doi.org/10.36721/PJPS.2024.37.3.REG.571-581.1

Cytotoxic, antioxidant and αlpha-amylase inhibitory activities of wild and Nabali olive leaf extracts from Jordan

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Abstract: Olive leaf extracts contain several phytochemical and pharmacological properties. This study evaluated the cytotoxic, antioxidant, α-amylase inhibitory activities of aqueous, ethanol and ethyl acetate extracts from the Nabali, Muhassan and wild olive leaves grown in Jordan. Total polyphenols, flavonoids and flavonols contents, chelating power activity, total antioxidant activity and DPPH free radical scavenging activity of each extract were evaluated. The α-amylase inhibitory activity of each extract was evaluated using CNP-G3 assay while cytotoxicity was assessed against viability of MCF7 and MB-MDA-231 breast cancer cell lines by MTT. The results showed that total polyphenol content was the highest in the ethanolic wild leaf extract (113.97 mg gallic acid equivalent/g of dry extract). At a concentration of 100µg/ml, the extracts from ethanolic wild leaf, ethyl acetate of wild leaf, and ethanolic Nabali leaf exhibited the highest chelating activity for ferrous ions (52.4%, 50.5%, and 47.2%). All olive leaf extracts significantly reduced MCF7 cell growth, while ethyl acetate wild leaf extract decreased MB-MDA-231 viability. The findings revealed a robust correlation between the antioxidant, cytotoxic, and α-amylase inhibitory activities of various olive leaf extracts. Further investigations are needed to identify cytoprotective effects of olive leaf extracts and the evaluation of its efficacy *in vivo*.

Keywords: *Olea europaea*, cytotoxicity, MTT, antioxidant activity, DPPH, CNP-G3, phenolic.

INTRODUCTION

The olive (*Olea europaea* L.) tree belongs to the family Oleaceae that is well known for its remarkable pharmacological properties. The presence of several phytochemical compounds present in olive leaf extracts (OLE) have been attributed for their beneficial effects (Rosa *et al*., 2019). These compounds are primarily involved in diverse functions showing antioxidant, anticancer, anti-inflammatory, antimicrobial, hypoglycemic and hypolipidemic properties (Acar-Tek and Ağagündüz, 2020; Selim *et al*., 2022).

Olive trees yield a significant quantity of leaves, which are mainly considered as by-product of olive industry. These leaves after thinning and pruning practices are often subjected to burning, grinding, landfill disposal and used as animal feed or organic fertilizer (Lama-Muñoz *et al*., 2020). However, bioactive compounds and in particular, phenolic compounds present in leaves are disposed of due to these conventional practices. Olive leaves are rich in polyphenols and phenolic acids like gallic, caffeic, vanillic and coumaric acids, as well as tyrosol, hydroxytyrosol, ligstroside and lignans, while oleuropein is considered as the main active phenolic compound present in olive leaf (fig. 1) (Özcan *et al*., 2019; Clodoveo *et al*., 2022; Selim *et al*., 2022; Ferreira *et al*., 2023).

Previous studies reported that bioactive phenolic compounds present in olive leaves have antidiabetic effect and play a beneficial role in the management of diabetes (Mansour *et al*., 2023; Da Porto *et al*., 2022). Indeed, Mansour *et al*. (2023) demonstrated the inhibition of α-glucosidase *in vitro* using three genotypes of Egyptian OLE. Diabetes is one of the most prevalent non-communicable diseases thus leading to other chronic complications (Ferreira *et al*., 2022). In this context, pancreatic α-amylase serves as a key enzyme in the digestive system, facilitating the hydrolysis of starch to produce glucose, maltose, oligosaccharides and dextrins, elevate post-meal glucose levels. Consequently, inhibiting enzymes like α-amylase plays a crucial role in the management of diabetes (Mansour *et al*., 2023).

In addition, the antioxidant and pharmacological properties of olives and olive leaves are well known for many diseases including cancer. Considering that, polyphenolic content of OLE exhibit glucose moiety which plays a major role for its bioavailability and intracellular transport via GLUT transporters into cancer cells. The anti-cancerous effects of OLE have been

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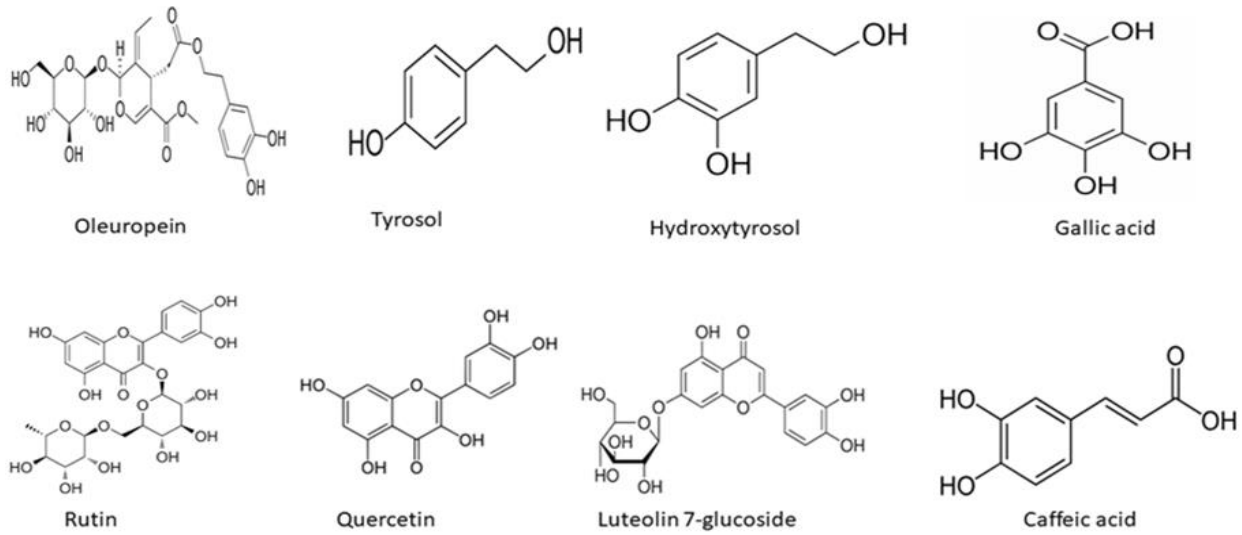


