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Neuroprotective Activity of *Catharanthus roseus* Ethanol Extract by Acetylcholinesterase Inhibition and Neurite Outgrowth Studies

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TVN and DHN designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors TVN and NTD managed the analyses of the study. Author NTD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate biological activities for neuroprotective effect of *Catharanthus roseus*.
Methodology: *Catharanthus roseus* was identified using DNA barcoding, utilizing *matK*, *trnH-psbA,* and *rbcL* markers. Additionally, thin-layer chromatography (TLC) method was used to analyze the phytochemistry compounds present in the *C. roseus* extracts. Moreover, acetylcholinesterase (AChE) inhibition activity was tested using a modified Ellman's method. Finally, neurite outgrowth activity was determined in rat glial C6 cells treated with varying concentrations of *C. roseus* extracts.

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Results: Overall, the plant samples which were collected in Laocai, Vietnam were successfully identified through DNA barcoding regions, using *trnH-psbA, matK*, and *rbcL* genes. Phytochemical analysis detected the presence of sterols, terpenoids, flavonoids, polyphenolic in the ethanol extract and its fraction from *C. roseus*. Additionally, the extracts of *C. roseus* displayed remarkably high acetylcholinesterase inhibitory activity. Moreover, the ethanol extract of *C. roseus* shown the most potent neurotrophic activity in a preliminary cell-based screening based on C6 cells neurite outgrowth.
Conclusion: These results demonstrate that *Catharanthus roseus* could be a strong candidate for developing pharmacological drugs to treat neurodegenerative diseases.

Keywords: Catharanthus roseus; neurological diseases; AChE inhibitory; neurite outgrowth.

1. INTRODUCTION

Recently, medicinal plants for addressing neurodegenerative diseases and neurological disorders capture the enormous attention from many research groups all over the world [1-3]. *Catharanthus roseus* (L.) G. Don, popularly called the Madagascar periwinkle, was classified as a species of Apocynaceae family [4]. *C. roseus* is cultivated not only for its ornamental beauty in parks, farms, and garden, but is also renowned for its medicinal properties [5]. Having more than 130 discovered compounds, this plant is major source of bioactive substances [6-8]. In traditional folklore systems of medicines, the whole plant consists of leaf, root, flower and seed are usually employed in the treatment of conditions such as hypertension, diabetes, anticancer, and menorrhagia [9]. The extracts of *C. roseus* contain numerous pharmacological features, consist of antimicrobial, anti-sterility, anthelmintic, antioxidant, antifeedant, and antidiarrheal [10]. Furthermore, vincristine and vinblastine are key compounds acquired from *C. roseus* and exhibit robust anti-neoplastic efficacy in conditions including acute lymphoblastic leukemia, non-Hodgkin's and Hodgkin's lymphomas, breast cancer, Ewing's sarcoma, and other diseases [11]. Additionally, two monomeric Monoterpenoid Indole Alkaloids, ajmalicine and serpentine, derived from its roots, find extensive use in clinical practice for managing circulatory and hypertension disorders [12-15]. In fact, *C. roseus* was revealed neuroprotective activity through acetylcholinesterase inhibition activity by David M. Pereira et al. [16, 17]. However, the molecular mechanisms involved in neuronal protection activity in this effect remain incompletely understood fully.

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 [18]. 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 [19]. Especially, AD is related to 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 it signaling role, ACh is hydrolyzed into choline and acetyl groups through the action of the enzyme acetylcholinesterase (AChE). The utilization of AChE inhibition has been suggested as a promising therapeutic strategy for the management of neurological disorders. The abundance of plants in nature offers a promising source of AChE inhibitors [20,21].

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 [22]. 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 [23-25].

The study aimed to investigate the identification *Catharanthus roseus* species, thin layer chromatography (TLC) was utilized to characterize of *C. roseus* extracts, elucidating their phytochemical profiles. Moreover, the present study also evaluated the acetylcholinesterase inhibitory activity and neurite outgrowth activity of *C. roseus* extracts. The results of this study more clarified molecular mechanisms of cognitive improvement.

