



RESEARCH ARTICLE

Isolation of Apigenin from Sungkai (*Peronema canescens*) Leaves and Its Immunomodulatory Effects: An In Vivo Study on Granzyme B, Interferon- γ , and Perforin Expression with Supporting In Silico Analysis

[version 1; peer review: 2 approved, 2 approved with reservations]

Dwisari Dillasamola¹, Yufri Aldi¹, Najmiatul Fitria¹, Biomechy Oktomalioputri², Uce Lestari³, Risma Multia¹

¹Pharmacy, Universitas Andalas, Padang, West Sumatra, Indonesia

²Medicine, Universitas Andalas, Padang, West Sumatra, Indonesia

³Medicine, Universitas Jambi, Jambi, Jambi, Indonesia

V1 First published: 08 Aug 2025, 14:774
<https://doi.org/10.12688/f1000research.167153.1>
Latest published: 08 Aug 2025, 14:774
<https://doi.org/10.12688/f1000research.167153.1>

Abstract

Background

Paronema canescens Jack., commonly known as sungkai, is a medicinal plant native to Southeast Asia, particularly abundant in the forests of Sumatra and Borneo, Indonesia. Traditionally, sungkai has been used to treat various ailments, likely due to its rich content of bioactive secondary metabolites. This study aimed to isolate, characterize, and evaluate the immunostimulatory and anti-inflammatory potential of compounds from sungkai leaves based on in silico and in vivo analyses.

Methods

Apigenin was isolated from *Paronema canescens* leaves via ethanol extraction, liquid-liquid partitioning, and chromatographic purification, then characterized by UV-Vis, FT-IR, and NMR spectroscopy. Molecular docking was conducted using MOE software to assess apigenin's binding to granzyme B, perforin, and IFN- γ , with levamisole as a reference. In vivo, 25 male mice were randomized into five groups and administered apigenin (1, 25, or 50 mg/kg BW) intramuscularly for seven days, alongside COVID-19 vaccination.

Open Peer Review

Approval Status    

	1	2	3	4
version 1				
08 Aug 2025	view	view	view	view

1. **Dharmalingam Kirubakaran**, Saveetha Institute of Medical and Technical Sciences, Chennai, India
2. **Valentina Mittova**, University Geomedi, Tbilisi, Georgia
3. **Bushra Hussain Shnawa**, Soran University, Kurdistan, Iraq
4. **Pitchaporn Wanyo**, Kalasin University, Kalasin, Thailand

Any reports and responses or comments on the article can be found at the end of the article.

Granzyme B and IFN- γ serum levels were quantified using ELISA. Statistical analysis employed one-way ANOVA with Duncan's test ($p < 0.05$).

Results

The *in silico* analysis demonstrated that apigenin exhibited favorable binding affinities and multiple stabilizing interactions with granzyme B, perforin, and interferon- γ , supporting its potential role in enhancing cellular immune responses through direct molecular modulation of key cytotoxic effector proteins. To assess its immunostimulatory activity *in vivo*, apigenin was orally administered to mice (*Mus musculus*) at doses of 1, 25, and 50 mg/kg body weight. Mice were pre-induced with a COVID-19 vaccine to simulate immune system activation. Immunological responses were evaluated through the measurement of granzyme B, perforin, and interferon- γ expression levels. The results demonstrated that apigenin significantly increased the expression of all three markers in a dose-dependent manner.

Conclusion

Collectively, the chemical, computational, and biological data confirm that apigenin from sungkai leaves holds strong immunostimulant and selective anti-inflammatory potential, supporting its development as a natural immune booster or vaccine adjuvant.

Keywords

Apigenin, granzyme, immunomodulatory, interferon-gamma, sungkai.



This article is included in the **Plant Science** gateway.

Corresponding author: Dwisari Dillasamola (dwisaridillasamola@phar.unand.ac.id)

Author roles: **Dillasamola D:** Conceptualization, Data Curation, Investigation, Methodology, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; **Aldi Y:** Formal Analysis, Software, Validation, Visualization, Writing – Review & Editing; **Fitria N:** Data Curation, Investigation, Resources; **Oktomalioputri B:** Formal Analysis, Visualization, Writing – Original Draft Preparation; **Lestari U:** Resources, Supervision, Writing – Original Draft Preparation; **Multia R:** Supervision, Validation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

Copyright: © 2025 Dillasamola D *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Dillasamola D, Aldi Y, Fitria N *et al.* **Isolation of Apigenin from Sungkai (*Peronema canescens*) Leaves and Its Immunomodulatory Effects: An In Vivo Study on Granzyme B, Interferon- γ , and Perforin Expression with Supporting In Silico Analysis [version 1; peer review: 2 approved, 2 approved with reservations]** F1000Research 2025, 14:774
<https://doi.org/10.12688/f1000research.167153.1>

First published: 08 Aug 2025, 14:774 <https://doi.org/10.12688/f1000research.167153.1>

Introduction

Indonesia, as a tropical country with extensive rainforest ecosystems, harbors one of the highest levels of biodiversity in the world.^{1–3} These forests support numerous ecological functions, such as nutrient cycling, energy flow, prevention of soil erosion, and facilitation of photosynthesis.^{4,5} However, the vast array of plant species within these ecosystems remains underexplored for their medicinal potential.^{6,7} One such underutilized plant is *Paronema canescens* Jack., commonly known as sungkai, a member of the Lamiaceae family. Sungkai is widely cultivated by local communities and naturally grows in forests, gardens, and residential areas.⁸ Ethnobotanical records indicate that this plant has traditionally been used in Sumatra and Borneo to treat various ailments, including malaria, fever, hypertension, intestinal worm infections, and hypercholesterolemia.^{8–10} Phytochemical analyses have identified the presence of flavonoids, alkaloids, and terpenoids in sungkai leaves.^{11–13}

Previous studies have successfully isolated apigenin, a major flavonoid from sungkai leaves, which demonstrated significant anti-inflammatory activity.¹⁴ Apigenin, a polyphenolic compound, has been reported to exhibit a wide range of pharmacological properties, including antioxidant, anti-inflammatory, antiviral, and immunomodulatory effects, primarily due to its phenolic structure.

The immune system plays a crucial role in protecting the body from infectious agents such as viruses. Impaired immune responses, which may be exacerbated by psychological stress and social isolation, can increase susceptibility to infections.^{15–18} Enhancing immune function is a key preventive strategy.^{19,20}

Granzyme B (GzmB), produced by cytotoxic T lymphocytes and natural killer (NK) cells, is an essential effector molecule that promotes the destruction of virus-infected or malignant cells.^{21,22} GzmB is also known to cleave viral proteins involved in replication, such as those of the herpes simplex virus.²³

While compounds such as stigmasterol and bis(2-ethylhexyl) phthalate from sungkai have shown immunostimulatory effects by increasing NK cell levels,²⁴ the potential of apigenin as an immunostimulant remains unexplored. Given apigenin's broad pharmacological profile and its presence as a major constituent of sungkai leaves, it is imperative to investigate its possible role in enhancing immune responses. Therefore, this study aims to evaluate the immunostimulatory activity of apigenin isolated from sungkai leaves by assessing its effects on key immune markers (Granzyme B, Interferon-gamma (IFN- γ), and Perforin) in a murine model.

Methods

Chemicals, reagents, and instruments

Analytical procedures utilized a variety of laboratory instruments and materials. Weighing was conducted using an analytical scale (Ohaus[®]), while absorbance readings were obtained via a UV–Vis spectrophotometer (Beckman Coulter[®]). Chromatographic separation was performed using radial chromatography with silica gel 60 PF254 containing gypsum (Merck, Cat. No. 1.07749.1000). For TLC profiling, aluminum-backed silica gel 60 F254 plates (Merck, Cat. No. 1.05554.0001; 0.25 mm thickness) were used and visualized under 254 nm UV light or after spraying with 10% H₂SO₄ (v/v) in methanol.

Advanced compound identification was conducted using a Vanquish[™] UHPLC Binary Pump (Thermo Scientific[™], Germany) coupled to a Q Exactive[™] Hybrid Quadrupole-Orbitrap[™] Mass Spectrometer. Separation was achieved using a Thermo Scientific[™] Accucore[™] Phenyl-Hexyl column (100 mm $\times$ 2.1 mm ID $\times$ 2.6 μ m). Solvents used for extraction, chromatography, and sample preparation included methanol (MeOH; Merck, Cat. No. 106009), ethanol (EtOH; Merck, Cat. No. 100983), ethyl acetate (EtOAc; Merck, Cat. No. 109623), and Milli-Q water (Merck Millipore). Additional laboratory tools comprised Pyrex[®] beakers, droppers, probes (Terumo[®]), microscope (Olympus[®]), ELISA microplate reader (Thermo Scientific[™] Multiskan), and standard laboratory glassware.

Reagents used in animal treatments included: Sodium acetate 1 M (Merck, Cat. No. 106268), Sodium carboxymethyl cellulose (NaCMC) 0.5% (Sigma-Aldrich, Cat. No. C4888) as a suspension vehicle, Aqua pro injection (Andeska Lab, Indonesia, Cat. No. AQPRO-INJ-500), Aquadest (Andeska Lab, Cat. No. AQ-DES-1000), COVID-19 vaccine (Indovac[®], Bio Farma, Indonesia).

Biological materials included male white mice (*Mus musculus*, 6–8 weeks old, 25–30 g) from PT Indoanilab (Indonesia), standard mouse pellet diet (Vita Feed[®]), and apigenin isolated from *Peronema canescens* leaves.

Plant materials

Leaves of *Paronema canescens* Jack. (sungkai) were collected from Aia Pacah, Padang City, West Sumatera, Indonesia. The plant was identified and authenticated by Dr. Nurainas at the Herbarium ANDA, Department of Biology, Universitas

Andalas, with voucher specimen number 717/K-ID/ANDA/X/2024. Samples were air-dried and ground to a fine powder before extraction.

Extraction

Seventeen kilograms of dried sungkai leaf powder were macerated in 96% ethanol (2:5 w/v) at room temperature for 72 hours. The extraction process was repeated every 24 hours by decanting the extract and re-macerating the residue. The combined filtrates were concentrated using a rotary evaporator to yield approximately 1.2 kg of crude extract.

Isolation and characterization

Five hundred grams of the concentrated extract were fractionated via liquid-liquid extraction using increasing polarity solvents: *n*-hexane, ethyl acetate, and ethanol. The ethanol-soluble fraction was further subjected to vacuum liquid chromatography using gradients of *n*-hexane: EtOAc (100:0 to 0:100) and methanol. This yielded 14 initial fractions, which were combined based on TLC spot profiles into five main fractions.

Fraction X (FX), displaying prominent UV-active spots, was purified using radial chromatography with silica gel-coated plates (0.5 mm thickness) and solvent gradients of *n*-hexane: EtOAc (6:4, 5:5, 3:7). Approximately 10 g of a yellow solid (Isolate 1) was obtained.

