# Molecular Phylogeny, Systematics, Antioxidant and Antibacterial Activities of Lichenized fungi genus *Usnea* in Northern Thailand

Arerat Saisong

A Thesis Submitted in Partial Fulfillment of Requirements for

degree of Master of Science in Biology

May 2025

ความสัมพันธ์เชิงวิวัฒนาการด้านชีวโมเลกุล ซิสเทมาติกส์ ฤทธิ์ต้านอนุมูลอิสระและต้านแบคทีเรีย
ของไลเคไนซ์ฟังไจสกุล *Usnea* ในภาคเหนือของประเทศไทย

วิทยานิพนธ์

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for Master of Science (Biology)

May 2025

The examining committee has unanimously approved this Thesis, submitted by Miss Arerat Saisong , as a partial fulfillment of the requirements for the Master of Science Biology at Mahasarakham University

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| | | | |
|---|---|---|---|
| **TITLE** | Molecular Phylogeny, Systematics, Antioxidant and Antibacterial Activities of<br>Lichenized fungi genus *Usnea* in Northern Thailand | | |
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## ABSTRACT

This research aims to 1) identify species of the genus *Usnea* from Northern Thailand by using morphological, anatomical, chemical characteristics, and molecular analysis 2) evaluate the potential of antioxidant and antibacterial activities, and 3) phytochemistry analysis within total phenolic compound, total flavonoid compound, and tannin. The studies of molecular phylogeny utilized ITS rDNA as a primer, and genomic DNA was extracted using plant DNA extraction mini kits. DNA sequencing was conducted at Macrogen, followed by alignment and phylogenetic tree reconstruction using the MEGA-X program. The crude extract of *Usnea himantodes* and *Usnea* cf. *pectinata* from 4 different solvents is macerated with 95% ethanol, acetone, and ethyl acetate, and boiling water. Antioxidant activities using DPPH, ABTS, and FRAP assays. Agar well diffusion using for antibacterial activity with 4 bacterial species, such as *B. cereus* TISTR 1449, *Staphylococcus epidermidis* TISTR 2162, *Escherichia coli* TISTR 527, and *Pseudomonas aeruginosa* TISTR 1287. Total phenolics and tannin content using the Folin-Ciocaltue method, whereas flavonoids using Aluminum chloride colorimetric assay. The results show 12 species identified including *U. aciculifera* Vain., *U. articulata* (L.) Hoffm., *U, baileyi* (Stirt.) Zahlbr., *U. ceratina* Ach., *Usnea* cf. *dendritica* Stirt, *U. himantodes* Stirt., *U. pangiana* Stirt., *Usnea* cf. *perhispidella J. Steiner*, *Usnea* cf. *pectinata*, *U. rubicunda* Stirt., *Usnea* cf. *rubicunda*, and *U. shimadae* Asahina. Only *U.* aciculifera Vain supported with molecular data of ITS region. The results of the antibacterial activities show that the extract of boiling water of *U. himantodes* and *Usnea* cf. *pectinata* had the lowest $IC_{50}$ of 0.759±0.036 and 0.741±0.021 mg/ml in DPPH assay, ABTS assay having an $IC_{50}$ of 0.765±0.018 and 1.001±0.005 mg/ml, and the FRAP value being 30.069±0.211 and 27.927±0.129 mg $FeSO_4$/g extract. The antibacterial properties show that ethanolic, acetone, and ethyl acetate extracts can inhibit *B. cereus* TISTR 1449, *S. epidermidis* TISTR 2162, and *E. coli* TISTR 527, but cannot effectively against *P. aeruginosa* TISTR 1287 with MIC and MBC values of 20 mg/ml. The phytochemistry analysis showed that ethanolic extract of *U. himantodes* and *Usnea* cf. *pectinata* contained the highest total phenolic content (84.430±1.385 and 93.772±2.847 mg Gallic acid/g

Extract), total flavonoid content (21.595±1.138 and 36.847±0.613 mg Quercetin/g Extract), and tannin content (81.418±1.341 and 90.467±2.784 mg Tannic acid/g Extract). This study demonstrates the antioxidant and antibacterial effects of *Usnea* lichen extracts, providing insights for potential applications in various industries. These findings provide valuable insights for future development and application in pharmaceutical and food industries, enhancing the use of natural products for health-related benefits.

Keyword : Antibacterial, Antioxidant, Crude extract, Flavonoid, Lichen

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Arerat Saisong

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# CHAPTER 1
# INTRODUCTION

## Introduction and Background

Lichens are organisms formed by symbiosis between fungi (mycobiont) and algae (photobiont), forming an adhesion structure called the "thallus". Lichens are a complex life form that is in a symbiosis relationship. In this relationship, the fungus is the dominant partner, which acts as a store the moisture from the atmosphere. Another is algae, The algae can be either green algae or blue-green algae (cyanobacteria) and many lichens contain both types. The algae are responsible for photosynthesis. Lichens can thrive in any habitat in terrestrial ecosystems and have distribution in all continents: Europe, America, Africa, Australia, Antarctica, and Asia. Although lichens can be observed in several areas, there are still few studies by biologists. For this reason, the diversity of lichens is still very little known (Lagostina et al., 2018).

Lichens have been studied and found to have numerous benefits. They can be used for dye (e.g. *Usnea barbata, Lecanora tartarea,Letharia vulpine*), as an indicator of air quality, as an ingredient in perfume (e.g., *Evernia prunastri*), as a source of pH paper, and even as food for reindeer (e.g., *Cladonia* sp.). In the past, lichens were even utilized as an ingredient in bread and soup for use as a medicine to aid digestion (e.g., *Cetraria islandica*) (Römpp, 1995; Nash III, 1996). People believe that lichens contain some bioactive substances, corresponding to nowadays research confirms that lichens produce a substance known as "Lichen substance" or "Secondary metabolite". These substances have been proven to be effective in studying their ability against bacteria and fungi. (Naksuwankul, 2015; Zhao et al., 2021). Many lichens can produce phenolic compounds, which have antioxidant properties and can eliminate free radicals and ions. In addition, phenolic compounds are also utilized as rancid preservatives for food preservation (Muangsan et al., 2018).

*Usnea* lichens belong to the phylum Ascomycota, one of the large genera in the family Parmeliaceae (Funk et al., 2018). Globally, there are around 600 species of *Usnea* (Jannah et al., 2021). The genus is identifiable by its fruticose trait, commonly referred to as Old Man's Beard, and its distinct yellowish-green color due to the production of Usnic acid. The *Usnea* species can be found on every continent with a cosmopolitan distribution. Previous studies have relied on morphology, anatomy, and chemistry to identify it. To identify different species of *Usnea*, one can consider the thickness ratio of the cortex, medulla, and central axis. Other factors like the presence or absence of soredia, color, shape, and smoothness of the surface of the branches (Lamb, 1964). To identify the secondary metabolites of lichen using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The study suggests that using molecular data to identify lichen species can provide valuable insights into their limits. Currently, the studies on identifying lichen species popularly use molecular analysis due to its highly reliable and accurate. This method can provide insight into data about DNA sequences.

At present, research on identifying *Usnea* is restricted in Europe, the Americas, and Asia, particularly in Thailand. To tackle this issue, a study was conducted in Northern Thailand that employed both molecular analysis and traditional methods, including morphology, anatomy, and chemical analysis, to identify the genus *Usnea*. The molecular analyses are based on ITS regions.  This research aims to conduct phylogenetic analyses of the lichen genus *Usnea* by combining examinations of morphology, anatomy, and chemistry with molecular analysis. Furthermore, also studies the biological properties including antioxidant and antibacterial activities of some lichen extracts. The evaluation of antioxidants using DPPH, ABTS, and FRAP assays, and analysis of the content of polyphenols which are phenolic and flavonoid compounds. Agar well diffusion, MIC, and MBC methods to assess antibacterial properties. The researcher is highly interested in studying *Usnea*, given its usefulness, to obtain valuable information on identifying lichens and medical applications in the future.



**Objectives of the Study**

1. To study the molecular phylogeny of *Usnea* by using ITS regions.

2. To identify the species based on morphological, anatomical, and chemical characteristics.

3. To study the properties of biological activities substances in antioxidants by using ABTS, DPPH, and FRAP assays.

4. To study the properties of bioactive compounds in antibacterial growth by Agar well diffusion, MIC, and MBC methods.

**Location of Research Conducting**

1. Department of Biology, Faculty of Science, Mahasarakham University
2. Laboratory Equipment Center of Mahasarakham University
3. Laboratory Equipment Center, Faculty of Science, Mahasarakham University
4. Molecular Biology Laboratory under BSL level 2, Faculty of Science, Mahasarakham University

**Research Plan and Procedures**

Table 1 shows the duration of the research plan procedure from February 2023 to April 2024.

**Table 1** The duration of the research plan and procedure

| Activities | Month | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| **1st Year** | | | | | | | | | | | | |
| 1. Coursework | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| 2. Preliminary reviews | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| **2nd Year** | | | | | | | | | | | | |
| 3. Coursework | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | | | | | |
| 4. Literature reviews | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| 5. Lichen collection | | | | | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| **3rd Year** | | | | | | | | | | | | |
| 6. Morphological studies | ↔ | ↔ | ↔ | | | | | | | | | |
| 7. Anatomical studies | | | ↔ | ↔ | ↔ | | | | | | | |
| 8. Chemical studies | | | | | ↔ | ↔ | ↔ | | | | | |
| 9. Molecular studies | | | | | | | | ↔ | ↔ | ↔ | ↔ | ↔ |
| **4th Year** | | | | | | | | | | | | |

| Activities | Month | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 10. Test of antioxidant activities | ↔ | ↔ | ↔ | | | | | | | | | |
| 11. Test of antibacterial activities | | | | ↔ | ↔ | ↔ | ↔ | | | | | |
| 12. Manuscript | | | | | | | | ↔ | ↔ | | | |
| 13. Published the paper | | | | | | | | | | ↔ | ↔ | ↔ |
| 14. Research summary and thesis writing | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |

**Research Expectations**

1. Species diversity of the genus *Usnea* in Thailand.
2. The biological activities of antioxidants using ABTS, DPPH, and FRAP assays.
3. The bacterial growth inhibition of bioactive compounds can inhibit bacterial growth by Agar well diffusion, MIC, and MBC methods.
4. The results of this study can be published in at least one national or international journal.

# CHAPTER 2
# LITERATURE REVIEW

## Lichens

Lichens are a unique composite organism that results from a mutually beneficial relationship between fungal (mycobionts) and green or blue-green algae (photobionts) in suitable environmental conditions (United States Department of Agriculture, 2021). The dominant partner is the fungus, which gives the lichen most of its characteristics. The algae provide the fungi with moisture and oxygen, while the fungi provide the algae with carbon dioxide to be used in photosynthesis (Forest Biodiversity Division, 2019).

## Lichen Growth Forms

Lichens can be classified into three main growth groups based on their appearance. (Forest Biodiversity Division, 2014)

1. The first group is crustose or crust-like, which appears as a flat sheet attached to the substrate where lichens grow (Fig 1A).

2. The second group is foliose, which usually has a leaf-like plate with distinguishable top and bottom sides (Fig 1B).

3. The third group, fruticose, is a fibrous lichen that looks like a beard or Old Man's Beard and typically hangs from the object it's attached to (Fig 1C).


**Figure 1** Lichen growth forms

(A) Crustose (B) Foliose (C) Fruticose

Ref: (Forest Biodiversity Division, 2014)

**Reproductive Structure of Lichen**

The reproductive can be classified into two main types: sexual and asexual.

1. Sexual Reproductive: only the partner of the fungus has established a sexual reproductive.

1.1 Apothecium: There are two types of fruiting structures produced by the fungal component of lichen. The first type is called an apothecium, which can be either cup- or disc-shaped (also known as disc-like apothecia, as shown in Figure 2A), or elongated and lip-shaped, with a branch called Lirellate apothecia (Figure 2B). Apothecia contain spores.

1.2 Perithecium: it is flask-shaped and often embedded in the thallus, which makes it somewhat inconspicuous. A small hole at the top of the perithecium releases spores (Figure 2C).


**Figure 2** Sexual reproductive

A) Disc-like apothecia B) Lirellate apothecia C) Perithecia

Ref: (Naksuwankul, 2015)

2. Asexual reproduction in lichens involves establishing reproductive propagation on the thallus. The thallus is easily broken or detached from the thallus surface. There are four main structures involved in asexual reproduction: soredia, isidia, lobules, and schizidia (as shown in Figure 3A).

2.1 Soredia are small powdery or granular structures that grow from a layer of algae and emerge from the thallus where there are fissures or holes on the thallus surface (Figure 3A).

2.2 Isidia are small structures found on the thallus consisting of a combination of mold and algae. The shape of isidia varies depending on the type of lichen. When the isidia break and fall away, they spread with the wind (Figure 3B).

2.3 Lobules are small, flat leaf-like structures with a cortex like the thallus. Most lobules are found around the edge of the thallus. Unlike isidia, lobules do not break off easily, and therefore, do not contribute to reproductive propagation. However, they can increase the area of photosynthesis of the thallus (Figure 3C).

2.4 Schizidia refers to a structure that grows from the top of the thallus. They are small scale-like shapes separated from the thallus lobe (Figure 3D).

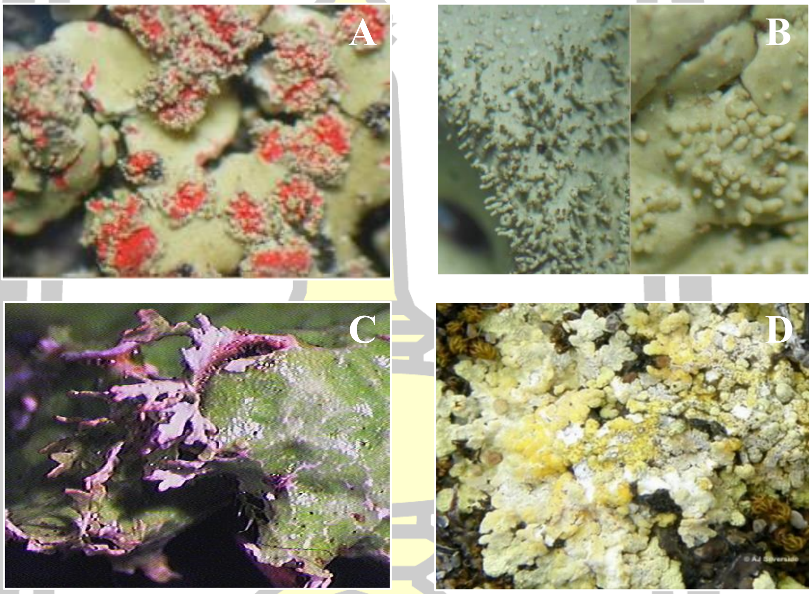


**Figure 3** Asexual reproductive

A) Soredia B) Isidia C) Lobules D) Schizidia

Ref: Ryan, Bungartz, & Nash III (2002)

**Lichen Substances**

Lichens can produce unique substance compounds from a fungal with primary and secondary metabolites. There are antimicrobial, antitumor, antipyretic, anticancer, antioxidant, anti-inflammatory effects, and antifungal properties (Römpp, 1995; Ullah et al., 2019; Zhao et al., 2021). The Primary substances include amino acids, protein, chitin, vitamins, and polysaccharides (Laxinamujila et al., 2013).

Most of the unique secondary substances are produced in lichen, have been reported more than 1000 substances compounds of lichen (Gonz΄alez-Burgos et al., 2019). The importance of secondary metabolites that are found in *Usnea* include usnic acid (produced in the cortex), salazinic acid, stictic acid, and constrictic acid (as shown in Figure 4) (there are produced in the medulla) (Elix et al., 2007).

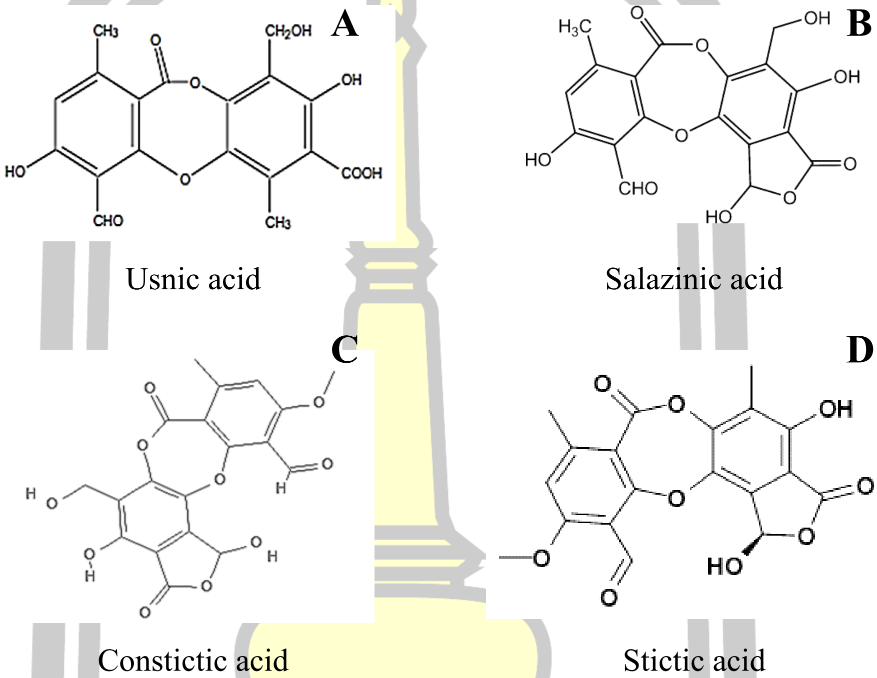


**Figure 4** Chemistry of *Usnea*

A) Usnic acid B) Salazinic acid C) Consticitic acid D) Stictic acid

Ref: Zhao et al., (2021)

**The genus *Usnea***

*Usnea* is a genus of fruticose lichen that is like a mini shrub or tassel. Thallus elongated pale grayish-green, either branched or not branched, and found growing on bark or branches. The genus is in the family Parmeliaceae. *Usnea* has a cosmopolitan distribution and is found on every continent. Members of the genus are commonly called old man's beard, or beard lichen. Among the 600 species known in the genus *Usnea* (Jannah et al., 2021).

Description: *Apothecia* lateral, subterminal or terminal, peltate, applanate, coriaceous, emarginate, periphery generally ciliate; thalline exciple glabrous, lacunose, echinate or ciliate, concolorous, pale stramineous or virescent, sometimes pruinose (rarely dichroic-red). *Asci* clavate, containing 8 spores; *paraphyses* gelatinous, filamentous. *Spores* monoblast, hyaline, ellipsoid. *Spermogones* lateral, immersed in shallow, colorless conceptacles. *Sterigmata* simple or subsimple. *Spermatia* fusiform or acicular-cylindrical, incrassate, apices truncate. *Soredia* normal (Figure 5C), occasional on all forms. *Cephalodia* lateral, concolorous, or darker, sometimes black. *Thallus* erect, subpendulous, or pendulous, branched (Figure 5A), fibrillose or efibrillose, terete, compressed or angulate, nitidous, glabrous, scabrous, squamose, or foveolate (Figure 5B), papillate or epapillate, pale stramineous, virescent, green, or tawny; cortex: subcrustose, subcontiguous, bambusaceous, or articulate; gonidia " *Protococcus*"; medulla cottonous, central indurated chondroid cord percurrent (Figure 5D) (Howe, 1910).

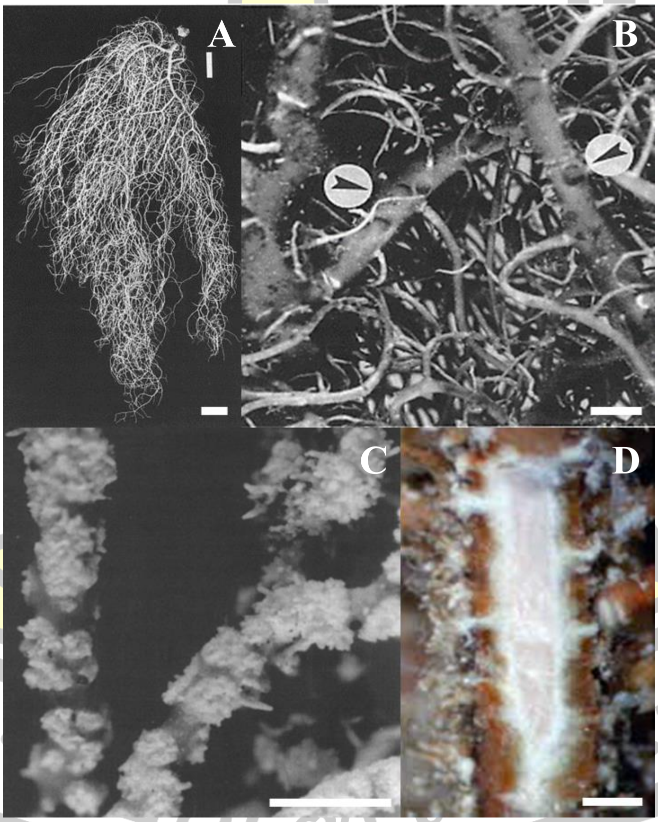


**Figure 5** Characteristics of genus *Usnea*

(A) Subpendent thallus of *Usnea*; scale = 1 cm. (B) Foveoles (pointed by arrows) on a branch; scale = 1.3 mm. (C) Expanded soralia with some isidia; scale = 0.5 mm. (D) Uninflated branch with medulla; scale = 0.5 mm.

Ref: (Howe, 1910; Halonen et al., 1999)

## The Identification Species of *Usnea*

The traditional identify species of *Usnea* using the combination of morphological, anatomical, and chemical characteristics to delimit the species (Paliya et al., 2016):

*Morphology:* The primary characteristics used to identify *Usnea* species include various morphological features such as the thallus habit, branching pattern, and pigmentation of the basal part. Additionally, the presence or absence of sorelia and their morphology, along with features like isidia, pseudocyphellae, papillae, tubercles, fibrils, faveolae, and the shape of branches, are also important for identification.

*Anatomy:* Measure the ratio of the thickness of cortex (C), medulla (M), and central axis (A), the compactness of fungal hyphae in the medulla

*Chemistry:* The lichen genus *Usnea* can produce specific primary and secondary metabolites in each species as shown in Table 2.

**Table 2** Primary and secondary substances of *Usnea* spp.

| Chemistry | *Usnea* spp. | References |
|---|---|---|
| **Polysaccharides** | | |
| Lichenan | *U. barbata, U. longissima, U. baileyi* | Shibata et al. (1968) |
| Isolichenin | *U. fasciata* | Periera et al. (1994) |
| **Fatty acid** | | |
| Muronic acid | *U. longissima* | Dobrescu et al. (1993) |
| Lichesterinic acid | *U. longissima* | Dobrescu et al. (1993) |
| Caperatic acid | *U. lapponica, U. angulata, U. nipparensis, U. orientalis, U. florida, U. sinensis* | Culberson et al. (1983) |
| **Depsides** | | |
| Aciculiferin A | *U. aciculifera* | Truong et al. (2014) |
| Atranorin | *U. aciculifera, U.articulata* | Truong et al. (2014); Sultana et al. (2011) |
| Baeomycesic acid | *U. pacificana* | Behara et al. (2012) |
| 4-O-demethylbarbatic acid | *U. longissima* | Okuyama et al. (1995) |
| Barbatic acid | *U. diplotypes, U. fulvoreaquens, U. lapponica, U. pacificana, U. substerilis, U. wasmuthii,* | Nishanth et al. (2015) |

| Chemistry | *Usnea* spp. | References |
|---|---|---|
| | *U. pangiana, U. dendritica, U. fragilis, U. norketti, U. nilgrica, U. certain* | |
| Diffractaic acid | *U. longissima, U. baileyi, U. aciculifera, U. ceratina, U. fulvoreagens, U. diffracta* | Nishanth et al. (2015); Sultana & Afolayan (2011); Okuyama et al. (1995) |
| Evernic acid | *U. madeirensis, U. longissima* | Sultana & Afolayan (2011) |
| Thamnolic acid | *U. subfloridana, U. hirta, U. florida* | Nishanth et al. (2015); Periera et al. (1994) |
| Squamatic acid | *U. pacificana, U. subfloridana, U. fragilescens, U. florida, U. longissima* | Nishanth et al. (2015) |
| 4-O-demethylbarbatic | *U. dendritica, U. longissima* | Koparal & Naturforsch (2015) |
| **Depsidones** | | |
| Menegazziaic acid | *U. aciculifera, U. undulate* | Sultana & Afolayan (2011) |
| Norstictic acid | *U. aciculifera, U. angulate, U.baileyi, U. cornuta, U. flammea, U. fragilescens, U. fulvoreagens, U. hakonensis, U. hirta, U. subfloridana, U. vulneraria, U. wirthi* | Sultana & Afolayan (2011); Truong et al. (2014) |
| Constictic acid | *U. aciculifera* | Koparal & Naturforsch (2015) |
| Protocetraric acid | *U. albopunctata, U. articulta, U. dasaea, U. firmula, U. glabrata, U. maculate, U. madeirensis* | Periera et al. (1994) |
| Psoromic acid | *U. bornmuelleri, U. complanata, U. dasaea, U. inermis, U. pseudosinensis, U. subfloridana* | Nishanth et al. (2015); Periera et al. (1994); Rankovic et al. (2012) |
| Salazinic acid | *U. rubrotincta, U. baileyi, U. trichodeoides, U. pangiana, U. longissima, U. complanata, U. compressa, U. corallina, U. dendritica, U. dasaea, U. himalayana, U. luridorufa, U. norketti,* | Periera et al. (1994); Sultana & Afolayan (2011) |

| Chemistry | *Usnea* spp. | References |
|---|---|---|
| | *U. orientalis, U. pangiana, U. perplexans, U. picta, U. rigidula, U. robusta, U. sordida, U. rubicunda, U. splendens, U. subfloridana, U. trichodeoides, U. undulate* | |
| Galbinic acid | *U. undulata* | Sultana & Afolayan (2011) |
| Stictic acid | *U. aciculifera, U. cornuta, U. flammea, U. frgilescens, U. fulvoreagens, U. bismolliuscula, U. complanata,U. dasaea, U. eumitrioides, U. fischeri, U. himalayana, U. himantodes, U. indica, U. lucea, U. luridorufa, U. picta, U. pectinata, U. nipparensis, U. pseudojaponica, U. rigidula, U. rubicunda, U. spinosula, U. stigmatoides, U. stigmata* | Nishanth et al. (2015); Periera et al. (1994); Troung et al. (2014) |
| **Benzofuran** | | |
| Usnic acid | *U. florida, U. barbata, U. longissima, U. rigida,U. hirta, U. subflorida, U. undulate* | Sultana & Afolayan (2011); Okuyama et al. (1995) |
| **Others** | | |
| Eumitrin B, Eumitrin A2, Eumitrin A1 | *U. baileyi* | Koparal & Naturforsch (2015) |

***Usnea* species in Thailand**

A checklist of lichenized fungi occurring in Thailand (Buaruang, 2017) had reported 13 species of *Usnea*. All species are listed in Table 3 and the morphology and chemical profiles found in lichen have been compiled in Table 4.

**Table 3** A checklist of lichenized fungi genus *Usnea* occurring in Thailand

| Species | Current name | Literature |
|---|---|---|
| *U. australis* Fr. | *U. australis* Fr. | Satô 1962 ; Vainio 1921 ; Wolseley et al., 2002 |

| Species | Current name | Literature |
|---|---|---|
| *U. baileyi* (Stirt.) Zahlbr. | *Eumitria baileyi* Stirt. | Wolseley et al., 2002 |
| *U. baileyi* subsp. *chrysopora* (Stein) Asahina | *Eumitria baileyi* Stirt. | Yoshimura 1978; Wolseley et al., 2002 |
| *U. baileyi* subsp. *septentrionalis* Asahina | *Eumitria baileyi* Stirt. | Yoshimura 1978; Wolseley et al., 2002 |
| *U. cinchonarum* (Fee) Zahlbr. | *U. cinchonarum* (Fee) Zahlbr. | Vainio 1921 |
| *U. dasycera* (Nyl.) Vain. | *U. dasycera* (Nyl.) Vain. | Wolseley et al., 2002 |
| *U. endochroa* var. *papillata* (Vain.) Zahlbr. | *U. endochroa* var. *papillata* (Vain.) Zahlbr. | Wolseley et al., 2002 |
| *U. endorhodina* (Vain.) Zahlbr. | *U. endorhodina* (Vain.) Zahlbr. | Wolseley et al., 2002 |
| *U. florida* (L.) Weber ex F. H. Wigg. | *U. florida* (L.) Weber ex F. H. Wigg. | Vainio 1921 |
| *U. florida* var. *subcomosa* Vain. | *U. florida* var. *subcomosa* Vain. | Wolseley et al., 2002 |
| *U. himantodes* Stirt. | *U. himantodes* Stirt. | Wolseley et al., 2002 |
| *U. hossei* Vain. | *Eumitria pectinata* (Taylor) Articus | Satô 1962 ; Vainio 1921 ; Alava 1988 ; Wolseley et al., 2002 |
| *U. leucospilodea* Nyl. | *U. leucospilodea* Nyl. | Asahina 1969; Yoshimura 1978; Wolseley et al., 2002 |
| *U. luridorufa* Stirt. | *U. luridorufa* Stirt. | Wolseley et al., 2002 |
| *U. neoguineensis* var. *Neoguineensis* Asahina | *U. himantodes* Stirt. | Yoshimura 1978 |
| *U. pectinata* Taylor | *Eumitria pectinata* (Taylor) Articus | Yoshimura 1978; Wolseley et al., 2002 |
| *U. perplexans* Stirt. | *U. perplexans* Stirt. | Wolseley et al., 2002 |
| *U. rubicunda* Stirt. | *U. rubicunda* Stirt. | Yoshimura 1978; Wolseley et al., 2002 |

**Table 4** The morphological and chemical present in genus *Usnea*

| Species | Morphology | Chemistry | References |
|---|---|---|---|
| *U. rubicunda* | Red pigment in cortex, Terminal and subterminal branches not elongated, soralia present mainly on thick branches | Stictic, Salazinic, Norstictic, Menegazziaic, Constictic, Psoromic, 2'-O-demethylpsoromic acids, Atranorin | Ohmura (2012); Truong et al. (2013) |
| *U. baileyi* | Wine red pigment present in the cortex-side of medulla, Serediate, shrubby to sub-pendulous thallus with cylindrical branches, red subcortical pigment | Atranorin, Salazinic, Norstictic, Protocetraric, Constictic, Diffractaic acids, Zeorin, Eumitrin | Ohmura (2012); Temu et al. (2019) |
| *U. pectinata* | Cortex fragile on main branches, Thallus with black to dark brown base, Soralia with short isidiomorphs, dark brown pigmented axis | Stitic, Constictic, Norstictic, Salazinic, Menegazziaic acids | Ohmura (2012); Temu et al. (2019) |
| *U. florida* | Thallus erect to subpendent, apothecia usually present Fibrils straight, Thallus with jet black base, Apothecia present, soredia absent | Usnic, Thamnolic acid | Articus et al. (2002); Ohmura (2012) |
| *U. dasaea* | Erect,Subpendent thallus, 2-15cm long, Inflated branches, Soredia present Isidiomorphs, Pseudocyphellae and Papillae present | Usnic, Psoromic, Norstictic acids | Mariraj et al. (2020) |
| *U. perplexans* | Sorediate present, non-pigmented, isidia absent, pseudocyphellae absent or sparse, slightly orange pigmented in axis | Salazinic acid | Swinscow & Krog. (1979); Clerc (1987) |
| *U. himantodes* | axis reddish brown, terete branches, slight angular cracking, a few papillae along the entire length of the branches, and an ochre- coloured axis | Stictic (major), Constictic (minor), Menegazziaic, Cryptostictic, Nor atictic acids (accessory) | Stevens (1990) |

**Forest in Thailand**

Thailand is located in the tropics. Most of the forests are broad-leaved forests. The classification of forest types varies depending on the distribution of rainfall, the duration of rainfall, and the amount of rainfall. As a result, each forest has different moisture levels. They can be classified into two main types: 1) Evergreen forests 2) Deciduous forests (Figure 6)

1. Evergreen Forests

This type of forest appears evergreen since almost all the trees that grow there are evergreen. The evergreen forest covers about 40 percent of the total forested area. It can be further classified into:

1.1 Tropical Rain Forest (Tropical Evergreen Rain Forest)

This forest is categorized as tropical rainforest located in an area with abundant rainfall throughout the year and high soil moisture. The tropical rainforest has a dense structure consisting of hundreds of species of trees, shrubs, herbal, and epiphytes.

Common species: *Dipterocarpus kerrii, D. grandiflorus, D. gracilis, D. chartaceus, Anisoptera curtisii, Neobalbanocarpus heimii, Cotylelobium lanceolatum, Hopea odorata, H. pedicellata, H. sangal, H. latifolia, Shorea glauca, S. gratissima, S. hypochra.*

1.2 Dry Evergreen Forest

Dry evergreen forest is a closed canopy forest in which the main canopy is predominantly evergreen forest trees with scattered individuals of deciduous trees. In southern Thailand, seasonal dry evergreen forests can be found on sandstone ridges, while in northern Thailand, these forests are limited to riparian galleries amid regions of deciduous forests. In other parts of Thailand, the seasonal dry evergreen forests are integrated into the landscape as part of a forest mosaic (Bunyavejchewin S., 1999).

Common species: *Hopea ferrea, H. odorata, Shorea henryana, S. roxburghii, Dipterocarpus turbinatus, D. alatus, Erythrina subumbrans, Sandoricum koetjape, Artocarpus lacucha, Legerstroemia calyculata, L. balansae, Anisoptera costata, Irvingia malayana.*

1.3 Hill Evergreen Forest

The hill evergreen forest is found on the highland parts (above 1,000 meters from the sea level) of the country where the climatic condition is the Humid Subtropical type. The presence of mosses and lichens on trees and rocks indicates this forest type. The predominant species are oaks and chestnuts.

Common species: *Castanopsis* spp, *Quercus* spp, and *Lithocarpus* spp.

1.4 Pine Forest

Pine forests consist of groups of softwood trees, such as conifers, that grow on the flat-topped sandstone mountains in the northeastern region, typically at an altitude of approximately 1,100-1,300 meters, including Phu Luang and Phu Kradueng.

Common species: *Calocedrus macrolepis*, *Podocarpus neriifolius, P. polytachyus, Dacrydium elatum, Lithocarpus fenestratus*, *Quercus* spp., *Aidia parvifolia, Syzygium oblatum, Nageia motleyi*

1.5 Mangrove Forest:

The mangrove forest has a dominant plant species is *Rhizophora mucronata*, which grows along the muddy coastline. It is found in abundance at the mouths of large rivers and canals that flow into the sea, and along the coastal channels of the Gulf of Thailand and the Andaman Sea on various islands.

Common species: *Avicennia marine, A. alba, Sonnerratia alba, Rhizophora mucronata, R. apiculata, Bruguiera* spp., *Ceriops* spp., *Xylocarpus granatum, Heritiera littoralis*

1.6 Swamp Forest:

Peat swamp forests occur in coastal areas in the lower southern region of Thailand, such as Narathiwat Province, where rainfall is abundant and the area is low-lying. The structure of peat swamp forests that are intact and undisturbed resembles a tropical rainforest, but the species of plants are very different. There are trees of various sizes and the canopy is close together continuously.

Common species: *Calophyllum teysmannii, Blumedendron kurzii, Eleiodoxa conferta, Neesia malayana, Parishia insignis, Elaeocarpus* *macrocerus, Myristica elliptica, Polyalthia lateriflora, P. sclerophylla, Aglaonema* spp. *Cyrtostachys renda, Nenga pumila, Macraranga pruinaos, Malaleuca cajuputi* (Santisuk, 2012)

2. Deciduous Forests

The remaining 60 percent belongs to the Deciduous Forest. which is comprised of species with leafless periods. During the rainy season, the forest appears evergreen, but in the summer, most trees lose their leaves, making the forest clearer and more susceptible to forest fire. The main types of forests can be categorized into:

2.1 Mixed Deciduous Forest

Mixed deciduous forest is a type of tropical seasonal forest found in Thailand. It covers extensive areas and exhibits considerable variation in composition and structure. In the northern regions of the country, this forest type typically occupies riparian zones and gentle slopes at elevations below 500 meters above mean sea level. During the dry season, forest fires can occur (Marod et al., 1999).

Common species: *Acacia tomentosa, A. harmandiana, Flacourtia indica, Cassia fistula, Shorea roxburghii, Millingtonia hortensis, Spondias pinnata, Melia azedarach, Phyllanthus emblica, Pterocarpus macrocarpus, Bambusa bambos, B. vulgaris, Thyrsostachys siamensis, Tectona grandis*

2.2 Deciduous Dipterocarp Forest

Deciduous Dipterocarp Forest is the most extensive forest type in Thailand found in the Northeast about 80% of the total. It is also commonly found in North thailand, both in the plains and ridges elevation range from 150-1300 meters above sea level (Eiadthong, 2009).

Common species: *Dipterocarpus intricatus, D. obtusifolius, D. tuberculatus, Shorea siamensis, S. obtusa, S. roxburghii, Pterocarpus macrocarpus, Sindora siamensis* var. *maritima, Lagerstroemia macrocarpa, Gluta usitata, Codariocalyx motorius, Vietnamosasa ciliata, V. pusilla, Cycas siamensis*

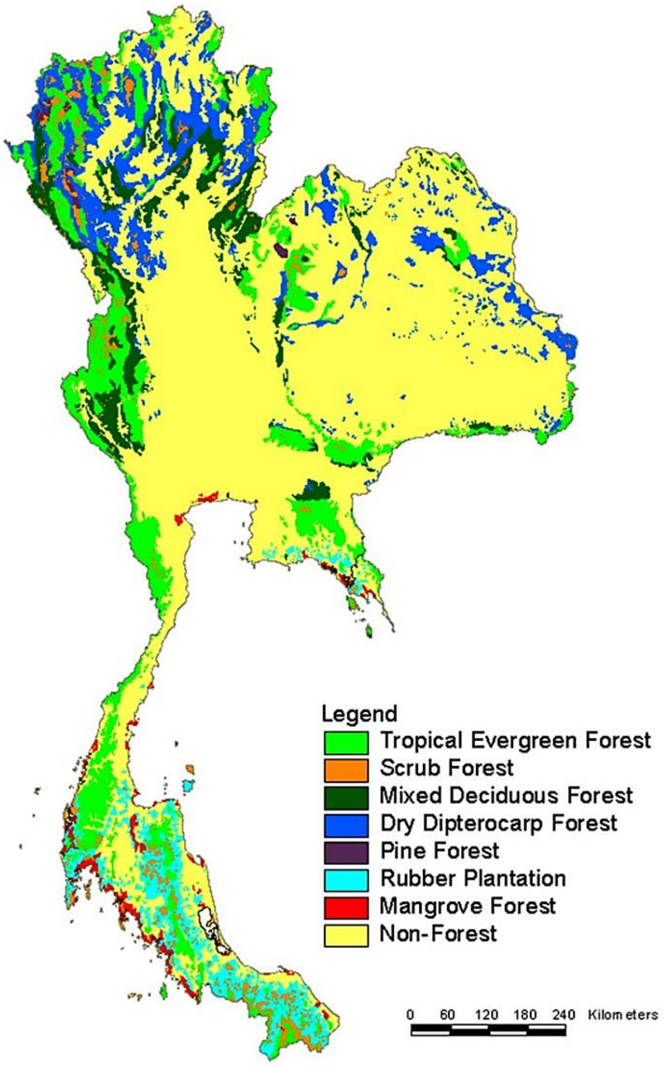


**Figure 6** Forest in Thailand

Ref: Beaver & Liu (2013)

**Forest in Northern Thailand**

the forest in North Thailand is not a rain forest. The tropical islands of Southeast Asia support dense rainforests. In Thailand, you can find rain forests in the South, close to the Malaysian border. In a rain forest, it rains every day: there is no dry season. In North Thailand, the dry season is from November until May. Forest types are evergreen forest, mixed deciduous forest, deciduous bamboo forest, deciduous dipterocarp forest, and pine forest. The dense montane rain forest was the original vegetation of North Thailand. Most of these old-growth forests have disappeared due to logging and swidden agricultural practices. The secondary forest has replaced the old-growth forest in most areas.

Bamboo deciduous dipterocarp, Deciduous trees, and bamboo dominate this type of forest. Deciduous trees lose their leaves seasonally and can be found in this forest type on Doi Suthep and in the Chiang Dao area. Dry deciduous dipterocarp forests are typical of mountainous areas and can be found in all national parks in Northern Thailand. In contrast, hill evergreen forests are located in Northern Thailand at altitudes exceeding 1,000 meters above sea level, just below the summit of Doi Inthanon. Additionally, pine forests are present at similar altitudes on Doi Inthanon as well as in Doi Pui-Doi Suthep and Huay Nam Dang National Parks (Figure 7).


**Figure 7** Forest in Northern Thailand

A) Bamboo deciduous dipterocarp forests B) Dry deciduous dipterocarp forests

C) Hill evergreen forests D) Pine forests

Ref: Green-tails (n.d.)

**Phylogenetic Studies**

Articus et al. (2002) studies the phylogenetic based on ITS and nuLSU regions of Ribosomal DNA and β-tubulin data of two *Usnea* species, *U. florida*, and *U. subfloridana*, together with morphological analysis. The consensus tree shows that both species form a monophyletic clade together. However, the morphological analysis has been separated by different reproductive. Therefore, both results are not corresponding.

Articus (2004) *U. florida* and *U. subflorida* were studied, Thise specimens are different dispersal strategies. Phylogenetic analyses based on data set of rDNA and β-tubulin data. The results of phylogeny show that *U. florida* and *U. subflorida* formed a monophyletic clade. The data in this study do not support a species pair relationship of *U. florida* and *U. subflorida.*

Temu et al. (2019) study phylogeny of the subgenus *Eumitria* in Tanzania based on ITS, nuLSU, *RPB1*, and *MCM7* regions. This research is the first reported for the generated sequence using nuLSU, *RPB1*, and *MCM7* of *U. baileyi.* The molecular data shows that *U. baileyi* and *U. pectinata* belong to *Eumitria* clade which this result is similar to the study of Jannah et al., (2021).

Gauslaa & Timdal (2020) Studies the phylogenetic analyses using the fungal barcode marker, nrITS of *U. rubicunda*. This species is reported as a new species in Scandinavia, southwest Norway. The result shows 99.8 - 100% similarity to specimens from British.

Jannah et al. (2021) study DNA barcoding and phylogenetic analysis to improve identify *Usnea* species collected at Mount Lawu Forest and Turgo Hill Forest based on ITS rDNA. The phylogenetic topology of this study showed that *U. pectinata* is very closely related to *U. baileyi* (Figure 8) and both were classified within the subgenus *Eumitria* clade. Therefore, it can be concluded that *U. pectinata* and *U. baileyi* shared a common ancestor to form one clade. All the foregoing can be summarized in Table 5.

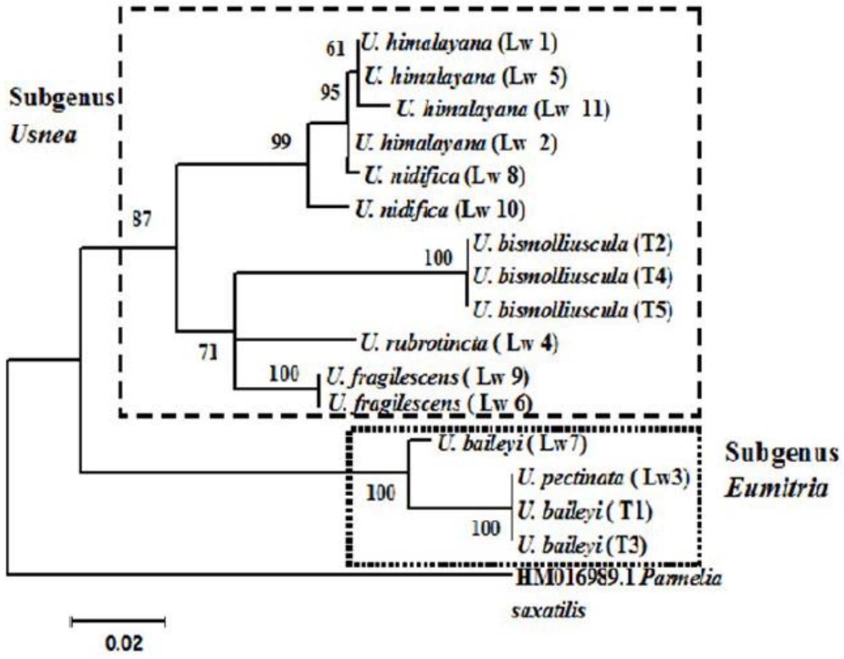


**Figure 8** Phylogenetic analysis of *Usnea* spp. Based on ITS rDNA
Ref: Jannah et al. (2021)

**Table 5** The studies of molecular analysis of *Usnea*

| Species | Country | DNA region | References |
|---|---|---|---|
| *U. florida*, *U. subflorida* | in Europe | ITS, nuLSU | Articus et al. (2002) |
| *U. florida*, *U. subflorida* | - | rDNA, β-tubulin | Articus, K. (2004) |
| *U. baileyi*, *U. pectinata* | Tanzania | ITS, nuLSU, RPB1, MCM7 | Temu et al. (2019) |
| *U. baileyi*, *U. pectinata* | Norway | ITS | Gauslaa & Timdal (2020) |
| *Usnea* sp. | Turkey | ITS rDNA | Jannah et al. (2021) |

**Bioactive Activity Studies**

Madamombe and Afolayan (2003) studied the antimicrobial activity of the *Usnea barbata* extract found in South America. Lichen was extracted with three solvents: acetone, methanol, and water. Ten bacteria and five fungi were tested using the dilution method on an agar culture medium. The extract showed significant inhibition of gram-positive bacteria. The minimum antibacterial concentration was 0.1 mg/ml. *Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus viradans*, and *Staphylococcus aureus* showed the highest potential for acetone extracts. Whereas aqueous extract showed the lowest potential for antimicrobial.

Oran et al. (2016) studied the antibacterial and antioxidant activity of extracts of 3 *Usnea* species, *Usnea intermedia*, *Usnea filipendula*, and *Usnea fulvoreagent*, extracted with three solvents: acetone, ethanol, and methanol. Antioxidants were tested by the ABTS method and total phenolic content was determined by the Folin-Ciocalteu method. The antibacterial efficacy is as follows: acetone > ethanolic > methanolic extracts. The methanolic extracts of 3 *Usnea* sp. had minimum inhibitory concentration (MIC), which was able to inhibit *Escherichia coli* O157:H7 at 64 ug/ml. The ABTS assay showed the highest activity in the methanolic extract of *Usnea fulvoreagent* (181.0 ± 4.4 mg TE/100g dry weight of lichen). While acetone extract of *Usnea filipendula* shows the highest of total phenolic (329 ± 18.5 mg GAE/100 g dry weight of lichen).

Fernández-Moriano et al. (2016) studies the antioxidant activity of the methanolic extract of *U. aurantiacoatra* and *U. contexta.* The studies were evaluated through DPPH, FRAP assays, and total phenolic content. The results exhibited that *U. contexta, and U. aurantiacoatra* displayed the lowest phenolic content. *U. aurantiacoatra* possessed low reducing ferric capacity in the FRAP method and lowest scavenging potential in the DPPH assay.

Jha et al. (2017) studied the inhibitory activity of bacteria and the DPPH-estimated antioxidant potential of a total of 84 lichen samples was studied, classified into 19 genera, 47 species, one of which was *Usnea pectinata* lichen. The samples were extracted with methanolic and dichloromethane (DCM). It was found that could not inhibit the bacteria and antioxidants. On the other hand, found to have very high cytotoxicity more than 80 percent of deaths.

Popovici et al. (2018) Studied the antioxidant activity of *Usnea barbata* evaluated by the scavenger DPPH method. The extract was prepared in acetone, 96% ethanol, and water. The results showed that the ethanolic extract has the greatest antioxidant activity and *Usnea* lichen 200mg/ml extract in ethanol had a DPPH scavenger activity over 50%.

Kocakaya et al. (2024) Evaluated the antioxidant potential of *Usnea florida* (L.) Weber ex F.H. Wigg., *Usnea intermedia* (A. Massal.) Jatta, and *Usnea lapponica* Vain. The Samples were extracted three times for 24 hours with 80% methanol. The results indicated that *U. lapponica* exhibited the highest DPPH radical scavenging activity, whereas the extract that scavenged ABTS radicals more potently was identified as *U. intermedia*.

The summary of the studied antioxidant activities is presented in Table 6.

**Table 6** The studies of antioxidant activity and polyphenol compound

| Species | Solvents | Method | Results | References |
|---|---|---|---|---|
| *U. pectinata* | Methanol, dichloro-methane | DPPH assay | no activity | Jha et al. (2017) |
| *U. imtermedia, U. filipemdula, U. fulvoreagent* | Acetone, ethanol, methanol | ABTS assay, Total phenolic contents | The methanolic extract of *U. fulvoreagent* showed the highest activity. While acetone extract of *U. filipendula* shows the highest total phenolic. | Oran et al. (2016) |

| Species | Solvents | Method | Results | References |
|---|---|---|---|---|
| *U. barbata* | Acetone, 96% ethanol and water | DPPH assay | The ethanolic extract has the greatest antioxidant activity. | Popovici et al. (2018) |
| *U. florida, U. intermedia, U. lapponica* | 80% methanol | DPPH, ABTS assays | *U. lapponica* exhibited the highest DPPH radical scavenging activity and *U. intermedia* had the highest ABTS scavenging | Kocakaya et al. (2024) |
| *U. aurantiacoatra, U. contexta* | Methanol | DPPH, FRAP assays, Total phenolic content | *U. contexta, and U. aurantiacoatra* displayed the lowest phenolic content. *U. aurantiacoatra* possessed low reducing ferric capacity in the FRAP method and lowest scavenging potential in the DPPH assay | Fernández-Moriano et al. (2016) |

Çobanoğlu et al. (2016) study to evaluate the antimicrobial activity of methanolic and chloroform extracts of lichen five species; *Melanohalea exasperata, Physcia aipolia, Usnea florida, Usnea.subfloridana, and Xanthoria parietina*. The chloroform extracts of *Usnea subfloridana* showed the highest activity against

*Escherichia coli* and *Pseudomonas aeruginosa*. The chloroform extracts of *Usnea florida* against *Escherichia coli, Pseudomonas aeruginosa, E. faecalis, Staphylococcus aureus,* and *Candida albicans* while the methanol extracts against *E. faecalis, S. aureus,* and *C. albicans.*

Maulidiyah et al. (2021) Studies on isolation and antibacterial activity of diffractaic acid compound from lichen *Usnea blepharea* Motyka. This specimen was collected from the mountains in South Sulawesi, Indonesia. Acetone was used to extract the lichen and isolate the diffractaic acid using gravity column chromatography. The results showed that the diffractaic acid very strongly inhibits the growth of *S. aureus* and also inhibits *E. coli*.

Popovici et al. (2021) studied on antibacterial and antifungal activity of *Usnea barbata* from Romani using an adapted disc diffusion test method. Bacterial tests include *Staphylococcus aureus*, *Enterococcus casseliflavus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Fungi species there are *Candida albicans* and *Candida parapsilosis*. The results indicated that the extract of *U. barbata* showed the highest level of inhibition of the growth of *Enterococcus casseliflavus* represented Gram-positive bacterium) and the most susceptible gram-negative bacteria was *P. aeruginosa.* The activity on *C. albicans* was slightly higher than *C. parapsilosis.*

Dela Cruz et al. (2023) studied on antimicrobial activities of five *Usnea* species from the Philippines there are *U. baileyi, U. diffracta, U. glabrata, U. longissimi,* and *U. rubicunda*. Microbial tests include *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter cloacae.* The results indicate that the crude extracts of *Usnea* species demonstrate significant in vitro inhibitory activities against standard antibiotic-sensitive *E. faecalis* and multidrug-resistant strains, including methicillin-resistant *S. aureus* and *E. faecalis.*

Adjeng et al. (2023) Studies on antibacterial activity of *Escherichia coli* and *Salmonella typhi* by acetone extract of the lichen *Usnea* sp. The specimen was extracted using acetone three times for 24 hours. The results of the antibacterial test indicate that the extract of *Usnea* sp. can inhibit *E. coli* with an inhibition zone of 21.25 mm at a concentration of 1.25% and 23.08 mm to inhibit *S. typhi.*

The summary of the studied antimicrobial activities is presented in Table 7.

