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Efficiency of olive (*Olea europaea* L.) leaf extract as antioxidant and anticancer agents

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Abstract

The present study was carried out to obtain an olive leaf extract rich in bioactive compounds. Antioxidant and anticancer activities of the extract were evaluated. Polyphenols and flavonoids were extracted from olive leaves by water and methanol 80%, at two extraction ratios for up to 120 min. Methanol was more efficient than water in extracting polyphenols. Polyphenols and flavonoids were analyzed by High Performance Liquid Chromatography. The antioxidant activity was evaluated using the diphenyl picryl hydrazyl method and the reducing power assay. The extract exhibited antioxidant potential superior to that of BHT. The anticancer effect of the methanolic extract of olive leaves in breast, colon, hepatocellular and cervical carcinoma cells was studied. The extract possessed high activity against hepatocellular carcinoma cells. These results pave the way for utilization of olive leaf as a source of natural antioxidants and anticancer agents.

Keywords: olive leaves, polyphenols, reducing power, antioxidant, anticancer

1. Introduction

Olea europaea L. (*Oleaceae*) is one of the most important fruit trees and has been widely used in folk medicine in Mediterranean countries. In the Mediterranean area, there are approximately eight million hectares of cultivated olive trees (98% of the world crop). This demonstrates the great economic and social importance of this crop and the possible benefit to be derived from utilization of any of it's by products. Olive leaves are one of the byproducts of farming of the olive grove and they accumulate during pruning of the olive trees [1]. Recently, O. europaea L. leaves have attracted growing interest in the scientific community. Hence, important scientific and technical efforts have been made to determine the value of this agricultural waste [2].

Furthermore, recent studies have highlighted the antitumor activity of olive-leaf extracts, inhibiting cell proliferation in breast-cancer cell lines MCF-7, SKBR3 and JIMT-1 [3].

However, neither the molecular mechanism nor the specific compound or mixture of compounds responsible for this bioactivity is known in detail [4]. In the past few years, the demand for olive leaf extract has increased for use in foodstuffs, food additives and functional food materials [5].

Although the antioxidant activities of some single phenolic compounds in olive leaf are well known, antioxidant activities of its extract form and solvent fractions have not been clearly investigated [6].

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The present study was carried out to establish a procedure to obtain an olive leaf extract rich in bioactive compounds and to evaluate its antioxidant and anticancer activities.

2.Materials and Methods

2.1. Plant material. Fresh green olive leaves (*O. europaea* L.) were collected during the 2012 season from the farm of Faculty of Agriculture, Cairo University, Giza, Egypt. The leaves were identified and confirmed by the Botany Department of the University. Cancer cell lines (breast, colon, hepatocellular and cervical carcinoma cells) were obtained from the American Type Culture Collection, USA.

2.2. Chemicals and reagents. Organic solvents (methanol and acetone) used were obtained from Merck, Darmstadt, Germany. HPLC grade solvents were used for HPLC analysis of polyphenols and flavonoids. Folin–Ciocalteau phenol reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), gallic acid (GA), protocatechuic acid, catechin, catechol, caffein, vanillic acid, ferulic acid, salycilic acid, chlorogenic acid, apigenin, benzoic acid, coumarin, quercetin(Q), chrysin, rutin, hesperetin, kampferol, and BHT (Butylated hydroxy toluene) were purchased from Sigma–Aldrich (St. Louis, USA).

2.3. Preparation of olive leaf extracts. Fresh olive leaves (200g) were selected randomly from around the tree and were washed with water, then, the leaves were dried at 40°C for 18 h in an air forced oven and then were ground in a hammer mill at 8000 rpm and 0.03 mm diameter sieve to obtain a fine powder and stored in a dry place in the dark for extraction.

2.4. Extraction methods of polyphenols. Extraction was carried out according to [7] by two types of solvents; water and methanol 80%, at room temperature. Solvent (100ml) was added to the olive leaf powder at two different material / solvent ratios (1:10 and 1:20). Extraction time was extended 15, 30, 60, 90 and 120 min. The mixture was allowed to stand on orbital shaker (at 250 rpm). After filtra¬tion, the extract was centrifuged at 3000 rpm for 15 min. Finally, the supernatant was stored in the dark at - 20 ºC.

Obtained extracts were analyzed for total phenolic and flavonoids contents and were used for in vitro evaluations.

