Thesis reference number: ……

University of Science and Technology of Hanoi

INTEGRATION OF METABOLOMICS AND ANTIOXIDANT ACTIVITY FOR THE VALORIZATION OF SEVERAL ENDEMIC GOLDEN *CAMELLIA* SPECIES OF VIETNAM

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Hanoi, 2026

1. Acceptance of Supervisors and Co-Supervisors (approve/reject): **approve**

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I approve all the modifications and corrections made to the revised version of the thesis.

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DECLARATION

I, the undersigned, confirm that this thesis is my work, except where stated otherwise. I also affirm that it has not been submitted for any other degree or professional qualification unless noted. This declaration confirms the authenticity and originality of the thesis.

Parts of this work have been published in the following papers:

1. **Nguyen, P. N**., Do, T. Y., Do, T. N., Gontier, E., Le Nguyen, H. T., Le Thi, V. A., ... & Nguyen Thi, K. O. (2024). Widely targeted metabolomics reveals the species-specific, matureness-specific and post-harvest-specific discriminations in the chemical profiles of Vietnamese endemic golden *Camellia*. *International Journal of Food Science and Technology*, *59*(10), 7873-7886. doi:10.1111/ijfs.17071
2. **Nguyen, P. N**., Le, H. L., Gontier, E., Hoang Thi, H. C., Le Thi, V. A., Mai, N. T., & Nguyen Thi, K. O. (2024). A Comprehensive Strategy for Metabolites Profiling of Flowers and Leaves from *Camellia tienii*, an Endemic Golden Tea of Vietnam. *Chemistry & Biodiversity*, *21*(10), e202400997
3. Van-Anh Le, T., Mai Nga, T. P., **Nhi Nguyen, P**., & Kieu-Oanh Nguyen, T. (2023). Genotypic and phenotypic diversity of endemic Golden *Camellia* collected from the north of Vietnam. *Chemistry & Biodiversity*, *20*(1), e202200843., 2023. doi.org/10.1002/cbdv.202200843.

01 proceeding of national conference

4. Nguyễn Phương Nhung, **Nguyễn Phương Nhị**, Mai Thị Phương Nga, Lê Thị Vân Anh, Nguyễn Thị Kiều Oanh. Proceeding Hội thảo Sinh lý thực vật - Đà Lạt 2024: Phân biệt hệ chất chuyển hóa theo khu vực địa lý của loài chè hoa vàng *Camellia euphlebia* thu hái ở Quảng Ninh.

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Research Topic: "**Integration of metabolomics and antioxidant activity for the valorization of several endemic golden camellia species of Vietnam".**

We have reviewed Ms. Nguyen Phuong Nhi's Ph.D. thesis and approved it for submission to the Doctoral School for processing towards her Ph.D. defense. Our evaluation covered the following:

- ✓ The Ph.D. research objectives are clear and well-suited to the relevant scientific field.
- ✓ The results were discussed logically and understandably. They are reasonable and consistent with the research goals. All data reported in the thesis were thoroughly reviewed and verified.
- ✓ The results from this thesis benefit in Research showed the major classes including flavonoids, alkaloids, tannins, amino acids, fatty acids, vitamins, and some minerals Cr, Fe, Mg, Ga, Mo, Co, Sr, Bi and Zn as good pharmacological components/nutrients for human, the content of these secondary metabolites or minerals differ depending on several factors including geographical location, species, and plant part, thus influencing to their bioactivities. Selecting the right species, i.e., giving the high content and diversity of natural compounds and minerals, is therefore the prerequisite criterion for growing and exploiting this medicinal plant. It is also important to know the responsible active compounds, minerals in the complex matrix, to develop high-value products such as standardized extracts/fractions/ essential oils that could be produced from these valuable plants.
- ✓ Ms. Nguyen Phuong Nhi has excellent writing capability, and she is highly independent in conducting experiments and analysing the data.

We have allowed Ms. Nguyen Phuong Nhi to use the data in the following articles for her PhD thesis.

01 proceeding of international conference

1. **Nguyen, P. N**., Do, T. Y., Do, T. N., Gontier, E., Le Nguyen, H. T., Le Thi, V. A., ... & Nguyen Thi, K. O. (2024). Widely targeted metabolomics reveals the species-specific, matureness-specific and post-harvest-specific discriminations in the chemical profiles of Vietnamese endemic golden *Camellia*. *International Journal of Food Science and Technology*, *59*(10), 7873-7886. doi:10.1111/ijfs.17071
2. **Nguyen, P. N**., Le, H. L., Gontier, E., Hoang Thi, H. C., Le Thi, V. A., Mai, N. T., & Nguyen Thi, K. O. (2024). A Comprehensive Strategy for Metabolites Profiling of Flowers and Leaves from *Camellia tienii*, an Endemic Golden Tea of Vietnam. *Chemistry & Biodiversity*, *21*(10), e202400997
3. Van-Anh Le, T., Mai Nga, T. P., **Nhi Nguyen, P**., & Kieu-Oanh Nguyen, T. (2023). Genotypic and phenotypic diversity of endemic Golden *Camellia* collected from the north of Vietnam. *Chemistry & Biodiversity*, *20*(1), e202200843., 2023. doi.org/10.1002/cbdv.202200843.

01 proceeding of national conference

4. Nguyễn Phương Nhung, **Nguyễn Phương Nhị**, Mai Thị Phương Nga, Lê Thị Vân Anh, Nguyễn Thị Kiều Oanh. Proceeding Hội thảo Sinh lý thực vật - Đà Lạt 2024: Phân biệt hệ chất chuyển hóa theo khu vực địa lý của loài chè hoa vàng *Camellia euphlebia* thu hái ở Quảng Ninh.

Approval of supervisor(s) and Co-Supervisor(s)

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ACKNOWLEDGEMENT

First, I would like to extend my sincere thanks to the University of Science and Technology of Hanoi (USTH), especially the Doctoral School of Pharmacological, Medical, and Agronomical Biotechnology, and the Vietnam University of Traditional Medicine (VUTM), which facilitated my work.

I would like to express my deepest gratitude to my supervisor, Associate Prof. Nguyen Thi Kieu Oanh, for her invaluable feedback at every stage of my work, her dedicated guidance, and her constant encouragement. She guided me with passion and inspired me to become a true researcher. She created valuable opportunities to strengthen my scientific foundation, leading to meaningful scholarships. I truly appreciate her belief in my educational journey and the respect she showed for my work and perspective. Her support boosted my motivation, allowing me to face challenges with confidence and optimism.

I extend my sincere thanks to my co-supervisor, Prof. Eric Gontier, for his guidance. His timely encouragement, valuable advice, and patient, enthusiastic support was crucial throughout the study, right up to the results.

To my colleagues, teachers, and friends at Life Science Lab, it has been a pleasure working alongside you. Your dedication, support, and the valuable lab experiences we've shared have meant very much to me. I also treasure the wonderful memories and support from USTH students during my time there

I would like to express my sincere gratitude to the French Embassy, University of Picardie Jules Verne, and USTH for sponsoring and supporting my study and experimental work at UPJV University in France. This opportunity allowed me to spend three months at the BIOPI/BioEcoAgro Lab, which was an incredible experience. Working directly with Professor Gontier, I gained valuable skills in Gas Chromatography-Mass Spectrometry (GC-MS) and developed my scientific thinking through our daily, insightful discussions. Every day at the BIOPI/BioEcoAgro lab was meaningful. I am especially grateful for Romain's technical support, Ms. Soléne’s assistance with tools and instruments, and the help from the BIOPI/BioEcoAgro staff and students, which contributed to the success of my research. This scholarship was instrumental in helping me complete my doctoral thesis with the best efforts.

Finally, I would like to express my gratitude to my family because they are always by my side to give me peace. This support encouraged me so much during the Ph.D. journey. My family is the most important part of my life, and I am deeply grateful for their love, support,

and encouragement. I want to thank my parents, children, extended family, and husband for always pushing me to excel. I'm also thankful to my friends for their constant inspiration.

I apologize to anyone I couldn't mention by name. I extend a heartfelt thank you to them and everyone who contributed to the successful completion of this thesis.

Thank the universe for always listening and blessing me and us!

ABSTRACT

Vietnam is one of the world's centers of diversity for golden *Camellia*, a group of yellow-flowered species within the genus *Camellia* (Theaceae). In recent years, the discovery of new species has increased scientific interest in Vietnamese *Camellia*. The thesis is structured into four chapters, in addition to references and annexes. Chapter 1 provides a comprehensive review of the literature on the Golden *Camellia*, and specifically six Golden *Camellia* species (*C. hakodae, C. phanii, C. tamdaoensis, C. tienii, C. petelotii,* and *C. euphlebia*) collected from Tam Dao - Vinh Phuc and Hai Ha - Quang Ninh. Chapter 2 outlines the materials and methodologies employed in the study, including LC-MS, GC-MS, and ICP-MS analyses, as well as DPPH and ABTS free radical scavenging assays. Chapter 3 presents and discusses the experimental results:

First, this study identified a shared metabolite profile of 131 compounds across six endemic Golden *Camellia* species using a widely targeted metabolomics approach based on ultra-performance liquid chromatography coupled with tandem mass spectrometry. The metabolite profiles were dominated by 41 flavonoids and 32 amino acids representing diverse aglycone skeletons, including anthocyanins, aurones, chalcones, flavan-3-ols, flavanones, flavones, and flavonols. Additional primary metabolites, such as amino acids and their derivatives, were also detected and are likely to contribute to the physicochemical properties as well as the taste and flavor of Golden Camellia tea products.

Multivariate analysis revealed clear separation between oven-dried and freeze-dried samples, as well as between young and mature leaves. Compounds such as catechin, epicatechin, L-glutamic acid, choline, and several bioactive flavonoids showed distinct accumulation patterns depending on species and thermal treatment. Amino acids were generally more abundant in young leaves, whereas flavonoids accumulated at higher levels in mature leaves. These findings demonstrate that both harvest stage and processing methods significantly influence metabolite composition, thereby affecting the sensory, nutritional, and pharmacological properties of Golden Camellia materials

Second, presents the potential of six golden *Camellia* species by examining the presence and abundance of chemical components in flowers and leaves. The thesis identified 42 metabolites in leaf samples and 43 metabolites in flower samples using an untargeted GC-MS analysis. The groups that appeared most abundant included esters, fatty acids, and terpenoids with a high probability. Besides, there are natural compounds that contribute to the medicinal and nutritional value of precious tea varieties endemic to Vietnam, such as citral, dl-α-tocopherol, phytol, and squalene. *Camellia* flowers accumulated more flavonoids, amino acids, and fatty acids, while leaves contain more terpenes, suggesting different pharmacological

properties of these materials. Overall, this pipeline can be applied to other *Camellia* species and the valorization of these valuable resources for health benefits.

Third, the elemental composition of six endemic Vietnamese Golden Camellia species was quantified using a microwave digestion ICP-MS method developed to quantify 23 elements. Both leaves and flowers were analyzed for macro- and micro-minerals, as well as trace elements, providing the first comprehensive elemental profile for these species in Vietnam. In leaves and flowers samples, macro-minerals (Na, Mg, K, Ca) constituted the largest proportion of total elements. Among micro-minerals, Mn, Fe, Cu, and Zn predominated and are closely associated with antioxidant activity. Importantly, heavy metals such as Pb, As, Cd, and Ag were not detected or were present below permissible limits, confirming the safety of Golden Camellia for human consumption.

Four, this study presents and discusses the antioxidant bioactivity of six endemic Vietnamese Golden *Camellia* species, and represents the first comprehensive evaluation of antioxidant capacity in both leaves and flowers of these species. Antioxidant activity was assessed using DPPH and ABTS radical-scavenging assays; all species exhibited strong antioxidant capacity. Flower extracts exhibited antioxidant activity similar to leaves. Mature leaves consistently outperformed young leaves, and oven-dried samples showed slightly stronger activity than freeze-dried samples. Phytochemical analysis revealed the presence of abundant antioxidant compounds, including phenolics, flavonoids, carotenoids, vitamin E, catechins, phytol, and stigmasterol, confirming a strong correlation between the chemical composition and antioxidant activity in Golden *Camellia.*

Chapter 4 presents the Conclusion and Perspectives section that summarizes the presence of valuable natural compounds, minerals, and antioxidants in six golden *Camellia* species. The antioxidant effects of six Golden Camellia species endemic to Vietnam were compared, and the relationships between their metabolite profiles and antioxidant activities were partially evaluated. It emphasizes their rich bioactive content and strong antioxidant effects in both leaves and flowers. In reality, the flowers bloom seasonally, while the leaves are available year-round, and the flowers are high-cost. Thus, using leaves is a practical choice for healthcare. In the future, continued research on other varieties of golden *Camellia* for comparison and widespread use will bring numerous benefits to healthcare. In the future, it's crucial to develop convenient products such as instant tea, hard capsules, and soft capsules using golden camellia leaf extracts. Research on the golden *Camellia* species plays a key role in conserving and developing Vietnam's precious medicinal resources.

TÓM TẮT

Chi trà hay chè (*Camellia* L.) là một chi thực vật có hoa trong họ chè Theaceae. Dựa vào hình thái màu sắc hoa thì chi này có số lượng rất lớn các loài trà hoa vàng (Golden *Camellia*). Trong những năm gần đây, việc liên tục phát hiện các loài mới đã làm gia tăng sự quan tâm của giới khoa học đối với chi *Camellia* ở Việt Nam. Vì vậy, các nghiên cứu chuyên sâu về hệ chất chuyển hóa của những loài trà quý này là hết sức cần thiết.

Luận án gồm 04 chương cùng phần tài liệu tham khảo và phụ lục. Chương 1 trình bày tổng quan tài liệu về họ chè (Theaceae), chi *Camellia* L., trà hoa vàng (Golden *Camellia*) và đặc biệt là sáu loài trà hoa vàng (*C.hakodae, C.phanii, C.tamdaoensis, C.tienii, C.petelotii và C. euphlebia*) thu hái tại Tam Đảo - Vĩnh Phúc và Hải Hà - Quảng Ninh. Chương 2 mô tả nguyên liệu và các phương pháp nghiên cứu trên sáu loài trà hoa vàng, bao gồm các phương pháp: LC-MS, GC-MS, ICP-MS và khả năng bắt gốc tự do DPPH và ABTS. Chương 3 trình bày kết quả và thảo luận cho toàn bộ các thực nghiệm, gồm các kết quả sau:

Thứ nhất, nghiên cứu đánh giá các hệ chất chuyển hóa hữu cơ trong trà hoa vàng bằng phương pháp sắc ký lỏng - khối phổ (LC-MS). Kết quả thu được dữ liệu chung của 131 chất chuyển hóa bằng cách sử dụng phân tích chuyển hóa hóa học có mục tiêu rộng rãi sử dụng sắc ký lỏng hiệu năng cao kết hợp với khối phổ. Hồ sơ chuyển hóa chủ yếu gồm 41 flavonoid và 32 axit amin, đại diện cho nhiều bộ khung aglycon khác nhau như flavan-3-ol, flavanone, flavone, flavonol, anthocyanin, aurone và chalcone. Ngoài ra, các chất chuyển hóa sơ cấp khác, bao gồm axit amin và các dẫn xuất của chúng, cũng được phát hiện và có khả năng góp phần tạo nên các đặc tính lý hóa cũng như hương vị của các sản phẩm trà Trà hoa vàng. Các hợp chất như catechin, epicatechin, L-glutamic acid, choline và một số flavonoid có hoạt tính sinh học thể hiện các xu hướng tích lũy khác nhau tùy theo loài và phương pháp xử lý nhiệt. Axit amin nhìn chung phong phú hơn trong lá non, trong khi flavonoid tích lũy ở mức cao hơn trong lá trưởng thành. Những kết quả này chứng minh rằng cả giai đoạn thu hoạch và phương pháp chế biến đều ảnh hưởng đáng kể đến thành phần chuyển hóa, từ đó tác động đến các đặc tính cảm quan, dinh dưỡng và dược lý của nguyên liệu Trà hoa vàng.

Thứ hai, luận án làm rõ tiềm năng của sáu loài trà hoa vàng thông qua việc khảo sát sự hiện diện và hàm lượng của các thành phần hóa học trong hoa và lá. Kết quả phân tích GC-MS cho thấy có 42 chất chuyển hóa được xác định trong mẫu lá và 44 chất chuyển hóa trong mẫu hoa. Các nhóm hợp chất chiếm ưu thế bao gồm este, axit béo và terpenoid. Bên cạnh đó, nhiều hợp chất tự nhiên quý của các loài trà quý đặc hữu ở Việt Nam đã được phát hiện, như citral, dl-α-tocopherol, phytol và squalene. Hồ sơ hóa học toàn diện thu được từ các kỹ thuật trên giúp làm rõ sự khác biệt đáng kể giữa lá và hoa. Hoa trà tích lũy nhiều flavonoid, axit amin và axit béo hơn, trong khi lá chứa hàm lượng terpen cao hơn, cho thấy các vật liệu này có thể sở hữu

những đặc tính dược lý khác nhau. Quy trình nghiên cứu này có thể được áp dụng cho các loài *Camellia* khác và góp phần nâng cao giá trị sử dụng của những nguồn tài nguyên quý này cho mục đích chăm sóc sức khỏe.

Thứ 3, nghiên cứu về thành phần khoáng chất có trong các loài trà hoa vàng. Đây là nghiên cứu đầu tiên đánh giá và so sánh tổng thế các nguyên tố có trong 6 loài trà hoa vàng ở cả lá và hoa. Phân tích sử dụng phương pháp phá mẫu vi sóng kết hợp ICP-MS để định lượng toàn diện 23 nguyên tố trong trà hoa vàng. Phân tích các nguyên tố trong lá và hoa cho thấy hàm lượng nguyên tố trong lá cao hơn đáng kể. Trong các mẫu, các nguyên tố đa lượng (Na, Mg, K, Ca) chiếm tỷ lệ lớn nhất trong tổng hàm lượng nguyên tố, trong đó Canxi là nguyên tố phong phú nhất. Trong nhóm vi lượng, Mn, Fe, Cu và Zn chiếm ưu thế và có mối liên hệ chặt chẽ với hoạt tính chống oxy hóa. Nhìn chung, hàm lượng khoáng chất trong lá cao hơn đáng kể so với hoa. Đáng chú ý, các kim loại nặng như Pb, As, Cd và Ag không được phát hiện hoặc nằm dưới ngưỡng cho phép, khẳng định tính an toàn của Trà hoa vàng đối với người sử dụng.

Thứ tư, đánh giá khả năng chống oxy hóa của sáu loài Trà hoa vàng đặc hữu của Việt Nam. Đây là công trình đầu tiên đánh giá một cách toàn diện khả năng chống oxy hóa ở cả lá và hoa của sáu loài này. Hoạt tính chống oxy hóa được xác định thông qua hai phép thử bắt gốc tự do DPPH và ABTS. Trong đó, tất cả các loài đều thể hiện khả năng chống oxy hóa mạnh ở cả lá và hoa và tương đương nhau giữa các loài. Phân tích hóa thực vật cho thấy sự hiện diện phong phú của các hợp chất có hoạt tính chống oxy hóa như polyphenol, flavonoid, carotenoid, vitamin E, catechin, phytol và stigmasterol có trong trà hoa vàng.

Chương 4, phần Kết luận và kiến nghị đã cho thấy sáu loài trà hoa vàng này đều chứa các hợp chất tự nhiên, các khoáng chất có hoạt tính chống oxy hóa rất mạnh. Đây là một nghiên cứu đầu tiên tích hợp, đánh giá tổng thể các chất chuyển hóa trong 6 loài trà hoa vàng cả về hệ chất hữu cơ và các thành phần nguyên tố. Đã so sánh tác dụng chống oxy hóa của 6 loài trà hoa vàng đặc hữu tại Việt Nam.

Thực tế hoa chỉ ra theo mùa nhưng lá có quanh năm và giá thành của hoa rất đắt, trong khi đó hoạt chất và tác dụng lại gần tương đương ở cả lá và hoa trên cả sáu loài. Do vậy việc sử dụng lá trà hoa vàng sẽ là một lựa chọn thiết thực cho việc chăm sóc sức khỏe. Việc tiếp tục nghiên cứu về các loài trà hoa vàng khác để so sánh và đưa vào sử dụng phổ biến có ý nghĩa rất quan trọng trong việc chăm sóc sức khỏe cộng đồng. Tiến xa hơn nữa là tạo ra các sản phẩm tiện lợi như trà hòa tan, viên nang cứng và viên nang mềm sử dụng chiết xuất từ lá cây trà hoa vàng trên nhiều loài là rất cần thiết. Việc nghiên cứu về các loài trà hoa vàng cũng đóng vai trò quan trọng để bảo tồn và phát triển nguồn dược liệu quý của Việt Nam.

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LIST OF ABBREVIATIONS

GC-MS	Gas Chromatography-Mass Spectrometry
EI	Electron ionization
QUAD	Quadrupole
TOF	Time-of-flight
LC-MS	Liquid Chromatography-Mass Spectrometry
TIC	Total ion chromatogram
EIC	Extracted Ion Chromatogram
UPLC	Ultra Performance Liquid Chromatography
DPPH	2-Diphenyl-1-picryl-hydrazyl
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
Sp.	Species
Spp.	Species pluralis
C.	*Camellia*
HAK	*C. hakodae*
PHA	*C.phanii*
TAM	*C. tamdaoensis*
TIE	*C. tienii*
PET	*C. petelotii*
EUP	*C.euphlebia*
AAS	atomic absorption spectroscopic
ICP-MS	inductively coupled plasma-mass-spectrometry
RF	Radio frequency
a.m.u	atomic mass unit
PC	Principal Component
PCA	Principal Component Analysis
RT	Retention time
RI	Retention index
LRI	Linear retention indices
TMS	trimethylsilylated

CSV	Comma-separated values
CPS	10-Camphorsulfonic acid
CG	(-)-catechin-3-gallate
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin-3-gallate
GCG	(-)-gallocatechin-3-gallate
LOD	Limits of Detection
LOQ	Limits of Quantification
UPW	Ultra-pure water
ROS	Reactive oxygen species

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INTRODUCTION

Vietnam is a tropical country with a rich diversity of plant species. Its natural conditions are well-suited for flowering plants in general and the *Camellia* genus in particular. *Camellia* is the largest genus in the family Theaceae, featuring various flower colours, including yellow, red, white, pink, and brown. Among them, Golden *Camellia*, distinguished by its yellow flowers, holds significant economic, scientific, and health-related value. In Vietnam, three key regions - Quang Ninh, Vinh Phuc, and Lam Dong - are the main suppliers of *Camellia* species and products.

Although several studies have been conducted on endemic *Camellia* species in Vietnam, systematic research on the chemical characteristics and biological activities of these species remains limited. Investigating the chemical and biological properties of these species across different locations, as well as under varying harvesting and processing methods, is essential for accurate species identification and for selecting the most promising varieties for large-scale domestic cultivation

This study aims to explore and enhance the value of Vietnam's endemic Golden *Camellia* species through the research project titled: "**Integration of metabolomics and antioxidant activity for the valorization of several endemic golden *Camellia* species of Vietnam."**

In this context, the primary goal of this project is to comprehensively investigate the chemical composition and antioxidant properties of the Vietnamese Golden *Camellia* species while establishing a metabolite-based classification for these endemic plants. Specifically, the study aims to achieve the following detailed objectives:

1. Metabolite profiling and discrimination of leaf and flower extracts from six endemic golden *Camellia* species - namely *Camellia hakodae*, *Camellia phanii*, *Camellia tamdaoensis*, *Camellia tienii*, *Camellia petelotii*, and *Camellia euphlebia* - based on species-specific, geographic-specific, and postharvest-specific characteristics, using liquid chromatography - mass spectrometry (LC-MS), gas chromatography - mass spectrometry (GC-MS), and inductively coupled plasma mass spectrometry (ICP-MS) techniques.
2. Determination of antioxidant activity of leaf and flower of endemic golden *Camellia* by applying two widely used assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

To achieve the above objectives, the study needs to carry out the following tasks:

1. Metabolite profiling and discrimination

- Conduct metabolite profiling of leaf and flower extracts from six endemic *Camellia* species- namely *Camellia hakodae, Camellia phanii, Camellia tamdaoensis, Camellia tienii, Camellia petelotii,* and *Camellia euphlebia,* using both liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) techniques.
- Differentiate among these six species based on their metabolite profile, including species-specific, tissue-specific, maturity-specific, and postharvest-specific discrimination.

2. Mineral and ion profiling

- Analyze the mineral and ion composition of the extracts by using inductively coupled plasma mass spectrometry (ICP-MS).
- Determine the presence and concentration of essential trace elements and minerals of these species.

3. Determination of antioxidant activity

- Assess the antioxidant potential of the extracts by applying two widely used assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).
- Compare the antioxidant capacities of different species and plant parts to identify those with the highest bioactivity

Chapter 1: LITERATURE REVIEW

1.1. General overview

Based on literature reviews, Vietnam is home to 95 species and two varieties of *Camellia* (Lu H, Jiang W, Ghiassi M, Lee S, 2012) (Le et al., 2020) (Chang, 1998);. Notably, seven endemic Camellia species have been identified in Tam Dao, Vinh Phuc province (Tran Ninh & Hakodae Naotoshi, 2009), while 18 endemic species have been recorded in the Central Highlands, particularly in Lam Dong province (Hoang et al., 2022).

Chemical studies on several golden *Camellia* species have revealed their key bioactive compounds. The leaves and flowers of Golden *Camellia* contain saponins, polyphenols, polysaccharides, phytosterols, triterpenoids, phenolic compounds, and flavonoids (Trinh Thi Diep, 2022).

Pharmacological studies have demonstrated that the total extracts, fractions, and isolated substances from the golden *Camellia* species exhibit antioxidant, anti-allergic, anxiolytic, antidepressant, hypolipidemic, hypoglycemic, hepatoprotective, anticancer, and neuroprotective activities. The Golden *Camellia* was found to inhibit the growth of transplanted cancer, lower blood lipids, reduce cholesterol levels, and prevent atherosclerosis. Species of *Camellia* have been highlighted as a source of antioxidants and a huge source of polyphenols (Trinh Thi Diep, 2022).

Due to the high value on human health, the commercial value of golden camellias is much higher compared to green tea. However, the basic biological and chemical diversity of the endemic golden camellia collection has still been considered underestimated in this country. On the other hand, even though previous research showed the major compound classes including flavonoids, tannins, coumarins, vitamins, and some minerals Ge, Se, Mo, and Zn as good pharmacological nutrients for human, the content of these secondary metabolites or minerals differ depending on several factors including geographical location, species, and plant part, thus influencing to their bioactivities. Selecting and determining the right species and major parts of plants with high concentrations of natural compounds and potential bioactivities is thus a prerequisite for growing and exploiting this medicinal plant.

The “omics” study refers to the branches of life science whose names end with the suffix “- omics,” such as genomics, transcriptomics, proteomics, and metabolomics. "Omics" aims to characterize and quantify pools of biological molecules under specific conditions, providing information about the structure, function, and dynamics of an organism or organisms. The advent of "omics" studies has significantly altered our approaches to investigating biological molecules, their interactions, and functional processes. Indeed, knowledge at the “omics” level provides an organism with an understanding of all its metabolic pathways, allowing for

comparisons and correlations among them. The name metabolomics was coined in the late 1990s. Metabolomics is defined as the comprehensive analysis of the small molecules (MW<1000 Da) in a biological system under a given set of conditions. The metabolomics approach, i.e., metabolite profiling, is considered a powerful technique for analyzing the chemical composition, chemotaxonomy, and quality control of herbal medicine. This approach recently has been applied in wide range of natural-product drug discovery and development fields including improve identification and dereplication steps, quality control of phytomedicines, link chemical profile and bioactivity pattern of phytomedicines, identification of active compounds and quantitative prediction of bioactivity, bioavailability and fate of natural compound assessment, safety and toxicity of herbal medicines assessment as well as proof of efficacy and mode of action identification of herbal medicines. Metabolomics plays a crucial role in chemotaxonomy by providing a comprehensive analysis of metabolites that serve as biochemical markers for taxonomic classification. Chemotaxonomy, also known as chemical taxonomy, relies on the qualitative and quantitative profiling of secondary metabolites to distinguish between species, subspecies, and even varieties within a genus. This approach is particularly valuable in plant systematics, where traditional morphological classification may be insufficient due to environmental influences on phenotypic traits.

The taxonomic complexity and morphological similarity among golden *Camellia* species present significant challenges for accurate identification and quality assessment, critical steps in their conservation and commercial utilization. Traditional classification methods, including morphological traits and DNA barcoding, often fall short due to environmental variability and low genetic divergence. In contrast, omics-based approaches such as metabolomics and metallomics offer deeper biological insights and higher resolution. Metabolomics enables the profiling of bioactive secondary metabolites, such as flavonoids and polyphenols, that contribute directly to therapeutic value. Metallomics complements this by revealing patterns of trace elements associated with physiological function and safety. Together, these methods provide a comprehensive chemical fingerprint for each species, facilitating more accurate classification, discovery of high-value taxa, and standardization of raw materials. This integrative approach not only addresses the shortcomings of conventional techniques but also supports the development of evidence-based strategies for the valorization of Camellia species in pharmaceuticals, nutraceuticals, and functional products.

On the other hand, metal ions are part of this system biology but have rarely been conjugated with other omics. For this new “omics” field, metallomics and metallome have been proposed and established. A shared definition of the metallome is difficult to reach on its own, due to the special complexity of the matter. Indeed, the definition of “metallome” is still an

open question, and extensive debate is ongoing. Metals in the metallome could be considered as the inorganic elements that interact with living systems. According to this definition, most metals enter this pool. Other inorganic elements could also be considered, such as selenium and phosphorus. Of all these inorganic elements, some are necessary for life, i.e., they are essential elements (mineral nutrients), while others are toxic, and some occur only in trace amounts. The metallome, therefore, encompasses the molecular mechanisms and metabolic pathways of metal-dependent life processes, as well as the entirety of metals and inorganic species at broad levels within cells and living organisms. Metallomics is related to the study of these aspects. Metallomics addresses various aspects and components, such as metal-induced proteomic and metabolomic profiles and their metal-dependent alterations. The interaction between omics data can provide precious information within the biological system.

1.2. Literature review

1.2.1. Overview of the genus *Camellia*

a. Taxonomic position of the genus *Camellia*

Botanists worldwide consider the *Camellia* species to be an extremely rare natural source of genes. According to Le Nguyet Hai Ninh (Le et al., 2020),the genus *Camellia* L. in Vietnam has 95 species and two varieties.

Golden Camellia (*Camellia spp*.), also known as Kim Hoa Tra or “Queen of teas” or “the queen of the tea family” or "giant pandas of the plant kingdom,” with yellow flowers of the genus *Camellia* L. Golden *Camellia* has long been used for kings because of its great value for health (He et al., 2018; Tran Ninh & Hakodae Naotoshi, 2009; Z. Wang et al., 2022; Zhaoran, 1995).

In the botanical taxonomy, the genus *Camellia* includes the following taxa: Taxonomic position (Takhtajan, 2009) and (IUCN red list, 2018)

Regnum: Plantae

Divisio: Tracheophyta

Classis: Magnoliopsida

Subclass: Dillenniidae

Ordo: Theales

Family: Theaceae

Genus: *Camellia*

Species: *Camellia* spp.

b. Botanical characteristics of the *Camellia* genus (Bartholomew, 2007)

The genus ***Camellia*** consists of evergreen shrubs or small trees, only rarely reaching the size of large trees. Leaves are generally petiolate, though they may occasionally be sessile and amplexicaul. The blades are leathery to thinly leathery, with margins that are serrate, serrulate, or, more rarely, entire.

Flowers are axillary or subterminal, typically solitary but sometimes appearing in clusters of up to three. They are clearly pedicellate. The bracteoles - distinct from the sepals - number 2-10, are spirally arranged, and may be persistent or caducous. Sepals are usually five (occasionally six), persistent, and either free or basally fused.

In many species, flowers appear sessile because the short, stout pedicel is entirely enclosed by bracteoles and sepals at anthesis; in such cases, these structures are not well differentiated and may be caducous or persistent. Petals number 5-8 and may be white, red, or yellow.

Stamens are numerous and arranged in 2-6 whorls. The outer whorl forms a tubular structure through fusion of the filaments and is adnate to the petals. Anthers are dorsifixed, two-loculed, and open both longitudinally and laterally. The ovary is superior, 3-5-loculed, with axile placentation.

c. Overview of Golden *Camellia*

Golden *Camellia* is a species belonging to the genus *Camellia* L., family. Theaceae (Tran Duc Manh et al., 2019). Golden *Camellia* includes a total of 69 species that have been described to date, of which 56 species are found in Vietnam (Hoi, Doudkin, et al., 2021; Hoi, Dung, et al., 2021; Hoi, Thin, et al., 2021; Le et al., 2020; Lu et al., 2008; Lu H, Jiang W, Ghiassi M, Lee S, 2012; Pham.H.H, 1999; Thanh et al., 2022; Tran Duc Manh et al., 2019, 2020; Tran Ninh & Hakodae Naotoshi, 2009; Trinh Thi Diep, 2022; Danh-Hùng Nguyễn et al., 2020; Quach et al., 2022; Trinh et al., 2023; L. T. Nguyen et al., 2018)Vietnam has become one of the countries possessing the most species of *Camellia* in the world. (Hoang et al., 2022)

Table 1.2. List of 56 golden *Camellia* species

No	Scientific name
1.	*Camellia achrysantha* Hung T. Chang & S. Ye Liang
2.	*Camellia aurea* H.T. Chang
3.	*Camellia cattienens*
4.	*Camellia chrysantha* (Hu) Tuyama
5.	*Camellia chrysanthoides* H.T. Chang (*C. xiashiensi; C. longzhouensis*)
6.	*Camellia crassiphylla* Ninh & Hakoda
7.	*Camellia cucphuongensis* Ninh & Rosmann
8.	*Camellia dalatensis* Luong, Tran & Hakoda
9.	*Camellia dilinhensis* Ninh & V.D. Luong
10.	*Camellia dormoyana* (Pierre) Sealy
11.	*Camellia euphlebia* Merr. ex Sealy
12.	*Camellia fascicularis* Hung T. Chang
13.	*Camellia flava* (Pit.) Sealy
14.	*Camellia flavida* H.T. Chang
15.	*Camellia flavida var. patens* (S.L.Mo & Y.C.Zhong) T.L.Ming
16.	*Camellia gilbertii* (A.Chev.) Sealy
17.	*Camellia gilbert* (Chev) Sealy
18.	*Camellia grandis* (C.F. Liang & S.L. Mo) H.T. Chang & S. Ye Liang *(C. ptilosperma)*
19.	*Camellia hakodae* M.Sealy
20.	*Camellia hamyenensis* M.Sealy
21.	*Camellia hirsuta* Hakoda et Ninh
22.	*Camellia huana* T. L. Ming & W. J. Zhang *(C. liberofilamenta)*
23.	*Camellia huulungensis* Rosmann & Ninh
24.	*Camellia impressinervis* Hung T. Chang & S. Ye Liang

25.	*Camellia indochinensis* Merrill
26.	*Camellia kirinoi* Ninh
27.	*Camellia leptopetala* Chang & S.Y.Liang
28.	*Camellia limonia* C.F. Liang & S.L. Mo *(C. limonia f. obovata* S.L. Mo & Y.C. Zhong)
29.	*Camellia longruiensis* S. Y. Liang & X. J. Dong
30.	*Camellia longzhouensis* J.Y. Luo
31.	*Camellia megasepala* Hung T. Chang & Tran Ninh
32.	*Camellia micrantha* S.Ye Liang & Y. C. Zhong
33.	*Camellia multipetala S.Ye Liang & C. Z. Deng*
34.	*Camellia murauchii* Ninh & Hakoda
35.	*Camellia nitidissima* C.W.Chi
36.	*Camellia nitidissima var. phaeopubisperma* S. Ye Liang & Z. H. Tang
37.	*Camellia parvifolia* Makino
38.	*Camellia parvipetala* J. Y. Liang & Z. M. Su
39.	*Camellia petelotii* (Merr.) Sealy
40.	*Camellia phanii* Hakoda et Ninh
41.	*Camellia pingguoensis* D. Fang
42.	*Camellia pubipetala* Y. Wan & S. Z. Huang
43.	*Camellia puhoatensis* N.S.Ly, V.D.Luong, T.H.Le, D.H.Nguyen & N.D.Do
44.	*Camellia quephongensis* Hakoda et Ninh
45.	*Camellia quynhii* Luong, Quach & Hoang
46.	*Camellia quinqueloculosa* S.L. Mo & Y.C. Zhong
47.	*Camellia rosmannii* Ninh
48.	*Camellia tamdaoensis* Ninh et Hakoda
49.	*Camellia terminalis* J.Y. Liang & Z.M. Su
50.	*Camellia thanxaensa* Hakoda et Kirino
51.	*Camellia tianeensis* S.Y. Liang & Y.T. Luo
52.	*Camellia tienii* Ninh
53.	*Camellia tonkinensis* (Pit.) Cohen-Stuart
54.	*Camellia tuyenquangensis*
55.	*C. vanlangensis V.D. Luong & V.T. Pham*
56.	*Camellia vuquangensis* Luong, Tran & L. T. Nguyen

In addition to conventional morphological classification, recent research has increasingly utilized genetic analysis for the classification of golden *Camellia* species, reflecting a contemporary direction in plant systematics for some golden species such as *Camellia phanii, C. tamdaoensis, C. tienii, C. flava, C. petelotii, C. euphlebia, C. huulungensis, C.euphlebia and C. nitidissima* (Liu et al., 2023; H. H. Nguyen et al., 2020; Tang et al., 2024; Van Viet, N., Trung, P. Q., Ha, T. V., & Douangmala, 2019; Van-Anh Le, T., Mai Nga, T. P., Nhi Nguyen, P., & Kieu-Oanh Nguyen, 2023; Wei et al., 2005; Zhou et al., 2024).

1.2.2. Six endemic Golden *Camellia* in Vietnam

Vietnam is home to approximately 56 endemic species of golden *Camellia*. This dissertation focuses on six representative species distributed across two regions: Tam Dao (Vinh Phuc) and Hai Ha (Quang Ninh).

***a. Camellia hakodae* Ninh, Tr.** (Tran Ninh & Hakodae Naotoshi, 2009)

A small tree reaching 3-4 m in height, with young branches that are brownish and glabrous. Leaves are petiolate, with glabrous petioles 8-15 mm long. The blades are thick and coriaceous, elliptic, broadly elliptic, or oblong, measuring 23.5-29 cm × 9-11.5 cm. The apex is acuminate and the base cuneate or rounded; the upper surface is glossy and deep green, while the lower surface is light green and densely dark-punctate. Margins are regularly denticulate. Lateral veins number 12-16 pairs, impressed above and raised below.

Flowers are yellow, 6-8 cm in diameter, and borne terminally or in the axils. Pedicels are approximately 1.2 cm long. Bracteoles (5-6) are nail-to-scale-shaped, 1-4 mm high and 2-7 mm wide, with pubescence on the margins and inner surface. The five sepals are scale-shaped to nearly rounded, 4-6 mm high and 7-12 mm wide, and pubescent along the margins and on the inner surface.

Petals (16-17) range from nearly rounded to elliptic, 2.0-5.3 cm long and 2.3-3.5 cm wide, with the inner surface pubescent, particularly on the innermost petals. The androecium is numerous and 4.0-4.5 cm long; outer filaments are connate for 1.4-2.1 cm, while the inner filaments are free and pubescent. The gynoecium is glabrous, with 4-5 ovaries and 4 or 5 styles, each 3.2-3.5 cm long and free to the base.

The fruit is a nearly globose capsule, 5-6 cm in diameter and 4-4.5 cm high, containing 3-4 seeds per locule. The pericarp is 4.5-6.5 mm thick. Seeds are about 2.2 cm long and pubescent. Flowering occurs from early winter to spring.

Figure 1.2.1: *Camellia hakodae* Ninh

***b. Camellia phanii* Hakoda et Ninh** (Tran Ninh & Hakodae Naotoshi, 2009)

Camellia phanii is distinguished by its smaller lateral organs, pubescent petals, more numerous stamens, and larger capsules. It is a small tree, 4.0-5.0 m tall, with young branches that are light brown and glabrous. Leaves are petiolate, the petioles 10-12 mm long and glabrous. Leaf blades are thick, coriaceous, oblong-elliptic to elliptic, 14.5-16.0 cm long and 6.8-7.7 cm wide, with an acuminate apex and a cuneate to broadly cuneate base. The adaxial surface is deep green, whereas the abaxial surface is light green and densely punctate. Margins are coarsely and irregularly denticulate. Lateral veins occur in 8-10 pairs.

Flowers are yellow, 4.0-6.0 cm in diameter, borne terminally or axially. Pedicels measure 1.0-1.5 cm. Bracteoles (5-6) are nail-shaped, 1.0-4.0 mm high and 2.0-6.0 mm wide, acyclic, with ciliate margins. The five sepals are scale-shaped to nearly rounded, about 7.0 mm high and 10.0-11.0 mm wide, with pubescent inner surfaces. Both bracteoles and sepals persist on the fruiting pedicel.

There are 14-19 petals, broadly ovate, 2.0-3.5 cm long and 1.3-2.0 cm wide, pubescent on both surfaces. Petals are united to each other and adnate to the androecium for 2-10 mm at the base. The numerous stamens form an androecium approximately 2.5 cm long; the outer filaments are fused for about 2.0 cm into a tube, whereas the inner filaments are free and pubescent.

The gynoecium is glabrous, comprising three ovaries and three styles, each 2.2-2.5 cm long and free to the base. The capsule is nearly globose, 5.5-7.0 cm in diameter and 3.5-4.5 cm high, 3-locular, containing 1-4 seeds per locule. The pericarp is 4.0-5.0 mm thick. Seeds are 1.0-1.8 cm long and hairy. Blooming season: winter to spring.

Figure 1.2.2: *Camellia phanii* Hakoda et Ninh

***c. Camellia tamdaoensis* Hakoda et Ninh** (Tran Ninh & Hakodae Naotoshi, 2009)

Shrub or small tree 2-4 m tall. Young branches are brownish and pubescent, becoming glabrous with age. Leaves petiolate; petioles 7-9 mm long, glabrous. Leaf blades oblong-elliptic to broadly elliptic, 14-15.5 cm long and 5-7 cm wide; adaxial surface deep green and glossy, glabrous; abaxial surface reddish-green with numerous dark punctae. Apex acuminate; base cuneate to nearly rounded; midrib sunken above and prominent below. Margins are bluntly to sharply denticulate, irregular, and becoming obscure toward the base. Lateral veins 7-9 pairs.

Flowers are yellow, 3.5-4 cm in diameter, terminal or axillary. Pedicels 5-7 mm long. Bracteoles 5. Sepals 5, nail-shaped to nearly rounded, with pubescent inner surfaces and margins. Petals 11-12, broadly rounded, ranging from round to obovate or elliptic, 1.4-2.2 cm long, pubescent on both surfaces, and connate with each other and adnate to the androecium for 1-5 mm at the base.

Androecium numerous, 1.5-1.7 cm long; outer filaments united for about 9 mm; inner filaments free and densely hairy at the base. Gynoecium glabrous; ovary 3 or 4 locular; styles 3 or 4, glabrous, ca. 2.2 cm long, free to the base.

Capsule flattened-globose, 3-locular, with 3 seeds per locule; pericarp 2 mm thick. Seeds variable in shape, semi-globose to cuneate and glabrous. Blooming season: winter.

Figure 1.2.3: *Camellia tamdaoensis* Hakode et Ninh

***d. Camellia tienii* Ninh** (Tran Ninh & Hakodae Naotoshi, 2009)

Small tree approximately 2.5 m tall, with glabrous young branches; emerging leaves and shoots are violet and opalescent. Leaves petiolate; petioles 9-18 mm long, glabrous. Blades thick and coriaceous, oblong to elliptic, 19.8-26.0 cm long and 7.2-11.3 cm wide; adaxial surface deep green and shining, abaxial surface light green with dense dark punctae, both surfaces glabrous. Apex acute to acuminate; base auriculate with a few teeth; margins sharply serrulate. Lateral veins 13-14 pairs.

Flowers are yellow, axillary, and 5-6 cm in diameter. Pedicels are about 9 mm long. Bracteoles 5, 2-3 mm long and 5-7 mm wide, margins pubescent. Sepals 5, nail-shaped to nearly rounded, 3-7 mm long and 8-10 mm wide, with pubescent margins. Petals 14; outer petals pubescent, middle petals sparsely pubescent, inner petals glabrous; shapes ranging from nearly round to broadly elliptic or oblong.

Androecium numerous; filaments about 3.3 cm long, with outer filaments united for about 1.8 cm and inner filaments free and glabrous. Gynoecium composed of 4-5 carpels; ovary 4-5-locular, glabrous; styles 4-5, free to the base and glabrous. Blooming season: winter to early spring.

Figure 1.2.4: *Camellia tienii* Ninh

***e. Camellia petelotii* (Merr.) Sealy** (Tran Ninh & Hakodae Naotoshi, 2009)

Shrub or small tree, 3-5 m high. Young branches are pale grey-brown and glabrous. Leaves petiolate; petioles 1.3-2.0 cm long, glabrous. Blades coriaceous, oblong-elliptic, occasionally oblong or elliptic, 13.5-17.0 cm long and 5.0-6.0 cm wide; adaxial surface deep green, abaxial surface light green with light brown punctae, glabrous on both sides. Apex acuminate; base cuneate to broadly cuneate; margins sharply denticulate. Lateral veins: 10-12 pairs.

Flowers are yellow, solitary at the ends of young branches, approximately 4.7 cm in diameter. Pedicel stout, 1.0-1.2 cm long. Bracteoles 9-10, closely imbricate, scale-shaped or broadly ovate, 1.5-3.0 mm high and 3.0-5.0 mm wide, with pubescent margins and inner surfaces. Sepals 5, obovate, 6.0-8.0 mm high and 5.0-9.0 mm wide, margins and inner surfaces pubescent; bracteoles and sepals persistent on the fruit.

Petals 14, broadly obovate to elliptic, 1.7-3.0 cm long and 1.5-1.8 cm wide, white-puberulous abaxially; all petals united with one another and adnate to the androecium for 8 mm at the base. Androecium numerous, about 2.3 cm long; outer filaments connate for 1.3 cm, inner filaments free and pubescent.

Gynoecium glabrous; ovary 3-locular; styles 3, 1.5-2.0 cm long, free to the base. Capsule depressed-globose, 4.0–5.0 cm in diameter and 2.8-3.2 cm high. Seeds 1-2 cm long, hairy. Blooming season: winter to early spring.

Figure 1.2.5: *Camellia petelotii* (Merr.) Sealy

***f. Camellia euphlebia* Merr.ex Sealy** (Ming and Bartholomew, 2007)

Shrubs or small trees, 2-5 m tall. Young branches grayish-brown; current-year branchlets purplish-brown, thick, glabrous. Leaves petiolate; petioles 0.9-1.3 cm long, stout, glabrous, adaxially grooved. Leaf blades elliptic to broadly elliptic, leathery; abaxial surface pale green with brown glandular punctae, turning pale brown when dry; adaxial surface dark green and slightly glossy; both surfaces glabrous. Midvein elevated abaxially, impressed adaxially; secondary veins 11-13 per side, abaxially raised, adaxially slightly impressed. Leaf base obtuse to subrounded; margin serrulate; apex abruptly shortly caudate to caudate with blunt tip.

Flowers axillary or subterminal, solitary or paired. Pedicels thick, ca. 5-7 mm long. Bracteoles 7-8, appressed, covering pedicel, semiorbicular, 1-3 × 3-5 mm, leathery; outer surface green and glabrous, inner surface brown with white sericeous hairs, margins ciliolate. Sepals 5, semiorbicular to broadly ovate, 4-5 × 5-7 mm, leathery; outer surface green and glabrous, inner surface brown and white sericeous, margins ciliolate. Petals 7-9, golden yellow, ± fleshy; outer 2-3 petals sepaloid, suborbicular, 1-1.5 cm, concave; inner petals broadly obovate to obovate-elliptic, 2.5-4 × 2-2.5 cm, glabrous, basally connate for 5-10 mm and adnate to androecium, apex rounded.

Stamens numerous, 2-3.5 cm long, glabrous; outer filament whorl basally connate for 1-1.5 cm. Ovary ovoid, ca. 2.5 mm in diameter, glabrous, 3-loculed; styles 3, distinct, 2-3.5 cm long. Capsule oblate, 3-loculed, 2-3 × 3.5-6 cm, apex sunken; 1-2 seeds per locule; pericarp 2-3 mm thick when dry. Seeds brown, globose or hemispherical, 1.5-2 cm in diameter, glabrous.

Figure 1.2.6: *Camellia euphlebia* Merr.ex Sealy

1.2.3. Distribution and diversity of Golden *Camellia* species in Vietnam

Golden *Camellia* consists of 69 species worldwide (Hoang et al., 2022; Hoi, Dung, et al., 2021; Le et al., 2020; Tran Duc Manh et al., 2019, 2020). *Camellia* species are distributed mainly in Vietnam and China, with 56 Vietnamese endemic species of yellow *Camellia* worldwide (Hoang et al., 2022; Tran Duc Manh et al., 2019; Trinh et al., 2023; Trinh Thi Diep, 2022). In Vietnam, the golden *Camellia* is distributed in three main regions: Vinh Phuc, Quang Ninh, and Lam Dong, in the highland mountains of Vietnam (Hoi, Dung, et al., 2021; Tran Duc Manh et al., 2020; Tran Ninh & Hakodae Naotoshi, 2009).

a. Species diversity in Vinh Phuc

The tea genus *Camellia* is both diverse and abundant in Tam Dao National Park. A total of 16 species are found in the park, with one species representing 28% of all *Camellia* species in Vietnam. Compared to other national parks and protected areas, Tam Dao National Park harbors the highest number of tea species in the country (Tran Ninh & Hakodae Naotoshi, 2009).

Based on the distribution of *Camellia* species, the tea species of Tam Dao can be classified according to their geographical origins. Vietnamese endemic species include those found exclusively within Vietnam or, in some cases, only recorded in Tam Dao National Park. Although a few of these species are now cultivated as ornamental plants in botanical gardens or private gardens abroad, they are naturally restricted to Vietnam. These endemic species are *Camellia amplexicaulis*, *C. crassiphylla*, *C. hakoda*, *C. hirsuta*, *C. petelotii*, *C. phanii*, *C. pubicosta*, *C. rubriflora*, *C. tamdaoensis*, and *C. tienii*, collectively accounting for 62% of the *Camellia* species in the park (Tran Ninh & Hakodae Naotoshi, 2009)

b. Species diversity in Quang Ninh

The genus *Camellia* in Quang Ninh Province is remarkably diverse, reflecting the region's distinctive ecological conditions. A total of 18 species have been recorded, with one species representing about 25% of all *Camellia* species in Vietnam. Compared with other provinces and protected areas, Quang Ninh ranks among the richest regions in the country in terms of *Camellia* diversity (Tran Ninh & Hakodae Naotoshi, 2009;.Hoi, Dung, et al., 2021)

Based on their distribution patterns, the *Camellia* species in Quang Ninh can be grouped by major geographical elements. The Vietnamese endemic group includes species restricted to Vietnam or recorded only from Quang Ninh, although some have recently been cultivated as ornamentals in botanical gardens. This group comprises *Camellia dilinhensis*, *C. euphlebia*, *C. cucphuongensis*, *C. krempfii*, *C. quangninhensis*, *C. trachyphylla*, *C. vidalii*, and *C. tuyenquangensis*, representing approximately 60% of all *Camellia* species in the province (Hoang et al., 2022; Tran Ninh & Hakodae Naotoshi, 2009;Tran Duc Manh et al., 2020)

c. Species diversity in the central highlands of Vietnam

The Central Highlands has emerged as a major center of golden *Camellia* diversity in Vietnam. Data show that 14 golden *Camellia* species from this region were published between 2010 and 2021. Before 2010, only four species from the Central Highlands had been formally described: *C. dormoyana*, *C. langbianensis*, *C. vidalii*, and *C. dongnaiensis* (Ming and Bartholomew, 2007). In total, the Central Highlands harbors 18 endemic *Camellia* species, representing about 37% of Vietnam's golden *Camellia* and 28% of all golden *Camellia* species worldwide - more than one-third of the national total and nearly one-quarter of the global total. (Hoang et al., 2022).

1.2.4. Chemical compounds of Golden *Camellia* species

Chemical analyses of several species, including *C. nitidissima*, *C. chrysantha*, and *C. euphlebia*, have revealed that they contain the same major classes of compounds found in *C. sinensis*, such as phenolic compounds, flavonoids, saponins, alkaloids, polysaccharides, amino acids, and essential oils. Among these constituents, flavonoids and phenolic compounds are the most intensively studied due to their abundance and biological activities. (Trinh Thi Diep, 2022; Wang et al., 2016; Zheng et al., 2022; Yang et al., 2017; (Tran Duc Manh, 2019)

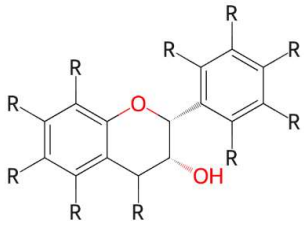
1.2.4.1. Flavonoid compounds

Flavonoids are the dominant secondary metabolites in yellow camellias, especially in leaves and flowers. Quantitative analyses have shown that the total flavonoid content in several yellow camellia species exceeds that of green tea (*C. sinensis*) (Trinh Thi Diep, 2022; J. Bin Wei et al., 2015). These compounds are closely associated with the yellow coloration of flowers and with antioxidant, anticancer, and anti-inflammatory activities.

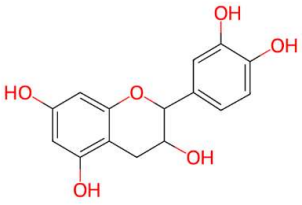
Representative flavonoids include catechin, epicatechin, quercetin, kaempferol, myricetin, rutin, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, apigenin, and luteolin (J. N. Lin et al., 2013; Nguyen et al., 2018; Trinh Thi Diep, 2022). Most flavonoids occur as glycosides, with glucose or rhamnose as the sugar moiety.

The following main flavonoids have been isolated and structurally characterized from yellow *Camellia,* which determines the yellow color and bioactivity of golden *Camellia*:

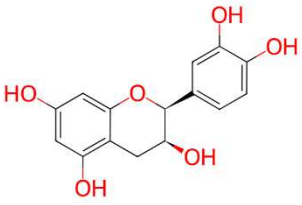
- **Flavan-3-ol structures:**



Flavan-3-ols

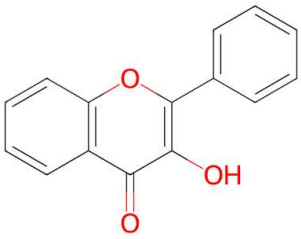


Catechin

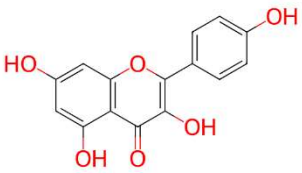


Epicatechin

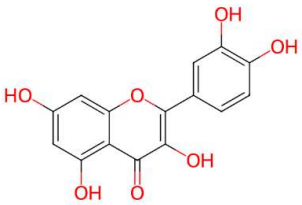
- **Flavonol structures:**



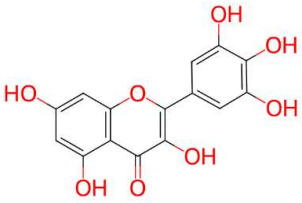
Flavonol



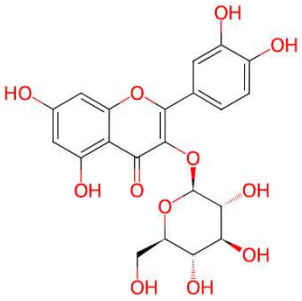
Kaempferol



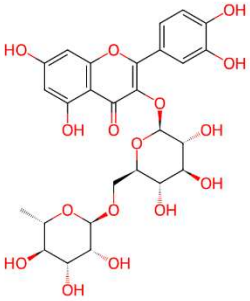
Quercetin



Myricetin

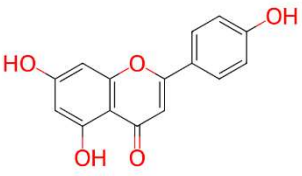


Quercetin-3-O-β-D-glucopyranoside

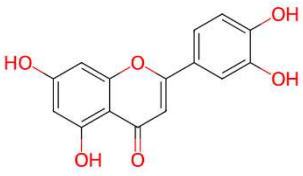


Quercetin-3-O-rutinoside

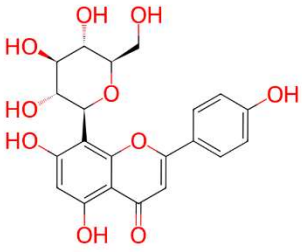
- **Flavone structures:**



Apigenin



Luteolin



Vitexin

Figure 1.2.7. Structures of flavonoids in yellow camellias

In the leaves of *C. chrysantha,* catechin, epicatechin, vitexin, isovitexin, kaempferol, and quercetin-7-O-β-D-glucopyranoside were identified and quantified. Catechin and epicatechin were the most abundant compounds, with concentrations ranging from 32.23 to 44.89 μg/g and from 36.93 to 55.56 μg/g, respectively (J. Bin Wei et al., 2015). In addition to vitexin and quercetin-7-O-β-D-glucopyranoside, three other flavonoid glycosides-quercetin-3′-O-β-D-glucopyranoside, quercetin-3-O-β-D-glucopyranoside, and quercetin-3-O-rutinoside, were isolated from the ethyl acetate and water-soluble fractions of the flowers of *C. chrysantha* collected in Vietnam (Nguyen et al., 2018)

From the ethanol extract of *Camellia nitidissima* Chi flowers, a new acylated flavonoid glycoside, quercetin 7-O-(6″-O-E-caffeoyl)-β-D-glucopyranoside, was isolated along with three known flavonoids: quercetin, quercetin 3-O-β-D-glucopyranoside, and quercetin 7-O-β-D-glucopyranoside (Peng et al., 2012)

Yang et al. (2018) reported the isolation and structural elucidation of twelve flavonoids from the flowers of *C. nitidissima*, including catechin, catechin-4α,8-catechin, quercetin, isoquercetin, kaempferol, kaempferol 3-O- [4-O-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-O-acetyl-α-L-rhamnopyranosyl-(1→6)]- β-D-glucopyranoside, kaempferol 3-O-[α-L-rhamnopyranosyl-(1→3)- 2,4-di-O-acetyl-α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside, kaempferol 3-O-β-D-glucopyranoside, kaempferol 3-O-[2,3,4-tri-O-acetyl-α-L-rhamnopyranosyl- (1→3)-4-O-acetyl-α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside, kaempferol-3-O-β-D-rutinoside, kaempferol 3-O-[2,3,4-tri-O-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-O-acetyl-α-L-rhamnopyranosyl -(1→6)]-β-D-glucopyranosid, and multiflorin C. (Yang R, Guan Y, Wang W, Chen H, He Z, 2018). Advanced analytical approaches also revealed potent antioxidant constituents such as gallic acid, catechin, salicylic acid, and a newly identified compound, okicamelliaside. Related ellagic acid glucosides have shown exceptional antihistaminic and antitumor activities, highlighting C. nitidissima as a valuable source of bioactive natural products with antioxidant, anti-inflammatory, antimicrobial, and anticancer potential (Cheng et al., 2021).

A total of 46 compounds were identified in the ethanol/water extract, while 20 compounds were detected in the ethyl acetate fraction of *Camellia quephongensis* Hakoda & Ninh collected in Que Phong, Nghe An Province. Among these, five flavonoids were identified: catechol, epicatechin, isorhamnetin, quercetin 3-O-β-D-glucopyranoside, and rutin (Nguyễn V. M. Khôi et al., 2017)

According to Tuyen et al., one new flavonoid - sexangularetin 3-O-(2″-O-(E)-p-coumaroyl-β-D-glucopyranoside), along with nine known flavonoid compounds such as

naringenin, kaempferol, quercetin, taxifolin, (-) epicatechin, epigallocatechin, epigallocatechin gallate, quercetin 3-*O*-β-dglucopyranoside, and quercetin 7-*O*-β-d-glucopyranoside was isolated from the flowers of *Camellia hakodae* Ninh (Tuyen et al., 2019).

According to Nguyen et al, *Camellia hakodae* Ninh was collected from Tam Dao, Vinh Phuc Province. The present study aims to isolate and characterize flavonoids from the leaves of *C. hakodae*. As a result, one new flavanone glycoside, camehakonin A, was identified along with six known flavonoids: (-)-epicatechin, naringenin, isoxanthohumol, (+)-taxifolin, macarangin, and 5,7,3′,4′-tetrahydroxy-6-geranylflavonol. The chemical structures of these compounds were elucidated based on comprehensive spectroscopic analyses and comparison of their NMR data with previously reported values (T. T. Nguyen et al., 2024)

1.2.4.2. Phenolic compounds

Phenolic compounds are considered the main constituents of all golden *Camellia* species and have attracted considerable attention. The most prominent are phenolic compounds such as gallic acid, ellagic acid, and chlorogenic acid, which exhibit strong antioxidant, anti-inflammatory, and anticancer activities (Fu et al., 2011; Yang R, Guan Y, Wang W, Chen H, He Z, 2018; Zhao et al., 2019).

Camellia nitidissima was reported for the first time that the antioxidant capacity and polyphenolic composition of six yellow camellia species vary substantially. Among them, dried leaves of *Camellia impressinervis* exhibited the highest oxygen radical absorbance capacity (2270.9 μmol TE/g), total phenolic content (475.6 μmol GAE/g), and proanthocyanidin content (PAC, 66.1 μmol CE/g). Compared with commonly consumed tea leaves, yellow camellia leaves contained a more diverse range of phenolic compounds, including ellagitannins, proanthocyanidins, taxifolin deoxyhexosides, apigenin derivatives, a kaempferol derivative, quercetin derivatives, glucosyl isorhamnetin, (epi)catechin-(epi) afzelechin polymers, and platphyllosides (Song, 2011)

In the ethanolic extract fraction of *C. nitidissima* flowers, 21 phenolic compounds by Yang et al. (Yang R, Guan Y, Wang W, Chen H, He Z, 2018).

In the *Camellia quephongensis* Hakoda & Ninh, five phenolics were identified, such as gallic acid, pyrogalol, piceatannol, and vanillin (Nguyễn V. M. Khôi et al., 2017).

1.2.4.3. Triterpenoid and phytosterol compounds

In 2013, Huang Ya conducted a study on *Camellia euphlebia* in Guangxi, China. The study successfully isolated several compounds, including β-sitosterol, α-spinasterol, oleanic acid, olibanumol-L, n-tetratriacontanol, and kaempferol. Among these, α-spinasterol, n-

tetratriacontanol, and olibanumol-L were identified for the first time in *C. euphlebia*, while β-amyrin and kaempferol were isolated for the first time from the genus *Camellia* (Huang, 2013).

Several triterpene compounds have been isolated from the leaves of *C. nitidissima*, including 3β-acetoxy-20-lupanol, A1-barrigenol-22α-angelate, and 3β,6α,13β-trihydroxyolean-7-one (Hou et al., 2018; Qi et al., 2016). Among these, A1-barrigenol-22α-angelate exhibited inhibitory activity against the EGFR-mutant lung cancer cell line NCI-H1975, indicating strong potential for antitumor drug development (Hou et al., 2018).

From the ethanol extract of leaves of *C. dalatensis*, an endemic yellow camellia species from Lam Dong Province, Nguyen et al. isolated and identified two phytosterols, spinasterol and stigmasterol, as well as one triterpene, oleanolic acid (Uyên et al., 2019).

1.2.4.4. Saponin compounds

The triterpene saponins in *C.nitidissma* possess a range of bioactivities, including antioxidant, cardioprotective, neuroprotective, and antidiabetic effects. These are 20(S)-O-protopanaxatriol 20-O-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside, 20-O-β-D-glucopyranosyl - 20(S) - protopanaxatriol and ginsenoside Rg1 [6,20-di-O-β-glucosyl-20(S)-protopanaxatriol (He et al., 2018; Trinh Thi Diep, 2022).

Two C-27 steroidal saponins - stigmasta-7,22-diene-3-O-[α-L-arabinopyranosyl-(1→2)]-β-D-galactopyranoside and α-spinasteryl-β-D-glucopyranoside - were successively isolated from the leaves of *C. nitidissima* (Qi et al., 2016; Hou et al., 2018). In addition, the sterol glycoside daucosterol was identified in the flowers of *C. nitidissima* (Peng, Yu et al., 2011), while ilexside II [3β-O-[β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl]-pomolic acid-(28→1)-β-D-glucopyranosyl ester] was isolated from its leaves.

Recent studies in Vietnam have identified several chemical constituents in the golden *Camellia* species, particularly saponin. The phytochemical investigation of the leaves of *C. petelotii* revealed the presence of oleanane-type saponins, particularly highly oxygenated derivatives bearing sulfonato functional groups such as chikusetsusaponin IVa, have been isolated. Their chemical structures were elucidated through analyses of HR-ESI-MS and NMR spectral data (Thi Cuc et al., 2023).

1.2.4.5. Other compounds

In addition to the compounds mentioned above, other groups of bioactive constituents have been identified in yellow camellia species, including alkaloids (such as 8-oxocaffeine, deoxythymidine, N-ethyl-5-hydroxy-2-pyrrolidinone, thymine, etc.), fatty compounds (docosane, 1-tricosanol, dodecanoic acid, *n*-tetratriacontanol, β-D-glucopyranoside, 2-[[(9Z,12Z,15Z)-1-oxo-9,12,15-octadecatrien-1-yl]oxy]-3-[(1-oxooctyl)oxy]propyl, etc.), and essential oils

(linalool, phytol, α-eudesmol, geranylacetone, etc.). Notably, several amino acids have also been detected, including glutamate, theanine, γ-aminobutyric acid (GABA), and aspartic acid. In addition, other chemical constituents such as quinic acid, daucic acid, arabinose, rhamnose, glucose, galacturonic acid, vitamin C, and vitamin E have been reported (He et al., 2018; Tran Duc Manh et al., 2019; Trinh Thi Diep, 2022)

Therefore, the golden *Camellia* species contains a wide range of organic compounds with high nutritional value and significant biological activity.

b. Trace elements

Mineral elements, namely Na, K, Mg, Ca, Cu, Zn, Mn, Al, Fe, Ni, P, N, Cr, Co, Ge, Se, Mo, and V, have been detected in *C. nitidissima and C. euphlebia* (He et al., 2018; Xiong et al., 2012). An analysis of leaf mineral composition in four Golden *Camellia* species from Vietnam - *C. gilbertii*, *C. hirsuta*, *C. hakodae*, and *C. petelotii* - showed that all species contained measurable amounts of vanadium (V), zinc (Zn), manganese (Mn), and selenium (Se) (Nguyễn, K. B., Nguyễn, T., & Trần, 2007; Trinh Thi Diep, 2022).

Trace elements, also called trace minerals, are chemical constituents naturally present in plants and animals in small amounts. They are vital for proper growth and metabolic functions, including cellular metabolism, immune system support, and reproductive health in humans. The roles and required balance of these elements vary across living organisms. According to WHO guidelines, 18 trace elements are recognized and classified into three categories: essential, probably essential, and potentially toxic. Some of these elements can also serve as useful diagnostic indicators for healthcare professionals and researchers. Minerals are generally divided into major minerals (macro-minerals) and trace minerals (micro-minerals). Major minerals include calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), chloride (Cl), phosphorus (P), and sulfur (S). Trace minerals include iodine (I), zinc (Zn), selenium (Se), iron (Fe), manganese (Mn), copper (Cu), cobalt (Co), molybdenum (Mo), fluoride (F), chromium (Cr), and boron (B) (Van Campen, 2018)

Minerals play essential roles in the body, supporting vital functions from building strong bones to transmitting nerve impulses, which are crucial for maintaining health and longevity. Certain minerals contribute to hormone production and help regulate a normal heartbeat. Some macro- and micro-elements, such as calcium (Ca), phosphorus (P), and fluoride (F), are integral to the structure of teeth, while calcium (Ca), magnesium (Mg), manganese (Mn), phosphorus (P), boron (B), and fluoride (F) are important for bone health. Many micro-elements, including copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), selenium (Se), and zinc (Zn), serve as critical structural components of numerous enzymes (Gharibzahedi & Jafari, 2017). Minerals

are vital for the body, supporting essential functions such as building strong bones and transmitting nerve impulses, both of which are crucial for overall health and longevity. Some minerals are involved in hormone production and help maintain a regular heartbeat. Macro- and micro-elements like calcium (Ca), phosphorus (P), and fluoride (F) are key components of teeth, while calcium (Ca), magnesium (Mg), manganese (Mn), phosphorus (P), boron (B), and fluoride (F) are essential for bone health. Additionally, micro-elements such as copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), selenium (Se), and zinc (Zn) play critical roles as structural components in many enzymes (FAO and WHO, 2024; Genève, 1998; Gharibzahedi & Jafari, 2017). Calcium is a crucial element for the human body. Calcium salts provide rigidity to the skeleton, while calcium ions are essential for a wide range of metabolic processes. Bone serves as the primary reservoir for calcium in the extracellular fluid. Calcium enters this fluid through intestinal absorption and bone resorption and exits via the gastrointestinal tract, kidneys, and skin, while also being incorporated into bone during formation. Additionally, calcium moves across all cell membranes. Maintaining ionized calcium levels in the extracellular fluid is critical for numerous neuromuscular and cellular functions. Cytoplasmic calcium is regulated by calcium pumps, which either store it in intracellular compartments or expel excess calcium entering by diffusion. Overall, calcium metabolism is primarily focused on sustaining extracellular ionized calcium, which is why deficiencies can lead to osteoporosis (D. D. Wu et al., 1990). Zinc deficiency can lead to a range of health problems, including growth impairments such as weight loss and hair loss, digestive disturbances like diarrhea, loss of appetite, and altered taste, weakened immune function resulting in slow wound healing and higher susceptibility to infections, as well as sexual and reproductive issues, including delayed sexual maturation, reduced sperm production, hypogonadism, and impotence (Badii et al., 2012). Iron deficiency decreases hemoproteins, such as hemoglobin, and impairs Fe-dependent enzymes, resulting in anemia or reduced red blood cell counts. Insufficient dietary iron can slow growth and cognitive development in children, increase pregnancy complications, and reduce work performance in adults (Martínez-Navarrete et al., 2002). Certain minerals, such as selenium (Se) and molybdenum (Mo), are involved in the production of specialized proteins called antioxidant enzymes, which help protect the body against the damaging effects of heavy metals, free radicals, and other harmful substances (Bieżanowska-Kopeć et al., 2016; Leung, 1998).

Additionally, these elements interact with organic compounds to influence the characteristic color and aroma of each golden *Camellia* tea variety. For instance, the deep yellow color of *C. chrysantha* flowers results from the complexation of aluminum ions with quercetin

derivatives, forming a distinctive “yellow-dyeing” system unique to these blossoms (Tanikawa et al., 2008).

1.2.5. Pharmacological activities of Golden *Camellia* species

Research on golden Camellia has primarily focused on their antioxidant and anticancer properties, along with evidence of hypolipidemic, hypoglycemic, antiallergic, immunomodulatory, hepatoprotective, neuroprotective, anxiolytic, and antidepressant effects. Among these, *C. nitidissima* and *C. euphlebia* have been the most extensively studied species. Additionally, investigations into the acute, genetic, and subchronic toxicities of leaf extracts from *C. nitidissima*, *C. euphlebia*, and *C. hakodae* have indicated favorable safety profiles (Huang et al., 2005; Teixeira & Sousa, 2021; Trinh Thi Diep, 2022; M. N. Truong et al., 2024; Wan et al., 2011; Z. Wang et al., 2022).

Research indicates that the flowers and leaves of *Camellia nitidissima* exhibit antioxidant and free radical-scavenging properties. Extracts obtained using different solvents show high oxygen radical absorbing capacity, along with elevated levels of total phenolics and proanthocyanidins, surpassing those found in other yellow-flowered camellia species. Strong correlations between antioxidant activity and phenolic as well as proanthocyanidin content suggest that these compounds are key contributors. Notably, the n-butanol fraction demonstrates the most powerful radical-scavenging effects, efficiently neutralizing DPPH and ABTS radicals, protecting DNA, and mitigating oxidative damage in cell-based models. Animal studies further support these findings. Aqueous and ethanol extracts of the flowers significantly increase antioxidant enzyme activities, such as SOD and GSH-Px, while reducing markers of lipid peroxidation in hyperlipidemic mice. Both polyphenol and saponin fractions display strong activity against a range of free radicals; saponins show antioxidant potency comparable to vitamin C. Overall, the antioxidant capacity of *C. nitidissima* is largely attributed to its rich content of saponins and phenolic compounds (He et al, 2018; Trinh Thi Diep, 2022).

Golden camellia tea (*Camellia nitidissima*) has been the subject of early pharmacological research in animal models, yielding highly encouraging outcomes. Studies suggest that the tea can suppress tumor growth by as much as 33.8%, surpassing the 30% benchmark commonly regarded as effective in cancer therapy. It has also been shown to lower blood cholesterol by 35% and reduce lipoprotein levels by 36.1%, outperforming many conventional pharmaceutical treatments. As a result, golden camellia tea is considered especially beneficial in supporting the management of atherosclerosis related to elevated blood lipid levels. In addition, the tea may help regulate and reduce blood pressure, alleviate constipation within one week, and decrease

blood glucose levels in individuals with diabetes when taken consistently for about three months. Research has further indicated its potential role in supporting cancer treatment, including advanced-stage lymphatic cancer, as well as in managing respiratory, urinary, and gynecological disorders. These wide-ranging health benefits are largely attributed to its high concentration of trace elements such as selenium (Se), germanium (Ge), molybdenum (Mo), manganese (Mn), vanadium (V), and zinc (Zn), which contribute to overall health protection, cancer prevention, and metabolic regulation (Tran Ninh & Hakodae Naotoshi, 2009).

Oxidation is a crucial physiological process in which oxygen is used to produce energy for cellular activities. However, it also generates free radicals that can damage cells and tissues. When free radical production surpasses the body's antioxidant defenses - a condition known as oxidative stress - it can accelerate aging and contribute to serious diseases, including cancer, cardiovascular disorders, diabetes, and neurodegenerative conditions such as Alzheimer's disease (Barry Halliwel, 1996; Frei & To, 2003; Gulcin, 2025; Katharine Brieger et al., 2012; Wypych, 2020).

Studies have reported that excessive production of reactive oxygen species (ROS) plays a significant role in the development of various chronic diseases, including diabetes, cancer, neurodegenerative disorders, and cardiovascular conditions (Katharine Brieger et al., 2012; Liguori I. et al, 2018).

Several studies have shown that consuming fruits and vegetables rich in polyphenols can help neutralize reactive oxygen species (ROS) (Middleton et al., 2000; K. G. and B. Xu, 2017). Therefore, *Camellia* species rich in polyphenolic compounds may offer potential strategies for preventing these diseases. Numerous studies have shown that the antioxidant activity of common tea is closely linked to the concentration of polyphenols in its extracts (Claudia Musial, 2021; Frei & To, 2003). In recent years, multiple studies have reported a similar relationship in Golden Camellia, a rare species of the family Theaceae.

Vietnam stands out as the global hotspot for *Camellia* diversity, hosting more species than any other country in the world (Tran Duc Manh et al., 2019; Tran & Le, 2013). However, despite the rich biodiversity of these plants, research on their biological activities remains very limited. Only a few recent studies have explored the chemical composition of certain Golden Camellia species (Tuyen et al., 2019). Besides, some reports about finding more species and determining the morphology and the distribution area (L. H. Truong et al., 2018; Tuan et al., 2019), the investigation into 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.

Golden Camellia species contain a variety of compounds, including polyphenols, catechins, theaflavins, theasinensins, thearubigins, flavonoids, and gallic acid. Among these, catechins have been shown to correlate strongly with antioxidant activity(W. Wang et al., 2023). Phenolic compounds are the main contributors to tea's antioxidant capacity. As a rich source of natural antioxidants, tea - particularly its phenolic constituents - holds great potential for development as functional beverages or dietary supplements. Furthermore, the antioxidant activity and bioactive compound profiles, including EGCG, theaflavins, and caffeine, vary among different tea varieties. Among them, green tea exhibits the highest antioxidant capacity and total phenolic content, making it the most abundant source of polyphenols, especially catechins (Zhao et al., 2019).

The antioxidant capacities and total phenolic contents of golden *Camellia* depend on various factors, including cultivar type, production location, planting conditions, harvest timing, leaf grade, and manufacturing process (Piboolpunthuwong et al., 2018; Sahar Roshanak, 2016; Yang R, Guan Y, Wang W, Chen H, He Z, 2018). The strong radical-scavenging ability of phenolic compounds may be attributed to their phenolic hydroxyl groups. Several, including salicylic acid, cinnamic acid, vanillic acid, protocatechuic acid, ferulic acid, and caffeic acid, have shown promising results in both in vitro and in vivo studies (Ge et al., 2019). DL-α-Tocopherol, a form of vitamin E, acts as an antioxidant that protects cell membranes from free radical damage, preserving their integrity. It works synergistically with vitamin C, selenium, vitamin A, and carotenoids, enhancing nutritional and therapeutic benefits (Rizvi et al., 2014a). Also, squalene, a triterpene, was detected in most leaves and flowers of *C. tienii* in this study. With a long history of research, this triterpenoid was revealed to exhibit potential therapeutic effects, such as antioxidant properties (Lou-Bonafonte et al., 2018). Furthermore, neophytadiene was the compound found in many plants and algae and is considered an anti-inflammatory and antimicrobial agent (Bhardwaj et al., 2020). Phytol, another diterpene that is a component of chlorophyll, is very well known as an aromatic ingredient, antioxidant, anti-inflammatory, antihyperalgesic, and antiarthritic. This compound was also detected as the most abundant accumulated component of *C. tunghinensis* and *C. euphlebia* leaves (Ge et al., 2019). Stigmasterol, an unsaturated phytosterol, belongs to the triterpene class and exhibits antioxidant, antifungal, anti-inflammatory, and anticancer characteristics (García et al., 1999; Y. Hara et al, 1995).

