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# Screening of different extraction methods for maximum production of total flavonoids, tannins, and antioxidants from *Centella asiatica*

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**Abstract**

The study was carried out to maximise the extraction efficiency of different extraction methods and evaluate the total flavonoid, tannin, and antioxidant activity of the well-known medicinal herb *Centella asiatica*. A total of nine extraction methods were used viz. squeezing of fresh leaves, extraction of fresh leaves and oven-dried powder using boiled water, ethanol, methanol, and acetone as solvents, respectively. Different *in vitro* assays were used to maximize the extraction of total flavonoid content, and total tannin content. The antioxidant activities in terms of total phenolic content (TPC), DPPH(1,1-Diphenyl-2-picrylhydrazyl) radical scavenging assay, and ferric reducing antioxidant power (FRAP) of *C. asiatica* were also evaluated. The extract from oven-dried powder using 70% acetone exhibited the maximum flavonoid (3.0533±0.1069 mg QE/g) and tannin content (0.7800±0.0100 mg TAE/g). The powder extract using 70% acetone also exhibited the highest total phenolic content (13.883±0.050 mg GAE/g), DPPH (70.630±2.310%) and FRAP value (166.670±2.260 mg AAE/100g). The acetone extract of dried *C. asiatica* powder was found to be the best extraction procedure for tannin, total flavonoids, and antioxidant production. The findings of this study might be helpful in extracting natural antioxidants from *C. asiatica* and improve the existing literature.

## 1. Introduction

*Centella asiatica L.* is a prostrate medicinal herb of the Apiaceae family and an indigenous plant found in Bangladesh. It has a broad array of applications that have been recorded in Southeast Asia and Bangladesh (Rahman *et al.*, 2013). *Centella asiatica* is one of the local herbs reportedly comprising multiple health effects. It could be used for the treatment of various diseases within the Ayurvedic medicine system. It has also been used for memory recovery, regeneration of tissue, mental fatigue management, bronchitis, asthma, dysentery, jaundice, leucorrhoea, kidney dysfunction, anti-allergic, anticancer, high fever and is frequently utilized as a food supplement for infants and babies in the fight against nutritional deficiencies (Orhan, 2012).

The lipid-containing foods are very prone to oxidative degradation during preparation and storage, which results in rancidity. This ultimately destroys the nutritional profile and overall organoleptic quality of foods because of the development of poisonous substances (Orhan, 2012). Antioxidant supplementation is an approach to increasing food shelf life. Numerous phenolic compounds, notably flavonoids, possess several biological functions namely germicidal, anti-allergic, anti-thrombotic, anti-inflammatory, and vasodilator actions. Many of these components are active free radical scavengers and are particularly useful in preventing arteriole sclerosis, arthritis, diabetes, cancer, Alzheimer's, and so on. The presence of these molecules has been directly linked to the beneficial role of foodstuffs rich in vegetables and fruits (Hossain, Disha, Shourove *et al.*, 2020; Sarkar *et al.*, 2021; Ahmed *et al.*, 2021; Roy *et al.*, 2022). There is increasing consumer awareness of the safety of food additives and the higher production costs and lower effectiveness of natural antioxidants, thereby creating the need to identify alternative natural and healthier sources of antioxidant foods (Hossain, Evan, Moazzem *et al.*, 2020; Zzaman *et al.*, 2021; Hossain, Mitra, Belal *et al.*, 2021; Hossain *et al.*, 2022). The search for natural plant-based antioxidants has dramatically increased in recent times. Antioxidant compounds derived from natural sources, including rice, olive seeds, beans, spices, fruits, and vegetables, have been studied (Alam *et al.*, 2020). Many of these bioactive components are present in many plants' parts, including wood, bark, stem, leaf, fruit, root, herb, and seed (Ahmed *et al.*, 2016; Rahman *et al.*, 2016,

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Ahmed *et al.,* 2021).

