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Variations in Phytochemicals and Antioxidant Activity of *Clitoria ternatea* Flowers and Leaves

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ABSTRACT

Clitoria ternatea L. (CT) is widely used as an herbal medicine and a food colorant in the Southeast Asian region. This study was performed to determine chemical constituents and antioxidant activity of aerial parts of the plant. The acetone extracts of fresh flowers and leaves of CT were analyzed for chlorophylls and carotenoids while their methanol extracts were used for qualitative analyses of phytochemicals, quantification of phenolics and evaluation of DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) free radical scavenging activities. The results showed the flowers were richer in carotenoids whereas the leaves abounded with chlorophylls. The phytochemical analyses showed the presence of alkaloids, saponins and tannins in both the extracts of flowers and leaves. Total flavonoid content of the flower extracts (34.88 ± 4.18 mg QE/g) was significantly higher ($p < 0.05$) while their total phenolic content (13.35 ± 1.34 mg GAE/g) was considerably lower compared to the leaf extracts (24.42 ± 3.15 mg QE/g and 54.77 ± 8.80 mg GAE/g). The findings indicated ferulic acid, p-coumaric acid and rutin were the most abundant compounds among the ten phenolics quantified by liquid chromatography. In addition, these compounds were shown to have higher concentrations in the flowers (6417.04, 2647.31 and 3917.08 μg/g, respectively). Finally, the free radical scavenging capacities differed significantly between the extracts of CT flowers and leaves. In conclusion, the aerial parts of CT are a rich source of phytochemical constituents, had strong antioxidant activity, and could be used for development of food and cosmeceutical ingredients.

Keywords: *Clitoria ternatea*, Methanol extract, Phytochemicals, Flavonoids, Antioxidant.

Introduction

Clitoria ternatea L. (CT), commonly known as butterfly pea or Asian pigeonwing, is a garden plant grown in most Southeast Asian countries. It is widely used as an herbal medicine in India for treatment of different ailments, such as body aches and infections. Seeds and leaves of the plant are often used as tonics for the brain and are believed to boost memory.[1] In Cuba, an infusion of the flowers has been traditionally used to treat menstrual problems.[2] Research into CT has shown that extracts of its plant parts possess a variety of pharmacological activities. For example, alcoholic extracts of aerial parts and roots of CT at 300 and 500 mg/kg doses reduced electroshock-induced amnesia in rats.[3] The leaves and flowers may also exert antihyperglycaemic and antihyperlipidaemic effects. The oral administration of aqueous extracts of these plant parts in alloxan-induced diabetic rats (400 mg/kg body weight) for 12 weeks considerably reduced serum glucose, total cholesterol and triglycerides.[4] Methanol extracts of CT aerial parts were reported to have anticonvulsant activity, suggesting that this plant may be useful in treatment of seizure.[5]

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These pharmacological properties are in part ascribable to the presence of phytochemicals, especially phenolics, in CT.

Phenolics are compounds whose molecules consist of hydroxyl groups attached to a benzene ring. Two subgroups of phenolics that are commonly known include phenolic acids and flavonoids. Research revealed that flavonoids may exert antioxidant activity, lessening negative effects of free radicals.[6] The free radicals are known to be able to increase oxidative stress which neurodegenerative diseases are linked to.[7] Because of this, it is believed that flavonoids could be explored as antioxidant strategies to fight against neuronal damage.[8] Prior research has indicated that CT flowers contain a diverse mixture of anthocyanidins, a subgroup of flavonoids, derived from cyanidin and delphinidin.[9,10] For example, cyanidin-3-(p-coumaroyl-glucoside), cyanidin 3-(6"-p-coumaroyl)-rutinoside and ternatins were identified as major anthocyanidins in CT flowers. In addition to those compounds, β-carotene was documented for different varieties of CT grown in India.[11] This compound class reportedly contributes to lowered risks of certain diseases, such as cardiovascular diseases, diabetes and cancers.[12]

In Vietnam, CT is usually planted in fences or trellises as an ornamental tree thanks to its beautiful blue flowers and ethnomedicinal values. The flowers are often used to prepare a caffein-free herbal tea or make a natural food colorant. A few studies have been performed to explore anthocyanidins of CT.[9, 13] Other than these, scarce information about phytochemicals and bioactivities of importance to human health is available in the literature. This study aimed to explore phenolics, carotenoids and free radical scavenging activities of CT aerial parts and compare the variations in levels of these constituents between flowers and leaves of the species.

