



OPEN Agarwood leaf ethanol extract provides neuroprotective properties and promotes cholinergic differentiation of HT22 hippocampal neurons

Kishoree K. Kumaree^{1,2}, James M. Brimson^{2,3}, Kanika Verma^{2,4}, Siriporn Chuchawankul⁵, Tewin Tencomnao^{2,4}✉ & Anchalee Prasansuklab^{1,2}✉

Neurodegenerative diseases, characterized by the loss or damage of neurons, represent a growing global health concern. Plants are a rich source of naturally occurring compounds with immense therapeutic potential. Among them, *Aquilaria crassna* (commonly known as agarwood) is a precious fragrant plant extensively used in cosmetics, perfumes, and traditional Asian medicine. However, its neuroprotective role, particularly in neuroregeneration, has been minimally explored. This study aimed to investigate the therapeutic potential of agarwood leaves in promoting neuroregeneration, with a focus on cholinergic function and neural differentiation. To identify bioactive compounds, a comprehensive LC–MS analysis was conducted on agarwood ethanolic extract (AWE). The phytochemicals detected were further evaluated using *in silico* methods to predict their interaction with receptor proteins linked to neurodegenerative diseases. Virtual screening revealed that several compounds in AWE exhibited strong binding affinities to receptors such as sigma-1, TrkB, Nogo-66, and p75NTR, providing insights into the potential mechanisms underlying its neuroprotective effects. The *in-silico* findings were validated through *in vitro* experiments using HT-22 mouse hippocampal cells as a model. AWE treatment led to a dose-dependent increase in the expression of marker proteins associated with neural differentiation and regeneration, including neuronal nuclei (NeuN), growth-associated protein 43 (GAP43), synaptophysin (Syn), brain-derived neurotrophic factor (BDNF), and the sigma-1 receptor. Additionally, AWE enhanced the expression of specific markers for cholinergic neurons, demonstrating its influence on neuronal development and synaptic function. These findings provide compelling evidence of AWE's neuroprotective properties, highlighting its potential as a therapeutic agent for neurodegenerative diseases.

Keywords *Aquilaria crassna*, Neuroprotection, Neurodifferentiation, Neurodegenerative diseases, Cholinergic neurons, *In silico*

Agarwood, from the *Aquilaria* tree, is a traditional medicinal herb used to treat infectious and inflammatory diseases¹. In addition, studies have reported the extensive use of agarwood in aromatic incense and traditional medicine its calming effects^{2,3}. Recent studies have shown several benefits of agarwood extract, which include anti-oxidative, anti-inflammatory, and neuroleptic capacities^{4,5}. Furthermore, its molecular derivative relieves neuropsychiatric symptoms via modulating central monoamine neurotransmitters in several brain regions⁶. Given the pressing need to harness the therapeutic potential of plants, we aim to investigate the significance of agarwood in addressing one of the major global health challenges: neurodegenerative diseases (NDDs).

¹College of Public Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand. ²Center of Excellence on Natural Products for Neuroprotection and Anti-ageing (Neur-Age NatChula), Chulalongkorn University, Bangkok 10330, Thailand. ³Research, Innovation and International Affairs, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand. ⁴Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand. ⁵Department of Transfusion Medicine and Clinical Microbiology, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand. ✉email: tewin.t@chula.ac.th; anchalee.pr@chula.ac.th

NDDs affect approximately 50 million people worldwide, posing a significant challenge to modern medicine. Furthermore, statistical projections indicate that the global population aged 60 years⁷ and over will rise to 1.5 billion by the year 2050⁸, suggesting a corresponding increase in the prevalence of NDDs in the coming years. Currently, there are limited therapies available for NDDs, and those that exist have a relatively low success rate, underscoring the urgent need for the development of effective therapeutics by the clinical and scientific communities.

Neurodegenerative disorders are characterised by significant neuronal cell death, identified as a critical factor in their pathogenesis. Studies have shown that an imbalance in the neurotransmitter system primarily contributes to synaptic loss and the subsequent degenerative cell death observed in these disorders⁹. Neurodegenerative diseases, including AD, are associated with various pathological changes, including the build-up of malformed protein aggregates (Amyloid- β in AD, α -synuclein in PD, and Huntingtin in HD), degeneration neuron signal transmission (loss of cholinergic neurons³ in AD, and loss of dopaminergic neurons in PD¹⁰), neuro-inflammation, oxidative stress (ROS), and reduced expression levels of the sigma-1 receptor¹¹ and brain-derived neurotrophic factor (BDNF)¹². These pathologies are considered significant contributors to the onset and progression of neurodegenerative diseases¹³.

Neurogenesis, the process of forming new neurons, is vital for maintaining cognitive and motor functions. A reduction in neurogenesis is linked to the neuronal loss and functional impairments seen in neurodegenerative disorders¹⁴. Once thought to cease after adulthood, neurogenesis has been shown to occur in the adult brain, particularly in the hippocampus¹⁵. This process, involving the proliferation and differentiation of neural stem cells, is regulated by various molecular factors, including neurotrophins and growth factors like BDNF, which promote neural differentiation^{16,17}.

Drug development for neurodegenerative diseases is time-consuming and costly, often exceeding \$2.6 billion for a New Molecular Entity (NME) or New Biological Entity (NBE)^{18,19}. However, plants provide a valuable resource for drug development, offering pre-existing compounds with evolved biological functions. Traditional medicinal plants have been sources of treatments for various ailments and show potential for neurodegenerative disease therapies^{20,21}. However, it is worth mentioning that the climatic and environmental conditions influence the bioavailability of bioactive compounds. For instance, variations in temperature, sunlight, rainfall, and other environmental conditions significantly alter the levels and types of phytochemicals in plants. For example, plants grown in warmer climates often produce higher quantities phenolic compounds, which contribute to their pharmacological properties²². This concept was supported by earlier findings, where variations in altitude affected the active compounds in *Potentilla fruticosa* L. across different regions of China²³. Understanding how environmental factors shape plant biochemistry not only helps in harnessing the full potential of natural resources but also guides research into their therapeutic applications. In line with this, our study explored the neuropharmacological potential of Agarwood (*Aquilaria crassna*) extract. Our preliminary in vitro study demonstrated that agarwood extracts have significantly protected HT-22 hippocampal neuronal cell line from glutamate excitotoxicity²⁴, which implies its neuropharmacological action in stress-related brain disorders. Encouraged by these findings, we explored the potential of *A. crassna* to induce cholinergic differentiation in HT-22 cells. No previous research has investigated a plant extract's ability to induce cellular differentiation in an in vitro experimental model. This study aims to elucidate agarwood extract's role in promoting neural differentiation and neurogenesis, contributing to its potential as a therapeutic for neurodegenerative diseases. Furthermore, agarwood extract could promote the expression of cholinergic markers in HT-22 cells, suggesting it could also potentially promote the proliferation of cholinergic neurons in vivo. The result of the present study may contribute to understanding the important contribution of agarwood towards new insight into its health benefits and potential therapeutic benefits for treating neurodegenerative diseases.

Materials and methods

Chemicals and reagents

Antibodies targeting Synaptophysin, Choline Acetyltransferase (ChAT), and β -actin were acquired from Cell Signalling Technology (Danvers, MA, USA). Antibodies targeting Neuronal Nuclei (NeuN), Growth Associated Protein 43 (GAP43), sigma-1 receptor, muscarinic Acetylcholine receptor 2 (mAChR-2), and BDNF were acquired from Abcam (Cambridge, UK). We obtained 2° antibodies, anti-rabbit-horseradish peroxidase (HRP) tagged and anti-mouse-HRP tagged, from Cell Signalling Technology (Danvers, MA, USA).

