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Evaluation of Phenolic Content and Free Radical Scavenging Activity of Indonesia Wild Honey Collected from Seven Different Regions

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Abstract

Several wild honey samples collected from seven different regions in Indonesia were investigated to determine their total phenolic content, flavonoid content, and free radical scavenging activity by analyzing the 1,1-diphenyl-2-picrylhydrazyl (DDPH) radical and phenolic profile. Rutin, (+)-catechin, ferulic acid, and galangin were found to be the major phenolic compounds of Indonesia wild honey. The total phenolic content significantly correlated with the total flavonoid content (p=0.000) and the percentage of DPPH radical scavenging activity (p=0.000). Results indicated that there are different polyphenol profiles among the different regions.

Keywords: wild honey, antioxidant, polyphenolic, flavonoid

1. Introduction

Honey is a natural food produced by honey bees using flower nectar or tree and plant exudates. Recently, honey has become extremely popular because of its potential beneficial effects for human health. It has been used as a common traditional drug since ancient times for treating burns, gastrointestinal problems, asthma, infection and wounds, skin ulcers, and cataracts and other eye diseases (Osés, et al., 2016; Ferreira, et al., 2009). The beneficial effects of honey can be partly attributed to its antibacterial and antioxidant activities. Studies have shown that honey exhibits activity against oxidation reactions in food such as enzymatic browning, lipid oxidation, and food deterioration(Ardehali, et al., 2017; Ferreira, et al., 2009; Antony, et al., 2000; Chen, et al., 2000).

The antioxidant activity of honey is due to a combination of compounds such as phenolic acids, flavonoids, ascorbic acid, carotenoids, enzymes, amino acids, and products derived from the Maillard reaction (Gül & Pehlivan, 2018; Moniruzzaman, et al. 2014, Khalil, et al. 2011, and Gheldof, et al. 2002).Recently, there has been a significant increase in research on the characterization of natural polyphenols especially their identification and quantification (Pyrzynska, & Biesaga, 2009). This research direction has been largely oriented toward identifying a promising marker compound (Kus, et al., 2014; Kaškoniene, & Venskutonis, 2010), and such studies have reported more than 150 polyphenol compounds from honey bee products (Ferreira, et al., 2009).

The concentration and the class of antioxidants are highly dependent on the floral source and the total capacity of the antioxidants, which comprises a combination of activity of compounds found in honey (Gheldof, et al., 2002). Earlier studies have demonstrated that the capacity of antioxidants in honey obtained from various flower sources significantly correlated with its phenolic and flavonoid contents and the origin of the flower (Alvarez-Suarez, et al., 2009; Baltrušaityte, et al., 2007; Gheldof, et al., 2002; Anklam, 1998). One study showed that the important components, including phenolic derivatives, present inplant nectar are transferred to honey (Idris, et al., 2011). The primary factor responsible for the antioxidant activity of honey is the substituent group present in its phenolic components, e.g., hydroxyl, methyl, acyl, or glycosyl groups (Gašic et al., 2014).

Regarding the flavonoids present in honey, previous studies have reported that pinobanksin, pinocembrin, quercetin, chrysin, galangin, luteolin, and kaempferol are the major ones, whereas luteolin, quercetin, 8-methoxykaempferol, is or hamnetin, kaempferol, and galangin are present in small concentrations in Manuka honey (Alvarez-Suarez, et al., 2014; Chan, et al., 2013; Kaškonienand Veskutonis, 2010). This characterization of polyphenols was useful for differentiating the source of honey and could be used as a botanical marker (Spilioti, et al., 2014; Alvarez-Suarez, et al 2014; Anklam, 1998; Tomas-Barberan, et al., 1993). Caffeic acid and *p*-coumaric acid found in chestnut honey and protocatechuic acid found in conifer tree honey have been used as flower markers in some previous studies (Haroun, et al., 2012; Tomas-Barberan, 2001).

