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Recovery of High Yield Flavonoids Rich Extract from Two-phase Chemlali Olive Pomace

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Abstract

Increasing interest in phenolic compounds in olive cake is due to their antioxidant and health-enhancing properties. In this study the flavonoids in two phase olive pomace of the Tunisian olive cultivar Chemlali were extracted by ethanol-water to obtain a flavonoid rich extract. The identification and quantification of flavonoids was based on separation by high-performance liquid chromatography equipped with a diode array detector followed by

liquid chromatography-mass spectrometry analysis. Five flavonoids were isolated and purified using HPLC apparatus. Hesperidin and quercetin-3-*O*-arabinoglucoside were in high amount (15.60 and 21.71 mg/kg of fresh weight, respectively) followed by quercetin and luteolin (8.12 and 11.01 mg/kg of fresh weight, respectively). The antioxidant activity of purified flavonoids was evaluated by measuring the radical scavenging effect on DPPH and by using ferric reducing antioxidant power assays. Bactericidal and fungicidal effects were determined using the NCCLS broth dilution. The minimal cidal concentrations (MCCs) values of ethyl acetate extract of two-phase Chemlali olive oil ranged from 1.825 to 14.6 mg/ml against tested pathogens and phytopathogens microbes. This activity was related to the richness of the extract of flavonoid compound especially quercetin-3-*O*-arabinoglucoside and hesperidin.

Keywords: flavonoids, Chemlali, hesperidin, olive pomace, antioxidant activity.

1. Introduction

During the last few years the production of olive oil has undergone important changes in the equipment used for the separation of the olive oil from the remaining components. The extraction of olive oil generates huge quantities of wastewater and/or solid residue (olive pomace) that may have a great impact on land and water environments because of their high phytotoxicity (Roig *et al.,* 2006; Rigane *et al.,* 2012a,b).

Olive materials contain many compounds with antioxidant activity, mostly polyphenols, and could therefore be used as sources of potentially safe natural antioxidants for the food industry. The antioxidant capacities of oleuropein and hydroxytyrosol in *O. europaea* L. tissues are well-known (Visioli *et al.,* 2002; Rigane *et al.,* 2012b), but few studies have been made on the antioxidant activities of other low molecular weight phenols, such as flavonoids, present in two-phase Chemlali olive pomace.

Flavonoids comprise a large group of naturally occurring, low molecular weight, polyphenolic compounds widely distributed in the plant kingdom as secondary metabolites. To date, more than 6000 flavonoids have been identified. They represent one of the most important and interesting classes of biologically active compounds and occur both in the free states and as glycosides (Garg *et al.,* 2001; Chebil *et al.,* 2007). Flavonoids have been observed to have biological in vitro effects, such as free radical scavenging, modulation of enzymatic activity, and inhibition of cellular proliferation. They showed also antibiotic activities and could be used as antiallergic, antidiarrheal, antiulcer, and anti-inflammatory agents (Cherng *et al.,* 2007; Ramos *et al.,* 2007).

The possible beneficial effects of flavonoids have resulted in their use as food supplements. A variety of flavonoid extracts from plants are available on the market. Recently, extracts of berries, grapes, pine barks, containing mainly flavonols and anthocyans, have been marketed by ingredients companies and used in beverages and food. Many of the large food companies have interests in flavonoids to meet consumers' demand for healthier food. Thus, flavonoids may carry many opportunities of food ingredient innovations and business developments (Chebil *et al.,* 2007).

The aim of this study is to identify, quantify, and evaluate the biological activities, including antioxidant and antimicrobial activities, of some flavonoids purified from the two-phase of the Tunisian olive cultivar Chemlali.

2. Materials and Methods

2.1 Chemicals

The solvents used for extraction and chromatographic separation (ethyl acetate, acetic acid and acetonitrile) were purchased from Riedel-deHaen (Buchs, Switzerland). Ethanol was obtained from Carlo Erba (Milan, Italy). Formic acid was purchased from Panreac Quimica SA (Barcelona, Spain). The solvents were of appropriate purity. Esculetin, quercetin, luteolin, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 6-di-*tert*-butyl-4-hydroxy-boxylic acid (BHT), 2, 4, 6-tri(2-pyridyl)-s-triazine (TPTZ) and 6-hydroxy-2, 5, 7,

8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (USA). Iron (III) chloride ($FeCl_3.6H_2O$) and sodium acetate ($CH_3COONa.3H_2O$) were purchased from Merck (Darmstadt, Germany). Double distilled water was used in the HPLC mobile phase.

