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Recovering Polyphenols in Aqueous Solutions from Olive Mill Wastewater and Olive Leaf for Biological Applications

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Abstract: Olive leaf and olive mill wastewater are wastes which are produced in olive industry and can be used to obtain value-added products such as polyphenols. In this work, extracts of polyphenols from olive mill wastewater and olive leaf in an aqueous medium are obtained and their composition in polyphenols were determined and compared with those obtained using the conventional extract agent (methanol–water). The antioxidant capacity of the aqueous extracts of olive mill wastewater and olive leaf were also analysed. It was observed that the olive mill wastewater extracted in water provides a similar content of phenolic compounds in the final extract compared to extraction with methanol–water, with the main polyphenols being hydrotyrosol and tyrosol (20.1 and 6.61 mg gdw^{-1}). In the case of leaf extraction, the methanol-free extract (recovery in water after extraction with methanol–water) had a lower total phenol content compared to the methanolic extract, with the main polyphenol being oleuropein (22.73 and 9.05 mg gdw^{-1}, for the methanol and methanol-free extract, respectively). However, both extracts obtained in aqueous solution present a similar antioxidant capacity at very diluted concentrations of the original extract, with IC50 values (half-maximal antioxidant concentration) of about 20 mg TS L^{-1}. The antioxidant capacity of the extract in aqueous solution facilitates its application as an antioxidant in biological systems, like animal food, where the use of extracts based on organic solvents, like methanol, are not suitable.

Keywords: oleuropein; hydroxytyrosol; polyphenols; olive leaf; olive mill wastewater; circular economy

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1. Introduction

Olive groves cover 2.75 million hectares of which 2.55 million hectares belong to olive oil mills (93% of the total olive grove). Olive cultivation has the largest area of integrated production in Spain with 477,606 hectares (2019 data), which represents 57% of the national total for this type of production and 18% of the total olive grove area in Spain. In addition, 217,864 hectares of olive oil mills are farmed organically (2020 data). Spanish olive oil production comprises 70% of EU production and 45% of the world's production. All these data reflect the great importance of the olive industry in Spain [1]. Olive leaves and olive mill wastewater represent two of the main by-products of the olive-processing industry. Out of the 5,227,770 tons of olives processed in Spain, a total of 329,233 tons of olive leaves were obtained from the cleaning process (averaged across three campaigns: 2012–2013, 2013–2014, and 2014–2015) [2]. Furthermore, the production of olive oil dominates the olive agroindustry, with nearly 1800 active olive mills processing over 3 million tons of olives annually to produce virgin olive oil [2]. Considering that 60% of the weight corresponds to wastewater from oil mills, 1.8 million tons of wastewater from oil mills would be generated. The significant and inexpensive quantity of these residues typically lacks practical applications and is often burned or discarded in landfills, or sometimes utilized

as animal feed [3]. Consequently, it is disposed of as a by-product, which can potentially lead to economic and environmental issues, as well as the wastage of a valuable resource. Producers face increasing costs associated with its removal, storage, and disposal [4]. However, there is potential to extract value-added products such as polyphenols from olive leaves and olive mill wastewater. This presents a promising opportunity to enhance the profitability of olive groves and promote more sustainable agricultural practices [5].

Over time, numerous proposals have emerged to assess and utilize agricultural and industrial remnants [6], which often contain higher levels of bioactive compounds compared to the final products [7]. Olive leaves, for instance, contain polyphenols that exhibit various pharmacological activities, including antioxidant, anti-inflammatory, anti-atherogenic, anticancer, antimicrobial, antiviral, hypolipidemic, and hypoglycemic effects [8]. Consequently, there is a growing interest in the importance of olive leaves and olive mill wastewater. Olive leaf powders or extracts hold potential for diverse industrial applications, including food supplements, cosmetics, and the pharmaceutical industry [9]. Olive leaves play a significant role as an abundant source of phenolic substances. These phenolic compounds include oleuropein, verbascoside, rutin, tyrosol, and hydroxytyrosol [10–15]. The quantitative and qualitative composition of phenolics in olive leaves can vary considerably due to agricultural factors such as olive variety or climate conditions [10,16–18].

Similarly, olive oil mill wastewater (OMW) is a by-product in the oil production industry, akin to olive leaves. OMW contains a significant amount of polyphenols, with hydroxytyrosol (HT) and tyrosol being the economically relevant components of the phenolic fractions. The specific composition of OMW varies depending on factors such as olive variety, fruit ripening, climate conditions, and storage time. The phenolic compounds identified in OMW include hydroxytyrosol, tyrosol, caffeic acid, ferulic acid, secoiridoid compounds, verbascoside, oleuropein, and their isomers/derivatives [19–26]. Oleuropein is the most abundant polyphenol in OMW. The concentration of oleuropein and its derivatives in OMW can vary significantly in different studies, primarily due to the degradation of oleuropein during the olive oil production process, particularly during malaxation [20–22].

