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Green extraction of polyphenolic-polysaccharide conjugates from *Pseuderanthemum palatiferum* (Nees) Radlk.: chemical profile and anticoagulant activity

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Abstract

In this study, pressurized liquid extraction (PLE) of polyphenolic-polysaccharide (PP) from *Pseuderanthemum palatiferum* (Nees) Radlk. leaves was carried out and compared with a conventional technique using 0.1 M sodium hydroxide. The extracts were purified according to the method reported previously to obtain PP conjugates which were further studied about chemical profiles and anticoagulant activity. Fourier-transform infrared spectroscopy (FTIR), UV-Vis, nuclear magnetic resonance (NMR), gel permeation chromatography (GPC), and spectrophotometry analysis were used to characterize the selected PP conjugates. The results showed that PP conjugates comprised of carbohydrate, phenolic, and protein constituents with the yield ranged from 2.76% to 14.34%. Seven mono sugars containing in all conjugates were determined using high-performance liquid chromatography (HPLC), namely, arabinose, fucose, galactose, glucose, mannose, rhamnose, and xylose. PP conjugates obtained from PLE at 150 °C (PP-PLE5) exhibited better anticoagulant activity than those found at 200 °C and comparable to that of the conventional technique. On gel permeation chromatography, PP-PLE5 showed a broad molecular mass from 6 to 642 kDa. From the obtained results, PLE can be used as a green effective technique for the recovery of PP conjugate from *P. palatiferum* leaves.

Keywords

Pressurized liquid extraction

Polyphenolic-polysaccharide conjugates

Anticoagulant activity

1. Introduction

Recently, polyphenolic–polysaccharide (PP) conjugates obtained from medicinal plants exhibited high biological activities, such as anticoagulant and platelet aggregation inhibitory activities [1-6]. Plant samples were isolated and purified through several stages using different organic solvents [3] to obtain PP conjugates. However, it has been reported that conventional extraction techniques require long extraction time, high energy consumption, and more organic solvents, of which the latter may not only cause negative effects on human health upon ingestion due to their residue in the final products but also be costly to purchase and dispose of [7-12]. In recent years, modern techniques have been commonly used for the extraction of nutraceuticals from foods and herbal plants. These techniques retain huge advantages, such as less toxic solvents, safe operation, ease of scale-up, short time, and low energy consumption compared with conventional methods. Therefore, the influences of “green” extraction techniques on the structures and biological activities of the PP conjugate from medicinal plants should be considered [13].

Among modern extraction methods, subcritical water extraction (SWE) or pressurized liquid extraction (PLE), which mainly uses water as an available resource and environmentally benign solvent, has exhibited the potential to replace conventional techniques [14]. SWE drives at temperatures above the boiling point of water (100 °C–374 °C) and at a pressure high enough to keep it in the liquid state (1–22 MPa). Under these thermodynamic conditions, the solubility of organic compounds in water increases with a small increase in temperature. The water properties affecting the solubility of organic solutes in water, controlled by fine-tuning water temperature, are polarity or dielectric constant, and water density [15]. The solubility of organic compounds in water increases significantly in the subcritical water state [16]. Thus, water has been used to

extract bioactive compounds from a variety of natural resources [17-20]. Besides, the use of solvent mixtures is also found to improve the extraction efficiency by enhancing the solubility and increasing the interaction between the solvent and targeted compounds. For instance, in a mixture of two solvents, one could enhance the soluble capacity, whereas the other would improve the desorption of the compound [8]. A mixture of water and ethanol has been used as a “green” solvent in PLE to extract bioactive compounds from grape marc [21], phenolic compounds and sugars from flower resources [19], and polyphenols from goldenberry [22]. In addition to solvent selection, extraction temperature is one of the most important factors which has a significant influence on the efficiency and selectivity in PLE. Elevated temperatures can reduce surface tension and viscosity of the solvent, resulting in the enhancement of the solvent penetration inside the matrix, thus causing the compounds to be dissolved faster by the solvent. The solvent flow rate is also a considerable factor that may affect the extraction efficiency of bioactive compounds from the sample [23].

P. palatiferum is a new medicinal plant that was first found in Vietnam in the late twentieth century. Thereafter, various bioactive compounds from the leaf of this plant, including phytosterols (stigmasterol and β -sitosterol), flavonoids (kaempferol 3-methyl ether 7-O- β -glucoside and apigenin 7-O- β -glucoside), saponins, and lipids [24], were determined. In recent studies, the crude extracts from the leaf exhibited various biological activities, such as anticancer [25] and antidiabetic activities [26], hypoglycemic [27] and hypertensive effect [28], anti-lipolytic and alpha-amylase inhibitory [29], anti-inflammatory [30], acetylcholinesterase inhibitory effect [31], and antimicrobial [17, 32] activities. Consequently, this plant is recommended in folk medicine in some Southeast Asian countries for promoting people's health and treating various diseases, including hypertension, diarrhea, arthritis, hemorrhoids,

stomachache, tumors, colitis, bleeding [27]. Owing to its significant biological activities and containing bioactive components, *P. palatiferum* could be a potential source of PP.

