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Mediterranean Journal of Chemistry **2016**, 5(6), 605-614

Chemical composition, antioxidant potential and phenolic profile of oil mill waste water from Tunisian olive varieties (Chetoui and Chemlali)

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Abstract: Oil mill waste water (OMWW) is of great interest due to the presence of valuable resources such as biophenols that can be recovered as food additives and pharmaceuticals. The aim of this study is to investigate the variation of physicochemical composition of OMWW from Chetoui and Chemlali varieties, to evaluate phenolic composition, antioxidant potential and phenolic profile of OMWW extracts under native and acidified conditions. Liquid-liquid extraction was performed for the extraction of polyphenols. Antioxidant activity was investigated by DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAP tests. Phenolic compounds content was determined by HPLC-DAD method. OMWW from Chetoui variety has been shown to contain an important amount of K, Ca and Na whereas Chemlali cultivar was rich in Mg. Phenolic extract from Chetoui fruit (COCt) has been shown to contain the highest amount of polyphenols (2.48 ± 0.21 g L^{-1}) as well as an appreciable content of flavonoids (9.39 ± 0.32 g L^{-1}). However, phenolic extract from Chemlali fruit (COCm) has been shown to have the highest content of proanthocyanidins (0.39 ± 0.00 g L^{-1}). Acidification treatment improved polyphenol recovery of extracts from both varieties. COCt was more active using DPPH (EC_{50} of 7.5 mg L^{-1}) and FRAP tests. However, COCt and COCm exhibited the same activity using ABTS test. In general, acidification treatment decreased antioxidant activity of extracts. COCt has been shown to contain higher amount of hydroxytyrosol when compared to COCm (157.16 ± 0.820 and 23.440 ± 0.440 mg g^{-1} D.W. of extract, respectively) as revealed by HPLC-DAD analysis.

Keywords: oil mill waste water; acidification; antioxidant activity; phenolic compounds; HPLC-DAD analysis.

Introduction

Olive cultivation constitutes one of the main strategic economic sectors for many regions in the world. Mediterranean countries are the main producers, including Tunisia with 6% of production, where olive potential is localized up to 30% in the north, 38% in the center and 32% in the south; it is distributed over very different climatic conditions [1]. However, olive production is focused on two main cultivars, Chetoui cultivar in the north, and Chemlali cultivar in the center and in the south of Tunisia.

Olive oil extraction, either by traditional discontinuous press process or by continuous centrifugation systems, generates large amount of liquid by-products named oil mill waste water (OMWW). Continuous three-phase centrifugation system, widely used in Tunisia, generates between 80-110% (v/weight of the olives) of OMWW. Traditional press generates roughly 50% of OMWW. However, recent two-phase system generates a solid waste but an increasing organic load concentration [2].

The disposal of this effluent causes serious environmental problems on soil microbial populations [3], aquatic ecosystems [4] and air through phenol and sulfur dioxide emissions [5]. This pollution is due to the high load of organic matter which mainly consists of polysaccharides, tannins, sugars, polyalcohols, polyphenols, pectins, proteins and lipids [6]. Polyphenols play a crucial role in the increase of the pollutant potential by their toxicity and antimicrobial activity [7]. However, natural phenolic compounds have many health benefits namely by preventing human diseases [8]. Numerous studies have demonstrated the antioxidant potential of phenolic compounds isolated from OMWW [9,10].

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DOI: http://dx.doi.org/10.13171/mjc56/01606121022/bouzouita

Over recent years, phenolic compounds such as phenolic acids, phenyl alcohols, secoiridoids and flavonoids isolated from OMWW have been identified [11–13]. However, the genetic and agronomic factors of olive cultivation as well as olive oil extraction-process technology play a crucial role in the content and phenolic profile of OMWW [14,15].

The aim of the present work was to investigate the variation of physicochemical composition of OMWW from two Tunisian varieties (Chetoui and Chemlali) ,cultivated in two different regions, to determine phenolic composition and antioxidant activity of OMWW extracts under native and acidified conditions and finally to identify and quantify individual biophenols.

Experimental Section

OMWW samples and physicochemical analyses

Fresh OMWW were collected in the ending of the season (December 2012) from a three-phase olive oil mill located in Sidi Thabet, North of Tunisia and in Sfax, Mid-South of Tunisia for Chetoui and Chemlali cultivars, respectively. Samples were directly subjected to physicochemical analyses. Minerals are quantified by using a flame spectrometer (Hitachi instruments engineering Co, Ibaraki-Ken, Japon). Exhausted samples were kept in dark at -20°C for further analyses.

Chemical reagents

HPLC standards (hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, gallic acid, protocatechuic acid, vanillic acid, luteolin, apigenin, rutin), Butylated Hydroxytoluene (BHT), ascorbic acid and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA); ethyl acetate, hexane, acetonitrile, methanol were HPLC-grade solvents purchased from Fluka Chemika (Buchs, Switzerland); 2.2'-azinobis (3-ethylenbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were purchased from Merck (Darmstadt, Germany).

Soluble sugars determination

Soluble sugars were determined by the phenol sulfuric method [16] by mixing 1 ml of the extract, obtained after a range of washing with ethanol of the dried sample, with 5% phenolic solution and 5 ml of concentrated sulfuric acid. A yellow-orange coloration was obtained and the optical density was measured after 20 min of reaction at 490 nm. Total sugars were determined from a calibration curve using glucose as a standard at concentrations ranging from 10 to 100 mg L^{-1}.

Phenolic compounds extraction

Oil mill waste water from Chetoui and Chemlali cultivars were acidified to pH 2 by HCl 1N [10], and then centrifuged at 6000 rpm for 30 min by a centrifuge (Hettich Zentrifugen, Germany) in order to remove suspended solids. The supernatant was filtered through Buchner flask to eliminate residual solids. The eluate was extracted two times by 10 mL of hexane to remove oil fraction. Extraction of polyphenols was carried out at room temperature by liquid-liquid extraction with 25 mL of ethyl acetate. The extraction was repeated successively a total of four times. The organic phase which mainly contained phenolic fraction was concentrated under vacuum. The dry residue was then dissolved in methanol 80% (v/v) and stored at -20°C. Different solutions (crude OMWW extract from Chetoui cultivar (COCt), crude OMWW extract from Chemlali cultivar (COCm), acidified OMWW extract from Chetoui cultivar (AOCt) and acidified OMWW extract from Chemlali cultivar (AOCm)) were used for quantification, identification and determination of antioxidant activity of phenolic compounds.

