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Original Article

Sauropus species containing eudesmin and their DNA profile

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Abstract

Some *Sauropus* and *Breynia* species were investigated for phytochemicals as well as their DNA profile for genetic relationships. *S. bicolor* and *S. thorelii* are important species that contain eudesmin concentrations of 36.445 mg and 32.190 mg from 2 g fresh leaves and 1.5 g dried leaves, respectively, and 10.620 mg from 2 g dried leaves was revealed via high-performance liquid chromatography (HPLC) analysis. The genetic relationships calculated via inter simple sequence repeat (ISSR) dendrogram construction, as shown by similarity indices, revealed *Breynia* and *Sauropus* separation accuracies, with S values of 0.71-0.81 between genera, S values of 0.76-0.85 at the interspecific level of *Breynia* and S values of 0.69-0.80 in the *Sauropus* species, in agreement with the criteria for category distinction. Barcodes with *rpo*B and *trn*H-*psb*A spacer regions can be used to identify some species of the two studied genera.

Keywords: Barcode, ISSR fingerprint, *Sauropus* species, Eudesmin, *Breynia* species

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Introduction

There are 27 *Sauropus* species in Thailand (Welzen and Chayamarit, 2007), and many of these species have been used as vegetables in local cuisine and as traditional medicine. For example, *S. androgynous* is a well-known and distinguished vegetable in Thai cuisine, both at home and in restaurants. Additionally, it is indigenous to Southeast Asia and is widely cultivated for traditional medicinal purposes in India (Gopalakrishnan, 2007), Laos (Culloty, 2010), Malaysia, Indonesia, the Philippines and Cambodia (Bunawan et al., 2015). It contains many substances, such as sterol, resins, tannins, saponins, alkaloids, flavonoids, terpenoids, glycosides, and phenols. These substances contain chemicals with anti-obesity effects, wound-healing activity, anti-inflammatory activity, lactation activity, antioxidant activity and antimicrobial activity. However, it should be noted that the consumption of raw *S. androgynous* leaf juice for weight loss resulted in an outbreak of bronchiolitis obliterans due to the presence of papaverine, which is responsible for the occurrence of pulmonary failure (Bunawan et al., 2015). Moreover, an *in vitro* study revealed that necrosis and apoptosis are associated with the toxic effect of *S. androgynous* in NIH3T3 fibroblasts. However, evidence is needed to clarify whether necrosis and apoptosis are related to the

associated bronchiolitis pathogenesis. It is evident that this species seems to have usages concurrent with toxicity.

The genus *Breynia* is also interesting due to its close relationship to the *Sauropus* genus; it has seven species distributed in Thailand (Chayamarit and Welzen, 2005). Plants of a single species growing in different areas with different climates may have different phenotypes, phytochemicals (Sirikhansaeng et al., 2017), characteristic variations (Sanubol et al., 2014) and related factors. Species usages and their growing areas must be determined before they can be classified as a targeted species. DNA fingerprints and barcodes have long been popularly used for plants for such classification (Chaveerach et al., 2016; Thooptianrat et al., 2017).

The phytochemicals of several species of the genus *Sauropus* were studied using gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC), and toxicity testing was undertaken to determine further usages of these plants. Additionally, the DNA profiles of the different species were compared to those of some *Breynia* species, and barcode constructions were assessed to determine classification criteria.

Material and Methods

Plant collection

Young and mature leaves of *Sauropus* species, *Breynia* species and *Claoxylon longifolium* were collected throughout distributed areas in Thailand. The young leaves were used for DNA extraction, DNA fingerprinting and DNA barcoding, and mature leaves of *Sauropus* species were used for phytochemical analysis by GC-MS and HPLC; some *Breynia* species and *Claoxylon longifolium*, a species in an identical family, Euphorbiaceae, as an outgroup were also included. The plants were identified by Professor Arunrat Chaveerach at the Department of Biology, Faculty of Science, Khon Kaen University, by using the Flora of Thailand Volume 8, Parts 1-2 (Chayamarit and Welzen, 2005; Welzen and Chayamarit, 2007).

Plant extracts and analysis of the constituents by GC-MS

The leaf samples were washed and air dried at room temperature. A 20-g dried sample was ground into a powder, mixed with 125 ml hexane or absolute ethyl alcohol solvents (analytical grade), incubated for 72 h, and then filtered through filter paper. The filtrate was used for the GC-MS analysis. An Agilent Technologies GC 6890 N/5973 Inert mass spectrometer fused with a capillary column (30.0 m × 250 µm) was used for the analysis of the extract constituents. The carrier was helium gas at a constant flow rate of 1 ml/min. The injection and mass-transferred line temperature was set at 280°C. The temperature of the oven was programmed from 70°C to 120°C at 3°C/min, held isothermally for 2 min, and then increased to 270°C at 5°C/min. A total of 1 µl extract was injected in split mode. The relative percentage of the extract constituents was expressed using peak area normalization. Constituent identification was determined by comparing the derived mass spectra with the reference substances in the Wiley 7N.1 library.

