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An Optimum Dose of Olive Leaf Extract Improves Insulin Receptor Substrate-1, Tyrosine Kinase, and Glucose Transporters, While High Doses Have Genotoxic and Apoptotic Effects

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Abstract

Type 2 diabetes is the most common type of diabetes. Conventionally many drugs are used for the treatment of diabetes such as biguanides, sulfonylureas, meglitinides, etc. But the desired effective treatment is still not to be achieved. So researches are going on for the development of effective alternative therapy against diabetes. Olive leaves are traditionally used in the treatment of the disease. However, studies on its mechanism of action are not yet enough. The aim of this study was to investigate whether olive leaf extract (OLE) improves insulin receptor substrate-1 (IRS-1), tyrosine kinase (TK), GLUT-2, and GLUT-4. Oleuropein levels were analyzed from OLE obtained by using four different solvents, and the highest content of methanol extract was selected for the study. Different concentrations of OLE (2.5 to 320 μg/mL) were incubated with hepatocellular carcinoma (HepG2) cells for 24 hours. After incubation, cell viability was assessed based on luminometric ATP cell viability assay kit. Intracellular reactive oxygen species (ROS) generating level was detected using 2,7dichlorodihydrofluorescein-diacetate (H_2DCF-DA) fluorescent probes. Apoptosis was evaluated by acridine orange/ethidium bromide double staining method. Genotoxicity was evaluated by alkaline single cell gel

electrophoresis assay (Comet Assay). Protein expression levels of IRS-1, TK, GLUT-2, and GLUT-4 were analyzed by western blotting technique from the obtained cell lysates. Although an optimum doses of OLE (10 μg/mL) maximally increased cell proliferation, decreased ROS generation improved IRS-1, TK, GLUT-2, and GLUT-4 protein expression levels (about fivefold), higher doses (10 to 320 μg/mL) markedly decreased the cell viability, increased DNA damage, apoptosis and ROS generation in a concentration-dependent manner. OLE can be used in the treatment of type 2 diabetes. However, in order to find the most effective and non-toxic concentration, dose optimization is required.

Keywords

Phytotherapy, Diabetes Mellitus, Olive Leaf Extract, Glucose Transporters, Insulin Receptors

1. Introduction

Diabetes is a metabolic disorder which is mainly characterized by hyperglycemia and arises by the defects in insulin secretion or insulin action or both. It is categorized into two types, type-I, and type-II diabetes. It is a common public health problem around the world, and more than 90% of patients with diabetes are type 2 diabetes mellitus (T2DM) [1]. The global prevalence of DM in adults was estimated to be 8.8% in 2015 and is predicted to rise to 10.4% in 2040 [2]. An unhealthy diet and a sedentary lifestyle are important roles in the development of T2DM [3]. It is characterized by disorders of carbohydrate, lipid and protein metabolism, impaired insulin secretion, insulin resistance, or a combination of both [4]. The main metabolic problem in insulin-resistant T2DM is a defect in glucose uptake due to inaccurate regulation of the glucose transporter proteins (GLUT) [5]. An inhibition of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) causes GLUT protein defects [5] [6].

T2DM is a disease that can be treated successfully, even if it is not fully cured. Lifestyle changes, such as diet and exercise, may facilitate the treatment of T2DM, but there is a need for anti-diabetic agents to regulate blood sugar levels in severe cases. Conventionally many drugs are used for the treatment of diabetes such as biguanides, sulfonylureas, meglitinides, etc. But the desired effective treatment is still not to be achieved. So researches are going on for the development of effective alternative therapy against diabetes. Medicinal plants are promising source and also very useful for the development of complementary therapy. For example, Subash-Babu *et al.* [7] demonstrated by an *in vitro* study that *Nymphaea stellata* extract increased glucose uptake and glucose-induced insulin secretion and they also showed by an *in vivo* study that it increased IRS-1 phosphorylation and GLUT-4 expression, which increased insulin response in liver and muscle in T2DM. The protective effects of *Astragalus membranaceus*

on nephropathy as a complication of diabetes have also been shown [8]. The olive tree leaf (*Olea europaea L.*), which is one of the medicinal plants, is widely used in traditional medicine, especially in European and Mediterranean basin. It has been used in different ways as extracts, herbal teas and powders and, potentially contain bioactive compounds such as oleuropein, tocopherol, caffeic acid and luteolin [9]. Studies indicate that biologically active compounds in olive leaf products can exhibit a variety of biological actions, such as antioxidant [10], antimicrobial [11], anticancer [12], anti-hypertensive and vasodilator [13] properties. It has also been shown as possible diabetes-preventive components that decrease blood glucose concentrations, recover hyperglycemic symptoms, and enhance anti-oxidative activity [14]. Liu, Y.N. *et al.* [15] have demonstrated with an animal study that olive leaf extract (OLE) attenuate insulin resistance by suppressing mRNA expression of pro-inflammatory cytokines and elevating of IRS-1 expression. However, there is no *in vitro* cell culture study investigating the effects of OLE on both insulin receptors and carrier proteins with genotoxic, cytotoxic, and apoptotic effects. In this study, we aimed to investigate the effects of OLE on IRS-1, TK, GLUT-2, and GLUT-4 protein expression levels and, cytotoxic, genotoxic, apoptotic and reactive oxygen species (ROS) generating potentials.