*2.5. Determination of total phenolic content.*Total polyphenols measurement was carried out according to [8] using the Folin-Ciocalteu method. The total polyphenols content was calculated from the standard curve of Gallic acid (100-1000 mgL^{-1}) plotted by using the same procedure. Results were expressed as mg Gallic acid equivalents (GAE) g^{-1} dried leaves.

2.6. Determination of total flavonoids. The total flavonoids content of the leaf extract was determined according to the colorimetric assay developed by [9]. The total flavonoids content was calculated from the standard curve of quercetin (10 - 250 mg L^{-1}) plotted by using the same procedure and total flavonoids was expressed as mg quercetin equivalents g^{-1} dried leaves.

2.7. Radical scavenging activity. The radical scavenging activity of olive extract towards DPPH radical was determined according to [10]. The initial absorbance of the DPPH solution (45 mg L^{-1}) was 1.2. BHT solution was used as a reference in concentrations of 25, 50 and 100 mg L^{-1}.

2.8. Reducing power assay. The reducing power activity of the extract was determined according to the method of [11]. BHT solution was used as a reference in 5, 10, 20 and 25 mg L^{-1}. IC50 was calculated from the graph of absorbance at 700 nm against extract concentration and compared with that of BHT.

2.9. Determination of pigments contents. The procedure was carried out at 4ºC. A leaf sample (0.25 g) was mashed in a mortar and pestle with 80% acetone (v/v), the extract was filtered through two layers of nylon and centrifuged in sealed tubes at 15,000 ×g for 5min. The supernatant was collected and the absorbance was read at 663 and 647 nm for chlorophyll a and chlorophyll b, respectively, and at 470 nm for carotenoid content. The concentrations of chlorophyll a, chlorophyll b, and the sum of leaf carotenoids (xanthophylls and carotenes) were given in mg L-1 extract solution according to [12].

2.10. Fractionation of phenolic acids and flavonoid compounds using HPLC. The phenolic and flavonoid compounds of the olive leaf extract were analyzed according to the method described by [13], using

HPLC instrument (Hewlett Packard, series 1050, country) composed of column C18 hypersil BDS with particle size 5µm. The separation was carried out with methanol and acetonitrile as a mobile phase, at a flow rate of 1 ml min^{-1}. The phenolic and flavonoid compounds were detected at 330 and 280 nm, respectively [14]. Quantification was carried out for a calibration based on the phenolic and flavonoid standards.

*2.11. Estimation of cytotoxic effects of olive leaf extract.*Cytotoxicity assay was carried out in the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The cells were propagated in Dulbecco's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50 mg L^{-1} gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured twice a week.

The methanolic extract of olive leaves was concentrated under vaccum below 45°C in a rotary evaporator before cytotoxicity evaluation.

Cell toxicity was monitored by determining the effect of the test samples on cell viability. For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1x104 cells per well in 100 µl of growth medium. Fresh medium containing different concentrations of olive leaf extract was added after 24h of seeding. Serial twofold dilutions of the tested chemical compound were added to confluent cell monolayer dispensed in 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO2 for a period of 48h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found to have no effect on the experiment. After incubation of the cells for 24 h at 37°C, various concentrations of olive leaf extract (100,50, 25, 12.5, 6.25, 3.125 and 1.56 mg GAE L-1) or vinblastine (50, 25, 12.5, 6.25, 3.125 and 1.56 mg L^{-1}) were added, and the incubation was continued for 48h and viable cells yield was determined by colorimetric method.

In brief, after the end of the incubation period, the media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 min. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates was measured after gently shaken on a microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compound [15,16].

2.12. Statistical analysis. Total phenolics and total flavonoids contents, radical scavenging activity, reducing power, pigments contents and cytotoxic activity of the olive leaf extract were carried out in triplicates. Results presented as mean values of each determination ± standard deviation (SD). Analysis of variance was performed by one-way ANOVA procedure. All statistical analyses were performed using the PC-STAT analysis of variance program [17]. $P<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Extraction optimization and determination of total phenolics. To optimize the extraction conditions for total phenolic compounds, the yield (mean ± SD) of total phenolics (mg GAEg-1dried leaves) as a function of the type of solvent, sample-to-solvent ratio and extraction time was assessed and data are illustrated in Figure 1.

