HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

الجمعية السعودية لعلوم الحياة
SAUDI BIOLOGICAL SOCIETY

Original article

Morphological, phytochemical and genetic characterization of *Centella asiatica* accessions collected throughout Vietnam and Laos

Hai Thi Hong Truong [a,*], Nhi Thi Hoang Ho [a], Han Ngoc Ho [a], Bao Le Quy Nguyen [a], Minh Hoang Duy Le [a], Thuy Thanh Duong [b]

[a] *The Institute of Biotechnology, Hue University, Thua Thien Hue, Viet Nam*
[b] *Hue University of Agriculture and Forestry, Hue University, Thua Thien Hue, Viet Nam*

ARTICLE INFO

Keywords:
Pennywort
Germplasm
Flavonoid
Phenolic compounds
SSR

ABSTRACT

Pennywort (*Centella asiatica* L.) is commonly grown in the tropical world for its nutritional and medicinal values. Valuable saponins in pennywort are extensively investigated for their anti-tumour activities. The diversity in morphology, phytochemical contents and genetics among pennywort accessions has been extensively studied to identify elite landraces for large-scale production. While pennywort is widely consumed in Vietnam, a systematic characterization of their diverse morphology, secondary metabolites and genetics is lacking. In this work, 26 pennywort accessions were collected across Vietnam and Laos. Their morphological features and yields were characterized under uniform agro-climatic conditions at Hue city in central Vietnam. The highest yield was obtained with HUIB_CA20 (478 g per tray), compared to the lowest yield in HUIB_CA19 (107 g per tray). Furthermore, a range of phytochemical markers, including vitamin C, reducing sugar, carotenoid, tannin, phenolic, flavonoid and saponin contents, were determined. Based on yield, phenolic and flavonoid contents, HUIB_CA20 and HUIB_CA27 were determined to be elite cultivars in this germplasm. Finally, microsatellite analysis was performed to explore the genetic diversity within the germplasm. Using fourteen SSR primer pairs, a total of 47 alleles were identified with 45 alleles (96 %) being polymorphic. These results will be useful for breeding programs aiming to create elite pennywort cultivars with enhanced properties.

1. Introduction

Pennywort (*Centella asiatica* L.) is a herb that is commonly consumed in South China, Southeast Asia and South Asia. For centuries, this herb is used to treat a range of ailments (Brinkhaus et al., 2000), including chronic venous insufficiency, striae gravidarum, wound healing and skin diseases (Gohil et al., 2010; Tanga et al., 2022). More than 130 secondary metabolites have been isolated from pennywort, including at least 18 flavonoids and 13 phenolic compounds (Kunjumon et al., 2022a). Some of these compounds display anti-inflammatory (Park et al., 2017), antioxidant, antibiotic and antiviral properties (Kunjumon et al., 2022a; Mudaliana, 2021; Pasri et al., 2023; Wong & Ramli, 2021). Nanoparticles prepared with pennywort extracts were also shown to display antibacterial effects (Saikia et al., 2015; Eze et al., 2019). Notable secondary metabolites from pennywort include asiatic acid, asiaticoside, madecassoside and madecassic acid (Sun et al., 2020). Madecassic acid and its derivatives displayed anti-tumour activities on a range of solid and hematological tumors (Hussin et al., 2014; Valdeira et al., 2019).

Efforts to identify elite genotypes of *C. asiatica* for industrial production require first to collate a germplasm of pennywort. A common theme is the morphological and genetic diversity among pennywort accessions in germplasms collected throughout India (Prasad et al., 2014; Ravi et al., 2019), Madagascar (Rakotondralambo et al., 2013), Iran (Nav et al., 2021) and Myanmar (Shukurova et al., 2021). Furthermore, morphological diversity is accompanied with variations in yield and quality of *Centella asiatica* (Rohini & Smitha, 2022). Combining morphological and molecular markers helps more accurately distinguish accessions. Different molecular markers have been used to assess genetic diversity of *C. asiatica* accessions such as RAPD (Krishnan et al., 2007; Padmalatha & Prasad, 2008), AFLP (Prasad et al., 2014), SSR (Sakthipriya et al., 2018; Rakotondralambo et al., 2013; Rakotondralambo et al., 2012; Rohini et al., 2019) and ISSR (Zhang et al., 2011).

Multiple efforts have been made to identify elite pennywort

* Corresponding author.
E-mail addresses: tthhai@hueuni.edu.vn (H.T.H. Truong), hothihoangnhi@hueuni.edu.vn (N.T.H. Ho), hongochan@hueuni.edu.vn (H.N. Ho), nlqbao@hueuni.edu.vn (B.L.Q. Nguyen), lhdminh@hueuni.edu.vn (M.H.D. Le), duongthanhthuy@huaf.edu.vn (T.T. Duong).

https://doi.org/10.1016/j.sjbs.2023.103895
Received 30 June 2023; Received in revised form 24 November 2023; Accepted 1 December 2023
Available online 3 December 2023

accessions that are high-yielding and contain elevated levels of beneficial phytochemicals (Prasad et al., 2014; Prasad et al., 2016; Singh et al., 2022; Kunjumon et al., 2022b). Similarly, several elite accessions with high centelloside contents were identified in Western Ghats and Deccan Plateau, India (Singh et al., 2022). More recently, Kunjumon and co-workers (2022b) described six elite lines of *C. asiatica* from south India, with their combined asiaticoside and madecassoside contents above the industrial benchmark (≥4%). On the other hand, endophytic fungi from *Centella asiatica* leaves were found to produce asiaticoside, raising the possibility that changing the microbiome associated with different ecotypes is responsible for different secondary metabolite levels measured in pennywort (Gupta et al., 2018).

Vietnam has a large pennywort production industry and pennywort is commonly used in cooking, making juice and in traditional medicine. However, each region in the country grows their own local landraces and systematic studies to identify elite *C. asiatica* accessions in Vietnam has been lacking. This hampers future efforts to breed advanced cultivars which are more high-yielding and richer in bioactive compounds. Towards these goals, this study aimed to build a germplasm of pennywort collected throughout Vietnam and its neighbouring country, Laos. The morphology, phytochemical contents and genetic diversity among the germplasm were studied. To our knowledge, this is the first systematic characterisation of pennywort accessions distributed in this region.

2. Materials and methods

2.1. Plant collection

The germplasm included 26 pennywort accessions (Table 1), collected from farms and meadows in seventeen different geographical locations across Vietnam (24 accessions) and Laos (2 accessions).

2.2. Soil preparation and plant cultivation

The soil was collected from Quang Tho, Thua Thien Hue (16°32′06.2″N, 107°31′39.7″E), a well-known pennywort production area in Vietnam. Measurements of electrolytic conductivity, pH, total C, available phosphorus, total N, water holding capacity, humidity and textural characteristics of the soil were performed as previously described (Ruíz-Valdiviezo et al., 2010). The soil had a pH of 6.0, low total N, P, K, available P, OM, Ca^{2+} and pH_{KCl} but medium available N, K and CEC (Table 2). To fill a W40 cm × L65 cm × H18 cm tray, soil was mixed with 5 g of N:P:K (30:10:10) (Binh Dien Company, Vietnam), 9 g of organic compost (MiNoRi 2, Japan) and 15 g of $CaCO_3$. The mother plant of each accession was grown in a tray, in full sun positions with regular watering. After three months, new plants derived from stem cuttings were transplanted to new W40 cm × L65 cm × H18 cm trays (three technical replicates). Plant-to-plant and row-to-row spacing in the trays was maintained at 15 × 15 cm and 20 × 20 cm, respectively.

2.3. Morphological characterization

Four months following transplantation, twenty morphological traits were studied at full foliage stage (three plants per accession). The qualitative traits included plant growth habit (PGH), plant regenerability (PR), leaf arrangement (LA), leaf size (LS), leaf shape (LSH), leaf surface (LSF), leaf margin (LM), leaf colour (LC), petiole thickness (PT), petiole pigmentation at the base (PPAB), stolon colour (SC), texture of stolon (TS) and flower colour (FC). Leaf size was calculated from leaf length, width and shape. The plant regeneration capacity was observed from transplanting to first harvest. The regeneration ability of each accession was the amount of time between sprouting and ground coverage. The leaf, flower, stolon colour and petiole pigmentation at the base were recorded using Royal Horticultural Society colour chart. On the other hand, the quantitative morphological traits included leaf

Table 1
The *Centella asiatica* germplasm collected across Vietnam and Laos.