2. Materials and Methods

2.1. Chemicals

The human hepatocellular carcinoma cells (HepG2) were purchased from ATCC (American Type Culture Collection, Manassas, VA 20110 USA). Fetal Bovine Serum (FBS), Penicillin-Streptomycin (10,000 U/mL), Trypsin-EDTA (0.25%) with phenol red and E'MEM—Eagle Minimum Essential Media were purchased from Gibco (CA, USA). IRS-1, TK, GLUT-2, and GLUT-4 primer antibodies were provided by Santa Cruz Biotechnologies (Santa Cruz, California, USA), and ATP-Glo cell viability assay kit was provided by Promega (Madison, Wisconsin, USA). Other chemicals such as 2,7′-Dichlorodihydrofluorescein-diacetate (H2DCF-DA), ethidium bromide (EB), acridine orange (AO), ninhydrin, acetic acid, aluminum chloride ($AlCl_3$), cadmium chloride hemi (pentahydrate), (+) quercetin, methanol, sodium hydroxide (NaOH), gallic acid, L-Leucine, 2,4,6-tripyridyl-S-triazine (TPTZ), sodium nitrite ($NaNO_2$), potassium persulphate (K_2SO_4), ferric chloride ($FeCl_3$), sodium chloride (NaCl), sodium carbonate (Na_2CO_3), ammonium ferrous sulphate, phosphoric acid (H_3PO_4), Coomassie Brilliant Blue, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and Oleuropein (purity 98% by HPLC) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). HPLC grade acetonitrile, methanol, ethyl acetate, and orthophosphoric acid were purchased from Merck Chemical (Darmstadt, Germany).

2.2. Preparation of Olive Leaf Extract

The fresh olive leaves (*Olea europaea* L.) were collected after fruit harvesting

during December 2017 from an olive tree located in Tekirdag, Turkey. Extraction of olive leaf was prepared according to the method of Zahkok *et al.* [16] with slight modifications. Thus, 500 g of fresh olive leaf was washed, dried at 30˚C for 5 days and crushed to a moderately-coarse powder. The fourth gram of resulting powder was divided into four equal parts and suspended in ethanol, methanol, acetonitrile, dichloromethane (70%) for 24 hours. The supernatant was filtered, and solvents were evaporated at 45˚C using rotary evaporator (Heidolph, Germany). Then, solvent-free extracts were dried by using a freeze drier system (Labconco; Kansas City, USA). The dry extracts obtained were kept away from the light (at room temperature) in amber-colored glass bottles until further analysis. The amount of OLE was expressed as milligram per gram of OLE. The OLE was dissolved in DMSO (0.1%) and filtered with a Millipore membrane filter (0.45 µm) prior to analysis.

2.3. Determination of Total Flavonoids and Phenolic Contents

The Folin-Ciocalteu method [17] was used to determine the total phenolic content of OLE. Fifty µL of filtered sample and 250 µL of 0.2 N Folin-Ciocalteu reagent was mixed with vortex and kept 5 min at room temperature. Then it was mixed with 200 µL of 0.7 $mol \cdot L^{-1}$ Na_2CO_3. After incubation at room temperature for 2 hours, the absorbance of the reaction mixture was measured at 760 nm against a blank using a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Scientific, USA). Gallic acid (0 - 300 $mg \cdot L^{-1}$) was used as a standard to produce the calibration curve. The mean of three readings was used, and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/100 g OLE.

The total flavonoid content of the OLE samples was determined according to the colorimetric assay method developed by Zhishen *et al.* [18]. Fifty µL of filtered OLE samples were mixed with 250 µL of distilled water and 15 µL of a 5% $NaNO_2$ solution. After 6 min, 30 µL of 10% $AlCl_3$ solution was added, then 100 µL 1 $mol \cdot L^{-1}$ NaOH was added, and the solution was incubated for a further 5 min at room temperature. The reaction mixture was mixed well, and the intensity of the red flavonoid-aluminum complex was measured at 510 nm using a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Scientific, USA). A standard curve of quercetin was drawn within a concentration range of 5 to 50 mg/L. Total flavonoid content was expressed as mg of quercetin equivalents per 100 g of OLE.

2.4. Total Antioxidant Capacity Measurement

The total antioxidant capacity (TAC) was determined according to the photometric method developed by Erel [19]. Briefly, 5 µL of OLE samples were added to 500 µL of $ABTS^+$ reagent and incubated at room temperature for 90 seconds. The color inhibition of the $ABTS^+$ radical was measured at 734 nm using a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Scientific, USA).

