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Determination of the Mutagenicity Potential of Supermint Herbal Medicine by Single Cell Gel Electrophoresis in Rat Hepatocytes

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ABSTRACT

Purpose: The increasing use of herbal drugs and their easy availability have necessitated the use of mutagenicity test to analyze their toxicity and safety. The aim of this study was to evaluate the genotoxicity of Supermint herbal medicine in DNA breakage of rat hepatocytes in comparison with sodium dichromate by single cell gel electrophoresis technique or comet assay. ***Methods:*** Hepatocytes were prepared from male wistar rats and were counted and kept in a bioreactor for 30 minutes. Then cells were exposed to the Supermint herbal medicine at doses of 125, 250 and 500 µl/ml. Buffer 4 (incubation buffer) and sodium dichromate were used as negative and positive control for one hour respectively. Then cell suspension with low melting point agarose were put on precoated slides and covered with agarose gel. Then lysing, electrophoresis, neutralization and staining were carried out. Finally the slides were analyzed with fluorescence microscope. The parameter under this analysis was the type of migration which was determined according to Kobayashi pattern. ***Results:*** With increased dose of Supermint herbal medicine the DNA damage was slightly increased (P<0001). ***Conlusion:*** In overall compared to the positive control significant differences is observed which convinced that the crude extract of Supermint in vitro did not have mutagenic effect.

Introduction

Fossils date human use of plants as medicine to approximately 60000 years ago. Today, almost 65% of the world's population relies on plants as an integral part of their primary health care. Also there have been many validations of traditional remedies through scientific research and in addition, the use of ethnomedical information has contributed to health care worldwide through the isolation of bioactive compounds for direct use in medicine. The adverse effects of widely used plants are not well documented in the literature. Based on their long-term use by human one might expect plants used in traditional medicine to have low toxicity. However, recent investigations have revealed that many plants used as food or in traditional medicine have mutagenic effects in *in vitro* assay this raises concern about the potential mutagenic hazards resulting from the long-term use of medicinal plants.[1-9]
In Iranian folk medicine, Supermint is an example of plants that are widely used and today, Supermint oral drop contains Essential oil of *Mentha spicata* which is widley used as Carminative, Gastrointestinal analgesic, antispasmodic, Dyspepsia treatment and Stomachic.[10] Therefore, one of the objectives of our study was to evaluate the saftey activity of Supermint as herbal medicine to evaluate the safety by in vitro method on hepatocytes.
In the evaluation of in vitro methods for natural products the biological activity determination has changed in the past few years, one of the recent developments is comet assay, which gives a ratio between the viable cells in the cell culture to total cells in the culture. These techniques are considered rapid and economical for the evaluation of mutagenicity or genotoxicity of compounds.[11-12] In view of the potential therapeutic use of Supermint herbal medicine and the absence of any data on its genetic toxicity in eukaryotes, the study described in this paper was under-taken to evaluate the potential in vitro mutagenic effects in terms of DNA damage in rat hepatocytes by Single Cell Gel Electrophoresis technique.

Materials and Methods

Animal used in this experiment was wistar rat (250 – 300 g weight) obtained from the animal house of Razi Institute, Iran. Rat was housed in polyethylene cage in a climate controlled environment with a 12 hours (07.00 to 19.00) day length and ad libitum access to food and water. Hepatocyte extraction was prepared by IP injection of ketamin 90 mg/kg and xylazine 10

