

Chemical Constituents of *Etlingera triorgyalis* (Baker) R.M.Sm.: Acetylcholinesterase Inhibitory Activity and Molecular Docking Insights

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The genus *Etlingera*, belonging to the family Zingiberaceae, is widely distributed throughout tropical Southeast Asia and has long been used in traditional medicine for the treatment of fever, inflammation, gastrointestinal disorders, infectious diseases, and wounds. This study focusses on analysing the phytochemicals from *Etlingera triorgyalis* rhizome extracts and assessing their acetylcholinesterase inhibitory activity. The phytochemicals were obtained using column chromatography techniques and characterised by spectroscopy methods (IR, MS, and NMR) and comparison with literatures. Acetylcholinesterase inhibitory activity was determined using acetylcholinesterase enzyme according to the Ellman colorimetric method. The compounds were extracted by cold extraction with solvents (*n*-hexane, ethyl acetate, methanol) and isolated by column chromatography. Spectroscopic analyses (IR, MS, and NMR) led to the identification of ten compounds, including pinostrobin (**1**), hesperetin (**2**), naringenin (**3**), vanillin (**4**), β -sitosterol (**5**), coniferyl alcohol (**6**), sinapyl alcohol (**7**), *p*-coumaryl alcohol (**8**), stearic acid (**9**), and capric acid (**10**). Compound (**3**) exhibited the strongest AChE inhibition (IC₅₀ value 45.0 μ M), followed by compound (**2**) (IC₅₀ value 60.8 μ M) and compound (**1**) (IC₅₀ value 85.2 μ M), compared with galantamine (IC₅₀ value 2.2 μ M). Molecular docking against human AChE (PDB ID: 4EY5) supported the activity of the flavanones, with naringenin, hesperetin, and pinostrobin recording binding scores of -8.514, -7.779, and -7.502 kcal/mol, respectively. In silico ADMET analysis predicted high intestinal absorption for all ten compounds and low mutagenicity probabilities for the three active flavanones. These findings identify the flavanone constituents of *E. triorgyalis* as promising natural AChE-inhibitory scaffolds for further investigation.

Keywords: *Etlingera triorgyalis*, Zingiberaceae, phytochemical, acetylcholinesterase, flavanone

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Acetylcholinesterase (AChE) is a key enzyme in the cholinergic nervous system that rapidly hydrolyses the neurotransmitter acetylcholine into acetate and choline, thereby terminating synaptic transmission. Proper regulation of acetylcholine levels is essential for normal cognitive processes, including learning, memory, and attention [1]. Reduced cholinergic neurotransmission resulting from excessive AChE activity has been closely associated with the progression of neurodegenerative disorders, particularly Alzheimer's disease (AD), the most common form of dementia worldwide.

Consequently, inhibition of AChE has become one of the principal therapeutic strategies for alleviating the cognitive symptoms of AD by increasing acetylcholine availability at cholinergic synapses [2].

Currently available AChE inhibitors, such as galantamine, donepezil, and rivastigmine, have demonstrated clinical efficacy in improving cognitive function; however, their long-term use is often associated with adverse effects including nausea, vomiting, diarrhoea, dizziness, and hepatotoxicity.

Moreover, these drugs provide symptomatic relief rather than preventing or reversing disease progression. These limitations have prompted increasing interest in the discovery of safer and more effective AChE inhibitors from natural sources [3,4]. Medicinal plants have attracted considerable attention because they produce a wide range of structurally diverse secondary metabolites with significant pharmacological activities and generally favourable safety profiles.

Phytochemical investigations have revealed that numerous classes of plant secondary metabolites possess acetylcholinesterase inhibitory activity. These include alkaloids, flavonoids, coumarins, terpenoids, phenylpropanoids, lignans, xanthenes, and phenolic compounds [5-8]. These phytochemicals inhibit AChE through different mechanisms, including competitive, non-competitive, and mixed-mode interactions with the catalytic active site and peripheral anionic site of the enzyme. In addition to direct enzyme inhibition, many of these compounds exhibit antioxidant, anti-inflammatory, and neuroprotective properties, which may provide synergistic benefits in the management of neurodegenerative diseases by mitigating oxidative stress and neuroinflammation, two major contributors to neuronal degeneration [9-11]. Recent studies have continued to identify structurally diverse plant-derived metabolites with measurable AChE inhibitory activity, reinforcing natural products as an active source of cholinesterase-inhibitor leads [5-7].

The genus *Etlingera* (Zingiberaceae) represents a valuable source of bioactive phytochemicals, including flavonoids, phenolic acids, diarylheptanoids, terpenoids, and essential oils, many of which have demonstrated antimicrobial, antioxidant, anti-inflammatory, and enzyme inhibitory activities [12]. *Etlingera triorgyalis* (Baker) R.M. Smith, locally known as *tepus gajah*, is an uncommon species native to Peninsular Malaysia. It is a rhizomatous perennial herb that grows in wet tropical forests, including limestone areas, and can reach up to 5 m in height. It is easily identified by its purple-red young leaves and rose-red inflorescences with greenish or whitish margins. This species has a limited distribution and has only been reported in Kelantan, Perak, Selangor, and Terengganu, Malaysia [13]. Recently, we have reported the chemical components of rhizome oil of *E. triorgyalis* [14]. The analysis of essential oil revealed the presence of 28 components, accounting for 94.1% of the total oil. The main components of essential oil were neral (48.5%), geranial (10.5%), α -cadinol (7.7%), methyl eugenol (6.5%), and (Z)-isocitral (4.6%). The essential oil showed notable enzyme inhibition, with IC₅₀ values of 133.2 μ g/mL (acetylcholinesterase), 118.5 μ g/mL (tyrosinase), and 128.4 μ g/mL (lipoxigenase).

As a continuation of our study, the primary objective of the present research was to evaluate the

AChE inhibitory activity of the compounds isolated from the rhizome extracts of *E. triorgyalis*. In addition, molecular docking studies were performed to investigate the binding interactions of the active compounds with the AChE active site, providing insights into their possible mechanism of enzyme inhibition and supporting the experimental findings.

EXPERIMENTAL

Plant Material

The rhizome of *E. triorgyalis* was collected from Gambang, Pahang in September 2019, and identified by a botanist, Shamsul Khamis from Universiti Kebangsaan Malaysia (UKM). The voucher specimen (SK28/19) was deposited at the Herbarium of the Universiti Kebangsaan Malaysia Bangi (Herbarium UKM).

