Original Article
DOI: 10.4274/tjps.galenos.2021.03271

Investigation of Genotoxic, Cytotoxic, Apoptotic, and Oxidant Effects of Olive Leaves Extracts on Liver Cancer Cell Lines

Zeytin Yaprağı Ekstrelerinin Karaciğer Kanseri Hücre Hatları Üzerine Genotoksik, Sitotoksik, Apoptotik ve Oksidan Etkilerinin Araştırılması

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02.12.2020
26.03.2021

Abstract
Objectives: Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third leading cause of tumor-related death worldwide. Mechanisms underlying tumor onset, progression, and metastasis in the case of HCC have not been adequately studied. In this study, we aimed to investigate the genotoxic, cytotoxic, apoptotic, and oxidant effects of olive leaf extract on HCC cells.
Materials and Methods: H4IIE Rattus norvegicus hepatoma cells and Rattus norvegicus healthy liver clone-9 cells were treated with increasing concentrations of olive leaf extracts (250-2000 ppm) in ethanol, acetone, dichloromethane, and methanol. ATP cell viability, intracellular reactive oxygen species (iROS) generation levels, double staining test with acridine

orange/ethidium bromide (AO/EB), comet assay, levels of IL-1β, IL-6, and TNF-α were applied. Significance was determined with ANOVA.
Results: Apoptotic, genotoxic, cytotoxic, and oxidative effects of olive leaf extracts increased with increasing concentrations as compared to controls in H4IIE cells ($p<0.001$).
Conclusion: This study is the first study to show a significant and selective cytotoxic activity of olive leaf extracts in selected hepatic human H4IIE cancer cell lines. Olea leaf extracts can selectively increase apoptotic damage and show antiproliferative and pro-apoptotic properties against H4IIE cells. They can be recommended as potential nutraceuticals in the prevention of cancer.
Keywords: Olive leaf, hepatocellular carcinoma, Oleuropein, oxidative stress, genotoxicity, apoptosis.

Öz
Giriş ve Amaç: Hepatoselüler karsinom (HSK), en yaygın yedinci kanser türüdür ve kanserle ilişkili ölümlerde üçüncü sırada yer almaktadır. HSK'da tümör başlangıcına, ilerlemesine ve metastazlara neden olan mekanizmalar tam olarak bilinmemektedir. Bu çalışmada zeytin yaprağı ekstresinin HSK hücreleri üzerine genotoksik, sitotoksik, apoptotik ve oksidan etkilerinin araştırılması amaçlanmıştır.
Yöntem ve Gereçler: Bu çalışmada hepatoma hücreleri (H4IIE) ve sağlıklı karaciğer hücrelerine (Clone-9), farklı çözücülerle hazırlanmış zeytin yaprağı ekstreleri farklı konsantrasyonlarda (250-2000 ppm) uygulandı. Ekstrelerin sitotoksisitesi ATP testiyle, hücre içi reaktif oksijen türlerinin (iROS) oluşumu florometrik yöntemlerle, genotoksik etkileri alkalen tekli hücre jel elektroforez (Comet Assay) yöntemiyle, apoptotik etkileri akridin turuncusu/etidyum bromür yöntemiyle ölçüldü. IL-1β, IL-6 ve TNF-α seviyeleri ELISA yöntemi ile belirlendi. İstatistiksel test olarak ANOVA testi kullanıldı.
Bulgular: Zeytin yaprağı ekstrelerinin apoptotik, genotoksik, sitotoksik ve oksidatif etkileri H4IIE hücrelerinde kontrole göre artan konsantrasyonlarla istatistiksel olarak anlamlı şekilde yükseldi ($p <0.001$).
Sonuç: Bu çalışma, H4IIE kanser hücre hatlarında zeytin yaprağı ekstrelerinin seçici sitotoksik aktivitesini gösteren ilk çalışmadır. Zeytin yaprağı ekstreleri apoptozu indüklemiş ve H4IIE hücrelerine karşı antiproliferatif ve pro-apoptotik özellikler göstermiştir. Zeytin yaprağı içeriğindeki sekonder metabolitler karaciğer kanserinin önlenmesinde ve tedavisinde ümit vadetmektedirler.
Anahtar Kelimeler: Zeytin yaprağı, hepatoselüler kanser, oleuropein, oksidatif stres, genotoksite, apoptoz.

1. Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third cause of cancer-related mortality worldwide. Despite significant advances in diagnosis and treatment, HCC remains a terminal disease. The most common cause for mortality in HCC is progression and metastasis. However, the molecular mechanisms underlying tumor onset, progression, and metastasis in the case of HCC have not been adequately studied [1]. The neoplastic development of HCC is related to many histological incidents. Hyperplastic nodules formed in hepatocyte regeneration observed in the liver in response to cell deaths due to risk factors are cytologically normal findings, which happens as the first step in HCC formation. These lesions can transform into pre-malignant dysplastic nodules and cause nuclear aggregation with cytologically well-

detected cell changes. As a result of molecular analysis in HCCs, various genetic and epigenetic changes have been detected [1].