2. MATERIALS AND METHODS

2.1 Collection and Identification of *Catharanthus roseus*

We grabbed the samples of *C. roseus* in Sapa, Laocai province in South-west Vietnam in April 2021. The identification of *C. roseus* samples was based on a comparative morphological method following Sunil Kumar *et al*.'s guidelines [10]. Additionally, DNA barcoding was used, and the nucleotide sequences of the *trnH-psbA, matK*, 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.

2.2 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 *C. roseus* [26]. Then, we performed the polymerase chain reaction (PCR) amplifications in 20 µL mixture using Phusa master mix 2x (Phusa Biochem, Vietnam). The primers utilized for sample identification were detailed as follows: *trnH-psbA* (F/R), *matK* (F/R), *rbcL* (F/R) (Table 1). 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 [27].

2.3 Preparation of *Catharanthus roseus* Extracts

The whole plant (flower, leave, stem, root, seed) of *C. roseus* 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

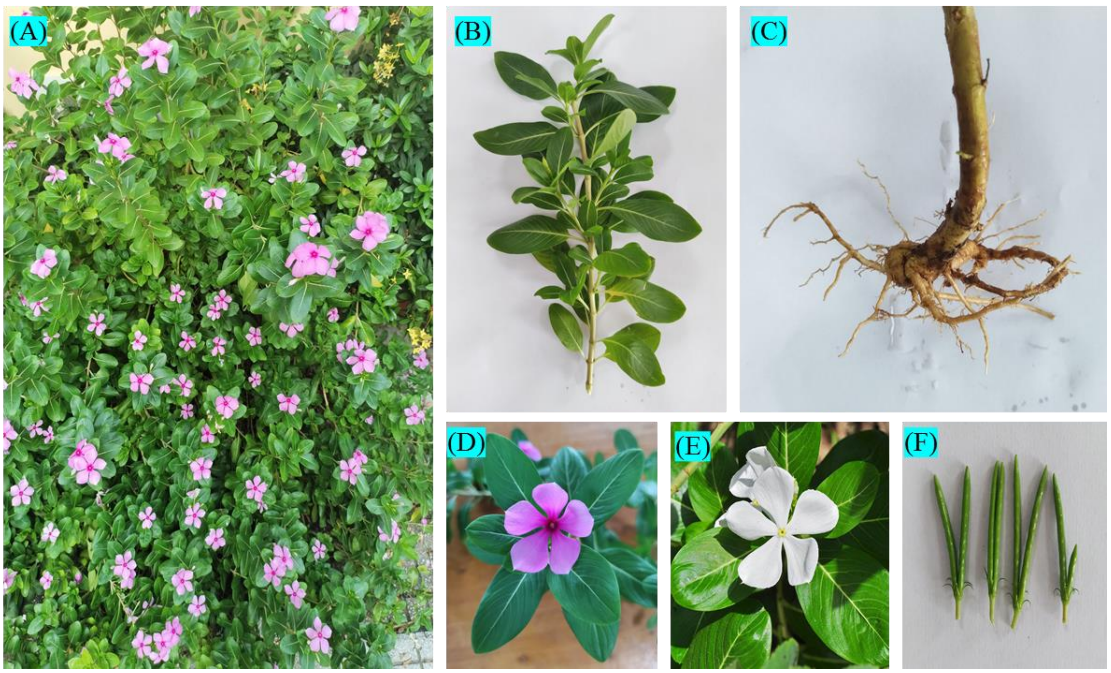

Fig. 1. Some morphological characteristic of *Catharanthus roseus*: (A) the whole *C. roseus* plant, (B) stems and leaves of plants at the mature stage, (C) root of *C. roseus*, (D-E) flowers and (F) fruits

Table 1. The specific primer for *matK*, *trnH-psbA* and *rbcL* genes in this study

Gene	Primer	Primer sequences (5'-3')	Approximate fragment size (bp)
matK	Forward (F)	ACCGTACTTTTATGTTTACGAGC	883
	Reverse (R)	TCCATCTGGAAATTTCGTTCA	
trnH-psbA	Forward (F)	CGCGCATGGTGGATTCACAATCC	513
	Reverse (R)	GTTATGCATGAACGTAATGCTC	
rbcL	Forward (F)	GCAAGTGTTGGATTCAAAGCTGGTG	573
	Reverse (R)	TGGTTGTGAGTTCACGTTCT	

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 [28]. 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. roseus* using a normal phase TLC plate, according to Supriya Tiwari *et al.* with minimal modification [29]. We utilized normal phase Silica gel 60 F254 HPTLC glass plates measuring 6cm x 6cm (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 positioned it on a hot plate preheated to 100 °C until the appearance of visible colored spots.