Fig. 1: The structure of the main phenolic compounds identified in *Olea europaea* L. extracts.

observed in breast, leukaemia, pancreatic, prostate, colorectal and ovarian cell lines (Rishmawi *et al*., 2022).

Olives and its extracted oil have been widely used in many Mediterranean regions including Jordan. Olive oil is an important component for Jordanian diet extensively consumed by lager population. In country, Jordan boasts of a diverse array of olive tree cultivars, primarily cultivated for olive oil production. Among the local olive cultivars, the most prevalent ones include Nabali Baladi, Nabali Muhassan, Alsoori, Nasoohi and Alshami. In addition, a variety of clones derived from these cultivars acquiring distinct common names according to the specific region of cultivation (Brake *et al*., 2014). However, the wild olive tree cultivar is mainly used as a source for propagation and rootstock for grafting. It yields a minimal amount of oil from its fruits. Also, the leaves are characterized by diminutive size, oblong shape, with a dark green upper surface and a paler underside (Filgueira-Garro *et al*., 2022).

In Jordanian folk medicine, the boiled aqueous extract of early-grown leaves in juvenile shrubs of the tree is used as a hypoglycemic and anticancer herbal drink. While the Jordanian aqueous OLE had antiglycemic and anticancer properties, the polar OLE potential anticancer and antidiabetic effects remain unknown. Hence, this study's objective is to evaluate the cytotoxic, antioxidant and α-amylase inhibitory activities of different polar extracts of early-grown olive leaf from Wild and Nabali Muhassan cultivars grown in Jordan. The antioxidant activities were assessed by free radical scavenging activities, chelating power activity and total antioxidant activity. Antidiabetic activity was evaluated using alpha-amylase inhibitory activity, while cytotoxic activity was assessed using an MTT assay on breast cancer cell lines.

MATERIALS AND METHODS

All the chemicals and used in the present study were of analytical grade. The chemicals included: Aluminum trichloride ($AlCl_3$ anhydrous sublimed, ≥ 99.8%), gallic acid, rutin, ferric chloride ($FeCl_3$), and L-ascorbic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Folin Ciocalteu reagent and trichloro acetic acid were purchased from AppliChem GmbH (Darmstadt, Germany), while 1,1-diphenyl-2-picrylhydrazyl (DPPH) was acquired from ICN Biomedicals Inc. (South Chillicothe Road Aurora, Ohio) and α-amylase from porcine pancreas was obtained from (Sigma-Aldrich, Switzerland). Potassium thiocyanate was taken from Tedia, Ohio USA, sodium carbonate (Na_2CO_3), and potassium ferricyanide ($K_3[Fe(CN)_6]$ 6) were purchased from E. Merck (Darmstadt, Germany). MCF7 and MDA MB 231 cancer cell lines were acquired from ECACC (Salisbury, UK), however, other chemicals were of reagent grade and purchased from local companies.

Olive leaf samples and preparation of extracts

The olive leaves (~500g) of matured and not wilted on shrubs from the Nabali Muhassan olive tree and wild olive tree grown in Jordan were collected in January 2022 with the help of botanist (Dr. Khaled Abu Laila) for botanical identification of olive leaf. A voucher specimen for Nabali Muhassan (OEnm-1-002) and for wild olive (OEw-1-003) leaves has been deposited in the Faculty of Agriculture, University of Jordan, Amman, Jordan. Further, collected Nabali Muhassan and wild olive tree leaves were washed with water, air-dried at room temperature (25C) under ventilation for 15 days. The leaves were grinded separately using stainless steel grinder. After allowing the powder to pass through a 0.5 mm mesh screen, the ground leaf powder (10g) was extracted with 25 ml of distilled water, absolute ethanol

and ethyl acetate for 15 mins using an ultrasonic bath (Elma S 30 H, Elmasonic Co., GmbH, Germany) at 45°C, separately. All extracts were filtered using Whatman filter paper No. 1 to remove particulate matter. The amount of dried matter in each extract was determined (mg/ml) and the volume of the filtered solutions was adjusted to 100 ml with its solvent and stored at 4°C until use.