No.	*C. asiatica* accession (Genbank accession)	Place of collection	Coordinates	Habitat
1	HUIB_CA01 (OM943922.1)	Hung Nguyen, Nghe An	18°40′35.4″N 105°36′50.9″E	Paddy field
2	HUIB_CA02 (OM943923.1)	Gio Linh, Quang Tri	16°59′39.1″N 107°03′14.7″E	Paddy field
3	HUIB_CA03 (OM943924.1)	Phu Ninh, Quang Nam	15°32′29.2″N 108°27′26.3″E	Paddy field
4	HUIB_CA04 (OM943925.1)	Song Kong, Thai Nguyen	21°27′25.5″N 105°50′35.8″E	Lowland plantation
5	HUIB_CA05 (OM943926.1)	Vu Quang, Ha Tinh	18°28′17.8″N 105°31′56.9″E	Highland plantation
6	HUIB_CA06 (OM943927.1)	Quang Dien, Thua Thien Hue	16°31′59.2″N 107°31′34.1″E	Lowland *C. asiatica* plantation
7	HUIB_CA07 (OM943928.1)	Thach Ha, Ha Tinh	18°20′22.4″N 105°50′46.6″E	Paddy field
8	HUIB_CA08 (OM943929.1)	Kong Chro, Gia Lai	13°37′55.4″N 108°43′03.5″E	Highland field
9	HUIB_CA09 (OM943930.1)	Phu Vang, Thua Thien Hue	16°29′42.8″N 107°36′24.2″E	Lowland plantation
10	HUIB_CA10 (OM943931.1)	Buon Ma Thuot, Đak Lak	12°40′33.5″N 108°02′57.8″E	Highland plantation
11	HUIB_CA11 (OM943932.1)	Yen Mo, Ninh Binh	20°08′21.5″N 106°00′21.5″E	Paddy field
12	HUIB_CA12 (OM943933.1)	Quang Hoa, Thanh Hoa	20°23′23.3″N 105°05′51.2″E	Paddy field
13	HUIB_CA13 (OM943934.1)	Le Thuy, Quang Binh	17°11′46.2″N 106°49′46.0″E	Paddy field
14	HUIB_CA15 (OM943935.1)	Hung Nguyen, Nghe An	18°39′57.8″N 105°38′53.4″E	Paddy field
15	HUIB_CA16 (OM943936.1)	Ngo May, Kon Tum	14°21′25.4″N 107°59′59.5″E	Highland field
16	HUIB_CA18 (OM943937.1)	Dong Trieu, Quang Ninh	21°05′23.9″N 106°27′38.1″E	Paddy field
17	HUIB_CA19 (OM943938.1)	Dong Trieu, Quang Ninh	21°05′23.9″N 106°27′38.1″E	Paddy field
18	HUIB_CA20 (OM943939.1)	Tuy Hoa, Phu Yen	13°04′36.4″N 109°18′18.0″E	Lowland *C. asiatica* plantation
19	HUIB_CA21 (OM943940.1)	Hung Nguyen, Nghe An	18°40′14.9″N 105°36′42.8″E	Paddy field
20	HUIB_CA25 (OM943941.1)	O Mon, Can Tho	10°07′54.9″N 105°37′16.1″E	Lowland *C. asiatica* plantation
21	HUIB_CA26 (OM943942.1)	Quang Dien, Thua Thien Hue	16°31′58.4″N 107°31′35.9″E	Lowland *C. asiatica* plantation
22	HUIB_CA27 (OM943943.1)	Quang Dien, Thua Thien Hue	16°32′39.3″N 107°30′36.5″E	Lowland *C. asiatica* plantation
23	HUIB_CA28 (OM943944.1)	Chau Thanh, Tien Giang	10°23′29.1″N 106°16′30.9″E	Lowland *C. asiatica* plantation
24	HUIB_CA29 (OM943945.1)	Cu Chi, HCMC	10°57′30.7″N 106°28′48.5″E	Lowland *C. asiatica* plantation
25	HUIB_CA30 (OP179632.1)	Savannakhet, Laos	16°32′41.6″N 104°49′41.3″E	Lowland plantation
26	HUIB_CA31 (OP179633.1)	Vientiane, Laos	18°08′04.3″N 102°49′45.0″E	Lowland plantation

Table 2
Characteristics of the soil used for growing the *C. asiatica* germplasm.

Soil mechanical composition	Limon (%)	31.5	P_2O_5 (mg/100 g)	3.0
	Clay (%)	22.1	Total K (%)	0.6
	Sand (%)	13.28	K_2O (mg/100 g)	15
	Fine sand (%)	33.12	OM (%)	1.86
pH_{KCl}		3.78	Ca^{2+} (ldl/100 g)	0.43
Total N (%)		0.09	Mg^{2+} (ldl/100 g)	0.22
N (mg/100 g)		4.35	CEC (ldl/100 g)	18
Total P (%)		0.057		

length, leaf width, petiole length, number of primary lateral veins, runner length, fresh yield, plant weight and dry matter. For each trait, the results represent averages of three repeats; for each repeat, ten leaves per plants at full foliage stage were randomly selected for measurement. The fresh yield at the first harvest for each tray was measured after being transplanted for four months.

2.4. Measurement of vitamin C content

The measurement of vitamin C content was performed as previously described (Satpathy et al., 2021) with modifications. Briefly, pennywort leaves (5 g) were finely ground in a mortar with distilled water (50 mL). The paste was centrifuged at 13,000 rpm for 10 min in 50-mL Falcon tubes. The supernatant (10 mL) was then transferred to a 250-mL conical flask containing 150 mL of distilled water and 1 mL of starch indicator solution (0.5 %). Next, 5 mM iodine solution was used to titrate samples with the first distinct trace of a dark blue-black colour indicating the titration endpoint. Results represent averages of three repeats.

2.5. Measurements of reducing sugar content

The reducing sugar content (RSC) was determined using the 3,5-dinitrosalicylic acid (DNSA) assay as previously described (Krivorotova & Sereikaite, 2014) with modifications. Pennywort leaves were washed and dried at room temperature. Dried leaves (5 g) were ground to coarse powder and macerated twice with 70 % ethanol at a ratio of 1:7 w/v at 25 °C for 48 h. To prepare 100 mL of DNSA reagent, 30 g of sodium–potassium tartaric acid and 1 g of DNSA were first dissolved in 80 mL of NaOH (0.5 M) at 45 °C. Once cooled to room temperature, distilled water was added to make up 100 mL. To determine RSC, 1 mL of DNSA reagent was added to 0.5 mL of *C. asiatica* leaf extract and the mixture was incubated at 95 °C for 5 min. After cooling to room temperature, 3.5 mL of distilled water were added to the solution and the absorbance at 540 nm was measured using a spectrophotometer (Multiskan GO, ThermoScientific, USA). RSC was calculated from the calibration curve of standard D-glucose (0.2—–1 mg/mL), and the results were expressed as D-glucose equivalents (GE) per dry weight. Results represent averages of three repeats.

2.6. Measurements of total carotenoid content

The total carotenoid content in *C. asiatica* leaves was determined using a colorimetric assay as previously described (Biswas et al., 2011). Pennywort leaves were ground in a mortar to obtain a fine paste. A portion of the paste (about 0.5 g) was weighed in a 50-mL Falcon tube. Next, the paste was mixed with 5 mL of chilled acetone for 15 min at 4 ± 1 °C with occasional shaking. The tubes were vortexed at high speed for 10 min and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to new tubes, and the extraction was repeated with another 5 mL of acetone, followed by centrifugation as above. The supernatant was pooled together and filtered using a Whatman filter paper (No. 42) and the absorbance of the extract at 449 nm was determined using a spectrophotometer (Multiskan GO, ThermoScientific, USA). The standard curve was constructed using β-carotene Type I (95 % purity, Sigma) that were serially diluted to 2, 4, 8, 16 and 32 mg/mL in acetone. Results represent averages of three repeats.

2.7. Measurement of total tannin content

Total tannin content in *C. asiatica* leaves was measured as previously described (Atanassova & Christova-Bagdassarian, 2009) with modifications. To prepare standard solutions of Indigo carmine, 6 g of Indigo carmine were dissolved in 500 mL of distilled water with heating. Following cooling to room temperature, 50 mL of 95–97 % H_2SO_4, and water were added to make up 1 L. The mixture was filtered. In 200 mL conical flasks, 5 mL of the leaf extracts were added to 2.5 mL of Indigo solution and 75 mL of water. The mixture was titrated with $KMnO_4$ solution (0.1 M) until the colour of the solution changed from blue to golden yellow. The blank tests were performed by titrating a mixture containing 2.5 mL of Indigo carmine solution and 75 mL of water. All samples were analysed in triplicates. The total tannin content (T, %) in the sample was calculated as previously described (Atanassova & Christova-Bagdassarian, 2009). Results represent averages of three repeats.

2.8. Measurements of total phenolic content

The total phenolic content of *C. asiatica* leaves was determined using the Folin–Ciocalteu assay as previously described (Singleton & Rossi, 1965) with modifications. Briefly, freeze-dried sample (0.5 g) was extracted with 10 mL of 70 % aqueous ethanol in an ultrasonic bath for 20 min. An aliquot (2 mL) of the extracts was centrifuged for 5 min at 14,000 rpm. Ethanol (70 %) was used to prepare serial dilutions of 2, 4, 6, 8, 10, 12 and 14 mg/L of gallic acid (Sigma). The extracts or standard solutions (0.5 mL) were added to 3 mL of distilled water in 10 mL volumetric flasks. Folin–Ciocalteu's reagent (0.25 mL) was added and mixed. After 5 min, 0.75 mL of 20 % Na_2CO_3 solution was added to the mixture, followed by distilled water to make up 5 mL. After incubation for 45 min at room temperature, the absorbance was measured at 758 nm using a spectrophotometer (Multiskan GO, ThermoScientific, USA). Total phenolic content of *C. asiatica* leaves was expressed as mg gallic acid equivalents (GAE) per gram of dry weight. Results represent averages of three technical repeats.

2.9. Measurements of total flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric assay as previously described (Zhishen et al., 1999) with modifications. Specifically, 0.5 mL of extracts or standard solutions containing catechin (45, 90, 180, 360 or 720 mg/mL of catechin in water) was added to 2 mL of distilled H_2O in 10 mL volumetric flasks, followed by the addition of 0.15 mL of 5 % $NaNO_2$. After 5 min, 0.15 mL of 10 % $AlCl_3$ was added to each flask, followed by 6 min incubation. Two mL of 1 M NaOH were added and the total volume was made up to 5 mL with distilled H_2O. The absorbance at 510 nm was measured using a spectrophotometer (Multiskan GO, Thermo Scientific, USA). Total flavonoid content of *C. asiatica* leaves was expressed as mg catechin equivalents (CE) per gram of dry weight. Results represent averages of three technical repeats.

