

Chemical Profiling, Anticholinesterase Inhibitory Activity and Molecular Docking Studies of Essential Oil from *Calophyllum canum* Hook.f. ex T.Anderson

Nur Nabilah Mohd Zaini¹, Wan Mohd Nuzul Hakimi Wan Salleh^{1*}, Abubakar Siddiq Salihu^{1,2},
Nurunajah Ab Ghani^{3,4}, Amira Muhammad⁵, Vinay Bharadwaj Tatipamula⁶
and Farkhod Eshboev⁷

¹Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris,
35900 Tanjung Malim, Perak, Malaysia

²Department of Pure and Industrial Chemistry, Faculty of Natural and Applied Science,
Umaru Musa Yar'adua University, Katsina, Nigeria

³Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Universiti Teknologi MARA,
Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor, Malaysia

⁴Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

⁵Faculty of Applied Sciences, Universiti Teknologi MARA Perak Branch, Tapah Campus,
35400 Tapah Road, Perak, Malaysia

⁶Center for Pharmaceutical Biotechnology, Duy Tan University, Da Nang, Vietnam

⁷Institute for Advanced Studies, New Uzbekistan University, Tashkent 100007, Uzbekistan

*Corresponding author (e-mail: wmnhakimi@fsmt.upsi.edu.my)

Medicinal plant essential oils are an important source of bioactive compounds with potential relevance to neurodegenerative disorders, including Alzheimer's disease. In this study, the chemical composition and anticholinesterase activity of the essential oil obtained from *Calophyllum canum* leaves collected in Malaysia were investigated. Gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analyses identified eleven constituents, representing 99.6% of the total oil composition. The oil was dominated by caryophyllene oxide (41.7%), spathulenol (31.8%), γ -eudesmol (6.0%), and β -eudesmol (5.8%). Anticholinesterase activity was assessed using Ellman's method. A moderate inhibitory effect was observed for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes with IC₅₀ values of 88.5 ± 0.22 and 92.2 ± 0.21 μ g/mL, respectively. Molecular docking of the two major constituents alongside galantamine (reference inhibitor) showed favorable binding interactions toward AChE and BChE, with predicted binding energies of -5.5 to -7.6 kcal/mol for the plant constituents and -8.5 – -8.0 kcal/mol (AChE/BChE) for galantamine, supporting their accommodation within the enzyme active sites. This study provides the first report on the chemical profile and anticholinesterase potential of *C. canum* essential oil, highlighting its promise as a natural source of neuroactive compounds and contributing to the pharmacological characterization of the *Calophyllum* genus.

Keywords: *Calophyllum canum*, essential oil, caryophyllene oxide, acetylcholinesterase, butyrylcholinesterase, docking

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Medicinal plants have long served as important sources of therapeutic agents and continue to play a significant role in modern drug discovery. Their medicinal properties are largely attributed to structurally diverse secondary metabolites, including alkaloids, flavonoids, phenolic compounds, terpenoids, coumarins, and volatile constituents, which exhibit a broad spectrum of pharmacological activities [1, 2]. In particular, increasing attention has been directed towards plant-derived compounds for the prevention and management of neurodegenerative disorders because of their potential antioxidant, anti-inflammatory, neuroprotective, and enzyme-inhibitory effects [3-5]. Unlike conventional single-target agents, many

natural products may interact with multiple biological pathways involved in neurodegeneration, making medicinal plants valuable sources for identifying new therapeutic candidates. Among these, compounds capable of modulating the cholinergic system through inhibition of cholinesterase enzymes have attracted considerable interest in the search for potential agents for the management of Alzheimer's disease and related cognitive disorders.

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are key enzymes involved in the regulation of cholinergic neurotransmission. AChE is primarily responsible for the

rapid hydrolysis of the neurotransmitter acetylcholine at synaptic clefts, thereby terminating nerve signal transmission [6]. In contrast, BChE, although less specific, acts as a complementary enzyme that hydrolyses choline esters and becomes increasingly important when AChE activity is reduced [7]. Dysregulation of the cholinergic system, particularly excessive degradation of acetylcholine, is a hallmark of neurodegenerative disorders such as Alzheimer's disease, leading to cognitive decline and memory impairment.