Although there are many reports about the use of PLE, studies about the recovery of polyphenolic–polysaccharide (PP) conjugate using PLE are scarce. Given the advantages of this green technique, there is a considerable gap in recovering PP conjugates from herbal plants. Therefore, in this study, PLE was used to isolate PP from *P. palatiferum* leaves and then extracts were purified to achieve PP conjugates. The obtained PP conjugates were compared with that of the conventional method using 0.1 M NaOH in terms of their anticoagulant activity and chemical profiles. Finally, the selected conjugates were characterized using advanced characterization techniques.

2. Materials and methods

2.1. Materials and chemicals

P. palatiferum leaves were collected in Western Highlands, Vietnam, in October 2017 and provided by Thanh Binh Co., Ltd (Ho Chi Minh, Vietnam). Dried leaves containing $9.96\% \pm 0.03\%$ (w/w) of moisture and 15.5% of ash were ground with a particle size ≤ 710 nm and stored at $-60\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for further use. The aPTT and PT reagents, including pooled human blood plasma from healthy donors, were purchased from Thermo Fisher Scientific, Pittsburgh, PA. Standard chemicals, including Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS⁺ [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], gallic acid, heparin, arabinose, fucose, galactose, glucose, mannose, xylose, and rhamnose, were all purchased from Sigma-Aldrich Chemical Co., USA. Other solvents and chemicals used in this study were of analytical grade.

2.2. Extraction of PP from *P. palatiferum* leaves

Pressurized liquid extraction (PLE) of PP from *P. palatiferum* leaves was performed using the semicontinuous system (Figure 1). In this study, two extraction solvents were water and 60% aqueous ethanol, extraction temperatures were 150 °C and 200 °C, and flow rates of 5 and 10 mL min⁻¹ were employed. The extracts were filtered under vacuum and evaporated to dryness.

The conventional technique using 0.1 M NaOH was performed according to the method described in the previous study [3] with some modifications. Briefly, the sample was extracted by 250 mL of 0.1 M NaOH at room temperature for 24 h, and then for 6 h at boiling point, under the reflux. Afterward, the mixture was centrifuged at 4000 rpm for 15 min to obtain the supernatant. Then, the supernatant was neutralized with 1 M HCl and evaporated to dryness. The sample (6 g) was used for all experiments.

2.2.2. Purification of PP from *P. palatiferum* extracts

The dryness parts obtained from PLE and 0.1 M NaOH techniques were further purified according to the method described in the previous study [3] with some modifications. Briefly, the dryness parts were dissolved in 250 mL of deionized water and extracted twice with n-hexane (water/hexane – 1:1 v/v) for 6 h at 69 °C. This step was followed by the extraction of the water part twice with diethyl ether at 34 °C for 6 h. The separated water parts were evaporated into a paste-like form. Then, the paste-like forms were treated with methanol (2 L) at room temperature, filtered in a glass funnel under reduced pressure, and dried at room temperature. Finally, the residues were dissolved in distilled water, intensively dialyzed against distilled water (4 L) for 5 days, and then freeze-dried to give dark isolates. The final PP conjugates obtained from different extraction methods were named PP-PLE1 (PP obtained from PLE at 5 mL/min–200 °C–60% ethanol), PP-PLE2 (PP obtained from PLE at 5 mL/min–200 °C–water), PP-PLE3 (PP obtained from PLE at 5 mL/min–150 °C–water), PP-PLE4 (PP obtained from PLE at 10 mL/min–150 °C–

60% ethanol), PP-PLE5 (PP obtained from PLE at 10 mL/min–150 °C–water), PP-PLE6 (PP obtained from PLE at 10 mL/min–200 °C–60% ethanol), PP-NaOH (PP obtained from conventional technique using 0.1 M NaOH).

2.3. Chemical characterization

The total carbohydrate content was measured by the phenol sulfuric acid method [33]; glucose was used as a standard and expressed as weight percentage (wt%). The determination of total phenolic and flavonoid contents was performed using the previous methods [34, 35], gallic acid and rutin were used as standard compounds. The total protein content was estimated according to the Pomory assay [36], and bovine serum albumin (BSA) was used as a reference. The content of uronic acids (UA) was established using the 96-well plate carbazole reaction assay [37] and D-glucuronic acid was used as a reference compound. The free radical scavenging activity of selected PP conjugates was also determined according to the method described in the previous study [38]. All the assays were measured using a multimode reader (BioTek Instruments, Inc., USA).

2.4. High-performance liquid chromatography (HPLC) of monosaccharides

For hydrolysis steps, sample (3 mg) was mixed with 72% of H₂SO₄ (30 µL) and incubated at 30 °C for 1 h before being diluted with 840 µL of deionized water. The mixture was then treated at 121°C for 30 min using an autoclave. The resulting mixture was neutralized by 42 mg of CaCO₃ and centrifuged to achieve the supernatant.

In 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization procedure [39], the supernatant (100 µL) was sequentially mixed with 0.6 M NaOH (100 µL) and 0.5 M PMP in methanol (200 µL) and thoroughly vortexed before incubating at 70 °C in an oven for 1.5 h. After being cooled down to room temperature, the mixture was neutralized with 0.3 M HCl (200 µL) and vigorously

vortexed. To the resulting mixture, chloroform (1 mL) was added and carefully shaken. The chloroform layer was discarded and this step was repeated five times. Finally, the aqueous layer was filtered using a 0.45 μm filter for HPLC analysis. Both hydrolyzed samples and standards were treated under the same procedure. HPLC analysis was done according to the method reported previously [39]. Briefly, a mixture of 0.1 M phosphate buffer (pH 6.7) and acetonitrile (83:17, v/v) was used as a mobile phase. The PMP derivatives elution was performed with the mobile phase through the Eclipse Plus C₁₈ column (250 $\times$ 4.6 mm i.d., 5 μm) with a flow rate of 1 mL/min. An injection volume of 20 μL and UV absorbance at 245 nm were set for the analysis. Seven mono sugars were used for establishing standard curves with concentrations from 50 to 1000 $\mu\text{g/mL}$.

