Using NMR, X-ray, and CD analysis in the study on natural products obtained from Vietnamese plant and fungi in terms of pharmaceutical product development

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Abstract:

NMR, X-ray analysis, and CD methods are powerful techniques for the study of absolute configuration of bioactive compounds from natural resources. This study presents the results of a joint-study between Vietnam and Taiwan on the bioactive compounds obtained from Vietnamese plants and fungi. Among the tested compounds, hexatenuin A displayed the most significant inhibition of superoxide anion generation and elastase release. These triterpenoids may be used as potential anti-inflammatory agents.

Keywords: ***absolute configuration, circular dichroism, NMR, X-ray analysis.***

Classification number: ***2.2***

Introduction

Natural products are an important source for drug discovery. The determination of absolute configuration is one of the most challenging tasks in the structure elucidation of chiral natural products, especially those with complex structures. The available methods include NMR spectroscopy/chiral derivatization, analytical chemistry, X-ray crystallography for crystalline compounds, chemical synthesis, and chiroptical approaches [1]. Among these, X-ray crystallography probably remains the most powerful and effective approach. However, the complete structure elucidation of new compound may require considerable effort and involve many different spectroscopic and, sometimes, computational techniques.

The purpose of this review is to use several examples, representing different classes of natural products, to illustrate the applicability of these approaches in determining the absolute configuration of natural products obtained from Vietnamese plants and fungi. Moreover, the purified constituents were examined for their anti-inflammatory activity. Among the tested compounds, hexatenuin A displayed the most significant inhibition of superoxide anion generation and elastase release. These triterpenoids may have potential to be used as anti-inflammatory agents.

Experimental

General experimental procedures

The optical rotations were measured with a JASCO P-2000 digital polarimeter in a 0.5 dm cell. The UV spectra were obtained with a Hitachi UV-3210 spectrophotometer while the IR spectra were measured with a Shimadzu FTIR Prestige-21 spectrometer. The ECD spectra were recorded on a JASCO J-720 spectrometer. The ^{1}H- and ^{13}C-NMR spectra were measured using Bruker AMX-400 and AV500 spectrometers with TMS as the internal reference, while the chemical shifts were expressed in δ (ppm). The ESIMS and HRESIMS were collected on a Bruker Daltonics APEX II 30e spectrometer. HPLC was performed on a Shimadzu LC-10ATVP (Japan) system, equipped with a Shimadzu SPD-M20A diode array detector at 250 nm, a Purospher STAR RP-8e c (5 µm, 250×4.6 mm), Cosmosil 5C18 ARII (250×4.6 mm *i.d.* Nacalai Tesque Inc.), and Astec Cellulose DMP (150×4.6 mm *i.d.* 5 µm) columns. The X-ray diffraction experiments were performed on a Bruker D8 Venture with a Photon 100 CMOS detector system equipped with a Cu Incoatec IµS microfocus source (λ = 1.54178 Å).

Preparation of human neutrophils

Neutrophils were isolated by a standard method of dextran sedimentation, prior to their centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Blood was drawn from healthy human donors (20-30 years old) by venipuncture into heparin-coated Vacutainer tubes, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital [2]. The blood samples were mixed gently with an equal volume of 3% dextran solution. After the sedimentation of the red cells for 30 min at room temperature, the leukocyte-rich plasma was collected,. The leukocyte-

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rich plasma was transferred on top of a 20 ml Ficoll solution (1.077 g/ml) and spun down at 400 g for 40 min at 20°C. The granulocyte/erythrocyte pellets were resuspended in ice-cold 0.2% NaCl to lyse the erythrocytes. After 30 s, the same volume of 1.6% NaCl solution was added to reconstitute the isotonic condition. Purified neutrophils were pelleted and then resuspended in a calcium (Ca^{2+})- free Hank's balanced salt solution (HBSS) buffer at pH 7.4 and maintained at 4°C before use [2].

Measurement of superoxide anion generation

The assay of the superoxide anion generation was based on the SOD-inhibitable reduction of ferricytochrome c [2]. Briefly, after supplementation with 0.5 mg/ml ferricytochrome c and 1 mM Ca^{2+}, the neutrophils (6×105 cells/ml) were equilibrated at 37°C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO, negative control) for 5 min. The cells were activated with 100 nM FMLP during the preincubation of 1 µg/ml cytochalasin B (FMLP/CB) for 3 min. Changes in the absorbance, with a reduction in ferricytochrome c at 550 nm, were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Then calculations were based on the differences in the reactions with and without SOD (100 U/ml), divided by the extinction coefficient for the reduction of ferricytochrome c (ε = 21.1/mM/10 mm) [2].