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mg/kg for anesthesia. Then rat was dissected and IV injection of heparin the canulation of liver was made. Liver was washed with washing buffer for 10 minutes and then with perfusion buffer (colagenase buffer) for 12 minutes. The isolated liver was transferred in to a petri dish containing washing buffer to separate hepatocytes, then the cell suspension filtered and the filtrate was centrifuged for 3 minutes at 1500 rpm. The upper layer was discarded and to the lower layer 10 ml of washing buffer was added, mixed well and then from this mixture 100 µl was mixed with 200 µl of incubation buffer and 300 µl of trypan blue for counting cells. From this cell suspension little amount poured on Neubar slide and start counting the cells. Mean of the viable cells and dead cells were calculated as follow; % of viable cells = (mean of viable cells/total cells) × 100. Then hepatocytes were counted until we got the ratio 10^6 cells.[13] From this cell suspension a desired amount were kept in bioreactor bath at 37 C° atmosphere of 10 % O_2, 85 % N_2 and 5 % CO_2 for 30 minutes and then to five bioreactor flasks containing hepatocytes. After this step the hepatocytes were exposed to Supermint herbal medicine (was purchased from barijessence . co. Iran) for one period of 60 minute at doses of 125, 250 and 500 µl/ml (test groups) respectively. Buffer 4 (which contains HEPES 0.75 g and Krebs 250 ml) was used as negative control and 1000 µM of sodium dichromate used as positive control. Then from each bioreactor flask 1 ml of the hepatocyte cell suspension was taken and diluted to 10 ml with low melting point agarose and then 100 µl of this suspension were poured on precoated slides and covered with cover slips. Slides were kept for 15 to 20 minutes in a horizontal placed iced tray till to become solidified. The next step, the cover slips were removed from the slides and kept them in a lysing solution for one hour and then they were removed from lysing solution and washed by D-ionized water, kept for 20 minutes in electrophoresis buffer and did electrophoresis at 25 V and 300 mA for 20 minutes and then washed with neutralized buffer for five minutes for three times. Slides were immersed in ethidium bromide solution for five minutes and according to the method described by Speit and Hartmann,[14] which is based on the original work of Singh *et al.*[15] slides were analyzed by fluorescence microscope. The extent and distribution of DNA damage indicated by comet assay was evaluated by examining cells. The cells were visually scored into comet classes according to tail size class[16-18] Class 0 = no tail, Class 1 = tail shorter than the diameter of the head (nucleous), Class 2 = tail length 1 to 2x the diameter of the head and Class 3 = tail longer than 2x the diameter of the head. Comet without head and those with nearly all the DNA in the tail or with a very wide tail were excluded from the evaluation because they probably represented dead cells[19-20] tail length and the mutagenic Index is calculated according the following formula MI = (0NMC+ 1SMC+ 2MMC+ 3LMC) /200, or we can express it such as NMC = No migration cells (score 0), SMC = Short migration cells (score) MMC = Medium migration cells (score 2), LMC = Long migration cells (score 3).

Results and Discussion

In Iran like other countries, the use of herbal medicine in the treatment of diseases is very common; therefore, their safety like the mutagenecity potential should be evaluate by techniques like single cell gel electrophoresis. This technique is a sensitive, reliable and an important tool for evaluating the in vitro and in vivo genotoxic potential of bioactive compounds.

Table 1 demonstrates the percentage of frequency of comet pattern of rat hepatocytes at different doses of Supermint herbal medicine in comparison with positive control (sodium dichromate) in 60 minutes after exposure. Similarly, as can be seen in Table 2 the percentage of mutagenic index (MI) and damaged cells treated with Supermint herbal medicine at different doses are significantly lower that of positive control (sodium dichromate) in 60 minutes after exposure ($P<0.0001$).

The microscopic photograph 1 to 4 present the comet in scoring pattern from 0 score to score 3 which indicate the comet length (Figure 1). Comets from the broken ends of a negatively charged DNA molecule became free and migrated towards the anode in the electric field. The assay provided direct determination of the extent of DNA damage in individual cells and the extent of DNA damage have been assessed from the length of DNA migration. As it was expected, the results obtained from positive control were statistically significant in comparison to the negative control.

Table 1. The percentage of rat hepatocytes comet pattern frequency at different doses of Supermint herbal medicine and its comparison with positive control (sodium dichromate) in 60 minutes after exposure ($P<0.0001$)

Substance	Score 0	Score 1	Score 2	Score 3
Sodium dichromate 1000 µM	8.5 ± 1.21	16 ± 1.3	28.5 ± 1.38	47 ± 1.67
Supermint 0 µl/ml	94 ± 1.62	5.5 ± 1.09	0.5 ± 0.57	0 ± 0
Supermint 125 µl/ml	87 ± 1.22	10 ± 0.81	3 ± 0.4	0 ± 0
Supermint 250 µl/ml	82 ± 0.81	12 ± 0.81	6 ± 0	0 ± 0
Supermint 500 µl/ml	81 ± 2.1	12 ± 1.7	7 ± 0.66	0 ± 0

Table 2. Mutagenic index (MI) and damaged cells treated by Supermint at different doses and its comparison with positive control (sodium dichromate) in 60 minutes after exposure (P<0.0001).

