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The Effects of Oleuropein on Epirubicin and Cyclophosphamide Induced Toxicity in Rats

Oleuropeinin Ratlarda Epirubisin ve Siklofosfamid ile Indüklenen Toksisitede Etkileri

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Öz

Amaç: Oleuropein zeytin yaprağı, zeytin meyvesi ve zeytinyağında bulunan başlıca biyoaktif polifenolik bileşiktir. Oleuropeinin anti-kanser, antioksidan ve anti-enflamatuar etkileri çeşitli *in vitro* ve *in vivo* çalışmalarla gösterilmiştir. Araştırmamızda oleuropeinin siklofosfamid ve epirubisin kaynaklı toksisite üzerindeki etkilerinin dişi ratlarda araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Ratlar her grupta sekiz hayvan olacak şekilde yedi eşit gruba ayrıldı. Daha sonra dört kür 16 mg/kg/ hafta siklofosfamid ve 2.5 mg/kg/hafta epirubisin intraperitoneal (i.p.) yolla verildi. Oleuropein (150 mg/kg/hafta) oral gavaj yolu ile eş zamanlı olarak ratlara uygulandı. Oleuropeinin etkileri tam kan örneklerinde hemogram testleri ve serum numunelerinde biyokimyasal analizlerle incelendi. Daha sonra serum örneklerinde Enzyme-Linked ImmunoSorbent Assay (ELISA) ile tümör nekrozis faktör-α (TNF-α) ve interlökin 6 (IL-6) analizi yapıldı. Ardından, lenfosit DNA'sı kullanılarak Comet Assay gerçekleştirildi. Son olarak, kalp, böbrek ve karaciğer dokularında oksidan [malondialdehit (MDA)] ve antioksidan [katalaz (CAT), süperoksit dismutaz (SOD) ve glutatyon (GSH)] parametreler ölçüldü.

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

Sonuç: Sonuçlar oleuropeinin epirubisin ve siklofosfamid kombinasyon tedavisinde gözlenen toksisiteye karşı koruyucu bir ajan olabileceğini göstermektedir. Oleuropeinin

antineoplastiklerle indüklenen toksisiteye karşı koruyucu etkilerinin kesin olarak ortaya konulabilmesi için daha fazla çalışmaya ihtiyaç bulunmaktadır.

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

Abstract

Objectives: Oleuropein is the major bioactive polyphenolic compound in the olive leave, olive and olive oil. The anti-cancer, antioxidant and anti-inflammatory effects of oleuropein have been demonstrated with several *in vitro* and *in vivo* studies. In our research, we aimed to investigate the effects of oleuropein on cyclophosphamide and epirubicin induced toxicity in female rats.

Materials and Methods: Seven groups were formed with eight rats in each group. Four cycles of 16 mg/kg/week of cyclophosphamide and 2.5 mg/kg/week of epirubicin were administered to the rats by intraperitoneal (i.p.) injection. Oleuropein (150 mg/kg/week) were applied via oral gavage simultaneously. The effects of oleuropein were examined by hemogram tests in the whole blood samples and biochemical analysis in the serum samples. Then, tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) analysis by Enzyme-Linked ImmunoSorbent Assay (ELISA) were conducted in the serum samples. Subsequently, Comet Assay was performed by using lymphocyte DNA. Finally, levels of oxidant [malondialdehyde (MDA)] and antioxidant [catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH)] parameters were measured in heart, kidney and liver tissues.

Results: We determined oleuropein can reduce the levels of DNA damage and proinflammatory cytokines such as TNF-α and IL-6. Furthermore, oleuropein ameliorated some hemogram and biochemical parameters that deteriorated due to antineoplastic drugs. Moreover, we detected oleuropein caused an increase in the amounts of antioxidant parameters (GSH, SOD and CAT) and reduced the level of MDA in heart, kidney and liver tissues.

Conclusion: The results indicate oleuropein might be a beneficial agent against toxicity caused by cyclophosphamide and epirubicin combination treatment. Further studies are needed to precisely demonstrate the protective effects of oleuropein against antineoplastic induced-toxicity.