Today, several methods are used to explore the antioxidant activity of honey, such as determination of active oxygen species, radical scavenging activity (RSA) (Alvarez-Suarez, et al., 2009; Gheldof, et al., 2005; Meda, et al., 2005; Gheldof, et al., 2002; Nagai, T, et al., 2001),measurement of the inhibition of lipid peroxidation by enzymatic or nonenzymatic reactions (Nagai, et al., 2001; Chen, et al., 2000), and ferric reducing antioxidant power assay (Bertoncelj, et al., 2007; Aljadi & Kamarudin,2004).

Chromatographic fingerprinting is an efficient and widely used method for determining the content of polyphenols or antioxidant compounds (Zhao, et al., 2016; Sun et al., 2014; Kuś, et al., 2014). Solid-phase extraction techniques have been successfully applied,followed by the identification of the compounds by capillary electrophoresis (CE), gas chromatography, or high-performance liquid chromatography (HPLC).The use ofdiode array detector (DAD) and mass spectrometry (MS) demonstrated greater effectiveness, especially withHPLC in cases of flavonoids and with GC in cases of phenolic acids (Alvarez-Suarez, et al., 2009). Several researchers have demonstrated that HPLC with DAD and GC-MS are useful for the characterization of honey from plant sources (Kus, et al., 2014; Soria, et al., 2009).Till date, only a few studies have reported about the determination of total phenolic content (TPC), antioxidant activity, and the phenolic profile of honey in Indonesia (Chayati, 2008; Kartika & Bertoni, 2014; Kustiawan 2014). Therefore, we conducted this study to determine the TPC, the total flavonoid content (TFC), the (RSA) (DPPH), and the phenolic profiling by HPLC – DAD using wild honey collected from seven different regions or islands in Indonesia.

2. Material and Method

2.1 Honey Samples

Indonesia wild honey (IWH) samples were collected between 2016 and 2018 from seven different regions in seven different islands in Indonesia. The regions wereas follows: Bangka Belitung, Sulawesi, Kalimantan, Sumatra, West Nusa Tenggara, East Nusa Tenggara, and Java (Figure 1). Their authenticity and freshness wereassured by collecting them directly from the forest areas, local beekeepers under the Indonesia Wild Honey Association, and/or from well-traced suppliers. The samples were collected in sealed glasscontainers and kept in dark conditions.