2.4. *In vitro* clotting assays

The *in vitro* anticoagulant activity of PP conjugates from *P. palatiferum* leaves was investigated using two different conventional diagnostic assays – the aPTT and PT tests using the procedures described by Pawlaczyk [5]. The concentrations of analyzed samples in deionized water ranged from 3.91 to 4000 $\mu\text{g mL}^{-1}$. The aPTT and PT tests were conducted in triplicate.

2.5. Gel permeation chromatography (GPC) analysis

The molecular weight of the selected PP was determined using gel permeation chromatography (GPC). Before the analysis, the sample was dissolved in deionized water (3 mg mL^{-1}), filtered through a 0.45- μm pore-size Nylon filter, and degassed. The injection volume was maintained at 100 μL , and 0.1 M of NaNO_3 was used as a developing solvent with a flow speed of 1 mL min^{-1} . The molecular mass of polysaccharide and its distribution were analyzed at 40 $^{\circ}\text{C}$ using Tosoh EcoSEC HLC-8320 GPC equipped with RI detector and TSKgel guard PW $\times$ 1 + 2 $\times$ TSKgel MPW $\times$ 1 + TSKgel G2500PW $\times$ 1 (7.8 $\times$ 300mm) column. The determination of the molecular

mass of the sample was based on the calibration curve of the pollutant standards (6.1 to $642 \times 10^3 \text{ g mol}^{-1}$). The results were analyzed using the EcoSEC software.

2.7. Fourier transform infrared (FTIR) spectroscopy

The spectra of KBr pellets, including 2 mg of PP and 250 mg of KBr, were determined using Cary 640 Fourier transform infrared (FTIR) spectrometer (Agilent Technologies Inc., USA). The wavenumber region and the number of scans used in this study were 4000 to 400 cm^{-1} and 16, respectively.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

The nuclear magnetic resonance (^1NMR) and heteronuclear single quantum coherence (HSQC) NMR spectra of samples (70 mg) were recorded in D_2O (1 mL) on FT-NMR 600 MHz (JEOL JNM-ECP600, Japan).

2.9. Ultraviolet-visible (UV-Vis) analysis

The UV spectra of the purified sample were measured in the wavelength range of 200 – 700 nm using a multi-microplate reader (Synergy HTX BioTek Instruments, Winooski, VT, USA) at 25°C .

2.10. Statistical analysis

Statistical analysis was conducted using Microsoft Office Excel 2010. The standard deviation of data was expressed as mean $\pm$ S.D.

3. Results and discussion

3.1. Isolation and chemical characterization of PP from *P. palatiferum*

PP conjugates were successfully isolated from various medicinal plants and exhibited high anticoagulant activity [1-4, 40, 41]. The previous extraction procedure [42] was also modified to improve the anticoagulant activity on the selection of enzymes of coagulant cascade [2].

In this study, PP conjugates from *P. palatiferum* leaves were isolated using PLE and compared with conventional methods using 0.1 M NaOH. According to the previous report, subcritical water at 150 °C and 200 °C and pressure of 5 MPa has dielectric constants of approximately 44.12 and 34.90, respectively [43]. Consequently, water was not appropriate to remove nonpolar and less polar compounds, as compared with 60% aqueous ethanol under the same conditions or alkaline at ambient temperature and pressure. No color appeared in the upper phase (hexane or diethyl ether) of the mixture containing the extracts obtained from PLE using water (Fig. S1). This indicated that there were no nonpolar and less polar compounds extracted. Therefore, the use of hexane and diethyl ether can be ignored in the purification procedure employing subcritical water as an extraction solvent. On the other hand, the yellow color was observed in the upper phase of the remaining mixtures demonstrating the presence of some unexpected compounds.

However, the thermodynamic properties of water at 150 °C and 200 °C are close to that of methanol at ambient temperature and pressure [44]. Consequently, some waxes, isoprenoids, and even some low molecular mass polyphenolics can be extracted [2]. To eliminate these unwanted components, the dryness part needed to be treated with methanol. From this careful observation, we proposed a new procedure for the recovery and purification of PP conjugates from medicinal plants using PLE (Figure 2).

The PP conjugates attained from different isolation techniques showed different colors (Figure S2). Specifically, PP-PLE1, PP-PLE4, and PP-PLE6 possessed beige and brown color; PP-PLE3 was blonde; whereas PP-PLE5 and PP-NaOH had a dark brown color. Moreover, texture between the conjugates was also diverse, for instance, PP-NaOH had a layer structure, whereas all PP conjugates achieved from the PLE technique had a soft fiber structure. The difference in

extraction conditions might result in a reduction of the molecular weight of PP conjugates, leading to variations in their structures.

