Prayogik Rasayan

Lycopodium Alkaloids from Thai Club Mosses

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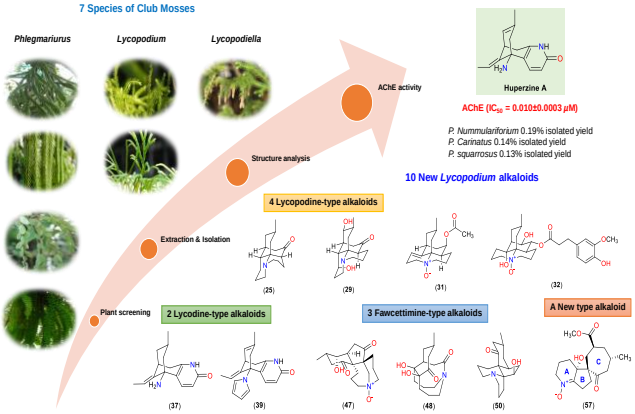
Received: May 8, 2021 | Accepted: May 26, 2021 | Published online: June 22, 2021

Abstract

The Lycopodiaceae family is the main source of *Lycopodium* alkaloids (LAs). The phytochemical constituents of alkaloid extract from seven species of Thai club mosses: *Phlegmariurus carinatus*, *P. squarrosus*, *P. nummularifolius*, *P. phlegmaria*, *Lycopodiella cernua*, *Lycopodium clavatum* and *L. complanatum*, were reviewed and classified into four major groups consisting of: lycopodine, lycodine, fawcettimine and phlegmarine (or miscellaneous group). The phytochemical relationships between the genera, *Phlegmariurus* (4 species), *Lycopodium* (2 species) and *Lycopodiella* (1 species) were investigated and reviewed based on their isolated *Lycopodium* alkaloids. The structures of 38 *Lycopodium* alkaloids are presented and some LAs are marked as key representatives for classification of the genera. The content of huperzine A in the club moss species was evaluated for biological activities. Some isolated LAs were potent acetylcholinesterase (AChE) inhibitors. This review focuses on the structural classification of the LAs isolated from Thai club mosses and their biological activities, especially acetylcholinesterase inhibitory activity.



Keywords: Lycopodiaceae, *Lycopodium* alkaloids, lycopodine, lycodine, fawcettimine, phlegmarine, acetylcholinesterase (AChE)

1. Introduction

Lycopodiaceae are terrestrial or epiphytic homosporous plants that are normally known as club mosses, comprising of 16 genera[1] and about 400 species around the world.[2-6] They are distributed particularly in the tropical areas that are humid with plentiful rainfall as in Southern China, India and Southeast Asia. There are a variety of club mosses that have been reported and are used in the 10 member countries of the Association of Southeast Asian Nations (ASEAN). Many club mosses are used as traditional herbal medicine for the treatment of: contusions, strains, scalds, swelling, schizophrenia, rheumatic fever and myasthenia gravis.[7] To date, studies of the Lycopodiaceae in ASEAN countries are scant. Of the 10 ASEAN countries, only Thailand, Malaysia and Vietnam have actively studied the phytochemistry of this family. In Malaysia 27 species were reported and grouped into three genera, i.e. *Huperzia*, *Lycopodium* and *Lycopodiella*.[8] Likewise, 14 species were found in Vietnam and classified into the same three genera.[9,10]

The Lycopodiaceae is an important source of the *Lycopodium* alkaloids (LAs). Alkaloids are known to possess important bioactivities including potent acetylcholinesterase inhibition activity. For example, huperzine A (HupA, **36**), an acetylcholinesterase inhibitor used for the treatment of Alzheimer's disease (AD),[11] was first isolated from Chinese club moss *H. serrata* Thunb., a traditional Chinese folk medicine, in 1986. The frequency of phytochemical studies of club mosses in ASIAN countries has increased in the last decade resulting in the discovery of new compounds. The club moss, *Huperzia goebelii*, that was collected in Malaysia, was found to contain a novel $C_{16}N$-type lycopodium alkaloid consisting of decahydroquinoline with an aminohexyl side chain, lycobelines A-C (**1-3**) as shown in Figure 1.[12] In addition, huperminone A (**4**)[13] and hupermine A (**5**)[14] were isolated from *H. phlegmaria* collected in Malaysia. Furthermore, *Lycopodium* alkaloids comprising six lycodine-type (**6, 7, 10, 13, 14,** and **15**), three fawettimine-type (**8, 9,** and **16**) and two phlegmarine-type (**11-12**) were isolated from the Malaysian club moss, *Lycopodium platyrhizoma*.[15] Two novel *Lycopodium* alkaloids, huperphlegmines A-B (**17-18**),[16] and one fawcettimine alkaloid, fawcettidine (**44**),[17] were

isolated from the aerial parts of *H. phlegmaria* collected in Vietnam.

