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Characterization of the bioactivities of an ethanol extract and some of its constituents from the New Zealand native mushroom *Hericium novae-zealandiae*

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In this study, we investigated the potential bioactivities of an ethanol extract of *Hericium novae-zealandiae* and four of its constituents, namely hericenone C, hericene B, ergosterol and ergosterol peroxide. The proliferation of three prostate cancer cell lines, namely DU145, LNCaP and PC3, was evaluated after treatment with the extract and constituents. It was found that both the ethanol extract and ergosterol peroxide possess anti-proliferative activities to the three prostate cancer cell lines. Ergosterol peroxide was considered likely to be one of the major compounds responsible for the anti-proliferative effect of the ethanol extract. Subsequently, the results of RT-qPCR assay showed two possible mechanisms for these anti-proliferative activities. One is apoptosis, supported by the up-regulation of *CASP3*, *CASP8*, *CASP9*, and an increase in the ratio of *Bax*/*Bcl2*. The other is anti-inflammation, indicated by the down-regulation of *IL6* and up-regulation of *IL24*. The ethanol extract also exhibited antioxidant and AChE inhibitory (though weak) activities. However, none of the four compounds were found to account for these latter two activities. This is the first report of the bioactivities, and the corresponding active ingredients of lipophilic constituents from *H. novae-zealandiae*.

Introduction

Medicinal mushrooms are valued in many countries as they are believed to play a role in the treatment of various degenerative diseases.[1] In recent decades, the utilization of medicinal mushrooms has expanded from being a food ingredient to being viewed as an important component of human nutraceuticals, and even pharmaceuticals.[1]

Hericium novae-zealandiae (Colenso) Chr. A. Sm. & J. A. Cooper,[2] known as Pekepekekiore in Te Reo Māori, is an edible, New Zealand (NZ) native mushroom traditionally collected and consumed by Māori.[3] Until recently, it was misidentified as *Hericium coralloides*, well known from the Northern Hemisphere. Recent phylogenetic studies distinguished the NZ species as distinct and indigenous. The well-known medicinal mushroom *Hericium erinaceus*, also known as Hou Tou Gu, Yamabushitake or Lion's Mane, has been used for centuries to treat neurasthenia and general debility.[4] Other reported activities of *H. erinaceus* include anti-proliferative effects,[5] neuroprotective,[6] antioxidant,[7] and anti-inflammatory activities.[8] Several compounds isolated from *Hericium* species show a wide range of bioactivities, including erinacine,[9,10] hericenone,[11,12] and hericene.[13]

In contrast to *H. erinaceus*, very little is known about the chemical composition or the potential bioactivities of the *H. novae-zealandiae*. In this study, the pharmacological properties of an ethanol extract of this species were investigated. In earlier publication on this species, we reported that a water extract of *H. novae-zealandiae* was rich in nucleoside compounds,[14] and that three polysaccharide fractions exhibited bioactivity in the following publication.[15] Several liposoluble compounds, namely hericene B, ergosterol, and ergosterol peroxide, have been isolated from the ethanol extract of *H. novae-zealandiae*. Additionally, hericenone C was also identified from the ethanol extract but was not successfully isolated (manuscript under review). Of these four compounds, hericene B and hericenone C are compounds unique to *Hericium*.

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Noting the pharmacological effects reported for *H. erinaceus*, we designed experiments to assess *H. novae-zealandiae* for related properties. Anti-proliferative activities of lipophilic constituents from *H. erinaceus* have been reported on the following cancer cell lines: gastric cancer cell TSGH 9201,[16] colorectal cancer cell lines, HCT-116 and DLD-1,[17] myelogenous leukaemia cell line K562 and prostate cancer (PCa) line LNCaP,[18] human acute promyelocytic leukaemia cell HL-60[19] and human esophageal squamous cell carcinoma EC109 cell line.[20] Our hypothesis was that the lipophilic constituents of *H. novae-zealandiae* would exhibit similar anti-proliferative activities to *H. erinaceus*.

PCa is the fourth most common cancer in men worldwide[21] and has a particularly high incidence in Australia and New Zealand. In 2016, the NZ Ministry of Health reported PCa frequency at 27% for male New Zealanders.[22] Various phytochemicals have been evaluated as synergistic or alternative cancer treatments to chemotherapeutic drugs.[23] For *in vitro* PCa experiments, LNCaP, DU145, and PC3 are the frequently used cell lines,[24] being transformed from PCa metastases in the lymph node, brain, and bone marrow respectively.[25] In the present study, the anti-proliferative activities of the ethanol extract and derived compounds from *H. novae-zealandiae*, were studied in LNCaP, DU145, and PC3 cells. Furthermore, the molecular mechanisms accounting for the observed activities were investigated by RT-qPCR assays.

In a number of studies the authors have indicated that the lipophilic constituents, particularly the compounds unique to *Hericium*, played a role in neuroprotection.[26–30] The cholinergic hypothesis claims that memory and learning impairment in Alzheimer's disease (AD) patients is initiated by acetylcholine (ACh) deficiency.[31] The levels of synaptic ACh are regulated by the enzyme acetylcholinesterase (AChE), which appears to be a promising target for the treatment of AD.[32] Furthermore, phytotherapy has been reported to alleviate AD symptoms by targeting the aforementioned cholinergic pathway.[33] Consequently, an additional hypothesis in this study was that the lipophilic constituents of *H. novae-zealandiae* could inhibit AChE, which is related to neurological disorders.