The yields of PP conjugates were also varied and depended on the extraction processes (Table 1). The technique using 0.1 M NaOH provided the highest yield (14.34%) as compared with those of PLE. With the treatment at a mild temperature, the conventional technique might not render any thermal degradation of PP. On the other hand, PLE is usually carried out under an elevated temperature that could decompose more polysaccharides. This results in the formation of low molecular weight compounds, such as glucose and other saccharides [45], leading to a decrease in the final yield after dialysis. Regarding the type of extraction solvents in PLE, water extracted more PP than 60% aqueous ethanol (PP-PLE1 and PP-PLE2 or PP-PLE3 and PP-PLE4). Also, a solvent flow rate of 10 mL min⁻¹ was more effective than that of 5 mL min⁻¹ in improving extraction efficiency (PP-PLE1 and PP-PLE6 or PP-PLE3 and PP-PLE5, Table 1). In the dynamic extraction mode, the efficiency increased with the increase in the flow rate owing to the promotion of mass transfer of the solutes from the sample matrix [46]. According to a previous study, pressurized hot water extraction of black tea at a flow rate of 12 mL min⁻¹ gave a higher yield than that found at 6 mL min⁻¹ [23].

As shown in Table 1, PP-PLE1, PP-PLE2, and PP-PLE6 comprised higher carbohydrate and protein content than those found in PP-NaOH, PP-PLE3, PP-PLE4, and PP-PLE5. This can be explained by the low viscosity and surface tension of water at elevated temperatures, thus promoting better penetration of water into the matrix particles to improve the extraction [46]. Moreover, under these conditions, cellulose, a major component in the plant cell wall and consisting of glucose units, linked by β -(1-4)-glycosidic bonds, is rapidly hydrolyzed to its constituents [45]. In a previous study, the total sugar and protein contents extracted from defatted

rice bran reached peaks at 200 °C and 240 °C, respectively [47]. However, low contents of uronic acid, TPC, and TFC were found in the conjugates obtained at 200 °C (Table 1). This might have resulted from the thermal degradation of these components under severe temperature treatment. The highest content of TPC and TFC was observed in the PP-PLE5 conjugate (264.66 ± 2.54 mg GAE g⁻¹ and 135.83 ± 1.66 mg RE g⁻¹, respectively). Besides, seven monosaccharides were determined using HPLC and presented in Table 1. Rhamnose, glucose, and galactose were dominant among the constituents of carbohydrates; whereas, fucose and xylose are the least. The formation of xylose depends upon the treating temperature during the extraction process. The high amount of xylose was observed with the application of temperature at 200 °C.

3.2. Anticoagulant activity

The examination of the anticoagulant activity of PP conjugates from *P. palatiferum* leaves was done and presented in Tables 2 and 3. In these tests, the use of pooled human blood plasma from healthy donors was followed according to the manufacturer's instruction. Heparin sodium salt from porcine intestinal mucosa (180 UI mg⁻¹) was used as a reference substance. The concentrations of PP conjugates in deionized water ranged from 3.91 to 4000 µg mL⁻¹.

For aPTT tests, PP-NaOH and PP-PLE5 conjugates at a concentration of 2000 µg mL⁻¹ strongly inhibited plasma clot formation for more than 10 min; whereas, the PP-PLE3 and PP-PLE4 conjugates required a higher concentration (4000 µg mL⁻¹) to reach that duration. We found that at the concentration above 1000 µg mL⁻¹, PP-NaOH conjugates extended the clotting time longer than those found in PP-PLE3, PP-PLE4, and PP-PLE5 conjugates. However, with concentrations no more than 500 µg mL⁻¹, the converse trend was observed when PP-PLE3, PP-PLE4, and PP-PLE5 conjugates showed better inhibition of plasma clot formation in the reacting mixture. This may be due to the carbohydrate contents in these conjugates were noticeable higher than that of

PP-NaOH (Table 1). Although the degradation of PP might slightly occur during hydrothermal treatment, their biological activity remained. Consequently, the conjugates with dense PP more effectively inhibited Factor Xa.

PP-PLE1 was the least active even though it contained the most carbohydrates (Table 1). Owing to the influence of elevated temperature during the PLE extraction process, PP structure was strongly fragmented, leading to a decrease in anticoagulant activity. It is reported that polysaccharides with higher molecular weight exhibited greater anticoagulant capacity [43].

PT test is utilized to perceive bleeding disorders. The extension of the PT test often implies the insufficiency in one or more of the factors in the extrinsic or common pathway of blood coagulation. This insufficiency can result from hereditary coagulation disorders, vitamin K shortage, liver disease or drug administration. The use of the PT test is common to examine oral anticoagulant therapy because it is sensitive to insufficiency in Factors II, VII, and X1,2 and for general pre-operative screening. However, the PT test is not sensitive to insufficiency in the intrinsic coagulation system (i.e. Factors VIII, IX, XI, and VII) or to platelet dysfunctions [41]. PP-NaOH and PP-PLE5 were observed as the most active anticoagulant which prolonged the clotting time two and a half min at the concentration of 4000 $\mu\text{g/mL}$ (Table 3). The inhibition of the coagulation process by the extrinsic pathway of the enzymatic cascade is usually associated with some side effects, for example, uncontrolled bleeding episodes [2].

