



Research article

Investigation of the *in vitro* anti-*Toxoplasma gondii* activity of the citrus-derived flavonoids hesperetin and naringin

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Abstract

Toxoplasma gondii is an important intracellular parasite that poses a substantial public health concern worldwide, particularly among immunocompromised individuals and pregnant women. Pyrimethamine and sulfadiazine are the standard treatments for toxoplasmosis. However, these drugs can cause adverse effects and teratogenicity, potentially leading to treatment failure. These limitations highlight the need to explore new drugs as alternative therapeutic agents. Hesperetin and naringin are citrus-derived flavonoids known to exhibit antiparasitic activity. However, evidence regarding their effects against *T. gondii* remains limited. This study evaluated their cytotoxicity against human foreskin fibroblast cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and assessed their anti-*T. gondii* (RH strain) activity using a plaque assay. Both hesperetin and naringin exhibited low cytotoxicity, with CC₅₀ values exceeding 100 µg/mL. Interestingly, hesperetin completely inhibited *T. gondii* plaque formation at 100 µg/mL, and this effect was comparable to that of the reference drug pyrimethamine. In contrast, naringin showed no inhibitory effect on plaque formation compared with the untreated group. Although hesperetin and naringin belong to the same chemical class, they demonstrated a clear difference in antiparasitic activity. In conclusion, hesperetin derived from citrus plants exhibited anti-*T. gondii* activity with low cytotoxicity *in vitro*. Further studies are required to elucidate its mechanism of action and evaluate its potential therapeutic applications.

Keywords: Flavonoids, Hesperetin, Naringin, Plaque assay, *Toxoplasma gondii*.

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite capable of infecting warm-blooded animals, including humans, and is the causative agent of toxoplasmosis (Sanchez and Besteiro, 2021). Human infection with *T. gondii* typically occurs through the ingestion of oocysts excreted in the feces of infected cats that subsequently contaminate the environment (e.g., water and food) or through the consumption of undercooked meat containing tissue cysts (Shapiro et al., 2019). In immunocompetent individuals, infection is typically asymptomatic or mild and self-limiting, whereas in immunocompromised patients (e.g., those with acquired immunodeficiency syndrome (AIDS) or those receiving immunosuppressive therapy), severe, life-threatening disease may develop (McLeod et al., 2020). In pregnant women, infection can lead to congenital toxoplasmosis, affecting the fetal nervous system and potentially causing developmental abnormalities or fetal death (Walana et al., 2026).

Globally, *T. gondii* is a ubiquitous parasite, and approximately one-third of the world's population is estimated to have been exposed to it (Qiu et al., 2025). A systematic review of 152 studies involving 648,010 individuals reported an average global seroprevalence of 25.7%, with wide regional variation (0.5%–87.7 %). The highest prevalence was observed in Africa, followed by Oceania, South America, Europe, North America, and Asia (Molan et al., 2019). Studies have documented the presence of *T. gondii* in various food animals, including pigs, sheep, and poultry (Guo et al., 2015). In Thailand, *T. gondii* infection has been continuously reported in both humans and animals. A study of HIV-infected patients in southern Thailand found a seroprevalence of approximately 36.3% (Chemoh et al., 2015). Moreover, *T. gondii* infection has been reported in other animals, such as stray cats in the Bangkok metropolitan area, with a prevalence of approximately 4.8% (Jittapalapong et al., 2010). These findings highlight the importance of surveillance and the development of infection-control strategies at the public health level.

Although treatments for toxoplasmosis, such as pyrimethamine in combination with sulfadiazine, are currently available, these therapies have several limitations, including long-term adverse effects such as bone marrow suppression and severe cutaneous reactions, including Stevens–Johnson syndrome and toxic epidermal necrolysis. Moreover, these drugs have limited efficacy against certain stages of the parasite life cycle, particularly bradyzoites, allowing the parasite to persist in the host (Farhab et al., 2025). Furthermore, treatment in certain patient groups often requires prolonged administration, leading to safety concerns and reduced treatment adherence. Therefore, the discovery and development of new active compounds that effectively inhibit the parasite while exhibiting low cytotoxicity toward host cells are considered highly important.