Ancient Egyptians initiated the use of antioxidants; they used plants with a high content of phenolic compounds to preserve dead bodies (Gutteridge and Halliwell, 2010). These curative plants have global importance due to the presence of phytoconstituents with effective pharmacological action. Today natural antioxidants contained in abundant amounts in plants, fruits, and vegetables have attained global concerns because of their functional, safety, and therapeutic value. In plants, naturally occurring antioxidants are found as phenolic compounds (Hossain and Hossain, 2021; Hossain, Mitra, Belal *et al*., 2021; Hossain, Dey and Joy, 2021; Sarkar *et al*., 2021). Scientific studies in the literature have indicated promising phytoconstituents that can be extracted from single or multiple plant extracts to develop herbal drugs (Anand *et al.*, 2010; Hasim, 2011). This is one of the important reasons that the quest for formulating a prime natural antioxidant has become a significant scientific research and technical challenge. Considering the advantages of natural antioxidants over synthetic antioxidants, this study was planned to compare the antioxidant potential of several *C. asiatica* extracts. The literature reveals that most previous studies were based on extracting antioxidants from *C. asiatica* by optimizing extracting temperature, solvent concentration, and solvent type (Pittella *et al*., 2009; Hashim *et al*., 2011; Hashim, 2011). However, before extraction, treating samples in various ways was not common in the literature. In this context, this study was carried out to evaluate the efficiency of different extraction methods for maximum production of the flavonoid, tannin, and antioxidant activities of *C. asiatica*.

## 2. Materials and methods

### *2.1 Sample collection and preparation*

The sample (*C. asiatica* L.) was procured from Bander Bazar, Sylhet, Bangladesh. About 1 kg of the sample was collected, and it was kept at an average room temperature (25±2°C). The samples were first washed with running tap water to remove the impurities, dust, soil, and plant debris. The collected samples were separated from undesirable materials or parts. Only leaves and petioles were used for analysis. There was a total of 9 samples prepared as stated in Table 1.

Table 1. Symbolization of the samples of *Centella asiatica*

| Sample Name | Symbol |
|---|---|
| Squeezing | A1 |
| Fresh leaves using boiled water | A2 |
| Fresh leaves extract using 70% ethanol | A3 |
| Fresh leaves extract using 70% methanol | A4 |
| Fresh leaves extract using 70% acetone | A5 |
| Powdered dry leaves using boiled water | A6 |
| Powder extract using 70% ethanol | A7 |
| Powder extract using 70% methanol | A8 |
| Powder extract using 70% acetone | A9 |

### *2.2 Squeezing*

The fresh foliage (25 g) of *C. asiatica* was cut into tiny slices and squeezed it with the mortar and pestle (Z247502, Sigma-Aldrich, Germany) to find the juice of this. Then the amount of fluid was measured and mixed with double-distilled water (1:10; v/v) to dilute it. Then the solution was filtered using a muslin cloth and filter paper (Whatman No. 1; Sigma-Aldrich, Germany), respectively, for the preparation of sample A1 (Vongsak *et al.*, 2013).

### *2.3 Boiling of fresh leaves*

The raw leaves (25 g) were cut into little pieces, then cooked in double-distilled water (1:10; w/v) at 100°C for 30 mins, and then filtered using a filter paper (Whatman No.1). The coir was extracted over and again until complete depletion (Vongsak *et al.*, 2013) to find extract for sample A2.

### *2.4 Extraction of fresh leaves using alcoholic solvents*

The raw *C. asiatica* leaves were cut into little slices (25g) and soaked in 70% extraction solvent of ethanol (A3), methanol (A4) and acetone (A5), respectively at the ratio of (1:10; w/v). The solvent concentration was selected based on previous literature (Rahman *et al*., 2016). They were kept for 72 hrs at normal environmental temperature (25±2°C) and shook occasionally for better extraction. The mixtures were extracted over and again by the same procedure until the complete exhaustion of the sample (Vongsak *et al.*, 2013).

