

Several Herbal Tea Plants: Antioxidant Profile of Non-polar, Semi-Polar and Polar Extract

Purwa Kurniasari ^{1*}, Rika Hartati ¹, Irda Fidrianny ¹

¹ Department of Pharmaceutical Biology, School of Pharmacy, Bandung Institute of Technology; purwa.kurniasari@gmail.com (P.K.); rikahar@gmail.com (R.H.); irdafidrianny@gmail.com (I.F.);

* Correspondence: purwa.kurniasari@gmail.com;

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Abstract: The extraction method and solvent used influence the composition of antioxidant compounds in a plant extract. Research on the antioxidant activity of various extracts from *Caesalpinia sappan* heartwood, *Camellia sinensis* leaves, *Clitoria ternatea* flowers, and *Hibiscus sabdariffa* flowers remains limited. This study aimed to evaluate the antioxidant activity of extracts with different polarities and to investigate their correlation with the total phenolic and total flavonoid contents. *C. sinensis* leaves exhibited the highest DPPH and FRAP values in semi-polar to polar extracts (797.664 ± 30.603 mg AEAC/g and 437.606 ± 41.580 mg AEAC/g, respectively). Meanwhile, *C. sappan* heartwood exhibited the highest CUPRAC value in the semi-polar extract (1017.620 ± 55.802 mg AEAC/g). The highest total phenolic content (TPC) was observed in the ethyl acetate extract of *C. sappan* heartwood (76.291 ± 1.495 g GAE/100 g), whereas the highest total flavonoid content (TFC) was found in its n-hexane extract (5.092 ± 0.202 g QE/100 g). The results suggest that phenolic and flavonoid compounds contributed significantly to the antioxidant activity.

Keywords: antioxidant; CUPRAC; DPPH; FRAP; herbal tea; phenolic.

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1. Introduction

Herbal teas are believed to have positive health effects. Unsurprisingly, the lifestyle of drinking herbal beverages is gaining popularity worldwide. Tea consumption has been shown to rank second only to water globally [1]. Herbal infusions typically consist of a single herb or a mixture of herbs with purported health benefits [2]. Phenolic compounds, which are rich in them, play an important role in human physiology due to their antioxidant activity [3-8]. *Caesalpinia sappan* heartwoods, *Camellia sinensis* leaves, *Clitoria ternatea* flowers, and *Hibiscus sabdariffa* flowers are examples of medicinal plants commonly consumed as herbal tea.

Due to growing interest in natural products, natural antioxidants have been extensively investigated in recent years. The phytochemical components of *C. sappan* heartwoods, *C. sinensis* leaves, *C. ternatea* flowers, and *H. sabdariffa* flowers extracts have been the subject of numerous studies. However, research on the antioxidant activity of various extracts was limited. Most previous studies have focused solely on polar extracts, using methanol, ethanol, or water as solvents, even though the extraction method and solvent type influence the

composition of antioxidant compounds in the extract [9-13]. Previous studies showed that the ethanolic extract had the greatest antioxidant potential among the extracts [14-15]. The other study also revealed that ethyl acetate extract exhibited the highest antioxidant activity [16]. The choice of solvent is crucial as it determines the biological activity and chemical profile of the extracts [17-19]. Therefore, the study aimed to analyze the phenolic compounds and antioxidant activity of *C. sappan* heartwood, *C. sinensis* leaves, *C. ternatea* flowers, and *H. sabdariffa* flowers, extracted with solvents of varying polarity. Furthermore, the study examined the correlations between total phenolic content (TPC)/ total flavonoid content (TFC) and antioxidant activity, as well as the correlations among three antioxidant assays (DPPH, CUPRAC, and FRAP). Samples were extracted by reflux using three solvents of increasing polarity (n-hexane, ethyl acetate, ethanol) sequentially, and also by a single solvent (direct ethanol). This extraction method effectively classified phytochemicals by polarity and enabled measurement of their antioxidant activities.

2. Materials and Methods

2.1. Materials.

Dried herbal plants were collected from various local farmers on Java Island, Indonesia. *H. sabdariffa* flowers var red and purple, were obtained from Kediri, East Java, *C. sappan* heartwoods from Blora, Central Java, *C. ternatea* flowers from Wonogiri, Central Java, and *C. sinensis* leaves from Ciwidey, West Java. All chemical compounds were of analytical grade. DPPH, neocuproine, 2,4,6-tripyridyl-S-triazine (TPTZ), gallic acid, rutin, and quercetin were purchased from Sigma-Aldrich

2.2. Sample preparation.

H. sabdariffa flowers var red were designated as RSM, *H. sabdariffa* flowers var purple as RSU, *C. sappan* heartwoods as SEC, *C. ternatea* flowers as TEL, and *C. sinensis* leaves as THU. Each sample was ground into a fine powder.

2.3. Extraction.

A 300 g powder sample was extracted sequentially with three solvents of differing polarity (n-hexane, ethyl acetate, ethanol), followed by a single direct solvent extraction using ethanol. In total, twenty extracts were obtained. These extracts were concentrated into a soft extract, ensuring consistent density across all samples. A pycnometer was used to determine the 1% density of each extract.

