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Ultrasound-Assisted Extractions for Improving the Recovery of Phenolics and Charantin from Bitter Melon and for Increasing the Antioxidant, Antidiabetic and Anti-Obesity Activities of Its Extracts

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Key words: bitter melon, ultrasound-assisted extraction, charantin, phenolic compounds, antioxidant activity, antidiabetic activity

Bitter melon is rich in bioactive compounds and has a significant potential for commercial use as a functional food material. Its bioactive compound-rich extract was prepared using probe- or bath-type ultrasound-assisted extraction (UAE) with 60% (*v/v*) ethanol or distilled water. The composition and bioactivity of the extracts prepared using UAE was compared with those obtained by conventional extraction methods, such as autoclave extraction, ethanol extraction, and hot-water extraction. Although the yield of the autoclave extraction was the highest, the extracts obtained using UAE and aqueous ethanol exhibited a higher total phenolic content, antioxidant activity, antidiabetic activity (α-amylase and α-glucosidase inhibitory activities), and pancreatic lipase inhibitory activity than the conventional extracts. In particular, UAE with probe system and aqueous ethanol (UAE-P-E) was effective for extracting bioactive compounds, such as phenolics (total phenolic content of 18.73 mg GAE/g extract) and charantin (28.56 mg/g extract). Among all extracts, this prepared by UAE-P-E showed the highest DPPH radical scavenging activity (IC_{50} of 0.55 mg/mL), ferric reducing antioxidant power (250.5 μmol TE/g extract), and pancreatic lipase inhibitory activity (76.38% at a concentration of 3.0 mg/mL). These results suggest that bioactive compound-rich extracts from bitter melon obtained using UAE, especially UAE-P-E, are expected to have high application potential as a functional food material, and are also expected to be used as natural antioxidant, antidiabetic, and anti-obesity agents.

INTRODUCTION

Bitter melon *(Momordica charantia* L.) is an annual vine plant belonging to the Cucurbitaceae family and is widely grown in tropical and subtropical regions, such as Asia, Africa, and central Europe [Jia *et al*., 2017]. Bitter melon is also called bitter gourd because of its characteristic bitter taste, as well as has various other names such as wild cucumber in Africa, karela in India, goya in Japan, and yoeju or bitter cucumber in South Korea [Grover & Yadav, 2004]. The large quantities of bioactive compounds, such as charantin, alkaloids, triterpenoids, phenolic acids, flavonoids, saponins, and carotenoids have been determined in this fruit [Day *et al*., 1990; Pugazhenthi & Murthy, 1995]. Among them, phenolic compounds were recognized as important. The type and content of phenolic compounds in bitter melon differs depending on the fruit part and its degree of ripening. The major phenolic compounds of green and ripe fruits are gallic acid, (+)-catechin, tannic acid, and caffeic acid [Kubola & Siriamornpun, 2008]. Horax *et al.* [2010] found that gallic acid, *p*-coumaric acid, quinic acid, 4-hydroxybenzyoic acid, caffeic acid, and gentisic acid were the main phenolic acids of pulp, peel, and seeds. The charantin is another main bioactive component of bitter melon. It exists as a mixture of two steroidal saponins, β-sitosteryl glucoside and stigmasteryl glucoside and is known for its hypoglycemic activity by acting on β-cells of the pancreas and promoting insulin secretion [Grover & Yadav, 2004]. The charantin content of bitter melon varies depending on the plant variety, genetic resource, fruit maturity, growing region, and cultivated environment [Goo *et al*., 2016; Lee *et al*., 2016; Mahwish *et al*., 2021].

The extraction method influences the content of bioactive compounds and the biological activity of the extract; thus, it is an important step in the process of recovering bioactive constituents from plant materials. Conventional extraction techniques, such as Soxhlet extraction, maceration, and steam distillation, have been used to extract bioactive compounds from plants [Ajila *et al*., 2011]. However, they have certain drawbacks, such as low yield, degradation of the target compounds, large solvent requirements, and long extraction times; therefore, elicit adverse impact on the environment and human health [Chemat *et al*., 2012]. Recently, green extraction methods, such as pressurized liquid extraction, pressurized hot-water extraction, supercritical extraction, microwave-assisted extraction, and ultrasound-assisted extraction (UAE), have been used as alternatives to the conventional methods [Ajila *et al.*, 2011; Lončarić *et al*., 2020].

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UAE is used for the efficient recovery of bioactive compounds, including phenolics, from various plant materials [Horzic *et al*., 2012]. There are two types of ultrasonic systems: bath and probe types, depending on the location of the ultrasound. Ultrasound generates cavitation bubbles in the extraction solvent, and the collapse of these bubbles leads to the extreme conditions of high temperature and high pressure [Rostagno *et al*., 2003]. During ultrasound treatment, the plant cell wall located near the bubble collapses, facilitating the penetration of the solvent into the plant tissue, and thereby increasing the release of organic compounds inside the plant cell [Sharmila *et al*., 2016]. The UAE methods provide a simple alternative to the conventional extraction methods, increase the extraction speed, and minimize the volume of solvent used, thereby enabling the efficient extraction of useful components [Vilkhu *et al.*, 2008]. The proposed study aimed to produce an extract rich in bioactive compounds from bitter melon for further application in the food industry as a functional ingredient with potent biological activities. For this purpose, bioactive compound-rich extracts were prepared using UAE (in bath and probe systems), a green extraction technique, and the bioactive compound contents and health-related properties, including antioxidant, antidiabetic, and pancreatic lipase inhibitory activities of the extract, were measured. In addition, the studied bioactive compound compositions and activities were compared with those of the extracts obtained by conventional extraction methods, such as autoclave extraction (AE-W), aqueous ethanol extraction (EE), and hot-water extraction (HWE).