2.10. Measurements of total saponins content

The total saponins content of pennywort leaves was determined using the vanillin-sulphuric acid assay as previously described (Le et al., 2018) with modifications. Briefly, 250 μL of extracts or standards containing gypenoside XVII (15, 30, 45, 60 and 75 μg/L) were added to 250 μL of 8 % (w/v) vanillin in ethanol, 2.5 mL of 72 % (v/v) sulfuric acid in water. The mixtures were incubated for 15 min at 60 °C with shaking. After cooling in water to ambient temperature (5 min), the absorbance of the standards and extracts was measured at 560 nm using a spectrophotometer (Multiskan GO, ThermoScientific, USA). The total saponins content of the samples was expressed as mg of gypenoside equivalents (GYE) per gram of dry weight. Results represent averages of three repeats.

2.11. SSR analysis

The SSR analysis was carried out in 15-μL PCR containing 10 ng of DNA, 7.5 μL of 2x MyTaq buffer (Meridian Bioscience, US) and 10 pmol SSR primers (Table 3). The thermocycling program (Applied Biosystems, USA) included an initial denaturation (94 °C for 2 min), followed by 35 cycles of 94 °C for 30 sec, 54 °C to 58 °C for 1 min, 72 °C for 2 min, and a

Table 3
SSR primer pairs used in this study. Following preliminary screen with 30 primer pairs, 14 SSR primer pairs (in bold) were used to analyse the genetic diversity of the entire *C. asiatica* germplasm.

No.	Primer	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Reference
1	TBG-Centa F1	AGGACTTGACACTGCTTTTGCT	TGCCTTCTCCTTCTTCATCTTC	Sakthipriya et al., 2018
2	TBG-Centa F2	CTACTCTATCCCGCAAATCCTT	CTCTCTCTCGTTTCTCGCC	
3	TBG-Centa F3	AGTGTTGATGATGATGACGAGG	CAGACTCATTTGCTTTTGCTTG	
4	TBG-Centa F8	AGAATCAATACATACAGCCCCG	AAACGAAAGATTGTGAGAAGGG	
5	TBG-Centa F10	CCAAAACCATTCTCTCCACTTC	CTCTTCTTTGTCGCCATCTTCT	
6	TBG-Centa F14	TCCTCCAAAATACCACCATACC	GACCAATGAGTGCCAAAAGAAT	
7	**TBG-Centa F15**	GAACTTTCGCCTCTTCTCTTGA	TCCTCATTTATCTCCCTCGGTA	
8	TBG-Centa F19	TTAGCATTTAGAAGGTCAGGGC	ATTTACAGCAATCAGAGACGCA	
9	TBG-Centa F26	ATGGGAGAGAAATAAAGGAGCC	GAAACGATAGTCAGGGATTGGA	
10	**TBG-Centa F31**	AGAGCACACCTTTATCCCTTTG	AGAAGAAGAAGGAGGATTTGGG	
11	**mCaCIR002**	CCACAGGTAACACCGAAT	GCACTTGCACTATCTGGAA	Rakotondralambo et al., 2012
12	**mCaCIR004**	GGGTGGTCTGCCTAAAGA	TGGAGATCAAGTTTCATGC	
13	**mCaCIR005**	GGCCTTCAATGTATGCTG	TTTGATTTGTTGGGTCTTG	
14	mCaCIR006	ACGGGCATTTATTCCATT	GCAAACCACCACAACTTC	
15	**mCaCIR007**	TGGAGGTGGTGTAACTGG	AGGGGATCAAACCTCATC	
16	mCaCIR009	TGCCTATCCTTTGAATGC	CAAACATGACATTCTTAAAACA	
17	mCaCIR010	AATGTAAAATTCCCGGTGT	TAAACAGGCGTTCCAAGT	
18	**mCaCIR011**	TTCATAAAAGTCCTTCCACA	TAGGTTGATGTGGCCTCT	
19	**mCaCIR012**	CACGAAAATTGGAAACAA	CATGTGAGTTTATGAGTTTCTATG	
20	mCaCIR013	CAAGTTCCTCCCACGAAT	GCCGAAATAATCGAAATATAAG	
21	mCaCIR018	TTGAGTTTAAGAAGTCCCAAAT	AATCCTTCACACTCCTAAAGC	
22	**mCaCIR019**	TTTCTTGTTAAATGCGATGA	AATGACATCACTGCTATGGA	
23	mCaCIR020	TTTAGGAAGTTGGATTTTGC	GGTTTAATTCAGGACGCTTA	
24	mCaCIR021	TGCCTAGATTTTGGGTTTT	TCTTACAATGCAATCAACCT	
25	**mCaCIR022**	AGGAGTATTGACAAGAGGTGA	GGATGGCAGTCCATTTTA	
26	mCaCIR024	TCTTTCGTTGATACATGCAC	AAAACTTAAAGAAGATACAAACTCC	
27	**mCaCIR027**	ACCCCAAGACCTTCAGTT	CCTTCTGCTTTCCCTTTT	
28	**mCaCIR028**	CAGAGTTTGGGCAGAAAA	GACGAGTGGAGGATAAGAAA	
29	**mCaCIR029**	GGTCTGAGGTCTGTTGAGG	CGCATTGACAGAACAAAA	
30	**mCaCIR030**	GGCAAATCGAGAGCAATA	ACGGAAAAGCCTAACAGC	

final extension (72 °C for 7 min). The PCR products were stained with SYBR Green, resolved on 5 % agarose gel (0.5X TBE buffer) and visualized under UV light. DNA bands of the same size represent the same allele, and data were analysed using POPGENE 1.32 and NTSYSpc 2.1 to determine the genetic distance among pennywort accessions. The genetic dendrogram was constructed in NTSYSpc 2.1. The number of alleles was used to calculate the expected heterozygosity, the observed heterozygosity and the polymorphism information content (PIC) using Cervus 3.0.7 (Kalinowski et al., 2007).

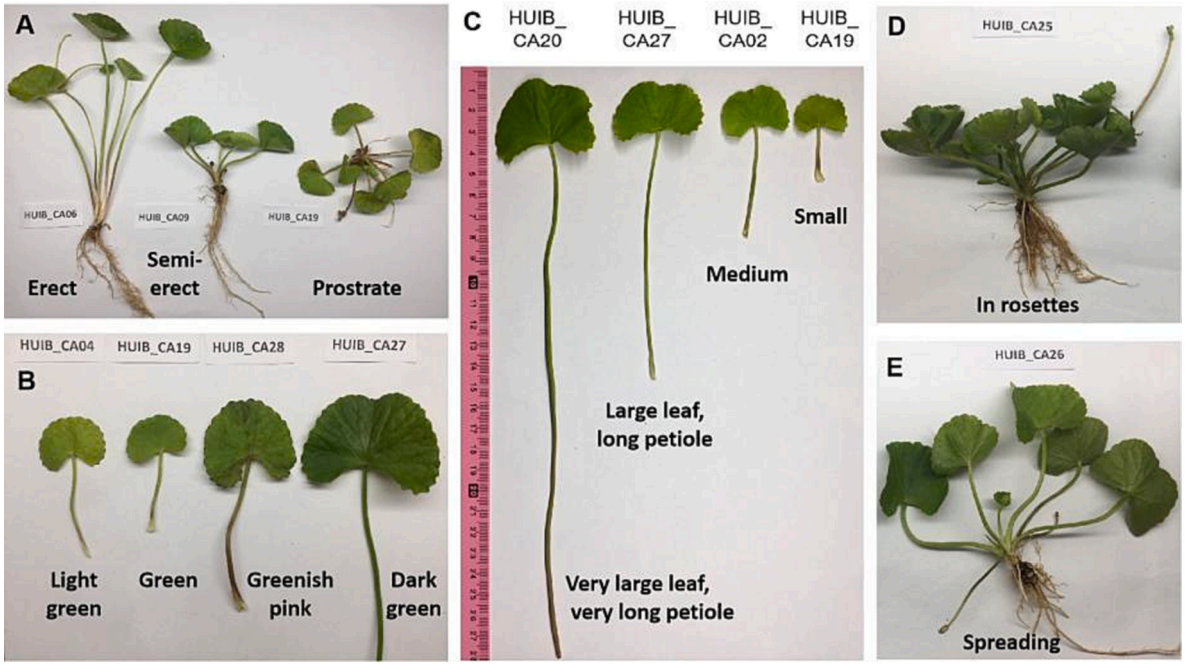

Fig. 1. Variations in plant morphological traits and growth habits of 26 *C. asiatica* accessions collected throughout Vietnam and Laos. (A) Plant growth habits vary from erect, semi-erect and prostrate. (B) Leaf colours vary from light green, green, greenish pink to dark green. (C) Variations in leaf sizes and petiole lengths. (D) Leaf arrangement in rosette. (E) Spreading leaf arrangement.

2.12. Statistical analysis

Data obtained on quantitative morphological traits and phytochemical profiling were expressed as means and standard deviations of three repeats. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's test in IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA). Data represented significant differences as $p < 0.05$.

3. Results

3.1. Morphological characterization

A total of twenty morphological traits were evaluated for four months following planting in the trays containing alluvial soil. Morphological diversity was observed among accessions in both qualitative and quantitative traits (Fig. 1, Tables 4 and 5). Nine accessions exhibited erect growth while twelve and five accessions demonstrated semi-erect and prostrate growth respectively (Fig. 1A, Table 4). A wide variation in leaf colour was observed among accessions: light green (6 accessions), green (9 accessions), greenish pink (2 accessions) and dark green (9 accessions) (Fig. 1B). Leaf sizes also varied: small (7 accessions), medium (11 accessions), large (7 accessions) and very large (one accession) (Fig. 1C). Another qualitative trait, plant regenerability (PR), is important for perennial crops as fast regenerability allows multiple harvests per year. In this germplasm, regenerability varied from good (11 accessions), medium (10 accessions) to poor (5 accessions). Leaves were arranged in rosette (18 accessions) or spreading (8 accessions) (Fig. 1D–E). The majority of accessions (19) displayed reniform leaves whereas only 7 accessions had orbicular leaf shape. Leaf margins in 22 accessions were dentate and only four accessions produced crenate margins. Among qualitative traits, only leaf surface was shared by all accessions: glabrous.