**Table 7** The previous studies of antimicrobial activity from crude extract of *Usnea* spp.

| Lichen species | Bacteria species | Results | References |
|---|---|---|---|
| *U. florida, U. subfloridana* | *E. coli, P. aeruginosa, E. faecalis, S. aereus, C. albicans* | showed the highest activity against all bacterial species | Çobanoğlu et al. (2016) |
| *U. barbata* | *S. aureus*, *E. casseliflavus*, *Streptococcus pneumoniae*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa* | showed the highest inhibition of *E. casseliflavus* and *P. aeruginosa.* | Popovici et al. (2021) |
| *U. baileyi, U. diffracta, U. glabrata, U. longissimi, U. rubicunda.* | *E. faecalis, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, Enterobacter cloacae* | crude extracts of all species demonstrate inhibitory activities against *E. faecalis*, *S.aureus,* and *E. faecalis.* | Dela Cruz et al. (2023) |
| *Usnea* sp. | *E. coli, Salmonella typhi* | the extract can inhibit *E. coli* and *S. typhi* | Adjeng et al. (2023) |
| *U. blepharea* | *S. aureus, E. coli* | the diffractaic acid from *U. blepharea* very strongly inhibits the growth of *S. aureus* and also inhibits *E. coli*. | Maulidiyah et al. (2021) |

# CHAPTER 3
# RESEARCH METHODOLOGY

## The Scope of the Studies

This research study focuses on identifying the species of *Usnea* spp. using morphological, anatomical, chemical characteristics, and molecular analysis. Another part is studying the bioactive activities of lichen crude extract which are antioxidant and antibacterial activities as shown in Figure 9.

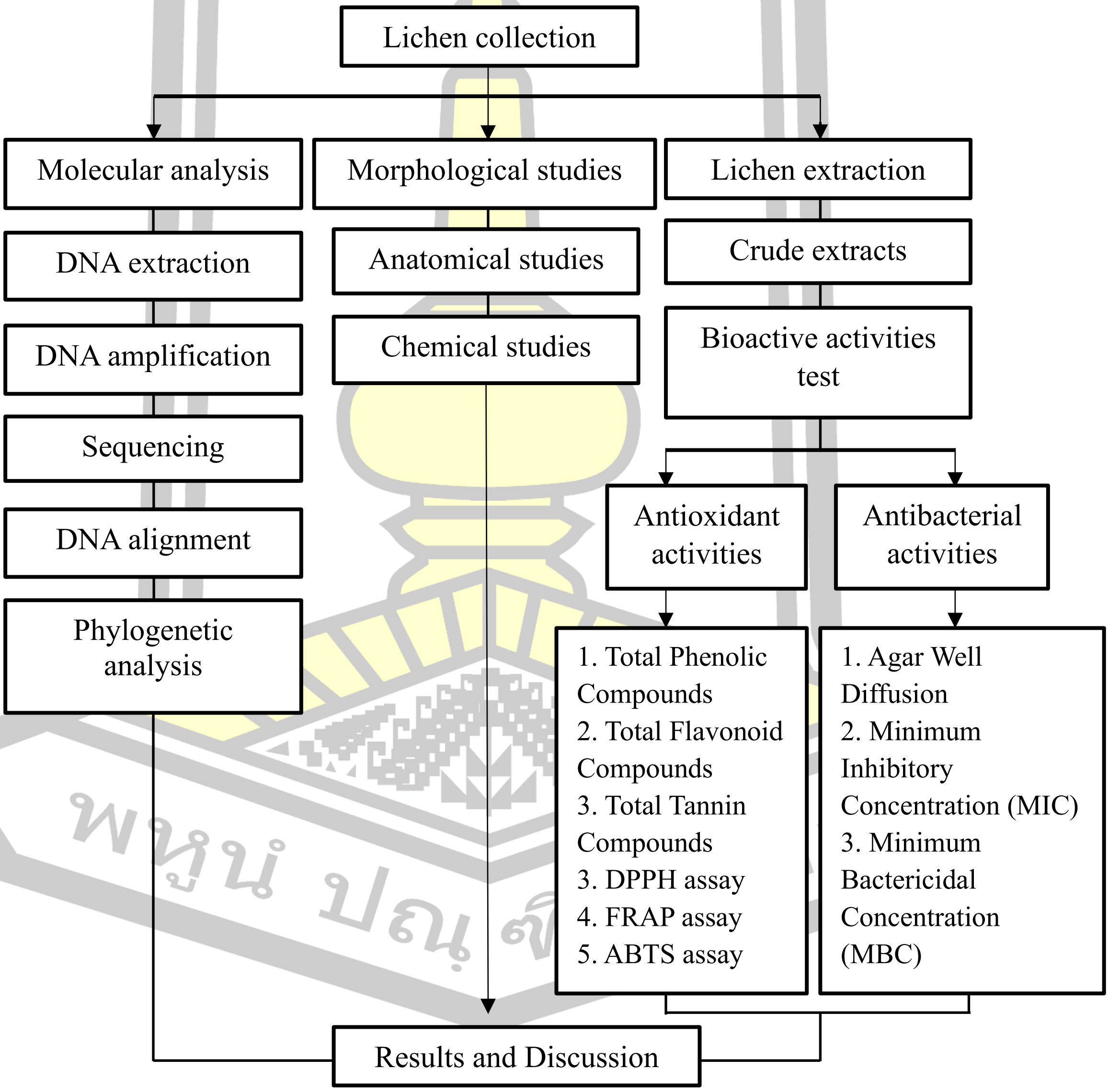


**Figure 9** The scope of the studies

**Instruments and other equipment**

Lichens Specimen, Stereo Microscope, Bright-field Microscope, Blade, Slide and Cover slide, TLC Chamber, TLC Plate, Capillary Tube, Hot Plate, Hot Air Oven, Plant Genomic DNA Extraction Mini Kit (FAVORGEN, USA), Centrifuge, Water Bath, Micropipette, Micropipette Tips, Eppendorf, Pestle, Electrophoresis Tank, Power Supply, Cylinder, Duran Bottle, Spatula, Digital Balance, Applied Biosystems Veriti 96 Well, Thermal Cycler, UV illumination, Petri Dish, Inoculating Loop, Needle, Cork Borer, Autoclave, BSC Class II, Cotton Swab, Microplate Reader, 96 Well Plate, Rotary Evaporator, Freeze Dryer

**Media and Chemicals**

Distilled Water, 1X TBE Buffer, Agarose (Invitrogen, USA), DNA marker, DNA product, Novel Juice Supplied In 6X Loading Buffer (GeneDireX, USA), Acetic acid, Acetone, Diethyl ether, Dioxane, Formic acid, Hexane, Methyl-tert butyl ether, 10% Sulfuric acid, Toluene, Gallic acid (Sigma-Aldrich, Germany), Ascorbic acid (Sigma-Aldrich, Germany), Trolox (Sigma-Aldrich, Switzerland), Quercetin (Sigma-Aldrich, India), Ferrous Sulfate ($FeSO_4$), Folin-Ciocalteu (Merck, Germany), Sodium Carbonate ($NaCO_3$), Aluminum Chloride ($AlCl_3$), Dimethyl Sulfoxide (DMSO), DPPH (Sigma-Aldrich, Germany), Ferric Chloride ($FeCl_2$), TPTZ (Sigma-Aldrich, Switzerland), ABTS (Sigma-Aldrich, China), Hydrochloric (HCl), Sodium Acetate, Gracial Acetic acid, Ethanol, Ethyl acetate, Sodium Chloride (NaCl), Mueller Hinton Agar, Mueller Hinton Broth, *Bacillus cereus* (TISTR 1449), *Staphylococcus epidermidis* TISTR 2162, *Escherichia coli* (TISTR 527), *Pseudomonas aeruginosa* (TISTR 1287), Tetracycline, Ciprofloxacin

**Experimental Design**

**1. Sample Collection**

The sample studies of genus *Usnea* total of 74 specimens (Table 8) were collected in 4 provinces (Figure 10) 9 locations such as Phu Hin Rong Kla National Park (Phitsanulok), Phu Thap Boek (Phetchabun), Mon Long (Chiang Mai), Doi Pui (Chiang Mai), Queen Sirikit Botanic Garden (Chiang Mai), Doi Ang Kang (Chiang

Mai), Hod District (Chiang Mai), Doi Inthanon National Park (Chiang Mai), and Chae Son National Park (Lampang) (Figure 11 and 12). Additional samples were found out of the study site, but it's fresh material and an interesting sample. About 5 specimens were identified in this study (4 specimens from Phu Ruae National Park, Loei Province; 1 specimen from Phu Khiao Wildlife Sanctuary, Chaiyaphum). The map are reconstructed by QGIS version 3.34.14 and details of the geography of each location are shown in Table 9.

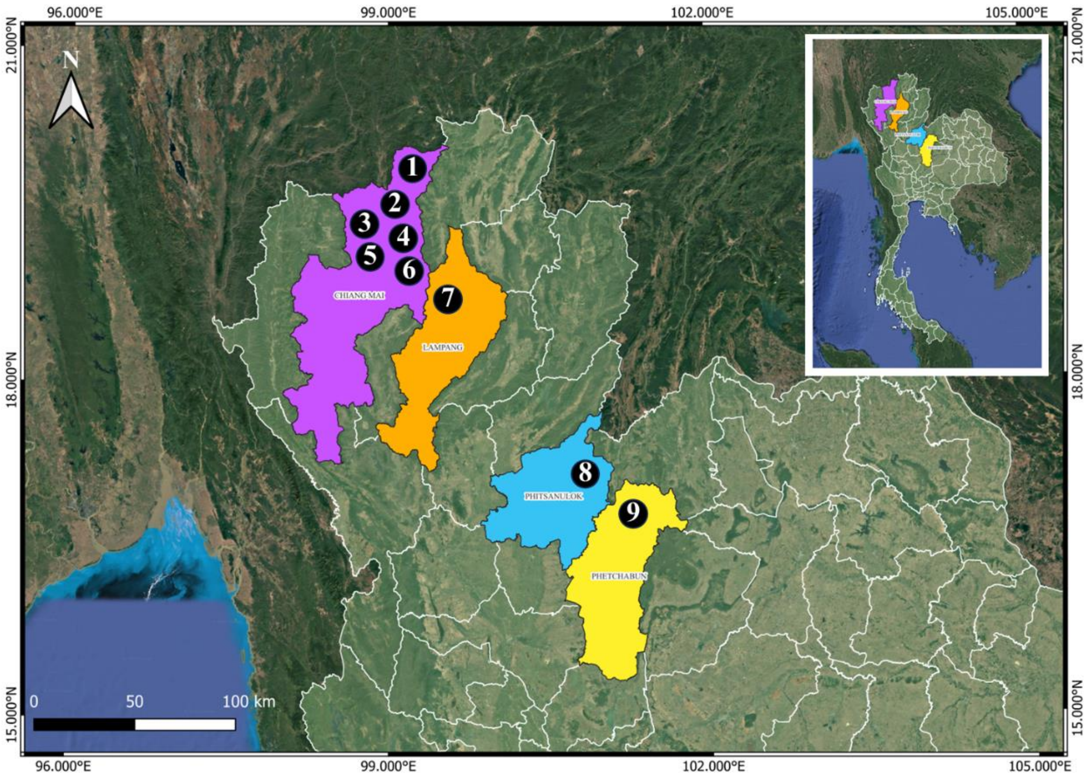


**Figure 10** Sampling sites in Northern Thailand

Notes: Purple = Chaing Mai Province, Orange = Lampang Province, Blue = Phitsanulok Province, Yellow = Phetchabun Province, Red = Loei Province, Green = Chaiyaphum Province, 1 = Doi Inthanon, 2 = Mon Long, 3 = Doi Pui, 4 = Queen Sirikit Botanic Garden, 5 = Doi Ang Kang, 6 = Hod District, 7 = Chae Son National Park, 8 = Phu Hin Rong Kla National Park, 9 = Phu Thap Boek

**Table 8** The specimens studied from various locations

| Specimens | Provinces | Locations |
|---|---|---|
| Fresh specimen | | |
| 1. ML01 | Chiang Mai | Mon Long |
| 2. ML02 | Chiang Mai | Mon Long |
| 3. ML03 | Chiang Mai | Mon Long |
| 4. ML04 | Chiang Mai | Mon Long |
| 5. ML05 | Chiang Mai | Mon Long |
| 6. ML06 | Chiang Mai | Mon Long |
| 7. ML07 | Chiang Mai | Mon Long |
| 8. ML08 | Chiang Mai | Mon Long |
| 9. ML09 | Chiang Mai | Mon Long |
| 10. ML10 | Chiang Mai | Mon Long |
| 11. ML11 | Chiang Mai | Mon Long |
| 12. ML12 | Chiang Mai | Mon Long |
| 13. ML13 | Chiang Mai | Mon Long |
| 14. ML14 | Chiang Mai | Mon Long |
| 15. ML15 | Chiang Mai | Mon Long |
| 16. ML16 | Chiang Mai | Mon Long |
| 17. ML17 | Chiang Mai | Mon Long |
| 18. ML18 | Chiang Mai | Mon Long |
| 19. ML19 | Chiang Mai | Mon Long |
| 20. ML20 | Chiang Mai | Mon Long |
| 21. PH01 | Phitsanulok | Phu Hin Rong Kla National Park |
| 22. PH02 | Phitsanulok | Phu Hin Rong Kla National Park |
| 23. PH03 | Phitsanulok | Phu Hin Rong Kla National Park |
| 24. PH04 | Phitsanulok | Phu Hin Rong Kla National Park |
| 25. PH05 | Phitsanulok | Phu Hin Rong Kla National Park |
| 26. PH06 | Phitsanulok | Phu Hin Rong Kla National Park |
| 27. PH07 | Phitsanulok | Phu Hin Rong Kla National Park |
| 28. PH08 | Phitsanulok | Phu Hin Rong Kla National Park |

| Specimens | Provinces | Locations |
|---|---|---|
| 29. PT01 | Phetchabun | Phu Thab Buek |
| 30. PT02 | Phetchabun | Phu Thab Buek |
| 31. PT03 | Phetchabun | Phu Thab Buek |
| 32. PT04 | Phetchabun | Phu Thab Buek |
| 33. PT05 | Phetchabun | Phu Thab Buek |
| 34. PT06 | Phetchabun | Phu Thab Buek |
| 35. PT07 | Phetchabun | Phu Thab Buek |
| 36. PT08 | Phetchabun | Phu Thab Buek |
| 37. PT09 | Phetchabun | Phu Thab Buek |
| Dried Specimens | | |
| 38. RAMK035261 | Chiang Mai | Queen Sirikit Botanic Garden |
| 39. RAMK035328 | Chiang Mai | Queen Sirikit Botanic Garden |
| 40. RAMK035437 | Chiang Mai | Queen Sirikit Botanic Garden |
| 41. RAMK035457 | Chiang Mai | Queen Sirikit Botanic Garden |
| 42. RAMK035462 | Chiang Mai | Queen Sirikit Botanic Garden |
| 43. RAMK035468 | Chiang Mai | Queen Sirikit Botanic Garden |
| 44. RAMK035744 | Chiang Mai | Queen Sirikit Botanic Garden |
| 45. RAMK040350 | Chiang Mai | Queen Sirikit Botanic Garden |
| 46. RAMK040353 | Chiang Mai | Queen Sirikit Botanic Garden |
| 47. RAMK040355 | Chiang Mai | Queen Sirikit Botanic Garden |
| 48. RAMK040357 | Chiang Mai | Queen Sirikit Botanic Garden |
| 49. RAMK040362 | Chiang Mai | Queen Sirikit Botanic Garden |
| 50. RAMK040372 | Chiang Mai | Queen Sirikit Botanic Garden |
| 51. RAMK025749 | Chiang Mai | Doi Ang Kang |
| 52. RAMK025747 | Chiang Mai | Hod District |
| 53. RAMK040354 | Chiang Mai | Doi Inthanon National Park |
| 54. RAMK040358 | Chiang Mai | Doi Inthanon National Park |
| 55. RAMK040367 | Chiang Mai | Doi Inthanon National Park |
| 56. RAMK040369 | Chiang Mai | Doi Inthanon National Park |
| 57. RAMK040368 | Lampang | Chae Son National Park |

| Specimens | Provinces | Locations |
|---|---|---|
| 58. RAMK040351 | Phitsanulok | Phu Hin Rong Kla National Park |
| 59. RAMK040352 | Phitsanulok | Phu Hin Rong Kla National Park |
| 60. RAMK040356 | Phitsanulok | Phu Hin Rong Kla National Park |
| 61. RAMK040359 | Phitsanulok | Phu Hin Rong Kla National Park |
| 62. RAMK040360 | Phitsanulok | Phu Hin Rong Kla National Park |
| 63. RAMK040361 | Phitsanulok | Phu Hin Rong Kla National Park |
| 64. RAMK040363 | Phitsanulok | Phu Hin Rong Kla National Park |
| 65. RAMK040364 | Phitsanulok | Phu Hin Rong Kla National Park |
| 66. RAMK040365 | Phitsanulok | Phu Hin Rong Kla National Park |
| 67. RAMK040366 | Phitsanulok | Phu Hin Rong Kla National Park |
| 68. RAMK040370 | Phitsanulok | Phu Hin Rong Kla National Park |
| 69. RAMK040371 | Phitsanulok | Phu Hin Rong Kla National Park |
| Additional Locations | | |
| 70. PR01 | Loei | Phu Ruea National Park |
| 71. PR02 | Loei | Phu Ruea National Park |
| 72. PR03 | Loei | Phu Ruea National Park |
| 73. PR04 | Loei | Phu Ruea National Park |
| 74. PK01 | Chaiyaphum | Phu Khiao Wildlife Sanctuary |

The details of the sample were recorded in a field as well as the habitat, sea level elevation of the area, location, collected date, collector, and other details of the sample. Take all samples that were collected to dry or incubate in a hot air oven. Keep it in a paper wrapper to use in a further experiment.

**Table 9** Detailed of sampling sites

| Province | Detailed |
|---|---|
| Chaing Mai | Mon Long is the highest mountain in Mae Rim District, Chiang Mai Province, with a height of approximately 1,450 meters above sea level. The forest types are hill evergreen forests, and pine forests. (Thailand Tourism Directory, n.d.) |
| Chaing Mai | Doi Pui is a pine forest area with abundant forest areas, plants, and birds. The area is mountainous at 1,685 meters above sea level. It is part of Doi Suthep-Pui National Park in Chiang Mai Province. It is a complex mountainous area in the Thanon Thongchai mountain range. Doi Pui is full of large trees, pine forests, and hill evergreen forests. (Office of Natural Resources and Environmental Policy and Planning, n.d.) |
| Chiang Mai | The general geography of Queen Sirikit Botanic Garden is located at the terminal of Doi Suthep-Pui National Park, on the mountain range that runs along the western side of Chiang Mai Province. The topology of this area is a complex mountain range. The highest of the area is approximately 550-1,270 meters above sea level. It has many types of forests including evergreen forests, deciduous forests, and semi-evergreen forests. (CMU Intellectual Repository, n.d.) |
| Chiang Mai | Doi Ang Khang is located in the Dan Lao Mountain range, Mae Ngon sub-district, Fang district, Chiang Mai province. The topography of Doi Ang Khang is part of the Dan Lao Mountain range, which is a mountain with the shape of a long valley. It is a high and complex mountain range with an average height of 1,400 meters above sea level and the highest peak is 1,928 meters. The vegetation is mostly temperate plants and fruit trees. (Office of Natural Resources and Environmental Policy and Planning, n.d.) |

| Province | Detailed |
| --- | --- |
| Chiang Mai | Hod District is a district in Chiang Mai Province located in the southern part of the province. Most of its area is mountainous. It has a variety of landscapes. The general characteristics are forests and high mountains, most of which are conserved forests and national parks. The forest areas are rich in pine forests and dry dipterocarp forests. |
| Chiang Mai | Doi Inthanon National Park covers the areas of Chom Thong District, Mae Chaem District, Mae Wang District, and Doi Lor District, Chiang Mai Province. The general topography consists of complex mountains, with Doi Inthanon as the highest peak, at 2,565 meters above sea level. There are many types of forests, such as tropical rainforests, pine forests, dry dipterocarp forests, and mixed deciduous forests. (Thai Heritage, n.d.) |
| Lampang | Chae Son National Park, the topography is a complex mountain range. It is part of the Phi Pan Nam West Ridge, stretching from north to southwest, continuing south to Mae Phrik District, which is the boundary between Lampang Province, Chiang Mai Province, and Lamphun Province. It has an elevation of 300-2,031 meters above sea level. There are various types of forest, including mixed deciduous forests, dry dipterocarp forests, hill evergreen forests, pine forests, and moist evergreen forests. (National Park Thailand, 2024) |
| Phitsanulok | Phu Hin Rong Kla National Park is a large national park covering the border of two provinces, Dan Sai District, Loei Province, and Nakhon Thai District, Phitsanulok Province. It is approximately 1,820 meters above sea level. The landscape is a complex mountain range, rich in nature, covered with three types of forest: dry dipterocarp forest, hill evergreen forest, and hill pine forest. (Thawatchai Santisuk, 2012) |

| Province | Detailed |
|---|---|
| Phetchabun | Phu Thap Boek is the highest peak in Phetchabun Province, with the highest approximately 1,768 meters above sea level. The landscape changes from a plain to a sandstone mountain. The east and south border Lom Sak District and Khao Kho District. The north and west border Loei Province. Forest types are found in this area such as dry dipterocarp forest, hill evergreen forest, and hill pine forest. (Thailand Tourism Directory, n.d.) |
| Additional Sites | |
| Loei | Phu Ruea National Park, the topography is a complex high mountain range consisting mostly of sandstone mountains, with some granite mountains interspersed. This creates a plateau alternating with high peaks. The highest peak is Phu Ruea, at 1,365 meters above sea level. Phu Ruea has many mixed forests, including mixed deciduous forests, dry dipterocarp forests, tropical forests, and pine forests. Phu Ruea's peak consists of pine forests alternating with natural rock gardens interspersed with low bushes and grasslands at intervals. (Loei Province, n.d.) |
| Chaiyaphum | Phu Khiao Wildlife Sanctuary, the topology of Phu Khiao Wildlife Sanctuary is a mountain surrounding the central highland and the highest peak is approximately 1,310 meters above sea level. Forest types found in this area include hill evergreen forest, dry evergreen forest, deciduous dipterocarp forest, deciduous dipterocarp-pine forest, hill pine forest, mixed deciduous forest, and moist evergreen forest. (National Park, Wildlife and Plant Conservation Department, n.d.) |


**Figure 11** Sampling sites in Chiang Mai A) Mon Long (Thailand Tourism Directory, n.d.) B) Doi Pui (Office of Natural Resources and Environmental Policy and Planning, n.d.) C) Queen Sirikit Botanic Garden (CMU Intellectual Repository, n.d.) D) Doi Ang Kang (Office of Natural Resources and Environmental Policy and Planning, n.d.) E) Hod District and F) Doi Inthanon National Park (Thai Heritage, n.d.)


**Figure 12** Sampling sites A) Chae Son National Park, Lampang Province (National Park Thailand, 2024) B) Phu Hin Rong Kla National Park, Phitsanulok Province (Thawatchai Santisuk, 2012) C) Phu Thap Boek, Phetchabun Province (Thailand Tourism Directory, n.d.) D) Phu Ruea National Park, Loei Province (Loei Province, n.d.) E) Phu Khiao Wildlife Sanctuary, Chaiyaphum Province (National Park, Wildlife and Plant Conservation Department, n.d.)

## 2. Morphological Analysis

The morphological characteristics of the *Usnea* were performed using a Stereo microscope. The sample was investigated for the components of the lichens including the central cord, base of the thallus, soredia, isidia, apothecia, pseudocyphellae, papillae, hyphae in the medulla, pigment, main branch, fibril, and lateral branches. (Figure 13).

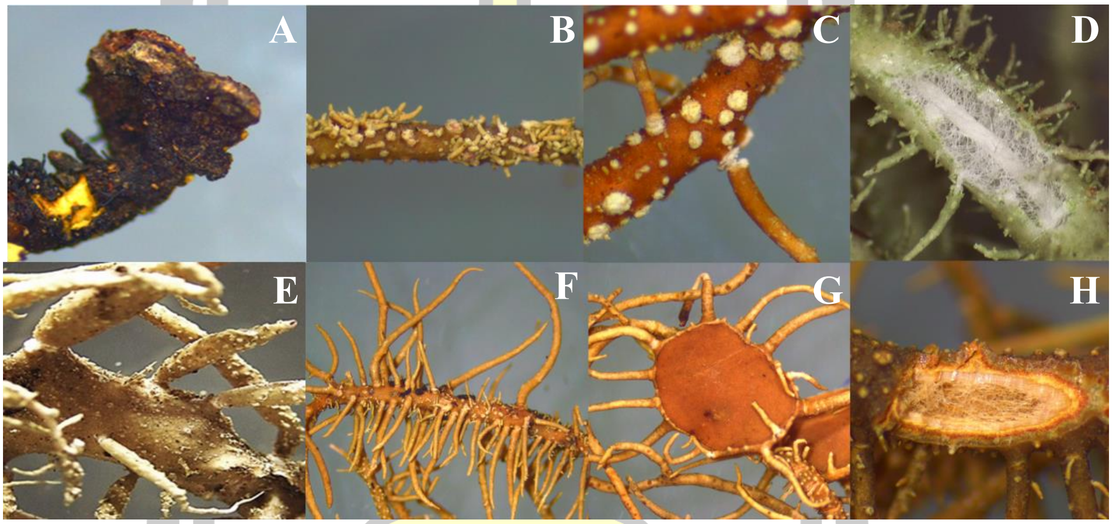


**Figure 13** The morphological characteristic used in the studies
A) Base of thallus B) Isidia C) Soredia D) Thallus layer E) Shape of branch F) Fibrils G) Apothecia H) Pigment in thallus
Ref: (Areerat, 2023)

## 3. Anatomical Analysis

The anatomical characteristics of the *Usnea* were performed using a Bright-field microscope with a hand cross-section by using the blade (Figure 14). Mounting the slide with the wet-mount technique. The character of the cortex, medulla, axis, the ratio of CMA (cortex, medulla, axis), and pigment in the fungal layer were investigated.

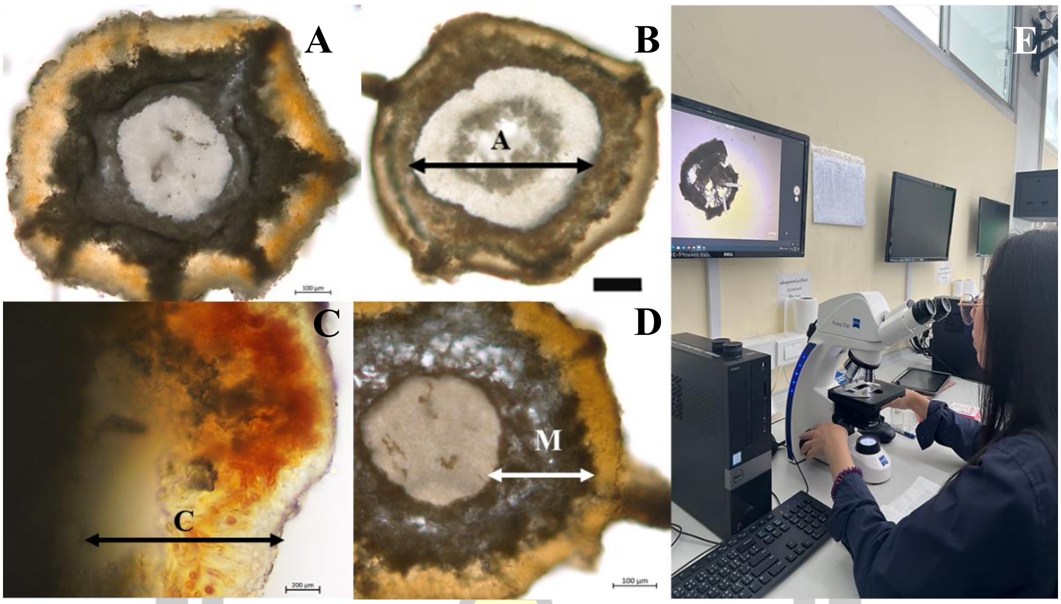


**Figure 14** Anatomical analysis

A) Thallus cross-section B) Axis C) Cortex D) Medulla

E) The study of anatomy by using a bright-field microscope

Ref: (Areerat, 2023)

**4. Chemical Analysis**

Lichen substances were investigated by High-Performance Thin Layer Chromatography (HPTLC) by following of Ohmura (2020) and Huneck & Yoshimura (1996). Solvent A, B, and C systems were used to analyze the lichen substances (Solvent A; Toluene: dioxane: acetic acid, 90: 25: 4, Solvent B; hexane: methyl tert-butyl ether: formic acid, 140: 72: 18, and Solvent C; n-Hexane: diethyl ether: formic acid = 5: 4: 1).

The extraction of lichen substances using acetone as a solvent and macerated for 24 hours. The analysis began by dropping the substance onto the TLC plate with a Capillary tube of about 50 µl. The TLC plate was dried at room temperature, then the plate was put into the TLC chamber with the mobile phase (systems A, C, and G). Bring the plate out from the chamber and let it dry again. After that, check under the UV illuminator with a wavelength of 245 and 365 nm. The TLC plate was sprayed with 10% sulfuric acid and then warmed in the hot air oven at 110 °C for 3-5 min. The color appears when provided under hot conditions. Finally, the Rf value was calculated and compared with the standard shown in Figure 15.

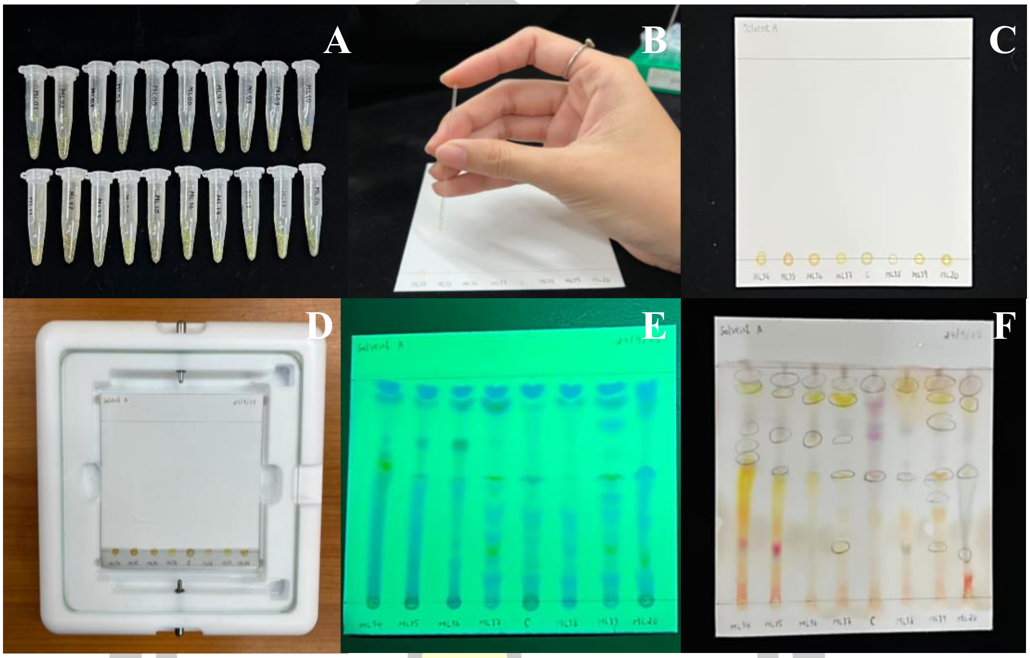


**Figure 15** Chemical analysis using High-Performance Thin Layer Chromatography A) Sample in acetone B-C) Drop sample on TLC plate D) TLC plate on the chamber with mobile phase E) Check under the UV illuminator with a wavelength of 245 and 365 nm F) Presence of secondary substance on TLC plate

Ref: (Areerat, 2023)

**5. Molecular Analysis**

Genomic DNA was extracted from the thallus of *Usnea* using a Plant Genomic DNA Extraction Mini Kit (FAVORGEN, USA) followed by the manufacturer's instructions. PCR amplification of the DNA product using Applied Biosystems Veriti 96 Well Thermal Cycler. The ITS region was used to amplify and performed using ITS1F/ITS4 as a primer. Total PCR reaction volume 50 µl includes 21 µl Nu-Free $H_2O$, 25 µl Taq DNA Polymerase (ONEPCR), 1 µl ITS1F (10 pmol/ µl), 1 µl ITS4 (10 pmol/ µl), and 2 µl genomic DNA. It was performed with an initial denaturation for 5 min at 94°C, followed by 39 cycles of 94°C for 30 s, 48 °C for 30 s, 72°C 1.30 min, and a final elongation step at 72°C for 5 min. The quality of the DNA product was examined using 2% agarose gel with 1X TBE buffer, setting the power supply to 100 volts for 30 min, and visualized through UV illumination and gelLITE Gel Documentation System. The sequencing process was carried out at Macrogen, Inc. in a South Korea (Figure 16).

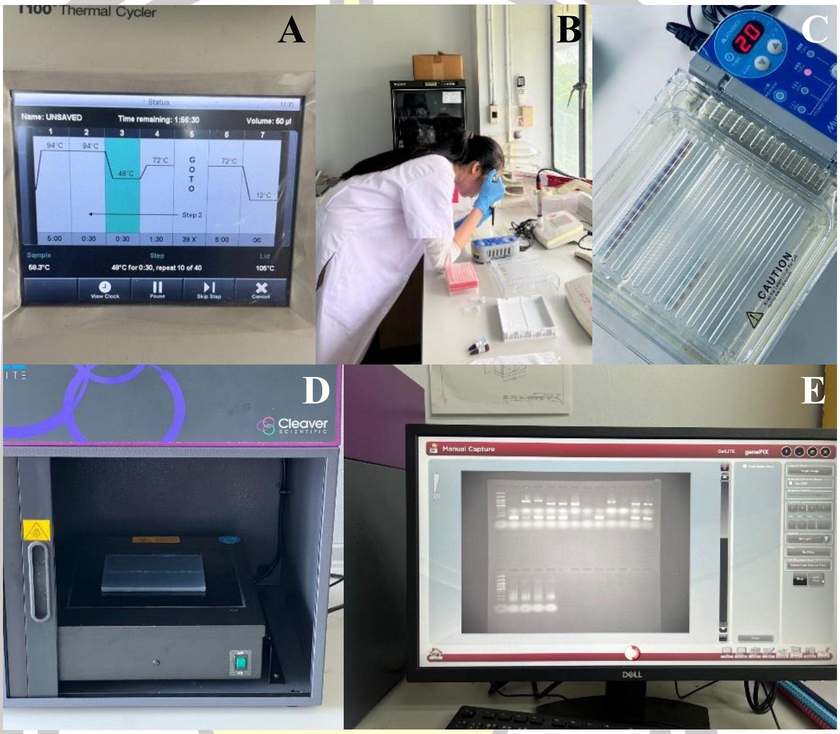


**Figure 16** DNA extraction

A) PCR amplification B) DNA loading C) DNA gel check D) Visualized through UV illumination E) DNA band

Ref: (Areerat, 2024)

The ITS rDNA sequences were analyzed and edited using BioEdit V. 7.2.5.0 and Chromas. The homology level was determined through BLAST online. The sequence alignment was analyzed using MEGA-X. The phylogenetic tree reconstruction was carried out using Maximum Likelihood (ML) and Neighbour Joining (NJ) with the Kimura 2-parameter model and 1000 bootstrap replications on MEGA-X. The genetic distance was analyzed using the Pairwise distance method in MEGA-X compared with twelve ITS rDNA sequences downloaded from the NCBI GenBank database (Table 10).

**Table 10** Voucher and accession number of *Usnea* spp.

| Specimens | Voucher | Accession No. |
|---|---|---|
| *U. aciculifera* | Japan; Y. Ohmura 4400 (TNS) | AB051049 |
| *U. aciculifera* | Taiwan; TNM L00004752 | FJ494923 |
| *U. aciculifera* | 5 | OM808704 |
| *U. aciculifera* | Thailand; Areerat | This study |
| *U. dasaea* | Japan; Y. Ohmura 2842 (TNS) | AB051056 |
| *U. dasaea* | Philippines; PU09 | OQ591849 |
| *U. bismolliuscula* | Japan; YO11225 | OQ591837 |
| *U. ceratina* | Taiwan; TNM L00004733 | FJ494928 |
| *U. ceratina* | Brazil; ceratina_143BR | MF669813 |
| *U. rubicunda* | Norway; O-L-227232 | MW174764 |
| *U. rubicunda* | Japan; TNS:YO:5826B | AB368487 |
| *Pleurosticta acetabulum* | Czech Republic; PRA-Vondrak26105 | OQ717576 |

## 6. Antioxidant Activities

### 6.1 Lichen extraction

The sample that collected up to 10 g was selected for extraction, including *U. himantodes* and *Usnea* cf. *pectinata*. They were dried in the hot air oven at 60 °C and were cut into small pieces to increase the surface area in contact with the solvent to improve the extraction efficiency with organic solvents. Then, the sample was extracted in the ratio of 10 g sample/150 ml solvent. The extraction is divided into two types: Maceration and boiling water. Three solvents, 95% ethanol, ethyl acetate, and acetone, were used for maceration for 24 hours, and distilled water was used to boil for 30 min. After that, the extract was filtered into the Duran bottle using filter paper No.1 and repeated three times. The extract solution was evaporated by using the Rotary Evaporator, and the samples were dried to powder using a Freeze dryer. A crude extract was obtained that looks like a powder and stored for further bioactivity testing (shown in Figure 17).

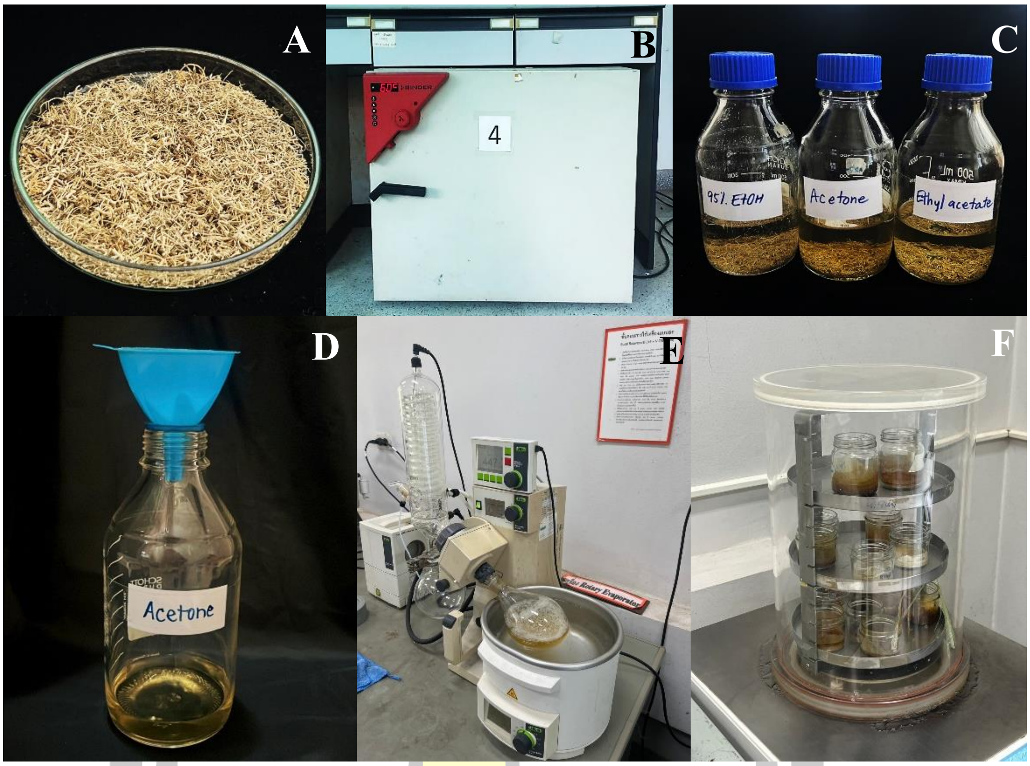


**Figure 17** Lichen substance extraction

A) Lichen sample B) Dry in hot air oven C) Extraction D) Filtration

E) Evaporation F) Freeze dry

Ref: (Areerat, 2023)

**6.2 Total Phenolic Compounds**

6.2.1 Preparation of 7.5% sodium carbonate solution

7.5% sodium carbonate solution was prepared by weighing $Na_2CO_3$ with 7.5 g, dissolving it in distilled water, and adjusting the volume to 100 ml.

6.2.2 Preparation of 10% Folin-Ciocalteu reagent

10% Folin-Ciocalteu reagent was prepared by mixing 10 ml of Folin-Ciocalteu reagent and adjusting the volume to reach 100 ml with distilled water.

6.2.3 Preparation of Gallic acid standard solution

The standard of Gallic acid was prepared to a concentration of 0.1 mg/ml in 10 ml by weighing 0.001 g, dissolving with distilled water, and adjusting the volume to complete 10 ml.

6.2.4 Graphing the standard curve of Gallic acid

The stock solution of Gallic acid was diluted to a concentration of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml for 50 µl. Distilled water was added to the well for 75 µl. Then, 10% Folin reagent was added for 25 µl, and 7.5% $Na_2CO_3$ was added for 50 µl. The microplate was shaken and incubated in the dark for 45 minutes. The absorbance was measured at a wavelength of 765 nm ($A_{765}$) using a Microplate Reader (Figure 18). The standard curve of Gallic acid was constructed using concentration (x) and absorbance (y).

6.2.5 Total phenolic content analysis in the sample extract.

The stock solution of the sample was diluted to a concentration of 0.5 and 1 mg/ml for 50 µl. Distilled water was added to the well for 75 µl. Then, 10% Folin reagent was added for 25 µl, and 7.5% $Na_2CO_3$ was added for 50 µl. The microplate was shaken and incubated in the dark for 45 minutes. The absorbance was measured at a wavelength of 765 nm ($A_{765}$) using a Microplate Reader. The content of total phenolic was calculated by comparing with the standard curve of Gallic acid and reported in mg Gallic acid/ g Extract (Rattana & Sungthong, 2017; Lertcanawanichakul, Chawawisit & Hiransai, 2019).


**Figure 18** Total phenolic content analysis using microplate reader

Ref: (Areerat, 2023)

**6.3 Total Flavonoid Compounds**

6.3.1 Preparation of 2% Aluminum chloride solution

2% Aluminum chloride solution was prepared by weighing $AlCl_3$ with 2 g, dissolving it with distilled water, and adjusting the volume to 100 ml.

6.3.2 Preparation of Quercetin Standard Solution

The standard of Quercetin was prepared to a concentration of 0.1 mg/ml for 10 ml by weighing 0.001 g, dissolving with distilled water, and adjusting the volume to 10 ml.

6.3.3 Graphing the standard curve of Quercetin

The stock solution of Quercetin was diluted to a concentration of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml for 80 µl. 2% $AlCl_3$ was added to the well for 50 µl. The microplate was shaken and incubated in the dark for 10 minutes. The absorbance was measured at a wavelength of 425 nm ($A_{425}$) using a Microplate Reader (Figure 19). The standard curve of Quercetin was constructed using concentration (x) and absorbance (y).

6.3.4 Total flavonoid content analysis in the sample extract.

The stock solution of Quercetin was diluted to a concentration of 0.5 and 1 mg/ml for 80 µl. 2% $AlCl_3$ was added to the well for 50 µl. The microplate was shaken and incubated in the dark for 10 minutes. The absorbance was measured at a wavelength of 425 nm ($A_{425}$) using a Microplate Reader. The content of total flavonoid was calculated by comparing with the standard curve of Quercetin and reported in mg Quercetin/ g Extract. (Daupor et al., 2017; Phonprapai and Oontawee, 2019).

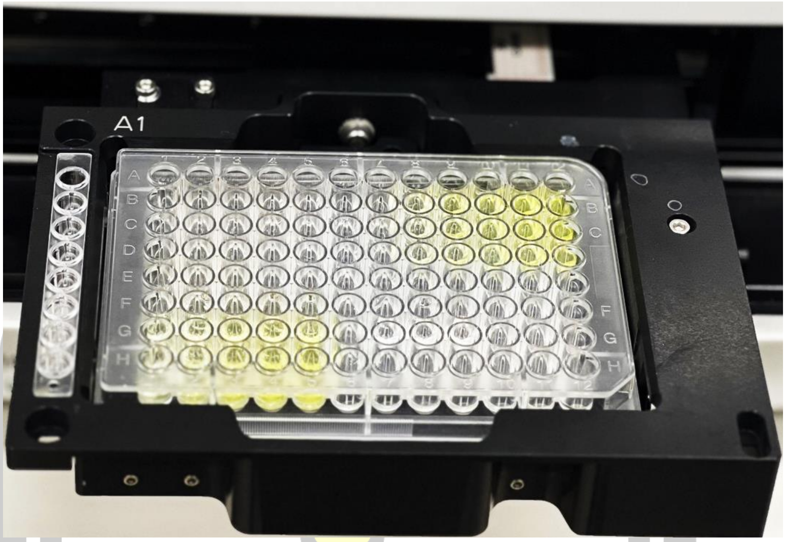

**Figure 19** Total flavonoid content analysis using microplate reader

Ref: (Areerat, 2023)

**6.4 Total Tannin Compounds**

6.4.1 Preparation of 7.5% sodium carbonate solution

7.5% sodium carbonate solution was prepared by weighing $Na_2CO_3$ with 7.5 g, dissolving it in distilled water, and adjusting the volume to 100 ml.

6.4.2 Preparation of 10% Folin-Ciocalteu reagent

10% Folin-Ciocalteu reagent was prepared by mixing 10 ml of Folin-Ciocalteu reagent and adjusting the volume to reach 100 ml with distilled water.

6.4.3 Preparation of Tannic acid standard solution

The standard solution of Tannic acid was prepared to a concentration of 0.1 mg/ml in 10 ml by weighing 0.001 g, dissolving in distilled water, and adjusting the volume to complete 10 ml.

6.4.4 Graphing the standard curve of Tannic acid

The stock solution of Tannic acid was diluted to a concentration of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml for 50 µl. Distilled water was added to the well for 75 µl. Then, 10% Folin reagent was added for 25 µl, and 7.5% $Na_2CO_3$ was added for 50 µl. The microplate was shaken and incubated in the dark for 45 minutes. The absorbance was measured at a wavelength of 765 nm ($A_{765}$) using a Microplate Reader (Figure 20). The standard curve of Tannic acid was constructed using concentration (x) and absorbance (y).

6.4.5 Total tannin content analysis in the sample extract.

The stock solution of the sample was diluted to a concentration of 0.5 and 1 mg/ml for 50 µl. Distilled water was added to the well for 75 µl. Then, 10% Folin reagent was added for 25 µl, and 7.5% $Na_2CO_3$ was added for 50 µl. The microplate was shaken and incubated in the dark for 45 minutes. The absorbance was measured at a wavelength of 765 nm ($A_{765}$) using a Microplate Reader. The content of tannin was calculated by comparing with the standard curve of Tannic acid and reported in mg Tannic acid/ g Extract (Rattana & Sungthong, 2017; Lertcanawanichakul, Chawawisit & Hiransai, 2019).

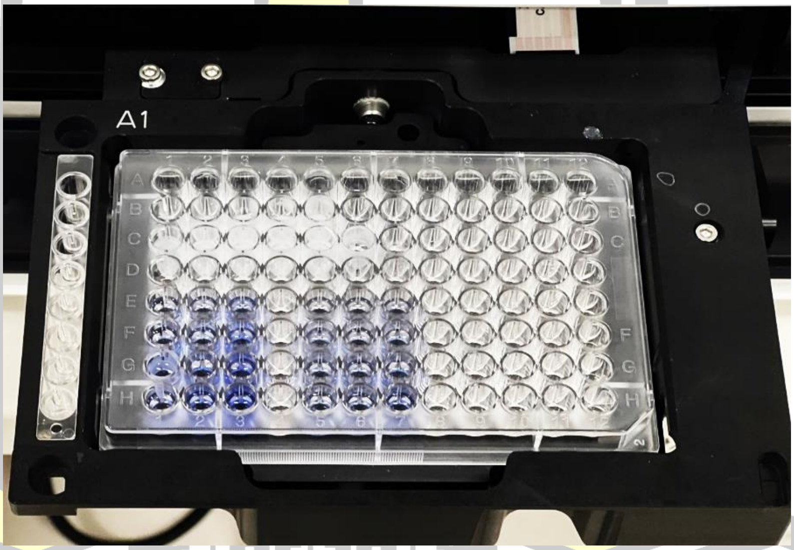


**Figure 20** Total tannin content analysis using microplate reader

Ref: (Areerat, 2023)

**6.5 DPPH assay (2,2-diphenyl-1-picrylhydrazyl)**

6.5.1 Preparation of 0.2 mM DPPH solution

The DPPH reagent was prepared by weighing 0.0039 g and dissolving it in 50 ml of ethanol.

6.5.2 Preparation of Ascorbic acid standard solution

The stock solution of Ascorbic acid was prepared to a concentration of 0.1 mg/ml by weighing 0.001 g of ascorbic acid, dissolving in ethanol, and adjusting the volume to 10 ml.

6.5.3 Ascorbic acid standardization graph

The stock solution of Ascorbic acid was diluted to a concentration of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml, volume 150 µl with distilled water. DPPH reagent was added to the well for 75 µl. The microplate was shaken and incubated in the dark for 30 minutes. The absorbance was measured at a wavelength of 516 nm ($A_{516}$) using a Microplate Reader. The % radical scavenging was calculated using the equation mentioned above, and the standard curve of Ascorbic acid was constructed using concentration (x) and % radical scavenging (y). The $IC_{50}$ value was calculated from the linear equation of the standard curve.

6.5.4 Antioxidant capacity analysis in sample extracts

The stock solution of the sample was prepared by weighing 0.1 g extract and dissolving it with 10 ml of the solvent used for extraction, and diluted to a concentration of 0, 1, 2, 3, 4, and 5 mg/ml, volume 150 µl. DPPH reagent was added to the well for 75 µl. The microplate was shaken and incubated in the dark for 30 minutes. The absorbance was measured at a wavelength of 516 nm ($A_{516}$) using a Microplate Reader (Figure 21. The % radical scavenging was calculated using the equation above, and the sample curve was constructed using concentration (x) and % radical scavenging (y). The $IC_{50}$ value was calculated from the linear equation of the sample curve. (Wannawet & Thiangphet, 2017; Lertcanawanichakul, Chawawisit & Hiransai, 2019).

$$\%\ \text{Radical scavenging} = 1 - \left(\frac{\text{O.D. sample}}{\text{O.D. control}}\right) x\ 100$$


**Figure 21** Antioxidant analysis of DPPH assay using microplate reader
Ref: (Areerat, 2023)

**6.6 ABTS assay [2,2’-azino bis-(3-ethylbenzothiazoline-6-sulfonate)]**

6.6.1 Preparation of 7 mM ABTS solution

The ABTS reagent was prepared to a concentration of 7 mM and 2.45 mM potassium persulfate ($K_2S_2O_8$) at a ratio of 1:2. ABTS and potassium persulfate were mixed and incubated for 12-16 hours. The absorbance was measured and adjusted to 0.7±0.02 before use.

6.6.2 Preparation of Trolox Standard Solution

The standard solution of Trolox was prepared for 10 ml of 0.1 mg/ml by weighing 0.001 g Trolox, dissolving in ethanol, and adjusting the volume to 10 ml.

6.6.3 Trolox standardization graph

The stock solution of Trolox was diluted to a concentration of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml, volume 20 µl with distilled water. ABTS reagent was added to the well for 180 µl. The microplate was shaken and incubated in the dark for 4 minutes. The absorbance was measured at a wavelength of 734 nm ($A_{734}$) using a Microplate Reader. The % radical scavenging was calculated using the equation mentioned above, and the standard curve of Trolox was constructed using concentration (x) and % radical scavenging (y). The $IC_{50}$ value was calculated from the linear equation of the standard curve.