2.2 Plant material

The two phase Chemlali olive pomace used in the experiment was collected from an olive oil factory located in Sfax, Tunisia, and kept at a temperature of -20°C in the dark until its use.

2.3 Extraction and pre-purification of two phase Chemlali olive pomace flavonoids

Two- phase Chemlali olive pomace (100 g) was extracted with Ethanol (80%, w/v), the mixture was left to stand under agitation for 24 h at room temperature. The solution was filtered using GF/F filter paper. The ethanol was removed by rotary evaporation at 35 °C under high vacuum. After removal of ethanol, the extract was diluted with 50 mL of distillated water and then was partitioned successively between petroleum ether to remove carotenoids, fats, waxes and residual olive oil (100 mL x 3) and ethyl acetate (EtOAc) (100 mL x 3) to obtain three fractions (petroleum ether soluble fraction: 240 mg, EtOAc soluble fraction: 60 mg and aqueous soluble fraction: 2.46 g). The EtOAc extract was concentrated by vacuum evaporation. The ethyl acetate extract (60 mg) was dissolved in a small amount of ethanol for HPLC analyses.

2.4 Determination of the total phenolic content

The phenolic content was measured according to the Folin-Ciocalteu method and expressed as grams of gallic acid equivalents per kilogram of fresh alperujo (Rigane et al., 2011, 2013).

2.5 Total flavonoids determination

The determination of total flavonoid compounds was carried out according to the method adapted by Rigane et al. (2011, 2013). One mL aliquot of the appropriately diluted sample or standard solution of quercetin was added to 10 mL volumetric flask containing 4 mL of water. At zero time, 300 µL of $NaNO_2$ (5 %, w/v) in water was added to the flask. After 5 min, 300 µL of 10 % $AlCl_3$ was added. At 6 min, 2 mL of aqueous NaOH (1mol. L^{-1}) was added to the mixture. Immediately; the mixture was diluted with water to 10 mL. The absorbance of the mixture, characterized by a pink colour, was determined at 510 nm compared to a water control. The total flavonoids were expressed as fresh weight g/kg of quercetin equivalents (QE). For quercetin, the curve absorbance against concentration was described by the equation $y = 0.0049x$ ($r^2 = 0.9984$).

2.6 Reverse-phase HPLC condition

The identification and quantification of flavonoids were carried out by HPLC. The assays were performed with an Agilent Technologies 1100 series system equipped with an automatic injector, a Spherisorb S3 ODS2 column (250 x 4.6 mm i.d., 5 µm particle size, Waters Co., Milford, MA, USA) oven and a diode array UV detector. A gradient of two solvents, A and B, was used. Solvent A consisted of 2% acetic acid in water and solvent B of methanol, acetic

acid, and water (18:1:1). The following proportions of solvent B were used for elution: 0-20 min, 25-100%; 20-24 min, 100%; and 24-30 min, 25%. The column temperature was maintained at 30 °C, and the flow rate was 1 mL/min. The injection volume was 20 µL. All samples were filtered through a 0.45 µm Minisart filter.

Detection and quantification were performed at 335 nm. Each flavonoid compound was expressed with its standard. Quantitative evaluation of individual flavonoids was performed by means of a four-point regression curve (r^2=0.989) using authentic external standards.

2.7 LC-MS analysis

Samples were analyzed on Agilent 1100 series LC-MSD consisting of a degasser, a binary pump, an auto sampler, and a column heater. For chromatographic conditions, the compounds were separated with a Zorbax 300 A° Extend-C-18 column (2.1 x 150 mm, particle size 5µm, Agilent Technology, INC, Wilmingtom, DE, USA). The column outlet was coupled with an Agilent MSD ion trap XCT mass spectrometer (Santa Clara, CA, USA) equipped with an ESI ion source. The capillary voltage was set to 3.5 KV, the temperature to 350°C, the nebulizer gas to 40 p.s.i, and the drying gas flow to 10 L/min. The maximum accumulation time was 50 ms, the scan speed was 26.000 m/z s^{-1} (ultra scan mode) and the fragmentation time was 30 ms. Formic acid (0.1%, v/v) in water and 0.1% of formic acid in acetonitrile served as solvents A and B, respectively. Analysis conditions were as described before by Rigane *et al.* (2011, 2012a) and involved a four-step linear gradient analysis for a total time of 18 min was used as follows: starting from 95% solvent A and 5% solvent B for 1 min, followed by an 11 min step gradient from 5% B to 100% B. Then, elution was conducted in the isocratic mode with 100% B for 4 min. Finally, the elution was achieved with linear gradient from 100% B to 5 % in 2 min. The flow rate was 200 µL/min. An aliquot (5 µL) of sample solution was injected

2.8 Chromatographic purification of flavonoids

Of the olive pomace extract, 1 g was chromatographed on a C-18 silica gel (liChroprep RP-18; 25–40 mm) column (25 x 150 mm) under high pressure using a preparative HPLC system equipped with a UV detector. Flavonoid compound elution was carried out with the same solvent gradient as was used in HPLC. The flow rate was adjusted to 0.7 mL/min. The first two separated peaks correspond to pure hesperidin and quercetin-3-*O*-arabinoglucoside.