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Materials and Methods

Chemicals

Analytical standards (chemical purity: ≥ 98%) include caffeic acid, chlorogenic acid, cinnamic acid, ferulic acid, cinnamic acid, p-coumaric acid, gallic acid, salicylic acid (Sigma–Aldrich, St. Louis, Missouri, USA), quercetin and rutin (Chengdu Biopurify Phytochemicals, Sichuan, China). HPLC-grade methanol and acetone were purchased from Fisher Scientific (Pittsburg, PA, USA). The two reagents used for antioxidant assays (DPPH and ABTS) were purchased from Sisco Research Laboratories (Maharashtra, India) and Sigma-Aldrich, respectively. The chemical used for total phenol content determination (Folin-Ciocalteu's reagent) was obtained from Merck KGaA (Darmstadt, Germany).

Sample collection

Blue flowers and leaves of CT were harvested in gardens in Ho Chi Minh city (latitude 10°49′49″N, longitude 106°49′3″E) in November 2021, Vietnam and a voucher specimen (47/CV-STHMN) was collected, and its identity was confirmed at the Southern Institute of Ecology, Ho Chi Minh city. The collected samples underwent careful washing and air-drying steps. All the washed and dried samples (moisture < 10%) were ground into coarse particles and kept in a freezer (-18 °C) for further use. The study was conducted in November and December 2021.

Total carotenoid and chlorophyll contents

The total contents of carotenoids (TCC) and chlorophylls were estimated using the method introduced by Lichtenthaler and Wellburn (1983).[14] The dried flowers or leaves (1 g) with 10 mL of acetone was placed in a tube. The mixture was vigorously shaken for 24 hours at ambient temperature in a shaker and then centrifuged at 5500 rpm for 10 min. The supernatant was collected and was spectrophotometrically measured at 470, 645 and 662 nm. Chlorophyll and total carotenoid contents (μg/g) were calculated using the following equation:

$$\text{Chlorophyll a} = (11.24 \times A_{662} - 2.04 \times A_{645}) \times V/W$$

$$\text{Chlorophyll b} = (20.13 \times A_{645} - 4.19 \times A_{662}) \times V/W$$

$$\text{Total carotenoid content} = [1000 \times A_{470} - (2.27 \times A + 81.4 \times B)]/227 \times V/W$$

where V and W are volume of the solvent (mL) and amount of the sample (g), respectively.

Preparation of crude extracts

An amount (10 g) of each dried CT aerial parts (flower or leaf) was ground and mixed with 100% methanol at a ratio of 1:10 (g/mL). After 24 hr of shaking on a horizontal shaker, the mixture was filtered through a Whatman filter paper (GE Healthcare, Illinois, USA), and the solvent in the filtrate was removed in a rotary evaporator. The crude extracts (874 mg of flower extract and 357 mg of leaf extract) were obtained and used for further analyses.

Phytochemical screening

The presence of alkaloids, anthraquinones, saponins and tannins in the crude extracts were examined using Wagner's, Bontrager's, frothing and ferric chloride tests.[15]