6.6.4 Antioxidant capacity analysis in sample extracts

The stock solution of the sample was diluted to a concentration of 0, 1, 2, 3, 4, and 5 mg/ml, volume 20 µl with distilled water. ABTS reagent was added to the well for 180 µl. The microplate was shaken and incubated in the dark for 4 minutes. The absorbance was measured at a wavelength of 734 nm ($A_{734}$) using a Microplate Reader (Figure 22). The % radical scavenging was calculated using the equation mentioned above, and the curve of the sample was constructed using concentration (x) and % radical scavenging (y). The $IC_{50}$ value was calculated from the linear equation of the sample curve. (Re et al., 1999; Lertcanawanichakul et al., 2019).

$$\% \text{ Radical scavenging} = 1 - \left(\frac{\text{O.D. sample}}{\text{O.D. control}}\right) x\ 100$$

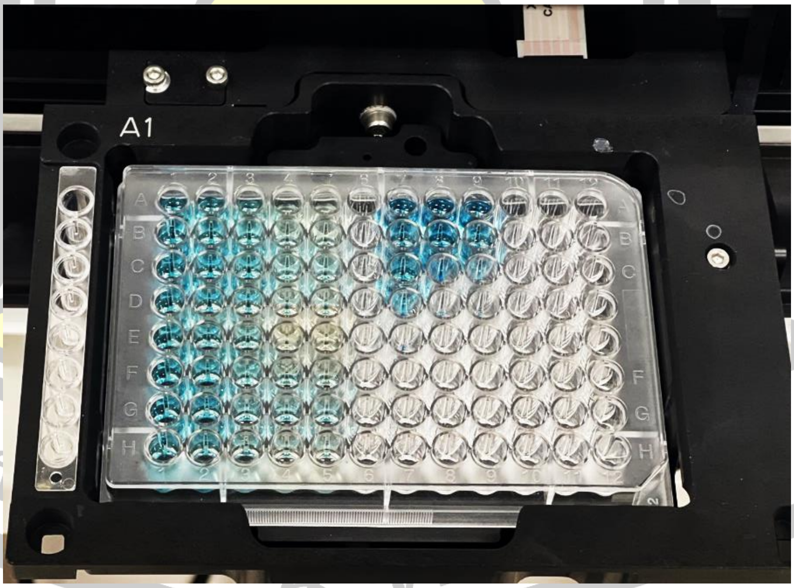


**Figure 22** Antioxidant analysis of ABTS assay using microplate reader

Ref: (Areerat, 2023)

**6.7 FRAP assay (Ferric reducing antioxidant power)**

6.7.1 Preparation of FRAP reagent

FRAP reagent was prepared by weighing 0.054 g of 20 mM ferric chloride and dissolving in 10 ml of distilled water, and weighing 0.031 g of 10 mM TPTZ and dissolving in 20 mM hydrochloric acid, volume 10 ml (at 50 °C). The ferric chloride and 10 mM of TPTZ were mixed with 100 ml of acetate buffer, and then 12 ml of distilled water was added and stored at 37 °C.

6.7.2 Preparation of 300 mM acetate buffer, pH 3.6.

Sodium acetate was weighed for 1.8 g, and 8 ml of glacial acetic acid was added. The volume was adjusted to 500 ml with distilled water. The pH was adjusted to 3.6 and stored at 4 °C.

6.7.3 Preparation of 4 mM concentrated hydrochloric acid.

Hydrochloric acid was pipetted for 0.0033 ml and the volume was adjusted with distilled water to 10 ml for use to dissolve 10 mM TPTZ.

6.7.4 Preparation of ferrous sulfate standard

Prepare a 2 mg/ml ferrous sulfate standard by weighing 0.02 g $FeSO_4$ and adjusting the volume to 10 ml with distilled water.

6.7.5 Ferrous sulfate standardization graph

The stock solution was diluted to a concentration of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml, volume 20 µl. FRAP reagent was added to the well for 180 µl. The microplate was shaken and incubated in the dark for 4 minutes. The absorbance was measured at a wavelength of 595 nm ($A_{595}$) using a Microplate Reader. The standard curve of Ferrous sulfate was constructed by using the concentration (x) and the absorbance (y).

6.7.6 Antioxidant capacity analysis in sample extracts

The stock solution of the sample was prepared by weigh 0.01 g extract and dissolve it with 10 ml of the solvent used for extraction. The stock solution was diluted to a concentration of 0, 0.5, and 1 mg/ml, volume 20 µl. FRAP reagent was added into the well for 180 µl. The microplate was shake and incubated in the dark for 4 minutes. The absorbance was measured at a wavelength of 595 nm ($A_{595}$) using a Microplate Reader (Figure 23). The antioxidant potential of lichen crude extract was calculated compared with the standard curve of Ferrous sulfate and report with mg $FeSO_4$/g extract. (Sae-chan et al., 2020; Xiao et al., 2020).

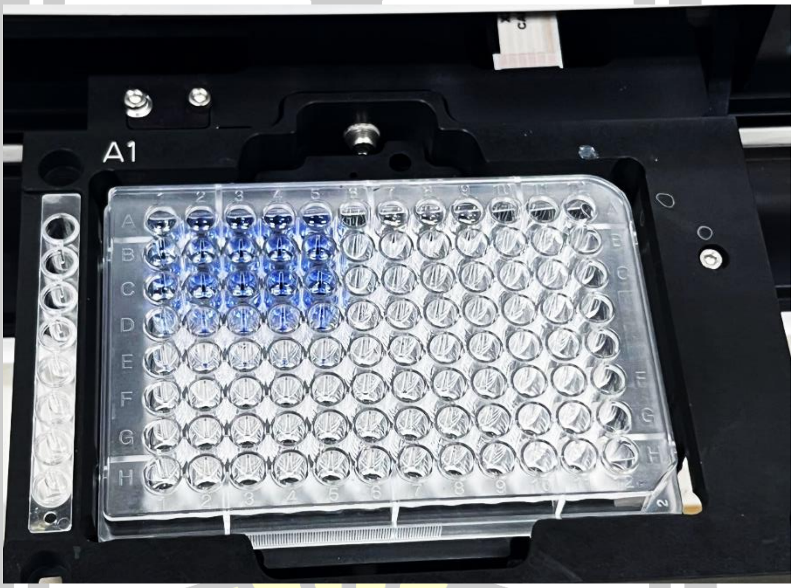


**Figure 23** Antioxidant analysis of FRAP assay using microplate reader

Ref: (Areerat, 2023)

## 6.8 Antibacterial activity

6.8.1 Preparation of the culture medium

MHA and MHB culture media were prepared by weighing the ingredients according to the media recipe. Distilled water was used to dissolve the culture medium, and then it was boiled under hot conditions to homogenize. The culture medium was poured into a Duran bottle and taken to an autoclave with a temperature of 121 °C and pressure of 15 pounds/square inch for 15 minutes.

When the culture medium has a temperature of about 45-50 °C, pour approximately 20 ml into a petri dish under the BSC class II and let the culture medium compactify before use.

6.8.2 Bacterial strains

Bacterial strains were used in this study belonging to Gram positive and Gram- negative species: *Bacillus cereus* (TISTR 1449), *Staphylococcus epidermidis* (TISTR 2162), *Escherichia coli* (TISTR 527), and *Pseudomonas aeruginosa* (TISTR 1287) (Figure 24), Tetracycline was used as a positive control for positive gram bacteria (*B. cereus*, *S. epidermidis*), Ciprofloxacin was used as a positive control for negative gram bacteria (*E. coli, P. aeruginosa*) and the negative control was 3% DMSO.

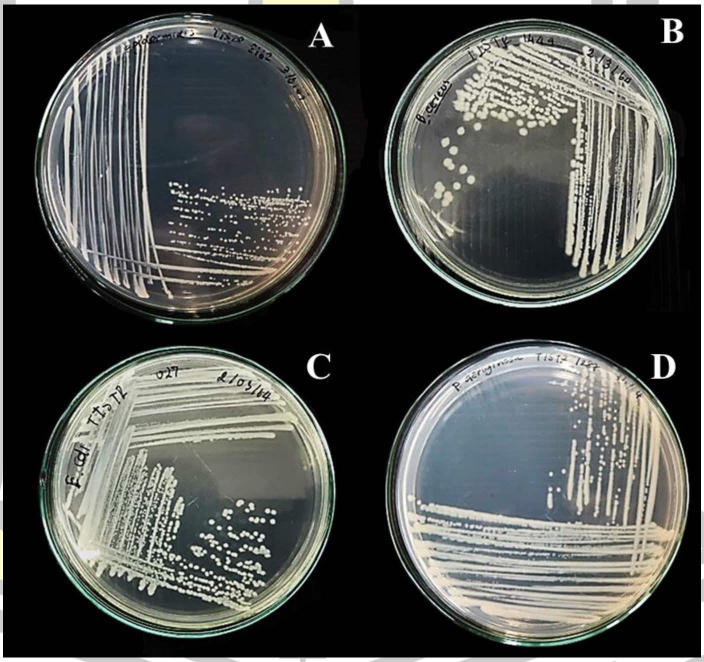


**Figure 24** Bacterial strains on Muller Hinton Agar plates

(A) *S. epidermidis* TISTR 2162 (B) *B. cereus* TISTR 1449

(C) *E. coli* TISTR 527 (D) *P. aeruginosa* TISTR 1287

Ref: (Areerat, 2023)

6.8.2 Agar well diffusion method

The antibacterial activity of lichen crude extracts was followed by Goel et al., (2021). The lichen extracts were dissolved in 3% DMSO with a concentration of 40 mg/ml. The bacterial inoculums containing $1.5 \times 10^8$ CFU/ml (prepared in sterile 0.85% saline until turbidity of 0.5 McFarland) were spread on the Mueller Hinton Agar plate. The well was drilled with a 6 mm Cork borer and 50 µl lichen extracts were loaded into the well. Incubated at 37 °C for 18-24 hours and measured the diameters of inhibition zones (Figure 25).


**Figure 25** Agar well diffusion test

A) Spreading bacterial inoculums B) Drilling the agar well C) Inhibition zone

Ref: (Areerat, 2023)

6.8.3 Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MIC) of the crude extract was determined by broth micro-dilution techniques in Mueller Hinton Broth (MHB), followed by Srivastava et al. (2013). The lichen extracts were prepared with concentrations from 1.25 to 40 mg/ml in MHB. Bacterial inoculum was added to 10 µl in all wells. The mixture was incubated at 37 °C for 24 hours. Resazurin (0.01%) was used as an indicator to observe the color change, and the MIC was determined as the lowest concentration that could inhibit bacterial growth (Figure 26).

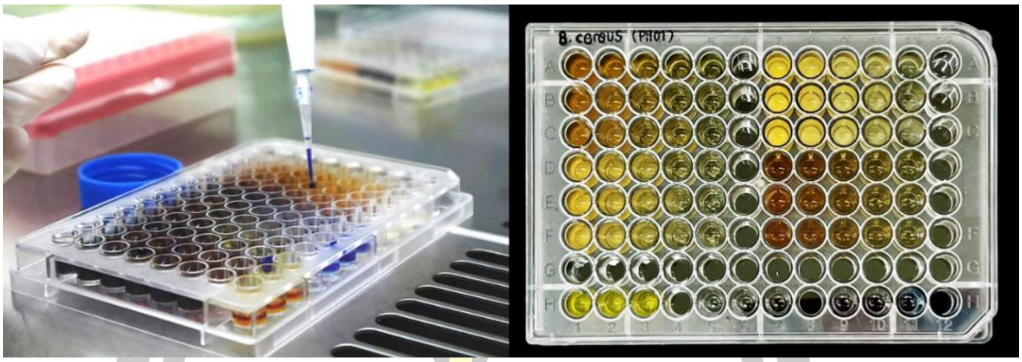


**Figure 26** Minimum Inhibitory Concentration test

Ref: (Areerat, 2023)

6.8.4 Minimum Bactericidal Concentration (MBC)

To determine the Minimum Bactericidal Concentration (MBC), the extracts with bacterial inoculum in the MIC test were streaked on the agar plates and incubated for 24 hours. The MBC was determined as the lowest concentration that killed bacterial growth on the agar plate (Figure 27) (Goel et al. 2021).


**Figure 27** Minimum Bactericidal Concentration test

Ref: (Areerat, 2023)

# CHAPTER 4

# RESULTS

## Sample Collection

The lichen specimens of *Usnea* are from nine different areas located in four provinces. Fresh materials were collected from Chiang Mai Province; Mon Long and Doi Pui, Phu Hin Rong Kla National Park (Phitsanulok Province), Phu Thab Buek (Phetchabun Province). Two location localities out off this study area; Phu Ruea National Park (Loei Province), and Phu Khiao Wildlife Sanctuary (Chaiyaphum Province). Dry specimens were loaned from RAMK (Ramkhamhaeng University). The specimens were collected from 4 locations in Chiang Mai Province; Queen Sirikit Botanic Garden, Doi Ang Kang, Hod District, and Doi Inthanon National Park, Chae Son National Park (Lampang Province), and Phu Hin Rong Kla National Park (Phitsanulok Province). In total 74 specimens were studied. The number and code of specimens from each location can be found in Table 11.

**Table 11** The number of samples collected in each area and the code showing the abbreviation of the sampling area

| Location | Number of specimens | Code |
|---|---|---|
| 1. Phu Hin Rong Kla National Park | 8 | PH |
| | 12 | RAMK |
| 2. Phu Thab Buek | 9 | PT |
| 3. Mon Long | 20 | ML |
| 4. Doi Pui | 0 | DP |
| 5. Phu Ruea National Park | 4 | PR |
| 6. Phu Khieo Wildlife Sanctuary | 1 | PK |
| 7. Queen Sirikit Botanic Garden | 13 | RAMK |

| Location | Number of specimens | Code |
|---|---|---|
| 8. Doi Ang Kang | 1 | RAMK |
| 9. Hod District | 1 | RAMK |
| 10. Doi Inthanon National Park | 4 | RAMK |
| 11. Chae Son National Park | 1 | RAMK |
| Total | 74 | |

In the sampling area of Doi Pui, it was impossible to collect the specimen because the entire forest was fire, leading to the demise of all living beings, including animals and plants. The survey revealed that the forest area comprises Hill Evergreen Forest and Coniferous Forest, with pine trees being the predominant tree species.

Thirty-two specimens from the Herbarium at Ramkhamhaeng University some specimens have been identified by scientific name but have not yet been described. In this study, the researcher also examined the morphological and anatomical characteristics, as well as the chemistry, and provided a detailed description of each.

**Morphological, Anatomical, and Chemical Analysis**

The morphological characteristics of *Usnea* sp. were examined using a stereo microscope. The examination included looking at various components of the specimens such as the central cord, base of the thallus, soredia, isidia, apothecia, papillae, hyphae in the medulla, pigment, main branch, fibril, and lateral branches.

The anatomical characteristics of *Usnea* sp. were studied using a light microscope and hand cross-section by blade. The cortex/medulla/axis ratio (CMA ratio) was measured.

Lichen substances of *Usnea* species were investigated by High Performance Thin Layer Chromatography (HPTLC). Solvent A (Toluene: dioxane: acetic acid, 90: 25: 4), Solvent B (hexane: methyl tert-butyl ether: formic acid, 140: 72: 18), and Solvent C (n-Hexane: diethyl ether: formic acid = 5: 4: 1) were used to analyze.

**Lichen Identification**

In this research, a total of 74 specimens can be classified 61 specimens into 12 species and 11 unidentified specimens (Table 12).

**Table 12** The numbers of each species of genus *Usnea* in Thailand

| Species | Number |
|---|---|
| 1. *Usnea aciculifera* Vain. | 3 |
| 2. *Usnea articulata* (L.) Hoffm. | 5 |
| 3. *Usnea baileyi* (Stirt.) Zahlbr. | 15 |
| 4. *Usnea ceratina* Ach. | 1 |
| 5. *Usnea* cf. *dendritica* Stirt. | 1 |
| 6. *Usnea himantodes* Stirt. | 2 |
| 7. *Usnea pangiana* Stirt. | 1 |
| 8. *Usnea* cf. *perhispidella* J. Steiner | 2 |
| 9. *Usnea* cf. *pectinata* Taylor | 4 |
| 10. *Usnea rubicunda* Stirt. | 27 |
| 11. *Usnea* cf. *rubicunda* Stirt. | 1 |
| 12. *Usnea shimadae* Asahina | 1 |
| 13. *Usnea* sp.1 | 1 |
| 14. *Usnea* sp.2 | 1 |
| 15. *Usnea* sp.3 | 1 |
| 16. *Usnea* sp.4 | 1 |
| 17. *Usnea* sp.5 | 1 |
| 18. *Usnea* sp.6 | 1 |
| 19. *Usnea* sp.7 | 1 |
| 20. *Usnea* sp.8 | 1 |
| 21. *Usnea* sp.9 | 1 |
| 22. *Usnea* sp.10 | 1 |
| 23. *Usnea* sp.11 | 1 |

The important characteristics used to reconstruct the key to species of *Usnea* in Thailand in this study, such as Base color, thallus color, thallus character, cortex, medulla, axis, presence of isidia, soredia, fibrils, pigmentation, and chemistry as shown in Table 13.

**Table 13** The important characteristics used to identify species of *Usnea* in Thailand

| Species | Axis | Medulla | Cortex | Thallus character | Base | Isidia | Soredia | Fibrils | Thallus color | Chemistry |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. *U. baileyi* (Stirt.) Zahlbr | Hollow | Red subcortical<br>Dense | Non-pigment | uninflated | Black | Present abundant | Present (some specimen) | Present (Fish-bone) | Nut brown (in herbarium) | Salazinic (Major), Constictic, Norstictic, Barbatic, Stictic, Baeomycesic, Diffractaic, Menegazziaic, Psoromic, Evernic, 4-O-demethylbarbatic, 2'-O-demethylpsoromic, Usnic acids, Atranorin |
| 2. *Usnea shimadae* Asahina | Hollow | Red subcortical<br>Dense | Non-pigment | Uninflated with apothecia | Lack | Absent | Absent | Present (abundant) | Dark liver or coffee (in herbarium) | Salazinic, 4-O-demethyl-barbatic, Evernic, Usnic acids, Atranorin, Eumitrin A |
| 3. *U. ceratina* Ach | Compact | Straw-berry Pink, Dense | Non-pigment | Uninflated | Dark brown | Absent | Absent | Present | Olive green | Barbatic (Major), 4-O-demethyl-barbatic, Menegazziaic, Usnic, Baeomycesic acids, Eumitrin A, Atranorin |
| 4. *U. pangiana* Stirt | Compact | Dense | Red | Uninflated | Dark brown | Absent | Absent | Uneven (not smooth) | Sepia brown (in herbarium) | Salazinic, Norstictic, Barbatic, Usnic acids, Atranorin |
| 5. *Usnea* cf. *perhispidella* J. Steiner | Compact | Dense | Red | Uninflated | Dark brown | Present abundant | Absent | Present | Chocolate brown (in herbarium) | Galbinic, Stictic, Salazinic, Barbatic, Diffractaic, Squamatic, Usnic acids, Atranorin |
| 6. *U. rubicunda* Stirt | Compact | Dense | Red | Uninflated | Dark brown | Present (Isidio-morph) | Present | Present | Green (Fresh), Turn red (Old) | Salazinic, Constictic, Stictic, Barbatic, Menegazziaic, Galbinic, 4-O-demethyl-barbatic, 2'-O-demethylpsoromic, Usnic acids, Atranorin |
| 7. *Usnea* cf. *rubicunda* Stirt | Compact | Dense | Red | Uninflated | Dark brown | Present (Isidio-morph) | Present | Present | Grayish-green (Fresh), Pale green (Old) | Barbatic, Norstictic, 4-O-demethyl-barbatic, Usnic acids, Atranorin, Eumitrin A |
| 8. *Usnea* sp.9 | Compact | Loose | Red | Uninflated | Black | Present abundant | Present | Absent | Grayish green | Stictic, Usnic acids, Atranorin |
| 9. *U. articulata* (L.) Hoffm | Compact | Loose | Non-pigment | Inflated | Black | Absent | Present | Present (inflated) | Yellow | Protocetraric (Major), Salazinic, Stictic, Psoromic, Menegazziaic, Norstictic, Notaic, Usnic acids, Eumitrin B |
| 10. *Usnea* sp.1 | Compact | Loose | Non-pigment | Inflated | Lack | Present (few) | Absent | Uneven (not smooth) | Beige brown (in herbarium) | Norstictic, Stictic, Psoromic, Galbinic acids, Atranorin |
| 11. *Usnea* sp.2 | Compact | Loose | Non-pigment | Inflated (slightly) | Black | Absent | Present | Present | Olive green | Stictic, Galbinic, Diffractaic, |

| Species | Axis | Medulla | Cortex | Thallus character | Base | Isidia | Soredia | Fibrils | Thallus color | Chemistry |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | (soredia on fibrils) | | Evernic, Usnic acids, Atranorin, Eumitrin B |
| 12. *Usnea* cf. *dendritica* | Compact | Dense | Non-pigment | Uninflated | Pale | Absent | Absent | Present | Pale brown (in herbarium) | Salaznic, Barbatic, Thamnolic, Baeomycesic, Usnic acids, Atranorin |
| 13. *U. himantodes* Stirt | Compact | Dense | Non-pigment | Uninflated, Ridged with segment | Dark brown | Absent | Absent | Absent | Olive yellow | Stictic, Usnic, Norstictic, Notaic, Evernic acids, Atranorin |
| 14. *Usnea* cf. *pectinata* | Compact | Dense | Non-pigment | Uninflated | Dark brown | Present | Absent | Absent | Pale brown (in herbarium) | 2'-O-demethylpsoromic, Galbinic, Evernic, Usnic, Constictic, Baeomycesic, Norstictic, Psoromic, Atranorin |
| 15. *Usnea aciculifera* Vain | Compact | Dense | Non-pigment | Uninflated | Dark brown | Present | Absent | Absent | Green | Stictic (Major), Diffractaic, Usnic acids, Atranorin |
| 16. *Usnea* sp.7 | Compact | Dense | Non-pigment | Uninflated | Black | Present | Present | Present (smooth) | Olive green | Barbatic, 4-O-demethylbarbatic, Baeomycesic, Squamatic, Physodalic, Usnic acids, Atranorin |
| 17. *Usnea* sp.3 | Compact | Loose | Non-pigment | Uninflated | Black | Absent | Present | Absent | Olive yellow | Diffractaic, Evernic, Usnic acids, Atranorin, Eumitrin B |
| 18. *Usnea* sp.4 | Compact | Loose | Non-pigment | Uninflated | Black | Absent | Present | Absent | Olive yellow | Usnic acid, Atranorin |
| 19. *Usnea* sp.5 | Compact | Loose | Non-pigment | Uninflated | Black | Absent | Present | Present (smooth) | Olive yellow | Stictic, Diffractaic, Usnic acids, Atranorin |
| 20. *Usnea* sp.6 | Compact, | Loose | Non-pigment | Uninflated | Black | Absent | Absent | Present (abundant) | Green (fresh), Turn red (old) | Stictic, Diffractaic, Usnic acids, Atranorin |
| 21. *Usnea* sp.8 | Compact | Loose | Non-pigment | Uninflated | Brown | Present | Absent | Present (abundant) | Olive green | Norstictic, Salazinic, Stictic, Usnic acids, Eumitrin A, Eumitrin B, Atranorin |
| 22. *Usnea* sp.10 | Compact | Loose | Non-pigment | Uninflated | Brown | Absent | Absent | Present | Yellowish green | Stictic, Usnic acids, Atranorin |
| 23. *Usnea* sp.11 | Compact | Loose | Non-pigment | Uninflated | Brown | Present | Present | Absent | Pale green | Physodalic, Menegazziaic, Psoromic, Barbatic, Galbinic, Usnic acids, Atranorin |

**Keys to species of *Usnea* in Northern Thailand**

1a. Thallus with hollow axis with red subcortical……………………………………… 2

1b. Thallus with compact axis with red subcortical…………………………………..3

2a. Isidia and soredia present, stictic acid and norstictic acid present…………………
…………………………..……………………………....***Usnea baileyi* (Stirt.) Zahlbr.**

2b. Isidia and soredia absent, stictic acid and norstictic acid absent………..…………
…………………………..…………………………........***Usnea shimadae* Asahina**

3a. Thallus with pigmentation……..…………………………………………………4

3b. Thallus without pigmentation ………………...……………………………………9

4a. Red pigment in the cortex……….……………………………..………………5

4b. Strawberry pink in whole medulla……..……….................***Usnea ceratina*** **Ach**

5a. Medulla filled with dense hyphae…...…………………………………………..…6

5b. Medulla filled with loose hyphae…………………………..………***Usnea*** **sp.9**

6a. Thallus with isidia, annulary crack absent……………………….…………….……7

6b. Thallus without isidia, annulary crack present …………..…***Usnea pangiana*** **Stirt**

7a. Isidiomorph present……..………………………………………………….……8

7b. Isidiomorph absent……………………………....***Usnea*** **cf.** ***perhispidella*** **J. Steiner**

8a. Stictic acid and salazinic acid present……………….……....***Usnea rubicunda*** **Stirt**

8b. Stictic acid and salazinic acid absent………………..….....***Usnea*** **cf.** ***rubicunda*** **Stirt**

9a. Thallus inflated like sausage…………………………..………………………....10

9b. Thallus uninflated……………………………………..……………………………12

10a. Isidia absent, soredia present……………….…………..………..……………11

10b. Isidia present, soredia absent……………………………………....…***Usnea*** **sp.1**

11a. Thallus yellow, protocetraric and salazinic present….....***U. articulata*** **(L.) Hoffm**

11b. Thallus olive green, protocetraric and salazinic acid absent………...***Usnea*** **sp. 2**

12a. Thallus with brown to black base…………………..……..………………………..13

12b. Thallus with pale base………………………….....……....***Usnea*** **cf.** ***dendritica*** **Stirt**

13a. Thallus pendent………...……………………………………………………………14

13b. Thallus erect to subpendulous…………………………………………………..…..15

14a. Thallus uninflated with segment, stictic acid present......***Usnea himantodes*** **Stirt**

14b. Thallus uninflated without segment, stictic acid absent…………………………….. ……………………………………………………..***Usnea*** **cf.** ***pectinata*** **Taylor**

15a. Medulla filled with dense hyphae……………………………………………...…16

15b. Medulla filled with loose hyphae ……………………..…………………………17

16a. Soredia and fibrils absent, stictic and barbatic acid absent………………….……… ……………………………………………………………………***Usnea aciculifera*** **Vain**

16b. Soredia and fibrils present, stictic and barbatic acid absent………..…***Usnea*** **sp.7**

17a. Both isidia and soredia absent or present………………………...……………18

17b. Either isidia or isidia absent……………………………………………………20

18a. Isidia and soredia absent……….......................................................................19

18b. Isidia and soredia present…………………………………………....***Usnea* sp.11**

19a. Thallus green when fresh, turn red when old, diffractaic acid present……...……
…………………………………………………….……………………..…….. ***Usnea* sp.6**

19b. Thallus yellowish green, diffractaic acid absent..…………………..….***Usnea* sp.10**

20a. Isidia absent, soredia present...………………………………………………….21

20b. Isidia present, soredia absent ……………………………………...***Usnea* sp.8**

21a. Thallus without fibrils…………...…………………….……………………..…22

21b. Thallus with smooth fibrils ……………………………………..….***Usnea* sp.5**

22a. Diffractaic acid, evernic acid and eumitrin B present…………...……***Usnea* sp.3**

22b. Diffractaic acid, evernic acid and eumitrin B absent………….……...***Usnea* sp.4**

**The species**

**1. *Usnea aciculifera* Vain.**

***Morphology:*** thallus fruticose, 2-4 cm long, 1 mm diameter, dark brown base, pale green when fresh, dark olive green when dry, thallus uninflated, thallus with a medullary thread, non-pigment in cortex, medulla filled with dense hyphae, compact axis, lateral branch plenty, fibrils absent, isidia present, soredia, absent, apothecia and pseudocyphellae not seen, papillae absent (Figure 28)

***Anatomy:*** Thallus cross section (n=10) C = (70.3-) 81.5 (-90.9) µm; M = (206.0-) 227.5 (-251.9) µm; A = (311.1-) 356.0 (-388.4) µm; CMA ratio %C = (10.5-) 12 (-14.2); %M = (32.4-) 33-34 (-37.5); %A = (49.2-) 53.6 (-56.6); A/M = (1.3-) 1.6 (-1.7)

***Chemistry:*** Stictic acid, Diffractaic acid, Usnic acid, and Atranorin detected by TLC

***Habitat:*** On twigs of an unidentified tree in attitude 1,768 metres above sea level

***Notes:*** The distinguished features of *U. aciculifera* Vain are (1) thallus uninflated (2) the presence of stictic acid as a major substance (3) medulla dense and thick axis (3) the presence of isidia

***Specimens examined:*** THAILAND. Phetchabun Province, Phu Thab Buek, Pha Hua Sing, 16°54′23″N 101°5′14″E, 3 December 2023, Areerat Saisong and Orathai Sertsri — (PT01, PT03, PT07)

***Distribution:*** Taiwan, Thailand

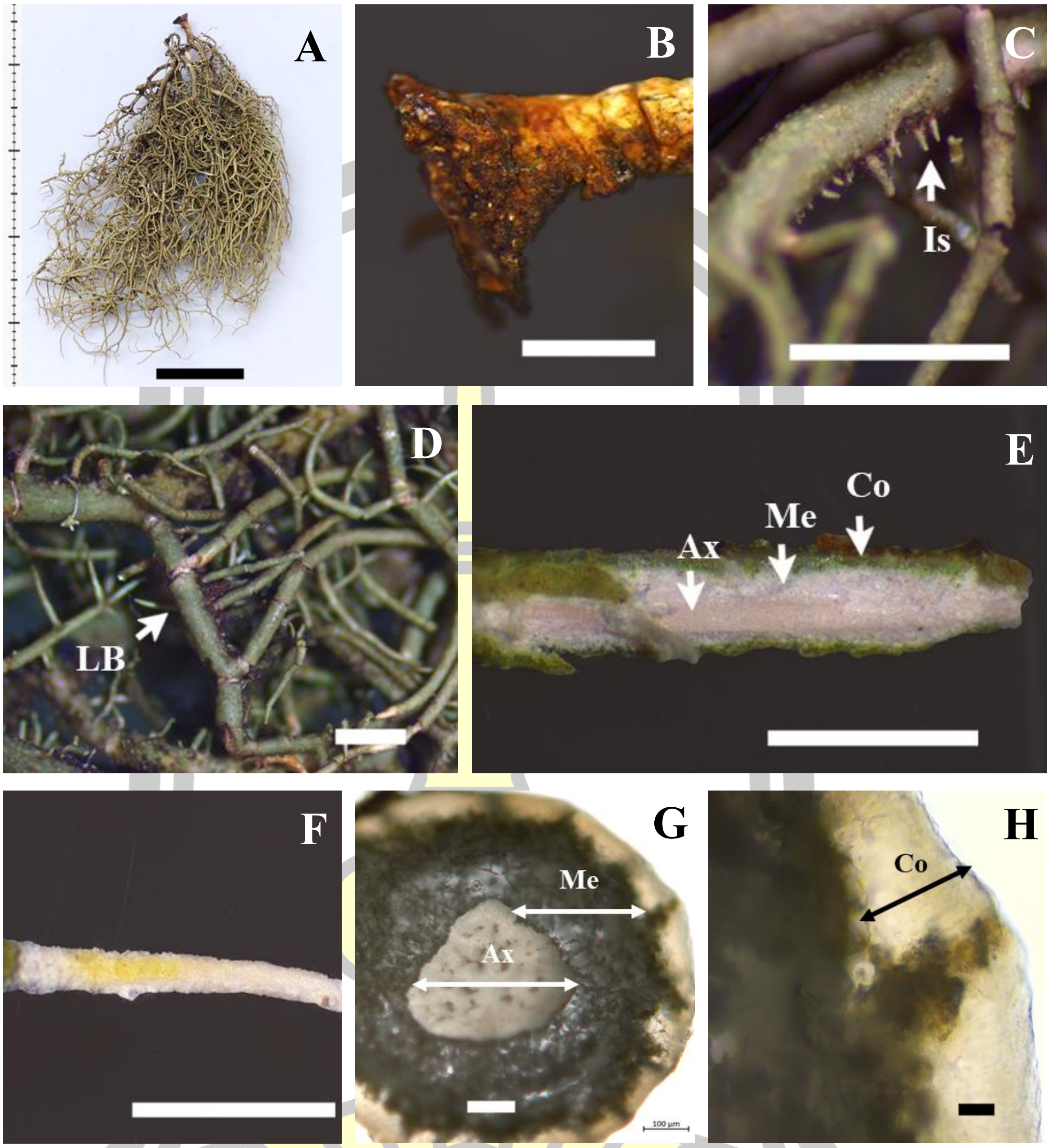


**Figure 28** Characteristics of *Usnea aciculifera* Vain (PT07)

A) Habitat (Scale = 1 cm) B) Base C) Isidia (Is) D) Lateral branch (LB) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and F) Medullary thread (Scale = 1 mm) G) Dense hyphae in the medulla (Me) and axis (Ax) (Scale = 100 µm) and H) cortex (Co) (Scale = 20 µm)

**2. *Usnea articulata* (L.) Hoffm. (PH07)**

***Morphology:*** Thallus fruticose, pendent, up to 11.5 cm long, 2-4 mm diameter, blackish or brownish base, fresh thallus greyish green, old thallus yellowish green, thallus inflated like sausage, thallus with a medullary thread visible by stretching the branches, fibrils sparse, thin cortex and hyaline, non-pigment in cortex, thick medulla filled with very loose hyphae, thin and compact axis, soredia present, isidia plenty to none, apothecia and pseudocyphellae not seen, papillae absent (Figure 29)

***Anatomy:*** Thallus cross section (n=10) C = (74.5-) 86.6 (-97.7) µm; M = (677.9-) 845.2 (-1092.8) µm; A = (328.3-) 450.6 (-571.9) µm; CMA ratio %C = (5.3-) 6.2 (-6.9); %M = (52-) 59 (-63); %A = (30-) 35 (-41); A/M = (1.3-) 1.4 (-2.1) — (PH07)

***Chemistry:*** Usnic acid, Evernic acid (±), Protocetraric acid (+), Salazinic acid (±), Stictic acid (±), Psoromic acid (±), Menegazziaic acid (±), Norstictic acid (±), Notatic acid (±), Atranorin (+), and Eumitrin B detected by TLC

***Habitat:*** On twigs of a *Schima wallichii* Choisy in attitude 1,314.6 metres above sea level — (ML03); On twigs of *Erythrina subumbrans* (Hassk.) Merr. in attitude 1,309.4 metres above sea level — (ML19)

***Notes:*** The distinguished features of *U. articulata* (L.) Hoffm. are (1) Thallus inflated like sausage with fibrils (2) medulla losoe (3) the presence of protocetraric acid as a major substance (4) soredia and isidia present

***Specimen examined:*** THAILAND. Phitsanulok Province, Phu Hin Rong Kla National Park, 26 January 2020, Areerat Saisong — (PH07); Chiang Mai Province, Mae Rim District, Mon Long, 18°93′25″N 98°83′23″E, 9 August 2022, Areerat Saisong, Orathai Sertsri, and Wattana Tanming — (ML03, ML19); Loei Province, Phu Ruea District, Phu Ruea National Park, 4 November 2020, Areerat Saisong — (PR03, PR04)

***Distribution:*** Taiwan, Australia (Ohmura, 2012), Thailand

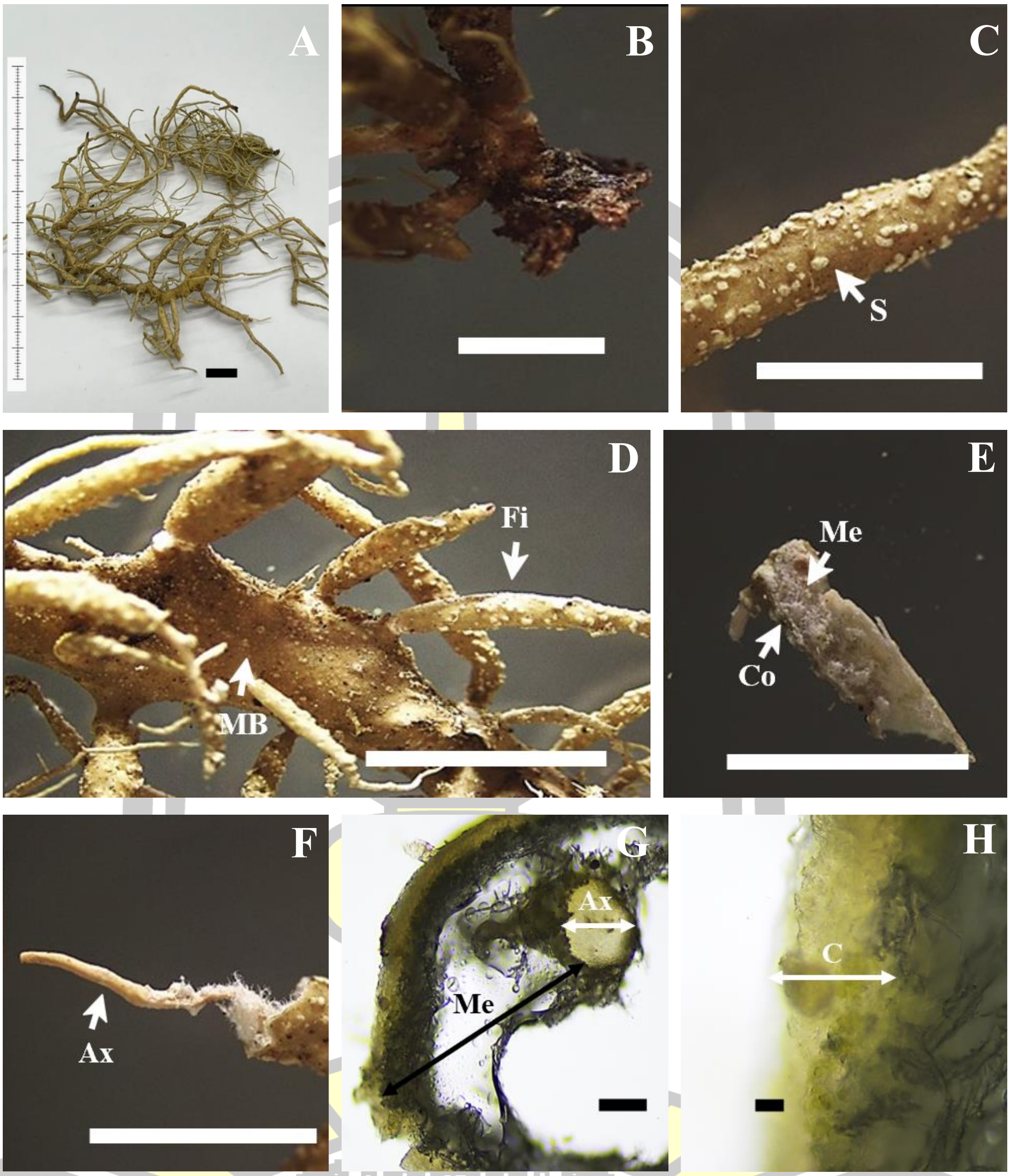


**Figure 29** Characteristics of *Usnea articulata* (L.) Hoffm. (PH07)

A) Habitat (Scale = 1 cm) B) Base C) Soredia (S) D) Main branch (MB) and Fibrils (Fi) E) Thallus layer (Co = Cortex; Me = Medulla) (Scale = 1 mm) F) Axis (Ax) (Scale = 200 µm) G) Loose hyphae in medulla (Me) and axis (Ax) H) Cortex (Co) (Scale = 100 µm)

**3. *Usnea baileyi* (Stirt.) Zahlbr.**

***Basionym:*** *Eumitria baileyi* Stirt. (1881)

***Morphology:*** Thallus fruticose, up to 11 cm long, 0.5-2 mm diameter, brownish to blackish base, nut brown in herbarium, thallus uninflated, thallus with medullary thread visible by stretching branches, hollow central axis and thick, medulla filled with dense hyphae, fibrils numerous like fish-bone, soredia present (some specimen), isidia abundant, apothecia and pseudocyphellae not seen, papillae absent, presence of red subcortical pigmentation, presence of lichenized fungi (some specimen) (Figure 30)

***Anatomy:*** Thallus cross section (n=10) C = (58.4-) 76.4 (-93.3) µm; M = (120.4-) 146.6 (-200.3) µm; A = (428.9-) 472.8 (-535.6) µm; CMA ratio %C = (9.2-) 11.1 (-14.3); %M = (17.8-) 20.2 (-27); %A = (62.6-) 69 (-70.2); A/M = (2.3-) 3.4 (-3.9)

***Chemistry:*** Salazinic acid (+), Constictic acid, Stictic acid, Barbatic acid, Norstictic acid (±), Baeomycesic acid, Diffractaic acid, Menegazziaic acid, Psoromic acid, Evernic acid, 4-O-Demethylbarbatic acid, 2'-O-Demethylpsoromic acid, Usnic acid, Atranorin, and Eumitrin A detected by TLC

***Habitat:*** on *Pittosporum ferrugineum* W. T. Aiton (Dry Dipterocarp Forest) — (RAMK035261); on *Quercus mespilifolioidea* A. Cumus, in attitude 650-730 metres above sea level — (RAMK035328, RAMK035457); on *Rhododendron* sp. in attitude 1,475 metres above sea level (Lower Montane Rain Forest) — (RAMK040351); on *Pinus* sp. (Lower Montane Rain Forest) — (RAMK040352); on *Pinus kesiya* Royle ex Gordon. in attitude 1,450 metres above sea level (Lower Montane Rain Forest) — (RAMK040355); on *Syzygium* sp. (Dry Dipterocarp Forest) — (RAMK040357); on *Pinus morkurii.* in attitude 1,100 metres above sea level (Lower Montane Rain Forest) — (RAMK040364); on *Castanopsis* sp. (Lower Montane Rain Forest) — (RAMK040365, RAMK040368); on Rock, in attitude 1,200 metres above sea level (Lower Montane Rain Forest) — (RAMK040371)

***Notes:*** The distinguished features of *U. baileyi* (Stirt.) Zahlbr. are (1) red subcortical pigmentation (2) hollow axis (3) fibrils like fish-bone (4) presence of lichenized fungi present in some specimens (5) the presence of stictic acid and salazinic acid (some specimens norstictic acid present similar to the specimen from India. (Clerc & Ohmura, 2023; Joshi et al., 2020; Temu et al., 2019; Troung & Clerc, 2013)

***Specimens examined:*** THAILAND. Chiang Mai Province, Fang District, Doi Ang Kang, May 1996, Siripen 290 — (RAMK025749); Mae Rim District, Queen Sirikit Botanic Garden, On the right side of the road headquater to 'Ban Maeo', forest than on the left side, 27 January 1994, Piboon Mongkolsuk et al., 1195.7 — (RAMK035261); opposite the Queen Sirikit Botanic Garden across the Highway, walk further up the tree, the grass underneath, 18°53′56″N 98°51′36.91″E, 3 November 1994, Piboon Mongkolsuk et al., 2938.1 — (RAMK035328); Forest on the hill behind office close to garden 'Mae Mae' Preserve Water Source Unit, 3 November 1994, Piboon Mongkolsuk et al., 2938.1 — (RAMK035457); Doi Pha Klong, 7 November 1994, Kansri Boonpragob et al., 3107 — (RAMK035477); 18°48′58.29″N 98°51′0.11″E, 8 November 1994, Kansri Boonpragob et al., 3351 — (RAMK040355); opposite Queen Sirikit Botanic Garden Station, 17 January 1995, Kansri Boonpragob et al., 3972 — (RAMK040357); Phitsanulok Province, Phu Hin Rong Kla National Park, The Area Office of the State Power, 21 October 1997, Natsurang Honmchantara, Kajohnsak Vongshewarat and Ratchada Noicharoen, 94870 — (RAMK040351); Lan Hin Taek, approximately 20-30 meters from the entrance, mound leading up to the shop beside Than-Patcharin, 1 June 2003, Chonnikan Thunyagun, Chutamat Phraphuchamnong and Natwida Daungphui, ND.235 — (RAMK040352); Pha Choo Thong, 23 February 1998, Ratchada Noicharoen, 10450 235 — (RAMK040361); Lan Hin Taek, 22 February 1998, Thitiporn Pooparng, Kajohnsak Vongshewarat, Wetchasart Polyiam and Ratchada Noicharoen, 10405 — (RAMK040364); The Area Office of the State Power, around the second rocky hill, intersection on the left before entering the air raid shelter, 4 April 2003, Natsurang Homchantara and Natwida Duangphui, ND.446 — (RAMK040365); in Air Road Shelter, 24 February 1998, Ratchada Noicharoen, 10737 — (RAMK040371); Chiang Mai Province, Mae Chaem District, Doi Inthanon National Park, 16 January 1995, Piboon Mongkolsuk et al., 4123.2B — (RAMK040358); Lampang Province, Muang Pan District, Chae Son Sub-District, Chae Son National Park, 7 November 2002, Kansri Boonpragob et al., 3354 — (RAMK040368);

***Distribution:*** Australia, India, China, Indonesia, Japan, Malaysia, Papua New Guinea, Taiwan, Thailand, Tanzania, Nepal, Central and South America, Africa, Philippines, and Australia (Clerc & Ohmura, 2023; Joshi et al., 2020; Temu et al., 2019; Troung & Clerc, 2013)

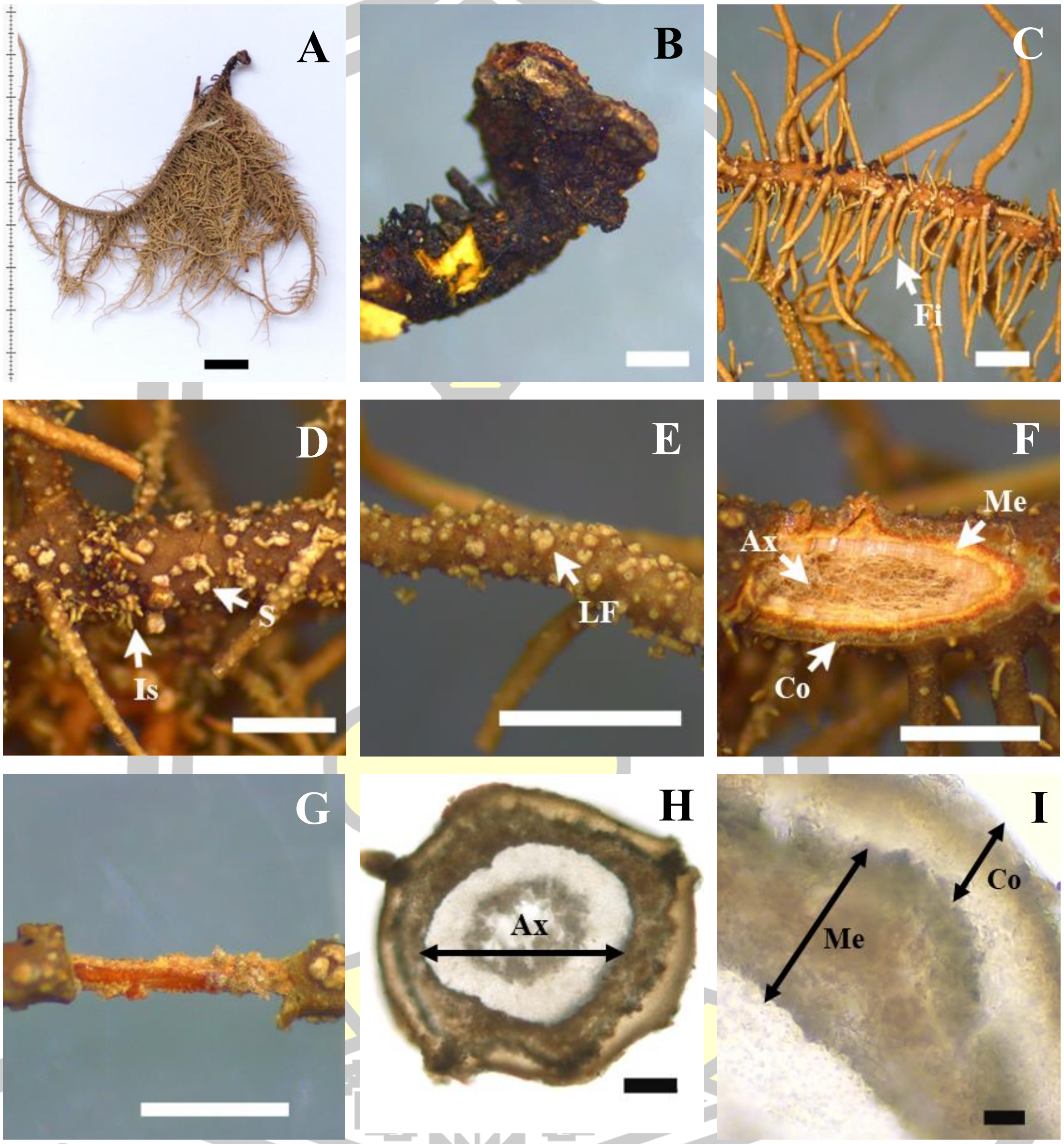


**Figure 30** Characteristics of *Usnea baileyi* (Stirt.) Zahlbr. (RAMK040358)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Isidia (Is) and Soredia (S) E) Lichenized fungi (LF) F) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and G) Medullary thread (Scale = 1 mm) H) Axis (Ax) I) Dense hyphae in medulla (Me) (Scale = 100 µm) and cortex (Co) (Scale = 20 µm)

**4. *Usnea ceratina* Ach.**

***Morphology:*** Thallus fruticose, very small, 2 cm long, dark brown base, olive green when fresh, thallus uninflated, thallus with medullary thread visible by stretching branches, thin cortex non-pigment, medulla filled with dense hyphae, strawberry pink in medulla and thick, axis compact, fibrils abundant, isidia absent, soredia absent, apothecia and pseudocyphellae not seen, papillae absent (Figure 31)

***Anatomy:*** Thallus cross section (n=10) C = (42.3-) 49.9 (-59.5) µm; M = (165.4-) 189.9 (-228.3) µm; A = (187.5-) 204.5 (-229.7) µm; CMA ratio %C = (8.7-) 11.2 (-13.8); %M = (40-) 41.5 (-46.6); %A = (42.9-) 44 (-49.9); A/M = (0.9-) 1.1 (-1.2)

***Chemistry:*** Barbatic acid, 4-O-Demethylbarbatic acid, Menegazziaic acid, Usnic acid, Baeomycesic acid, Eumitrin A, and Atranorin detected by TLC

***Habitat:*** On twigs of a *Melia azedarach* Linn. in attitude 1,307.4 metres above sea level, 18°92′69″N 98°83′30″E — (ML11)

***Notes:*** The distinguished features of *U. ceratina* Ach. are (1) strawberry pink pigmentation in the medulla thallus (2) fibrils abundant without soredia and isidia (3) the presence of barbatic acid, diffractaic acid, and baeomycesic acid as major substances. However, the morphology is distinguished from the specimen from Taiwan by the absence of soredia and isidia.

***Specimen examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Mon Long, 9 August 2022, Areerat Saisong, Orathai Sertsri, Wattana Tanming — (ML11)

***Distribution:*** Taiwan, Thailand

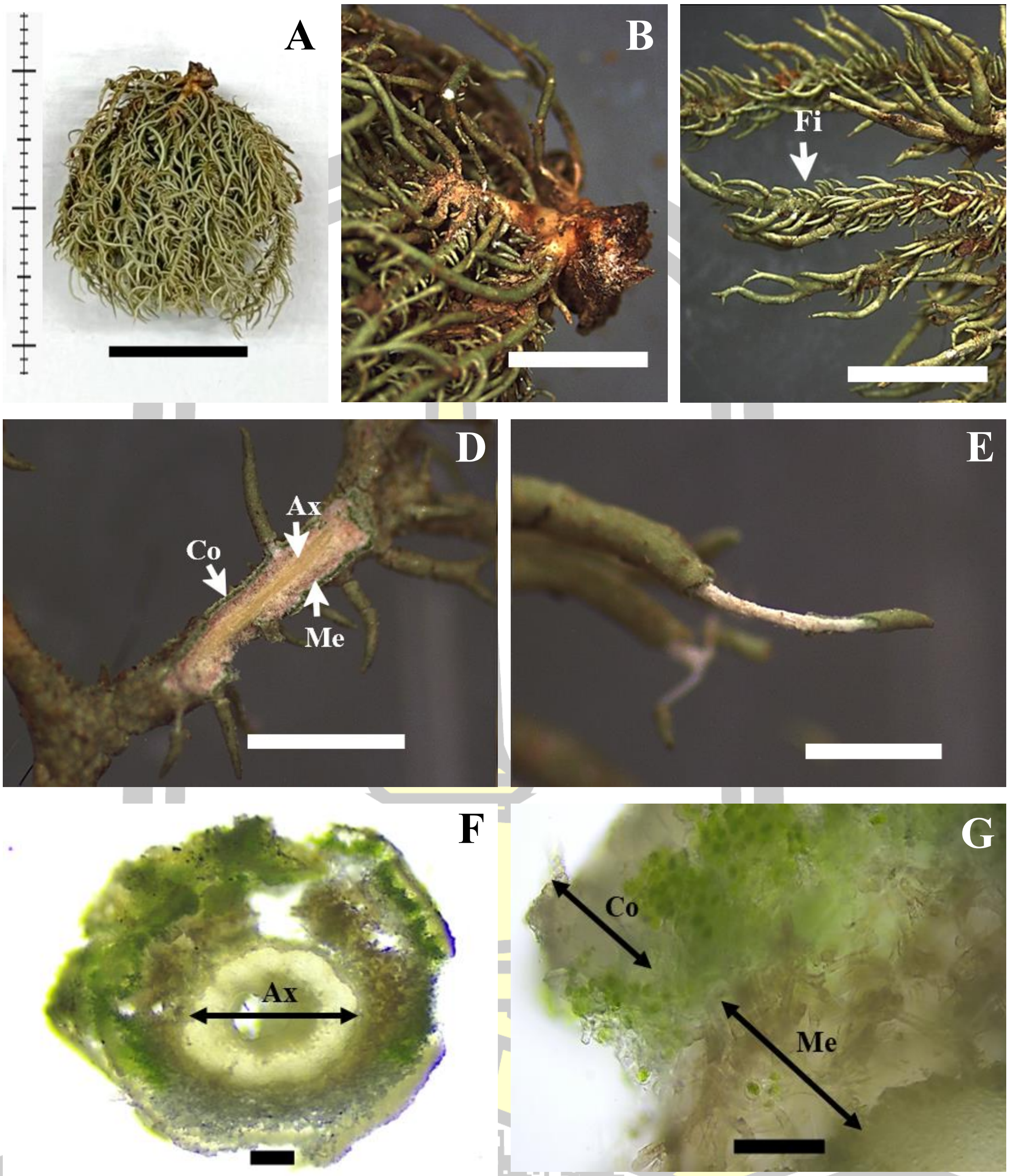


**Figure 31** Characteristics of *Usnea ceratina* Ach. (ML11)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) E) Medullary thread (Scale = 1 mm) F) Loose hyphae in medulla (Me) and thin cortex (Co) (Scale = 100 µm) G) Axis (Ax) (Scale = 50 µm)

**5. *Usnea* cf. *dendritica* Stirt.**

***Morphology:*** Thallus fruticose, small, 2.5 cm long, 0.5 mm diameter, pale base, blackish-brown base, pale brown in herbarium, unknown when fresh, thallus uninflated and uneven, thallus with a medullary thread visible by stretching branches, non-pigment in cortex, medulla filled with dense hyphae, central axis compact and thick, lateral branches and fibrils numerous, isidia absent, soredia absent, apothecia and pseudocyphellae not seen, papillae absent (Figure 32)

***Anatomy:*** Thallus cross section (n=10) C = (50.0-) 58.0 (-64.4) µm; M = (97.7-) 113.1 (-132.3) µm; A = (392.8-) 389.8 (-456.7) µm; CMA ratio %C = (8.2-) 10.6 (-11.2); %M = (15.9-) 20.8 (-23); %A = (65.8-) 68.4 (-75.1); A/M = (2.9-) 3.3 (-4.6)

***Chemistry:*** Salazinic acid, Barbatic acid, Thamnolic acid, Baeomycesic acid, Usnic acid and Atranorin detected by TLC

***Habitat:*** On *Melia azedarach* Linn. In attitude 1,170 metres above sea level, 18°53′56.46″N 98°51′36.91″E (Hill Evergreen (moist) Forest) — (RAMK035437)

***Notes:*** The distinguished features of *Usnea* cf. *dendritica* are (1) thallus uninflated (2) medulla dense and compact axis (3) fibrils numerous (4) without soredia and isidia (5) the presence of salazinic acid and barbatic acid, but it must be confirmed by DNA checking.

