Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

LC-ESI-QTOF-MS/MS characterization of phenolic compounds from *Pyracantha coccinea* M.Roem. and their antioxidant capacity

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Received August 20, 2020; **Accepted** December 3, 2020; **Published** January 31, 2021
Doi: http://dx.doi.org/10.14715/cmb/2021.67.1.29

Abstract: *Pyracantha coccinea* M.Roem. is considered as an important medicinal plant contributing remarkably to health and medicinal benefits. This is attributed to the presence of abundant polyphenols with powerful antioxidant properties. However, little research has been studied on the comprehensive identification and characterization of the phenolic compounds in areal parts of *P. coccinea*. This study aimed to investigate, characterize, and quantify the phenolic profiles of *P. coccinea* through liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS/MS) and high-performance liquid chromatography-photodiode array (HPLC-PDA. Further, it showed a significantly higher value in total phenolic content (TPC) than that of total flavonoids (TFC) and tannins (TTC). As for antioxidant capacities, *P. coccinea* presented the highest activity in ABTS (7.12 ± 0.25 mg AAE/g dw) compared with DPPH, FRAP, and TAC assays. The LC-ESI-QTOF-MS/MS analysis detected 28 phenolic compounds, including phenolic acids (12), flavonoids (13), other polyphenols (2), and lignans (1) in *P. coccinea* samples. The results from HPLC–PDA indicated the chlorogenic acid (11.49 ± 1.89 mg/g) was the most abundant phenolic acid, while kaempferol (14.67 ± 2.17 mg/g) was the predominant flavonoid in *P. coccinea*. This research confirms the benefits of the *P. coccinea* plant as a potential source of natural antioxidants for the food and pharmaceutical industries.

Key words: *Pyracantha coccinea* M.Roem.; Polyphenols; Antioxidant potential; LC-ESI-QTOF-MS/MS; HPLC-PDA.

Introduction

It is widely recognized that medicinal plants make a great contribution to human health. *Pyracantha coccinea* M.Roem. is a thorny evergreen shrub found in Central and Southern Italy. Their aesthetic characteristics and dense thorny structure make them an alternative to artificial fences and as ornamental plants grown in gardens. In late autumn, it produces red or orange cherries that can be used in jam, jellies, and sauces (1). The fruits of *P. coccinea* have been used in traditional medicine owing to their diuretic, cardiac and tonic properties (2-4). According to epidemiological studies, the consumption of foods rich in natural antioxidants strengthens the antioxidant capacity of organisms and eliminates the risk of chronic diseases (5). Previous studies have shown that *P. coccinea* is rich in bioactive compounds such as polyphenols, fatty acids, and vitamins, and so forth (3, 4). In particular, Keser (3) demonstrated that the presence of phenolic acids and flavonoids shows significant free radical scavenging and metal-chelating capacities. It serves not only as a natural antioxidant in the food and pharmaceutical industries but also as a potential candidate for monitoring heavy metals in the environment (6).

Polyphenols being a secondary metabolite of plants, exist in various phases, such as plant growth, promoter, lignification, and environmental threats. Chemically, a great number of polyphenols derived from edible plants can be categorized as flavonoids and non-flavonoid constituents (7). Flavonoids can be divided into several subfamilies according to the degree of oxidation of the oxygenated heterocycle, being flavanols, flavanones, flavones, flavonols (essentially, flavan-3-ols), isoflavones, and anthocyanidins (8). According to Rentzsch, Wilkens (9), non-flavonoid polyphenols mainly include phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavan-3-ols (catechin, epicatechin), and stilbenes (aglycones and glucosides). Previously, it is reported that the number of flavonoid compounds increased in the subsequent phases of growth for *Pyracantha coccinea*; young plantlets showed the presence of pyracanthoside and rutin, while one-year-old plants have hyperoside and isoquercitrin (2).

There are several *in vitro* methods used to evaluate the antioxidant properties of medicinal plants, including 2,2'-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid)(ABTS), 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging radical, ferric reducing antioxidant power (FRAP), and total antioxidant capacity (TAC) (10). However, the antioxidant capacity results may be affected by extracting solvent and type of sample (11). For example, Keser (3) indicated that ethanol extract of *P. coccinea* had higher scavenging potential on DPPH radical, ABTS radical cation, superoxide anion, and nitric oxide than the water extract. Further, the phenolic profiles of diverse plant materials can be determined by combining liquid chromatography with tandem mass

spectrometers (LC-MS/MS). LC-MS/MS is a sensitive analytical technique used to analyze biochemical, organic, and inorganic compounds commonly found in complex samples of environmental and biological origin (12). Additionally, the high-pressure liquid chromatography (HPLC) coupled with ultraviolet-visible (UV) or photodiode array detector (PDA) is also considered to be a highly effective tool for the quantitative analysis of specific polyphenols (13).

Numerous studies have previously focused on the phytochemistry of *P. coccinea,* but limited research being conducted on the identification, characterization, and quantification of polyphenols from this plant. Therefore, this research aimed to identify, characterize, and quantity the polyphenols from *P. coccinea* through LC-ESI-QTOF-MS/MS and HPLC-PDA. Also, phenolic content (TPC, TFC, and TTC) were determined, followed by the measurement of antioxidant potential (DPPH, FRAP, ABTS, and TAC) for targeted polyphenols.