Spectroscopic analyses identified Isolate 1 as apigenin (Table 1), based on the following data:

- UV-Vis (MeOH): λ_{max} 210, 267, and 336 nm
- FT-IR: ν_{OH} 3286 cm^{-1} ; $\nu_{\text{C=O}}$ 1653 cm^{-1} ; $\nu_{\text{C=C}}$ aromatic 1587–1446 cm^{-1} ; $\nu_{\text{C-O}}$ 1298–1031 cm^{-1}
- $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ (DMSO- d_6 , 500/125 MHz): Confirmed the flavonoid structure with corresponding HMBC and COSY correlations.

In Silico study

Ligand and protein preparation

The 3D structure of apigenin (CID: 5280443) and Levamisole (CID: 26879) was retrieved from the PubChem website (<https://pubchem.ncbi.nlm.nih.gov/>). Crystal structures of granzyme B (PDB: 1FQ3), perforin (PDB: 3NSJ), and interferon- γ (PDB: 1FG9) were retrieved from the Protein Data Bank (<https://www.rcsb.org/>). Protein and ligand were

Table 1. MS (m/z 268.992 [$M-H$][−]) for $C_{15}H_{10}O_5$.

No	DEPT-135°	^{13}C (ppm)*	HSQC (<i>mult.</i> , <i>J</i> in Hz, ΣH)**	HMBC	COSY
2	C	164.2	-	-	-
3	CH	102.9	6.78 (<i>s</i> , 1H)	C-2, C-4, C-10, C-1'	-
4	C	181.8	-	-	-
5	C	161.5	-	-	-
6	CH	98.9	6.18 (<i>d</i> , <i>J</i> = 2.05 Hz, 1H)	C-5, C-7, C-8, C-10	-
7	C	163.8	-	-	-
8	CH	94.0	6.47 (<i>d</i> , <i>J</i> = 2.1 Hz, 1H)	C-6, C-7, C-9, C-10	-
9	C	157.3	-	-	-
10	C	103.7	-	-	-
1'	C	121.2	-	-	-
2', 6'	CH	128.5	7.92 (<i>d</i> , <i>J</i> = 8.75 Hz, 2H)	C-2, C-4', C-3', 5'	H-3', 5'
3', 5'	CH	116.0	6.92 (<i>d</i> , <i>J</i> = 8.75 Hz, 2H)	C-1', C-4', C-2', 6'	H-2', 6'
4'	C	161.2	-	-	-

** (DMSO- d_6 ; $^1\text{H-NMR}$ 500 MHz; $^{13}\text{C-NMR}$ 125 MHz).

* (DMSO- d_6 ; $^{13}\text{C-NMR}$ 125 MHz).

optimized with MOE 2022.02 (licensed proprietary software, license was obtained) to a gradient convergence of 0.001 kcal/mol/Å².

Molecular docking

The active sites of each target protein were identified using the Site Finder tool in MOE. The Molecular docking simulations were carried out using the default settings of the MOE applications. Ligand–protein interactions were evaluated based on binding affinity (kcal/mol); more negative binding affinities were considered to exhibit favorable binding stability and orientation. Levamisole, a known immunomodulatory agent, was used as a reference compound for comparison. Following docking, ligand–protein interactions were analyzed through 2D and 3D visualizations using the Biovia Discovery 2022 application.

In vivo assay of expression of Granzyme B

Experimental design

This study was a randomized controlled in vivo study conducted to evaluate the immunostimulatory effect of apigenin, isolated from *Paronema canescens* (sungkai) leaves, in male mice immunized with the COVID-19 vaccine. This study utilized 25 healthy mice (*Mus musculus*), each weighing between 25 and 40 grams. Only healthy male mice aged 6–8 weeks and weighing between 25 and 40 grams were included. Mice were obtained from a certified vendor and allowed a 7-day acclimatization period before treatment. Mice showing signs of illness, injury, or abnormal behavior during the acclimatization period were excluded. No animals were excluded after allocation, and all animals that began treatment completed the protocol and were included in the final analysis.

The animals were randomly assigned to five groups (n = 5 per group) as follows:

- Group 1 (Negative control): Received only the drug carrier, 0.5% sodium carboxymethyl cellulose (NaCMC).
- Group 2 (Positive control): Received the COVID-19 vaccine along with 0.5% NaCMC as the carrier.
- Group 3 (Apigenin 1 mg/kg BW): Received the COVID-19 vaccine and apigenin at a dose of 1 mg/kg body weight, formulated in 0.5% NaCMC.
- Group 4 (Apigenin 25 mg/kg BW): Received the COVID-19 vaccine and apigenin at a dose of 25 mg/kg body weight, formulated in 0.5% NaCMC.
- Group 5 (Apigenin 50 mg/kg BW): Received the COVID-19 vaccine and apigenin at a dose of 50 mg/kg body weight, formulated in 0.5% NaCMC.

All mice underwent a 7-day acclimatization period to allow adaptation to the laboratory environment and reduce stress-related variability. On Day 1, all groups except the negative control (Group 1) were immunized with the COVID-19 vaccine. Apigenin was administered intramuscularly once daily for 7 consecutive days (Days 1–7) to Groups 3, 4, and 5 according to their respective dosages. On Day 8, a booster dose of the COVID-19 vaccine was administered to all mice in Groups 2 through 5. On Day 9, before blood collection, animals were sedated using diethyl ether as an inhalation anesthetic. The anesthetic was administered by placing a cotton pad soaked in diethyl ether into a sealed treatment chamber. Each mouse was then gently placed into the chamber and monitored until sedation was achieved. Standard equipment used during animal procedures included feeding sonde, sterile disposable syringes, and surgical blades (scalpels). Blood samples were collected following euthanasia by guillotine decapitation targeting the carotid artery. This method was selected to ensure rapid and humane termination under deep sedation, minimizing animal distress and ensuring optimal blood volume recovery for downstream analyses. All procedures were performed by trained personnel under the supervision of a laboratory veterinarian to ensure animal welfare and procedural consistency. After blood collection, the samples were allowed to clot at room temperature and subsequently centrifuged to separate the serum. The isolated serum was aliquoted and stored under appropriate conditions until further analysis.

Granzyme B expression assay

The level of granzyme B was quantified using an ELISA kit (BT Lab[®], Cat. No. E0846Mo) according to the manufacturer's instructions. Standards were prepared at concentrations of 1600, 800, 400, 200, 100, 50, and 0 pg/mL. Each well received 50 µL of the granzyme B standard or sample, followed by 50 µL of detection antibody.

The plate was incubated at room temperature (25°C) for 1 hour, then washed three times with 350 µL of wash buffer (BT Lab[®], provided in the kit). After washing, 100 µL of TMB substrate (BT Lab[®], supplied with the kit) was added per well and incubated in the dark for 10 minutes. The reaction was stopped with 100 µL of stop solution (BT Lab[®]), and absorbance was read at 450 nm using a microplate reader. Granzyme B concentrations were calculated based on the standard curve generated from known concentrations.

Interferon gamma level examination

Serum IFN-γ levels were measured using a commercial ELISA kit (BT Lab[®], Cat. No. E0002Mo) according to the manufacturer's instructions. Each well was loaded with 50 µL of serum or standard, followed by antibody incubation and washing steps as recommended. Subsequently, 100 µL of TMB substrate was added to initiate the enzymatic reaction, followed by 10 minutes of incubation. The reaction was stopped with 100 µL of stop solution, and absorbance was measured at 450 nm. The concentrations were derived from a standard curve generated using IFN-γ standards provided in the kit.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA). Post hoc comparisons were performed using Duncan's multiple range test to determine statistically significant differences among groups. Statistical significance was defined as $p < 0.05$. All analyses were carried out using SPSS Statistics version 15.0 (SPSS Inc., Chicago, IL, USA).

Ethical considerations

All animal procedures were conducted following the ethical standards for animal experimentation and complied with institutional and national guidelines. Ethical approval for this study was obtained from the Health Research Ethics Committee of the Faculty of Pharmacy, Andalas University, under approval number: 93/UN16.10.D.KEPK-FF/2024. All efforts were made to minimize animal suffering, and only the minimum number of animals required to achieve statistical significance was used. Sedation and euthanasia procedures were performed to ensure humane treatment throughout the experimental protocol.

Results

Compounds isolations

Isolate 1 was successfully obtained from fraction FX of the sungkai leaf extract, yielding 10 grams of yellow powder solid that was soluble in ethanol. The TLC profile showed a single spot under UV light at 254 and 365 nm, indicating the presence of a single compound.

UV-Visible spectroscopy (UV-Vis)

The UV spectrum of Isolate 1 exhibited three maximum absorption peaks at λ_{max} 210, 267, and 336 nm. These peaks suggested the presence of a conjugated chromophore system (Figure 1).

Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectrum showed characteristic peaks at ν_{max} 3286 cm^{-1} (O–H stretch), 1653 cm^{-1} (C=O stretch), 1587–1446 cm^{-1} (aromatic C=C), and 1298–1031 cm^{-1} (C–O stretch), indicating the presence of hydroxyl, carbonyl, and aromatic functional groups (Figure 2).

Proton Nuclear Magnetic Resonance (¹H-NMR)

The ¹H-NMR spectrum revealed five proton signals in the δ H 6–8 ppm region. Signals at δ H 7.92 and 6.92 ppm appeared as doublets with $J = 8.75$ Hz, suggesting ortho-coupled aromatic protons. Signals at δ H 6.47 and 6.18 ppm showed meta coupling ($J \approx 2$ Hz), while a singlet at δ H 6.78 ppm was attributed to an isolated proton between two quaternary carbons (Figure 3).

Carbon-13 NMR (¹³C-NMR)

The ¹³C-NMR spectrum exhibited 13 carbon signals, all in the sp^2 region. Six signals between δ C 157.3–181.8 ppm corresponded to =C–OH and –C=O carbons, while the remaining seven signals (δ C 94.0–128.5 ppm) represented sp^2 methine and quaternary carbons (Figure 4).

COSY spectrum

COSY analysis confirmed proton-proton correlations, notably between δ H 7.92 and 6.92 ppm, supporting ortho substitution patterns. No direct coupling was observed among the remaining three protons (Figure 5).



Figure 1. Spectrum UV-Vis isolate 1.

