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ANTIOXIDANT CAPACITIES OF THE EXTRACTS FROM *PERILLA FRUTESCENS* L. LEAVES USING PRESSURIZED LIQUID EXTRACTION AND CONVENTIONAL TECHNIQUES

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SUMMARY

Perilla frutescens L. is an edible plant used as a food item as well as a folk medicine for human diseases in some Asian countries. Due to limited studies in the extraction of total phenols responsible for its high bio-functional properties, this study is aimed to compare the yields, total phenolic content (TPC), and antioxidant capacities of the extracts between pressurized liquid extraction (PLE) at 100°C with or without ultrasonic treatment and conventional extraction techniques. A batch mode of the PLE system was utilized with the use of water and 50% aqueous ethanol as extraction solvents. Hot water extraction at 80°C and soxhlet extraction were employed as conventional techniques. The extraction yields from each technique were determined before their TPC and antioxidant capacities were examined. The results showed that PLE with and without ultrasonic treatment enhanced the extraction process of TPC as compared with conventional ones. Consequently, PLE resulted in higher antioxidant capacities and lipid peroxidation-inhibiting activity than conventional extraction technique ($p < 0.05$). However, PLE of bioactive components from *Perilla frutescens* L. leaves with higher thermal treatment should be considered in future studies.

Keywords: *Perilla frutescens* L., bioactive components, pressurized liquid extraction

INTRODUCTION

Extraction is one of the most crucial methods in the production of food, pharmaceutical, chemical, and cosmetic items since it is paramount to recover the targeted materials of interest. Recently, there has been an increase in studies of new emerging green technologies (Belwal *et al.*, 2018) since conventional extraction techniques use high amount of toxic solvents which compromise the safety of extracts, requiring extra-purification steps for their removal, to avoid health risks that they may cause upon ingestion by human beings or animals. Among modern extraction methods, pressurized liquid extraction (PLE), which mainly uses water as an available resource and environmentally benign solvent, has demonstrated the potential to replace conventional extraction techniques (Plaza, Turner, 2015).

Pressurized hot water extraction (PHWE) or subcritical water is defined as liquid water at temperatures above the boiling point of water (100-374°C) and pressure (0.1-22MPa) (Cvjetko Bubalo *et al.*, 2018). Under these thermodynamic conditions, the solubility of organic compounds in

water increases with a small increase in temperature (Naffati *et al.,* 2017). Water properties affecting the solubility of organic solutes in water and controlled by fine-tuning water temperature are polarity, dielectric constant, and density (Shitu *et al.,* 2015). Organic compound solubility in water increases significantly in the subcritical water state (Carr *et al.,* 2011). Therefore, there is a surge in the utilization of PLE in the extraction of valuable industrial materials with several applications (Cvjetko Bubalo *et al.,* 2018; Pereira *et al.,* 2019). PHWE has been used to extract phenolic compounds from *Pseuderanthemum palatiferum* leaves (Ho, Kiddane *et al.,* 2020; Ho, Kim *et al.,* 2020), flower resources (Trinh *et al.,* 2018), citrus by-products (Barrales *et al.,* 2018), green tea (Bindes *et al.,* 2019), and goldenberry (Corazza *et al.,* 2018).

Perilla frutescens L., a family of Labiatae, is an edible plant known as "tía tô" in Vietnam. *Perilla frutescens* L. is usually used as garnishes and colorants in some Asian countries such as China and Japan. The leaves of this plant are utilized as a folk medicine for the treatment of intestinal disorders and allergies as they exhibit as a detoxicant, antitussive, antibiotic, and antipyretic (Peng *et al.,* 2005). Recently, there have been numerous studies on the chemical compositions and biological properties reported. Much research is interested in *Perilla frutescens* L. as a result of its high amount of total phenolic content correlated with antioxidant capacities. However, few studies are carried out on the use of extraction techniques to recover bioactive components from this highly valuable plant. This study, therefore, examines the effectiveness of pressurized liquid extraction with and without ultrasonic-assisted (UA) treatment in comparison with some conventional extraction techniques.