MATERIALS AND METHODS

Materials and chemicals

Bitter melon (15–20 cm), cultivated in Gyeungsan (South Korea), was purchased, washed, and had its seed removed. The pericarp was lyophilized and ground into a fine powder (approximately 0.2 mm). Ground samples were stored at –40°C in a deep freezer (MDF, Sanyo, Tokyo, Japan).

HPLC standards of phenolic compounds (gallic acid, chlorogenic acid, (–)-epicatechin, (+)-catechin, caffeic acid), naringin, Folin–Ciocalteu phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, porcine pancreatic α-amylase (≥5 units/mg solid), *p*-nitrophenyl-α-D-glucopyranoside (*p*-PNG), α-glucosidase (from *Saccharomyces cerevisiae,* ≥5 units/mg protein using *p*-PNG), porcine pancreatic lipase (100–650 units/mg protein using olive oil), Trolox, ascorbic acid, and acarbose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Charantin (mixture of stigmasta-5,25-dien-3β-yl β-D-glucopyranoside and β-sitosteryl glucoside; $C_{35}H_{60}O_6$ and $C_{35}H_{58}O_6$; molecular weight 576.85 and 574.83; purity: 91.2% *w/w*) used as an HPLC standard was purchased from ChromaDex (Santa Ana, CA, USA). Orlistat was purchased from Tokyo Chemical Co. (Tokyo, Japan). Ethanol and other chemicals were of analytical grade.

Extract preparation

The extracts of freeze-dried and ground bitter melon were prepared using ultrasound-assisted extraction with a probe system and 60% (*v*/v) ethanol (UAE-P-E) or water (UAE-P-W), ultrasound-assisted extraction with an ultrasonic bath and 60% (v/v) ethanol (UAE-B-E), autoclave extraction with water (AE-W), 60% (*v*/v) ethanol extraction (EE), and hot-water extraction (HWE). The extraction conditions were selected based on previously reported optimized methods for the extraction of bioactive ingredients from various natural materials [Lee & Yoon, 2021; Suh *et al.*, 2017]. For UAE with the probe system, 2 g of ground sample was added to a certain amount of solvent (60% (*v/v*) ethanol or distilled water) corresponding to 1:20 (*w/v*), and extraction was performed using an ultrasonic probe device (KFS-600N, Korprotech, Seoul, South Korea) for 15 min at a constant frequency of 20 kHz and an ultrasonic intensity of 270 W. For UAE-B-E, a ground sample of bitter melon (2 g) was mixed with 40 mL of 60% (*v*/v) ethanol (1:20, *w*/v) in an Erlenmeyer flask (100 mL). The flask was placed in an ultrasonic bath (5510-DTH, Brason, Danbury, CT, USA), with circulating water, set at 40°C and 20 kHz, with an ultrasound intensity of 270 W for 15 min. To prepare the extract using AE-W, the sample and distilled water were mixed in a ratio of 1:20 (*w/v*), and the mixture was autoclaved at 121°C at 0.13 MPa for 15 min. For EE and HWE, a ground sample (2 g) was mixed with 60% (*v/v*) ethanol or distilled water in an Erlenmeyer flask at a 1:20 (*w/v*), and the mixture was then placed in a shaking water bath (BS-11, JeioTech, Seoul, South Korea) for 3 h at 60°C and 90°C, respectively. The mixture obtained using each extraction method was centrifuged at 12,000×*g* and 4°C for 20 min, and the supernatant was condensed using an evaporator (N-1000, EYELA, Tokyo, Japan) at 35°C and freeze-dried. All the above extraction procedures were performed in triplicate. The extract powders were stored in a deep freezer until further use, and then dissolved in distilled water to prepare a constant concentration and used as an analytical sample.

Determination of total phenolic content and total flavonoid content

The total phenolic content (TPC) of the extracts was measured using the Folin–Ciocalteu method [Folin & Ciocalteu, 1927] with gallic acid as the standard. Briefly, 0.1 mL of the extract was mixed with the Folin–Ciocalteu's phenol reagent (0.1 mL) and allowed to react for 3 min, followed by the addition of 2 mL of distilled water and 0.2 mL of 10% sodium bicarbonate. The absorbance of the mixture was measured at 725 nm (U-2000 spectrophotometer, Hitachi, Tokyo, Japan) after incubation at room temperature for 1 h. The results were expressed as mg gallic acid equivalents (GAE)/g extract.