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

Introduction

Breast tumours are the most frequently diagnosed cancer type and the leading cause of cancer-related death among women worldwide.[1] Chronic inflammation-induced oxidative stress is an important factor in the development of cellular changes which lead to an enhancement in reactive oxygen species (ROS) production and cell proliferation. Inflammation induced by cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) have been linked to the elevated production of reactive oxygen species and breast tumour formation.[2,3]

Chemotherapy is the most commonly used method in the treatment of breast cancer. Although some antineoplastic drugs can be used as a single agent for chemotherapy, to enhance their

effectiveness, two or three antineoplastic agents are generally administered together as a combination therapy regimen.[4] 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 tumour types. It inhibits DNA, RNA and protein synthesis via the intercalation of DNA, the inhibition of topoisomerase II activity and the generation of reactive oxygen radicals. Both epirubicin and cyclophosphamide can be used in breast cancer chemotherapy as single agents. Moreover, epirubicin and cyclophosphamide combination treatment is among the frequently used chemotherapy regimens in both early and metastatic stages of breast tumour development.[6,7] Nonetheless, these agents have been frequently reported to be associated with an elevated risk of hepatotoxicity, nephrotoxicity, cardiotoxicity and hematologic toxicity in breast cancer patients.[8-10] These toxic effects may reduce the patient's quality of life and affect the success of their treatment. Therefore, it is necessary to explore novel remedies to reduce the toxicity in breast cancer patients undergoing epirubicin and cyclophosphamide combination chemotherapy in order to minimize the morbidity and improve their quality of life.

Some plant-derived polyphenols have pharmacological effects like anti-inflammatory, antioxidant, and anti-tumour properties. Therefore, in recent years, researchers has been focused on the use of natural dietary antioxidants to alleviate the toxic effects of antineoplastic drugs.[11] Oleuropein (3,4-dihydroxyphenylelenolic acid) is a nontoxic secoiridoid glycoside and the major polyphenolic compound in the olive tree (*Olea europaea* L.) and in olive oil. It is the compound responsible for the bitter taste of the leaves and fruit of the olive tree. Oleuropein and its bioactive derivatives, such as hydroxytyrosol, have been reported to have antioxidant, anti-inflammatory, anti-cancer, cardioprotective, neuroprotective and hepatoprotective effects via modulating several mechanisms.[12,13] Furthermore, in a novel study, it was reported oleuropein did not lead to a decrease in the efficacy of anthracycline-based chemotherapy in breast tumour-induced female BALB/c mice. On the contrary, it was demonstrated it showed a synergistic anti-tumoural effect with the antineoplastic agents.[14] Therefore, in the study we aimed to investigate 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 by different methods. First of all hemogram tests were performed in the whole blood samples and biochemical analysis in the serum samples. Then, tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) analysis by Enzyme-Linked ImmunoSorbent Assay (ELISA) were conducted in the serum samples. Subsequently, Comet Assay was performed by using lymphocyte DNA. Finally, levels of oxidant [malondialdehyde (MDA)] and antioxidant [catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH)] parameters were measured in heart, kidney and liver tissues.

Chemicals

Oleuropein (HPLC grade ≥ 98%) was obtained from Santa Cruz Biotechnology® (Santa Cruz Biotechnology Inc, California, USA). Epirubicin (Pirucin®- Saba İlaç AŞ., İstanbul, Turkey), cyclophosphamide (Endoxan®- Baxter Oncology GmbH, Frankfurt, Germany), ketamine (Ketalar®- Pfizer Inc.,İstanbul, Turkey) and xylazine (Rompun®- Bayer LLC., İstanbul, Turkey) were purchased as commercially available products. All other chemicals were used in the study obtained from Sigma-Aldrich (Sigma Aldrich Inc., Missouri, USA).

Animals
Fifty-six healthy female Sprague-Dawley rats, three months old and weighing 220 g ± 20 g, were purchased from the XXX University Experimental Animals Research and Application Center (Aydın, Turkey). Animals were kept inside polycarbonate cages in an air conditioned room (23 °C ± 2 °C) and relative humidity of 50%-55% with 12 hours light/dark cycle. Standard rat chow and water were provided *ad libitum*. The rats were held in the room for acclimatize to the laboratory environment for a week before the drug administration phase. The study were approved by the Local Ethics Committee for Experiments on Animals of XXX University (Ethics committee permission no: 64583101/2017/117) and conformed to the ethical standards of the Helsinki Declaration.

Experimental design
Rats were separated into seven equal groups (n= 8 in each group):
Group I (Control): 1 milliliter (ml) saline was administered once a week for 4 cycles.
Group II (epirubicin: "E"): 2.5 mg/kg/week epirubicin was administered for 4 cycles.
Group III (cyclophosphamide "C"): 16 mg/kg/week cyclophosphamide was administered for 4 cycles.
Group IV (epirubicin + cyclophosphamide: "EC"): 2.5 mg/kg/week epirubicin + 16 mg/kg/week cyclophosphamide was administered for 4 cycles.
Group V (epirubicin + oleuropein: "EO"): 2.5 mg/kg/week epirubicin + 150 mg/kg/week oleuropein was administered for 4 cycles.
Group VI (cyclophosphamide + oleuropein: "CO"): 16 mg/kg/week cyclophosphamide + 150 mg/kg/week oleuropein was administered for 4 cycles.
Group VII (epirubicin + cyclophosphamide + oleuropein: "ECO"): 2.5 mg/kg/week epirubicin + 16 mg/kg/week cyclophosphamide + 150 mg/kg/week oleuropein was administered for 4 cycles.