The yield of the phenolic compounds extracted by aqueous extraction is not altered significantly when the sample-to-solvent ratio decreases from 1:10 to 1:20. Extending extraction time from 15 min to 120 min decreases the yield of the phenolic compounds in the aqueous extract. The yield of phenolic compounds extracted by methanol increases by extending the extraction time to 90 min, with a higher rate during the first 15 min of extraction. Extending extraction time from 90 to 120min causes a remarkable reduction in the extracted polyphenols. Excessive extraction time is not useful to extract more phenolic compounds and prolonged extraction

process may lead to phenolic oxidation attributable to light or oxygen exposure [18].

The highest yield of phenolic compounds (90.48 ± 0.16 mg GAE g^{-1} dried leaves) is obtained in the methanol extraction medium. Therefore, methanolic extract of olive leaves (90 min extraction using material: solvent ratio of 1:20) was chosen for further evaluations. A significant linear correlation between the values for the total phenolic content and antioxidant activity of herb of *Marrubium peregrinum* L. extracts was confirmed by [19]. Polyphenolic compounds are secondary metabolites present in many plant species. Their content depends on various factors, such as cultivar, climatic and cultivation conditions [20].

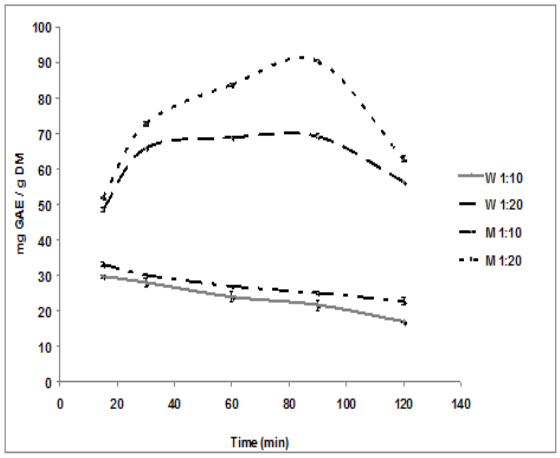

Figure 1. Total phenolics content (mean ± SD, n=3) of olive leaf extracts in water (W) and methanol (M) (mg GAE g^{-1} DM)

Flavonoids content of this extract was found to be 21.45 ± 1.2 mg of quercetin equivalents g-1 dried leaves. This result is in agreement with that reported by [21].

3.2. HPLC analysis of phenolic compounds. Analysis of the olive leaf extract by HPLC illustrated a complex mixture of phenolic compounds. The concentration of the identified phenolic compounds of olive leaves extracted by 80% methanol is shown in Table 1. Rutin, hesperetin and quercetin are the major flavonoid compounds, and apigenin and kampferol are the minor flavonoid compounds. Benzoic acid, coumarin and catechol represent 34.5%, 15.8% and 14.8% of the identified polyphenolic compounds in olive leaf extract. The types and levels of phenolic compounds greatly varied with cultivar [22]. Nevertheless, drying process may affect the matrix structure and the bioactive components [23]. Oleuropein in olive leaf extract ranged from not detected to 539 mg Kg^{-1} extract according to extraction conditions [24].

Table 1. Flavonoids and phenolic compounds identified in the methanolic extract of dried olive leaf

Compound	**mg kg^{-1} dried olive leaf**
Flavonoids	
Oleuropein	540
Rutin	3242
Hesperetin	2177.4
Quercetin	994.7
Kampferol	35.3
Apigenin	199.3
Phenolic compounds	
Gallic acid	37.6
Protocatechuic acid	228.6
Catechin	289.2
Chlorogenic acid	522.0
Catechol	953.0
Caffein	335.3
Vanillic acid	51.5
Ferulic acid	182.4
Salycilic acid	550.9
Benzoic acid	2210.7
Coumarin	1013.0
Chrysin	30.1

3.3. Pigments contents. Results revealed that chlorophyll a, chlorophyll b and carotenoids contents in the olive leaf extract were 1.882 ± 1.18, 2.816 ± 1.17 and 2.705 ± 1.18 mg L^{-1}, respectively. Chlorophylls and their derivatives have antioxidant properties [25].

3.4. DPPH radical scavenging activity. In order to evaluate the in vitro antioxidant activity of olive leaf extract, the DPPH radical scavenging activity of the leaf extract was measured and compared with that of BHT, Figure2. The DPPH test is based on the capability of stable free radical, 2, 2-diphenyl-1-picrylhydrazyl (Nitrogen centered radicals), to react with H-donors compounds including phenolics forming a stable compound. Depending on the DPPH• method it was possible to determine the anti-radical power of an antioxidant by measuring the decrease in the absorbance of DPPH• at 517 nm [26]. Increasing the concentration of olive leaf extract

from 10.29 mg GAE L-1 to 38 mg GAE L-1 causes a continuous increase in the antioxidant efficiency to >80%.

