (90 centigrade) and were put in water bath and mixed for 30 minutes. Then these were filtered and freeze dried.[21]

Cell culture

In the present study, we used human hematoma ($hepG_2$) cells to investigate the genotoxicity of *E. amoenum* and *N. jatamansi*. The $hepG_2$ cell line was obtained from Pasture Institute of Iran and was cultured in RPMI medium (contain 10% fetal bovine serum) under 5% CO_2 at 37°C in micro filter plates.

Cells were incubated with different concentrations of the extraction of *E. amoenum* and *N. Jatamansi* or media for 24 hours. Then the upper medium of each well was thrown away and 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 to use for next stages of the comet assay.

Comet assay

Only cell suspensions with viabilities of more than 90% were used for determination of DNA damage. 0.4 ml cell suspension was added to 1.2 ml of one percent low melting point agarose. After solidification, the slides were submerged in a cold fresh lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100, and 200 mM NaOH with pH 10) for 40 minutes. Then, the slides were put in an alkaline solution (NaOH 10 N, EDTA 200 mM with pH 13) for 40 min to permit unwinding of DNA. Electrophoresis was done in the same buffer for 40 min at 25 V and 300 mA. After this stage, the slide was neutralized by 0.4 M Tris (pH 7.5). Afterwards the slides were stained using 100 µl of ethidium bromide (EtBr, 20 µg/ml). Finally, the images were taken by fluorescence microscope and at least 100 randomly selected cells (33 cells from each of the

three replicate slides) were analyzed per sample. Parameters of Tail moment (% DNA in tail × Tail length), Tail length, and percent of DNA in tail, the most frequently used parameters in the comet assay, were used in this study.[22]

Statistical analysis

Results are presented as mean ± SD from at least three separate experiments. Statistical analysis of data was carried out by one-way ANOVA followed by Tukey *post hoc* test. All statistical analyses were performed using the statistical software package Sigma Stat 3.1. A difference was considered significant at $P < 0.05$.

RESULTS

Genotoxic effects of hydro-alcoholic and aqueous extracts of *E. amoenum* and *N. jatamansi* were studied. Aqueous extract of *N. jatamansi* was incubated with $hepG_2$ in cell culture. This test was done at two concentrations of 1 and 5 mg/ml and was compared with control group. Three parameters of Tail length, percent of DNA in tail, and Tail moment were checked. Our findings showed that the aqueous extract of *N. jatamansi* at a concentration of 5 mg/ml increased Tail length, %DNA in tail, and Tail moment with a significant difference compared with the control group. For concentration of 1 mg/ml only Tail length and Tail moment were significantly increased, so this extract would be genotoxic at a concentration of 5 mg/ml. ($P < 0.05$) [Figure 1]. For hydro-alcoholic extract of *N. jatamansi*, different concentrations were in contact with $hepG_2$. The results are shown in Figure 2. This diagram demonstrates that at concentrations of 1, 5, and 10 mg/ml, all parameters are significantly increased, concentration dependently, and the concentrations of 5 mg/ml and 10 mg/ml have significant difference when compared with the control group. According to these results this extract would be genotoxic at concentration of greater than 5 mg/ml ($P < 0.05$) [Figure 2].

Aqueous extract of *E. amoenum* significantly and concentration dependently increase all the three parameters. The diagrams of Tail length, %DNA, Tail moment against concentration of aqueous extract of *E. amoenum* were reviewed. A significant difference is observed between concentration of 10 µg/ml, 100 µg/ml, 500 µg/ml, 10 mg/ ml, 25 mg/ml for tail moment (Tail moment = %DNA in tail × Tail length) when compared with the control, similar results can be seen for %DNA and the most significant one is seen at concentration of 25 mg/ml. Consequently, the genotoxic effect was seen at concentrations greater than 25 mg/ml ($P < 0.05$) [Figure 3].

Also exposure of $hepG_2$ cells to hydro-alcoholic extract of *E. amoenum* significantly and concentration dependently increased all the three parameters tested. The diagram of tail length demonstrates the significant difference between the concentration of 25 mg/ml and control group. Therefore, the hydro-alcoholic extract of *E. amoenum* is genotoxic at concentrations above 25 mg/ml ($P < 0.05$) [Figure 4].

