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# Assessment of the cytotoxic, genotoxic, and antigenotoxic potential of Pycnogenol® in *in vitro* mammalian cells

Gökçe Taner [a], Sevtap Aydın [b], Zeki Aytaç [a], Arif Ahmet Başaran [c], Nurşen Başaran [b,*]

[a] Department of Biology, Science Faculty, Gazi University, 06500 Ankara, Turkey
[b] Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey
[c] Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey

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ABSTRACT

Pycnogenol® (PYC), a standardized plant extract obtained from the bark of the French maritime pine *Pinus pinaster*, has been suggested to exert strong antioxidant activity and used as a phytochemical remedy for various diseases. In this study, we investigated the antioxidant capacity of PYC by the trolox equivalent antioxidant capacity (TEAC) assay and the cytotoxicity by neutral red uptake (NRU) test in Chinese Hamster Ovary (CHO) cells. The genotoxic and antigenotoxic effects of PYC were evaluated by the cytokinesis-blocked micronucleus (CBMN) and alkaline comet assays in human peripheral blood lymphocytes. At the concentrations of 2–200 μg/ml, PYC was found to have antioxidant activity. The viability of CHO cells during 24 h exposure were not affected at the concentrations of 5–150 μg/ml of PYC. $IC_{50}$ value of PYC was found to be 285 μg/ml. At the concentrations above 100 μg/ml, PYC alone induced DNA damage and increased MN frequency, although PYC at all concentrations in a dose dependent manner revealed a reduction in the frequency of MN and the extent of DNA damage induced by $H_2O_2$. These results suggest PYC might reduce $H_2O_2$ induced chromosome breakage and loss and DNA damage in cultured human lymphocytes.



## 1. Introduction

Oxygen-derived free radicals, produced in the course of several biochemical reactions, are extremely reactive intermediates and can cause damage to various biologic targets, such as proteins, DNA, and lipids (Basaga, 1990; Southorn and Powis, 1988). The damage caused by free radicals can be protected by natural or synthetic scavenger antioxidants. Since epidemiological data provide evidence that it can be possible to prevent cancer and some chronic diseases, some of which share common pathogenetic mechanisms, such as DNA damage, oxidative stress, and chronic inflammation. The identification and the usage of well-known antimutagens is a valid complementary strategy for improving human health (Anderson, 1996; Ferguson, 1994; Garcia et al., 2006; Hernandez-Ceruelos et al., 2002). Phenolic phytochemicals are a large group of substances that occur as secondary metabolites in plants and found in significant quantities in vegetables, fruits, spices, and seeds. They have been regarded as possible antioxidants, so their roles in food industry and in chemoprevention of diseases resulting from oxidative stress have become an area of active research in many fields (Block et al., 1992; Nakatani, 2000; Noguchi and Niki, 2000; Ross and Fuster, 1996; Steinmetzer and Potter, 1991). Nevertheless, there are still many phenolic compounds with unclear or unidentified prooxidant/antioxidant properties (Cemeli et al., 2009). Also questions concerning the safety of these compounds have encouraged more detailed studies of plant polyphenols (Ferguson, 2001).

Pycnogenol® (PYC) is a standardized natural plant extract obtained from the bark of the French maritime pine *Pinus pinaster* (formerly known as *Pinus maritime*) (Krizkova et al., 2008). PYC is a complex extract containing monomeric phenolic compounds (catechins, epichatechins and taxofolins) and condenced flavonoids (procyanidines and proanthocyanidines). Between 65% and 75% of PYC are procyanidins comprising of catechin and epicatechin subunits with varying chain lengths (D'Andrea, 2010; Packer et al., 1999). Studies indicate that PYC components are highly bioavailable and it is assumed to display greater biologic effects as a mixture than its individual purified components indicating that there has been synergistical interactions between its components (Masquelier et al., 1979; Packer et al., 1999). PYC has been used extensively in European countries as a dietary food supplement because of its strong antioxidant activity and capacity to efficiently scavenge reactive oxygen and nitrogen species (D'Andrea, 2010).

In this study, we aimed to evaluate the antioxidant activity of PYC by the trolox equivalent antioxidant capacity (TEAC) assay, the cytotoxicity by neutral red uptake (NRU) test in Chinese

* Corresponding author. Address: Department of Toxicology, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey. Tel.: +90 312 3052178.
*E-mail address:* nbasaran@hacettepe.edu.tr (N. Başaran).

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Hamster Ovary (CHO) cells. The genotoxicity and antigenotoxicity of PYC against hydrogen-peroxide ($H_2O_2$) were also evaluated by the cytokinesis-blocked micronucleus (CBMN) and alkaline comet assays in human lymphocytes.