Materials and Methods

Chemicals

Most of the chemicals, reagents, and standards utilized for extraction and characterization were analytical grade and purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). To estimate the polyphenols and their potential antioxidant capability, Folin-Ciocalteu reagent, gallic acid, L-ascorbic acid, vanillin, hexahydrate aluminium chloride, sodium phosphate, iron(III) chloride hexahydrate ($Fe[III]Cl_3.6H_2O$), hydrated sodium acetate, hydrochloric acid, sodium carbonate anhydrous, ammonium molybdate, quercetin, catechin, 2,2'-diphenyl-1-picrylhy-drazyl (DPPH), 2,4,6tripyridyl-s-triazine (TPTZ), and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from the Sigma-Aldrich (Castle Hill, NSW, Australia). RCI Labscan (Rongmuang, Thailand) is the main supplier of 98% sulphuric acid (H_2SO_4). The HPLC standards for quantitative analysis involving gallic acid, p-hydroxybenzoic acid, caftaric acid, caffeic acid, protocatechuic acid, sinapinic acid, chlorogenic acid, syringic acid, ferulic acid, coumaric acid, catechin, quercetin, quercetin-3-galactoside, diosmin, quercetin-3-glucuronide, epicatechin gallate, quercetin-3-glucoside, kaempferol and kaempferol-3-glucoside, which were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). HPLC and LC-MS/MS grade reagents were bought from Thermo Fisher Scientific Inc. (Scoresby, VIC, Australia); in detail, those reagents include methanol, ethanol, acetonitrile, formic acid, and glacial acetic acid. The 1mL HPLC vials were purchased from Agilent Technologies (Mulgrave, VIC, Australia). In addition, 96 well-plates were purchased from Thermo Fisher Scientific (VIC, Australia) for *vitro* bioactivities and antioxidant assays.

Sample preparation and extraction

A total of 3 kg, areal parts of *Pyracantha coccinea* M. were collected from the Mohammad-Shahr region in Alborz, Iran, in the maturing stage (September 2019). They were dried under shade at room temperature. A control herbarium specimen was prepared, and the identity was confirmed by a positive comparison of its characteristics with in-house plant reference material. This herbarium specimen, identified by A botanist from the Shahid Beheshti University of Medical Sciences, Tehran, Iran (SBMU-1150), is kept in the Phytochemistry Research Center at Shahid Beheshti University of Medical Sciences. Leaves were washed three times with distilled water and then dried at 25 ± 5 °C for one week. The dried leaves were powdered by a mechanical grinder, and then 400 g of powdered leaves were dissolved in 800 mL of 85% ethanol using a shaking water bath (90 rpm) at room temperature for 24 h. Then, filtering was performed (Whatman No. 1 filter paper), and this was concentrated with a rotary evaporator (Laborota 4000, Heidolph, Germany) at 40 °C for 30 min. This act was performed three times to achieve maximum extraction with ethanol. The solid residue (i.e., the extract) was stored at 4 °C until further analysis.

Estimation of polyphenols and antioxidant assays

TPC, TFC, and TTC assays were performed to evaluate the phenolic content in targeted *P. coccinea* samples. Further, four different types of antioxidant assays (FRAP, DPPH, ABTS, and TAC) were conducted by utilizing the method of Tang et al. (2020) with some modifications. The data was measured by a Multiskan® Go microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA).

Determination of total phenolic content (TPC)

The total phenolic content of the extracts was analyzed by modifying the spectrophotometric method described by Samsonowicz, Regulska (14). On the whole, 25 µL of extracts, 25µL Folin–Ciocalteu reagent solution ((1:3 diluted with water) and 200 µL water were mixed and added to a 96-well plate ((Corning Inc., Midland, NC, USA), and then incubated at 25°C for 5 mins. Subsequently, further incubation for 1h at 25°C was needed after adding 25 µL 10% (w/w) sodium carbonate. Then, the absorbance was measured at 765 nm by a spectrophotometer plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The gallic acid standard with concentration ranging from 0 to 200 µg/mL was prepared for the calibration curve to quantify the total phenolic content. The results were represented as mass (mg) of gallic acid equivalents (GAE) per sample weight.

Determination of total flavonoid content (TFC)

Total flavonoid constituents were measured according to the aluminum chloride colorimetric method as described by (15). The mixture of 80 µL 2% aluminium chloride and 120µL, 50g/L sodium acetate solution were added into a 96-well plate and then incubated at 25°C for 2.5h. Carrying out the absorbance measurement at the wavelength of 440nm. Meanwhile, quercetin (0 - 50 µg/mL) methanolic solution was applied to draw the standard curve. Each sample was analyzed at least 3 times, and the unit was showed by mg quercetin equivalents (QE) per weight of the sample.

Determination of total tannins content (TTC)

Total tannins were analyzed as reported by Zou, Dong (16) with some modification. Mixing 25µL of

sample extract and 150 µL 4% (w/v) vanillin solution, followed by 25 µL 32% sulfuric acid on a 96-well plate. Then, incubating them at 25 °C for about 15min to measure the absorbance at 500nm. Using catechin solution with concentrations ranging from 0 to 1000 µg/mL to prepare the standard curve, in which the unit of extracts was presented as mg catechin equivalents (CE) per weight of the sample.

2,2'-Diphenyl-2-picryl-hydrazyl (DPPH) antioxidant assay

The method of DPPH radical scavenging activities is in line with Vella, Cautela (17). For determination, 25 µL of sample extract and 260 µL of 0.1 M DPPH radical methanol solution were mixed and added into a 96-well plate and incubated at 25 °C for half an hour. The absorbance was determined at 517 nm, and concentrations of 0 - 50 µg/mL ascorbic acid aqueous solution was prepared for the standard curve. The results were calculated as parameters of ascorbic acid equivalent (mg AAE/g).

Ferric reducing-antioxidant power (FRAP) assay

The FRAP assay was conducted by adopting the method of Sogi, Siddiq (18) with some modification. The FRAP reagent was prepared freshly by mixing 300 mM sodium acetate solution (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution, and 20 mM ferric chloride solution in 10:1:1(v/v/v) ratio, respectively. Afterward, 280 µL of FRAP reagent was mixed with 20 µL of extract, and the mixture was transferred into a 96-well plate and incubated at 37 °C for 10 min, followed by the measurement of absorbance at 593 nm. A standard curve was generated using 0 - 50 µg/mL ascorbic acid aqueous solution, and the results were converted to mg of ascorbic acid equivalents per g (mg AAE/g) of the sample.