Epirubicin and cyclophosphamide were administered by intraperitoneal (i.p.) injection and oleuropein was administered by 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 stated in the United States National Comprehensive Cancer Network (NCCN) Guidelines (Available at: https://www.nccn.org). Dose conversions between human and rat were calculated as described in the United States Food and Drug Administration (FDA) Guidelines (Available at: https://www.fda.gov). The dose of oleuropein used in the study was determined from previous studies.[15-19]

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

Hemogram tests
Blood samples were collected in ethylene diamine tetra acetic acid (EDTA) containing tubes. Samples were analyzed within the first hour after received from the rats. Routine hematological parameters such as leukocyte (WBC), lymphocyte (LYM), monocyte

(MONO), granulocyte (GRA), lymphocyte% (LY%), monocyte% (MONO%), granulocyte% (GR%), erythrocyte (RBC), hemoglobin (HGB), mean cell volume (MCV), hematocrit (HCT), mean cell hemoglobin concentration (MCHc), mean cell hemoglobin (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 automated Diatron® Abacus Junior Vet (Diatron medical instruments plc, Hungary) hematology analyzer.

Biochemical analysis

The plasma was separated by centrifugation from the whole blood and used for determining biochemical parameters in rat serums such as urea, uric acid, creatinine, aspartate aminotransferase (AST), creatinine kinase (CK), alanine aminotransferase (ALT), creatinine kinase isoenzyme 3 (CK-MB), gamma glutamyl transferase (GGT), direct biluribin, and total bilirubin. Analysis were began within the first hour after the blood samples taken from the rats. Analysis performed by using Roche® Cobas c501 autoanalyzer (Roche Diagnostics, Switzerland) and Roche® commercial kits.

Serum levels of IL-6 and TNF-α were determined by commercially available Thermo Fisher® (Thermo Fisher Scientific co., USA) Enzyme-Linked ImmunoSorbent Assay (ELISA) kits according to the manufacturer's instructions.

Comet assay

The comet assay protocol was performed as described in Singh *et al*.[20] Briefly, cells (lymphocytes) were included in the low-melting agarose [0.7% in Phosphate-Buffered Saline (PBS)] and placed to a lysis solution [2.5 molar (M) NaCl, 100 millimolar (mM) Na_2EDTA, 10 mM Tris-HCl, pH 10, containing freshly added before use 1% Triton X-100 and 10% dimethyl sulfoxide] for one hour at 4 °C. Then, the samples were applied onto slides.

The slides were placed on a Cleaver® horizontal gel electrophoresis tank which 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 the alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13) until the liquid level just covers samples. Then, the lid of the tank was closed and waited for 30 minutes at 4 °C. Electrophoresis was conducted at 4 °C in the dark for 30 minutes at 25 Volt and approximately 300 mA, and then the slides were rinsed with 400 mM Tris buffer with pH 7.5 for seven minutes to neutralize the excess alkali. The neutralized slides were kept in ethanol for five minutes and then allowed to dry at room temperature. Slides stained with 70 µL (10 µg/mL) 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) just before microscopic examination.

The comets were analyzed after staining at 400X magnification using a Leica® fluorescence microscope (Leica microsystems GmbH, Germany) equipped with a 50 Watt mercury lamp. 100 cells were randomly counted for each slide. The extension of each comet was analyzed by the Comet assay® IV (Perceptive Instruments Ltd, UK) computerized image-analysis software. The damage ratio for each sample was expressed as ‘‘tail moment’’ and "%tail intensity" as described in Collins *et al.*[21]

Measurement of oxidant/antioxidant parameters

0.5 g tissue samples were taken from each organ (kidneys, heart and liver) for homogenization. Tissues were homogenized [2000 round per minute (rpm) for one minute] using a teflon-glass stirrer (IKA Overhead Stirrer; IKA-Werke GmbH & Co. KG, Germany) in a 10-fold volume of ice-cold 10% 150 mM phosphate buffer (pH 7.4). The homogenate was centrifuged (Hettich Zentrifugen, Mikro 200 R, Germany) at 12000 rpm for 10 minutes at 4 °C. The supernatants, referred as homogenate were stored at -80 °C (Glacier Ultralow Temperature Freezer, Japan) until analysis of CAT, SOD, GSH, and MDA levels. Spectrophotometric measurements for all oxidant/antioxidant level analysis in the study were performed by a Shimadzu® UV-1601 (Shimadzu Scientific Instruments, Japan) spectrophotometer. Protein concentrations in supernatants were measured by the Biuret method by spectrophotometer and using commercially available kits (Archem Diagnostic Ind. Ltd., Turkey). The results were expressed as (mg/ml protein).