3.3. Antioxidant activities

DPPH and ABTS radical scavenging assays of PP-PLE5, PP-NaOH conjugates, and Trolox were investigated and shown in Figure 3. In these assays, analyzed samples were dissolved in deionized water with concentrations from 15.6 to 250 $\mu\text{g mL}^{-1}$. Concentrations of Trolox in methanol were between 1.5 and 9.5 $\mu\text{g mL}^{-1}$. Sample and standard solutions were used to

scavenge against 0.05 M DPPH and 0.05 M ABTS solutions with the absorbance of approximately 1.4. The IC_{50} values of PP-PLE5 were 151.5 and 111.5 $\mu\text{g mL}^{-1}$ for DPPH and ABTS, respectively. Those values of PP-NaOH were 237.7, and 152.1 $\mu\text{g mL}^{-1}$, for DPPH and ABTS radicals, respectively. They were higher than that found in Trolox (7.76 and 6.72 $\mu\text{g mL}^{-1}$, for DPPH and ABTS radicals, respectively), indicating that the samples had lower antioxidant capacity than the standard compound. The differences in the antioxidant capacity of the two conjugates can be explained by their TPC and carbohydrate contents. These values of the PP-PLE5 conjugate were noticeable greater than that of PP-NaOH (Table 1).

3.5. FTIR, UV-Vis, and NMR analysis

Fourier-transform infrared analysis (FTIR) spectra of PP-PLE5 and PP-NaOH conjugates were measured and presented in Figure 4A. In general, the spectra of the two samples indicated some similarities and differences from each other. In the frequency region of approximately 3420 cm^{-1} , the two spectra displayed wide intensive bands, which are representative of the stretching vibrations (O–H) of hydroxyl groups of saccharides, as well as phenolic groups [3]. Two low-intensity bands were observed in both spectra at frequencies of $2933\text{--}2854\text{ cm}^{-1}$ and $2927\text{--}2852\text{ cm}^{-1}$ derived from the stretching vibrations of (C–H). The first band is antisymmetric stretching vibrations of ($-\text{CH}_2-$), whereas the second one is characteristic of symmetric interactions of such bonds, both of them confirmed the presence of ($-\text{CH}_2-$) group which is typically for aromatic and aliphatic structures, respectively [2, 3]. Similarly, symmetric stretching vibrations of (C=O) bonds at a frequency of 1421 cm^{-1} observed in both spectra were also recognizable [3]. A band at the region of 1330 and 1340 cm^{-1} , which confirmed the presence of phenyl O–H structure, was also visible in both spectra [3]. Other intensive bands, which are characteristic of C=O and C=C bonds, at the same frequency region of 1639 cm^{-1} in both analyzed spectra, were detected [2].

Two bands at $1560\text{--}1542\text{ cm}^{-1}$ and $1560\text{--}1544\text{ cm}^{-1}$ related to phenolic fragments were observed in both spectra [3]. Other visible bands of the two spectra representative of the stretching vibrations (C–O–C) were recognized at 1238 and 1251 cm^{-1} [3]. FTIR bands at the frequency region of 1143 and 1145 cm^{-1} are dominated by the glycosidic linkage (C–O–C) contribution [48]. Bands at frequencies of $1051\text{--}1047\text{ cm}^{-1}$ were typically for the presence of mannose, arabinose, and rhamnose constituents [48]. The band shape is influenced by galactan from the side chain, which has a band at around 1072 cm^{-1} ; however, the band at 1095 could not be explained yet [48]. Bands at lower frequencies of 887 and 896 cm^{-1} in both spectra confirmed the presence of β -anomer [48].

The UV–Vis spectrum of the isolates was reported in $250\text{ }\mu\text{g mL}^{-1}$ solutions at a wavelength range of $200\text{--}700\text{ nm}$ and was presented in Figure 4B. The peaks at the absorbance of 230 nm observed in both spectra indicated the presence of protein [49]. The maximum absorbance at 260 nm might be attained from the aromatic structures of phenolic origin (C=C bonds of aromatic rings). Another wide band with low intensity at the absorbance of approximately 330 nm is typically for the presence of subunits of phenolic conjugates to each other (to the C=C bonds of the aromatic structures) [1].

The ^1H NMR spectra of PP-PLE5 and PP-NaOH conjugates containing three visible regions of signals were presented in Figure 5A. First, in the region at $\delta\ 3.0\text{--}0.5\text{ ppm}$, broad signals were mostly caused by the aliphatic fragments ($-\text{CH}_2-$) and ($-\text{CH}_3$) of phenolic compounds, protein content, and $-\text{CH}_3$ of C6 of deoxyhexose (Rha). The powerful signals in the region at $\delta\ 5.5\text{--}3.3\text{ ppm}$ typically constituted the carbohydrate part, and signals at $\delta\ 7.5\text{--}6.5\text{ ppm}$ attained the aromatic segments of phenolic fragments [2]. Specifically, two signals in the region between 1.0 and 1.25 ppm obtained from $-\text{CH}_3$ protons of O-2 and O-2,4 linked L-rhamnose. Another two

signals at approximately 1.12 and 1.18 ppm were designated for the acetyl groups binding at 2- and 3-O-galacturonic acid (GalA), respectively [50]. The powerful peaks at around 3.68 ppm derived from methoxyl groups attached to carboxyl (R-COOH) groups of esterified GalA residues [50]. These groups are also correlated with the COO group of phenolic compounds (δ 176.4) as shown in ^{13}C NMR spectra (Figure 5B) [51]. The second region containing five intensive peaks of both spectra were allocated the esterified GalA, including H-1 4.95, H-2 3.64, H-3 3.86, H-4 4.05, and H-5 4.95 ppm, proposing that GalA was the major constituent [52]. The chemical shift of H-1 observed at the region around 4.95 ppm is characteristic of the α -glycoside linkage of GalA, whereas an anomeric region with signals at approximately 5.09 and 5.17 ppm was designated to the H-1 of Ara and Rha, respectively [50].

