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Original Research Article

Isolation and determination of lipophilic mycochemicals from a New Zealand edible native mushroom *Hericium novae-zealandiae*

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ABSTRACT

Hericium novae-zealandiae is a native mushroom consumed by indigenous Māori people in New Zealand. The lipophilic mycochemicals of the mushroom were isolated using a normal column chromatography combined with a preparative HPLC. Structural characterisation based on spectroscopic methods, namely UV, MS, NMR and single crystal XRD have identified three lipophilic compounds as hericene B (a compound unique to *Hericium*), ergosterol and ergosterol peroxide. Following this, an HPLC-DAD method was developed and validated to quantify the hericene B and ergosterol. The method showed excellent selectivity, linearity, precision, accuracy and robustness. The content of hericene B was determined as 28.53 mg/g by dry weight of *H. novae-zealandiae* (approximately 3%). This discovery indicates the potential utilisation of *H. novae-zealandiae* as a natural source of hericene B. Current research revealed for the first time, the lipophilic constituents of *H. novae-zealandiae* and the method development for quantification of hericene B in the above species.

1. Introduction

Edible mushrooms have been consumed since ancient times in Asian countries including China and Japan (Zhang et al., 2007) because of their distinctive taste and nutritional values. These mushrooms have also been extensively studied for their medicinal characteristics including hypotensive, immuno-modulatory and antitumor activities (Rathee et al., 2012).

Hericium novae-zealandiae is a New Zealand (NZ) native wood decay mushroom, traditionally consumed by the indigenous Māori people who named it as pekepekekiore (Fuller et al., 2004). This New Zealand species was earlier incorrectly identified as *H. coralloides* as both species share similarities in their overall morphological features. Recent phylogenetic studies confirmed that the NZ mushroom, as used in this study, is a separate species of *Hericium*, and thus, it is named as *Hericium novae-zealandiae* (Colenso) (Smith and Cooper, 2019). The genus *Hericium* (from Family *Hericiaceae*) contains some edible mushroom species. Among them, *H. erinaceus*, has been consumed since ancient time in Eastern Asia, for its culinary and nutritional properties, and as a folk medication for treating dyspepsia, gastric ulcers and enervation (Büssing and Hübner, 2009). There have been considerable research in this species and the following bioactivities were reported: neuro-protection (Lee et al., 2014), liver-protection (Kim et al., 2012a, b), anti-oxidant character (Charumathy et al., 2016), antimicrobial (Okamoto et al., 1993), anti-cancerous, immuno-modulating (Sheu et al., 2013), and anti-inflammatory activities (Kim et al., 2012a, b). The chemical constituents associated with the bioactivities have been explored, with major focus on polysaccharides as immune enhancers (Sheu et al., 2013). Other isolated bioactive compounds from the species have been found to promote the biosynthesis of nerve growth factor (NGF) and further studies revealed that the compounds responsible for the above effect are unique to the genus *Hericium*. At present, some unique *Hericum* compounds have been reported as below: hericerin (Kimura et al., 1991), hericene (Arnone et al., 1994), erinacol (Kenmoku et al., 2004), erinacine (Shimbo et al., 2005), erinacerin ((Yaoita et al., 2005), hericenone (Ma et al., 2010a,b) and erinarol (Li et al., 2015). Extensive studies have confirmed the role of erinacines and hericenones in promoting the biosynthesis of NGF (Kawagishi et al., 1996, 1994;

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Kawagishi and Zhuang, 2008). In addition to the NGF promoting property, these compounds have been examined for other medicinal effects including their anti-cancer and anti-inflammation properties. Erinacine A was studied against ischemia-injury-induced neuronal cell death (Lee et al., 2014) and was found to inhibit amyloid β production to ameliorate the Alzheimer's disease (AD)-related pathologies in mice (Tzeng et al., 2018). This compound was also found to generate apoptosis in gastric cancer cell line TSGH 9201 (Kuo et al., 2017) and colorectal cancer cell lines HCT-116 and DLD-1 (Lee et al., 2017). Hericenone L showed cytotoxic activity against the human esophageal squamous cell carcinoma (ESCC) EC109 cell line (Ma et al., 2012). Furthermore, hericenone F and erinarols were reported to inhibit lipopolysaccharide (LPS)-induced inflammatory activity in RAW264.7 cells (Lee et al., 2016; Li et al., 2015).

On the other hand, much less is known about the New Zealand's *H. novae-zealandiae*. In earlier studies, we found that the water extract of *H. novae-zealandiae* was a good source of nucleoside compounds (Chen et al., 2019a) and discovered some bioactivities from three polysaccaride fractions extracted from this species (Chen et al., 2009b). We also reported that the ethanol extract possessed meaningful anti-proliferative and antioxidant activities as well as weak AChE inhibitory effect (Chen et al., 2019c). Apart from these, no published literature is available regarding the isolation and determination of any lipid soluble compounds from the ethanol extract of this species. Since *H. novae-zealandiae* and *H. erinaceus* are from the same genus, their chemical profiles could be similar. Therefore, the unique *Hericium* compounds which were found in the *H. erinaceus* may also be presence in the *H. novae-zealandiae*. To test this hypothesis, we designed experiments to isolate the unique *Hericium* compounds using chromatography techniques. A preliminary screening was then conducted to check the presence of those compounds. Although dozens of compounds unique to *Hericium* were isolated, there were no relevant commercial reference standards. We obtained three compounds, namely erinacine A, hericenone C and hericenone D as reference standards. From literature, erinacines were found in trace amounts in the fruiting bodies but could be present at higher concentrations in the mycelia of the submerged cultures, while hericenones were present only in the fruiting bodies of *H. erinaceus* (Bhandari et al., 2014; Ma et al., 2010a). Our preliminary investigation (using the above three reference standards) showed that only hericenone C was detected from the ethanol extract of the fruiting bodies of *H. novae-zealandia*. Based on this, we aimed to isolate lipophilic compounds, particularly the compounds unique to *Hericium*, from *H. novae-zealandiae* using hericenone C as a tracking reference. Column chromatography, thin liquid chromatography (TLC) and preparative high performance liquid chromatography (HPLC) were applied for compound isolation. The structure of the isolated compounds was elucidated by spectral analyses. An HPLC method was then developed and validated for quantifying the isolated compounds.