General Experimental Procedures

A cold extraction technique was applied to extract phytochemicals from the dried roots using different polarity solvents (*n*-hexane, ethyl acetate, and methanol). All reagents used (*n*-hexane, ethyl acetate, dichloromethane, chloroform, diethyl ether, and methanol) were of analytical grade (Merck). Column chromatography (CC) was performed using Merck silica gel 60 (70-230 mesh) as the stationary phase. Thin layer chromatography (TLC) analysis was performed on Merck precoated silica (SiO₂) gel F₂₅₄ plates (0.22 mm thickness) to detect and monitor the presence of compound samples. The spots were visualized under UV light (254 and 365 nm) and included with spraying reagent vanillin sulphuric acid in MeOH followed by heating. Melting points were measured by comparing them with other literature. The ¹H NMR (500 MHz) and ¹³C NMR (100 MHz) spectra were recorded on a Bruker Avance 500 Spectrometer. Chemical shifts were reported in ppm and DMSO/MeOD/CDCl₃ used as solvent. The residual solvent was used as an internal standard. The IR spectra were recorded on the Perkin Elmer ETR and 1600 spectrophotometer series as KBr discs or thin film of NaCl discs. Mass spectral data were obtained from Orbitrap Exploris 240 Mass Spectrometer.

Extraction and Isolation

The dried rhizome of *E. triorgyalis* (500 g) were ground into powder and sequentially extracted with *n*-hexane, ethyl acetate and methanol sequentially by cold extraction. The extract was concentrated using rotary evaporation to afford the crude extracts. The *n*-hexane extract (ETRH, 8.2 g) was fractionated by VLC and eluted with *n*-hexane: DCM: EtOAc to afford 7 fractions (ETRH A-G). The ETRH-A was purified by CC and eluted with *n*-hexane: DCM to afford compound (**9**) (11.7 mg) and compound (**10**)

(12.1 mg). The combined fraction ETRH-E was purified followed by PTLC to obtain compound (5) (10.5 mg). Purification of the EtOAc extract (ETRE, 10.1 g) by CC eluted with *n*-hexane: EtOAc: MeOH yielded 11 fractions (ETRE A-K). The ETRE-C fraction was crystallized to afford compound (4) (29.5 mg). The ETRE-D-F was submitted to CC to afford compound (6) (10.3 mg), compound (7) (10.4 mg), and compound (8) (11.8 mg). Purification of the MeOH extract (ETRM, 12.9 g) by CC eluted with *n*-hexane: EtOAc: MeOH yielded 5 fractions (LSRM A-E). The combined fraction LSRM C was purified by PTLC with CHCl₃: MeOH to obtain compound (1) (20.6 mg). The combined fraction LSRM D-E was purified and washed with Et₂O to produce compound (2) (30.6 mg) and compound (3) (10.2 mg).

Structural Elucidation

Pinostrobin (5-Hydroxy-7-methoxyflavanone) (1). Colourless crystalline needles (20.6 mg); The *R_f* value of 0.48 was obtained using *n*-hexane:EtOAc (6:4). ¹H NMR (CDCl₃, 500 MHz): δ 2.84 (1H, dd, *J* = 2.9 and 14.8 Hz, H-3a), 3.10 (1H, dd, *J* = 13.0 and 15.0 Hz, H-3b), 3.84 (3H, s, OCH₃), 5.44 (1H, dd, *J* = 2.9 and 13.0 Hz, H-2), 6.10 (1H, d, *J* = 2.3 Hz, H-6), 6.11 (1H, d, *J* = 2.3 Hz, H-8), 7.42-7.48 (5H, m, H-2' – H-6'), 12.05 (1H, s, OH); ¹³C NMR (CDCl₃, 100 MHz): δ 43.4 (C-3a/C-3b), 55.7 (OCH₃), 79.3 (C-2), 94.3 (C-8), 95.2 (C-6), 103.2 (C-10), 126.2 (C-2'/C-6'), 128.9 (C-3'/C-4'/C-5'), 138.4 (C-1'), 162.8 (C-9), 164.2 (C-5), 168.0 (C-7), 195.8 (C-4); EIMS: *m/z* 270 [M⁺ for C₁₆H₁₄O₄].

Hesperetin (3',5,7-Trihydroxy-4'-methoxyflavanone) (2). Pale yellow crystalline solid (30.6 mg); The *R_f* value of 0.50 was obtained using *n*-hexane:EtOAc (5:5). ¹H NMR (DMSO, 500 MHz): δ 2.71 (1H, dd, *J* = 17.1, 3.2 Hz, H-3b), 3.21 (1H, dd, *J* = 17.1, 12.5 Hz, H-3a), 3.78 (3H, s, 4'-OCH₃), 5.44 (1H, dd, *J* = 12.4, 3.1 Hz, H-2), 5.89 (1H, d, *J* = 2.1 Hz, H-8), 5.90 (1H, d, *J* = 2.1 Hz, H-6), 6.87 (1H, dd, *J* = 8.3, 2.1 Hz, H-6'), 6.93 (1H, d, *J* = 2.1 Hz, H-2'), 6.94 (1H, d, *J* = 8.6 Hz, H-5'), 9.13 (1H, s, 3'-OH), 10.81 (1H, s, 5-OH), 12.15 (1H, s, 7-OH); ¹³C NMR (DMSO, 150 MHz): δ 42.1 (C-3a), 42.1 (C-3b), 55.6 (4'-OCH₃), 78.2 (C-2), 95.0 (C-8), 95.8 (C-6), 101.8 (C-4a), 111.9 (C-2'), 114.0 (C-5'), 117.9 (C-6'), 131.1 (C-1'), 146.9 (C-4'), 147.9 (C-3'), 162.8 (C-8a), 163.5 (C-5), 166.6 (C-7), 196.3 (C-4); EIMS *m/z* 303 [M+H⁺ for C₁₆H₁₄O₆].