Epidemiological studies and animal experiments have revealed that some nutritional compounds play a role in the incidence rates of various cancers, including HCC. One-third of all known human cancers may be related to specific components of nutrients [1–3]. Many research groups are investigating the effects of nutritional components on cancer processes and state that dietary fats increase the risk of brain, colon, breast, and prostate cancer. In addition, the incidence of the brain, cardiovascular diseases, and cancers such as colon and breast is lower in the Mediterranean Region than in Europe [4,5]. Olive tree leaf (*Olea europaea L.*) is widely used in traditional medicine in the European and Mediterranean regions. Olive leaf extract (OLE) has been reported to show anti-aging, immune system enhancing, and antibiotic effects [6]. In studies, olive leaf antioxidants have been shown to be effective in the treatment of cancers such as liver, prostate, and breast [7,8]. Olive leaf contains substantial secoiridoids, flavonoids, phenolic acids, and lignans. Of these, Oleuropein is an important phenolic compound in the secoiridoid group [7]. Oleuropein and its metabolite hydroxytrizole (HT) have antioxidant activity *in vivo* and *in vitro* [6,7]. The antioxidant activity of Oleuropein and HT has also been demonstrated on cellular models and animals [6,7]. In recent epidemiological studies, it was shown that olive leaf extracts rich in phenolic compounds correlated with decreased cardiovascular risk, neurodegenerative disease, and cancer [6,7,9]. Besides different phenolics and flavonoids olive leaves contain the most oleuropein content when compared with other parts of the plan such as root, bark, fruit, etc. [9]. In this study, it was aimed to investigate the *in vitro* genotoxic, cytotoxic, apoptotic, and oxidant effects of olive leaf extract on HCC cells.

2. Materials and Methods

2.1. Collection of plant samples

The plants have been purchased commercially as medical drugs with batch number of 26/0/2019. Plant leaves of *Olea europaea L.* were dried at room temperature, then shredded with a plant shredder and stored at room temperature until they were used in the study.

2.2. Preparation of methanol, acetone, dichloromethane, and aqueous-ethanol extracts

The preparation of OLEs was performed for the methanol extract (O-MeOH), acetone (O-ACE), dichloromethane (O-DCM), and aqueous-ethanol extract (O-EtOH), 70 g of the plant sample was ground with a laboratory blender, extracted in the solvent, and after the extraction, the extract has been filtered, followed by the evaporation of the solvent. The amount of Oleuropein was expressed as milligram per gram of OLE. The Oleuropein was dissolved in DMSO (0.1%) prior to analysis.

2.3. Determination of total phenolic and flavonoid content

The total amount of phenolic compounds found in the extracts was determined by Folin-Ciocalteu Reagent FCR [10]. Gallic acid was used as the standard phenolic compound. The total amount of phenolic compounds found in all OLEs was evaluated according to the study conducted by Gulcin et al. [12]. The findings were given in gallic acid equivalent (GAE) and micrograms.

The total amount of flavonoids found in all OLEs was determined by the method of Park et al. [11]. Quercetin was used as the standard. Total flavonoid concentration was calculated as quercetin equivalent (QE).

2.4. Determination of free radical activity

The free radical removal activities of the extracts were determined using the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical [13]. The absorbance was measured at 517 nm, values of the

samples were evaluated against control. As standard, Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT), and α-Tocopherol (α-Toc) were used.

2.5. Determination of ABTS cation radical scavenging activity

This method was used to assess the potential of the extracts to sweep the ABTS cation radical. The absorbance of each solution was detected at 734 nm, and the inhibition % was calculated [14].

2.6. Determination of reduction force

The cupric ion (Cu^{2+}) reduction capacities of the extracts were measured with the CUPRAC method [15]. As standard, BHA, BHT, and α-Toc were used.

2.7. HPLC analysis

The amount of oleuropein in leaf extracts was measured by HPLC according to the method developed by Al-Rimawi [16]. HPLC analysis was done with Shimadzu (Japan) LC-20A model, and the peak areas of automatic injections of 20 µL with UV detector were calculated with LC solution computer software. Chromatographic separation was attempted to be optimized using C18 (5 µm, 150 x 4.6 mm) and C8 (5 µm, 150x4.6 mm) columns.

The calibration coefficient was calculated from the calibration curve created by the concentration-dependent linear mathematical equation of the field data, and linearity was calculated (Figure 1G). The UV detection of the separation was performed using the mobile phase of water (pH: 3): acetonitrile (70:30) at 1 mL min-1 flow over the C18 ODS-3 (150mm, 4.6×5mm) column at 280 nm. The linear coefficient of the linear calibration curve created in the range of 100-1000 µg/mL was found to be 0.99.

2.8. Cell culture

H4IIE (ATCC ® CRL-1548 ™) *Rattus norvegicus* hepatoma cells were obtained from the American Cell Cultures Collection (ATCC, Middlesex, UK). Cells were grown in Eagle's Minimum Essential Medium (E'MEM) containing 10% FBS and 1% P / S in a humidified incubator with 5% CO_2 at 37°C.

Healthy rat liver cell clone 9 (ATCC® CRL-1439 ™) was obtained from the American Cell Cultures Collection (ATCC, Middlesex, UK). Cells were grown in an F12K medium containing 10% FBS, and 1% P / S in a humidified incubator with 5% CO_2 at 37° C. Samples were prepared at different doses and left for 24-hour incubation in cells. Each dose was analyzed with a minimum of four replicates.

2.9. Cytotoxicity assay

To measure cytotoxicity in cancer and healthy cells, 1.5 x 10^4 cells per well were added to 96 opaque white plates. Samples of different concentrations were added and incubated for 24 hours. After incubation, the ATP solution was added to the wells and measured luminometrically using the Cell Titer-Glo® Luminescent Cell Viability Test Kit.

