Journal of Medicinal Materials, 2022, Vol. 27, No. 4 (pp. 235 - 239)

MINERAL CONTENT AND ANTIOXIDANT ACTIVITY IN LEAVES OF THREE ENDEMIC GOLDEN *CAMELLIA* SPECIES OF TAM DAO, VINH PHUC, VIETNAM

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(Received June 27th, 2022)

Summary

Mineral Content and Antioxidant Activity in Leaves of Three Endemic Golden *Camellia* Species of Tam Dao, Vinh Phuc, Vietnam

The aim of this study is to determine the mineral content and antioxidant properties of the leaf of 3 endemic Golden Camellias, *Camellia phanii* Hakoda & Ninh, *Camellia tamdaoensis* Hakoda & Ninh, and *Camellia tienii* Ninh collected in Tam Dao district, Vinh Phuc province. The element constituents were quantified by using both Inductively coupled plasma mass spectrometry (ICP-MS) and atomic absorption spectroscopy (AAS). The leaves used for analysis possess good mineral content for both macro and microelements such as Zn, Fe, Cu, Mn, Ca, Mg. The content of non-essential heavy metals has been lower than maximum limit values suggested that these materials could use safely as tea and tea products. On the other hand, the DPPH radical scavenging assay performed for leaves methanolic extracts of three species indicated the great potential antioxidant of the investigated extracts with IC_{50} values ranged from 7.280 to 7.985 µg/mL. Hence, these species can be used for not only cosmetics and therapeutics purposes but also as nutritional supplements.

Keywords: *Element content, Camellia phanii, Camellia tamdaoensis, Camellia tienii, ICP-MS, AAS.*

1. Introduction

Golden *Camellia* species, a group of yellow-flowering species, have recently been used as a food, cosmetic and traditional medicine in China and Vietnam. Forty-two species have natural distribution in Vietnam, of which thirty-two species have been found only there and were then considered endemic species of this country [1],[2]. The flowers and leaves of these Golden *Camellias* species can be used traditionally to treat high blood pressure, high cholesterol, obesity, sore throat, dysentery, diarrhea, faucitis, irregular menstruation, as well as liver protection and cancer prevention. The previous pharmacological studies reported antioxidant [3],[4], antibacterial [5], antidepressant [6] properties, inhibition of transplanted cancer, prevention of atherosclerosis and chemotherapeutic activities of Golden *Camellia* species [7],[8]. Due to the high value on human health, the commercial value of Golden Camellias is much higher than green tea (*Camellia sinensis*). As consequence, the cultivated areas of these Golden Camellias have been increasing dramatically from north to south, especially in Quang Ninh, Vinh Phuc and Lam Dong provinces.

However, the basic research on the chemical diversity of the endemic Golden Camellias Vietnam Collection has still been considered an underestimate in this country. Previous investigations reported that *C. chrysantha* and *C. nitissdima,* two common species found in China, can produce flavonoids, tannins, coumarins, vitamins and some minerals as good pharmacological components/nutrients for human [5],[8],[9]. Such kind of study has limited in Vietnam while our country has been one of the countries owning the highest number of Golden Camellias species in the world [10]. In this research, we investigated the mineral/heavy metals content and antioxidant ability of three endemic species of Tam Dao, Vinh Phuc province including *Camellia phanii*, *Camellia tamdaoensis* and *Camellia tienii*. Using ICP-MS, the nutritional values were evaluated through the macronutrients (Ca, Mg, Fe, Zn) micronutrients (Cu, Cr, Se, Mn, Mo, Co) level and the toxicity was assessed by toxic heavy metals content (Al, Ni, As, Ag, Cd, Sn, Sb, Ba, Pb). The preliminary antiradical activities of the methanolic extracts against DPPH were determined to evaluate the antioxidant capacity of these species.

2. Materials and methods

2.1. Materials

Plant collection

Three endemic Golden *Camellia* species used in this study were respectively *Camellia phanii* (PHA, specimen code PHA-06-2021/USTH), *Camellia tamdaoensis* (TAM, specimen code TAM-06-2021/USTH), and *Camellia tienii* (TIE,

specimen code TIE-06-2021/USTH). For each species, the leaves of three individual plants were initially collected in the garden of a local farmer in Hop Chau ward, Tam Dao district, Vinh Phuc province in June 2021 on an area of 100m^2. All samples were identified by Dr. Nguyen The Cuong from the Institute of Ecology and Biological Resources. The specimen dossiers were deposited in the Herbarium of this Institute to store and serve for further investigation.

