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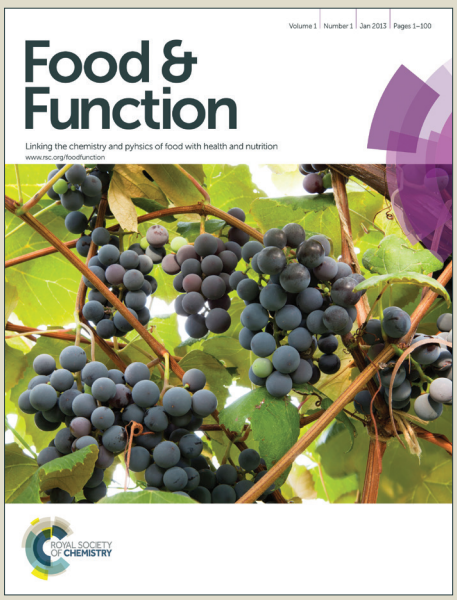

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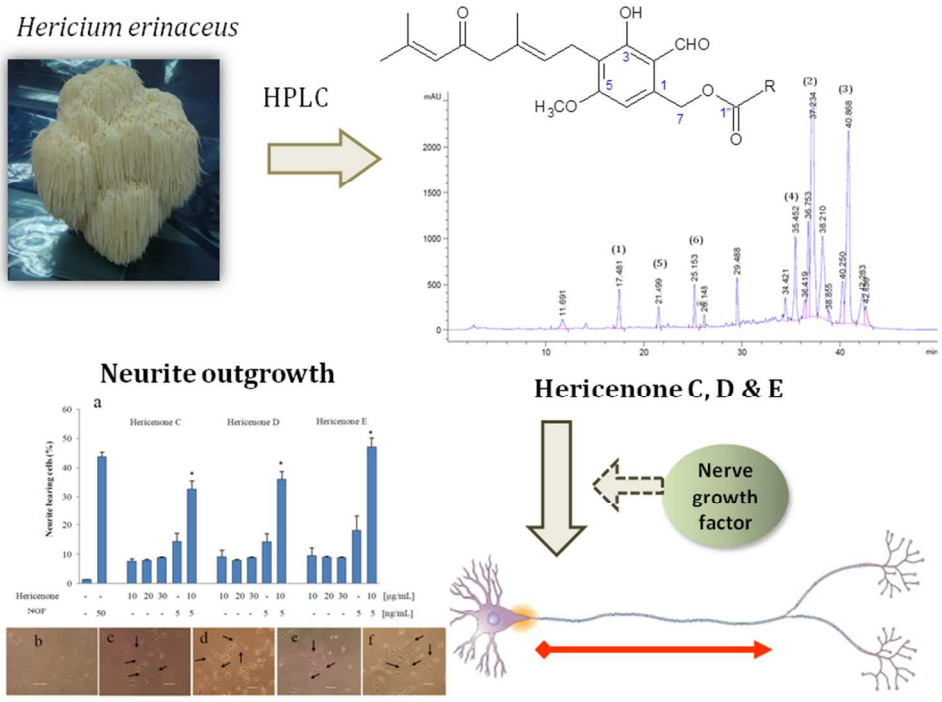

Graphical Abstract
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Food & Function

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PAPER

Hericium erinaceus (Bull.:Fr) Pers. cultivated in tropical conditions: Isolation of hericenones and demonstration of NGF-mediated neurite outgrowth in PC12 cells *via* MEK/ERK and PI3K-Akt signaling pathways

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Chia-Wei Phan, [a, b] Guan-Serm Lee,[a,b] Sok-Lai Hong,[a,b] Yuin-Teng Wong,[a,b] Robert Brkljača,[c] Sylvia Urban,[c] Sri Nurestri Abd Malek[a, b] and Vikineswary Sabaratnam[*,a, b]

Hericium erinaceus (Bull.: Fr.) Pers. is an edible and medicinal mushroom used traditionally to improve memory. In this study, we investigated the neuritogenic effects of hericenones isolated from *H. erinaceus* and the mechanisms of action involved. *H.erinaceus* was cultivated and the secondary metabolites were elucidated by high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR). The secondary metabolites were tested for neurite outgrowth activity (if any). Rat pheochromocytoma cell (PC12) cells were employed and the nerve growth factor (NGF) level was also determined. The signaling pathways involved in the mushroom-induced neuritogenesis were investigated by using several pharmacological inhibitors. Hericenones B-E (**1-4**), erinacerin A (**5**) and isohericerin (**6**) were isolated from the basidiocarps of *H. erinaceus*. The hericenones did not promote neurite outgrowth but when induced with a low concentration of NGF (5 ng/mL), the neuritogenic activity was comparable to that of the positive control (50 ng/mL of NGF). Hericenone E was able to stimulate NGF secretion which was two-fold higher than that of the positive control. The neuritogenesis process was partially blocked by the tyrosine kinase receptor (Trk) inhibitor, K252a; suggesting that the neuritogenic effects was not solely due to NGF. Hericenone E also increased the phosphorylation of extracellular-signal regulated kinases (ERKs) and protein kinase B (Akt). Taken together, this study suggests that hericenone E potentiated NGF-induced neuritogenesis in PC12 cells *via* MEK/ERK and PI3K/Akt pathways.

Introduction

Nerve growth factor (NGF) plays a pivotal role in supporting neuronal survival, inducing differentiation and neuroregeneration.[1] The most notable role of NGF is to maintain the functionality of cholinergic neurons of the basal forebrain complex (BFC). The neurons in BFC are important for memory, consciousness, learning, and attention. Since BFC neurons degenerate during the progression of Alzheimer's disease (AD), NGF has been targeted as the first line treatment/protection agent against AD. Besides that, NGF has been shown to counteract another two major hallmarks of AD, i.e. tau hyperphosphorylation and beta-amyloid neurotoxicity.[2]

The most difficult challenge of NGF-intended treatment is its mode of delivery to the brain. First, being a large polypeptide molecule, NGF does not cross the blood brain barrier when peripherally administered.[3] For this reason, intra-cerebro-ventricular (ICV) way of NGF delivery has been pursued but it often causes adverse effects like pain and weight loss in patients.[4] Furthermore, NGF must be delivered in sufficient quantities in order to stimulate neurite outgrowth. Therefore, the use of an NGF-potentiating substance with a low molecular weight, or a small chemical that can mimic the effect of NGF has been proposed to be a promising alternative strategy to overcome the shortage of NGF.