Collection and extraction of plant materials

Leaves of agarwood (*A. crassna*) were harvested on 25 June 2022 with prior approval from the concerned authority at the Princess Maha Chakri Sirindhorn Herbal Garden in Rayong Province, Thailand, and subsequently verified by Mrs. Parinyanoot Klinratana. The specimen was placed in the Professor Dr. Kasin Suvatabhandhu Herbarium (Department of Botany, Faculty of Sciences, Chulalongkorn University, Thailand) under voucher specimen number A17634(BCU). The plant material was taken with the permission from the authorities on behalf of the herbal garden through the formal letter issued by the principal investigator, and the protocols for plant access and collection were performed in accordance with relevant Thai national regulations, notably the Plant Variety Protection Act (1999), in obtaining appropriate permits and maintaining ethical academic standards throughout the process. The leaves were desiccated in the shade, pulverised into a fine powder, and subsequently macerated with ethanol at ambient temperature for 72 h before the collection of the supernatant, which was filtered, and evaporated using a rotary evaporator at 45 °C. The final extract was dissolved in dimethylsulfoxide (DMSO) creating a working stock solution (20 mg/mL) and preserved at –20 °C until utilised. The ethanol extract of *A. crassna* is referred to as AWE.

LC–MS/MS analysis

LC–MS analysis is more effective for detecting drug-like substances, whereas GC–MS is employed to identify volatile chemicals. This study conducted LC–MS/MS analysis on the ethanol extract of AW to identify bioactive components. Separation was conducted with a Thermo Scientific C18 column (Acclaim™ Polar Advantage II, 3 × 150 mm, 3 µm particle size) on an UltiMate 3000 UHPLC system (Dionex). Gradient elution was conducted at a flow rate of 0.4 mL/min and a column temperature of 40 °C, utilising H₂O with 0.1% formic acid (A) and 100% acetonitrile (B), with a total run time of 22 min. The sample injection volume was 3 µL. The gradient commenced at 5% B (0–3 min), transitioned to 80% B (3–10 min), maintained 80% B (10–15 min), and concluded at 5% B (15–22 min). Under the following conditions, high-resolution mass spectrometry was carried out using a MicroTOF QIII Bruker Daltonics equipped with ESI positive ionisation under the following conditions: Nebuliser pressure is 2.0 bar, capillary voltage is 4500 V, and drying gas is 8 L per minute at 250 degrees Celsius. The mass ranged from 50–1000 m/z. Compass Data Analysis software (Bruker Daltonik. GmbH), was utilised in order to do an analysis on the precise mass data of the molecular ions that were provided by the TOF analyser.

Molecular docking

Metabolite from the agarwood ethanolic extract were identified using the online tools of MetFrag and Metlin^{25,26}. The phytochemicals isolated from the Agarwood leaf extract were extracted from the PubChem database²⁷. The 3D protein structures of Nogo-66 Receptor, TRK B receptor, sigma-1 receptor, p75NTR associated with neuronal development and neuroprotection were retrieved from RCSB PDB database (<https://www.rcsb.org/>). The corresponding PDB IDs were 1PBT (Nogo-66 Receptor), 1WWB (TRK B receptor), 5HK1 (sigma-1 receptor), and 7CSQ (p75NTR). We have employed DockThor online server to calculate the binding affinities of the phytochemicals against the target protein. It is a grid-based docking method which computes different binding modes of ligands on the protein structure²⁸.

Evaluation of ADMET properties and drug-likeness

The chemical properties and drug-like attributes of the phytochemicals were evaluated based on "Lipinski's Rule of Five"²⁹. The phyto-compounds were examined for their physicochemical properties, drug-likeness, toxicity, and ADMET characteristics, utilizing the ADMETlab 2.0 accessible at admetmesh.scbdd.com. The ADME/Tox profiling focused on essential parameters such as lipophilicity (log P), Hydrogen bond donors, Hydrogen bond acceptors, polar surface area (PSA) and molecular weight to identify suitable candidates with drug-like properties. SMILES of the phytochemicals were used as input in the server, was obtained from the PubChem database²⁷. In addition, we have also analysed the Blood Brain Barrier (BBB) for each phytochemicals as it plays an important role in neuronal therapeutics designing.

MTT cell viability assay

The mouse hippocampal neuronal cell line, HT-22, was kindly gifted by Prof. David Schubert at the Salk Institute (San Diego, CA, USA). To ascertain the optimal working concentration of AW extract with respect to the lowest cellular toxicity. HT-22 cells were added onto 96-well plates (5000 cells per well) and subjected to treatment for 24 h with AWE at different doses (0.001, 0.01, 0.1, 1, 10, and 100 µg/mL). Following incubation, each well was supplemented with Dulbecco's Modified Eagle's Medium (DMEM) containing 0.5 mg/mL of MTT ((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The cells were treated for 3 to 4 h, and the formazan salt was solubilised in DMSO. Absorbance was measured at a wavelength of 570 nm utilising the EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Results are shown as a percentage compared to the untreated control.

Glutamate toxicity assay

The glutamate toxicity experiment was conducted as previously outlined³⁰. HT-22 cells were seeded in 96-well plates and permitted to adhere overnight. On the subsequent day, the cells were co-treated with new DMEM media with quantities of AWE ranging from 0 to 100 µg/mL, in conjunction with 5 mM of Glutamate. The cells were cultured for 24 h, after which the media was discarded, the cells were rinsed with PBS, and DMEM containing MTT was added to each well. The absorbance was recorded at 570 nm.

Cell treatment for neurite growth analysis

HT-22 cells were seeded in six-well plates (about 2000 cells/cm²) in 1 mL of full DMEM culture medium and permitted to adhere to the surface overnight. The subsequent day, the media was substituted with 2 mL of fresh media containing 0–100 µg/mL AWE. The cells were subsequently cultured for 24 h in a humidified incubator at 5% CO₂ and 37 °C. Cellular images were acquired utilising a Zeiss Axio Observer A1 Inverted Fluorescence/Phase Contrast Microscope. Neurite outgrowth was evaluated through Image J, which facilitated the enumeration of differentiated cells (cells exhibiting an outgrowth at least twice the diameter of the cell) and non-differentiated cells, as well as the measurement of neurite lengths via the Neuron J plugin for Image J³¹.

Western blotting

HT-22 cells plated in 6-well plates were treated with varying concentrations (0–100 µg/mL) of AWE extract. The cells were lysed using NP-40 lysis buffer, incubated on ice for 30 min, then centrifuged at 12,000 × g for 15 min at 4 °C, followed by a supernatant collection for SDS-PAGE running. An equal amount of protein (20 µg) from each treatment was denatured by heating in Laemmli buffer at 95 °C for 10 min. The denatured protein was separated on 10% SDS polyacrylamide gel (SDS-PAGE) before protein transfer to the PVDF membrane. Following the membrane blocking for 1 h with 5% BSA (bovine serum albumin) in TBS-T (Tris-buffered saline with 0.1% Tween 20), the membranes were then incubated overnight at 4 °C with primary antibodies Syn

(1:1000), sigma-1 (1:1000), ChAT (1:1000), GAP-43 (1:1000), NeuN (1:1000), mACh-2 (1:2000), BDNF (1:2000) and β -actin (1:5000). HRP signals were detected by enhancing chemical luminol reagent (ECL[™] Select Western blotting detection reagent: GE Healthcare, Piscataway, NJ, USA) on Chemidoc IQ800 (Thermo Fisher Scientific). The intensity of the bands were quantified using Image-J software.

Free cellular choline and acetylcholine measurement

The Choline/Acetylcholine assay kit from Abcam (Cambridge UK) measured free cellular Choline and acetylcholine. The kit relies on the oxidation of Choline to betaine which generates products that react with the probe producing a color that can be read at Abs 570 or fluorometrically (Ex/Em 535/587). The samples are incubated with and without AchE to give the total free cellular Choline, Choline, and acetylcholine. Cellular acetylcholine can then be inferred by subtracting one from the other. HT-22 cells were plated in 6-well plates at a density of 500,000 cells per well ($\approx 50,000$ cells/cm²) in complete 1 mL of DMEM and allowed to adhere overnight.

The following day the media was replaced with 2 mL of DMEM containing 0–100 μ g/mL AWE, and the cells were again incubated for a further 24 h in a humidified incubator (5% of CO₂ at 37 °C). The cells were washed in ice-cold PBS and harvested by scraping them in the Choline assay buffer. The cells were lysed on ice in the choline assay buffer by vigorous pipetting and vortexing for 10 min. The cell lysate was centrifuged at 12,000 rpm to remove any non-solubilized materials, and the supernatant was transferred to a clean tube. The supernatant was added to 96 well plates in duplicate, to which the mix of choline enzyme and choline probe was added in the presence and absence of AchE. The plate was then incubated at room temperature for 1–3 h, protected from light. The plate was read at Abs 570, and the data normalized to the untreated HT-22 cells.

