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The Protective Effects of Some Phenylethanoid Glycosides on the Mitomycin C Induced DNA Strand Breakage

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Introduction

A growing number of scientific concern is focusing on the significance of natural compounds that can act as protectants against diseases. Phenolic phytochemicals are a large group of substances ubiquitous in plants and found in significant quantities in vegetables, fruits and beverages such as teas. They have a number of biological effects *in vivo* and *in vitro* and have been regarded as possible antioxidant, anti-inflammatory, antiviral and antiallergic agents[1,2]. Some of the phenolic phytochemicals such as flavonoids have been suggested to inhibit oxidative damage and effectively protect cells and tissues against the deleterious effects of reactive species[3,4]. However, the full chemical properties and effects of phenolic phytochemicals have not completely been examined and it is also unclear whether all phenolic phytochemicals have beneficial effects. There is also considerable evidence that some phenolic phytochemicals are mutagenic and clastogenic in both mammalian and bacterial experimental systems[5,6]. Phenylethanoid glycosides are a group of phenolic compounds mainly spread in several botanical families of Tubiflorae (Verbenaceae, Lamiaceae, Scrophulariaceae)[7,8]. Considering the widespread occurrence of phenylethanoids in plants, relatively little has been done on the mutagenic and/or antimutagenic activity of this group of compounds. Further studies are warranted with respect to evaluation of their activity and/or their toxicity.

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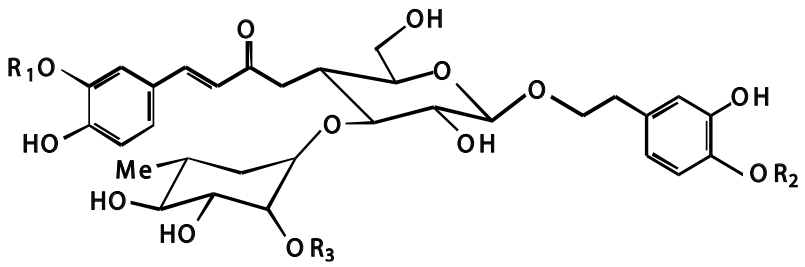
On the other hand, single cell alkaline gel electrophoresis (Comet) assay is a sensitive and rapid technique for the detection of DNA single strand breaks and alkali-labile damage in individual cells. It is considered to be a good predictor of mutagenic and carcinogenic activity of chemicals in animal and human cells. In this technique, a suspension of cells is embedded in agarose on microscope slides, lysed and subjected to electrophoresis, and then stained with a fluorescent DNA binding dye. During alkaline gel electrophoresis, single stranded DNA fragments can migrate out of the nucleus towards the anode and form a comet tail according to the extent of damage[9,10].

Stachys species are rich in flora of Turkey and some of them are known as herbal teas. The preliminary phytochemical studies show that these species contain mainly phenolic compounds especially phenylethanoids and flavonoids. In continuation of our studies on the mutagenic and/or antimutagenic activities of natural products isolated from plants widely grown in Anatolia[11,12,13,14]. *Stachys macrantha* rich in phenylethanoid glycosides was fractionated and investigated at a wide range of different concentrations with and without mitomycin C which is a known mutagenic anticancer drug using comet assay in human lymphocytes.

Materials and Methods

Chemicals: The fraction containing 4 phenylethanoid glycosides were obtained after treating the methanolic extract of *Stachys macrantha* (Labiatae) with n-buthanol (Merck) and applying the buthanolic fraction to column chromatography over polyamide (Fluka) and silica gel 60 (Merck 7734) respectively using appropriate solvent systems. The compounds from the fraction were separated as published formerly[15] and determined as lavandulifolioside, verbascoside, leucosceptoside A and martynoside (Figure 1) by direct chromatographic comparison with authentic samples obtained from the early studies carried out in Pharmacognosy Department of the Faculty[16,17,18]. The other chemicals used in the comet assay were purchased from the following suppliers; normal melting agarose (NMA) and low melting agarose (LMA) from Boehringer Manheim (Germany); sodium chloride (NaCl) and sodium hydroxide (NaOH) from Merck Chemicals (Darmstadt, Germany); dimethyl-sulfoxide (DMSO), ethidium bromide (EtBr); Triton x-100 and phosphate buffered saline (PBS) tablets from Sigma (St. Louis, USA); ethylenediamine tetra acetic acid disodium