Petiole pigmentation at the base also varied from green (1 accession), greenish pink (18 accessions), light pink (5 accessions) and pink (1 accession) (Table 4). Fourteen accessions exhibited thick petioles while thin petioles were observed in the rest. Also, stolon colours ranged from light pink (2 accessions), greenish pink (2 accessions), pink (10 accessions) to dark pink (12 accessions). The texture of the stolon was hard across 26 accessions. In terms of flower colour (FC), greenish pink flowers (5 accessions), pink flowers (11 accessions) and dark pink flowers (10 accessions) were observed.

On the other hand, quantitative morphological traits showed significant differences among 26 accessions in the germplasm (Table 5). Leaf length ranged from 2.28 cm (HUIB_CA19) to 5.82 cm (HUIB_CA20), while leaf width ranged from 1.38 cm (HUIB_CA19) to 3.27 cm (HUIB_CA20). In terms of leaf size, leaves from HUIB_CA20 were the largest among the 26 accessions. HUIB_CA09 had the longest runner (14.07 cm), while HUIB_CA05 had the shortest (5.20 cm). The longest petiole was observed in HUIB_CA20 (24.53 cm), while the shortest was found in HUIB_CA05 (2.45 cm). The number of primary lateral veins was found to be different among accessions as well as plants within the same accession, and varied from 6.36 (HUIB_CA19) to 8.43 (HUIB_CA20). Furthermore, fresh yield per tray at the first harvest differed among

Table 4
Qualitative morphological traits observed in 26 *C. asiatica* accessions.

No.	Accession code	PGH	PR	LA	LS	LSH	LSF	LM	LC	PPAB	PT	SC	TS	FC
1	HUIB_CA01	2	2	1	3	2	1	1	3	3	2	3	1	2
2	HUIB_CA02	3	2	1	2	1	1	1	3	2	2	3	1	3
3	HUIB_CA03	2	2	1	2	1	1	2	2	2	1	4	1	2
4	HUIB_CA04	2	2	1	2	1	1	1	1	2	2	4	1	3
5	HUIB_CA05	2	3	1	1	1	1	2	1	2	1	4	1	3
6	HUIB_CA06	1	1	2	3	2	1	1	3	2	2	2	1	1
7	HUIB_CA07	2	1	2	2	1	1	1	2	3	2	4	1	3
8	HUIB_CA08	3	1	2	2	1	1	1	1	2	1	4	1	2
9	HUIB_CA09	2	1	2	2	1	1	1	2	2	1	4	1	1
10	HUIB_CA10	1	1	2	2	1	1	2	1	2	1	3	1	1
11	HUIB_CA11	2	2	1	1	1	1	2	1	3	1	3	1	1
12	HUIB_CA12	3	2	1	3	1	1	1	2	2	1	4	1	2
13	HUIB_CA13	1	1	1	2	1	1	1	2	2	2	4	1	3
14	HUIB_CA15	3	3	1	1	1	1	1	2	2	2	4	1	3
15	HUIB_CA16	2	3	1	1	1	1	1	3	2	1	2	1	3
16	HUIB_CA18	2	2	1	1	2	1	1	2	2	1	3	1	2
17	HUIB_CA19	3	3	1	1	1	1	1	2	3	1	4	1	2
18	HUIB_CA20	1	1	1	4	1	1	1	2	3	2	3	1	2
19	HUIB_CA21	2	3	2	1	2	1	1	1	2	1	3	1	2
20	HUIB_CA25	1	2	1	3	1	1	1	1	2	2	3	1	3
21	HUIB_CA26	1	1	2	3	1	1	1	3	2	2	3	1	1
22	HUIB_CA27	1	1	2	3	1	1	1	3	2	2	3	1	2
23	HUIB_CA28	1	1	1	2	2	1	1	4	4	2	4	1	2
24	HUIB_CA29	1	1	1	2	2	1	1	4	4	2	4	1	2
25	HUIB_CA30	2	2	1	2	2	1	1	1	1	1	1	1	3
26	HUIB_CA31	2	2	1	3	1	1	1	1	2	2	1	1	3

PGH; Plant growth habit: Erect (1), Semi-erect (2), Prostrate (3).
PR; Plant regenerability: Good (1), Medium (2), Poor (3).
LA; Leaf arrangement: In rosettes (1), Spreading (2).
LS; Leaf size: Small (1), Medium (2), Large (3), Very large (4).
LSH; Leaf shape: Reniform (1), Orbicular (2).
LSF; Leaf surface: Glabrous (1).
LM; Leaf margin: Dentate (1), Crenate (2).
LC; Leaf colour: Light green (1), Green (2), Dark green (3), Greenish pink (4).
PPAB; Petiole pigmentation at the base: Green (1), Greenish pink (2), Light pink (3), Pink (4).
PT; Petiole thickness: Thin (1), Thick (2).
SC; Stolon colour: Greenish pink (1), Light pink (2), Pink (3), Dark pink (4).
TS; Texture of stolon: Hard (1).
FC; Flower colour: Greenish pink (1), Pink (2), Dark pink (3).

Table 5
Quantitative morphological traits observed in 26 *C. asiatica* accessions.