2.9 Antioxidant activities

DPPH assay. The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging effect was evaluated following the procedure described in a previous study (Rigane *et al.,* 2011; 2012a, 2013) and expressed as IC_{50} (effective concentration, µg/mL), calculated from a calibration curve using linear regression for each antioxidant. The synthetic antioxidant reagent butyl-hydroxytoluene (BHT) was used as positive control and all tests were carried out in triplicate.

Ferric reducing antioxidant power (FRAP) assay. The reducing power assay was performed according to the procedure described in a previous study (Rigane *et al.,* 2011; 2012a, 2013).

The assay was calibrated using 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), and the results were expressed in Trolox equivalent mM per 1g of extract. The calibration curve was y (absorbance) = 0.2595 x (mM TE/g of extract) (r^2=0.9965). All of the samples were analyzed in triplicate.

2.10. Bactericidal Activities

In order to evaluate the antimicrobial activities of the ethyl acetate extract of two phase olive pomace of the Tunisian olive cultivar Chemlali, pure cultures of authenticated bacteria were obtained from the international culture collections (ATCC: The American Type Culture Collection and CIP: The Collection of Pasteur institute) and from laboratory collection. Standard bacterial strains: *Pseudomonas aeruginosa* CIPA22, *Escherichia coli* ATCC 1053, *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* CIP80.39, *Agrobacterium tumefaciens*, *Erwinia* sp. and *Pseudomonas savastanoa*.

The bacteria were cultivated in LB broth at the appropriate temperature at 30°C. The cultures of bacteria were maintained in their appropriate agar slants at 4 °C throughout the study and used as stock cultures. Inocula were prepared by adjusting the turbidity of each bacterial culture to approximately 10^6 CFU/mL.

2.11. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC) of ethyl acetate extract of two phase olive pomace of the Tunisian olive cultivar Chemlali were determined by the broth dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The test was performed in sterile 96-well microplates. The inhibitory activity of the ethanolic extract were properly prepared and transferred to each microplate well in order to obtain a twofold serial dilution of the original sample. The inocula (10 µL) containing 10^6 CFU of bacteria were added to each well. While the negative control wells contained bacteria only in their adequate medium, the positive ones contained 50 µg L-1 of ampicillin antibiotic. Subsequently, 20 µL of 0.1% MTT were added. Plates were aerobically incubated at 30°C for 16-20 h. After incubation, the wells were observed for a colour change from yellow to blue. MIC was defined as the lowest concentration of test samples that inhibited the bacterial growth.

2.12. Statistical Analysis

Results were expressed as mean ± standard deviation (SD) of 3 measurements for the analytical determination. Statistical differences were calculated using a one-way analysis of variance (ANOVA), followed by Student's t-test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1 Phenolic and flavonoid content in olive pomace extract

The total phenolic and flavonoid contents of ethyl acetate fraction of alperujo are shown in Table 1. The total phenol concentration of alperujo was 218.20 g as gallic acid equivalents/kg

of fresh alperujo, while, flavonoid content attaint 102.20 g quercetin equivalents/kg of fresh weight. These values were in agreement with results reported by Lesage-Meessen *et al.* (2001) who found that total phenol content were 240 and 204 g gallic acid equivalents/kg in other olive oil residues originating from the three- and two-phase system, respectively) from French olive mills.

Table 1. Total phenolic content and total flavonoid content in Ethyl acetate extract.

	EtOAc extract
Phenolic content (g GAE/kg of fresh alperujo)	218.20±0.13
Flavonoids content (g QE/kg of fresh alperujo)	102.20±0.31

Results are expressed as mean ± standard deviation of 3 determinations.

2.2 Identification and quantification of flavonoid compounds in olive pomace extract

The HPLC analysis of the ethyl acetate fraction (Figure 1) of two-phase Chemlali olive pomace showed the presence of peaks with flavonoid-type UV spectra (two bands, λ max of band 1 between 320 and 370 nm and λ max of band 2 between 220 and 280 nm). Table 2 lists the identified compounds in order of elution. The structure assignment of flavonoids for which no standards were available, was based on a systematic search for molecular ions using extracted ion mass chromatograms and comparing them with the data in the literature (Obied *et al.,* 2007; Ryan *et al.,* 1998; 2002).

