

Original Research Paper

Evaluating the effects of galbanic acid on hydrogen peroxide-induced oxidative DNA damage in human lymphocytes

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Abstract

Objective: *Ferula szowitsiana* has been widely used for medicinal purposes around the world. The anti-oxidant effect of *F. szowitsiana* had been proved. The current study aims to determine the protective effects of galbanic acid, a sesquiterpene coumarin from *F. szowitsiana*, against hydrogen peroxide (H₂O₂) - induced oxidative DNA damage in human lymphocytes.

Materials and Methods: Human lymphocytes were incubated with H₂O₂ (0, 25, 50, 100, and 200 μM), galbanic acid (200 and 400 μM) and a combination of galbanic acid (200 and 400 μM) and H₂O₂ (25 μM) at 4 C for 30 minutes. Solvents of galbanic acid without H₂O₂ were used as negative controls.

Results: The findings of this study demonstrated that H₂O₂ exposure leads to a significant concentration-dependent increase in DNA damage. Galbanic acid did not cause DNA damage compared with the control cells. Data showed that galbanic acid does not have a protective effect against H₂O₂-induced oxidative DNA damage in human lymphocytes.

Conclusion: According to the results, it is concluded that the capability of *F. szowitsiana* in reducing reactive oxygen species and the anti-inflammatory property of its methanolic extract may be due to its other ingredients.

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Introduction

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free

radicals) and antioxidant defenses. It may have a part in pathogenesis of various diseases, including cancer, diabetes, aging and other degenerative diseases (Uttara et

al., 2009, Lobo *et al.*, 2010). Antioxidants are substances capable of inhibiting or slowing the oxidation of other molecules. They are different in shape, physicochemical properties and their site of action (Flora 2009). Antioxidants can be natural or synthetic (Yeo *et al.*, 2010).

Some synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are believed to be responsible for liver damage and cancer in laboratory animals (Biswas, Haldar *et al.*, 2010). As a result, there is renewed interest in the increased use of naturally occurring antioxidants. Because they occur in nature and in many cases are derived from plant sources, natural antioxidants are presumed to be safe (Ji *et al.*, 2009). Phytochemicals are found in fruits, vegetables, and plant-derived beverages and may prevent some of the damage caused by free radicals (Stevenson and Hurst 2007, Lin *et al.*, 2013).

Several studies have been done on the antioxidant compounds from *Ferula* species (Gholitabar and Roshan 2013, Kavooosi and Rowshan 2013, Kavooosi *et al.*, 2013). One of the most important compounds of *F. szowitsiana* are sesquiterpenes particularly sesquiterpene coumarins (Shahverdi *et al.*, 2007, Bazzaz *et al.*, 2010). Coumarins represent a wide spectrum of biological activity such as antibacterial, antifungal, anti-inflammatory, anticoagulant, anti-HIV and antitumor (Wu *et al.*, 2009, Riveiro *et al.*, 2010). Galbanic acid is an isolated sesquiterpene coumarin from *Ferula* species with antibacterial, anti-tumor and anti-proliferation activities (Kim *et al.*, 2011). The aim of this study was to assess the ability of galbanic acid in protecting human lymphocytes against H₂O₂-induced oxidative DNA damage.

Materials and Methods

Chemicals

Low melting point agarose (LMA) and normal melting agarose (NMA) were purchased from Fermentas International,

Inc. (Canada). All the remaining chemicals were obtained from Merck (Germany).

Galbanic acid

Galbanic acid was isolated from *F. szowitsiana* described previously (Iranshahi *et al.*, 2007).

Isolation of human lymphocytes

Fasting blood was obtained from volunteer donors. Five milliliters of the whole blood was diluted 1:1 with PBS, and then carefully layered on top of a lymphocyte separation medium [aqueous solution of Ficoll, 57 g/L; density of 1.077 g/mL] in a centrifugation tube in a 1:1 ratio. After centrifugation for 20 minutes at 2000 rpm, gradient-separated lymphocytes were recovered, diluted 1:1 with PBS, and centrifuged a second time at 1500 rpm for 10 minutes. The cell pellets were resuspended in 500 mL of PBS, and the cells counted in a Neubauer chamber. The cell concentration was adjusted to 5000 cells/mL in preparation for the comet assay. Cell viability was determined using the trypan blue dye exclusion method, and only cell suspensions with viabilities of more than 96% were used for determination of DNA damage.

