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Olive Leaf Extract Downregulates the Protein Expression of Key SARS-CoV-2 Entry Enzyme ACE-2, TMPRSS2, and Furin

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Running Head: Olive Leaf Extract Downregulates SARS-Cov2 Entry Enzymes

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Abstract

Severe acute respiratory syndrome coronavirus 2 poses ongoing global health challenges due to its propensity for mutations, which can undermine vaccine efficacy. With no definitive treatment available, urgent research into affordable and biocompatible therapeutic agents is extremely urgent. Angiotensin converting enzyme-2 (ACE-2), transmembrane protease serine subtype 2 (TMPRSS2), and Furin enzymes, which allow the virus to enter cells, are particularly important as potential drug targets among scientists. Olive leaf extract (OLE) has garnered attention for its potential against COVID-19, yet its mechanism remains understudied. In this study, we aimed to investigate the effects of OLE on ACE-2, TMPRSS2, and Furin protein expressions by cell culture study. Total phenol, flavonoid content, and antioxidant capacity were measured by photometric methods, and oleuropein levels were measured by liquid LC-HR-MS. Cell viability was analyzed by ATP levels using a luminometric method. The Western Blotting method analyzed ACE-2, TMPRSS2, and Furin expressions. ACE-2, TMPRSS2, and Furin protein expression levels were significantly lower in a dose dependent manner and the highest inhibition was seen at 100 μg/ml OLE. The results showed that OLE may be a promising treatment candidate for COVID-19 disease. However, further studies need to be conducted in cells co-infected with the virus.

Keywords: Severe acute respiratory syndrome coronavirus 2; olive leaf extract; angiotensin converting enzyme-2; transmembrane protease serine subtype 2; Furin.

Abbreviations

ACE-2	Angiotensin Converting Enzyme II
COVID-19	Coronavirus Disease-2019
LC-HR-MS	Liquid Chromatography-High Resolution Mass Spectrometry
OFE	Olive Fruit Extract
OLE	Olive Leaf Extract
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
TAC	Total Antioxidant Capacity
TF	Total Flavonoid
TLRs	Toll-Like Receptors
TMPRSS2	Transmembrane Serine Protease 2
TP	Total Phenol

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Introduction

Coronavirus Disease-2019 (COVID-19) is caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged in China in late 2019, leading to a pandemic in early 2020. Because the virus is an RNA virus and is prone to frequent mutation, most vaccines developed are sometimes inadequate [1]. The virus can multiply by entering the epithelial and endothelial cells of organs and smooth muscles through the angiotensin converting enzyme-2 (ACE-2) receptor [2]. Furin and transmembrane serine protease 2 (TMPRSS2) play an important role in S-protein cleavage and activation by the host cell ACE-2 receptor, entering cells through the endosomal pathway and creating replication and pathogenicity [3]. Although several vaccines have been developed that are effective in most cases, preventing the viral disease from developing into a more serious illness, the effectiveness of the vaccine against the virus may decrease over time[4]. Therefore, research has focused on the development of antiviral drugs or strengthening natural immunity. In this context, although the therapeutic effectiveness of some drugs such as remdesivir, chloroquine, hydroxychloroquine, lopinavir, and corticosteroids has been demonstrated in some clinical studies, serious side effects have also been reported [5]. Therefore, there is an urgent need to find biocompatible and more effective therapeutics with fewer side effects. Besides of therapeutic molecules, some herbal formulas are also used for the prophylaxis and treatment of SARS-CoV-2 [6], including OLE [7].

