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A Modulator Against Gliotoxin-Induced Genotoxic Damage: *Pseudovernia furfuracea* (L.) Zoph.

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Abstract

Gliotoxin (GTX) is one of the toxic, carcinogenic and mutagenic seconder metabolite which is produced by *Gliocladium, Aspergillus* and *Penicillum* species. Many *in vivo* and *in vitro* studies were carried out to minimize these harmfull effects of mycotoxins. To our best knowledge, the effects of the lichen, *Pseudovernia furfuracea*, against genetic damage induced by GTX on human lymphocytes were not evaluated. Therefore, in the present study, we assessed the genotoxic effects of GTX at different concentrations (50, 75 and 100 ng/mL) and the role of *P. furfuracea* (5 and 10 µg/mL) in GTX-treated cultures (n=2). The sister chromatid exchange (SCE) test was used to monitor the genetic damage. Our results indicated that, GTX caused statistically significant increases in SCE frequency as compared to control group. However, the SCE rates induced by different GTX concentrations were alleviated by the presence of *P. furfuracea*. As conclusion, the results of present study revealed for the first time that the lichen *P. furfuracea* provided increased resistance of DNA against GTX-induced genetic damage on human lymphocytes.

INTRODUCTION

Lichens have been used for sources of natural drugs, possible food supplement and pharmaceutical industry [1]. This organism was especially effective in treatment of hemorrhoids, bronchitis, dysentery, and tuberculosis [2]. Biological activities and chemical composition of lichens have long been investigated for antimicrobial, antitumor, antiviral, allergenic, plant growth inhibitory, antiherbivore, and enzyme inhibitory till today [3]. Pharmaceutical studies were also carried out about anti-inflammatory and antioxidant activities of aqueous extracts of lichenes [1,4,5].

GTX is the most known seconder metabolites produced by diverse of fungi like *Gliocladium, Penicillium* and especially *Aspergillus* [6,7]. Biosynthesis or primary role of GTX production in *Aspergillus* species are not illustrated fully. Interestingly, not all Aspergillus species have ability of producing GTX [8]. GTX is a member of the epipolythiodioxopiperazine (ETP) class of toxins which has supressive effects on different cell types of immune system e.g inhibition of lymphocyte proliferation, antigen presentation, NF-k-B (transcription factor) activation and reduction of the activity of cytotoxic T-cells [9-11]. Furthermore, ETP

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compounds also cause apoptotic cell death in macrophages and monocytes [12]. GTX can be located in the sera of patients with invasive aspergillosis providing evidence that GTX is also produced in invasive aspergillosis in humans [13]. In addition, GTX was found to be genotoxic in the bacterial repair assay [14]. Nevertheless, the mechanisms of GTX toxicity and its molecular targets in eukaryotic cells have not been fully characterized [15].

The aim of the present work was to evaluate the role of *P. furfuracea* on GTX-induced genotoxic effects in human lymphocyte cells by using SCE test. The SCE assay is an ideal genotoxicity and cytotoxicity assay, since peripheral lymphocytes are easily accessible. Moreover, SCE is much more sensitive as a mutagenic biomarker than chromosome aberration and micronucleus [16].

MATERIAL AND METHODS

Experimental design

P. furfuracea samples were collected from the vicinity of Inci village, Oltu district, Erzurum, Turkey. The samples were identified using various flora books and papers [17-23]. Identified samples were air dried and kept in the herbarium of Kazım Karabekir Education Faculty, until they were used. For water extraction of the lichen, 20 g sample was mixed with 400 mL distilled and boiling water using magnetic stirrer for 15 min. Then the extract was filtered over Whatman No. 1 paper.

The heparinized blood samples obtained from two healthy non-smoking donors. Human peripheral blood lymphocyte cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan [24]. The heparinized blood (0.5 ml) was cultured in 5 ml of culture medium (Chromosome Medium B, Biochrom, Leonorenstr. 2-6.D-12247, Berlin) with 5 µg/mL of phyto-hemagglutinin (Biochrom). GTX ($C_{13}H_{14}N_2O_4S_2$) (Sigma Chemical Co., St Louis, MO. USA) (in concentrations of 50, 75 and 100 ng/mL) and lichen extract (in concentrations of 5 and 10 µg/mL) were added to the cultures just before incubation, separately and together. Each individual lymphocyte culture without GTX and lichen extract was studied as a control group.

