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Evaluation of *In Vitro* Acetylcholinesterase Inhibitory Potential in *Ficus adenosperma* Warb. Leaf Extracts Using the Ellman Method

Evaluasi Potensi Penghambatan Asetilkolinesterase secara *In Vitro* pada Ekstrak Daun *Ficus adenosperma* Warb. Menggunakan Metode Ellman

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ABSTRACT

Alzheimer's disease involves progressive loss of nerve cell function and affected about 50 million people worldwide in 2018, a figure projected to triple by 2050. Current acetylcholinesterase (AChE) inhibitors cause nausea and gastrointestinal disturbance, so natural alternatives are being sought. This study evaluated the AChE inhibitory activity of *Ficus adenosperma* Warb. leaf extracts prepared by sequential reflux extraction with n-hexane, ethyl acetate and ethanol, using the Ellman assay. The n-hexane extract yielded the highest total phenolic content (43.85 ± 3.48 mg GAE/g extract), while the ethyl acetate extract showed the highest total flavonoid content (114.27 ± 10.59 mg QE/g extract) and the greatest AChE inhibitory activity (IC_{50} = 1776 µg/mL). Pearson correlation analysis showed a positive but statistically non-significant association between total flavonoid content and AChE inhibition (r = *0.99*, p = *0.10*, n = 3). These preliminary findings suggest that flavonoids may contribute to the observed *in vitro* inhibition.

ABSTRAK

Penyakit Alzheimer ditandai dengan hilangnya fungsi sel saraf secara progresif dan menyerang sekitar 50 juta orang di dunia pada 2018, angka yang diproyeksikan meningkat tiga kali lipat pada 2050. Penghambat asetilkolinesterase (AChE) sintetis menimbulkan mual dan gangguan gastrointestinal sehingga alternatif alami diperlukan. Penelitian ini mengevaluasi aktivitas penghambatan AChE ekstrak daun *Ficus adenosperma* Warb. yang diperoleh melalui metode refluks bertingkat menggunakan pelarut n-heksan, etil asetat, dan etanol, dilanjutkan dengan uji Ellman. Ekstrak n-heksan menghasilkan kandungan fenolik total tertinggi (43,85 ± 3,48 mg GAE/g ekstrak), sedangkan ekstrak etil asetat menunjukkan kandungan flavonoid total tertinggi (114,27 ± 10,59 mg QE/g ekstrak) dan aktivitas penghambatan AChE terbesar (IC_{50} = 1776 µg/mL). Analisis korelasi Pearson menunjukkan hubungan positif yang tidak signifikan secara statistik antara kandungan flavonoid total dan penghambatan AChE (r = *0,99*; p = *0,10*; n = 3). Temuan awal ini menunjukkan bahwa konstituen flavonoid mungkin berkontribusi pada penghambatan enzim *in vitro*.

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1. INTRODUCTION

Alzheimer's disease is a prominent type of neurodegenerative disease, a class of disorders characterized by the progressive loss of function and structure of nerve cells. Alzheimer's disease can be caused by damage to the brain's cells, resulting in a decline in memory, attention, and personality (Reubun et al., 2020). The disease is no longer a strange disease, as Alzheimer's disease reached 50 million people worldwide in 2018, and is expected to triple by 2050 (Scheltens et al., 2021). Alzheimer's can occur due to brain cell damage caused by aging. In this case, the cholinergic

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neurons undergo a decrease in function, which leads to decreased levels of acetylcholine, leading to Alzheimer's disease (Marucci et al., 2021). Acetylcholine will be degraded to acetyl and choline by the degrading enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Richbart et al., 2021). While treatments targeting amyloid-β accumulation, tau hyperphosphorylation, and immunotherapy have been extensively explored, many of these approaches have faced significant clinical challenges and have yet to yield a definitive cure for the disease. Therefore, the therapy used is to inhibit acetylcholinesterase, preventing acetylcholine from degrading. Donepezil, Rivastigmine, and galantamine are synthetic drugs commonly used to inhibit AChE. These drugs have side effects such as nausea, diarrhea, abdominal cramps, and vomiting for the first 3 months, and will gradually decrease as tolerance develops (Madav et al., 2019). In addition to having side effects as already mentioned, donepezil and tacrine have other side effects, such as hepatotoxicity, as well as digestive disorders (Saputra et al., 2015). Other side effects of donepezil also affect the cardiovascular system (Jia et al., 2017).

Given the adverse effects of synthetic drugs, this research explores alternative treatments derived from natural sources. Flavonoids can inhibit acetylcholinesterase by binding to the active site of the acetylcholinesterase so that the acetylcholinesterase cannot bind to its substrate (Karmakar et al., 2019). Several plants known to contain flavonoids have demonstrated potential as natural AChE inhibitors, but much remains to be explored. Within the genus *Ficus* itself, phenolic- and flavonoid-rich extracts of *Ficus sur* have been reported to inhibit both acetylcholinesterase and butyrylcholinesterase (Sieniawska et al., 2022), lending support to the rationale for examining other *Ficus* species such as *F. adenosperma*. *Ficus adenosperma* Warb., a plant belonging to the Moraceae family, is one such candidate. This plant has not yet been studied extensively for its ability to inhibit acetylcholinesterase, but its flavonoid content suggests it may possess therapeutic potential. The Moraceae family, to which *F. adenosperma* belongs, has already been recognized for its pharmacological properties. Another research identified several plants within this family as having active compounds with AChE inhibitory activity, highlighting the Moraceae family as a valuable source of natural AChE inhibitors (Bhadra et al., 2022). Given this background, the present study evaluates the AChE inhibitory activity of *F. adenosperma* leaf extracts and examines the correlation between total phenolic and flavonoid content and inhibitory activity, with the aim of identifying this plant as a promising natural candidate for Alzheimer's disease management.