The olive leaf extract shows antioxidant potential superior to BHT. IC50 values refer to the concentration of the sample that is required to scavenge 50% of DPPH free radicals. IC50 values for the extract and BHT are 19.31 mg GAE L^{-1} and 83 mg L^{-1}, respectively. A higher IC50 value indicates a low antioxidant activity. The IC50 of BHT against DPPH radicals was 75 mg L-1[27]. The antioxidant activity of plant materials is correlated well with their phenolic compounds content [28].

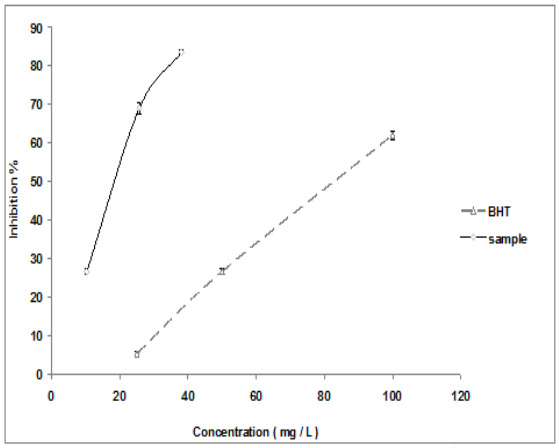

Figure 2. Scavenging activity (mg GAE L^{-1}) of olive leaf methanolic extract using the DPPH assay (mean ± SD, n=3)

3.5. Reducing power activity. Antioxidants may be explained as reductant substances in the sample that cause the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} may be monitored by measuring the formation of Perl's Prussian blue at 700 nm [29]. Reducing power will increase accordingly with the increase in absorbance [30]. Figure 3 shows the reducing power of leaves' methanolic extract, as a function of its concentration. Reducing power of olive leaf extract increases from 0.567 to 0.743 with the increase of it's concentration from 5.14 to 25.7 mg GAE L^{-1}. IC50 values (the effective concentration at which the absorbance was 0.5 for reducing power) for olive leaf extract and BHT are 2.45 mg GAE L^{-1} and 5.7 mg L^{-1}, respectively.

IC50 of reducing power of BHT was found to be 4.07 mg L^{-1}[31]. Higher absorbance of the reaction mixture indicates higher reductive potential.

The reducingcapacity of a compound may serve as a significant indicator of its potential antioxidant activity [32]. This indicates the high antioxidant activity of the investigated extract.

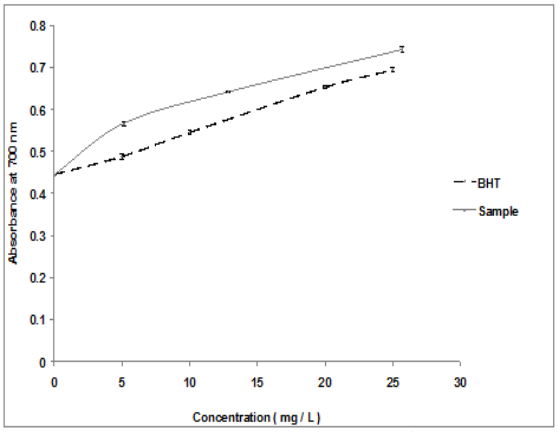

Figure 3. Reducing power (mg GAE L^{-1}) of olive leaf methanolic extract (mean ± SD, n=3)

3.6. Cancer cell lines. Four cancer cell lines (breast, colon, hepatocellular and cervical carcinoma cells) were used to determine the anticancer effect of the methanolic extract of olive leaves. Results were compared with those of standard drug, vinblastine. Results in Figures 4-5 show the cytotoxic activity of olive leaf extract as an anticancer agent for different cancer cell lines. IC50 values (50% of growth inhibition) of olive leaf methanolic extract against MCF-7, HCT, HEPG-2 and HELA cancer cell lines are 81.6, 43, 21.5 and 77.9 mg GAE L^{-1}, respectively.