Oxidation is a biological process which results in the formation of highly reactive, oxygen-derived free radicals containing one or more unpaired electrons. Free radicals attack living organisms during oxidative stress, which is an imbalanced state that can trigger the development of aging and chronic degenerative diseases, including coronary heart disease, and cancers.[32] In addition, oxidative stress, through the generation of both free radicals and their associated cellular redox stress, has also been considered a major mechanism in the pathogenesis of dementia and other neurodegenerative diseases.[34] As a result, a therapy combining the inhibition of AChE and oxidation has been developed to modulate this kind of cognitive decline.[35] Correspondingly, since oxidation is related to the development of both neurodegenerative diseases and cancer, three antioxidant capacity measurements were performed in this study to explore the antioxidant potential of *H. novae-zealandiae*.

The objective of the present study was to screen the anti-proliferative, AChE inhibitory, and antioxidant activities of an ethanol extract of *H. novae-zealandiae*. In addition, an investigation into the active compounds underlying these activities was also carried out.

Materials and methods

Materials

All the chemicals used in this study are of analytical grade, unless stated otherwise. The absolute ethanol used for the ethanol extraction was from ECP Ltd, Auckland, NZ. The chemicals used for the proliferation inhibition experiment were: trichloroacetic acid (TCA), sulforhodamine B (SRB), glacial acetic acid (Emsure®), phosphate buffered saline (PBS), disodium ethylenediamine-tetraacetic acid (EDTA) from Sigma-Aldrich, Auckland, NZ; tris(hydroxymethyl)aminomethane (Tris, Invitrogen™), trypsin (Gibco™), trypan blue dye (Gibco™) from Thermo Fisher Scientific, Auckland, NZ. The Milli-Q water for buffer solutions were prepared using the Milli-Q water purification system (Millipore Corporation, Burlington, USA). The chemicals used for the antioxidant experiment were: trolox ((*S*)-(2)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) from Sigma-Aldrich, Auckland, NZ; acetic acid, ferric chloride ($FeCl_3$), sodium acetate (Na_2CO_3) from ECP Ltd, Auckland, NZ; hydrochloric acid (HCl, Avantor, PA, USA). The chemicals used in the AChE experiment were: AChE enzyme (*Electrophorus electricus* (electric eel) EC, Auckland, NZ); Trizma Base, Trizma HCl, Eserine, DTNB, BSA, NaCl, $MgCl_2{\cdot}6H_2O$, SDS, and ATCI from Sigma-Aldrich, Christchurch, NZ. Pure water (H_2O) was obtained from a Milli-Q purification system, which was purchased from Thermo Fisher, Waltham, MA, USA. EnSpire Multimode Reader (PerkinElmer, MA, USA). Freshly cultivated specimens of *H. novae-zealandiae* (from ICMP 21483, Auckland, May 2016) were provided by a mushroom grower in Napier, NZ. The samples were freeze-dried and powdered prior to analysis.

Ethanol extract and the chemical compounds for the study

Two kilograms of freeze-dried *H. novae-zealandiae* powder were dispersed in 6 L of absolute ethanol overnight, followed by a reflux extraction at 60 °C. The extract was concentrated under reduced pressure until no more solvent could be removed. The yield of the extract was approximately 18%. Hericenone C was provided by Professor Hirokazu Kawagishi from Shizuoka University, Japan. Isolation of metabolites from the ethanol extract of *H. novae-zealandiae* was performed using silica gel column chromatography, followed by a preparative high-performance liquid chromatography. Three compounds were isolated and identified through MS/NMR and single crystal X-Ray diffraction, as hericene B, ergosterol, and ergosterol peroxide

(manuscript submitted). The ethanol extract and the four above-mentioned compounds were dissolved in ethanol at different concentrations, depending on the requirements of each assay.

Anti-proliferation assay

The cryopreserved human PCa cell lines PC3, DU145, and LNCaP, as well as a non-cancer HEK293 cell line (as control), were supplied by the Auckland Cancer Society Research Centre (ACSRC). These cell lines were cultured in minimum essential medium supplemented with 5% Fetal Calf Serum and 1% penicillin/streptomycin/glutamine in Becton Dickinson Falcon™ cell culture flasks (BD Biosciences) at 37 °C with a 5% carbon dioxide atmosphere in a humidified incubator.

All four cell lines were seeded at a density of 2500 cells per well in a 96-well plate and incubated under the previously described conditions to allow cells to adhere to the bottom of the wells. The cells were treated with the ethanol extract and the above mentioned four compounds at various concentrations through a series of serial dilutions. The final dosing regimen of this experiment is shown on Table 1. Following treatment, the cells were incubated under the same conditions for four doubling cycles. Cell proliferation was measured by an sulforhodamine B-based assay.[36] Dose–response curves were generated using SPSS. The half-maximal inhibitory concentrations (IC_{50}) of the ethanol extract and four compounds were determined for each of the cell lines through the dose–response curves. The experiments were performed for four technical repeats and two biological repeats.