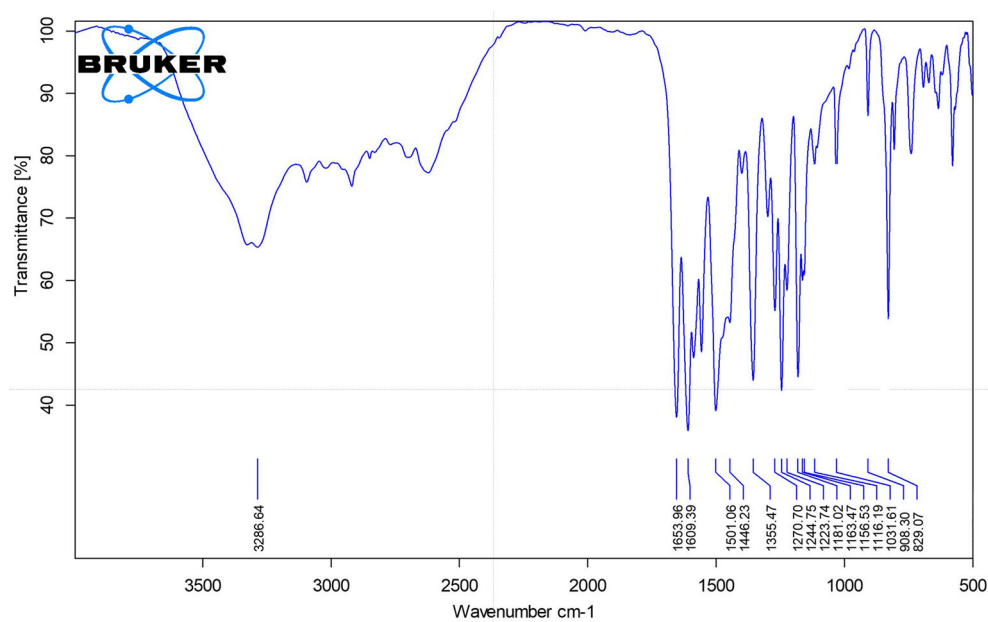


Figure 2. Spectrum FT-IR isolate 1.

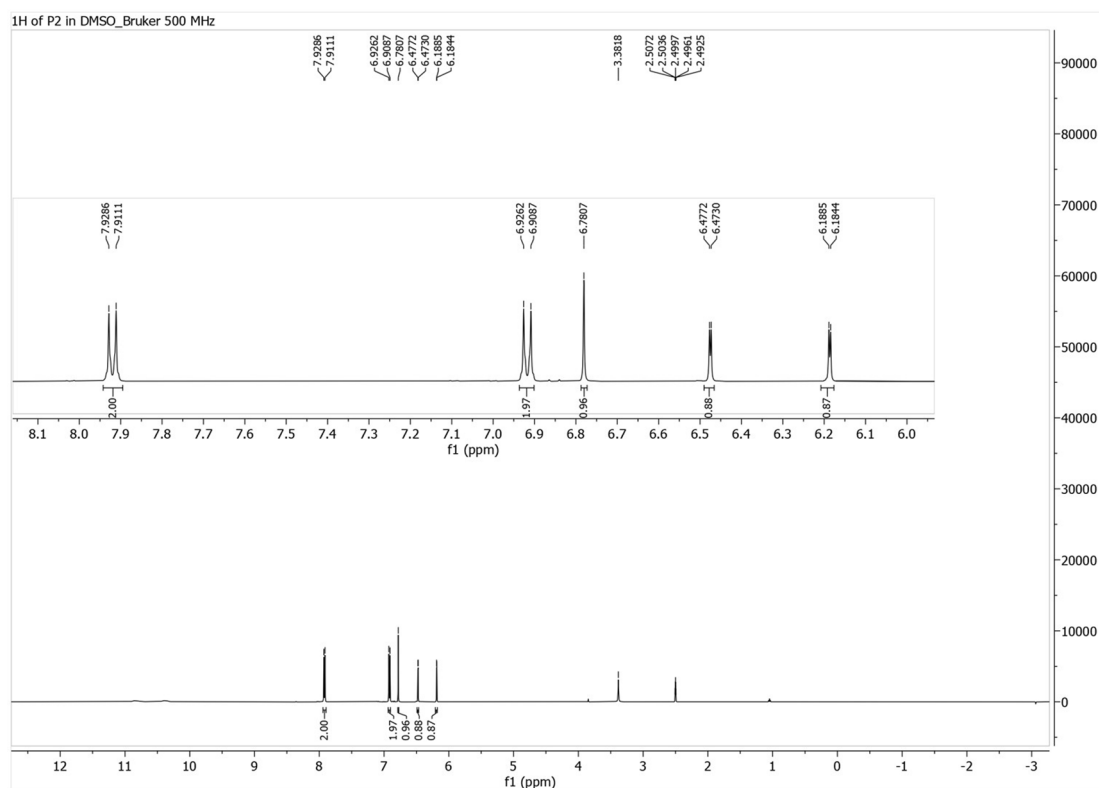


Figure 3. ^1H NMR spectrum for isolate 1 in DMSO- d_6 (500 MHz).

HMBC spectrum

Long-range correlations observed in the HMBC spectrum confirmed the positioning of key functional groups. δH 6.78 ppm correlated with C-2, C-4, C-10, and C-1', indicating carbonyl at C-4 and phenolic group at C-2. δH 6.18 and 6.47 ppm showed meta correlations to C-5, C-6, C-7, C-8, C-9, and C-10, confirming OH substitution at C-5 and C-7 (Figure 6).

Mass Spectrometry (MS)

Using UPLC-MS/MS in negative ion mode, the molecular ion peak $[\text{M}-\text{H}]^-$ was detected at m/z 268.992, corresponding to the molecular formula $\text{C}_{15}\text{H}_{10}\text{O}_5$. This confirmed the identity of the compound as apigenin (Figure 7).

Index of Hydrogen Deficiency (IHD)

The IHD value was calculated to be 11, which supports a structure containing three rings and eight additional degrees of unsaturation, consistent with a flavonoid backbone (Figure 8).

Molecular docking analysis of apigenin with granzyme B, perforin, and interferon- γ

The docking results demonstrated that apigenin exhibited binding affinities of -5.56 kcal/mol with both granzyme B and perforin, and -4.95 kcal/mol with IFN- γ . These values were comparable to those of levamisole, which showed binding affinities of -5.08 kcal/mol (granzyme B), -5.36 kcal/mol (perforin), and -4.82 kcal/mol (IFN- γ) (Table 2). In terms of molecular interactions, apigenin formed hydrogen bonding with His151 in granzyme B, and additional interactions including Pi-alkyl (Leu39, Lys40), sulfur-X (Lys149), and van der Waals (Phe57). For perforin, interactions included Pi-alkyl (Ala8, Leu11, Ile73), Pi-Pi stacking (Tyr53), and Pi-sulfur (Met77). For IFN- γ , apigenin exhibited multiple hydrogen bonds (Arg143, Arg372, Gln374, Asp544), Pi-alkyl interaction (Pro546), and ionic interactions including Pi-cation/anion (Arg378, Gly551). Levamisole, in comparison, exhibited hydrogen bonding with Ser195 (granzyme B), Arg372 (IFN- γ), and various hydrophobic and Pi-based interactions with all three proteins (Table 3).

Immunostimulatory effects of apigenin on granzyme B, Perforin, and Interferon- γ expression in mice induced with COVID-19 vaccine

The administration of apigenin isolated from *Peronema canescens* (sungkai) leaves was evaluated for its immunostimulant effects in male white mice induced with the COVID-19 vaccine. The study involved five experimental groups: a

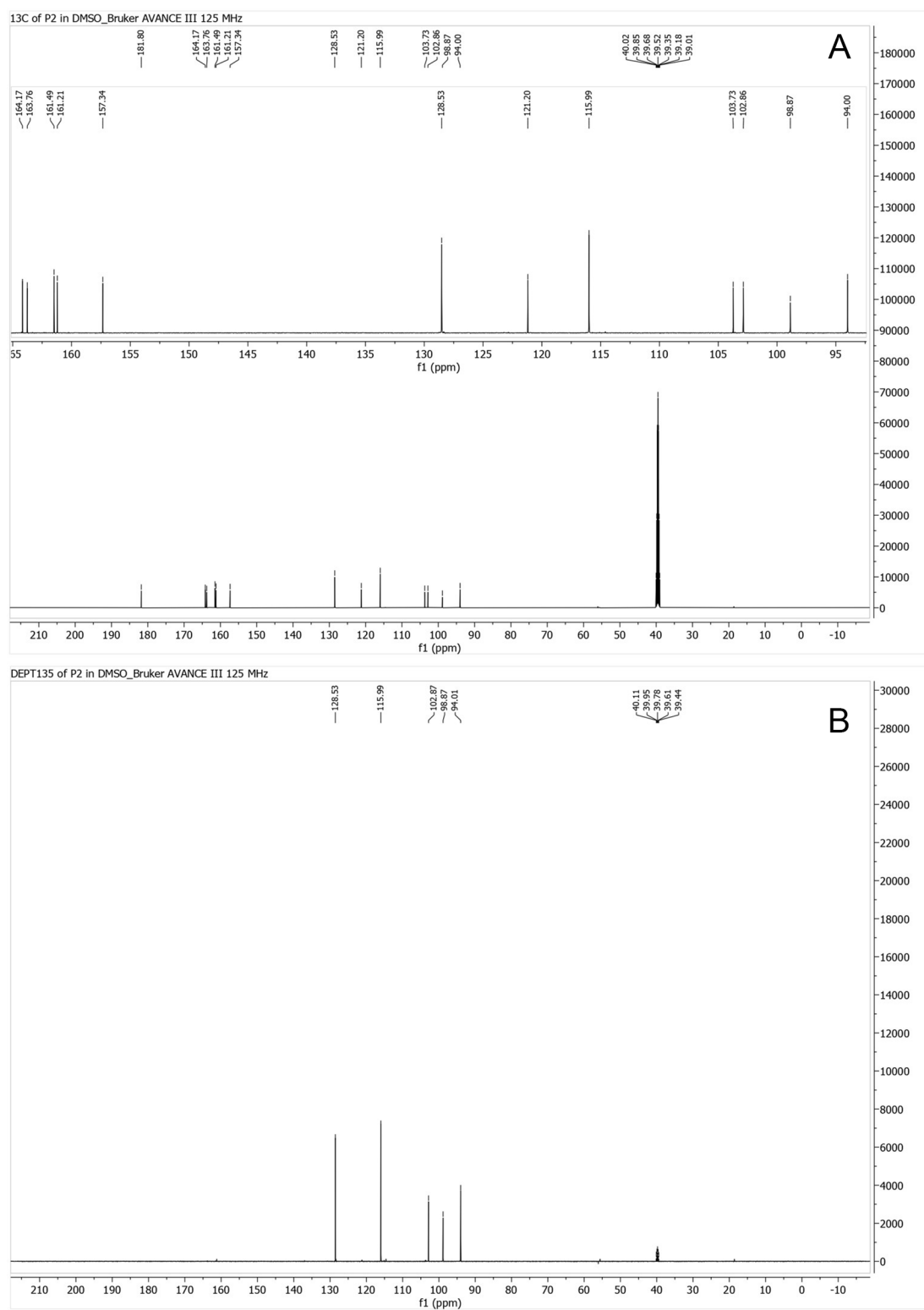


Figure 4. ^{13}C NMR spectrum for isolate 1 in DMSO- d_6 (125 MHz).

