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Research Article

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Phytochemical composition and biological activities of *Dorcoceras uthongensis* (Gesneriaceae) - A new species from the limestone karst of Suphan Buri, Thailand

Jaran Prajanban[1,4], Wichai Patumchartpat [2,4], Radeekorn Akkarawongsapat[3], Chanita Napaswad[3], Sathit Panvilai[1,4]*

[1]Program in Medical Science, Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand, [2]Program in Agricultural Science, Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand, [3]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand, [4]Center of Biodiversity and Indigenous Culture, Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand

ABSTRACT

Dorcoceras uthongensis, a new species of the genus *Dorcoceras* Bunge, is described. This new species is endemic to Uthong district, Suphan Buri province, Thailand, and differs from the other species by exhibiting capitate glandular hairs with globose unicellular head on the abaxial surface of the leaf. Additionally, it is classified as an endangered species (EN) according to IUCN criteria. The phylogenetic analysis based on nuclear ITS1-5.8S-ITS2 confirmed its placement within *Dorcoceras*. Moreover, we sought to explore the potential biological activities of the crude extract of this new species. We evaluated the aqueous extract of leaves which revealed antioxidant activity and no cytotoxicity indicating potential safety for further research and utilization. To examine the phytochemical composition, we performed an analysis using LC-MS/MS-QTOF. The result revealed the presence of flavonoids, alkaloids, phenolic compounds, and terpenes.

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***Corresponding author:**
Sathit Panvilai
E-mail: scsathit@bsru.ac.th

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INTRODUCTION

The genus *Dorcoceras* Bunge is distributed in China, Vietnam, Thailand, Cambodia, the Philippines, and Indonesia. It was resurrected from *Boea* by observing the distinctive morphology and genetic diversity. Four species were ascribed to it, including *Dorcoceras wallichii* (R.Br.) C.Puglisi, *Dorcoceras geoffrayi* (Pellegr.) C.Puglisi, *Dorcoceras hygrometricum* Bunge, and *Dorcoceras philippense* (C.B.Clarke) Schltr. (Puglisi *et al.*, 2016). A recent revision of *Dorcoceras* Bunge in Thailand revealed two new species, *Dorcoceras glabrum* C.Puglisi and *Dorcoceras brunneum* C.Puglisi (Puglisi & Middleton, 2017). The key characters of this genus include an obliquely campanulate, ventricose, pale lilac corolla with reflexed upper two lobes, and a broad throat, while species can be identified by the vegetative features and indumentum (Puglisi *et al.*, 2016; Puglisi & Middleton, 2017). While conducting biodiversity research on the limestone karst in Uthong district, Suphan Buri province (Figure 1), we found a putative new species of *Dorcoceras* with distinct morphological features. For the taxonomic treatment of this new species, we focused on the multicellular hair types (trichomes) that can be observed in the fresh specimens. We also conducted a phylogenetic analysis to assess whether our morphological observations would be supported by genetic data.

Various species of *Gesneriaceae* have been utilized for medicinal uses against fever, respiratory diseases, inflammation, and infectious diseases (Verdan & Stefanello, 2012; Yang *et al.*, 2023). The phytochemical constituents as natural antioxidant compounds in plants mostly consist of carotenoids, vitamins, phenolic compounds, and flavonoids, which prevent cellular damage caused by inflammation and pathogen infection (Emsen *et al.*, 2023). The acetone extract of *Sinningia bullata* (*Gesneriaceae*) exhibited cytotoxic, antibacterial, and