2. Materials and methods

2.1. Materials, reagents and instruments

Freshly cultivated fruiting bodies of *H. novae-zealandiae* (culture ICMP 21483, from Landcare Research, Auckland, New Zealand, May 2016), was provided by a mushroom grower at Napier, New Zealand. Mushroom fruiting bodies were lyophilised and powdered prior to analysis. Reference standards including erinacine A, hericenone C and hericenone D were kindly given by Professor Hirokazu Kawagishi from Shizuoka University. Silica gel (200–300 mesh) for column chromatography and the pre-coated silica gel thin-layer chromatography (0.25 mm, TLC) plate were purchased from Merck, Darmstadt, Germany. Methanol (High performance liquid chromatography (HPLC grade), was obtained from Macron Chemicals, PA, USA; Formic Acid (chromatograph grade), cyclohexane, ethyl acetate, petroleum ether (60 − 90 °C), and sulfuric acid were obtained from Sigma-Aldrich, Auckland, New Zealand; purified water (H_2O) was prepared using a Milli-Q purification system (Thermo Fisher, Waltham, MA, USA). The preparative HPLC was performed using a Biotage Isolera One Chromatography with a Biotage SNAP Cartridge KP-Sil column (Uppsala, Sweden).

2.2. Compound extraction and isolation

The freeze-dried powder of *H. novae-zealandiae* (1 kg) was dispersed in 6 L absolute ethanol over night before reflux extraction at 60 °C. The extract solution was condensed until no more solvent could be collected. Silica-gel was added into the ethanol extract and the dried mixture was applied into a silica gel column for normal-phase chromatography using a gradient of cyclohexane-ethyl acetate solution to give 60 fractions, with the volume of 500 mL each. All the fractions were subjected to thin-layer chromatography (TLC) using a mixture of petroleum ether (60 − 90 °C), cyclohexane, ethyl acetate, acetone and formic acid as the mobile phase. The fractions with the same TLC profiles were combined and concentrated for subsequent separation on the preparative high performance liquid chromatography (HPLC). The sub-fractions were obtained by a preparative HPLC at the wavelengths of 295 nm and 254 nm by eluting with cyclohexane-ethyl acetate having a gradient of 10:2-10:5, at a flow rate of 5.0 mL/min to give another 60 sub-fractions. All sub-fractions were checked by TLC and those with the characteristics of dominant single compound were selected for the next confirmation. Three groups of sub-fractions were identified with the high potential of consisting single constituent. The solvent was evaporated from sub-fraction 8, yielding compound **1** (10 mL in room temperature). Fractions 30–35 were combined and concentrated, and then subjected to recrystallization from cyclohexane to give compound **2** (52 mg). Similarly, fractions 51–55 was also subjected to recrystallization from petroleum ether to give compound **3** (33 mg).

2.3. Spectral analysis

Three isolated compounds were subjected to an ultraviolet (Lalanne et al., 2009) spectrometer (Shimadzu UV-1280, Kyoto, Japan) and an diode array detectors (DAD) to check their UV absorption wavelengths. $CDCl_3$ (Sigma-Aldrich, Auckland, New Zealand) was used as the Nuclear magnetic resonance (NMR) solvent to dissolve all the three compounds. NMR spectra were acquired from a Bruker AV-400 spectrometer using tetramethylsilane (TMS) as the internal standard. The coupling constants was presented in *J* Hz. High resolution-electron spray ionization (HR-ESI) MS spectra were obtained by infusing the sample into the Bruker (Bremen, Germany) MicrO-TOF Q2 mass spectrometer in positive ion mode, with capillary voltage at 4.5 kV and desolvation gas temperature at 180 °C. Tandem ESI-MS-MS spectra were acquired by collision-induced dissociation (CID), with argon as the buffer gas and the collision energy setting between 5 to 45 eV. Mass selection were performed by quadrupole 1 using a unitary *m/z* window, and collisions were conducted in the radio frequency (RF)-only quadrupole collision cell, followed by time-of-flight (TOF) mass analysis. Spectra were recorded over a *m/z* range of 80–1000 amu. In addition to the traditional MS/NMR spectra for structure elucidation, a single crystal X-ray diffraction technique was also applied to acquire the stereo-structures of the two compounds in crystal forms. Compound **2** and compound **3** were collected at 100 K on a Rigaku Oxford Diffraction Supernova Dual Source, Cu Kα radiation equipped with an AtlasS2 CCD detector (Tokyo, Japan) to obtain the single crystal X-ray diffraction spectra. Compound **1** was not analysed by single crystal X-ray diffraction as it was not in a crystal form.

