EVALUATION OF THE ANTIOXIDANT ACTIVITY OF TWO ENDEMIC GOLDEN CAMELLIA SPECIES (*CAMELLIA TAMDAOENSIS NINH ET HAKODA* AND *CAMELLIA TIENII NINH*) IN VIETNAM

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ABSTRACT

Vietnam is one of the countries that possesses the most numbers of *Camellia* species worldwide. However, the number of publications regarding this plant in comparison to their biodiversity is very limited up to the present time. Therefore, we aim to measure the total phenolic content of methanol extracts obtained from the leaves of two endemic Golden Camellia species including *Camellia tamdaoensis Ninh et Hakoda* (CTNH) and *Camellia tienii Ninh* (CTN) and then evaluate their antioxidant activity by using DPPH and ABTS methods. We found that the leaves extracts from CTNH and CTN species contain high amounts of phenolic compounds at 492.3 ± 129.1 and 644.7 ± 116.2 mg GAE/g, respectively. The antioxidant assays indicated that CTNH and CTN species had a great antioxidant activity with IC_{50} 9.00 ± 1.72 and 15.28 ± 3.62 µg/mL, respectively, for DPPH and IC_{50} were 6.75 ± 1.31 and 7.15 ± 0.89 µg/mL, respectively, for ABTS assays. These findings suggest that CTNH and CTN can be considered as a potential antioxidant source.

ĐÁNH GIÁ HOẠT TÍNH CHỐNG OXY HÓA CỦA HAI LOÀI TRÀ HOA VÀNG ĐẶC HỮU (*CAMELLIA TAMDAOENSIS NINH ET HAKODA* VÀ *CAMELLIA TIENII NINH*) TẠI VIỆT NAM

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Camellia tamdaoensis Ninh et Hakoda
Camellia tienii Ninh
DPPH

TÓM TẮT

Việt Nam là một trong những quốc gia sở hữu số lượng loài hoa trà nhiều nhất thế giới. Tuy nhiên, số lượng công bố về loài thực vật này so với đa dạng sinh học của chúng tính đến thời điểm hiện tại còn rất hạn chế. Do đó, nghiên cứu tiến hành đánh giá hàm lượng phenolic tổng của dịch chiết metanol thu được từ lá của hai loài Trà vàng đặc hữu của Việt Nam là *Camellia tamdaoensis Ninh et Hakoda* (CTNH) và *Camellia tienii Ninh* (CTN), sau đó tiến hành đánh giá hoạt tính chống oxy hóa bằng phương pháp DPPH và ABTS. Kết quả nghiên cứu cho thấy dịch chiết từ hai loài CTNH và CTN chứa lượng hợp chất phenolic cao lần lượt là 492,3 ± 129,1 và 644,7 ± 116,2 mg GAE/g. Các phân tích chống oxy hóa chỉ ra rằng hai loài CTNH và CTN có hoạt tính chống oxi hoá cao với IC_{50} lần lượt là 9,00 ± 1,72 và 15,28 ± 3,62 µg/mL cho thí nghiệm DPPH và IC_{50} tương ứng là 6,75 ± 1,31 và 7,15 ± 0,89 µg/mL, cho thí nghiệm ABTS. Những phát hiện này cho thấy CTNH và CTN có thể được coi là nguồn chống oxy hóa tiềm năng.

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1. Introduction

Literature has reported that the overproduction of reactive oxygen species (ROS) has a high implication in the pathogenesis of numerous chronic diseases including diabetes, cancer, neurodegenerative, and cardiovascular diseases [1], [2]. Several studies have demonstrated that the consumption of polyphenol-rich fruits and vegetables could remove ROS [3], [4]. Therefore, *Camellia* species containing a high amount of polyphenolic compounds might provide potential interventions for the prevention of these diseases. It has been widely published that the antioxidant activity of common tea is strongly associated with the amount of polyphenolic compounds in their extract [5], [6]. In recent years, several studies have revealed a similar association in Golden Camellia – a rare species belonging to the family Theaceae. In a study that set out to evaluate the antioxidant capacity of *Camellia nitidissima Chi* leaves extracts, Chun and colleagues have found that the high level of total phenolic content in the leave extract was well correlated with the antioxidant capacity [7]. Another study has also assessed the antioxidant capacity of *Camellia chrysantha* leaves extracts and found the remarkable antioxidant activity in their leaves extract [8]. Notably, Vietnam possesses the most numbers of Camellia species worldwide [9], [10]. However, the number of studies regarding the biological activities of this plant in comparison to their biodiversity is very limited. Recently, few studies have discussed the chemical components of some Golden Camellia species [11]. Besides, some reports about finding more species and determining the morphology and the distribution area [12]–[14], the investigation about their pharmacological properties has not progressed significantly. Therefore, a study regarding the biological activity of endemic *Camellia* species in Vietnam collected from different geographical regions is extremely important to provide fundamental data in finding a solution for the preservation and development of these valuable plants in the future. Therefore, we carried out this study to measure the total phenolic content of methanol extract obtained from the leaves of two endemic Golden Camellia species in Vietnam including *Camellia tamdaoensis Ninh et Hakoda* and *Camellia tienii Ninh* and then assess the antioxidant activity by using DPPH and ABTS methods.

