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3B QUANG TRUNG - HOAN KIEM - HANOI - VIETNAM
TEL: (+84-24) 38247058 FAX: (+84-24) 39348740
Email: tapchiduoclieu@nimm.org.vn; tapchiduoclieu@gmail.com

CONTENTS VOL. 30(2), 2025
(Pages 65 - 144)

Page	SCIENTIFIC RESEARCH

Journal of Medicinal Materials, 2025, Vol. 30, No. 2 (pp. 107 - 112)

SERRATANE TRITERPENOIDS AND CAFFEIC ACID DERIVATIVES FROM WHOLE PLANTS OF *LYCOPODIUM CLAVATUM* AND THEIR POTENTIAL DPPH AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES

Nguyen Ngoc Linh[1], Vu Thi Thu Thuy[1], Nguyen Cao Cuong[2], Le Ba Vinh[2,3,*]

[1]*Faculty of Pharmacy, Thanh Do University, Hanoi, Vietnam;*

[2]*Faculty of Medicine and Pharmacy, Yersin University of Da Lat, Lam Dong, Vietnam;*

[3]*Department of Chemistry, University of Bergen, Bergen, Norway*

*Corresponding author: vinh.ba@uib.no

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Summary

Serratane Triterpenoids and Caffeic Acid Derivatives from Whole Plants of *Lycopodium clavatum* and their Potential DPPH and Acetylcholinesterase Inhibitory Activities

Lycopodium clavatum is used in traditional medicine to treat conditions such as rheumatism, digestive disorders, and skin diseases. As part of our ongoing research on bioactive compounds from Vietnamese medicinal plants, phytochemical investigations of whole plants led to the isolation of five compounds (**1–5**). Using advanced analytical techniques, including electrospray ionization mass spectrometry (ESI-MS) and one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy, the structures of these compounds were successfully elucidated as 21-*epi*-serratenediol (**1**), serratenediol (**2**), tohogenol (**3**), methyl ferulate (**4**), and methyl *p*-coumarate (**5**). The *in vitro* acetylcholinesterase (AChE) inhibitory activities of these compounds were evaluated, revealing that compound **3** exhibited an IC_{50} value of 8.3 ± 0.6 μM, which is comparable to that of the positive control, tacrine (IC_{50} = 2.5 ± 0.2 μM). Additionally, compounds **4** and **5** exhibited potent antioxidant activity in the DPPH assay, comparable to those of L-ascorbic acid (vitamin C). The results suggest that triterpenoids from *L. clavatum* are responsible for the AChE inhibitory activities, while caffeic acid derivatives contribute to the antioxidant activity. This study represents the first investigation into the chemical constituents, antioxidant properties, and AChE inhibitory effects of *L. clavatum* collected in Vietnam.

Keywords: *Lycopodium clavatum*; *Serratane triterpenoid; Caffeic acid derivative; Acetylcholinesterase inhibition.*

1. Introduction

Alzheimer's disease (AD) is the leading cause of dementia among the elderly, representing a significant neurodegenerative disorder characterized by progressive cognitive decline, memory loss, and behavioral alterations [1]. This condition places a considerable strain on both individuals and healthcare systems, especially as the population ages. A central factor in the pathophysiology of AD is a deficiency of acetylcholine, a vital neurotransmitter essential for memory and learning [2]. This cholinergic deficit has prompted the investigation of various therapeutic approaches aimed at increasing acetylcholine levels, particularly through the inhibition of acetylcholinesterase (AChE), the enzyme responsible for breaking down acetylcholine in the synaptic cleft. According to the World Health Organization (WHO), a new diagnosis of Alzheimer's disease occurs every three seconds, and projections indicate that the number of cases could triple over the next 50 years [3]. In Vietnam, the prevalence of Alzheimer's disease is rising, particularly as the population continues to age. In recent years, there has been a growing interest in natural products and herbal medicines as promising sources of therapeutic agents for the management of AD [4]. As researchers seek alternative treatments beyond conventional pharmaceuticals, the exploration of bioactive compounds derived from plants has gained significant attention. These natural substances are being investigated for their potential neuroprotective, anti-inflammatory, and cognitive-enhancing properties, which could provide novel approaches to alleviate the symptoms and progression of AD [5]. This renewed focus on herbal remedies not only highlights the importance of traditional medicine but also opens avenues for innovative drug development and improved patient care in the context of Alzheimer's disease.