Naringenin (4',5,7-Trihydroxyflavanone) (3). Pale yellow crystalline solid (10.2 mg); The *R_f* value of 0.54 was obtained using *n*-hexane:EtOAc (4:6). ¹H NMR (CD₃OD, 500 MHz): δ 2.67 (1H, dd, *J* = 17.2, 3.0 Hz, H-3b), 3.09 (1H, dd, *J* = 17.2, 13.0 Hz, H-3a), 5.31 (1H, dd, *J* = 13.0, 3.0 Hz, H-2), 5.88 (1H, d, *J* = 2.2 Hz, H-6/H-8), 6.81 (1H, d, *J* = 6.6 Hz, H-3'/H-5'), 7.30 (1H, d, *J* = 6.6 Hz, H-2'/H-6'); ¹³C NMR (CD₃OD, 125 MHz): δ 44.0 (C-3), 80.5 (C-2), 96.2 (C-8), 97.0 (C-6), 103.3 (C-4a), 116.3 (C-3'), 116.3 (C-

5'), 129.1 (C-2'), 129.1 (C-6'), 131.0 (C-1'), 159.0 (C-4'), 164.9 (C-8a), 165.5 (C-5), 168.3 (C-7), 197.8 (C-4); EIMS *m/z* 273 [M+H⁺ for C₁₅H₁₂O₅].

Vanillin (4-Hydroxy-3-methoxybenzaldehyde) (4). White crystalline solid (29.5 mg); The *R_f* value of 0.49 was obtained using *n*-hexane:EtOAc (6:4). ¹H NMR (CDCl₃, 500 MHz): δ 3.97 (3H, s, 3-OCH₃), 6.46 (1H, s, 4-OH), 7.06 (1H, d, *J* = 8.6, H-5), 7.43 (1H, dd, *J* = 8.6, 2.4 Hz, H-6), 7.45 (1H, d, *J* = 2.4 Hz, H-2), 9.84 (1H, s, CHO); ¹³C NMR (CDCl₃, 125 MHz): δ 56.1 (3-OCH₃), 108.9 (C-2), 114.4 (C-5), 129.8 (C-1/C-6), 147.2 (C-3), 151.8 (C-4), 191.0 (CHO); GC-MS *m/z* 152 [M⁺ for C₈H₈O₃].

β-Sitosterol (5). White solid (10.5 mg); The *R_f* value of 0.85 was obtained using *n*-hexane:EtOAc (7:3). ¹H NMR (CDCl₃, 500 MHz): δ 0.70 (3H, s, H-18), 0.83 (3H, d, *J* = 6.8 Hz, H-27), 0.85 (3H, d, *J* = 6.8 Hz, H-26), 0.86 (3H, d, *J* = 7.8 Hz, H-29), 0.94 (3H, d, *J* = 6.5 Hz, H-21), 1.02 (3H, s, H-19), 1.20-2.33 (29H, m, overlapping CH and CH₂), 3.55 (1H, m, H-3), 5.37 (1H, d, *J* = 5.2 Hz, H-6); ¹³C NMR (CDCl₃, 100 MHz): δ 11.8 (C-29), 11.9 (C-18), 18.7 (C-21), 19.0 (C-27), 19.3 (C-19), 19.7 (C-26), 21.0 (C-11), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 28.2 (C-16), 29.2 (C-25), 31.6 (C-2), 31.9 (C-8/C-7), 33.9 (C-22), 36.1 (C-20), 36.5 (C-10), 37.5 (C-1), 39.8 (C-12), 42.3 (C-4/C-13), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.7 (C-14), 71.8 (C-3), 121.7 (C-6), 140.7 (C-5); EIMS: *m/z* 414 [M⁺, C₂₉H₅₀O].

Coniferyl alcohol (6). Pale yellow crystalline solid (10.3 mg); The *R_f* value of 0.54 was obtained using *n*-hexane:EtOAc (5:5). ¹H NMR (DMSO, 500 MHz): δ 3.34 (1H, s, 3'-OH), 3.78 (3H, s, 3-OCH₃), 4.07 (2H, td, *J* = 5.4, 1.7 Hz, H-3'), 6.18 (1H, dt, *J* = 15.9, 5.4 Hz, H-2'), 6.42 (1H, dt, *J* = 15.9, 1.7 Hz, H-1'), 6.71 (1H, d, *J* = 8.1 Hz, H-5), 6.79 (1H, dd, *J* = 8.1, 1.8 Hz, H-6), 6.99 (1H, d, *J* = 1.8 Hz, H-2), 8.99 (1H, s, 4-OH); ¹³C NMR (DMSO, 125 MHz): δ 55.5 (3-OCH₃), 61.7 (C-3'), 109.6 (C-2), 115.4 (C-5), 119.4 (C-6), 127.4 (C-2'), 128.4 (C-1), 128.9 (C-1'), 146.1 (C-4), 147.6 (C-3); EIMS *m/z* 180 [M⁺ for C₁₀H₁₂O₃].

Sinapyl alcohol (7). Pale yellow crystalline solid (10.4 mg); The *R_f* value of 0.62 was obtained using *n*-hexane:EtOAc (4:6). ¹H NMR (DMSO, 500 MHz): δ 3.76 (3H, s, 3/5-OCH₃), 4.08 (2H, td, 5.4, 1.7 Hz, H-3'), 4.77 (1H, s, 3'-OH), 6.22 (1H, *J* = dt, 15.9, 5.4 Hz, H-2'), 6.41 (1H, dt, *J* = 15.9, 1.7 Hz, H-1'), 6.68 (1H, s, H-2/6), 8.36 (1H, s, 4-OH); ¹³C NMR (DMSO, 125 MHz): δ 56.3 (3/5-OCH₃), 62.1 (C-3'), 104.6 (C-2/6), 127.8 (C-2'), 128.3 (C-1), 129.6 (C-1'), 135.6 (C-4), 148.4 (C-3/5); EIMS *m/z* 210 [M⁺ for C₁₁H₁₄O₄].

***p*-Coumaryl alcohol (8).** White crystalline solid (11.8 mg); The *R_f* value of 0.60 was obtained using *n*-hexane:EtOAc (4:6). ¹H NMR (DMSO, 500 MHz): δ 3.34 (1H, s, 3'-OH), 4.06 (2H, td, *J* = 5.4, 1.7 Hz, H-3'), 6.13 (1H, dt, *J* = 15.9, 5.4 Hz, H-2'), 6.42 (1H, dt, *J* = 15.9, 1.7 Hz, H-1'), 6.71 (1H, d, *J* = 8.7 Hz, H-3/H-

5), 7.23 (1H, d, $J = 8.7$ Hz, H-2/H-6), 9.43 (1H, s, 4-OH); ^{13}C NMR (DMSO, 125 MHz): δ 61.7 (C-3'), 115.3 (C-3/C-5), 127.1 (C-2'), 127.3 (C-2/C-6), 127.8 (C-1), 128.6 (C-1'), 156.8 (C-4); EIMS m/z 150 $[\text{M}]^+$ for $\text{C}_9\text{H}_{10}\text{O}_2$.