Accession code	Leaf length (cm)	Leaf width (cm)	Runner length (cm)	Leaf petiole length (cm)	Number of primary lateral veins	Fresh yield at the first harvest (g)	Plant weight (g)	Dry matter (%)
HUIB_CA01	4.75 ± 0.06^{b}	2.85 ± 0.09^{bc}	10.43 ± 0.27^{b}	11.83 ± 1.50^{b}	8.10 ± 0.17^{ab}	150 ± 12^{hij}	1.08 ± 0.08^{ghi}	17.933^{b} ± 0.757
HUIB_CA02	3.79 ± 0.05^{cdef}	2.27 ± 0.61^{efgh}	8.94 ± 0.23^{bcd}	5.10 ± 1.06^{fghi}	7.73 ± 0.50^{bcdef}	157 ± 5^{hij}	1.45 ± 0.03^{fghi}	16.380^{a} ± 0.430
HUIB_CA03	3.38 ± 0.18^{fg}	2.02 ± 0.10^{fghij}	7.56 ± 1.39^{cdefghi}	6.86 ± 0.08^{def}	7.80 ± 0.26^{bcde}	149 ± 16^{hij}	1.9 ± 0.4^{defgh}	17.333^{ab} ± 0.611
HUIB_CA04	3.46 ± 0.55^{efg}	2.07 ± 0.34^{fghi}	7.54 ± 1.38^{cdefghij}	5.51 ± 0.40^{efg}	7.57 ± 0.15^{cdefgh}	176 ± 36^{ghij}	2.3 ± 0.3^{defg}	16.867^{ab} ± 0.611
HUIB_CA05	2.44 ± 0.01^{jk}	1.63 ± 0.09^{jklm}	5.20 ± 0.74^{k}	2.45 ± 0.08^{j}	7.20 ± 0.20^{ghijk}	147 ± 6^{ij}	1.13 ± 0.09^{fghi}	16.600^{ab} ± 0.917
HUIB_CA06	4.21 ± 0.09^{c}	2.51 ± 0.07^{cde}	8.36 ± 1.31^{bcdef}	10.46 ± 0.89^{bc}	7.30 ± 0.26^{fghijk}	242 ± 48de	3.9 ± 1.2^{bc}	16.867^{ab} ± 0.643
HUIB_CA07	3.33 ± 0.43^{fgh}	2.05 ± 0.34^{fghij}	9.68 ± 1.76^{bc}	6.80 ± 2.12^{def}	7.03 ± 0.12^{ijk}	258 ± 14^{cd}	1.9 ± 0.6^{defgh}	17.267^{ab} ± 0.416
HUIB_CA08	3.28 ± 0.08^{ghi}	1.85 ± 0.06^{hijkl}	8.07 ± 0.23^{cdefg}	5.83 ± 1.06^{efg}	7.43 ± 0.25^{defghij}	249 ± 33^{cde}	1.75 ± 0.05^{defghi}	16.333^{a} ± 0.503
HUIB_CA09	3.91 ± 0.50^{cde}	2.33 ± 0.33^{egf}	14.07 ± 5.33^{a}	7.62 ± 3.20^{de}	7.67 ± 0.40^{bcdefg}	217 ± 19^{ef}	2.0 ± 0.5^{defgh}	17.000^{ab} ± 0.600
HUIB_CA10	3.52 ± 0.17^{efg}	2.01 ± 0.10^{fghij}	8.34 ± 0.64^{bcdef}	8.43 ± 1.12^{cd}	7.83 ± 0.12^{bcd}	278 ± 4^{bc}	1.9 ± 0.5^{defgh}	16.933^{ab} ± 0.413
HUIB_CA11	2.82 ± 0.10^{ij}	1.67 ± 0.13^{ijklm}	6.69 ± 0.83^{efghijk}	4.68 ± 0.44^{fghij}	7.20 ± 0.56^{ghijk}	204 ± 7^{fg}	2.4 ± 0.2^{defg}	16.733^{ab} ± 0.503
HUIB_CA12	4.13 ± 0.58^{cd}	2.20 ± 0.63^{efgh}	8.10 ± 0.89^{cdefg}	4.40 ± 0.29^{ghij}	7.07 ± 0.35^{ijk}	155 ± 8^{hij}	1.52 ± 0.02^{fghi}	16.533^{ab} ± 0.306
HUIB_CA13	3.26 ± 0.37^{ghi}	1.93 ± 0.22^{ghijk}	6.79 ± 0.30^{defghijk}	5.23 ± 1.43^{fgh}	7.70 ± 0.53^{bcdef}	222 ± 30^{ef}	2.5 ± 0.6^{def}	16.333^{a} ± 2.610
HUIB_CA15	2.60 ± 0.36^{jk}	1.56 ± 0.36^{klm}	6.45 ± 0.70^{fghijk}	2.87 ± 0.30^{ij}	7.03 ± 0.25^{ijk}	166 ± 12^{hij}	1.59 ± 0.03^{efghi}	16.467^{a} ± 0.416
HUIB_CA16	2.59 ± 0.36^{jk}	1.42 ± 0.25^{m}	5.75 ± 0.93^{hijk}	3.81 ± 0.48^{ghij}	7.17 ± 0.35^{hijk}	168 ± 7^{hij}	0.77 ± 0.02^{hi}	16.333^{a} ± 0.306
HUIB_CA18	2.85 ± 0.11^{hij}	1.45 ± 0.13^{lm}	5.41 ± 0.26^{ijk}	4.60 ± 0.45^{fghij}	7.47 ± 0.25^{defghi}	176 ± 4^{ghij}	0.43 ± 0.22^{i}	16.667^{ab} ± 0.416
HUIB_CA19	2.28 ± 0.29^{k}	1.38 ± 0.16^{m}	5.98 ± 0.49^{ghijk}	3.06 ± 1.12^{hij}	6.37 ± 0.21^{l}	107 ± 26^{k}	2.0 ± 0.4^{defgh}	16.667^{ab} ± 0.306
HUIB_CA20	5.82 ± 0.24^{a}	3.27 ± 0.12^{a}	12.68 ± 0.65^{a}	24.53 ± 1.67^{a}	8.43 ± 0.12^{a}	478 ± 48^{a}	8.9 ± 2.7^{a}	17.933^{b} ± 0.917
HUIB_CA21	2.62 ± 0.04^{jk}	1.44 ± 0.07^{lm}	5.38 ± 0.26^{ijk}	3.95 ± 0.06^{ghij}	6.93 ± 0.15^{k}	145 ± 9^{ij}	1.27 ± 0.02^{fghi}	16.600^{ab} ± 0.721
HUIB_CA25	4.21 ± 0.34^{c}	2.42 ± 0.14^{def}	8.21 ± 0.34^{cdef}	6.00 ± 0.17^{efg}	6.97 ± 0.06^{jk}	176 ± 16^{ghij}	4.4 ± 1.2^{b}	16.200^{a} ± 0.200
HUIB_CA26	4.01 ± 0.16^{cd}	2.23 ± 0.13^{efgh}	6.99 ± 0.16^{defghijk}	12.45 ± 3.81^{b}	7.90 ± 0.26^{bcd}	223 ± 17^{ef}	3.0 ± 0.8^{cd}	16.400^{a} ± 0.721
HUIB_CA27	5.00 ± 0.27^{b}	2.84 ± 0.41^{bcd}	8.69 ± 0.43^{bcde}	11.98 ± 1.57^{b}	8.00 ± 0.69^{abc}	295 ± 24^{b}	4.9 ± 0.6^{b}	16.400^{a} ± 0.529
HUIB_CA28	3.90 ± 0.22^{cde}	2.42 ± 0.03^{def}	7.90 ± 1.05^{cdefgh}	8.74 ± 0.37^{cd}	7.77 ± 0.21^{bcdef}	180 ± 39^{ghi}	4.47 ± 0.02^{b}	16.333^{a} ± 0.115
HUIB_CA29	3.81 ± 0.08^{cdef}	2.50 ± 0.04^{cde}	7.93 ± 0.64^{cdefg}	7.69 ± 0.43^{de}	7.90 ± 0.26^{bcd}	181 ± 27^{gh}	4.7 ± 2.1^{b}	16.600^{ab} ± 0.200
HUIB_CA30	3.65 ± 0.02^{defg}	2.03 ± 0.03^{fghij}	7.61 ± 0.37^{cdefgh}	5.11 ± 2.30^{fghi}	7.33 ± 0.15^{efghijk}	167 ± 17^{hij}	1.8 ± 0.1^{defhi}	16.533^{ab} ± 0.231
HUIB_CA31	4.86 ± 0.13^{b}	2.97 ± 0.06^{ab}	7.74 ± 0.95^{cdefgh}	4.87 ± 0.51^{fghi}	7.50 ± 0.00^{defghi}	160 ± 13^{hij}	3.0 ± 0.2^{cde}	16.667^{ab} ± 0.231

The same lower-case letters within columns indicate the lack of significant difference ($p \geq 0.05$). Error bars represent standard deviation.

accessions: from 107 g (HUIB_CA19) to 478 g (HUIB_CA20). Plant weight varied from 0.43 g (HUIB_CA18) to 8.91 g (HUIB_CA20). Finally, dry matter varied from 16.2 to 17.9 % among pennywort accessions (Table 5).

3.2. Phytochemical analysis

The vitamin C content varied from 0.31 to 0.48 % of dry weight, with HUIB_CA26 and HUIB_CA18 containing the highest and lowest amounts respectively (Table 6). The reducing sugar content varied from 7.0 to 8.7 % of dry weight, with HUIB_CA29 and HUIB_CA12 containing the highest and lowest amounts respectively. On the other hand, the carotenoid content varied from 0.78 to 0.90 mg per 100 g of dry weight, with HUIB_CA02 and HUIB_CA30 containing the highest and lowest carotenoid amounts respectively.

The highest and lowest tannin contents were found in HUIB_CA03 and HUIB_CA21 (4.6 % and 2.8 % of dry weight respectively) (Table 7). The accessions with the highest and lowest saponin contents were HUIB_CA06 (2.1 mg GYE/g of dry weight) and HUIB_CA21 (1.5 mg GYE/g of dry weight) respectively. The accessions with the highest phenolic content were HUIB_CA13 and HUIB_CA26 (19.6 mg GAE/g of dry weight), and the lowest was HUIB_CA04 (15.9 mg GAE/g of dry weight). In terms of flavonoid, the accession containing the highest amount of flavonoid was HUIB_CA20 (6.5 mg CE/g of dry weight), and the lowest was HUIB_CA10 (4.1 mg CE/g of dry weight).

3.3. SSR analysis

To pre-screen for SSR primers that yielded polymorphism in this germplasm, thirty primer pairs were used to amplify eight randomly selected accessions (Table 3, Fig. 2). Fourteen of these pairs yielded polymorphic products (Table 3). Next, these fourteen SSR primer pairs

Table 6
Vitamin C content, reducing sugar and carotenoid contents in 26 *C. asiatica* accessions.

No.	Accession	Vitamin C content (% dry weight)	Reducing sugar (% dry weight)	Carotenoid (mg/100 g dry weight)
1	HUIB_CA01	0.347^{ab} ± 0.020	8.493^{a} ± 0.602	0.892 ^{fg} ± 0.021
2	HUIB_CA02	0.341^{ab} ± 0.004	7.883^{a} ± 0.614	0.896 ^{g} ± 0.020
3	HUIB_CA03	0.352^{ab} ± 0.009	7.395^{a} ± 0.672	0.843^{bcdefg} ± 0.032
4	HUIB_CA04	0.342^{ab} ± 0.009	7.517^{a} ± 0.812	0.844^{bcdefg} ± 0.033
5	HUIB_CA05	0.344^{ab} ± 0.024	7.965^{a} ± 0.462	0.830^{abcdef} ± 0.012
6	HUIB_CA06	0.408^{c} ± 0.013	8.453^{a} ± 0.428	0.885^{efg} ± 0.029
7	HUIB_CA07	0.334^{a} ± 0.009	7.558^{a} ± 1.375	0.814^{abcd} ± 0.031
8	HUIB_CA08	0.336^{a} ± 0.017	7.070^{a} ± 1.220	0.825^{abcde} ± 0.020
9	HUIB_CA09	0.416^{c} ± 0.016	8.371^{a} ± 0.577	0.805^{abc} ± 0.060
10	HUIB_CA10	0.336^{a} ± 0.014	7.883^{a} ± 1.093	0.858^{bcdefg} ± 0.018
11	HUIB_CA11	0.338^{a} ± 0.009	7.680^{a} ± 0.610	0.833^{abcdefg} ± 0.052
12	HUIB_CA12	0.383^{bc} ± 0.042	7.029^{a} ± 0.745	0.859^{bcdefg} ± 0.031
13	HUIB_CA13	0.345^{ab} ± 0.026	7.273^{a} ± 0.932	0.812^{abcd} ± 0.039
14	HUIB_CA15	0.334^{a} ± 0.012	7.965^{a} ± 0.508	0.805^{abc} ± 0.045
15	HUIB_CA16	0.315^{a} ± 0.008	8.087^{a} ± 1.714	0.798^{ab} ± 0.027
16	HUIB_CA18	0.306^{a} ± 0.018	8.656^{a} ± 0.880	0.810^{abcd} ± 0.065
17	HUIB_CA19	0.310^{a} ± 0.002	8.371^{a} ± 0.493	0.823^{abcde} ± 0.008
18	HUIB_CA20	0.329^{a} ± 0.002	8.046^{a} ± 1.202	0.865^{cdefg} ± 0.045
19	HUIB_CA21	0.331^{a} ± 0.017	8.046^{a} ± 0.559	0.829^{abcdef} ± 0.014
20	HUIB_CA25	0.318^{a} ± 0.005	8.331^{a} ± 0.614	0.833^{abcdefg} ± 0.018
21	HUIB_CA26	0.484^{d} ± 0.088	8.656^{a} ± 0.742	0.873^{defg} ± 0.012
22	HUIB_CA27	0.408^{c} ± 0.010	8.416^{a} ± 1.753	0.826^{abcde} ± 0.032
23	HUIB_CA28	0.329^{a} ± 0.009	7.721^{a} ± 0.672	0.796^{ab} ± 0.031
24	HUIB_CA29	0.340^{ab} ± 0.006	8.737^{a} ± 0.510	0.794^{ab} ± 0.021
25	HUIB_CA30	0.397^{c} ± 0.006	8.656^{a} ± 0.646	0.776^{a} ± 0.057
26	HUIB_CA31	0.346^{ab} ± 0.020	8.534^{a} ± 1.084	0.857^{bcdefg} ± 0.020

The same lower-case letters within columns indicate the lack of significant difference ($p \geq 0.05$). Error bars represent standard deviation.