Olea europaea L. is a fruit tree that grows in Mediterranean countries, Iran, North Africa, and Asia [8]. It has been reported that OLE has anti-inflammatory, analgesic, antipyretic, immunomodulatory, and antithrombotic activities, and with these activities, it can be of great benefit in the control of COVID-19 disease [7]. In a recent study, we also showed that drinking olive leaf tea boosts natural immunity and leads to a milder course of the disease in COVID-19 patients [9]. However, the mechanisms of action that strengthen the natural immunity or prevent the entry of SARS-CoV-2 into host cells have not been clarified yet. In fact, *in silico* studies have shown that olive leaf active molecules affect SARS-CoV-2 proteases such as 3C-like proteinase protein (3CLPro/Mpro), PLpro, Toll-like receptors (TLRs), ACE-2, non-structural protein (NSP15), TMPRSS2, S protein and Furin [10]. In addition to antiviral molecules, the antiviral effects of some medicinal plants and active substances against SARS-CoV-2 have been demonstrated by *in vitro* and *in silico* studies [11]. However, there was no *in vitro* cell culture study investigating the effects of OLE on TMPRSS2 and Furin protease enzymes and ACE-2 receptor protein expressions, which are required for virus entry into cells. Therefore, the aim of this study is to investigate the effect of OLE on ACE-2 receptor, TMPRSS2, and Furin protease expression levels by an *in vitro* cell culture study. It was shown in the literature that TMPRSS2 and ACE-2 are co-expressed in colon tissues [12], and intestinal HT29 cells were characterized by the appearance of additional ACE-2 fragment [13]. Therefore, we employed HT-29, a colon adenocarcinoma cell line, in this study which endogenously expresses ACE-2, TMPRSS2, and Furin.

Results and Discussion

There are several potential therapeutic targets for the treatment of SARS-CoV-2, such as inhibition of functional enzymes or proteins necessary for the survival of the virus, destruction of the protein structures of the virus, inhibition of the receptors that allow the virus to enter the cells or stimulation of the immunity of the host [14]. Blocking the entry of the virus into the cells by blocking the binding of the SARS-CoV-2 spike protein to the ACE-2 receptor in the human cell is the first and most promising approach to inhibit SARS-CoV-2 infection [15]. Since the virus reproduces by using the organelles of the cell after it enters the cell, all methods that will prevent the virus from multiplying can directly affect the metabolic pathways of the cell [16]. Therefore, we researched the protein expression levels of ACE-2, Furin, and TMPRS2 proteins which have a role in the entry of SARS-CoV-2 into cells. We showed for the first time that OLE significantly reduced the expression of Furin and TMPRSS2 protease enzymes and slightly reduced the expression levels of the ACE-2 receptor protein, which is important for SARS-CoV-2 entry into the cells. Recent studies have shown that ACII

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overexpression is frequently observed in gastrointestinal tissues and colon cell lines, and this rate is relatively higher than in other tissues, including lung tissues [3]. Therefore, in this study, HT-29, a colon adenocarcinoma cell line, was used to investigate the ACE-2 inhibitory effect of the test samples *in vitro*.

Previous studies have shown that phenolic compounds of natural origin are effective at different stages of entry or spread of SARS-CoV-2 [17]. For this reason, it may be an excellent approach to investigate herbal products with high phenolic content used in complementary medicine instead of antiviral drugs in the treatment of COVID-19 patients. People have used olive leaves and fruits for medicinal purposes such as antipyretic, anti-diabetic, anti-malarial, and anti-infective since ancient times [18]. The antiviral activity of OLE has also been demonstrated in several studies [7, 19]. Recent research shows that olive leaves have been selected as a potential co-therapy supplement for the treatment and improvement of clinical symptoms in COVID-19 patients [20]. With our previous clinical study, we have also shown that olive leaf tea can increase natural immunity and can prevent COVID-19 infection or make it milder [9]. We carried out this study to investigate the antiviral effect mechanisms of olive leaf, apart from its natural immunity strengthening effect. Although the molecular mechanism of its attenuation of SARS-CoV-2 is not fully understood, phenolic compounds such as oleuropein, the main component of olive leaf, hydroxytyrosol, and tyrosol are thought to play important roles [21]. Therefore, we measured TP, TF, TAC, and oleuropein levels in OLE and found relatively high levels of TP, TF, TAC, and oleuropein in OLE. The amounts of TP, TF, and TAC levels of OLE and OFE are given in Figure 1. TP, TF, TAC and oleuropein contents of OLE and UFE samples were different and, the highest TP, TF, TAC, and oleuropein contents were found in the samples extracted with methanol (Figure 1), and further experiments were continued with methanol extraction of olive leaves.