MATERIALS AND METHODS

Materials

Perilla frutescens L. leaves were collected at the University of Cuu Long in April 2020. The leaves were cut into small size before oven drying at 40^{o}C and crushed to powder form with particle size less than 710 μm using a Standard testing sieve (Korea) and stored at -60±1^{o}C until use.

Soxhlet extraction

The sample (2 g) was extracted with 140 mL of 50% aqueous ethanol for 12 hours in a Soxhlet system. Then, the extracts were transferred to brown glass bottles and stored at 4^{o}C until use. Soxhlet extraction was performed in triplicate.

Pressurized liquid extraction (PLE)

The flow diagram of the subcritical water experimental apparatus is shown in Fig. 1. First, the 800 mL volume reactor (7) was filled with 400 mL of distilled water or 50% aqueous ethanol. Sample (6 g) was added into the reactor which was then sealed and pressurized using nitrogen gas to keep extraction solvents in the liquid state. The electric heater (9) was started to heat the reactor from room temperature to 100^{o}C. This step took approximately 45 min. The extraction parameters including temperature, time, pressure, stirring speed, solid/liquid ratio used in all experiments were 100^{o}C, 30 min, 5 bar, 400 rpm, and ~1/70, respectively. After completing the extraction, the mixtures or hydrolysates were quickly cooled down by the water line established inside the reactor. Then the hydrolysates were collected and filtered under a slight vacuum through a Buchi vacuum pump V100 with F1113 grade filter paper, pooled, and lyophilized using freeze dryer HyperCool from Gyrozen Co., Ltd. (Daejeon, Korea). The experiment was repeated three times. For comparison purposes, ultrasonic-assisted (UA) treatment for 15 min and power 50% was applied to the sample before PHWE or PEtOH (pressurized ethanol extraction) and named UA-PHWE and UA-PEtOH, respectively.

Hot water extraction (HWE)

HWE was carried out following the method described by Ho, 2019. The sample (2 g) was introduced into a 500 mL beaker containing 150 mL HPLC-grade distilled water and heated at

80±1°C for 30 min with 250 rpm stirring. The slurry was filtered under a slight vacuum through a Buchner funnel lined with F1113 grade filter paper, and the solid residue was extracted twice more under the same conditions. The extracts were pooled, lyophilized, weighed, transferred to brown glass bottles, and stored at 4°C for further analysis. Hot water extraction was performed in triplicate.

Determination of total phenolic content (TPC)

TPC was measured using the method described by Ho, 2019. Briefly, the sample (0.5 mL) was mixed with 2.5 mL of 0.2 N Folin Ciocalteu reagent (Sigma Alrich Chemical, USA) for 5 min followed by the addition of 2 mL of 75 g/L Na_2CO_3 to the mixture. After incubation for 2 h at room temperature, the absorbance of the reaction mixture was measured at 760 nm. Gallic acid (Sigma Alrich Chemical, USA) was used as a standard, and TPC was expressed as milligram gallic acid equivalent per gram sample (mg GAE/g).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging activity was determined according to the method described by Kolodziejczyk-Czepas et al., 2015, with some modifications. Sample (100 µL) of different concentrations (62.25, 125, 250, 500, 1000, and 2000 µg/mL) was added into a 96-microplate. Then, 500 µM DPPH solutions were added with the same volume to obtain the final concentrations of samples from 31.13, 62.25, 125, 250, 500, and 1000 µg/mL. Next, the mixtures were incubated in a dark place for 30 min before measuring at 517 nm using a multimode reader (BioTek Instruments, Inc., USA). The measurement was done in triplicate.

$$\% \text{ DPPH scavenging activity} = [(A_c - A_s) / A_c] \times 100$$

Where A_c is the absorbance of the control, and A_s is the absorbance of the sample solutions.

