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Investigation of Cytotoxic and Genotoxic Potential of *Cinnamomum Cassia* Bark Water Extract

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ÖZET

Cinnamomum cassia kabuğu su ekstresinin sitotoksik ve genotoksik potansiyelinin araştırılması

Amaç: Son zamanlarda, tarçın ile ilgili çalışmalar zengin polifenol içeriğine bağlı antioksidan aktivitesi üzerinde yoğunlaşmıştır. Polifenollerin aynı zamanda pro-oksidan olarak hareket ettikleri ve DNA'da oksidatif zincir kırıklarına neden oldukları bildirilmiştir. Bu çalışmada *Cinnamomum cassia* su ekstresinin insan periferal kan lenfositlerindeki sitotoksik ve genotoksik potansiyelini araştırdık.

Yöntemler: *Cinnamomum cassia* su ekstresi, toz haline getirilmiş tarçın kabuğunun ultra saf suda 72 saat maserasyonu ile hazırlandı. Ekstrenin lenfositlerdeki sitotoksik etkisi WST-1 yöntemi ile araştırıldı. Ekstrenin DNA'da hasar oluşturucu potansiyelini değerlendirmek için alkali Komet yöntemi uygulandı. DNA hasarı kuyruk DNA yüzdesi ve kuyruk momenti şeklinde ifade edildi.

Bulgular: Lenfositlerin canlılığı ekstre ile 24 saat muamele sonrası konsantrasyona bağımlı olarak azaldı. Ekstre ≥400 µg/ml konsantrasyonlarda negatif kontrole göre anlamlı düzeyde DNA hasarına neden oldu.

Sonuç: Çalışma sonuçlarımız *Cinnamomum cassia* kabuğu su ekstresinin *in vitro* olarak sitotoksik ve genotoksik etki potansiyelini göstermektedir. Ekstrenin özellikle yüksek dozlarda ya da uzun süreli kullanımında güvenliliği açısından bu bulgu önemli görünmektedir ve bu nedenle *in vivo* çalışmalarla aydınlatılmalıdır.

Anahtar sözcükler: *Cinnamomum cassia*, alkali Komet yöntemi, sitotoksisite, pro-oksidan aktivite

ABSTRACT

Investigation of cytotoxic and genotoxic potential of *cinnamomum cassia* bark water extract

Objective: Recently many investigations on cinnamon have focused on its powerful antioxidant activity due to its rich polyphenol content. Polyphenols have also been reported to act as pro-oxidants, causing oxidative strand breaks in DNA. In the present study, we investigated the cytotoxic and genotoxic potential of *Cinnamomum cassia* water extract in human peripheral blood lymphocytes.

Methods: *Cinnamomum cassia* water extract was prepared from grounded bark of cinnamon by maceration with ultrapure water for 72 h. Cytotoxicity of the extract on lymphocytes was determined by WST-1 assay. Alkaline Comet assay was conducted to evaluate the DNA damaging potential of the extract. DNA damage was expressed as DNA percentage in the tail and tail moment.

Results: The viability of lymphocytes was decreased by treatment with the extract for 24 h, as a concentration-dependent manner. At the concentrations ≥400 µg/ml, the extract induced significant DNA damage compared to negative control.

Conclusion: Our results show the cytotoxic and genotoxic potential of *Cinnamomum cassia* bark water extract *in vitro*. This finding seems a significant safety concern, particularly in high doses or long term use of the extract, and therefore needs to be clarified by *in vivo* studies.

Key words: *Cinnamomum cassia*, alkaline Comet assay, cytotoxicity, pro-oxidant activity