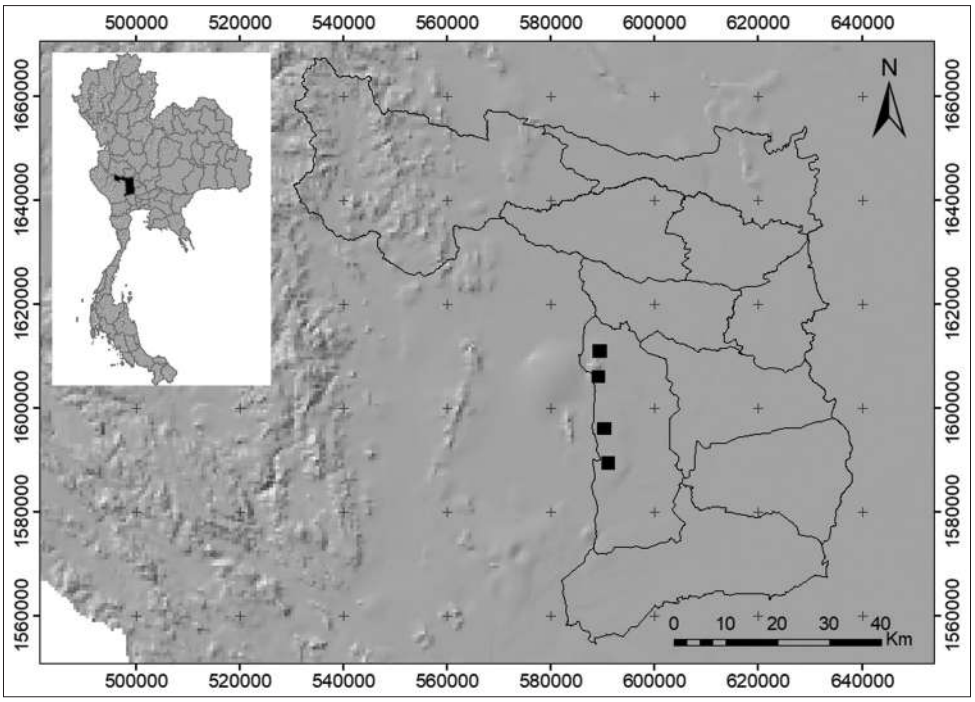

Figure 1: Distribution of *Dorcoceras uthongensis* in Uthong District, Suphan Buri Province, Thailand. The map was generated using ArcGIS 10.2

antioxidant activities whereas the water extract showed no cytotoxic and antibacterial activities (Chen *et al.*, 2023). Besides, the extract of *Haberlea rhodopensis* Friv. (*Gesneriaceae*) displayed antioxidant activity (Kondeva-Burdina *et al.*, 2013). The endemic plant, adapted to limestone habitats, has been investigated for its phytochemical composition and biological properties, highlighting the production of unique compounds influenced by its specific environmental conditions (Di Simone *et al.*, 2023; Kok *et al.*, 2023). To explore the significance of phytochemicals in plants adapted to limestone habitats, this study investigated the biological activities and chemical compositions of the aqueous extract from this new species through cytotoxicity testing, antioxidant assays, and LC-MS/MS-QTOF analysis. These assays will provide valuable information and be useful for further utilization of human health as a medicinal plant.

MATERIALS AND METHODS

Plant Specimens

The known species of *Dorcoceras* were collected from the locations previously reported by Puglisi and Middleton (2017). The fresh specimens were subjected to a comprehensive morphological study, employing the terminology as described previously. The examination of the morphological characteristics was carried out by macroscopic and microscopic observations using a digital camera and stereoscope (Olympus, SZX2-ILLTQ).

DNA Extraction, PCR Amplification, and Phylogenetic Analysis

The fresh leaves were rinsed with sterile ddH_2O and grounded by liquid nitrogen. The plant genomic DNA was extracted using DNeasy Plant Mini Kit (QIAGEN). The DNA concentration was determined by NanoDrop spectrophotometer (DeNoix DS-11). The nuclear ITS1-5.8S-ITS2 was used for phylogenetic analysis, and the PCR amplification was performed using ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White *et al.*, 1990) and the PCR condition was set as follows: 95 °C for 3 min, 35 cycles of 95 °C 2 min, 50 °C 45 sec, 68 °C 1 min, and a final extension at 68 °C for 5 min. The PCR products were visualized by 2% agarose gel electrophoresis (AGE) which then was stained with SYBR GOLD (Invitrogen). The DNA bands were subsequently purified by QIAquick Gel Extraction Kit (QIAGEN). The DNA purity was determined by 2% AGE and subjected to DNA sequencing (MACROGEN, South Korea). The sequences were manipulated with SnapGene®Viewer and a nucleotide BLAST was performed in GenBank. The ITS sequence of *Dorcoceras uthongensis* was deposited in the GenBank (Accession no.; PP059122.1). For the phylogenetic analysis, ITS sequences of *Dorcoceras* or *Gesneriaceae* available in GenBank were downloaded and subsequently aligned using MUSCLE (Edgar, 2004) via MEGA7 (Kumar *et al.*, 2016). The phylogenetic reconstructions were executed by maximum likelihood (ML) and following a previous study by Puglisi *et al.* (2016) for Bayesian inference approach. The best fitting of the evolutionary model was inferred by jModelTest 2.1.10 (Darriba *et al.*, 2012) based on the Akaike information criterion (AIC) (Akaike, 1974). The ML was first analyzed with MEGA 7 using K2+G as the best fit model, and bootstrap analysis (1000 replicates). Bayesian analysis was executed with MrBayes 3.2.7a (Ronquist *et al.*, 2012). The evolutionary model was set as nst=1, rates=gamma, and the Markov Chain Monte Carlo (MCMC) was run for 10,000,000 generations in which one tree was sampled every 1000th generation. Once the average

standard deviation (SD) of the split frequencies reached 0.01, the MCMC analysis was terminated. The burn-in of 2000 trees was discarded and the output tree was displayed and edited in FigTree v.1.4.4 (Rambaut, 2022).