Lycopodium clavatum L., commonly known as Thông đá, is a perennial herbaceous plant belonging to the family Lycopodiaceae [6]. This species is widely distributed across various regions, often found in forests and mountainous areas. Traditionally, *L. clavatum* has been utilized in Vietnamese and Chinese folk medicine for its reputed healing properties [7]. In particular, the

plant has been employed to treat ailments such as respiratory disorders, wounds, and inflammation [8]. Previous research has shown that this plant contains a rich array of chemical constituents, notably alkaloids, flavonoids, and terpenoids, which contribute to its pharmacological activity [6]. Among the significant alkaloids identified in *L. clavatum* is lycopodine, which has demonstrated potential anti-inflammatory and analgesic effects [9]. Pharmacological studies have highlighted the diverse therapeutic effects of *L. clavatum*, including anti-inflammatory, antimicrobial, and cytotoxic activities [6]. Research indicates that extracts from the plant can modulate various biological pathways, making it a candidate for further investigation in drug development [9]. Furthermore, the use of *L. clavatum* in traditional medicine underscores its potential as a natural remedy, warranting systematic studies to explore and validate its medicinal properties. However, there have been limited studies on secondary metabolites and their acetylcholinesterase inhibitory activities of *L. clavatum*. Herein we reported the first-time chemical constituents and their acetylcholinesterase and DPPH inhibitory activities of *L. clavatum*,

2. Materials and methods

2.1. General experimental procedures

NMR data were measured using a Bruker 600 MHz NMR spectrometer. TMS was employed as an internal standard, with coupling constants (*J* in Hz) and chemical shift values (δ) expressed in ppm. Structural assignments were supported by additional information from HSQC and HMBC experiments. High-resolution electrospray ionization mass spectrometry (HRESIMS) data were acquired using a Thermo Fisher LC-LTQ-Orbitrap XL spectrometer (Thermo Fisher, Palo Alto, CA, USA) in positive ion mode. For open column chromatography (CC), YMC*GEL (ODS-A, 12 nm S-150 mm, YMC) and *silica gel* (Kieselgel 60, 70-230 mesh, and 230-400 mesh, Merck, Darmstadt, Germany) were used. Thin-layer chromatography (TLC) was performed using Merck precoated *silica gel* 60F_{254} (1.05554.0001), Sephadex LH-20 (GE Healthcare Bio-Science AB), and RP-C_{18} F_{254S} plates (1.15685.0001). Compounds on the TLC plates were identified under UV light (254 and 365 nm), and 10% (v/v) aqueous H_2SO_4 solution was sprayed to visualize them.

2.2. Plant Material

The entire plant sample of *L. clavatum* was collected from Lam Dong in August 2023. The plant was authenticated by Prof. Ki Young Lee at the College of Pharmacy, Korea University, and a voucher specimen (TD 003) has been deposited in the herbarium of the College of Pharmacy, Korea University, Sejong, Korea.

2.3. Extraction and isolation

The extraction and isolation process in this study was conducted following our previously reported method, with slight modifications. The entire dried plant material of *L. clavatum* (3.8 kg) underwent extraction with methanol (MeOH) at room temperature, using three consecutive rounds of maceration (5 L each). The collected MeOH extracts were concentrated under reduced pressure, resulting in a crude MeOH residue (680 g). This residue was subsequently suspended in water and partitioned sequentially with *n*-hexane, and ethyl acetate (EtOAc), yielding fractions of *n*-hexane (85 g), and EtOAc (150 g), alongside an aqueous layer that was set aside for further analysis.

The EtOAc fractions were pooled and subjected to *silica gel* column chromatography (CC) using a stepwise gradient of *n*-hexane–acetone (100:1 to 0:1, v/v), followed by $CHCl_3$–MeOH (8:1 to 1:1, v/v), yielding ten subfractions (E1–E10). Among these, fraction E3 (15 g) was further fractionated by reversed-phase C_{18} column chromatography (RP-C_{18} CC) with a gradient elution of MeOH–$H_{22}O$ (1:1 to 8:1, v/v), resulting in nine subfractions labeled E3A–E3I.

Subfraction E3B (400 mg) was subjected to *silica gel* CC using *n*-hexane–EtOAc (5:1, v/v) as the mobile phase, followed by purification on Sephadex LH-20, which led to the isolation of compounds **4** (9.5 mg) and **5** (1.8 mg). Meanwhile, E3D (4.6 g) was further purified on a *silica gel* column with *n*-hexane–$CHCl_3$–EtOAc (3:2:0.5, v/v/v) and processed through RP-C_{18} CC using MeOH–$H_{22}O$ (10:1, v/v), affording compounds **1** (3.2 mg) and **2** (5.7 mg). Finally, fraction E3E (900 mg) was subjected to *silica gel* CC, eluted with *n*-hexane–acetone (3:1, v/v), yielding compound **3** (3.3 mg).