Regarding the biological application of polyphenols, they seem to be a promising strategy for improving animal product quality through animal diets such those of as ruminants, chickens, and pigs. In fact, the number of publications indexed by Web of Science on polyphenols and their use as feed additives has increased exponentially from over 200 in the 2000–2005 period to over 1500 in the 2016–2020 period [27]. Furthermore, polyphenols have proved to have an important impact on human health according to their antioxidant, antimicrobial, immunomodulatory, antihypertensive, anticancer, and anti-inflammatory properties [28].

The predominant extraction methodologies employed by industrial sectors for the retrieval of high-value compounds from olive by-products include the utilization of organic solvents mixtures, such as ethanol–water or methanol–water mixtures. However, the utilization of compounds with residues of organic solvents constrains their application in biological systems due to their adverse ecological repercussions and associated human health concerns. In this sense, other studies have proposed water as a solvent for polyphenols extractions in olive by-products, although they report a lower efficiency in the extraction of phenolic compounds compared to the values reported for organic solvents [29–34]. As an alternative, in the present work, the efficacy of a new method was analysed. This method combines an initial ultrasound-assisted extraction in methanol–water mixture due to its recognized extracting capacity [35,36], followed by a removal of the organic solvent residue by evaporation, and finally a recovery of the extract in water as a harmless application medium. Moreover, in the case of OMW, the standard method for the determination of biophenols in olive oils and olive by-products also proposes an ultrasound-assisted extraction in a methanol–water mixture [36]. However, considering the aqueous nature of the OMW, in the present work the direct analysis of OMW after centrifugation was evaluated.

In this work, extracts of polyphenols from olive oil mill water and olive leaf in aqueous medium are obtained, their composition in polyphenols has been characterized and their

antioxidant power has been determined and compared to those obtained via extraction in methanol–water medium. Specifically, the most important polyphenols of these residues, that is, hydroxytyrosol, tyrosol, and oleuropein, have been quantified. Furthermore, the total polyphenols have also been quantified by the Folin–Ciocalteu method and by HPLC. The antioxidant capacity of the obtained extracts has also been determined, as well as other physicochemical properties of the extracts such as their pH, ionic conductivity, or oxidation-reduction potential.

2. Materials and Methods

2.1. Materials and Reference Compounds

Folin–Ciocalteu's phenol reagent, gallic acid, sodium carbonate, 1,1-diphenyl-2-dipicrylhydrazyl (DPPH), quercetin, oleuropein, 2-(4-hydroxyphenyl)ethanol (tyrosol), 2-(3,4-Dihydroxyphenyl)etanol (hydroxytyrosol), syringic acid, Griess reagent (modified), sodium nitrite, dicyandiamide (DCD), and phosphate-buffered saline were purchased from Sigma-Aldrich. HPLC-grade solvents such as acetonitrile, methanol, water, and orto-phosphoric acid were purchased from Panreac ApplyChem ITW Reagents (Barcelona, Spain).

2.2. Olive Leaves and Olive Mill Wastewater Raw Materials

In this study, olive leaves (OL) and olive mill wastewater (OMW) were selected as typical natural by-products from the Spanish olive oil industry. These by-products were supplied by a local company of "Región de Murcia" (Spain). OMW was stored at 4 °C until the preparation of the extracts, while OL samples were air dried at room temperature and then the leaves were milled using a Mixer grinder TR2 (Optic Ivymen Systems, COMECTA S.A., Barcelona, Spain) and stored at 4 °C until the preparation of the extracts.

2.3. Natural Extract Preparation

The sample preparation followed a modified version of the standard method based on the International Olive Council's methodology for determining biophenols in olive oils (2009), as described by Goldsmith et al. (2014) [36]. In this following method, a few adjustments were made. For the olive leaf (OL) samples, 2 g was mixed with 12 mL of a methanol–water solution (80:20 v/v). For the olive mill wastewater (OMW) samples, 5 g was combined with 15 mL of a methanol–water solution (80:20 v/v). The samples were then subjected to vortex for 2 min, followed by extraction in an ultrasonic bath for 15 min. Afterward, centrifugation was performed at 3000× g for 25 min at 4 °C. The extracts were separated via filtration with No 40 Whatman paper.

For OMW, this extraction method was compared with a direct analysis in which the samples were only centrifuged and filtrated.

For OL, this extraction method was compared with a modification in which the sample was added to methanol–water (80:20 v/v), vortexed, sonicated, centrifugated, and filtrated as commented before, and then the extracts were concentrated with a rotary evaporator Büchi R-200, equipped with a thermostatic bath and refrigerated condenser and connected to a vacuum pump Laboport N816 (KNF Neuberger GmbH, Freiburg, Germany). The thermostatic bath was set at 38–40 °C until total evaporation of the solvent. The extract was collected in 10 mL of ultrapure water and centrifuged again at 3000× g for 25 min at 4 °C. Finally, the extracts were separated via filtration with No 40 Whatman paper, and then they were made up to a total volume of 10 mL in ultrapure water.

