Analysis of genetics and chemical contents relation compared to commonly used *Cissus quadrangularis* L. and barcode markers of some Thailand *Cissus* species

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Abstract: Several *Cissus* species are commonly used as traditional and modified medicines, and their chemical constituents are major point for precise usage. However, *C. quadrangularis* is the only species for which the usages and the chemical composition have been reported. These data should be investigated for other species in the genus. Eight species namely *C. assamica*, *C. carnosa*, *C. elongata*, *C. hastata*, *C. javana*, *C. pteroclada*, *C. quadrangular* is and *C. repens* were evaluated for genetic relationships and chemical composition. Constructed dendrogram shows high-powered efficiency of inter-simple sequence repeat (ISSR) data used which can clearly identify different and identical species. Genetic similarity (S) value of the identical species is 0.86, whereas for different species the value can vary from 0.53 to 0.75. Four highly related species (S=0.64-0.72), *C. assamica*, *C. carnosa*, *C. hastata* and *C. repens* were selected to undergo chemical study by gas chromatography-mass spectrometry (GC-MS) on the methanol crude extract. Only one compound, β-sitosterol, found in the four species is identical to the compound reported from *C. quadrangular* is, where there were five identical chemicals found in the selected species. Species-specific barcode with *rbc*L region was constructed. Nucleotide variation was evaluated indicating genetic distance value of 0.025 to 0.072.

Keywords: *Cissus*; inter-simple sequence repeat (ISSR); medicinal plant; phytochemicals; DNA barcode; *rbc*L region

INTRODUCTION

Cissus is a genus of the family Vitaceae, which has not reported a number of species by Flora of Thailand except for progress. *Cissus quadrangular* is a well-known and very important species in Thailand, which is used as medication in both traditional and modified forms including tablets, capsules, or pure extracted substances. The properties are weight loss for overweight and obese people, and the improvement of cardiovascular health (Oben *et al*., 2007). Diseases which have long been treated with *C. quadrangularis* in both its native and modernized forms are hemorrhoids and bone fracture. The effects associated with hemorrhoids are analgesic properties, anti-inflammatory activities, as well as, a reduction in the size of the hemorrhoids (Panthong *et al*., 2007). *Cissus quadrangular* is has also been proven to be of use in the treatment of bone fractures or in hastening bone healing by a faster initiation of healing by Ruangwitthayanon and Leeanansaksiri (2009), Deka *et al*. (1994), and Shirwaikar *et al*. (2010). Mishra *et al*. (2010) reviewed many publications and reported chemical compounds in *C. quadrangular* is that were found in different plant parts such as the roots and stems by using different methods and extract buffers, and in different countries. Phytochemical studies of *C. quadrangularis* have shown the presence of several major constituents such as vitamin C, triterpene, β-sitosterol, ketosteroid, two asymmetrical tetracyclic triterpenoids (onocer-7ene3α,21βdiol ($C_{30}H_{52}O_2$) and onocer-7ene-3β,21αdiol ($C_{30}H_{52}O_2$), and calcium. In addition, many others including resveratrol, piceatannol, pallidol perthenocissine, and phytosterols, two steroidal principles ($C_{27}H_{45}O$ and $C_{23}H_{41}O$), δ amyrin, δ amyrone, and a new asymmetric tetracyclic triterpenoid as 7-oxo-onocer-8-ene-3β21αdiol ($C_{30}H_{50}O_3$) have been found. Seven new compounds have also been reported which are as follows: a) 4-hydroxy2methyl-tricos-2ene-22-one, b) 9-methyl-octadec-9-ene, c) hepadecyl-octadecanoate, d) icosanyl-icosanoate, e) 31-methyl tritiacontan-1-ol, f) 7-hydroxy-20-oxo-docosanyl cyclohexane, and g) 31-methyl tritiacontanoic acid. Additionally, the following compounds have also been found and reported: taraxeryl acetate, friedelan-3-one, taraxerol, isopentacosanoic acid, 3,3′,4,4′-tetrahydroxybiphenyl (a water-soluble glycoside, which produces a fall in blood pressure in anaesthetized cats), and three new stilbene derivatives, namely quadrangularins A, B, and C. More recently, Kokilavani *et al.* (2014) have proved the valuable properties of *C. quadrangular* is that it significantly recovered the alterations in antioxidant profiles and prevent male infertility by preventing the inhibition of steroid genesis.

Also, some of the other *Cissus* species have been studied. The alkaloids of the leaves of *C. rheifolia* are cryptopleurine, kayawongine, isomeric terpenoid vomifoliol, romalea allene, and flavonoid vitexin (Saifah *et al*., 1983). β-Sitosterol and sitosterol-β-D-