INTRODUCTION

Oxidative stress has been implicated in onset and development of several chronic diseases. Therefore, there is a growing interest on herbs as natural antioxidants sources to prevent the development of diseases, by decreasing the oxidative stress caused by reactive oxygen species. Cinnamon has extensively been used as a source of traditional remedies for thousands of years (1,2). Recently, diverse biological activities of cinnamon species including

antidiabetic and antitumor activities have been shown by several *in vivo* and *in vitro* studies (3-8). *Cinnamomum cassia* (*C. cassia*), also known as Chinese cinnamon, is one of the major cinnamon species. The less expensive and the most common cinnamon variety sold in the United States and European countries is *C. cassia* and it has been reported as the only one which has a significant effect on glycemic control (9,10). Most of the activities of *C. cassia* were suggested to be involved in antioxidant activities of polyphenols which are the bioactive components of cinnamon water extract (5,6,11-13). Phytochemical studies have been well conducted on *C. cassia* revealed the presence of water-soluble polyphenols consisting of flavonoids, mainly procyanidins and phenolic compounds. Specific antioxidants that have been identified in cinnamon include epicatechin, camphene, eugenol, γ-terpinene, phenol, and tannins (14). These compounds have been shown to protect cells against oxidative damage and function as antioxidants, potentiate insulin action, improve glucose, insulin, and lipid metabolisms and improve inflammation (14-17). On the other hand, polyphenols including flavonoids have been shown to act as pro-oxidant under the conditions that favor their autoxidation, causing damage to DNA, protein, lipids, and subsequent cell death (15). Some polyphenols have been reported to have genotoxic or carcinogenic effects at high concentrations (18-20).

According to our knowledge, there is no *in vivo/in vitro* study conducted to determine pro-oxidant potential of *C. cassia* bark water extract on healthy cells. Furthermore, adverse effects of cinnamon have been poorly documented, as most of the researches focused on safety and efficacy of cinnamon and its polyphenols (18-20). Starting from this viewpoint, in this study we aimed to investigate the cytotoxic and DNA damaging potential of *C. cassia* bark water extract in human peripheral blood lymphocytes.

MATERIALS AND METHODS

Chemicals and Reagents

A commercial product of *C. cassia* bark was used in the study. The product was authenticated by Prof. Dr. Bijen Kıvçak from Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir. All chemicals used were of analytical grade. The chemicals used in the experiments were purchased from the following suppliers: Normal melting point agarose (NMA) and low melting point agarose (LMA), ethidium bromide (EtBr), Triton X-100, phosphate buffered saline (PBS) tablets, ethylenediaminetetraacetic acid (EDTA) disodium, Tris, anhydrous sodium carbonate, Histopaque, methanol from Sigma-Aldrich (St. Louis, USA); dimethylsulfoxide (DMSO), sodium chloride and sodium hydroxide from Merck Chemicals (Darmstadt, Germany); WST–1 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) from Roche (Switzerland); RPMI-1640, penicillin/streptomycin, L-glutamine and fetal bovine serum (FBS) from Biological Industries (Israel).

Preparation of *Cinnamomum Cassia* Bark Water Extract

Cinnamon bark was ground into a fine powder and kept airtight in cool, dry and dark conditions. For preparation of the extract, cinnamon powder was macerated with distilled water at 40°C by continuous stirring. After 72 h, the extract was centrifuged at 10,000 rpm for 5 min. The supernatant was filtered using filter paper (Whatman no: 4). The filtrate was concentrated under reduced pressure using a rotary evaporator, and finally freeze-dried (Labconco/Freezone 6, Kansas City, MO). The extract was kept at +4°C, and protected from sun light throughout the study.

Preparation of Lymphocytes

In the present study, human peripheral blood lymphocytes were used as they are primary, non-invasive cells as representative of the actual body state. Peripheral venous blood samples from healthy male donors were drawn into heparinized tubes and protected from light. Lymphocytes were separated by density centrifugation over a layer of Histopaque and washed in PBS. After centrifugation, the supernatant was removed carefully without disturbing the pellet. The pellet was resuspended by adding one ml of PBS. Cell viability was performed using trypan blue dye exclusion technique.

This study was approved by Ege University, Faculty of Medicine, Clinical Research Ethical Committee, Izmir, Turkey (18.06.2009, 09-5.1/14) and performed in accordance with Declaration of Helsinki. Informed donor consent was also obtained.

Cell Counts and Viability

Cell counts were determined with a Thoma cell counting chamber. Cell viability was assessed by trypan blue dye exclusion method (21). Trypan Blue dye (0.4%) was added to lymphocytes in a ratio of 1:1 and examined under the light microscope (Olympus, UK) in 3-5 min. Trypan blue penetrates the damaged membrane of dead cells and stains the nucleus. The number of viable and dead cells was counted using a hemocytometer chamber. The experiments were run in triplicate.