Measurement of elastase release

The degranulation of azurophilic granules was determined by the elastase release, as described previously [2]. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 µM), the neutrophils (6×105 cells/ml) were equilibrated at 37°C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO, negative control) for 5 min. The cells were activated by 100 nM FMLP and 0.5 µg/ml cytochalasin B while the changes in absorbance at 405 nm were continuously monitored to assay the elastase release. The results were expressed as the percentage of elastase release in the FMLP/CB-activated, drug-free control system [2].

Hexagonin A (**16**): white powder ($CHCl_3$); mp 184-185°C; $[\alpha]^{25}_D$ +57 (c 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 262 (2.65) nm; IR (neat) ν_{max} 2946, 1759, 1693, 1455, 1376, 1256, 1219, 1156 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) (δ ppm): 4.71 (1H, *br s*, H-3), 4.32 (1H, *ddd*, *J* = 11.5, 11.5, 5.0 Hz, H-16), 3.72 (3H, *s*, CH_3-4'), 3.40 (2H, *s*, CH_3-2'), 2.27 (1H, *dd*, *J* = 14.0, 11.5 Hz, H-15), 2.18 (1H, *m*, H-20), 2.05 (2H, *m*, H-6, -11), 1.89 (1H, *m*, H-2), 1.84 (1H, *m*, H-12), 1.71 (1H, *m*, H-2), 1.60 (3H, *m*, H-7, -12, -22), 1.49 (3H, *m*, H-1, -7, -22), 1.41 (3H, *m*, H-1, -5, -17), 1.20 (1H, *dd*, *J* = 14.0, 5.0 Hz, H-15), 1.94 (3H, *d*, *J* = 0.5 Hz, CH_3-31), 1.81 (3H, *d*, *J* = 0.5 Hz, CH_3-27), 1.08 (3H, *s*, CH_3-30), 1.00 (3H, *s*, CH_3-19), 0.93 (3H, *s*, CH_3-29), 0.95 (3H, *d*, *J* = 6.5 Hz, CH_3-21), 0.88 (3H, *s*, CH_3-28), 0.68 (3H, *s*, CH_3-18); ^{13}C-NMR (125 MHz, $CDCl_3$) (δ ppm): 172.2 (C-26), 165.9 (C-1'), 167.2 (C-3'), 157.4 (C-24), 135.1 (C-9), 133.8 (C-8), 125.2 (C-25), 108.2 (C-23), 79.8 (C-16), 79.6 (C-3), 54.6 (C-17), 52.3 (C-4'), 48.6 (C-14), 45.3 (C-5), 43.5 (C-13), 41.8 (C-2'), 41.1 (C-22), 37.1 (C-10), 36.8 (C-4), 35.4 (C-15), 30.7 (C-20), 30.5 (C-1), 30.1 (C-12), 28.0 (C-30), 27.6 (C-28), 26.5 (C-6), 23.1 (C-2), 21.7(C-29), 20.2 (C-11), 19.4 (C-21), 18.8 (C-19), 17.9 (C-7), 16.5 (C-18), 10.8 (C-31), 8.5 (C-27); ESIMS m/z 621 ($[M+K]^+$, 60), 605 ($[M+Na]^+$, 26), 521 (33), 505 (100), 483 (48); HRESIMS m/z 605.3451 ($[M + Na]^+$, calcd for $C_{35}H_{50}O_7Na$, 605.3454).

Results and discussions

A joint-study between Vietnam and Taiwan on bioactive compounds from the Vietnamese plant, *Clausena lansium* Skeels (Rutaceae), was conducted.

The methanol extract from the dried leaves of *C. lansium* was partitioned between H_2O and $CHCl_3$. The purification of the $CHCl_3$ fraction by a combination of column chromatographic methods afforded eight new lactams, including γ-lactams (**1-3**), δ-lactams (**4-7**), and amide (**8**), along with seven known lactams (**9-15**), which were characterized from the leaves of *C. lansium* (Fig. 1). Their structures were elucidated using spectroscopic methods [3] and the absolute configurations were determined using electronic circular dichroism (ECD) and single-crystal X-ray diffraction analyses with Cu Kα radiation.

