



# Identification and Evaluation of Neuroprotective Effect of a Butanol Extract from *Centella asiatica* (L.) URB

Tru Van Nguyen <sup>a\*</sup>, Nguyet Thi Doan <sup>b</sup>, Nhi Hanh Ngo <sup>a</sup>,  
Hanh Van Nguyen <sup>a</sup> and Duong Hoang Nguyen <sup>b\*</sup>

<sup>a</sup> Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, 100000, Vietnam.

<sup>b</sup> Center for High Technology Development, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, 100000, Vietnam.

## Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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## ABSTRACT

In recent years, herbal plants have been widely used in the treatment of neurodegenerative diseases. *Centella asiatica* (L.) urb is a significant medicinal plant renowned for its diverse ethnomedicinal applications. The present study evaluated biological activities for neuroprotective effect of *C. asiatica*. In summary, *Centella asiatica* (L.) urb was identified using DNA barcoding such as matK and rbcL genes. The sequences obtained were then compared through a similarity search using the NCBI GenBank database. Notably, the extracts of *Centella asiatica* exhibited high acetylcholinesterase inhibitory activity. Furthermore, the butanol extract of *C. asiatica* demonstrated

\*Corresponding author: E-mail: nvtru@ibt.ac.vn;

the most significant neurotrophic effects in preliminary cell-based screenings, particularly in promoting neurite outgrowth in C6 cells. These findings suggest that *C. asiatica* holds considerable promise as a potential candidate for the development of pharmacological treatments for neurodegenerative diseases.

**Keywords:** *Centella asiatica* (L.) urb; neurodegenerative diseases; AChE inhibitory; neurite outgrowth.

## 1. INTRODUCTION

Neurodegenerative diseases are the major cause of physical and cognitive disability worldwide, recently affecting about 15% of the global population (The Lancet Public Health 2019). The number of patients has increased significantly in the last 30 years. According to WHO, approximately 55 million individuals globally were diagnosed with dementia in 2019. It is projected that by the year 2050, this number will rise to 139 million (WHO 2023). In addition, the burden of chronic neurodegenerative diseases is estimated to double at least in the next two decades (Wong 2020). Because of this development, which can be largely attributed to the aging of population, it will be very difficult to maintain access to neurological care for all.

In recent years, medicinal plants have received much attention in the treatment of neurodegenerative diseases due to their natural compounds with low side effects, and complementary to conventional therapies. Many studies demonstrated bioactive compounds from medical plants using for treatment of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Rajaram et al., 2015, Perera et al., 2011, Kabra et al., 2019, Bhangale & Acharya 2016, Chen et al., 2017). Bioactive compounds from medical plants showed antioxidant, anti-inflammatory, neuroprotective, and neuroregenerative effects (Kuo et al., 2017, Wu et al., 2017, Hiep et al., 2017, Shagirtha et al., 2017, Yang et al., 2011, Huang et al., 2016).

*Centella asiatica* (L.) Urban also known as Gotu kola, *Syn. Centella coriacea* Nannfd., Pegaga, Madukaparni and belongs to family Apiaceae. It is a tropical medicinal plant, and found many Southeast Asian countries including Vietnam, Malaysia, Indonesia, Sri Lanka, China, and India, as well as South Africa and Madagascar (Gajbhiye et al., 2016). It was commonly used as traditional medicines. *Centella asiatica* (L.) Urban has been showed many biological activities such as desired for human health such as

neuroprotection, antioxidant, anti-inflammatory (Singh et al., 2014, Choi et al., 2016, Dewi & Maryani 2015, Hafiz et al., 2020, Ho et al., 2018). wound healing (Diniz et al., 2023, Witkowska et al., 2024) cardioprotective, anticancer, hepatoprotection (Hafiz et al., 2020), and antibacterial (Idris & Nadzir 2017). Its biological activities come from its secondary metabolites. Triterpenoids and their glycosides are the main groups of compounds in *Centella asiatica* (L.) Urban (Gajbhiye et al., 2016, Diniz et al. 2023, Witkowska et al., 2024, Rafi et al., 2018, Torbati et al., 2021). In addition, *Centella asiatica* (L.) Urban also provide essential oils dominated as sesquiterpenes (Joshi et al., 2007). According to a literature review, *Centella asiatica* (L.) Urban has been reported to provide comprehensive neuroprotection through multiple mechanisms of action, such as enzyme inhibition, prevention of amyloid plaque formation in Alzheimer's disease, dopamine neurotoxicity in Parkinson's disease, and reduction of oxidative stress (Gajbhiye et al., 2016, Singh et al., 2014, Choi et al., 2016, Dewi & Maryani 2015).

