ORIGINAL RESEARCH

Investigation of the protective effect of *Cinnamomum cassia* bark extract against H_2O_2-induced oxidative DNA damage in human peripheral blood lymphocytes and antioxidant activity

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ABSTRACT: Cinnamon, one of the most widely used spices in the world, has been shown to have various biological functions including antidiabetic and antitumor activities. Its antidiabetic and antitumor effects were linked with its strong antioxidant activity. In the present study we aimed to investigate the antioxidant activity and possible protective effect of *Cinnamomum cassia* bark water extract against H_2O_2-induced oxidative DNA damage.

Viability of lymphocytes was determined by Trypan Blue test. For the evaluation of the antioxidant activity, total phenol and flavonoid contents and 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibitory activity of the extract were determined. DNA damage was determined by alkaline comet assay in human peripheral blood lymphocytes.

Lymphocytes exhibited >86 % survival up till the concentration of 800 μg/ml of the extract. Total phenol and flavonoid contents were calculated as 10.6 g ± 0.001 gallic acid equivalents/100 g dry weight and 2.25±0.004 g quercetin equivalents/100 g dry weight of the extract, respectively. The extract concentration providing 50 % inhibition of DPPH was found as 76.68 μg/ml.

***Cinnamomum cassia* bark water extract at ≥100 μg/ml concentrations caused significant protection against H_2O_2-induced oxidative DNA damage in lymphocytes.**

Our results support the suggestions that *Cinnamomum cassia* bark water extract could be beneficial as a prophylactic agent in prevention of oxidative stress-related diseases.

KEYWORDS: *Cinnamomum cassia;* DPPH; total phenol; total flavonoid; alkaline comet assay.

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INTRODUCTION

Living cells have antioxidant defense potential to protect themselves from damages caused by reactive oxygen species (ROS) and there is a balance between production and scavenging of ROS. When free radicals exceed the cellular antioxidant defense, oxidative stress occurs and consequently may cause the oxidative damage to lipids, proteins, and DNA, leading development of chronic diseases (1-8). DNA is probably the most biologically significant target of oxidative attack, and it is widely thought that continuous oxidative damage to DNA is a significant contributor to the development of the cancer (7,8).

The implication of oxidative stress in the etiology and progression of clinical disorders has led to the researchers to investigate the health benefits of natural antioxidants as prophylactic agents (6,9). Several studies have shown that most of the plants including spices have antioxidant potential due to their polyphenol rich contents and that they play protective or therapeutic role in diseases associated with oxidative stress (10-15). *Cinnamomum cassia* (*C. cassia*) is one of the major

cinnamon species and also known as Chinese cinnamon. *In vitro* and *in vivo* studies have reported the diverse biological activities of cinnamon including antiulcerogenic, antiinflammatory, antipyretic, antimicrobial, antioxidant, antidiabetic and antitumor effects (16,17). Both antidiabetic and antitumor activities were suggested to be involved in antioxidant activities of polyphenols which are the bioactive components of cinnamon aqueous extracts (18-25). We aimed to investigate the antioxidant potential of *C. cassia* water extract in conditions associated with oxidative stress, *in vitro*. For this aim we evaluated the antioxidant activity and possible protective effect of the extract against H_2O_2-induced oxidative DNA damage.

MATERIAL AND METHODS

A commercial product of *C. cassia* bark was used in the study and 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, ethanol, DPPH, quercetin, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), resveratrol and Trypan Blue from Sigma-Aldrich (St. Louis, USA); gallic acid, Folin–Ciocalteu phenol reagent, dimethylsulfoxide (DMSO), aluminium chloride, potassium acetate, sodium chloride and sodium hydroxide from Merck Chemicals (Darmstadt, Germany).

Preparation of cinnamon 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 ultrapure water at 40°C stirring continuously. After 72 h, extract centrifuged at 10.000 rpm for 5 min. The supernatant was filtered using Whatman no.4 filter paper. Filtrate was concentrated under reduced pressure using a rotary evaporator, and finally freeze-dried (Labconco/Freezone 6, Kansas City, MO). 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 representative of the actual body state. Peripheral venous blood samples from healthy male donor were drawn into heparinized tubes and were 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. An aliquot of 1 ml of PBS was added and the pellet was re-suspended. 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), performed in accordance with Declaration of Helsinki, and informed donor consent was also obtained.

Cell counts and viability

Cell counts were determined with a Thoma cell counting chamber and adjusted to $2X10^6$ cells/ml. Cell viability was assessed by Trypan Blue dye exclusion method (26). 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 were counted using a hemocytometer chamber. Experiment was run in triplicate.

