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Research Article

Hexane extract of *Telosma cordata* enhances neurite outgrowth via the epigenetically regulated genes expression in neuronal cells

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Abstract

Telosma cordata has long been consumed as foods and herbal medicine. The aim of this research is to investigate the potential biological activities of *Telosma cordata* flower (TCF) extracts and their molecular mechanisms in the treatment of neurological diseases, especially dementia. In general, the plant samples were successfully identified through DNA barcoding regions, utilizing *matK*, *trnH-psbA,* and *rbcL* markers. The ethanol extract and its fraction exhibited significantly high acetylcholinesterase inhibitory activity. Moreover, hexane extract of *T. cordata* displayed the most potent neurotrophic activity in a preliminary cell-based screening based on C6 cells neurite outgrowth. Additionally, hexane extract of *T. cordata* demonstrated the highest antioxidant activity. Finally, the hexane extract of *T. cordata* upregulated the expression of BDNF, NGF and acetyl H3 in C6 cells, affecting both mRNA and protein levels. These findings indicate that *Telosma cordata* could be a strong candidate for developing pharmacological drugs to treat dementia and neurodegenerative diseases.

Keywords: AChE inhibitory; *BDNF;* neurite outgrowth; neurological diseases; *NGF* genes; *Telosma cordata*

Introduction

Dementia is a clinical syndrome primarily that predominantly manifests as a decline in cognitive functions such as memory, learning, thinking and social abilities. It has emerged as a significant social and

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medical challenge that demands urgent attention to understand its underlying pathogenesis (Alzobaidi *et al*., 2021; Wang *et al*., 2010). According to the World Alzheimer Report 2022, the number of individuals worldwide suffering from dementia was estimated to be over 55 million, and this quantity is predicted to grow by about 50% in 2030 and further escalate to 139 million by 2050 (International, 2018). The percentage of citizens above the age of 65 with dementia stands at approximately 7%, while in developed countries, this rate is slightly higher, ranging from 8% to 10% due to longer life spans (Prince *et al*., 2013; WHO, 2022).

Alzheimer's disease (AD) stands as the predominant manifestation of dementia, characterized by the accumulation of beta-amyloid (forming amyloid plaques) and the gradual deterioration of microtubules. This leads to the loss of synaptic connections, impaired communication, and the apoptosis of neuronal cells (Alzobaidi *et al*., 2021). Although not entirely comprehended, the progression of the disease is believed to be connected to the presence of neurofibrillary tangles and senile plaques. These aggregates are composed of hyperphosphorylated tau protein and amyloid β (Aβ) of varying sizes, respectively (Balkrishna *et al*., 2019). Especially, AD is associated with a substantial reduction in the levels of acetylcholine (ACh) due to increased breakdown. ACh is a crucial neurotransmitter responsible for transmitting signals across synapses. After fulfilling its signaling role, ACh is hydrolyzed into choline and acetyl groups through the action of the enzyme acetylcholinesterase (AChE). The utilization of AChE inhibition as a promising therapeutic approach for the management of neurological disorders has been proposed. The abundance of plants in nature offers a promising source of AChE inhibitors (Pagliosa *et al*., 2010; Seong *et al*., 2017).

The implementation of neural-regeneration strategies aiming at reconstructing neuronal and synaptic networks holds potential as a therapeutic approach for AD. Neurogenesis, characterized by neurite outgrowth, is one of the neural-regeneration processes crucial for this purpose. It involves the branching of neurites, subsequent axonal, and dendritic elongation in maturing neurons. This fundamental process plays a vital role in constructing functional neuronal networks and is regarded as a hallmark of neuronal differentiation (Rangsinth *et al*., 2021). Neurite outgrowth serves as a crucial initial step in the formation of the neuronal network. Therefore, drug discovery and development efforts targeted at promoting neurite outgrowth are an essential for understanding molecular mechanisms and developing effective treatments for axonal and synaptic damages (Gao *et al*., 2019; Mitre *et al*., 2017; Rigby *et al*., 2020). Neurotrophic factors, which encompass Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF) and acetyl H3 are known to extend neurite outgrowth in neuronal cells and play critical roles in the differentiation, survival, and function of neurons (Park *et al*., 2015).

Recently, studies on natural compounds derived from medicinal plants, which are considered a new therapeutic agent for managing neurodegenerative diseases and neurological disorders, it has shown a dramatic increase. Many of these compounds have demonstrated their neurotrophic properties by inhibiting acetylcholinesterase and stimulating the growth of neurites in neuronal cells (Duangjan *et al*., 2021; Li *et al*., 2022; More *et al*., 2012).

Telosma cordata (Burm. F.) Merr., commonly known as 'Thien ly' in Vietnam, is classified as a member of Apocynaceae family. This plant is primarily grown in Southeast Asia, with significant cultivation in countries such as Vietnam, Thailand, and Malaysia (Jing, 2014; Lim, 2014). *T. cordata* is not only collected for use as foods, but is also renowned for its medicinal properties (Wang *et al*., 2022). It holds a significant place in traditional medicine, where its essential oils extracted from leaves are utilized for pain-removing, wound healing, scabies treatment, ulcer management, headache, and nerve relaxation (Jing, 2014). Moreover, *T. cordata* has been reported to possess antimicrobial (Buathong and Duangsrisai, 2023), antidiabetic (Cajuday and Amparado, 2014), and antioxidant (Ngoitaku, 2016) properties. Studies have also suggested its capabilities to mitigate the danger of cardio-vascular disease, anti-cancers, and treating conjunctivitis (Huang *et al*., 2010). Furthermore, in Vietnam, native people have used this plant as herbal tea to improve sleep quality, which suggests its potential as a supplement with sedative and neuroprotective effects. Chen Li *et al*. (2022)

demonstrated that the ethanol extract from *T. cordata* flowers (TCF) contained a total phenolic content of 40.51 mg GAE/g and indicated significant antioxidant activities. However, there was no mention of its neuronal protection activity, and the underlying molecular mechanisms involved in this effect remain incompletely understood elucidated.