2.5 Acetylcholinesterase (AChE) Inhibitory Activity

Each *C. roseus* extract was evaluated AChE inhibition by the modified Ellman's method [30,31]. 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 and 25 µL of 15 mM ACTI were mixed. 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:

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

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 determined by observing the impact of increasing sample concentrations on inhibition values in the experiment. The positive control used in the experiments was berberine chloride. All the assays were repeated three times [32].

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), the cell culture conditions were described by Samuel Salazar-García *et al.* [33].

2.7 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 *C. roseus* extracts. Ethanol, ethyl acetate, butanol and hexane extracts of *C. roseus* were added at final concentrations of 5 µg/mL and 2.5 µg/mL. After a 24-hour incubation period, 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. The experiments were repeated three times [22].

2.8 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 conduct statistical evaluations, within GraphPad Prism 10. The statistical significance of the p-value was considered below 0.05.

3. RESULTS

3.1 The Identification of *Catharanthus roseus* 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 Fig. 1 (A-F), the obtained samples were identified as the *Catharanthus roseus* species through a comparative morphological method following by Sunil Kumar et al. [10]. 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 for the three regions of the *matK, trnH-psbA,* and *rbcL* genes in the plant samples 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 Fig. 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 *Catharanthus*. Specifically, the *trnH-psbA* region possessed the highest identification efficiency of 99.74% with *Catharanthus roseus* voucher Trotta950331 (GenBank accession number **MH621819.1**), and *Catharanthus roseus* voucher A2424, partial sequence – **MH069885.1**- (99.74%) based on Genbank on the NCBI website. In addition, when comparing the *matK* sequences extracted from the Sapa samples with the *matK* sequence of *Catharanthus roseus* (GenBank accession