MDA levels was determined according to the method as described previously by Ohkawa *et al*.[22] The MDA concentration (nmol/mg of tissue protein) was calculated by the absorbance complex [absorbance coefficient (ε) = $1.56x10^5$/M/cm]. CAT activity (k/mg tissue protein) was evaluated according to the method described by Aebi.[23] SOD activity (U/mg of tissue protein) was determined according to the method of Sun *et al*.[24] GSH levels (mg/g tissue protein) was spectrophotometrically determined at 412 nm using the method described by Tietze.[25]

Statistical analysis

Statistical analysis were performed using Statistical Package for the Social Sciences, version 22.0 (SPSS 22.0) package program. All parameters were checked for normal distribution using the Shapiro-Wilk test and for homogeneity of variance with Levene's test. Friedman test was used to evaluate intra-group repeated measurements of rat weights. The results of the shapiro-wilk test showed the data did not match the normal distribution. Therefore, the data were compared between groups using Kruskal-Wallis analysis of variance (ANOVA). *Post hoc* binary comparisons were performed using the Mann-Whitney U test with Bonferroni correction. Differences were considered statistically significant if $p<0.05$. All data were expressed as mean ± standard deviation (X±SD).

Results

Body Weight Changes

In the study, a significant weight reduction ($p<0.05$) was observed only in the combination therapy (EC) group, while considerable increases were noticed in all other groups ($p<0.05$). Although there were no differences among the groups in terms of initial body weights, significant differences were observed between the control group and 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 on which the study was terminated (day 35), are presented in Table 1.

[Table 1. is here]

Hemogram Tests

There were no significant differences between the groups in terms of the MONO, GRA, MONO%, GRA%, RDWc, PCT, MPV and PDWc parameters ($p>0.05$). To simplify the results table, these data are not shown. The results of the hemogram tests and the statistical differences between the experimental groups are given in Table 2.

[Table 2. is here]

In a general summary of the hemogram results, the majority of the parameters were observed to have deteriorated in all of the groups administered antineoplastic drugs alone in both single

and combination therapy, and there were significant differences between them and the control group ($p<0.05$). In parameters such as WBC, LYM, RBC, HGB, HCT and PLT, which are related to neutropenia and bone marrow suppression, significant differences were detected between the groups treated with antineoplastic drugs alone (E, C and EC) and the oleuropein + antineoplastic drug-treated groups (EO, CO and ECO) ($p<0.05$). However, the hemogram test results in the oleuropein + antineoplastic drug-treated groups were generally found to be closer to the results of the healthy control group when compared to the groups treated with antineoplastic drugs alone.

Biochemical Analysis

The biochemical parameters and the statistical differences between the experimental groups are given in Table 3. According to the results, there were no significant differences between the groups in terms of the urea, GGT, direct bilirubin and CK parameters ($p>0.05$). Therefore, these data are not shown in order to simplify the results table.

[Table 3. is here]

In parameters such as AST and ALT, which are important biochemical indicators of liver damage, significant differences ($p<0.05$) were detected between the groups treated with antineoplastic drugs alone (E, C and EC) and the oleuropein + antineoplastic drug-treated (EO, CO and ECO) groups. Treatment with oleuropein caused an amelioration in these parameters and decreased them to levels similar ($p>0.05$) to the healthy control group. According to the biochemical parameters, oleuropein was found to be beneficial for reducing liver damage. Although, oleuropein showed partial benefits in some parameters which are related with heart and kidney damages, no considerable effects was observed in most of other parameters.

It was determined the administered antineoplastic agents as single or in combination significantly increased the serum TNF-α and IL-6 levels when compared to the control group ($p<0.05$). On the other hand, these levels were decreased and found to be similar to those of the healthy control group in all oleuropein-administered groups ($p>0.05$). The results of the ELISA tests are presented in Figure 1.

[Figure 1. is here]

Comet Assay

The tail moment and %tail intensity results of the comet assay are presented in Figure 2. According to the results, the level of DNA damage was considerably high in all groups when compared to the control group. However, it was determined the considerable DNA damage caused by combination chemotherapy (EC) was significantly decreased in the combination chemotherapy + oleuropein treatment group (ECO) ($p<0.001$). Fluorescent microscope images of some DNA samples are presented in Figure 3.