Total phenolic content determination

The total phenolic content (TP) was determined by the Folin-Ciocalteau reagent according to the method of [17] with some modifications. To 0.4 mL of each extract, 10 mL of diluted Folin-Ciocleteau was added. The content was thoroughly mixed and then incubated for 1 min. 8 mL of sodium carbonate (75 g L^{-1}) was added and the mixture was incubated for 60 min. The absorbance was measured at 765 nm versus a blank by an ultraviolet-visible (UV-Vis) spectrophotometer (Jenway, UK). Caffeic acid (20-200 mg L^{-1}) was used as a standard for the calibration curve. TPC was expressed as gram of caffeic acid equivalent (CAE) per liter of OMWW. All measurements were repeated in triplicate.

Total flavonoids content determination

Total flavonoids content (TFC) was carried out by a calorimetric method [18]. Rutin was used as a standard for the calibration curve. TFC was expressed as Rutin equivalent (RE). All measurements were repeated in triplicate.

Total proanthocyanidins content determination

Total proanthocyanidins content (TPC) was determined by HCl/butan-1-ol assay [19]. Results were expressed as catechin equivalent (CE). All measurements were repeated in triplicate.

DPPH• radical scavenging assay

The antiradical potential of OMWW extracts (5, 12.5, 25, 50, 100, 200 µg mL^{-1}) was estimated by radical scavenging capacity using DPPH• as a stable radical [20]. BHT and ascorbic acid were used as positive controls. The percentage inhibition of radical DPPH by phenol extracts was expressed by the following formula:

$$\%\, \boldsymbol{Inhibition} = [(\boldsymbol{A_{control}} - \boldsymbol{A_{sample}})/\boldsymbol{A_{control}}] \times \mathbf{100}$$

Where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the tested extract.

Radical scavenging capacity of phenolic extracts was expressed in terms of EC_{50}, which is the effective concentration of sample at which 50% of initial amount of DPPH$^{\bullet}$ were scavenged. The lower the EC_{50} was, the better the extract to scavenge radicals would be. Experiments were performed in triplicate.

$ABTS^{+\bullet}$ radical scavenging assay

The antioxidant activity of OMWW extracts was determined by the $ABTS^{\bullet+}$ radical cation scavenging method [21]. Radical scavenging activity was performed by mixing 100 µL of each extract at different concentrations (5, 12.5, 25, 50, 100, 200 µg mL^{-1}) with 900 µL of $ABTS^{\bullet+}$ solution. After 10 min of reaction in dark, the absorbance was read at 734 nm against methanol. Experiments were performed in triplicate.

Ferric reducing antioxidant power assay (FRAP)

The antioxidant power assay of different samples (5, 12.5, 50, 100, 150, 200, 400 µg mL^{-1}) was tested [22]. Ascorbic acid (6-115 µM) was used as a standard. The increase of the absorbance of the reaction mixture indicates an increase of the reducing power activity. Experiments were performed in triplicate.

HPLC-DAD analysis

Phenolic compounds in the different OMWW extracts were investigated by reversed phase HPLC (Agilent 1100, Series, USA) equipped with a diode array detector. The chromatographic separation was achieved on a 5 µm Shim-pack VP-ODS (250 mm × 4.6 mm) C18 column (Phenomenex, Shimadzu, Japan). The solvent system used was a gradient of solvent A (water), and solvent B (methanol). A linear gradient from 10% to 90% B for a total run time of 60 min was applied at a flow rate of 1 mL min^{-1}. Phenolic acids and flavonoids were identified at 280 nm and 340 nm respectively at a temperature of 25°C and a pressure of 138 bar. The main phenolic compounds were identified by comparison with relative retention times and UV spectra of pure compounds. The equilibration of the system between runs was carried out for 60 min.

Statistical analysis

All experiments were performed in triplicate (n=3); the results are presented as means ± standard deviations (SD). Data were subjected to the one-way analysis of variance (ANOVA) using SPSS program, release 17.0 for windows (SPSS, Chicago, IL, USA) and the significance was accepted at $p < 0.05$.

Results and Discussion

Physicochemical characterizations

General characterization

Many factors such as olive species, origin of the olives and climate conditions play a crucial role in the variation of chemical composition of OMWW [23]. The average physicochemical composition of OMWW recovered from three-phase centrifugation system from Chetoui and Chemlali cultivars is summarized in Table 1. Results revealed OMWW to be a mildly acidic liquid with pH value of 5.03 ± 0.01 and 4.75 ± 0.01 for OMWW from Chetoui and Chemlali fruits, respectively. The dry matter content was higher in OMWW from Chetoui variety (12.64 ± 0.32 %). A noticeable content of proteins (4.55 ± 0.06 and 3.74 ± 0.05 % for Chetoui and Chemlali, respectively) was observed. Sugars were also identified in a significant amount especially for OMWW from Chetoui cultivar (10.56 ± 0.05 %). OMWW is a natural source of nutrients. A Recent study [24] used OMWW as the sole carbon source to produce biosurfactants. OMWW are fluids of high conductivity and they exhibit appreciable content of minerals. The analysis showed that OMWW from Chetoui variety was richer in minerals (11.25 ± 0.24 %) than OMWW from Chemlali cultivar (7.94 ± 0.29 %). Mineral nutrients analysis showed that calcium, sodium, potassium and magnesium were the major elements in OMWW. Phosphor was also detected in a significant amount (371.06 ± 2.14 and 204.3 ± 0.84 mg/L in Chetoui and Chemlali cultivar, respectively). The abundance of these valuable resources in OMWW is of a great interest as some elements can be useful for agronomic applications, in particular potassium, which could be potentially used as fertilizer [23]. The protein fraction of OMWW may be of interest in several industrial applications regarding their nutritionaln value [25] and their potential use as food stabilizer [26] and allergens [27].

Table 1. Physicochemical composition of oil mill waste water; values are expressed as means ± standard deviation (SD), n = 3.