Gene expression

The cells were treated with the ethanol extract and ergosterol peroxide at the calculated IC_{50} dosage. After four doubling cycles, RNA was isolated using an RNeasy Plus Mini Kit (Qiagen®). The RNA samples were converted to cDNA using a Quantitect Reverse Transcription Kit (Qiagen®), and the cDNA samples were diluted four times for use in the RT-qPCR assays. RT-qPCR assays were performed in accordance with the manufacturer's recommendations (ref. TaqMan® FA Mix manual). cDNA was amplified through a PCR process with the aid of TaqMan® Fast Advanced Master Mix (Applied Biosystems) and TaqMan® primer-sets (Applied Biosystems). Details of the twelve TaqMan® primer-sets used in this study are listed in Table 2. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *hypoxanthine phosphoribosyltransferase (HPRT)1* were selected as the housekeeping genes based on their cycle threshold (CT) values relative to the CT values of the genes of interest. Additionally, meaningful differential expression was set at a cut-off value of a 2-fold change in gene expression.[37]

AChE inhibition

The AChE inhibition assay was modified from Ellman and Benabent's methods,[38] by applying SDS to stop the reaction.[39] Briefly, sample (or standard), AChE, Buffer A, and DTNB were added to each well of the 96-well plates at volumes of 30 μL, 30 μL, 30 μL, and 50 μL, respectively. The plates were incubated for 10 min at 37 °C and the absorbance was then measured at 412 nm and was regarded as the initial reading (EnSpire Multimode Reader (PerkinElmer, MA, USA)). Subsequently, 30 μL of ATCI were added to the wells to initiate the reaction. The plates were incubated at 37 °C for 20 min. Lastly, 30 μL of SDS were pipetted into the wells to end the reaction. The absorbance was measured as before. This was regarded as the second reading.

$$A = \text{the second reading} - \text{the initial reading}$$

$$\text{AChE Inhibition } (\%) = ((A\ (\text{Blank}) - A\ (\text{Sample}))/(A\ (\text{Blank})) \times 100$$

Table 2 The assay IDs, target chromosome locations, and amplicon lengths of the TaqMan® primer-sets (Applied Biosystems) utilized in this study

Gene	Assay ID	Human target chromosome location	Amplicon length (bp)
Bax	Hs00180269_m1	Chr.19: 48 954 825–48 961 798	62
Bcl2	Hs00608023_m1	Chr.18: 63 123 346–63 319 778	81
CASP3	Hs00991555_m1	Chr.4: 184 627 696–184 649 475	118
CASP8	Hs01018160_m1	Chr.2: 201 233 443–201 287 711	124
CASP9	Hs00962278_m1	Chr.1: 15 491 401–15 524 912	84
GAPDH	Hs99999905_m1	Chr.12: 6 534 405–6 538 375	122
HPRT1	Hs99999909_m1	Chr.*X*: 134 460 145–134 500 668	100
IL24	Hs01114274_m1	Chr.1: 206 897 404–206 904 139	67
IRF1	Hs00971965_m1	Chr.5: 132 481 609–132 490 789	76
IL6	Hs00174131_m1	Chr.7: 22 725 889–22 732 002	95
TLR2	Hs02621280_s1	Chr.4: 153 684 080–153 710 643	112
NFkB1	Hs00765730_m1	Chr.4: 102 501 329–102 617 302	66

Table 1 The dosing regimen used in the anti-proliferation assay (LNCaP, DU145 and PC3)

Extract/compounds	1	2	3	4	5	6	7	8	9	10
EtOH ext (10^{-1} mg mL^{-1})	6.67	5.00	3.34	2.50	1.88	1.25	0.94	0.70	0.47	0.35
Hericenone C (μM)	8.20	6.15	4.10	3.08	2.05	1.54	1.03	0.77	0.51	0.38
Hericene B (μM)	30.00	22.50	15.00	11.30	7.50	5.60	3.80	2.80	1.90	1.40
Ergosterol (μM)	11.00	8.25	5.50	4.12	2.75	2.06	1.38	1.03	0.69	0.52
Ergosterol peroxide (μM)	39.70	29.78	19.85	14.89	9.92	7.44	4.96	3.72	2.48	1.86
EtOH control (%)	0.33	0.25	0.17	0.12	0.08	0.06	0.04	0.03	0.02	0.02

EtOH ext refers to the ethanol extract; EtOH control refers to the ethanol solvent control.

The IC_{50} values (concentrations of test samples that inhibit 50% of AChE) were determined by applying the two equations above. The activity of eserine was also measured as a positive control. To calculate the IC_{50} value, both the samples (3.2–20 mg mL^{-1}) and positive control (0.0625–1 μM) were analyzed at five different concentrations. IC_{50} values were obtained from dose-effect curves generated through linear regression.

Determination of antioxidant capacity

The antioxidant assay of Trolox equivalent capacity was used to measure the radical scavenging activities of the ethanol extract and compounds relative to the antioxidant reference Trolox. For each test below, a calibration curve was prepared by using Trolox as a standard ranging from 15.625 to 1000 μM. The results of these experiments were expressed as micromole Trolox equivalent per gram sample (μmol TE per g extract).

DPPH assay

The assay was performed in accordance with the method applied by Tang's group[40] with minor modifications. Briefly, 200 μl of DPPH solution (40 μg mL^{-1} in ethanol) and 10 μL of sample, standard, or ethanol (blank), were added to each well of a 96-well plate. The plate was incubated in the dark at room temperature for 60 min. DPPH scavenging activities of the ethanol extract, and compounds, were assessed by measuring the absorbance of DPPH at 517 nm (EnSpire Multimode Reader (PerkinElmer, MA, USA)).

FRAP

The FRAP assay was performed using the method modified from Benzie's report.[41] An amount of 200 μl of the radical FRAP reagent and 10 μL of sample, standard or ethanol (blank) was added to each well of a 96-well plate, which was incubated in the dark at room temperature for 60 min. The absorbance of the solution was measured at 593 nm.

ABTS

The ABTS assay was modified from Du's description.[42] Briefly, 200 μl of ABTS solution and 10 μL of sample, standard, or blank were added to each well of a 96-well plate. The plate was incubated in the dark at room temperature for 60 min. The decolorization caused by the radical scavenging activity was then measured at 734 nm.