Research on the health benefits of natural products and especially as an integral part of the human diet in disease prevention has dramatically increased in the recent years. It is now well established that neuroactive compounds from natural resources, especially mushrooms, can also provide neuroprotective effects and potentially reduce the risk of developing age-related neurodegenerative diseases such as AD.[5,6] We recently discovered several ethnopharmacologically important mushroom species that showed neuritogenic properties across various neuronal cells *in vitro*. Such mushrooms include the morning glory mushroom, *Pleurotus*

[a] *Mushroom Research Centre, University of Malaya, 50603 Kuala Lumpur, Malaysia.*viki@um.edu.my; Tel: +603-7967 4349; Fax: +603-7967 6703*
[b] *Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[c] *School of Applied Sciences (Discipline of Chemistry), Health Innovations Research Institute (HIRi), RMIT University, GPO Box 2476V Melbourne, Victoria 3001, Australia*

giganteus,[7] tiger's milk mushroom, *Lignosus rhinocerotis*;[8] and purple Reishi, *Ganoderma neo-japonicum*.[9] Preliminary toxicity assessment showed that these mushroom extracts did not exert undesirable cytotoxicity against cultured embryonic fibroblasts and neuroblastoma cells.[10]

Hericium erinaceus (Bull.: Fr.) Pers. (lion's mane mushroom, monkey head mushroom or hedgehog mushroom) is a very popular edible and medicinal mushroom and it remains the prime choice for neuronal health promoting activities. It is a temperate mushroom and is very popular in China and Japan. *Hericium erinaceus* has also been reported to have wound healing properties, anti-oxidant, anti-hypertensive, anti-diabetic, among other therapeutic potentials.[11] Extracts of the tropical grown *H. erinaceus* had neuroprotective[12] and neuroregenerative effects[13]. It was shown that an aqueous extract of *H. erinaceus* fresh basidiocarps was able to promote neurite outgrowth and nerve regeneration; and accelerate motor functional recovery of rodents *in vivo* after crush injury.[14,15]

In the early 1990's, hericenone C, D and E from *H. erinaceus* had been reported to stimulate NGF synthesis in mouse astroglial cells [16]. However, the effect of hericenones on neurite outgrowth is poorly understood, let alone the detailed mechanisms by which these agents work to induce neuritogenesis. Furthermore, the chemical components reported are mainly from basidiocarps grown at low temperatures (18- 24°C). It has been a decade since this mushroom was successfully domesticated and grown in the tropical climate in Malaysia. The extracts of these basidiocarps have been shown to have neurite outgrowth stimulatory activities.[12,17] However, the chemical components for the activities have not been investigated. The objectives of this study were to (a) elucidate the chemical profiles of the *H. erinaceus* basidiocarps grown in Malaysia and (b) to screen the isolated compounds for neurite outgrowth activity using rat pheochromocytoma (PC12) cells. We selected the PC12 cell line as it has been extensively employed as a model system to study neuritogenesis *in vitro*. Further the signaling pathways involved were investigated by using several pharmacological inhibitors. Our hypothesis is that the bioactive components in basidiocarps of *H. erinaceus* grown in tropical temperatures can interact with NGF to enhance neurite outgrowth by up-regulating common pathways linked to the induction of NGF signaling for example TrkA-associated tyrosine kinase (TrkA), which leads to a sustained activation of extracellular signal-regulated kinases (ERKs) and phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) necessary for neurite outgrowth.

Materials and methods

Mushroom species

Fresh basidiocarps (fruiting bodies) of *H. erinaceus* were obtained from Ganofarm Sdn. Bhd., Tanjung Sepat, Malaysia (2°39'42.7"N 101°33'36.4"E). A voucher specimen of the mushroom (KLU-M 1232) was deposited in the Herbarium of University of Malaya.

Chemicals and reagents

Nerve growth factor (NGF) from murine submaxillary gland, U0126, PD98059, LY294002 and K252a were purchased from Sigma (St. Louis, MO, USA). Gradient grade solvents, ethanol, hexane, ethyl acetate, HPLC grade methanol (MeOH) and acetonitrile (CH_3CN) were purchased from Merck (Germany). Water (H_2O) was obtained from Milli-Q Type 1 (Ultrapure) water coupled with Millipak filter (0.22 μm). All solvents were filtered through a membrane filter (0.45 μm, Sartorius) before use in the HPLC system.

Cultivation of *H. erinaceus*

A mycelium culture of *H. erinaceus* was maintained on potato dextrose agar (PDA) at 4-8°C and subcultured regularly. Polypropylene bags were used for bagging. The substrate formulation was as follows (% w/w): rubber wood sawdust (88), rice bran (10), and calcium carbonate (2). Moisture content was kept at 65%. After spawn inoculation, full colonization of the bags occurred in about 50-60 days at a temperature and humidity at 27-34°C and 80-90%; respectively. Fruiting occurred around 100-120 days from the day of inoculation. Formation of pinhead was observed 10-12 days after opening the bags and the basidiocarps grew to its maturity after 7-10 days. Fully grown basidiocarps with the formation of spine-like structures were harvested. Each bag (containing one kg of substrate) was expected to flush 3-4 flushes and a total yield of 300-350 g of fresh basidiocarps were obtained per bag. Biological efficiency was 60-75% based on dry weight of sawdust used.