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glucopyranoside have been isolated from *C. sicyoides* and have shown antibacterial activity against *Bacillus substilis*, but have not shown any anti-diabetic activity (Beltrame *et al*., 2002). Chemical contents in the aerial parts of *C. repens* are four new stilbene C-glucosides as trans-3-O-methyl-resveratrol-2-C-beta-glucoside, cis-3-O-methyl-resveratrol-2-C-beta-glucoside, trans-3-O-methyl-resveratrol-2-(2-p-coumaric)-C-beta-glucoside (cissuside A), and trans-3-O-methyl-resveratrol-2-(3-p-coumaric)-C-beta-glucoside (cissuside B), along with three known trans-resveratrol, trans-resveratrol-2-C-beta-glucoside, and cis-resveratrol-2-C-beta-glucoside (Wang *et al*., 2009). Xie *et al*. (2009) isolated eight compounds from *C. assamica* namely ursolic acid, lupeol, n-hexacosinc acid, isolariciresino-9-o-β-D-glucopyranoside, dauco sterin, 3,3′-dimethy ellagicacid, β-sitosterol, and berginin. James *et al*. (2010) concluded that distilled water extracts of *C. multistriata* leaves contain a mixture of many powerful antioxidant compounds and that may be one of the potential mechanisms responsible for claiming its diverse therapeutic effects. Recently, more phytochemicals and their properties have been reported for *Cissus* species. Subramani *et al*. (2014) showed the antimicrobial activity and some bioactive compounds, such as steroids, triterpenoids, glycosides, sugar, alkaloids, flavonoids, tannins, amino acid, and coumarin from the *C. vitiginea* leaf extract. Crude extracts, aqueous (Shi *et al*., 2014), alcoholic extracts (Ahmad *et al*., 2014; Bakht *et al*., 2014), or hexane extracts (Chaveerach *et al*., 2014) have been successfully tested for their activities, therefore, leading for further applications.

Cissus carnosa locally called '*hun*' in Loei Province in Thailand has been used as an anti-inflammatory and inflammatory treatment on hands and feet. Aerial parts of the plant are boiled in water for 15 minutes, left to sit until warm, and then hands or feet are soaked in the warm water for 30 minutes to an hour. The wounded areas will recover (corresponding author).

Thailand is expected to have 25 to 30 *Cissus* species (Flora of Thailand Project, http://web3.dnp.go.th/botany/index.aspx; Smitinand, 2001). However, only *C. quadrangularis* has been extensively reported for its chemical contents and properties. In regard to discovering its usefulness for healing bone fracture and hemorrhoids, the genus should be studied to reveal the diversity of the species in order to find its uses in traditional medicine and as important chemicals both in local and industrial products instead of consuming *C. quadrangularis* solely. The genetic relationships of the group of *Cissus* species will imply which of the species have properties similar to *C. quadrangularis*. Various molecular approaches called DNA markers, such as DNA fingerprints based on polymerase chain reaction (PCR) and DNA barcodes are essential in precisely analyzing the relationships. The fingerprints as inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) methods are generally used to effectively indicate genetic relationships. The banding patterns reveal genetic variations/relationships by cladogram construction. The concept critical to cladistics is homology, which can be defined as a similarity/distance resulting from common ancestry (Simpson, 2006). These markers for genetic variation are generally independent of environmental factors and are more numerous than phenotypic characteristics. As a result, they provide a clearer indication of the underlying variations in the genome.

DNA barcoding is a popular method for species identification and can be used in samples which have only a short reliable DNA region or in samples such as processed food, fossil remains, or herbarium specimens in which the DNA has been highly degraded. In plant groups there have been many studies that have tested standard regions that have aimed to provide rapid, accurate, and automatable species identification by using a standardized DNA region as a tag (Hebert and Gregory, 2005). Chase *et al*. (2007) proposed using two barcoding region options as a standard protocol for barcoding all land plants: the three combined regions of the *rpo*C1, *mat*K, and *psb*A-*trn*H intergenic spacers, or the *rpo*B, *mat*K, and *psb*A-*trn*H regions. Newmaster *et al*. (2008) proposed using *mat*K and *psb*A-*trn*H to identify plants in Myristicaceae. More recently, CBOL Plant Working Group (CBOL Plant Working Group, 2009) recommended using *rbc*L+*mat*K as the core DNA barcode regions for land plants. Many plant groups such as *Dendrobium* species (Asahina *et al*., 2010), mosses (Liu *et al*., 2010), *Nymphaea* species (Chaveerach *et al*., 2011), medicinal plants of *Smilax* and *Cissus* (Kritpetcharat *et al*., 2011), medicinal *Senna* species (Monkheang *et al*., 2011), and *Cymbidium* species (Siripiyasing *et al*., 2012) have all had their barcodes recorded in the GenBank for identification of plant parts.

It is clear that *C. quadrangularis* has served human beings as a natural source for traditional treatments and chemicals from ancient times to the present. There is a high biodiversity of *Cissus* species in Thailand. Other species in the group may be used in the same ways as *C. quadrangularis*. Therefore, the aim of this research is to analyze the relationship of the genetics and the chemical contents of some *Cissus* species by comparing them to *C. quadrangularis*. Additionally, each species-specific marker has been constructed as a barcode by *rbc*L sequencing.

MATERIALS AND METHODS

Plant materials

Eight *Cissus* species, namely *C. assamica* Craib, *C. carnosa* Lam., *C. elongata* Roxb., *C. hastata* Miq., *C. javana* DC., *C. pteroclada* Hayata, *C. quadrangularis* L., and *C. repens* Lam., were collected and identified following the Flora of China (Hui and Wen, 2007). Young leaves were collected and immediately kept at -20°C for DNA extraction for DNA fingerprinting and

DNA barcoding. Mature leaves were kept for chemical extraction.

DNA extraction

Genomic DNA was extracted from all collected samples using the Plant Genomic DNA Extraction Kit (RBC Bioscience). The extracted DNA was examined by subjecting it to 0.8% agarose gel electrophoresis with ethidium bromide staining. The extracted DNA was used as a DNA template in the PCR reaction.