The external morphology of *C. asiatica* is similar to that of *Hydrocotyle umbellata* L (Laksanara et al., 2023). Shared morphological characteristics and common local names make it difficult to distinguish between similar species, especially when processed into commercial products. Today, DNA barcoding has greatly facilitated the identification of species of organisms ranging from bacteria to higher eukaryotes (plants and animals). A combination of DNA barcoding methods has emerged as a promising tool for identifying species in local flora and ecological communities based on *rbcL* and *matK* sequences (Idris & Nadzir 2017, Rafi et al., 2018, Torbati et al., 2021). Burgess et al. 2011). Previously, DNA barcoding including *rbcL*, *matK*, ITS2, *trnL-psbA* has been used to identifying *C. asiatica* from commercial products (Joshi et al., 2007, Chen et al., 2010, Wanrachon et al., 2023).

The present study focused to identify *Centella asiatica* (L.) Urban using DNA barcoding. In addition, the acetylcholinesterase inhibitory

activity and neurite outgrowth activity of *C. asiatica* extracts were evaluated. These results have provided insights neuroprotective effect of *Centella asiatica* (L.) urb. extracts.

## 2. MATERIALS AND METHODS

### 2.1 Collection and Identification of *Centella asiatica* (L.) Urban

*C. asiatica* were collected in Sapa, Lao cai province in South-west Vietnam in April 2021. The identification of *C. asiatica* samples was based on morphological characterization including leaves, roots. Additionally, DNA barcoding was used, and the nucleotide sequences of the *matK* and *rbcL* genes were alimented for identification purposes. The collected plant samples were dried to a constant weight and stored at temperature of -20 °C for the next experiments.

### 2.2 DNA Extraction, PCR Amplification, and Sequencing for Identification of *Centella asiatica* (L.) Urban

DNA total was extracted by modified CTAB method (Laksanara et al. 2023). Polymerase chain reaction (PCR) was conducted in 20 µL of the mixture using Phusa Master Mix 2x (Phusa Biochem, Vietnam). The primers used for sample identification were as follows: trnH-psbA (F/R), *matK* (F/R), *rbcL* (F/R) (Table 1). PCR products were examined by electrophoresis on a 1% agarose gel and purified with 100% ethanol. The nucleotide sequences of *matK* and *rbcL* fragments were determined using the Sanger method and analyzed using BLAST in NCBI.

### 2.3 Preparation of *Centella asiatica* (L.) Urban Extracts

The whole plant (flower, leave, stem, root, seed) of *C. asiatica* 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 Asmah et al. protocol (Asmah et al., 2020). Following that, each extract underwent low-pressure vaporization utilizing a rotary evaporator at 55 ± 2 °C).

### 2.4 Thin Layer Chromatography (TLC)

We conducted thin layer chromatography (TLC) analysis of all extracts of *C. asiatica* using a normal phase TLC plate, according to Kowalska et al. with minimal modification (Kowalska et al., 2022). We utilized 6 cm x 6 cm normal phase Silica gel 60 F254 HPTLC glass plates (Merck, Darmstadt, Germany) for TLC separations. Chromatographic plates were dried in at 115 °C for 5 min before use. Following, 10 µL of each extract was applied manually as spots to silica gel plates using fine capillary tube. The mobile phase solution for the TLC analysis was optimized by altering its polarity. This was achieved by commencing with the highly non-polar solvent, and subsequently augmenting the polarity. The separated compounds were then visualized under UV light at 254 nm, marked on each TLC plate with a pencil and captured images. Finally, TLC plates were immersed in the Vanillin solution and placed on a hot plate heated to 100 °C until the color visible spots appeared.