Compound **1**. White amorphous powder, ^{1}H (600 MHz in CD_3OD + DMSO) and ^{13}C-NMR (150 MHz in CD_3OD + DMSO): see Table 1.

Compound **2**. White amorphous powder, ^{1}H (600 MHz in CD_3OD + DMSO) and ^{13}C-NMR (150 MHz in CD_3OD + DMSO): see Table 1.

Compound **3**. White amorphous powder, ^{1}H (600 MHz in CD_3OD + $CDCl_3$) and ^{13}C-NMR (150 MHz in CD_3OD + $CDCl_3$): see Table 1.

Compound **4**. Yellow solid, ^{1}H NMR (600 MHz in CD_3OD) δ_H 7.18 (1H, d, J = 2.0 Hz, H-2′), 6.82 (1H, d, J = 8.0 Hz, H-5′), 7.08 (1H, dd, J = 8.0, 2.0, Hz, H-6′), 3.78 (3H, s, OCH_3), 3.80 (3H, s, OCH_3). ^{13}C-NMR (150 MHz in CD_3OD): δ_C 51.9 (C-1), 169.7 (C-2), 115.2 (C-3), 146.8 (C-4), 127.7 (C-1′), 111.7 (C-2′), 149.4 (C-3′), 150.6 (C-4′), 116.5 (C-5′), 124.1 (C-6′).

Compound **5**. Yellow solid, ^{1}H NMR (600 MHz in CD_3OD) δ_H 7.46 (2H, dd, J = 7.0, 2.0 Hz, H-3′, H-5′), 6.83 (2H, dd, J = 7.0, 1.5 Hz, H-2′, H-6'), 7.62 (1H, d, J = 16.0 Hz, H-4), 6.33 (1H, d, J = 16.0 Hz, H-3), 3.78 (3H, s, OCH_3); ^{13}C-NMR (150 MHz in CD_3OD): δ_C 51.9 (C-1), 169.8 (C-2), 114.9 (C-3), 146.5 (C-4), 127.1 (C-1′), 116.8 (C-2′ and C-6′), 131.1 (C-3′ and C-5′), 161.3 (C-4′).

2.5. In vitro acetylcholinesterase enzyme assay

AChE inhibition experiments were performed as previously described with a few minor modifications [10]. Briefly, a 96-well plate was prepared by adding 20 µL of either methanol (MeOH) or the test material dissolved in MeOH, followed by the addition of 130 µL of AChE (approximately 0.05 U/mL) in 50 mM phosphate buffer (pH 7.4). The mixture was then supplemented with 25 µL of acetylthiocholine chloride (5 mM), and 25 µL of DTNB (1 mM) was added. The generation of the product was monitored at 475 nm for 20 minutes using a UV-Vis spectrophotometer, starting with the initiation of the AChE reaction at 37°C. Percentage inhibition was calculated using the following formula:

$$\text{Inhibitory activity \%} = [(\Delta\text{control} - \Delta\text{sample}) / \Delta\text{control}] \times 100,$$

where Δcontrol and Δsample represent the absorbance intensities of the control and sample wells, respectively, after 20 minutes.

2.6. DPPH assay

Measurements of DPPH radical-scavenging activity were conducted in accordance with our previous publication, with slight modifications [11]. In brief, each well contained 100 µL of the sample, diluted in distilled water to final concentrations of 100, 50, 25, 12.5, and 6.25 µM. A 100 µL solution of DPPH, prepared at a concentration of 100 µM in ethanol, was then mixed with the sample. Control wells included the same components as the experimental wells, but water was added instead of the samples. The contents of the wells were mixed thoroughly and incubated in the dark at room temperature for 30 minutes. DPPH reacts with antioxidant compounds, resulting in a color change from deep violet to light yellow, which was measured at 517 nm using a microplate reader.

The radical-scavenging capacity was expressed as a percentage effect (EC%) and calculated using the following equation:

Percentage effect (EC%) = (A control - A sample) / (A control) × 100

where A sample is the absorbance of the sample and A control is the absorbance of the positive control. DPPH radical scavenging curves were generated using various sample concentrations to determine EC_{50} values. L-ascorbic acid (vitamin C) was chosen as the positive control.