Plant extracts and quantitative analysis of eudesmin by HPLC

The leaf samples were washed and divided into two groups for HPLC analysis: fresh and dried leaves, which were air dried at room temperature. A 20-g fresh or dried sample was ground into a powder, mixed with 125 ml hexane (HPLC grade) and then filtered through filter paper. The filtrate was used for the HPLC analysis. The plant species details are listed below. A total of 1 mg of eudesmin (Sigma Aldrich) was dissolved in 1 ml methanol (1 mg/ml) and was 2-fold diluted with five concentrations, using 20 µl each for the standard solutions. The samples were analyzed by HPLC using a Shimadzu LC-20AD (Japan) model with a quaternary pump, a PAD (SPD-M20A) detector, and an Inertsil ODS-3 C18 column, 4.6×250 mm, 5 microns (GL Sciences Inc.). A 20-µl sample was injected. The mobile phase consisted of two solutions: acetonitrile and 1% acetic acid in deionized water at 40:60 v/v. The elution was carried out at a flow rate of 1 ml/min. The detection UV wavelength was 280 nm.

DNA analysis

DNA extraction

The young leaves were subjected to DNA extraction using the Plant Genomic DNA Extraction Kit (RBC Bioscience) following the kit's protocols. Genomic DNA was further used for DNA fingerprint and DNA barcode construction.

DNA fingerprinting by Inter Simple Sequence Repeat (ISSR) marker and dendrogram construction

DNA amplification mixtures contained GoTaq Green

Master Mix (Promega) with 50 µM primer and 5 ng DNA template following the kit's protocols. Sixty-three ISSR primers were screened, and 17 primers produced clear bands. The sequences are listed as follows (5′ to 3′):

CACACACACACAAC,
CACACACACACAGT,
CACACACACACAAG,
GAGAGAGAGAGAGG,
GTGTGTGTGTGTCC,
ACACACACACACACACG,
ACACACACACACACACG,
CCCCGTGTGTGTGTGT,
AGAGAGAGAGAGAGAAC,
CAGCAGCAGCAGCAG,
CCACCACCACCACCA,
CTCTCTCTCTCTCTCTGTC,
CACACACACACACACAA,
ACACACACACACACACG,
ACAGTGTGTGTGTGTGT,
CACTGTGTGTGTGTGTG,
GGGTGGGGTGGGGTG.

The amplification reactions were conducted in a thermal cycler (Swift™ Maxi Thermal Cycler, Esco Micro Pte. Ltd.) with initial denaturation for 3 min at 94°C, followed by 35 cycles (denaturing for 30 sec at 94°C, annealing for 45 sec at 57°C, extending for 1 min at 72°C) and a final extension for 7 min at 72°C. The amplified products were subjected to agarose gel (1.2%) electrophoresis in tris-acetate EDTA (TAE) buffer and visualized using ethidium bromide staining. The resulting ISSR bands were used to construct a dendrogram using NTSY Spc version 2.1 (Rohlf, 1998).

DNA barcoding of the rpoB gene region and psbA-trnH spacer region

Barcoding fragments were amplified with the following primer pairs (5′ to 3′): AAGTGCATTGTTGGAACTGG and CCGTATGTGAAAAGAAGTATA for the *rpo*B gene and TTATGCATGAACGTAATGCTC and CGCGCATGGTGGATTCACAATCC for the *psbA-trnH* spacer region. The amplification and product screening were performed as previously described (Sudmoon et al., 2016).

The target amplified fragments were sent for sequencing at the Macrogen INC, Geumcheon-GU, Seoul, Korea. The sequences were then analyzed using MEGA7 (Kumar et al., 2016), annotated and submitted to GenBank as previously described (Thooptianrat et al., 2017).

Results

Phytochemical constituents

After identification of the plant species collected, there were *Sauropus amoebiflorus*, *S. androgynus*, *S. bicolor*, *S. brevipes*, *S. elegantissimus*, *S. kerrii*, *S. quadrangularis* and *S. thorelii*. The phytochemical analysis by GC-MS indicated that there were several substances divided into two groups: major and minor. The major group contained concentrations greater than 10%, and the amounts from lowest to the highest were 13.35% phytol acetate in the ethanol *S. brevipes* extract, 15.26% δ-tocopherol in the hexane *S. androgynus* extract, 11.39% and 15.37% stigmast-4-en-3-one in the hexane *S. brevipes* and *S. quadrangularis* extracts, 19.22% phytol in the ethanol *S. brevipes* extract, 19.70% palmitic acid in the ethanol *S. kerrii* extract, 21.43% 6,7-dimethoxy-2-methyl-1H-quinolin-4-one in the ethanol *S. quadrangularis* extract, 25.33% cubebinolide in the hexane *S. thorelii* extract, 27.22% squalene in the hexane *S. thorelii* extract, 29.80% γ-sitosterol in the hexane *S. brevipes* extract, 36.98% (9E,12E,15E)-octadeca-9,12,15-trienal in the *S. androgynous* extract, 64.70% (3S,4S)-3,4-bis(1,3-benzodioxol-5-ylmethyl)oxolan-2-one in the ethanol *S. thorelii* extract and 78.13% eudesmin in the ethanol *S. bicolor* extract. All the substances in both the major and minor groups are shown in Table 1. GC-MS chromatograms showing the retention time and the peak area of each substance are shown in Figure 1.

Table 1: Phytochemical constituents according to the GC-MS analysis of the extracts of the eight *Sauropus* species.