DNA fingerprinting by ISSR marker and dendrogram construction

Amplifications were carried out in 25µl reactions consisting of GoTaq Green Master Mix (Promega), 0.5 µM primers, and a 5 ng DNA template. A set of 38 ISSR primers were screened and the 12 primers that successfully amplified clear bands are as follows (5′ to 3′): $(CT)_8TG$, $(CT)_8GC$, $(CA)_6AC$, $(ACTG)_4$, $(GT)_8C$, $(AG)_8G$, $(AG)_8T$, $(CA)_8CC$, $(CA)_9A$, $(CA)_9T$, $(CA)_9G$, and $(AG)_7AAA$. The reaction mixtures were incubated at 94°C for 3 min and the amplification was performed with the following 35 thermal cycles: denaturation for 1 min at 94°C, annealing for 2 min at 50°C, extension for 2 min at 72°C, and final extension for 7 min at 72°C using a Swift Maxi Thermal Cycler (Esco Micro Pte. Ltd.). Amplified products were detected by 1.2% agarose gel electrophoresis in TAE buffer and visualized using ethidium bromide staining. The resulting ISSR bands from the successfully amplified primers and discerned from the agarose gel were documented as diallelic characters: present=1, absent=0, for the ISSR are considered as the dominant markers. These resulting bands were used to construct a dendrogram by the NTSYS-pc 2.1p software (Rohlf, 1998).

DNA barcode amplification and sequence analysis

PCR was performed using primer pairs 5′-ATGTCACCA CAAACAGAGACTAAAGC-3′ and 5′-GTAAAATCAA GTCCACCRCG-3′ of the *rbc*L gene region, 5′-TAATTT ACGATCAATTCATTC-3′ and 5′-CTTCCTCTGT AAAGAATTC-3′ of the *mat*K gene region (http://www.kew.org/barcoding/update.html; 28 January 2009). The reaction mixture was done in 25µl consisting of the GoTaq Green Master Mix (Promega), 0.25 µM each primer, and a 10ng DNA template. The reaction mixture was incubated at 94°C for 1 minute and amplification was performed with the following 35 thermal cycles: denaturation for 30 s at 94°C, annealing for 40 s at 53°C, extension for 40 s at 72°C, and the final extension for 5 min at 72°C. The amplified products were detected by 1.2% agarose gel electrophoresis in TAE buffer and visualized with ethidium bromide staining. Sequences were analyzed and annotated using online tools namely BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ExPASy (http://www.expasy.org). The amplified specific fragments were sequenced and the sequences were submitted to the GenBank database. The sequences were compared (sequence alignment) using MEGA software version 5 (Tamura *et al*., 2011) indicating the genetic distances simply shown in tables.

Preparation of Chemical Extracts

Four studied species namely *C. assamica*, *C. carnosa*, *C. hastata*, and *C. repens* were extracted by the following procedure. Air-dried leaves weighing 25 g were ground and mixed with 120 mL methanol solvent (analytical grade), and were then filtered through a Whatman No.1 filter paper at room temperature. The filtrate was stored at -20°C until it was used for the GC-MS analysis.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis and Identification of Components

The GC-MS analysis of the crude extracts was performed using an Agilent Technologies GC 6890 N/5973 inert MS fused with a capillary column (30.0m x 250µm x 0.25 µm). Helium gas was used as the carrier gas at a constant flow rate of 1µL/min. The injection and the mass-transferred line temperature were set at 280°C. The oven temperature was programmed for 70°C to 120°C at 3°C/min, was then held isothermally for 2 min, and was finally raised to 270°C at 5°C/min. A 1 µL aliquot of the crude extract was injected in the split mode. The relative percentage of the crude constituents was expressed as the percentage using peak area normalization. Identification of the components of the crude was assigned by a comparison of the mass spectra obtained with those of the reference compounds stored in the Wiley 7N.1 library.

RESULTS

The eight *Cissus* species studied are easily found as climbing plants in both forests and in communities. Some species like *C. carnosa* are considered weeds and can always be found growing on the roofs of building. Others can be seen as ornaments on the roofs of buildings or on fences such as *C. pteroclada*, or as ornamental potted plants like *C. javana*, which is sold at plant markets. *Cissus quadrangularis* is planted in the home garden for ornamentation and is also used as traditional medicine. Yet another, *C. hastata*, is used to provide a sour flavor to Thai curry. The other two species were collected from forests near a national park in Loei Province located in Northeastern Thailand. One more species, *C. repens*, which is used in traditional medicine, was collected from Khon Kaen Province. *Cissus repens* is a wild plant that has been moved so that it can be cultivated on farms and used in traditional medical settings under the local names of '*khao-yen nuer*' and '*khao-yen tai*'.

All collected samples of the eight species were studied by DNA analysis using the ISSR method. The 38 primers were screened and 12 primers successfully produced 407 banding patterns. The sample of DNA bands are shown in

fig. 1. A total of 407 DNA band data were used for the dendrogram construction as shown in fig. 2.