Statistical analysis

All experiments were carried out in two biological repeats and at least three technical repeats, unless stated otherwise, and the results were expressed as the mean of replicates (including all the biological and technical repeats) ± pooled standard deviation (SD). The data were subjected to a one-way ANOVA performed with IBM SPSS Statistics 23. Differences between mean values were considered as statistical significance at $p < 0.05$ (*i.e.* to be inconsistent with the null-hypothesis).

Results and discussion

Inhibition of cell proliferation in LNCaP, DU145, PC3 and HEK293 cells

The ethanol extract and ergosterol peroxide showed differing levels of inhibition on cell proliferation. Anti-proliferative activities were also observed in response to hericenone C, hericene B and ergosterol, but were insufficient to reach the IC_{50} values. Table 3 shows the calculated IC_{50} values of the ethanol extract and tested compounds in the three PCa cell lines and the non-cancer control HEK293 cell line.

Relative to the other cell lines, the IC_{50} values of both the ethanol extract and ergosterol peroxide were the lowest in the LNCaP cells. Specifically, the IC_{50} values of the ethanol extract were 0.33, 0.23, and 0.19 mg mL^{-1} for the DU145, PC3 and LNCaP cells, respectively. As seen in Table 3, the ethanol extract had a greater impact on cell proliferation in HEK293 cells than in the PCa cells. Ergosterol peroxide showed a promising anti-proliferative effect, suggesting that this compound may be largely responsible for the anti-proliferative activities of the ethanol extract. Ergosterol peroxide has been reported to induce apoptosis in human colon cancer cell lines (HCT116, HT-29, and SW620) and in the human colorectal cancer cell line (DLD-1).[43] In a related study, the anti-proliferative activity of ergosterol peroxide was assessed in PC3 cells and reported as 38 μM.[44] This value is comparable to our current result (IC_{50} of 42 μM). Therefore, it is not unexpected for ergosterol peroxide to have greater anti-proliferative activities than the other compounds tested in this proliferation experiment of prostate cancer cell line.

Results show that both the ethanol extract and ergosterol peroxide were also showing anti-proliferative activities to the non-cancer control HEK293 cells (Table 3). This observation is unexpected. As such, to determine whether the molecular mechanism underlying the anti-proliferative activity of the three PCa cell lines also applies to the HEK293 cells, the RT-qPCR assay was also performed on HEK293 cells.

The HEK293 cell line is not a non-cancer prostate cell line, but was selected as the only non-cancer cell line currently available to us. In future studies, non-cancer prostate cells

Table 3 The IC_{50} values of LNCaP, DU145, PC3 and HEK293 cells treated with ethanol extract of *H. novae-zealandiae* and four compounds found in ethanol extract

Extract/compounds	DU145	PC3	LNCaP	HEK293
EtOH ext (mg mL^{-1})	0.33 ± 0.03	0.23 ± 0.02	**0.19 ± 0.02***	0.15 ± 0.02
Hericenone C (μM)	≫8.2	≫8.2	≫8.2	≫8.2
Hericene B (μM)	≫30	≫30	≫30	≫30
Ergosterol (μM)	≫11	≫11	≫11	≫11
Ergosterol peroxide (μM)	21 ± 3	42 ± 3	**15 ± 2***	24 ± 2

The results are expressed as mean value ± pooled SD; ≫ indicates that IC_{50} was not achieved; **bold** print indicates the strongest anti-proliferation in all three PCa cell lines; * indicates significant difference at $p < 0.05$ for cell observed as having the strongest anti-proliferation compared to the other cell lines; EtOH ext refers to the ethanol extract.

would serve as a better control. Examples of commercially available, non-cancer prostate cell lines include RWPE-1 and PNT-2, both of which have been used to assess anti-proliferative activities.[45]

Fig. 1 and 2 show the dose–response curves of the four cell lines treated with the ethanol extract and ergosterol peroxide, respectively. When treated with the solvent control (ethanol), the cells lines showed negligible responses. Thus, it can be inferred that the inhibitory activities of the ethanol extract were not attributed to the solvent content.

RT-qPCR

Table 4 shows the gene expression results of the 12 genes of interest following treatment of cells with the ethanol extract or ergosterol peroxide. These results suggest that the anti-proliferative activities of the ethanol extract and ergosterol peroxide are likely to be mediated through at least two different pathways; those of apoptosis and inflammation, both of which are related to cancer. According to Hanahan,[46] apoptosis is a hallmark of cancer, while inflammation is an enabling characteristic of cancer. Based on the results from the gene expression experiments, the apoptotic pathway is not influenced by the treatment of HEK293 cells with extract or the compounds under investigation. An increase of apoptosis in non-cancer cells is an undesirable property, therefore this result is ideal. Further investigation should be carried out to study the effect of ethanol extract and ergosterol peroxide on the gene expression of HEK293 cells, to define the mechanism underlying the anti-proliferative activity and to assess if they are suitable to be applied for cancer treatment in human.

The normalized relative quantities (NRQs) of all cell lines treated with ethanol extract and ergosterol peroxide are presented in Table 4. Results indicate that ethanol extract from *H. novae-zealandiae* and the compound isolated from the extract, ergosterol peroxide, both have good anti-proliferative effects toward the three PCa cell lines. As such, they have a potential to be considered as suitable candidates in prostate cancer treatment. Two possible mechanism could be applied to explain the anti-proliferative effect of the ethanol extract and ergosterol peroxide as detailed below.