2. Materials and methods

2.1. Materials

2.1.1. Plant material

In our study, leaves of two Golden Camellia species *Camellia tamdaoensis Ninh et Hakoda* (CTNH) and *Camellia tienii Ninh* (CTN) were collected in at least 10 individual plants in Tam Dao district, Vinh Phuc province. All samples were botanically identified by Dr. Nguyen The Cuong from the Institute of Ecology and Biological Resources. The specimen dossiers were stored in the Herbarium of this Institute for further investigation.

2.1.2. Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ascorbic acid, Folin-Ciocalteu reagent, gallic acid, trolox, quercetin, were purchased from TCI-SU (Tokyo Japan), potassium persulfate, sodium carbonate, DMSO (Dimethyl Sulfoxide) and all solvents were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China); the absorbance measurements were performed using a spectrophotometer (SpectraMax® iD5 Multi-Mode Microplate Readers, VWR, Germany).

2.2. Methods

2.2.1. Sample preparation

Golden Camellia tea leaves (Fig. 1) were cleaned to remove dust, sorted. The fresh leaves samples were placed in liquid nitrogen, while the dried leaves samples were dried in the thermostatic oven at 50°C for several days to obtain the unchanged weight. The leaves then were shaken with zirconium beads at a frequency of 25 times per second in 2 mins into a fine powder by MM400 homogenizer (Retsch, Germany). The Camellia leaves powder was kept in a falcon tightly closed, stored at 4°C and protected from humid air. Extracts from tea leaves were prepared by mixing 100 mg of leaf power and 1 mL methanol 80%. The extracts were sonicated for 20 minutes at 70°C before being filtered and evaporated by a speed-vac (Labconco, Fisher Scientific, Austria) until the constant weight. These extracts were stored at 4°C for further experiments.

(a)

(b)

Figure 1. *Leaf samples collected from CTNH (a) and CTN (b) species.*

2.2.2. Determination of total phenolic content

The total phenolic content (TPC) was evaluated using the Folin-Ciocalteu assay according to the protocol adapted from Singleton and Rossi (1965) with some modification [15]. Briefly, the extracts were dissolved and diluted by methanol in a range of concentrations from 0.5 to 4 mg/mL. Then 40 µL of each extract was mixed with 480 µL Folin-Ciocalteu agent. After thoroughly mixing, 480 µL of Na_2CO_3 6% was added, the mixtures then were kept at 40°C for 15 minutes. Parallelly, gallic acid (GA), which was used as the positive control, was prepared in the same manner as extract samples but with the concentrations of 0.125 - 2 mg/mL. After incubation time, the absorbance of each mixture was measured at 765 nm. The phenolic content was calculated from the calibration curve of gallic acid. All the tests were carried out with three replications and the TPC were presented as milligrams of gallic acid equivalents (GAE) per g of dried extract.

2.2.3. Determination antioxidant activity

a) DPPH radical scavenging assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was performed using the methods published by Blois (1958) and Braca et al., (2001) [16], [17]. The DPPH solution (0.1 mM) was prepared in methanol and mixed at a ratio of 1:19 with the plant extracts (the final concentrations from 1.0625 to 25 µg/mL). The reaction mixture was incubated for 20 mins at room temperature. In this assay, ascorbic acid dissolved in DMSO at different concentrations (0.625 to 10 µg/mL) was used as the positive control. After incubation time, the absorbance of the mixture of DPPH solution and extract samples or ascorbic acid was measured at 517 nm by a spectrophotometer to evaluate the DPPH radical scavenging capacity. All the samples were performed in triplicate and the entire process must be kept away from the light. The radical inhibition percentage was calculated as the following equation:

$$\% \text{ scavenging capacity (or \% inhibition)} = [(Ac-As)/Ac]\times 100 \quad (1)$$

where Ac is the absorbance value of the control (DMSO solution without the extract samples) and As is the absorbance value of the tested samples.