2.4. Total phenolic content (TPC).

The total phenolic content (TPC) was quantified using the Folin-Ciocalteu reagent [20]. A series of gallic acid solutions at various concentrations (60–135 µg/mL) was prepared to construct a calibration curve. To 50 µL of the gallic acid solution, 500 µL of 10% Folin-Ciocalteu reagent and 400 µL of 1 M Na₂CO₃ were added, followed by a 30-minute incubation. Absorbance was measured using a UV spectrophotometer at 765 nm. Sample extracts were dissolved in pro-analytical methanol to prepare the test solutions. A volume of 50 µL from each sample was processed in the same manner as the standard solutions. Absorbance for each

extract was measured in six replicates. The total phenolic content (TPC) was expressed as grams of gallic acid equivalent per 100 grams of sample (g GAE/100 g).

2.5. Total flavonoid content (TFC).

The total flavonoid content (TFC) was determined using a modified version of Chang's method [21]. Various concentrations of quercetin solution (40–110 µg/mL) were prepared to construct a calibration curve. To 100 µL of the quercetin solution, 300 µL of methanol, 20 µL of 10% AlCl₃, 20 µL of 1 M sodium acetate, and 560 µL of distilled water were added, followed by incubation for 30 minutes. Absorbance was measured using a UV spectrophotometer at 415 nm. Sample extracts were dissolved in pro-analytical methanol to prepare the test solutions. A volume of 50 µL from each sample was processed in the same manner as the standard solutions. Absorbance for each extract was measured in six replicates. The total flavonoid content (TFC) was expressed as grams of quercetin equivalent per 100 grams of sample (g QE/100 g).

2.6. Antioxidant activity measured by DPPH assay.

The DPPH assay was carried out using a modified version of Celep's method [22]. A stock solution of DPPH was prepared at a concentration of 50 µg/mL. The ascorbic acid stock solution was prepared at a concentration of 200 µg/mL in pro-analysis-grade methanol. To generate a calibration curve, a series of at least six ascorbic acid concentrations was prepared by diluting 15–35 µL of the stock solution with pro-analytical methanol to a final volume of 125 µL. Each dilution was then mixed with 750 µL of the DPPH solution and incubated for 30 minutes. Absorbance was measured using a UV-visible spectrophotometer at 517 nm. Sample extracts were dissolved in pro-analytical methanol to prepare the test solutions. A volume of 12.5 µL from each sample was processed identically to the standard solutions. Absorbance for each extract was measured in six replicates. The antioxidant activity was expressed as milligrams of ascorbic acid equivalent antioxidant capacity per gram of sample (mg AEAC/g).

2.7. Antioxidant activity measured by CUPRAC assay.

The CUPRAC assay was conducted with slight modifications to Özyürek's method [23]. A CUPRAC reagent at a concentration of 100 µg/mL was prepared by mixing CuCl₂, neocuproin, and ammonium acetate buffer (pH 7). A standard stock solution of ascorbic acid was prepared at a concentration of 200 µg/mL in methanol pro-analysis. A series of at least six ascorbic acid concentrations was prepared to generate a calibration curve by diluting 20–50 µL of the stock solution with ammonium acetate buffer to a final volume of 250 µL. Each dilution was then mixed with 750 µL of the CUPRAC reagent and incubated for 30 minutes. Absorbance was measured using a UV-visible spectrophotometer at 450 nm. Sample extracts were dissolved in pro-analytical methanol to prepare the test solutions. A volume of 12.5 µL from each sample was processed identically to the standard solutions. Absorbance for each extract was measured in six replicates. The antioxidant activity was expressed as milligrams of ascorbic acid equivalent antioxidant capacity per gram of sample (mg AEAC/g).

2.8. Antioxidant activity measured by FRAP assay.

The FRAP assay was performed following a slightly modified version of Özyürek's method [23]. The FRAP reagent was prepared by mixing FeCl₃, tripyridyltriazine (TPTZ), and sodium acetate buffer (pH 3.6) in a 1:1:10 ratio. A standard stock solution of ascorbic acid was

prepared at 200 µg/mL in methanol of pro-analysis grade. Subsequently, at least six different concentrations of ascorbic acid were prepared by diluting 2.5-15 µL of the stock solution with distilled water to a final volume of 500 µL, creating a calibration curve. Following this, 500 µL of the FRAP reagent was added to each dilution, and the mixture was incubated for 30 minutes. Absorbance was measured at 595 nm using a UV-visible spectrophotometer. Sample extracts were dissolved in pro-analytical methanol to prepare the test solutions. A volume of 12.5 µL from each sample was processed identically to the standard solutions. Absorbance for each extract was measured in six replicates. The antioxidant activity was expressed as milligrams of ascorbic acid equivalent antioxidant capacity per gram of sample (mg AEAC/g).