Consequently, inhibition of AChE and BChE remains one of the most established therapeutic strategies for the symptomatic management of Alzheimer's disease [8]. Current clinically used cholinesterase inhibitors, including donepezil, rivastigmine, and galantamine, are effective in improving cognitive function but are often associated with adverse effects and limited long-term efficacy. This has driven sustained interest in the discovery of alternative cholinesterase inhibitors from natural sources that may offer improved safety profiles and multi-target activity [9]. In this context, plant-derived secondary metabolites have attracted considerable attention due to their structural diversity and biological relevance.

Essential oils, complex mixtures of volatile terpenoids and oxygenated compounds, represent a promising class of natural cholinesterase inhibitors. Numerous studies have demonstrated that sesquiterpenes and oxygenated terpenes exhibit significant inhibitory effects against AChE and BChE. Beyond direct enzyme inhibition, essential oils may exert neuroprotective effects through antioxidant, anti-inflammatory, and membrane-modulating mechanisms, which are highly relevant to the multifactorial nature of neurodegenerative diseases [10-12]. Therefore, investigating the cholinesterase inhibitory activity of essential oils not only contributes to the identification of new bioactive compounds but also supports the development of plant-based therapeutic agents for neurodegenerative disorders.

Calophyllum canum Hook.f. ex T.Anderson (Calophyllaceae), locally known in Malaysia as *bintangor* or *penaga hutan*, is a tropical evergreen tree distributed across Southeast Asia, including Malaysia, Thailand, Indonesia, and Borneo, where it grows in lowland to hill dipterocarp forests [13]. Although *C. canum* itself remains poorly documented ethnomedicinally, numerous species within the *Calophyllum* genus are traditionally used to treat inflammation, wounds, skin infections, rheumatism, and microbial-related diseases, indicating shared therapeutic relevance within the genus [14]. Phytochemical investigations of *Calophyllum* species have revealed a rich diversity of secondary metabolites, notably xanthenes, coumarins, chromanones, flavonoids, triterpenoids, and terpenoids, many of which exhibit antioxidant,

antimicrobial, anti-inflammatory, anticancer, and neuroprotective activities [15-18].

Despite the growing pharmacological interest in the genus, data on the volatile composition and biological activities of *C. canum* are extremely limited, underscoring the importance of chemical profiling and bioactivity evaluation of its essential oil to expand chemotaxonomic knowledge and explore its potential therapeutic applications. As a continuation of our systematic evaluation of the aromatic flora of Malaysia, the current study reports the chemical composition and anticholinesterase activity of the essential oil obtained from *C. canum* leaves, as well as molecular docking studies.

EXPERIMENTAL

Plant Material

The leaves of *C. canum* was collected at Fraser Hill, Malaysia (3.7113°N, 101.7364°E; altitude 1200 m) in February 2024. The plant material was identified through both morphological and taxonomic comparison with authenticated specimens. Fresh plant samples were examined by a qualified taxonomist, Dr Sahmsul Khamis, and the species identity was verified based on key diagnostic features (leaf shape, flower structure, and fruit characteristics). The voucher specimen (SA35-63) was prepared and deposited at the Herbarium of UKM, for future reference.

Isolation of Essential Oil

The fresh leaves (300 g) was cut into small pieces and hydrodistilled using a Clevenger-type apparatus for 4 h. The essential oil obtained was dried over anhydrous magnesium sulphate, filtered, and stored in sealed brown glass vials at 0°C until analysis. On a fresh weight basis, the oil yield (w/w) was 0.27% and the average moisture content was 85-89%.