The DPPH scavenging activity (% inhibition) was then plotted against concentrations and from the graph IC_{50} value (µg/mL) was calculated as the concentration at which the % scavenging capacity was 50%.

b) ABTS radical scavenging assay

The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay was conducted following the procedure published by Re et al., (1999) [18] with some modifications. The ABTS solution was dissolved in distilled water to have the absorbance 0.7 ± 0.02 at 734 nm. After the addition of the ABTS solution to the prepared samples at a ratio of 1:19, the extract samples obtained the final concentration from 1.0625 to 25 µg/mL. Trolox was dissolved in ethanol and was used as the reference. The same adjustment was conducted to the Trolox to get a final concentration that gradually decreases from 0.625 to 10 µg/mL. The absorbance of the solution then was read after 10 mins of incubation at room temperature by a spectrophotometer at 734 nm to measure the ABTS radical scavenging activity. All the tests were performed at three different times and the whole process was done in the dark. The radical inhibition rate was calculated using the following formula:

$$\% \text{ scavenging capacity } (\% \text{ inhibition}) = [(Ac-As)/Ac]\times 100 \quad (2)$$

where Ac is the absorbance value of the control (without extracts) and As is the absorbance value of the tested samples.

The ABTS scavenging activity (% inhibition) was then plotted against concentration and from this dose-response curve the IC_{50} value (µg/mL) was determined as the concentration at which the % inhibition was 50%.

2.2.4. Data analysis

The results were presented as the means ± standard deviation. The IC_{50} values and the differences between means of each group were analyzed using *t*-test and one-way Analysis of Variance (ANOVA) by Graphpad Prism 9.2.0. The p value < 0.05 was considered as statistical significance.

3. Result and discussion

3.1. Total phenolic content of methanolic leaves extracts from Camellia tamdaoensis Ninh et Hakoda (CTNH) and Camellia tienii Ninh (CTN) species

Literature has shown that a considerable amount of the phenolic compounds could be found in many medicinal plants. The phenolic compounds, which are characterized by the presence of hydroxyl groups attached to aromatic rings, have shown a strong association with the antioxidant capacity of plant extracts [19]. Therefore, the measurement of the total phenolic content served as the primary data to assess the antioxidant capacity of our samples. Leaves of *Camellia tamdaoensis Ninh et Hakoda* (CTNH) and *Camellia tienii Ninh* (CTN) species were collected in Tam Dao district, Vinh Phuc province (Fig. 1). The total phenolic content of CTNH and CTN leaves extracts was estimated by using the Folin-Ciocalteu reagent (Fig. 2).

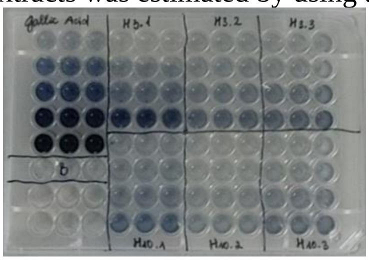


Figure 2. *A colorimetric reaction between the Folin reagent and samples extracts/GA standards*

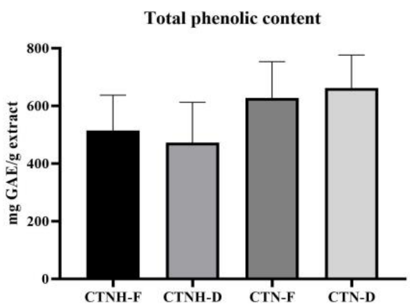


Figure 3. *Comparison of the phenolic content between fresh (F) and dried (D) leaves extracts obtained from CTNH and CTN species. The data values were the mean ± SD (n = 5,6)*

This method was used very extensively in order to evaluate the total amount of phenolic content from plant extracts due to its simplicity, accuracy, and sensitivity. The result of total phenolic content from our two Camellia leaves extracts was shown in Table 1. Based on the obtained result, both two species have been shown to contain high amounts of phenolic compounds. Data from Table 1 showed that the methanol extract obtained from CTN leaves containing 644.7 ± 116.2 mg GAE/g the phenolic content, which was significantly higher in comparison to the one of CTNH containing 492.3 ± 129.1 mg GAE/g. Some previous studies regarding Chinese golden Camellia species have revealed that the total phenolic content in the ethanol extract of *Camellia nitidissima* was determined as 281.04 ± 6.87 mg GAE/g [20] and the one of *Camellia nitidissima* Chi was 170.74 ± 1.99 mg GAE/g [21]. Our findings suggested that CTNH and CTN species can be considered as a rich source of phenolic compounds.