The total flavonoid content (TFC) was determined using the method reported by Sembiring *et al.* [2018]. Extract (0.5 mL, 10 mg/mL) was added to 0.1 mL of 10% aluminum nitrate and 0.1 mL of 1 M potassium acetate. Then, 4.3 mL of 80% (*v/v*) ethanol was added, the sample was then mixed and left in the dark for 40 min, and its absorbance was measured at 415 nm (U-2000 spectrophotometer, Hitachi). Naringin was used as the standard, and the results were expressed as mg naringin equivalents (NE)/g extract.

HPLC ANALYSIS OF CHARANTIN

The charantin content of the extracts was determined by the HPLC method described previously by Goo *et al*. [2016] with some modifications. The Waters 2695 HPLC device (Waters Co., Milford, MA, USA) equipped with a Waters 2489 UV detector and an Atlantis dC18 column (4.6×150 mm, 5 μm; Waters Co.) was used. The extract dissolved in distilled water was passed through membrane filters (0.45 μm pore size, Millipore, Billerica, MA, USA) and injected into the HPLC column. The column temperature was maintained at 30°C, and the injection volume was 10 μL. The mobile phase consisted of water (solvent A) and 100% acetonitrile (solvent B). The following gradient program was used for the separation of charantin: 85–95% B (0–15 min), 95% B (15–20 min), 95–85% B (20–35 min), 85% B (35–45 min). The analysis was performed at a flow rate of 1 mL/min, with the detection wavelength set at 197 nm. Peak identification was done by comparing the retention time with that of a standard solution, and the charantin content was expressed as mg/g extract.

HPLC ANALYSIS OF PHENOLIC COMPOUNDS

The content of phenolic compounds in the extract was measured according to the method described in the study of Nour *et al*. [2013]. The phenolic compounds were analyzed using HPLC under the same conditions as those used for the charantin analysis described above, except for the mobile phase, which consisted of 1% (*v/v*) phosphoric acid (solvent A) and 100% acetonitrile (solvent B), and the detection wavelength which was 280 nm. The following gradient program was used for the separation of phenolic compounds: 10–30% B (0–27 min), 30–44% B (27–55 min), 44–10% B (55–60 min). Peak identification was performed using phenolic standards, and the content of phenolic compounds was expressed as mg/g extract.

Determination of antioxidant activity

The DPPH radical scavenging activity of extracts was determined as described by Brand-Williams *et al.* [1995]. The bitter melon extracts were dissolved in distilled water with different concentrations ranging from 250 to 2,500 μg/mL. Sample solution (100 μL) and 0.2 mM DPPH radical solution (200 μL) were added to each well of a 96-well-plate. The plate was incubated at 37°C for 30 min, and the absorbance was measured at 517 nm using a microplate reader (Epoch, BioTek Instrument Inc., Winooski, VT, USA). Distilled water was used in the control sample instead of the sample solution, and ascorbic acid was used as a positive control. The following formula was used to calculate the DPPH radical scavenging activity:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100 \quad (1)$$

where: A_{sample} is the absorbance with the test sample, and $A_{control}$ is the absorbance with distilled water instead of test sample. DPPH radical scavenging activity was expressed as half-maximal inhibitory concentration (IC_{50}) defined as the extract concentration, that is required to inhibit 50% of the DPPH radicals.

Ferric reducing antioxidant power (FRAP) was measured using the method described by Benzie & Strain [1996] with some modifications. Sodium acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, 10 mM), and ferric chloride (20 mM) were prepared and mixed in a ratio of 10:1:1 (*v/v/v*) and used as the FRAP reagent. Next, 175 μL of FRAP reagent was added to 25 μL of extract solution (concentration of 250–2,000 μg/mL), and after reacting at 37°C for 30 min, the absorbance was measured at 590 nm using a microplate reader (Epoch, BioTek Instrument Inc.). Standard curve was prepared using different concentrations (0.63, 0.125, 0.250, 0.5, 1.0. μM) of Trolox. The results were expressed as μmol Trolox equivalents (TE)/g extract.

Determination of antidiabetic activity

α-Amylase inhibitory activity

The α-amylase inhibitory activity of the extract was evaluated according to the method described by Kazeem *et al*. [2013]. Sample solution (20 μL; 250–2,000 μg/mL), phosphate buffer (50 μL, 100 mM, pH 6.8), and 0.1 mL of α-amylase (from porcine pancreas, 1 U/mL) were placed in a tube. This mixture was pre-incubated at 37°C for 5 min, after which 0.1 mL of 1% (*w/v*) soluble starch was added, and the mixture was incubated at 37°C for 5 min. The reaction was terminated by adding 0.1 mL of aliquot of 3,5-dinitrosalicylic acid color reagent solution and then boiled at 95°C for 15 min. Finally, 0.9 mL of distilled water was added to the reaction mixture and vortexed, and the absorbance was measured at 540 nm. Acarbose (concentration of 100––1,000 μg/mL) was used as a positive control. Inhibitory activity was calculated using equation (2) and curves of inhibitory activity *vs* concentration were plotted. α-Amylase inhibitory activity was expressed as IC_{50} defined as the extract concentration, which is required to inhibit 50% of the enzyme activity.