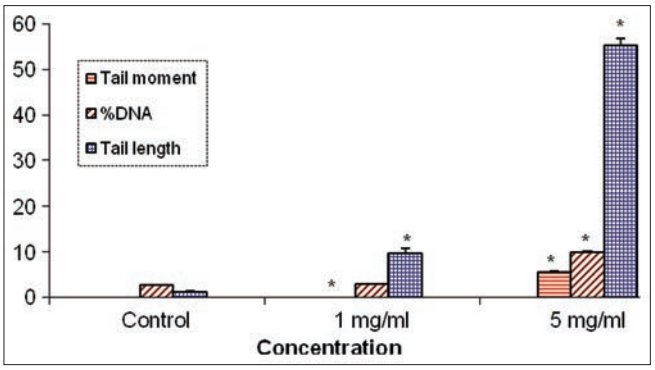

Figure 1: The effect of aqueous extract of *N. jatamansi* on Tail length, %DNA in tail, and Tail moment of $hepG_2$ cells. Results are presented as mean ± SD from at least three separate experiments, *= $P < 0.05$

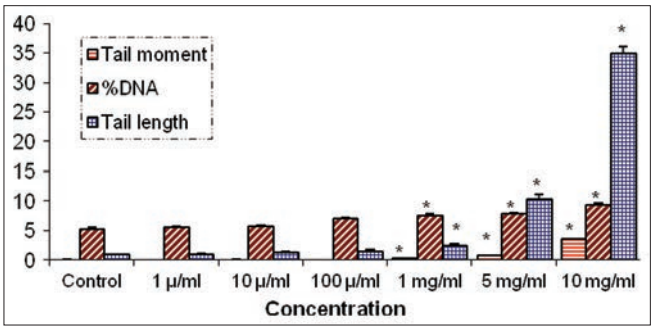

Figure 2: The effect of hydro-alcoholic extract of *N. jatamansi* on Tail length, %DNA in tail, and Tail moment of HepG2 cells. Results are presented as mean ± SD from at least three separate experiments, *= $P < 0.05$

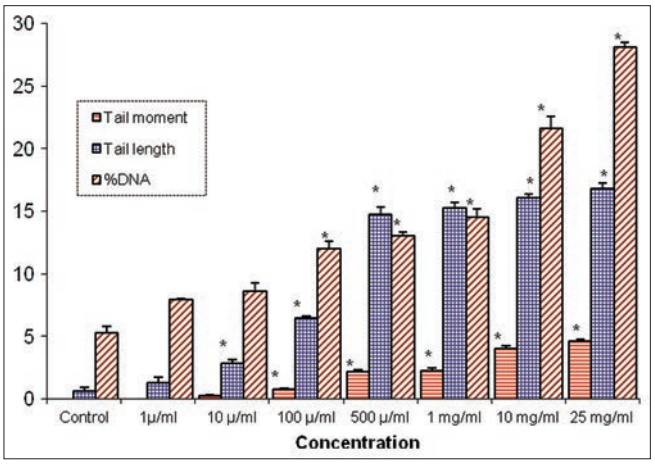

Figure 3: The effect of aqueous extract of *E. amoenum* on Tail length, %DNA in tail, and Tail moment of $hepG_2$ cells. Results are presented as mean ± SD from at least three separate experiments, *= $P < 0.05$

DISCUSSION

DNA damage could be detected by the single cell gel electrophoresis (SCGE) or comet assay, a fast, flexible, simple,

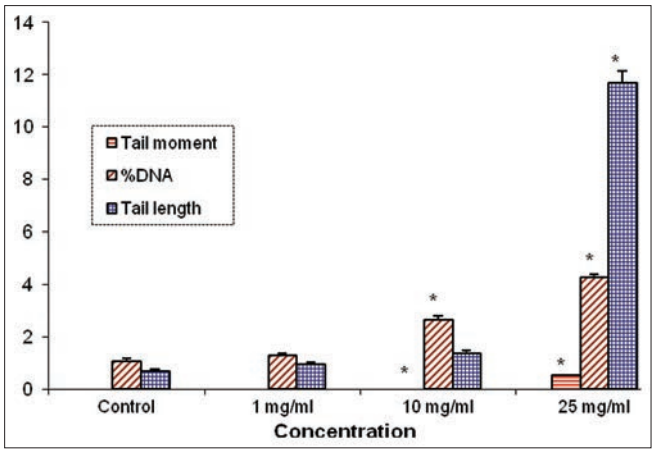

Figure 4: The effect of hydro-alcoholic extract of *E. amoenum* on Tail length, %DNA in tail, and Tail moment of hepG$_2$ cells. Results are presented as mean ± SD from at least three separate experiments, *= $P < 0.05$