Apoptotic pathways

As seen in Table 4, the expression of the caspase-encoding genes *CASP3*, *CASP8*, and *CASP9* were greatly upregulated in the PCa cell lines that were treated with the ethanol extract and ergosterol peroxide. An increase of *Bax* : *Bcl2* ratio was observed in the treated PC3 cells: 4.2 (using *GAPDH* as the normalisation gene) and 2.8 (using *HPRT1* as the normalisation gene) times increment for the ethanol extract compared to the solvent control; 4.7 (using *GAPDH* as the normalisation gene) and 2.7

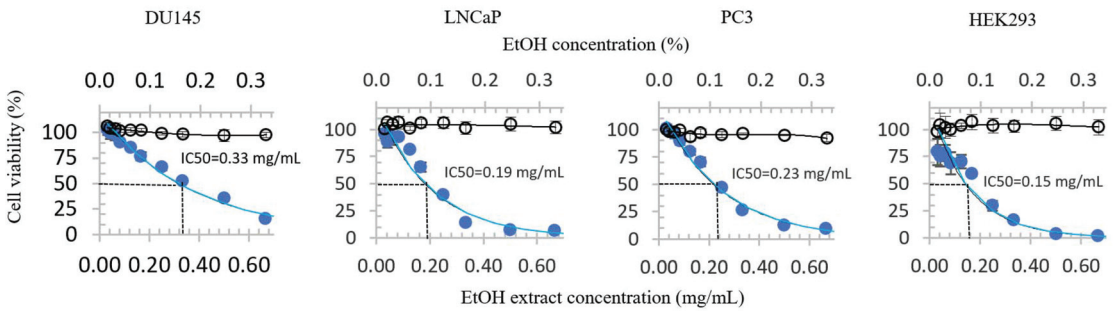

Fig. 1 Dose–response curves of ethanol extract in the DU145, LNCaP, PC3 and HEK293 cell lines. The blue circles and black circles represent the growth inhibitory properties of the ethanol extract and the solvent, ethanol, respectively. A lower IC_{50} value indicates a stronger anti-proliferative effect.

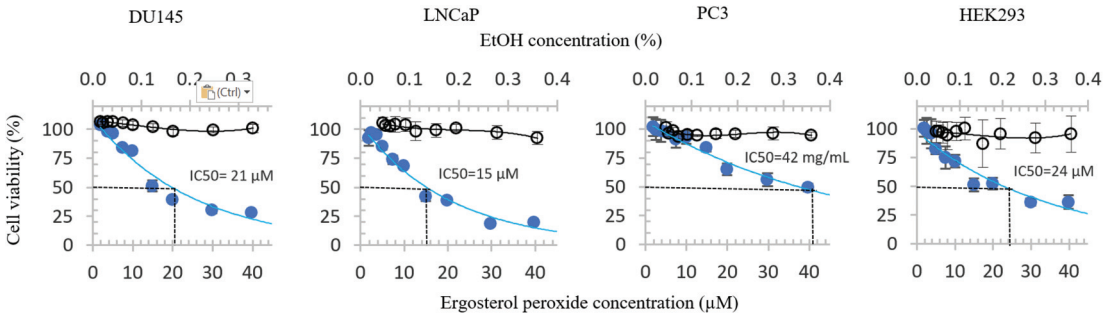

Fig. 2 Dose–response curves of ergosterol peroxide in the DU145, LNCaP, PC3 and HEK293 cell lines. The blue circles and white circles represent the growth inhibitory properties of ergosterol peroxide and the solvent ethanol respectively. A lower IC_{50} value indicates a stronger anti-proliferative effect.

Table 4 Normalised relative quantities of gene expression in cell lines after treatment with the ethanol extract of *H. novae-zealandiae* and ergosterol peroxide. Results for each of the housekeeping genes utilized are presented