2.10. Intracellular reactive oxygen generation

The intracellular ROS levels were measured using the H_2DCF fluorescence probe. Cancer and healthy cells seeded on opaque black plates, and samples of different concentrations (250-2000 ppm) were incubated for 24 hours. After incubation, cells were washed three times with dPBS and incubated at 37°C in the dark with 5µM H_2DCFH-DA. After the cells were washed, fluorometric measurements were taken at Ex: 482 nm/Em: 512 nm. Results were normalized according to the ATP level.

2.11. Apoptosis

Acridine orange (AO) / ethidium bromide (EB) dye was used to measure apoptosis [17]. After mixing AO / EB and cell pellet in a 1:1 ratio, images were taken under a fluorescence microscope (Leica DM 1000, Solms, Germany). Apoptotic cells are separated from living cells by morphological changes in the nuclei. While AO is taken by both living and dead cells, EB stains

only the apoptotic cell nucleus. AO spreads green fluorescence at 480-490 nm from living cells while spreading to dsDNA. Green cells were evaluated as healthy; yellow-orange ones were apoptotic and red ones were necrotic. Briefly, 2×10^5 H4IIE and clone-9 cells/well were seeded in 6 well-plate and incubated for 24 hours. Cells seeded in 6-well plates under IC_{50} doses were washed for 24 hours after incubation with samples. Following treatments, the cells were collected and washed with PBS, followed by staining with 1:1 mixture of AO/EB (100 µg/mL). Triplicate samples of 100 cells each were counted and scored for the incidence of apoptotic chromatin condensation using a fluorescent microscope (Leica DM 1000, Solms, Germany) [17, 18]. Apoptotic cells were calculated relative to the total number of cells [18].

2.12. Genotoxicity

Genotoxicity analysis was done according to the comet assay method in our previous study. DNA damage in cells was given as tail intensity % [19]. Alkaline single cell gel electrophoresis assay (Comet Assay) was carried out with a slight modification of Singh et al. to assess the genotoxic effects of olive leave extracts on H4IIE and clone-9 cells [19]. The cells were plated on 6-well cell culture plates (approximately $2x10^5$ cells per well) containing cell culture medium and incubated at 37°C. Then, the OLE samples below IC_{50} concentrations were added and incubated for 24 hours. The cells were collected using Trypsin-EDTA (0.25%), phenol red for 2-3 min in the incubator, and centrifuged at 1500xrpm for 5 min at 4˚C. The supernatant was aspirate, and the cell density was adjusted to $2x10^5$ cells/mL using cold dPBS. 85 µL of 0.6% low melting point agarose (LMPA) and 15 µL cell suspension were mixed and placed on 1% normal melting point agarose (NMPA) pre-coated slides. They were allowed to solidify on a cold tray for a 2 min, and the slides were then placed in lysis buffer, pH 10.0 (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, Sigma-Aldrich) for overnight in dark conditions. The slides were then incubated in an alkaline solution (0.3 M NaOH, 1 mM EDTA, Sigma-Aldrich) for 40 min in the dark in the presence of cooling blocks to unwind the DNA. Electrophoresis was performed at 0.72 V/cm (26 V, 300 mA) for 25 min in an electrophoresis tank at 4°C 0.72 V/cm (26 V, 300 mA) for 25 min. Tris buffer (0.4 M Tris, pH: 7.5) for 5 min and then dehydrated with ethanol before staining. The slides were then stained with ethidium bromide (EB) (2 µg/mL in distilled H_2O, 70 µL/slide) coated with a coverslip and scored with a fluorescence microscope (Leica DM 1000, Solms, Germany) using the Comet assay IV software (Perceptive Instruments, Suffolk, UK).

2.13. Inflammation assays

Cancer and healthy cells were incubated at concentrations below IC_{50} for 24 hours. The levels of Interleukin-1β (IL-1β), Interleukin-6 (IL-6), and Tumor Necrosis Factor-α (TNF-α) in the medium were measured by commercially available ELISA kits (Elabscience, Texas, USA) by photometric methods. Levels of IL-1β, IL-6, and TNF-α in cell medium after 24-hour incubation were measured by the ELISA method according to the protocol provided by the manufacturer. All ELISA kits were purchased from Elabscience (Texas, USA). IL1β, IL6, and TNFα levels were read at 450 nm using an ELISA plate reader (Thermo Scientific, Massachusetts- USA), and their concentrations were calculated based on standard curves obtained from serially diluted external standards.

2.14. Statistical analysis

All experiments were repeated a minimum of 4 times. Results are given as mean ± standard deviation (Mean ± SD). Variance analysis was done with one-way ANOVA. $p < 0.05$ level was considered statistically significant. Regression analysis was done for IC_{50} values. All analyzes were done with the Statistical Package Program for Social Sciences (IBM, Inc version 25).

3. Results

3.1. The amount of flavonoids, phenolic, free radical activity, ABTS cation radical scavenging activity, reduction force, and oleuropein level analysis

Polyphenolic compounds, namely flavonoids, phenolic acids, and their derivatives, are typical phytochemicals for olive leaves. Total flavonoid levels in O-DCM, O-ACE, O-MeOH, and O-EtOH were found 8.52, 7.18, 3.29, and 1.73 mg QUE/mL, respectively (Figure 1A), while total phenolic levels in O-DCM, O-ACE, O-MeOH, and O-EtOH were 11.24, 6.92, 9.96, and 9.81 mg GAE/mL, respectively (Figure 1B).

The free radical removal activities of the extracts are determined using the DPPH free radical (Figure 1C). O-DCM showed inhibitions of %0.13, %0.13, %18.72 and %18.85 with different concentrations. O-ACE showed inhibitions of %9.49, %18.72, %41.54 and %81.85 with different concentrations. O-MeOH showed inhibitions of %6.63, %19.76, %42.78 and %81.85 with different concentrations. BHA was used as a standard.