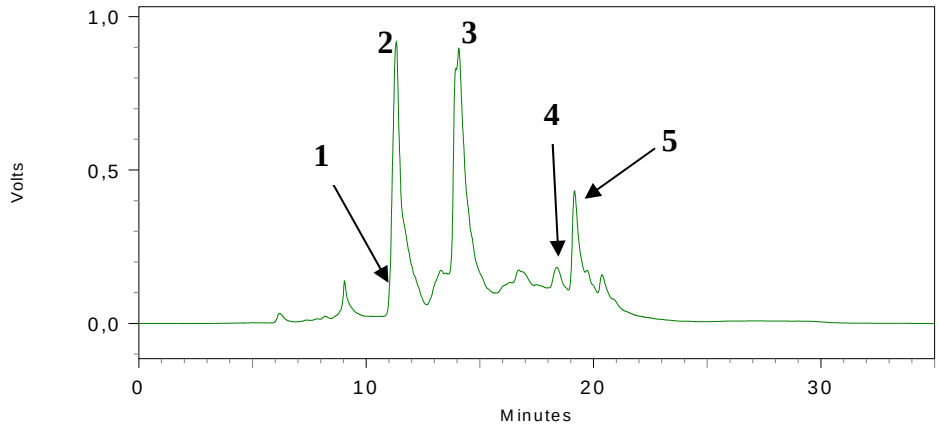

Figure 1. HPLC chromatogram of ethyl acetate extract: **1**: Esculetin, **2**: Hesperindin, **3**: Quercetin-3-*O*-arabinoglucoside, **4**: Quercetin, **5**: Luteolin

For example, the ESI mass spectrum (Figure not shown) in positive mode of compound **2** exhibited a peak $[M +CH_3CN]^+$ at m/z 651 and fragment ions at m/z 621 $[M-CH_3O+CH_3CN]^+$, 543 $[M-C_3H_9O_4+CH_3CN]^+$, 502 $[M-C_9H_{10}O_2+CH_3CN]^+$ and 435 $[M-C_{10}H_{15}O_5+CH_3CN]^+$. On the other hand, the λ max of the UV spectrum at 283 and 327 suggests that compound **2** is hesperetin-7-*O*-sugar (Obied et al., 2007), and the combined results of the MS and UV spectra suggest that compound **2** could be hesperetin-7-O-rhamnoglucoside (Hesperidin). In addition, the pseudomolecular ion $[M+NH_3]^+$ of compound **3** was also at m/z 613 (Table 2), but there was an intermediate ion at m/z 448 and an aglycon ion at m/z 303. The loss of 149 amu from the pseudomolecular ion represents the sugar arabinose, and the loss of 162 amu from the intermediate ion is due to the loss of glucose. The λ_{max} of the UV spectrum at 355 and 256 nm suggests that flavonoid **3** is quercetin-3-*O*-arabinoglucoside (Schieber et al., 2003). In the same way, compounds **1, 4** and **5** were identified as flavonoids: Esculetin (**1**), quercetin (**4**) and luteolin (**5**). To the best of our knowledge, compounds **1** and **2** were identified for the first time in two-phase Chemlali olive pomace, while, quercetin-3-O-arabinoglucoside (compound **3**) was found only in Chemlali olive leaves cultivated in Sfax (Tunisia), moreover, compounds **4** and **5** were identified in leaves and fruit of some Tunisian and Australian cultivars (Obied *et al.,* 2007; Rigane *et al.,* 2011).

The analysis of flavonoids substances using reversed phase-HPLC from two-phase Chemlali olive pomace, as described in the experimental section, allowed to the quantification of the identification of flavonoids compounds. As shown in Figure 2 and Table 2, flavonoid compounds identified and quantified in the two-phase Chemlali olive pomace belong to three classes: coumarin (Esculetin: t_R= 10.92 min), flavanone glucoside (Hesperidin: t_R= 11.33 min), flavonols (quercetin 3-arabino-glucoside: t_R= 14.07 min and quercetin: t_R= 18.37 min) and flavones (luteolin: t_R= 19.12 min). It appears that flavonoid glucosides were dominant. The main flavonoid compounds quantified in our study were heperidin and quercetin-3-O-arabino-glucoside (Table 2), followed by a free flavonoids such as luteolin (11.01 g/kg of fresh olive pomace). According to Vinha *et al.,* (2005), free flavonoids appear at the end of the maturation stage as a consequence of hydrolytic processes, i.e, one possibility is that luteolin is a product of luteolin-7-O-glucoside transformation by glycosidase activities (Bouaziz et al., 2004). In addition, quercetin content was noticeably lower (8.12 g/kg of fresh olive pomace), this may be explained by the fact that *O. europaea* L. fruit appears to accumulate only glycosylated derivatives because they are probably less toxic than aglycons (Amiot *et al.,* 1989; Bouaziz *et al.,* 2004).