Hence, in this report, our purpose was to investigate the acetylcholinesterase inhibitory activity, neurite outgrowth activity, and antioxidant activity of TCF extracts. Additionally, we examine the influence of TCF extracts on the expression of BDNF, NGF and acetyl H3 markers in C6 neuronal cells. The present results have provided insights into the molecular mechanisms related to cognitive improvement.

Materials and Methods

Collection and identification of T. cordata

We grabbed the samples of *T. cordata* in Sapa, Laocai province in South-west Vietnam in April 2021. The identification of *T. cordata* samples was based on a comparative morphological method following Endress *et al*.'s guidelines (Endress *et al*., 2014). Additionally, DNA barcoding was used, and the nucleotide sequences of the *matK*, *trnH-psbA,* and *rbcL* genes were employed for identification purposes. The collected plant samples were dried to a constant weight and stored at temperature of -20 °C for the next experiments.

DNA extraction, PCR amplification, and sequencing for identification of samples

We employed the CTAB method with a slight modification to extract the total DNA of *T. cordata* (Aboul-Maaty and Oraby, 2019). Then, we conducted the polymerase chain reaction (PCR) amplifications in 20 µL mixture using Phusa master mix 2× (Phusa Biochem, Vietnam). The primers utilized for sample identification were detailed as follows: *matK* (forward) 5'- ACCGTACTTTTATGTTTACGAGC -3' (reverse) 5'- TCCATCTGGAAATTTCGTTCA-3', *trnH-psbA* (forward) 5'- CGCGCATGGTGGATTC-ACAATCC -3' (reverse) 5'- GTTATGCATGAACGTAATGCTC -3', *rbcL* (forward) 5'-GCA-AGTGTTGGATTCAAAGCTGGTG -3' (reverse) 5'- TGGTTGTGAGTTCACGTTCT -3'. The electrophoresis on a 1% agarose gel was carried out to examine the PCR products and purified using 100% ethanol. The *matK*, *trnH-psbA,* and *rbcL* fragments' nucleotide sequences were determined using Sanger method and analyzed based on the BLAST in NCBI (NCBI.).

Preparation of T. cordata extracts

The fresh flowering bodies of *T. cordata* were cut off and then freeze-dried for 48 hours. After drying, the samples were soaked in 90% ethanol. The ethanol extract was carried out through refluxing (55 °C-65 °C) and repeated three times. The ethanol solvent was subsequently removed by employing a rotary evaporator to yield the ethanol extract. The liquid-liquid extraction method was used to extract hexane, ethyl acetate, and butanol fractions from the samples following the modified Kwon et al. protocol (Seo *et al*., 2016). Following that, each extract underwent low-pressure vaporization utilizing a rotary evaporator at 55 ± 2 °C).

Maintenance of neuronal cells

The C6 cell line, drived from a rat glial tumor and procured from the American Type Culture Collection (ATCC; MD, USA), was nurtured in Dulbecco′s Modified Eagle′s Medium (Sigma-Aldrich). The medium was added 10% FBS (Gibco) and 1% penicillin/streptomycin (100× concentration) under standard conditions of 37 °C and 5% CO_2 in a humidified environment.

Acetylcholinesterase (AChE) inhibitory activity

Each *T. cordata* extract was evaluated AChE inhibition by the modified Ellman's method *(El-Sayed et al*., 2020; Youdim, 2022). Briefly, 10 µL of the sample with different concentrations, 15 µL of 0.1 M phosphate buffer (pH 7.7), 125 µL of 3 mM DTNB (17.838 mg of DTNB in 15 mL phosphate buffer, pH 7.7) and 25 µl of 15 mM ACTI (21.675 mg in 5 mL of phosphate buffer, pH 7.7) were mixed. The mixture was was placed in an incubator at a temperature of 37 °C for 10 minutes. After the pre-incubation, 25 µL of enzyme AChE (0.22 U/mL) was added to the solution and incubated at 37 °C for 15 min. Enzyme activity was measured in a 96-well plate at 410 nm. The inhibition rate was calculated by following formula:

$$\text{Inhibition rate (\%)} = \frac{A_S - A_B}{A_C - A_B} x\ 100, \qquad (1)$$

where A_S, A_B, A_C were the absorbance of the investigated extract sample, blank and control samples, respectively. The inhibitory concentrations (IC_{50}) were estimated based on monitoring the effect of increasing concentrations of these samples in the experiment on the inhibition values. The positive control used in the experiments was berberine chloride. All the assays were repeated three times (Leimann *et al*., 2023).

Neurite outgrowth

The human glial C6 cell line was introduced into 24-well plates to reach the population of 8000 cells in each well. The plates, afterwards, was incubated overnight with various non-toxic concentrations of *T. cordata* extracts. Ethanol, ethyl acetate, butanol and hexane extracts of *T. cordata* were added at final concentrations of 2.5 µg/mL and 5 µg/mL. After a 24-hour incubation period, neurite length was observed and measured under 20× magnification using a Nikon Eclipse Ti-U microscope from Japan). At least 5 randomly selected areas (100-200 cells/well) were captured in each well under the microscope (Nikon Eclipse Ti-U, Japan). Within these chosen regions, the length of neurite was examined in a total of 100 cells, employing ImageJ software. The experiments were repeated three times (Rangsinth *et al*., 2021).

