BIODIVERSITAS
Volume 26, Number 7, July 2025
Pages: 3144-3159

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d260706

Characterization of phytochemicals of *Clitorea ternatea* and the potention as neuroprotectant to prevent Alzheimer's disease

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Manuscript received: 20 September 2024. Revision accepted: 18 June 2025.

Abstract. *Sanjaya WBT, Tirtosari DR, Widayanti R, Nugrahaningsih DAA, Saragih GR, Wihadmadyatami H, Kusindarta DL. 2025. Characterization of phytochemicals of* Clitorea ternatea *and the potention as neuroprotectant to prevent Alzheimer's disease. Biodiversitas 26: 3144-3159. Clitoria ternatea* (Fabaceae), known by most as butterfly pea, is a significant medicinal plant recognized for its diverse bioactive compounds and therapeutic potential. The ethanolic extract of *C. ternatea* blume has shown promising neuroprotective effects, particularly in enhancing memory and reducing behavioural impairments. This plant, especially the section of roots and leaves, is recognized in traditional medicine and has been studied for its potential in treating neurodegenerative conditions. Meanwhile, there's still a lack of information for the therapeutic activities of its blume. Consequently, it is necessary to characterize the phytochemical compound of *C. ternatea* blume ethanolic extract to determine the neuroprotective potential. The phytochemical compounds were characterized by Ultraviolet-Visible spectroscopy (UV-VIS), Thin Layer Chromatography (TLC), Gas Chromatography-Mass Spectrometry (GC-MS), Fourier Transform Infra-Red (FT-IR), in silico molecular docking, and proliferation evaluation using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on TMT-induced neurotoxicity Pheochromocytoma rat cell (PC-12). The result presented total phenol (12.28%b/b), total tannin (10.39%b/b), total flavonoid (4.54%b/b), total saponin (1.2%b/b), total steroids (8,019.81 µg/mL), total alkaloids (617.9 µg/mL), 33 active phytochemical constituents with palmitic acid composition reaching 17%, strong appearance displays in many functional groups including hydroxy compound, unsaturated/aromatic compound, saturated aliphatic (alkene/alkyl), skeletal C-C vibrations, and ether compound, in silico molecular docking shows that palmitic acid inhibits the accumulation of β-amyloid, and *C. ternatea* reaches the optimum dose-dependent level of PC-12 cell proliferation at 31.25 µg/mL. Based on the results, *C. ternatea* ethanolic extract has significant potential as a neuroprotectant.

Keywords: Butterfly pea, ethanolic extract, medicinal plant, neurodegenerative, neuroprotective

INTRODUCTION

Alzheimer's Disease (AD) is one major worldwide health issue, with its incidence projected to increase significantly due to the aging population. Studies indicate that the occurrence of AD is closely linked to age, with the likelihood of developing the disease doubling approximately every five years after the age of 65 (Ridge et al. 2013; Ahmed et al. 2021). With the increasing aging population, the prevalence of AD is expected to increase, and it will affect an estimated 35 million people worldwide. These projections suggest this number could rise to 1 in 85 individuals by 2050. The prevalence of Alzheimer's disease in Indonesia is also a growing concern. Recent studies indicate that the prevalence of dementia, which includes AD, is significant, with estimates suggesting that over 4.2 million individuals may be affected by dementia in the country (Farina et al. 2023). By 2050, it is anticipated that this number will have risen sharply, with an estimated 3.98 million cases of dementia (Turana et al. 2019). Factors such as improved healthcare access and increased life expectancy contribute to this trend, highlighting the need for effective prevention and treatment strategies (Lane et al. 2018; Ramadan 2023). Current treatment options for Alzheimer's disease primarily focus on symptomatic relief, as there is no cure available. The most common pharmacological treatments include cholinesterase inhibitors, which aim to enhance cholinergic function in the brain (Topcu and Khusman 2014). Recent studies have proposed the potential of combining medications, such as aripiprazole and olanzapine, to manage behavioral symptoms associated with AD (Zheng et al. 2020)

Herbal medicine has gained attention as a potential therapeutic approach for Alzheimer's disease, primarily due to its multifaceted mechanisms that may address various pathophysiological aspects of the disease. Research indicates that certain herbal treatments exhibit neuroprotective effects due to their anti-inflammatory, antioxidant, and

anti-apoptotic properties. These effects can help protect neurons from amyloid-β toxicity, inhibit amyloid-β secretion, and lower oxidative stress and apoptosis associated with AD (Lee et al. 2020; Gregory et al. 2021). One of the herbal medicines is *Clitoria ternatea* L. *C. ternatea* is a common tropical flower in the Fabaceae family. It usually grows spontaneously in the wild or gardens. The plant is indigenous to Indonesia, Malaysia, Guinea, Ghana, and Zimbabwe. In several nations, *C. ternatea* is referred to as "butterfly pea" or "blue pea," while in Indonesia and Malaysia, it is called "Bunga Telang." In Southeast Asia, like Indonesia, the blue flower pigment is traditionally used as a food coloring, and the flower is used as a tea for drinks, but it is also extensively grown as a decorative plant and employed as a revegetation species (Jamil et al. 2018; Jeyaraj et al. 2021). Naturally, it thrives in full sun or slightly shaded areas, where flowering takes place after four weeks and the growth of seeds takes around 1-2 weeks.

In the Ayurvedic system of Indian medicine, *C. ternatea* is frequently mentioned as an antidepressant, antipyretic, and nootropic herb that serves as an effective nerve tonic that improves memory (Mukherjee et al. 2008). Memory loss is a consequence of the progressive decrease of certain neuron populations during neurodegenerative conditions. Proteotoxic stress, oxidative stress, apoptosis, and neuroinflammation are key physiological processes that are abnormally altered in neurodegeneration. This causes gradual neuronal dysfunction and cell death (Sahoo et al. 2022). Studies using *C. ternatea* root extract as an herbal remedy have demonstrated that in vivo administration increased acetylcholine level in the rat hippocampal region along with potential neuroprotectant and neuroprotection using behavioral learning tests, Novel Object Recognition (NOR) (Rai et al. 2002; Jiji and Muralidharan 2021). The bio-enhanced fraction of *C. ternatea* significantly restored cognitive deficits and neuroplasticity in models of chronic cerebral hypoperfusion, with doses of 40 mg/kg showing notable effects on amyloid-beta plaque reduction. Meanwhile, Clitorienolactones A and B from *C. ternatea* have been shown to improve long-term potentiation in rat models of chronic cerebral hypoperfusion by sustaining intracellular calcium levels, which is crucial for synaptic plasticity (Ahad et al. 2023; Ahad et al. 2024). Meanwhile, the ethanolic extract of *C. ternatea*'s flower needs further investigation to determine its neuroprotective capability due to the limited information available on its phytochemical and neuroprotective potentials. Therefore, this study aimed to identify the active compound in the ethanolic extract of *C. ternatea* that could act as a potential neuroprotectant agent and proliferation against TMT-induced neurotoxicity in Rat pheochromocytoma PC-12 cells.

MATERIALS AND METHODS

Ethanolic extract of *Clitoria ternatea* characterization

Simplicia of *C. ternatea* flower was purchased from CV Merapi Herbal, Yogyakarta, Indonesia. The method used for extracting was maceration. 300 g of a total of 1 kg of *C. ternatea* flower simplicial were dissolved in 4 L of 96% ethanol (Merck, Darmstadt, Germany). Rotary vacuum evaporator (Heidolph, Schwabach, Germany) was used to distil the filtrate, the final form of the extract is Ethanolic extract *C. ternatea* (EECT) in the form of pasta.

Characterization of the EECT was carried out in stages using Ultraviolet-Visible Spectroscopy (UV-VIS) (Shimadzu UV 1800-1700, Tokyo, Japan), Thin Layer Chromatography (CAMAG TLC Scanner 4, Muttenz, Switzerland), Gas Chromatography-Mass Spectrometry (Thermo Fisher Scientific, Massachusetts, USA), and Fourier Transform Infra-Red (Shimadzu 8201, Tokyo, Japan).