The inhibitory activity of the tested vinblastine standard drug against MCF-7, HCT, HEPG-2 and HELA carcinoma cells with IC50=6.1, 2.6, 4.6, 5.2mg L^{-1}, respectively is detected under the same experimental conditions. Results indicate the potent effect of olive leaf extract against the HEPG-2 cancer cell line, since, the criterion of cytotoxicity for the crude extracts, as established by the US National Cancer Institute is an IC50 < 30 mg L^{-1} [33]. Dietary hesperetin possessed antiproliferative ability against chemically induced colon tumourigenesis [34]. Results in Table 1 indicate that hesperetin content in olive leaf extract represent 2177.4 mg Kg^{-1} dried olive leaf.

The therapeutic properties of chlorophyll include the ability to help prevent cancer and is being used in cancer therapy [35].

These results proved the in vitro antioxidant and anticancer activities of the olive leaf extract. These results should be taken into consideration in further studies concerning biological evaluation of olive leaf extract in animal models.

4. Conclusions

Methanol was found to be an appropriate solvent for production of olive leaf extract with high polyphenols content. Methanolic extract exhibited a good anti-radical activity and a reduced power compared to BHT. The obtained extract displayed anticancer properties against hepatocarcinoma cells.

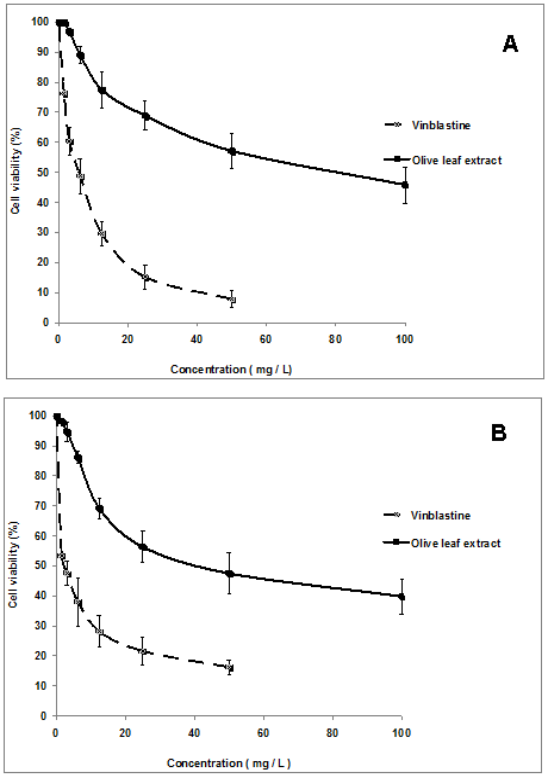

Figure 4. Cytotoxic activity of olive leaf extract and vinblastin compound used as positive control on MCF-7, Breast (A) and HCT, Colon (B) carcinoma cells (mean ± SD, n=3).

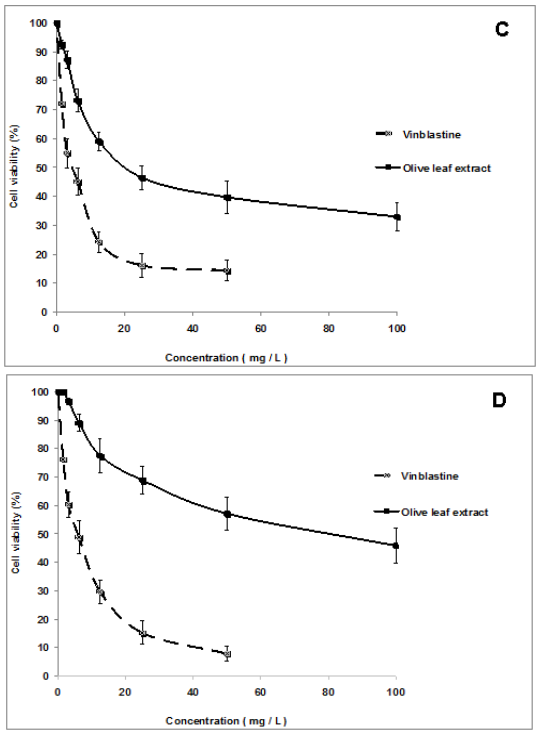

Figure 5. Cytotoxic activity of olive leaf extract and vinblastin compound used as positive control on HEPG-2, Hepatocellular (C) and HELA, Cervical (D) carcinoma cells (mean ± SD, n=3).

Compliance with Ethics Requirements: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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