3. Results and discussion

The MeOH extract of *L. clavatum* was partitioned based on increasing polarity using *n*-hexane, and ethyl acetate (EtOAc). A combination of chromatographic separation techniques was employed to obtain five compounds (**1–5**) through repeated CC on *silica gel*, RP-C_{18}, and Sephadex LH-20.

Compound **1** was isolated as a white amorphous powder. The HR-ESI-MS spectrum of **1** revealed a sodium adduct-ion peak at *m/z* 465.3698 $[M+Na]^+$ (calcd. for $C_{30}H_{50}NaO_2^+$, 465.3703), corresponding to the molecular formula $C_{30}H_{50}O_2$. The ^{1}H-NMR spectrum of **1** showed signals of seven methyl groups (each 3H, s) at δ_H 0.91 (CH_3-23), 0.70 (CH_3-24), 0.78 (CH_3-25), 0.83 (CH_3-26), 0.65 (CH_3-28), 0.82 (CH_3-29) and 0.84 (CH_3-30), an olefinic proton at δ_H 5.31 (1H, d, J = 2.0 Hz, H-15), and two oxygenated methines at 3.25 (1H, d, J = 3.0 Hz, H-21), and 3.00 (1H, m, H-3). The ^{13}C-NMR and HSQC spectra exhibited clearly 30 carbon signals, comprising two olefinic carbons at δ_C 137.9 (C-14) and 121.6 (C-15), two oxygenated methines at δ_C 76.5 (C-3) and 73.8 (C-21), and a characteristic serratane methylene evidence at δ_C 55.6 (C-27) (**Table 1**). The evidence from the ^{1}H and ^{13}C NMR signals indicates that **1** is a serratane triterpenoid, one of the major components already reported in *L. clavatum*. A thorough analysis of the NMR data for **1** demonstrated a high degree of similarity to the NMR profile of serratenediol [12]. The assignment of the C-3 position was supported by the HMBC cross-peak of H-23 (δ_H 0.91) with C-3 (δ_C 76.5), and H-3 (δ_H 3.00) with C-24 (δ_C 15.3) (**Fig. 2**). Additionally, HMBC correlations from both H-29 (δ_H 0.82) and H-30 (δ_H 0.84) to C-

21 (δ_C 73.8) confirmed the presence of a hydroxy group at the C-21 position. Correlations from H-25 (δ_H 0.78) to C-1 (δ_C 38.1), C-9 (δ_C 62.2), and C-10 (δ_C 37.5) were also observed, as well as from H-26 (δ_H 0.83) to C-8 (δ_C 36.8), C-27 (δ_C 55.6), and C-9 (δ_C 62.2). Thus, the complete planar structure of compound **1** was fully established through the analysis of cross-peaks in the HSQC, and HMBC spectra (**Fig. 2**). The β-orientation of H-3 was verified by comparing the ^{1}H and ^{13}C NMR data of compound **1** with the corresponding values of serratenediol [12]. Specifically, the chemical shift at the C-21 position in the ^{13}C NMR data for serratenediol is δ_C 78.4, which differs significantly from the ^{13}C NMR data of **1** (δ_C 73.8). This evidence suggests that the relative configuration of **1** also has the β-orientation at H-21. Consequently, the structure of compound **1** was identified as 21-*epi*-serratenediol.

Compound **2** was also yielded as a white amorphous powder. The HR-ESI-MS spectrum of **2** revealed a deprotonated ion peak at *m/z* 441.3736 $[M-H]^-$ (calcd. for $C_{30}H_{49}O_2^-$, 441.3738), corresponding to the molecular formula $C_{30}H_{50}O_2$. The NMR data for compound **2** (**Table 1**) were similar to those of compound **1**, except for the difference in the *E*-ring (from C-17 to C-22) as deduced from its characteristic chemical shift values. This suggests that the *E*-rings of compounds **1** and **2** differ in their chair and boat conformations. Additionally, the structure of compound **2** was further analyzed in detail based on 2D NMR (HSQC and HMBC spectra). The HMBC correlations from both H-29 (δ_H 0.75) and H-30 (δ_H 0.89) to C-21 (δ_C 76.8) confirmed the presence of a 21-OH. Correlations from H-25 (δ_H 0.78) to C-1 (δ_C 38.0), C-9 (δ_C 62.0), and C-10 (δ_C 37.5) were also observed, as well as from H-26 (δ_H 0.81) to C-8 (δ_C 35.4), C-27 (δ_C 55.4), and C-9 (δ_C 62.0). Moreover, the NMR data for compound **2** were compared to published spectroscopic data [12]. This evidence suggests that the structure of compound **2** has been identified as serratenediol.