Ultraviolet -Visible Spectroscopy (UV-VIS)

Flavonoids

Fifty milligrams of dried sample of EECT and 0.3 mL 5% $NaNO_2$ (Merck, Darmstadt, Germany) were added to a test tube are combined. After five minutes, 0.6 mL of 10% aluminum chloride (Merck, Darmstadt, Germany) was added to the mixture. Add 2 mL of sodium hydroxide (1 M) (Merck, Darmstadt, Germany) after waiting for five minutes. Finally, 10 mL of deionized water was added. Once the liquid had been diluted as necessary, it was put into a cuvette and measured at 510 nm.

Phenols

Ethanolic extract of *C. ternatea* at 50 mg was mix with 0.5 mL of Folin-Ciocalteu (Sigma, Burlington, MA, USA) and 7.5 mL of aquadest, then 10 min rest period was given to the mixture prior to the addition of 1.5 mL of 20% sodium carbonate (Merck, Darmstadt, Germany). Water was added to get the volume up to 10 mL. The final solution was tested for wavelength at 760 nm.

Tannins

Fifty milligrams of a dried sample of EECT were filtered after being submerged in 10 mL of diethyl ether (Merck, Darmstadt, Germany) for 20 hours, 10 mL of distilled water was added to the mixture after the remaining diethyl ether (Merck, Darmstadt, Germany) had evaporated. Then, 10 mL of Folin-Ciocalteu (Sigma, Burlington, MA, USA) was added to the sample solution, vortexed, and incubated for five minutes. Finally, 2 mL of 20% sodium carbonate (Merck, Darmstadt, Germany) was added. The mixture was put at room temperature and incubated for 30 minutes, and then measured absorbance was measured at 760 nm.

Alkaloids

Ethanolic extract of *C. ternatea* at 50 mg was mix with 2N hydrochloric acid (Sigma, Burlington, MA, USA). Then the solution was rinsed three times with 10 mL of chloroform NaOH (0.1 M) (Sigma, Burlington, MA, USA) was then added to the solution to neutralize it. After neutralization procedures, 5 mL of phosphate buffer and 5 mL of Bacillus Calmette-Guerin (BCG) (Sigma, Burlington, MA, USA) solution were added, followed by 5 mL of chloroform. Agitation was performed for 15 minutes at 500 rpm using a magnetic stirrer. After gathering the

chloroform phase and evaporating it with nitrogen gas, more chloroform was added to make the volume reach 5 mL. A wavelength of 470 nm was used to measure the absorption.

Saponins

The combination of 50 mg and 2 mL of 25% sulfuric acid (Sigma, Burlington, MA, USA) was made, followed by 120 min autoclave at 110°C, and ether was used to extract the mixture, and the filtrate was dried. After adding 1 mL of water, vortex extraction took place for five minutes. After homogenizing the mixture and adding 50 mL of 4-anisaldehyde (Sigma, Burlington, MA, USA), the mixture was allowed to stand for 10 minutes. After adding 2 mL of 50% sulfuric acid (Sigma, Burlington, MA, USA), the liquid was heated for 10 minutes at 60°C in a water bath. Using a measuring flask, water was then added to get the volume up to 10 mL, and the mixture was diluted five times. A wavelength of 435 nm was used to measure the absorption.

Thin Layer Chromatography

Thin-layer chromatography was performed to analyze steroids and terpenoids. 1 mL of ethanol was mixed with a 50 mg sample of EECT. After an hour of sonication, the mixture was vortexed and then centrifuged. Maceration went on for 24 h. On silica gel 60 F_{254}, 100 µL of spot sample containing standard beta sitosterol (60% purity) (Sigma, Burlington, MA, USA) was applied. Then it was placed in the saturated chamber containing the mobile phase mixture of ethyl acetate (Sigma, Burlington, MA, USA) and toluene (93:7) (Sigma, Burlington, MA, USA), sprayed with Lieberman-Burchard reagent (Sigma, Burlington, MA, USA), and heated for two minutes at 110°C. The substance was detected at the wavelength of 340 nm.

Gas Chromatography-Mass Spectrometry

The 0.5 g of *C. ternatea* ethanol extract was combined with 1 mL of ethanol, and then the combination underwent centrifuging at 9,500 rpm for three minutes at room temperature. Subsequently, 1 mL of the solution was introduced into the Gas Chromatography-Mass Spectrometry (Thermo Fisher Scientific, Massachusetts, USA), which was initially calibrated to operate at 230°C for injection. The operating mode was split less, 50mL/min split flow, transfer line's degree of 250°C, the degree of the ion supply at 200°C, and 3mL/min purge flow. Agilent HP-5MS UI columns were utilized, while FID detectors with helium gas as the carrier gas and a maximal temperature setting of 350°C were employed. In order to determine potential substances contained in the sample, the obtained spectrum was compared with the databases on MAINLIB mass spectra.

Fourier transform infrared

Potassium bromide and a powder sample of EECT were combined in the proper ratio (1:100) for the FTIR analysis. The combination created pellets; each pellet was created at the same pressure in order to ensure that they have the same thickness. An FTIR spectrophotometer (Shimadzu 8201, Tokyo, Japan) was used to record FTIR spectra in the range of 400 -4000 cm^{-1}.

Cell culture maintenance (PC12 cell line)

PC12 cell line (ATCC, Manassas, VA, USA) was grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, Auckland, New Zealand) supplemented with one percent Penicillin-Streptomycin (Capricorn, Ebsdorfergrund, Germany), 0.5% Amphotericin B (Gibco, Auckland, New Zealand), 2% MycoXpert (Capricorn, Ebsdorfergrund, Germany), five percent Horse Serum, and 10% Fetal Bovine Serum (Capricorn, Ebsdorfergrund, Germany). The cells were then cultured in an incubator with 5% CO_2 at 37°C.

IC_{50} and proliferation assay

To determine the IC_{50} value, A 96-well plate was seeded with PC12 (2×10^4 cells). The cells were given to 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 µg/mL EECT and 1 µM donepezil HCl (Sigma, Burlington, MA, USA) after an hour treatment of 50 µM trimethyltin (TMT) (Sigma, Burlington, MA, USA) for 24 hours. Following the aspiration of the medium, Dulbecco's phosphate-buffered saline (DPBS) was used to rinse. Subsequently, each well received 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (Roche, Mannheim, Germany) and was incubated for 4 hours. After aspirating the MTT reagent, 100 µL of DMSO was applied to each well.

The wavelength of 595 nm is used to determine absorbance. Similar to the IC_{50} determination method, to determine the proliferation rate of the in vitro AD model with TMT administration, A 96-well plate was seeded with PC12 (2×10^4 cells); the cells were then divided into seven groups, namely the negative control group (non-treated), 50 µM TMT group (as a positive AD control), AD model group given the commercial drug Donepezil (Sigma, Burlington, MA, USA), and AD model group given EECT with graded doses of 7.81 µg/mL, 15.63 µg/mL, 31.25 µg/mL and 62.5 µg/mL (dose determination is determined based on the IC_{50} value). The proliferation rate is calculated using the following formula (Kustiati et al. 2021).

$$Proliferation\ rate\ (\%) = \frac{(Treated\ Cell\ Absorbance - Control\ Media\ Absorbance)}{(Control\ Cells\ Absorbance - Control\ Media\ Absorbance)} X100\%$$

In silico molecular docking

Docking preparation and simulation

The palmitic acid structure was downloaded from the PubChem NCBI database, CID 985. Donepezil, as a control, was carried out using the structure from the PubChem database with CID 3152. B-amyloid precursor protein (PDB ID 1AAP), acetylcholinesterase (PDB ID 4EY7), and Brain-Derived Neurotrophic Factor (BDNF) (PDB ID 1BND) were downloaded from the protein data bank. Protein structures were imported into Molegro Virtual Docker version 5.1 (Bitencourt-Ferreira and De Azevedo, 2019) and predicted the binding cavities using van der Waals expansion as a docking grid protein.