Chemicals

Dimethyl sulfoxide (DMSO) was provided by Thermo Fisher Scientific, USA. DPPH (2,2-diphenyl-1-picrylhydrazyl), ascorbic acid was purchased from Sigma Aldrich, Singapore. Methanol was acquired by Thermo Fischer, USA. HNO_3, H_2SO_4, $HClO_4$ were provided by Merck, Germany.

2.2. Methods

Sample extraction

The mature leaves of three plant species were collected and washed gently with distilled water to remove dust. After drying at 50°C until constant weight, leave tissues were shaken with zirconium bead at frequency of 25 times per second in 2 min into a fine powder by MM400 homogenizer (Retsch, Germany). The moisture of leaf powders was measured by using Infrared Moisture Analyzer MA37 (Satorius, Germany). Afterward, 100 mg of each leave powder was macerated in 1 mL of 80% methanol, ultrasonic at 70°C in 15 min. The extraction process was repeated in triplicate. All the extracts were then collected into a container at the last filtration and evaporated in a speed-vac (Labconco, Fisher Scientific, Austria) until the weight of obtained extracts did not change. The products were dry extracts with the amount of 8.7 mg, 9.8 mg and 10.5 mg corresponding to *C. phanii*, *C. tamdaoensis, C. tienii,* respectively.

Elemental composition analysis

Sample preparation was performed by transferring 0.2000 g powder of each species into a microwave PPFE vessel. Approximately 2 mL of concentrated HNO_3 (65%) and 1 mL H_2O_2 (34%) were added to the vessel and subjected to sample digestion by microwave oven (Multi-wave PRO, Anton PAAR, Graz, Austria). Samples were then cooled to room temperature, filtered through 0.45 µL membrane, and transferred to 50 mL volumetric flasks and made up to volume with deionized water. These solutions were then injected into ICP-MS system using the standard solution for multi-elements diluted in 1 mol/L HNO_3 to get the final solution of 10 ng/mL.

The mineral content (Al, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Ag, Cd, Sn, Sb, Ba, Pb) in leaves samples was quantified by using PerkinElmer®'s NexION 2000 ICP-MS. The main optimized instrument conditions were set up with the radio frequency (RF) power of 1600 W; plasma gas flow of 15 L/min; auxiliary gas flow of 1.2 L/min; nebulizer gas flow of 1 L/min; monitored ion m/z $^{75}As^{16}O^+$(m/z 91); $^{111}Cd^+$; $^{59}Co^+$; $^{52}Cr^+$; $^{65}Cu^+$; $^{60}Ni^+$; $^{208}Pb^+$; $^{78}Se^{16}O^+$ (m/z 94); $^{66}Zn^+$ and $^{115}In^+$. To eliminate polyatomic ion interferences, the elements like Al, Cr, Mn, Co, Ni, Cu, Zn, Mo, Ag, Sn, Sb, Ba, Pb were measured in kinetic energy discrimination and dynamic reaction cell mode employing helium as collision gas. For As and Se measurement, the reaction gas used in dynamic reaction cell mode was oxygen. The standard mode was employed for Cd. The quantification was performed by a calibration curve prepared from a high purity ICP-multi element calibration standard (Perkin Elmer Life and Analytical Sciences). The data were treated and illustrated in mg/kg of dry leaves. Each sample was performed in triplicate and the average value with a relative standard deviation lower than 10% was representative of the sample in further analysis.

The elements Fe, Ca and Mg were quantified by flame atomic absorption spectrometry (AA900, Perkin Elmer, Ottawa, Canada) using acetylene and compressed air as fuel gas and oxidation gas, respectively. To do that, 0.1000 g powder was weighed and added 1 mL of deionized water, 2 mL mixture of concentrated HNO_3 and $HClO_4$ (1/1: v/v), and 5 mL of H_2SO_4. The reaction was heated for 30 min at 230°C, then cooled at room temperature. Subsequently, this solution was filtered through 0.45 µm membrane and transferred into 50 mL volumetric flask and made up to 50 mL by deionized water. The operating conditions for AAS instrument were set up as follows: C_2H_2 flow rate of 2.2 L/min; compressed air flow rate of 10 L/min. HCL current values were chosen as 25 mA, 12 mA and 20 mA for Fe, Mg and Ca, respectively. The slit width was set at 0.2 nm. Wavelengths were chosen as 248.3 nm, 285.2 nm and 422.7 nm corresponding to Fe, Mg and Ca, respectively. Quantification was performed by taking absorbance and using the calibration curve of each element.