^1H - ^{13}C hetero-correlated HSQC spectra of the purified conjugates (Figure 5C and 5D) provided more information on cross-peak signal assignments based on the literature data. The cross-peak located at 1.24/17.2 in the HSQC NMR spectra due to the rhamnose (Rha) component in the conjugates. Besides, the signals were at approximately 4.55/103.5 and 4.65/105.5 due to β -galactose (terminal β Gal) and internal 1,4-linked β Gal and/or internal 1,3-linked β Gal. Another cross-peak was observed at around 5.16/107.3, which is characteristic of α Araf demonstrating its origin in 1,2,5- or 1,3,5-linked α Araf, primarily due to its branching by β Gal and/or its oligosaccharides [41].

3.6. Gel permeation chromatography (GPC)

The molecular mass of the PP-PLE5 was distributed in a wide range from Mp 0.18 to >642 kDa (Figure 6). There were three peaks with a dominance of low molecular peak at approximately 8 kDa, which could have resulted from the hydrothermal degradation of PP under subcritical water conditions. The second peak was observed at 21.5 min, with a molecular weight of about 83.5

kDa. The third low-intensity peak observed at 19 min was characteristic of high molecular mass with >642 kDa. Under the subcritical water state, the carbohydrates were converted into monosaccharides, which are mainly glucose [45], resulting in a decrease in molecular weight. PP-PLE5 was rich in carbohydrate content (26.58%, w/w) and phenolic content (~265 mg GAE g⁻¹) but low in protein content (1.48%, w/w). Galactose, glucose, and rhamnose were the main neutral sugars comprised of carbohydrates part with 22.87%, 20.16%, and 29.22%, respectively.

4. Conclusion

PP conjugates were successfully isolated from *P. palatiferum* using both conventional and green techniques. The biological activity and chemical profiles depended on the extraction conditions. PP-PLE conjugates obtained at 200 °C possessed the most carbohydrate; however, due to the thermal degradation of PP, they had the lowest anticoagulant activity. On the other hand, the anticoagulant effects of PP conjugates achieved from PLE at 150 °C were comparable to those of the conventional technique. In this study, we found that subcritical water is more appropriate to recover PP as compared with 60% aqueous ethanol under the same conditions. The results of this study revealed that PLE can be a potential candidate for the recovery of PP conjugates from medicinal plants. A new procedure for the isolation and purification of PP conjugates using this green effective technique was also proposed.

Declarations of interest: none

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Figures **Figure 1.** Diagram of the pressurized liquid extraction system

Figure 2. Isolation and purification steps of *P. palatiferum* conjugates

Figure 3. Antioxidant activity of PP-PLE5, PP-NaOH conjugates, and Trolox

Figure 4. FT-IR (A) and UV-Vis (B) spectra of PP-PLE5 and PP-NaOH conjugates

Figure 5. ^1H -NMR and ^{13}C -NMR NMR spectra of PP conjugates from *P. palatiferum* leaves: A - ^1H NMR of PP-
PLE5
and PP-NaOH; B - ^{13}C NMR of PP-PLE5 and PP-NaOH; C and D – HSQC NMR of PP-PLE5 and PP-NaOH,
respectively.

Figure 6. Gel permeation chromatography (GPC) of PP-PLE5 conjugates. Molecular weight was expressed as g/mol.