	R1	R2	R3
Lavandulifolioside	H	H	Arabinose
Verbascoside	H	H	H
Leucosceptoside A	CH_3	H	H
Martynoside	CH_3	CH_3	H

Figure 1
The Formula of the Isolated Phenylethanoids From Stachys macrantha

salt dihydrate (EDTA), N-lauroyl sarcosinate and Tris from ICN Biochemicals (Aurora, OH, USA).

Comet Assay
Blood Samples and Cell Preparation:

For each experiment 5 ml heparinized (50 units/mol sodium heparin) whole blood was collected by venepuncture from a non-smoking female donor not exposed to radiation or drugs. Lymphocytes were isolated by Ficoll-Hypaque density gradient[19] and washed with PBS. Cell concentrations were adjusted to approximately 2 x10^5 cells/ml in the buffer. The cells were suspended in a total volume of 1 ml and each reaction contained 50µl suspension (≈ 10^4 cells), varying micro liter amounts of the test agent (phenolic phytochemicals) with or without mitomycin C, and PBS buffer in a total volume of 1 ml.

In this study, the fraction containing these 4 phenylethanoid glycosides were investigated for the genotoxic and the protective properties against mitomycin C at the concentrations of 10, 50, 100, 200, 500, 1000 and 2000 µg/ml. The cells were incubated for 0.5 h at 37 ^{0}C in an incubator together with untreated control samples. All test substances were

dissolved in DMSO with a maximum solvent concentration in the culture medium of 1%. Control incubations contained the same concentrations of DMSO. After incubation the lymphocytes were harvested by centrifugation at 800 x g for 3 min at 4 ^{0}C and the cells were suspended in 75 μl low melting point agarose (LMA) for embedding on slides. The replicate experiments were carried out with blood samples from the same donor collected at different time intervals. The cells used in each concentration of the fraction were checked for viability by trypan blue exclusion.

Slide Preparation:

The basic alkaline technique of Singh *et al.*[9], as further described by Anderson *et al.*[20] and Collins *et al.*[21] was followed. Fully frosted microscopic slides (Surgipath, Winnipeg, Wanitoba) were each covered with 110 μl of 0.5% NMA at about 45^{0}C in Ca^{2+} and Mg^{2+} free PBS. They were immediately covered with a large no.1 coverslip and kept at room temperature for about 5 min to allow the agarose to solidify. This layer was used to promote the attachment of the second layer of 0.5% LMA. Approximately 10 000 cells were mixed with 75 μl of 0.5% LMA. After gently removing the coverslip, the cell suspension was rapidly pipetted onto the first agarose layer, spread using a coverslip and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the coverslip, the third layer of 0.5% LMA (75 μl) at 37^{0}C was added, spread using a coverslip, and again allowed to solidify on ice for 5 min. After removal of the coverslip the slides were immersed in cold lysing solution, (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10), with 1% Triton-X 100 and 10% DMSO added just before use for a minimum of 1 h at 4^{0}C.

Electrophoresis:

The slides were removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank side by side, avoiding spaces and with the agarose ends facing each other, nearest the anode. The tank was filled with fresh electrophoresis solution (1mM Na_2EDTA and 300 mM NaOH, pH 13) to a level approximately 0.25 cm above the slides. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali labile damage. Electrophoresis was conducted at a low temperature (4^{0}C) for 20 min using 24

V and adjusting the current to 300 mA by raising or lowering the buffer level and using a compact power supply (Power Pack P 25 Biometra Analytic GmbH). All of these steps were conducted under dimmed light (tank was covered with a black cloth) to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were taken out of the tank. Tris buffer (0.4 M Tris, pH 7.5) was added dropwise and gently to neutralize the excess alkali and the slides were allowed to sit for 5 min. The neutralizing procedure was repeated 3 times.