Docking was simulated by Molegro Virtual Docker version 5.0 (Bitencourt-Ferreira and De Azevedo, 2019) at the active sites of the protein. The B-amyloid precursor protein active site was set at center X= 11.99; Y= 19.01; Z= 36.73; radius 12. The Acetylcholinesterase docking site was X=-12.88; Y=-43.96; Z=28.80; Radius 13, while the BDNF site for docking was set on the center X=10.09; Y=-13.09; Z=36.80, radius 8. The docking setup used MolDock SE with specific parameters and optimization settings. The docking model was saved in mol2 format and visualized with PyMol 2.3. Ligand-protein interactions were analyzed in Discovery Studio ver. 21.1.1.

RESULTS AND DISCUSSION

The morphology of the *Clitoria ternatea* flower

This study shows that the blooms of *C. ternatea* have funnel-shaped petals and a butterfly-shaped crown, and the cylindrical stalks that support them are about 1.5 cm long. The perennial herbaceous plant is recognized for its medicinal properties and ornamental value. *C. ternatea* is a climbing plant and often utilizes nearby structures for support, which allows it to thrive in various environments (Figure 1). Early flowering occurs after 36 to 43 days, and pod lengths range from 3 to 8 cm. The flowers of *C. ternatea* are known to be 4-5 cm long with several variations of color: light blue, dark blue, white, and mauve (Nguyen et al. 2011). Flowers are borne in axillary racemes, enhancing their visibility and accessibility to pollinators. In the present study, the *C. ternatea* have a mauve color (Figure 1.D). The mauve color of the petals of *C. ternatea* is due to the presence of secondary metabolites, namely anthocyanins with their derivatives, namely delphinidin 3,5', 5'-triglucoside, which is also known as ternatin. Anthocyanin content is known to be one of the sources of natural antioxidants (Siti et al. 2017; Suarna and Wijaya 2021). The flowers used in this study were well intact, not wilted, and not infected by pests.

UV-Vis and TLC analysis

The phytochemical compounds of EECT blume as flavonoid, phenol, tannin, saponin, alkaloid, steroid, and terpenoid, are measured (Table 1). Phenol is the highest compound found in the extract, with a total percentage of 12.28%(w/w). Phenolic compounds, including phenol and eugenol, possess various pharmacological functions, such as antioxidant and anti-inflammatory, making them valuable in medical research (Liu et al. 2023). Due to its neuroprotective qualities against neurodegenerative illnesses, phenol and its derivatives, especially polyphenols, have attracted interest. Studies show that these substances can lessen inflammation, oxidative stress, and neuronal cell death, which are important components of Alzheimer's Disease. Polyphenols can scavenge Reactive Oxygen Species (ROS), reducing oxidative stress that contributes to neurodegeneration (Kusindarta et al. 2018; Alifia et al. 2023; Khairnar and Jadhav 2023). It acts as a neuroprotectant by crossing the blood-brain barrier, reducing reactive oxygen species, chelating metal ions, increasing neurotrophic factors, and activating the signaling cascade for neuronal cell health (Arias-Sánchez et al. 2023). Through regulation of the conversion of pro-inflammatory (M1) to anti-inflammatory (M2) macrophage responses, aiding in neuroprotection (Yuan et al. 2024).

Given a proportion of 10.39% (w/w), tannin is the second-highest component. It is present in botanical gardens and belongs to polyphenolic secondary metabolites (Table 1) (Agrippina et al. 2024). Tannins, particularly tannic acid, have emerged as a promising neuroprotectant due to their multifaceted mechanisms, including antioxidant and anti-inflammatory properties. Tannic acid has demonstrated significant antioxidant activity, reducing oxidative damage in models of traumatic brain injury and Parkinson's disease (Salman et al. 2020; Hemalatha 2022). It effectively chelates zinc ions, which are implicated in neurotoxicity, thereby protecting neurons from ischemic damage (Kim et al. 2022). Tannic acid activates neuroprotective pathways, such as PGC-1α/Nrf2/HO-1, which enhance cellular resilience against stressors (Salman et al. 2020).

Following tannin with a percentage of 10.39% (w/w), flavonoids come in third place. Flavonoids belong to a broad class of chemical substances mostly present in fruits, vegetables, cereals, tea, and wine. They are distinguished by their phenolic structures (Table 1) (Hasnat et al. 2024). Flavonoid has a total percentage of 4.54% (Table 1). Many flavonoids can cross the blood-brain barrier, ensuring their bioavailability in the central nervous system, which enhances their therapeutic potential (Hasan et al. 2023). Flavonoids like catechin and quercetin exhibit strong antioxidative effects, reducing oxidative stress in neuronal cells, which is crucial for preventing neurodegeneration (Kalra et al. 2024; Belenichev et al. 2024).

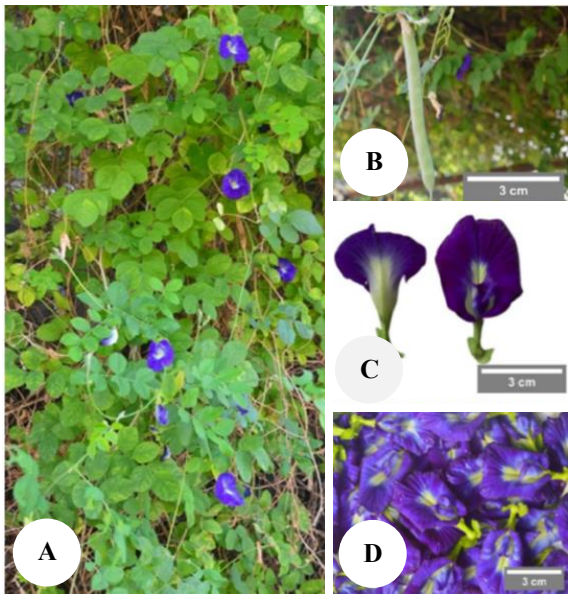


Figure 1. *Clitoria ternatea*. A. Living collection of *C. ternatea* that grows in the wild; B. The fruit; C. The flower is characterized by its blue pea and butterfly-shaped crown; D. Pile of flowers with the mauve color

Saponins are a diverse group of naturally occurring amphiphilic glycosides, primarily found in plants and some marine organisms. Total saponin in *C. ternatea* extract reaches 1.2% (Table 1). They consist of a hydrophilic sugar moiety linked to a lipophilic aglycone, which can be either a triterpene or a steroid (Timilsena et al. 2023). Saponins reduce inflammatory markers and cytokines, contributing to neuroprotection in models of diseases like Parkinson's (Chen et al. 2015; Wu et al. 2023). They influence neurotransmitter systems, enhancing neurotrophic factors that support neuronal health (Sun et al. 2015). Steroids (8,019.81 µg/g) are essential for many biological activities, such as cellular activity and hormone control (Table 1). Novel neurosteroids have been identified that inhibit NMDA receptor-mediated excitotoxicity, significantly reducing calcium influx and Reactive Oxygen Species (ROS) release (Smidkova et al. 2019). Known for their solubility in both organic solvents and acidic aqueous solutions, alkaloids are a class of naturally occurring compounds (Walsh and Tang 2022). Alkaloids have noteworthy biological actions, such as antimicrobial, anti-inflammatory, and anti-cancer effects (Rajput et al. 2022). Alkaloid only shows a small amount of 617.9 µg/g in this extract.

Terpenoids, which are commonly utilized in food and pharmaceutical items, are molecules that are formed from isoprene units present in plants (Table 1). They have a variety of biological actions, including antibacterial, antiviral, and anti-cancer characteristics (Fan et al. 2023). Terpenoids enhance neurotransmitter synthesis and release, improving cognitive functions and motor skills, particularly in Alzheimer's and Parkinson's diseases. Moreover, it suppresses pro-inflammatory mediators linked to neurodegenerative illnesses, including cyclooxygenase-2 (COX-2) and inducible Nitric Oxide Synthase (iNOS) (Lahyaoui et al. 2024).