Results were expressed in mmol Trolox equivalents per 100 g of OLE.

2.5. HPLC Analysis of Oleuropein

Oleuropein concentration from OLE was determined according to the method of Al-Rimawi, F. [20]. The HPLC system (Thermo Fisher Dionex Ultimate 3000 UHPLC, USA) consisted of a quaternary gradient pump, UV-vis detector, and a reversed-phase C18 column (250 × 4.6 mm particle size 5 μm, Betasil-Thermo Scientific, USA) were used. Phosphate buffer (0.05 mol/L and pH 3 adjusted with orthophosphoric acid) and acetonitrile (70:30, v/v) were used as mobile phase. Injection volume was 5 μL for both standard and sample solution, flow rate was 1 mL/min, and column heat was adjusted to 24˚C. UV detector at 240 nm was used for oleuropein determination. Identification of oleuropein was based on retention times in comparison with standard of oleuropein. The concentration of oleuropein in the extracts was calculated using peak area. Oleuropein was dissolved in 100% methanol to prepare a stock standard at a concentration of 4000 mg/L. Then, deionized water was used to dilute this and obtain standard solutions with concentrations between 100 and 800 mg/L. **Figure 1** shows chromatogram and calibration curve for oleuropein standard.

2.6. Cell Viability Assay

Cell Titer-Glo® Luminescent Cell Viability Test Kit (Promega, Madison, WI, USA) was used to measure cell viability level. This method determines the degree of cell viability in proportion to the amount of ATP. For analysis, HepG2 cancer cells (1.5×10^3 cells·well^{-1}) were plated on 96-well plates. After 24 hours, the cells were incubated with different concentrations of Olive leave extract (2.5 to 320 μg/mL). After incubation, the luciferin derivative and cell lysis solution were added as substrates. The luciferin derivative converts a light signal proportional to the current amount of ATP. Luminescence was measured using a Varioskan Flash Multimode Reader (Thermo Scientific, USA) and normalized to control.

2.7. Intracellular Reactive Oxygen Species Measurement

The intracellular reactive oxygen species (ROS) production levels were measured by the fluorometric method using a prob, H_2DCF-DA. 1.5×10^5 cells/well were seeded in each well of 96 wells. After 24 hours, they were treated with OLE at different concentrations (2.5 to 320 μg/mL) and incubated for another 24 hours. The cells were washed with PBS and incubated with 5 μM H_2DCF-DA for 30 min at 37˚C in the dark. The cells were then washed, resuspended in PBS, and measured for the ROS contents using a fluorimeter (Varioskan Flash Multimode Reader, Thermo Scientific, USA) and normalized to control.

2.8. Genotoxicity Analysis

Alkaline single cell gel electrophoresis assay (Comet Assay) was carried out with a slight modification of Singh *et al.* [19] to assess the genotoxic effects of olive