Table 7
Tannin, phenolic, flavonoid and saponin contents in 26 *C. asiatica* accessions.

No	Accession	Tannin content (% dry weight)	Phenolic content (mg GAE/g of dry weight)	Flavonoid content (mg CE/g of dry weight)	Saponin content (mg GYE/g of dry weight)
1	HUIB_CA01	4.035^{cdef} ± 0.381	18.205^{bc} ± 2.011	4.842^{abcde} ± 1.391	1.692^{a} ± 0.050
2	HUIB_CA02	4.334 ^{fg} ± 0.437	19.300^{bcd} ± 0.126	5.429^{abcdefg} ± 1.011	1.898^{a} ± 0.051
3	HUIB_CA03	4.645 ^{g} ± 0.144	18.062^{b} ± 0.218	6.362 ^{fg} ± 0.572	1.783^{a} ± 0.086
4	HUIB_CA04	4.201^{efg} ± 0.072	15.887^{a} ± 0.477	5.714^{bcdefg} ± 0.594	1.735^{a} ± 0.089
5	HUIB_CA05	3.758^{bcde} ± 0.000	18.586^{bcd} ± 0.297	4.876^{abcde} ± 1.765	1.586^{a} ± 0.106
6	HUIB_CA06	4.312 ^{fg} ± 0.144	18.332^{bcd} ± 2.503	6.248^{efg} ± 0.508	2.139^{c} ± 0.142
7	HUIB_CA07	3.813^{bcdef} ± 0.190	18.110^{b} ± 0.298	5.162^{abcdefg} ± 0.564	1.695^{a} ± 0.085
8	HUIB_CA08	3.869^{cdef} ± 0.260	19.411^{bcd} ± 0.180	6.076^{cdefg} ± 0.368	1.762^{a} ± 0.055
9	HUIB_CA09	4.035^{cdef} ± 0.191	19.602 ^{cd} ± 0.271	4.762^{abcd} ± 1.062	2.088^{bc} ± 0.159
10	HUIB_CA10	3.647^{bcd} ± 0.072	18.125^{b} ± 0.235	4.133^{a} ± 0.119	1.477^{a} ± 0.048
11	HUIB_CA11	4.002^{cdef} ± 0.188	18.760^{bcd} ± 0.167	5.067^{abcdefg} ± 1.173	1.772^{a} ± 0.138
12	HUIB_CA12	4.312 ^{fg} ± 0.144	18.141^{b} ± 0.509	6.229^{defg} ± 0.249	1.820^{a} ± 0.048
13	HUIB_CA13	3.758^{bcde} ± 0.125	19.633^{b} ± 0.390	4.533^{ab} ± 0.699	1.648^{a} ± 0.105
14	HUIB_CA15	4.146^{defg} ± 0.191	18.570^{bcd} ± 0.225	5.181^{abcdefg} ± 0.809	1.696^{a} ± 0.121
15	HUIB_CA16	3.536^{bc} ± 0.402	18.110^{b} ± 0.126	4.686^{abc} ± 0.151	1.603^{a} ± 0.072
16	HUIB_CA18	3.503^{bc} ± 0.144	18.078^{b} ± 0.198	4.762^{abcd} ± 0.119	1.583^{a} ± 0.180
17	HUIB_CA19	3.292^{b} ± 0.288	19.300^{bcd} ± 0.126	5.162^{abcdefg} ± 0.216	1.580^{a} ± 0.022
18	HUIB_CA20	3.913^{cdef} ± 0.113	18.475^{bcd} ± 0.225	6.533 ^{g} ± 0.119	2.050^{bc} ± 0.144
19	HUIB_CA21	2.794^{a} ± 0.000	18.379^{bcd} ± 0.335	5.562^{abcdefg} ± 0.611	1.470^{a} ± 0.083
20	HUIB_CA25	3.703^{bcde} ± 0.260	18.205^{bc} ± 0.265	5.086^{abcdefg} ± 0.314	1.618^{a} ± 0.048
21	HUIB_CA26	3.924^{cdef} ± 0.000	19.633^{d} ± 0.784	5.581^{abcdefg} ± 0.216	2.050^{bc} ± 0.314
22	HUIB_CA27	3.758^{bcde} ± 0.000	19.078^{bcd} ± 0.291	5.771^{bcdefg} ± 0.580	1.994^{ab} ± 0.087
23	HUIB_CA28	3.714^{bcde} ± 0.194	18.760^{bcd} ± 0.453	4.838^{abcde} ± 0.543	1.671^{a} ± 0.082
24	HUIB_CA29	3.924^{cdef} ± 0.125	19.379^{bcd} ± 0.303	4.990^{abcdef} ± 0.611	1.724^{a} ± 0.057
25	HUIB_CA30	3.326^{b} ± 0.132	18.284^{bcd} ± 0.055	4.610^{abc} ± 0.335	1.576^{a} ± 0.055
26	HUIB_CA31	3.658^{bcd} ± 0.180	18.951^{bcd} ± 0.548	5.562^{abcdefg} ± 0.929	1.939^{a} ± 0.076

The same lower-case letters within columns indicate that the lack of significant difference ($p \geq 0.05$). GAE: gallic acid equivalents, CE: catechin equivalents, GYE: gypenoside XVII equivalents. Error bars represent standard deviation.

were used to explore the genetic diversity within the *C. asiatica* germplasm (Table 8). The amplified products were in the range of 140 bp to 300 bp. A total of 47 alleles were identified with 45 alleles (96 %) being polymorphic, the average allele number was 3.357. All fourteen primer pairs yielded highly polymorphic products (50–100 %), with TBG-Centa F15 resulting in 67 % polymorphism, mCaCIR004 resulting in 50 % polymorphism and the rest yielding 100 % polymorphism. Genetic diversity analysis using Cervus (version 3.0.7) showed that the average observed and expected heterozygosity ratios were 0.138 and 0.4958 respectively. The mean PIC was found to be 0.4364, indicating that these primer pairs were useful to demonstrate the genetic diversity among pennywort accessions (Table 8). Furthermore, analysis using POPGENE 1.32 showed that the genetic diversity was high within the germplasm (Shannon index averaged at 0.8723). Finally, NTSYSpc 2.1 was employed to obtain the genetic relationship within the germplasm. Based on this analysis, the germplasm was divided into three groups (Fig. 3). Group I contained only one accession: HUIB_CA02, whereas group IIA included HUIB_CA06, HUIB_CA08 and HUIB_CA11. The rest belonged to group IIB. While a few accessions collected from proximal geographical locations such as HUIB_CA01 and HUIB_CA15, HUIB_CA05 and HUIB_CA07 were genetically close (coefficients larger than 0.6), others displayed larger genetic distances (HUIB_CA06, HUIB_CA09, HUIB_CA26 and HUIB_CA27).

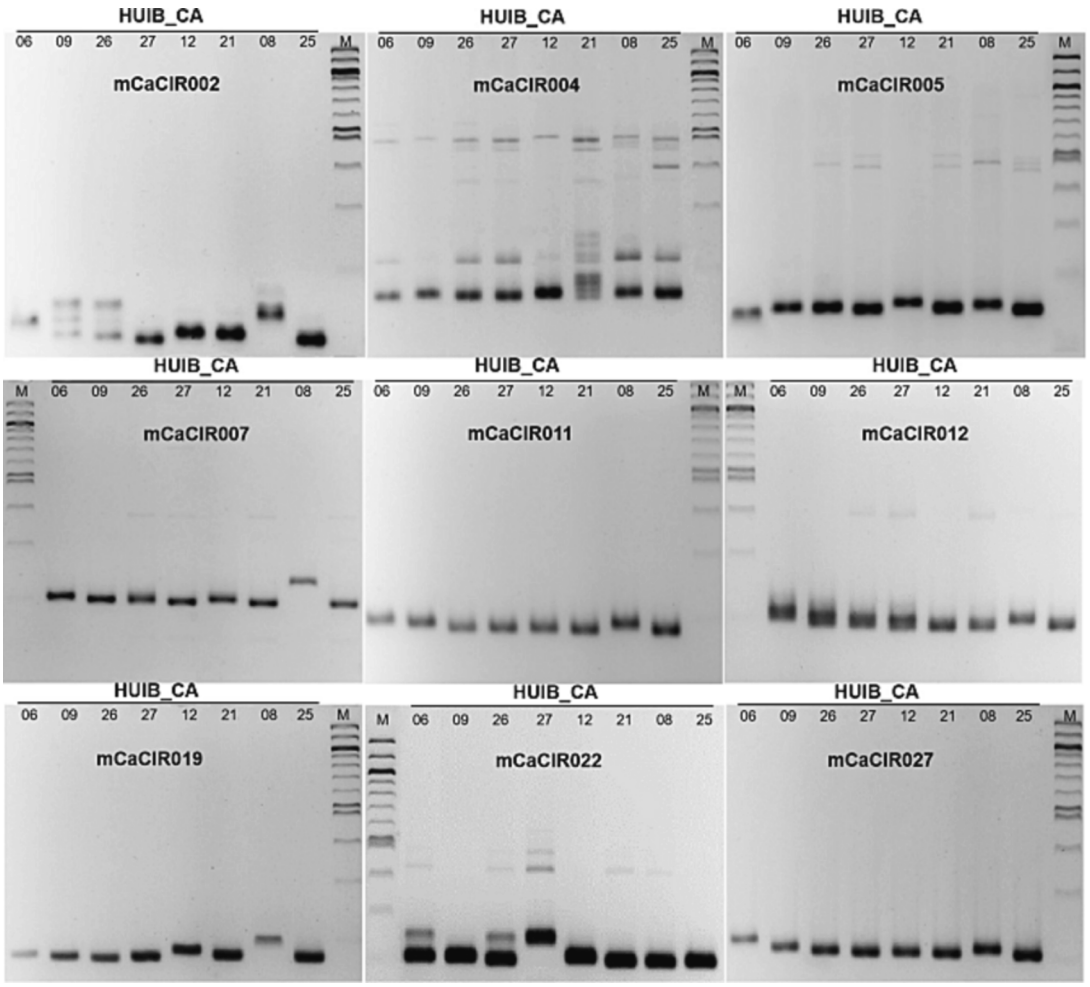

Fig. 2. Representative gel showing PCR products obtained using nine pairs of SSR primers: mCaCIR002, mCaCIR004, mCaCIR005, mCaCIR007, mCaCIR011, mCaCIR012, mCaCIR019, mCaCIR022 and mCaCIR027. M: 100 bp DNA ladder (NEB, US).