ABTS cation radical scavenging activity of extracts was showed in Figure 1D. O-DCM showed inhibitions of %9.96, %0.13, %18.72 and %18.85 with different concentrations. O-ACE showed inhibitions of %30.32, %65.82, %70.54 and %87.14 with different concentrations. O-MeOH showed inhibitions of %18.13, %50.43, %85.49 and %85.93 with different concentrations. O-EtOH showed inhibitions of %42.30, %69.89, %87.36 and %86.92 with different concentrations.

The determination of reduction force was showed as absorbance levels of extracts (Figure 1E). O-DCM showed absorbance of 0.14, 0.24, 0.31 and 0.52 with different concentrations. O-ACE showed absorbance of 0.20, 0.43, 0.70, and 1.24 with different concentrations. O-MeOH showed absorbance of 0.17, 0.39, 0.60 and 1.04 with different concentrations. BHA is used as standard.

Oleuropein level determination was conducted with HPLC as three parallels (Figure 1E). Mean values of these three were 19.56 mg/mL (O-DCM), 97.97 mg/mL (O-ACE) and 72.20 mg/mL (O-MeOH).

3.2. Inhibition of cell viability

Cytotoxicity results of OLEs to liver hepatoma (H4IIE) and healthy liver clone-9 cells are presented in Figure 2A. These results showed a robust concentration-response relationship with cytotoxicity of OLEs. In Figure 2A, a gradual decrease was seen in the viability of H4IIE cells, a statistically significant difference obtained $p<0.001$ with increased concentrations.

3.3. Intracellular reactive oxygen species (iROS) generation levels

The results of the iROS of OLEs to liver carcinoma (H4IIE) and healthy liver clone-9 cells are presented in Figure 2B. There is a gradual increase in the iROS level of H4IIE cells, with all concentrations of O-MeOH and O-EtOH of 250, 500, 1000, and 2000 ppm showed increased iROS compare to control cells ($p<0.001$) (Figure 2B).

3.4. Induction of apoptosis

OLEs induce Apoptosis of H4IIE cells in a concentration-dependent manner, showing a gradual increase of ethidium bromide positive cells in OLEs-treated cells compared to the control, and a statistically significant difference was obtained $p<0.001$ (Figure 2C, 2D).

3.5. Induction of DNA damage

Extracts at all concentrations lead to the breaking of DNA single-strand in H4IIE cells, and there is a gradual concentration-response relationship and a statistically significant difference of $p<0.001$. Comet assay images of control and OLEs-treated H4IIE cells are presented in Figures 3A and 3B.

3.6. Anti-inflammatory activity of OLEs and its phenolic compounds

Results showed that all extracts at the concentrations of 250, 500, 1000, and 2000 ppm causes elevated levels of IL-1β, IL-6, and TNF-α in H4IIE and Clone-9 cells (Figure 4A, 4B and 4C).

The OLEs had a significant activity on IL-1β, IL-6, and TNF-α production in a concentration-dependent manner.

4. Discussion

Studies on *Olea europaea L.* generally include data on the chemical and physical properties of the oil extracted from fruits and their samples. There are few studies on the antioxidant activities of leaf extracts in the literature since the oil of the fruits is very valuable. Ethanol and acetone have been found to be highly effective solvents in extracting phenolic compounds. Phenolic compounds are one of the main secondary metabolites of olive leaves [20–23]. It has also been found that water / aqueous-ethanol / acetone mixtures are generally more effective solvents to extract polyphenolic compounds of olive leaves [20–23].

The antioxidant activity of olive leaf extracts may directly be related to the polyphenol content [21, 23]. Oleuropein, is the major secondary metabolite and has been extensively studied, but available information is limited for different olive leaf extracts [21–23]. Oleuropein is an important metabolite for the antioxidant ability of olive leaf extracts [9]. For the olive leaf extracts, acetone and ethanol extracts exhibit higher antioxidant capacity in both methods (DPPH and ABTS). Our results were consistent with the literature, and acetone and ethanol extracts with the high oleuropein content were effective in antioxidant scavengers [23-26]. In a study using solid-liquid extraction technique and three different extraction solvents (petroleum ether, water, methanol (80%)), suggested that flavonoids (from 3.33-17.64 mg catechin equivalents (CE)/g), phenolics (from 3.64-21.47 mg gallic acid equivalents (GAE)/g) were abundant in the content of olive leaf extracts and their analysis correlated with antioxidant capacity [24]. These data are consistent with our results presented in this study.

Olive leaf extracts caused cytotoxicity in many different cancer cells such as human breast cancer cells - MCF7, human thyroid cancer cells - TAD2, human promyelocytic leukemia cells - HL60 [25-27]. Kimura et al. investigated the effects of olive leave extract and oleuropein on the skin cancer animal model. According to their investigations, oleuropein and olive leave extract were superior to the control group in skin thickness and tumor incidence [26]. Barbaro et al. suggested in their systematic review that olive leaves and oleuropein anti-tumor activity might be related with ROS scavenging effect, antiproliferative effect, apoptosis induction, angiogenesis inhibition, and anti-migration effect [28]. Our study is coherent with previous studies, and we found that olive leave extracts at different concentrations inhibit H4IIE cancer cells more than clone-9 healthy cells (Figure 2). In fact, at 0.25 mg/mL concentrations, acetone and methanolic extracts positively affected cell viability in terms of RLU in the healthy clone-9 cell line. This indicates that OLE causes selective cytotoxicity in rat hepatoma cell lines, but not in healthy hepatocytes. This selectivity can be a result of the susceptibility of cancer cells to intracellular ROS formation. Cancer cells have increased ROS producing capacity, and therefore the selective activity of OLE on H4IIE may be due to their increased susceptibility to iROS. The effects of OLE on H4IIE cells may offer opportunities for new chemoprevention studies for liver cancer.