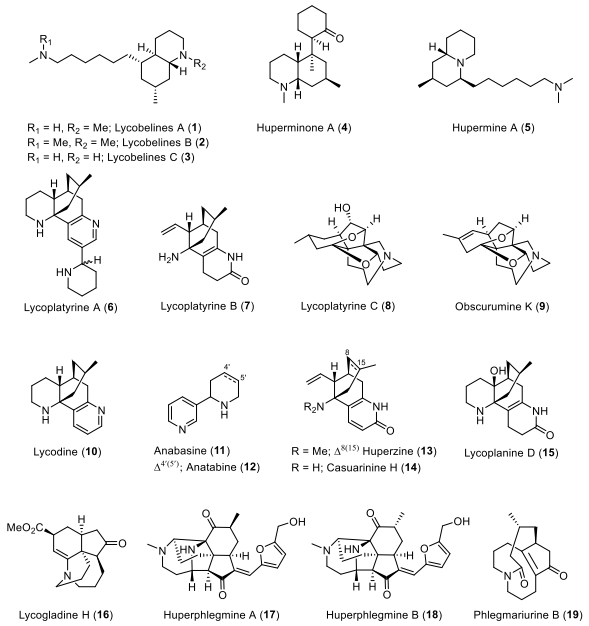


Figure 1. Structures of compounds **1-19** isolated from the aerial parts of *H. phlegmaria*, *H. goebelii*, *L. platyrhizona* collected from Malaysia and Vietnam.

To date, more than 300 *Lycopodium* alkaloids have been isolated from the Lycopodiaceae family.[18-22] Due to continued attention to the search for potentially new and bioactive natural products from the Lycopodiaceae, many reviews on *Lycopodium* alkaloids have been written dealing with club mosses of Chinese, Japanese and Canadian origins covering a number of areas of research progress concerning *Lycopodium* alkaloids.[7,23,24] However, phytochemical investigations concerning *Lycopodium* alkaloids in ASEAN countries have not yet been reviewed. Therefore, this review focuses on the isolation and biological activity of *Lycopodium* alkaloids from Thai club mosses.

To establish a platform study to search for bioactive *Lycopodium* alkaloids in this area, our group has focused on screening for potential acetylcholinesterase inhibitors (AChEI) such as HupA derivatives and other LAs. Fifteen samples belonging to seven species of club mosses including: *Phlegmariurus carinatus* (previously reported as *Huperzia carinata*), *P. squarrosus* (previously reported as *H. squarrosa*), *P. nummularifolius*, *P. phlegmaria* (previously reported as *H. phlegmaria*), *Lycopodiella cernua*, *Lycopodium clavatum* and *L. complanatum* were studied. The relationships between members of the genera, *Phlegmariurus* (4 species), *Lycopodium* (2 species) and *Lycopodiella* (1 species), were investigated and reviewed based on the isolated *Lycopodium* alkaloids. The structures of 38 *Lycopodium* alkaloids were presented and some LAs were marked as a key representative of genera. Some *Lycopodium* alkaloids were shown to be potent inhibitors of acetylcholinesterase.

This review will place particular emphasis on the structural classification and acetylcholinesterase inhibitory activity of *Lycopodium* alkaloids isolated Thai club mosses. Ten new LAs were isolated, including 4-epilycopodine (**25**), 8β,11α-dihydroxylycopodine (**29**), acetyllycophlegmarianol (**31**), lycocarinatine A (**32**) classified as the lycodoline-type alkaloids. 8,15-Dihydrohuperzine (**37**) and pyrrolhuperzine A (**39**) were two new LAs in lycodine-type alkaloid. Lycoposerramine U N-oxide (**47**), lycoclavatumide (**48**), and squarrosine A (**50**) were classified as fawcettimine-type alkaloids. In addition, squarrosinoxide (**57**) was a novel type LA containing an unprecedented 6/5/7 tricyclic spiro-system. Twenty-eight known *Lycopodium* alkaloids from our previous studies are also mentioned in this review.

2. Sample collection and general method for extraction of *Lycopodium* alkaloids.

Fifteen samples from seven species of club mosses from three genera of Lycopodiaceae including: *Phlegmariurus* (4 species), *Lycopodium* (2 species) and *Lycopodiella* (1 species) were studied. Their sources were as follows: an ornamental *P. carinatus* (I) was purchased from Chatuchak Market in Bangkok, Thailand in March of 2011. Other batches, *P. carinatus* (II) and (III) were purchased from a farmer in Chanthaburi province, Thailand in January of 2016 and 2017, respectively. Another ornamental, *P. squarrosus* samples (I) and (II) were collected from an author's house in BaanFa-PaFern, Bangkok, and Chatuchak Market in Bangkok, Thailand in March of 2011 and April of 2013, respectively. For the Philippines, *P. squarrosus* (III) was collected from Northern Luzon in June 2013. *P. nummularifolius* (I) and (II) were purchased from Chatuchak Market in Bangkok and a farmer in Chanthaburi province, Thailand, in October of 2012 and June of 2017, respectively. *P. phlegmaria* was purchased from Chatuchak Market in Bangkok in October of 2013. Thai *Lycopodiella cernua* (I) and (II) were collected from Kang Krachan, Petchburi province and Pha Tang, Chiang Rai province, Thailand in February of 2013 and November of 2014, respectively. Philippine *L. cernua* (III) was collected from Quezon, Philippines in May 2015. *Lycopodium clavatum* (I) and (II) and *L. complanatum* were collected from Doi Ang Khang and Doi Pha Hom Pok, Chiang Mai province in December of 2013.

Alkaloid extracts were prepared by soaking the aerial part of the club mosses in MeOH at room temperature. After removal of the solvent, the dry alcohol extract was partitioned between 3-5 % tartaric acid solution and EtOAc or CH_2Cl_2. The aqueous layer was adjusted to pH 10 with Na_2CO_3 and exhaustively extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to yield the alkaloid-rich extract.