### 2.5 Acetylcholinesterase (AChE) Inhibitory Activity

Each *C. asiatica* extract was evaluated AChE inhibition by the modified Ellman's method (Pohanka et al., 2011). 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.

Table 1. The specific primer for *matK* and *rbcL* genes in this study

Gene	Primer	Primer sequences (5'-3')	Approximate fragment size (bp)
<i>matK</i>	Forward (F)	ACCGTACTTTTATGTTTACGAGC	850
	Reverse (R)	TCCATCTGGAAATTCGTTCA	
<i>rbcL</i>	Forward (F)	GCAAGTGTGGATTCAAAGCTGGTG	550
	Reverse (R)	TGGTTGTGAGTTCACGTTCT	

The mixture was placed in an incubator at a temperature of 37 °C for 10 minutes. After the pre-incubation, we added 25 µL of enzyme AChE (0.22 U/mL) 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 using the following formula:

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

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 (Pohanka et al., 2011).

## 2.6 Maintenance of Neuronal Cells

The C6 cell line, derived from a rat glial tumor and procured from the American Type Culture Collection (ATCC; MD, USA), was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich). The medium was added 10% FBS (Gibco) and 1% penicillin/streptomycin (100x concentration) under standard conditions of 37 °C and 5% CO<sub>2</sub> in a humidified environment.

## 2.7 Neurite Outgrowth

Glial C6 cell line was seeded into 24-well plates at 8000 cells in each well, and incubated overnight to get stable cell. Then, cells were treated with various non-toxic concentrations of *C. asiatica* extracts at final concentrations of 5 µg/ml and 2.5 µg/ml. for 24h. Neurite length was observed and measured under 20x 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. DMSO was used as control. The experiments were repeated three times.

## 2.8 Statistical Analysis

The results (mean ± standard deviation (SD)) were obtained from three separate measurement. Significance between two groups

determined in this study was used unpaired two tailed Student's t tests or a one way ANOVA followed by a Bonferroni's post hoc test, followed by GraphPad Prism 10 software. The p-value below 0.05 was considered statistically significant.

## 3. RESULTS

### 3.1 The Identification of *C. asiatica* Using DNA Barcoding

To identify collected sample, DNA total was used to amplify target genes with special primer pairs. The results showed that the *matK* and *rbcl* genes were successfully amplified with the band size of 850, 550 bp, respectively. Comparison of gene sequence by BLAST tool demonstrated high similarity to species *C. asiatica*. As showed in Fig. 1, sequence of MatK marker has high similarity to *Centella asiatica* Sequence ID: GQ434230.1 (99.87%), and *Centella erecta* Sequence ID: ON341148.1 (99.75%). The phylogenetic tree of different *Centella* species based on matk sequences indicated that our collected sample has a close genetic relationship to *Centella asiatica* (Fig. 1). Similar results showed with *rbcl* gene, which share the sequence similarity of 99.27% to *Centella asiatica* Sequence ID: MK905035.1 (99.82%), and *Centella villosa* sequence ID: KP110227.1 (99.46%). Taken together, morphological features and DNA barcoding results demonstrated that the collected sample was *Centella asiatic* species, belongs to the genus *Centella* (Fig. 1).