On the other hand, the higher concentration of OLE was induced cytotoxicity and iROS for both healthy and cancer cell lines. These results indicate that the dose of the extracts have a crucial role in the pharmacological effect. According to concentration, OLE may induce or inhibit cytotoxicity and apoptosis. According to Park et al., mice fed with an oleuropein-supplemented diet attenuated hepatic steatosis in microscopic and macroscopic scale [29]. According to the literature and our results, olive leave extract or oleuropein-supplemented diet have protective effects on liver diseases.

In literature, via various mechanisms, olive leaves can induce apoptosis and inhibit cell proliferation. Oleuropein stops cell growth and promotes apoptosis in HT29 colorectal cancer

cells via a p53-dependent pathway [25,26]. This apoptotic effect with a similar mechanism was also reported in breast cancer cells [30]. In addition, on doxorubicin-induced cardiomyopathy, major phenolic of olive leave oleuropein showed a protective effect through AMPK activation and iNOS suppression [27]. This study revealed that olive leaves extract, which contains oleuropein as major secondary metabolites has antitumor activity in hepatoma cell lines. These results are partly consistent with the apoptosis mechanism described in MCF-7 cells treated with oleuropein [30]. iROS is thought to be below the activation of AKT [31,32]. Oleuropein can suppress the phosphorylation of AKT. Apoptotic effects of olive leave extract may be related PI3K / AKT pathway, which is a critical pathway for H4IIE cells.

Antigenotoxic agents often show expected therapeutic effects that may be effective in to control cancer. Our results were promising that OLE reduced cell viability in H4IIE cells and were in line with literature showing that OLE can inhibit the proliferation of malignant cells *in vivo* [29-34]. This feature of OLE can be explained by its capacity to act as a strong free radical scavenger, as shown in Figure 1.

Our results pointed out, OLE have induced the IL-1β, IL-6, and TNF-α production in a concentration-dependent manner. A significant decrease in pro-inflammatory cytokine levels was found in cells treated with different OLE. Our results were also consistent with the literature in terms of anti-inflammatory effects [9]. In comparison with healthy and cancer cell lines, anti-inflammatory effects were significantly higher in healthy cell lines. On the other hand, the increased inflammatory response of cancer cell lines is desirable, which starts apoptosis cascades. The possible anti-inflammatory mechanism might be related with Arachidonic Acid Pathway, Mitogen-activated protein kinase (MAPK), and MAPK enzymes such as p38, extracellular signal-related kinase (ERK), and c-Jun N terminal, kinase (JNK) induce transcriptional and post-transcriptional, activation of COX enzymes. Anti-inflammatory effects of *Olea europaea L may be* accomplished through NFκB, activation. Inhibition of these proinflammatory cytokines production could be related either with oleuropein or other secondary metabolites [35]. Oleuropein is the fundamental OLE component that exhibits anti-inflammatory effects at a concentration of 20 µg/mL [9]. In another study, OLE reduced mRNA expression of E-selectin in human coronary artery endothelial cells stimulated with serum amyloid A, as well as decreased IL-6 and IL-8 protein levels and reduced matrix metalloproteinase 2 levels in non-stimulated cells [34]. In both studies, the amount of OLE used was 20 µg/mL and 0.5-1 µg/mL, respectively. Compared with our OLE (0.25-2 mg/mL) concentrations, these results show that high amounts of OLE show prooxidant activity.

5. Conclusion

To the best of our knowledge, this is the first study in the literature investigating the cytotoxic, genotoxic, and apoptotic activity of olive leaf extracts on liver cancer cell lines. Olive leaf extracts activated cytotoxic and apoptotic mechanisms on cancer cells. For this reason, olive leaf extracts can be a potential drug for cancer treatment. More detailed *in vivo* studies are needed in the future.

6. Acknowledgment

The authors would like to thank Bezmialem Vakif University, Istanbul, Turkey, for financial support. We are also grateful to Humeyra SAHIN-BEKTAY for her valuable contribution during her undergraduate education to our research.

This study has been supported by the Scientific Research Unit of Bezmialem Vakif University with a grant number of 9.2015/22. The authors declare that there are no conflicting interests.

