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Phenolic composition and antimicrobial activity of Algerian olive products and by-products

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1. Introduction

Olea europaea L. represents one of the most important crops in Mediterranean countries. According to the International Olive Oil Council, Algeria is the ninth largest producer country of olive oil in the world, with around 87.5 tons in the 2015/16 season (IOC, 2017). For olive oil **production**, fruits are harvested from the tree from November to February, crushed in a grinder and malaxed to increase the released oil yield. The oil **extraction** from the olive paste can be conducted according to different processes: (i) traditional discontinuous press, (ii) 3-phase centrifugal, and (iii) 2-phase centrifugal extraction systems (Klen & Vodopivec, 2012). Three different products are generated with the traditional discontinuous press and the 3-phase system, (olive oil, pomace, and olive mill wastewater (OMWW)), and only two (olive oil and pomace) with the 2-phase system.

Virgin olive oil, unlike other vegetable oils, is consumed directly without refining, preserving its characteristic aroma and organoleptic properties. Olive oil is appreciated worldwide for its nutritional value, flavor and for its healthy properties. The increasing popularity of olive oil is due to its high **concentration** in monounsaturated **fatty acid (oleic acid)**, but the presence of minor compounds such as phenolic compounds also contributes to the nutritional valorization of this fat; in particular, these substances possess antioxidant, antimicrobial and antitumoral activities, among others (**Brenes, Medina, Romero & de Castro, 2007; D'Angelo et al., 2005; Visioli et al., 2005**). The phenolic profile and the quality of olive oils depends on several factors such as cultivar (Laincer et al., 2016), maturation of the fruits, time of harvesting, and the oil extraction process used (**Bengana et al., 2013; Laribi et al., 2009**).

Even though less eco-friendly, traditional press process and 3-phase centrifugal processes are still in use in many olive mills in Algeria that generate large amounts of OMWW. The seasonal production of OMWW receives insufficient treatment in the plant with a final destination in large ponds for evaporation or dumping in the soil or rivers, with the concomitant environmental and management problems due to their high polluting load and toxicity (Chatzistathis & Koutsos, 2017; McNamara, Anastasiou, O’Flaherty & Mitchell, 2008). The chemical characteristics of these OMWW can vary from different origins, but they present a high concentration of organic substances such as sugars, proteins, carotenoids, tocopherols (Dermeche, Nadour, Larroche, Moulti-Mati & Michaud, 2013) and phenolic compounds (El-Abbassi, Kiai & Hafidi, 2012), and could be considered as a potential source of valuable natural biocompounds with a feasible recovery (Rubio-Senent, Martos, Lama-Muñoz, Fernández-Bolaños, Rodríguez-Gutiérrez & Fernández-Bolaños, 2015a). Precisely, olive products and by-products possess an antimicrobial activity against phytopathogenic bacteria and fungi which have been associated with their phenolic compounds concentration (Brenes et al., 2011). The presence of polyphenols in this waste product open up the possibility of using it in agriculture as fertilizer or for restoration of degraded soils making this by-product more valuable (Chatzistathis & Koutsos, 2017; Medina et al., 2011).

The objective of the present work was i) to study the polyphenols composition present in virgin olive oil, pomace and OMWW from Algerian olive oil mills, considering extraction processes (traditional discontinuous press and 3-phase centrifugal system) and olive cultivars (Sigoise, Blanquette and Rougette); ii) to determine *in vitro* the antimicrobial activity of Algerian virgin olive oil and OMWW and their bioactive phenolic compounds against bacteria, in order to highlight their bactericidal potential.

2. Material and methods

2.1. Olive samples and extraction process

A total of 54 samples of olive oil (18), pomace (18) and olive mills waste waters (OMWW) (18) were collected in December from various mills located in Roknia, Bouati Mahmoud, Guelma, Bouchegouf and Medjez Sfa, all of them in the region of eastern Algeria (36° 28' 00" N, 7° 27' 00" E) during the season 2016/2017. Samples were from three different olive cultivars (cv. Rouquette (R), cv. Blanquette (B) and cv. Sigoise(S)) and were obtained by two different extraction methods, traditional by pressure (P) and 3-phase continuous by centrifugation (C). Three lots of each combination were studied. Samples of olive oil were filtrated through cellulose paper and kept at ambient temperature until analyses. Pomace samples were extracted immediately with dimethyl sulfoxide (DMSO), and OMWW were acidified with phosphoric acid at pH 2 in order to avoid the oxidation of the phenolic compounds. Both were kept frozen at -30 °C for further analysis after two months of storage.