Antioxidant activity using DPPH method

The antioxidative potential of *T. cordata* extracts was assessed using the DPPH (1,1-diphenyl-2-pycrylhydrazile) radical scavenging method as described in (Chen *et al*., 2020), with the following modification. Different concentrations of *T. cordata* extracts (e.g. 10, 100, 250, 500, 1000 µg/mL) were prepared in DMSO. Ascorbic acid (Vitamin C, from Sigma Co.) served as the standard antioxidant, and four different concentrations of vitamin C (4, 20, 50, 100 µg/mL) were prepared in distilled deionized water. Each extract sample was mixed with 0.25 µM DPPH solution in methanol. Subsequently, we incubated the resulted mixture in a dark chamber at room temperature for 30 min, prior to measuring the absorbance at 517 nm. The blank consists of 100 µl of methanol and 100 µl of DPPH. Finally, we determined the rate of inhibition by the following equation (2):

$$Inhibition\ DPPH\ (\%) = \frac{A_C - A_S}{A_C} \times 100, \qquad (2)$$

where A_C, A_S were the absorbance of the investigated control sample and extract sample, respectively.

Quantitative real-time PCR (qRT-PCR)

To analyze the relative expression levels of *T. cordata* extract samples on the target genes, we conducted the qRT-PCR reaction. Total RNA from C6 cells was extracted using the Trizol method (Sigma Aldrich). A total of 1 µl RNA was used in a 15 µL first-strand cDNA synthesis with the GoScript™ Reverse Transcription System (Promega, Madison, USA). Relative gene expression was measured using the GoTaq® qPCR Master Mix (Promega, Madison, USA). The RT-PCR primer sequences were as follows: BDNF (forward) 5'- ACC CTG AGT TCC ACC AGG TG -3' (reverse) 5'- TGG GCG CAG CCT TCA T -3'; NGF (forward) 5'- TGG ACC CAA GCT CAC CTCA -3' (reverse) 5'- GGA TGA GCG CTT GCT CCT -3'; RPL (forward) 5'- TCAGACAGTGATTACACCGAGTTC -3' (reverse) 5'- GCCAGTAGAGACAAAAAGGCAAGA -

3'; GAPDH (forward) 5'-ATCACCATCTTCCAGGAGCGA-3' and (reverse) 5'-AGTTGTCATGGATGACCTTGGC-3'. The reaction was conducted following the manufacturer's instructions. qRT-PCR reactions and analyses were performed using Rotor-Gene Q (Qiagen, Düsseldorf, Germany) (Ashouri and Farshbaf Pourabad, 2021).

Western blotting

The hexane TCF extract was treated in C6 cells with different concentrations for 48 h. After that, this protein was collected by utilizing lysis buffer. To determine protein concentration, the Coomassie Protein Assay Reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA) was employed. The following antibodies were used to detect neural factors: acetyl H3 (1:2000) (AH01432; Invitrogen, Waltham, MA, USA), BDNF (1:5000) (MA5-34960; Invitrogen, Waltham, MA, USA), NGF (1:1000) (N5415; SigmaAldrich, St. Louis, Missouri, USA). We used the α-tubulin antibody (ab7291; Abcam, Cambridge, MA, USA) as a reference control. To detect the antibodies, we employed a secondary antibody that was linked to horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA). This was followed by visualization using the PierceTM ECL Western Blotting Substrate Kit (Thermo Fisher Scientific, Waltham, MA, USA). Following, the luminescence imaging was performed with the Image Quant LAS 500 system. Quantitation of bands was performed using ImageJ software. And then, the ratio between the density bands in each sample relative to the density of the reference control band was measured. All results were replicated in a minimum of three separate assays.

Statistical analysis

The results (mean ± standard deviation (SD)) were obtained from three separate measurement. We then used both Student's t-test and one-way analysis of variance (ANOVA) to perform statistical evaluations, followed by GraphPad Prism 10 software. The p-value below 0.05 was considered statistically significant.

Results

The identification of Telosma cordata species from collected samples in Sapa, Laocai, Vietnam

Plant samples were collected from Sapa, Laocai, Vietnam, in different areas based on morphological characteristics. As shown in Figure 1 (A-C), the obtained samples were identified as the *Telosma cordata* species through a comparative morphological method following by Endress *et al*. (2014). Moreover, the samples were molecularly identified using DNA barcoding. The results demonstrated successfully amplification of the medicinal plant samples using primer pairs specific to the three DNA barcoding regions. The nucleotide sequence lengths of the plant samples for the three regions of the *matK, trnH-psbA,* and *rbcL* genes are 883 bp, 513 bp, and 573 bp, respectively. The obtained sequences from the collected samples were tested for similarity with the available sequences on Genbank using the BLAST tool. The Figure 2 (A-B) showed that the sample sequences of the three barcode genes closely aligned with the reference database, indicating high similarity to species in genus *Telosma*. Specifically, the *matK* region possessed the highest identification efficiency of 99.42% with *Telosma cordata plastid* species, partial genome (GenBank accession number **KF539853.1**), and *Telosma cordata maturase (matK)* gene, partial cds – DQ660551.1 - (99.42%) based on Genbank on the NCBI website (Figure 2B). In addition, when comparing the *rbcL* sequences extracted from the Sapa samples with the *rbcL* sequence of *Telosma cordata* (GenBank accession number KF539853.1), a notable similarity percentage of 99.46% was uncovered. Moreover, similar results were observed with *trnH-psbA* barcode, with a a sequence similarity of 97.63% to *Telosma cordata plastid* species, partial genome (KF539853.1), *Telosma accedens* – MW226392.1 (90.00%), and *Telosma pallida* – MW226393.1 (86.50%). These results conclude that the collected medicinal plant samples are members of the genus *Telosma*, specifically belonging to the species *Telosma cordata*.