2.4. Quantification of the isolated compounds

2.4.1. Preparation of standard stock and working solutions

Hericene B and ergosterol isolated form *H. novae-zealandiae* were used as reference standards for their quantification. Hericene B and

ergosterol were dissolved into hexane to prepare the stock standard containing 2056 μg of hericene B and 1270 μg of ergosterol per mL. Five concentration of calibration standards were prepared by diluting the stock solution with hexane. The stock standard solution was stored at 4 °C after preparation and the calibration working solutions were freshly prepared before the experiments.

2.4.2. Instrument conditions

The quantification experiment were carried out using an Agilent 1100 series HPLC system consisting of an in-line vacuum degasser G1322A, quaternary pump G1311A, auto-sampler G1313A, column oven G1316A, and a diode array detector G1315A. A reversed phase (Berven et al., 2015) Waters Symmetry C_{18} column (250 × 4.6 mm, 5 μm) was chosen for separation at 40 °C. A linear gradient mobile phase system consisting of methanol and 0.1 % formic acid was applied; starting from 50:50 for 20 min, then moving to 100:0 linearly in 10 min, and remaining for 25 min in this ratio, finally returning to the initial 50:50. Detection was conducted at a wavelength of 293 nm. The injection volume was 8 μL and the flow rate was 1.0 mL/min. The quantification of the two compounds was carried out using external calibration curves and all concentrations are expressed as mg/g dried weight. The data acquisition and analysis was implemented by Agilent ChemStation software (Agilent Technologies, Waldbronn, Germany).

2.4.3. Sample preparation for quantification

Samples (110 mg) were dispersed and immersed in 20 mL of absolute ethanol overnight and then treated in an ultrasonic bath (Type DT106, Bandelin, Berlin, Germany) for 30 min. The extraction solutions were filtered and passed through the 0.45 μm membrane (MicroAnalytix, Auckland, New Zealand). Samples were stored in the dark at 4 − 5 °C prior to HPLC analysis. All samples were prepared and tested in triplicate.

2.5. Validation of HPLC method

Of the several guidelines issued for analytical method validation, we adopted the International Conference on Harmonization (ICH, 2005); Association of Official Analytical Chemists (AOAC, 2002); and US Food and Drug Administration (FDA, 2000) for this research.

2.5.1. Selectivity and system suitability

Peak purity was checked by comparing the DAD spectra at three different points of the peak (rising, apex and declining) for the samples and standards. The System Suitability was assessed in terms of selectivity; resolution; number of theoretical plates; USP tailing; and the relative standard deviation (RSD) of the retention times of the peaks obtained.

2.5.2. Linearity

The mixed solutions containing the two standards were prepared at five levels ranging from 67.84–452.25 μg/mL for hericene B and 4.77–31.75 μg/mL for ergosterol, respectively. The five levels of mixed standard solutions were injected into HPLC from the lowest to the highest concentration to obtain the calibration curves. The peaks of hericene B and ergosterol were integrated to get the peak area using the built-in ChemStation software. All standards were injected and analysed in two repeats. The regression analysis was conducted on data obtained using the least squares method.

2.5.3. Precision

Inter-day precision, also named as intermediate precision was studied on the same day. Intra-day variation was also checked by comparing the analysis performed on two different days. RSD (relative standard deviation) of the peak area for the six sample repeats was calculated to express the precision of the method.

2.5.4. Accuracy

The accuracy of the method was measured as percent recovery (100 % × calculated concentration / theoretical concentration). Recovery was determined by spiking known quantities of the standards into the sample before extraction, at three different concentration levels falling within the range of the calibration curve. Three repeating samples containing the spiking standards were prepared independently using the method developed.

2.5.5. Limit of quantitation (LOQ) and limit of detection (LOD)

LOD and LOQ were determined using the formulas: LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the y-intercept of the regression lines and S is the slope of the calibration curve.

2.5.6. Method robustness

Six replicate samples were prepared in parallel according to conditions as stated in Section 2.4 with slight, yet deliberate variations in chromatography parameters as follows: applying an alternative column (Waters SymmetryShield RP-C18 (250 mm × 4 mm, 5 mm)); an altered column temperature (30 °C); various mobile phase pH (0.05 % and 0.2 % formic acid); a different gradient of mobile phase composition by changing 50 % methanol to 60 % methanol and a diverse flow rate (0.85 and 1.20 mL/ min). The content of the two compounds was examined by ANOVA and Tukey test to indicate method robustness during normal usage.

2.6. Statistical analysis

All results were expressed as the mean ± standard deviation (SD) of at least three independent experiments. One-way ANOVA and Excel 2013 (Microsoft Office) were used to assess the data, and statistical significant was considered at $p < 0.05$ level.