Table 2. Flavonoid compound compositions of the studied extract.

N°	Flavonoid compound	Rt (min)	UV(λ_{max})	MW	EtOAc extract *
1	Esculetin	10.92	228, 257, 349	178	0.05±0.00
2	Hesperidin	11.33	283, 327	610	15.60±0.01
3	Quercetin-3-*O*-arabinoglucoside	14.07	256, 355	596	21.71±0.03
4	Quercetin	18.37	255, 371	302	8.12±0.01
5	Luteolin	19.16	254, 266, 351	286	11.01±0.00

* Concentration expressed as mg/kg of fresh alperujo. Results are expressed as mean ± standard deviation of 3 determinations. EtOAc: Ethyl Acetate

On the other hand, the quantification of the two flavonols identified in olive pomace was dominated by quercetin-3-*O*-arabinoglucoside (21.71 mg/kg of fresh weight). The last flavonol was found as the main flavonols in Chemlali olive fruit at the 9th harvest (170 mg/kg of fresh olive) then decreased during the last stage of maturation (up to 60 mg/kg of fresh olive) (Bouaziz *et al.,* 2004). In addition, heperidin was found also for the first time in two phase Chemlali olive pomace as second main flavonoids (15.60 mg/kg of fresh weight), this compound was detected only in olive leaves and pulp (Baldi *et al.,* 1995; Ryan *et al.,* 1998; 2002).

In Tunisian olive pomace analyzed, esculetin was present in very low concentrations (0.05 g/kg of fresh olive pomace). This compound was identified and quantified as minor components for the first time in two-phase Chemlali olive pomace.

2.3. Olive pomace flavonoid antioxidant activity verification

The flavonoids rich extract and the major flavonoid compounds (hesperidin, quercetin-3-*O*-arabinoglucoside, quercetin and luteolin) purified from two-phase Chemlali olive pomace were subjected to in vitro tests to evaluate their antioxidant activities. In particular, we carried out two tests: the DPPH radical-scavenging and FRAP assays.

The standard reference compound for all tests was BHT (Table 3). This table shows that the most effective flavonoid for scavenging the DPPH radicals and FRAP assay was the quercetin (Table 3), which could be used as natural antioxidants and might substitute synthetic antioxidants that produced many undesirable secondary effects (Yanishlieva *et al.,* 2001), followed by the luteolin and quercetin-3-*O*-arabinoglucoside (Table 3), while heperidin has the lowest antioxidant activity (Table 3). These results confirm the importance of the flavonoid B-ring catechol structure, the presence of a 3-hydroxyl free or glycosylated group and the 2, 3-double band conjugated with the 4-oxo function. For example, all purified flavonoids, which characterized by a 3', 4'-dihydroxylation of the B ring, have a high

antioxidant activity when compared with a synthetic antioxidant. These results suggest that further hydroxylation at the C-3' position contributes to a greater inhibition and increase of the antioxidant activity. On the other hand, the hydroxylation of the C-3 of the non-phenolic C ring seems to be important, as the flavonol quercetin, which differs from the flavone luteolin in being 3-hydroxylated, has a stronger antioxidant activity than the latter compound (Table 3). In addition, the glucosylation of the 7-hydroxyl group and the absence of 2, 3-double band conjugated with 4-oxo function of flavonoids (such as hesperindin) could reduce the antioxidant activity respect to their aglycones (quercetin and luteolin). The 7-*O*-glucosylation produces conformational changes in the flavonoid molecule that might make electronic delocation difficult as well as decreasing the electron donor capacity of the 7-hydroxyl group (Benavente-Garcia *et al.,* 2000). These results are similar to those reported by Rice-Evans *et al.,* (1996). Moreover, many authors note that in most, hesperidin was found to be inactive or only moderately active in comparison with other flavonoids antioxidants (Limasset *et al.,* 1993; Malterud and Rydland, 2000).

Table 3. Antioxidant activity of the studied extract.