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS radical scavenging activity was determined using an ABTS decolorization assay (18). To obtain the fresh $ABTS^+$ dye, 5 mL of 7 mmol/L of ABTS solution was mixed with 88 µL of 140 mmol/L potassium persulfate solution. Then Free radicals were produced after 16 hours of incubation in the dark at room temperature. Furthermore, analytical grade ethanol was utilized to dilute the prepared $ABTS^+$ dye for obtaining an absorbance of 0.70 at 734 nm. Adding 290 µL ABTS radical cation reagent to 10µL sample solution and incubating the mixture for 6 min in a dark room at room temperature. Then, the absorbance was operated at 734 nm, and a standard curve was plotted using concentrations of 0-150 µg/mL ascorbic acid. The antioxidant property was expressed as mg ascorbic acid equivalents per g sample weight (mg AAE/g).

Total antioxidant capacity (TAC)

The total antioxidant capacity assay followed the procedure described in (19). Briefly, 260 µL of phosphomolybdate reagent was prepared by 0.6 M H_2SO_4, 0.028 M sodium phosphate, and 0.004 M ammonium molybdate, then mixed with 40 µL of sample extract. The mixture was incubated at 95 °C for 10 min, and the absorbance was measured at 695 nm after being cooled at room temperature. Concentrations of 0 to 200 µg/mL of ascorbic acid were prepared to describe the standard curve, and the antioxidant ability was expressed as mg of ascorbic acid equivalents per g (mg AAE/g) of the sample.

LC-ESI-QTOF-MS/MS analysis

The LC-ESI-QTOF-MS/MS analysis was conducted by modifying the method of previous studies (20) with some modifications. Agilent 1200 series HPLC equipped with Agilent 6520 I Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA) was applied to identify polyphenolic compounds. Utilizing a Synergi Hydro-RP 80 Å, reverse phase column (250 mm x 4.6 mm, 4 µm particle size) with protected C18 ODS (4.0 × 2.0 mm) guard column (Phenomenex, Lane Cove, NSW, Australia) to separate the comprehensive constituents. Mobile phase A was made in the ratio of water/acetic acid (98:2, v/v), and mobile phase B consisted of acetonitrile/ acetic acid/ water (50:0.5:49.5, v/v/v). The flow rate was set to be 0.8 mL/min, and the injection volume was 6 µL of each sample. The gradient elution was performed in the following program: 10–25% B (0–25 min), 25–35% B (25–35 min), 35–40% B (35–45 min), 40–55% B (45–75 min), 55–80% B (75–79 min), 80–90% B (79–82 min), 90–100% B (82–84 min), 100–10% B (84–87 min), isocratic 10% B (87–90 min). When the capillary and nozzle voltage set to 3.5 kV and 500 V, respectively, peaks could be identified in both positive and negative ion modes. Besides, nitrogen gas temperature 300 °C with the flow rate 5 L/min, sheath gas temperature 250 °C with the flow rate 11 L/min, nebulizer gas pressure 45 psi were set to meet the mass spectrometry condition. Using a complete mass scan ranging from m/z 50 to 1300 and analyzing MS/MS in automatic mode with collision energy (10, 15, and 30 eV) for fragmentation. Peak identification was carried out in both positive and negative modes with the controlled instrument. LC-ESI-QTOF-MS/MS MassHunter workstation software (Qualitative Analysis, version B.03.01, Agilent Technologies, Santa Clara, CA, USA) was regarded as the main source n and identify data.

HPLC analysis

The quantification of phenolic compositions was conducted HPLC analysis, and the method was referred to the research of (21). Each sample was prepared with 20 µL injection volume consistent with the same conditions characterized in LC-ESI-QTOF-MS/MS. Some of the polyphenol representatives, such as hydroxybenzoic acids and flavonols were identified by the UV detection at three different wavelengths (280 nm, 320 nm, and 370 nm) with 1.25 scan/s (peak width = 0.2 min) spectral acquisition rate. Data collection and processing were performed using Agilent MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis

All the analyses were performed in triplicate, and the values were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was applied to determine the mean differences between different samples and Tukey's honestly significant differences

Table 1. Polyphenol content in *P. coccinea* plant.

TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg CE/g)
14.48 ± 1.09	0.13 ± 0.01	4.56 ± 0.31

All data are presented as mg/g mean ± standard deviation (n = 3) and measured on a dry weight basis. TPC, Total phenolic content; TFC, total flavonoid content; TTC, total tannins content; GAE, gallic acid equivalents; CE, catechin equivalents; QE, quercetin equivalents.

(HSD) multiple rank test at $p \leq 0.05$. Besides, Pearson's correlation coefficient at $p \leq 0.05$ was used to explain the correlations between polyphenol content and antioxidant activities.

Results and discussion

Polyphenol estimation (TPC, TFC, and TTC)

Pyracantha contains a variety of phenolic compounds, such as phenolic acids, flavonoids, and tannins. The phenolic contents in *P. coccinea* samples were measured by TPC, TFC, and TTC assays, and the results were summarized in Table 1.

The TPC value of *P. coccinea* was 14.48 ± 1.09 mg GAE/g of dry weight, which was significantly higher than the value observed by Sarikurkcu and Tepe (4), with 1.31 ± 0.20 mg GAE/g fresh fruit. Previously, Chang, Lin (22) reported that the dry samples might contain more total phenols than the fresh ones because drying treatments accelerate complex phenolic compounds to be broken into part of cellular constituents in date fruit (*Phoenix dactylifera L.)*. Another reason may be that high temperatures cause larger cells to disintegrate, break apart, and release phenolic compounds (23). In addition, Sultana, Anwar (24) and Que, Mao (25) found that new phenolic compounds may be generated from the Maillard reaction precursor products during thermal treatment. However, Popoviciu, Negreanu-Pîrjol (26) demonstrate approximately 79% loss of total phenolics in *Cotoneaster salicifolius* Franch. fruits may be caused by heat-drying. Therefore, the effect of drying treatments on phenolic compounds varies from different materials.

