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ULTRASOUND ASSISTED EXTRACTION OF POLYPHENOLS WITH HIGH ANTIOXIDANT ACTIVITY FROM OLIVE POMACE (*Olea europaea* L.)

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ABSTRACT

Olive pomace is an industrial by-product resulted from the olive oil production process. This study was carried out to optimize the extraction of polyphenols with high antioxidant activity from olive pomace using different techniques. The extraction was performed using homogenization and ultrasonic techniques at different solvent/pomace ratios till polyphenols reached a plateau. Total phenolic content was determined with the Folin-Ciocolteau method. Extracts were analyzed by HPLC for polyphenol and flavonoid contents. Scavenging activity of the extracts was determined against 1,1-diphenyl-2-picryl-hydrazyl and hydrogen peroxide radicals. The highest yield of the polyphenols (86.13±0.80 mg gallic acid equivalents/g dried defatted pomace) was recorded after 30 min of extraction using ultrasonic technique and 40/1 methanol (80%)/pomace (v/w) ratio. Extracts obtained by the methanol/sample ratio of 20/1 and high ultrasonic intensity for 7 min possessed higher antioxidant activity than the synthetic antioxidant, butylated hydroxytoluene.

1. Introduction

Olive pomace is the main agricultural by-product of the olive industry. It represents a particular environmental problem. The types and concentrations of polyphenols in olive pomace depend on the cultivar, agro-climatic conditions, fruit maturity, fruit storage, and extraction method. About 90% of the total phenolic compounds in olive products are present in the free form (Alu'datt *et al*., 2010). A two-phase extraction system accumulated metabolites in the solid pomace (Boskou, 2015). The majority of polyphenols present in olive pomace are hydroxytyrosol, oleuropein, and tyrosol, in addition to, caffeic acid, ρ-coumaric acid, vanillic acid and rutin (Ciriminna *et al*., 2016). Many of the phenolic compounds showed antioxidant activity. Recent investigations are focussed on the isolation and evaluation of antioxidant phenolics from plant wastes. Acoustic cavitation of ultrasound-assisted extraction (UAE) technique causes molecular movement of solvent and sample. Advantages of the UAE include high efficiency, reduced extraction time and low solvent consumption versus conventional extraction techniques (Jerman *et al*., 2010; Wong Paz *et al*., 2015).

Most methods of extracting polyphenols from olive pomace use a solvent/material ratio of 5/1 up to 25/1 and an extraction time of 15 min to 12 hours , with temperatures from 25 to 70 °C or higher. These different techniques have a yield that ranges from 1.29 to 60 mg gallic acid equivalent/g dried pomace (Alu'datt *et al*., 2010; Lafka *et al*., 2011; Aliakbarian *et al*., 2012; Ramos *et al*., 2013). Accordingly, this study was carried out to optimize the extraction conditions of the polyphenols from olive pomace using homogenization, and ultrasound techniques and

to evaluate the antioxidant activities of the obtained extracts.

2. Materials and methods

2.1. Materials

2.1.1. Samples

Olive pomace Two-phase Maraki variety used in the experiment was collected from an olive oil factory by-product (Mini Frantoio Oliomio-50-60 Centrifuge, Italy) located in Agricultural Research Centre, Giza, Egypt. The obtained pomace was dried in an oven at 70 °C under vacuum (70 mm Hg). Solid samples were ground using a laboratory mixer. Dried samples were extracted with petroleum ether (b.p. 40-60 °C) as a solvent to remove the residual oil using a Soxhlet apparatus for 4 h.

2.1.2. Reagents and standards

HPLC-grade solvents were purchased from Merck (Darmstadt, Germany). Folin–Ciocalteau phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), polyphenol reference standards: syringic acid, gallic acid, pyrogallol, 4-aminobenzoic acid, 3-hydroxytyrosol, protocatechuic acid, catechin, chlorogenic acid, catechol, epicatechin, caffeine, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, ρ-coumaric acid, ferulic acid, oleuropein, ellagic acid, benzoic acid, coumarin, naringin, rutin, hesperidin, rosmarinic acid, quercitrin, quercetin, naringenin, hesperetin were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Methods

2.2.1. Extraction of polyphenols

Dried, defatted and milled pomace samples (2.0±0.05 g) were extracted with aqueous methanol (methanol/water, 80/20, v/v) to pomace ratios (v/w) of 20/1 and 40/1, at room temperature. Extraction used UAE technique according to Jerman *et al.* (2010). Homogenization technique was performed according to Arslan and Ozcan (2011). Alcoholic extracts were centrifuged using Hettich Universal Centrifuge D7200, Germany at 327 *g*/min for 5 min.

