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RESEARCH NOTE

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Antioxidant, Antimicrobial, and Antiproliferative Activities of Olive (*Olea europaea* L.) Leaf Extracts

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Abstract Total phenol, total flavonoid, reducing powder, electron donating activity, ascorbic acid equivalent antioxidant capacity, antimicrobial and antiproliferative activities of olive leaf extracts were investigated. The contents of total phenol and flavonoid were 257.48 and 92.33 mg in 100 g of olive leaf extract, respectively. The reducing power of the olive leaf extract increased with concentration increasing. Electron donating activity was high in 100 μg/mL treated olive leaf extract as 95.20%. The ascorbic acid equivalent antioxidant capacity of the olive leaf extract was 68.93 mg/g olive leaf extract. The olive leaf extracts showed relatively high antimicrobial activity against *Escherichia coli*, *Salmonella typhimurium*, *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes,* and *Pseudomonas aeruginosa*. All of the cancer cell lines including MKN45, HCT116, NCI-H460, and MCF7 have 70-81% as effective growth inhibition.

Keywords: antioxidant, antimicrobial, antiproliferative, olive leaf extract

Introduction

Olives are usually used for direct consumption at meal (1). Olive leaf is one of the by-product of farming of the olive grove (2). Popular medicine and phyto therapy use olive leaves to treat and prevent hypertension, and for their hypoglycemic, antiseptic, and diuretic properties (3). Several reports have shown that olive leaf extract has the effect on the lower blood pressure in animals and increase blood flow in the coronary arteries (4), relieve arrhythmia and prevent intestinal muscle spasms (5). There is an increasing interest in the phenol compounds in olive by-products, due to their biological properties. Specially, olive leaf (5,6) and oil (1,7) as by-product are a source of several antioxidants. Olive flavonoids, phenols, and oleuropeosides have been shown to possess important antioxidant activity towards reactive species such like superoxide, hydroxyl, and lipidic peroxides which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (8,9). In the past few years, the suspected toxicity of some synthetic compounds used in food has raised interest in natural products. Some industrials, such as those related to food additive production, cosmetics, and pharmaceuticals have increased their efforts in obtaining bioactive compounds from natural products by extraction and purification. Thus, a need for identifying alternative natural and safe sources of food antioxidants has been created, and the search for natural antioxidants, especially of plant origin, has notably increased in recent years.

In the present study, we have evaluated the antioxidant and antimicrobial activities of olive leaf extract, and its cytotoxicity effect on cancer cell proliferation. Total phenolics, total flavonoids, reducing power, the scavenging effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and 2,2'-azino-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals, antimicrobial activities, and antiproliferative effect of the olive leaf extracts were investigated.

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Materials and Methods

Materials The product olive leaf extract was provided by olive leaf Australia Pty. Ltd., (Coominya, QLD, Australia). It contained 50% water-extracted olive leaf extract (from fresh *Olea europaea* leaves) and 50% vegetable glycerin. The product was aqueous and could be use to directly before analysis.

Determination of total phenol content Total phenol content was determined according to the modified a Folin-Ciocalteu method (10). A 2 mL of 2% Na_2CO_3 solution to the sample 100 μL was stirred with 100 μL of 50% Folin-Ciocalteu reagent and allowed to stand at room temperature for 3 min. The absorbance was measured with spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) at 750 nm after 30 min. The results are expressed as gallic acid (Sigma-Aldrich, St. Louis, MO, USA) equivalents, the sample total phenol content showed in mg gallic acid.

Determination of total flavonoid content Total flavonoid content was determined according to the modified method by Maria *et al.* (11). A 50 μL of sample was added to test tubes containing 100 μL of 1 M CH_3COOK, 100 μL of 10% $Al(NO_3)_3$, and 1 mL of distilled water and then stirred. The absorbance was determined spectrophotometrically at 415 nm after 40 min. Total flavonoid concentration was calculated using quercetin as standard. The total flavonoid content of the sample showed in mg quercetin equivalent/100 g sample.

Determination of reducing power The reducing power of sample was determined according to the method of Yen and Chen (12). The concentration of sample extract was 100 mg/mL, the 250 μL of this was mixed with 250 μL of 200 mM sodium phosphate buffer (pH 6.6) and 250 μL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 250 μL of the 1% trichlorocaetic acid were added, the mixture was centrifuged at 2,090×g for 10 min. The 5 mL of upper layer was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride and the absorbance was measured spectrophotomtrically at 700 nm. The higher absorbance indicates higher reducing powder of the samples.