Statistical analysis

The data were expressed as standard error of the mean (SEM). The experiments were conducted in triplicates. The data were analysed with GraphPad Prism version 9 (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses between different groups were performed by using unpaired *t*-test or One-way ANOVA, which were succeeded by Dunnett's *post-hoc* comparison. *p* values less than 0.05 were deemed statistically significant.

Results

Phytochemicals detection from AWE through LC–MS/MS

To identify the major bioactive components of agarwood extract that might be contributing to its neuroprotective efficiency, an LC–MS/MS analysis was performed. The major compounds detected based on their *m/z* peak intensity were analyzed and mentioned in Table 1 and Supplementary Fig. S1.

In silico analysis of phytochemicals from AWE against different neuronal receptors/proteins

This study aimed to explore the potential role of phytochemicals derived from agarwood ethanol extract (AWE) in neuronal signaling and development through molecular docking analyses focused on specific neuronal receptor proteins. The receptors selected for this investigation included the Nogo-66 receptor (1PBT), Tropomyosin Receptor Kinase-B (TrkB, 1WWB), sigma-1 receptor (5HK1), and p75 Neurotrophin Receptor (p75 NTR, 7CSQ). The binding affinities of the identified phytochemicals were evaluated and compared to reference molecules (Table 1, Supplementary Table S1–S4): α -D-GlcNAc for the 1PBT receptor, 1-N,3-N,5-N-tris(2-hydroxyethyl)benzene-1,3,5-tricarboxamide for the 1WWB receptor, Pentazocine for the 5HK1 receptor, and (2S,3S)-2-Amino-3-methyl-N-(2-morpholinoethyl)-pentanamide for the 7CSQ receptor (Supplementary Fig. S3). A lower docking score indicates the compound's stronger binding affinity against the target. Thus, the compounds with more negative values exhibit reduced binding energy and are considered to have better binding affinity. The reference molecules exhibited binding energies of –6.43, –6.59, –7.91, and –6.28 kcal/mol for the 1PBT, 1WWB, 5HK1, and 7CSQ receptors. However, the top five hits against each protein were tabulated in Table 2 with the energies in the –7 to –9.5 kcal/mol range.

Furthermore, to understand the binding confirmations of the top hits Villinol, Angustine, Homaline, Tiliroside, and 7-Hydroxy-2,4,5-trihydroxyisio-flavone against each receptor protein were analyzed for the interaction study (Supplementary Figure S2 A–D). Demonstrated significant binding affinities across several targets, suggesting potential similarities in their interaction characteristics. These visual representations illustrate the interactions contributing to the ligand binding in the protein binding pocket for their strong binding affinities. The findings underscore the significance of these compounds and indicate their potential as multi-target ligands that can effectively engage with a range of neuronal receptors.

ADMET profiling of the identified phytochemical from AWE

ADMET analysis and drug-likeness evaluation were conducted to predict the properties of the compounds based on their chemical structure. A drug-likeness assessment was conducted on the compounds, and the results presented to the drug-likeness evaluation for the compounds are detailed in Supplementary Table S5. Results from this analysis indicated that 28 compounds out of 32 met the criteria outlined by Lipinski's rule of Five. In addition, BBB permeability scores stated the ability of ligands to cross the blood–brain barrier, and all the phytochemicals identified hold the property to be able to cross the BBB.

AWE dose–response in HT-22 cells

The toxicity of AWE extract was assessed using the MTT assay. HT-22 cells subjected to AWE had no toxicity between 0.001 to 10 μ g/mL. However, at 100 μ g/mL some reduction was observed (mean % viability 82.76 ± 14.32); this reduction was not statistically significant (ANOVA *p* = 0.1792) (Fig. 1).

	Component in AW	Mol weight (g/mol)	Molecular Formula	Retention time (min)	m/z Ratio
1	Tridecane	184.4	C ₁₃ H ₂₈	1.3	207.21
2	Linamarin	247.2	C ₁₀ H ₁₇ NO ₆	1.4	248.11
3	Villinol	438.4	C ₂₄ H ₂₂ O ₈	1.8	439.14
4	Quinic acid	192.2	C ₇ H ₁₂ O ₆	2.3	193.07
5	Guanine	151.1	C ₅ H ₅ N ₅ O	2.5	174.04
6	L-Phenylalanine	165.2	C ₉ H ₁₁ NO ₂	3.5	166.09
7	Gambirinin C	562.5	C ₃₀ H ₂₆ O ₁₁	7.7	585.14
8	3-Dehydroshikimic Acid	172.1	C ₇ H ₈ O ₅	8.0	195.03
9	Khellol glucoside	408.4	C ₁₉ H ₂₀ O ₁₀	8.0	409.11
10	Tiliroside	594.5	C ₃₀ H ₂₆ O ₁₃	8.1	595.16
11	1'-Acetoxyeugenol Acetate	266.3	C ₁₄ H ₁₈ O ₅	8.1	265.11
12	Quercetin	302.2	C ₁₅ H ₁₀ O ₇	8.5	303.05
13	Geranyl diphosphate	314.2	C ₁₀ H ₂₀ O ₇ P ₂	9.1	315.08
14	Biochanin A	284.3	C ₁₆ H ₁₂ O ₅	9.2	285.07
15	Procyanidin B1	578.5	C ₃₀ H ₂₆ O ₁₂	9.2	579.17
16	C16 Sphinganine	273.5	C ₁₆ H ₃₅ NO ₂	9.2	274.27
17	N-Carbamoyl-L-aspartic acid	176.1	C ₅ H ₈ N ₂ O ₅	9.3	177.05
18	7-Hydroxy-2',4',5'-trimethoxyisoflavone	328.3	C ₁₈ H ₁₆ O ₆	9.3	329.10
19	Actinodaphnine	311.3	C ₁₈ H ₁₇ NO ₄	9.3	312.12
20	Petunidin 3-galactoside	479.4	C ₂₂ H ₂₃ O ₁₂	10.0	479.11
21	4,6,2',4'-Tetramethoxychalcone 2'-beta-glucoside	506.5	C ₂₅ H ₃₀ O ₁₁	10.0	507.18
22	Angustine	313.4	C ₂₀ H ₁₅ N ₃ O	10.1	314.14
23	Phenylacetic acid	136.2	C ₈ H ₈ O ₂	10.6	137.06
24	3-Tert-Butyl-5-methylcatechol	180.2	C ₁₁ H ₁₆ O ₂	11.0	181.12
25	Cucurbitacin S	498.6	C ₃₀ H ₄₂ O ₆	11.1	499.30
26	Cuscohygrine	224.3	C ₁₃ H ₂₄ N ₂ O	11.7	225.19
27	L-2,4-Diaminobutyric Acid	118.1	C ₄ H ₁₀ N ₂ O ₂	11.9	119.08
28	Homaline	490.7	C ₃₀ H ₄₂ N ₄ O ₂	12.0	513.33
29	Montanol	352.5	C ₂₁ H ₃₆ O ₄	12.6	353.27
30	α-Licanic acid	292.4	C ₁₈ H ₂₈ O ₃	13.2	293.21
31	Ornithine	132.2	C ₅ H ₁₂ N ₂ O ₂	14.8	133.10
32	α-Linolenic Acid	278.4	C ₁₈ H ₃₀ O ₂	17.2	279.23

Table 1. LC–MS/MS analysis of compounds isolated from the ethanol extract of agarwood leaf.

AWE protected against glutamate-induced toxicity

Glutamate-induced toxicity at 5 mM is a well-characterized assay in HT-22 cells that results in oxidative stress causing cellular toxicity³². AWE (10 µg/mL) significantly reduced the adverse effects of 5 mM glutamate from 50 ± 7.9% to 97 ± 1.5% cell viability (measured via MTT assay). ANOVA followed by Dunnett's post hoc test for statistical significance showed a statistically significant difference ($p < 0.05$) between control and 5 mM glutamate-treated HT-22 cells as well as; there was a significant difference between cells only treated with 5 mM of Glutamate and AWE plus 5 mM glutamate-treated HT-22 cells. The data also indicates a bell-shaped dose-response curve, as 100 µg/mL appears to have a less protective effect than 10 µg/mL (Fig. 2).