3. Structural classification of *Lycopodium* alkaloids

Ayer and Trifonov divided the *Lycopodium* alkaloids into four major classes; phlegmarine, lycodine, lycopodine and fawcettimine.[23] Skeletons of *Lycopodium* alkaloids (LAs) are shown in Figure 2.[7] The LAs are made up from two units of 2-propylpiperidine (**I**). First, construction of the D ring by C7-C12 and C8-C15 bond formation gives phlegmarine (**II**). The bond formation between C-4 and C-13 forms the B ring and gives a lycodane skeleton followed by oxidation of the A ring to produce lycodine (**III**). Detachment of C-1 from N_α and reattachment to N_β formed the lycopodine (**IV**). The A ring rearrangement of C-4 moved from C-13 to C-12 yields fawcettimine (**V**) (Figure 2).

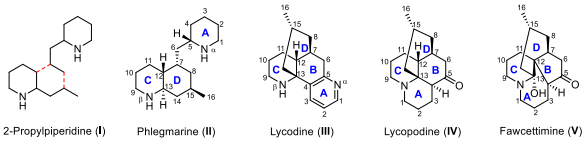


Figure 2. Representative examples of *Lycopodium* alkaloid classes.

Prayogik Rasayan. 2021, *05(1)*, 7 –22 (not final page nos.)
DOI: 10.53023/p.rasayan-20210526

This review describes the phytochemical constituents of *Lycopodium* alkaloid extracts from seven species of club mosses (*P. carinatus*, *P. squarrosus*, *P. nummulariforlius*, *P. phlegmaria*, *L. cernua*, *L. clavatum* and *L. complanatum*) comprising fifteen samples. Ten new Lycopodium alkaloids were isolated from these club mosses together with 28 known LAs as reported in our previous works.[25-28] Thirty-eight Isolated LAs were grouped into four main classes as mentioned above and a novel compound was classified as a new group.

The lycodine-type alkaloids were the most common LAs isolated from genus *Phlegmariurus*. Huperzine A was isolated as a major component from the alkaloid extract of *P. nummularifolius* (II), *P. carinatus* (I) and *P. squarrosus* (III), data not shown. The content of the isolated lycodine-type alkaloid was found to be highest in *P. squarrosus* (III) (up to 92%) yield followed by *L. clavatum* (II) (75.9% yield), *P. nummularifolius* (II) (68.3% yield), *P. carinatus* (II) (44.5% yield), and *P. phlegmaria* (36% yield) as shown in Figure 3. The lycopodine-type alkaloids were the most common LAs found in the genus, *Lycopodium*. A set of lycopodine-type LAs was isolated at high content in *L. complanatum* (82% yield), and *L. clavatum* (I) (77.3%) yield. Two different sources of *L. clavatum*, (I) and (II), gave dissimilar results likely due to the environment of the plants that may have affected the production of the metabolites. Lycopodine-type alkaloids were also found in *P. nummularifolius*, *P. phlegmaria*, *P. carinatus* with an average yield of 38-58%.

In addition, the fawcettimine-type alkaloid was found in both *Phlegmariurus* and *Lycopodium* genera albeit with varying yields (2.5-68.0% yield). *P. squarrosus* (I) alkaloid extract gave the highest yield of the fawcettimine-type alkaloid at 68% yield. The fawcettimine-type alkaloid was also isolated from *P. carinatus* in 18-36% yield. Interestingly. the phlegmarine-type alkaloid was the major LA isolated from the genus *Lycopodiella*. Cernuine and lycocernuine were isolated from the alkaloid extract of all *L. cernua* (I-III) collected from different locations and time periods. This could be an important marker to classify the genus *Lycopodiella* in terms of chemotaxonomy. Surprisingly, squarrosinoxide was isolated from *P. squarrosus* and identified as belonging to another class of LAs, which was found in low content in club moss genus *Phlegmariurus* (Figure 3).

