الفعالية المضادة للأكسدة والمضادة للبكتريا للمستخلصات الكحولية لأوراق الزيتون من الأصناف المحلية الموجودة في سورية

Antioxidant and Antibacterial Activities of Alcoholic Leaf Extracts of Local Olive Varieties in Syria

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الملخص

أجريت هذه الدراسة بين عامي 2014 و2015 في مختبرات الهيئة العامة للتقانة الحيوية (دمشق، سورية)، إذ تم اختبار 13 مستخلصاً كحولياً لأوراق الزيتون، تم جمعها في شهر تموز/ يوليو عام 2014 من الأصناف المحلية المختلفة (أبو شوكة، دان، جلط، استانبولي، نبالي، تفاحي، طلياني، أرابكوين، وفيلاماتيا) من حيث فعاليتها المضادة للميكروبات، وفعاليتها المضادة للأكسدة. تم في القسم الأول من هذه الدراسة تقدير القدرة النسبية المضادة للأكسدة المعبر عنها بمحتوى الفينولات الكلي لأوراق الزيتون على شكل مكافئ حمض الغاليك، ووجد أنها تراوحت بين 1.10-1.69 غ/100 غ من الوزن الجاف. في حين تم في القسم الثاني من الدراسة تطبيق المستخلصات الكحولية على 4 أنواع بكتريا إيجابية غرام (*Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *B. cereus*)، و4 أنواع بكتريا سلبية غرام (*Enterocobacter cloacae*, *Eschrichia coli*, *Proteus mirabilis*, *Salmonella enterica*) لتحديد الفعالية المضادة للبكتريا. وجد أن نوعي البكتريا الأكثر مقاومة هما: *Bacillus subtilis* و*Eschrichia coli*، بينما كان النوعان *Enterobacter cloacae* و*Staphylococcus epidermidis* هما الأكثر حساسيةً من بين البكتريا إيجابية غرام وسلبية غرام.

الكلمات المفتاحيّة : أوراق الزيتون، الفعالية المضادة للأكسدة، الفعالية المضادة للبكتريا، البكتريا سلبية غرام، البكتريا إيجابية غرام.

Abstract

The current study was carried out in the period of 2014-2015 in the laboratories of The National Commission for Biotechnology (NCBT), Damascus-Syria. Thirteen ethanolic extracts of olive leaves (OLE), which were collected in July 2014 from different local varieties (*Abou-Shouki, Dan, Gilit, Istanbouli, Nibali, Toufahi, Touliani, Arabquine, Sourani and Vilamatia*) were examined for their antimicrobial and antioxidant activities. In the first part of the study, relative antioxidant capacity expressed as total phenol contents of the olive leaves were determined as gallic acid equivalent, and found to be 1.10-1.69 g/100 g of dry weight. In the second part, the alcoholic extracts were applied on 4 Gram positive bacteria (*Staphylococcus aureus, S. epidermidis, Bacillus subtilis, B. cereus*) and 4 Gram negative bacteria (*Enterocobacter cloacae, Eschrichia coli, Proteus mirabilis, Salmonella enterica*) to determine the antibacterial activity. The most resistant bacteria were *Bacillus subtilis* and *Eschrichia coli* whereas *Enterobacter cloacae* and *Staphylococcus epidermidis* were the most sensitive species among the negative and positive Gram species.

Keywords: Olive leaves, Antioxidant activity, Antibacterial activity, Gram-negative bacteria, Gram-positive bacteria.

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Introduction

The number of studies on use of plant polyphenols in food researches is increasing day by day. Olive leaf extract (OLE) is one of the potent source of plant polyphenols having antioxidant and antimicrobial properties due to its rich phenolic content. The antimicrobial activity of OLE against a wide range of bacteria, fungi, and viruses has been investigated since the late 1980s and it showed variable activity against different microorganisms (Benavente-Garcõa *et al*., 2000; Ritchason, 2000). Many researchers have studied the antibacterial activity of OLE against gram-positive and gram-negative bacteria. They found that OLE had antibacterial activity against a wide range of gram-positive strains but had limited or no activity against gram-negative strains (Korukluoglu *et al*., 2010; Markin *et al*., 2003; Pereira *et al*., 2007).

