Turk J Pharm Sci 2021;18(4):420-429
DOI: 10.4274/tjps.galenos.2020.69008

ORIGINAL ARTICLE

Effects of Oleuropein on Epirubicin and Cyclophosphamide Combination Treatment in Rats

Oleuropeinin Sıçanlarda Epirubisin ve Siklofosfamid Kombinasyon Tedavisi Üzerine Etkileri

Metin Deniz KARAKOÇ[1]*, Selim SEKKİN[2]

[1]Aydın Adnan Menderes University Health Sciences Institute, Department of Pharmacology and Toxicology, Aydın, Turkey
[2]Aydın Adnan Menderes University Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Aydın, Turkey

ABSTRACT

Objectives: Oleuropein is the main bioactive polyphenolic compound in olive leaves, olive, and olive oil. Its anticancer, antioxidant, and antiinflammatory effects have been proven through several *in vitro* and *in vivo* studies. This study aimed to explore the effects of oleuropein on cyclophosphamide- and epirubicin-induced toxicity in female rats.

Materials and Methods: Seven groups containing eight rats in each group were formed. Four cycles of 16 mg/kg/week of cyclophosphamide and 2.5 mg/kg/week of epirubicin were administered to the rats through intraperitoneal injection. Oleuropein (150 mg/kg/week) was simultaneously applied via oral gavage. The effects of oleuropein were examined with hemogram tests in whole blood samples and biochemical analysis in serum samples. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in the serum samples were analyzed through enzyme-linked immunosorbent assay. Subsequently, a comet assay was performed using lymphocyte DNA. The levels of oxidant [i.e., malondialdehyde (MDA)] and antioxidants [i.e., catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD)] were measured in the heart, kidney, and liver tissues.

Results: Oleuropein could reduce DNA damage and serum TNF-α and IL-6 levels. It also ameliorated some hemogram and biochemical parameters that deteriorated due to antineoplastic drugs. It increased the amounts of antioxidants (GSH, SOD, and CAT) and reduced the level of MDA in the heart, kidney, and liver tissues.

Conclusion: Oleuropein might be a beneficial agent against toxicity caused by the combination treatment of cyclophosphamide and epirubicin. Further studies should be performed to demonstrate the protective effects of oleuropein against antineoplastic induced-toxicity precisely.

Key words: Oleuropein, epirubicin, cyclophosphamide, toxicity, oxidative stress

ÖZ

Amaç: Oleuropein zeytin yaprağı, zeytin meyvesi ve zeytinyağında bulunan başlıca biyoaktif polifenolik bileşiktir. Antikanser, antioksidan ve antienflamatuvar etkileri birçok *in vitro* ve *in vivo* çalışmayla doğrulanmıştır. Bu çalışma, oleuropeinin siklofosfamid ve epirubisin kaynaklı toksisite üzerindeki etkilerini dişi sıçanlarda araştırmayı amaçlanmıştır.

Gereç ve Yöntemler: Her grupta sekiz sıçan içeren yedi grup oluşturulmuştur. Sıçanlara intraperitoneal enjeksiyon yoluyla dört döngü 16 mg/kg/hafta siklofosfamid ve 2,5 mg/kg/hafta epirubisin uygulanmıştır. Oleuropein (150 mg/kg/hafta) eş zamanlı olarak oral gavaj yoluyla verilmiştir. Oleuropeinin etkileri tam kan örneklerinde hemogram testleri ve serum örneklerinde biyokimyasal analizlerle incelenmiştir. Serum numunelerindeki interlökin-6 (IL-6) ve tümör nekroz faktörü-α (TNF-α), enzime bağlı immünosorbent yöntemi ile analiz edilmiştir.

Ardından, lenfosit DNA'sı kullanılarak Comet yöntemi gerçekleştirilmiştir. Son olarak, kalp, böbrek ve karaciğer dokularında oksidan [malondialdehit (MDA)] ve antioksidan [katalaz (CAT), süperoksit dismutaz (SOD) ve glutatyon (GSH)] parametreler ölçülmüştür.

Bulgular: Oleuropeinin, DNA hasarını ve TNF-α ile IL-6 gibi proenflamatuvar sitokinlerin serum düzeylerini azaltabildiği belirlenmiştir. Ayrıca, oleuropeinin antineoplastik ilaçlara bağlı olarak bozulan bazı hemogram ve biyokimyasal parametrelerini düzelttiği tespit edilmiştir. Ayrıca, oleuropeinin kalp, böbrek ve karaciğer dokularında antioksidan parametrelerde (GSH, SOD ve CAT) artışa neden olduğu ve MDA miktarını azalttığı belirlenmiştir.

Sonuç: Oleuropein, siklofosfamid ve epirubisinin kombinasyon tedavisinin neden olduğu toksisiteye karşı faydalı bir ajan olabilir. Oleuropeinin antineoplastik kaynaklı toksisiteye karşı koruyucu etkilerini tam olarak göstermek için daha ileri çalışmalar yapılmalıdır.

Anahtar kelimeler: Oleuropein, epirubisin, siklofosfamid, toksisite, oksidatif stres

***Correspondence:** mdkarakoc@gmail.com, **Phone:** +90 258 263 93 11, **ORCID-ID:** orcid.org/0000-0003-3188-8738

Received: 25.06.2020, **Accepted:** 24.09.2020

INTRODUCTION

Breast tumors are the most frequently diagnosed cancer type and the leading cause of cancer-related deaths among women worldwide.[1] Oxidative stress induced by chronic inflammation is an important determinant in the progression of cellular changes, which help enhance in the production of reactive oxygen species (ROS) and the proliferation of cells. Inflammation caused by cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), has been linked to the increased production of ROS and breast tumor formation.[2,3]

Chemotherapy is a frequently used method to treat breast cancer. Although some antineoplastic drugs can be used as a single agent for chemotherapy, two or three antineoplastic agents are generally administered together as a combination therapy regimen to enhance their effectiveness.[4] For instance, cyclophosphamide is a frequently used alkylating agent in the treatment of various types of cancers.[5] Epirubicin is an anthracycline used in the treatment of various tumor types. It inhibits DNA, RNA, and protein synthesis through the intercalation of DNA, the hindering of topoisomerase II activity, and the production of reactive oxygen radicals. Epirubicin and cyclophosphamide can be used as single agents in breast cancer chemotherapy. Moreover, the combination treatment of epirubicin and cyclophosphamide is one of the frequently used chemotherapy regimens in the early and metastatic stages of breast tumor development.[6,7] Nonetheless, these agents are associated with an increased risk of hepatotoxicity, nephrotoxicity, cardiotoxicity, and hematologic toxicity in patients with breast cancer.[8-10] These toxic effects may reduce the quality of life of patients and affect the success of their treatment. As such, novel remedies should be investigated to reduce the toxic effects on patients with breast cancer under the combination chemotherapy of epirubicin and cyclophosphamide; through such treatments, morbidity can be minimized, and patients' quality of life can be improved.