Extraction and fractionation

Fresh basidiocarps of *H. erinaceus* (1.3 kg) were freeze-dried, blended and stored at -20°C prior to assay. A total of 200.0 g of powdered freeze-dried basidiocarps of *H. erinaceus* was soaked in 80% (v/v) aqueous ethanol for three days at room temperature. The solvent containing extract was decanted, filtered and then concentrated under vacuum using a rotary evaporator. The residue was re-soaked in 80% (v/v) aqueous ethanol and the extraction and filtration process was repeated three times. The crude ethanol extract (52.3 g) obtained was successively fractionated with hexane, ethyl acetate and water. All the fractions (hexane, ethyl acetate and water) were filtered separately and concentrated under vacuum using a rotary evaporator to give hexane (3.8 g), ethyl acetate (0.7 g) and aqueous fractions (44.3 g). Both hexane and ethyl acetate fractions were found to exhibit similar activities after preliminary screening. These fractions were combined and subjected to flash column chromatography to yield 7 fractions, which were E1 (384.0 mg), E2 (780.8 mg), E3 (438.2 mg), E4 (62.4 mg), E5 (39.7 mg), E6 (183.1 mg) and E7 (1068.2 mg). Fraction E2 was selected for further investigation as it showed the highest enhancement of neurite outgrowth. Fraction E2 (based on neurite outgrowth activity) was thus subjected to HPLC analysis for isolation of pure compounds.

High performance liquid chromatography (HPLC) analysis

HPLC was performed on a Agilent 1260 infinity HPLC system consisting of a quaternary pump equipped with a 1260 autosampler (ALS), 1290 thermostat, 1260 thermostatted column compartment (TCC), 1260 diode array detector (DAD VL+), 1260 fraction

collector (FC-AS) and Agilent OpenLAB CDS Chemstation for LC software. The analytical scale analysis was carried out using a binary eluent of chromatographic grade MeOH and ultrapure H_2O under the following gradient conditions: 0 to 5 minutes isocratic 65% MeOH; 5 to 30 minutes linear gradient from 65 to 100% MeOH; 30 to 40 minutes isocratic 100% MeOH; 40 to 50 minutes linear gradient from 100 to 65% MeOH at a flow rate of 1.0 mL/min. The column used was a ZORBAX Eclipse XDB-C18 (4.6 x 250 mm, 5 µm) and the temperature was set to 30°C. Fraction E2 was prepared to a concentration of5.0 mg/mL in methanol and filtered through a membrane filter (0.45 µm, Sartorius). The sample (5.0 µL) was injected onto the column and peaks were detected by monitoring the UV absorbance at 214 nm. Subsequently fraction E2 was prepared at a concentration of 40.0 mg/mL in methanol and from this an100.0 µL aliquot was injected onto the semi-preparative column, ZORBAX Eclipse XDB-C18 (9.4 x 250 mm, 5 µm) operating with a flow rate of 4.18 mL/min. Selected peaks in the resultant chromatogram were repeatedly collected using a fraction collector. Similar fractions from each round of separation were combined and the mobile phases were evaporated using a rotary evaporator at 40°C.

Liquid chromatography-mass spectrometry (LC-MS) analysis

ESI mass spectra of the isolated compounds were obtained on a Micromass Platform II mass spectrometer equipped with a LC-10AD Shimadzu solvent delivery module (50% CH_3CN/H_2O at a flow rate of 0.2 mL/min) in both the positive and negative ionisation modes using cone voltages between 20 and 30 V.

Nuclear magnetic resonance (NMR) analysis

^1H (500 MHz) and ^{13}C (125 MHz) spectra of the isolated compounds were acquired in $CDCl_3$ on a 500 MHz Agilent DD2 spectrometer with referencing to solvent signals (δ 7.26 and 77.0 ppm). Two-dimensional NMR experiments recorded included gCOSY, HSQCAD, and gHMBCAD experiments.

Cell culture

Rat pheochromocytoma cell (adherent variant, PC-12Adh) was purchased from American Type Culture Collection (ATCC; MD, USA). The cells were cultured in F-12K medium (Sigma) supplemented with 2.5% (v/v) heat-inactivated fetal bovine serum (PAA), 15% (v/v) horse serum (PAA), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37°C in a humidified environment of 5% CO_2 and 95% air and were routinely passaged every 3-4 days.

Neurite outgrowth assay

PC12 cells were seeded at a density of 5×10^3 cells in growth medium per well in 24-well culture plates and incubated overnight. Compounds isolated from *H. erinaceus* were added to the cells and further incubated for 3 days. Cells treated with nerve growth factor (50 ng/mL) were used as a positive control. After 3 days, the cells were examined using an inverted light microscope (Nikon Eclipse TS100). Five random fields (200-300 cells/well) were examined in each well. The number of neurite outgrowth (axon-like processes), defined as extensions longer than twice the cell body diameter, was recorded. Neurite length was measured using Image-Pro Insight image analysis software (MediaCybernetics, MD, USA). The mean number of neurite-bearing cell was quantified by scoring the total number of neurite-bearing cells over the total number of viable cells per field. Three independent experiments were conducted for each compound.

Treatment with specific inhibitors of signaling pathways

MAPK inhibitor (U0126, PD98059), PI3K inhibitor (LY294002) and TrkA inhibitor (K252a) were prepared in DMSO and stored at -20 °C in the dark. Each of the inhibitors [i.e. U0126 (10 µM), PD98059 (40 µM), LY294002 (50 µM), and K252a (100 nM)] was prepared by diluting it in medium before use. PC12 cells were either incubated with or without inhibitors for 1 h. The cells were then added with respective compounds for 3 days prior to scoring neurite bearing cells and ELISA assay.

Measurement of NGF levels

ChemiKine™ NGF sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit (Merck Millipore, Germany) was used to determine the NGF concentration in the cell-free culture medium. PC12 cells were incubated with hericenone C-D with or without NGF (5 ng/mL) for 3 days. The culture medium was then collected and added into a microplate pre-coated with sheep anti-mouse NGF polyclonal antibody. Anti-mouse NGF monoclonal antibody was then added to each well as a detection antibody. After 2 h, horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG polyclonal antibody was added. The samples were incubated for 30 mins after which the TMS solution was added and the colour intensity of the sample was measured at 450 nm. The level of NGF was determined from a standard curve plotted with known concentrations of NGF (r^2= 0.9741-0.9899)