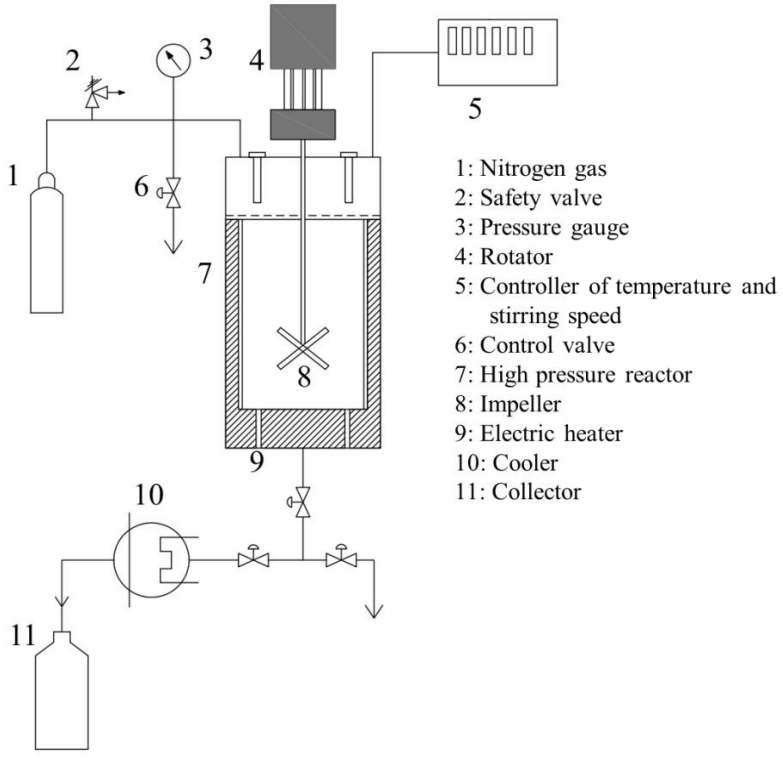

Figure 1. Batch pressurized liquid extraction apparatus.

The radical scavenging activity (SC_{50}) was calculated from the regression equation of the curve of % inhibition versus extract concentration. The experiment was done in triplicate. The results were expressed as mean SC_{50} ± SD. Trolox was used as a reference.

Trolox equivalent antioxidant capacity (TEAC)

TEAC assay was performed according to the previously reported method (Arts et al., 2004). Briefly, the concentrated $ABTS^+$ solution was diluted with phosphate-buffered saline (PBS), pH 7.4 to a final absorbance of ~0.70 ± 0.02 at 734 nm. Ten microliters of extracts were added to 990 µl $ABTS^+$ solution and the absorbance was measured after incubation for 6 min in a dark place. This was compared to a blank where 10 µL of the solvent was added to 990 µL of the ABTS solution. Trolox was used to establish the standard curve.

Lipid peroxidation inhibition

The anti-lipid peroxidation activity of the extracts obtained from different extraction techniques was examined according to the previous method (K. Chayarop, 2011). Briefly, 150 µL of the sample (1 mg/mL) was mixed with 500 µL of 0.6% v/v linoleic acid in 5% sodium dodecyl sulfate (SDS), 450 µL of tris-HCl buffer (pH 7.5), and 100 µL of 4 mM FeSO4.7H2O solution. Linoleic acid peroxidation was initiated by the addition of 200 µL of 2 mM ascorbic acid to the reaction mixtures. After incubating at 37°C for 30 min, trichloroacetic acid (5.5%) was added to terminate the peroxidation reaction. Then 1 mL of 1% (w/v) thiobarbituric acid was added to the reaction mixture, followed by boiling for 15 min. The mixture was centrifuged at 3500 rpm for 10 min, after which the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was read at 532 nm using a Synergy HTX multi-mode reader (USA). The malondialdehyde (MDA) content was evaluated using a standard curve of malondialdehyde bis (dimethyl acetal) 0.006-3 mM). The percentage of linoleic acid peroxidation inhibition was calculated using the following equation:

$$\% \text{ linoleic acid peridation inhibition} = [(M_c - M_s) / M_c] \times 100$$

Where M_c = MDA equivalent of the control, and M_s = MDA equivalent of the samples solution. The IC_{50} value was determined from the regression equation of the curve of % linoleic acid peroxidation inhibition versus extract concentration. The lipid peroxidation inhibitory activity of the extract was expressed as mean IC_{50} SD. Trolox (200 µg/mL) was used as a reference.