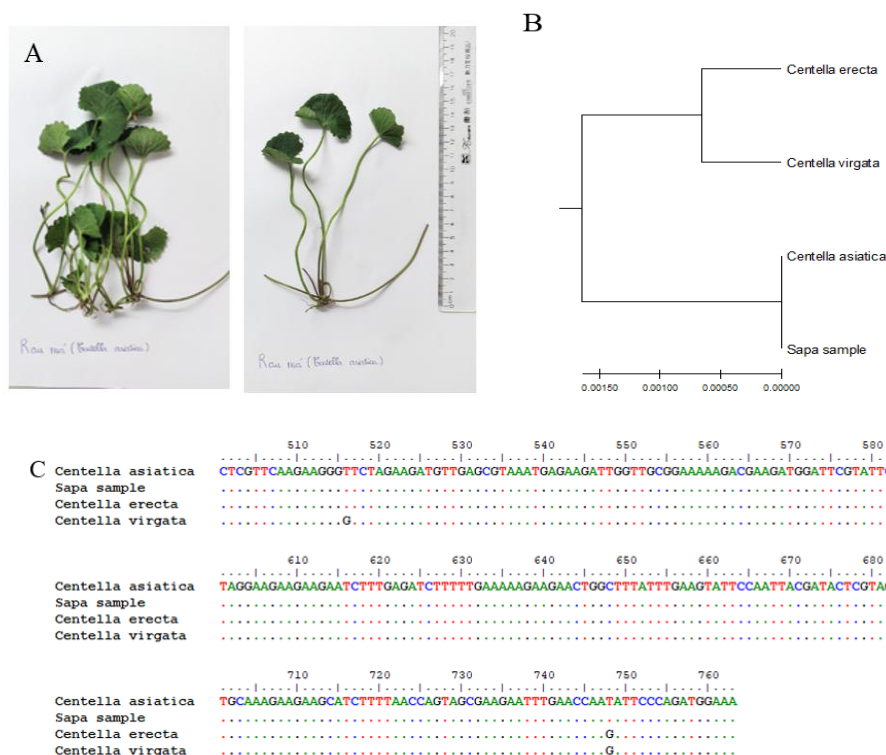
### 3.2 *C. asiatica* Extracts Inhibited Acetylcholinesterase Activity

To evaluate the acetylcholinesterase inhibition of *C. asiatica* extracts, *in vitro* Ellman assay was performed using berberine chloride as a positive control. The results showed that *C. asiatica* extracts inhibited the activity of acetylcholinesterase in a dose-dependent manner. Butanol extract of *C. asiatica* showed the most potency in the inhibition effect of acetylcholinesterase with  $IC_{50}$  value of 270 µg/ml. The weakest inhibition effect of acetylcholinesterase was observed in the hexan extract of *C. asiatica* with  $IC_{50}$  value of 420 µg/ml. The difference in the inhibitory Effect of acetylcholinesterase among extracts could be come from the phytochemical component of extracts (Fig 2).

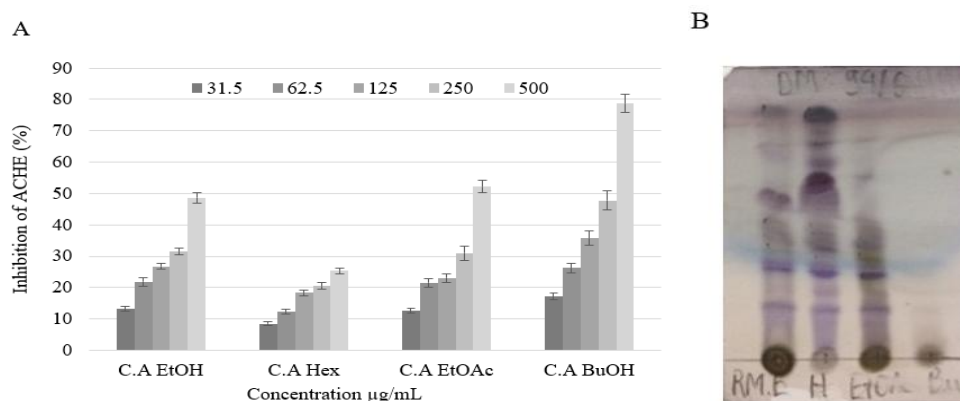
### 3.3 Extracts of *C. asiatica* Induces Neurite Outgrowth

To measure the effect of inducing neurite outgrowth of *C. asiatic* extracts, which were added to C6 cell culture medium at two distinct concentrations (5 µg/ml and 10 µg/ml). Neurite outgrowth was observed using a confocal microscope at 20x magnification after 24h incubation. DMSO was used as a negative control. As shown in Fig. 3, treatment with *C. asiatica* extracts significantly increased the average neurite length compared to 0.1% DMSO

