RESEARCH ARTICLE Asian Journal of Dairy and Food Research, Volume 41 Issue 2: 237-241 (June 2022)

Phenolic Compounds and Antimicrobial Activity of Olive (*Olea europaea* L.) Leaves

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10.18805/ajdfr.DR-240

ABSTRACT

Background: Olive leaves are of great interest, especially in traditional medicine. The polyphenols contained in olive leaves play an important role in this respect, as they have anti-carcinogenic, anti-inflammatory and anti-microbial properties. Olive leaves share phenolic compounds with other plants, but they also contain phenolic compounds belonging to the Oleaceae family.

Methods: We report the determination of phenolic compounds in olive leaves by HPLC and the evaluation of their *in vitro* activity against several microorganisms that may be causal agents of human intestinal and respiratory tract infections, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumonia*, *Enterobacter cloacae*, *Proteus mirabilis* and *Salmonella typhimurium*.

Result: The results reveal that the olive leaves may constitute a good source of antimicrobial agents. The high performance liquid chromatography (HPLC) analysis showed the presence of five phenolic compounds: oleuropein, ascorbic acid, rutin, catechinand verbascoside and for the first time ascorbic acid. At low concentrations, olive leaf extracts showed an unusual antibacterial action, which suggests their great potential as nutraceuticals, particularly as a source of phenolic compounds.

Key words: Antimicrobial activity, Antimicrobial, Ascorbic acid, Catechin, HPLC, Olive leaves, Phenols, Rutin.

INTRODUCTION

The olive (*Olea europaea* L.) is an evergreen tree requires chilling for fruiting. It is mostly grown for oil extraction and it holds numerous biological and medicinal values (Kumar and Sharma, 2016).

Olive tree (*Olea europaea* L.) is one of the most important fruit trees in Mediterranean countries, where they cover 8 million ha, accounting for almost 98% of the world crop (Guinda *et al.* 2004). (*Olea europaea* L.) is widely studied for its alimentary use, whereas the leaves are important for their secondary metabolites such as the secoiridoid compounds oleacein and oleuropein, the former responsible for hypotensive activity (Hansen *et al.* 1996). Several reports have shown that olive leaf extract has the capacity to lower blood pressure in animals (Samuelsson, 1951) and increase blood flow in the coronary arteries (Zarzuelo *et al.* 1991), relieve arrhythmia and prevent intestinal muscle spasms (Garcia *et al.* 2000).

Fungal pathogens are mainly responsible for the decay of fruits and vegetables during the postharvest period (Pathak, 1997; Debjani *et al.* 2018). *Aspergillus*, *Fusarium* and Penicilliumare are responsible for spoilage of many foods and causes decay on stored fruits damaged by insects, animals, early splitsand mechanical harvesting. Apart from causing diseases in plants, many species of *Aspergillus*, *Penicillium* and *Alternaria* can also synthesize mycotoxins (Rojas *et al.* 2004, Agrios, 1997, Alkooranee *et al.* 2020). The main aim of this work was to evaluate the antifungal properties of the extract of olive leaves against phytopathogens that cause several diseases in olive, such as *Aspergillus solani*, *Aspergillus niger*, *Penicillium digitatum* and *Mucor hiemalis*.

In this paper, HPLC was used to evaluate the qualitative composition of the phenolic compounds in olive leaves with the aim to identify new compounds and we evaluated the extracts' antimicrobial activity against bacteria and fungi.

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How to cite this article: Bensehaila, S., Ilias, F., Saadi, F. and Zaouadi, N. (2022). Phenolic Compounds and Antimicrobial Activity of Olive (*Olea europaea* L.) Leaves. Asian Journal of Dairy and Food Research. 41(2): 237-241. DOI: 10.18805/ajdfr.DR-240.

Submitted: 10-05-2021 **Accepted:** 11-02-2022 **Online:** 29-03-2022

MATERIALS AND METHODS

Plant materials

The olive leaves (Sigoise variety of olive fruit) were collected from Tlemcen (West of Algeria), in autumn and dried away from direct sunlight. Dried plant material was then crushed into a mortar and stored at very low temperature until further use.

Extraction of phenolic compounds

The dried powder of olive leave (10 g) was extracted in triplicate, using EtOH (96% v/v) at room temperature, under stirring. The aqueous suspension of the concentrated EtOH extract was evaporated to dryness and used for all investigations (Kukic *et al.* 2008).