***Specimens examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Queen Sirikit Botanic Garden, a Forest on the hill behind the office close to the garden 'Mae Mae' Preserve Water Source Unit, 30 June 1994, Kansri Boonpragob et al., 2209 — (RAMK035437)

***Distribution:*** Taiwan, Thailand (Ohmura, 2001; Ohmura, 2012)

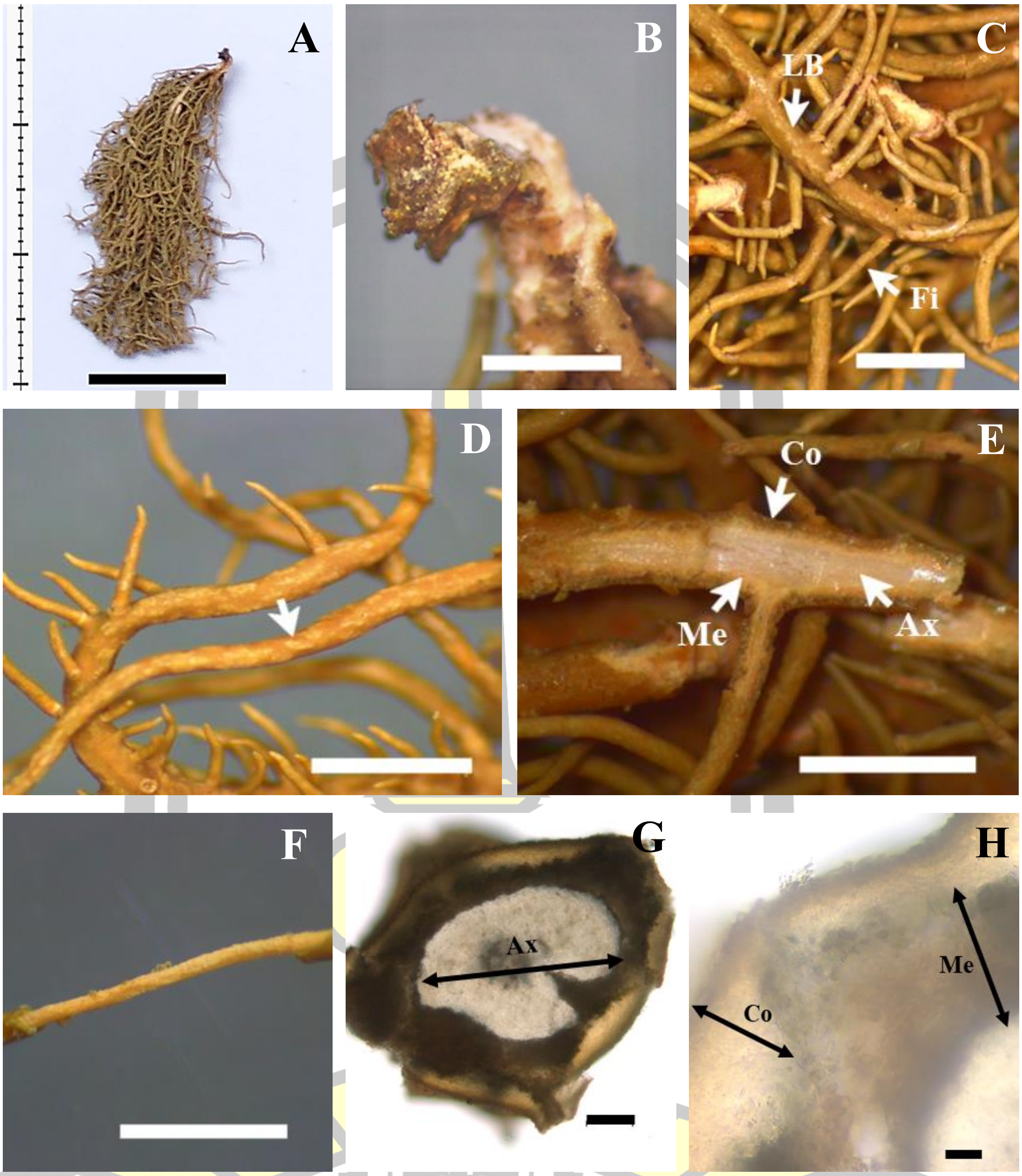


**Figure 32** Characteristics of *Usnea* cf. *dendritica* Stirt. (RAMK035437)

A) Habitat (Scale = 1 cm) B) Base C) Lateral branch (LB) and fibrils (Fi) D) uneven branches E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and F) Medullary thread (Ax = Axis) (Scale = 1 mm) G) Axis (Ax) (Scale = 100 µm) H) Dense hyphae in medulla (Me) and cortex (Co) (Scale = 20 µm)

**6. *Usnea himantodes* Stirt.**

***Synonymy:*** *Usnea neoguineensis* Asahina

*Usnea neoguineensis* var. *gracilior* Asahina

***Morphology:*** Thallus fruticose, pendent, rigid, 55-84.5 cm long, 1.5 mm diameter, brownish base, thallus pale green when fresh, olive-yellow in herbarium, thallus uninflated with ridged branches and segment, non-pigment in cortex, medulla filled with dense hyphae, compact central axis, lateral branches numerous with segment to none, fibrils absent, soredia absent, isidia absent, apothecia and pseudocyphellae not seen, papillae present (Figure 33)

***Anatomy:*** Thallus cross section (n=10) C = (76.4-) 97.3 (-127.7) µm; M = (123.8-) 194.9 (-302.4) µm; A = (699.0-) 765.7 (-846.1) µm; CMA ratio %C = (8.0-) 8.5-9.5 (-16.4); %M = (13-) 18-19 (-24); %A = (67-) 71-73-74 (-78); A/M = (0.2-) 0.3 (-0.4)

***Chemistry:*** Usnic acid, Atranorin, Norstictic acid, Notaic acid, Evernic acid, Stictic acid detected by TLC

***Habitat:*** on an unidentified tree

***Notes:*** The distinguished features of *U. himantodes* Stirt. Are (1) the presence of ridged branches with segment (2) uninflated branches, and pendent thallus. (3) medulla dense and cortex axis (4) the presence of stictic acid and norstictic acid as major substances.

***Specimen examined:*** THAILAND. Phitsanulok Province, Phu Hin Rong Kla National Park, 24 November 2020, Areerat Saisong, Chanthanaphat Champatasi, Nucharee Chamnantab, and Khwanyaruan Naksuwankul — (PH01, PH05)

***Distribution:*** Australia, Philippines, East Africa, Japan, Taiwan, Thailand, and India (Stevens, 1990; Ohmura, 2001; Ohmura, 2012).

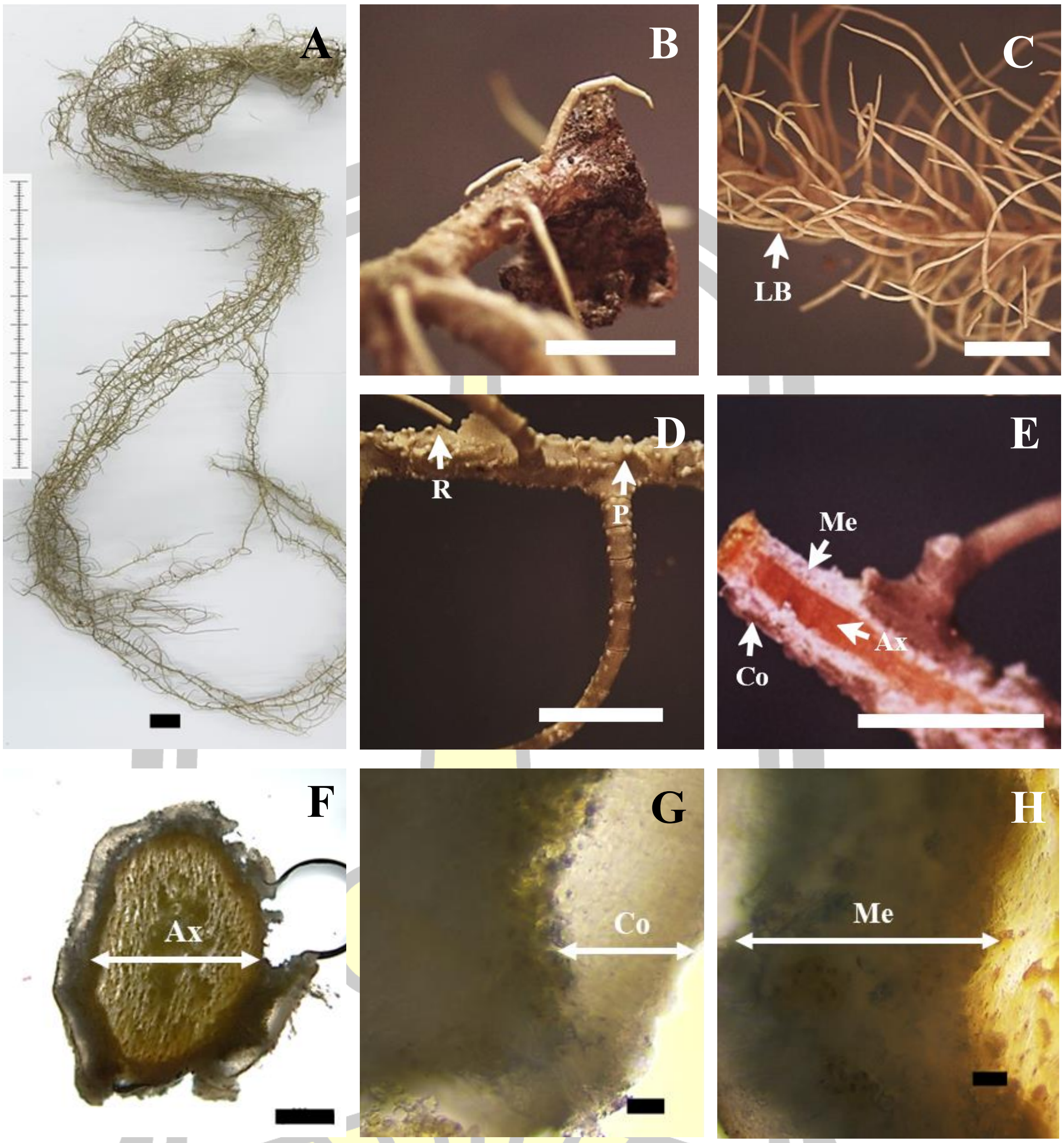


**Figure 33** Characteristics of *Usnea himantodes* Stirt. (PH05)

A) Habitat (Scale = 1 cm) B) Base C) Lateral branch (LB) D) Ridged branch (R) and Papillae (P) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) (Scale = 1 mm) F) Axis (Ax) (Scale = 200 µm) G) Cortex (Co) and H) Dense hyphae in medulla (Me) (Scale = 100 µm)

**7. *Usnea pangiana* Stirt.**

***Morphology:*** Thallus fruticose, shrub-like, small, 2.5 cm long, 2 mm diameter, base attached to twig, sepia brown thallus, thallus uninflated, red pigment in cortex, medulla filled with dense hyphae, thallus with a compact central axis, numerous lateral branches, fibrils abundant, fibrils uneven (not smooth), presence of annulary cracked on branches, isidia absent, soredia absent, apothecia absent and pseudocyphellae not seen, papillae present (Figure 34)

***Anatomy:*** Thallus cross section (n=10) C = (84.9-) 99.7 (-111.1) µm; M = (162.8-) 190.3 (-210.6) µm; A = (302.6-) 320.9 (-352.1) µm; CMA ratio %C = (13.9-) 16.5 (-18.6); %M = (28.6-) 31.1 (-34.1); %A = (50.1-) 52.5 (-55.2); A/M = (1.5-) 1.7 (-1.9)

***Chemistry:*** Salazinic acid, Norstictic acid, Barbatic acid, Usnic acid and Atranorin detected by TLC

***Habitat:*** On *Shorea obtusa* Wall. ex Blume. in attitude 830 metres above sea level, Mixed Deciduous Forest

***Notes:*** The distinguished features of *U. pangiana* Stirt. are (1) red pigment in cortex (2) cracks on branches (3) medulla dense, compact axis (4) the presence of papillae (5) the presence of usnic acid, salazinic acid, norstictic acid, barbatic acid, and atranorin

***Specimens examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Queen Sirikit Botanic Garden, on a slope (rather steep), undergrowth bamboo and grass, 18°53′56.46″N 98°51′36.91″E, 27 June 1994, Kansri Boonpragob et al., 1926.1 — (RAMK040362)

***Distribution:*** Taiwan, Thailand

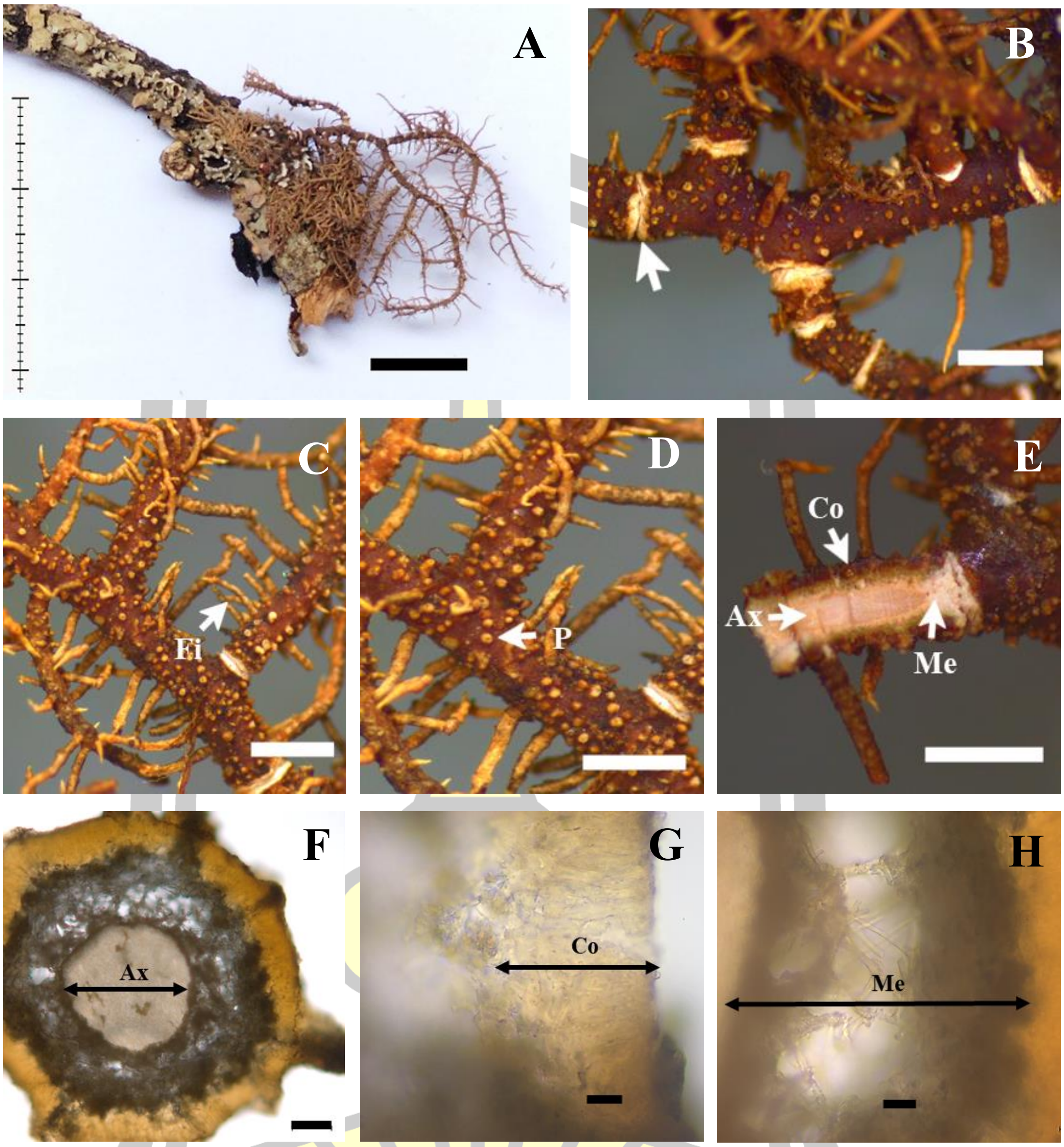


**Figure 34** Characteristics of *Usnea pangiana* Stirt. (RAMK040362)

A) Habitat (Scale = 1 cm) B) Main branch with segment C) Fibrils (Fi) D) Papillae (P) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) (Scale = 1 mm) F) Axis (Ax) (Scale = 100 µm) G) Red pigment in cortex (Co) and H) Dense hyphae in medulla (Me) (Scale = 20 µm)

**8. *Usnea* cf. *perhispidella* J. Steiner**

***Morphology:*** Thallus fruticose, beard-like, 6.5 cm long, 1 mm diameter, blackish-brown base, chocolate brown in herbarium, unknown when fresh, thallus uninflated, red pigment in cortex, thallus with a medullary thread visible by stretching branches, medulla filled with dense hyphae, compact central axis, numerous lateral branches, fibrils present, isidia abundant, soredia absent, apothecia and pseudocyphellae not seen, papillae absent (Figure 35)

***Anatomy:*** Thallus cross section (n=10) C = (71.6-) 78.8 (-89.3) µm; M = (136.1-) 172.7 (-225.0) µm; A = (288.6-) 310.8 (-338.4) µm; CMA ratio %C = (11.7-) 14.1 (-15.4); %M = (26.6-) 29.8 (-35.8); %A = (50.1-) 56.4 (-58.9); A/M = (1.4-) 1.9 (-2.2)

***Chemistry:*** Galbinic acid, Stictic acid, Salazinic acid, Barbatic acid, Diffractaic acid, Squamatic acid, Usnic acid, Atranorin detected by TLC

***Habbitats:*** On *Dipterocarpus obtusifolius* Teijsm.ex Miq. (Dry Diterocarp Frorest) 1125 — (RAMK035468); on *Prunus cerasoides* D. Don. (Lower Montane Rain Forest) — (RAMK040363)

***Notes:*** The distinguished features of *U.* cf. *perhispidella* J. Steiner are (1) red pigment in cortex (2) numerous isidia and soredia (3) the presence of stictic acid (4) the presence of galbinic acid similar to the specimen from East African, but it must be confirmed by DNA checking.

***Specimens examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Queen Sirikit Botanic Garden, 3 February 1994, Piboon Mongkolsuk et al., 1125 — (RAMK035468); Phitsanulok Province, Phu Hin Rong Kla National Park, Phu Man Khao, a paved roadside walkway before reaching the parking lot, about 100-200 meters, 31 March 2004, Sitthiporn Parnmen and Natwida Duangphui, ND.544 — (RAMK040363)

***Distribution:*** East Africa, Taiwan, Thailand

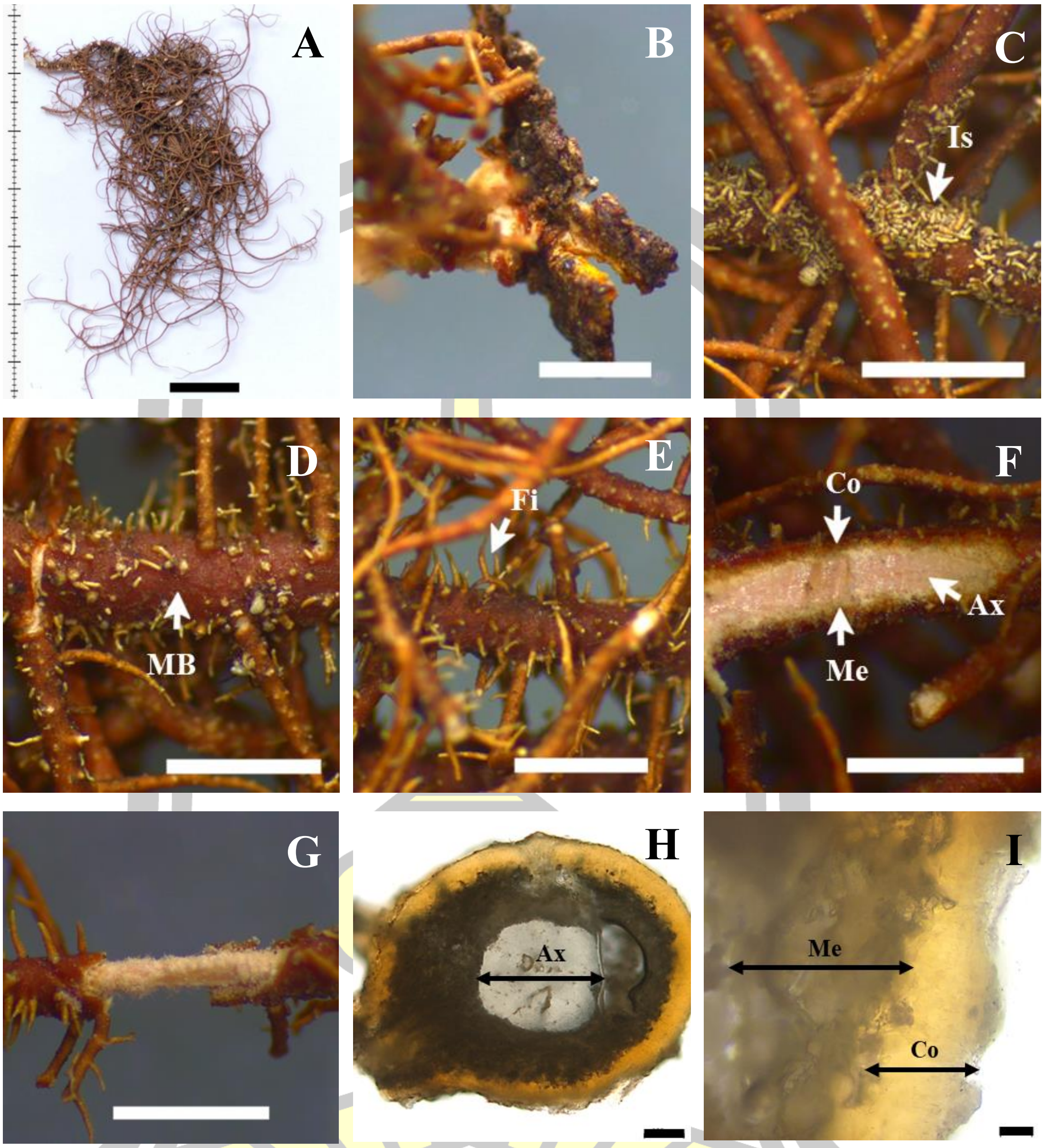


**Figure 35** Characteristics of *Usnea perhispidella* J. Steiner (RAMK040363)

A) Habitat (Scale = 1 cm) B) Base C) Isidia (Is) D) Main branch (MB) E) Fibrils (Fi) F) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and G) Medullary thread (Scale = 1 mm) H) Axis (Ax) (Scale = 100 μm) I) Dense hyphae in medulla (Me) and orange pigment in cortex (Co) (Scale = 20 μm)

**9. *Usnea* cf. *pectinata* Taylor**

***Morphology:*** Thallus fruticose, pendent, up to 68 cm long, 0.5 mm diameter, brownish base, pale brown or olive yellow when dry, pale green when fresh, thallus uninflated, thallus uneven and smooth (some branches), thallus rigid, thallus with a medullary thread visible by stretching branches, non-pigment in cortex, medulla filled with dense hyphae and thin, compact central axis and thick, lateral branches with segment, fibrils abdsent, isidia present, soredia present to none, apothecia and pseudocyphellae not seen, papillae absent (Figure 36)

***Anatomy:*** Thallus cross section (n=10) C = (45.2-) 56.1 (-75.6) µm; M = (74.1-) 115.1 (-153.6) µm; A = (350.7-) 379.9 (-399.2) µm; CMA ratio %C = (7.6-) 9.6 (-14.6); %M = (14.7-) 21 (-25.9); %A = (66.5-) 68.2 (-73.7); A/M = (2.6-) 3.3 (-5.0)

***Chemistry:*** 2'-O-Demethylpsoromic acid, Galbinic acid, Evernic acid, Usnic acid, Consticti acid, Baeomycesic acid, Norstictic acid, Psoromic acid, Atranorin detected by TLC

***Habitat:*** On *Castanopsis* sp., in attitude 1,400 metres above sea level — (RAMK035462); On an unidentified tree in attitude 1,200 metres above sea level (Lower Montane Rain Forest) — (RAMK040356);

***Notes:*** This specimen resembles *U. pectinata* in having (1) decorticated thallus (2) the presence of maculae on branches (3) the absence of ridged branches, but it is distinguished by the absence of stictic acid as a major substance, but it must be confirmed by DNA checking. (Ohmura, 2012; Nadel et al., 2022; Gerlach et al., 2023)

***Specimens examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Queen Sirikit Botanic Garden, Doi Pha Klong, 7 November 1993, Piboon Mongkolsuk et al., 3146 — (RAMK035462); Phitsanulok Province, Phu Hin Rong Kla National Park, Air Raid Shelter, 24 February 1998, Ratchada Noicharoen, 10660 — (RAMK040356); Politics and Military School, 24 November 2020, Areerat Saisong, Chanthanaphat Champatasi, Nucharee Chamnantab, and Khwanyuruan Naksuwankul — (PH04); Kang Hun Nam (water turbine), 24 November 2020, Areerat Saisong — (PH08)

***Distribution:*** Japan, Thailand, Taiwan, Philippines, Eastern Africa (Ohmura, 2001; Ohmura, 2012)

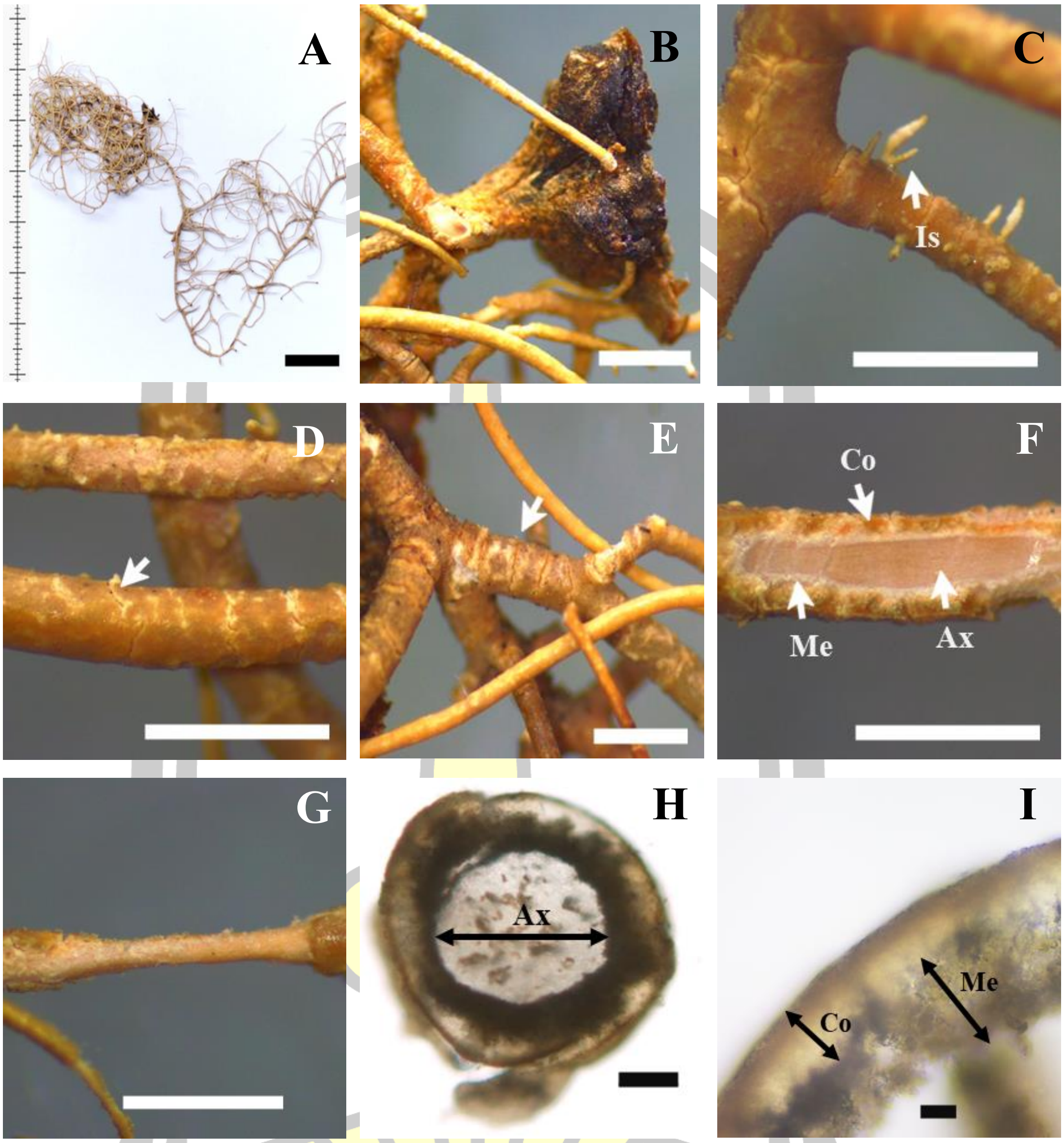


**Figure 36** Characteristics of *Usnea* cf. *pectinata* Taylor (RAMK040356)

A) Habitat (Scale = 1 cm) B) Base C) Isidia (Is) D) Cracking on branch E) Thallus uneven F) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and G) Medullary thread (Scale = 1 mm) H) Cross section (Ax = Axis) I) Dense hyphae in medulla (Me) (Scale = 100 µm) and thin cortex (Co) (Scale = 20 µm)

**10. *Usnea rubicunda* Stirt.**

***Synonymy:*** *Usnea protensa* Stirt. (1883)

*Usnea rubicunda* var. *spilota* (Stirt.) G.N. Stevens (1999)

*Usnea spilota* Stirt. (1882)

*Usnea sublurida* Stirt. (1898)

***Morphology:*** Thallus fruticose, shrub-like, small, 4.5 cm long, 0.5 mm diameter, dark brown base, olive green when fresh, turn red when old, thallus uninflated, thallus with a medullary thread visible by stretching branches, red pigment in cortex, medulla filled with dense hyphae (specimen from Queen Sirikit Botanic Garden, Doi Inthanon, Mon Long, Chiang Mai; Phu Hin Rong Kla National Park, Phitsanulok), medulla loose (specimen from Hod District, Chaing Mai; Phu Thab Buek, Phetchabun; Phu Ruea National Park; Loei), compact axis, fibrils present, many isidiomorphs develops on soredia, apothecia and pseudocyphellae not seen, papillae absent (Figure 37)

***Anatomy:*** Thallus cross section (n=10) C = (94.8-) 113.8 (-130.5) µm; M = (156.6-) 180.5 (-210.1) µm; A = (316.3-) 336.9 (-355.8) µm; CMA ratio %C = (15.2-) 18.1 (-20.9); %M = (25.5-) 28.5 (-32.9); %A = (49.6-) 53.8 (-56.3); A/M = (1.5-) 1.9 (-2.2)

***Chemistry:*** Salazinic acid (+), Constictic acid (±), Stictic acid (+), Barbatic acid (±), Menegazziaic acid (±), Galbinic acid (±), 4-O-Demethylbarbatic acid (±), 2’-O-Demethylpsoromic acid (±), Usnic acid, Atranorin detected by TLC

***Habitat:*** On *Conifer* sp. in attitude 1,700 metres above sea level — (RAMK025747); On *Pinus kesiya* Royle ex Gordon. In attitude 1,450 metres above sea level, Lower Montane Rain Forest — (RAMK040353); on *Tabebuia chrysantha* (Jacq.) G.Nicholson — (RAMK040367); On *Shorea obsuta* Wall ex Blume. (Mixed Deciduous Forest) in attitude 830 metres above sea level — (RAMK040372); On *Schima wallichii* Choisy in attitude 1,296.4 metres above sea level — (ML01, ML02); On *Pinus kesiya* Royle ex Gordon in attitude 1,371.3 metres above sea level — (ML08, ML09, ML10); On *Gluta laccifera* (Pierre) Ding Hou in attitude 1,413.0 metres above sea level — (ML13); On twigs of a *Zanthoxylum limonella* Alston in attitude 1,413.4 metres above sea level — (ML14); On twigs of a *Castanopsis* sp. in attitude 1,413.9 metres above sea level — (ML16); On *Erythrina subumbrans* (Hassk.) Merr. in attitude 1,292.4 metres above sea level — (ML18); On *Erythrina subumbrans* (Hassk.) Merr. in attitude 1,309.4 metres above sea level — (ML20)

***Notes:*** The distinguished features of *U. rubicunda* Stirt. Are (1) red pigment in cortex (2) presence of isidiomorphs (3) medulla dense in specimens from Queen Sirikit Botanic Garden, Doi Inthanon, Mon Long, Chiang Mai; Phu Hin Rong Kla National Park, Phitsanulok, medulla loose in specimens from Hod District, Chaing Mai; Phu Thab Buek, Phetchabun; Phu Ruea National Park; Loei) (4) the presence of usnic acid, stictic acid, constictic acid, menegazziaic acid, and atranorin as major substances.

***Specimens examined:*** THAILAND. Chiang Mai Province, Hod District. 10 August 1996, Aruni Chantarasanit — (RAMK025747); Chiang Mai Province, Mae Rim District, Queen Sirikit Botanic Garden, Doi Pha Klong. 7 November 1994, Kansri Boonpragob et al., 3107 — (RAMK035477); 8 November 1994, Kansri Boonpragob et al., 3348 — (RAMK040353); on a slope (rather steep), undergrowth bamboo and grass, 18°53′56.46″N 98°51′36.91″E, 27 June 1994, Kansri Boonpragob et al., 19311 — (RAMK040372); Chiang Mai Province, Doi Inthanon National Park, 16 January 1995, Piboon Mongkolsuk et al., 4122.1A — (RAMK040354, RAMK040369); Siripoom Waterfall, 9 August 2005, Nirun Janthawong et al., 290 — (RAMK040367); Phitsanulok Province, Phu Hin Rong Kla National Park, 24 February 1998, Kajohnsak Vongshewarat, Wetchasart Polyiam and Ratchada Noicharoen, 10541 — (RAMK040370); Man Daeng Waterfall, 3 March 2004, Natwida Duangphui and Sitthiporn Parnmen, ND.778 — (RAMK040360); Kang Han Nam (water turbine) waterfall in lower montane rain forest, 5 June 2003, Natwida Duangphui, ND.456 — (RAMK040366); 25 January 2020, Areerat Saisong, Chanthanaphat Champatasi, and Nucharee Chamnantab — (PH02, PH03, PH06); Chiang Mai Province, Mae Rim District, Mon Long, 18°92′92″N 98°83′32″E, 9 August 2022, Areerat Saisong, Orathai Sertsri, and Wattana Tanming — (ML01, ML02, ML04); 18°92′31″N 98°83′33″E, 10 August 2022, Areerat Saisong, Orathai Sertsri, and Wattana Tanming — (ML05, ML06, ML08, ML09, ML10, ML13, ML14, ML15, ML16, ML18, ML20); Phetchabun Province, Pha Hua Sing, Phu Thab Buek, 3 December 2023, Areerat Saisong and Orathai Sertsri — (PT08); Loei Province, Phu Ruea District, Phu Ruea National Park, 4 November 2020, Areerat Saisong — (PR03, PR04)

***Distribution:*** Thailand, Taiwan, Japan (Ohmura, 2001; Ohmura, 2012)

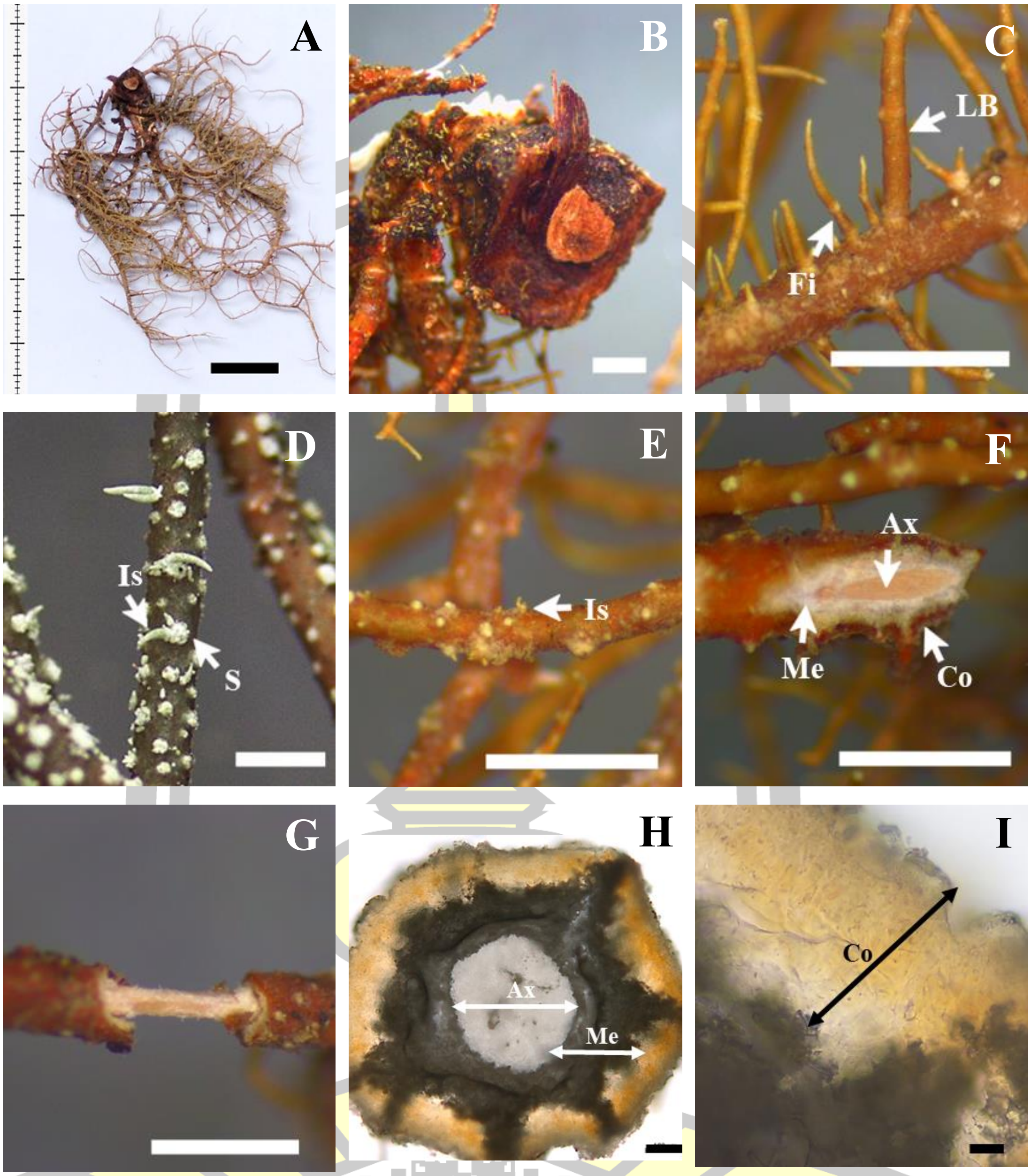


**Figure 37** Characteristics of *Usnea rubicunda* Stirt. (RAMK040366)

A) Habitat (Scale = 1 cm) B) Base C) Lateral branch (LB) and Fibrils (Fi) D) Soredia (S) E) Isidia (Is) F) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and G) Medullary thread (Scale = 1 mm) H) Axis (Ax) and dense hyphae in medulla (Me) (Scale = 100 µm) I) Red pigment in cortex (Co) (Scale = 20 µm)

**11. *Usnea* cf. *rubicunda* Stirt.**

***Morphology:*** Thallus fruticose, beard-like, small, 5-7 cm long, brownish base, beige grayish green when fresh, dry specimens pale green, thallus uninflated, thallus with a medullary thread visible by stretching branches, red pigment in cortex, medulla filled with dense hyphae and thin, fibrils present, compact axis, fibrils present, many isidiomorphs develops on soredia, apothecia and pseudocyphellae not seen, papillae absent (Figure 38)

***Anatomy:*** Thallus cross section (n=10) C = (39.8-) 46.5 (-57.3) µm; M = (74.9-) 90.1 (-107.6) µm; A = (363.9-) 372.5 (385.8) µm; CMA ratio %C = (7.5-) 9.2 (-11.3); %M = (15.6-) 17.5 (-20.2); %A = (70.6-) 73 (-76); A/M = (3.5-) 4 (-4.9)

***Chemistry:*** Barbatic acid, Norstictic acid, 4-O-Demethylbarbatic acid, Usnic acid, Atranorin, and Eumitrin A detected by TLC

***Habitat:*** On twigs of a *Schima wallichii* Choisy in attitude 1,403.4 metres above sea level

***Notes:*** This species resembles *U. rubicunda* in having (1) red pigment in cortex (2) thallus uninflated (3) isidiomorphs present, but it is distinguished from *U. rubicunda* by the absence of stictic and salazinic acid as major substances

***Specimen examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Mon Long, 18°92′08″N 98°83′98″E. 10 August 2022, Areerat Saisong, Orathai Sertsri, and Wattana Tanming — (ML07)

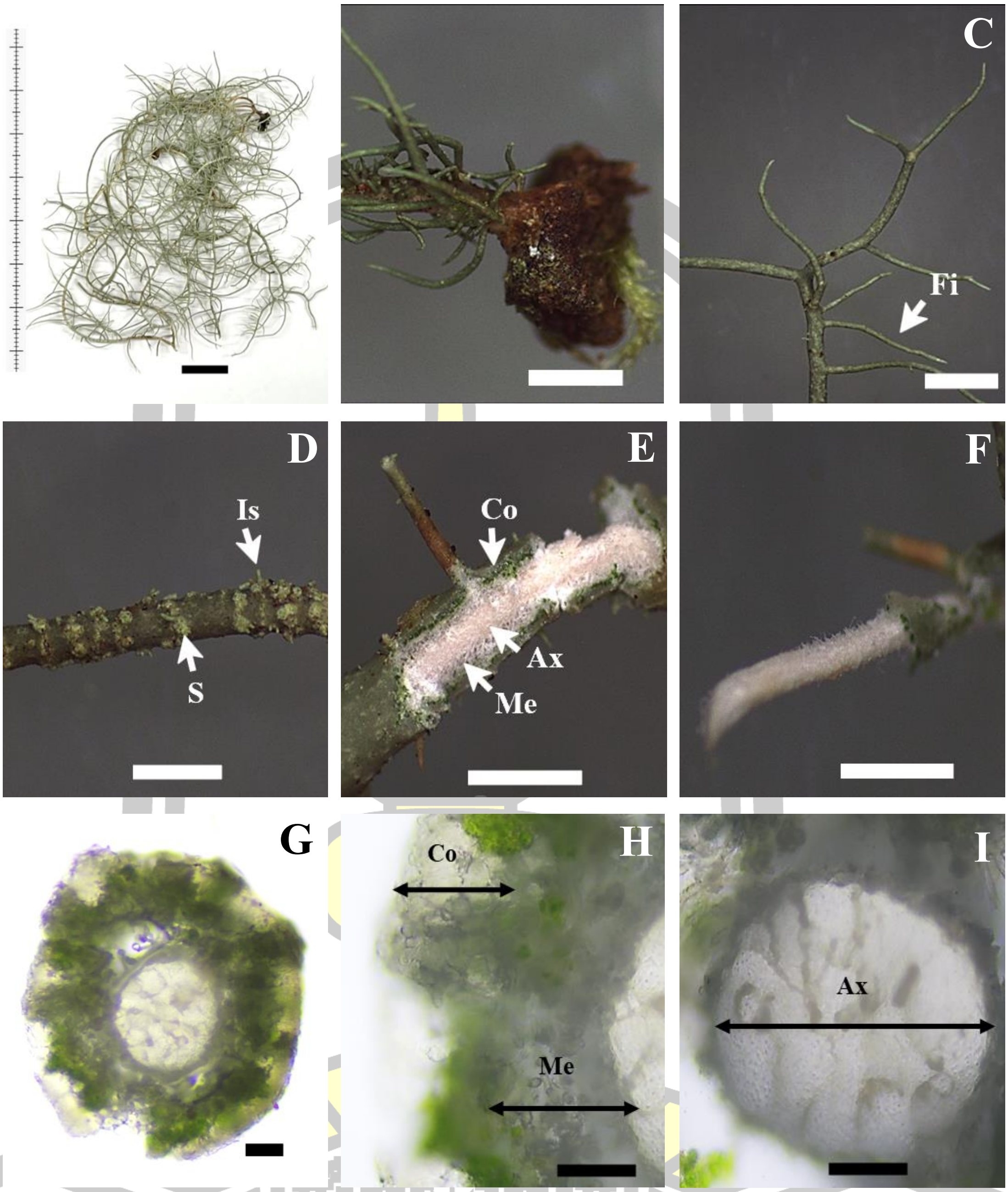


**Figure 38** Characteristics of *Usnea* cf. *rubicunda* Stirt. (ML07)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Soredia (S) and Isidia (Is) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) F) Medullary thread (Scale = 1 mm) G) Thallus cross section (Scale = 100 µm) H) Hyaline cortex (Co) and dense hyphae in medulla (Me) I) Axis (Ax) (Scale = 50 µm)

**12. *Usnea shimadae* Asahina**

***Morphology:*** Thallus fruticose, shrub-like, 1.3-1.5 cm long, 1 mm diameter, lacking base, dark liver or coffee in herbarium, unknown when fresh, thallus uninflated, red pigment in subcortical, thallus with a medullary thread visible by stretching branches, medulla filled with dense hyphae, hollow central axis and thick, fibrils abundant, isidia absent, soredia absent, reddish brown disc-like apothecia present, fibrils on exciple of apothecia, pseudocyphellae absent, papillae present (Figure 39)

***Anatomy:*** Thallus cross section (n=10) C = (56.7-) 66.9 (-84.0) µm; M = (81.9-) 96.0 (-114.4) µm; A = (714.9-) 797.3 (-866.7) µm; CMA ratio %C = (6.0-) 6.6 (-9.2); %M = (8.8-) 9.6 (-12.4); %A = (81.0-) 83.1 (-85.2); A/M = (6.5-) 8.7 (-9.7); 1 Ascus consists of 5-7 ascospores, about 4.6 x 3.5 µm.