2. METHODS

2.1. Materials

Equipment used included a reflux apparatus (Iwaki, Tokyo, Japan), analytical balance (Ohaus, Parsippany, USA), rotary evaporator with Rotachill and vacuum pumping unit (Hei-VAP Value, Heidolph, Schwabach, Germany), water bath (Merck, Darmstadt, Germany), oven (Memmert UF 55, Memmert, Schwabach, Germany), micropipette, ELISA spectrophotometric reader (ALLSHENG, Hangzhou, China), micropipettes (Eppendorf). Chemicals and reagents included simplicia powder and leaf extracts of *F. adenosperma*; n-hexane, ethyl acetate, and ethanol 96% (Brataco, Bandung, Indonesia); chloroform, toluene, glacial acetic acid, magnesium powder, $FeCl_3$, acetic anhydride, H_2SO_4, gallic acid (Merck, Darmstadt, Germany); Folin-Ciocalteu 10%, NaOH, quercetin 99%, $AlCl_3$, sodium acetate (Merck, Darmstadt, Germany); Acetylcholinesterase Inhibitor Screening Kit Colorimetric (Cat. No. MAK 324, Sigma-Aldrich, St. Louis, USA); Acetylcholinesterase enzyme from *Electrophorus electricus* (Sigma-Aldrich, Cat. No. C3389); Physostigmine (Cat. No. SA1530, Merck, Darmstadt, Germany); and distilled water.

2.2. Leaf Extraction

F. adenosperma leaves were collected at Kebun Raya Bogor (determination number: 3616/IPH 3/KS/X/2019). Leaf extraction was performed using the reflux method with three sequentially applied solvents of increasing polarity: n-hexane (non-polar), ethyl acetate (semi-polar), and 96% ethanol (polar). A total of 151.6 g of the sample was extracted at 60 °C for 3 h with a sample-to-solvent ratio of 1:2 (w/v), using 300 mL of solvent (Kurniawati et al., 2022). The reflux process was repeated for three cycles for each solvent. Between steps, the marc was filtered through Whatman No. 1 filter paper, dried thoroughly at room temperature, and then subjected to the next solvent. Each obtained filtrate was independently concentrated using a rotary evaporator at 50 °C (Shafaei et al., 2014). The final thick extracts were stored in airtight containers until further use. The yield of each fraction was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract fraction (g)}}{\text{Weight of initial dried powder (g)}} \times 100$$

2.3. Standardization of Simplicia

The determination of water-soluble and ethanol soluble extractable matter was conducted in triplicate according to the standard method. Water-soluble content was determined by placing a 5-gram sample in a sealed bottle, and 100 mL of chloroform-saturated water was added. The bottle was shaken for 6 h and then left to stand for the next 18 h. The solution was then filtered. 20 mL of the filtrate was pipetted and evaporated to a constant weight at 105 °C. For ethanol-soluble content, a 5-gram sample was placed in a sealed bottle, and 100 mL of ethanol was added. The bottle was shaken for 6 h and then left to stand for the next 18 h. The solution was then filtered. 20 mL of the filtrate was pipetted and evaporated to a constant weight at 105 °C (Kementerian Kesehatan Republik Indonesia, 2017). The results were expressed as percentage extractable matter (% w/w) and reported as mean ± standard deviation (SD). The acceptance criteria for both extractable contents were aligned with official monographs to ensure quality and authenticity. The calculation formulas for determination of water soluble and ethanol soluble extractable matter were as follows:

$$\text{Extractable matter content (\%)} = \frac{\text{Weight of residue (g)} \times \frac{\text{Volume total (mL)}}{\text{Filtrate volume (mL)}}}{\text{Weight of initial dried simplicia (g)}} \times 100$$

The moisture content of the sample was determined in triplicate was performed using a moisture balance device: 1 g of the sample was weighed into the device, and the temperature was set to 105 ˚C and the process was maintained until a constant weight (stable equilibrium) was achieved (Ningtyas & Erwiyani, 2022). The moisture content was directly recorded as a percentage and reported as mean ± standard deviation (SD). The maximum tolerance for moisture content was set at ≤10% to prevent microbial degradation.

2.4. Thin-Layer Chromatography

Analytical TLC was performed on silica gel 60 F_{254} aluminum sheets size 2.0 × 7.0 cm. The *F. adenosperma* extract was dissolved in each solvent to achieve a final concentration of 10% (w/v). A 1 µL volume of each samples was spotted 1 cm above the lower edge of the TLC plate using a capillary tube. The chamber was saturated with the mobile phase of toluene:ethyl acetate:glacial acetic acid (7:2:0.5) for 30 min at room temperature. The spotted plate was placed in the chamber in a vertical position once the chamber was saturated. The chamber was covered, and the mobile phase was allowed to ascend to a distance of 5 cm from the spotting line. The spots were observed under UV light at 254 nm and 366 nm, followed by spraying with citroborate and ammonia (NH_3) (Akhtar et al., 2022). The retention factor (R_f) values were calculated by dividing the distance traveled by the compound by the total distance traveled by the solvent front.

2.5. Phytochemical Screening

The extract was screened for its constituent compounds, including alkaloids, flavonoids, phenolics, saponins, steroids, terpenoids, and tannins (Farnsworth, 1966; Kementerian Kesehatan Republik Indonesia, 2017). Alkaloid: two grams of the sample were added to 5 mL of 25% ammonia and 20 mL of chloroform, ground thoroughly, and filtered. The filtrate was then dropped onto filter paper and treated with Dragendorff's reagent. The formation of an orange color indicated the presence of alkaloids in the sample. The remaining filtrate was extracted twice with 10% HCl. The acid layer formed was separated into two test tubes for testing with Mayer's and Dragendorff's reagents. The formation of a white precipitate with Mayer's reagent and a red precipitate with Dragendorff's reagent indicated a positive result. Flavonoids: the extract was dissolved in ethanol. The extract was then added to 10% $AlCl_3$ and 1 M sodium acetate. The formation of a yellow color in the solution indicated that the extract contains flavonoids. Phenolic: one milliliter of the test solution dissolved in methanol was added with 10% Folin-Ciocalteu reagent and 1% NaOH. The formation of a deep blue color in the solution indicated the presence of phenolic compounds in the sample. Saponin: the extract was dissolved in water, heated for 15 min, and filtered. The filtrate was shaken vertically for 10 s. The foam formed (about 1-10 cm) was treated with 2N HCl. The foam that did not disappear indicated that the sample contained saponins. Steroid and terpenoid: one gram of the sample was macerated with ether and filtered. Five milliliters of the filtrate were evaporated. Liebermann-Burchard reagent was added to the evaporated filtrate. The formation of a blue-green or reddish-purple color indicated the presence of steroids and terpenoids in the sample. Tanin: the extract was added to water and heated for 15 min. Three reaction tubes were each filled with 5 mL of the extract. In the first tube, the filtrate was added with 1% $FeCl_3$, and the appearance of a dark blue color indicated the presence of phenolic compounds in the sample. Gelatin was added to the second tube, and the formation of a white precipitate indicated the presence of tannins in the sample. In the third tube, Stiasny's reagent was added and heated in a water bath. The formation of a pinkish-red precipitate indicated the presence of catechol tannins. The filtrate was then filtered, and sodium acetate and $FeCl_3$ were added. The formation of a dark blue precipitate indicated a positive result for gallotannins.