Measurement of phospho-ERK (Thr202/Tyr204) and phospho-Akt levels

The compounds were added to PC12 cells with or without pretreatment with specific inhibitors. At indicated time points, the cells were harvested and lysates were prepared. To harvest the cells in a non-denaturing condition, the cells were rinsed with ice-cold PBS after which 0.5 mL of ice-cold cell lysis buffer (Cell Signaling Technology, Inc) with 1 mM of phenylmethylsulfonyl fluoride (PMSF) (Sigma) was added. The cell lysis buffer (1X) consists of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA,1 mM EGTA 1% Triton, 2.5 mM sodium pyrophosphate,1 mM b-lycerophosphate, 1 mM Na_3VO_4, and 1 µg/ml leupeptin. The cells were scraped off, sonicated on ice, and microcentrifuged at 4°C. The cell lysates were incubated in microwells pre-coated with phospho-ERK (Thr202/Tyr204) rabbit antibody and Akt (Thr308) antibody; respectively. Following washing, detection antibody was added. Anti-rabbit IgG, HRP-linked antibody was then added to recognize

the bound detection antibody. HRP substrate, TMB, was then added for colour development. Absorbance at 450 nm was recorded and the magnitude of absorbance is proportional to the quantity of phospho-ERK (Thr202/Tyr204), and phospho-Akt (Thr308); respectively.

Statistical analysis

Results were expressed as a mean ± standard deviation (SD) (n=3). Analysis of variance (ANOVA) followed by Duncan's test was performed to test for differences between means by employing Statgraphics Plus for Windows 7.0 (Statistical Graphics Corp., Herndon, VA). The statistical significance of mean differences was based on a p value of < 0.05.A Pearson correlation test was also conducted to study the relationship between the neurite-bearing percentage, concentrations of hericenone E, and NGF levels. The Pearson correlation coefficient (r_p) ranged between −1 (strong negative correlation) to +1 (strong positive correlation), and these were calculated by using SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA).

Results and discussion

Isolation and identification of secondary metabolites

The semi-preparative HPLC chromatogram of fraction E2 of *H. erinaceus* showed the presence of eight (8) major peaks at retention times of 17.481, 21.499, 25.153, 29.488, 35.452, 37.234, 38.210 and 40.868 minutes respectively (Fig. 1a). Each peak was collected using a fraction collector and the eluting solvent was evaporated using rotary evaporator. The fractions corresponding to the various peaks were subjected to LCMS and NMR analysis. Six (6) of the isolated compounds were identified as hericenones B (1.3 mg), C (22.4 mg), D (13.9 mg) and E (16.9 mg) (corresponding to peaks **1** to **4**, with retention times 17.481, 37.234, 40.868 and 35.452 minutes, respectively), erinacerin A (0.8 mg) (peak **5**, retention time 21.499 minutes) and isohericerin (0.7 mg) (peak **6**, retention time 25.153 minutes). The remaining two isolated compounds corresponding to peaks at retention times 29.488 and 38.210 minutes (Fig. 1a) could not be identified due to the insufficient quantities isolated and their lack of purity. Compounds **1-6** (Fig. 1b) were identified by comparison of their mass and NMR spectra with published data. [16,18–20]

H. erinaceus is known to be a temperate rather than a tropical mushroom. It requires relatively low temperatures (18-24°C) for fruiting to occur.[21] However, in Malaysia, given the tropical geographical zone with temperatures recorded at 27-34°C in the low land, *H. erinaceus* was also successfully domesticated and cultivated. To the best of our knowledge, this is the first report of the isolation of secondary metabolites (specifically the hericenones) from the basidiocarps of *H. erinaceus* cultivated in Malaysia. Hericenone B which is a phenol derivative was reported to exhibit cytotoxicity effects against HeLa cells.[18] Hericenone B was also a promising preventive or therapeutic agent of thrombosis and vascular diseases as it potently inhibited platelet aggregation induced by collagen.[22] Hericenones C-E have been reported to be effective stimulators of NGF synthesis.[16] There was, however, no report on the biological activity of erinacerin A. Further, isohericerin, which is an isoindolinone alkaloid was reported to be cytotoxic to various cancer cell lines .[23]

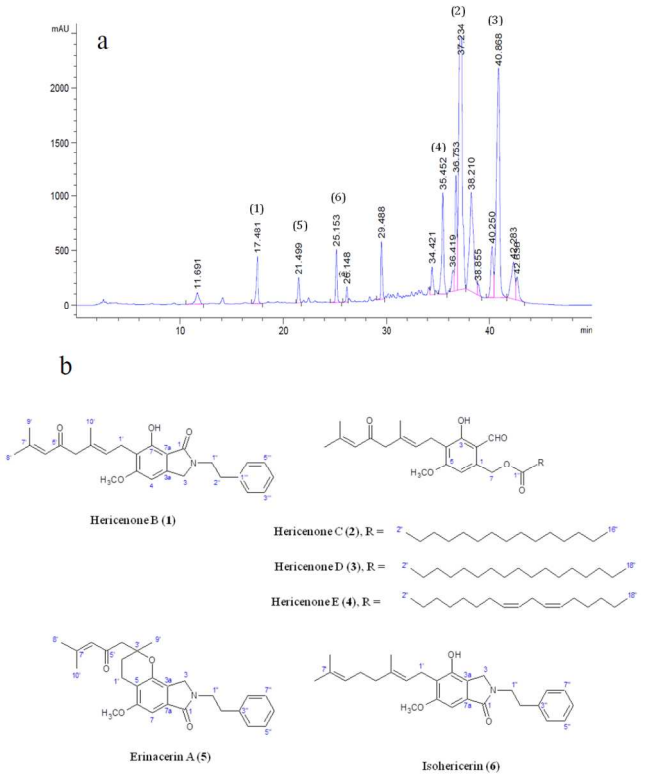

Fig. 1 (a) Semi-preparative HPLC chromatogram of fraction E2 of *H. erinaceus* at UV wavelength 214 nm showing the six secondary metabolites identified. **(b)** Structures of compounds 1-6.