Staining:

To each slide, 65 μl EtBr (20 μg/ml) was added. The slides were covered with a cover slip, placed in a humidified airtight container to prevent drying of the gel, and analysed within 3-4 h.

Slide Scoring:

For visualization of DNA damage, slides were examined at an x 1000 magnification using a 100 x objective (oil immersion) on a fluorescence microscope (Zeiss, Germany). Images of 200 randomly selected lymphocytes, i.e. 100 cells from each of two replicate slides, were analyzed from each sample and the DNA damage was scored visually as described by Collins *et al.*[21]. Breaks in the DNA molecule disturbed its complex supercoiling, allowing liberated DNA to migrate towards the anode. Staining showed the DNA as 'comets' with a briefly fluorescent head and a tail streaming away from it. Randomly selected lymphocytes were graded visually for each slide. A total damage score for the slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and summing over all grades giving a maximum possible score of 300, corresponding to 100 cells of grade 3 i.e. grade 0 (no damage), grade 1 (low damage), grade 2 (medium damage), grade 3 (high damage).

Statistical Analysis:

The SPSS for Windows 10.0 computer programme was used for statistical analysis. Statistical comparison of the results from controls, mitomycin C and phenylethanoids groups were carried out by one-way analysis of variance (ANOVA) test and post hoc analysis of group differences was performed by LSD test. Results are expressed as mean ± SD.

Results and Discussion

The effects of phenylethanoid glycosides on DNA strand breakage in human lymphocytes with and without mitomycin C is shown in Table I. According to the data obtained from 3 separate experiments no additional DNA strand breakage in human lymphocytes was seen at the

TABLE I

The Number of Damaged Cells Exposed to Phenylethanoids (PE) and Phenylethanoids (PE) + Mitomycin C (MMC)

Treatment-Concentration	**Number of Cells** [a]			
	Undamaged	**Low Damaged**	**Damaged**	**High Damaged**
Negative Control (1 % DMSO)	80	10	7	3
MMC (0.1 µg/ml)	52 [c]	15 [c]	18 [c]	15 [c]
PE 10 µg/ml	83 [f]	8 [f]	7 [f]	3 [f]
PE 50 µg/ml	89 [f]	6 [f]	3 [f]	2 [f]
PE 100 µg/ml	77 [e]	10 [e]	7 [e]	6 [e]
PE 200 µg/ml	72 [d]	13 [d]	8 [d]	7 [d]
PE 500 µg/ml	29 [c,f]	9 [c,f]	17 [c,f]	45 [c,f]
PE 1000 µg/ml	21 [c,f]	14 [c,f]	24 [c,f]	41 [c,f]
PE 2000 µg/ml	28 [c,f]	12 [c,f]	19 [c,f]	41 [c,f]
PE 10 µg/ml + MMC	80 [f]	9 [f]	6 [f]	5 [f]
PE 50 µg/ml + MMC	81 [f]	11 [f]	5 [f]	3 [f]
PE 100 µg/ml +MMC	78 [e]	10 [e]	8 [e]	5 [e]
PE 200 µg/ml + MMC	68 [d]	17 [d]	7 [d]	8 [d]
PE 500 µg/ml +MMC	38 [c,d]	12 [c,d]	19 [c,d]	31 [c,d]
PE 1000 µg/ml +MMC	40 [c]	12 [c]	19 [c]	29 [c]
PE 2000 µg/ml + MMC	21 [c,f]	9 [c,f]	27 [c,f]	43 [c,f]

* Values are the mean of 3 separate studies

[a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ compared with the negative control (1% DMSO) or