Determination of yield content in extracts

The yield of extracts (%) was determined in triplicate for each extract using a conventional oven (Memmert, Model UFE 500, Schwabach, Germany) at 105°C until constant weight was achieved.

Determination of total phenolic contents

The total phenolic contents present in each extract were measured using the Folin-Ciocalteau reagent (Duha and Yed, 1997). Slowly, 0.5ml of each extract (100mg/ml) was added to 2.5ml of distilled water in 10ml volumetric flask and was mixed thoroughly with 250µl of the Folin-Ciocalteu reagent. After 3min, 0.5ml of 10% sodium carbonate (10g/100ml) was added and the final volume was adjusted to 10ml using distilled water. The absorbance was measured at 760 nm spectrophotometer (Lambomed, model UVD-2900, USA). Gallic acid was used as the standard for the calibration curve. The total phenolic compound content (mg/g of dry extract) was expressed as gallic acid equivalent (GAE). The determination was conducted using the established calibration curve and the content was calculated using the following regression equation:

$$Y=0.0677X - 0.0145, R^2 = 0.9837$$

Where Y is the absorbance and X is the gallic acid concentration in ppm. All measurements were done in triplicate.

Determination of total flavonoids and flavonols

The flavonoids content for each extract was determined by the colorimetric method using the Miliauskas method with slight modifications (Miliauskas *et al*., 2004). Rutin was used as the standard for a calibration curve. To proceed the methods, 1ml of each extract (100 mg/ ml) was mixed with 1ml of 2% aluminum trichloride in absolute ethanol. Later, the mixture was diluted with absolute ethanol to 25 ml and allowed to stand for 40 min at 20°C. The absorbance was measured at 415nm (Lambomed, model UVD-2900, USA). In the blank, a mixture of 1 ml of each extract and 1 drop of acetic acid was diluted with absolute ethanol to 25ml, followed by a measurement of the absorbance as mentioned earlier. The total amount of flavonoids (mg/g of dry extract) was expressed as rutin equivalent (RE). The determined was conducted using the established calibration curve, and the content was calculated using the following regression equation:

$$y = 0.0295x - 0.0297, R^2= 0.9942$$

Where Y is the absorbance and X is the rutin concentration in ppm. All measurements were made in triplicate.

The amount of flavonols was determined by the colorimetric method. Slowly, 1 ml of each plant extract (100 mg/ ml) was transferred to 25 ml volumetric flask, and then 2 ml of 2 % $AlCl_3$ in absolute ethanol and 6 ml of 5 % sodium acetate were added and mixed thoroughly. The final volume was adjusted to 25ml using absolute ethanol and was allowed to stand for 2.5 hr at 20°C, its absorbance was measured at 440 nm using a UV-Visible spectrophotometer (Labomed, Model UVD-2900, CA, USA). In the blank, the absorbance of a mixture consisting of 1 ml of the olive leaf extract, 2 ml of ethanol and 6 ml of sodium acetate was measured adopting the above- mentioned procedure. The total amount of flavonols (mg /g of dry extract) was expressed as rutin equivalent (RE) determined from the following regression equation based on the calibration curve.

$$Y=0.0021X, R^2 = 0.97$$

Where Y is the absorbance and X the rutin concentration in mg/l. All measurements were done in triplicate.

Determination of antioxidant activities DPPH free radical scavenging assay

The antioxidant effectiveness of OLE was estimated using DPPH (2,2 dipheny1-1- picrylhydrazyl) free radical scavenging activities to determine the IC_{50} for each extract (Hatano *et al*., 1998). The reaction mixture consisted of 0-1000µl (1000µg/ml) of aqueous, ethanol and ethyl acetate of each extract. An aliquot from each extract was diluted to exactly 1ml from its solvent and vortexed, then 3ml of methanolic solution of DPPH ($6X10^{-5}M$) was added. Each solution was mixed by Vortex and the absorbance of each extract and control was measured at 517 (Labomed spectrophotometer, model UVD-2900, Labomed, USA) after 30 min against a blank. All measurements were done in triplicate. The percentage of the DPPH free radical inhibition was calculated using the following equation:

$$\text{DPPH free and radical inhibition activity (\%)} = \frac{\text{Control absorbance (sample absorbance} - \text{Blank absorbance}}{\text{Control absorbance}} \times 100$$

Determination of metal ion chelating activity

Chelating power of the extracts was measured according to the method described by Kumar *et al*. (2008). For this purpose, 100µg/ml of each olive leaf extract was mixed with 0.1ml of 2mM $FeCl_2$ and 0.2ml of 5mM ferorozine solutions. Afterward, the mixture was incubated at 25ºC for 10min. The absorbance of the resulting solution was measured at 562 nm for each extract against its blank. The $FeCl_2$ and ferrozine mixture was used as control solution. All measurements were done in triplicate. The percentage inhibition of the ferrous ion of extracts was determined using the following equation:

$$\text{Chelating activity (\%)} = \frac{=[1 - (\text{absorbance of extract extract blank absorbance})]}{\text{Control absorbance}} \times 100$$

Determination of total antioxidant activity

The total antioxidant activities of the OLE were determined by phosphomolybdate method and ascorbic acid was used as a standard (Umamaheswari and Chatterjee, 2008). Briefly, to 0.1ml (100µg/ml) of each extract, 1ml of reagent solution (0.6 M H_2SO_4, 28Mm sodium phosphate and 4mM ammonium molybdate) was added. The tubes were capped and incubated in a boiling water bath at 95 °C for 90 minutes. After the mixture was cooled at room temperature, the absorbance of each extract solution was measured at 695 nm against each extract blank using spectrophotometer. The results of antioxidant activity were expressed as mg equivalent to ascorbic acid per gram-dried extract. All measurements were done in triplicate.