Hericenones C, D and E potentiated NGF-induced neurite outgrowth in PC12 cells

Hericenones C-E were selected to be tested on neurite outgrowth assay as it is well documented that these compounds enhanced neuritogenesis.[16,24] PC12 cells treated with 50 ng/mL of NGF were used as a positive control as previously reported.[8] The neurite bearing score for NGF (50 ng/mL)-treated cells was 43.8 ± 1.6% (Fig. 2a). The ability of hericenones C-E to induce neurite outgrowth without the presence of NGF was tested. Hericenones alone (10–30 µg/mL) did not trigger neurite outgrowth as reflected by the low neurite bearing scores, i.e. 7.7% - 9.2%. Neurite outgrowth activity with the presence of both NGF and hericenones was then tested. By varying the concentration of NGF from 1 to 5 ng/mL, the percentage of neurite-bearing cells increased in a dose-dependent manner (data not shown). In the presence of 5 ng/mL NGF, hericenones C-E (10 µg/mL) significantly ($P < 0.05$) increased the percentage of neurite-bearing cells when compared to control cells which had either hericenones alone or 5 ng/mL of NGF (Fig. 2a). Hericenone E, in particular enhanced neurite outgrowth by 47% when compared to cells cultured in medium only (Fig. 2a). This showed that hericenones are neurotrophic compounds that potentiate NGF-induced neurite outgrowth in PC12 cells. Fig. 2b-f shows the morphological changes of PC12 cells at day-3 of different

treatments. Phenotypic changes associated with NGF-induced PC12 differentiation include the biosynthesis of neurotransmitters, the acquisition of electrical excitability, along with the growth of axon-like extensions during neuritogenesis [25].

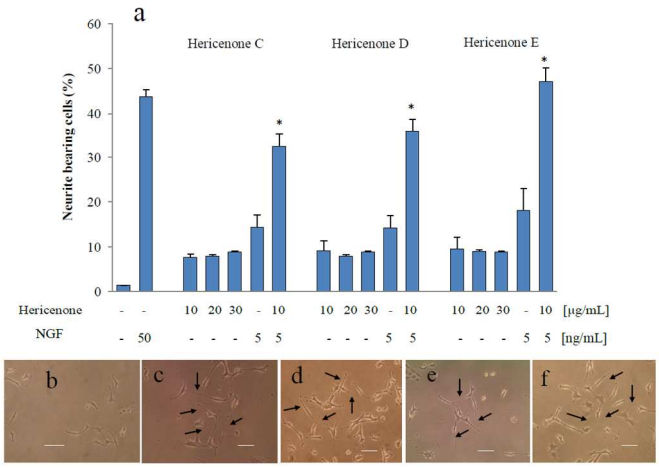

Fig. 2 (a) Effects of hericenone C, D, and E on the potentiation of neurite-bearing PC12 cells in the presence or absence of NGF. NGF (50 ng/mL) was used as the positive control. Cells with medium only served as negative control. Values are mean ± SD from three independent experiments. * $P< 0.05$ represents a significant difference from the control with NGF (5 ng/mL); and hericenones (10 µg/mL). Phase contrast photomicrographs of: **(b)** PC12 cells in growth medium only, **(c)** Cells treated with NGF (50 ng/mL), **(d)** Cells treated with NGF (5 ng/mL) and hericenone C (10 µg/mL), **(e)** Cells treated with NGF (5 ng/mL) and hericenone D (10 µg/mL), and **(f)** Cells treated with NGF (5 ng/mL) and hericenone E (10 µg/mL).Scale bar=20 µm. Arrows indicate neurite extensions.

Derived from rat adrenal medullary tumour (pheochromocytoma), PC12 cells have been immortalised and widely employed as an *in vitro* model for neuronal differentiation and neurite outgrowth studies.[26] PC12 cells require NGF to differentiate and when triggered, stop to proliferate, extend neurites, and become electrically excitable. In our study, hericenones alone did not induce neurite outgrowth in PC12 cells. When the cells were primed with a low concentration of NGF (5 ng/mL), neurite outgrowth activity was enhanced by hericenones C-E. This observation is consistent with other studies. Littora chalcone isolated from seashore vervain, *Verbena littoralis* H. B. K. (Verbenaceae), did not cause any morphological changes in PC12 cells in the absence of NGF but markedly enhanced the NGF (2 ng/mL)-induced neurite outgrowth activity.[27] Similarly, verbena chalcone from *V. littoralis* also markedly potentiated the NGF-induced neurite outgrowth activity in the presence of NGF.

In contrast to that, a polysaccharide purified from *H. erinaceus* mycelium was found to enhance neurite outgrowth of PC12 cells and its efficacy was higher than that of NGF alone.[28] An earlier study also showed that the crude aqueous extract of *H. erinaceus* cultivated under tropical condition stimulated neurite outgrowth of neural hybrid clone NG108-15 cells without the presence of NGF.[17] The same observation was recorded for aqueous extract of tiger's milk mushroom, *L. rhinocerotis* (basidiocarps and mycelium);[8,29] and for the morning glory mushroom, *P. giganteus.*[7] This suggests that pure compounds that induce neuritogenesis often need initial priming of neuronal cells with low concentration of neurotrophic factor like NGF. Although pure compounds in general are much more favorable as it poses pharmacological merit, an aqueous extract of a medicinal mushroom is important as this may contain compound/s which is/are not active independently but may affect the pharmacological parameters of other bioactive compounds and provide a synergistic or antagonistic action when combined with active fractions.

Hericenones C, D and E increased NGF level in culture medium

To investigate whether hericenones potentiate NGF-induced neurite outgrowth by stimulating NGF synthesis or as substitutes for NGF (NGF-mimicking activity), the NGF levels in the culture medium were measured. PC12 cells that were treated with 50 ng/mL of NGF (positive control) secreted 157 ± 12 pg/mL of NGF in the culture medium while hericenones C-E alone did not promote NGF synthesis (20–57 pg/mL). Upon addition of 5 ng/mL of NGF, hericenones C-E (10 µg/mL) stimulated the production of NGF (pg/mL) to 100 ± 1, 143 ± 9, and 319 ± 12; respectively (Fig. 3). The result of this study is in agreement with the study by Kawagishi et al[16]. Hericenones C-E were reported to stimulate NGF biosynthesis in mouse astroglial cells at 33 µg/mL. However, in another study, hericenones C-E did not increase NGF mRNA expression at 10-100 µg/mL in 1321N1 human astrocytoma cells and primary cultured rat astroglial cells.[30] Interestingly, the degree of NGF biosynthesis effect of hericenone E (319 ± 12 pg/mL) was almost two times higher than that of positive control and it was the highest among the hericenones ($P< 0.05$). In contrast, Kawagishi et al[16] reported that hericenone D was the most effective and its efficacy was almost the same as epinephrine (adrenaline). Therefore, it is noteworthy to highlight that the difference of the activity among these compounds is dependent on the chain length and the double bond of the fatty acid (Fig. 1).