2.6. Total Phenolic Content

10 mg of gallic acid is weighed and placed into a 25 mL volumetric flask, with methanol added to the mark. Dilutions are prepared with concentrations of 80, 75, 70, 65, 60, and 55 µg/mL. One hundred milligrams of extract are weighed, and methanol is added up to the 10 mL mark to prepare the sample. For the operating time test, 1 mL of the standard solution is mixed with 5 mL of Folin-Ciocalteu reagent and allowed to stand at room temperature for 8 min, then 4 mL of 1% NaOH is added. Absorbance is measured every 5 min over a wavelength range of 400–800 nm to determine the time to a constant absorbance. In the procedure, 1 mL of the sample is added to the mixture, followed by 5 mL of 10% Folin-Ciocalteu reagent. The mixture is allowed to stand for 8 min, after which 4 mL of 1% NaOH is added. The solution is incubated for 1 h, and absorbance is measured at 740 nm. (Kementerian Kesehatan Republik Indonesia, 2017; Tommy et al., 2022).

2.7. Total Flavonoid Content

10 mg of quercetin is weighed and placed into a 25 mL volumetric flask, with ethanol added to the mark. Dilutions are prepared with concentrations of 80, 70, 60, 50, 40, and 30 µg/mL. One hundred milligrams of extract are weighed, and ethanol is added up to the 10 mL mark to prepare the sample. For the operating time test, 0.5 mL of the standard solution is mixed with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M sodium acetate, and 2.8 mL of water. Absorbance is measured every 5 min over a wavelength range of 400–800 nm to determine the time to a constant absorbance. In the procedure, 0.5 mL of the test solution is combined with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M sodium acetate, and 2.8 mL of water, and the mixture is incubated for 30 min. Absorbance is measured at 435 nm to determine the total flavonoid content (Kementerian Kesehatan Republik Indonesia, 2017; Tommy et al., 2022).

2.8. Acetylcholinesterase Inhibition Activity

AChE inhibitory activity was determined using Acetylcholinesterase Assay Kit based on the Ellman method. To ensure assay validity, physostigmine was used as a reference inhibitor, yielding an experimentally determined IC_{50} comparable to the manufacturer's reported value (46.7 nM). The assay was performed in a 96-well microplate. The total reaction mixture volume per well was 245 µL, consisting of 45 µL of AChE (final concentration of 400 U/L in assay buffer), 5 µL Test Extract/Physostigmine/ Solvent as negative control, 45 µL Assay Buffer and 150 µL substrate (the substrate mixture contains 154 µL of assay buffer, 1 µL of acetylthiocholine iodide (ATCI) substrate, and 0.5 µL of DTNB chromogenic reagent. This mixture must be freshly prepared immediately before use). The assay was performed in a 96-well microplate. First, 45 µL of AChE solution, 45 µL of assay buffer, and 5 µL of the test compound (extract or physostigmine) were added to the sample wells. For the negative control wells, the 5 µL test compound was replaced with 5 µL of the pure extraction solvent. To account for the intrinsic absorbance of the plant extracts at 412 nm, sample blanks were also prepared by replacing the 45 µL AChE solution with an additional 45 µL of assay buffer. The plate was then incubated at room temperature (25 ± 2 °C) for 15 min. Subsequently, the enzymatic reaction was initiated by adding 150 µL of the freshly prepared substrate reaction mixture to all wells. Finally, absorbance was measured at 412 nm using a microplate reader immediately after substrate addition (T = 0) and after exactly 10 min of incubation (T = 10). All measurements were performed in technical triplicate for each preparation. The percentage of AChE inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\Delta A_{\text{test}}}{\Delta A_{\text{no inhibitor}}}\right) \times 100$$

Where:

ΔA_{test} is the absorbance of the test sample at 0 min subtracted from the absorbance of the same well at 10 min and,

$\Delta A_{\text{no inhibitor}}$ is the absorbance of the no inhibitor control at 0 min subtracted from the absorbance of the same well at 10 min.

For each extract, percentage AChE inhibition was plotted against extract concentration, and the IC_{50} (the concentration producing 50% inhibition) was estimated by linear regression of percentage inhibition against $\log_{10}$-transformed concentration in Microsoft Excel, using the fitted equation $y = a + bx$ ($R^2 = 0.97$), by interpolating the concentration corresponding to $y = 50$. Because IC_{50} was derived from technical triplicates of a single extract batch, a 95% confidence interval could not be calculated from independent replicates; this is acknowledged as a limitation. A nonlinear (sigmoidal dose–response) model was considered but was not applied because the tested concentration range did not span a full sigmoidal response; this choice is noted as a limitation of the current IC_{50} estimate.

2.9. Statistical Analysis

Data are reported as mean ± standard deviation (sample standard deviation, $n - 1$) of three technical replicates. Differences among the three extracts in total phenolic content, total flavonoid content and acetylcholinesterase inhibition at 500 µg/mL were tested by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for all pairwise comparisons. The analyses were computed in Python 3.11 with SciPy 1.17 from the raw replicate values supplied by the authors. Because the three replicates per extract are technical rather than independent biological batches, the ANOVA compares measurement-level means within the batch tested and does not substitute for biological replication; the outcome is therefore read as a description of this batch rather than as a generalisable inference.