A B C

Figure 1. Some morphological characteristic of (A) *T. cordata* flower: the *T. cordata* flower after harvested, (B) flowers, leaves and trunk of plants at the mature stage, (C) dried flowers

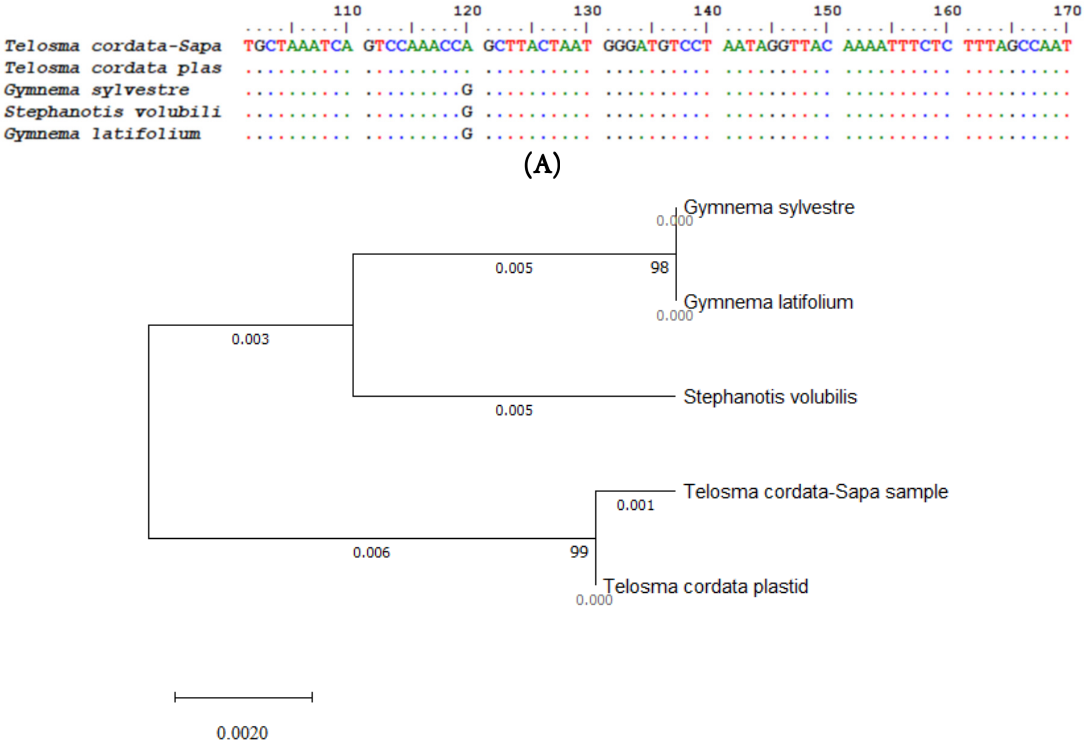


Figure 2. (A) Alignment of maturase (matK) barcode region of five species (B) Phylogenetic tree of 4 species from a comparison of sample nucleotide sequences using MEGA method

Inhibition of acetylcholinesterase (AChE) by Telosma cordata extracts

To screen for inhibitors from *TCF* extracts, we carried out testing of the AChE inhibitory activities of the extracts using *in vitro* Ellman assay, with berberine chloride as a positive control. The results are presented in Figure 3. The inhibitory activity of *T. cordata* extracts against AChE demonstrated a dose-dependent pattern. The IC_{50} determinations confirmed that all four extracts were able to inhibit AChE to different levels,

with the following order of potency: EtOAc > BuOH > EtOH > hexane > berberine chloride. As shown in Figure 3, *T. cordata* (EtOH) and *T. cordata* (BuOH) extracts inhibited AChE at IC_{50} values of 8.352 ± 0.12 µg/mL and 13.474 ± 0.48 µg/mL, respectively. *T. cordata* (hexane) extract exhibited the strongest AChE inhibitory potency, displaying an IC_{50} value of 6.196 ± 0.02 µg/mL. Conversely, the AChE inhibitory activity of T. cordata (EtOAc) extract was the weakest, demonstrating an IC_{50} value of 67.546 ± 3.78 µg/mL. These results reveal that *TCF* extracts have a good activity for AChE inhibition.

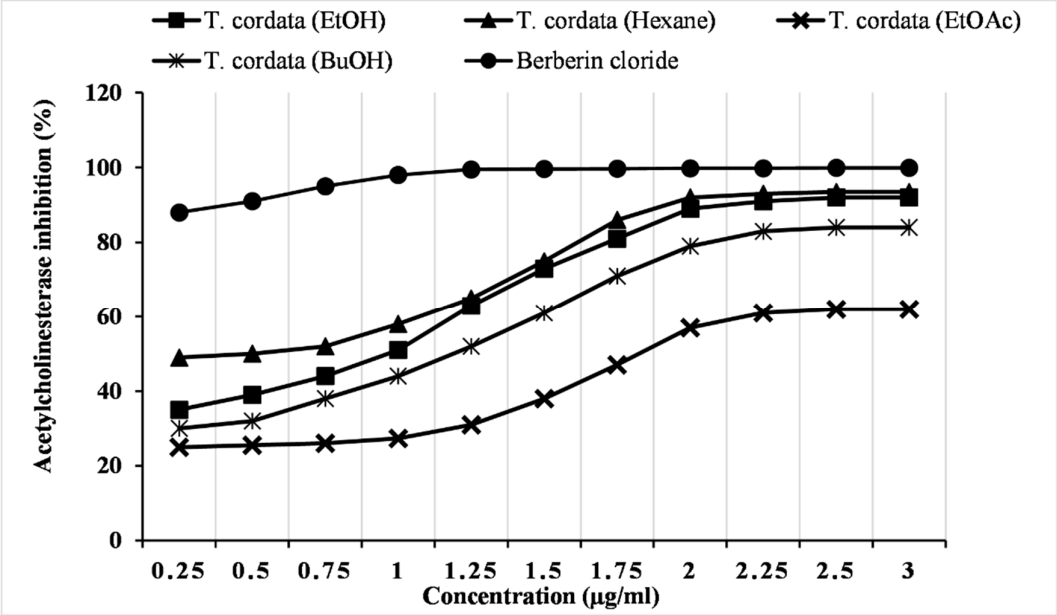


Figure 3. Percentage inhibition of acetylcholinesterase by the different fraction of the *T. cordata* extracts and Berberine chloride