Scanning Electron Microscopy

The seeds were obtained from desiccated capsules and stored in a moisture-free environment to prevent damage. To eliminate dust, the dried seeds were promptly immersed in absolute ethanol and then left to air-dry at room temperature. For the scanning electron microscopy (SEM) analysis, the samples were mounted on the stubs without first undergoing dehydration. The seeds were sputter-coated with a layer of gold-palladium before being examined morphologically using the SEM instrument (JEOL; JSM-IT300).

Aqueous Extraction of Crude Extract

Leaves of *Dorcoceras uthongensis*, 100 g of fresh weight, were carefully cleaned using tap water and subsequently rinsed twice with sterile ddH_2O. Once cleaned, the leaves were blended with 500 mL of pre-chilled ddH_2O at 4 °C. The mixture was centrifuged at 6000 rpm for 15 minutes to separate cell debris. The supernatant was filtered through Whatman No.1 filter paper and the filtrate was subsequently frozen at -80 °C overnight. The frozen mixture was then dried using a freeze dryer (OPERON, Korea). The crude extract was denoted as *Dorcoceras uthongensis* aqueous extact (DUAE). To prepare the DUAE stock, the crude extract was dissolved in type I distilled water to achieve a concentration of 10 mg/mL. The extract was further filtered through a 0.45 µm syringe filter and the stock solution was stored at -20 °C until further use (Panvilai *et al.*, 2020).

Toxicity Testing

Cytotoxicity assay

To assess the potential toxicity of the DUAE extract on human cells, a colorimetric XTT assay was performed using 1A2 cells, specifically T-cell lymphoblasts. The experimental procedure involved seeding the cells into the wells of a 96-well plate, along with various concentrations of the DUAE extract ranging from 250 µg/mL to 7.8 µg/mL. The plate was incubated at 37 °C in a 5% CO_2 environment. After three days of incubation, a mixture of XTT tetrazolium salt (1 mg/mL) and 1% phenazine methosulfate was added to each well (50 µL/well) and incubated for three hours. The viable cells metabolized the XTT salt, resulting in the formation of a yellowish-brown formazan product. The absorbance of this formazan was measured at a wavelength of 450 nm (A450) using a microplate reader (TECAN F50). The cytotoxicity of the extracts was determined by calculating the concentration that inhibited the metabolic activity of 50% of the cells, known as the IC_{50} value (Panvilai *et al.*, 2020).

Bacterial toxicity testing

To determine whether the extract of this new species is toxic to bacteria that colonize the human body as a normal flora, we tested toxicity toward Gram-positive and Gram-negative bacteria, *Bacillus subtilis* and *Escherichia coli*, respectively. The agar disk-diffusion assay was performed according to Bauer *et al.* (1966). Briefly, bacterial cultures were grown overnight at 35 °C and subsequently adjusted turbidity to 0.5 McFarland (approximately 1.5 x 10^8 CFU/mL). The bacterial cells were spread onto the Mueller-Hinton agar (MHA). The filter disc (6 mm diameter) was soaked with 300 µg/disc of DUAE and placed on the MHA. The culture plates were incubated at 35 °C for 24 hours. The inhibition zone was observed to determine toxicity against bacteria.

DPPH Antioxidant Assay

The scavenging ability of free radicals of DUAE, ranging from 0.195-12.5 mg/mL, was evaluated using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay kit (Dojindo-D678) according to the manufacturer's instruction. Absorbance was measured at 517 nm using SPECTROstar Nano (BMG LABTECH). The IC_{50} of the DUAE was calculated to represent the extract's ability to decrease the initial DPPH by 50% (Koyama *et al.*, 2022).