***Chemistry:*** Salazinic acid, 4-O-Demethylbarbatic acid, Evernic acid, Usnic acid, Atranorin, and EumitrinA detected by TLC

***Habitat:*** Open canopy mise with Pinus and Fern other sp., on *Castanopsis costata* Miq. (Dry Dipterocarp Forest)

***Notes:*** The distinguished features of *U. shimadae* Asahina are (1) red pigmentation in subcortical (2) dense hyphae and hollow axis (3) fibrils abundant, apothecia present with fibrils on exciple (4) presence of salazinic acid as a major substance

***Specimens examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Queen Sirikit Botanic Garden, the tree is very close to dirt road about 5 meters, facing south. 23 November 1998, P. Suksathan, 1537 — (RAMK040350)

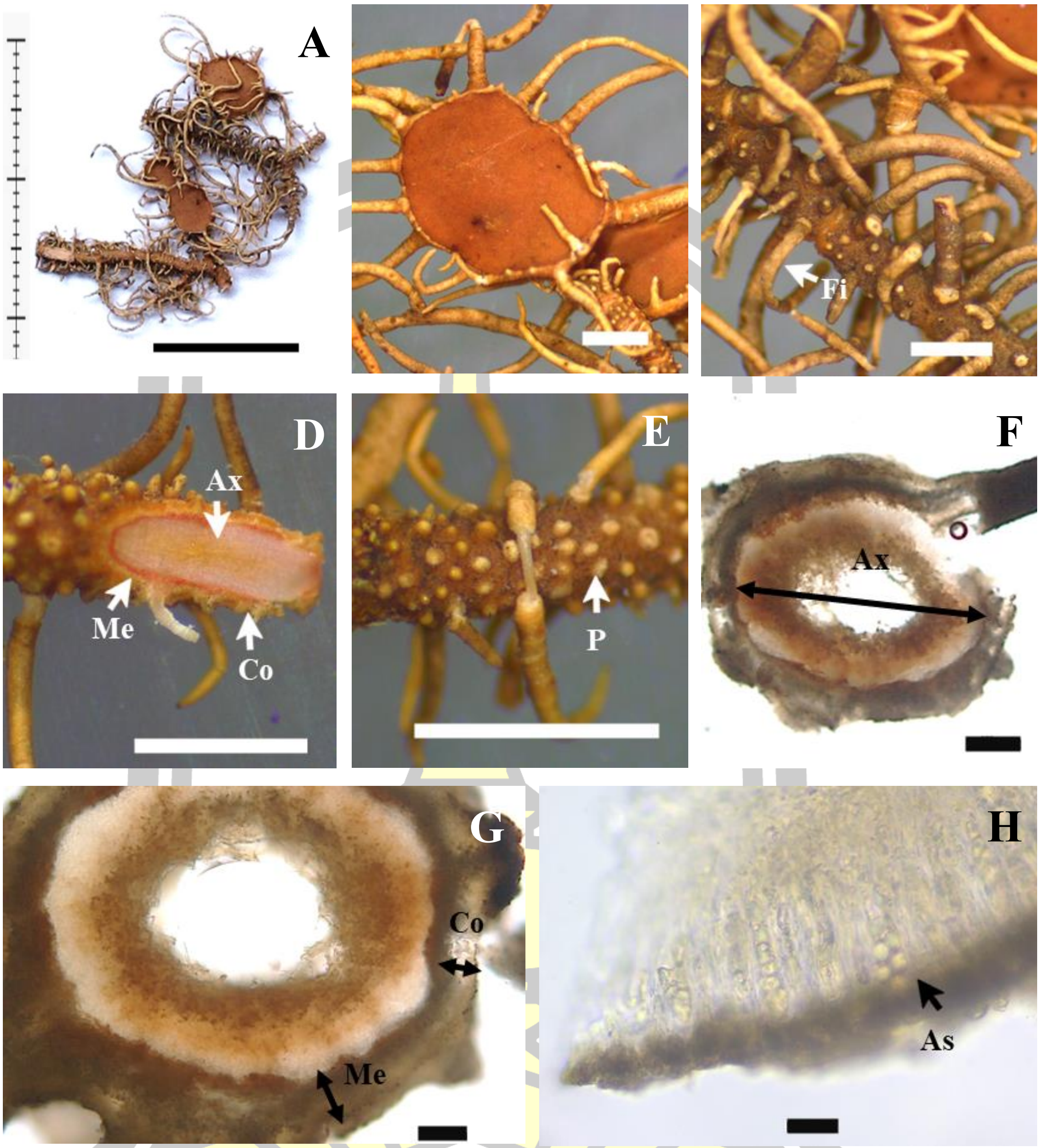


**Figure 39** Characteristics of *Usnea shimadae* Asahina (RAMK040350)

A) Habitat (Scale = 1 cm) B) Disk-like apothecia C) Fibrils (Fi) D) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and E) Papillae (P) (Scale = 1 mm) F) Cross section (Ax = Axis) G) Thin medulla (Me) (Scale = 100 µm) and cortex (Co) (Scale = 20 µm) and H) Ascospore (AS) (Scale = 10 µm)

**13. *Usnea* sp.1 (RAMK040359)**

***Morphology:*** Thallus fruticose, thallus incomplete, lacking base, 4 cm long, 1 mm diameter, beige brown in herbarium, unknown when fresh, thallus inflated like sausage, thallus with a medullary thread visible by stretching branches, thin cortex non-pigment, medulla filled with very loose hyphae, compact axis and thin, fibrils uneven (not smooth), few of isidia present, soredia absent, apothecia and pseudocyphellae not seen, papillae absent (Figure 40)

***Anatomy:*** Thallus cross section (n=10) C = (60.1-) 72.7 (-85.6) µm; M = (443.8-) 476.3 (-507.3) µm; A = (204.8-) 220.5 (-242.0) µm; CMA ratio %C = (8.3-) 9.4 (-10.4); %M = (59.3-) 61.8 (-64.4); %A = (26.3-) 28.7 (-31.3); A/M = (0.4-) 0.5 (-0.5)

***Chemistry:*** Norstictic acid, Stictic acid, Psoromic acid, Galbinic acid acid, and Atranorin detected by TLC

***Habitat:*** On *Pinus* sp. (Lower Montane Rain Forest)

***Notes:*** The specimen of *Usnea* sp.1 (RAMK040359) resembles *U. articulata* (L.) Hoffm. in having (1) thallus inflated like sausage (2) medulla loose (3) presence of isidia and soredia, but it is distinguished by (1) the absence of protocetraric acid and fumarprotocetraric acid (2) Fibrils uneven

***Specimens examined:*** THAILAND. Phitsanulok Province, Phu Hin Rong Kla National Park, Lan Hin Taek, approximately 20-30 meters from the entrance, mound leading up to the shop beside Than-Phatcharin. 1 June 2003, Chonnikan Thunyagun, Chutamat Phraphuchamnong and Natwida Duangphui, ND.241 — (RAMK040359)

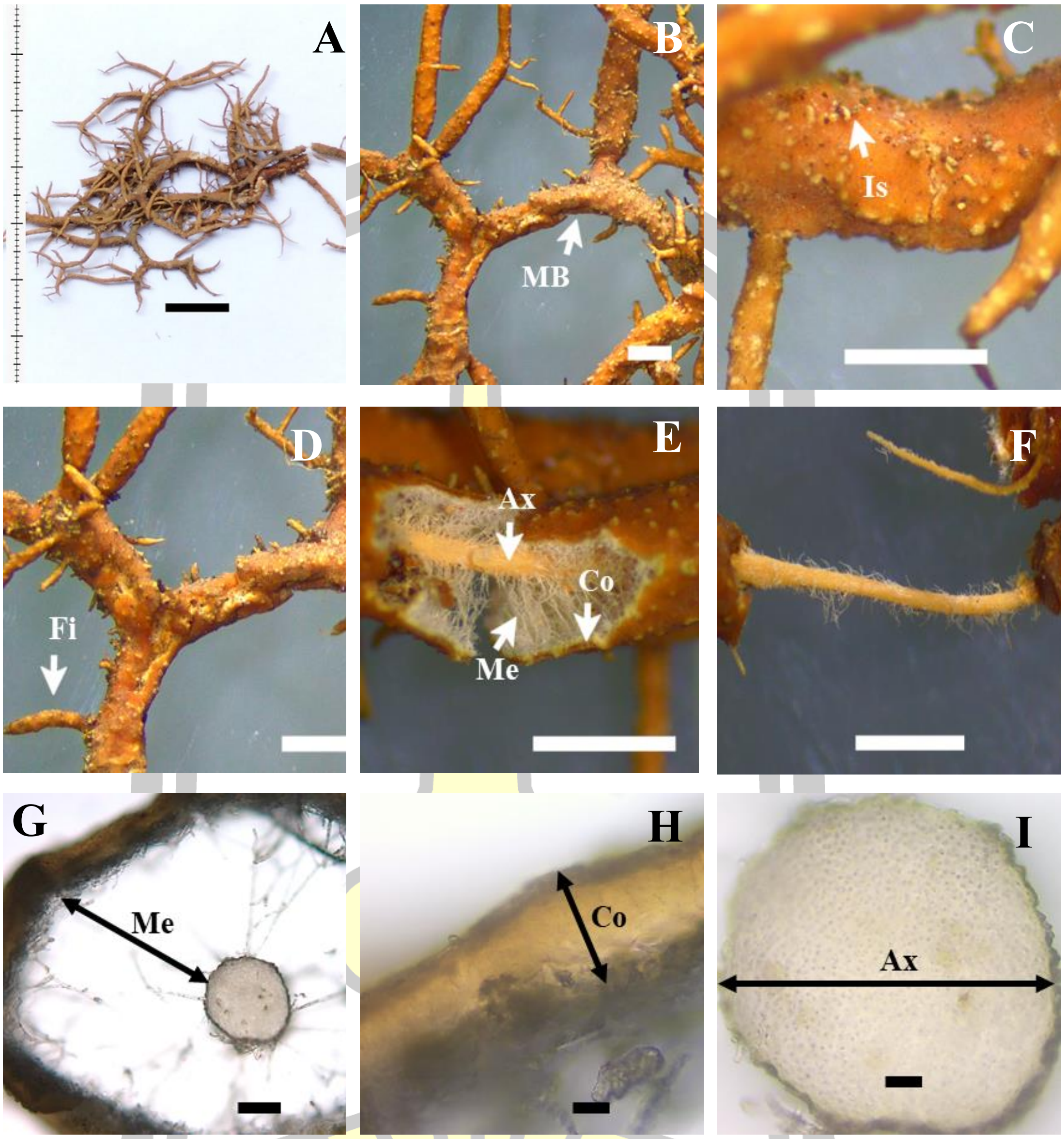


**Figure 40** Characteristics of *Usnea* sp.1 (RAMK040359)

A) Habitat (Scale = 1 cm) B) Main branch bulbous C) Isidia (Is) D) Fibrils (Fi) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and F) Medullary thread (Scale = 1 mm) G) Loose hyphae in medulla (Me) (Scale = 100 µm) H) Cortex (Co) and I) Axis (Ax) (Scale = 20 µm)

**14. *Usnea* sp.2 (PT02)**

***Morphology:*** Thallus fruticose, shrub-like, 2-3 cm long, blackish base, yellowish green to moderate olive green, thallus slightly inflated, thallus with a medullary thread visible by stretching branches, thin cortex non-pigment, medulla filled with loose hyphae and thick, thin compact axis, fibrils abundant with soredia, isidia absent, soredia present, apothecia and pseudocyphellae not seen, papillae absent (Figure 41)

***Anatomy:*** Thallus cross section (n=10) C = (76.4-) 87.0 (-95.6) µm; M = (248.9-) 298.9 (-377.7) µm; A = (155.3-) 277.2 (-369.3) µm; CMA ratio %C = (11.1-) 12 (-16.7); %M = (38.2-) 38-43 (-53.8); %A = (29.5-) 29-49.2 (-49.2); A/M = (0.5-) 1.1 (-1.3)

***Chemistry:*** Stictic acid, Galbinic acid, Diffractaic acid, Evernic acid, Usnic acid, Atranorin, and Eumitrin B detected by TLC

***Habitat:*** On twigs of an unidentified tree in attitude 1,768 metres above sea level

***Notes:*** The specimen of *Usnea* sp.2 (PT02) resembles *U. dasaea* in having (1) branches inflated with soredia (2) the presence of usnic, galbinic acid, and atranorin (3) dense medulla (Ohmura, 2012), but it is distinguished by the absent of spinulous fibrils

***Specimen examined:*** THAILAND. Phetchabun Province, Pha Hua Sing, Phu Thab Buek, 16°54′23″N 101°5′14″E. 8 April 2023, Areerat Saisong, Sutthiporn Chotinate, Primprapa Poosongsee — (PT02)

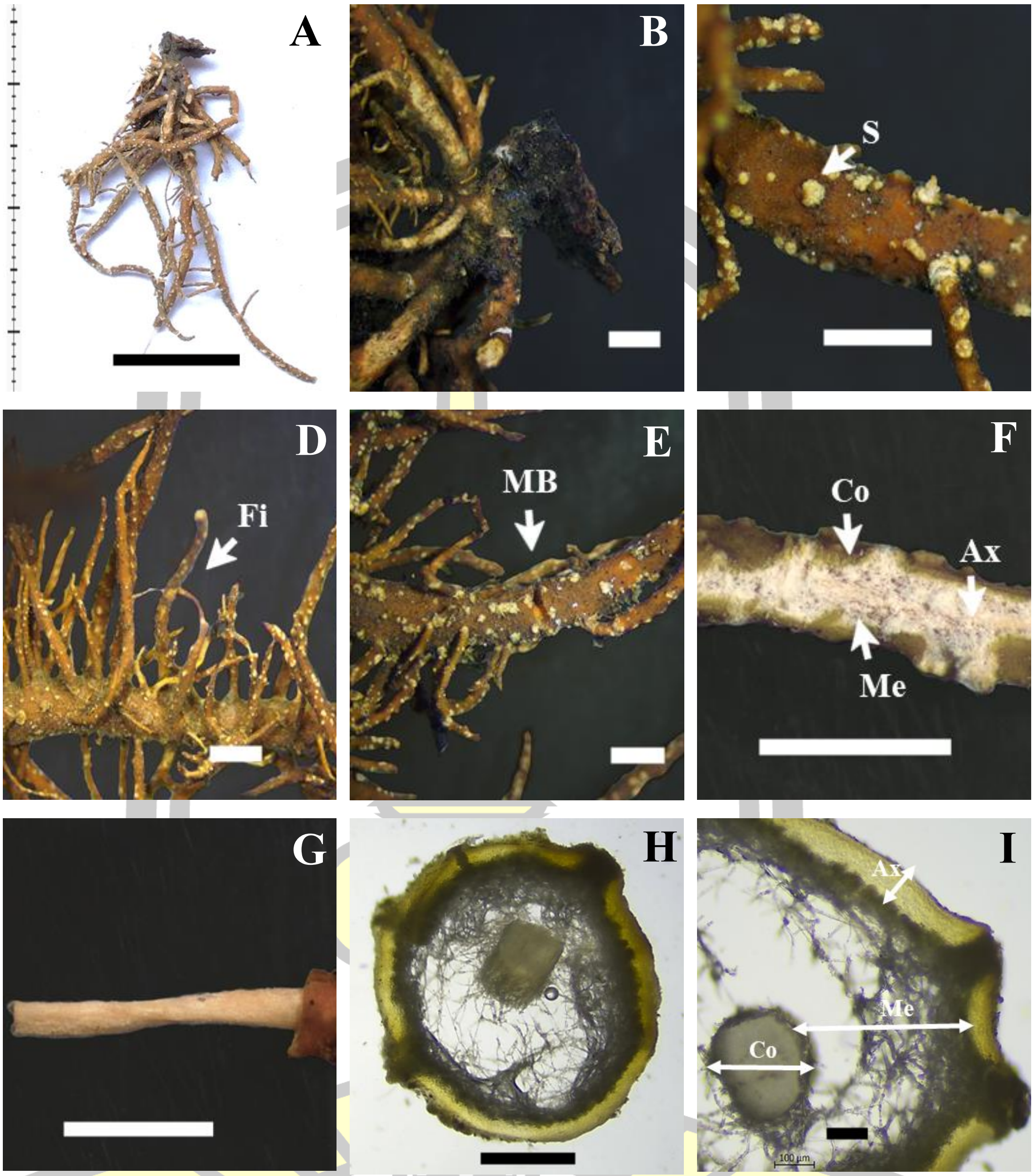


**Figure 41** Characteristics of *Usnea* sp.2 (PT02)

A) Habitat (Scale = 1 cm) B) Base C) Soredia (S) D) Fibrils (Fi) E) Main branch (MB) F) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and G) Medullary thread (Ax = Axis) (Scale = 1 mm) H) Thallus cross section (Scale = 500 µm) I) Cortex (Co) dense hyphae in medulla (Me) and axis (Ax) (Scale = 100 µm)

**15. *Usnea* sp.3 (PT04)**

***Morphology:*** Thallus fruticose, erect, tapered, 3-5 cm long, 0.5 mm diameter, blackish base, olive-yellow when fresh, pale brown in herbarium, thallus uninflated, thallus with a medullary thread visible by stretching branches, cortex non-pigment, thick medulla filled loose hyphae, thin compact axis, isidia absent, soredia present, apothecia absent and pseudocyphellae not seen, papillae absent (Figure 42)

***Anatomy:*** Thallus cross section (n=10) C = (67.5-) 83.1 (-101.1) µm; M = (194.8-) 221.4 (-246.4) µm; A= (159.6-) 185.2 (-217.7) µm; CMA ratio %C = (13.3-) 17 (-21); %M = (40.4-) 43 (-44.6); %A = (32.1-) 37 (-40.7); A/M = (0.7-) 0.7 (-1)

***Chemistry:*** Diffractaic acid, Evernic acid, Usnic acid acid, Atranorin, and Eumitrin B detected by TLC

***Habitat:*** On twigs of an unidentified tree in attitude 1,768 metres above sea level,

***Specimen examined:*** THAILAND. Phetchabun Province, Pha Hua Sing, Phu Thab Buek, 16°54′23″N 101°5′14″E. 8 April 2023, Areerat Saisong, Sutthiporn Chotinate, Primprapa Poosongsee — (PT04)

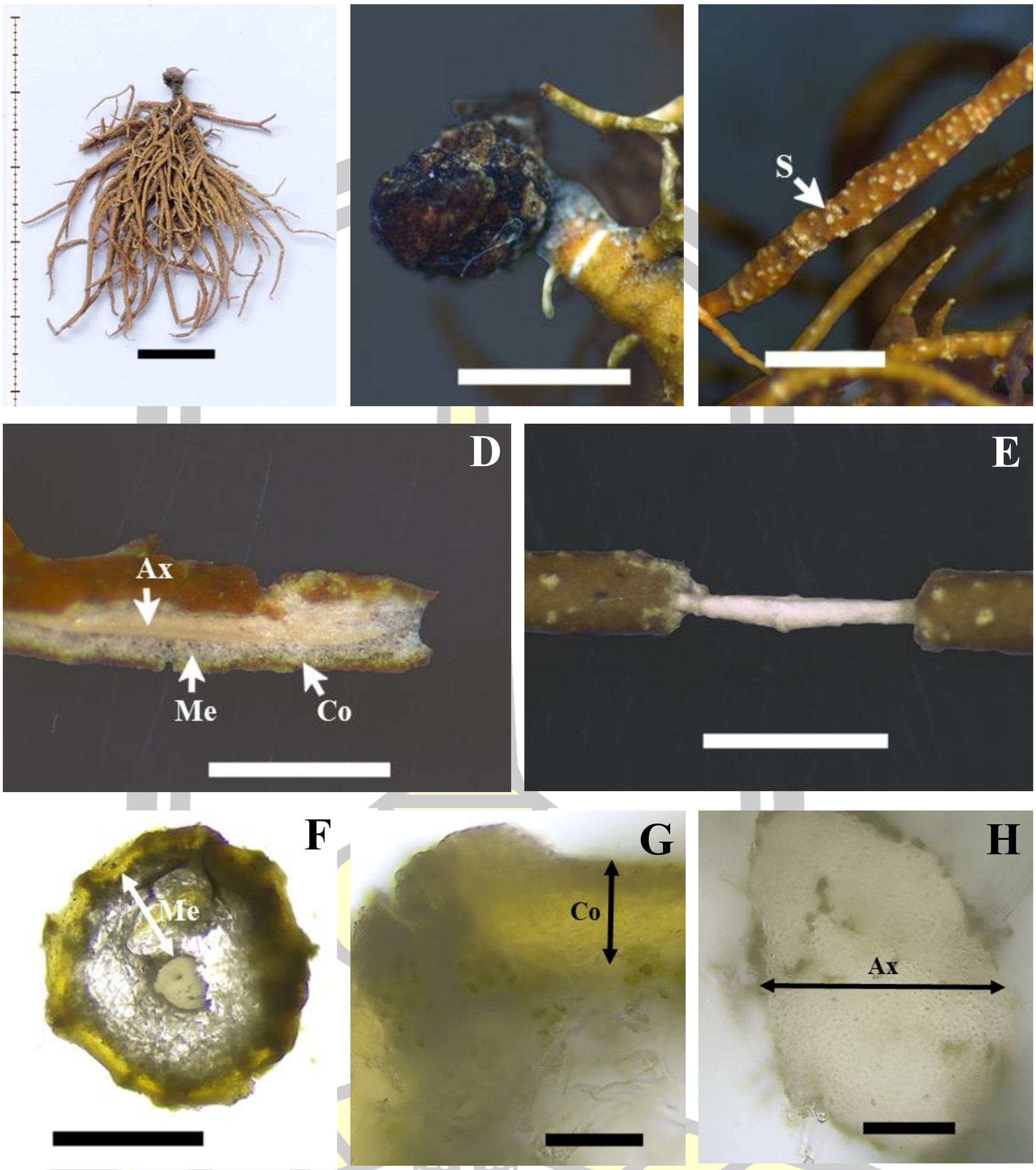


**Figure 42** Characteristics of *Usnea* sp.3 (PT04)

A) Habitat (Scale = 1 cm) B) Base C) Soredia (S) D) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and F) Medullary thread (Ax = Axis) (Scale = 1 mm) F) Loose hyphae in medulla (Me) (Scale = 500 µm) G) Cortex (Co) H) Axis (Ax) (Scale = 50 µm)

**16. *Usnea* sp.4 (PT05)**

***Morphology:*** Thallus fruticose, erect, tapered, 3-5 cm long, 0.5 mm diameter, blackish base, olive-yellow when fresh, pale brown in herbarium, thallus with a medullary thread visible by stretching branches, thin cortex non-pigment, thick medulla filled with loose hyphae, thin compact axis, fibrils absent, isidia absent, soredia present, apothecia and pseudocyphellae not seen, papillae absent (Figure 43)

***Anatomy:*** Thallus cross section (n=10) C = (74.8-) 95.6 (-114.9) µm; M = (337.4-) 396.0 (-492.0) µm; A = (170.4-) 238.6 (-317.1) µm; CMA ratio% C = (10.6-) 12 (15.4); %M = (47.3-) 57 (-58.2); %A = (28.8-) 29 (-40.7); A/M = (0.5-) 0.7 (-0.9)

***Chemistry:*** Usnic acid and Atranorin detected by TLC

***Habitat:*** On twigs of an unidentified tree in attitude 1,768 metres above sea level

***Notes:*** The specimen *Usnea* sp.4 (PT05) resembles *U. bismolliuscula* in having (1) inflated branches (2) the presence of irregular soredia (3) lax medulla and thin axis. It is distinguished by (1) the absence of stictic acid as a major substance (Ohmura, 2012)

***Specimen examined:*** THAILAND. Phetchabun Province, Pha Hua Sing, Phu Thab Buek, 16°54′23″N 101°5′14″E. 3 December 2023, Areerat Saisong and Orathai Sertsri — (PT05)

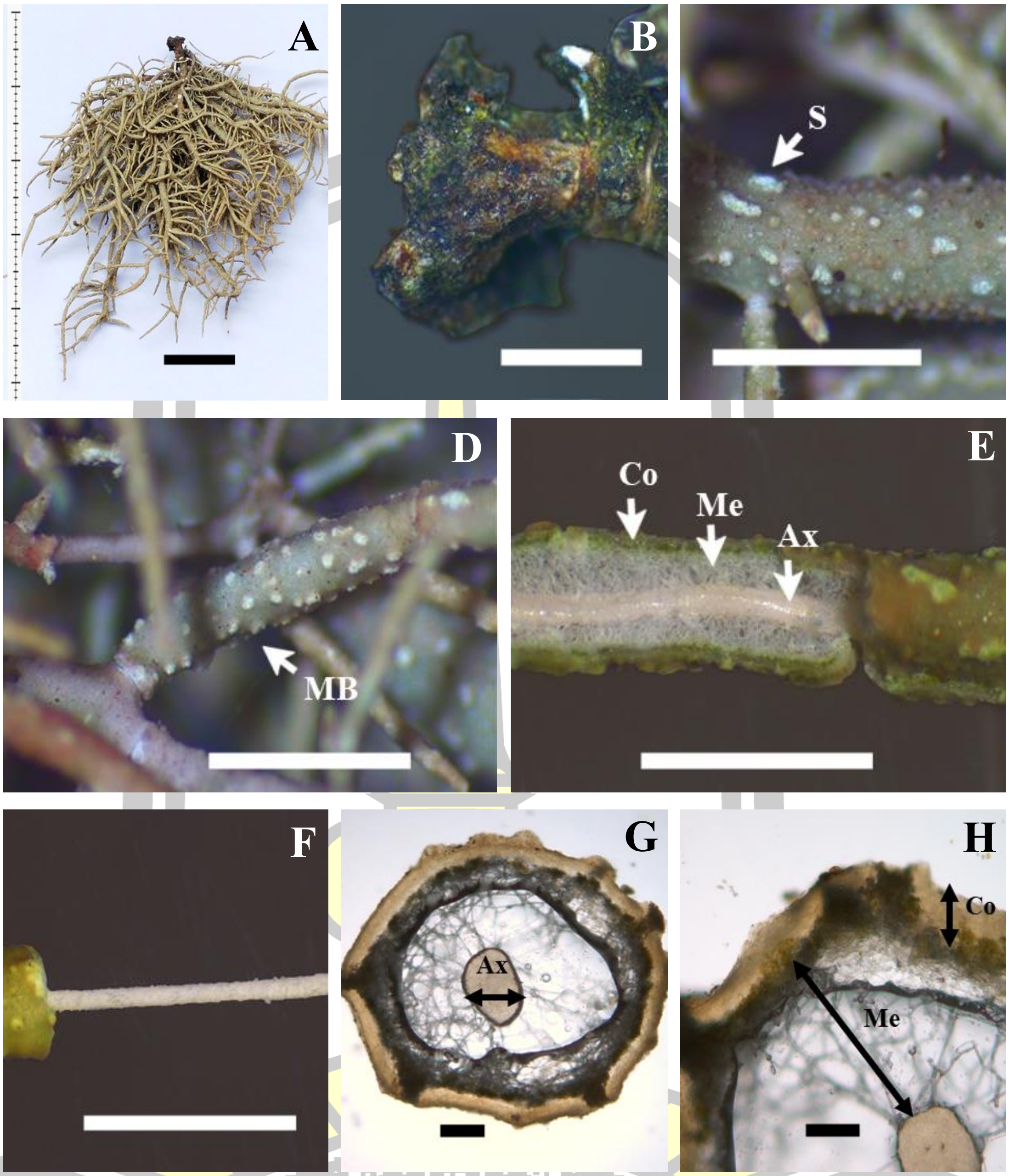


**Figure 43** Characteristics of *Usnea* sp.4 (PT05)

A) Habitat (Scale = 1 cm) B) Base C) Soredia (S) D) Main branch (MB) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) and F) Medullary thread (Ax = Axis) (Scale = 1 mm) G) Axis (Ax) (Scale = 200 µm) H) Loose hyphae in medulla (Me) I) Cortex (Co) (Scale = 100 µm)

**17. *Usnea* sp.5 (PT06)**

***Morphology:*** Thallus fruticose, 3-5 cm long, 1 mm diameter, blackish base, olive-yellow to green when fresh, pale brown in herbarium, thallus uninflated, thallus with a medullary thread visible by stretching branches, cortex non-pigment, medullar filled with loose hyphae, compact axis, smooth fibrils present, isidia absent, irregular soredia present, apothecia and pseudocyphellae not seen, papillae absent (Figure 44)

***Anatomy:*** Thallus cross section (n=10) C = (63.3-) 79.7 (-98.0) µm; M = (202.6-) 236.3 (-267.1) µm; A = (174.2-) 205.3 (-237.3) µm; CMA ratio %C = (11.7-) 15 (-18.6); %M = (38.8-) 47-48 (51.2); %A = (33.6-) 38 (-45.1); A/M = (0.7-) 0.8 (-1.2)

***Chemistry:*** Stictic acid, Diffractaic acid, Usnic acid, and Atranorin detected by TLC

***Habitat:*** On twigs of unidentified tree in attitude 1,768 m

***Notes:*** The specimen of *Usnea* sp.5 (PT06) resembles *U. bismolliuscula* (Y. Ohmura 6222, TNS) in having (1) the presence of usnic and stictic acid as a major substance (2) soralia irregular in shape (3) medulla lax It is distinguished by (1) the absence of perforation on branches (Ohmura, 2012)

***Specimens examined:*** THAILAND. Phetchabun Province, Pha Hua Sing, Phu Thab Buek, 16°54′23″N 101°5′14″E. 3 December 2023, Areerat Saisong and Orathai Sertsri — (PT05)

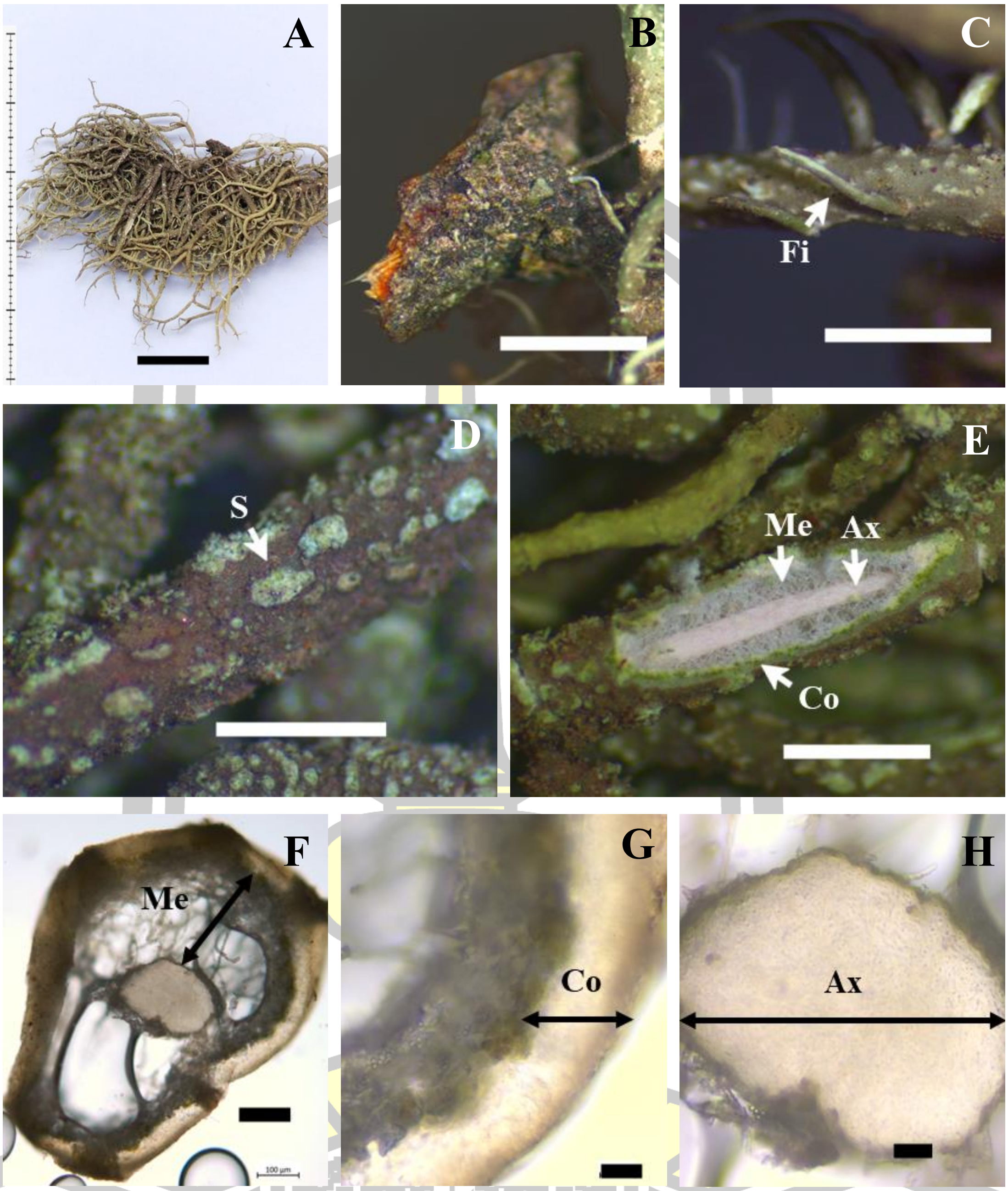


**Figure 44** Characteristics of *Usnea* sp.5 (PT06)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Soredia (S) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) (Scale = 1 mm) F) Loose hyphae in medulla (Me) (Scale = 100 µm) G) Cortex (Co) and H) Axis (Ax) (Scale = 20 µm)

**18. *Usnea* sp.6 (PT09)**

***Morphology:*** thallus fruticose, shrubby, 2-6 cm long, 1 mm diameter, blackish base, dark khaki to olive yellow when fresh, turn red when old, thallus uninflated, thallus with a medullary thread visible by stretching branches, cortex non-pigment, medulla filled with loose hyphae, thick compact axis, fibrils abundant covering the branches, isidia absent, soredia absent, apothecia and pseudocyphellae not seen, papillae present (Figure 45)

***Anatomy:*** Thallus cross section (n=10) C = (76.3-) 86.5 (-98.9) µm; M = (246.9-) 275.4 (-305.7) µm; A = (334.3-) 366.0 (-432.8) µm; CMA ratio %C = (10.7-) 11.7 (-13.2); %M = (32.7-) 36 (-41.9); %A = (46.4-) 51.5 (-56.4); A/M = (1.1-) 1.4 (-1.7)

***Chemistry:*** Stictic acid, Diffractaic acid, Usnic acid, and Atranorin detected by TLC

***Habitat:*** On twigs of an unidentified tree in attitude 1,768 metres above sea level

***Notes:*** The specimen of *Usnea* sp.6 (PT09) resembles *U. steineri* in having (1) fibrils densely covering the branches (2) soredia and isidia absent (3) papillae numerous (4) medulla filled with loose to dense hyphae (Truong et al., 2011). It is distinguished by (1) the absence of salazinic acid (2) apothecia absent

***Specimen examined:*** THAILAND. Phetchabun Province, Pha Hua Sing, Phu Thab Buek, 16°54′23″N 101°5′14″E. 3 December 2023, Areerat Saisong and Orathai Sertsri — (PT09)

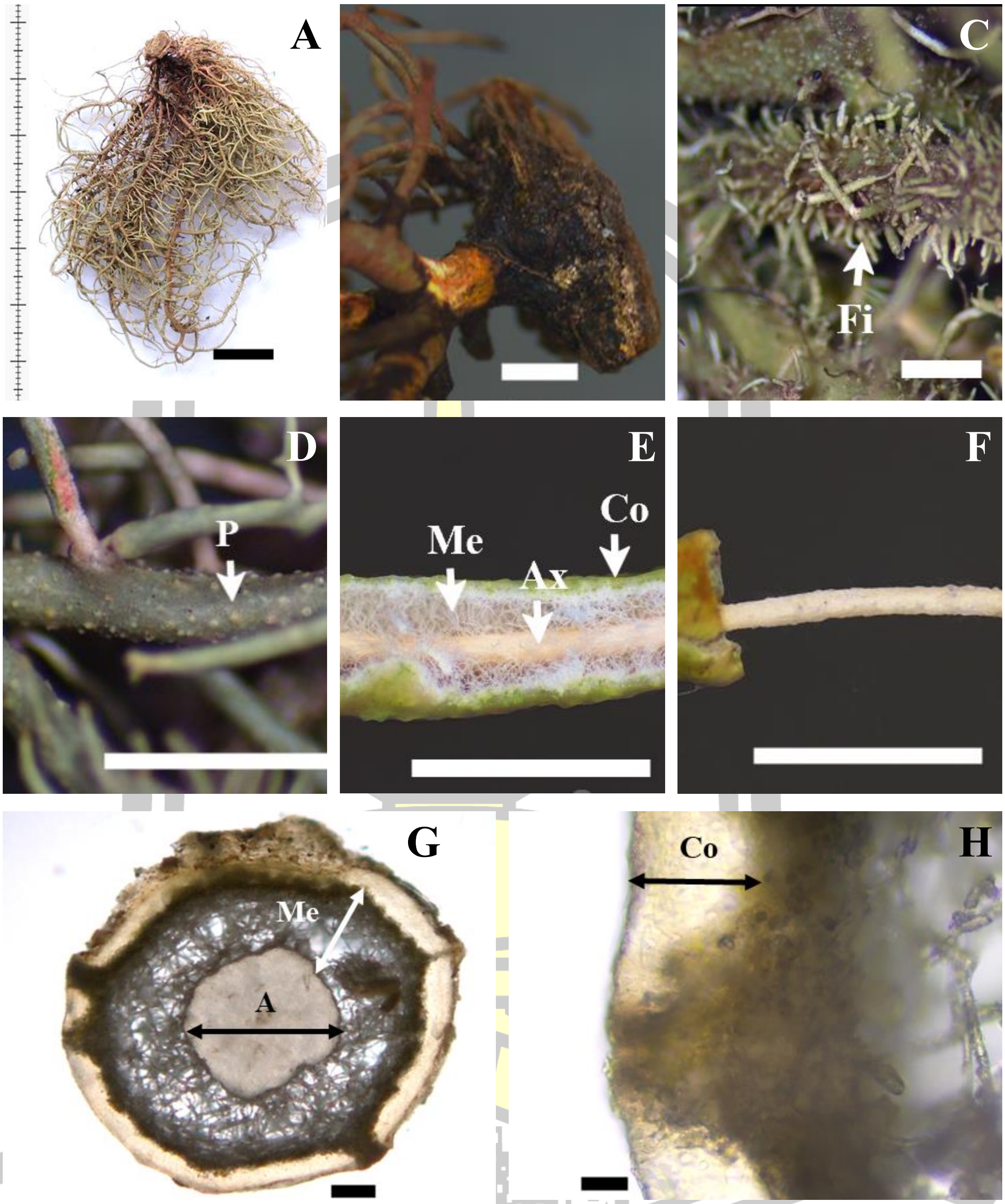


**Figure 45** Characteristics of *Usnea* sp.6 (PT09)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Papillae (P) E) Thallus layer (C = Cortex; Me = Medulla; Ax = Axis) and F) Medullary thread (Ax = Axis) (Scale = 1 mm) G) Dense hyphae in medulla (Me) and axis (Ax) (Scale = 200 µm) H) Cortex (C) I) Cortex (Co) (Scale = 20 µm)

**19. *Usnea* sp.7 (ML12)**

***Morphology:*** Thallus fruticose, beard-like, 6-7 cm long, 0.5 mm diameter, blackish base, olive green when fresh, pale green in herbarium, thallus uninflated, thallus with a medullary thread visible by stretching branches, cortex non-pigment, medulla filled with dense hyphae, compact axis, smooth fibrils present, isidia present, soredia abundant, apothecia absent and pseudocyphellae not seen, papillae absent (Figure 46)

***Anatomy:*** Thallus cross section (n=10) C = (89.7-) 110.2 (-131.8) µm; M = (212.4-) 257.2 (-316.6) µm; A = (303.9-) 344.2 (-392.2) µm; CMA ratio %C = (13-) 15.7 (-17.6); %M = (30.3-) 35.6 (-41.5); %A = (43-) 50 (-56); A/M = (1.0-) 1.1-1.5 (-1.8)

***Chemistry:*** Barbatic acid, 4-O-Demethylbarbatic acid, Baeomycesic acid, Squamatic acid, Physodalic acid, Usnic acid, and Atranorin detected by TLC

***Habitat:*** On twigs of a *Melia azedarach* Linn. in attitude 1,351.3 metres above sea level

***Notes:*** The specimen of *Usnea* sp.7 (ML12) resembles *U. rubicunda* Stirt. In having (1) thallus uninflated (2) isidiomorphs abundant, but it is distinguished by the absence of red pigment in cortex and stictic acid and salazinic absent

***Specimen examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Mon Long, 18°92′55″N 98°83′25″E. 10 August 2022, Areerat Saisong, Orathai Sertsri, and Wattana Tanming — (ML12)

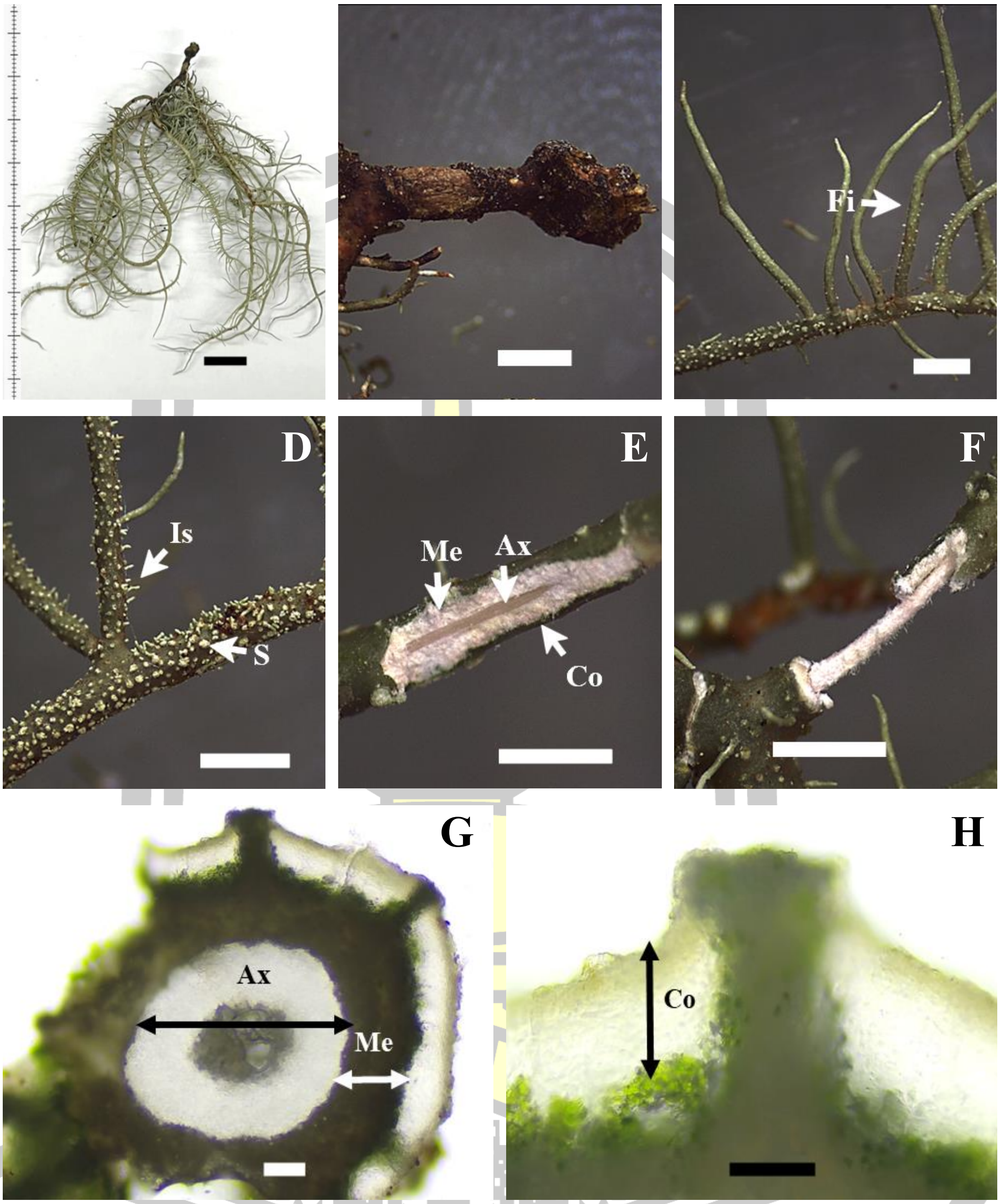


**Figure 46** Characteristics of *Usnea* sp.7 (ML12)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Soredia (S) and Isidia (Is) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) F) Medullary thread (Scale = 1 mm) G) dense hyphae in medulla (Me) and axis (Ax) (Scale = 100 µm) H) Hyaline cortex (Co) (Scale = 50 µm)

**20. *Usnea* sp.8 (ML17)**

***Morphology:*** Thallus fruticose, 3 cm long, 0.5 mm diameter, brownish base, olive green when fresh, pale green to brown in herbarium, thallus uninflated, thallus with a medullary thread visible by stretching branches, thin cortex non-pigment, thick medulla filled with loose hyphae, thin compact axis, fibrils abundant, isidia abundant, soredia absent, apothecia and pseudocyphellae not seen, papillae absent (Figure 47)

***Anatomy:*** Thallus cross section (n=10) C = (76.5-) 88.6 (-111.6) µm; M = (398.3-) 493.2 (-580.9) µm; A = (321.6-) 347.1 (-378.1) µm; CMA ratio %C = (7.6-) 9.7 (-11.1); %M = (48.9-) 53.5 (-57.2); %A = (34.3-) 37.1 (-41.3); A/M = (0.6-) 0.7 (-0.8)

***Chemistry:*** Norstictic acid, Salazinic acid, Stictic acid, Usnic acid, Eumitrin A, Eumitrin B, and Atranorin detected by TLC

***Habitat:*** On twigs of a *Craibiodendron stellatum* W.W. Smith in attitude 1,439.8 metres above sea level

***Specimen examined:*** THAILAND. Chiang Mai Province, Mae Rim District, Mon Long, 18°91′93″N 98°83′48″E. 10 August 2022, Areerat Saisong, Orathai Sertsri, and Wattana Tanming — (ML17)

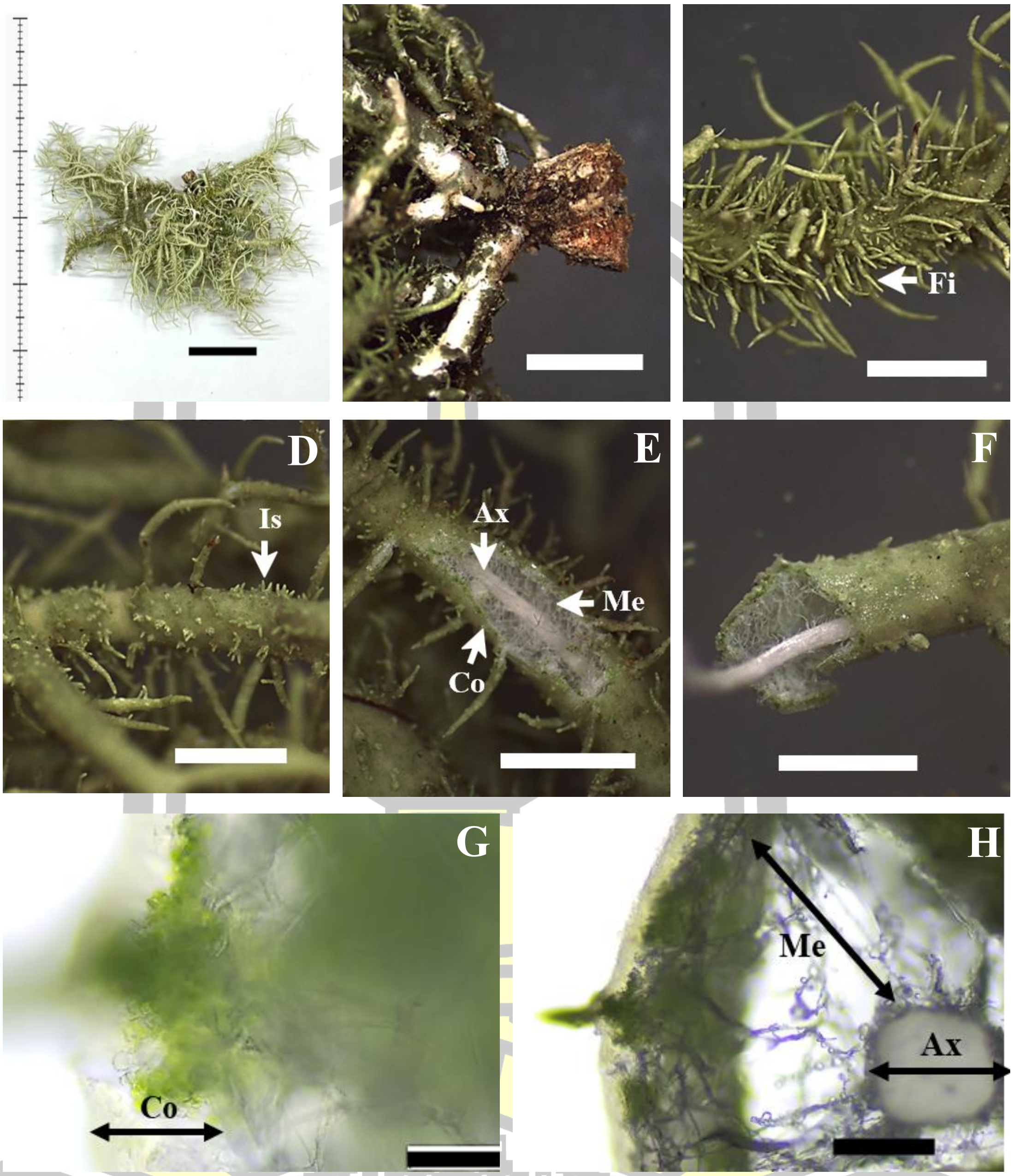


**Figure 47** Characteristics of *Usnea* sp.8 (ML17)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Isidia (Is) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) F) Medullary thread (Scale = 1 mm) G) Hyaline cortex (Co) H) Loose hyphae in medulla (Me) and axis (Ax) (Scale = 50 µm)

**21. *Usnea* sp.9 (PR01)**

***Morphology:*** Thallus fruticose, small, 4 cm long, 1 mm diameter, blackish brown base, grayish-green when fresh, pale brown when old, thallus uninflated, thallus with a medullary thread visible by stretching branches, red pigment in cortex, thick medulla filled with loose hyphae, thin compact axis, isidia abundant, soredia present, apothecia and pseudocyphellae not seen, papillae absent (Figure 48)

***Anatomy:*** Thallus cross section (n=10) C = (82.1-) 115.6 (-145.9) µm; M = (364.5-) 412.6 (-452.9) µm; A = (264.1-) 291.393 (-316.5) µm; CMA ratio %C = (10.4-) 14 (-16.4); %M = (46.3-) 52 (-53.6); %A = (32.5-) 34-37 (-39.1); A/M = (0.6-) 0.7 (-0.8)

***Chemistry:*** Stictic acid, Usnic acid, and Atranorin detected by TLC

***Habitat:*** On an unidentified tree in attitude 1,365 metres above sea level

***Notes:*** This species resembles *U. rubicunda* Stirt. in having (1) red pigment in cortex (2) thallus uninflated (3) isidia and soredia present, but it is distinguished by the absence of salazinic acid as a major substance

***Specimen examined:*** THAILAND. Loei Province, Phu Ruea District, Phu Ruea National Park, 17.5142° N 101.3449° E. 4 November 2020, Areerat Saisong — (PR01)

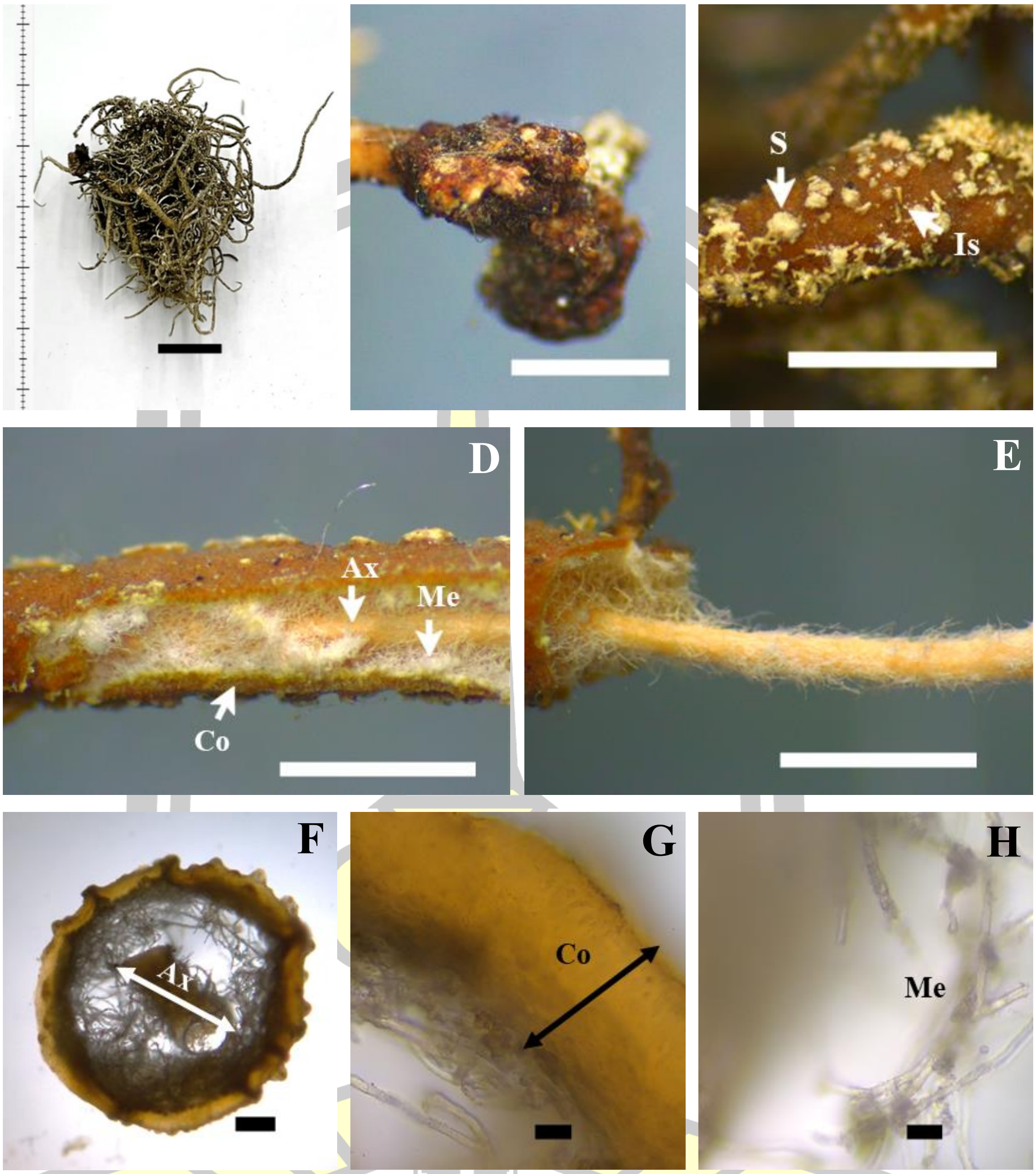


**Figure 48** Characteristics of *Usnea* sp.9 (PR01)

A) Habitat (Scale = 1 cm) B) Base C) Soredia (S) and Isidia (Is) D) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) E) Medullary thread (Scale = 1 mm) F) Axis (Ax) (Scale = 200 µm) G) Cortex (Co) H) Loose hyphae in medulla (Me) (Scale = 20 µm)

**22. *Usnea* sp.10 (PR02)**

***Morphology:*** Thallus fruticose, 3.5 cm long, 0.5 mm diameter, brown base, yellowish green when old, unknown when fresh, thallus uninflated, thallus with a medullary thread visible by stretching branches, cortex non-pigment, medulla filled with loose hyphae, compact axis, fibrils present, isidia absent, soredia absent, apothecia and pseudocyphellae not seen, papillae present (Figure 49)

***Anatomy:*** Thallus cross section (n=10) C = (75.7-) 89.7 (-100.7) µm; M = (327.9-) 398.6 (-501.0) µm; A = (146.9-) 203.4 (-300.2) µm; CMA ratio %C = (10.8-) 12 (-16.3); %M = (49-) 55 (-64.2); %A = (22.7-) 24-25 (-39); A/M = (0.4-) 0.4 (0.8)