2.4. Physico-Chemical Characteristics of Natural Extracts

The physico-chemical parameters, including electrical conductivity (EC), oxidation-reduction potential (ORP), and pH, were assessed using a multimeter sensION+ MM150. To analyse the total solids content, 10 mL of extract samples were transferred to pre-dried and weighed crucibles. The crucibles were then placed in a vacuum oven (Vaciotem-TV, J.P. Selecta S.A., Barcelona, Spain) and dried at 70 °C until a constant weight was attained (approximately 72 h). The weight loss was utilized to determine the total solids content in

grams per litre (g L^{-1}) of the sample. These physico-chemical characteristics were analysed in 4 replicates for each type of natural extract.

2.5. Biophenols Analysis

2.5.1. Determination of Total Phenol Content

Total phenolic compounds analysis was carried out using the Folin–Ciocalteu method based on Cicco et al. (2009) with slight modifications [37]. Each sample, appropriately diluted, along with a blank (pure water), was mixed with 300 µL of Folin–Ciocalteu's reagent and equilibrated for 2 min. Subsequently, 2.4 mL of a 5% (w/v) Na_2CO_3 solution was added to each preparation, and the mixtures were left to react in the dark at room temperature for 1 h. The absorbance was measured at a wavelength of 760 nm using a T80 UV-Visible Spectrophotometer (PG Instruments Limited, Woodway Lane, Alma Park, UK). Results were expressed as mg gallic acid (standard) equivalents (GAE) per L and per gram of dry weight (gdw) of sample. A total of 3 replicates were carried for each type of natural extract.

2.5.2. Biophenols Analysis Using the HPLC Method

The prepared samples underwent filtration using a 0.45 µm Nylon PVF filter (Merk Millipore, Tullagrenn, Carrigtwohill, Co., Cork, Ireland) prior to further analysis. The analysis was conducted using an Agilent 1220 Infinity Series. The chromatographic conditions were described by Martínez-Navarro et al., 2021 [38]. A ZORBAX Eclipse XDB-C18 column with a guard column Eclipse XDB-C18 was used. The column temperature and the flow rate were set at 30 °C, and at 1 mL min^{-1}, respectively. The mobile phase consisted of water acidified to pH 2.2 with ortho-phosphoric acid. Acetonitrile was used as solvent B. The elution gradient for solvent B was described in [38]. A diode array detector was used in the analysis (λ = 280 nm). Syringic acid was used as the internal standard. Values for the total HPLC peaks were determined using a tyrosol standard curve in methanol, and the sum of all values was expressed as the total phenolic compounds in g Tyrosol Equivalents (TRE) per L and per gdw of sample [39]. The HPLC peaks corresponding to oleuropein, tyrosol, and hydroxytyrosol were identified and their content in the extracts were quantified using the corresponding standard curves of those prepared in methanol. The results were expressed as g of the corresponding biophenol per L and per gdw of sample. Biophenols analysis by HPLC method was carried out in 3 replicates for each type of natural extract.

2.6. DPPH Assay

The antioxidant activities of different extract samples were determined using a DPPH free radical scavenging assay as described by Mishra and Ojha (2012) [38], with a few modifications. A fresh solution of DPPH was prepared in methanol. For each sample, 500 µL of different concentrations of extract samples was mixed with 500 µL of 100 µM methanolic solution of DPPH and kept at room temperature for 30 min. The control was carried out with water instead of an extract sample, while methanol instead of DPPH was used as blank. Absorbance was then read on a T80 UV-Visible Spectrophotometer (PG Instruments Limited, Woodway lane, Alma park, UK) at a wavelength of 515 nm. DPPH scavenging effects was calculated using Equation (1).

$$\text{DPPH scavenging effects } (\%) = \frac{\text{Sample absorbance} - \text{Control Absorbance}}{\text{Control Absorbance}} \times 100 \quad (1)$$

IC50 was calculated by fitting the best function by linear and non-linear regression to the curves of DPPH. Scavenging effects versus extract concentration was expressed as mg of total solids (TS) L^{-1}. IC50 values of the tested extracts were compared with the IC50 values obtained from a quercetin standard curve (prepared each day). Antioxidant activity analyses by DPPH method were carried out in 2 replicates for each type of natural extract.