Table 8
Details of the genetic diversity at various loci in the *C. asiatica* germplasm using fourteen SSR primer pairs.

Primer pairs	Number of alleles	Number of polymorphic alleles	Polymorphism percentage (%)	Size (bp)	PIC	H_o	H_e	n_e	I
mCaCIR002	6	6	100	140–170	0.667	0.2500	0.7429	3.6688	1.4773
mCaCIR004	2	1	50	170–190	0.037	0.0385	0.0385	1.0392	0.0950
mCaCIR005	3	3	100	150–170	0.343	0.0000	0.3861	1.6095	0.6871
mCaCIR007	6	6	100	170–230	0.685	0.0400	0.7404	3.6443	1.4789
mCaCIR011	3	3	100	190–210	0.521	0.0000	0.6184	2.5311	0.9899
mCaCIR012	3	3	100	200–220	0.562	0.0000	0.6465	2.7293	1.0512
mCaCIR019	4	4	100	175–210	0.603	0.1923	0.6780	2.9845	0.8763
mCaCIR022	3	3	100	200–250	0.454	0.1538	0.5196	2.0392	0.8763
mCaCIR027	2	2	100	200–210	0.311	0.0400	0.3927	1.6255	0.5731
mCaCIR028	2	2	100	180–190	0.164	0.0400	0.1837	1.2195	0.3251
mCaCIR029	4	4	100	240–300	0.492	0.2692	0.5400	2.1258	1.0123
mCaCIR030	3	3	100	200–250	0.570	0.6538	0.6569	2.8108	1.0647
TBG-CentaF 15	3	2	67	180–220	0.140	0.1538	0.1478	1.1696	0.3245
TBG-CentaF 31	3	3	100	170–190	0.561	0.1000	0.6500	2.7304	1.0504
All	**47**	**45**	**50–100**	**140–300**					
Average					**0.4364**	**0.1380**	**0.4958**	**2.2805**	**0.8723**
Standard deviation						**0.1747**	**0.2311**	**0.8724**	**0.4213**

PIC: Polymorphism information content, H_0: Observed heterozygosity, H_e: Expected heterozygosity, n_e: number of effective alleles, I: Shannon index.

4. Discussion

Morphological characterisation has been useful for plant breeders to breed cultivars with advanced agronomic traits. In this study, morphological features of 26 *Centella asiatica* accessions collected in Vietnam and Laos were studied. Apart from the glabrous leaf surface and hard stolon, these accessions displayed a wide range of qualitative and quantitative morphological features. Fresh yield per tray at the first

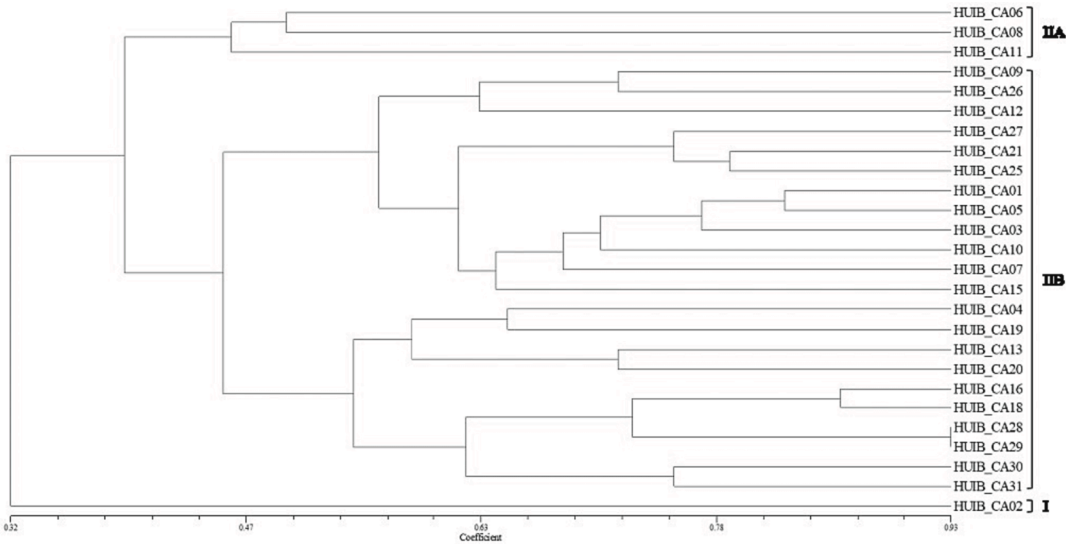

Fig. 3. Dendrogram showing the genetic relationship between 26 *C. asiatica* accessions collected throughout Vietnam and Laos.

harvest varied from 107 g (HUIB_CA19) to 478 g (HUIB_CA20) whereas plant weight varied from 0.43 g (HUIB_CA18) to 8.9 g (HUIB_CA20). HUIB_CA20 was the accession with the highest yield in this germplasm. Given the tray's area was 0.26 m^2, the yield of HUIB_CA20 was extrapolated to more than 18 tons of fresh weight per ha, which was higher than those of reported pennywort accessions from India (13–14 tons per ha) (Rohini & Smitha, 2022). Furthermore, HUIB_CA20 exhibited good regenerability, the largest leaf size and erect growth habit. Typically, erect growth facilitates harvesting and enhances resistance to soil-borne diseases. In terms of bioactive compounds, HUIB_CA20 also had the highest flavonoid content and third highest saponins content. Another accession, HUIB_CA27 also displayed erect growth, good regenerability, the second highest fresh yield and high flavonoid and saponin contents. Therefore, HUIB_CA20 and HUIB_CA27 can be considered elite lines for future breeding programs aiming to develop high-yielding *C. asiatica* cultivars with high flavonoid/saponin contents.

The diversity in morphology among plant populations is often attributed to environmental and genetic factors. Here, we ensured that the environmental conditions were identical and therefore, the observed morphological diversity was largely genetic. This is corroborated by the fact that pennywort plants are cross-pollinated by insects (Duara & Kalita, 2013), giving rise to diverse pools of genetic materials. Consistent with this, our SSR analysis demonstrated the large genetic diversity among pennywort accessions in Vietnam and Laos (Fig. 3, Table 8). The range of PIC observed in this study (0.037 to 0.685) was comparable to those reported previously (Rakotondralambo et al., 2013; Rohini et al., 2019). Of the 14 SSR primer pairs, PIC values of mCaCIR002, mCaCIR007 and mCaCIR019 were larger than 0.6, indicating their usefulness in studying the genetic diversity among *C. asiatica* accessions in Vietnam and Laos. However, mCaCIR002 and mCaCIR019 did not yield 100 % polymorphism rate in other studies, suggesting genetic diversity among accessions collected in different geographic regions (Rakotondralambo et al., 2013; Rohini et al., 2019).

Despite the variations in morphology and genetics, the phytochemical contents were quite similar between accessions grown under the same agro-climatic conditions. The phenolic contents were similar to those reported previously (19.9 mg GAE/g of dry weight) but flavonoid contents were lower than published values (Seong et al., 2023). Future work will focus on further characterising the phytochemical contents of leaf extracts from elite lines (HUIB_CA20 and HUIB_CA27), with a focus on notable saponins: asiatic acid, asiaticoside, madecassoside and madecassic acid for their potential use as cancer therapies (Kraft et al., 2022).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by the Ministry of Science and Technology of Thua Thien Hue Province (Grant No. TTH.2020-KC.09) using Thua Thien Hue province state budget. The authors acknowledge partial support from the Core Research Program, Hue University (Grant No. NCM.DHH.2019.01) and Quang Tho 2 Agricultural Cooperative. We thank Dr. Nguyen Quang Co for sample collection, Dr. Dang Thanh Long for help with the phytochemical analysis, Sonexay Rasphone, Nguyen Van Hoan and Hatsadong Chanthanousone for plant care.

References

Atanassova, M., Christova-Bagdassarian, V., 2009. Determination of tannins content by titrimetric method for comparison of different plant species. J. Univ. Chem. Technol. Metal. 44, 413–415.

Biswas, A., Sahoo, J., Chatli, M.K., 2011. A simple UV-Vis spectrophotometric method for determination of β-carotene content in raw carrot, sweet potato and supplemented chicken meat nuggets. LWT Food Sci. Technol. 44, 1809–1813.

Brinkhaus, B., Lindner, M., Schuppan, D., Hahn, E.G., 2000. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. Phytomedicine 7, 427–448.