Some plant-derived polyphenols have pharmacological effects, such as antiinflammatory, antioxidant, and antitumor properties. Therefore, studies have focused on the use of natural dietary antioxidants to alleviate the toxic effects of antineoplastic drugs.[11] Oleuropein (3,4-dihydroxyphenylelenolic acid) is a non-toxic secoiridoid glycoside and the major polyphenolic compound in olive tree (*Olea europaea* L.) and olive oil. It is responsible for the bitter taste of the leaves and fruits of olive tree. Oleuropein and its bioactive derivatives, such as hydroxytyrosol, have antioxidant, cardioprotective, anticancer, antiinflammatory, neuroprotective, and hepatoprotective effects by modulating several mechanisms.[12,13] In a novel study, oleuropein does not lead to a decrease in the efficacy of anthracycline-based chemotherapy in breast tumor-induced female BALB/c mice. On the contrary, oleuropein elicits a synergistic antitumoral effect with antineoplastic agents.[14] Therefore, our study aimed to explore the effects of oleuropein on epirubicin- and cyclophosphamide-induced toxicity in rats.

MATERIALS AND METHODS

The effects of oleuropein on cyclophosphamide- and epirubicin-induced toxicity in rats were examined using different methods. Whole blood samples were examined through hemogram tests, and serum samples were subjected to biochemical analysis. IL-6 and TNF-α in the serum samples were analyzed through enzyme-linked immunosorbent assay (ELISA). Subsequently, a comet assay was performed by using lymphocyte DNA. The levels of oxidant [i.e., malondialdehyde (MDA)] and antioxidants [i.e., catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD)] were measured in the heart, kidney, and liver tissues.

Chemicals

The following commercially available chemicals were used in this study: Oleuropein (high-performance liquid chromatography grade ≥98%; Santa Cruz Biotechnology®, Santa Cruz Biotechnology Inc., California, USA); epirubicin (Pirucin®, Saba Ilaç AŞ, Istanbul, Turkey); cyclophosphamide (Endoxan®, Baxter Oncology GmbH, Frankfurt, Germany); ketamine (Ketalar®, Pfizer Inc., Istanbul, Turkey); and xylazine (Rompun®, Bayer LLC, Istanbul, Turkey). All the other chemicals used in this study were obtained from Sigma-Aldrich Inc. (Missouri, USA).

Animals

Fifty-six healthy 3-month-old female Sprague-Dawley rats weighing 220 g±20 g were obtained from the Aydın Adnan Menderes University Experimental Animals Research and Application Center (Aydın, Turkey). They were kept inside polycarbonate cages in an air-conditioned room (23°C±2°C) and relative humidity of 50-55% with 12 h/12 h light/dark cycle. Standard rat food and water were provided ad libitum. The rats were held in the room and acclimatized to the laboratory environment for a week before the drug administration phase. This study was approved by the Local Ethics Committee for Experiments on Animals of Aydın Adnan Menderes University (Ethics Committee permission no: 64583101/2017/117) and performed in accordance with the ethical rules of the Helsinki Declaration.

Experimental design

The rats were separated into seven equal groups (n=8 in each group): Group I (control), 1 mL of saline administered once a week for four cycles; group II (epirubicin, "E"), 2.5 mg/kg/week epirubicin administered for four cycles; group III (cyclophosphamide "C"), 16 mg/kg/week cyclophosphamide administered for four cycles; group IV (epirubicin + cyclophosphamide, "EC"), 2.5 mg/kg/week epirubicin + 16 mg/kg/week cyclophosphamide administered for four cycles; group V (epirubicin + oleuropein, "EO"), 2.5 mg/kg/week epirubicin + 150 mg/kg/week oleuropein administered for four cycles; group VI (cyclophosphamide + oleuropein, "CO"), 16 mg/kg/week cyclophosphamide + 150 mg/kg/week oleuropein administered for four cycles; and group VII (epirubicin + cyclophosphamide + oleuropein, "ECO"), 2.5 mg/kg/week epirubicin + 16 mg/kg/week cyclophosphamide + 150 mg/kg/week oleuropein administered for four cycles.

Epirubicin and cyclophosphamide were administered via intraperitoneal (i.p.) injection, and oleuropein was administered through oral gavage (p.o.). Epirubicin, cyclophosphamide, and oleuropein were freshly prepared in saline and administered at the same time of the day in every cycle. The doses of epirubicin and cyclophosphamide administered to the rats in the study were determined by converting the human doses, which were stated in the United States National Comprehensive Cancer Network Guidelines (available at https://www.nccn.org). Dose conversions between humans and rats were calculated as described in the United States Food and Drug Administration Guidelines (available at https://www.fda.gov). The dose of oleuropein used in the study was determined from previous studies.[15-19]

The rats were treated for 4 weeks and anesthetized with 50 mg/kg ketamine (i.p) and 5 mg/kg xylazine (i.p.) 1 week after the last treatment. Blood samples were taken by cardiac puncture for comet assay, ELISA, hemogram tests, and biochemical analysis. Then, the rats were sacrificed, and the heart, liver, and kidneys were taken for the analysis of oxidant/antioxidant parameters. The organs were removed immediately and kept frozen (-80°C) until they were analyzed.

Hemogram tests

Blood samples were taken into tubes containing ethylene diamine tetra acetic acid. The samples were analyzed within the first hour after they were received from the rats. Routine hematological parameters such as leukocyte (WBC), lymphocyte (LYM), monocyte (MONO), granulocyte (GRA), lymphocyte %, monocyte % (MONO %), granulocyte % (GRA %), hemoglobin (HGB), erythrocyte (RBC), hematocrit (HCT), mean cell volume, mean cell hemoglobin concentration (MCHc), MCH, erythrocyte distribution width concentration (RDWc), platelet (PLT), platelet count/the values of other cells % ratio (PCT), platelet distribution width (PDWc), and platelet/cell number ratio (MPV) were analyzed using an automated Diatron® Abacus Junior Vet (Diatron Medical Instruments Plc, Hungary) hematology analyzer.

Biochemical analysis

Plasma was separated through centrifugation from the whole blood and used to determine biochemical parameters in rat serum: Urea, uric acid, creatinine, aspartate aminotransferase (AST), creatinine kinase (CK), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), CK-myocardial band (CK-MB) isoenzyme 3, direct biluribin, and total bilirubin. Analysis was initially performed within the first hour after the blood samples were taken from the rats by using a Roche® Cobas c501 autoanalyzer (Roche Diagnostics, Switzerland) and Roche® commercial kits.

Serum IL-6 and TNF-α levels were determined using a commercially available Thermo Fisher® (Thermo Fisher Scientific Co., USA) ELISA kits in accordance with the manufacturer's instructions.

Comet assay

The comet assay protocol was implemented as described in Singh et al.[20] In brief, lymphocytes were placed to low-melting agarose (0.7% in phosphate-buffered saline) and placed in a lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris-HCl, pH 10, containing freshly added before use 1% Triton X-100 and 10% dimethyl sulfoxide) for 1 h at 4°C. Then, the samples were placed on slides.

The slides were placed on a horizontal Cleaver® gel electrophoresis tank connected to a Cleaver® Scientific CS 300 power supply (Cleaver Scientific Ltd., UK) and a Julabo® FL300 recirculating cooler (Julabo GmbH, Germany). The tank was filled with alkaline solution (1 mM Na_2EDTA and 300 mM NaOH, pH 13) until the liquid level covered the samples. Then, the lid of the tank was closed and waited for 30 min at 4°C. Electrophoresis was continued at 4°C in the dark for 30 min at 25 V and 300 mA, and the samples were rinsed with 400 mM Tris buffer with pH 7.5 for 7 min to neutralize surplus alkali. The neutralized slides were kept in ethanol for 5 min and then dried at room temperature. The slides were stained with 70 µL (10 µg/mL) 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) before microscopic examination.