[d]$p<0.05$; [e]$p<0.01$; [f]$p<0.001$ compared with the Mitomycin C (MMC)

phenylethanoid concentrations of 10, 50, 100 and 200 μg/ml but at the concentrations of 500, 1000, and 2000 μg/ml a significant increase in DNA damage was observed ($p<0.001$) when compared with negative control and mitomycin C. The strand breakages in human lymphocytes at the highest concentrations of phenylethanoid glycosides were even higher than the mitomycin C (0.01 μg/ml) induced-DNA damage. The numbers of damaged cells were significantly reduced when lymphocytes were incubated with mitomycin C and phenylethanoids ($p<0.01$) and as seen in Figure 2 it seemed that phenylethanoid glycosides protected lymphocytes in a dose-dependent manner from the mutagenic effects of mitomycin C. Since at lower concentrations (i.e. 10, 50, 100 and 200 μg/ml) a significant reduction in the DNA damage was seen, however at highest concentrations no protective effect was observed.

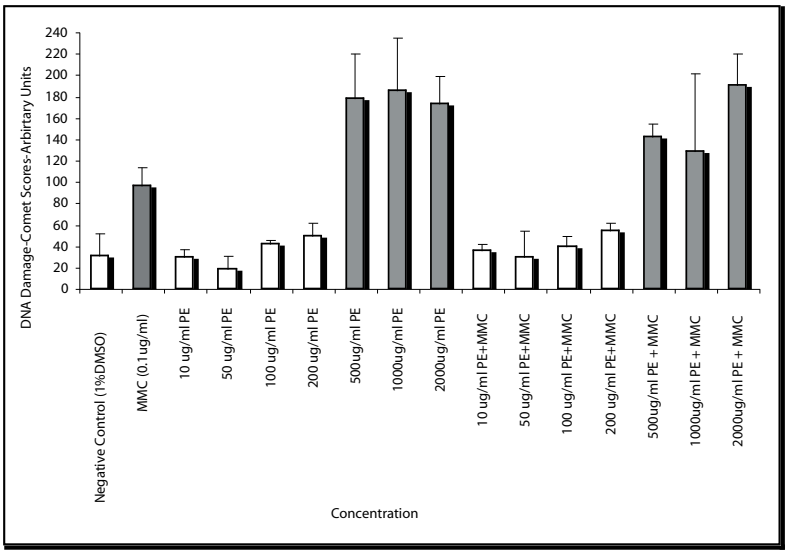


Figure 2
Effects of Phenylethanoids (PE) and Phenylathanoids (PE)+ Mitomycin C on DNA in Human Lymphocytes in the Comet Assay

Phenolic phytochemicals, which are present in many edible plants and medicinal herbs, have become an important issue since they are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against free oxygen radicals. Due to their diverse chemical structures phenolic phytochemicals likely possess different degrees of activity or toxicity. Most of the studies dealing with

phenolic phytochemicals have focused on flavonoids. It has also been reported that flavonoids show cancer preventive effects in several animal models[22,23]. Although the mechanisms for their chemopreventive effects have not been fully understood several studies suggest that flavonoids may act as antioxidants, free radical scavengers, or radioprotectors[24,25,26]. On the other hand certain flavonoids have been shown to be both mutagenic and clastogenic in short-term in vitro systems and flavonoids have been found to be capable of inducing strand breaks in human DNA and inhibiting normal cellular proliferation[27]. But it was also found that pretreating HepG2 liver cells and human peripheral lymphocytes with low doses of quercetin inhibited H_2O_2 and ter-butylhydroperoxide induced oxidative DNA damage[4,28]. The data on the genotoxicity of the flavonoids are incomplete and conflicting results have been obtained with respect to mutagenicity observed with mammalian cell tests[29,30]. The mechanism of genotoxicity of mitomycin C is apparently rather different including the generation of reactive oxygen species. Mitomycin C is a cross linker, but its genotoxicity can also be explained by other mechanisms, including one-electron reduction to a semiquinone free radical in which some reductases are involved. This reduction pathway is dependent on the presence of oxygen, with production of superoxide anion, hydrogen peroxide and hydroxyl radicals[31,32,33]. Our results are in agreement with the findings of Olivera *et al.*[34], who have recently found a significant decrease by quercetin in mitomycin C-induced frequency of micronuclei, which is also good biomarker of genotoxicity, in human peripheral blood lymphocytes. The results of the present study also suggest that phenylethanoid glycosides may protect tissues and cells from oxidative DNA damage.

