

APPLICATION OF ENZYME AND ULTRASOUND ASSISTED EXTRACTION OF POLYPHENOLS FROM AVOCADO (*PERSEA AMERICANA* MILL.) PEEL AS NATURAL ANTIOXIDANTS

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ABSTRACT

Background. Hass avocado (*Persea americana* Mill.) peel is a rich source of natural antioxidants. The present work aims to evaluate ultrasound and enzyme-assisted extraction methods of polyphenols with antioxidant properties from avocado peel.

Materials and methods. The impact of extraction parameters on the extraction yield of polyphenols was assessed using the Folin-Ciocalteu reagent method, while the antioxidant activity of the extracts was evaluated using DPPH and FRAP assays. The polyphenolic compounds were identified by HPLC.

Results. The major polyphenolic compounds identified in the investigated extracts were benzoic acid, vanillic acid, resveratrol and syringic acid. The highest yield of polyphenols ~35.4 mg GAE/g of dried peel was obtained with a solid-to-solvent ratio of 1:20 (w/v) using 20% ultrasonic intensity for 30 min or by treatment with viscozyme at a 1% level for 60 min. The IC₅₀ values by DPPH and FRAP in the ultrasound assisted extract were statistically lower than those in the enzyme assisted extract. The avocado peel extract is a promising source of antioxidants.

Conclusion. The ultrasound assisted extraction proved to be more efficient than enzyme aided extraction in terms of the antioxidant activity of the extractable phenolic compounds.

Keywords: avocado peel, ultrasound, enzyme, extraction, polyphenols, natural antioxidants

INTRODUCTION

Avocado (*Persea americana*, Mill., family Lauraceae Juss.) is a tropical fruit that matures on the tree and ripens after its harvest. It is increasingly cultivated around the world due to global interest and rising consumption. The fruit has nutritional benefits since it contains high levels of vitamins (A, B, C and E) and phenolic compounds. The plant possesses medicinal properties, such as anticancer, antihypertensive, anti-inflammatory and antidiabetic (Arackal and Parameshwari, 2021). Hass is the most common cultivar

of avocado worldwide (Bhuyan et al., 2019). The peel of the ‘Hass’ avocado represents about 14% of fruit’s weight. An enormous quantity of peels is discarded as waste during avocado processing (Melgar et al., 2018; Salazar-López et al., 2020). The avocado by-product as agricultural waste is considered to be an important raw material in food and non-food applications (Colombo and Papetti, 2019).

Agro-industrial by-products can be used as raw materials for the production of high added value

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products (Calderón-Oliver et al., 2016). Avocado by-products have been evaluated in just a few studies in the literature, and the existing research articles used solvents that are not allowed by the FDA (2017), such as methanol and hexane (Morais et al., 2015; Hürkül et al., 2021).

Recently, several environmentally friendly technologies have been reported to reduce the negative effects of extraction conditions on the bioactivity of bioactive compounds (Carciochi et al., 2017). Therefore, nonconventional extraction methods, such as ultrasound-assisted extraction (U) and enzyme-based extraction (E), have been adopted to extract valuable compounds from plant processing byproducts with high efficiency. Efficiency of U is attributed to acoustic cavitation that leads to high mass transfer across cell membranes (Pan et al., 2012; Ramić et al., 2015). On the other hand, the E technique depends on the ability of an enzyme to degrade the cell wall and release the intracellular components (Zhu et al., 2014). Therefore, in order to valorize avocado peels, this investigation was carried out to study the enzyme and ultrasound assisted extraction conditions required to obtain a high yield of polyphenols. In addition, qualitative and quantitative analysis (HPLC method) and the antioxidant properties of the extracts with the highest content of polyphenols were also evaluated.

MATERIALS AND METHODS

Materials

Ten kg of Hass avocado fruits at ready-to-eat ripeness were purchased from a local market in Cairo, Egypt. The ripened avocado fruits were manually peeled. The peels were dried in a conventional oven at 45°C for 48 h until the moisture content reached less than 10%. The dried peels were then ground and milled to a particle size less than 0.45 mm before being stored at 4°C for further use.