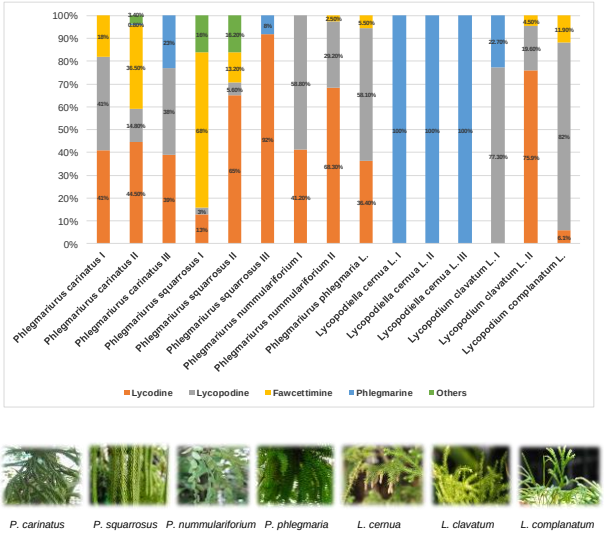


Figure 3. Percent content of LAs in 15 samples from members of the Lycopodiaceae

3.1. Lycopodine-type alkaloids

Four new lycopodine-type alkaloids were isolated from four different species of club mosses including 4-epilycopodine (**25**), 8β,11β-dihydroxylycopodine (**29**), acetyllycophlegmarianol (**31**) and lycocarinatine A (**32**) (Figure 4). 4-Epilycopodine (**25**)[29] was a previously undescribed lycopodine-related alkaloid, isolated from *P. nummulariforium* I. The ^{1}H and ^{13}C NMR data showed similarities with the data of lycopodine (**20**)[30], a known alkaloid, with the exception of the ^{13}C NMR signals of carbonyl (C-5) and stereocenter (C-13). The configuration of C-4 was assigned to be 4*R* that was confirmed by the CD spectrum of **25** showing a contrasting curve ($[\theta]_{232}$ +32, $[\theta]_{292}$–17) to the spectrum of lycopodine (**20**) ($[\theta]_{216}$–27, $[\theta]_{300}$ +20). These data revealed that **25** was a C-4 diastereomer of **20,** which was concluded to be a 4β-epimer of lycopodine (**20**) and named 4-epilycopodine (**25**). 8β,11α-Dihydroxylycopodine (**29**)[25] was isolated from *L. clavatum* II. The structure of **29** is also very similar to 8β-acetoxy-11α-hydroxylycopodine[31] in which position C-8 was replaced by a hydroxy group. The relative configuration was proposed from the NOESY spectrum. A cross-peak observed between H-8/H-12 revealed that the hydroxyl group at C-8 was oriented in the β-configuration. A cross-peak spotted between H-11/H-7 implied that the hydroxyl group at C-11 was oriented in the α-configuration.

Acetyllycophlegmarianol (**31**)[26], isolated from *P. phlegmaria*, was an undescribed *N*-oxide lycopodine-type alkaloid. The low field of quaternary carbon (C-13) and the two methylene carbons (C-1 and C-9) were all ascribed to be connected to a nitrogen atom of *N*-oxide. The HMBC correlations of an oxymethine proton (H-5) and a singlet methyl proton (H_3-18) correlated to an ester carbon indicating that an acetoxy group was attached at C-5. The relative stereochemistry of **31** revealed the relative configurations as 4*S*, 5*R*, 15*R*. Lycocarinatine A (**32**),[27,32-34] isolated from *P. carinatus* I, was the first example of an *N*-oxide of lycopodine-type decorated with the 3-methoxy-4-hydroxy dihydrocinnamoyl unit at position C-5. The relative configuration was elucidated by using the HMBC correlation.

Along with four new compounds, twelve known lycopodine-type alkaloids including: lycopodine (**20**)[28], lycodoline (**21**)[35-37], lycopodatine C (**22**)[38], lycoposerramine K (**23**)[39], 6α-hydroxylycopodine (**24**)[40], lycopodine N-oxide (**26**)[41], 12-epilycodine *N*-oxide (**27**)[42], clavolonine (**28**)[43], gnidioidine (**30**)[27,37], anhydrolycodoline (**33**)[38], lycofoline (**34**)[44,45], and lycopocarinamine F (**35**)[46] were isolated from the genera *Phlegmariurus* and *Lycopodium* (Figure 4).

Acetylcholinesterase inhibitory activity assays of the isolated lycopodine-type alkaloids were negative for 4-epilycopodine (**25**), acetyllycophlegmarianol (**31**) and lycocarinatine A (**32**) (% inhibition < 50%). Lycodoline (**21**), lycoposerramine K (**23**) and gnidioidine (**30**) possessed a moderate inhibitory activity with IC_{50s} of 13.6±0.78, 11.6±0.60 and 3.44±0.20 μM, respectively. Among this group, 12-epilycodine *N*-oxide (**27**) showed the most potent inhibitory activity against AChE with an IC_{50} of 0.59±0.06 μM (see Table 1).[26,27]

3.2. Lycodine-type alkaloids

Two new lycodine-type alkaloids, 8,15-dihydrohuperzine A (**37**)[27] and pyrrolhuperzine A (**39**),[28] were isolated from *P. carinatus* I and *P. squarrosus* III, respectively, together with five known compounds: huperzine A (**36**),[11] huperzine B (**38**),[11] α-obscurine (**40**),[43] des-*N*-methyl α-obscurine (**41**),[43] and des-*N*-methyl β-obscurine (**42**) (Figure 5).[43] All known compounds were identified by comparing their spectroscopic data with those reported in the

literature. Huperzine A (**36**),[11] a lycodine-type alkaloid, was isolated in high yield from several species of the genus, *Phlegmariurus*, such as, *P. nummulariforium* (up to 0.19% isolated yield), *P. carinatus* (up to 0.14% isolated yield), and *P. squarrosus* (up to 0.13% isolated yield).