Compounds	Formula	Relative contents (%)															
		S. amoebiflorus (Ethanol)	*S. amoebiflorus* (Hexane)	*S. androgynus* (Ethanol)	*S. androgynus* (Hexane)	*S. bicolor* (Ethanol)	*S. bicolor* (Hexane)	*S. brevipes* (Ethanol)	*S. brevipes* (Hexane)	*S. elegantissimus* (Ethanol)	*S. elegantissimus* (Hexane)	*S. kerrii* (Ethanol)	*S. kerrii* (Hexane)	*S. quadrangularis* (Ethanol)	*S. quadrangularis* (Hexane)	*S. thorelii* (Ethanol)	*S. thorelii* (Hexane)
Eudesmin	$C_{22}H_{26}O_6$	-	-	-	-	78.13	27.76	-	-	-	-	-	-	-	-	-	-
(3S,4S)-3,4-Bis(1,3-benzodioxol-5-ylmethyl)oxolan-2-one	$C_{20}H_{18}O_6$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	64.70	-
(9E,12E,15E)-Octadeca-9,12,15-trienal	$C_{18}H_{30}O$	-	-	36.98	-	-	-	-	-	-	-	-	-	-	-	-	-
γ-Sitosterol	$C_{29}H_{50}O$	3.32	10.79	0.98	0.54	0.28	3.04	17.28	29.80	2.78	4.01	-	-	9.25	7.40	-	1.02
Squalene	$C_{30}H_{50}$	-	9.66	-	-	-	-	-	-	-	-	-	-	-	-	-	27.22
Cubebinolide	$C_{20}H_{18}O_6$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.50	25.33
6,7-Dimethoxy-2-methyl-1H-quinolin-4-one	$C_{12}H_{13}No_3$	-	-	-	-	-	-	-	-	-	-	-	-	21.43	-	-	-
Palmitic acid	$C_{16}H_{32}O_2$	6.55	-	3.88	4.77	2.70	1.52	9.45	5.84	3.52	1.28	19.70	0.71	4.83	0.72	0.61	4.44
Phytol	$C_{20}H_{40}O$	2.15	6.87	-	-	0.36	0.28	19.22	3.49	4.59	3.49	1.07	0.36	1.15	0.73	3.87	3.28
Stigmast-4-en-3-one	$C_{29}H_{48}O$	0.83	4.08	-	-	-	9.72	4.01	11.39	-	-	-	-	-	15.37	-	-
δ-Tocopherol	$C_{27}H_{46}O_2$	3.23	-	-	15.26	0.05	0.58	0.83	-	-	0.41	-	-	-	3.66	0.36	3.77
Phytol acetate	$C_{22}H_{42}O_2$	2.54	-	2.84	-	1.40	-	13.35	-	4.65	-	-	-	4.60	-	2.03	0.87
γ-Tocopherol	$C_{28}H_{48}O_2$	1.31	4.13	1.73	5.80	0.65	6.10	3.60	8.89	2.27	4.05	0.78	0.43	5.60	5.24	-	1.33
Stigmasterol	$C_{29}H_{48}O$	-	-	-	-	-	-	2.49	-	1.09	-	-	-	-	-	-	8.44
Linolenic acid	$C_{18}H_{30}O_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.03
Vitamin E	$C_{29}H_{50}O_2$	0.16	-	-	-	-	-	-	-	-	-	1.83	-	6.18	-	-	-
Octadecanoic acid	$C_{18}H_{36}O_2$	2.26	-	-	-	0.52	-	-	-	0.85	-	6.06	0.22	1.28	-	-	1.17
β-Tocopherol	$C_{28}H_{48}O_2$	1.56	5.80	-	1.86	-	-	-	-	0.39	0.62	-	-	1.09	0.99	-	-
Fargsin		-	-	-	-	4.81	5.65	-	-	-	-	-	-	-	-	-	-
Lanosta-8,24-dien-3-ol	$C_{30}H_{50}O$	-	-	-	4.01	-	-	-	-	1.07	2.83	4.85	5.31	-	-	-	-
Lupenone	$C_{30}H_{48}O$	-	-	-	4.96	-	-	-	-	-	-	-	-	-	-	-	-
4-Hydroxy-2-cyclohexen-1-one	$C_6H_8O_2$	-	-	-	-	0.50	-	-	-	-	-	4.64	-	-	-	-	-
1,6-Anhydro-beta-D-glucopyranose	$C_6H_{10}O_5$	-	-	-	-	-	-	-	-	4.54	-	-	-	-	-	-	-
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	-	-	-	4.00	0.22	-	2.99	-	0.55	-	-	-	-	-	-	-
5-(Hydroxymethyl)furfural	$C_6H_6O_3$	-	-	-	-	-	-	-	-	-	-	3.72	-	-	-	-	-
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	0.88	-	0.69	-	0.69	-	3.68	-	1.24	-	1.80	-	-	-	0.24	-
Lanosterol	$C_{30}H_{50}O$	-	-	-	-	-	2.89	-	-	-	-	-	-	3.55	3.02	-	-
Simiarenol	$C_{30}H_{50}O$	0.84	3.24	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(4,5-Dimethoxy-2-styryl-phenyl)-methyl-dimethyl-amine	$C_{19}H_{23}NO_2$	-	-	-	-	-	-	-	-	-	-	-	2.98	-	-	-	-
Linoleic acid	$C_{18}H_{32}O_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.71
4-Propylphenol	$C_9H_{12}O$	-	-	-	-	2.62	-	-	-	-	-	-	-	-	-	-	-
Methyl 7,11,14-eicosatrienoate	$C_{21}H_{36}O_2$	-	2.62	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cholest-5-en-3-ol, 24-propylidene-, (3beta)-	$C_{27}H_{46}O$	-	-	-	-	-	-	-	-	2.42	-	-	-	-	-	-	-