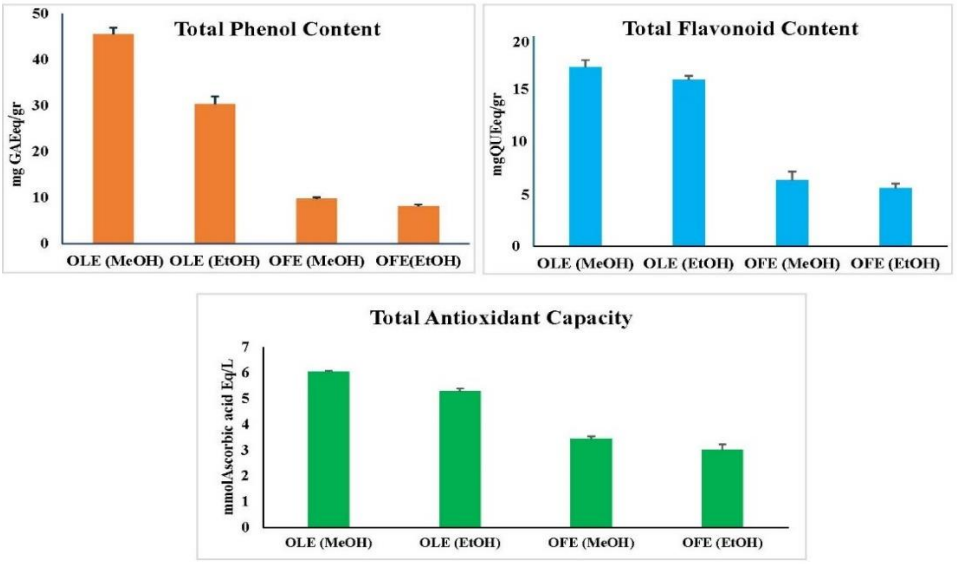

Figure 1. Total phenol content, total flavonoid content, and total antioxidant capacity of olive leaf and olive fruit extract samples were obtained by using methanol (MeOH) and ethanol (EtOH).

Oleuropein contained in OLE was analyzed with *LC-HR-MS*. Oleuropein concentration of OLE-MetOH was found 12.43 mg/kg. The results are given in Figure 2.

10.1002/cbdv.202400717

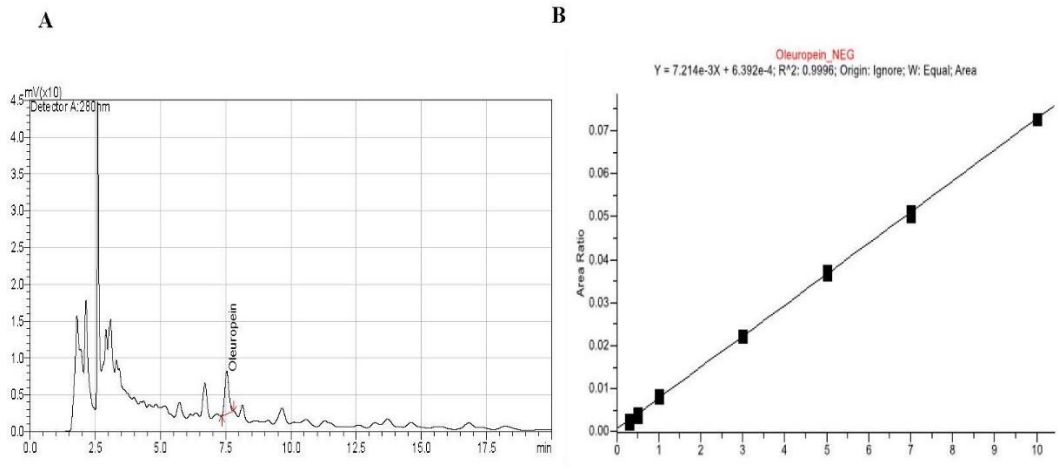

Figure 2. The chromatogram (A) and calibration curve (B) of oleuropein analyzed.