Determination of DPPH radical scavenging activity The effect of olive leaf on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was estimated according to the method of Andreasen *et al.* (13). A 50 μL of sample was added test tube containing 1 mL of 0.2 mM methanolic DPPH solution. The mixture was vortexed and left to stand at room temperature for 10 sec. A tube containing 50 μL of methanol and 1 mL of 0.2 mM methanolic DPPH solution served as the blank. The absorbance of solution was measured spectrophometrically at 517 nm. The percentage of DPPH scavenging was obtained from the equation thus, electron donating activity (EDA, %)=100 $(1-A_{sample}/A_{blank})$.

Determination of ABTS radical scavenging activity The 2,2'-azino-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) cation decolorization assay was modified method by Robert *et al.* (14) based on the abilities of different substances to scavenge the ABTS radical cation compared with standard antioxidant (ascorbic acid) in a dose response curve. ABTS was dissolved in water to a 7.4 mM concentration. ABTS radical cation was made by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 24 hr before analysis. The concentration of ABTS radical solution was diluted with ethanol to an absorbance of 1.4 at 734 nm using the molar extinction coefficient of ABTS radical cation ($\varepsilon=3.6\times10^4$/mol/cm). A sample was added to test tube containing ABTS radical solution and left to stand at room temperature for 1 hr. The determination of sample was using the spectrophotometer at 734 nm. The results are expressed as ascorbic acid equivalents thus,

AEAC (ascorbic acid equivalent antioxidant capacity)

$$=\frac{\Delta A}{\Delta A_{aa}}\times C_{aa}\times V\times\frac{100}{W_s}$$

ΔA: change of absorbance using the sample
ΔA_{aa}: change of absorbance using the L-ascorbic acid
C_{aa}: standard concentration of L-ascorbic acid (mg/mL)
V: extract volume (mL)
W_s: weight of sample for the extract (g)

Determination of antimicrobial activity The *Escherichia coli* KFRI 836, *Sallmonella typhimurium* KFRI 191, *Bacillus cereus* KFRI 181, *Staphylococcus aureus* KFRI 240, *Pseudomonas aeruginosa* KFRI 252, and *Listeria monocytogenes* KFRI 799 were obtained from a Korea Food Research Institute (KFRI, Seongnam, Korea). Antimicrobial test was carried out by disc diffusion method. The microorganism were grown in a nutrient broth (Difco, Benton Dickenson Co., Sparks, MD, USA) and incubated at 35°C for 48 hr. A suspension of the tested microorganism (100 μL) was spread on the plate count agar (Difco, Benton Dickenson Co.) and the 8-mm diameter disc (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) were impregnated with 2, 5, 10, 20, 30, 50, and 70 μL of the olive leaf extract on the inoculated plates. The inoculated plated were incubated at 37°C for 24 hr, antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Antiproliferative activity *Cell proliferation*: The test cell lines of MKN45 (stomach cancer), HCT116 (colon cancer), NCI-H460 (lung cancer), and MCF7 (breast cancer) were purchased from Korean Cell Line Bank (KCLB). The test cancer cell lines of MKN45, HCT116, NCI-H460, and MCF7 were adapted in a Rosewell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS) and 1% of 100 unit/mL penicillin at 37°C in a 5% CO_2 incubator and used for proliferation (15).

***Anti-cancer effect by the MTT assay*:** Inhibition effect of the proliferation of the cancer cells by the sample was determined following the procedure of Young *et al.* (16). Each cancer cell such as 5×10^4 (MKN45), 2.5×10^4 (HCT116), 1.5×10^4 (NCI-H460), and 2×10^4 cell/mL (MCF7) was inoculated into a 96-well plate. After 48 hr of incubation at 37°C in a 5% CO_2 incubator, the The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL) 20 μL was added and incubated for another 4 hr. The growth media was removed and dimethyl sulfoxide (DMSO) 150 μL was added into each well and dissolved. The absorbance of each well was determined by using an enzyme-linked immunosorbent assay (ELISA) microplate reader with a 550 nm (17). The growth inhibition rate was calculated as follows;

Antiproliferative activity (%)

$$=100\left(1-\frac{A_s}{A_c}\right)$$

A_s: absorbance of the sample
A_c: absorbance of the blank

Statistical analysis Each experiment was performed 3 times and the data are expressed as mean±standard deviation (SD). Statistical analyses were performed by Duncan's multiple-range tests using SAS (SAS Institute, Cary, NC, USA) software. The level of statistical significance was defined at $p<0.05$.