Determination of DNA damage [comet assay]

The comet assay was performed under alkaline conditions according to the technique described in 1988 (Singh *et al.*, 1988). Human lymphocytes were incubated in different concentrations of H₂O₂ (50, 100, and 200 μM) as a positive control and different concentrations of galbanic acid (200 and 400 μM) alone. In the test groups, lymphocytes were exposed to H₂O₂ (25 μM) and 200 or 400 μM of galbanic acid at 4°C for 30 minutes. In addition, we used the galbanic acid solvents without H₂O₂ as negative controls.

Samples were then centrifuged at 3000 rpm for 10 minutes and the cells washed with PBS. The cell pellets were mixed with

Effects of galbanic acid on oxidative DNA damage

100 mL of 0.75% (w/v) low melting point agarose (LMA), and then distributed onto microscope slides coated with 100 mL of 1% (w/v) normal melting agarose, covered with a cover slip, and kept for 10 minutes at 4°C to solidify. After the cover slips were removed, the slides were covered with another 100 mL of (0.75% w/v) LMA, covered with a cover slip, and kept for 10 minutes at 4°C. After solidification, the slides were submerged in a cold fresh lysing solution [2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% (v/v) triton X-100, 10% DMSO, pH 10.0] for at least 2 hours. After the lysis, the slides were placed in an alkaline solution [1 mM Na₂EDTA, 0.3 N NaOH, pH 13.0] for 40 min to permit unwinding of the DNA.

Electrophoresis was run at 25 V for 45 minutes at 4°C. To prevent additional DNA damage all procedural steps were performed under yellow light conditions. The slides were then neutralized with 0.4 M Tris-HCl buffer, pH 7.5, stained with ethidium bromide (20 µg/mL). The slides were studied using a fluorescent microscope (Nikon100) connected to a CCD camera and a personal computer. Fifty individual cells were selected for calculations for each analysis, and four separate experiments [four slides for each experimental point] were conducted for each series. Single cells were analyzed with "Casp 1.2.2." software. The DNA damage was expressed as % Tail DNA, where % Tail DNA = [Tail DNA / [Head DNA + Tail DNA]] x 100. A higher % Tail DNA indicated a higher level of DNA damage.

Statistical analysis

All statistical analyses were performed using SPSS analysis software (version 17.0) and the data are represented as mean ± SEM. ANOVA followed by Tukey test was used to compare the results obtained for the groups treated with galbanic acid and the negative control group. The difference was considered significant when *p value* was less than 0.05.

Results

Data showed that H₂O₂ exposure (0, 25, 50, 100, and 200 µM) leads to a significant concentration-dependent increase in DNA damage when compared to control cells (Figure 1). Galbanic acid (200 and 400 µM) showed no toxicity against human lymphocytes (Figure 2).

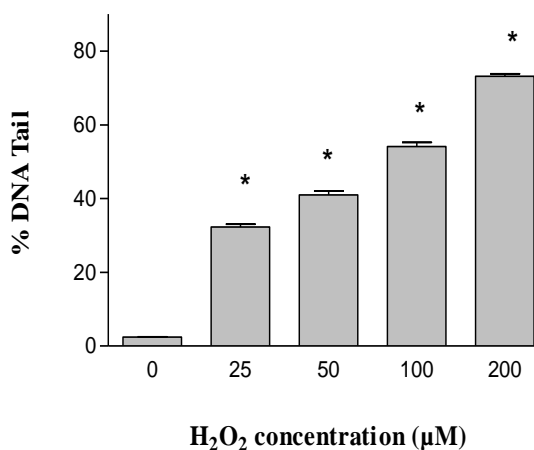


Figure 1. Level of DNA damage in H₂O₂-treated human lymphocytes. Lymphocytes were incubated for 30 minutes at 4°C with different concentrations of H₂O₂. Results are presented as mean ± SEM (n=4 slides × 50 lymphocytes). ANOVA was used for comparison and *p value* <0.05 was considered as significant and marked by *

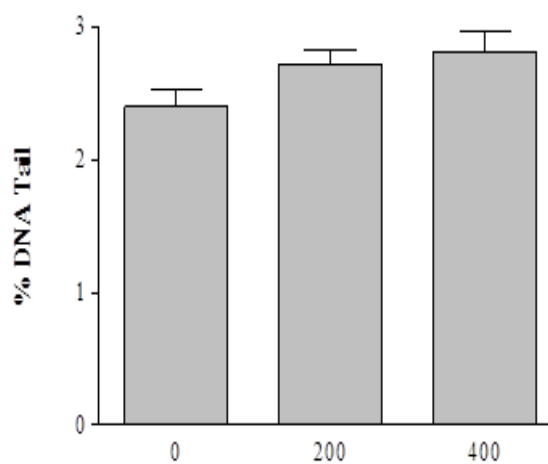


Figure 2. Level of DNA damage in human lymphocytes upon treatment with different concentrations of galbanic acid compare to negative control. Lymphocytes were incubated for 30 minutes at 4°C with galbanic acid. Results are mean ± SEM (n=4 slides × 50 lymphocytes). ANOVA was used for group comparison in significance level of *p value* <0.05.