To investigate the effect of OLE on cell viability, different concentrations of OLE (12.5 to 500 µg/mL) were added to the HT-29 cells and incubated for 24 hours. Cell viability was measured by the luminometric ATP method. Control cells were considered to have 100% cell viability. At low OLE concentration, cell viability increased to 106.8 % at the concentration of 100 µg/ml. At higher doses, cell viability decreased in a concentration-dependent manner. The IC_{50} was found to be 480 µg/mL after 24 hours of exposure (Figure 3). Our findings show that OLE may increase cell viability at low concentrations and decreased the cell viability at higher concentrations in a dose-dependent manner.

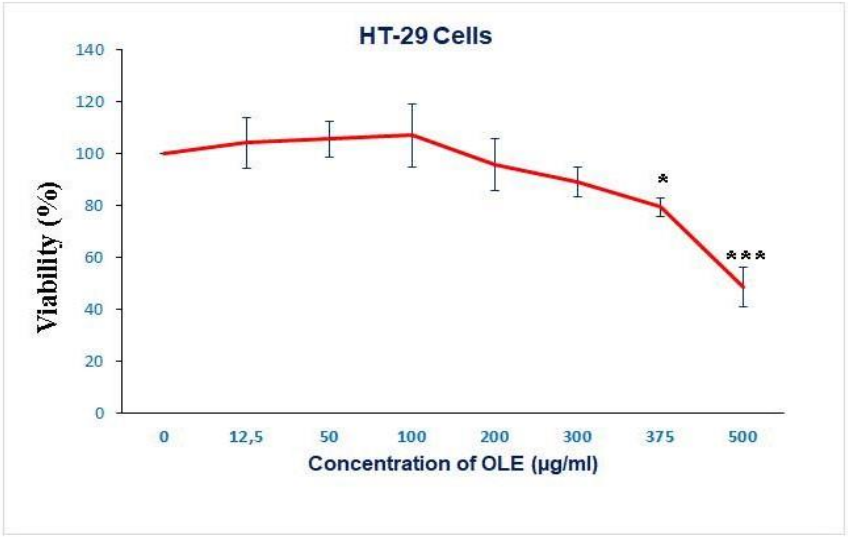

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Figure 3. Effect of OLE on cell viability. HT-29 cells were treated with OLE at different concentrations for 24 h. The ATP test was used to determine cell viability. Percent cell viability was calculated by normalizing with a control panel. Data are expressed as the Mean ± SD. The significant difference compared to the control is indicated by *p <0.05 and *** p < 0.001

The effect of OLE on the protein expression levels of ACE-2, Furin, and TMSPR2 in HT-29 cells was determined by western blot analysis. Cells were seeded on six-well plates and treated with OLE at 50 and 100 µg/mL for 24 hours. OLE treatment reduced Furin and TMPSPR2 levels by approximately 60% while reducing ACE-2 levels by approximately 20% (Figure 4). These results suggest that OLE specifically affects signal transduction pathways that control the synthesis of Furin and TMPRSS2, which are responsible for the virus's degradation of the S protein for recognition by ACE-2 receptors.