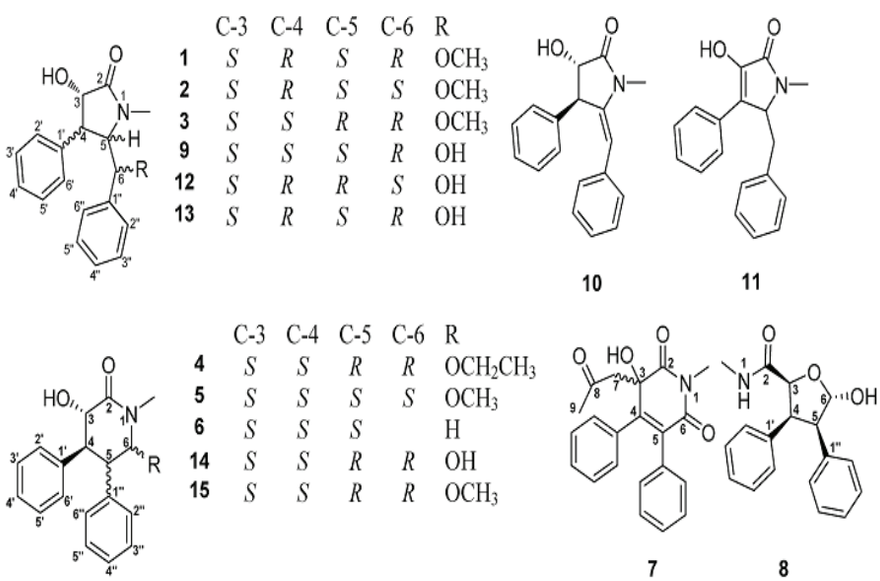


Fig. 1. The lactam compounds 1-15.

The ECD sign and red shift of the Cotton effect were shown to experimentally determine the C-3 configuration as well as the sign and the magnitude of the n → π* Cotton effect, which are sensitive to the nature of the C-3 substituent [4]. Therefore, the C-3 configuration of compound **1** with a hydroxyl functionality was determined as S, because it displayed a positive Cotton effect near 230 nm. The absolute configuration of compound **1** was unambiguously defined, by a single-crystal X-ray diffraction analysis with Cu Kα radiation, as 3S, 4R, 5S, and 6R (Fig. 2). Consequently, the structure of the 6-O-methylneoclausenamide (**1**) was characterized, as shown in Fig. 1. The 2D structure of compound **2** was similar to compound **1**, while the relative configuration of the lactam ring was assigned as being similar to compound **1,** through the analysis of their NOESY spectra (Fig. 3). In addition, the absolute configurations at C-4, C-5, and C-6 were determined by the single-crystal X-ray diffraction pattern using the anomalous scattering of Cu Kα radiation (Fig. 2). Therefore, the absolute configuration was determined as 3S, 4R, 5S, and 6S. In effect, the structure of 6-O-methyl-epi-neoclausenamide (**2**) was assigned as shown. The 2D structure of compound **3** was assigned to be identical to those of compounds **1** and **2** by a comparison of their UV, IR, MS, and NMR data [2]. The ECD spectrum of compound **3** showed a low-amplitude positive Cotton effect near 236 nm. The ECD spectrum of compound **12** showed a high-amplitude positive Cotton effect at 230 nm. Thus, the low-amplitude positive Cotton effect at 238 nm in the ECD spectrum of compound **3** (Fig. 4) suggested 3S and 4S absolute configurations [5]. By comparing the specific rotation and absolute configuration of compound **3** with the 16 stereoisomers of clausenamide, the 3S, 4S, 5R, 6S and 3S, 4S, 5R, 6R configurations could be further considered [3]. Therefore, the absolute configuration of 6-O-methyl-epi-cisneoclausenamide (**3**) was established as 3S, 4S, 5R, and 6R. The absolute configuration of C-3 in compound **4** was deduced by the ECD spectrum. In this case, the ECD spectrum of compound **4** (Fig. 4) showed a positive Cotton effect at 231 nm, which evidenced a 3S absolute configuration. Consequently, the absolute configuration of compound **4** was deduced as 3S, 4S, 5R, and 6R, the structure of which was illustrated as shown. To determine the absolute configuration, compound **5** was subjected to a single-crystal X-ray diffraction analysis with Cu Kα radiation (Fig. 2) which confirmed the structure unambiguously. Therefore, the absolute configuration was established as 3S, 4S,