Macro-minerals and trace minerals also influence the antioxidant capacity of medicinal herbs. Trace elements such as Fe, Cu, Zn, and Mn play a crucial role in regulating free radical reduction. Certain metals, including Fe^{2+} and Cu^{+}, act as pro-oxidant catalysts by participating in the Fenton and Haber-Weiss type reactions, generating hydroxyl radicals (•OH), the most reactive form of ROS. Consequently, unbound ('free') iron and copper in the body can cause severe oxidative damage to DNA, lipids, and proteins (Faustina Barbara Cannea and Alessandra Padiglia, 2025; H. Guenane et al., 2016; Muhammad Junaid Rao et al., 2025; Robert A. Jacob, Ph.D., 1995)

In contrast, several trace elements are indispensable cofactors of the antioxidant enzyme system. Mn, Cu, and Zn are required for superoxide dismutase (SOD): Cu/Zn-SOD functions in the cytoplasm, while Mn-SOD operates in mitochondria - the primary site of ROS production - making it especially important (Mariani E et al., 2008; Robert A. Jacob, Ph.D., 1995; Vishnu D. Rajput et al., 2021)

Transition metals such as iron (Fe) and copper (Cu) have a dual role: they function as catalytic centers in protective enzymes but can act as "double-edged swords" in their free ionic form by promoting radical-generating reactions. Overall, essential trace elements - including selenium (Se), zinc (Zn), manganese (Mn), iron (Fe), and copper (Cu) - are critical components of the body's endogenous antioxidant defense system. The balance between their enzyme- or protein-bound forms and free ionic forms is a key determinant of their antioxidant activity (Mariani E et al., 2008; Paradorn Ngamdee et al., 2016; Vishnu D. Rajput et al., 2021)

Finally, studies on acute and sub-chronic toxicity in several *Golden Camellia* species have demonstrated their safety. For instance, research on *C. hakodae* collected from Tam Dao National Park showed that the species is safe and exhibits no toxic effects (H. H. Nguyen et al., 2020). Similarly, studies on the acute and sub-chronic toxicity of leaf extracts from *C. euphlebia* have also demonstrated their safety (He & Xu, 2022)

1.2.6. Metabolomics approach

Golden *Camellia* is an attractive member of the genus *Camellia*, within the Theaceae family. The most well-known morphological characteristic that distinguishes these species from other *Camellia* species is the yellowish flowers. Phytochemical profile studies show that golden camellia is a valuable reservoir of bioactive compounds, including amino acids, polysaccharides, flavonoids, volatile compounds, and many other mineral elements. In terms of pharmacological effect, numerous investigations have demonstrated that the crude extracts, fractions, and isolated substances from the Chinese golden camellia species possess antioxidant, anticancer,

hypolipidemic, hypoglycaemic, anti-allergic, hepatoprotective, neuroprotective, anxiolytic, and antidepressant activities (Ana Margarida Teixeira and Clara Sousa, 2021; Zheng et al., 2022). However, only a humble number of studies on Vietnamese domestic golden camellias have been conducted recently.

Metabolomics approaches, including untargeted and targeted metabolomics, aim to measure as many small molecules within an organism as possible. They are often used in combination for the discovery and accurate content determination of metabolites (Lelli et al., 2021). Targeted analysis characterizes defined groups of compounds (H. Wang et al., 2021). Untargeted analysis comprehensively and systematically analyzes all the measurable analytes in a sample, including unknown metabolites. Both of these approaches are thus very necessary to provide information about the comprehensive chemical profile of plant tissues because, until now, no ideal universal technique has reached this purpose. Metabolomics analysis typically requires a variety of specialized techniques. Indeed, an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system with an electrospray ionization (ESI) source has been widely used as an efficient method to determine specific elements in tea. Rapid LC can lower solvent consumption, making the procedure more repeatable, reproducible, and sensitive. This method furthermore reduces the risk of compound degradation, which is specifically suitable for investigating a thermally sensitive compound like catechins (the main bioactive component in tea) (Nguyen et al., 2022). Another interesting group compound in tea is amino acids, which can be characterized by UPLC-MS/MS by adding more special post-column derivatization (Salazar et al., 2012). Using conventional HPLC, the complete separation and quantification of 23 amino acids typically takes over 35 minutes for a single chromatographic analysis (Paramás et al., 2006). UPLC offers superior peak capacity, resolution, and sensitivity, along with a markedly increased analysis speed (Nováková et al., 2006), so it helps to shorten analysis time. It requires only 8 minutes to achieve efficient separation of 26 amino acids in royal jelly, representing just one-fourth of the HPLC analysis time, while providing superior resolution (Liming et al., 2009). The hyphenated system of UPLC-MS/MS is also powerful for widely targeted metabolomics analysis, in which a large number of references were injected into the system, turning it into an identification tool by quantifying each compound within the list of references (Sawada et al., 2009). Besides, gas chromatography-mass spectrometry (GC-MS) is a relatively low-cost but high-throughput detection technique (with high resolution, high sensitivity, and strong identification ability) that is more suitable for untargeted volatile compounds profiling (Manickam et al., 2023).

Minerals are important elements for humans. The methods used to determine it are especially important experimentally. The Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) technique is a highly advanced analytical method widely applied for the determination of trace elements and heavy metals in medicinal plant materials. ICP-MS operates on the principle of sample ionization by high-frequency argon plasma, after which the generated ions are separated and quantified according to their mass-to-charge ratio (m/z) using a mass spectrometer. This technique offers exceptional sensitivity, enabling the detection of elements at extremely low concentrations (from parts per billion to parts per trillion) and allowing the simultaneous quantification of multiple elements in a single analysis. The analytical procedure generally includes several stages: sample preparation (commonly through acid digestion), nebulization**,** ionization**,** ion separation**,** and signal detection. ICP-MS is considered the gold standard for elemental analysis and quality control of medicinal plant materials, particularly for determining toxic heavy metals such as Pb, Cd, As, and Ag, as well as essential elements such as Fe, Zn, Cu, Mn, and other elements (Wilschefski & Baxter, 2019).

The trace elements Cu, Zn, and Mn are critical components for antioxidant processes, and a deficiency of any one of these elements can result in an impairment of the functioning of the overall antioxidant system (Zidenberg-Cherr and Keen, 1991). Several exogenous dietary micronutrients contribute significantly to the body's antioxidant defense system. Vitamin C (ascorbic acid) acts as a potent scavenger of reactive oxygen species in aqueous environments, including blood plasma and the cytosol of cells. Vitamin E (tocopherol) provides antioxidant protection within lipid environments, particularly by safeguarding the polyunsaturated fatty acids of cellular membranes from oxidative damage. Carotenoids, abundantly present in fruits and vegetables, are also believed to protect lipid-rich tissues against oxidative stress. In addition, a wide range of plant-derived compounds, such as flavonoids, polyphenols, and other phytochemicals, exhibit strong antioxidant activity and contribute to the overall maintenance of redox balance in the human body (Robert A. Jacob, Ph.D., 1995).

The evaluation of antioxidant activity in medicinal plants is vital for assessing their ability to neutralize free radicals and reduce oxidative stress–related damage. Among *in vitro* assays, DPPH and ABTS methods are the most frequently used because of their simplicity, accuracy, and reproducibility. The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay measures the electron- or hydrogen-donating capacity of antioxidants by monitoring the reduction of the purple DPPH radical to a yellow compound, with absorbance measured at 517 nm (Brand-Williams et al., 1995). The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay similarly quantifies the scavenging of the blue-green $ABTS^{+}$ radical cation, detected at 734 nm (Re et al.,

1999). While DPPH is limited to lipophilic systems, ABTS can assess both hydrophilic and lipophilic antioxidants. The combined use of these assays provides a comprehensive and reliable evaluation of the total antioxidant potential in plant extracts and natural products.

Chapter 2: MATERIALS AND METHODS

2.1. Materials

Materials

Plant collection: The endemic *Camellia hakodae, Camellia phanii, Camellia tamdaoensis, and Camellia tienii* were collected in Tam Dao, and two species commonly cultivated in Quang Ninh province, including *Camellia petelotii* and *Camellia euphlebia*. They were initially collected at Hop Chau ward, Tam Dao district, Vinh Phuc province (GPS: 21°26'18.0'' N 105°36'17.3'' E) in December 2021, and Quang Minh ward, Hai Ha district, Quang Ninh province (GPS: 21°28'45.9'' N 107°46'07.8'' E) in April 2022. These samples were identified by Dr. Nguyen The Cuong from the Institute of Ecology and Biological Resources (Institute of Biology) (Annex 1: Plant identification). The specimen dossiers were deposited in the Herbarium of this Institute to store and serve for further investigation.

Sample preparation

The leaves were cut into small pieces, each 1 cm in length, along the tissue. With mature leaves, these pieces were equally divided to ensure that the two parts contained homogenous tissues. One part was immersed immediately in liquid nitrogen, stored at -80°C, and freeze-dried in a lyophiliser for 48h. The remaining tissue was dried in the oven at 45°C until no change in sample weight was detected. For young tissues, all pieces were treated by the oven-drying method. The flower tissue was dried in the oven at 45°C until no change in sample weight was observed. Dried leaves and flowers were then ground initially by a rough homogenizer before being weighed for analysis.

List of Chemicals

Chemicals such as 10-Camphorsulfonic acid (CPS) and lidocaine were purchased from Thermo Fisher Scientific, USA. A catechins mix including 7 catechins ((+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin-3-gallate (EGCG), (-)-gallocatechin-3-gallate (GCG) and (-)-catechin-3-gallate (CG)), was purchased from Cerilliant® (Round Rock, Texas, USA), with the concentration of 100 µg/mL, the accuracy was validated by certified reference material

Oxidized hydrolysate standard, including 23 amino acids (L-theanine, gamma amino butyric acid, L-glutamine, L-cysteic acid, taurine, D, L-methionine sulfoxide, L-methionine sulfone, L-aspartic acid, L-threonine, L-serine, L-glutamic acid, L-proline, glycine, L-cystine, L-valine, L-methionine, L-isoleucine, L-tyrosine, L-phenylalanine, L-histidine, L-ornithine, L-lysine, L-arginine) with 2.5 mM concentration of each component, all loading buffers and reagents for derivatization were provided by Biochrom, United Kingdom.

ICP-MS tuning solution ICP multi-element standard VI from Merck - Germany. The standard solution of the analyzed elements includes Na, Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Sr, Mo, Ag, Cd, Ba, Pb, and Bi from the concentration of 1.00 - 2000 ppb, used in the smart calibration process.

Pentadecane was used as an internal standard and was purchased from Aladdin Company (China). An internal standard is a compound having similar physicochemical properties to the analytes and should not be present in the sample. It was added in a constant amount to blank, calibration standards, and samples. An internal standard was used to monitor the recovery of the whole analytical procedure. An alkane mixture (C_8-C_{40}) was used as the analytical standard. Alkane standard solution C_8-C_{40} was prepared and used for the measurement of linear retention indices (LRI) in the identification of volatile components in samples using GC/MS.

Formic acid (HPLC grade), Methanol (HPLC grade), Chloroform ($CHCl_3$) (HPLC grade), and Acetonitrile solvents (HPLC grade) are from Thermo Fisher Scientific-USA.

Nitric acid of superior grade (65%-68%) was purchased from Scharlab S.L., Spain. Hydrogen peroxide 33% was bought from VWR-BDH - France. Use double-distilled water prepared in the USTH laboratory on the device to dilute samples.

DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), and ascorbic acid were purchased from Aladdin, China. Potassium persulfate, sodium carbonate, DMSO (Dimethyl Sulfoxide), trolox, and other solvents were purchased from Sinopharm Chemical Reagent Co.Ltd (Shanghai, China).

2.2. Methods

Metabolomics analysis are comprehensive analyses of small molecules (metabolites, organic molecule detectable in the body with a MW < 1500 Da) in plant tissues and cells. In this study using untargeted analysis, targeted analysis and widely targeted.

Therefore, the research contents were carried out according to the following diagram:

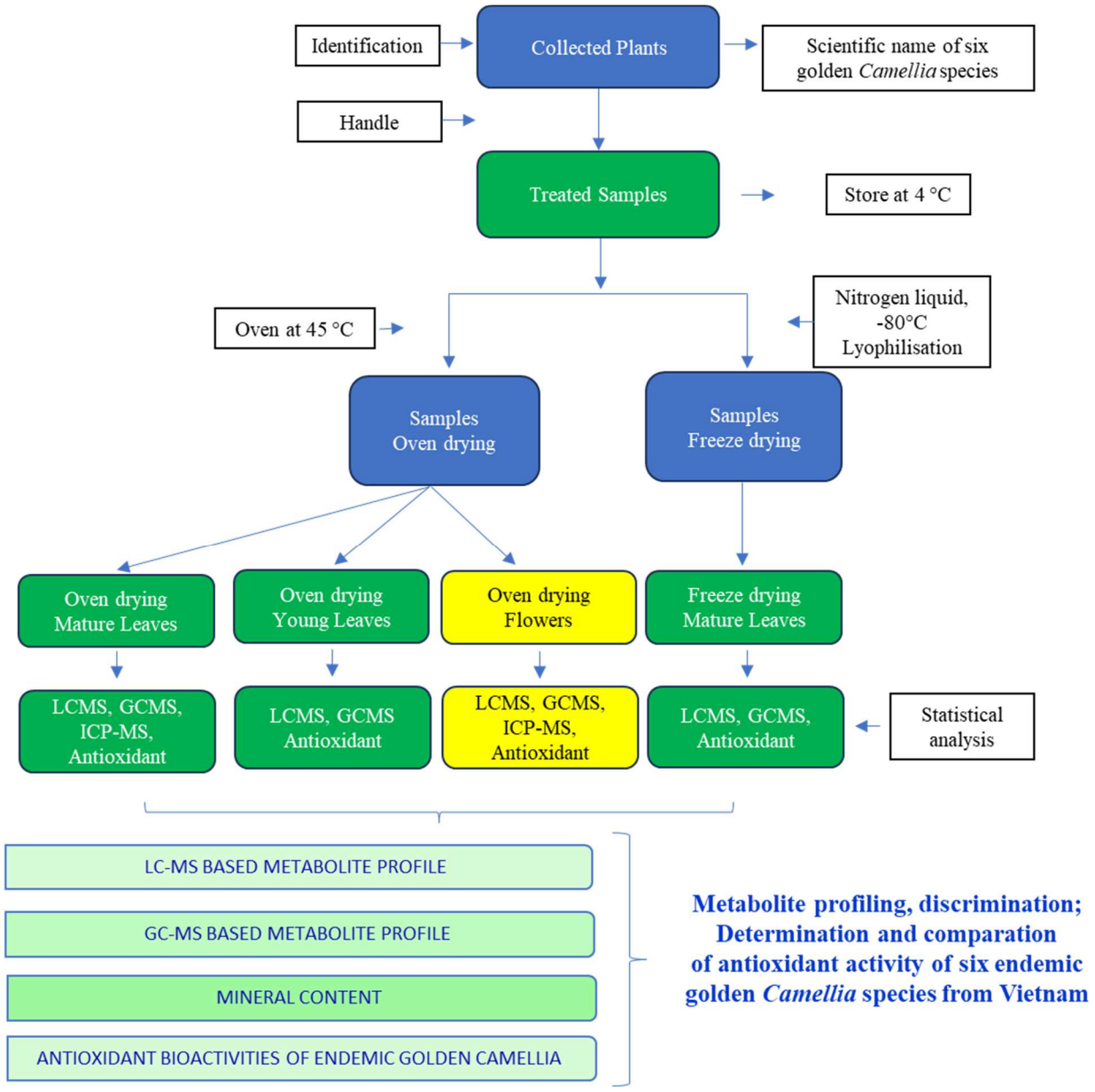


Figure 2.1. Research diagram of metabolomics and antioxidant activity of golden *Camellia*

2.2.1. Methods of LC-MS-based metabolite profile

Samples

Table 2.1 Sampling information and sample size (LC-MS)

Sample code	Species	Sample type	Sample size	Sample location
LGT1.1-1.10	*C. hakodae*	Freeze-drying mature leaves	10	Tam Dao, Vinh Phuc
LGK1.1-1.10	*C. hakodae*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc

LN1.1-1.10	*C. hakodae*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
LGT2.1-2.10	*C. phanii*	Freeze-drying mature leaves	10	Tam Dao, Vinh Phuc
LGK2.1-2.10	*C. phanii*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
LN2.1-2.10	*C. phanii*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
LGT3.1-3.10	*C. tamdaoensis*	Freeze-drying mature leaves	10	Tam Dao, Vinh Phuc
LGK3.1-3.10	*C. tamdaoensis*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
LN3.1-3.10	*C. tamdaoensis*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
LGT4.1-4.10	*C. tienii*	Freeze-drying mature leaves	10	Tam Dao, Vinh Phuc
LGK4.1-4.10 (& L1-25)	*C. tienii*	Oven-drying mature leaves	10 (& 25)	Tam Dao, Vinh Phuc
LN4.1-4.10	*C. tienii*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
HHLK1.1-1.10	*C. petelotti*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHLT1.1-1.10	*C. petelotti*	Freeze-drying mature leaves	10	Hai Ha, Quang Ninh
HHLK2.1-2.10	*C. euphlebia*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHLT2.1-2.10	*C. euphlebia*	Freeze-drying mature leaves	10	Hai Ha, Quang Ninh
HTDD3.1-3.12	*C. tamdaoensis*	Oven-drying flower	10	Tam Dao, Vinh Phuc
H1-H25	*C. tienii*	Oven-drying flower	25	Tam Dao, Vinh Phuc

**** Targeted analysis***

A liquid chromatography (ACQUITY UPLC H-Class, Waters, US) coupled to a mass spectrometer (Xevo TQD Triple Quadrupole, Waters, US) system was used for targeted analysis, including catechins, caffeine, and amino acid profile (K. O. T. Nguyen et al., 2022; Salazar et al., 2012).

To target the major group of Camellia genus, a protocol to quantify simultaneously seven catechins ((+)-catechin, (-)-catechin 3-gallate, (-)-epicatechin, (-)-epicatechin-3-gallate, (-)-epigallocatechin 3-gallate, (-)-gallocatechin (-)-gallocatechin) 3-gallate and caffeine in methanolic extracts of leaves and flowers of six species were developed. Sample preparation, sample extraction, UPLC-MS/MS analysis, and data treatment followed the previous procedure mentioned in Nguyen et al.'s protocol (Nguyen et al., 2022).

The catechin profile of leaves was quantified by using ultra-performance liquid chromatography coupled to a tandem mass spectrometer. For sample extraction, 10 mg powder leaves were added to 1 mL MeOH 80% in an Eppendorf tube under ultrasonic treatment for 15 min, before being filtered through a 0.22 µm membrane and injected into an ACQUITY UPLC H-Class system (Waters Corporation, US).

The mass spectrometry was carried out on a Xevo TQD MS/MS triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. The mixture of catechin standards was infused directly into a triple quadrupole mass spectrometer to optimize the cone voltage (CV) in Q1 and collision cell energy (CE) in Q2 using an automatic tuning process by

the IntelliStart tool (Waters Corporation, US). The following parameters of ESI source were respectively set up for negative ion detection mode: desolvation temperature of 350°C, capillary voltage of 2.4 V, extractor voltage of 3 V, RF lens (hexapole) voltage of 2.5 V, nebulization gas flow rate of 650 L/h, cone gas flow rate of 150 L/h, and CV of 35 V. SRM was performed with auto dwell time in transitions for negative ion detection mode: 304.97→124.88 (CV 50 V, CE 18 eV), 288.97→108.88 (CV 50 V, CE 28 eV), 457.03→168.88 (CV 46 V, CE 16 eV), 441.03→168.88 (CV 50 V, CE 18 eV). For separation, the analytical column C18 BEH (100 mm× 2.1 mm i.d; 1.7 µm, Waters Corporation, US) was kept at constant temperature (40°C) during chromatographic separation, with an injection volume of 1.5 µL. The samples were stored at 4 °C in a sample manager. The UPLC condition was as in Table 2.2. The data were acquired and processed using MassLynx software version 4.1 (Waters Corporation, US).

Table 2.2. UPLC Parameters: Gradient Table of UPLC-QqQ-MS/MS Analysis for catechins.

No	Time (min)	Flow (mL/min)	Solvent A (%)	Solvent B (%)
1	Initial	0.3	75	25
2	1	0.3	70	30
3	2.50	0.3	70	30
4	2.51	0.3	75	25
5	5.51	Stop	75	25

Solvent A: 0.01% (v/v) formic acid in water. Solvent B: Methanol 100%.

For the amino acids, 26 amino acids, including L-theanine, gamma-aminobutyric acid, L-glutamine, L-cysteic acid, taurine, D, L-methionine sulfoxide, L-methionine sulfone, L-aspartic acid, L-threonine, L-serine, L-glutamic acid, L-proline, glycine, L-cystine, L-valine, L-methionine, L-isoleucine, L-tyrosine, L-phenylalanine, L-histidine, L-ornithine, L-lysine, ammonia, L-arginine, L-alanine, L-leucine were quantified in the extracts by the method of Salazar (Salazar et al., 2012). The free amino acids in the sample were derivatized using a 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate kit (Waters, US) before their separation and detection.

For sample extraction, the infusion was achieved by adding 1 mL of boiling water to 100 mg of finely powdered sample before ultrasonic extraction for 15 min at 70°C. Then, the mixture was extracted for 45 min while shaking and heating using a ThermoMixer® C (Eppendorf, Germany). After centrifugation, the supernatants were filtered through 0.22 µm membranes and loaded into vials. The samples were injected through the column Acquity UPLC BEH C18 130A, 1.7 µm, 1x100 mm at 55 °C, using mobile phase of eluent A (acetonitrile 10%, formic acid 6%, ammonium formate in water 84%) and eluent B (acetonitrile). Gradient elution was applied, starting with 99.9 % A at 0.54 min, ramping to 90.0% at 5.74 min, followed by 78.8% at 7.74 min, 40.4 % at 8.04-8.64 min, then increased again to 99.9% at 8.73-9.50 min, with a flow rate

of 0.3 mL/min. The sample chamber was set at 25 °C. The analysis procedure was managed using MassLynx 4.1 software, and then the data acquisition, processing, and reporting for quantitative results were performed using the TargetLynx application. The UPLC condition was as in Table 2.3

Table 2.3. UPLC parameters: gradient table of UPLC-QqQ-MS/MS analysis for amino acid.

No	Time (min)	Flow (mL/min)	Solvent A (%)	Solvent B (%)
1	Initial	0.3	99.9	0.1
2	0.54	0.3	99.9	0.1
3	5.74	0.3	90.0	10.0
4	7.74	0.3	78.8	21.2
5	8.04	0.3	40.4	59.6
6	8.64	0.3	40.4	59.6
7	8.73	0.3	99.9	0.1
8	9.50	Stop	99.9	0.1

Solvent A (acetonitrile 10%, formic acid 6%, ammonium formate in water 84%) and eluent B (acetonitrile).

**** Widely targeted metabolomics analysis***

Widely targeted metabolomic analysis was performed according to the protocols described by Sawada and Uchida et al (Sawada et al., 2009; Uchida et al., 2020). Mass spectrometry conditions, including cone voltage, collision energy, and polarity optimized for authentic reference standards, were applied for metabolite identification using automated flow injection analysis with a triple quadrupole detector. The peak area values were determined using MRMPROBS (Tsugawa et al., 2013).

Sample extraction

Precisely 4 mg of material was weighed into a 2 mL tube. Consequently, 1 mL of solvent mixture including 0.1% (v/v) formic acid, 80% (v/v) methanol, and two internal standards (8.4 nM of lidocaine for negative ionisation and 210 nM of 10-camphorsulfonic acid for positive ionisation) was adjusted to the tube. The extraction was performed in a bead-shocker for 2 min at 1000 rpm. The supernatant solution was separated from solid contaminants by centrifugation at 10 000 rpm for 1 min.

The extracted solutions were suspended in the extract solvent to a final volume of 100 µL. One-quarter of the solution was evaporated, and the residual extract was dissolved in 250 µL LC-MS grade water. The obtained solution was filtered through a 0.45 µm membrane (MZHVN0W50; Merck Millipore, Darmstadt, Germany), and 1 µL of the final solution was injected into the LCQqQ-MS system (Nexera MP/LCMS-8050; Shimadzu Corporation). The

separation was conducted on the ACQUITY UPLC HSS T3 Column, 100Å, 1.8um, 1mm X 50mm column with parameters in the table below:

Table 2.4. UPLC parameters: gradient table of UPLC-QqQ-MS/MS for widely targeted metabolomics analysis.

No	Time (min)	Flow (mL/min)	Solvent A (%)	Solvent B (%)
1	Initial	0.24	99.9	0.1
2	0.25	0.24	99.9	0.1
3	0.40	0.24	91.0	9
4	0.80	0.24	83.0	17
5	1.90	0.24	0.1	99.9
6	2.10	0.24	0.1	99.9
7	2.11	0.24	99.9	0.1
8	2.70	Stop	99.9	0.1

Solvent A: 0.1% formic acid in water. Solvent B: 0.1% formicacid in acetonitrile (v/v)

The MS parameter was set as follows: interface voltage 4.0 and 3.0 kV for positive and negative mode, interface temperature 300 °C, DL temperature 250 °C, heat block temperature 400 °C. nebulizer gas flow 3.0 L/min, drying and heating gas flow 10.0 L/min, dwell time 0.006s.

Statistical analysis

MetaboAnalyst 6.0 software was applied to reveal the species-specific, maturity-specific, and post-harvesting-specific discriminations of golden *Camellia* leaves. The score plots illustrated the difference among the chemical profiles of samples, and VIP score plots visualized markers contributing to the discrimination. These plots were conducted using MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca). The Principal Component Analysis (PCA) was used to visualize the discrimination of the metabolite profiles of flower and leaves samples using FactoMineR and ggplot2, along with Factoextra packages (John Maindonald and W. John Braun, 2010; Sebastien Le, 2008; Sekhar et al., 2018)

Partial least squares discriminant analysis (PLS-DA) was performed under the mixOmics R-package (Kassambara, 2017; Rohart et al, 2017).

PLS-DA (Partial Least Squares - Discriminant Analysis) is a supervised classification method that adapts PLS regression to separate predefined groups. It works well for high-dimensional, correlated data, common in metabolomics and other omics fields. The model identifies latent variables that both capture variation in the predictors (X) and maximize discrimination in the response (Y).

VIP (Variable Importance in Projection) scores indicate how much each variable contributes to the PLS-DA model. A higher VIP value means the variable plays a stronger role in class separation. As general guidance, VIP > 1.0 suggests an important variable, values between 0.8 - 1.0 are moderately relevant, and values below 0.8 typically contribute less. VIP

scores are relative and should be interpreted alongside loadings, coefficients, and proper model validation to avoid overfitting. Together, PLS-DA and VIP help identify key variables that drive differences between groups in complex datasets.

2.2.2. Methods of GC-MS-based metabolite profile

Samples

Table 2.5: Sampling information and sample size (GC-MS)

Sample code	Species	Sample type	Sample size	Sample location
LGK1.1-1.10	*C. hakodae*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
HTD1.1-1.5	*C. hakodae*	Oven-drying flowers	05	Tam Dao, Vinh Phuc
LGK2.1-2.10	*C. phanii*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
HTD2.1-2.5	*C. phanii*	Oven-drying flowers	05	Tam Dao, Vinh Phuc
LGK3.1-3.10	*C. tamdaoensis*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
HTD3.1-3.5	*C. tamdaoensis*	Oven-drying flowers	05	Tam Dao, Vinh Phuc
LGK4.1-4.10	*C. tienii*	Oven-drying leaves	10	Tam Dao, Vinh Phuc
HTD4.1-4.5	*C. tienii*	Oven-drying flowers	05	Tam Dao, Vinh Phuc
HHLK1.1-1.10	*C. petelotti*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHH1.1-1.5	*C. petelotti*	Oven-drying flowers	05	Hai Ha, Quang Ninh
HHLK2.1-2.10	*C. euphlebia*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHH2.1-2.5	*C. euphlebia*	Oven-drying flowers	05	Hai Ha, Quang Ninh

To broaden the identification of volatile metabolites in the sample, an untargeted analysis was performed by a hyphenated system including a Thermo Fisher Scientific™ DSQ / TRACE™ GC Ultra with a TriPlus autosampler linked to a DSQ II mass spectrometer. GC-MS-based metabolite profile analysis was performed according to the protocols described by Wang and Yang (H. Wang et al., 2021; Yang et al., 2012)

Sample extraction

The samples were extracted according to the following procedure: for each sample, 200 mg of powder was weighed into a glass vial. A volume of 1.3ml of Chloroform containing internal standard (Pentadecane) with the final concentration of 0.046 mg/mL and 75 µL of tetramethylammonium hydroxide (TMAH) was added to each sample. Samples were extracted using an ultrasonic extractor for 30 minutes at room temperature, then centrifuged for 5 minutes at 4000 rpm. Extraction was then filtered through Pasteur pipettes, including small pieces of Kimtech paper, and transferred to a vial for GC-MS injection. All samples and the alkanes mixtures (C_8- C_{40}) were injected into the GC-MS system for profiling analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

All samples and the alkane mixture solution were injected into the GC-MS system with 1 µL. Profiling analysis was performed using a Thermo Scientific™ TRACE™ 1310 GC system equipped with a Thermo Scientific™ 1310 autosampler connected to the ISQ 7000 mass spectrometer. A GC column (30 m × 0.25 mm × 0.25 µm; Thermo Scientific™) was used with purified Helium as the carrier gas at a constant flow rate of 1.2 ml/min with mode splitless. The oven temperature was held at 50 °C for 5 min, increased to 220 °C at a rate of 3 °C/min, and held at 220 °C for 5 minutes; and increased to 240 °C at a rate of 10º C/min, and finally kept at 240 °C for 5 minutes. The injector and ion source temperatures were set at 200 °C, respectively, and MS was scanned at 45 eV over the range of 50 - 800 a.m.u. GC-MS was operated in the full scan mode, and the peak areas were determined by Xcalibur software (Thermo Scientific™).

Statistical analysis

The statistical analysis was conducted in the R language with packages in R Studio. Peak finding, peak integration, and retention time correction of the GC-MS chromatograms were performed with Xcalibur or the XCMS R-package (R version 4.0.3) (http://www.R-project.org). The alignment of peaks was carried out by comparing the retention time and mass fragments. The peak selection was performed based on the criteria: each peak should exist in at least three samples of one species. The n-alkane mixture (C_8 to C_{20}) was used to obtain the Kovats' retention indices for each peak. Compound identification in gas chromatography mass spectrometry (GC-MS) is currently achieved by comparing a query mass spectrum with reference mass spectra in a library via spectrum matching. Identification of the selected peaks was made by searching AMDIS and the NIST2017 data library. Selected peaks were quantified by the relative ratio by taking the peak area of this peak divided by the sum of peak areas of all detected peaks in the tea leaf sample.

Integrated peaks (output of XCMS package) of the mass (m/z) fragments were normalised across all the samples by internal standard peak area. The information on analytes and peak areas for each analyte was saved in files as comma-separated values (csv) files, which served as input data for statistical analysis. A PLS-DA model was used to discriminate the metabolite profile according to species and tissues.

The statistical analysis was conducted in the R language with packages in R Studio (http://www.Rproject.org). The Principal Component Analysis (PCA) was used to visualize the discrimination of the metabolite profiles of flower and leaves samples using FactoMineR (Sebastien Le, 2008) and ggplot2, along with Factoextra packages (Kassambara, 2017). Heatmap and volcano plots were created by using MetaboAnalysis 6.0

(https://www.metaboanalyst.ca) (John Maindonald and W. John Braun, 2010; Kabacoff, 2020; Kassambara, 2017; Rohart et al, 2017; Sebastien Le, 2008)

2.2.3. Methods of mineral content

a. Samples

Table 2.6: Leaf and flower samples of six golden *Camellia* species for running IPC-MS

Sample code	Species	Sample type	Sample size	Sample location
LGK1.1-1.10	*C. hakodae*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
HTD1.1-1.3	*C. hakodae*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
LGK2.1-2.10	*C. phanii*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
HTD2.1-2.3	*C. phanii*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
LGK3.1-3.10	*C. tamdaoensis*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
HTD3.1-3.3	*C. tamdaoensis*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
LGK4.1-4.10	*C. tienii*	Oven-drying leaves	10	Tam Dao, Vinh Phuc
HTD4.1-4.3	*C. tienii*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
HHLK1.1-1.10	*C. petelotti*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHH1.1-1.3	*C. petelotti*	Oven-drying flowers	03	Hai Ha, Quang Ninh
HHLK2.1-2.10	*C. euphlebia*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHH2.1-2.3	*C. euphlebia*	Oven-drying flowers	03	Hai Ha, Quang Ninh

b. Preparation of samples

The procedure concerning preparations of samples was adopted from Ye and Zang (Ye et al., 2017; Zhang, 2019) with modifications. 200 mg of powder sample was transferred into a Teflon digestion vessel with the previously added 5 ml HNO_3. After setting overnight, samples were then digested using 1 ml H_2O_2 until solutions were transparent using the Multiware 5000 microwave digestion apparatus (Anton Paar, Italy). The vessels were sealed and placed in the turntable of the microwave oven for digestion under the setup conditions and controlled by the system software. Two stages of temperature were installed, including a first 20 min for increasing the temperature to 200 °C (P=100 bar) and then maintaining for 20 min before automatically refreshing to room temperature.

When the digestion had finished, the vessels were carefully uncapped under the fume hood to avoid the acid fumes. The samples were then transferred to a 50 mL volumetric flask and diluted with UPW. The prepared solution was filtered through a cellulose nitrate membrane 0.22 µm before being injected into the ICP-MS system. For the long-term analysis, the digested sample was stored at 4 °C in a fridge.

A digestion solution prepared without plant material was used as a blank. Blank samples were prepared concurrently with the experimental samples to monitor background levels during extraction. Except for the omission of the golden *Camellia* powder addition step, the preparation

procedures for the blank samples were identical to those of the golden *Camellia* samples. All samples were digested in a randomized order.

c. Determination of mineral content

ICP-MS with Model iCAP RQ - Thermo Scientific - Germany are the instruments that were used for element analysis. The ICP RF power was set to be 1550 W, the plasma gas flow rate was 14 L/min, and the carrier/nebulizer gas flow rate was 0.99 L/min. The position of the torch, carrier gas flow rate, quadrupole ion deflector (QID) voltage, and dual-mode correction of the detector of ICP-MS were all optimized. ICP-MS tuning solution ICP multi-element standard VI from Merck, Germany, was used in the smart tune procedure. The main technique indicators all met the requirements.

d. Statistical analysis

Qtegra intelligent scientific data solution software was used for concentration processing of the detected elements. The raw data of response intensity (counts per second, cps) of each element were collected, and then the calibration curves were plotted by using standards working solution to obtain the corresponding concentrations of all elements in all samples.

GraphPad Prism 10 was used to analyze data, statistical analysis, perform statistical tests like t-tests and ANOVA

2.2.4. Methods of antioxidant activity

a. Samples

Table 2.7: Leaf and flower samples of six golden *Camellia* species for study antioxidant

Sample code	Species	Sample type	Sample size	Sample location
LGK1	*C. hakodae*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
LGT1	*C. hakodae*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
HTD1	*C. hakodae*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
LGK2	*C. phanii*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
LGT2	*C. phanii*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
HTD2	*C. phanii*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
LGK3	*C. tamdaoensis*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
LGT3	*C. tamdaoensis*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
HTD3	*C. tamdaoensis*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
LGK4	*C. tienii*	Oven-drying mature leaves	10	Tam Dao, Vinh Phuc
LGT4	*C. tienii*	Oven-drying young leaves	10	Tam Dao, Vinh Phuc
HTD4	*C. tienii*	Oven-drying flowers	03	Tam Dao, Vinh Phuc
HHLK1	*C. petelotti*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHH1	*C. petelotti*	Oven-drying flowers	03	Hai Ha, Quang Ninh
HHLK2	*C. euphlebia*	Oven-drying mature leaves	10	Hai Ha, Quang Ninh
HHH2	*C. euphlebia*	Oven-drying flowers	03	Hai Ha, Quang Ninh

b. Sample preparation

Extracts from tea leaves and flowers were prepared by mixing medicinal plant powder and methanol (ratio 1:10 W/V). The extracts were sonicated for 30 minutes at 50°C before being filtered and evaporated by a speed-vac (Labconco, Fisher Scientific, Austria) until a constant weight. These extracts were stored at 4°C for further.

c. Determination of antioxidant activity

The absorbance measurements were performed using a spectrophotometer (SpectraMax® iD5 Multi-Mode Microplate Readers, VWR, Germany).

**** Method for determining antioxidant activity by DPPH radical scavenging assay***

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was conducted following the methods published by (Abramovič et al., 2018; Braca, A., De Tommasi, N., Di Bari, L., Pizza, C., Politi, M., & Morelli, 2001; Brand-Williams et al., 1995).

The test samples were first prepared as stock solutions in methanol and then diluted with deionized water to obtain a series of test concentrations. L-ascorbic acid was used as the reference control and was similarly diluted with deionized water to prepare a corresponding concentration range.

DPPH dissolved in 100% methanol at a concentration of 0.25 mM. Add 100 µL of each sample concentration to the wells of a 96-well plate, and subsequently introduce an equal volume of the DPPH solution, maintaining a 1:1 ratio. The control wells contained no test sample, consisting only of 100 µL of water and 100 µL of DPPH. Additional wells containing 100 µL of the methanol-dissolved samples at the corresponding concentrations were also prepared. The final extract concentrations ranged from 4.00 to 500.00 µg/mL (4.00, 20.00, 100.00, and 500.00 µg/mL). The same adjustment was made to Ascorbic acid to achieve a final concentration ranging from 0.8.00 to 100.00 µg/mL (0.80, 4.00, 20.00, and 100.00 µg/mL)

The reaction mixture was incubated at room temperature for 20 minutes. After incubation, the absorbance of the DPPH solution mixed with extract samples or ascorbic acid was measured at 517 nm using a spectrophotometer to assess DPPH radical scavenging activity. All samples were tested in triplicate, with the entire process conducted in darkness. 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 plotted against concentrations, and the IC_{50} value (µg/mL) was determined as the concentration required to achieve 50% scavenging capacity.

**** Method for determining antioxidant activity by ABTS radical scavenging assay***

The 2,2'- azinobis - (3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay was performed following the procedure published by (Re et al., 1999; Saeed et al., 2012) with some modifications. The samples were diluted in deionized water to obtain concentrations of 10,000, 2,000, 400, and 80 µg/mL. Trolox, used as the reference control, was similarly prepared in deionized water at concentrations of 2,000, 400, 80, and 16 µg/mL.

ABTS (7 mM) was mixed with potassium persulfate (2.45 mM) and allowed to stand in the dark for 16 hours at room temperature. Before the assay, the resulting $ABTS^{\bullet+}$ solution was diluted with acetate buffer to achieve an absorbance of 0.70 ± 0.05 at 734 nm. Subsequently, 190 µL of $ABTS^{\bullet+}$ was added to 10 µL of each prepared sample, yielding final concentrations of 500, 100, 20, and 4 µg/mL per well. A 1% DMSO solution served as the control, and wells containing only deionized water were used as blanks.

The same adjustment was made to Trolox to achieve a final concentration ranging from 0.8.00 to 500.00 µg/mL (0.80, 4.00, 20.00, and 100.00 µg/mL). The solution's absorbance was measured using a spectrophotometer at 734 nm to assess the ABTS radical scavenging activity. The tests were conducted at three different times, and the entire process was carried out in the dark. The radical inhibition rate was calculated using the following formula:

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

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 plotted against concentration, and the IC_{50} value (µg/mL) was determined from the dose-response curve as the concentration at which the inhibition reached 50%.

d. ***Data analysis***

The results are expressed as the mean ± standard deviation. The IC_{50} values and the differences between means of each group were analyzed using a *t*-test and Analysis of Variance (ANOVA) by GraphPad Prism 10. The p-value of < 0.05 was considered statistically significant.

Chapter 3. RESULTS AND DISCUSSION

PART 3.1. STUDY ON LC-MS BASED METABOLITE PROFILE OF ENDEMIC GOLDEN *CAMELLIA*

3.1.1. LC-MS-based metabolite profiling

A total of 440 compounds were detected in at least one sample by a widely targeted metabolomics analysis. Regarding structural elucidation, this method provided the highest reliability level as the selected reaction monitoring conditions were tuned and optimised with reference standards. The rigour of metabolite identification in this study is 'identified compound' as proposed by the Chemical Analysis Working Group (Sumner et al., 2007). After selecting the peaks with signal-to-noise more than 3 and RSD <30%, and peaks appeared in at least three samples, 131 metabolites were chosen as the mutual profile of leaf and flower extracts.

Table 3.1.1: Identification of 131 metabolites in golden *Camellia* **leaves** and **flowers** methanolic extracts by widely targeted metabolomics, followed by the ionisation mode, precursor and product ion used for screening, the RT (retention time), and the classification of each metabolite

ID	Identification	Ionisation mode	Precursor ion	Product ion	RT	Classification
1	(-) Shikimic acid	Negative	173.05	93.10	0.291	Carboxylic acid
2	5-Methylcytosine	Positive	126.05	109.10	0.279	Other
3	Adenine	Positive	136.05	119.05	0.349	Nucleosides
4	Adenosine	Positive	268.10	136.05	1.213	Nucleosides
5	Glycine	Positive	76.05	30.10	0.190	Amino acid
6	L-Histidine	Positive	156.10	110.15	0.188	Amino acid
7	L-(-)-Phenylalanine	Positive	166.10	120.10	1.267	Amino acid
8	L-Tryptophane	Positive	205.10	188.15	1.353	Amino acid
9	L-Tyrosine	Positive	182.10	165.10	0.597	Amino acid
10	Sucrose	Negative	341.10	88.95	0.256	Sugar
11	Leupeptin	Positive	427.30	409.25	1.446	Peptide
12	2-Aminoethylphosphonic acid	Positive	126.05	109.05	0.196	Phosphonic acid
13	Cytidine	Positive	244.10	112.10	0.380	Nucleosides
14	Diethanolamine	Positive	106.10	88.15	0.190	Amines
15	DL-2,3-Diaminopropionic acid	Positive	105.05	88.10	0.178	Carboxylic acid
16	Guanine	Positive	152.05	135.05	0.358	Nucleosides
17	Guanosine	Positive	284.10	152.05	1.259	Nucleosides
18	L-Asparagine	Positive	133.05	87.10	0.193	Amino acid
19	L-Aspartic acid	Positive	134.05	74.05	0.189	Amino acid
20	L-Glutamic acid	Positive	148.05	84.10	0.203	Amino acid
21	L-Glutamine	Positive	147.10	130.05	0.170	Amino acid
22	L-Homocarnosine	Positive	241.15	110.10	0.194	Amino acid
23	L-Ornithine	Positive	133.10	70.15	0.177	Amino acid
24	L-Proline	Positive	116.05	70.15	0.222	Amino acid
25	L-Pyroglutamic acid	Positive	130.05	84.10	0.466	Amino acid
26	L-Serine	Positive	106.05	60.15	0.193	Amino acid
27	3,4-Dihydroxybenzoic acid	Negative	153.00	109.05	1.336	Phenol
28	Pyridoxine	Positive	170.10	134.05	0.567	Pyridines

29	Succinic acid	Negative	117.00	73.00	0.547	Carboxylic acid
30	Uridine	Positive	245.10	113.10	0.733	Nucleosides
31	Uridine-5'-monophosphate	Positive	325.05	97.05	0.329	Nucleosides
32	α-Lactose	Negative	341.10	161.05	0.206	Sugar
33	3-Guanidinopropionic acid	Positive	132.10	72.10	0.234	Carboxylic acid
34	Gamma-Amino-n-butyric acid	Positive	104.05	87.10	0.202	Amino acid
35	Naringenin	Positive	273.10	153.00	1.629	Flavonoid (flavanone)
36	Apigenin-7-O-glucoside	Positive	433.10	271.05	1.477	Flavonoid (flavone)
37	Rhoifolin	Positive	579.15	271.05	1.459	Flavonoid (flavone)
38	Isorhamnetin-3-O-glucoside	Positive	479.10	317.00	1.471	Flavonoid (flavonol)
39	Kaempferol-3-O-glucoside	Positive	449.10	287.05	1.470	Flavonoid (flavonol)
40	Homoorietin	Positive	449.10	299.05	1.400	Flavonoid (flavone)
41	Naringenin-7-O-glucoside	Positive	435.15	273.05	1.486	Flavonoid (flavanone)
42	Hyperoside	Positive	465.10	303.00	1.440	Flavonoid (flavonol)
43	4-Coumaric acid	Positive	165.05	91.10	1.471	Hydroxycinnamic acid
44	Kaempferol-7-O-α-L-rhamnoside	Positive	433.10	287.05	1.553	Flavonoid (flavonol)
45	N-Acetyl-D-mannosamine	Positive	222.10	126.10	0.209	Sugar
46	O-Acetyl-L-serine	Positive	148.05	88.10	0.218	Amino acid
47	L-Saccharopine	Positive	277.05	84.05	0.192	Sugar
48	D-Glucoheptose	Negative	209.05	89.05	0.199	Sugar
49	D-(-)-Quinic acid	Negative	191.05	85.05	0.228	Carboxylic acid
50	Chlorogenic acid	Negative	353.10	191.10	1.377	Phenol
51	5'-Deoxy-5'-Methylthioadenosine	Positive	298.10	136.00	1.337	Nucleosides
52	L-allo-threonine	Positive	120.05	56.10	0.198	Amino acid
53	Methyl jasmonate	Positive	225.15	151.10	1.780	Carboxylic acid
54	Vanillin	Positive	153.05	65.15	1.490	Phenol
55	5-Aminovaleric acid	Positive	118.10	55.10	0.255	Carboxylic acid
56	D-Sorbitol-6-phosphate	Negative	261.05	96.95	0.211	Sugar
57	L-Threonic acid	Negative	135.05	75.00	0.214	Carboxylic acid
58	2,2',2"-Nitrilotriethanol	Positive	150.10	70.15	0.191	Polyol
59	Luteolin-3',7-di-O-glucoside	Positive	611.15	287.00	1.400	Flavonoid (flavone)
60	Luteolin-4'-O-glucoside	Positive	449.10	287.05	1.478	Flavonoid (flavone)
61	Neoeriocitrin	Negative	595.15	151.00	1.431	Flavonoid (flavanone)
62	Phloridzin	Negative	435.15	273.10	1.500	Flavonoid (dihydrochalcone)
63	Poncirin	Negative	593.20	285.05	1.552	Flavonoid (flavanone)
64	Saponarin	Positive	595.15	283.00	1.390	Flavonoid (flavone)

65	Vitexin	Positive	433.10	313.05	1.437	Flavonoid (flavone)
66	S-(5'-Adenosyl)- L-methionine	Positive	399.15	250.10	0.210	Amino acid
67	Kaempferol-3-O-alpha-L-arabinoside	Positive	419.10	287.05	1.491	Flavonoid (flavonol)
68	2,5-dihydroxy benzoic acid	Negative	153.00	108.05	1.407	Phenol
69	Salicylic acid	Negative	137.00	93.05	1.577	Phenol
70	Trimethylamine N-oxide	Positive	76.10	58.15	0.205	Amines
71	DL-5-Hydroxylysine	Positive	163.10	82.10	0.176	Amino acid
72	L-Anserine	Positive	241.05	109.15	0.184	Amino acid
73	(-)-Riboflavin	Positive	377.15	243.1	1.391	Nucleosides
74	Choline	Positive	104.10	60.15	0.193	Other
75	Kynurenic acid	Positive	190.05	144.05	1.374	Carboxylic acid
76	Trigonelline	Positive	138.05	92.10	0.226	Alkaloid
77	DL-Pipecolinic acid	Positive	130.10	84.15	0.312	Carboxylic acid
78	D-(+)-Raffinose	Negative	503.15	179.05	0.321	Sugar
79	Betaine	Positive	118.10	58.15	0.205	Alkaloid
80	Procyanidin C1	Negative	865.20	125.00	1.404	Flavonoid (flavan-3-ols)
81	Quercetin-3,4'-O-di-β-glucopyranoside	Negative	625.15	463.10	1.396	Flavonoid (flavonol)
82	E-3,4,5'-trihydroxy-3'-glucopyranosylstilbene	Negative	405.10	243.00	1.412	Stilbenoid
83	Esculin	Positive	341.10	179.05	1.366	Coumarin
84	Stachyose	Negative	665.20	383.05	0.305	Sugar
85	L-Canavanine	Positive	177.10	76.10	0.184	Amino acid
86	L-Leucine	Positive	132.20	30.10	0.514	Amino acid
87	L-Isoleucine	Positive	132.10	69.10	0.528	Amino acid
88	Methionine sulfoxide	Positive	165.95	74.05	0.196	Amino acid
89	Aureusidin	Positive	287.05	153.05	1.473	Flavonoid (aurone)
90	Glutamine	Positive	147.10	84.10	0.180	Amino acid
91	Isorhamnetin-dihexose	Positive	625.20	317.05	1.453	Flavonoid (flavonol)
92	Kaempferol-dihexose	Negative	593.15	285.05	1.451	Flavonoid (flavonol)
93	Cyanidin-3,5-dihexose	Positive	611.15	287.05	1.303	Flavonoid (anthocyanin)
94	Kaempferol-3,7-dihexose	Negative	577.15	285.00	1.431	Flavonoid (flavonol)
95	Kaempferol-3-O-α-L-rhamnoside	Negative	431.10	285.00	1.517	Flavonoid (flavonol)
96	Maritimein or luteolin-7- O-glucoside	Positive	449.10	287.05	1.440	Flavonoid (flavone)
97	Quercetin-3-Arabinoside or Quercetin-3-D-xyloside	Positive	435.10	303.05	1.473	Flavonoid (flavonol)
98	Quercetin-3-Rhamnoside or Quercetin-3-O-alphaL-rhamnopyranoside	Negative	447.10	300.00	1.481	Flavonoid (flavonol)
99	Myricitrin or Myricetin-3-Rhamnoside	Negative	463.10	316.15	1.446	Flavonoid (flavonol)
100	(+)-Catechin or (+)-Epicatechin	Positive	291.10	139.00	1.417	Flavonoid (flavan-3-ols)

101	Sodium pantothenate or D-Pantothenic acid or Calcium (+)-pantothenate	Positive	220.10	90.10	1.322	Polyol
102	D(+)-Galactosamine or D-(+)-Glucosamine	Positive	180.10	72.10	0.182	Sugar
103	D-(+)-Cellobiose or Lactulose	Negative	341.10	161.10	0.232	Sugar
104	Melibiose or D-(+)-Turanose or Isomaltose or Gentiobiose or Melibiose Palatinose	Negative	341.10	179.10	0.214	Sugar
105	L-Iditol or D-Sorbitol or D-(-)-Mannitol	Positive	183.10	69.10	0.202	Sugar
106	L(+)-Arginine or N-alpha-Acetyl-L-ornithine or L-Citrulline	Positive	175.10	70.15	0.187	Amino acid
107	Histamine or Cytosine	Positive	112.10	95.15	0.173	Nucleosides
108	Nicotinamide or Niacinamide	Positive	123.05	80.10	0.409	Pyridines
109	L-Alanine or Sarcosine	Positive	90.05	44.10	0.195	Amino acid
110	L-Norvaline or L-Valine	Positive	118.10	72.10	0.291	Amino acid
111	L-Threonine or alpha- Methyl-DL-serine or L-Homoserine	Positive	120.05	74.10	0.198	Amino acid
112	L-2-Aminobutyric acid or N, N-Dimethylglycine or N-Methyl-DL-Alanine	Positive	104.05	44.15	0.201	Amino acid
113	(S)-(+)-1-Aminoethylphosphonic acid or Taurine	Positive	126.05	44.15	0.197	Amino acid
114	DL-Malic acid	Negative	133.00	115.00	0.287	Carboxylic acid
115	L-Carnitine	Positive	162.10	60.20	0.204	Amino acid
116	Adipic acid or 2-Methylglutaric Acid	Negative	145.05	83.00	1.360	Carboxylic acid
117	(-)-Citramalic acid	Negative	147.05	87.00	0.576	Carboxylic acid
118	L-Lysine	Positive	147.10	84.10	0.18	Amino acid
119	Nicotinic acid or Isonicotinic acid	Positive	124.05	78.05	0.355	Carboxylic acid
120	Rutin or Quercetin-3-O-b-glucopyranosyl-7-O-a-rhamnopyranoside	Negative	609.15	300.00	1.432	Flavonoid (flavonol)
121	D-(+)-Melezitose or 1-Kestose	Negative	503.15	89.00	0.348	Sugar
122	Cyanidin 3-(6"-malonylglucoside)	positive	535.10	287.10	1.334	Flavonoid (anthocyanin)
123	Schaftoside	Positive	565.15	547.05	1.391	Flavonoid (flavone)
124	(-)-Epigallocatechin	Positive	307.05	139.05	1.302	Flavonoid (flavan-3-ols)
125	(-)-Gallocatechin	Positive	307.05	139.05	1.302	Flavonoid (flavan-3-ols)
126	Delphinidin 3-Rutinoside	Positive	612.15	304.00	1.337	Flavonoid (anthocyanin)
127	Delphinidin 3-Galactoside	Negative	464.10	301.05	1.330	Flavonoid (anthocyanin)
128	Cyanidin 3-Galactoside	Negative	448.10	285.00	1.344	Flavonoid (anthocyanin)

129	Vicenin-1	Positive	565.15	427.15	1.356	Flavonoid (flavone)
130	Vicenin-2	Negative	593.15	353.05	1.337	Flavonoid (flavone)
131	Vicenin-3	Positive	565.15	547.15	1.358	Flavonoid (flavone)

Table 3.1.1 shows the identification of these metabolites and their corresponding MS parameters. Interestingly, mainly forty-one flavonoids and thirty-two amino acids dominate the profiles of golden *Camellia* samples. Forty-one flavonoids detected in these samples correspond to different aglycone skeletons such as anthocyanin, aurone, chalcone, flavan-3-ol, flavanone, flavone, and flavonol. Among them, flavonols dominated the flavonoid profile with fourteen diverse glycosides of kaempferol (**39**, **44**, **67**, **92**, **94**, **95**), quercetin (**42**, **81**, **97**, **98**, **120**), isorhamnetin (**38**, **91**), and myricetin (**99**). All flavonols are oxygenated glycosides, usually in the position 3 and/or 7, which are attached to glucose, rhamnose, arabinose, or rutinose. Besides, flavones are the second most abundant group in the profile with twelve representatives. Most flavones detected in the tested samples are apigenin derivatives (**36**, **37**, **65**, **123**, **129**, **130**, **131**) and luteolin (**40**, **59**, **60**, **96**). Apigenin glycosides consist of 7-O-glycosides of glucose and neohesperidose. Interestingly, five flavonoids contain an apigenin skeleton belonging to C-glycosides at the position of 6 and/or 8. Indeed, homoorientin brings one hexose while schaftoside, vicenin 1, vicenin 2, and vicenin 3 are dihexose glycosides. Flavanones are aglycones and O-glycosides of naringenin, eriodictyol, and isosakuranetin at position 7 of the skeleton (**35**, **41**, **61**, **63**). Anthocyanins are mostly oxygenated derivatives of cyanidin and delphinidin at positions 3 and 5 (**93**, **122**, **126**, **127**, **128**). Notably, these flavonoids were commonly well-known for numerous health benefits as antioxidant, anti-inflammatory, anti-microbial, and anti-cancer activities (Aumeeruddy & Mahomoodally, 2021; Safe et al., 2021; Zhang et al., 2020). The complication in the structure of aglycons and the abundant combinatorial manner between these skeletons with numerous glycoside units brings a rich diversity in flavonoid profiles of golden *Camellia*. The catechin profiles of golden *Camellia* leaf extracts are dominated by three flavan-3-ols, including catechin/epicatechin (**100**), epigallocatechin (**124**), and gallocatechin (**125**) in widely targeted metabolomes. Catechin 3-gallate, epicatechin-3-gallate, and gallocatechin 3-gallate were detected in not many samples, even though these catechins were abundant in *C. sinensis* leaf extracts (K. O. T. Nguyen et al., 2022). In a previous study, gallocatechin was not detected in *C. murauchii, C. impressinervis, C. euphlebia, C. tunghinensis, C. nitidissima var. microcarpa, and C. nitidissima* (J. N. Lin et al., 2013). This method cannot distinguish catechin and its epimer; therefore, to have an overview of the

distribution of each catechin in the profile, a targeted analysis was conducted, focusing on caffeine and seven catechins in 160 samples of leaves. Similarly, the targeted analysis demonstrated that catechin and epicatechin were abundant in these extracts, followed by gallocatechin, while catechin 3-gallate, epicatechin-3-gallate, epigallocatechin, and gallocatechin 3-gallate were minor. More interestingly, caffeine, a commonly detected compound in green tea, was under the detection limit in all tested golden *Camellia* by both methods, targeted and widely targeted. This result suggests that these teas may not stimulate the central nervous system and do not cause insomnia, thus they can be suitable to make an alternative herbal tea for evening consumption. This observation is consistent with other studies on the chemical profile of other golden *Camellia* species, such as *C. chrysantha* (Bach et al., 2020), on Chinese species, including *Camellia impressinervis, C. euphlebia, C. microcarpa, C. nitidissima, and C. tunghinensis* (J. N. Lin et al., 2013; Song et al., 2011).

Other nitrogen-containing compounds, such as trigonelline (**76**) and betaine (**79**), also contribute to the chemical profile of the studied golden *Camellia*. In previous research, these substances showed attractive therapeutic potential for antidiabetic, anticancer, and neuroprotective effects, thus offering a good source of nutrients for golden tea products (Liang et al., 2023; Ueland, 2011).

Besides, several phenols, not limited to benzoic acid and hydrocinnamic acid derivatives, coumarin, and stilbenoids, were detected in these extracts. Vanillin (54), 3,4-dihydroxybenzoic acid (**27**), chlorogenic acid (50), 2,5-dihydroxybenzoic acid (**68**), salicylic acid (**69**), 4-coumaric acid (**43**), esculin (**83**), and E-3,4,50-trihydroxy-30-glucopyranosylstilbene (**82**) are biologically important phenolic compounds present in many plant species. They are well-known for various biological properties such as antioxidant, anti-bacterial, anti-inflammatory, anti-obesity, and antidiabetic activities, and improve cardiovascular function (Kabir et al., 2014; C. xiao Li et al., 2022; C. Wang et al., 2022).

Amino acids are bioactive compounds in green tea *C. sinensis*, which contribute to the taste and flavour of tea products and pharmacological effects on multiple targets (Hung et al., 2010; Y. Xu et al., 2020). Plants have two main types of amino acids: amino acids released from protein and free amino acids. In our work, the widely targeted analysis revealed the presence of 32 free amino acids in the PRIMe library for all samples. GABA, glutamine, and glutamic acid were mainly found in the methanolic extract of golden *Camellia*. These amino acids are analogues and are considered responsible for the umami taste of tea (Kaneko, S., Kumazawa, K., Masuda, H., Henze, A. & Hofmann, 2006). The combination of these components can trigger neuroprotective effects, especially against the stimulatory effect of caffeine in the central nervous system (C.

Wang et al., 2022; Yoneda et al., 2019). In addition, certain amino acids, such as phenylalanine, tryptophan, and tyrosine, were considered precursors of flavonoid biosynthesis, playing a significant role in phenolic accumulation (L. Wang et al., 2020). The wide range of amino acid profiles, which is relevant to flavonoid metabolism, is a typical feature of *Camellia* species and is conserved in golden teas.

Other primary metabolites, such as amines, carboxylic acids, nucleosides, polyols, pyridine derivatives, or sugar, were also observed in these extracts, contributing to the physicochemical properties and the taste and flavour of golden tea products. Overall, the comprehensive metabolome data revealed by the widely targeted metabolomics approach indicated that this method is suitable for high-throughput descriptions of golden *Camellia*, and this complex data could be mined from many perspectives.

3.1.2. Species-specific discrimination of golden *Camellia*

a. Leaf extracts

The semi-quantitative profile of 131 peaks identified in the methanolic extract of oven-drying mature leaves of six species, *C. hakodae, C. phanii, C. tamdaoensis, C. tienii, C. euphlebia,* and *C. petelotii* were subjected to PLS-DA analysis after a normalisation process with internal standards. Figure 3.1.1 shows the score plot of PLS-DA with the combination of the first two components, which explained 27% of the total data variance. In the plot, there is a clear discrimination observed among three species (*C. euphlebia, C. petelotii, and C. tienii*) while the other three species were moderately clustered (*C. tamdaoensis, C. phanii, and C. hakodae*). From an applied point of view, this graph demonstrated that the leaves of these species are well distinguished in terms of chemical profiles, even though their appearance is quite similar. Otherwise, a shared region between *C. phanii and C. tamdaoensis* exists, indicating that these two leaves produce closer metabolomes. *C. hakodae's* leaves were well grouped in the plot except for one point, HAK6; however, this cluster is near *C. tienii and C. phanii* samples. The chemical difference is consistent with the neighbour-joining phylogenetic tree using matk, rbcL, and trnH-psbA genes built for *C. phanii, C. tamdaoensis, C. petelotii, C. tienii and C. euphlebia* in our previous article (Van-Anh Le et al., 2023). In this phylogeny tree, *C. phanii and C. tamdaoensis* were close in the first branch, whereas *C. tienii and C. euphlebia* were located apart in the second branch. Significantly similar diversity between genotypic and metabolic profiles of the investigated golden *Camellia* species suggested that the analysis method can be used for the chemotaxonomy of these golden *Camellia* and provide deep insight into bioactive compounds in these plant materials.

Looking at the chemical markers that contribute to the PLS-DA model, we selected ten metabolites representing the most important VIP scores, including vicenin-3, schaftoside, vicenin-1, vitexin, rutin or quercetin-3-O-β-glucopyranosyl-7-O-alpha-rhamnopyranoside, quercetin-3-rhamnoside or quercetin-3-O-alpha-L-rhamnopyranoside, choline, kaempferol-3-O-glucoside, (+)-catechin or (+)- epicatechin, maritimein or luteolin-7-O-glucoside. Of the ten compounds, nine flavonoids suggested that this group is responsible for the chemical discrimination of these six golden *Camellia*.

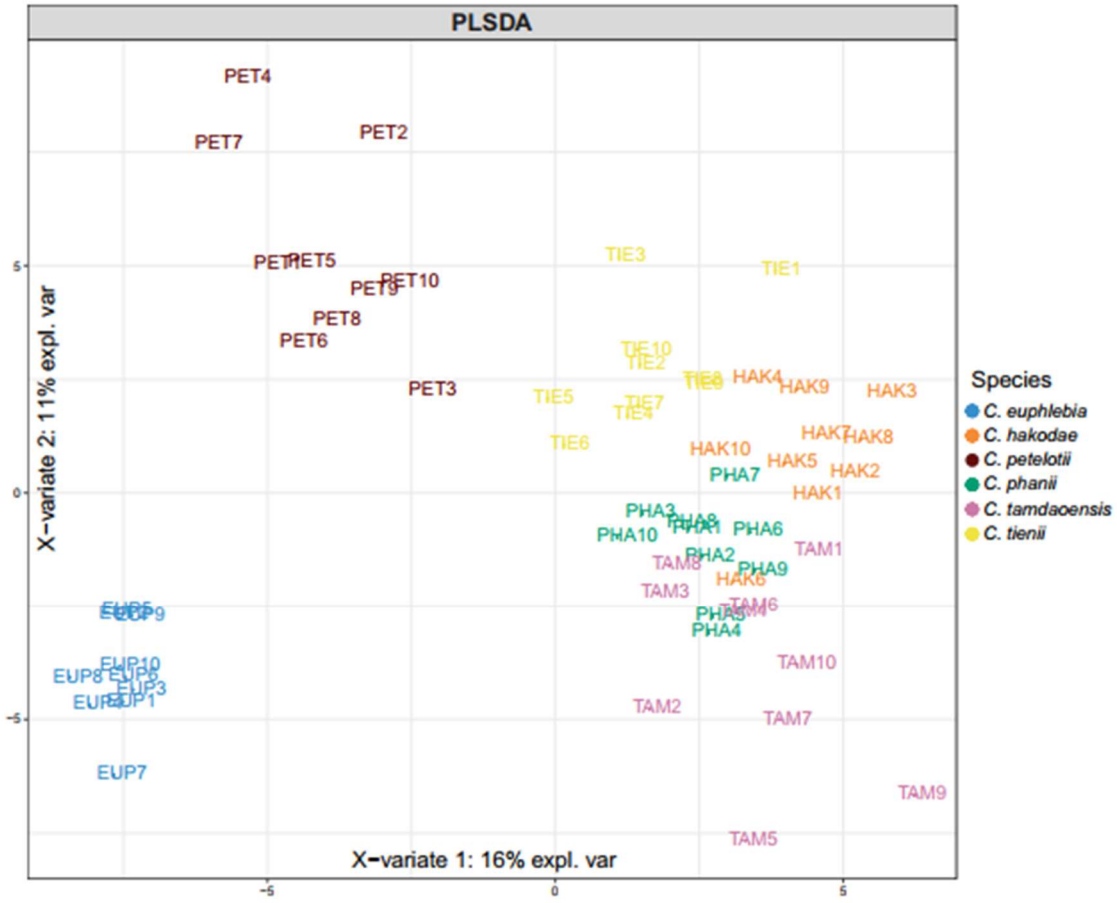


Figure 3.1.1. PLS-DA score plot of metabolomes produced by the methanolic extract of the oven-drying leaves of six Vietnamese golden *Camellia*.

Figure 3.1.2 also shows the relative content of these differential metabolites in six species by color. Herein, three flavones, vicenin-3, schaftoside, and vicenin-1, which are all C-glycosides of apigenin, were accumulated with discriminative levels in the decreasing order from the high to low content of *C. euphlebia* > *C. petelotii* > *C. phanii* > *C. hakodae* > *C. tienii* > *C. tamdaoensis*. Supportively, vitexin, another flavone that is also a C-glycoside of apigenin, appeared as the fourth compound in the VIP scores figure. Otherwise, rutin or quercetin-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside, quercetin-3-rhamnoside, or quercetin-3-O-α-L-

rhamnopyranoside, and kaempferol-3-O-glucoside are three O-glycosides of flavonol aglycon that also contribute to the distinction of the six leaf extracts profile. These observations may support the hypothesis that the metabolic pathway of flavonoid derivatives is enormously diverse among those investigated golden *Camellia* and, particularly in the case of apigenin C-glycosides, a series of natural products with numerous interesting pharmacological effects. Thus, the biosynthetic pathway of apigenin C-glycosides may be the key differentially regulated pathway contributing to both chemical and biological activity discrimination of those species.

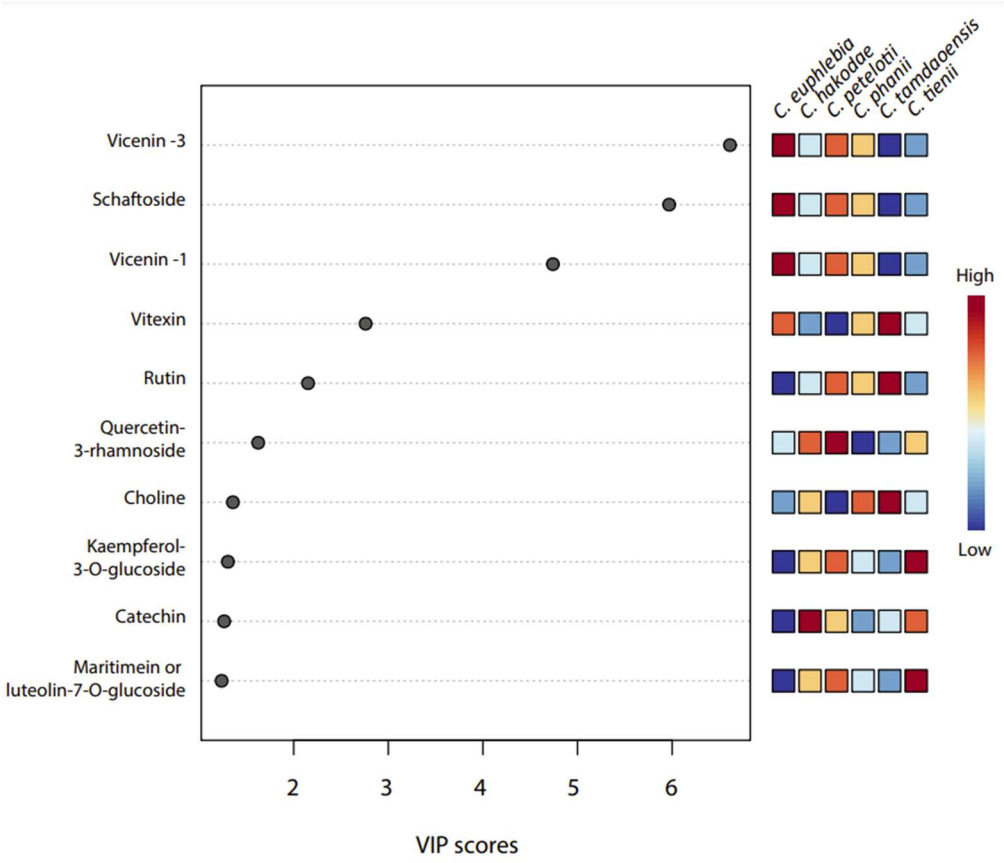


Figure 3.1.2 The VIP scores of differential metabolites contributing the most to discriminate chemical profiles of six golden *Camellia* leaves

b. Flower extracts

With flower samples, there are only two species, *C. tienii* and *C. tamdaoensis*, collected from Tam Dao, Vinh Phuc. The PLS-DA analysis demonstrated that the LC-MS profiles of these extracts were distinguished. The X- and Y-axes represent the first two principal components (X-variate 1 and X-variate 2), which explain 22% and 18% of the total variance, respectively. It can be seen that a clear separation between species in the PLS-DA plots. The red spots (*C. tienii*) are clustered in the lower-left and right parts of the plot. The blue spots (*C. tamdaoensis*) are grouped in the upper part of the plot. This separation suggests distinct metabolite compositions between the two species. In terms of intraspecies variability, *C. tamdaoensis* has a tighter clustering,

indicating relatively consistent metabolite profiles within the species, while *C. tienii* shows a broader spread, suggesting higher variability in its metabolite composition.

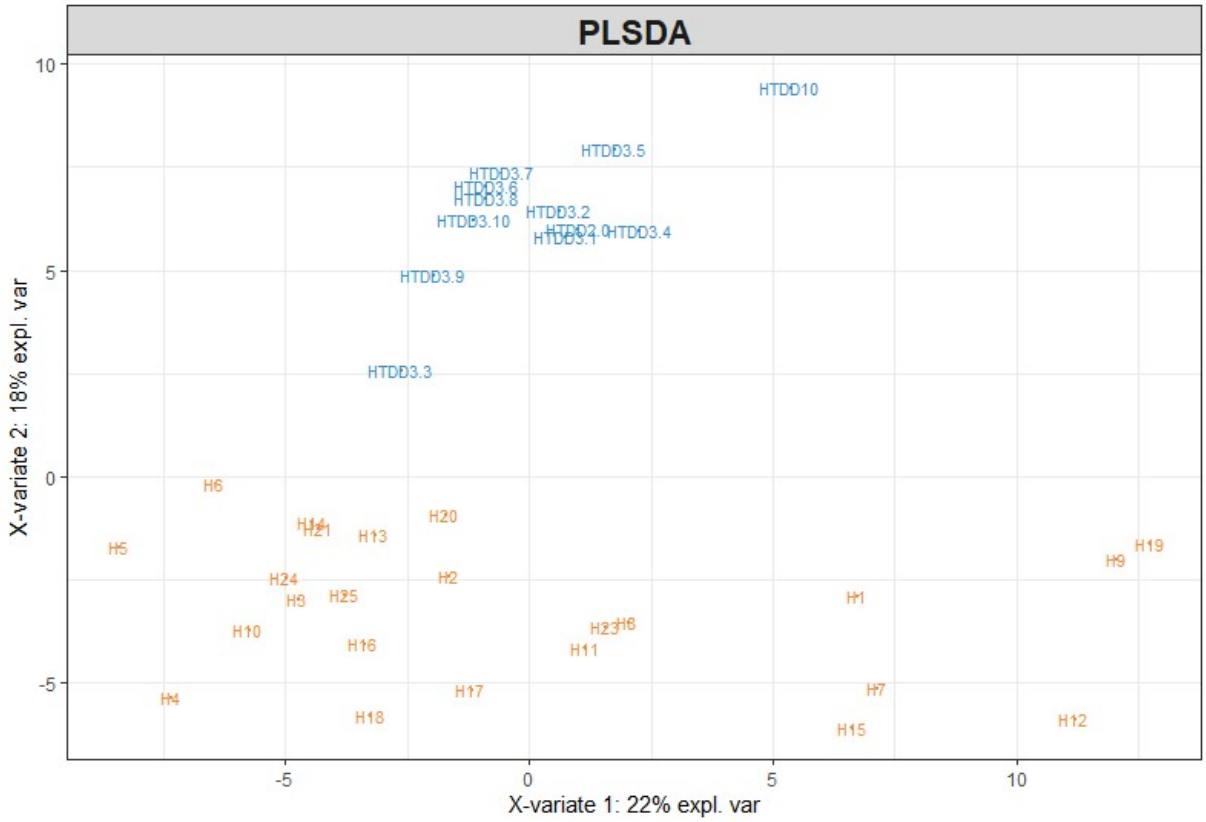


Figure 3.1.3: PLS-DA score plot of metabolomes produced by the methanolic extract of the oven-drying flowers of two Vietnamese golden *Camellia*.

The VIP (Variable Importance in Projection) score plot from a PLS-DA model identified the most significant metabolites distinguishing *Camellia tamdaoensis* (TAM) and *Camellia tienii* (TIE). Metabolites with higher VIP scores contribute more to the separation between the two species. From this figure, hyperoside has the highest VIP score, suggesting it plays the most significant role in differentiating the species. This compound was detected in *C. tienii* with very high concentrations, whereas its content in *C. tamdaoensis* was very low. Neoeriocitrin has the second-highest VIP score. However, this metabolite shows an inverse trend compared to hyperoside. It is more abundant in *C. tamdaoensis* than in *C. tienii* flowers. Other notable metabolites include (+)-catechin, L-proline, rutin, DL-malic acid, and D-(-)-quinic acid, delphinidin, kaempferol-3-O, L-arginine, quercetin-3-rhamoside, choline, adenosine, L-glutamic acid, and kaempferol-dihexose. *C. tamdaoensis* appears to have a higher concentration of most key metabolites, while *C. tienii* has lower levels (predominantly blue). These differences may be related to species-specific metabolic pathways, environmental adaptation, or genetic variation, making them important markers for chemotaxonomic classification.

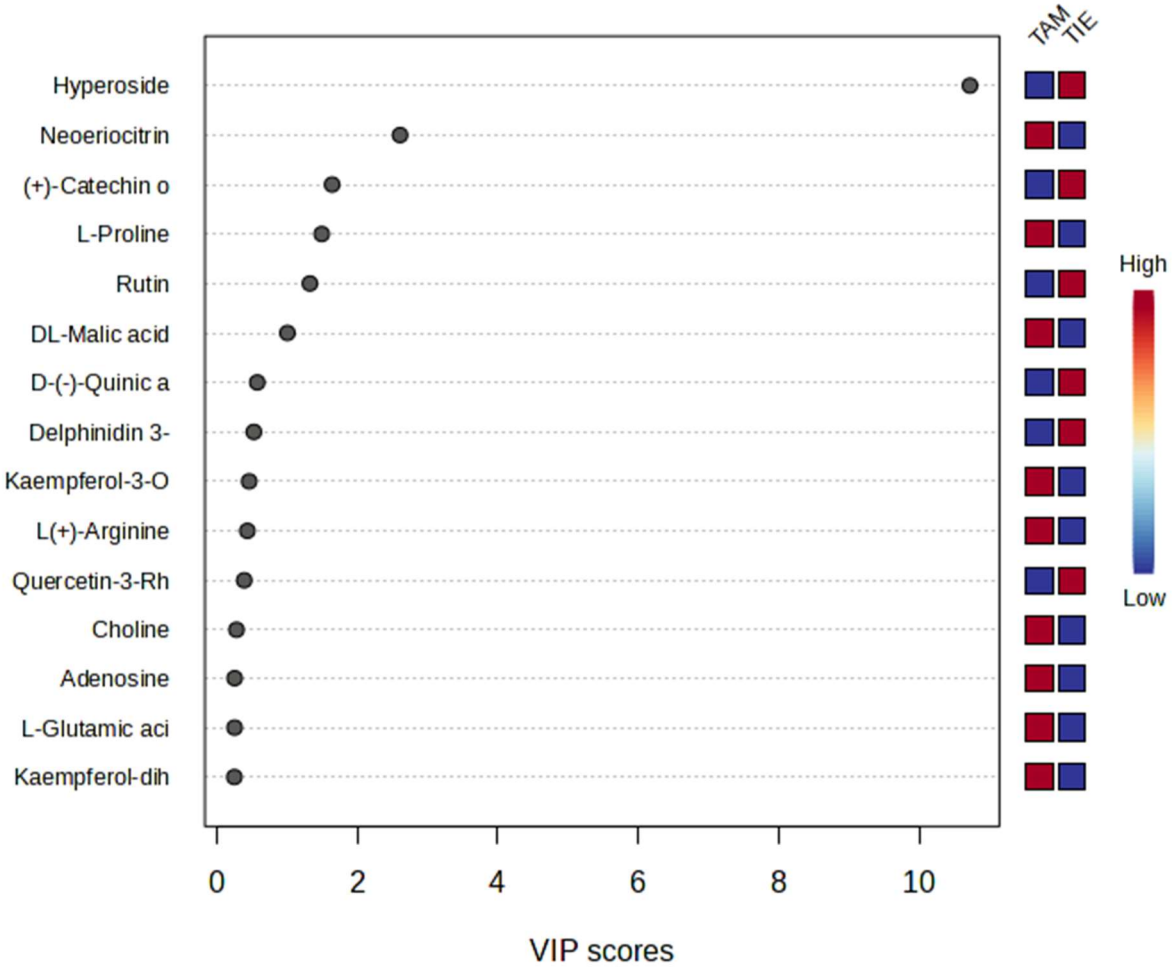


Figure 3.1.4: The VIP Scores plot of *C. tienii* and *C. tamdaoensis* flowers. The color scale (blue to red) represents metabolite abundance in *C. tamdaoensis* (TAM) vs. *C. tienii* (TIE). Red indicates higher abundance, and blue indicates lower abundance.