Phytochemical Composition by LC-MS/MS-QTOF

The LC-MS/MS Triple-TOF 6600+ (SCIEX, USA) was carried out to identify the chemical constituents in both a positive and negative mode, according to Sun *et al.* (2023). In brief, the mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was set as follows: 0-3 min 22% B; 3-5 min 22-30% B; 5-9 min 30-35% B; 9-11 min 35-40% B; 11-13 min 40-48% B; 13-18 min 48-55% B; 18-22 min 55-70% B; 22-25 min 70-90% B; 25-26 min 90% B. The flow rate was set at 350 µL/min. The MS analysis was conducted with an electrospray ionization (ESI) source and implemented with full scan mode. The parameters were set as follows: the iron spray voltage floating (ISVF) was 5,500 V (ESI+) or -4,500 V (ESI-); the temperature of the ion source was 600 °C: the ion source gas 1 was 50 psi, the ion source gas 2 was 60 psi, the curtain gas was 30 psi, and the scanning range of TOF masses was set between *m/z* 100-1500. The conditions of IDA-MS/MS were set as follows: the high resolution was determined; declustering potential (DP) was -80/80V; collision energy was 40 eV (ESI+) or -40 eV (ESI-); collision energy spread (CES) was 10 eV. The data acquisitions were analyzed by SCIEX OS 3.1.0.

RESULTS AND DISCUSSION

Taxonomic Treatment

Dorcoceras uthongensis Prajanban, Patumchartpat & Panvilai, *sp. nov.* (Figures 2, 3 & 4).

TYPE: THAILAND, Uthong district, Suphan Buri province, 14°22'30.4"N, 99°51'41.3"E, alt. 80 m limestone karst, 12 September 2020, *Prajanban J. & Panvilai S. 001* (holotype: BKF; isotype: BSRU).

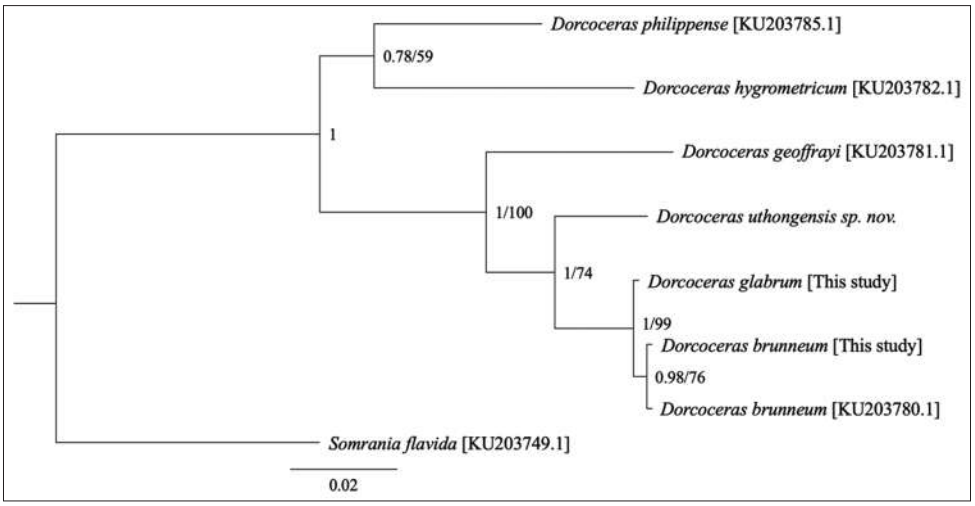

Figure 2: Phylogenetic analysis of the ITS region. The posterior probability and bootstrap support (>50%) are represented at the nodes

Figure 3: *Dorcoceras uthongensis* Prajanban, Patumchartpat & Panvilai, *sp. nov*. a) Habitat on the boulder of limestone karst, b) Lateral side of the flower is shown, c) Oblong unicellular head outside the corolla, d) Pale pink stigma, e) Detail of the reproductive organs, f) Pistil, g) Twisted capsule, h) Flower bud with pale purple petals, dorsally white, i) Glandular multicellular hairs on pedicel, j) Three staminodes are shown and k) Gland-tipped and eglandular multicellular hairs on calyx

ETYMOLOGY: The specific epithet refers to its type locality Uthong district.

DIAGNOSIS: *Dorcoceras uthongensis* is similar to *D. geoffrayi* and *D. wallichii* in colorless indumentum on the abaxial leaf surface but consisting of capitate glandular hairs with globose unicellular head (absent in *D. geoffrayi* and *D. wallichii*), as shown in Figure 5. Moreover, the sessile glandular trichomes observed in *D. wallichii* are absent in *D. uthongensis*. Also similar to *D. brunneum* in the leaf shape (Figure 4) but differing in the colorless indumentum abaxially (rusty brown in *D. brunneum*). The stigma of *D. uthongensis* is pale pink bilabiate, which is only found in this new species (Figure 3).