Cai, Luo (27) found that the total phenolic content of 112 medicinal herbs had a significant variation, ranging from 0.19 to 50.20 g of gallic acid equivalents (GAE)/100 g dry weight (DW) with an average value of 2.93 g/100 g DW for aqueous extracts, and from 0.22 to 50.30 g/100 g DW with an average value of 3.87 g/100 g DW for methanolic extracts. Chinese Galls from *Rhus chinensis/R. potaninii* contained the most phenolic compounds (aqueous/methanolic: 50.20/50.30 g/100 g), followed by dried extracts (Catechu) from branches and stems of *Acacia catechu* (aqueous/methanolic: 41.94/45.70 g/100 g).

The TFC value of *P. coccinea* was 0.13 ± 0.01 mg QE/g, which was relatively low compared with that of previous studies (28, 29). The inconsistency might be due to cultivar, growth phases, and extraction process. As the related genera, like *Cotoneaster* sp. reported by Mohamed, Sokkar (30), the flavonoid content was around 6.8 mg /g in *Cotoneaster horizontalis*, while up to 5.4 mg/Kg dw in *Cotoneaster multiflorus* fruit (31). Flavonoids are the essential phenolic compounds in *Pyracantha coccinea*, especially pyracanthoside and rutin. These two flavonoids are the main constituents of flavonoids and present in all the growth stages of *P. coccinea* (2). They also found that the concentration and distribution of flavonoids in the aerial parts increased with plant growth. In addition, the flavonoid content in different parts (roots, leaves, fruits, and seeds) also varies during the reproductive period; for example, apigenin, kaempferol, and prunin are present in the leaves but not detected in the fruit (2). Han and Zhang (28) optimized the extraction process of total flavonoids from the *Pyracantha* and obtained 2.10 mg/g of total flavonoid content. They also reported that the optimum extraction conditions were as follows: ethanol concentration of 70%, the solid-liquid ratio of 1:10 g/ml, and extraction time of 3h.

The TTC value of *P. coccinea* was 4.56 ± 0.31 mg CE/g, which was higher than previously reported fruits of *P. coccinea* in Konya Turkey, with 0.46 ± 0.00 mg CEs/g fresh fruit of total condensed tannins (4). Different breeding areas and climatic conditions may influence the variation of tannin content. In this study, *P. coccinea* was collected from Iran, and the geographical location and climatic conditions of the two countries resulted in different tannin content, even though both were extracted with ethanol.

Antioxidant activities (DPPH, FRAP, ABTS, and TAC)

The antioxidant capacities of *P. coccinea* were determined by DPPH, FRAP, ABTS, and TAC assays. DPPH and ABTS assays were based on evaluating the radical scavenging ability of extracts, while FRAP and TAC assays were designed for analyzing the reducing power of extracts. Figure 1 shows that the results were expressed as mg ascorbic acid (AAE) equivalents per gram of samples (mg AAE/g).

The DPPH assay is conducted by combing the hydrogen-donating antioxidant of extract and the stable free radical DPPH· to produce the detectable non-radical DPPH-H (32). In this study, the DPPH value of *P.*

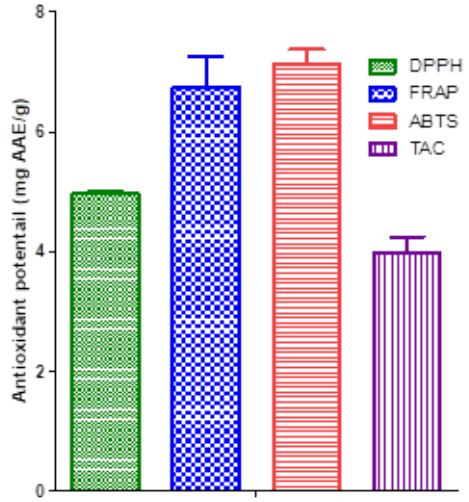

Figure 1. Antioxidant activities in *P. coccinea.* DPPH, 2,2'-Diphenyl-2-picryl-hydrazyl; FRAP, Ferric Reducing-Antioxidant Power; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); TAC, Total Antioxidant Capacity. Data of antioxidant activities in *P. coccinea* are presented as mg ascorbic acid (AAE) equivalents per gram of samples (mg AAE/g).

coccinea was 4.96 ± 0.04 mg AAE/g dw. Previously, few studies on the antioxidant potential in leaves of *Pyracantha coccinea* but some reports on related genera fruits. Saklani, Chandra (29) analyzed that the DPPH value was 21.10 ± 0.20 mm AAE/100g fw in *P. crenulata* fruits from Karanprayag city (Dist- Chamoli, Uttarakhand). The influence of extraction reagent is a key factor in DPPH experimental results. As stated by Sarikurkcu and Tepe (4) ethanol extracts of *P. coccinea* showed remarkable free radical scavenging capacity (6.12 ± 0.292 mg TEs/g fresh fruit) compared to water extracts (2.05 ± 0.101mg TEs/g fresh fruit).

The FRAP assay is based on the ability of the antioxidant in the extract to reduce Iron (III) to iron (II) through electron transfer (32). The FRAP value of *P. coccinea* was 6.75 ± 0.51 mg AAE/g dw, which was not consistent with a previous study (4). Different extraction methods and variety may explain it. Saklani, Chandra (29) also found the ferric reducing power of *P. crenulata* was within the range (4.23 ± 0.05 mm AAE/100g fw). The total antioxidant capacity of 70 medicinal plants was determined as FRAP, Katalinic, Milos (33) found that medicinal plant infusions with considerable antioxidant potential and high PAC can be considered as a rich dietary source of potent antioxidants. *Melissae folium* infusions had high phenolic content, very high FRAP (>20 mM/L) and PAC > 3.