	DU145		LNCaP		PC3		HEK293	
	GAPDH	*HPRT1*	*GAPDH*	*HPRT1*	*GAPDH*	*HPRT1*	*GAPDH*	*HPRT1*
The ethanol extract								
CASP3	1.69 ± 0.17	**3.15 ± 0.23***	1.78 ± 0.18	**2.41 ± 1.15***	1.44 ± 0.11	**2.10 ± 0.15***	1.25 ± 0.11	1.72 ± 0.20
CASP8	1.25 ± 0.11	**5.25 ± 0.59***	1.85 ± 0.19	**9.01 ± 1.11***	1.12 ± 0.13	0.79 ± 0.09	1.42 ± 0.15	0.89 ± 0.12
CASP9	0.72 ± 0.08	0.87 ± 0.10	1.73 ± 0.16	**2.95 ± 0.26***	0.89 ± 0.09	0.79 ± 0.09	0.63 ± 0.05	0.76 ± 0.08
Bax	1.26 ± 0.14	0.97 ± 0.10	1.31 ± 0.13	0.78 ± 0.09	1.31 ± 0.02	1.25 ± 0.14	0.72 ± 0.07	0.79 ± 0.08
Bcl2	1.14 ± 0.12	1.03 ± 0.09	1.21 ± 0.11	1.34 ± 0.12	**0.31 ± 0.02***	**0.45 ± 0.05***	1.04 ± 0.08	1.20 ± 0.11
IL24	**2.71 ± 0.22***	1.85 ± 0.17	x	x	**8.86 ± 0.61***	**7.49 ± 0.71***	x	x
IRF1	1.76 ± 0.09	1.36 ± 0.10	**3.01 ± 0.21***	**2.56 ± 0.21***	1.05 ± 0.09	**1.96 ± 0.18***	1.21 ± 0.10	1.15 ± 0.08
IL6	**0.11 ± 0.01***	**0.10 ± 0.01***	x	x	0.95 ± 0.09	0.67 ± 0.08	x	x
TLR-2	1.26 ± 0.11	1.45 ± 0.13	x	x	0.92 ± 0.10	1.28 ± 0.11	1.05 ± 0.09	0.98 ± 0.10
NFkB1	1.31 ± 0.12	1.11 ± 0.13	1.16 ± 0.08	1.19 ± 0.11	0.85 ± 0.07	1.24 ± 0.14	1.49 ± 0.16	1.38 ± 0.12
Ergosterol peroxide								
CASP3	1.29 ± 0.15	**2.96 ± 0.31***	1.51 ± 0.16	**2.09 ± 0.22***	1.37 ± 0.19	**3.38 ± 0.32***	1.71 ± 0.19	1.61 ± 0.18
CASP8	1.54 ± 0.16	**2.56 ± 0.18***	**2.01 ± 0.18***	**2.35 ± 0.17***	0.98 ± 0.10	0.82 ± 0.10	**2.21 ± 0.21***	1.36 ± 0.12
CASP9	0.63 ± 0.08	0.81 ± 0.09	1.75 ± 0.15	**2.52 ± 0.20***	0.95 ± 0.10	0.69 ± 0.08	0.62 ± 0.04	0.79 ± 0.07
Bax	0.78 ± 0.08	1.47 ± 0.14	0.98 ± 0.11	0.89 ± 0.10	1.42 ± 0.15	1.39 ± 0.15	1.00 ± 0.09	0.82 ± 0.09
BcL2	1.13 ± 0.12	1.42 ± 0.15	1.35 ± 0.12	1.17 ± 0.16	**0.30 ± 0.02***	**0.52 ± 0.05***	1.29 ± 0.12	1.41 ± 0.14
IL-24	**11.62 ± 1.21***	**4.95 ± 0.47***	x	x	**2.86 ± 0.21***	**2.89 ± 0.21***	x	x
IRF1	**2.15 ± 0.18***	1.24 ± 0.11	**3.25 ± 0.21***	**3.05 ± 0.31***	1.11 ± 0.10	**2.05 ± 0.18***	1.35 ± 0.12	1.29 ± 0.12
IL-6	**0.45 ± 0.04***	**0.49 ± 0.05***	x	x	1.45 ± 0.13	1.39 ± 0.15	x	x
TLR-2	**10.13 ± 0.85***	**7.28 ± 0.71***	x	x	**2.75 ± 0.21***	**3.86 ± 0.21***	**2.18 ± 0.20***	**4.27 ± 0.31***
NFkB1	0.90 ± 0.10	0.86 ± 0.09	0.97 ± 0.10	1.06 ± 0.07	**0.48 ± 0.05***	0.67 ± 0.07	1.41 ± 0.12	0.94 ± 0.10

The results are normalized to ethanol solvent control and are expressed as mean value ± pooled SD; **bold** print indicates mean values that have exceeded the differential expression threshold of 2-fold; x indicates that the gene primer was not amplified; * indicates significant difference at $P < 0.05$.

(using *HPRT1* as the normalisation gene) folds increase for ergosterol peroxide compared to the solvent control (Table 4).

Three inferences can be drawn from these results. Firstly, the caspase-mediated apoptosis pathway (Fig. 3a) is affected by the treatment with both the ethanol extract of *H. novae-zealandiae* and the ergosterol peroxide isolated from the extract. Secondly, the results imply that the mitochondrial apoptotic pathway (Fig. 3a) in PC3 may also be affected by the treatment. Finally, the results also suggest that ergosterol peroxide is likely to be a major compound responsible for the anti-proliferative activity of the ethanol extract mediated through this apoptotic pathway.

Research shows that activation of *CASP3* can trigger cell death through both inactivation of vital cellular proteins like poly-adenosine diphosphate-ribose polymerase (PARP), as well as activation of nucleases that trigger DNA fragmentation.[47] The 'initiator caspases' CASP8 and CASP9 may be activated either directly through cleavage of their respective procaspases by the ethanol extract/ergosterol peroxide, or through the cytochrome c produced in the mitochondrial apoptotic pathway. It has been theorized that these initiator caspases cleave and convert procaspase3 into CASP3, which is the active form of the protein.[48]

The mitochondrial apoptotic pathway is believed to regulate the caspase-mediated pathway through the activation of *CASP9*. Mitochondria release cytochrome c that triggers cell death by cleaving procaspase9 into CASP9, which would trigger the pathway explained above. However, the release of cytochrome c from the mitochondria is thought to be regulated by the Bcl2 family of proteins, including Bcl2 and Bax. While *Bcl2* has been reported to play an anti-apoptotic role by blocking the release of cytochrome c, *Bax* is thought to play a pro-apoptotic role by forming heterodimers with *Bcl2* and altering its activities.[49] As such, the results of *Bax* and *Bcl2* expression levels are often reported as a ratio.