Furthermore, an exploratory Pearson's product-moment correlation analysis was conducted to evaluate the relationships between total phenolic content (expressed as mg GAE/g extract), total flavonoid content (expressed as mg QE/g extract) and acetylcholinesterase inhibitory activity (expressed as percentage inhibition). All statistical tests were two-tailed, and a p-value below 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Leaf Extraction

Extraction of *F. adenosperma* leaves was performed using the heat reflux method with three different solvents of varying polarity: n-hexane, ethyl acetate, and ethanol. From 151.6 g of dried simplicia, the ethanol extract gave the highest yield (2.35 g; 1.55%), followed by the n-hexane extract (1.88 g; 1.24%) and the ethyl acetate extract (1.07 g; 0.71%); all three were greenish-black, dry and of characteristic odour. Based on the prior characterization of the herbal material, polar compounds were found to be more abundant than nonpolar compounds. Therefore, this yield outcome is consistent with the herbal material's characterization, as the water-soluble extract content exceeded the ethanol-soluble extract content, suggesting a higher concentration of polar compounds than non-polar compounds (Febrianti et al., 2019). Ethanol, as a universal solvent, has an affinity for compounds that were not attracted to previous solvents. N-hexane exhibits non-polar properties, thus attracting non-polar compounds (Fransiska et al., 2021). Ethyl acetate, conversely, possesses semi-polar properties, attracting semi-polar compounds. By selectively extracting non-polar and semi-polar compounds, ethanol effectively extracts polar compounds. This interpretation should be regarded as provisional: because extraction was performed sequentially (n-hexane, then ethyl acetate, then 96% ethanol on the same marc), the final yield of each fraction may also have been influenced by the order of extraction, extraction temperature and duration, particle size of the dried simplicia, solvent-to-sample ratio, drying conditions, and losses during filtration and transfer between steps, in addition to solvent polarity. The extraction yield alone should therefore not be

equated with the abundance of any specific phytochemical class (e.g., phenolics or flavonoids); the measured total phenolic and flavonoid contents (Sections 3.4 and 3.5) are discussed separately as the more direct indicators of those compound classes.

3.2. Standardization of Simplicia

Measuring the water-soluble and ethanol-soluble extract content helps determine the amounts that dissolve in water and ethanol (Jayani & Handojo, 2021). The water-soluble extractable matter of the simplicia, 11.51 ± 0.18% (w/w), exceeded the ethanol-soluble extractable matter, 2.84 ± 0.09% (w/w); both are means of three replicates. This implies that polar compounds in *F. adenosperma* herbal material are more prevalent than non-polar compounds (Warnis et al., 2021). The water content of herbal material can affect the growth of fungi and bacteria during storage; hence, understanding this content is crucial to maintaining its quality and longevity without compromising the active ingredients (Wandira et al., 2023). In this research, *F. adenosperma* herbal material has a water content of 3.37 ± 0.06%, which meets the necessary standards. As per PerBPOM No. 32 regarding Safety and Quality Requirements for Traditional Medicines, the water content standard for herbal materials is set at below 10%. With the water content meeting the specified criteria, this herbal material is of high quality and suitable for research purposes and long-term storage.

Thin-layer chromatography (TLC) test provides initial insight into the compounds present in the extract, based on their R_f values and the colors of the spots on the plate. TLC testing was carried out using a mobile phase of toluene: ethyl acetate: glacial acetic acid in a ratio of 7:2:0.5. A non-polar solvent was chosen due to the polar nature of the TLC plate itself (Dyera & Arnida, 2020). Visualization with Citroborate and NH_3 was performed under UV light at 254 nm and 366 nm. A citroborate stain is used to identify flavonoid compounds in the extract. In this test, color changes on the TLC plate are observed before and after the addition of the citroborate stain when viewed under UV light at 366 nm. A blue color appears when viewed under UV light at 366 nm after the addition of citroborate (**Figure 1**). The R_f results for TLC test are presented in **Table 1**. Spraying the plate with citroborate stain yields a bluish-green color under UV light at 366 nm, providing presumptive evidence of flavonoid-type compounds (Kurnia et al., 2022). Citroborate works by reacting with the ortho-dihydroxy groups in flavonoid compounds, thus indicating the presence of flavonoid content (Santosa & Haresmita, 2015). Another stain added to the TLC plate is the NH_3 stain. There is a color change in the n-hexane extract part from light blue fluorescence to greenish-blue fluorescence. While literature suggests this specific color shift can be characteristic of flavonols without a free 5-OH but substituted at 3-OH, this finding serves only as a preliminary indication. The parts of the extract extracted with n-hexane, ethyl acetate, and ethanol exhibit light blue fluorescence that does not change upon addition of the NH_3 stain. Although this behavior is sometimes associated with isoflavonoid compounds lacking free 5-OH groups (Andersen & Markham, 2005), these observations must be interpreted with caution. Because this preliminary analysis was conducted without authentic reference standards or further analytical confirmation (e.g., LC-MS or NMR), these findings represent presumptive indications of flavonoid-type derivatives rather than definitive structural identifications.