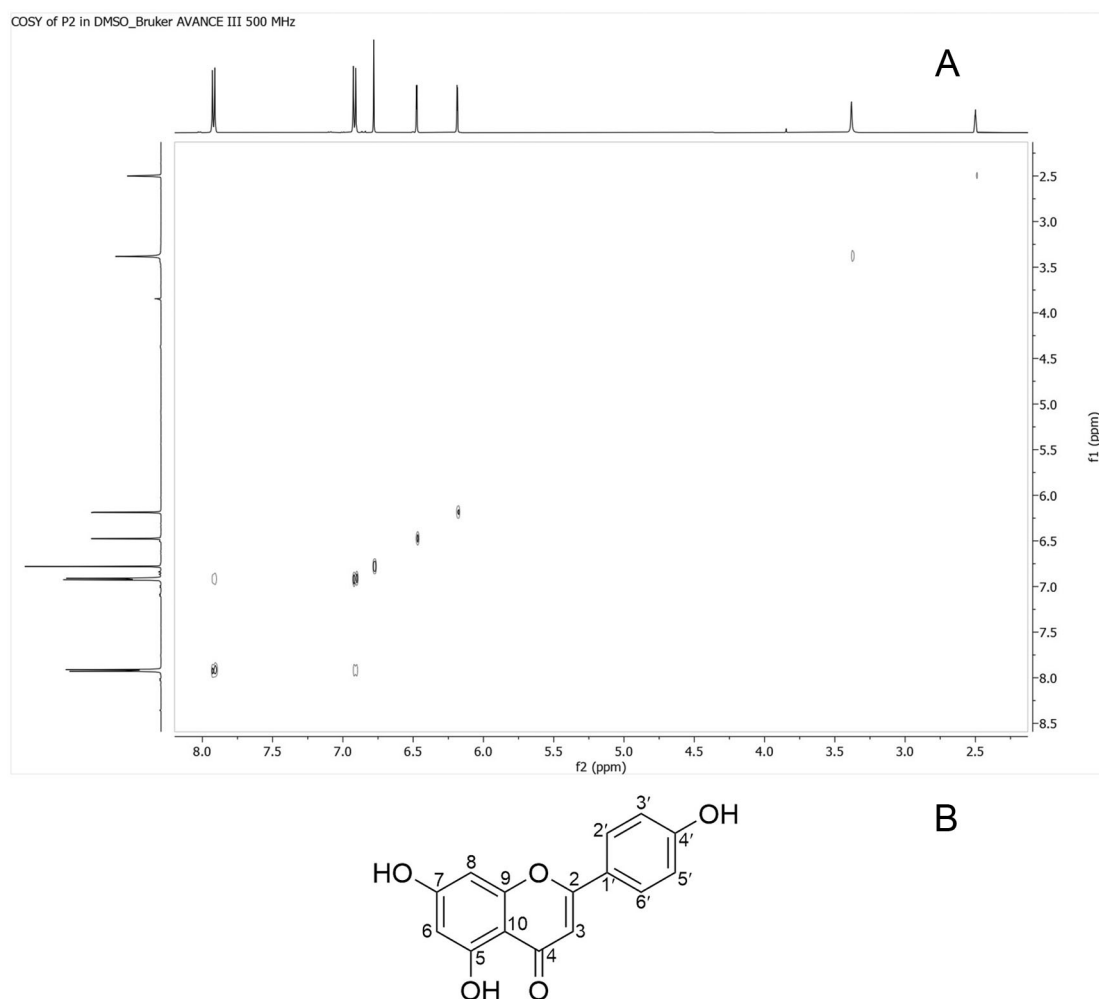


Figure 5. COSY spectrum of isolate 1.

negative control group receiving 0.5% NaCMC, a positive control group receiving the COVID-19 vaccine, and three treatment groups receiving apigenin at doses of 1 mg/kgBW, 25 mg/kgBW, and 50 mg/kgBW, respectively. The ELISA results indicated a dose-dependent increase in granzyme B levels across the groups. The negative and positive control groups showed relatively lower granzyme B levels, with mean values of 150.83 and 151.98, respectively. In contrast, the treatment groups demonstrated higher values, reaching 160.75 at 1 mg/kgBW, 161.38 at 25 mg/kgBW, and 162.09 at 50 mg/kgBW (Figure 9a). Statistical analysis using one-way ANOVA confirmed significant differences among the groups ($p < 0.05$), and Duncan's post hoc test revealed that all treatment groups formed a separate subset from the controls, indicating a significant stimulatory effect of apigenin on granzyme B production.

Similarly, perforin levels showed a notable increase with escalating doses of apigenin. The negative and positive control groups had lower mean values of 1,602 and 1,789, respectively, whereas the treatment groups exhibited higher values of 1,846 at 1 mg/kgBW, 2,917 at 25 mg/kgBW, and 3,086 at 50 mg/kgBW (Figure 9b). Statistical validation through one-way ANOVA revealed a significant increase in perforin levels ($p < 0.05$), and Duncan's test indicated a consistent trend with the granzyme B data, supporting the conclusion that apigenin administration enhances perforin expression.

For interferon- γ , the ELISA data also demonstrated a dose-dependent increase. The negative and positive control groups exhibited mean levels of 107.18 and 109.10, respectively, while the treatment groups showed elevated levels of 114.20, 130.32, and 130.57 for doses of 1, 25, and 50 mg/kgBW, respectively (Figure 9c). Although body weight was not significantly different among the groups, interferon- γ levels increased significantly with higher doses of apigenin. Normality testing with the Shapiro-Wilk test ($p > 0.05$) and homogeneity testing ($p > 0.05$) confirmed the appropriateness of one-way ANOVA, followed by Duncan's test, which indicated the highest stimulation occurred at the 50 mg/kgBW dose.

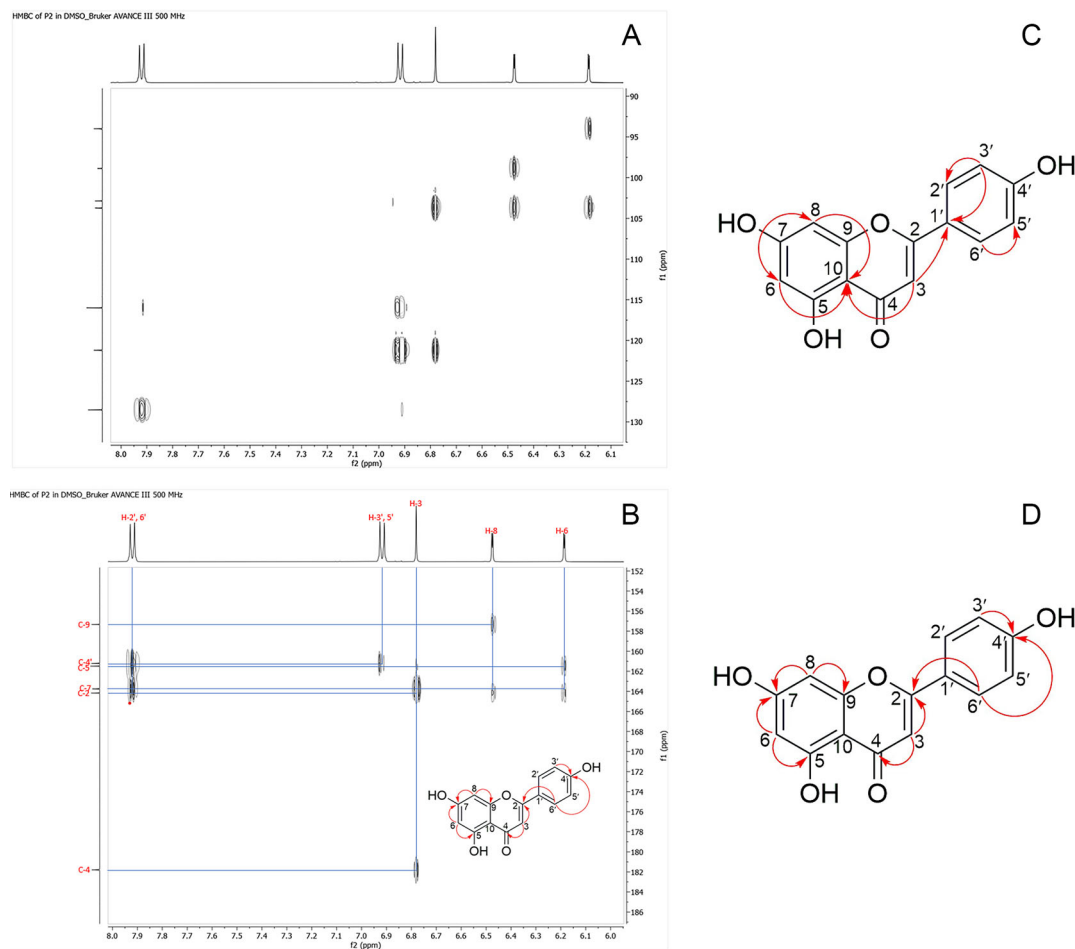


Figure 6. HMBC (Heteronuclear Multiple Bond Correlation) spectrum of isolate 1.

Discussion

The comprehensive characterization of the isolate from *Peronema canescens* (sungkai) leaves using spectroscopic and spectrometric techniques confirmed its identity as apigenin, a well-known flavonoid. The UV-Vis spectral data revealed the presence of a conjugated chromophore system typical of flavonoids,²⁴ while FTIR spectra confirmed the existence of phenolic hydroxyl groups and a conjugated carbonyl moiety. Further structural elucidation using ^1H -NMR and ^{13}C -NMR indicated the presence of a flavone backbone, with specific proton and carbon signals corresponding to methine and quaternary carbons within an aromatic system.^{25,26} DEPT-135 and HSQC spectra allowed the differentiation of protonated from non-protonated carbons, while COSY and HMBC spectra elucidated substitution patterns on the flavone rings, confirming ortho and meta relationships and assigning the phenolic hydroxyl groups to C-5, C-7, and the para-position on the B-ring. Mass spectrometry validated the molecular formula ($\text{C}_{15}\text{H}_{10}\text{O}_5$) and molecular weight (268.992), consistent with the known structure of apigenin. The Index of Hydrogen Deficiency (IHD) of 11 further supported the proposed flavonoid structure, composed of three aromatic rings and multiple degrees of unsaturation. These findings are consistent with those of a previous study on the apigenin compound in *Gentiana veitchiorum*.²⁶ Based on all this data, the compound was identified as 5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one, commonly known as apigenin.