Total phenol and flavonoid contents

Total phenol content was determined by Folin-Ciocalteu colorimetric method using gallic acid as a standard (27). Briefly, an aliquot (0.2 ml) of the extract or standard solution of gallic acid in ethanol:water (1:10 v/v) was mixed with 1 ml Folin-Ciocalteu reagent (previously diluted with water; 1:10 v/v). After incubation at room temperature for 4 min, 800 ml of saturated sodium carbonate (75 g/l) was added and the absorbance was read at 765 nm with microplate reader after 2 h. The standard calibration curve was obtained with gallic acid (0.3-100 mg/l), and the total phenol content was expressed as g gallic acid equivalents (GAE) per 100 g dried weight (DW) of the extract. Experiments were run in triplicate.

Total flavonoid content was determined according to the method of Chang et al., using quercetin as reference standard (28). Briefly, 0.1 ml of 10 % $AlCl_3$ and 1 M potassium acetate (0.1 ml) were added to 2 ml of cinnamon extract in methanol. Total volume of the mixture was made up to 5 ml with distilled water. After incubation at room temperature for 40 min the absorbance was measured against blank at 415 nm using microplate reader. The standard calibration curve was obtained using quercetin (10-200 μg/ml) and the total flavonoid content was expressed as g quercetin equivalents (QE) per 100 g DW of the extract. Each experiment was run in triplicate.

DPPH radical scavenging activity

Free radical scavenging activity of the extract was evaluated by DPPH method using Trolox as reference standard (29). This method is a widely applied, reliable assay for the evaluation of antioxidant activity. The principle of the assay is the color change of DPPH solution from purple to yellow as the radical is quenched by antioxidants present in the extract (30,31). The degree of discoloration of DPPH indicates the scavenging activity of the antioxidant in terms of hydrogen donating ability. Briefly, 1 ml of 1 mM DPPH in methanol was added to 1 ml of various concentrations of the extract in methanol. The mixture was left for 30 min at room temperature in the dark for transformation of DPPH radical to its reduced form. The decrease in absorbance against blank was read at 517 nm. Each experiment was run as triplicate. The DPPH radical scavenging activity of the extract was calculated according to the following equation (30):

Scavenging activity (%) = $(A_{DPPH}-A_{EXTRACT+DPPH}) / (A_{DPPH} - A_{EXTRACT}) \times 100$ where A_{DPPH} is the absorbance of the control solution containing only DPPH; and $A_{EXTRACT+DPPH}$ is the absorbance of DPPH solution containing extract; and $A_{EXTRACT}$ is the absorbance of only extract solution without DPPH.

Extract concentration providing 50% inhibition (IC_{50}) of DPPH was calculated by a linear regression equation of the dose-inhibition curve obtained by plotting the extract concentrations versus the corresponding radical scavenging activity.

The scavenging activity of Trolox on DPPH was also tested at the same time; linear regression equation was obtained and IC_{50} value of Trolox was calculated. The results were corrected for dilutions.

Alkaline comet assay

The alkaline comet assay was performed on the day of sampling by modification of the methods described by Collins et al. and Singh et al. (32,33).

Lymphocytes suspended in PBS, were incubated with different concentrations of the extract for 60 min at 37ºC. At the end of the incubation, the medium was replaced with cold PBS containing H_2O_2 (100 µM) and then incubated for 5 min at +4°C. A negative control (PBS) and a positive control (100 µM H_2O_2) samples were also included. Resveratrol (50 µM) was used as reference antioxidant standard.

Alkaline comet assay protocol: Treated lymphocytes were suspended in 0.65 % (w/v) LMA at 37°C and were rapidly pipetted onto the microscope slides precoated with a layer of 1.5 % (w/v) NMA. The slides were covered with coverslips and agarose layer was allowed to solidify at +4ºC for 5 min. After removal of the coverslips, slides were immersed into cold, freshly made lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 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 to allow unwinding of DNA, 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). In order to visualize DNA damage, slides were examined at 40X.Two slides per sample, with 50 randomly selected cells per slide, were analyzed for each experiment. Experiment was repeated five times. DNA damage was scored by use of 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 (34).

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

Statistical Analysis

Statistical analysis were carried out using SPSS for Windows software, version 15.0. Differences between the means of data were evaluated using one-way ANOVA. Differences at $p < 0.05$ were considered as significant.

RESULT

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

The effect of the extract on cell viability was shown in Figure 1.