The ABTS assay is used to assess the antiradical scavenging capacity of extracts through hydrogen atom conversion reactions (34). The ABTS value of *P. coccinea* was 7.12 ± 0.25 mg AAE/g dw, which differs from previous studies of Sarikurkcu and Tepe (4). *P. crenulata* had a higher ABTS value (5.40 ± 0.05mm AAE/100g fw) than the other two wild edible fruits, *Ficus palmata* and *Pyrus pashia* (29). According to Cai, Luo (27), analyzed by the improved ABTS. $^{+}$ method, Chinese Galls from *Rhus chinensis/R. potaninii* contained the highest antioxidant concentration (aqueous/methanolic: 17,674/17,323 Amol TEAC/100 g), followed by dried extracts (Catechu) from branches and stems of *Acacia catechu* (aqueous/methanolic: 10,049/11,197 Amol TEAC/100 g).

The TAC assay is based on reducing molybdenum (VI) to molybdenum (V) by transferring electrons from phenols (35). In this study, the TAC value of *P. coccinea* was 3.97 ± 0.28 mg AAE/g dw, which is significantly higher than the average TAC in the leaves of Kaliningrad oblast (0.94-2.9 mg/g), a medicinal plant of the botanical garden (36). The total antioxidant capacity is related to the concentration of the extract. As reported by Pal, Kumar (37), the total antioxidant activity of *Pyracantha crenulata* increased with the increase of extract concentration from 0.5 to 2 mg/mL. Llesuy, Evelson (38) reported that harvesting techniques and stages, as well as storage conditions, have important effects on the total antioxidant capacity of plants.

LC-ESI-QTOF-MS/MS characterization of the phenolic compounds

LC-MS/MS has been widely used to identify and characterize the phenolic compounds across various plant species (39). Qualitative analysis of the phenolic compounds from *P. coccinea* leaves has been achieved by using LC-ESI-QTOF-MS/MS. The *P. coccinea* leaves were tentatively characterized by their *m/z* value and MS spectra in both positive and negative ionization modes. The software used was Agilent LC-MS qualitative software and personal compound database and library (PCDL). Compounds with mass error < ± 10 ppm and PCDL library score > 80 were selected to further analyze MS/MS identification and *m/z* characterization.

In this study, LC-MS/MS has characterized 28 compounds, including phenolic acids (12), flavonoids (13), other polyphenols (2), and lignans (1). The results were shown in Table 2 and the structures of compounds in the figure 2.

Phenolic acids

Phenolic acids are the abundant subclass of the phenolic compounds in plants (40, 41). In our study, 12 phenolic acids were characterized, including hydroxybenzoic acids (5) and hydroxycinnamic acids (7).

Gallic acid (Compound 1) was tentatively characterized in the negative mode of ionization, precursor ion at *m/z* 169.0135 and characterized based on the product ion at *m/z* 125 (M-H-44, loss of CO_2) (42). Previously, gallic acid has been extracted from the seed, stem/bark, fruit, and leaves (43). The keen interest shown in gallic acid is due to the presence of pharmacological properties (44) including anti-inflammatory (45) and antitumor properties (46). Gallic acid has the pro-oxidant property in the presence of metal ions that induces apoptosis of cancer cell lines (47). Verma, Singh (48) study showed that gallic acid was effective and toxic to cancer cells and least toxic to normal cells. Previously gallic acid was identified in fresh fruit of P. coccinea (49) and high concentration in green tea (50). Ayurvedic medicine uses equal proportions of *Phyllanthus emblica*, *Terminalia belerica,* and *Terminalia chebula* containing a high concentration of gallic acid to treat illness including constipation, anaemia, jaundice, asthma, and fever (51). Gallic acid detected in *Rhus chinensis* samples had the ability to induce apoptosis in lymphoma cell line U937 (52).

Compound 3 ($[M-H]^-$ *m/z* at 299.075) was identified as 4-hydroxybenzoic acid 4-*O*-glucoside, while the product ions at *m/z* 137 (M-H-162, loss of hexosyl moiety) (42). Previously, 4-Hydroxybenzoic acid 4-*O*-glucoside was found in *Sargassum* sp. of seaweed (53). Methanolic extraction of needles *of Cedrus brevifolia,* a narrow endemic tree of *Cyprus flora*, found the presence of the compound (54). Blackcurrant, gooseberry, and blueberries also showed the presence of the compound (55). 4-Hydroxybenzoic acid 4-*O*-glucoside was also detected in the carrot juice treated with high pressure processing treatments (56).

Compound 7 and Compound 11 were tentatively characterized in negative mode of ionization with precursor ions at *m/z* 179.0347 and *m/z* 193.0505, respectively. Upon MS/MS analysis, caffeic acid was identified by the product ions at *m/z* 143 and *m/z* 133 due to the loss of two H_2O (36 Da) and HCOOH (46 Da) from the precursor ion, respectively (57). Characterization of ferulic acid, product ions at *m/z* 178, *m/z* 149, and *m/z* 134 due to the corresponding loss of CH_3(15 Da), CO_2 (44 Da), and CH_3-H_2O (59 Da) (58). Caffeic acid, its esters, and amide derivatives exhibit biological activities, including anti-inflammatory and protect the

Table 2. LC-MS/MS characterization of phenolic compounds from *P. coccinea* plant.