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8. Figure legends

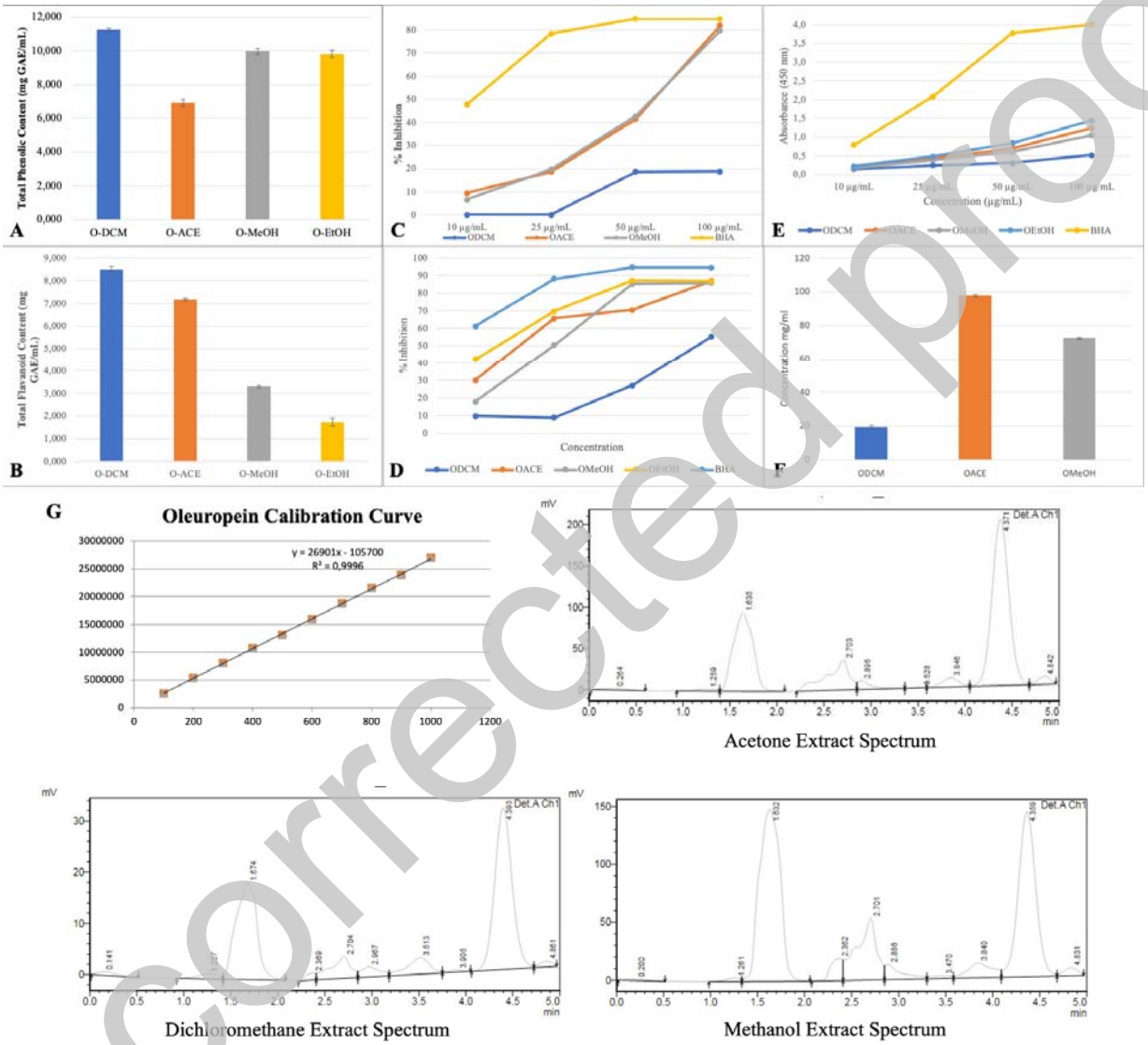

Figure 1. Evaluation of The Amount of Flavonoids, Phenolic, Free Radical Activity, ABTS Cation Radical Scavenging Activity, Reduction Force, and Oleuropein Level Analysis in Olea Leaf Extracts. (A) Total phenolic compounds of extracts; (B) Total flavonoid compounds of extracts; (C) Free radical activity of extracts for different concentrations; (D) ABTS cation radical scavenging activity of extracts for different concentrations; (E) Reduction force of extracts; (F) Oleuropein concentration in extracts. (G) Oleuropein calibration curve and extract spectrums of acetone, dichloromethane, and methanol. Abbreviations: GAE, gallic acid; QUE,

quercetin; DCM, OLE with dichloromethane; ACE, OLE with acetone; MeOH, OLE with methanol; EtOH, OLE with ethanol; BHA, Butylated Hydroxyanisole

