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-\textit{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-\textit{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 anthocyanins, 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-butyric 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$_2$O) and sodium acetate (CH$_3$COONa·3H$_2$O) 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 distilled 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, Wilmington, 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 IC₅₀ (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 attained 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.

<table>
<thead>
<tr>
<th></th>
<th>EtOAc extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic content (g GAE/kg of fresh alperujo)</td>
<td>218.20±0.13</td>
</tr>
<tr>
<td>Flavonoids content (g QE/kg of fresh alperujo)</td>
<td>102.20±0.31</td>
</tr>
</tbody>
</table>

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: Hesperidin, 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 $\left[ M + CH_3CN \right]^+$ at m/z 651 and fragment ions at m/z 621 $\left[ M-CH_3O+CH_3CN \right]^+$, 543 $\left[ M-C_3H_5O_4+CH_3CN \right]^+$, 502 $\left[ M-C_9H_{10}O_2+CH_3CN \right]^+$ and 435 $\left[ M-C_{10}H_{15}O_5+CH_3CN \right]^+$. On the other hand, the $\lambda_{\text{max}}$ 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 $\left[ M+NH_3 \right]^+$ 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 $\lambda_{\text{max}}$ 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.

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

* 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 hesperidin) 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.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>DPPH (IC_{50} \mu g/mL)</th>
<th>FRAP (mM TE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperidin</td>
<td>9.70±0.12\textsuperscript{a}</td>
<td>1.15±0.00\textsuperscript{A}</td>
</tr>
<tr>
<td>Quercetin-3-O-arabino-glucoside</td>
<td>3.53±0.31\textsuperscript{b}</td>
<td>3.19±0.02\textsuperscript{B}</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.92±0.01\textsuperscript{c}</td>
<td>12.18±0.08\textsuperscript{C}</td>
</tr>
<tr>
<td>Luteolin</td>
<td>2.19±0.04\textsuperscript{d}</td>
<td>5.15±0.07\textsuperscript{D}</td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
<td>4.66±0.14\textsuperscript{e}</td>
<td>3.08±0.12\textsuperscript{E}</td>
</tr>
<tr>
<td>BHT</td>
<td>8.30±0.23\textsuperscript{f}</td>
<td>1.34±0.02\textsuperscript{F}</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MIC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>3.65-1.825</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>14.6-7.3</td>
</tr>
<tr>
<td>Erwinia sp.</td>
<td>3.65-1.825</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>3.65-1.825</td>
</tr>
<tr>
<td>Pseudomonas savastanana</td>
<td>3.65-1.825</td>
</tr>
<tr>
<td>Salmonella Enterica</td>
<td>7.3-3.65</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>7.3-3.65</td>
</tr>
</tbody>
</table>

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 microbes. 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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