### *2.5 Drying of fresh leaves*

After cleaning, a small amount (25 g) of fresh *C. asiatica* leaves were dried in a hot air oven (D-056, Binder, Danmark) at 60°C for 8 hrs. The parched leaves were then kept in a sealed container (Vongsak *et al.*, 2013).

### *2.6 Boiling of dried leaves*

The dried leaves found from the oven drying were powdered using a Blender (MJ-M176P, Panasonic, Malaysia). The powdered leaves were then boiled with distilled water at 100°C for 30 mins and then strained with filter paper (Whatman No. 1; Sigma-Aldrich, Germany). The coir was extracted over and again until complete depletion (sample A6).

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*2.7 Extraction of dried leaves using alcoholic solvents*

The blended dried leaves from the blender were soaked in 70% extraction solvent of ethanol (A7), methanol (A8), and acetone (A9), respectively. The ratio of solute: solvent was maintained at 1:10 (w/v). Then the mixture was centrifuged with Benchtop Centrifuge (416G, Gyrozen, Korea) at 3000 rpm for 15 mins. The extracted portion was then collected from the tube after centrifuge (Vongsak *et al.*, 2013).

*2.8 Estimation of total flavonoids content*

The modified aluminium chloride colorimetric assay of Pothitirat *et al.* (2009) was used for the evaluation of total flavonoids. In a 10 mL volumetric flask, approximately 1.5 mL of the extract was placed. The suspension was then added with 2% $AlCl_3$ solution, added 6 mL of water and vortexed. After 30 mins, the absorbances of the supernatant of the solutions were measured. A T60-U Spectrophotometer was used at 415 nm against a blank. The equation $y = 0.003x + 0.025$; $R^2 = 0.999$ was used as standard curve to determine the total flavonoid compounds and expressed as milligram quercetin equivalent per gram (mg QE/g).

*2.9 Estimation of total tannin contents*

The Folin-Ciocalteu phenol methods described by Amorim *et al.* (2008) was used to evaluate the total tannins. An amount of 0.5 mL of sample was taken, and after thawing, 8 mL of double-distilled water was added to it. This was followed by the addition of 0.5 mL Folin-Ciocalteu reagent. After holding the solution for 5 mins, 1 mL of 35% sodium carbonate was mixed with the solution. Then it was appropriately mixed by a vortex machine for a few seconds and kept for another 20 mins for the development of colour. The absorbance was taken at 725 nm by a T60-U spectrophotometer. Instead of sampling Blank was formulated with water. Standard tannic acid solutions of different concentrations were read against a blank. The tannin findings have been presented in mg/g of dry extract in terms of tannic acid. The estimation of total tannin content was done by using the equation $y = 0.008x+0.002$; $R^2 = 0.999$. This equation was obtained from a standard tannic acid calibration curve.

*2.10 Determination of antioxidant activity*

*2.10.1 Total phenolic compounds*

The modified method of Hossain, Mitra, Belal *et al.* (2021); Hossain, Sifat, Hossain *et al*. (2021) was used to evaluate the total phenolic compound. Approximately 0.5 mL of the extracts were placed in a 10 mL volumetric flask followed by the addition of 0.5 mL Folin-Ciocalteu reagent and shaking (3 mins). Then, 1 mL of $Na_2CO_3$ was poured into the flask, and the flask was made up to the brim with double-distilled water. After 1h, the absorbances of the supernatant of the solutions were measured using a Jenway 6405 Spectrophotometer (Germany) estimated at 725 nm adverse to a reagent blank. The Gallic acid (GA) calibration curve was utilized as a standard. The result was expressed in mg GAE/ gm w/w using the equation $y = 0.0004x + 0.0013$; $R^2 = 0.999$.