***Chemistry:*** Stictic acid, Usnic acid, and Atranorin detected by TLC

***Habitat:*** On the unidentified tree in attitude 1,365 metres above sea level

***Specimen examined:*** THAILAND. Loei Province, Phu Ruea District, Phu Ruea National Park, 17.5142° N 101.3449° E. 4 November 2020, Areerat Saisong — (PR02)

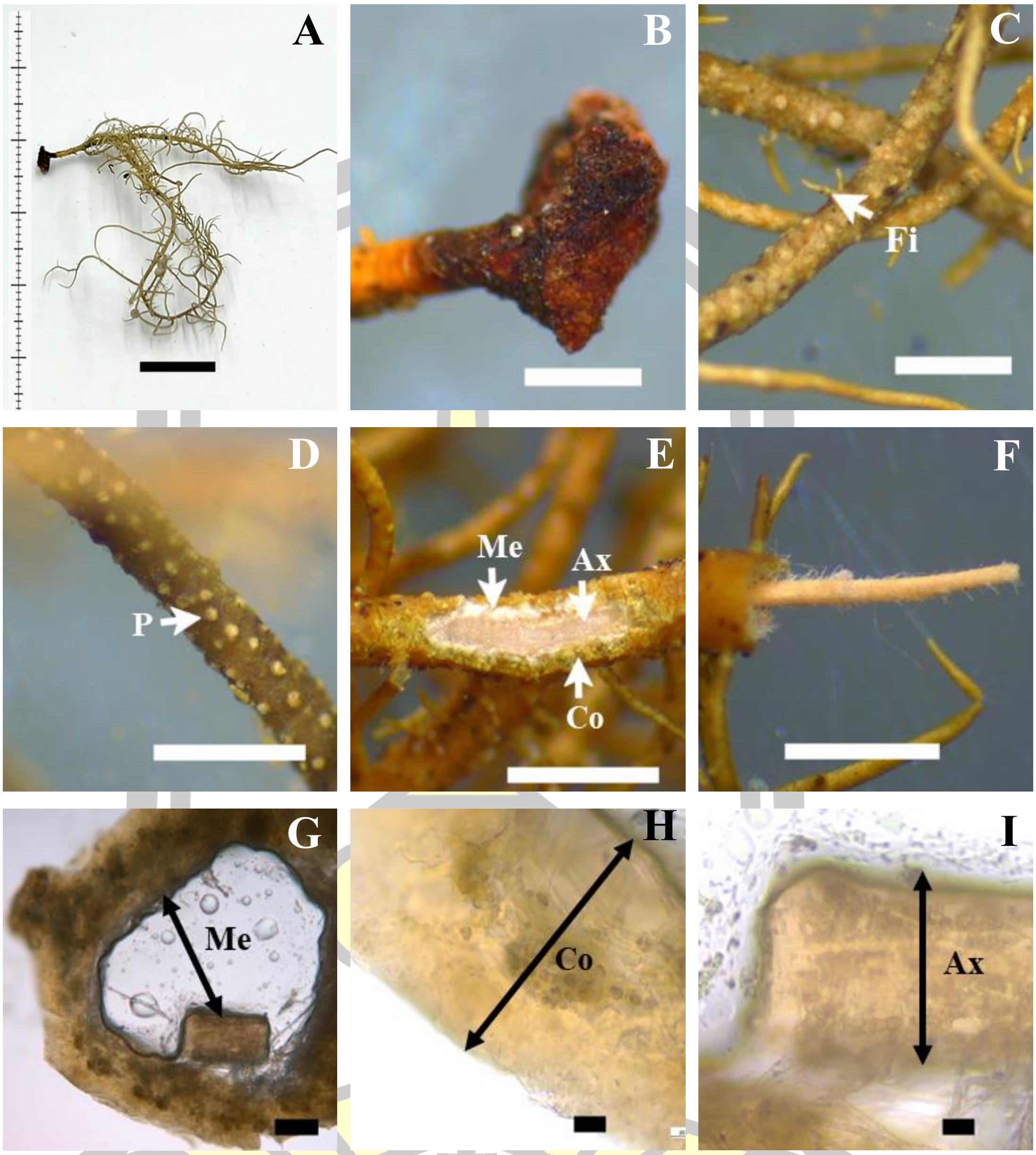


**Figure 49** Characteristics of *Usnea* sp.10 (PR02)

A) Habitat (Scale = 1 cm) B) Base C) Fibrils (Fi) D) Papillae (P) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) F) Medullary thread (Scale = 1 mm) G) Loose hyphae in medulla (Me) (Scale = 100 µm) H) cortex (Co) I) Axis (Ax) (Scale = 20 µm)

**23. *Usnea* sp. 11 (PK01)**

***Morphology:*** Thallus fruticose, small, 3-5.5 cm long, 1 mm diameter, brownish base, pale green when fresh, turn pale brown when old, thallus uninflated, thallus with a medullary thread visible by stretching branches, cortex non-pigment, thick medulla filled with loose hyphae, thin compact axis, fibrils absent, isidia present, soredia present, apothecia and pseudocyphellae not seen, papillae absent (Figure 50)

***Anatomy:*** Thallus cross section (n=10) C = (46.8-) 59.3 (-71.0) µm; M = (265.5-) 302.6 (-359.8) µm; A = (203.6-) 266.8 (-328.7) µm; CMA ratio %C = (7.2-) 9.6 (-11.8); %M = (41.9-) 47.3 (-55.0); %A = (35.1-) 43 (-50.9); A/M = (0.6-) 0.7 (-1.2)

***Chemistry:*** Physodalic acid, Menegazziaic acid, Psoromic acid, Barbatic acid, Galbinic acid, Usnic acid, and Atranorin detected by TLC

***Habitat:*** On *Pinus kesiya* Royle ex Gordon in attitude 1,200 metres above sea level

***Notes:*** The species *Usnea* sp.11 (PK01) resembles *U. jezoformosana* in having (1) soredia and isidia (2) lacking pseudocyphellae and papillae (3) the presence of barbatic acid as a major substance (Ohmura & Clerc, 2023)

***Specimen examined:*** THAILAND. Chaiyaphum Province, Khon San District, Phu Khieo Wildlife Sanctuary, 16°30′17.1″N 101°41′23.1″E. 16 October 2023, Areerat Saisong and Maneesila Potiwatnangun — (PK01)

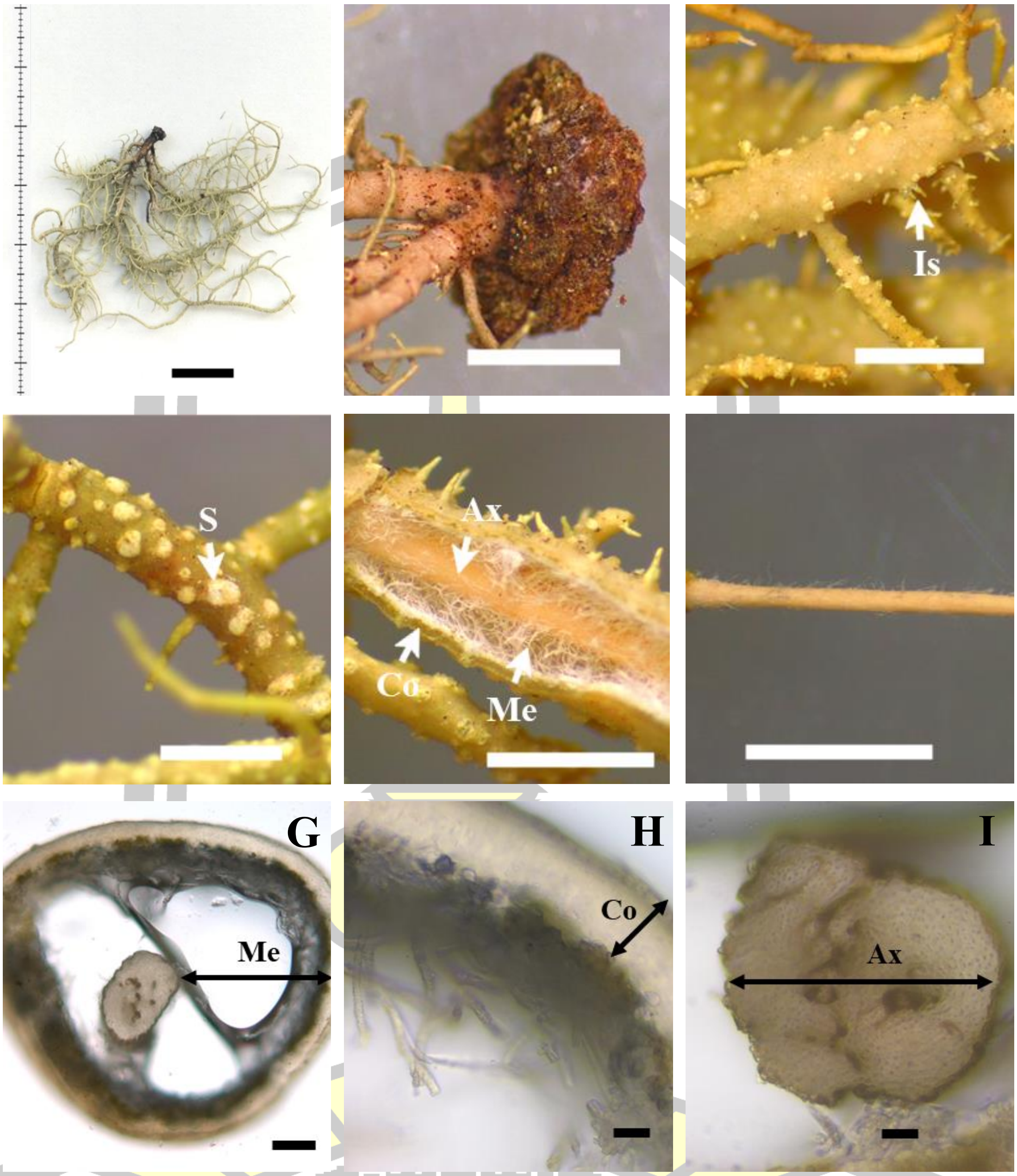


**Figure 50** Characteristics of *Usnea* sp. 11 (PK01)

A) Habitat (Scale = 1 cm) B) Base C) Isidia (Is) D) Soredia (S) E) Thallus layer (Co = Cortex; Me = Medulla; Ax = Axis) F) Medullary thread (Scale = 1 mm) G) Loose hyphae in medulla (Me) (Scale = 100 µm) H) Hyaline cortex (Co) I) Axis (Ax) (Scale = 20 µm)

The distribution of the specimens in this study is shown in Figure 51 which shows that 12 species are distributed in Chiang Mai Province, 7 species in Phitsanulok and Phetchabun Provinces, 4 species in Loei Province, and 1 species in Lampang and Chaiyaphum Provinces. Among These, they are distributed in Evergreen forests; Lower Montane Forest, Dry Dipterocarp Forest, Hill Evergreen Forest, and Hill Pine Forest as shown in Table 14.

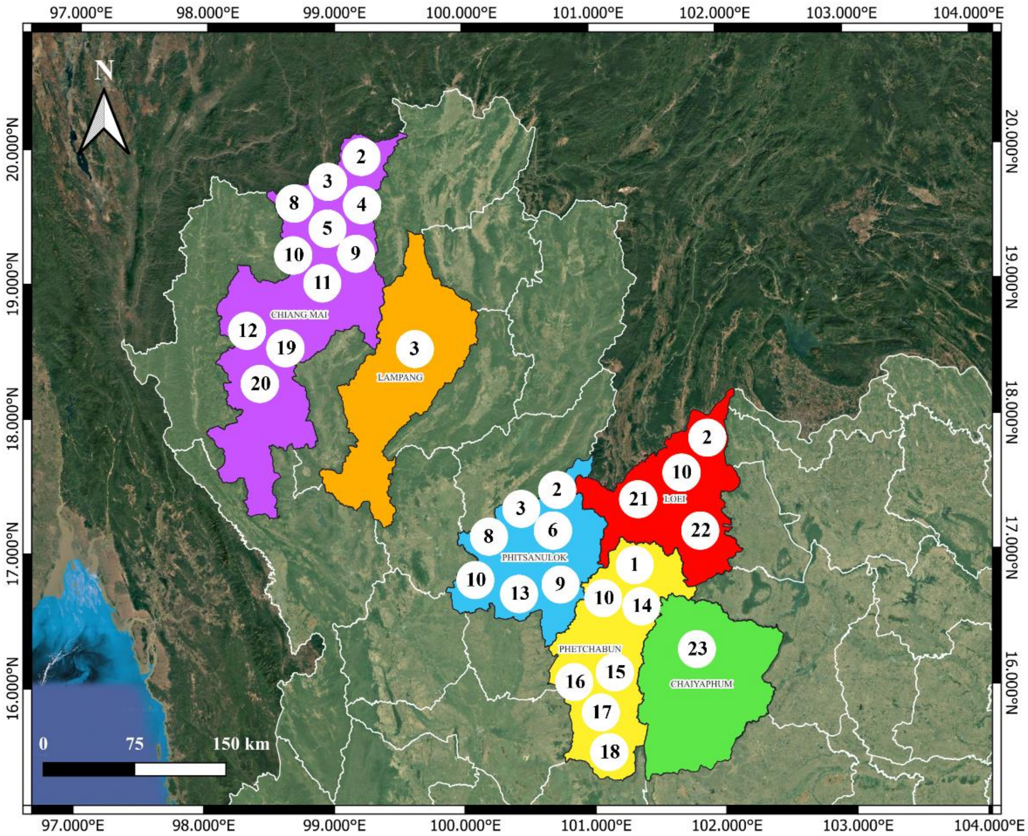


**Figure 51** The Distribution of the genus *Usnea* in Thailand

Purple = Chiang Mai Province, Orange = Lampang Province, Blue = Phitsanulok Province, Yellow = Phetchabun Province, Red = Loei Province, and Green = Chaiyaphum Province; 1 = *U. aciculifera,* 2 = *U. articulata*, 3 = *U. baileyi*, 4 = *U. ceratina*, 5 = *Usnea* cf. *dendritica*, *U. himantodes*, 7 = *U. pangiana*, 8 = *U.* cf. *perhispidella*, 9 = *Usnea* cf. *pectinata*, 10 = *U. rubicunda*, 11 = *Usnea* cf. *rubicunda*, 12 = *U. shimadae*, 13 = *Usnea* sp.1, 14 = *Usnea* sp.2, 15 = *Usnea* sp.3, 16 = *Usnea* sp.4, 17 = *Usnea* sp.5, 18 = *Usnea* sp.6, 19 = *Usnea* sp.7, 20 = *Usnea* sp.8, 21 = *Usnea* sp.9, 22 = *Usnea* sp.10, 23 = *Usnea* sp.11. * Two location localities out of this study area Loei and Chaiyaphum Provinces, but have specimens. Therefore, the researcher uses this identification as well.

**Table 14** The distribution of *Usnea* in various ecology

| Type of Forests | Species |
|---|---|
| **Chiang Mai Province** | |
| 1. Dry Dipterocarp Forest<br>2. Lower Montane Rain Forest<br>3. Hill Evergreen Forest<br>4. Pine Forest | *U. articulata, U. baileyi, U. ceratina, Usnea* cf. *dendritica, U. pangiana,* Usnea cf. *perhispidella, U. rubicunda*, *Usnea* cf. *rubicunda, U. shimadae, Usnea* sp. 7, *Usnea* sp.8 |
| **Lampang Province** | |
| 1. Lower Montane Rain Forest | *U. baileyi* |
| **Phitsanulok Province** | |
| 1. Lower Montane Rain Forest<br>2. Hill Pine Forest | *U. articulata*, *U. baileyi*, *U. himantodes, Usnea* cf. *perhispidella, Usnea* cf. *pectinata,* *U. rubicunda*, *Usnea* sp.1 |
| **Phetchabun Province** | |
| 1. Hill Pine Forest<br>2. Hill Evergreen Forest<br>3. Dry Dipterocarp Forest | *U. aciculifera, U. rubicunda, Usnea* sp.2, *Usnea* sp.3, *Usnea* sp.4, *Usnea* sp.5, *Usnea* sp.6 |

**Molecular Analysis**

The resulting amplification of ITS rDNA of *U. aciculifera* (PT07) was successful, and the sequence length was about 400 bp. The sequencing result was compared with the BLAST in the NCBI GenBank Database, which showed 100% identity with *U. aciculifera* (accession no. AB051049) specimens from Taiwan.

The phylogenetic tree was constructed using the Maximum Likelihood method. The reliability of each branch was analyzed by Maximum Likelihood (ML) and Neighbor-Joining (NJ) analysis with 1000 replicates. The sample of *Usnea aciculifera* Vain. (PT07) forms a monophyletic clade together with *Usnea aciculifera* from the 3 voucher specimens of the GenBank accession numbers AB051049, FJ494923, and OM808704 with high bootstrap values (99/88). However, the results of the phylogenetic tree relationship are confirmed by the genetic distance analysis shown in Figure 52.

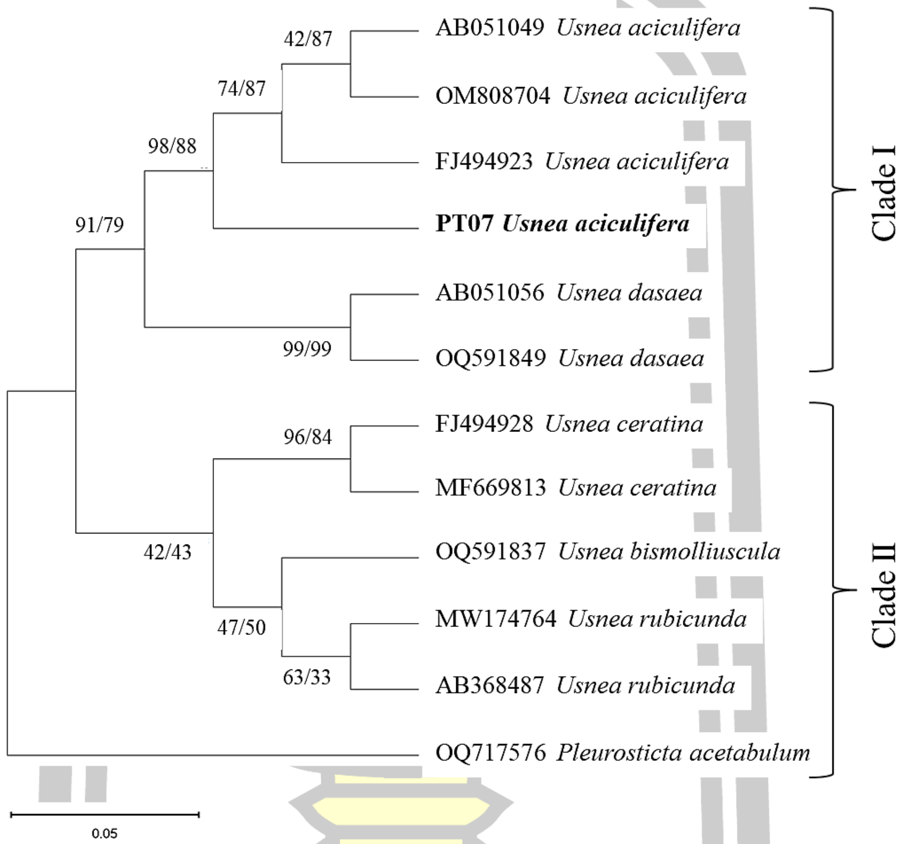


**Figure 52** Phylogenetic tree obtained from Maximum Likelihood and Neighbor-Joining analysis. The number above the nodes represents bootstrap support of ML/NJ

The genetic distance analysis of *Usnea* spp. based on ITS rDNA sequences were analyzed within genus *Usnea* using the Pairwise Distance method on MEGA-X. Ten sequences within the genus *Usnea* were downloaded from the NCBI GenBank database and the results in Table 15 in the red frame showed the intraspecific distance in *U. aciculifera* with the minimum genetic distance of 0.0758 (7.58%) and the highest genetic distance of 0.0938 (9.38%). The green frame showed the interspecific distance with the lowest genetic distance of 0.0052 (0.52%), and the blue frame showed the interspecific distance with the highest genetic distance of 0.1645 (16.45%)

**Table 15** Genetic distance of *Usnea* spp. based on ITS rDNA sequences

| No. | Species* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ***Usnea aciculifera*** | | | | | | | | | | | |
| 2 | *Usnea aciculifera* | 0.0758 | | | | | | | | | | |
| 3 | *Usnea aciculifera* | 0.0938 | 0.0105 | | | | | | | | | |
| 4 | *Usnea aciculifera* | 0.0906 | 0.0131 | 0.0262 | | | | | | | | |
| 5 | *Usnea dasaea* | 0.0935 | 0.0206 | 0.0318 | 0.0346 | | | | | | | |
| 6 | *Usnea dasaea* | 0.0926 | 0.0211 | 0.0322 | 0.0294 | 0.0052 | | | | | | |
| 7 | *Usnea bismolliuscula* | 0.1645 | 0.0816 | 0.0944 | 0.0912 | 0.0843 | 0.0814 | | | | | |
| 8 | *Usnea ceratina* | 0.1116 | 0.0395 | 0.0510 | 0.0568 | 0.0394 | 0.0403 | 0.0816 | | | | |
| 9 | *Usnea ceratina* | 0.1025 | 0.0422 | 0.0537 | 0.0417 | 0.0421 | 0.0422 | 0.0857 | 0.0109 | | | |
| 10 | *Usnea rubicunda* | 0.1082 | 0.0444 | 0.0561 | 0.0442 | 0.0387 | 0.0388 | 0.0695 | 0.0359 | 0.0334 | | |
| 11 | *Usnea rubicunda* | 0.1264 | 0.0565 | 0.0689 | 0.0565 | 0.0505 | 0.0507 | 0.0856 | 0.0478 | 0.0485 | 0.0304 | |

Intraspecific distance in *U. aciculifera*

Interspecific distance in the genus *Usnea* (lowest genetic distance)

Interspecific distance in the genus *Usnea* (highest genetic distance)

* ***Usnea aciculifera*** *(Specimen studies;* ***bold****), Usnea aciculifera (Accession No. AB051049), Usnea aciculifera (Accession No. FJ494923), Usnea aciculifera (Accession No. OM808704), Usnea dasaea (Accession No. AB051056), Usnea dasaea (Accession No. OQ591849), Usnea bismolliuscula (Accession No. OQ591837), Usnea ceratina (Accession No. FJ494928), Usnea ceratina (Accession No. MF669813), Usnea rubicunda (Accession No. MW174764), Usnea rubicunda (Accession No. AB368487), Pleurosticta acetabulum (Accession No. OQ717576)*

**Bioactive Compounds Extraction**

In this study, the lichen *U. himantodes* and *Usnea* cf. *pectinata* were extracted with two methods (maceration and boiling) with four different solvents including 95% ethanol, acetone, ethyl acetate, and boiling with water. The results indicate that all samples produced crude extracts with a dry powder texture. However, the yield, color, and texture of the extracts varied depending on the solvent used. Table 13 and Figure 53 provide a clear overview of the differences between the solvents used.


**Figure 53** The powder and color of lichen extraction

Lichen extracts of *U. himantodes* (PH01) (A-D); A = 95% ethanol, B = acetone, C = ethyl acetate, D = boiling water. Lichen extracts of *Usnea* cf. *pectinata* (PH04) (E-H); E = 95% ethanol, B = acetone, C = ethyl acetate, D = boiling water

According to Table 16, the highest yield was obtained from the lichen crude extract that was boiled with water, with a yield of 13.55% (*U. himantodes*) and 24.73% (*Usnea* cf. *pectinata*). For *U. himantodes*, the next highest yields were obtained from ethyl acetate, acetone, and ethanol, whereas in *Usnea* cf. *pectinata,* the highest yields were obtained from ethyl acetate, ethanol, and acetone, respectively.

**Table 16** Extraction yields and color of lichen genus *Usnea* with four different solvents

| Sample | Solvent | %Yield | Color |
|---|---|---|---|
| *U. himantodes* | 95% Ethanol | 6.86 | Greenish brown |
| | Acetone | 9.65 | Creamy |
| | Ethyl acetate | 10.13 | Pale yellow |
| | Boil | 13.55 | Brown |
| *Usnea* cf. *pectinata* | 95% Ethanol | 14.37 | Reddish brown |
| | Acetone | 9.32 | Pale yellow |
| | Ethyl acetate | 13.78 | Creamy |
| | Boil | 24.73 | Brown |

**Phytochemistry Analysis**

**1. Total Phenolic Content Analysis**

A total phenolic content analysis was performed on extracts of *U. himantodes* and *Usnea* cf. *pectinata* with four different solvents: 95% ethanol, ethyl acetate, acetone, and boiling with water. The Gallic acid standard curve was used for comparison (Figure 54).

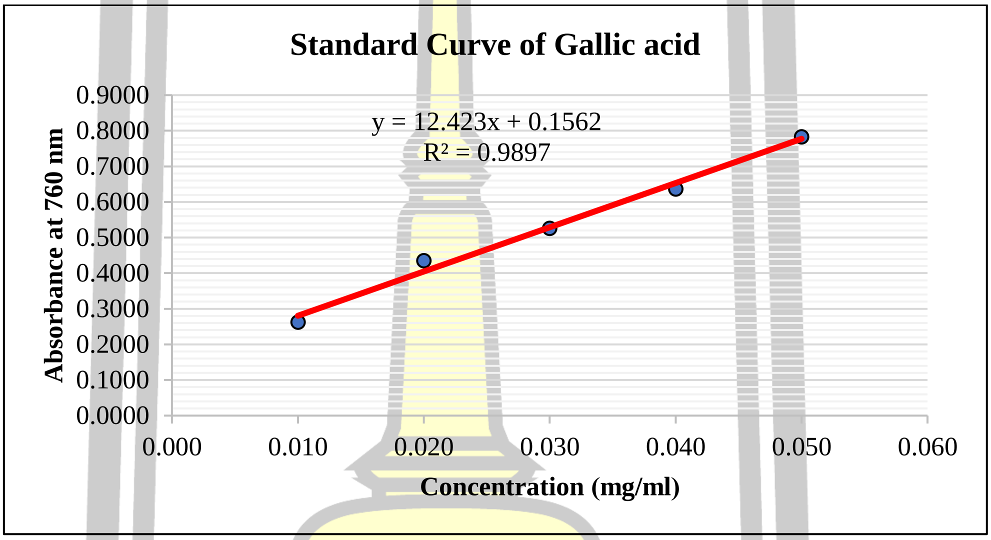


**Figure 54** Standard curve of Gallic acid

In terms of total phenolic content, the *U. himantodes* extract had the highest total phenolic content with ethanolic extract of 84.430±1.385 mg GAE/g Extract, followed by ethyl acetate, boiled water, and acetone, with 67.391±2.858, 32.531±2.543, and 27.567±2.999 mg GAE/g Extract, respectively. Meanwhile, the *Usnea* cf. *pectinata* extract had the highest total phenolic content with ethanolic extract of 93.772±2.847 mg GAE/g Extract, followed by ethyl acetate, acetone, and boiled water, with 53.809±0.523, 48.247±2.299, and 15.750±1.223, respectively, as shown in Table 17 and Figure 55.

**Table 17** Total phenolic content in lichen extracts with 4 different solvents

| Lichen Extracts | Total Phenolic Contents (mgGAE/gExtract) | |
|---|---|---|
| | Mean±SD | |
| | *U. himantodes* | *Usnea* cf. *pectinata* |
| 95% Ethanol | 84.430±1.385[a] | 93.772±2.847[a] |
| Acetone | 27.567±2.999[d] | 48.247±2.299[c] |
| Ethyl Acetate | 67.391±2.858[b] | 53.809±0.523[b] |
| Boiling water | 32.531±2.543[c] | 15.750±1.223[d] |

*Note: a,b,c,d represent statistically significant differences in data sets ($p<0.05$)

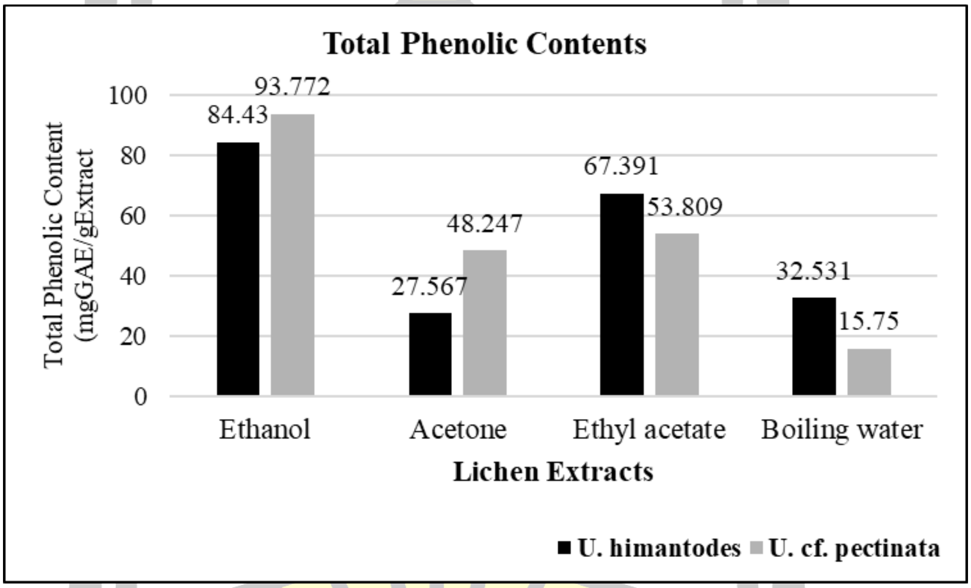


**Figure 55** Total phenolic content

**2. Total Flavonoid Content Analysis**

A total flavonoid content analysis was performed on extracts of *U. himantodes* and *Usnea* cf. *pectinata* with four different solvents: 95% ethanol, ethyl acetate, acetone, and boiling with water. The Quercetin standard curve was used for comparison (Figure 56).

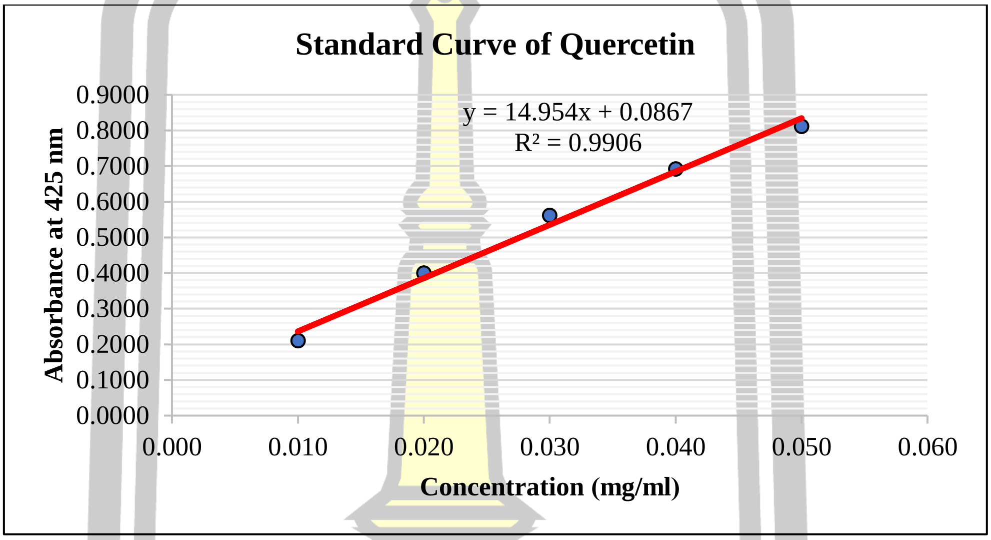


**Figure 56** Standard curve of Quercetin

In terms of total flavonoid content, the *U. himantodes* extract had the highest total flavonoid content with ethanolic extract of 21.595±1.138 mg QE/g Extract, followed by acetone, ethyl acetate, and boiling with water with 15.244±0.768, 14.736±0.892, and 8.836±0.669 mg QE/g Extract, respectively. Meanwhile, the *Usnea* cf. *pectinata* extract had the highest total flavonoid content with ethanolic extract of 36.847±0.613 mg QE/g Extract, followed by acetone, ethyl acetate, and boiling with water, with 24.294±0.240, 11.839±0.393, and 9.318±0.399 mg QE/g Extract, respectively, as shown in Table 18 and Figure 57.

**Table 18** Total flavonoid content in lichen extracts with 4 different solvents

| Lichen Extracts | Total Flavonoid Contents (mgQE/gExtract) | |
|---|---|---|
| | Mean±SD | |
| | *U. himantodes* | *Usnea* cf. *pectinata* |
| 95% Ethanol | 21.595±1.138[a] | 36.847±0.613[a] |
| Acetone | 15.244±0.768[b] | 24.294±0.240[b] |
| Ethyl Acetate | 8.836±0.669[d] | 9.318±0.399[d] |
| Boiling water | 14.736±0.892[c] | 11.839±0.393[c] |

*Note: a,b,c,d represent statistically significant differences in data sets ($p<0.05$)

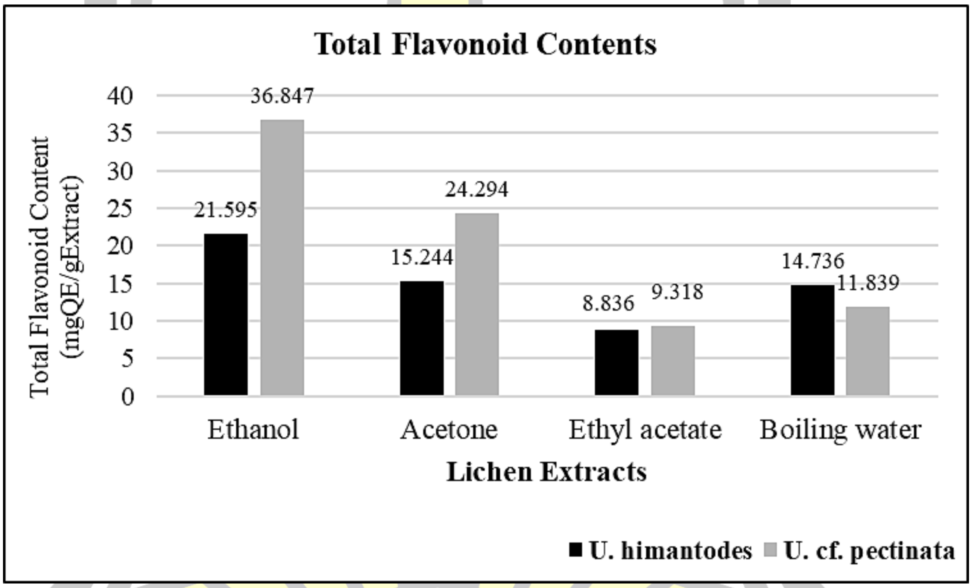


**Figure 57** Total flavonoid content

**3. Tannin Content Analysis**

A tannin content analysis was performed on extracts of *U. himantodes* and *Usnea* cf. *pectinata* with four different solvents: 95% ethanol, ethyl acetate, acetone, and boiling with water. The Tannic acid standard curve was used for comparison (Figure 58).

In terms of total tannin content, the *U. himantodes* extract had the highest total tannin content with ethanolic extract of 81.418±1.341 mg Tannic acid /g Extract, followed by ethyl acetate, boiling with water, and acetone, with 26.342±2.905, 64.915±2.769, and 31.150±2.463 mg Tannic acid /g Extract, respectively. Meanwhile, the *Usnea* cf. *pectinata* extract had the highest total tannin content with ethanolic extract of 90.467±2.784 mg Tannic acid /g Extract, followed by ethyl acetate, acetone, and boiling with water, with 46.385±2.245, 51.759±0.507, and 15.564±0.112 mg Tannic acid/g Extract, respectively, as shown in Table 19 and Figure 59.

**Table 19** A tannin content in lichen extracts with 4 different solvents

| Lichen Extracts | Total Tannin Contents (mgTannic acid/gExtract) | |
|---|---|---|
| | Mean±SD | |
| | *U. himantodes* | *Usnea* cf. *pectinata* |
| 95% Ethanol | 81.418±1.341$^{a}$ | 90.467±2.784$^{a}$ |
| Acetone | 26.342±2.905$^{d}$ | 46.385±2.245$^{c}$ |
| Ethyl Acetate | 64.915±2.769$^{b}$ | 51.759±0.507$^{b}$ |
| Boiling water | 31.150±2.463$^{c}$ | 15.564±0.112$^{d}$ |

*Note: a,b,c,d represent statistically significant differences in data sets (p<0.05)

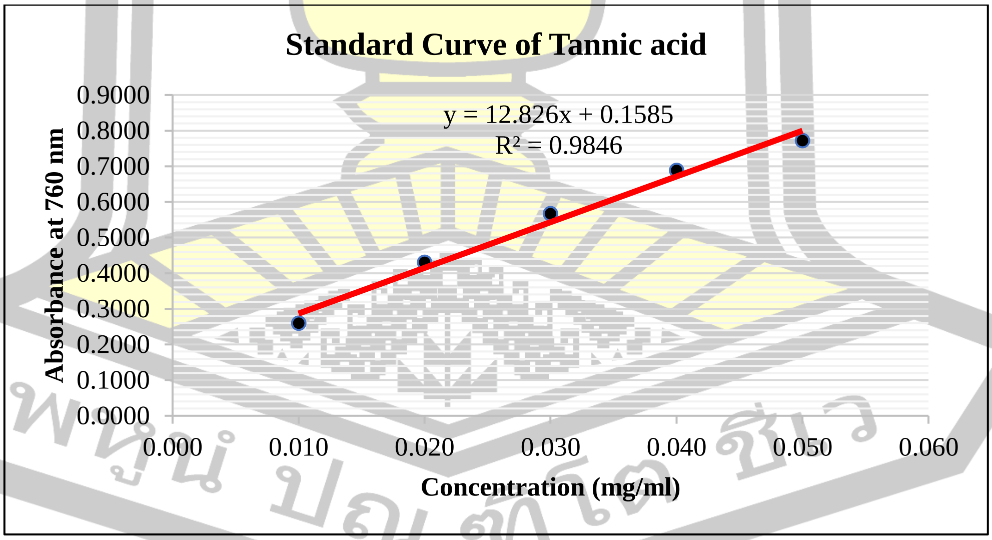


**Figure 58** Standard curve of Tannic acid

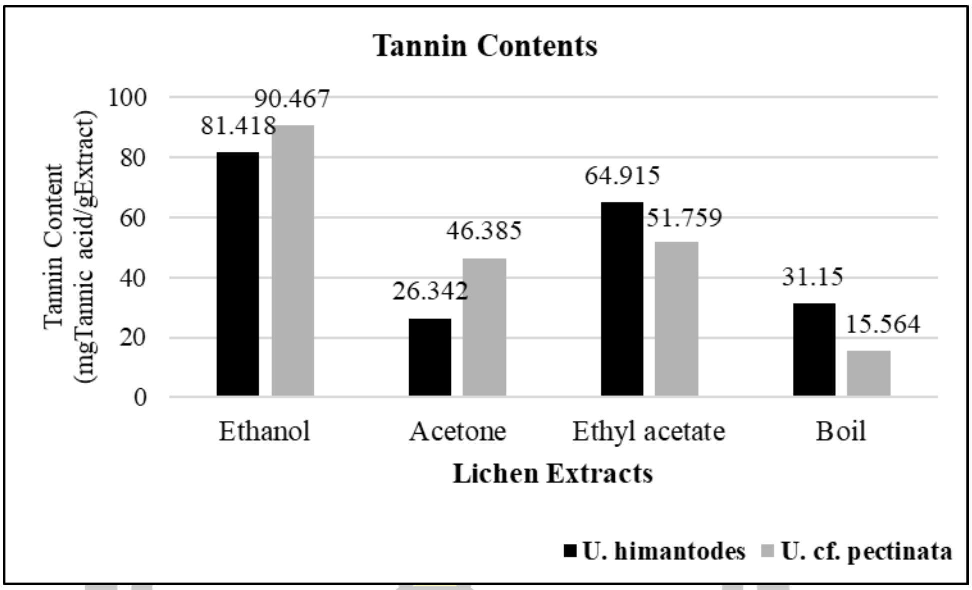


**Figure 59** Total tannin content

Result of the phytochemistry analysis of *U. himantodes* and *Usnea* cf. *pectinata* showed that the 95% ethanolic and acetone extract of *Usnea* cf. *pectinata* contained higher levels of phenolic, flavonoid, and tannin compounds than the extract from *U. himantodes*. On the other hand, the ethyl acetate and boiling water extract from *U. himantodes* greater contents of total phenolics, flavonoids, and tannins than those in *Usnea* cf. *pectinata*. Notable, the highest polyphenol content was recorded in 95% ethanolic extract for both *U. himantodes* and *Usnea* cf. *pectinata*.

**Antioxidant Activity**

**1. Antioxidant Analysis by DPPH Assay**

The result of antioxidant evaluation using a DPPH assay of lichen extract with 4 solvents including 95% ethanol, ethyl acetate, acetone, and boiling with water was reported with $IC_{50}$. The results, compared to the Ascorbic acid standard curve (Figure 60), showed that the crude extract of *U. himantodes* in boiling water had the highest antioxidant potential with the lowest $IC_{50}$ of 0.756±0.036 mg/ml.

The extract of ethanolic, acetone, and ethyl acetate, respectively, followed this. Similarly, in *Usnea* cf. *pectinata*, the boiling water extracts had the highest antioxidant potential with the lowest $IC_{50}$ of 0.741±0.021 mg/ml, followed by the extract of ethyl acetate, acetone, and ethanolic, respectively. The details are shown in Table 20 and Figure 61.

**Table 20** The percentage of DPPH free radical inhibition at 50% ($IC_{50}$)

| Lichen Extracts | $IC_{50}$ (mg/ml) | |
|---|---|---|
| | Mean±SD | |
| | *U. himantodes* | *Usnea* cf. *pectinata* |
| 95% Ethanol | 1.666±0.046[b] | 1.945±0.044[a] |
| Acetone | 1.284±0.083[c] | 1.304±0.034[b] |
| Ethyl Acetate | 2.002±0.029[a] | 1.143±0.048[c] |
| Boiling water | 0.756±0.036[d] | 0.741±0.021[d] |
| Ascorbic acid | 0.012±0.000 | 0.012±0.000 |

*Note: a,b,c,d represent statistically significant differences in data sets ($p<0.05$)

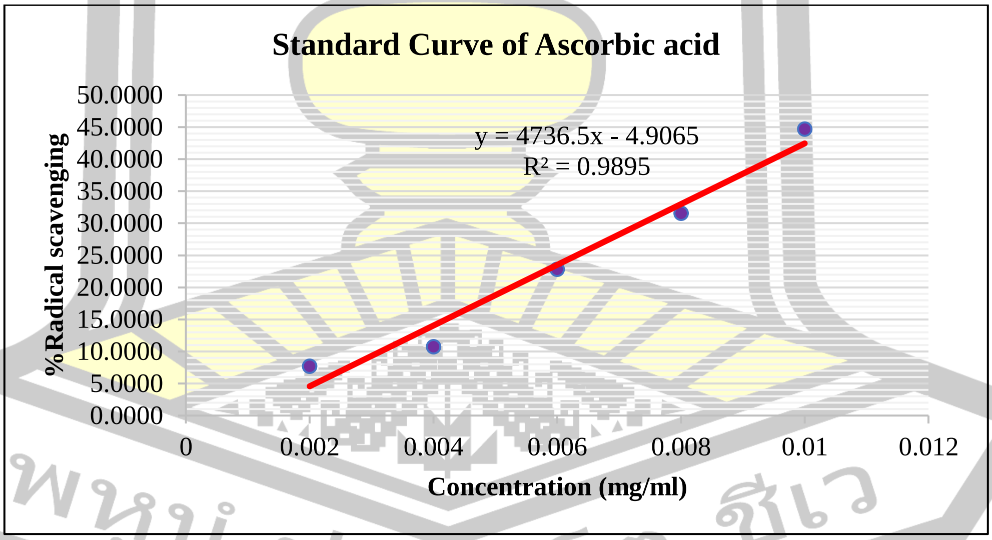


**Figure 60** Standard curve of Ascorbic acid

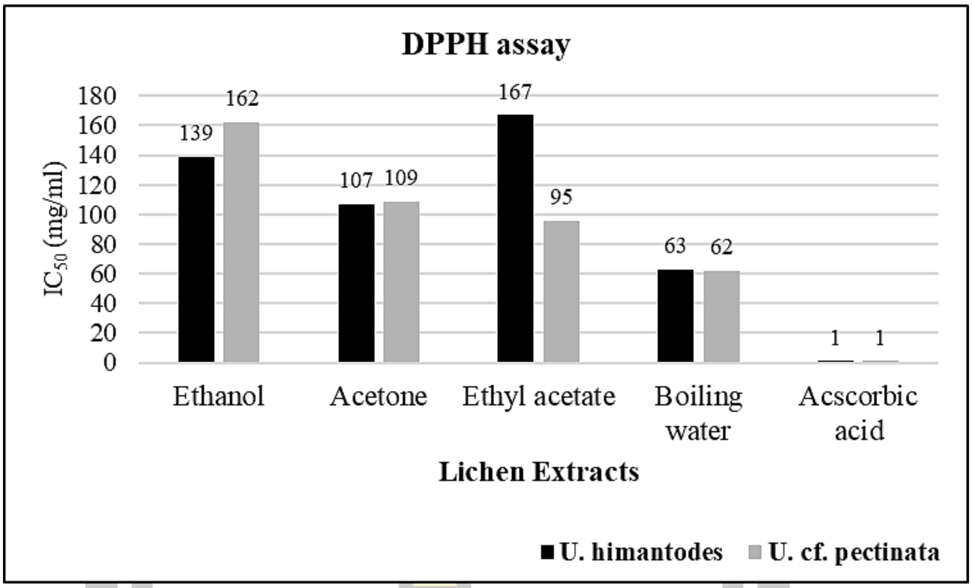


**Figure 61** Antioxidant potential of lichens compare to Ascorbic acid

**2. Antioxidant Analysis by ABTS Assay**

The result of antioxidant evaluation using an ABTS assay of lichen extract with 4 solvents including 95% ethanol, ethyl acetate, acetone, and boiling with water was reported with $IC_{50}$. The results, compared to the Trolox standard curve (Figure 62), showed that the crude extract of *U. himantodes* in boiling water had the highest antioxidant potential with the lowest $IC_{50}$ of 0.765±0.018 mg/ml. The extract of ethanolic, ethyl acetate, and acetone, respectively, followed this. Similarly, in *Usnea* cf. *pectinata*, the boiling water extracts had the highest antioxidant potential with the lowest $IC_{50}$ of 1.001±0.005 mg/ml, followed by the extract of acetone, ethyl acetate, and ethanolic, respectively. The details are shown in Table 21 and Figure 63.

**Table 21** The percentage of ABTS free radical inhibition at 50% ($IC_{50}$)

| Lichen Extracts | $IC_{50}$ (mg/ml) | |
|---|---|---|
| | Mean±SD | |
| | *U. himantodes* | *Usnea* cf. *pectinata* |
| 95% Ethanol | 3.068±0.006[c] | 3.250±0.015[a] |
| Acetone | 4.700±0.012[a] | 3.151±0.009[b] |
| Ethyl Acetate | 3.627±0.008[b] | 3.107±0.012[c] |
| Boiling water | 0.765±0.018[d] | 1.001±0.005[d] |
| Trolox | 0.039±0.000 | 0.039±0.000 |

*Note: a,b,c,d represent statistically significant differences in data sets (p<0.05)

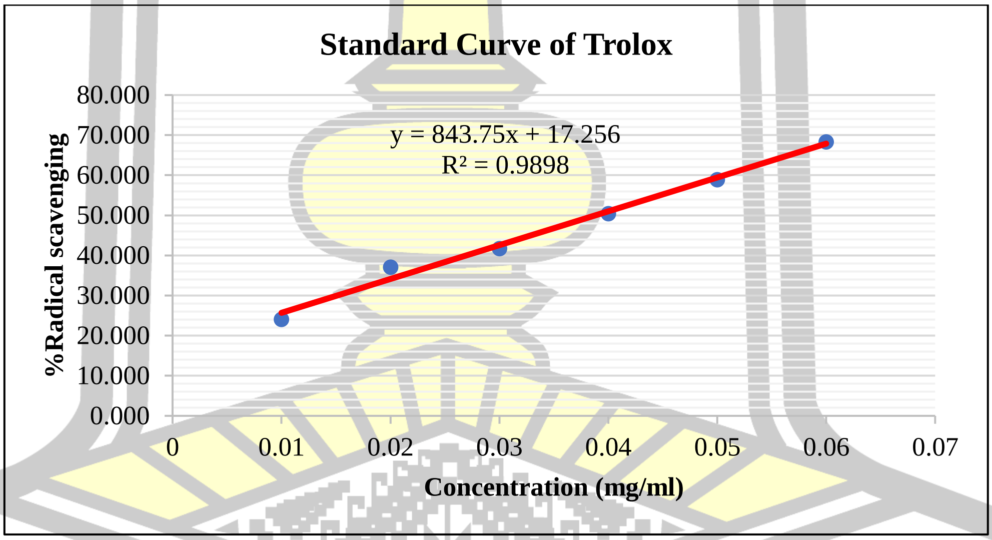


**Figure 62** Standard curve of Trolox

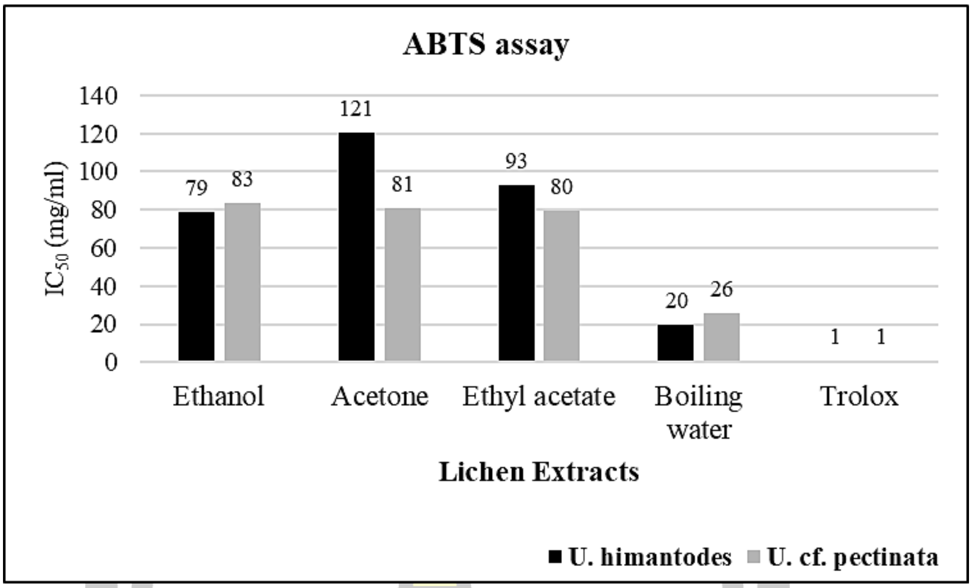


**Figure 63** Antioxidant potential of lichens compare to Trolox

**3. Antioxidant Analysis by FRAP Assay**

The result of antioxidant evaluation using a FRAP assay of lichen extract with 4 solvents including 95% ethanol, ethyl acetate, acetone, and boiling with water was reported with mg $FeSO_4$/g Extract. The result compared with the Ferrous Sulfate standard curve (Figure 64) revealed that the lichen *U. himantodes* extract had the highest antioxidant potential with boiling water extract of 30.069±0.211 mg $FeSO_4$ /g Extract, followed by acetone, ethyl acetate, and ethanol, with 21.463±0.673, 19.490±0.589, and 15.848±0.251 mg $FeSO_4$ /g Extract, respectively. Meanwhile, the *Usnea* cf. *pectinata* extract had the highest antioxidant potential with boiling water extract of 27.972±0.129 mg $FeSO_4$ /g Extract, followed by ethyl acetate, ethanolic, and acetone with 18.978±0.117, 17.921±0.386, and 13.626±0.614 mg $FeSO_4$ /g Extract, respectively, as shown in Table 22 and Figure 65.