2.9. The levels of flavonoid compounds determination.

High-performance liquid chromatography (HPLC) was used to quantify the concentrations of flavonoid constituents in the extracts that demonstrated the highest yield and antioxidant activity, specifically the direct-ethanol extracts of SEC and THU rutin and quercetin as standard reference compounds. The HPLC system used was an HPLC-20AD equipped with a Shimadzu SPD-20A UV-visible detector set at a wavelength of 360 nm. Chromatographic separation was carried out using a LiChrospher® 100 RP-C18 5 µm column (100 mm in length, 4 mm in diameter, with a 20 mm precolumn, Merck) as the stationary phase. The mobile phases consisted of water (eluent A) and methanol (eluent B). A linear gradient elution was applied, starting from 40% to 60% eluent B over the first five minutes, increasing to 70% by the tenth minute, and returning to 40% by the fifteenth minute. The sample injection volume was 20 µL, with a flow rate of 1 mL/min (CTO-20A pump, Shimadzu, Japan), and the column temperature was maintained at 30°C (Oven CTO-20A, Shimadzu, Japan). Standard solutions of rutin and quercetin were prepared at a concentration of 50 µg/mL each. The direct-ethanol SEC extract was prepared at a concentration of 2000 µg/mL, while the direct-ethanol THU extract was prepared at a concentration of 500 µg/mL. Flavonoid levels in the samples were determined by comparing the area under the curve (AUC) of the sample peaks with those of the standards, using the one-point method for quantification [24].

3. Results and Discussion

3.1. Antioxidant activity, total phenolic and flavonoid content.

The antioxidant activity of plant extracts is influenced by several factors, including the extract's composition and the method used to assess antioxidant activity. Therefore, a single method is insufficient. The multifunctionality of natural antioxidants necessitates the use of reliable testing methods to accurately assess their activity. Therefore, it is crucial to assess antioxidant activity using multiple methods [25]. Different testing methods applied to the same sample may yield varying results. Additionally, the composition of antioxidant compounds within an extract is affected by the extraction method and solvent used, due to the differing chemical properties and polarities of antioxidant compounds [26-28].

In the current study, antioxidant activity was measured by the DPPH, CUPRAC, and FRAP methods (Figures 1-3), and the results were expressed as mg AEAC/g (mg ascorbic acid equivalent antioxidant capacity per gram of sample). Variations were observed in the antioxidant activity results across methods. In most samples, the antioxidant activity of the ethyl acetate extract was higher than that of the ethanolic extract. This was likely due to the

presence of glycosylated flavonoids in polar solvents, which exhibit lower antioxidant activity compared to aglycone flavonoids.

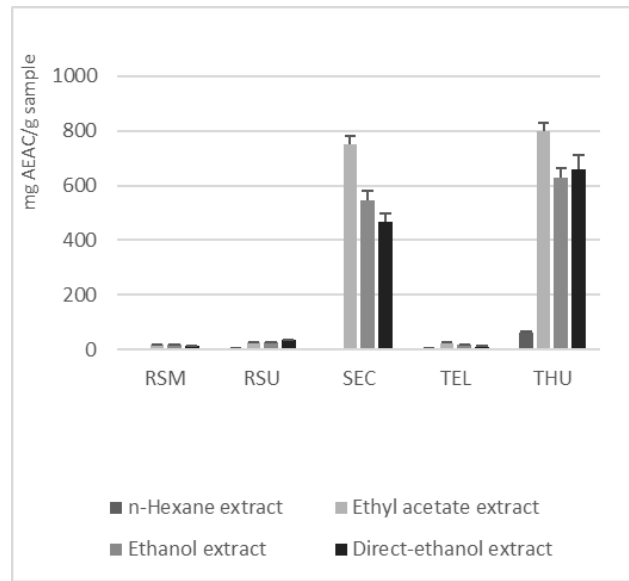


Figure 1. Antioxidant activity measured by DPPH assay.

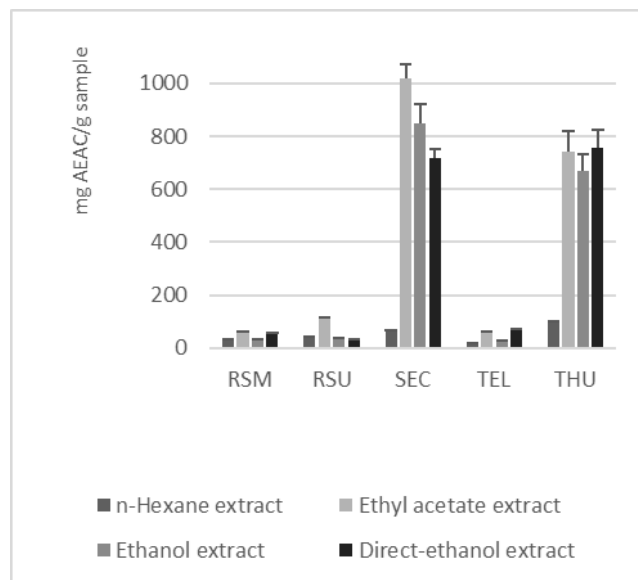


Figure 2. Antioxidant activity measured by CUPRAC assay.

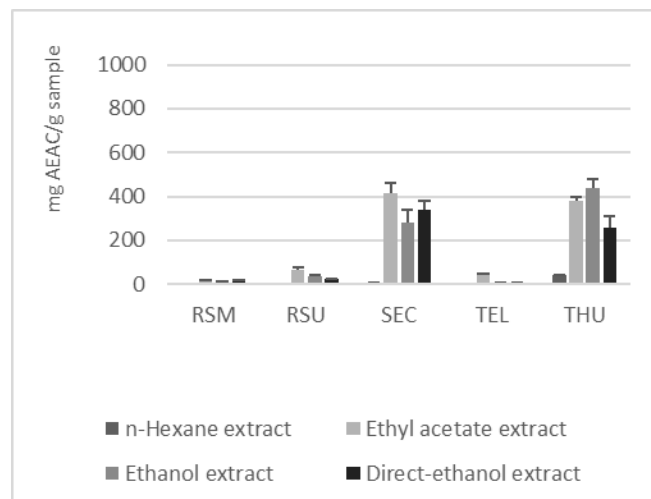


Figure 3. Antioxidant activity measured by FRAP assay.