*2.10.2 DPPH radical scavenging assay*

The slightly modified method of Brand-Williams *et al.* (1995) was used for the DPPH radical scavenging assay. Approximately 1 mL of the extract was added with 4 mL DPPH solution in a tube. The tubes were vortexed well and placed in a standing position for 30 mins in the dark environment. Hereafter, the absorbance reading of the mixtures was estimated at 517 nm by Jenway 6405 Spectrophotometer (Germany). A DPPH solution without adding the aliquot was considered as a control. The DPPH radical scavenging effect was estimated using the following calculation, where the results were expressed as a percentage

$$\text{DPPH radical scavenging effect (\%)} = (A_0\text{-}A_s) \times 100$$

*2.10.3 Ferric reducing antioxidant power assay*

The modified method of Oyaizu (1986) was used to measure the total antioxidant activity of *C. asiatica* extracts by means of ferric reducing antioxidant power (FRAP). From the extracts, 0.3 mL was added with 0.85 mL of 0.2 M phosphate buffer (pH 6.6) and 0.85 mL of potassium ferricyanide (1%), and vortexed well. After incubating the mixture for 20 mins at 50°C, 0.85 mL of trichloroacetic acid (10%) was mixed and vortexed. Finally, 2.85 mL of double-distilled water and 0.57 mL $FeCl_3$ (1%) were mixed in it, and the solution was then incubated at 25°C for half an hour. After the second incubation, absorbance was taken at 700 nm by operating a Jenway 6405 Spectrophotometer (Germany). A Blank was prepared in parallel, where distilled water was added instead of the aliquot. The standard ascorbic acid was developed by a serial aqueous dilution of stock solution. The standard curve was prepared by fitting the absorbance versus its corresponding standard ascorbic solutions. The outcomes were estimated in mg ascorbic acid correspondent antioxidant capability/100g w/w (mg AAE/100g w/w) using the equation $y = 0.175x + 0.005$; $R^2 = 0.999$.

*2.11 Statistical analysis*

The result was described as mean±standard deviation (SD) for triplicate measurements of each treatment. The data were analysed statistically by Minitab-19 statistical

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software. Mean and standard deviation were measured by the Tukey One Way ANOVA Test. The least significant differences were measured for the significant data at a 95% confidence level ($P < 0.05$).

## 3. Results and discussion

The total flavonoids, tannins, and antioxidant activities of *C. asiatica* were calculated after extraction and the absorbances by comparing them with the standard curves. After calculating the parameters in a similar volume of plant materials, all the amounts were compared. For dried leaves, the parameters were calculated based on the original weight of the fresh leaves.

### *3.1 Total flavonoid contents*

Flavonoids such as quercetin help in functioning as hydrogen donors (Cervato *et al.*, 2000). Sample A9 gave the highest output of 3.0533±0.1069 mg QE/g, while A1 showed the lowest output of 1.7500±0.0917 (mg QE/g) TFC from raw extracts of leaves. A5 contains a higher amount of flavonoid 2.9400±0.0656 (mg QE/g) among fresh leaves extracts (Table 2). This study revealed that a high amount of flavonoids were found in the 70% Acetone extract. Additionally, acetone is polar solvent and flavonoids obtained using acetone and ethanol extracts are also polar in nature. Do *et al.* (2014) found that the 75% methanol extract contained 22.51±0.97 (mg QCE/g), the 75% ethanol extract contained 19.47±0.35 (mg QCE/g) and the 75% acetone extract contained 29.34±0.64 (mg QCE/g) in *Limnophila aromatic* powder extract. The findings of the present study have similarities with their findings.

Table 2. Total flavonoid and tannin content of different samples.

| Samples | Total flavonoid content (mg QE/g) | Total tannin content (mg TAE/g) |
|---|---|---|
| A1 | 1.7500±0.0917$^{e}$ | 0.3833±0.0153$^{d}$ |
| A2 | 2.6033±0.0451$^{bc}$ | 0.4367±0.0306$^{d}$ |
| A3 | 2.6467±0.0513$^{bc}$ | 0.4533±0.0153$^{d}$ |
| A4 | 2.5467±0.0493$^{c}$ | 0.6766±0.0116$^{bc}$ |
| A5 | 2.9400±0.0656$^{a}$ | 0.7067±0.0351$^{ab}$ |
| A6 | 2.1300±0.1609$^{d}$ | 0.6100±0.0458$^{c}$ |
| A7 | 2.8233±0.1050$^{ab}$ | 0.7266±0.0153$^{ab}$ |
| A8 | 2.8600±0.1200$^{ab}$ | 0.7500±0.0400$^{ab}$ |
| A9 | 3.0533±0.1069$^{a}$ | 0.7800±0.0100$^{a}$ |

Values are presented as mean±SD. Values with different superscript within the same column are statistically significantly different ($P<0.05$) according to Tukey test.