Stearic acid (Octadecanoic acid) (9). White crystalline solid (11.7 mg); The R_f value of 0.58 was obtained using *n*-hexane:EtOAc (5:5). ^1H NMR (CDCl_3 , 500 MHz): δ 0.88 (3H, t, $J = 6.9$ Hz, H-18), 1.27-1.38 (28H, m, overlapping CH_2 , H-4 – H-17), 1.63 (2H, m, H-3), 2.35 (2H, t, $J = 7.5$, H-2); ^{13}C NMR (CDCl_3 , 125 MHz): δ 14.1 (C-18), 22.6 (C-9), 24.6 (C-3), 29.0 (C-4), 29.2 (C-5), 29.2 (C-7), 29.4 (C-6), 31.8 (C-8) 34.0 (C-2), 180.2 (C-1); GC-MS m/z 284 $[\text{M}^+]$ for $\text{C}_{18}\text{H}_{36}\text{O}_2$.

Capric acid (Decanoic acid) (10). White crystalline solid (12.1 mg); The R_f value of 0.58 was obtained using *n*-hexane:EtOAc (5:5). ^1H NMR (CDCl_3 , 500 MHz): δ 0.88 (3H, t, $J = 7.0$ Hz, H-10), 1.27-1.35 (12H, m, overlapping CH_2 , H-4 – H-9), 1.63 (2H, m, H-3), 2.35 (2H, t, $J = 7.5$, H-2); ^{13}C NMR (CDCl_3 , 125 MHz): δ 14.1 (C-10), 22.6 (C-9), 24.6 (C-3), 29.0 (C-4), 29.2 (C-5), 29.2 (C-7), 29.4 (C-6), 31.8 (C-8) 34.0 (C-2), 180.2 (C-1); GC-MS m/z 172 $[\text{M}^+]$ for $\text{C}_{10}\text{H}_{20}\text{O}_2$.

Acetylcholinesterase Inhibitory Activity

The isolated compounds were evaluated for their acetylcholinesterase activity by assessing their AChE inhibitory effects. The spectrophotometric method was used with modifications [15,16]. AChE from *Electrophorus electricus* (Sigma-Aldrich) and acetylthiocholine iodide were used as substrates. The acetylcholinesterase activity was determined using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). In brief, a 96-well microplate was used to combine 140 μL of sodium phosphate buffer (pH 8.0), 20 μL of DTNB, 20 μL of compounds (at final concentrations of 5, 10, 25, 50, and 100 μM), and 20 μL of AChE (0.22 U/mL) solution. This mixture was incubated at 25°C for 15 min before adding 10 μL of acetylthiocholine iodide to initiate the reaction. The hydrolysis of acetylthiocholine iodide was measured by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm using a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Ethanol (EtOH) was used to dissolve the test compounds, and a blank sample containing only EtOH in phosphate buffer (without compounds) served as the solvent control to correct for any effect of the solvent on enzyme activity. The percentage inhibition (%I) of AChE was calculated by comparing the reaction rates relative to the blank sample using the formula:

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

where E is the enzyme activity without the test sample and S is the enzyme activity with the test sample. All tests were carried out in triplicate, and IC_{50} values were reported as means $\pm$ SD of triplicate. Galantamine was used as a positive control at same concentrations of samples. IC_{50} values were determined from dose response curves generated by non-linear regression analysis using data obtained at multiple concentrations (5, 10, 25, 50, and 100 μM), and results are expressed as mean $\pm$ SD from triplicate experiments.

Molecular Docking Studies

Molecular docking was performed using AutoDock Vina 1.2 against the crystal structure of human acetylcholinesterase (hAChE) co-crystallised with huperzine A (PDB ID: 4EY5) [17-21]. Chain A was retained, while water molecules, the co-crystallised ligand, and other heteroatoms were removed. Polar hydrogens were added, and the ligands were converted to energy-minimised three-dimensional structures and prepared in PDBQT format. A docking box of $22 \times 22 \times 22$ Å was centred on the huperzine A binding site at $x = -9.453$, $y = -43.660$, and $z = 31.879$, with exhaustiveness set to 8. The docking protocol was validated by redocking huperzine A, which reproduced the crystallographic orientation with a heavy-atom RMSD of 0.26 Å. The most favourable poses were selected for interaction analysis. Huperzine A was used as the reference ligand because it is the co-crystallised inhibitor in 4EY5; therefore, it provides an experimentally defined binding site and enables validation of the docking protocol by redocking.

ADMET Prediction

The in-silico absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles of the ten isolated compounds were predicted using ADMET-AI [22]. The evaluated endpoints included human intestinal absorption (HIA), Caco-2 permeability, blood-brain barrier (BBB) penetration, P-glycoprotein substrate probability, plasma protein binding (PPB), CYP2D6 and CYP3A4 substrate probabilities, hERG cardiotoxicity risk, and AMES mutagenicity.

RESULTS AND DISCUSSION

In this study, we successfully isolated ten compounds from the rhizome of *E. triorgyalis*, which were characterised as flavanones, phenolics, fatty acids, steroid, and phenylpropanoid alcohols. They were all identified by analysing their spectroscopic data and comparing them with the reported literature. Their chemical structures are shown in **Figure 1**.