Total phenolic and flavonoid contents

The crude extracts of CT flower and leaf were analyzed for total phenolic and flavonoid contents using the methods of Rana et al. (2019).[16] In detail, a mixture containing 60 μL of the samples (10 mg/1.6 mL), 2.34 mL of distilled water and 150 μL of Folin – Ciocalteu solution (10%) was placed in a screw-capped tube and then well shaken for 8 mins. 450 μL Na_2CO_3 (0.3 g/mL) were added and then vortexed for 1 min and incubated for 30 mins. The mixture was spectrophotometrically measured at 750 nm. Gallic acid was used as a reference standard, with the concentration ranging between (10 – 400 mg/mL in methanol). The calculation of TPC was based on the calibration curve: $y = 0.0009x + 0.0599$ ($R^2 = 0.99$). To determine total flavonoid content, the extract (100 μL, 10 mg/1.6 mL) was mixed with distilled water (4.9 mL) and $NaNO_2$ (0.3 μL, 5%) and incubated for 5 mins. Into these, $AlCl_3$ (300 μL, 10%) was added and then left to stand still for 6 min incubation. NaOH (2 mL, 1 M) and water was added in to make a final volume of 10 mL. Finally, the mixture was spectrophotometrically measured at 510 nm after 15 min incubation. Quercetin was used as a reference standard, with the concentration ranging from 1 to 100 mg/mL. The calculation of TFC was based on the calibration curve ($y = 0.0003x + 0.0028$; $R^2 = 0.96$).

Determination of phenolics

Phenolics in the CT samples were determined on a Shimadzu LC-2030C high performance liquid chromatography system equipped with a diode-array detector (HPLC-DAD). The analysis was carried out on a C18 reverse-phase column (250 × 4.6 mm, 5.0 μm particle size), based on the method previously described by Vu et al. (2021).[17] The mobile phase comprised methanol (A) and 1% formic acid in water (B). The gradient profile of the mobile phase was: 25% A for 3 min, 25 – 40% A for 5 min, hold 40% A for 4 min, 40 – 60% A for 4 min, hold 60% A for 4 min, 60 – 80% A for 3 min, hold 80% A for 3 min, 80% – 85% A for 4 min and hold 80% for 2 min, then reduced to 25% A at the 35th min. The flow rate was 0.8 mL/min and the column temperature was set at 40°C. The detection was performed at a wavelength of 295 nm for phenolic acids and 340 nm for flavonoids. The phenolics were quantified using their calibration curves with concentration range from 1 to 30 μg/mL. The correlation coefficients (R^2) of the calibration were above 0.99.

Antioxidant activity

The capacity of the extracts to trap free radical (DPPH) was evaluated using the approach described by Xiao et al. (2014).[18] A DPPH solution (40 μg/mL) was prepared in methanol/water (4:1, v/v). The reaction mixture consisting of the test sample (0.5 mL) and the DPPH solution (0.75 mL) was shaken for 30 mins at room temperature in darkness. Finally, the change in absorbance was recorded at 517 nm using 80% methanol as a blank. Ascorbic acid was employed as a positive standard and IC_{50} representing DPPH activity was calculated using the relationship between the concentrations (μg/mL) against the percent inhibition.

In addition, ABTS assay was employed to determine antioxidant activity of the extracts based on the method previously described by Leung et al. (2009).[19] ABTS (7 mM) was mixed with potassium persulfate (2.45 mM) in phosphate-buffered saline at a ratio of 1:1 (v/v), and the mixture underwent a 12 – 16 hr incubation at ambient temperature in darkness. The test sample (100 μL) was mixed with the above ABTS solution (3 mL), and the change in absorbance was determined at 734 nm.

The DPPH or ABTS scavenging capacities was estimated as follows:

$$\%\ \text{scavenging activity} = \frac{A_0 - A}{A_0} \times 100\%$$

where, A_0 and A are the absorbance of the blank and samples. Ascorbic acid was used as a standard. The sample concentration required to neutralize 50% of DPPH or ABTS radicals is IC_{50} and is used to compare the capacities between the samples.

Statistical analysis

Data analysis was carried out using Minitab 2019 (State College, Pennsylvania, USA). Bar graphs were presented using Microsoft Excel 365. Data comparison was made via one-way ANOVA and statistically significant differences ($p < 0.05$) among means were assessed using Tukey's test.