2.2. Phenolic compounds analysis by HPLC

The phenolic composition of olive oils was studied according to Medina, de Castro, Romero & Brenes (2006). Briefly, an extraction of 0.6 mL of olive oil was obtained using 3 x 0.6 mL of *N, N*- dimethylformamide (DMF). The extract was washed with hexane, and N₂ was bubbled into the DMF extract to eliminate the residual hexane. Then, the extract was filtered (0.2 µm pore size) and 20 µL were injected into the chromatograph. The extraction of phenolic compounds from pomace was based on methodology reported elsewhere (Romero, Medina, Mateo & Brenes, 2017). Around 10

g of pomace was mixed in an Ultra-Turrax homogenizer (Ika, Breisgau, Germany) with 30 mL DMSO. After 30 min of resting contact, the mixture was centrifuged at $6000 \times g$ for 5 min (22 °C). Then, an aliquot of 250 μ L of DMSO extract was mixed with 250 μ L of internal standard (0.2 mM syringic acid in DMSO) and 500 μ L of DMSO. For the case of OMWW, 250 μ L of samples were centrifuged and mixed with 250 μ L of internal standard (2 mM syringic acid in distilled water) and 500 μ L of distilled water. Finally, all samples were filtered through a 0.22 μ m pore size nylon filter and an aliquot (20 μ L) was injected into the chromatograph. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module and a Waters 996 photodiode array detector (PDA). For olive oils samples was also used a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan). They were connected in series and operated with Empower 2.0 software (Waters Inc.). A 25 cm $\times$ 4.6 mm i.d., 5 μ m Spherisorb ODS-2 (Waters) column at a flow rate of 1 mL min⁻¹ and a temperature of 35 °C was used in all experiments. Separation was achieved by gradient elution using (A) water (pH 2.5 adjusted with 0.15% phosphoric acid) and (B) methanol described by Ramírez, Medina, Brenes & Romero (2014). Phenolic compounds were monitored at 240, 280 and 320 nm with the PDA detector and the fluorescence detector only for olive oil samples. The evaluation of each compound was performed using a regression curve with the corresponding standard. Hydroxytyrosol (Hy), luteolin, luteolin 7-glucoside, rutin and apigenin were purchased from Extrasynthese SA (Genay, France). Hydroxytyrosol glycol, tyrosol (Ty), 4-ethylphenol, caffeic, *p*-coumaric and vanillic acids were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hydroxytyrosol 1-O-glucoside, salidroside, ester of caffeic acid and comsologoside were quantified using the response factors of Hy, Ty, caffeic acid, and *p*-coumaric acid, respectively. Hydroxytyrosol 4-O-glucoside, pinoreosinol, 1-acetoxypinoreosinol, the elenolic acid

(EA), the oleuropein aglycon (HyEA) and ligustroside (TyEA), and the dialdehydic form of decarboxymethyl elenolic acid free (EDA) or linked to hydroxytyrosol (HyEDA) or tyrosol (TyEDA) were obtained by semi-preparative HPLC (Medina, Brenes, Romero, García & de Castro, 2007; Medina, Romero, Brenes, de Castro & García, 2008). The analyses were performed in duplicate.

The identification of the isolated phenolic compounds from the extract was made using a HPLC-MS system described by Susamci, Romero, Tuncay & Brenes (2017). The chromatographic system consisted of a Waters 2695 Alliance with a pump, column heater and autosampler modules, and the detection was carried out with a Waters 2998 photodiode array detector and a mass single-quadrupole detector (QDa, Waters, USA). The QDa mass detector was operated in the negative mode (ESI-), the capillary voltage was set at 0.8 kV, the cone voltage to 15 V, and nitrogen was used as nebulizer gas with de-solvation temperature set at 600°C. The flow rate was 1 mL/min, and the column, solvent and gradient conditions were the same as mentioned above.