Table 1. *Total phenolic content in methanol extract of CNTN and CTN leaves*

Samples	TPC (mg GAE/g)[a]	P value
CTNH leaves	492.3 ± 129.1*	P = 0.0039
CTN leaves	644.7 ± 116.2*	
CTNH – F	514.6 ± 122.5	Ns
CTNH – D	472.7 ± 139.8	
CTN – F	627.8 ± 125.5	Ns
CTN – D	661.6 ± 115.2	

*[a]The total phenolic content (TPC) was expressed as mg of gallic acid equivalent (GAE)/g of methanol extract. Values were means of 5-10 replicates ± SD, * within the same column indicate significant difference at $p < 0.05$ by t test*

3.2. Phytochemical content between fresh and dry leaves of CTNH and CTN species

The samples from both species were divided into fresh and dried leaves in order to clarify the differences in the phytochemical content in each subgroup. However, there was no significant difference in the total phenolic content between the fresh and dried leaves extracts obtained from both species. Data were shown in Table 1 and Fig. 3. In contrast, in another study, the total phenolic content has been observed higher in dried leaf extracts than in fresh one of *Camellia sinensis* and *Camellia assamica* [22]. Notably, it has been reported that the fresh young leaves of green tea species contain a greater amount of phenolic compounds in comparison with the old mature leaves of the same species [23]. In contrast, the total flavonoid content seems to be higher for mature leaves [24]. These data suggest that the inconsistency found between our findings and literature might be due to the different number of young and mature leaves used to conduct the experiment. In addition, comparing between two species, the phenolic content in CTN extract seemed higher than the one in CTNH, but no remarkable difference has been observed between these two species.

3.3. Antioxidant capacity of CTNH and CTN leaves extracts

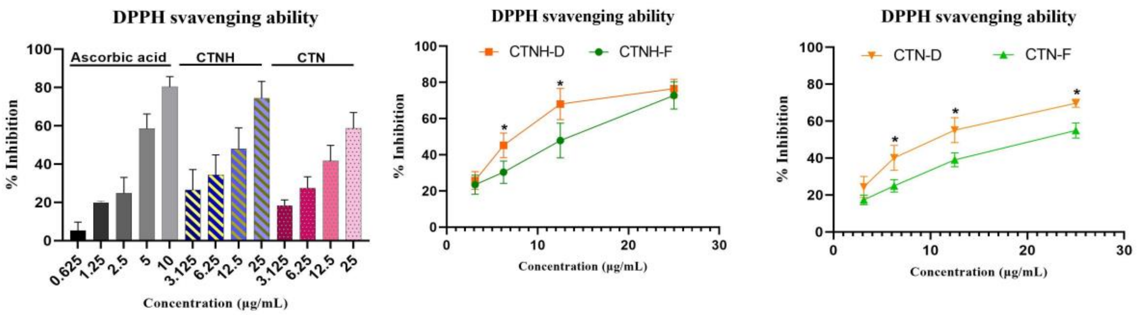
DPPH and ABTS assays have been intensively applied for the assessment of the antioxidant capacity of plant extracts due to their sensitivity, simplicity and lower interference. The presence of phenolic compounds in the plant extracts is strongly correlated with the DPPH/ABTS radical scavenging percentage inhibition. Our data also showed the great antioxidant capacity of the methanol extract of CTNH and CTN leaves in the scavenging reaction with DPPH (Fig 4. a) and ABTS (Fig 5. a) radicals, which were comparable to the positive control, ascorbic acid and trolox, respectively. According to the IC_{50} values shown in Table 2, the lower the IC_{50} value of sample is, the stronger its scavenging DPPH and ABTS radicals' ability are. We found that CTN leaves extract significantly showed a greater IC_{50} value in comparison to the one from CTNH species in DPPH radical scavenging activity, 15.28 ± 3.62 and 9.00 ± 1.72 µg/ml, respectively. However, there was no remarkable difference in ABTS radical scavenging activity between the

CTN and CNTH leaf extracts, 7.15 ± 0.89 and 6.75 ± 1.31 μg/ml, respectively. These results suggest that CTNH leaf extract had a greater capacity in scavenging DPPH radicals in comparison to CTN leaf extract. Recently, it has been reported that the IC_{50} value of the leaf extract obtained from *C. nitidissima* was 17.4 and 28.8 μg/mL in the DPPH and ABTS tests, respectively [20]. The approximate value has been also reported for other two golden Camellia species including *Camellia tunghinensis*, and *Camellia euphlebia* [25]. In comparison with other golden Camellia species found in these studies, our two endemic species had shown a greater DPPH and ABTS scavenging capacity.