Results and Discussion

Qualitative analyses of phytochemicals

In this study, phytochemicals, including alkaloids, anthraquinones, saponins, terpenoids and tannins in the methanol extracts of CT flower and leaf were screened. The results showed the presence of alkaloids, saponins, terpenoids and tannins in CT extracts except for anthraquinones (Table 1). Of these groups, the CT leaf extracts tested negative for terpenoids while both the aerial parts of CT showed positive for the other classes of compounds. Previously, *Clitoria*

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ternatea has been reported to contain bioactive phytochemicals, such as kaempferol, taraxerol, β-sitosterol and clitorienolactones.[20-22] In addition, alkaloids and tannins have been detected in roots of CT derived from India.[23, 24] Research also showed geographical variations in phytochemical levels of CT. For example, Ali et al. (2013) revealed that kaempferol was detected at different concentrations among the CT samples harvested from different regions in India.[20]

Total carotenoid and chlorophyll contents

In this study, the analytical results showed that TCC and levels of chlorophylls differed considerably between the two examined CT aerial parts (Table 2). Furthermore, the TCC of the flowers (352.98 ± 8.00 μg/g) was found to be twice as much as that of the leaves (150.70 ± 1.00 μg/g). In contrast, chlorophyll levels of the leaves were significantly higher than those of the flowers and this can be quite understandable as leaves are the plant part containing most of these pigments. Previous research reported chlorophylls and β-carotene obtained from different varieties of CT.[11, 25] Carotenoids are effective antioxidants with the capacity to trap free radicals, prevent against carcinogens as well as slow down the aging process of the body. Scarce information about these pigments and their variations in concentrations between flowers and leaves of CT is available in the literature. The results of this study will give a better understanding of chlorophylls and carotenoids besides the common pigments (i.e., anthocyanidins) of CT.

Total phenolic and flavonoid contents

Table 2 presented total phenolic and flavonoid contents in the methanol extracts of the CT flowers and leaves. The results showed that the flower extracts contained 13.35 ± 1.34 mg GAE/g. Prior research showed that average TPC of methanol CT flower extracts obtained after 24 hour extraction was 13.7 mg GAE/g.[26] Perhaps, similar results that were observed for the two studies could be in part due to a similar extraction method used. The leaf extracts appeared to be richer in phenolics, with their average TPC (54.77 mg GAE/g) four times those of the flower extracts. The results were comparable with those reported by a recent study which revealed methanol extracts of CT leaves had 64.8 mg GAE/g.[27] Similarly, a study on CT collected in Sri Lanka revealed leaves of CT plants with different phenotypes had significantly higher TPC compared to flowers.[28] Flowers of CT is commonly known as a rich source of anthocyanidins, a subgroup of flavonoids. In this study, it is understandable that the average TFC determined in the CT flowers was 42% higher than that of the CT leaves (Table 2). Reportedly, TFC of CT flowers and leaves generally varied remarkably and this variation could be attributed to multiple factors, such as geography, morphotypes and extraction and analytical methods.[28-30] In general, significant differences in TPC and TFC between these two plant parts were noted. It is also noted that the extracts of the CT aerial parts with higher TFC do not always have high TPC (Table 2). Such an observation was previously reported in studies on plants by Kaisoon et al. (2011) and Stanković (2011).[31, 32]

Phenolics

Unlike the measurement of TPC and TFC above, the quantification of phenolic constituents facilitates a detailed look at individual phenolics. In this study, nine compounds were monitored, and the results indicated that they were all detected in the examined samples (Table 3). A representative HPLC-DAD chromatogram of the CT flower extract was displayed in Figure 1. Of the phenolic acids, gallic acid, p-coumaric acid, cinnamic acid, caffeic acid and ferulic acid were found to be more abundant in flower. In particular, the concentrations of gallic acid, p-coumaric acid and cinnamic acid measured in the flower extract were approximately 30 – 155 times as high as those in the leaf extract. Conversely, chlorogenic acid and salicylic acid were detected at higher levels in the leaf (1182.60 and 962.65 μg/g, respectively). Prior research showed that gallic acid and chlorogenic acid were present in lyophilized extracts of CT flower at average levels of 670 and 540 μg/g, respectively whereas the other phenolic acids were not detected.[29] In this study, two flavonoids found in the CT flower and leaf extracts include quercetin and rutin. No significant difference in concentrations of quercetin between the two plant parts was noted (12.83 vs. 16.14 μg/g) and it also indicated that this compound was present at very low levels in CT. In contrast, the results revealed that rutin was mainly present in flower at a level of 3917.08 μg/g. These concentration values were much higher than those previously reported for CT flower in the literature[29] In general, the results indicated that among the monitored phenolics ferulic acid was the most abundant compound both in flower and leaf.