The molecular docking results support the hypothesis that apigenin possesses immunomodulatory potential by interacting with key effector proteins involved in cytotoxic immune responses. The relatively low binding affinity of apigenin toward granzyme B and perforin (-5.56 kcal/mol) suggests a possible stabilizing interaction that may influence their expression or activity in vivo. This finding is in line with the in vivo results, which demonstrate a dose-dependent increase in the expression of these proteins following apigenin administration. Notably, apigenin showed multiple interaction types with interferon- γ , including hydrogen bonds and ionic contacts. The presence of multiple stabilizing interactions (e.g., Arg143, Arg372, Gln374) suggests that apigenin may play a role in enhancing or modulating IFN- γ signaling pathways. In comparison with levamisole, a known immunostimulant, apigenin showed similar or slightly better docking

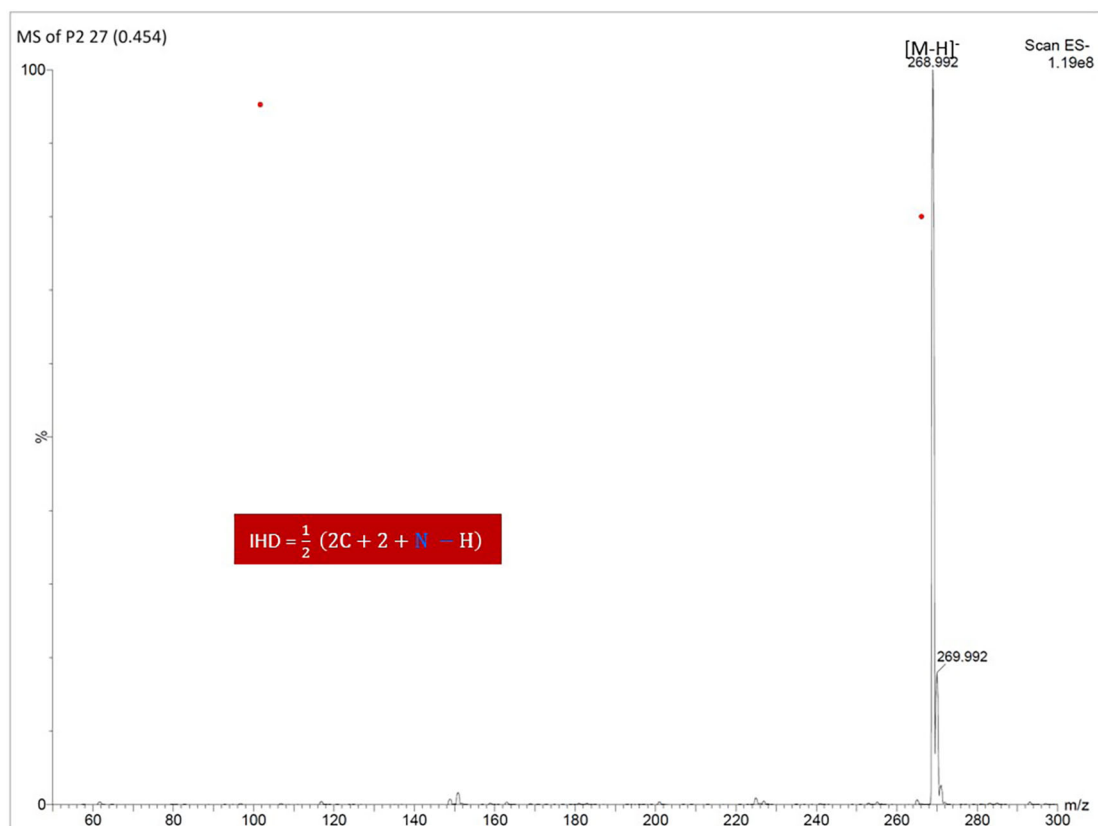


Figure 7. Mass spectrum of isolate 1 Using (-) mode.

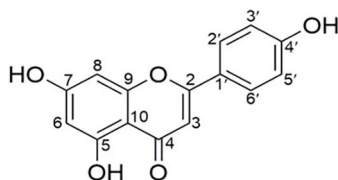


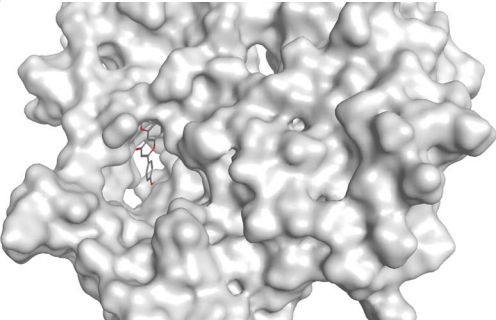
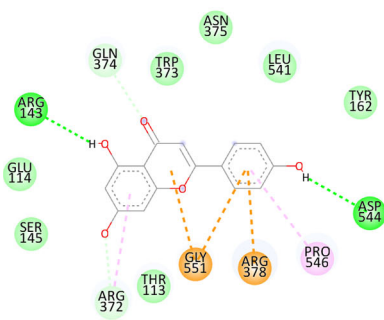
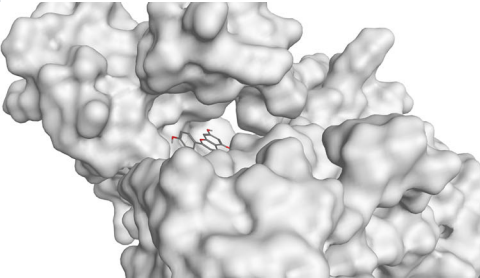
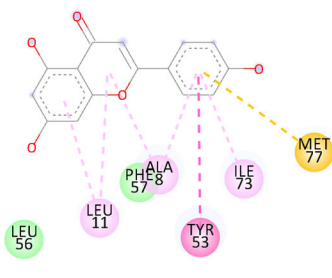
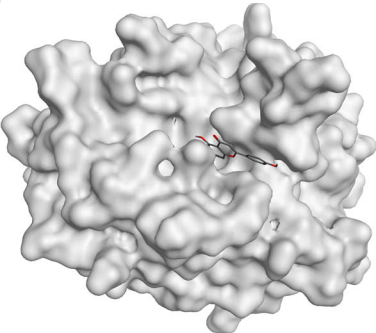
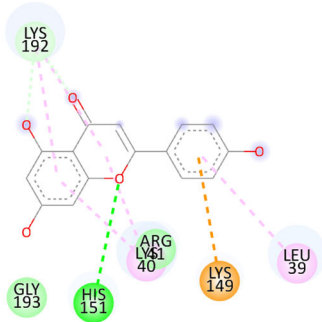
Figure 8. Molecular structure of isolate 1 (Apigenin).

Table 2. Binding affinities of apigenin with granzyme B, perforin, and IFN- γ .

No	Binding affinity (kcal/mol)		
	Granzyme B	Interferon- γ	Perforin
Apigenin	-5.56	-4.95	-5.56
Levamisole	-5.08	-4.82	-5.36

scores and comparable interaction patterns across the three proteins. This suggests that apigenin may exert its effects through a mechanism partially overlapping with levamisole, yet possibly more selective or synergistic in enhancing cytotoxic immune responses without broadly modulating other inflammatory mediators, such as IL-10. The molecular interactions observed, including hydrogen bonding, π -alkyl stacking, and electrostatic forces, further reinforce the specificity of apigenin toward cytotoxic effector proteins. These findings are consistent with previous reports describing the ability of flavonoids to modulate T cell and NK cell function through direct or indirect modulation of granzyme B and perforin expression.^{27,28}

Table 3. Binding interaction of apigenin with granzyme B, perforin, and IFN- γ .

Name	3D interaction	2D interaction
Apigenin Perforin		
Apigenin Interferon- γ		
Apigenin Granzyme B		

The *in vivo* findings of this study further confirm that apigenin isolated from sungkai leaves possesses potent immunostimulatory activity. The observed increases in granzyme B, perforin, and interferon- γ levels in apigenin-treated mice support its role in enhancing cellular immune responses. The significant elevation in granzyme B levels aligns with previous studies reporting that apigenin can upregulate granzyme B expression through the activation of the JNK and ERK signaling pathways, thereby enhancing the cytotoxic function of natural killer (NK) cells, particularly in their response to tumors or virally infected cells.^{29,30} Granzyme B and perforin are crucial cytolytic proteins secreted by cytotoxic T lymphocytes (CTLs) and NK cells to induce apoptosis in infected or malignant cells, and the marked increase in their levels in this study highlights apigenin's capacity to potentiate the immune defense mechanisms of the host.³¹

Furthermore, the elevation of interferon- γ levels suggests that both innate and adaptive immune responses are enhanced. Interferon- γ is a pivotal cytokine produced predominantly by activated T cells and NK cells and plays a central role in orchestrating the immune response against viral infections.³² The increasing trend in interferon- γ production across the treatment groups suggests that apigenin enhances cytokine-mediated signaling, which is crucial for the activation and proliferation of immune effector cells. These results are in agreement with the existing literature, which suggests that flavonoids, including apigenin, exert immunomodulatory effects through their antioxidant properties and interactions with immune signaling pathways.³²

The presence of other phytochemical constituents in sungkai leaves, such as tannins, alkaloids, and phenolics, may further contribute to the immunostimulatory potential observed. However, among these, apigenin appears to be the most

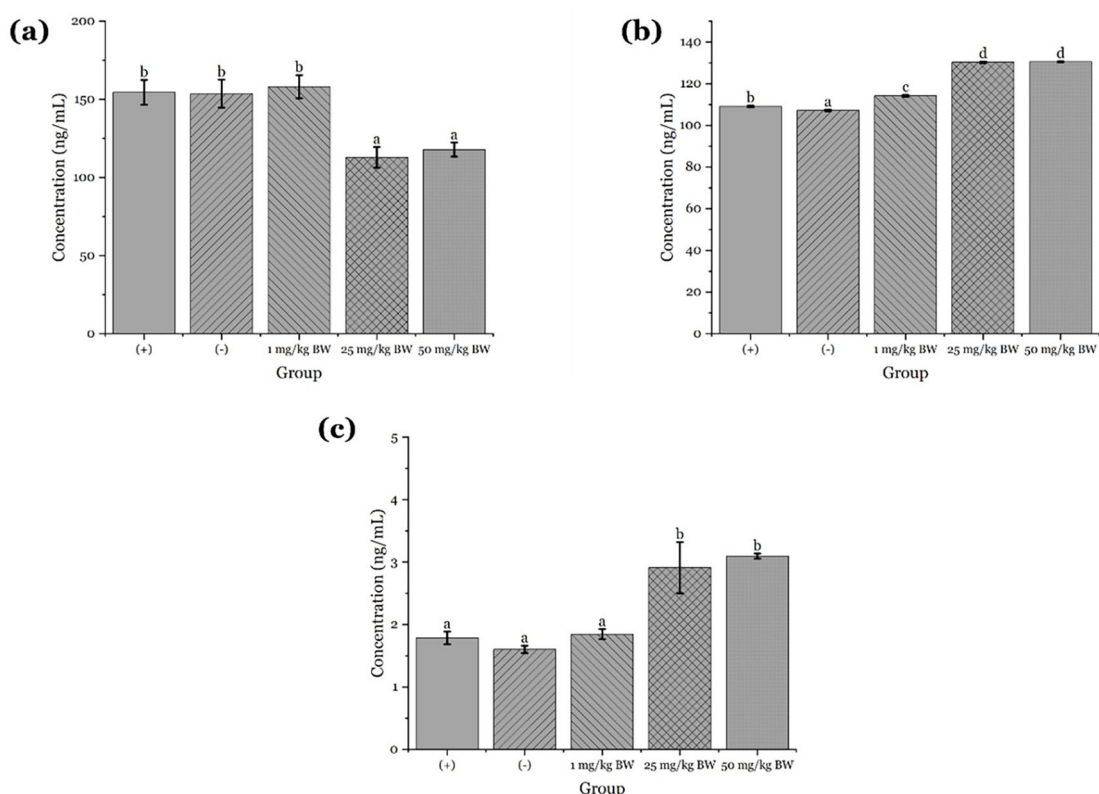


Figure 9. The effect of apigenin administration on the concentrations of: (a) granzyme; (b) interferon- γ ; and (c) perforin. Different letters on the graphs indicate significant differences ($P < 0.05$) based on Duncan's test.