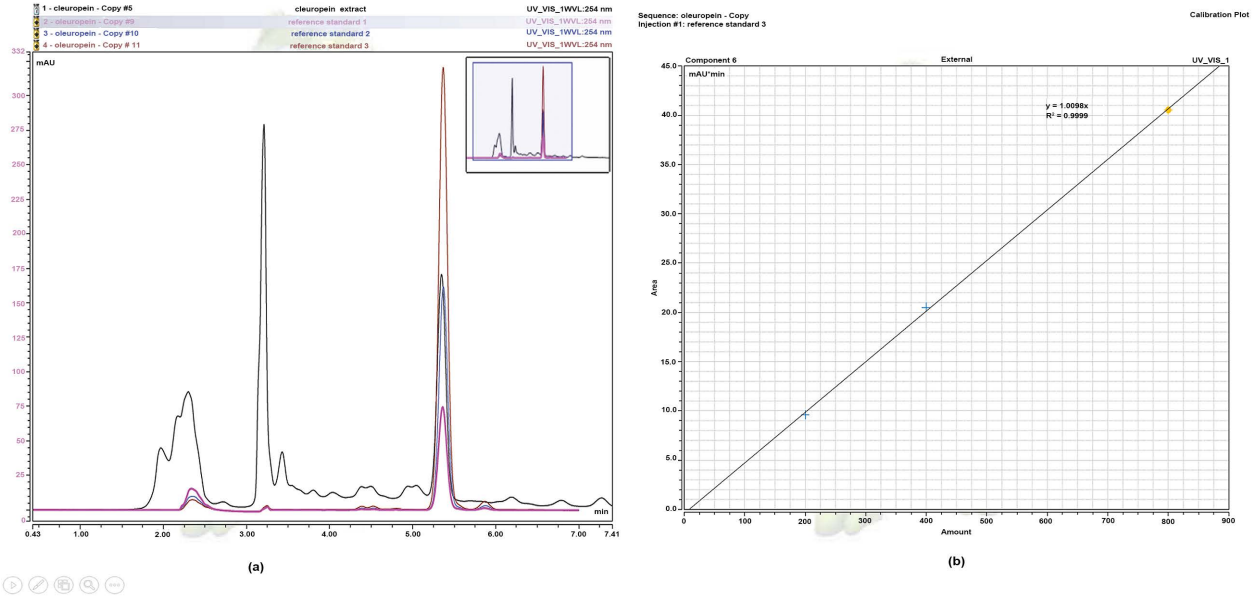

Figure 1. Chromatogram (a) and calibration curve (b) of oleuropein analyzed.

leaf extract on HepG2 cells. The cells were plated on 6-well cell culture plates (approximately 2×10^5 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 another 24 hours. The cells were collected using trypsin/EDTA for 4 min at 4˚C and centrifuged at 400 ×g for 5 min at 4˚C. The supernatant was drained, and the cell density was adjusted to 2×10^5 cells/mL using cold PBS. Ninety µl of 0.6% low melting point agarose (LMPA) and 10 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 few min, and the slides were then placed in lysis buffer, pH 10 (1% Triton X-100, 2.5 M NaCl, 10 mmol·L^{-1} Tris, 0.1 mol·L^{-1} EDTA, Sigma-Aldrich) for 1 hour on the ice in the dark conditions. The slides were then incubated in an alkaline solution (0.3 mol·L^{-1} NaOH, 1 mmol·L^{-1} EDTA, Sigma-Aldrich) for 40 min at dark conditions 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. The slides were neutralized in 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).

2.9. Apoptosis Measurement

Acridine orange/ethidium bromide (AO/EB), double staining test, was developed by Kaibhatla *et al.* [21] 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 apoptotic cell nucleus [22]. AO spreads green fluorescence

at 480 - 490 nm from living cells while spreading to dsDNA. Briefly, 2×10^5 cells/well were seeded in 6 well-plate and incubated for 24 hours. Then, the olive leaf samples below IC_{50} concentrations were added and incubated for another 24 hours. Following OLE treatment, 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).

2.10. Immunoblotting Measurements

HepG2 cells were seeded on six-well plates at 1.5×10^5 cells per well and incubated for 24 hours. It was then treated with olive leaf extract according to their IC_{50} values. After 24 hours of incubation, the cells were harvested and prepared in NP-40 cell lysis buffer (2 mmol·L^{-1} Tris-HCl pH 7.5, 150 mmol·L^{-1} NaCl, 10% glycerol and 0.2% NP-40 plus a protease inhibitor cocktail) for 30 min on ice and centrifuged at 14,000 ×g (Beckman Coulter, Krefeld, Germany) for 10 min at 4˚C. The final supernatant was then used as the cytosolic fraction. The protein concentration of the supernatant was determined using the Bradford protein assay method [23]. Proteins from cellular supernatants were separated on 8% to 10% polyacrylamide gel and transferred to a nitrocellulose membrane using the Trans-blot SD semi-permeable electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). Tris-HCl buffered saline with Tween 20 (TBST) with 5% non-fat-milk was used for blocking the membrane. The primary antibodies, IRS-1, TK, GLUT-2, and GLUT-4 (1/500 dilution) were used after a night incubation (4˚C). All samples were also blotted for β-actin to normalize protein amounts. TBST were used for washing the membrane and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for another 1 hour. Immunolabelled proteins were visualized using ECL Western staining substrate (Thermo Scientific) and captured with an imaging system (Vilber Lourmat Sté, Collégien, France).

2.11. Statistical Analysis

The experiments were repeated 3 times, and the results were expressed as mean value ± standard deviation (Mean ± SD). Statistical evaluation was performed using the analysis of variance (One-way ANOVA). Differences with a probability value of $p < 0.05$ were accepted statistically significant. IC_{50} values of OLE over the cell line were calculated by nonlinear regression analysis. The statistical analysis was performed by using Statistical Package for Social Sciences (SPSS) version 21.

3. Results

3.1. Total Phenol, Flavonoids, Antioxidant and Oleuropein Contents of Olive Leaf Extracts

Total phenol, flavonoid, antioxidant and OLE contents of four different ex-

tracted samples were different and, the highest total phenol, flavonoid, antioxidant, and OLE contents were found in the samples extracted with methanol (Figure 2).

3.2. Cell Culture Studies

3.2.1. Cell Viability Assessment of Olive Leaf Extract

To assess the effect of OLE on cell growth, HepG2 cells were treated with different concentrations of OLE (2.5 to 320 µg/mL) for 24 hours. After incubation, the cytotoxic effect of OLE was measured by ATP cell viability test. The cell viability of the control cells was 100%. Cell viability was increased to 120% at a concentration of 10 µg/mL compared to the control after 24 hours of addition of the OLE. At doses above this concentration, cytotoxic activity increased in a concentration-dependent manner ($p < 0.001$). The 50% inhibitory concentration (IC50) was found 88 µg/mL (Figure 3). These findings demonstrated that OLE has a proliferative effect at lower concentrations, but it shows cell dead effect at higher concentrations.