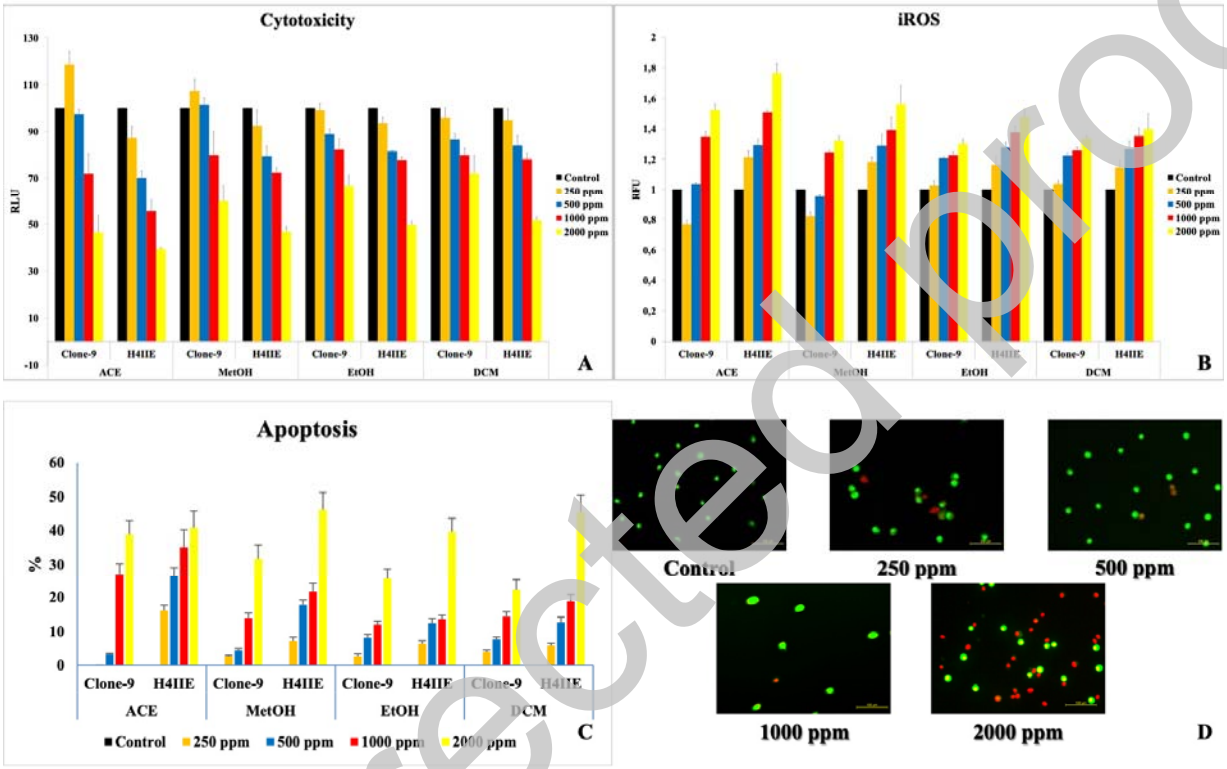

Figure 2. Cytotoxicity, iROS, and Apoptosis assays with increasing concentrations (250, 500, 1000, and 2000 ppm) of *Olea europaea L.* extract on Clone-9, and H4IIE cells. (A) Cytotoxic effect of *Olea europaea L.* extracts. (B) Intracellular reactive oxygen species (iROS) generation levels of *Olea europaea L.* extracts. (C) Determination of apoptotic and living cells using acridine orange/ethidium bromide double staining test. (D) Representative immunofluorescence images in H4IIE cells. The level of apoptosis increased with increasing concentrations of O-MeOH. O-MeOH showed the highest apoptotic damage among all extracts. Differences between the groups were tested for significance using one-way ANOVA (*p <0.05, **Pp <0.01, ***P <0.001, $^{+}$p <0.05, $^{++}$p <0.01, $^{+++}$p <0.001). Abbreviations: RLU, Relative Luminescence Units; RFU, Relative Fluorescent Units; DCM, OLE with dichloromethane; ACE, OLE with acetone; MeOH, OLE with methanol; EtOH, OLE with ethanol.