Components	Chetoui	Chemlali
pH	5.03 ± 0.01a	4.75 ± 0.01b
Dry matter (%)	12.64 ± 0.32a	8.58 ± 0.21b
Residual oil (g/L)	10.83 ± 0.09a	10.71 ± 0.51a
Proteins (%)	4.55 ± 0.06a	3.74 ± 0.05a
Total soluble sugars (%)	10.56 ± 0.05a	6.73 ± 0.05b
DCO (g/L)	174.09 ± 0.50a	151.85 ± 0.42b
DBO (g/L)	45.63 ± 0.58a	37.75 ± 0.46b
Conductivity (25°C) (mS/cm)	11.80 ± 0.27a	11.22 ± 0.30b
Total suspended solids (g/L)	8.43 ± 0.11a	7.20 ± 0.20b
Salinity (g/L)	7.64 ± 0.05a	6.51 ± 0.01b
Ash (%)	11.25 ± 0.24a	7.94 ± 0.29b
Ca (mg/L)	3808 ± 2.08a	1966 ± 0.26b
Na (mg/L)	3792 ± 3.10a	1220 ± 0.09b
K (mg/L)	4044.53 ± 1.15a	867.80 ± 0.94b
Mg (mg/L)	3759.28 ± 3.48a	5160.02 ± 5.20b
P (mg/L)	371.06 ± 2.14a	204.3 ± 0.84b
Cu (mg/L)	7.70 ± 0.25a	5.83 ± 0.14b
Mn (mg/L)	14.01 ± 0.64a	8.59 ± 0.35b
Zn (mg/L)	8.96 ± 0.42a	4.13 ± 0.11b
Fe (mg/L)	10.68 ± 0.51a	4.05 ± 0.07b

(%): g/100g of dry matter. Different letters in the same row indicate significantly different mean ± standard deviation of triplicates ($p < 0.05$).

Total phenolics, flavonoids and proanthocyanidins content

The chemical composition of extracts from crude and acidified OMWW from Chetoui and Chemlali varieties was cited in Table 2. Results indicated that phenols content of crude OMWW extract from Chetoui variety was roughly 4-fold higher (2.48 ± 0.21 g L^{-1} OMWW) than that from Chemlali variety (0.56 ± 0.09 g L^{-1} OMWW). Moreover, results showed that acidification of OMWW increased total phenols content of both extracts (3.69 ± 0.21 and 0.98 ± 0.11 g L^{-1} for respectively AOCt and AOCm). These results were in accordance with previous reports [10,28] who showed that acidification enhances the release of phenols. In addition, data showed that OMWW extract from Chetoui variety exhibited an important amount of flavonoids (9.39 ± 0.32 g L^{-1} OMWW) compared to OMWW extract from Chemlali variety (0.88 ± 0.01 g L^{-1} OMWW). Acidification of OMWW contributed to an increase of flavonoids content for Chemlali cultivar extract (1.89 ± 0.33 g L^{-1} OMWW). Flavonoids may be exploited in many foods, cosmetics and pharmaceutical applications. They are a group of natural substances with several biological functions such as anti-inflammatory, antioxidant, cardioprotective, antiallergic and anticarcinogenic properties [29]. Proanthocyanidins are presented in lower amount as phenols and flavonoids with 0.21 ± 0.00 g L^{-1} for COCt and 0.39 ± 0.00 g L^{-1} for COCm. TPC was significantly increased with the acidification treatment of OMWW from Chemlali variety (1.73 ± 0.04 g L^{-1} OMWW). The variation of phenolic composition of OMWW between varieties is due to many factors such as olive cultivar, climatic and agronomic conditions [23]. The impact of acidification treatment of OMWW on the increase of phenolic compounds content may be explained by the following reasons: (1) the aggregation of proteins at acidic pH, (2) the release of phenolic compounds bounded by either covalent or non-covalent bonds to polysaccharides and/or proteins and (3) the improvement of phenolic compounds solubility in the extraction solvent [30].

Table 2. Content of different biophenolic classes of extracts from crude and acidified OMWW from Chetoui and Chemlali varieties; values are expressed as means ± standard deviation (SD), n = 3. COCt, crude OMWW extract from Chetoui cultivar; AOCt, acidified OMWW extract from Chetoui cultivar; COCm, crude OMWW extract from Chemlali cultivar; AOCm, acidified OMWW extract from Chemlali cultivar.

Phenolic compounds (g L^{-1} OMWW)	COCt	AOCt	COCm	AOCm
Polyphenols content[a]	2.48 ± 0.21c	3.69 ± 0.21d	0.56 ± 0.09a	0.98 ± 0.11b
Flavonoids content[b]	9.39 ± 0.32c	9.25 ± 0.00c	0.88 ± 0.01a	1.89 ± 0.33b
Proanthocyanidins content[c]	0.21 ± 0.00a	0.27 ± 0.01b	0.39 ± 0.00c	1.73 ± 0.04d

Expressed as: [a]CAE, [b]RE and [c]CE. Different letters in the same raw indicate significantly different mean ± standard deviation of triplicates ($p < 0.05$).

Antioxidant activity

The antioxidant activity of natural polyphenols is a useful tool to assess their beneficial health effects. OMWW extracts were individually assessed for their antioxidant activity using three different spectrophotometric tests.

DPPH• radical scavenging assay

Radical scavenging activity of different OMWW extracts was determined by scavenging activity (EC_{50}) using DPPH• test and compared to positive controls, BHT and ascorbic acid. The ability of OMWW extracts to scavenge the stable free radical DPPH is shown in Fig. 1a. Results showed that COCt was more active (EC_{50} of 7.5 mg L^{-1}) than COCm (EC_{50} of 23 mg L^{-1}). In addition, scavenging activity of COCt was significantly similar to that of the synthetic antioxidant, the BHT (EC_{50} of 7.1 mg L^{-1}). However, Ascorbic acid, used as another positive control, was more potent antioxidant with an EC_{50} of 4.72 mg L^{-1} than OMWW extracts. Phenolic extract from Chetoui variety (COCt) exhibited higher content of phenols and flavonoids than extract from Chemlali variety (COCm) (Table 2). Therefore, we can conclude that phenolic fraction could influence the antioxidant potential of each extract. Several studies have shown that phenols present in OMWW exert potent biological activities, including, antioxidant capacity, free radical scavenging activity and therefore prevention against oxidative stress, which is associated with the pathogenesis of several diseases. Indeed, these bioactive compounds are known to prevent or reduce, either directly or indirectly, cancer [8], diabetes mellitus, coronary heart disease and inflammatory diseases such as osteoarthritis [9,31].