Determination of α-amylase inhibitory activity by CNP-G 3

The α-amylase inhibitory activity was assessed by measuring the ability of porcine pancreas α- amylase to release 2-chloro-4-nitrophenol (CNP) from CNP-G 3 according to the previous study of Suganuma *et al*. (1997). The level of CNP was determined by measuring an increase of the absorbance at 405 nm during the reaction against blank. A reaction mixture consisting of 0.15mM CNP-G 3, 0.2M potassium thiocyanate solution in 0.05 M phosphate buffer (pH=7.0) and 50µl of aqueous or ethanol or ethyl acetate (100µg/ml) from OLE, then 100 µl of freshly prepared α-amylase solution (1 mg/ml) in phosphate buffer (pH=7.0) was added.

$$\text{The inhibitory activity (\%)} = [(A-B)/A] \times 100$$

where A was an increase in the absorbance during the reaction in the absence of the extract, and B was in its presence. All measurements were done in triplicate.

Cell culture

MCF7 and MDA MB 231 cancer cell lines were obtained from ECACC (Salisbury, UK). MCF7 cells were maintained and cultured in EMEM media, supplemented with 10% fetal bovine serum, while MDA MB 231 cells were grown in DMEM media/15% fetal bovine. All media were supplemented with 2mM glutamine, 1% non-essential amino acids (NEAA) and antibiotics. Cells were maintained at 37°C in an atmosphere with 5% CO_2 and subcultured every 2-4 days. Experiments were conducted with cells in the exponential (log) growth phase.

Cytotoxicity assay

Viability assays were performed using MTT proliferation assay (ab211091) according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate at a density of 5.5 x 104 and allowed to attach overnight. Media was removed 24 hrs post-plating and replaced with fresh media with or without the OLE at a 100µg/ml concentration for 72 hrs at 37°C. The controls were treated with the solvents used for extractions. After the 72 hrs had elapsed, MTT reagent was added to each well and incubated at 37°C for ~3hrs. MTT solvent was then added per well and left overnight. The 590-nm absorbance (O.D.) in each well was determined using Synergy H1. The equation that determined the percentage viability was:

$$\text{\% cells viability} = \frac{\text{mean O.D. of extract wells}}{\text{mean O.D. of control wells}} \times 100\ \%$$

STATISTICAL ANALYSIS

Statistical calculations were performed using the statistical analysis system, SPSS program, version 20. Significant differences among means of treatment were determined using the LSD test. Differences at $P<0.05$ were considered significant. All treatments were conducted in triplicate. The correlation coefficient was determined (calculated) using Microsoft Excel 2016.

RESULTS

The yield content of OLE obtained from each solvent is shown in table 1. The highest extraction yield was obtained from the aqueous OLE of both Nabali and wild (15.5 and 19.6 %, respectively), while the lowest yield was observed for ethyl acetate extract (7.6 and 7.8%, respectively). The different extraction yields were highly affected by solvent polarity used as olive leaf components are more soluble in distilled water than other solvents due to more extraction of non-phenolic compounds, such as carbohydrates, salts, and others than other organic solvents.

The total phenolics, flavonoids and flavonols of different polar extracts obtained from OLE are shown in table 1. The total phenolic compound (TPC) contents in OLE varied from 113.97 to 58.09 mg GAE/g of dry extract. The ethanolic wild leaf extract (WE) showed the highest TPC (113.97 mg GAE/g of dry extract), followed by the ethyl acetate extract of Nabali leaf (NEa), the ethyl acetate of wild leaf (WEa) extract, the ethanolic Nabali leaf (NE) extract, wild aqueous (WA) and Nabali aqueous leaf (NA) extracts in decreasing order.

The total flavonoid compound (TFC) contents of WEa extract were significantly the highest (123.07±6.5 RE mg/g of dry extract) followed by WE extract (118.3±3.3 RE mg/g of dry extract) and NEa extract (107.97±2.4 RE mg/g of dry extract) and the lowest was in NA extract (38.34±2.8 RE mg/g of dry extract). The flavonols content varied from 91.3±0.8 RE mg/g of dry extract in the WEa extract to 12.7±1.8 RE mg/g of dry extract in the NA extract.

Table 1: Average yields (%), phenolic, flavonoids and flavanols contents (mg/g of dry extract) of Nabali and wild type OLE.