Analysis of Essential Oil

The analysis of essential oil was performed using gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). GC-FID analysis was conducted on an Agilent Technologies 7890B equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 0.7 mL/min. The injector and detector temperatures were maintained at 250°C and 280°C, respectively. The oven temperature was initially set at 50°C, then increased to 280°C at a rate of 5°C/min and held isothermally for 15 minutes. Diluted samples (1/100 in diethyl ether, v/v) of 1.0 µL were injected manually with a split ratio of 50:1. The percentage of peak areas was calculated using GC HP Chemstation software (Agilent Technologies).

For GC-MS analysis, an Agilent Technologies 7890A gas chromatograph coupled with an Agilent

5975 GC MSD was used, fitted with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness). Helium served as the carrier gas at a flow rate of 1.0 mL/min. The injector temperature was set at 250°C. The oven temperature program started at 50°C (held for 5 min), increased to 280°C at 5°C/min, and was held isothermally for 15 min. An electron ionization (EI) system with an ionization energy of 70 eV was used for detection. The scan rate was 0.5 s, covering a mass range from 40 to 400 amu.

Identification of essential oil components was based on co-injection which was performed with standards (caryophyllene oxide, spathulenol, γ -eudesmol, and β -eudesmol), retention indices (calculated relative to *n*-alkane series), and mass spectral library comparison of mass spectra with established databases, including Wiley (Wiley-VCH, Germany), Adams (Allured Publishing Corporation, USA), FFNSC2 (Shimadzu, Japan), and NIST08 (National Institute of Standards and Technology, USA) [19-21]. Semi-quantification of essential oil components was performed by peak area normalization, assuming equal response factors for all volatile components. Standards were obtained from Sigma-Aldrich, USA.

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) Inhibitory Activities

AChE and BChE inhibitory activities of the essential oil were measured according to reported spectrophotometric method with minor modifications [8]. Electric eel AChE/horse serum BChE (0.22 U/mL; Sigma-Aldrich, USA) was used, while acetylthiocholine iodide (Sigma-Aldrich, USA) and butyrylthiocholine chloride (Sigma-Aldrich, USA) were employed as substrates of the reaction. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich, USA) was used for measurement of anticholinesterase activity. Briefly, in this method, 140 µL of sodium phosphate buffer (50 mM, pH = 8.0), 20 µL of DTNB (0.33 mM), 20 µL of essential oil (conc. of 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 µg/mL) and 20 µL of AChE/BChE solution were added by multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 µL of acetylthiocholine iodide/butyrylthiocholine chloride (0.5 mM each). Hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion because of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of inhibition of AChE/BChE (%) was determined by comparison of samples reaction rates relative to a blank sample (EtOH in phosphate buffer (pH = 8.0) using the formula:

$$I\% = [(E - S)/E] \times 100;$$

where E is the activity of enzyme without test sample and S is the activity of the enzyme with the test sample. Galantamine was used as a positive control. The IC₅₀ values, defined as the concentration of the sample required to inhibit 50% of enzyme activity, were calculated from concentration–response curves by nonlinear regression analysis using GraphPad Prism version 10.0. All experiments were performed in triplicate, and the results are expressed as mean ± standard deviation (SD).

Molecular Docking Studies

The 3D crystallographic structures of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were retrieved from the Protein Data Bank (PDB IDs: 4EY5 and 4BDS). Protein preparation and energy minimization were performed using UCSF Chimera with the AMBER force field. The structures of caryophyllene oxide, spathulenol, and galantamine (included as reference inhibitor) were obtained from the PubChem database and energy-minimized using PyRx (OpenBabel tool). Molecular docking was carried out using AutoDock Vina implemented in PyRx, with grid boxes centered on the active-site regions and dimensions sufficient to cover the binding pockets. Semi-flexible docking was applied, with rigid protein structures and flexible ligands. The best docking poses were selected based on the lowest binding energy (kcal/mol) and further analyzed for ligand-enzyme interactions [22]. Visualization and interaction analysis were performed using Discovery Studio Visualizer 2021.