In this study, we test the effect of a prior acid treatment of OMWW on the antioxidant activity of the resulting extract. Several previous studies have applied the acid treatment to improve the extraction yield of polyphenols [10,11]. It is therefore appropriate to examine the effect of such treatment on the antioxidant properties of these natural substances. Results of Fig. 1a showed that the acidification of OMWW did not affect statistically the antioxidant activity of Chetoui extract (EC_{50} of 8.9 mg L^{-1}); however, this treatment decreased significantly ($p < 0.05$) the antioxidant activity of Chemlali extract with an EC_{50} of 34 mg L^{-1}. The decrease of radical scavenging activity of Chemlali extract may be explained by structural change of some bioactive compounds, such as oleuropein, due to degradation reactions under acidified conditions. Indeed, antioxidant activity can be assigned especially on the nature of mono- or polyphenols i.e, to the number and the position of hydroxyl groups [12].

ABTS•+ radical scavenging assay

Phenolic extracts were tested for their antioxidant activity by testing their ability to trap another stable free radical ABTS. Literature data on the analysis of the anti-radical activity by DPPH• and ABTS•+ tests have shown that these tests are not always well correlated and they often don't give the same results [32]. Indeed, these two tests treat two different mechanisms of action using two different radicals. It is for this reason that the two tests were considered.

Results of Fig. 1b showed that COCt and COCm exhibited statistically similar scavenging activity toward the stable free radical ABTS with an EC_{50} of 55 and 60 mg L^{-1}, respectively. This classification is not similar to DPPH scavenging activity. Moreover, OMWW extracts have been shown to be more effective scavenger of DPPH radical (Fig. 1a). These results could be explained by the nature of phenolic compounds present in the *Olea europaea* fruits, which can react with DPPH• better than ABTS•+.

The impact of the prior acidification of OMWW on the ability of the extracts to scavenge ABTS radical cation is shown in Fig.1b. In this respect, antiradical activity of OMWW extracts decreased significantly ($p < 0.05$) with an EC_{50} of 88.5 and 157.5 mg L^{-1} for respectively Chetoui and Chemlali cultivars. However, the anti-radical activity of ascorbic acid, used as positive control, was relatively more efficient than that of the phenolic extracts obtained from crude and acidified OMWW with an EC_{50} of 24 mg L^{-1}. Previous work [9] have demonstrated that pure phenolic compounds and phenolic complexes affect the antioxidant level. In our work, although polyphenols content was increased by acidification of OMWW to pH 2, scavenging activity was decreased by ABTS•+ test.

We may conclude that, besides polyphenols, the antioxidant potential is a function of *in vitro* conditions. This finding may be of great interest in view of acidic pH of *in vivo* conditions.

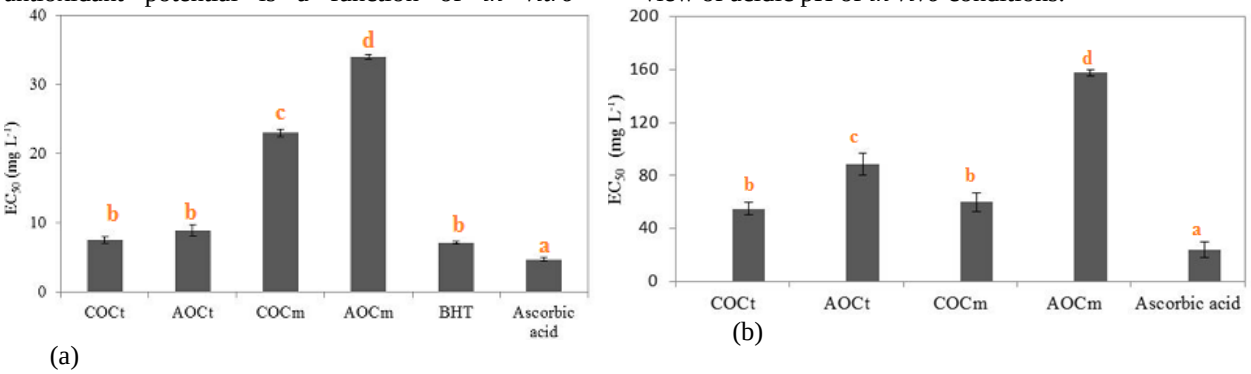

Figure 1. Radical scavenging (EC_{50}) of different OMWW extracts compared to BHT and ascorbic acid as positive controls for respectively $DPPH^{\bullet}$ test (a) and $ABTS^{+\bullet}$ test (b). Different letters indicate significantly different results ($p < 0.05$).

FRAP assay

The different extracts from crude and acidified OMWW were tested for their abilities to reduce Fe (III) to Fe (II). Previous research has shown the existence of a direct correlation between the antioxidant activity and reducing power of plant extracts [33]. Reducing power is another important parameter for the evaluation of antioxidant activity. The reducing power of phenolic extracts was measured at 50 mg L^{-1} and compared to a reference standard (ascorbic acid) (Fig. 2). Results were expressed as mM ascorbic acid equivalent/g dry weight of extract (mM AAE/g D.W.). As shown, COCt (1.33 ± 0.11 mM AAE/g D.W.) was roughly three times higher than COCm (0.38 ± 0.01 mM AAE/g D.W.). Therefore, the extract of Chetoui variety exhibits more effective antioxidants than the extract of Chemlali variety. The pronounced greenish blue color formed during the addition of COCt to the iron solution suggests the power of ions chelation phenomenon [34]. As shown in Fig. 2, acidification treatment of OMWW from Chetoui variety decreased significantly ($p < 0.05$) the reducing power of the phenolic extract (AOCt) (0.93 ± 0.04 mM AAE / g D.W.). However, acid treatment does not affect the power of Chemlali extract (AOCm) at 50 mg L^{-1}. Therefore, acidification of OMWW may influence differently the quantitative-qualitative profile of phenolic compounds in OMWW.