SCE assay

With the aim of providing a better visualization of SCEs, 5-bromo-2′-deoxyuridine (Sigma, final concentration 20 µM) was added after culture initation. The cultures were incubated in complete darkness for 72 h at 37°C. Exactly 70 h and 30 min after beginning of the incubations, colcemid (Sigma) was added to the cultures to achieve a final concentration of 0.5 µg/L. After hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation and resuspension, the cell suspension was dropped onto chilled and grease-free microscopic slides, air-dried, aged, and then differentially stained for inspection of the SCE rate according to the fluorescence plus Giemsa (FPG) procedure [25]. For each treatment condition, 25 well-spread second division metaphases were scored and the values obtained were calculated as SCEs per cell.

Statistics

The statistical analysis of experimental values in the SCE test was performed by Student's *t*-test and using the S.P.S.S. 12.0 software. Statistical decisions were made with a significance level of 0.05.

RESULTS AND DISCUSSION

The effects of GTX and lichen extracts on the number of SCEs in human whole blood cultures are shown in Figure 1. GTX caused significant increases of SCE frequencies on human peripheral lymphocytes compared with the controls in a clear dose dependent manner. However, the lichen extracts at both applied concentrations did not indicate significantly difference (*P<0.05*) in number of SCEs statistically. Moreover, the positive effect of lichen extracts was established on GTX-induced SCEs. And the incidence of SCEs was decreased in comparison with GTX-treated group. Also, this situation was related as depending on concentrations of *P. furfuracea*.

Our results indicated that one of the targets of GTX in human cells was DNA. In similar to our finding, a dose-related increase in DNA damage was observed in mouse RAW264.7 macrophages exposed to gliotoxin for 2 h in plain medium in the single cell gel (SCG) electrophoresis assay [14]. Likewise, Golden et al. [26] measured DNA adduct formation after exposure to GTX in HeLa cell line, and found increases in 6-hydro-5,6-dihydroxy-thymidine monophosphate, 8-hydroxy-2'-deoxy guanidine monophosphate, deoxynucleotide diphosphate and other as yet unidentified adducts. The genotoxicity of GTX was also assessed in bacterial test systems including bacterial repair assay and reported to be positive [14]. On the contrary, GTX was found to be non-genotoxic in the in the Salmonella test and SOS-chromotest. GTX did not induce SCEs in Chinese hamster ovarian (CHO) cells after the 4 h pulse treatment with rat liver S9-mix [14]. However, previous observation made by electron micrographs demostrated that the condensation of chromatin on macrophages treated with GTX [12]. DNA-reactivity of GTX has also been examined in some in vitro studies, which demonstrated that the reactive oxygen species (ROS) produced by GTX were capable of evoking DNA damage [26-28]. Otherwise, some authors have suggested that ROS may be implicated in the production of high basal SCE frequencies in chromosome-instability syndromes [16,29,30].