RESULTS AND DISCUSSIONS

The essential oil obtained from the fresh leaves of *C. canum* yielded 0.27% (w/w). The identified components are summarized in Table 1. GC-FID and GC-MS (Figure 1) analyses resulted in the identification of 11 components, representing 99.6% of the total oil composition. Oxygenated sesquiterpenes predominated, accounting for 98.0% of the oil. The major constituents were caryophyllene oxide (41.7%), spathulenol (31.8%), γ -eudesmol (6.0%), and β -eudesmol (5.8%). Comparative analysis with other *Calophyllum* species shows that β -caryophyllene is a major constituent in the essential oils of *C. inophyllum* [23,24] and *C. soulattri* [25], whereas the oil of *C. coriaceum* is dominated by β -selinene [26]. Importantly, this study provides the first evidence identifying caryophyllene oxide and spathulenol as the predominant constituents in the leaf essential oil of *C. canum*, highlighting their potential utility as chemotaxonomic markers for this species. Variations in the chemical profiles observed among *Calophyllum* species are likely attributable to multiple factors, including extraction techniques, seasonal influences, developmental stage of the plant, and local environmental conditions at the site of collection. Such variables play a crucial role in regulating secondary

metabolite biosynthesis, leading to marked differences in the qualitative and quantitative composition of essential oils [27].

Caryophyllene oxide, a bicyclic sesquiterpene commonly occurring in the essential oils of various medicinal and edible plants and widely used as a flavoring agent, has been reported to exhibit significant central and peripheral analgesic effects in addition to pronounced anti-inflammatory activity [28]. It functions as a potent anti-inflammatory compound and modulates emerging therapeutic

targets, including 15-lipoxygenase [29]. Furthermore, caryophyllene oxide has demonstrated inhibitory effects against multiple myeloma, breast, and prostate cancer cell lines and is suggested to induce apoptosis through the suppression of NF- κ B activation [30]. Spathulenol, another oxygenated sesquiterpene, has been reported to attenuate cardiac fibrosis by inhibiting the transforming growth factor- β /Smad signaling pathway [31]. Additionally, spathulenol exhibits antiproliferative activity in lymphocytes and induces apoptosis, potentially *via* a caspase-3-independent mechanism [32].

Table 1. Chemical components identified from *C. canum* essential oil.

No	Components	KI ^a	KI ^b	Percentage (%)	Identification ^c
1	β -Caryophyllene	1415	1417	1.6 $\pm$ 0.05	RI, MS
2	Elemol	1549	1548	3.1 $\pm$ 0.06	RI, MS
3	Globulol	1578	1577	2.4 $\pm$ 0.04	RI, MS
4	Caryophyllene oxide	1580	1582	41.7 $\pm$ 0.12	RI, MS, Std
5	Spathulenol	1590	1590	31.8 $\pm$ 0.11	RI, MS, Std
6	γ -Eudesmol	1632	1630	6.0 $\pm$ 0.03	RI, MS, Std
7	<i>epi</i> - α -Cadinol	1640	1638	1.5 $\pm$ 0.01	RI, MS
8	β -Eudesmol	1648	1649	5.8 $\pm$ 0.08	RI, MS, Std
9	α -Cadinol	1652	1652	1.7 $\pm$ 0.05	RI, MS
10	α -Eudesmol	1654	1655	2.7 $\pm$ 0.05	RI, MS
11	(<i>E,E</i>)- α -Farnesol	1745	1742	1.3 $\pm$ 0.02	RI, MS
Sesquiterpenes hydrocarbon				1.6	
Oxygenated sesquiterpenes				98.0	
Total identified (%)				99.6	

^a Linear retention index experimentally determined using homologous series of C₆-C₃₀ alkanes

^b Linear retention index taken from Adams and literatures

^c Identification methods: Std, based on comparison with authentic compounds; RI, MS - based on comparison with Wiley, Adams, FFNSC2 and NIST08 MS databases