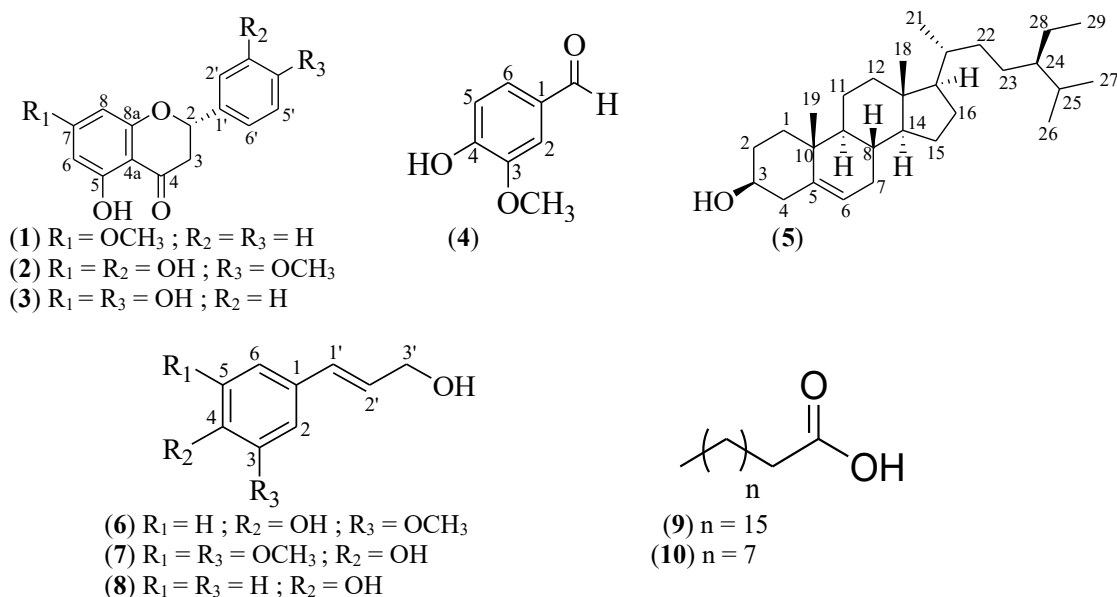


Figure 1. Chemical structures of constituents from *E. triorgyalis*.

Compound (1) revealed the presence of hydroxyl group in the ^1H NMR spectrum through a downfield singlet signal at δ 12.02, which is characteristic of a hydrogen-bonded phenolic proton. Another singlet resonance observed at δ 3.80 was assigned to the methoxy (7- OCH_3) group. Two *meta*-coupled aromatic proton signals were detected at δ 6.06 ($J = 2.3$ Hz), corresponding to H-6 and H-8. Furthermore, three sets of doublet signal, each integrated for one proton were observed at δ 2.82 ($J = 17.2$ Hz and 3.0 Hz), δ 3.08 ($J = 17.2$ and 13.0 Hz) and δ 5.41 ($J = 13.0$ Hz and 3.0 Hz) were allocated for H-3a, H-3b and H-2 respectively. In addition, a multiplet signal resonating at δ 7.40-7.48 integrating for five protons was attributed to the aromatic protons, H-2' – H-6' of ring B. Meanwhile, the ^{13}C NMR spectrum revealed sixteen distinct carbons signals, consisting of into one methyl, one methylene, one carbonyl carbon, eight methine and five quaternary carbons.

The ^1H NMR spectrum of compound (2) exhibited proton resonances characteristic of a flavanone framework. The spectrum showed a deshielded methine proton signal at δ 5.44 (1H, dd, $J = 12.5$ and 3.2 Hz), which was assigned to H-2 of the heterocyclic C-ring. The diastereotopic protons H-3a and H-3b appeared as doublet of doublet at δ 3.21 ($J = 17.1$ and 12.5 Hz) and 2.71 ($J = 17.1$ and 3.2 Hz), respectively. In ring A, two aromatic proton signals were detected as doublet at δ 5.90 ($J = 2.1$ Hz, H-6) and 5.89 ($J = 2.1$ Hz, H-8). The aromatic region of ring B displayed three proton signals, appeared as doublet at δ 6.93 ($J = 2.1$ Hz), 6.94 ($J = 8.6$ Hz), 6.87 ($J = 8.3$ and 2.1 Hz), which were assigned to H-2', H-5' and H-6', respectively. The doublet of doublets pattern observed for H-6' further supported its

coupling with both H-5' and H-2' within the substituted aromatic ring system. Furthermore, a deshielded singlet observed at δ 9.13 was assigned to the phenolic hydroxyl proton at 3'-OH. Additionally, two strongly deshielded singlet signals appearing at δ 10.81 and 12.15 were assigned to the hydroxyl protons at 7-OH and 5-OH, respectively.

The ^1H NMR spectrum for compound (3) displayed a distinctive methine proton signal assigned to H-2 at δ 5.31 as a doublet of doublets ($J = 13.0$ and 3.0 Hz). This splitting pattern indicates coupling with two non-equivalent methylene protons at C-3. The two diastereotopic methylene protons, H-3a and H-3b, resonated as doublet of doublet at δ 3.09 ($J = 17.2$ and 13.0 Hz) and 2.67 ($J = 17.2$ and 3.0 Hz), respectively. The aromatic proton signal appeared as doublet at δ 5.88 ($J = 2.2$ Hz) were assigned to H-6 and H-8 of ring A. In ring B, two aromatic doublets were detected at δ 7.30 ($J = 6.6$ Hz) and 6.81 ($J = 6.6$ Hz), corresponding to H-2'/H-6' and H-3'/H-5', respectively. The similar coupling constants suggested *ortho*-coupling interactions between adjacent aromatic protons within the *para*-substituted benzene ring. The ^{13}C NMR spectrum exhibited fifteen carbon resonances, which were consistent with the carbon framework of a flavanone derivative.

The ^1H NMR spectrum for compound (4) exhibited displayed several characteristic proton signals that support the proposed aromatic aldehyde structure. A downfield singlet at δ 9.84 (1H, s) was assigned to the aldehyde proton. A singlet observed at δ 3.97 corresponded to the methoxy protons (3- OCH_3) attached to the aromatic ring. In addition, a singlet at δ 6.46 was attributed to the hydroxyl proton. The

signal at δ 7.45 (J = 2.4 Hz) appeared as a doublet corresponded to H-2. Meanwhile, the signal at δ 7.43 (J = 2.4, 8.6 Hz) appeared as a doublet of doublets corresponded to H-6. The ^{13}C NMR spectrum displayed eight carbon signals corresponding to an oxygenated aromatic aldehyde structure.