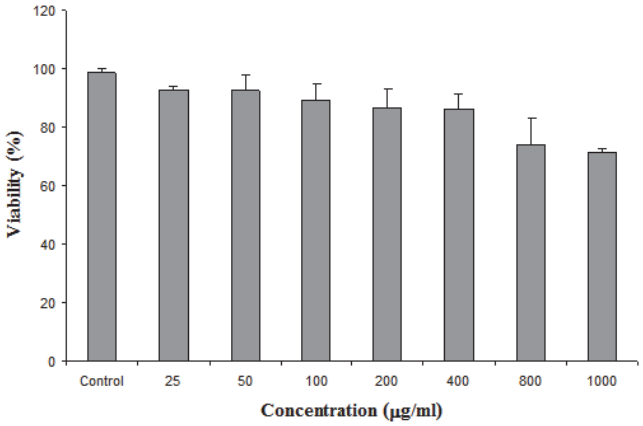

FIGURE 1. The effects of C. cassia extract on cell viability. Each value represents the mean ± standard deviation (SD) of three independent counting.

Gallic acid and quercetin calibration curves are presented in Figure 2. Total phenol and flavonoid contents were calculated as 10.6 g ± 0.001 GAE/100 g DW, and 2.25±0.004 g QE/100 g DW of the extract, respectively. Percentage inhibitions of DPPH by different concentrations of the extract or Trolox were shown in Figure 3. Calibration curves of the extract and Trolox were linear with R^2 values of 0,9925 and 0,9868, respectively. The IC_{50} value of the extract (76.68 µg/ml) was found higher than that of Trolox standard (15.14 µg/ml).

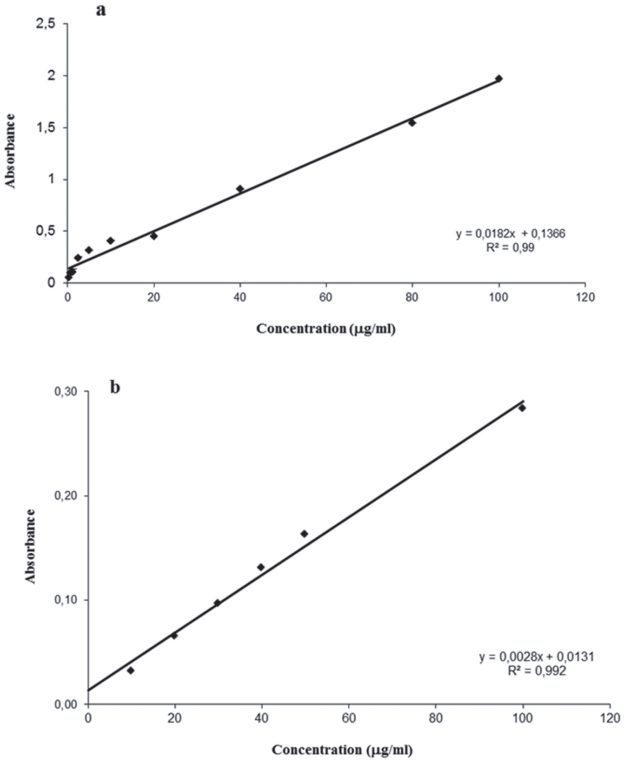

FIGURE 2. The calibration curves of gallic acid (a), and quercetin (b) standards. Each data point represents the mean ± SD of three independent experiments.

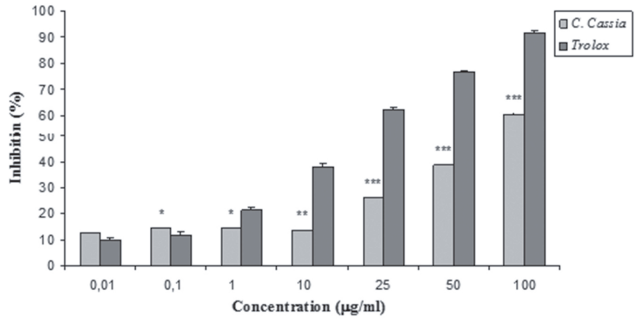

FIGURE 3. Percentage inhibition of DPPH by various concentrations of C. cassia extract and Trolox. *p< 0.05; **p< 0.001; ***p< 0.0005, significantly different from Trolox within each group. Bars represent the means ± SD of three independent experiments.

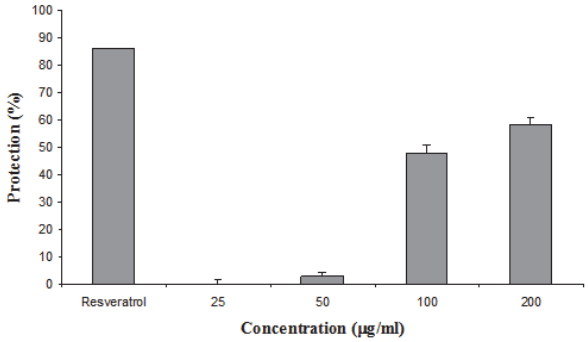

FIGURE 5. The percentage protective effects of the various concentrations of C. cassia water extract and resveratrol (50 μM) against H2O2-induced DNA damage.