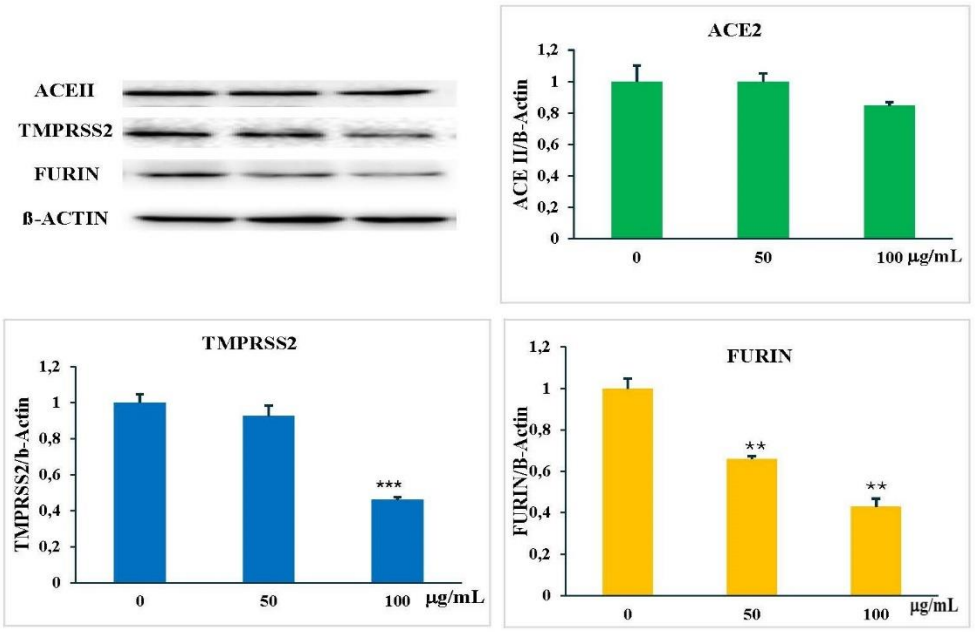

Figure 4. Western blot analyses were performed to determine ACE-2, TMPRRS2, and Furin protein expression levels. HT-29 cells were treated with different doses of OLE (50 and 100 µg/mL) for 24 hours. Changes in the protein expression levels of ACE-2, TMPRSS2, and Furin according to the control were given by graphics. Significant differences according to the control indicated by **p < 0.01, ***p < 0.001

Most researchers have investigated phenolic compounds found in plants rather than plant extracts with in silico and in vitro studies. As a matter of fact, recent in silico experiments have investigated the binding pattern of oleuropein against the main protease 3CLpro target of SARS-CoV-2 and have shown that there is a strong binding between oleuropein and the active site amino acid residues of the target proteins [22] . Istifli FS [23] also investigated the inhibitory potentials of oleuropein and its hydrolysis product 3-hydroxytyrosol against the spike glycoprotein, papain-like protease, major protease, and RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2 using molecular modeling simulations and found that oleuropein binds tightly to the active site of RdRp and could inhibit this enzyme. However, there are no in silico, in vitro, or in vivo studies showing the inhibitory effects of oleuropein on ACE-2, Furin, and TMPRSS2 protein expressions. In an in vitro cell culture study using the Vero E6 cell model infected with SARS-CoV-2, oleuropein was found to have no antiviral effect [21]. These studies' results show that an active ingredient that appears to be effective in silico may not be effective

in in vitro, and while whole plant extracts have antiviral effects, one of their active ingredients may not be effective. Indeed, in the antiviral effect, plant extracts have generally been proven to be more active than their most abundant isolated compounds, hypothesizing synergistic mechanisms [24].

Conclusions

The evidence presented in this study supports the idea that OLE has promising therapeutic potential for herbal products against COVID-19 infections. Further research on the mechanisms by which OLE exerts its antiviral effects will allow the development of successful target-specific drug delivery systems. In this study, we were unable to ensure that OLE reached the viruses directly or the correct structures within the cell. Ideally, the aim is to demonstrate the effectiveness of OLE on virally infected cells and its activity on specific molecules. In the future, we hope to conduct higher quality preclinical and clinically relevant studies that will shed sufficient light on the full mechanisms of antiviral action of OLE.

Experimental Section

Cells and Chemicals

The human colon adenocarcinoma cell line (HT-29) was obtained from ATCC (American Type Culture Collection, Manassas, VA 20110 USA). McCoy's 5A Medium, with L-Glutamine, fetal bovine serum (FBS), Penicillin-Streptomycin (10,000 U/mL), Trypsin-EDTA (0.25%) with phenol red were purchased from Capricorn Scientific GmbH, Germany. ACE-2, Furin, and TMPRRS2 primary antibodies were purchased commercially (Santa Cruz, California, USA), beside the cell viability assay kit was purchased commercially (Promega, Madison, WI, USA). Aluminum nitrate ($Al(NO_3)_3$), potassium acetate (CH_3CO_2K), sodium carbonate (Na_2CO_3), tris-HCl, quercetin, gallic acid, and oleuropein (purity 98% by HPLC) were obtained commercially (Sigma–Aldrich, Steinheim, Germany). Also, methanol and orthophosphoric acid (HPLC grade) were obtained commercially (Merck Chemical, Darmstadt, Germany).