3.1.3. Post-harvest-specific discrimination

To explore the conversion of metabolites according to the post-harvesting treatment, we examined the variation in the relative abundance of 131 compounds between freeze-drying and oven-drying samples by widely targeted analysis. In all species, the PLS-DA model showed a clear separation between the two groups with a very high % explaining data variance (Figure 3.1.5), which indicates the significant impact of the drying method on the chemical profiles of these leaf materials.

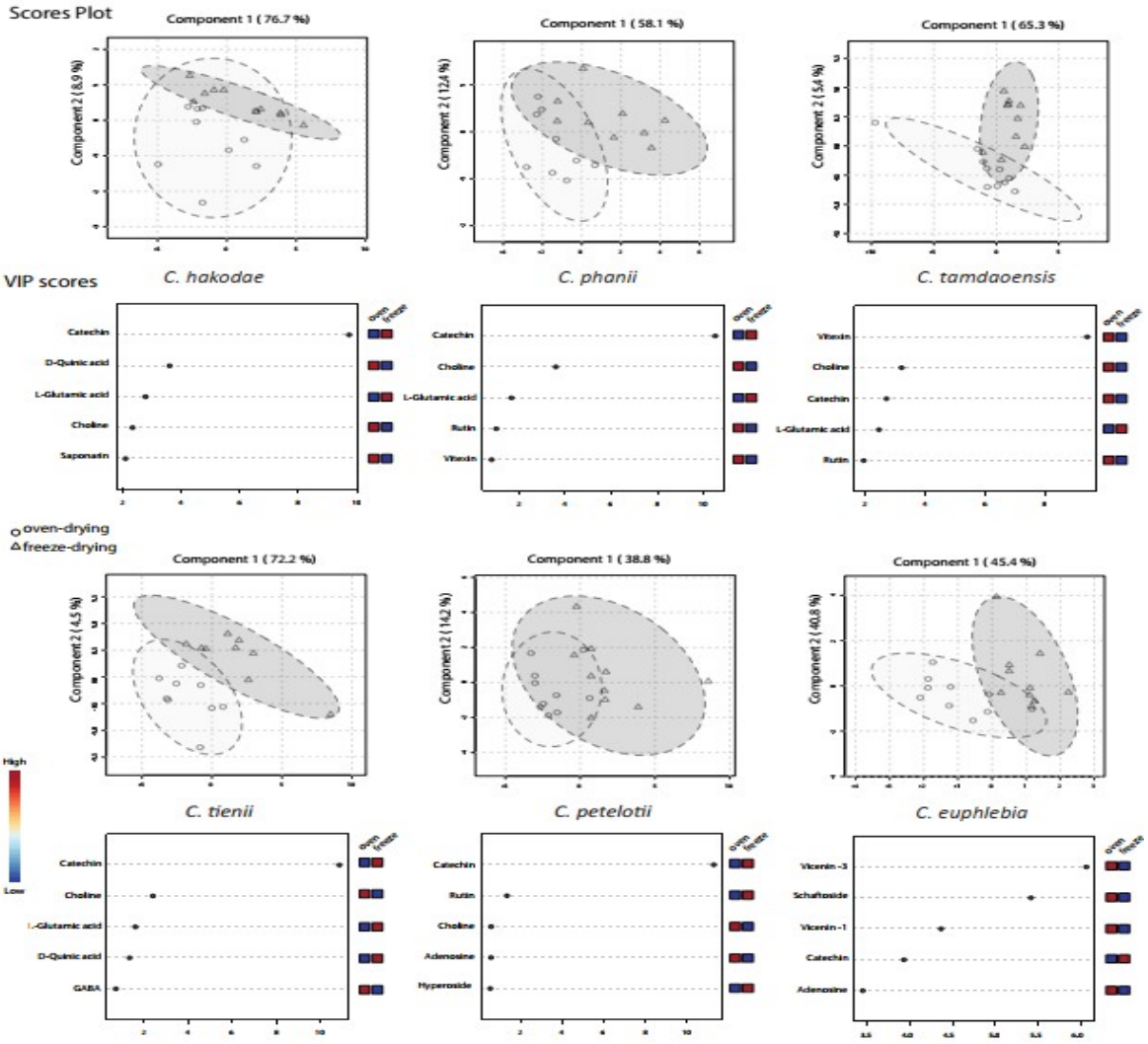


Figure 3.1.5. The PLS-DA scores plots distinguish the metabolomes of oven-drying and freeze-drying leaves of *C. hakodae, C. phanii, C. tamdaoensis, C. tienii, C. petelotii, C. euphlebia,* and VIP scores of differential metabolites contributing to the discrimination.

Regarding the metabolites responsible for the discrimination between two processing conditions, Fig. 3.1.5 of the VIP scores plot illustrates five key differential accumulated compounds of each species. Overall, six species share some common metabolite markers such as catechin/epicatechin, L-glutamic acid, choline, rutin, or quercetin-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside. In detail, catechin or epicatechin accumulated in significantly higher content in freeze-drying samples of most leaves, including *C. hakodae*, *C. phanii*, *C. tienii*, and *C. petelotii*. In contrast, *C. tamdaoensis* produces more of this component in oven-drying conditions. Regarding the content of sole catechin, this compound accumulated at a higher level in oven-drying conditions for five out of six leaves except *C. euphlebia*. Inversely in the case of epicatechin, drying by lyophillisation method led to producing a larger amount of epicatechin than by oven, except for *C. tamdaoensis*. Indeed, the formation of catechin and epicatechin within the *Camellia* genus has been proposed to happen by distinct enzymatic reactions, leucoanthocyanidin reductase and anthocyanidin reductase, respectively, utilizing different substrates, leucocyanidin and cyanidin, respectively. Following the synthetic pathway, both catechin and epicatechin act as precursors of proanthocyanidins, a class of flavonoids. The observation suggested that temperature may induce the enzymes involved in the catechins pathway and/or alter the abundance of products. Consequently, thermal processing could influence the biosynthesis or conversion of catechins into flavonoids, the prominent group of the *Camellia* genus, and this effect is a species-specific difference.

L-glutamic acid, an amino acid that affects the taste of tea infusion, was also revealed to exist at a higher level in freeze-dried materials of *C. hakodae*, *C. phanii*, *C. tamdaoensis*, and *C. tienii*. Inversely, choline, an intermediate product of amino acids, was found with a more considerable content in the oven-drying samples of *C. hakodae*, *C. phanii*, *C. tamdaoensis*, *C. tienii*, and *C. petelotii*. While the human body can synthesize this component, an additional amount is still required in our diet to improve physiological function. Hence, the upregulation of this active agent in temperature-based processing samples suggests a suitable preparation method to increase the creation of choline in the product. These contrasting trends indicate the need for different thermal processing methods tailored to specific products to enrich bioactive compounds.

Notably, rutin or quercetin-3-O-b-glucopyranosyl-7- O-a-rhamnopyranoside represented the inverse regulated trend in different species. On one side, the accumulation of this compound increases in oven-drying samples of *C. phanii* and *C. tamdaoensis*; however, it is down-regulated in *C. petelotii*. Some active flavonoids, such as saponarin, vitexin, vicenin-1, vicenin-3 and schaftoside were also up-regulated in oven-drying samples in *C. hakodae*, *C. phanii*, *C.*

tamdaoensis, and *C. euphlebia*. This proposed a hypothesis on the impact of thermal conditions on the transformation of these apigenin C-glycoside derivatives that were reported to have remarkable health benefits. The heat can induce the formation of these compounds, an issue that needs to be taken into account in post-harvesting practice.

Traditionally, leaves of golden *Camellia* were usually dried naturally under the atmospheric ambiance or under the sunlight, which is more similar to oven-drying conditions. The observation of this study provides a good suggestion for the specific post-harvesting processing method for these materials corresponding to different purposes.

3.1.4. Matureness-specific discrimination

The difference in chemical composition between young and mature leaves of green tea, *C. sinensis,* was revealed in much previous research (C. N. Chen et al., 2003; Y. L. Lin et al., 1996; K. O. T. Nguyen et al., 2022). In agricultural activities, young buds of green tea were normally collected because they are rich in antioxidant catechins. Herein, we examined the widely targeted metabolomes of young leaves and mature leaves of the same plant individual plants *C. hakodae*, *C. phanii*, *C. tamdaoensis,* and *C. tienii*. Under the same preparation process, the profiles of these methanolic extracts were subjected to a PLS-DA model. For all species, the scores plot of PC1 and PC2 indicated visible separation between young and mature samples with a significant percentage of explained data variance (Fig. 3.1.6). These results indicate a considerable variation of metabolites based on the maturity of the leaf.

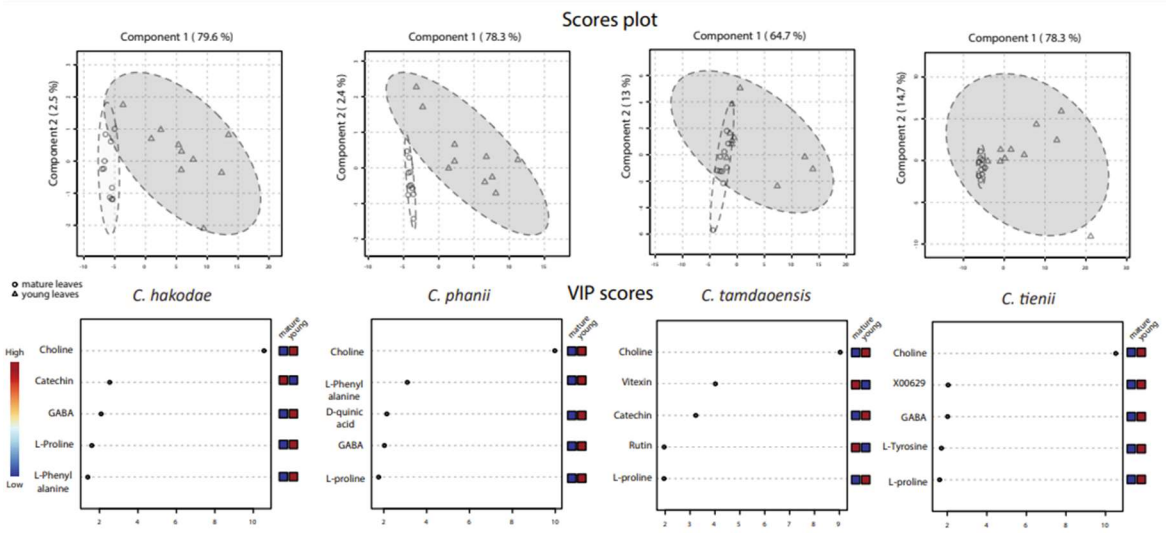


Figure 3.1.6. The PLS-DA scores plots distinguish the metabolomes of mature and young leaves of *C. hakodae, C. phanii, C. tamdaoensis, C. tienii,* and VIP scores of differential metabolites contributing to the discrimination.

Regarding the five key metabolites that represented the most important VIP scores in the PLS-DA components of each species (Fig. 3.1.6), amino acids and derivatives were dominant in the figures. Indeed, choline contributed the most to the metabolic difference between young and mature leaves for all studied species, with very high scores (more than 10). This metabolite was produced in significantly greater amounts in young leaves of all four species. Other amino acids, GABA (34), L-proline (24), L-phenylalanine (7), and L-tyrosine (9), were also up-regulated in the accumulation in young leaves of these species. These amino acids are well known for their activity on the central nervous system and other human physiological functions (Delwing-Dal Magro et al., 2016; Hinton et al., 2019; C. Wang et al., 2022) and also affect much on the taste and flavours of the *Camellia* genus (Scharbert & Hofmann, 2005).

In contrast, flavonoids, including rutin or quercetin- 3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside (120), vitexin (65), and catechin/epicatechin (100) existed at a higher level in mature leaves than young ones of *C. tamdaoensis* and *C. hakodae*, respectively. Indeed, the targeted analysis showed that the content of sole catechin was produced much more by mature leaves than by young leaves in four species, while this trend is not significant in the case of epicatechin (Figures 3.1.5, 3.1.6). Regarding the common flavonoid pathway, each class of anthocyanins, flavonols, and flavan-3-ols is biosynthesised by enzyme reactions, and their precursors are amino acids such as L-phenylalanine. The regulation in flavonoid biosynthesis was described at different developmental stages in green tea *C. sinensis*, indicating the differential expression of some enzymes between young and mature leaves, like cinnamic acid 4-hydroxylase, flavanone 3-hydroxylase, flavonoid 3',5'-hydroxylase, anthocyanidin reductase,

and leucocyanidin reductase (C. F. Li et al., 2015). The enzymatic reactions that regulate the conversion of three differential components should be targeted in further research to reveal the global mechanism of this metabolic change.

The observation is logically consistent with plant physiology because the young leaf contains more primary metabolites, while the older leaf produces more secondary metabolites. However, golden *Camellia* represented an opposite regulation to green tea leaves in the biosynthesis of catechins. Therefore, the young leaves and buds are selected for processing green tea-related products. In contrast, in the case of golden tea, people traditionally utilise the mature leaves for consumption. This result also explains the different tastes between immature and mature leaf infusions and may suggest the other pharmacological profiles of these two types. Moreover, the results highlight the effect of the growth and development stage of tissue on the bioactive component production. From an applied point of view, this can facilitate the optimisation of harvest time in agricultural activities.

3.1.5. Tissue-specific discrimination

a. *Camellia tienii*

Regarding the chemical difference between the two tissues, a heatmap was used to illustrate the discriminative components (Figure 3.1.7). The level of the difference in each metabolite's concentration was demonstrated based on the color intensity in each sample. In Figure 3.1.7, the group of metabolites overexpressed in flowers is shown in red color. More precisely, the plot demonstrated that metabolites accumulated significantly more in flower include mostly flavonoids (rutin, kaempferol-7-O-α-L-rhamnoside, maritimein, catechin or epicatechin, hyperoside, delphinidin 3-galactoside, procyanidin C1, kaempferol 3-rhamnoside-7-rhamnoside, quercetin 3-arabinoside, delphinidin 3-rutinoside, quercetin 3-rhamnoside, poncirin…) and amino acids (L-anserine, L-proline, DL-malic acid, L-glutamic acid, L-tryptophan, L-phenylalanine, choline…). In contrast, the group of compounds that were produced much more in leaves consists of L-pyroglutamic acid, esculin, stachyose, and 2,5-dihydroxy benzoic acid (in blue color). The variation of these compounds in two tissues contributes to explaining the typical taste of tea made by leaf and flower, as water-soluble compounds, like free amino acids, caffeine, polyphenols, and polysaccharides, are generally accountable for the taste of tea infusions. From a more pharmacological point of view, this discrimination could suggest a distinguished biological profile between the two types of medicinal materials. Typically, flowers of the golden *Camellia* are commercialized at a higher price than leaves because it is believed that flowers offer more biological health benefits. Our research findings align with this

hypothesis as most of the compounds in the profile, which are mainly active, dominate significantly in flowers rather than leaves.

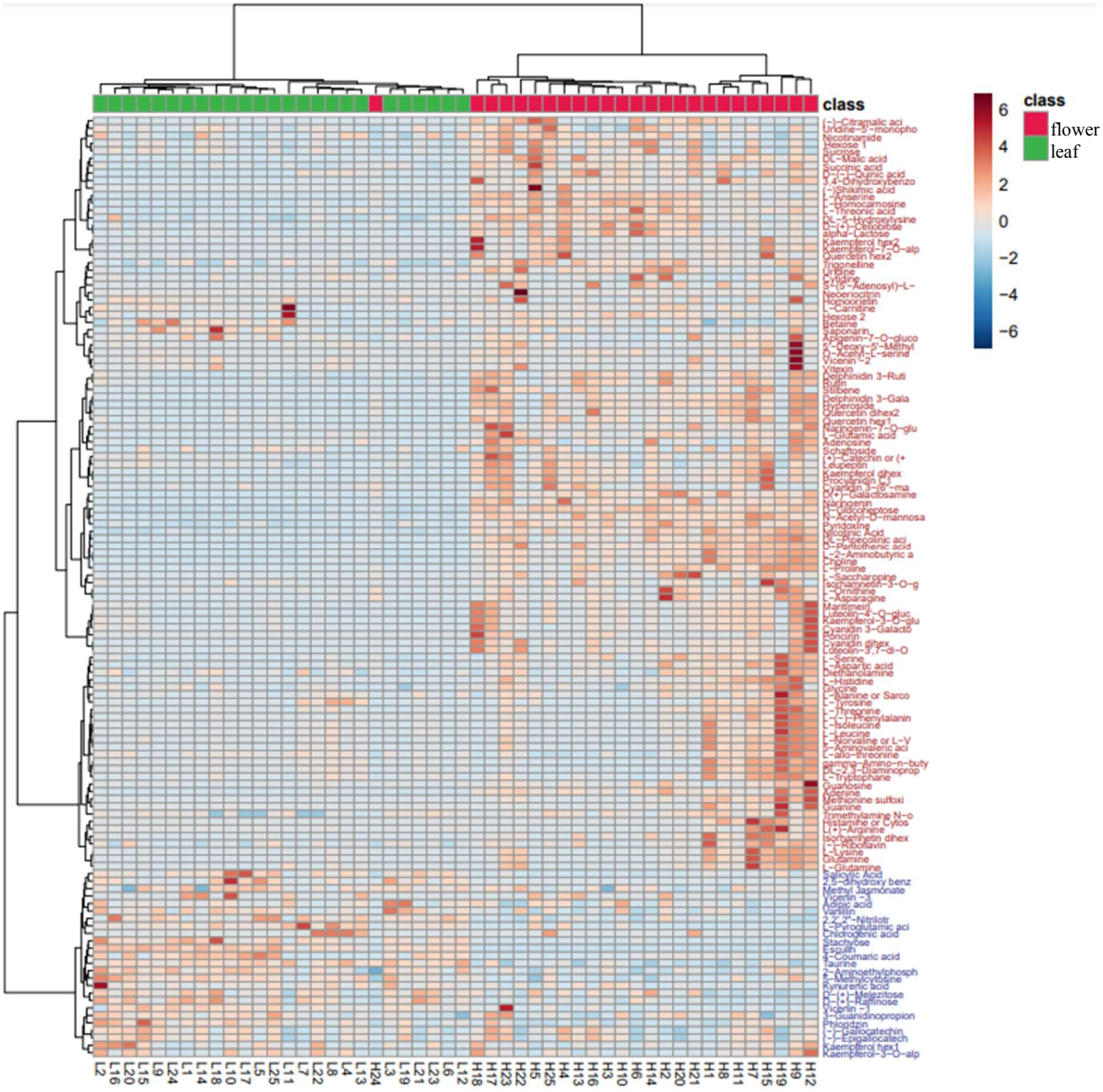


Figure 3.1.7: Heatmap of the intensity of metabolites detected by widely targeted metabolomics of flowers and leaves of *C. tienii* samples.

b. *Camellia tamdaoensis*

Leaf and flower samples of *C. tamdaoensis* have been characterized by a widely targeted metabolomics analysis. The heatmap represents the metabolite profiles of flowers (red) and leaves (green) of the *Camellia* species. The hierarchical clustering at the top group samples based on their metabolic similarity, while the left-side clustering groups metabolites based on their abundance patterns. The red-labeled flower samples cluster together, indicating they share similar metabolic compositions. The green-labeled leaf samples form a separate cluster,

suggesting significant metabolic differences between leaves and flowers. The clustering pattern suggests that flowers and leaves have distinct biochemical roles, likely influenced by their physiological functions and environmental interactions.

Certain compounds (e.g., flavonoids, phenolic acids) appear to be more concentrated in flowers, possibly contributing to pigmentation, scent, and defense. Leaves might have higher levels of specific amino acids, organic acids, or other secondary metabolites essential for photosynthesis and stress resistance. The significantly different metabolites between leaves and flowers of *Camellia* tamdaoensis ($p < 0.05$) were: taurine, nicotinamide, sugars such as sucrose, hexose, D-glucoheptose, kaempferol-dihexose, neoeriocitrin, adipic acid, D-(+)-cellobiose, guanosine ($p = 1.53 \times 10^{-7}$). These metabolites show significant differences between the two tissues, indicating potential metabolic variations between leaves and flowers in *C. tamdaoensis*.

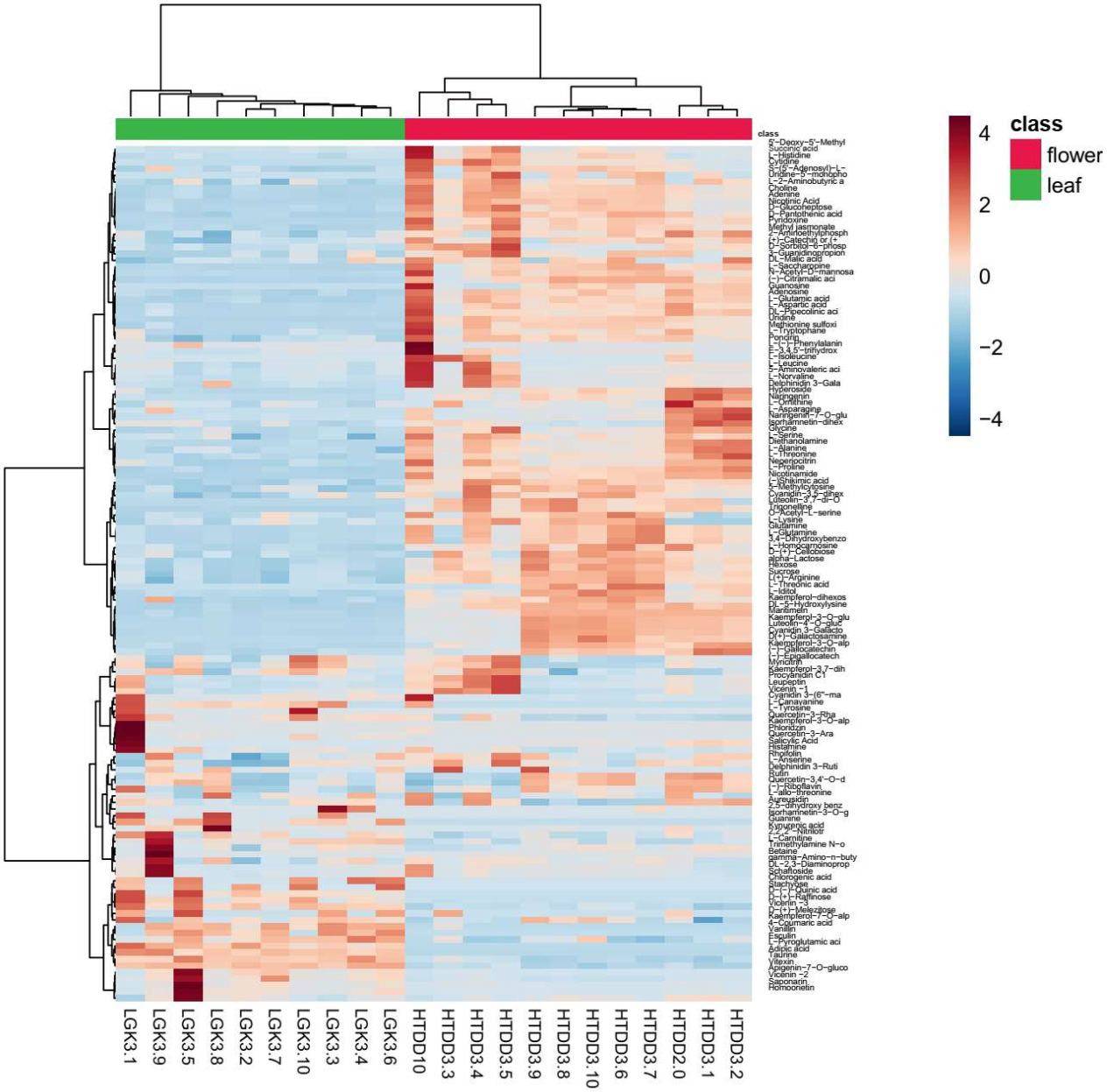


Figure 3.1.8: Heatmap of the intensity of metabolites detected by widely targeted metabolomics of flowers and leaves of *C. tamdaoensis* samples.

Discussion

This study has successfully identified a mutual profile of 131 metabolites, which was obtained by using a widely targeted metabolomics analysis using ultra-performance liquid chromatography coupled with tandem mass spectrometry with a high level of structural elucidation. This comprehensive analysis provides a good source of information for species discrimination, which can be used for chemotaxonomy. Flavonoids, that is, apigenin C-glycosides, may contribute to the chemical diversity of these six species. Otherwise, our results showed a clear separation between the clusters of oven-drying and freeze-drying leaves and young and mature leaves on the PLS-DA score plots. Catechin and epicatechin, L-glutamic acid, choline, and some active flavonoids were accumulated with distinct trends in six species with or without thermal treatment. While some amino acids were upregulated in young leaves, flavonoids were found with higher content in mature leaves. This suggests an undeniable effect of preparation treatment and harvest time on the metabolite composition, which decides these materials' taste, nutritional, and pharmacological properties.

PART 3.2: STUDY ON GC-MS BASED METABOLITE PROFILE OF ENDEMIC GOLDEN *CAMELLIA*

3.2.1. GC-MS-based metabolite profiling of leaf and flower extracts

The contents of volatile compounds in leaf samples were determined by a GC-MS untargeted metabolomics approach. After alignment of peaks detected in all samples, the major peaks were selected because they were detected in at least three biological samples with a relatively high peak area (peak ratio based on IS) and with a suitable match and reverse match (>700). The annotation of compounds was conducted based on matching their spectra and relevance with their retention index (RI) with those referenced by the software. Indeed, the theoretical RI, which was forecast from AMDIS/NIST, must be approximately calculated RI and should not differ from 5%. A total of 43 signals (including the IS peak) corresponding to compounds from various chemical classes. Details of compounds detected with retention time, retention index, and probability in leaf and flower samples were represented in Tables 3.2.1 and 3.2.2.

Among the compounds identified, fatty acids, carboxylic acids, and their esters are most commonly found with almost a high probability. Saturated fatty acids like butanoic acid, hexenoic acid, myristic acid, hexadecanoic acid, heptadecanoic acid, eicosanoic acid, docosanoic acid, tetracosanoic acid, and hexacosanoic acid dominate the GC-MS-based profile. Specifically, unsaturated fatty acids commonly possess potential bioactivity, such as 9,12-octadecadienoic acid (Z, Z) and 9,12,15-octadecatrienoic acid (Z, Z, Z), which were found with high intensity in leaves and flowers of golden *Camellia* species.

Other classes, such as ketones and terpenes, were less abundant groups but showed an interesting profile of pharmacological effects. Terpenoids play a role in traditional herbal remedies. Terpenoids contribute to the scent of eucalyptus, the flavors of cinnamon and cloves, the yellow color in sunflowers, and the red color in tomatoes. Terpenoids play a role as prophylaxis against pathogens in plants. In addition, dl-α-Tocopherol is a natural vitamin E compound. α-Tocopherol is a fat-soluble vitamin, widely distributed in food, especially vegetable oils, wheat germ oil, sunflower oil, cereals, and eggs. Indeed, a compound was found at RT 51.44 minutes, showing the similarity with preliminary literature, and can be annotated as dl-α-tocopherol, a form of vitamin E. This compound has an antioxidant effect, protecting cell membranes from free radical attack, thereby preserving the integrity of cell membranes. Vitamin E has an antioxidant effect, protecting cell membranes from free radical attack, thereby preserving the integrity of cell membranes. Vitamin E has a synergistic effect with vitamin C,

selenium, vitamin A, and carotenoids. Vitamin E protects vitamin A from oxidation and stabilizes vitamin A. Vitamin E synergizes with vitamin C, selenium, vitamin A, and carotenoids, contributing to the nutritional and therapeutic benefits (Rizvi et al., 2014b).

Also, squalene, a triterpene, was detected in most leaves and flowers in this study. With a long history of research, this triterpenoid was revealed to exhibit potential therapeutic effects such as antioxidant, anti-inflammatory, and lipid-lowering properties (Lou-Bonafonte et al., 2018). Furthermore, at RT 33.42, a diterpene, namely neophytadiene, was annotated. This compound has been found in many plants and algae and is considered an anti-inflammatory and antimicrobial agent (Bhardwaj et al., 2020). Recently, some research revealed the capability of neophytadiene in anxiolytic-like activity, soothing properties, and antidepressant-like actions (Gonzalez-Rivera et al., 2023). Phytol, another diterpene that is a component of chlorophyll, is very well known as an aromatic ingredient, antioxidant, anti-inflammatory, antihyperalgesic, and antiarthritic. This compound was also detected as the most abundant accumulated component of *C. tunghinensis* and *C. euphlebia* leaves (Ge et al., 2019). Stigmasterol, an unsaturated phytosterol, belongs to the triterpenes class with antioxidant, antifungal, anti-inflammatory, and anticancer characteristics (García et al., 1999; S. Panda, M. Jafri, A. Kar, 2009).

Table 3.2.1: Tentative annotation of the compounds detected in leaf samples by GC-MS.

No	Compound	RT	Formula	Prob	RI	RI*
1	Butanoic acid, 2-methyl-, methyl ester	5.14	$C_6H_{12}O_2$	87.1	775	807
2	2-Butenoic acid, 2-methyl-, methyl ester	7.39	$C_6H_{10}O_2$	32.4	873	885
3	2-Hexenoic acid, methyl ester, (E)-	8.84	$C_7H_{12}O_2$	51.8	966	987
4	trans-Linalool oxide (furanoid)	13.65	$C_{10}H_{18}O_2$	25.2	1086	1080
5	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	18.83	$C_7H_9NO_2$	98.3	1239	1255
6	2-Propenoic acid, 3-phenyl-, methyl ester	20.79	$C_{10}H_{10}O_2$	31.2	1380	1326
7	2-Propenoic acid, 3-phenyl-, methyl ester, (E)-	23.03	$C_{10}H_{10}O_2$	49.5	1380	1408
8	Phenol, 2-methoxy-4-(1-propenyl)-	24.65	$C_{10}H_{12}O_2$	44.8	1450	1474
9	2,4-Di-tert-butylphenol	25.84	$C_{14}H_{22}O$	49.6	1519	1523
10	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	26.85	$C_{11}H_{16}O_2$	76.4	1532	1564
11	Unknown 1	29.95				
12	Methyl tetradecanoate	30.67	$C_{15}H_{30}O_2$	82.6	1725	1732
13	Myristic acid	31.53	$C_{14}H_{28}O_2$	76.0	1768	1771
14	Octadecane	32.13	$C_{18}H_{38}$	29.1	1800	1798
15	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	32.42	$C_{11}H_{16}O_3$	93.3	1784	1812
16	(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-	32.59	$C_{13}H_{18}O_3$	29.8	1800	1821

	1-en-1-yl)cyclohex-2-enone					
17	Neophytadiene	33.42	$C_{20}H_{38}$	78.3	1837	1862
18	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	33.64	$C_{11}H_{12}O_4$	75.5	1855	1873
19	(Z)-Methyl hexadec-11-enoate	34.43	$C_{17}H_{32}O_2$	28.8	1913	1913
20	Hexadecanoic acid, methyl ester	34.84	$C_{17}H_{34}O_2$	80.7	1928	1933
21	Hexadecanoic acid, ethyl ester	36.13	$C_{18}H_{36}O_2$	77.1	1993	1998
22	Heptadecanoic acid, methyl ester	36.77	$C_{18}H_{36}Q_2$	80.3	2028	2033
23	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	38.08	$C_{19}H_{34}O_2$	31.3	2092	2104
24	9,12,15-Octadecatrienoic acid, methyl ester	38.21	$C_{19}H_{32}O_2$	35.1	2098	2111
25	Phytol	38.33	$C_{20}H_{40}O$	80.0	2114	2118
26	Methyl stearate	38.62	$C_{19}H_{38}O_2$	82.7	2128	2134
27	9,12-Octadecadienoic acid (Z,Z)-	38.93	$C_{18}H_{32}O_2$	32.9	2133	2151
28	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	39.37	$C_{18}H_{30}O_2$	48.8	2139	2175
29	Unknown 2	41.68				
30	Eicosanoic acid, methyl ester	42.10	$C_{21}H_{42}O_2$	73.0	2329	2335
31	Oxazole, 2-(8Z,11Z,14Z)-8,11,14-heptadecatrien-1-yl-4,5-dihydro-	42.32	$C_{20}H_{37}NO$	46.6	2329	2348
32	4,8,12,16-Tetramethylheptadecan-4-olide	42.62	$C_{21}H_{40}O_2$	86.1	2364	2366
33	Unknown 3	43.03				
34	Docosanoic acid, methyl ester	45.29	$C_{23}H_{46}O_2$	77.8	2528	2536
35	15-Tetracosenoic acid, methyl ester, (Z)-	47.84	$C_{25}H_{48}O_2$	54.9	2710	2727
36	Tetracosanoic acid, methyl ester	48.10	$C_{25}H_{50}O_2$	89.8	2728	2748
37	Squalene	48.92	$C_{30}H_{50}$	64.4	2832	2823
38	Unknown 4	49.82				
39	Hexacosanoic acid, methyl ester	49.99	$C_{27}H_{54}O_2$	86.3	2935	2957
40	Stigmasterol	50.31	$C_{29}H_{48}O$	56.0	3000	2997
41	dl-α-Tocopherol	51.44	$C_{29}H_{50}O_2$	27.6	3150	3172
42	Olean-12-en-3-ol, acetate, (3β)	53.58	$C_{32}H_{52}O_2$	40.0	3438	3454

Note: RT (Retention Time), RI (Retention Index - theoretical), RI*(Retention Index - calculate) and Prob (Probability)

Table 3.2.2: Tentative annotation of the compounds detected in flower samples by GC-MS.

No	Compound	RT	Formula	Prob	RI	RI*
1	Butanoic acid, 2-methyl-, methyl ester	5.16	$C_6H_{12}O_2$	87.1	775	786
2	Unknown 1	5.44				
3	Methyl tiglate	7.42	$C_6H_{10}O_2$	48.5	866	875
4	Benzoic acid, methyl ester	14.67	$C_8H_8O_2$	33.4	1094	1114

5	Unknown 2	20.82				
6	2-Propenoic acid, 3-phenyl-, methyl ester, (E)-	23.02	$C_{10}H_{10}O_3$	60.8	1379	1409
7	2,4-Di-tert-butylphenol	25.81	$C_{14}H_{22}O$	54.4	1519	1522
8	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	26.85	$C_{11}H_{16}O_2$	77.3	1538	1564
9	2-Cyclohexen-1-one, 4-(3-hydroxy-1-butenyl)-3,5,5-trimethyl-	29.25	$C_{13}H_{20}O_2$	72.5	1647	1668
10	Methyl tetradecanoate	30.66	$C_{15}H_{30}O_2$	81.0	1725	1732
11	2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)	31.35	$C_{15}H_{22}O$	92.0	1734	1763
12	Tetradecanoic acid	31.53	$C_{14}H_{28}O_2$	80.4	1768	1771
13	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	32.41	$C_{11}H_{16}O_3$	92.6	1784	1812
14	(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone	32.56	$C_{13}H_{18}O_3$	29.5	1800	1819
15	Hexadecanoic acid, methyl ester	34.83	$C_{17}H_{34}O_2$	70.2	1926	1933
16	n-Hexadecanoic acid	35.66	$C_{16}H_{32}O_2$	80.4	1968	1975
17	Hexadecanoic acid, ethyl ester	36.12	$C_{18}H_{36}O_2$	74.5	1993	1998
18	Heptadecanoic acid, methyl ester	36.77	$C_{18}H_{36}O_2$	87.1	2028	2033
19	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	38.07	$C_{19}H_{34}O_2$	33.7	2092	2105
20	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	38.22	$C_{19}H_{32}O_2$	55.0	2098	2113
21	Methyl stearate	38.62	$C_{19}H_{38}O_2$	80.3	2128	2135
22	9,12-Octadecadienoic acid (Z,Z)-	38.95	$C_{18}H_{32}O_2$	33.3	2133	2153
23	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	39.08	$C_{18}H_{30}O_2$	53.7	2139	2160
24	Octadecanoic acid	39.39	$C_{18}H_{36}O_2$	77.5	2172	2177
25	9-Octadecenoic acid (Z)-, methyl ester	41.13	$C_{19}H_{36}O_2$	65.0	2103	2105
26	Unknown 3	41.50				
27	Trichloroacetic acid, pentadecyl ester	41.60	$C_{15}H_{28}O_2$	26.7	2301	2306
28	Cis-methyl 11-eicosenoate	41.68	$C_{21}H_{40}O_2$	30.5	2306	2310
29	Eicosanoic acid, methyl ester	42.09	$C_{21}H_{42}O_2$	77.1	2329	2336
30	13-Docosenoic acid, methyl ester, (Z)-	44.83	$C_{23}H_{44}O_2$	47.2	2510	2510
31	Docosanoic acid, methyl ester	45.30	$C_{23}H_{46}O_2$	71.8	2528	2538
32	Unknown 4	46.25				
33	Tricosanoic acid, methyl ester	46.82	$C_{24}H_{48}O_2$	85.5	2628	2645
34	Unknown 5	47.66				

35	15-Tetracosenoic acid, methyl ester, (Z)-	47.85	$C_{25}H_{48}O_2$	54.9	2710	2729
36	Tetracosanoic acid, methyl ester	48.10	$C_{25}H_{50}O_2$	86.4	2728	2749
37	Unknown 6	48.71				
38	Squalene	48.89	$C_{30}H_{50}$	62.8	2832	2821
39	Pentacosanoic acid, methyl ester	49.08	$C_{26}H_{52}O_2$	59	2813	2818
40	Hexacosanoic acid, methyl ester	49.91	$C_{27}H_{54}O_2$	86.3	2935	2957
41	Unknown 7	51.07				
42	Vitamin E	51.41	$C_{29}H_{50}O_2$	29.5	3136	3168
43	Chondrillasterol	53.02	$C_{29}H_{48}O$	34.4	3295	3295
44	Olean-12-en-3-ol, acetate, (3β)	53.57	$C_{32}H_{52}O_2$	40.0	3438	3454

Note: RT (Retention Time), RI (Retention Index - theoretical), RI*(Retention Index - calculate), and Prob (Probability)

3.2.2. Species-specific discrimination

a. Flower samples of six species

The GC-MS-based metabolite profiles of flower samples were also used in discrimination analysis. The PLS-DA score plot shows the discrimination of flower metabolite profiles among six *Camellia* species. Component 1 (38.8%) explains most of the variance, strongly separating *C. tienii* from the others, while the second component accounts for less variation, 5.3%, but still contributes to species discrimination. Each species forms distinct clusters, indicating significant differences in its metabolite profiles. *C. tienii* (TIE, yellow) is the most distinct, forming a well-separated cluster on the right side. *C. tamdaoensis* (TAM, pink) overlaps slightly with *C. phanii* (PHA, purple) and *C. hakodae* (HAK, dark blue), suggesting some similarity in their chemical composition. Some species (EUP, PET, HAK, and PHA) exhibit slight overlap, indicating a degree of shared metabolites.

The clear separation among species suggests that flower metabolites are species-specific, which can be useful for classification and identification. *C. tienii* has a highly unique metabolite composition, making it a potential target for further phytochemical studies. The partial overlap among *C. tamdaoensis*, *C. hakodae*, and *C. phanii* suggests closer biochemical relationships, possibly due to shared biosynthetic pathways or ecological adaptation.

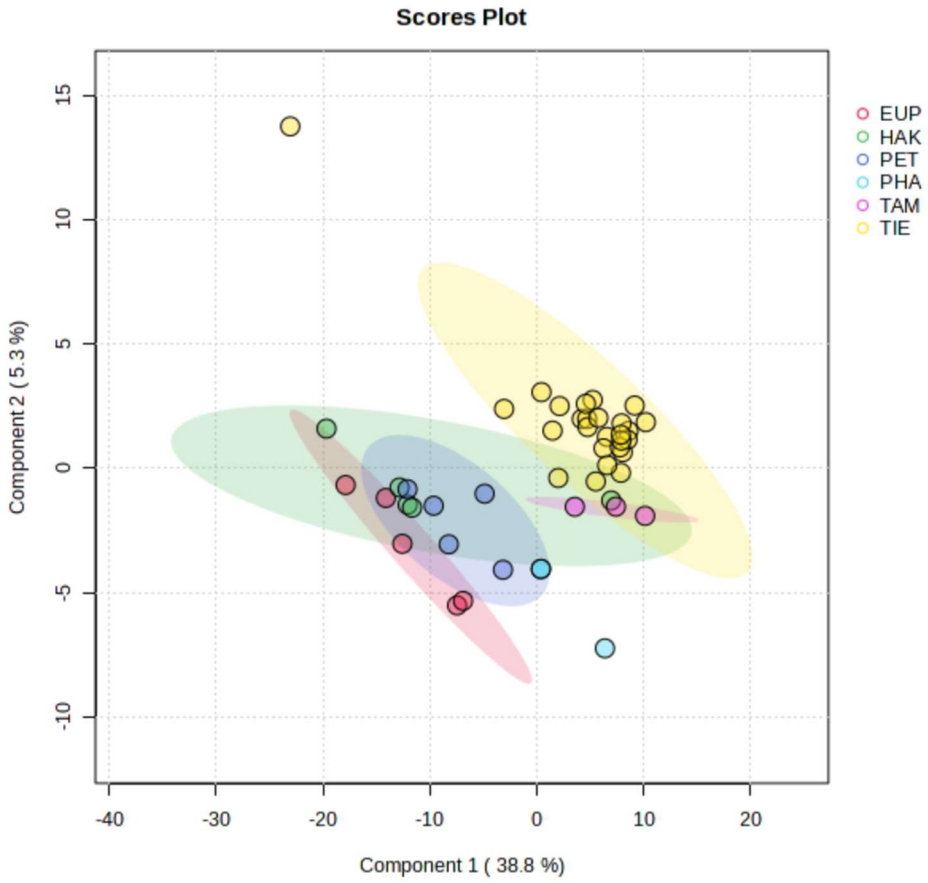


Figure 3.2.1. The PLS-DA score plot shows the discrimination of flower metabolite profiles among six *Camellia* species.

b. Leaf samples of six species

For leaf samples, the PLS-DA score plots show the metabolic differentiation of leaf profiles among six *Camellia* species. While the first component 1 accounts for most of the variation with 44.8%, effectively separating different species, the second component 2 contributes further to the discrimination, 22.1%. Together, these components explain 66.9% of the total variance, indicating a strong differentiation among species. In which *C. tienii* (TIE, yellow) remains distinct but has some overlap with *C. phanii* (PHA, purple). *C. tamdaoensis* (TAM, pink) is more spread out, showing high variability in its metabolite profile. *C. euphlea* (EUP, red) and *C. petelotii* (PET, light blue) form compact clusters, indicating more uniform metabolite compositions. *C. hakodae* (HAK, green) shows overlap with PHA and PET, suggesting some metabolic similarity. The plot suggests species-specific metabolite signatures, useful for chemotaxonomy. Overall, *C. tienii* and *C. tamdaoensis* are distinct from the others, possibly due to unique biosynthetic pathways, which should be subjected to further investigation. Overlapping clusters (HAK, PET, and PHA) may indicate shared ecological or evolutionary adaptations.

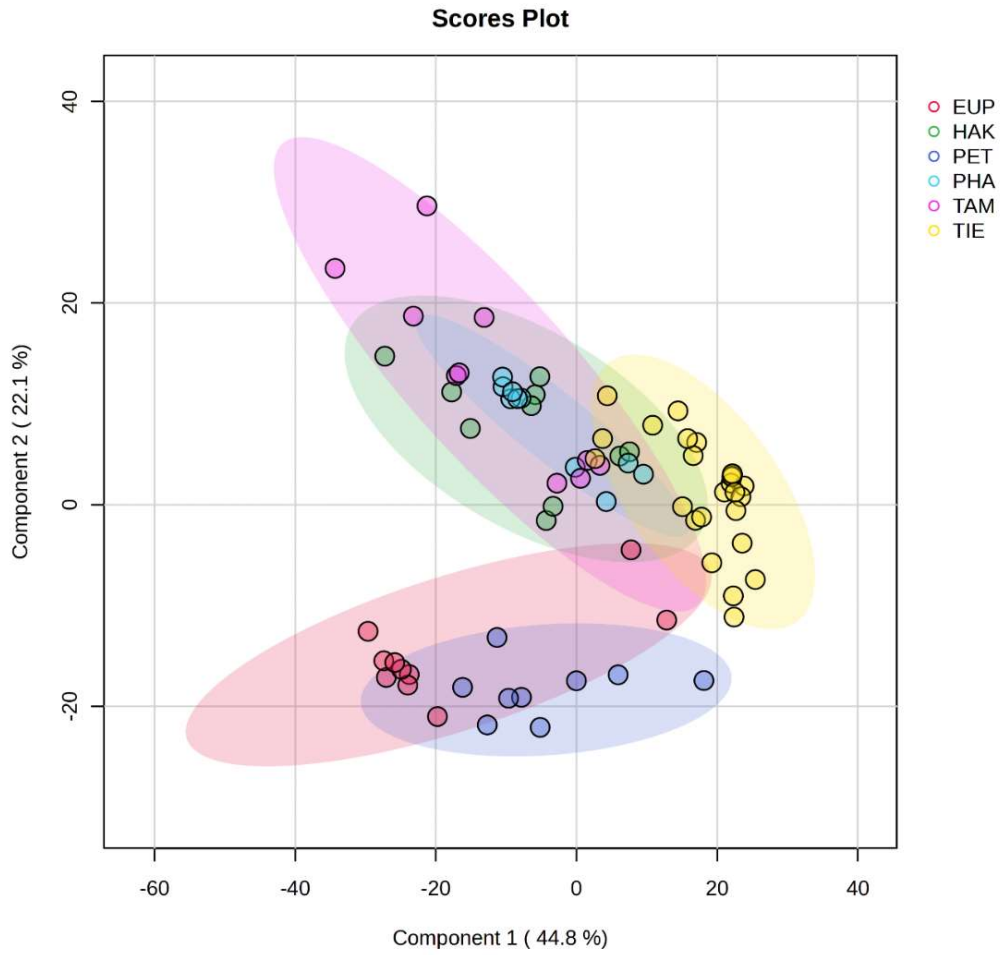


Figure 3.2.2. The PLS-DA score plots show the metabolic differentiation of leaf profiles among six *Camellia* species

3.2.3. Tissue-specific discrimination

The PCA method was applied to the data set formed by leaf and flower *C. tienii* samples, jointly accumulating 53.1% of the total variance, in which PC1 and PC2 explained 32.6% and 20.5%, respectively (Figure 3.2.3). A clear separation is observed in the PCA score plots, indicating a clear discrimination in volatile compounds between leaves and flowers of *C. tienii.* This plot may contribute to explaining the difference in the flavour of golden *Camellia* infusion from these tissues, as the *Camellia*'s popularity often relies on its satisfying aroma from volatile compounds (Y. Hara et al, 1995).

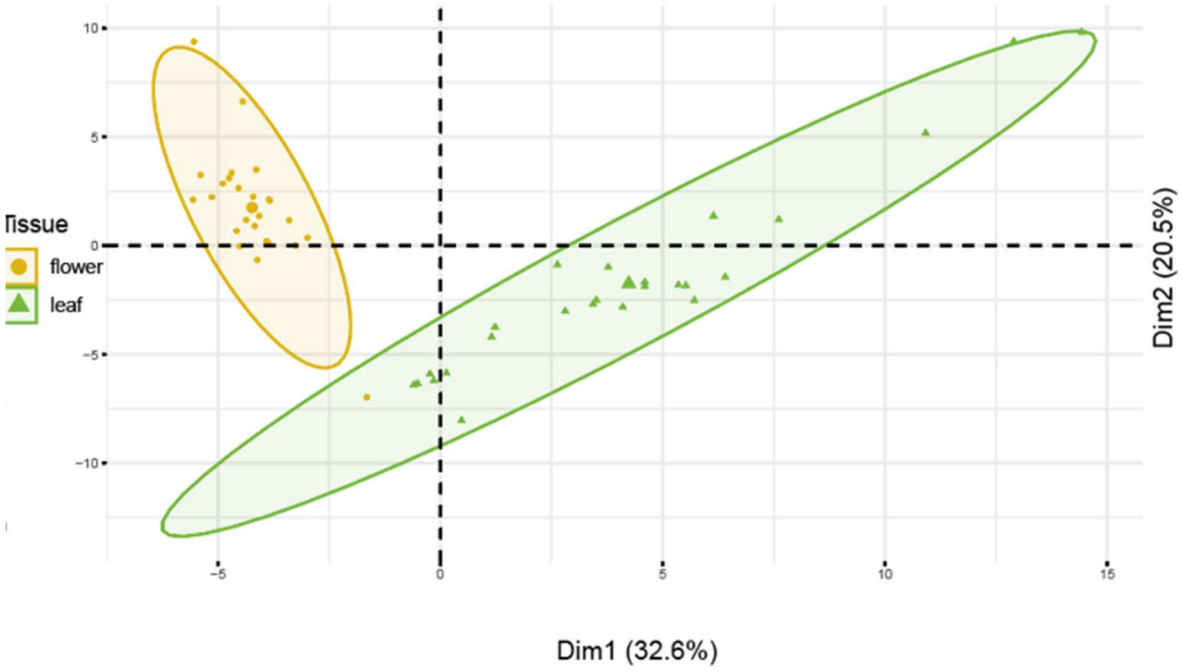


Figure 3.2.3. The PLS-DA score plots show the metabolic differentiation of leaf profiles among leaf and flower *C. tienii* samples.

The heatmap (Figure 3.2.4) shows the dominance of terpenoids such as neophytadiene, squalene, phytol, and DL-α-tocopherol in leaves, while fatty acids in both saturated and unsaturated forms were produced more in flowers of *C. tienii*. These results will serve as the basis for additional research on these materials' chemical composition and biological activity.

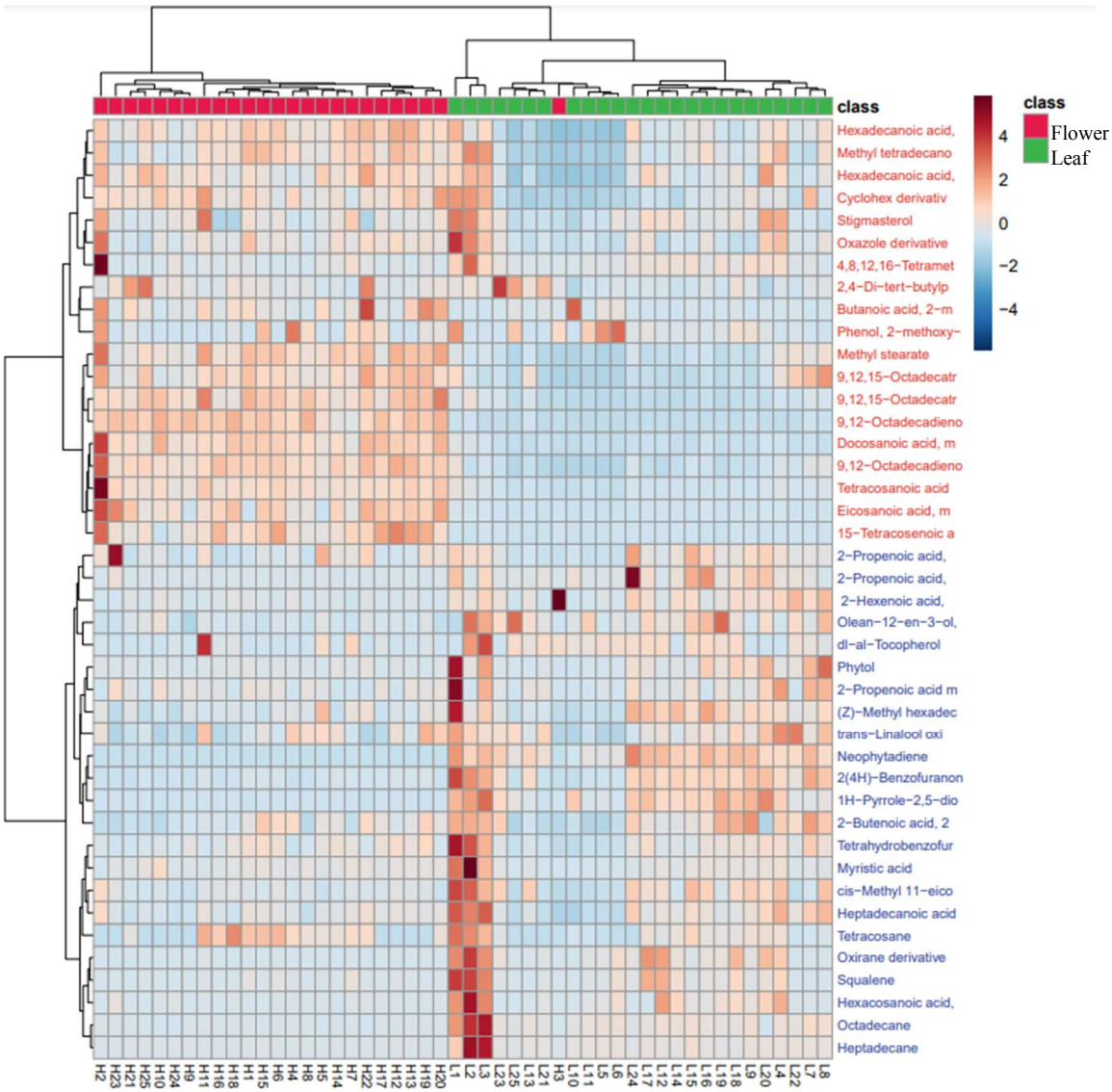


Figure 3.2.4: Heatmap of the intensity of 42 metabolites detected by GC-MS of flowers and leaves of *C. tienii* samples

Discussion

This study has successfully identified volatile compounds in the endemic species. Among compounds analyzed by the GC-MS method, the groups that appeared most abundant included esters, fatty acids, and terpenoids with a high probability. Besides, there are natural compounds that contribute to the medicinal and nutritional value of precious tea varieties endemic to Vietnam, such as citral and dl-α-tocopherol, phytol, and squalene. PLS-DA can discriminate the GC-MS-based metabolite profiles of leaves and flowers from six species, providing a feasible method for chemotaxonomy and a biomarker for quality control of these materials. PCA was successfully used to discriminate the parts of the species *Camellia tienii* clearly. The results of this study demonstrate that GC-MS and PCA are very suitable methods for the identification and

discrimination of tea species. Further studies on other types of tea in Vietnam are necessary to discover new natural compounds with potential medical and nutritional values.

PART 3.3. STUDY ON MINERAL CONTENT OF ENDEMIC GOLDEN *CAMELLIA*

3.3.1. Mineral content of leaves

A series of standard solutions and analytical elements was prepared, including the elements Na, Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Sr, Mo, Ag, Cd, Ba, Pb, and Bi from concentrations of 1.00 - 2000 ppb. The intensity of the analytical signal (Y) varied with the concentration of the standard substance (X) to construct the calibration curves. The spectrometric experiment was based on the selection of isotopes ^{23}Na, ^{24}Mg, ^{27}Al, ^{39}K, ^{40}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{64}Zn, ^{71}Ga, ^{75}As, ^{77}Se, ^{88}Sr, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{137}Ba, ^{208}Pb, and ^{209}Bi to avoid mass duplication phenomena.

Table 3.3.1: Standard curve of elements determined by ICP-MS

No	Elements	Standard curves	Correlation coefficient (R)
1	^{23}Na	f(x) = 45100.5094*x + 148335.4194	0.9953
2	^{24}Mg	f(x) = 23631.9510*x + 167560.9200	0.9970
3	^{27}Al	f(x) = 33484.6399*x + 39442.3046	0.9968
4	^{39}K	f(x) = 48154.8508*x + 2124208.2870	0.9957
5	^{40}Ca	f(x) = 624.5126*x + 8397071.6421	0.9922
6	^{51}V	f(x) = 49424.4185*x + 27630.6492	0.9929
7	^{52}Cr	f(x) = 45758.8904*x + 20346.6530	0.9939
8	^{55}Mn	f(x) = 75820.1980*x + 10034.0955	0.9948
9	^{56}Fe	f(x) = 7864.5965*x + 1320.0769	0.9890
10	^{59}Co	f(x) = 50087.0730*x + 540.0138	0.9951
11	^{60}Ni	f(x) = 10006.5806*x + 5311.1690	0.9956
12	^{63}Cu	f(x) = 22640.2381*x + 3640.5371	0.9966
13	^{64}Zn	f(x) = 12510.6010*x + 24684.4538	0.9965
14	^{71}Ga	f(x) = 37189.0275*x + 410.0089	0.9954
15	^{75}As	f(x) = 778.2577*x + 70.0004	0.9969
16	^{77}Se	f(x) = 570.7533*x + 1080.0531	0.9991
17	^{88}Sr	f(x) = 113625.6487*x + 2360.2257	0.9952
18	^{95}Mo	f(x) = 16413.8956*x + 870.0324	0.9948
19	^{107}Ag	f(x) = 42800.7570*x + 10954.8576	0.9970
20	^{111}Cd	f(x) = 10331.4159*x + 20.0001	0.9962
21	^{137}Ba	f(x) = 17277.1827*x + 510.0118	0.9971
22	^{208}Pb	f(x) = 78830.9545*x + 1790.1331	0.9962
23	^{209}Bi	f(x) = 112163.2288*x + 260.0034	0.9967

After mineralization, the materials were analysed with a spectrometer to identify and measure the sample's mineral components. More than 20 minerals were identified in the sample, including: Ca, Na, Al, K, Mg, V, Cr, Fe, Co, Ni, Cu, Zn, Sr, Mo, Ga, Se, Ag, Cd, Pb, Bi, and Ba. The elemental composition data of the samples are presented in Table 3.3.2. Metal concentrations in the samples are expressed as arithmetic mean values along with their corresponding standard deviations (SD).

Table 3.3.2: The content of the minerals in **leaves** of six *Camellia* species

No	Elements	HAK		PHA		TAM		TIE		PET		EUP	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1.	$^{23}Na^{c}$	0.0007	± 0.0001	0.0035	± 0.0032	0.0004	± 0.0004	0.0004	± 0.0007	0.0022	± 0.0013	0.0006	± 0.0006
2.	$^{24}Mg^{c}$	0.0173	± 0.0045	0.0169	± 0.0052	0.0150	± 0.0036	0.0138	± 0.0034	0.0098	± 0.0022	0.0096	± 0.0023
3.	$^{27}Al^{c}$	0.0553	± 0.0147	0.0412	± 0.0151	0.0323	± 0.0185	0.0429	± 0.0128	0.0619	± 0.0127	0.0698	± 0.0114
4.	$^{39}K^{c}$	0.0738	± 0.0171	0.0883	± 0.0223	0.0659	± 0.0109	0.0659	± 0.0159	0.0426	± 0.0114	0.0408	± 0.0084
5.	$^{40}Ca^{c}$	0.1963	± 0.0464	0.1804	± 0.0686	0.0916	± 0.0846	0.1587	± 0.0566	0.1108	± 0.0291	0.0941	± 0.0226
6.	$^{51}V^{a}$	0.0146	± 0.0003	ND	ND	ND	ND	ND	ND	ND	ND	0.1860	± 0.0432
7.	$^{52}Cr^{b}$	0.0072	± 0.0048	0.0057	± 0.0040	0.0074	± 0.0056	0.0137	± 0.0089	0.0055	± 0.0037	0.0093	± 0.0105
8.	$^{55}Mn^{b}$	2.4137	± 0.8388	3.7093	± 1.5398	1.9275	± 1.7937	3.5149	± 2.2610	1.6061	± 0.5395	1.7158	± 0.5679
9.	$^{56}Fe^{b}$	0.5370	± 0.1613	0.4671	± 0.1468	0.3765	± 0.1892	0.5933	± 0.2309	0.4675	± 0.1775	0.6748	± 0.3886
10.	$^{59}Co^{a}$	0.4342	± 0.2448	0.5991	± 0.5516	0.5502	± 0.2670	0.8389	± 0.3122	0.3153	± 0.0814	0.3710	± 0.0941
11.	$^{60}Ni^{a}$	7.2651	± 2.8631	6.8537	± 2.8913	8.8176	± 2.0603	7.8470	± 3.0473	9.2670	± 2.2665	7.5290	± 1.6547
12.	$^{63}Cu^{b}$	0.0277	± 0.0104	0.0227	± 0.0052	0.0290	± 0.0095	0.0188	± 0.0056	0.0276	± 0.0297	0.0224	± 0.0037
13.	$^{64}Zn^{b}$	0.0781	± 0.0764	0.0785	± 0.0369	0.0564	± 0.0167	0.1461	± 0.1839	0.0467	± 0.0500	0.0944	± 0.0885
14.	$^{71}Ga^{a}$	0.5850	± 0.7159	1.2987	± 1.1521	0.1344	± 0.0339	1.4363	± 1.1180	ND	ND	0.0287	± 0.0879
15.	$^{75}As^{a}$	ND	ND	1.3568	± 5.5021	ND	ND	1.8427	± 0.2660	ND	ND	ND	ND
16.	$^{77}Se^{a}$	ND	ND	ND	ND	ND	ND	1.0027	± 0.8012	ND	ND	ND	ND
17.	$^{88}Sr^{b}$	0.1994	± 0.0666	0.1796	± 0.0711	0.0838	±0.1265	0.1931	± 0.0821	0.1303	± 0.0925	0.0958	± 0.0416
18.	$^{95}Mo^{a}$	2.8408	± 0.0816	2.3755	± 0.2204	ND	ND	ND	ND	ND	ND	ND	ND
19.	$^{107}Ag^{a}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
20.	$^{111}Cd^{a}$	ND	ND	ND	ND	0.1159	± 0.4263	ND	ND	0.0106	± 0.0282	0.0511	± 0.0337
21.	$^{137}Ba^{b}$	0.6326	± 0.2124	0.6472	± 0.3723	0.2604	± 0.3715	0.7043	± 0.2544	0.3544	± 0.2001	0.3295	± 0.1861
22.	$^{208}Pb^{a}$	4.1305	± 1.2550	3.7021	± 2.3265	4.3081	± 4.299	4.5659	± 1.0263	1.9445	± 0.8704	2.9322	± 1.1179
23.	$^{209}Bi^{b}$	0.0117	± 0.0161	0.0050	± 0.0074	0.0002	± 0.0037	0.3851	± 0.4080	ND	ND	ND	ND

Note: ND: not detected; a: ppb (ng/g); b: ppm (µg/g); c: ‰ (mg/g)

The elemental profile comprised macro-minerals (Na, Mg, K, Ca), micro-minerals (Mn, Fe, Cu, Zn, Mo, Se, Co, Cr), and other elements (V, Al, Ni, Sr, Ga, Ba, Bi). Heavy metals, including Ag, As, Pb, and Cd, were analyzed but were not detected or were present at levels below permissible limits.

The macro-minerals (Na, Mg, K, Ca) constitute the largest proportion of the elemental composition in these *Camellia* species (Figure 3.3.1).

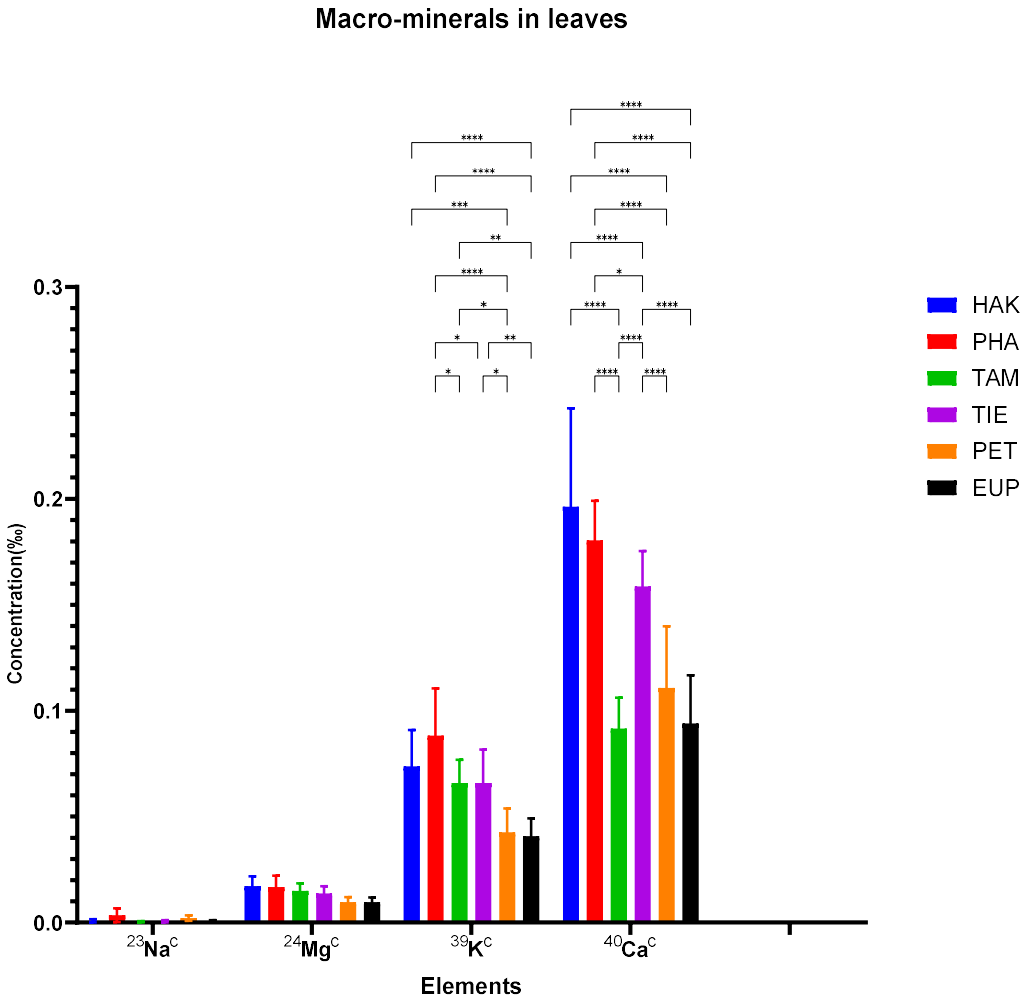


Figure 3.3.1: Macro-Mineral Contents in Leaves of Six Golden Camellia Species

*[Note: **** $p \leq 0.0001$ (*extremely significant*), *** $p \leq 0.001$ (highly significant), ** $p \leq 0.01$ (more significant), * $p \leq 0.05$ (statistically significant), NS $p > 0.05$ (not significant)]*

Among all elements, calcium exhibits the highest concentration, with 0.1963 mg/g in *C. hakodae* and the lowest value of 0.0916 mg/g in *C. tamdaoensis*. This corresponds to approximately 0.9160 - 1.9630 mg of calcium per 10 g of dried leaves in golden *Camellia*. (Table 3.3.3 and Figure 3.3.1)

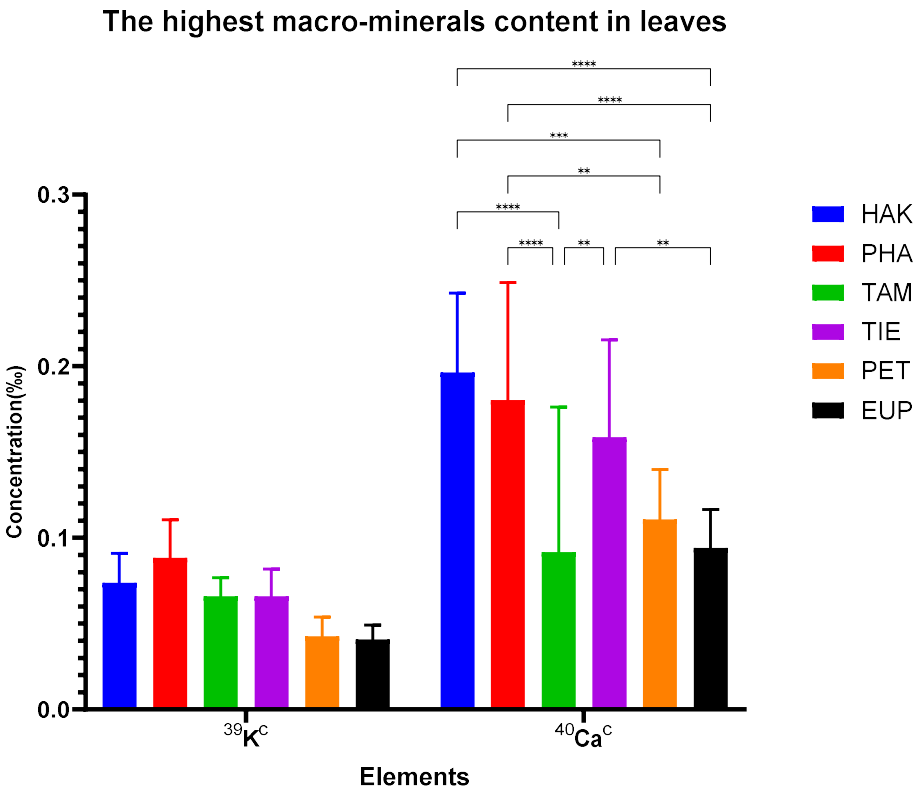


Figure 3.3.2: Highest Macro-Mineral Contents in Leaves of Six Golden *Camellia* Species

Concentration of potassium is the highest in *C.phanii* (0.0883 mg/g) and lowest in *C.euphlebia* (0.0408 mg/g). It is similar in three species (*C.hakodae, C.tamdaoensis* and *C.tienii*) and the same in two species (*C.petelotii and C.euphlebia*) (Table 3.3.3 and Figure 3.3.2)

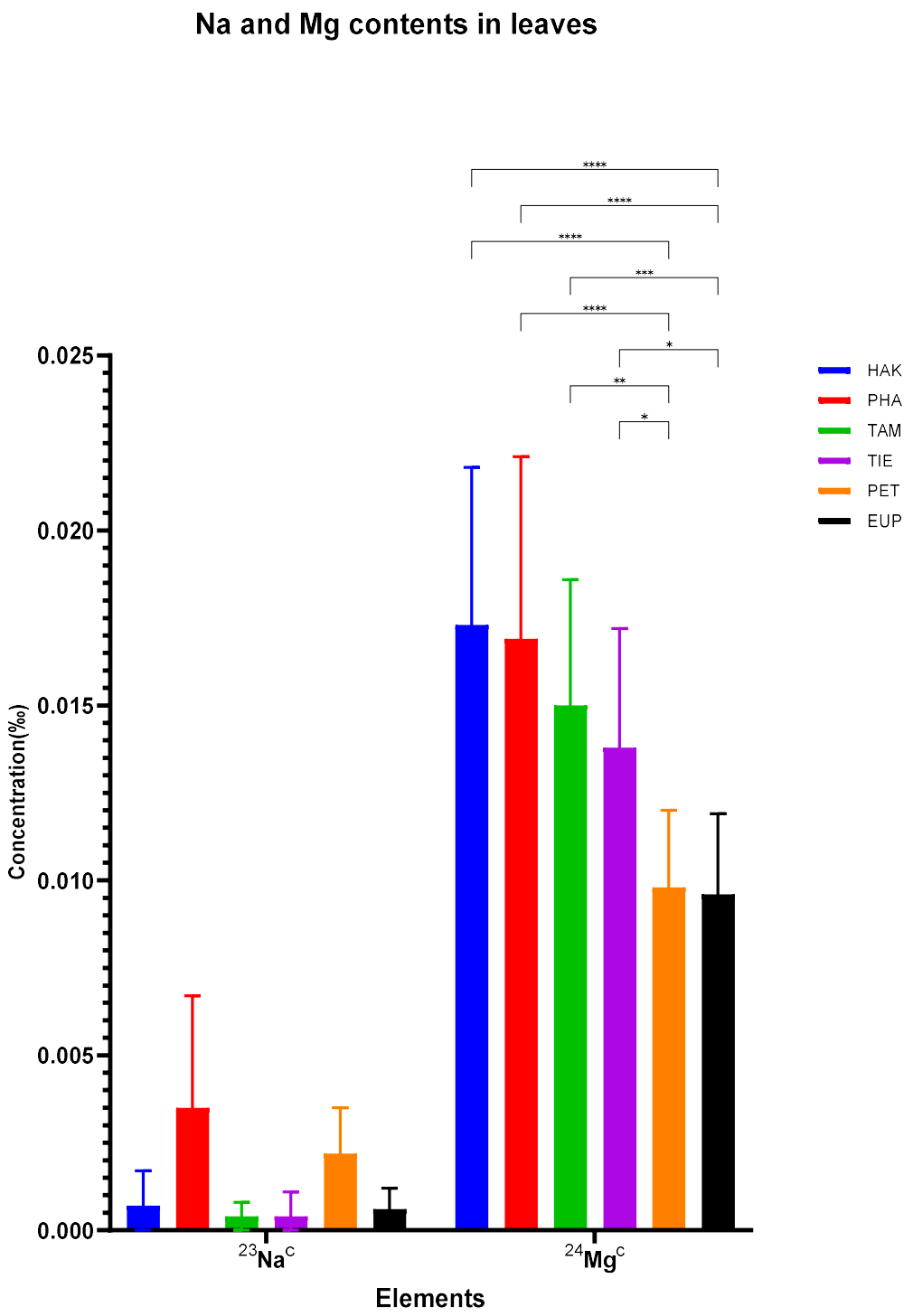


Figure 3.3.3: Na and Mg Content in Leaves of Six Golden *Camellia* Species

Figure 3.3.3 illustrates that the Mg content between the two species in Tam Dao did not differ significantly, and similarly, no significant difference was observed among the four species in Quang Ninh. In contrast, a significant difference in Mg content was detected between the two regions, highlighting a clear regional distinction in the Mg content of golden *Camellia*

Micro-mineral contains Cr, Mn, Fe, Co, Cu, Zn, Se, and Mo. Trace element analysis of six species of golden *Camellia* revealed that the predominant elements in the leaves are Mn, Fe, Cu, and Zn, which are the four principal elements closely associated with antioxidant activity (Figure 3.3.4).