Chemicals

The chemicals used were of analytical grade and supplied by Merck Chemical Company (Darmstadt, Germany). Viscozyme L. (A cellulolytic enzyme mixture including arabinase, cellulase, β -glucanase, hemicellulase and xylanase; 100 FBGU/g), Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol

(HPLC grade), acetonitrile (HPLC grade), standards of polyphenols (Quinol, gallic acid, p -hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, p -coumaric acid, benzoic acid, ferulic acid, ellagic acid, o -coumaric acid, resveratrol, cinnamic acid, rosmarinic acid, catechol, catechin, rutin, quercetin, naringin, myricetin and kampherol) and butylated hydroxytoluene (BHT) were purchased from Sigma Aldrich, USA.

Methods

Ultrasound assisted extraction (U). The U was performed by extracting the avocado peel powder (5 g) with 80% ethanol at solid-to-solvent ratios of 1:10 and 1:20 (w/v) and ultrasonic intensity (10% and 20% of the maximum power (300 W)) for extraction times of 15, 30, 45 and 60 min. Extraction was carried out at room temperature with magnetic stirring and an ultrasonicator (Fisher Sonic Dismemberator, Model 300, maximum power 300 W, 50 Hz, USA) equipped with a 19 mm diameter tip.

The solvent of the extracts with the highest polyphenol content was evaporated using a rotary evaporator (EYELA rotary evaporator N-1000, Japan) at 40°C before freeze-drying (Edward freeze dryer, England).

Enzyme assisted extraction (E). Avocado peel powder (5 g) was mixed with citrate buffer (0.1 M, pH 5) at solid-to-buffer ratios of 1:10 and 1:20 (w/v). Viscozyme L. was used at solutions of 0.5 mL and 1 mL/100 mL. Incubation was carried out for 30 and 60 min at 50°C in a shaker (G-25, New Brunswick Scientific Company, New Jersey) at 120 rpm. After incubation time, the enzyme was inactivated by immersion of the samples in a water bath at high temperature (90°C) for 5 min. Then the mixture was filtrated through Whatman No. 1 filter paper and the resultant filtrate (E) was centrifuged at 4,000 rpm for 10 min. The supernatant was collected and stored at 4°C for further analysis.

Freeze dried extracts were used for the identification of polyphenolic compounds by HPLC.

Identification of polyphenols by High Performance Liquid Chromatography (HPLC). The avocado peel lyophilized extracts were analyzed using Agilent 1260 infinity HPLC Series (Agilent, USA), equipped with a Quaternary pump, and the column used was

a Kinetex® 5 µm EVO C₁₈ 100 mm × 4.6 mm, (Phenomenex, USA), operated at 30°C. The separation was achieved under a gradient elution with (A) HPLC grade water 0.2% H₃PO₄ (v/v), (B) methanol and (C) acetonitrile as follows: 0–11 min (96% A, 2% B, 2% C); 11–13 min (50% A, 25% B, 25% C); 13–17 min (40% A, 30% B, 30% C); 17–20.5 min (50% B, 50% C), and 20.5–30 min (96% A, 2% B, 2% C). The VWD detector was set at 284 nm. The sample volume injected was 20 µL and the flow rate used was 0.7 mL/min. Identification of the phenolic compounds was carried out by comparing the retention times (RT) and UV spectra with those of standards stored in a database. Quantification was performed using an external standard method with reference samples of polyphenols.

Determination of total phenolic content (TPC).

The TPC was determined using the Folin-Ciocalteu reagent according to Rodríguez-Pérez et al. (2016). Briefly, 10 µL of the diluted extract was mixed with 600 µL of water and 50 µL of Folin-Ciocalteu reagent. After 1 min of reaction, 150 µL of Na₂CO₃ (20%, w/v) was added and mixed by vortex. The mixture was left for 2 h at room temperature in darkness. The absorbance of the resulting solution was read at 750 nm using a spectrophotometer Jenway (6800 UV/VIS, UK). Gallic acid (10–150 µg/mL) was used as the standard. The results were expressed in mg gallic acid equivalent (GAE)/g of dry sample as a mean of three replicates.