Extract (Leaf)	Yield (%)	Phenolic (mg GAE/g)	Flavonoids (mg RE/g)	Flavanols (mg RE/g)
Nabali (N)				
Aqueous (A)	15.5 ± 0.3[b]	58.1 ± 1.0[e]	38.3 ± 1.8[e]	12.7 ± 1.8[e]
Ethanol (E)	11.4 ± 0.5[c]	87.5 ± 1.2[d]	91.2 ± 1.6[c]	62.3 ± 1.2[c]
Ethyl acetate (Ea)	7.7 ± 0.2[d]	102.2 ± 1.5[b]	107.9 ± 2.4[b]	82.4 ± 1.7[b]
Wild type				
Aqueous (A)	19.6 ± 0.6[a]	75.7 ± 0.9[e]	45.1 ± 0.9[d]	17.6 ± 1.9[d]
Ethanol (E)	14.7 ± 0.4[b]	113.9 ± 0.5[a]	118.0 ± 2.3[a]	88.3 ± 2.1[a]
Ethyl acetate (Ea)	7.8 ± 0.2[d]	96.3 ± 1.7[c]	123.1± 6.5[a]	91.3 ± 0.8[a]

*Means within columns followed by different letters are significantly different ($P < 0.05$)

Table 2: DPPH free radicals scavenging activities (IC_{50}), chelating power and average total antioxidant activity of various extracts from Nabali and wild olive leaf at concentration of 100µg/ml.

Extracts	DPPH IC_{50} (µg/ml)	Chelating power (Inhibition %)	Total antioxidant activity (µg ascorbic acid equivalent/mg of dry extract)
Nabali			
Aqueous	510 ± 0.2[e]	28.5 ± 1.9 [e]	138.2 ± 6.1 [d]
Ethanol	223 ± 0.2[b]	40.3 ± 1.6 [c]	182.6 ± 4.4 [b]
Ethyl acetate	192.2 ± 0.8[a]	47.2 ± 0.7 [a,b]	201.7 ± 7.0 [a]
Wild type			
Aqueous	403.2 ± 0.4[d]	34.5 ± 1.3 [d]	155.1 ± 8.3 [c]
Ethanol	192.1 ± 0.6[a]	52.4 ± 1.5 [a]	202.3 ± 5.7 [a]
Ethyl acetate	277± 0.3[c]	50.5 ± 0.8 [a]	197.5 ± 4.2 [a]
BHT (Standard)	55.4 ± 0.7[a]		

* Means within columns followed by different letters are significantly different ($P < 0.05$) according to LSD.

Table 3: Correlation between Nabali and wild type OLE content (100µg/ml) with their antioxidant activities

	Correlation coefficient with Phenolic	Correlation coefficient with flavonoids	Correlation coefficient with flavanols
DPPH (IC_{50})	-0.93	-0.89	-0.90
Chelating power (Inhibition %)	0.96	0.97	0.97
Total antioxidant activity	0.96	0.97	0.98

Table 4: α-amylase inhibition activities of various extracts from Nabali and wild olive leaf at concentration of 100µg/ml

Extracts	Inhibition of α-amylase (%)
Nabali	
Aqueous	45.6 ± 3.1 [d]
Ethanol	62.3± 5.3 [a]
Ethyl acetate	53.8± 2.7 [b,c]
Wild type	
Aqueous	51.8± 4.6 [c, d]
Ethanol	65.1 ± 6.1 [a]
Ethyl acetate	56.7± 3.8 [a,b]

*Means within columns followed by different letters are significantly different ($P < 0.05$) according to LSD.

Table 5: Correlation between Nabali and wild type OLE content (100µg/ml) with their α-amylase inhibitory activities

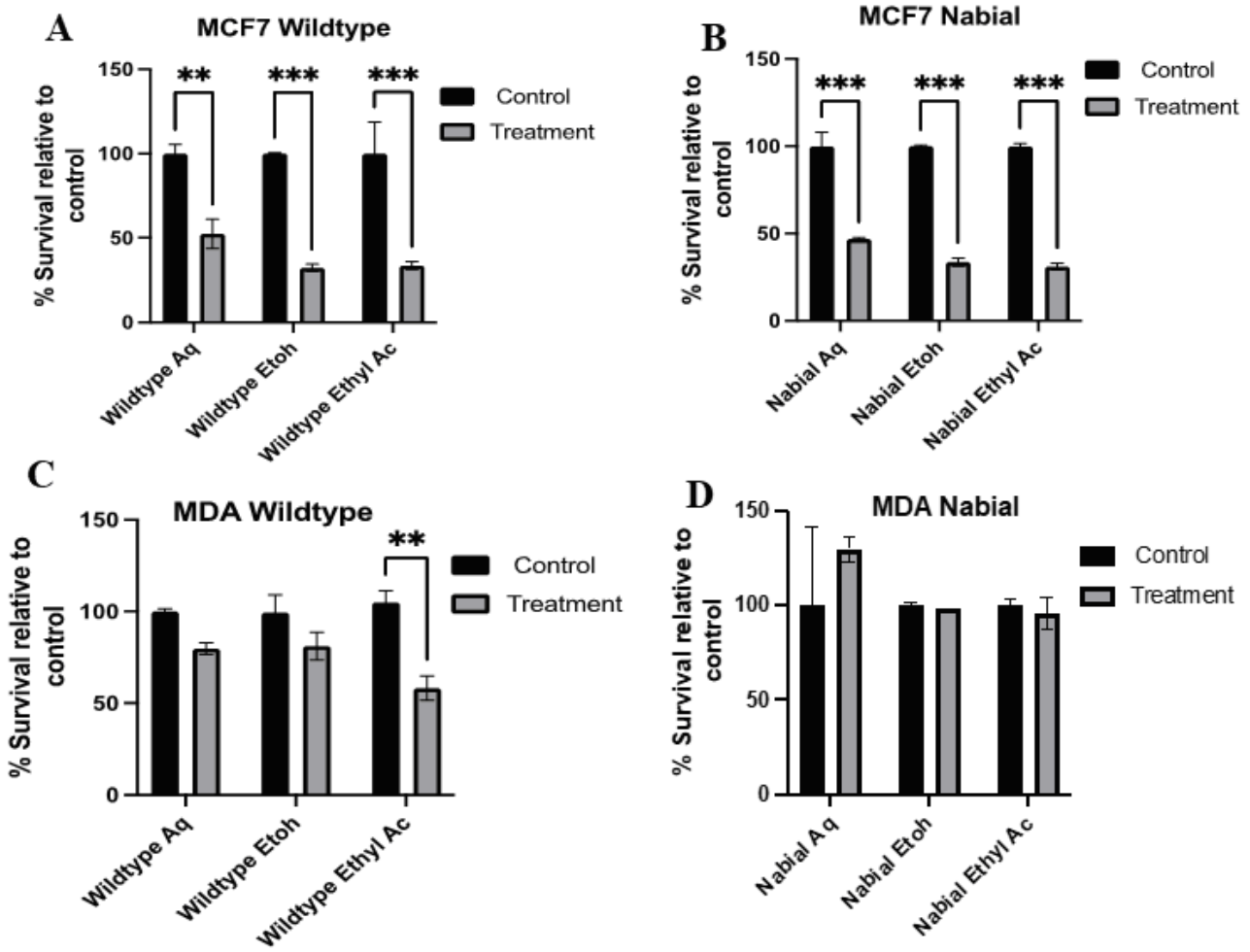
	Correlation Coefficient with Phenolic	Correlationcoefficient with flavonoids	Correlation coefficient with flavanols
Inhibition of α-amylase (%)	0.80	0.74	0.72