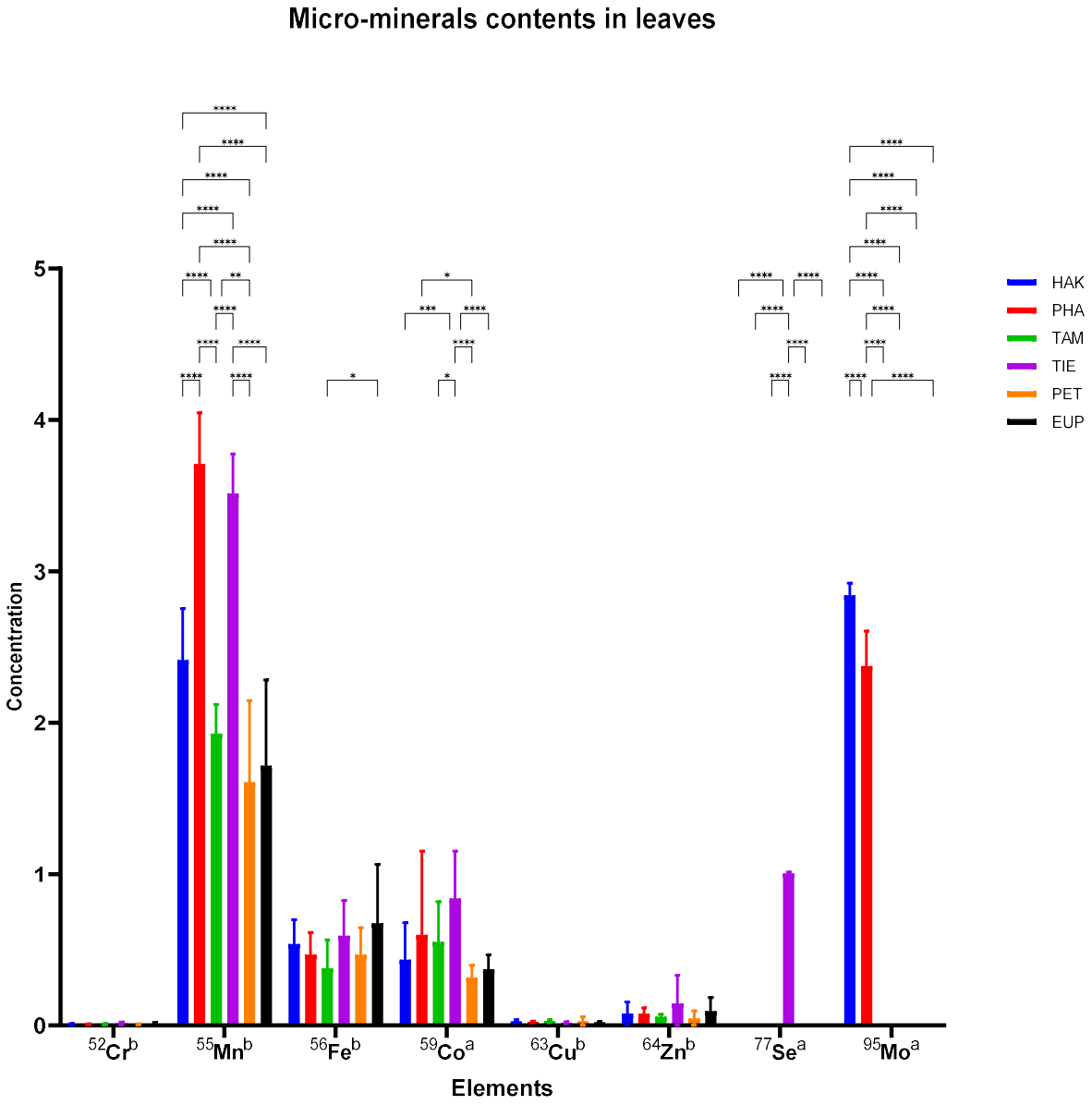


Figure 3.3.4: Micro-mineral Content in Leaves of Six Golden *Camellia* Species

Analysis deeply about four micro-minerals (Mn, Fe, Cu, Zn) is shown in Figure 3.3.5 below

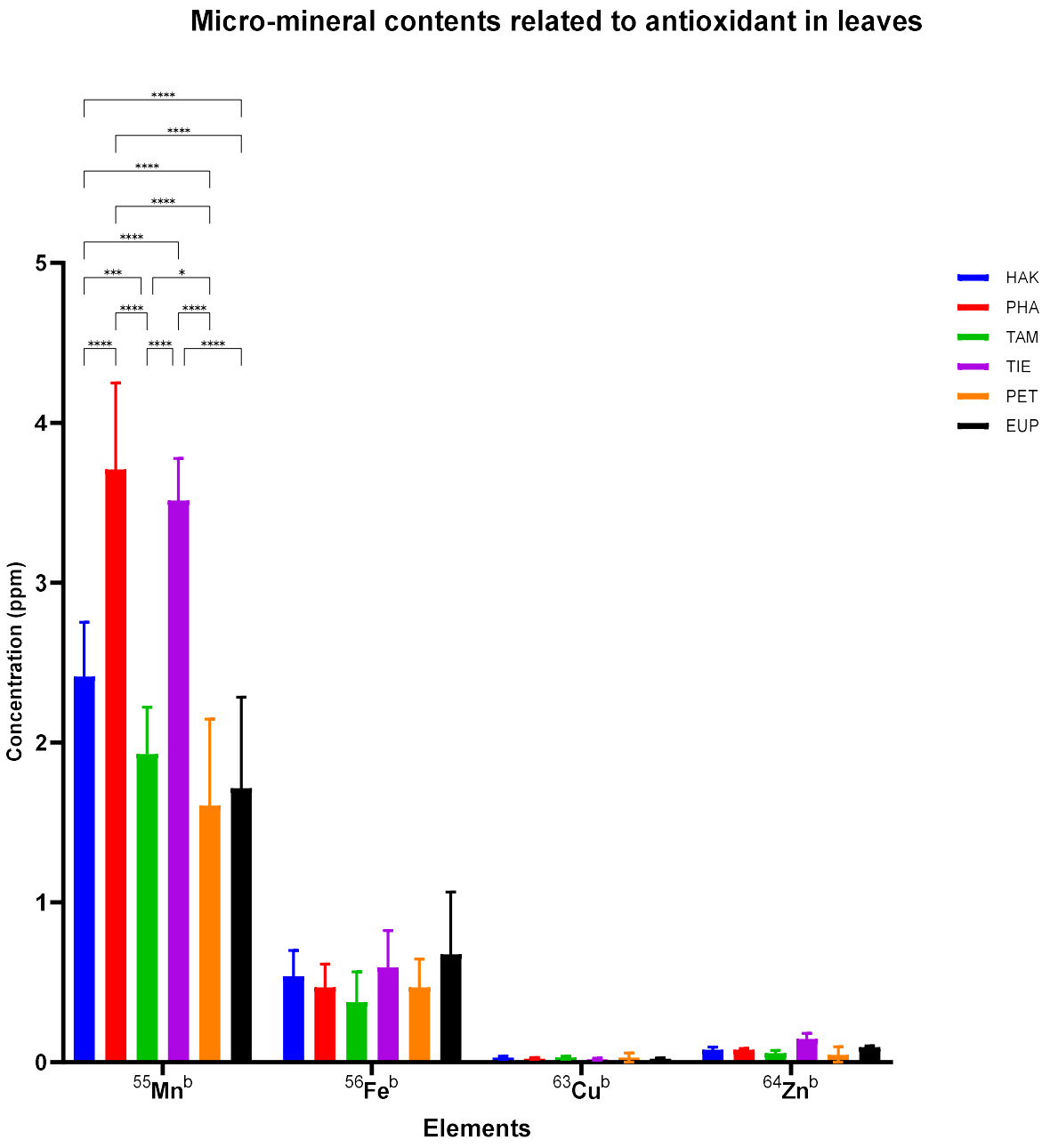


Figure 3.3.5: Micro-mineral contents related to antioxidants in Leaves of Six Golden *Camellia* Species

Mn was the most abundant trace element, with high contents in *C. phanii* and *C. tienii* (not significantly different from each other but significantly higher than the other four species). *C. hakodae* also had intermediate concentrations of Mn, while *C. petelotii* and *C. euphlebia* (both from Quang Ninh) had lower levels. Fe concentrations did not differ significantly among the six species. Mn and Fe distributions are shown in Figure 3.3.6 below

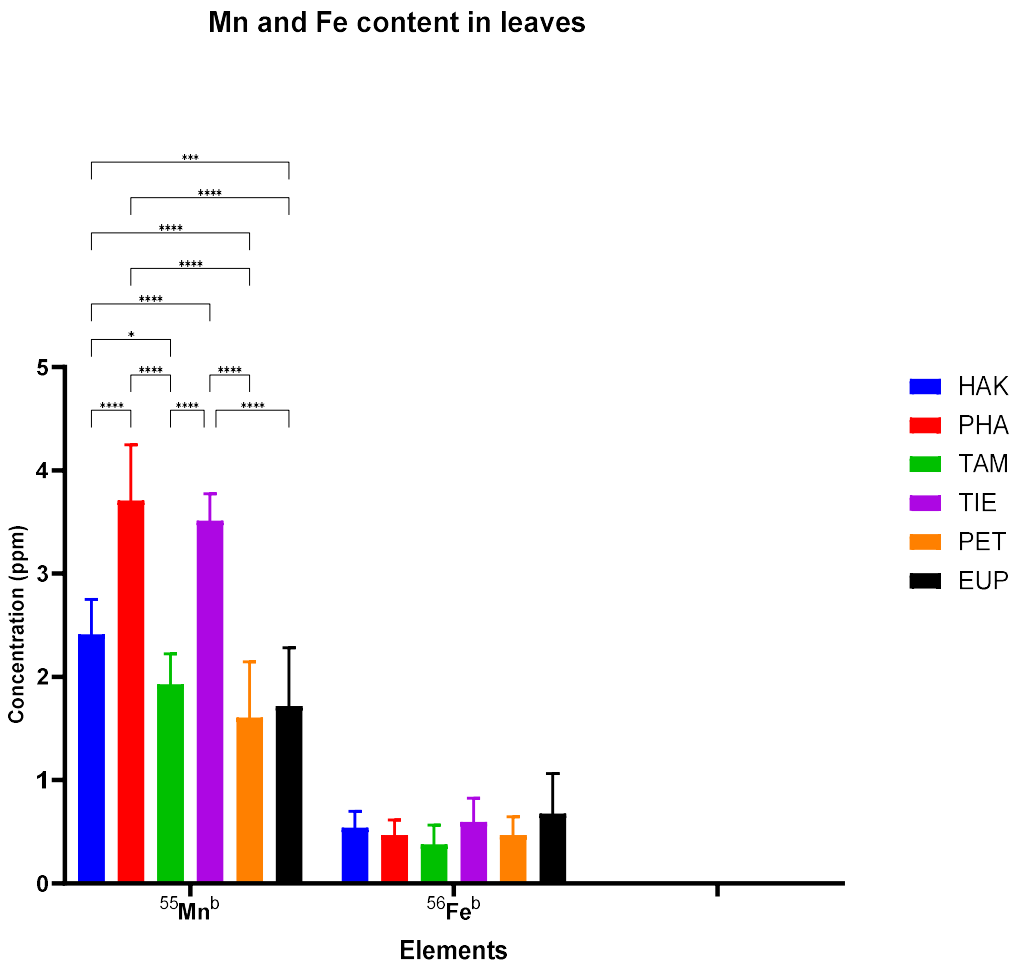


Figure 3.3.6: Mn and Fe Content in Leaves of Six Golden *Camellia* Species

As shown in Figure 3.3.6, *C. tienii* exhibited the highest Cu concentration, which is likely closely associated with the antioxidant activity of this species. The contents of Cu and Zn are presented in Figure 3.3.7

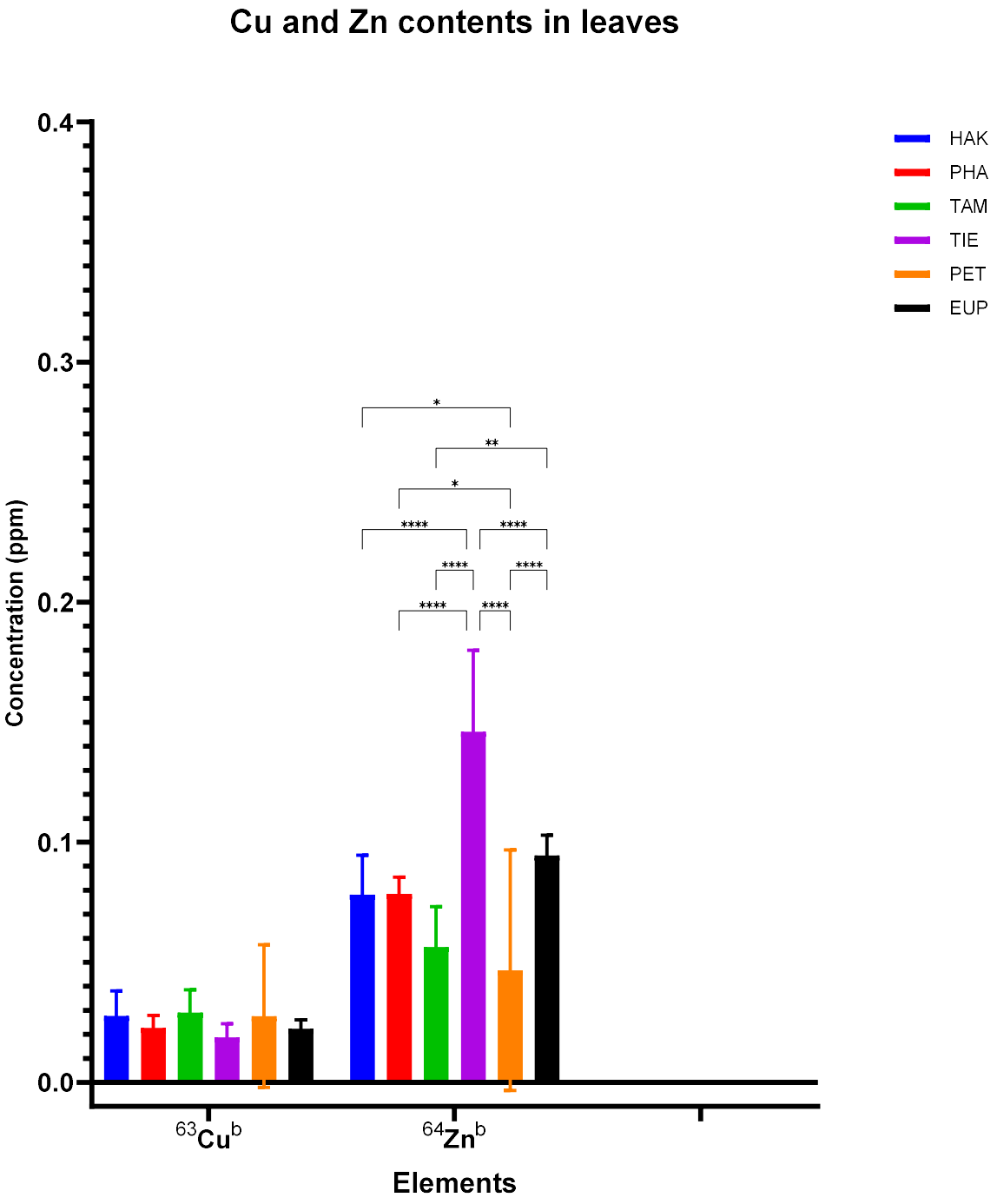


Figure 3.3.7: Cu and Zn Content in Leaves of Six Golden *Camellia* Species

The concentration of Zn was higher than that of Cu across all six species. *C. tienii* exhibited the highest Zn content, while *C. petelotii* showed the lowest. Meanwhile, the Cu content was relatively similar among all six species, with no significant differences observed.

Concentration of Aluminum was detected at relatively high levels in the leaf samples.

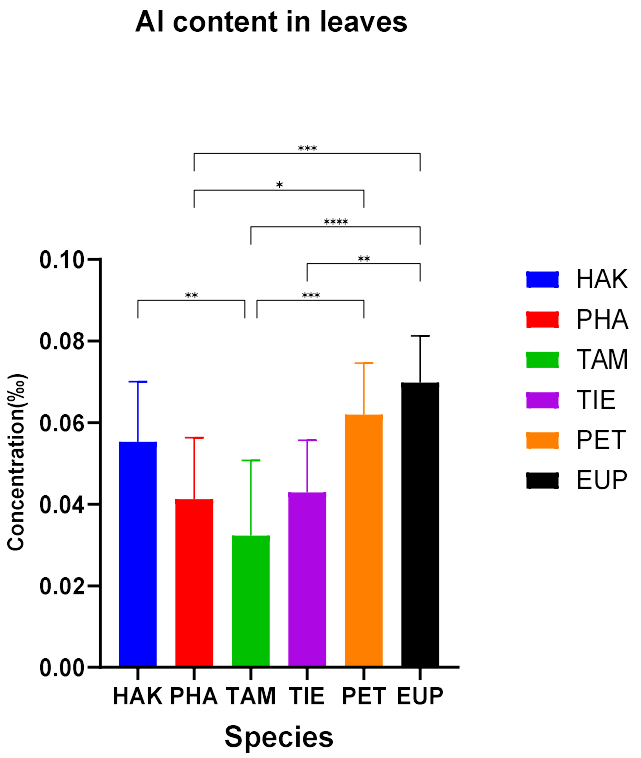


Figure 3.3.8: Al Content in Leaves of Six Golden *Camellia* Species

Several studies have indicated that Alzheimer's disease is associated with the amount of Al intake (Karak & Bhagat, 2010). However, aluminum also influences flower coloration through its interactions with flavonoid compounds (Tanikawa et al., 2008).

Based on Table 3.3.1, the Ca content is the highest among all minerals. After quantifying and obtaining the results shown in Table 3.3.2 above, it was found that there are nine minerals with biological effects, such as Ca, Mg, Na, K, Mn, Fe, Cu, Zn, and Cr. Evaluating and comparing the nine elements shown in the following Figures 3.3.1 and 3.3.5

In the leaf samples, *C. hakodae* species contains the highest concentrations for the following elements: Mg, Ca, Fe, Sr, while *C. phanii* represented the highest concentrations of Na, K, and Mn. Copper has been detected the most in *C. tamdaoensis,* whereas *C. tienii* species contain the highest concentrations of Cr, Co, and Zn. *C. euphlebia* species demonstrated the highest level of Al and Fe. The results showed that the content of heavy elements was not detected or was very low under limited conditions, such as Pb, As, Ag, and Cd. Therefore, the leaf of the golden *Camellia* is safe for humans.

3.3.2. Mineral content of flowers

Flowers are the highest value and are useful for health. Studying flower samples is significant. After mineralization, the digested samples were analyzed using a spectrometer to identify and quantify their mineral components. Many elements were detected, including Ca, Na, Al, K, Mg, Cr, Fe, Co, Ni, Cu, Zn, Sr, Mo, Ga, Se, Ag, Cd, Pb, Bi, and Ba. The elemental composition data are presented in Table 3.3.3, with metal concentrations expressed as arithmetic mean values and their corresponding standard deviations (SD).

Examination of the data in Table 3.3.3 shows that macro-elements (Na, Mg, K, and Ca) are present at relatively high levels across all six species. Among the trace elements analyzed (Cr, Mn, Fe, Co, Cu, Zn, Se, and Mo), Mn, Fe, Cu, and Zn were the most abundant, whereas Se was detected only in trace amounts. Heavy metals were either not detected or found at concentrations within acceptable safety limits.

Table 3.3.3: The content of the minerals in **flowers** of the golden *Camellia* species

No	Elements	HAK		PHA		TAM		TIE		PET		EUP	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1.	$^{23}Na^{c}$	0.0018	± 0.0003	0.0023	± 0.0001	0.0024	± 0.0008	ND	ND	ND	ND	ND	SD
2.	$^{24}Mg^{c}$	0.0072	± 0.0020	0.0126	± 0.0003	0.0119	± 0.0032	0.0089	± 0.0008	0.0052	± 0.0013	0.0070	± 0.0013
3.	$^{27}Al^{c}$	0.0064	± 0.0013	0.0056	± 0.0002	0.0052	± 0.0025	0.0025	± 0.0012	0.0049	± 0.0017	0.0046	± 0.0011
4.	$^{39}K^{c}$	0.0624	± 0.0107	0.0841	± 0.0206	0.0941	± 0.0054	0.0874	± 0.0083	0.0498	± 0.0143	0.0611	± 0.0062
5.	$^{40}Ca^{c}$	0.0359	± 0.0069	0.0360	± 0.0090	0.0330	± 0.0123	0.0388	± 0.0087	0.0345	± 0.0092	0.0360	± 0.0028
6.	$^{51}V^{a}$	ND		ND		ND		ND		ND		ND	ND
7.	$^{52}Cr^{b}$	0.0053	± 0.0010	0.0056	± 0.0001	0.0054	± 0.0016	0.0023	± 0.0013	0.0083	± 0.0055	0.0086	± 0.0084
8.	$^{55}Mn^{b}$	0.7824	± 0.0392	0.2267	± 0.0049	0.5812	± 0.3284	0.1383	± 0.0766	0.1905	± 0.0681	0.1817	± 0.0295
9.	$^{56}Fe^{b}$	0.2482	± 0.0304	0.3020	± 0.0223	0.2708	± 0.0327	0.2084	± 0.0216	0.1945	± 0.0758	0.2372	± 0.0335
10.	$^{59}Co^{a}$	0.2570	± 0.0001	0.1750	± 0.0120	0.3001	± 0.0670	0.3831	± 0.3320	0.0771	± 0.0041	0.0722	± 0.0290
11.	$^{60}Ni^{a}$	10.0941	± 0.0025	13.8040	± 0.3001	16.6101	± 0.8410	7.6390	± 0.1300	9.1640	± 0.8671	15.8890	± 0.0330
12.	$^{63}Cu^{b}$	0.0454	± 0.0060	0.0116	± 0.0011	0.0391	± 0.0251	0.0153	± 0.0051	0.0432	± 0.0221	0.0388	± 0.0281
13.	$^{64}Zn^{b}$	0.0664	± 0.0231	0.1813	± 0.0570	0.0649	± 0.0242	0.0689	± 0.0280	0.0499	± 0.0100	0.0981	± 0.0761
14.	$^{71}Ga^{a}$	ND		ND		ND		ND		ND		ND	ND
15.	$^{75}As^{a}$	ND		ND		ND		ND		ND		ND	ND
16.	$^{77}Se^{a}$	ND		ND		ND		ND		ND		ND	ND
17.	$^{88}Sr^{b}$	0.0574	± 0.0060	0.0122	± 0.0010	0.0121	± 0.0042	0.0106	± 0.0030	0.0189	± 0.0089	0.0148	± 0.0261
18.	$^{95}Mo^{a}$	ND		0.4220	± 0.2120	0.3600	± 0.5110	ND		ND		ND	ND
19.	$^{107}Ag^{a}$	ND		ND		ND		ND		ND		ND	ND
20.	$^{111}Cd^{a}$	ND		ND		ND		0.5590	± 0.0520	0.8600	± 0.7080	0.2480	ND
21.	$^{137}Ba^{b}$	0.0768	± 0.0104	0.0089	± 0.0004	0.0197	± 0.0098	0.0237	± 0.0108	0.0305	± 0.0071	0.0271	± 0.0162
22.	$^{208}Pb^{a}$	0.5300	± 0.0010	1.0693	± 0.3230	0.9790	± 0.8481	6.529	± 0.2294	8.794	± 0.6674	4.1190	± 0.3320
23.	$^{209}Bi^{b}$	0.0110	± 0.0190	0.0001	± 0.0010	0.0010	± 0.0030	0.0133	± 0.0198	ND	ND	0.0266	± 0.0477

Note: ND: not detected; a: ppb (ng/g); b: ppm (µg/g); c:‰ (mg/g)

The macro-minerals (Na, Mg, K, Ca) constitute the largest proportion of the elemental composition in these *Camellia* species. Potassium (K) was found to be the most abundant element (content from 0.0498 to 0.0841 mg/g), followed by calcium (Ca) (from 0.0330 to 0.0388 mg/g) (Table 3.3.3 and Figure 3.3.9). Among the species studied, *C. tamdaoensis* exhibited markedly higher levels of both elements, which may contribute to biological effects related to joint, neurological, muscular, and cardiovascular functions (Brylinski Lukasz et al, 2025; F. Chen et al., 2024; Pethő et al., 2024)

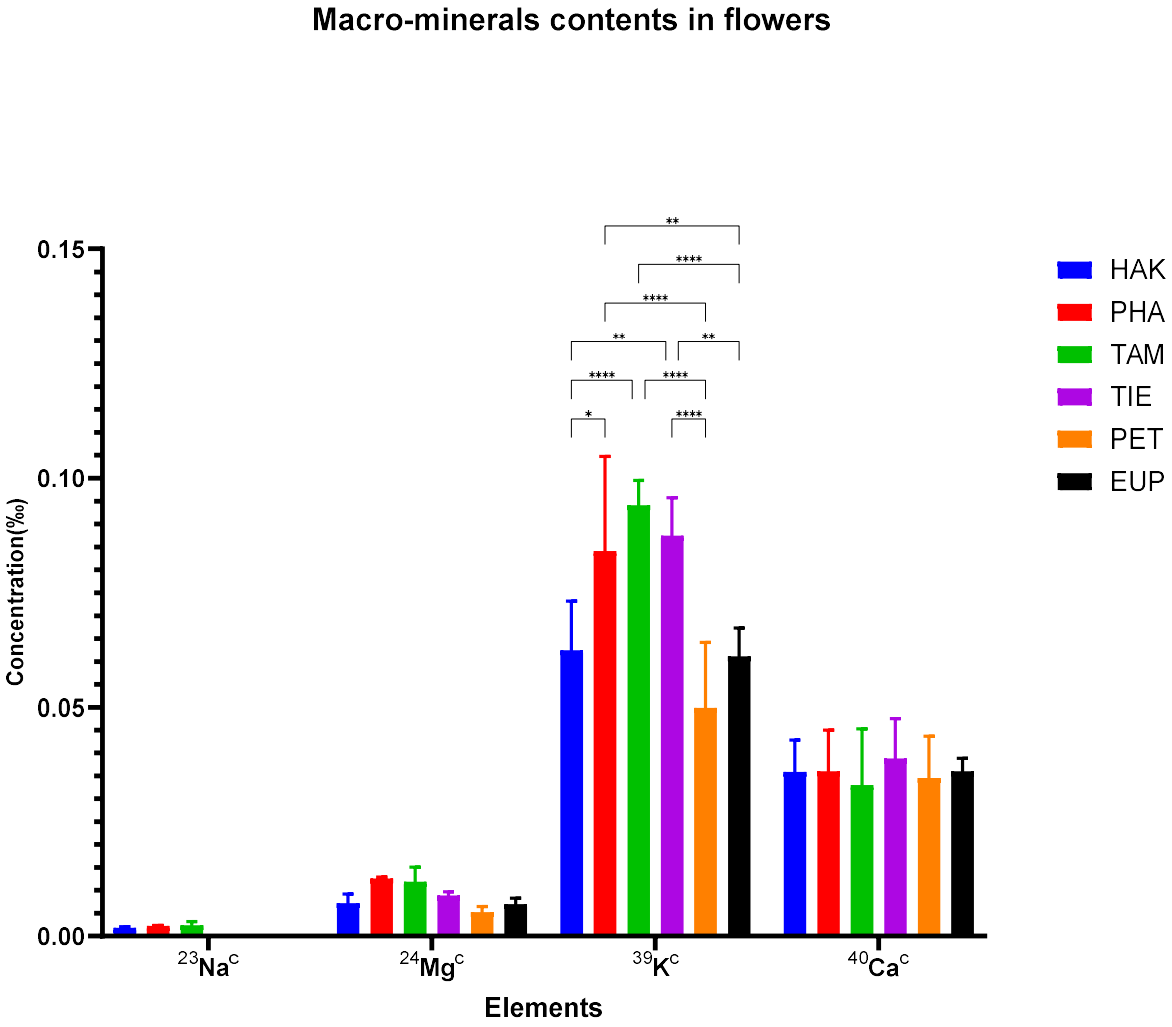


Figure 3.3.9: Macro-mineral content in flowers of Six Golden *Camellia* Species

*[Note: **** $p \leq 0.0001$ (*extremely significant*), *** $p \leq 0.001$ (highly significant), ** $p \leq 0.01$ (more significant), * $p \leq 0.05$ (statistically significant), NS $p > 0.05$ (not significant)]*

The concentration of Na and Mg is also high in the flowers of six species (Figure 3.3.10). Magnesium is essential for stabilizing neuronal activity, promoting synaptic communication, and safeguarding the integrity of the blood-brain barrier (Maier et al., 2023)

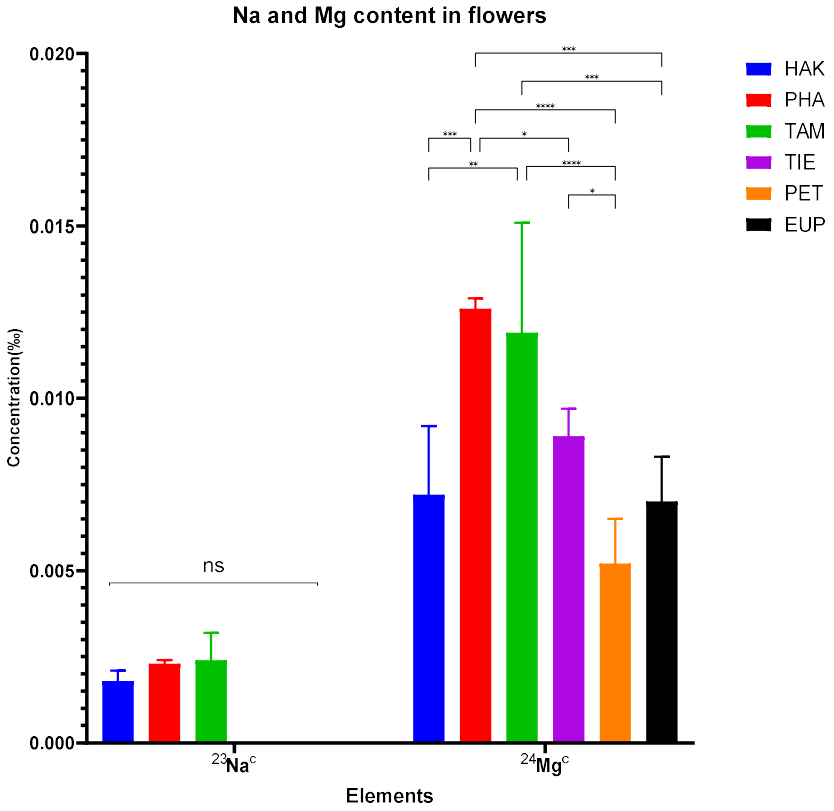


Figure 3.3.10: Na and Mg content in flowers of Six Golden *Camellia* Species

Analysis of micro-mineral (Cr, Mn, Fe, Co, Cu, Zn, Se, and Mo) in the flowers of six species is shown in Figure 3.3.11 below.

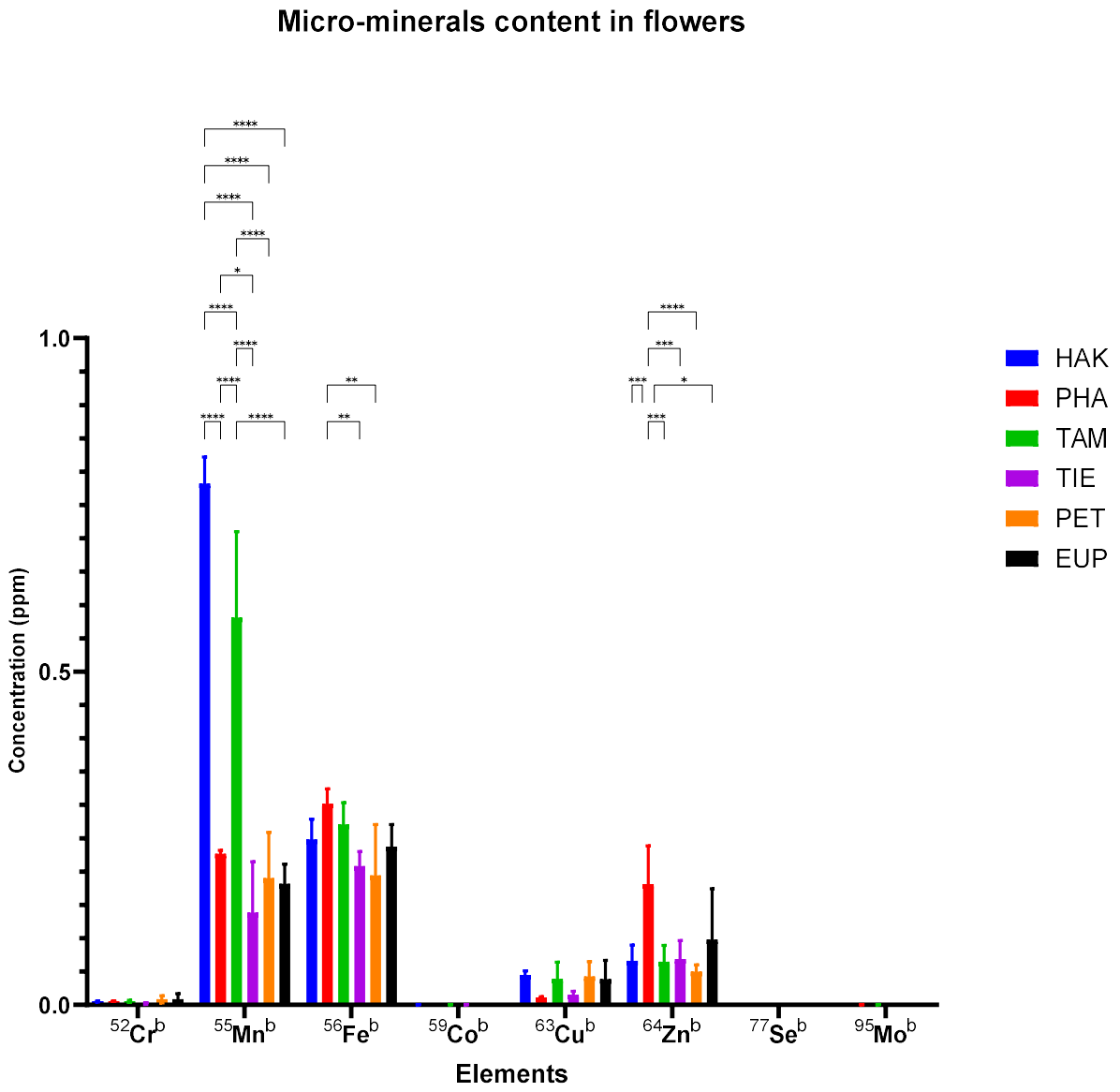


Figure 3.3.11: Micro-mineral content in flowers of Six Golden *Camellia* Species

The main elements are Mn, Fe, Zn, and Cu with high concentration. These elements are related to the antioxidant. Analysis data of this micro-mineral (Mn, Fe, Cu, Zn) concentration of Mn in *C.hakodae* is the highest, and then *C.tamdaoensis.*

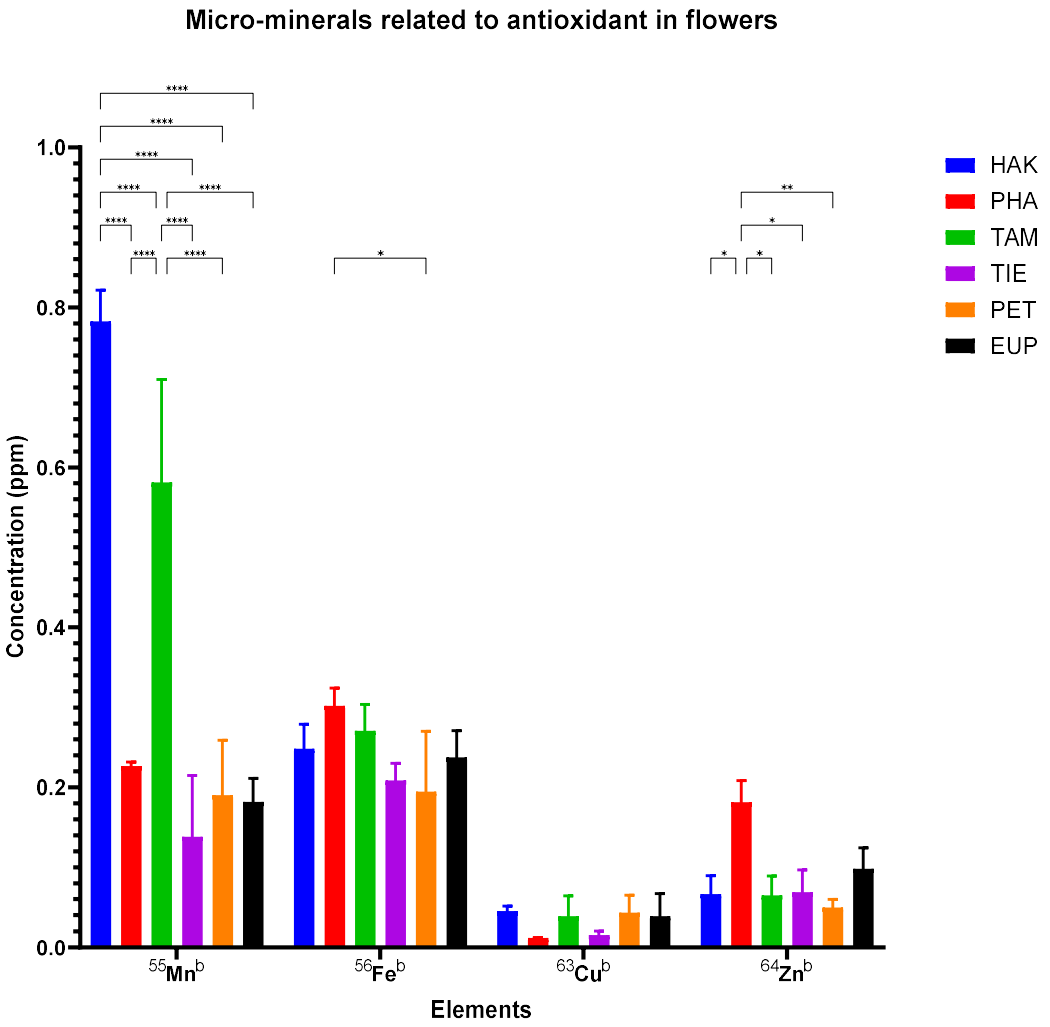


Figure 3.3.12: Mn, Fe, Cu, and Zn content in flowers of Six Golden *Camellia* Species

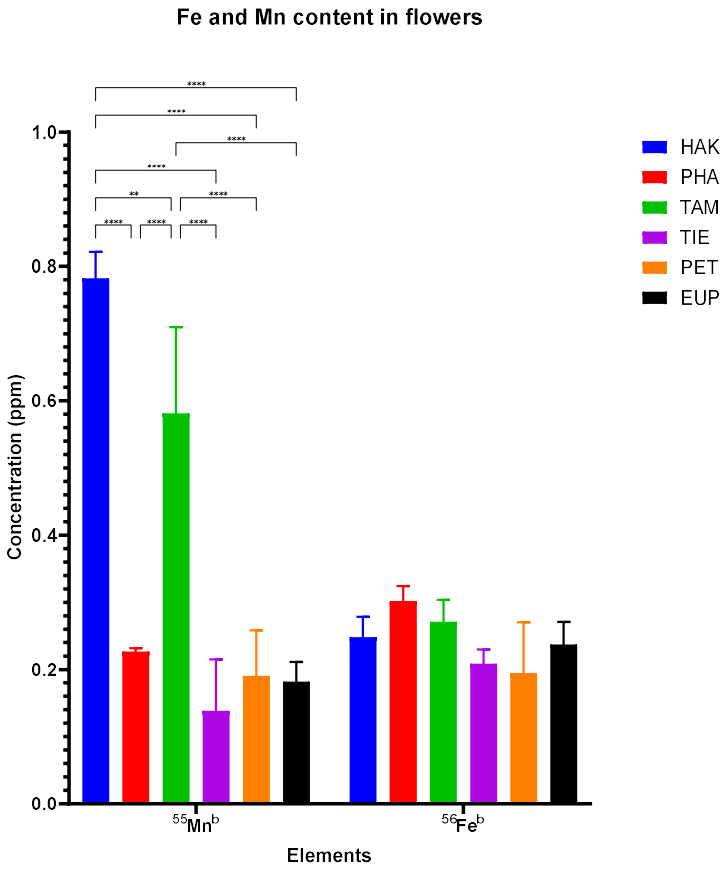


Figure 3.3.13: Mn and Fe content in flowers of Six Golden *Camellia* Species

Mn content in the flowers of *C. hakodae* and *C. tamdaoensis* was significantly higher than in the other four species, while Fe content showed no significant differences among species. This supports the strong link between Mn levels and antioxidant activity.

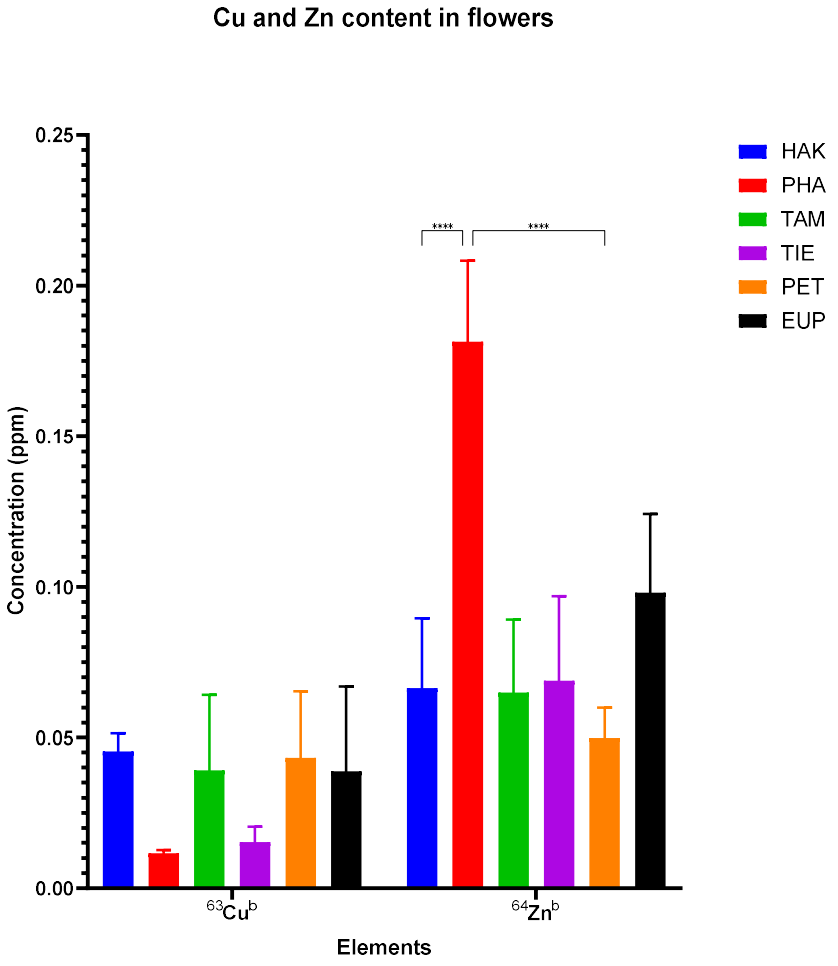


Figure 3.3.14: Cu and Zn content in flowers of Six Golden *Camellia* Species

Cu content in the flowers of all six species showed no statistically significant differences, and its levels were generally low. In contrast, Zn content was consistently higher than Cu across all six species, with the highest levels observed in *C. phanii*, followed by *C. euphelebia*. This correlates with the stronger antioxidant activity of these two species compared with the others

In the flowers, Mn content was highest in *C. hakodae*, followed by *C. tamdaoensis*, while Zn content was highest in *C. phanii*, followed by *C. euphelebia*. Accordingly, the antioxidant activity of the flowers in these four species was higher than in the remaining two, with the overall order decreasing as follows: *C. tamdaoensis* < *C. euphelebia* < *C. phanii* < *C. tienii* < *C. hakodae* < *C. petelotii*.

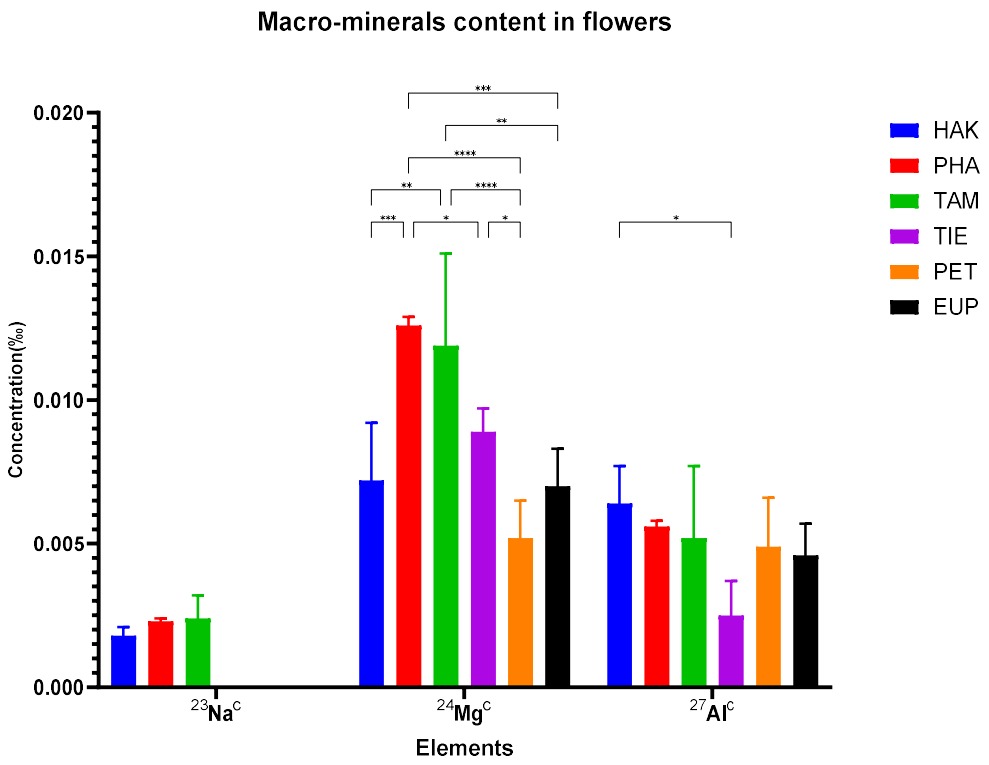


Figure 3.3.15: Na, Mg, and Al content in flowers of Six Golden *Camellia* Species

Based on Table 3.3.3, calcium (Ca) has the highest content among all measured minerals. The quantification results presented in Table 3.3.3 indicate the presence of nine biologically significant minerals: Ca, Mg, Na, K, Mn, Fe, Cu, Zn, and Cr. The evaluation and comparison of these nine elements are detailed in the following section.

In the flower samples, *C. hakodae* had the highest concentrations of the following elements among the six surveyed species: Al, Ca, Mn, Cu, Sr, and Ba. *C. phanii* exhibited the highest levels of Mg, Fe, and Zn, while *C. tamdaoensis* contained the highest concentrations of Na, K, and Ni. *C. tienii* had the highest levels of Ca and Co, whereas *C. euphlebia* showed the highest concentrations of Cr and Bi. Among these species, *C. hakodae* had the highest overall elemental content, which may influence the antioxidant activity of *Camellia* species.

All samples showed that the content of heavy elements was not detected or very low under limited conditions, such as Pb, As, Ag, and Cd. Therefore, the flower of the golden *Camellia* is safe for humans.

3.3.3. Compare mineral content between leaves and flowers

Both the leaves and flowers of golden *Camellia* contain various essential minerals, including Ca, Mg, Na, K, Mn, Fe, Cu, Zn, and Cr. The mineral concentration in the leaves is higher than in the flowers (Figures 3.3.16 and 3.3.19). Among the surveyed species, *C. hakodae* had the highest overall elemental content, which may influence the antioxidant properties of golden *Camellia*. All samples revealed that heavy elements, including Pb, As, Ag, and Cd, were either undetectable or present at trace levels within permissible limits, indicating that the flowers and leaves of golden *Camellia* are safe for human consumption.

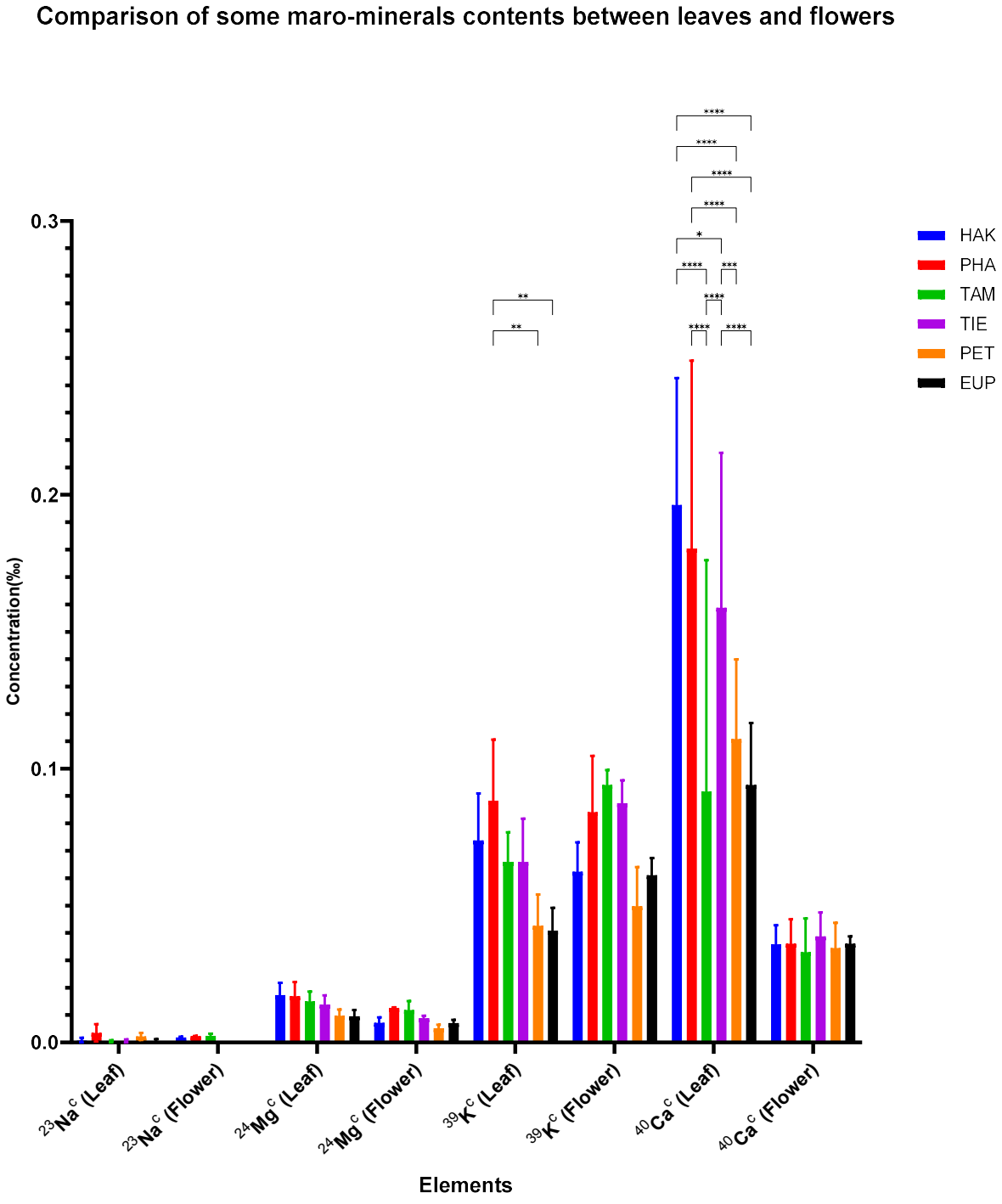


Figure 3.3.16: Content of macro-mineral in leaves and flowers of six species

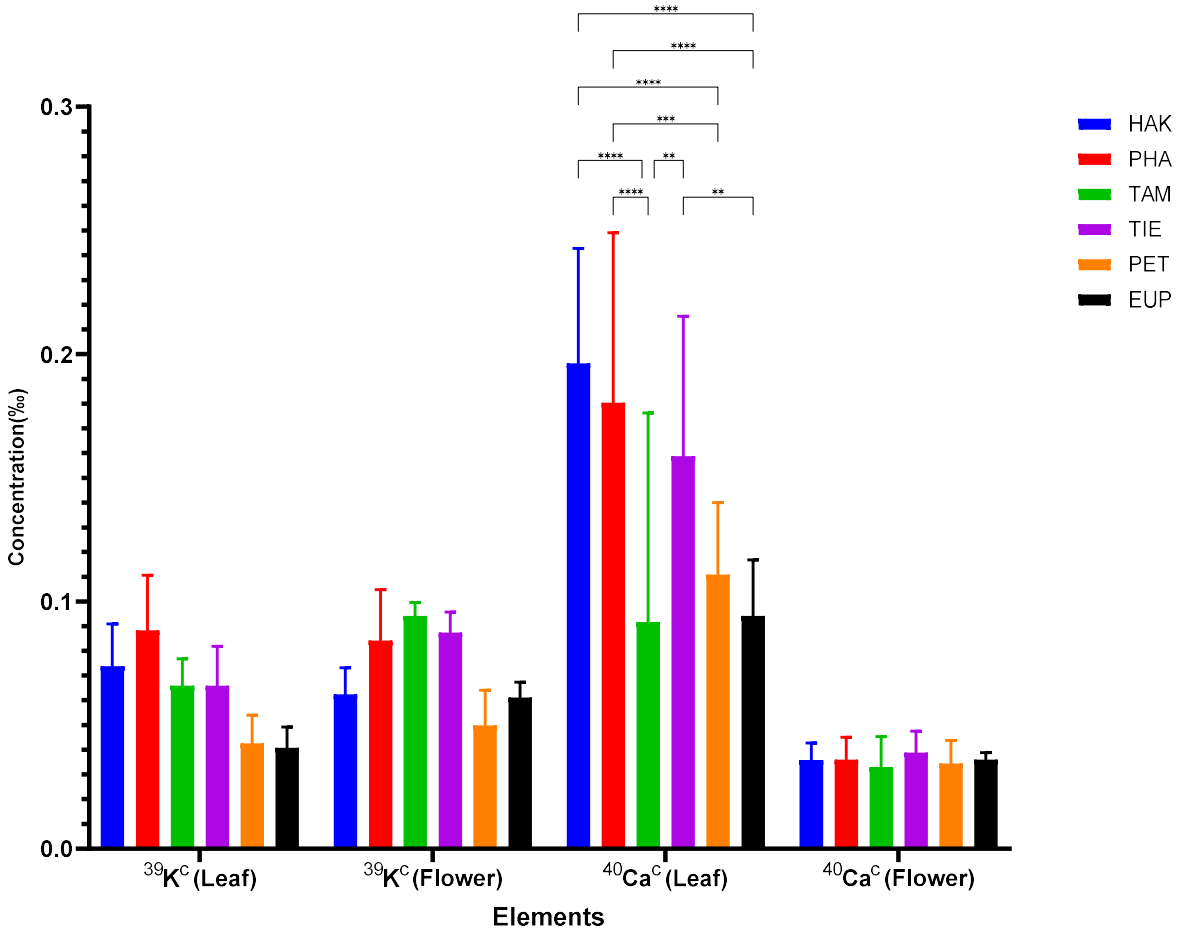


Figure 3.3.17: Content of Ca and K in leaves and flowers of six species

The leaf samples of *C. hakodae* showed high levels of Ca (0.1963 mg/g). In the leaf samples of all six species, the K content showed no statistically significant differences, whereas the Ca content differed significantly among most species. The contents of K and Ca in the flowers showed no statistically significant differences between the six species.

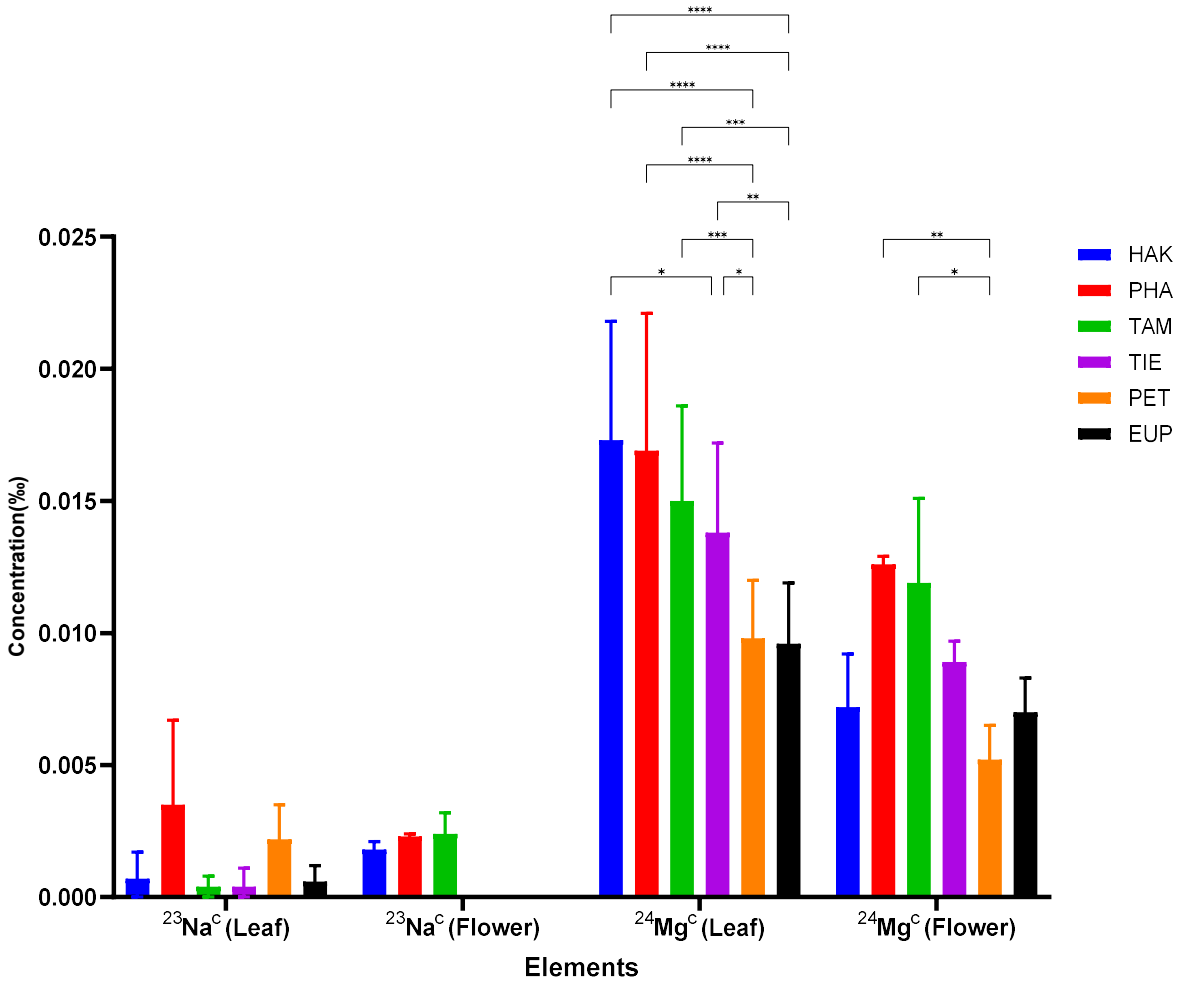


Figure 3.3.18: Content of Na and Mg in leaves and flowers of six species

The high levels of trace elements in both leaves and flowers play a crucial role in determining the biological effects of golden *Camellia*, such as antioxidant activity, anticancer potential, and cholesterol-lowering effects. This highlights the intrinsic relationship between trace element composition and the pharmacological properties of golden *Camellia*.

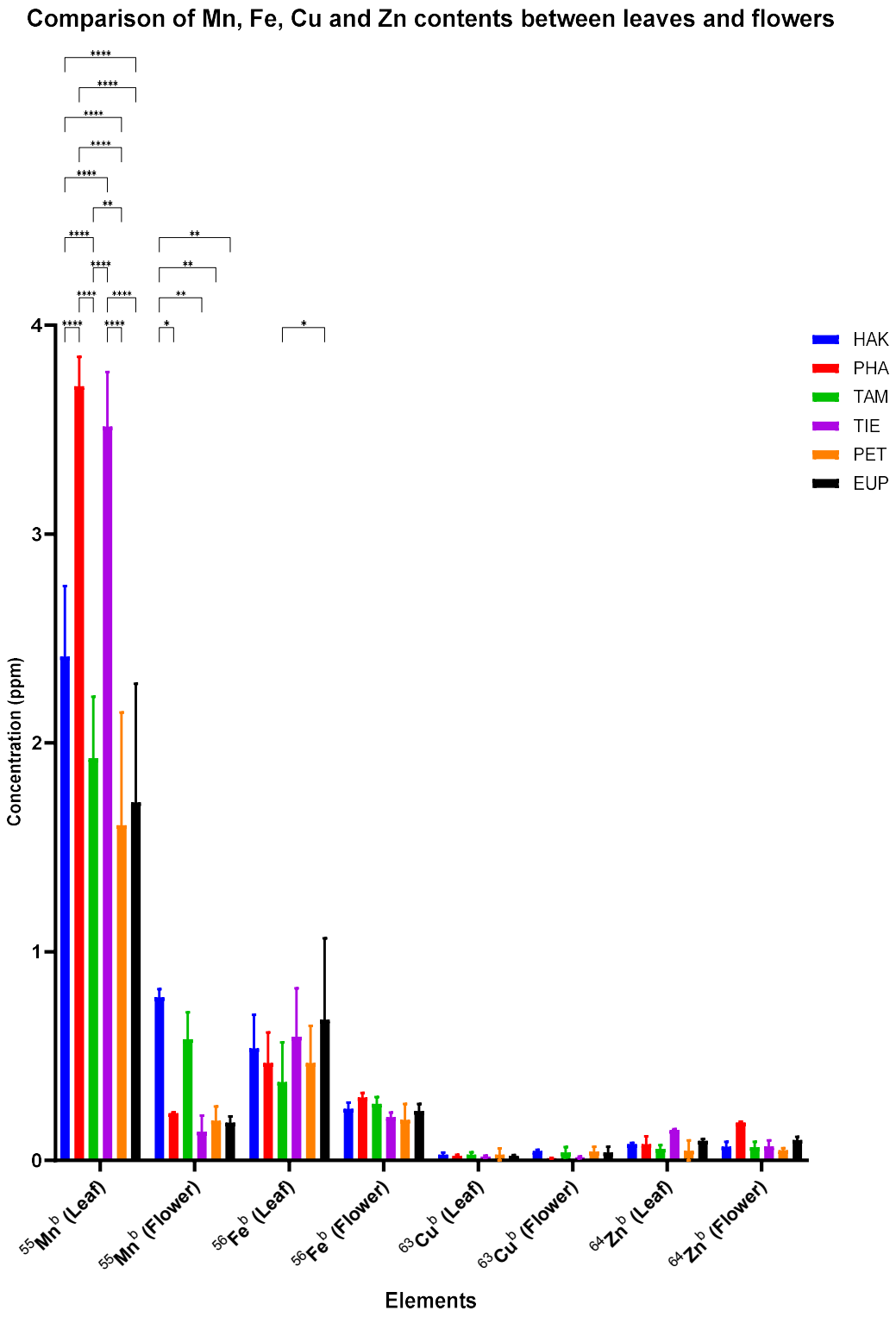


Figure 3.3.19: Content of micro-mineral related to antioxidant in leaves and flowers of six species

The concentrations of trace elements in the leaves were generally higher than those in the flowers. The content of Mn in leaves is clearly higher than in flowers, especially in *C.phanii* and *C.tienii.* Meanwhile, the Fe content in the leaves was slightly higher than in the flowers, while the levels of Cu and Zn were comparable between the two tissues. Among these, Cu was highest in the leaves of *C. tienii*, and Zn was highest in the flowers of *C. phanii*. These four elements are closely associated with natural antioxidant activity. This indicates that the comparable antioxidant effects observed in the flowers and leaves of the four species are closely related to the similar levels of Fe, Cu, and Zn present in both tissues.

Minerals are required in relatively large amounts and are designated as macro-minerals (Na, Mg, K, Ca). Minerals needed in smaller amounts are called microminerals (Cu, Fe, Mn, Zn, Mo, Ni, Co, Si). Plants require macro and micro-elements, each of which is essential for a plant to complete its life cycle; therefore, plants take up mineral elements from the soil solution in ionic form. 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 for research on the beneficial elements of food products (Farag et al., 2023).

All six Golden *Camellia* species in this study supply a sufficient amount of minerals that are available for humans' nutritional needs, especially K, Ca, Mg, Mn, Fe, Zn, and Cu. The minerals were reported to induce immunological reactions in the human body, such as the Zn element, represented in these species in high amounts.

Discussions

Minerals are essential for maintaining overall human health and physiological function. The findings of this study indicate that the golden *Camellia* species possesses notably high concentrations of both trace and macro elements. Consequently, the regular consumption of golden *Camellia* may confer significant health benefits, particularly for the elderly. Among these minerals, calcium is present in the highest concentration. Calcium (Ca^{2+}) is the most abundant mineral in the human body, with approximately 99% residing in bones and teeth, where it provides structural integrity and mechanical strength. In addition to its skeletal role, calcium functions as a ubiquitous intracellular messenger that regulates essential physiological processes, including muscle contraction, neurotransmitter release, and blood coagulation (Beto, 2015; Clapham, 2007). Calcium homeostasis is tightly controlled by the coordinated actions of parathyroid hormone (PTH), calcitonin, and vitamin D, which collectively maintain the balance between extracellular and intracellular calcium concentrations. Inadequate calcium intake,

particularly in older adults, is associated with an increased risk of osteoporosis and bone fractures (Rizzoli et al., 2008).

According to WHO/FAO, the daily intake of calcium is 300-1300 mg/day (FAO & World Health Organization, 1998). Golden Camellia contains 0.0916-0.1963 mg/g in leaves and 0.0388-0.0842 mg/g in flowers. This indicates that every 100 grams of golden *Camellia* tea supplies, on average, approximately 1/50 of the recommended daily calcium intake.

Potassium (K) is an essential mineral and the major intracellular cation in humans. It plays a crucial role in maintaining electrolyte balance, osmotic pressure, and normal cellular function. Through the Na^+/K^+-ATPase pump, potassium regulates membrane potential, supports nerve impulse transmission, and enables muscle contraction, including that of the heart (Giebisch, 1998; Saed, B., Kattan, A., & Rosenhouse-Dantsker, 2025; Srinivasa, 2019). Potassium also contributes to vasodilation and promotes renal sodium excretion, thereby reducing blood pressure and lowering the risk of cardiovascular diseases and stroke (Filippini et al., 2020). In addition, potassium is involved in acid–base balance, neuromuscular function, and kidney regulation to maintain proper ion homeostasis (Palmer & Clegg, 2016). Potassium deficiency (hypokalemia) may result in muscle weakness, fatigue, and cardiac arrhythmias, whereas potassium excess (hyperkalemia), often associated with renal dysfunction or potassium-sparing diuretics, can lead to life-threatening disturbances in cardiac conduction (Srinivasa, 2019). Therefore, maintaining adequate potassium intake through a diet rich in fruits, vegetables, and legumes is essential for cardiovascular and neuromuscular health.

Magnesium (Mg) is an essential mineral that plays a critical role in regulating metabolic pathways and maintaining physiological homeostasis across all tissues (Maier et al., 2023). Within the central nervous system, Mg is particularly important for modulating neuronal excitability, facilitating synaptic transmission, and preserving the structural and functional integrity of the blood–brain barrier (Maier et al., 2023; Romeo et al., 2019). Magnesium deficiency has been increasingly associated with systemic low-grade inflammation, a key pathological mechanism contributing to the onset and progression of various chronic diseases (Nielsen, 2018; Zheltova et al., 2016). Notably, neuroinflammation, an inflammatory response within the central nervous system, has been identified as a hallmark of several neurodegenerative disorders, underscoring the potential link between disrupted magnesium homeostasis and the pathogenesis of diseases such as Alzheimer's, Parkinson's, and multiple sclerosis (Veronese et al., 2016). Magnesium also affects neurological functioning and manifests dementia and cognitive (Chen et al., 2024).

The main micro-minerals are related to antioxidants such as Mn, Fe, Zn, and Cu, with high concentration in six golden *Camellia* species. The Mn content was highest in *C. hakodae*, followed by *C. tamdaoensis*. Overall, the combined levels of the four elements - Mn, Fe, Zn, and Cu - were greatest in *C. hakodae*, followed in descending order by *C. tamdaoensis*, *C. tienii*, *C. phanii*, *C. petelotii*, and *C. euphlebia*. This pattern is closely related to the antioxidant potential of these species. Generally, species with higher concentrations of elements involved in antioxidant mechanisms tend to exhibit stronger antioxidant activity. This has a significant impact on the antioxidant activity of the species, both in the leaves and the flowers. The elemental concentrations in the leaves are higher than in the flowers, which in turn influences the antioxidant capacity of the species.

In this part, the mineral/heavy metal content of six endemic species, including *C. hakodae*, *C. phanii*, *C. tamdaoensis, C. petelotii, C. euphlebia,* and *C. tienii*. Using ICP-MS, the nutritional values were evaluated through the macro nutrients (Ca, Mg, K, and Na) micronutrients (Cu, Cr, Se, Mn, Fe, Zn Mo, Co) level and the toxicity was assessed by toxic heavy metals content (Al, Ni, As, Ag, Cd, Sn, Sb, Ba, Pb).

The microwave digestion-ICP-MS-based analytical method for the comprehensive measurement of 23 elements in six golden *Camellia* was developed. Element analysis in golden *Camellia* leaves and flowers showed that the element content was significantly higher in leaves. The content of metal elements has become a potential indicator for the identification of raw tea leaves used in golden *Camellia* products. Moreover, chemometrics, especially the multivariate techniques applied, appear to be useful tools in the assessment of the distribution and enrichment of numerous chemical elements in golden *Camellia* with respect to the classification of their flowers and leaves.

Heavy metals such as Pb, Cd, As, Ag, … present in plant tissues with low levels or not detected are safe for human use. This research is the first to analyse and study the mineral contents of six endemic Golden *Camellia* in Vietnam, proving the nutritional value of these species as well as their potential to be applied in manufacturing beverage products and dietary supplements.

PART 3.4: STUDY ON ANTIOXIDANT ACTIVITIES OF SIX ENDEMIC GOLDEN *CAMELLIA*

The study examined six golden *Camellia* species, such as *C. hakodae, C. phanii, C. tamdaoensis, C. tienii, C. petelotti,* and *C. euphlebia*. Testing antioxidant effects on both oven-drying and freeze-drying mature leaves. Four species collected in Tam Dao, Vinh Phuc (*C. hakodae, C. phanii, C. tamdaoensis,* and *C. tienii*) were further analysed using dried young leaves.

3.4.1. DPPH scavenging activity

The antioxidant activity is shown when the antioxidant donates a hydrogen atom, reducing the purple DPPH free radical to yellow DPPH-H. This change is measured by absorbance at 517 nm. The effectiveness of the leaves and flowers extract is evaluated based on its ability to neutralize the DPPH free radical, with results presented as follows.

Table 3.4.1. Antioxidant capacity on DPPH of six Golden *Camellia* species

Species	**Oven-drying mature leaves (μg/ml)**	**Freeze-drying mature leaves (μg/ml)**	**Oven-drying young leaves (μg/ml)**	**Oven-drying flowers (μg/ml)**
	IC50 values (μg/ml) of radical scavenging			
C. hakodae	5.21±0.53	8.28±0.70	8.34±0.64	9.19± 0.64
C. phanii	8.52±0.68	11.39±0.98	12.50±1.13	6.97±0.74
C. tamdaoensis	5.89±0.52	9.22±0.72	8.88±0.61	6.84± 0.47
C. tienii	7.84±0.72	8.92±0.78	11.31±1.09	7.42±0.49
C. petelotii	11.04±0.89	13.06±1.04		15.89± 1.68
C. euphlebia	10.06±0.90	12.48±1.02		9.58± 0.74
Ascorbic acid	4.97±0.78			

Table 3.4.1 shows that all six species exhibit strong antioxidant activity with IC_{50} from 5.21 to 15.89 μg/ml, while the IC_{50} of Ascorbic acid is 4.97 μg/ml. The four species from Tam Dao - Vinh Phuc showed slightly superior antioxidant effects in both leaves and flowers compared to the two from Quang Ninh (Figure 3.4.1). Antioxidant effects depend on post-harvest processing methods on leaves, with oven-drying samples yielding IC_{50} values of 5.21-11.04 μg/ml and freeze-drying sample of 8.28-13.06 μg/ml. Oven-dried samples exhibit slightly higher antioxidant capacity than freeze-dried ones, though the difference is minimal. The leaves of *C. hakodae* have the strongest antioxidant activity (IC_{50} = 5.21 μg/ml), while *C. petelotii* is the weakest (IC_{50} = 11.04 μg/ml). The flower samples showed antioxidant activity similar to

the leaves, with IC_{50} values from 6.84 µg/ml to 15.89 µg/ml. The Tam Dao species displayed marginally higher activity than the two species from Quang Ninh.

The study assessed antioxidant capacity by leaf maturity, finding that mature leaves outperformed young ones across four species in Tam Dao, Vinh Phuc (Figure 3.4.1). The comparison of antioxidant effects between leaves and flowers also shows that their effects are equivalent, with no significant difference (Table 3.4.2 and Figure 3.4.1).

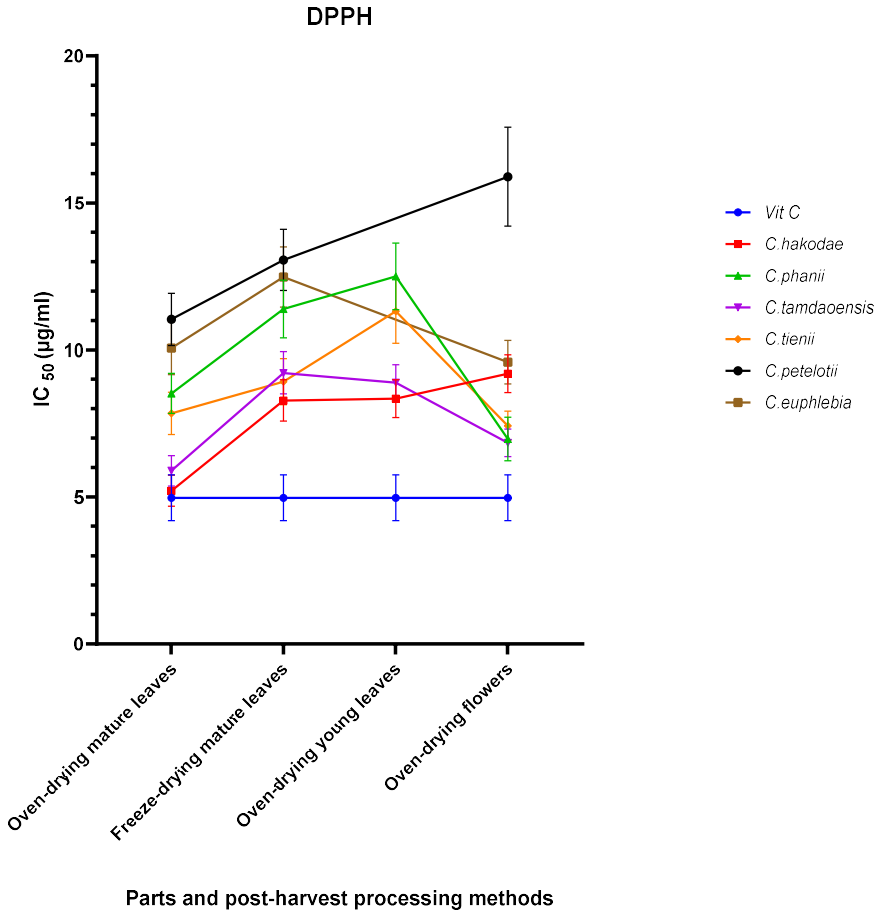


Figure 3.4.1. DPPH scavenging reactions of the extract from six species of Golden *Camellia*

The strongest antioxidant activity of the flower is observed in *C. tamdaoensis* with an IC_{50} value of 6.84 µg/ml (DPPH), while the weakest antioxidant activity is found in *C. petelotii* with an IC_{50} value of 15.89 µg/ml (DPPH).

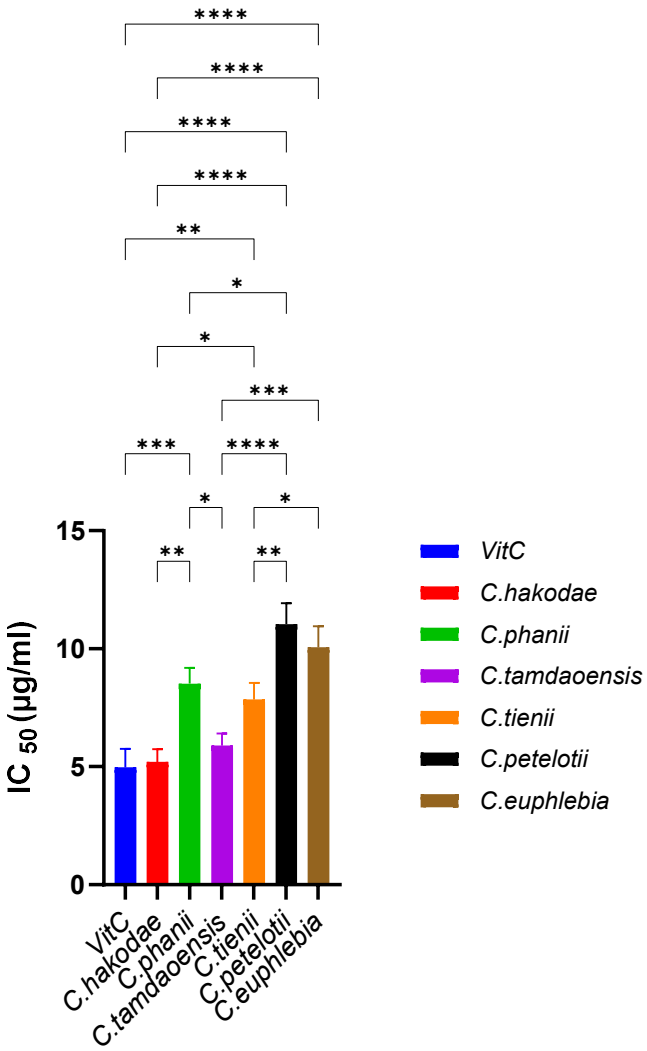


Figure 3.4.2: DPPH scavenging reactions of the extract from leaves of Golden *Camellia*

*[Note: **** $p \leq 0.0001$ (*extremely significant*), *** $p \leq 0.001$ (highly significant), ** $p \leq 0.01$ (more significant), * $p \leq 0.05$ (statistically significant), NS $p > 0.05$ (not significant)]*

The figure above indicates that the leaves of *C. hakodae* have the strongest antioxidant activity (IC_{50}= 5.21 µg/ml), while *C. petelotii* shows the weakest (IC_{50} = 13.46 µg/ml). The four species collected in Vinh Phuc have similar IC_{50} values, suggesting comparable antioxidant activities. While two species collecting in Quang Ninh have similar IC_{50} values.

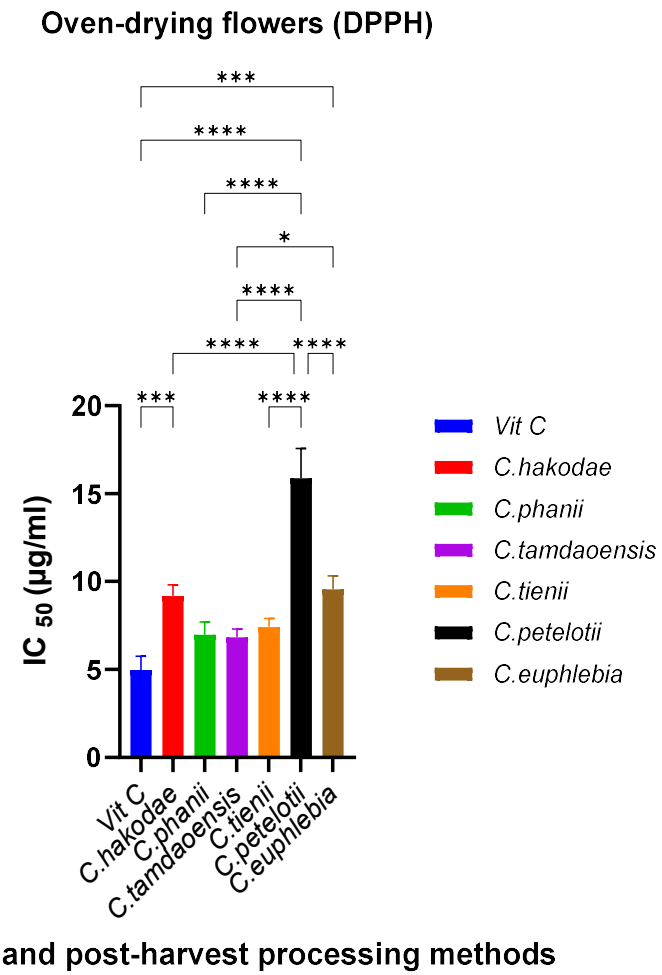


Figure 3.4.3: DPPH scavenging reactions of the extract from flowers of Golden *Camellia*

Figure 3.4.3 shows that the strongest antioxidant activity of the flower is observed in *C. tamdaoensis* and *C. phanii* with an IC_{50} value of 6.84 µg/ml and 6.97 µg/ml, while the weakest antioxidant activity is found in *C. petelotii* with an IC_{50} value of 15.89 µg/ml. The species *C. hakodae* and *C. euphlebia* exhibit similar antioxidant activity.