Total phenolic compounds in olive oils is the sum of Hy, Hy glycol, Tyr, 4-ethylphenol, pinorelinol, 1-acetoxypinorelinol, apigenin, vanillin, luteolin, *p*-coumaric acid, EDA, HyEDA, TyEDA, EA, HyEA and TyEA. Total phenolic compounds in pomace is the sum of Hy, Hy 1-O-glucoside, Hy 4-O-glucoside, Ty, salidroside, HyEDA, rutin, luteolin, luteolin 7-glucoside, *p*-coumaric acid, vanillic acid, caffeic acid, ester of caffeic acid, and comselogoside. Total phenolic compounds in OMWW is the sum of Hy, Hy glycol, Hy 1-glucoside, Hy 4-glucoside Ty, salidroside, EDA-like, HyEDA, EA, caffeic acid, ester of caffeic acid and comselogoside.

2.3. Isolation of phenolic compounds

OMWW SP (generated from Sigoise cultivar by a traditional extraction by pressure) was selected because it contained less solids in suspension and was easier to handle. A 1:1 (v:v) mixture of OMWW SP and ethyl acetate was vortexed for one minute and then centrifuged at 9.000 g for five minutes. This step was repeated five times. Ethyl acetate extract was evaporated under vacuum in a rotavapor at 35°C and suspended in distilled water (pH 5) using the same initial volume. A blank of ethyl acetate extract with water was also performed. Furthermore, the OMWW (SP) was centrifuged at 9.000 g for five minutes and ultrafiltered through a membrane of 3000 Daltons (Whatman, Sigma-Aldrich).

Phenolic compounds (Hy 1-O-glucoside, EDA- like and EA) were isolated from the ethyl acetate extract by analytical HPLC. The column, gradient and equipment were the same as those used for the phenolic compound analysis. Aqueous mobile phase was acidified with 2N HCl (522 µL/L). Fractions were collected peak-by-peak manually. The pooled extract for each peak was evaporated under vacuum to dryness, and the residue was dissolved in acetic acid/sodium acetate buffer (pH 5.0). Finally, the concentration of each compound was measured by HPLC. A control run of HPLC was also performed injecting deionized water and collecting the corresponding fractions.

2.4. Bacterial strains and culture conditions

Pseudomonas fluorescens (CECT 378), *Staphylococcus aureus* (CECT 239), *Escherichia coli* (CECT 434), and *Enterococcus faecalis* (CECT 481) were obtained from the Spanish Type Culture Collection (Burjasot, Valencia, Spain). *P. fluorescens*, *S. aureus*, and *E. coli* were cultured in nutrient broth prepared with (g/L) “Lab-Lemco” powder (Oxoid) 5, Neutralized bacteriological peptone (Oxoid) 10, NaCl 5 and agar 15

for solid medium (pH 7.2). *E. faecalis* was cultured in Brain Heart Infusion (Oxoid Ltd., Basingstoke, Hampshire, England) with or without 1.5% agar. *P. fluorescens* was cultured at 29°C, and other strains were cultured at 37°C. These strains were chosen as they are commonly used in antimicrobial testing (European Standard EN 1276, 1997). All strains were kept at - 80°C in the adequate culture broths with glycerol (15-20%) until use. Every target strain was cultured twice in its corresponding broth, from the frozen stock, before testing, and overnight cultures were routinely used for inoculum preparation.

2.5. Antimicrobial assays

Three olive oils and OMWW (RC, SP and BC) were selected according to the concentration of total polyphenols: low, intermediate and high phenolic concentration, respectively. RC was obtained from Rougette cultivar by 3-phase continuous extraction, BC from Blanquette cultivar by 3-phase continuous extraction, and SP from Sigoise cultivar by traditional pressure extraction.

An antimicrobial test was performed with all strains against the selected OMWW. The pH of the OMWW were adjusted to 5.5, filtered through 0.22 µm of diameter, and diluted with sterile water at pH 5.5 before use. One mL of OMWW (at concentrations of 0.25, 0.50, 1.25, 2.5, 5, 10, 20, 40 and 60%) was inoculated and mixed with 50 µL of an overnight culture of the target microorganism diluted with saline solution (0.85 g/100mL NaCl) to get an initial population between 5.51 – 6.07 log CFU/mL. The mixture was incubated at room temperature for 30 minutes and vortexed occasionally. After treatment, culturable survivors were determined by plating these mixtures or the corresponding decimal dilutions (0.1% peptone water) on the appropriate solid media

plating with a Spiral Plater (Don Whitley Sci. Ltd., model WASP 2, Shipley, U.K.). Colonies were enumerated with an automated counter (Counterstat, IUL Instruments, Barcelona, Spain). A control with water was performed and the antimicrobial test was performed in duplicate. The minimal bactericidal concentrations (MBC) of OMWW under these experimental conditions were determined as the lowest concentrations at which 3-log drops in CFU per milliliter were obtained (National Committee for Clinical Laboratory Standards, 1999).