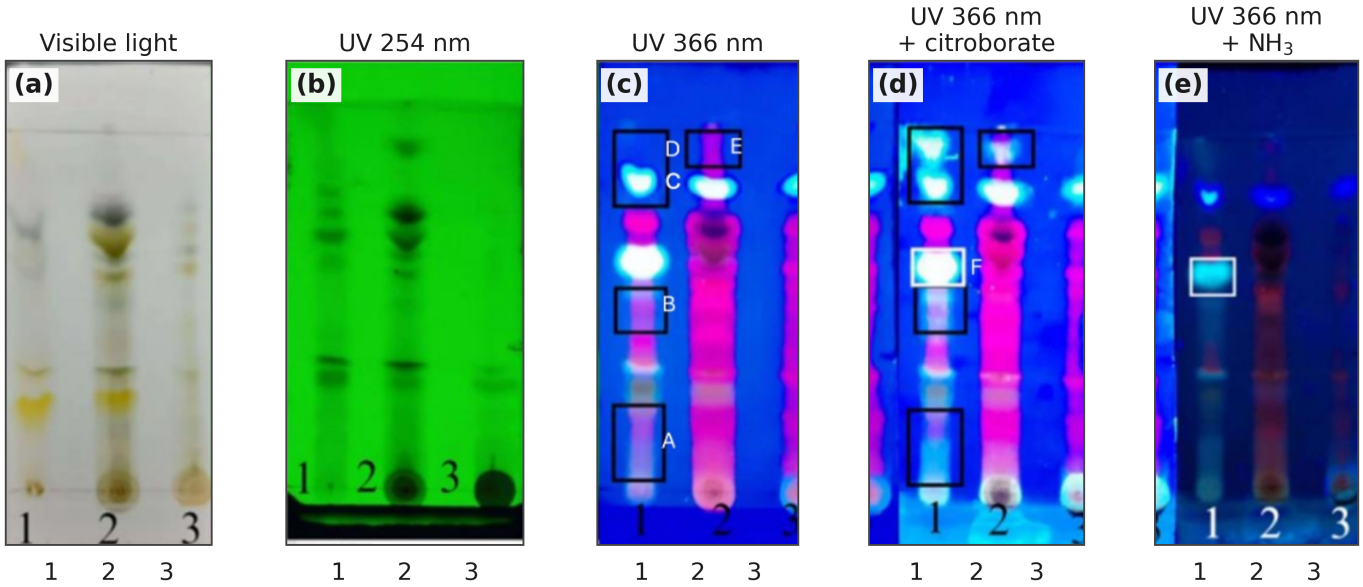


Figure 1. Thin-layer chromatograms of the *F. adenosperma* leaf extracts. Lanes: 1, n-hexane extract; 2, ethyl acetate extract; 3, 96% ethanol extract. Boxed spots A–F are the spots listed in Table 1

3.3. Phytochemical Screening

Phytochemical screening tests are conducted as preliminary steps to identify compounds present in the leaves of *F. adenosperma*. The metabolite classes screened were alkaloids, flavonoids, phenolics, saponins, tannins, quinones, and steroids. The summary of the phytochemical screening results is presented in **Table 2**. The flavonoid test is performed with $AlCl_3$ and sodium acetate reagents; a positive result is indicated by a yellow color change in the solution. $AlCl_3$ will turn the solution yellow while sodium

acetate will maintain the yellow color formed in the sample (Suwartini et al., 2021). This test obtained positive results in n-hexane, ethyl acetate, ethanol extracts, and the crude drug. The phenolic test is carried out using 10% Folin-Ciocalteu reagent and NaOH. A positive result is indicated by a color change of the solution to blue or dark blue after adding the reagent. The 10% Folin-Ciocalteu reagent will detect phenolate ions present in the sample. However, phenolate ions can form under basic conditions, so NaOH must be added to the solution to create a basic environment. This test obtained positive results in n-hexane, ethyl acetate, ethanol extracts, and the crude drug. The saponin test is performed by shaking the solution vertically and then adding HCl. Saponins are glycosides of sapogenin that have polar properties and can produce foam when shaken in water. The foam formed indicates the presence of glycosides, which then hydrolyze into glucose and other compounds. HCl is added to increase the polarity of the saponin group, forming micellar structures that produce foam as a sign of saponin compounds (Putri & Lubis, 2020). A positive result was obtained if the foam persisted after adding HCl. The crude drug and ethanol extract showed positive results for saponins.

Table 1. Thin-layer chromatographic profile of the *F. adenosperma* leaf extracts

Spot	R_f	UV light at 366 nm			Interpretation
		Without reagent	After citroborate	After NH_3	
A	0.167	Red	Bluish-green	-	Flavonoid
B	0.533	Red	Bluish-green	-	Flavonoid
C	0.867	Blue	Bluish-green	-	Flavonoid
D	0.967	Red	Bluish-green	-	Flavonoid
E	0.967	Red	Bluish-green	-	Flavonoid
F	0.633	Light blue	Light blue	Greenish-blue	Flavonoid substituted at 3-OH

Note. R_f, retention factor; NH_3, ammonia vapor. –, no color change observed.

3.4. Total Phenolic Content

Total phenolic content was investigated to determine the amount of phenolics present in the *F. adenosperma* extract. This test utilizes the Folin-Ciocalteu reagent and UV-Vis spectrophotometry. The intensity of the blue color formed is directly related to the concentration of phenolic compounds present (Ismail et al., 2012). The blue hue developed indicates the formation of phenolate ions, which reflects the phenolic content. Before the test, the operational duration and the appropriate wavelength are established to determine the reaction time of the sample with the reagent, ensuring optimal testing conditions. Gallic acid serves as the reference standard in this test due to its stability as a phenolic compound (Rojas-Ocampo et al., 2021). A standard curve is constructed to determine the regression equation for calculating phenolic content by inputting the sample absorbance values. This standard curve ensures method standardization, enabling precise measurement outcomes (Mapoung et al., 2021). The regression derived from the standard curve is y = 0.0064x + 0.0101 with a correlation coefficient (r) of 0.9996. After obtaining the regression value, the sample's absorbance is substituted into the regression equation to obtain the y-value. The highest calculated phenolic content in the *F. adenosperma* extract was observed in the n-hexane extract at 43.85 ± 3.48 mg GAE/g extract, followed by the ethyl acetate extract at 41.96 ± 0.20 mg GAE/g extract, and the lowest in the ethanol extract at 18.96 ± 1.29 mg GAE/g extract (**Figure 2**). The three extracts differed significantly in total phenolic content (one-way ANOVA, $F(2,6) = 125.1$, $p < 0.001$); by Tukey's HSD the n-hexane and ethyl acetate extracts did not differ from each other ($p = 0.56$), while both exceeded the ethanol extract ($p < 0.001$ for both).

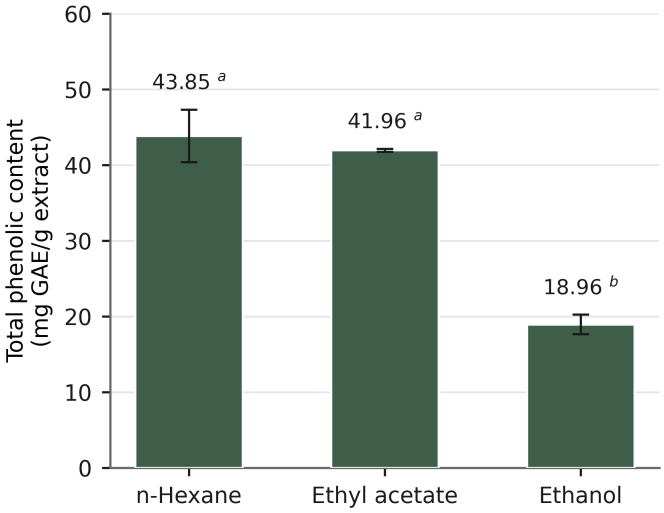


Figure 2. Total phenolic content of the *F. adenosperma* leaf extracts. Bars are mean ± SD of three technical replicates; bars sharing a letter do not differ (Tukey HSD, $p < 0.05$)