Cytotoxic effect of the extract was determined by WST-1 assay, according to the manufacturer's protocol. WST-1 is a water soluble tetrazolium salt. The assay principle is based on the reduction of WST-1 to dark yellow colored formazan by cellular dehydrogenases, which directly correlates to the cell number. Lymphocytes were suspended in RPMI-1640 supplemented with 10% FBS and 1% (v/v) penicillin-streptomycin, and plated in 96-well plates at a concentration of $1x10^5$ cells/well. Cells were incubated with different concentrations (25-1000 µg/ml) of the extract in a humidified CO_2 incubator (Nuaire 5510E, USA) at 37°C for 24 h. Then, WST-1 reagent (10 µl/well) was added to each well. After 4 h incubation at 37°C, the absorbance was read at 450 nm using a microplate reader (Thermo Scientific, Rockford, USA), and expressed as percentages of the untreated control (control was considered as 100%). Results were analyzed using GraphPad Prism 5.0 software.

Alkaline Comet Assay

The alkaline Comet assay was performed on the day of sampling according to the methods described by Collins et al. and Singh et al. with some modifications (22-23). Isolated lymphocytes, suspended in one ml of PBS, were incubated with different concentrations of the extract for 60 min at 37°C. A negative control (PBS) and a positive control (100 µM H_2O_2) samples were also included.

Briefly, treated lymphocytes were suspended in 0.65% (w/v) LMA at 37°C and rapidly pipetted onto the microscope slides pre-coated with a layer of 1.5% (w/v) NMA. The slides were covered with coverslips and agarose layer was allowed to solidify at +4°C for 5 min. After removal of the coverslips, the slides were immersed into cold, freshly made lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris; pH 10; 1% Triton X-100, and 10% DMSO were added just before use) for 1 h at +4°C. Then, the slides were removed from the lysing solution and placed in a horizontal gel electrophoresis tank (Cleaver Scientific, Model CSL-COM20, UK) filled with fresh electrophoresis buffer (300 mM NaOH, 1mM EDTA; pH 13). The slides were left for 20 min at +4°C, and then electrophoresed for 20 min at 20 V (1 V/cm) and 300 mA at +4°C, using a compact power supply (Biorad Power PacTM Basic, Singapore). After neutralization in buffer (0.4 M Tris HCl; pH 7.5), the slides were rinsed, dried and fixed in cold methanol for 5 min. The dried slides were stained with EtBr (20 µg/ml in distilled water). DNA damage was evaluated using a fluorescent microscope (BAB image analyzing systems Bs200ProP, Turkey). One-hundred cells from two replicate slides were analyzed for each experiment. Experiment was run in triplicate. DNA damage was scored by using Comet score 15 image analysis program (Tritek Corp., USA) and expressed as DNA percentage in the tail because it is linearly related to DNA break frequency over a wide range of damage; the results are also presented as tail moment for comparison (24).

All steps of Comet assay were carried out under dimmed light to avoid induction of additional DNA damage.

Statistical Analysis

The statistical analysis was carried out using SPSS for Windows software, version 15.0. Differences between the means of data were compared by the one-way analysis of variance (ANOVA) test. Differences at $p<0.05$ were considered as significant.

RESULTS

Extraction Efficiency

The extraction yield of the *C. cassia* bark water extract was calculated as 7.27% by the following formula:

Extraction yield (%) = Weight of the freeze-dried extract/ weight of the sample x 100

Cell Viability

The viability of isolated lymphocytes was checked before experiments by trypan blue dye exclusion test and

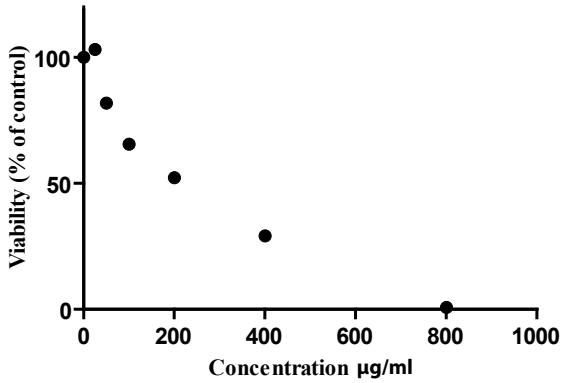

Figure 1: Effects of *C. cassia* water extract on viability of human peripheral lymphocytes. Cell viability was plotted as percentage of control. Each value represents the mean ± standard deviation (SD) of three independent experiments.