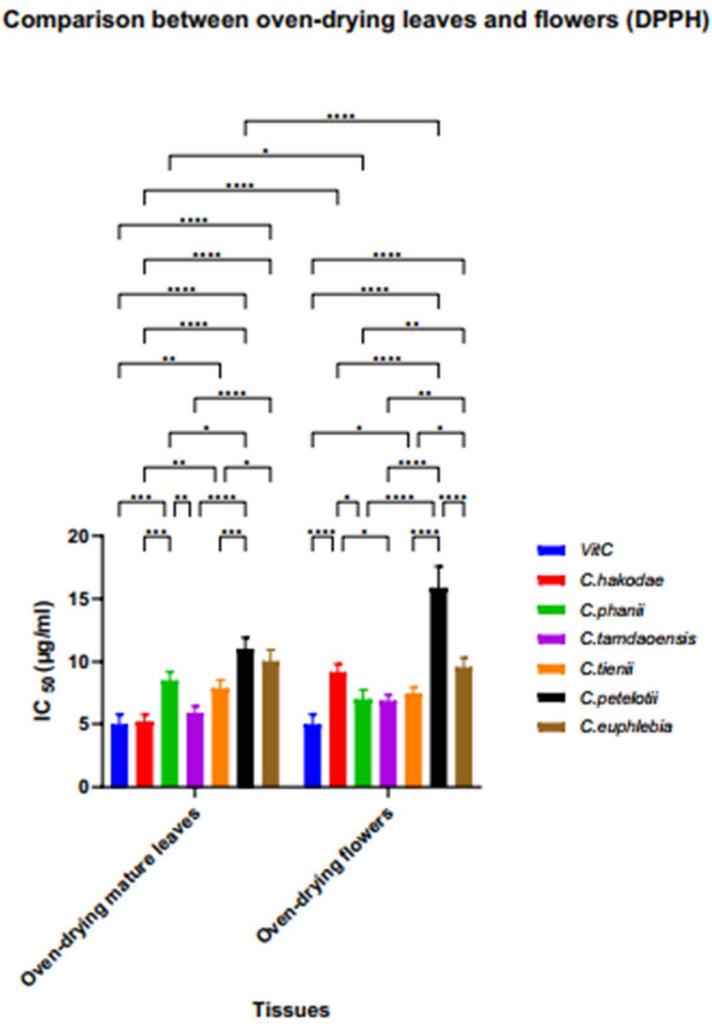


Figure 3.4.4. Comparing DPPH scavenging reactions of the extract from leaves and flowers of Golden *Camellia*

Figures 3.4.4, comparing the antioxidant capacity of leaves and flowers of each species, it was found that the effect of flowers and leaves was excellent in all species. The difference in free radical inhibition ability was most evident in the *C. hakodae* species showed the highest difference. *C. euphlebia* is the weakest antioxidant in both leaves and flowers.

Comparison of the effects of post-harvest processing methods on antioxidant activity

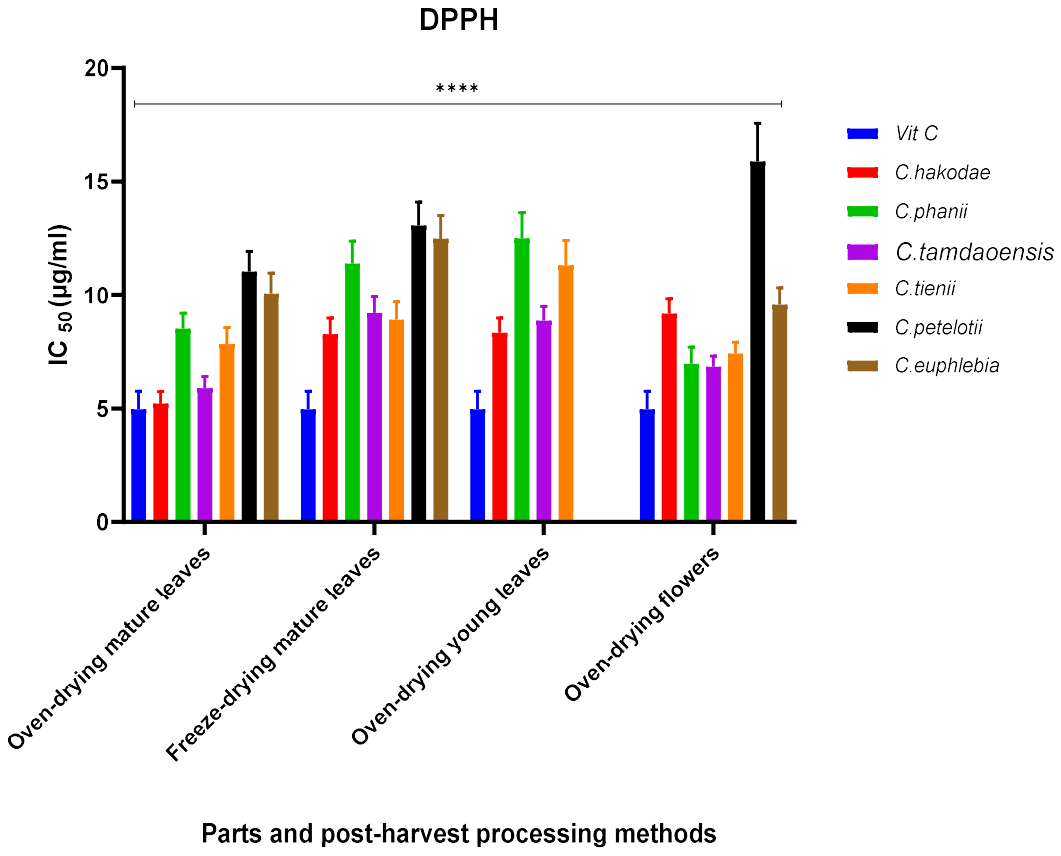


Figure 3.4.5: DPPH scavenging reactions of the extract from six species of Golden *Camellia*

The chart above shows that oven-drying resulted in the lowest IC_{50} values for both leaves and flowers, indicating the highest antioxidant activity. In contrast, freeze-drying generally produced higher IC_{50} values, meaning its antioxidant activity was lower compared with the oven-dried materials.

3.4.2. ABTS scavenging activity

The results of antioxidants in the radical scavenging reactions are shown in the table and figure below

Table 3.4.2. Antioxidant capacity on ABTS of six Golden *Camellia* species

Species	Oven-drying mature leaves (μg/ml)	Freeze-drying mature leaves (μg/ml)	Oven-drying young leaves (μg/ml)	Oven-drying flowers (μg/ml)
	IC50 values (μg/ml) of radical scavenging			
C. hakodae	6.91 ± 0.52	8.76 ± 0.69	9.55 ± 0.62	10.41 ± 0.66
C.phanii	12.58 ± 0.71	13.72 ± 0.86	13.44 ± 0.80	9.98 ± 0.66
C.tamdaoensis	8.69 ± 0.63	11.35 ± 0.68	13.74 ± 0.82	7.52 ± 0.47
C.tienii	7.12 ± 0.65	11.48 ± 0.78	13.26 ± 0.79	10.03 ± 0.62
C.petelotii	13.46 ± 1.40	19.26 ± 1.22		17.50 ± 1.23
C.euphlebia	11.63 ± 0.63	16.39 ± 0.87		9.78 ± 0.53
Trolox	5.84 ± 0.24			

Table 3.4.2 shows that all six species exhibit strong antioxidant activity with IC_{50} from 6.91 to 17.50 μg/ml in both leaves and flowers. The four species from Tam Dao-Vinh Phuc exhibited slightly stronger antioxidant effects in both leaves and flowers than the two species from Quang Ninh. Antioxidant effects depend on post-harvest processing methods on leaves, with oven-drying yielding IC_{50} values of 6.91-13.46 μg/ml and freeze-drying from 8.76 to 19.26 μg/ml. Oven-dried samples exhibit slightly higher antioxidant capacity than freeze-dried ones, though the difference is minimal.

The study evaluated antioxidant capacity by leaf maturity, revealing that mature leaves surpassed young ones in all four species examined in Tam Dao, Vinh Phuc. The ABTS radical scavenging reactions of leaves and flowers are equivalent, showing no significant difference. The leaves of *C. hakodae* have the strongest antioxidant activity (IC_{50} = 6.91 μg/ml), while *C. petelotii* shows the weakest (IC_{50} = 13.46 μg/ml). The strongest antioxidant activity of the flower is observed in *C. tamdaoensis* with an IC_{50} value of 7.52 μg/ml (ABTS), while the weakest antioxidant activity is found in *C. petelotii* with an IC_{50} value of 17.50 μg/ml (ABTS).

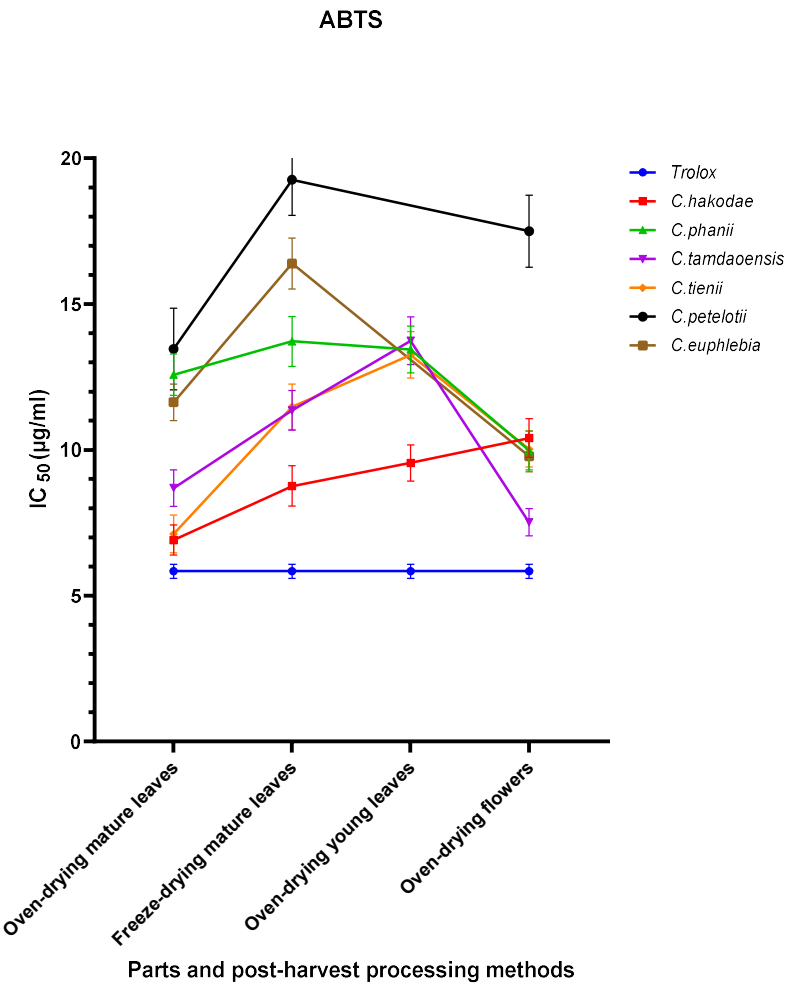


Figure 3.4.6. ABTS scavenging reactions of the extract from six species of Golden *Camellia*

The results are clearly presented in the figures below, focusing primarily on oven-dried leaves and flowers.

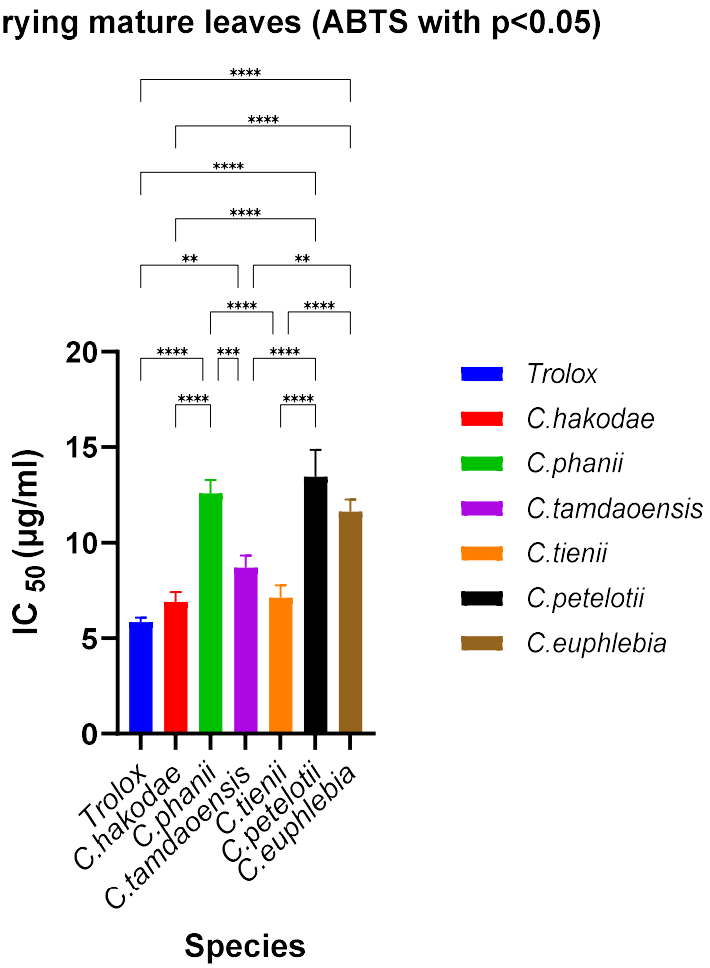


Figure 3.4.7. ABTS scavenging reactions of the extract from the leaves of Golden *Camellia*

Table 3.4.1 and figures above indicate that the leaves of *C. hakodae* have the strongest antioxidant activity (IC_{50} = 6.91 µg/ml), while *C. petelotii* shows the weakest (IC_{50} = 13.46 µg/ml). The other three species have similar IC_{50} values, suggesting comparable antioxidant activities.

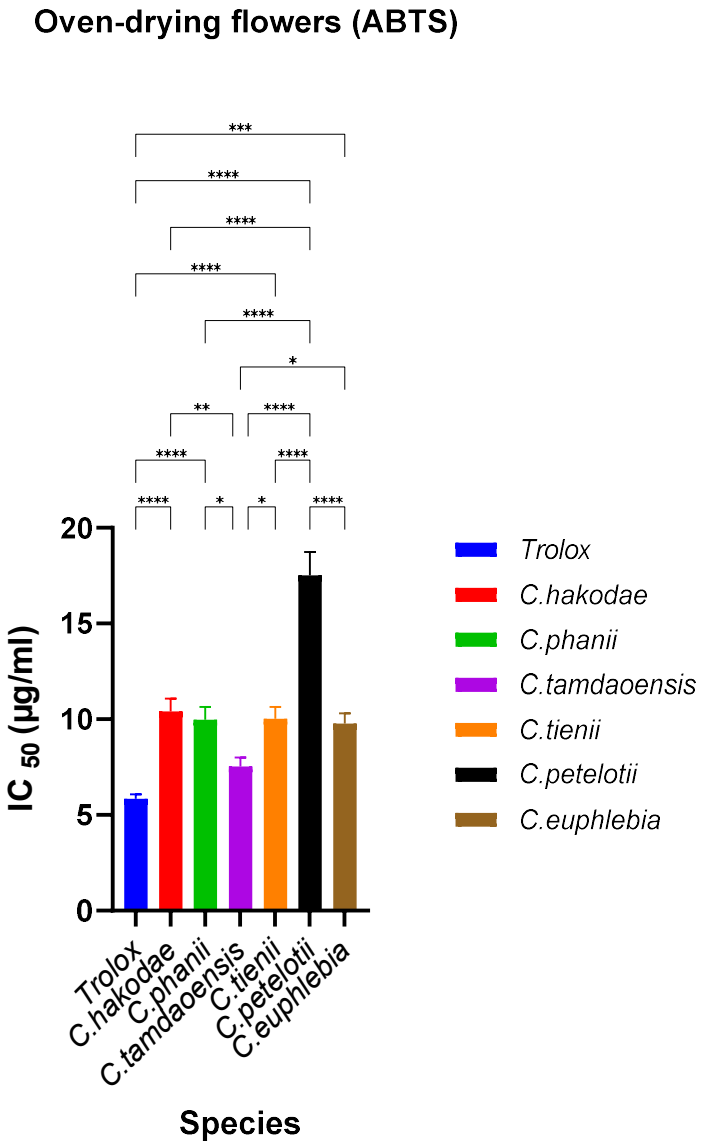


Figure 3.4.8. ABTS scavenging reactions of the extract from the flowers of Golden *Camellia*

Table 3.4.7 and Figure 3.4.8 show that the strongest antioxidant activity of the flower is observed in *C. tamdaoensis* with an IC_{50} value of 7.52 µg/ml, while the weakest antioxidant activity is found in *C. petelotii* with an IC_{50} value of 17.50 µg/ml. The species *C. hakodae, C. tienii,* and *C. euphlebia* exhibit similar antioxidant activity.

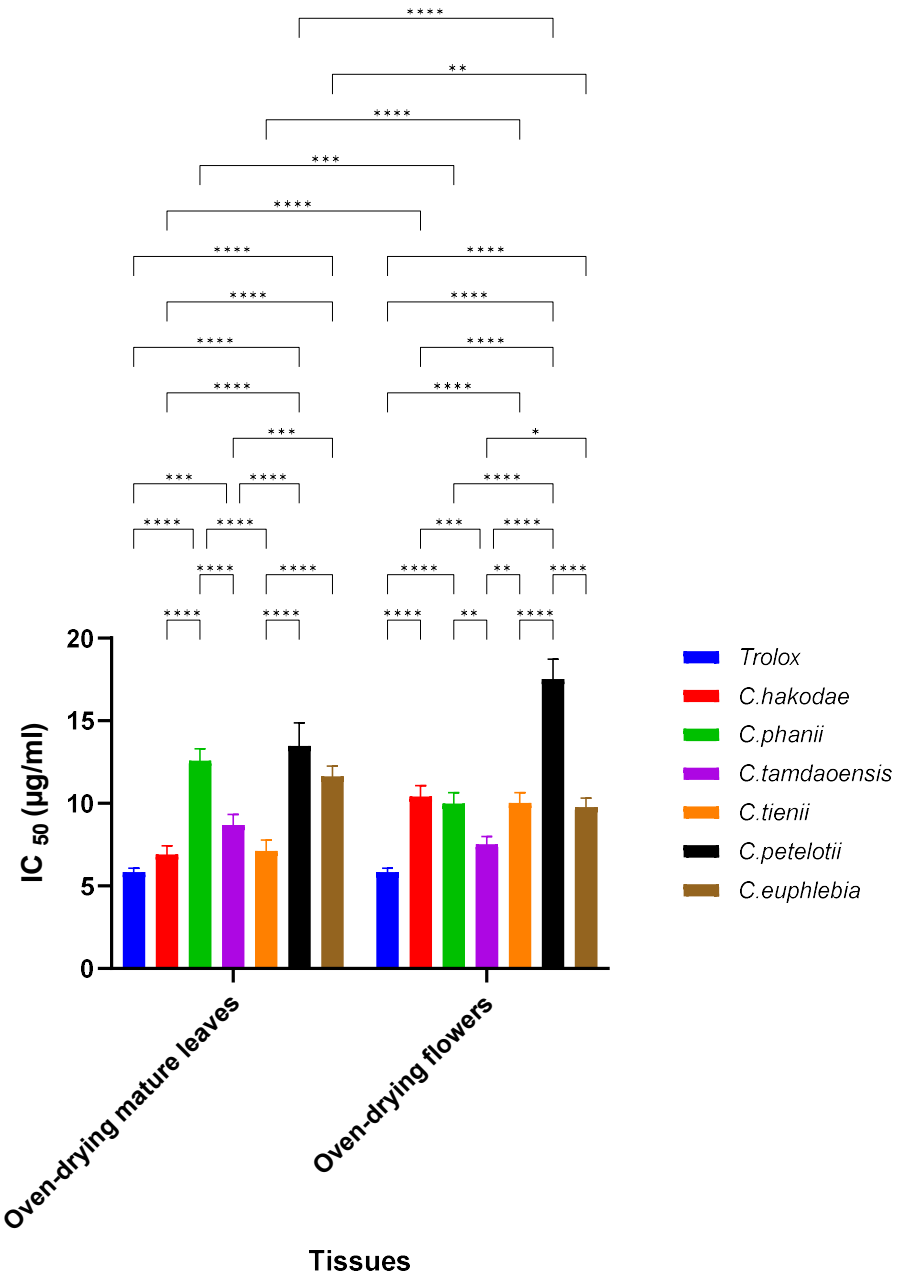


Figure 3.4.9. ABTS scavenging reactions of the extract from leaves and flowers of Golden *Camellia*

Comparing the antioxidant capacity of leaves and flowers of each species, it was found that the effect of flowers and leaves was excellent in all species. The difference in free radical inhibition ability was most evident in *C.euphlebia* species, and *C.phanii* species showed the least difference.

The species *C. phanii*, *C. tamdaoensis*, *C. tienii*, and *C. euphlebia* exhibit better antioxidant activity in their flowers compared to their leaves, as indicated by lower IC_{50} values in the flowers. For the other two species, the IC_{50} values of the flowers are higher than those of the leaves, but the difference is not significant. Overall, the antioxidant activity is nearly equivalent between the flowers and leaves of most golden *Camellia* species.

The leaves of *C. hakodae* have the strongest antioxidant activity (IC_{50} = 5.21 µg/ml and 6.91 µg/ml in DPPH and ABTS, respectively), while *C. petelotii* shows the weakest (IC_{50} = 11.04 and 13.46 µg/ml in DPPH and ABTS, respectively). The strongest antioxidant activity of the flower is observed in *C. tamdaoensis* with an IC_{50} value of 6.84 µg/ml (DPPH) and 7.52 µg/ml (ABTS), while the weakest antioxidant activity is found in *C. petelotii* with an IC_{50} value of 15.89 µg/ml (DPPH) and 17.50 µg/ml (ABTS).

Discussions

Evaluation of the antioxidant capacity of medicinal materials is based on their ability to inhibit free radicals, which is clearly reflected in the IC_{50} value. This means that the higher the IC_{50} value, the lower the antioxidant capacity, and vice versa. A lower IC_{50} value indicates that a smaller amount of the material is needed to inhibit 50% of free radicals, signifying higher antioxidant capacity (Brand-Williams et al., 1995).

The study reveals that all six species exhibit strong antioxidant activity, with low IC_{50} values in both DPPH and ABTS. The four species from Tam Dao-Vinh Phuc showed slightly superior antioxidant effects in both leaves and flowers compared to the two from Quang Ninh. In the previous chapter, the results of studies on organic and inorganic transformations in golden *Camellia* species were presented. All golden *Camellia* species contain numerous organic compounds with antioxidant properties, such as phenolic compounds, flavonoids, and anthocyanins. This highlights the close relationship between the natural compound composition and the antioxidant effects of golden *Camellia*. This underscores the intrinsic and interconnected relationship between their natural compound composition and antioxidant effects (X. Wu, 2021).

The antioxidant effects vary with post-harvest processing methods, such as oven-drying or freeze-drying. Dried samples show slightly higher antioxidant capacity than freeze-dried ones, though the difference is minimal.

The antioxidant capacity was also evaluated based on leaf maturity, with mature leaves showing better performance than young leaves. This study was conducted on four species in Tam Dao-Vinh Phuc. Before research found that while some amino acids were upregulated in young leaves, phenolic and flavonoids were found with higher content in mature leaves. This

confirms that the high levels of phenolic and flavonoid compounds enhance the antioxidant capacity of golden *Camellia*.

The comparison of antioxidant effects between leaves and flowers also shows that their effects are equivalent. The species *C. phanii*, *C. tamdaoensis*, *C. tienii*, and *C. euphlebia* exhibit better antioxidant activity in their flowers compared to their leaves, as indicated by lower IC_{50} values in the flowers. For the other two species, the IC_{50} values of the flowers are higher than those of the leaves, but the difference is not significant. Overall, the antioxidant activity is nearly equivalent between the flowers and leaves of most golden *Camellia* species. This suggests that leaves similar to those of the golden *Camellia* flower can be used in health care and protection.

Trace elements, including copper, manganese, selenium, and zinc, function as critical cofactors of antioxidant enzymes that safeguard the organism against oxygen-free radicals (OFRs) generated during oxidative stress. The maintenance of equilibrium between pro-oxidant species and antioxidant defenses is essential for cellular homeostasis. Among the redox-active trace elements, copper exemplifies this delicate balance: although it can catalyze the initiation of free radical reactions, it concurrently serves as a cofactor for Cu/Zn-superoxide dismutase, an enzyme responsible for the detoxification of superoxide radicals. Metal-binding proteins such as ceruloplasmin are indispensable for sequestering reactive copper ions, thereby mitigating their potential toxicity. Likewise, transferrin and its receptor regulate iron homeostasis, restricting the availability of free iron and consequently limiting the Fenton reaction that produces highly reactive hydroxyl radicals. Selenium, frequently observed to be severely depleted in critically injured patients, necessitates adequate supplementation during parenteral micronutrition to sustain the activity of glutathione peroxidase, a pivotal antioxidant enzyme, and to support optimal immune function (Leung, 1998)

Manganese (Mn), iron (Fe), zinc (Zn), and copper (Cu) are important micro-minerals associated with antioxidant activity and are found at high concentrations in six golden camellia species. *C. hakodae* has the highest overall concentration, followed by *C. tamdaoensis*, *C. tienii*, *C. phanii*, *C. petelotii*, and *C. euphlebia*. Higher levels of these elements are associated with stronger antioxidant activity, with leaves generally containing more than flowers, contributing to greater antioxidant capacity. However, antioxidant compounds such as flavonoids and polyphenols appear to be higher in flowers than in leaves. Due to these two factors, the evaluated antioxidant activities of both leaves and flowers are relatively similar.

In summary, this study represents the first report regarding the evaluation of the antioxidant capacity of methanol leaves extracted from six golden *Camellia* species, which are

endemic *Camellia* species in Vietnam. The results showed that these six endemic *Camellia* species have great antioxidant activity in both leaves and flowers. The antioxidant effect is better on mature leaves when using the oven-drying method. The antioxidant effects of both the leaves and flowers are good and similar. Meanwhile, the leaves are the part that produces in large quantities and can be harvested year-round, so it is recommended to harvest the leaves to create valuable products from the golden *Camellia*.

The previous study on the compounds found in six species of golden *Camellia* demonstrated the presence of numerous organic compounds, including phenolics, flavonoids, carotenoids, vitamin E, phytol, stigmasterol, and catechins. These are all natural compounds with strong antioxidant properties. This confirms a close relationship between the active ingredients and the antioxidant effects of the studied golden *Camellia* species.

Chapter 4. CONCLUSIONS AND PERSPECTIVES

4.1. CONCLUSIONS

This study represents the most comprehensive and integrated investigation to date of the metabolite systems in six endemic golden *Camellia* species of Vietnam: *C. hakodae*, *C. phanii*, *C. tamdaoensis*, *C. tienii*, *C. petelotii*, and *C. euphlebia.* The dissertation employed metabolomics research methods, including both untargeted and targeted approaches, to identify unknown metabolite systems and to measure relative differences among the six golden *Camellia* species in commonly used parts such as flowers and leaves; between mature and young leaves; as well as between different post-harvest processing methods, oven-drying, and freeze-drying.

This work has successfully identified a mutual profile of 131 metabolites of six Vietnamese golden *Camellia,* which was obtained by using a widely targeted metabolomics analysis using ultra-performance liquid chromatography coupled with tandem mass spectrometry. Interestingly, mainly forty-one flavonoids and thirty-two amino acids dominate the profiles of golden *Camellia* samples, corresponding to different aglycone skeletons such as anthocyanin, aurone, chalcone, flavan-3-ol, flavanone, flavone, and flavonol. Other primary metabolites, ex, amino acids and derivatives, were also observed in these extracts, contributing to the physicochemical properties and the taste and flavour of golden tea products. The comprehensive metabolome data revealed by the widely targeted metabolomics approach indicated that this method is suitable for high-throughput descriptions of golden *Camellia*, and this complex data could be mined from many perspectives.

Otherwise, our results showed a clear separation between the clusters of oven-drying and freeze-drying leaves and young and mature leaves on the PLS-DA score plots. Catechin and epicatechin, L-glutamic acid, choline, and some active flavonoids were accumulated with distinct trends in six species with or without thermal treatment. While some amino acids were upregulated in young leaves, flavonoids were found with higher content in mature leaves. This suggests an undeniable effect of preparation treatment and harvest time on the metabolite composition, which decides these materials' taste, nutritional, and pharmacological properties.

Secondly, the thesis has revealed 42 metabolites in leaf samples and 44 metabolites in flower samples by using an untargeted GC-MS analysis. The groups that appeared most abundant included esters, fatty acids, and terpenoids with a high probability. Besides, there are natural compounds that contribute to the medicinal value and nutritional value of precious tea varieties endemic to Vietnam such as citral and dl-α-tocopherol, phytol, and squalene. Discrimination analysis, such as PLS-DA and PCA, can distinguish the GC-MS-based

metabolite profiles of leaves and flowers, species-specific and tissue-specific. This result provides a tool for chemotaxonomy of these species and quality control of these materials.

Thirdly, a microwave digestion ICP-MS method was established to analyse 23 elements in golden *Camellia* comprehensively. Element analysis of golden *Camellia* leaves and flowers revealed significantly higher element content in the leaves. The content of metal elements has the potential to be an indicator for the identification of raw tea leaves used in the golden *Camellia* production. In detail, *C. hakodae* had the highest overall elemental content in both leaves and flowers among the surveyed species, which may influence the antioxidant properties of golden *Camellia*. In leaves, it showed the highest levels of Mg, Ca, Fe, and Sr, while in flowers, it had the highest concentrations of Al, Ca, Mn, Cu, Sr, and Ba. Other species also exhibited varying elemental dominance, with *C. phanii* leading in Na, K, and Mn (leaves) and Mg, Fe, and Zn (flowers); *C. tamdaoensis* in Cu (leaves) and Na, K, and Ni (flowers); *C. tienii* in Cr, Co, and Zn (leaves) and Ca and Co (flowers); and *C. euphlebia* in Al and Fe (leaves) and Cr and Bi (flowers).

Finally, this study represents the first report regarding the evaluation of the antioxidant capacity of methanol-extracted leaves from six golden *Camellia* species, which are endemic in Vietnam. The results showed that these six endemic *Camellia* species have great antioxidant activity in both leaves and flowers. The antioxidant effect is better on mature leaves when using the oven-drying method. The antioxidant effects of both the leaves and flowers are good and similar. Meanwhile, the leaves are the part that produces in large quantities and can be harvested year-round, so it is recommended to harvest the leaves to create valuable products from the golden *Camellia*. The difference in antioxidant effects aligns perfectly with studies on the metabolic systems of six golden *Camellia* species. Dried processed mature leaf samples contain higher levels of flavonoids, particularly catechins, which are natural compounds with excellent antioxidant properties. Therefore, the correlation with higher antioxidant effects is entirely reasonable.

4.2. PERSPECTIVES

The study explored organic and inorganic compounds in six endemic golden *Camellia* species in Vietnam, identifying both similarities and differences. Bioactive analysis revealed a rich composition in leaves and flowers, with comparable antioxidant properties. Since flowers are seasonal but leaves are available year-round, leaves offer a practical option for health care.

Golden *Camellia* is a medicinal herb with powerful health benefits, primarily due to its antioxidant properties. It helps prevent diseases like cancer, cardiovascular issues, and

metabolic disorders such as diabetes and high cholesterol. These effects come from its rich content of bioactive compounds, including flavonoids, polyphenols, tannins, coumarins, vitamins, and many minerals - found in few herbs as abundantly. Additionally, it contains essential amino acids vital for human health, supporting overall well-being. Thus, golden *Camellia* not only helps prevent diseases but also supports the treatment of many serious illnesses.

Vietnam has an abundance of golden *Camellia* species, but research on their inorganic and organic metabolism, as well as their antioxidant and anticancer properties, remains limited. To raise awareness of this medicinal plant and develop health products for the public, my research team and I will continue studying other golden *Camellia* species. Our goal is to create convenient products such as tea, hard capsules, and soft capsules, with a focus on extracts from golden *Camellia* leaves.

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ANNEX 1: PLANT IDENTIFICATION

VIỆN HÀN LÂM
KHOA HỌC VÀ CÔNG NGHỆ VN
VIỆN SINH THÁI VÀ
TÀI NGUYÊN SINH VẬT

CỘNG HOÀ XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập - Tự do - Hạnh phúc

Hà Nội, ngày 10 tháng 05 năm 2023

KẾT QUẢ XÁC ĐỊNH TÊN KHOA HỌC MẪU THỰC VẬT

Kính gửi: TS. Nguyễn Thị Kiều Oanh.

Trường Đại học Khoa học Công nghệ Hà Nội.

Chúng tôi nhận được đề nghị xác định tên khoa học cho mẫu tiêu bản thực vật của TS. Nguyễn Thị Kiều Oanh, Trường Đại học Khoa học Công nghệ Hà Nội, ngày 05 tháng 5 năm 2023, có gửi theo mẫu vật và toàn bộ thông tin liên quan đến mẫu.

Kết quả xác định tên khoa học

Sau khi tiến hành phân tích và giám định, chúng tôi có kết luận như sau:

- **Mẫu TĐ1**: nơi thu Hợp Châu, Tam Đảo, Vĩnh Phúc; ngày thu 22/6/2021; người thu: Nguyễn Thị Kiều Oanh, Nguyễn Phương Nhị & Lê Thị Vân Anh; tên khoa học: *Camellia hakodae* Ninh, thuộc họ Chè (Theaceae).
- **Mẫu TĐ2**: nơi thu: Hợp Châu, Tam Đảo, Vĩnh Phúc; ngày thu: 22/6/2021; người thu: Nguyễn Thị Kiều Oanh, Nguyễn Phương Nhị & Lê Thị Vân Anh; tên khoa học: *Camellia phaoli* Hakoda & Ninh, thuộc họ Chè (Theaceae).
- **Mẫu TĐ3**: nơi thu: Hợp Châu, Tam Đảo, Vĩnh Phúc; ngày thu: 22/6/2021; người thu: Nguyễn Thị Kiều Oanh, Nguyễn Phương Nhị & Lê Thị Vân Anh; tên khoa học: *Camellia tamdaoensis* Hakoda & Ninh, thuộc họ Chè (Theaceae).
- **Mẫu TĐ4**: nơi thu: Hợp Châu, Tam Đảo, Vĩnh Phúc; ngày thu: 22/6/2021; người thu: Nguyễn Thị Kiều Oanh, Nguyễn Phương Nhị & Lê Thị Vân Anh; tên khoa học: *Camellia tienii* Ninh, thuộc họ Chè (Theaceae).
- **Mẫu TĐ5**: nơi thu ở Hợp Châu, Tam Đảo, Vĩnh Phúc; ngày thu: 10/11/2021; người thu: Nguyễn Thị Kiều Oanh, Nguyễn Phương Nhị & Lê Thị Vân Anh; tên khoa học: *Camellia petelotii* (Merr.) Sealy, thuộc họ Chè (Theaceae).

- **Mẫu TĐ6**: nơi thu: Hợp Châu, Tam Đảo, Vĩnh Phúc; *ngày* thu: 10/11/2021; người thu: Nguyễn Thị Kiều Oanh, Nguyễn Phương Nhị & Lê Thị Vân Anh; tên khoa học: *Camellia flava* (Pit.) Sealy, thuộc họ Chè (Theaceae).
- **Mẫu HH1**: nơi thu: Quang Minh, Hải Hà, Quảng Ninh; ngày thu 10/4/2022; người thu: Nguyễn Thị Kiều Oanh; tên khoa học: *Camellia euphlebia* Merr. ex Sealy, thuộc họ Chè (Theaceae).
- **Mẫu HH2**: nơi thu: Quang Minh, Hải Hà, Quảng Ninh; ngày thu: 10/4/2022; người thu: Nguyễn Thị Kiều Oanh; tên khoa học: là *Camellia petelotii* (Merr.) Sealy, thuộc họ Chè (Theaceae).
- **Mẫu QN1**: nơi thu: Ba Xã, An Sinh, Đông Triều, Quảng Ninh; ngày thu: 22/04/2023; người thu: Nguyễn Thị Kiều Oanh & Nguyễn Phương Nhị; tên khoa học: *Camellia flava* (Pit.) Sealy, thuộc họ Chè (Theaceae).
- **Mẫu ĐL1**: nơi thu: Mê Linh, Lâm Hà, Lâm Đồng; ngày thu: 20/04/2023; người thu: Nguyễn Thị Kiều Oanh & Nguyễn Phương Nhị; tên khoa học: *Camellia puhoatensis* N. S. Ly, V. D. Luong, T. H. Le, D. H. Nguyen & N. D. Do, thuộc họ Chè (Theaceae).
- **Mẫu ĐL2**: nơi thu: Mê Linh, Lâm Hà, Lâm Đồng; ngày thu: 20/04/2023; người thu: Nguyễn Thị Kiều Oanh & Nguyễn Phương Nhị; tên khoa học: *Pyrenaria jonquieriana* Laness., thuộc họ Chè (Theaceae).

Xin gửi kết quả xác định tên khoa học mẫu thực vật đến TS. Nguyễn Thị Kiều Oanh, Trường Đại học Khoa học Công nghệ Hà Nội.

Xác nhận của Viện Sinh thái và Tài nguyên sinh vật

VIỆN SINH THÁI VÀ TÀI NGUYÊN SINH VẬT

PHÓ VIỆN TRƯỞNG

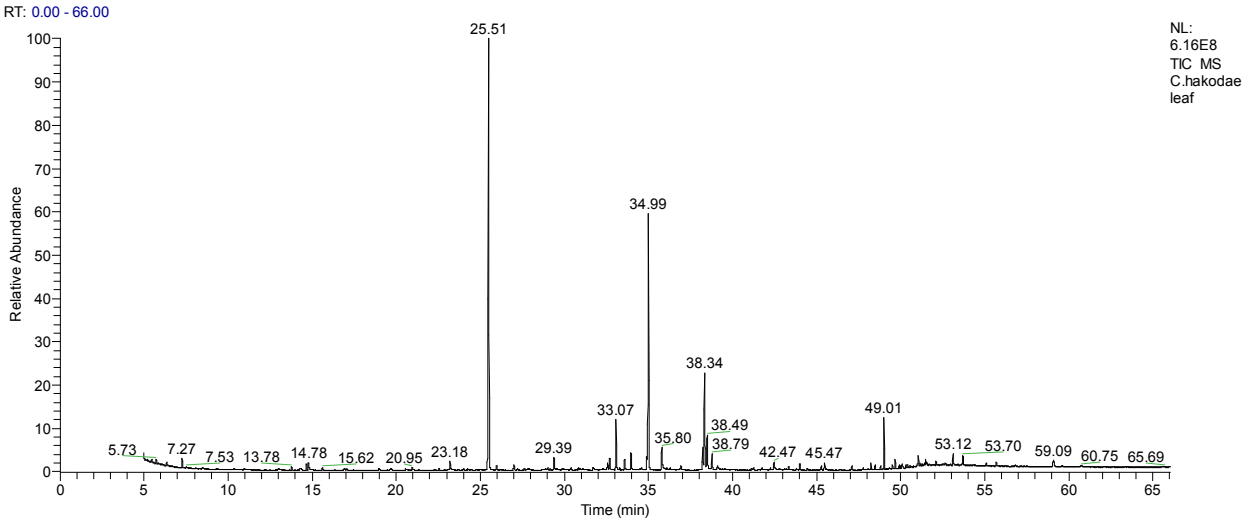
Nguyễn Quảng Trường

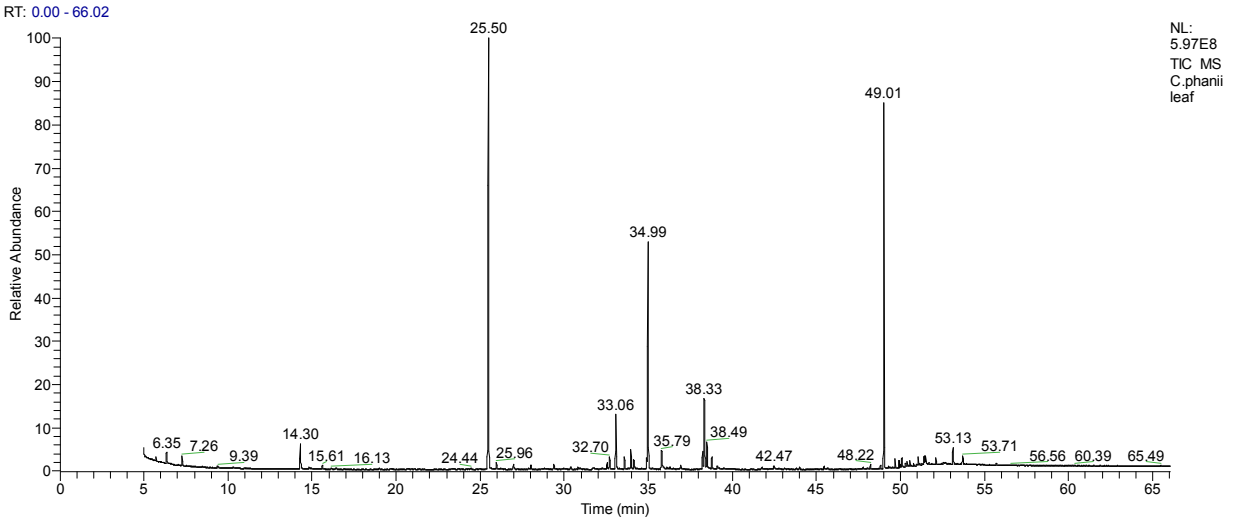
Người giám định

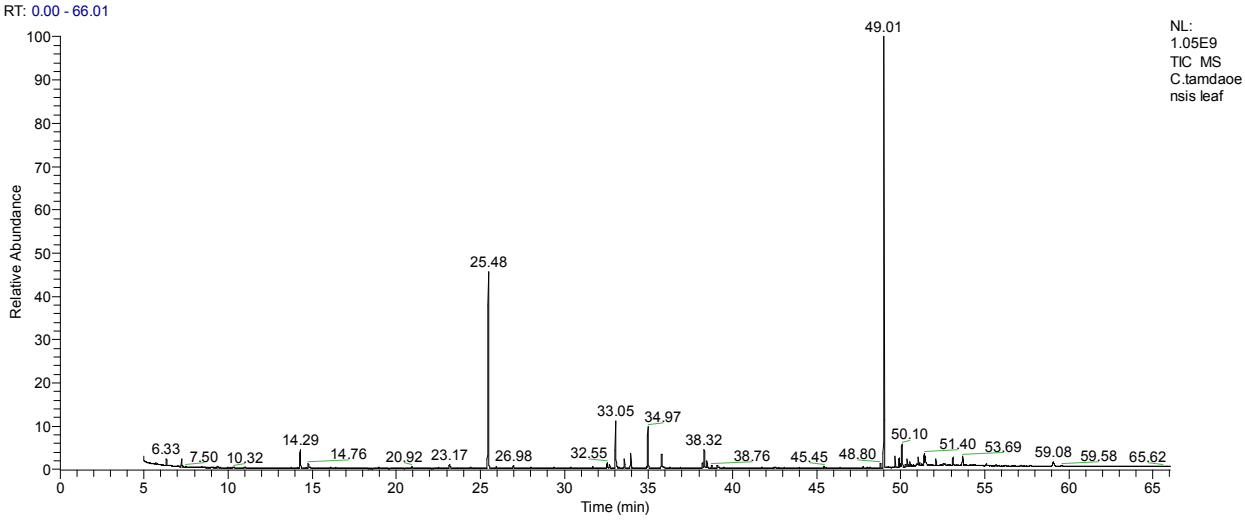
Nguyễn Thế Cường

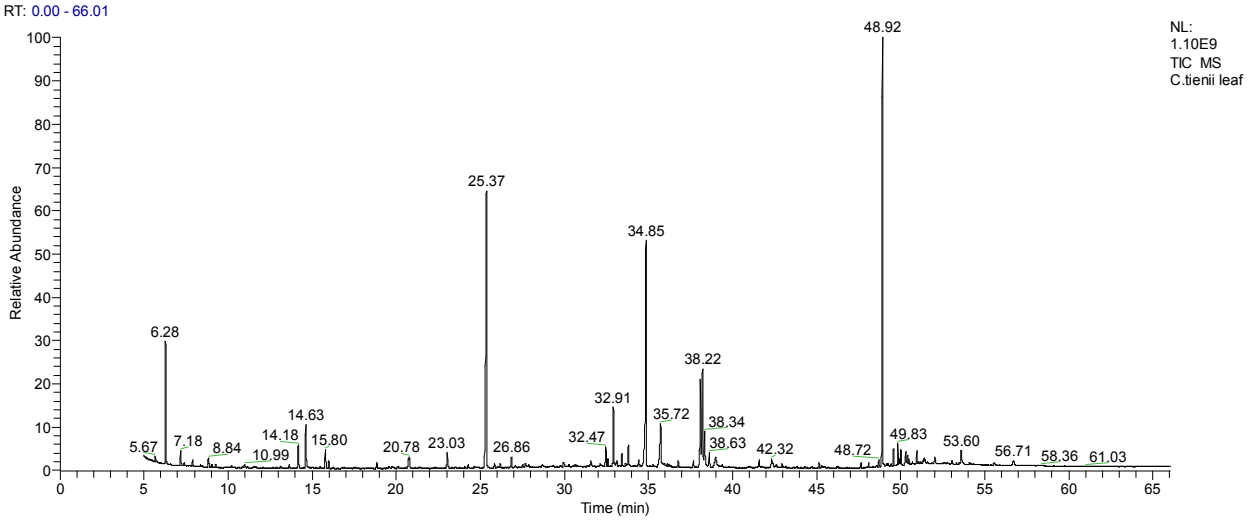
ANNEX 2: GC-MS CHROMATOGRAMS

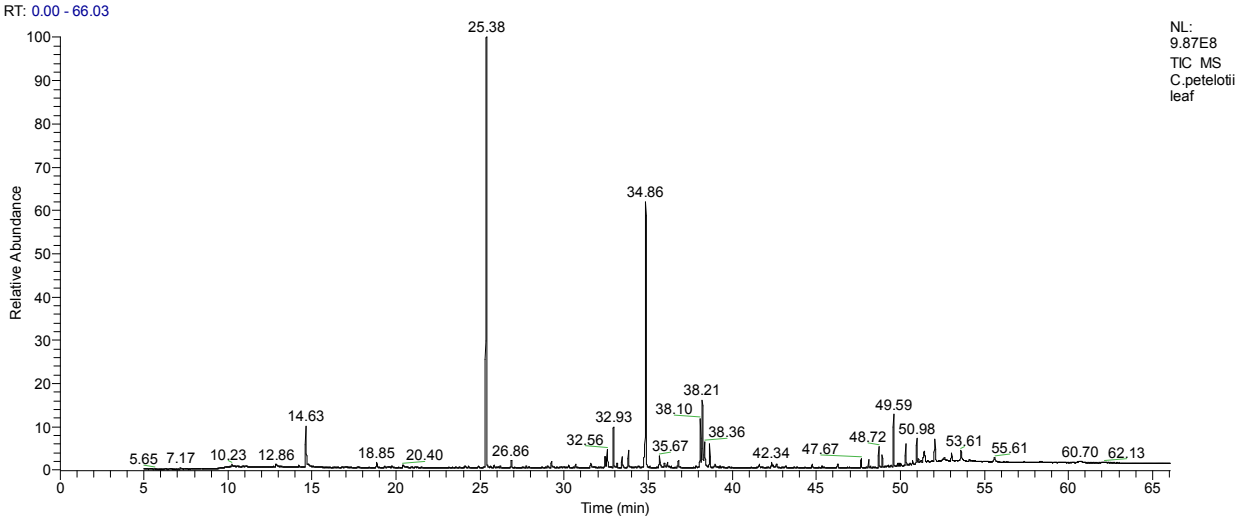
GC-MS chromatograms of six endemic golden *Camellia* leaves

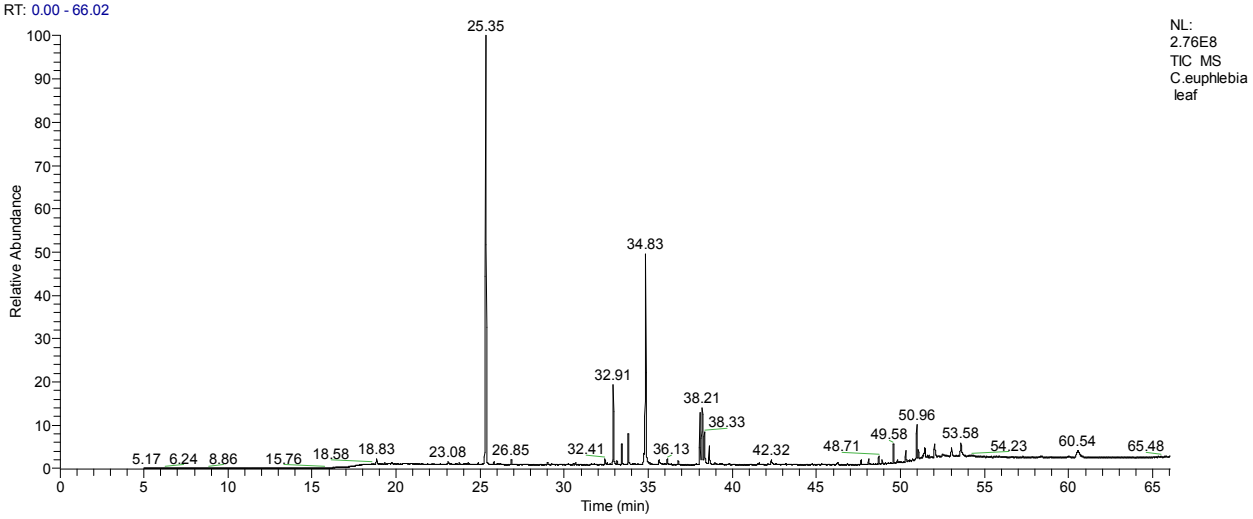


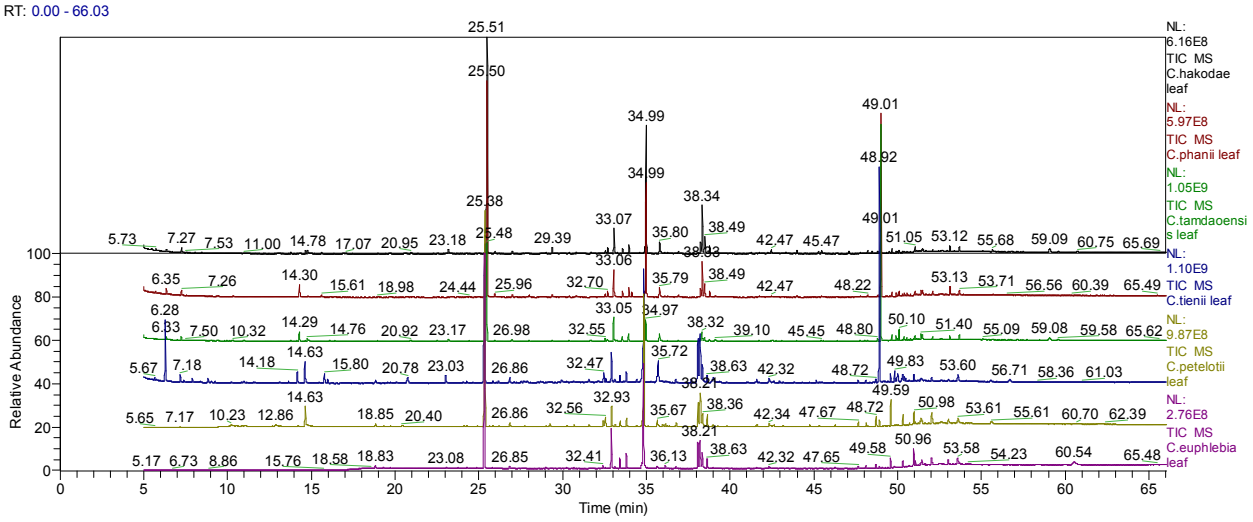


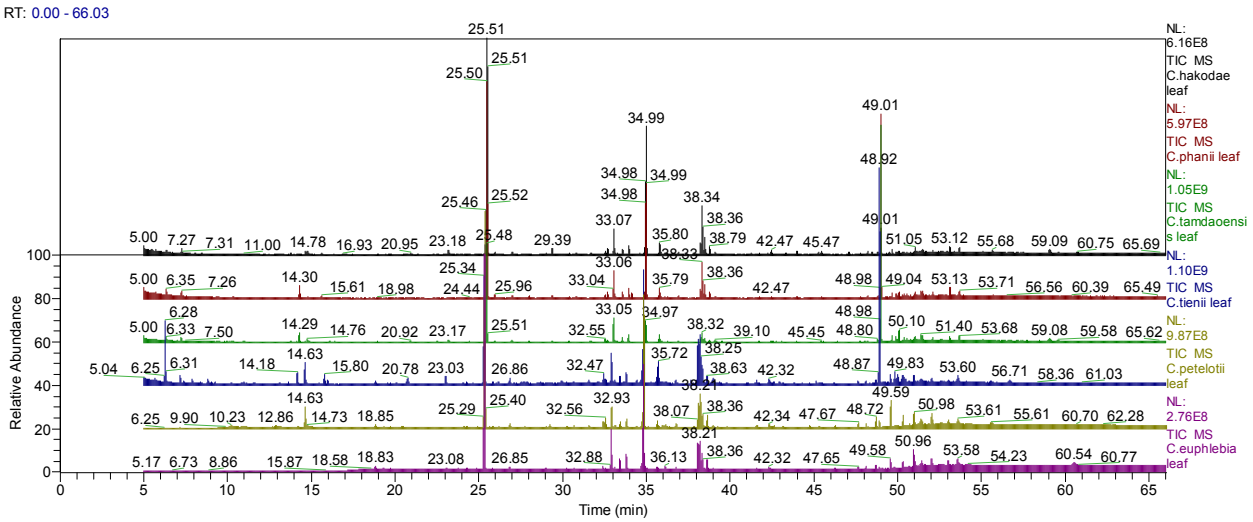


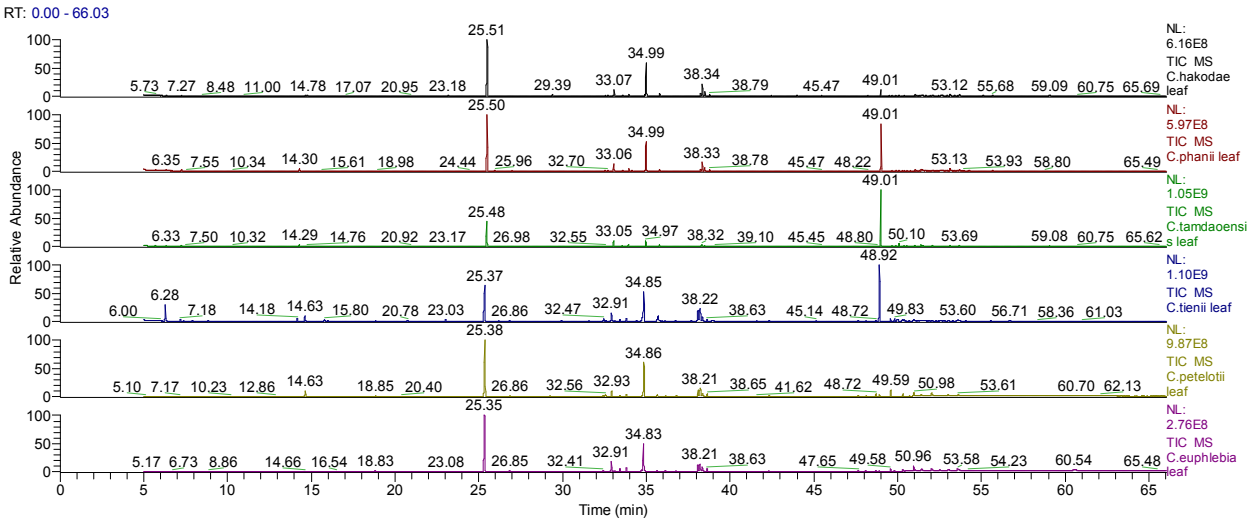

RT: 0.00 - 66.01
NL:
1.10E9
TIC MS
C.tienii leaf
Relative Abundance
Time (min)
6.28
25.37
34.85
48.92
38.22
32.91
14.63
35.72
38.34
14.18
15.80
38.63
32.47
49.83
5.67
7.18
8.84
10.99
20.78
23.03
26.86
42.32
48.72
53.60
56.71
58.36
61.03


RT: 0.00 - 66.03
NL:
9.87E8
TIC MS
C.petelotii
leaf
Relative Abundance
Time (min)
25.38
34.86
38.21
38.10
14.63
32.93
49.59
50.98
32.56
38.36
35.67
48.72
53.61
55.61
5.65
7.17
10.23
12.86
18.85
20.40
26.86
42.34
47.67
60.70
62.13

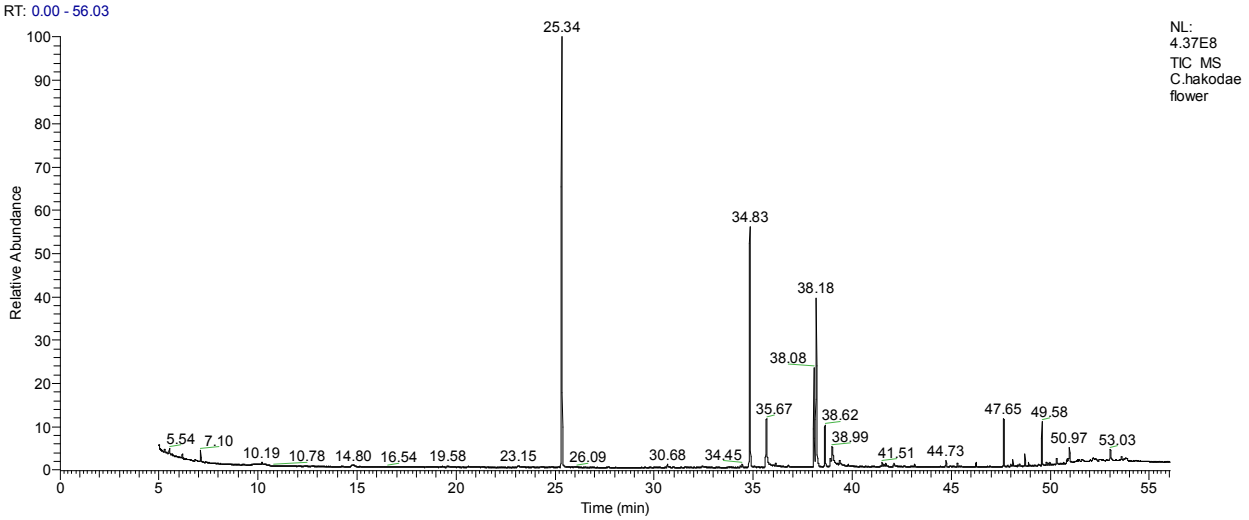

RT: 0.00 - 66.02
NL:
2.76E8
TIC MS
C.euphlebia
leaf
Relative Abundance
Time (min)
25.35
34.83
32.91
38.21
38.33
50.96
49.58
53.58
5.17
6.24
8.86
15.76
18.58
18.83
23.08
26.85
32.41
36.13
42.32
48.71
54.23
60.54
65.48

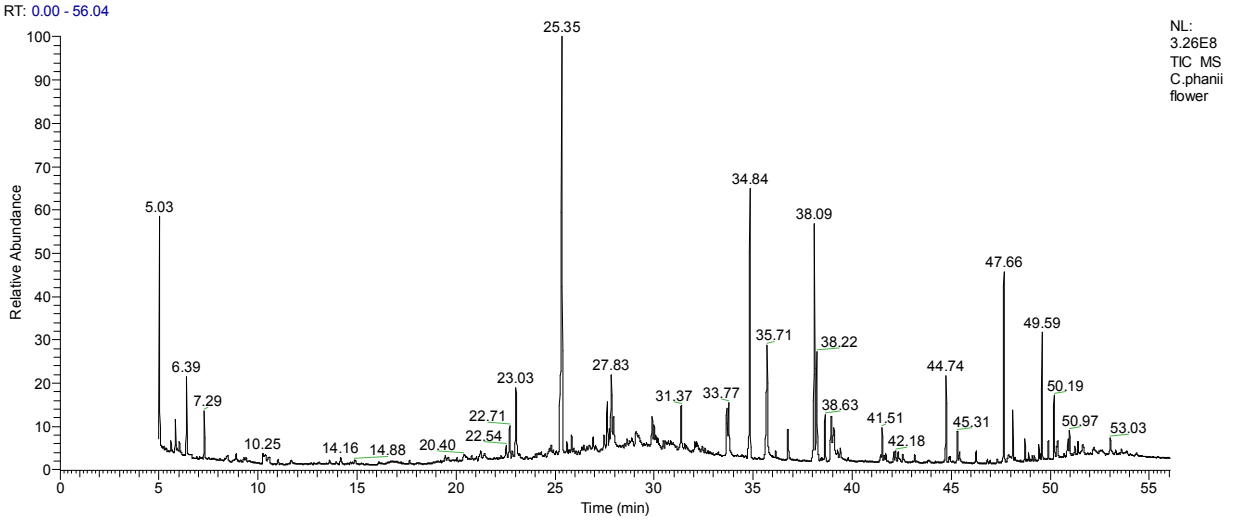

RT: 0.00 - 66.03
Relative Abundance
Time (min)
NL: 6.16E8 TIC MS C.hakodae leaf
NL: 5.97E8 TIC MS C.phanii leaf
NL: 1.05E9 TIC MS C.tamdaoensis leaf
NL: 1.10E9 TIC MS C.tienii leaf
NL: 9.87E8 TIC MS C.petelotii leaf
NL: 2.76E8 TIC MS C.euphlebia leaf

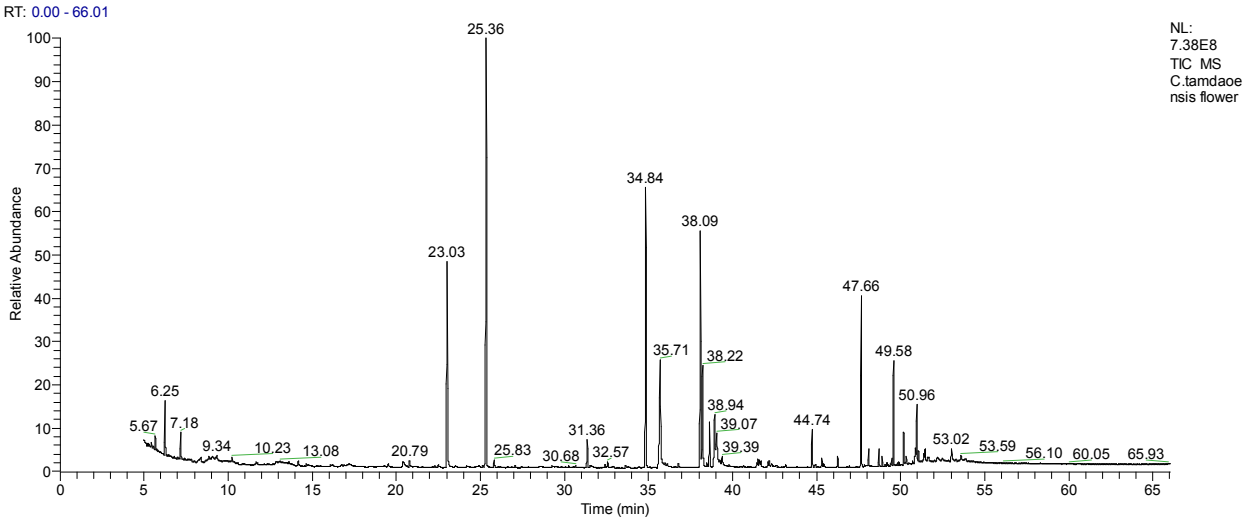

RT: 0.00 - 66.03
Relative Abundance
Time (min)
NL: 6.16E8 TIC MS C.hakodae leaf
NL: 5.97E8 TIC MS C.phanii leaf
NL: 1.05E9 TIC MS C.tamdaoensis leaf
NL: 1.10E9 TIC MS C.tienii leaf
NL: 9.87E8 TIC MS C.petelotii leaf
NL: 2.76E8 TIC MS C.euphlebia leaf

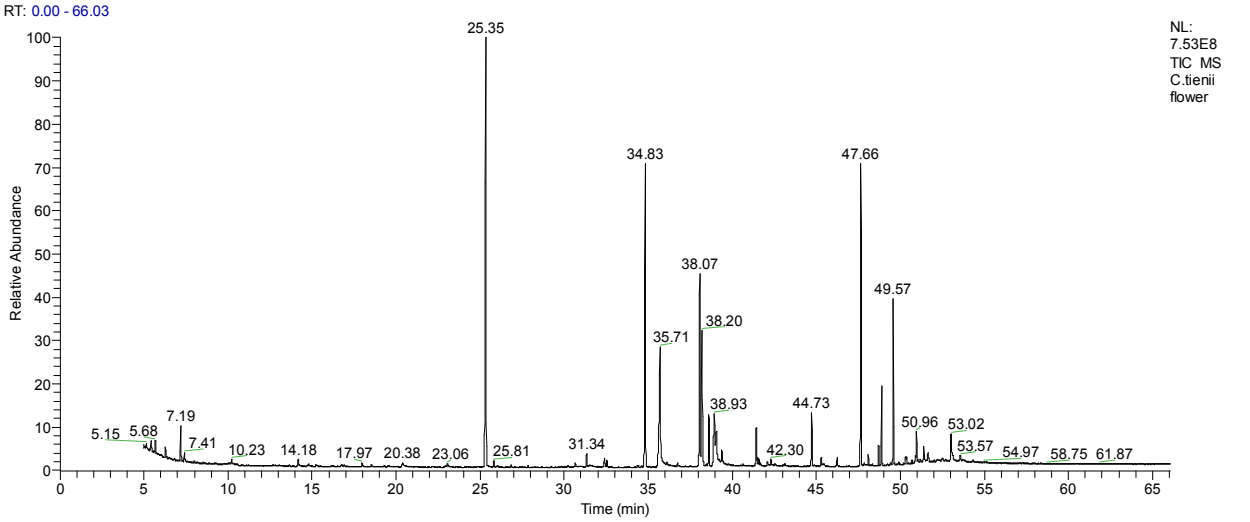

RT: 0.00 - 66.03
Relative Abundance
Time (min)
NL: 6.16E8 TIC MS C.hakodae leaf
NL: 5.97E8 TIC MS C.phanii leaf
NL: 1.05E9 TIC MS C.tamdaoensis leaf
NL: 1.10E9 TIC MS C.tienii leaf
NL: 9.87E8 TIC MS C.petelotii leaf
NL: 2.76E8 TIC MS C.euphlebia leaf

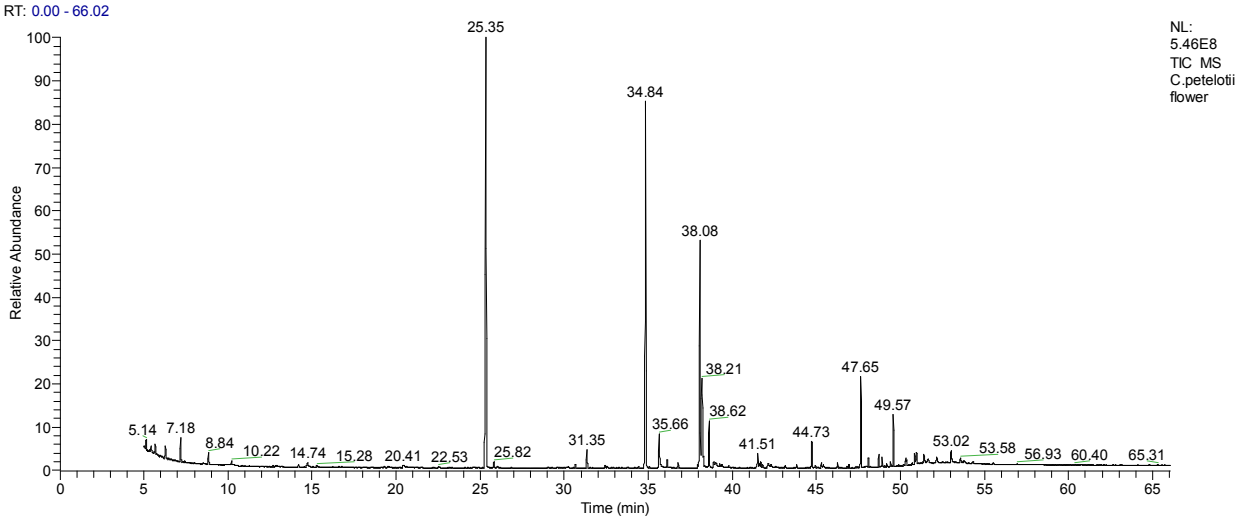
GC-MS chromatograms of six endemic golden *Camellia* flowers

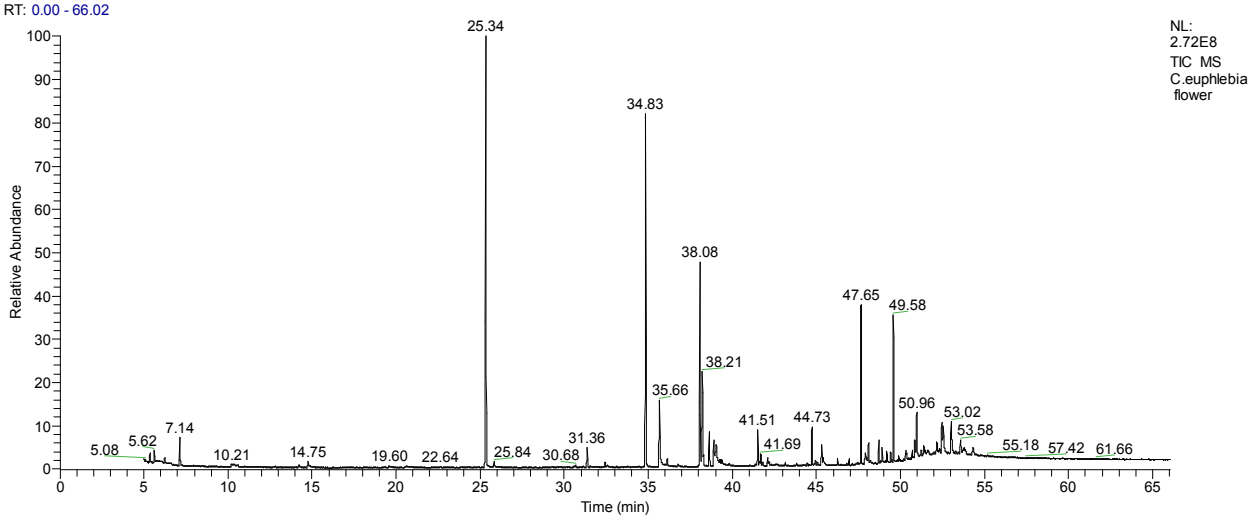







RT: 0.00 - 66.03
NL:
7.53E8
TIC MS
C.tienii
flower
Relative Abundance
Time (min)
25.35
34.83
47.66
38.07
49.57
38.20
35.71
38.93
44.73
50.96
53.02
7.19
5.15
5.68
7.41
10.23
14.18
17.97
20.38
23.06
25.81
31.34
42.30
53.57
54.97
58.75
61.87


RT: 0.00 - 66.02
NL:
5.46E8
TIC MS
C.petelotii
flower
Relative Abundance
Time (min)
25.35
34.84
38.08
38.21
47.65
49.57
38.62
35.66
44.73
41.51
53.02
53.58
56.93
60.40
65.31
5.14
7.18
8.84
10.22
14.74
15.28
20.41
22.53
25.82
31.35


RT: 0.00 - 66.02
NL:
2.72E8
TIC MS
C.euphlebia
flower
Relative Abundance
Time (min)
25.34
34.83
38.08
47.65
49.58
38.21
35.66
50.96
53.02
53.58
41.51
44.73
41.69
55.18
57.42
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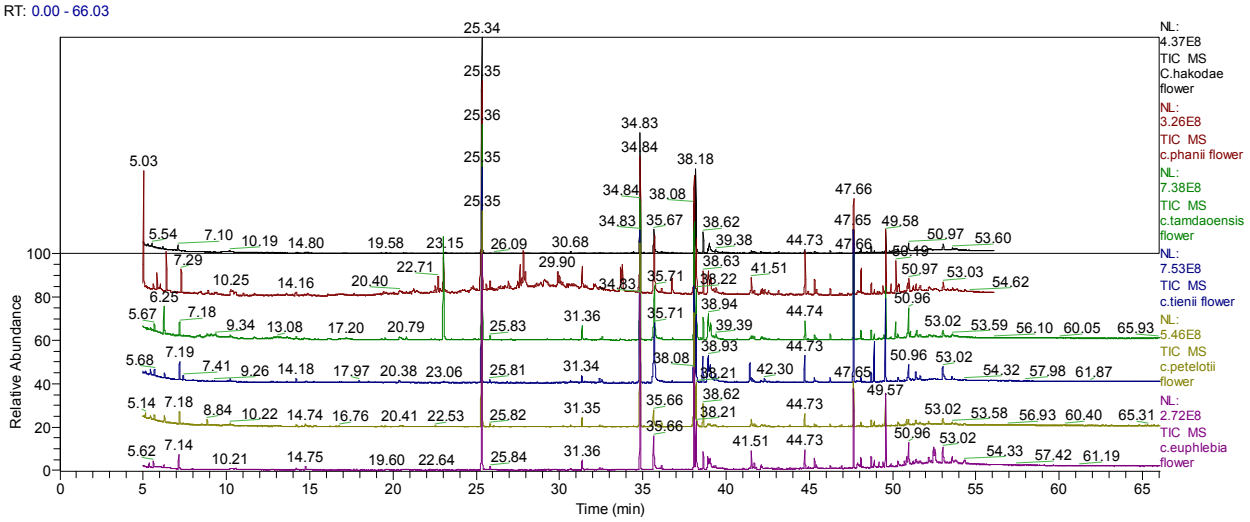
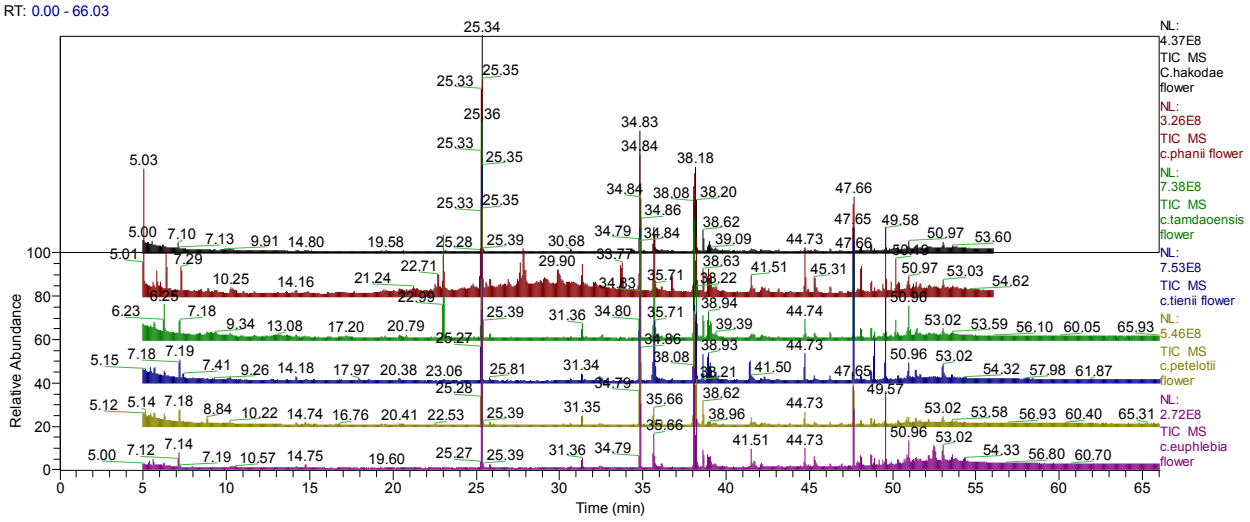
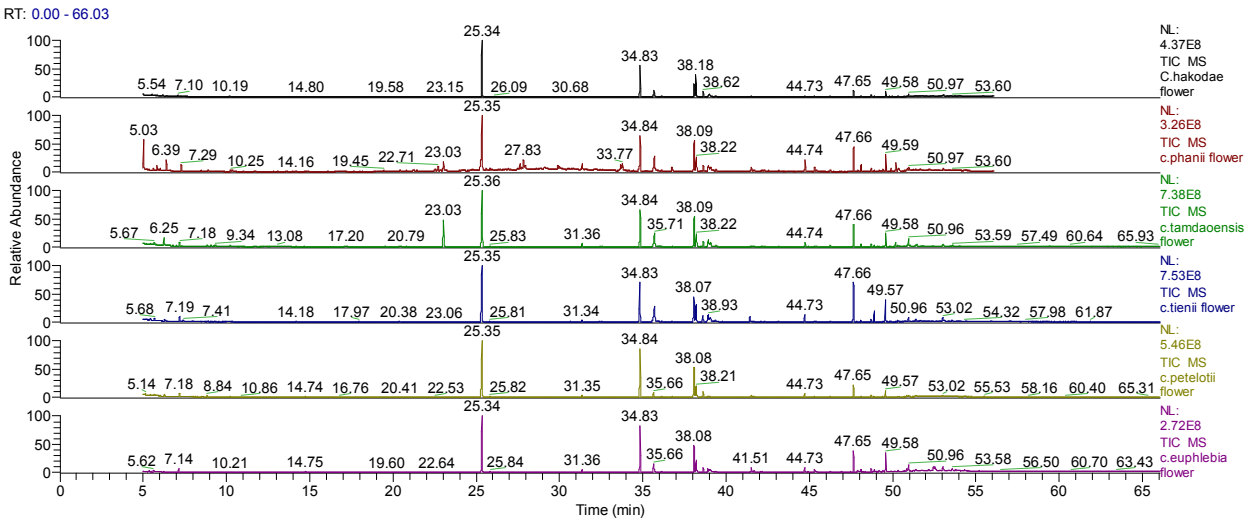
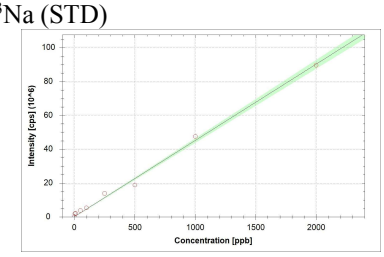
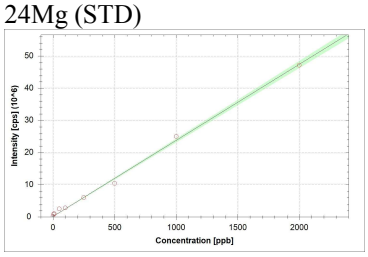
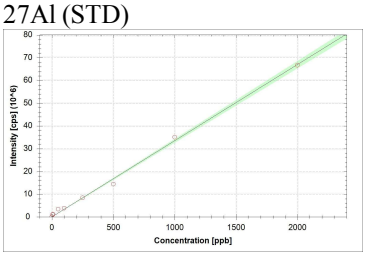
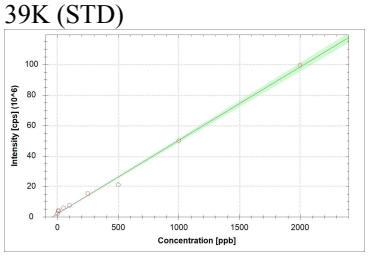
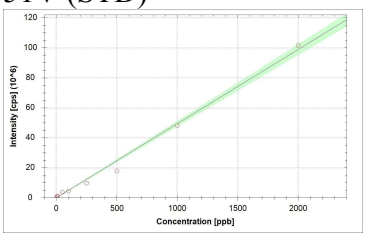
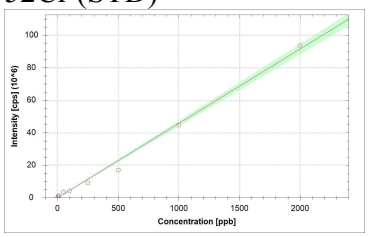
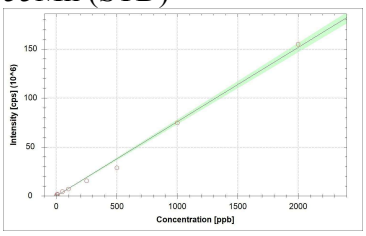
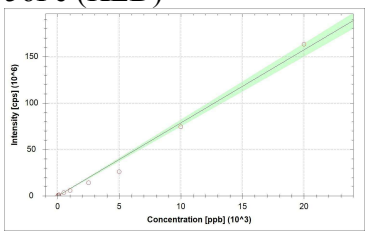
5.62
10.21
14.75
19.60
22.64
25.84
30.68
31.36