Statistical analysis

Statistical significance was determined among various treatments with one-way ANOVA and Duncan test using IBM® SPSS® Statistics version 20; $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Extraction yield

Results of extraction yields of different extraction techniques (Fig. 2) showed that conventional methods recovered less amount of bioactive compounds than those of advanced extraction techniques. In the latter, ultrasonic treatment demonstrated that it could improve the extraction process due to cavitation effects which can break down the cell wall of the sample matrix and release the solutes to the media.

Total phenolic content (TPC)

TPC, the most abundant antioxidant in our diet, is a widespread constituent of fruits, vegetables, cereals, olive, dry legumes, chocolate, and beverages, such as tea, coffee, and wine. As antioxidants, TPC may protect cell constituents from oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress (D Archivio et al., 2007). Fig. 3 showed the TPC of the extracts from *Perilla frutescens* L. leaves using different extraction techniques. PHWE, UA- PHWE, PEtOH, and UA-PEtOH recovered more TPC than soxhlet and HWE at 80°C. The range can be displayed as follows: soxhlet<HWE<PHWE<UA-

PHWE<PetOH <UA-PetOH. High TPC was observed in the extract using PetOH may be due to the similarity in dielectric constant or polarity of solvent and solutes as that value of ethanol at normal conditions is approximately 25. Besides, with ultrasonic treatment, TPC tended to improve as compared with the extracts without ultrasonic treatment (PHWE and UA- PHWE or PEtOH and UA-PetOH). This can be explained by the cavitation effect that can break down the cell walls of the sample matrix leading to the improvement of mass transfer of solutes.

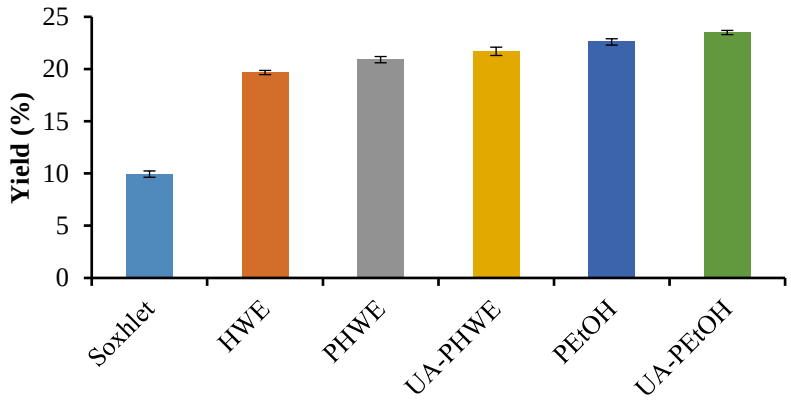

Figure 2. Yields of different extraction methods.

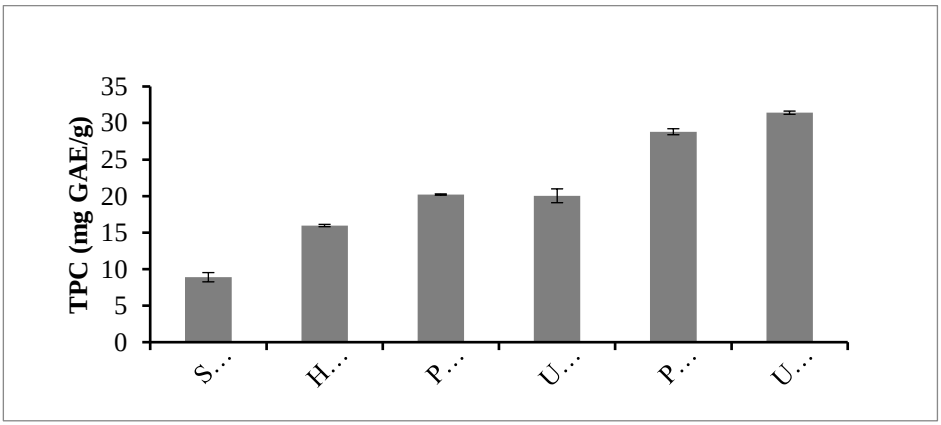

Figure 3. Total phenolic content of the extracts from different extraction methods (mg GAE/g).