The aim of this study was to determine the antibacterial activities of 13 olive leaves samples collected from 2 various geographical regions of Syria against 8 important food-borne pathogens, 4 gram-positive bacteria (*Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *B. cereus*) and 4 gram-negative bacteria (*Enterocobacter cloacae*, *Eschrichia coli*, *Proteus mirabilis*, *Salmonella euterica*).

Materials and methods

1-Olive leaves samples:

The olive leaves samples used in preparing the extractions were collected from 7 cultivars (Abou-Shouki, Dan, Gilit, Istanbouli, Nibali, Toufahi and Touliani) in July 2014 in an olive grove located in Qunitera, Syria (Table 1), the orchard has a planting destiny of 6×6 m. The trees are twenty-five years old, being pruned when necessary, irrigated and no phytosanitary treatments had been applied in the last year. Also, we collected leaves from 3 cultivars (Arabquine, Sourani and Vilamatia) from arboretum, Abou-Jarash orchard, Damascus, Syria. The collected samples were put in plastic bags and immediately dried.

Table 1. Olive leaves cultivars used in this study.

Location	Olive cultivars	Symbol
Qunitera	Abou-Shouki	**QASH**
Qunitera	Dan	**QD**
Qunitera - Waste water	Dan	**QDW**
Qunitera - Al-Baa'ith city	Dan	**QDB**
Qunitera - Ayin Al-Thaoura	Dan	**QDATH**
Qunitera	Gilit	**QG**
Qunitera	Istanbouli	**QI**
Qunitera	Nibali	**QN**
Qunitera	Toufahi	**QTA**
Qunitera	Touliani	**QTI**
Damascus	Arabquine	**DA**
Damascus	Sourani	**DS**
Damascus	Vilamatia	**DV**

2-Total polyphenols determination

The concentration of total phenolics was measured according to the Folin-Ciocalteu (FC) method. This method is based on colorimetric oxidation/reduction reaction where Folin-Ciocalteu reagent is used as oxidising agent. The olive leaf extracts (0.25 mL) were transferred to a 25 mL volumetric flask, containing 15 mL of distilled water and 1.25 mL of Folin-Ciocalteu reagent. The solution was neutralized by adding 20% sodium carbonate (3.75 mL). The volume was made up with distilled water to 25 mL and after 2 hours the absorbance was measured at 765 nm (Mekinić *et al*., 2014).

3-Microorganisms and culture conditions

The ethanolic extracts were applied on four Gram-positive bacteria (*Bacillus cereus*, *B. subtilis*, *Staphylococus aureus*, *S. epidermidis*) and four Gram-negative bacteria (*Enterocobacter cloacae*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella enterica*). Microorganisms were obtained from National Commission for Biotecnology (NCBT), Laboratory of Food and Industrial Technique, Damascus, Syria.

4-Antimicrobial activity test

4-1- Extract preparation

Olive leaves were washed to remove impurities such as dust and then dried in an air oven for 3 days at 38 ºC. A standardized solvent extraction protocol was used for the plant material. The air dried plant materials were ground in a blender with a particular size to ensure the plant powders in identical size. 10 g of each plant powder was soaked for 2 hrs with 200 ml of 70% (v/v) aqueous ethanol at 38 ºC by a thermo-shaker (New Burnswick Scientific, NJ, USA) which is fixed to 180 rpm. Then the samples were centrifuged at 5000 rpm for 15 minutes and the supernate parts of the samples were carried to a rotary evaporator to remove ethanol under reduced pressure at 38 ºC, 120 rpm until completely dryness. The crude extracts were kept in refrigerator in glass bottles until the further experiments. And a series of double dilution concentrations ranged from 200 to 10 mg/mL (w/v in water) was prepared to be used for MIC determination.

4-2- Minimum Inhibition Concentrations (MIC) Assay

A macro-broth-dilution technique (Pereira *et al*., 2007) was used (with some modifications) to determine the growth inhibition of the Gram+ (*B. subtilis*, *B. cereus*, *S. aureus*, *S. epidermidis*) and Gram– (*Enterocobacter cloacae*, *Eschrichia coli*, *Proteus mirabilis*, *Salmonella euterica*) bacteria to olive leaves extracts. Mueller Hinton Broth (MHB) medium (17.5 g Acidicase Peptone, 1.5 g starch, water to 1 L) was used to prepare the inocula after being sterilized in an autoclave (JRS, Jsac 80) at 121 ºC for 15 min. Bacterial strains were grown in MHB tubes for 24 h. at 37 ° C (Memmert, Germany). The concentration of bacterial suspension was adjusted to 0.5 McFarland using densimeter (Densemat, bioMerieux), to obtain concentration equal to 1×108 CFU/mL.