always found as >95%. In WST-1 assay, the viability of lymphocytes was decreased by treatment with the extract for 24 h, as a concentration-dependent manner (Figure 1). Concentration required to reduce 50% (IC_{50}) of that in the control wells was calculated as 253.2 µg/ml.

Alkaline Comet Assay

Figure 2 shows the DNA damaging effects of different concentrations of *C. cassia* extract on lymphocytes. One-hundred µM H_2O_2 was used as the positive control of DNA damage. The extract caused significant increase in DNA damage compared to negative control at ≥400 µg/ml concentrations (Figure 2). The DNA damage at the highest concentration of the extract was not significantly different from the damage induced by 100 µM H_2O_2.

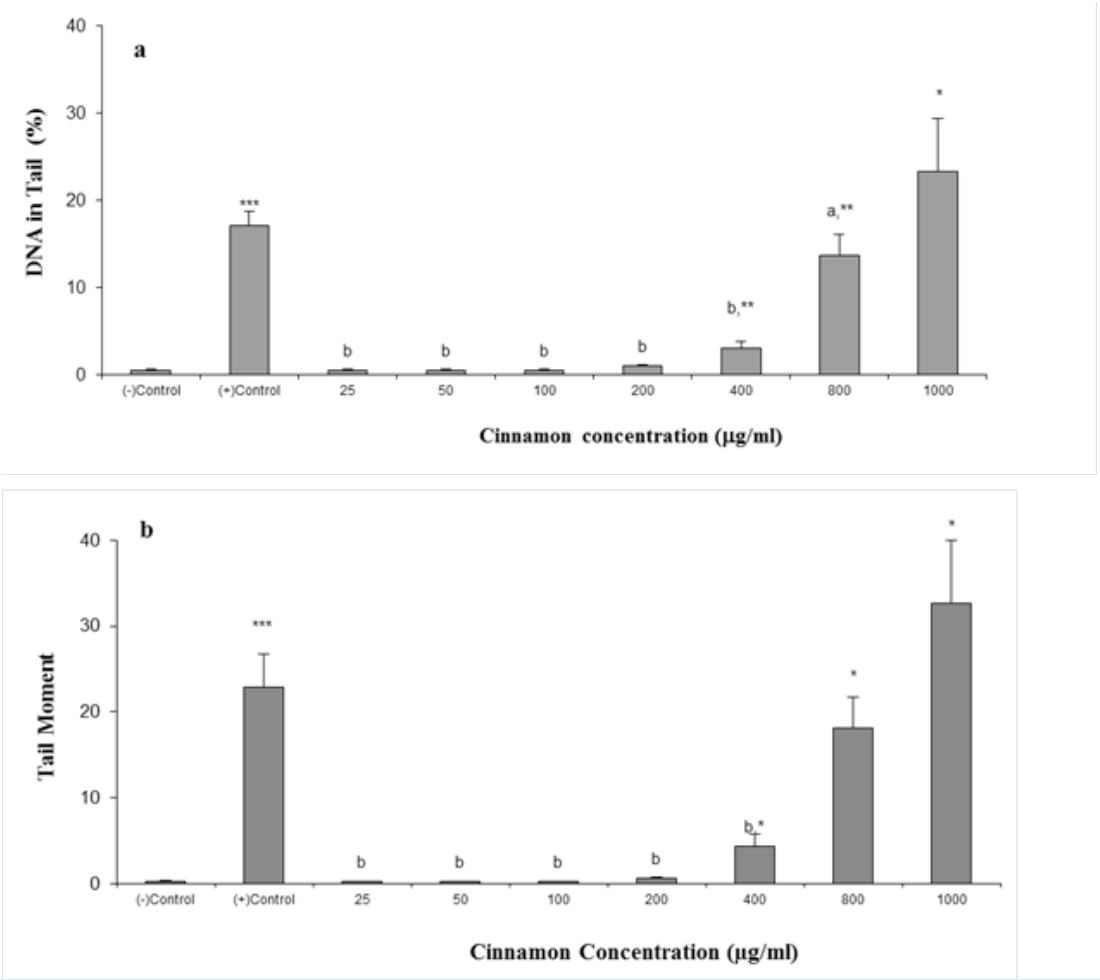

Figure 2: DNA damages expressed as DNA percentage in tail (a) and tail moment (b) in lymphocytes treated with *C. cassia* water extract. *p<0.01, **p<0.005, ***p< 0.0005, significantly different from negative control (PBS). [a]p<0.05, [b]p<0.0005, significantly different from positive control (100 µM H_2O_2). Each bar represents the mean ± SD of three independent experiments.

DISCUSSION

C. cassia bark water extract has been used as natural antioxidant supplement due to high polyphenols content (5,6,11-13). It has been reported that polyphenols may act as pro-oxidants under certain circumstances such as high doses (25). As a result of pro-oxidant activity, there is a possibility for damaging biomolecules such as DNA, proteins and lipids, and outcome of cellular death (15). Yen et al. reported that the phenolic compounds were highly cytotoxic and capable of inducing DNA damage (26), which is a major primary cause of cancer.

In another study, we evaluated the antioxidant activity of *C. cassia* water extract by determining the total phenol and flavonoid contents, and DPPH (2,2-diphenyl-b-picrylhydrazyl) scavenging activity (33). In the present study, we investigated the cytotoxic and genotoxic potential of the same extract. For this purpose human peripheral blood lymphocytes were used as they are primary, non-invasive cells representative of actual body state.