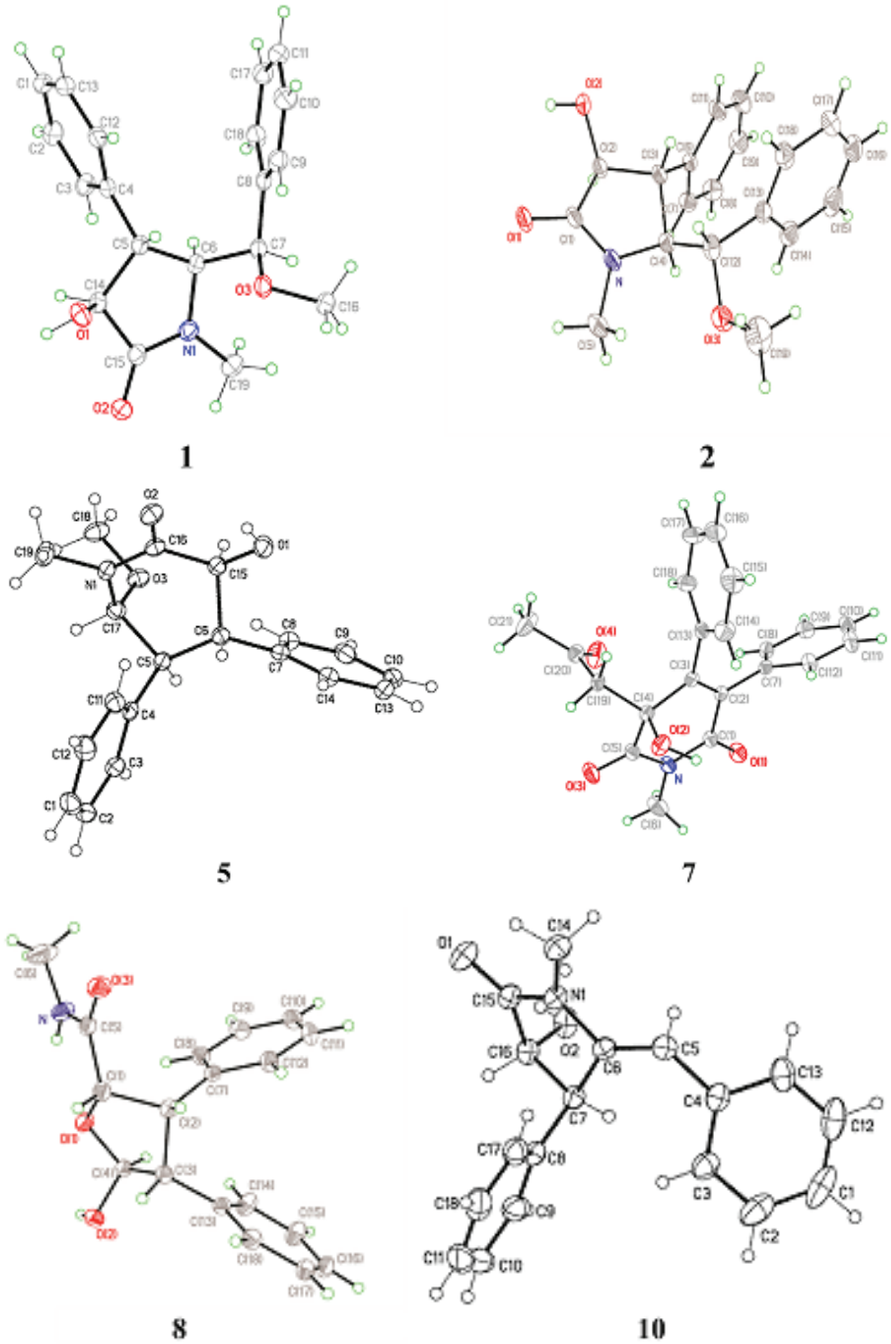


Fig. 2. ORTEP drawings of compounds 1, 2, 5, 7, 8, and 10.

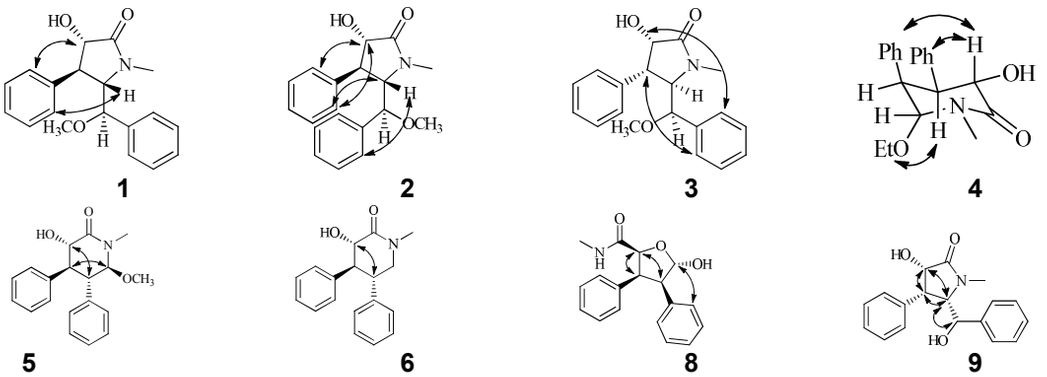


Fig. 3. Selected NOESY (↔) correlations for compounds 1-6, 8, and 9.