Hexane extract of Telosma cordata enhances neurite outgrowth

To evaluate the impact of herbal extracts on neurite outgrowth, the C6 cells were exposed to two distinct concentrations of *T. cordata* extracts (2.5 µg/mL and 5 µg/mL) and observed using a confocal microscope at 20× magnification. As a negative control, dimethyl sulfoxide (DMSO) was employed. Neuronal morphology was observed following treatment with varying concentrations for 24 hours, as depicted in Figure 4A and B. The results showed that *T. cordata* extracts significantly increased the average neurite length compared to 0.1% DMSO control. At a concentration of 2.5 µg/mL, the hexane extract displayed the highest neurite outgrowth-promoting activities, while the BuOH extract showed the lowest neurite outgrowth-promoting activities, with average neurite length of 41.74 ± 4.26 µm and 25.71 ± 1.49 µm, respectively. Similarly, at a concentration of 5 µg/mL, the hexane extracts still had the highest neurite outgrowth-promoting activities, while the EtOH extract showed the lowest neurite outgrowth-promoting activities, with average neurite length of 43.18 ± 5.62 µm and 23.54 ± 2.43 µm, respectively (Figure 4B). The data shown in Figure 5 performs that the C6 cell numbers treated with T. cordata extracts were found to be considerably increased about neurite lengths in comparison with control cells. The herbal medicinal extracts exhibited a concentration-dependent enhancement in neurite outgrowth-promoting activity. Therefore, the hexane extract demonstrated the strongest neurite outgrowth-extending activities at both concentrations of 2.5 µg/mL and 5 µg/mL. Hence, we selected *T. cordata* (hexane) for the subsequent assays.

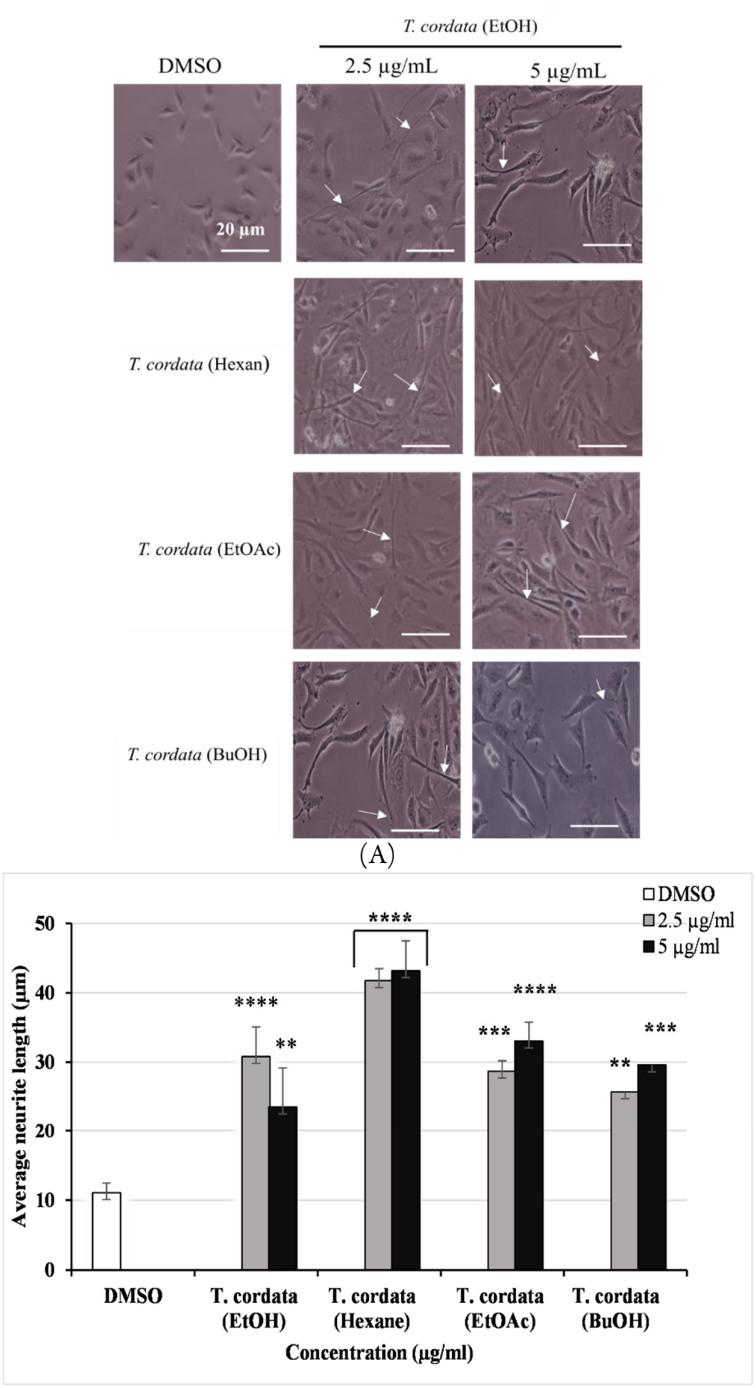


Figure 4. *Telosma cordata* extracts for neurite promoting activity in C6 cells. A: Immuno-stained image of C6 cells showed neurite outgrowth following treatment with *T. cordata* extracts (at different concentrations), and 0.1% DMSO (vehicle)

Scale bar represents 20 µm. Photomicrographs of representative microscope fields were taken with a 20× objective. B: Graph describing the average length of neurites and optimized concentration of *T. cordata* extracts. Neurites were measured using ImageJ software on bright-field images of C6 cells, taken 48h after treatment. Statistical significance compared with vehicle: *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$ and ****$p < 0.0001$ (ANOVA). Data points represent the mean ± SD, N=100.