8,15-Dihydrohuperzine A (**37**)[27] is related to huperzine A (**36**),[11] except for the absence of a double bond at position C8-C15. The ^{13}C NMR and DEPT spectra of positions C-8 and C-15 in the high field region indicated that the C8-C15 double bond of **36** was reduced to a saturated single bond. This corresponded with the absence of doublet H-8 in the ^{1}H NMR spectra and appeared as a multiplet of methylene protons in the high field region. Interestingly, 8,15-dihydrohuperzine A (**37**) was previously synthesized by Kosikowski[47,48] and was recently synthesized by Sarpong in 2021.[49]

The structure of pyrrolhuperzine A (**39**)[28] was similar to the gross structure of huperzine A, but there is a difference in the heterocyclic connection at C-13. The ^{1}H NMR signals of H-3, H-11 and H-14 together with the ^{13}C NMR of C-13 were also remarkably shifted, indicating that the C-13 amino group was incorporated into the pyrrole ring. To test this assumption, huperzine A (**36**) was reacted with 2,5-dimethoxytetrahydrofuran via the Clauson-Kaas reaction to generate a pyrrolyl derivative.[50] All of the spectroscopic data were in full agreement with those of the natural product.[28] Therefore, it was confirmed as the structure of pyrrolhuperzine A (**39**). The AChE inhibitory activity of the new compounds, **37** and **39,** were determined to be moderate and showed IC_{50}s of 2.63±0.37 and 8.91±0.83 µM, respectively. However, they still showed a lower inhibitory activity than huperzine A.

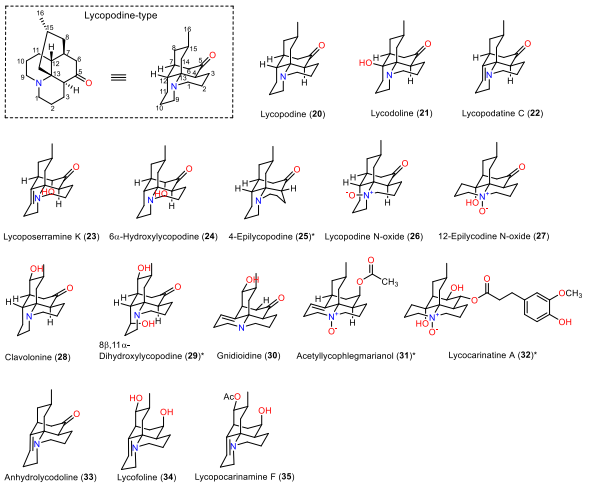


Figure 4. Sixteen isolated lycopodine-type alkaloids. An asterisk (*) indicates a new compound.

3.3. Fawcettimine-type alkaloids

Eight fawcettimine-type alkaloids were isolated and found to be distributed in both genera *Phlegmariurus* and *Lycopodium*. Three new LAs: lycoposerramine U *N*-oxide (**47**),[27] lycoclavatumide (**48**),[25] and squarrosine A (**50**),[28] were isolated from *P. squarrosus* I, *L. clavatum* I, and *P. squarrosus* II, respectively. Whereas five known compounds including fawcettimine (**43**),[51-54] fawcettidine (**44**),[52,55] phlegmariurine B (**45**),[56] lycoflexine *N*-oxide (**46**),[51] and lycophlegmarine (**49**)[52] were also isolated from the genera, *Phlegmariurus* and *Lycopodium* (Figure 6). All known

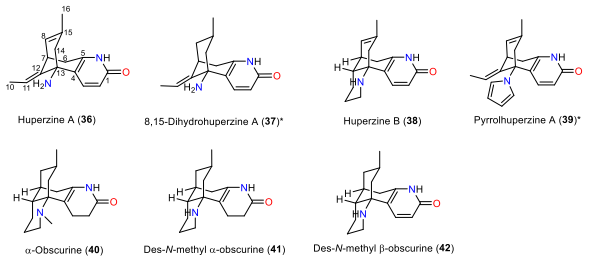


Figure 5. Seven isolated lycodine-type alkaloids. An asterisk (*) indicates a new compound.

structures of the LAs were identified by comparing their spectroscopic data with those reported in the literature.

Lycoposerramine U *N*-oxide (**47**),[27] isolated from *P. squarrosus* I (*H. squarrosa* I), was similar to lycoflexine *N*-oxide (**46**)[51] except for the low field shifted methine at C-8 that was hydroxylated. The ^{13}C NMR and DEPT spectra revealed 17 carbon signals together with HMBC correlation indicating the presence of a hydroxyl group at position C-8. The selected NOESY correlation of H-8/H-6b indicated that the hydroxyl group could be assigned to possess an α-orientation.

Lycoclavatumide (**48**),[25] isolated from *L. clavatum* I, is an unprecedented C-4/C-7 linkage fawcettimine-type LA. The unusual cleavage of the C-7 and C-12 bond might allow the C-4 of lycoclavatumide to form a unique bond with C-7. Cleavage of C-4 and C-5, may be another possibility that could permit this unprecedented C-4 and C-7 linkage. Hydroxyl groups were assigned on C-4 and C-12 and the NOESY cross peak revealed that the two hydroxyl groups at C-4 and C-12 pointed in the same configuration. The biogenetic pathway of **48** may begin from a hydroxylated fawcettimine such as alopecuridine (Ayer *et al*., 1968)[57] after retro-Michael reaction and cyclization followed by rearrangement could generate lycoclavatumide (**48**).

Squarrosine A (**50**),[28] isolated from *P. squarrosus* II (*H. squsrrosa* II), has the unprecedented C-4/N linkage 5/5/6/6 tetracyclic structure of a fawcettimine-type LA. Its structure was constructed and assigned by the HMBC correlations from H-3, H-6, H-8, H-11 and H-14 to C-12, which suggested the connectivity of all substructures to quaternary carbon C-12. The hydroxyl group at C-5 was confirmed by HMBC correlation between H-3 and C-5. The NMR data of the 5-hydroxyl proton showed a broad singlet peak that was assigned to the 5-hydroxyl proton hydrogen bonded with the nitrogen atom and the relative configuration of 5-OH was deduced to be β-oriented by NOESY correlations.