### *3.2 Total tannin contents*

The powder extract using 70% acetone (A9) gave the highest (0.7800±0.0100 mg TAE/g) and Extracts obtained by squeezing (A1) gave the lowest amount of tannin content (0.3833±0.0153 mg TAE/g) in *C. asiatica*. Among fresh leaves extracts, 70% acetone extraction (A5) gave a comparatively higher tannin quantity (0.7067±0.0351 mg TAE/g). It might be due to the polarity of that extract. This study found that ethanol is not as effective as other solvents for the extraction of tannin from *C. asiatica* (Table 2). The tannin content in *C. asiatica* was higher than in the Moringa leaves extract (Hossain *et al*., 2020). Mailoa *et al.* (2013) found that 50% ethanol extract produced 1.728 (mg/g) and 50% acetone extract produced 1.774 (mg/g) for *Psidium guajava* leaves. This study's finding has similarities with their findings.

### *3.3 Antioxidant activity of Centella asiatica*

#### *3.3.1 Total phenolic compound*

Polyphenols are available as secondary metabolites in plants and are a critical index for antioxidant activity determination (Khanavi *et al.*, 2009). Polyphenols act as reducing factors, metal chelators, and single-oxygen relievers (Javanmardi *et al.*, 2003). This redox property of polyphenols is a good indicator of being a potent antioxidant. The effect of solvents on total flavonoid content is close to that of total phenolic content. Sample A9 gave the maximum output of 13.883±0.050 mg GAE/g, while A1 gave the lowest raw extract output. Among four extraction samples of fresh leaves, A5 contained the highest amount of 13.043±0.216 mg GAE/g of phenolic content. It was found that ethanol is not as useful as other solvents for the extraction of phenolics from *C. asiatica* (Table 3). It can be attributed to the presence of water extract of more non-phenol compounds, along with carbohydrate and terpene than in other extracts. The potential complex formation can also trigger this in extracting different phenolic compounds that are soluble in alcohols. The phenolics that are alcohol soluble can have higher molecular weights or more classes of phenols than the phenolic compounds extracted by water. The results of TPC indicated that better results were obtained when the extracting solvents were acetone and ethanol.

Do *et al.* (2014) found that the 75% ethanol extract contained a total phenolic content of 30.60±1.36 mg GAE/g 75% acetone extract contained 39.10±0.87 mg GAE/g and 75% methanol extract contain 35.70±1.95 mg GAE/g in *L. aromatic* powder extract. Our study findings have similarities with their finding.

#### *3.3.2 DPPH radical scavenging assay*

At 517 nm of the absorption band, DPPH (2, 2-diphenyl-1-picrylhydrazyl) acts as a steady natural free radical. While receiving an electron, it loses this

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Table 3. Antioxidant activities of different samples

| Samples | Total phenolic content (mg GAE/g) | DPPH (%) | FRAP (mg AAE/100g) |
|---|---|---|---|
| A1 | 7.926±0.110$^{e}$ | 44.170±0.437$^{e}$ | 51.467±0.763$^{f}$ |
| A2 | 9.956±0.140$^{d}$ | 49.730±0.334$^{d}$ | 63.963±0.355$^{h}$ |
| A3 | 10.587±0.307$^{c}$ | 61.630±0.338$^{c}$ | 74.657±0.617$^{e}$ |
| A4 | 10.970±0.111$^{c}$ | 63.073±0.679$^{bc}$ | 81.077±0.287$^{c}$ |
| A5 | 13.043±0.216$^{b}$ | 64.590±0.389$^{b}$ | 88.097±1.060$^{g}$ |
| A6 | 8.350±0.120$^{e}$ | 47.620±0.380$^{d}$ | 55.593±0.667$^{d}$ |
| A7 | 12.750±0.231$^{b}$ | 69.800±0.656$^{a}$ | 110.180±1.830$^{b}$ |
| A8 | 13.767±0.226$^{a}$ | 69.863±0.492$^{a}$ | 124.453±1.407$^{f}$ |
| A9 | 13.883±0.050$^{a}$ | 70.630±2.310$^{a}$ | 166.670±2.260$^{a}$ |