2.2.1.1. Homogenization extraction technique

Homogenization (T1 and T2 used solvent/pomace ratios (v/w) of 20/1 and 40/1, respectively) was conducted using Heidolph type ST1 homogenizer (Germany) at the maximum power (264 W) for 10-60 min, at intervals of 10 min.

2.2.1.2. Ultrasonic-assisted extraction (UAE) technique

UAE (T3- T6, a solvent/pomace ratio (v/w) of 20/1) used a Fisher-Sonic, Dismemberator Model 300, USA at 10, 20, 40 and 50% of the maximum output power (300 W), respectively, for 10 min, at one minute intervals. In T7, extraction was performed using a solvent/pomace ratio of 40/1 at 50% of the maximum output power for 10 min, at one minute intervals and continued for 60 min, at intervals of 10 min.

2.2.2. Determination of total polyphenols

Total polyphenols (TP) content was determined using the Folin-Ciocalteu reagent and Unico UV-2000 Spectrophotometer, USA at 750 nm according to Zhou *et al.* (2017). A standard calibration curve was prepared using gallic acid (50–800 mg/L). Total polyphenol concentration was calculated from a calibration curve (r^2 = 0.9951). Spectrophotometric analysis was carried out for each extract in triplicate. Results were expressed as mg gallic acid equivalents (GAE)/g dried defatted pomace (ddp) ± standard deviation (SD) and μg GAE/mL of the olive pomace extract (OPE) ± SD.

2.2.3. HPLC analyses of polyphenols

HPLC was used for the identification and quantification of polyphenols for the extract with the highest yield that obtained by each extraction technique. The assays were performed with Agilent Technologies 1200 HPLC series, USA equipped with Agilent

1200 Series quaternary pump, vacuum degasser, and Agilent UV-VIS detector. Five microliters of the extract were injected into a column (Zorbax ODS, 250 mm×4.6 mm inner diameter, Agilent, USA) at room temperature. The solvent system used a gradient of A (8% CH_3COOH) and B (acetonitrile). The separation was obtained with the following gradients: at 0 min, 5% A and 95% B; at 5 min, 25% A and 75% B; at 10 min, 45% A and 55% B; at 15 min, 65A and 35% B; at 20 min, 85% A and 15% B; and from 25 to 30 min, 99% A and 1% B. The solvent flow rate was 1 mL/min, and separation was performed at 35°C. Wavelength of the UV-VIS detector was set at 330 nm for polyphenols and 280 nm for flavonoids. Identification was accomplished by comparing the retention time of the analyte with that of a reference standard. The results were expressed as mg/g ddp. Quantification of the identified compounds was performed using the calibration curves of the reference standards.

2.2.4. Antioxidant assays

2.2.4.1. DPPH radical scavenging activity

DPPH free radical-scavenging activity of the extracts was determined, according to Zhou *et al.* (2017). The absorbance was measured at 517 nm against methanol (blank) using Unico UV-2000 Spectrophotometer, USA. The synthetic antioxidant BHT was used as a reference compound (positive control) at 50, 100, 150 and 200 µg/mL. The inhibition percentage of DPPH radicals was calculated according to the following formula:

$$\% \text{ inhibition} = [A_b - A_s / A_b] * 100 \quad \text{Eq. (1)}$$

Where A_b and A_S stand for the absorbance of blank and sample or reference, respectively. The concentration of the test extract or reference providing 50% inhibition (IC_{50}, expressed in µg/mL) was calculated from the graph plotted with inhibition percentage against the concentration. Assays were carried out in triplicate and the results were expressed as mean values ± SD.

2.2.4.2. Hydrogen peroxide scavenging activity

The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Amessis-Ouchemoukh *et al*. (2017). The absorbance of the reaction mixture was recorded after 10 min at 230 nm using Unico UV-2000 Spectrophotometer, USA against a blank solution containing the phosphate buffer without hydrogen peroxide. The inhibition percentage of H_2O_2 was calculated according to Eq. (1)

2.2.5. Statistical analyses

Polyphenol extraction and evaluation of the antioxidant activity of the extracts were carried out in triplicate. The data were analyzed using Costat statistical software version 6.4. The significance of the differences of the means at a 5% level used one-way analysis of variance (ANOVA) and Duncan's multiple-range test. The IC_{50} values were obtained with Origin 2016 software (Origin Lab Corporation, USA).