3.5. Total Flavonoid Content

The total flavonoid content test, similar to the total phenolic content test, is conducted to quantify the flavonoid compounds present in *F. adenosperma* extract. This test uses $AlCl_3$ as the reagent, which binds to the C-4 ketone group and the C-3 and C-5 hydroxyl groups of flavonoids in the sample. After adding $AlCl_3$, sodium acetate is also introduced to stabilize the wavelength within the visible region and to detect the presence of the 7-OH group (Suwartini et al., 2021). To optimize the test, both the operating time and maximum wavelength are determined to ensure complete reaction between the sample and reagent, yielding accurate results. Quercetin is used as the standard in this test, as it possesses a ketone group at C-4 and hydroxyl groups at

C-3 and C-5, which allow it to bind with $AlCl_3$ (Aminah et al., 2017). The flavonoid standard is measured to generate the linear regression, which is then used to calculate the flavonoid content in the sample. The standard curve obtained yields the equation y = 0.0081x + 0.0211, with a correlation value (r) of 0.9974. After obtaining the regression value, the absorbance of the sample is substituted as the y-value. The extracts are measured at the same wavelength as the standard. The results show that the ethyl acetate extract contains the highest flavonoid content, with a value of 114.27 ± 10.59 mg QE/g extract, followed by the n-hexane extract (43.22 ± 7.33 mg QE/g extract) and the ethanol extract (10.33 ± 1.32 mg QE/g extract) (**Figure 3**). These results suggest that the flavonoids in *F. adenosperma* are primarily semi-polar in nature. Total flavonoid content also differed among the extracts ($F(2,6) = 151.6$, $p < 0.001$), and here every pairwise comparison was significant (Tukey's HSD, p ≤ 0.004): ethyl acetate > n-hexane > ethanol.

Table 2. Phytochemical screening of the *F. adenosperma* leaf extracts and simplicia

Secondary metabolite	Extract of *F. adenosperma*			
	n-Hexane	Ethyl acetate	Ethanol	Simplicia
Alkaloid	-	-	-	-
Flavonoid	+	+	+	+
Phenolic	+	+	+	+
Saponin	-	-	+	+
Tannin	-	-	-	-
Quinone	-	-	-	-
Steroid	-	-	-	-
Terpenoid	-	-	-	-

Note. +, detected; –, not detected.

3.6. Acetylcholinesterase Inhibition Activity

The acetylcholinesterase inhibition test is the core test of this study. The acetylcholinesterase inhibition test is conducted to determine the extent to which the sample can inhibit acetylcholinesterase. This test is performed using the Ellman method with a 96-well plate. The testing method follows the guidelines provided in the Acetylcholinesterase Inhibitor Screening Kit manual. The principle of this test is that the enzyme hydrolyzes the substrate acetylthiocholine to thiocholine. The thiocholine will then react with DTNB to produce TNB, a yellow product. The absorbance is then measured with an ELISA reader at 412 nm (Nuria et al., 2019). In this test, it is expected that the extract will inhibit acetylcholinesterase activity, preventing it from hydrolyzing acetylthiocholine to thiocholine; thus, DTNB will not react, and no yellow color will form. The positive control is physostigmine, which has the same mechanism of action as *F. adenosperma* leaves, namely, inhibition of the active site of its substrate (Triggle et al., 1998). The results obtained are shown in **Figure 4**. From these results, it can be seen that the positive control has an IC_{50} of 47.701 nM, which is consistent with the certificate of analysis provided with the kit test. This value is consistent with the potency reported in the manufacturer's certificate of analysis, confirming assay validity and reproducibility. Because physostigmine potency is reported here in nanomolar units, the ppm-based IC_{50} potency classification (very strong < 50 ppm) used elsewhere in this manuscript for the plant extracts does not apply to physostigmine and was not used to describe it. (Fauziah et al., 2021).

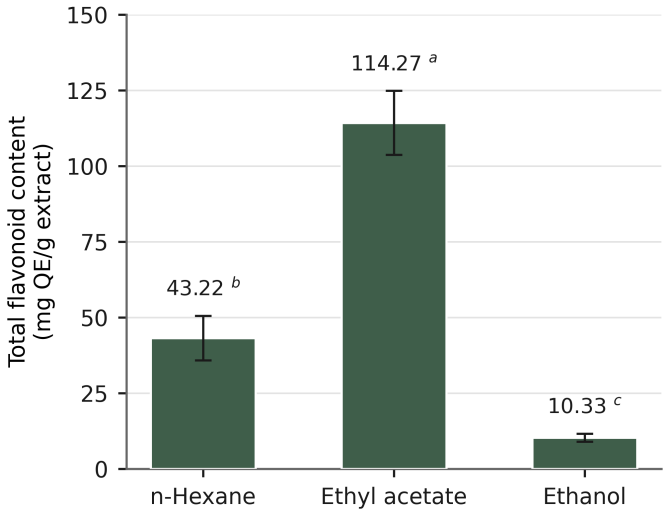


Figure 3. Total flavonoid content of the *F. adenosperma* leaf extracts. Bars are mean ± SD of three technical replicates; bars sharing a letter do not differ (Tukey HSD, $p < 0.05$)

The three extracts were first screened at a single concentration of 500 µg/mL (**Figure 5**a). The ethyl acetate extract gave the highest inhibition, 20.60 ± 4.62%, followed by the n-hexane extract at 5.14 ± 0.23% and the ethanol extract at 2.00 ± 0.94%. This variation in inhibition is attributed to the presence of secondary metabolites, specifically flavonoids, which are responsible for acetylcholinesterase inhibition (Khan et al., 2018). This observation is consistent with previous total flavonoid testing, which indicated that ethyl acetate had the highest flavonoid content. The three extracts differed significantly in inhibition ($F(2,6) = 40.2$, $p < 0.001$); the ethyl acetate extract exceeded both the n-hexane extract (Tukey's HSD, $p < 0.001$) and the

ethanol extract ($p < 0.001$), whereas the n-hexane and ethanol extracts did not differ from each other ($p = 0.39$).