dl-α-Tocopherol	$C_{29}H_{50}O_2$	-	-	1.89	-	-	-	1.70	2.55	-	-	1.10	-	-	-	-	1.69
Ergosterol	$C_{28}H_{44}O$	-	-	-	-	-	-	2.25	-	-	-	-	-	-	-	-	-
Marinobufagenin	$C_{24}H_{32}O_5$	-	-	-	-	2.00	-	-	-	-	-	-	-	-	-	-	-
Lupeol	$C_{30}H_{50}O$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.37
Oleanitrile	$C_{18}H_{33}N$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.20
Lupeol acetate	$C_{32}H_{52}O_2$	-	-	-	1.03	-	-	-	-	-	-	-	-	-	-	-	-
Caryophyllene	$C_{15}H_{24}$	-	-	-	1.00	-	-	-	-	-	-	-	-	-	-	-	-
4-Methoxybenzene-1,2-diol	$C_7H_8O_3$	-	-	-	-	0.96	-	-	-	-	-	-	-	-	-	-	-
Tetradecanoic acid	$C_{24}H_{28}O_2$	0.51	-	0.24	-	0.24	-	-	0.89	0.36	-	0.80	-	-	-	-	-
1,2-Benzenediol	$C_6H_6O_2$	-	-	-	-	-	-	-	-	0.86	-	-	-	-	-	-	-
Tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	-	-	-	0.83	-	-	-	-	-	-	-	-	-	-	-	-
Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	-	-	-	-	-	-	-	0.73	-	-	-	-	-	-	-	-
1,2-Bis[1-(2-hydroxyethyl)-3,6-diazahomo		-	-	-	-	0.55	-	-	-	-	-	-	-	-	-	-	-
9,12,15-Octadecatrienal	$C_{18}H_{30}O$	-	-	-	-	-	-	-	-	-	-	-	0.54	-	-	-	-
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	-	-	-	0.48	-	-	-	-	-	-	-	-	-	-	-	-
Benzamide	C_7H_7NO	0.48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl 4-ethoxybenzoate	$C_{11}H_{14}O_3$	-	-	0.44	-	-	-	-	-	-	-	-	-	-	-	0.43	-
Pluchidiol	$C_{13}H_{20}O_2$	0.33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8-Pentadecanone	$C_{15}H_{30}O$	-	0.32	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eicosanoic acid	$C_{20}H_{40}O_2$	-	-	-	-	-	-	-	-	-	-	0.31	-	-	-	-	-
Yangambin	$C_{24}H_{30}O_8$	-	-	-	-	0.23	-	-	-	-	-	-	-	-	-	-	-
2-(2-Ethoxyethoxy)ethyl acrylate	$C_9H_{16}O_4$	0.20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1,1'-Methylene-bis(di-2-propenylamine)	$C_{13}H_{22}N_2$	-	-	0.20	-	-	-	-	-	-	-	-	-	-	-	-	-
Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	-	-	-	-	0.05	-	-	-	-	-	-	-	-	-	-	-
Unknown		72.40	52.49	50.13	55.46	3.04	42.46	17.70	38.04	68.82	83.31	53.32	89.45	27.42	62.87	25.26	9.13

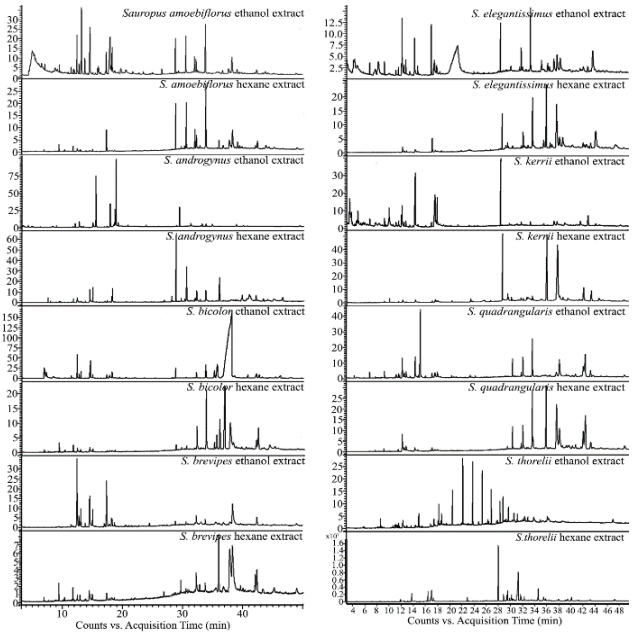

Figure 1. GC-MS chromatograms showing each phytochemical constituent by retention time and peak of hexane and ethanol of the extracts of the eight *Sauropus* species

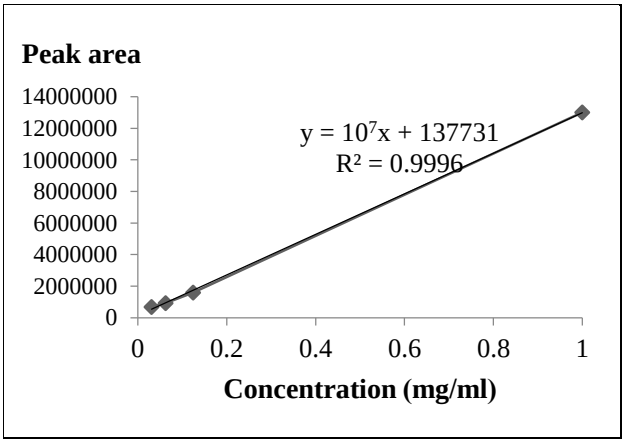

Figure 2. Linearity equation of eudesmin standard derived from graph plotting by area, and the five tested concentrations produced the calibration equation and correlation coefficient (R^2)