$$\text{Inhibitory activity (\%)} = \left(1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right) \times 100 \quad (2)$$

where: A_{sample} is the absorbance with the test sample, A_{blank} is the absorbance with distilled water instead of substrate, and $A_{control}$ is the absorbance with distilled water instead of test sample.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined using the method reported by Kim *et al.* [2004]. The sample solution (50 μL; 250–2,000 μg/mL) was blended with 50 μL of α-glucosidase (0.2 U/mL) dissolved in 0.2 M potassium phosphate buffer (pH 6.8), followed by pre-incubation at 37°C for 15 min. Then, 0.1 mL of 3 mM *p*-NPG was added to initiate the enzymatic reaction, and the mixture was incubated at 37°C for 10 min. NaOH (50 μL, 0.1 M) was added to stop the reaction, and the absorbance was measured at 405 nm. Acarbose (concentration of 100–1,000 μg/mL) was used as a positive control. The inhibitory activity was calculated using equation (2), as described above. α-Glucosidase inhibitory activity was expressed as IC_{50} – the extract concentration that required to inhibit 50% of the enzyme activity.

Determination of pancreatic lipase inhibitory activity

The pancreatic lipase inhibitory activity was determined to investigate the anti-obesity effect of the extract. Porcine pancreatic lipase (6 μL, 10 mg/mL) dissolved in enzyme buffer (10 mM 3-(*N*-morpholino)propanesulfonic acid, 1 mM ethylene-diamine-tetraacetic acid, pH 6.8), 170 μL of Tris buffer (100 mM Tris, 5 mM $CaCl_2$, pH 7.0), and 20 μL of each concentrated sample (0.5, 1.0, 2.0, and 3.0 mg/mL) was added to a 96 well-plate, the mixture was mixed well and incubated at 37°C for 15 min. Next, 4 μL of 10 mM *p*-nitrophenyl butyrate was added to the reaction solution, which was incubated at 37°C for 60 min. The absorbance of the reaction solution was measured at 400 nm using a microplate reader (Epoch, BioTek, Instrument Inc.). Orlistat (0.5 mg/mL) was used as a positive control and the lipase inhibitory activity was calculated using equation (2) as described above.

Statistical analysis

All experiments were performed in triplicate, and the experimental results were expressed as the mean± and standard deviation. One-way analysis of variance (ANOVA) was performed using the SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) and Duncan's multiple range test comparisons were performed at $p<0.05$ to determine the statistically significant differences.

RESULTS AND DISCUSSION

Extraction yield

The yields of extraction of bitter melon by various methods are shown in Figure 1. The yield of AE-W was the highest (50.22%), followed by HWE (42.60%), UAE-P-W (37.72%), UAE-P-E (34.84%), UAE-B-E (34.38%), and EE (33.69%). There was no significant ($p\geq0.05$) difference between the yields of UAE-P-E and UAE-B-E. The yields of extraction with water were higher than those with aqueous ethanol. The extraction efficiency of functional ingredients from plants is affected by various factors, such as the solvent type, material to solvent ratio, temperature, and pH [Vilas-Boas *et al*., 2020]. In particular, the type and concentration of solvent are important factors that have the most significant influence on the extraction efficiency [Gunathilake *et al*., 2019]. Water is the most polar solvent and it can easily extract many polar plant components that dissolve in it. There are bioactive compounds among them. However, undesirable compounds, such as carbohydrates and proteins, can also be eluted into the water together with bioactive compounds [Do *et al.*, 2014]. The combination of water and organic solvents can facilitate the extraction of bioactive compounds that are soluble in water and/or organic solvents. Therefore, aqueous ethanol is most often used to extract bioactive components, including phenolic compounds, from various plants [Sun *et al*., 2020]. Temperature also significantly affects the extraction efficiency, as higher temperatures increase solubility of the solute in the solvent and increase the rate at which the solute diffuses into the solvent bulk, resulting in a higher mass transfer rate [Cacace & Mazza, 2003]. In our study, the highest extraction yield of AE-W could be due to the high temperature and high dissolving ability of water.

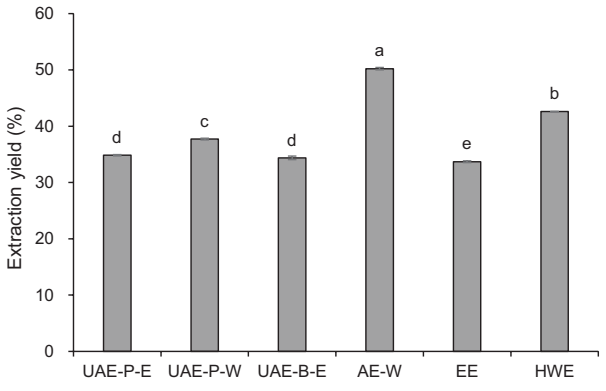

FIGURE 1. Yield of extraction of bitter melon by various extraction methods. UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v*/v) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v*/v) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v*/*v*) ethanol extraction; HWE, hot-water extraction. Bar represents the mean and standard deviation ($n=3$). Values with different letters are significantly different at $p<0.05$.

