Protective effects of olive oil phenolics oleuropein and hydroxytyrosol against hydrogen peroxide-induced DNA damage in human peripheral lymphocytes

This study investigates antioxidant capacity and protective effects of phenolic compounds oleuropein (OLP) and hydroxytyrosol (HT), present in olive oil and olive leaves, against H$_2$O$_2$-induced DNA damage in human peripheral lymphocytes. Antioxidant potency was determined using the measurement of radical-scavenging activity (ABTS∙$^-$ assay), ferric reducing power (FRAP assay) and cupric reducing antioxidant capacity (CUPRAC assay). Both substances were found to be potent antioxidant agents due to their free radical-scavenging activities. Antigenotoxic effects of oleuropein and hydroxytyrosol against H$_2$O$_2$-induced damage in human lymphocytes were evaluated in vitro by alkaline comet assay. At tested concentrations (1, 5, 10 µmol L$^{-1}$), oleuropein and hydroxytyrosol did not induce a significant increase of primary DNA damage in comparison with the negative control. Pretreatment of human lymphocytes with each of the substances for 120 min produced a dose-dependent reduction of primary DNA damage in the tested cell type. Hydroxytyrosol showed a better protective effect against H$_2$O$_2$-induced DNA breaks than oleuropein which could be associated with their free radical-scavenging efficacy.

Keywords: oleuropein, hydroxytyrosol, olive, antioxidant activity, DNA damage, human lymphocytes, comet assay

Phenolic compounds present in olive tree products have beneficial health properties including antimicrobial, antioxidant and anti-inflammatory (1, 2). Olive oil phenolics have been found to decrease reactive oxygen species (ROS) production and elicit free radical-scavenging effects (3, 4). An excess of free radicals can cause oxidative damage to biomolecules increasing the risk of developing chronic diseases (5). Oxidative stress is involved in the pathology of oxidation-linked diseases such as cancer, heart diseases, rheumatoid arthritis and neurodegenerative diseases. Polyphenols can act as antioxidants by a number of potential pathways (1). They are known to have the ability to scavenge various radicals such as DPPH and ABTS∙$, as well as radicals generated by aerobic metabolism in organ-
isms (OH and O$_2^-$) (6). These compounds possess antioxidant activity against radicals due to the redox properties of their phenolic groups and the structural relationship between different parts in their chemical structures. Mechanism of action of a compound with antioxidant capacity depends on structural factors, such as the presence or absence of glycosidic moiety in the polyphenol, the glycosylation site and the number and position of free and esterified hydroxyl groups (7). Further, olive oil phenolic compounds are able to bind to LDL which decreases LDL oxidation associated with a major risk factor for the development of atherosclerosis cardiovascular diseases (8). Intake of phenol-rich olive oil (up to 592 mg kg$^{-1}$) decreases oxidative DNA damage 	extit{in vivo} in humans up to 30 % (9, 10).

The main polyphenol of olive fruit, oil and leaf, oleuropein (OLP) and its metabolite hydroxytyrosol (HT) (Fig. 1) are reported to have scavenging activity against stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH test) (11). Anter et al. (12) assessed antigenotoxicity of oleuropein with the 	extit{Drosophila} wing spot test (SMART) which showed that oleuropein reduced the frequencies of H$_2$O$_2$-induced single and total spots at high concentrations. Oleuropein was found to inhibit the H$_2$O$_2$-induced oxidative damage (inhibition percentage of 73.7 % at 555 µmol L$^{-1}$). Additionally, oleuropein showed cytotoxic effect with $IC_{50}$ of 170 µmol L$^{-1}$ against HL-60, human promyelocytic leukemia cells. Recently, it was identified that hydroxytyrosol generates H$_2$O$_2$ in the RPMI medium that was responsible for the induction of apoptosis in HL-60 cells (13). Fabiani et al. (14) measured the effect of olive phenols on hydrogen peroxide-induced DNA damage in human peripheral blood mononuclear cells (PBMC) and promyelocytic leukemia cells (HL-60) using a comet assay. Hydroxytyrosol reduced the DNA damage at a concentration as low as 1 µmol L$^{-1}$ when co-incubated in the medium with H$_2$O$_2$ (40 µmol L$^{-1}$). Oleuropein also protected the cells against hydrogen peroxide-induced DNA damage although with a lower efficacy (range of protection 25–75 %).