bioactive compound. Previous research has demonstrated that apigenin can enhance NK cell activity by modulating the expression of key effector molecules.³³ This was also evidenced by a previous study that highlighted the importance of hydroxylation patterns in the flavone structure, which influence the immunostimulatory potency of such compounds. Additionally, a previous study demonstrated that total flavonoids from *Hippophae rhamnoides* can enhance NK cell cytotoxicity by upregulating the expression of granzyme and perforin, which further supports the proposed mechanism of action for apigenin.³³

Conclusion

Sungkai (*Peronema canescens* Jack.) leaves are known to contain various secondary metabolites, among which flavonoids, particularly apigenin, have shown promising immunostimulatory potential. In this study, Isolate 1 obtained from the ethyl acetate extract was identified as apigenin through comprehensive spectroscopic and spectrometric analysis. Molecular docking simulations were performed to explore the potential immunomodulatory mechanism of apigenin against granzyme B, perforin, and interferon- γ (IFN- γ). Subsequent *in vivo* assays in mice demonstrated that apigenin, administered at doses of 1 mg/kg BW, 25 mg/kg BW, and 50 mg/kg BW, significantly enhanced the expression of key immune effector molecules—granzyme B, perforin, and interferon- γ —which play critical roles in cellular immune responses.

Taken together, the integrated chemical, computational, and biological findings confirm that apigenin isolated from sungkai leaves possesses potent immunostimulant and anti-inflammatory properties. Its ability to modulate immune signaling pathways and enhance the expression of cytolytic proteins highlights its potential as a natural therapeutic agent. These results support further investigation and development of apigenin as an immune-enhancing and antiviral compound, especially for applications in immunocompromised individuals or as an adjuvant in vaccine formulations.

Ethical considerations

This study has obtained approval from the code of ethics for health research, Faculty of Pharmacy, Andalas University with the number: 93/UN16.10.D.KEPK-FF/2024.

Underlying data

Zenodo: Isolation of Apigenin from Sungkai (*Peronema canescens*) Leaves and Its Immunomodulatory Effects: An in vivo Study on Granzyme B, Interferon- γ , and Perforin Expression with Supporting In Silico Analysis. Doi: [10.5281/zenodo.15980341](https://doi.org/10.5281/zenodo.15980341)³⁴

This project contains the following underlying data:

- ARRIVE Checklist
- Data Granzyme
- Data Interferon- γ
- Data Perforin

Reporting guidelines

ARRIVE checklist for “Isolation of Apigenin from Sungkai (*Peronema canescens*) Leaves and Its Immunomodulatory Effects: An in vivo Study on Granzyme B, Interferon- γ , and Perforin Expression with Supporting In Silico Analysis”, Doi: [10.5281/zenodo.15980341](https://doi.org/10.5281/zenodo.15980341)³⁴

Data availability

Isolation of Apigenin from Sungkai (*Peronema canescens*) Leaves and Its Immunomodulatory Effects: An in vivo Study on Granzyme B, Interferon- γ , and Perforin Expression with Supporting In Silico Analysis © 2025 by Dwisari Dillasamola, M.Farm, Apt is licensed under Creative Commons Attribution 4.0 International.

Acknowledgements

The author would like to thank Universitas Andalas for Research Excellence Expertise Pathway (PUJK) Batch I, Universitas Andalas, Fiscal Year 2024 for facilitating and funding this research, Number: 340/UN16.19/PT.01.03/PUJK/2024, signed on July 17, 2024.

References

1. Maskun M, Assidiq H, Mukarramah NHA, *et al.*: **Threats to the sustainability of biodiversity in Indonesia by the utilization of forest areas for national strategic projects: A normative review.** *IOP Conf. Ser. Earth Environ. Sci.* 2021; **886**: 012071. [Publisher Full Text](#)
2. Afriwardi A, Aldi Y, Dillasamola D, *et al.*: **Immunostimulatory Activities of Pegagan Embun (*Hydrocotyle sibthorpioides* Lam.) in White Male Mice.** *Pharm. J.* 2021; **13**(2): 368–375.
3. Syofyan S, Almahdy A, Wulandari A, *et al.*: **Effects of Ethanol Extract of Sungkai (*Peronema canescens* Jack.) on Fertility of Female Wistar Mice (*Mus musculus* L.).** *Trop. J. Nat. Prod.* 2023; **7**(3): 2547–2550.
4. Huntington ND, Cursons J, Rautela J: **The Cancer–natural killer cell immunity cycle.** *Nat. Rev. Cancer.* 2020; **20**(8): 437–454. [PubMed Abstract](#) | [Publisher Full Text](#)
5. Taniguchi M, Seiro K, Nakayama T: **The NKT cell system: bridging innate and acquired immunity.** *Nat. Immun.* 2003; **4**: 1164–1165. [Publisher Full Text](#)
6. Stern BF, Marliyana SD, Wibowo FR, *et al.*: **Stigmasterol and Stigmasterone from Methanol Extract of H.** *J. Undip.* 2021; **24**(4): 108–113.
7. Rizal DF, Muharni M, Yohandini H, *et al.*: **Standardization in ethanolic extract of *Peronema canescens* leaves.** *Indones. J. Fundamental Appl. Chem.* 2022; **7**: 136–142. [Publisher Full Text](#)
8. Yani AP, Putranto AMH: **Examination of the Sungkai's young leaf extract (*Peronema Canescens*) as an antipiretic, immunity, antiplasmodium and teratogenity in mice (*Mus. mucus*).** *Int. J. Sci. Eng.* 2014; **7**: 30–34. [Publisher Full Text](#)
9. Dillasamola D, Wahyuni FS, Rita RS, *et al.*: **Immunostimulating Study of Active Agent Fraction from Sungkai (*Peronema canescens* Jack.) Leaf from SARS-COV-2 Virus Antigen Exposure to NK and CD8+T Cells.** *Pharm. J.* 2022; **14**(4): 344–351.
10. Dillasamola D, Aldi Y, Wahyuni FS, *et al.*: **Study of Sungkai (*Peronema canescens*, Jack) leaf extract activity as an immunostimulators with in vivo and in vitro methods.** *Pharm. J.* 2021; **13**(6): 1397–1407.
11. Pratiwi U, Muharni M, Ferlinahayati F, *et al.*: **Antihyperlipidemia activity of sungkai (*Peronema canescens*) leaves extract in albino rats *Rattus novericus* (Wistar strain) with propylthiouracil-induced hyperlipidemia.** *Med. Plants - Int. J. Phytomed. Rel. Ind.* 2022; **14**: 589–596. [Publisher Full Text](#)
12. Kanedi M: **Therapeutic potentials of sungkai (*Peronema canescens* Jack.) an Indonesian luxurious woody plant.** *World Journal of Biology Pharmacy and Health Sciences.* 2022; **11**: 069–073. [Publisher Full Text](#)
13. Tarigan IL, Aini IPS, Latief M: **Isolation of a Flavone Apigenin and a Steroids Squalene from *Peronema canescens* Jack Leaves with Anti-Inflammatory Activities.** *Pharm. J.* 2022; **14**: 744–752. [Publisher Full Text](#)
14. Hannoodde S, Nasuruddin DN: **Acute inflammatory response.** 2020.
15. Bennett JM, Reeves G, Billman GE, *et al.*: **Inflammation–nature's way to efficiently respond to all types of challenges: implications for understanding and managing “the epidemic” of chronic diseases.** *Front. Med. (Lausanne).* 2018; **5**. [Publisher Full Text](#)