The ^1H NMR spectrum for compound (5) displayed characteristic signal consistent with a sterol-type compound. Two singlet methyl resonance observed at δ 0.67 and 0.99 were assigned to H-18 and H-19, respectively. These angular methyl groups are attached to the quaternary carbons C-13 and C-10 of the tetracyclic steroid nucleus, resulting in the absence of *vicinal* proton coupling and therefore appearing as singlets. Additional methyl resonances as doublet at δ 0.91 (J = 6.7 Hz), 0.81 (J = 6.4 Hz), 0.79 (J = 6.4 Hz), and 0.83 (J = 7.5 Hz) were attributed to H-21, H-26, H-27, and H-29, respectively. A multiplet signal at δ 3.51 (1H, m) was assigned to H-3, corresponding to a proton attached to an oxygenated carbon and supporting the presence of a hydroxyl substituent in the structure. Furthermore, an olefinic proton signal was observed at δ 5.34 (J = 5.3 Hz) and assigned to H-6, confirming the presence of a double bond within the ring system. The remaining proton resonances between δ 0.83–2.24 were reported collectively as overlapping multiplets corresponding to aliphatic protons. The ^{13}C NMR spectrum showed 29 distinct carbon resonances, classified into six methyl carbons, eleven methylene carbons, nine methine carbons and three quaternary carbons.

The ^1H NMR spectrum for compound (6) displayed signals characteristic of a substituted aromatic ring, a *trans*-alkene side chain, a methoxy group, and hydroxyl functionalities. In the aromatic region, three proton signals were observed at δ 6.99 (d, J = 1.8 Hz, H-2), 6.79 (dd, J = 8.1, 1.8 Hz, H-6), and 6.71 (d, J = 8.1 Hz, H-5). Accordingly, H-6 appeared as a doublet of doublets due to coupling with both H-2 and H-5, supporting a 1,3,4-trisubstituted aromatic ring system. The olefinic protons resonated at δ 6.42 (dt, J = 15.9, 1.7 Hz, H-1') and 6.18 (dt, J = 15.9, 5.4 Hz, H-2'). The large coupling constant (J = 15.9 Hz) is characteristic of a *trans*-disubstituted double bond, while the additional smaller couplings arise from interactions with the adjacent methylene group. The signal at δ 4.07 (dt, J = 5.4, 1.7 Hz, H-3') was assigned to the oxygenated methylene group attached to the hydroxyl substituent. Its doublet of triplet multiplicity results from coupling with H-2' (J = 5.4 Hz) and weaker long-range coupling with H-1' (J = 1.7 Hz). A singlet at δ 3.78 was attributed to the methoxy group attached to C-3. The hydroxyl protons appeared as singlets at δ 8.99 (4-OH) and 3.34 (3'-OH).

The ^1H NMR spectrum for compound (7) showed two equivalent aromatic protons at δ 6.68 (H-2/H-6) appeared as a singlet. The olefinic proton

H-1' appeared at δ 6.41 (J = 15.9 and 1.7 Hz), while H-2' resonated at 6.22 (J = 15.9 and 5.4 Hz). The oxygenated methylene protons H-3' were observed at δ 4.08 (J = 5.4 and 1.7 Hz), due to coupling with H-2' and weak long-range coupling across the double-bond system. Meanwhile, the hydroxyl proton at C-3' resonated at δ 4.77 (J = 5.4 Hz). Two methoxy groups attached at C-3 and C-5 appeared together as a singlet at δ 3.76. The phenolic hydroxyl proton resonated at δ 8.36 (4-OH), reflecting the deshielded nature of a hydroxyl group bonded to an aromatic ring.

The ^1H NMR spectrum for compound (8) displayed a downfield singlet at δ 9.43, assigned to the hydroxyl proton at C-4. In the aromatic region, the signal at δ 7.23 (1H, d, J = 8.7 Hz) was attributed to H-2 and H-6, while the signal at δ 6.71 (1H, d, J = 8.7 Hz) was assigned to H-3 and H-5. The olefinic proton H-1' resonated at δ 6.42 (J = 15.9, 1.7 Hz), where the larger coupling constant arise from coupling with H-2'. Proton H-2' appeared at δ 6.13 (J = 15.9, 5.4 Hz), showing coupling with both H-1' and the adjacent H-3' methylene group. The oxygenated methylene protons H-3' resonated at δ 4.06 (J = 5.4, 1.7 Hz), consistent with its position as an oxygenated methylene group adjacent to the olefinic proton H-2'. A singlet at δ 3.34 was assigned to the hydroxyl proton attached to C-3'.

The ^1H NMR spectrum for compound (9) showed resonances characteristic of a long-chain saturated fatty acid. The triplet signal at δ 2.35 was assigned to proton H-2, corresponding to the methylene protons adjacent to the carboxylic acid group. The methylene protons from H-4 to H-17 appeared as an overlapping multiplet at δ 1.27–1.38 (28H), which is typical for methylene groups. The ^{13}C NMR spectrum exhibited eighteen carbon resonances, consistent with a long-chain saturated fatty acid structure. The ^1H NMR spectrum for compound (10) displayed a triplet signal at δ 2.35 (J = 7.5 Hz) was assigned to H-2, which is the methylene group directly next to the carboxylic acid carbonyl. Meanwhile, the strong overlapping signal at δ 1.27–1.35 (12H) was assigned to the internal methylene protons H-4 to H-9. The terminal methyl group H-10 appeared as singlet at δ 0.88. The ^{13}C NMR spectrum displayed ten carbon resonances, which matched the number of carbons present in the structure.

The AChE inhibitory activities of the isolated compounds were evaluated using the Ellman colorimetric assay, and the results are presented as IC_{50} values (Table 1). Of the isolated compounds, naringenin (3) showed the highest AChE inhibitory activity (IC_{50} value 45.0 μM), followed by hesperetin (2) (IC_{50} value 60.8 μM) and pinostrobin (1) (IC_{50} value 85.2 μM). The superior activity of the flavanones can be attributed to their rigid benzopyran skeleton and multiple hydroxyl groups, which promote hydrogen bonding and π – π interactions

with amino acid residues lining the catalytic gorge of AChE [23]. Among these compounds, naringenin (**3**) displayed the greatest potency, likely because its three free hydroxyl groups enhance enzyme binding compared with pinostrobin, which contains only one hydroxyl group and one methoxy substituent, while the methoxy group in hesperetin may slightly reduce its inhibitory activity despite the presence of three hydroxyl groups [24]. Similar structure-activity relationships have been reported for naturally occurring flavonoids, where increased hydroxylation generally improves cholinesterase inhibition. The moderate activity of β -sitosterol (**5**) is likely associated with hydrophobic interactions arising from its steroidal nucleus, although the lack of multiple hydrogen-bonding functionalities limits its binding affinity [25]. Likewise, the phenylpropanoid alcohols and vanillin (**4**) possess simpler molecular frameworks with fewer interaction sites, resulting in weaker inhibition [26], while the aliphatic structures of stearic acid (**9**) and capric acid (**10**) lack aromatic rings necessary for effective interaction with the active-site gorge [27].