Antioxidant activity

Sample extracts were also prepared by diluting in absolute DMSO to get the stocks of 500.000, 250.000, 125.000, 62.500 and 31.250 mg/mL. Subsequently, 5 µl of each solution was mixed with 195 µl DPPH (2,2-Diphenyl-1-picrylhydrazyl) 0.1 mM to obtain the final

concentrations of 25.000, 12.500, 6.250, 3.125, and 1.563 µg/mL. The same adjustment to the ascorbic acid standard was made to get a range from 10.000, 5.000, 2.500, 1.250, 0.625 µg/mL. The reactions were incubated in a 96-well plate in the dark at room temperature for 20 min and then measure the absorbance with a spectrometry machine (SpectraMax® iD5 Multi-Mode Microplate Readers, VWR, Germany) at 517 nm.

The radical inhibition rate was evaluated using the following formula: % scavenging capacity (%SC) = (Ac-As)/Ac×100 where Ac and As are the absorbance of the blank (5 µl DMSO + 195 µl DPPH 0.1 mM) and the extracted sample, respectively. The DPPH scavenging activity was exhibited as IC_{50} (µg/mL) which was defined as the concentration at which the % scavenging capacity of 50%. In this study, this value was determined by Graphpad prism 9.2.0 software.

Data analysis

All experiments were performed in triplicate. The results were taken from the average of independent experiments and illustrated as the means ± standard deviation. One-way Analysis of Variance (ANOVA) test was applied to determine statistically the significance of differences among the mean values (scavenging activity of IC_{50}) by using Graphpad Prism 9.2.0.

3. Results

3.1. Mineral composition

The mineral composition of samples from these Golden Camellias varied greatly according to the species in this study. Among three species, *C. phanii* contained the highest concentration of Pb, Zn, Mo, Mn, Sb, Ba, Hg, Ca and the lowest concentration of Co, Ni, Cr, Fe, Mg. Meanwhile, *C. tamdaoensis* showed a higher concentration of Cu, Co, Ni, Mg and lower concentration of Pb, Zn, Mn,As, Al, Ba, Hg and Ca in comparison to other species. *C. tienii* was found to have the highest concentration of Cr, As, Al, Fe and lowest Cu and Mo concentration. Cd, Ag, Sn and Se concentrations among all three plant samples are < 0.1 mg/kg.

In detail, we experienced that the concentration of Ca was high in three Golden Camellias species with values in the range of 0.260 to 2.160 g/100g dry leaves. The recommended dietary allowance (RDA) of Ca for adults is 2.000 - 2.500 g, which suggested that these plant materials can be useful as Ca supplement source.

All three species contain less than 0.1 mg/kg each in Se concentration while the RDA of this element is 55 µg. Meanwhile, the concentration of Zn was found in plant leaves with moderate levels, ranging from 6.820 to 12.490 mg/kg. The RDA of Zn is 8 - 11 mg which indicated that these leaves can be a good source for this element in the human regime. The species with the highest and lowest Zn concentration among the three Golden Camellias species was *C. phanii* and *C. tamdaoensis*, respectively. *C. tienii* contains 9.42 mg/kg of Zn in its ionic form. The Mo, a critical mineral aiding the breakdown of toxic substances entering the body as well as the process of proteins and genetic materials, concentration was found to be highest in *C. phanii* (0.13 mg/kg) and lowest in *C. tienii* (0.06 mg/kg). The RDA of this metal is 45 µg, suggesting that these leaves have been suitable for their use as tea products.