Antioxidant properties of natural compounds are commonly determined with a combination of different methods. Therefore, in the present study, we assessed antioxidant capacity using ABTS$^-$ scavenging assay, reducing power assay and cupric reducing antioxidant capacity assay. Further, the aim of the study was to investigate the protective effects of oleuropein and hydroxytyrosol 	extit{in vitro} against H$_2$O$_2$-induced DNA damage in isolated human lymphocytes by the alkaline single-cell gel electrophoresis.

**EXPERIMENTAL**

**Chemicals and reagents**

Oleuropein (HPLC purity ≥ 98 %, Extrasynthese, France) and hydroxytyrosol (HPLC purity ≥ 98 %, Extrasynthese) were dissolved separately in pH 7.4 phosphate buffer (PBS) to
prepare stock solutions of 50 mg mL$^{-1}$. All other chemicals and reagents except FeSO$_4 \times 7\text{H}_2\text{O}$ (Kemika, Croatia) were purchased from Sigma Chemical Co. (USA). Absorbance measurements were performed on a double-beam UV-VIS spectrophotometer Agilent 8453E (Hewlett Packard, Germany).

**ABTS$^\cdot+$ scavenging activity**

The radical-scavenging activity of the compounds was evaluated according to the modified version of the method by Re et al. (15). ABTS$^\cdot+$ radical solution was prepared by mixing ABTS$^\cdot+$ stock solution (7 mmol L$^{-1}$) with 2.45 mmol L$^{-1}$ potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The obtained radical solution was further diluted with ethanol to an absorbance of 0.70 ($\pm$ 0.02) at 734 nm (16). Twenty (20) $\mu$Ls of each compound (1–3 $\mu$mol L$^{-1}$) were added into 2 mL of ABTS$^\cdot+$ solution and the absorbance was read at 734 nm after exactly 6 min. Solvent blanks were run in parallel. An aqueous solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used for calibration. Trolox stock standard (2 mmol L$^{-1}$) was prepared in PBS. Fresh working standards were prepared by diluting a stock solution of Trolox with PBS. Abilities of phenolic compounds to scavenge the ABTS$^\cdot+$ radical cation compared with a standard antioxidant (Trolox) were derived from the dose-response curve.

**Ferric reducing power (FRAP assay)**

The reducing power was determined according to the method by Benzie et al. (17) with slight modifications. FRAP assay is based on the conversion of ferric tripyridyltriazine [Fe(III)-TPTZ] complex to the ferrous tripyridyltriazine [Fe(II)-TPTZ] at low pH. The formation of the blue Fe(II)-TPTZ complex can be monitored spectrophotometrically at 593 nm. FRAP working solution contained 25 mL of 300 mmol L$^{-1}$ acetate buffer, 2.5 mL of 10 mmol L$^{-1}$ 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 2.5 mL of 20 mmol L$^{-1}$ FeCl$_3 \times 6$ H$_2$O. Briefly, 50 $\mu$L of each compound was mixed with 950 $\mu$L of freshly prepared FRAP working solution. The reaction mixture was kept for 4 min at room temperature and the absorbance was recorded at 593 nm. A calibration curve was plotted for a standard solution of FeSO$_4 \times 7$ H$_2$O. Stock solution (1 mmol L$^{-1}$) of FeSO$_4 \times 7$ H$_2$O was diluted with PBS to obtain working standards. The reducing power ability of phenolics was measured at three different concentrations which had been determined to be within the range of the dose-response curve (1, 2 and 3 $\mu$mol L$^{-1}$).