**Table 22** Antioxidant analysis by FRAP assay

| Lichen Extracts | Antioxidant analysis by FRAP assay (mg $FeSO_4$/g Extract) | |
|---|---|---|
| | Mean±SD | |
| | *U. himantodes* | *Usnea* cf. *pectinata* |
| 95% Ethanol | 15.848±0.251$^{d}$ | 17.921±0.386$^{c}$ |
| Acetone | 21.463±0.673$^{b}$ | 13.626±0.614$^{d}$ |
| Ethyl Acetate | 19.490±0.589$^{c}$ | 18.978±0.117$^{b}$ |
| Boiling water | 30.069±0.211$^{a}$ | 27.972±0.129$^{a}$ |

*Note: a,b,c,d represent statistically significant differences in data sets ($p<0.05$)

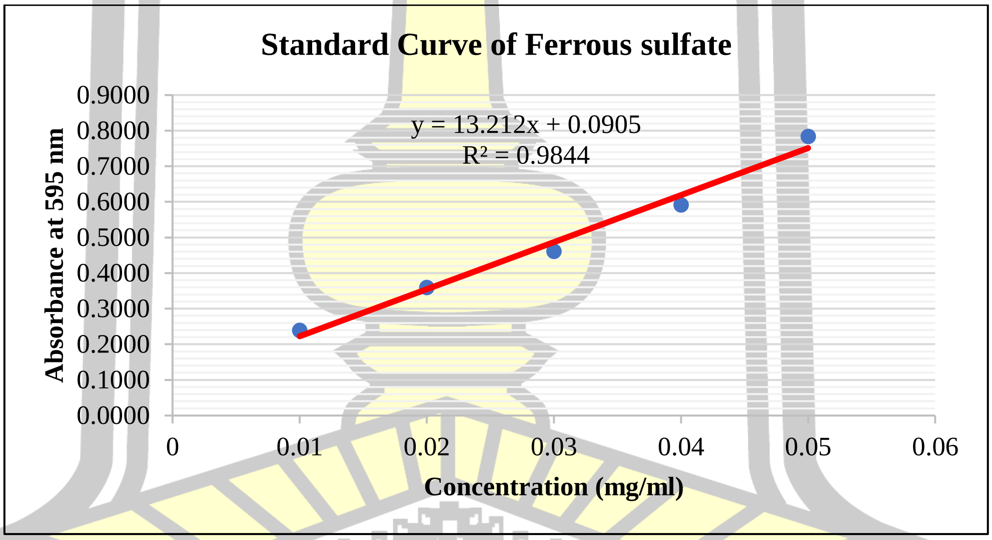


**Figure 64** Standard curve of Ferrous sulfate

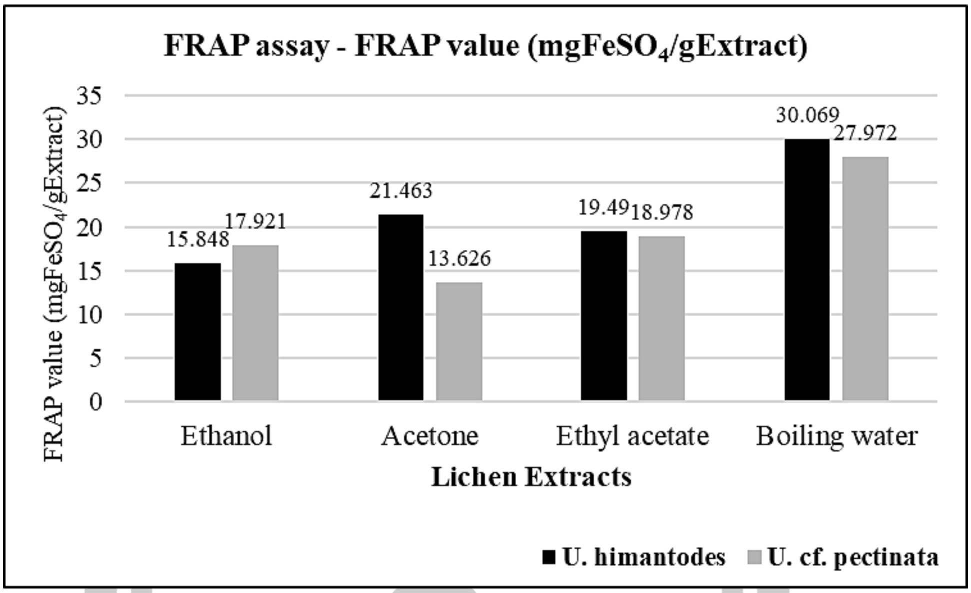


**Figure 65** Antioxidant potential of lichens with FRAP assay

In conclusion, the antioxidant analysis reveals that both *U. himantodes* and *Usnea* cf. *pectinata* extracts from boiling water exhibited the highest effectiveness in antioxidant activity across all DPPH, ABTS, and FRAP assays.

**Antibacterial Activities**

**1. Agar Well Diffusion**

The evaluation of antibacterial activity using the Agar well diffusion method of lichen genus *Usnea* against bacteria *Bacillus cereus* TISTR 1449, *Staphylococcus epidermidis* TISTR 2162, *Escherichia coli* TISTR 527, and *Pseudomonas aeruginosa* TISTR 1287. Lichens were extracted with four different solvents; 95% ethanol, ethyl acetate, acetone, and boiled water for 30 min. Tetracycline was used for a positive control for gram positive bacteria (*B. cereus* TISTR 1449, *S. epidermidis* TISTR 2162), Ciprofloxacin was used for a positive control for gram negative bacteria (*E. coli* TISTR 527, *P. aeruginosa* TISTR 1287) and 3% DMSO was used for a negative control.

**Table 23** Diameters of inhibition zone (mm) of lichen extracts

| Lichen extracts | | Diameters of inhibition zone (mm) | | | |
|---|---|---|---|---|---|
| | | *B. cereus* | *S. epidermidis* | *E. coli* | *P. aeruginosa* |
| *U. himantodes* | Ethanol | 20.67±0.29 | 14.83±1.44 | 21.17±1.53 | - |
| | Acetone | 20.33±0.58 | 14.17±0.29 | 21.67±0.58 | - |
| | Ethyl acetate | 19.50±0.50 | 14.00±0.87 | 19.83±1.26 | - |
| | Boiling water | 18.82±0.29 | - | - | - |
| *Usnea* cf. *pectinata* | Ethanol | 24.00±0.87 | 20.67±0.57 | 18.89±1.15 | - |
| | Acetone | 23.67±0.76 | 19.83±0.76 | 19.17±0.58 | - |
| | Ethyl acetate | 21.50±0.87 | 19.83±0.29 | 18.83±0.58 | - |
| | Boiling water | 16.17±2.31 | - | - | - |
| Positive control | | 38.50±0.87 | 47.17±4.16 | 49.50±0.58 | 47.33±1.15 |

- : No inhibition zone observed

**1.1 *B. cereus* TISTR 1449 antibacterial activity tests**

According to Table 23, all extracts of *U. himantodes* showed similar antibacterial activity against *B. cereus* TISTR 1449 with no statistically significant differences. Among the various extracts tested, the ethanolic extract exhibited the highest antibacterial activity with an inhibition zone diameter of 20.67±0.29 mm followed by the extracts of acetone, ethyl acetate, and boiling water, respectively.

As well as the ethanolic, acetone, and ethyl acetate extracts of *Usnea* cf. *pectinata* showed similar antibacterial potential with no statistically significant differences. The ethanolic extract exhibited the highest antibacterial activity with an inhibition zone diameter of 24.00±0.87 mm which is better than *U. himantodes*. Followed by the extracts of acetone, ethyl acetate, and boiling water, respectively.

However, in comparison to the positive control tetracycline (10 mg/ml) that exhibited a diameter of inhibition zone of 38.50±0.87 mm, the extracts were only able to inhibit bacteria at a rate two to three times lower as shown in Figure 66.

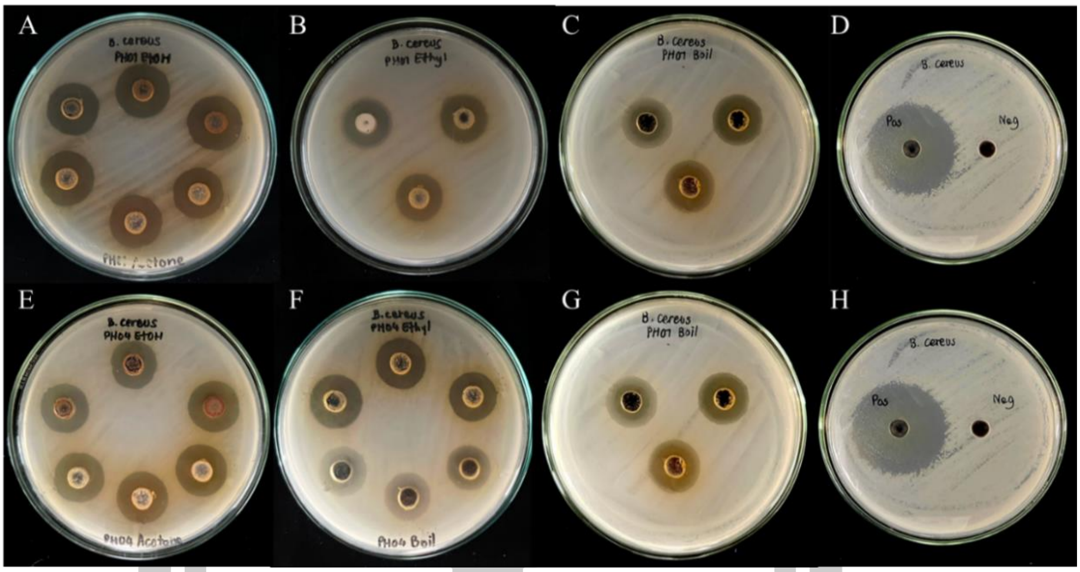


**Figure 66** Inhibition zone of lichen extracts

(A-C) extracts of *U. himantodes* (E-G) extracts of *Usnea* cf. *pectinata* (D, H) control

**1.2 *S. epidermidis* TISTR 2162 antibacterial activity tests**

Both *U. himantodes* and *Usnea* cf. *pectinata* extracts, obtained using ethanol, acetone, and ethyl acetate, exhibited similar antibacterial activity against *S. epidermidis* TISTR 2162, with no statistically significant differences. Among the various extracts tested, the ethanolic extract showed the highest antibacterial activity, with inhibition zone diameters of 14.83±1.44 mm (*U. himantodes*) and 20.67±0.57 mm (*Usnea* cf. *pectinata*), which was better than the ethanolic extract of *U. himantodes*. The acetone and ethyl acetate extracts followed in antibacterial activity, respectively. The extract obtained through boiling water was not effective in inhibiting the growth of *S. epidermidis* TISTR 2162.

However, in comparison to the positive control tetracycline (10 mg/ml) that exhibited a diameter of inhibition zone of 47.17±4.16 mm, the extracts were only able to inhibit bacteria at a rate two to three times lower as shown in Figure 67.

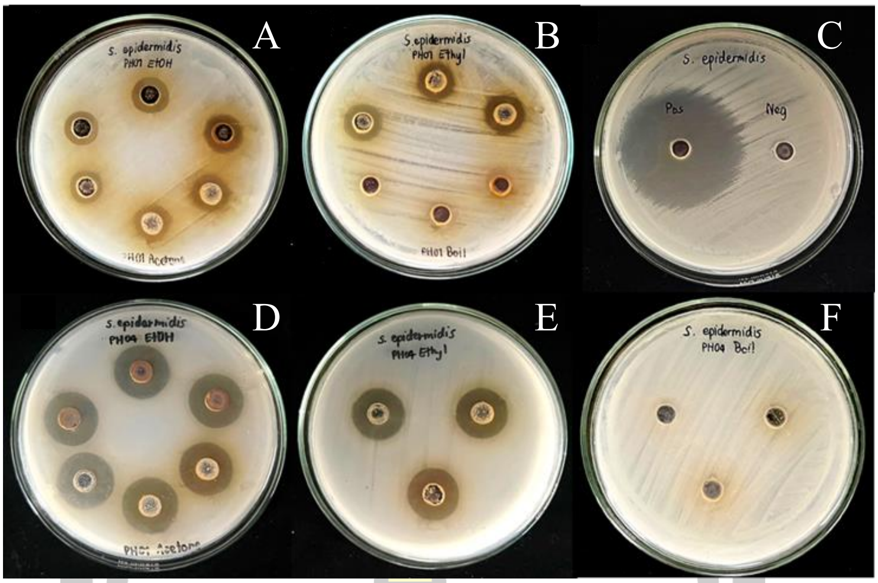


**Figure 67** Inhibition zone of lichen extracts

(A-B) extracts of *U. himantodes* (D-F) extracts of *Usnea* cf. *pectinata* (C) control

**1.3 *E. coli* TISTR 527 antibacterial activity tests**

Based on the results presented in Table 21, *U. himantodes* extracts obtained using ethanolic, acetone, and ethyl acetate solvents showed similar levels of antibacterial activity against *E. coli* TISTR 527. However, the acetone extract demonstrated the highest activity, with an inhibition zone diameter of 21.17±1.53 mm, followed by the ethanolic and ethyl acetate extracts, respectively. On the other hand, the extract of the boil did not have any effect on the growth of *E. coli* TISTR 527

The ethanolic, acetone, and ethyl acetate extracts of *Usnea.* cf. *pectinata* exhibited similar antibacterial potential with no statistically significant differences. However, the acetone extract showed the highest antibacterial activity with an inhibition zone diameter of 18.89±1.15 mm, followed by the extracts of ethyl acetate and ethanol, respectively. It is worth noting that the extract of boiling water was unable to inhibit the growth of *E. coli* TISTR 527.

In comparison to the positive control ciprofloxacin (10 mg/ml) that exhibited a diameter of inhibition zone of 49.50±0.58 mm, the extracts were only able to inhibit bacteria at a rate two to three times lower as shown in Figure 68.

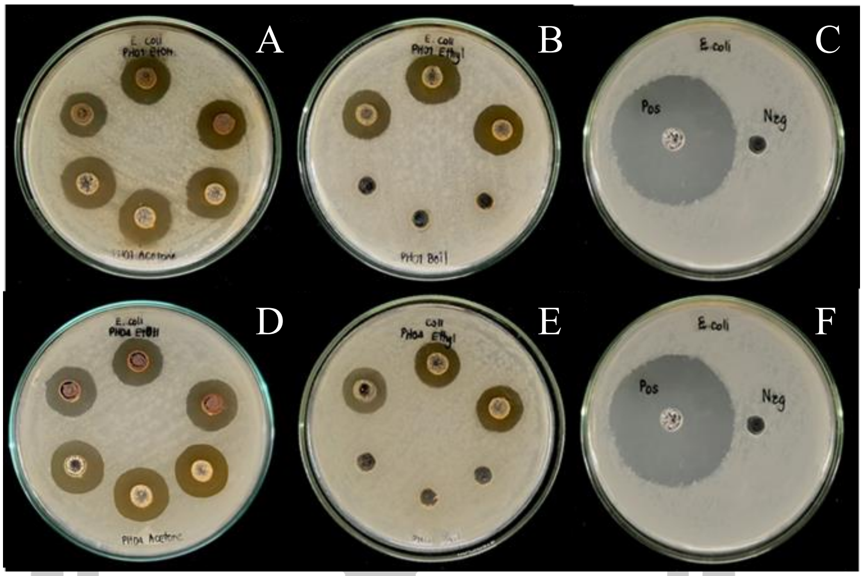


**Figure 68** Inhibition zone of lichen extracts

(A-B) extracts of *U. himantodes* (D-E) extracts of *Usnea* cf. *pectinata* (C, F) control

**1.4 *P. aeruginosa* TISTR 1287 antibacterial activity tests**

Antibacterial activity tests reveal that all extracts both *U. himantodes* and *Usnea* cf. *pectinata* cannot inhibit the growth of *E. coli* TISTR 527. Ciprofloxacin used as a positive control showed the diameter of the inhibition zone of 47.33±1.15 mm as shown in Figure 69.

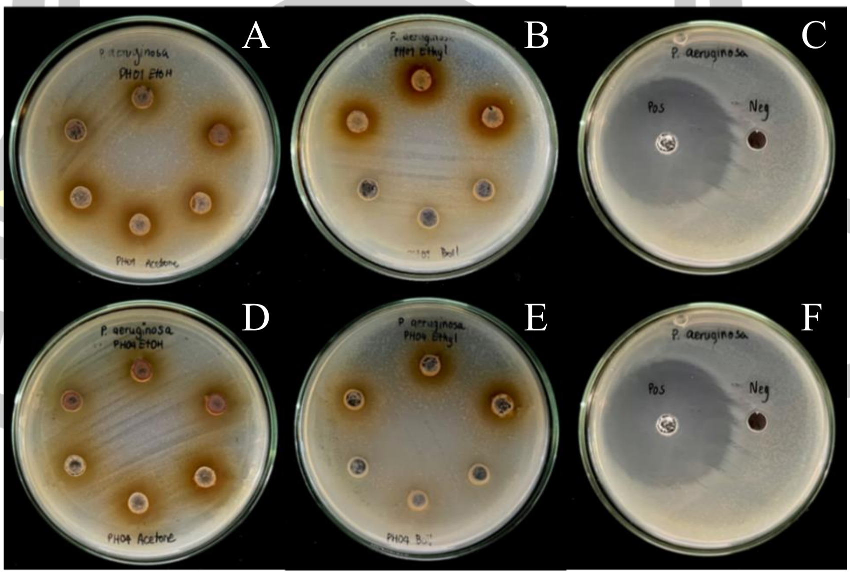


**Figure 69** Inhibition zone of lichen extracts

(A-B) extracts of *U. himantodes* (D-E) extracts of *Usnea* cf. *pectinata* (C, F) control

**2. Minimum Inhibitory Concentration (MIC)**

The evaluation of minimum inhibitory concentration of lichen genus *Usnea* using the two-fold microdilution method against bacteria *Bacillus cereus* TISTR 1449, *Staphylococcus epidermidis* TISTR 2162, *Escherichia coli* TISTR 527, and *Pseudomonas aeruginosa* TISTR 1287. Lichens were extracted with four different solvents; 95% ethanol, ethyl acetate, acetone, and boiled water. Tetracycline was used for a positive control for gram-positive bacteria (*B. cereus* TISTR 1449, *S. epidermidis* TISTR 2162), Ciprofloxacin was used for a positive control for gram-negative bacteria (*E. coli* TISTR 527) and 3% DMSO was used for a negative control. The extracts were diluted to a concentration ranging from 1.25-40 mg/ml. The results are reported in Table 24.

**Table 24** Minimum inhibitory concentration test against the bacterial strains

| Lichen extracts | | MICs (mg/ml) | | |
|---|---|---|---|---|
| | | *B. cereus* | *S. epidermidis* | *E. coli* |
| *U. himantodes* | Ethanol | 20 | 20 | 40 |
| | Acetone | 40 | 20 | 20 |
| | Ethyl acetate | 20 | 20 | 40 |
| | Boiling water | 40 | - | - |
| *Usnea* cf. *pectinata* | Ethanol | 20 | 20 | 20 |
| | Acetone | 40 | 20 | 40 |
| | Ethyl acetate | 40 | 20 | 20 |
| | Boiling water | 40 | - | - |

- : No MICs observed

**2.1 Minimum inhibitory concentration tests of *B. cereus* TISTR 1449**

In the evaluation of the minimum inhibitory concentration (MIC) of lichen extract to inhibit the growth of *B. cereus* TISTR 1449 (Figure 70), it was found that the ethanolic and ethyl acetate extracts of *U. himantodes* showed the lowest concentration of 20 mg/ml. On the other hand, the acetone and boiled extract required a higher concentration of 40 mg/ml to inhibit.

According to Table 24, the extract of ethanolic showed strong inhibit the growth of *B. cereus* TISTR 1449 with a concentration of 20 mg/ml. However, the other extracts required a higher concentration of 40 mg/ml to exhibit the same level of inhibition against the bacterial test.

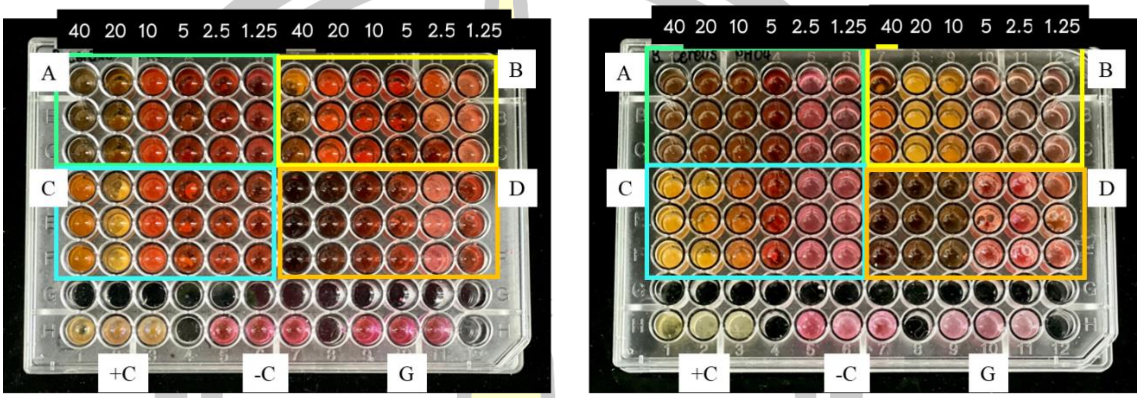


**Figure 70** Minimum Inhibitory Concentration Test against *B. cereus* TISTR 1449

Left = extract of *U. himantodes*; (A) ethanol (B) acetone (C) ethyl acetate (D) boiling water (+C) tetracycline (-C) 3% DMSO (G) growth control

Right = extract of *Usnea* cf. *pectinata*; (A) ethanol (B) acetone (C) ethyl acetate (D) boiling water (+C) tetracycline (-C) 3% DMSO (G) growth control

**2.2 Minimum inhibitory concentration tests of *S. epidermidis* TISTR 2162**

In evaluating the minimum inhibitory concentration (MIC) of lichen extract to restrain the growth of *S. epidermidis* TISTR 2162, it was observed that both *U. himantodes* and *Usnea* cf. *pectinata* extracts showed similar results. All samples indicated strong inhibition with a concentration of 20 mg/ml as shown in Figure 71.

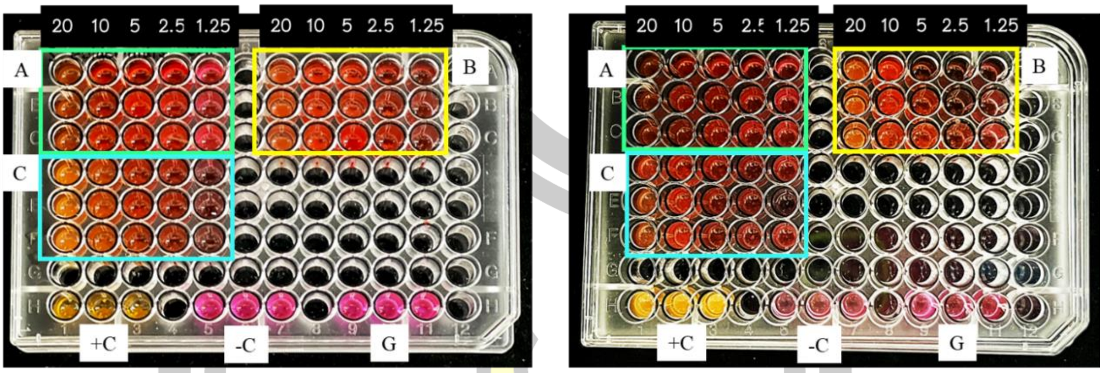


**Figure 71** Minimum Inhibitory Concentration Test against *S. epidermidis* TISTR 2162
Left = extract of *U. himantodes*; (A) ethanol (B) acetone (C) ethyl acetate (+C) tetracycline (-C) 3% DMSO (G) growth control
Right = extract of *Usnea* cf. *pectinata*; (A) ethanol (B) acetone (C) ethyl acetate (+C) tetracycline (-C) 3% DMSO (G) growth control

**2.3 Minimum inhibitory concentration tests of *E. coli* TISTR 527**

The Minimum Inhibitory Concentration (MIC) of lichen extracts was evaluated using a microdilution method, with concentrations ranging from 1.25-40 mg/ml. The results showed that the acetone extracts of *U. himantodes* had strong antibacterial properties with the lowest concentration of 20 mg/ml, whereas the ethanolic and ethyl acetate extracts required a higher concentration of 40 mg/ml to inhibit the growth of *E. coli* TISTR 527.

In the case of *Usnea* cf. *pectinata*, both the ethanolic and ethyl acetate extracts exhibited the greatest inhibition of *E. coli* TISTR 527 with the lowest concentration of 20 mg/ml. However, the acetone extract showed weak antibacterial potential and required a higher concentration of 40 mg/ml to inhibit the growth of *E. coli* TISTR 527 (Figure 72).

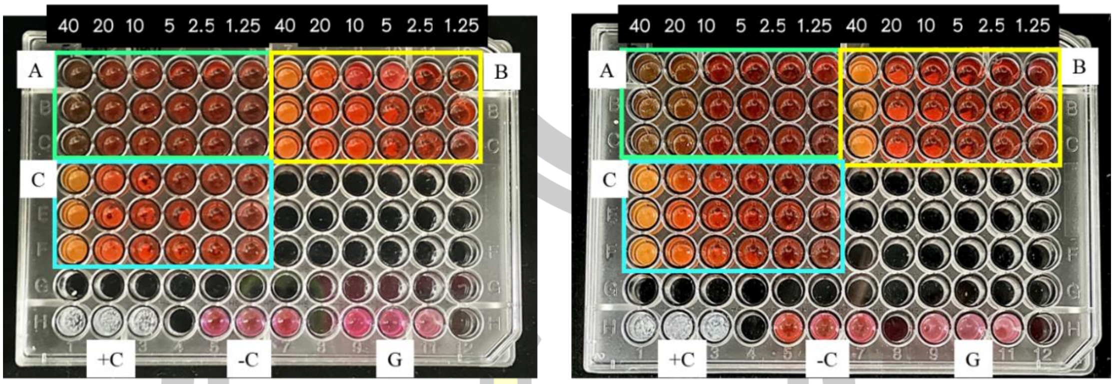


**Figure 72** Minimum Inhibitory Concentration Test against *E. coli* TISTR 527

Left = extract of *U. himantodes*; (A) ethanol (B) acetone (C) ethyl acetate (+C) tetracycline (-C) 3% DMSO (G) growth control

Right = extract of *Usnea* cf. *pectinata*; (A) ethanol (B) acetone (C) ethyl acetate (+C) tetracycline (-C) 3% DMSO (G) growth control

**3. Minimum Bactericidal Concentration (MBC)**

The evaluation of the minimum bactericidal concentration of the lichen genus *Usnea* against three bacterial strains, namely *Bacillus cereus* TISTR 1449, *Staphylococcus epidermidis* TISTR 2162, and *Escherichia coli* TISTR 527 was performed on MHA. The extracts of *U. himantodes* and *Usnea* cf. *pectinata* were dissolved in 3% DMSO and diluted to a concentration ranging from 1.25-40 mg/ml. The results are reported in Table 25 with the lowest concentration that can kill bacterial strains.

**Table 25** Evaluation of minimum bactericidal concentrations (mg/ml)

| Lichen extracts | | MBCs (mg/ml) | | |
|---|---|---|---|---|
| | | *B. cereus* | *S. epidermidis* | *E. coli* |
| *U. himantodes* | Ethanol | 20 | 20 | 40 |
| | Acetone | 40 | 20 | 20 |
| | Ethyl acetate | 20 | 20 | 40 |
| | Boiling water | 40 | - | - |
| *Usnea* cf. *pectinata* | Ethanol | 20 | 20 | 40 |
| | Acetone | 40 | 20 | 40 |
| | Ethyl acetate | 40 | 20 | 40 |
| | Boiling water | 40 | - | - |

- : No MBCs observed

In this experiment, tetracycline was utilized as a positive control for gram-positive bacteria, specifically *B. cereus* TISTR 1449 and *S. epidermidis* TISTR 2162. Ciprofloxacin was also used as a positive control for gram-negative bacteria, specifically *E. coli* TISTR 527. Meanwhile, 3% DMSO was used as the negative control. The diameters of inhibition zones can be seen in Figure 73.

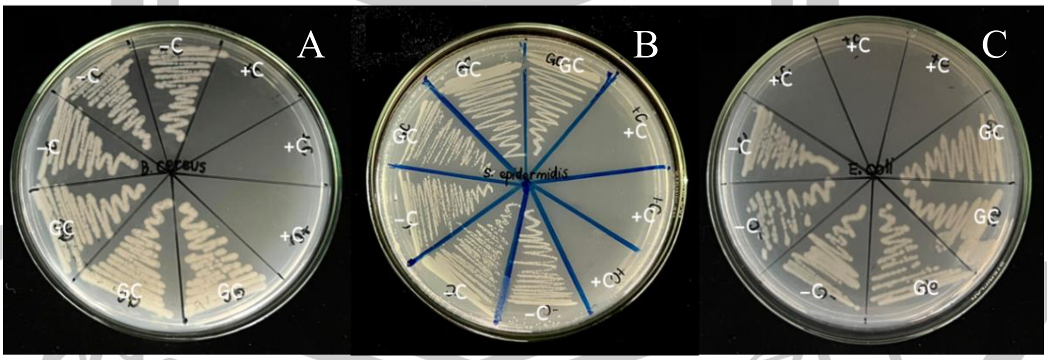


**Figure 73** The controls were used for the minimum bactericidal concentration test (A) *B. cereus* (B) *S. epidermidis* (C) *E. coli*

** (+C = antibiotic; -C = 3%DMSO; GC = growth control)

**3.1 Minimum bactericidal concentration tests of *B. cereus* TISTR 1449**

Evaluation of MBCs of lichen extracts against *B. cereus* TISTR 1449 was tested with concentrations ranging from 1.25-40 mg/ml. The results of *U. himantodes* extracts revealed that ethanolic and ethyl acetate extract showed the greatest killing of this bacterial strain with the lowest concentration of 20 mg/ml. Whereas the extract of acetone and boil required a higher concentration of 40 mg/ml.

In the case of *Usnea* cf. *pectinata* extracts, ethanolic extract showed the strong potential to kill the *B. cereus* TISTR 1449 with 20 mg/ml concentration. On the other hand, acetone, ethyl acetate, and boiled extract kill these bacteria tested with a higher concentration of 40 mg/ml (Figure 74).

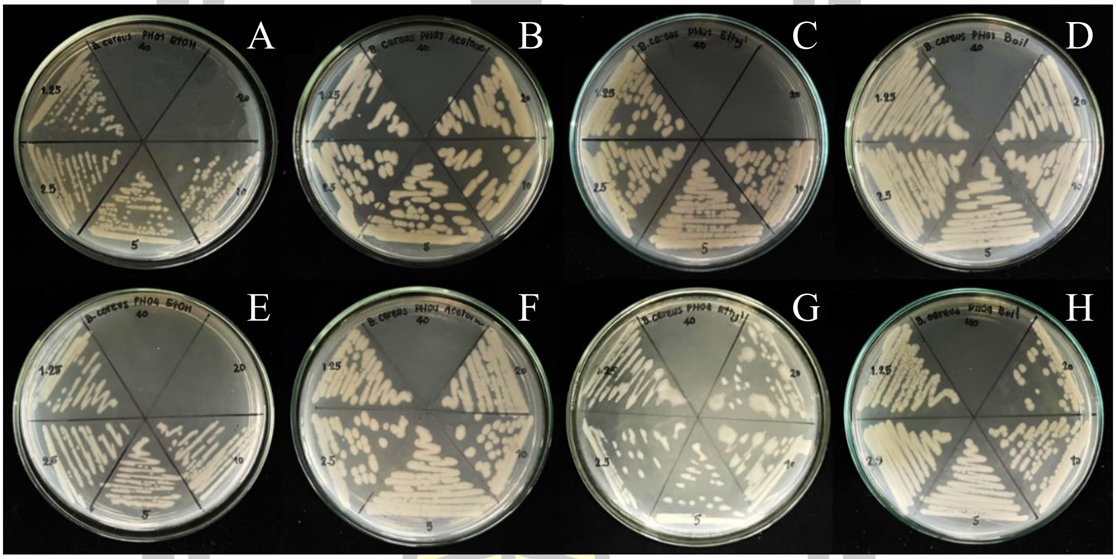


**Figure 74** Minimum Bactericidal Concentration of *B. cereus* TISTR 1449

A-D; *U. himantodes* (A) ethanol (B) acetone (C) ethyl acetate (D) boiling water

E-H; *Usnea* cf. *pectinata* (E) ethanol (F) acetone (G) ethyl acetate (H) boiling water

**3.2 Minimum bactericidal concentration tests of *S. epidermidis* TISTR 2162**

During the experiment, lichen extracts were tested for their Minimum Bactericidal Concentration (MBC) using varying concentrations of 1.25, 2.5, 5, 10, and 20 mg/ml. The results indicate that both *U. himantodes* and *Usnea* cf. *pectinata* extracts had similar bactericidal effects. All extracts could kill the *S. epidermidis*

TISTR 2162 at the lowest concentration of 20 mg/ml. The findings are visualized in Figure 75.

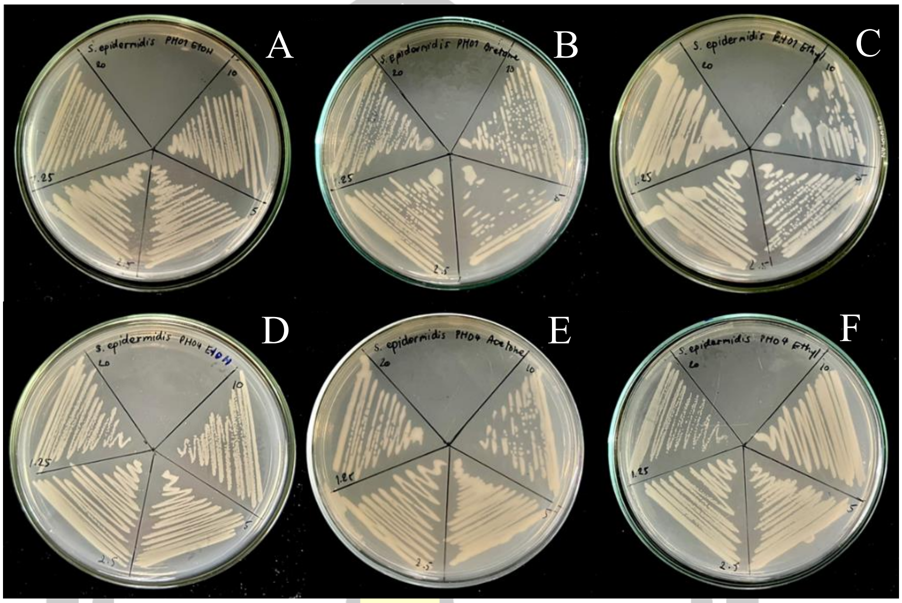


**Figure 75** Minimum Bactericidal Concentration of *S. epidermidis* TISTR 2162

A-C; *U. himantodes* (A) ethanol (B) acetone (C) ethyl acetate

D-E; *Usnea* cf. *pectinata* (D) ethanol (E) acetone (F) ethyl acetate

**3.3 Minimum bactericidal concentration tests of *E. coli* TISTR 527**

The Minimum Bactericidal Concentration of lichen extracts was evaluated with concentrations ranging from 1.25-40 mg/ml. The results showed that the acetone extracts of *U. himantodes* had strong antibacterial properties with the lowest concentration of 20 mg/ml to kill *E. coli* TISTR 527, whereas the ethanolic and ethyl acetate extracts required a higher concentration of 40 mg/ml. In terms of *Usnea* cf. *pectinata* extracts, all extracts exhibited similar results in killing these bacteria with a higher concentration of 40 mg/ml as shown in Figure 76.

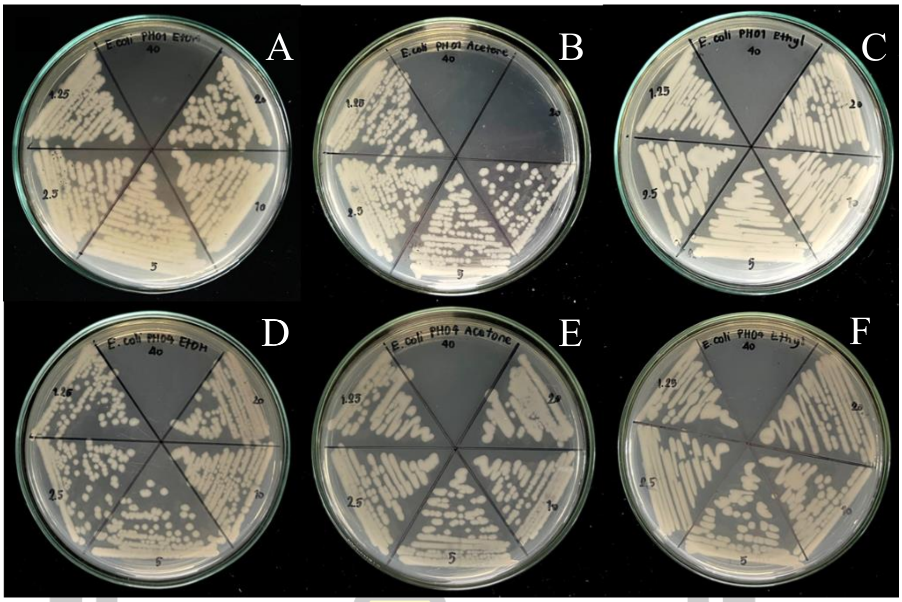


**Figure 76** Minimum Bactericidal Concentration of *E. coli* TISTR 527

A-C; *U. himantodes* (A) ethanol (B) acetone (C) ethyl acetate

D-F; *Usnea* cf. *pectinata* (D) ethanol (E) acetone (F) ethyl acetate

The results of the antibacterial activity indicate that the ethanolic, acetone, and ethyl acetate extracts of *U. himantodes* and *Usnea* cf. *pectinata* can effectively inhibit the growth of three bacterial species such as *B. cereus* TISTR 1449, *S. epidermidis* TISTR 2162, and *E. coli* TISTR 527. In contrast, boiling water extract is only able to inhibit *B. cereus* TISTR 1449. Additionally, neither *U. himantodes* nor *Usnea* cf. *pectinata* extracts were effective against *P. aeruginosa* TISTR 1287.

# CHAPTER 5
# DISCUSSION

## Morphological, Anatomical, and Chemical Analysis

The studies of morphological, anatomical characteristics of *Usnea* spp. collected in Thailand performed by using a microscope and chemical analysis conducted by High-Performance Thin Layer Chromatography. The specimens were compared with specimens published in the global database, including the studies of Ohmura (2021); Troung & Clerc (2013); Joshi et al. (2020); Clerc & Ohmura (2023); Temu & Clerc (2012); Ohmura (2012); Clerc (2016); Troung et al (2011); Nadel & Clerc (2022); Gauslaa & Timdal (2020); Ohmura & Clerc (2023); Gerlach et al. (2023); Shukul et al. (2014); Temu et al. (20 19); Ohmura (2001), that studies from Taiwan, Philippines, Japan, India, Australia, and Africa. The studies of *Usnea* spp. in Thailand in this research total 74 specimens, 42 specimens were newly collected in various locations in Thailand, and 32 specimens from the herbarium of Ramkhamhaeng University (RAMK). Among these can be identified 63 specimens into 12 species, including *U. aciculifera* Vain.,*U. articulata* (L.) Hoffm., *U, baileyi* (Stirt.) Zahlbr., *U. ceratina* Ach., *Usnea* cf. *dendritica* Stirt, *U. himantodes* Stirt., *U. pangiana* Stirt., *Usnea* cf. *perhispidella* J. Steiner, *Usnea* cf. *pectinata* Taylor, *U. rubicunda* Stirt., *Usnea* cf. *rubicunda* Stirt., *U. shimadae* Asahina, and 11 unidentified specimens (*Usnea* sp.1-*Usnea* sp.11). This research is the first to identify the species of *Usnea* spp. using morphological, anatomical, and chemical characteristics, and the first to report in Thailand of *U. aciculifera* Vain., *U. articulata* (L.) Hoffm., *U. ceratina* Ach., *Usnea* cf. *dendritica* Stirt, *Usnea* cf. *himantodes* Stirt., *U. pangiana* Stirt., *U.* cf. *perhispidella* J. Steiner, *Usnea* cf. *pectinata* Taylor, *U. shimadae* Asahina.

However, 23 species have been identified, and showed the differences in morphology, anatomy, and substances. Specimens of each species collected from different locations showed slight distinctions but retained the dominant characteristics of their species. This reflected notable diversity within the genus *Usnea*. The distribution of *Usnea* in this study was observed primarily in Evergreen Forests, especially in the Lower Montane Rain Forest. It was not found in Deciduous Forests, which tend to be more open and receive a lot of sunlight. This excessive light leads to the dehydration of the lichen's thallus, making the environment unsuitable for lichen growth.

**Molecular studies**

The molecular phylogeny studies of *Usnea* spp. involved a total of 74 specimens. Out of these, 73 specimens were unsuccessful in extracting the DNA. The failure was likely due to contamination by other microorganisms, as well as the advanced age of the specimens, some of which were over 25 years old. Only one specimen was successfully analyzed which was *Usnea aciculifera* (PT07). The ITS rDNA sequence was analyzed through the BLAST option on the NCBI database. The result shows a homology level of 100% identity with *U. aciculifera* (Accession No. FJ494923) specimen from Taiwan. The phylogeny was constructed using Maximum Likelihood and Neighbor-Joining with high bootstrap support (99/88). This demonstrates a very close relationship between *U. aciculifera* from Taiwan (Ohmura, 2021) and *U. aciculifera* (PT07) from Thailand. In addition, the genetic distance analysis confirms their relationship with a lowest distance value of 9%. This research is the first to identify *Usnea* using molecular data and the first report of *U. aciculifera* in Thailand. The *Usnea* sp.1- *Usnea* sp.11 also need molecular data to confirm species. Therefore, new techniques with used to get DNA from the materials.

**Antioxidant Activity**

The findings of this research study indicated that the crude lichen extract, which was extracted using 95% ethanol, contained the highest concentration of phenolic compounds, flavonoids, and tannins. This is likely because ethanol is a polar solvent that can extract secondary metabolites in lichen, which is also a polar substance, according to the principle of like dissolve like. These results are consistent with the study conducted by Popovici, V. et al. (2022) which evaluated the total phenolic content of *U. barbata* extracts. The effect had the highest for dry ethanolic (573.234 ± 42.308 mg PyE/g Extract). Likewise, the results of total flavonoid content are similar to the study of Aydin (2018) which found that the ethanolic extract had the highest TFC with 20.85 ± 0.005 µg QE/g lichen. According to Sinha's (2013) research, the present study observed a higher activity in the ethanol extract of *U. longissima* collected from India. The total phenolic content of the ethanol extract was 115 ± 0.58 mg GAE/g dry lichen, while the total flavonoid content was 1.625 ± 0.001 mg rutin equivalent/g dry lichen. Importantly, this is the first report on the evaluated content of the tannin compound in the lichen genus *Usnea*.

The evaluated the antioxidant activity of the lichen crude extract genus *Usnea* revealed that the extract with boiling water exhibited the highest antioxidant potentials of 3 assays: DPPH, ABTS, and FRAP assay. This finding is different from Pavithra et al. (2013) studied the antioxidant properties of the genus *Usnea pictoides* using the DPPH and FRAP assay and found that *Usnea* lichens extracted with acetone had the highest free radical inhibition value. In the same way, this research is dissimilar to the study of Londoñe-Bailon et al. (2019), which studied the antioxidant activity in lichens of the genus *Usnea* that were extracted with methanol-acetone using the ABTS assay method. It was observed that the lichens could inhibit free radicals with the $IC_{50}$ value being 19.42±0.32 mg GAE/g extract.

In this study, the extract with boiling water showed the highest antioxidant potential, despite not having the highest levels of phenolic compounds, flavonoids, and tannins. Possibly because the lichen crude extract is extracted with boiling water, it has the capability to pull out the primary substances that have soluble properties, such as proteins and polysaccharides. Recently, it was reported that polysaccharides significantly affect antioxidant potential. The polysaccharides inhibit the free radical chain reaction, acting as free radical scavengers and electron acceptors, resulting in a decrease in free radicals (Fernandes & Coimbra, 2023).

**Antibacterial Activity**

The study evaluated the antibacterial activity of crude extracts from the lichen *U. himantodes* and *Usnea* cf. *pectinata* extracts, indicating that it can inhibit bacterial test *B. cereus* and *S. epidermidis* which are gram-positive bacteria better than gram-negative bacteria such as *E. coli* and *P. aeruginosa.* This is likely because the structure of the cell wall of gram-negative bacteria is more complex than that of gram-positive bacteria (Suwanphinij and Suwanphinij, 1998). The cell wall of negative gram bacteria comprises a thin peptidoglycan layer between the inner and outer membranes. The outer membrane, consisting of lipopolysaccharides, protects the cell and makes it more resistant to damage than gram-positive bacteria (Srisukong et al, 2016). In addition, the previous study on metabolites substances in lichens showed that the lichen consists of tannin, the structure is a negative charge that can bind with substances that have a positive charge found in lichen, this interaction likely affects the breakdown of chromosomes of bacteria (Farha *et al*., 2020). Tannin can inhibit the cell wall synthesis of bacteria by making enzymes inactive (Trentin et al., 2013). In terms of the study of antibacterial activity 2 strains; *E. coli* and *P. aeruginosa* of the lichen extract shown that the lichen extract cannot inhibit the growth of *P. aeruginosa*, this may be because *P. aeruginosa* is a gram-negative bacterial that can produce the capsule and biofilm formation (Nunez et al., 2023). The biofilm structure comprises polysaccharides that affect the adaptation, survival rate, and growth of bacteria, making them more resistant to inhibition (Laverty et al., 2014). However, this finding is consistent with the study of Dieu et al. (2020) who studied the antimicrobial activity of the acetone extract of the lichen *U. florida*, their research showed that the extract can inhibit the growth of *S. aureus*, *C. albicans*, and *A. brasiliensis*, but it cannot inhibit *P. aeruginosa*.

**Conclusion**

The results of this study based on morphological, anatomical, chemical characteristics, and molecular studies, show species is the first report from Thailand including *U. aciculifera* Vain, *U. articulata* (L.) Hoffm., *U. ceratina* Ach., *Usnea* cf. *dendritica* Stirt, *U. pangiana* Stirt., *Usnea* cf. *perhispidella* J. Steiner, and *U. shimadae* Asahina. In addition, this research is a report on testing the antioxidant and antibacterial effects of lichens of the genus *Usnea*. These findings provide valuable insights for future development and application in pharmaceutical and food industries, enhancing the use of natural products for health-related benefits.

**Limitations of This Study**

This research study found limitations in sample collection because *Usnea* lichen samples are only found in environments with clean air. The specimens are usually found in high mountains, specifically on the canopy of trees, which makes collection challenging. There is also a low amount of samples in each area, which affects the extraction of substances. This may not be sufficient for testing.

**Suggestions**

This research focuses on studying the active substances present in lichen extracts only. To be a guideline for more specific studies, there may be studies to classify active substances found in lichen for further study of their effects. This can be achieved through chemical analysis techniques, such as analyzing the active substances using the High-Performance Liquid Chromatography (HPLC) technique. In addition, DNA extraction requires other techniques for old specimens in the museum and the specimens collected from the ground may be contaminated by other microorganisms, which can affect DNA extraction.