HT-22 cells exhibited improved neurogenesis and differentiation properties on agarwood ethanol extract treatment

On microscopic examination of HT-22 cells treated with AWE, it was observed that at the higher concentrations of AWE (Fig. 3A,B), there was a change in the morphology of the cells. Therefore, we quantified these morphological changes by counting the number of differentiated cells and measuring the length of the neurites (Fig. 3C,D). The neurite length increased with increased AWE dose, and the differentiation percentage in the HT-22 cells also increased with the dose of AWE.

Studies have established a link between increased NeuN, GAP43, and BDNF protein expression and neuronal differentiation. Therefore, we assessed these marker proteins' expression changes after the AWE. In the Western blot analysis presented in Fig. 4A,B, treatment with Agarwood Extract (AWE) significantly elevated NeuN levels at concentrations of 10 µg/mL and 100 µg/mL compared to the untreated control group. Additionally, a notable increase in GAP43 expression was observed at a concentration of 100 µg/mL, indicating enhanced neuronal development and axonal growth in HT-22 cells. Furthermore, BDNF a pivotal neurotrophic factor essential for neuronal survival, differentiation, and synaptic plasticity, significantly increased in HT-22 cells treated with 10 µg/mL and 100 µg/mL of AWE. Additionally, the sigma-1 receptor, vital for reducing cellular stress, enhancing neural protection, and promote neurite outgrowth, showed significant upregulation in its protein expression

Receptors	Selected ligands	Kcal/mol
1PBT (Nogo-66 Receptor)	Positive ligand (α -D-GlcNAc)	−6.43
	Villinol	−8.34
	Angustine	−8.12
	Homaline	−7.76
	7-Hydroxy-2'	−7.96
1WWB (TrkB receptor)	Positive ligand (1-N,3-N,5-N-tris(2-hydroxyethyl)benzene-1,3,5-tricarboxamide)	−6.59
	Tiliroside	−8.62
	Villinol	−8.16
	Homaline	−7.80
	Angustine	−7.54
5HK1 (Sigma-1 receptor)	Positive ligand (Pentazocine)	−7.91
	Tiliroside	−9.07
	Villinol	−9.06
	Homaline	−8.71
	7-Hydroxy-2'	−8.51
7CSQ (p75NTR)	Positive ligand ((2S,3S)-2-Amino-3-methyl-N-(2-morpholinoethyl) pentanamide)	−6.28
	7-Hydroxy-2'	−8.31
	Homaline	−8.21
	Villinol	−7.50

Table 2. The binding affinity of the selected ligands from agarwood ethanol extract with their respective receptors.

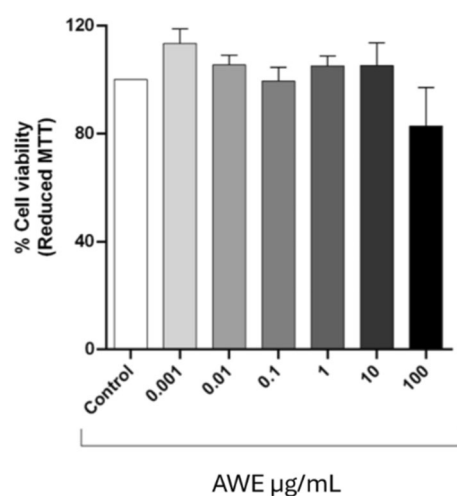


Fig. 1. Effect of different agarwood extract on cell viability of HT-22 cells. Cell viability determination using MTT assay for HT-22 cells treated with AWE. Data are presented as the means \pm SEM; (n = 3).

within HT-22 cells treated with 100 $\mu\text{g/mL}$ of AWE. This upregulation of mentioned proteins underscores the potential neuroprotective benefits of the extract, which appear to augment with the dose (Fig. 4A,B).

Expression of cholinergic markers in HT-22 cells during AWE treatment

This study measured choline/acetylcholine production using a colorimetry assay kit. Compared to the control group, the results show a dose-dependent increase in choline/acetylcholine production in AWE-treated HT-22 cells (Fig. 5A). However, only the free Choline and choline/acetylcholine showed a significant increase in AWE-treated HT-22 cells at 100 $\mu\text{g/mL}$ doses.

Furthermore, we investigated the cholinergic properties of HT-22 cells upon treatment with AWE at different doses (1, 10, and 100 $\mu\text{g/mL}$). To assess the cholinergic activity, we examined the expression levels of marker proteins, including Muscarinic Acetylcholine Receptor 2 (M2/AChR-2) and Synaptophysin. Western blot analysis revealed a dose-dependent increase in AChR-2 expression in AWE-treated HT-22 cells compared to the control group (Fig. 5B,C). In addition, we found that the ChAT expression level demonstrated a significant increase in AWE-treated HT-22 cells (Fig. 5B,C). This indicates enhanced synaptic activity and potentially

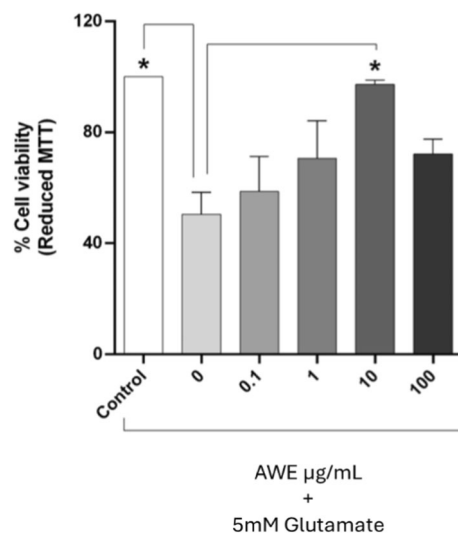


Fig. 2. The protective effect of AW extract on HT-22 cells when the cells were exposed to the toxicity of 5 mM Glutamate. The percentage cell viability of HT-22 was determined through MTT assay when treated with various doses (0.1 to 100 $\mu\text{g/mL}$) of AWE. Data are presented as the means \pm SEM. One-Way ANOVA, followed by Dunnett's post hoc test, evaluated the statistical differences; * $p < 0.05$, ** $p < 0.01$; ($n = 3$).

increased cholinergic neurotransmission. Our results demonstrate that agarwood treatment in HT-22 cells promotes cholinergic properties.

Discussion

Neuronal damage, loss of neurons, imbalance in cholinergic signalling, and several other similar factors contribute toward the progression of neurodegenerative diseases, such as AD, PD, and HD³³. Therefore, current research focuses on developing neuroprotective approaches to prevent neuronal damage, promoting neuronal survival, and exploring regenerative strategies to replace damaged neurons³⁴. A crucial aspect of this research is investigating how compounds can induce neuronal differentiation. This holds promise for protecting existing neurons and generating new functional ones, potentially leading to improved outcomes for patients with neurodegenerative conditions.

Numerous studies have highlighted the potential of plants and their isolated compounds in promoting neurite outgrowth and providing neuronal protection, making them attractive candidates for treating neurodegenerative disorders^{35,36}. Previous research has also demonstrated the neuroprotective effects of *Aquilaria crassna* (Agarwood)³⁷. Agarwood has been utilized for centuries as incense and traditional medicine across East, South, and Middle Asia^{38,39}. Traditional Chinese herbal medicine's use is well-documented, particularly for its potential to prevent cardiovascular and neurological disorders⁴⁰.

In line with these findings, our study aimed to identify the compounds from agarwood extract and investigate the potential effects on neural differentiation and cholinergic activity using an in vitro model of HT-22 cells. Given the critical role of neuronal differentiation and regeneration in repairing the nervous system, particularly in neurodegenerative diseases⁴¹, we first conducted in silico analysis to predict the potential interactions of agarwood extract compounds with key neurodegenerative disease-associated receptors. Encouraged by these findings, we proceeded to validate the neuroprotective potential of agarwood extract through in vitro experiments using HT-22 mouse hippocampal cells. Agarwood extract AWE, exhibited no cytotoxic effects on the growth or proliferation of HT-22 mouse hippocampal cells, as shown in Fig. 1. However, under co-treatment with glutamate, a well-established neurotoxic agent, AWE provided significant neuroprotective effects (Fig. 2).