Results and Discussion

Total phenol and flavonoid contents In this study, the content of total phenol and flavonoid contained 257.48 and 92.33 mg, respectively in 100 g of olive leaf extract. According to the Benavente-Garcia *et al.* (5), the HPLC profiles of phenolic compounds in olive leaf was shown that 5 groups of compounds were present: oleuroside (oleuropein, verbascoside), flavones (luteolin, dismetin, apigenin-7-glucose, luteolin-7-glucose, and diometin-7-glucose), flavonol (rutin), flavan-3-ol (catechin), and

Table 1. Reducing power and electron donating ability (EDA) of olive leaf extract[1)]

Concentration (μg/mL)	Reducing power	EDA (%)
10	0.75±0.006[c]	37.50±1.744[c]
100	1.44±0.008[b]	91.00±0.917[b]
1000	2.78±0.115[a]	95.20±0.900[a]

[1)]Different letters within the same column differ significantly ($p<0.05$).

substituted phenols (tyrosol, hydroxytyrosol, vaniliin, vanillic acid, and caffeic acid). The most abundant compound in olive leaf is oleuropein. The antioxidant effects of pure qurecetin, myristin, luteolin, apigenin, and kaempferol, as well as plant extracts were reported (18).

Reducing power The result of reducing power on the olive leaf extract was shown in Table 1. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The reducing power of the olive leaf extract increased with concentration. It was shown that the reducing power were 0.75, 1.44, and 2.78 in 10, 100, and 1,000 μg/mL, respectively. The result of high reducing power was related to result of high antioxidant activities of olive leaf extract.

DPPH radical scavenging effect The reduction of the stable radical DPPH by natural antioxidant such like olive leaf can be monitored by following the disappearance of the absorption at 517 nm (8,19). This purple color generally disappears when an antioxidant is present in the sample. Antioxidant molecules can scavenge DPPH free radicals and convert them to a colorless/bleached product (20). EDA of olive leaf by different concentration was shown in Table 1.

It was shown higher 95.20% of 1,000 μg/mL and 91.00% in 100 μg/mL than 37.50 of 10 μg/mL in olive leaf extract as natural antioxidant. The EDA was high so that the concentration of olive leaf extract increased.

Compared to the other research results of the DPPH radical scavenging effect on olive leaf extract, a 100 μg/mL of olive leaf extract concentration shows high activity as 92.35% while Al-Tardeh (21) reported EC_{50}=1.87 mg/mg of the olive leaf extraction shown the activity is lower than the our experiment result. And also, Akinmoladunl *et al.* (22) showed 84.6% in 250 μg/mL of the methanol extraction of the olive leaf.

ABTS radical scavenging effect A method of antioxidant activity on the olive leaf extract is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants. The preformed radical monocation of $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen donating antioxidants. Addition of antioxidant to the preformed radical cation reduces it ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus, the extent of decolorization as percentage inhibition of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated reactive to the reactivity of ascorbic acid as a standard. The ability of ABTS radical scavenging effect on the olive leaf extract was 68.93±0.485 mg AEAC/g sample.

Table 2. Antimicrobial activities of olive leaf extract on microorganisms[1)]

Concentration (μL/disc)	ECO	LIS	SAL	BAC	STA	PSE
2	ND[2)]	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND
10	9.80[3)]	ND	ND	ND	ND	ND
20	11.20	ND	ND	ND	ND	ND
30	15.15	ND	9.60	ND	ND	ND
50	15.15	ND	10.00	10.00	10.00	ND
70	20.15	11.00	11.50	11.25	11.00	ND

[1)]ECO, *Escherichia coli* KFRI 836; LIS, *Listeria monocytogenes* KFRI 799; SAL, *Salmonella typhimurium* KFRI 191; BAC, *Bacillus cereus* KFRI 181; STA, *Staphylococcus aureus* KFRI 240; PSE, *Pseudomonas aeruginosa* KFRI 252,

[2)]Not dectected.