Total phenolic, total flavonoid, and charantin contents

The contents of total phenolics, total flavonoids, and charantin in the extracts obtained from bitter melon by various methods were determined, and the results are shown in Table 1. Phenolics are a diverse group of compounds with various structures and molecular weights that exist in abundance as secondary metabolites in plants. Phenolic compounds are found in various plants and exhibit various biological activities such as anticancer, anti-allergic, antibacterial, anti-inflammatory, and antithrombotic, as well as acting as antioxidants [Sen *et al*., 2013]. The TPC of the bitter melon extract was in the range of 11.08–18.73 mg GAE/g extract, and the highest TPC was found in the extract obtained using UAE-P-E (Table 1). For the remaining extracts, TPC decreased in the following order of extraction methods: UAE-B-E > UAE-P-W > EE > AE-W > HWE. These results are consistent with findings published by Dobrincic *et al.* [2020], who reported that the TPC of extracts obtained from olive leaves using UAE was higher than those of extracts obtained by high pressure-assisted extraction and conventional heat-reflux extraction. The TFC of extracts obtained using ethanol was higher than that of the extract obtained using water as a solvent (Table 1). The highest TFC was found in the extract obtained using UAE-P-E (8.29 NE/g extract). In other extracts, TFC decreased in the following order of extraction methods: UAE-B-E > EE > UAE-P-W = AE-W = HWE.

Generally, UAE produced the extract with higher TPC and TFC than the other extraction methods. This phenomenon can be explained by the destruction of the cell wall by the bubbles generated by cavitation caused by ultrasonic waves during the UAE, which increased the elution and solubility of substances inside the cell [Horzic *et al.*, 2012]. In addition, UAE-P-E produced the extract with higher TPC and TFC than UAE-B-E. This could be because the ultrasonic generator was located at the bottom of the ultrasonic bath and indirectly transmitted energy to the sample through

TABLE 1. Total phenolic, total flavonoid, and charantin contents of bitter melon extracts obtained by various extraction methods.

Extraction method	Total phenolics (mg GAE/g extract)	Total flavonoids (mg NE/g extract)	Charantin (mg/g extract)
UAE-P-E	18.73±0.18[a]	8.29±0.12[a]	28.56±0.36[a]
UAE-P-W	14.45±0.26[c]	3.58±0.03[d]	Nd
UAE-B-E	16.34±0.24[b]	7.39±0.13[b]	17.79±0.45[b]
AE-W	12.66±0.30[e]	3.99±0.12[d]	Nd
EE	13.53±0.22[d]	5.37±0.29[c]	14.60±0.24[c]
HWE	11.08±0.06[f]	3.62±0.11[d]	Nd

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction; GAE, gallic acid equivalent; NE, naringin equivalent; Nd, not detected.
Values with different letters in the same column are significantly different at $p<0.05$.

the medium during UAE-B, whereas in UAE-P, energy was directly transferred to the sample by ultrasonic waves generated from the probe, which increased the ultrasonic efficiency [Capelo *et al*., 2005].

The content of charantin was the highest in the bitter melon extract obtained using UAE-P-E (28.56 mg/g extract), followed by UAE-B-E and EE (Table 1). Charantin was detected only in the aqueous ethanolic extract, presumably because charantin, a saponin component, has a higher affinity for ethanol than water. Kole *et al*. [2013] reported a difference in charantin content of 5.01–8.29 mg/g of lyophilized and powdered bitter melon depending on the extraction conditions, and Lee *et al.* [2016] reported charantin content in the range of 13.3–24.7 mg/g dry weight (DW), depending on the harvest period. Mahwish *et al*. [2021] also reported that the charantin content of bitter melon differed depending on the part of the fruit, and was higher in the flesh part (0.16 mg/g DW) than in the whole fruit (0.11 mg/g DW) and skin (0.08 mg/g DW). As mentioned earlier, charantin demonstrates insulin-like activity by increasing insulin release from the pancreas and decreasing gluconeogenesis [Wang *et al.*, 2014]. Therefore, the high charantin extract obtained using UAE-P-E is expected to exhibit a significant antidiabetic activity.