Comets were analyzed after the specimens were stained at 400x magnification by using a Leica® fluorescence microscope (Leica Microsystems GmbH, Germany) equipped with a 50 W mercury lamp. Then, 100 cells were randomly counted for each slide. The extension of each comet was examined through Comet Assay® IV (Perceptive Instruments Ltd., UK) computerized image analysis. The damage ratio of each sample was expressed as "tail moment" and "% tail intensity," as described in Collins et al.[21]

Measurement of oxidant/antioxidant parameters

In this procedure, 0.5 g of tissue samples was taken from each organ (kidneys, heart, and liver) for homogenization. Tissues were homogenized at 2.000 rpm for 1 min by using a Teflon-glass stirrer (IKA Overhead Stirrer; IKA-Werke GmbH & Co., KG, Germany) in a 10-fold volume of ice-cold 10% of 150 mM phosphate buffer (pH 7.4). The homogenate was centrifuged (Hettich Zentrifugen, Mikro 200 R, Germany) at 12.000 rpm for 10 min at 4°C. The supernatants, referred to as homogenate, were stored at -80°C (Glacier Ultralow Temperature Freezer, Japan) until CAT, SOD, GSH, and MDA levels were examined. All oxidant/antioxidant levels were analyzed through spectrophotometric measurements by using a Shimadzu® ultraviolet-1601 (Shimadzu Scientific Instruments, Japan) spectrophotometer. Protein concentrations were measured in accordance with the Biuret method by using a spectrophotometer and commercially available kits (Archem Diagnostic Ind., Ltd., Turkey). The results were expressed in milligrams per milliliter of protein.

MDA levels were determined as described previously by Ohkawa et al.[22] The MDA concentration (nmol/mg of tissue protein) was calculated on the basis of absorbance: Absorbance coefficient (ε)=1.56×10^5/M/cm. CAT activity (k/mg tissue protein) was evaluated as described by Aebi.[23] SOD activity (U/mg of tissue protein) was determined as described by Sun et al.[24] GSH levels (mg/g tissue protein) were spectrophotometrically determined at 412 nm by using the method described by Tietze.[25]

Statistical analysis

Data were statistically analyzed using SPSS 22.0. All the parameters were examined for the homogeneity of variance with Levene's test and normal distribution with the Shapiro-Wilk test. The intra-group repeated measurements of rat weights were evaluated with the Friedman test. Because of the Shapiro-Wilk test results did not match normal distribution, data were compared between groups by using Kruskal-Wallis ANOVA. Post-hoc binary comparisons were performed using the Bonferroni-corrected Mann-Whitney U test. Differences were considered statistically significant if $p<0.05$. All data were expressed as mean ± standard deviation.

RESULTS

Body weight changes

In this study, the weight of the rats in the combination therapy (EC) group significantly decreased, whereas the weight of the rats in all the other groups significantly increased ($p<0.05$). Although the initial body weights did not vary among the groups, the weights of the control group significantly differed from those of the E, C, EC, and CO groups at the end of the study ($p<0.05$). The results of the body weight measurements of the rats from the day of the determination of the groups (day 0) until the day the study was terminated (day 35) are presented in Table 1.

Hemogram tests

MONO, GRA, MONO %, GRA %, RDWc, PCT, MPV, and PDWc did not significant vary ($p>0.05$), but these data were not shown to simplify the results in the table. The results of the hemogram tests and their statistical differences between the experimental groups are given in Table 2.

Most of the parameters generally deteriorated in all the groups administered with the antineoplastic drugs alone in the single and combination therapies. These results significantly differed from those observed in the control group ($p<0.05$). WBC, LYM, RBC, HGB, HCT, and PLT, which are related to neutropenia and bone marrow suppression, significantly varied between the groups treated with antineoplastic drugs alone (E, C, and EC) and the groups treated with oleuropein + antineoplastic drug (EO, CO, and ECO; $p<0.05$). However, the hemogram test results of the groups treated with oleuropein + antineoplastic drug were generally closer to those of the healthy control group than to the groups treated with antineoplastic drugs alone.

Biochemical analysis

The biochemical parameters and their significant differences between the experimental groups are given in Table 3. Urea, GGT, direct bilirubin, and CK parameters did not significantly differ among the groups ($p>0.05$). Therefore, these data were not shown to simplify the results in the table.

AST and ALT, which are important biochemical indicators of liver damage, significantly differed ($p<0.05$) between the groups treated with antineoplastic drugs alone (E, C, and EC) and the groups treated with oleuropein + antineoplastic drugs (EO, CO, and ECO). Oleuropein ameliorated these parameters and decreased them to levels similar to those of the healthy control group ($p>0.05$). These biochemical parameters indicated that oleuropein helped alleviate liver damage. Although oleuropein was beneficial to some parameters, which are related to heart and kidney damages, no considerable effects were observed in most of the other parameters.

The administered antineoplastic agents as single or in combination significantly increased the serum IL-6 and TNF-α levels compared with those in the control group ($p<0.05$). These levels decreased and were similar to those of the healthy control group in all oleuropein-treated groups ($p>0.05$). The results of the ELISA tests are presented in Figure 1.

Comet assay

The tail moment and % tail intensity results of the comet assay are presented in Figure 2. The degree of DNA damage was considerably higher in the experimental groups than in

Table 1. Body weight measurement results

Groups (n=8)	Day 0	Day 7*	Day 14	Day 21	Day 28	Day 35**	p***	X^2
Control	210.0±0.007	216.1±0.012	224.5±0.011$^{a, b}$	232.1±0.013$^{a, b, c, d}$	240.0±0.014$^{a, b, c, d, e}$	247.7±0.018$^{a, b, c, d}$	p<0.001	39.014
E	209.2±0.009	212.6±0.008	214.8±0.007	218.8±0.006^{a}	223.5±0.006^{a}	222.3±0.013^{a}	p<0.001	35.180
C	218.5±0.013	221.1±0.014	223.6±0.011^{c}	225.2±0.009	228.3±0.006^{b}	228.0±0.014^{b}	p<0.05	18.157
EC	216.5±0.003	221.3±0.008	217.1±0.007^{d}	212.6±0.006$^{b, e}$	206.2±0.005^{cf}	200.2±0.005^{ce}	p<0.001	36.025
EO	207.3±0.003	209.7±0.006	214.2±0.007^{a}	217.3±0.008^{c}	228.0±0.010^{d}	235.0±0.012	p<0.001	37.929
CO	209.1±0.008	212.8±0.007	214.3±0.010$^{b, c}$	219.2±0.010^{d}	225.1±0.010^{e}	232.8±0.010^{d}	p<0.001	36.577
ECO	215.5±0.009	218.7±0.009	227.8±0.009^{d}	232.0±0.010^{e}	236.5±0.009^{f}	241.2±0.011^{e}	p<0.001	38.000
p****	p>0.05	p>0.05	p<0.05	p<0.05	p<0.05	p<0.05	-	-
X^2	11.266	10.616	15.524	22.423	31.647	30.044		

*Day of the first drug administration, **Day of sacrification, ***Intra-group Friedman test results, ****Kruskal-Wallis analysis of body weight changes between groups. $^{a, b, c, d, e}$The same superscript letters in the columns indicate significant differences with one another. Differences were considered statistically significant if $p<0.05$. Weight is expressed in grams. E: Epirubicin, C: Cyclophosphamide, EC: Epirubicin + cyclophosphamide, EO: Epirubicin + oleuropein, CO: Cyclophosphamide + oleuropein, ECO: Epirubicin + cyclophosphamide + oleuropein. The results are given as mean ± standard deviation