Anti-inflammatory pathway

The results in Table 4 show that expression of the pro-inflammatory cytokine-encoding gene *IL6* was downregulated in the treated DU145 cells. Furthermore, the expression of the anti-inflammatory cytokine-encoding gene *IL24* was upregulated in the treated DU145 and PC3 cells. Current theory holds that the toll-like receptor 2 (*TLR2*) -mediated inflammatory pathway (Fig. 3b) is one of the pathways that can regulate the expression levels of *IL6* and *IL24*. However, it was found that *TLR2* expression was unaffected by supplementation with the ethanol extract (Table 4), but was upregulated in cells that were treated with ergosterol peroxide. Nonetheless, *NFkB1* expression in the same cells was unaffected by either treatment; this means that treatment with ergosterol peroxide upregulates the expression of *TLR2* in PCa cells, but potentially 'blocks' its cascade signaling pathway. This blockade is likely to be the cause of the down-regulation of *IL6* expression. A possible cause for the inhibition of the *TLR2*-induced *NFkB* activation may be due to an increased *IRF1* expression.[50] The mechanisms of how the ethanol extract and ergosterol peroxide up-regulate *IRF1* expression requires further investigation. Ultimately, it should be noted that inflammation is not

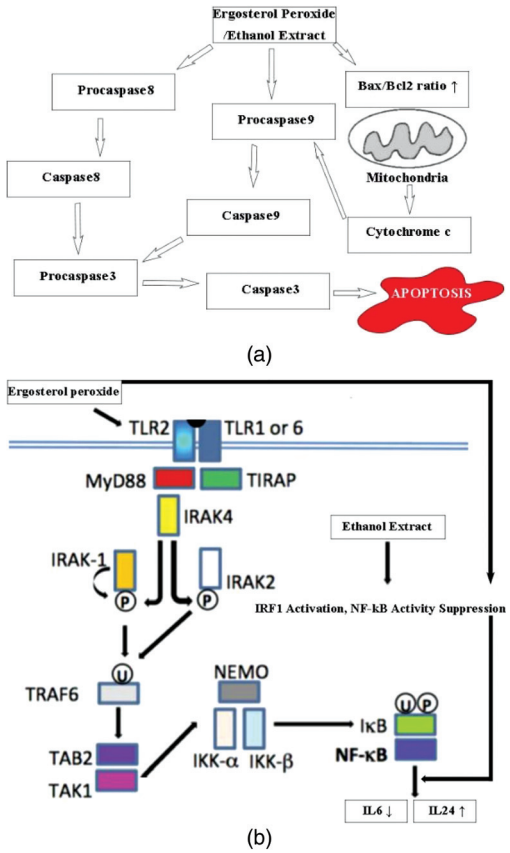

Fig. 3 (a) Proposed mechanism of the ethanol extract/ergosterol peroxide-induced apoptosis in PCa cells, based on the results of present study. (b) Proposed mechanism of the ethanol extract/ergosterol peroxide-induced anti-inflammatory response in PCa cells, based on the results of present study. The figure was modified from Oliveira-Nascimento *et al.*[51]

directly related to cancer, but it governs many pathways related to various hallmarks of cancer.[46]

AChE inhibition

It was observed that the ethanol extract exhibited AChE inhibition in a concentration-dependent manner. The AChE inhibition of the ethanol extract increased from 9.11% to 40.34% with its concentration increased from 0.48 mg mL^{-1} to 3.00 mg mL^{-1}. The results of the AChE inhibitory assay are shown in Table 5. The IC_{50} value of the positive control (eserine) was found to be 0.073 μM, which was comparable with the values reported by several authors, such as 0.04 μM[52] and 0.025 μg mL^{-1} (equivalent to 0.091 μM).[53]

The activity of the ethanol extract and several compounds in acetylcholine accumulation was indirectly assessed *via* their ability to inhibit AChE. The screening confirmed that the ethanol extract exerted a weak AChE inhibitory activity, with an IC_{50} values of 5.19 mg mL^{-1}. Furthermore, it was found that the four compounds hericenone C, hericene B, ergosterol and ergosterol peroxide did not exhibit any meaningful inhibitory effects on AChE even at concentrations as high as 26, 26, 38, 35 μM as shown in Table 5. Thus, these four compounds are not promising candidates for inhibiting AChE activities. Ultimately, it implies that the above compounds from *H. novae-zealandiae* may not be useful for neuroprotection as hypothesized or that the role of *H. novae-zealandiae* in neuroprotection, if indeed it plays such a role, is not mediated through the AChE inhibitory pathway.

Antioxidant activities

The antioxidant activities of the ethanol extract, hericene B, ergosterol and ergosterol peroxide were assessed using three antioxidant assays. Hericenone C was not assessed due to limited amount of compound obtained.

Concentration-dependent results were observed for the ethanol extract in all the three assays. In the DPPH assay, the antioxidant activities of the ethanol extract increased from 15.83 to 141.67 μM TE per g whey its concentration increased from 0.5015 to 5.0150 mg mL^{-1}. In the FRAP and ABTS assays, the values increased from 14.83 to 109.67 μM TE per g and 29.29 to 148.57 μM TE per g, respectively. Table 5 shows the DPPH, FRAP, and ABTS scavenging activities of the ethanol extract and the three compounds of interest. For the ethanol extract, the ABTS assay generated the highest antioxidant capacity (58.87 μmol TE per g) than the other two assays. The DPPH assay showed the second highest antioxidant capacity with a value of 34.45 μmol TE per g, followed by the FRAP assay (29.57 μmol TE per g). In the case of the three tested compounds, no antioxidant effects were observed at the concentrations of 340 μM, 300 μM and 280 μM for hericene B, ergosterol, and ergosterol peroxide, respectively. Their antioxidant activities were weaker than the minimal concentration of Trolox (15.625 μM). This implies that these three com-

Table 5 AChE inhibitory and radical scavenging activities of ethanol extract and compounds isolated from *H. novae-zealandiae*

Bioactivities	EtOH ext	Hericenone C	Hericene B	Ergosterol	Ergosterol peroxide
AChE inhibition (IC_{50})	5.19 ± 0.49 mg/mL	≫26 μM	≫26 μM	≫38 μM	≫35 μM
DPPH (μmol TE per μmol)	34.45 ± 1.12 μmol TE/g	x	<0.044	<0.052	<0.056
FRAP (μmol TE per g)	29.57 ± 0.90	x	<0.044	<0.052	<0.056
ABTS (μmol TE per g)	58.87 ± 1.78	x	<0.044	<0.052	<0.056