Description

Rosulate herb. *Leaves* petiolate; petiole ca. 1 cm long, lamina 10.5-14 cm long, 2.8-4 cm wide; narrowly elliptic to spathulate with narrowly acute apex, attenuate base, serrate margin; adaxial surface green, with subulate eglandular hair with multicellular jointed stalk, multicellular base; abaxial pale green, with colorless capitate glandular hairs with globose unicellular head slightly scattered throughout the lamina; 3-4 pairs of secondary veins, tertiary venation absent. *Inflorescence* a compound dichasium with 10-15 flowers, with pale purple glandular and egladular hairs; peduncles 8.5-11.5 cm long,

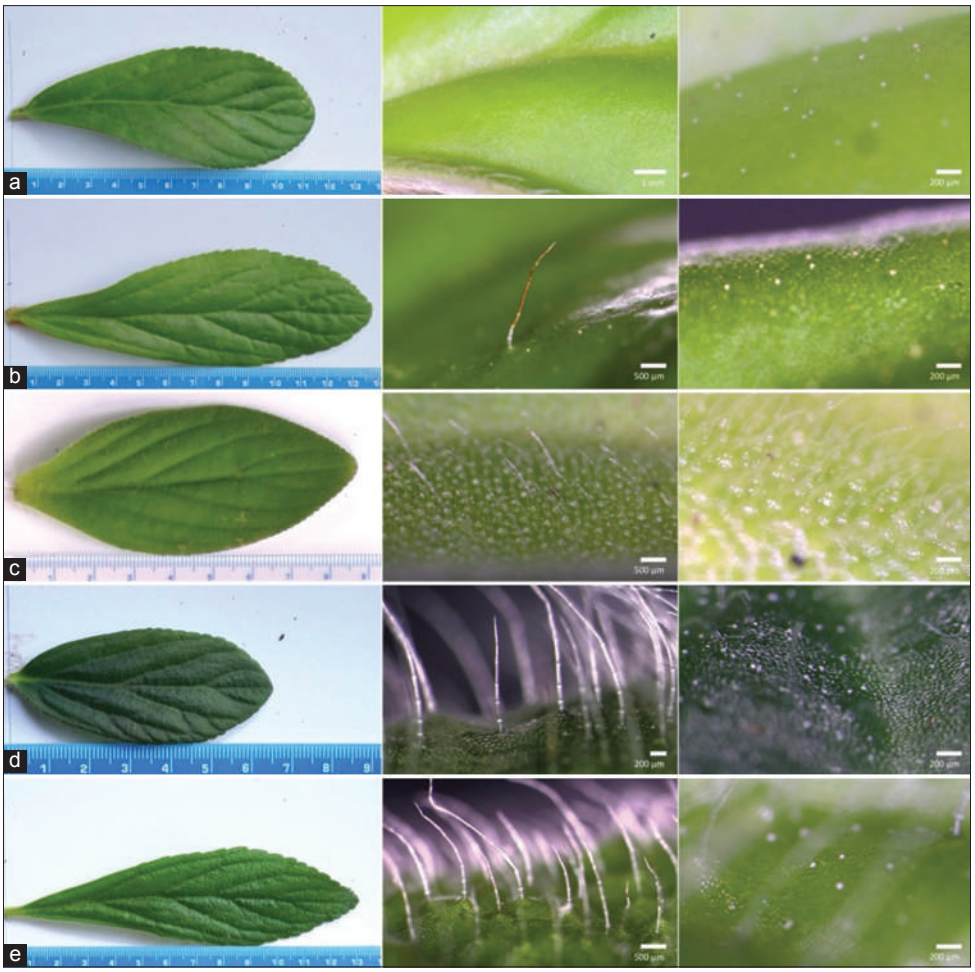

Figure 4: Upper leaf surface of genus *Dorcoceras* collected in this study. All species have sessile glands on the surface. a) *Dorcoceras glabrum*, the only species that has no hairs. b) *Dorcoceras brunneum*, rusty brown hair exhibited on the surface. c) *Dorcoceras geoffrayi*, d) *Dorcoceras wallichii*, and e) *Dorcoceras uthongensis* exhibited colorless indumentum. The scale bars are indicated in each figure taken from the stereoscope (Prajanban & Panvilai, 2021a, b, c, 2022)