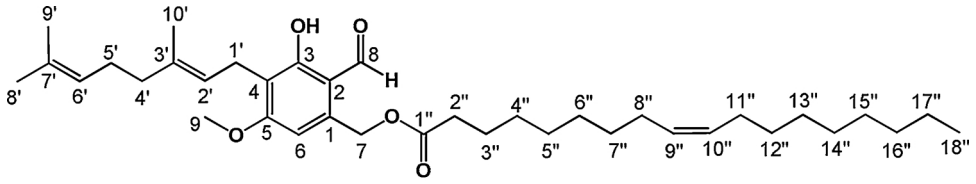
3. Results and discussion

3.1. Structural elucidation of the isolated compounds

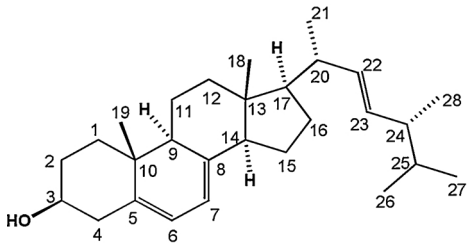
One of the most challenging parts of this research was to obtain samples in sufficiently large quantities for isolation of compounds. *H. novae-zealandiae* is originally a wild species, and its in-house cultivation has just started not long ago. Thereafter, the production yield was low and this has limited the availability of samples for isolation of *Hericium* unique compounds. Thus, hericenone C, despite being identified in our primary experiment, has not been isolated successfully due to its relatively lower content in *H. novae-zealandiae* and the sample size limitation (only 1 kg freeze-dried powder was available). Three other compounds were isolated from the ethanol extract of *H. novae-zealandiae*. Both compounds 1 and 2 showed maximum absorption at 293 nm, whereas compound **3** displayed no absorption from 200 nm to 400 nm. Their chemical structures were identified as hericene B (1), ergosterol (2) and ergosterol peroxide (3) in accordance with their spectra, chemical evidence such as colour reaction and previous reports as described below for each individual compound. The structures of these three compounds are shown in Fig. 1.

3.1.1. Hericene B

Compound **1** was obtained as a light yellow oily substance. The compound showed yellowish-brown spot when exposed to 10 % sulfuric acid solution in TLC. Its UV λ_{max} were at 225 and 293 nm as determined by HPLC-DAD. The molecular ion [M + Na] + at *m/z* 605.4126 amu shown inthe HR-ESI-MS spectrum indicated that the molecular formula of compound **1** was $C_{37}H_{58}O_5$ (calculated molecular weight 582.43). The core structure of compound **1** was revealed by the 1D NMR and 2D NMR spectroscopy (through Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments). A benzyl alcohol group was inferred from the signals at δ 138.6



(1)



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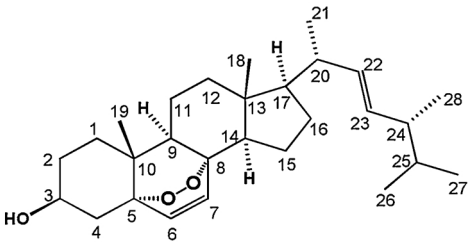

(3)

Fig. 1. Chemical structures of compound 1-3 from the ethanol extract of *H. novae-zealandiae* with structure 1) for of hericene B; 2 for ergosterol B; 3 for ergosterol peroxide.

(C-1), 113.0 (C-2), 163.0 (C-3), 118.2 (C-4),163.6 (C-5), and 105.7 (C-6) with carbinol group signals at δ 63.0 (C-7) and δ_H 5.32 (H-7). A formyl group was linked to C-2 from the correlations of δ_H 10.10 (H-8) and C-2, 3, 4, 8 in HMBC. A hydroxy group was linked to C-3 from the correlations of δ_H 12.37 (HO-3) and C-2, 3, 4, and a methoxy group was linked to C-5 from the correlations of δ_H 3.91 (MeO-9) and C-5. A residue of 3′,7′-dimethyl-2′,6′-octadienyl was linked to C-4 through C-1′ from the correlations of δ_H 3.33 (H-1′) and C-3, 2′, 4, in addition to the carbon signals at δ_C 21.5 (C-1′), 121.4 (C-2′), 131.2 (C-3′), 39.9 (C-4′), 26.8 (C-5′), 124.5 (C-6′), 135.8 (C-7′), 25.8(C-8′), 17.7 (C-9′), and 16.2 (C-10′) with their proton signals at δ_H 3.33 (H-1′), 5.17 (H-2′), 1.95 (H-4′), 2.02 (H-5′), 5.05 (H-6′), 1.62 (H-8′), 1.56 (H-9′), and 1.76 (H-10′). The core structure was determined as 4-(3′, 7′-dimethyl-2′, 6′-octadienyl)-2-formyl-3-hydroxy-5-methoxybenzyl alcohol, which is similar to that of mycophenolic acid. Moreover, an oleic acid residue was indicated by the remaining carbon signals at δ_C 173.2 (C-1″), 34.3 (C-2″), 32.1 – 22.7 (C-3″ ~ C-8″, C-11″ ~ C-17″), 130.1 (C-9″), 129.8 (C-10″), and 14.2 (C-18″) with their proton signals at δ_H 2.32 (H-2″), 5.33 (H-9″, 10″), 1.25 (H-3″ ~ 8″,11″ ~ 17″), and 0.87 (H-18″). In addition, this oleic acid residue was inferred to link to C-7 by ester bond according to the correlation between δ_H 5.32 (H-7) and C-1, 2, 6, 1″. Compound **1** was thus identified as hericene B based on the spectral evidence and by comparison with literatures (Arnone et al., 1994; Kobayashi et al., 2018). The ^{1}H and ^{13}C NMR data of compound **1** were summarised in Table 1, and the raw UV, HR-ESI-MS and NMR spectral data were present in Supplementary Material 1.

Table 1
NMR data of compound 1 in $CDCl_3$.