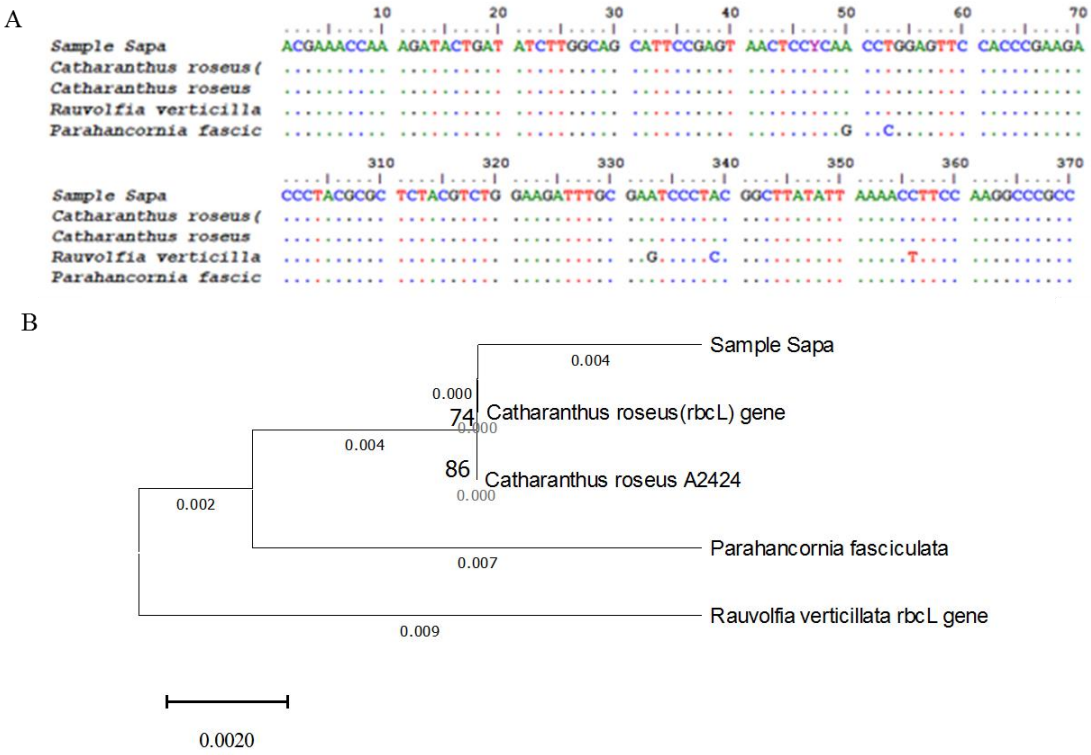

Fig. 2. (A) Alignment of ribulose-1,5-bisphosphate carboxylase (*rbcL*) barcode region of five species. (B) The phylogenetic tree was constructed using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 5 nucleotide sequences. Evolutionary analyses were conducted in MEGA11

number **DQ660507.1**), a notable similarity percentage of 99.64% was uncovered. Moreover, similar results were observed with *rbcL* barcode, with a sequence similarity of 99.27% to *Catharanthus roseus (rbcL) gene partial cds* (**MN125628.1**), *Catharanthus roseus* voucher A2424 – **MH069755.1** (99.27%), and *Catharanthus sp.* yangApY002– **KX910826.1** (99.27%) (**Figs. 2A-B**). These results conclude that the collected medicinal plant samples are members of the genus *Catharanthus*, specifically belonging to the species *Catharanthus roseus*.

3.2 Thin-Layer Chromatography Analysis of Different Fractions of *Catharanthus roseus*

To verify the presence of compounds in *C. roseus* extracts by TLC analysis, five mobile phases were selected for the analyses of extracts: Dichloromethane (DCM): Methanol (MeOH) (7:3, *v/v*); DCM: MeOH (8:2, *v/v*) for the more polar butanol extract; DCM: MeOH (9:1, *v/v*); DCM: Acetone (95:5, *v/v*); Hexane: Ethanol (9:1, *v/v*) for the hexane, ethyl acetate and ethanol extract. We utilized Vanillin reagent to assess the content of polyphenols, phytosterols, and terpenoids in the extracts. As shown in Fig.3A, we examined the developed plates under the UV light with a wavelength of 254 nm before undergoing derivatization. When the plate was exposed to UV light with the 254 nm wavelength, we observed aromatic compounds and highly conjugated systems referring to dark zones against the light-green fluorescence background of the TLC plate. We employed the Vanillin reagent for derivatization to identify a broad range of natural products within the extracts. After the treatment of Vanillin, the plates exhibited violet, blue, red, or green emission, referring to the existence of terpenes, phenols, steroids and sugars in *C. roseus* extracts (Fig. 3B). Based on the various colors of bands on the TLC plate after derivatization with Vanillin, we can predict the compounds present in the extract samples. In mobile phase (DCM: MeOH (9:1, *v/v*), a purple band under visible light at 254 nm after derivatization with Vanillin reagent suggest the presence of sterols, terpenoids in extracts at Rf = 0.54, whereas a pink band reveal the existence of flavonoids in *C. roseus* extracts at Rf = 0.75 (Fig. 3B). Hence, the extracts of *Catharanthus roseus* exhibit compounds such as sterols, terpenoids, flavonoids, polyphenolic.