Phenolics constitute one of the most important groups of phytochemicals in plants. These compounds have attracted much interest in scientific community as well as food and cosmeceutical industries. Among the major phenolics identified in the present study, ferulic acid and p-coumaric acid have been shown to possess multiple bioactivities of importance to human health. Evidence indicated that ferulic acid may be useful for the prevention and treatment of age-related diseases, such as neurodegenerative disorders, diabetes and cardiovascular diseases.[33]

Table 1: Phytochemicals in the methanol extracts of *Clitoria ternatea*

Phytochemicals	Test methods	Indicator	
		Fresh flower	Fresh leaf
Alkaloids	Wagner's test	+	+
Anthraquinones	Borntrager's test	–	–
Saponins	Frothing test	+	+
Terpenoids	Salkowski's test	+	–
Tannins	Ferric chloride test	+	+

+: present; –: absent

Table 2: Chlorophyll, total carotenoid, phenolic and flavonoid contents*

	Fresh flower	Fresh leaf
Chlorophyll a (μg/g FW)	120.79 ± 1.58 [b]	805.84 ± 5.90 [a]
Chlorophyll b (μg/g FW)	2.94 ± 0.26 [b]	228.03 ± 2.80 [a]
TCC (μg/g FW)	352.98 ± 8.00 [a]	150.70 ± 1.00 [b]
TPC (mg GAE/g extract)	13.35 ± 1.34 [b]	54.77 ± 8.80 [a]
TFC (mg QE/g extract)	34.88 ± 4.18 [a]	24.42 ± 3.15 [b]

* Data are expressed as mean ± standard deviation of 3 independent replicates. Means with different lowercase letter were significantly different at $p < 0.05$ within the row.

Table 3: Phenolic composition (μg/g of extract) of *Clitoria ternatea* flower and leaf

Phenolics	Fresh flower	Fresh leaf
Gallic acid	2320.10	73.35
Chlorogenic acid	813.58	1182.60
Caffeic acid	502.10	229.84
p-Coumaric acid	2647.31	17.77
Ferulic acid	6417.04	1906.21
Salicylic acid	289.62	962.65
Cinnamic acid	693.29	24.18
Rutin	3917.08	16.31
Quercetin	16.14	12.83

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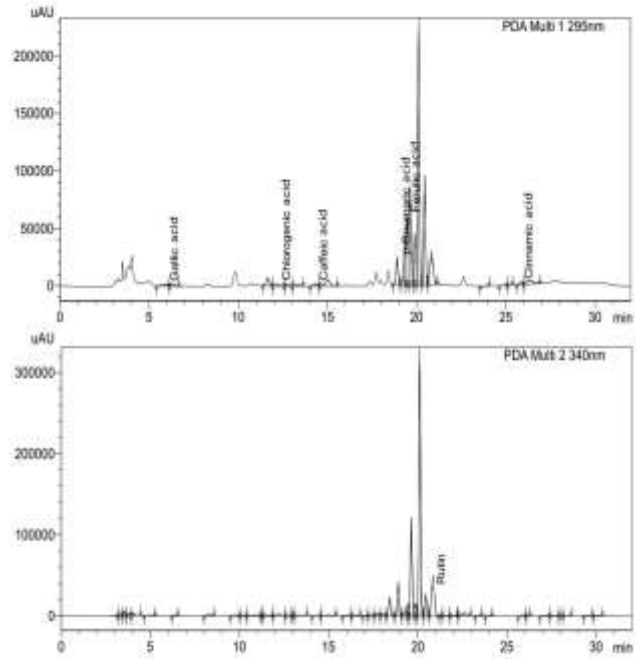

Figure 1: Representative chromatograms of the CT flower extract monitored at 295 nm (the upper) and 340 nm (the lower).