The influence of methoxy substitution on AChE inhibition should, however, be interpreted cautiously. Tamfu et al. [24] reported that methoxy substituents were associated with reduced cholinesterase inhibition in several flavonoid comparisons, although the effect was not universal and depended on the substitution pattern. A similar trend was observed among the present flavanones: naringenin (**3**), which

contains three free hydroxyl groups and no methoxy substituent, was more active than hesperetin (**2**), which contains a 4'-methoxy group, and pinostrobin (**1**), which contains a 7-methoxy group and only one free hydroxyl group. Methoxylation may reduce the number or accessibility of hydrogen-bond donors while also altering electronic and lipophilic properties. Nevertheless, because these compounds differ at more than one substitution position, the observed potency differences cannot be attributed to the methoxy groups alone, rather, the combined hydroxylation and methoxylation pattern is likely to influence AChE inhibition.

The molecular docking results are summarised in **Figure 2**, which presents the physicochemical properties and docking profiles of the compounds included in the computational analysis. The flavanones and phenylpropanoid alcohols generally occupied favourable drug-like regions, whereas β -sitosterol and octadecanoic acid showed high lipophilicity and lower QED values. In the docking-score profile, naringenin showed the most favourable score (-8.514 kcal/mol), followed by hesperetin (-7.779 kcal/mol) and pinostrobin (-7.502 kcal/mol), consistent with their comparatively stronger experimental AChE inhibition. Stearic acid, sinapyl alcohol, *p*-coumaryl alcohol, and coniferyl alcohol showed intermediate docking scores, whereas vanillin and β -sitosterol showed weaker calculated binding. The stronger binding of the flavanones was associated with their ability to form hydrogen-bonding and aromatic interactions within the active-site gorge.

Table 1. Acetylcholinesterase activity of constituents of *E. triorgyalis*.

Compounds	IC ₅₀ value (μM)
Pinostrobin (1)	85.2
Hesperetin (2)	60.8
Naringenin (3)	45.0
Vanillin (4)	220.3
β -Sitosterol (5)	95.2
Coniferyl alcohol (6)	140.8
Sinapyl alcohol (7)	120.5
<i>p</i> -Coumaryl alcohol (8)	180.2
Stearic acid (9)	350.6
Capric acid (10)	500.4
Galantamine	2.2

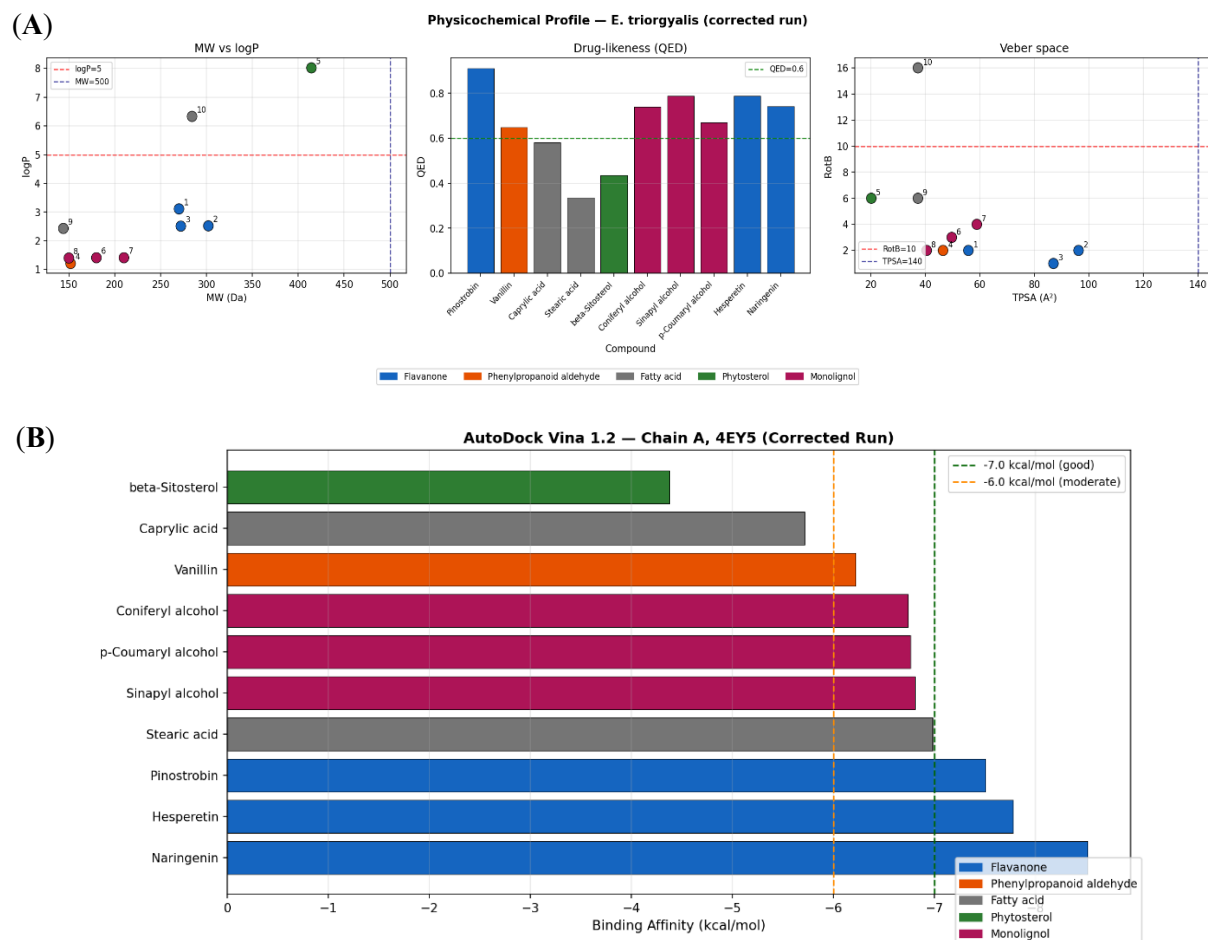


Figure 2. Physicochemical and molecular docking profiles of the compounds included in the computational analysis. The upper panels (A) show molecular weight versus logP, quantitative estimate of drug-likeness (QED), and Veber-space distributions, while the lower panel (B) presents ranked AutoDock Vina binding scores against human acetylcholinesterase (PDB ID: 4EY5).