Given a certain amount of existing eudesmin and measured in mg/g of the dominant substance, the plant with the highest amount of eudesmin was *S. bicolor*, and the other four species that lacked eudesmin, *S.*

androgynous, *S. elegantissimus*, *S. quadrangularis* and *S. thorelii*, were further investigated by the HPLC method. When the HPLC for the standards was performed for each concentration, the peak areas of the chromatogram were shown. The graph created from standard concentrations and peak areas showed the calibration equation, $y = 10^7x + 137731$ (the calibration equation was $y = mx + c$, where y is the peak area, m is the slope, c is the intercept of the linear curve and x is the derived area of the targeted substance). The correlation coefficient (R^2) was 0.9996, which indicated the reliability of the standard curve and the calibration equations (Figure 2).

The targeted substance was detected by peak area characteristics coupled with retention times. The HPLC analysis exhibited eudesmin in *S. bicolor*, *S. androgynus* and *S. thorelii*, with different peak areas of 73037854 and 107438321 in fresh and dried leaves of *S. bicolor*, 147235 in *S. androgynous* and 17825880 in *S. thorelii*, as shown by HPLC chromatograms (Figure 3).

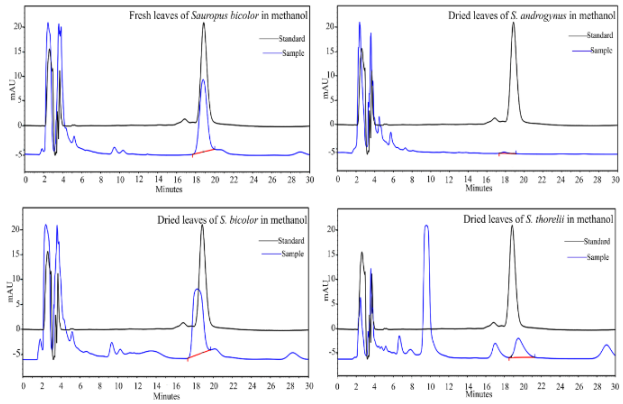

Figure 3. Chromatograms revealing peak areas of eudesmin of the studied samples (blue line) compared to the eudesmin standard (black line)

The peak areas were substituted in calibration equation to calculate the eudesmin content in the studied samples, resulted in the amounts of 7.289 mg/ml and 10.73 mg/ml in *S. bicolor*, 0.0008504 mg/ml in *S. androgynus* and 1.77 mg/ml in S. *thorelii* (Table 2). Whereas, eudesmin was not detected in the other two species, *S. elegantissimus* and *S. quadrangularis*.

Genetic diversity based on DNA fingerprinting

The ISSR amplification successfully produced DNA fingerprints with 17 primers (Figure 4) from 63 screening primers and with 400 derived characters of the total 802 clear bands. These total bands were used for dendrogram construction using the NTSY Spc 2.1 program (Figure 5). The dendrogram revealed the separation of each *Breynia* and *Sauropus* species, while two individuals of a species were set in a branch, and the outgroup was distinguished. These results confirmed that sufficient banding data were included in the analysis and the dendrogram reliability evaluation.

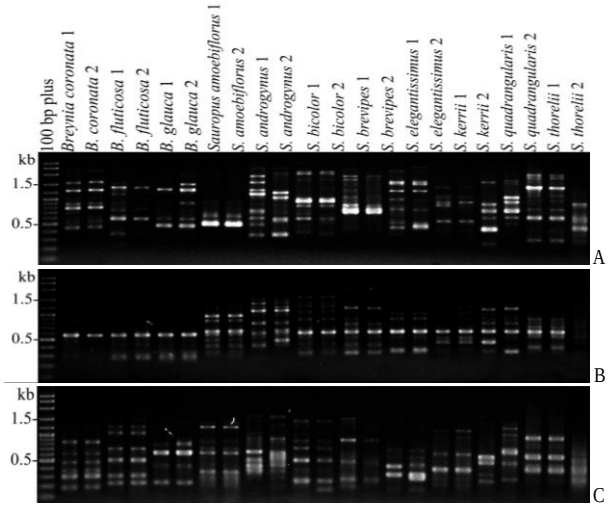

Figure 4. Examples of ISSR fingerprints from the primers GTGTGTGTGTGTCC (A), CCACCACCACCACCA (B) and CACTGTGTGTGTGTGTG (C) of the *Breynia* and *Sauropus* species and *Claoxylon longifolium* outgroup.

Table 2: Eudesmin quantity in leaf samples of *Sauropus* species (D = dried leave, F = fresh leaves) according to the starting weight of leaves and filtrate volume and calculated from peak area obtained by HPLC analysis compared to the standard curve.