Glycosylated flavonoids are more soluble in polar solvents such as ethanol than in semi-polar solvents like ethyl acetate. In addition, due to the presence of O-methylated flavonoids, n-hexane extracts of all samples exhibited the lowest antioxidant activity among all extracts. The reduction of the hydroxyl group through methylation is known to diminish the antioxidant activity of flavonoids. Consequently, both glycosylated and O-methylated flavonoids possess lower antioxidant activity than aglycone flavonoids [29].

Antioxidant activity is influenced by the chemical structure and functional moieties of phenolic and flavonoid compounds. Phenols are essential plant constituents with strong radical-scavenging ability due to the presence of hydroxyl functional groups [30]. Flavonoids are a class of polyphenolic compounds with a benzopyrone structure, and also exhibit antioxidant properties through interactions between their hydroxyl functional groups and free radicals [31]. Structure-activity relationships studies have shown that flavonoids exhibit higher antioxidant activity when they possess an OH group at C-3, an ortho di-OH group at C-3'-C-4', a double bond at C-2 and C-3, and a ketone group at C-4 [32-33]. According to Table 1, total flavonoid content (TFC) was higher than total phenolic content (TPC) in all n-hexane extract samples, except for *C.sinensis* leaves (THU). When the hydroxyl group of flavonoids is reduced, their lipophilicity increase, enhancing the solubility in non-polar solvents such as n-hexane [34]. Additionally, the O-methylated flavonoid is a derivative of flavonoid that is soluble in n-hexane. In this study, TFC was measured using a colorimetric assay based on the formation of a flavonoid-AlCl₃ complex. Prior research demonstrated that O-methylated flavonoids could still form chelation with AlCl₃ [35]. On the other hand, TPC, expressed as grams of gallic acid equivalent per 100 grams of sample (g GAE/100 g), was measured using the Folin-Ciocalteu assay, which is sensitive to simple phenol, citric acid, various amines, amino acids, and sugars. Interestingly, this assay was unstable for lipophilic compounds because the molybdenum-phosphotungstate heteropolyanion, the Folin chromophore, is (4-) charged and forms strong ion-dipole interactions with water molecules [36].

Table 1. TPC (mg GAE/ 100 g sample) and TFC (mg QE/ 100 g sample) in several herbal tea plant extracts.

Sample	Content	n-Hexane	Ethyl acetate	Ethanol	Direct ethanol
RSM	TPC	0.241 ± 0.006 ^{aw}	2.107 ± 0.040 ^{ax}	2.734 ± 0.078 ^{ay}	1.725 ± 0.051 ^{az}
	TFC	0.553 ± 0.049 ^{aw}	1.179 ± 0.040 ^{ax}	0.609 ± 0.010 ^{aw}	0.837 ± 0.059 ^{ay}
RSU	TPC	0.507 ± 0.010 ^{bw}	3.721 ± 0.100 ^{ax}	3.217 ± 0.66 ^{ay}	3.857 ± 0.076 ^{az}
	TFC	1.268 ± 0.110 ^{aw}	0.967 ± 0.052 ^{ax}	0.726 ± 0.015 ^{ay}	0.876 ± 0.025 ^{ax}
SEC	TPC	1.011 ± 0.025 ^{cw}	76.291 ± 1.495 ^{bx}	50.598 ± 0.904 ^{by}	50.808 ± 0.705 ^{by}
	TFC	5.092 ± 0.202 ^{bw}	2.761 ± 0.058 ^{bx}	1.861 ± 0.037 ^{by}	2.100 ± 0.095 ^{bz}
TEL	TPC	0.348 ± 0.007 ^{abw}	7.227 ± 0.188 ^{ax}	1.479 ± 0.029 ^{ay}	2.333 ± 0.098 ^{az}
	TFC	1.081 ± 0.057 ^{aw}	3.621 ± 0.083 ^{cx}	0.824 ± 0.020 ^{ay}	1.694 ± 0.042 ^{bz}
THU	TPC	1.696 ± 0.014 ^{dw}	45.033 ± 1.079 ^{bx}	37.188 ± 0.961 ^{by}	38.241 ± 0.617 ^{by}
	TFC	1.201 ± 0.067 ^{aw}	3.151 ± 0.079 ^{cx}	2.119 ± 0.031 ^{cy}	2.622 ± 0.089 ^{cz}

RSM: *Hibiscus sabdariffa* var. red; RSU: *Hibiscus sabdariffa* var. purple; SEC: *Caesalpinia sappan*;

TEL: *Clitoria ternatea*; THU: *Camellia sinensis*; n = 6; a-c = different letters in the same column showed significant difference (P<0.05); w-z = different letters in the same row showed significant difference (P<0.05);

GAE = Gallic Acid Equivalent; QE = Quercetin Equivalent.