3. Results and discussions

3.1. The effect of extraction conditions on the yield of total polyphenols

Aqueous methanol was an efficient solvent to extract lower molecular weight polyphenols (Pintać *et al*., 2018). The effects of solvent/pomace ratio, time and technique on the TP of OPE are shown in Fig. 1.

The contents of polyphenols extracted by homogenization at a solvent/pomace ratio (v/w) of 20/1 (T1) reached a maximum level (21.3±0.30 mg GAE/g ddp) after 30 min (Fig. 1a). Further increases in extraction time did not significantly (p>0.05) increase the yield of the extracted polyphenols. Increasing the solvent/pomace ratio to 40/1, (T2) increased significantly (P < 0.05) polyphenol yields to 74.35±0.93 mg GAE/g ddp during the same extraction time (Fig. 1a). Beyond 30 min, the yield of the polyphenols decreased sharply (p<0.05) and reached a minimum at 60 min, possibly because of the decomposition of the active compounds during the prolonged

homogenization time. These results are in agreement with the findings of Zhu *et al*. (2016).

On the other hand, increasing the ultrasonic intensity during the extraction of polyphenols from 10% to 50% of the maximum ultrasonic output power using a methanol (80%)/pomace ratio 20/1 (v/w) caused a remarkable increase in the recovery of polyphenols (T3-T6, Fig. 1b). The extracted polyphenols using 50% of the maximum ultrasonic output power were twice as high as those at 10% at identical extraction times. The significantly (p<0.05) highest yield of the polyphenols (86.13±0.80 mg GAE/g ddp) was recorded after 30 min of extraction using UAE at 50% of the maximum output power and 40/1 methanol (80%)/pomace (v/w) ratio (T7, Fig. 1c). The yield of T7 was higher than that obtained by T6 that conducted for a short time (7 min) using low solvent/pomace ratio (20/1). This could be due to the distribution of pomace in the solution is rather low and diluted, since it needs more time before decomposition of polyphenols by oscillation.