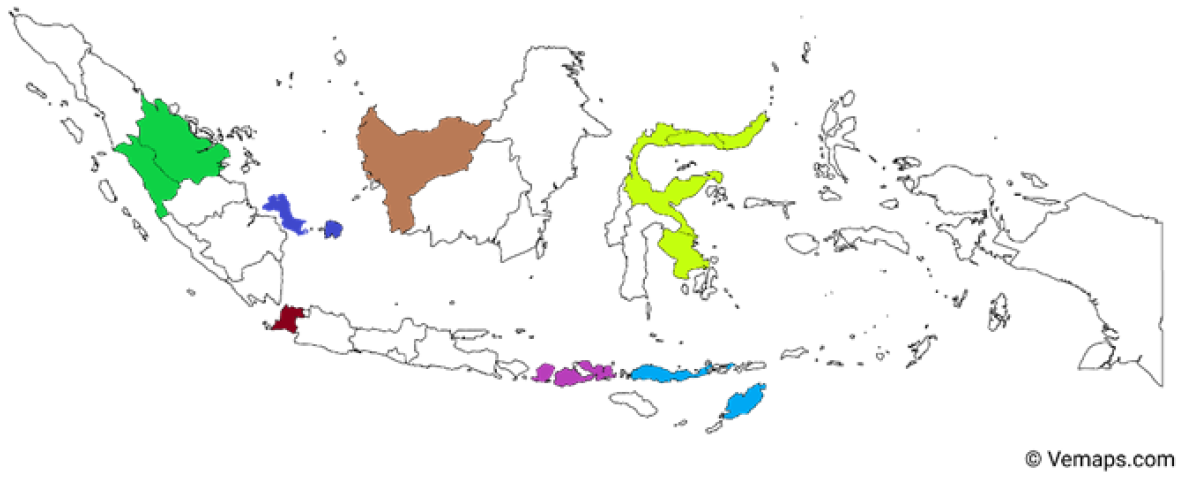

Figure 1. Sampling area for Wild Honey (= Sumatera island (Riau& Jambi) =Bangka & Belitung Island = Java (Banten province) = Kalimantan Island (West Kalimantan Province = Sulawesi Island (South Sulawesi, Central Sulawesi, Gorontalo & North Sulawesi) = West Nusa Tenggara (Sumbawa Province) =East Nusa Tenggara (Kupang, Timor Tengah Selatan , Flores Timur &Sikka)

2.2 Analysis of TPC

TPC was determined using the Folin–Ciocalteu method at a wavelength of 750 nm with gallic acid as the standard (Ferreira, et al 2009; Socha, et al., 2007; Prior, et al., 2005; Singleton, et al., 1999; Singleton, et al., 1965). About 0.5 mL of honey solution (100 mg/mL) that was previously homogenized was added to 0.3 mL of 10% Folin–Ciocalteu reagent. Then, 2 mL of 15% sodium carbonate solution (Na_2CO_3) was added after 6 min and the volume was made up to 5 mL using distilled water. This solution was incubated for 20 min in the dark before measuring using a spectrophotometer. A calibration curve was plotted at a predetermined concentration of 0–300 mg/L, and the results were expressed in milligrams of gallic acid equivalent (mg/kg GAE).

2.3 Analysis of TFC

The TFC was measured using the method developed by Zhishen et al. (1999). Briefly, 2 mL of honey solution in water (1 g/4 mL) was mixed with 0.3mL of 5% $NaNO_2$ (w/v). After 5 min, 0.3 mL of 10% w/v $AlCl_3$ was added to the solution, followed by the addition of 2mLofNaOH and then 10 mL of distilled water after 6 min. The absorbance of the solution was read in a Thermo Scientific Genesys 10 S UV-Vis spectrophotometerat 425 nm. A calibration curve was plotted with quercetinas the standard at a concentration range of 0–8 mg/L. The results were expressed as milligrams of quercetine quivalent (QEQ) per 100 g of honey.

2.4 Radical Scavenging Activity

The antioxidant activity of all honey samples was measured using the scavenging activity for the radical DPPH as developed by Meda et al (2005). The homogenized sample was weighed and dissolved in methanol (150 mg/mL). The honey solution (0.075 mL) was mixed with the DPPH reagent solution (0.025 mg/mL) and left for 15 min at room temperature in the dark condition. The absorbance of the mixture was measured at 517 nm against methanol blank. The RSAwas expressed as % inhibition according to the following equation:

$$\%\ \text{inhibition} = \frac{\text{Blank absorbance} - \text{Control absorbance)}}{\text{Blank absorbance}} \times 100\%$$

2.5 Analysis of Phenolic Compound Profile

2.5.1 Sample Extraction

The homogenized sample (0.5 g) was dissolved in 10 mL of acidified deionized water (pH 3.5) and inserted into the SPE column Bond Elut octadecyl C18 (500 mg) from Agilent Technology (Santa Clara, CA, USA)whichwas previously conditioned using 4mL of methanol and 2mL of deionized water. Subsequently, the column was washed with 6 mL of deionized water (pH 3.5) and the desirable fraction was eluted by passing 2 mL of absolute methanol. Before injecting into the HPLC system, the sample extracts were filtered through a 0.45-µm membrane filter (Millipore) (Gašić et al., 2014; Beretta et al., 2005).