No.	Proposed compounds	Molecular Formula	RT (min)	Mode of ionization	Molecular Weight	Theoretical (m/z)	Observed (m/z)	Error (ppm)	MS/MS Product ion
Phenolic acid									
Hydroxybenzoic acids									
1	Gallic acid	$C_7H_6O_5$	11.133	$[M-H]^-$	170.0215	169.0142	169.0135	-4.14	125
2	2-Hydroxybenzoic acid	$C_7H_6O_3$	11.414	$[M-H]^-$	138.0317	137.0244	137.0244	0.00	93
3	4-Hydroxybenzoic acid 4-*O*-glucoside	$C_{13}H_{16}O_8$	11.414	$[M-H]^-$	300.0845	299.0772	299.075	-7.36	255,137
4	3-*O*-Methylgallic acid	$C_8H_8O_5$	12.93	$[M+H]^+$	184.0372	185.0445	185.0452	3.80	170, 142
5	2,3-Dihydroxybenzoic acid	$C_7H_6O_4$	15.201	$[M-H]^-$	154.0266	153.0193	153.0187	-3.92	109
Hydroxycinnamic acids									
6	1,5-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	8.838	$[M-H]^-$	516.1268	515.1195	515.1224	5.63	353,335,191,179
7	Caffeic acid	$C_9H_8O_4$	32.032	$[M-H]^-$	180.0423	179.0350	179.0347	-1.70	143, 133
8	Caffeic acid 3-*O*-glucuronide	$C_{15}H_{16}O_{10}$	13.999	$[M-H]^-$	356.0743	355.0670	355.0661	-2.53	179
9	Ferulic acid 4-*O*-glucuronide	$C_{16}H_{18}O_{10}$	19.56	$[M-H]^-$	370.09	369.0827	369.0805	-5.96	193
10	m-Coumaric acid	$C_9H_8O_3$	19.473	$[M-H]^-$	164.0473	163.0400	163.0395	-3.10	119
11	Ferulic acid	$C_{10}H_{10}O_4$	23.366	$[M-H]^-$	194.0579	193.0506	193.0505	-0.50	178, 149, 134
12	3-*p*-Coumaroylquinic acid	$C_{16}H_{18}O_8$	31.351	$[M-H]^-$	338.1002	337.0929	337.0917	-3.6	265, 173, 162
Flavonoids									
Flavanols									
13	(+)-Catechin	$C_{15}H_{14}O_6$	13.897	** $[M-H]^-$	290.079	289.0717	289.0707	-3.46	245, 205, 179
14	Procyanidin dimer B1	$C_{30}H_{26}O_{12}$	16.769	$[M-H]^-$	578.1424	577.1351	577.1316	-6.06	451
15	(+)-Gallocatechin	$C_{15}H_{14}O_7$	20.183	** $[M-H]^-$	306.074	305.0667	305.0666	-0.33	261, 219
Flavones									
16	Apigenin 6-C-glucoside	$C_{21}H_{20}O_{10}$	28.131	$[M-H]^-$	432.1056	431.0983	431.0965	-4.2	413, 341, 311
17	7,4'-Dihydroxyflavone	$C_{15}H_{10}O_4$	39.854	$[M+H]^+$	254.0571	255.0644	255.0641	-1.18	227, 199, 171
Flavonols									
18	Myricetin 3-*O*-rutinoside	$C_{27}H_{30}O_{17}$	24.938	$[M-H]^-$	626.1483	625.141	625.1415	0.8	301
19	Myricetin 3-*O*-galactoside	$C_{21}H_{20}O_{13}$	26.531	$[M-H]^-$	480.0904	479.0831	479.0819	-2.50	317
20	Kaempferol 3,7-*O*-diglucoside	$C_{27}H_{30}O_{16}$	28.41	$[M+H]^+$	610.1506	611.1579	611.1587	1.31	447, 285
21	Myricetin 3-*O*-rhamnoside	$C_{21}H_{20}O_{12}$	30.09	$[M-H]^-$	464.0955	463.0882	463.0869	-2.8	317
22	Quercetin 3-*O*-(6"-malonyl-glucoside)	$C_{24}H_{22}O_{15}$	31.68	$[M+H]^+$	550.0934	551.1007	551.1008	0.18	303
Dihydrochalcones									
23	3-Hydroxyphloretin 2'-*O*-glucoside	$C_{21}H_{24}O_{11}$	13.897	** $[M-H]^-$	452.1319	451.1246	451.1245	-0.22	289, 273
24	Phloridzin	$C_{21}H_{24}O_{10}$	26.592	$[M-H]^-$	436.1369	435.1296	435.1301	1.1	273
Isoflavonoids									
25	5,6,7,3',4'-Pentahydroxyisoflavone	$C_{15}H_{10}O_7$	30.029	$[M+H]^+$	302.0416	303.0489	303.0488	-0.33	285, 257
Other polyphenols									
Hydroxycoumarins									
26	Coumarin	$C_9H_6O_2$	16.163	$[M+H]^+$	146.0366	147.0439	147.0438	-0.68	103, 91
Hydroxybenzaldehydes									
27	4-Hydroxybenzaldehyde	$C_7H_6O_2$	9.521	$[M-H]^-$	122.0368	121.0295	121.0297	1.7	77
Lignans									
28	Schisandrol B	$C_{23}H_{28}O_7$	5.936	$[M+H]^+$	416.1837	417.1910	417.1926	3.84	224, 193, 165