High performance liquid chromatography (HPLC)

Total phenolics analyses of methanolic extract of infected olive leaves were carried out using Jasco HPLC, consisting of a pump (PU-2089 Plus) and UV detector model UV-2077 Plus with ChromNAV on a XBridge analytical column (RP-C18: 5 µm, 4.6×150 mm) (Waters Inc. USA) with gradient solvent system and parameter condition as shown in Table 1. The chromatograms were observed at wavelengths of 254, 270, 280 and 329 nm. All the analyses were carried out at sample concentration of 1 mg/ml and injection volume of 20 µl.

Pathogenic fungi

Four fungal isolates causing olive rot. *Aspergillus niger*, *Aspergillus solani*, *Penicillium digitatum* and *Mucor hiemalis* were isolated directly from rotten Olea europaea fruits. All isolated fungal species were transferred to sterilized triplicate 9 cm Petri dishes containing fresh potato dextrose agar medium (PDA: potato 200, dextrose 20 g and agar 15 g/L in distilled water at 25°C) in the presence of a quantity of lactic acid (25%) to stop the growth of bacteria. The plates were incubated at 25±2°C for 8 days, in darkness. The developing fungal colonies were purified and identified up to the species level by microscopic examination through the help of published materials (Barnett, 2006).

Antifungal assay

The antifungal activity of essential oil and extracts was tested using the radial growth technique (Zambonelli *et al.* 1996, Bajpai *et al.* 2007). Appropriate volumes of the essential oil and extracts were added to the PDA medium immediately before it was poured into the Petri dishes (9.0 cm diameter) (at 40-45°C) to obtain a series of concentrations (0.01 to 5500 µg/mL). Each concentration was tested in triplicate. The discs of mycelial felt (0.5 cm diameter) of the plant pathogenic fungi, taken from 8-day-old cultures on PDA plates, were transferred aseptically to the center of Petri dishes. Amphotericin B was used as a reference fungicide. The treatments were incubated at 27°C in the dark. Colony growth diameter was measured after the fungal growth in the control treatments had completely covered the Petri dishes.

Antibacterial activity

Growth inhibition activities for sample extracts against: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumonia*, *Enterobacter cloacae*, *Proteus mirabilis* and *Salmonella typhimurium* were tested using disc diffusion method (Berghe *et al.* 2001). The suspension of bacteria of about 1.5×10^6 CFU/ml. Colony forming units per milliliter obtained following a 0.5 McFarland turbidity standard, which was standardized by adjusting the optical density to 0.1 at 600 nm (JENWAY 6405UV/Vis spectrophotometer) (Tereschck *et al.* 1997). One milliliter of inoculums suspensions were used to inoculate by flooding the surface of Mueller-Hinton Agar plates. Excess liquid was air dried under a sterile hood. Dried extracts were dissolved in DMSO at the concentration 25, 30 and 50 mg/ml for aqueous methanol extract and aqueous acetone extract and 10, 15 and 20 mg/ml of ethyl acetate fraction, butanolic fraction. After, sterilized discs (Whatman N°1, 6 mm diameter) were impregnated with 5 µl of each extract (equivalent to 125, 150 and 250 µg/disc for aqueous methanol extract and aqueous acetone extract, respectively and equivalent to 50, 75 and 100 µg/disc for ethyl acetate fraction and butanolic fraction, respectively) and placed on the agar surface. DMSO was used as a negative control. The plates were left for 30 min at room temperature to allow the diffusion of extractand then they were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone and presented in millimeters.

RESULTS AND DISCUSSION

Phenolic compounds in olive leaves

The data (retention time, λ max in the visible regionand tentative identification) obtained for the phenolic compound peaks in the HPLC-DAD analysis are presented in Table 1 and Fig 1. HPLC studies point to five phenolic compounds determined in olive leaves extracts: Ascorbic acid (*Rt*=1.964 min, maximum absorbance at 243 nm), Verbascoside (*Rt*=16.23 min, maximum absorbance at 251 nm), Oleuropein (*Rt*=16.95 min, maximum absorbance at 242 nm), Rutin (*Rt*=18.535 min, maximum absorbance at 250 nm) and catechin (*Rt*=19,549 min, maximum absorbance at 252 nm).