2.5.2 Preparation of Polyphenolic Standards

All standards were prepared using methanol to make 500 mg/L of standard stock solution. Working standards were prepared by diluting the stock solution with methanol to serial concentrations of 0.10, 0.25, 0.50, 0.75, and 1.00 mg/L. A calibration curve was drawn by plotting the analyte peak areas against the serial concentration of the working standard solutions. The calibration curve was considered to have good linearity if the R^2 values were >0.99. The standards of ferulic acid, *p*-coumaric acid, vanillic acid, gallic acid, quercetin, tricine,4-hydroxybenzoic acid, (+)-catechin, syringaldehyde, rutin trihydrate, chrysin, galangin, epigallocatechin, and 3,4-dihydroxy benzaldehydewere purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Methanol was of HPLC grade purchased from Merck. All solutions were filtered and degassed before use.

2.5.3 HPLC Analysis

The honey samples were analyzed using a Shimadzu liquid chromatographic system equipped with binary pumps LC20-A, degasser DGU-20A5, autosampler SIL-20AC, photodiode array detector set to a wavelength of 280 nm, and a Shimadzu Shim pack GIST column (5µm, 4.6 × 15 mm.). The mobile phase consisted of a combination of water–acetic acid (phase A) and methanol–acetic acid (phase B). The system was run with a gradient elution with the following conditions: time 0.02 min, 18.3% phase B; time 10 min, 100% phase B; time 13.1 min, 18.3% phase B. The flow rate was set to1mL/min with an injection volume of 20 µL.

2.6 Statistical Analysis

Statistical analyses were conducted using the computer programs SPSS 24.0 Statistics software (SPSS Inc., 2016), Minitab® 18.1 (State College, Pennsylvania, USA) and Microsoft Excel 2016 (Microsoft Corp.). Data were presented as mean ± S.D. The Pearson correlation was calculated to determine the relationship between the

phenolic acid content and antioxidant properties. The level of significance was set at $p<0.01$.

3. Results and Discussion

3.1 TPC and TFC

The TPC (mg/kg of honey) exhibited significant variations among the various samples of IWH (Table 1), which might be due to the differences in the botanical and regional origin (Tahir, et al 2017; Mamary et al., 2002). The TPC of the IWH samples varied from 188.03(mg/kg GAE) in Kalimantan to 467.84 (mg/kg GAE) inEast Nusa Tenggara (Table 1) with standard linearity (R^2 = 0.9990). This value was still higher than previously reported, namely, lime honey (83.7mg/kg), Slovenia honey (44.8 mg/kg), and honey from Burkina Faso (74.38mg/kg) (Moniruzzaman, et al., 2014;Bertoncelj, et al., 2007;Meda, et al 2005).

In this study, the TFC was evaluated using an aluminum chloride reagent, which was specific to only flavones and flavonols, so that the test results would provide a lower estimation of the number of flavonoids because they ignored the flavanone compounds (Meda, et al., 2015). The TFC of the IWH samples ranged from 0.81 mg QEQ/100 gin honey from Kalimantan to 3.09 mg QEQ/100 g in honey from Sumatra (Table 1), made by calibration curve of quercetin with R^2 = 0.9999. These results were closely similar to the values of other honey samples in the world such as those from Bangladesh(2.57 mg QEQ/100 g) (Moniruzzaman, et al., 2011), Burkina Faso(2.57 ± 2.09 mg QEQ/100 g), Europe (0.5–2 mg QEQ/100 g) (Amiot et al., 1989; Martos et al., 2000), Czech (1.9–15.74 mg QEQ/100g) (Vit, et al., 2008),andFrance (<1 mg QEQ/100 g).The most significant positive linear correlation was observed between the phenolic and flavonoid parameters (r = 0.533, p = 0,000, Table 2). It has been reported that honey samples with a higher polyphenol content produce high levels of flavonoids (Moniruzzaman, 2014, Khalil, 2012).