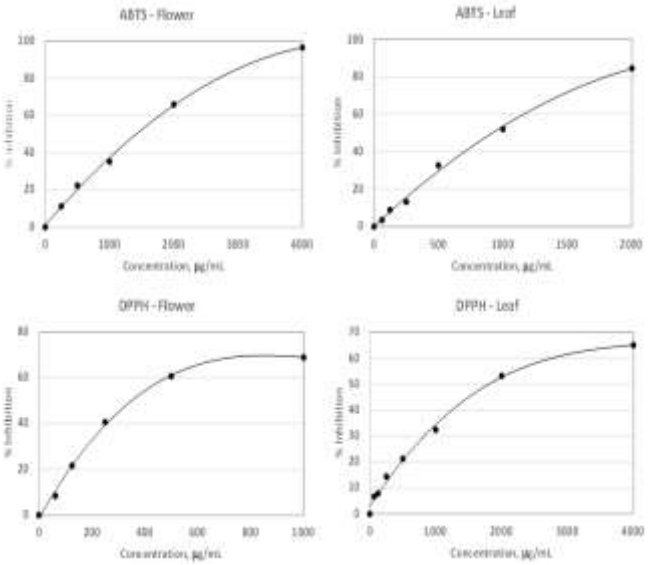

Figure 2: ABTS and DPPH free radical scavenging activities of the CT flower and leaf extracts.

Research suggested that p-coumaric acid can be recommended as promising adjuvant agents against brain neurodegeneration in diabetics.[34] As natural constituents of CT, phenolics helps underscore the nutritionally and medicinally important values of CT as well as CT-containing products.

Antioxidant activity

In this study, free radical scavenging capacity of methanol extracts of CT flower and leaf were assessed using ABTS and DPPH assays. As depicted in Figure 2, the ABTS IC_{50} values of the flower and leaf were 1770.6 and 1084.9 µg/mL, respectively. The results showed that the leaf extracts expressed higher antioxidant activity measured by the ABTS assay. As described above, the leaf extracts had higher TPC than the flower extracts, hinting at a possible positive correlation between phenolics and ABTS scavenging activity of CT. In contrast, the DPPH assay revealed CT flower extracts displayed a much stronger antioxidant activity compared to CT leaf, with IC_{50} values of 384.9 and 1787.4 µg/mL, respectively. The IC_{50} values for ascorbic acid employed as a positive control in both assays were 35.69 and 5.22 µg/mL, respectively.

Similar findings in which methanol and aqueous extracts of CT flowers possessed higher antioxidant activity determined by the DPPH assay than those of CT leaves were previously reported by Rabeta and An Nabil (2013).[27] Furthermore, our study showed that the free radical scavenging activities of CT leaf extracts were comparable with those discovered in prior research.[35] Nevertheless, few investigations into CT reported that flowers of the plant appeared to have lower antioxidant activity. For example, a study by Buddika et al. (2021) showed blue colored flowers of CT expressed lower DPPH scavenging potential compared to leaves.[28] It could be due to differences in growing condition and geography resulting in the variations among the studies.

Phenolics have been identified as making significant contributions to antioxidant activity of plants.[36] As discussed above, there were remarkable differences in levels of these constituents between the flowers and leaves. This may suggest the variations in antioxidant activity of CT in the present study could be affected by individual phenolic acids and flavonoids present in the CT studied aerial parts.

Conclusion

This study is the first work to explore the variations in phenolic acids, flavonoids, carotenoids and chlorophylls as well as free radical scavenging capacities between CT flowers and leaves. Multiple classes of phytochemicals were found to be present in these aerial parts. Flowers of CT had much higher carotenoid content whereas leaves of CT contained much more chlorophylls. The findings also showed flowers of CT were richer in flavonoids while its leaves had a higher TPC. Free radical scavenging potentials determined by DPPH and ABTS assays varied significantly between CT flowers and leaves. Finally, p-coumaric acid, ferulic acid and rutin were the most abundant phenolics identified and quantified in the CT samples. Further research should be focused on assessment of how phenolic profiles from CT samples of different genotypes, maturity stages, and storage conditions have impacts upon antioxidant activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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