Although the results concern only in vitro experiments with human cells which means additional animal studies should be performed but also it can be recommended that cancer patients as a part of their chemotherapy should eat a balanced diet with plant components containing phenolic phytochemicals that might promote antimutagenic effects against any mutagens/carcinogens. Herbal medicines and/or plants containing phenolic phytochemicals such as phenylethanoid glycosides have seemed to protect cells from DNA damage and reduce the side effects of chemicals or drugs such as mitomycin C.

In conclusion, the combination of mitomycin C with low doses of phenylethanoid glycosides can be used in cancer therapy in order to avoid the unwanted DNA damaging properties of this mutagenic anticancer drug, but further studies with other cells and tissues of humans are needed in order to give a definite conclusion.

Summary

Phenylethanoid glycosides represent a group of natural products, which have been reported to have various biological activities. In this study the mutagenic and/or antimutagenic activities of lavandulifolioside, verbascoside, leucosceptoside A and martynoside isolated from *Stachys macrantha* were investigated in human lymphocytes by single cell gel electrophoresis or 'comet' assay. The lymphocytes incubated with different concentrations of phenylethanoid glycosides with or without mitomycin C and DNA strand breakage was measured by comet assay. At the phenylethanoid glycoside concentrations of 10, 50, 100, and 200 μg/ml no additional DNA damage was observed whereas the frequency of damaged cells induced by mitomycin C was significantly decreased by phenylethanoid glycoside treatment. However at the highest concentrations above 200 μg/ml no protective effect was observed. It seemed that in human lymphocytes phenylethanoid glycosides showed protective effects on DNA damage induced by mitomycin C in a concentration dependent manner.

Key Words: phenylethanoid glycosides, lavandulifolioside, verbascoside, leucosceptoside A, martynoside, mitomycin C, single cell gel electrophoresis

Özet

Bazı Feniletanoid Glikozitlerin Mitomisin C ile İndüklenen DNA Zincir Kırıklarına Koruyucu Etkisi

Feniletanoit glikozitler, çeşitli biyolojik aktivitelere sahip olduğu bildirilen doğal bileşiklerdir. Bu çalışmada *Stachys macrantha*'dan izole edilen lavandulifoliozit, verbaskozit, leukoseptozit A and martinozit'in insan lenfositlerinde mutajenik ve/veya antimutajenik aktiviteleri tek hücre jel elektroforez veya "comet" yöntemi ile incelendi. Mitomisin C varlığında veya yokluğunda lenfositler feniletanoit glikozitlerin farklı konsantrasyonları ile inkübe edildi ve DNA zincir kırıkları comet yöntemi ile ölçüldü. Feniletanoit glikozitlerin 10, 50, 100 ve 200 μg/ml konsantrasyonlarında artan DNA hasarı gözlenmemiştir, oysa mitomisin C ile indüklenen hasarlı hücre sıklığı feniletanoit glikozitlerin düşük konsantrasyonları ile muamelesinde anlamlı olarak azalmıştır. Ancak 200 μg/ml konsantrasyonların yukarısında koruyucu etki gözlenmemiştir.

İnsan lenfositlerinde feniletanoit glikozitlerin mitomisin C ile indüklenen DNA hasarına karşı konsantrasyona bağımlı olarak koruyucu etki gösterdiği bulunmuştur.

Anahtar Kelimeler: feniletanoit glikozitler, lavandulifoliozit, verbaskozit, leukoseptozit A, martinozit, mitomisin C, tek hücre jel elektroforez

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