Olive Leaf and Fruit Extract Preparation

Olive leaves were collected after the fruit harvest in 2022 in Balıkesir province in the Aegean region of Turkey. OLE and olive fruit extract (OFE) were obtained with minor modifications to the method of Zahkok et al.[25]. 100 g of fresh olive leaves and fruits were washed, dried in the shade at 30˚C for 5 days, and then ground into powder. About 20 grams of olive leaf and fruits were separated into four equal parts and extracted with methanol and ethanol (70%) at room temperature for 24 hours. After filtering the supernatant, the solvent was evaporated at 45˚C using a rotary evaporator (Heidolph, Germany). The remaining aqueous extract was dried using a lyophilizer (Labconco; Kansas City, USA). The obtained lyophilized extracts were stored in amber colored glass bottles at -20ºC until analysis. Prior to analysis, OLE and OFE were dissolved in DMSO (0.1%) and passed through a membrane filter (Millipore, 0.45 µm).

Determination of the total phenol (TP) contents of OLE and OFE were made by the Folin-Ciocalteu method [26]. After mixing 50 µL of extract and 250 µL of 0.2 N Folin-Ciocalteu reagent, it was incubated at room temperature for 5 minutes. Then, 200 µL Na_2CO_3 (200 g/L) was added. The absorbance of the color formed after a two-hour incubation was measured at 760 nm in a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Scientific, USA). Gallic acid (0 - 300 mg/L^{-1}) was used as a standard. TP is expressed in mg gallic acid equivalents (GAE) per g OLE.

The determination of the total flavonoid (TF) content of the extracts was analyzed by photometric method according to the method of Meda et al. [27]. According to the method, 192 µL of the sample was dissolved in 80% ethanol and mixed with 4 µL of 1 M potassium

acetate and 4 µL of 10% aluminum nitrate solution. After 40 minutes of incubation protected from the light, the absorbance of the reaction was measured at 425 nm in a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Scientific, USA). Using quercetin as a standard, TFC was expressed as mg quercetin equivalents per 100 g of OLE and OFE.

Total Antioxidant Capacity (TAC) was determined by the photometric method [28]. 5 µL of OLE and OFE were added to 500 µL of ABTS+ reagent and incubated for 90 seconds at room temperature. The color change resulting from the reaction was measured at 734 nm in a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Scientific, USA). Results were expressed in mmol ascorbic acid equivalents per 100 g of OLE and OFE.

Measurement of Oleuropein by liquid chromatography-high resolution mass spectrometry (LC-HR-MS)

Analyses of the amount of oleropein in OLE was performed with an LC-HR-MS instrument (Thermo ORBITRAP Q-EXACTIVE mass spectrometry) using a Troyasil HS C18 column (150×3 mm i.d., 3 µm particle size). Phosphate buffer (0.05 mol/L and pH adjusted to 3 with orthophosphoric acid) and acetonitrile (70:30, v/v) were used as mobile phase. The column temperature was set to 22°C and the flow rate of the mobile phase was 0.35 mL/min. The ambient temperature was set as 22.0 ±5.0°C and relative humidity (50±15)% rh. For standard and sample solutions, the injection volume was 5 µL, the flow rate was 1 mL/min, and the column temperature was 24˚C'. The amount of oleuropein in the extracts was calculated by the peak area. To prepare the stock standard, oleuropein was dissolved in 100% methanol and dilute deionized water was used. Figure 1 shows the chromatogram and calibration curve for the oleuropein standard.

Cell Viability Assay

Cytotoxic activity of OLE on the HT-29 cells was examined by ATP levels measured with a luminescence test called Cell-Titer-Glo Luminescent Cell Viability Assay (Promega). Cells (1.5×10^3 cells) were seeded on 96-well plates and incubated overnight at 37 °C in 5% CO_2. After incubation, the medium was then changed with fresh complete medium and, cells were treated with different concentrations of OLE (12.5 to 500 µg/mL). Control cells were treated with 0.1% DMSO. Cells were left for incubation under humidified 5% CO_2 and 95% O_2 at 37 °C for 24 hours. Afterwards, the cells were rinsed with the culture medium and the luciferin and cell lysis solution were added as substrates to the wells. After the reaction of luciferin to ATP, luminescence intensity was measured using a Multiplate Reader (Varioskan Multimode Reader, Thermo Scientific, USA) and normalized to control.