According to this study, the ethyl acetate of *C.ternatea* flower (TEL) had the highest TPC (7.227 ± 0.188 g GAE/100 g) and TFC (3.621 ± 0.083 g QE/100 g) among ethyl acetate extracts of *H.sabdariffa* flower var. red (RSM) and *H.sabdariffa* flower var. purple (RSU). Based on the DPPH assay, the antioxidant activity of ethyl acetate TEL extract was comparable to that of RSM and RSU. It was hypothesized that the ethyl acetate extract of RSM and RSU contained flavonoids with high antioxidant activity characterized by the presence of an OH group at carbon 3, an ortho di-OH group at carbon 3'-carbon 4', a double bond at carbons 2 and

3, and a ketone group at carbon 4. On the other hand, flavonoids in the ethyl acetate TEL extract were predicted to have distinct structures with low antioxidant activity. Similarly, the direct ethanol TEL extract showed higher TPC and TFC levels than the direct ethanol RSM extract; however, their antioxidant activity was comparable.

On the other hand, Figures 1-3 indicate that CUPRAC values reflected higher antioxidant activity than FRAP values. It was feasible because the latter had a narrower detection range. The univalently charged CUPRAC chromophore $(\text{Cu}(\text{Nc})_2)^+$ has a higher affinity for both aqueous and organic solvents, enabling the simultaneous detection of hydrophilic and lipophilic antioxidants. In contrast, FRAP is limited in its ability to detect lipophilic antioxidants because the trivalently charged $\text{Fe}(\text{III})\text{-TPTZ}$ complex has a stronger affinity for the aqueous phase than organic solvents. Furthermore, the electron-transfer (ET)-based assay relies on the redox potential to detect antioxidant compounds. The standard redox potential (E°) of the $\text{Cu}(\text{II/I})\text{-neocuproine}$ complex used in CUPRAC is 0.6 V, whereas the FRAP-TPTZ complex is 0.582 V. Antioxidant compounds can be detected if their redox potential is lower than that of the reference compound [37].

The TPC of the ethanol and direct ethanol extract of *C.sappan* (SEC) did not differ substantially in terms of GAE content (50.598 ± 0.904 g GAE/100 g and 50.808 ± 0.705 g GAE/100 g, respectively). However, the TFC of direct-ethanol SEC extract (2.100 ± 0.095 g QE/100 g) was higher than that of ethanol SEC extract (1.861 ± 0.037 g QE/100 g). The CUPRAC assay showed that the direct-ethanol SEC extract (716.080 ± 34.310 mg AEAC/g) exhibited lower antioxidant activity than the ethanol SEC extract (846.418 ± 75.235 mg AEAC/g). This suggests that most antioxidant compounds in the ethanol SEC extract likely had a redox potential below 0.6 V (E° $\text{Cu}(\text{II/I})\text{-neocuproine}$ complex), whereas the majority of antioxidant compounds in the direct ethanol SEC extract had a redox potential above 0.6 V. Furthermore, the TPC of direct-ethanol RSU extract (3.857 ± 0.076 g GAE/100 g) was significantly higher ($p < 0.05$) than ethyl acetate RSU extract (3.721 ± 0.100 g GAE/100 g). However, the TFC of both extracts were not significantly different (0.876 ± 0.025 g QE/100 g and 0.967 ± 0.052 g QE/100 g, respectively). Antioxidant activity analysis using CUPRAC and FRAP assays revealed that ethyl acetate RSU extract was more potent than the direct-ethanol RSU extract (31.270 ± 0.722 mg AEAC/g and 25.696 ± 2.104 mg AEAC/g, respectively; CUPRAC and FRAP assays). It was concluded that most of the antioxidant compounds in the ethyl acetate RSU extract had redox potentials below 0.582 V.

In this study, the RSM extracts with the highest TPC and TFC were the ethanol extract (2.734 ± 0.078 g GAE/100 g) and the ethyl acetate extract (1.179 ± 0.040 g QE), respectively. In both the DPPH and CUPRAC assays, the RSM extract derived from ethyl acetate exhibited the most potent antioxidant activity. However, based on the FRAP assay, no significant differences in antioxidant activity among the ethanol, ethyl acetate, and direct-ethanol RSM extracts. Meanwhile, the TPC of direct-ethanol RSU extract (3.857 ± 0.076 g GAE/100 g) was higher than that of ethyl acetate RSU extract (3.721 ± 0.100 g GAE/100 g). Nevertheless, the TFC of the two extracts did not differ significantly. The ethyl acetate RSU extract exhibited superior antioxidant activity compared to the direct-ethanol RSU extract, as indicated by the CUPRAC and FRAP assays. Previous research demonstrated that the polar extract of *H.sabdariffa* possessed the highest TPC compared to the semi-polar and non-polar extracts. Yagi *et al.* [17] showed that the TPC obtained from water extracts (21.67 mg GAE/g) was greater than that obtained from methanol, ethyl acetate, and n-hexane extracts at 50°C for 10 min. An 80% methanolic extraction also yielded a significantly higher ($P \leq 0.05$) concentration

of phenolic compounds compared to an aqueous extraction [19]. Additionally, another study also found that among the ethyl acetate, aqueous, and ethanolic extracts, the ethanolic extract exhibited the strongest antioxidant activity in the FRAP, DPPH, and nitric oxide (NO) assays, as indicated by its lowest IC₅₀ values of 2.8 µL/mL, 3.3 µL/mL, and 9.2 µL/mL, respectively [38]. These contrast with the findings of the present study, which indicated that ethyl acetate (semi-polar) extracts had greater antioxidant activity than ethanolic (polar) extracts.