2.7. Statistical Analysis

All data were analysed using PASW Statistics 28 for Windows. To compare the different extracts analysed, a Student *t*-test (independent two-sample *t*-test) was conducted with the significance level set at $p < 0.05$. Bilateral correlations were determined using Pearson's correlation with a confidence interval of 95%. Regression analysis was performed using both linear and nonlinear regression analysis with Sigma Plot 12.5. The estimated data were accompanied by confidence intervals established at a 95% confidence level.

3. Results

3.1. Physico-Chemical Characteristics and Biophenolic Composition of Natural Extracts

As commented above, the extraction of polyphenols and their characterization is usually carried out in methanol medium or other organic solvents media. We are interested in the application of extracts in biological systems in which methanol is harmful. For that, extracts of polyphenols from olive oil mill water and olive leaf were obtained and characterized in aqueous medium and these values were compared to the extracts obtained with the conventional methanol–water extraction medium. Figures 1 and 2 show the HPLC profiles of oil mill water extract and olive leaf extract obtained via the conventional method, respectively, and they are compared to the HPLC profiles of the modified extracts based on the standard method compared to the proposed modified methods.

Regarding the biophenolic composition, examination of the extracts from the samples using HPLC analysis showed qualitative and quantitative differences between the two types of extracts (Figures 1 and 2). Hydroxytyrosol and tyrosol were the main peaks detected in OMW, although their contents were very low in the case of OL extracts. In the latter extracts, oleuropein was the main peak detected, but this compound was not individually quantifiable in the case of OMW extracts. Furthermore, it was observed that the standardized area of the peaks of the extracts recovered in water is lower than that of the peaks extracted with methanol. Figure 3 shows the chemical structure of main polyphenols which are found in the extract such as tyrosol, hydroxytyrosol, and oleuropein.

Table 1 summarizes the physico-chemical characteristics of the extracts obtained by the different extraction methods. Regarding the total phenolic compounds, the values were 10 times higher in OMW extracts compared with OL extracts using both HPLC quantification methods or Folin–Ciocalteu's method, with significant differences detected by the statistical analysis ($p < 0.01$).

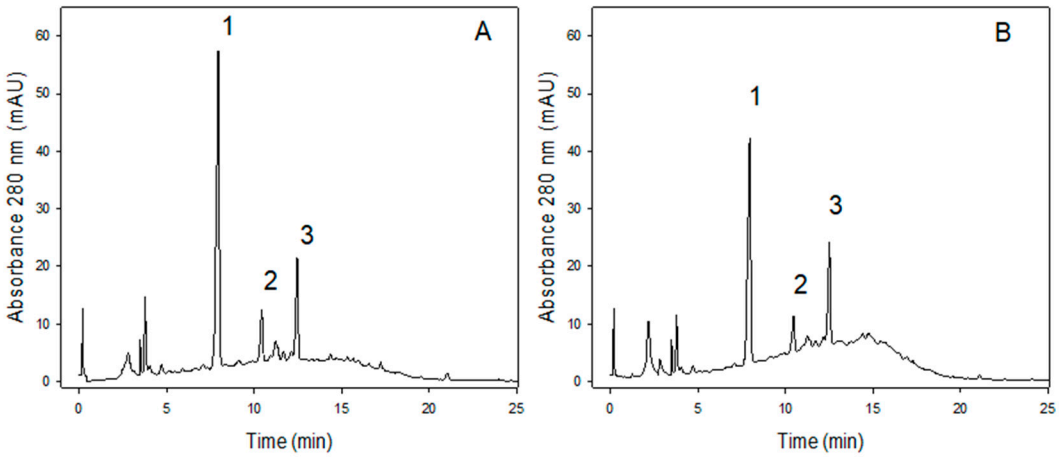

Figure 1. HPLC chromatograms of OMW extract obtained via conventional method in methanol–water (**A**), and via direct analysis of OMW after centrifugation and filtration (**B**). Dilution factor 50. Peaks identified: (1) hydroxytyrosol, (2) tyrosol, (3) internal standard.