Substance	MI (%)	Damaged cells (%)
Sodium dichromate 1000 µM	53.5 ± 1.38	75.5 ± 1.67
Supermint 0 µl/ml	1.6 ± 0.51	0.5 ± 0.57
Supermint 125 µl/ml	4 ± 0.81	3 ± 0.4
Supermint 250 µl/ml	6 ± 0.81	6 ± 0
Supermint 500 µl/ml	6. 5 ± 1.66	7 ± 0

The data are expressed as the means ± SE

The results obtained in this study indicated that the mutagenicity index of positive control was 53.5 % and the mutagenicity index for the lower dose of Supermint herbal medicine after 60 minutes was 4 % and the mutagenicity index of higher dose of the Supermint herbal medicine after 60 minutes was 6.5 %. These findings indicated that with the increase of the dose of the Supermint herbal medicine mutagenicity index was increased. However it was not significant as compared with positive control P<0.0001. Based on genotixicity study by comet assay the present study provided additional confirmation to our previous study, that high doses of Supermint herbal medicine did not produce significant DNA damage.

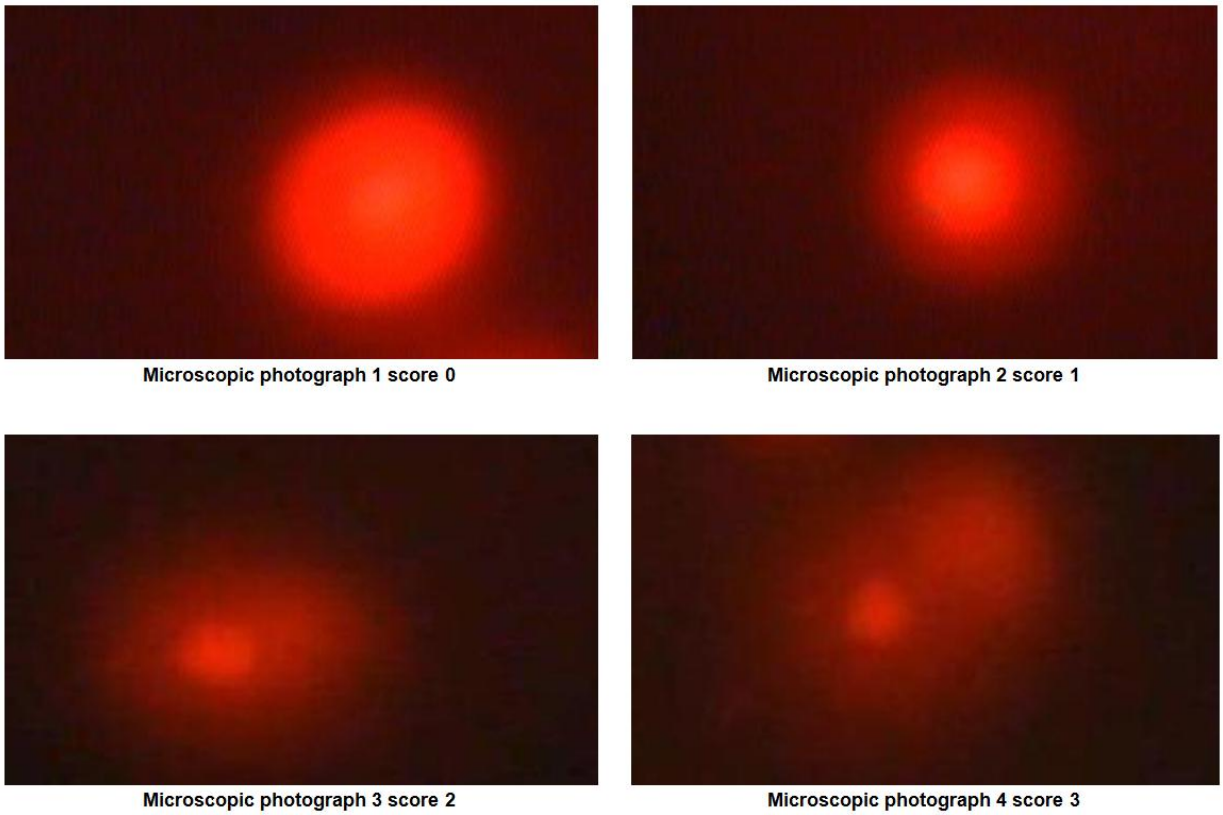

Figure 1. The extent and distribution of DNA damage indicated by comet assay.