3.2.2. Reactive Oxygen Species Generation Assessment

We measured intracellular ROS formation by the fluorometric method using the H_2DCF-DA probe. Low OLE dose (10 µg/mL) significantly reduced ROS production in HepG2 cells ($p < 0.05$). ROS levels were significantly increased when the dose of OLE was over 40 µg/mL (Figure 3).

3.2.3. Genotoxic Assessment of Olive Leaf Extract

For DNA damage analysis, HepG2 cells were treated with different doses of OLE

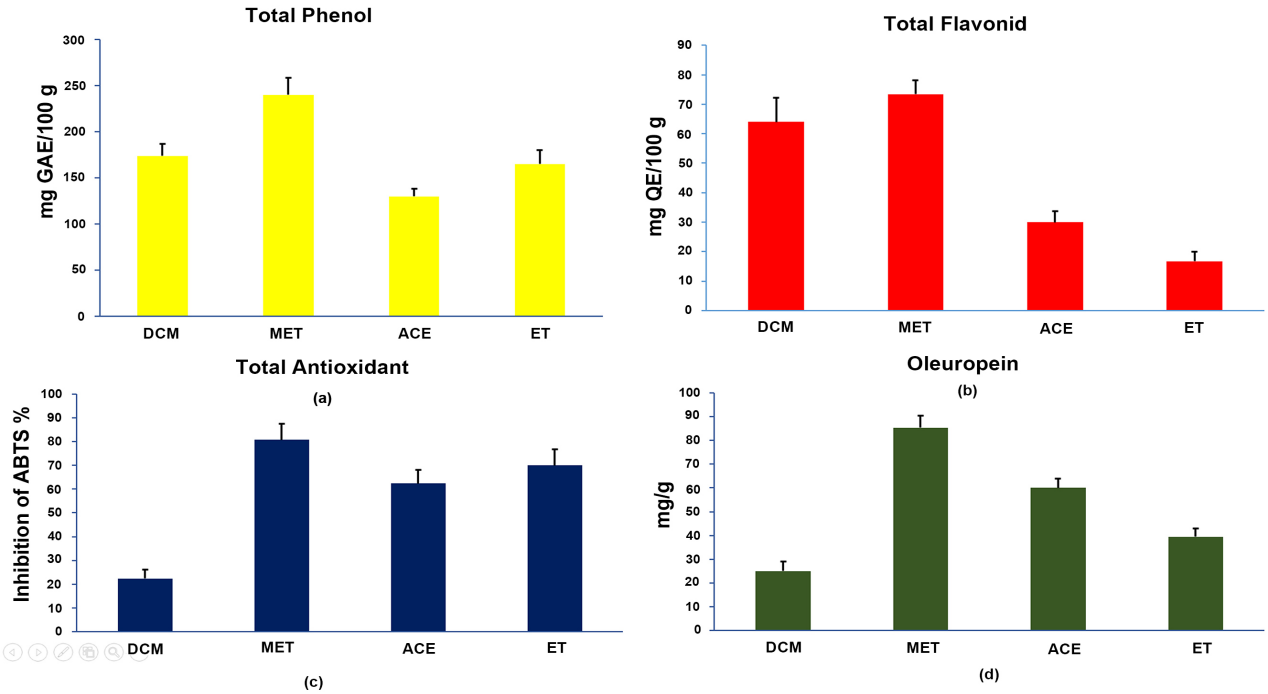

Figure 2. Total phenol (a), flavonoid (b), antioxidant capacity (c) and oleuropein content (d) of olive leaf extract samples obtained by using four different solvents (dichloromethane (DCM), Methanol (MET), acetonitrile (ACE), ethanol (ET).