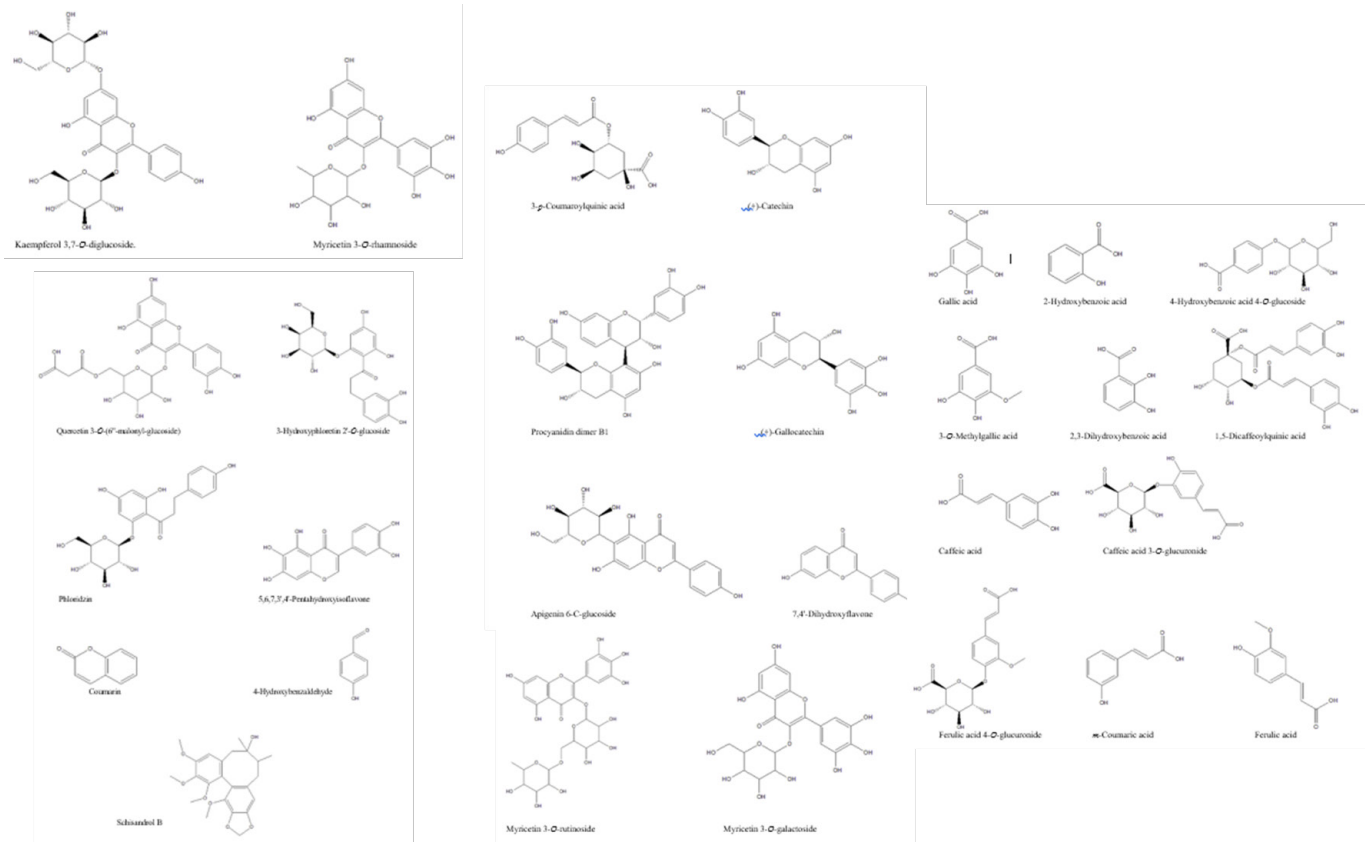

Figure 2. Structures of phenolic compounds from *P. coccinea* extract by LC-ESI-QTOF-MS/MS.

liver from Ni induced oxidative damage (59). Previously, caffeic acid was identified in the phenolic extract of *Clerodendrum volubile* leaves (60), an underutilized vegetable native to Nigeria. The plant is traditionally used in the treatment of arthritis, diabetes, and analgesic (61). Caffeic acid has antiviral properties against herpes simplex virus, severe fever with thrombocytopenia syndrome, and influenza virus (62). Caffeic acid can inhibit 5-lipoxygenase, which biosynthesized to form leukotrienes as it causes asthma, inflammation, and various allergic conditions (63). *Carum carvi* L., *Tussilago farfara* L., *Taraxacum officinale,* and *Morus alba* have high concentration of caffeic acid derivatives (64). Other study show the presence of caffeic acid in Mangifera indica and Coffea canephora (65). Maize and wheat bran have higher concentration of ferulic acid compared to rye and oat bran (66). Ferulic acid detected in pomegranate peels and leaves of pomegranate, olive, fig and guava (67). *Cola nitida* and *Garcinia kola* had compound ferulic acid (68).

m-Coumaric acid (Compound 10) with $[M-H]^-$ and precursor ion at *m/z* at 163.039. Upon MS/MS analysis, the product ions at *m/z* 119 due to the corresponding loss of CO_2 (44 Da) from the precursor ion (58). Previously *m*-coumaric acid was found in *Pyracantha crenulate* (69). *Mentha longifolia,* a medicinal plant is native to Europe, Asia, and Africa was detected with compound m-coumaric acid (70). The compound was also identified in Mabseeli date seeds (71) and in the ethyl acetate extract of Moringa oleifera leaves, commonly called drumstick tree (72).

Flavonoids

Flavonoids are subclass of the phenolic compounds (73) with antioxidant and free radical scavenging properties. On LCMS/MS analysis, 13 flavonoids were characterized including flavanols (3), flavones (2), flavonols (5), dihydrochalcones (2) and, isoflavonoids (1).

Compound 13 and Compound 15 were tentatively characterized in both mode of ionization, precursors at *m/z* 289.0707 and *m/z* 305.0666, respectively. On MS/MS analysis, catechin was identified based on the product ions at *m/z* 245 (M-H-44, loss of CO_2), *m/z* 205 (M-H-84 loss of flavonoid A ring), and *m/z* 179 (M-H-110, loss of flavonoid B ring) (42). In MS/MS analysis, the product ions at *m/z* 261 and *m/z* 219 due to the corresponding loss of CO_2 (44 Da) and loss of C_3O_2 and H_2O (86 Da) (74), identified as gallocatechin. Green tea has a high concentration of catechin and gallocatechin, and upon oxidation of the tea leaves for the production of black tea, the catechin level lowers and converts into theaflavins and thearubigins (75). Previously the compounds were reported in banana peels (76), apple skin (77), apple pomace, apple leaves (78) and coconut water (79). Consumption of catechin, particularly via cocoa reduces blood pressure and reduces platelet aggregation and thrombus formation (80).

Other polyphenols

Compound 26 was tentatively characterized in a positive mode of ionization with precursor ion at *m/z* 147.0438. Upon MS/MS analysis, the product ions at *m/z* 103 and *m/z* 91 identified coumarin due to the loss of CO_2 (44 Da) and loss of 2CO (56 Da), respectively (81). Coumarin is one among the volatile compounds present in plants (82). Previously, coumarin was reported in cinnamon sticks and ground cinnamon, where cinnamon sticks had a higher concentration (83). Coumarin was isolated from *Amburana cearensis* bark and had biological activity against leishmaniasis (84). Coumarin is a

good compound for anti-viral drug since it can inhibit the growth of viruses including HIV, influenza, dengue and chikungunya virus (85). Bergamot peel has higher content of coumarin when compared to orange and clementine (86).