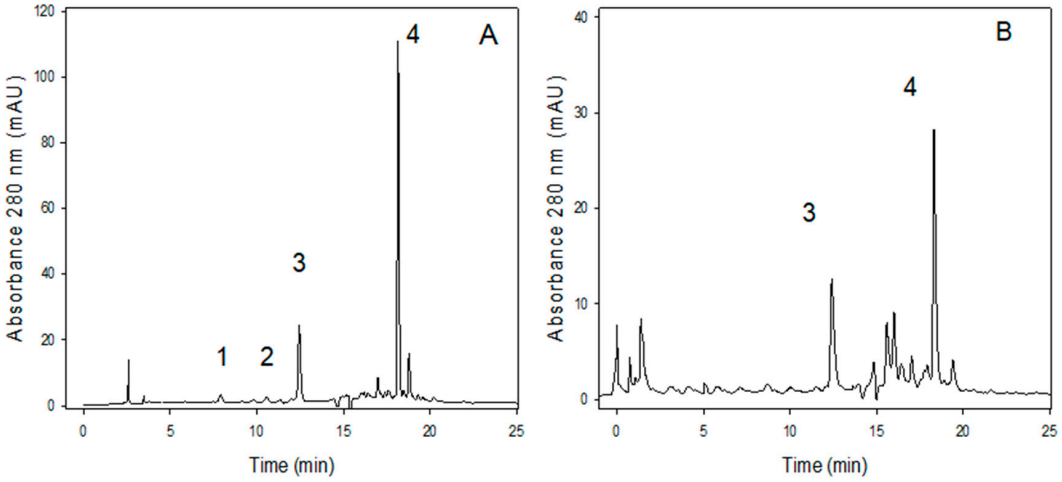

Figure 2. HPLC chromatograms of OL extracts obtained via conventional method in methanol–water (**A**), and via modified standard method with a subsequent recovery in water (**B**). Dilution factor 25. Peaks identified are: (1) hydroxytyrosol, (2) tyrosol, (3) internal standard, (4) oleuropein.

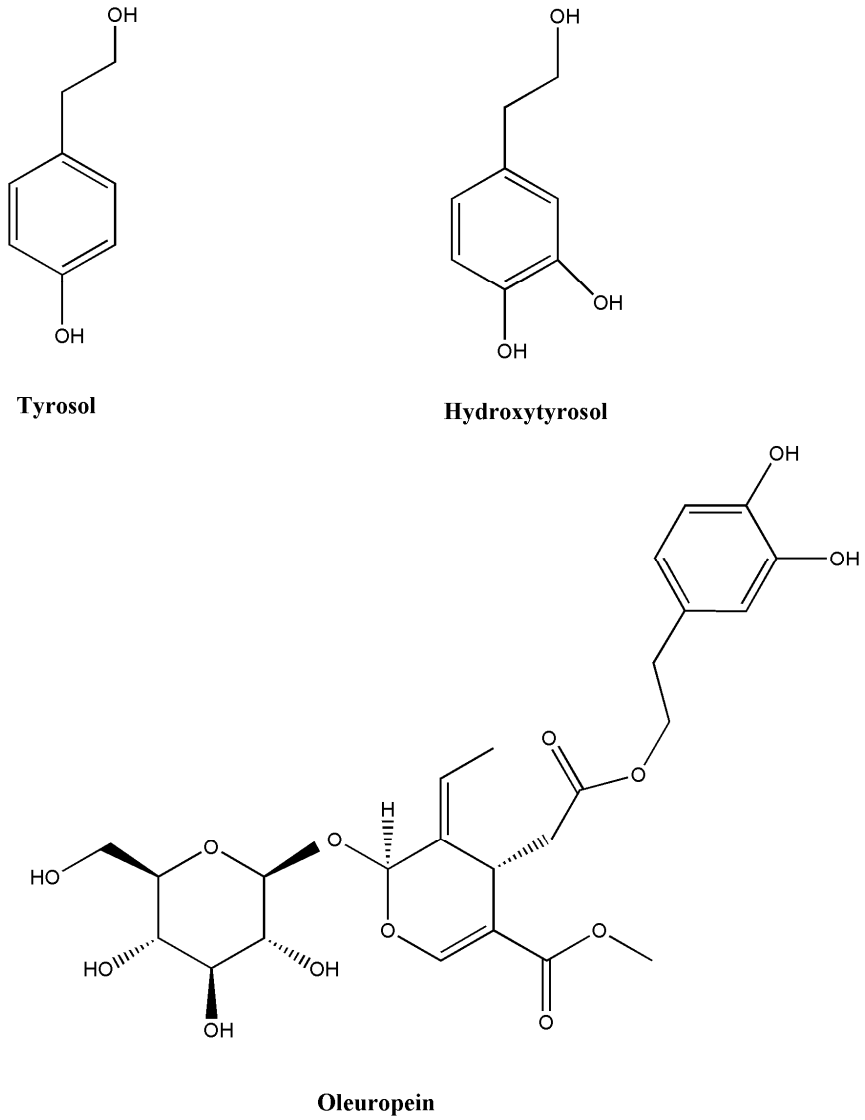

Figure 3. Chemical structure of polyphenols.

Table 1. Physico-chemical characteristics of the extracts obtained via different extraction methods. ND, not detected.