Table 1

Chemical profiles of PP conjugates isolated from *P. palatiferum* leaves

Compositions		PP-PLE1	PP-PLE2	PP-PLE3	PP-PLE4	PP-PLE5	PP-PLE6	PP- NaOH
Yield (wt%)		3.63	6.84	3.93	2.76	6.06	6.20	14.34
Carbohydrate content (wt%)		31.94± 2.35	27.10± 1.45	23.77 ± 2.29	18.41± 1.15	26.58 ± 0.54	23.85± 2.17	15.58 ± 0.58
Protein content (wt%)		1.72±0.50	4.02± 1.18	1.30 ± 1.00	1.16± 0.40	1.48 ± 0.48	1.90± 0.85	2.14 ± 1.40
Uronic acid (wt%)		13.43± 0.43	16.77± 1.99	19.79 ± 1.38	17.91± 1.39	18.14 ± 1.04	18.50± 1.59	20.86 ± 1.11
Monosaccharides composition [wt%]	Ara	9.89 ± 0.02	8.76 ± 0.02	8.45 ± 0.06	9.56 ± 0.31	10.22 ± 1.21	9.21 ± 0.01	10.27 ± 0.02
	Fuc	1.71 ± 0.03	2.84 ± 0.01	3.1 ± 0.09	3.51 ± 0.51	3.64 ± 0.79	3.12 ± 0.04	3.26 ± 0.03
	Gal	20.61± 0.01	19.23± 0.01	23.86± 0.01	23.65 ± 0.01	22.87 ± 0.01	18.62 ± 0.01	21.33 ± 0.11
	Glu	25.92 ± 0.01	23.65 ± 0.04	20.34 ± 0.01	19.25 ± 0.01	20.16 ± 0.01	23.3 ± 0.01	22.11 ± 0.11
	Man	7.66± 0.01	8.25± 0.01	9.21± 0.01	9.47 ± 0.06	8.93 ± 0.11	8.26 ± 0.11	10.24 ± 0.13
	Rha	27.68 ± 0.01	28.21 ± 0.01	30.26 ± 0.01	29.17 ± 0.01	29.22 ± 0.01	29.11 ± 0.01	28.19 ± 0.11
	Xyl	7.12 ± 0.03	8.25 ± 0.1	3.24 ± 0.21	4.28 ± 0.12	4.38 ± 1.32	8.74 ± 1.12	5.36 ± 0.13
Total phenolic content (mg GAE g ⁻¹)		157.32± 0.49	151.95± 0.85	172.29± 0.91	201.99± 0.98	264.66 ± 2.54	180.48± 0.74	216.92± 1.29
Total flavonoid content (mg RE g ⁻¹)		75.23± 1.01	99.82± 1.32	111.97± 2.33	115.98± 2.72	135.83 ±1.66	99.09±2.15	118.03 ± 2.37

PP-PLE1: PP extracted at 5 mL/min – 200 °C – 60% ethanol

PP-PLE2: PP extracted at 5 mL/min – 200 °C – water

PP-PLE3: PP extracted at 5 mL/min – 150 °C – water

PP-PLE4: PP extracted at 10 mL/min – 150 °C – 60% ethanol

PP-PLE5: PP extracted at 10 mL/min – 150 °C – water

PP-PLE6: PP extracted at 10 mL/min – 200 °C – 60% ethanol

PP-NaOH: PP extracted at with 0.1 M NaOH

Table 2. Measurements of activated thromboplastin time (aPTT) tests of conjugates using in vitro experiments in human pooled plasma. Heparin sodium salt from porcine intestinal mucosa (180 UI mg⁻¹) was used as a reference substance. Measurements are performed in triplicates and standard deviation ($\pm$ S.D.) was reported.

Sampl e	aPTT test (s)											Contro 1 - 0
	Concentration of conjugates (µg/mL)											
	4000	2000	1000	500	250	125	62.5	31.25	15.62	7.81	3.91	
Hepar in	-	-	-	-	-	-	-	>600	>600	350 ± 2.0	270.6 ± 3.1	34.3 ± 0.6
PP- PLE1	365.1 ± 3.2	139.2 ± 1.8	105.6 ± 2.1	86.2 ± 1.6	61.3 ± 1.1	49.6 ± 0.8	39.6 ± 0.7	36.5 ± 0.6	35.1 ±1.0	35.6 ±0.5	34.2 ± 0.8	34.5 ± 0.5
PP- PLE2	570.3 ± 2.5	240.6 ± 2.4	140.3 ± 2.3	100.2 ± 1.3	70.2 ± 1.5	56.3 ± 0.9	44.3 ± 0.6	40.2 ± 0.9	36.6 ±0.7	35.4 ±0.7	35.2 ± 0.7	35.6 ± 0.7
PP- PLE3	>600	545.7 ± 2.8	456.1 ± 3.6	301.7 ±2.1	199.4 ± 1.6	157.4 ± 2.5	98.7 ± 1.4	71.4 ± 0.8	54.3 ± 0.9	37.6 ± 0.8	34.6 ± 0.4	35.2 ± 0.8
PP- PLE4	>600	500.2 ± 2.7	430.4 ± 2.5	296.3 ± 2.9	196.7 ± 2.3	152.1 ± 2.6	96.2 ± 1.1	69.7 ± 1.1	52.2 ± 0.6	36.3 ± 0.6	34.8 ± 0.5	34.6 ± 0.6
PP- PLE5	>600	>600	492.7 ± 2.8	335.4 ± 2.7	226.4 ± 2.6	162.5 ± 2.3	104.5 ± 1.8	73.3 ± 1.2	54.6 ± 0.7	41.7 ± 0.4	34.3 ± 0.7	34.8 ± 0.6
PP- PLE6	451.6 ± 2.8	190.3 ± 2.1	109.7± 1.8	95.5 ± 1.3	68.4 ± 0.8	51.1 ±0.7	42.6 ± 0.6	37.6 ± 0.8	35.1 ± 0.3	35.5 ± 0.7	34.6 ± 0.6	35.3 ± 0.4
PP- NaOH	>600	>600	498.1 ± 3.3	295.2 ± 2.8	189.7 ± 1.6	136.5 ± 1.6	87.4 ± 1.3	59.4 ± 0.5	45.4 ± 0.6	35.8 ± 0.9	34.2 ± 0.4	34.6 ± 0.8

PP-PLE1: PP extracted at 5 mL/min – 200 °C – 60% ethanol

PP-PLE2: PP extracted at 5 mL/min – 200 °C – water

PP-PLE3: PP extracted at 5 mL/min – 150 °C – water

PP-PLE4: PP extracted at 10 mL/min – 150 °C – 60% ethanol

PP-PLE5: PP extracted at 10 mL/min – 150 °C – water

PP-PLE6: PP extracted at 10 mL/min – 200 °C – 60% ethanol

PP-NaOH: PP extracted at with 0.1 M NaOH

Table 3. Prothrombin time (PT) assay of PP conjugates obtained from *P. palatiferum* leaves, made in vitro experiments in human pooled plasma