**Cupric reducing antioxidant capacity (CUPRAC method)**

Measurement of the cupric reducing antioxidant capacity was determined according to the method by Apak et al. (18). For CUPRAC testing copper(II) chloride solution was prepared from CuCl$_2 \times 2$H$_2$O (0.4262 g) dissolved in water and diluted to a final volume of 250 mL. Further, 250 mL of ammonium acetate, pH 7.0, was prepared by dissolving 19.27 g of NH$_4$Ac in the water whereas 25 mL of neocuproine (2,9-dimethyl-1,10-phenanthroline) solution was prepared by dissolving 0.039 g of neocuproine in 96 % ethanol. Reaction mixtures contained each tested compound (1 or 2 $\mu$mol L$^{-1}$), copper(II) chloride solution (10$^{-2}$ mol L$^{-1}$), neocuproine alcoholic solution (7.5 $\times$ 10$^{-3}$ mol L$^{-1}$) and ammonium acetate, pH 7.0. After 30 min, the absorbance of the sample was measured at 450 nm against a
Protective effects measured by the method of alkaline single-cell electrophoresis

Blood sampling. – A blood sample was obtained from a healthy male donor (age 45, non-smoker). The donor had not been exposed to diagnostic or therapeutic irradiations or known genotoxic chemicals for 1 year before blood sampling. Venous blood (40 mL) was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson, USA) containing lithium heparin as the anticoagulant. Ethical Committee Approval has been issued by the Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

Experimental procedure. – Three independent experiments were performed for each tested concentration. The concentrations of oleuropein and hydroxytyrosol were 1, 5 and 10 µmol L\(^{-1}\).

Lymphocytes were isolated using the Histopaque-1077 reagent according to the manufacturer’s instructions (Sigma Chemical Co.). Human lymphocytes were preincubated for 120 min with oleuropein and hydroxytyrosol in RPMI-1640 culture medium before exposure to H\(_2\)O\(_2\) (5 µmol L\(^{-1}\)) for 30 min. Negative and positive controls were incubated simultaneously. Hydrogen peroxide (100 µmol L\(^{-1}\) for 10 min on ice) and bleomycin (10 µg mL\(^{-1}\) for 3 h) served as positive controls in the test of potential genotoxic effects.

The comet assay. – The comet assay was carried out under alkaline conditions according to the method by Singh et al. (19). Agarose microgels were prepared on microscopic slides covered with 1 and 0.6 % of normal melting point agarose (NMP). After polymerisation at room temperature, a new layer has been added containing 100 µL of 0.5 % low melting point agarose (LMP) mixed with 10 µL of lymphocytes in RPMI. The tray was kept for 10 min on ice and an additional layer of 100 µL of 0.5 % LMP agarose has been added. Preparation of the slides has been protected from light in order to prevent the additional incidence of DNA damage in the samples. The slides were placed in the cold lysis solution (pH 10.0) freshly prepared according to the standard protocol (19). The lysis of all microgels lasted for 1 h at 4 °C. Following lysis, standard denaturation (20 min, protected from light) and electrophoresis (25 V, 300 mA) were carried out at 4 °C under dim light in a freshly prepared electrophoretic buffer (pH 13.0) and lasted for 20 min each. After electrophoresis, the slides were neutralised in three changes of buffer (0.4 µmol L\(^{-1}\) Tris-HCl, pH 7.5) at 5-min intervals. Slides were stained with 100 µL of ethidium bromide (20 µg mL\(^{-1}\)) and stored at 4 °C in humified sealed containers until analysis. The scoring of alkaline comet assay slides was performed using an image analysis system attached to an epifluorescence microscope (Olympus BX 51, Japan) under 200× magnification. Per each tested sample, a total of 150 comets were scored (3 × 50). As a measure of DNA damage, tail intensity (percentage of DNA in the comet tail), tail length and tail moment were chosen.