Position	δ_C, mult.	δ_H (J in Hz)[a]	Position	δ_C, mult.	δ_H (J in Hz)[a]
1	138.6, C	–	7′	135.8, C	–
2	113.0, C	–	8′	25.8, CH_3	1.62, s
3	163.0, C	–	9′	17.7, CH_3	1.56, s
4	118.2, C	–	10′	16.2, CH_3	1.76, s
5	163.6, C	–	1″	173.2, C	–
6	105.7, CH	6.52, s	2″	34.3, CH_2	2.32, t (8.0)
7	63.0, CH_2	5.32, s	3″	25.0, CH_2	1.62, s
8	193.2, CH	10.10, s	4″–7″	32.1 ~ 22.7, CH_2	1.25, m
9	56.0, CH_3	3.91, br s	12″–17″	32.1 – 22.7, CH_2	1.25, m
1′	21.5, CH_2	3.33, d (5.3)	8″	27.3, CH_2	2.02, m
2′	121.4, CH	5.17, t (7.0)	11″	27.3, CH_2	2.02, m
3′	131.2, C	–	9″–10″	130.1–129.8, CH	5.33, m
4′	39.9, CH_2	1.95, d (7.7)	18″	14.2, CH_3	0.87, t (7.0)
5′	26.8, CH_2	2.02, m	3-OH	–	12.37, s
6′	124.5, CH	5.05, br s			

Spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

[a] Indiscernible signals owing to overlapping or having complex multiplicity are reported without designating multiplicity. Reference peak 7.26 ppm for $CDCl_3$.

3.1.2. Ergosterol & ergosterol peroxide

Compound **2** was obtained as a white acicular crystal in cyclohexane. It showed deep green spot when exposed to 10 % sulfuric acid solution in TLC. The UV λ_{max} were 272, 282 and 293 nm determined by HPLC-DAD. The [M + Na]+ ion at *m/z* 419.3276 amu obtained from the HR-ESI-MS spectrum indicated that the molecular formula of compound **2** was $C_{28}H_{44}O$ (calculated molecular weight 396.34).

Compound **3** was obtained as a white flake crystal in petroleum ether (60-90℃) showing no UV λ_{max} by the UV spectrometer. The [M + Na]+ ion at *m/z* 451.3181 amu given by the HR-ESI-MS spectrum indicated that the molecular formula of compound **3** was $C_{28}H_{44}O_3$ (calculated molecular weight is 428.33).

As both compound **2** and **3** are in crystal form, single crystal X-ray diffraction analysis were carried out on the compounds. The data obtained are as below. Crystal data of compound **2**: $C_{28}H_{46}O_2$ (containing one molecular H_2O, which might be introduced into the crystal from the solvents containing trace moisture during crystallization), M = 414.65 g/mol, monoclinic, space group P21 (no. 4), a = 9.7966(2) Å, b = 7.50230(10) Å, c = 34.4295(7) Å, β = 94.390(2)°, V = 2523.04(8) Å3, Z = 4, T = 100.00(10) K, μ(*CuK*$_\alpha$) = 0.500 mm-1, Dcalc = 1.092 g/cm3. The integration of the data yielded a total of 21611 reflections (5.148° ≤ 2Θ ≤ 147.578°), of which 8628 were unique (Rint = 0.0502, Rsigma = 0.0507) and were used in all calculations. The final R1 was 0.0622 (I > 2σ(I)) and wR2 was 0.1623 (all data). The size of the crystal used for measurements was 0.12 × 0.1 × 0.08 mm. Crystal data of compound **3**: $C_{28}H_{44}O_3$, M = 428.63 g/mol, monoclinic, space group

P21 (no. 4), a = 15.53490(12) Å, b = 10.62703(8) Å, c = 15.70339(14) Å, β = 102.3342(8)°, V = 2532.63(4) Å3, Z = 4, T = 100.00(10) K, μ(CuKα) = 0.546 mm-1, Dcalc =1.124 g/cm3. The integration of the data yielded a total of 18529 reflections ($5.76^{\circ} \leq 2\Theta \leq 146.966^{\circ}$), of which 9438 were unique (Rint = 0.0294, Rsigma = 0.0354) which were used in all calculations. The final R1 was 0.0403 ($I > 2\sigma(I)$) and wR2 was 0.1083 (all data). The size of the crystal used for measurements was 0.14 × 0.11 × 0.09 mm. The ^{1}H and ^{13}C NMR spectral data of compound **2** and **3** are consistent with those reported in the previous studies (Zhang, Bao, & Tolgor, 2012; Zhang et al., 2002). Compound **2** and **3** were determined as ergosterol and ergosterol peroxide, respectively. The raw spectral data of UV, HR-ESI-MS, NMR and single crystal X-Ray diffraction analyses were shown in the Supplementary Material 1, 2 and 3.