0.6-1.5 mm wide, reddish brown, with mixed eglandular and glandular hairs; bracts 1.5-2 mm long, ca. 0.5 mm wide, narrowly lanceolate, apex acute, adaxial side glabrous, abaxial side with multicellular eglandular hairs; pedicels 1.1-2.5 mm long, 0.8-1.0 mm wide, pale green, with multicellular glandular hairs (purple globose unicellular head). *Calyx* lanceolate sepals with apex acute, 1.5 mm long, 0.7 mm wide, indumentum with gland-tipped and eglandular multicellular hairs on the abaxial surface, adaxial glabrous. *Corolla* broadly campanulate, thickened at the base, pale purple, ventrally white, outer side with oblong unicellular head hair, especially on the lateral and ventral sides, glabrous inside; tube 5-7 mm long, upper two lobes rounded, ca. 6 mm long, 4-6 mm wide, lateral lobes orbicular, 6-8 x 6-8 mm, ventral lobe orbicular, 6-8 x 6-8 mm. *Stamens* with filament curved, pale yellow, ca. 1.5-2 mm long, rising at the corolla base; anthers yellow, sagittate, ca. 1.5-2 mm long, 1-1.5 mm wide; two lateral staminodes, 0.8-1 mm long, central staminode ca. 0.5 mm long. *Ovary* red-brown, ca. 3 mm long, with pale pink, glandular hairs; style white, 4-5 mm long, slightly bent downwards, glabrous; stigma pale pink bilabiate, ca. 0.5 mm wide. *Capsule* strongly twisted, 1.5-2.8 cm long, with dark purple to brown glandular hairs scattered throughout the surface. *Seeds* (Figure 6) elliptic, brown, ca. 300-406 µm long, ca. 193-221 µm wide, epidermal cells irregularly reticulate with granules, cell wall straight to slightly undulate. *Phenology*; flowering August-September.

Distribution and Habitat

Dorcoceras uthongensis is endemic to the limestone karst of Uthong district, Suphan Buri province (Figure 1).

Conservation Status

IUCN assessment was conducted that based on the Geospatial Conservation Assessment Tool (GeoCAT) (Bachman *et al.*, 2011). The calculated extent of occurrence (EOO) of 107 km^2 and area of occupancy (AOO) of 16 km^2 for *Dorcoceras uthongensis* indicated that the species qualified as endangered (EN) under criterion B of the IUCN Red List.