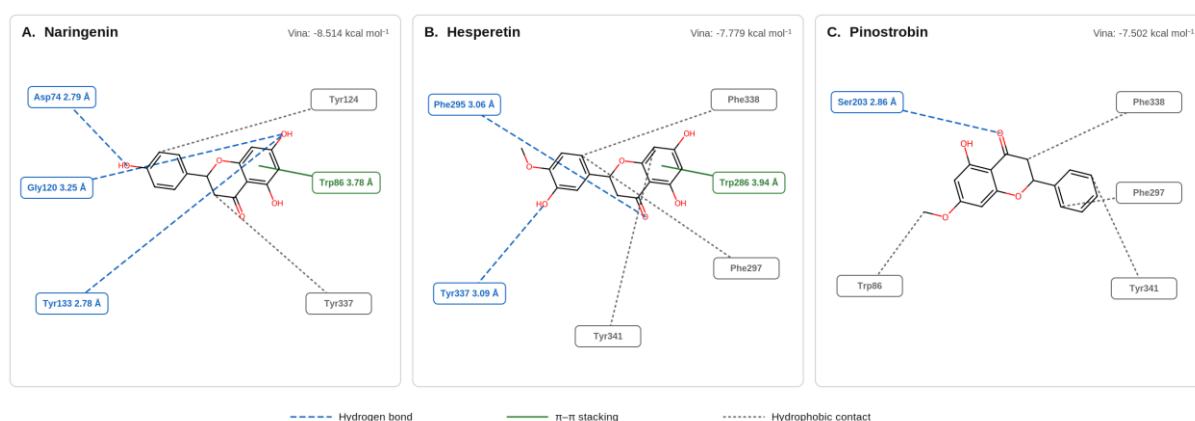


Figure 3. Two-dimensional interaction diagrams of (A) naringenin (3), (B) hesperetin (2), and (C) pinostrobin (1) within the active-site gorge of human acetylcholinesterase (PDB ID: 4EY5). Blue dashed lines represent hydrogen bonds, green solid lines represent π - π stacking interactions, and grey dotted lines represent hydrophobic contacts.

Table 2. Predicted in silico ADMET profiles of the ten isolated compounds.

Compound	Caco-2 (log cm/s)	HIA	BBB	P-gp substrate	PPB (%)	CYP2D6 substrate	CYP3A4 substrate	hERG	AMES
Pinostrobin (1)	-4.64	1.000	0.762	0.641	≈100	0.098	0.538	0.482	0.190
Hesperetin (2)	-5.11	1.000	0.194	0.322	97.5	0.033	0.377	0.435	0.154
Naringenin (3)	-5.11	1.000	0.104	0.172	95.9	0.018	0.279	0.304	0.137
Vanillin (4)	-4.39	1.000	0.838	0.007	56.5	0.094	0.301	0.056	0.309
β-Sitosterol (5)	-5.09	1.000	0.901	0.739	≈100	0.632	0.725	0.845	0.067
Coniferyl alcohol (6)	-4.58	0.998	0.674	0.021	56.2	0.134	0.361	0.143	0.356
Sinapyl alcohol (7)	-4.60	0.999	0.769	0.021	61.1	0.173	0.425	0.111	0.309
p-Coumaryl alcohol (8)	-4.56	0.996	0.509	0.016	51.1	0.075	0.289	0.108	0.298
Stearic acid (9)	-4.50	1.000	0.943	0.247	≈100	0.299	0.631	0.280	0.005
Capric acid (10)	-4.34	0.997	0.886	0.039	77.6	0.228	0.383	0.082	0.012

Values are probabilities from 0 to 1 unless otherwise stated. Higher BBB values indicate greater predicted penetration; lower hERG and AMES values indicate lower predicted cardiac and mutagenicity risks, respectively. PPB predictions exceeding 100% were reported as approximately 100%.

As shown in Figure 3, naringenin (3) (Figure 3A) formed hydrogen bonds with Asp74, Gly120, and Tyr133 together with π - π stacking against Trp86. Hesperetin (2) (Figure 3B) interacted through hydrogen bonding with Phe295 and Tyr337 and π - π stacking with Trp286, while pinostrobin (1) (Figure 3C) formed a hydrogen bond with Ser203 and hydrophobic contacts with Trp86, Phe297, Phe338, and Tyr341. These results support the experimental activity trend, although docking scores should be interpreted as comparative computational estimates rather than direct measurements of binding affinity [28].

The predicted ADMET profiles are summarised in Table 2. All ten compounds showed high predicted intestinal absorption (HIA = 0.996-1.000). Among the three most active flavanones, pinostrobin (1) showed the highest BBB penetration probability (0.762), whereas hesperetin (2) (0.194) and naringenin (3) (0.104) showed lower predicted CNS penetration, consistent with their greater hydroxylation. The three flavanones showed low AMES probabilities (0.137-0.190) and hERG probabilities below 0.50. Octadecanoic acid showed high predicted BBB penetration (0.943), but its high lipophilicity and approximately complete plasma protein binding may limit its suitability as a drug-like AChE inhibitor. β-Sitosterol (5) also showed high BBB and PPB predictions together with the highest hERG probability (0.845). These results provide preliminary pharmacokinetic and safety guidance and require experimental confirmation.

CONCLUSION

In conclusion, phytochemical investigation of the rhizome extracts of *E. triorgyalis* yielded ten compounds belonging to the flavanone, phenolic, phytosterol, phenylpropanoid alcohol, and fatty-acid classes. Naringenin exhibited the strongest AChE inhibitory activity, followed by hesperetin and pinostrobin. Molecular docking against human AChE supported the experimental activity trend and showed that the active flavanones were stabilised within the enzyme gorge through hydrogen bonding, π - π stacking, and hydrophobic interactions. ADMET prediction indicated high intestinal absorption for all ten compounds and generally low predicted mutagenicity, while differences in BBB penetration, plasma protein binding, and hERG risk highlighted the need for further pharmacokinetic and safety evaluation. Overall, the flavanone constituents of *E. triorgyalis* represent promising natural scaffolds for further investigation as AChE inhibitors.

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