Extract	Extraction Method	Total Phenolic Content using Folin–Ciocalteu's Method (mg GAE gdw^{-1})	Total Phenolic Content Using HPLC Method (mg TRE gdw^{-1})	Hydroxityrosol (mg gdw^{-1})	Tyrosol (mg gdw^{-1})	Oleuropein (mg gdw^{-1})
Olive mill waste water	Methanol extraction	105.08 ± 2.79	103.99 ± 5.50	22.45 ± 1.13	6.99 ± 0.31	ND
	Direct analysis	98.27 ± 3.89	101.27 ± 6.93	20.41 ± 0.79	6.61 ± 0.21	ND
Olive leaves	Methanol extraction	10.38 ± 1.75	16.65 ± 1.13	0.14 ± 0.01	0.20± 0.01	22.73 ± 1.14
	Methanol extraction + water recuperation	7.88 ± 0.19	12.89 ± 2.14	ND	ND	9.05 ± 1.66

Values are the mean ± standard deviation of four replicates. GAE: gallic acid (standard). Gdw: gram of dry weight. TRE: Tyrosol Equivalents. ND: not detected.

In the OMW extracts, hydroxytyrosol was the main phenolic compound detected, whose peaks showed a relative area up to 50% of total peak areas detected from the chromatograms in both type of extraction methods (see Figure 1). In these extracts, oleuropein was not detected using the HPLC method. The total phenolic concentration and the concentration of hydroxytyrosol and tyrosol were slightly higher in the OMW extracts with the use of methanol. The analysis of the total phenolic compounds of the OMW using HPLC and Folin–Ciocalteu's methods did not show significant differences in the biophenolic composition (Table 1).

In the case of the OL extracts, in both types of extractions analysed the main phenolic compound detected was oleuropein, whose peaks showed a relative area of up to 40 and 25% of total peak areas detected from the chromatograms in standard extraction and aqueous extraction, respectively (Figure 2). Moreover, the analysed extracts obtained by the two different extractions (methanol and water) showed quantitative differences in biophenolic composition. So, the content of total phenolic compounds were 25% lower in the case of the modified method with a subsequent recovery in water compared to the original standard method with methanol–water, with significant differences detected by statistical analysis ($p < 0.01$). Similarly, oleuropein content was up to 50% lower in the case of aqueous solution compared to the extract obtained only with methanol–water (see Table 1), with significant differences detected by statistical analysis ($p < 0.01$). Hydroxytyrosol and tyrosol were only detected in the OL extract obtained with methanol alone, but with a very low content. Moreover, in OL extracts, the Pearson's correlation analysis showed that the measure of the total phenolic compounds via HPLC and via Folin–Ciocalteu's method showed a statistically significant correlation ($p < 0.05$, Pearson correlation coefficient of 0.9), and in both cases the total phenolic content showed a significant correlation with the oleuropein content (($p < 0.01$, Pearson correlation coefficient > 0.9).

The data corresponding to the extractions in aqueous solutions were also analysed by expressing the results in concentration per volume, in order to assess their potential in their final application form. When both aqueous extracts were compared the results showed that the total solids content was three times higher in the aqueous extract obtained from OMW compared to the OL extract, and significant differences ($p < 0.001$) among the evaluated extracts were observed (see Table 2). Both types of extract showed acidic properties, with a pH close to 4.5, and similar ORP levels (Table 2).

Table 2. Physico-chemical characteristics of the extracts in aqueous solution.

Extract	pH	EC (mS cm^{-1})	ORP (mV)	Total Solids Content (g L^{-1})	Total Phenolic Content Using Folin–Ciocalteu's Method (mg GAE L^{-1})	Total Phenolic Content Using HPLC Method (mg TRE L^{-1})
Olive mill waste water	4.73 ± 0.01	15.09 ± 1.33	157.55± 1.34	48.81 ± 0.13	5064 ± 41	4645 ± 355
Olive leaves	4.50 ± 0.01	0.93 ± 0.01	190.60 ± 1.41	16.97 ± 0.31	1501 ± 37	2455 ± 408

Values are the mean ± standard deviation of four replicates. EC: Electrical conductivity. ORP: oxidation-reduction potential. GAE: gallic acid (standard). Gdw: gram of dry weight. TRE: Tyrosol Equivalents.

Regarding biophenolic composition, the values observed for hydroxytyrosol and tyrosol from OMW extracts (mean values ± SD) were 1468 ± 110 and 506 ± 35 mg L^{-1}, respectively. In the case of OL extracts, the values for oleuropein (mean values ± SD) were 1722 ± 316 mg L^{-1}. The total phenolic compounds expressed as concentration per volume of extract also showed significant differences between both types of extracts ($p < 0.01$), and the content was higher in OMW extracts compared to OL extracts, both in the analysis via HPLC and Folin–Ciocalteu's method (Table 2). Moreover, the Pearson's correlation analysis showed that the measure of total phenolic compounds showed a statistically significant correlation with the total solids content measurements ($p < 0.001$)

3.2. Antioxidant Activity Analysis via DPPH Assay

The antioxidant activities of different methanol-free extract samples were determined using the DPPH free radical scavenging assay. The results of this study showed that the curves of the DPPH scavenging effects versus extract concentration were best fitted to a logarithmic regression function in the case of the OMW extract, in contrast to the data of the OL extract, which were best fitted to a linear regression function (see Figure 4).