[Figure 2. is here]

[Figure 3. is here]

Oxidant/Antioxidant Levels

The levels of MDA and GSH and the measurement of CAT and SOD activities in the heart, liver and kidneys of the experimental groups are given in Table 4. According to the results, in the combination chemotherapy + oleuropein treatment (ECO) group, the SOD activity were significantly higher than in the combination chemotherapy (EC) group ($p<0.05$). Moreover,

there were no significant differences between the ECO and the control groups in terms of the SOD activity in the heart ($p>0.05$).

[Table 4. is here]

We detected oleuropein showed protective effects in heart tissue by increasing GSH levels and decreasing MDA levels. It was determined the increased MDA levels due to antineoplastic drugs decreased ($p<0.05$) in the oleuropein-treated groups and reduced to levels similar to that of the control group ($p>0.05$). However, while GSH levels decreased in the groups treated with antineoplastic drugs alone (E, C and EC), these levels were increased significantly ($p<0.05$) in the oleuropein-treated groups (EO, CO and ECO) and reached levels similar to that of the control group ($p>0.05$).

It was determined the SOD and CAT activities in the kidneys were significantly decreased antineoplastic drugs alone treated groups (E, C and EC) when compared to the control group ($p<0.05$). On the other hand, the administration of oleuropein led to an amelioration of SOD and CAT activities in kidneys and enabled them to reach levels similar to those of the healthy control group.

The MDA levels in kidneys were lower in the ECO group when compared to the EC group ($p<0.05$). However, there were not any significant differences between the healthy control group and the ECO group ($p>0.05$). This result indicates oleuropein can significantly lower the elevated MDA level which is a marker of oxidative damage, and it showed cell-protective effects in kidneys.

The results of the GSH, SOD and CAT analysis in liver tissues were found to be generally compatible with each other. In most of the oleuropein-treated groups, the antioxidant parameter levels were similar ($p>0.05$) to those of the healthy control group and significantly higher ($p<0.05$) when compared to the groups treated with antineoplastic drugs alone. Oleuropein treatment increased the antioxidant capacity in liver tissues to levels similar to those of the control group and decreased the oxidative stress and degradation products. However, it is important to note that unlike cardiac and renal tissues, measurements of SOD, GSH, and CAT in the liver were even higher in the oleuropein-treated groups than in the healthy control group.

Discussion

In the study, the efficacy of oleuropein against the toxic effects of epirubicin and cyclophosphamide combination therapy, which is a frequently used chemotherapy regimen in the treatment of breast tumours, was investigated using healthy female rats. This is the first study to demonstrate the effects of oleuropein against toxicity induced by an anthracycline and alkylating agent-based combination chemotherapy. Although many parameters were investigated and important data were obtained in the study, a histopathological examination of the tissues and an investigation of the other serum cytokine levels, such as interleukin 1 β (IL-1β) and interleukin 8 (IL-8), which have roles in the inflamation, could not be performed. These were the limits of our study.

Weight loss, vomiting, loss of appetite, anorexia and neutropenia are the important side effects of epirubicin and cyclophosphamide combination therapy.[26] In our study, a remarkable weight loss ($p<0.05$) was observed in the combination chemotherapy (EC) group in terms of initial and final body weights. On the other hand, similar weight gain results were detected in

the oleuropein + combination chemotherapy (ECO) group when compared to the healthy control group ($p>0.05$). These results suggest oleuropein might be a useful agent to prevent the weight loss in this combination chemotherapy. However, it was reported in a study which a fat-rich diet was administered to the rats, oleuropein-treated groups gained less weight than the other groups ($p<0.05$).[27] It was reported in another study the oleuropein + bisphenol A (BPA) administered rats gained less weight than the BPA-alone treated group ($p<0.05$).[28] Considering the results of the studies, the administration of oleuropein was thought to play a regulatory role in various mechanisms for maintaining an ideal weight rather than being a weight loss or weight gain agent.

The number of studies in which the effects of oleuropein or other olive products (leaf, fruit, olive oil and olive mill waste water extracts) were examined in terms of the hemogram parameters is extremely limited. In a study, oleuropein was administered to cisplatin-induced toxicity in rats. It was reported the hemogram parameters were ameliorated and reached similar levels to those of the healthy control group.[29] These consistent results indicates oleuropein may have beneficial effects in ameliorating most of the hemogram parameters which deteriorated due to epirubicin and cyclophosphamide toxicity.

The results of the comet assay showed oleuropein might be a beneficial agent to reduce the oxidative DNA damage caused by antineoplastics. In the literature search, no data were found on the effects of oleuropein in terms of a comet assay for epirubicin and cyclophosphamide toxicity. Therefore, the results were compared oleuropein-rich olive plant (leaf, fruit, olive mill waste water extracts) and olive oil extracts in mice and human peripheral mononuclear blood cells. It was reported in the studies these compounds significantly reduce the oxidative damage levels and showed DNA protective activities.[30-34] In this regard, our results are consistent with the previous studies.