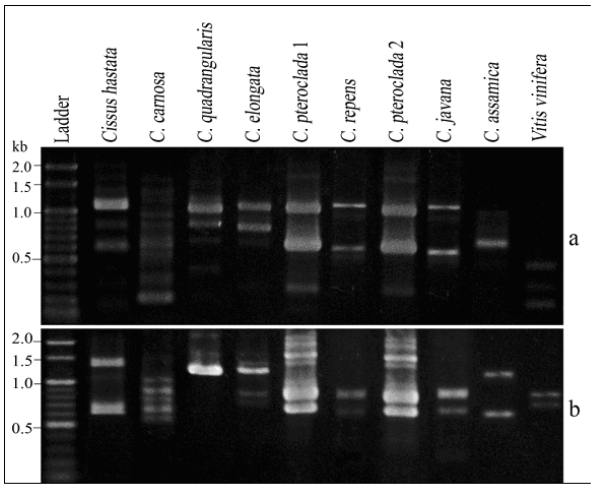

Fig. 1: Two examples of ISSR banding patterns from primers $(CT)_8$ TG (a) and $(CT)_8$GC (b)

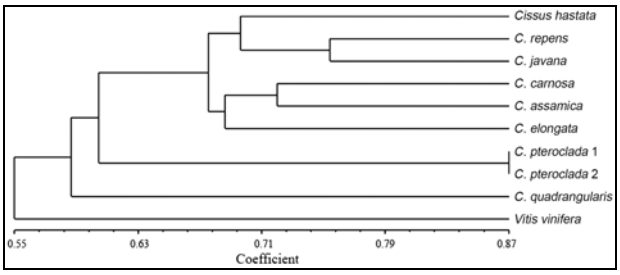

Fig. 2: The dendrogram constructed from total ISSR bands from twelve primes of the eight species of *Cissus* by NTSYS-pc 2.1p.

The dendrogram shows the high-powered efficiency of the ISSR data used, which has clearly distinguished each of the different species, identify identical species and has also separated out *Vitis vinifera*, the outgroup. The genetic similarity (S) value shown in table 1 indicates that the relationship of the identical species, *C. pteroclada* 1 and *C. pteroclada* 2, is 0.86. The S value of the different species is 0.53 between *C. pteroclada* 1 and *C. elongata*, and between *C. pteroclada* 2 and *C. elongata*, and is 0.75 between *C. javana* and *C. repens*.

Based on the DNA profiles, the S values of the four species; *C. assamica*, *C. carnosa*, *C. hastata*, and *C. repens*; are 0.64 (*C. carnosa* and C. *hastata*; *C. carnosa* and *C. repens*) to 0.72 (*C. assamica* and *C. carnosa*). These were higher than the S values of *C. assamica*, *C. carnosa*, *C. hastata*, and *C. repens* when compared to *C. quadrangularis*, 0.59, 0.57, 0.59 and 0.60, respectively. So, these four species were selected to undergo primarily chemical study by GC-MS with the major point as a higher S level in the group and a lower S level of the group when compared to *C. quadrangularis*.

The preliminary phytochemical screening on the methanol crude extract of the four *Cissus* species showed the presence of different chemical compounds. Chemical compounds, retention times, and relative contents for each plant are revealed in table 4. The total ion chromatographs (TIC), showing the peak identities of the compounds identified within each of the species studied, are shown in fig. 3.

The results of barcode performance with *mat* K and *rbc*L regions in the nine species of *Cissus* studied and the outgroup have been reported. The amplification was successful in only the *rbc*L gene region. The fragment sizes are about 550bp. Sequence alignments were compared for evaluation of nucleotide variations indicating genetic distance values as shown in table 2. Nucleotide variations of the *rbc*L region range from 0.025 (*C. assamica* and *C. repens*) to 0.072 (*C. javana* and *C. elongata*) and (*C. elongata* and *C. quadrangularis*), respectively. The tag sequences are kept at GenBank database with Gen Bank accession numbers shown in table 3.

DISCUSSION

The major characteristics for grouping plants are the reproductive parts, so many plant species have been classified as *Cissus* because of the aforementioned characteristics. In regard the vegetative characteristics of *C. quadrangularis*, although its stem is a climber and it has characteristics similar to those of other *Cissus* species, there are differences in structure. The *C. quadrangularis* stem is quadrilateral and looks very different from the other species in the group. Accordingly, *C. quadrangularis* shows a far distance on the similarity index as compared to *C. assamica*, *C. carnosa*, *C. hastata*, and *C. repens* with 0.59, 0.57, 0.59, and 0.60, respectively. However, these similarity values remain within the range for the difference levels given the identical level of the value is 0.86 for *C. pteroclada* 1 and *C. pteroclada* 2. Morphological characteristics are expression characters, and ISSR data studies the whole genome. Therefore, it is better to use both morphological character states and ISSR markers to assist in identifying different or identical species.

This research has used individuals of a species, however it is possible to use small sample sizes in molecular studies as quoted by Hillis (1987). The sizes used in molecular studies are usually much smaller than in morphological studies (often as small as a single individual) because the analyses of large sample sizes are often limited by the availability of specimens and/or the expense of the analysis. However, the *Cissus* samples that were studied have been randomly collected which has led to having realistic results. Morphological characteristics and expression characters are a subset of genomic study.