Nerve growth factor is essential for the development and maintenance of neurons in the peripheral nervous system (PNS) and for the functional integrity of cholinergic neurons in the central nervous system (CNS)[3]. Therefore, maintaining a healthy NGF supply in PNS and CNS is of utmost importance. A study showed that *H. erinaceus* exhibited a neuroprotective effect against ischemic brain damage in a middle cerebral artery occlusion model in mice through stimulation of NGF biosynthesis[31]. Taken together, *H. erinaceus* and its components (hericenones) could be useful as potent protective and/or curative agents for strokes and neurodegenerative disorders. In this study, as hericenone E showed a significantly higher neurite bearing score and NGF stimulating activity as compared to hericenones C and D, it was selected for subsequent tests.

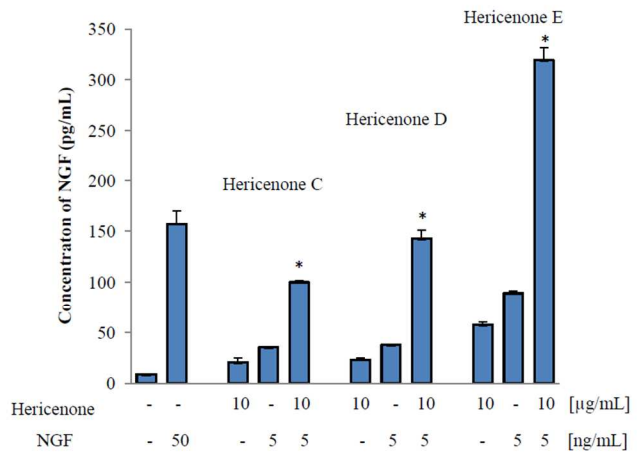

Fig. 3 Effects of hericenone C, D, and E on the stimulation of NGF secretion by neurite- bearing PC12 cells in the presence or absence of NGF. * $P< 0.05$ represents a significant difference from the controls, i.e. NGF (5 ng/ mL) and hericenones (10 µg/mL).

Correlation between the levels of hericenone E, NGF, and percentage of neurite-bearing cells

To show that NGF levels play a role in affecting the percentage of neurite-bearing cells, the Pearson correlation between the two variables was measured. The degree of correlation between the concentrations of hericenone E, NGF, as well as percentage of neurite-bearing cells, was also determined. As a result, given the range of 0-10 µg/mL of hericenone E, NGF levels in the culture medium is strongly correlated (r = +0.833) to neurite outgrowth activity in PC12 cells (Table 1). In PC12 cells, neurite outgrowth can be stimulated by different growth factors namely NGF, brain derived growth factor (BDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF).[26] Our results showed that elevated NGF biosynthesis leads to more neurite outgrowth activity and this agreed with Barde[1] who stated that the increase in neurite number is closely related to the NGF concentrations. Furthermore, the increasing concentration of hericenone E exhibited a strong correlation on the stimulation of NGF synthesis (r = +.969) and neurite outgrowth activity (r = +0.889).

Table 1 Correlation between the concentration of hericenone E, NGF levels and percentage of neurite-bearing cells.

	Correlations, r_p		
	Hericenone E (µg/mL)	NGF (pg/mL)	Neurite-bearing cells (%)
Hericenone E (µg/mL)	1.00	+0.97**	+0.89**
NGF (pg/mL)	+0.97**	1.00	+0.83**
Neurite-bearing cells (%)	+0.89**	+0.83**	1.00

A correlation study between the concentration of hericenone E, NGF level, and the percentage of neurite-bearing cell was conducted. **Correlation is significant at the 0.01 level (1-tailed). r = Pearson correlation coefficient. Strength of the correlation: 0.00-0.19 "very weak"; 0 .20-0.39 "weak"; 0.40-0.59 "moderate"; 0.60-0.79 "strong"; and 0.80-1.00 "very strong".

NGF-induced neurite outgrowth potentiated by hericenone E was partially mediated by TrkA

Specific inhibitors of key intermediates involved in neurite outgrowth signaling pathways were used to explore the mechanism of NGF-induced neurite outgrowth potentiated by hericenone E. PC12 cells were pre-treated with inhibitors for one hour, and then cultured in the presence or absence of hericenone E and/or NGF for 3 days. The percentage of neurite bearing cells was determined. The antagonist of NGF receptor tyrosine protein kinase A (TrkA), K252a significantly reduced the percentage of hericenone E-potentiated neurite bearing cells by approximately 46% (Fig. 4). However, the percentage of neurite bearing cells (32 ± 2%) was still significantly higher as compared to the control with medium only or with hericenone and/or NGF alone. Furthermore, K252a-treated cells incubated with hericenone E (10 µg/mL) alone showed no significant difference in terms of neurite bearing percentage when compared to cells without K252a (Fig.4). This observation suggested that NGF-induced neurite outgrowth potentiated by hericenone E was not entirely Trk-dependant even though neurotrophin like NGF was necessary for the differentiation and neurite outgrowth in PC12 cells.[32] There might be other TrKA-independent signaling pathways that are involved in the interaction of hericenone E as the signaling ligand (rather than NGF) to trigger neuritogenesis.