Data are represented graphically (Fig. 3) at different concentrations of OMWW extracts (5-400 mg L^{-1}). Reducing power activity increased with increasing sample concentration. This result is in agreement with previous works [34]. In this study, antioxidants (reducers) were able to reduce the yellow-coloured Fe^{3+}/ferricyanide complex to the green ferrous form. Besides, results revealed that COCt exhibited the highest reducing power activity compared to COCm (Fig. 3). The same order of reducing power activity for the extracts was maintained as for DPPH radical scavenging activity. The high antioxidant activity of COCt may be attributed to the high levels of phenolic compounds, mainly the substantial amount of flavonoids measured in OMWW extract from Chetoui variety (Table 2).

The impact of acidification of OMWW on reducing power activity was tested (Fig. 3). This treatment significantly reduced the power of the Chetoui extract (AOCt) regardless of the concentration. While extracts from Chemlali variety (COCm and AOCm) had the same reducing power until the concentration of 200 mg L^{-1}. However, this power decreased at 400 mg L^{-1}. In our study, although the polyphenolic composition of OMWW extracts was substantially improved by acidification with HCl 1N (Table 2), antioxidant activity was significantly decreased (Fig.1, Fig. 3). This finding was not in accordance with previous research [35] who demonstrated that acidification of the extracting distilled water by 5% formic acid has led to a significant increase in the total phenolic content, total flavonoids content and antioxidant activity of blueberry extract as measured by FRAP and ORAC assays. However, our findings are in accordance with a previous work who revealed that antioxidant activity of the extracted phenols from OMWW decreased at pH 2 [28]. The decrease of antioxidant level of OMWW extracts under acidic conditions may be due to the extraction condition, in deed, antioxidant activity depends on the type of the extracting process as well as on the polarity of the organic solvent. Therefore, various classes of simple and complex phenolic compounds may be made up [28]. The antagonist effect between these compounds may result in a lower antioxidant activity. Hence, the variation of natural antioxidants glycosides, the abundance of the isomers and their combined action can play an important role in their antioxidant activity.

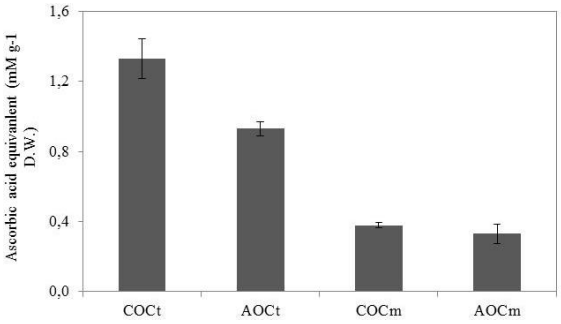

Figure 2. Reducing power activity of the different OMWW extracts at 50 mg/L expressed as mM AAE/g D.W. Different letters indicate significantly different results ($p < 0.05$).

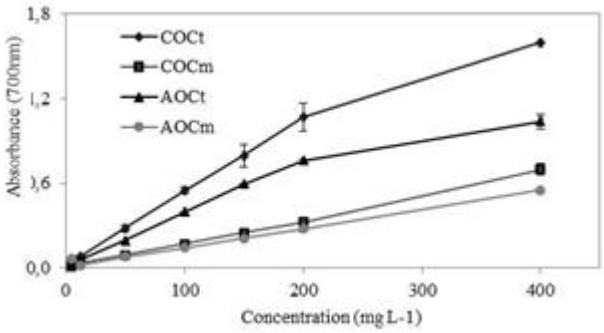

Figure 3. Reducing power of different OMWW extracts.

In this study, COCt extract showed the best antioxidant level in all the tests used. The other OMWW extracts showed different antioxidant power depending on the used assay. These results are due to the variation of redox potentials, solvent dependencies and mechanisms of the antioxidant assays [36].

The correlation between phenolic composition of the extracts and their antioxidant activity, using the three methods considered in this study, was tested (Table 3). The relationship between phenolic content compounds and antioxidant activities revealed no correlation between total phenols or total flavonoids and radical scavenging activity using DPPH test for OMWW extracts from Chetoui variety. However, a positive correlation between the different classes of polyphenols and antioxidant activity using DPPH test was observed for OMWW extracts from Chemlali cultivar similarly to previous research [9]. Various interactions of different natures, such as synergy, antagonism or additive effect may occur between polyphenols when they are present in mixture [43]. These interactions could explain the change of antioxidant activity between varieties. The correlation between antioxidant activity using ABTS test and different phenolic classes was relatively more important compared to the other two tests. A negative correlation was observed between total phenols or total proanthocyanidins and antioxidant level of OMWW extract from Chetoui variety using FRAP test. However, results showed no correlation between flavonoids content and antioxidant activity. Table 3 showed no correlation between the content of different phenolic groups and antioxidant level of OMWW extract from Chemlali cultivar using FRAP test. This result correlates with a recent report which has demonstrated a poor correlation between polyphenols content and antioxidant capacity of adzuki bean sprouts [37].

Table 3. Pearson Correlation coefficient (r^2) between the total content of phenolic groups in OMWW extracts and the antioxidant activities using DPPH (EC_{50}), ABTS (EC_{50}) and FRAP (mM AAE/g D.W.) tests.

	DPPH		ABTS		FRAP	
Correlation coefficient (r^2)	Chetoui	Chemlali	Chetoui	Chemlali	Chetoui	Chemlali
Total phenols	0.658	0.895*	0.840*	0.930**	-0.881*	-0.555
Total flavonoids	0.394	0.921*	-0.584*	0.960**	-0.173	-0.609
Total Proanthocyanidins	0.820*	0.972**	0.907*	0.997**	-0.961**	-0.767

* $p < 0.05$.
** $p < 0.01$.

