Acta Pharm. **71** (2021) 131–141
https://doi.org/10.2478/acph-2021-0003

Original research paper

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

NATAŠA ZORIĆ[1]
NEVENKA KOPJAR[2]
JADRANKA VUKOVIĆ RODRIGUEZ[3]
SINIŠA TOMIĆ[1]
IVAN KOSALEC[4,*]

[1] *Agency for Medicinal Products and Medical Devices of Croatia (HALMED) 10000 Zagreb, Croatia*

[2] *Institute for Medical Research and Occupational Health, 10000 Zagreb Croatia*

[3] *University of Zagreb Faculty of Pharmacy and Biochemistry Department of Pharmaceutical Analysis 10000 Zagreb, Croatia*

[4] *University of Zagreb Faculty of Pharmacy and Biochemistry Department of Microbiology, 10000 Zagreb, Croatia*

Accepted March 10, 2020
Published online July 3, 2020

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_2O_2-induced DNA damage in human peripheral lymphocytes. Antioxidant potency was determined using the measurement of radical-scavenging activity ($ABTS^{\cdot+}$ 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_2O_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_2O_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 $ABTS^{\cdot+}$, as well as radicals generated by aerobic metabolism in organ-

* Correspondence; e-mail address: ivan.kosalec@pharma.hr

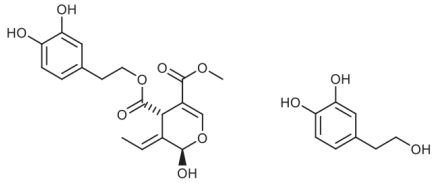

Fig. 1. Chemical structures of oleuropein (OLP) aglycon and hydroxytyrosol (HT).

isms (OH and $O_2^{\cdot -}$) (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 *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 *Drosophila* wing spot test (SMART) which showed that oleuropein reduced the frequencies of H_2O_2-induced single and total spots at high concentrations. Oleuropein was found to inhibit the H_2O_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_2O_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_2O_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 $ABTS^{\cdot +}$ 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 *in vitro* against H_2O_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 $FeSO_4 \times 7H_2O$ (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 (± 0.02) at 734 nm (16). Twenty (20) μLs of each compound (1–3 μmol 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_2O. Briefly, 50 μL of each compound was mixed with 950 μL 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_2O. Stock solution (1 mmol L^{-1}) of $FeSO_4 \times 7$ H_2O 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 μmol 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 $CuCl_2 \times 2H_2O$ (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_4Ac 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 μmol L^{-1}), copper(II) chloride solution (10^{-2} mol L^{-1}), neocuproine alcoholic solution (7.5×10^{-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

reagent blank. An aqueous solution of Trolox was used for calibration. The CUPRAC antioxidant capacity expressed as Trolox equivalents was derived from the slope of the calibration line.

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_2O_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

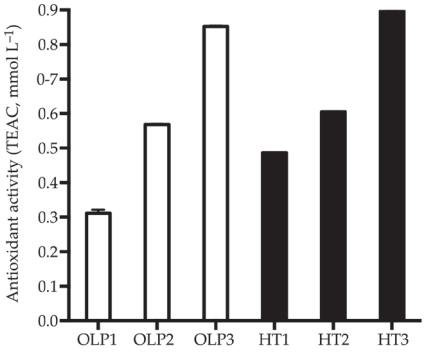

Fig. 2. Antioxidant capacities of phenolics oleuropein (OLP) and hydroxytyrosol (HT) determined by ABTS·$^+$ method and expressed as Trolox equivalent antioxidant capacities (TEAC); OLP1, HT1 – 1 μmol L^{-1}, OLP2, HT2 – 2 μmol L^{-1}, OLP3, HT3 – 3 μmol L^{-1}. Mean ± SD, n = 3.

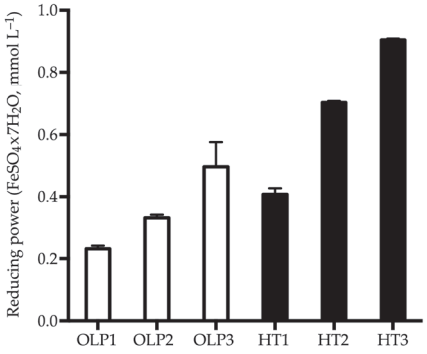

Fig. 3. Reducing power of phenolics oleuropein (OLP) and hydroxytyrosol (HT) expressed as $FeSO_4 \times 7\ H_2O$. OLP1, HT1 – 1 μmol L^{-1}, OLP2, HT2 – 2 μmol L^{-1}, OLP3, HT3 – 3 μmol L^{-1}. Mean ± SD, n = 3.