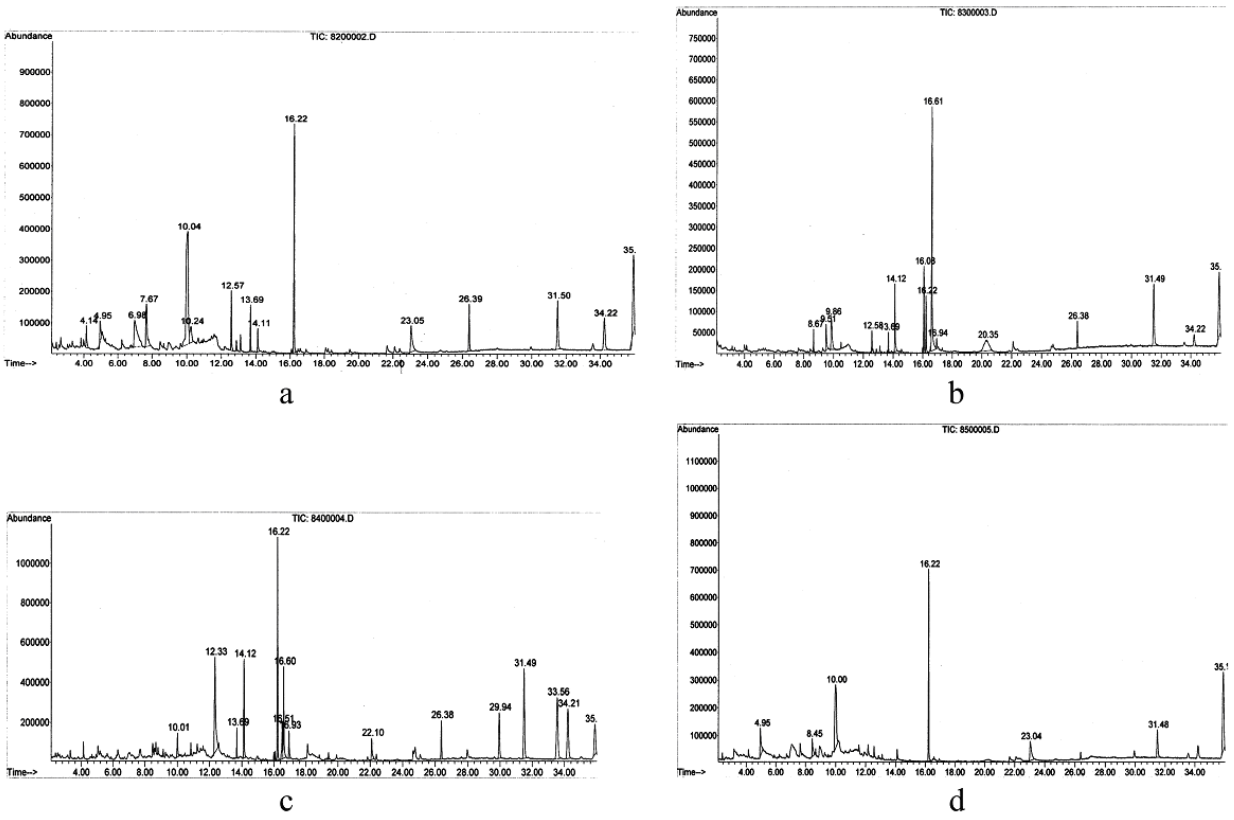

Fig. 3: GC-MS chromatogram of methanol crude extracts on the leaves of the four *Cissus* species, *C. assamica* (a), *C. carnosa* (b), *C. hastata* (c) and *C. repens* (d).

Additionally, at the intra-specific and inter-specific levels, there has actually been limited variation following the Weier *et al*. (1982) proposal.

Barcode markers of some of Thailand's *Cissus* species are beneficial for worldwide usage of the plant species, and methods for sustainable uses should be constructed. Since many *Cissus* species are medicinal plants that are economically profitable when sold as potted plants, or in modified forms like capsules, tablets or plant parts, there need to be specific markers to provide further rapid, automatable, and accurate species identification. The DNA barcoding has served its purpose. Therefore, after morphological and fingerprint identification, the species-specific card called barcodes were done to support the evidence previously mentioned. Besides the plants mentioned above, DNA barcodes can be used to verify plants that lack flowers or have incomplete morphological characteristics. DNA barcodes with *mat*K and *rbc*L for each species were performed following the guidelines set by CBOL Plant Working Group (2009). They proposed the use of two regions of plastid DNA as a standard protocol for the core barcoding of land plants. However, the *mat*K region has not been successful in the *Cissus* species studied and in the out-group. Therefore, there is a region of powerfully efficient *rbc*L sequences with nucleotide variations/genetic distances of 0.025-0.072, which is in agreement with Chaveerach *et al*. (2011). Kritpetcharat *et al*. (2011) used only one region as *psb*A-*trn*H intergenic spacer, and this would be potentially possible for some species. Also, CBOL Plant Working Group (2009) suggested that the *psb*A-*trn* H spacer region is a strong candidate for plant barcoding aside from core barcode. A summary has been added; one more region, the *rbc*L gene, becomes suitable for *Cissus* species barcodes and can be used as a single region.

In each species, chemical constituents are shown in different substances and in the same substances. A summary of the substances found in the four studied species and *C. quadrangularis* is shown in the table 5. Only one compound, β-sitosterol, was found in both the species studied and in *C. quadrangularis*. Five chemicals were found in all four of the species studied, four chemicals were found in three of the species studied, and eleven chemicals were found in two of the species studied. One unknown chemical found in *C. repens* is contained in high amounts up to 26.248%. The results of genetics of the four selected species ranges from 0.64-0.72 which is higher in comparison to *C. quadrangularis* (0.57-0.61) and which are in accordance with the kinds of compounds found.