HPLC-DAD analysis

Phenolic compounds of different extracts from crude and acidified OMWW from Chetoui and Chemlali cultivars were identified by HPLC-DAD. Concentration of biophenols in OMWW extracts was calculated based on 92 mg ml^{-1} of methanolic extracts using peak area of the external standards (Table 4). Gallic acid, hydroxytyrosol, tyrosol, catechol, chlorogenic acid, caffeic acid, p-coumaric acid, hydroxycinnamic acid, oleuropein and ellagic acid are among identified phenolic compounds in crude OMWW from Chetoui (COCt) and Chemlali cultivar (COCm) (Fig. 4). COCt exhibited higher total phenol content than COCm as determined by Folin-Ciocalteau reagent (Table 2) and by HPLC analysis (Table 4). Individual biophenols contents were quantitatively different in COCt and in COCm extracts. Hydroxytyrosol was the main phenolic compound identified with 83% and 75 % abundance in COCt and COCm, respectively. Based on the dry weight of the extracts (D.W.), hydroxyl-tyrosol content was seven times higher in COCt (157.11 ± 0.820 mg g^{-1} D.W.) than in COCm (23.440 ± 0.440 mg g^{-1} D.W.). Hydroxytyrosol

(3,4-dihydroxyphenylethanol) is an orthodiphenol released naturally during olive oil extraction. It has been demonstrated that hydroxytyrosol is a powerful antioxidant compound with potential human health benefit [38]. Previous works [9] reveal a good correlation ($r^2 = 0.778$) between hydroxytyrosol and antioxidant activity. The high content of hydroxytyrosol in COCt could explain the high antioxidant activity of Chetoui extract as measured by DPPH and FRAP tests. Tyrosol was detected in COCt in a significant amount (24.076 ± 0.127 mg g^{-1} D.W.) compared to COCm (4.060 ± 0.090 mg g^{-1} D.W.). The variation of polyphenols level and antioxidant activity of Chetoui and Chemlali extracts depends on the olive variety [23]. It has been shown that the tyrosol is not considered as a powerful antioxidant [9]. Tyrosol and hydroxytyrosol are structurally identical except that hydroxytyrosol has an extra hydroxyl group in the meta position [39]. Oleuropein is among the phenolic compounds identified in significant amounts in OMWW extracts. As shown in table 4, oleuropein content ranges from 3,932 ± 0,187 to 3,238 ± 0,668 mg g^{-1} D.W for COCm and COCt, respectively. Oleuropein is the main biophenol commonly found in fruits of *Olea europaea* [40] and it is a pharmacologically active molecule due to its potential application as an antimicrobial agent [41]. Flavonoids such as luteolin 7-O-glucoside (3.586 ± 0.305 mg g^{-1} D.W.) and luteolin (0.130 ± 0.010 mg g^{-1} D.W.) were also present at a significant amount in COCt extract. However, these compounds were not detected in the Chemlali extract (COCm). Apigenin has been identified in both crude extracts with 0.171 ± 0.010 and 0.320 ± 0.005 mg g^{-1} D.W. for COCt and COCm, respectively.

We have demonstrated that acidification treatment decreases antioxidant activity as shown by ABTS and FRAP tests (Fig. 1a, Fig. 3). Phenolic profile of OMWW extracts could explain these results. Acidification of OMWW showed to release some phenolic compounds such as Luteolin 7-O-glucoside and luteolin in AOCm and p-coumaric acid, hydroxycinnamic acid and ellagic acid in AOCt (Table 4). However, the amount of hydroxytyrosol (135.11 ± 0.130 and 20.28 ± 0.720 mg g^{-1} D.W. for AOCt and AOCm, respectively) decreases significantly after the acidification treatment of OMWW.

Table 4. Main phenolic compounds identified and quantified by HPLC-DAD in the extracts from crude and acidified OMWW from each variety. Different letters in the same raw indicate significantly different mean ± standard deviation of triplicates ($p < 0.05$). n.d. the compound was not detected.

			Quantification (mg/g D.W. of extract)			
Peak number	Retention time (min)	Identification	COCt	AOCt	COCm	AOCm
1	8.762	Gallic acid	0.031 ± 0.001b	0.033 ± 0.005b	0.030 ± 0.002b	0.013 ± 0.005a
2	10.703	Hydroxytyrosol	157.16 ± 0.820d	135.11 ± 0.130c	23.440 ± 0.440b	20.28 ± 0.720a
3	12.908	Catechol	0.366 ± 0.036a	0.391 ± 0.009a	0.523 ± 0.025b	0.352 ± 0.018a
4	13.942	Tyrosol	24.076 ± 0.127d	18.548 ± 0.443c	4.060 ± 0.090b	2.376 ± 0.008a
5	16.258	Chlorogenic acid	0.592 ± 0.020c	0.305 ± 0.007b	0.099 ± 0.014a	0.076 ± 0.004a
6	18.931	Caffeic acid	0.078 ± 0.005c	0.029 ± 0.006a	0.504 ± 0.008d	0.061± 0.012b
7	24.181	p-coumaric acid	0.142 ± 0.004b	0.178 ± 0.024c	0.053 ± 0.005a	0.042 ± 0.006a
8	27.450	Hydroxycinnamic acid	0.018 ± 0.002a	0.043 ± 0.019b	0.020 ± 0.004a	0.012 ± 0.004a
9	29.506	Luteolin 7-O-glucoside	3.586 ± 0.305c	0.772 ± 0.078b	n.d.	0.099 ± 0.021a
10	32.960	Oleuropein	3.238 ± 0.668b	3.844 ± 0.095bc	3.932 ± 0.187c	1.172 ± 0.026a
11	33.996	Ellagic acid	0.311 ± 0.020b	0.714 ± 0.024c	0.150 ± 0.020a	0.144 ± 0.013a
12	42.832	Luteolin	0.212 ± 0.015c	0.130 ± 0.010b	n.d.	0.034 ± 0.015a
13	46.914	Apigenin	0.171 ± 0.010b	n.d.	0.320 ± 0.005c	0.074 ± 0.006a

This result is in discordance with researchers of previous work [10] who showed that acidification of OMWW enhances the release of hydroxytyrosol. Numerous studies have demonstrated the decrease of the extraction yield of phenolic compounds under different pH conditions which correlate with our findings. A research study [42] has demonstrated the decreased recovery of quercetin aglycone from the shallot digestate under in-vitro digestion (pH 2 by HCl 1N) condition. Recently, chemical structure and antioxidant activity of verbascoside, which is a phenolic antioxidant compound of OMWW, were demontrated to be affected by simulated digestive conditions [43]. Despite hydroxytyrosol, acidification treatment decreased other phenolic compounds such as tyrosol and caffeic acid (Table 4). Moreover, some phenolic compounds such as caffeic and gallic acid was shown to be unstable under acid condition [44]. The decrease of hydroxytyrosol and other biophenolic compounds content in acidified OMWW may be attributed to the auto-oxidation occurring under acid conditions, to the antagonist effect by the simultaneous presence of some compounds or to the instability of these natural compounds which explain the decrease of antioxidant activity of the extracts from acidified OMWW.