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-

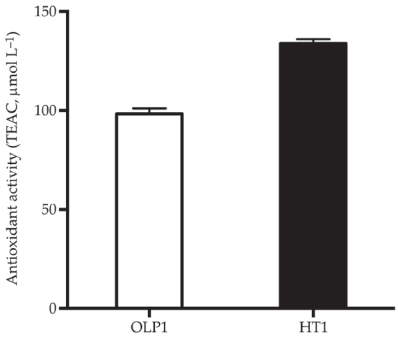

Fig. 4. Antioxidant capacities of phenolics oleuropein (OLP) and hydroxytyrosol (HT) determined by the CUPRAC method and expressed as Trolox equivalent antioxidant capacity (TEAC). OLP1, HT1 – 1 μmol L^{-1}. Mean ± SD, $n = 3$.

dant activity was also determined with the CUPRAC method. In comparison with other assays, the advantage of the cupric reducing method is that the measurement of antioxidant capacity is carried out at nearly physiological pH; therefore, it mimics the physiological action of the antioxidants. Additionally, it is suitable for the determination of hydrophilic and lipophilic antioxidants (24). Potent antioxidant capacity of tested compounds was confirmed with the cupric reducing method; results are presented in Fig. 4 ($R^2 = 0.996$).

Comet assay

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):

$$H_2O_2 + Fe^{2+} \rightarrow OH\cdot + OH^- + Fe^{3+}$$

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-

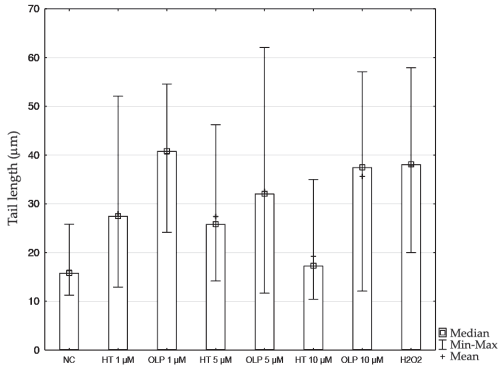

Fig. 5. Comet tail lengths measured by the comet assay in isolated peripheral blood lymphocytes treated *in vitro* for 150 min with oleuropein (OLP) and hydroxytyrosol (HT) (applied at three concentrations: 1, 5 and 10 μmol L^{-1}). After 120 min of pretreatment, lymphocytes were treated with 5 μmol L^{-1} H_2O_2 for 30 min at 37 °C. Negative, positive control – NC, H_2O_2.

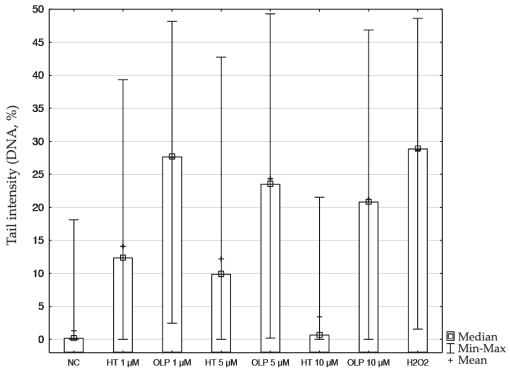

Fig. 6. Comet tail intensities measured by the comet assay in isolated peripheral blood lymphocytes treated *in vitro* for 150 min with oleuropein (OLP) and hydroxytyrosol (HT) applied at three concentrations (1, 5 and 10 μmol L^{-1}). After 120 min of pretreatment, lymphocytes were treated with 5 μmol L^{-1} H_2O_2 for 30 min at 37 °C. Negative, positive control – NC, H_2O_2.