DPPH

Free radicals play a crucial role in the pathogenesis of several human diseases, such as cancer, rheumatoid arthritis, and various neurodegenerative and pulmonary diseases. Antioxidants protect against these radicals and are therefore important tools for obtaining and preserving good health (Arts et al., 2004). In this study, DPPH scavenging capacity, as well as their SC_{50} of the extracts obtained from different extraction techniques, were examined and presented in Fig. 4 and Table 1, respectively**.** Due to the correlation between TPC and antioxidant capacity, the results of DPPH scavenging of PHWE, UA-PHWE, PEtOH, and UA-PEtOH were also higher than those obtained from soxhlet and HWE at 80°C. In Table 1, SC_{50} values of the extracts showed the same order indicating that the extracts using PHWE, UA-PHWE, PEtOH, and UA-PEtOH possessed higher antioxidant capacity than those using conventional techniques. These results once again illustrated the effectiveness of the new extraction techniques in the recovery of bioactive compounds from natural resources as compared to conventional ones.

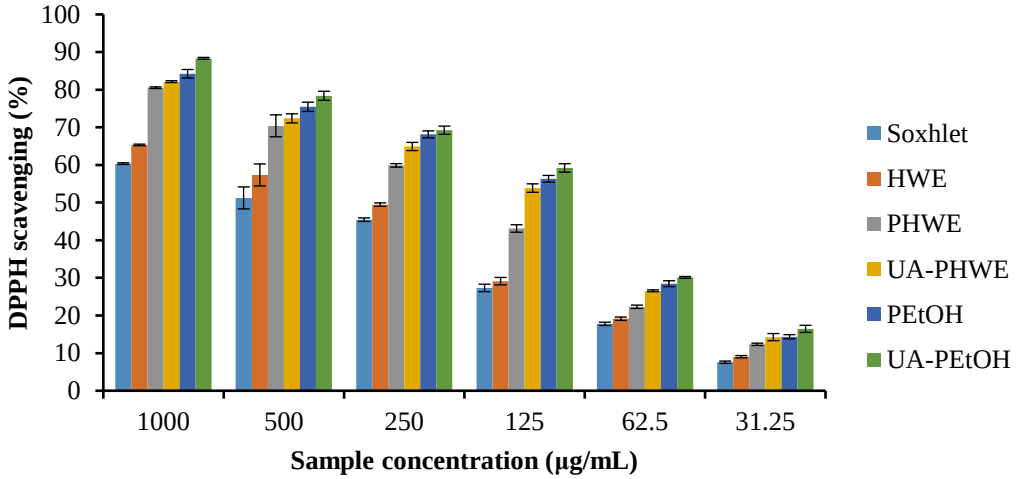

Figure 4. DPPH scavenging capacity of the extracts.

Table 1. SC_{50} values of DPPH scavenging.

Samples	Soxhlet	HWE	PHWE	UA-PHWE	PEtOH	UA-PEtOH	Trolox
SC_{50} [µg/mL]	564.1 ± 10.3 ^{f}	496.8 ± 11.8 ^{e}	350.6 ± 10.1 ^{d}	301.7 ± 8.5 ^{c}	279.8 ± 7.6 ^{b,c}	253.5 ± 6.5 ^{b}	7.76 ± 0.3 ^{a}

TEAC

Due to its operational simplicity, the TEAC assay has been used in many research laboratories for studying antioxidant capacity, and TEAC values of many compounds and food samples are reported. The TEAC values for pure antioxidant compounds do not show a clear correlation between TEAC values and the number of electrons an antioxidant can give away (Dejian Huang, 2005). HWE showed a higher TEAC value than UA-PHWE although the convert trend was observed in the DPPH scavenging assay (Fig. 5).