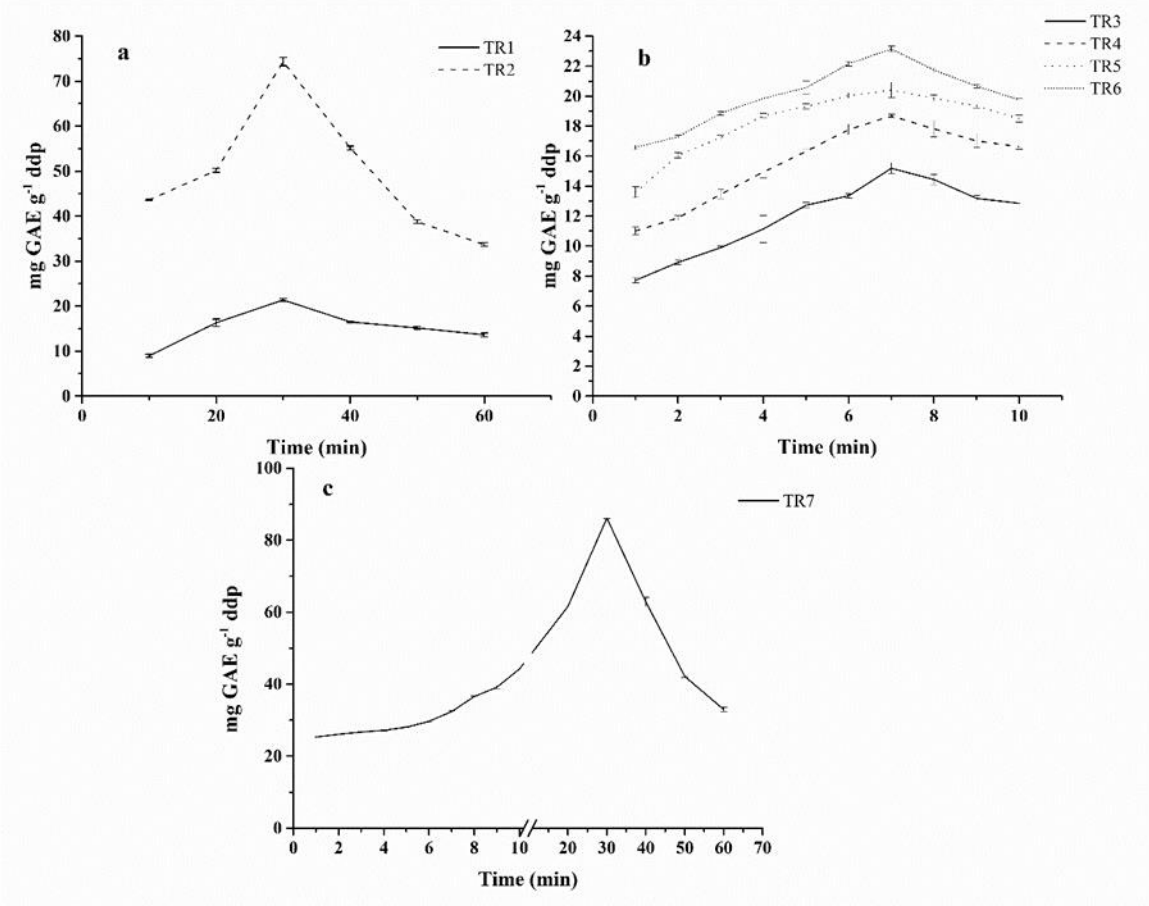

Figure 1. Yield of the polyphenols (mg GAE/g ddp ± SD) during (a) homogenization using solvent/ pomace ratios 20/1 (T1) and 40/1 (T2); (b) ultrasonic assisted extraction using solvent/ pomace ratio 20/1 at ultrasonic intensity of 10%, 20%, 40% and 50% of the maximal output power (T3-T6, consecutively); (c) ultrasonic assisted extraction using solvent/pomace ratio 40/1 at ultrasonic intensity of 50% of the maximal output power (T7). Error bars indicate the standard deviation of triplicate values (p<0.05)

These results exceeded those obtained by other investigators (Aliakbarian *et al*., 2012; Neviani *et al*., 2019) who found that the TP yield of OPE ranged from 9.1 to 68 mg/g dried pomace. The results indicated that extending UAE time, under T7 conditions, to 60 min was accompanied by a significant (p<0.05) reduction in the yield of TP to 30.13±0.15 mg GAE/g ddp (Fig. 1c). This could be due to the degradation of polyphenols by excessive Ultrasonic. These results are in agreement with previous studies (Zhang *et al*., 2015; Sun *et al*., 2016). They found that increasing the extraction time increased the recovery of TP until it reached a plateau, but further increases in extraction time caused a drop in the extracted polyphenols. Long extraction time increased the chances of polyphenol oxidation. Selecting an efficient extraction method to maintain the stability of the polyphenols is critically important because conventional extraction methods such as maceration have low efficiency and require long extraction times (Plazzotta and Manzocco, 2018).

Increasing solvent/pomace ratio (v/w) from 20/1 to 40/1 provided significantly (p<0.05) higher yield of extracted polyphenols at each extraction time regardless of the extraction technique used (Fig. 1). This could be due to mass transfer principles. The extraction efficiency of analytes in the sample depends on the intensity of the ultrasound transmitted to the medium and the number of cavitation bubbles produced. Ultrasonic waves create expansion-compression cycles in extracting media. These generate strong liquid jets that rupture the cells (Rodsamran and Sothornvit, 2019). During high-intensity ultrasound waves, the implosion of gas bubbles in liquid generates intense pressure within the material, causes plant tissue disruption, enhances penetration of the solvent into cellular materials, facilitates the transfer of components from the cell into the solvent and improves the mass transfer rate (Boskou, 2015). This could explain why ultra-sonication is more effective in extractability than homogenization.

3.2. HPLC analyses of polyphenols

HPLC analyses of the resulting extracts of each extraction technique with the highest yield of polyphenols are illustrated in Figures 2, 3 and tabulated in Table 1.