A comprehensive LC-MS analysis of AWE identified diverse bioactive constituents, including flavonoids, rotenoids, and terpenoids (Table 1; Supplementary Fig. S1). Several bioactive components identified in AWE in our current study have also been previously linked for their neuroprotective properties, suggesting a potential role in neurodegenerative disease intervention. Flavonoids like quercetin, tiliroside, and biochanin-A have antioxidant, anti-inflammatory, and cholinergic-enhancing effects, helping to prevent tau hyperphosphorylation and amyloid-beta toxicity in Alzheimer's disease^{42–45}. Similarly, alkaloids such as actinodaphnine and angustine have demonstrated neuroinflammation by modulating neurotransmitter systems and reducing neuroinflammation^{46,47}. Fatty acids such as α -linolenic acid and sphinganine are essential for neuronal membrane integrity and synaptic plasticity, playing a key role in cognitive function⁴⁸. Research has shown that α -linolenic can enhance brain plasticity and exert an anti-depressant effect also the sub-chronic treatment with α -linolenic increasing neurogenesis⁴⁹. Additionally, other polyphenols like procyanidin B1 have been linked to protect neuronal cells from oxidative stress and apoptosis⁵⁰.

In silico docking studies revealed strong binding affinities of phyto-compounds to key receptors, including sigma-1, TrkB, Nogo-66, and p75NTR, suggesting their potential role in neuroprotection and neuro-

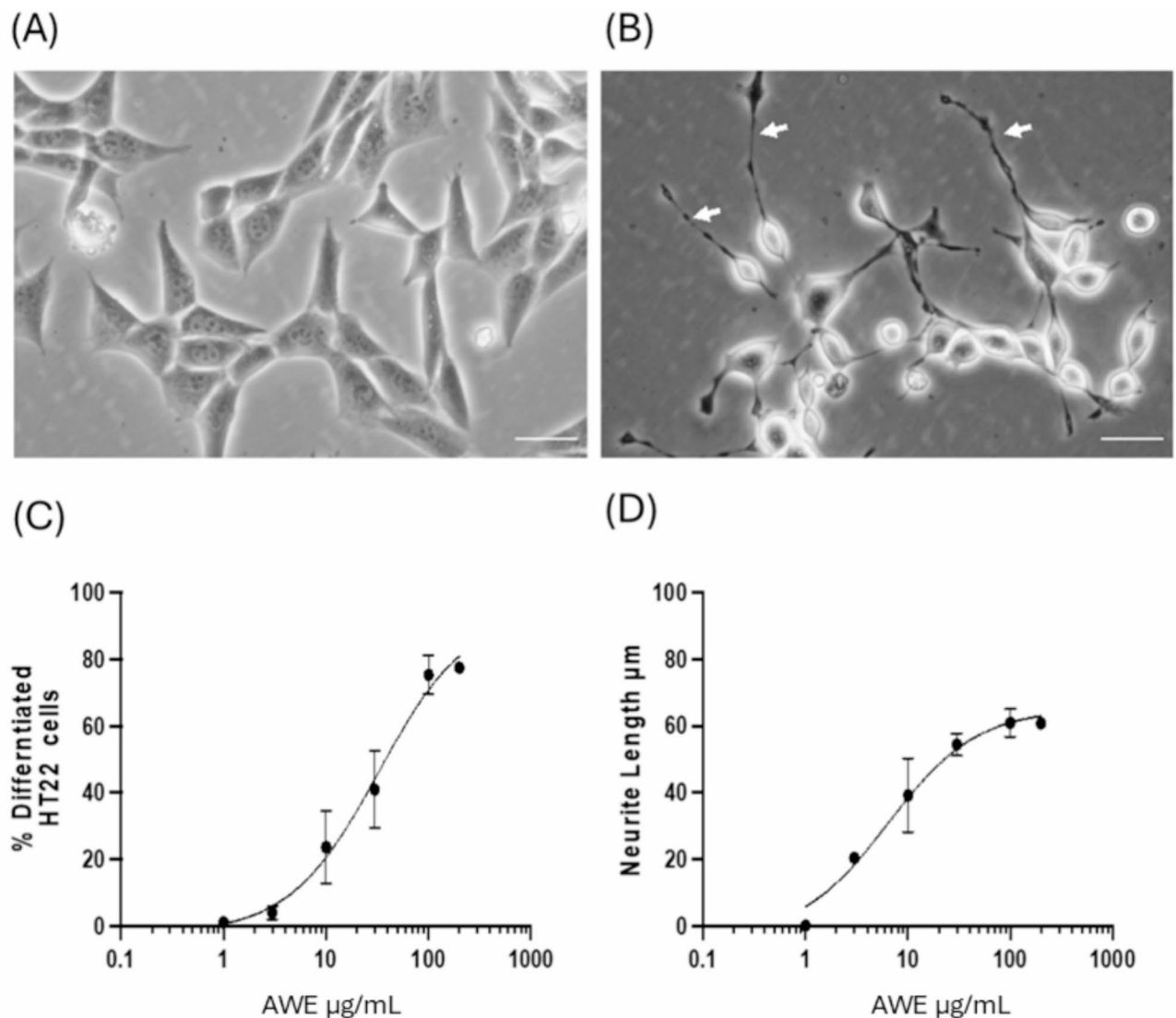


Fig. 3. The neural differentiation-inducing effect of AWE. (a) HT-22 control cells. (b) HT-22 cells treated with 10 µg/ml of AWE, the white arrows (↑) signifies the neurite outgrowth. (c) Analysis of % cell differentiation of HT-22 cells treated with AWE from three independent experiments where at least five fields of view were selected and counted presented here are the means \pm SEM. (d) Analysis of neurite length HT-22 cells treated with AWE from three independent experiments where at least five fields of view were selected and measured using Neuron J. The data presented here are the means \pm SEM; (n = 3).

regeneration. Additionally, *in silico* ADMET analysis predicted favorable metabolic profiles for these compounds (Supplementary Table S5), supporting their suitability for CNS drug development.

Moreover, the docking studies identified several promising interactions. For instance, Villinol exhibited exceptional hydrogen bond formation with the TrkB receptor, involving residues such as Asp, His, and Ser, which are known for TrkB ligand binding (Supplementary Fig. S2 B). This suggests that AWE may exert neuroprotective and differentiation-promoting effects through TrkB activation. Furthermore, *in silico* analysis highlighted the potential of AWE compounds to target the Nogo-66 receptor, a key inhibitor of neurite outgrowth and axon regeneration⁵¹, providing a molecular basis for AWE's role in neuro-regeneration. Our findings, in conjunction with previous studies on curcumin⁵², suggest that components of agarwood extract may serve as ligands for the Nogo-66 receptor, thereby promoting neurite outgrowth and facilitating neuro-regeneration (Fig. 3). This highlights their potential as promising therapeutic agents for neurodegenerative disorders.

Additionally, docking results also indicated interactions between AWE phytochemicals and sigma-1 receptors, which mitigate cellular stress and promote neurite outgrowth. These findings align with previous studies showing the ability of plant extracts to modulate sigma-1 receptor expression and exert neuroprotective effects⁵³.