The AChE inhibitory activity of some fawcettimine-type alkaloids was evaluated. The AChE inhibitory activity of compounds **45** and **50** were determined to be moderate and showed IC_{50}s of 26.4±6.48 and 7.30±0.12 *µ*M, respectively.

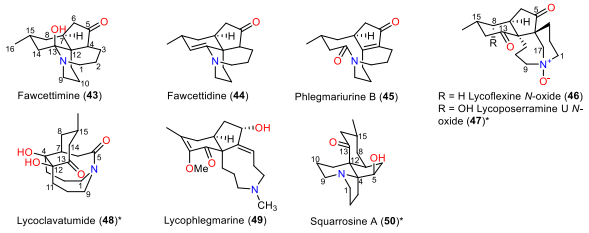


Figure 6. Eight isolated fawcettimine-type alkaloids. An asterisk (*) indicates as a new compound.

3.4. Phlegmarine-type (Miscellaneous) alkaloids

Cernuine (**51**) and lycocernuine (**52**),[58] isolated from both species *Lycopodiella cernua* and *Lycopodium clavatum*, are members of the quinolizidines, which were derived from phlegmarine-type alkaloids by C-9/Nα bond formation (Figure 7). Serratezomine E (**53**),[59] a known *N*-acdetylphlemarin-type alkaloid was isolated from *Phlegmariurus squarrosus.* 2-Piperidineacetic acid (**54**),[7,60] a known C_7N_1 alkaloid precursor, was first isolated from *P. squarrosus*, which was the first reported isolation of this compound from a natural source. From a biosynthetic point of view, **54** could be derived from either pelletierine or 4-(2-piperidyl) acetoacetate (4PAA/4PAACoA), both plausible precursors of *Lycopodium* alkaloids. Lycofargesiine F (**55**)[61] and huperzine J (**56**),[59] recently obtained from the club moss, *P. carinatus*, are phlegmarine-type alkaloids bearing two nitrone moieties.

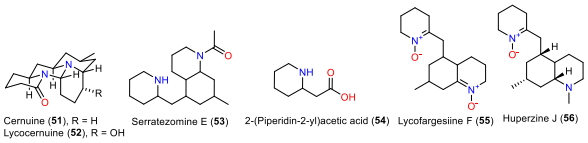


Figure 7. Six isolated phlegmarine-type (miscellaneous) alkaloids.

3.5. A new type of *Lycopodium* alkaloid

Squarrosinoxide (**57**), isolated from *P. squarrosus*, was an undescribed *Lycopodium* alkaloid possessing an unprecedented 6/5/7 tricyclic-spiro system.[26] The structure of **57** was confirmed by spectroscopic techniques and chemical correlations. The HMBC correlations indicated the connectivities of fragments A and B through quaternary carbons C-4 and C-5 followed by fragments B and C through quaternary carbons C-12 and C-13 that form a hexahydro-*2H*-cyclopenta[*b*]pyridine oxide (ring A/B) and cycloheptanone (ring C) linked through a single carbon (C-12).

The most plausible biosynthetic pathway for **57** suggests it could be derived from L-lysine to pelletierine, a common precursor of *Lycopodium* alkaloids, and then a phlegmarine skeleton (C-7/C-12 and C-8/C-15 bond formations) (Ma and Gang, 2004).[7] Bond formation at C-4/C-12 would give ring B (Liu *et al*., 2014).[62] C-8 hydroxylation and C-9/C-10 imine/enamine isomerization would give a key intermediate of the C-8/C-10 bond formation. Bond cleavage at C-7/C-8 would give azaspirobicyclo [3.2.2] nonadiene. The C-13/N bond could be hydrolyzed as proposed in the biosynthesis of lycopladine A.[63] Further transamination, oxidation and esterification reactions would give squarrosinoxide (**57**) as shown in Figure 8.

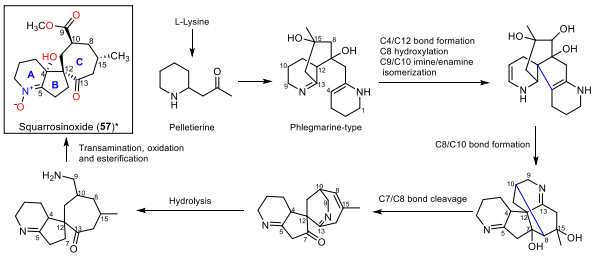


Figure 8. A plausible biosynthetic pathway of squarrosinoxide (**57**). The asterisk (*) indicates a new compound.

Investigation of the acetylcholinesterase inhibitory activities of **57** indicated a moderate inhibitory activity (IC_{50} = 3.12 ± 0.028 μM).