The data obtained from DNA damage in lymphocytes exposed to H₂O₂ (25 μM) and galbanic acid (200 and 400 μM) revealed that H₂O₂-induced DNA damage was unaffected due to galbanic acid treatment (Figure3)

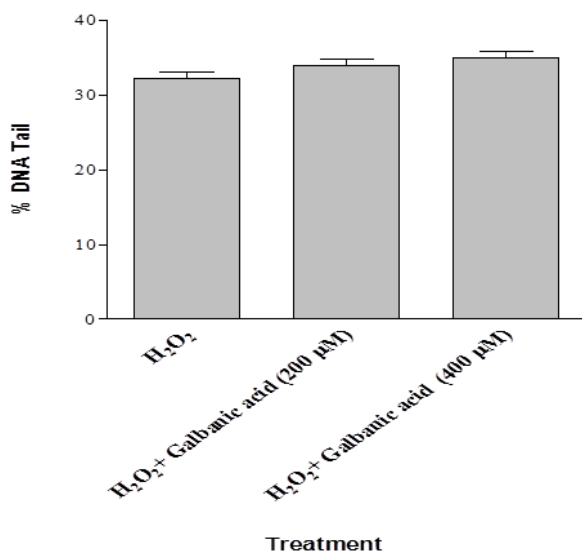


Figure 3. Effect of galbanic acid on lymphocyte DNA damage induced by H₂O₂. Human lymphocytes were incubated for 30 minutes at 4°C with a combination of H₂O₂ (25 μM) with different concentrations of Galbanic acid 200 and 400 μM). Results are mean±SEM (n=4 slides × 50 lymphocytes). ANOVA was used for group comparison in significance level of *p* value <0.05.

Discussion

The genus *Ferula* belongs to the Umbelliferae family with approximately 130 species distributed throughout the Mediterranean area and central Asia where many species have been traditionally used in folk medicine (Kim et al., 2011). *F. szowitsiana* is one of the ethnomedicinal plants in the genus that is widely used in Azerbaijan, Iran, Turkey and Afghanistan. Many studies reported a wide range of pharmacological effects of the plant, including anti-cancer, anti-inflammation, antimicrobial and antibiotic activities (Gholami et al., 2013, Paydar et al., 2013). Galbanic acid as an ingredient of *F. szowitsiana*, showed various biological properties, including anticancer, cancer

chemopreventive, anticoagulant, antiviral, and antileishmanial activities (Kasaian et al., 2013). In this study we used the comet assay to find if this compound has any protective effect against H₂O₂-induced oxidative DNA damage in human lymphocytes.

The capability of *F. szowitsiana* in reducing ROS and its anti-inflammation property had been recognized. Soltani et al demonstrated antioxidant capacity in auraptene isolated from *F. szowitsiana*. Auraptene was proved to be more effective than ascorbic acid to decrease genotoxicity of H₂O₂. They suggested that prenyl moiety in auraptene may suppress the generation of superoxide anion (Soltani et al., 2010).

In another study, gooshchi et al evaluated antioxidant activity of n-hexan, dichloromethane and methanolic extracts of *F. szowitsiana* by 2, 2-diphenyl para-1-picryl hydrazyl (DPPH) method. The methanolic extract has shown significant antioxidant activity which was comparable with rutin. They demonstrated that *F. szowitsiana* is a rich source of coumarin and furanocomarin derivatives and these compounds may pose potent antioxidant property (Gooshchi et al., 2012). According to our results, it is concluded that galbanic acid does not have a protective effect against H₂O₂-induced oxidative DNA damage in human lymphocytes at the tested concentrations. The lack of efficacy may be due to the use of inappropriate concentrations. Therefore, the antioxidant properties of the methanolic extract of *F. szowitsiana* may be due to other compounds such as: umbelliferon-7-apiosyl β (16) glucoside, p-hydroxy phenylethanoid glucoside, umbelliferon β-D [6' (ferolye)-glucoside] and umbelliferon. Further research should be done to investigate the antioxidant activity and antigenotoxic effects of these compounds (Gooshchi et al., 2012).

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Conflict of interest

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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