The SEC extract with the highest TPC was the ethyl acetate extract (76.291 ± 1.495 g GAE/100 g), followed by ethanol, direct ethanol, and n-hexane. In contrast, n-hexane had the highest TFC value (5.092 ± 0.202 g QE/100 g), followed by ethyl acetate, direct ethanol, and ethanol extract. A previous study revealed that higher TPC in SEC extract correlates with stronger antioxidant activity [39]. The present study showed that the ethyl acetate extract had the highest TPC among all samples and demonstrated the most potent antioxidant activity, followed by ethanol, direct ethanol, and n-hexane, as determined by DPPH and CUPRAC assays. However, the FRAP assays yielded different results, showing that the antioxidant activity of the direct ethanol extract was higher than that of the ethanol extract. Consistent with the present findings, previous studies reported that the ethyl acetate fraction of the SEC extract exhibited strong antioxidant activity, with scavenging values of 2.24 µg/mL in the DPPH assay and 2.38 µg/mL in the ABTS assay [40-42]. This study aligned with those demonstrating that the ethyl acetate extract exhibited superior antioxidant activity, as measured by the DPPH, CUPRAC, and FRAP assays, with values of 749.445 ± 30.603 mg AEAC/g, 1017.620 ± 55.802 mg AEAC/g, and 412.331 ± 48.731 mg AEAC/g, respectively.

On the other hand, the ethyl acetate fraction of the TEL extract had the highest TPC and TFC, with values of 7.227 ± 0.188 g GAE/100 g and 3.621 ± 0.083 g QE, respectively, among all TEL extracts. Additionally, the ethyl acetate TEL extract exhibited the highest antioxidant activity as measured by DPPH (25.204 ± 0.455 mg AEAC/g) and FRAP (45.01 ± 5.404 mg AEAC/g) assays. There are limited studies examining the TPC, TFC, and antioxidant activity of TEL using solvents other than water or alcohol. In the present research, both DPPH and FRAP assays demonstrated that the antioxidant activity of the ethyl acetate extract was greater than that of the ethanolic extract. In contrast, previous studies found that water extract exhibited higher antioxidant activity than alcoholic extracts [43,44]. Additionally, in earlier research, a water extract of *Clitoria* flower obtained via ultrasound-assisted extraction yielded up to 26.9 ± 0.6 g GAE/kg of TPC and showed DPPH radical scavenging activity of 22.6 ± 1.0 g TE/kg [45]. Another study reported that the ethanolic extract of TEL exhibited antioxidant activity with an IC₅₀ of 2.77 ± 0.020 mg/g (DPPH assay), and a TPC value of 28.75 ± 1.215 mg GAE/g [46].

Similar to TEL, the ethyl acetate THU extract yielded the highest TPC and TFC concentrations among all THU extracts, with values of 45.033 ± 1.079 g GAE/100 g and 3.151 ± 0.079 g QE, respectively. In the DPPH assay, the ethyl acetate THU extract exhibited the highest antioxidant activity (797.664 ± 30.603 mg AEAC/g). However, in the CUPRAC and FRAP assays, the direct ethanol extract and the ethanol extract showed the highest antioxidant activity, with values of 758.385 ± 65.016 mg AEAC/g and 437.606 ± 41.580 mg AEAC/g, respectively. Previous research also reported that the ethyl acetate fraction demonstrated the highest antioxidant activity, followed by the water and ethanolic fractions, with IC₅₀ values of 3.926 µg/mL, 7.408 µg/mL, and 9.017 µg/mL, respectively, measured by the DPPH method [47]. In line with this study, several studies have shown that *C. sinensis* is better extracted with

hydroalcoholic solvents to obtain phenolic-rich extracts with the strongest antioxidant activity [48,49].

Many potent antioxidant compounds in plants, such as phenolics, flavonoids, and tannins, are polar molecules. Polar solvents (such as water, methanol, and ethanol) are more effective at dissolving and extracting these compounds, thereby enhancing the antioxidant activity of the extracts [26,27]. However, semi-polar solvents (such as ethyl acetate and acetone) can sometimes yield extracts with even higher antioxidant activity, particularly when targeting specific compounds such as certain flavonoids or phenolic acids, for example, flavonoids with an OH group at C-3, an ortho di-OH group at C-3'-C-4', a double bond at C-2 and C-3, and a ketone group at C-4. In some cases, ethyl acetate extracts outperform both polar and non-polar extracts in antioxidant assays [28].

Based on this study, the ethyl acetate extracts (semi-polar) of *H. sabdariffa* flowers, *C. ternatea* flowers, and *C. sappan* heartwood exhibited higher antioxidant activity compared to the n-hexane extracts (non-polar) and ethanol extracts (polar). In contrast, the ethanol extract (polar) of *C. sinensis* leaves showed higher antioxidant activity than its n-hexane (non-polar) and ethyl acetate (semi-polar) counterparts. These findings have implications for tea preparation and nutraceutical formulation. Tea prepared using polar or semi-polar solvents (such as hot water or hydroalcoholic solvents) may offer greater health benefits by maximizing the extraction of phenolic and flavonoid compounds. In nutraceuticals, using semi-polar solvents such as ethyl acetate during extraction may yield formulations with higher antioxidant activity. However, different plant parts and species (e.g., *C. sinensis* vs. *C. sappan*) exhibit varying optimal extraction profiles. Therefore, formulations should be tailored by selecting the appropriate solvent and plant combination to achieve the desired antioxidant therapeutic effects.