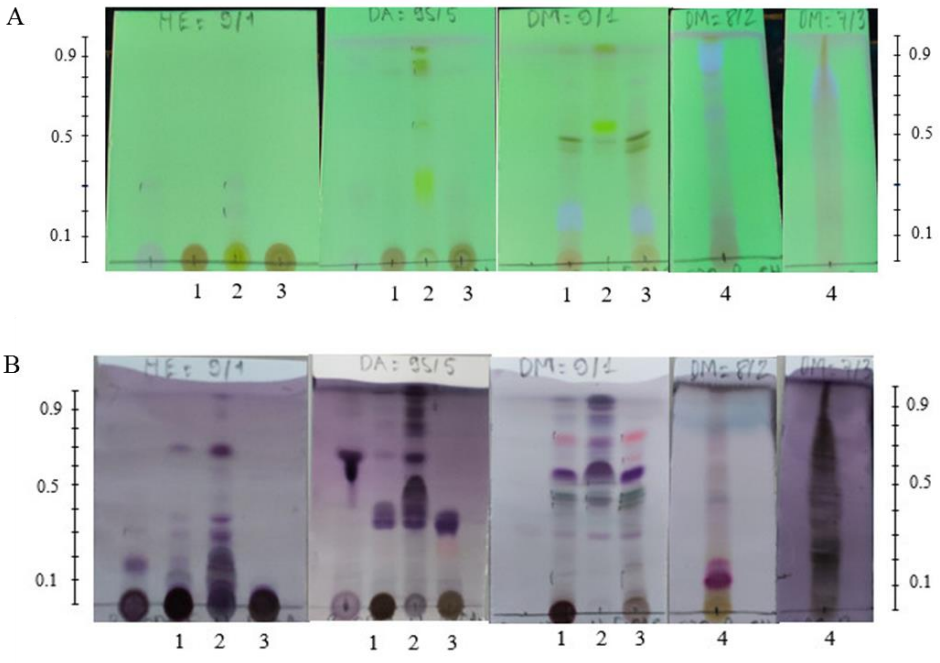

Fig. 3. (A) *Catharanthus roseus* extract samples chromatograms viewed under 254 nm. (B) Thin layer chromatography analysis conducted on (1) ethanol, (2) hexane, (3) ethyl acetate, and (4) butanol extract from *Catharanthus roseus* in different solvent systems. H: Hexane; E: Ethyl acetate; D: Dichloromethane; A: Acetone; M: Methanol

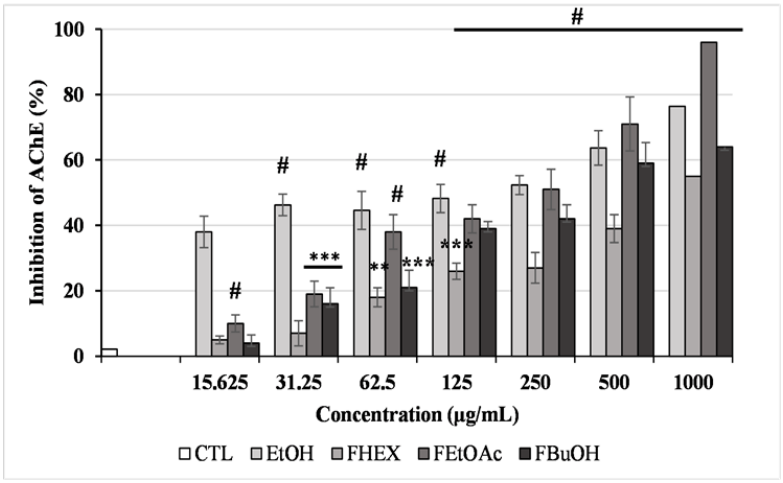

Fig. 4. AChE inhibitory activity of *Catharanthus roseus* extracts. The AChE inhibitory activity was evaluated by Ellman's method in presence of ethanol extract (EtOH), hexane fraction (FHEX), ethyl acetate fraction (FEtOAc) and butanol fraction (FBuOH). Data were analysed using the ANOVA test followed by Graphpad prism. Data represented the mean ± SD. *p < 0.05, **p < 0.01; ***p < 0.001 and #p < 0.0001 is significant differences and percentage inhibition of AChE calculated relative to control (CTL).