GC-MS analysis

The GC-MS chromatogram of the ethanolic extract of *C. ternatea* showed a total of 36 peaks (Figure 2). Meanwhile, the identified phytochemical substances that were obtained are 33 (Table 2). Ethanolic extract of *C. ternatea* has a total of 33 compounds uncovered by GC-MS. Some phytochemicals show biological activity as neuroprotectives. Palmitic acid had the highest relative abundance of 17.27%. This compound is a saturated fatty acid that acts as a 5-alpha reductase inhibitor in addition to having antioxidant properties. It has free radical detoxification and inhibition activity, also lessens the neurotoxicity of amyloid beta, and has antidepressant and anticonvulsant qualities. (Margret et al. 2015). Following palmitic acid, 4H-Pyran-4-one. 2.3-dihydro-3.5-dihydroxy-6-methyl had the second highest relative abundance with a total of 13%. The 4H-Pyran-4-one. 2.3-dihydro-3.5-dihydroxy-6-methyl, commonly referred to as DDMP, is a compound of significant interest due to its presence in various food products and its potential biological activities (Chen et al. 2021). It is defined as a part of flavonoids, which are referred to as phytoestrogens when plants produce them. Through activation of the dependent ER pathway, phytoestrogen substances prevent neuronal death (Margret et al. 2015; Muslikh et al. 2022).

Table 1. Profile of ethanolic extract *Clitoria ternatea* flower phytochemical compound using spectrophotometry UV-Vis and Thin Layer Chromatography

Compound name	Concentration	Unit	Metode
Phenol	12.28	%(w/w)	UV-Vis
Tanin	10.39	%(w/w)	UV-Vis
Flavonoid	4.54	%(w/w)	UV-Vis
Saponin	1.20	%(w/w)	UV-Vis
Steroid	8,019.81	µg/g	UV-Vis
Alkaloid	617.9	µg/g	TLC
Terpenoid	Positive	-	TLC

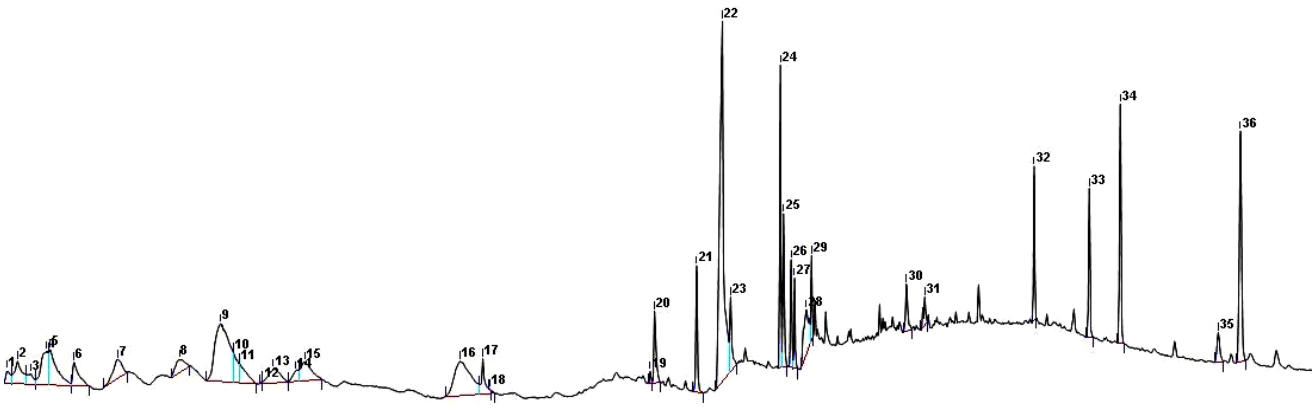
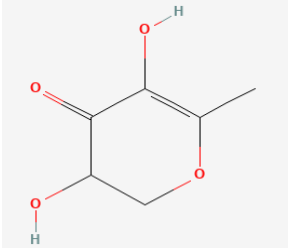
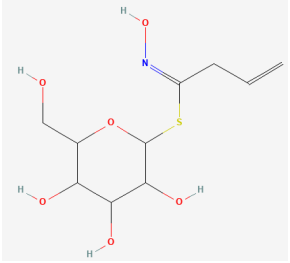
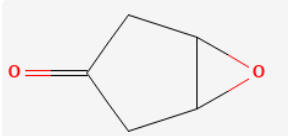
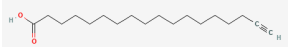
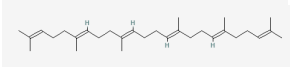
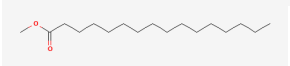
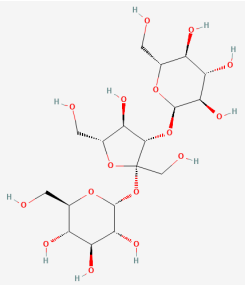
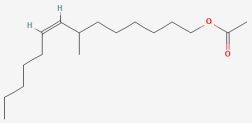
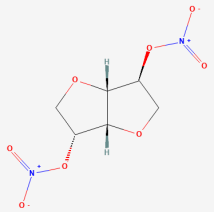
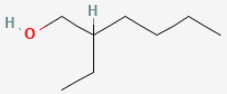
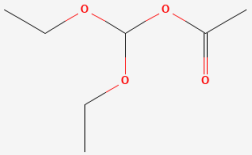
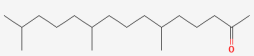
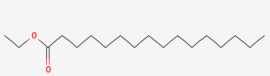
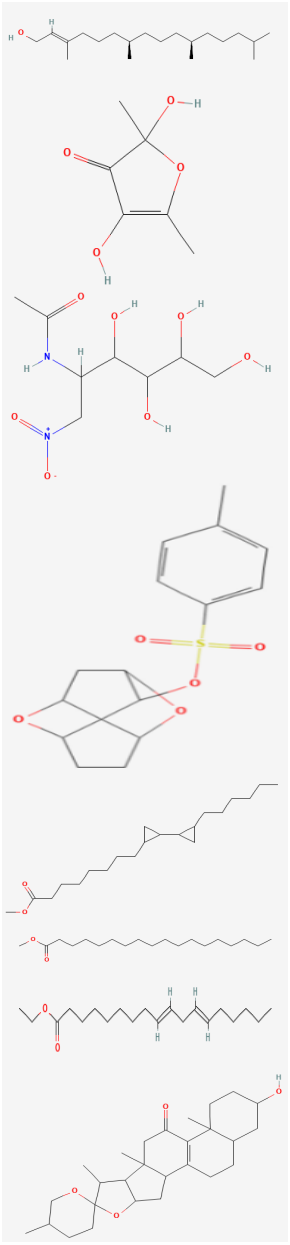


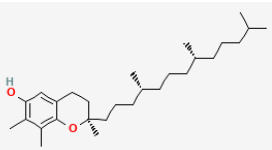
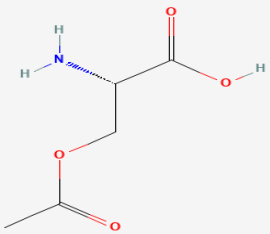
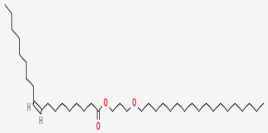
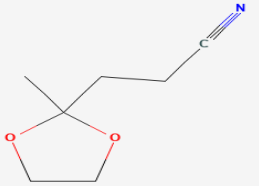
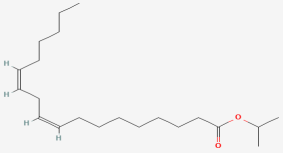
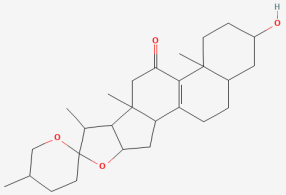
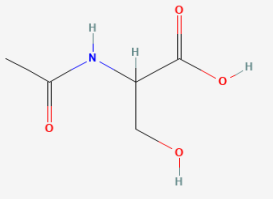
Figure 2. GC-MS chromatogram of ethanolic extract of *C. ternatea*, with Palmitic acid had the highest relative abundance of 17.27%