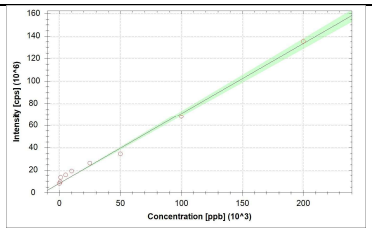
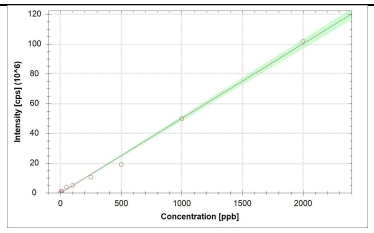
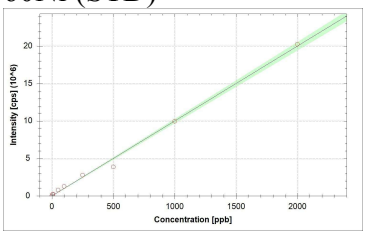
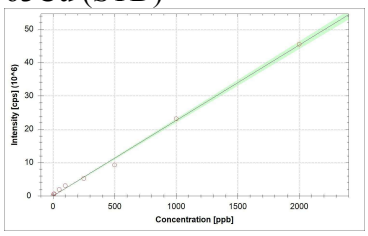
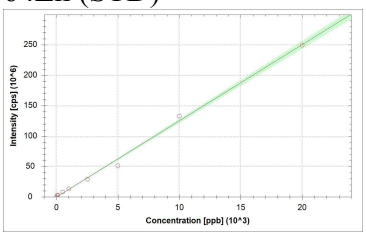
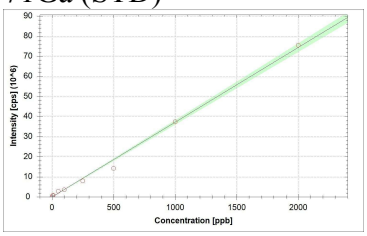
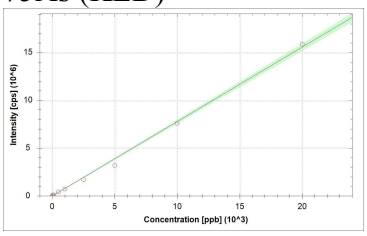
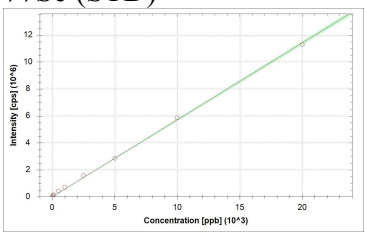

RT: 0.00 - 66.03
Relative Abundance
Time (min)
NL: 4.37E8 TIC MS C.hakodae flower
NL: 3.26E8 TIC MS c.phanii flower
NL: 7.38E8 TIC MS c.tamdaoensis flower
NL: 7.53E8 TIC MS c.tienii flower
NL: 5.46E8 TIC MS c.petelotii flower
NL: 2.72E8 TIC MS c.euphlebia flower

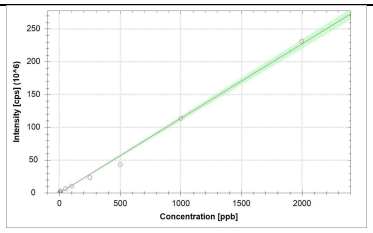

RT: 0.00 - 66.03
Relative Abundance
Time (min)
NL: 4.37E8 TIC MS C.hakodae flower
NL: 3.26E8 TIC MS c.phanii flower
NL: 7.38E8 TIC MS c.tamdaoensis flower
NL: 7.53E8 TIC MS c.tienii flower
NL: 5.46E8 TIC MS c.petelotii flower
NL: 2.72E8 TIC MS c.euphlebia flower


RT: 0.00 - 66.03
Relative Abundance
Time (min)
NL: 4.37E8 TIC MS C.hakodae flower
NL: 3.26E8 TIC MS c.phanii flower
NL: 7.38E8 TIC MS c.tamdaoensis flower
NL: 7.53E8 TIC MS c.tienii flower
NL: 5.46E8 TIC MS c.petelotii flower
NL: 2.72E8 TIC MS c.euphlebia flower

ANNEX 3: CALIBRATION CURVES

^{23}Na (STD)  f(x) = 45100.5094*x + 148335.4194 R² = 0.9953 BEC = 3.289 ppb LoD = N/A	24Mg (STD)  f(x) = 23631.9510*x + 167560.9200 R² = 0.9970 BEC = 7.090 ppb LoD =N/A
27Al (STD)  f(x) = 33484.6399*x + 39442.3046 R² = 0.9968 BEC = 1.178 ppb LoD = N/A	39K (STD)  f(x) = 48154.8508*x + 2124208.2870 R² = 0.9957 BEC = 44.112 ppb LoD = N/A
51V (STD)  f(x) = 49424.4185*x + 27630.6492 R² = 0.9929 BEC = 0.559ppb LoD = N/A	52Cr (STD)  f(x) = 45758.8904*x + 20346.6530 R² = 0.9939 BEC = 0.445 ppb LoD =N/A
55Mn (STD)  f(x) = 75820.1980*x + 10034.0955 R² = 0.9948 BEC = 0.132 ppb LoD = N/A	56Fe (KED)  f(x) = 7864.5965*x + 1320.0769 R² = 0.9890 BEC = 0.168 ppb LoD =N/A
40Ca (KED)	59Co (STD)

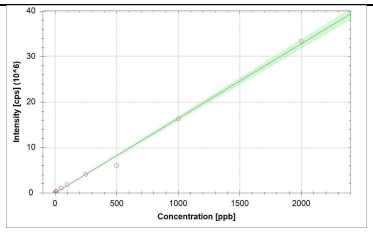
 f(x) = 624.5126*x + 8397071.6421 R² = 0.9922 BEC = 13445.800 ppb LoD = N/A	 f(x) = 50087.0730*x + 540.0138 R² = 0.9951 BEC = 0.011 ppb LoD = N/A
60Ni (STD)  f(x) = 10006.5806*x + 5311.1690 R² = 0.9956 BEC = 0.531ppb LoD = N/A	63Cu (STD)  f(x) = 22640.2381*x + 3640.5371 R² = 0.9966 BEC = 0.161 ppb LoD =N/A
64Zn (STD)  f(x) = 12510.6010*x + 24684.4538 R² = 0.9965 BEC = 1.973 ppb LoD = N/A	71Ga (STD)  f(x) = 37189.0275*x + 410.0089 R² = 0.9954 BEC = 0.011 ppb LoD = N/A
75As (KED)  f(x) = 778.2577*x + 70.0004 R² = 0.9969 BEC = 0.090 ppb LoD = N/A	77Se (STD)  f(x) = 570.7533*x + 1080.0531 R² = 0.9991 BEC = 1.892 ppb LoD = N/A
88Sr (STD)	95Mo (STD)



f(x) = 113625.6487*x + 2360.2257

R^2 = 0.9952

BEC = 0.021 ppb LoD = N/A

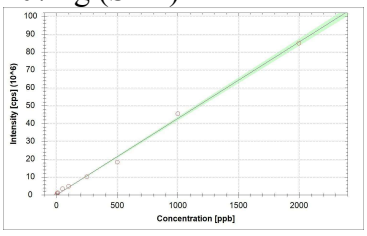


f(x) = 16413.8956*x + 870.0324

R^2 = 0.9948

BEC = 0.053 ppb LoD = N/A

107Ag (STD)

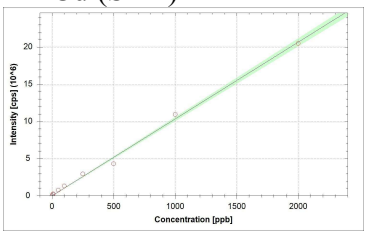


f(x) = 42800.7570*x + 10954.8576

R^2 = 0.9970

BEC = 0.256 ppb LoD = N/A

111Cd (STD)

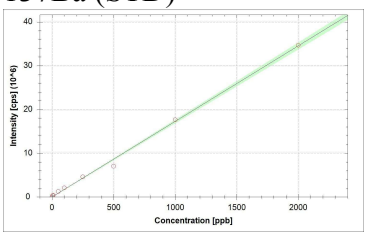


f(x) = 10331.4159*x + 20.0001

R^2 = 0.9962

BEC = 0.002 ppb LoD = N/A

137Ba (STD)

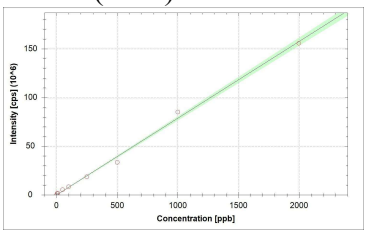


f(x) = 17277.1827*x + 510.0118

R^2 = 0.9971

BEC = 0.030 ppb LoD = N/A

208Pb (STD)

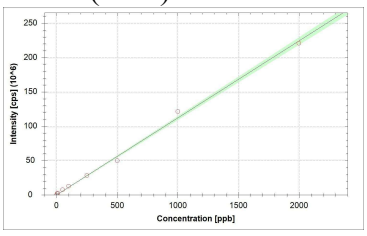


f(x) = 78830.9545*x + 1790.1331

R^2 = 0.9962

BEC = 0.023 ppb LoD = N/A

209Bi (STD)

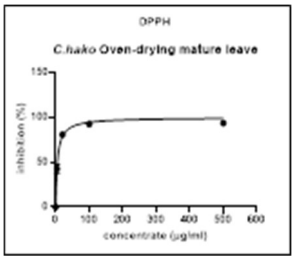


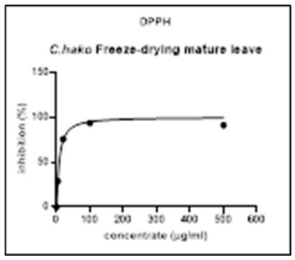
f(x) = 112163.2288*x + 260.0034

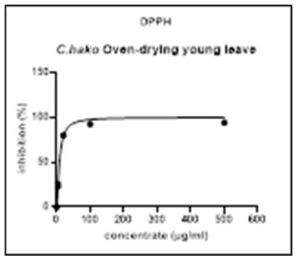
R^2 = 0.9967

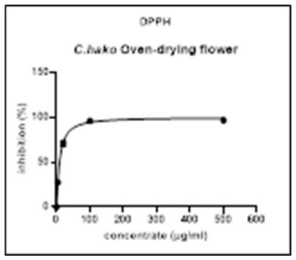
BEC = 0.002 ppb LoD = N/A

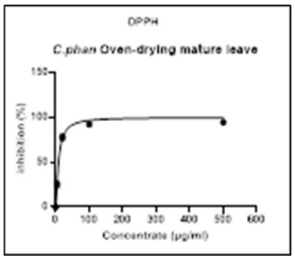
ANNEX 4. DPPH STANDARD CURVES

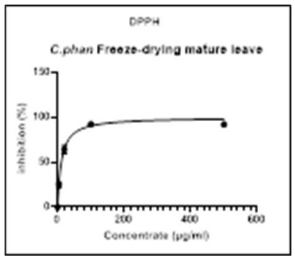


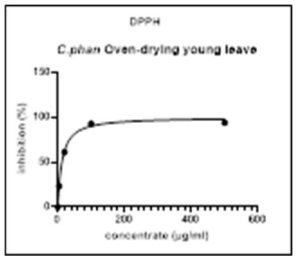


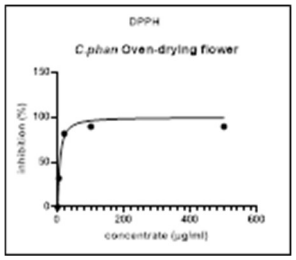


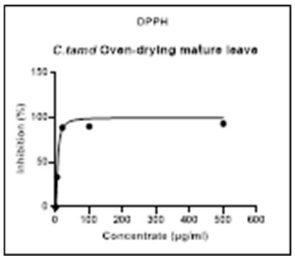


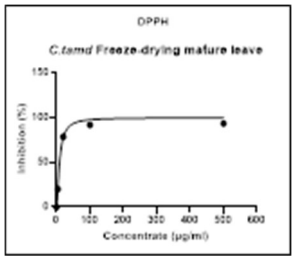


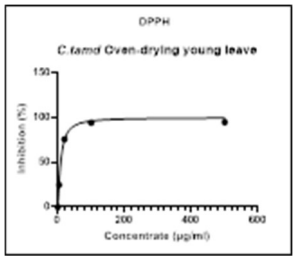


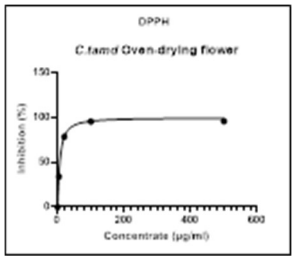


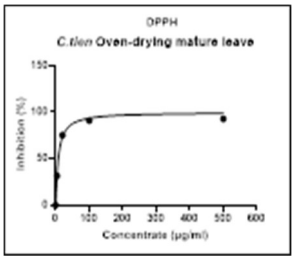


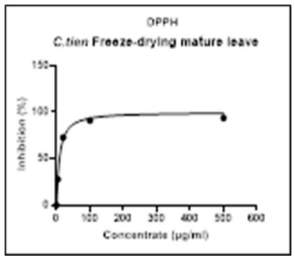


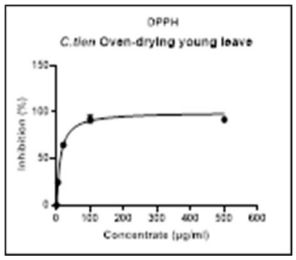


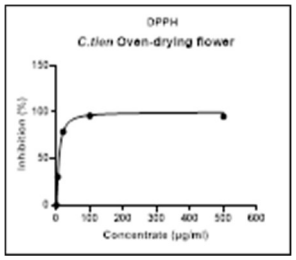


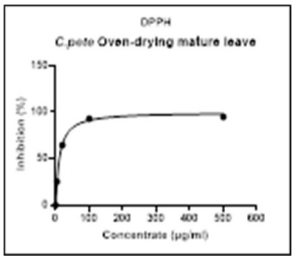


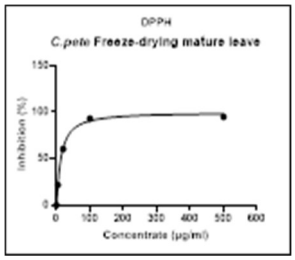


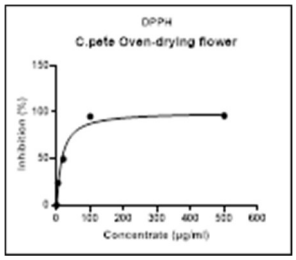


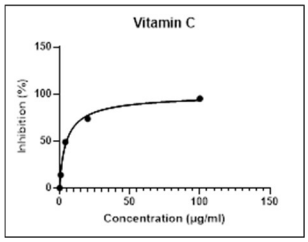


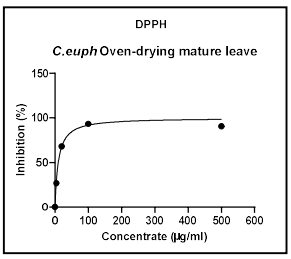


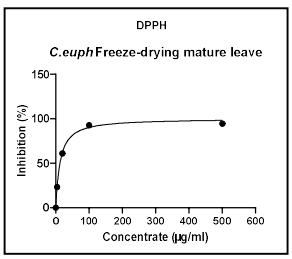


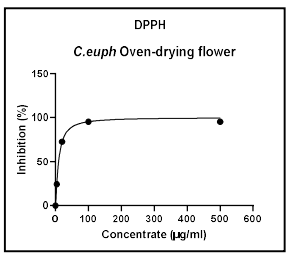




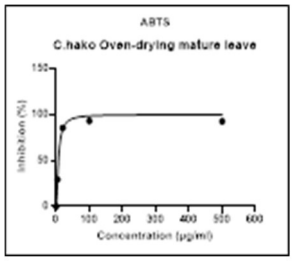


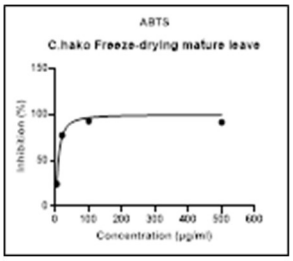


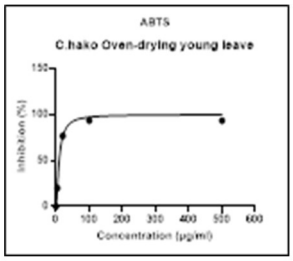


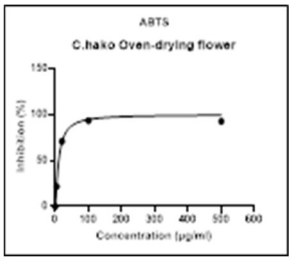


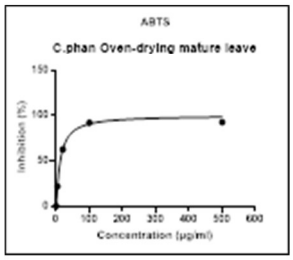
ANNEX 5: ABTS STANDARD CURVES

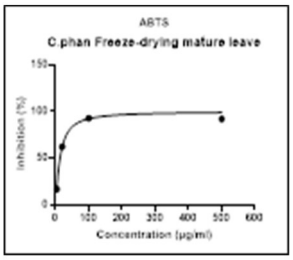


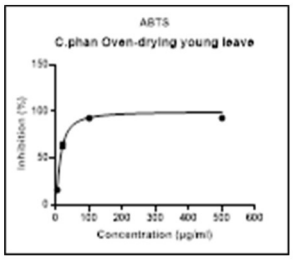


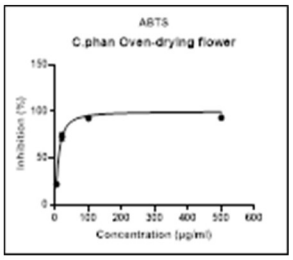


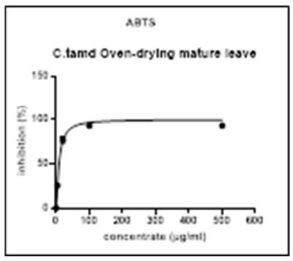


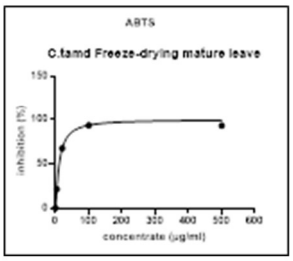


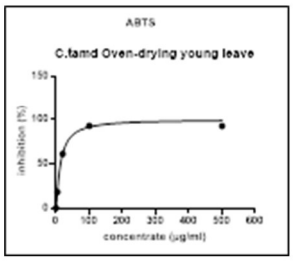


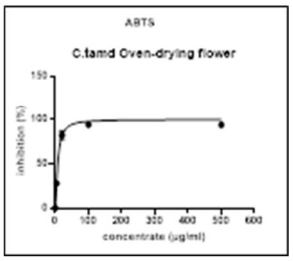


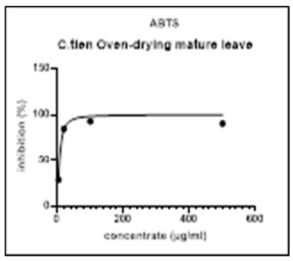


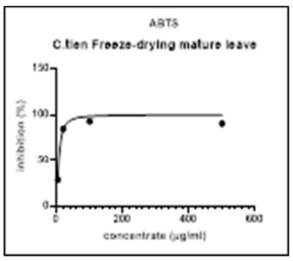


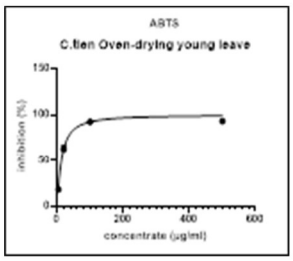


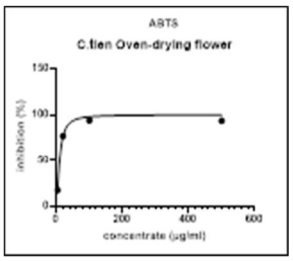


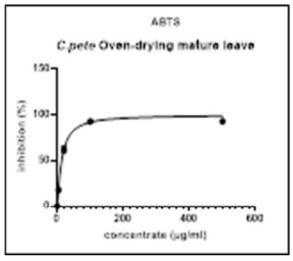


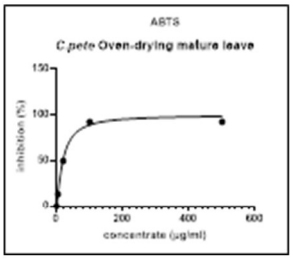


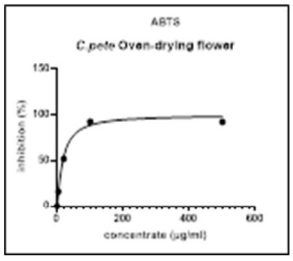


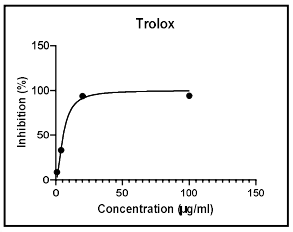


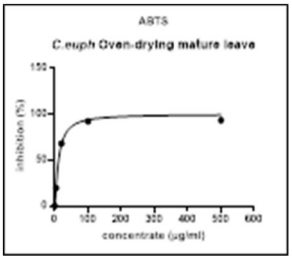


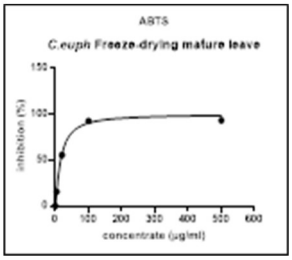


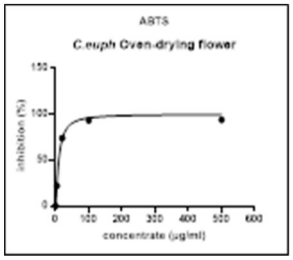












PUBLICATION LIST

03 publications in international journal:

1. **Nguyen, P. N**., Do, T. Y., Do, T. N., Gontier, E., Le Nguyen, H. T., Le Thi, V. A., ... & Nguyen Thi, K. O. (2024). Widely targeted metabolomics reveals the species-specific, matureness-specific and post-harvest-specific discriminations in the chemical profiles of Vietnamese endemic golden camellias. *International Journal of Food Science and Technology*, *59*(10), 7873-7886. doi:10.1111/ijfs.17071
2. **Nguyen, P. N**., Le, H. L., Gontier, E., Hoang Thi, H. C., Le Thi, V. A., Mai, N. T., & Nguyen Thi, K. O. (2024). A Comprehensive Strategy for Metabolites Profiling of Flowers and Leaves from Camellia tienii, an Endemic Golden Tea of Vietnam. *Chemistry & Biodiversity*, *21*(10), e202400997.
3. Van-Anh Le, T., Mai Nga, T. P., **Nhi Nguyen, P**., & Kieu-Oanh Nguyen, T. (2023). Genotypic and phenotypic diversity of endemic Golden camellias collected from the north of Vietnam. *Chemistry & Biodiversity*, *20*(1), e202200843., 2023. doi.org/10.1002/cbdv.202200843

01 proceeding of national conference

4. Nguyễn Phương Nhung, **Nguyễn Phương Nhị**, Mai Thị Phương Nga, Lê Thị Vân Anh, Nguyễn Thị Kiều Oanh. Proceeding Hội thảo Sinh lý thực vật - Đà Lạt 2024: Phân biệt hệ chất chuyển hóa theo khu vực địa lý của loài chè hoa vàng *Camellia euphlebia* thu hái ở Quảng Ninh.

doi.org/10.1002/cbdv.202200843

RESEARCH ARTICLE

CHEMISTRY & BIODIVERSITY

Genotypic and Phenotypic Diversity of Endemic Golden Camellias Collected from the North of Vietnam

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Golden Camellias 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 were considered endemic species of this country. The morphology of leaves and flowers of these species were similar; therefore, their taxonomic identification usually needed experts and the authentication has often been confused among species. Our study aims to describe the genetic diversity and the relationship of six species *Camellia phanii*, *Camellia tamdaoensis*, *Camellia tienii*, *Camellia flava*, *Camellia petelotii* and *Camellia euphlebia* by using three chloroplast DNA-barcodes: *matK*, *rbcL* and *trnH-psbA*. We also clarified the significant differences in anatomical characteristics of midvein and blade of their leaves, which suggested the possibility to use these criteria in taxonomy. In addition, preliminary chemical profiles of the methanolic extracts of leaves from six Golden Camellias such as total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC) and chlorogenic acids content (TCGAs) also showed the diversity among them. Interestingly, the discrimination on the catechins profile among six species followed the same tendency with the genetic distance on the phylogeny tree suggesting that catechins (i.e., discriminative catechins) can be biomarkers for the chemotaxonomy of these six Golden Camellias.

Keywords: golden camellia, genetic diversity, leaf anatomy, TPC, TFC, TAC, TCGAs.

Introduction

Golden Camellias is a group of species belonging to the genus Camellia L. (Theaceae family) that have golden yellow flowers, generally small, evergreen, and shade tolerant. Although this group comprises about fifty species, they are rarely distributed in the South of China and North of Vietnam and many of them were listed in the Red Data Book of Threatened Species.[1–2] In Vietnam, nearly forty species have natural distribution, of which thirty-two species have been considered endemic plants. This country has thus become one of the countries owning the highest number of Golden Camellias species in the world.[3] Both leaves and flowers of these species have been used daily as tea. In addition, they are often used traditionally to treat hypertension, high cholesterol and obesity, to support/protect the liver and prevent cancer. There are many studies demonstrating that the natural products from the leaves of Golden Camellias possess antioxidant activity,[4–5] anticancer activity,[6] and cytotoxicity.[7] These species also could inhibit the development of tumors, reduce blood pressure, lower blood cholesterol, and prevent cardiovascular diseases such as atherosclerosis.[8–9]

Due to these above-mentioned pharmacological activities, Golden Camellias are being paid much attention to and present a great potential in developing healthcare products. These species have thus been cultivated in many provinces of Vietnam such as Quang Ninh, Vinh Phuc, Ninh Binh, Lam Dong… Even though the growing areas have been increasing dramatically in recent years, limited information was reported about the biological and chemical diversity of these native species, especially at molecular levels. In previous studies, the identification was mostly

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202200843

based on the traditional characterization such as growth habitat and flower/leaf/fruit morphologies which showed many problems of misidentification and mislabeling.[10] Due to the similarity in the whole appearance of Golden Camellias, the morphological identification usually needs the competence of botanist experts by compulsorily using their flowers/fruits while access to these tissues was sometimes limited. Moreover, the number of newly discovered Golden Camellias species has been increasing in recent years while species are transported from one region to another, which modified their natural distribution. That causes interference and mis-delimitation in the morphology-based taxonomy. In addition, there was no chemical investigation that can be applied for the classification of these herbal medicines, whereas the chemical-based taxonomy has been crucial for quality control as the variation in metabolite profile cannot be sufficiently reflected by the genetic variation.[11–12]

The development of molecular biology and chemotaxonomy may provide a comprehensive solution for the accurate identification and delimitation of these Golden Camellias. Indeed, the core plant barcodes have been used to provide information related to taxonomic classification and phylogenetics of closely and distantly plant species according to the recommendation of the Consortium for the Barcode of Life (CBOL) Plant Working Group. The principle of the DNA barcoding techniques is based on the matching of the sequences of unidentified samples against a database of know-DNA sequences.[13–15] DNA markers were selected according to barcoding objectives and generally met the requirements that allowed for the design of universal primers such as high interspecific polymorphism, little or no intraspecific divergence, and the presence of conserved flanking regions.[16–18] On the other side, the science of chemotaxonomy, also known as chemical taxonomy, is used to categorize plants according to their chemical components. It is supposed that the secondary metabolites produced by all living things are generated from the primary metabolites. Secondary metabolite profiles and related biosynthesis pathways are frequently specialized and common in similar taxonomic groups, which makes them helpful for classification. The chemotaxonomy approach is based on the similarity and dissimilarity in the profile of certain chemicals and is key for successful phytochemistry research.

This study aims to provide systemic information on the genotypic and phenotypic diversity of six Golden Camellias collected in northern Vietnam that could be used in the breeding and conservation of these precious biological resources. For genotypic discrimination, different molecular markers have been used to identify the relationship and origin of the studied species. For phenotypic diversity, both anatomical and chemical characteristics of these species were described. The anatomical structure of leaves was observed by using histological techniques. Chemical indexes such as total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and chlorogenic acids content (CGAs) were determined by colorimetric and chromatographic assays. Catechins, a specific class of secondary metabolites in the Camellia genus were quantified and discriminated among six species. The combination of molecular markers, phenotypic description, and chemotaxonomy thus provided the most accurate classification of Golden Camellias and useful information for further valorization strategies of these precious medicinal plants.

Results and Discussion

Genetic Diversity Using the DNA Barcodes

For conventional genotypic discrimination, different molecular markers such as Restriction Fragments Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP), Cleaved simple sequence repeats SSR (CAPS) and Amplicon Length Polymorphisms (ALPs) have been used to identify the relationship and origin of the studied species. Besides these markers, new DNA markers have been proposed and used to efficiently detect the genetic diversity of the Camellia genus (A DNA barcode for land plants). These barcodes have been used to provide information related to taxonomic classification and phylogenetics of closely and distantly plant species because they are relatively stable and not easily affected by the environment.[19] Indeed, twenty-nine samples of yellow Camellias were analyzed using simple sequence repeats (SSR) markers to detect their genetic diversity and relationship.[20] Recently, numerous studies have used the internal transcribed spacers (ITS) marker to identify and distinguish Camellia species.[21] However, one marker seems to be not sufficient to significantly reveal the small difference in the genetic diversity of numerous species. Therefore, some reports have thus suggested using of more than one marker to discriminate a big number of species in a complex genus.[22–23] In this study, we use the combination of three locus *rbcL*,

matK, and *trnH-psbA* (chloroplast genes) as the core plant barcodes to understand the genetic relationship of six Golden Camellias species that originated from Vietnam.

The results of successful PCR amplification of chloroplast *matK*, *rbcL*, and *trnH-psbA* genes of six investigated Camellia species were shown in *Figure 1*. The size of obtained bands for *matK*, *rbcL*, and *trnH-psbA* are approximately 850 bp, 650 bp, and 500 bp, respectively, which is consistent with other studies.[24–25] The Sanger sequencing of the PCR products results in the formation of the polymorphisms of these three genes in six species and was used to identify the relationship among them.

The multi-alignment of *matK*, *rbcL*, and *trnH-psbA* genes were displayed in *Figure 2*. The results showed that there were some point mutations in the *matK* gene in six yellow Camellia species. In *C. euphlebia*, a one-point mutation replacing nucleotide G with nucleotide A at position number 153 and a one-point mutation adding one more nucleotide T at position 790 were identified. In position 495, there was another point mutation of nucleotide T instead of C in *C. tienii* (*Figure 2A*). These results indicated that using the *matK* gene, *C. euphlebia* is far more different from five other species. Among the remaining five species, *C. tienii* is far different from other *Camellia* species.

Similar to the *matK* gene, the sequence alignment of the *rbcL* gene indicated the greatest difference of *C. euphlebia* compared to six studied *Camellia* species. Indeed, there was only one mutation changing nucleotide T by C at position 504 in *C. euphlebia* (*Figure 2B*). With this number of mutations, this *rbcL* gene showed limited phylogenetic information and cannot be used as a maker for the classification of six species.

The most differential marker was found in the *trnH-psbA* gene with several informative sites in the sequence among six species. In *C. euphlebia*, the data showed that at position 57 the nucleotide T was replaced by C. At position 102, nucleotide C took the place of nucleotide A in three species *C. euphlebia*, *C. tienii*, and *C. flava*. Especially, mutations leading to the deletion of 5 nucleotides in *C. tamdaoensis* occurred from position 222 to 227. Lastly, at position 261, a nucleotide C was replaced by T in *C. tienii* (*Figure 2C*). This result suggested that this gene was feasible to utilize as a marker for genetics discrimination of four out of six studied species including *C. euphlebia*, *C. tienii*, *C. flava*, and *C. tamdaoensis*.

We have combined three DNA barcode markers including *matK*, *rbcL*, and *psbA-trnH* to build the phylogenetic tree of six investigated Camellias. The results from the phylogenetic tree showed that we have two main clades. Clade 1 (bootstrap 80) includes *C. petelotti* as the sister to the clade of *C. tamdaoensis* and *C. phanii* (bootstrap 92). Clade 2 (bootstrap 65) includes *C. tienii* as the sister to the clade *C. flava* and *C. euphlebia* (bootstrap 70). The reference species *C. danzaiensis* H.T.Chang & K.M.Lan belongs to another branch of origin with our six investigated species (*Figure 3*).

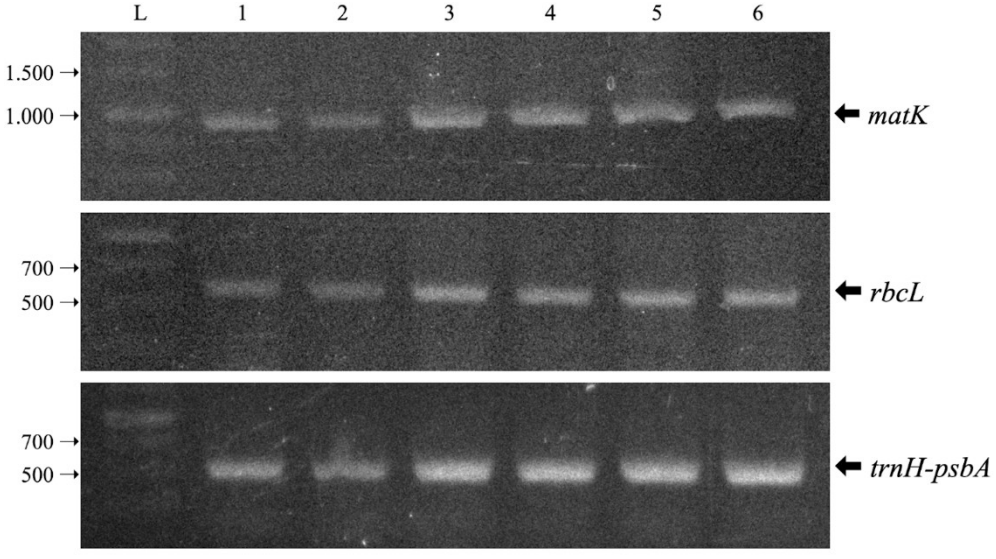

Figure 1. PCR amplification of three selected genes in six investigated *Camellia* species. *matK*, *rbcL*, *trnH-psbA.* are three chloroplast genes. (1) *C. euphlebia*, (*2*) *C. phanii*, (*3*) *C. tamdaoensis*, (*4*) *C. petelotii*, (*5*) *C. C. flava*, (*6*) *C. tienii*, (L) Ladder.

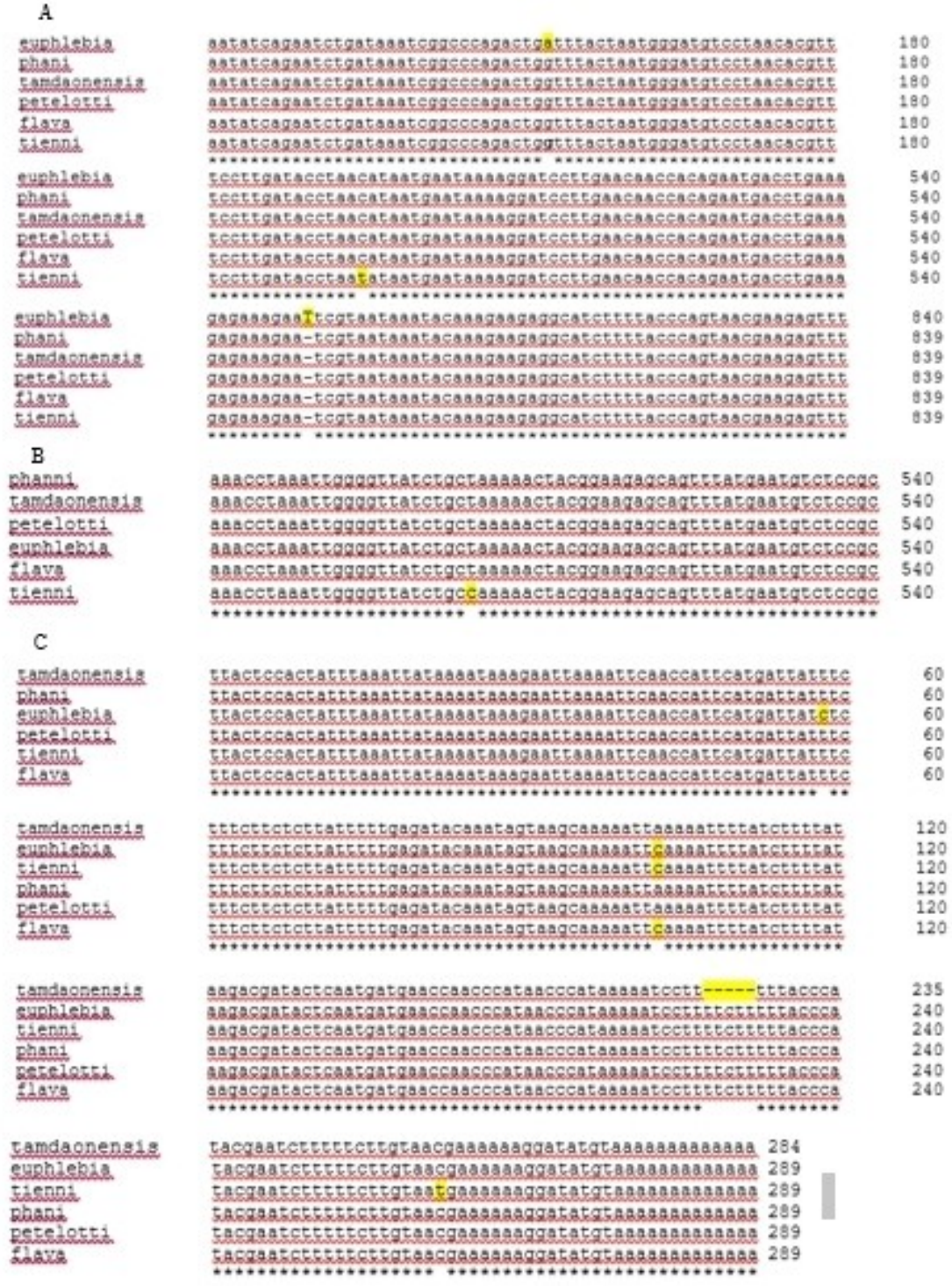

Figure 2. Chloroplast DNA alignment of six investigated Camellia species. (A) *matk* gene alignment, (B) *rbcL* gene aligment, (C) *trnH-psbA* intergenic spacer alignment. Yellow colors indicate the nucleotides difference.

In the literature, several investigations showed the phylogenetic tree of Golden Camellias, mainly on Chinese Golden Camellias. In a study using electrochemical fingerprints for species identification in the *Camellia* genus, scientists showed that *C. euphlebia* belonged to the second clade including *C. tunghinensis*, *C. euphlebia*, and *C. wumingensis*.[26] Fang et al. successfully classified twenty-eight native species of

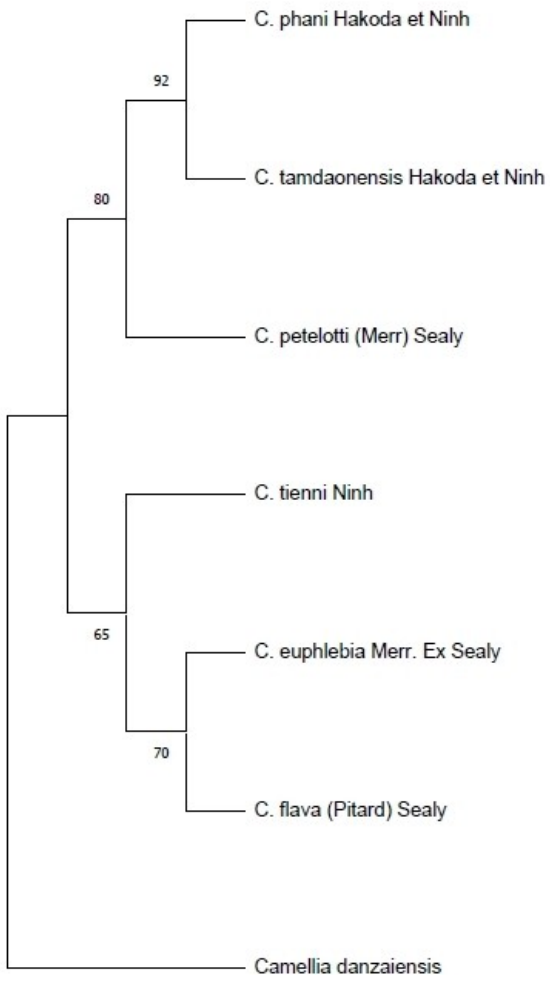

Figure 3. The Neighbor-joining phylogenetic tree conducted in MEGA v11 of six investigated and one reference Camellia species using *matk*, *rbcL* and *trnH-psbA* genes.

Camellia using four chloroplast DNA regions including rpl 16, psbA-trnH, trnL–F & rpl 32- trnL.[27] Concerning Vietnamese endemic species of Vietnam, most research studied the genetic relationship among two or three species, thus cannot be used for building a phylogeny tree. In a comparative research of *C. tamdaoensis* and *C. petelotii*, two species represented the difference in position 613, nucleotide C was replaced by A in the *matK* gene.[28] Recently, Nguyen et al. successfully used three genes, including *matK*, *rbcL*, and *ITS2*, to identify the relationship between *Camellia euphlebia* and *Camellia chrysantha*. The nucleotide difference between them ranged from 0–0.82%, suggesting that *Camellia chrysantha* from Quang Ninh is a derivative of *Camellia euphlebia* from Bac Giang.[29] This study demonstrated for the first time the phylogenetic tree of five endemic Golden Camellias species of Vietnam in which *C. petelotii*, *C. tamdaoensis*, and *C. tienii* were considered endemic to Tam Dao region, Vinh Phuc province.[30] *C. flava* is endemic from Cuc Phuong, Ninh Binh province,[31] *C. phanii* is native species of Thai Nguyen province,[32] and *C. euphlebia* which was found in both Vietnam and China.[33] It might provide useful information for breeding and conservation programs of these endangered genetic resources.

Leaf Morphology and Anatomy

The leaves structures and characteristics are an important criterion for taxonomy identification.[34] Recently, the leaf morphology and anatomy of 60 species of the Camellia genus were described, in which leaf thickness and the area of the midrib showed significant diversity.[35] In this study, we enlarge the number of examined parameters related to the anatomical construction of the midvein (midvein width, depression angle, number of collenchyma cell layer, phloem area, bundle sheath area, xylem area) and blade (palisade/spongy ratio) to validate the above-mentioned genetic discrimination. *Figure 4* showed both the slice anatomy of midvein blades and the bar charts illustrating the statistical analysis of measured parameters.

Overall, our findings demonstrated a substantial difference in leaf morphology of six analyzed Golden Camellias. Interestingly, the area of bundle sheath and phloem in midvein showed a significant difference in all six groups of samples while five remaining phenotypes witnessed diversity among four or five species (p-value < 0.05). For the former property, *C. tienii*'s leaf represented the largest area of bundle sheath, followed by the leaves of *C. flava*, *C. petelotii*, *C. tamdaoensis*, *C. phanii*, and *C. euphlebia*. For the phloem, it was measured with the biggest area in *C. flava*'s leaves, then those of *C. tamdaoensis*, *C. tienii*, *C. petelotii*, *C. phanii*, and *C. euphlebia*. This observation suggested that these two parameters might be considered discriminative anatomical phenotypes and can be used for the identification of these six species while the five remaining indexes can support this identification.

Regarding the appearance of the midrib, *C. euphlebia* and *C. tienii* leaves were nearly flat while those of four other species were concave toward the adaxial-abaxial side. The bundle sheath of all six samples displayed with convex form, however, varied in a wide range of the depression angle from *C. flava* (79°) to *C. euphlebia* (163°). In terms of midvein width,

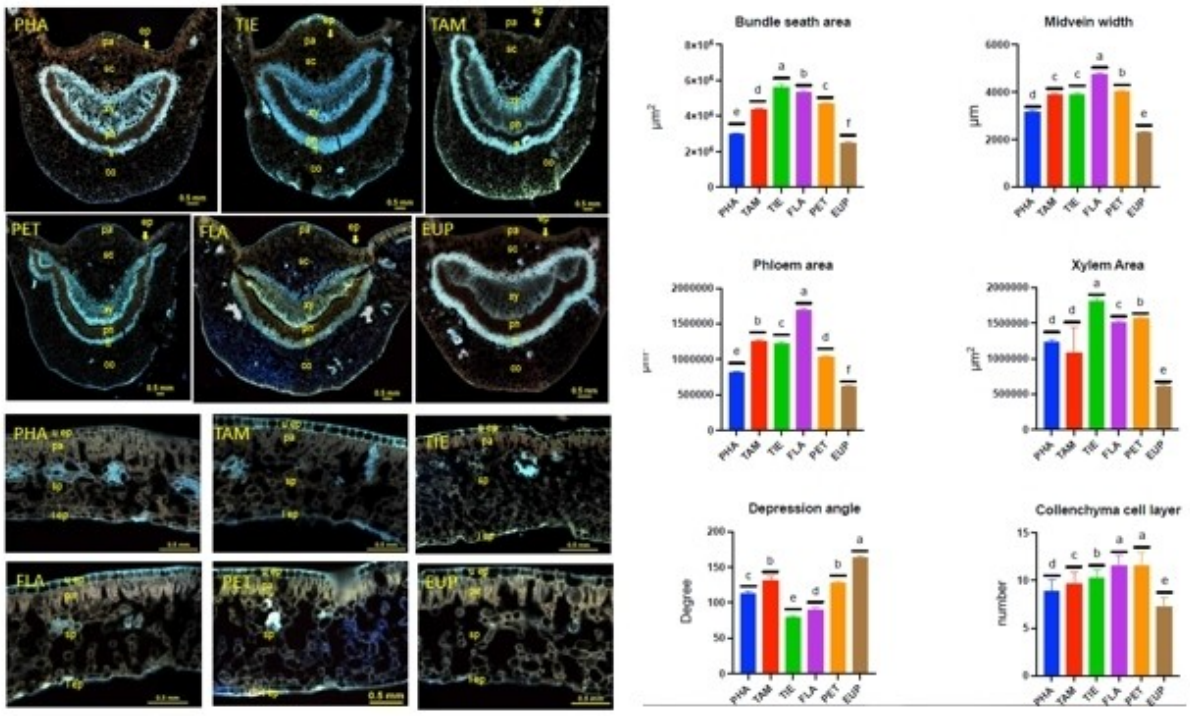

Figure 4. The cross-section images of midvein blade under fluorescence; and the anatomical parameters of six Golden Camellias species. *ep: epidermis*; *pa: parenchyma*; *sc: sclerenchyma*; *xy: xylem*; *ph: phloem*; *fi: fiber*; *co: collenchyma.* Different letters in the same column indicate significantly differences using Student's t-test ($p < 0.05$). PHA – *C. phanii*, TIE – *C. tienii*, TAM – *C. tamdaoensis*, PET – *C. petelotii*, EUP – *C. euphlebia*, FLA – *C. flava.*

the leaves of *C. flava* represented the highest value, followed by *C. petelotii* while *C. phanii* and *C. euphlebia's* leaves showed the lowest values. *C. tamdaoensis* and *C. tienii* did not show any significant difference in midvein width.

The xylem area exposed a slightly different trend compared to the latter parameters. Indeed, *C. tienii* exhibits the highest data (approximately 6×10^6) which was twice as high as *C. euphlebia* (about 3×10^6). The number of collenchyma cell layers also exhibited a clear diversity which ranged between roughly 7 in *C. euphlebia* and 12 for *C. petelotti* and *C. flava.*

The leaf blades of six species shared a similar histological structure including an upper epidermis cell layer and two primary mesophyll tissues, which are composed of a vertically elongated palisade, loosely packed spongy, and a lower epidermis cell layer. Only *C. euphlebia* had 2 cells layer in palisade tissue. Consequently, the palisade/spongy ratio of this species showed the highest value of 0.46 followed by *C. phanii*, *C. flava*, *C. petelotii*, *C. tamdaoensis*, and *C. tienii* with 0.28; 0.24; 0.19; 0.15 and 0.13, respectively. The number of palisade cell layers as well as the palisade/spongy ratio was specific for each species; moreover, these parameters were strongly affected by the rainfall amount.[34] In our study, *C. euphlebia* was gathered in Hai Ha – Quang Ninh where the annual rainfall is much higher while the others were collected from Tam Dao – Vinh Phuc province.[36] This geographical characteristic contributes to the great discrimination in the palisade/spongy ratio of *C. euphlebia* compared to other species.

To see the specificity of all anatomical factors in the discrimination of species, six parameters of these fifty-four samples were put in Principal Component Analysis (PCA) analysis. *Figure S2* (*Supporting Information*) showed that there is a clear separation of six species. In which, *C. tamdaoensis*, *C. petelotti*, and *C. phanii* samples were close to each other. Regarding the above phylogeny result, the histological parameters showed consistency with the genetic relationship of these three species. The others, *C. tienii*, *C. flava*, and *C. euphlebia* showed the different discrimination trends between phylogeny tree and PCA-based histological profiles which can be explained by the interaction of genetic and environmental factors during their growth and development.

Even though the size shape and appearance of Golden Camellias leaves are very similar, the anatomical analysis of leaf morphology confirmed the diversity in midvein blades of six species, both pictorial and statistical analysis. This result provided an opportunity to use leaf morphology in classifying Golden Camellias species while the conventional identification required flowers/fruits which were sometimes not available. From a more applied point of view, as leaves were also used as a tea and medicinal plants, this diversity might be applied to authentication, traceability, and quality control of these herbal materials.

Chemical Profiles

Total Phenolic and Flavonoid Content, Total Anthocyanin and Total Chlorogenic Acids Content

Phenolics and flavonoids are plant secondary metabolites containing at least one aromatic ring and hydroxy group attached to the ring. These compounds play an important physiological role in protecting against the action of free radicals; thus can be a good source of antioxidants for the human body in preventing cardiovascular diseases, cancer, diabetes, and neurodegenerative diseases.[37–38] Total phenolic content (TPC) and total flavonoid content (TFC) were measured in each extract using colorimetric assays with gallic acid (GAE) and quercetin (QE) as the standards. As shown in *Table 1*, *C. phanii* leave extract contained a higher amount of phenolics (309.22 ± 61.46 mgGAE/g), followed by *C.tienii* (261.17 ± 50.96 mgGAE/g), *C. tamdaoensis* (224.09 ± 66.51 mgGAE/g). Other species *C. euphlebia* and *C. petelotii* have presented moderate content of phenolics which were found lower than those of the same species reported in the literature.[4,6] The content of phenolics in tea leaves varied greatly depending on plant varieties, the extraction solvent, the intact properties and the growth stage.[6] On the other hand, TFC did not follow the same tendency as TPC. In detail, *C. euphlebia* represented the highest TFC values with 630.84 ± 179.77 mgQE/g dry extracts, followed by *C. petelotii* and *C. tamdaoensis* with 540.79 ± 98.39 and 539.73 ± 53.11 mgQE/g, respectively. *C. phanii* and *C. tienii* samples contained insignificant differences in TFC levels of 495.13 ± 23.35 and 480.98 ± 19.13 mgQE/g. *C. flava* demonstrated the lowest concentration of TFC with 74.70 ± 8.76 mgQE/g dry extracts.

Anthocyanins are naturally occurring color substances that belong to the flavonoid group. In a similar trend with TFC, TAC varied significantly from 4.62 ± 0.07 to 9.84 ± 0.08 mgCGE/g. *C. euphlebia* showed the highest concentration of anthocyanin, followed by *C. tamdaoensis*, *C. petelotii*, *C. tienii*, *C. phanii*, and *C. flava*.

Chlorogenic acids are important phenolics that decide generally the flavor formation and biological effect of coffee and tea products. They are esters formed between caffeic and quinic acids and possess many pharmacological benefits such as antioxidant, antimicrobial, antiviral, and anti-inflammatory abilities.[39–41] In this study, the total chlorogenic acids content of leaf tissues of six species ranged from 10.52 ± 0.86 to 16.86 ± 0.54 mg chlorogenic acid equivalent per g of leaves dry weight, which was relatively equivalent to that in other *Camellia* species.[42] *C. tienii* represented the highest level of total chlorogenic acids content, followed by *C. flava*, *C. tamdaoensis*, *C. euphlebia*, *C. phanii*, and *C. petelotii*. These significant differences among species suggested that these indices can contribute to the discrimination of the studied Golden Camellias.

This is the first report on the TPC, TFC, TAC and TCGAs of six species, which contributed to the chemotaxonomy of these rare Camellias. In addition, phenolics, flavonoids, anthocyanins, and chlorogenic acids are good sources of bioactive compounds. This preliminary study showed the potential to use these

Table 1. Total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC) and chlorogenic acids content (CGAs) in methanolic extracts of six Golden Camellias. Different letters in the same column represent results with a statistical difference, according to Student t-test ($p \leqslant 0.05$).

Sample	TPC (mgGAE/g) (n = 3)	TFC (mgQE/g) (n = 3)	TAC (mgCGE/g) (n = 3)	TCGAs (mgCGAE/g) (n = 3)
C. phanii	309.22 ± 61.46^{a}	495.13 ± 23.35^{a}	5.09 ± 0.18^{a}	10.86 ± 0.69^{a}
C. tamdaoensis	224.09 ± 66.51^{b}	539.73 ± 53.11^{b}	6.71 ± 0.86^{b}	13.82 ± 0.11^{b}
C. tienii	261.17 ± 50.96^{c}	480.98 ± 19.13^{a}	6.56 ± 0.14^{b}	16.86 ± 0.54^{c}
C. euphlebia	127.50 ± 3.80^{d}	630.84 ± 179.77^{c}	9.84 ± 0.08^{c}	11.16 ± 0.97^{a}
C. flava	123.66 ± 1.50^{d}	74.70 ± 8.76^{d}	4.62 ± 0.07^{d}	15.08 ± 1.02^{d}
C. petelotii	68.87 ± 5.33^{e}	540.79 ± 98.39^{b}	6.60 ± 0.19^{b}	10.52 ± 0.86^{a}

leaves in developing healthcare products or cosmetics. However, the content of these groups varied in six investigated species implying the importance of taxonomy and traceability especially in the case that these leaves showed similar appearance.

Catechins Profile

Catechins are common and specific groups of secondary metabolites in Camellia genus. Catechins are derivatives of a flavan-3-ol, a subgroup of polyphenols providing antioxidant roles in plants. Song et al. reported that the polyphenolic constituents of six China-origin Golden Camellias varied widely in terms of qualitative analysis.[4] Other research showed a significant difference in the content of catechins among these six species.[6] In this study, seven catechins including (+)-Catechin (C), (−)-Catechin 3-gallate (CG), (−)-Epicatechin (EC), (−)-Epicatechin-3-gallate (ECG), (−)-Epigallocatechin 3-gallate (EGCG), (−)-Gallocatechin (GC), (−)-Gallocatechin 3-gallate (GCG) were determined in six Vietnamese Golden Camellias using the LC/MS/MS method. The catechins profile released by the analysis of a great number of samples can be used to discriminate among these Golden Camellias.

The retention times (in min) for the studied compounds were as follows: 1.31 (C), 1.94 (EC), 0.65 (EGC), 2.81 (CG), 3.05 (ECG), 1.61 (GCG), 1.87 (EGCG) (*Figure 1S*, *Supporting Information*). The peak area for each analyte in sixty samples has been used directly for Principal Component Analysis (PCA) to visualize the discrimination of catechins profiles in six species (*Table 1S*, *Supporting Information*). The measured data's highest part of explainable variability is represented by the first PC (Dim1), and the next largest component is represented by PC2 (Dim2). Thus, those principal components allowed for simply analyze complicated data with an advantage of a lesser dimensionality.[43]

Figure 5 showed the clear separation of six clusters corresponding to six species of Golden Camellias in the PCA plot. Indeed, two principal components explained up to 63.6% of the total variance. It suggested that PCA still preserved most of the information in the large set of data and guarantees the strong existence of the discriminant factors among

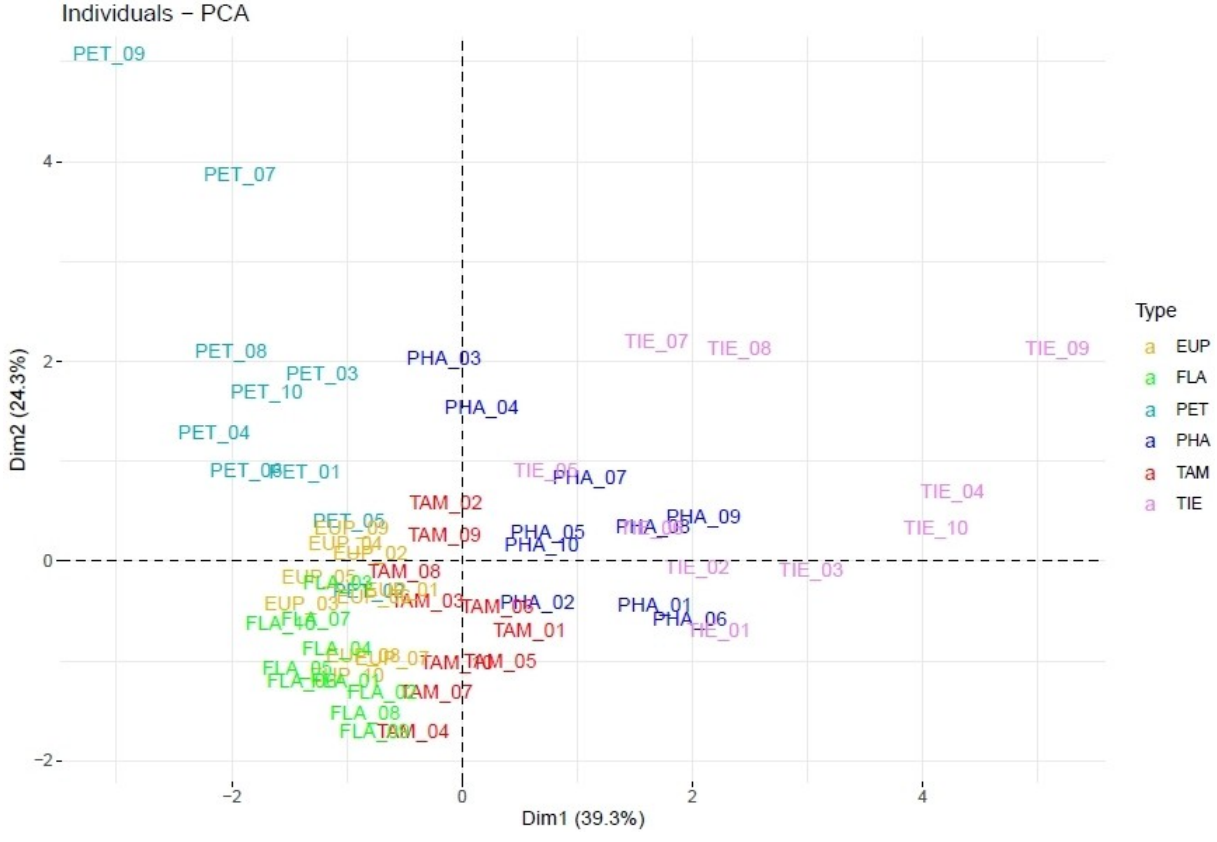

Figure 5. PCA score plot of the two major components of catechins in six Golden Camellias samples. EUP = *C. euphlebia*; FLA = *C. flava*; PET = *C. petelotii*, PHA = *C. phanii*; TAM = *C. tamdaoensis*, TIE = *C. tienii*.

six studied species. *C. petelotii* and *C. tienii* presented mainly in the upper left and right sides, which showed that these species were rather discriminant against the remaining 4 species. In contrast, *C. euphlebia* and *C. flava* were separately scattered, however, next to each other and undifferentiable in a small area in the plot. Similarly, *C. tamdaoensis* and *C. phanii* localized in close positions. The relative coordinates of the scatter in the plot indicated that the leaves of six species were discriminant based on the catechins profile. Interestingly, the distance between these clusters followed the same tendency as the phylogeny tree in *Figure 3*. Indeed, *C. petelotii* was far from other species; *C. tienii* was located in a different branch from two close species *C. euphlebia* and *C. flava*; *C. tamdaoensis* and *C. phanii* belong to the same branch. All the above-mentioned arguments allowed us to suggest that the catechins profile was an important parameter for the chemotaxonomy of Golden Camellias.

The contribution level of the variables on the factor map was illustrated by a bar plot of variables cos2 (square cosine, squared coordinates) on two first dimensions (*Figure 6*). This chart shows how strongly each catechin contributes to the definition of components. As can be seen in this Figure, GC contributed the most to the variability explanation in the PCA plot, followed by C and EC.

Conclusions

The present study showed the diversity in terms of anatomical, genetic, and chemical characteristics of six Camellia species of which four species are endemic to Vietnam. The histological discrimination was indicated by seven parameters related to the leaf vein and blade. Our study also points out that using three DNA barcodes, including *rbcL*, *matK*, and *trnH-psbA* can distinguish well these Camellias and can be used to build the genetic relationship in a phylogeny tree. The total phenolic/flavonoid/anthocyanin/chlorogenic acids contents of these species represented significant differences suggesting that a more delicate analysis of secondary metabolites should be performed. The results from our study provide new insight into the diversity of Camellia in Vietnam and open new

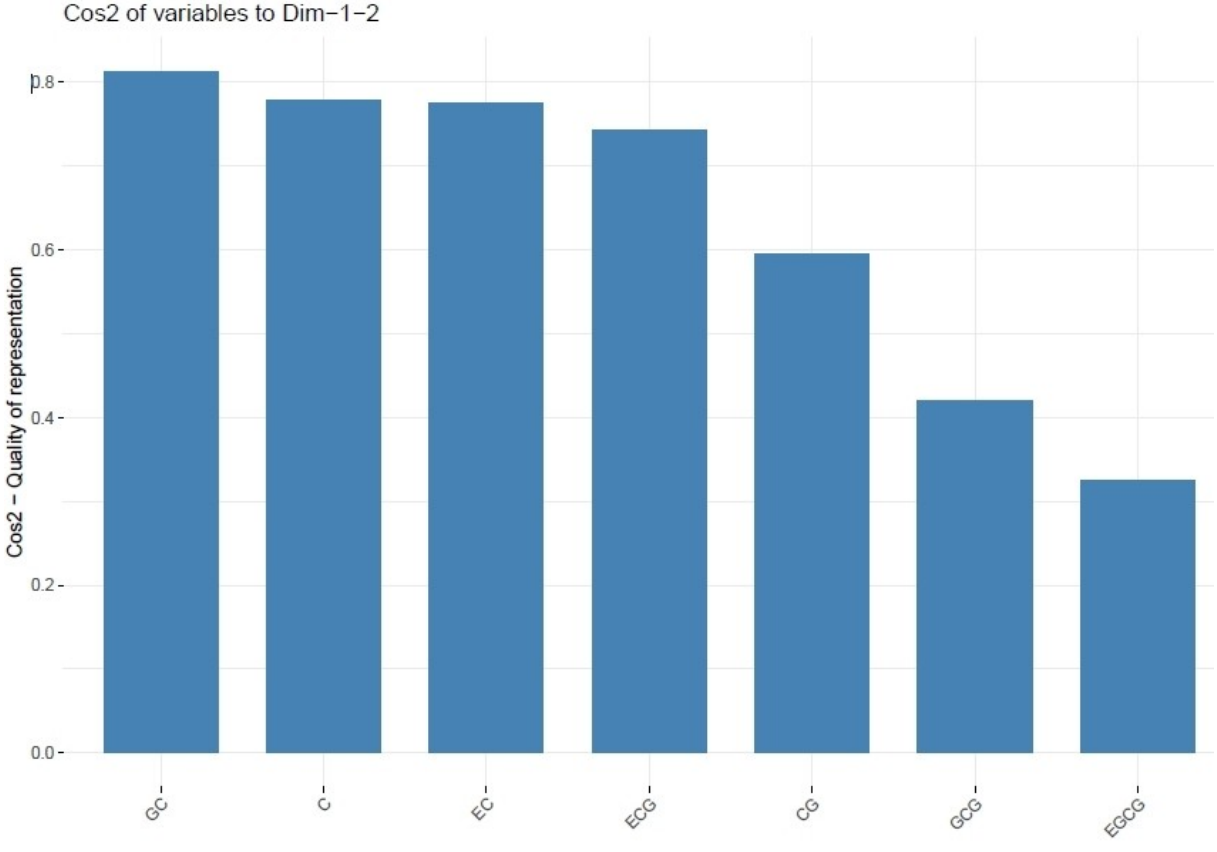

Figure 6. Loading plots of contribution level of each variable to the PCA discrimination analysis.

avenues for deeper studies about these endemic species.

Experimental Section

Plant Materials

Six endemic Golden Camellias species: *Camellia phanii* (PHA, specimen code PHA-12-2021), *Camellia tam-daoensis* (TAM, specimen code TAM-12-2021), *Camellia tienii* (TIE, specimen code TIE-12-2021), *Camellia flava* (FLA, specimen code FLA-12-2021), *Camellia petelotii* (PET, specimen code PET-12-2021) were collected at Hop Chau ward, Tam Dao district, Vinh Phuc province (GPS: 21°26′18.0″N 105°36′17.3″E) on December 21st 2021. *Camellia euphlebia* (EUP, specimen code EUP-04-2022) was collected in Quang Minh ward, Hai Ha district, Quang Ninh province (GPS: 21°28′45.9″N 107°46′07.8″E) on April 29th 2022. All plant materials were identified by Dr. Nguyen The Cuong from the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The specimen dossiers were deposited in the Department of Life Sciences, University of Science and Technology of Hanoi to store and serve for further investigation.

DNA Extraction

To prepare samples for PCR reaction, DNA was extracted from wild-type leaves. First, 0.5 g of leaf material was ground in a 1.5 ml Eppendorf tube in liquid nitrogen. Then, 800 µl of 2X CTAB buffer (2% (w/v) cetyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris HCl pH 8.0, 20 mM EDTA) was added and the tube was incubated at 65 °C for 30 min. After that, 300 µl of chloroform and 20 µl potassium acetate 5 M was added and the tube was thoroughly vortex. The mixture was centrifuged (Centrifuge 5702, Eppendorf, Germany) at 10000 rpm for 10 min to separate phases. Subsequently, the upper aqueous phase was transferred into a new Eppendorf tube. Three hundred µl of 2-propanol was added and the tube was mixed well before being stored at −20 for 2 h. Next, the mixture was centrifuged at 12000 rpm for 15 min to pellet DNA. The pellet was kept and washed with 300 µl of 70% ethanol. The mixture was centrifuged at 13000 rpm for 1 min. This washing step was repeated twice. Finally, ethanol was removed, and the pellet was air-dried pellet until transparency. The pellet was dissolved in 30 µl of TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA). The quantity and quality of the DNA were estimated with nanodrop (NanoDrop 2000 Spectrophotometers, Thermo Scientific) as well as by electrophoresis on 1.0% agarose gel in 1×TAE buffer.

PCR and Sequencing

Genomic DNA from the leaves of six species was used as plant materials for polymerase chain reaction (PCR). The PCR was performed to amplify the sequence of two chloroplast genes including *matK* and *rbcl* and one intergenic sequence *trnH-psbA* which have been proposed by the Consortium for the Barcode of Life (CBOL) Plant Working Group as the core barcodes of plant species. The sequence of the forward and reverse primers used in this study were presented in *Table 2*. The PCR protocol was followed in our previous study.[44] The high-quality PCR products were sent for Sanger sequencing.

Sequence Alignment and Phylogeny Tree

The DNA sequence between six investigated and one reference Camellia species was aligned using Clustal Omega[47] to see how different in sequences of the three selected genes are. The phylogenetic tree was elaborated using Mega11 software,[48] the neighbor-Joining method.[49] The bootstrap consensus tree was inferred from 1000 replicates. The *p*-distance method was used to calculate the evolutionary distances.

Morphological Analysis

Samples measuring 1 cm×1 cm were immediately obtained from the midvein and blade area as illustrated in *Figure 7* and processed as previously reported in plant microtechnique and microscopy to produce paraffin blocks.[50] A vibrating HM 340E Rotary

Table 2. List of DNA barcode primers used in this study.

No.	Gene	Forward primer	Reverse primer	Expected size	Ref.
1	*matk*	CGTACAGTACTTTTGTGTTTACGAG	ACCCAGTCCATCTGGAAATCTTGGTTC	846 to 852 bp	[45]
2	*rbcL*	ATGTCACCACAAACAGAGACTAAAGC	CTTCTGCTACAAATAAGAATCGATCTC	654 bp	[46]
3	*trnH-psbA*	CGCGCATGGTGGATTCACAATCC	GTTATGCATGAACGTAATGCTC	500 bp	[25]

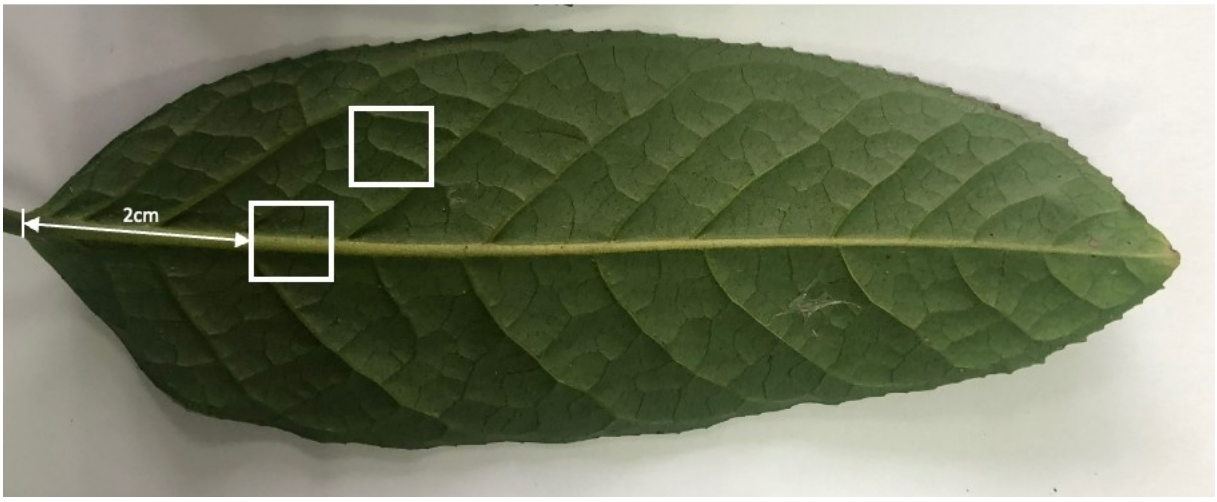

Figure 7. The method of sampling for anatomical analysis.

Microtome (Thermo Scientific, USA) was used to get each 10 μm cross-section, which was spread out on glass plates, and heated to dry at 50 °C for a few hours. After that, the plant tissue-containing slides then were deparaffinized in a 100% xylene solution (Merck, USA), subsequently dried for 1 h, and finally covered with glass slips. The images were observed by using laser light, under a Nikon Eclipse Ni–U microscopy (Nikon, Japan), and cooperated with a DS–Ri2/DS–Qi2 camera (Nikon, Japan).

The white square shows the region of the leaf containing midvein and blade that were collected for further analysis. The following variables were then measured: the area of bundle sheath, the area of xylem, the area of phloem, the area of midvein, the depression angle, the number of abaxial collenchyma cell layers, the leaf thicknesses, the ratio of palisade and spongy. For replication, three leaves from three 3 plant individuals were collected and three cross-sections of each leaf were used for the measurement.

For imaging, NIS-ELEMENTS software provided with the DS–Ri2/DS–Qi2 camera (Nikon, Japan) was used to take all measurements and do all counts.

Chemical Profiling

Total Phenolic Content

The total phenolic content of dry leaf extracts of the above-mentioned Golden Camellias was determined by using colorimetric assay following the published article of Baba and Malik.[51] Briefly, the extracts were dissolved in methanol to a concentration range of 0.4–4 mg/mL. For derivatization, 40 μl of samples were incubated with 480 μl Folin-Ciocalteu agent for 1 min, then added with 480 μl of Na_2CO_3 6%. The reaction was allowed to stand for another 15 min at 40 °C. Parallelly, gallic acid, which was an external standard, was prepared in the same way as for samples in the concentrations of 0.125–2 mg/mL. After incubation, the optical density of each mixture was read by using a spectrophotometer at the wavelength of 765 nm (SpectraMax® iD5 Multi-Mode Microplate Readers, VWR, Germany). The phenolic content was drawn based on the standard curve of gallic acid and the results were displayed as mg of gallic acid equivalent per g dried weight (mgGAE/g).

Total Flavonoid Content

The flavonoid content in the dry leaf extracts was examined by following the article of Chang et al. with slight modifications.[52] The crude extracts were diluted in methanol for the concentrations of 0.5–2 mg/mL. 240 μl of this methanolic extract was mixed well with 40 μl of NaNO2 10% for 6 min. Following that, 40 μl of AlCl3 10% was added to the mixture which was vortexed well for another 6 min. In the final step, 400 μl NaOH 1 M and 280 μl ethanol 30% were added and the reaction was incubated at room temperature for 0.5 h. The calibration curve was constructed by using quercetin with its initial concentrations of 0.25–0.4 mg/mL. The absorbance of these solutions was measured spectrometrically at the wavelength of

510 nm (SpectraMax® iD5 Multi-Mode Microplate Readers, VWR, Germany). The flavonoid content was drawn based on the standard curve of quercetin and the results were displayed as mg of quercetin equivalent per g dried weight (mgQE/g).

Total Anthocyanin Content

The total anthocyanin content was measured following the instruction from Pedro et al.[53] In detail, 1 gram of dry powder leaves was weighed in a 2 ml tube. The sample was added with 1 mL of the solution mixture of citric acid 1 M: ethanol absolute (20:80). The suspension was left at 30 °C for 80 min, then centrifuged at 12000 rpm in 5 min using Centrifuge 5702 (Eppendorf, Germany). The aqueous phase was measured by spectrophotometer at a wavelength of 535 nm (SpectraMax® iD5 Multi-Mode Microplate Readers, VWR, Germany). The total anthocyanin content was calculated by A535 × dilution factor/98.2 and expressed as mg cyanidin-3-glucoside equivalents (CGE) per 100 g dry weight sample (mg CGE/100 g).

Total Chlorogenic Acids Content

The chlorogenic acids content was determined in the fresh leaves of the Golden Camellias by using Dionex UltiMate 3000 HPLC coupled to a diode array detector (DAD) (Thermo Scientific, MA, US) following the method of Magdalena et al. with slight modification.[54] In detail, 10 mg of fresh tissue was weighed and ground into a 2-ml Eppendorf tube by Mixer Mill MM 400 (Retsch, Germany). The tissue was then mixed with 1 ml methanol under ultrasonic for 15 min and centrifuged at 12000 rpm at 4 °C in 5 min by using Centrifuge 5702 (Eppendorf, Germany). About 1 ml of the supernatant was then collected by filtering the extract through a 0.22 μm membrane. 20 μl of samples were injected into an HPLC Column Hypersil GOLD C18 column (250x4.6 mm 5 μm) from Thermo Scientific, MA, US at room temperature. Solvent A (0.1% formic acid in ultrapure water and solvent B (acetonitrile) were used as mobile phase with a gradient (*Table 2S*, *Supporting Information*). Detection was performed with a DAD lamp at 325 nm. The total chlorogenic acids content was calculated by comparing the area of all peaks with a calibration curve established by a range of chlorogenic acid dilution series (with concentrations from 1, 2.5, 5, 7.5, and 10 mg/L).