Table 1. Mean and standard deviation of antioxidant parameters

Antioxidant Parameters		Origin of Indonesia Wild Honey						
		Bangka Belitung	*Sulawesi*	*Kalimantan*	*Sumatra*	*West Nusa Tenggara*	*East Nusa Tenggara*	*Java*
TPC*	mean	254.92 ±	423.17 ±	188.03 ±	422.9528 ±	467.84 ±	343.15 ±	250.96 ±
	±SD	83.26^{ac}	55.45^{b}	6.74^{ca}	64.95^{d}	45.66^{e}	84.18^{f}	66,46^{g}
TFC**	Mean ±SD	1.09 ±0.38^{a}	2.57 ±1.35^{bd}	0.81 ±0.21^{c}	3.09 ±0.79^{db}	2,22 ±0.57^{e}	1.46 ±0.29^{fg}	1,13 ±0.64^{gf}
% Inhibition	Mean ±SD	28.23 ±6,68^{a}	80.74 ±19.00^{bd}	49.24 ±5.15^{ceg}	69.85 ±9.76^{db}	51.24 ±10.61^{ecg}	68.65 ±12.23^{f}	50.42 ±4.6^{gec}
Number of samples		7	12	10	41	29	30	20

*Total Phenolic Content (mg of gallic acid equivalent (GAE) per kg)

**Total Flavonoid Content (mg quercetin(QEQ)/100 g)

3.2 Radical Scavenging Activity

The results of RSA are summarized in Table 1. The percentage inhibitory activity of IWH ranged from 28.23%to 80.74%. The highest mean value was found in honey collected from Sulawesi, whereas the lowest was observed in honey collected from Bangka Belitung. These results were higher to those of Portuguese honey collected from several vegetation sources such as rosemary (4.5%–59.3%), orange (8.8%–23.2%), thyme (35.8%–47.3%), and eucalyptus (27.7%) (Alves, et al., 2013). Other studies have reported that Lithuania honey has an RSA value of 43.0%–95.7% (Baltrušaityte, et al., 2005),dark honey samples have a DPPH inhibitory value of69.2%, and light honey samples have an inhibitory value of 37% (Estevinho et al.,2008). Similarly, rhododendron honey samples showed inhibitory values between 2.30% and 90.73% (Silici et al., 2010).

A positive correlation was observed among the RSA, TPC, and TFC of the honey samples, with a Pearson coefficient<0.001. The TPC showed a weak correlation with the antioxidant activity, whereas the TFC exhibited a moderate correlation with RSA (Table 2).These results were similar to those of previous studies that reported that the phenolic acid and flavonoid contents and the antioxidant potential of honey were significantly correlated, which was influenced by the origin of the sample (Jarom ŕ, et al 2010; Beretta, et al., 2005). This correlation indicates that the phenolic compounds have an effect on the antioxidant potential.

Table 2. Correlation of Total Phenolic, Total Flavonoid, and % Inhibition

		Total Phenolic	Total Flavonoid	% Inhibition
Total Phenolic	Pearson Correlation	1	.533**	.336**
	Sig. (2-tailed)		.000	.000
	N	145	145	138
Total Flavonoid	Pearson Correlation	.533**	1	.504**
	Sig. (2-tailed)	.000		.000
	N	145	149	142
% Inhibition	Pearson Correlation	.336**	.504**	1
	Sig. (2-tailed)	.000	.000	
	N	138	142	142

**. Correlation is significant at the 0.01 level (2-tailed).

3.3 Profile of Phenolic Compounds

In general, the analysis procedure for determining a single phenolic compound needs the sample preparation step intended for extraction from the honey matrix, followed by separation, identification, and quantification steps. The extraction step generally involves SPE or solvent extraction using various solvents. Separation is generally done by HPLC or CE because of the presence of electroactive phenolic hydroxyl groups with simple oxidation potential. Some authors had conducted SPE procedures to extract phenolic components from honey (Aljadi & Kamarudin, 2004). Quantitation was done on 13 phenolic compounds, but only 10 of them were able to produce linear equations with R^2=0.99 (Table 3). Other flavonoids such as syringaldehyde and rutin, although they were detected, could not produce linear equations that met the requirements for quantification.