Determination of DPPH radical scavenging activity.

The DPPH radical scavenging activity of the avocado peel extracts was measured according to Liu et al. (2009). An aliquot of 0.1 mL of the tested extract was mixed with 3.9 mL of DPPH solution (2.5 mg DPPH in 100 mL methanol), then vortexed and left to stand for 30 min in darkness. The absorbance of the reaction mixture was measured against a blank with the same spectrophotometer mentioned above at 515 nm. The inhibition percentage was calculated according to the following formula:

$$\text{Inhibition, \%} = \frac{A_c - A_s}{A_c} \cdot 100$$

where:

A_s – the sample absorbance,

A_c – the absorbance of the control.

The IC₅₀ value was expressed as the concentration of the sample corresponding to an absorbance of 0.5. Butylated hydroxyl toluene (BHT) was used as a standard. The values are expressed as the mean of triplicate analyses.

Determination of reducing power. The reducing power of the extract samples was determined according to Hinneburg et al. (2006). The extracts (1.0 mL) were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of K₃[Fe(CN)₆] (1%). The mixture was incubated at 50°C for 20 min. 2.5 mL of 10% trichloroacetic acid was added to the mixture and it was centrifuged at 3000 rpm for 10 min. 2.5 mL of the upper layer was mixed with the same volume of distilled water, after which 0.5 mL FeCl₃ (0.1%) was added. Finally, the absorbance was measured with the same spectrophotometer mentioned above at 700 nm. The BHT was used as a reference standard. The extract concentration providing 0.5 of the absorbance (IC₅₀) was calculated using the graph showing absorbance against extract concentration. Measurements were made in triplicate.

Statistical analysis

Determination of the polyphenol content and the antioxidant activity assays were carried out in triplicate. The data are presented as mean values ±SD. The data were subjected to analysis of variance (ANOVA) followed by Tukey's test using XLSTAT (significance level $P < 0.05$).

RESULTS AND DISCUSSION

Effects of extraction techniques on the yield of polyphenols

Extraction with ultrasound technique. Figure 1 shows the effects of ultrasonic intensity, extraction time and solid-to-solvent ratio on the extraction yield of polyphenols in ultrasonic assisted extraction. The yield of polyphenols that recorded ~30 mg GAE/g of dried peel was obtained by extraction with a solid-to-solvent ratio of 1:10 (w/v) at 20% ultrasonic intensity after 15 min instead of 30 min with lower ultrasonic intensity (10% of the maximal output power). Extending the ultra-sonication time after reaching the highest recovery caused a significant ($P < 0.05$) decrease in