Table 2. Profile of secondary metabolites of the ethanolic extract of *Clitoria ternatea* by GC-MS

RT (min)	Molecular name	Molecular formula	Molecular structure	MW	RA (%)	Biological activity
17.96	Palmitic Acid	$C_{16}H_{32}O_2$		256.42 g/mol	17.27%	It protects amyloid beta and hypoxic stressors
8.27 8.64 8.52	4H-Pyran-4-one. 2.3-dihydro-3.5-dihydroxy-6-methyl-	$C_5H_8O_4$		144.12 g/mol	9.89% 1.62% 1.49%	Prevent neuronal death
12.90 9.71	Desulphosinigrin	$C_{10}H_{17}NO_6S$		279.31 g/mol	6.81% 0.75%	Anti-inflammatory, Anti-cancer, and anti-asthmatic
27.97	Tetratriacontane	$C_{34}H_{70}$		478.9 g/mol	6.52%	Antioxidant, Anti-cancer, and antibacterial.
25.65	Tritetracontane	$C_{43}H_{88}$		605.2 g/mol	4.46%	Antibacterial
19.08	9.12-Octadecadienoic acid (Z.Z)-. Methyl ester	$C_{19}H_{34}O_2$		294.5 g/mol	4.03%	Protect the membranes of neurons from oxidative damage
4.98 4.91	6-Oxa-bicyclo[3.1.0]hexan-3-one	$C_5H_6O_2$		98.10 g/mol	3.35% 2.98%	Antibacterial and antifungal
19.59	17-Octadecynoic acid	$C_{18}H_{32}O_2$		280.4 g/mol	2.76%	Antihypertensive
19.15	9.12.15-Octadecatrienoic acid. methyl ester	$C_{19}H_{32}O_2$		292.5 g/mol	2.64%	Anti-inflammatory and antimicrobial
25.05	Squalene	$C_{30}H_5O$		410.7 g/mol	2.61%	Increased the amount of acetylcholine (Ach) while lowering the levels of acetylcholinesterase (AChE) activity
17.47	Methyl Palmitate	$C_{17}H_{34}O_2$		270.5 g/mol	2.5%	Anti-inflammatory and anti-fibrotic.

9.90 13.47	Melezitose	$C_{18}H_{32}O_{16}$		504.4 g/mol	2.5% 0.01%	Suppression of lung cancer cell growth.
23.99	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$		268.4 g/mol	2.08%	Anti-cancer, anti-inflammatory, and hepatoprotective properties.
9.29	Isosorbide Dinitrate	$C_6H_8N_2O_8$		236.14 g/mol	2.07%	Increasing acetylcholine activity.
6.29	1-Hexanol. 2-ethyl-	$C_8H_{18}O$		130.23 g/mol	2.07%	Antibacterial.
4.35	Diethoxymethyl acetate	$C_7H_{14}O_4$		162.18 g/mol	1.97%	Used in the synthesis of theophylline nucleosides D-mannosyl, D-galactosyl, and D-glucosyl.
16.65	Hexahydrofarnesyl acetone	$C_{18}H_{36}O_2$		268.5 g/mol	1.94%	Antibacterial, antinociceptive, and anti-inflammatory activities.
18.13	Ethyl Palmitate	$C_{18}H_{36}O_2$		284.5 g/mol	1.94%	Anti-inflammatory.

19.29	Phytol	$C_{20}H_{40}O$		296.5 g/mol	1.91%	Reduced ROS production and Aβ deposition. Downregulated the expression of genes and proteins related to AD.
5.43	2.4-Dihydroxy-2.5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$		144.12 g/mol	1.76%	Antimicrobial, anti-inflammatory, and antioxidant.
7.47	-Mannitol	$C_8H_{16}N_2O_7$		252.22 g/mol	1.49%	Antimicrobial.
13.34	Toluene-4-sulfonic acid. 2.7dioxatricyclo[4.3.1.0(3.8)]dec-10-yl ester	$C_{15}H_{18}O_5S$		310.4 g/mol	1.42%	-
21.52 16.57	[1.1'-Bicyclopropyl]-2-octanoic acid. 2'-hexyl-. methyl ester	$C_{21}H_{38}O_2$		322.5 g/mol	1.29% 0.19%	Antibacterial.
19.36	Methyl stearate	$C_{19}H_{38}O_2$		298.5 g/mol	1.26%	Antibacterial and Antifungal activity.
19.69	Octadecenoic acid. methyl ester	$C_{20}H_{36}O_2$		308.5 g/mol	1.25%	Antihistaminic, cancer preventative, hepatoprotective, and anti-inflammatory.
30.97	3-Hydroxyspirost-8-en-11-one #	$C_{27}H_{40}O_4$		428.6 g/mol	1.1%	Antibacterial.

27.55	Tocopherol	$C_{28}H_{48}O_2$		416.7 g/mol	0.98%	Regulate the APP gene expression of non-amyloidogenic and amyloidogenic.
4.60	o-Acetyl-L-serine	$C_5H_9NO_4$		147.13 g/mol	0.84%	Anti-inflammatory and neuroprotective properties.
31.40	Oleic acid. 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$		593.0 g/mol	0.75%	Antifungal.
4.15	4.4-Ethylenedioxy-pentanenitrile	$C_7H_{11}NO_2$		141.17 g/mol	0.75%	-
21.87%	Isopropyl linoleate	$C_{21}H_{38}O_2$		322.5 g/mol	0.74%	Antioxidant.
31.14	Spirost-8-en-11-one. 3-hydroxy-. (3ß.5a.14ß.20ß.22ß.25R)-	$C_{27}H_{40}O_4$		428.6 g/mol	0.04%	Antioxidant, antimicrobial, and anti-inflammatory.
9.08	N-Acetyl-DL-serine	$C_5H_9NO_4$		147.13 g/mol	0.0016%	Antimicrobial.

The sixth highest phytochemical, 9.12-Octadecadienoic acid (Z.Z)-. Methyl ester, with a relative abundance of 4.03% is a fatty acid that functions as an anti-inflammatory mediator in the brain as well as an antioxidant and protects the membranes of neurons from oxidative damage (Lei et al. 2016). Squalene, a natural triterpenoid hydrocarbon, is primarily sourced from shark liver oil and various plant seeds (Zhang et al. 2023). This compound, reaching a relative abundance of 2.61% proved to significantly increase the amount of acetylcholine (Ach) while lowering the levels of acetylcholinesterase (AChE) activity in the region of the hippocampal and cerebral cortex, which is essential for treating AD. It protects the brain from Alzheimer's disease, which results from D-galactose and leads to an increase in the cholinergic neurotransmitter in the rat brain's cerebral cortex and hippocampal regions (Yellamma and Pravee 2023).

Isosorbide dinitrate, with a relative abundance of 2.07%, has a neuroprotective effect by increasing acetylcholine activity in the rat brain (Abdel-Salam et al. 2012). Phytol (1.91%) is a diterpene alcohol primarily recognized as a component of chlorophyll, playing a significant role in various biological processes and exhibiting potential health benefits (Narai-Kanayama et al. 2023). In transgenic *Caenorhabditis elegans* models of AD, in vivo investigation showed that phytol increased lifespan. Chemotaxis behavior and reduced ROS (Reactive oxygen species) production and Aβ deposition. The gene associated with nematode longevity (dnj-14) was upregulated during phytol treatment. Phytol also decreased the protein level of Aβ peptide expression and reduced the expression of genes linked to AD, including ace-1, hsp-4, and Aβ (Sathya et al. 2020).