A sensitive and accurate test for evaluation of cytotoxicity, WST-1 assay was used for determining reduced viability of human primary lymphocytes. The principal advantage of WST-1 over XTT and MTT tests is the production of water-soluble reduced product, which can be measured without an additional solubilization step (27). After incubation of cells with different concentrations of extract for 24 h, IC_{50} value was found as 253.2 µg/ml. Koppikar et al determined the viability of human cervical cancer cells exposed to 10-320 µg/ml of aqueous *C. cassia* extract by MTT dye uptake and found that cells exhibited 100% survival within 24 h at those concentrations (28). Kwon et al. investigated the effect of cinnamon water extract (0.5 mg/ml) on cell viability of mouse primary lymphocytes and did not find any decrease after 48 h and 72 h (29).

Alkaline Comet assay is a widely-used, rapid, and sensitive technique for the measurement of DNA damage both *in vitro* and *in vivo* (30-32). In the present study, we applied this technique for the evaluation of DNA damaging potential of the extract. The extract caused significant increase in DNA damage at ≥400 µg/ml concentrations as compared to negative control. Furthermore, the DNA damage at the highest concentration of the extract was not significantly different from the damage induced by 100 µM H_2O_2. In our another study, we found that pretreatment of human peripheral blood lymphocytes with lower concentrations of *C. cassia* water extract protected the cells against H_2O_2-induced oxidative DNA damage (in press). Yen et al. reported the DNA damage with increasing concentrations of flavonoids, which was attributed to their stimulation of oxidative stress (26). This pro-oxidant action has been suggested to play an important role in the prevention of certain types of cancer (34). Although this anticancer effect potential, it seems difficult to predict the consequences of genotoxic potential of cinnamon in normal, healthy cells, *in vivo*. According to our knowledge, there is not any study showing the genotoxic activity of *C. cassia* water extract. On the other hand, Sharma et al. demonstrated antimutagenic potential of *C. cassia* against two mutagens, viz. benzo[a]pyrene and cyclophosphamide (35).

CONCLUSION

Our results suggest the cytotoxic and genotoxic potential of *C. cassia* bark water extract *in vitro*. This finding seems a significant safety concern, particularly in high doses or long term use of the extract. There is a need of further studies determining *in vivo* antioxidant and pro-antioxidant activities of *C. cassia* bark water extract in terms of concentrations and conditions.

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