The bactericidal activity assay of olive oil was based on the method reported by Medina et al. (2006). A 2 mL mixture (1:1) of olive oil with sterilized phosphate-buffered saline with Tween 20 (Sigma-Aldrich) was inoculated with 50 μ L of the refreshed target strain previously diluted with saline solution to obtain an initial inoculum between 5.90 – 5.70 log CFU/mL. The mixture was incubated at room temperature for 30 minutes with occasional shaking and then plated to count survivors on appropriate media. Controls with no oil were also performed. Trials were carried out in duplicate. This test was performed with the oils RC, BC and SP against all strains.

The strain *S. aureus* CECT 239 was chosen for testing the effect of the ethyl acetate extract of OMWW SP, the OMWW extracted, ultrafiltered and isolated compounds. A mixture of 200 μ L of isolated compounds at different concentrations was inoculated with a 50 μ L of an overnight culture of the microorganism diluted with saline solution (0.85 g/100mL NaCl) to get an initial population of 5.86 log CFU/mL. The mixture was incubated at room temperature for 30 minutes and viable count was determined by plating as mentioned before. Isolated compounds were tested at a concentrations range of 8 – 69 mg/L for Hy, 19 – 150 mg/L for EDA, and 40 – 320 mg/L for EA. They corresponded to concentrations found in the OMWW SP diluted at 2.5 – 20 %. Isolated

compound solutions were prepared diluting with sterile acetic acid/sodium acetate buffer at pH 5.5. The experiments were carried out in duplicate and a blank with mobile phases from HPLC was performed as a control.

2.6. Statistical analysis

Data were expressed as mean values and standard deviations (SD) were calculated. Comparison among mean variables was carried out by the Duncan's multiple range tests and the differences considered significant when $p < 0.05$. Statistica software version 7.0 was used for data processing (Statistica for Windows, Tulsa, OK, USA).

3. Results and discussion

3.1. Phenolic compounds in olive products

The phenolic composition in olive products obtained by traditional discontinuous and 3-phase centrifugal systems for the three Algerian cultivars studied is shown in Figure 1, Figure S1 and S2. Virgin olive oil obtained from cv. Blanquette by 3-phase centrifugal system showed the highest concentration of total phenols (395 mg/kg of oil), significantly higher than for traditional pressure extraction (202 mg/kg of oil). By contrast, Rougette and Sigoise cultivars presented higher phenolic concentrations extracted by traditional system (300 and 267 mg/kg of oil, respectively) than 3-phase (187 and 150 mg/kg of oil, respectively), although this difference was only significant for the cv. Rougette. The same tendency was observed for the phenolic composition in olive pomace (Figure 1). The highest phenolic concentration was found for the cv. Blanquette by the 3-phase extraction (661 mg/kg of pomace) and the lowest for the cv.

Rouquette with the same system (165 mg/kg of pomace). There were no significant differences between the extraction systems for the cv. Sigoise. The virgin olive oils analyzed presented a phenolic content in line with those obtained by Douzane, Tamendjari, Abdi, Daas, Mendid & Bellal (2013). However, other authors have quantified higher concentrations of phenolic compounds for the Blanquette cultivar (Laincer et al., 2016). This variability of concentration among the same cultivars depends on many factors, including geography, agronomy, seasons and environmental factors. According to Klen & Vodopivec (2012), the traditional press system provides a higher transfer rate of phenols to oil than the 3-phase centrifuge. This corresponds to the Rouquette and Sigoise cultivars studied, whereas it is not fulfilled for the Blanquette cultivar. Regarding the oil extraction system in this study, there were no conclusive differences between the different oil extraction processes.