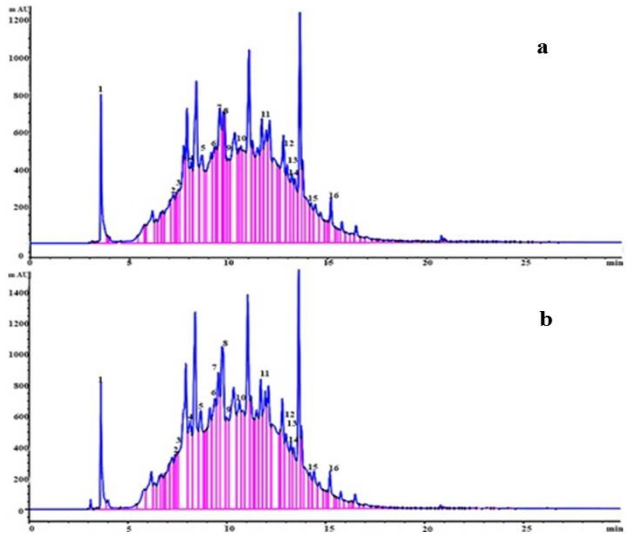

Figure 2. HPLC chromatogram of olive pomace extract polyphenols using (a) homogenization; (b) ultrasonic techniques with solvent/pomace ratio of 40/1. Peaks:1, syringic; 2, gallic acid; 3, pyrogallol; 4, 3-OH-Tyrosol; 5, protocatchuic; 6, catechins; 7, chlorogenic; 8, catechol; 9, caffeine; 10, 4-OH-benzoic; 11, vanillic; 12, ferulic acid; 13, oleuropein; 14, ellagic acid; 15, benzoic acid; 16, coumarin.

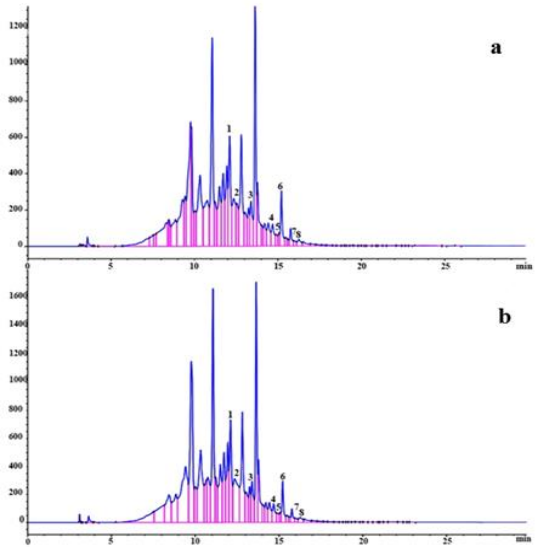

Figure 3. HPLC chromatogram of olive pomace extract flavonoids using (a) homogenization; (b) ultrasonic technique with solvent/pomace ratio of 40/1. Peaks:1, naringin; 2, rutin; 3, hesperidin; 4, rosmarinic acid; 5, quercetrin; 6, quercetin; 7, narengenin; 8, hespertin.

Table 1. Identified phenolic compounds (mg/g dried defatted pomace) of olive pomace extracts obtained by different extraction techniques

Compounds	Extraction Techniques	
	Homogenization	Ultrasonic
Polyphenols		
Syringic acid	0.368	0.574
Gallic acid	0.150	0.196
Pyrogallol	8.607	12.834
4-Aminobenzoic acid	0.441	0.484
3-Hydroxytyrosol	7.894	13.028
Protocatechuic acid	0.961	1.213
Catechin	0.310	1.347
Chlorogenic acid	0.058	0.909
Catechol	2.67	4.976
Epicatechin	0.531	1.675
Caffeine	0.419	0.254
4-Hydroxybenzoic acid	2.867	3.991
Caffeic acid	0.311	0.179
Vanillic acid	0.808	1.862
ρ-coumaric acid	0.547	0.833
Ferulic acid	0.544	1.181
Oleuropein	9.139	13.112
Ellagic acid	1.88	2.374
Benzoic Acid	8.37	2.992

Coumarin	0.159	0.169
Salicylic acid	1.912	0.630
Flavonoids		
Naringin	2.390	2.342
Rutin	0.266	0.570
Hesperidin	3.760	4.413
Rosmarinic acid	0.161	0.178
Quercitrin	0.310	0.031
Quercetin	0.337	0.230
Naringenin	0.214	0.274
Hesperetin	1.811	1.671
Kaempferol	0.060	0.064
Rhamnetin	0.069	0.065
Apigenin	0.047	0.031

Many polyphenols were detected in the OPE and include oleuropein, hydroxytyrosol, pyrogallol, catechol, ellagic acid and benzoic acid. The OPE obtained via homogenization was characterized by higher concentrations of benzoic acid, caffeine and caffeic acid versus other extracts. Sonication resulted in the highest recoveries for the other polyphenols. Sonication extracted ≥1.5 fold more syringic acid, pyrogallol, 3-hydroxytyrosol, chlorogenic acid, catechol, epicatechin, 4-hydroxybenzoic acid, vanillic acid, ρ-coumaric acid, ferulic acid and oleuropein versus other technique.