16. Harvanová G, Duranková S, Bernasovská J: **The role of cytokines and chemokines in the inflammatory response.** *Alergologia Polska-Polish Journal of Allergology.* 2023; **10**: 210–219.
[Publisher Full Text](#)
17. Cicchella A, Stefanelli C, Massaro M: **Upper respiratory tract infections in sport and the immune system response. A review.** *Biology (Basel).* 2021; **10**: 362.
[Publisher Full Text](#)
18. Paul S, Hmar HKSEB: **Strengthening immunity with immunostimulants: a current trend.** *Pharm. Res.* 2020; **7**.
19. Guarnieri G, Olivieri B, Senna G, et al.: **Relative humidity and its impact on the immune system and infections.** *Int. J. Mol. Sci.* 2023; **24**: 9456.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
20. Tibbs E, Cao X: **Emerging canonical and non-canonical roles of granzyme B in health and disease.** *Cancers (Basel).* 2022; **14**: 1436.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
21. Gleave A, Granville DJ: **Granzyme B in autoimmune skin disease.** *Biomolecules.* 2023; **13**: 388.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
22. You Y, Cheng AC, Wang MS, et al.: **The suppression of apoptosis by α -herpesvirus.** *Cell Death Dis.* 2017; **8**: e2749–e2749.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
23. Shoubaky GAE, Abdel-Daim MM, Mansour MH, et al.: **Isolation and identification of a flavone apigenin from marine red alga *Acanthophora spicifera* with antinociceptive and anti-inflammatory activities.** *J. Exp. Neurosci.* 2016; **10**: JEN-S25096.
[Publisher Full Text](#)
24. Tofighi Z, Molazem M, Doostdar B, et al.: **Antimicrobial activities of three medicinal plants and investigation of flavonoids of *Tripleurospermum disciforme*.** *Iran J. Pharm. Res.* 2015; **14**: 225–231.
[PubMed Abstract](#)
25. Sianturi GLR, Trisnawati EW, Koketsu M, et al.: **Chemical constituents and antioxidant activity of Britton's wild petunia (*Ruellia brittoniana*) flower.** *Biodiversitas.* 2023; **24**: 24.
[Publisher Full Text](#)
26. Dou X, Zhou Z, Ren R, et al.: **Apigenin, flavonoid component isolated from *Gentiana veitchiorum* flower suppresses the oxidative stress through LDLR-LCAT signaling pathway.** *Biomed. Pharmacother.* 2020; **128**: 110298.
[PubMed Abstract](#) | [Publisher Full Text](#)
27. Cai SQ, Tang ZM, Xiong C, et al.: **The anti-inflammatory effects of apigenin and genistein on the rat intestinal epithelial (IEC-6) cells with TNF- α stimulation in response to heat treatment.** *Curr. Res. Food Sci.* 2022; **5**: 918–926.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
28. Al-Khayri JM, Sahana GR, Nagella P, et al.: **Flavonoids as Potential Anti-Inflammatory Molecules: A Review.** *Molecules.* 2022; **27**(9): 2901.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
29. Spicer JA, Huttunen KM, Jose J, et al.: **Small molecule inhibitors of lymphocyte perforin as focused immunosuppressants for infection and autoimmunity.** *J. Med. Chem.* 2022; **65**: 14305–14325.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
30. Wang D, Wan X: **Progress in the study of molecular mechanisms of cell pyroptosis in tumor therapy.** *Int. Immunopharmacol.* 2023; **118**: 110143.
[PubMed Abstract](#) | [Publisher Full Text](#)
31. Cao Y, Zhang J, Wang D, et al.: **Granzyme B secreted by T cells is involved in anti-bacterial immune response of tilapia.** *Fish Shellfish Immunol.* 2024; **153**: 109865.
[PubMed Abstract](#) | [Publisher Full Text](#)
32. Feng YB, Chen L, Chen FX, et al.: **Immunopotential effects of apigenin on NK cell proliferation and killing pancreatic cancer cells.** *Int. J. Immunopathol. Pharmacol.* 2023; **37**: 3946320231161174.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
33. Hou D, Wang D, Ma X, et al.: **Effects of total flavonoids of sea buckthorn (*Hippophae rhamnoides* L.) on cytotoxicity of NK92-MI cells.** *Int. J. Immunopathol. Pharmacol.* 2017; **30**: 353–361.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
34. Dillasamola D, et al.: **Isolation of Apigenin from Sungkai (*Peronema canescens*) Leaves and Its Immunomodulatory Effects: An in vivo Study on Granzyme B, Interferon- γ , and Perforin Expression with Supporting In Silico Analysis.** [Data set]. *Zenodo.* 2025.
[Publisher Full Text](#)

Open Peer Review

Current Peer Review Status:    

Version 1

Reviewer Report 07 October 2025

<https://doi.org/10.5256/f1000research.184242.r411394>

© 2025 Wanyo P. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Pitchaporn Wanyo

Kalasin University, Kalasin, Kalasin, Thailand

The manuscript is scientifically relevant and contributes to the understanding of the immunomodulatory properties of *Peronema canescens* (sungkai) and its flavonoid apigenin. The integration of in silico docking with in vivo immunological evaluation is commendable. However, several areas require clarification, refinement of experimental justification, and improvement in scientific presentation and discussion depth.

1. Abstract

The Abstract should emphasize the novelty of isolating apigenin, integration of in silico-in vivo design, and key quantitative findings.

2. Introduction

2.1 Clarify the research gap—specifically, that apigenin's immunostimulatory role from *Peronema canescens* has not been previously demonstrated.

2.2 Add recent (2023–2025) references about apigenin and flavonoid-based immunomodulators.

2.3 Justify selection of granzyme B, perforin, and IFN- γ as immune markers representing cytotoxic activity.

3. Materials and Methods

3.1 Reduce redundancy in brand and catalog details; focus on replicability.

3.2 Clarify animal randomization, blinding, and justification for sample size ($n=5$).

3.3 Explain rationale for using COVID-19 vaccine as an immune activator in mice, and provide ethical justification.

3.4 Include an apigenin-only control group to assess independent effects.

3.5 Mention toxicity or LD50 data or cite prior literature confirming safety at tested doses.

3.6 Include effect sizes or confidence intervals for ANOVA results.

4. Results

4.1 Summarize spectroscopic data in a single table or composite figure.

4.2 Report RMSD, hydrogen bonds, and binding pocket residues in docking results.

4.3 Improve figure resolution, axis labeling, and statistical annotations.

4.4 Discuss biological relevance of small increases (≈ 5 –20%) in immune markers.

5. Discussion

5.1 Focus more on mechanism—describe apigenin's modulation of JNK/ERK or NF- κ B signaling

pathways.

5.2 Discuss study limitations: lack of interleukin profiling, no apigenin-only control, and untested toxicity.

5.3 Compare results with other flavonoid immunomodulators (quercetin, luteolin).

5.4 Include recent references (2024–2025) to enhance depth.

6. Conclusion

6.1 Rephrase to indicate potential immunostimulatory effects rather than definitive proof.

6.2 Suggest future work: cytokine profiling (IL-2, IL-10), NK cell assays, or mechanistic pathway analysis.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Natural products, bioactive compounds

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 11 Oct 2025

Dwisari Dillasamola

We sincerely thank the reviewer for the positive evaluation and constructive feedback. All suggested revisions have been carefully addressed to enhance the clarity, depth, and overall quality of the manuscript. Detailed responses to each comment are provided below.

1. Abstract

The Abstract should emphasize the novelty of isolating apigenin, integration of in silico–in vivo design, and key quantitative findings.

Response:

The Abstract has been fully rewritten to highlight the novel isolation of apigenin from *Peronema canescens*, the integration of *in silico* molecular docking and *in vivo* immunological assays, and to include key quantitative outcomes (7–20% increases in immune markers). The revised version now better reflects the study's originality, scope, and results.

2. Introduction**2.1 Clarify the research gap—specifically, that apigenin's immunostimulatory role from *Peronema canescens* has not been previously demonstrated.****Response:**

This clarification has been incorporated. The Introduction now explicitly states that although *P. canescens* is traditionally recognized for medicinal properties, its apigenin component has never been scientifically confirmed to exhibit immunostimulatory effects, establishing the study's novelty.

2.2 Add recent (2023–2025) references about apigenin and flavonoid-based immunomodulators.**Response:**

We have added several recent references (2023–2025) to strengthen the contextual foundation, including:

- Allemailem et al., *Biomed J*, 2024
- Kurek-Górecka et al., *Pharm J (Basel)*, 2025
- Mohammadkhanizadeh et al., *Brain Disord*, 2025
- Naponelli et al., *Int J Mol Sci*, 2024
- Hsieh et al., *J Cell Mol Med*, 2024

2.3 Justify selection of granzyme B, perforin, and IFN- γ as immune markers representing cytotoxic activity.**Response:**

Justification has been added to the Introduction. These markers were chosen because they directly represent cytotoxic effector function of NK and CD8⁺ T cells, reflecting the cellular arm of immune activation. This rationale is now clearly stated in the text.

3. Materials and Methods**3.1 Reduce redundancy in brand and catalog details; focus on replicability.****Response:**

Redundant catalog and brand information have been condensed. Only essential details for reproducibility are retained.

3.2 Clarify animal randomization, blinding, and justification for sample size (n=5).**Response:**

Details regarding random allocation of animals, blinding during ELISA measurement, and justification of sample size (n=5) based on prior immunological pilot studies have been added.

3.3 Explain rationale for using COVID-19 vaccine as an immune activator in mice, and provide ethical justification.**Response:**

The use of the COVID-19 vaccine was intended to provide a controlled immune challenge

mimicking a clinically relevant activation of CTL and NK cell responses. Ethical approval and humane treatment statements have been clarified and referenced to institutional guidelines.

3.4 Include an apigenin-only control group to assess independent effects.

Response:

We acknowledge that an apigenin-only control group was not included in this preliminary study. This limitation has been explicitly discussed in the *Discussion* section, and future studies will incorporate apigenin-only and cytokine-profiling groups.

3.5 Mention toxicity or LD₅₀ data or cite prior literature confirming safety at tested doses.

Response:

Toxicity information from previous studies have been cited (Allemailem et al., 2024; El Shoubaky et al., 2016), confirming the safety of the selected dose range. This addition is reflected in the revised Methods and Discussion.

3.6 Include effect sizes or confidence intervals for ANOVA results.

Response:

We have added effect size (η^2) and 95% confidence intervals for all ANOVA tests in the revised Results section to improve statistical transparency.

4. Results

Report RMSD, hydrogen bonds, and binding pocket residues in docking results.

Response:

Docking results were expanded to include RMSD values, hydrogen bond, and binding pocket residues (Table 2) for granzyme B, perforin, and IFN- γ .

Improve figure resolution, axis labeling, and statistical annotations.

Response:

All figures were re-rendered at ≥ 300 dpi. Figure legends were revised for clarity.

Discuss biological relevance of small increases (≈ 5 –20%) in immune markers.

Response:

This aspect has been elaborated in the Discussion. We emphasized that even small increases in granzyme B, perforin, and IFN- γ can substantially enhance CTL and NK function, demonstrating the biological importance of such modest changes.

5. Discussion

5.1 Focus more on mechanism—describe apigenin's modulation of JNK/ERK or NF- κ B signaling pathways.

Response:

The Discussion now provides a detailed mechanistic explanation of apigenin's modulation of MAPK (JNK/ERK) and NF- κ B pathways, supported by recent references.

5.2 Discuss study limitations: lack of interleukin profiling, no apigenin-only control, and untested toxicity.

Response:

The study limitations has been added explicitly addressing these three points, along with recommendations for future studies.

5.3 Compare results with other flavonoid immunomodulators (quercetin, luteolin).

Response:

A comparative discussion with quercetin and luteolin has been added, emphasizing

structural-functional relationships and mechanistic similarities/differences (Oo et al., 2022; Kurek-Górecka et al., 2025).

5.4 Include recent references (2024–2025) to enhance depth.

Response:

Recent studies (2024–2025) have been incorporated to strengthen discussion depth and update mechanistic perspectives.

6. Conclusion

6.1 Rephrase to indicate potential immunostimulatory effects rather than definitive proof.

Response:

The Conclusion has been rewritten to use cautious and scientifically appropriate phrasing, stating that apigenin shows potential immunostimulatory activity rather than definitive proof.

6.2 Suggest future work: cytokine profiling (IL-2, IL-10), NK cell assays, or mechanistic pathway analysis.

Response:

Future research directions now include cytokine profiling, NK-cell functional assays, and pathway validation (MAPK/NF- κ B) to confirm mechanisms inferred from this study.