The majority of phenols of the olive fruits were flushed away with the wastewater generated in olive oil production, only 0.3 – 1.5 % from total polyphenols are found in the olive oil (Klen & Vodopivec, 2012). The highest phenolic concentration was found in the OMWW, which ranged from 10,030 to 1,184 mg/L for the Rouquette cultivar, 11,155 and 9,690 mg/L for the Blanquette cultivar, and 5,760 and 5,469 mg/L for the Sigoise cultivar by traditional pressure system and 3-phase respectively. OMWW from Rouquette and Blanquette cultivars exhibited much higher polyphenols by traditional than 3-phase extraction systems, unlike that observed for other Algerian olive cultivars (Aggoun, Arhab, Cornu, Portelli, Barkat & Graulet, 2016). Otherwise, there were no significant differences for the Sigoise cultivar.

3.2. Antimicrobial activity of olive products

268 Three OMWW (RC, BC and SP) were selected for antimicrobial tests according to their
269 concentration in total polyphenols (Table 1). The OMWW generated from cv.
270 Blanquette BC showed the highest phenolic content (10,828 mg/L), ten times more than
271 OMWW RC from cv. Rougette (1,096 mg/L). Both were obtained by a 3-phase
272 continuous olive oil extraction system. The OMWW from cv. Sigoise generated by
273 traditional pressure system (SP) showed an intermediate concentration in total
274 polyphenols (5,762 mg/L). Among all the phenolic compounds, Hy, Hy 4-O-glucoside,
275 EDA-like and EA composed around 90% of the total phenols and derivatives. To a
276 lesser extent, hydroxytyrosol glycol, Hy 1-O-glucoside, tyrosol and salidroside showed
277 lower concentrations, being very low or null for caffeic acid and caffeic acid ester
278 (Table 1). No oleuropein concentration was found in any samples.

279 Several dilutions of these OMWWs were inoculated with selected microbial strains to
280 get the MBC shown in Table 2. After 30 minutes of incubation at room temperature, the
281 highest antimicrobial activity corresponded to OMWW BC, which also presented the
282 highest phenolic content. This OMWW reduced 3 log units of the 4 strains tested when
283 the concentration of total polyphenols was 2.5 %. The OMWW SP showed a MBC of
284 10% for *E. coli* and 5% for the rest of the microorganisms. The lowest activity was
285 observed for OMWW RC that showed a MBC of 60% for *S. aureus* and *E. coli* and
286 more than 60% for *E. faecalis* and *P. fluorescens*.

287 As mentioned, OMWWs are sources of phenolic compounds which are known to
288 exhibit antimicrobial effects (Leouifoudi, Harnafi & Zyad, 2015; Thielman, Kohnen &
289 Hauser, 2017), but this activity cannot be assigned to a particular substance or to the
290 polymeric fraction of this liquid. To elucidate the compounds responsible for this
291 antimicrobial activity, OMWW SP was subjected to a phenolic extraction with ethyl

acetate. The extract was enriched in Hy, Tyr, EDA-like and EA (Table 1), and the extracted OMWW held Hy 4-O-glucoside and salidroside. Only the Hy glycol was found in both phases at similar concentration. In parallel, the OMWW SP was ultrafiltered through a membrane of 3000 Da to remove the polymers so that simple phenols remained in the filtered OMWW at the same concentration than the original (Table 1). The SP extract of phenolic compounds, the extracted OMWW SP and the ultrafiltered OMWW SP were inoculated with *S. aureus* and the antimicrobial activity is shown in Figure 2A. After 30 minutes of contact, the ultrafiltered OMWW SP exerted the same MBC (5%) as the non-filtered. The extract of OMWW SP at 5% reduced around 2 log units and reached the MBC at 10% presenting good bactericidal properties. Nevertheless, the extracted OMWW SP reached the MBC at a concentration of 40%. During the extraction, certain phenolic compounds such as Hy, EDA-like and EA diffused to the ethyl acetate extract and, consequently, the antimicrobial activity in the extracted OMWW decreased. Some of these compounds were isolated from the extract by HPLC, identified by MS-HPLC. The MS spectra of isolated compounds exhibited a signal at m/z 153 and 241 for hydroxytyrosol and EA respectively, as previously described by Medina et al. (2007). EDA-like exhibited a signal at m/z 199 (Figure S3) as previously described for the first time by Medina et al. (2011), and subsequently characterized as (E)-3-(1-oxobut-2-en-2-yl) glutaric acid (Rubio-Senent, Martos, García, Fernández-Bolaños, Rodríguez-Gutiérrez & Fernández-Bolaños, 2015b).