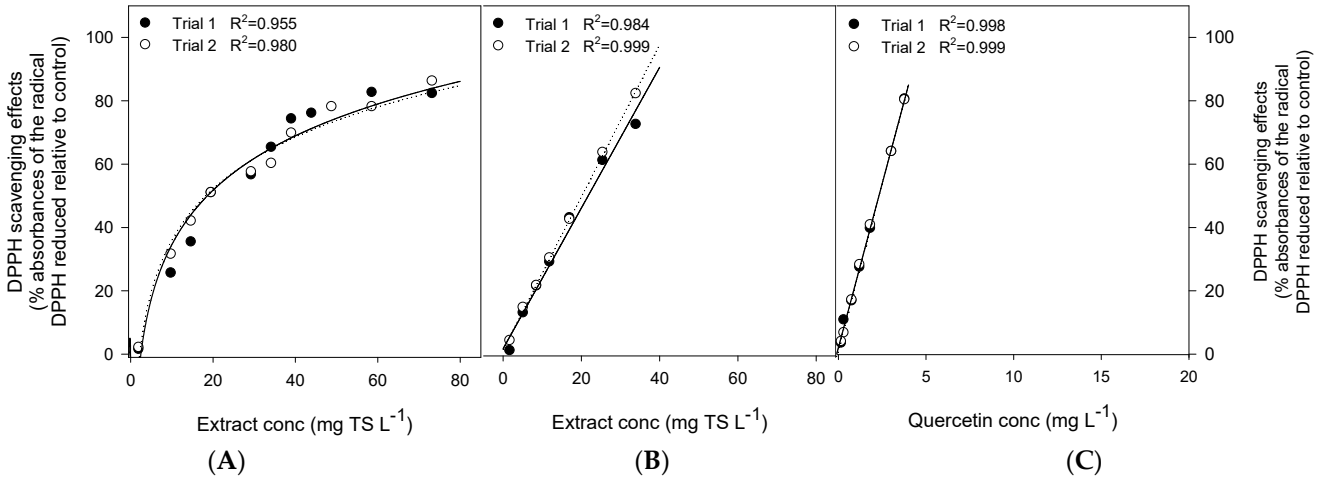

Figure 4. DDPH scavenging effects analysis of olive mill wastewater extract (**A**) olive leaf extract (**B**) and (**C**) quercetin. R^2: correlation coefficient of the regression analysis for the experimental data of each study.

In all cases, the regression coefficients of the regression's analysis were up to 0.95, with a high reproducibility between the replicates of both analyses (Figure 4).

Based on the best function obtained, IC50 values were calculated. The values of IC50 (mean values ± SD) were 18.41 ± 0.33 mg TS L^{-1} and 20.93 ± 1.13 mg TS L^{-1} for OMW and OL extract samples, respectively, with significant differences between them ($p < 0.05$).

Although the two types of extracts have a different phenolic profile, both showed a similar response in terms of DPPH radical scavenging activity, with IC50 values with very similar concentrations in both extracts. The positive control was carried out with quercetin which showed an IC50 value of 2.31 $\pm$ 0.01 mg L^{-1}.