The protective effect of various concentrations of the extract on H_2O_2-induced oxidative DNA damage was presented in Figure 4. Regarding our previous studies conducted to determine the proper concentration of H_2O_2 to induce DNA damage, we used 100 µM of H_2O_2 in this study (data are not shown). Percent protection based on the percentage of DNA in tail was calculated by the following formula and presented in Figure 5.

Protection (%) = $(T_{H2O2} - T_{extract+H2O2}) / (T_{H2O2} - T_{control}) \times 100$ where T_{H2O2} = tail DNA (%) induced by H_2O_2, and $T_{extract+H2O2}$= tail DNA (%) of extracts in the presence of H_2O_2, and $T_{control}$ = tail DNA (%) of negative control (PBS). The effect of resveratrol (50 µM) was also presented for comparison.

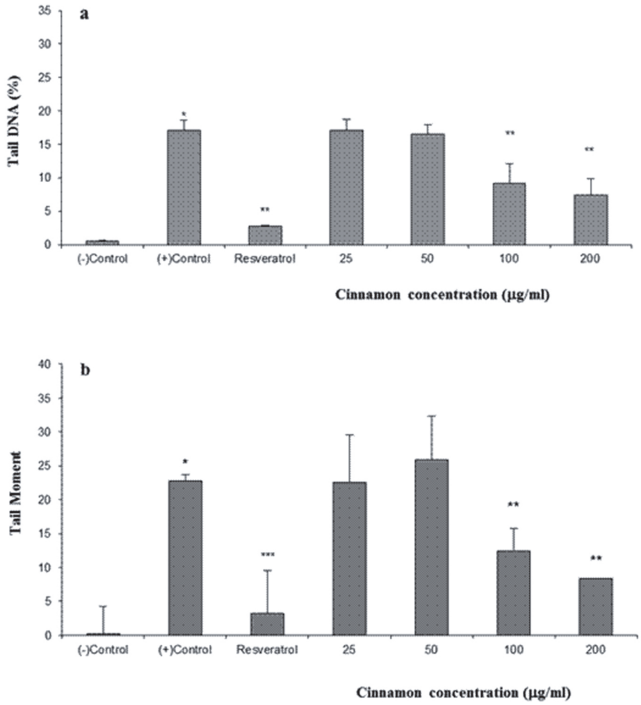

FIGURE 4. Effects of C. cassia extract on H2O2-induced oxidative DNA damage in lymphocytes, expressed as the percentage of DNA in tail (a), and tail moment (b). Each bar represents the mean ± SD of five independent experiments. *p<0.005 statistically difference of positive control (100 μM H2O2) from negative control (PBS). ** p< 0,01; *** p< 0,005 statistically differences of resveratrol (50 μM) and extract from positive control.

DISCUSSION

In this study antioxidant activity of *C. cassia* water extract and protective effect aginst H_2O_2-induced oxidative damage were investigated. In alkaline comet assay, human peripheral blood lymphocytes were used to evaluate oxidative DNA damage as they are primary, non-invasive cells representative of the actual body state. Cell viability was not effected by incubation with cinnamon extract in accordance with other studies (10,35). The cells exhibited >86 % survival up till the concentration of 800 µg/ml.

It has been suggested that polyphenols including flavonoids in spices contribute to their antioxidant capacities (11). In the present study, total phenol and flavonoid contents were calculated as 10.6 g ± 0.001 GAE/100 g DW and 2.25 ± 0.004 g QE/100 g DW of cinnamon, respectively. There are limited numbers of studies about total phenol and flavonoid contents of *C. cassia* bark and results vary considerably among different extracts. The total phenol content of acetone and methanol extracts of *C. cassia* was reported as 9.6 and 7.1 mM GAE/L, respectively (36). Shan et al. found that the total phenol content of *C. cassia* bark methanol extract was 6.34 g GAE/100 g DW (11). Yang et al. reported the total phenol and flavonoid contents of the *C. cassia* bark ethanol extracts as 9.534 ± 0.26 g GAE/100 g DW and 2.030 g QE/100 g DW, and of supercritical fluid extracts as 0.398 ± 0.01 g GAE/100 g DW and 0.031–2.504 g QE/100 g DW, respectively (29).