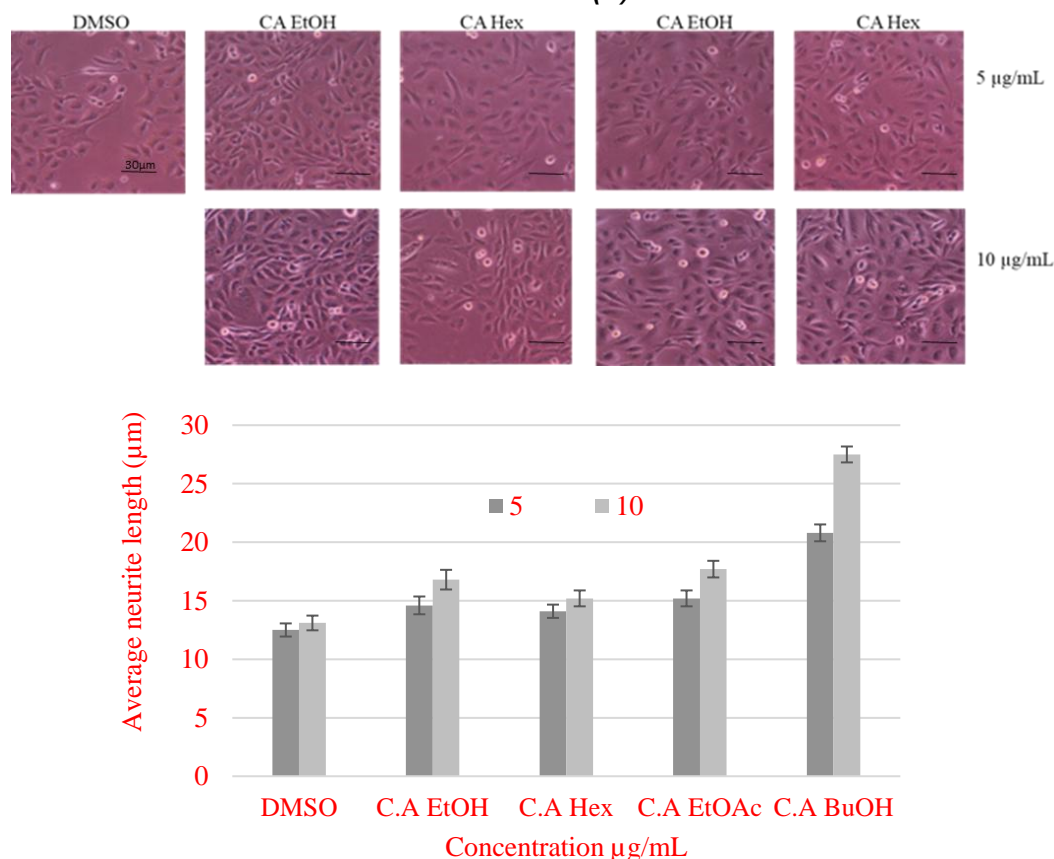
control. Butanol extract of *C. asiatica* showed the most acceleration in neurite outgrowth, and dose-dependent manner. The average neurite length was 1.7 and 2.2 times longer than that of control group, when cells treated with butanol extract of *C. asiatica* at a concentration of 5.0 and 10.0 µg/ml, respectively. The lowest neurite outgrowth-inducing activities was observed in hexan extract of *C. asiatica* Fig. 3. Thess results suggested that butanol extract of *C. asiatica* possesses neurite outgrowth-inducing activity.



**Fig. 1. Morphological characterization of *Centella asiatic* including leaves, roots (A), the phylogenetic tree of genus *Centella* based on matk sequences using MEGA software 11 (B), multiple sequence alignment of four matk sequences from genus *Centella* (C).**



**Fig. 2. The ACHE inhibition effect of *C. asiatica* extracts (A), Phytochemistry of *C. asiatica* extracts by TLC, E: ethanol extract, H: hexan extract, EtOAc: ethyl acetate extract, Bu: Butanol extract (B)**



**Fig. 3. *C. asiatica* extracts induced neurite outgrowth. C6 cell morphology (A), average neurite length treated with *C. asiatica* extracts (B)**