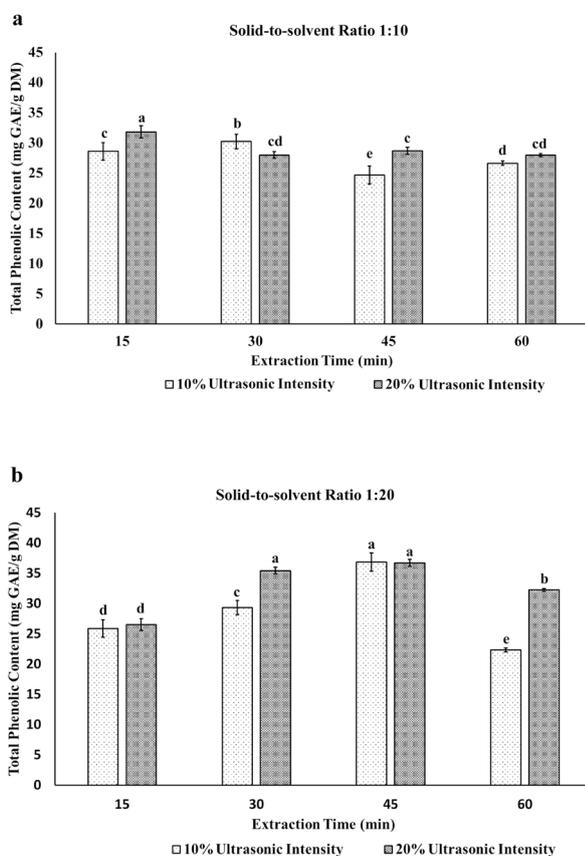


Fig. 1. Total phenolic content (mg GAE/g dry avocado peel) of the ultrasound-assisted extracts (U) obtained by (a) a solid-to-solvent ratio of 1:10 (w/v) and (b) a solid-to-solvent ratio of 1:20 (w/v) at 10% and 20% ultrasonic intensities and different extraction times. The results are represented as average values of three replicates \pm SD. Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's test

the polyphenol yield (Fig. 1a). However, a continual increase of the extraction time to 60 min was accompanied by a significant increase in the yield of polyphenols, but it did not reach the maximum yield of polyphenols that was obtained after 30 min. This increment could be due to extraction of polyphenols highly bound to polymers (polysaccharides or proteins) that need more time and/or higher ultrasonic intensity to liberate them. A prolonged extraction time may lead to oxidation of the phenolic compounds, which is attributable to light and oxygen exposure (Silva et al., 2007).

Extending the extraction time from 15 to 30 min using 20% ultrasonic intensity and 1:20 solid-to-solvent

ratio caused a significant ($P < 0.05$) increase in polyphenol yield (Fig. 1b). After 30 min, the yield of polyphenols reached a plateau. Increasing extraction time from 45 to 60 min after equilibrium caused a significant decrease in the yield due to decomposition and decreased extraction rate, as reported by Wang et al. (2018). Extraction of polyphenols using the same solid-to-solvent ratio but with 10% ultrasonic intensity required 45 times to reach maximum yield, after which a significant ($P < 0.05$) decrease in polyphenols occurred. Extraction with high ultrasonic intensity (20%) for 60 min caused a slight increase in the medium temperature, thereby enhancing the liberation of phenolic compounds bound to polymers and slowing down the rate of the polyphenols compared to that performed with 10% ultrasonic intensity. Ultrasound irradiation facilitates the mass transfer of the solute within the solvent (Samaram et al., 2015).

At a low solid-to-solvent ratio (1:10 w/v), high viscosity of the solution causes stronger cohesive forces among the molecules that has to be overcome by negative pressure in the rarefaction phase. With an increase in solvent volume (1:20 w/v), the viscosity of the extraction medium decreases, which increases the cavitation effect and the diffusivity of the extractable material in the solvent (Altemimi et al., 2015). The yield of polyphenols increases with an increase in solvent to solid ratio till it reaches a peak, after which it decreases.

Figure 1 shows that total polyphenol yield is somewhat higher for a solid-to-solvent ratio of 1:10 (w/v) than for 1:20 (w/v) after 15 min. This could be due to the short extraction time. It suppresses bubbles initiated by ultrasonic waves from growing to a size suitable for disruption, resulting in low yield levels, as reported by Altemimi et al. (2015).

The results in the same figure illustrate that increasing ultrasonic intensity did not significantly ($P > 0.05$) increase the yield of polyphenols at 45 min of extraction with a solid-to-solvent ratio of 1:20 (w/v) because the recovered polyphenols reached a peak.

Extending the extraction time to 60 min using a solid-to-solvent ratio of 1:20 (w/v) decreased the extracted polyphenols significantly ($P < 0.05$), regardless of the ultrasonic intensity used (Fig. 1b). The polyphenols extracted from brown algae increased when the liquid to solid ratio to was increased to 15:1 (w/v),

after which the yield decreased significantly (Han et al., 2011).

The results in Figure 1a and Figure 1b illustrate that the highest polyphenol yield (35.4 ± 1.08 mg GAE/g of dried peel) was reached using a solid-to-solvent ratio of 1:20 (w/v) at high ultrasonic intensity (20%) for 30 min or at low ultrasonic intensity (10%) for 45 min.