## 2. Materials and methods

### 2.1. Chemicals

The chemicals used in the experiments were purchased from the following suppliers: Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), trypsin–EDTA, RPMI 1640, penicillin–streptomycin, L-glutamine, phytohaemagglutinin-M (PHA-M) from Biological Industries (Kibbutz Beit-Haemek, Israel), normal melting point agarose (NMA) and low melting point agarose (LMA) from Boehringer Mannheim (Mannheim, Germany), sodium chloride (NaCl), sodium hydroxide (NaOH), glacial acetic acid and giemsa from Merck Chemicals (Darmstadt, Germany), dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), Triton X-100, phosphate buffered saline (PBS), methanol, ethanol, formaldehyde, potassium chloride (KCl), cytochalasin B (Cyt-B), neutral red (NR) (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid radical) (ABTS), potassium peroxodisulfat, and (±)6-hydoxy-2,5,7,8-tetramethylchromon-2-carboxylic acid (trolox) (purity >97%) from Sigma (St. Louis, USA), ethylenediamine tetra acetic acid disodium salt dihydrate ($Na_2$-EDTA), N-lauroyl sarcosinate, and tris from ICN Biomedicals Inc. (Aurora, Ohio, USA). Pycnogenol® (PYC), a registered trade mark of Horphag Research Ltd., Geneva, Switzerland), was provided by HenkelCorporation (La Grange, IL, U.S.A.).

### 2.2. Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant activity of PYC was determined in a cell-free system as used for other phytochemicals (Riedl and Hagerman, 2001; Virgilio et al., 2004). The trolox equivalent antioxidant capacity (TEAC) was measured spectrophotometrically by analysing the decolourisation of stable radical cation 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid radical) (ABTS) in the presence of the different concentrations of PYC (2, 2.5, 5, 7.5, 10, 25, 50, 100, and 200 μM) or the same concentrations of the synthetic antioxidant trolox (Dicko et al., 2005; Prior et al., 2005). The experiment was repeated four times.

### 2.3. Determination of the cytoxicity of PYC by neutral red uptake (NRU) assay

The cytotoxicity of PYC was performed in Chinese Hamster Ovarium (CHO) epithelial cells (CHO-K1 Americal Type Culture Collection, Rockville, Md.) by NRU assay following the protocols described by Virgilio et al. (2004) and Saquib et al. (2012). The CHO cell line is chosen because of its highly sensitivity to chemicals, high cloning efficiency (95%), and excellent colony-forming properties (Wininger and Kulik, 1979).

Cells were seeded in 25 $cm^2$ flasks in 5 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2% Penicillin–streptomycin and then grown for 2 days in an incubator at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. Following disaggregation with trypsin/EDTA and resuspension of cells in medium, a total of $1 \times 10^5$ cells/well were plated in 96 well tissue-culture plates. After 24 h incubation, the different concentrations of PYC (25, 50, 100, 125, 150, 200, 250, and 300 μg/ml) in medium were added. The cells were incubated for 18 h (1.5 cell cycle) at 37 °C in 5% $CO_2$ in air, then the medium was aspirated. The cells were washed twice with PBS and incubated for an additional 3 h in the medium supplemented with NR (50 μg/ml). After the medium was discarded, the cells were rinsed five times with warm PBS (pH 7.4) to remove the nonincorporated excess dye and 200 μl of 'destain solution' (50% ethanol, 1% acetic acid, and 49% distilled water) was added to each well to fix the cells and bring the NR into solution. The plates were shaken for 20 min, and the absorbance of the solution in each well was measured in a microplate reader at 540 nm and compared with wells containing untreated cells. Results were expressed as the mean percentage of cell growth from three independent experiments.