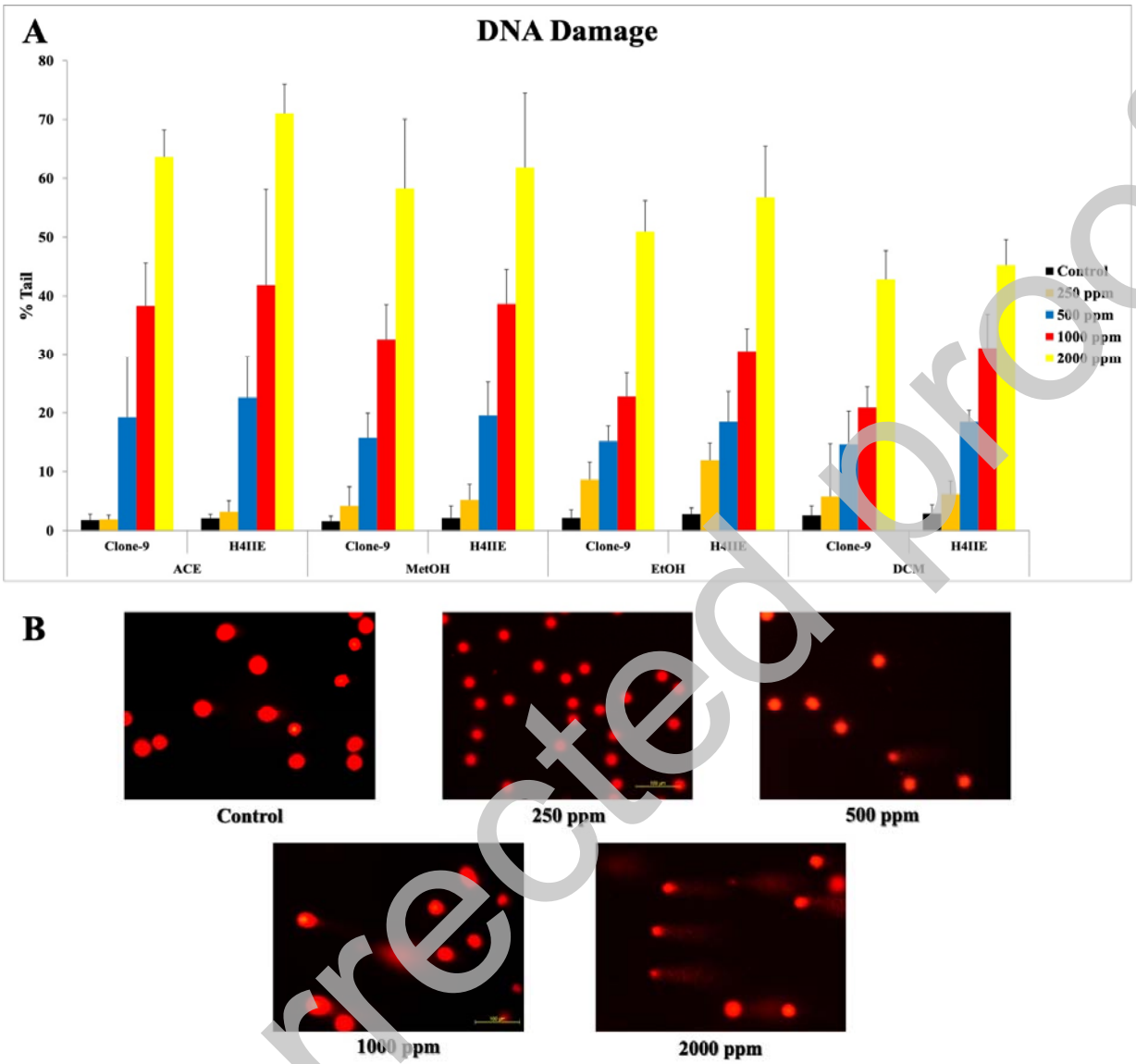

Figure 3. Olive leave extracts cause DNA damage with increasing concentrations (250, 500, 1000, and 2000 ppm) on H4IIE cells. (A) Genotoxic effects of olive leaf extract with increasing concentrations (250, 500, 1000, and 2000 ppm) using Comet Assay after 24-hour incubation. (B) Representative immunofluorescence images in H4IIE cells. The level of DNA damage increased with increasing concentrations of O-ACE. O-ACE showed the highest genotoxic damage among all extracts. Differences between the groups were tested for significance using one-way ANOVA (*p <0.05, **Pp <0.01, ***P <0.001, $^{+}$p <0.05, $^{++}$p <0.01, $^{+++}$p <0.001). Abbreviations: DCM, OLE with dichloromethane; ACE, OLE with acetone; MeOH, OLE with methanol; EtOH, OLE with ethanol.

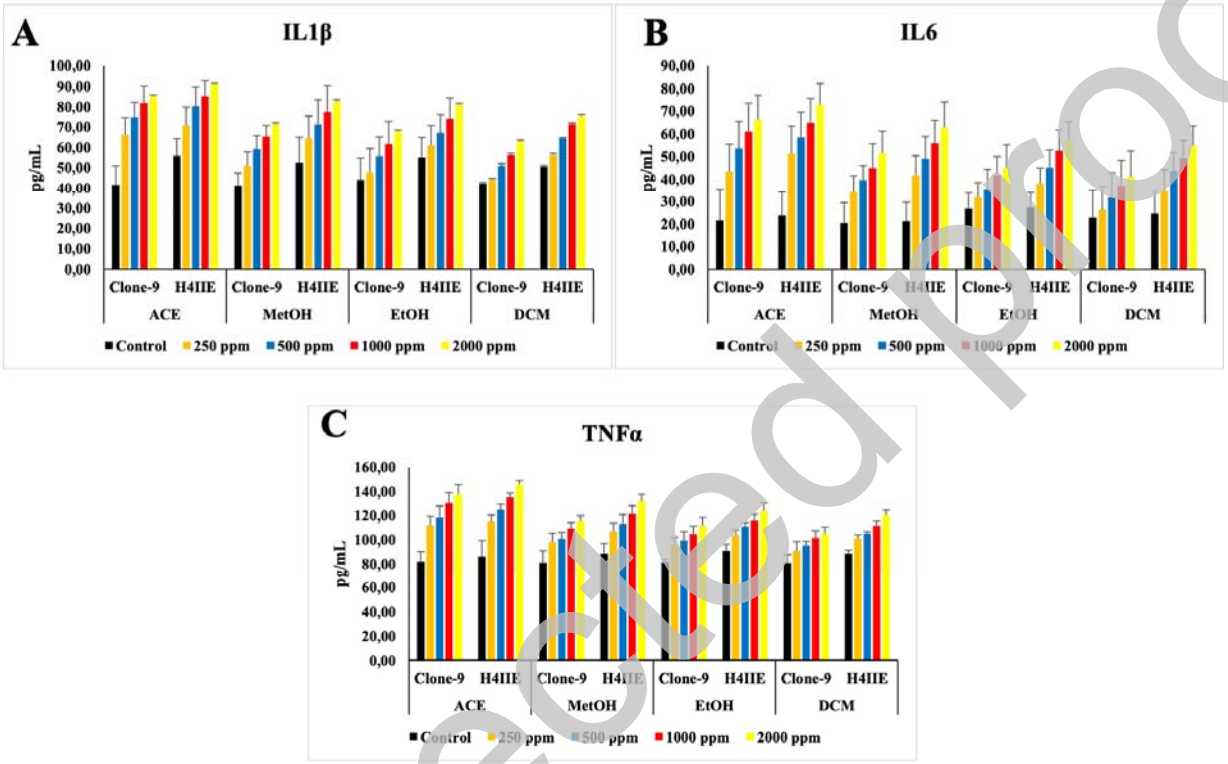

Figure 4. Levels of IL-1β, IL-6, and TNF-α with increasing concentrations (250, 500, 1000 and 2000 ppm) of OLE on Clone-9 and H4IIE cells after 24-hour incubation. (A) IL-1β levels (B) IL-6 levels (C) TNF-α levels. Differences between the groups were tested for significance using one-way ANOVA (*p <0.05, **Pp <0.01, ***P <0.001, $^{+}$p <0.05, $^{++}$p <0.01, $^{+++}$p <0.001). Abbreviations: DCM, OLE with dichlorometane; ACE, OLE with acetone; MeOH, OLE with methanol; EtOH, OLE with ethanol.