All values are reported as means ± polled SD (n = 6). Antioxidant activity was expressed as micromole Trolox equivalents per gram of extract/compounds (μmol TE per g). x indicates that the experiment was not performed due to limited amount of available compound; ≫ indicates that activity was not achieved as the value would be far above the concentration shown.

pounds may not contribute to the antioxidant capacity of the ethanol extract. However, it is possible that the antioxidant activity of the ethanol extract is contributed to by the synergy of these compounds or by some other constituents yet to be identified. Ultimately, further study is required to identify the active compounds responsible for the antioxidant activity of the ethanol extract.

We have applied three of the frequently used methods, namely DPPH, FRAP, and ABTS to determine antioxidant activity as each method has specific mechanisms and pH scope. For example, the FRAP and DPPH assays were carried out at acidic and neutral pH respectively. This is potentially meaningful as the reducing capacity may be suppressed at acidic conditions due to protonation of the antioxidant components, or conversely it may be enhanced in alkaline conditions due to dissociation of phenolic constituents.[54] As such, a single antioxidant activity assessment method is not sufficient and more than one type of antioxidant capacity measurement needs to be performed to account for these variables.

The results across the DPPH, FRAP, and ABTS experiments were consistent with respect to trend. Thus, it is reasonable to conclude that the ethanol extract has antioxidant potential. Nevertheless, it is worth mentioning that all the selected antioxidant assays involved *in vitro* chemical reactions, which cannot account for the complexities of the biological systems. The results, therefore, should be interpreted with caution. Consequently, the antioxidant activity of the ethanol extract should be further validated beyond chemical reaction models, such as by performing an oxidative stress biomarker assay. This would facilitate further investigation into any possible impact given by the ethanol extract of *H. novae-zealandiae* on oxidative stress and its implication for oxidative-related diseases.

Limitations and future study

We acknowledge that our preliminary study has limitations. Firstly, a positive control should be included in the cell proliferation assay once the other bioactive compounds from the species have been identified and purified. In addition, the apparent cytotoxicity of the ethanol extract/ergosterol peroxide on the HEK293 cells should be further investigated. While similar effects have been observed in at least one anticancer drug, etoposide, on a non-cancer cell line,[55] further understanding on the potential role of the ethanol extract/ergosterol peroxide in cancer treatment is required. Although the SRB assay is a cost effective method for rapidly screening the cytotoxicity of extracts/compounds, it cannot be used to distinguish if the extract is cytotoxic or cytolytic. Since being cytolytic is the ideal mechanism, we recommend that cell cycle analysis be performed in future study.

Furthermore, to avoid misinterpretation, it is noted that the effect of both the ethanol extract and ergosterol peroxide on the proposed pathways were identified at the genetic level. The changes in gene expression should be confirmed at the protein level, such as through western blots. Additionally, since our results strongly imply that caspase-mediated apoptosis is one of the potential pathways underlying the anti-proliferative activity of the extracts/ergosterol peroxide, we suggest further validation be performed. Various methods for validating the apoptosis pathway exist, including using annexin V staining, cell cycle analysis (flow cytometry), and use of a caspase activity assay to confirm and expand on present findings.

Conclusions

This publication is, to the best of our knowledge, the first scientific report on bioactivities of lipophilic constituents of *H. novae-zelandiae*. Both the ethanol extract and the four tested compounds, namely hericenone C, hericene B, ergosterol and ergosterol peroxide identified from this fungus, were assessed for their anti-proliferative activity, AChE inhibition, and antioxidant potential. The ethanol extract exhibited suppression of cell proliferation in DU145, LNCaP and PC3 cell lines with the lowest IC_{50} value of 0.28 mg mL^{-1} in LNCaP. The strongest anti-proliferative activity was seen following the treatment of LNCaP (IC_{50} = 15 μM) with the compound ergosterol peroxide. The two possible pathways underlying this activity are thought to be apoptosis and anti-inflammation, indicated by changes in the expression of *CASP3*, *CASP8*, *CASP9*, *Bax*/*Bcl2*, *IL6* and *IL24*. It should be noted that anti-inflammatory effects might not be directly responsible for the anti-proliferative effects, yet it may suppress the signaling pathway of various cancer hallmarks. Weak AChE inhibition and antioxidant activities were observed from the ethanol extract of *H. novae-zealandiae.* However, these two activities were not due to any of the four tested compounds. The results presented here support the hypothesis that *H. novae-zealandiae* inhibits, to some extent, PCa cell proliferation, AChE and oxidation. As this is the first report on the bioactivities of *H. novae-zealandiae* extracts, further *in vitro* studies are required before moving onto *in vivo* experiments. However, current findings do suggest that *H. novae-zealandiae* may have potential health benefits and enhance cancer treatments.

Author contributions

The listing authors contributed to this work as follows: Siew Young Quek, Karen Suzanne Bishop and Zhixia (Grace) Chen are responsible for conceptualization. Zhixia (Grace) Chen is responsible for data curation and formal analysis. Chris Smith is responsible for species identification and writing review. Zhixia (Grace) Chen and Hartono Tanambell are responsible for the original draft preparation. Karen Suzanne Bishop, Peter Buchanan and Siew Young Quek are responsible for supervision and writing—review and editing. Siew Young Quek was responsible for securing the funding for this work. All authors approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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