### 2.4. Determination of the genotoxicity of PYC by the CBMN assay

The presence of MN in a binucleated cell using the protocol of Fenech (2000) was determined with minor modifications. Human peripheral blood cultures were used for the CBMN test. Blood samples were obtained from three healthy and non-smoking female donors aged 23–33. Briefly, 0.5 ml of heparinized blood samples were placed in sterile culture tubes containing 5 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 2% L-glutamine, 2% penicillin–streptomycin antibiotic mixture, and 2.5% phytohaemaglutinin. The samples were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ in air for 72 h. After 24 h (one cell cycle), 6 different concentrations of PYC (5, 10, 25, 50, 100, and 150 μg/ml) were added to the samples alone and also in combination with100 μM $H_2O_2$ and they were kept for another 48 h at 37 °C. In all sets of experiments, an untreated negative control, as well as a positive control (100 μM $H_2O_2$) was also run simultaneously. Cytochalasin B (Cyt-B), an inhibitor of the mitotic spindle that prevents cytokinesis, were added to the samples at a final concentration of 6 μg/ml at 44 h of the incubation. After a total of 72 h incubation the samples were centrifuged at 1000 rpm for 10 min. Supernatant was discarded and the cell pellets were treated with a ice-cold hypotonic solution (0.075 M KCl) for 5 min at 37 °C. The cells were fixed with cold methanol: glacial acetic acid (3:1, v/v) for 15 min and fixation procedure wasrepeated twice. The last methanol: glacial acetic acid (3:1, v/v) fixation was supplemented with 1% formaldehyde. The fixed cells were dropped onto slides previously cleaned with nitric acid. The slides were air-dried and stained in 5% Giemsa for 13 min. 1000 binucleated cells surrounded by well-preserved cytoplasm per donor (total 3000 binucleated cells per concentration) were scored for the presence of MN. 500 lymphocytes from per donor (total 1500 lymphocytes) were scored to evaluate the percentages of the cells with 1–4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated according to Surrales et al. (1995) as follows: $[1 \times N1] + [2 \times N2] + [3 \times (N3 + N4)]/N$, where $N1$–$N4$ represent the number of cells with 1–4 nuclei, respectively, and $N$ is the total number of cells scored.

### 2.5. Determination of the genotoxicity of PYC by the alkaline comet assay

The basic alkaline technique of Singh et al. (1988), as further described by Anderson et al. (1998) and Collins et al. (1997) was followed. Lymphocytes from whole heparinized blood were separated by Ficoll-Hypaque density gradient and centrifugation (Boyum, 1976). Then the cells were washed with PBS buffer. The $1 \times 10^4$ cells in 50 μl were treated with the increasing concentrations of PYC (5–150 μg/ml) for 30 min at 37 °C for the assessment of DNA damage. After the pretreatment of PYC (5–150 μg/ml) for 30 min, oxidative damage was induced by replacing the medium with PBS containing 50 μM $H_2O_2$ and then incubating for 5 min on ice to assess the antigenotoxicity of PYC, Then the lymphocytes were centrifuged and washed with PBS for removing the $H_2O_2$ solution. A negative control sample (PBS) and positive control 50 μM $H_2O_2$ were also included in the experiments. After centrifugation at 3000 rpm, supernatant was discarded and the cells were suspended in 75 μl of 0.65% LMA. Then the suspensions were embedded on the slides pre-coated with a layer of 1% NMA. The slides were allowed to solidify on ice for 5 min. Coverslips were then removed. The slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium sarcosinate, pH 10.0), with 1% Triton X-100 and 10% DMSO added just before use for a minimum of 1 h at 4 °C. Then they were removed from the lysing solution, drained and were left in the electrophoresis solution (1 mM sodium EDTA and 300 mM NaOH, pH 13.0) for 20 min at 4 °C to allow unwinding of the DNA and expression of alkali-labile damage. Electrophoresis was conducted also at a low temperature (4 °C) for 20 min using 25 V and adjusting the current to 300 mA by rising or lowering the buffer level. The slides were neutralized by washing 3 times in 0.4 M Tris–HCl (pH 7.5) for 5 min at room temperature. After neutralization, the slides were incubated in 50%, 75%, and 99% of alcohol for 5 min, successively. The dried microscope slides were stained with ethidium bromide (EtBr 20 μg/ml in distilled water, 35 μl/slide), covered with a cover-glass prior to analysis with a Leica fluorescence microscope under green light. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize DNA damage, slides were examined at 40×. The experiment was repeated four times. One-hundred cells from two replicate slides were assayed for each experiment. Results were expressed as tail length, tail intensity, and tail moment.

### 2.6. Statistical analysis

For statistical analysis of the CBMN assay results, the $z$-test were applied for the percentage of MN and CBPI. The results were given as the mean ± standard error. For alkaline comet assay and TEAC assay statistical analysis was performed by SPSS for Windows 15.0 computer program. The results were expressed as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. $P$ value of less than 0.05 was considered as statistically significant.

## 3. Results

### 3.1. Antioxidant capacity of PYC

The antioxidant capacity of the different concentrations of PYC as mesasured by the TEAC assay was shown in Fig. 1. According to the results compared to the same concentrations of reference antioxidant trolox, PYC was found to have significantly more antioxidant activity than trolox at the studied concentrations of 2–200 μM.