Table 1: Genetic relationships shown in similarity index of the eight species of *Cissus* studied as evaluated by NTSYS-pc 2.1p using total ISSR bands

	C. hastata	*C. carnosa*	*C. quadrangularis*	*C. elongata*	*C. pteroclada* 1	*C. pteroclada* 2	*C. repens*	*C. javana*	*C. assamica*	*V. vinifera*
Cissus hastata	1.00									
C. carnosa	0.64	1.00								
C. quadrangularis	0.59	0.57	1.00							
C. elongata	0.66	0.67	0.61	1.00						
C. pteroclada 1	0.60	0.58	0.58	0.53	1.00					
C. pteroclada 2	0.59	0.57	0.55	0.53	0.86	1.00				
C. repens	0.68	0.64	0.60	0.70	0.57	0.60	1.00			
C. javana	0.71	0.62	0.57	0.67	0.65	0.70	0.75	1.00		
C. assamica	0.70	0.72	0.59	0.70	0.68	0.61	0.68	0.73	1.00	
Vitis vinifera	0.60	0.53	0.51	0.56	0.45	0.43	0.63	0.58	0.64	1.00

Table 2: Nucleotide variations of sequence divergences of the *rbc*L region in all species studied as conducted in MEGA5

	C. hastata	*C. carnosa*	*C. quadrangularis*	*C. elongata*	*C. pteroclada*	*C. repens*	*C. javana*	*C. assamica*	*V. vinifera*
Cissus hastata	0.000								
C. carnosa	0.043	0.000							
C. quadrangularis	0.041	0.051	0.000						
C. elongata	0.070	0.064	0.072	0.000					
C. pteroclada	0.045	0.045	0.045	0.066	0.000				
C. repens	0.027	0.029	0.031	0.057	0.031	0.000			
C. javana	0.029	0.043	0.045	0.072	0.045	0.027	0.000		
C. assamica	0.037	0.043	0.033	0.070	0.039	0.025	0.039	0.000	
Vitis vinifera	0.057	0.055	0.061	0.074	0.057	0.051	0.061	0.053	0.000

Table 3: GenBank accession numbers of the *rbc*L barcoding region of the all the *Cissus* species studied

Scientific name	Accession number	Scientific name	Accession number
Cissus hastata	JX262269	*C. pteroclada*	JX262273
C. carnosa	JX262270	*C. repens*	JX262274
C. quadrangularis	JX262271	*C. javana*	JX262278
C. elongata	JX262272	*C. assamica*	JX262279

The various chemicals contained in the plants are in agreement with their various medicinal usages. *Cissus quadrangularis* has properties that help in the treatment of weight loss for overweight and obese people, and help to improve cardiovascular health, hemorrhoids and bone fracture, as well as having anti-inflammatory properties (Oben *et al*., 2007; Panthong *et al*., 2007; Ruangwitthayanon and Leeanansaksiri, 2009; Deka *et al*., 1994; Shirwaikar *et al*., 2010). Whereas, the other species that were studied such as *C. repens* are used as medical treatments in Thailand by superseding or sharing with *Smilax china* and *S. glabra* for many disease treatment mentioned by Kritpetcharat *et al*. (2011). All three species are used in the traditional medicine setting under the local names of '*khao-yen nuer'* and '*khao-yen tai*'. In addition, *C. carnosa* is used for anti-inflammatory and inflammatory treatments on the hands and feet, locally called '*hun'* in Thai (corresponding author).

Table 4: The preliminary phytochemicals identified in the methanol crude extracts on the leaves of the four examined *Cissus* species