5S, and 6S (Fig. 2). Hence, compound **5** was characterized as lansamide-6. A positive Cotton effect at 223 nm in the ECD spectrum (Fig. 4) suggested a 3S absolute configuration. The absolute configuration was established as 3S, 4S, and 5S, while the structure of lansamide-7 (**6**) was characterized as shown. Based on these results and the single-crystal X-ray diffraction analyses using Cu Kα radiation (Fig. 2), the structure of lansamide-8 (**7**) was identified as shown. The crystals of compound **7** were orthorhombic and belonged to the space group, *Pbca*. As shown in the ORTEP drawing (Fig. 2), the X-ray analysis revealed that compound **7** was a racemic mixture presumably originating from the reaction between pyridine-2,3,6-trione and acetone. From the spectroscopic analysis and the single-crystal X-ray diffraction data (Fig. 2), the absolute configuration was confirmed by the Flack parameter 0.0(2) and defined as 3S, 4S, 5R, and 6S. The structures of compounds **9** and **10** were confirmed by the HRESIMS data and single-crystal X-ray diffraction analysis (Fig. 2). These structures have been reported as synthetic products, but they were isolated from their natural sources for the first time. Compounds **12** and **13** were identified as (-)-clausenamide and (-)-neoclausenamide through the ^{1}H and ^{13}C NMR [1], the positive Cotton effect in the ECD spectrum [at 230 and 229 nm] (Fig. 4), single-crystal X-ray diffraction analysis (Fig. 2), and its negative specific rotation [-148.5 (c 0.8, MeOH) and -71.8 (c 1.8, MeOH)]. Compounds **14** and **15** were reported as racemates in a previous study [5], but the negative specific rotation [-107.8 (c 1.4, MeOH) and -117.1 (c 0.7, MeOH)] and a high-amplitude Cotton effect (Fig. 4) confirmed that they were pure enantiomers. Their structures were confirmed by the positive Cotton effects in their ECD spectra [at 230 and 231 nm] (Fig. 4) and single-crystal, X-ray diffraction analyses (Fig. 2).

Some relationships between the ECD spectra and the absolute configurations could be found from the above results. In the ECD spectra, δ-lactams **4**, **14**, and **15,** with 3S, 4S, and 5R absolute configurations, exhibited negative and positive Cotton effects near 210 and 230 nm, respectively. Compound **5** and **6,** possessing 3S, 4S, and 5S absolute configurations, displayed ECD spectra with a positive Cotton effect at 220 nm. For the γ-lactam group, compounds **1**, **12**, and **13**, with 3S and 4R stereochemistry, exhibited similar ECD spectra. However, the absolute configurations of compound **12** at C-5 and C-6 were different from those of compounds **1** and **13**. This implied that the absolute configuration of C-5 and C-6 had little contribution to the ECD spectra. In contrast, compounds **3** and **9** possessed 3S and 4S absolute configurations and showed different ECD spectra, as compared to those of compounds **1**, **12**, and **13**. This indicated that the C-4 phenyl group may have a significant influence on the Cotton effect near 230 nm. Furthermore, a comparison of the ECD spectra of compounds **3** and **9** showed that the absolute configuration at C-5 may influence the wavelength of the Cotton effect.

In the other joint-study, air-dried and powdered fruiting bodies of *H. apiaria* were extracted with methanol and the combined extracts were concentrated under reduced pressure to produce a deep brown syrup. The crude extract was suspended in water and partitioned with ethyl acetate to afford ethyl acetate and water-soluble fractions. Purification of the ethyl acetate fraction by a conventional combination of column chromatographies yielded four new triterpenoids (**16-19**) and hexatenuin A [6].

Compound **16** was obtained as an optically active white powder, with $[\alpha]^{25}_{D}$ +57 (c 0.6, MeOH). The positive-mode HRESIMS of compound **16** showed a pseudo-molecular ion peak at *m/z* 605.3451 ([M+Na]$^{+}$, calcd for $C_{35}H_{50}O_7Na$, 605.3454), corresponding to the molecular formula of $C_{35}H_{50}O_7$ with 11 indices of hydrogen deficiency (IHD). The UV spectrum of compound **16** exhibited an absorption maxima at 262 nm, compatible with an α,β-unsaturated carbonyl chromophore [7]. The IR absorption bands at 2946, 1759, and 1693 cm^{-1} suggested the presence of aliphatic C-H, lactonic carbonyl, and carbon-carbon double bond functionalities. The ^{1}H NMR spectrum of compound **16** displayed five methyl singlets at δ 0.68 (3H, CH_3-18), 0.88