Statistical analysis

All values are means of three measurements ± SD. Results were analysed using commercial programmes Excel and Statistica 12.0 (statSoft Inc.). The comparisons between samples were done using one-way analysis of variance (ANOVA). The level of statistical significance was set at \(p < 0.05\).
RESULTS AND DISCUSSION

Antioxidant and radical-scavenging activity of oleuropein and hydroxytyrosol

Results presented in Fig. 2 show the antioxidant activity of OLP and HT determined with ABTS$^+$ method. Their scavenging effect on ABTS$^+$ radical was concentration-dependent ($R^2 = 0.982$) with lower activity of OLP than HT. Results obtained in our study are in accordance with the previously published reports (20). The reducing capacity of OLP and HT in our study are shown in Fig. 3 ($R^2 = 0.988$). We observed differences between antioxidant activities measured by these assays; however, these differences could be attributed to different mechanisms of action of tested phenolic compounds.

In general, the antioxidant and radical-scavenging activity of phenolic compounds such as oleuropein and hydroxytyrosol is based on the presence of free hydroxyl groups in their chemical structure, catechol phenolic group being a characteristic pattern in both OLP and HT (Fig. 1) (21). Important for antioxidative activity are the number and location of aromatic hydroxyl groups of natural polyphenols, as well as stability of the formed aroxyl radicals (22, 23). The increased reducing power is likely to be correlated with the antioxidant properties of natural compounds due to their hydrogen-donating ability (24). Fe$^{3+}$ reduction is an indicator of electron-donating activity which is an important antioxidant mechanism of action of phenolic compounds (7). It has been reported that the antioxidant capacity of hydroxytyrosol is due to its H-donor ability and the formation of a highly stable aromatic ring radical when losing a hydrogen atom for conjugation. On the other hand, oleuropein shows antioxidant properties due to the o-dihydroxy (catechol) structure present in its moiety.

Several studies have investigated the ability of OLP and HT to scavenge DPPH radicals and ABTS$^+$; however, limited data exist against other radicals (1, 3, 21). Therefore, antioxi-
The comet assay is a sensitive method that detects a variety of DNA lesions. This assay can detect both direct a breakage of the DNA phosphodiester backbone and damage to DNA bases. The method can be used with almost any cell type. The present study used lymphocytes to evaluate the protective effects of tested compounds; lymphocytes have been extensively used in genotoxicity testing of bioactive compounds (25). Measurement of more than one parameter gives the best explanation on the real levels of primary DNA damage (26).

Hydrogen peroxide causes DNA strand breakage by the generation of the hydroxyl radical (OH•) close to the DNA molecule, via the Fenton reaction, which may result in DNA instability, mutagenesis and carcinogenesis (27):

$$\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{OH}^- + \text{Fe}^{3+}$$

The genotoxic effect of hydrogen peroxide and the protective effect of OLP and HT were assessed in human lymphocytes using the comet assay and the results of the measured parameters (tail length, tail intensity and tail comet) are shown in Figs. 5–7. OLP and HT, at tested concentrations (1, 5, 10 µmol L\(^{-1}\)), did not induce a significant increase of primary DNA damage in comparison with the negative control. Pretreatment of human lymphocytes with each of the substances for 120 min produced a dose-dependent reduction of primary DNA damage in the tested cell type. Fig. 8 shows the photomicrographs of lymphocyte nuclei observed after the alkaline comet assay procedure.

Oleuropein and hydroxytyrosol reduce hydrogen peroxide-induced DNA damage in isolated human lymphocytes; HT showed a higher level of protection against hydrogen per-
oxide-mediated DNA strand breaks than OLP. This could be associated with their free radical-scavenging efficacy or, the compound with protective effect could chelate iron ions present in the cells and thus depress the Fenton reaction, i.e., $H_2O_2$-induced formation of hydroxyl radicals (4). The results obtained with the alkaline comet assay have shown that the treatment with the tested compounds prevents primary DNA damage at very low concentrations (1, 5, 10 µmol L$^{-1}$) which could be reached with ordinary intake of 50 g olive oil daily (14).