4-Hydroxybenzaldehyde (Compound 27) with the negative mode of ionization and precursor ion at *m/z* 121.0297. 4-hydroxybenzaldehyde was characterized based on the product ions at *m/z* 92 [M-H-29, loss of CH,O] and *m/z* 77 [M-H-44, loss of CO_2] (87). Previously found in Syzygium aqueum leaf extract, grown in Malaysia and Indonesia and has been used in traditional medicine as it possesses antibiotic activity (88) and also found in hops (89). 4-Hydroxybenzaldehyde compound was found in buckwheat honey (90).

HPLC quantification

The high-pressure liquid chromatography (HPLC) coupled with photodiode array detector (PDA) is regarded as a highly effective technique to identify and quantify the phenolic compounds in different plants (13). In this study, 10 representative polyphenols in *P. coccinea* were quantitatively analyzed by HPLC-PDA, consisting of 5 phenolic acids (gallic acid, protocatechuic acid, p-hydroxybenzoic acid chlorogenic acid, caffeic acid) and 5 flavonoids (catechin, epicatechin, epicatechin gallate, quercetin and kaempferol). They are selected from targeted polyphenols screened by previous antioxidant activities and LC-ESI-QTOF-MS/MS characterization. The results are reported as mg/g dry weight of *P. coccinea* samples through assessing their retention time with reference standards (Figure 3).

According to the figure 2, chlorogenic acid contained the most predominant phenolic compound among the other phenolic acids, with the concentration of 11.49 ±1.89 mg/g sample. Similarly, Mocan, Zengin (91) found that chlorogenic acid was the dominant compound in Goji (*Lycium barbarum L.*) leaves, which acted as medicinal vegetables consumed worldwide. In China, the amount of chlorogenic acid in wild-growthing *L. barbarum* leaves range from 2.09 mg/g dw to 4.28 mg/g dw (92), which was significantly lower than that of *P. coccinea* in the present study. Then, p-hydroxybenzoic acid was detected to be the second abundant phenolic acid in *P. coccinea* (6.98±1.40 mg/g), followed by gallic acid (4.39±1.27 mg/g), protocatechuic acid (4.67±1.39 mg/g), and caffeic acid (4.78±1.96 mg/g), respectively. Previously, Wang, Ye (93) quantified the phenolic acid in *P. fortuneana* fruit extracts by HPLC-QTOF-MS/MS, in which protocatechuic acid and chlorogenic acid were determined through multiple extractions of methanol, ethanol, and acetone. Also, various phenolic acids were reported in *Melissa officinalis*, which is another medicinal plant widely used in pharmacy. Karasová and Lehotay (94) presented that gallic acid, protocatechuic acid, and p-Hydroxybenzoic acid were achieved the highest amounts with 56.3 µg/g with methanol extraction, 75.3 µg/g using methanol–water (80: 20, v/v) and 11.7µg/g in the case of methanol–water (60: 40, v/v), respectively.

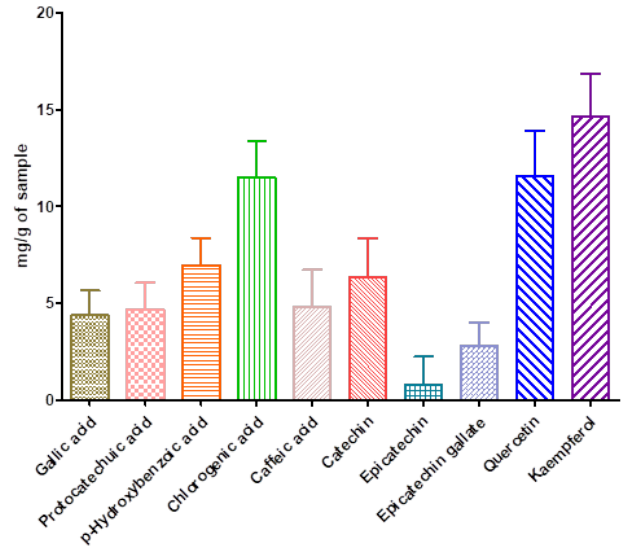

Figure 3. Quantification of polyphenolic compounds in *P. coccinea* samples by HPLC-PDA.

As for flavonoids, kaempferol was quantified to be the predominant component in *P. coccinea* leaves, with 14.67 ± 2.17 mg/g, followed by quercetin (11.59 ± 2.34 mg/g) and catechin (6.37 ± 1.98 mg/g). These results were consistent with the trends that reported by Mocan, Zengin (91). They quantified that kaempferol and quercetin were the two main components of flavonoids in Goji (*Lycium barbarum L.)* leaves. Another medicinal halophyte *Mesembryanthemum edule L.*, quercitrin (a flavonol-3-O-glycoside), and (+)-catechin were the major flavonoids in leaves (95). In addition, Cai, Luo (27) also found that quercetin and kaempferol were the most abundant flavonols in numerous medicinal herbs, such as aerial parts of *Artemisia annua*, *Crataegus pinnatifida* and *Alpinia officinarum*.

Overall, *P. coccinea* leaves were identified to have considerable amounts of phenolic compounds with prominent vitro antioxidant potential. A total of 28 phenolic compounds were tentatively screened from *P. coccinea* by LC-ESI-QTOF-MS/MS analysis. Further, 10 individual polyphenols were quantified through HPLC-PDA, which demonstrated abundant flavonoids and phenolic acids in *P. coccinea*. Especially, chlorogenic acid and kaempferol were characterized the most abundant polyphenols. The total phenolic acid content in the samples was significantly higher than the total flavonoids and tannins. Four antioxidant assays were applied for evaluation of an overall antioxidant capacity in *P. coccinea* samples. The obtained results indicate that *P. coccinea* leaves have a relatively abundant phenolic content and antioxidant capacity compared to some other medicinal plants. The presence of the rich polyphenols with high antioxidant capacity verified that *P. coccinea* might be a potential source of natural antioxidants and widely used in food and pharmaceutical industries.

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