Furthermore, *in vitro*, experiment showed that AWE treatment significantly enhanced neuronal differentiation and regeneration, as indicated by the upregulation of key markers, including NeuN (mature neurons), GAP43 (axonal growth), synaptophysin (synaptic vesicles), and sigma-1 receptor (neuroprotection

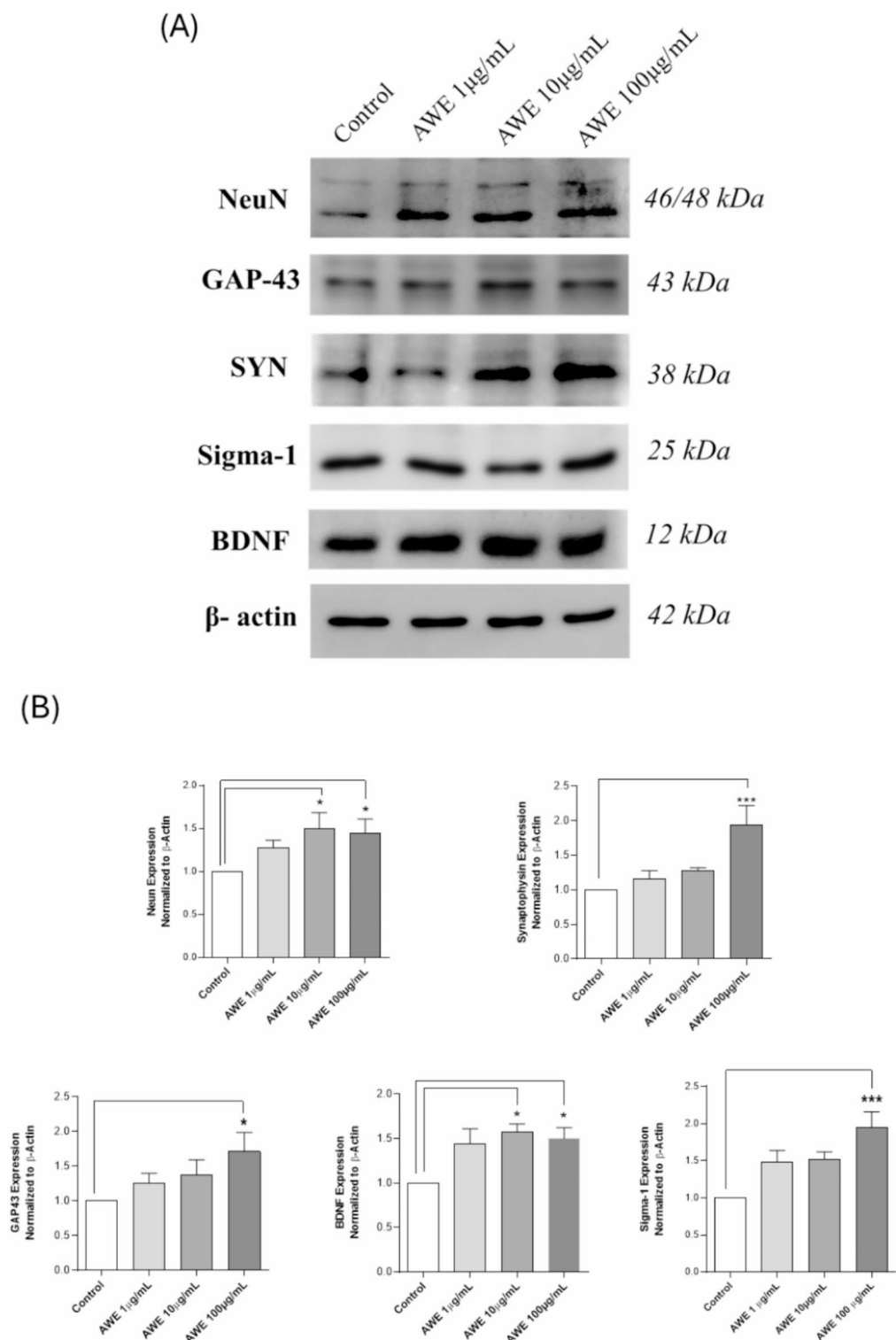
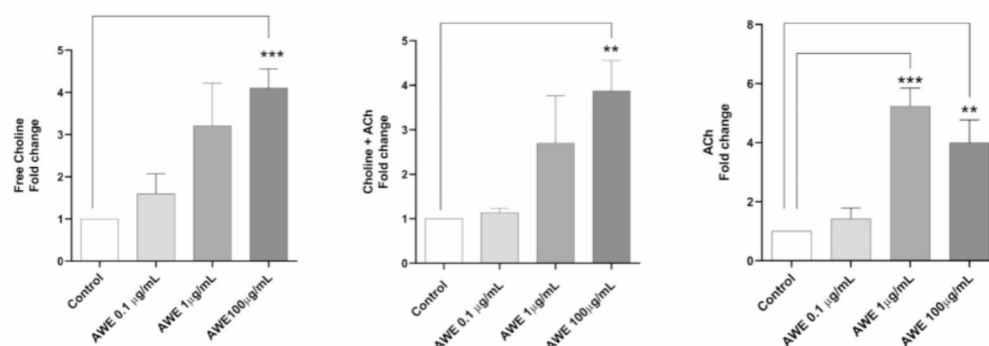
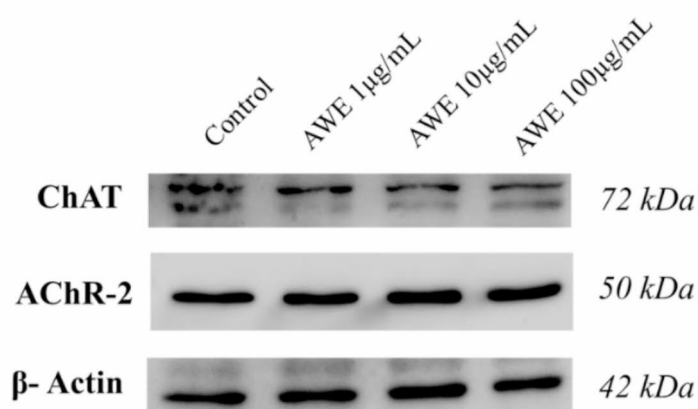


Fig. 4. The expression level of cellular differentiation marker proteins in HT-22 cells during the treatment of agarwood ethanol extract. (A) Representative western-blot images of NeuN, BDNF, Syn, Sigma-1 and GAP43 in HT-22 cells. (B) The graph represents the semi-quantified data using Image J. The data are obtained from three independent experiments and expressed as the means \pm SEM ($n = 3$). One-Way ANOVA, followed by Dunnett's post hoc test, evaluated the statistical differences; * $p < 0.05$, *** $p < 0.001$ compared with control group.

(A)



(B)



(C)

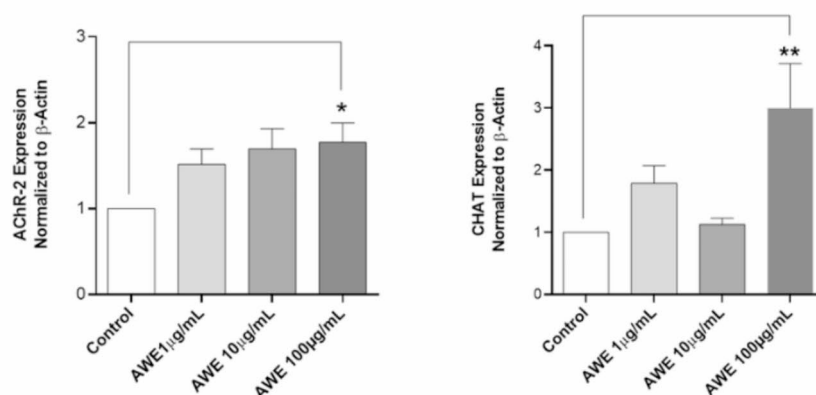


Fig. 5. The expression level of cholinergic marker proteins in HT-22 cells during the treatment of agarwood ethanol extract. (A) Bar graph representing the amount of choline and acetylcholine content in HT-22 cells treated with AWE and compared to control cells; (n = 4) (B) Representative western-blot images of ChAT and muscarinic acetylcholine receptor (mAChR/M2) in HT-22 cells. (C) The graph represents the semi-quantified data using Image J. The data are obtained from three independent experiments and expressed as the means \pm SEM (n = 3). One-Way ANOVA, followed by Dunnett's post hoc test, evaluated the statistical differences. * $p < 0.05$, ** $p < 0.01$, compared with control group.

and neuroplasticity). Moreover, AWE promoted cholinergic function, a critical aspect of neurodegenerative diseases characterized by cholinergic neuron loss. A dose-dependent increase in mAChR/M2 expression was observed (Fig. 4A,B), along with a significant upregulation of ChAT expression (Fig. 5A,B), suggesting its ability to modulate cholinergic signaling pathways. These findings are particularly relevant in studies showing decreased ChAT and M2 levels in Alzheimer's disease (AD) patients⁵⁴. Additionally, AWE significantly increased BDNF expression, a neurotrophin that supports cholinergic neurons and promotes neurogenesis (Fig. 4A,B). BDNF's role in neuroprotection and neuro-regeneration further emphasizes AWE's therapeutic potential in combating neurodegenerative diseases^{55,56}.