3.2. The levels of flavonoid compounds determination.

In addition, HPLC was used to identify flavonoid compounds in direct-ethanol extracts of *C. sappan* (SEC) and *C. sinensis* (THU), which demonstrated superior antioxidant activity in this study. Among flavonoids, flavonols are a subclass commonly found in numerous edible and medicinal plants.

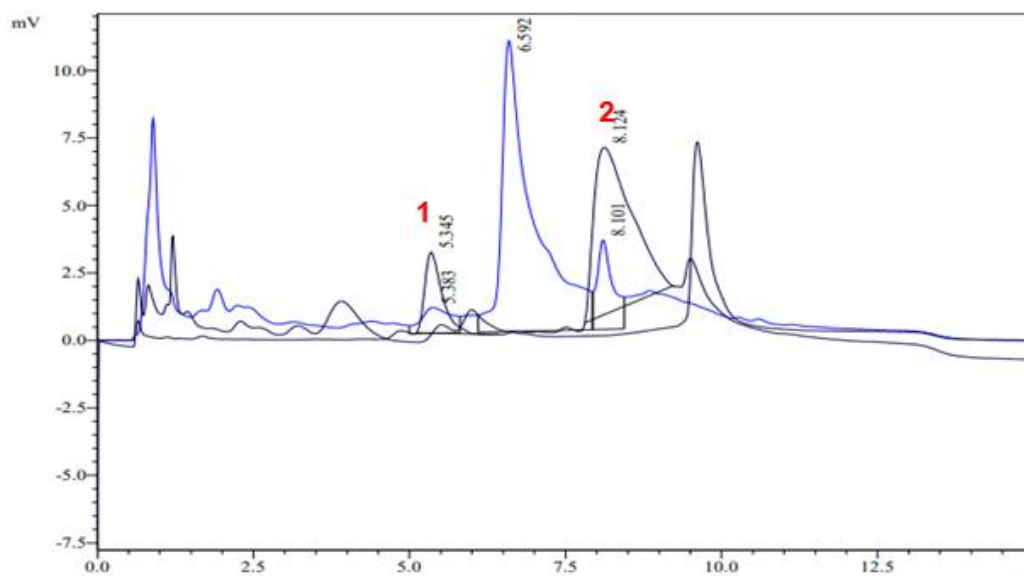


Figure 4. HPLC chromatogram of direct-ethanol SEC and standards, 1: rutin, 2: quercetin, black line chromatogram = standard, blue line chromatogram = SEC.

One of the most well-known flavonol compounds is quercetin and its derivatives [50], with rutin being a glycoside form of quercetin [51]. Previous research has reported the presence of both quercetin and rutin in SEC and THU extracts [52-55]. As shown in Figure 4, the direct-ethanol SEC extract contained both rutin and quercetin. Based on one-point calibration, the concentrations were 1.427% for rutin and 0.579% for quercetin. Meanwhile, Figure 5 showed that the direct-ethanol THU extract contained 3.312% rutin. Quercetin and rutin exhibit strong antioxidant activity due to their structural features, including an OH group at C-3, an ortho di-OH group at C-3'-C-4', double bonds at C-2 and C-3, and a ketone group at C-4.

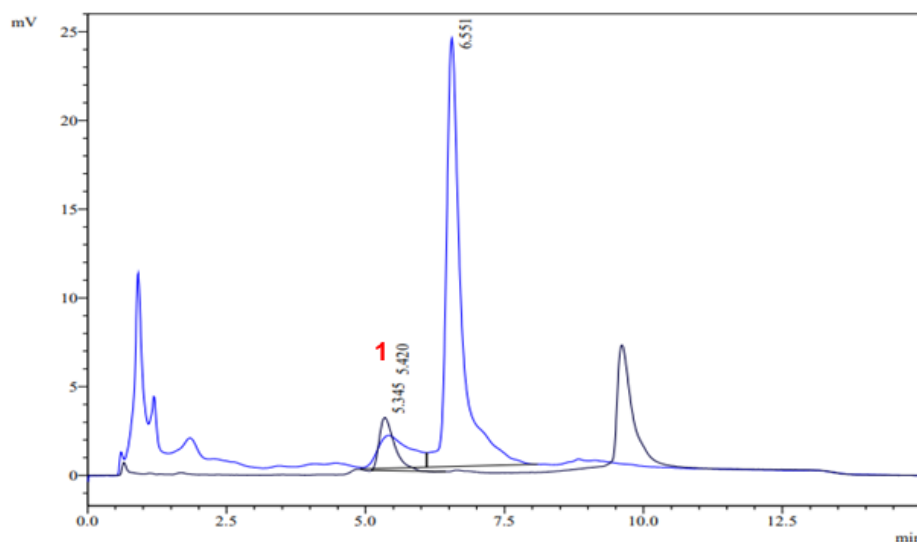


Figure 5. HPLC chromatogram of direct-ethanol THU and standards, 1: rutin, black line chromatogram = standard, blue line chromatogram = THU.