4. Biological activities of *Lycopodium* alkaloids

Alzheimer's disease (AD) is a chronic neurodegenerative disease which leads to the gradual loss of neuronal cells.[64] The pathogenesis of Alzheimer's disease is still unclear due to the complex and multifactorial nature of the disease. The anti-AD drugs currently on the market include: donepezil, rivastigmine, galantamine, and tacrine, which are acetylcholinesterase (AChE inhibitors), and memantine, a *N*-methyl-D-aspartate receptor (NMDA) inhibitor.[65] As a natural product, huperzine A (Hup A) is a licensed anti-Alzheimer's disease drug in China and is available as a nutraceutical in the US.[66] Hup A acts as a cholinesterase inhibitor. Compared to other AChEIs, Hup A shows higher AChE inhibitory activity (IC_{50}) than tacrine, physostigmine and galantamine, while doneperzil exhibits higher than Hup A.[67]

The AChE inhibitory activities of isolated *lycopodium* alkaloids were evaluated by a colorimetric Ellman's assay[68] with donepezil as the positive control. The assay solution was added to a 96-well microtiter plate containing 100 μl of phosphate buffer pH 8.0, 20 μL of 5,5′-dithiobis (2-nitrobenzoic acid (DTNB), 40 μL of Electrophorus electricus acetylcholinesterase (EeAChE, 0.75 U/mL in 0.1 M phosphate buffer pH 7) and 20 μL of test compound in methanol, was pre-incubated at 37 °C for 20 min. After that, 20 μL of acetylcholine iodide (ATCI, 5 mM in water) was added into each well. The enzymatic reaction rate (maximal velocity, Vmax) was determined by measuring the change in absorbance at 412 nm. 12-Epilycodine *N*-oxide (**27**), 8,15-dihydrohuperzine (**37**), squarrosinoxide (**57**) and gnidioidine (**30**) showed moderate inhibitory activity against AChE (see Table 1). Huperzine A showed highly potent inhibitory activity against acetylcholinesterase.

Table 1. *Lycopodium* alkaloids corresponding *Lycopodium* plant sources and their acetylcholinesterase inhibitory activity.

Compound	Name	Sources	Acetylcholinesterase inhibitory (IC_{50}) μM	References
Lycopodine-type alkaloids				
20	Lycopodine	*P. nummulariforium* *L. complanatum* L.	N/A[b]	[28]
21	Lycodoline	*P. carinatus* *L. clavatum* L. *L. complanatum* L.	AChE (IC_{50} = 13.6±0.78 *μ*M)	[25]
22	Lycopodatine C	*P. nummulariforium* *P. carinatus* *L. phlegmaria* L.	N/A[b]	[34]
23	Lycoposerramine K	*P. nummulariforium* *P. carinatus* *L. complanatum* L.	AChE (IC_{50} = 11.6±0.60 *μ*M)	[25]
24	6α-Hydroxylycopodine	*P. nummulariforium*	N/A[b]	[35]
25	4-Epilycopodine	*P. nummulariforium*	AChE (Inactive)[a]	[24]
26	Lycopodine *N*-oxide	*P. nummulariforium* *L. complanatum* L.	N/A[b]	[36]
27	12-Epilycodine *N*-oxide	*P. squarrosus* *P. nummulariforium* *P. carinatus* *P. phlegmaria* L. *L. clavatum* L. *L. complanatum* L.	AChE (IC_{50} = 0.59±0.06 *μ*M)	[25]
28	Clavolonine	*L. clavatum* L.	N/A[b]	[38]

Compound	Name	Sources	Acetylcholinesterase Inhibitory (IC_{50}) μM	References
29	8β,11α-Dihydroxylycopodine	*L. clavatum* L.	N/A[b]	[23]
30	Gnidioidine	*P. carinatus*	AChE (IC_{50} = 3.44±0.20 *μ*M)	[25]
31	Acetyllycophlegmarianol	*P. phlegmaria* L.	AChE (Inactive)[a]	[24]
32	Lycocarinatine A	*P. carinatus*	AChE (Inactive)[a]	[25]
33	Anhydrolycodoline	*P. phlegmaria* L.	N/A[b]	[33]
34	Lycofoline	*P. carinatus*	N/A[b]	[39]
35	Lycopocarinamine F	*P. carinatus*	N/A[b]	[40]
Lycodine-type alkaloids				
36	Huperzine A	*P. carinatus* *P. squarrosus* *P. nummulariforium* *P. phlegmaria* L.	AChE (IC_{50} = 0.010±0.0003 *μ*M)	[26]
37	8,15-dihydrohuperzine A	*P. carinatus*	AChE (IC_{50} = 2.63±0.37 *μ*M)	[25]
38	Huperzine B	*P. squarrosus*	N/A[b]	[9]
39	Pyrrolhuperzine A	*P. squarrosus*	AChE (IC_{50} = 8.91±0.83 *μ*M)	[26]
40	α-Obscurine	*L. clavatum* L.	N/A[b]	[38]
41	Des-N-methyl α-obscurine	*L. clavatum* L. *L. complanatum* L.	N/A[b]	[38]
42	Des-N-methyl β-obscurine	*L. complanatum* L.	N/A[b]	[38]
Fawcettimine-type alkaloids				
43	Fawcettimine	*P. carinatus*	AChE (Inactive)[a]	[25]
44	Fawcettidine	*P. carinatus* *P. squarrosus* *P. phlegmaria* L.	N/A[b]	[45, 47]
45	Phlegmariurine B	*P. carinatus* *P. squarrosus*	AChE (IC_{50} = 26.4±6.48 *μ*M)	[25]
46	Lycoflexine *N*-oxide	*P. squarrosus*	N/A[b]	[44]
47	Lycoposerramine U *N*-oxide	*P. squarrosus*	N/A[b]	[25]
48	Lycoclavatumide	*L. clavatum* L.	N/A[b]	[23]
49	Lycophlegmarine	*P. phlegmaria* L.	N/A[b]	[49]
50	Squarrosine A	*P. squarrosus*	AChE (IC_{50} = 7.30±0.12 *μ*M)	[26]
Phlegmarine-type (Miscellaneous) alkaloids				
51	Cernuine	*L. cernua* L.	N/A[b]	[50]
52	Lycocernuine	*L. complanatum* L. *L. cernua* L.	N/A[b]	[50]
53	Serratezomine E	*P. squarrosus*	N/A[b]	[51]
54	2-(Piperidin-2-yl) acetic acid	*P. squarrosus*	N/A[b]	[6, 52]
55	Lycofargesiine F	*P. carinatus*	N/A[b]	[53]
56	Huperzine J	*P. carinatus*	N/A[b]	[51]
A new type of *Lycopodium* alkaloid				
57	Squarrosinoxide	*P. squarrosus*	AChE (IC_{50} = 3.12±0.028 *μ*M)	[24]
Positive control	Donepezil	N/A[b]	0.021±0.0002 *μ*M	[23]