DPPH method is a widely applied, reliable assay for the evaluation of antioxidant activity. In the present study, *C. cassia* water extract exhibited a significant concentration-dependent inhibition of DPPH activity (R^2=0,9925). This DPPH inhibitory activity was found relatively lower than that of Trolox standard, which might be explained by use of crude cinnamon extract in the study. Kamleshiya et al. observed that the DPPH radical scavenging activity of *C. cassia* water extract was changed between 32.69 to 62.08 % at the concentrations of 50-250 µg/ml (30). Yang et al. found the IC_{50} value of cinnamon bark ethanol extract as 72 µg/ml (29).

The alkali comet assay has become a standard method for the measurement of oxidative DNA damage both *in vitro* and *in vivo* (32,33). In our study, we used H_2O_2 to induce oxidative stress in lymphocytes. Hydrogen peroxide causes DNA strand breakage by generating the hydroxyl radical (OH·) via the Fenton reaction (37). In our another study, *C. cassia* water extract alone was found to cause high DNA damage at ≥ 400 µg/ml concentra-

tions in human peripheral lymphocytes (in press). Therefore 25-200 µg/ml concentrations of the extract were used for the evaluation of its protective effect against oxidative DNA damage in this study. Pretreatment of lymphocytes with the extract for 60 min significantly reduced the DNA damage induced by H_2O_2 at ≥100 µg/ml concentrations. The highest protection was 58.3 % at 200 µg/ml, whereas 47.9 % at 100 µg/ml. These results revealed the protective potential of C. *cassia* water extract against to oxidative DNA damage. The high total phenol and flavonoid contents and DPPH inhibitory activity of the extract determined in this study also supported this finding.

CONCLUSION

Regarding the results of this study, we concluded that *Cinnamomum cassia* bark water extract is a potential natural antioxidant source and it could be beneficial as a prophylactic agent in prevention of oxidative stress-related conditions. Analysis of major polyphenol components of the extract and further *in vivo* studies would be complementary.

Cinnamomum cassia kabuğu ekstresinin insan periferik kan lenfositlerinde H2O2 ile oluşturulan oksidan DNA hasarına karşı koruyucu etkilerinin ve antioksidan aktivitesinin araştırılması

ÖZET: *Cinnamomum cassia* Kabuğu Su Ekstresinin İnsan Periferal Lenfositlerinde H_2O_2 ile İndüklenen Oksidatif DNA Hasarına Karşı Koruyucu Etkisi ve Antioksidan Aktivitesinin Araştırılması

Dünyada en fazla kullanılan baharatlardan birisi olan tarçının antidiyabetik ve antitümör aktiviteleri de içeren çeşitli biyolojik aktivitelere sahip olduğu gösterilmiştir. Tarçının antidiyabetik ve antitümör etkileri güçlü antioksidan aktivitesi ile ilişkilendirilmiştir. Bu nedenle çalışmamızda *Cinnamomum cassia* kabuğu su ekstresinin antioksidan aktivitesini ve H_2O_2 ile indüklenen oksidatif DNA hasarına karşı olası koruyucu etkisini araştırmayı amaçladık.

Lenfositlerin canlılığı Tripan Mavisi testiyle belirlenmiştir. Antioksidan aktivitenin değerlendirilmesi için ekstrenin total fenol ve flavonoid içeriği ile 2,2-difenil-1-pikrilhidrazil (DPPH)' i inhibe edici aktivitesi tayin edilmiştir. DNA hasarı insan periferal kan lenfositlerinde alkali komet yöntemi ile değerlendirilmiştir.

Lenfosit canlılığı, ekstrenin 800 µg/ml konsantrasyonuna kadar >% 86 olarak saptanmıştır. Total fenol ve flavonoid içeriği ekstrenin 100 g kuru ağırlığı için sırasıyla 10.6 g ± 0.001 gallik asit eşdeğeri ve 2.25±0.004 g kersetin eşdeğeri olarak hesaplanmıştır. % 50 DPPH inhibisyonuna neden olan ekstre konsantrasyonu 76.68 µg/ml olarak bulunmuştur.

***Cinnamomum cassia* kabuğu su ekstresi lenfositlerde H_2O_2 ile indüklenen oksidatif DNA hasarına karşı ≥100 µg/ml konsantrasyonlarda anlamlı düzeyde koruyucu etki göstermiştir.**

Çalışma sonuçlarımız *Cinnamomum cassia* kabuğu su ekstresinin profilaktik bir ajan olarak oksidatif stresle ilişkili hastalıkların önlenmesinde yararlı olacağı görüşlerini desteklemektedir.

ANAHTAR SÖZCÜKLER: *Cinnamomum cassia*; DPPH; total fenol; total flavonoid; alkali komet yöntemi.

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