Several natural products have been identified to have the ability to potentiate the action of NGF to induce neurite outgrowth *in vitro*. Green tea polyphenols (GTPP) at low concentrations (0.1 µg/mL) potentiate NGF (2 ng/mL) to induce neuritogenesis at a level comparable to that induced by NGF (50 ng/mL) alone.[33] Iridoidcatalpol from Chinese herbs *Rehmannia glutinosa* (Shengdihuang) was found to increase brain-derived neurotrophic factor (BDNF) expression. When the action of BDNF was inhibited by K252a in a primary culture of forebrain neurons, the neurite outgrowth effect of catalpol was completely abolished.[34] Furthermore, tuftelin protein (a glycoprotein found in tooth enamel) significantly enhanced NGF-mediated PC12 differentiation and neurite outgrowth and this induction was also found to be partially blocked by K252a.[35]

On the other hand, neuritogenic effects of some compounds were not blocked by K252a suggesting that the effects are not due solely to the classical neurotrophic factors. The extract of a medicinal mushroom, *Ganoderma lucidium* (Lingzhi) was found to induce neurite outgrowth with no direct involvement of TrkA.[36] It is thus predicted that stimulatory factors other than classical neurotrophins present in the extract, stimulated neurite outgrowth and that activation of TrKA may not be necessary. Other TrKA-independent neuritogenic compounds include lysophosphatidylethanolamine from a medicinal mushroom, *Grifola frondosa*;[37] 5-Hydroxy-3,6,7,8,39,49-hexamethoxyflavone (5-OH-HxMF) from peels of sweet orange;[38] cyanine dye NK-4;[39] and α-phenyl-N-tert-butylnitron (PBN).[40]

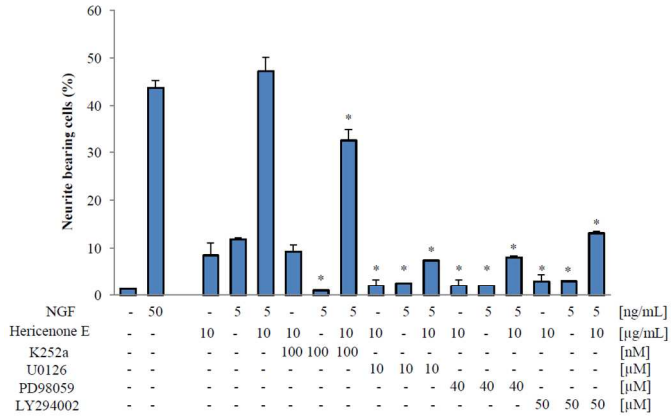

Fig. 4 Effects of specific signaling pathway inhibitors on the potentiation of neurite- bearing PC12 cells by hericenone E. NGF (50 ng/mL) was used as the positive control. PC12 cells were seeded in culture medium containing hericenones (10 µg/mL), NGF (5 ng/ml), and/or K252a (100 nM), U0126 (10 µM), PD98059 (40 µM), and LY294002 (50 µM). Data sets are expressed as percentage neurite bearing cells and are the mean ± SD of three independent experiments. * $P< 0.05$ represents a significant difference from the respective non-inhibitors treated cells.

NGF-induced neurite outgrowth potentiated by hericenone E is MEK-and PI3K/Akt-dependant

The major signaling pathways activated by TrkA include MAPK, ERK, PI3K/Akt, and phospholipase C (PLC)-γ.[41] Since the neurite outgrowth activity was found to be partially mediated by TrKA, MEK/ERK and PI3K/Akt pathways were investigated. NGF-induced neurite outgrowth in PC12 cells was inhibited after treatment with MEK inhibitors (U0126 and PD98059) (Fig. 4). Both U0126 and PD98059 reduced hericenone E-potentiated neurite outgrowth by almost 6-fold. Based on these findings, we tested the ability of hericenone E to activate p44/42 by measuring the phosphorylation of the downstream target proteins. MEK activity (Fig. 5a) was enhanced significantly ($P < 0.05$) by 50 ng/mL of NGF. The presence of hericenone E (10 µg/mL) alone also triggered significantly ($P < 0.05$) higher phospho-p44/42 (Thr202/Tyr204) levels when compared to cells with medium only (Fig. 5a). Upon addition of 5 ng/mL of NGF, hericenone E afforded a significant ($P<0.05$) increase of p44/42 phosphorylation. As expected, ERK phosphorylation was significantly ($P <0.05$) suppressed in the presence of inhibitors U0126 and PD98059.

Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine or threonine protein kinases which regulate various cellular programs such as cell proliferation, differentiation, motility, and survival.[42] The Erk1/2 (p44/42) signaling pathway can be activated in response to many extracellular stimuli, for example mitogens and growth factors. Upon stimulation, a chronological protein kinase cascade is initiated. The three-part kinase consist of a MAP kinase kinase kinase (MAPKKK or MAP3K), a MAP kinase kinase (MAPKK or MAP2K), and a MAP kinase (MAPK). MEK1 and MEK2 which are MAPKKs, activate p44 and p42 through phosphorylation of activation loop residues Thr202/ Tyr204 and Thr185/Tyr187, respectively. Here, we demonstrated phosphorylation of ERK1/2 by hericenone E at 10 µg/mL concentration. However, hericenone E (10 µg/mL)-treated cells had a low neurite-bearing cells percentage as shown in Fig. 2a. Therefore, it is postulated that ERK activation by hericenone E was mainly for cell differentiation rather than neurite extension and outgrowth. After being induced by a low concentration of NGF, we observed an increase and prolonged hericenone E-potentiated ERK activation. Previous studies have shown that induction of ERK activation by some medicinal mushrooms was consistent with their ability to stimulate neurite outgrowth and that treatment with specific inhibitors resulted in inhibition of neuritogenesis by *P. giganteus*, [7] *G. lucidum*,[36] *G. neo-japonicum*,[9] and *G. frondosa*.[37]