Table 2. Hemogram results of the experimental groups

	Groups (n=8)							p
Parameters	Control	E	C	EC	EO	CO	ECO	X^2
WBC	6.02±0.39 $^{a, b, c, d}$	3.45±0.29$^{a, e}$	3.41±1.48$^{b, c}$	3.10±0.48$^{c, e}$	4.65±0.51$^{d, e}$	5.73±0.98^{c}	5.70±0.29^{e}	p<0.001 40.491
LYM	3.53±0.41$^{a, b, c}$	1.98±0.88$^{a, d}$	1.83±0.50$^{b, e}$	1.71±0.41$^{c, f}$	3.54±0.37^{d}	3.42±0.18^{e}	3.37±0.63^{f}	p<0.001 34.950
LY %	73.86±17.43$^{a, b, c, d, e}$	56.38±17.14^{a}	47.04±17.54^{b}	44.70±15.09^{c}	70.24±15.11	58.91±22.67^{d}	59.49±17.13^{e}	p<0.05 17.538
RBC	6.94±0.34$^{a, b, c, d}$	5.76±0.79$^{a, e}$	5.91±0.36$^{b, f}$	5.58±1.13$^{c, g}$	6.70±0.25^{e}	6.77±0.47^{f}	6.57±0.31$^{d, g}$	p<0.001 31.528
HGB	11.70±0.49$^{a, b, c, d}$	9.66±1.34$^{a, e}$	10.73±0.88$^{b, f}$	9.41±0.99$^{c, g}$	11.25±0.30$^{d, e}$	11.77±0.44^{f}	11.64±0.50^{g}	p<0.001 35.829
HCT	40.85±2.74$^{a, b, c, d}$	33.99±4.32$^{a, e}$	36.80±3.37$^{b, f}$	33.07±5.44$^{c, g}$	39.40±1.30^{e}	43.15±3.72^{f}	36.80±7.16$^{d, g}$	p<0.001 33.134
MCV	58.00±0.75$^{a, b, c}$	59.13±1.13	65.13±5.82^{a}	59.88±4.48	58.75±1.22	64.00±5.40^{b}	61.25±4.20^{c}	p<0.05 17.034
MCH	17.03 ± 0.42^{a}	21.99±14.56	23.73±15.11^{a}	17.26±1.24	16.61±0.51	17.65±1.07	17.24±1.21	p<0.05 13.259
MCHc	29.28±0.69$^{a, b, c, d, e}$	28.36±0.56^{a}	28.08±0.84^{b}	28.83±0.72	28.24±0.48^{c}	27.71±1.09^{d}	28.23±0.96^{e}	p<0.05 14.908
PLT	705.48±214$^{a, b, c, d}$	509.37±174$^{a, e}$	483.63±122$^{b, f}$	674.50±163^{g}	860.63±117$^{c, e}$	675.25±131^{f}	1006.38±110$^{d, g}$	p<0.001 33.510
MPV	9.43±3.60$^{a, b, c, d}$	9.16±5.78$^{a, e}$	8.81±2.57$^{b, f}$	9.80±7.55^{g}	9.76±5.30$^{c, e}$	9.08±5.59^{f}	9.08±8.21$^{d, g}$	p>0.05 2.371

WBC, LYM, and PLT are expressed in 10^9/L; RBC is expressed in 10^{12}/L; other parameters are presented in percentage (%).$^{a, b, c, d, e, f, g}$The same superscript letters in the lines indicate significant differences with one another. Differences were considered statistically significant if p<0.05. E: Epirubicin, C: Cyclophosphamide, EC: Epirubicin + cyclophosphamide, EO: Epirubicin + oleuropein, CO: Cyclophosphamide + oleuropein, ECO: Epirubicin + cyclophosphamide + oleuropein. The results are given as mean ± standard deviation. WBC: Leukocyte, LYM: Lymphocyte, LY %: Lymphocyte %, RBC: Erythrocyte, HGB: Hemoglobin, HCT: Hematocri, MCV: Mean cell volume, MCH: Mean cell hemoglobin, MCHc: Mean cell hemoglobin, PLT: Platelet, MPV: Mean platelet volume

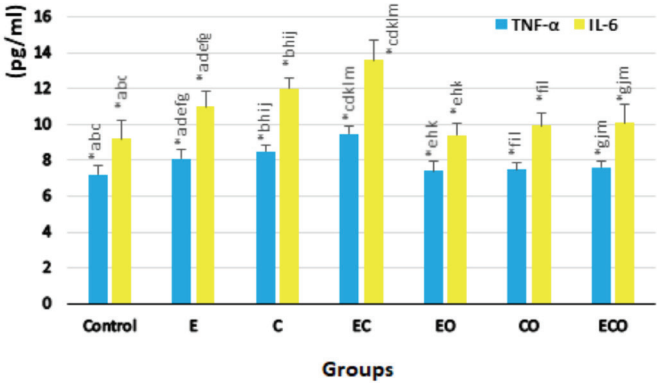

Figure 1. Serum TNF-α and IL-6 levels of the experimental groups; results were expressed as mean ± standard deviation. *Whiskers on the boxes indicates standard deviation. The same superscript letters on the boxes indicates significant statistical difference with each other. Differences were considered statistically significant if p<0.05. E: Epirubicin, C: Cyclophosphamide, EC: Epirubicin + cyclophosphamide, EO: Epirubicin + oleuropein, CO: Cyclophosphamide + oleuropein, ECO: Epirubicin + cyclophosphamide + oleuropein, TNF-α: Tumor necrosis factor-α, IL-6: Interleukin-6.

the control group. However, the considerable DNA damage caused by EC significantly decreased in the ECO (p<0.001). The fluorescent microscope images of some DNA samples are shown in Figure 3.

Oxidant/antioxidant levels

The levels of MDA and GSH and the measurement of SOD and CAT activities in the liver, heart, and kidneys of the experimental groups are given in Table 4. The SOD activity was considerably higher in the ECO group than in the EC group (p<0.05). Moreover, the SOD activity in the heart did not significantly vary between the control and ECO groups (p>0.05).

Oleuropein showed protective effects on the heart tissues by increasing GSH levels and decreasing MDA levels. The increased MDA levels were due to the decrease in the antineoplastic drugs (p<0.05) in the oleuropein-treated groups, but these levels decreased to levels similar to those of the control group (p>0.05). Although the GSH levels in the groups treated with antineoplastic drugs alone (E, C, and EC) decreased, these levels increased remarkably (p<0.05) in the oleuropein-treated groups (EO, CO, and ECO) and reached levels similar to those of the control group (p>0.05).