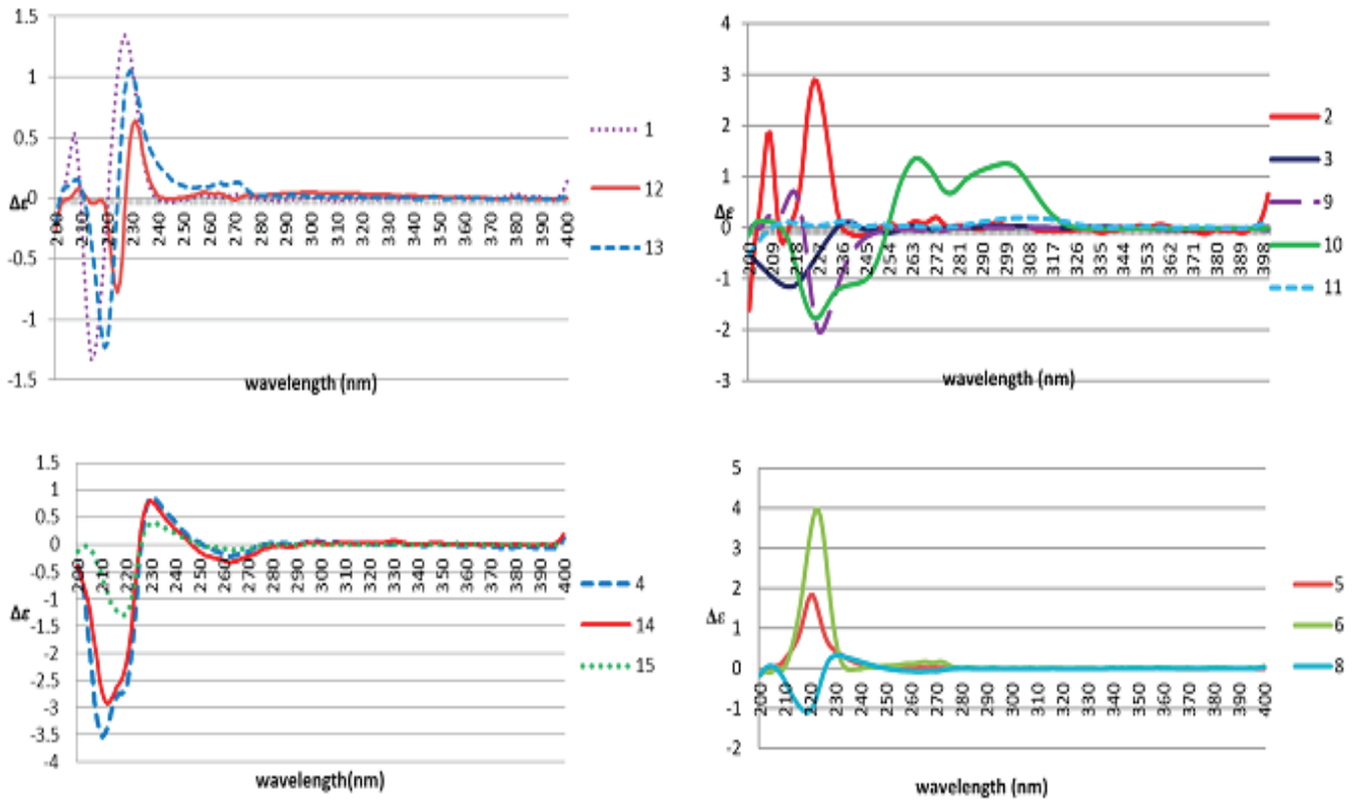
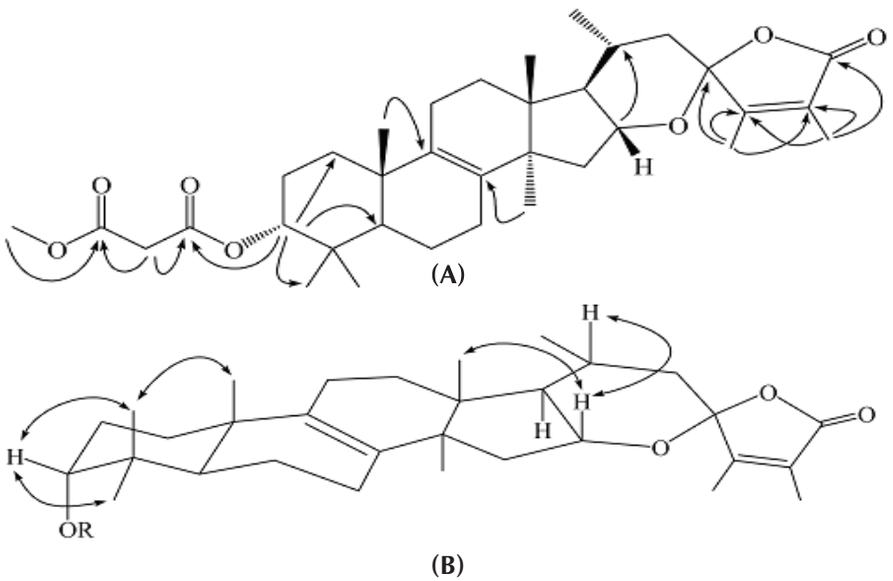


Fig. 4. ECD spectra of compounds 1-6 and 8-15.