Table 1. The mineral elements content (in mg/kg or in %) of three endemic *Camellia* species collected in Tam Dao, Vinh Phuc

No	Element	Unit	*C. phanii*	*C. tamdaoensis*	*C. tienii*	Allowable limit
1	Cu	mg/kg	3.940 ± 0.030	6.500 ± 0.310	3.910 ± 0.020	
2	Pb	mg/kg	2.020 ± 0.001	0.570 ± 0.030	1.960 ± 0.001	2
3	Cd	mg/kg	< 0.100	< 0.100	< 0.100	1
4	Zn	mg/kg	12.490 ± 0.150	6.820 ± 0.030	9.420 ± 0.010	
5	Co	mg/kg	0.060 ± 0,001	0.130 ± 0.001	0.110 ± 0.002	
6	Mo	mg/kg	0.130 ± 0.010	0.110 ± 0.003	0.060 ± 0.001	
7	Ni	mg/kg	0.560 ± 0.030	2.140 ± 0.070	0.640 ± 0.003	
8	Sn	mg/kg	< 0.100	< 0.100	< 0.100	
9	Mn	mg/kg	701.310 ± 3.400	307.230 ± 2.080	491.860 ± 0.020	
10	Sb	mg/kg	0.340 ± 0.020	0.230 ± 0.001	0.230 ± 0.003	
11	Cr	mg/kg	1.800 ± 0.120	4.210 ± 0.230	4.320 ± 0.010	
12	As	mg/kg	0.280 ± 0.040	0.190 ± 0.002	0.390 ± 0.010	1
13	Al	%	1.340 ± 0.070	0.890 ± 0.060	1.890 ± 0.040	
14	Ba	mg/kg	196.840 ± 1.360	19.490 ± 0.230	176.800 ± 0.020	
15	Ag	mg/kg	< 0.100	< 0.100	< 0.100	
16	Se	mg/kg	< 0.100	< 0.100	< 0.100	
17	Fe	mg/kg	98.200 ± 0.130	98.620 ± 0.110	143.500 ± 0.120	
18	Mg	mg/kg	1572.040 ± 2.030	2616.850 ± 0.230	2168.820 ± 0.180	
19	Hg	mg/kg	0.062 ± 0.001	0.017 ± 0.003	0.031 ± 0.001	0.05
20	Ca	%	2.160 ± 0.240	0.260 ± 0.001	1.417± 0.002	

According to the National technical regulation on the limits of heavy metals contamination in food QCVN 8-2:2011/BYT, the maximum limit of As, Cd, Pb and Hg in tea and tea products are 1, 1, 2 and 0.05 mg/kg or mg/l, respectively. Regarding the concentration of these heavy metals in different Golden Camellias, these four elements existed with an average concentration which was lower than maximum limit values except the Hg content in *C. phanii*.

Minerals are required in relatively large amounts and are designated as macrominerals or macronutrients (Ca, P, Mg). Minerals needed in smaller amounts are called microminerals or micronutrients (Cu, Fe, Mn, Zn, Mo, Ni, Co, Si...). Plants require macro- and micronutrients, each of which is essential for a plant to complete its life cycle; therefore, plants take up mineral elements from soil solution in the ionic form [11]. Interestingly, minerals also play a vital role in the human body and are responsible for regulating processes such as neuromuscular transmission, blood coagulation, oxygen transport, and enzyme activity as well as structural processes involving the skeleton and soft tissues. People take up minerals through food and supplement products. That leads to the need to research on the beneficial elements of food products.

All three Golden Camellias species in this study supply enough minerals as the ionic form that is available for human nutritional needs, especially Ca, Mg, Mn, Fe, Zn, Cu. Minerals that were reported to induce immunology reactions of the human body such as Zn represented in three studied species with high amounts. Heavy metals such as Hg, Cd, As, Cr... present in plant tissues with moderate levels that are safe for human use. This research is the first to analyse and study the mineral contents of different Golden Camellias endemic to Vietnam, proving the nutritional value of these species as well as their potential to be applied in manufacturing beverage products and dietary supplements.

3.2. Antioxidant capacity determination by DPPH scavenging assay

The IC_{50} values which indicate the concentration of each methanolic leaf extract needed to inhibit 50% DPPH radicals were represented in Table 2. Compared with IC_{50} value obtained from the ascorbic acid standard curve (5.466 ± 0.025 µg/mL), all plant extracts illustrated relatively strong antioxidant properties. In turn, the descending order of DPPH radical scavenging capacity as follows: *C. tamdaoensis* (7.280 ± 0.035 µg/mL) > *C. phanii* (7.753 ± 0.055 µg/mL) > *C. tienii* (7.985 ± 0.086 µg/mL). This result is consistent with other studies revealing the potential antioxidant capacities of the two most studied Golden Camellias, *C. chrysantha* and *C. nitidissima* [4],[9],[12].