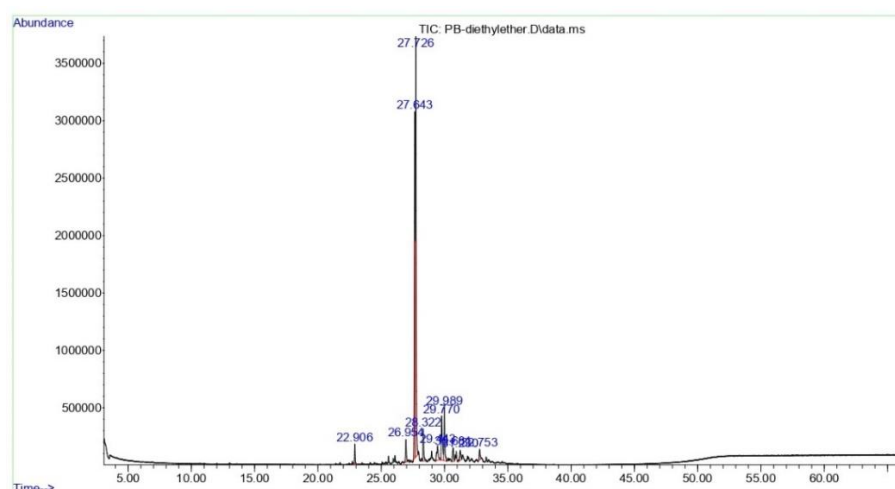


Figure 1. Chromatogram of *C. canum* essential oil.

Plant-derived essential oils possess a wide range of pharmacological activities attributable to their structurally diverse bioactive constituents and are increasingly explored for their anticholinesterase potential [33]. Aazza et al. [34] documented significant cholinesterase inhibitory activities in commercial essential oils from *Citrus aurantium*, *Cupressus sempervirens*, *Eucalyptus globulus*, *Foeniculum vulgare*, and *Thymus vulgaris*. Despite growing evidence supporting the anticholinesterase effects of numerous plant-derived compounds, in vitro investigations focusing on essential oils from tropical plant species remain scarce. Furthermore, the essential oil was initially evaluated for its inhibitory potential against AChE and BChE. AChE is the primary enzyme responsible for the breakdown of acetylcholine, a key neurotransmitter in the central nervous system. Reversible inhibition of AChE is a common therapeutic approach for the treatment of Alzheimer's disease [35]. BChE is another important cholinesterase that is present not only in the brain but also in the liver and plasma. Increased BChE activity has been linked to the progression of amyloid plaques, which may contribute to neurodegeneration [36].

In this study, anticholinesterase activity was evaluated using Ellman's method, which is based on the enzymatic hydrolysis of acetylthiocholine iodide and butyrylthiocholine iodide. The reaction produces a yellow 5-thio-2-nitrobenzoate anion through the interaction of thiocholines with DTNB, and the absorbance was measured at 412 nm using a spectrophotometer or microplate reader. Acetylthiocholine iodide and butyrylthiocholine iodide served as substrates, while DTNB was employed as the chromogenic reagent. Enzyme inhibition was calculated from the change in absorbance relative to the control. The inhibitory activity of the essential oil was compared with galantamine, a standard drug used in Alzheimer's disease therapy. The essential oil indicated moderate AChE (IC_{50} value 88.5 ± 0.21 $\mu\text{g/mL}$) and BChE (IC_{50} value 92.2 ± 0.22 $\mu\text{g/mL}$) inhibitory activity at 1,000 $\mu\text{g/mL}$ concentration, compared to galantamine (AChE: IC_{50} value 2.5 ± 0.12 $\mu\text{g/mL}$, BChE: IC_{50} value 17.5 ± 0.15 $\mu\text{g/mL}$) inhibition at the same concentration. The pronounced anticholinesterase activity observed in the essential oil may be largely attributed to its high content of caryophyllene oxide, which accounted for 41.7% of the total composition. Caryophyllene oxide is an oxygenated sesquiterpene known to interact with enzyme active sites through hydrophobic interactions and hydrogen bonding, thereby potentially interfering with substrate access to the catalytic gorge of cholinesterases [37]. Previous studies have reported moderate to strong AChE and BChE inhibitory activities for essential oils and isolated fractions rich in caryophyllene oxide, supporting its role as a key