Table 6: Correlation between wild type and Nabial olive plant content with survival (%) ofMCF7 breast cancer cell line.

	Correlation coefficient with Phenolic	Correlation coefficient with flavonoids	Correlation coefficient with flavanols
% MCF7 Survival	-0.81	-0.92	-0.93

Table 7: Correlation between wild type and Nabial olive plant antioxidant activities with survival (%) of MCF7 breast cancer cell line.

	Correlationcoefficient with DDPH	Correlationcoefficient With chelatingpower	Correlationcoefficient with total antioxidant activities
% MCF7 Survival	0.88	-0.83	-0.90



Data represented as Mean ± SD. Aq: Aqueous, EtoH: Ethanol; Ethyl Ac. Ethyl acetate. **P ≤ 0.01, ***P ≤ 0.001.

Fig. 2: Olive plant wild type and Nabial extracts reduced cell viability of MCF7 cells while wild type ethyl acetate extract reduced survival of MD MB 231 cells. (A-D) MCF7 and MDA MB231 breast cancer cell lines were treated with 100ug/ml of olive plant wild type and Nabial extracts and incubated for 72 hours. MTT assay was then conducted to determine cell viability.

The DPPH free radical-scavenging activity of various extracts from olive is shown in table 1. The results showed that BHT exhibited the highest DPPH radical scavenging activity (IC_{50} value 55µg/ml), while NEa and WE extracts exhibited significantly (P<0.05) the highest DPPH scavenging activity among investigated extracts in a dose dependent manner (IC_{50} value 192.2 and 192.1 µg/ml). The IC_{50} values of various extracts from olive leaf cultivars on DPPH free radical scavenging activity were in the following decreasing order: NE > WEa > WA > NA (table 1). It is important to note that the lower the IC_{50} value of the extract the higher its antioxidant activity.

Table 2 shows the chelating power of different extracts at concentration of 100µg/ml. The WE, WEa and NEa extracts have significantly the highest chelation power activity on ferrous ion (52.4, 50.5 and 47.2%, respectively), followed by NE, WA, and NA extracts (40.3, 34.5 and 28.5%, respectively). Table 2 shows the total antioxidant activities of different OLE expressed as number of equivalents of ascorbic acid per mg of dried extract at concentration of 100 g/ml. The WE, NEa and WEa extracts have significantly the highest total antioxidant activity (202.3, 201.7 and 197.5µg ascorbic acid equivalent/ mg extract, respectively) followed by NE, WA, and NA extracts (182.6, 155.1 and 138.2µg ascorbic acid equivalent/ mg extract, respectively). With further data analysis, we found that there was a positive correlation between TPC, TFC and total flavonol contents of extracts and their total antioxidant activities (R=0.96, 0.97 and 0.98, respectively) as shown in table 3.

Table 4 shows porcine pancreas α-amylase inhibitory activity (%) of different OLE at concentration of 100 µg/ml. The WE, NE, WEa and NEa extracts have significantly the highest α-amylase inhibitory activity (65.1, 62.3, 56.7 and 53.8 (%), respectively) followed by WA and NA extracts (51.8 and 45.6 %). A positive correlation between α-amylase inhibitory activity and TPC, TFC and total flavonol contents of extracts (R = 0.80, 0.74 and 0.72, respectively) (table 5).