Competing Interests: The authors declare no competing financial or personal interests that could have influenced the work reported in this manuscript. All experimental design, data collection, analysis, and interpretation were conducted independently and without any external bias.

Reviewer Report 29 September 2025

<https://doi.org/10.5256/f1000research.184242.r411397>

© 2025 Shnawa B. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Bushra Hussain Shnawa

Soran University, Kurdistan, Kurdistan, Iraq

The manuscript is novel, well organized and designed, as well as well written. However, minor comments need to be addressed.

- 1- What about the toxicity of the apigenin, which is classified as a flavonoid, to determine its LD50?
- 2- The authors didn't use another standard immunostimulatory material to compare with it. To determine the levels of the immune stimulation of apigenin in comparison with others.
- 3- The proposed mechanisms of the immunostimulatory potential of apigenin need to be presented in a schematic diagram.
- 4- Why did the authors select granzyme B, perforin, and interferon- γ (IFN- γ)? No interleukin included.

Good luck

.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical biosciences, Immunology, Medical parasitology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 11 Oct 2025

Dwisari Dillasamola

We sincerely thank the reviewer for the positive evaluation and constructive feedback. All suggested revisions have been carefully addressed to enhance the clarity, depth, and overall quality of the manuscript. Detailed responses to each comment are provided below.

What about the toxicity of apigenin, which is classified as a flavonoid, to determine its LD50?

Response:

We appreciate this important observation. Experimental determination of apigenin's LD₅₀ was beyond the scope of this preliminary study; however, we have incorporated relevant toxicity data from recent literature. Previous reports indicate that apigenin has a high safety margin, with Acute oral toxicity studies revealing no mortality or signs of toxicity for the isolated apigenin at doses up to 5000 mg/kg in either mice or rats (El Shoubaky et al., 2016). This information has been added to the *Discussion* (Study Limitations section), and we have acknowledged the need for future in vivo toxicity and safety assessments under our specific experimental conditions.

The authors didn't use another standard immunostimulatory material to compare with it. To determine the levels of the immune stimulation of apigenin in comparison with others.

Response:

We acknowledge this limitation and thank the reviewer for the suggestion. In our *in silico* docking study, levamisole, a known immunostimulant, was used as a reference ligand for granzyme B, perforin, and IFN- γ , providing a baseline for molecular interaction comparison. However, in the *in vivo* experiment, a reference immunostimulant was not included to focus the investigation on apigenin's independent effects. We have now clarified this rationale in the *Methods* and *Discussion* sections, and we note that future studies will incorporate both levamisole and plant-derived comparators (e.g., quercetin or luteolin) for quantitative benchmarking.

Why did the authors select granzyme B, perforin, and interferon- γ (IFN- γ)? No interleukin included.

Response:

We thank the reviewer for this valuable question. Granzyme B, perforin, and IFN- γ were selected because they are key cytotoxic effector molecules directly associated with natural killer (NK) cell and CD8⁺ T-cell activity, which represent the innate and adaptive cellular immune arms of host defense.

Our study aimed to determine whether apigenin could enhance cell-mediated immunity via these effectors. We agree that inclusion of interleukin markers (e.g., IL-2, IL-10, IL-12) would provide a broader immunological profile. This limitation has been explicitly acknowledged in the *Discussion* and *Conclusion*, with a note that future experiments will include cytokine profiling to assess Th1/Th2 balance and helper T-cell responses.

Competing Interests: The authors declare no competing financial or personal interests that could have influenced the work reported in this manuscript. All experimental design, data collection, analysis, and interpretation were conducted independently and without any external bias.

Reviewer Report 22 September 2025

<https://doi.org/10.5256/f1000research.184242.r411398>

© 2025 Mittova V. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Valentina Mittova

University Geomedi, Tbilisi, Tbilisi, Georgia

The article presents interesting results on the isolation of apigenin from plant material and its immunostimulatory effect. However, more substantiation should be given to experiments with the administration of apigenin.

1. Why was the COVID-19 vaccine chosen as an immunostimulator?
2. Why was the control group, which received apigenin and was not vaccinated, not used in the experiments?
3. In Fig. 9 signs like "+" and "-" should be explained.
4. The conclusion section should be rephrased; the statement "apigenin significantly enhanced the expression of key immune effector molecules—granzyme B, perforin, and interferon- γ " is not accurate, since it was revealed only in preliminary vaccinated mice.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antioxidant and antibacterial properties of medicinal plants.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 11 Oct 2025

Dwisari Dillasamola

We sincerely thank the reviewer for the positive evaluation and constructive feedback. All suggested revisions have been carefully addressed to enhance the clarity, depth, and overall quality of the manuscript. Detailed responses to each comment are provided below.

Why was the COVID-19 vaccine chosen as an immunostimulator?

Response:

We thank the reviewer for this insightful question. The COVID-19 vaccine was selected as an immunostimulatory model because it reliably induces a measurable cell-mediated immune response, particularly the activation of cytotoxic T lymphocytes (CTLs) and natural killer (NK)

cells, which are key producers of granzyme B, perforin, and interferon- γ . This allowed us to evaluate whether apigenin could potentiate or modulate vaccine-induced immune activation under physiologically relevant conditions.

Additionally, the vaccine model reflects a clinically relevant immune challenge, enabling translational insights into how apigenin might serve as a potential vaccine adjuvant or immune-supportive compound. These details have been clarified in the revised *Materials and Methods* section.

Why was the control group, which received apigenin and was not vaccinated, not used in the experiments?

Response:

We acknowledge this limitation and have discussed it in the revised *Discussion* (Study Limitations section). The initial design aimed to explore the immunomodulatory role of apigenin in the context of vaccine-induced immune activation, rather than its baseline effects. Hence, a non-vaccinated apigenin-only group was not included in this preliminary study.

In Fig. 9, signs like "+" and "-" should be explained.

Response:

We appreciate this observation.

"-" indicates the negative control group (NaCMC 0.5%).

"+" indicates the positive control group (COVID-19 vaccine only).

The conclusion section should be rephrased; the statement "apigenin significantly enhanced the expression of key immune effector molecules—granzyme B, perforin, and interferon- γ " is not accurate, since it was revealed only in preliminary vaccinated mice.

Response:

We fully agree with this important correction. The Conclusion section has been rewritten to reflect the preliminary and context-specific nature of our findings. The revised statement now reads:

"Preliminary in vivo assays in vaccinated mice indicated dose-dependent increases in the expression of these cytotoxic markers following apigenin administration. Although these findings suggest an immunostimulatory trend, they should be interpreted cautiously as indicative rather than conclusive evidence of efficacy."

This phrasing avoids overstatement and accurately conveys the scope of the experimental findings.

Competing Interests: The authors declare no competing financial or personal interests that could have influenced the work reported in this manuscript. All experimental design, data collection, analysis, and interpretation were conducted independently and without any external bias.

Reviewer Report 16 September 2025

<https://doi.org/10.5256/f1000research.184242.r405596>

© 2025 Kirubakaran D. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Dharmalingam Kirubakaran

Saveetha Institute of Medical and Technical Sciences, Chennai, Tamilnadu, India

The manuscript titled "Isolation of Apigenin from Sungkai (*Peronema canescens*) Leaves and Its Immunomodulatory Effects: An In Vivo Study on Granzyme B, Interferon- γ , and Perforin Expression with Supporting In Silico Analysis" presents a study with strong potential and scientific relevance. However, following a thorough evaluation, I recommend minor revision and after acceptance for publication

1. Revise grammar throughout the manuscript and correct all errors.
2. Strengthen and improve the Discussion section.
3. Rewrite the Abstract for clarity and refine the Conclusion.
4. Ensure all figures are of high quality and appropriately corrected.
5. Overall, the manuscript is good, but it requires the addition of recent references and a thorough grammar check across all sections.
 - We have relevant references that can be cited to improve the Discussion section. (refer to 1,2,3,4)

References

1. Julius A, Renuka R, Malakondaiah S, Ramalingam S, et al.: Radiolabeled nanoparticles in multimodal nuclear imaging, diagnostics and therapy. *Journal of Radioanalytical and Nuclear Chemistry*. 2025; **334** (7): 4403-4418 [Publisher Full Text](#)
2. <https://www.scopus.com/pages/publications/84864012845>.
3. Elangovan M, Santhoshkumar M, Selvaraj K, Sathishkumar K, et al.: Sunlight-driven photocatalytic and anticancer properties of biogenic synthesized gold nanoparticles (AuNPs) employing *Polygala elongata*. *Journal of King Saud University - Science*. 2024; **36** (5). [Publisher Full Text](#)
4. <https://www.scopus.com/pages/publications/77956130065>.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Good data and research

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 11 Oct 2025

Dwisari Dillasamola

We sincerely thank the reviewer for the positive evaluation and constructive feedback. All suggested revisions have been carefully addressed to enhance the clarity, depth, and overall quality of the manuscript. Detailed responses to each comment are provided below.

1. Revise grammar throughout the manuscript and correct all errors.

Response:

We have thoroughly revised the manuscript for grammar, syntax, and language accuracy. All sections were edited for clarity and conciseness, with attention to tense consistency and technical phrasing. The final version has been proofread by a professional language editor.

2. Strengthen and improve the Discussion section.

Response:

The Discussion section has been comprehensively revised to enhance scientific interpretation.

- We added detailed explanations on **apigenin's modulation of JNK/ERK and NF-κB signaling pathways**, as suggested.
- A new comparative discussion with other flavonoid immunomodulators (e.g., **quercetin and luteolin**) has been included to highlight mechanistic similarities and differences.
- The **biological relevance of modest ($\approx 5\text{--}20\%$) increases** in immune markers was elaborated using recent evidence.
- Several **recent references (2024–2025)** were added to support these updates (e.g., Allemailem et al., 2024; Kurek-Górecka et al., 2025; Mohammadkhanizadeh et al., 2025; Hsieh et al., 2024).

3. Rewrite the Abstract for clarity and refine the Conclusion.

Response:

Both the Abstract and Conclusion have been rewritten to improve clarity and logical flow.

- The **Abstract** now emphasizes the novelty of isolating apigenin from *Peronema canescens* and integrates the *in silico-in vivo* design, highlighting key quantitative findings (7–20% increases in immune markers).
- The **Conclusion** has been revised to indicate **potential immunostimulatory effects** rather than definitive claims, aligning with the study's preliminary scope. It now also

suggests directions for **future research** (cytokine profiling, NK cell assays, pathway validation).

4. Ensure all figures are of high quality and appropriately corrected.

Response:

All figures have been re-rendered at high resolution (≥ 300 dpi), with standardized labeling and improved contrast. Figure legends were rewritten for accuracy and clarity. The corresponding raw image data were reviewed to confirm fidelity.

Competing Interests: The authors declare no competing financial or personal interests that could have influenced the work reported in this manuscript. All experimental design, data collection, analysis, and interpretation were conducted independently and without any external bias.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research