Isolated compounds were tested individually against *S. aureus* (Figure 2B). Neither hydroxytyrosol nor EA showed antimicrobial activity at concentrations studied. The same results were obtained for hydroxytyrosol, its glucosides, and tyrosol against lactic acid bacteria (Medina et al., 2007). Only the EDA-like compound showed

antimicrobial activity at a concentration of 150 mg/L, which corresponded to the content found in the OMWW extract when it was diluted to around 13%. Hence, this antimicrobial activity agrees with the MBC found for the extract.

The antimicrobial activity of OMWW has widely been studied and several compounds have been attributed as responsible (Capasso, Evidente, Schivo, Orru, Marcialis & Cristinzio, 1995; Obied, Bedgood, Prenzler & Robards, 2007) but any individual compound explained this bioactivity by itself. However, it has been demonstrated that the most bactericidal compounds in olive products are glutaraldehyde-like compounds such as HyEDA and EDA (Medina, Brenes, García, Romero & de Castro, 2009). EDA is an oleuropein derivative well studied in table olive elaborations (Medina et al., 2008). The presence of a dialdehydic structure in the molecule of EDA confers a strong antimicrobial activity, which is similar to that of the commercial biocide glutaraldehyde (Medina et al., 2009). In alperujo, the compound EDA-like is detected in high concentrations (Medina et al., 2011) and its origin has been suggested to be the antimicrobial EDA by a hydrolytic reaction during the storage and/or processing. The MS spectra differ in an oxygen atom from m/z 183 and 199 for EDA and EDA-like respectively. Although EDA-like had less antimicrobial activity against *Pseudomonas savastanoi* (Medina et al., 2011), the high amount of EDA-like compound present in the OMWW explains their bactericidal properties against bacteria and other phytopathogenic microorganisms (Medina et al., 2011; Rubio-Senent et al., 2015b).

3.3. Antimicrobial activity of virgin olive oil

In the same way as OMWW, three virgin olive oils were selected for antimicrobial activity testing against target microorganisms. BC oil showed the highest concentration

in total polyphenols (452 mg/Kg of oil), followed by SP (370 mg/Kg of oil) and RC (126 mg/Kg of oil) (Table 3). Among the phenolic compounds, Hydroxytyrosol and tyrosol derivatives are the most abundant in the olive oil, where HyEA, HyEDA, TyEA and TyEDA are the majority. Also, the lignans pinoresinol and 1-acetoxypinoresinol were found at high concentrations in all the olive oils (Table 3). After inoculation and 30 minutes of contact with the oils (50%), only BC showed antimicrobial activity against *S. aureus* and *E. faecalis* (Figure 3). This oil reduced the initial inoculum by more than 3 log units and no effect was observed for the rest of the oils and strains. Lainer, Laribi, Tamendjari, Arrar, Rovellini & Venturini, (2014) found a high antimicrobial activity in Algerian virgin olive oils against *S. aureus* and *Bacillus subtilis*. This bactericidal activity was attributed to the phenolic compounds of olive oil as being mainly responsible (Brenes et al., 2007). The effect of each individual phenolic compound was studied *in vitro* and showed that TyEDA and HyEDA presented a potent antimicrobial activity (Romero, Medina, Vargas, Brenes & de Castro, 2007) due to the presence of the dialdehydic structure in their molecules (Medina et al., 2009). Both compounds possess the EDA structure linked to the phenols tyrosol and hydroxytyrosol respectively, that increases their lipophilic character and antibacterial efficacy. The results obtained in this work are in agreement with the previous studies. The BC olive oil presented higher concentrations of the oleuropein and ligustroside derivatives than RC and SP oils (Table 3) which is related to a higher antimicrobial activity.

4. Conclusions

The results obtained in the present work have characterized the phenolic profile of products generated during olive oil extraction of three Algerian olive cultivars. Regarding the phenolic composition of olive products, no significant differences were

found between the traditional discontinuous press and 3-phase centrifugal olive oil extraction processes. The antimicrobial test with isolated phenolic compounds revealed that EDA-like possesses a high bactericidal activity *in vitro*, and the high concentrations in the OMWW can explain the antimicrobial properties of this by-product. Moreover, the presence of glutaraldehyde-like compounds such as TyEDA and HyEDA in the virgin olive oils confer them with a strong antimicrobial activity. These results support that the high amount of antimicrobial compounds in the products derived from olive oil extraction such as virgin olive oil, pomace and OMWW, are a good source of natural antimicrobials with potential in healthcare, animal feed, the food industry or as pesticides in agriculture.