Plant sample	Sample weight (g)	Filtrate volume (ml)	Peak area	Eudesmin	
				mg/ml	mg
S. androgynus D	2	6	147235	0.00085	0.005
S. bicolor D	1.5	3	107438321	10.73000	32.190
S. bicolor F	2	5	73037854	7.28900	36.445
S. elegantissimus D	2	5	-	-	-
S. quadrangularis D	2	5	-	-	-
S. thorelii D	2	6	17825880	1.77000	10.620

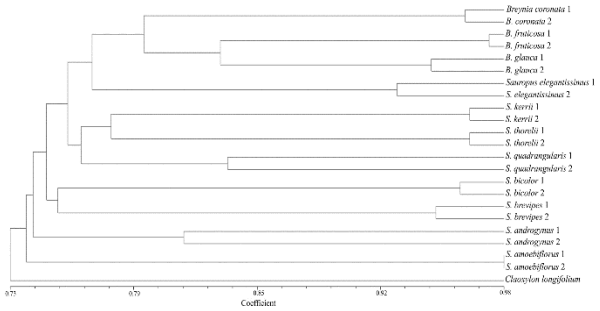

Figure 5. Dendrogram constructed from 17 ISSR primers using NTSY Spc 2.1 program of the *Breynia* and *Sauropus* species and *Claoxylon longifolium* outgroup

The similarity indices (S) of the genera were as follows: 0.71 to 0.81 for the pairs *S. brevipes* 1 and *B. glauca* 2 and *S. kerrii* 1 and *B. glauca* 1; for the *Breynia* species, 0.76 to 0.85 for the pairs *B. glauca* 2 and *B. coronata* 1, *B. glauca* 1 and *B. fruticosa* 1, and *B. glauca* 1 and *B. fruticosa* 2; and for the *Sauropus* species, 0.69 to 0.80 for the pairs *S. bicolor* 1 and *S. amoebiflorus* 2, *S. thorelii* 2 and *S. kerrii* 1 (Table 3).

Table 3: Genetic distances derived from ISSR fingerprinting of 17 primers calculated by NTSY Spc 2.1 indicating similar indices of the *Breynia* and *Sauropus* species

	B. coronata 1	*B. coronata* 2	*B. fruticosa* 1	*B. fruticosa* 2	*B. glauca* 1	*B. glauca* 2	*S. amoebiflorus* 1	*S. amoebiflorus* 2	*S. androgynus* 1	*S. androgynus* 2	*S. bicolor* 1	*S. bicolor* 2	*S. brevipes* 1	*S. brevipes* 2	*S. elegantissimus* 1	*S. elegantissimus* 2	*S. kerrii* 1	*S. kerrii* 2	*S. quadrangularis* 1	*S. quadrangularis* 2	*S. thorelii* 1	*S. thorelii* 2	*C. longifolium*
B. coronata 1	1.00																						
B. coronata 2	0.96	1.00																					
B. fruticosa 1	0.79	0.82	1.00																				
B. fruticosa 2	0.80	0.82	0.97	1.00																			
B. glauca 1	0.79	0.81	0.85	0.85	1.00																		
B. glauca 2	0.76	0.78	0.82	0.82	0.94	1.00																	
S. amoebiflorus 1	0.72	0.75	0.75	0.75	0.77	0.75	1.00																
S. amoebiflorus 2	0.72	0.75	0.75	0.75	0.77	0.75	0.98	1.00															
S. androgynus 1	0.73	0.77	0.74	0.73	0.75	0.73	0.74	0.74	1.00														
S. androgynus 2	0.74	0.76	0.74	0.73	0.76	0.73	0.71	0.71	0.82	1.00													
S. bicolor 1	0.72	0.75	0.74	0.73	0.74	0.72	0.70	0.69	0.71	0.76	1.00												
S. bicolor 2	0.75	0.78	0.76	0.76	0.77	0.75	0.72	0.71	0.74	0.78	0.96	1.00											
S. brevipes 1	0.72	0.74	0.74	0.74	0.75	0.71	0.72	0.72	0.71	0.73	0.74	0.76	1.00										
S. brevipes 2	0.74	0.76	0.76	0.76	0.78	0.74	0.73	0.73	0.72	0.73	0.74	0.77	0.95	1.00									
S. elegantissimus 1	0.77	0.80	0.75	0.76	0.79	0.75	0.74	0.74	0.74	0.74	0.75	0.76	0.73	0.75	1.00								
S. elegantissimus 2	0.76	0.79	0.75	0.76	0.79	0.75	0.73	0.73	0.74	0.74	0.72	0.74	0.73	0.75	0.93	1.00							
S. kerrii 1	0.77	0.80	0.77	0.77	0.81	0.78	0.77	0.77	0.73	0.75	0.74	0.78	0.75	0.78	0.78	0.77	1.00						
S. kerrii 2	0.76	0.79	0.76	0.76	0.80	0.76	0.76	0.76	0.72	0.73	0.73	0.77	0.73	0.75	0.76	0.75	0.96	1.00					
S. quadrangularis 1	0.72	0.75	0.74	0.74	0.76	0.72	0.72	0.72	0.73	0.71	0.70	0.73	0.73	0.73	0.76	0.75	0.77	0.76	1.00				
S. quadrangularis 2	0.77	0.79	0.76	0.76	0.79	0.76	0.74	0.74	0.76	0.77	0.74	0.78	0.77	0.79	0.78	0.77	0.79	0.78	0.84	1.00			
S. thorelii 1	0.74	0.74	0.73	0.73	0.74	0.72	0.70	0.71	0.72	0.73	0.72	0.75	0.71	0.72	0.70	0.70	0.77	0.76	0.73	0.75	1.00		
S. thorelii 2	0.75	0.76	0.76	0.76	0.77	0.74	0.73	0.74	0.75	0.75	0.74	0.77	0.73	0.75	0.73	0.73	0.80	0.79	0.74	0.78	0.96	1.00	
Claoxylon longifolium	0.70	0.73	0.72	0.72	0.76	0.73	0.71	0.72	0.71	0.72	0.70	0.73	0.70	0.73	0.71	0.72	0.77	0.76	0.73	0.76	0.71	0.75	1.00