(3H, CH_3-28), 0.93 (3H, CH_3-29), 1.00 (3H, CH_3-19), and 1.08 (3H, CH_3-30), respectively. In addition, one doublet methyl group at δ 0.95 (3H, J = 6.5 Hz, CH_3-21) suggested the presence of the lanostane skeleton. Two vinyl methyl signals at δ 1.81 (3H, *d*, J = 0.5 Hz, CH_3-27) and 1.94 (3H, *d*, J = 0.5 Hz, CH_3-31), along with the ^{13}C NMR signals at δ 8.5 (C-27), 10.8 (C-31), 108.2 (C-23), 125.2 (C-25), 157.4 (C-24), and 172.2 (C-26), indicated a γ-lactone ring cyclized between C-23 and C-26. This was verified by the HMBC correlations from CH_3-31 to C-23, -24, and -25 as well as from CH_3-27 to C-24, -25, and -26, respectively. In the downfield region of the ^{13}C NMR spectrum, there were two oxygenated methines at δ 79.6 (C-3) and 79.8 (C-16), one set of tetra-substituted double bonds at δ 133.8 (C-8) and 135.1 (C-9), and two ester carbonyl carbons at δ 165.9 (C-1′) and 167.2 (C-3′). The location of the tetra-substituted double bond at C-8/C-9 was determined by the ^3J-HMBC correlations between CH_3-19 and C-9 and between CH_3-30 and C-8. The HMBC cross-peaks from H-16 (δ 4.32, 1H, *ddd*, J = 11.5, 11.5, 5.0 Hz) to C-20 (δ 30.7), from H-3 (δ 4.71, 1H, *br s*) to C-29 (δ 21.7), C-1 (δ 30.5), C-5 (δ 45.3), C-1′; from CH_2-2′ (δ 3.40, 2H, *s*) to C-1′ and C-3′; and from CH_3-4′ (δ 3.72, 3H, *s*) to C-3′ evidenced that the C-16 had been oxygenated while the C-3 had been acetylated by the carbomethoxyacetyloxy group. The elucidations provided above constructed the chemical skeleton of 1 with 10 IHDs. The last IHD was afforded by the cyclization between C-16 and C-23 through the ether linkage with a spiro structure. These spectra evidenced that compound **16** was very similar to the reported compound hexatenuin A [8], with the only difference being that compound **16** was the methyl derivative of hexatenuin A. The coupling constants of H-3 (*br s*) and H-16 (11.5, 11.5, 5.0 Hz) indicated their orientations to be equatorial and axial. The stereochemical configurations of H-3 and H-16 were further established as β and β, according to the NOESY analysis and comparison of the spectral data of compound **16** and hexatenuin A [8]. The successive two-dimensional spectral experiments, including COSY, NOESY, HMQC, and HMBC accomplished the assignments of all the proton and carbon signals of compound **16**, and therefore its chemical structure was established as shown in Fig. 5 and named trivially as hexagonin A.

Compounds **17-19** were all obtained as optically active white powder, displaying similar UV spectra and IR absorption bands as those of compound **16**. Moreover, the proton resonances for the eight methyl groups, characteristic of the triterpenoid basic skeleton, were all observed in their ^{1}H NMR spectra. These data indicated that compounds **16-19** were structurally similar compounds (Fig. 6).

The purified triterpenoids, which were isolated in sufficient quantity,



(A)

(B)

Fig. 5. Significant HMBC (A) and NOESY (B) correlations of compound 16.

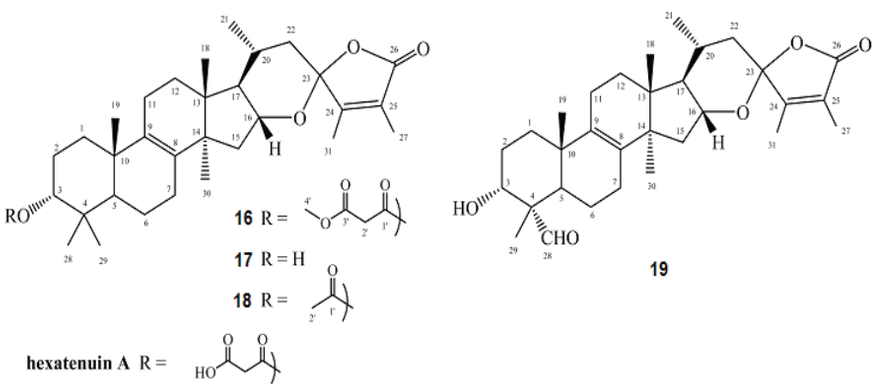


Fig. 6. Chemical structures of all the purified compounds.