Duara, P., Kalita, J., 2013. An investigation on the pollinating insects of medicinally important plants. Intl. J. Life Sci. Biotechnol. Pharma Res. 2, 318–324.

Eze, F.N., Tola, A.J., Nwabor, O.F., Jayeoye, T.J., 2019. *Centella asiatica* phenolic extract-mediated bio-fabrication of silver nanoparticles: characterization, reduction of industrially relevant dyes in water and antimicrobial activities against foodborne pathogens. RSC Adv. 9, 37957–37970.

Gohil, K., Patel, J., Gajjar, A., 2010. Pharmacological Review on *Centella asiatica*: A Potential Herbal Cure-all. Indian J. Pharm. Sci. 72, 546–556.

Gupta, S., Bhatt, P., Chaturvedi, P., 2018. Determination and quantification of asiaticoside in endophytic fungus from *Centella asiatica* (L.). Urban. World J Microbiol Biotechnol 34, 111.

Hussin, F., Eshkoor, S.A., Rahmat, A., Othman, F., Akim, A., 2014. The *Centella asiatica* juice effects on DNA damage, apoptosis and gene expression in hepatocellular carcinoma (HCC). BMC Complement Altern Med 14, 32.

Kalinowski, S.T., Taper, M.L., Marshall, T.C., 2007. Revising how the computer program cervus accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16, 1099–1106.

Kraft, O., Hartmann, A.-K., Hoenke, S., Serbian, I., Csuk, R., 2022. Madecassic Acid - A New Scaffold for Highly Cytotoxic Agents. Int. J. Mol. Sci. 23, 4362.

Krishnan, P.G.R., Chethala, V.N., Rajmohan, K., Soni, K.B., 2007. RAPD Analysis of *Centella asiatica* from different locations of Kerala. In: R. Keshavachandran, P. A. Nazeem, D. Girija, P. S. John and K. V. Peter, (eds.), Recent Trends in Horticultural Biotechnology, Vol. III, pp. 593-597.

Krivorotova, T., Sereikaite, J., 2014. Determination of fructan exohydrolase activity in the crude extracts of plants. Electron. J. Biotechnol. 17, 329–333.

Kunjumon, R., Johnson, A.J., Baby, S., 2022a. *Centella asiatica*: Secondary metabolites, biological activities and biomass sources. Phytomedicine plus 2, 100176.

Kunjumon, R., Johnson, A.J., Remadevi, R., Baby, S., 2022b. Assessment of major centelloside ratios in *Centella asiatica* accessions grown under identical ecological conditions, bioconversion clues and identification of elite lines. Sci. Rep.

Le, A.V., Parks, S.E., Nguyen, M.H., Roach, P.D., 2018. Improving the Vanillin-Sulphuric Acid Method for Quantifying Total Saponins. Technologies 6.

Mudaliana, S., 2021. Antimicrobial activity of *Centella asiatica* and *Gigantochloa apus*. J Basic Clin Physiol Pharmacol 32, 755–759.

Nav, S.N., Ebrahimi, S.N., Sonboli, A., Mirjalili, M.H., 2021. Variability, association and path analysis of centellosides and agro-morphological characteristics in Iranian *Centella asiatica* (L.) Urban ecotypes. S. Afr. J. Bot. 139, 254–266.

Padmalatha, K.V., Prasad, M.N.V., 2008. Genetic diversity in *Centella asiatica* (L.) Urb., a memory-enhancing neutraceutical herb, using RAPD markers. Med. Arom. Plants Sci. Biotechnol. 2, 90–95.

Park, J.H., Choi, J.Y., Son, D.J., Park, E.K., Song, M.J., Hellström, M., Hong, J.T., 2017. Anti-inflammatory effect of titrated extract of *Centella asiatica* in phthalic anhydride-induced allergic dermatitis animal model. Int. J. Mol. Sci. 18, 738.

Pasri, P., Mermillod, P., Khempaka, S., 2023. Antioxidant properties and cytotoxic effects of selected edible plants in Southeast Asia for further use as phytogenic antioxidant additives. Saudi J. Biol. Sci. 30, 103631.

Prasad, A., Dhawan, S.S., Mathur, A.K., Prakash, O., Gupta, M.M., Verma, R.K., Lal, R.K., Mathur, A., 2014. Morphological, chemical and molecular characterization of *Centella asiatica* germplasms for commercial cultivation in the Indo-Gangetic plains. Nat. Prod. Commun. 9 (6).

Prasad, A., Yadav, K.S., Yadav, N.P., Mathur, A., Sreedhar, R.V., Lal, R.K., Mathur, A.K., 2016. Biomass and centellosides production in two elite *Centella asiatica* germplasms from India in response to seasonal variation. Ind. Crop. Prod. 94, 711–720.

Rakotondralambo, S.O.R., Lussert, A., Rivallan, R., Danthu, P., Noyer, J.L., Baurens, F.C., 2012. Microsatellite markers isolated from the wild medicinal plant *Centella asiatica* (Apiaceae) from an enriched genomic library. Am. J. Bot. 99, e176–e178.

Rakotondralambo, S.O.R., Rodier-Goud, M., Rivallan, R., Lussert, A., Danthu, P., Lamotte, F., Ralambofetra, E., Ramavovololona, P., Noyer, J.L., Baurens, F.C., 2013. Insight into the biology, genetics and evolution of the *Centella asiatica* polyploid complex in Madagascar. Ind. Crop. Prod. 47, 118–125.

Ravi, C., Umesha, K., HimaBindu, K., Shetty, R., Kumar, G., 2019. Collection and Morphological Variability in Ecotypes of Indian Pennywort (*Centella asiatica* L.) of Hill Zone of Karnataka, India. Int. J. Curr. Microbiol. App. Sci. 8, 994–1008.

Rohini, M.R., Sane, A., Chaudhary, R., Himabindu, K., 2019. Molecular characterization and DNA fingerprinting of *Centella asiatica* using SSR markers. Int. J. Chem. Stud. 7, 705–710.

Rohini, M.R., Smitha, G.R., 2022. Studying the effect of morphotype and harvest season on yield and quality of Indian genotypes of *Centella asiatica*: A potential medicinal herb cum underutilized green leafy vegetable. S. Afr. J. Bot. 145, 275–283.

Ruíz-Valdiviezo, V., Luna-Guido, M., Galzy, A., Gutiérrez-Miceli, F., Dendooven, L., 2010. Greenhouse gas emissions and C and N mineralization in soils of Chiapas (México) amended with leaves of *Jatropha curcas* L. Appl. Soil Ecol. 46, 17–25.

Saikia, D., Gogoi, P.K., Phukan, P., Bhuyan, N., Borchetia, S., Saikia, J., 2015. Green synthesis of silver nanoparticles using asiatic pennywort and bryophyllum leaves extract and their antimicrobial activity. Adv. Mater. Lett. 6, 260–264.

Sakthipriya, M., Vishnu, S.S., Sujith, S., Kumar, P., Sabu, K.K., 2018. Analysis of genetic diversity of *Centella asiatica* using SSR markers. Int. J. Appl. Sci. Biotechnol. 6, 103.

Satpathy, L., Pradhan, N., Dash, D., Baral, P.P., Parida, S.P., 2021. Quantitative determination of vitamin C concentration of common edible food sources by redox titration using iodine solution. Lett. Appl. Biosci. NanoBioSci. 10, 2361–2369.

Seong, E., Heo, H., Jeong, H.S., Lee, H., Lee, J., 2023. Enhancement of bioactive compounds and biological activities of *Centella asiatica* through ultrasound treatment. Ultrason. Sonochem. 94, 106353.

Shukurova, M., Myint, D., Yi, S., Saw, O., Watanabe, K., 2021. Morphological description and ethnobotanical review of the orphan crop Myin-Hkwa *(Centella asiatic*a L.) from Myanmar. Front. Sustain. Food Syst. 5, 680862.

Singh, S.P., Misra, A., Kumar, B., Adhikari, D., Srivastava, S., Barik, S.K., 2022. Identification of potential cultivation areas for centelloside-specific elite chemotypes of *Centella asiatica* (L.) using ecological niche modeling. Ind. Crop. Prod. 188, 115657.

Singleton, Y.I., Rossi, J.A., 1965. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Amer. J. Enol. Vitic 16, 144–158.

Sun, B., Wu, L., Wu, Y., Zhang, C., Qin, L., Hayashi, M., Kudo, M., Gao, M., Liu, T., 2020. Therapeutic potential of *Centella asiatica* and its triterpenes: A review. Front. Pharmacol. 11.

Tanga, B.M., Bang, S., Fang, X., Seo, C., De Zoysa, M., Saadeldin, I.M., Lee, S., Park, S.U., Chung, S.O., Lee, G.J., Cho, J., 2022. *Centella asiatica* extract in carboxymethyl cellulose at its optimal concentration improved wound healing in mice model. Heliyon 8, e12031.

Valdeira, A.S.C., Darvishi, E., Woldemichael, G.M., Beutler, J.A., Gustafson, K.R., Salvador, J.A.R., 2019. Madecassic acid derivatives as potential anticancer agents: synthesis and cytotoxic evaluation. J. Nat. Prod. 82, 2094–2105.

Wong, J.X., Ramli, S., 2021. Antimicrobial activity of different types of *Centella asiatica* extracts against foodborne pathogens and food spoilage microorganisms. LWT 142, 111026.

Zhang, X.G., Han, T., He, Z.G., Zhang, Q.Y., Zhang, L., Rahman, K., Qin, L.P., 2011. Genetic diversity of *Centella asiatica* in China analyzed by inter-simple sequence repeat (ISSR) markers: Combination analysis with chemical diversity. J. Nat. Med. 66, 241–247.

Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559.