contributor to cholinesterase inhibition. For instance, essential oils from *Syzygium aromaticum*, *Salvia officinalis*, and *Artemisia* species, in which caryophyllene oxide is a major component, have demonstrated comparable inhibitory profiles against both AChE and BChE [38].

In comparison with earlier reports, the inhibitory effects of the *C. canum* leaf essential oil are consistent with sesquiterpene-rich oils that typically exhibit moderate inhibition relative to standard drugs such as galantamine. While galantamine remains markedly more potent, the activity displayed by the present essential oil is noteworthy given its complex mixture and natural origin. Moreover, the coexistence of other oxygenated sesquiterpenes, including spathulenol and eudesmol isomers, may enhance the overall activity through additive or synergistic effects, as previously suggested for multicomponent essential oil systems [39].

Molecular docking was performed to evaluate the interactions of the two major constituents, caryophyllene oxide and spathulenol, with acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Figure 2). Caryophyllene oxide exhibited predicted binding affinities of $-6.0/-7.3$ kcal/mol for AChE/BChE, respectively, while spathulenol showed $-5.5/-7.6$ kcal/mol. In AChE, both ligands occupied the catalytic gorge and formed predominantly hydrophobic and van der Waals contacts, including π -alkyl/ π -sigma interactions with Trp86 together with additional close contacts involving Ser203, His447, Gly448, Glu202, and several tyrosine and leucine residues (Fig. 2A). In BChE, binding occurred within the broader active-site cavity, where the ligands interacted mainly with Trp82 and surrounding hydrophobic residues such as Phe329, Phe398, His438, Ala328, and Met437 (Fig. 2B), while spathulenol additionally displayed a weak hydrogen-bond contact with Ser198. Overall, both compounds remained stably positioned within the enzyme pockets through predominantly non-covalent interactions. Galantamine, included as reference inhibitor, exhibited predicted binding affinities of -8.5 kcal/mol for AChE and -8.0 kcal/mol for BChE, values more favourable than those of caryophyllene oxide and spathulenol, consistent with its established potency as a dual cholinesterase inhibitor. In AChE, galantamine occupied the catalytic gorge with interactions involving Trp86, Glu202, Tyr337, and His447, while in BChE it engaged Trp82, His438, Phe329, and Gly439. These reference docking energies confirm that the protocol correctly reproduced the established pharmacological profile of galantamine and provide a meaningful benchmark for interpreting the binding affinities of the plant constituents.