3.2. HPLC method development

Both hericene B and ergosterol have the maximum UV absorption wavelength at 293 nm. This makes developing an HPLC-DAD method to quantify them at 293 nm possible. As ergosterol peroxide shows no UV absorption, it will not be included in this method, instead a different detector such as mass spectroscope could be applied to determine its content. No report on quantification of hericene was found. We referred to the quantification of ergosterol from other mushrooms (Martin et al., 1990) for analytical method development. As both compounds have low polarity, mobile phase with high percentage of organic phase was tried initially. A constant ratio of methanol: water of 80:20 was applied as mobile phase initially, but the two target peaks were neither sharp nor symmetrical. Subsequently, the gradient of the HPLC mobile phase was optimized using selected Waters columns. Several combinations of mobile phase were trialled in search for the best separation with the shortest analysis time. Among them, MeOH containing 0.1 % formic acid was found to give the optimal result as compared to others (e.g. MeOH: water and MeOH: KH_2PO_4 solution). Two Waters C_{18} columns, Symmetry and SymmetryShield were tested for their efficiency. The results show no significant difference between the two columns ($p > 0.05$). Fig. 2a and b show representative chromatograms of the standards and the ethanol extract of *H. novae-zealandiae*, respectively.

3.3. Validation of HPLC method

3.3.1. Selectivity and system suitability

The peak purity of the two compounds present in the ethanol extract of *H. novae-zealandiae* was checked by observing the DAD spectra of the peak at three points (rising, apex and declining). The spectrograms from the three points were identical, indicating that no other compounds were co-eluted with the targeted two compounds. The system suitability was satisfied by the outcomes of Resolution > 2; Selectivity > 1; USP tailing < 1.5; Number of theoretical plates > 8000 according to the US FDA (FDA, 1994) (Supplementary Material 4).

3.3.2. Linearity, LOQ and LOD, precision and accuracy

Five levels of mixed working standard solutions were prepared and injected into HPLC for hericene B (67.83–452.25 μg/mL) and ergosterol (4.76–31.75 μg/mL) in duplicate to check the linearity. Table 2 shows the linear regression formula and the correlation coefficient. A linear model was obtained according to the figure of the calibration curve (compound concentration versus peak area), as shown in the Supplementary Material 5. Furthermore, the residuals were randomly scattered, which met the requirement of homoscedasticity (Supplementary Material 5).

3.3.3. Precision and accuracy

Two levels of precision under the same conditions, namely over one day (repeatability) and two different days (intermediate precision) were assessed and calculated. A good level of precision of this developed HPLC method was achieved based on the values of % RSD as all results were within 5% in terms of inter- and intra-day variation (Table 3).

This HPLC method developed has a high level of accuracy as indicated by the recovery rates of 95%–105% with RSDs less than 5% (Table 3). To date, there is no formal criterion set for recovery rate in an HPLC analytical method as it is dependent on the analyte content in the sample, and the lower the concentration, the greater the tolerance. The AOAC guideline (AOAC, 2002) has stated that the reasonable recovery rates ranging from 95 to 102 %, 92–105%, 90 to 108 % and 85–110% must be attained for analyte having concentrations of 10 %, 1%, 0.1 % and 0.01 %, respectively. The results of recovery rates obtained from this study therefore satisfied this above requirement.

3.3.4. Method robustness

The robustness of this new HPLC method was challenged by its capacity to remain stable under small yet intentional changes in the analytical parameters. Slight variations of column type, column temperature, mobile phase pH, mobile phase composition, and flow rate were evaluated. The resulting impacts on the chromatograph performance and compound content were calculated and subjected to statistical analysis. No significant differences ($p > 0.05$) were observed in the concentration of the two compounds when minor changes occurred in column type, column temperature, pH of mobile phase, and flow rate. While these results indicated that the method is reasonably robust, it was noted that the peaks of the two compounds became asymmetric, producing a poor chromatograph performance if the mobile phase was fixed to a constant ratio of methanol and 0.1 % formic acid such as 50:50. This finding suggested that gradient elution is the preferable method as stated in Section 2.4 to ensure reliability of the results.

3.4. Quantification of the isolated compounds

Table 4 shows the content of the two compounds isolated from the ethanol extract of *H. novae-zealandiae* as determined by the validated HPLC method. The contents of hericene B and ergosterol were determined as 28.53 ± 0.89 and 2.09 ± 0.08 mg/g, respectively. Information concerning hericene B is limited. This compound was being isolated from the culture extracts of *H. erinaceus* for the first time in 1994 by Arnone et al. (Arnone et al., 1994). The authors had stated that research to evaluate the promoting activity of NGF synthesis was in progress but follow up publication couldn't be found. One recent research has reported the isolation of hericene B from the primordium of *H. erinaceus* (Corana et al., 2019). There were reports on other form of hericenes (hericene A, C and D) being isolated from *H. erinaceus* and *H. coralloides* (Ma et al., 2010b; Zhang et al., 2012). In addition, some researchers have successfully synthesized hericenes A–C and tested their bioactivities, among which hericene B and C have been found to show moderate protective activity against thapsigargin-induced endoplasmic reticulum (ER) stress-dependent cell death (Kobayashi et al., 2018). Hericene D was reported to exert a weak cytotoxic activity against esophageal cancer cell line EC109 and a weak antibacterial activity *in vitro* against five bacteria, namely *Staphylococcus aureus, Bacillus thuringiensis, Escherichia coli, Bacillus megaterium and Bacillus subtilis* (Ma et al., 2010a,b). Hericenes and hericenones share the basic structure and the latter have been reported to show positive bioactivity *in vitro*, with biosynthesis of NGF being the most prominent one. It's possible that hericene B might exhibit similar bioactivities as hericenones since they share similarity in molecular structure. From the current findings, *H. novae-zealandiae* could be an important natural source of hericene B since it contains as high as 3% of this compound. Further research is required to give insight into the biological properties of hericene B isolated from *H. novae-zealandiae*. In addition, future work could be conducted to determine the content of hericene B in other *Hericium* sp. to give better understanding regarding the distribution of this compound in different species.