Values are presented as mean±SD. Values with different superscript within the same column are statistically significantly different (P<0.05) according to Tukey test.

absorption, which causes a visually prominent colour change from purple to yellow. DPPH can retain several specimens for a short time and is sufficiently capable of detecting active compounds at low concentrations (Kumoro *et al.*, 2009). A8 gave the highest 70.630±2.310 %, and A1 gave the lowest output of raw extracts 44.170±0.437% in *C. asiatica* leaves. Among four leaf extracts, the A5 sample showed a high amount 64.590±0.389% of DPPH (Table 3). This study found that methanol and acetone are productive extraction solvents of DPPH from *C. asiatica*. The linear regression plots of TPC, DPPH and FRAP were created to rationalize the antioxidant activity of *C. asiatica* juice extracts by means of their phenolic component, and the Pearson correlation coefficient was estimated. A strong positive correlation (0.947) between DPPH and TPC was found (Figure 1). This can be summarized as that the highest DPPH activity was found in the extracts that have the highest phenolic compound and *vice versa*.

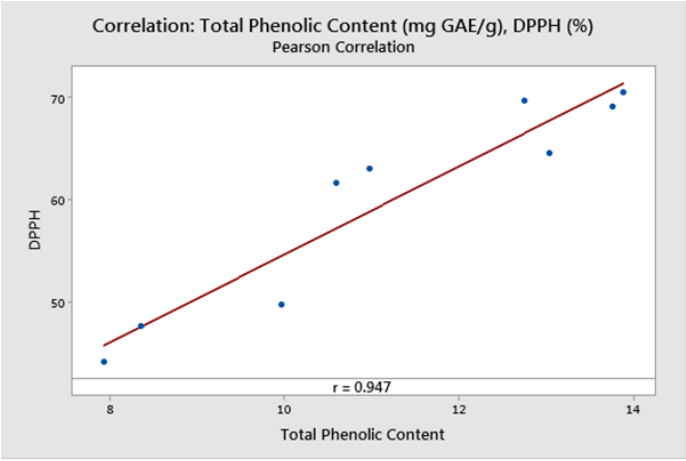


Figure 1. Correlation between DPPH and TPC.

Do *et al.* (2014) studied DPPH radical scavenging activity in *L. aromatic* powder extract and found that 75% methanol contained 80.98±2.5 $IC_{50}$ µg/mL, 75% ethanol contained 106.18±1.2 $IC_{50}$ µg/mL and 75% acetone contain 79.98±1.6 $IC_{50}$ µg/mL. Our study finding has similarities with their finding.

*3.3.3 Ferric reducing antioxidant power*

Sample A9 showed the highest output of raw extracts (166.670±2.260mg AAE/100g), and A1 had the lowest production (51.467±0.763 mg AAE/100g) during FRAP analysis. Among four fresh leaf extracts, A5 gave the highest quantity 88.097±1.060 (mg AAE/100g) (Table 3). The extract's reduction power was measured using a modified ferric to ferrous ion reduction analysis, in which the changing of yellow to green and blue colour was used as the indicator of the sample's reduction capacity. The antioxidant properties in the sample cause the ferric ferricyanide complex to be reduced to the ferrous form, which is investigated by evaluating Perl's Prussian blue formation (Yang *et al.*, 2010).