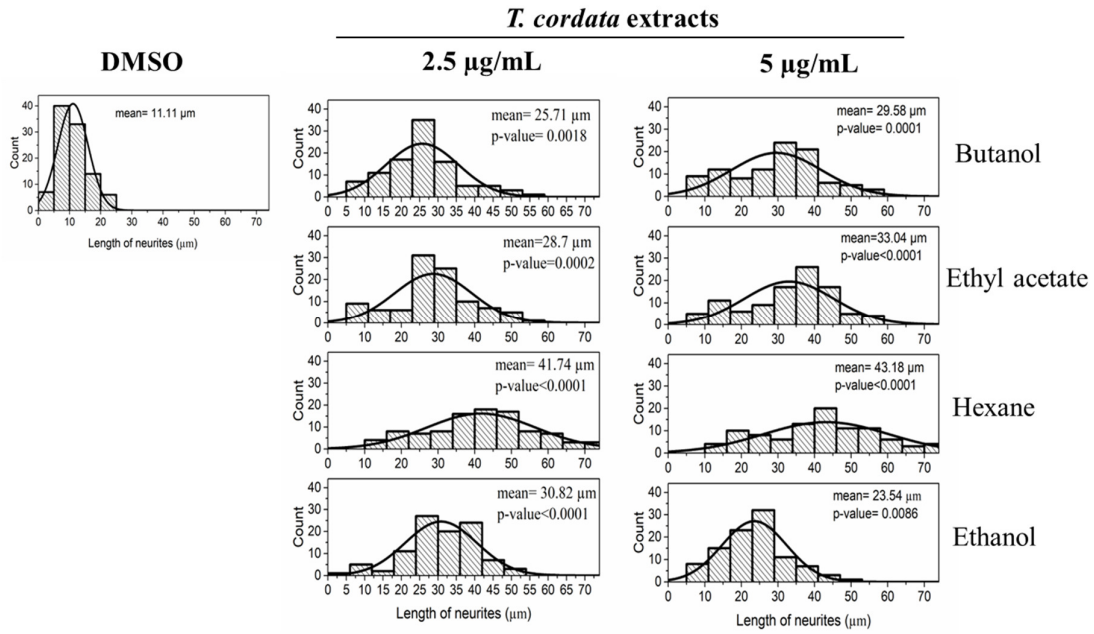


Figure 5. Histograms of neurite length of C6 cells following treatment with *T. cordata* extracts at different concentrations, and 0.1% DMSO (vehicle). The values of mean length ± SD (n = 3) are shown in parentheses

Differences between groups were examined for statistical significance using the Graphpad prism method

Antioxidant activity of Telosma cordata extracts

The antioxidant activity of Telosma cordata extracts was assessed using the DPPH method. Figure 6 illustrate the antioxidant capacity of *T. cordata* hexane, ethyl acetate and butanol extracts, respectively, for scavenging DPPH radicals. All three *T. cordata* extract samples (hexane, ethyl acetate, and butanol) showed lower IC_{50} values of 0.713, 0.781, and 0.827 mg/mL, respectively, compared to the reference standard, vitamin C (0.012 mg/mL). Amongst the extracts, the hexane extract displayed the highest antioxidant activity, as evidenced by its lowest IC_{50} value (0.713 mg/mL). The significant antioxidant activity of these *T. cordata* extracts suggests that they are rich in antioxidants that effectively inhibit the DPPH free radicals. These antioxidants are likely polyphenolic compounds, well-known for their efficient free radical scavenging abilities (Platzer *et al*., 2022). This result indicates that *Telosma cordata* extracts hold significant potential as a valuable and potent natural source of antioxidants.

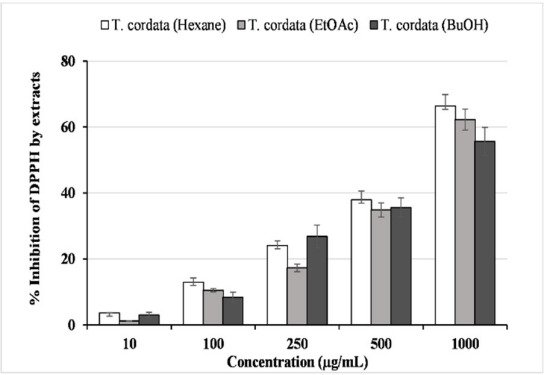


Figure 6. Antioxidant activity of *T. cordata* extracts at different concentration based on DPPH method

Ascobic acid was used as a positive control

Hexane extract of Telosma cordata enhanced the neural-related gene expression

To investigate the effective neuroprotective activity of *T. cordata* (hexane) extract, we further examined its ability to modulate the expression of neuroprotective genes, including NGF, BDNF, and RPL. The relative gene expression levels were quantified as fold changes in the treated groups relative to the untreated control using RT-PCR (Figure 7). The RT-PCR results revealed that the expression of NGF exhibited a dose-dependent augmentation in the treated groups subjected to *T. cordata* (hexane) extract, with fold changes of 1.27±0.24, 1.54±0.28 and 3.39±0.41 for concentrations of 1 µg/mL, 2.5 µg/mL, and 5 µg/mL, respectively, compared to controls (DMSO), which showed a fold change of 1.07±0.15 (Figure 7). Following 24-hour treatment of the cells with different concentrations of *T. cordata* (hexane) extract, there was a substantial increase in NGF expression. The results of the gene expression analysis are presented in Figure 7.