Tocopherol (0.98%), commonly known as vitamin E, is a group of lipid-soluble compounds with significant antioxidant properties, primarily produced by photosynthetic organisms (Bora et al. 2022). Through the activation and suppression of gene expression related. Regulation of APP non-amyloidogenic and amyloidogenic processing pathways may protect the cells against Aβ toxicity (Pahrudin-Arrozi et al. 2022). The o-Acetyl-L-serine (0.84%) is a significant intermediate in the biosynthesis of D-cycloserine (Robbins et al. 2023). It is essential for the synthesis of proteins, growth and division of cells, and the production of sphingolipids in the central nervous system. It works by activating glycine receptors and upregulating PPAR-γ, which produces neurotransmitters and has anti-inflammatory and neuroprotective properties (Phone-Myint and Sun 2023).

FTIR analysis

The ethanolic extract of *C. ternatea's* FTIR analysis results are displayed in 25 peaks (Figure 3). The result showed several specific peaks with the highest wavelength of 3,385.81 cm^{-1} and the lowest is 419.48 cm^{-1}. Strong appearance displays in many functional groups, including Hydroxy compounds, Unsaturated/aromatic compounds, Saturated Aliphatic (alkene/alkyl) compounds, Skeletal C-C vibrations, and ether and oxy compounds (Table 3). These data also conform to the UV-vis result showed which shows a higher percentage of phenol, tannin, and flavonoid than saponin, steroid, and alkaloid (Table 1).

Phenol is classified as an aromatic compound due to its benzene structure, which contributes to its unique chemical properties (Rasheedy 2022). Phenol exhibits hydrogen bonding, influencing its solubility and boiling point, making it a significant compound in organic chemistry (Singh et al. 2021). Given that phenol has a hydroxyl or -OH functional group. The chemical structure of tannins is complex, primarily characterized by their polyphenolic nature, which includes various ring structures (Omura et al. 2021). Tannins typically feature a phenolic backbone, with the B-ring being particularly significant. The B-ring's chemistry is akin to that of simple phenols, allowing for reactions such as esterification and oxidation, which contribute to their biological activity (Table 1; Figure 2; Table 3).

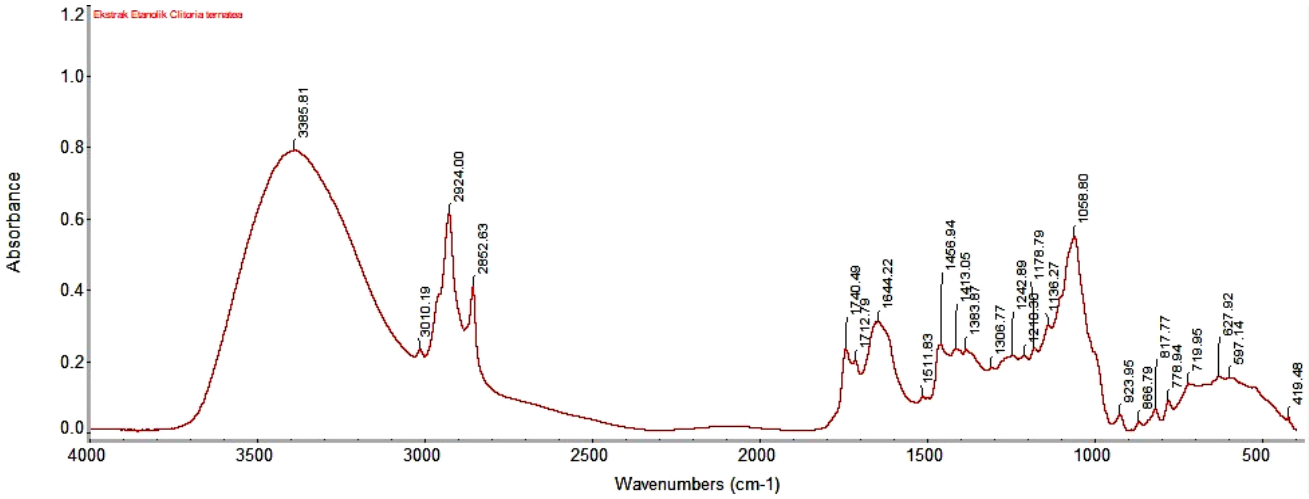


Figure 3. The spectra peaks of the Fourier Transform Infrared (FTIR) (wave number in cm^{-1}) of *Clitoria ternatea* ethanolic extract

Table 3. Phytochemical functional groups of ethanolic extract of *Clitoria ternatea* by FTIR

Functional group	Absorbance	Appearance	Specific peaks
Hydroxy compound			
Polymeric OH stretch	3200-3400	Strong	3385.81
Hydroxy group (H-OH stretch)	3200-3570 (broad)	Strong	3385.81
Alcohol. OH out-of-plane bend	590-720	Weak	419.48
Unsaturated/aromatic compound			
Olefinic	3010-3040	Medium	3010.19
Aromatic ring			
Aromatic ring (C-H)	3000-3150	Medium	3010.19
Aromatic ring stretch (C=C-C)	1450-1510	Strong	1456.94
Meta structure (C-H 1.3)	860-900	Weak	866.79
Para structure (C-H 1.4)	800 -860	Weak	817.77
Saturated aliphatic (alkene/alkyl)			
Methylene (>CH2)			
Methylene C-H asym/sym. stretch	2915 -2935	Strong	2924.00
		Strong	2852.63
Methylene C-H bend	1445 -1485	Strong	1456.94
Methyl (−CH3)			
gem-Dimethyl or ‘‘iso’’- (doublet)	1380-1385	Medium	1383.87
Methyne (>CH−)			
Skeletal C-C vibrations	700 -1300	Medium	1242.89
		Medium	1210.30
		Medium	1178.79
		Medium	1136.27
		Strong	1058.80
		Weak	923.95
		Weak	866.79
		Weak	817.77
		Weak	778.94
		Weak	719.95
Carbonyl compound			
Ester	1725-1750	Medium	1740.49
Ketone	1705-1725	Medium	1712.79
Carboxylic acid	1700-1725	Medium	1712.79
Amide	1630-1680	Medium	1644.22
Carboxylate (carboxylic acid salt)	1300-1420	Medium	1413.05
		Medium	1383.87
		Medium	1306.77
Aromatic compounds			
Double bonds (C=C)	1600-1650 (sharp)	Medium	1644.22
Basic hetero-oxy compounds			
Nitrogen-oxy compounds			
Aromatic nitro compounds	1485-1555	Weak	1511.83
Sulfur-oxy compounds			
Dialkyl/aryl sulfones	1300-1335	Medium	1306.77
Olefinic (alkene)			
Vinyl C-H in-plane bend	1410-1420	Medium	1413.05
Ether and oxy compound			
Alkyl-substituted ether. C-O stretch	1050-1150	Strong	1058.80
Tertiary amino			
Tertiary amine. CN stretch	1150-1210	Medium	1178.79
Secondary amino			
Secondary amine. CN stretch	1130-1190	Medium	1136.27
Acetylenic (alkyne)			
Alkyne C-H bend	610 -680	Medium	627.92
Thiols and thio-substituted compounds			
Disulfides (C-S stretch)	570-705	Weak	597.14
Aliphatic organohalogen compound			
Perchlororthylene	419	Weak	419.48

Flavonoids are a diverse group of polyphenolic compounds characterized by their complex structures, which include various functional groups and phenolic rings. Typically, flavonoids have a 15-carbon skeleton made up of one heterocyclic ring (C) and two rings of aromatic compounds (A and B) (Abhinav et al. 2024). These results support the FTIR finding that demonstrated a strong appearance in a normal "polymeric" OH stretch hydroxy group (3200-3570 cm^{-1}) and C=C-C aromatic ring stretch (1450-1510 cm^{-1}) (Table 1; Figure 2; Table 3).