	DPPH (IC_{50} µg/mL)	FRAP (mM TE)
Hesperidin	9.70 ± 0.12^{a}	1.15 ± 0.00^{A}
Quercetin-3-*O*-arabino-glucoside	3.53 ± 0.31^{b}	3.19 ± 0.02^{B}
Quercetin	0.92 ± 0.01^{c}	12.18 ± 0.08^{C}
Luteolin	2.19 ± 0.04^{d}	5.15 ± 0.07^{D}
Ethyl acetate extract	4.66 ± 0.14^{e}	3.08 ± 0.12^{E}
BHT	8.30 ± 0.23^{f}	1.34 ± 0.02^{F}

Results are expressed as mean ± standard deviation of 3 determinations. Means with different letters were significantly different at $p < 0.05$.

The antioxidant activity of the ethyl acetate extract (Table 3) is probably due to the presence of several chemical components, such as high content of quercetin-3-*O*-arabinoglucoside and luteolin that may act as radical scavenging agents. It seems to be a general trend that the ethyl acetate extract, containing a high level of flavonoid compounds, have high antioxidative properties. The results suggested that flavonoids rich extract from two-phase Chemlali olive pomace can serve as substitutes for synthetic antioxidants.

2.4. Antimicrobial activity

The antimicrobial activity of ethyl acetate extract of two phase olive pomace of the Tunisian olive cultivar Chemlali was evaluated against bacterial strains. The MICs values of ethyl

acetate extract of two phase olive pomace examined by the broth micro-dilution susceptibility assay NCCLS (2002) on the tested microorganisms were showed in Table 4. The MIC values for bacterial strains which were sensitive to the ethyl acetate extract of two-phase Chemlali olive oil and were in the range of 1.825-3.65 mg/mL to 7.3-14.6 mg/mL. The strong antimicrobial activity of the ethyl acetate extract of two-phase Chemlali olive oil against almost all the susceptible microorganisms could be attributed to the presence of high content of polyphenols and flavonoids content (218.2 g GAE/kg and 102.2 g QE/kg of fresh two phase Chemlali olive pomace, respectively) having antibacterial and antifungal potential. In fact, the major compounds of flavonoids were quercetin-3-*O*-arabinoglucoside, hesperidin followed by luteolin and quercetin (21.7, 15.6, 11.03 and 8.2 mg/kg of fresh two phase Chemlali olive pomace, respectively).

Table 4. Minimum inhibitory (MIC) and cidal concentration (MCC) of ethyl acetate extract of TPCOP and quercetin as pure standard in two phase Chemlali olive pomace against standard microbial strains

Bacterial strain	MIC (mg/m
Agrobacterium tumefaciens	[3.65-1.825]
Escherichia coli	[14.6-7.3]
Erwinia sp.	[3.65-1.825]
Pseudomonas aeruginosa	[3.65-1.825]
Pseudomonas savastanoa	[3.65-1.825]
Salmonella Enterica	[7.3-3.65]
Staphylococcus aureus	[7.3-3.65]

TPCOP: Two Phase Chemlali Olive Pomace

It has been reported that pure phenolic compounds isolated from olive cake could completely inhibit the growth of studied bacterial strains at concentrations ranged between 0.2 mg/mL to 0.4 mg/mL (Aziz *et al.*, 1998). These results were strongly lower than our results obtained by ethyl acetate extract of two-phase Chemlali olive oil against tested miroorganims. Also, Winkelhausen *et al*. (2005) reported that phenolic compounds extracted from olive pomace were added to a medium to reach phenol concentration of 0.1 and 0.2 % (w/v) and tested for their antifungal activity against *Alternaria solani*, *Botrytis cinerea* and *Fusarium culmorum*. A strong antifungal activity was obtained against the three fungi.

The strong antimicrobial activity of the ethyl acetate extract of two-phase Chemlali olive oil against almost all the susceptible microorganisms can be attributed to the presence of high concentration of quercetin-3-*O*-arabinoglucoside (Garg *et al.,* 2001) and hesperidin (Kohno *et al.,* 2001) having antibacterial and antifungal potential. The major compounds, as well as other minor constituents of ethyl acetate extract of two-phase Chemlali olive oil have been described to have antimicrobial activity. In fact, the synergistic effects as well as the diversity of major and minor constituents present in the ethyl acetate extract of two-phase Chemlali olive oil should be taken into consideration to account for their biological activity.

Genera including *Escherichia*, *Pseudomonas*, *Staphylococcus*, *Salmonella, Agrobacterium* and *Salmonella* are responsible for numerous human and plant diseases. All bacteria are sensitive and may be destroyed by ethyl acetate extract of two-phase Chemlali olive oil. Additional, CMIs data show that ethyl acetate extract of two-phase Chemlali olive oil may be a good candidate for its employment as an antimicrobial agent against numerous pathogenic bacteria.