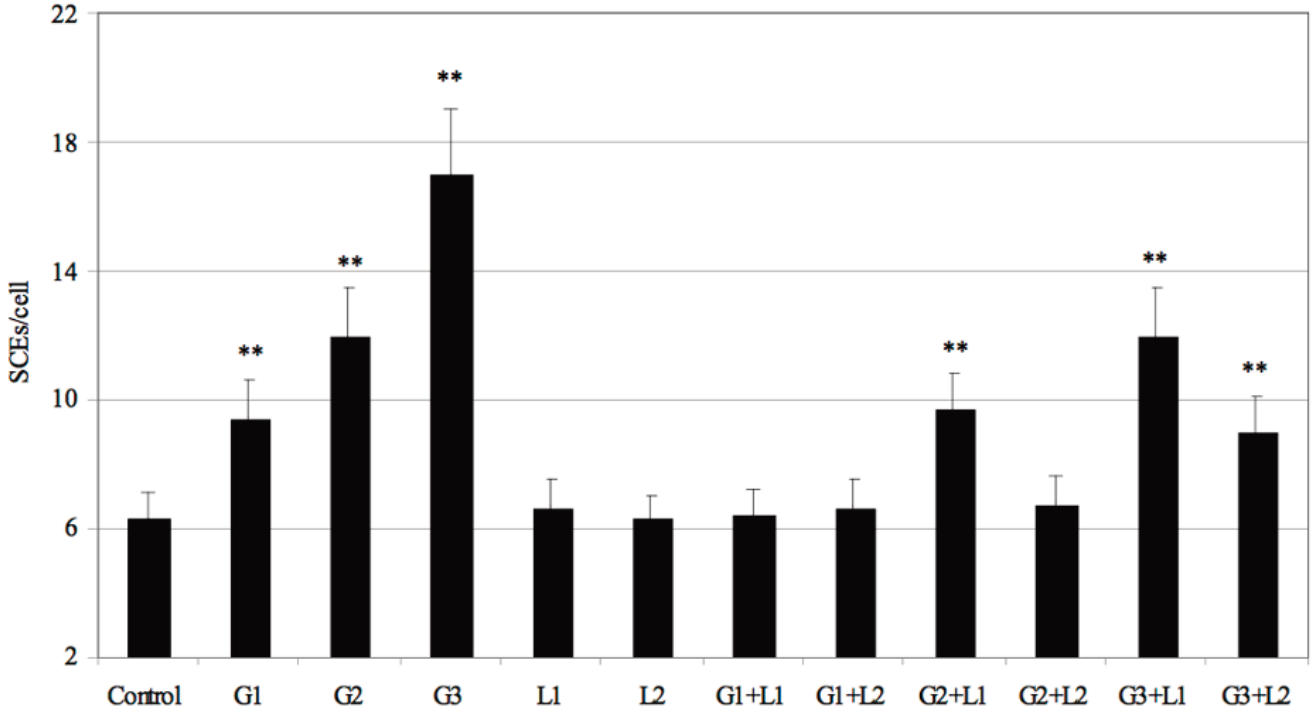

Figure 1. The SCE frequencies after exposure to GTX and lichen extracts on human whole blood cultures for 72 h.

G1=50 ng/mL of GTX, G2=75 ng/mL of GTX, G3=1000 ng/mL of GTX, L1= 5 µg/mL of *P. furfuracea*, L2= 10 µg/mL of *P. furfuracea*, ** means significant differences from the control group at the level of 5%; values are means ± standard deviation.

Thus, the increases of SCE rates after GTX exposure could be explained by possible pro-oxidant effect of this mycotoxin.

The results of the present study revealed that treatment with *P. furfuracea* extracts provide anti-genotoxic effects against GTX at different concentrations. There is considerable evidence that the lichen presents positive effects with increasing concentrations without leading to any genetic damage on human blood cells. It was established that *P. furfuracea* extracts alone were non-genotoxic. Moreover, the treatment with *P. furfuracea* water extracts significantly decreased the ratios of the SCEs when the values were comparable with that of the GTX-treated groups. The biologically fundamental macromolecules such as nucleic acids and proteins in mammalian cells defense themselves with antioxidants [31]. At this context, *P. furfuracea* could support the antioxidant defense mechanism against GTX. As a matter of fact, *P. furfuracea* was found to have significant antioxidant activity [32]. Previous studies demostrated presence of atranorin, chloroatranorin, physodic acid, tetrahydroxy fatty acids, ergosterol, fungisterol, erythritol, arabitol, lichenin, mannitol, folic acid-, folinic acid-, and vitamin B_{12}-group factors in this lichen [33]. Atranorin, physodic acid, oxyphysodic acid and virensic acid are the components of *P. furfuracea* [34]. Many of these compounds are poly-phenolic substances, which are known to have more or less antioxidant activity. Likewise, it was reported that N-acetylcysteine, a well-known antioxidant, completely abolished the GTX-induced cytotoxicity and ROS [35]. Thus, the results of this study may be, at least in part, attributed to antioxidant activity of the lichen extracts, as GTX is known to induce mutagenicity through oxidative stress.

In conclusion, the findings of this research clearly indicated that *P. furfuracea* modulated GTX-induced genetic damage in human blood cultures due to its antioxidant and detoxifying nature. So, the lichens can be a new resource of therapeutics as recognized in this study against oxidative DNA damages.

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