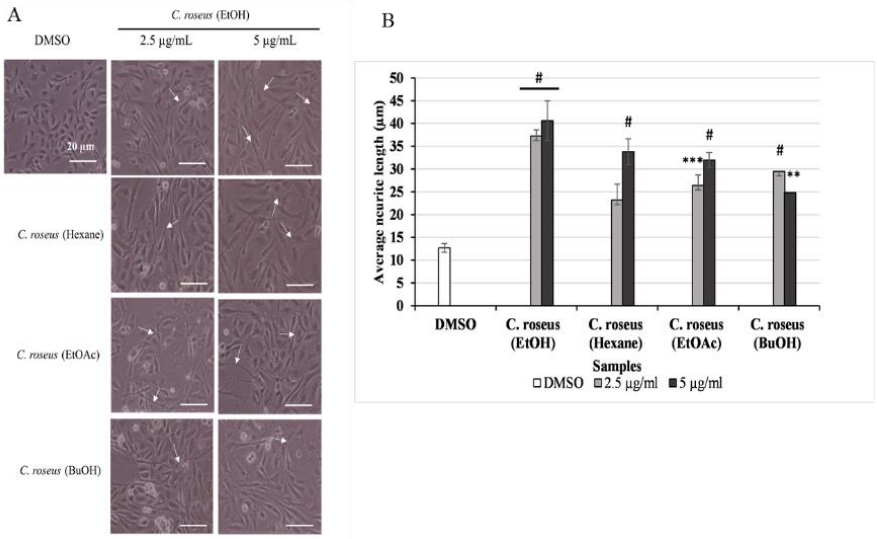

Fig. 5. *Catharanthus roseus* extracts for neurite promoting activity in C6 cells. A: Immuno-stained image of C6 cells showed neurite outgrowth following treatment with *C. roseus* extracts (at different concentrations), and 0.1% DMSO (vehicle). Scale bar represents 20 µm. Photomicrographs of representative microscope fields were taken with a 20x objective. B: Graph describing the average length of neurites and optimized concentration of *C. roseus* extracts. Neurites were measured using ImageJ software on bright-field images of C6 cells, taken 24h 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

3.3 Inhibition of Acetylcholinesterase (AChE) by *Catharanthus roseus* Extracts

To assess for inhibitors from *C. roseus* 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 Fig. 4. The inhibitory activity of *C. roseus* extracts against AChE showed 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:Hexane > Butanol > Ethyl acetate > Ethanol > Berberine chloride. As shown in Fig. 4, *C. roseus* (ethyl acetate) and *C. roseus* (butanol) extracts inhibited AChE at IC_{50} values of 212.11 ± 5.24 µg/mL and 331.59 ± 8.47 µg/mL, respectively. *C. roseus* (ethanol) extract exhibited the strongest AChE inhibitory potency, displaying an IC_{50} value of 10.715 ± 0.82 µg/mL. In contrast, the AChE inhibitory activity of *C. roseus* (hexane) extract was the weakest, demonstrating an IC_{50} value of 67.546 ± 3.78 µg/mL. These results reveal that *C. roseus* extracts possess activity for AChE inhibition.

4. DISCUSSION

In our study, we present a fast and convenient identification system for plants based on DNA barcoding analysis. DNA barcoding is a genomics-based technique introduced by Hebert et al. in 2003 for taxonomic identification, provides a precise and universally applicable platform for unambiguous plant species identification. Based on molecular and computational information, it uses standardized DNA regions named DNA barcodes, to ascertain the identification of a species or taxon. This method not only facilitates the authentication of raw plant materials but also serves as a quality control measure [34]. DNA barcoding has found wide application in the identification of animals. A portion of the mitochondrial cytochrome c oxidase 1 (CO1 or cox1) gene sequence has been used as a universal barcode for identifying several groups of animals, such as birds [35], fishes [36], and mammals [37]. Moreover, DNA barcoding is also beneficial to the authentication of various herbal plants. In 2009, the Consortium for the Barcode of Life Plant Working Group (CBOL) proposed a combination of *matK* and *rbcL* as a 'core barcode' for plant identification across land plants [38]. Currently, DNA barcoding is a prominent topic in the field of bio taxonomy. However, there is an ongoing debate regarding the selection of a standardized DNA region to serve as the universal barcode for land plants. Various markers from the chloroplast genome or plastid DNA regions, such as *trnH-psbA*, *atpF-atpH*, *rpoB*, *psbK-psbIr*, and *rpoC16*, have been explored as potential DNA barcodes. In addition, several nuclear ribosomal DNA sequences, including the internal transcribed spacer (*ITS*), *internal transcribed spacer 1* (*ITS1*), *internal transcribed spacer 2* (*ITS2*), and others, have also been under evaluation [39]. In this study, we chose three DNA fragments (*trnH-psbA, matK*, and *rbcL*), previously proposed as DNA barcodes for identifying Catharanthus medicinal plants. In this study, we selected three standard barcodes, *trnH-psbA*, *matK* and *rbcL* achieved good species resolution. Through the utilizing of BLAST on NCBI Genbank, the percentage of similarity between the collected sample and the sequences in the NCBI GenBank when employing specific DNA barcoding was *trnH-psbA* > *matK* > *rbcL* at species level. These results indicate that *trnH-psbA* outperforms the other candidate barcodes due to its higher similarity percentage compared to *matK* and *rbcL.* Despite its relatively short sequence length (approximately 513 bp), *trnH-psbA* is recognized as the most polymorphic plastid region in angiosperms. It can be efficiently amplified across a wide spectrum of terrestrial plants, making it capable of distinguishing among a vast quantity of plant species for barcoding applications [40-42]. In previous research, Jian Zhang et al. (2015) indicated the discriminatory capacity of four frequently utilized DNA barcoding markers (*ITS*, *trnH-psbA*, *matK*, and *rbcL*) and their respective multi-locus combinations were investigated using 135 individuals from 33 species of Schisandraceae. The findings revealed that the trnH-psbA gene exhibited a greater species-resolving capability compared to the two coding genes. [43]. Furthemore, Maloukh et al. studied DNA barcode on 51 plant species in United Arab Emirates, the *rbcL* marker demonstrated a perfect identification rate, successfully classifying all 51 plant species, which included 11 monocots and 40 eudicots. In contrast, *matK* achieved a correct species identification rate of only 24.45% (14 out of 51) [44]. In a separate study involving Casuarinaceae, it was observed that the *matK* gene provided enhanced resolution compared to *rbcL* [45]. In this study, *trnH-psbA* emerged as the most effective molecular barcode for the authentication of *Catharanthus roseus*, with *matK* or *rbcL* serving as supplementary markers. Hence, DNA barcoding has been used to be suitable for this study.