Figuroa et al. (2021) used microwave-assisted extraction for the recovery of polyphenols (37 mg GAE/g of avocado peel) at a peel powder-to-ethanol of 75% (v/v) and a ratio of 1:20 (w/v) for 15 min at a high temperature (110°C).

Enzymatic extraction with viscozyme L. The main factors in the enzyme-assisted extraction include enzyme concentration, extraction time and reaction temperature (Liyana-Pathirana and Shahidi, 2005).

The results in Figure 2 illustrate that the addition of the enzyme at both investigated levels (0.5% and 1%) using a solid-to-buffer ratio of 1:10 (w/v) for 30 min of extraction significantly increased ($P < 0.05$) the yield of polyphenols. However, decreasing the

solid-to-buffer ratio from 1:10 to 1:20 (w/v) significantly increased ($P < 0.05$) the yield of polyphenols at both investigated levels of the enzyme at each extraction time. This could be due to the low water content in the system (solid-to-buffer ratio of 1:10 w/v) that negatively affected enzyme performance since water is the crucial factor that enhances the function of enzymes (Rezaei et al., 2007).

Increasing enzyme concentration from 0.5% to 1% did not significantly ($P > 0.05$) increase the total polyphenol yield during the first 30 min of the enzymatic reaction, regardless of the solid-to-buffer ratio used. An increase in the enzyme amount at a low solid-to-solvent ratio increased the viscosity of the mixture and reduced mass transfer (Lin et al., 2016).

On the other hand, increasing the reaction time to 60 min and enzyme concentration to 1% using a solid-to-buffer ratio of 1:20 (w/v) caused a significant increase ($P < 0.05$) in the total polyphenol yield. The highest yield (35.1 ± 0.45 mg GAE/g of dried peel) was obtained by adding 1% enzyme using a solid-to-buffer ratio of 1:20 (w/v) after incubation for 60 min.

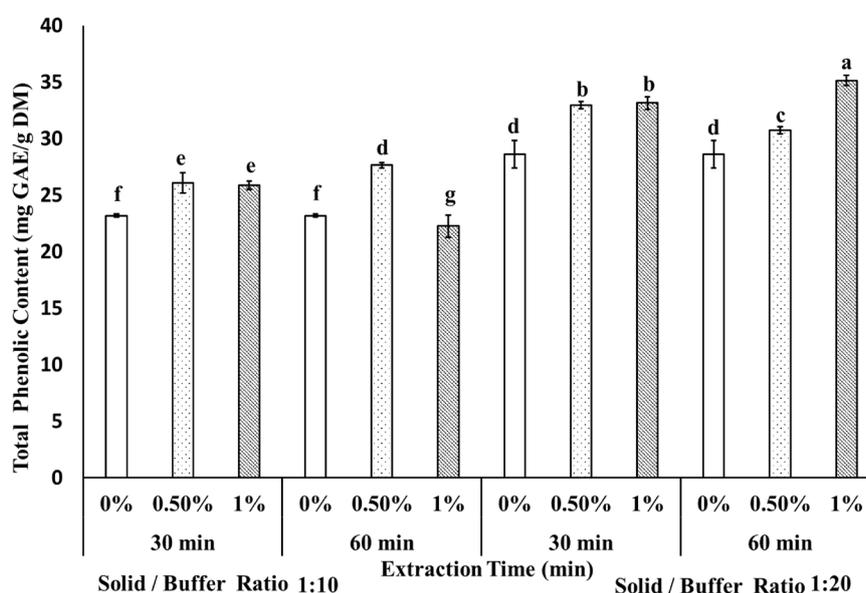


Fig. 2. Total phenolic content (mg GAE/g dry avocado peel) of the enzyme-assisted extracts (E) obtained by a solid-to-buffer ratio of 1:10 and 1:20 (w/v) at 0, 0.5 and 1% viscozyme concentration and different extraction times. The results are represented as average values of three replicates \pm SD. Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's test

Polyphenol profile of the avocado (U and E) extracts using HPLC

The main bioactive compounds in avocado fruit are polyphenols, since, avocado peels possess the highest content of total polyphenol (1252.31 ± 165.62 mg GAE/100 g of dried material) compared to the pulp

(297.72 ± 85.44 mg GAE/100 g of dried material) and seeds (155.30 ± 29.65 mg GAE/100 g of dried material) (Morais et al., 2015).