As the herbal medicine contains antioxidant agents may protect the DNA damage by chemicals and prevent mutagenicity. In conclusion these results indicated that by increasing the dose of Supermint herbal medicine the DNA damage is lightly increases but in overall as compared to the positive control significant differences is observed which convinced that the Supermint herbal medicine in vitro did not have mutagenic effect.

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Conflict of interest
The authors declare there is no Conflict of interest in the content of this study.

References

1. Ahn HS, Jeon TI, Lee JY, Hwang SG, Lim Y, Park DK. Antioxidative activity of persimmon and grape seed extract: in vitro and in vivo. Nutr Res 2002;22(11):1265-73.
2. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environ Health Persp 2001;109(Suppl 1):69-75.
3. Higashimoto M, Purintrapiban J, Kataoka K, Kinouchi T, Vinitketkumnuen U, Akimoto S, et al.

Mutagenicity and antimutagenicity of extracts of three spices and a medicinal plant in Thailand. Mutat Res 1993;303(3):135-42.
4. Kassie F, Parzefall W, Musk S, Johnson I, Lamprecht G, Sontag G, et al. Genotoxic effects of crude juices from Brassica vegetables and juices and extracts from phytopharmaceutical preparations and spices of cruciferous plants origin in bacterial and mammalian cells. Chem Biol Interact 1996;102(1):1-16.
5. Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr 2003;78(3 Suppl):517S-20S.
6. McGaw LJ, Jager AK, van Staden J. Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. J Ethnopharmacol 2000;72(1-2):247-63.
7. Rabe T, Van Staden J. Screening of Plectranthus species for antibacterial activity. S Afr J Botany 1998;64(1):62-5.
8. Schimmer O, Hafele F, Kruger A. The mutagenic potencies of plant extracts containing quercetin in Salmonella typhimurium TA98 and TA100. Mutat Res 1988;206(2):201-8.
9. Schimmer O, Kruger A, Paulini H, Haefele F. An evaluation of 55 commercial plant extracts in the Ames mutagenicity test. Pharmazie 1994;49(6):448-51.
10. Barijessence. Supermint. [Internet].[Update 2012 May 3]. Available from http://www.barijessence.com.
11. Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, et al. Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J Natl Cancer Inst 1990;82(13):1113-8.
12. Moneef MA, Sherwood BT, Bowman KJ, Kockelbergh RC, Symonds RP, Steward WP, et al. Measurements using the alkaline comet assay predict bladder cancer cell radiosensitivity. Br J Cancer 2003;89(12):2271-6.
13. Berry MN, Phillips JW. The isolated hepatocyte preparation: 30 years on. Biochem Soc Trans 2000;28(2):131-5.
14. Speit G, Hartmann A. The comet assay (single-cell gel test). A sensitive genotoxicity test for the detection of DNA damage and repair. Methods Mol Biol 1999;113:203-12.
15. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175(1):184-91.
16. Jalali M, Hatami A, Kalantari H, Kalantar E. Mutagenicity Assessment of Two Herbal Medicines, Urtan and Carmint in Human Leukocytes by Single Cell Gel Electrophoresis. Saudi Pharm J 2006;14(2):129.
17. Junqueira APF, Perazzo FF, Souza GHB, Maistro EL. Clastogenicity of Piper cubeba (Piperaceae) seed extract in an in vivo ma mmalian cell system. Genet Mol Biol 2007;30:656-63.
18. Kalantari H, Jalali M, Moein E. In Vitro Evaluation of Mutagenic Effect of Vitagnus and Shirafza in Human Leukocytes by Single Cell Gel Electrophoresis. Jundushapur J Nat Pharm Prod 2007;1(1):26-31.
19. Hartmann A, Speit G. The contribution of cytotoxicity to DNAeffects in the single cell gel test (comet assay). Toxicol Lett 1997;90(2-3):183-8.
20. Kalantari H, Panahi M, Izadinajafabadi A, Mohammadi E. Genotoxicity Determinations of Coriander Drop and Extract of Coriander Sativum in Cultured Fibroblast of Rat Embryo by CometAssay. Saudi Pharm J 2008;16(1):85.