Table 3. Biochemical parameters of the experimental groups

	Groups (n=8)							p
Parameter	Control	E	C	EC	EO	CO	ECO	X^2
Creatinine	0.46±0.03[a]	0.41±0.05[b]	0.49±0.07	0.51±0.07	0.51±0.05[b]	0.48±0.04	0.58±0.11[a]	p<0.05 17.707
Uric acid	1.31±0.34[a, b, c, d, e, f]	1.93±0.57[a]	2.32±0.46[b]	2.51±0.71[c]	2.05±0.22[d]	2.08±0.77[e]	2.55±0.65[f]	p<0.05 20.705
AST	169.63±29.97[a, b, c, d]	211.87±43.00[a, e]	219.25±35.09[b, f]	210.88±30.38[c, g]	141.00±22.86[e]	135.88±25.39[d, f]	152.38±29.57[g]	p<0.001 32.313
ALT	54.25±5.65[a, b, c, d]	63.25±4.06[a, e]	62.38±7.13[b, f]	64.63±4.98[c, g]	48.25±4.98[d, e]	52.13±5.80[f]	52.88±7.04[g]	p<0.001 32.844
Total bilirubin	0.03±0.01[a, b]	0.03±0.01	0.06±0.01[a]	0.03±0.01	0.02±0.01	0.04±0.01[b]	0.03±0.01	p<0.05 19.559
CK-MB	1207.30±142.56[a, b, c, d, e]	1287.50±240.28	746.75±216.14[a]	819.25±147.39[b]	893.88±149.67[c]	987.13±308.33[d]	792.88±170.75[e]	p<0.001 27.203

Creatine kinase myocardial band (CK-MB), total bilirubin, alanine transaminase (ALT), and aspartate transaminase (AST) are expressed in units per liter. Uric acid and creatinine are expressed in milligrams per deciliter. [a, b, c, d, e, f, g]The same superscript letters in the lines indicate significant differences with one another. Differences were considered statistically significant if p<0.05. E: Epirubicin, C: Cyclophosphamide, EC: Epirubicin + cyclophosphamide, EO: Epirubicin + oleuropein, CO: Cyclophosphamide + oleuropein, ECO: Epirubicin + cyclophosphamide + oleuropein. The results are given as mean ± standard deviation

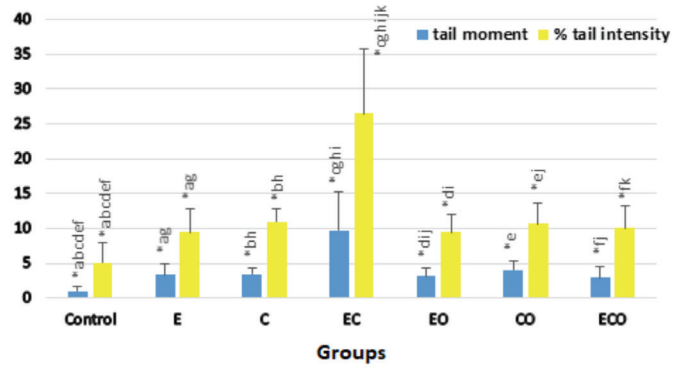

Figure 2. Tail moment and % tail intensity results of the comet assay; results were expressed as mean ± standard deviation. *Whiskers on the boxes indicates standard deviation. The same superscript letters on the boxes indicates significant statistical difference with each other. Differences were considered statistically significant if p<0.05. E: Epirubicin, C: Cyclophosphamide, EC: Epirubicin + cyclophosphamide, EO: Epirubicin + oleuropein, CO: Cyclophosphamide + oleuropein, ECO: Epirubicin + cyclophosphamide + oleuropein.

The SOD and CAT activities in the kidneys of the rats treated with the antineoplastic drugs alone (E, C, and EC) significantly decreased compared with those of the rats in the control group (p<0.05). The administration of oleuropein led to an amelioration of SOD and CAT activities in the kidneys, thereby reaching levels similar to those of the healthy control group.

The MDA levels in the kidneys were lower in the ECO group than in the EC group (p<0.05). However, the differences between the healthy control group and the ECO group were not significant (p>0.05). This result indicated that oleuropein could significantly lower the elevated MDA level, which is a marker of oxidative damage. Therefore, it showed cell-protective effects on the kidneys.

The results of the GSH, SOD, and CAT analysis in the liver tissues were generally compatible with one another. In most of the oleuropein-treated groups, the antioxidant parameters were similar (p>0.05) to those of the healthy control group and significantly higher (p<0.05) than those of the groups treated with antineoplastic drugs alone. Oleuropein increased the antioxidant capacity in the liver tissues to levels similar to those

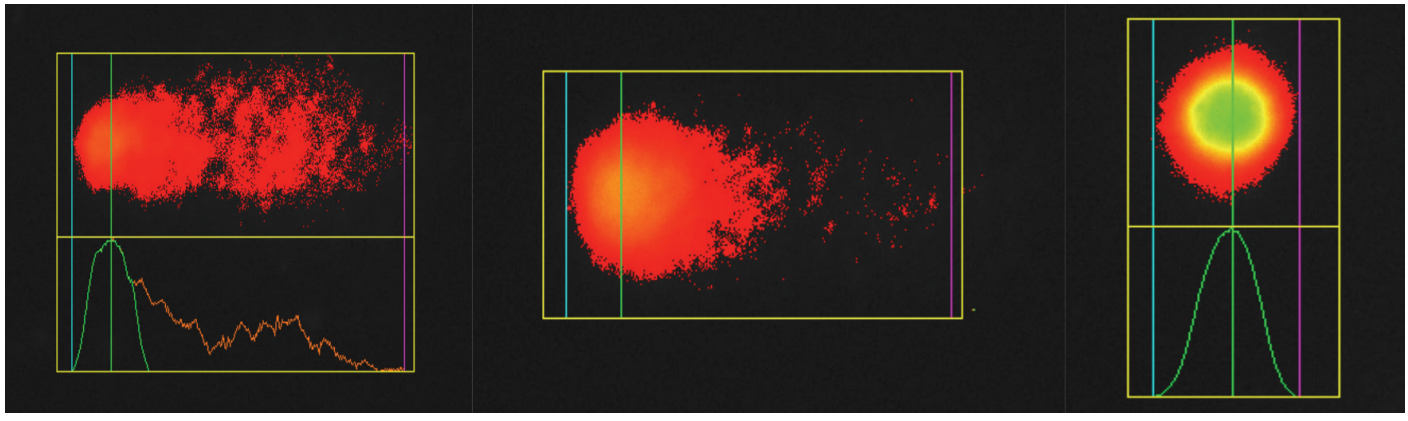

Figure 3. Fluorescence microscopy images of highly damaged (left), moderately damaged (middle), and intact (right) DNA samples in comet analysis

Table 4. Measurement results of MDA and GSH levels and CAT and SOD activities in the heart, kidney, and liver tissues of the experimental groups