Flavonoids are plant-derived secondary metabolites found in citrus plants and play important roles in determining their color, aroma, and taste. Moreover, flavonoids exhibit a wide range of biological activities, including anti-inflammatory, antioxidant, antiviral, anticancer, antibacterial, and antiparasitic effects, highlighting their potential applications in medicine and pharmacology (Xue et al., 2023; Baeza-Jiménez et al., 2026). Among the flavonoids of interest, naringin and hesperetin are key compounds found in citrus plants. Naringin is commonly present in the fruit and peel of grapefruit and contributes to the bitter taste of citrus juice (Xue et al., 2023). In contrast, hesperetin is found in Chinese medicinal herbs such as chenpi and zhishi, which are key components of traditional Chinese medicine formulations (Ramachandran et al., 2025). Furthermore, studies have reported the antiparasitic potential of both compounds, with hesperetin showing marked inhibitory activity against *Leishmania donovani* (Parente Rocha et al., 2025) and naringin exhibiting activity against *Plasmodium* spp. (Bhatt et al., 2024). However, data regarding their effects against *T. gondii* remain limited.

Therefore, this study aimed to evaluate the cytotoxicity of naringin and hesperetin in human foreskin fibroblast (HFF) cells and to investigate their inhibitory

effects on the growth of *T. gondii* *in vitro*, thereby providing foundational data for the future development of natural antiparasitic agents.

MATERIALS AND METHODS

Cell culture

HFF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% fetal bovine serum (FBS) and 1% penicillin–streptomycin and were maintained in a humidified incubator at 37°C under 5% CO₂.

Parasite culture

Tachyzoites of the *T. gondii* RH strain (Department of Protozoology, Faculty of Tropical Medicine, Mahidol University) were cultured in HFF cells. Parasites were collected following host cell rupture and purified by passage through a 27-gauge needle to disrupt host cells, followed by filtration through a 5.0-µm-pore-size filter to remove cell debris and contaminants before use in subsequent experiments.

Preparation of test compounds

Naringin and hesperetin (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions at a concentration of 10 mg/mL. The stock solutions were subsequently diluted in cell culture medium to a final concentration of 100 µg/mL, with the DMSO concentration maintained at 1%, prior to use in the experiments. For cytotoxicity assessment, the compounds were further serially diluted from 100 µg/mL to 3.13 µg/mL, resulting in a proportional reduction in the DMSO concentration.

Evaluation of cytotoxicity

The cytotoxicity of naringin and hesperetin against HFF cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HFF cells were cultured in 96-well plates at a density of 2×10^4 cells per well and incubated for 24 h, or until a confluent monolayer had formed, at 37°C under 5% CO₂. The cells were then treated with twofold serial dilutions of naringin and hesperetin at concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL to cover a broad concentration range and enable the evaluation of dose-dependent cytotoxic effects. The plates were subsequently incubated for an additional 24 h. After incubation, MTT reagent (Sigma-Aldrich) was added to each well, and the plates were incubated for a further 4 h to allow the formation of formazan crystals. The crystals were then dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader (Azure Biosystems, Dublin, CA, USA). Cell viability was calculated using the following formula: HFF cell viability (%) = $\frac{[\text{absorbance of treated cells}]}{[\text{absorbance of untreated control cells}]} \times 100$ (Mahmood et al., 2023). The percentage of cell viability was plotted against compound concentration to generate dose–response curves, and the 50% cytotoxic concentration (CC₅₀) was determined using nonlinear regression analysis in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Cytotoxicity was evaluated according to the criteria described by Gruber and Nickel (2023), whereby a reduction in cell viability of more than 30% compared with the control was considered cytotoxic (Gruber and Nickel, 2023).