The most common flavonoids in the extracts were hesperidin, naringin, and hesperetin. Rutin, quercetin, rosmarinic acid, naringenin, and quercitrin were also found in considerable concentrations. The sonicated extract had the highest concentrations of rutin, hesperidin, and rosmarinic acid. On the other hand, the homogenized extract was characterized by higher concentrations of quercitrin and naringin versus the other investigated extracts. These results are consistent with those reported by other researchers (Gomez-Rico *et al*., 2009; Boskou, 2015).

3.3. Radical scavenging activity of pomace extracts

Polyphenols and flavonoids are the most common antioxidants in olives. Two assays based on different radicals (DPPH and hydrogen peroxide) assessed the antioxidant activity of the extracts during the upward part of the polyphenolic yield for each extraction technique. Hydrogen peroxide activity comes from its potential to produce a highly reactive hydroxyl radical through Fenton reaction (Kerins and Ooi, 2018). The DPPH and H_2O_2 radical-scavenging activities were recorded in terms of % inhibition (Fig. 4-5); IC_{50} values were deduced from the graphs. The results were compared to BHT as a reference standard in concentrations from 50 to 200 μg/mL to deduce the IC_{50} for BHT (Fig. 5f).

The lowest IC_{50} values for DPPH and H_2O_2 (98 μg GAE/mL and 105.8 μg GAE/mL, respectively) of the homogenized extracts were recorded by T1 (Fig. 4a). However, the highest antiradical activity was recorded for the homogenized extracts obtained by T2 (75.39 ± 0.99% and 71.07 ± 0.5% against DPPH and H_2O_2 radicals, respectively) (Fig. 4b).

The extract obtained by UAE (T3) at 10% of the maximum output power (300 W) showed a lower ability to reduce free radicals (Fig. 5a). On the other hand, the extracts obtained by UAE (T4, Fig. 5b) exhibited lower IC_{50} values against DPPH and H_2O_2 radicals than those of T5 (Fig. 5 c).

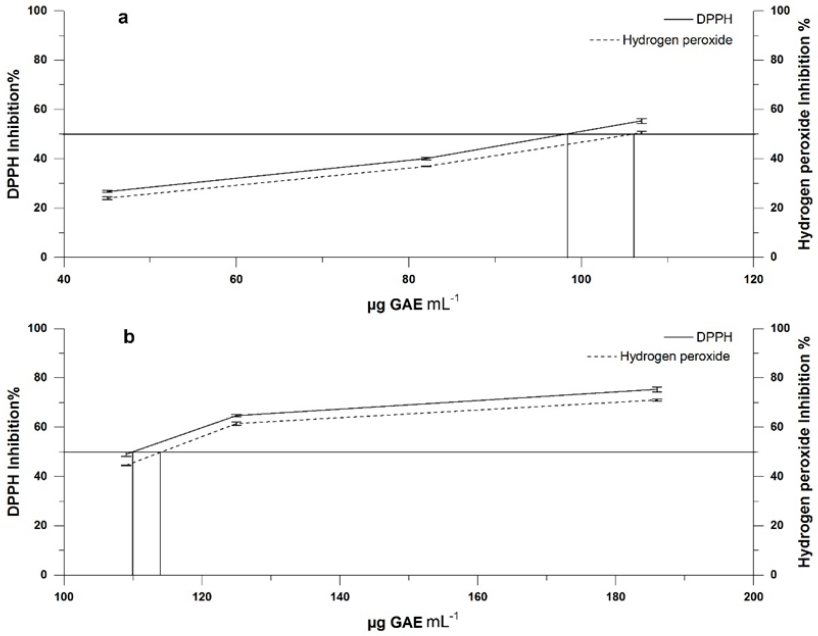

Figure 4. Inhibition percentage of DPPH and H_2O_2 radicals ± SD and IC_{50} values of extracts obtained by homogenization as a function of solvent/pomace ratio (20/1 (a); 40/1(b)) and performed at different polyphenol concentrations. Error bars indicate the standard deviation of triplicate values ($p<0.05$)