and sensitive technique. During the last 20 years, the comet assay has been used to discover primary DNA damages such as double-strand breaks, single-strand breaks, alkali-labile sites in complete repair sites, and crosslinks.[23]

In this study, we used comet assay to find if *E. amoenum* and *N. jatamansi* have any genotoxicity. The comet assay has expanded an internationally well-known method to assay DNA damage in various cell types during the last two decades. It is believed that the comet assay is still growing in use and has high potential to be use in clinical and cancer research.[13] According to the frequent use of *E. amoenum* and *N. jatamansi* and evidence of their active toxic compounds, we made an effort to assess probable effects of aqueous and hydro-alcoholic extracts of *E. amoenum* and *N. jatamansi* on DNA of hepG$_2$ cells using the comet assay. Regarding the fact that in extraction by water and alcohol, different compounds are separated, obtained result for each type of extract could be different from each other. Our findings showed that aqueous and hydro-alcoholic extracts of *N. jatamansi* increased Tail length and Tail moment significantly and concentration dependently indicating the genotoxicity of this plant and its capability of DNA damage. Similarly, aqueous and hydro-alcoholic extracts of *E. amoenum* increased Tail length, %DNA and Tail moment of hepG$_2$ cells significantly and concentration dependently. This *in vitro* study was done on hepG$_2$ cell culture and there is a long way to be generalized to *in vivo* and determine the amount of plant that would affect human hepatoma. It is important to perform an *in vivo* study to recognize the amount of the plant should be consume orally to create concentrations of the herb extract that is in genotoxic for hepG$_2$ cells and to conclude if the amount of herb which is normally consumed is equal to amount of herb that is needed to make genotoxic effect and on the basis of this case decide about that normal use are genotoxic or not.

This study showed that mentioned concentrations of these herbs are toxic but more studies are required to determine the toxic effects of these herbs when they are used according to their traditionally common amounts. So our finding should serve as scientific guidance for clinical therapy of these two herbs preparation and more studies should be done about their harmlessness or their harmfulness in common dose but the obvious matter is that it is better to use them cautiously.

CONCLUSION

In conclusion, our findings revealed that hydro-alcoholic and aqueous extracts of *E. amoenum* and *N. Jatamansi* can cause DNA damage which make it necessary to reconsider the safety of using these plants and need of more toxicological studies in this regard.

ACKNOWLEDGMENTS

This study with research project number 389236 was financially supported by Research Vice-Chancellery of Isfahan University of Medical Sciences. We would like to thank Mina Mirian for her helps in cell culture.