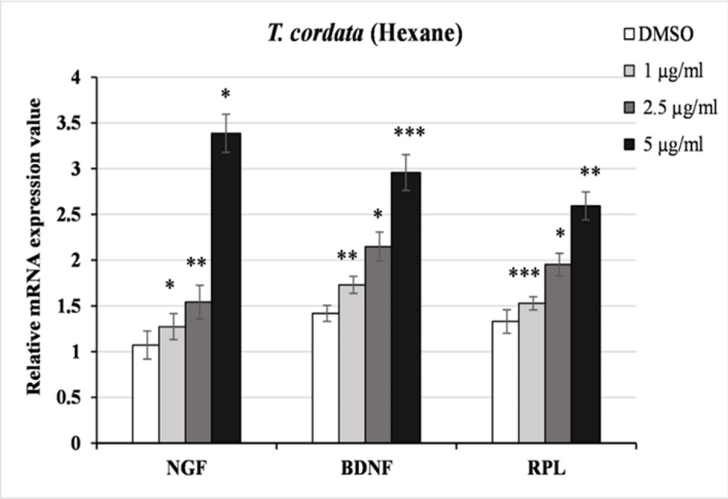
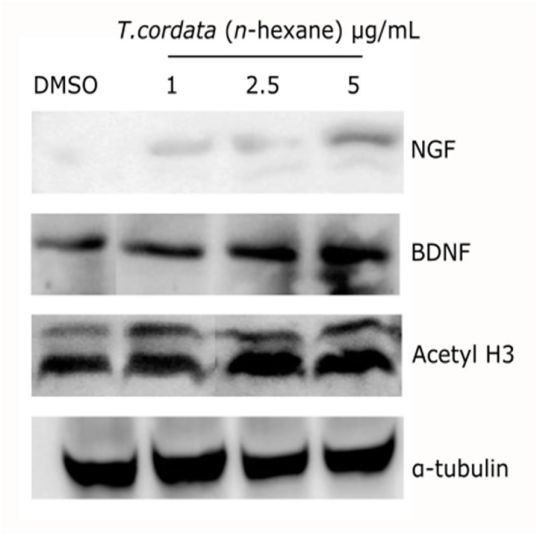


Figure 7. Extension of mRNA transcript levels of neurotrophic factor genes by *T. cordata* extracts (*T. cordata* hexan 1 µg/ml; *T. cordata* hexan 2.5 µg/ml; *T. cordata* hexan 5 µg/ml)

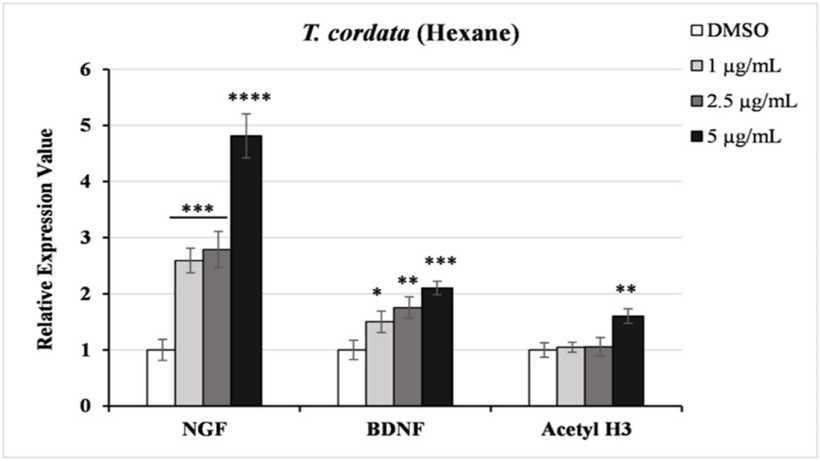
Relative mRNA expression of markers NGF, BDNF and RPL on C6 cells after treatment with different concentrations of hexane extract of *T. cordata*. Quantitative data were obtained from three independent experiments. Data are presented as the Mean ± SD. Statistical significance between control treated samples was analyzed using ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and ****$p<0.0001$ vs. DMSO-treated C6 cells.

Hexane extract of Telosma cordata promoted the neural-related protein expression

To determine whether hexane extract of *Telosma cordata* affects the expression of neural cell markers, we assessed the protein levels of BDNF, NGF, and acetyl H3 in C6 cells. As depicted in Figure 8A-B, the NGF levels increased dramatically by approximately five-fold in cells treated with TCF (hexane) extract at 5 µg/mL. Likewise, other neural cell markers, BDNF and histone H3, showed significant growth at the same concentration, increasing approximately two-fold and 1.6-fold, respectively. These findings demonstrate that the herbal extract significantly influences the expression of all these markers. Thus, these results indicated that TCF (hexane) extract can enhance the expression of neurology-related proteins.



(A)



(B)

Figure 8. Effects of hexane extract of *T. cordata* on neural-related protein expression. (A) Western blot analysis of neural cell markers nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and acetyl H3 in C6 cells treated with hexane extract of *T. cordata* with different concentration. (B) Quantitative analysis of the protein expression levels of neural cell markers NGF, BDNF, and acetyl H3 in C6 cells treated with different concentration of hexane extract of *T. cordata*

Quantitative data were obtained from three independent experiments. Data are presented as the Mean ± SD. Statistical significance between control treated samples was analyzed using ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001; ****p<0.0001 vs. DMSO-treated C6 cells.

Discussion

In our study, we present a fast and convenient identification system for plants based on DNA analysis. DNA barcoding demonstrates potential as a practical and standardized method for species-level identification of both fungal and plant species. It is effectiveness relies heavily on the establishment of high-quality sequence databases (Bruni *et al*., 2010; Fernández Moriano *et al*., 2015). As part of our DNA barcode identification process, we used three different DNA barcode regions: *mat K, rbcL,* and *trnH-psbA,* to successfully identify multiple species (Begerow *et al*., 2010). These findings from this study suggest that the combination of

conventional core-barcode markers with the *trnH-psbA* spacer can be a valuable strategy for differentiating between closely related taxa (Lima *et al*., 2018). The *trnH-psbA* region proves useful in characterizing honey from specific geographic areas with familiar flora. This region's limited representation in public databases, which play a key role in comprehensive analyses across various taxa, is noted (Loera-Sánchez *et al*., 2020). Furthermore, the *mat K* gene demonstrated seamless amplification and alignment in the species under study, revealing strong discriminatory abilities across different groups (Figure 1A). Saha *et al*. (2020) recognized the *matK* gene as a universal DNA barcode for flowering plants. In recent times, the previously study further supported its universality and suggested combining *mat*K with *rbc*L as a universal DNA barcode for plants. Additionally, both *rbcL* and *trnH-psbA* markers demonstrated easy amplification and sequencing, confirming their suitability for scientific applications in plant identification and diversity studies (Bruni *et al*., 2015).