Content of individual phenolics

The composition of phenolic compounds of bitter melon extracts was shown in Table 2. Five phenolic compounds (gallic acid, chlorogenic acid, (-)-epicatechin, (+)-catechin, and caffeic acid) were identified. Gallic acid was present in the highest content in all extracts, and chlorogenic acid had the second highest content. All extracts showed the highest gallic acid content, followed by chlorogenic acid content. The gallic acid content in the extracts obtained using UAE-P-E, UAE-B-E, UAE-P-W, EE, AE-W, and HWE was 4.55, 3.68, 3.35, 3.05, 2.12, and 2.11 mg/g extract, respectively, and the chlorogenic acid content was 1.08, 0.86, 0.76, 0.69, 0.69, and 0.52 mg/g extract, respectively. The content of total phenolic compounds detected by HPLC was 7.05 mg/g extract (UAE-P-E), 5.73 mg/g extract (UAE-B-E), 5.19 mg/g extract (UAE-P-W), 4.59 mg/g extract (EE), 3.30 mg/g extract (AE-W), and 3.28 mg/g extract (HWE). Thus, the total phenolic content in the extract obtained using UAE-P-E was 2.2 times higher than that in the extract obtained using HWE. Horax *et al.* [2010] reported that the main phenolics of the 80% ethanolic extract of bitter melon were catechin (1.54 mg/g extract), gentisic acid (0.72 mg/g extract), gallic acid (0.49 mg/g extract), chlorogenic acid (0.66 mg/g extract), epicatechin (0.29 mg/g extract), *o*-coumaric acid (0.27 mg/g extract), and procatechuic acid (0.12 mg/g extract). Thus, most of the identified phenolic compounds were similar to those found in this study, but their content was lower. Lopes *et al.* [2020] reported that a wider variety of phenolic compounds were extracted from bitter melon by UAE than by conventional extraction, and the content was also found to be higher than that in conventional extracts. Gallic acid is a representative phenolic that is widely distributed in plants and exhibits antioxidant, anti-inflammatory, and antibacterial activities [Bai *et al.*, 2020]. Chlorogenic acid has various bioactivities such as antibacterial, anti-inflammatory, antipyretic,

TABLE 2. Content of phenolic compounds of bitter melon extracts (mg/g extract) obtained using various extraction methods.

Extraction method	Gallic acid	Chlorogenic acid	(–)-Epicatechin	(+)-Catechin	Caffeic acid
UAE-P-E	4.55±0.28[a]	1.08±0.08[a]	0.61±0.06[a]	0.65±0.02[a]	0.16±0.01[a]
UAE-P-W	3.35±0.15[bc]	0.76±0.08[b]	0.51±0.03[a]	0.45±0.01[c]	0.12±0.01[c]
UAE-B-E	3.68±0.02[b]	0.86±0.04[ab]	0.54±0.04[a]	0.51±0.01[b]	0.14±0.01[b]
AE-W	2.12±0.09[d]	0.69±0.07[bc]	0.22±0.02[b]	0.19±0.02[f]	0.08±0.02[de]
EE	3.05±0.14[c]	0.69±0.05[bc]	0.43±0.02[b]	0.32±0.01[d]	0.10±0.01[d]
HWE	2.11±0.14[d]	0.52±0.02[c]	0.28±0.02[b]	0.26±0.01[e]	0.06±0.01[e]

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with an probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction.
Values with different letters in the same column are significantly different at $p<0.05$.

TABLE 3. Antioxidant activity of bitter melon extracts obtained using various extraction methods.

Extraction method	IC_{50} of DPPH radical scavenging activity (mg/mL)	Ferric reducing antioxidant power (μmol TE/g extract)
UAE-P-E	0.55±0.03^{e}	250.5±2.7^{a}
UAE-P-W	1.17±0.01^{bc}	170.0±2.9^{c}
UAE-B-E	1.01±0.03^{d}	214.3±6.2^{b}
AE-W	1.20±0.10^{b}	168.8±7.4^{c}
EE	1.07±0.04^{cd}	176.7±3.8^{c}
HWE	2.19±0.11^{a}	130.1±2.5^{d}
Ascorbic acid	0.12±0.12^{f}	–

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with a ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction; TE, Trolox equivalent.
Values with different letters in the same column are significantly different at $p<0.05$.

neuroprotective, anti-obesity, antiviral, antibacterial, and antihypertensive as well as antioxidant activity [Naveed *et al*., 2018]. In turn, (+)-catechin is a strong antioxidant cholesterol esterase inhibitor, arteriosclerosis preventing agent, as well as antibacterial and antiulcer agent [Pedro *et al.*, 2020]. Therefore, the phenolic-rich extracts of bitter melon, specially these obtained using UAE, are expected to exhibit high biological activity, including antioxidant activity.

Antioxidant activity

To evaluate the antioxidant activity of extracts of bitter melon, the DPPH radical scavenging activity and FRAP were determined and results are shown in Table 3. The IC_{50} values of the extract for DPPH radical scavenging activity ranged from 0.55 to 2.19 mg/mL, with the lowest value determined for the extract obtained using UAE-P-E followed by UAE-B-E, EE, UAE-P-W, AE-W, and HWE. IC_{50} of ascorbic acid was 0.12 mg/mL. These results of antioxidant activity was very high compared to that reported by Aljohi *et al.* [2016], who found that the DPPH radical scavenging activity of bitter melon extract was 50% at a concentration of 15 mg/mL. Nam & Kim [2015] reported that the ethanolic extract of dried unripe bitter melon demonstrated higher DPPH radical scavenging ability than the hot water extract at all tested concentrations, which was consistent with the results of this study.

The effect of the extraction method on FRAP of the extracts was similar as on DPPH radical scavenging activity; the highest FRAP was found for the extract obtained by UAE-P-E (250.5 μmol TE/g extract), followed by UAE-B-E (214.3 μmol TE/g extract), and the lowest by HWE (130.1 μmol TE/g extract). The FRAP values for EE, UAE-P-W, and AE-W samples were in the range from 168.8 to 176.7 μmol TE/g extract and did not differ significantly ($p\geq0.05$). A study by Alothman *et al*. [2009] found that ethanolic extracts of pineapples, banana, and guava had higher FRAP than those prepared using water. Ahmad-Qasem *et al.* [2013] reported that the extract obtained from olive leaves by UAE with ethanol had significantly higher FRAP than that obtained by EE, which is consistent with the results of this study.