REFERENCES

1. Vo LT, Chan D, King RG. Investigation of the effects of peppermint oil and valerian on rat liver and cultured human liver cells. Clin Exp Pharmacol Physiol 2003;30:799-804.
2. Ahmad M, Yousuf S, Khan MB, Hoda MN, Ahmad AS, Ansari MA, *et al*. Attenuation by Nardostachys jatamansi of 6-hydroxydopamine-induced parkinsonism in rats: Behavioral, neurochemical, and immunohistochemical studies. Pharmacol Biochem Behav 2006;83:150-60.
3. Amit S, Shashi T, Sharad S, Rawat A. HPTLC method for quantification of valerenic acid in ayurvedic drug jatamnsi and its substitutes. J Liq Chromatogr Relat Technol 2010;33:1679-88.
4. Evans WC. Trease and Evans Pharmacognosy. In: Miscellaneous Isoprenoids. London: WB Saunders; 2002. p. 316-8.
5. Heidari MR, Azad EM, Mehrabani M. Evaluation of the analgesic effect of Echium amoenum Fisch & C.A. Mey. Extract in mice: Possible mechanism involved. J Ethnopharmacol 2006;103:345-9.
6. Stegelmeier BL. Pyrrolizidine alkaloid-containing toxic plants (Senecio, Crotalaria, Cynoglossum, Amsinckia, Heliotropium, and Echium spp.). Vet Clin North Am Food Anim Pract 2011;27:419-28.
7. Mehrabani M, Raftari SH, Nabipour, Heidari MR, Mahdavi Z, Sadeghi Rad B. Evaluation of hepatotoxicity of common doses of decoction of *Echium amoenum* Fish and C.A. Mey in rats. J Kerman Uni of Med Sci 2007;14:44-5.
8. Sayyah M, Boostani H, Pakseresht S, Malaieri A. Efficacy of aqueous extract of *Echium amoenum* in treatment of obsessive-compulsive disorder. Prog Neuropsychopharmacol Biol Psychiatry 2009;33:1513-6.
9. Mehrabani M, Ghannadi A, Sajjadi SE, Ghassemi N, Shams-Ardakani M. Toxic pyrrolizidine alkaloids of *Echium amoenum* Fish and Mey. Daru 2006;14:122-7.
10. Timothy ST, Richard LK. Herbal products, toxicology and clinical pharmacology. In: Isetts BJ, Berkner ED, Sioris L, editors. New Jersey: Humana Press; 2007. p. 55-70, 97-109.
11. Gensler HL, Bernstein H. DNA damage as the primary cause of

aging. Q Rev Biol 1981;56:279-303.
12. Mersch-Sundermann V, Knasmuller S, Wu XJ, Darroudi F, Kassie F. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. Toxicology 2004;198:329-40.
13. Collins AR. The comet assay for DNA damage and repair: Principles, applications, and limitations. Mol Biotechnol 2004;26:249-61.
14. Wu Y, Chen H, Li Z, Sun L, Qu M, Li M, *et al*. Genotoxicity evaluation of drinking water sources in human peripheral blood lymphocytes using the comet assay. J Environ Sci (China) 2008;20:487-91.
15. Knopper LD, McNamee JP. Use of the comet assay in environmental toxicology. Methods Mol Biol 2008;410:171-83.
16. Fenech M. The micronucleus assay determination of chromosomal level DNA damage. Methods Mol Biol 2008;410:185-216.
17. Belloir C, Singh V, Daurat C, Siess MH, Le Bon AM. Protective effects of garlic sulfur compounds against DNA damage induced by direct- and indirect-acting genotoxic agents in HepG2 cells. Food Chem Toxicol 2006;44:827-34.
18. Steenkamp V, Grimmer H, Semano M, Gulumian M. Antioxidant and genotoxic properties of South African herbal extracts. Mutat Res 2005;581:35-42.
19. Helma C, Uhl M. A public domain image-analysis program for the single cell gel electrophoresis (comet) assay. Mutat Res 2000;466:9-15.
20. Samsam Shariat SH. Extraction of effective substances of medicinal plants. Isfahan: Isfahan Mani Publications; 1992. p. 2-5.
21. Iranian Herbal Pharmacopoeia. Vol 1, 2. Tehran: Food and drug deputy of health ministry; 2002. p. 22, 457, 667.
22. Jiang L, Cao J, An Y, Geng C, Qu S, Jiang L, *et al*. Genotoxicity of acrylamide in human hepatoma G_2 ($HepG_2$) cells. Toxicol *In Vitro* 2007;21:1486-92.
23. Ladeira MS, Rodrigues MA, Freire-Maia DV, Salvadori DM. Use of comet assay to assess DNA damage in patients infected by helicobacter pylori: Comparisons between visual and image analyses. Mutat Res 2005;586:76-86.

How to cite this article: Etebari M, Zolfaghari B, Jafarian-Dehkordi A, Rakian R. Evaluation of DNA damage of hydro-alcoholic and aqueous extract of *Echium amoenum* and Nardostachys jatamansi. J Res Med Sci 2012;17:782-6.

Source of Support: This study with research project number 389236 was financially supported by Research Vice-Chancellery of Isfahan University of Medical Sciences., **Conflict of Interest:** None declared.