In this report, our TLC method allows to detect the presence of substances in the extracts of *C. roseus* using Vanillin as a derivatization reagent. TLC has consistently demonstrated its superiority as the method of choice, owing to its flexibility, affordability, convenience, and faster analytical capabilities when compared to alternative techniques [46]. TLC profiling of extracts yields compelling indications of the presence of

numerous phytochemicals. Different phytochemicals exhibit distinct Rf values in various solvent systems. This diversity in the Rf values of phytochemicals offers valuable insights into their polarity and aids in selecting an optimal solvent system for the purification of pure compounds through column chromatography [47].

Acetylcholine (ACh) serves as a vital neurotransmitter with widespread distribution in the nervous system [48]. AChE is an enzyme that hydrolyzes acetylcholine, occurring in both synapses and neuromuscular junctions, leading to the termination of nerve impulses [49]. Inducing higher levels of ACh in the synaptic cleft through the use of AChE inhibitors stands out as one of the most promising approaches for treating neurological diseases [50]. In this study, *C. roseus* ethanol extract displayed strong AChE inhibition, was measured IC_{50} at 10.715 ± 0.82 µg/mL (Fig. 4). The activity showed with the ethanol extracts of *C. roseus* was significantly greater than that identified in any plant part in previous studies: stems IC_{50} = 442; leaves IC_{50} = 422; petals IC_{50} = 2683 and roots = 25.5 µg/mL [16,17]. These results indicate a potency that is 2.5 to 250 folds higher.

Our study has only reached the *in vitro* level, and further research is needed to investigate the efficacy of *Catharanthus roseus* extract in treating neurological diseases *in vivo*. In the future, we recommend conducting research to isolate the main active compounds from the ethanol extract of *C. roseus*, which may be responsible for improving neurodegenerative diseases.

5. CONCLUSION

In conclusion, the present study has successfully identified morphological characteristics and DNA barcoding of samples belonging to the *Catharanthus roseus* species. Analysis of the *C. roseus* extracts in various solvents through TLC revealed the presence of major compounds in fractions. Furthermore, these results demonstrated that *C. roseus* extracts have significant potential to improve neuronal survival and exhibited substantial anti-acetylcholinesterase and neurite outgrowth activities. These findings show that *Catharanthus roseus* has the potential to be promising candidate for developing of pharmacological drugs that can facilitate neuronal regeneration. In the future, our aim is to evaluate the expression of genes and proteins related to its neuroprotective effects in treating 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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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