Table 1. Inhibitory effects of purified samples from *H. apiaria* on superoxide Anion generation and elastase release by human neutrophils, in response to N-Formyl-Lmethionyl-phenylalanine/Cytochalasin B (FMLP/CB).

Compound	IC_{50} (μM)[a]	
	Superoxide anion generation	*Elastase release*
16	>10	-[b]
17	>10	-[b]
18	>10	-[b]
19	6.0±1.0***	>10
hexatenuin A	1.9±0.2***	4.3±1.4***
LY294002[c]	0.4±0.02***	1.5±0.3***

[a]Concentration necessary for 50% inhibition. Results are presented as mean ± SD (n = 3-4). ***p < 0.001 compared with the control value. [b]Increasing effects were observed. [c]A phosphatidylinositol-3-kinase inhibitor was used as a positive control for superoxide anion generation and elastase release.

were examined for their inhibition of superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB (Table 1). Among the examined constituents, hexatenuin A displayed the most significant inhibition of superoxide anion generation and elastase release, with IC_{50} values of 1.9±0.2 and 4.3±1.4 μM, as compared to the reference compound LY294002,12 with IC_{50} values of 0.4±0.02 and 1.5±0.3 μM for superoxide anion generation and elastase release, respectively. In addition, the following structure-activity relationships could be deduced from the bioactivity data. Hexagonins B (**17**) and D (**19**), which possess the basic triterpenoid skeleton without the malonyl substitution at C-3, did not show any anti-inflammatory bioactivity. Comparatively, hexagonin A (**16**), with its triterpenoid skeleton and malonyl and methyl ester functions, also failed to exhibit significant activity. Hexatenuin A, which had the triterpenoid skeleton as well as a free malonic acid group, displayed the most significant inhibitory effects in the bioactivity examination. Consequently, the free malonic acid function was important for anti-inflammatory activity. From the above data, it was concluded that the purified triterpenoids of *H. apiaria* are new potential leads for anti-inflammatory drug development and the starting fungus can be used as a health food with a possible and known mechanism of action.

Therefore, it is not surprising that intrinsic anti-inflammatory properties demonstrated in vitro with *H. apiaria* can be transferred in vivo after mushroom consumption as food or nutraceutical food. This study has identified the ability for food processing to anti-inflammatory. The process extraction for *H. apiaria* identified a five-step process that would address certain critical aspects in the design and development of functional food (Fig. 7).

Conclusions

A total of 15 lactams were isolated

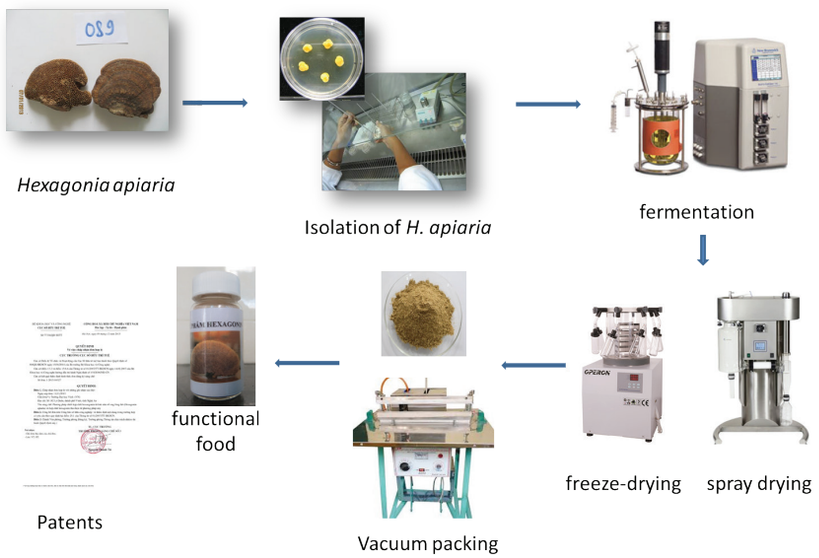


Fig. 7. The process of extraction for *H. apiaria*.

from the methanolic extract of *C. lansium*. This research work enabled the determination of the absolute configuration of these classes of compounds using MS, NMR, electronic circular dichroism (ECD), and single-crystal X-ray diffraction analyses with Cu Kα radiation. In the other study, a chemical investigation of the fruiting bodies of *H. apiaria* resulted in the identification of five compounds, hexagonins A-D (**16-19**) and hexatenuin A. The purified constituents were examined for their anti-inflammatory activity. Among the tested compounds, hexatenuin A displayed the most significant inhibition of superoxide anion generation and elastase release. These triterpenoids may have the potential to be used as anti-inflammatory agents. This study has identified abilities from food processing to anti-inflammatory. The process extraction for *H. apiaria* identified a five-step process that would address certain critical aspects in the design and development of functional food.

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