4. Discussion

The results of this study relate the new extraction methods analysed and show that in the case of OMW extract, the direct analysis of the phenolic composition of OMW after centrifugation and filtration did not show qualitative or quantitative differences compared to the standard method recommended by the literature (ultrasound-assisted extraction in methanol–water mixture) [36]. For the conventional extraction of OMW in methanol–water, unfiltered OMW (with suspended solids) was used and after sonication in a methanol–water solution the sample was centrifugated (to separate solids from liquid) and filtrated to refine the extract. For the new and direct method to obtain the OMW extract, the unfiltered OMW was centrifugated a step (to separate solids from liquid) and filtrated to refine the removal of solids. In this way, whether the extraction with methanol achieved a higher extraction of compounds present in the suspended solids was compared to the simplest direct method which recovers the polyphenol dissolved in the liquid phase. The results show that the use of methanol does not provide a significant advantage in recovering the polyphenols adsorbed to the solid particles of OMW. However, a slight increase in concentration was achieved with methanol extraction, maybe due to the recovery of the adsorbed polyphenol on the solid particle of the extract. Both treatments yielded a phenolic profile similar to that described by Azaizeh et al. (2012) [21] and Goldsmith et al. (2014) [36] in extractions with organic solvents, with hydroxytyrosol being the main phenolic compound detected. Moreover, for OMW, the hydroxytyrosol content and the total phenolic compounds contents in our study showed similar values to those described in previous studies [21,36]. However, the new method proposed in our study for extraction of oleuropein (OL) showed less extraction power compared to the standard method (ultrasound-assisted extraction in methanol–water mixture). Although both methods result in a similar qualitative phenolic profile, the steps taken in the new method after the extraction in the methanol-water mixture (evaporation of solvent and recovery of the extract in water) resulted in a less concentrated phenolic extract. The OL extract obtained via the new method proposed in our study showed about a 25% and 50% reduction in total phenolic compounds and oleuropein compared with the standard method, respectively. In both treatments analysed, oleuropein was the main phenolic compound detected in OL extracts, as was also reported by several studies with organic or aqueous solvents [29–33,35]. Japón-Luján et al. (2006) [40] and Şahin and Samli (2013) [41] reported similar results in oleuropein content and total phenolic content in extractions with organic solvents compared to our results when we used the standard method. In the case of the new method analysed, our results were similar to or better than reports in the literature, in the case of studies which analysed water as a solvent for extraction in OL [29–35]. In this sense, our results with a total phenolic content of about 10 mg GAE gdw^{-1} was similar to that described by Papoti et al. (2018) [33] obtained with ultrasound-assisted extraction using water as the solvent. On the other hand, the content of oleuropein obtained by the proposed new method (about 10 mg gdw^{-1}) was similar to the results described by Ansari et al. (2011) [29] and Ghomari et al. (2019) [31] in extracts obtained in distilled water at 60 °C in acidic conditions, and also similar to the results described by Huguet-Casquero et al. (2020) [32] in aqueous extraction into a pre-heated water bath at 80 °C. Moreover, our results were better than those described by Yateem et al., 2014 [34] and Benincasa et al. (2019) [30] with concentrations of oleuropein below 0.8 mg g^{-1} when aqueous extraction with maceration was used. Therefore, the poor results obtained when water is used as final solvent may be due to a lower water solubility of certain phenolic compounds present in the olive leaf. In fact, after centrifugation of the aqueous extract a sediment was observed in the centrifuge tube.

The comparison of OMW extract with OL extracts obtained in aqueous solution showed that the total phenolic content in OMW was 10 times higher compared to OL, when the data referenced the dry weight of the extracts. These differences are reduced when the data are analysed in reference to their concentration in extracts, expressed as mgL^{-1}. The differences between OMW and OL are greater in the dry weight data because the data are corrected for the water content of the raw material. In this case, as the water content is much higher in OMW compared to olive leaf, the differences are more pronounced when the data are expressed in dry weight. When discussing the data regarding their bioactivity, we prefer to compare them with their parameters expressed in the concentration unit mg L^{-1} in the extract, because it is closer to the comparable content of the bioactive compounds in their possible direct application form. So, the total phenolic content was approximately still two times higher in the case of OMW compared to OL when the data are compared as concentrations in mg L^{-1}. Furthermore, a significant correlation of concentration expressed in mg L^{-1} with total solids content of the extracts was found (see Table 2).

Despite these observed differences, the analysis of their antioxidant capacity showed that both have a similar potential with similar EC 50 values, around 20 mg TS L^{-1}. These EC 50 values are similar to those described by Azaizeh et al. (2012) [21] and Lins et al. (2014) [42] for OMW and OL extracts, respectively, in extracts obtained in organic solvents and which according to these studies represent a high antioxidant capacity. Furthermore, Carrasco-Pancorbo et al. (2005) studied the antioxidant capacity of hydroxytyrosol, tyrosol, and oleuropein at two different concentrations of the isolated phenolic compounds via DPPH test. They found hydroxytyrosol had the highest capacity followed by oleuropein and tyrosol. Additionally, the antioxidant activity was dependent on polyphenol concentration. In spite of the similar EC50 at around 20 mg TS L^{-1} for both extracts, the antioxidant capacity at low polyphenols concentration was higher for OMW, for A, than for B (see Figure 4). This could be explained by the higher antioxidant activity of hydroxytyrosol (the main component in OMW aqueous extract) with respect to oleuropein (the main component on OL extract) as it was studied by Carrasco-Pancorbo et al. [43].

5. Conclusions

Despite the fact that several methods recommend extraction of olive oil mill wastewater using methanol, which could extract a higher content of suspended solids together with the dissolved ones, this study confirms that the direct use of the OMW, without extraction via methanol–water, provides a similar content of phenols in the final extract. On the other hand, in the case of leaf extraction, recovery in water after extraction with methanol–water reduces the total phenol content of the extract, compared to the methanolic extract, but with similar results that were reported in other studies using water as the solvent.

However, the extracts obtained in aqueous solution from OMW and the olive leaf in water, although they differ in qualitative and quantitative content of polyphenol, both present a similar antioxidant capacity.

Both extracts obtained in aqueous solution have high antioxidant capacity, similar to reports by previous studies of extraction in organic solvents, which facilitates extract application in biological systems in which an extract based on a water solution is needed.

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