Anti-*T. gondii* activity assay using a plaque assay

HFF cells at a density of 4×10^5 cells/mL were cultured in 12-well plates for 24 h, or until a confluent monolayer had formed, at 37°C under 5% CO₂. Each well was infected with *T. gondii* RH strain tachyzoites at a density of 1×10^3 parasites per well, and the plates were incubated for 4 h to allow parasite invasion. The medium was then removed to eliminate non-invaded parasites, and naringin or hesperetin was added to each well at a concentration of 100 µg/mL. The plates

were further incubated for 24 h under the same conditions. Following treatment, the compounds were removed, and the cells were overlaid with DMEM containing 1.5% carboxymethyl cellulose (CMC) supplemented with 1% FBS and incubated for 4 days. The cells were subsequently stained with naphthol blue-black, and plaques were manually counted by visual inspection to assess parasite growth. The control groups included a positive control (reference drug), consisting of infected HFF cells treated with pyrimethamine (Sigma-Aldrich; 2.49 µg/mL); a negative control, consisting of infected HFF cells without treatment; and a blank control, consisting of uninfected HFF cells cultured in medium alone without exposure to the tested compounds. The percentage of parasite growth inhibition (plaque inhibition percentage) was calculated as follows: $\text{Plaque inhibition (\%)} = (1 - [\text{mean number of plaques in treated wells} / \text{mean number of plaques in infected untreated wells}]) \times 100$ (Ahmadi et al., 2021).

Statistical analysis

All experiments were performed in triplicate, and the results are presented as the mean $\pm$ standard error of the mean (SEM). Differences between experimental groups in the anti-*T. gondii* assay were analyzed using Student's t-test, with statistical significance set at $p < 0.05$. All analyses were performed using GraphPad Prism 8.

RESULTS

Cytotoxicity of hesperetin and naringin against HFF cells

The cytotoxic effects of hesperetin and naringin on HFF cells were evaluated using the MTT assay, a standard method for assessing cell viability. HFF cells were incubated with hesperetin and naringin at concentrations ranging from 3.125 to 100 µg/mL for 24 h. These concentrations were selected to cover a broad range for the evaluation of dose-dependent cytotoxic effects. Absorbance was measured to calculate the percentage of HFF cell viability. The results showed that HFF cell viability remained above 70% at all tested concentrations, indicating that the CC_{50} values of both compounds were greater than 100 µg/mL (Table 1 and Figure 1). Thus, both compounds exhibited low cytotoxicity against HFF cells under the experimental conditions.

Table 1 Viability and optical density of HFF cells treated with serial concentrations of hesperetin and naringin, as determined by the MTT assay.

Treatment	Concentration (µg/mL)	Optical density (mean $\pm$ SEM)	Percentage growth of HFF cells (mean $\pm$ SEM)
Hesperetin	3.125	2.4700 $\pm$ 0.2	104.69 $\pm$ 8.5
	6.25	1.9563 $\pm$ 0.1	82.92 $\pm$ 4.1
	12.5	2.2850 $\pm$ 0.2	96.85 $\pm$ 6.4
	25	2.2063 $\pm$ 0.2	93.52 $\pm$ 10.3
	50	1.9590 $\pm$ 0.3	83.03 $\pm$ 11.0
	100	1.7080 $\pm$ 0.3	72.39 $\pm$ 12.9
Naringin	3.125	2.3870 $\pm$ 0.2	135.52 $\pm$ 12.4
	6.25	2.3257 $\pm$ 0.1	132.04 $\pm$ 8.1
	12.5	2.3633 $\pm$ 0.1	134.18 $\pm$ 3.2
	25	2.3840 $\pm$ 0.1	135.35 $\pm$ 4.8
	50	2.2230 $\pm$ 0.2	126.21 $\pm$ 10.1
	100	2.2463 $\pm$ 0.2	127.54 $\pm$ 9.8
Non-treatment (control group)	N/A	1.7613 $\pm$ 0.2	100 $\pm$ 10.7

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM, standard error of the mean; HFF, human foreskin fibroblast.