[3)]8.0-9.0 mm, slight low activity; 9.1-10.0 mm, middle activity; 10.1-11.0 mm, slight high activity; 11.1-12.0 mm, moderate high activity; over 12.1 mm, very high activity.

Antimicrobial activity The antimicrobial effect of olive leaf extract results was shown in Table 2. There was no antimicrobial activity against tested all microorganism at low concentration (2 and 5 μL) of olive leaf extracts. From the concentration of 10 μL of olive leaf extract was shown antimicrobial activity against *E. coli*. However, same concentration of olive leaf extract showed no effect on other microorganisms. Antimicrobial activity against *E coli* was showed at 9.80 and 20.15 at 10 and 70 μL of olive leaf extracts, respectively. There was no effect on the antimicrobial activity with 2-20 μL concentration and from the concentration of 30 μL of olive leaf extract was shown antimicrobial activity against *S. typhimurium*. Antimicrobial activities of concentration with 50 μL of olive leaf extract were shown against *S. typhimurium*, *B. cereus*, and *S. aureus*. From the 70 μL of concentration of extract was showed against *L. monocytogenes* on the antimicrobial activity. However, there was no antimicrobial activity against *P. aeruginosa* of olive leaf extract. According to the Banavente-Garcia *et al.* (5) the olive leaf extract was showed as the powerful antimicrobial effect which elenolic acid in oleuropein. The olive leaf possessed that various phenolic compounds such like verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, rutin, catechin, oleuropein, and oleuroside, the most abundant compound in olive leaf is oleuropein (5). In this study, the antimicrobial effect of the olive leaf extract presumed to be linked to a phenolic compound showing antioxidant activity.

Antiproliferative activity MTT is commonly used to measure the number of metabolically active cells *in vitro* assays. It is based on the conversion of yellow, water-soluble MTT to the purple, water insoluble end product, formazan by mitochondrial dehydrogenase. The amount of formazan formed is proportional to the number of metabolically active cells. The key steps in the assay are to

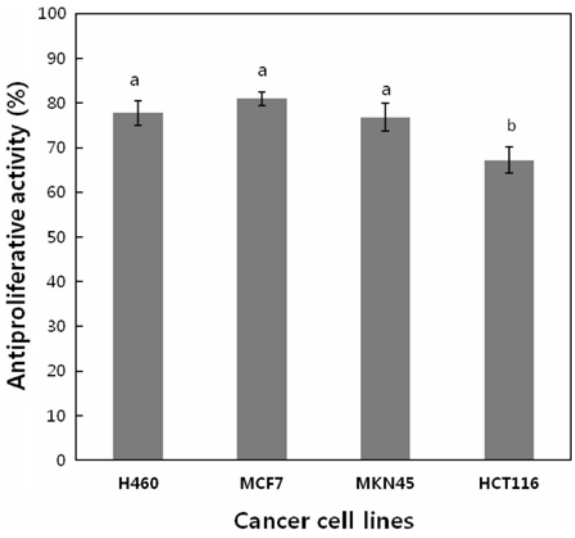

Fig. 1. Antiproliferative activity of olive leaf extracts against cancer cell (100 μg/mL).

incubate cells with MTT, extract the formazan crystals from the cells, dissolve the formazan and measure absorbance at 570 nm (23).

Cancer cell lines including MKN45 (stomach cancer), HCT116 (colon cancer), NCI-H460 (lung cancer), and MCF7 (breast cancer) were used to investigate the cell growth inhibition effect of olive leaf extract by concentrations (Fig. 1). In the results, the concentration of 100 μg/mL treated olive leaf extract was high effect on the growth inhibition of all the cell lines. These results showed us that all of the cancer cell lines has 70-81% as effective growth inhibition. The many research results were mainly described to effect on the antioxidant and antimicrobial of the olive leaf extract, according to the Briante *et al.* (24) reported that olive leaf contains bioactivities materials including various polyphenols such as and flavonoids. Also, olive leaves are a source of several antioxidants (25,26). Even the experiment result of our research was shown to reason in an olive leaf included various bioactive substances on the cancer cell inhibition effects on the olive leaf showing high activity.

In conclusion, the potential antioxidant, antimicrobial, and antiproliferative activities of the olive leaf extract could be provided as a fundamental data to be utilized for functional food and pharmaceutical industries. The findings on these functional activities showed that the olive leaf extract can be used for preparing functional materials such as antioxidant, antimicrobial agent, and anticancer effect agent.

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