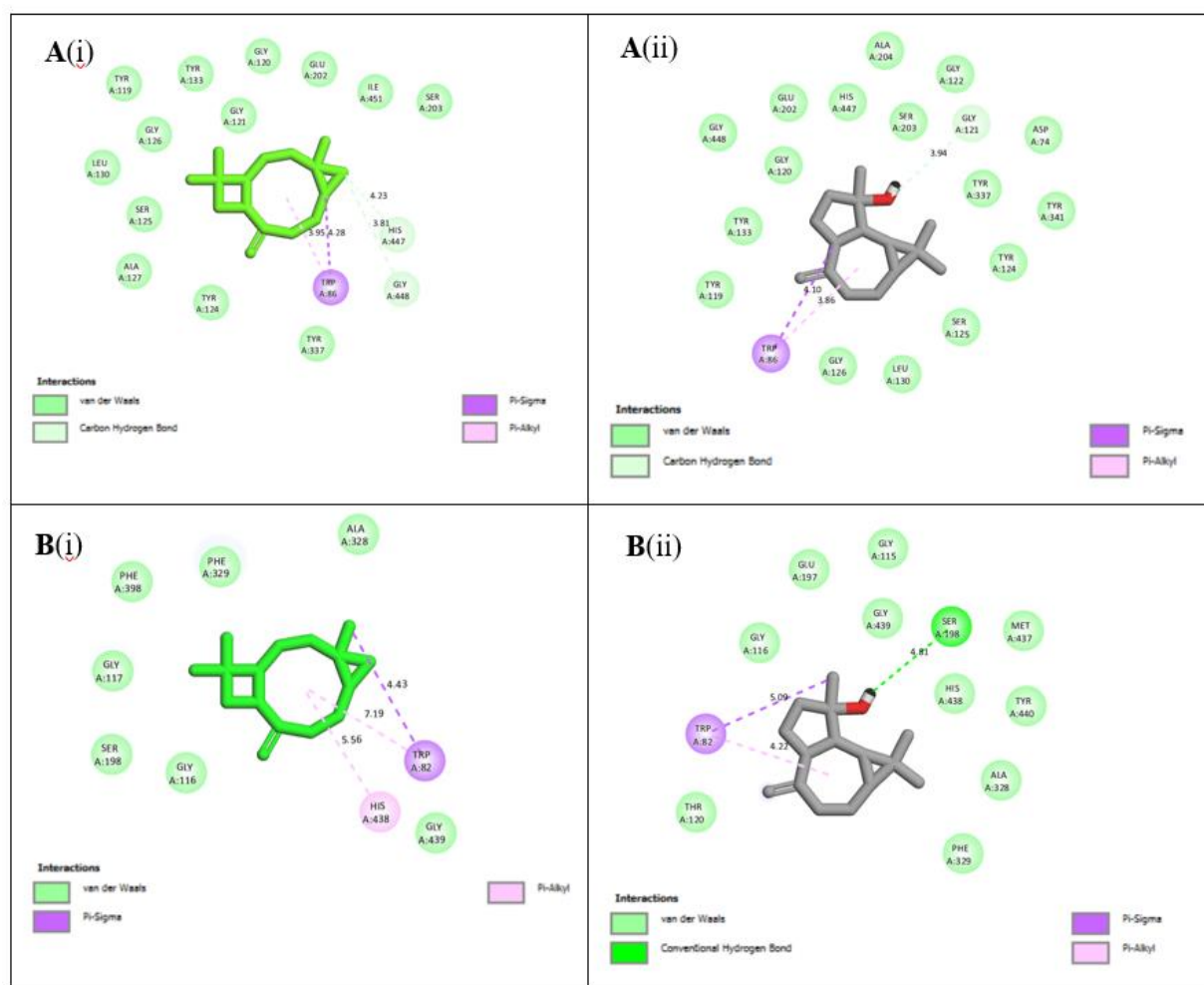


Figure 2. Two-dimensional docking interaction diagrams of caryophyllene oxide (i) and spathulenol (ii) with **(A)** acetylcholinesterase (AChE) and **(B)** butyrylcholinesterase (BChE).

CONCLUSION

The present study provides the first comprehensive report on the chemical composition and anticholinesterase activity of the leaf essential oil of *C. canum*. GC-FID and GC-MS analyses revealed a sesquiterpene-rich oil dominated by oxygenated sesquiterpenes, with caryophyllene oxide and spathulenol identified as the principal components. Biological evaluation demonstrated that the essential oil exhibited notable inhibitory activity against both acetylcholinesterase and butyrylcholinesterase, indicating its relevance as a natural source of anticholinesterase agents. Overall, these findings highlight the pharmacological potential of *C. canum* leaf essential oil and expand current knowledge on the genus *Calophyllum*, particularly for species with limited prior phytochemical and bioactivity data. The results warrant further investigations, including bioactivity-guided fractionation, mechanistic studies, and in vivo evaluations, to better elucidate the therapeutic

prospects of this essential oil in the context of neurodegenerative disorders.

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