Table 1. Identified phenolic compounds of avocado peel extracts

Compounds	E	U
Phenolic compounds, $\mu\text{g/g}$ dried extract		
Quinol	ND	332.86
Gallic acid	6.43	79.80
<i>p</i> -hydroxybenzoic acid	16.64	204.50
Chlorogenic acid	8.34	255.33
Vanillic acid	138.27	5 386.57
Caffeic acid	57.47	ND
Syringic acid	53.44	1 528.88
<i>p</i> -coumaric acid	20.31	630.44
Benzoic acid	389.27	14 395.2
Ferulic acid	28.41	116.87
Ellagic acid	11.51	213.48
<i>o</i> -coumaric acid	32.79	63.93
Resveratrol	172.09	1 671.74
Cinnamic acid	1.65	35.93
Rosmarinic acid	58.79	373.86
Flavonoid compounds, $\mu\text{g/g}$ dried extract		
Catechol	45.48	451.90
Catechin	13.16	173.50
Rutin	50.74	570.96
Quercetin	190.881	171.51
Naringin	164.84	ND
Myricetin	78.46	ND
Kampherol	34.47	ND

E – enzyme extract, U – ultrasound extract.
ND – not detected.

The results in Table 1 indicate the major polyphenols identified in the investigated extracts with the highest polyphenol contents. The main identified polyphenols obtained by both extraction techniques were benzoic acid, vanillic acid, resveratrol and syringic acid. However, the levels of chlorogenic acid, vanillic acid, syringic acid, *p*-coumaric acid and benzoic acid in the enzyme assisted (E) extract were less than 3.5% of the levels of the same compounds in the ultrasonic assisted (U) extract. The levels of gallic acid, *p*-hydroxybenzoic acid, resveratrol, catechol, catechin and rutin in the U extract were ~ten times those in the E extract. The quercetin level (U and E) was nearly the same in both investigated extracts. The polyphenol with the highest level in both extracts was benzoic acid. Its level was 3 times that of vanillic acid. Melgar et al. (2018) reported that catechin and chlorogenic derivatives represented the main avocado peel polyphenols. Figueroa et al. (2018) found that 4-hydroxybenzoic acid, vanillic acid and quercetin were among the major phenolic compounds in avocado peel extract.

The results in Table 1 prove that caffeic acid, naringin, myricetin and kampherol were not detected in the U extract. The absence of these compounds in the U extract could be due to their insolubility in 80% ethanol because they are bound to polysaccharides in the cell wall and released by enzymatic degradation of the peel. Meanwhile, the quinol was not identified in the E extract. These differences in the composition of the extracts could be due to the variation of the extraction techniques (E and U) and the type of solvent used (buffer solution in the case of E extraction and ethanol in the U extraction).

Antioxidant activity of the (U and E) extracts

DPPH radical scavenging activity. Hass is the most explored avocado cultivar in terms of its antioxidant properties (Bhuyan et al., 2019). The results in Figure 3a indicate that the DPPH radical scavenging activity of the U extract ($IC_{50} = 38.77 \pm 0.45$ $\mu\text{g/mL}$) was superior to that obtained by enzymatic reaction E ($IC_{50} = 53.48 \pm 1.2$ $\mu\text{g/mL}$). The IC_{50} value of the BHT solution for DPPH radicals was 65 ± 2 $\mu\text{g/mL}$ (Fig. 3b). Antasionasti et al. (2017) found that the IC_{50} values