Immunoblotting Measurements

HT-29 cells were seeded on six-well plates (1.5×10^5 cells per well) and incubated for 24 hours. Cells were then treated with OLE at concentrations below the IC_{50} values. After the cells were harvested, protein isolation was performed by using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, USA) on ice. The lysate was centrifuged at 14000 x g for 15 minutes at 4˚C (Beckman Coulter, Krefeld, Germany). By separating from the supernatant, the protein concentration was determined by the Bradford method using Coomassie Plus Assay Kit (Thermo Scientific Waltham, MA, USA). Protein samples were separated by vertical electrophoresis with 8% and 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Tris-HCl buffered saline with Tween 20 (TBST) containing 5% skim milk was used to block its membrane. Transferred proteins were incubated overnight (4˚C) with ACE-2, Furin, and TMPRSS2 primary antibodies (1/500 dilution). All samples were also stained for β-actin to normalize protein amounts. TBST was used to wash the membrane. After washing the membrane, it was incubated for an additional 1 hour with horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology). Antigen-antibody complex was visualized using a Western

staining substrate (ECL, Thermo Scientific). Images were photographed using Fusion FX imaging system (Fusion FX, Vilber, France). The intensity of protein bands was measured by the Image J software program.

Statistical Analysis

All analysis results were expressed as mean ± standard deviation (Mean ± SD). Comparison of group parameters was performed by analysis of variance (One-way ANOVA). IC_{50} values of OLE on cells were calculated by nonlinear regression analysis. Experiments were repeated three times. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 26.

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Declaration of financial/other relationships

The Authors declare that there is no conflict of interest.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution

AK contributed to the conception, analyzed the data, and wrote the manuscript. VBY contributed to the acquisition of the financial support for the project. EK, VBY, ZO, FBB, ED, and OY performed experiments. All authors read and approved the manuscript.

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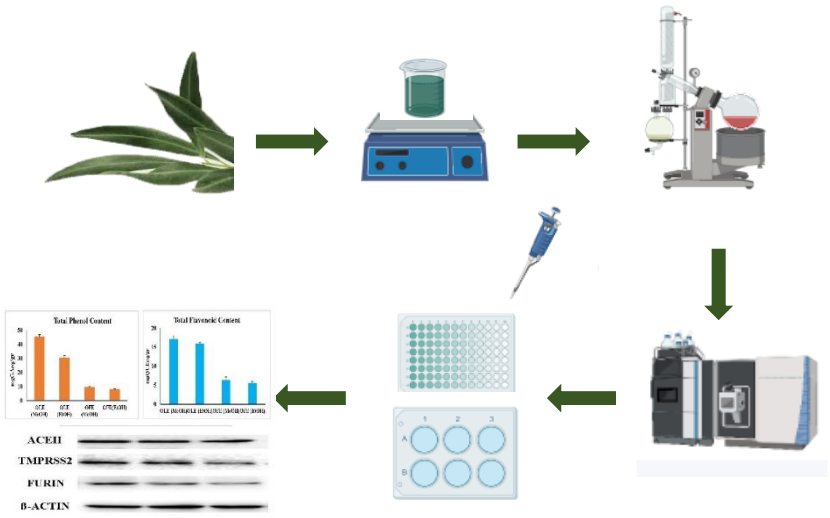

Total phenol (TP), flavonoid (TF), and antioxidant capacity (TAC) were measured by photometric methods, and oleuropein levels were measured by LC-HR-MS. Highest TP, TF, TAC, and oleuropein contents were found in the methanol extract of OLE. ACE-2, TMPRSS2, and Furin protein expression levels were significantly lower in a dose-dependent manner.