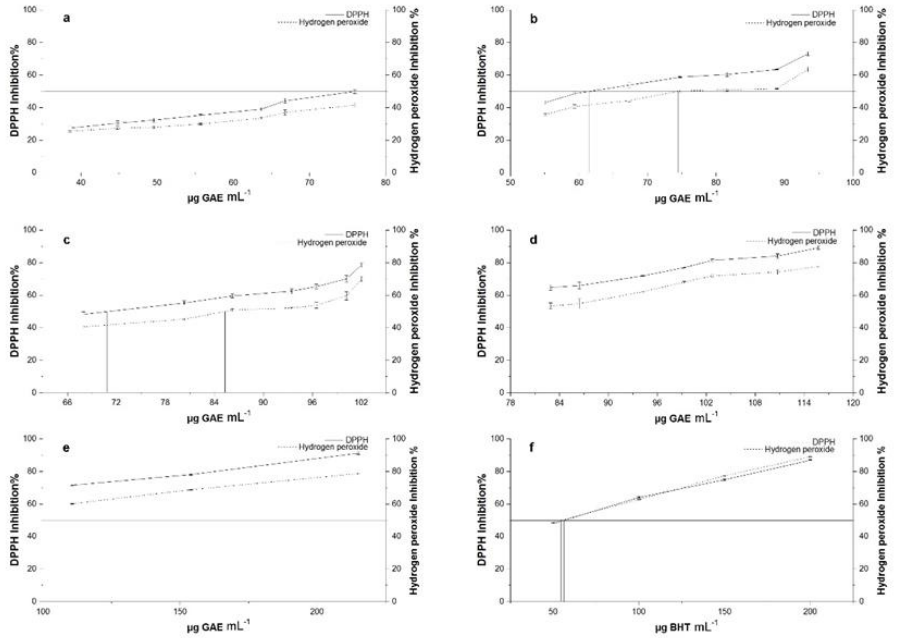

Fig 5. Inhibition percentage of DPPH and H_2O_2 radicals ± SD and IC_{50} values of extracts as a function of ultrasonic intensity (10%, 20%, 40% and 50%, (a-d)) at solvent/pomace ratio of 20/1, and at 50% of ultrasonic intensity and solvent/pomace ratio of 40/1 (e) and performed at different polyphenol and BHT (f) concentrations. Error bars indicate the standard deviation of triplicate values ($p<0.05$)

At high ultrasonic intensity, the sonicated extracts (T6, 7 min, 115.75 μg GAE/mL, Fig. 5d) and (T7, 30 min, 213.3 μg GAE/mL, Fig. 5e) displayed the significantly highest (p<0.05) antioxidant activity against DPPH radicals (DPPH % inhibition 89.20% and 91.30%, respectively) among all the investigated extracts. However, the sonicated extract (T6) showed a significantly higher (p<0.05) level of free radical-sequestering activity than the sonicated extract of T7, at the same concentration. This result may be attributed to the individual polyphenols present in each extract. The efficacies of the extracts could be classified in the following order: extract obtained by UAE > extract obtained by homogenization. The sonicated extract was rich in hydroxytyrosol, oleuropein, pyrogallol and catechol as illustrated in Table 1. These polyphenols have a significant DPPH-quenching ability (Xie and Schaich, 2014). The scavenging ability of olive pomace extracts obtained by homogenization or UAE at low intensity (< 50%) was found to be polyphenols concentration dependent.

The IC_{50} values of DPPH and H_2O_2 of the reference antioxidant BHT were 52.96 μg/mL and 53.56 μg/mL, respectively (Fig. 5f). These values for BHT agree with those reported by Xu *et al*. (2009). The results illustrate that olive pomace sonicated extract (T6) possessed higher antioxidant activity than that of the synthetic antioxidant BHT (Fig. 5f). At 115.75 μg GAE/mL of OPE (T6), 89.20% of the DPPH radicals were inhibited. The same effect required ~200 μg BHT/mL.

4. Conclusions

This study showed that extraction of the polyphenols from pomace is not favored at times longer than 30 min during homogenization or UAE. The UAE maximized the extracted polyphenols from olive pomace and increased the antioxidative activities in the extract. The scavenging activities against DPPH and H_2O_2 radicals reflected the unique antioxidant activity of the olive pomace extract obtained by the UAE.

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