Table 4: The genetic distances of the *Breynia* and *Sauropus* species derived from *rpo*B sequence alignment using MEGA7

	B. coronata	*B. fruticosa*	*B. glauca*	*S. amoebiflorus*	*S. androgynus*	*S. bicolor*	*S. brevipes*	*S. elegantissimus*	*S. kerrii*	*S. quadrangularis*	*S. thorelii*
B. coronata	0.000										
B. fruticosa	0.000	0.000									
B. glauca	0.004	0.004	0.000								
S. amoebiflorus	0.004	0.004	0.008	0.000							
S. androgynus	0.004	0.004	0.008	0.008	0.000						
S. bicolor	0.000	0.000	0.004	0.004	0.004	0.000					
S. brevipes	0.002	0.002	0.006	0.006	0.006	0.002	0.000				
S. elegantissimus	0.000	0.000	0.004	0.004	0.004	0.000	0.002	0.000			
S. kerrii	0.000	0.000	0.004	0.004	0.004	0.000	0.002	0.000	0.000		
S. quadrangularis	0.000	0.000	0.004	0.004	0.004	0.000	0.002	0.000	0.000	0.000	
S. thorelii	0.002	0.002	0.006	0.006	0.006	0.002	0.004	0.002	0.002	0.002	0.000

Genetic distance based on DNA sequencing of barcoding regions

Amplification of the DNA fragments in the *rpo*B and *psb*A-*trn*H spacer regions of the *Breynia* and *Sauropus* species showed sizes of approximately 500 bp and 400 bp, respectively, as shown in Figure 6. With sequence alignments of the MEGA7, genetic distances of the two regions in the two genera revealed that the distances were 0.000-0.008 at the intergeneric level, 0.000-0.004 between the interspecific *Breynia* species and 0.000-0.008 between the interspecific *Sauropus* species in the *rpo*B region (Table 4); 0.000-0.099 at the intergeneric level, 0.003-0.019 between the interspecific *Breynia* species and 0.000-0.120 between the interspecific *Sauropus* species in the *psb*A-*trn*H spacer region (Table 5). The dendrogram constructed from these two sequences indicated that the groups *S. kerrii*, *S. quadrangularis*, *S. elegantissimus*, *S. bicolor*, *B. fruticose* and *B. coronata* of the *rpo*B region, as well as the groups *S. bicolor*, *S. kerrii* and *B. coronata* and a pair *B. fruticosa* and *S. elegantissimus* of the *psb*A-*trn*H spacer, could not use these sequences for their identification (Figures 7, 8). The sequences were submitted to GenBank, and the corresponding accession numbers are given (Table 6).

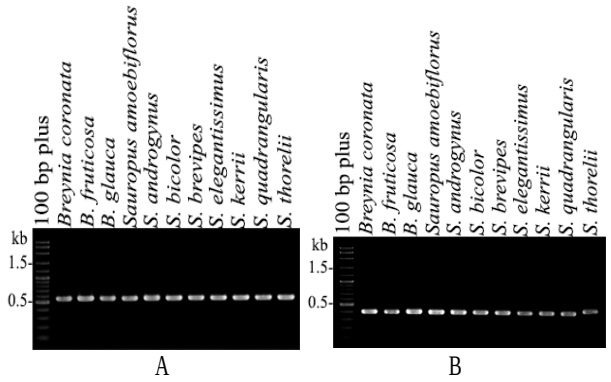

Figure 6. DNA fragments amplified for barcode construction derived from *rpo*B (A) and *trn*H-*psb*A spacer (B) of the studied *Breynia* and *Sauropus* species

Table 5: The genetic distances of the *Breynia* and *Sauropus* species derived from *trn*H-*psb*A spacer sequence alignment using MEGA7

	B. coronata	*B. fruticosa*	*B. glauca*	*S. amoebiflorus*	*S. androgynus*	*S. bicolor*	*S. brevipes*	*S. elegantissimus*	*S. kerrii*	*S. quadrangularis*	*S. thorelii*
B. coronata	0.000										
B. fruticosa	0.016	0.000									
B. glauca	0.019	0.003	0.000								
S. amoebiflorus	0.009	0.025	0.028	0.000							
S. androgynus	0.003	0.019	0.022	0.006	0.000						
S. bicolor	0.000	0.016	0.019	0.009	0.003	0.000					
S. brevipes	0.025	0.041	0.045	0.016	0.022	0.025	0.000				
S. elegantissimus	0.016	0.000	0.003	0.025	0.019	0.016	0.041	0.000			
S. kerrii	0.000	0.016	0.019	0.009	0.003	0.000	0.025	0.016	0.000		
S. quadrangularis	0.022	0.006	0.009	0.019	0.019	0.022	0.035	0.006	0.022	0.000	
S. thorelii	0.099	0.085	0.088	0.102	0.095	0.099	0.120	0.085	0.099	0.085	0.000

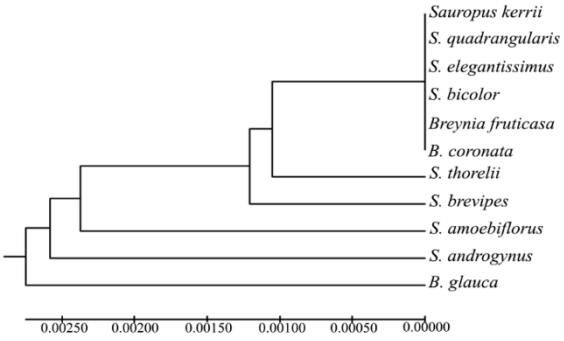

Figure 7. Dendrogram constructed from the *trn*H-*psb*A spacer of the *Breynia* and *Sauropus* species using MEGA version 7