The retention time and absorption spectrum (obtained by means of a UV/vis DAD) were identical to those obtained for the corresponding standards. The HPLC analysis of the studied sample revealed different chemical profiles, in which five phenolic compounds were identified and quantified: oleuropein, ascorbic acid,rutin, catechinand verbascoside (Fig 1, Table 1). All these compounds were previously reported to occur in olive leaf except ascorbic acid (Benavente-Garcia *et al.* 2000; Meirinhos *et al.* 2005; Pereira *et al.* 2007).

The differences found in the phenolic composition are not surprising, considering that a different extractive method was applied (Romero *et al.* 2004). According to the literature, these compounds are present in the olive fruit (Blekas *et al.* 2002; Romero *et al.* 2004; Pereira *et al.* 2006). The phenolics content of olive depends on several factors, such as cultivar

Table 1: Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax) and tentative identification of phenolic compounds in olive.

Peak	Rt (min)	Λmax (nm)	Tentative identification
1	1.964	243	Ascorbic acid
2	16.23	251	Verbascoside
3	16.95	242	Oleuropein
4	18.535	250	Rutin
5	19.549	252	Catechin
6	19.944	249	N.D

(Vinha *et al.* 2005; Esti *et al.* 1998), climate (Salvador *et al.* 2001), irrigation regimes (Romero *et al.* 2002), degree of ripeness of the fruit (Gutierrez *et al.* 2005) and elaboration process (Romero *et al.* 2004; Das *et al.* 2021).

Antibacterial and antifungal activities

The inhibitory effects of olive leaf extracts were evaluated against eight four bacteria: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumonia*, *Enterobacter cloacae*, *Proteus mirabilis* and *Salmonella typhimurium* and against four fungi: *Aspergillus niger*, *Aspergillus solani*, *Penicillium digitatum* and *Mucorhiemalis.* The results obtained from assays of antibacterial activity at different concentrations of olive leaf extracts by the radial growth technique are reported in Table 2 and 3.

The results indicate that the inhibition of the mycelial growth of each strain was significantly influenced by the extracts concentration. This study revealed the significant antimicrobial activity of olive leaf extracts.

Some researchers have also demonstrated that biocompounds present in olive products, such as oleuropein (Furneri *et al.* 2002; Battinelli *et al.* 2006) and hydroxytyrosol (Furneri *et al.* 2002) and aliphatic aldehydes (Battinelli *et al.* 2006), inhibit or delay the rate of growth of a range of bacteria and microfungi. In this study, the antimicrobial activity of extracts of olive leaves was evaluated against fungi isolated from olive and bacteria.

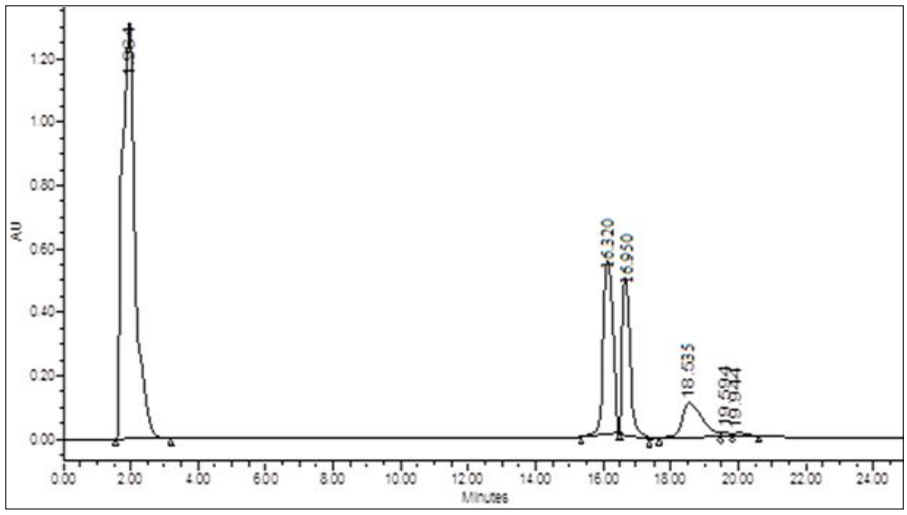

Fig 1: Chromatogram (zoom) recorded at 254 nm showing the phenolic compounds profiles identified and not identified of olive leaves (*Olea europea* L.).