According to the biochemical parameter results, oleuropein was found to be beneficial to reduce the elevated AST and ALT levels which are associated with liver damage. On the other hand, no significant results was observed for most parameters. In a study, it was reported oleuropein administration considerably decreased the elevated levels of AST, ALT, urea and creatinine in rats with BPA-induced toxicity.[35] In another study, it was reported oleuropein significantly decreased the elevated ALT and AST levels in a hepatic fibrosis mice model and might be a pharmacologically useful agent in hepatic fibrosis.[36]

Increased risk of myocardial infarction is an important side effect of anthracycline-derived drugs.[26] CK-MB is an important biochemical marker in monitoring myocardial infarction risk.[37] In our study, conflicting results were obtained on the effects of oleuropein in CK-MB parameter. It was thought these results may be related with the dose of oleuropein administered, as reported in Janahmadi *et al*.[16] It may be possible to observe pharmacological effects in this parameter by administration of higher doses.

Proinflammatory cytokines, such as IL-6 and TNF-α, have roles in both the formation and progression of breast tumours.[2,38] Moreover, several studies demonstrated most antineoplastic agents used in the treatment of breast tumours cause an increase in both tissue and serum IL-6 and TNF-α levels.[39-41] In a study, the effects of different doses of (5, 10 and 20 mg/kg) oleuropein were investigated against cisplatin-induced toxicity in mice. It was reported nuclear factor kappa B (NF-κB), cyclooxygenase-2 (COX-2) and TNF-α levels in kidneys decreased in proportion to the administered dose of oleuropein, and it may be beneficial in reducing cisplatin-induced kidney toxicity.[40] In another study conducted in human synovial

sarcoma cells (SW982), IL-6 and TNF-α levels increased as a result of the induction of IL-1β-mediated inflammation and significantly decreased after oleuropein administration when compared to the control group.[42] In several studies, oleuropein was investigated against toxicity induced by different agents in terms of serum and tissue cytokine levels in rats. It was reported in the studies, oleuropein can significantly reduce the IL-6 and TNF-α levels in both serum and tissues and show protective effects on the organs. It is reported in the studies, oleuropein is able to decrease the elevated IL-6 and TNF-α levels induced by various compounds in direct proportion with the dose.[39,43-45] In this regard, the previous studies and our results are consistent with each other. According to the results of animal experiments, it was determined oleuropein is able to decrease the 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) was also decreased the levels of these cytokines to the healthy control group. It was observed similar results were obtained even at different doses in these studies. This situation might be related with the purity of the oleuropein used in the studies.

Oxidative stress plays an important role in the pathogenesis of the hepatotoxic, nephrotoxic and cardiotoxic effects of both 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 as well as anti-inflammatory and antioxidant activities.[12,13,39] Determinations of reduced GSH, CAT, SOD and MDA levels in kidney, hepatic and cardiac tissues as indicators of oxidative stress and organ damage were performed in our study. Reduced GSH plays a major role in cellular defence against toxicity and scavenging the ROS. The SOD enzyme converts the highly reactive superoxide anions to H_2O_2. Subsequently, CAT is the enzyme responsible for the conversion of the H_2O_2 formed in cellular processes into molecular oxygen and water. Moreover, MDA is a lipid peroxidation product and an indicator of oxidative damage resulting from ROS generation.[47,48]

Our study showed oleuropein can able to decrease the level of MDA and increase the GSH level with the SOD and CAT activity in the liver, heart and kidneys when compared to antineoplastic drugs alone treated rats. However, no data were found in the literature on the effects of oleuropein in terms of the levels of endogenous oxidant/antioxidant parameters in epirubicin and cyclophosphamide combination chemotherapy. Therefore, our findings were compared to the results of the studies conducted in rats on the effects of oleuropein against toxicity induced by cyclophosphamide, doxorubicin and other chemical agents. In these studies, it was observed oleuropein was administered in a wide dose range of between 5 and 2000 mg/kg/day. These studies were reported oleuropein decreased the amount of MDA and increased the amount of antioxidant markers (GSH, SOD and CAT) in many organs, including liver, heart and kidneys, at the dose of 10 mg/kg/day and more.[15-19,33,35,39] Our findings are parallel with the results of these studies. Furthermore, it was reported in the studies in which the different doses were examined together, total antioxidant capacity also increased in direct proportion with the oleuropein administration doses.[16,18,39,48]

Conclusions

Recent studies have shown that oleuropein is a promising compound with anti-inflammatory and antioxidant effects against chemical-toxicity. Moreover, its anti-tumoral properties and synergistic effects with some antineoplastics increase the importance of oleuropein, especially in patients with cancer. The present study showed oleuropein can reduce the levels of DNA damage and the serum proinflammatory cytokines such as TNF-α and IL-6. Furthermore, we

determined oleuropein ameliorated some of the deteriorated hemogram (WBC, LYM and HGB) and biochemical parameters (AST, ALT and total bilirubin) due to antineoplastic drugs. Finally, our study showed oleuropein can 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 suggest oleuropein might have protective effects against the toxicity induced by epirubicin and cyclophosphamide combination chemotherapy. Further studies are needed to precisely demonstrate the protective effects of oleuropein against antineoplastic induced-toxicity.