The double concentration diluents of OLEs were mixed with equal volumes of MHB to reach the normal concentrations (10-200 mg/mL). Each tube containing the normal concentration of OLE was inoculated with 10 µL of the 0.5 McFarland bacterial suspension, and the mixture was incubated at 37° C for 24 h. After the incubation period was passed, 10 µL of incubated mixture was cultured on LBA plate, which was re-incubated at the same conditions (37° C for 24 h.). Then the LBA plates were investigated for growth. Controls were carried out in the same manner but in the absence of OLEs.

Results and discussion

Total phenol contents were dominantly detected in the OLs (Table 2). They were the shared component found in all OL samples with different concentration (1.10-1.69 g/ 100 g). These contents of total phenol were very close to that recorded in previous researches and, 2.48 and 1.66-1.79 g/ 100 g, respectively in the ethanol: water (1:1) extracts (Lafka *et al*., 2013; Mekinić *et al*., 2014). However, the values recorded in the current study were lower than they found in other researches and, 19.7 and 21.82 g/100 g, respectively (Aytul, 2010; Yahyaoui, 2014).

However, there was no clear correlation between the amount of total phenol contents and the antibacterial effect of OLE samples, perhaps due to the differences among antimicrobial effect of individual phenolic compounds.

In this study, the antibacterial activities of 13 different OLEs were tested at 5 different concentrations (200, 100, 50, 25 and 10 mg/mL) against some foodborne pathogenic bacteria, namely *B*. cereus, *B. subtilis*, *S. aureus*, *S. epidermidis* (Gram +) and *Enterocobacter cloacae*, *Eschrichia coli*, *Proteus mirabilis*, *Salmonella enterica* (Gram -) bacteria.

Table 2. Total phenol contents of the OLE samples.

Sample symbol	Absorbance*	Total phenols (g/100 g)*
DV	1.840±0.03	1.69±0.03
QG	1.679±0.02	1.55±0.02
QDB	1.670±0.02	1.53±0.02
DS	1.646±0.02	1.50±0.02
QD	1.560±0.04	1.42±0.03
QDATH	1.516±0.01	1.38±0.01
DA	1.480±0.04	1.34±0.04
QN	1.465±0.03	1.33±0.03
QDW	1.440±0.04	1.30±0.03
QI	1.331±0.03	1.19±0.03
QTI	1.274±0.02	1.14±0.02
QTA	1.272±0.02	1.14±0.02
QASH	1.237±0.04	1.10±0.04
* mean±standard deviation of three replications		

The results showed that almost all OLEs at higher concentration (200 mg/mL) showed a strong bactericidal effect on bacterial species, i.e. no viable bacteria were determined after 24 h of incubation of the inoculated medium, with some exceptions. However, OLE samples at lower concentrations (10 mg/mL) had variable inhibitory action on the growth of the bacterial species. *B. subtilis* seemed to be the most resistant species among all other species, while the most susceptible species was *E. cloacae* (Table 3).

The antibacterial activity of OLEs were higher in the samples DS, DV and DA, while the antibacterial activities were the lowest in the samples QI, QTI and QASH.

The lowest MICs of OLEs for *S. aureus* and *E. coli* were 10 and 100 mg/mL, respectively. It was higher than that recorded in a previous research (Markin *et al*., 2003) that investigated the minimal bactericidal concentration of olive leaf water extract against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *K. pneumoniae* and *E. coli*, and defined the minimal bactericidal concentration of the OLE as 6 mg/mL for S. aureus and *E. coli*, and observed a complete destruction of *S. aureus* within 2 h. Moreover increasing OLE concentration from 3 mg/mL to 6 mg/mL caused a decrease the inhibition time from 24 h to 3 h for *E. coli*.