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,

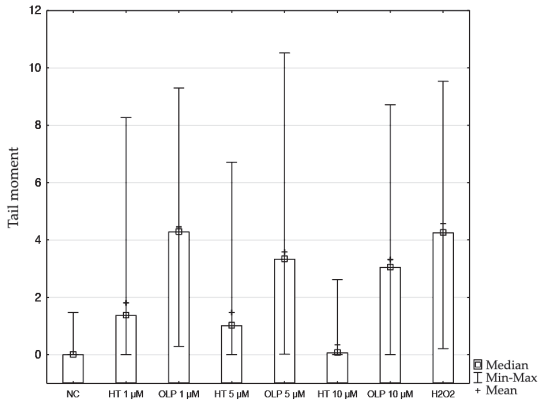

Fig. 7. Comet tail moments measured by the comet assay in isolated peripheral blood lymphocytes treated *in vitro* for 150 min with oleuropein (OLP) and hydroxytyrosol (HT) applied at three concentrations (1, 5 and 10 μmol L^{-1}). After 120 min of pretreatment, lymphocytes were treated with 5 μmol L^{-1} H_2O_2 for 30 min at 37 °C. Negative, positive control – NC, H_2O_2.

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).

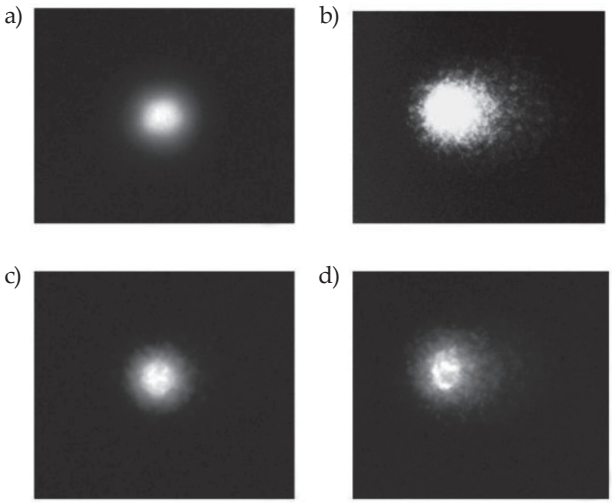

Fig. 8. Photomicrographs of lymphocyte nuclei observed after the alkaline comet assay procedure: a) negative control, b) nucleus with highly damaged DNA in H_2O_2-treated cell, c) nucleus of a lymphocyte after 120 min pre-treatment with 10 μmol L^{-1} hydroxytyrosol and 30 min treatment with 5 μmol L^{-1} H_2O_2, 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} H_2O_2. 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*-butyl-hydroperoxyde-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, ß-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

1. O. Lee and B. Lee, Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract, *Bioresour. Technol.* **101** (2010) 3751–3754; https://doi.org/10.1016/j.biortech.2009.12.052
2. N. Zorić, I. Horvat, N. Kopjar, A. Vučemilović, D. Kremer, S. Tomić and I. Kosalec, Hydroxytyrosol expresses antifungal activity *in vitro*, *Curr. Drug Targets* **14** (2013) 992–998.
3. S. Cicerale, L. J. Lucas and R. S. J. Keast, Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil, *Curr. Opin. Biotechnol.* **23** (2011) 1–7; https://doi.org/10.1016/j.copbio.2011.09.006
4. A. Aherne and N. M. O'Brien, Protection by the flavonoid myricetin, quercetin, and rutin against hydrogen peroxide-induced DNA damage in Caco-2 and Hep G2 cells, *Nutr. Cancer* **34** (1999) 160–166; https://doi.org/10.1207/S15327914NC3402_6
5. B. Uttara, A. V. Singh, P. Zamboni and R. T. Mahajan, Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options, *Curr. Neuropharmacol.* **7** (2009) 65–74; https://doi.org/10.2174/157015909787602823
6. J. M. Mates and F. M. Sanchez-Jimenez, Role of reactive oxygen species in apoptosis: Implications for cancer therapy, *Int. J. Biochem. Cell Biol.* **32** (2000) 157–170; https://doi.org/10.1016/S1357-2725(99)00088-6

7. P. Xie, L. Huang, C. Zhang and Y. Zhang, Phenolic compositions, and antioxidant performance of olive leaf and fruit (*Olea europaea* L.) extracts and their structure-activity relationship, *J. Funct. Foods* **16** (2015) 460–471; https://doi.org /10.1016/j.jff.2015.05.005