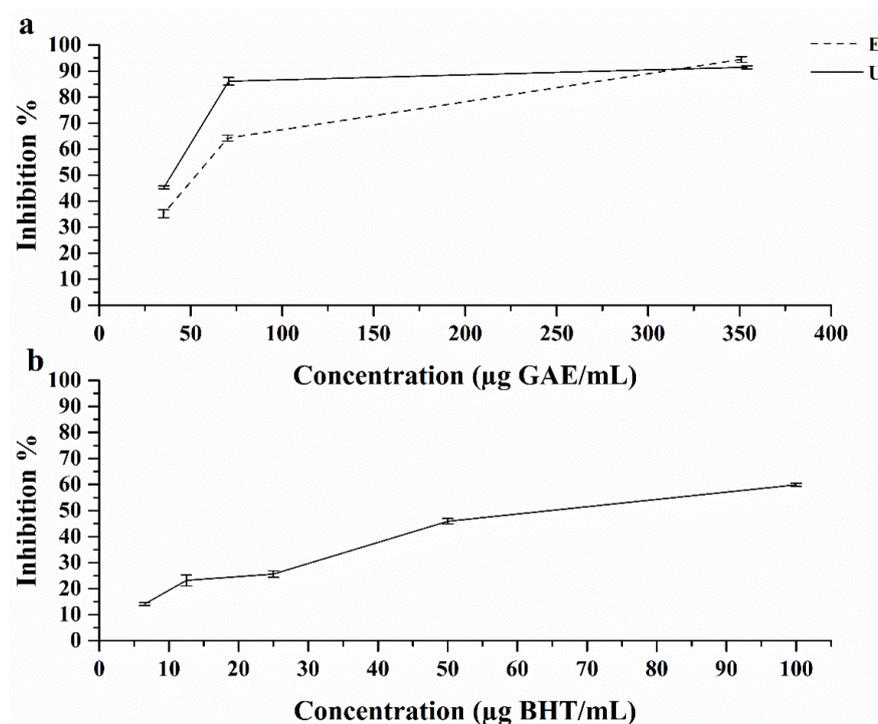


Fig. 3. DPPH radical-scavenging effects: **a** – ultrasound-assisted extract (U) and enzyme-assisted extract (E) of avocado peel, **b** – BHT. Each value is expressed as mean \pm SD ($n = 3$)

of DPPH for methanol, ethyl acetate and petroleum ether of the avocado peel extracts were 9.467 ± 0.045 , 18.387 ± 0.022 and 78.331 ± 0.210 $\mu\text{g/mL}$, respectively. The difference in antioxidant activity could be due to the different solvents used and the different composition of the extracted constituents.

Extracts with an IC_{50} value for DPPH radicals lower than 50 $\mu\text{g/mL}$ are considered strong antioxidants (Phongpaichit et al., 2007). Therefore, the ultrasonic extract (U) could be considered to be a strong antioxidant while the enzyme extract (E) has an intermediate level of activity. Nguyen et al. (2014) found that the antioxidant activity of the ultrasound extract of mulberry mash was higher than that obtained by enzyme-assisted extraction. This could be due to the increase in total phenolic and anthocyanin contents of the ultrasound extract. Morais et al. (2015) found that avocado dried peels had the highest content of total polyphenols (1252.31 ± 165.62 mg GAE/100 g of dried material) compared to the same part of the fruit of pineapple

(374.38 ± 78.19 mg GAE/100 g of dried material), banana (385.83 ± 8.58 mg GAE/100 g of dried material), papaya (315.45 ± 36.27 mg GAE/100 g of dried material) and passion fruit (86.74 ± 19.67 mg GAE/100 g of dried material). They studied the antioxidant activity of methanol extracts of the dried peels of the preceding fruits. Among them, avocado was the highest, followed by banana, papaya, passion fruit and pineapple. Their IC_{50} values for DPPH radicals were 18.22 ± 1.45 , 163.66 ± 14.32 , 328.46 ± 10.18 , 371.14 ± 13.05 and 407.15 ± 21.09 $\mu\text{g/mL}$, respectively.