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Figure captions

Fig. 1. Total phenolic compounds in olive products from cv. Rougette, cv. Blanquette and cv. Sigoise. See section 2.2 for the sum of single phenolic compounds. Three samples of each cultivar were run in duplicates. P corresponds to traditional discontinuous by pressure, and C corresponds to 3-phase continuous by centrifugation. Bars show standard deviation. Same letters within the same columns designates no statistically significant differences ($p < 0.05$) according to the Duncan's New Multiple Range Test.

Fig. 2. Antimicrobial activity of OMWW **SP** extract (panel A) and isolated phenolic compounds (panel B) against *S. aureus* after 30 minutes of contact. Bars means standard deviations. See section 2.1 for abbreviations.

Fig. 3. Antimicrobial activity of olive oils against targeted microorganisms after 30 minutes of contact. Test was run in duplicates. Bars means standard deviations.

496 Table 1. Phenolic composition (mg/L) of OMWWs and their extracts used for the antimicrobial assays.

Compound	RC	BC	SP	SP extract	Extracted SP	SP ultrafiltered
Hy Glycol	150 ^a (21)	230 (4)	258 (0)	132 (2)	159 (8)	325 (22)
Hy 1-O-Glucoside	27 (5)	23.8 (0)	23 (4)	-	-	-
Hydroxytyrosol	108 (5)	1409 (63)	427 (11)	348 (2)	102 (3)	465 (10)
Hy 4-O-Glucoside	211 (11)	3150 (73)	1748 (92)	54 (3)	1897 (142)	1927 (91)
Salidroside	33 (3)	265 (2)	125 (5)	-	119 (9)	135 (1)
Tyrosol	14 (3)	76 (7)	425 (4)	53 (2)	-	54 (0)
Caffeic acid	-	3 (1)	1 (0)	-	-	-
Ester of caffeic	-	1 (0)	1 (0)	-	-	-
Comselogoside	-	1 (1)	2 (1)	1 (0)	-	-
EDA-like	465 (31)	3828 (359)	1252 (46)	1118 (23)	0 (0)	1237 (13)
EA	87 (2)	1840 (131)	1884 (4)	1582 (15)	0 (0)	1805 (6)
Total phenols	1096 (60)	10828 (344)	5762 (118)	3287 (16)	2277 (162)	5949 (129)

497
498 ^aData are the mean of duplicates. Standard deviation shown in parentheses. Abbreviation: cv. Rouquette (R), cv. Blanquette (B), cv. Sigoise(S),
499 traditional by pressure (P) and 3-phase continuous by centrifugation (C). See section 2.2 for phenolic compounds abbreviations.

Table 2. MBCs of OMWWs against target bacteria.

OMWW	MBC (%)			
	<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>	<i>P. fluorescens</i>
RC	60	>60	60	>60
SP	5	5	10	5
BC	2.5	2.5	2.5	2.5

513 Table 3. Phenolic compounds (mg/kg of oil) of olive oils used in the antimicrobial
514 assays.
515

Compounds	Olive Oil		
	RC	BC	SP
Hydroxytyrosol	11 ^a (1)	5 (0)	9 (0)
Hy Glicol	-	1 (0)	2 (0)
Hy- EDA	-	27 (0)	16 (3)
Hy- EA	11 (1)	99 (11)	66 (2)
Tyrosol	1 (0)	11 (1)	19 (2)
Ty - EDA	18 (4)	38 (1)	28 (0)
Ty- EA	7 (0)	185 (21)	142 (4)
4-ethylphenol	8 (0)	-	-
Pinoresinol	52 (3)	62 (1)	62 (6)
1- Acetoxypinoresinol	16 (1)	22 (2)	24 (2)
Luteolin	2 (0)	2 (0)	2 (0)
Apigenin	1 (0)	1 (0)	1 (0)
Total phenols	126 (8)	452 (35)	371 (13)

516

517 ^aData are the mean of duplicates. Standard deviation shown in parentheses. See section
518 2.1 and 2.2 for abbreviations.

Fig 1.

Fig 2.

Fig 3.

Supplementary Material

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