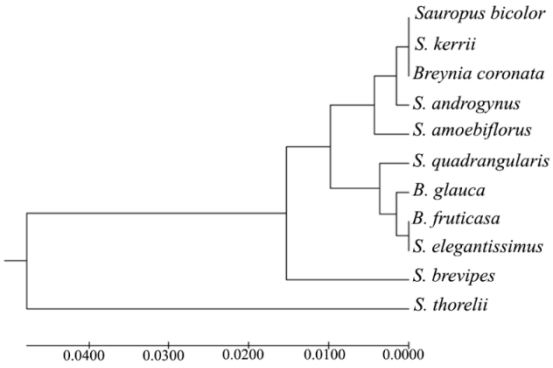

Figure 8. Dendrogram constructed from the *rpo*B region of the *Breynia* and *Sauropus* species using MEGA version 7

Table 6: GenBank accession numbers of DNA barcodes from two regions of *rpo*B and *trn*H-*psb*A spacer regions in *Breynia and Sauropus* species.

Plant	GenBank accession number	
	*rpo*B	*trn*H-*psb*A spacer
B. coronata	N/A	N/A
B. fruticosa	MH052648	MH052657
B. glauca	MH052649	MH052658
S. amoebiflorus	MH052650	MH052659
S. androgynus	N/A	MH052660
S. bicolor	MH052651	MH052661
S. brevipes	MH052652	MH052662
S. elegantissimus	MH052653	MH052663
S. kerrii	MH052654	MH052664
S. quadrangularis	MH052655	MH052665
S. thorelii	MH052656	MH052666

Discussion

Many species of the genus *Sauropus* have not been studied, and little scientific information is available. Many phytochemicals that at high concentrations affect human health were revealed in the eight studied *Sauropus* species, including squalene in *S. thorelii* and γ-sitosterol and phytol in *S. brevipes*. The most exciting is 78.13% eudesmin, of which 36.445 mg and

32.190 mg is produced from 2 g fresh leaves and 1.5 g from dried leaves in *S. bicolor*.

Eudesmin functions include anti-inflammatory (Cho et al., 1999; Yang et al., 2018) and anticonvulsant effects, and it has a sedative effect (Liu et al., 2015), while it also has antitumor effects on lung cancer (Jiang et al., 2017). The high amount of eudesmin will be expected to be used in the forms of traditional medicine, crude extracts or purity for human wellbeing.

The DNA profiles of *Breynia* species were compared with those of the studied *Sauropus* species. The genetic relationships via dendrogram produced by ISSR fingerprints indicated the accuracy of genera separation, with the S between genera calculated in the range of 0.71-0.81, of 0.76 - 0.85 at the interspecific level of *Breynia*, and of 0.69 - 0.80 in the *Sauropus* species, in agreement with the genus and species criteria created by Weier et al. (1982) that have been used many times in many publications, including those of Chaveerach et al. (2008), Sudmoon et al. (2011), Siripiyasing et al. (2013), and Chaveerach et al. (2014). The same barcode markers in various regions, for example, the *mat*K, *rpo*B, *rbc*L, *rpo*C1, and *trn*H-*psb*A spacer, are found in many publications. For example, Thooptianrat et al. (2017) found the *trn*H-*psb*A spacer in the *Dillenia* species after publishing the early barcode principle by Hebert et al. (2003) and Chase et al. (2007). Thus, to identify species in a specific area, barcodes were constructed for the studied *Breynia* and *Sauropus* species. With the *rpo*B and *trn*H-*psb*A spacer regions, some species of the two genera cannot be identified using these regions, as revealed by the 0.000 distance in any pair of species (Table 4, 5) and by the dendrogram producing species in identical branches, namely, *S. kerrii*, *S. quadrangularis*, *S. elegantissimus*, *S. bicolor*, *B. fruticose* and *B. coronata* in the *rpo*B region as well as the groups *S. bicolor*, *S. kerrii* and *B. coronata* and the pair *B. fruticosa* and *S. elegantissimus* in the *psb*A-*trn*H spacer region; these sequences cannot be used for their identification (Figures 7, 8). It is noted that the species pairs that cannot be identified by the barcode sequence of the *rpo*B region also cannot be identified by the *psb*A-*trn*H spacer.

Conclusion

Sauropus bicolor and *S. thorelii* show high content of eudesmin, an important phytochemical functioning as an anti-inflammatory and antitumor agent. Its high amount containing in the plant leaves indicates that the plants can be efficiently used as natural sources for traditional medicine or other health products. The related species in genus *Breynia* which show similar morphological characteristics can be distinguished by DNA profiles based on ISSR fingerprinting. Additionally, the barcode markers of *rpo*B and *trn*H-*psb*A regions can be partially used for distinguishing some species of these two genera.

Contribution of Authors

Sawasdee N: Data Collection, Literature Review, Designed Research Methodology, Statistical Analysis, Manuscript Writing, Manuscript final reading and approval

Chaveerach A: Data Interpretation, Manuscript final reading and approval

Tanee T: Data Interpretation, Statistical Analysis

Suwannakud KS: Data Collection, Literature Search

Ponkham P: Data Collection, Literature Search

Sudmoon R: Conceived Idea, Data Collection, Literature Review, Designed Research Methodology, Data Interpretation, Manuscript final reading and approval

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