Our study shows that agarwood extract has significant potential for therapeutic applications. The phytochemicals identified in AWE could also have significant clinical and translational implications. As previous studies have demonstrated that bioactive compounds from plants like Ashwagandha (*Withania somnifera*) could improve cognitive functions during the clinical trial in humans⁵⁷, similarly *Ginkgo biloba* extract EGB 761 has shown efficacy in enhancing cognitive performance and neurological outcomes in post-ischemic stroke patients⁵⁸. These findings provide strong support for the therapeutic potential of plant-derived compounds in neurodegenerative conditions. Inspired by such evidence, our identified bioactive compounds may also possess the potential to be translated into clinical trials.

The present study significantly advances our understanding of the potent neuroprotective effects of AWE and its identified bioactive phytochemicals. In summary, our in vitro findings revealed that AWE effectively mitigates glutamate-induced toxicity in HT-22 cells, a critical mechanism implicated in neurodegenerative disorders like Alzheimer's and Parkinson's diseases. Additionally, AWE promoted neurite outgrowth and enhanced neuronal differentiation, indicating its role in neurogenesis. Moreover, the LC-MS analysis identified key compounds known for their antioxidant, anti-inflammatory, and cholinergic-enhancing properties, which likely contribute to the observed neuroprotective effects. However, future research should focus on elucidating the molecular mechanisms of these bioactive compounds, particularly their interactions with neurotransmitter systems, blood-brain barrier integrity, or epigenetic regulation. In silico approach conducted in the current study provided a preliminary insight into the binding affinity and interactions of phytochemicals with key receptors involved in neuronal differentiation and neuroprotection. However, advancing into in vivo studies will be crucial for determining optimal dosages, assessing biological responses, and ensuring the regulated use of these phytochemicals. A deeper understanding of the neuropharmacological potential of agarwood derived compounds could facilitate the development of novel therapeutic strategies for cognitive enhancement, neurogenesis, and the prevention of neurodegenerative disorders.

Data availability

The data generated or analyzed during the current study that are relevant to the results presented here have been included in this article and its supplementary information file.

Received: 22 December 2024; Accepted: 6 March 2025

Published online: 25 March 2025

References

1. Alam, J. et al. An insight of pharmacognostic study and phytopharmacology of *Aquilaria agallocha*. *J. Appl. Pharm. Sci.* **5**, 173–181 (2015).
2. Wang, S. et al. Agarwood essential oil displays sedative-hypnotic effects through the GABAergic system. *Molecules* **22**, 2190 (2017).
3. Chen, X. et al. Chemical composition and potential properties in mental illness (anxiety, depression and insomnia) of agarwood essential oil: A review. *Molecules* **27**, 4528 (2022).
4. Miraghaee, S. S., Karimi, I. & Becker, L. A. Psychobiological assessment of smoke of agarwood (*Aquilaria* spp.) in male rats. *J. Appl. Biol. Sci.* **5**, 45–53 (2011).
5. Dahham, S. S. et al. The anticancer, antioxidant and antimicrobial properties of the sesquiterpene β -caryophyllene from the essential oil of *Aquilaria crassna*. *Molecules* **20**, 11808–11829 (2015).
6. Lee, H.-Y. et al. The ethanol extract of *Aquilariae Lignum* ameliorates hippocampal oxidative stress in a repeated restraint stress mouse model. *BMC Complement. Altern. Med.* **17**, 1–12 (2017).
7. Lilienfeld, D. E. & Perl, D. P. Projected neurodegenerative disease mortality in the United States, 1990–2040. *Neuroepidemiology* **12**, 219–228 (1993).
8. Kumar, D., Ashraf, G. M., Bilgrami, A. L. & Hassan, M. I. Emerging therapeutic developments in neurodegenerative diseases: A clinical investigation. *Drug Discov. Today* **27**, 103305 (2022).
9. Paula-Lima, A. C., Brito-Moreira, J. & Ferreira, S. T. Deregulation of excitatory neurotransmission underlying synapse failure in Alzheimer's disease. *J. Neurochem.* **126**, 191–202 (2013).
10. Minakaki, G., Krainc, D. & Burbulla, L. F. The convergence of alpha-synuclein, mitochondrial, and lysosomal pathways in vulnerability of midbrain dopaminergic neurons in Parkinson's disease. *Front. Cell Dev. Biol.* **8**, 580634 (2020).
11. Malar, D. S. et al. Targeting sigma receptors for the treatment of neurodegenerative and neurodevelopmental disorders. *CNS Drugs* **37**, 399–440. <https://doi.org/10.1007/s40263-023-01007-6> (2023).
12. Fumagalli, F., Racagni, G. & Riva, M. The expanding role of BDNF: A therapeutic target for Alzheimer's disease?. *The Pharmacogenom. J.* **6**, 8–15 (2006).
13. Gil-Bea, F. J. et al. Cholinergic hypofunction impairs memory acquisition possibly through hippocampal Arc and BDNF downregulation. *Hippocampus* **21**, 999–1009 (2011).
14. Gonçalves, J. T., Schafer, S. T. & Gage, F. H. Adult neurogenesis in the hippocampus: from stem cells to behavior. *Cell* **167**, 897–914 (2016).
15. McKay, R. Stem cells in the central nervous system. *Science* **276**, 66–71 (1997).
16. Oliveira, S. L. et al. Functions of neurotrophins and growth factors in neurogenesis and brain repair. *Cytom. Part A* **83**, 76–89 (2013).
17. Lee, J., Seroogy, K. B. & Mattson, M. P. Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice. *J. Neurochem.* **80**, 539–547 (2002).
18. DiMasi, J. A., Feldman, L., Seckler, A. & Wilson, A. Trends in risks associated with new drug development: Success rates for investigational drugs. *Clin. Pharmacol. Ther.* **87**, 272–277 (2010).