Ferric reducing antioxidant power (FRAP). The antioxidant activity of polyphenols is related to their electron transfer ability that quenches free radicals by donating a hydrogen atom (Duan et al., 2007). The results in Figure 4a show that the FRAP of the U extract was higher ($\text{IC}_{50} = 16.2 \pm 0.5$ $\mu\text{g/mL}$) than that of the E extract ($\text{IC}_{50} = 27.11 \pm 1.1$ $\mu\text{g/mL}$). The reduction capacity of BHT against FRAP (Fig. 4b) was lower

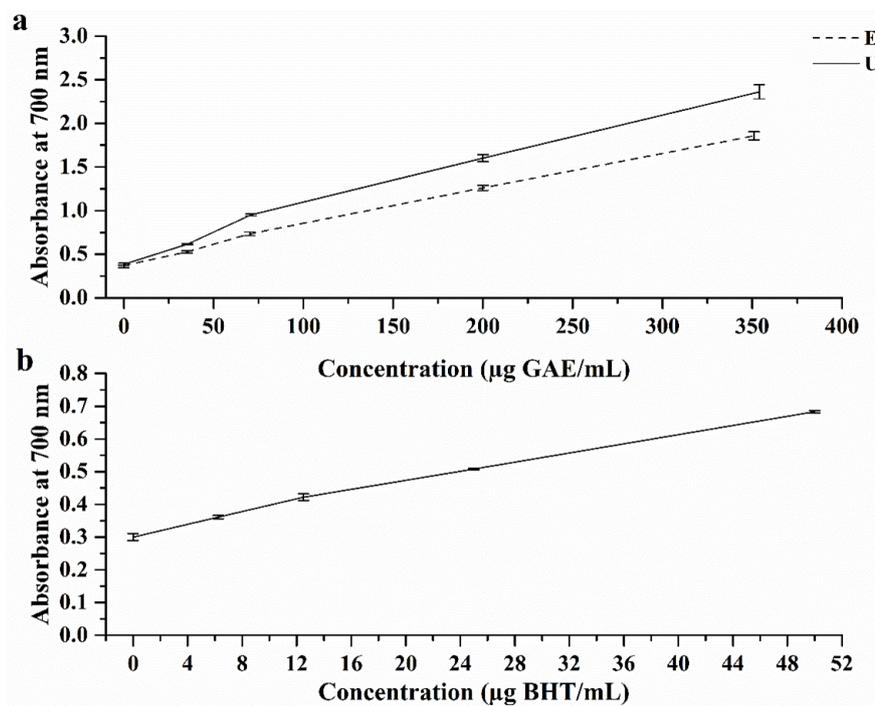


Fig. 4. Ferric reducing antioxidant power: **a** – enzyme-assisted extract (E) and ultrasound-assisted extract (U) of avocado peel, **b** – BHT. Each value is expressed as mean \pm SD ($n = 3$)

($IC_{50} = 23.82 \pm 0.75 \mu\text{g/mL}$) than that of the U extract. These results indicate the potent antioxidant activity of the U extract. Antasionasti et al. (2017) reported that the reducing power of avocado peel methanol extract expressed as IC_{50} was $25.63 \mu\text{g/mL}$. The peel extract possesses more compounds that confer high antioxidant capacity (Calderón-Oliver et al., 2016).

CONCLUSION

Hass avocado peel is a powerful source of polyphenols. Viscozyme L (E) and the ultrasound-assisted process were performed to increase the polyphenol yield extraction of avocado peel. The ultrasound-assisted extraction allowed phenolic extraction yields equal to enzyme-aided extraction. Avocado peels contain a variety of phenolic compounds which possess strong antioxidant capacities, and the U extract has a higher antioxidant activity than the E extract due to the type of polyphenols and their concentration. Furthermore, the ultrasound-assisted process (U) can be

recommended as a green extraction method of the active constituents from avocado peel.

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