4. Conclusions

This work is the first report on the identification and quantification of flavonoid compounds in olive pomace of a Tunisian cultivar, Chemlali, most widespread cultivar in Tunisia. Five flavonoids were isolated and purified and studied for their antioxidant activities. Flavonoids such as quercetin-3-*O*-arabinoglucoside which was found in high content in two-phase Chemlali olive pomace could be used as natural antioxidants and might substitute synthetic antioxidants that produce many undesirable secondary effects (Yanishlieva *et al.,* 2001). On the other hand, this paper demonstrates that flavonols and flavones structures are the most efficient olive phenolic compound quenchers for the radicals and this ability is greater as more free hydroxyl groups are present in the flavonoid structure. The results showed that the relative abilities of the flavonoids are influenced by the presence of functional groups in their structures, mainly the B-ring catechol, the 3-hydroxyl group and the 2, 3-double bonds conjugated with the 4-oxo function. In addition, the antimicrobial activity showed that the ethyl acetate extract of alperujo exerted an interesting inhibitory activity against all challenge bacteria strains.

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References

Amiot, M.J., Fleuriet, A., & Macheix, J.J. (1989). Accumulation of oleuropein derivative during olive maturation. *Phytochemistry*, *28,* 67-69. http://dx.doi.org/10.1016/0031-9422(89)85009-5

Aziz, N.H., Farag, S.E., Mousa, L.A.A., & Abo Zaid, M.A. (1998). Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios, 93,* 43–54.

Benavente-Garcia, O., Castillo, J., Lorente, J., Ortuno, A., & Del Rio, J.A. (2000). Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chemistry, 68,* 457-462. http://dx.doi.org/10.1016/S0308-8146(99)00221-6

Bouaziz, M., Chamkha, M., & Sayadi S. (2004). Comparative Study on Phenolic Content and Antioxidant Activity during Maturation of the Olive Cultivar Chemlali from Tunisia. *Journal of Agricultural and Food Chemistry, 52,* 5476-5481. http://dx.doi.org/10.1021/jf0497004

Chebil, L., Anthoni, J., Humeau, C., Gerardin, C., Engasser, J.M., & Ghoul, M. (2007). Enzymatic Acylation of Flavonoids: Effect of the Nature of the Substrate, Origin of Lipase, and Operating Conditions on Conversion Yield and Regioselectivity. *Journal of Agricultural and Food Chemistry, 55,* 9496–9502. http://dx.doi.org/10.1021/jf071943j

Cherng, J.M., Shieh, D.E., Chiang, W., Changn M.Y., & Chiang, L.C. (2007). Chemopreventive effects of minor dietary constituents in common foods on human cancer cells. *Bioscience, Biotechnology, and Biochemistry, 71,* 1500–1504. http://dx.doi.org/10.1271/bbb.70008

Garg, A., Garg, S., Zaneveld, L.J.D., & Singla, A.K. (2001). Chemistry and Pharmacology of The Citrus Bioflavonoid Hesperidin. *Phytotherapy Research, 15,* 655–669. http://dx.doi.org/10.1002/ptr.1074

Harborne, J.B., & Williams, C.A. (2000). Advances in flavonoid research since 1992. *Phytochemistry,* 55, 481–504. http://dx.doi.org/10.1016/S0031-9422(00)00235-1

Kohno, H., Taima, M., Sumida, T., Azuma, Y., Ogawa, H., Tanaka, T. (2001). Inhibitory effect of mandarin juice rich in b-cryptoxanthin and hesperidin on 4 (methylnitrosamino)-1-(3-pyridyl)-1-butanoneinduced pulmonary tumorigenesis in mice. *Cancer Letters, 174*, 141–150. http://dx.doi.org/10.1016/S0304-3835(01)00713-3

Lesage-Meessen, L., Navarroa, D., Mauniera, S., Sigoillota, J.C., Lorquin, J., Delattrea, M., Simonc, J.L., Asthera, M., & Labat, M. (2001). Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chemistry, 75*, 501–507. http://dx.doi.org/10.1016/S0308-8146(01)00227-8

Limasset, B., Le Doucem, C., Dore, J.C., Ojasout, T., Damon, M., Crastes de Paulet, A. (1993). Effects of flavonoids on the release of reactive oxygen species by stimulated human neutrophils. *Biochemical Pharmacology, 46,* 1257-1271. http://dx.doi.org/10.1016/0006-2952(93)90476-D

Malterud and Rydland (2000). Inhibitors of 15-lipoxygenase from orange peel. *Journal of Agricultural and Food Chemistry, 48,* 5576-5580. http://dx.doi.org/10.1021/jf000613v

NCCLS (National Committee for Clinical Laboratory Standards), 2002. Perfomance standards for antimicrobial susceptibility testing. Twelfth International Supplement, Wayne, PA; M100 (S12).