Among the UAE samples, aqueous ethanolic extracts demonstrated higher antioxidant activity than that obtained using water. It is assumed that the phenolic compounds have a high affinity for ethanol compared to water, and the high antioxidant activity of the ethanolic extracts were due to the high phenolic content. In addition, the highest radical scavenging activity and reducing power of the extract produced with UAE-P-E were likely due to the high gallic acid and caffeic acid contents, which, according to literature reports, provide excellent radical scavenging activity [Chalas *et al.*, 2011].

Anti-diabetic activity

α-Amylase is an enzyme that breaks down α-D-(1,4)-glucoside bonds of polysaccharides composed of α-linked glucose, such as starch or glycogen, and is an essential enzyme for carbohydrate metabolism. Intestinal α-glucosidase is a membrane-bound enzyme located in the epithelium of the small intestine and breaks down starch and disaccharides into absorbable monosaccharides [Patel & Ghane, 2021]. For the treatment of diseases, such as non-insulin-dependent diabetes mellitus and hyperglycemia, in which carbohydrate metabolism does not proceed normally and is manifested by high blood sugar, due to insulin resistance, it is necessary to control the digestion and absorption of sugar [Nam & Kim, 2015]. Blood glucose is regulated when the activity of enzymes, which play an important role in carbohydrate digestion, is inhibited, and the breakdown and absorption of starch entering the body through meals decreases, causing the amount of glucose released into the blood to decrease [Kajaria *et al.*, 2013]. Therefore, in patients with non-insulin-dependent diabetes mellitus, obesity, and hyperglycemia, the activity levels of α-amylase and α-glucosidase serve as indicators of the suppression of blood glucose levels.

The antidiabetic activity of bitter melon extract was determined by α-amylase and α-glucosidase inhibitory activity assays, and results are shown in Table 4. The IC_{50} values for the α-amylase inhibitory activity of the bitter melon extract were 0.81 and 0.83 mg/mL for UAE-P-E and UAE-B-E samples, respectively. In increasing order, the IC_{50} values for other extracts were 0.92 mg/mL (UAE-P-W), 1.02 mg/mL (EE), 1.49 mg/mL (AE-W), and 1.62 mg/mL (HWE). The IC_{50} value of acarbose, a positive control, was 0.25 mg/mL, and the extract obtained using UAE-P-E with the highest α-amylase inhibitory activity, had approximately 31% of the activity of acarbose. α-Glucosidase inhibitory activity of extracts exhibited the same trend as the α-amylase inhibitory activity, and the UAE-P-E extract demonstrated the lowest value at 0.96 mg/mL, which corresponds to 74% of the activity of acarbose (0.71 mg/mL). These results were higher than those reported by Kang *et al.* [2018], who found that α-glucosidase inhibitory activity of hot water and ethanolic extracts from bitter melon were 29.65% and 66.88%, respectively, at a concentration of 2.5 mg/mL. In turn, Nam & Kim [2015] reported that the ethanolic extract obtained from

TABLE 4. Antidiabetic activity of bitter melon extracts obtained using various extraction methods.

Extraction method	α-Amylase inhibitory activity	α-Glucosidase inhibitory activity
	IC_{50} (mg/mL)	
UAE-P-E	0.81 ± 0.02^{e}	0.96 ± 0.02^{b}
UAE-P-W	0.92 ± 0.05^{cd}	1.26 ± 0.02^{d}
UAE-B-E	0.83 ± 0.01^{e}	1.02 ± 0.00^{bc}
AE-W	1.49 ± 0.04^{b}	1.66 ± 0.05^{e}
EE	1.02 ± 0.04^{c}	1.06 ± 0.03^{cd}
HWE	1.62 ± 0.03^{a}	1.88 ± 0.04^{f}
Acarbose	0.23 ± 0.01^{f}	0.71 ± 0.02^{a}

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with an probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with an probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction.
Values with different letters in the same column are significantly different at $p<0.05$.

immature dried bitter melon demonstrated significantly higher α-glucosidase inhibitory activity than the hot water extract, which was consistent with the results of the present study.

These results demonstrate that the antidiabetic activity of bitter melon extracts was correlated with TPC, suggesting that the TPC may have a significant effect on the antidiabetic activity. Polyphenols can be used to prevent and manage diabetes mellitus *via* insulin-dependent and independent approaches: the former protects pancreatic islet β-cells, reduces β-cell apoptosis, promotes β-cell proliferation, activates insulin signaling, and stimulates pancreas to secrete insulin, the latter inhibits glucose absorption, inhibits digestive enzymes, regulates intestinal microflora, and inhibits the formation of advanced glycation end products [Sun *et al.*, 2020]. The mechanism of the high inhibitory activity of the ultrasonicated extracts involves modifying the α-amylase molecular structure by the hydroxyl radical generated by the ultrasound action [Kadkhodaee & Povey, 2008]. The extract obtained using aqueous ethanol as a solvent exhibited a higher antidiabetic activity than the water extract, presumably due to the effect of charantin, an antidiabetic component of bitter melon.