Table 2: Values for bacterial growth rate in the presence of different olive leaf extract concentrations.

Extract (mg/mL)	*S.a*	*B.c*	*E.c*	*P.a*	*K.p*	*En.c*	*P.m*	*S.t*
5	0.9±0.1	1.14±0.06	1.2±0.11	0.8±0.07	1.11±0.09	0.94±0.11	0.87±0.09	1.05±0.13
10	1.8±0.12	1.53±0.11	2.15±0.01	1.23±0.11	1.65±0.07	1.17±0.13	1.03±0.11	1.65±0.11
15	2.15±0.09	1.87±0.03	2.65±0.20	1.5±0.03	1.9±0.03	1.39±0.08	1.85±0.06	1.78±0.08
20	3.01±0.02	2.08±0.20	2.89±0.11	1.87±0.20	2.13±0.21	1.87±0.09	2.09±0.17	2.13±0.06
25	3.76±0.08	3.15±0.15	3.22±0.04	2.03±0.13	22.54±0.13	2.65±0.15	2.55±0.14	2.86±0.05
30	3.98±0.13	3.68±0.20	3.56±0.15	2.15±0.05	2.81±0.08	2.78±0.2.	2.86±0.08	3.01±0.11

S.a= Staphylococcus aureus, *B.c= Bacillus cereus*, *E.c= Escherichia coli.*

P.a= Pseudomonas aeruginosa, K.p= Klebsiella pneumonia, *En.c= Enterobacter cloacae*.

P.m= Proteus mirabilis and *S.t= Salmonella typhimurium.*

Table 3: Values for fungal growth rate in the presence of different olive leaf extract concentrations.

Extract (mg/mL)	*Aspergillus niger*	*Aspergillus solani*	*Penicillium digitatum*	*Mucor hiemalis*
5	1.31±0.02	1.19±0.11	3.19±0.01	2.18±0.03
10	2.76±0.14	2.02±0.2	4.78±0.06	3.47±0.09
15	3.15±0.3	3.39±0.13	5.03±0.14	5.12±0.11
20	4±0.04	3.44±0.08	5.49±0.08	6.17±0.18
25	4.30±0.11	4.07±0.01	6.17±0.02	6.31±0.2
30	5.02±0.06	4.75±0.12	6.22±0.11	6.54±0.09

The response for each microorganism tested was different. The mycelial growth of colonies in the presence of the extracts of olive leaves showed that it effectively controlled all the fungi tested. This efficiency can be explained by the presence of active molecules that inhibited the growth of the five phytopathogenic fungi. Several authors have attributed the antifungal capacity of olive (Pereira *et al.* 2006). Oleuropein and hydroxytyrosol have shown antimicrobial activity against *Salmonella* spp., *Vibrio* spp. and *Staphylococcus aureus* (Pereira *et al.* 2006).

In addition, some reports (Ruiz-barba *et al.* 1991; Marsilio *et al.* 1998) have shown that some phenolic substances of olive trees may inhibit the growth of bacteria, such as *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and fungi like *Phytophthora* (Delrio *et al.* 2003). Similarly, the phenolic metabolism of the olive tree is considered as a plant-response to the infection by *Verticilliumdahliae* (Daayf, 1993).

The chemical composition of olive leaf extracts impacted the antimicrobial effects observed. In fact, the mode of action of phenolics has been shown to be concentration dependent (Battinelli *et al.* 2006; Cowan, 1999). Additionally, the antimicrobial action of these compounds is well-known and is related to their ability to denature proteins, which in general renders them to be classified as surface-active agents (Denyer *et al.* 1998). These results are important against several pathogenic microorganisms resistant to a number of phytochemicals.

CONCLUSION

This study strongly suggests that some phenolic compounds present in olive leaves play a role in the natural defense mechanism, as it has been established for other phenolic secondary metabolites in different plant materials infected by pathogens. Regarding the part of the evaluation of the antibacterial activity of olives *in vitro*, it turns out that the bactericidal effect varies considerably depending on the nature and concentration of the polyphenol. Overall, olives are a source of valuable natural bio-phenols, endowed with remarkable antibacterial activity, which can serve in the pharmaceutical, food and agriculture industries.

Conflict of interest: None.

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