Obied, K.H., Danny, R., Bedgood, Jr., Renzler, D.P., & Robards, K. (2007). Chemical screening of biophenol extracts by hyphenated liquid chromatography. *Analytica Chimica Acta, 603,* 176-189. http://dx.doi.org/10.1016/j.aca.2007.09.044

Ramos, S. (2007). Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *The Journal of Nutritional Biochemistry, 18,* 427–442. http://dx.doi.org/10.1016/j.jnutbio.2006.11.004

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1996). Structureantioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine, 20,* 933-956. http://dx.doi.org/10.1016/0891-5849(95)02227-9

Rigane, G., Ben Salem, R., Sayadi, S., & Bouaziz, M. (2011). Phenolic composition, isolation and structure of a new deoxyloganic acid derivative from Dhokar and Gemri-Dhokar olive cultivars. *Journal of Food Science, 76,* 965-973. http://dx.doi.org/10.1111/j.1750-3841.2011.02290.x

Rigane, G., Bouaziz, M., Sayadi, S., & Ben Salem, R. (2012a). Identification and characterization of a new iridoid compound from two phase Chemlali olive pomace. *European Food Research and Technology, 234,* 1049–1054. http://dx.doi.org/10.1007/s00217-012-1722-y

Rigane, G., Bouaziz, M., Baccar, N., Abidi, S., Sayadi, S., & Ben Salem, R. (2012b). Recovery of Hydroxytyrosol Rich Extract from Two-Phase Chemlali Olive Pomace by Chemical Treatment. *Journal of Food Science, 77,* 1077-1083. http://dx.doi.org/10.1111/j.1750-3841.2012.02898.x

Rigane, G., Ben Younes, S., Ghazghazi, H. Ben Salem, R. (2013). Investigation into the biological activities and chemical composition of Calendula officinalis L. growing in Tunisia. *International Food Research Journal, 20,* 3001-3007.

Roig, A., Cayuela, M.L., Sanchez-Monedero, M.A. (2006). An overview on olive mill wastes and their valorisation methods. *Waste Management, 26,* 960–969. http://dx.doi.org/10.1016/j.wasman.2005.07.024

Ryan, D., & Robards, K. (1998). Phenolic compounds in olives. *Analyst, 123,* 31–44. http://dx.doi.org/10.1039/a708920a

Ryan, D., Antolovich, M., Prenzler, P.D., Robards, K., & Lavee, S. (2002). Biotransformations of phenolics compounds in Olea europaea L. *Horticultural Science, 92,* 147–176. http://dx.doi.org/10.1016/S0304-4238(01)00287-4

Schieber, A., Berardini, N., & Carle, R. (2003). Identification of flavonol and xanthone glucosides from mango (Mangifera indica L. Cv. "Tommy Atkins") peels by high-performance liquid chromatography-electrospray ionization mass spectrometry. *Journal of Agricultural and Food Chemistry, 51,* 5006-5011. http://dx.doi.org/10.1021/jf030218f

Vinha, A.F., Ferreres, F., Silva, B.M., Velentao, P., Gonc¸alves, A., Pereira, J.A., Oliveira, M.B., Seabra, R.M., & Andrade, P.B. (2005). Phenolic profiles of Portuguese olive fruits (Olea europaea L.): influence of cultivar and geographical origin. *Food Chemistry, 89,* 561–568. http://dx.doi.org/10.1016/j.foodchem.2004.03.012

Visioli, F., Poli, A., & Galli, C. (2002). Antioxidant and other biological activities of phenols from olives and olive oil. Medical Care Research and Review, 22, 65-75.

Winkelhausen, P., Pospiech, R., & Laufenberg, G. (2005). Antifungal activity of phenolic compounds extracted from dried olive pomace. *Bulletin of the Chemists and Technologists of Macedonia, 24*, 41-46.

Yanishlieva, N., & Marinova, E. (2001). Stabilisation of edible oils with natural antioxidants. *European Journal of Lipid Science and Technology, 103,* 752-767. http://dx.doi.org/10.1002/1438-9312(200111)103:11<752::AID-EJLT752>3.0.CO;2-0