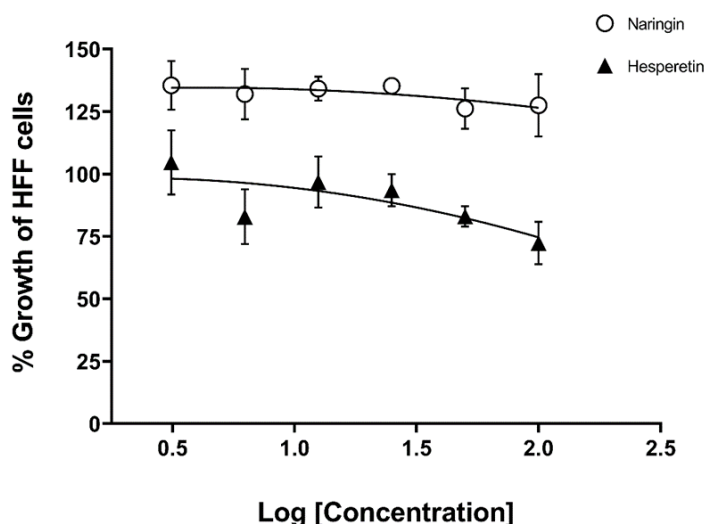


Figure 1 Effect of hesperetin and naringin on HFF cell viability, as determined by the MTT assay. HFF cells were treated with various concentrations of hesperetin and naringin for 24 h. Cell viability is expressed as a percentage relative to that of untreated control cells. Data are presented as the mean $\pm$ SEM. HFF, human foreskin fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM, standard error of the mean.

Anti-*T. gondii* activity in vitro

In the cytotoxicity assay, both compounds exhibited low cytotoxicity against HFF cells. Therefore, they were considered safe for host cells under the experimental conditions and suitable for evaluating anti-*T. gondii* activity without affecting HFF cell viability. Accordingly, the highest tested concentration was selected for the anti-*T. gondii* assay. The anti-*T. gondii* activities of hesperetin and naringin were evaluated using a plaque assay to assess their inhibitory effects on parasite plaque formation in HFF cells. Assessment of the number of *T. gondii* plaques in each group revealed that no plaque formation was detected in the hesperetin- or pyrimethamine-treated groups. In contrast, the naringin-treated group had 127 ± 2.3 plaques, whereas the negative control group had 106 ± 12.1 plaques. The results demonstrated that hesperetin significantly inhibited *T. gondii* plaque formation ($p < 0.001$). At a concentration of 100 $\mu\text{g/mL}$, hesperetin completely inhibited plaque formation in HFF cells, corresponding to 100% inhibition. A similar result was observed with pyrimethamine, which also achieved 100% inhibition. In contrast, naringin at the same concentration (100 $\mu\text{g/mL}$) showed no inhibitory effect on *T. gondii*, exhibiting a pattern similar to that of the negative control group (Table 2). Plaque formation and quantitative comparisons among the treatment groups are presented in Figures 2 and 3, respectively. These findings indicate that, under the experimental conditions, hesperetin exhibited anti-*T. gondii* activity, whereas naringin did not demonstrate inhibitory effects.

Table 2 Number of *Toxoplasma gondii* plaques observed in the plaque assay.

Treatment	Replicate	Number of plaques (plaques)	Plaques (mean $\pm$ SEM)	Plaque inhibition (%)
Hesperetin (100 μg /mL)	1	0	0	100 $\pm$ 0.0
	2	0		
	3	0		
Naringin (100 μg /mL)	1	123	127 $\pm$ 2.3	No inhibitory effect
	2	127		
	3	131		
Pyrimethamine (2.49 μg/mL)	1	0	0	100 $\pm$ 0.0
	2	0		
	3	0		
Non-treatment	1	127	106 $\pm$ 12.1	No inhibitory effect
	2	85		
	3	106		

Abbreviation: SEM, standard error of the mean.

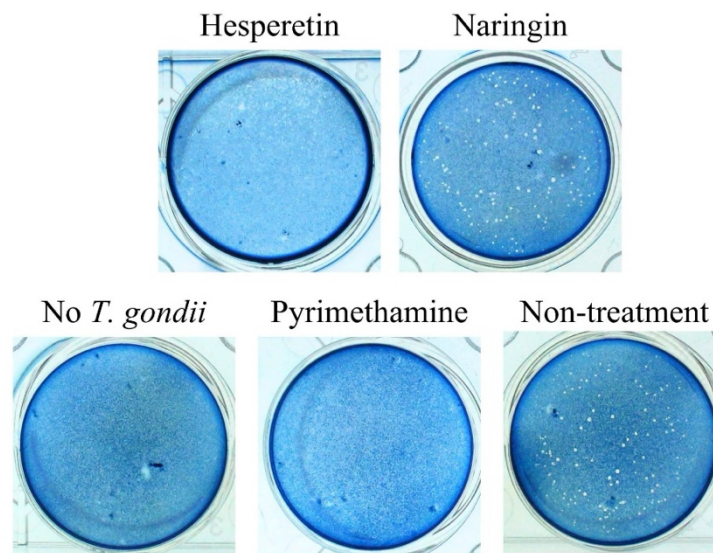


Figure 2 Representative images of *Toxoplasma gondii* plaque formation in HFF cells following treatment with hesperetin (100 μ g/mL), naringin (100 μ g/mL), and pyrimethamine (2.49 μ g/mL). Uninfected HFF cells (no *T. gondii*) were cultured in medium alone without treatment. Infected, untreated HFF cells (infection control) were used as the negative control. HFF, human foreskin fibroblast.