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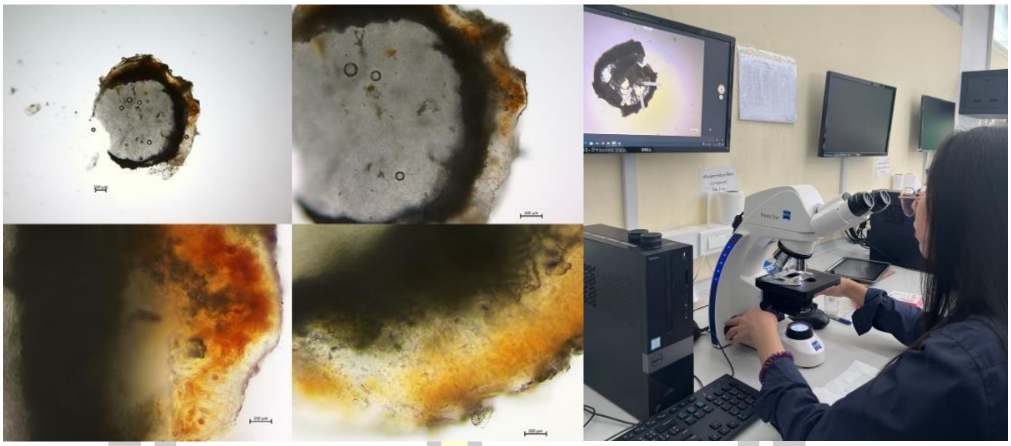
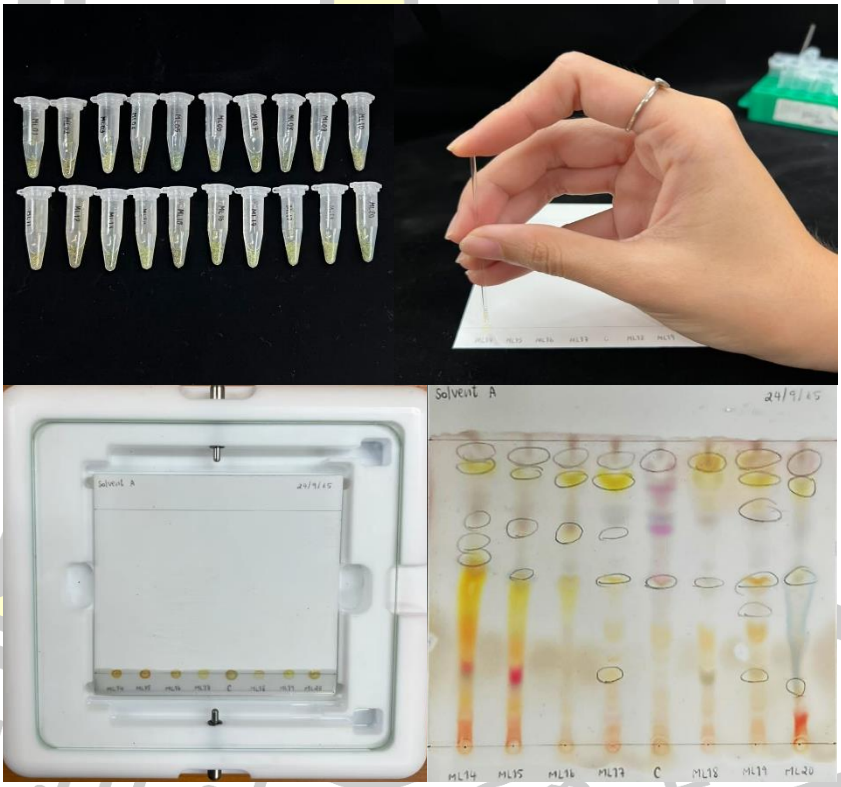
# APPENDIX

Sample Collection

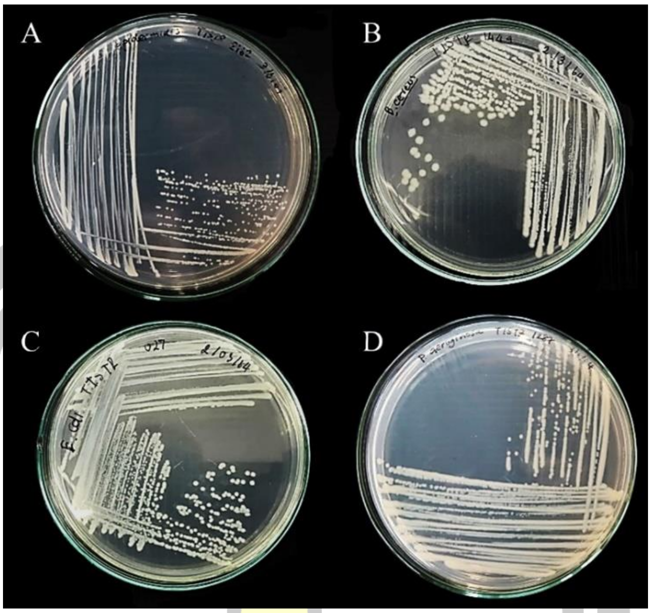
Morphological Analysis



Anatomical Analysis



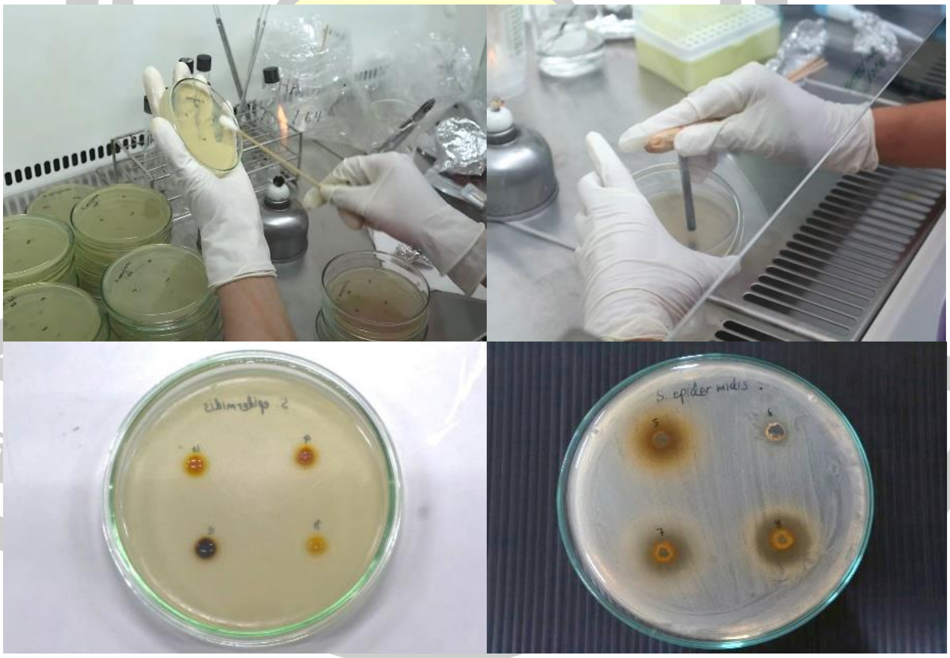
Chemical Analysis



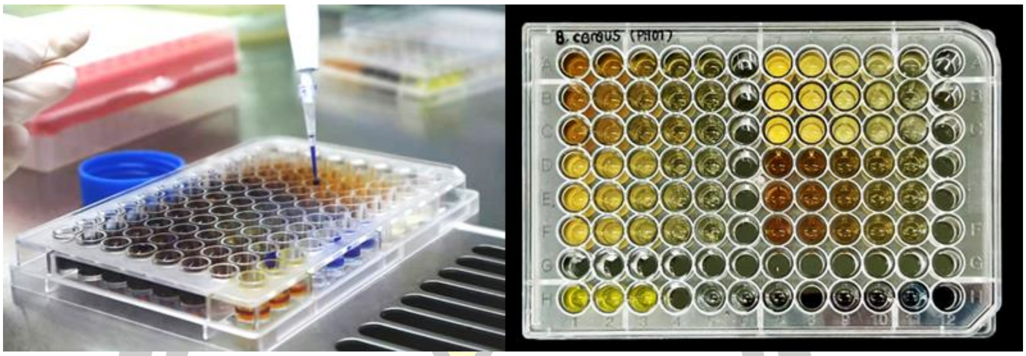

Bacterial cells were used in the experiment

(A) *S. epidermidis* TISTR 2162 (B) *B. cereus* TISTR 1449

(C) *E. coli* TISTR 527 (D) *P. aeruginosa* TISTR 1287



Agar Well Diffusion Test




Minimum Inhibitory Concentration Test


Minimum Bactericidal Concentration Test

The species identified

**Appendix Table 1** CMA ratio of *Usnea* sp.

| Specimen | %C | %M | %A | A/M |
|---|---|---|---|---|
| **Phu Hon Rong Kla** | | | | |
| PH01 | (6.6-) 7.3 (-9.3) | (17.6-) 23 (-31) | (63.1-) 69 (-74.8) | (2.1-) 3 (-3.7) |
| PH02 | (8.4-) 11 (-14.7) | (15.4-) 18 (-25.8) | (63.4-) 68 (-73.9) | (2.5-) 4 (-4.8) |
| PH03 | (6.8-) 10 (-12) | (20-) 26.8 (-31.4) | (58.7-) 63.4 (-70.1) | (1.9-) 2 (-3.5) |
| PH04 | (6.4-) 8 (-12.7) | (16.1-) 23.9 (-27.1) | (63.5-) 68 (-75.6) | (2.4-) 2.8 (-4.7) |
| PH05 | (7.9-) 8.5 (-13) | (12.8-) 18 (-23.8) | (66.6-) 71 (-77.9) | (2.8-) 3.8 (-6.1) |
| PH06 | (20.3-) 21 (-26.4) | (18.9-) 22 (-25.4) | (48.4-) 59 (-59.8) | (1.9-) 2.3 (-3.2) |
| PH07 | (5.4-) 6.5 (-6.9) | (52.4-) 63 (-66.1) | (27.4-) 30 (-40.8) | (0.4-) 0.5 (-0.8) |
| PH08 | (6.4-) 8.5 (-9.8) | (17.2-) 21 (-22) | (68.5-) 71 (-75.9) | (3.1-) 3.6 (-4.3) |
| **Phu Tab Buek** | | | | |
| PT01 | (9-) 14.3 (-17.2) | (25.7-) 28 (-31.7) | (51.4-) 59 (-65.3) | (1.6-) 2 (-2.5) |
| PT02 | (11.1-) 12 (-16.7) | (38.2-) 43 (-53.8) | (29.5-) 49.2 (-49.2) | (0.5-) 1.1 (-1.3) |
| PT03 | (15.9-) 19.6 (-21.6) | (46.1-) 53 (-56.4) | (24-) 31 (-35) | (0.4-) 0.6 (-0.8) |
| PT04 | (13.3-) 17 (-21) | (40.4-) 43 (-44.6) | (32.1-) 37 (-40.7) | (0.7-) 0.7 (-1) |
| PT05 | (10.6-) 12 (15.4) | (47.3-) 57 (-58.2) | (28.8-) 29 (-40.7) | (0.5-) 0.7 (-0.9) |
| PT06 | (11.7-) 15 (-18.6) | (38.8-) 47-48 (51.2) | (33.6-) 38 (-45.1) | (0.7-) 0.8 (-1.2) |
| PT07 | (10.5-) 12 (-14.2) | (32.4-) 34 (-37.5) | (49.2-) 53.6 (-56.6) | (1.3-) 1.6 (-1.7) |
| PT08 | (8.4-) 11.5 (-12) | (54.7-) 61 (-65.7) | (22.8-) 29.1 (-36.9) | (0.3-) 0.5 (-0.7) |
| PT09 | (10.7-) 11.7 (-13.2) | (32.7-) 36 (-41.9) | (46.4-) 51.5 (-56.4) | (1.1-) 1.4 (-1.7) |
| **Mon Long** | | | | |
| ML01 | (10.9-) 11 (-24.4) | (26.7-) 29 (-31.1) | (56.5-) 58 (-62.4) | (1.9-) 2 (-2.3) |
| ML02 | (9.8-) 10 (-12) | (16.9-) 19 (-21.3 | (66.7-) 71 (-73.3) | (3.1-) 3.7 (-4.3) |
| ML03 | (8.8-) 11 (-11.9) | (46.2-) 52 (-53.3) | (35-) 39.6 (-44.1) | (0.7-) 0.8 (-1.0) |
| ML04 | (11.8-) 14.4 (-16) | (37.9-) 46.1 (-48.4) | (37.5-) 45.5 (47.7) | (0.8-) 1.1 (-1.3) |
| ML05 | (-9.3) 15.4 (-16.7) | (-41.3) 46.2 (-51.4) | (-33.2) 40.8 (-47.4) | (0.7-) 0.9 (-1.1) |
| ML06 | (16.3-) 19.6 (-22.4) | (23.4-) 29.1 (-31.3) | (49.6-) 52.4 (-56.9) | (1.6-) 1.7 (-2.4) |
| ML07 | (7.5-) 9.2 (-11.3) | (15.6-) 17.5 (-20.2) | (70.6-) 73 (-76) | (3.5-) 4 (-4.9) |
| ML08 | (12-) 6.4 (-18.1) | (28.4-) 33.8 (-39.7) | (45-) 50.1 (53.8) | (1.1-) 1.5 (-1.9) |
| ML09 | (15-) 18.1 (-21.1) | (25.4-) 32.5 (-38) | (42.5-) 32.5 (-53.5) | (1.1-) 1.6 (-2.1) |
| ML10 | (5.5-) 9.6 (-13) | (57.5-) 60.3 (-64.8) | (25.7-) 29.7 (-36.2) | (0.4-) 0.5 (-0.6) |
| ML11 | (8.7-) 11.2 (-13.8) | (38.1-) 40 (-52.3) | (42.9-) 44 (-49.9) | (0.8-) 1.1 (-1.2) |
| ML12 | (13-) 15.7 (-17.6) | (30.3-) 35.6 (-41.5) | (43-) 50 (-56) | (1.0-) 1.1-1.5 (-1.8) |
| ML13 | (12.3-) 13.9 (-19.5) | (34.3-) 38.8 (-43.1) | (43.6-) 45.3 (-50.1) | (1.0-) 1.1 (-1.4) |
| ML14 | (15.9-) 18.5 (-21.5) | (24.7-) 26.6 (-31.9) | (51.1-) 54.7 (-58.6) | (1.6-) 2 (-2.3) |
| ML15 | (12.3-) 16 (-18.4) | (26.1-) 27.7 (-32.1) | (52.7-) 55.9 (-58.7) | (1.6-) 2.1 (-2.2) |
| ML16 | (11.8-) 12.9 (-15.5) | (25.9-) 29.4 (-32.5) | (54.6-) 56.4 (-61.5) | (1.7-) 2.0 (-2.4) |
| ML17 | (7.6-) 9.7 (-11.1) | (48.9-) 53.5 (-57.2) | (34.3-) 37.1 (-41.3) | (0.6-) 0.7 (-0.8) |
| ML18 | (13.4-) 17.1 (-18.7) | (24-) 28 (-29.6) | (53.3-) 55.7 (-61.4) | (1.8-) 1.9 (-2.4) |
| ML19 | (7.3-) 9 (-13.6) | (58.8-0 66.6 (-70.5) | (19.4-) 24.3 (-31.5) | (0.3-) 0.4 (-0.5) |
| ML20 | (16.6-) 19 (-21) | (30.2-) 34.8 (-39.2) | (42.4-) 46.6 (-51.2) | (1.1-) 1.2 (-1.7) |
| **Phu Ruea** | | | | |
| PR01 | (10.4-) 14 (-16.4) | (46.3-) 52 (-53.6) | (32.5-) 37 (-39.1) | (0.6-) 0.7 (-0.8) |
| PR02 | (10.8-) 12 (-16.3) | (49-) 55 (-64.2) | (22.7-) 24-25 (-39) | (0.4-) 0.4 (0.8) |
| PR03 | (7.6-) 9.7 (-13.4) | (37-) 49 (-60.6) | (27-) 38.3 (-49.5) | (0.4-) 0.9 (-1.3) |
| PR04 | (7.6-) 10.5 (-12.7) | (48-) 55 (-63.9) | (28.4-) 34.9 (-40.7) | (0.4-) 0.6 (-0.8) |

| Specimen | %C | %M | %A | A/M |
|---|---|---|---|---|
| **Phu Khieo** | | | | |
| PK01 | (7.2-) 9.6 (-11.8) | (41.9-) 47.3 (-55.0) | (35.1-) 43 (-50.9) | (0.6-) 0.7 (-1.2) |
| RAMK | | | | |
| RAMK025747 | (14-) 16.3 (-19.9) | (51.5-) 54.3 (-59.6) | (25.9-) 29.6 (-59.6) | (0.4-) 0.5 (-0.6) |
| RAMK025749 | (9.2-) 10.8 (-12.8) | (12.1-) 15.2 (-18.4) | (70.8-) 73.9 (-78.4) | (3.5-) 4.9 (-6) |
| RAMK035261 | (9.9-) 11.6 (-15.0) | (16.9-) 24.4 (-32.5) | (57.5-) 63 (-70.8) | (1.8-) 2.6 (-4.2) |
| RAMK035328 | (7.7-) 11.1 (-14.6) | (16.5 -) 22.3 (-26.4) | (64.1-) 66.5 (-68.9) | (2.4-) 3.0 (-4.2) |
| RAMK035437 | (8.2-) 10.6 (-11.2) | (15.9-) 20.8 (-23) | (65.8-) 68.4 (-75.1) | (2.9-) 3.3 (-4.6) |
| RAMK035457 | (7.8-) 11.4 (-14.5) | (14.3-) 17.5 (-23.4) | (64.3-) 71 (-74.9) | (2.7-) 4.2 (-5.0) |
| RAMK035462 | (7.6-) 9.6 (-14.6) | (14.7-) 21 (-25.9) | (66.5-) 68.2 (-73.7) | (2.6-) 3.3 (-5.0) |
| RAMK035468 | (5.0-) 6.6 (-7.9) | (7.2-) 11.1 (-14.6) | (77.5-) 81.8 (-86.5) | (5.3-) 7.4 (-12) |
| RAMK035477 | (9.2-) 11.1 (-14.3) | (17.8-) 20.2 (-27) | (62.6-) 69 (-70.2) | (2.3-) 3.4 (-3.9) |
| RAMK040350 | (6.0-) 6.6 (-9.2) | (8.8-) 9.6 (-12.4) | (81.0-) 83.1 (-85.2) | (6.5-) 8.7 (-9.7) |
| RAMK040351 | (11.9-) 12.9 (-16.0) | (16.8-) 20.1 (-24.5) | (63-) 66.7 (-70.8) | (2.6-) 3.3 (-4.2) |
| RAMK040352 | (7.2-) 7.7 (-10.3) | (13.8-) 16.0 (-17.2) | (74.5-) 76 (-77.5) | (4.4-) 4.8 (-5.6) |
| RAMK040353 | (12.2-) 16 (-21.1) | (23.8-) 25.8 (-29.2) | (53-) 57.8 (-62.2) | (1.8-) 2.2 (-2.5) |
| RAMK040354 | (11.2-) 14.4 (-16.4) | (14.8-) 16.8 (-21.2) | (65.3-) 69 (-71.8) | (3.1-) 4.1 (-4.8) |
| RAMK040355 | (9.3-) 11.2 (-12.4) | (12.7-) 14.4 (-17.6) | (71.1-) 75 (-76.2) | (4.0-) 5.2 (-6.0) |
| RAMK040356 | (9.4-) 12.2 (-16.8) | (13.4-) 17.9 (-21.7) | (66.5-) 68.5 (-75) | (3.1-) 3.9 (-5.6) |
| RAMK040357 | (9.4-) 10.9 (-13.0) | (17.2-) 24.4 (-29.7) | (60.9-) 65.4 (-70.3) | (2.1-) 2.8 (-3.8) |
| RAMK040358 | (9.6-) 11.7 (-14.1) | (17-) 20.7 (-23.5) | (64.4-) 67.8 (-71.9) | (2.8-) 3.2 (-4.2) |
| RAMK040359 | (8.3-) 9.4 (-10.4) | (59.3-) 61.8 (-64.4) | (26.3-) 28.7 (-31.3) | (0.4-) 0.5 (-0.5) |
| RAMK040360 | (9.7-) 10.8 (-14.6) | (50-) 57.8 (-61.2) | (29.1-) 30.9 (-35.3) | (0.5-) 0.5 (-0.7) |
| RAMK040361 | (10.8-) 12.4 (-15) | (28.5-) 31.5 (-39.8) | (49.1-) 55.5 (-58.4) | (1.2-) 1.8 (-2.0) |
| RAMK040362 | (13.9-) 16.5 (-18.6) | (28.6-) 31.1 (-34.1) | (50.1-) 52.5 (-55.2) | (1.5-) 1.7 (-1.9) |
| RAMK040363 | (11.7-) 14.1 (-15.4) | (26.6-) 29.8 (-35.8) | (50.1-) 56.4 (-58.9) | (1.4-) 1.9 (-2.2) |
| RAMK040364 | (9.6-) 10.3 (-12.3) | (15.7-) 18.4 (-23) | (67.1-) 70.3 (-74.2) | (2.9-) 3.8 (-4.7) |
| RAMK040365 | (6.5-) 7.7 (-10.4) | (9.8-) 11.2 (-15.7) | (77-) 80.4 (-82.9) | (4.9-) 7.3 (-8.5) |
| RAMK040366 | (15.2-) 18.1 (-20.9) | (25.5-) 28.5 (-32.9) | (49.6-) 53.8 (-56.3) | (1.5-) 1.9 (-2.2) |
| RAMK040367 | (11.8-) 15.1 (-20.8) | (14.4-) 20.1 (-23.6) | (56.9-) 65 (-70) | (2.4-) 3.2 (-4.9) |
| RAMK040368 | (6.0-) 8.4 (-11.2) | (12.5-) 15.2 (-17.5) | (74.5-) 75.8 (-78.6) | (4.3-) 4.9 (-6.2) |
| RAMK040369 | (14.1-) 16.2 (-19.5) | (22.7-) 28.2 (-31.5) | (51-) 55.1 (-60) | (1.7-) 1.9 (-2.6) |
| RAMK040370 | (8.5-) 9.4 (-10.3) | (54.1-) 57.6 (-62.2) | (28.5-) 32.5 (-36.3) | (0.5-) 0.6 (-0.7) |
| RAMK040371 | (5.9-) 7.3 (-9.1) | (9.4-) 12 (-13.5) | (78.4-) 80.7 (-84) | (5.8-) 6.7 (-8.9) |
| RAMK040372 | (10.6-) 12.1 (-13.2) | (22.4-) 28.9 (-32.7) | (54.6-) 69.3 (-64.4) | (1.7-) 2.1 (-2.9) |

**Abbreviation :** %C = cortex; %M = medulla; %A = axis; A/M = axis/medulla

**Appendix Table 2** Rf value of specimens

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| PH01 | A | 43 | 67 (Us) | 71 (At) | | | | | | | |
| | C | 32 (Nor) | 35 | 38 (Not) | 43 (Ever) | 63 | | | | | |
| | G | 35 | 42 | 64 | 75 | | | | | | |
| PH02 | A | 44 | 54 | 67 (Us) | 73 (At) | | | | | | |
| | C | 30 (Nor) | 36 (4dBar) | 41 (Pso) | 63 | | | | | | |
| | G | 27 | 31 | 42 | 59 | 73 | | | | | |
| PH03 | A | 67 (Us) | 73 (At) | | | | | | | | |
| | C | 36 (4dBar) | 41 (Pso) | 59 | | | | | | | |
| | G | 12 | 27 | 40 | 60 | 73 | | | | | |
| PH04 | A | 67 (Us) | 74 (At) | | | | | | | | |
| | C | 32 (Nor) | 36 (4dBar) | 41 (Pso) | 61 | | | | | | |
| | G | 25 | 38 | 58 | 71 | | | | | | |
| PH05 | A | 67 (Us) | 73 (At) | | | | | | | | |
| | C | 35 | 43 (Ever) | 62 | | | | | | | |
| | G | 25 | 38 | | | | | | | | |
| PH06 | A | 67 (Us) | 74 (At) | | | | | | | | |
| | C | 36 (4dBar) | 43 (Ever) | | | | | | | | |
| | G | 38 | | | | | | | | | |
| PH07 | A | 33 | 69 (Us) | 75 (At) | | | | | | | |
| | C | 43 (Ever) | | | | | | | | | |
| | G | 25 | 31 | 38 | 61 | 70 | | | | | |
| PH08 | A | 68 (Us) | 75 (At) | | | | | | | | |
| | C | 11 | 29 (Nor) | 34 | 37 | 41 (Pso) | 63 | | | | |
| | G | 14 | 24 | 30 | 42 | 67 | 75 | | | | |
| PT01 | A | 23 | 39 (Ever/ 4dBar) | 51 (Eum B) | 65 (Us) | 68 (At) | | | | | |
| | C | 50 (Dif) | 56 | | | | | | | | |
| | G | 35 (Stic) | 40 (Gal) | 50 | 62 | 70 | | | | | |
| PT02 | A | 25 | 40 (Ever/ Nor) | 45 (Dif/ Bar) | 52 (EumB) | 66 (Us) | 67 (At) | | | | |
| | C | 44 (Ever) (EumB) | 50 (Dif) | 57 | | | | | | | |

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | G | 35 (Stic) | 40 (Gal) | 52 | 61 | 69 | | | | | |
| PT03 | A | 24 | 40 (Ever/ Nor) | 45 (Dif/ Bar) | 52 (EumB) | 66 (Us) | | | | | |
| | C | 43 (Ever) | 48 (Dif) | 59 | | | | | | | |
| | G | 32 | 39 (Gal) | 52 | 65 | | | | | | |
| PT04 | A | 24 | 40 (Ever/ Nor) | 45 (Dif/ Bar) | 52 (EumB) | 67 (Us) | | | | | |
| | C | 42 (Ever) | 50 (Dif) | 63 | | | | | | | |
| | G | 31 | 40 (Gal) | 53 | 65 | | | | | | |
| PT05 | A | 56 | 66 (Us) | 73 (At) | | | | | | | |
| | C | 54 | 59 | 72 | | | | | | | |
| | G | 46 | 50 | 61 | 75 | | | | | | |
| PT06 | A | 33 (Stic) | 56 | 66 (Us) | 73 (At) | | | | | | |
| | C | 22 (Stic) | 51 (Dif) | 59 | 72 | | | | | | |
| | G | 44 | 51 | 62 | 75 | | | | | | |
| PT07 | A | 33 (Stic) | 56 | 67 (Us) | 73 (At) | | | | | | |
| | C | 22 (Stic) | 50 (Dif) | 59 | 71 | | | | | | |
| | G | 44 | 51 | 62 | 75 | | | | | | |
| PT08 | A | 35 (Stic) | 55 | 67 (Us) | 74 (At) | | | | | | |
| | C | 22 | 50 (Dif) | 59 | 73 | | | | | | |
| | G | 44 | 51 | 62 | 75 | | | | | | |
| PT09 | A | 34 (Stic) | 56 | 67 (Us) | 74 (At) | | | | | | |
| | C | 50 (Dif) | 59 | 73 | | | | | | | |
| | G | 44 (Gal) | 51 | 62 | 75 | | | | | | |
| ML01 | A | 41 (Nor) | 44 (Dif/ Bar) | 55 | 68 (Us) | 75 (At) | | | | | |
| | C | 34 | 37 (Not) | 41 (Pso) | 60 | | | | | | |
| | G | 25 (Sal) | 38 | 46 | 65 | 75 | | | | | |
| ML 02 | A | 16 (2dPso/ 2mSqu) | 41 (Nor) | 44 (Dif/ Bar) | 55 | 75 (At) | | | | | |
| | C | 21 (Leca) | 34 | 38 (Not) | 41 (Pso) | 60 | | | | | |
| | G | 23 (Sal) | 38 | 46 | 65 | 75 | | | | | |
| ML 03 | A | 10 (Sal) | 32 (Stic) | 36 (Pso) | 43 | 61 | 70 (Us) | 75 (At) | | | |
| | C | 11 (Mene) | 18 (Stic) | 32 (Nor) | 37 (Not) | 42 (Pso) | 62 | | | | |

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | G | 12 | 23 (Sal) | 27 | 31 (Stic) | 35 (Mene) | 38 | 42 | 57 | 65 | 75 |
| ML 04 | A | 9 (Sal) | 16 (2dPso/ 2mSqu) | 40 (Nor) | 56 | 68 (Us) | 75 (At) | | | | |
| | C | 36 (4dBar) | 41 (Pso) | 58 | | | | | | | |
| | G | 12 | 23 (Sal) | 31 (Stic) | 37 | 45 | 62 | 75 | | | |
| ML 05 | A | 15 (2dPso/ 2mSqu) | 45 (Dif/ Bar) | 70 (Us) | 75 (At) | | | | | | |
| | C | 37 (Not) | 41 (Pso) | 58 | | | | | | | |
| | G | 11 (Con) | 23 (Sal) | 34 (Stic) | 43 | 62 | 75 | | | | |
| ML 06 | A | 75 (At) | | | | | | | | | |
| | C | 8 | 38 (Not) | 43 | 60 | | | | | | |
| | G | 12 | 27 (Sal) | 30 | 32 (Stic) | 37 | 65 | 75 | | | |
| ML 07 | A | 46 (Dif/ Bar) | 71 (Us) | 75 (At) | | | | | | | |
| | C | 10 | 31 (Nor) | 36 (4dBar) | 40 (EumA) | 60 | | | | | |
| | G | 29 | 37 | 62 | 75 | | | | | | |
| ML 08 | A | 16 | 47 (Dif/ Bar) | 71 (Us) | 75 (At) | | | | | | |
| | C | 39 (Not) | 59 | | | | | | | | |
| | G | 13 | 24 | 30 | 41 (Gal) | 60 | 73 | | | | |
| ML 09 | A | 71 (Us) | 75 (At) | | | | | | | | |
| | C | 35 | 39 (Not) | 61 | | | | | | | |
| | G | 12 | 29 | 37 | 62 | 73 | | | | | |
| ML10 | A | 11 (Sal) | 20 | 24 | 32 (Stic) | 71 (Us) | 75 (At) | | | | |
| | C | 8 | 29 | 35 | 40 (Bae) | 60 | | | | | |
| | G | 13 | 27 | 30 | 37 | 62 | 75 | | | | |
| ML11 | A | 44 (Dif/Bar) | 71 (Us) | 75 (At) | | | | | | | |
| | C | 35 (4dBar) | 40 (EumA/ Bae) | | | | | | | | |
| | G | 22 | 30 | 36 (Mene) | 60 | 73 | | | | | |
| ML 12 | A | 45 (Dif/Bar) | 50 | 55 | 57 | 71 (Us) | 75 (At) | | | | |
| | C | 35 (4dBar) | 42 (Bae) | | | | | | | | |
| | G | 23 | 33 | 37 | 40 (Squa) | 45 (Phys) | 60 | 74 | | | |

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| ML 13 | A | 71 (Us) | 75 (At) | | | | | | | | |
| | C | 38 (Not) | 43 (Bae) | | | | | | | | |
| | G | 11 | 23 | 30 | 33 | 41 | 60 | 72 | | | |
| ML 14 | A | 47 (EumA) | 51 (EumB) | 56 | 69 (Us) | 73 (At) | | | | | |
| | C | 41 (Pso/Bae) | | | | | | | | | |
| | G | 45 (2dPso) | 49 | 67 | 79 | | | | | | |
| ML 15 | A | 42 | 55 | 67 (Us) | 72 (At) | | | | | | |
| | C | 43 (Pso/Bae) | | | | | | | | | |
| | G | 46 (2dPso) | 66 | 80 | | | | | | | |
| ML 16 | A | 54 | 66 (Us) | 71 (At) | | | | | | | |
| | C | 41 (Pso) | 56 | | | | | | | | |
| | G | 46 (2dPso) | 63 | 80 | | | | | | | |
| ML 17 | A | 19 | 41 (Nor) | 52 | 66 (Us) | 71 (At) | | | | | |
| | C | 10 | 40 (EumA) | 45 (EumB) | 59 | | | | | | |
| | G | 14 | 22 | 26 (Sal) | 31 | 33 (Stic) | 43 | 68 | 80 | | |
| ML 18 | A | 40 (Nor) | 70 (At) | | | | | | | | |
| | C | 62 | | | | | | | | | |
| | G | 12 | 18 | 31 (Fum Pr) | 42 | 61 | 74 | | | | |
| ML 19 | A | 18 | 34 | 40 | 60 | 66 (Us) | 71 (At) | | | | |
| | C | 11 | 37 | 45 (EumB) | 60 | | | | | | |
| | G | 14 | 20 | 28 (ProC) | 34 (Stic) | 40 | 43 | 59 | 67 | 80 | |
| ML20 | A | 15 (2mSqu) | 43 (Dif/Bar) | 65 (Us) | 71 (At) | | | | | | |
| | C | 13 | 47 | 63 | | | | | | | |
| | G | 31 | 47 (2dPso/EumA) | 67 | 80 | | | | | | |
| PR01 | A | 26 | 57 | 68 (Us) | 74 (At) | | | | | | |
| | C | 38 | 45 | 52 | 61 | | | | | | |
| | G | 33 (Stic) | 51 | 62 | 71 | | | | | | |
| PR02 | A | 26 | 57 | 60 | 69 (Us) | 74 (At) | | | | | |
| | C | 38 | 45 | 47 | 51 | 58 | | | | | |
| | G | 33 (Stic) | 51 | 62 | 72 | | | | | | |
| PR03 | A | 27 | 43 | 56 | 64 | 69 (Us) | 74 (At) | | | | |

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | C | 41 | 45 | 51 | 58 | | | | | | |
| | G | 22 | 33 (Stic) | 51 | 62 | 72 | | | | | |
| PR04 | A | 44 | 71 (Us) | 74 (At) | | | | | | | |
| | C | 44 | 55 | | | | | | | | |
| | G | 22 | 34 (Stic) | 52 | 63 | 73 | | | | | |
| PK01 | A | 10 (Phy) | 21 (Mene) | 67 (Us) | 73 (At) | | | | | | |
| | C | 10 (Mene) | 42 (Pso) | 52 (Bar) | 62 | | | | | | |
| | G | 11 | 40 (Gal) | 43 | 53 | 72 | | | | | |
| RAMK 040351 | A | 15 (2DePso) | 43 | 55 | 61 (Us) | 67 (At) | | | | | |
| | C | 47 | | | | | | | | | |
| | G | 28 | 48 | 56 | 67 | | | | | | |
| RAMK 040352 | A | 14 (2DePso) | 38 (4dBar) | 55 | 59 (Us) | 67 (At) | | | | | |
| | C | 47 | | | | | | | | | |
| | G | 26 (Sal) | 48 | 54 | 66 | | | | | | |
| RAMK 040356 | A | 16 (2DePso) | 41 (Nor) | 59 (Us) | 66 (At) | | | | | | |
| | C | 16 (Stic) | 37 | 41 (Pso) | 47 | | | | | | |
| | G | 40 (Gal) | 47 | 52 | 64 | | | | | | |
| RAMK 040359 | A | 18 | 42 (Nor) | 67 (At) | | | | | | | |
| | C | 15 (Stic) | 37 | 41 (Pso) | 47 | | | | | | |
| | G | 41 (Gal) | 47 | 53 | 66 | | | | | | |
| RAMK 040360 | A | 17 | 42 (Nor) | 67 (At) | | | | | | | |
| | C | 15 (Stic) | 37 | 41 (Pso) | 47 | | | | | | |
| | G | 41 (Gal) | 46 | 56 | 67 | | | | | | |
| RAMK 040361 | A | 40 (Nor) | 53 | 67 (At) | | | | | | | |
| | C | 41 (Pso) | 47 | | | | | | | | |
| | G | 31 | 48 | 59 | 71 | | | | | | |
| RAMK 040363 | A | 30 (Gal) | 38 | 66 (At) | | | | | | | |
| | C | 19 (Stic) | 30 | 59 | | | | | | | |
| | G | 33 (Stic) | 41 (Gal) | 67 | | | | | | | |
| RAMK 040364 | A | 8 (Const) | 14 | 39 (Bae/4dBar) | 53 | 67 (At) | | | | | |

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | C | 10 | 14 (Mene) | 38 (Not) | 48 | 59 | | | | | |
| | G | 27 (Sal) | 40 (Gal) | 44 | 67 | | | | | | |
| RAMK 040365 | A | 15 (2DePso) | 40 (Nor) | 53 | 57 | 66 (At) | | | | | |
| | C | 10 | 39 (Not) | 48 | 60 | | | | | | |
| | G | 25 (Sal) | 41 (Gal) | 45 | 50 | 66 | | | | | |
| RAMK 040366 | A | 8 (Const) | 19 (Mene) | 31 (Stic) | 66 (At) | | | | | | |
| | C | 20 | 30 | 60 | | | | | | | |
| | G | 23 | 33 (Stic) | 40 (Gal) | 66 | | | | | | |
| RAMK 040370 | A | 10 (Sal) | 31 (Stic) | 39 (Bae/4dBar) | 66 (At) | | | | | | |
| | C | 20 | 24 | 30 | 60 | | | | | | |
| | G | 25 | 34 (Stic) | 40 (Gal) | 65 | | | | | | |
| RAMK 040371 | A | 10 (Sal/Phy) | 14 | 32 | 40 | 59 | 66 (At) | | | | |
| | C | 35 | 40 | 51 (Dif) | 61 | | | | | | |
| | G | 30 | 33 (Stic) | 40 (Gal) | 47 | 65 | | | | | |
| RAMK 035261 | A | 9 (Sal) | 14 | 38 (Bae/4dBar) | 52 | 57 (Us) | 65 (At) | | | | |
| | C | 32 | 36 (4dBar) | 43 (Ever) | 50 | | | | | | |
| | G | 32 | 37 | 51 | 56 | 64 | | | | | |
| RAMK 035477 | A | 8 (Const) | 14 | 37 (Bae/4dBar) | 52 | 57 (Us) | 65 (At) | | | | |
| | C | 32 | 35 (4dBar) | 43 (Ever) | 50 | | | | | | |
| | G | 32 | 51 | 56 | 64 | | | | | | |
| RAMK 035462 | A | 8 (Const) | 16 (2dPso) | 29 (Gal) | 37 (Bae/4dBar) | 57 (Us) | 65 (At) | | | | |
| | C | 27 | 40 | 43 (Ever) | 50 | | | | | | |
| | G | 50 | 56 | 65 | | | | | | | |
| RAMK 035328 | A | 8 (Const) | 13 | 37 (Bae/4dBar) | 52 | 57 (Us) | 65 (At) | | | | |
| | C | 36 (4dBar) | 43 (Ever) | 50 | | | | | | | |
| | G | 31 | 49 | 56 | 65 | | | | | | |
| RAMK 035350 | A | 10 (Sal) | 39 (Bae/4dBar) | 58 (Us) | 65 (At) | | | | | | |
| | C | 35 | 39 (EumA) | 43 (Ever) | 49 | | | | | | |
| | G | 33 | 42 | 47 | 57 | 65 | | | | | |

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| RAMK 035457 | A | 8 (Const) | 14 | 38 (Ever) | 51 | 58 (Us) | 65 (At) | | | | |
| | C | 35 (4dBar) | 43 (Ever) | 50 | | | | | | | |
| | G | 32 | 37 | 49 | 57 | 66 | | | | | |
| RAMK 040353 | A | 16 | 40 (Nor) | 46 | 59 (Us) | 65 (At) | | | | | |
| | C | 13 (Tham) | 43 (Ever) | 51 (Dif) | 56 | | | | | | |
| | G | 29 | 51 | 54 (Us) | 61 (At) | | | | | | |
| RAMK 040355 | A | 15 (2DePso) | 40 (Nor) | 45 (Bar/Dif) | 47 | 50 | 59 (Us) | 65 (At) | | | |
| | C | 43 (Ever) | 51 (Dif) | 57 | | | | | | | |
| | G | 29 | 52 | 54 | 61 (At) | | | | | | |
| RAMK 040357 | A | 9 (Sal) | 17 | 40 (Nor) | 45 | 54 | 60 (Us) | 65 (At) | | | |
| | C | 15 | 43 (Bae) | 51 (Dif) | 59 | | | | | | |
| | G | 26 (Sal) | 51 | 54 (Us) | 61 (At) | | | | | | |
| RAMK 040362 | A | 16 | 40 (Nor) | 60 (Us) | 66 (At) | | | | | | |
| | C | 15 | 43 | 52 (Bar) | 59 | | | | | | |
| | G | 26 (Sal) | 51 | 54 (Us) | 61 (At) | | | | | | |
| RAMK 040372 | A | 9 (Sal) | 40 (Nor) | 59 (Us) | 66 (At) | | | | | | |
| | C | 42 (Bae) | 52 (Bar) | 58 | | | | | | | |
| | G | 27 (Sal) | 30 | 51 | 54 (Us) | 62 (At) | | | | | |
| RAMK 035437 | A | 9 (Sal) | 16 | 42 (Bar/Dif) | 55 | 60 (Us) | 66 (At) | | | | |
| | C | 13 (Tham) | 42 (Bae) | 52 (Bar) | 58 | | | | | | |
| | G | 30 | 51 | 55 (Us) | 63 (At) | | | | | | |
| RAMK 035468 | A | 11 (Sal) | 21 | 43 (Bar/Dif) | 45 (Bar/Dif) | 62 (Us) | 67 (At) | | | | |
| | C | 9 | 34 | 54 (Bar) | 59 | | | | | | |
| | G | 27 (Sal) | 40 (Squa) | 51 | 55 (Us) | 65 (At) | | | | | |
| RAMK 025747 | A | 12 (Sal) | 34 (Stic) | 59 (Us) | 66 (At) | | | | | | |
| | C | 10 | 26 | 35 | 54 | 60 | | | | | |
| | G | 16 | 33 (Stic) | 38 | 47 | 55 | 64 | | | | |
| RAMK 025749 | A | 17 | 43 (Bar/Dif) | 47 (EumA) | 49 | 59 (Us) | 66 (At) | | | | |
| | C | 43 (Bae) | 51 (Dif) | 54 | 60 | | | | | | |

| Sample | Solvent system | RF value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | G | 27 (Sal) | 51 | 54 | 63 | | | | | | |
| RAMK 040368 | A | 17 | 43 (Bar/Dif) | 47 (Eum A) | 49 | 52 | 59 (Us) | 66 (At) | | | |
| | C | 44 (Bae) | 51 (Dif) | 60 | | | | | | | |
| | G | 51 | 54 | 63 | | | | | | | |
| RAMK 040354 | A | 60 (Us) | 67 (At) | | | | | | | | |
| | C | 53 | 60 | | | | | | | | |
| | G | 54 | 63 | | | | | | | | |
| RAMK 040358 | A | 17 | 43 (Bar/ Dif) | 46 (Eum A) | 48 | 52 | 59 (Us) | 66 (At) | | | |
| | C | 15 (Stic) | 43 (Ever) | 51 (Dif) | 60 | | | | | | |
| | G | 52 | 54 | 63 | | | | | | | |
| RAMK 040367 | A | 22 | 61 (Us) | 66 (At) | | | | | | | |
| | C | 9 | 23 (2mSqu) | 34 | 52 (Bar) | 58 | | | | | |
| | G | 37 (Stic) | 50 | 54 | 63 | | | | | | |
| RAMK 040369 | A | 45 | 50 | 61 (Us) | 67 (At) | | | | | | |
| | C | 15 (Stic) | 44 (EumB) | 52 (Bar) | 59 | | | | | | |
| | G | 31 (Stic) | 52 | 55 | 64 | | | | | | |

**Abbreviation :** At = atranorin; Bae = Baeomycesic acid; Bar = barbatic acid; Con = constictic acid; Dif = diffractaic acid; 4dBar = 4-O-demethylbarbatic acid; 2dPso = 2’-O-demethylpsoromic acid; EumA = eumitrin A; EumB = eumitrin B; Ever = evernic acid; Gal = galbinic acid; Mene = menegazziaic acid; 2mSqua = 2-O-methylsquamatic acid; Not = notatic acid; Phy = physodalic acid; ProC = protocetraric acid; Pso = psoromic acid; Stic = stictic acid; Squa = squamatic acid; Tham = thamnolic acid; Us = usnic acid; Nor = norstictic acid; Sal = salazinic acid

**Appendix Table 3** Total Phenolic Content

**Descriptives**

**TPC**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| PH01 Ethanol | 3 | 84.4833 | 1.43590 | .82902 | 80.9164 | 88.0503 | 82.88 | 85.65 |
| PH01 Acetone | 3 | 27.5670 | 2.99889 | 1.73141 | 20.1173 | 35.0167 | 24.79 | 30.75 |
| PH01 Ethyl acetate | 3 | 67.3910 | 2.85816 | 1.65016 | 60.2909 | 74.4911 | 65.70 | 70.69 |
| PH01 Boil | 3 | 32.5310 | 2.54320 | 1.46832 | 26.2133 | 38.8487 | 30.51 | 35.39 |
| PH04 Ethanol | 3 | 93.7723 | 2.87350 | 1.65902 | 86.6342 | 100.9105 | 90.90 | 96.64 |
| PH04 Acetone | 3 | 48.2600 | 2.31847 | 1.33857 | 42.5006 | 54.0194 | 45.92 | 50.55 |
| PH04 Ethyl acetate | 3 | 53.8087 | .52348 | .30223 | 52.5083 | 55.1091 | 53.40 | 54.40 |
| PH04 Boil | 3 | 16.4390 | .11527 | .06655 | 16.1527 | 16.7253 | 16.34 | 16.57 |
| Total | 24 | 53.0315 | 26.32422 | 5.37341 | 41.9158 | 64.1473 | 16.34 | 96.64 |

**ANOVA**

**TPC**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 15858.966 | 7 | 2265.567 | 457.554 | .000 |
| Within Groups | 79.224 | 16 | 4.951 | | |
| Total | 15938.190 | 23 | | | |

**TPC**

Tukey HSD[a]

| sample | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| PH04 Boil | 3 | 16.4390 | | | | | |
| PH01 Acetone | 3 | | 27.5670 | | | | |
| PH01 Boil | 3 | | 32.5310 | | | | |
| PH04 Acetone | 3 | | | 48.2600 | | | |
| PH04 Ethyl acetate | 3 | | | 53.8087 | | | |
| PH01 Ethyl acetate | 3 | | | | 67.3910 | | |
| PH01 Ethanol | 3 | | | | | 84.4833 | |
| PH04 Ethanol | 3 | | | | | | 93.7723 |
| Sig. | | 1.000 | .182 | .105 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

**Appendix Table 4** Total Flavonoid Content

**Descriptives**

**TFC**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| PH01 Ethanol | 3 | 21.5950 | 1.13759 | .65679 | 18.7691 | 24.4209 | 20.34 | 22.55 |
| PH01 Acetone | 3 | 15.2443 | .76777 | .44327 | 13.3371 | 17.1516 | 14.75 | 16.13 |
| PH01 Ethyl acetate | 3 | 8.8360 | .66879 | .38612 | 7.1746 | 10.4974 | 8.26 | 9.57 |
| PH01 Boil | 3 | 14.2950 | .38385 | .22162 | 13.3415 | 15.2485 | 13.89 | 14.65 |
| PH04 Ethanol | 3 | 36.8467 | .61312 | .35398 | 35.3236 | 38.3697 | 36.33 | 37.52 |
| PH04 Acetone | 3 | 24.2943 | .24042 | .13881 | 23.6971 | 24.8916 | 24.03 | 24.51 |
| PH04 Ethyl acetate | 3 | 9.3283 | .38147 | .22024 | 8.3807 | 10.2759 | 8.89 | 9.61 |
| PH04 Boil | 3 | 11.8387 | .39289 | .22683 | 10.8627 | 12.8147 | 11.42 | 12.19 |
| Total | 24 | 17.7848 | 9.05036 | 1.84740 | 13.9632 | 21.6064 | 8.26 | 37.52 |

**ANOVA**

**TFC**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1877.485 | 7 | 268.212 | 668.067 | .000 |
| Within Groups | 6.424 | 16 | .401 | | |
| Total | 1883.909 | 23 | | | |

**TFC**

Tukey HSD[a]

| sample | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| PH01 Ethyl acetate | 3 | 8.8360 | | | | | |
| PH04 Ethyl acetate | 3 | 9.3283 | | | | | |
| PH04 Boil | 3 | | 11.8387 | | | | |
| PH01 Boil | 3 | | | 14.2950 | | | |
| PH01 Acetone | 3 | | | 15.2443 | | | |
| PH01 Ethanol | 3 | | | | 21.5950 | | |
| PH04 Acetone | 3 | | | | | 24.2943 | |
| PH04 Ethanol | 3 | | | | | | 36.8467 |
| Sig. | | .975 | 1.000 | .608 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

**Appendix Table 5** Total Tannin Content

**Descriptives**

**Tannin**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| PH01 Ethanol | 3 | 81.4700 | 1.39082 | .80299 | 78.0150 | 84.9250 | 79.92 | 82.60 |
| PH01 Acetone | 3 | 26.3423 | 2.90524 | 1.67734 | 19.1253 | 33.5594 | 23.66 | 29.43 |
| PH01 Ethyl acetate | 3 | 64.9153 | 2.76867 | 1.59849 | 58.0376 | 71.7931 | 63.28 | 68.11 |
| PH01 Boil | 3 | 31.1503 | 2.46282 | 1.42191 | 25.0324 | 37.2683 | 29.19 | 33.92 |
| PH04 Ethanol | 3 | 90.4670 | 2.78350 | 1.60706 | 83.5524 | 97.3816 | 87.68 | 93.25 |
| PH04 Acetone | 3 | 46.3850 | 2.24545 | 1.29641 | 40.8070 | 51.9630 | 44.11 | 48.60 |
| PH04 Ethyl acetate | 3 | 51.7593 | .50704 | .29274 | 50.4998 | 53.0189 | 51.36 | 52.33 |
| PH04 Boil | 3 | 15.5640 | .11166 | .06447 | 15.2866 | 15.8414 | 15.47 | 15.69 |
| Total | 24 | 51.0067 | 25.49699 | 5.20455 | 40.2402 | 61.7731 | 15.47 | 93.25 |

**ANOVA**

**Tannin**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 14877.888 | 7 | 2125.413 | 457.504 | .000 |
| Within Groups | 74.331 | 16 | 4.646 | | |
| Total | 14952.218 | 23 | | | |

**Tannin**

Tukey HSD[a]

| sample | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| PH04 Boil | 3 | 15.5640 | | | | | |
| PH01 Acetone | 3 | | 26.3423 | | | | |
| PH01 Boil | 3 | | 31.1503 | | | | |
| PH04 Acetone | 3 | | | 46.3850 | | | |
| PH04 Ethyl acetate | 3 | | | 51.7593 | | | |
| PH01 Ethyl acetate | 3 | | | | 64.9153 | | |
| PH01 Ethanol | 3 | | | | | 81.4700 | |
| PH04 Ethanol | 3 | | | | | | 90.4670 |
| Sig. | | 1.000 | .182 | .105 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

**Appendix Table 6** DPPH assay

**Descriptives**

**DPPH**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| PH01 Ethanol | 3 | 1.6643 | .00153 | .00088 | 1.6605 | 1.6681 | 1.66 | 1.67 |
| PH01 Acetone | 3 | 1.2877 | .00252 | .00145 | 1.2814 | 1.2939 | 1.29 | 1.29 |
| PH01 Ethyl acetate | 3 | 2.0040 | .00200 | .00115 | 1.9990 | 2.0090 | 2.00 | 2.01 |
| PH01 Boil | 3 | .7520 | .00265 | .00153 | .7454 | .7586 | .75 | .76 |
| PH04 Ethanol | 3 | 1.9123 | .00737 | .00426 | 1.8940 | 1.9306 | 1.90 | 1.92 |
| PH04 Acetone | 3 | 1.3100 | .00889 | .00513 | 1.2879 | 1.3321 | 1.30 | 1.32 |
| PH04 Ethyl acetate | 3 | 1.2047 | .00551 | .00318 | 1.1910 | 1.2183 | 1.20 | 1.21 |
| PH04 Boil | 3 | .7520 | .00361 | .00208 | .7430 | .7610 | .75 | .76 |
| Total | 24 | 1.3609 | .45451 | .09278 | 1.1690 | 1.5528 | .75 | 2.01 |

**ANOVA**

**DPPH**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4.751 | 7 | .679 | 27654.589 | .000 |
| ithin Groups | .000 | 16 | .000 | | |
| Total | 4.751 | 23 | | | |

**DPPH**

Tukey HSD[a]

| sample | N | Subset for alpha = 0.05 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| PH01 Boil | 3 | .7520 | | | | | | |
| PH04 Boil | 3 | .7520 | | | | | | |
| PH04 Ethyl acetate | 3 | | 1.2047 | | | | | |
| PH01 Acetone | 3 | | | 1.2877 | | | | |
| PH04 Acetone | 3 | | | | 1.3100 | | | |
| PH01 Ethanol | 3 | | | | | 1.6643 | | |
| PH04 Ethanol | 3 | | | | | | 1.9123 | |
| PH01 Ethyl acetate | 3 | | | | | | | 2.0040 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

**Appendix Table 7** ABTS assay

**Descriptives**

**ABTS**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| PH01 Ethanol | 3 | 3.0647 | .00153 | .00088 | 3.0609 | 3.0685 | 3.06 | 3.07 |
| PH01 Acetone | 3 | 4.7003 | .00351 | .00203 | 4.6916 | 4.7091 | 4.70 | 4.70 |
| PH01 Ethyl acetate | 3 | 3.6347 | .00208 | .00120 | 3.6295 | 3.6398 | 3.63 | 3.64 |
| PH01 Boil | 3 | .7503 | .00351 | .00203 | .7416 | .7591 | .75 | .75 |
| PH04 Ethanol | 3 | 3.2520 | .00436 | .00252 | 3.2412 | 3.2628 | 3.25 | 3.26 |
| PH04 Acetone | 3 | 3.1450 | .00400 | .00231 | 3.1351 | 3.1549 | 3.14 | 3.15 |
| PH04 Ethyl acetate | 3 | 3.1060 | .00100 | .00058 | 3.1035 | 3.1085 | 3.11 | 3.11 |
| PH04 Boil | 3 | 1.0033 | .00153 | .00088 | .9995 | 1.0071 | 1.00 | 1.00 |
| Total | 24 | 2.8320 | 1.26229 | .25766 | 2.2990 | 3.3651 | .75 | 4.70 |

**ANOVA**

**ABTS**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 36.648 | 7 | 5.235 | 601191.627 | .000 |
| Within Groups | .000 | 16 | .000 | | |
| Total | 36.648 | 23 | | | |

**ABTS**

Tukey HSD[a]

| sample | N | Subset for alpha = 0.05 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| PH01 Boil | 3 | .7503 | | | | | | | |
| PH04 Boil | 3 | | 1.0033 | | | | | | |
| PH01 Ethanol | 3 | | | 3.0647 | | | | | |
| PH04 Ethyl acetate | 3 | | | | 3.1060 | | | | |
| PH04 Acetone | 3 | | | | | 3.1450 | | | |
| PH04 Ethanol | 3 | | | | | | 3.2520 | | |
| PH01 Ethyl acetate | 3 | | | | | | | 3.6347 | |
| PH01 Acetone | 3 | | | | | | | | 4.7003 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

**Appendix Table 8** FRAP assay

**Descriptives**

**FRAP**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| PH01 Ethanol | 3 | 15.8517 | .34516 | .19928 | 14.9942 | 16.7091 | 15.46 | 16.09 |
| PH01 Acetone | 3 | 21.4630 | .38639 | .22308 | 20.5032 | 22.4228 | 21.09 | 21.86 |
| PH01 Ethyl acetate | 3 | 19.4897 | .58947 | .34033 | 18.0253 | 20.9540 | 18.81 | 19.83 |
| PH01 Boil | 3 | 30.0687 | .21126 | .12197 | 29.5439 | 30.5935 | 29.88 | 30.30 |
| PH04 Ethanol | 3 | 16.7140 | 1.96965 | 1.13718 | 11.8211 | 21.6069 | 14.48 | 18.19 |
| PH04 Acetone | 3 | 13.6820 | .08345 | .04818 | 13.4747 | 13.8893 | 13.59 | 13.76 |
| PH04 Ethyl acetate | 3 | 18.9727 | .11751 | .06785 | 18.6807 | 19.2646 | 18.86 | 19.10 |
| PH04 Boil | 3 | 27.9567 | .13972 | .08067 | 27.6096 | 28.3037 | 27.88 | 28.12 |
| Total | 24 | 20.5248 | 5.55897 | 1.13472 | 18.1774 | 22.8721 | 13.59 | 30.30 |

**ANOVA**

**FRAP**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 701.589 | 7 | 100.227 | 175.056 | .000 |
| Within Groups | 9.161 | 16 | .573 | | |
| Total | 710.750 | 23 | | | |

**FRAP**

Tukey HSD[a]

| sample | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| PH04 Acetone | 3 | 13.6820 | | | | |
| PH01 Ethanol | 3 | | 15.8517 | | | |
| PH04 Ethanol | 3 | | 16.7140 | | | |
| PH04 Ethyl acetate | 3 | | | 18.9727 | | |
| PH01 Ethyl acetate | 3 | | | 19.4897 | 19.4897 | |
| PH01 Acetone | 3 | | | | 21.4630 | |
| PH04 Boil | 3 | | | | | 27.9567 |
| PH01 Boil | 3 | | | | | 30.0687 |
| Sig. | | 1.000 | .847 | .988 | .082 | .054 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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| | |
|---|---|
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| **Research output** | Kritsada Champatasi, Nucharee Chamnantap, Areerat Saisong & Khwanyuruan Naksuwankul. (2022). The Evaluation of Potentials of Antioxidant Activities, Total Phenolic, Flavonoid, and Tannin Contents from Selected Species in Amanita Crude Extract. Journal of Thai Traditional & Alternative Medicine, 20(2), 282-294. (in Thai)<br><br>Khwanyuruan Naksuwankul, Areerat Saisong, Orathai Sertsri, Thantip Supama, Fuenglada Niyanan, Pongthep Suwanwaree, Kawisara Hengtanarat & Somsuk Kanchanakhot. (2023). Species Diversity of Mushrooms in Ecotourism Area at Pha Hin Ngam National Park, Chaiyaphum Province. Journal of Science & Technology, Ubon Ratchathani University, 25(2), 97-108. (in Thai)<br><br>Khwanyuruan Naksuwankul, Nucharee Chamnantap, Areerat Saisong & Kritsada Champatasi. (2023). Antioxidant Activity, Total Phenolic, Flavonoid, and Tannin Contents from Phlebopus colossus (R.Heim) Singer and Boletus griseipurpureus Corner Crude Extract with Various Solvents. KKU Research Journal (Graduate Studies), 23(1), 132-148. (in Thai)<br><br>Areerat Saisong & Khwanyuruan Naksuwankul. (2023). Antioxidant Activity, Total Phenolic Content and Total |

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