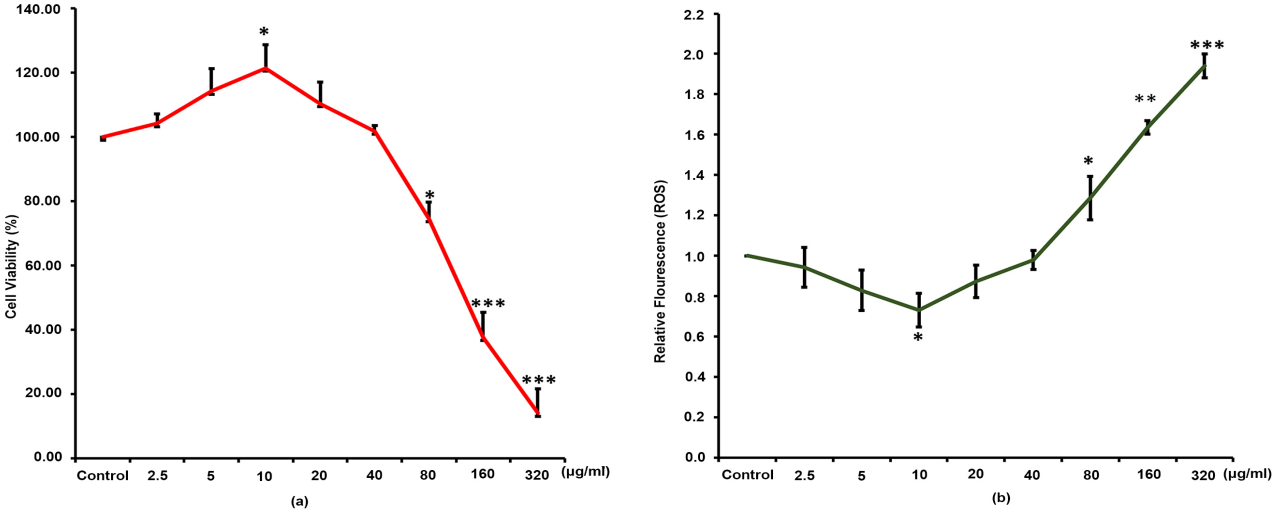

Figure 3. HepG2 cells were treated with 2.5, 5, 10, 20, 40, 80 160 and 320 µg/mL olive leaf extract and incubated for 24 hours. (a) cell viability assay was performed to determine cytotoxicity. The percentage of cell viability was calculated as a control of 100%; (b) Reactive oxygen generating assay was performed to determine pro-oxidant activity of Olive leaf extract. Significant differences compared to the control are indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$.

samples (2.5 to 160 µg/mL) for 24 hours, and the DNA damage was measured via the Comet Assay method. Damaged DNA appears in a bright head and comet, while undamaged DNA appears to be only round. After 24 hours incubation, the % tail intensity did not increase up to 80 µg/mL doses. It increased significantly with the increasing doses over 80 µg/mL (**Figure 4**).

3.2.4. Acridine Orange/Ethidium Bromide Double Staining

Apoptosis is important in determining tumor formation and resistance to treatment. In our study, we performed AO/EB double staining to evaluate the apoptotic effects of different concentrations of OLE in cancer cells. HepG2 cells were incubated with OLE for 24 hours to demonstrate the morphological characteristics of apoptosis on cells. It was then stained with AO/EB double staining and examined under fluorescence microscopy. After 24 hours of incubation, as the administered dose increased, the green-looking viable cell ratios decreased, and the yellow-orange-looking apoptotic cell ratios increased. Apoptotic cells increased significantly after 40 mg/mL doses, while necrotic cell ratios increased after 100 µg/mL (**Figure 4**).

3.2.5. Western Blotting Results

In order to investigate the relationship between the olive leaf extract, insulin receptors and transfer proteins, the expression levels of IRS-1, TK, GLUT-2, and GLUT-4 were analyzed by western blotting method. For this purpose, HepG2 cells were treated with OLE at different concentrations for 24 hours. Cell extracts were used for western blotting. The β-actin was used as a control. The results showed that both insulin receptors and transport protein expression levels increased up to 10 µg/mL. All protein expression levels increased approximately fivefold according to the control. Their levels decreased significantly with increasing doses after 10 µg/mL (**Figure 5**).