In Table 3, all the extracts display certain degrees of concentration-dependent electron-donating efficiency. The Powder extract using 70% acetone produced the highest reduction power, which is significantly higher at $p < 0.05$ than all other sample extracts. This trend was followed by that of the powder extract using 70% methanol, 70% ethanol, and the fresh leaves extracts derived from different aqueous acetone, methanol, and ethanol. The water extract showed the lowest reducing activity.

There was a strong positive correlation between FRAP and TPC, which was found to be 0.879 (Figure 2). There was also a strong positive correlation between DPPH and FRAP value, which was 0.843 (Figure 3). These correlations revealed that phenolic content in the *C. asiatica* plant extracts correlates significantly with their DPPH and anti-radical activity. The study found that methanol and acetone are suitable extraction solvents of FRAP from *C. asiatica*. These results comply with the findings of Rafat *et al.* (2010).

Several parameters were examined in this study, such as total flavonoid, tannin content, and antioxidant activities in terms of total phenolic content, DPPH, and FRAP. Overall, powder extracts showed better results

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than other extracts. Among the solvents, acetone extraction produced the maximum level of antioxidants.

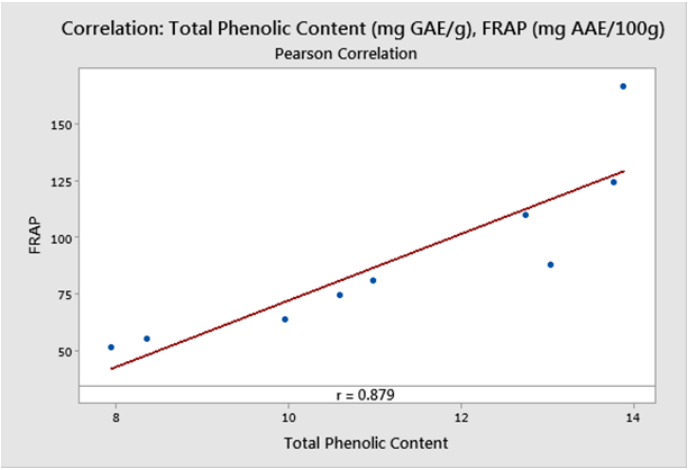


Figure 2. Correlation between TPC content and FRAP.

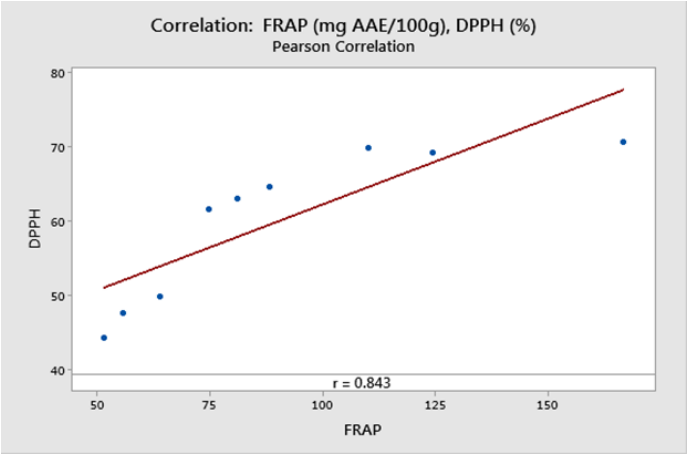


Figure 3. Correlation between DPPH and FRAP.

## 4. Conclusion

The antioxidant potentials of *C. asiatica* were estimated using total phenolic compound, DPPH radical scavenging assay, and FRAP (Ferric reducing antioxidant power). Powder extract using 70% methanol and 70% acetone showed the highest antioxidant activities, flavonoid and tannin content. Methanol and acetone were found to be productive extraction solvents for *C. asiatica,* which contained a high amount of antioxidant activity, phenolic, and flavonoid content. This would be an excellent medicinal plant in the future for its high antioxidant activity. Further study could be done to optimize the solvent concentration for maximizing the antioxidant activity of *C. asiatica*.

## Conflict of Interests

The authors declare no conflict of interest.

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