Pancreatic lipase inhibitory activity

Pancreatic lipase, secreted from the pancreas and stomach, plays an important role in fat digestion by decomposing triglycerides into 2-monoacylglycerol and two fatty acids. Therefore, by inactivating pancreatic lipase activity, triglycerides and cholesterol are excreted outside the body without being digested and absorbed in the body, thereby preventing the accumulation of fat [Bitou *et al.*, 1999]. Orlistat, mainly used as a treatment for obesity, irreversibly binds to pancreatic lipase and inactivates the enzyme. Due to the enzyme inhibitory action of Orlistat, it reduces intestinal triglyceride and cholesterol absorption and increases excretion, thereby acting as an anti-obesity agent, but causing side effects such as abdominal pain, diarrhea, and headache [El-Korany *et al.*, 2020]. Therefore, pancreatic lipase inhibitors as dietary components are still being searched for.

The pancreatic lipase inhibitory activity of bitter melon extracts obtained using various extraction methods and Orlistat as a positive control, is shown in Figure 2. The pancreatic lipase inhibitory activity of Orilstat was 87.38% at a concentration of 0.5 mg/mL. For extracts, the inhibitory activity increased as the sample concentration increased. The extract obtained using UAE-B-E showed the highest pancreatic lipase inhibitory activity ($p<0.05$) between samples at a concentration of 0.5 mg/mL. In turn, values for UAE-P-E, UAE-B-E, and EE (40.43%-41.47%) were significantly ($p<0.05$) higher than those for other extracts at 1.0 mg/mL. In addition, at concentrations of 2.0 and 3.0 mg/mL, the highest pancreatic lipase inhibitory activity ($p<0.05$) was demonstrated by the extract obtained using UAE-P-E (62.93 and 76.38%,

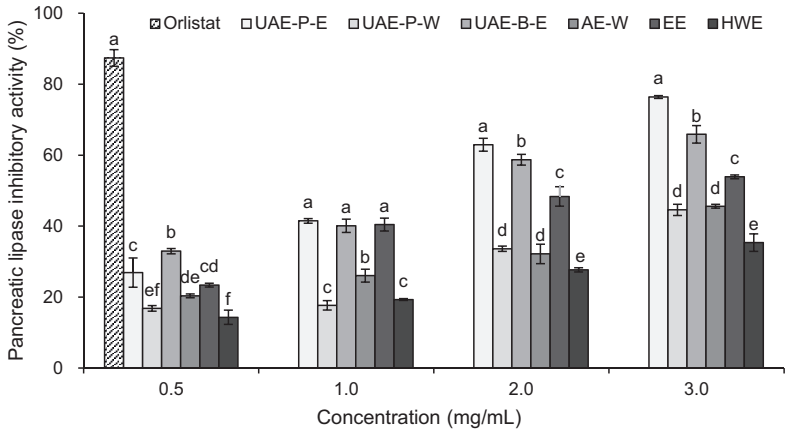

FIGURE 2. Pancreatic lipase inhibitory activity of bitter melon extracts obtained by various extraction methods. UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction. Pancreatic lipase inhibitory activity of Orlistat was tested at a concentration of 0.5 mg/mL. Bar represents the mean and standard deviation (n=3). Values with different letters separately for each concentration are significantly different at $p<0.05$.

respectively). However, all water extracts exhibited low pancreatic inhibitory activity at all sample concentrations. Fan *et al*. [2019] reported that the major bioactive compounds of bitter melon showing the anti-obesity activity were triterpenoids, saponins, and phenolics, which inhibit fat synthesis, promote glucose utilization, and stimulate auxiliary lipid-lowering activity. In turn, McDougall *et al*. [2009] found that polyphenol fractions of strawberries and raspberries exhibited lipase inhibitory activity and reported that this was due to the polyphenol compounds, such as tannin, contained in berries. Reports also state that the hydroxyl groups present in polyphenol compounds enter into hydrophobic interactions with pancreatic lipase and inhibit enzyme activity [Deavile *et al.*, 2007].

CONCLUSIONS

In the present study, we compared the TPC, TFC, phenolic composition, charantin content, antioxidant, antidiabetic, and pancreatic lipase inhibitory activities of extracts of bitter melon obtained using five extraction methods. The results demonstrate the feasibility of producing extracts with high bioactivity. In particular, the extract obtained using an ultrasonic probe with 60% ethanol (UAE-P-E) showed not only high TPC and TFC, but also excellent DPPH radical scavenging activity, FRAP and pancreatic lipase inhibitory activity. In addition, it had a high charantin content and showed α-glucosidase inhibitory activity equivalent to 74% of acarbose used in diabetes treatment. Therefore, the extract obtained from bitter melon using UAE-P-E is a practical new material with antioxidant, antidiabetic and anti-obesity activities, and has the potential to be applied to manufacture functional foods in the food industry. Furthermore, the UAE-P-E extract is expected to be a useful agent in diabetes treatment; however, extensive research is needed for effective drug development in the future.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests

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