The MICs of *B. subtilis* were in general higher than that of *B. cereus*, while the MIC values for *E.coli* were higher than that for *B. cereus* and lower than that for B. subtilis, as well as *S. aureus* had the lowest MIC values among these four species, and E. cloacae was the most sensitive species. These results were in agreement with other research (Hussain *et al*., 2014) in which the authors used OLEs in a concentration of 15 mg/mL and investigated inhibitory zones equal to 11.32, 2.11 and 13.27 mm for *S. aureus*, *B. cereus* and *E. coli*, respectively; while no inhibitory effect was recorded for *B. subtilis* as well as these results agree with that found in the research (Aliabadi *et al*., 2012) that concluded that *E. coli* was the most resistant species when compared with *S. aureus*, B. cereus and S. typhimurium with inhibitory zones 8.2, 9, 9.5 and 11.5 mm, respectively. However, these results did not resemble an another research (Pereira *et al*., 2007), that reported the antimicrobial capacity order for several concentrations of OLE as follows; *B. cereus* > *E. coli*> *S. aureus*> *B. subtili*, and revealed that the growth rates of *S. aureus* and *E. coli* were decreased while OLE concentration increased and the OLE showed a IC25 (25% inhibitory concentration) value of 2.68 and 1.81 mg/mL for *S. aureus* and *E. coli*, respectively. Moreover increasing OLE concentration from 0.05 mg/mL to 5 mg/mL caused a significant decrease in optical densities

of OLE added S. aureus and E. coli cultivars decreased 51% and 46%, respectively. Also, these results did not agreed with the research (Keskın *et al*., 2012), that used a concentration of 30 µg per disk, and recorded inhibitory zones 12, 10 and 9 mm for B. subtilis, S. aureus and E. coli, respectively; while no inhibitory zones were noticed for B. cereus and E. cloacae.

When we refer to table 2, we can concluded that the MIC values for S. enterica were lower than that for P. mirabilis. This result agree with a previous research (Faiza *et al*., 2011), that found 9 and 11 mm inhibitory zones for P. mirabilis and S. typhimurium, respectively, when it used concentration of 50 mg/mL of OLEs.

The study investigated the effect of the extraction solvent on the antimicrobial efficiency of S. aureus, E. coli, S. enteritidis, S. thypimurium and some others (Korukluoglu et al., 2010). It reported that solvent type affected the phenolic distribution and concentration in extracts, and antimicrobial activity against tested bacteria. As ethanol extracted OLE showed the highest antimicrobial efficiency against *E. coli* and *S. enteritidis*, acetone extracted OLE showed the highest antimicrobial efficiency against S. thypimurium.

In conclusion, OLE samples have strong antibacterial effect on both food-borne pathogens, 4 gram-negative bacteria (Enterocobacter cloacae, Eschrichia coli, Proteus mirabilis, Salmonella euterica) and 4 gram-positive bacteria (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *S. epidermidis*), if an appropriate OLE concentration is used. Antibacterial activity of olive leaves depends on the chemical composition and especially the concentration of the active components and compounds of the samples. These findings confirm that the antibacterial activity of olive leaves may be attributed to the total phenol contents of OLE samples. This study offers useful information for the usage of olive leaves as a natural antimicrobial agent to control microbial growth in food products and might provide an alternative to chemical preservatives.

Table 3. Antibacterial activity of OLEs against bacteria.

Bacterial Species	Olive Leaves Extracts concentration (mg/mL)												
	QI	QTI	DS	QG	QDW	QDB	DV	QDATH	QD	QN	DA	QASH	QTA
B. cereus	200	200	-	200	200	100	25	100	25	200	100	200	200
B. subtilis	-	200	100	200	-	-	200	200	-	-	-	-	200
S. aureus	-	200	10	200	25	100	10	25	200	200	100	200	10
S. epidermis	200	-	10	200	100	100	10	100	10	200	100	200	10
S. enterica	200	200	100	100	100	200	200	200	200	200	200	200	200
E. cloacae	200	200	10	200	100	200	10	200	10	100	10	200	200
E. coli	200	200	100	200	200	-	200	200	200	200	200	200	200
P. mirabilis	200	-	100	200	200	200	200	100	200	200	200	200	200

Conclusion

There is a large amount of olive leaves, which is produced in Syria annually. Olive leaves usually considered as useless by-product of olive oil production. However, olive leave is a good resource of phenolic compounds that have antibacterial activity against some food-borne pathogenic bacteria.

Recommendations

We recommend utilizing olive leaves in extraction of useful phenolic compounds, which showed a good antibacterial activity against food-borne pathogenic bacteria.

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