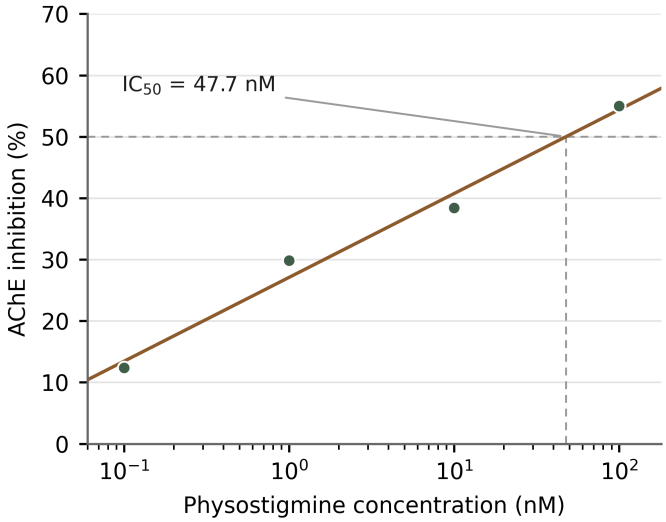


Figure 4. Concentration–response of the reference inhibitor physostigmine, with the fitted regression on $\log_{10}$ concentration, the 50% guide line and the interpolated IC_{50}. Concentrations are expressed in nM (10^{-10} to 10^{-7} mol/L in the source data)

Subsequent testing focused solely on the ethyl acetate extract with various concentrations. The results are shown in Figure 5b. From the results obtained, the IC_{50} value of the ethyl acetate extract is 1776 µg/mL. This relatively high IC_{50} value indicates weak inhibitory activity under the *in vitro* experimental conditions. Therefore, the result should be interpreted as preliminary evidence of acetylcholinesterase inhibition. In comparison, the ethyl acetate extract of *F. benghalensis* leaves reportedly exhibited acetylcholinesterase inhibitory activity with an IC_{50} value of 606.6 µg/mL (Hassan et al., 2020). The lower IC_{50} value reported for *F. benghalensis* indicates stronger inhibitory activity than that observed for *F. adenosperma* in the present study. Column semi-purified fractions of *F. benghalensis* have similarly been reported to possess anti-cholinesterase activity alongside anti-diabetic and anti-inflammatory effects (Rauf et al., 2024), further supporting the genus's potential as a source of AChE-inhibitory constituents. Nevertheless, the current findings show that the ethyl acetate extract of *F. adenosperma* possesses measurable *in vitro* acetylcholinesterase inhibitory activity. Further fractionation and isolation of active compounds are required to identify the constituents responsible for this effect.

The association between phytochemical content and inhibitory activity was examined by an exploratory Pearson correlation across the three extract types ($n = 3$; Figure 6). Total phenolic content was not correlated with AChE inhibition ($r = 0.58$, *exact* $p = 0.61$, $n = 3$). Total flavonoid content showed a positive coefficient, but the relationship was not statistically significant ($r = 0.99$, *exact* $p = 0.10$, $n = 3$). Because only three paired extract-level observations were available, both correlations are presented as exploratory descriptive observations and are not used to support inferential conclusions. Flavonoid constituents may contribute to the observed acetylcholinesterase inhibitory activity, potentially through binding at or near the enzyme's active site; however, the extract is a complex mixture, and the total flavonoid content assay does not identify specific active compounds, so this observation does not establish flavonoids as the causal agent. Thin-layer chromatography spot behavior (light-blue fluorescence unchanged after NH_3 spraying) is presumptive evidence consistent with isoflavonoid-type compounds, but does not constitute definitive structural identification, since no authentic reference standards or additional analytical confirmation (e.g., LC-MS or NMR) were used. Confirmation of the specific constituent(s) responsible for the observed AChE inhibition would require fractionation, compound isolation, structural elucidation, and testing of purified compounds. For context, isoflavones have previously been

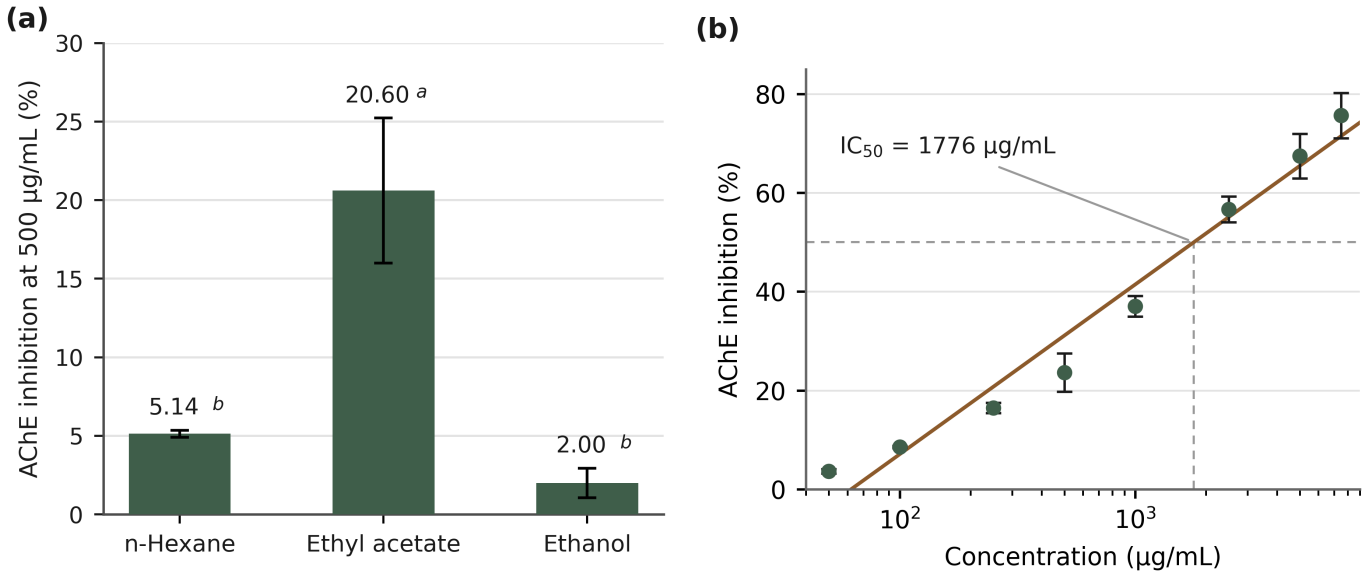


Figure 5. Acetylcholinesterase inhibition by the *F. adenosperma* leaf extracts: (a) inhibition by the three extracts at a single screening concentration of 500 µg/mL, and (b) concentration–response of the ethyl acetate extract, with the fitted regression on $\log_{10}$ concentration and the interpolated IC_{50}. Points and bars are mean ± SD of three technical replicates; in (a), bars sharing a letter do not differ (Tukey HSD, $p < 0.05$)