	Groups (n=8)							p	X^2
Parameters*	Control	E	C	EC	EO	CO	ECO		
Heart									
SOD	7.04±0.63[a, b, c, d, e]	6.39±0.14[a]	5.36±2.17[b]	3.40±2.17[c, f]	6.44±0.32[d]	6.16±0.35[e]	6.74±0.16[f]	p<0.001	28.420
CAT	3.88±1.23[a, b, c, d, e, f]	1.86±0.71[a]	1.75±0.53[b]	1.68±0.53[c]	2.28±0.65[d]	1.69±0.45[e]	1.74±0.38[f]	p<0.001	21.767
GSH	13.70±2.53[a, b, c]	9.58±2.53[a, d]	9.88±2.54[b, e]	9.43±2.54[c, f]	14.37±3.59[d]	13.70±3.02[e]	13.47±1.35[f]	p<0.001	23.871
MDA	70.08±6.08[a, b, c]	93.65±4.78[a, d]	90.88±13.43[b, e]	99.98±13.43[c, f]	75.99±7.18[d]	76.93±16.47[e]	77.92±5.52[f]	p<0.001	32.860
Kidneys									
SOD	7.40±0.22[a, b, c, d]	6.69±0.27[a, e]	6.31±1.20[b, f]	5.96±1.20[c, g]	7.24±0.39[e]	7.10±0.28[d, f]	7.16±0.21[g]	p<0.001	36.481
CAT	3.74±1.93[a, b]	2.82±0.88[c]	3.65±1.01[d]	2.49±1.01[e]	6.80±1.88[a, c]	6.29±1.34[b, d]	4.95±2.20[e]	p<0.001	30.219
GSH	20.43±2.53[a, b, c, d, e, f]	13.77±2.35[a, g]	11.90±3.02[b, h]	8.98±3.02[c, i]	17.07±2.88[d, g]	16.62±3.00[e, h]	16.39±2.61[f, i]	p<0.001	33.858
MDA	49.11±2.93[a, b, c, d, e]	61.10±5.77[a]	70.87±9.04[b, f]	74.62±9.04[c, g]	54.38±5.83[d]	60.34±8.00[e, f]	52.30±2.86[g]	p<0.001	32.409
Liver									
SOD	7.04±0.15[a, b, c, d, e]	6.39±0.33[e]	5.36±0.20[b, f]	6.54±0.02[c, g]	7.49±0.02[e]	7.27±0.13[d, f]	7.37±0.15[g]	p<0.001	41.283
CAT	3.88±7.22[a]	1.86±5.55[b]	1.75±5.65	6.18±5.65[c]	7.90±3.49[b]	21.34±16.96[a]	15.07±7.74[c]	p<0.05	20.521
GSH	13.70±3.81[a, b, c, d]	9.58±2.35[a, e]	9.88±2.30[b, f]	11.90±2.30[c, g]	21.78±3.38[e]	19.76±3.59[d, f]	19.98±2.02[g]	p<0.001	33.699
MDA	70.08±2.08[a, b, c, d, e, f]	93.65±7.80[a, g]	90.88±12.04[b, h]	88.25±12.05[c, i]	72.01±2.63[d, g]	77.54±5.86[e, h]	78.39±8.01[f, i]	p<0.001	31.320

*MDA is expressed in nanomole per milligram of protein; GSH is expressed in milligrams per gram of protein; CAT is expressed in k per milligram of protein; SOD is expressed in units per milligram of protein. [a, b, c, d, e, f, g, h, i]The same superscript letters in the lines indicate significant differences with one another. Differences were considered significant if p<0.05. SOD: Superoxide dismutase, CAT: Catalase, GSH: Reduced glutathione, MDA: Malondialdehyde; E: Epirubicin, C: Cyclophosphamide, EC: Epirubicin + cyclophosphamide, EO: Epirubicin + oleuropein, CO: Cyclophosphamide + oleuropein, ECO: Epirubicin + cyclophosphamide + oleuropein. The results are given as mean ± standard deviation

of the control group. By contrast, it decreased oxidative stress and degradation products. Unlike the SOD, GSH, and CAT levels in the cardiac and renal tissues, their levels in the liver were even higher in the oleuropein-treated groups than in the healthy control group.

DISCUSSION

In this study, the efficacy of oleuropein against the toxic effects of the combination therapy of epirubicin and cyclophosphamide, which are frequently used to treat breast tumors, was investigated using healthy female rats. This study was the first to demonstrate the effects of oleuropein against toxicity induced by a combination chemotherapy based on anthracycline and alkylating agents. Many parameters were investigated and important data were obtained in this study, but this study had limitations because it did not involve a histopathological examination of the tissues and an investigation of other serum cytokine levels, such as IL-8 and IL-1β, which are involved in inflammation.

Weight loss, vomiting, appetite loss, anorexia, and neutropenia are the important side effects of the combination therapy of epirubicin and cyclophosphamide.[26] In our study, the initial and final body weights of the rats in the EC group significantly differed (p<0.05). Similar weight gain results were detected in the ECO group compared with those in the healthy control group (p>0.05). These results suggested that oleuropein might be a useful agent to prevent the weight loss of the rats in this combination chemotherapy group. However, a previous study showed that rats in oleuropein-treated groups that receive a fat-rich diet gain less weight than those in other groups (p<0.05).[27] Another study has demonstrated that rats administered with oleuropein + bisphenol A (BPA) have less weight gains than those treated with BPA alone (p<0.05).[28] Based on the outcomes of these studies, our conclusion was that oleuropein treatment likely had a regulatory role in various mechanisms by which an ideal weight could be maintained rather than inducing weight loss or weight gain.

A limited number of studies on the effects of oleuropein or other olive products (leaves, fruits, olive oil, and olive mill waste water extracts) have examined the hemogram parameters. In one study, oleuropein administered to rats with cisplatin-induced toxicity ameliorates the hemogram parameters that reach levels similar to those of the healthy control group.[29] These consistent results indicated that oleuropein might have beneficial ameliorating effects on most of the hemogram parameters, which deteriorated because of epirubicin and cyclophosphamide toxicity.

The comet assay showed that oleuropein might be a beneficial agent that could reduce the oxidative DNA damage caused by antineoplastics. In literature search, no data were found in the effects of oleuropein in terms of a comet assay for epirubicin and cyclophosphamide toxicity. As such, studies

have compared oleuropein-rich olive plant (leaf, fruit, and olive mill waste water extracts) and olive oil extracts between mouse and human peripheral mononuclear blood cells. These compounds significantly reduce oxidative damage levels and show DNA protective activities.[30-34] In this regard, our results were consistent with previous findings.

The biochemical parameter results indicated that oleuropein could reduce the elevated AST and ALT levels, which are associated with liver damage. However, no significant differences were observed in most parameters. In a previous study, oleuropein administration considerably decreased the elevated levels of AST, ALT, urea, and creatinine in rats with BPA-induced toxicity.[35] In another study, oleuropein significantly decreased the increased ALT and AST levels in a hepatic fibrosis mouse model and may be a pharmacologically useful agent in hepatic fibrosis.[36]

The increased risk of myocardial infarction is an important side effect of anthracycline-derived drugs.[26] CK-MB is an important biochemical marker in monitoring the risk of myocardial infarction.[37] In our study, conflicting results were obtained on the effects of oleuropein in the CK-MB parameter. These results might be related to the dose of oleuropein administered.[16] Therefore, the pharmacological effects on this parameter might be observed through the administration of higher doses.

Proinflammatory cytokines, such as TNF-α and IL-6, are involved in the formation and progression of breast tumors.[2,38] Most antineoplastic agents used in the treatment of breast tumors cause an increase in tissue and serum IL-6 and TNF-α levels.[39-41] In another study, the effects of different doses of oleuropein (5, 10, and 20 mg/kg) are investigated against cisplatin-induced toxicity in mice. Cyclooxygenase-2, nuclear factor kappa B, and TNF-α levels in the kidneys decreased compared with the administered dose of oleuropein, which may help reduce cisplatin-induced toxicity in the kidneys.[40] In another study on human synovial sarcoma cells (SW982), IL-6 and TNF-α levels increase as a result of the induction of IL-1β-mediated inflammation and significantly decrease after oleuropein administration compared with those in the control group.[42] Several studies have investigated the effect of oleuropein against the toxicity induced by different agents in terms of serum and tissue cytokine levels in rats. They have shown that oleuropein can significantly reduce IL-6 and TNF-α levels in serum and tissues and elicit protective effects on organs. In other studies, oleuropein can decrease the elevated IL-6 and TNF-α levels induced by various compounds in direct proportion with doses.[39,43-45] In this regard, previous findings were consistent with our results. Animal experiments have also revealed that oleuropein can decrease IL-6 and TNF-α levels in a wide dose range (10-2000 mg/kg/day). The selected dose in our study (150 mg/kg/week) also decreased the levels of these cytokines compared with that in the healthy control group. Similar results were obtained even at different doses in these studies. This situation might be related to the purity of oleuropein used in previous studies.