Regarding anti-cancer effects of OLE, all olive plant extracts for both the wild type and Nabial resulted in a significant drastic reduction of MCF7 cancer cell viability. For the wild type aqueous, ethanol and acetate extracts, MCF7 inhibition of growth was 52.5%, 32.3% and 33.7%, respectively, relative to control (100% viable). Similarly, the NA, NE and NEa extracts increased MCF7 cell death by 2, 2.9 and 3.2-fold, respectively (fig. 2). Furthermore, there was a strong correlation between the reductions of MCF7 (%) survival and the phenolic, flavonoid and flavonol contents in dose-dependent matter for OLE (table 6). Regarding our MDA-MB-231 findings, only WEa extract resulted in a marked reduction of cancer cell survival compared to untreated control (58.2% vs 100%) (fig. 1). Interestingly, there was a negative correlation between the chelating power and total antioxidant of the extracts and the % survival of MCF7 cells (-0.83 and -0.90, respectively, table 7), which suggests that the higher the antioxidant activity, the lesser the MCF7 survival.

DISCUSSION

Extraction yields, total phenolic, flavonoids and flavonols contents of OLE

The highest extraction yield was obtained from the aqueous OLE of both Nabali and wild. In agreement to our findings, previous studies have reported a high extraction yields from olive leaf samples with distilled or deionized water (Benincasa *et al*., 2019; Cho *et al*., 2020). The ethanolic wild leaf extract (WE) showed the highest TPC. Similarly, Cho *et al*. (2020) reported that the highest total polyphenol contents of olive leaf were obtained from the extraction of leaf with 90% ethanol or methanol, while Martín-García *et al*. (2022) observed that 100% ethanolic extract of olive leaf was found to contain 24.9-29.2 mg GAE/g dry matter according to the solvent used for extraction (Martín-García *et al*., 2022). Total flavonoid compound content extract was significantly the highest in WEa. Note that Flavonoids represent the major polyphenolic compounds present in the olive leaf (Borjan *et al*., 2020). The flavonols content was higher in WEa in comparison to NA extract.

Our findings align with those of Medina *et al*. (2019), who observed higher variability in the total phenolic content (TPC) of olive leaf aqueous extracts, which ranged from 7.5 to 250mg/g. Similarly, Filgueira-Garro *et al*. (2022) reported varying phenolic compound contents in aqueous extracts from air-dried leaf of five different olive cultivars, ranging from 29.2 to 45.4mg GAE/g (Filgueira-Garro *et al*., 2022). In contrast, Fing *et al*. (2021) found that air-drying of olive leaf was the most effective method for retaining TPC and TFC with values of 51.48mg GAE/g and 28.59mg rutin equivalent (RE)/g. In our study, the TPC in air-dried leaf of the NA and WA cultivars was 58.09 and 74.74 mg GAE/g of dry extract, respectively. Additionally, the TFC values for these extracts were 38.34 and 45.05 mg RE/g of dry extract, respectively. The higher TPC and TFC results has been previously reported in some literature in different extracts that can be attributed for explanation. Our calculations are based on the quantification of bioactive compounds per gram of dried matter in each extract, rather than per gram of dried leaf.

Overall, although aqueous extracts showed the highest yield of extraction, the TPC, TFC and total flavonols contents had the lowest value due to higher extraction of non-phenolic compounds from the aqueous leaf extracts. Also, the highest levels of TPC, TFC and total flavonol contents in ethanol and ethyl acetate leaf extracts

appeared to enhance solubility in organic solvents used. This increase in solubility can be attributed to the higher organic content of the extraction solvents, which leads to improved extraction of these compounds (Cho *et al*., 2020; Khelouf *et al*., 2023). It is evident from the results that OLE contains considerable amounts of phenolic, flavonoids and flavonols. Many previous studies have been suggested that the antioxidant properties of OLE are directly related to the contents of these phenolic compounds which act by donating hydrogen from the phenolic hydroxyl groups (Borjan *et al*., 2020; Ferreira *et al*., 2020; Grubić Kezele and Ćurko-Cofek, 2022). Therefore, the relationships between values obtained using different models to evaluate cytotoxic and antioxidant activities and the content of phenolic, flavonoids and flavonols compounds were evaluated.

DPPH radical-scavenging activity of OLE

The results of antioxidant activities showed a positive correlation (R = 0.93) between the total phenolic content of extracts and their DPPH radical scavenging activities, whereas the correlation with the total flavonoids and flavonols was R = 0.88 and 0.89, respectively. These findings in agreement with the results of Cho *et al*. (2022), which indicated that the highest DPPH scavenging activity values were observed in ethanolic OLE, while the lowest values were found in olive leaf distilled water extracts. Furthermore, strong correlation (R = 0.82) between phenolic extracts of olive leaf and DPPH free radical scavenging activities was reported (Orak *et al*., 2019). Different polar extracts derived from olive leaf have exhibited promising free radical scavenging capabilities against DPPH radicals. The finding suggests the potential of these extracts to contribute to the prevention of diseases mediated by free radicals.