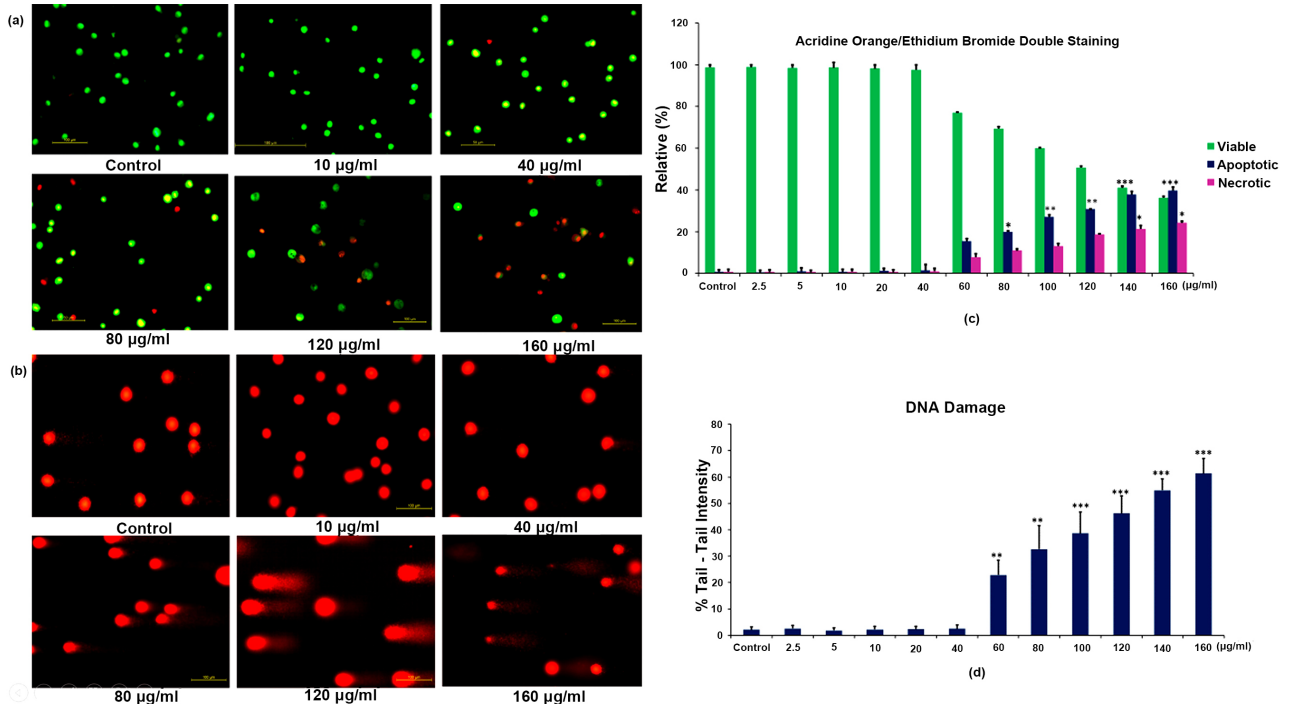

Figure 4. HepG2 cells were treated with different concentrations of OLE (2.5 to 160 µg/mL) for 24 hours, the genotoxic effect was analyzed with comet assay method (a) and apoptotic activity was analyzed with acridine orange/ethidium bromide double staining methods (b). There were significant changes in the tail % of DNA according to the control with the increasing concentrations (c), Increased percentage of apoptotic cells according to the control with the increasing concentrations of extract (d) Significant differences indicated by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

4. Discussion

Olive tree leaves have been widely used in traditional medicine in European and Mediterranean countries, and antidiabetic effects of OLE have been shown in several studies [14] [24] [25]. The bioactivity of olive leaf extracts appears to be due to the antioxidant and phenolic compounds such as oleuropein, hydroxytyrosis, oleuropein aglycone, and tyrosol [25]. Specifically, oleuropein has been reported to have an anti-hyperglycemic effect in diabetic rats [26]. However, the molecular mechanism of OLE to alleviate hyperglycemia remains unclear.

Two possible mechanisms of hypoglycemic effect of OLE are 1) enhanced glucose-induced insulin secretion, and 2) increased peripheral glucose uptake [9]. Insulin requires insulin receptors and transfer proteins to increase glucose uptake in peripheral tissues [27]. Binding of insulin to its receptor activates the phosphorylation of insulin receptor TK and the family of insulin receptor substrates (IRS), especially IRS-1 and IRS-2 [28]. The phosphorylated IRS proteins bind to intracellular signal molecules, phosphatidylinositol 3 kinase (PI3K) and serine/threonine kinase PI3K-linked protein kinase B (Akt/PKB) and, activate them. The activation of Akt/PKB stimulates GLUT-4, which results in enhanced glucose uptake [29]. Previous studies have shown that insulin resistance is most likely due to a defect in the insulin receptor IRS 1/PI3K cascade [30]. The present study demonstrated that OLE increased expressions of insulin receptors