#### 4. DISCUSSION

The concept of "DNA barcode" was first introduced by Paul Hebert from the University of Guelph in 2003 (Hebert et al., 2003). DNA barcodes are standardized sequences of DNA that ideally possess unique characteristics. These sequences typically range in length from 400 to 800 base pairs and serve as a means to identify and classify groups of organisms. This process involves amplifying the DNA, sequencing it, and comparing the results with a reference database that includes relevant sequences from various species. DNA barcoding provides a fast and precise method for identifying various groups. Notably, at the species level, it can be utilized to characterize new or previously unidentified species, as well as cryptic species (Jiang et al., 2020, Jourdan et al., 2023, Struck et al., 2018). DNA barcoding has been widely used

in the identification of various plant species. The Plant Working Group of the Consortium for the Barcode of Life (CBOL) proposed the chloroplast genes *matK* and *rbcL* as promising candidates for plant DNA barcoding (Wilson et al., 2024, Li, et al., 2021). In this study, we used genes *matK* and *rbcL* to identify our collected sample. Collected samples showed high similarity to *Centella asiatica* species, belongs to the genus *Centella*. The *matK* region is known for its rapid evolution and strong capability for interspecific identification, although the primers used are not universally applicable (Arca et al., 2012). On the other hand, *rbcL* offers high generality, straightforward amplification, and good comparability; however, it lacks efficiency in distinguishing between species. Consequently, other chloroplast regions such as *trnH-psbA*, *trnL*, and *trnL-F* (Wilson et al., 2024, Li, et al., 2021), as well as the nuclear ribosomal Internal

Transcribed Spacer (ITS) region, are commonly utilized as supplementary barcodes in conjunction with matK and rbcL (Fazekas et al., 2008). In our study, the chloroplast genes matK and rbcL is useful for identification *Centella asiatic* species.

Acetylcholine (ACh) plays a crucial role as a neurotransmitter, widely distributed throughout the nervous system. They were hydrolyzed by enzyme acetylcholinesterase, acting in both synapses and neuromuscular junctions. This hydrolysis is essential for terminating nerve impulses, ensuring proper communication within the nervous system. Imbalances in its levels can play a role in the development of neurodegenerative diseases and depressive disorders (Van et al. 2015). Furthermore, AChE inhibitors available on the market not only block the enzyme but also possess several additional properties that may aid in slowing the progression of these diseases (Walczak-Nowicka & Herbert 2021). In our study showed that butanol extraction of *C. asiatica* provided the inhibitory effect of acetylcholinesterase. While other extracts showed poorly activities. The difference could comes from different phytochemistry of the extracts. This biological activity also confirmed by several study (Hafiz et al., 2020, Ho et al., 2018, Torbati et al., 2021, Chen et al. 2016). However, the IC50 is still high. In this study, we also revealed the inducing effect of neurite outgrowth of *C. asiatic* butanol extract. Encouraging and enhancing neurite outgrowth plays a vital role in restoring neuronal function following injury or in the context of neurodegenerative diseases. A promising approach for developing drugs aimed at conditions like Alzheimer's and Parkinson's disease involves fostering the regeneration of neurites. Consequently, emerging therapies are concentrating on identifying molecules that can effectively promote neurite outgrowth (Yuan et al., 2024, Kiss et al. 2024). The challenge of utilizing plant extracts in the treatment of these diseases lies in their phytochemical variability. This variability leads to inconsistent results among individual extracts, as they differ in their potency for acetylcholinesterase (AChE) inhibition, as well as their antioxidant and anti-inflammatory properties. Therefore, it is crucial to identify the most promising extracts, isolate their components, and investigate the specific compounds responsible for the observed effects. Once these compounds are identified, they can be modified to optimize their pharmacodynamic and pharmacokinetic properties for better therapeutic outcomes.

## 5. CONCLUSION

In conclusion, the present study has successfully identified *C. asiatica* using DNA barcoding. Moreover, these results highlight the promising potential of *C. asiatica* extracts in enhancing neurite outgrowth. They also exhibit notable anti-acetylcholinesterase activity. These indicates that *C. asiatica* could be potential candidates for the development of pharmacological drugs aimed at facilitating neuronal regeneration. Moving forward, we plan to investigate the expression of genes and proteins associated with their neuroprotective effects of *C. asiatica* butanol extract for treating neurodegenerative diseases. Moreover, active compound isolation for neuroprotective activities will also be conducted.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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