The acetylcholinesterase enzyme (AChE) which exists in two different forms within the brain, known as monomer (G1) and tetramer (G4), responsible for hydrolyzing acetylcholine in the nervous system. The G4 isoform, which makes up the majority of total AChE, is closely tied to cognitive function (Behl *et al*., 2020; Ferlemi *et al*., 2014; Martini *et al*., 2018). Within the cholinergic anti-inflammatory pathway (CAP), AChE plays a vital role in modulating the interaction between ACh and α7nAChR (Abdullah *et al*., 2019; Benfante *et al*., 2021), which regulates immune responses in the brain. AChE inhibitors have been formulated and developed as potential therapeutic agents for managing dementia. In the previous study, another medicinal plant extract, Pei Li *et al.* indicated that the whole herbal extracts of *Dichocarpum auriculatum* also performed inhibitory activity of AChE by Ellman method (Li *et al*., 2019). While our *T. cordata* extracts were evaluated for AChE inhibitory activity, we found that hexane extract of *T. cordata* showed a higher inhibition (IC_{50} = 6.196 ± 0.02 µg/mL) than the previous result for the *D. auriculatum* extract (IC_{50} = 150 µg/mL) (Li *et al*., 2019). This result expresses the activity inhibitory of AChE and was lower than the positive control - berberine chloride (IC_{50} = 0.289 ± 0.03 µg/mL). Thus, neuroprotective activity of *T. cordata* is interested and needs to be investigated further for neurological-related disease treatment. In this study, for the first time, we suggest that extracts isolated from the flowers of *Telosma cordata* induce neurite outgrowth in rat C6 cell lines. Especially, the average length of neurite was increased five time when cell treat with hexane extract of *T. cordata* compared with control. This effect could help to connect cells that are very far apart. Numerous previous studies have shown that neurite outgrowth can be induced by natural compounds alone or in combination with neurotrophic factors (Duangjan *et al*., 2021; More *et al*., 2012). Our study demonstrates that hexane extract of *T. cordata* possess strong neurite outgrowth-promoting activities in C6 cells.

NGF has a vital function in facilitating neuronal cell differentiation, survival, and the stimulation of neurite outgrowth (Bradshaw *et al*., 2017). Due to its neurotrophic properties, NGF has emerged as a promising candidate for potential therapeutic interventions in neurodegenerative diseases, including dementia (Do Carmo *et al*., 2021; Eu *et al*., 2021; Triaca *et al*., 2021; Xu *et al*., 2019). Belonging to the neural family of growth factors, BDNF operates as a secreted protein that holds significance in bolstering the survival and expansion of neurons within both the central and peripheral nervous systems. Furthermore, BDNF signaling plays a pivotal role in the initial stages of development, governing the proliferation and differentiation of neuronal progenitors, cell survival, neurite outgrowth, as well as the dynamic establishment and refinement of synaptic connections (Allen *et al*., 2013; Amen *et al*., 2016; Arévalo and Deogracias, 2023). There is evidence supporting the involvement of histone modifications, particularly H3 and H4 acetylation, in neural stem or progenitor cell differentiation and neurotrophic. Furthermore, these neural differentiations are associated with histone deacetylase inhibitor (HDACi) induced neural differentiation and neurotrophic activity (Ngubo *et al*., 2022). In our study, we observed a remarkable increase in BDNF, NGF and acetyl H3 mRNA and protein levels in C6 cells following treatment with TCF extracts, compared to vehicle treatment. These findings suggest that the TCF extracts may have a positive impact on neurotrophic factors and histone modifications, which could be beneficial for neuronal function and potential therapeutic strategies in neurodegenerative conditions.

Moreover, antioxidant activity plays significant importance role in the prevention or postponement of major degenerative diseases. Hence, we opted to assess the antioxidative potential of *T. cordata* extracts using the DPPH radical method. Our investigation revealed noteworthy antioxidant activity in all three extracts from *T. cordata*. The DPPH assay stands as a suitable tool for gauging the antioxidative capacity of extracts with lower polarity (Mercado-Mercado *et al*., 2013). Flavonoids and terpenoids, prevalent in pants, are recognized as the main antioxidant compounds, and pivotal for their roles as radical scavengers, agents with reducing power, and chelators of metal ions (Saleem *et al*., 2022). The observed antioxidant activity of the *T. cordata* extracts is likely attributable to the presence of flavonoids and terpenes in the samples. These compounds are commonly found in various plants known to exhibit antioxidant properties, as demonstrated in previous studies utilizing the DPPH method (Nguyen, 2020). Overall, our results indicate that *T. cordata* extracts possess significant antioxidant potential, which could contribute to their potential therapeutic benefits in combating oxidative stress and related degenerative diseases.

Conclusions

In conclusion, this study demonstrated that *Telosma cordata* flower extracts have significant potential to improve neuronal survival and exhibited substantial anti-acetylcholinesterase, neurotrophic, and antioxidant activities. The treatment of C6 cells with *T. cordata* extracts resulted in enhanced gene expressions of BDNF, NGF, and RPL, as well as increased protein expression levels of BDNF, NGF and histone H3. These findings indicate that *T. cordata* has the potential to be promising candidate for developing of pharmacological drugs that can facilitate neuronal regeneration. In the future, we aim to isolate and identify the specific phytochemical compounds in *T. cordata* responsible for its neuroprotective effects in treating dementia and neurodegenerative diseases. This will provide valuable insights into the mechanisms responsible for its therapeutic properties, potentially paving the way for novel therapeutic interventions targeting neurological disorders.

Authors' Contributions

Conceived and designed the experiments: TVN, DHN and NTD; Performed the experiments: NTD, TLTB, HTTN and HMN; Analyzed the data: TVN, DHN, NTD and HTTN; Wrote the paper: TVN, DHN, NTD and HMN. All authors read and approved the manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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