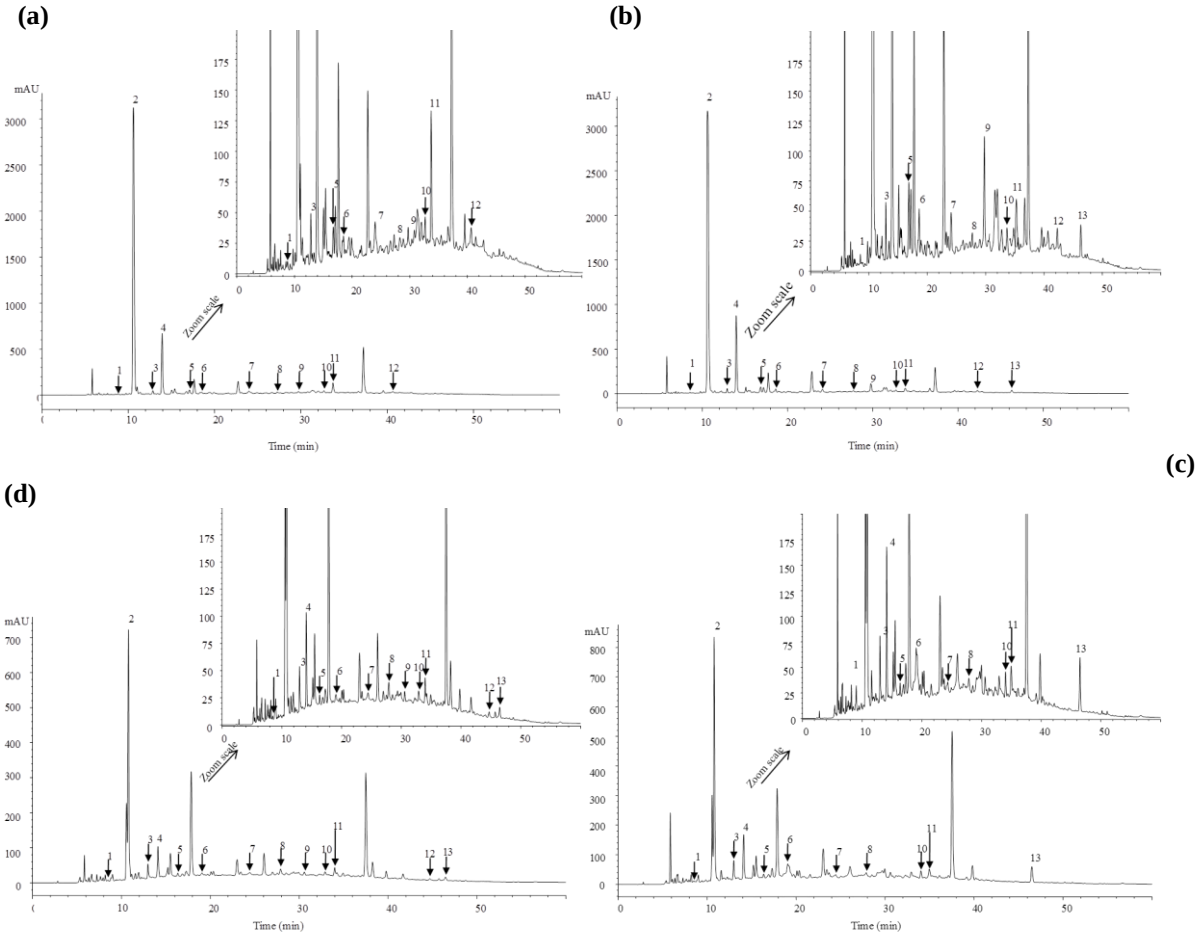

Figure 4. HPLC-DAD chromatographic profile of OMWW extracts at 280 nm. (a) COCt; (b) AOCt; (c) COCm; (d) AOCm. Main identified phenolic compounds: 1 gallic acid, 2 hydroxytyrosol, 3 catechol, 4 tyrosol, 5 chlorogenic acid, 6 caffeic acid, 7 p-coumaric acid, 8 hydroxycinnamic acid, 9 luteolin 7-O-glucoside, 10 oleuropein, 11 ellagic acid, 12 luteolin, 13 apigenin.

Conclusion

OMWW from two Tunisian varieties were investigated for the variation of their physicochemical composition, phenolic profile and antioxidant activity under native and acidified conditions. Acidification treatment to pH 2 has been shown to improve total phenol content, total flavonoids and total proanthocyanidins in OMWW extracts. OMWW extracts showed variable antioxidant levels depending on the used test where COCt was the most powerful. Acidification treatment decreased antioxidant activity of OMWW extracts in most cases. This finding may be attributed (1) to the decrease of individual phenolic compounds content in OMWW extracts, especially hydroxytyrosol; (2) to the instability of these compounds under acidic conditions; (3) to the antagonist effect of the various phenolic compounds made up with the decreasing of pH. Investigation of the impact of acidification on the phenolic composition and on the antioxidant activity is important, especially when considering the digestion condition using these natural biophenols in food and pharmaceutical industries. Encapsulation of valuable compounds may be a solution for preventing the

decrease of antioxidant activity under acidified conditions.

Acknowledgments

This work was supported by grants from The Institution for Research and Higher Education in Agriculture (Ministry of Agriculture, Tunisia). The authors would like to express their gratitude to Dr. F. Kachouri from the Higher School of Food Industries, Tunisia, for valuable assistance and helpful discussion.