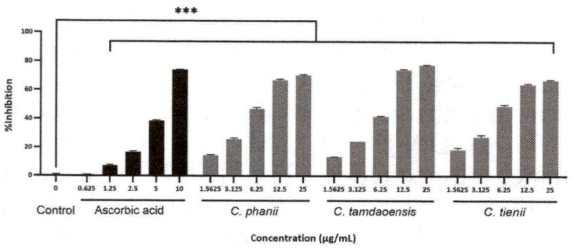

Fig. 1. Scavenging activity (SC%) of leaves extracts and ascorbic acid as positive control. The significant differences were obtained (***p-value < 0.001) between the tested samples and negative control.

Table 2. DPPH free radical scavenging activity of leaves extracts represented by IC_{50} values. ***P <0.001 versus IC_{50} of positive control by one-way ANOVA with Dunnett's test.

Sample	IC_{50}
Ascorbic acid	5.466 ± 0.025 µg/mL
C. phanii	7.753 ± 0.055 µg/mL***
C. tamdaoensis	7.280 ± 0.035 µg/mL***
C. tienii	7.985 ± 0.086 µg/mL***

The oxidative stress was known to come from the unequal level of reactive oxygen species/reactive nitrogen species and antioxidants in the body. Therefore, natural antioxidants in food or beverage may play a role in neutralizing these above-mentioned species thus protecting against heart disease, cancer, and other diseases. With the potential DPPH radical scavenging capacity, three endemic studied Camellia species can be subjected to further investigations on antioxidant activities as well as other pharmacological effects related to free radicals such as anti-aging or anticancer abilities.

4. Conclusion

The leaf of three endemic species *C. phanii, C. tamdaoensis, C. tienii* collected from Tam Dao district, Vinh Phuc province exposed the mineral values in terms of both macronutrients, micronutrients. The results also indicated the potential antioxidant activities of the methanolic extracts of these leaves compared to ascorbic acid. In summary, our study successfully revealed the preliminary investigation, proved that the leaf samples of *C. phanii, C. tamdaoensis* and *C. tienii* can

provide a good source of minerals and antioxidants. This is the first report on the chemical basis of these species, which provides new insight into the possibility to use these plants as food supplements.

Acknowledgments: *This study was supported by Vietnam Academy of Science and Technology fund for young researchers with grant number: THTEXS.05/21-24 and University of Science and Technology of Hanoi with grant number USTH.LS.01/22.*

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Journal of Medicinal Materials, 2022, Vol. 27, No. 4 (pp. 239 - 244)

STABILITY STUDY OF LIGNAN CONTENT IN *HERBA PHYLLANTHI AMARI* AND IN PREPARATIONS THEREOF

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(Received July 25th, 2022)

Summary

Stability Study of Lignan Content in *Herba Phyllanthi amari* and in Preparations Thereof

The study was conducted with the aim of evaluating the stability of lignans content including phyllanthin and hypophyllanthin - major active compounds in crude plant materials of *Phyllanthus amarus* and in preparations thereof in several conditions by HPLC. Quantification of phytomarkers was based on USP 41 by using an elution system including Acetonitrile (A)/0.14 g KH_2PO_4 and 500 µL H_3PO_4 in 1000 mL water (B) (A/B = 4/6) equipped with InertSustain C_{18} column (250 x 4.6 mm; 5 µm) from GL science and guard column (Phenomenex) at a flow rate 1.5 mL/min and 10 µL of injection volume with UV detection at 230 nm for HPLC analysis. The results indicated a significant reduction of lignan contents in raw materials of *Phyllanthus amarus*, but it was a slight in extract and tablet in the conditions of investigations. These may have implications for shelf-life determination of medicinal herbs, and the preparation of dosage forms from *Phyllanthus amarus*.

Keywords: *Phyllanthus amarus, Phyllanthin, Hypophyllanthin, Stability.*

1. Introduction

Phyllanthus amarus (Schum. et Thonn), belonging to the Euphorbiaceae family, is a medicinal herb with abundant chemical constituents including lignans, flavonoids, hydrolysable tannins, polyphenols, triterpenes, sterols, alkaloids and exhibits a wide range of pharmacological properties such as antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephroprotective and diurectic properties [1]. It has been utilized in folk remidies in quite a few tropical countries to effectively cure various ailments involving aundice, constipation, diarrhea, kidney ailments, ringworm, ulcers, malaria, genito - urinary infections, hemorrhoids and gonorrhea [2].