Samples	PT assay
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	Concentration of conjugates ($\mu\text{g/mL}$)											Control - 0
	4000	2000	1000	500	250	125	62.5	31.25	15.62	7.81	3.91	
PP-PLE1	64.7 $\pm$	43.2 $\pm$	24.3 $\pm$	14.2 $\pm$	13.3 $\pm$	12.2 $\pm$	13.6 $\pm$	13.5 $\pm$	13.2 $\pm$	13.7 $\pm$	13.3 $\pm$	13.3 $\pm$
	1.6	1.7	1.5	1.5	1.6	1.1	0.8	0.6	0.6	0.6	1.1	0.7
PP-PLE2	77.3 $\pm$	48.2 $\pm$	26.5 $\pm$	15.2 $\pm$	13.3 $\pm$	12.4 $\pm$	13.3 $\pm$	13.6 $\pm$	13.2 $\pm$	13.6 $\pm$	13.5 $\pm$	13.6 $\pm$
	1.8	1.5	1.6	1.6	1.5	1.3	0.9	0.8	0.6	0.8	0.5	0.6
PP-PLE3	134.8 $\pm$	69.8 $\pm$	49.2 $\pm$	33.6 $\pm$	24.4 $\pm$	13.5 $\pm$	13.7 $\pm$	13.3 $\pm$	13.4 $\pm$	12.3 $\pm$	13.3 $\pm$	13.4 $\pm$
	2.2	1.8	1.7	1.4	1.6	0.9	1.0	1.0	1.0	0.8	0.7	0.7
PP-PLE4	137.5 $\pm$	68.3 $\pm$	48.1 $\pm$	34.3 $\pm$	20.7 $\pm$	13.3 $\pm$	13.6 $\pm$	13.6 $\pm$	13.5 $\pm$	13.5 $\pm$	13.2 $\pm$	13.3 $\pm$
	1.9	1.9	1.6	1.5	1.6	1.1	0.4	0.5	0.9	0.7	1.0	0.9
PP-PLE5	151.3 $\pm$	73.5 $\pm$	52.3 $\pm$	35.5 $\pm$	24.4 $\pm$	14.6 $\pm$	13.2 $\pm$	13.3 $\pm$	13.3 $\pm$	13.3 $\pm$	13.7 $\pm$	13.2 $\pm$
	1.8	1.6	1.1	1.4	1.4	1.5	1.1	1.0	0.7	0.6	0.9	0.9
PP-PLE6	67.9 $\pm$	54.5 $\pm$	25.2 $\pm$	14.3 $\pm$	13.2 $\pm$	12.7 $\pm$	13.3 $\pm$	13.3 $\pm$	13.4 $\pm$	13.1 $\pm$	13.8 $\pm$	13.6 $\pm$
	1.2	1.3	1.8	1.6	1.3	1.6	0.8	0.7	1.0	1.2	0.8	0.4
PP-PPLE6	152.6 $\pm$	76.3 $\pm$	53.6 $\pm$	36.1 $\pm$	25.6 $\pm$	13.3 $\pm$	13.2	13.7 $\pm$	13.6 $\pm$	13.5 $\pm$	13.8 $\pm$	13.4 $\pm$
	2.1	1.4	1.8	1.8	1.7	1.4	± 1.1	0.6	0.8	0.7	0.4	0.8
PP-NaOH	2.1	1.4	1.8	1.8	1.7	1.4	± 1.1	0.6	0.8	0.7	0.4	0.8

PP-PPLE1: PP extracted at 5 mL/min – 200 °C – 60% ethanol

PP-PPLE2: PP extracted at 5 mL/min – 200 °C – water

PP-PPLE3: PP extracted at 5 mL/min – 150 °C – water

PP-PPLE4: PP extracted at 10 mL/min – 150 °C – 60% ethanol

PP-PPLE5: PP extracted at 10 mL/min – 150 °C – water

PP-PPLE6: PP extracted at 10 mL/min – 200 °C – 60% ethanol

PP-NaOH: PP extracted at with 0.1 M NaOH

Authorship contribution statement

Truc Cong Ho: Conceptualization, Resources, Methodology, Investigation, Formal analysis,

Writing - Original draft preparation; **Anley Teferra Kiddane:** Investigation; **Saravana**

Periaswamy Sivagnanam: Conceptualization; **Jin-Seok Park, Yeon-Jin Cho:** Investigation;

Adane Tilahun Getachew: Visualization; **Thanh-Tuyen Thi Nguyen:** Resources; **Gun-Do**

Kim: Resources; **Byung-Soo Chun:** Resources, Supervision, Funding acquisition.

Highlights

- PP conjugates from *P. palatiferum* were successfully isolated using PLE and 0.1 M NaOH.
- The anticoagulant activity of PP-PPLE at 150 °C was comparable to that of 0.1 M NaOH.
- The obtained PP conjugates using PLE at 200 °C showed low anticoagulant activity.

- In PLE, water recovered more PP than 60% aqueous ethanol under the same conditions.