Plant	RT (min)	Compound	Formula	MW	Relative content (%)
Cissus assamica	10.023	(1R, 3R, 4R, 5R)-(-)-Quinic acid	$C_7H_{12}O_6$	192	27.238
	16.219	Phytol isomer	$C_{20}H_{40}O$	296	12.508
		2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-	$C_{20}H_{40}O$	296	
	35.891	Stigmasterol, 22,23-dihydro-	$C_{29}H_{50}O$	414	10.095
		γ-stigmasterol	$C_{29}H_{50}O$	414	
		(23s)-Ethyl cholest-5-en-3beta .-ol	$C_{29}H_{50}O$	414	
		Stigmast-5-en-3-ol, (3.beta)-	$C_{29}H_{50}O$	414	
		β-sitosterol	$C_{29}H_{50}O$	414	
		1,5-Dimethyl-6-(1,5-dimethylhexyl)-15,16	$C_{28}H_{46}O_2$	414	
	31.501	Vitamin E	$C_{29}H_{50}O_2$	430	6.049
	34.218	Stigmasta-5,22-dien-3-ol	$C_{29}H_{48}O$	412	4.937
		Stigmasterol	$C_{29}H_{48}O$	412	
	12.573	Neophytadiene	$C_{20}H_{38}$	278	3.577
		2-Hexadecene, 3,7,11,15-tetramethyl-	$C_{20}H_{40}O$	296	
	26.384	2,6,10,14,18,22-Tetracosahexaene	$C_{30}H_{50}$	410	3.52
	13.686	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	2.332
	4.946	4-Vinylphenol	C_8H_8O	120	1.774
	14.107	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.533
		Hexadecanoic acid	$C_{16}H_{32}O_2$	256	
		Unknown			26.437
C. carnosa	16.605	9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	26.617
		Ethyl linoleate	$C20H_{36}O_2$	308	
	35.879	Gamma-sitosterol	$C_{29}H_{50}O$	414	13.536
		Stigmasterol, 22,23-dihydro-	$C_{29}H_{50}O$	414	
		1,5-Dimethyl-6-(1,5-dimethylhexyl)-15,16	$C_{28}H_{46}O_2$	414	
		24(Z)-Methyl-25-homocholesterol	$C_{29}H_{50}O$	414	
	31.489	Vitamin E	$C_{29}H_{50}O_2$	430	11.635
		D-α-Tocopherol	$C_{29}H_{50}O_2$	430	
	16.075	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292	6.877
		9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	
	14.113	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	6.122
		n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	
		Tetradecoic acid	$C_{14}H_{28}O_2$	228	
	16.219	Phytol isomer	$C_{20}H_{40}O$	296	4.862
		2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-	$C_{20}H_{40}O$	296	
	26.384	Squalene	$C_{30}H5_0$	410	2.972
		2,6,10,14,18,22-Tetracosahexaene	$C_{30}H5_0$	410	
	12.579	Neophytadiene	$C_{20}H_{38}$	278	1.61
	13.686	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	1.36
		Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	
		Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	288	
		Unknown			24.409
C. hastata	12.325	4-Methoxy-3,5-dihydroxybenzoic acid	C_8H_8O5	184	14.664
		6-Fluoro veratraldehyde	$C_9H_9FO_3$	184	
	16.219	Phytol isomer	$C_{20}H_{40}O$	296	14.378
		Phytol	$C_{20}H_{40}O$	296	
		2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-	$C_{20}H_{40}O$	296	
	33.56	Ergost-5-en-3-ol,(3.beta.,24R)-	$C_{28}H_{48}O$	400	11.6
		(E)-5,10-Secocholest-1(10)-en-3,5-dione	$C_{27}H_{44}O_2$	400	
		5-Cholestene-3-ol, 24-methyl	$C_{28}H_{48}O$	400	

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Table 4: The preliminary phytochemicals identified in the methanol crude extracts on the leaves of the four examined *Cissus* species

Plant	RT (min)	Compound	Formula	MW	Relative content (%)
C. hastata		23-R-Methylcholesterol	$C_{28}H_{48}O$	400	
		5,10-Seco-cholestan-1(10)-en-3,5-dione	$C_{27}H_{44}O2$	400	
	31.483	Vitamin E	$C_{29}H_{50}O_2$	430	11.24
		D-α-Tocopherol	$C_{29}H_{50}O_2$	430	
		1,8-Bis(3,4-dicyanophenyl)anthracene	$C_{30}H_{14}N_8$	430	
	34.212	Stigmasta-5,22-dien-3-ol	$C_{29}H_{48}O$	412	8.659
		Stigmasta-5,22-dien-3.beta.ol	$C_{29}H_{48}O$	412	
		1,5-Dioxa-8,11-diazacyclotridecane-7,12-	$C_{23}H_{44}N_2O_4$	412	
	16.599	7,10,13-Hexadecatrienoic acid, methyl ester	$C_{17}H_{28}O_2$	264	7.435
		9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	
	14.125	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	6.672
		Hexadecanoic acid	$C_{16}H_{32}O_2$	256	
		Tetradecoic acid	$C_{14}H_{28}O_2$	228	
	35.885	(23s)-Ethyl cholest-5-en-3 β-ol	$C_{29}H_{50}O$	414	5.41
		γ-Sitosterol	$C_{29}H_{50}O$	414	
		(22R,24S)-22,24-Dimethylcholesterol	$C_{29}H_{50}O$	414	
		Stigmasterol, 22,23-dihydro-	$C_{29}H_{50}O$	414	
		24(Z)-Methyl-25-homocholesterol	$C_{29}H_{50}O$	414	
		1,5-Dimethyl-6-(1,5-dimethylhexyl)-15,16	$C_{28}H_{46}O_2$	414	
		β-Sitosterol	$C_{29}H_{50}O$	414	
	29.943	γ-Tocopherol	$C_{28}H_{48}O_2$	416	5.338
	16.513	9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	3.166
	26.378	2,6,10,14,18-Pentamethyl-2,6,10,14,18-Eicosapentaene	$C_{25}H_{42}$	342	3.035
		2,6,10,14,18,22-Tetracosahexaene	$C_{30}H_{50}$	410	
		Docosa-2,6,10,14,18-pentaen-22-al, 2,6,10,15,18-Pentamethyl-	$C_{27}H_{44}O$	384	
	16.934	Octadecanoic acid	$C_{18}H_{36}O_2$	284	2.23
	13.686	Hexadecanoic acid,methyl ester	$C_{17}H_{34}O_2$	270	1.658
		Pentadecanoic acid, 14-methyl	$C_{17}H_{34}O_2$	270	
		Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	288	
		Unknown			4.515
C. repens	35.885	γ-Sitosterol	$C_{29}H_{50}O$	414	25.165
		Stigmasterol, 22,23-dihydro-	$C_{29}H_{50}O$	414	
		β-Sitosterol	$C_{29}H_{50}O$	414	
		(22R,24S)-22,24-Dimethylcholesterol	$C_{29}H_{50}O$	414	
		1,5-Dimethyl-6-(1,5-dimethylhexyl)-15,16	$C_{28}H_{46}O_2$	414	
		4H-3,8a-Methanofuro(2′,3′:6,7)azonino (5,4-b)indol	$C_{23}H_{30}N_2O_5$	414	
		(23s)-Ethyl cholest-5-en-3 β-ol	$C_{29}H_{50}O$	414	
	16.219	Phytol	$C_{20}H_{40}O$	296	22.464
		Phytol isomer	$C_{20}H_{40}O$	296	
		2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-	$C_{20}H_{40}O$	296	
	4.946	2,3-Dihydrobenzofuran	C_8H_8O	120	7.174
		4-Vinylphenol	C_8H_8O	120	
	31.489	Vitamin E	$C_{29}H_{50}O_2$	430	7.01
		α-Tocopherol-β-D-mannoside	$C_{35}H_{60}O_7$	592	
		Unknown			38.187