Compound **3** was also obtained as a white amorphous powder. Similarly, a comparison of the NMR data of compound **3** with those of compounds **1** and **2** (**Table 1**) indicated that compound **3** is a serratane triterpenoid. The results showed that the structure of compound **3** lacks double bonds at the C-14 and C-15 positions. This is evidenced by the absence of an olefinic proton signal in the ^{1}H NMR spectrum and the shift of the chemical shifts for C-14 and C-15 from the low-field to the high-field region. Additionally, the structure of compound **3** was confirmed by 2D NMR (HSQC and HMBC spectra) as well as by comparison with NMR data in the literature [13]. All these observations confirmed the structure of compound **3** as tohogenol.

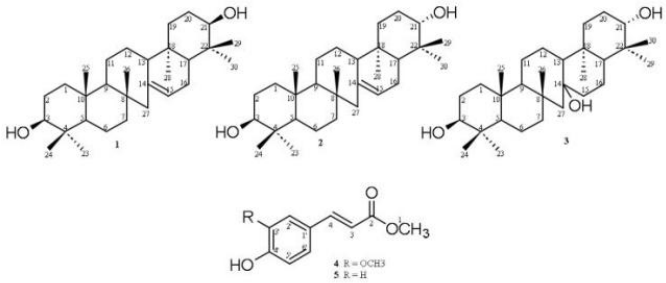

Fig. 1. Structures of isolated compounds (**1–5**) from *L. clavatum*

Compound **4** was obtained as a yellow solid. The ^{1}H-NMR spectrum of compound **4** displayed a set of ABX signals at δ_H 6.82 (1H, d, J = 8.0 Hz, H-5′), 7.18 (1H, d, J = 2.0 Hz, H-2′) and 7.08 (1H, dd, J = 8.0, 2.0 Hz, H-6′) indicating the presence of a trisubstituted benzene ring. The doublet at δ_H 7.61 (1H, d, J = 16.0 Hz, H-4) and δ_H 6.34 (1H, d, J = 16.0 Hz, H-3) correspond to two mutually coupled methine groups, and two methoxy signals at δ_H 3.87 and 3.80 were also observed. A comparison of these data with those reported [14] led to the identification of compound **4** as methyl ferulate.

Compound **5** was obtained as a yellow solid. The ^{1}H-NMR spectrum of compound **5** exhibited similar signals to those of compound **4**, except for the absence of signals for a methoxy group. The presence of aromatic proton resonances as doublets of doublets at δ_H 6.83 (2H, dd, J = 7.0, 1.5 Hz, H-2', H-6') and 7.46 (2H, dd, J = 7.0, 2.0 Hz, H-3′, H-5′) suggests that compound **5** is a derivative of compound **4** formed by the removal of a methoxy group attached at C-3′. These data allowed for the identification of compound **5** as methyl *p*-coumarate [14].