The results obtained by FTIR and GC-MS could confirm one another. Based on the GC-MS analysis result, there are several phytochemical compounds having their unique functional group that have neuroprotective potential. Palmitic acid, a saturated fatty acid, features a carboxyl functional group (-COOH) at one end of its long hydrocarbon chain, which is 16 carbon atoms long (Yu et al. 2020). The carboxylic acid functional group (1700-1725 cm^{-1}) is identified and shows a medium appearance by FTIR. The chemical structure of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) is characterized by a six-membered ring containing one carbonyl group and multiple hydroxyl groups. Carbonyl compounds show a medium appearance, while hydroxyl compounds show a strong appearance; that's because a lot of the phytochemicals belong to the phenol family; the 9.12-Octadecadienoic acid (Z.Z)-. Methyl ester contains a methyl ester functional group (-$COOCH_3$). The carbon chain has two cis double bonds located at the 9th and 12th carbon atoms, contributing to its unsaturated nature (Klok et al. 1980) (Table 2; Figure 2; Table 3).

Squalene's structure includes multiple methyl groups and double bonds, which are essential for its reactivity and biological functions (Klok et al. 1980); both 9.12-Octadecadienoic acid (Z.Z)-. Methyl ester and squalene have methyl functional groups (1445-1485 cm^{-1}) that show strong appearance, whereas the ester functional group that only present in 9.12-Octadecadienoic acid (Z.Z)-. Methyl ester (1725-1750 cm^{-1}) is identified as having medium appearance. Isosorbide dinitrate (ISDN) is a compound characterized by its dinitrate functional group (Guo et al. 2015). Nitrogen-oxy functional group shows a weak appearance, confirming the GC-MS ISDN result that only shows 2.07% relative abundance. Phytol is a fatty alcohol that contains a hydroxyl functional group, which shows a strong appearance. This group is responsible for its solubility in organic solvents and its surfactant capabilities (Prabha et al. 2019). Tocopherol contains one to three methyl groups, influencing the antioxidant capacity (Munne-Bosch and Falk 2004). Based on the FTIR result, methyl shows a strong appearance. The functional group of o-Acetyl-L-serine (OAS) plays a crucial role in biochemical reactions, particularly in the synthesis of L-cysteine. This compound features an acetyl group attached to the hydroxyl group of serine, which is essential for its reactivity in enzymatic processes (Table 2; Figure 2; Table 3).

In silico molecular docking assay

Palmitic acid presented the same binding sites as donepezil against β-amyloid. The binding energy of palmitic acid against β-amyloid was higher than donepezil as a control. The hydrogen bond complex surface of palmitic acid performed dominantly as a hydrogen acceptor among proteins, while the hydrophobicity of palmitic acid showed the same profile toward β-amyloid. The interaction of palmitic acid and donepezil with β-amyloid protein showed two major bonds, hydrogen bonds and hydrophobic bonds (Figure 4).

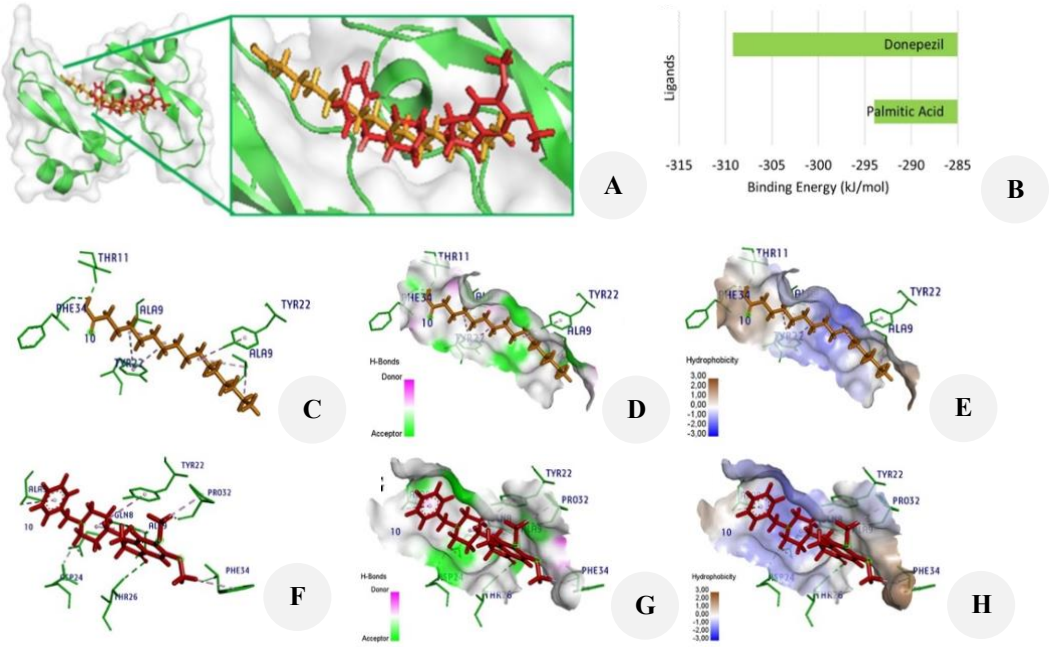


Figure 4. Interaction of palmitic acid and donepezil with β-amyloid protein. A. 3D structure of ligands-protein complex, B. binding energy of complex, C. Binding sites of Palmitic acid, D. hydrogen bond surface of palmitic acid, E. hydrophobicity of palmitic acid, F. Binding sites of donepezil, G. hydrogen bond surface of donepezil, H. hydrophobicity of donepezil

Proliferation assay

Neuronal loss in Alzheimer's disease is a critical aspect of its pathology, characterized by a significant decrease in neuron numbers across various brain regions, especially the hippocampus. This loss is linked to several mechanisms, including the accumulation of amyloid-β (Aβ) and neurofibrillary tangles, which contribute to neurodegeneration (Wirths and Zampar 2020). The average neuron loss at the most advanced stages (AD severe dementia) was over 80%. The underlying mechanism of TMT-induced neuronal loss involves multiple interrelated pathways, primarily centered around calcium dysregulation, apoptosis, and mitochondrial dysfunction. These mechanisms contribute to the neurotoxic effects observed in various neuronal cell types. A study showed that a dose of 50 µM TMT induced about 80% neuronal loss (Song et al. 2021). The research conducted in this study also shows similar results of TMT toxicity at a dose of 50 µM (Figure 5). Therefore, the proliferation assay will be using 50 µM as the induced dose. Research by Jeyaraj et al. (2022) showed that the ethanolic extract of *C. ternatea* flower doesn't show any cytotoxic activities up to a dose of 156.3 µg/mL, and it was also confirmed in this study, showing no cytotoxic activities up to 250 µg/mL.

The ability of EECT to induce cell proliferation is illustrated in the form of viable and dead cells (Figure 5)**.** In a recent study, the result showed that in PC-12 cells induced by TMT undergoing oxidative stress leading to apoptosis of the cell, the morphology was rounder than cells that were still adherent and viable. Meanwhile, cells treated with EECT showed more viable cells than ones treated with donepezil, suggesting that EECT has a significant role in inducing the proliferation of TMT-induced PC-12 cells. This ability is an important component for Alzheimer's disease prevention due to EECT significantly decreasing neuronal loss.

The neuroprotective potential of EECT is evaluated by a proliferation assay on TMT-induced neurotoxicity in PC-12 cells. The result showed that administration of EECT extract had significantly higher proliferation activity as same as to donepezil as a control of commercial used medication. In contrast, there is a very low proliferation rate of the cells, which is induced as an in vitro model of AD by TMT (Figure 6).

The highest proliferation activity was shown in a concentration of 31.25 µg/mL. Ten different concentrations of *C. ternatea* ethanolic extract were used to determine the IC_{50} value of the extract, which resulted in 54.46 µg/mL (Figure 7). IC_{50} Values between 10 and 100 µg/mL indicate moderate inhibition, commonly seen in various drug assays (Gong et al. 2014). Phytochemical constituents which assumed to play a role in neuroprotection and promote proliferation are ones that belong to phenol, tannin, and flavonoid. These substances can lessen inflammation, oxidative stress, and neuronal cell death, which are important components to treat neurodegenerative diseases (Khairnar and Jadhav 2023). Meanwhile, a more specific type of phytochemical that has neuroprotective including palmitic acid, 4H-Pyran-4-one. 2.3-dihydro-3.5-dihydroxy-6-methyl-, squalene, isosorbide dinitrate, phytol, and tocopherol, which mostly have antioxidant activities and increase the amount of neurotransmitter (Margret et al. 2015).