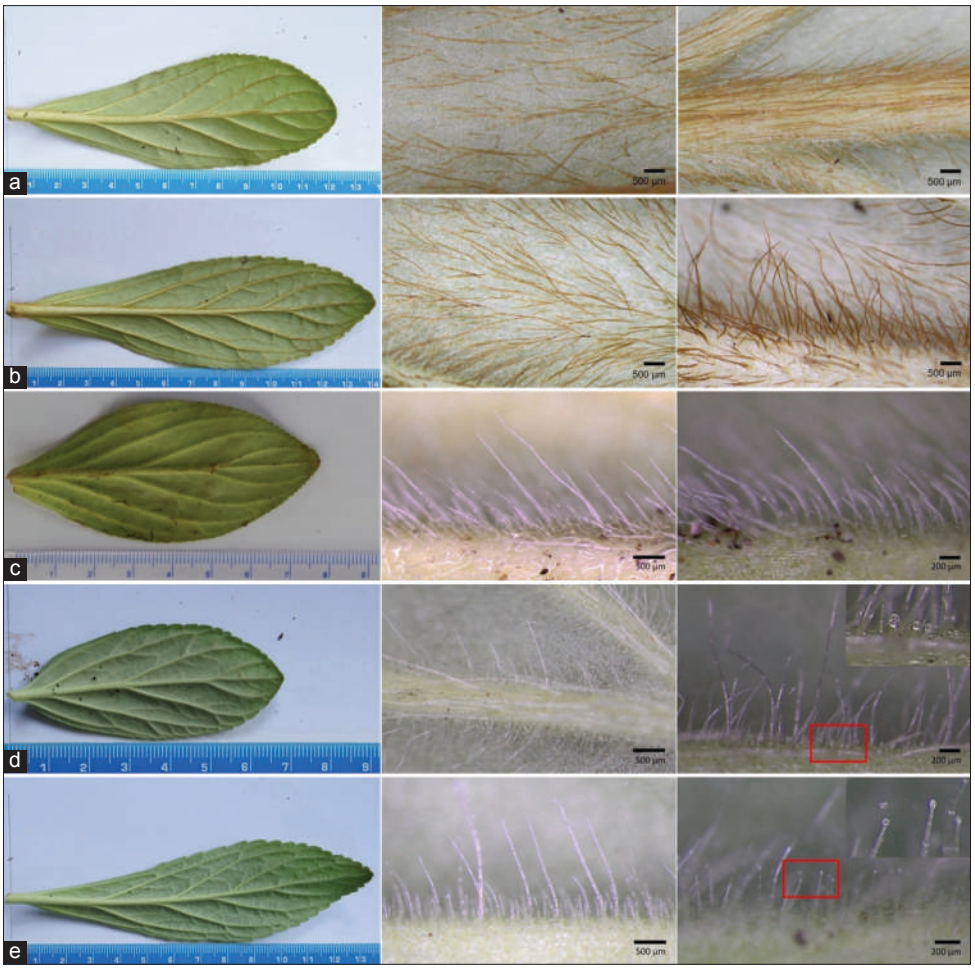

Figure 5: Lower leaf surface morphology of genus *Dorcoceras*. a) *Dorcoceras glabrum* and b) *Dorcoceras brunneum* have rusty brown indumentum, whereas c) *Dorcoceras geoffrayi*, d) *Dorcoceras wallichii*, and e) *Dorcoceras uthongensis* have colorless indumentum. Sessile glands are present (enlarged figure d) in *D. wallichii*, while hairs with globose unicellular heads are observed in *D. uthongensis* (enlarged figure e). The scale bars indicated in each figure are taken from the stereoscope (Prajanban & Panvilai, 2021a, b, c, 2022)

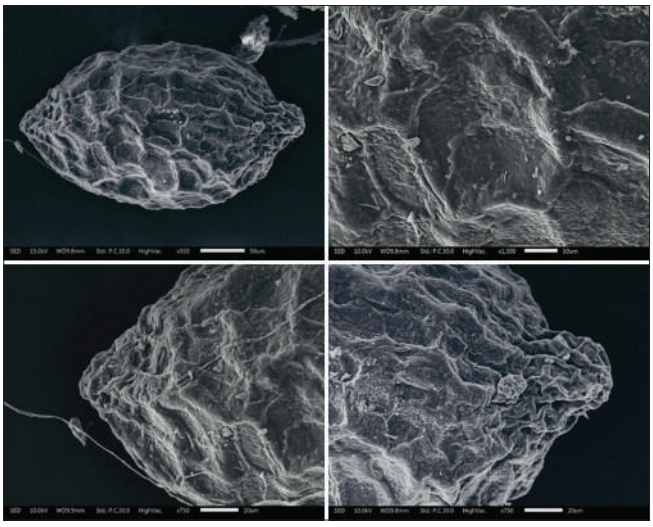

Figure 6: Seed SEM micrographs of *Dorcoceras uthongensis* Prajanban, Patumchartpat & Panvilai, *sp. nov*. The surface of the seed displayed an irregular reticulate epidermal cell shape with granules (Prajanban & Panvilai, 2020)

Key to New Species of *Dorcoceras*

1.	Adaxial surface glabrous	*D. glabrum*
1.	Adaxial surface with multicellular eglandular hairs	2
2.	Abaxial surface with rusty brown indumentum	*D. brunneum*
2.	Abaxial surface with colorless indumentum	3
3.	Glandular indumentum and sessile gland absent	*D. geoffrayi*
3	Glandular indumentum or sessile gland present	4
4.	Sessile gland present	*D. wallichii*
4.	Glandular indumentum present	*D. uthongensis*

Phylogenetic Analysis

The phylogenetic inference of the ITS marker supports the placement of the new species (*Dorcoceras uthongensis*) in the genus

Table 1: Phytochemical composition obtained from LC-MS/MS-QTOF analysis performing both positive and negative ionization modes