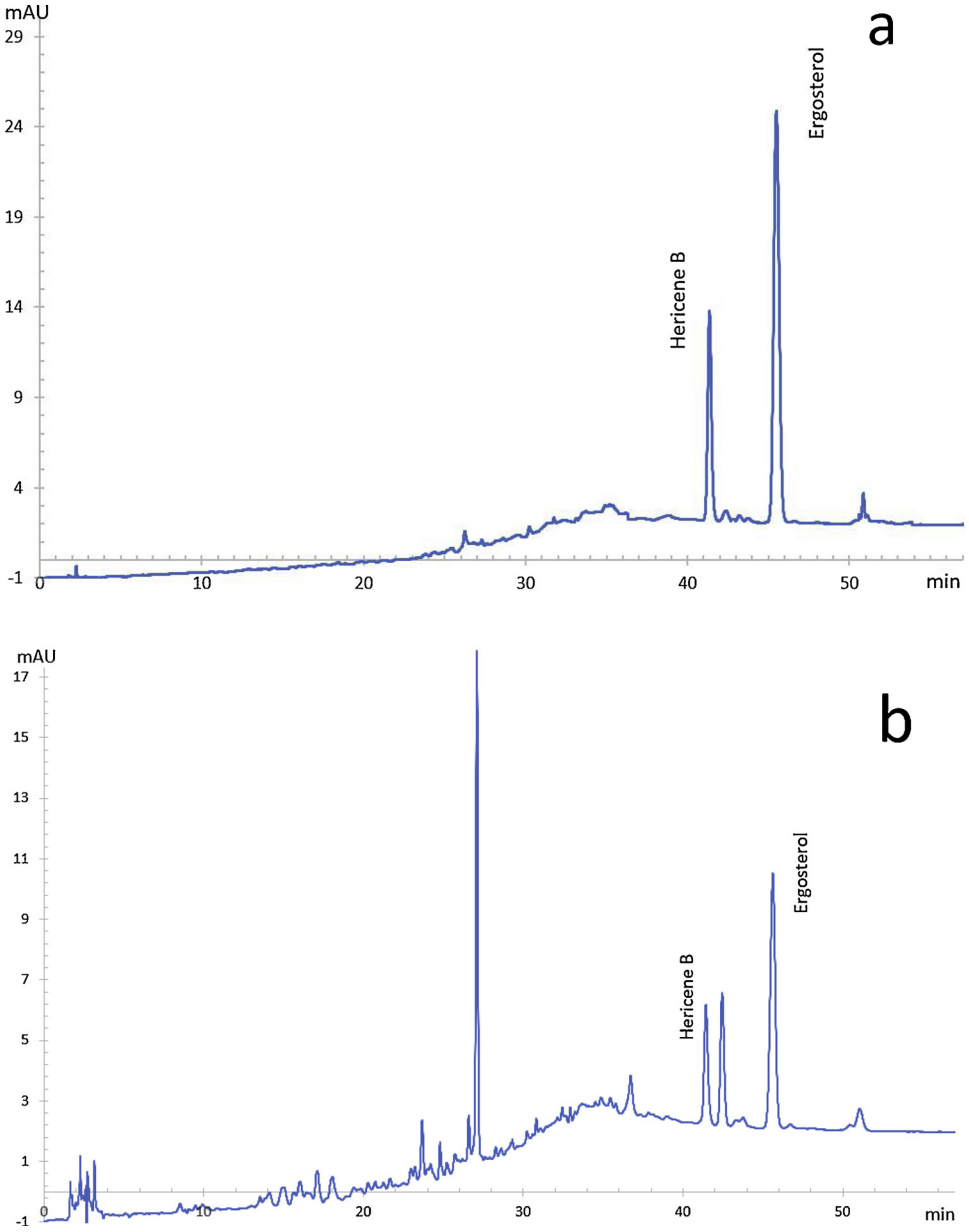

Fig. 2. Chromatogram of: a) reference standards of Hericene B and Ergsterol, and b) the two identified compounds in the extract of *H. novae-zealandiae*. Chromatographic conditions as specified in follows: Waters Symmetry C_{18} column (250 × 4.6 mm, 5 μm) was chosen for separation at 40 °C. A linear gradient mobile phase system consisting of methanol and 0.1 % formic acid was applied; starting from 50:50 for 20 min, then moving to 100:0 linearly in 10 min, and remaining for 25 min in this ratio, finally returning to the initial 50:50. Detection was conducted at a wavelength of 293 nm. The injection volume was 8 μL and the flow rate was 1.0 mL/min.

Table 2
Regression analysis on the linearity, LOQ, LOD, precision and accuracy of the two isolated monomers.

Parameter	Hericene B	Ergsterol
Linearity range of standards (μg/mL)	67.8375-452.2500	4.7625-31.7500
Correlation coefficient	0.9999	0.9999
Confidence limit of slope	1.187-1.202	5.959-6.074
p for the slope	3.23E-18	9.47E-17
SE of slope	0.0032	0.0248
Confidence limit of intercept	−4.025-0.031	−2.527-0.339
SE of intercept	0.879	0.474
p for the intercept	0.053	0.016
LOQ (ng)	3.6	1.12
LOD (ng)	1.2	0.38

Ergosterol is a predominant sterol of the fungal cell membrane (Czub and Baginski, 2006; Tantapakul et al., 2018), and its content is often correlated to the fungal dry mass (Pasanen et al., 1999). Ergosterol is a precursor of vitamin D and can be transformed into vitamin D when exposed to UV (Göring, 2018). It has been found to have a

Table 3
Precision and recoveries (%) of the two monomer compounds spiked at three concentration levels (high, medium, and low).