Table 5: A summary of chemical constituents in each studied *Cissus* species as compared to *C. quadrangularis*, letter symbols are a=*C. assamica*, b=*C. carnosa*, c=*C. hastata*, d=*C. repens* and e=*C. quadrangularis*; aa, bb, cc, and dd are % chemical constituents of a, b, c, and d, respectively*

Chemicals	a	aa	b	bb	c	cc	d	dd	e
1,5-Dimethyl-6-(1,5-dimethylhexyl)-15,16	/	10.095	/	13.536	/	5.410	/	25.165	-
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-	/	12.508	/	4.862	/	14.378	/	22.464	-
Phytol, phytol isomer	/	12.508	/	4.862	/	14.378	/	22.464	-
Stigmasterol, 22,23-dihydro-	/	10.095	/	13.536	/	5.410	/	25.165	-
Vitamin E	/	6.049	/	11.635	/	11.24	/	7.010	-
2,6,10,14,18,22-Tetracosahexaene	/	3.52	/	2.972	/	3.035	-	-	-
Gamma-sitosterol	-	-	/	13.536	/	5.41	/	25.165	-
Hexadecanoic acid, methyl ester	/	2.332	/	6.122	/	6.672	-		-
β-Sitosterol	/	10.095	-	-	/	5.410	/	25.165	/
(22R,24S)-22,24-Dimethylcholesterol	-	-	-	-	/	5.410	/	25.165	-
(23s)-Ethyl cholest-5-en-3beta .-ol	/	10.095	-	-	-	-	/	25.165	-
4-Vinylphenol	/	1.774	-	-	-	-	/	7.174	-
9,12,15-Octadecatrien-1-ol	-	-	/	26.617	/	7.435	-	-	-
D-α-Tocopherol	-	-	/	11.635	/	11.24	-	-	-
Hexadecanoic acid	-	-	/	6.122	/	6.672	-	-	-
Neophytadiene	/	3.577	/	1.61	-	-	-	-	-
n-Hexadecanoic acid	/	1.533	/	6.122	-	-	-	-	-
Stigmasta-5,22-dien-3.beta.ol/ Stigmasta-5,22-dien-3-ol	/	4.937	-	-	/	8.659	-	-	-
Tetradecoic acid	-	-	/	6.122	/	6.672	-	-	-
Tetradecoic acid, methyl ester	-	-	/	1.360	/	1.658	-	-	-

* /means detection of the chemical, - means no detection

Possibly, the various chemicals contained in identical species are dependent upon various growing regions, different geographical areas and/or weather, as well as the extraction buffer. However, this research used methanol extract, which is a polar substance that actually dissolves out polar phytochemicals. The method of using an extraction buffer will derive some extracted substances from all substances contained in the plant. Therefore, their medicinal usages should be concentrated in a region. However, the same disease-treating properties of *C. quadrangularis* are also indicated and are currently in agreement with many research projects quoted.

In the category of genus, the major related topic was which of the species may display similar properties to *C. quadrangularis*, and this topic was solved by linkage of genetic relationships and the chemical constituents of *Cissus* species. Despite the far genetic distances of the four studied species to *C. quadrangularis* (S=0.57-0.61), different chemicals are contained within them, and there is only one identical substance that is contained in all of them. Accordingly, there is a higher S value in the four species (0.64-0.72), which shows some identical species as in the table 5.

One more interesting, unknown substance is contained in *C. repens* in amounts as high as 26.248% and should be further clarified to discover its worthwhile uses.

CONCLUSION

Cissus species are commonly used as traditional and modified medicines, and their chemical constituents are major point for precise usage. However, *C. quadrangularis* is the only species for which the usages and the chemical constituents have been reported. This research investigates usages and chemicals content for other species in the genus. These include eight species, namely *C. assamica*, *C. carnosa*, *C. elongata*, *C. hastata*, *C. javana*, *C. pteroclada*, *C. quadrangularis*, and *C. repens*. The species were evaluated for genetic relationships based on inter-simple sequence repeat markers. The analysis showed low genetic relationships of the experimented species to the well-studied species, *C. quadrangularis*. Four closely related species, *C. assamica*, *C. carnosa*, *C. hastata*, and *C. repens* were selected for chemical constituent analysis. From the gas chromatography-mass spectrometry analysis, only one compound found in the four species is identical to the compound reported from *C. quadrangularis*, where there were five identical chemicals found in the selected species. The genetic relationships level is in accord with the chemical contents in the studied species.

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