Oxidative stress plays a crucial role in the pathogenesis of the hepatotoxic, nephrotoxic, and cardiotoxic effects of epirubicin and cyclophosphamide.[7,10] The protective effects of oleuropein have been attributed to several mechanisms, such as the reduction of nitrosative and oxidative stress and antiinflammatory and antioxidant activities.[12,13,39] In our study, the reduced GSH, CAT, SOD, and MDA levels in the kidney, hepatic, and cardiac tissues were determined as indicators of oxidative stress and organ damage. Reduced GSH plays a major role in cellular defense against toxicity and ROS scavenging. SOD converts highly reactive superoxide anions to H_2O_2. Subsequently, CAT is the enzyme responsible for the conversion of H_2O_2 formed in cellular processes into molecular oxygen and water. Moreover, MDA is a lipid peroxidation product and indicator of oxidative damage caused by ROS generation.[46-48]

Our study showed that oleuropein could decrease the MDA level and increase the GSH level and SOD and CAT activities in the liver, heart, and kidneys of the rats compared with those of the rats treated with antineoplastic drugs alone. However, studies have yet to reveal the effects of oleuropein on endogenous oxidant/antioxidant parameters in the combination chemotherapy of epirubicin and cyclophosphamide. Therefore, our findings were compared with the results of studies on the effects of oleuropein against toxicity induced by cyclophosphamide, doxorubicin, and other chemical agents in rats. In these studies, oleuropein was administered in a wide dose range of between 5 and 2000 mg/kg/day. These studies reported that oleuropein decreased the amount of MDA and increased the amount of antioxidant markers (GSH, SOD, and CAT) in many organs, including the liver, heart, and kidneys, at a dose of ≥10 mg/kg/day.[15-19,33,35,39] Our findings were parallel to previous results. Furthermore, studies have explored different doses and demonstrated that total antioxidant capacity also increases as the administration doses of oleuropein increase.[16,18,39,48]

CONCLUSION

Oleuropein is a promising compound with antiinflammatory and antioxidant effects against chemical toxicity. Its anti-tumoral properties and synergistic effects with some antineoplastics increase the importance of oleuropein, especially in patients with cancer. This study showed that oleuropein could reduce the levels of DNA damage and serum proinflammatory cytokines, such as TNF-α and IL-6. It ameliorated some of the deteriorated hemogram (WBC, LYM, and HGB) and biochemical parameters (AST, ALT, and total bilirubin) because of antineoplastic drugs. Oleuropein could also increase the amounts of antioxidant parameters (SOD, CAT, and GSH) in the tissues and caused a decrease in the levels of MDA, which is a cellular degradation product. These results suggested that oleuropein might have protective effects against the toxicity induced by the combination chemotherapy of epirubicin and cyclophosphamide. Further studies should be performed to demonstrate the protective effects of oleuropein against antineoplastic induced-toxicity precisely.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394-424.
2. Alokail MS, Al-Daghri NM, Mohammed AK, Vanhoutte P, Alenad A. Increased TNF α, IL-6 and ErbB2 mRNA expression in peripheral blood leukocytes from breast cancer patients. Med Oncol. 2014;31:38.
3. Esquivel-Velázquez M, Ostoa-Saloma P, Palacios-Arreola MI, Nava-Castro KE, Castro JI, Morales-Montor J. The role of cytokines in breast cancer development and progression. J Interferon Cytokine Res. 2015;35:1-16.
4. Jiang L, Jing C, Kong X, Li X, Ma T, Huo Q, Chen J, Wang X, Yang Q. Comparison of adjuvant ED and EC-D regimens in operable breast invasive ductal carcinoma. Oncol Lett. 2016;12:1448-1454.
5. Rehman MU, Tahir M, Ali F, Qamar W, Lateef A, Khan R, Quaiyoom A, Oday-O-Hamiza, Sultana S. Cyclophosphamide-induced nephrotoxicity, genotoxicity, and damage in kidney genomic DNA of Swiss albino mice: the protective effect of Ellagic acid. Mol Cell Biochem. 2012;365;119-127.
6. Mirzaei HR, Nasrollahi F, Mohammadi Yeganeh L, Jafari Naeini S, Bikdeli P, Hajian P. Dose-dense epirubicin and cyclophosphamide followed by weekly paclitaxel in node-positive breast cancer. Chemother Res Pract. 2014;2014:259312.
7. Li H, Hu B, Guo Z, Jiang X, Su X, Zhang X. Correlation of UGT2B7 polymorphism with cardiotoxicity in breast cancer patients undergoing epirubicin/cyclophosphamide-docetaxel adjuvant chemotherapy. Yonsei Med J. 2019;60:30-37.
8. Domercant J, Polin N, Jahangir E, Cardio-oncology: a focused review of anthracycline-, human epidermal growth factor receptor 2 inhibitor-, and radiation-induced cardiotoxicity and management. Ochsner J. 2016;16:250-256.
9. Ejlertsen B, Tuxen MK, E. H. Jakobsen EH, Jensen MB, Knoop AS, Højris I, Ewertz M, Balslev E, Danø H, Vestlev PM, Kenholm J, Nielsen DL, Bechmann T, Andersson M, Cold S, Nielsen HM, Maae E, Carlsen D, Mouridsen HT. adjuvant cyclophosphamide and docetaxel with or without epirubicin for early TOP2A-normal breast cancer: DBCG 07-READ, an open-label, phase III, randomized trial. J Clin Oncol. 2017;35:2639-2646.
10. Kurauchi K, Nishikawa T, Miyahara E, Okamoto Y, Kawano Y. Role of metabolites of cyclophosphamide in cardiotoxicity. BMC Res Notes. 2017;10:406.
11. León-González AJ, Auger C, Schini-Kerth VB. Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. Biochem Pharmacol. 2015;98:371-380.
12. Shamshoum H, Vlavcheski F, Tsiani E. Anticancer effects of oleuropein. Biofactors. 2017;43:517-528.
13. Ahmad Farooqi A, Fayyaz S, Silva AS, Sureda A, Nabavi SF, Mocan A, Nabavi SM, Bishayee A. Oleuropein and cancer chemoprevention: the link is hot. Molecules. 2017;22:705.
14. Elamin MH, Elmahi AB, Daghestani MH, Al-Olayan EM, Al-Ajmi RA, Alkhuriji AF, Hamed SS, Elkhadragy MF. Synergistic anti-breast-cancer effects of combined treatment with oleuropein and doxorubicin *in vivo*. Altern Ther Health Med. 2019;25;17-24.
15. Alirezaei M, Dezfoulian O, Neamati S, Rashidipour M, Tanideh N, Kheradmand A. Oleuropein prevents ethanol-induced gastric ulcers via elevation of antioxidant enzyme activities in rats. J Physiol Biochem. 2012;68:583-592.
16. Janahmadi Z, Nekooeian AA, Moaref AR, Emamghoreishi M. Oleuropein offers cardioprotection in rats with acute myocardial infarction. Cardiovasc Toxicol. 2015;15:61-68.
17. Alirezaei M, Rezaei M, Hajighahramani S, Sookhtehzari A, Kiani K. Oleuropein attenuates cognitive dysfunction and oxidative stress induced by some anesthetic drugs in the hippocampal area of rats. J Physiol Sci. 2017;67;131-139.
18. Janahmadi Z, Nekooeian AA, Moaref AR, Emamghoreishi M. Oleuropein attenuates the progression of heart failure in rats by antioxidant and antiinflammatory effects. Naunyn Schmiedebergs Arch Pharmacol. 2017;390;245-252.
19. Sherif IO. The effect of natural antioxidants in cyclophosphamide-induced hepatotoxicity: role of Nrf2/HO-1 pathway. Int Immunopharmacol. 2018;61;29-36.
20. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175:184-191.
21. Collins AR, Ma AG, Duthie SJ. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res. 1995;336:69-77.
22. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351-358.
23. Aebi H. Catalase *in vitro*. Methods Enzymol. 1984;105:121-126.
24. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem. 1988;34:497-500.
25. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem.1969;27:502-522.
26. Ejlertsen B. Adjuvant chemotherapy in early breast cancer. Dan Med J. 2016;63:B5222.
27. Hadrich F, Mahmoudi A, Bouallagui Z, Feki I, Isoda H, Feve B, Sayadi S. Evaluation of hypocholesterolemic effect of oleuropein in cholesterol-fed rats. Chem Biol Interact. 2016;252:54-60.
28. Mahmoudi A, Hadrich F, Feki I, Ghorbel H, Bouallagui Z, Marrekchi R, Fourati H, Sayadi S. Oleuropein and hydroxytyrosol rich extracts from olive leaves attenuate liver injury and lipid metabolism disturbance in bisphenol A-treated rats. Food Funct. 2018;9:3220-3234.
29. Geyikoğlu F, Çolak S, Türkez H, Bakır M, Koç K, Hosseinigouzdagani MK, Çerığ S, Sönmez M. Oleuropein ameliorates cisplatin-induced hematological damages via restraining oxidative stress and DNA injury. Indian J Hematol Blood Transfus. 2017;33:348-354.
30. Fuccelli R, Sepporta MV, Rosignoli P, Morozzi G, Servili M, Fabiani R. Preventive activity of olive oil phenolic compounds on alkene epoxides induced oxidative DNA damage on human peripheral blood mononuclear cells. Nutr Cancer. 2014;66:1322-1330.
31. Kalaiselvan I, Dicson SM, Kasi PD. Olive oil and its phenolic constituent tyrosol attenuates dioxin-induced toxicity in peripheral blood mononuclear cells via an antioxidant-dependent mechanism. Nat Prod Res. 2015;29:2129-2132.
32. Topalović DŽ, Živković L, Čabarkapa A, Djelić N, Bajić V, Dekanski D, Spremo-Potparević B. Dry olive leaf extract counteracts L-thyroxine-