Results of the phenolic profile are represented in Table 4 and Figure 1. Gallic acid, (+)-catechin, quercetin, epigallocatechin, pinocembrin, 4-hydroxybenzoic acid, chrysin, galangin, vanillic acid, and ferulic acid were detected in all types of honey samples collected from various regions, whereas3,4-dihydroxy benzaldehyde was detected only in the honey samples collected from Sumatra, East Nusa Tenggara, and Java. Syringaldehyde was detected only in Sumatra and Java honey samples, andrutin was found only in Sumatra and East Nusa Tenggara honey samples. An earlier study reported that pinocembrin, pinobanksin, chrysin, and luteolin represented approximately61% of the flavonoid content of Manuka honey (Chan, et al., 2013). In this study, the levels of each phenolic compound in each region varied significantly, which may be due to variation in the types of vegetation or multiflora. The dominant phenolic compound found in honey collected from Bangka Belitung was galangin (75.43–103.88 mg/kg), which was also detected in honey collected from Sulawesi but at a lower level (14.68–26.52 mg/kg). Epigallocatechin was the most dominant phenolic compound in honey collected from Kalimantan (3.027–34.617 mg/kg), whereas catechin was the dominant phenolic compound found in honey collected from Java. Ferulic acid was the most dominant phenolic compound in honey collected from West Nusa Tenggara (51.64–286.43 mg/kg), whereas the honey samples collected from East Nusa Tenggara and Sumatra showeda lower concentration of this compound.Polyphenolic content in several honey were than their TPC and TFC. This condition allows the exploration of other dominant phenolic compounds,which could not be identified in these experiments. It has been reported that the variation in the composition of phenolic compounds depends on the floral source transfer to the nectar, climate and other environmental factors, bee variety, and the processing technologies, handling, and storage (Kaskonienė and Venskutonis, 2010; Bertoncelj, et al., 2007). This variation in the profile of phenolic compounds was considered to be responsible for their diverse response in protecting against oxidative reactions. This compound could also be used as an indicator in studies analysing the flower and geographic origins of honey (Idris, Mariod, & Hamad, 2011; Alvarez-Suarez, et al., 2009).

Table 3. HPLC data for polyphenol standards at λ280nm

Polyphenols	T_R (min)	Regression Equation	R^2
Gallic acid	2.74	Y= 45617.3*x-10073.4	0.99
(+)-catechin	5.82	Y= 10181.8*x-2078.88	0.99
3,4-dihydroxy benzaldehyde	6.06	Y=55287.5*x-10670.5	0.99
Quercetin	6.41	Y= 29263.2*x-2762.52	0.99
Epigallocatechin	6.74	Y= 26279.1*x-2731.40	0.99
Pinocembrin	7.06	Y=34401.9*x-6223.43	0.99
4-hydroxybenzoic acid	7.46	Y= 31344.3*x-6814.47	0.99
Chrysin	7.78	Y= 132703*x-5297.36	0.99
Galangin	8.02	Y=2500.68*x+2802.56	0.99
Vanillic acid	9.03	Y= 21305.2*x-8083.04	0.99
Ferulic acid	9.93	Y=1138.20*x-239.447	0.99