Lipid peroxidation inhibition

Polyunsaturated fatty acids are highly susceptible to peroxidation and that, once initiated, the process proceeds as a free radical chain reaction. Peroxidation is initiated by the attack of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. The resulting carbon center radical undergoes molecular rearrangement, followed by a reaction with O_2 to give a peroxyl radical. Peroxyl radicals can combine to cause singlet oxygen formation. Hydrogen abstraction from adjacent membrane lipids leads to the formation of lipid hydroperoxide (Gutteridge & Halliwell, 1990). In this study, the extracts PEtOH, UA-PEtOH showed similar lipid peroxidation inhibition activity and were significantly different from PHWE and UA-PHWE ($p < 0.05$) (Fig. 6).

In general, the pressurized liquid extraction techniques with and without ultrasonic assistance (PEtOH, UA-PEtOH, PHWE, and UA-PHWE) provided extracts with higher lipid peroxidation inhibition activity as compared with that obtained from conventional extraction techniques such as Soxhlet and HWE ($p < 0.05$). These results were also consistent with antioxidant activity and total phenolic content in these extracts. A previous study demonstrated that there was a significant correlation between anti-lipid peroxidation and total phenolic and flavonoid contents (Shabbir, Khan, & Saeed, 2013).

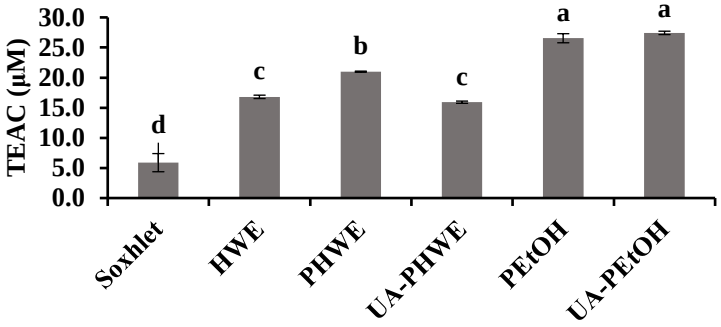

Figure 5. TEAC activity of the extracts from different extraction methods.

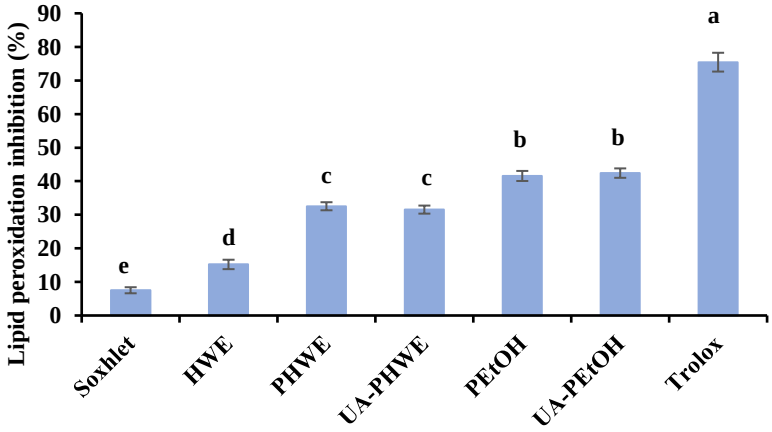

Figure 6. Lipid peroxidation inhibition activity of the extracts obtained from different extraction methods and Trolox.

CONCLUSION

The present study investigated the extraction of TPC from *Perilla frutescens* L. leaves using PLE and compared it with conventional ones. The outcomes indicated that PLE was more effective than conventional extraction techniques due to the change in the thermodynamic properties of solvents. Consequently, the antioxidant capacities such as DPPH scavenging, TEAC were higher in the extracts using PLE except for UA-PHWE than those in conventional ones. These results indicated that PLE has a potential application in the recovery of bioactive compounds from not only *Perilla frutescens* L. leaves but also other natural sources. The use of PLE for the extraction of bioactive components from *Perilla frutescens* L. leaves at higher temperatures should be considered in future studies.

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Conflict of interest: *The authors declare no conflict of interest.*

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