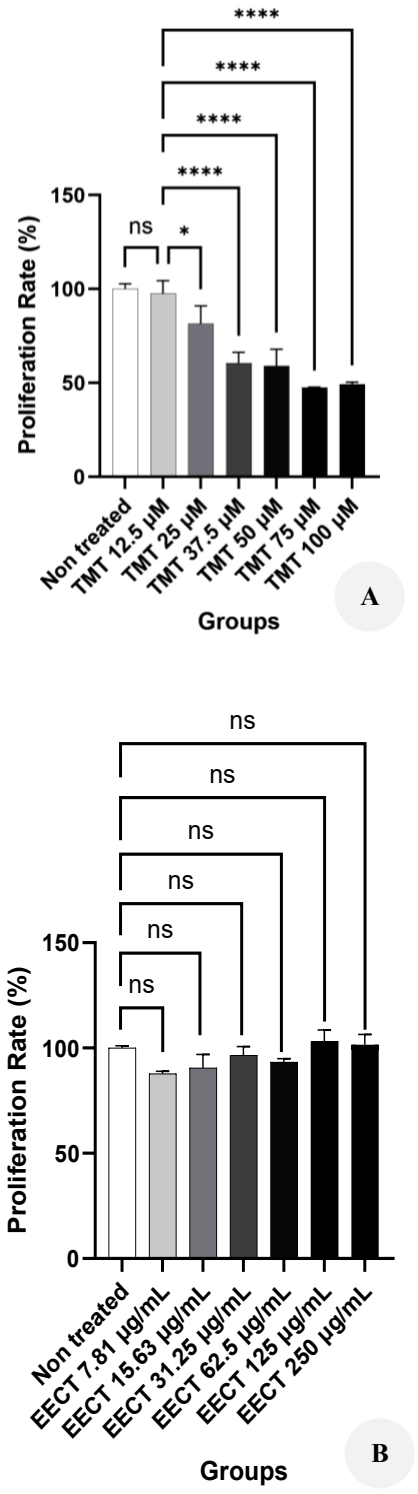


Figure 5. A. Other option despite ref optimization TMT by Wilda. B. Other option despite ref cytotoxic by Wilda

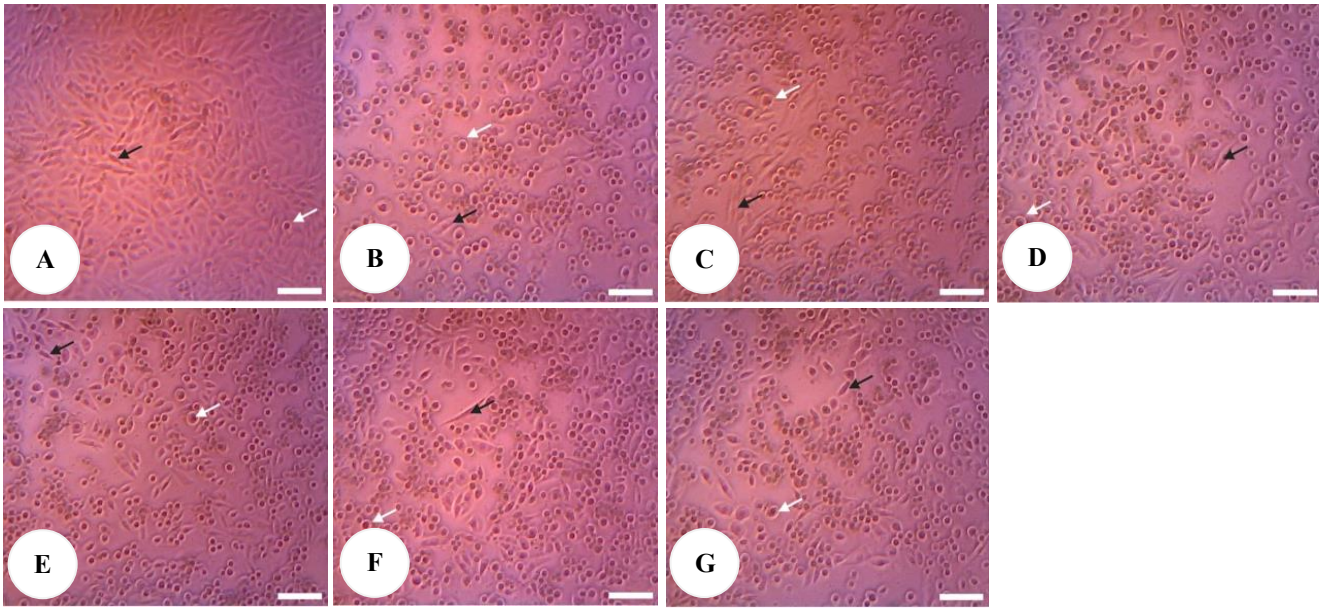


Figure 6. Ethanolic extract of *C. ternatea* maintains PC-12 cell proliferation: A. Non-treated cell, B. TMT-induced cell, C. Donepezil treated cell, D. TMT-induced and treated with EECT 7.81 µg/mL, E. TMT-induced and treated with EECT 15.63 µg/mL, F. TMT-induced and treated with EECT 31.25 µg/mL, G. TMT-induced and treated with EECT 62.5 µg/mL. Black arrows: Viable cells; White arrows: Dead cells. Scale bar: 50 µm

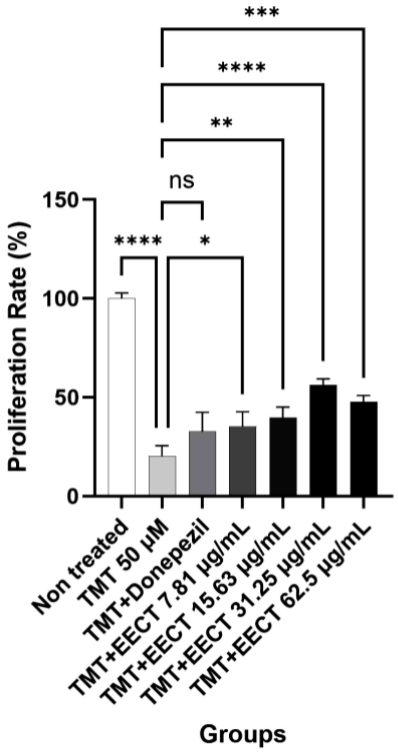


Figure 7. Ethanolic extract of *C. ternatea* preserved viability and encouraged proliferation of TMT-induced PC-12 cells. Tukey's HSD test: *0.05≥p>0.01; **0.01≥p>0.001; ***0.001≥p>0.0001; ****p≤0.0001

In conclusion ethanolic extract of *C. ternatea* contained phytochemicals that have neuroprotective biological activities, including phenol, tannin, and flavonoid. GC-MS analysis resulted in more specific compounds such as palmitic acid, 4H-Pyran-4-one. 2.3-dihydro-3.5-dihydroxy-6-methyl-, squalene, isosorbide dinitrate, phytol, tocopherol, and o-Acetyl-L-serine. Each of the compounds has its unique functional groups that help it perform its biological activities. Proliferation assay on TMT-induced neurotoxicity in PC-12 cells showed that *C. ternatea* ethanolic extract maintained viability and promoted proliferation in an in vitro model of AD. In silico molecular docking showed the activity of *C. ternatea* to prevent the accumulation of β-amyloid. Based on the study result, the ethanolic extract of *C. ternatea* has significant potential as a drug candidate to prevent Alzheimer's disease. However, further research needs to be conducted to learn the mechanism of this extract as a neuroprotectant agent.

ACKNOWLEDGEMENTS

The authors wish to thank of Integrated Laboratory for Research and Testing (LPPT) Universitas Gadjah Mada, Yogyakarta, Indonesia, for the use of analysis facilities. Ministry of Education, Research, and Technology supported this work through the Program towards a doctoral degree for outstanding undergraduates (PMDSU 2024).

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