8. E. Gimeno, K. de la Torre-Carbot, R. M. Lamuela-Raventos, A. I. Castellote, M. Fito, R. de la Torre, M. I. Covas and M. Carmen Lopez-Sabater, Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial, *Br. J. Nutr.* **98** (2007) 1243–1250; https://doi.org/10.1017/S0007114507778698

9. S. Salvini, F. Sera, D. Caruso, L. Giovannelli, F. Visioli, C. Saieva, G. Masal, M. Ceroti, V. Giovacchini, V. Pitozzi, C. Galli, A. Romani, N. Mulinacci, R. Bortolomeazzi, P. Dolara and D. Palli, Daily consumption of a high-phenol extra-virgin olive oil reduces oxidative DNA damage in postmenopausal women, *Br. J. Nutr.* **95** (2006) 742–751; https://doi.org/10.1079/BJN20051674

10. A. Machowetz, H. E. Poulsen, S. Gruendel, A. Weimann, M. Fito, J. Marrugat, R. de la Torre, J. T. Salonen, K. Nyyssonen, J. Mursu, S. Nascetti, A. Gaddi, H. Kiesewetter, H. Baumler, H. Selmi, J. Kaikkonen, H. J. Zunft, M. I. Covas and C. Koebnick, Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans, *FASEB J.* **21** (2007) 45–52; https://doi.org/10.1096/fj.06-6328com

11. S. Grasso, L. Siracusa, C. Spatafora, M. Renis and C. Tringali, Hydroxytyrosol lipophilic analogues: enzymathic sythesis, radical-scavenging activity and DNA oxidative damage protection, *Bioorg. Chem.* **35** (2007) 137–152; https://doi.org/10.1016/j.bioorg.2006.09.003

12. J. Anter, Z. Fernandez-Bedmar, M. Villatoro-Pulido, S. Demyda-Peyras, M. Moreno-Millan, A. Alonso-Moraga, A. Munoz-Serrano and M. D. Luque de Castro, A pilot study on the DNA-protective, cytotoxic, and apoptosis-inducing properties of olive-leaf extracts, *Mutat. Res.* **723** (2011) 165–170; https://doi.org/10.1016/j.mrgentox.2011.05.005

13. E. M. Odiatou, A. L. Skaltsounis and A. I. Constantinou, Identification of the factors responsible for the in vitro proxidant and cytotoxic activities of the olive polyphenols oleuropein and hydroxytyrosol, *Cancer Lett.* **330** (2013) 113–121; http://doi.org/10.1016/j.canlet.2012.11.035

14. R. Fabiani, P. Rosignoli, A. De Bartolomeo, R. Fuccelli, M. Servili, G. F. Montedoro and G. Morozzi, Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells, *J. Nutr.* **138** (2008) 1411–1416; https://doi.org/10.1093/jn/138.8.1411

15. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation delorization assay, *Free Radic. Biol. Med.* **26** (1999) 1231–1237; https://doi.org/ 10.1016/s0891-5849(98)00315-3

16. M. N. Asghar, I. U. Khan, I. Zia, M. Ahmad and F. A. Qureshi, Modified 2,2'-azinobis(3-ethylbenzo thiazoline)-6-sulphonic acid radical cation decolorization assay for antioxidant activity of human plasma and extracts of traditional medicinal plants, *Acta Chim. Slov.* **55** (2008) 408–418.

17. I. F. Benzie and J. J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of „antioxidant power": the FRAP assay, *Anal. Biochem.* **239** (1996) 70–76; https://doi.org/10.1006/abio.1996.0292

18. R. Apak, K. Guclu, M. Ozyurek and S. E. Karademir, Novel total antioxidant index for dietary polyphenols and vitamin C and E using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method, *J. Agric. Food Chem.* **52** (2004) 7970–7981; https://doi.org/10.1021/jf048741x

19. N. P. Singh, M. T. McCoy, R. R. Tice and E. L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp. Cell Res.* **175** (1988) 184–191; https://doi.org/10.1016/0014-4827(88)90265-0

20. W. Bors, W. Heller, C. Michel and M. Saran, Flavonoids as antioxidants: Determination of radical-scavenging efficiencies, *Methods Enzymol.* **186** (1990) 343–355; https://doi.org/10.1016/0076-6879(90)86128-I