Conflict of Interest
The authors declare no conflicts of interest.

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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^{ab}	232.1±0.013^{abcd}	240.0±0.014^{abcde}	247.7±0.018^{abcd}	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^{be}	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^{bc}	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**** **X²**	p>0.05 11.266	p>0.05 10.616	p<0.05 15.524	p<0.05 22.423	p<0.05 31.647	p<0.05 30.044	

*Day of first drug administration, ** Day of sacrification, *** Intra-group Friedman Test results, ****Kruskal-Wallis analysis of body weight changes between groups. The same superscript letters in the columns indicates significant statistical difference with each other. Differences were considered statistically significant if p<0.05. The unit of weight is 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 (X±SD).

Table 2. Hemogram results of experimental groups.

	Groups (n=8)							**p**
Parameters	**Control**	**E**	**C**	**EC**	**EO**	**CO**	**ECO**	**X²**
WBC	6.02±0.39^{abcd}	3.45±0.29^{ae}	3.41±1.48^{bc}	3.10 ±0.48^{ce}	4.65±0.51^{de}	5.73± 0.98^{c}	5.70±0.29^{e}	p<0.001 40.491
LYM	3.53±0.41^{abc}	1.98±0.88^{ad}	1.83±0.50^{be}	1.71±0.41^{cf}	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^{abcde}	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^{abcd}	5.76±0.79^{ae}	5.91±0.36^{bf}	5.58±1.13^{cg}	6.70±0.25^{e}	6.77± 0.47^{f}	6.57±0.31^{dg}	p<0.001 31.528
HGB	11.70±0.49^{abcd}	9.66±1.34^{ae}	10.73± 0.88^{bf}	9.41 ±0.99^{cg}	11.25± 0.30^{de}	11.77 ±0.44^{f}	11.64 ±0.50^{g}	p<0.001 35.829
HCT	40.85±2.74^{abcd}	33.99± 4.32^{ae}	36.80± 3.37^{bf}	33.07± 5.44^{cg}	39.40± 1.30^{e}	43.15 ±3.72^{f}	36.80± 7.16^{dg}	p<0.001 33.134
MCV	58.00±0.75^{abc}	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^{abcde}	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^{abcd}	509.37±174^{ae}	483.63±122^{bf}	674.50±163^{g}	860.63±117^{ce}	675.25±131^{f}	1006.38±110^{dg}	p<0.001 33.510
MPV	9.43±3.60^{abcd}	9.16±5.78^{ae}	8.81±2.57^{bf}	9.80±7.55^{g}	9.76±5.30^{ce}	9.08±5.59^{f}	9.08±8.21^{dg}	p>0.05 2.371

Unit for wbc, lym and plt is 10^9/l; Unit for rbc is 10^12/l; Units of other parameters given as percentage (%). The same superscript letters in the lines 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 results are given as mean ± standard deviation (X±SD).

Table 3. Biochemical parameters of experimental groups.

	Groups (n=8)							***p*** **X^2**
Parameter	**Control**	**E**	**C**	**EC**	**EO**	**CO**	**ECO**	
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^{abcdef}	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^{abcd}	211.87±43.00^{ae}	219.25±35.09^{bf}	210.88±30.38^{cg}	141.00±22.86^{e}	135.88±25.39^{df}	152.38±29.57^{g}	*p*<0.001 32.313
ALT	54.25±5.65^{abcd}	63.25±4.06^{ae}	62.38±7.13^{bf}	64.63±4.98^{cg}	48.25±4.98^{de}	52.13±5.80^{f}	52.88±7.04^{g}	*p*<0.001 32.844
Total Bilirubin	0.03±0.01^{ab}	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^{abcde}	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

Unit for creatine kinase myocardial band (CK-MB), total bilirubin, alanine transaminase (ALT) and aspartate transaminase (AST) is (U/L). Unit for uric acid and creatinine is (mg/dl). The same superscript letters in the lines 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 results are given as mean ± standard deviation (X±SD).