Table 2. *IC_{50} of antioxidant activity of the CTNH and CTN leaves extract*

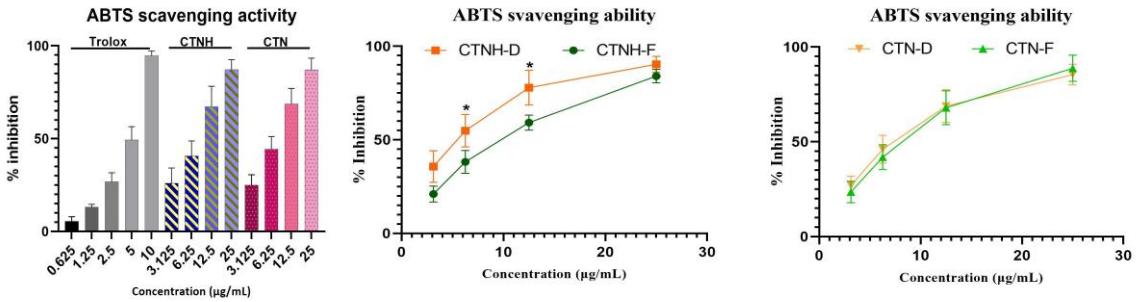
Samples	Scavenging activity ^{a}			
	DPPH (IC50 μg/ml)	P value	ABTS (IC50 μg/ml)	P value
CTNH	9.00 ± 1.72 *	P < 0.0001	6.75 ± 1.31	Ns
CTN	15.28 ± 3.62 *		7.15 ± 0.89	
CTNH – F	11.96 ± 2.1* #	P = 0.0013	8.77 ± 0.85 #	P < 0.0001
CTNH – D	7.4 ± 1.06 #		5.06 ± 0.85 #	
CTN – F	20.42 ± 2.47* #	P < 0.0001	7.39 ± 0.91	Ns
CTN – D	10.02 ± 0.6 #		6.85 ± 0.85	

*a: Data were expressed as the mean ± standard deviation (n=6-16); * significant difference between two species; # significant difference between the dried and fresh leaf extract*



(a) *(b)* *(c)*

Figure 4. *The DPPH scavenging activity (a) of methanol leaf extract obtained from CTNH and CTN and the comparison between fresh (F) and dried (D) leaves extract of CTNH (b) and CTN (c). Each value was presented as the mean ± SD (n = 10-12). Statistical difference between two groups was done by a One-way ANOVA test (*P<0.05).*



(a) *(b)* *(c)*

Figure 5. *The ABTS scavenging activity (a) of methanol leaf extract obtained from CTNH and CTN and the comparison between fresh (F) and dried (D) leaves extract of CTNH (b) and CTN (c). Each value was presented as the mean ± SD (n = 10-12). Statistical difference between two groups was done by a One-way ANOVA test (*P<0.05).*

Notably, the antioxidant activity of dried leaves extract has been reported significantly higher than the one of fresh leaves from *Camellia sinensis* and *Camellia assamica* [22]. Therefore, it is worth to compare the differences in the antioxidant activity between fresh and dried leaves from our two endemic Golden Camellia species. Similarly, we found that the methanolic extract obtained from dried leaves from both two species had a moderately higher potential in DPPH radical scavenging action in comparison to the one from fresh leaves as data shown in Fig 4b and 4c. Regarding ABTS radical scavenging activity, our data showed that the significant difference between dried and fresh leaves extract has only been observed in CTNH species (Fig. 5b). In contrast, both dried and fresh leaves extract obtained from CTN species were similar (Fig. 5c).

It has been published that the two phenolic hydroxyl groups in the ortho-position on the B-ring of flavonoid more strongly contributed to its antioxidant activity over other forms [26]. In our study, the difference observed in two antioxidant assays might be due to the participation of different antioxidants in the radical scavenging reactions. Therefore, further study needs to be conducted to set up a more sensitive method for the antioxidant assessment of our samples from different extraction procedures.

4. Conclusion

In summary, this study represents the first report regarding the evaluation of phenolic content and antioxidant capacity of methanol leaves extracted from *Camellia tamdaoensis Ninh et Hakoda* and *Camellia tienii Ninh* species, which are two endemic camellia species in Vietnam. The results showed that these two endemic Camellia species contained an abundant amount of phenolic compounds, which were attributed to their great antioxidant activity. Further studies need to be conducted to clarify active components which are responsible for the antioxidant activity.

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