Chelating power and total antioxidant activities

The 50% chelating activity of olive leaf ethanolic extracts was reported to be 152.4 µg/ml (Ivanov *et al*., 2018) and this value was observed to be lower than chelating power of EDTA (Ivanov *et al*., 2018). The reported value in our study is slightly higher, which can be attributed to differences in the way the activity presented, whether it is on per gram of dried matter basis or on per gram of dried leaf basis for the extract. Strong correlations were observed between TPC, TFC and total flavonol contents in the extracts and their chelating activity (R=0.96, 0.97 and 0.96, respectively). The ability of OLE to chelate metal ions, which play a role in catalyzing the generation of free radicals, is most likely attributed to their phenolic contents. These phenolic compounds may give the extracts the potential to reduce the production of reactive oxygen species and, consequently, lower the associated cardiovascular risks (Ivanov *et al*., 2018).

The results suggested that the phenolic compounds contributed significantly to the total antioxidant activity of extracts. The antioxidant activity of phenolic compounds in OLE is mainly related to their redox properties. These properties enable them to function as singlet oxygen quenchers, reducing agents, metal chelators and hydrogen donors. The variations in correlations between individual phenolic compounds and antioxidant activities in different assays can be attributed to the diverse ways in which these compounds respond to different antioxidant reaction mechanisms (Lfitat *et al*., 2021; Martín-García *et al*., 2022).

Antidiabetic activity

Phenolic compounds from extra virgin olive oil and OLE have been reported to exhibit α-amylase inhibitory activity, both in *vivo* and *in vitro*. Some of these compounds have been identified as inhibitors of enzymes linked to diabetic complications, potentially offering possible health benefits (Figueiredo-González *et al*., 2019; Zhang *et al*., 2022; Mansour *et al*., 2023). Additionally, the synergistic effects of other bioactive compounds, when combined with phenolics in olive leaf or olive fruit oil, may also contribute to the inhibitory activities against alpha-amylase (Collado-González *et al*., 2017). The results suggested that the phenolic compounds contributed significantly to α-amylase inhibitory activity of extracts. While the aqueous extracts from both cultivars exhibited a relatively modest inhibitory effect compared to other extracts, they still demonstrated a significant impact on α-amylase inhibition, offering potential health benefits in the management of diabetic complications. The inhibition of the α-amylase enzyme in the intestine plays a crucial role in controlling type 2 diabetes. This control is achieved by slowing down starch hydrolysis by α-amylase, resulting in a reduction in blood glucose levels.

Cytotoxic activity

All OLE resulted in the reduction of MCF7 viability. Similar to our findings, a recent study by Bal *et al*. (2023) demonstrated MCF7 cytotoxicity, cell cycle arrest, and apoptosis treated with Turkish OLE. Furthermore, the cytotoxic effect of the OLE microcapsules was not observed in the BEAS 2B healthy cell line (Bal *et al*. 2023). Also, another study showed that MCF7 treated with three polyphenols, oleocanthal, hydroxytyrosol and oleuropein (China), resulted in apoptosis, G1 cell cycle arrest and reduction of migration (Han *et al*., 2023). Furthermore, Oleuropein (USA) was shown to induce cytotoxicity, an effect that was not observed in non-cancer MCF-10A cells in another study (Junkins, Rodgers and Phelan, 2023). Regarding our MDA-MB-231 findings, only WEa extract resulted in a marked reduction of cancer cell survival. Similarly, oleuropein and its hydrolysate were previously reported to be cytotoxic to the MB-MDA-231 cell line. Also, anti-apoptotic and S phase cycle arrest was observed (Han *et al*., 2023). Also, OLE of Italian olive leaf cultivar reduced MDA MB 231 cell

viability with an IC_{50} of 200 µg/ml post 24 hrs incubation (Benot-Dominguez *et al*., 2021). The lack of significant effect by the remaining extracts could be attributed to the relatively low dosage utilized in our study, which was 100 µg/ml.

We found that there is a correlation of high antioxidant activity with reduction of MCF7 survival. Oxidative stress plays an essential role in the development and progression of breast cancer by affecting hallmarks of cancer, such as proliferation and metastasis stimulation (Zhong and Tang, 2024). Several studies investigating olive polyphenols have supported Nrf2 activation (master regulator of cellular antioxidant enzymes) and consequently, a mirage of antioxidant enzymes, including SOD and CAT (Bucciantini *et al*., 2021). Also, Oleuropein (USA) induced cytotoxicity and increased the expression of the antioxidant enzyme peroxiredoxin family in MCF7 (Junkins, Rodgers and Phelan, 2023). Overall, OLE's antioxidant effects could have contributed to the reduction of MCF7 viability in our study.

CONCLUSION

Olive leaf extract is a promising source of bioactive compounds with significant antioxidant, anti-inflammatory, and antimicrobial properties. Our study showed that OLE from Nabali and wild cultivars showed that phenolic, flavonoids and flavonol contents appear to have a significant correlation with their antioxidants, antidiabetic, and cytotoxic activities. Its potential therapeutic applications make it an emerging domain for ongoing and future research. In addition, the possibility of utilization of OLE with common medications may contribute to reduce the effect of certain diseases.

ACKNOWLEDGEMENTS

The P.I. of the study “Maher M Al-Dabbas” has performed this research during the sabbatical leave from the University of Jordan at Al Ain University (UAE) for the academic year 2023. The authors would like to thank deanship of scientific research at Al Ain University for their financial support.

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