reported with an AChE IC_{50} value of 47.0 µM (Karmakar et al., 2019).

This study has several limitations that should be considered when interpreting these findings: (1) each extract was prepared as a single batch and evaluated only in technical triplicate, meaning independent biological replication was not achieved; (2) crude, unfractionated extracts were tested, thus the specific compound(s) responsible for AChE inhibition remain unidentified; (3) the correlation analysis between phytochemical content and inhibitory activity was based on only three extract-level observations and should be regarded as exploratory rather than confirmatory; (4) no cytotoxicity testing was performed, leaving the safety of these extracts in cellular or animal systems unknown; and (5) no cellular or *in vivo* confirmation of AChE inhibitory activity was obtained. Addressing these limitations through independent extraction batches, compound isolation and structural identification, cytotoxicity assays, and *in vivo* or cell-based validation will be necessary before any therapeutic or biological relevance of *F. adenosperma* leaf extracts can be established.

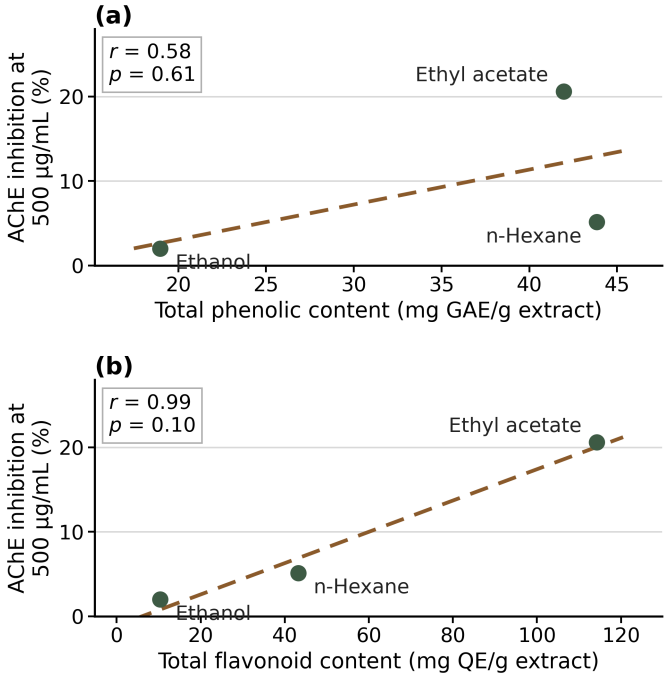


Figure 6. Relationship between phytochemical content and acetylcholinesterase inhibition across the three *F. adenosperma* leaf extracts: (a) total phenolic content and (b) total flavonoid content. The dashed line is the least-squares fit; Pearson r and its two-tailed p, recomputed from these three extract-level values, are printed on each panel ($n = 3$)

4. CONCLUSION

The research conducted demonstrates that extracts from *F. adenosperma* leaves contain measurable amounts of total phenolics and flavonoids. The n-hexane extract exhibited the highest total phenolic content, measuring 43.85 ± 3.48 mg GAE/g extract, while the ethyl acetate extract displayed the highest total flavonoid content at 114.27 ± 10.59 mg QE/g extract. Preliminary acetylcholinesterase inhibition assays with three different solvent extracts revealed the greatest inhibitory activity in the ethyl acetate extract. Further investigation focused exclusively on this extract, which yielded an IC_{50} value of 1776 µg/mL indicating relatively weak acetylcholinesterase inhibitory activity under the *in vitro* experimental conditions. An exploratory, non-significant positive correlation was observed between total flavonoid content and AChE inhibition ($r = 0.99$, $p = 0.10$, $n = 3$); this observation suggests that flavonoid constituents may contribute to the inhibitory activity but, given the very limited statistical power of a three-point correlation, does not establish a causal or significant relationship. Importantly, these findings do not imply any clinical or therapeutic relevance. Independent extraction batches, compound isolation and structural identification, cytotoxicity assays and cell-based or *in vivo* validation are the next steps required before any biological relevance of *F. adenosperma* leaf extracts can be established.

AUTHOR CONTRIBUTIONS

Conceptualization, F.R.C.S.; methodology, F.R.C.S. and Y.K.H.; validation, F.R.C.S., Y.K.H. and E.Z.A.; formal analysis, F.R.C.S. and Y.K.H.; investigation, Y.K.H. and E.Z.A.; resources, F.R.C.S.; data curation, Y.K.H. and E.Z.A.; writing—original draft preparation, F.R.C.S.; writing—review and editing, F.R.C.S., Y.K.H. and E.Z.A.; visualization, E.Z.A.; supervision, F.R.C.S.; project administration, F.R.C.S.; funding acquisition, F.R.C.S. All authors have read and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable because this *in vitro* study did not involve humans or animals.

INFORMED CONSENT STATEMENT

Not applicable because this study did not involve human participants.

DATA AVAILABILITY STATEMENT

The raw absorbance data, calibration curves, IC_{50} calculations, and phytochemical screening records are available from the corresponding author upon reasonable request.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) USE

During the preparation of this manuscript, the authors used Grammarly and Quillbot to assist in improving the clarity, structure, or readability of the text. These tools were not used to generate data, conduct statistical analyses, interpret the findings, or verify references. After using this tool, the authors thoroughly reviewed, edited, and verified the entire content to ensure it accurately represents their own ideas and interpretations. The authors take full responsibility for the integrity and originality of the published work.

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