[a] Inactive; %inhibition <50 at assay concentration of 10 mM. [b] N/A; not available

5. Conclusions

In summary, ten new *Lycopodium* alkaloids including 4-epilycopodine (**25**), 8β,11α-dihydroxylycopodine (**29**), acetyllycophlegmarianol (**31**), lycocarinatine A (**32**), 8,15-dihydrohuperzine (**37**), pyrrolhuperzine A (**39**), lycoposerramine U *N*-oxide (**47**), lycoclavatumide (**48**), squarrosine A (**50**) and squarrosinoxide (**57**) were isolated from members of the genera, *Phlegmariurus* and *Lycopodium*. Compounds **25**, **29**, **31** and **32** were grouped as lycopodine-type alkaloids. Compounds **37** and **39** belonged to the lycodine-type alkaloids, whereas compounds **47**, **48** and **50** were classified as fawcettimine-type alkaloids. In addition, squarrosinoxide (**57**) was classified as a novel type of (6/5/7) tricyclic spiro *Lycopodium* alkaloid. Twenty-eight known alkaloids including: lycopodine (**20**), lycodoline (**21**), lycopodatine C (**22**), lycoposerramine K (**23**), 6α-hydroxylycopodine (**24**), lycopodine *N*-oxide (**26**), 12-epilycodine *N*-oxide (**27**), clavolonine (**28**), gnidioidine (**30**), anhydrolycodoline (**33**), lycofoline (**34**), lycopocarinamine F (**35**), huperzine A (**36**), huperzine B (**38**), α-obscurine (**40**), des-*N*-methyl α-obscurine (**41**), des-*N*-methyl β-obscurine (**42**), fawcettimine (**43**), fawcettidine (**44**), phlegmariurine B (**45**), lycoflexine *N*-oxide (**46**), lycophlegmarine (**49**), cernuine (**51**), lycocernuine (**52**), serratozomine E (**53**), 2-(piperidin-2-yl) acetic acid (**54**), lycofargesiine F (**55**), and huperzine J (**56**), were also isolated from seven species of Thai club moss (*Phlegmariurus nummulariforium*, *P. phlegmaria*, *P. carinatus*, *P. squarrosus*. *Lycopodiella cernua, Lycopodium clavatum*, and *L. complanatum*). Some isolated compounds were also evaluated for anti-acetylcholinesterase inhibitory activity. 12-Epilycodine *N*-oxide (**27**), 8,15-dihydrohuperzine (**37**), squarrosinoxide (**57**) and gnidioidine (**30**) showed moderate inhibitory activity against AChE with IC_{50}s of 0.59±0.06, 2.63±0.37, 3.12±0.028 and 3.44±0.20 μM, respectively. For comparison, huperzine A (**36**) (IC_{50} = 0.010±0.0003 μM) and donepezil (IC_{50} = 0.021±0.0002) were used as positive controls.

Due to their biological activity as AChE inhibitors and unique skeleton characteristics as reported in this review, the *Lycopodium* alkaloids have attracted the attention of not only natural product and organic synthesis chemists but also plant taxonomists, biologists, and pharmacologists. Further phytochemical investigations of club mosses will shed increasing light on the structural variation of the *Lycopodium* alkaloids and their other biological activities. This review could be beneficial to the further study on *Lycopodium* alkaloids in many other disciplines.

6. Acknowledgements

This work was partially supported by the Thailand Research Fund (BRG6080013), the Thailand Science Research and Innovation, Grant number FEB640035 Project code 50184 for Chulabhorn Royal Academy and Grant number 313 Project code 2220 for Chulabhorn Research Institute, and the Royal Project Foundation (3040-A013). Support from the Center of Excellence on Environmental Health and Toxicology, Science & Technology Postgraduate Education and Research Development Office (PERDO), Ministry of Education is also gratefully acknowledged.

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DOI: 10.53023/p.rasayan-20210526

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Article Note: A collection of invited papers based on Award Lecture presentations in the CRS® International Symposium on Advanced Renewable and Functional Materials 'Rasayan4' held at Raja Narendra Lal Khan Womens College, Midnapore, West Bengal, India held during December 20-21, 2019.