Catechins Profile

The catechins including (+)-Catechin, (−)-Catechin 3-gallate, (−)-Epicatechin, (−)-Epicatechin-3-gallate, (−)-Epigallocatechin 3-gallate, (−)-Gallocatechin, (−)-Gallocatechin 3-gallate were quantified in plant samples by using ultra-performance liquid chromatography (ACQUITY UPLC H-Class system, Waters Corporation, US) coupled to tandem mass spectrometry (Xevo TQD mass spectrometer equipped with an electrospray ionization (ESI) interface, Waters Corporation, US). In detail, 10 mg of powdered leaves were weighed in 2 ml Eppendorf tubes and extracted with MeOH 80% in 15 min of ultrasonic. The tubes were centrifuged at 10 rpm for 5 min. The supernatants were collected in a syringe and filtered through a 0.22 μm membrane before being injected into the system. The separation was performed on the C18 BEH column (100 mm × 2.1 mm i.d.; 1.7 μm, Waters Corporation, US) at room temperature. Solvent A (0.01% formic acid in ultrapure water) and solvent B (methanol) were used as mobile phases with a gradient (*Table 2S*, *Supporting Information*). For the detector, Electrospray negative ionization source was set up as follows: desolvation temperature of 350 °C, capillary voltage of 2.4 V, extractor voltage of 3 V, RF lens (hexapole) voltage of 2.5 V, nebulization gas flow rate of 650 L/h, cone gas flow rate of 150 L/h, and cone voltage of 35 V. Selected reaction monitoring (SRM) was performed for each catechin with auto dwell time in transitions: 304.97→124.88 (CV 50 V, CE 18 eV), 288.97→108.88 (CV 50 V, CE 28 eV), 457.03→168.88 (CV 46 V, CE 16 eV), 441.03→168.88 (CV 50 V, CE 18 eV). The data were automatically processed by Target lynx (Masslynx 4.1, Waters Corporation, US). The response of each catechin in samples was used as input data for discrimination analysis while the absolute concentration of catechins was calculated based on the calibration curves.

Data Analysis

Generally, each experiment was performed in triplicate. The reported values were calculated as the mean ± standard deviation of three replications. Means for the different variables were compared using Student's t-test for independent samples, with the significance level $P < 0.05$. All calculations were done using Excel (Microsoft Office 2022, US). Graphs were created by using Graphpad prism 9.4.0.

The principal component analysis (PCA) was used to illustrate the data trends. This was implemented by

applying RStudio version 4.2.1[48] by fviz_pca function in *FactoMineR* R-package and visualized by *ggplot2* R-package.[49–50] The reliability of the PCA is assessed based on the sum of the percentage of explained variance. The high level of this sum explained the variation with the better performance.

Acknowledgements

This research was supported by Vietnam Academy of Science and Technology (grant number THTEXS.05/21-24).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Author Contribution Statement

VA. L. T. and N. T. P. M. are equal contributors to this work and designated as co-first authors. They performed the experiments, analyzed the data, and wrote the genetic and morphological diversity part. N. N. P. contributed samples treatment and LCMSMS analysis. KO. N. T analyzed the data related to chemical composition and wrote/edit the whole manuscript. KO. N. T, VA. L. T. and N. T. P. M. conceived and designed the experiments.

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Received September 6, 2022
Accepted December 13, 2022

International Journal of Food Science +Technology

Institute of Food Science +Technology ifst

International Journal of Food Science and Technology 2024

Original article

Widely targeted metabolomics reveals the species-specific, matureness-specific and post-harvest-specific discriminations in the chemical profiles of Vietnamese endemic golden camellias

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(Received 16 January 2024; Accepted in revised form 28 February 2024)

Summary Vietnam is one of the countries with the highest diversity of golden camellias in the world, with more than forty endemic species. However, studies on Vietnamese golden camellias need to be expanded to fill a knowledge gap on these species' chemical profiles and pharmacological properties. This qualitative study focuses on six golden camellias: four endemic species from Tam Dao district, Vinh Phuc province, including *Camellia hakodae*, *Camellia phanii*, *Camellia tamdaoensis*, *Camellia tienii* and two species commonly cultivated in Quang Ninh province including *Camellia petelotii* and *Camellia euphlebia*. A mutual profile of 131 metabolites was obtained by using widely targeted metabolomics analysis using ultra-performance liquid chromatography coupled with tandem mass spectrometry. Comparative metabolomics was performed among leaf extracts of six golden teas, between young and mature leaves and between freeze-drying and oven-drying leaves to discriminate these species, and determine the effect of developmental stage and post-harvesting method on the plant metabolism. The PLS-DA model successfully illustrated a clear separation of the six species and suggested apigenin C-glycoside derivatives as chemical markers for the diversity. Young and mature leaves of all six species also produced distinguished metabolomes, differentiated by mostly amino acid derivatives. The multivariate analysis also indicated the conversion of some flavonoids and amino acids induced by heat in the drying process. These results offer metabolite markers for quality control of Vietnamese golden camellias and recommendations for their usage and preparation.

Keywords *Camellia euphlebia*, *Camellia hakodae*, *Camellia phanii*, *Camellia tamdaoensis*, *Camellia tienii*, *Camellia petelotii*, chemotaxonomy, PLS-DA, post-harvest treatment, UPLC-MSMS, Vietnamese golden camellias, widely targeted metabolomics.

Introduction

Golden camellias are attractive members of the genus *Camellia*, within the Theaceae family. They are distributed popularly in southern China and Vietnam, as sixty-nine species have been found in this area (Trinh, 2022). Among them, forty-six endemic species were listed in the Vietnamese golden tea collection, showcasing a generous land of aspects to explore. The most well-known morphological characteristic that distinguishes these species from other *Camellia* species is the yellowish flowers. For many years, their flowers and leaves have been used not only as a beverage but also in traditional practices for addressing conditions such as dysentery, hypertension, diarrhoea, faucitis, hepatitis with jaundice, liver cirrhosis, sores, malignant tumours and irregular menstruation (He *et al.*, 2018). In the last 10 years, a massive wave of phytochemical and pharmacological investigations on golden camellias has occurred in China, usually focused on a few species, of which *C. nitidissima* was the most concerning (Zheng *et al.*, 2022). Phytochemical profile studies showed that golden camellia is a 'gold' storage of bioactive compounds, including amino acids, polysaccharides, flavonoids, volatile compounds and many other mineral elements. In terms of pharmacological effect,

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doi:10.1111/ijfs.17071

numerous investigations have demonstrated that the crude extracts, fractions, and isolated substances from Chinese golden camellia species possess antioxidant, anticancer, hypolipidemic, hypoglycaemic, anti-allergic, hepatoprotective, neuroprotective, anxiolytic and antidepressant activities (Feng *et al*., 2015; Lu *et al*., 2015). However, a humble number of research on Vietnamese domestic golden camellias has just been conducted recently, warranting more targeted research efforts to broaden the knowledge of these resources and explore a new local source for food, cosmetics and medicine development.

Our study focused on the following prominent golden tea *C. hakodae*, *C. phanii*, *C. tamdaoensis*, *C. tienii* (endemic species of Tam Dao, Vinh Phuc, Vietnam) and *C. euphlebia*, *C. petelotii* (cultivated widely in Hai Ha and Ba Che district, Quang Ninh, Vietnam) (Ninh & Hai-Ninh, 2014). Even though they were taxonomically identified as different species, the morphological appearance of their leaves is highly similar, and they cannot be distinguished without the botanical competencies of experts. In the growing areas of golden camellias from the northern to the central highland, these species are often intermixed without standardised harvesting and post-harvesting conditions, leading to problems in commercialization activities (Do *et al*., 2020). The metabolic discrimination between these species performed by the metabolomics approach plays a vital role in chemotaxonomy and brings the base for determining the particular pharmacological potential of each species, traceability, and geographical indication for quality control practices. In addition, the distinguished metabolite profile of one plant tissue depends also on the growth stage. Unlike green tea, farmers often collect the mature leaves of golden camellias as raw materials for making tea infusions. There is thus a lack of basic knowledge about the comparative metabolome data on different leaf maturity levels of these above-mentioned most consumable golden teas (Veeramohan *et al*., 2023). Otherwise, the effect of processing techniques, that is, thermal conditions, on metabolic changes should be addressed to reveal the best post-harvesting protocol from a more practical point of view (Yang *et al*., 2023). Therefore, we employ a widely targeted metabolomics analysis in this investigation to gain a deep understanding of the metabolomes of six Vietnamese golden camellias for unravelling the metabolic change species-specific, maturity-specific and thermal-processing-specific. This approach significantly improved the number of identified compounds, the reliability of structural elucidation and thus offering insights into quality assurance protocols for golden camellia products (Sawada *et al*., 2009). Through this investigation, we aim to contribute to the developing field of Vietnamese plant resources while exploring new avenues for agricultural products, therefore underlining the significance of our study within both academic and practical domains.

Materials and methods

Plant material

The leaves of endemic golden camellias species: *Camellia hakodae* Ninh (specimen code TDHAK-06-2021, TDHAK-12-2022), *Camellia phanii* Hakoda et Ninh (TDPHA-06-2021, PHA-12-2021), *Camellia tamdaoensis* Hakoda et Ninh (TDTAM-06-2021, TAM-12-2022), *Camellia tienii* Ninh, Tr. (TDTIE-06-2021, TIE-12-2022) were collected at Hop Chau ward, Tam Dao district, Vinh Phuc province (GPS: 21°26′18.0″ N 105°36′17.3″ E) in June and December 2021. In addition, leaves of *Camellia petelotii* (Merr.) Sealy (QNPET-04-2022) and *Camellia euphlebia* Merr. ex Sealy (QNEUP-04-2022) were collected in Quang Minh ward, Hai Ha district, Quang Ninh province (GPS: 21°28′45.9″ N 107°46′07.8″ E) on April 2022. Both leaves, flowers and fruits (if any) were attributed to Dr. Nguyen The Cuong from the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology for plant taxonomy. The voucher specimens were placed in the Department of Life Sciences, University of Science and Technology of Hanoi and were preserved for further investigation. The code, sample type, sample size and other information were provided in the Table 1.

With each species of *C. hakodae*, *C. phanii*, *C. tamdaoensis* and *C. tienii*, mature and young leaves were harvested on 10 plant individuals at the exact location. Indeed, the mature tissues were collected in June, and the young one in December 2021. For *C. petelotii* and *C. euphlebia*, only mature leaves were also collected from 10 individuals on the same farm. The leaves were cut into small pieces of 1 cm along the length of the tissue. With mature leaves, these pieces were equally divided to ensure that the two parts contained homogenous tissues. One part was immersed immediately in nitrogen liquid, stored at −80 °C and freeze-dried in a lyophilisator for 48 h. The remaining tissue was dried in the oven at 45 °C until no change in sample weight was detected. For young tissues, all pieces were treated by oven-drying method. Dried leaves were then ground initially by a rough homogeniser before being weighed for analysis.

Methods

Targeted analysis of catechins and caffeine profile

To target the major group of Camellia genus, a protocol to quantify simultaneously seven catechins (+)-catechin, (−)-catechin 3-gallate, (−)-epicatechin, (−)-epicatechin-3-gallate, (−)-epigallocatechin 3-gallate, (−)-gallocatechin,

Table 1 Sampling information and sample size

Sample code	Species	Sample type	Sample size	Sample location
LGT1.1–1.10	*C. hakodae*	Freeze-drying mature leave	10	Tam Dao, Vinh Phuc
LGK1.1–1.10	*C. hakodae*	Oven-drying mature leave	10	Tam Dao, Vinh Phuc
LN1.1–1.10	*C. hakodae*	Oven-drying young leave	10	Tam Dao, Vinh Phuc
LGT2.1–2.10	*C. phanii*	Freeze-drying mature leave	10	Tam Dao, Vinh Phuc
LGK2.1–2.10	*C. phanii*	Oven-drying mature leave	10	Tam Dao, Vinh Phuc
LN2.1–2.10	*C. phanii*	Oven-drying young leave	10	Tam Dao, Vinh Phuc
LGT3.1–3.10	*C. tamdaoensis*	Freeze-drying mature leave	10	Tam Dao, Vinh Phuc
LGK3.1–3.10	*C. tamdaoensis*	Oven-drying mature leave	10	Tam Dao, Vinh Phuc
LN3.1–3.10	*C. tamdaoensis*	Oven-drying young leave	10	Tam Dao, Vinh Phuc
LGT4.1–4.10	*C. tienii*	Freeze-drying mature leave	10	Tam Dao, Vinh Phuc
LGK4.1–4.10	*C. tienii*	Oven-drying mature leave	10	Tam Dao, Vinh Phuc
LN4.1–4.10	*C. tienii*	Oven-drying young leave	10	Tam Dao, Vinh Phuc
HHLK1.1–1.10	*C. petelotti*	Oven-drying mature leave	10	Hai Ha, Quang Ninh
HHLT1.1–1.10	*C. petelotti*	Freeze-drying mature leave	10	Hai Ha, Quang Ninh
HHLK2.1–2.10	*C. euphlebia*	Oven-drying mature leave	10	Hai Ha, Quang Ninh
HHLT2.1–2.10	*C. euphlebia*	Freeze-drying mature leave	10	Hai Ha, Quang Ninh

(−)-gallocatechin 3-gallate and caffeine in methanolic leave extract of six species were developed. Sample preparation, sample extraction, UPLC-MSMS analysis and data treatment followed the previous procedure mentioned in Nguyen *et al.* (2022).

Widely targeted metabolomics analysis

Dried samples were powdered using a bead-shocker (BMS-M10N21, Biomedical Science, Inc., Japan). Precisely 4 mg of material was weighed into a 2 mL tube containing a 5 mm zirconia bead. Consequently, 1 mL of solvent mixture including 0.1% (v/v) formic acid, 80% (v/v) methanol and two internal standards (8.4 nM of lidocaine for negative ionisation and 210 nM of 10-camphorsulfonic acid for positive ionisation) was adjusted to tube. The extraction was performed in a bead-shocker for 2 min at 2000 × ***g*** (1000 rpm). The supernatant solution was separated from solid contaminants by centrifugation at 20 000 × ***g*** (10 000 rpm) for 1 min.

The extracted solutions were suspended in extract solvent to a final volume of 100 µL. One-quarter of the solution was evaporated, and the residual extract was dissolved with 250 µL LC–MS grade water. The obtained solution was filtered through 0.45 µm membrane (MZHVN0W50; Merck Millipore, Darmstadt, Germany) and 1 µL of the final solution which contains 100 ng of sample was injected to the LC-QqQ-MS system (Nexera MP/LCMS-8050; Shimadzu Corporation).

Widely targeted metabolomics analysis was conducted following Sawada and Uchida et al. protocols (Sawada *et al.*, 2009; Uchida *et al.*, 2020). The MS conditions including cone voltage, collision energy and polarity acquired for authentic reference standards were applied to identify metabolites by automated flow injection analysis using triple quadrupole detector. The peak area values were determined using MRMPROBS (Tsugawa *et al.*, 2013).

Statistical analysis

The Partial Least Square Discrimination Analysis (PLS-DA) was applied to reveal the species-specific, matureness-specific, and post-harvesting-specific discriminations of golden camellias leaves. The scores plots illustrated the difference among chemical profiles of samples and VIP scores plots visualised makers contributing to the discrimination. These plots were conducted using MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca/).

Result and discussion

Metabolomes of golden camellias leaves methanolic extracts

A total of 440 compounds were detected in at least one sample by widely targeted metabolomics analysis. Regarding structural elucidation, this method provided the highest reliability level as the selected reaction monitoring conditions were tuned and optimised with reference standards. The rigour of metabolite identification in this study is 'identified compound' as proposed by Chemical Analysis Working Group (Sumner *et al.*, 2009). After selecting the peaks with signal to noise more than 3 and RSD <30%, and peaks appeared in at least three samples, 131 metabolites were chosen as the mutual profile of 160 extracts. Table 2 shows the identification of these metabolites and their corresponding MS parameters. Interestingly, mainly forty-one

Table 2 Identification of 131 metabolites in golden camellias leaves methanolic extracts by widely targeted metabolomics, followed by the ionisation mode, precursor and product ions used for screening, the RT (retention time) and the classification of each metabolite

ID	Identification	Ionisation mode	Precursor ion	Product ion	RT	Classification
1	(−)Shikimic acid	Negative	173.05	93.1	0.291	Carboxylic acid
2	5-Methylcytosine	Positive	126.05	109.1	0.279	Other
3	Adenine	Positive	136.05	119.05	0.349	Nucleosides
4	Adenosine	Positive	268.1	136.05	1.213	Nucleosides
5	Glycine	Positive	76.05	30.1	0.19	Amino acid
6	L-Histidine	Positive	156.1	110.15	0.188	Amino acid
7	L-(−)-Phenylalanine	Positive	166.1	120.1	1.267	Amino acid
8	L-Tryptophane	Positive	205.1	188.15	1.353	Amino acid
9	L-Tyrosine	Positive	182.1	165.1	0.597	Amino acid
10	Sucrose	Negative	341.1	88.95	0.256	Sugar
11	Leupeptin	Positive	427.3	409.25	1.446	Peptide
12	2-Aminoethylphosphonic acid	Positive	126.05	109.05	0.196	Phosphonic acid
13	Cytidine	Positive	244.1	112.1	0.38	Nucleosides
14	Diethanolamine	Positive	106.1	88.15	0.19	Amines
15	DL-2,3-Diaminopropionic acid	Positive	105.05	88.1	0.178	Carboxylic acid
16	Guanine	Positive	152.05	135.05	0.358	Nucleosides
17	Guanosine	Positive	284.1	152.05	1.259	Nucleosides
18	L-Asparagine	Positive	133.05	87.1	0.193	Amino acid
19	L-Aspartic acid	Positive	134.05	74.05	0.189	Amino acid
20	L-Glutamic acid	Positive	148.05	84.1	0.203	Amino acid
21	L-Glutamine	Positive	147.1	130.05	0.17	Amino acid
22	L-Homocarnosine	Positive	241.15	110.1	0.194	Amino acid
23	L-Ornithine	Positive	133.1	70.15	0.177	Amino acid
24	L-Proline	Positive	116.05	70.15	0.222	Amino acid
25	L-Pyroglutamic acid	Positive	130.05	84.1	0.466	Amino acid
26	L-Serine	Positive	106.05	60.15	0.193	Amino acid
27	3,4-Dihydroxybenzoic acid	Negative	153	109.05	1.336	Phenol
28	Pyridoxine	Positive	170.1	134.05	0.567	Pyridines
29	Succinic acid	Negative	117	73	0.547	Carboxylic acid
30	Uridine	Positive	245.1	113.1	0.733	Nucleosides
31	Uridine-5′-monophosphate	Positive	325.05	97.05	0.329	Nucleosides
32	Alpha-Lactose	Negative	341.1	161.05	0.206	Sugar
33	3-Guanidinopropionic acid	Positive	132.1	72.1	0.234	Carboxylic acid
34	Gamma-Amino-n-butyric acid	Positive	104.05	87.1	0.202	Amino acid
35	Naringenin	Positive	273.1	153	1.629	Flavonoid (flavanone)
36	Apigenin-7-O-glucoside	Positive	433.1	271.05	1.477	Flavonoid (flavone)
37	Rhoifolin	Positive	579.15	271.05	1.459	Flavonoid (flavone)
38	Isorhamnetin-3-O-glucoside	Positive	479.1	317	1.471	Flavonoid (flavonol)
39	Kaempferol-3-O-glucoside	Positive	449.1	287.05	1.47	Flavonoid (flavonol)
40	Homoorietin	Positive	449.1	299.05	1.4	Flavonoid (flavone)
41	Naringenin-7-O-glucoside	Positive	435.15	273.05	1.486	Flavonoid (flavanone)
42	Hyperoside	Positive	465.1	303	1.44	Flavonoid (flavonol)
43	4-Coumaric acid	Positive	165.05	91.1	1.471	Hydroxycinnamic acid
44	Kaempferol-7-O-alpha-L-rhamnoside	Positive	433.1	287.05	1.553	Flavonoid (flavonol)
45	N-Acetyl-D-mannosamine	Positive	222.1	126.1	0.209	Sugar
46	O-Acetyl-L-serine	Positive	148.05	88.1	0.218	Amino acid
47	L-Saccharopine	Positive	277.05	84.05	0.192	Sugar
48	D-Glucoheptose	Negative	209.05	89.05	0.199	Sugar
49	D-(−)-Quinic acid	Negative	191.05	85.05	0.228	Carboxylic acid
50	Chlorogenic acid	Negative	353.1	191.1	1.377	Phenol

Table 2 (*Continued*)

ID	Identification	Ionisation mode	Precursor ion	Product ion	RT	Classification
51	5′-Deoxy-5′-Methylthioadenosine	Positive	298.1	136	1.337	Nucleosides
52	L-allo-threonine	Positive	120.05	56.1	0.198	Amino acid
53	Methyl jasmonate	Positive	225.15	151.1	1.78	Carboxylic acid
54	Vanillin	Positive	153.05	65.15	1.49	Phenol
55	5-Aminovaleric acid	Positive	118.1	55.1	0.255	Carboxylic acid
56	D-Sorbitol-6-phosphate	Negative	261.05	96.95	0.211	Sugar
57	L-Threonic acid	Negative	135.05	75	0.214	Carboxylic acid
58	2,2′,2″-Nitrilotriethanol	Positive	150.1	70.15	0.191	Polyol
59	Luteolin-3′,7-di-O-glucoside	Positive	611.15	287	1.4	Flavonoid (flavone)
60	Luteolin-4′-O-glucoside	Positive	449.1	287.05	1.478	Flavonoid (flavone)
61	Neoeriocitrin	Negative	595.15	151	1.431	Flavonoid (flavanone)
62	Phloridzin	Negative	435.15	273.1	1.5	Flavonoid (dihydrochalcone)
63	Poncirin	Negative	593.2	285.05	1.552	Flavonoid (flavanone)
64	Saponarin	Positive	595.15	283	1.39	Flavonoid (flavone)
65	Vitexin	Positive	433.1	313.05	1.437	Flavonoid (flavone)
66	S-(5′-Adenosyl)-L-methionine	Positive	399.15	250.1	0.21	Amino acid
67	Kaempferol-3-O-alpha-L-arabinoside	Positive	419.1	287.05	1.491	Flavonoid (flavonol)
68	2,5-dihydroxy benzoic acid	Negative	153	108.05	1.407	Phenol
69	Salicylic acid	Negative	137	93.05	1.577	Phenol
70	Trimethylamine N-oxide	Positive	76.1	58.15	0.205	Amines
71	DL-5-Hydroxylysine	Positive	163.1	82.1	0.176	Amino acid
72	L-Anserine	Positive	241.05	109.15	0.184	Amino acid
73	(−)-Riboflavin	Positive	377.15	243.1	1.391	Nucleosides
74	Choline	Positive	104.1	60.15	0.193	Other
75	Kynurenic acid	Positive	190.05	144.05	1.374	Carboxylic acid
76	Trigonelline	Positive	138.05	92.1	0.226	Alkaloid
77	DL-Pipecolinic acid	Positive	130.1	84.15	0.312	Carboxylic acid
78	D-(+)-Raffinose	Negative	503.15	179.05	0.321	Sugar
79	Betaine	Positive	118.1	58.15	0.205	Alkaloid
80	Procyanidin C1	Negative	865.2	125	1.404	Flavonoid (flavan-3-ols)
81	Quercetin-3,4′-O-di-beta-glucopyranoside	Negative	625.15	463.1	1.396	Flavonoid (flavonol)
82	E-3,4,5′-trihydroxy-3′-glucopyranosylstilbene	Negative	405.1	243	1.412	Stilbenoid
83	Esculin	Positive	341.1	179.05	1.366	Coumarin
84	Stachyose	Negative	665.2	383.05	0.305	Sugar
85	L-Canavanine	Positive	177.1	76.1	0.184	Amino acid
86	L-Leucine	Positive	132.2	30.1	0.514	Amino acid
87	L-Isoleucine	Positive	132.1	69.1	0.528	Amino acid
88	Methionine sulfoxide	Positive	165.95	74.05	0.196	Amino acid
89	Aureusidin	Positive	287.05	153.05	1.473	Flavonoid (aurone)
90	Glutamine	Positive	147.1	84.1	0.18	Amino acid
91	Isorhamnetin-dihexose	Positive	625.2	317.05	1.453	Flavonoid (flavonol)
92	Kaempferol-dihexose	Negative	593.15	285.05	1.451	Flavonoid (flavonol)
93	Cyanidin-3,5-dihexose	Positive	611.15	287.05	1.303	Flavonoid(anthocyanin)
94	Kaempferol-3,7-dihexose	Negative	577.15	285	1.431	Flavonoid (flavonol)
95	Kaempferol-3-O-alpha-L-rhamnoside	Negative	431.1	285	1.517	Flavonoid (flavonol)
96	Maritimein or luteolin-7-O-glucoside	Positive	449.1	287.05	1.44	Flavonoid (flavone)
97	Quercetin-3-Arabinoside or Quercetin-3-D-xyloside	Positive	435.1	303.05	1.473	Flavonoid (flavonol)

Table 2 (*Continued*)

ID	Identification	Ionisation mode	Precursor ion	Product ion	RT	Classification
98	Quercetin-3-Rhamnoside or Quercetin-3-O-alpha-L-rhamnopyranoside	Negative	447.1	300	1.481	Flavonoid (flavonol)
99	Myricitrin or Myricetin-3-Rhamnoside	Negative	463.1	316.15	1.446	Flavonoid (flavonol)
100	(+)-Catechin or (+)-Epicatechin	Positive	291.1	139	1.417	Flavonoid (flavan-3-ols)
101	Sodium pantothenate or D-Pantothenic acid or Calcium (+)-pantothenate	Positive	220.1	90.1	1.322	Polyol
102	D(+)-Galactosamine or D-(+)-Glucosamine	Positive	180.1	72.1	0.182	Sugar
103	D-(+)-Cellobiose or Lactulose	Negative	341.1	161.1	0.232	Sugar
104	Melibiose or D-(+)-Turanose or Isomaltose or Gentiobiose or Melibiose Palatinose	Negative	341.1	179.1	0.214	Sugar
105	L-Iditol or D-Sorbitol or D-(−)-Mannitol	Positive	183.1	69.1	0.202	Sugar
106	L(+)-Arginine or N-alpha-Acetyl-L-ornithine or L-Citrulline	Positive	175.1	70.15	0.187	Amino acid
107	Histamine or Cytosine	Positive	112.1	95.15	0.173	Nucleosides
108	Nicotinamide or Niacinamide	Positive	123.05	80.1	0.409	Pyridines
109	L-Alanine or Sarcosine	Positive	90.05	44.1	0.195	Amino acid
110	L-Norvaline or L-Valine	Positive	118.1	72.1	0.291	Amino acid
111	L-Threonine or alpha-Methyl-DL-serine or L-Homoserine	Positive	120.05	74.1	0.198	Amino acid
112	L-2-Aminobutyric acid or N, N-Dimethylglycine or N-Methyl-DL-Alanine	Positive	104.05	44.15	0.201	Amino acid
113	(S)-(+)-1-Aminoethylphosphonic acid or Taurine	Positive	126.05	44.15	0.197	Amino acid
114	DL-Malic acid	Negative	133	115	0.287	Carboxylic acid
115	L-Carnitine	Positive	162.1	60.2	0.204	Amino acid
116	Adipic acid or 2-Methylglutaric Acid	Negative	145.05	83	1.36	Carboxylic acid
117	(−)-Citramalic acid	Negative	147.05	87	0.576	Carboxylic acid
118	L-Lysine	Positive	147.1	84.1	0.18	Amino acid
119	Nicotinic acid or Isonicotinic acid	Positive	124.05	78.05	0.355	Carboxylic acid
120	Rutin or Quercetin-3-O-b-glucopyranosyl-7-O-a-rhamnopyranoside	Negative	609.15	300	1.432	Flavonoid (flavonol)
121	D-(+)-Melezitose or 1-Kestose	Negative	503.15	89	0.348	Sugar
122	Cyanidin 3-(6″-malonylglucoside)	positive	535.1	287.1	1.334	Flavonoid (anthocyanin)
123	Schaftoside	Positive	565.15	547.05	1.391	Flavonoid (flavone)
124	(−)-Epigallocatechin	Positive	307.05	139.05	1.302	Flavonoid (flavan-3-ols)
125	(−)-Gallocatechin	Positive	307.05	139.05	1.302	Flavonoid (flavan-3-ols)
126	Delphinidin 3-Rutinoside	Positive	612.15	304	1.337	Flavonoid (anthocyanin)
127	Delphinidin 3-Galactoside	Negative	464.1	301.05	1.33	Flavonoid (anthocyanin)

Table 2 (*Continued*)

ID	Identification	Ionisation mode	Precursor ion	Product ion	RT	Classification
128	Cyanidin 3-Galactoside	Negative	448.1	285	1.344	Flavonoid (anthocyanin)
129	Vicenin-1	Positive	565.15	427.15	1.356	Flavonoid (flavone)
130	Vicenin-2	Negative	593.15	353.05	1.337	Flavonoid (flavone)
131	Vicenin-3	Positive	565.15	547.15	1.358	Flavonoid (flavone)

flavonoids and thirty-two amino acids dominate the profiles of golden camellias samples.

Forty flavonoids detected in these samples correspond to different aglycone skeletons such as anthocyanin, aurone, chalcone, flavan-3-ol, flavanone, flavone and flavonol. Among them, flavonols dominated the flavonoid profile with fourteen diverse glycosides of kaempferol (**39**, **44**, **67**, **92**, **94**, **95**), quercetin (**42**, **81**, **97**, **98**, **120**), isorhamnetin (**38**, **91**) and myricetin (**99**). All flavonols are oxygenated glycosides, usually in the position 3 and/or 7, which are attached to glucose, rhamnose, arabinose, or rutinose. Besides, flavones are the second most abundant group in the profile with twelve representatives. Most flavones detected in tested samples are apigenin derivatives (**36**, **37**, **65**, **123**, **129**, **130**, **131**) and luteolin (**40**, **59**, **60**, **96**). Apigenin glycosides consist of 7-O-glycosides of glucose and neohesperidose. Interestingly, five flavonoids contain an apigenin skeleton belonging to C-glycosides at the position of 6 and/or 8. Indeed, homoorientin brings one hexose while schaftoside, vicenin 1, vicenin 2 and vicenin 3 are dihexose glycosides. Flavanones are aglycones and O-glycosides of naringenin, eriodictyol and isosakuranetin at position 7 of the skeleton (**35**, **41**, **61**, **63**). Anthocyanins are mostly oxygenated derivatives of cyanidin and delphinidin at position 3 and 5 (**93**, **122**, **126**, **127**, **128**). Notably, these flavonoids were commonly well-known for numerous health benefits as antioxidant, anti-inflammatory, anti-microbial and anti-cancer activities (Zhang *et al*., 2020; Aumeeruddy & Mahomoodally, 2021; Safe *et al*., 2021). The complication in the structure of aglycons and the abundant combinatorial manner between these skeletons with numerous glycoside units bring a rich diversity in flavonoid profiles of golden camellias.

The catechin profiles of golden camellias leaf extracts are dominated by three flavan-3-ols, including catechin/epicatechin (**100**), epigallocatechin (**124**), and gallocatechin (**125**) in widely targeted metabolomes. Catechin 3-gallate, epicatechin-3-gallate, and gallocatechin 3-gallate were detected in not many samples, even though these catechins were abundant in *C. sinensis* leaf extracts (Nguyen *et al*., 2022). In previous study, gallocatechin was not detected in *C. murauchii*, *C. impressinervis*, *C. euphlebia*, *C. tunghinensis*, *C. nitidissima* var. microcarpa, and *C. nitidissima* (Lin *et al*., 2013). This method cannot distinguish catechin and its epi form; therefore, to have an overview of the distribution of each catechin in the profile, a targeted analysis was conducted, focusing on caffeine and seven catechins in 160 samples (Table S1). Similarly, the targeted analysis demonstrated that catechin and epicatechin were abundant in these extracts, followed by gallocatechin, while catechin 3-gallate, epicatechin-3-gallate, epigallocatechin and gallocatechin 3-gallate were minor. More interestingly, caffeine, a commonly detected compound in green tea, was under the detection limit in all tested golden camellias by both methods, targeted and widely targeted. This result suggests that these teas may not stimulate the central nervous system and does not cause insomnia, thus can be suitable to make an alternative herbal tea for evening consumption. This observation is consistent with other studies on the chemical profile of other golden camellias such as *C. chrysantha* (Bach, 2020), on Chinese species, including *Camellia impressinervis*, *C. euphlebia*, *C. microcarpa*, *C. nitidissima*, and *C. tunghinensis* (Song *et al*., 2011; Lin *et al*., 2013).

Other nitrogen-containing compounds, such as trigonelline (76) and betaine (79), also contribute to the chemical profile of studied golden camellias. In previous research, these substances showed attractive therapeutic potential for antidiabetic, anticancer, and neuroprotective effects, thus offering a good source of nutrients for golden tea products (Ueland, 2011; Liang *et al*., 2023).

Besides, a number of phenols, not limited to benzoic acid and hydrocinnamic acid derivatives, coumarin and stilbenoid, were detected in these extracts. Vanillin (**54**), 3,4-dihydroxybenzoic acid (**27**), chlorogenic acid (**50**), 2,5-dihydroxy benzoic acid (**68**), salicylic acid (**69**), 4-coumaric acid (**43**), esculin (**83**) and E-3,4,5′-trihydroxy-3′-glucopyranosylstilbene (**82**) are biologically important phenolic compounds present in many plant species. They are well-known for various biological properties such as antioxidant, anti-bacterial, anti-inflammatory, anti-obesity and antidiabetic activities and improve cardiovascular function (Kabir *et al*., 2014; Li *et al*., 2022; Wang *et al*., 2022).

Amino acids are bioactive compounds in green tea *C. sinensis*, which contribute to the taste and flavour of tea products and pharmacological effects on multiple targets (Hung *et al*., 2010; Xu *et al*., 2020). Plants

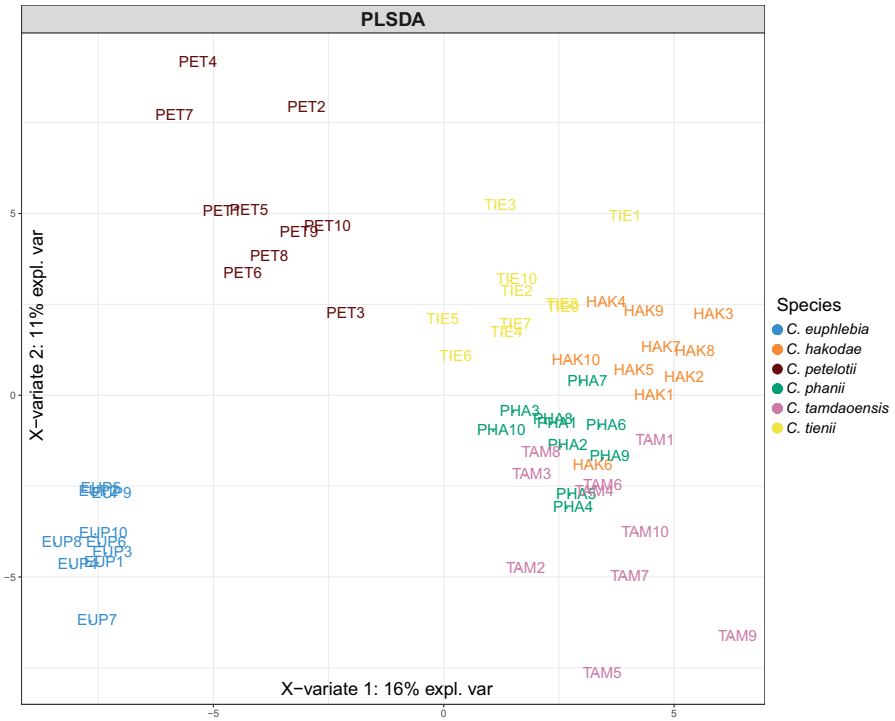

Figure 1 PLS-DA score plot of metabolomes produced by the methanolic extract of the oven-drying leaves of six Vietnamese golden camellias.

have two main types of amino acids: amino acids released from protein and free amino acids. In our work, the widely targeted analysis revealed the presence of thirty-two free amino acids in the library of PRIMe for all samples. GABA, glutamine and glutamic acid were mainly found in the methanolic extract of golden camellias. These amino acids are analogues and are considered responsible for the umami taste of tea (Kaneko *et al.*, 2006). The combination of these components can trigger neuroprotective effects, especially against the stimulatory effect of caffeine in the central nervous system (Yoneda *et al.*, 2019; Wang *et al.*, 2022). In addition, certain amino acids were considered precursors of flavonoid biosynthesis, that is, phenylalanine, tryptophan and tyrosine, by presenting a significant role in phenolics accumulation (Wang *et al.*, 2020). The wide range of amino acid profiles, which is relevant to flavonoid metabolism, is a typical feature of *Camellia* species and is conserved in golden teas.

Other primary metabolites, that is, amines, carboxylic acid, nucleosides, polyol, pyridines derivatives or sugar, were also observed in these extracts, contributing to the physicochemical properties and the taste and flavour of golden tea products. Overall, the comprehensive metabolome data revealed by the widely targeted metabolomics approach indicated that this method is suitable for high-throughput descriptions of golden camellias, and this complex data could be mined from many perspectives.

Species-specific discrimination of golden camellias leaves methanolic extracts

The semiquantitative profile of 131 peaks identified in the methanolic extract of oven-drying mature leaves of six species *C. hakodae*, *C. phanii*, *C. tamdaoensis*, *C. tienii*, *C. euphlebia* and *C. petelotii* were subjected to PLS-DA analysis after normalisation process with internal standards. Figure 1 shows the score plot of PLS-DA with the combination of two first components, which explained 27% of the total data variance. In the plot, there is a clear discrimination observed among three species (*C. euphlebia*, *C. petelotii* and *C. tienii*) while the other three species were moderately clustered (*C. tamdaoensis*, *C. phanii* and *C. hakodae*). From an applied point of view, this graph demonstrated that the leaves of these species are well distinguished in terms of chemical profiles, even though their appearance is quite similar. Otherwise, a shared region between *C. phanii* and *C. tamdaoensis* exists, indicating that these two leaves produce closer metabolomes. *C. hakodae*'s leaves were well grouped in the plot excepted one point HAK6; however, this cluster is near *C. tienii* and

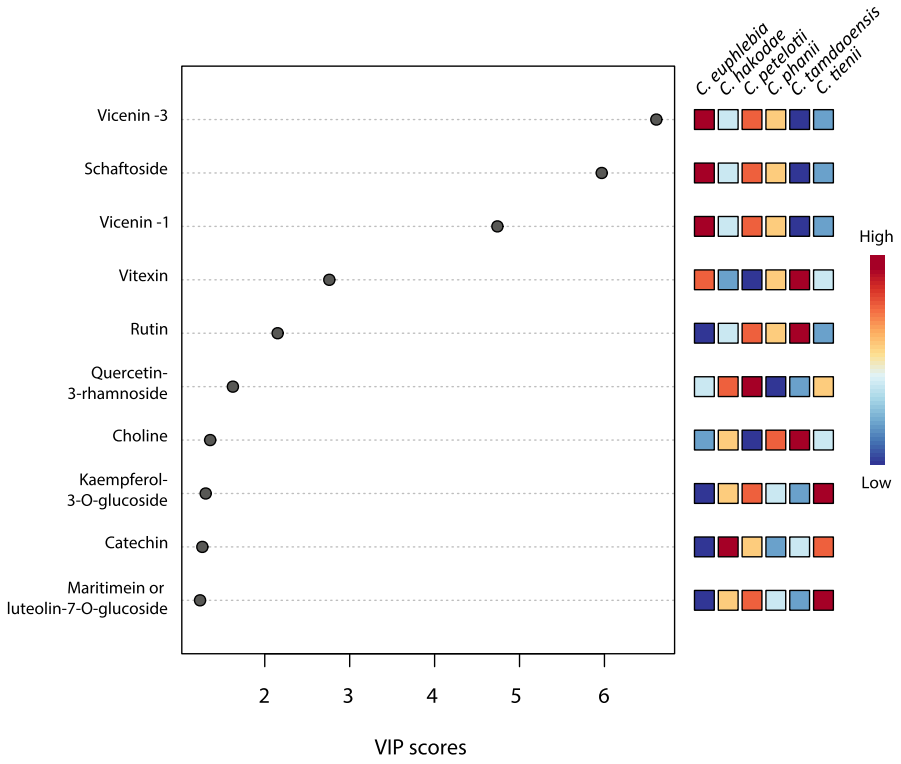

Figure 2 The VIP scores of differential metabolites contributing the most to discriminate chemical profiles of six golden camellias leaves.

C. phanii samples. The chemical difference is consistent with the neighbour-joining phylogenetic tree using *matk*, *rbcL*, and *trnH-psbA* genes built for *C. phanii*, *C. tamdaoensis*, *C. petelotii*, *C. tienii* and *C. euphlebia* in our previous article (Le *et al*., 2023). In this phylogeny tree, *C. phanii* and *C. tamdaoensis* were close in the first branch, whereas *C. tienii* and *C. euphlebia* were located apart in the second branch. Significant similar diversity between genotypic and metabolic profiles of the investigated golden camellia species suggested that the analysis method thus can be used for the chemotaxonomy of these golden camellias and provide deep insight into bioactive compounds in these plant materials.

Looking at the chemical markers that contribute to the PLS-DA model, we selected ten metabolites representing the most important VIP scores include vicenin-3 (**131**), schaftoside (**123**), vicenin-1 (**129**), vitexin (**65**), rutin or quercetin-3-O-beta-glucopyranosyl-7-O-alpha-rhamnopyranoside (**120**), quercetin-3-rhamnoside or quercetin-3-O-alpha-L-rhamnopyranoside (**98**), choline (**74**), kaempferol-3-O-glucoside (**39**), (+)-catechin or (+)-epicatechin (**100**), maritimein or luteolin-7-O-glucoside (**96**). Of the ten compounds, there are nine flavonoids, suggesting that this group is responsible for chemical discrimination of these six golden camellias. Figure 2 also shows the relative content of these differential metabolites in six species by colour. Herein, three flavones vicenin-3 (**131**), schaftoside (**123**) and vicenin-1 (**129**), which are all C-glycoside of apigenin, were accumulated with discriminative levels in the decreasing order from the high to low content of *C. euphlebia* > *C. petelotii* > *C. phanii* > *C. hakodae* > *C. tienii* > *C. tamdaoensis*. Supportively, vitexin, another flavone that is also a C-glycoside of apigenin, appeared as the fourth compound in the VIP scores figure. Otherwise, rutin or quercetin-3-O-beta-glucopyranosyl-7-O-alpha-rhamnopyranoside (**120**), quercetin-3-rhamnoside or quercetin-3-O-alpha-L-rhamnopyranoside (**98**) and kaempferol-3-O-glucoside (**39**) are three O-glycosides of flavonol aglycon also contribute to the distinguish of six leaf extracts profile. These observations may support a hypothesis that the metabolic pathway of flavonoid derivatives is enormously diverse among these investigated golden camellias and, particularly in the case of apigenin C-glycosides, a series of natural products with numerous interesting pharmacological effects (Ali *et al*., 2020; Mutha *et al*., 2021). Thus, the biosynthetic pathway of apigenin C-glycosides may be the key differentially regulated pathway contributing to both chemical and biological activity discrimination of these species.

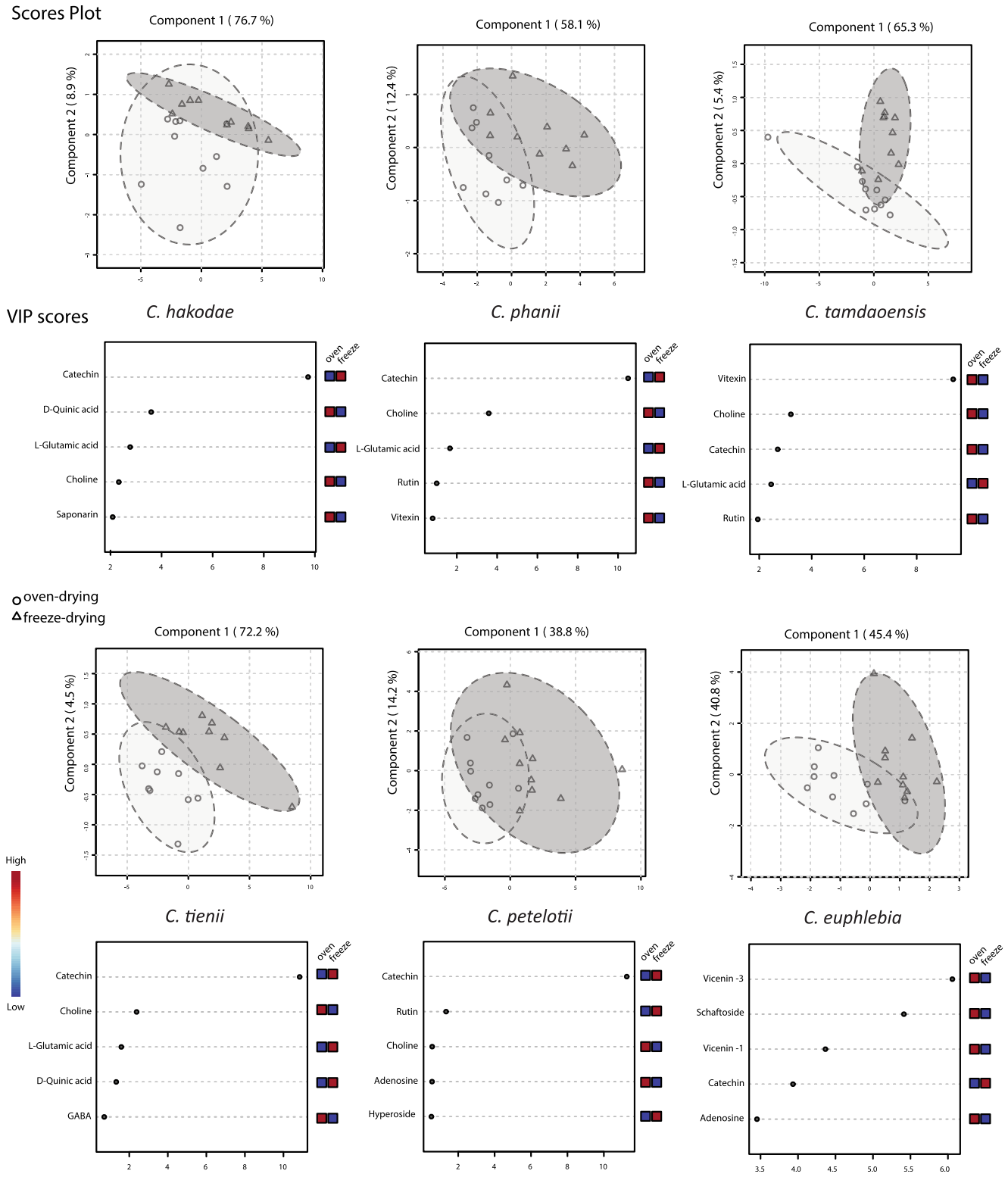

Figure 3 The PLS-DA scores plots distinguish the metabolomes of oven-drying and freeze-drying leaves of *C. hakodae*, *C. phanii*, *C. tamdaoensis*, *C. tienii*, *C. petelotii*, *C. euphlebia* and VIP scores of differential metabolites contributing to the discrimination.

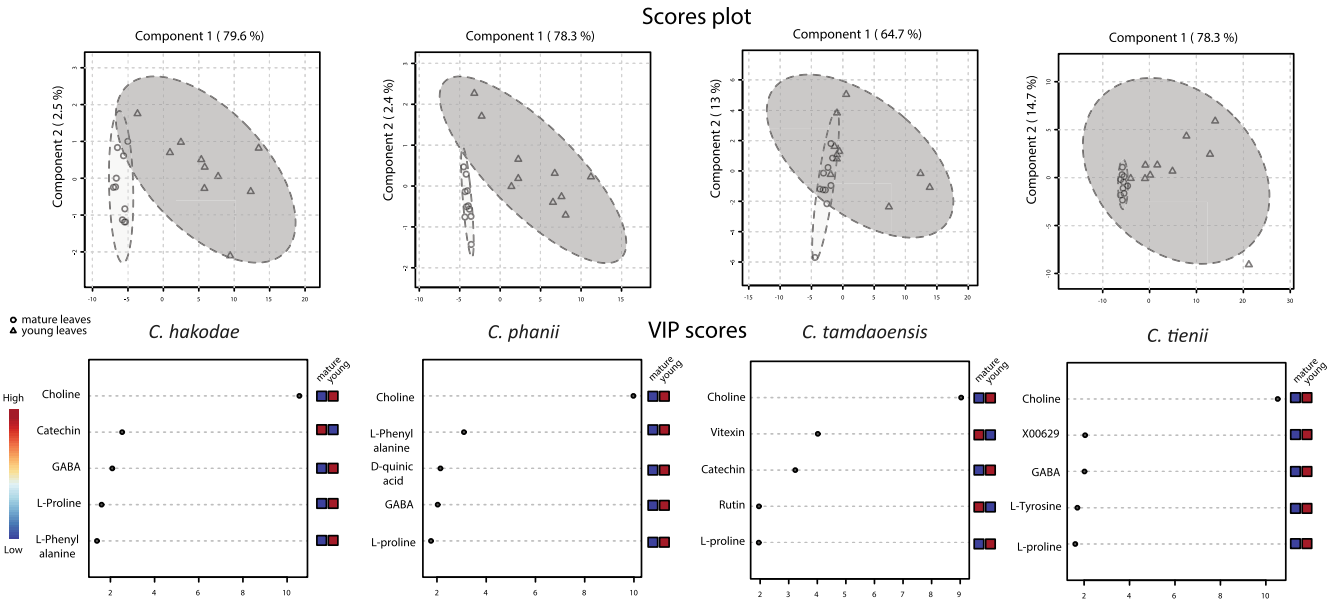

Figure 4 The PLS-DA scores plots distinguish the metabolomes of mature and young leaves of *C. hakodae*, *C. phanii*, *C. tamdaoensis*, *C. tienii* and VIP scores of differential metabolites contributing to the discrimination.

Post-harvest-specific discrimination

To explore the conversion of metabolites according to the post-harvesting treatment, we examined the variation in the relative abundance of 131 compounds between freeze-drying and oven-drying samples by widely targeted analysis. In all species, the PLS-DA model showed a clear separation between two groups with a very high % explaining data variance (Fig. 3), which indicates the significant impact of drying method on the chemical profiles of these leaf materials.

Regarding the metabolites responsible for the discrimination between two processing conditions, Fig. 3 of the VIP scores plot illustrates five key differential accumulated compounds of each species. Overall, six species shared some common metabolite markers such as catechin/epicatechin (**100**), L-glutamic acid (**20**), choline (**74**), rutin or quercetin-3-O-b-glucopyranosyl-7-O-a-rhamnopyranoside (**120**). In detail, catechin or epicatechin accumulated in a significantly higher content of freeze-drying samples of most leaves, including *C. hakodae*, *C. phanii*, *C. tienii* and *C. petelotii*. In contrast, *C. tamdaoensis* produces more of this component in oven-drying conditions. Regarding the content of sole catechin, this compound accumulated with a higher level in oven-drying conditions for five out of six leaves except *C. euphlebia* (Figure S1). Inversely in the case of epicatechin, drying by lyophillisation method led to producing a larger amount of epicatechin than by oven except *C. tamdaoensis* (Figure S2). Indeed, the formation of catechin and epicatechin within the *Camellia* genus has been proposed to happen by distinct enzymatic reactions, leucoanthocyanidin reductase and anthocyanidin reductase, respectively, utilising different substrates, leucocyanidin and cyanidin, respectively (Punyasiri *et al.*, 2004). Following the synthetic pathway, both catechin and epicatechin act as precursors of proanthocyanidins, a class of flavonoid. The observation suggested that temperature may induce the enzymes involving in the catechins pathway and/or alter the abundance of products. Consequently, the thermal processing could influence the biosynthesis or conversion of catechins into flavonoids, the prominent group of the *Camellia* genus, and this effect is species-specific difference.

L-glutamic acid, an amino acid that affects the taste of tea infusion, was also revealed to exist at a higher level in freeze-dried materials of *C. hakodae*, *C. phanii*, *C. tamdaoensis*, and *C. tienii*. Inversely, choline (**74**), an intermediate product of amino acids was found with a more considerable content in the oven-drying samples of *C. hakodae*, *C. phanii*, *C. tamdaoensis*, *C. tienii* and *C. petelotii*. While the human body can synthesise this component, an additional amount is still required in our diet for improving physiological function (Ueland, 2011). Hence, the up-regulation of this active agent in temperature-based processing samples suggests a suitable preparation method to increase the creation of choline in the product. These contrasting trends indicate the need for different thermal processing methods tailored to specific products to enrich bioactive compounds.

Notably, rutin or quercetin-3-O-b-glucopyranosyl-7-O-a-rhamnopyranoside (**120**) represented the inverse regulated trend in different species. On one side, the accumulation of this compound increases in oven-drying samples of *C. phanii* and *C. tamdaoensis*; however, it is down-regulated in *C. petelotii*. Some active flavonoids, such as saponarin (**64**), vitexin (**65**), vicenin-1 (**129**), vicenin-3 (**131**) and schaftoside (**123**) were also up-regulated in oven-drying samples in *C. hakodae*, *C. phanii*, *C. tamdaoensis* and *C. euphlebia*. That proposed a hypothesis on the impact of thermal conditions on the transformation of these apigenin C-glycoside derivatives that were reported with remarkable health benefits. The heat can induce the formation of these compounds, an issue that needs to be taken into account in post-harvesting practice.

Traditionally, leaves of golden camellias were usually dried naturally under the atmospheric ambiance or under the sunlight, which is more similar to oven-drying conditions. The observation of this study provides a good suggestion for the specific post-harvesting processing method for these materials corresponding to different purposes.

Matureness-specific discrimination

The difference in chemical composition between young and mature leaves of green tea *C. sinensis* was revealed in much previous research (Lin *et al.*, 1996; Chen *et al.*, 2003; Nguyen *et al.*, 2022). In agricultural activities, young buds of green tea were normally collected because they are rich in antioxidant catechins. Herein, we examined the widely targeted metabolomes of young leaves and mature leaves of the same plant individual plants *C. hakodae*, *C. phanii*, *C. tamdaoensis* and *C. tienii*. Under the same preparation process, the profiles of these methanolic extracts were subjected to PLS-DA model. For all species, the scores plot of PC1 and PC2 indicated visible separation between young and mature samples with a significant percentage of explained data variance (Fig. 4). These results indicate a considerable variation of metabolites based on the matureness of the leaf.

Regarding the five key metabolites that represented the most important VIP scores in the PLS-DA components of each species (Fig. 4), amino acids and derivatives were dominant the figures. Indeed, choline contributed the most to the metabolic difference between young and mature leaves for all studied species with very high scores (more than 10). This metabolite was produced with a significantly greater amount in young leaves of all four species. Other amino acids, GABA (**34**), L-proline (**24**), L-phenylalanine (**7**) and L-tyrosine (**9**), were also up-regulated accumulation in young leaves of these species. These amino acids are well known for their activity on the central nervous system and other human physiological functions (Magro *et al.*, 2016; Hinton *et al.*, 2019; Wang *et al.*, 2022) and also affect much on the taste and flavours of the *Camellia* genus (Scharbert & Hofmann, 2005).

In contrast, flavonoids, including rutin or quercetin-3-O-beta-glucopyranosyl-7-O-alpha-rhamnopyranoside (**120**), vitexin (**65**) and catechin/epicatechin (**100**) existed at a higher level in mature leaves than young ones of *C. tamdaoensis* and *C. hakodae*, respectively. Indeed, the targeted analysis showed that the content of sole catechin was produced much more by mature leaves than by young leaves in four species, while this trend is not significant in the case of epicatechin (Figures S3 and S4). Regarding the common flavonoid pathway, each class of anthocyanins, flavonols and flavan-3-ols is biosynthesised by enzyme reactions and their precursors are amino acids such as L-phenylalanine. The regulation in flavonoid biosynthesis was described at different developmental stages in green tea *C. sinensis*, indicating the differential expression of some enzymes between young and mature leaves like cinnamic acid 4-hydroxylase, flavanone 3-hydroxylase, flavonoid 3′,5′-hydroxylase, anthocyanidin reductase, and leucocyanidin reductase (Li *et al.*, 2015). The enzymatic reactions that regulate the conversion of three differential components should be targeted in further research to reveal the global mechanism of this metabolic change.

The observation is logically consistent with plant physiology because the young leaf contains more primary metabolites while the older produces more secondary metabolites. However, golden camellias represented an opposite regulation to green tea leaves in the biosynthesis of catechins. Therefore, the young leaves and buds are selected for processing green tea-related products. In contrast, in the case of golden tea, people traditionally utilise the mature leaves for consumption. This result also explains the different tastes between immature and mature leaf infusions and may suggest the other pharmacological profiles of these two types. Moreover, the results highlight the effect of the growth development stage of tissue on the bioactive component production. From an applied point of view, this can facilitate the optimisation of harvest time in agricultural activities.

Conclusion

This investigation successfully conducted for the first time a comprehensive metabolomics analysis to observe the chemical profile of six Vietnamese golden camellias. A total of 131 metabolites were characterised in 160 methanolic extracts of leaves samples with a high level of structural elucidation that provides a good source of information for species discrimination, then can be used for chemotaxonomy. Flavonoids, that is, apigenin

C-glycosides may contribute to the chemical diversity of these six species. Otherwise, our results showed a clear separation between the clusters of oven-drying and freeze-drying leaves and young and mature leaves on the PLS-DA score plots. Catechin and epicatechin, L-glutamic acid, choline and some active flavonoids were accumulated with distinct trends in six species with or without thermal treatment. While some amino acids were up-regulated in young leaves, flavonoids were found with higher content in mature leaves. This suggests an undeniable effect of preparation treatment and harvest time on the metabolite composition, which decides these materials' taste, nutritional and pharmacological properties.

Acknowledgments

This research was supported by Vietnam Academy of Science and Technology (grant number THTEXS.05/21-24).

Author contributions

Phuong Nhi Nguyen: Methodology; writing – original draft. **Thi Yen Do:** Formal analysis; visualization. **Thi Nhung Do:** Formal analysis; visualization. **Eric Gontier:** Writing – review and editing; supervision. **Ha Trang Le Nguyen:** Writing – original draft. **Van Anh Le Thi:** Resources; investigation. **Nga T.P. Mai:** Resources; investigation. **Muneo Sato:** Methodology; data curation. **Masami Yokota Hirai:** Methodology; data curation. **Kieu Oanh Nguyen Thi:** Conceptualization; funding acquisition; project administration; writing – original draft; writing – review and editing; supervision; resources; visualization.

Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Ethical approval

There is no animal and human subjects in this article.

Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ijfs.17071.

Data availability statement

All data available within the article or its supplementary materials.

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This paper gave the good indication for metabolite identification by showing the chemical component of *C. nitidissima*, an example of Chinese golden camellias.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Boxplots of catechin content in oven-drying (left) and freeze-drying (right) leaves of six golden camellias.

Figure S2. Boxplots of epicatechin content in oven-drying (left) and freeze-drying (right) leaves of six golden camellias.

Figure S3. Boxplots of catechin content in mature leaves (left) and young leaves (right) of six golden camellias.

Figure S4. Boxplots of epicatechin content in mature leaves (left) and young leaves (right) of six golden camellias.

Table S1. Catechins profile of 160 golden camellias extracts revealed by the area under the curve of catechin (C), epicatechin (EC), gallocatechin (GC), catechin 3-gallate (CG), epicatechin-3-gallate (ECG), gallocatechin 3-gallate (GCG), epigallocatechin 3-gallate (EGCG).

Chem. Biodiversity

A Comprehensive Strategy for Metabolites Profiling of Flowers and Leaves from *Camellia tienii*, an Endemic Golden Tea of Vietnam

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Abstract: Golden camellia is defined as a species of the *Camellia* genus with yellow flowers, which have long been used as a medicine, food, and cosmetic in many Asian countries. To date, more than 50 golden camellia species are considered endemic in Vietnam; however, more information is needed about its chemical constituents and biological activity. This work aims to unveil the potential of *Camellia tienii* Ninh, a golden camellia species, as an herbal beverage by examining the presence and abundance of chemical components in flowers and leaves. A comprehensive strategy has been developed using both liquid and gas chromatography coupled with mass spectrometry. Specifically, LC-MS-based widely targeted analyses were opted to characterize 158 polar metabolites belonging mainly to flavonoids, catechins, and amino acids classes, and an untargeted approach using GC-MS annotated 42 major volatile compounds such as terpenes and fatty acids. The extensive profile revealed by these techniques could help understand the significant discrimination between two organs. *C. tienii* flowers accumulated more flavonoids, amino acids, and fatty acids, while leaves contain more terpenes, suggesting different pharmacological properties of these materials. Overall, this pipeline can be applied for other *Camellia* species and valorization of these valuable resources for health benefits purposes.

Keywords: amino acids • *Camellia tienii* • catechins • flavonoids • mass spectrometry

Introduction

Tea, the processed leaves of *Camellia* species, is the second most commonly consumed beverage worldwide, next to water.[1] Among diverse tea species, golden camellias (yellow camellias) have been lauded as precious resources in Vietnam for beverage, cosmetic, and traditional medicine, as numerous golden camellias were recorded as endemic species of this country.[2][3] Unlike green tea, both flowers and leaves in fresh and dry forms of golden camellias have long been traditionally used to prevent and treat hypertension, obesity, sore throat, dysentery, diarrhea, faucitis, and irregular menstruation.[4] These materials represented multiple health benefits in previous investigations, including antioxidant activity,[5][6] anticancer,[7][8] blood pressure lowering, triglyceride and cholesterol-lowering, and preventing atherosclerosis.[9][10] Recently, golden camellias have attracted increasing attention in the cosmeceutical industry, with a variety of commercialized products from their leaf and flower such as golden silk oil, organic golden camellia oil, and facial cream.[11]

Regarding chemical composition, emerging evidence reveals many bioactive compounds, including flavonoids, saponins, polysaccharides, amino acids, and volatile compounds from golden camellia.[11] However, a comprehensive profiling of primary and secondary metabolites in these species still needs to be improved. A complete set of chemical compounds in leaves and flowers could be a good source of information that contributes to explaining the pharmacological effects of these materials.

Metabolomics approaches, including untargeted and targeted metabolomics, aim to measure as many small molecules within an organism as possible. They are often used in combination for the discovery and accurate content determination of metabolites.[12] Targeted analysis characterizes defined groups of compounds.[13] Untargeted analysis comprehensively and systematically analyzes all the measurable analytes in a sample,

including unknown metabolites. Both of these approaches are thus very necessary to provide information about the comprehensive chemical profile of plant tissues because, until now, no ideal universal technique could reach this purpose.

Metabolomics analysis typically requires a variety of specialized techniques. Indeed, an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system with an electrospray ionization (ESI) source has been widely used as an efficient method to determine specific elements in tea. Rapid LC can lower solvent consumption, making the procedure more repeatable, reproducible, and sensitive. This method furthermore reduces the risk of compound degradation, which is specifically suitable for investigating a thermally sensitive compound like catechins (the main bioactive component in tea).[14] Another interesting group compound in tea is amino acids, which can be characterized by UPLC-MS/MS by adding more special post-column derivatization.[15] The hyphenated system of UPLC-MS/MS is also very powerful for widely targeted metabolomics analysis in which a large number of references were injected into the system, turning it into an identification tool by quantifying each compound within the list of references.[16] Besides, gas chromatography-mass spectrometry (GC-MS) is a relatively low-cost but high-throughput detection technique (with high resolution, high sensitivity, and strong identification ability) that is more suitable for untargeted volatile compounds profiling.

Herein, we aim to develop an analytical strategy to examine the presence and abundance of compounds in flowers and leaves (the two most common organs to be used for tea production) of golden camellias, applied firstly for *Camellia tienii* Ninh (*C. tienii*), one of the endemic species cultivated explicitly in Tam Dao district, Vinh Phuc province, Vietnam. This species' tea leaf and flower samples were exposed to untargeted analysis for volatile compounds using gas chromatography-mass spectrometry (GC-MS). Additionally, targeted analysis utilizing liquid chromatography-mass spectrometry (LC-MS) opted to characterize catechins, amino acids, and other polar compounds. In addition, large datasets were used to evaluate the similarities and discrimination among the two organs, which was carried out by principal component analysis (PCA). The metabolome of *C. tienii* contributes to an uninvestigated database of golden camellias. The methods developed in this paper can be used intensively for other *Camellia* species and can serve as chemotaxonomy for these valuable teas.

Results and Discussion

Morphological properties and distribution of C. tienii

C. tienii Ninh distributes abundantly along stream under leaf canopy in evergreen forest at altitudes of 250 m in Tam Dao which located in the North of Viet Nam, with the altitude ranging from 100 m to 1590 m above the sea level with a cool climate.[16] It is small tree, 2.5m high, opalescent, young branches and young leaves violet, glabrous. Leaves stalked, petioles 9-18 mm long, glabrous. Leaf blade thick coriaceous, oblong or elliptic, above, glabrous on both sides, apex acute, base auriculate with some teeth, margins sharply serrulate, lateral vein 13-14 pairs. Flowers yellow, axillary. Pedicels about 9 mm long. Bractioles 5, 2-3 mm long, 5-7 mm wide, margins pubescent. Sepals 5, nail shape or nearly rounded, margins pubescent. Petals 14, outer petals pubescent, the rest sparsely pubescent, inner ones glabrous, petal shapes vary from almost round, wide elliptic to oblong. Androecium numerous, filaments about 3.3 cm long, outer filaments united about 1.8 cm, inner ones free, glabrous. Gynoecium 3-5, ovaries 3-5 loculi, glabrous, styles 3 or 5, free to the base, glabrous (**Figure 1**). Capsules globose, scabrous. Seeds semi globose or cuneate. Blooming season: winter to early spring.[17]

LC-MS based widely targeted metabolomics analysis

Table 1 shows the identification of 158 metabolites and their corresponding MS parameters by both widely targeted (126 identified compounds) and targeted approaches (31 identified compounds). The intensity of each metabolite in the profiles of 50 *C. tienii* samples was reported in **Table S1**. The widely targeted analysis reveals a total of 126 metabolites in 50 flower and leaf *C. tienii* samples after filtering the peaks with the signal to noise > 3, RSD < 30%, and appeared in at least five samples. In terms of robustness, the widely targeted metabolomics approach has been considered the best method, which compromised between the reliability of identification (as

based on the screening of seleted reaction monitoring condition of compounds through those of reference standards) and the number of identified compounds. The profile revealed by this approach can cover the wide range of common bioactive compounds in tea such as flavonoids, phenolics, amino acids...

Below leaf

Above leaf

Fruit

Adult tree

Branches with flower

Branches with leaf and flower

Figure 1. Leaf, fruit, tree, branches and flower of *C. tienii* Ninh

A large range of flavonoids were detected in *C. tienii,* including 45 compounds by widely targeted analysis, which covers the diverse structures of aglycones, such as flavonol (quercetin, kaempferol, isorhamnetin, myricetin); anthocyanin (cyanidin and delphinidin); flavones (apigenin, luteolin), flavanones (naringenin, eriodictyol and isosakuranetin); flavan-3-ols (catechins). The glycoside units attached to aglycone are also varied, including glucose, rhamnose, arabinose, rutinose, and neohesperidose. This structural diversity of flavonoid profile in these plant tissues provides a good chemical source for pharmacological effects as these compounds showed various bioactivities. [18]

Besides, some phenylprepanoids in the subclass of hydroxycinnamic acids, benzoic acids, stilbenoids, and coumarins were also described in *C. tienii*. These phenolic compounds have a wide range of therapeutic effects relevant to antioxidant, neuroprotective, cardiovascular protective, gastrointestinal protective, and anticancer effects. [19]

Primary metabolites such as sugars, carboxylic acids, and nucleoside derivatives also play an essential role in the profile revealed by widely targeted analysis. Even though these compounds exhibit limited capabilities for bioactive exploration, they contribute to the typical taste and flavor of the tea infusion made by flowers or leaves of *C. tienii*.

Regarding targeted analysis, the catechin profiles of *C. tienii* flowers and leaves are dominated by three flavan-3-ols, including catechin, epicatechin and gallocatechin. Epigallocatechin, catechin 3-gallate, epicatechin-3-gallate, gallocatechin 3-gallate were detected in only a few samples, while these catechins were abundant in *Camellia sinensis* leaves extracts.[14] This profiling explains the non-astringent taste of leaves or flower tea extracts, which is distinguished from those of *C. sinensis*. More interestingly, caffeine, a commonly detected compound in *C. sinensis* green tea, was under the detection limit in all tested samples. Consequently, the infusion of leaves and flowers of this yellow tea species may not stimulate the central nervous system and cause insomnia, so it can be used to make an herbal tea for evening consumption. This result is consistent with other studies on the chemical composition of *Camellia chrysantha* as well as other golden camellias. [20][21]

Amino acids are bioactive compounds in green tea *C. sinensis,* which contribute to the taste and flavor of tea products as well as pharmacological effect on multiple targets.[13-14] Indeed, the variation of amino acids profile of tea samples contribute to explain typical characteristics of tea. Therefore, free amino acid profile has been considered important quality indicator for *Camellia* species.[14] In *C. tienii*, the widely targeted analysis revealed the presence of 32 amino acids in the library of PRIMe. We then confirmed the above identifications of 18 amino acids by targeted analysis and enlarged for another 7 compounds, including L-theanine, beta-alanine, L-cysteine, L-hydroxy-L-proline, L-taurine, L-citrulliline, L-cystine.[22] Notably, L-theanine, a unique amino acid of the Camellia genus, which was reported as an important amino acid with a relaxation effect on the brain, was then detected in *C. tienii*.[23] In addition, GABA, glutamine, and glutamic acid were found in both leaves and flowers of *C. tienii* in this study. These amino acids are analogs and are responsible for the umami taste of tea.[24] Combining these components can trigger neuroprotective effects [25], especially against the stimulatory effect of caffeine on the central nervous system.[26][27] It is the first time these amino acids were characterized in *C. tienii*, suggesting that some pharmacological bioassays should be conducted on these tissues.

Table 1. Targeted-based profile of *C. tienii* leaves and flowers by LC-MS.