21. O. Benavente-Garcia, J. Castillo, J. Lorente, A. Ortuno and J. A. Del Rio, Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves, *Food Chem.* **68** (2000) 457–462; https://doi.org/10.1016/S0308-8146(99)00221-6

22. O. Benavente-Garcia, J. Castillo, F. R. Marin, A. Ortuno and J. A. del Rio, Uses and properties of *Citrus* flavonoids, *J. Agric. Food Chem.* **45** (1997) 4504–4515; https://doi.org/10.1021/jf970373s

23. Z. Xiang and Z. Ning, Scavenging and antioxidant properties of compound derived from chlorogenic acid in South-China honeysuckle, *LWT-Food Sci. Technol.* **41** (2008) 1189–1203; https://doi.org/10.1016/j.lwt.2007.08.006

24. M. Özyürek, K. Guclu and R. Apak, The main and modified CUPRAC methods of antioxidant measurement, *Trends Anal. Chem.* **30** (2011) 652–664; https://doi.org/10.1016/j.trac.2010.11.016

25. A. Lucić Vrdoljak, S. Žunec, B. Radić, R. Fuchs, D. Želježić and N. Kopjar, Evaluation of the cyto/genotoxicity profile of oxime K048 using human peripheral blood lymphocytes: An introductory study, *Toxicol. In Vitro* **28** (2014) 39–45; https://doi.org /10.1016/j.tiv.2013.06.007

26. A. Vulić, K. Durgo, J. Pleadin, L. Herceg and N. Kopjar, Mutagenicity and DNA-damaging potential of clenbuterol and its metabolite 4-amino-3,5-dichlorobenzoic acid *in vitro*, *Food Chem. Toxicol.* **77** (2015) 82–92; https://doi.org/10.1016/j.fct.2014.12.023

27. S. J. Duthie, A. R. Collins, G. G. Duthie and V. L. Dobson, Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes, *Mutat Res.* **393** (1997) 223–231; https://doi.org/10.1016/s1383-5718(97)00107-1

28. M. Czerwinska, A. K. Kiss and M. Naruszewicz, A comparison of antioxidant activity of oleuropein and its dialdehydic form oleacein, *Food Chem.* **131** (2012) 953–960; https://doi.org/10.1016/j.foodchem.2011.09.082

29. S. Bulotta, R. Corradino, M. Celano, M. D'Agostino, J. Maiuolo, M. Oliverio, A. Procopio, M. Iannone, D. Rotiroti and D. Russo, Antiproliferative and antioxidant effects of oleuropein and its semisynthetic peracetylated derivatives on breast cancer cells, *Food Chem.* **127** (2011) 1609–1614; https://doi.org/10.1016/j.foodchem.2011.02.025

30. A. Čabarkapa, L. Živković, D. Žukovec, N. Djelić, V. Bajić, D. Dekanski and B. Spremo-Potparević, Protective effect of dry olive leaf extract in adrenaline induced DNA damage evaluated using in vitro comet assay with human peripheral leukocytes, *Toxicol. In Vitro* **28** (2014) 451–456; https://doi.org/10.1016/j.tiv.2013.12.014

31. K. Kyriakopoulou, E. Katsanou, K. Machera and A. Charistou, Oleuropein protects Hep G2 cells against oxidative stress-induced DNA damage, *Toxicol Lett.* **211** (2012) S106; https://doi.org/10.1016/j.toxlet.2012.03.396

32. H. F. Al-Azzawie and M. S. lhamdani, Hypoglycemic and antioxidant effect of oleuopein in alloxan--diabetic rabbits, *Life Sci.* **78** (2006) 1371–1377; https://doi.org/10.1016/j.lfs.2005.07.02934

33. H. Jemai, I. Fki, M. Bouaziz, Z. Bouallaqui, A. El Feki, H. Isoda and S. Sayadi, Lipid-lowering and antioxidant effects of hydroxytyrosol and its triacetylated derivative recovered from olive tree leaves in cholesterol-fed rats, *J. Agric. Food Chem.* **56** (2008) 2630–2636; https://doi.org/10.1021/jf072589s

34. D. Kotyzova, A. Hodkov and V. Eybl, The effect of olive oil phenolics-hydroxytyrosol and oleuropein on antioxidant defence status in acute arsenic exposed rats, *Toxicol. Lett.* **205** (2011) S222; https://doi.org/10.1016/j.toxlet.2011.05.761