In addition to $H_2O_2$ treatment, positive control cells were also treated with bleomycin, and they had the most pronounced DNA damage, as bleomycin induces both, DNA single- and double-strand breaks. Health-protecting properties of olive oil and olive leaf compounds could be explained by various mechanisms including blocking the activation of carcinogens,

Fig. 7. Comet tail moments measured by the comet assay in isolated peripheral blood lymphocytes treated in vitro for 150 min with oleuropein (OLP) and hydroxytyrosol (HT) applied at three concentrations (1, 5 and 10 µmol L⁻¹). After 120 min of pretreatment, lymphocytes were treated with 5 µmol L⁻¹ H₂O₂ for 30 min at 37 °C. Negative, positive control – NC, H₂O₂.

increasing the level of enzymes involved in the detoxification of carcinogens (glutathione S-transferase), stimulation of error-free DNA repair and interception of carcinogens before their reaction with DNA (12).

Fig. 8. Photomicrographs of lymphocyte nuclei observed after the alkaline comet assay procedure: a) negative control, b) nucleus with highly damaged DNA in H₂O₂-treated cell, c) nucleus of a lymphocyte after 120 min pre-treatment with 10 µmol L⁻¹ hydroxytyrosol and 30 min treatment with 5 µmol L⁻¹ H₂O₂, d) nucleus of a lymphocyte after 120 min pre-treatment with 1 µmol L⁻¹ oleuropein and 30 min treatment with 5 µmol L⁻¹ H₂O₂. Stained with ethidium bromide. The scoring of alkaline comet assay slides was performed using an image analysis system attached to an epifluorescence microscope (Olympus BX 51, Japan) under 200× magnification.
So far, in vitro studies demonstrated that hydroxytyrosol prevents the tert-butylhydroperoxide-induced death of HepG2 cells, acts against the low-density lipoprotein oxidation produced chemically and cell-mediated and protects different cell types such as Caco-2, erythrocytes and PC12 from hydrogen peroxide-induced cytotoxicity (14).

Oleuropein was found to suppress the production of reactive oxygen and nitrogen species in in vitro cell-free systems and cellular screening systems (human neutrophil oxidative burst, monocytes' nitric oxide production) (28). Bulotta et al. (29) reported that oleuropein inhibited hydrogen peroxide-induced ROS increase in breast human cancer cell lines MCF-7 and T-47D. It was reported, using a comet assay, that oleuropein reduced hydrogen peroxide-induced DNA damage in human peripheral leukocytes and hepatocellular carcinoma (HepG2) cells (30, 31). Also, oleuropein was shown to increase the level and activities of enzymatic antioxidants in vivo, e.g., superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, and enhance the level of some non-enzymatic antioxidants such as glutathione, α-tocopherol, β-carotene and ascorbic acid in diabetic rabbits (32), rats on the cholesterol-rich diet (33) and in rats exposed to acute arsenic (34).

Genoprotective effects of HT and other olive phenolic compounds could be attributed to their potential radical-scavenging activity, metal-ion chelating properties and the activation of indigenous antioxidant defence and DNA repair systems (10, 14).

CONCLUSIONS

From the evidence provided in this study, it can be confirmed that oleuropein and hydroxytyrosol are strong antioxidants with the ability to scavenge various radicals, and their intake in OLP- and HT-rich food may reduce the body’s need for certain antioxidants. Additionally, these phenolic compounds have promising genoprotective properties against hydrogen peroxide-induced oxidative DNA damage in human peripheral blood lymphocytes. However, further studies investigating their mechanisms of action are required.

REFERENCES