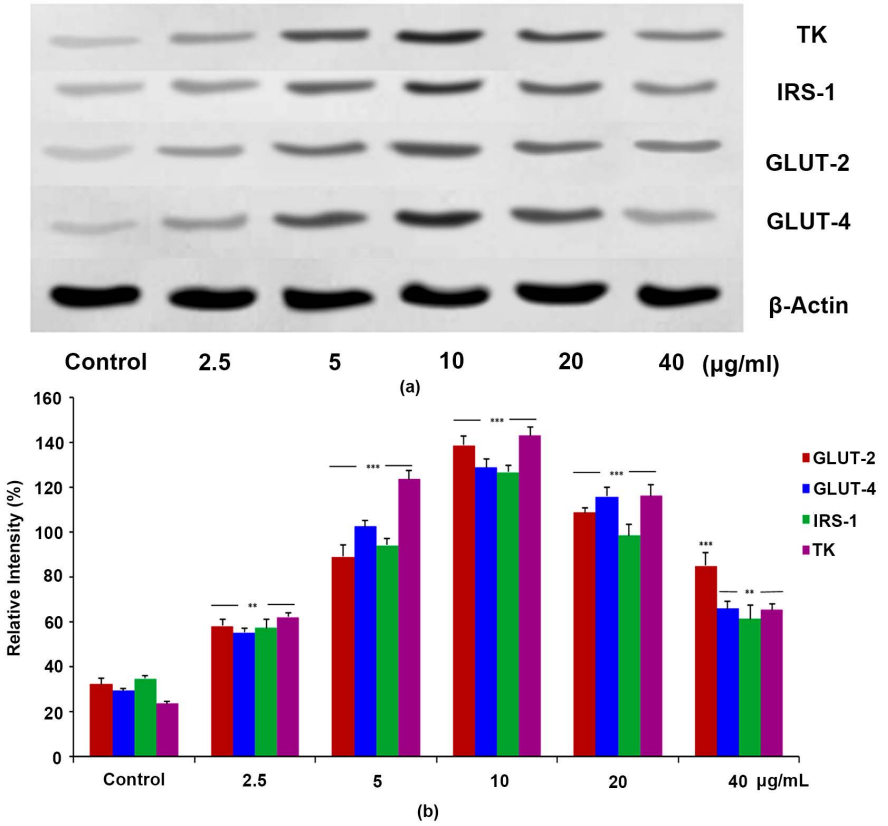

Figure 5. (a) Western blot analyses were performed to determine IRS-1, TK, GLUT-2, and GLUT-4 protein expression levels. HepG2 cells were treated with different doses of OLE (2.5 to 40 μg/mL) for 24 hours. (b) Changes in the protein levels were given in % according to the control. Significant differences according to the control indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

IRS-1 and TK at the optimum dose (10 μg/mL) in HepG2 cells. The present study also demonstrated that OLE upregulated the expression transport proteins GLUT-2 and GLUT-4 at optimum dose. However, the expression levels of these proteins decreased under and above this optimal dose. To the best of our knowledge, this is the first *in vitro* study to show that OLE increases insulin expression pathways with direct effect insulin signaling pathways. In addition, the present findings have shown that it is important to determine the dose at which insulin signal receptors and transfer proteins are expressed at the maximum levels.

In fact, OLE is known for its antioxidant, antihypertensive, anti-atherogenic, anti-inflammatory, anticancer, hypoglycemic, and hypocholesterolemia properties [31] [32]. OLE is also known for its anti-genotoxic properties. The protective effects of OLE by decreasing the level of oxidative stress-induced DNA damage in peripheral blood cells have been previously demonstrated [33] [34]. In fact, OLE has been recently accepted as a safe product in the category of food additives, approved by the European Food Safety Authority (EFSA) [35]. However, OLE has recently been shown to have cytotoxic, genotoxic, and apoptotic action in an *in vitro* cell culture medium, depending on dose and time [36] [37] [38].

Therefore, we researched genotoxic, apoptotic, cytotoxic, and ROS generating effects of different concentrations of OLE on HepG2 cells. In our study, we demonstrated that OLE proliferates to 120% at a concentration of 10 μg/mL, then it has an anti-proliferative effect in a dose-dependent manner. ROS production also decreased significantly at concentrations of 10 μg/mL and increased with dose increase.

Similarly, we observed that DNA damage and apoptosis increased significantly at doses above 80 μg/mL. These results showed that OLE has a proliferative effect with its antioxidant activity at low doses, whereas it has anti-proliferative, genotoxic, and apoptotic effect with its pro-oxidant activity at higher doses. It has previously been found that high concentrations of phenolic compounds found in olive products show genotoxic and pro-oxidant effects [39]. It was demonstrated that their pro-oxidant or antioxidant activity depends on the concentration and duration of exposure [40]. Cabarkapa *et al.* [38] claimed that olive leaves increased DNA damage and this damage was due to pro-oxidant rather than antioxidant effect. Vizza *et al.* [41] also demonstrated that in HeLa cells OLE exerts pro-apoptotic effects. Studies have shown that OLE contains much more antioxidant activity composition than the fruit, and oleuropein, flavonoids such polyphenols are the main active ingredients in olive leaf [42]. Normally, antioxidants inhibit oxidative damage as a consequence of their ability to inhibit ROS but exhibit pro-oxidant activity at high doses in the presence of transition metals such as iron (III) and copper (II) [43]. According to our knowledge, this study is the first *in vitro* study showing both antioxidant and pro-oxidant effects of OLE, and the results of previous studies support our findings.

5. Conclusion

Olive leaf extract may have the potential to treat T2DM as it increases expression levels of IRS-1, TK and glucose transport proteins. However, it is very important to determine the optimum and safe dose of OLE to obtain the best results. Therefore, experimental animal and clinical researches are required to find the most effective and non-toxic therapeutic concentrations.

Limitation

Our limitation for this study: the effects of high and basal glucose conditions on IRS-1, TK and GLUT protein expressions were not investigated.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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