Identified compounds (ESI Positive mode)	Library Score	Identified compounds (ESI Negative mode)	Library Score
Pantothenic acid	99.1	D-Saccharic acid	96.8
Ethyl glycolate	82.0	D-Gluconic acid	99.7
Phenylethylamine	100.0	4-O-beta-Galactopyranosyl-D-mannopyranose	99.5
Pentaethylene glycol	94.4	L-Malic acid	99.2
Hexaethylene glycol	100.0	Maleic acid	99.6
Lipoxin A5	100.0	D-(+)-Raffinose	84.6
Pinacolyl methylphosphonic acid	83.5	L-2-Hydroxyglutaric acid	82.7
2-Linoleoylglycerol	100.0	3-Furancarboxylic acid	100.0
Apigenin 7-O-neohesperidoside	95.0	Citric acid	99.2
3-Cyclohexyl-1,1-dimethylurea	100.0	Pipecolinic acid	100.0
Ingenol	89.5	Vitamin B6	97.9
Apigenin	99.2	Amber acid	96.2
2,6-Di-tert-butyl-4-hydroxymethylphenol	95.3	Adenosine 2',3'-cyclic monophosphate	91.4
N-(tert-Butyl)benzenesulfonamide	97.1	2-Penten-1-ol, (Z)-	100.0
1,3-Dicyclohexylurea	99.6	Guanosine	95.1
Anthraquinone	93.0	Pantothenic acid	93.8
Tuberostemonine	95.8	2-Hydroxy-3-methoxybenzoic acid	81.1
Ethyl 4-hydroxybenzoate	96.4	L-Tryptophan	95.8
2-(2-Hydroxy-5-methylphenyl) benzotriazole	89.2	4-Imidazoleacrylic acid	100.0
Cinnamic acid	100.0	4'-Hydroxyacetophenone	96.2
2,6-Di-tert-butylbenzoquinone	97.7	Kaempferol-7-O-neohesperidoside	97.4
Laurylguanidine	83.6	Apigenin 7-O-beta-D-glucuronide	100.0
Palmitamide	81.3	Vanillic acid	96.4
Cyanidin 3-O-lathyroside	80.1	trans-Glutaconic acid	100.0
1-Stearoyl-rac-glycerol	93.0	Syringic acid	87.5
Mono-2-ethylhexyl phthalate	86.8	Azelaic acid	95.8
Dioctyl phthalate	92.4	4-Hydroxybenzoic acid	100.0
Decamethylcyclopentasiloxane	80.3	Apigenin	98.3
3,5-Lutidine	96.3	Decanoic acid	98.4
(+)-Dihydrocarvone	96.9	Dodecyl sulfate	100.0
Dodecamethylcyclohexasiloxane	90.9	(+-)-9(10)-Epoxy-12Z-octadecenoic acid	90.2
		Ethylene glycol dodecyl ether sulfate	95.8
		3-tert-Butyl-2-hydroxybenzaldehyde	92.7

Dorcoceras Bunge. The best phylogram of maximum likelihood, with statistical bootstrap support (BS) and Bayesian posterior probability (PP value), is depicted in Figure 2. The DNA sequence of the new species represents a noticeable difference from other species of *Dorcoceras*, determining its recognition as a distinct species. *Dorcoceras uthongensis* is sister to *Dorcoceras glabrum* and *Dorcoceras brunneum* with strong support from bootstrap (BS=92%) and Bayesian posterior probability (PP=1), indicating its genetic divergence from the two closely related species.

Biological Activities

The cytotoxicity of DUAE, even at the highest concentration (250 µg/mL) tested, showed no toxicity towards 1A2 cell lines. Similarly, bacterial toxicity testing showed no toxicity in both Gram-positive and negative bacteria by which the zones of inhibition were absent (data not shown). The IC_{50} of antioxidant activity of DUAE was 2.3 mg/mL, suggesting that its constituent possessed an antioxidant compound.

Phytochemical Composition

The secondary metabolites found in the aqueous extract include flavonoids, alkaloids, phenolic compounds, and terpenes, which are common in plants (Table 1). Some compounds exhibited biological activities, such as apigenin, a flavonoid found in many fruits and vegetables which is well-known for its strong antioxidant properties (Salehi *et al.*, 2019) and show broad antiviral activity (Lee *et al.*, 2023), kaempferol-7-O-neohesperidoside, a flavonoid glycoside that exhibits significant antioxidant activity by neutralizing free radicals and reducing oxidative damage in cells (Ibrahim & Mohamed, 2015), and anthraquinone, which possesses antioxidant properties and antiviral activity (Schinazi *et al.*, 1990; Zhao & Zheng, 2023).

CONCLUSION

In the present study, we reported the new species of *Dorcoceras* Burnge, namely *Dorcoceras uthongensis* Prajanban, Patumchartpat & Panvilai, sp. nov. Its key character that differs from other species is multicellular glandular hair on the abaxial surface of the leaf. The limestone karst landscape is its natural habitat. The aqueous extract of *D. uthongensis* exhibited no cytotoxicity to either human lymphocyte or bacterial cells and demonstrated free radical scavenging activity. The major secondary metabolite consists of flavonoids, alkaloids, phenolic compounds, and terpenes.

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