Table 4. Quantification data of phenolic compounds in Indonesia wild honey

Polyphenols (μg/mL)	Bangka Belitung	Sulawesi	Kalimantan	Sumatra	West Nusa Tenggara	East Nusa Tenggara	Java
Gallic acid	0.25–4.92	0.25–0.78	0.29–1.57	0.41–4.0	0.36–6.09	0.27–4.43	0.27–5.67
(+)-catechin	42.59–69.52	1.69–17.09	0.84–7.14	4.71–29.00	6.28–22.86	0.60–16.40	1.02–27.77
3,4 dihydroxy benzaldehyde	ND	ND	ND	0.19–9.61	Not detected	0.20–0.19	0.21–0.81
Quercetin	6.57–17.10	0.14–23.86	0.78–6.58	0.31–4.76	1.10–7.11	0.41–2.22	0.09–2.02
Epigallocatechin	6.64–65.81	1.25–48.09	3.03–34.62	0.24–5.83	4.69–16.64	0.23–8.62	0.098–3.99
Pinocembrin	2.10–9.36	0.24–4.86	1.15–6.16	1.51–15.20	1.46–22.39	0.48–3.70	0.34–2.42
4-hydroxybenzoic acid	1.56–3.66	0.27–0.57	5.65–8.82	0.25–9.66	1.18–4.75	0.30–4.09	0.25–15.79
Chrysin	8.56–8.75	0.16–2.72	0.37–2.63	1.35–12.19	0.23–2.29	0.57–3.73	0.32–3.57
Galangin	75.43–103.88	14.68–26.53	0.492–23.22	0.64–20.38	0.88–15.54	1.13–51.27	0.45–44.67
Vanillic acid	9.05–33.52	0.44–0.45	0.52–4.89	0.47–1.86	2.02–13.50	0.57–9.77	0.44–18.56
Syringaldehyde	ND	ND	ND	Detected	ND	ND	Detected
Ferulic acid	1.58–2.47	1.59–43.39	0.78–34.77	10.22–41.71	51.64–286.43	1.01–108.73	2.82–11.45
Rutin	ND	ND	ND	Detected	ND	Detected	ND

ND: Not Detected

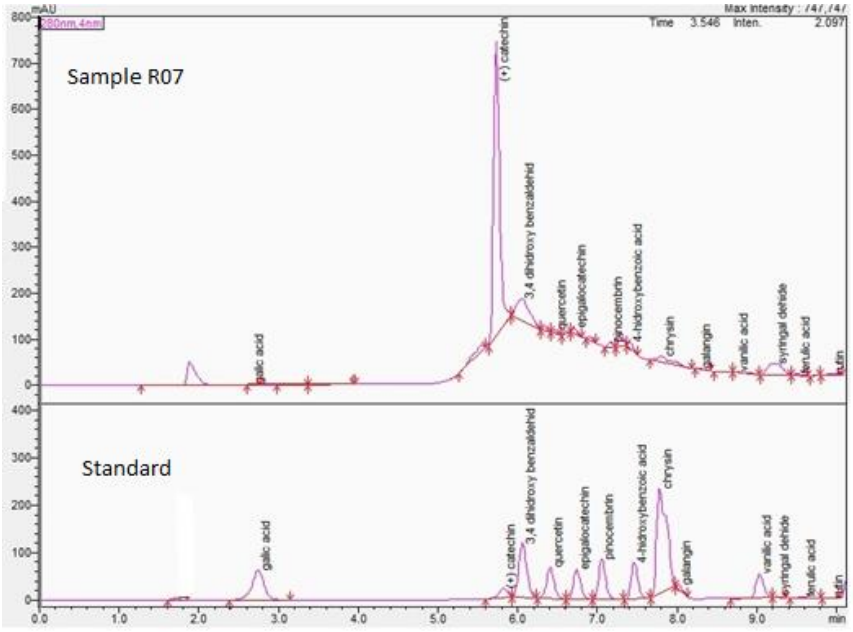

Figure 2.HPLC Chromatogram for Sample from Sumatra region and polyphenolic standard

4. Conclusion

This study demonstrated that a correlation exists among the TPC, TFC, and RSA values of the IWH samples. The phenolic profile varied significantly at both intra- or inter-region levels, which could be due to the variation in the planttype as a source of nectar. The dominant phenolic compounds in the IWH samples were (+)-catechin, ferulic acid, and galangin, whereas some compounds were found only in honey collected from certain regions; for example, 3, 4-dihydroxy benzaldehyde was detected only in honey collected from Sumatra, East Nusa Tenggara, and Java; syringaldehyde was found in honey collected from Sumatra and Java; and rutin was detected in honey samples collected from Sumatra and East Nusa Tenggara.

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