References

1- H. Hannachi, M. Msallem, S. Ben Elhadj, M. El Gazzah. Comptes Rendus - Biol. 2007,130, 135-142.
2- A. Roig, M.L. Cayuela, M.A. Sánchez-Monedero. Waste Manag. 2006, 26(9), 960-969.
3- M.J. Paredes, E. Moreno, A. Ramos-Cormenzana, J. Martinez. Chemosphere, 1987, 16(7), 1557-1564.
4- M. DellaGreca, P. Monaco, G. Pinto, A. Pollio, L. Previtera, F. Temussi. Bull. Environ. Contam. Toxicol. 2001, 67(3), 352-359.
5- G. Rana, M. Rinaldi, M. Introna. Agric. Ecosyst. Environ. 2003, 96(1-3), 49-58.
6- D. Mantzavinos, N. Kalogerakis. Environ. Int. 2005, 31(2), 289-295.
7- S. Khoufi, F. Aloui, S. Sayadi. J. Hazard. Mater. 2008, 151, 531-539.
8- R.W. Owen, A. Giacosa, W.E. Hull, R. Haubner, B. Spiegelhalder, H. Bartsch. Eur. J. Cancer. 2000, 36(10), 1235-1247.
9- H. Azaizeh, F. Halahlih, N. Najami, D. Brunner, M. Faulstich, A. Tafesh. Food Chem. 2012, 134(4), 2226-2234.
10- E. De Marco, M. Savarese, A. Paduano, R. Sacchi. Food Chem. 2007, (104), 858-867.
11- N. Allouche, I. Fki, S. Sayadi. J. Agric. Food. Chem. 2004, 52(2), 267-273.
12- J. He, M. Alister-Briggs, T. Lyster De, G.P. Jones. Food Chem. 2012, 131, 1312-1321.
13- L. Lesage-Meessen, D. Navarro, S. Maunier, et al. Simple phenolic content in olive oil residues as a function of extraction systems. Food Chem. 2001, 75(4), 501-507.
14- M. Servili, S. Esposto, R. Fabiani, et al. Inflammopharmacology, 2009, 17(2), 76-84.
15- M. Servili, R. Selvaggini, S. Esposto, A. Taticchi, G. Montedoro, G. Morozzi. J. Chromatogr. A. 2004, 1054(1-2), 113-127.
16- M. Dubois, K.A. Gilles., J.K. Hamilton, P.A. Rebers, F. Smith. Anal. Chem. 1956, 28(3), 350-356.
17- E.D. Tzika, T.G. Sotiroudis, V. Papadimitriou, A. Xenakis. Eur. Food Res. Technol. 2009, 228(3), 487-495.
18- V. Dewanto, W. Xianzhong, K.K. Adom, R.H. Liu. J. Agric. Food Chem. 2002, 50(10), 3010-3014.
19- L.J. Porter, L.N. Hrstich, B.G. Chan. Phytochemistry, 1985, 25(1), 223-230.
20- W. Brand-Williams, M.E. Cuvelier, C. Berset. LWT - Food Sci. Technol. 1995, 28(1), 25-30.
21- R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans. In: Free Radical Biology and Medicine, 1999, 26, 1231-1237.
22- G.C. Yen, P. Der Duh, H.L. Tsai. Food Chem. 2002, 79(3), 307-313.
23- S. Dermeche, M. Nadour, C. Larroche, F. Moulti-Mati, P. Michaud. Process Biochem. 2013, 48(10), 1532-1552.
24- I. Moya-Ramírez, K. Tsaousi, M. Rudden, et al. Bioresour. Technol. 2015, 198, 231-236.
25- G. Rodríguez, A. Lama, R. Rodríguez, A. Jiménez, R. Guillén, J. Fernández-Bolaños. Bioresour. Technol. 2008, 99(13), 5261-5269.
26- A. Koidis, D. Boskou. Eur. J. Lipid Sci. Technol. 2006, 108, 323-328.
27- C. Esteve, C. Montealegre, M.L. Marina, M.C. García. Talanta, 2012, 92, 1-14.
28- T.I. Lafka, A.E. Lazou, V.J. Sinanoglou, E.S. Lazos. Food Chem. 2011, 125(1), 92-98.
29- A. Djebbar, C. Nassima, A. Dina, B. Meriem, D. Nadjet, B. Hania. Curr. Nutr. Food Sci. 2009, 5(4), 225-237.
30- H.K. Obied, M.S. Allen, D.R. Bedgood, P.D. Prenzler, K. Robards, R..Stockmann. J. Agric. Food Chem. 2005, 53(4), 823-837.
31- C.M. Bitler, K. Matt, M. Irving, et al. Nutr. Res. 2007, 27(8), 470-477.
32- F. Rubio-Senent, G. Rodríguez-Gutiérrez, A. Lama-Muñoz, J. Fernández-Bolaños. LWT - Food Sci Technol. 2013, 54(1), 114-124.
33- P-D. Duh. J. Am. Oil Chem. Soc. 1998, 75(4), 455-461.
34- H.K. Obied, P.D. Prenzler, K. Robards. Food Chem. 2008, 171-178.
35- J. Vuthijumnok, A. Molan, J.A. Heyes. J. Pharm. Biol. Sci. 2013, 8(1), 42-48.
36- R. Apak, S. Gorinstein, V. Böhm, K.M. Schaich, M. Özyürek, K. Güçlü. Pure Appl. Chem. 2013, 85(5), 957-998.
37- U. Złotek, U. Szymanowska, B. Baraniak, M. Karaś. Acta Sci. Pol. Technol. Aliment. 2015, 14(1), 55-63.
38- K. Hamden, N. Allouche, M. Damak, A. Elfeki. Chem. Biol. Interact. 2009, (180), 421-432.
39- K.L. Tuck, P.J. Hayball. J. Nutr. Biochem. 2002, 13(11), 636-644.
40- E.D. Tzika, V. Papadimitriou, T.G. Sotiroudis, A. Xenakis. Food Biophys. 2008, 3(1), 48-53.
41- M. Bouaziz, S. Sayadi. Eur. J. Lipid Sci. Technol. 2005, 107, 497-504.
42- J. Boyer, D. Brown, R.H. Liu. Nutr. J. 2005, 4, 1.
43- M. D'Imperio, A. Cardinali, I. D'Antuono, et al. Food Res. Int. 2014, 66, 373-378.
44- M. Friedman, H.S. Jürgens. J. Agric. Food Chem. 2000, 48(6), 2101-2110.