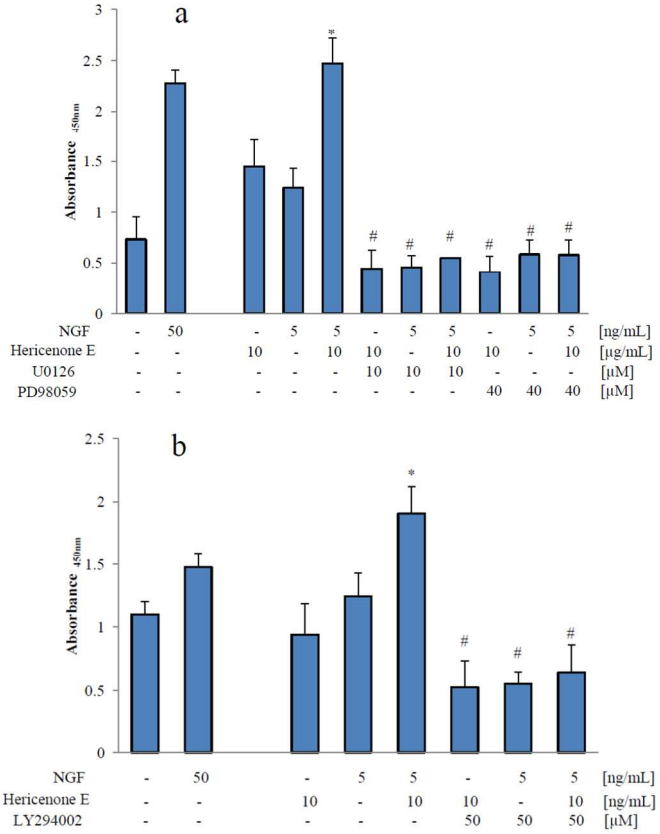

Fig. 5 Enhancement of NGF-induced neurite outgrowth by hericenone E is MEK- and PI3K/Akt-dependent. **(a)** Activation of ERK1/2 (p44/p42) was detected using antibody specific for phospho-ERK1/2. **(b)** Activation of PI3K/Akt (Thr308) was detected using antibody specific for phospho-Akt. * $P< 0.05$ represents a significant difference from the hericenone and NGF controls. # $P< 0.05$ represents a significant difference from the non-inhibitors treated cells.

Akt plays a critical role in controlling cell survival and apoptosis; and is activated by phospholipid binding and activation loop phosphorylation at Thr308 by pyruvate dehydrogenase lipoamide kinase 1 (PDK1).[43] PI3K/Akt has been proposed as a potential therapeutic target in neurodegenerative diseases since activation of Akt inhibits stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) that causes oxidative stress during neuronal

degeneration.[44] LY294002, a specific inhibitor of Akt has been used widely to elucidate PI3K/Akt pathway. We hypothesized that NGF-induced neuritogenesis potentiated by hericenone E was regulated by PI3K/Akt pathway. As a result, hericenone E supplemented with NGF (5 ng/mL), exhibited a significantly higher (P<0.05) Akt (Thr308) phosphorylation when compared to positive control (50 ng/mL) (Fig. 5b). Similarly, PI3K/AKT inhibitor LY294002-treated cells added with hericenone alone, and/or NGF was incapable of activating PI3K/Akt (Fig. 4).

Our result is consistent with the other reported studies. Dilong extracts (Chinese medicinal preparation from the earthworm species *Lumbricus rubellus*) were found to promote neuron regeneration.[45] Treatment with extract of Dilong induced the phosphorylation of the insulin-like growth factor-I (IGF-I)-mediated PI3K/Akt pathway and resulting in cell proliferation and survival of RSC96 Schwann cells. Besides, sargaquinoic acid isolated from a marine brown alga *Sargassum macrocarpum*, was found to promote NGF-dependent neurite outgrowth in PC12 cells and that inhibition of PI3K by wortmannin significantly suppressed the neuritogenic activity of sargaquinoic acid.[46] More recently, luteolin (3′,4′,5,7-tetrahydroxyflavone) isolated from rosemary, *Rosmarinus officinalis* (Lamiacea), has been reported to induce PC12 cell differentiation.[47] Luteolin treatment significantly enhanced acetylcholinesterase (AChE) activity and increased the level of total choline and acetylcholine in PC12 cells. In addition, treatment with U0126 and LY294002 also attenuated luteolin-induced AChE activity and neurite outgrowth in PC12 cells, suggesting that the neuritogenic properties of luteolin was regulated by activation of ERK1/2 and PI3K/Akt signalings.

Conclusion

In summary, four benzyl alcohol derivatives namely hericenones B-E (**1-4**), together with erinacerin A (**5**) and isohericerin (**6**) were isolated from the basidiocarps of *H. erinaceus* cultivated in tropical conditions in Malaysia. We concluded that hericenone E exerts neurotrophic activity in PC12 cells, and that this activity may be mediated partially through TrKA phosphorylation by NGF. Fig. 6 shows the schematic presentation of a possible mechanism by which hericenone E potentiates NGF-induced neurite outgrowth in PC12 cells. Analysis of additional signaling events revealed that the ERK1/2 and PI3K/Akt signaling cascades were triggered by hericenone alone with or without NGF. Therefore, NGF-induced neurite outgrowth potentiated by hericenone E is mediated, at least in part, through ERK1/2 and PI3K/Akt.

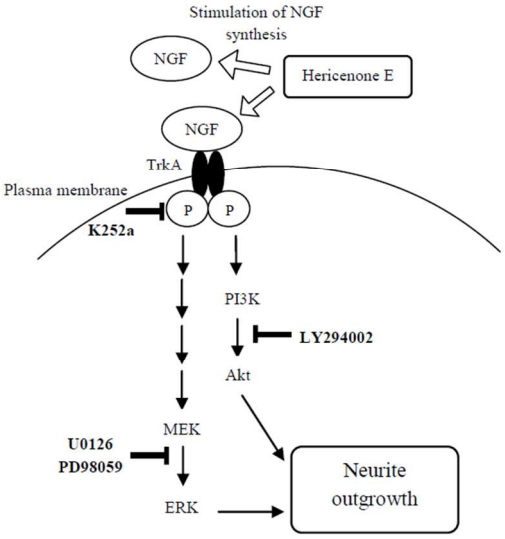

Fig. 6 Schematic presentation of a possible mechanism by which hericenone E potentiates NGF-induced neurite outgrowth. The role of TrkA, ERK, and Akt in the hericenoneE potentiation of NGF action was investigated by using K252a, U0126 and PD98059; and LY294002; respectively. Under basal condition, TrkA is phosphorylated by NGF and triggered downstream signaling. Hericenone E stimulates biosynthesis of NGF and further increases the phosphorylation of TrkA and subsequent activation of ERK and Akt. Hericenone E-induced ERK activation without NGF also suggests the involvement of additional pathways regulated by hericenone E directly to complement the ERK and Akt pathways to induce neuronal differentiation and neuritogenesis.

Conflict of interest

The authors declare no conflict of interest. This article does not contain any studies with human or animal subjects.

Acknowledgement

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