ID	Identification mode	Ionization mode	Precursor ion	Product ion	Classification	Method
1	(-)Shikimic acid	negative	173.05	93.1	Carboxylic acids	Widely targeted
2	5-Methylcytosine	positive	126.05	109.1	Other	Widely targeted
3	Adenine	positive	136.05	119.05	Nucleosides	Widely targeted
4	Adenosine	positive	268.1	136.05	Nucleosides	Widely targeted
5	Glycine	positive	76.05	30.1	Amino acid	Widely targeted
6	L-Histidine	positive	156.1	110.15	Amino acid	Widely targeted
7	L-(-)-Phenylalanine	positive	166.1	120.1	Amino acid	Widely targeted
8	L-Tryptophane	positive	205.1	188.15	Amino acid	Widely targeted
9	L-Tyrosine	positive	182.1	165.1	Amino acid	Widely targeted
10	Sucrose	negative	341.1	88.95	Sugar	Widely targeted
11	Leupeptin	positive	427.3	409.25	Peptide	Widely targeted
12	2-Aminoethylphosphonic acid	positive	126.05	109.05	Phosphonic acid	Widely targeted
13	Cytidine	positive	244.1	112.1	Nucleosides	Widely targeted
14	Diethanolamine	positive	106.1	88.15	Amines	Widely targeted
15	DL-2,3-Diaminopropionic acid	positive	105.05	88.1	Carboxylic acid	Widely targeted
16	Guanine	positive	152.05	135.05	Nucleosides	Widely targeted
17	Guanosine	positive	284.1	152.05	Nucleosides	Widely targeted
18	L-Asparagine	positive	133.05	87.1	Amino acid	Widely targeted
19	L-Aspartic acid	positive	134.05	74.05	Amino acid	Widely targeted
20	L-Glutamic acid	positive	148.05	84.1	Amino acid	Widely targeted

21	L-Glutamine	positive	147.1	130.05	Amino acid	Widely targeted
22	L-Homocarnosine	positive	241.15	110.1	Amino acid	Widely targeted
23	L-Ornithine	positive	133.1	70.15	Amino acid	Widely targeted
24	L-Proline	positive	116.05	70.15	Amino acid	Widely targeted
25	L-Pyroglutamic acid	positive	130.05	84.1	Amino acid	Widely targeted
26	L-Serine	positive	106.05	60.15	Amino acid	Widely targeted
27	3,4-Dihydroxybenzoic acid	negative	153	109.05	Phenol	Widely targeted
28	Pyridoxine	positive	170.1	134.05	Pyridines	Widely targeted
29	Succinic acid	negative	117	73	Carboxylic acid	Widely targeted
30	Uridine	positive	245.1	113.1	Nucleosides	Widely targeted
31	Uridine 5'-monophosphate	positive	325.05	97.05	Nucleosides	Widely targeted
32	alpha-Lactose	negative	341.1	161.05	Sugar	Widely targeted
33	3-Guanidinopropionic acid	positive	132.1	72.1	Carboxylic acid	Widely targeted
34	gamma-Amino n-butyric acid	positive	104.05	87.1	Carboxylic acid	Widely targeted
35	Naringenin	positive	273.1	153	Flavonoid (flavanone)	Widely targeted
36	Apigenin 7-O-glucoside	positive	433.1	271.05	Flavonoid (flavone)	Widely targeted
37	Isorhamnetin 3-O-glucoside	positive	479.1	317	Flavonoid (flavonol)	Widely targeted
38	Kaempferol 3-O-glucoside	positive	449.1	287.05	Flavonoid (flavonol)	Widely targeted
39	Homoorietin	positive	449.1	299.05	Flavonoid (flavone)	Widely targeted
40	Naringenin 7-O-glucoside	positive	435.15	273.05	Flavonoid (flavanone)	Widely targeted
41	Hyperoside	positive	465.1	303	Flavonoid (flavonol)	Widely targeted
42	4-Coumaric acid	positive	165.05	91.1	Hydroxycinnamic acid	Widely targeted
43	Kaempferol 7-O-alpha-L-rhamnoside	positive	433.1	287.05	Flavonoid (flavonol)	Widely targeted
44	N-Acetyl D-mannosamine	positive	222.1	126.1	Sugar	Widely targeted
45	O-Acetyl L-serine	positive	148.05	88.1	Amino acid	Widely targeted
46	L-Saccharopine	positive	277.05	84.05	Sugar	Widely targeted
47	D-Glucoheptose	negative	209.05	89.05	Sugar	Widely targeted
48	D-(-)-Quinic acid	negative	191.05	85.05	Carboxylic acid	Widely targeted
49	Chlorogenic acid	negative	353.1	191.1	Phenol	Widely targeted

50	5'-Deoxy-5'-Methylthioadenosine	positive	298.1	136	Nucleosides	Widely targeted
51	L-allo-threonine	positive	120.05	56.1	Amino acid	Widely targeted
52	Methyl jasmonate	positive	225.15	151.1	Carboxylic acid	Widely targeted
53	Vanillin	positive	153.05	65.15	Benzoic acid	Widely targeted
54	5-Aminovaleric acid	positive	118.1	55.1	Carboxylic acid	Widely targeted
55	L-Threonic acid	negative	135.05	75	Carboxylic acid	Widely targeted
56	2,2',2''-Nitrilotriethanol	positive	150.1	70.15	Polyol	Widely targeted
57	Luteolin 3',7-di-O-glucoside	positive	611.15	287	Flavonoid (flavone)	Widely targeted
58	Luteolin 4'-O-glucoside	positive	449.1	287.05	Flavonoid (flavone)	Widely targeted
59	Neoeriocitrin	negative	595.15	151	Flavonoid (flavanone)	Widely targeted
60	Phloridzin	negative	435.15	273.1	Flavonoid (dihydrochalcone)	Widely targeted
61	Poncirin	negative	593.2	285.05	Flavonoid (flavanone)	Widely targeted
62	Saponarin	positive	595.15	283	Flavonoid (flavone)	Widely targeted
63	Vitexin	positive	433.1	313.05	Flavonoid (flavone)	Widely targeted
64	S-(5'-Adenosyl)-L-methionine	positive	399.15	250.1	Amino acid	Widely targeted
65	Kaempferol 3-O-alpha-L-arabinoside	positive	419.1	287.05	Flavonoid (flavonol)	Widely targeted
66	2,5-dihydroxy benzoic acid	negative	153	108.05	Phenol	Widely targeted
67	Salicylic Acid	negative	137	93.05	Phenol	Widely targeted
68	Trimethylamine N-oxide	positive	76.1	58.15	Amines	Widely targeted
69	DL-5-Hydroxylysine	positive	163.1	82.1	Amino acid	Widely targeted
70	L-Anserine	positive	241.05	109.15	Amino acid	Widely targeted
71	(-)-Riboflavin	positive	377.15	243.1	Nucleosides	Widely targeted
72	Choline	positive	104.1	60.15	Other	Widely targeted
73	Kynurenic acid	positive	190.05	144.05	Carboxylic acid	Widely targeted
74	Trigonelline	positive	138.05	92.1	Alkaloid	Widely targeted
75	DL-Pipecolinic acid	positive	130.1	84.15	Carboxylic acid	Widely targeted
76	D-(+)-Raffinose	negative	503.15	179.05	Sugar	Widely targeted
77	Betaine	positive	118.1	58.15	Alkaloid	Widely targeted
78	Procyanidin C1	negative	865.2	125	Flavonoid (flavan-3-ols)	Widely targeted
79	Quercetin 3,4'-O-di-beta-	negative	625.15	463.1	Flavonoid	Widely targeted

	glucopyranoside				(flavonol)	
80	E-3,4,5'-trihydroxy-3'-glucopyranosylstilbene	negative	405.1	243	Stilbenoid	Widely targeted
81	Esculin	positive	341.1	179.05	Coumarin	Widely targeted
82	Stachyose	negative	665.2	383.05	Sugar	Widely targeted
83	L-Leucine	positive	132.2	30.1	Amino acid	Widely targeted
84	L-Isoleucine	positive	132.1	69.1	Amino acid	Widely targeted
85	Methionine sulfoxide	positive	165.95	74.05	Amino acid	Widely targeted
86	Glutamine	positive	147.1	84.1	Amino acid	Widely targeted
87	Isorhamnetin 3-O-rutinoside or Isorhamnetin 3-glucoside-6''-rhamnoside or Isorhamnetin 3-galactoside-6''-rhamnoside	positive	625.2	317.05	Flavonoid (flavonol)	Widely targeted
88	Kaempferol 3-O-rutinoside or Datiscin or Kaempferol 3-O-beta-D-glucoside-7-O-alpha-L-rhamnoside or Kaempferol 3-O-beta-D-galactoside-7-O-alpha-L-rhamnoside or Kaempferol 3-O-b-glucopyranosyl-7-O-a-rhamnopyranoside or Kaempferol 7-O-neohesperidoside or Kaempferol-3-Gluco	negative	593.15	285.05	Flavonoid (flavonol)	Widely targeted
89	Cyanidin 3,5-di-O-glucoside or Cyanidin 3-O-(2''-O-beta-glucopyranosyl-beta-glucopyranoside)	positive	611.15	287.05	Flavonoid (anthocyanin)	Widely targeted
90	Kaempferol 3-rhamnoside-7-rhamnoside or Vitexin 2''-O-rhamnoside or Kaempferol 3,7-O-bis-alpha-L-rhamnoside or Kaempferol 3,7-O-di-rhamnopyranoside	negative	577.15	285	Flavonoid (flavonol)	Widely targeted
91	Kaempferol 3-O-alpha-L-rhamnoside or Kaempferol 3-rhamnoside	negative	431.1	285	Flavonoid (flavonol)	Widely targeted
92	Maritimein or Luteolin 7-O-glucoside	positive	449.1	287.05	Flavonoid (flavone)	Widely targeted

93	Quercetin 3-arabinoside or Quercetin 3-D-xyloside	positive	435.1	303.05	Flavonoid (flavonol)	Widely targeted
94	Quercetin 3-rhamnoside or Quercetin 3-O-alpha-L-rhamnopyranoside	negative	447.1	300	Flavonoid (flavonol)	Widely targeted
95	(+)-Catechin or (+)-Epicatechin	positive	291.1	139	Flavonoid (flavan-3-ols)	Widely targeted
96	Sodium pantothenate or D-Pantothenic acid or Calcium (+)-pantothenate	positive	220.1	90.1	Polyol	Widely targeted
97	D(+)-Galactosamine or D-(+)-Glucosamine	positive	180.1	72.1	Sugar	Widely targeted
98	D-(+)-Cellobiose or Lactulose	negative	341.1	161.1	Sugar	Widely targeted
99	Melibiose or D-(+)-Turanose or Isomaltose or Gentiobiose or Melibiose or Palatinose	negative	341.1	179.1	Sugar	Widely targeted
100	L-Iditol or D-Sorbitol or D-(-)-Mannitol	positive	183.1	69.1	Sugar	Widely targeted
101	L(+)-Arginine or N-alpha-Acetyl-L-ornithine or L-Citrulline	positive	175.1	70.15	Amino acid	Widely targeted
102	Histamine or Cytosine	positive	112.1	95.15	Nucleosides	Widely targeted
103	Nicotinamide or Niacinamide	positive	123.05	80.1	Pyridines	Widely targeted
104	L-Alanine or Sarcosine	positive	90.05	44.1	Amino acid	Widely targeted
105	L-Norvaline or L-Valine	positive	118.1	72.1	Amino acid	Widely targeted
106	L-Threonine or alpha-Methyl-DL-serine or L-Homoserine	positive	120.05	74.1	Amino acid	Widely targeted
107	L-2-Aminobutyric acid or N,N-Dimethylglycine or N-Methyl-DL-Alanine	positive	104.05	44.15	Amino acid	Widely targeted
108	(S)-(+)-1-Aminoethylphosphonic acid or Taurine	positive	126.05	44.15	Amino acid	Widely targeted
109	DL-Malic acid	negative	133	115	Carboxylic acid	Widely targeted
110	L-Carnitine	positive	162.1	60.2	Amino acid	Widely targeted
111	Adipic acid or 2-Methylglutaric Acid	negative	145.05	83	Carboxylic acid	Widely targeted
112	(-)-Citramalic acid	negative	147.05	87	Carboxylic acid	Widely targeted
113	L-Lysine	positive	147.1	84.1	Amino acid	Widely targeted

114	Nicotinic Acid or Isonicotinic acid	positive	124.05	78.05	Carboxylic acid	Widely targeted
115	Rutin or Quercetin 3-O-b-glucopyranosyl-7-O-a-rhamnopyranoside	negative	609.15	300	Flavonoid (flavonol)	Widely targeted
116	D-(+)-Melezitose or 1-Kestose	negative	503.15	89	Sugar	Widely targeted
117	Cyanidin 3-(6''-malonylglucoside)	positive	535.1	287.1	Flavonoid (anthocyanin)	Widely targeted
118	Schaftoside	positive	565.15	547.05	Flavonoid (flavone)	Widely targeted
119	(-)-Epigallocatechin	positive	307.05	139.05	Flavonoid (flavan-3-ols)	Widely targeted
120	(-)-Gallocatechin	positive	307.05	139.05	Flavonoid (flavan-3-ols)	Widely targeted
121	Delphinidin 3-rutinoside	positive	612.15	304	Flavonoid (anthocyanin)	Widely targeted
122	Delphinidin 3-galactoside	negative	464.1	301.05	Flavonoid (anthocyanin)	Widely targeted
123	Cyanidin 3-galactoside	negative	448.1	285	Flavonoid (anthocyanin)	Widely targeted
124	Vicenin -1	positive	565.15	427.15	Flavonoid (flavone)	Widely targeted
125	Vicenin -2	negative	593.15	353.05	Flavonoid (flavone)	Widely targeted
126	Vicenin -3	positive	565.15	547.15	Flavonoid (flavone)	Widely targeted
127	L-Theanine	positive	345.1	171	Amino acid	Targeted
128	GABA	positive	274.1	171	Amino acid	Targeted
129	L-Glutamine	positive	317.1	171	Amino acid	Targeted
130	L-Glutamic acid	positive	318.1	171	Amino acid	Targeted
131	B-Alanine	positive	260.1	171	Amino acid	Targeted
132	L-Alanine	positive	260.2	171	Amino acid	Targeted
133	L-Tryptophan	positive	375.2	171	Amino acid	Targeted
134	L-Glycine	positive	246.1	171	Amino acid	Targeted
135	L-Serine	positive	276.1	171	Amino acid	Targeted
136	L-Proline	positive	286.1	171	Amino acid	Targeted
137	L-Valine	positive	288.2	171	Amino acid	Targeted
138	L-Cysteine	positive	292.1	171	Amino acid	Targeted
139	L-hydroxy-L-proline	positive	302.1	171	Amino acid	Targeted
140	L-Taurine	positive	296.1	171	Amino acid	Targeted
141	L-Isoleucine	positive	302.2	171	Amino acid	Targeted

142	L-Asparagine	positive	301.1	171	Amino acid	Targeted
143	L-Ornithine	positive	303.2	171	Amino acid	Targeted
144	L-Aspartic Acid	positive	304.1	171	Amino acid	Targeted
145	L-Lysine	positive	317.2	171	Amino acid	Targeted
146	L-Methionine	positive	320.1	171	Amino acid	Targeted
147	L-Histidine	positive	326.1	171	Amino acid	Targeted
148	L-Phenylalanine	positive	336.1	171	Amino acid	Targeted
149	L-Arginine	positive	345.2	171	Amino acid	Targeted
150	L-Citrululiline	positive	346.2	171	Amino acid	Targeted
151	L-Cystine	positive	411.4	171	Amino acid	Targeted
152	Catechin	negative	288.97	108.88	Flavonoid (flavan-3-ols)	Targeted
153	Epicatechin	negative	288.97	108.88	Flavonoid (flavan-3-ols)	Targeted
154	Gallocatechin	negative	304.97	124.88	Flavonoid (flavan-3-ols)	Targeted
155	Catechin 3-gallate	negative	457.03	168.88	Flavonoid (flavan-3-ols)	Targeted
156	Epicatechin 3-gallate	negative	457.03	168.88	Flavonoid (flavan-3-ols)	Targeted
157	Epigallocatechin 3-gallate	negative	441.03	168.88	Flavonoid (flavan-3-ols)	Targeted
158	Gallocatechin 3-gallate	negative	441.03	168.88	Flavonoid (flavan-3-ols)	Targeted

GC-MS based untargeted analysis

To delve into *C. tienii* metabolites variations in leaves and flowers, an untargeted metabolomics approach was used, which annotated a total of 43 signals (including the IS peak) corresponding to compounds from various chemical classes. **Figure 2** illustrated two chromatograms of a flower and a leaf samples. Each component was chosen because it was detected in at least three biological samples with a relatively high peak area (peak ratio based on IS) and with a suitable match and reverse match (>700). The annotation of the semi-volatile compounds (**Table 2**) was conducted based on matching their spectra and relevance with their retention index (RI) with those referenced by software. Indeed, the theoretical RI, which was forecast from AMDIS/NIST, must be approximately calculated RI and should not differ from 5%.

Among the compounds annotated, fatty acids, carboxylic acids, and their esters are most found with almost a high probability. Saturated fatty acids like butanoic acid, hexenoic acid, myristic acid, hexadecanoic acid, heptadecanoic acid, eicosanoic acid, docosanoic acid, tetracosanoic acid, hexacosanoic acid dominate the GC-MS based profile. Specifically, unsaturated fatty acids commonly possess potential bioactivity, such as 9,12-octadecadienoic acid (Z,Z); 9,12,15-octadecatrienoic acid

(Z,Z,Z) were found with high intensity in leaves and flowers of *C. tienii*.

Other classes, such as ketones and terpenes, were less abundant groups but showed an interesting profile of pharmacological effects. Indeed, a compound was found at RT 51.44 minutes, showing the similarity with preliminary literature, and can be annotated as dl-α-tocopherol, a form of vitamin E. This compound has an antioxidant effect, protecting cell membranes from free radical attack, thereby preserving the integrity of cell membranes. Vitamin E synergizes with vitamin C, selenium, vitamin A, and carotenoids, contributing to the nutritional and therapeutic benefits. [28]

Also, squalene, a triterpene, was detected in most leaves and flowers of *C. tienii* in this study. With a long history of research, this triterpenoid was revealed to exhibit potential therapeutic effects such as antioxidant, anti-inflammatory, and lipid-lowering properties. [29] Furthermore, at RT 33.42, a diterpene, namely neophytadiene was annotated. This compound has been found in many plants and algae and is considered an anti-inflammatory and antimicrobial agent. [30] Recently, some research revealed the capability of neophytadiene in anxiolytic-like activity, soothing properties, and antidepressant-like actions. [31] Phytol, another diterpene that is a component of chlorophyll, is very well known as an aromatic ingredient, antioxidant, anti-inflammatory, antihyperalgesic, and antiarthritic effects. This compound was also detected as the most abundant accumulated component of *C. tunghinensis* and *C. euphlebia* leaves. [32] Stigmasterol, an unsaturated phytosterol, belongs to the triterpenes class with antioxidant, antifungal, anti-inflammatory, and anticancer characteristics. [33][34]

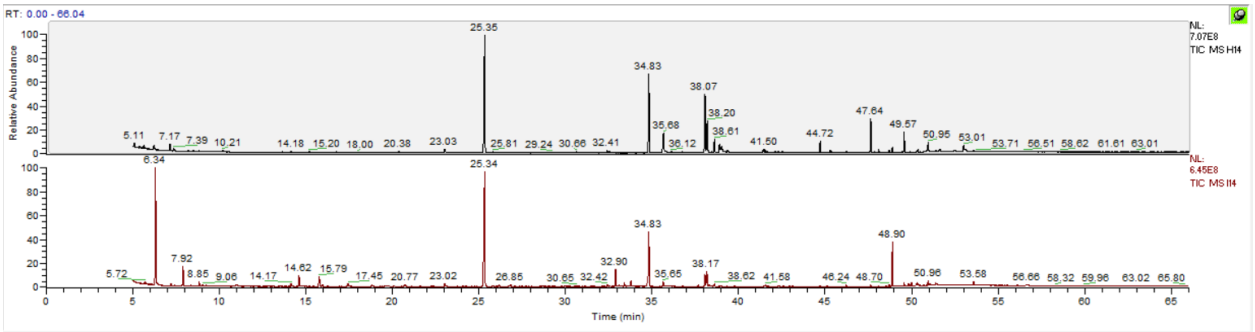

Figure 2. GC-MS based chromatograms of flower and leaf *C. tienii.*

Table 2. Tentative annotation of the compounds detected in *C. tienii* by GC-MS.

No	Name chemical	RT	Formula	MW	Probability	RI	RI*
1	Butanoic acid, 2-methyl-, methyl ester	5.14	$C_6H_{12}O_2$	116	87.1	775	ND
2	2-Butenoic acid, 2-methyl-, methyl ester	7.39	$C_6H_{10}O_2$	114	32.4	873	ND
3	2-Hexenoic acid, methyl ester, (E)-	8.84	$C_7H_{12}O_2$	128	51.8	966	ND
4	trans-Linalool oxide (furanoid)	13.65	$C_{10}H_{18}O_2$	170	25.2	1086	1080
5	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	18.83	$C_7H_9NO_2$	139	98.3	1239	1255
6	2-Propenoic acid, 3-phenyl-, methyl ester	20.79	$C_{10}H_{10}O_2$	162	31.2	1380	1326
7	2-Propenoic acid, 3-phenyl-, methyl ester, (E)-	23.03	$C_{10}H_{10}O_2$	162	49.5	1380	1408
8	Phenol, 2-methoxy-4-(1-propenyl)-	24.65	$C_{10}H_{12}O_2$	164	44.8	1450	1474
9	Pentadecane (IS)	25.37	$C_{15}H_{32}$	212	43.4	1500	1503
10	2,4-Di-tert-butylphenol	25.84	$C_{14}H_{22}O$	206	49.6	1519	1523

11	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	26.85	$C_{11}H_{16}O_2$	180	76.4	1532	1564
12	Heptadecane	29.95	$C_{17}H_{36}$	240	18.1	1700	1700
13	Methyl tetradecanoate	30.67	$C_{15}H_{30}O_2$	242	82.6	1725	1732
14	Myristic acid	31.53	$C_{14}H_{28}O_2$	228	76	1768	1771
15	Octadecane	32.13	$C_{18}H_{38}$	254	29.1	1800	1798
16	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	32.42	$C_{11}H_{16}O_3$	196	93.3	1784	1812
17	(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone	32.59	$C_{13}H_{18}O_3$	222	29.8	1800	1821
18	Neophytadiene	33.42	$C_{20}H_{38}$	278	28.3	1837	1862
19	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	33.64	$C_{11}H_{12}O_4$	208	75.5	1855	1873
20	(Z)-Methyl hexadec-11-enoate	34.43	$C_{17}H_{32}O_2$	268	28.8	1913	1913
21	Hexadecanoic acid, methyl ester	34.84	$C_{17}H_{34}O_2$	270	80.7	1928	1933
22	Hexadecanoic acid, ethyl ester	36.13	$C_{18}H_{36}O_2$	284	77.1	1993	1998
23	Heptadecanoic acid, methyl ester	36.77	$C_{18}H_{36}Q_2$	284	80.3	2028	2033
24	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	38.08	$C_{19}H_{34}O_2$	294	31.3	2092	2104
25	9,12,15-Octadecatrienoic acid, methyl ester	38.21	$C_{19}H_{32}O_2$	292	35.1	2098	2111
26	Phytol	38.33	$C_{20}H_{40}O$	296	80	2114	2118
27	Methyl stearate	38.62	$C_{19}H_{38}O_2$	298	82.7	2128	2134
28	9,12-Octadecadienoic acid (Z,Z)-	38.93	$C_{18}H_{32}O_2$	280	32.9	2133	2151
29	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	39.37	$C_{18}H_{30}O_2$	278	48.8	2139	2175
30	cis-Methyl 11-eicosenoate	41.68	$C_{21}H_{40}O_2$	324	11.4	2306	2310
31	Eicosanoic acid, methyl ester	42.1	$C_{21}H_{42}O_2$	326	73	2329	2335
32	Oxazole, 2-(8Z,11Z,14Z)-8,11,14-heptadecatrien-1-yl-4,5-dihydro-	42.32	$C_{20}H_{37}NO$	307	46.6	2329	2348
33	4,8,12,16-Tetramethylheptadecan-4-olide	42.62	$C_{21}H_{40}O_2$	324	86.1	2364	2366
34	Tetracosane	43.03	$C_{24}H_{50}$	338	13	2400	2391
35	Docosanoic acid, methyl ester	45.29	$C_{23}H_{46}O_2$	354	77.8	2528	2536
36	15-Tetracosenoic acid, methyl ester, (Z)-	47.84	$C_{25}H_{48}O_2$	380	54.9	2710	2727
37	Tetracosanoic acid, methyl ester	48.1	$C_{25}H_{50}O_2$	382	89.8	2728	2748
38	Squalene	48.92	$C_{30}H_{50}$	410	64.4	2832	2823
39	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-, (all-E)-	49.82	$C_{30}H_{50}O$	426	3.52	2963	2936
40	Hexacosanoic acid, methyl ester	49.99	$C_{27}H_{54}O_2$	410	86.3	2935	2957
41	Stigmasterol	50.31	$C_{29}H_{48}O$	412	56	3000	2997
42	dl-α-Tocopherol	51.44	$C_{29}H_{50}O_2$	430	27.6	3150	3172

43	Olean-12-en-3-ol, acetate, (3β)-	53.58	$C_{32}H_{52}O_2$	468	16.3		3438	3454

RT: Retention time, MW: Molecular weight. RI*: retention index (calculated), RI: retention index (theoretical)

LC-MS based profile discrimination among leaves and flowers

The PCA score plot was constructed on the widely targeted dataset among two tissues, leaves and flowers, to provide an insightful overview of the chemical constituent variations (**Figure 3**). The first (Dim-1) and the second (Dim-2) principal components attributed 32.7% and 13.2%, respectively, to the total variance of 45.9%. In this figure, leaf and flower samples were successfully clustered and separated, indicating that the chemical profiles in the leaves and flowers were remarkably different. The metabolic profiles of twenty-five leaf samples are relatively consistent, while more significant variations were observed in those of flowers.

Regarding the chemical difference between the two tissues, a heatmap was used to illustrate the discriminative components (**Figure 4**). The level of the difference in each metabolite's concentration was demonstrated based on the color intensity in each sample. In **Figure 4**, the group of metabolites overexpress in flowers (in the red color). More precisely, the volcano plot (**Figure S1**) demonstrated that metabolites accumulated significantly more in flower include mostly flavonoids (rutin, kaempferol-7-O-alpha-L-rhamnoside, maritimein, catechin or epicatechin, hyperoside, delphinidin 3-galactoside, procyanidin C1, kaempferol 3-rhamnoside-7-rhamnoside, quercetin 3-arabinoside, delphinidin 3-rutinoside, quercetin 3-rhamnoside, poncirin…) and amino acids (L-anserine, L-proline, DL-malic acid, L-glutamic acid, L-tryptophan, L-phenylalanine, choline…). In contrast, the group of compounds that were produced much more in leaves consists of L-pyroglutamic acid, esculin, stachyose, and 2,5-dihydroxy benzoic acid (in blue color). The variation of these compounds in two tissues contributes to explaining the typical taste of tea made by leaf and flower as water-soluble compounds, like free amino acids, caffeine, polyphenols, and polysaccharides, are generally accountable for the taste of tea infusions. From a more pharmacological point of view, this discrimination could suggest a distinguished biological profile between two types of materials. Typically, flowers of golden camellias are commercialized at a higher price than leaves because it is believed that flowers offer more biological health benefits. Our research findings align with this hypothesis as most of the compounds in the profile, which are mainly active, dominate significantly in flowers rather than in leaves.

GC-MS based profile discrimination among leaves and flowers

The PCA method was applied to the data set formed by leaf and flower samples, jointly accumulating 53.1 % of the total variance, in which the PC1 and PC2 explained 32.6% and 20.5%, respectively (**Figure 3**). A clear separation is observed in the PCA score plots, indicating a clear discrimination in volatile compounds between leaves and flowers of *C. tienii*. This plot may contribute to explaining the difference in the flavor of tea infusion from these tissues as the tea's popularity often relies on its satisfying aroma by volatile compounds. [35]

The heatmap (**Figure 4**) and the volcano plot (**Figure S2**) show the dominance of terpenoids such as neophytadiene, squalene, phytol, DL-α-tocopherol in leaves while fatty acids in both saturated and unsaturated forms were produced more in flowers of *C. tienii*. These results will serve as the basis for additional research on these materials' chemical composition and biological activity.

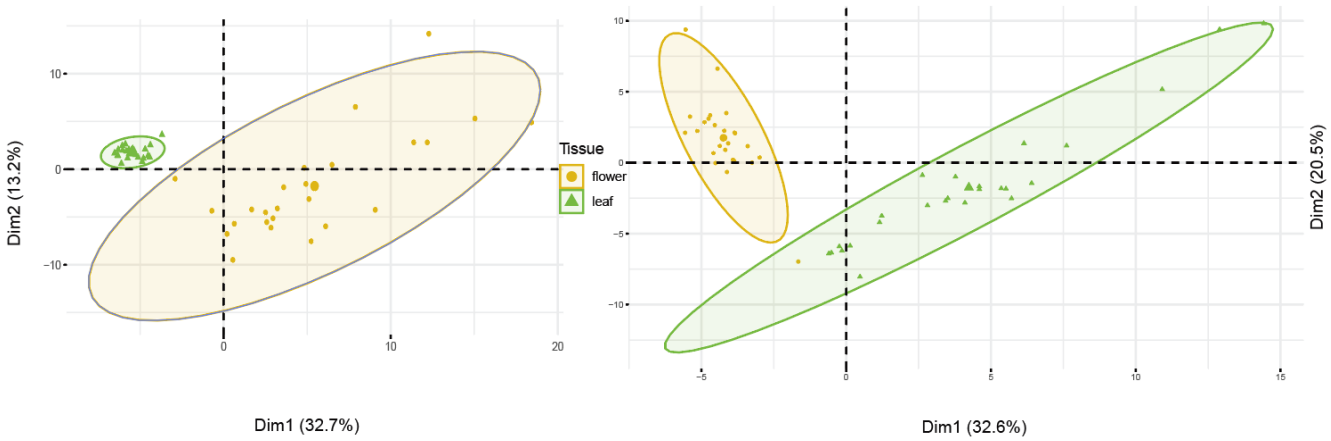

Figure 3: PCA of LC-MS based widely targeted (on the left) and GC-MS based untargeted (on the right) metabolomes from *C. tienii*'s flower

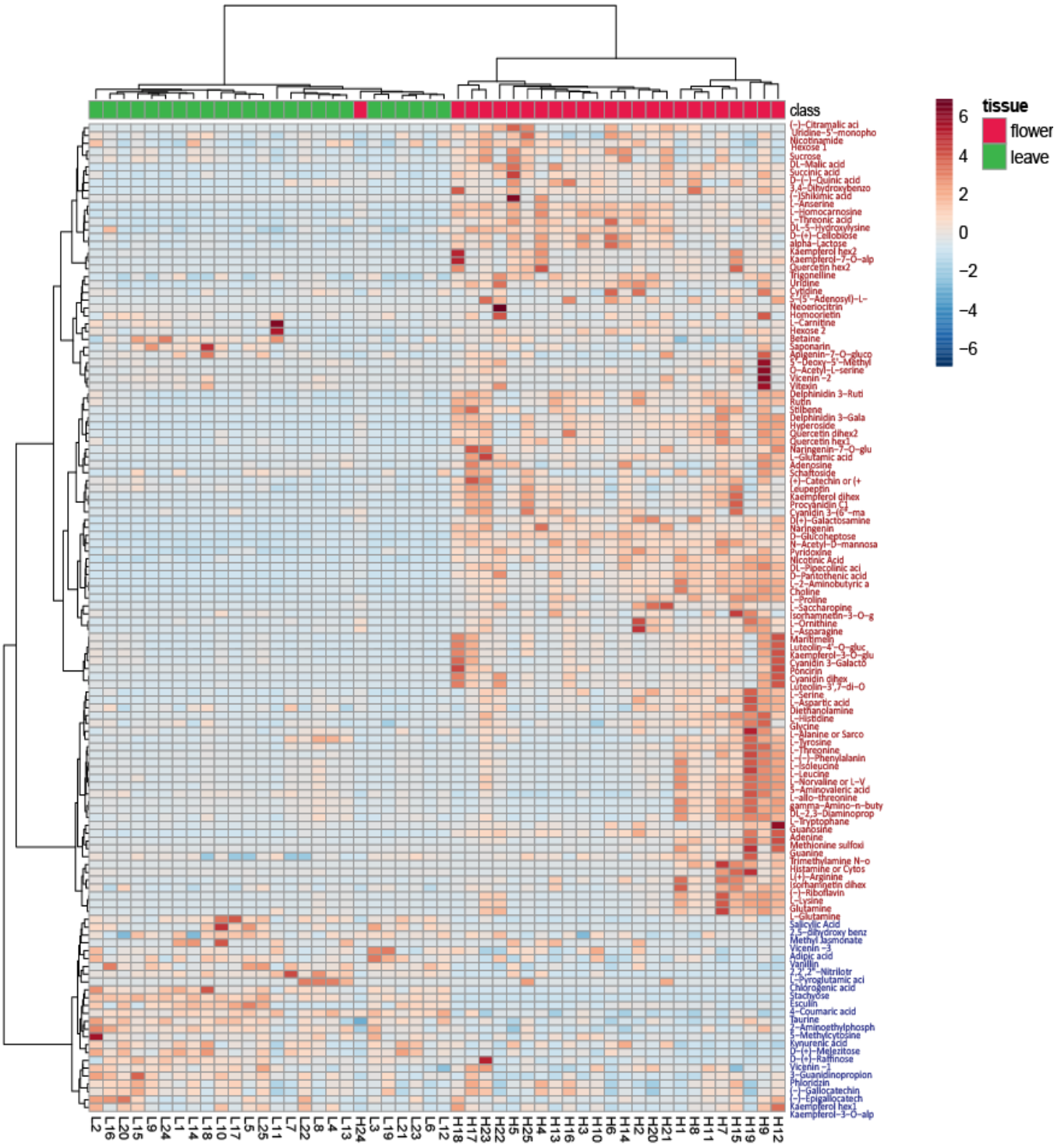

Figure 4: Heatmap of the intensity of 126 metabolites detected by widely targeted metabolomics of flowers and leaves *C. tienii* samples.

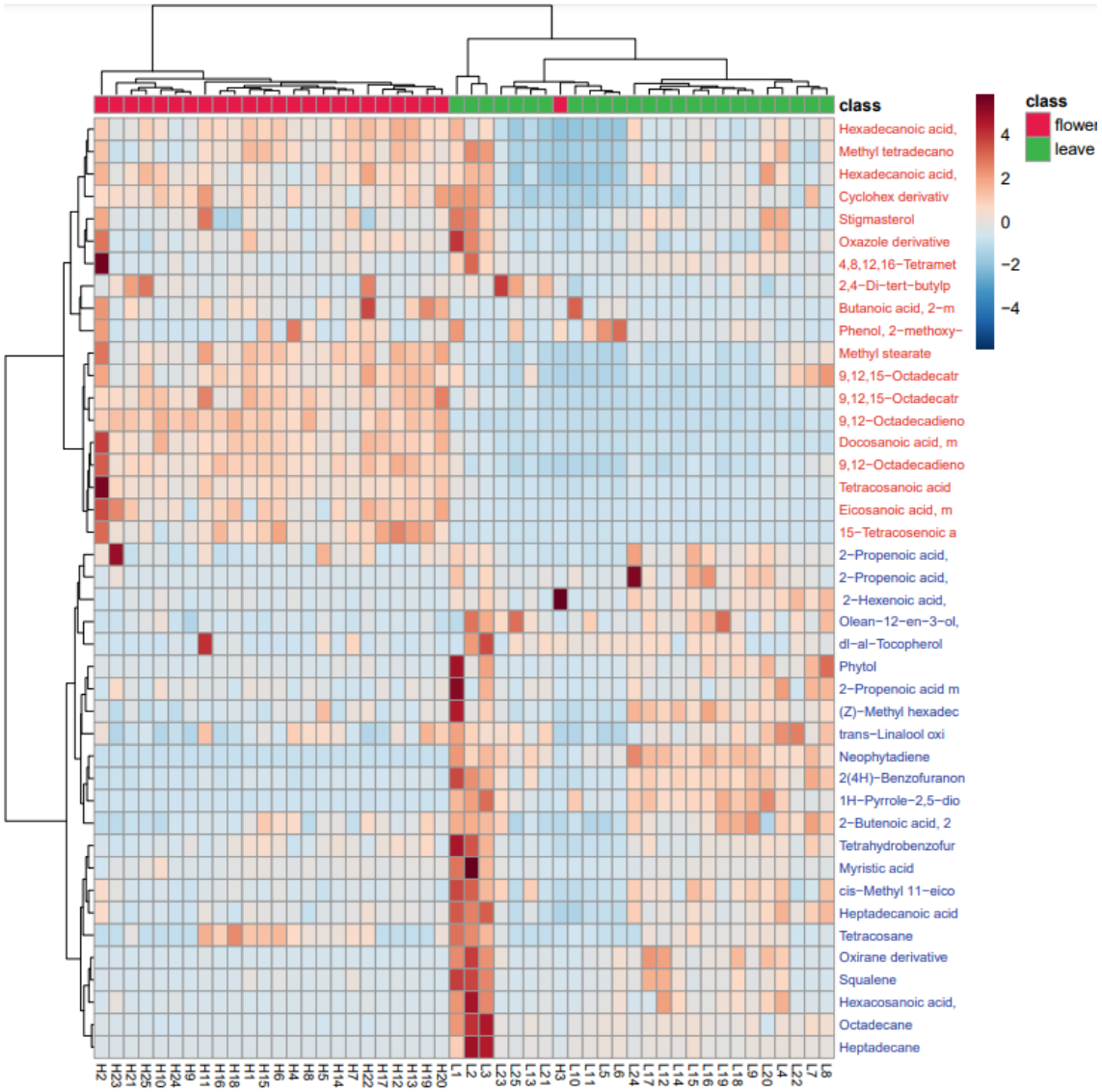

Figure 5: Heatmap of the intensity of 42 metabolites detected by GC-MS of flowers and leaves *C. tienii* samples.

Conclusions

This study shows the first report on the metabolome profiles of *C. tienii* leaves and flowers using a comprehensive approach gathering both targeted and untargeted strategies. We have examined and semi-quantitated 158 compounds in methanolic extracts of *C. tienii* flowers and leaves samples and annotated the presence of 42 metabolites in their chloroform extracts. These whole metabolome profiles were used to demonstrate the remarkable differences between leaves and flowers for the level of taste-maker compounds like amino acids, flavonoids, and aroma-contributing compounds in the based profile. A simple yet effective way for the mass spectrometry data was provided, which may offer a comprehensive pipeline to apply for other golden camellia species. These results will also advance the development of these tea materials for health benefits.

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Experimental Section

Plant materials

The endemic *C. tienii* (TIE, specimen code TIE-06-2021/USTH) were collcted at Hop Chau ward, Tam Dao district, Vinh Phuc province in June 21st 2021 (GPS: 21°26′18.0″N 105°36′17.3″E) and identified by Dr. Nguyen The Cuong from the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The specimen dossiers (code TIE21062021) were deposited in the Department of Life Sciences, University of Science and Technology of Hanoi to serve for further investigation.

Sample preparation

Twenty-five plant individuals were selected to harvest their flowers and leaves. These tissues were dried in the oven at 45°C until their weights were stable. Consequently, the samples were homogenized in

zirconium bead during 2 minutes at a frequency of 25 times per second by a Mixer Mill MM 400 (Retsch, Germany), then stored at temperature -80°C until analysis.

Targeted analysis

A liquid chromatography (ACQUITY UPLC H-Class, Waters, US) coupled to a mass spectrometer (Xevo TQD Triple Quadrupole, Waters, US) system was used for targeted analysis, including catechins and amino acid profile. The characterization of catechins (catechin, epicatechin, catechin 3-gallate, epicatechin-3-gallate, epigallocatechin 3-gallate, gallocatechin, gallocatechin 3-gallate) and caffeine in the methanolic extracts of leaves and flowers of *C. tienii* followed exactly our protocol reported in the previous articles.[36] For the amino acids, 26 amino acids including L-theanine, gamma amino butyric acid, L-glutamine, L-cysteic acid, taurine, D, L-methionine sulfoxide, L-methionine sulfone, L-aspartic acid, L-threonine, L-serine, L-glutamic acid, L-proline, glycine, L-cystine, L-valine, L-methionine, L-isoleucine, L-tyrosine, L-phenylalanine, L-histidine, L-ornithine, L-lysine, ammonia, L-arginine were quantified in the extracts by the method of Salazar.[14] The free amino acids in the sample were derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate kit (Waters, US) before its separation and detection. Amino acids were separated through the column Acquity UPLC BEH C18 130A, 1.7 µm, 1x100 mm in 55°C, using mobile phase of eluent A (acetonitrile 10%, formic acid 6%, ammonium formate in water 84%) and eluent B (acetonitrile). Gradient elution was applied, starting with 99.9 % A at 0.54 min, ramping to 90.0% at 5.74 min, followed by 78.8% at 7.74 min, 40.4 % at 8.04-8.64 min, then increased again to 99.9% at 8.73-9.50 min, with a flow rate of 0.3 mL/min. Sample chambre was set at 25°C. The analysis procedure was managed by MassLynx 4.1 software, and then the data acquisition, processing and reporting for quantitative results were performed by the TargetLynx application.

Widely targeted analysis

For analysis of polar metabolites, widely targeted analysis of methanolic flower and leaf extracts was conducted on liquid chromatography hybrid to a triple quadrupole mass spectrometer (Nexera MP/LCMS-8050; Shimadzu Corporation), following the protocol in our previous articles.[16][37] In detail, 4 mg of powdered material was extracted in the solvent mixture of 0.1% formic acid, 80% methanol, and two internal standards, lidocaine 8.4 nM and 10-camphorsulfonic acid 210 nM, using homogenizer at 1000 rpm for 2 min. The separation was conducted on the ACQUITY UPLC HSS T3 Column, 100Å, 1.8um, 1mm X 50mm column, with a mobile phase consisting of eluent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient started with 99.9 % A at 0.25 min, then decreased to 91% at 0.40 min, 83% at 0.80 min, 0.1% at 1.90 min, and held until 2.10 min, then back to 99.9% at 2.11 min and stop at 2.70 min, with a flow rate of 0.24 ml/min. The MS parameter was set as follows: interface voltage 4.0 and 3.0 kV for positive and nagative mode, interface temperature 300 °C, DL temperature 250 °C, heat block temperature 400 °C. nebulizer gas flow 3.0 L/min, drying and heating gas flow 10.0 L/min, dwell time 0.006 s.

Untargeted analysis

To broader the identification of volatile metabolites in the sample, an untargeted analysis was performed by a hyphenated system including a Thermo Fisher Scientific™ DSQ / TRACE™ GC Ultra with a TriPlus autosampler linked to a DSQ II mass spectrometer. 200 mg of the sample powder was extracted with 1.3 mL of $CHCl_3$ containing internal standard (IS) (Pentadecane) with the final concentration of 0.046 mg/mL and 75 µL of tetramethylammonium hydroxide (TMAH) into a glass vial. Samples were extracted using an ultrasonic extractor for 30 minutes at room temperature, then centrifuged for 5 minutes at 4000 rpm. 500 µL of extraction was then filtered through Pasteur pipettes, including small pieces of Kimtech paper, and transferred to a vial for GC-MS injection.

Concerning the GCMS parameter, purified helium (Alphagaz 2) was used as the carrier gas in the gas chromatography with a constant flow rate of 1.2 mL/min in the column TR-5MS (Thermo Scientific, France) with a capacity of 30 m x 0.25 mm x 0.25 µm. The oven temperature was maintained at 50°C for 3 minutes, then increased to 270°C at a rate of 5°C/min, and then increased to 330°C at a rate of 15°C/min and lastly held at 330°C for 15 minutes. The injector and ion source temperatures were set to 250°C and 230°C, respectively. The transfer line was set to 250°C. Ionization was done at 70eV, respectively, while the MS was scanned over the 50-800 a.m.u. range. The mass spectrometer was run in full scan mode, and Xcalibur software was used to calculate the area of each peak (Thermo Technologies).

The processing method was built manually by using Xcalibur 2.2 (Thermo Fisher Scientific, USA) to perform peak detection, integration, and retention time (RT) correction. Peaks were collected and categorized across samples based on retention duration and mass fragment mutuality. The chosen peaks should be present in at least three samples and have an average relative content of greater than 0.001% on average. From the raw GC-MS data (.raw or .cdf), an output including representative analytes with m/z fragments and RT, the number of peaks per group, and fragment intensity per sample was produced in the Microsoft Excel format (.xlsx) for the following

identification and filtration. The analyte was identified by comparing its query mass spectra to the reference one in the NIST98 MS data library using its most extensive fragments and the correlation between its RT and retention indices. To determine the analytes retention index (RI), a standard retention indices curve must be generated using the Alkanmix (C_{10} to C_{40}). The automatic RI prediction in the NIST98 MS library version 2.3 was compared to the computed RI and the annotation, which was chosen if the difference between these two RI values was less than 5 units.

Statistical analysis

The statistical analysis was conducted in R language with packages in R studio (http://www.Rproject.org/). The Principal Component Analysis (PCA) was used to visualize the discrimination of the metabolite profiles of flower and leave samples using FactoMineR[38] and ggplot2 along with Factoextra packages.[39] Heatmap and volcano plots were created by using MetaboAnalysis 6.0 (https://www.metaboanalyst.ca/).

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

Acknowledgements

This work was supported by the University of Science and Technology of Hanoi in the framework of emerging research group BEAM.

Author Contribution Statement

P.N.N, H.L.L and E.G performed the experiments including sample collection, analyzed the data, and wrote the morphology and GC-MS based profiling. VA. L. T., N. T. P. M., H.C.H.T contributed to samples treatment and edited the manuscript. KO. N. T performed the experiments, analyzed the data related to LC-MS based chemical composition and wrote/edit the whole manuscript. KO. N. T, E.G conceived and designed the experiments. All authors revised the manuscript.

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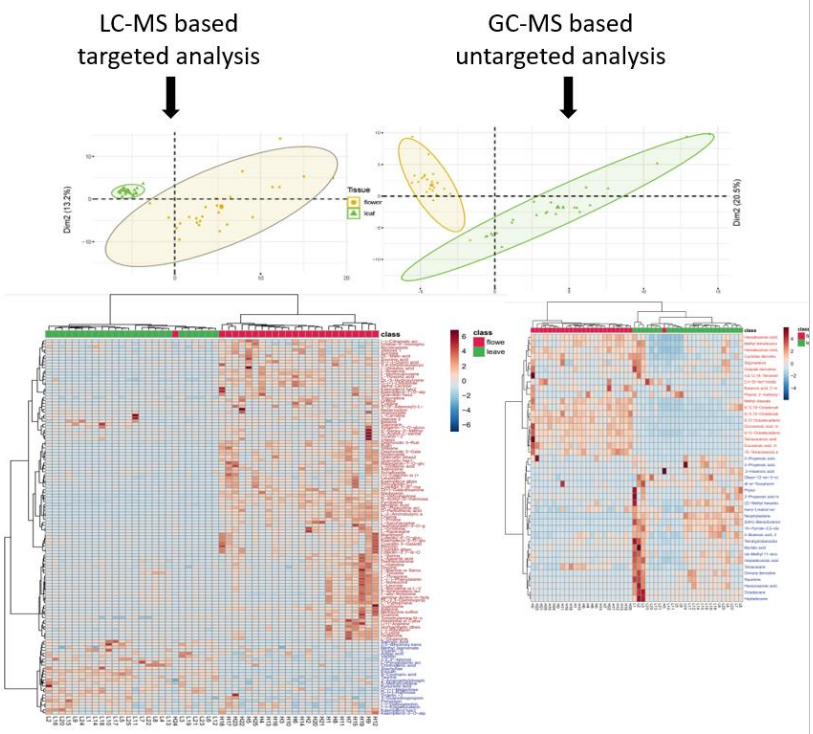
Entry for the Graphical Illustration

Camellia tienii Ninh
leaves and flowers



PHÂN BIỆT HỆ CHẤT CHUYỂN HÓA THEO KHU VỰC ĐỊA LÝ CỦA LOÀI CHÈ HOA VÀNG *CAMELLIA* EUPHLEBIA THU HÁI Ở QUẢNG NINH

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TÓM TẮT

Chè hoa vàng là tên gọi chung cho các loài trà có hoa màu vàng thuộc chi *Camellia* L., họ Chè (Theaceae). Ngoài công dụng làm trà, chè hoa vàng thường được dùng làm thuốc đông y chữa bệnh tăng huyết áp, hạ cholesterol, chống béo phì, bảo vệ gan, giảm đau họng và ngăn ngừa ung thư. Do có giá trị cao đối với sức khỏe con người nên giá trị thương phẩm của chè hoa vàng cao hơn nhiều so với trà xanh. Theo đánh giá của một số nhà thực vật học nước ngoài, vùng Đông Bắc Việt Nam (Quảng Ninh) được coi như là nơi phát sinh của chi *Camellia* nói chung và của các loài chè hoa vàng nói riêng của thế giới. Vài năm gần đây, các mô hình trồng nguyên liệu trà hoa vàng được nhân rộng ở nhiều huyện của tỉnh Quảng Ninh. Mặc dù diện tích trồng đang tăng lên đáng kể, thông tin về thành phần hóa học của các loài được trồng và được thương mại hóa ở các địa phương trên còn nhiều hạn chế. Trong bối cảnh đó, nghiên cứu được thực hiện với mục tiêu áp dụng cách tiếp cận phân tích toàn diện hệ chất chuyển hóa để mô tả thành phần hóa học của các dịch chiết lá chè hoa vàng. Bằng sắc ký lỏng siêu áp kết nối khối phổ ba lần tứ cực UPLC-QqQMS, chúng tôi đã mô tả được 88 chất trong các mẫu *Camellia euphlebia*, cây trồng chủ lực của hai vùng trồng nổi tiếng thuộc huyện Hải Hà và huyện Ba Chẽ, Quảng Ninh. Thêm nữa, nghiên cứu ảnh hưởng của nhiệt độ đến hệ chất chuyển hóa cũng được triển khai để làm cơ sở cho các phương pháp chế biến nguyên liệu. Phân tích thành phần chính PCA cho thấy sự khác biệt có ý nghĩa giữa các mẫu *C. euphlebia* thu ở Hải Hà và Ba Chẽ, mặc dù hai địa phương này thuộc cùng một tỉnh nhưng có khí hậu và thổ nhưỡng tương đối khác biệt. Sự ảnh hưởng của nhiệt độ lên hệ chất chuyển hóa thể hiện tương đối rõ ràng ở các mẫu thu ở Hải Hà, trong khi khác biệt không có ý nghĩa giữa mẫu sấy bằng nhiệt độ với mẫu sấy thăng hoa thu ở Ba Chẽ. Nghiên cứu thăm dò này đã cung cấp phương pháp phân tích có độ tin cậy cao để có thể làm cơ sở cho truy xuất nguồn gốc và chỉ dẫn địa lý, cũng như tối ưu cách chế biến nguyên liệu chè hoa vàng có giá trị kinh tế cao này.

Từ khóa: *chè hoa vàng, Camellia euphlebia, UPLC-QqQMS, PCA, phân tích hệ chất chuyển hóa.*

MỞ ĐẦU

Chi Chè (*Camellia* L.) là một chi thực vật có hoa trong họ Chè Theacea, có nguồn gốc ở khu vực miền đông và miền nam châu Á. Hiện nay, chi này có khoảng 100-250 loài tùy theo hệ thống phân loại, nổi tiếng nhất là các loài chè xanh *Camellia sinensis*, bao gồm 4 thứ: chè Ấn Độ lá to *C. sinensis* var. *assamica*, *C. sinensis* var. *dehungensis*, chè Trung Quốc lá nhỏ var. *sinensis*, chè Shan Tuyết var. *shan*, var. *waldenae*. Ngoài ra, nếu dựa vào hình thái màu sắc hoa thì chi này còn sở hữu số lượng rất lớn các loài chè hoa vàng (Yellow *Camellia* - Golden *Camellia*) là tên gọi chung cho các loài chè có hoa màu vàng. Trên thế giới, các nhà thực vật học đã thống kê có khoảng 52 loài Chè hoa vàng, chủ yếu phân bố ở miền nam Trung Quốc và miền bắc Việt Nam (Liang, 2007). Cũng do đặc tính phân bố tự nhiên của các loài này, nghiên cứu về Chè hoa vàng chủ yếu đến từ các nhà khoa học Trung Quốc và Việt Nam.

Theo đánh giá của một số nhà thực vật học, vùng Đông Bắc Việt Nam được coi như là nơi phát sinh của chi *Camellia* nói chung và của các loài Chè hoa vàng nói riêng của thế giới. Mặc dù các loài đặc hữu thuộc chi Chè rất được quan tâm trong những năm gần đây ở Việt Nam, thông tin về sự đa dạng di truyền và thành phần hóa học của các loài này vẫn còn hạn chế so với tiềm năng chúng mang lại. Một số nghiên cứu ở Việt Nam sử dụng kỹ thuật chiết xuất và phân lập để phân tích thành phần hóa học của 2 loài này đã chỉ ra một số chất như α-spinasteryl, stigmasta-7,22-diene glycoside, kaempferol 3-*O*-[2-*O*-(transp-coumaroyl)-3-*O*-α-D-glucopyranosyl]-α-D-glucopyranoside, aromadendrin, catechin, phlorizin 4'-*O*-β-D-glucopyranoside, (3R, 6R, 7E) -3-hydroxy-4,7-megastigmadien-9-one, dodecanoic acid, 3β-acetoxy-20-lupanol và 3β, 6α, 13β-trihydroxyolean-7-one, (+)-catechin, (-)-epicatechin, quercetin, quercetin-3-*O*-methyl ether và kaempferol (Nguyen *et al*., 2019). Ngoài ra, các lớp chất chính như saponin, flavonoid, polyphenol được định tính đơn giản bằng các phản ứng màu (Manh *et al*., 2019, Trinh *et al*., 2022). Gần đây, nghiên cứu của Ninh Khắc Thanh Tùng đã chỉ ra 3 flavonoid glycosides mới được phân

lập từ lá của loài *C. phanii* và các chất này thể hiện hoạt tinh ức chế α-glucosidase (Ninh Khắc Thanh Tùng *et al*., 2024).

Vài năm gần đây, các mô hình trồng nguyên liệu chè hoa vàng được nhân rộng ở nhiều địa phương trên cả nước như Ba chẽ (Quảng Ninh), Lục Ngạn (Bắc Giang), Tam Đảo, Tam Dương (Vĩnh Phúc) Đà Lạt (Lâm Đồng) với diện tích lên đến hàng ngàn hecta. Tuy nhiên, hiện chưa thấy có công bố khoa học nào về thành phần hóa học của các loài Chè hoa vàng đang được trồng và được thương mại hóa ở các địa phương. Bên cạnh công tác bảo tồn nguồn gen thì việc nghiên cứu sâu về hệ chất chuyển hóa trong các loài chè quý này là rất quan trọng và cung cấp thông tin cơ bản cho nhiều định hướng như phân loại thực vật dựa vào thành phần hóa học, làm cơ sở chọn vùng phát triển nguồn nguyên liệu.

NGUYÊN VẬT LIỆU VÀ PHƯƠNG PHÁP

Nguyên vật liệu

Camellia euphlebia Merr. ex Sealy (mã HH2) thu ở vùng trồng thuộc xã Quang Minh, huyện Hải Hà, tỉnh Quảng Ninh (GPS: 21°28'45.9"N 107°46'07.8"E). *Camellia euphlebia* Merr. ex Sealy (mã BC1) được thu ở xã Đạp Thanh, huyện Ba Chẽ, tỉnh Quảng Ninh (GPS: 21.287036N, 107.113351E). Với mỗi mẫu trên, các bộ phận lá non, lá trưởng thành, hoa, quả, hạt được thu thập để phục vụ định danh bởi TS Nguyễn Thế Cường, Viện Sinh thái và Tài nguyên sinh vật, Viện Hàn lâm Khoa học và Công nghệ Việt Nam. Hồ sơ tiêu bản được lưu trữ tại Khoa Khoa học Sự sống, Trường Đại học Khoa học và Công nghệ Hà Nội để phục vụ cho các nghiên cứu tiếp theo.

Xử lý mẫu

Lá của các loài chè được thu hái và rửa bằng nước cất để loại bỏ hoàn toàn bụi bẩn. Để phân biệt hệ chất chuyển hóa theo cách chế biến lá sau thu hoạch, 2 cách xử lý mẫu được áp dụng với mỗi nửa phần lá. Sau khi lau, mỗi lá được cắt đoạn 1 cm và chia thành 2 phần. Phần sấy khô ở 50°C, phần được sấy thăng hoa cho đến khi khối lượng không thay đổi. Sau đó, các mẫu được lắc bằng hạt zirconium với tần số 25 lần/giây trong 2 phút thành bột mịn bằng máy đồng nhất MM400 (Retsch, Đức), rồi lưu ở nhiệt độ -80^{o}C để dùng cho các phân tích tiếp theo.

Bảng 1. Thông tin mẫu lá chè hoa vàng.

Mã	Loài	Cách xử lý	Số lượng	Nơi thu mẫu
HHLK2.1-2.10	*C. euphlebia*	Lá sấy khô ở nhiệt độ cao	10	Hải Hà, Quảng Ninh
HHLT2.1-2.10	*C. euphlebia*	Lá sấy thăng hoa	10	Hải Hà, Quảng Ninh
BCLK1.1-1.10	*C. euphlebia*	Lá sấy khô ở nhiệt độ cao	10	Ba Chẽ, Quảng Ninh
BCLT1.1-1.10	*C. euphlebia*	Lá sấy thăng hoa	10	Ba Chẽ, Quảng Ninh

Phân tích hệ chất chuyển hóa bằng sắc ký lỏng khối phổ UPLC-QqQMS

Quá trình chiết xuất các chất chuyển hóa được thực hiện bằng cách cân chính xác 4 mg các mẫu bột lá vào ống 2 ml chứa hạt zirconia 5 mm. Sau đó, 1 ml hỗn hợp dung môi bao gồm axit formic 0,1% (v/v), metanol 80% (v/v) và hai chất chuẩn nội (8,4 nM lidocain cho ion hóa âm và 210 nM acid 10-camphorsulfonic cho ion hóa dương) được thêm vào mỗi ống. Quá trình chiết được thực hiện trong 2 phút ở tốc độ 2.000 × g (1.000 vòng/phút) bằng máy đồng hóa mẫu. Dịch chiết được ly tâm ở tốc độ 20.000 x g (10.000 vòng/phút) trong 1 phút để loại bỏ cặn. Dung dịch thu được được lọc qua màng 0,45 µm (MZHVN0W50; Merck Millipore, Đức) và 1 µl dung dịch được bơm vào hệ thống sắc ký lỏng siêu áp kết nối phổ khối ba lần tứ cực (UPLC-QqQ-MS). Phân tích hệ chất chuyển hóa được thực hiện theo phương pháp của Sawada và Uchida (Sawada *et al*., 2009; Uchida *et al*., 2020). Gradient pha động được cài đặt như trình bày ở Bảng 2, với tốc độ dòng 0.3 ml/phút. Các điều kiện phát hiện bằng khối phổ bao gồm điện áp CV, năng lượng va chạm CE và kiểu ion hóa của mỗi chất chuẩn trong thư viện đã được áp dụng để xác định các chất chuyển hóa trong các mẫu chè. Giá trị diện tích cực đại của mỗi chất được xác định bằng phần mềm MRMPROBS (Tsugawa *et al*., 2013).

Xử lý dữ liệu và phân tích đa biến

Phân tích thành phần chính (PCA) và phân tích bình phương tối thiểu (PLS-DA) biểu thị sự phân biệt hệ chất chuyển hóa của các mẫu lá theo vùng địa lý thu hái và ảnh hưởng của nhiệt độ lên hệ chất chuyển hóa được thực hiện bằng cách sử dụng MetaboAnalysis 6.0 (https://www.metaboanalyst.ca/).

Bảng 2. Điều kiện phân tách bằng sắc ký lỏng UPLC. Dung môi A : H2O+0.1%FA, Dung môi B: MeOH.

Thời gian (phút)	Tốc độ dòng (ml/phút)	Dung môi A (%)	Dung môi B (%)
0	0,3	75	25
1	0,3	70	30
2,50	0,3	70	30
2,51	0,3	75	25
5,51	Stop	75	25

KẾT QUẢ VÀ THẢO LUẬN

Hệ chất chuyển hóa của *C. petelotii* và *C. euphlebia*

Tổng cộng có 440 hợp chất đã được phát hiện trong ít nhất 1 mẫu bằng phương pháp phân tích chuyển hóa mục tiêu (widely targeted metabolomics). Cách tiếp cận này mang lại mức độ tin cậy cao vì các điều kiện nhận dạng ion và phân mảnh đã được điều chỉnh và tối ưu hóa với các chất chuẩn tham chiếu. Sau khi chọn các peak có tỷ lệ tín hiệu trên đường nền (S/N) lớn hơn 3 và trị số sai số tương đối (RSD) < 30%, đồng thời các peak cần xuất hiện trong ít nhất 3 mẫu, 88 chất chuyển hóa đã được chọn làm hệ chất chuyển hóa chung của 40 dịch chiết lá chè hoa vàng. Bảng 3 biểu thị các chất chuyển hóa và các thông số phổ khối tương ứng của chúng.

Bảng 3. Danh mục chất chuyển hóa được phát hiện trong các mẫu lá chè hoa vàng bằng phương pháp phân tích UPLC-QqQMS.

TT	Tên chất	Kiểu ion hóa	Mảnh mẹ	Mảnh con	RT	Nhóm chất
1	L-(-)-Phenylalanine	positive	166,1	120,1	1,267	Amino acid
2	L-Tryptophane	positive	205,1	188,15	1,353	Amino acid
3	L-Tyrosine	positive	182,1	165,1	0,597	Amino acid
4	Sucrose	negative	341,1	88,95	0,256	Sugar
5	L-Asparagine	positive	133,05	87,1	0,193	Amino acid
6	L-Aspartic acid	positive	134,05	74,05	0,189	Amino acid
7	L-Glutamic acid	positive	148,05	84,1	0,203	Amino acid
8	L-Glutamine	positive	147,1	130,05	0,17	Amino acid
9	L-Proline	positive	116,05	70,15	0,222	Amino acid
10	L-Pyroglutamic acid	positive	130,05	84,1	0,466	Amino acid
11	L-Serine	positive	106,05	60,15	0,193	Amino acid
12	3,4-Dihydroxybenzoic acid	negative	153	109,05	1,336	Phenol
13	Succinic acid	negative	117	73	0,547	Carboxylic acid
14	alpha-Lactose	negative	341,1	161,05	0,206	Sugar
15	gamma-Amino-n-butyric acid	positive	104,05	87,1	0,202	Amino acid
16	Naringenin	positive	273,1	153	1,629	Flavonoid (flavanone)
17	Apigenin-7-O-glucoside	positive	433,1	271,05	1,477	Flavonoid (flavone)
18	Rhoifolin	positive	579,15	271,05	1,459	Flavonoid (flavone)

19	Isorhamnetin-3-O-glucoside	positive	479,1	317	1,471	Flavonoid (flavonol)
20	Kaempferol-3-O-glucoside	positive	449,1	287,05	1,47	Flavonoid (flavonol)
21	Homoorietin	positive	449,1	299,05	1,4	Flavonoid (flavone)
22	Naringenin-7-O-glucoside	positive	435,15	273,05	1,486	Flavonoid (flavanone)
23	Hyperoside	positive	465,1	303	1,44	Flavonoid (flavonol)
24	4-Coumaric acid	positive	165,05	91,1	1,471	Hydroxycinnamic acid
25	Kaempferol-7-O-alpha-L-rhamnoside	positive	433,1	287,05	1,553	Flavonoid (flavonol)
26	D-(-)-Quinic acid	negative	191,05	85,05	0,228	Carboxylic acid
27	Chlorogenic acid	negative	353,1	191,1	1,377	Phenol
28	L-allo-threonine	positive	120,05	56,1	0,198	Amino acid
29	Methyl jasmonate	positive	225,15	151,1	1,78	Carboxylic acid
30	Vanillin	positive	153,05	65,15	1,49	Phenol
31	5-Aminovaleric acid	positive	118,1	55,1	0,255	Carboxylic acid
32	Luteolin-3',7-di-O-glucoside	positive	611,15	287	1,4	Flavonoid (flavone)
33	Luteolin-4'-O-glucoside	positive	449,1	287,05	1,478	Flavonoid (flavone)
34	Neoeriocitrin	negative	595,15	151	1,431	Flavonoid (flavanone)
35	Phloridzin	negative	435,15	273,1	1,5	Flavonoid (dihydrochalcone)
36	Poncirin	negative	593,2	285,05	1,552	Flavonoid (flavanone)
37	Saponarin	positive	595,15	283	1,39	Flavonoid (flavone)
38	Vitexin	positive	433,1	313,05	1,437	Flavonoid (flavone)
39	S-(5'-Adenosyl)-L-methionine	positive	399,15	250,1	0,21	Amino acid
40	Kaempferol-3-O-alpha-L-arabinoside	positive	419,1	287,05	1,491	Flavonoid (flavonol)
41	2,5-dihydroxy benzoic acid	negative	153	108,05	1,407	Phenol
42	Salicylic Acid	negative	137	93,05	1,577	Phenol
43	Kynurenic acid	positive	190,05	144,05	1,374	Carboxylic acid
44	Trigonelline	positive	138,05	92,1	0,226	Alkaloid
45	DL-Pipecolinic acid	positive	130,1	84,15	0,312	Carboxylic acid
46	D-(+)-Raffinose	negative	503,15	179,05	0,321	Sugar
47	Betaine	positive	118,1	58,15	0,205	Alkaloid
48	Procyanidin C1	negative	865,2	125	1,404	Flavonoid (flavan-3-ols)
49	Quercetin-3,4'-O-di-beta-glucopyranoside	negative	625,15	463,1	1,396	Flavonoid (flavonol)
50	Esculin	positive	341,1	179,05	1,366	Coumarin
51	Stachyose	negative	665,2	383,05	0,305	Sugar
52	L-Leucine	positive	132,2	30,1	0,514	Amino acid

53	L-Isoleucine	positive	132,1	69,1	0,528	Amino acid
54	Methionine sulfoxide	positive	165,95	74,05	0,196	Amino acid
55	Aureusidin	positive	287,05	153,05	1,473	Flavonoid (aurone)
56	Glutamine	positive	147,1	84,1	0,18	Amino acid
57	Isorhamnetin-dihexose	positive	625,2	317,05	1,453	Flavonoid (flavonol)
58	Kaempferol-dihexose	negative	593,15	285,05	1,451	Flavonoid (flavonol)
59	Kaempferol-3,7-dihexose	negative	577,15	285	1,431	Flavonoid (flavonol)
60	Kaempferol-3-O-alpha-L-rhamnoside	negative	431,1	285	1,517	Flavonoid (flavonol)
61	Maritimein	positive	449,1	287,05	1,44	Flavonoid (flavone)
62	Quercetin-3-Arabinoside	positive	435,1	303,05	1,473	Flavonoid (flavonol)
63	Quercetin-3-Rhamnoside	negative	447,1	300	1,481	Flavonoid (flavonol)
64	Myricitrin	negative	463,1	316,15	1,446	Flavonoid (flavonol)
65	(+)-Catechin/(+)-Epicatechin	positive	291,1	139	1,417	Flavonoid (flavan-3-ols)
66	D-(+)-Cellobiose	negative	341,1	161,1	0,232	Sugar
67	Melibiose	negative	341,1	179,1	0,214	Sugar
68	L-Iditol	positive	183,1	69,1	0,202	Sugar
69	L(+)-Arginine	positive	175,1	70,15	0,187	Amino acid
70	L-Alanine or Sarcosine	positive	90,05	44,1	0,195	Amino acid
71	L-Norvaline or L-Valine	positive	118,1	72,1	0,291	Amino acid
72	L-Threonine	positive	120,05	74,1	0,198	Amino acid
73	L-2-Aminobutyric acid	positive	104,05	44,15	0,201	Amino acid
74	DL-Malic acid	negative	133	115	0,287	Carboxylic acid
75	Adipic acid	negative	145,05	83	1,36	Carboxylic acid
76	(-)-Citramalic acid	negative	147,05	87	0,576	Carboxylic acid
77	L-Lysine	positive	147,1	84,1	0,18	Amino acid
78	Nicotinic Acid	positive	124,05	78,05	0,355	Carboxylic acid
79	Rutin	negative	609,15	300	1,432	Flavonoid (flavonol)
80	Schaftoside	positive	565,15	547,05	1,391	Flavonoid (flavone)
81	(-)-Epigallocatechin	positive	307,05	139,05	1,302	Flavonoid (flavan-3-ols)
82	(-)-Gallocatechin	positive	307,05	139,05	1,302	Flavonoid (flavan-3-ols)
83	Delphinidin 3-Rutinoside	positive	612,15	304	1,337	Flavonoid (anthocyanin)
84	Delphinidin 3-Galactoside	negative	464,1	301,05	1,33	Flavonoid (anthocyanin)

85	Cyanidin 3-Galactoside	negative	448,1	285	1,344	Flavonoid (anthocyanin)
86	Vicenin -1	positive	565,15	427,15	1,356	Flavonoid (flavone)
87	Vicenin -2	negative	593,15	353,05	1,337	Flavonoid (flavone)
88	Vicenin -3	positive	565,15	547,15	1,358	Flavonoid (flavone)

Hệ chất chuyển hóa lá chè hoa vàng *C. euphlebia* được chiếm ưu thế bởi các flavonoid tương ứng với các khung aglycone khác nhau như anthocyanin, aurone, chalcone, flavan-3-ol, flavanone, flavone và flavonol. Trong số đó, flavonol chiếm ưu thế trong thành phần flavonoid với 14 chất là dẫn xuất đường của khung kaempferol, quercetin, isorhamnetin và myricetin. Tất cả các flavonol đều là glycoside ở vị trí nguyên tử O, thường ở vị trí 3 và/hoặc 7, được gắn với glucose, rhamnose, arabinose hoặc rutinose. Ngoài ra, flavones là nhóm có hàm lượng ưu thế thứ hai trong hệ chất này với 12 đại diện. Hầu hết các flavon được phát hiện trong các mẫu thử nghiệm đều là dẫn xuất apigenin và luteolin. Apigenin glycoside bao gồm dẫn xuất 7-O-glycoside của glucose và neohesperidose. Đặc biệt, 5 flavonoid chứa khung apigenin thuộc loại C-glycoside ở vị trí 6 và/hoặc 8. Các flavanone là dẫn xuất O-glycoside của khung naringenin, eriodictyol và isosakuranetin ở vị trí số 7. Anthocyanin chủ yếu là dẫn xuất oxy hóa của cyanidin và delphinidin ở vị trí 3 và 5. Sự phức tạp trong cấu trúc của aglycon và cách kết hợp đa dạng với các đơn vị glycoside chứng tỏ con đường sinh tổng hợp các flavonoid diễn ra khá phức tạp trong lá chè hoa vàng *C. euphlebia* với sự tham gia của nhiều enzyme.

Ngoài ra, một số phenol thuộc loại dẫn xuất acid benzoic và acid hydrocinnamic, coumarin và stilbenoid, đã được phát hiện trong các dịch chiết này. Vanillin, 3,4-dihydroxybenzoic acid, chlorogenic acid, 2,5-dihydroxy benzoic acid, salicylic acid, 4-coumaric acid, esculin, E-3,4,5'-trihydroxy-3'-glucopyranosylstilbene là các hợp chất phenolic quan trọng về mặt sinh học có trong nhiều loài thực vật. Các chất này được biết đến với nhiều vai trò sinh lý trong thực vật như chống oxy hóa, kháng khuẩn do vậy cũng được sử dụng như các tác nhân chống viêm, chống béo phì, trị đái tháo đường và cải thiện chức năng tim mạch cho con người (Wang *et al*., 2022, Kabir *et al*., 2014, Li *et al*., 2022).

Một nhóm chất phổ biến trong chè hoa vàng *C. euphlebia* là các amino acid. Đây cũng là nhóm chất quan trọng của chi *Camellia* và được coi là đóng vai trò quan trọng trong việc tạo ra mùi vị và hương thơm cho các sản phẩm chè. Cấu trúc và hàm lượng các amino acid tự do không chỉ góp phần trực tiếp tạo nên hương vị của trà mà còn ảnh hưởng đến các hoạt tính của trà thành phẩm bởi các sản phẩm biến đổi của chúng. Vì amino acid là tiền chất của các aldehyd dễ bay hơi và các hợp chất khác trong quá trình pha trà nên chúng đóng vai trò quan trọng trong quá trình hình thành mùi thơm của trà. Ngoài ra, amino acid là các đơn vị cơ bản của protein, cũng là những tiền chất quan trọng tạo nên các enzyme có hoạt tính và các hợp chất thứ cấp có hoạt tính sinh học khác (Tzin, Galili, 2010). Trong nghiên cứu này, chúng tôi đã phát hiện 23 amino acid và các dẫn xuất trong các mẫu lá *C. euphlebia*, bao gồm một số loại axit amin có hoạt tính sinh học trên hệ thần kinh trung ương như gamma amino butyric acid, L-glutamine và một số loại acid amin thiết yếu khác như L-proline, glycine, leucin, isoleucin, L-valine, L-methionine, L-isoleucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-arginine,...

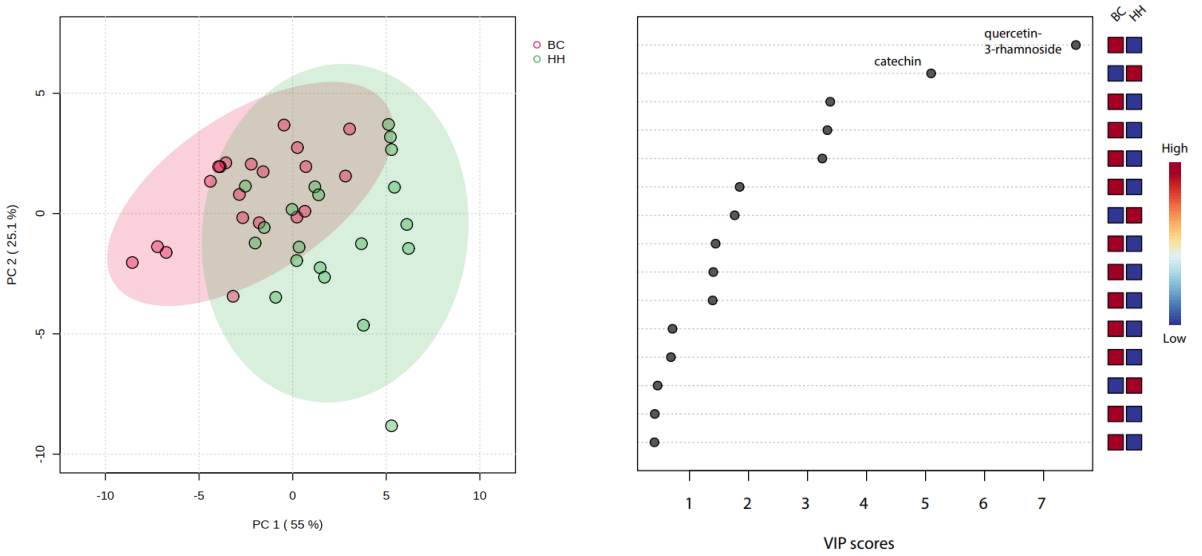
Sự có mặt đa dạng của các loại đường đơn trong hệ chất chuyển hóa của chè hoa vàng *C. euphlebia* cũng góp phần giải thích hương vị của sản phẩm được tạo ra từ lá chè hoa vàng. Các hợp chất chứa nitơ khác, chẳng hạn như trigonelline và betaine, cũng có mặt trong hệ chất chuyển hóa của *C. euphlebia*. Trong nghiên cứu trước đây, những chất này thể hiện tác dụng chống đái tháo đường, chống ung thư và bảo vệ thần kinh, do đó có thể cung cấp nguồn hoạt chất tốt cho các sản phẩm từ chè hoa vàng (Liang *et al*., 2023, Ueland, 2011).

Phân biệt hệ chất chuyển hóa của *C. euphlebia* theo vùng địa lý

Hệ chất chuyển hóa của các mẫu lá cùng loài *C. euphlebia* nhưng thu ở các địa điểm khác nhau (huyện Hải Hà và huyện Ba Chẽ, thuộc tỉnh Quảng Ninh) được đưa vào mô hình PCA để phân biệt. Kết quả cho thấy tuy cùng loài và địa điểm thu hái ở cùng một tỉnh, nhưng hệ chất chuyển hóa của các mẫu này tương đối khác biệt (Hình 1). Thành phần chính 1 thể hiện 55% độ dao động dữ liệu, thành phần chính 2 thể hiện 25.1% độ dao động dữ liệu, chứng tỏ mô hình có thể mô tả tốt sự phân biệt giữa các lá được thu hái từ huyện Hải Hà và huyện Ba Chẽ. Kết quả gợi mở sự khác biệt về độ cao và thổ nhưỡng, khí hậu của từng vùng trồng khác nhau có thể đem đến sự khác biệt về mặt

chuyển hóa và hàm lượng các chất có tác dụng sinh học.

Hơn nữa, mô hình phân tích bình phương tối thiểu PLS-DA cho thấy sự phân biệt tốt hơn giữa hai nhóm mẫu thu ở Hải Hà và Ba Chẽ. Chỉ số VIP thể hiện sự đóng góp của 2 chất quercetin-3-rhamnoside, catechin vào mô hình là lớn nhất, cho thấy hàm lượng các chất này chính là tiêu chí để phân biệt loài này theo vùng trồng.

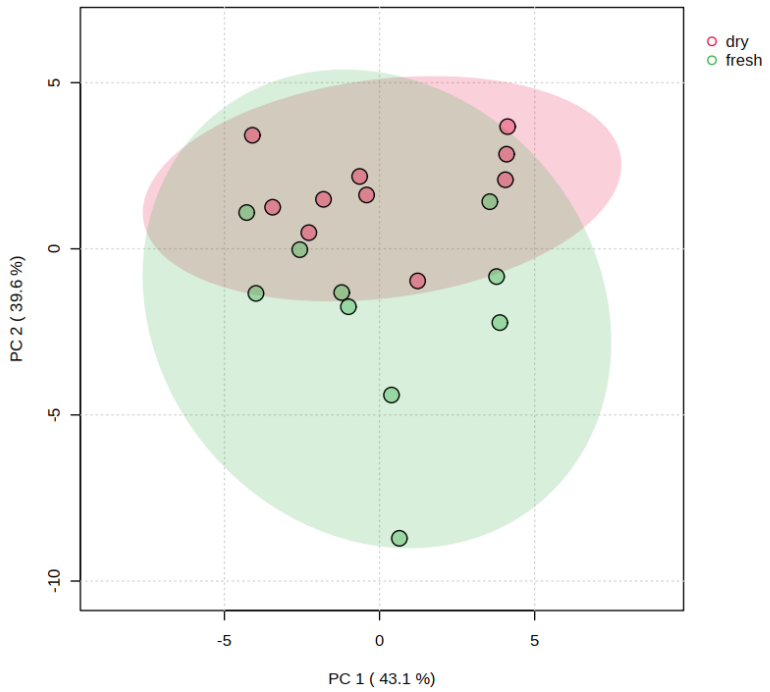


Hình 1. Biểu đồ PCA các mẫu lá *C. euphlebia* thu ở Hải Hà và Ba Chẽ (Quảng Ninh) và biểu đồ VIP scores các chất đóng góp cho mô hình phân biệt.

Phân biệt hệ chất chuyển hóa của *C. euphlebia* theo nhiệt độ chế biến

Để xem xét ảnh hưởng của cách chế biến lá tươi lên hệ chất chuyển hóa của *C. euphlebia*, phân tích thành phần chính PCA được áp dụng cho các mẫu lá thu ở Hải Hà và Ba Chẽ. Điều thú vị là đối với cùng loài thu ở Hải Hà, sự khác biệt tương đối có ý nghĩa thống kê, với giá trị p-value < 0,05 được chỉ ra ở biểu đồ Hình 2 trong khi các mẫu thu ở Ba Chẽ, sự khác biệt giữa hai nhóm mẫu là không có ý nghĩa (P-value > 0,05). Điều này có nghĩa là nhiệt độ có ảnh hưởng đến con đường chuyển hóa của các nhóm chất ở các mẫu *C. euphlebia* ở Hải Hà, trong khi lại ít ảnh hưởng hơn đến cùng loài này thu ở Ba Chẽ. Tuy nhiên đây mới chỉ là những kết quả thăm dò ban đầu và cần sử dụng cỡ mẫu lớn hơn để khẳng định giả thuyết đưa ra.

Sấy thăng hoa và sấy ở nhiệt độ cao là hai cách chế biến/bảo quản thường gặp sau thu hoạch các sản phẩm nông nghiệp nói chung và chè hoa vàng nói riêng. Trong đó, sấy thăng hoa là phương pháp hiện đại, hầu như giữ nguyên được màu sắc, hương vị của nguyên liệu còn sấy khô ở nhiệt độ cao là phương pháp rẻ tiền, đơn giản và hiệu suất cao. Thông thường, sấy thăng hoa được áp dụng cho các nguyên liệu nhạy cảm với nhiệt độ còn sấy ở nhiệt độ cao được áp dụng cho hầu hết các loại nguyên liệu và nhất là khi được chứng minh rằng nhiệt độ giúp tăng cường chuyển hóa các hợp chất thứ cấp có hoạt tính sinh học. Ở trong trường hợp cây chè hoa vàng *C. euphlebia* ở Ba Chẽ, kết quả phân tích chỉ ra rằng nhiệt độ không ảnh hưởng đến hệ chất chuyển hóa, gợi ý rằng chỉ cần sử dụng các phương pháp sấy ở nhiệt độ cao mà vẫn mang lại hiệu quả tốt. Ngược lại với nhóm mẫu thu ở Hải Hà, cần có những nghiên cứu sâu hơn về cơ chế tác động của nhiệt độ lên hệ chất chuyển hóa của lá chè hoa vàng, liệu có những con đường chuyển hóa nào bị tác động bởi nhiệt độ, từ đó đưa ra các chỉ dẫn cho bà con nông dân về cách chế biến sau thu hoạch. Đây là nghiên cứu đầu tiên đánh giá tác động của nhiệt độ lên hệ chất chuyển hóa của *C. euphlebia* tiến hành ở các khu trồng chè hoa vàng lớn nhất của tỉnh Quảng Ninh.



Hình 2. Biểu đồ PCA các mẫu lá *C. euphlebia* thu ở Hải Hà (Quảng Ninh) theo hai cách sấy khô khác nhau. Màu hồng là các mẫu sấy ở nhiệt độ cao, màu xanh là các mẫu sấy thăng hoa.

KẾT LUẬN

Sử dụng cách tiếp cận phân tích hệ chất chuyển hóa bằng sắc ký lỏng siêu áp kết nối phổ khối, nghiên cứu đã chỉ ra hệ chất gồm 88 chất bao gồm phần lớn là các flavonoid và amino acid trong các mẫu lá chè *C. euphlebia* thu được từ hai vùng trồng nổi tiếng thuộc huyện Hải Hà và huyện Ba Chẽ của tỉnh Quảng Ninh. Chúng tôi đã so sánh hệ chất chuyển hóa của loài chè hoa vàng này theo vị trí địa lý và cho thấy ảnh hưởng tương đối rõ ràng của điều kiện thổ nhưỡng và khí hậu đến hàm lượng hai chất chuyển hóa quercetin-3-rhamnoside và catechin đóng vai trò là chất chỉ thị phân biệt. Ngoài ra, nghiên cứu thăm dò này cũng mô tả tác động của nhiệt độ lên con đường chuyển hóa của một số hợp chất trong lá *C. euphlebia* thu ở Hải Hà dẫn đến hệ chất chuyển hóa có sự thay đổi đáng kể. Nghiên cứu này góp phần cung cấp cơ sở cho việc phát triển các vùng trồng trọt tối ưu cho cây chè hoa vàng C. euphlebia và cách chế biến phù hợp để đảm bảo hàm lượng hoạt chất trong nguyên liệu có giá trị này.

Lời cảm ơn

Nghiên cứu được thực hiện bằng kinh phí tài trợ của Trường Đại học Khoa học và Công nghệ Hà Nội, nhóm nghiên cứu tiềm năng BEAM.

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GEOGRAPHICAL DISCRIMINATION OF METABOLITE PROFILING OF CAMELLIA EUPHLEBIA COLLECTED IN QUANG NINH

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SUMMARY

Golden *Camellia* is the general name for tea species with yellow flowers belonging to the genus *Camellia* L., family Theaceae. In addition to its use as tea, golden *Camellia* is often used traditionally to treat high blood pressure, lower cholesterol, obesity, liver protection, relieve sore throat, and prevent cancer. Due to its high value to human health, the commercial value of golden *Camellia* is much higher than that of green tea. The Northeast region of Vietnam (Quang Ninh) is considered the birthplace of the genus *Camellia* and of golden *Camellia* in the world. In recent years, many golden *Camellia* growing field have been widely developing in this region. Although the cultivation area is increasing significantly, little information on the chemical composition of these species has been found in literature. In that context, the study aims to apply a comprehensive metabolomics approach to describe the chemical composition of tea leaf extracts. Using ultra performance liquid chromatography coupled with triple quadrupole mass spectrometry UPLC-QqQMS, we characterized 88 compounds in *Camellia euphlebia*, a major crop in two famous growing areas in Hai Ha and Ba Che districts, Quang Ninh. Principal component analysis (PCA) showed significant differences between *C. euphlebia* samples collected in Hai Ha and Ba Che, although these two localities are in the same province but have relatively different climates and soils. In addition, the impact of processing temperature on metabolite profile of these raw materials has been determined. The influence of temperature on the metabolomics was relatively clear in samples collected in Hai Ha, while there was no significant difference between the oven-dried and the freeze-dried samples of Ba Che. This exploratory study has provided a highly reliable analytical method that can serve as a basis for traceability and geographical indications, as well as optimizing the processing of this valuable golden flower raw material.

Keywords: *golden Camellia, Camellia euphlebia, UPLC-QqQMS, PCA, metabolomics.*