induced genotoxicity in human peripheral blood leukocytes *in vitro*. Oxid Med Cell Longev. 2015;2015:762192.

33. Rosignoli P, Fuccelli R, Sepporta MV, Fabiani R. *In vitro* chemo-preventive activities of hydroxytyrosol: the main phenolic compound present in extra-virgin olive oil. Food Funct. 2016;7:301-307.
34. Fuccelli R, Fabiani R, Rosignoli P. Hydroxytyrosol exerts anti-inflammatory and anti-oxidant activities in a mouse model of systemic inflammation. Molecules. 2018;23:3212.
35. Mahmoudi A, Ghorbel H, Bouallegui Z, Marrekchi R, Isoda H, Sayadi S. Oleuropein and hydroxytyrosol protect from bisphenol A effects in livers and kidneys of lactating mother rats and their pups'. Exp Toxicol Pathol. 2015;67:413-425.
36. Kim SW, Hur W, Li TZ, Lee YK, Choi JE, Hong SW, Lyoo KS, You CR, Jung ES, Jung CK, Park T, Um SJ, and Yoon SK. Oleuropein prevents the progression of steatohepatitis to hepatic fibrosis induced by a high-fat diet in mice. Exp Mol Med. 2014;46:e92.
37. Fan J, Ma J, Xia N, Sun L, Li B, Liu H. Clinical value of combined detection of CK-MB, MYO, cTnI and Plasma NT-proBNP in diagnosis of acute myocardial infarction. Clin Lab. 2017;63:427-433.
38. Katanov C, Lerrer S, Liubomirski Y, Leider-Trejo L, Meshel T, Bar J, Feniger-Barish R, Kamer I, Soria-Artzi G, Kahani H, Banerjee D, Ben-Baruch A, Regulation of the inflammatory profile of stromal cells in human breast cancer: prominent roles for TNF-α and the NF-κB pathway. Stem Cell Res Ther. 2015;6:87.
39. Andreadou I, Mikros E, Ioannidis K, Sigala F, Naka K, Kostidis S, Farmakis D, Tenta R, Kavantzas N, Bibli SI, Gikas E, Skaltsounis L, Kremastinos DT, Iliodromitis EK. Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism. J Mol Cell Cardiol. 2014;69:4-16.
40. Potočnjak I, Škoda M, Pernjak-Pugel E, Peršić P, Domitrović R. Oral administration of oleuropein attenuates cisplatin-induced acute renal injury in mice through inhibition of ERK signaling. Mol Nutr Food Res. 2016;60:530-541.
41. Fan C, Georgiou KR, Morris HA, McKinnon RA, Keefe DMK, Howe PR, Xian CJ. Combination breast cancer chemotherapy with doxorubicin and cyclophosphamide damages bone and bone marrow in a female rat model. Breast Cancer Res Treat. 2017;165:41-51.
42. Castejón ML, Rosillo MÁ, Montoya T, González-Benjumea A, Fernández-Bolaños JG, Alarcón-de-la-Lastra C. Oleuropein down-regulated IL-1β-induced inflammation and oxidative stress in human synovial fibroblast cell line SW982, Food Funct. 2017;8:1890-1898.
43. Ryu SJ, Choi HS, Yoon KY, Lee OH, Kim KJ, Lee BY. Oleuropein suppresses LPS-induced inflammatory responses in RAW 264.7 cell and zebrafish. J Agric Food Chem. 2015;63:2098-2105.
44. Sun W, Wang X, Hou C, Yang L, Li H, Guo J, Huo C, Wang M, Miao Y, Liu J, Kang Y. Oleuropein improves mitochondrial function to attenuate oxidative stress by activating the Nrf2 pathway in the hypothalamic paraventricular nucleus of spontaneously hypertensive rats. Neuropharmacology. 2017;113:556-566.
45. Koc K, Cerig S, Ozek NS, Aysin F, Yildirim S, Cakmak O, Hosseinigouzdagani M, Geyikoglu F. The efficacy of oleuropein against non-steroidal anti-inflammatory drug induced toxicity in rat kidney. Environ Toxicol. 2019;34:67-72.
46. Dunning S, Ur Rehman A, Tiebosch MH, Hannivoort RA, Haijer FW, Woudenberg J, van den Heuvel FA, Buist-Homan M, Faber KN, Moshage H. Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death. Biochim Biophys Acta. 2013;1832:2027-2034.
47. Lemire J, Alhasawi A, Appanna VP, Tharmalingam S, Appanna VD. Metabolic defence against oxidative stress: the road less travelled so far. J Appl Microbiol. 2017;123:798-809.
48. Khalili A, Nekooeian AA, Khosravi MB. Oleuropein improves glucose tolerance and lipid profile in rats with simultaneous renovascular hypertension and type 2 diabetes. J Asian Nat Prod Res. 2017;19:1011-1021.