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

	Groups (n=8)							**p**
Paramete	**Control**	**E**	**C**	**EC**	**EO**	**CO**	**ECO**	**X^2**
Heart								
SOD	7.04±0.63^{ab}	6.39±0.1	5.36±2.17	3.40±2.17	6.44±0.3	6.16±0.35	6.74±0.1	p<0.0
CAT	3.88±1.23^{ab}	1.86±0.7	1.75±0.53	1.68±0.53	2.28±0.6	1.69±0.45	1.74±0.3	p<0.0
GSH	13.70±2.53	9.58±2.5	9.88±2.54	9.43±2.54	14.37±3.	13.70±3.0	13.47±1.	p<0.0
MDA	70.08±6.08	93.65±4.	90.88±13.	99.98±13.	75.99±7.	76.93±16.	77.92±5.	p<0.0
Kidneys								
SOD	7.40±0.22^{ab}	6.69±0.2	6.31±1.20	5.96±1.20	7.24±0.3	7.10±0.28	7.16±0.2	p<0.0
CAT	3.74±1.93^{ab}	2.82±0.8	3.65±1.01	2.49±1.01	6.80±1.8	6.29±1.34	4.95±2.2	p<0.0
GSH	20.43±2.53	13.77±2.	11.90±3.0	8.98±3.02	17.07±2.	16.62±3.0	16.39±2.	p<0.0
MDA	49.11±2.93	61.10±5.	70.87±9.0	74.62±9.0	54.38±5.	60.34±8.0	52.30±2.	p<0.0
Liver								
SOD	7.04±0.15^{ab}	6.39±0.3	5.36±0.20	6.54±0.02	7.49±0.0	7.27±0.13	7.37±0.1	p<0.0
CAT	3.88±7.22^{a}	1.86±5.5	1.75±5.65	6.18±5.65	7.90±3.4	21.34±16.	15.07±7.	p<0.0
GSH	13.70±3.81	9.58±2.3	9.88±2.30	11.90±2.3	21.78±3.	19.76±3.5	19.98±2.	p<0.0
MDA	70.08±2.08	93.65±7.	90.88±12.	88.25±12.	72.01±2.	77.54±5.8	78.39±8.	p<0.0

*Unit for MDA is (nmol/mg protein); Unit for GSH is (mg/g protein); Unit for CAT is (k/mg protein); Unit for SOD is (U/mg protein). The same superscript letters in the lines indicates significant statistical difference with each other. Differences were considered statistically 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 (X±SD).

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

	Groups (n=8)							p
Paramete	**Control**	**E**	**C**	**EC**	**EO**	**CO**	**ECO**	**X^2**
Heart								
SOD	7.04±0.63[ab]	6.39±0.1	5.36±2.17	3.40±2.17	6.44±0.3	6.16±0.35	6.74±0.1	p<0.0
CAT	3.88±1.23[ab]	1.86±0.7	1.75±0.53	1.68±0.53	2.28±0.6	1.69±0.45	1.74±0.3	p<0.0
GSH	13.70±2.53	9.58±2.5	9.88±2.54	9.43±2.54	14.37±3.	13.70±3.0	13.47±1.	p<0.0
MDA	70.08±6.08	93.65±4.	90.88±13.	99.98±13.	75.99±7.	76.93±16.	77.92±5.	p<0.0
Kidneys								
SOD	7.40±0.22[ab]	6.69±0.2	6.31±1.20	5.96±1.20	7.24±0.3	7.10±0.28	7.16±0.2	p<0.0
CAT	3.74±1.93[ab]	2.82±0.8	3.65±1.01	2.49±1.01	6.80±1.8	6.29±1.34	4.95±2.2	p<0.0
GSH	20.43±2.53	13.77±2.	11.90±3.0	8.98±3.02	17.07±2.	16.62±3.0	16.39±2.	p<0.0
MDA	49.11±2.93	61.10±5.	70.87±9.0	74.62±9.0	54.38±5.	60.34±8.0	52.30±2.	p<0.0
Liver								
SOD	7.04±0.15[ab]	6.39±0.3	5.36±0.20	6.54±0.02	7.49±0.0	7.27±0.13	7.37±0.1	p<0.0
CAT	3.88±7.22[a]	1.86±5.5	1.75±5.65	6.18±5.65	7.90±3.4	21.34±16.	15.07±7.	p<0.0
GSH	13.70±3.81	9.58±2.3	9.88±2.30	11.90±2.3	21.78±3.	19.76±3.5	19.98±2.	p<0.0
MDA	70.08±2.08	93.65±7.	90.88±12.	88.25±12.	72.01±2.	77.54±5.8	78.39±8.	p<0.0

*Unit for MDA is (nmol/mg protein); Unit for GSH is (mg/g protein); Unit for CAT is (k/mg protein); Unit for SOD is (U/mg protein). The same superscript letters in the lines indicates significant statistical difference with each other. Differences were considered statistically 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 (X±SD).