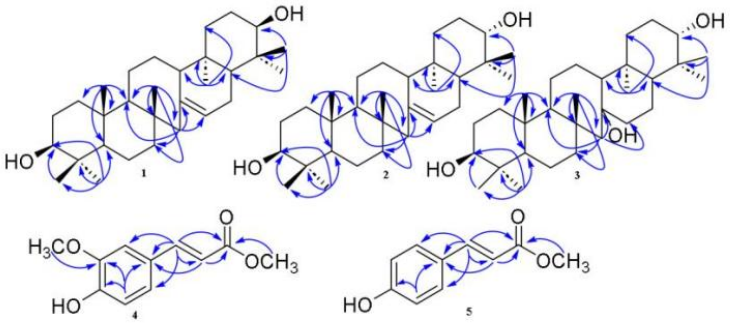

Fig. 2. Key HMBC correlations of **1**-**5**

The *in vitro* AChE inhibitory activity was assessed based on our previous protocol with slight modifications [15]. The AChE conversion rate in the presence of compounds **1–5** was measured to evaluate their AChE inhibitory activity. Each compound was initially screened for anti-AChE activity at a concentration of 100 μM. Among the tested compounds, only compound **3** exhibited significant inhibition, with an IC_{50} value of 8.3 ± 0.6 μM, which is relatively higher compared to the positive control, tacrine (IC_{50} = 2.5 ± 0.2 μM). The other compounds were inactive (IC_{50} > 100 μM). Furthermore, compounds **4** and **5** demonstrated strong DPPH radical-scavenging effects, with ED_{50} values of 15.3 ± 0.4 and 5.6 ± 0.2 μM, respectively, compared to an ED_{50} of 21.9 ± 0.8 for the control. These findings suggest that the triterpenoid component from *L. clavatum* may possess therapeutic potential for managing conditions such as Alzheimer's disease, while phenolic compounds are responsible for the DPPH inhibitory activity. This study also provides two potential antioxidant inhibitors and one compound for Alzheimer's disease from *L. clavatum*. This is also the first study on the chemical constituents of *L. clavatum* and their acetylcholinesterase and DPPH inhibitory activities.

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of **1–3** (^{1}H: 600 MHz; ^{13}C: 150 MHz).

	1		**2**		**3**	
Position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	38.1	0.90 m/1.70 m	38.0	0.90 m/1.70 m	39.1	1.00 m/1.80 m
2	27.0	1.50 m	27.2	1.50 m	27.9	1.60 m/1.65 m
3	76.5	3.00 m	76.5	3.00 m	79.4	3.15 m
4	38.3	-	38.3	-	39.9	-
5	55.1	0.75 dd (2.5, 11.5)	55.1	0.75 dd (2.5, 11.5)	56.7	0.80 m
6	18.3	1.45 m	18.3	1.42 m	19.6	1.50 m/1.68 m
7	44.6	1.19 m /1.28 m	44.6	1.16 m /1.37 m	46.3	1.19 m/1.28 m
8	36.8	-	35.4	-	38.5	-
9	62.2	0.82 m	62.0	0.80 m	62.2	1.18 m
10	37.5	-	37.5	-	39.5	-
11	22.5	1.00 m /1.70 m	24.6	1.03 m /1.70 m	25.5	1.30 m/1.70 m
12	26.6	1.10 m /1.98 m	26.9	1.50 m	27.2	1.14 m/1.84 m
13	56.2	1.80 m	56.4	1.73 m	60.5	1.13 m
14	137.9	-	137.6	-	76.1	-
15	121.6	5.31 d (2.0)	121.5	5.31 br s	45.2	1.55 m/1.65 m
16	23.3	1.80 m /1.90 m	23.4	1.89 m /2.03 m	19.6	1.50 m/1.68 m
17	42.6	1.63 m	48.9	1.18 m	56.5	0.80 m
18	35.2	-	36.4	-	39.6	-
19	30.6	1.42 m /1.50 m	36.5	1.05 m /1.78 m	39.2	1.00 m/1.80 m
20	25.2	1.50 m /1.80 m	26.3	1.10 m /1.87 m	27.7	1.60 m/1.65 m
21	73.8	3.25 d (3.0)	76.8	3.00 m	79.6	3.15 m
22	36.5	-	38.2	-	39.8	-
23	27.6	0.91 s	27.8	0.90 s	28.5	0.95 s

	1		2		3	
24	15.3	0.70 s	15.3	0.70 s	15.8	0.76 s
25	15.2	0.78 s	15.2	0.78 s	16.8	0.83 s
26	19.3	0.83 s	19.3	0.81 s	22.4	0.99 s
27	55.6	1.70 m /2.20 m	55.4	1.73 m /2.21 m	63.3	1.40 m/1.50 m
28	12.9	0.65 s	12.9	0.64 s	16.5	0.96 s
29	21.5	0.82 s	14.2	0.75 s	28.7	0.97 s
30	27.5	0.84 s	27.3	0.89 s	16.2	0.78 s

*Compounds **1** and **2** were analyzed in CD_3OD and DMSO, while compound **3** was measured in CD_3OD and $CDCl_3$. Assignments were identified based on HSQC and HMBC experiments.*

3. Conclusion

In conclusion, our ongoing phytochemical investigation of *L. clavatum* has led to the isolation of five compounds as 21-*epi*-serratenediol (**1**), serratenediol (**2**), tohogenol (**3**), methyl ferulate (**4**), and methyl *p*-coumarate (**5**). The AChE inhibitory activities of the isolated compounds were also evaluated. Additionally, compounds **4** and **5** demonstrated strong activity in the DPPH assay compared to L-ascorbic acid (vitamin C). These results suggest that the bioactive compounds from the whole plant of *L. clavatum* may have significant potential for Alzheimer's disease through AChE inhibition and antioxidant mechanisms.

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