19. DiMasi, J. A., Grabowski, H. G. & Hansen, R. W. Innovation in the pharmaceutical industry: New estimates of R&D costs. *J. Health Econ.* **47**, 20–33 (2016).
20. Brimson, J. M., Prasanth, M. I., Plaingam, W. & Tencomnao, T. Bacopa monnieri (L.) wettst. Extract protects against glutamate toxicity and increases the longevity of *Caenorhabditis elegans*. *J. Tradit. Complement. Med.* **10**, 460–470 (2020).
21. Kumaree, K. K., Anthikapalli, N. V. A. & Prasansuklab, A. In silico screening for potential inhibitors from the phytochemicals of *Carica papaya* against Zika virus NS5 protein. *F1000 Res.* **12**, 655 (2023).
22. Kabtni, S. et al. Influence of climate variation on phenolic composition and antioxidant capacity of *Medicago minima* populations. *Sci. Rep.* **10**, 8293 (2020).
23. Liu, W. et al. Influence of environmental factors on the active substance production and antioxidant activity in *Potentilla fruticosa* L. and its quality assessment. *Sci. Rep.* **6**, 28591 (2016).
24. Pattarachotananant, N. et al. *Aquilaria crassna* leaf extract ameliorates glucose-induced neurotoxicity in vitro and improves lifespan in *Caenorhabditis elegans*. *Nutrients* **14**, 3668 (2022).
25. Ruttkies, C., Schymanski, E. L., Wolf, S., Hollender, J. & Neumann, S. MetFrag relaunched: Incorporating strategies beyond in silico fragmentation. *J. Cheminf.* **8**, 1–6 (2016).
26. Zhu, Z.-J. et al. Liquid chromatography quadrupole time-of-flight mass spectrometry characterization of metabolites guided by the METLIN database. *Nat. Protoc.* **8**, 451–460 (2013).
27. Wang, Y. et al. PubChem: A public information system for analyzing bioactivities of small molecules. *Nucleic Acids Res.* **37**, W623–W633 (2009).
28. Guedes, I. A. et al. Drug design and repurposing with DockThor-VS web server focusing on SARS-CoV-2 therapeutic targets and their non-synonym variants. *Sci. Rep.* **11**, 5543 (2021).
29. Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **23**, 3–25 (1997).
30. Brimson, J. M., Brimson, S. J., Brimson, C. A., Rakkhitawathana, V. & Tencomnao, T. *Rhinacanthus nasutus* extracts prevent glutamate and amyloid- β neurotoxicity in HT-22 mouse hippocampal cells: Possible active compounds include lupeol, stigmaterol and β -sitosterol. *Int. J. Mol. Sci.* **13**, 5074–5097. <https://doi.org/10.3390/ijms13045074> (2012).
31. Meijering, E. et al. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytom. Part A J. Int. Soc. Anal. Cytol.* **58**, 167–176 (2004).
32. Brimson, J. M., Safrany, S. T., Qassam, H. & Tencomnao, T. Dipentylammonium binds to the sigma-1 receptor and protects against glutamate toxicity, attenuates dopamine toxicity and potentiates neurite outgrowth in various cultured cell lines. *Neurotox. Res.* **34**, 263–272. <https://doi.org/10.1007/s12640-018-9883-5> (2018).
33. Kapogiannis, D. & Mattson, M. P. Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease. *Lancet Neurol.* **10**, 187–198 (2011).
34. Rasool, M. et al. Recent updates in the treatment of neurodegenerative disorders using natural compounds. *Evid. Based Complement. Altern. Med.* **2014** (2014).
35. Mandel, S. A., Amit, T., Weinreb, O., Reznichenko, L. & Youdim, M. B. Simultaneous manipulation of multiple brain targets by green tea catechins: A potential neuroprotective strategy for Alzheimer and Parkinson diseases. *CNS Neurosci. Ther.* **14**, 352–365 (2008).
36. Chen, B. et al. Neuroprotective effects of natural compounds on neurotoxin-induced oxidative stress and cell apoptosis. *Nutr. Neurosci.* **25**, 1078–1099 (2022).
37. Supasuteekul, C. et al. Neuritogenic and neuroprotective constituents from *Aquilaria crassna* leaves. *J. Food Biochem.* **41**, e12365 (2017).
38. Borris, R. P., Blaskó, G. & Cordell, G. A. Ethnopharmacologic and phytochemical studies of the Thymelaeaceae. *J. Ethnopharmacol.* **24**, 41–91 (1988).
39. Hashim, Y.Z.H.-Y., Kerr, P. G., Abbas, P. & Salleh, H. M. *Aquilaria* spp.(agarwood) as source of health beneficial compounds: A review of traditional use, phytochemistry and pharmacology. *J. Ethnopharmacol.* **189**, 331–360 (2016).
40. Ma, J. et al. 2-(2-Phenylethyl) chromone-enriched extract of Chinese agarwood (*Aquilaria sinensis*) inhibits atherosclerosis progression through endoplasmic reticulum stress-mediated CD36 expression in macrophages. *J. Ethnopharmacol.* **320**, 117411 (2024).
41. Hussain, R., Zubair, H., Pursell, S. & Shahab, M. Neurodegenerative diseases: Regenerative mechanisms and novel therapeutic approaches. *Brain Sci.* **8**, 177 (2018).
42. Cui, Z. et al. Therapeutic application of quercetin in aging-related diseases: SIRT1 as a potential mechanism. *Front. Immunol.* **13**, 943321 (2022).
43. Grewal, A. K. et al. Mechanistic insights and perspectives involved in neuroprotective action of quercetin. *Biomed. Pharmacother.* **140**, 111729 (2021).
44. Alkholfi, F. K. et al. Effects of tiliroside and lisuride co-treatment on the PI3K/Akt signal pathway: Modulating neuroinflammation and apoptosis in Parkinson's disease. *Biomedicines* **11**, 2735 (2023).
45. Singh, L., Kaur, N. & Bhatti, R. Neuroprotective potential of biochanin-A and review of the molecular mechanisms involved. *Mol. Biol. Rep.* **50**, 5369–5378 (2023).
46. Chaurasiya, N. D., Leon, F., Muhammad, I. & Tekwani, B. L. Natural products inhibitors of monoamine oxidases—Potential new drug leads for neuroprotection, neurological disorders, and neuroblastoma. *Molecules* **27**, 4297 (2022).
47. Konrath, E. L., Passos, C. D. S., Klein-Júnior, L. C. & Henriques, A. T. Alkaloids as a source of potential anticholinesterase inhibitors for the treatment of Alzheimer's disease. *J. Pharm. Pharmacol.* **65**, 1701–1725 (2013).
48. Alaamery, M. et al. Role of sphingolipid metabolism in neurodegeneration. *J. Neurochem.* **158**, 25–35 (2021).
49. Blondeau, N. et al. Subchronic alpha-linolenic acid treatment enhances brain plasticity and exerts an antidepressant effect: A versatile potential therapy for stroke. *Neuropsychopharmacology* **34**, 2548–2559 (2009).
50. Xu, Q. et al. Neuroprotective effects of b-type cinnamon procyanidin oligomers on mpp+-induced apoptosis in a cell culture model of parkinson's disease. *Molecules* **26**, 6422 (2021).
51. GrandPré, T., Li, S. & Strittmatter, S. M. Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature* **417**, 547–551 (2002).
52. Yin, H. et al. Effects of curcumin on hippocampal expression of NgR and axonal regeneration in A β -induced cognitive disorder rats. *Genet. Mol. Res.* **13**, 2039–2047 (2014).
53. Brimson, J. M., Prasanth, M. I., Isidoro, C., Sukprasansap, M. & Tencomnao, T. *Cleistocalyx nervosum* var. *paniala* seed extracts exhibit sigma-1 antagonist sensitive neuroprotective effects in PC12 cells and protects *C. elegans* from stress via the SKN-1/NRF-2 pathway. *Nutr. Healthy Aging* **6**, 131–146 (2021).
54. Mash, D. C., Flynn, D. D. & Potter, L. T. Loss of M2 muscarinic receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. *Science* **228**, 1115–1117 (1985).
55. Pringle, A., Sundstrom, L., Wilde, G. & Williams, L. Brain-derived neurotrophic factor, but not neurotrophin-3, prevents ischaemia-induced neuronal cell death in organotypic rat hippocampal slice cultures. *Neurosci. Lett.* **211**, 203–206 (1996).
56. Almeida, R. et al. Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death Differ.* **12**, 1329–1343 (2005).
57. Abedon, B., Auddy, B., Hazra, J., Mitra, A. & Ghosal, S. A standardized *Withania somnifera* extract significantly reduces stress-related parameters in chronically stressed humans: A double-blind, randomized, placebo-controlled study. *Jana* **11**, 50–56 (2008).

58. Cui, M. et al. Ginkgo biloba extract EGb 761® improves cognition and overall condition after ischemic stroke: Results from a pilot randomized trial. *Front. Pharmacol.* **14**, 1147860 (2023).

Acknowledgements

K.K.K. and K.V. wishes to thank the Second Century Fund (C2F) Postdoctoral Fellowship, Chulalongkorn University, Thailand, for the support. We would like to express our sincere thanks to Supakorn Yuenyongwannachot of Siam Agarwood (2020) Co., Ltd., Thailand for providing the knowledge of agarwood that inspired us to develop this project.

Author contributions

Conceptualization, J.M.B. and T.T.; Investigation and Formal analysis, K.K.K., K.V., and J.M.B.; Writing – Original Draft Preparation, K.K.K., J.M.B. and A.P.; Writing – Review & Editing, K.K.K., J.M.B., K.V., S.C., T.T. and A.P.; Methodology, K.K.K., J.M.B. and K.V.; Visualization, K.K.K., J.M.B. and K.V.; Project administration, A.P.; Resources and Funding acquisition, T.T. and A.P. All authors have read and approved the final version of the manuscript.

Funding

This research is funded by the Thailand Science Research and Innovation Fund Chulalongkorn University.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-93462-y>.

Correspondence and requests for materials should be addressed to T.T. or A.P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025