3.3. Correlation between TPC and TFC to the antioxidant activity and correlation between antioxidant activity assays.

Pearson's method was used to analyze the correlation using the Pearson correlation coefficient (r). Schober *et al.* [56] proposed stratifications with varying cut-off points for interpreting the strength of the correlation: $r = 0.40 - 0.69$ indicates a moderate correlation, $r = 0.70 - 0.89$ indicates a strong correlation, and $r = 0.90 - 1.00$ indicates an extremely strong correlation. The results are presented in Table 2. Pearson's correlation coefficients between TPC and antioxidant activity, and between TFC and antioxidant activity, showed a positive relationship across all samples. The correlation values ranged from 0.689 to 0.994 for TPC and antioxidant activity, and from 0.727 to 0.993 for TFC and antioxidant activity. These findings indicate that the phenolic and flavonoid compounds are the primary contributors to antioxidant activity.

In addition, the correlation among the three antioxidant assays used in this study (DPPH, CUPRAC, and FRAP) was evaluated. The results showed a positive correlation between the method, with r values ranging from 0.642 to 0.985. Consequently, these assays produced consistent, linear results for determining the antioxidant activity of the five selected herbal tea extracts.

Table 2. Pearson's correlation coefficient between TPC, TFC, and their antioxidant activities.

Sample	Pearson's correlation coefficient (r) for DPPH		Pearson's correlation coefficient (r) for CUPRAC		Pearson's correlation coefficient (r) for FRAP	
	TPC	TFC	TPC	TFC	TPC	TFC
RSM ¹	0.914****	0.923****	0.787***	0.844***	0.883***	0.974****
RSU ¹	0.934****	0.881***	0.913****	0.919****	0.958****	0.875***

Sample	Pearson's correlation coefficient (r) for DPPH		Pearson's correlation coefficient (r) for CUPRAC		Pearson's correlation coefficient (r) for FRAP	
	TPC	TFC	TPC	TFC	TPC	TFC
SEC ¹	0.858***	0.946****	0.968****	0.889***	0.946****	0.822***
TEL ¹	0.983****	0.973****	0.956****	0.949****	0.982****	0.937****
THU ¹	0.864***	0.935****	0.962****	0.961****	0.871***	0.964****
RSM ²	0.925****	0.833***	0.987****	0.890****	0.982****	0.965****
RSU ²	0.940****	0.931****	0.834***	0.788***	0.969****	0.943****
SEC ²	0.891****	0.881***	0.921****	0.951****	0.904****	0.846***
TEL ²	0.985****	0.970****	0.918****	0.949****	0.820***	0.795***
THU ²	0.975****	0.944****	0.934****	0.939****	0.945****	0.917****
RSM ³	0.924****	0.928****	0.982****	0.975****	0.897****	0.867***
RSU ³	0.745***	0.727***	0.782***	0.796***	0.917****	0.941****
SEC ³	0.898****	0.980****	0.689**	0.867***	0.782***	0.864***
TEL ³	0.886***	0.944****	0.970****	0.868***	0.813***	0.986****
THU ³	0.764***	0.932****	0.839***	0.934****	0.808***	0.966****
RSM ⁴	0.940****	0.905****	0.916****	0.993****	0.893****	0.866***
RSU ⁴	0.893****	0.961****	0.934****	0.908****	0.904****	0.972****
SEC ⁴	0.994****	0.988****	0.874***	0.898****	0.978****	0.949****
TEL ⁴	0.942****	0.989****	0.915****	0.845***	0.984****	0.973****
THU ⁴	0.992****	0.943****	0.961****	0.929****	0.944****	0.906****

1 = n-hexane extract; 2 = ethyl acetate extract; 3 = ethanol extract; 4 = direct-ethanol extract;

** = moderate correlation; *** = strong correlation; **** = very strong correlation.

4. Conclusions

The ethyl acetate (semi-polar) extracts of *H. sabdariffa* flowers, *C. ternatea* flowers, and *C. sappan* heartwoods exhibited higher antioxidant activity compared to n-hexane (non-polar) extracts and ethanol (polar) extracts, except for *C. sinensis* leaves extracts. Among all samples, *C. sinensis* leaves exhibited the highest DPPH and FRAP values in semi-polar to polar extracts (797.664 ± 30.603 mg AEAC/g and 437.606 ± 41.580 mg AEAC/g, respectively). Meanwhile, *C. sappan* heartwood exhibited the highest CUPRAC value in the semi-polar extract (1017.620 ± 55.802 mg AEAC/g). The highest total phenolic content (TPC) was observed in the ethyl acetate extract of *C. sappan* heartwood (76.291 ± 1.495 g GAE/100 g), and total flavonoid content (TFC) was found in its n-hexane extract (5.092 ± 0.202 g QE/100 g). Both TPC and TFC were positively correlated with antioxidant activity, indicating that phenolic and flavonoid compounds contributed significantly to the observed antioxidant effects.

Author Contributions

Conceptualization, P.K.; methodology, P.K., R.H., I.F.; validation, R.H. and I.F.; investigation, P.K.; resources, P.K.; data curation, R.H. and I.F.; writing—original draft preparation, P.K.; writing—review and editing, R.H. and I.F.; visualization, P.K.; supervision, R.H. and I.F.; project administration, P.K.; funding acquisition, I.F. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

No new data were created or analyzed in this study. Data sharing is not applicable.

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Conflicts of Interest

The authors declare no conflict of interest.

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