Compound	Precision-RSD (%) intra-day	Precision-RSD (%) inter-day	Spiked amount (mg/g)	Recovery replicates (%) (n = 3)	RSD (%)
Hericene B	2.99	3.28	14.3	103 ± 3	3.51%
			28.5	101 ± 3	3.19%
			42.7	98.1 ± 3.0	3.02%
Ergosterol	3.76	3.98	1.04	97.9 ± 4.0	4.53%
			2.08	99.2 ± 4.0	4.08%
			3.12	103 ± 4	3.99%

Table 4
The hericene B and ergosterol content in *H. novae-zealandiae*.

Compound	Working range (mg/g)	Content (mg/g) (n = 6)	RSD (%)
Hericene B	12.33-82.23	28.53 ± 0.89	2.99
Ergosterol	0.866-5.772	2.086 ± 0.079	3.76

number of pharmacological activities including: reducing inflammation related pain, reducing the incidence of cardiovascular diseases, inhibiting cyclooxygenase enzyme, and involving in antioxidant, antimicrobial, anticomplementary and antitumor activities (Yazawa et al., 2000; Zhang et al., 2002; Okuda et al., 2001).

Ergosterol peroxide is a natural steroid found in a variety of fungi, including lichens, and in sponges (Kahlos, 1996). The first isolation of ergosterol peroxide from a biological source (*Aspergillus fumigatus*) was reported by Wieland and Prelog in 1947 (Wieland and Prelog, 1947). From then on, ergosterol peroxide has been found in medicinal mushrooms such as *Ganoderma*, *Hericium*, and *Inonotus* (Merdivan and Lindequist, 2017). Ergosterol peroxide contains an oxygen bridge (O−O) in which each oxygen atom is connected to another oxygen and a carbon atom. Organic peroxides are often very reactive molecules, exhibiting important physiological functions such as defence against pathogens (Shetty et al., 2007). There have been many reports revealing the medicinal significance of ergosterol peroxide. Some examples include showing a strong trypanocidal activity toward the intracellular form of *Trypanosoma cruzi* (Ramos-Ligonio et al., 2012), giving anti-inflammatory activity through suppression of LPS-induced inflammatory responses (Kobori et al., 2007) and exerting anti-mycobacterial activity against mycobacterium tuberculosis H37Rv and *Mycobacterium avium* (Rugutt and Rugutt, 2012). Much more importantly, what makes ergosterol peroxide distinctive is its anti-cancer activity. It has been reported that ergosterol peroxide demonstrated anti-tumour activities in multiple human tumour cell lines, including human prostate cancer cells (Russo et al., 2010), human hepatoma and colorectal tumour cells (Rhee et al., 2012), human renal carcinoma cells (He et al., 2018), and human lung adenocarcinoma (Wu et al., 2018). All these observations suggest the potential to explore the use of ergosterol peroxide as a therapeutic candidate for treating cancer complications. The content of ergosterol peroxide in *H. novae-zealandiae*is has yet to be determined, it is estimated to be slightly lower than ergosterol (> 2 mg/g) according to the yields of isolated compounds of this study. An HPLC analytical method coupling with detectors other than DAD (or UV) such as MS could be considered for quantification of ergosterol peroxide in the future work.

4. Conclusion

This is the first study on the lipophilic compounds of *H. novae-zealandiae*, an edible native mushroom in New Zealand. Normal column chromatography combined with preparative HPLC allowed the isolation of three compounds from the ethanol extract. Their structures were characterized as hericene B, ergosterol and ergosterol peroxide based on spectroscopic methods and comparison with literature. The discovery of hericene B supported the hypothesis that *H. novae-zealandiae* contains compounds unique to *Hericium*. An analytical method using HPLC coupled with DAD detector was developed and validated (based on selectivity, linearity, precision, accuracy and robustness) to determine the contents of hericene B and ergosterol. The method involved the use of a reverse phase C18 column, with gradient elution consisting of methanol and 0.1 % formic acid as mobile phase and detection at 293 nm. Both hericene B and ergosterol were quantified simultaneously using the newly developed method. Their contents were determined as 28.53 and 2.09 mg/g dry weight of the mushroom, respectively. This is a first report on the quantification of hericine B since its isolation from *H. erinaceus* in 1994. Furthermore, the data obtained also indicates that *H. novae-zealandiae* is a rich natural source of hericene B (~3% of dry weight), which sparked further investigation of its potential pharmacological effects. Overall, current study has provided significant scientific findings for discovery of lipophilic mycochemical constituents in *H. novae-zealandiae*.

Author contributions

The listing authors contributed to this work as follows: conceptualization, Siew Young Quek and Zhixia Chen; monomer isolation, Zhixia Chen and Xujiang Yuan; monomer structure elucidation, Xujiang Yuan; method development and validation, Zhixia Chen; writing—original draft preparation, Zhixia Chen; writing—review and editing, Xujiang Yuan, Peter Buchanan and Siew Young Quek; supervision, Siew Young Quek and Peter Buchanan. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2020.103456.

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