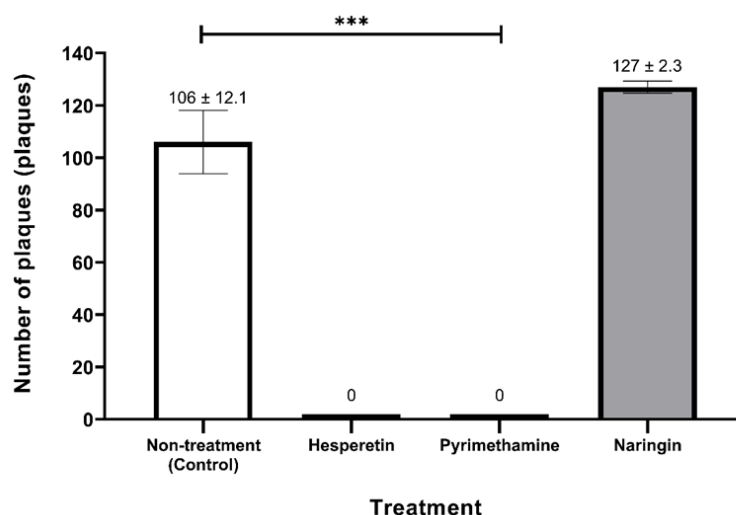


Figure 3 Effects of hesperetin (100 µg/mL), naringin (100 µg/mL), and pyrimethamine (2.49 µg/mL) on *Toxoplasma gondii* plaque formation in HFF cells. No plaque formation was observed in the hesperetin- or pyrimethamine-treated groups, whereas the naringin-treated group showed 127 ± 2.3 plaques and the untreated group showed 106 ± 12.1 plaques. Data are presented as the mean $\pm$ SEM. Statistical significance was determined at $p < 0.001$. HFF, human foreskin fibroblast; SEM, standard error of the mean.

DISCUSSION

In this study, the cytotoxicity of hesperetin and naringin against HFF cells was evaluated using the MTT assay. The results demonstrated that both compounds exhibited low cytotoxicity, with CC_{50} values greater than 100 µg/mL, indicating their safety toward HFF cells within the tested concentration range. Notably, naringin maintained a high level of cell viability even at the highest tested concentration (100 µg/mL), suggesting that it did not adversely affect cell survival. In a previous study, hesperetin and naringin exhibited low cytotoxicity against Vero cells ($CC_{50} = 110.3 \pm 0.32$ µg/mL and 230.3 ± 0.19 µg/mL, respectively) using the MTT assay over a 96-h period (Zandi et al., 2011), and these findings are consistent with our results. In addition, a previous study reported that treatment of HFF cells with 1% DMSO did not induce cytotoxicity, as cell viability remained comparable to that of the control group (Namięcińska et al., 2019). However, CC_{50} values could not be precisely determined in the present study because of limitations in the maximum achievable concentrations of the test compounds. Higher concentrations would have required DMSO levels exceeding 1%, which could potentially affect cell viability. In the anti-*T. gondii* assay, hesperetin completely inhibited plaque formation at a concentration of 100 µg/mL, and this effect was comparable to that observed with pyrimethamine, the reference drug. In contrast, naringin did not exhibit inhibitory activity, as the number of plaques observed was similar to that in the untreated control group. These findings indicate a clear difference in anti-*T. gondii* activity between hesperetin and naringin, despite both compounds belonging to the flavonoid class. Hesperetin has been reported to exhibit significant antiparasitic activity against *Leishmania donovani*, strongly inhibiting the promastigote stage and targeting key enzymes essential for parasite survival, including acetylcholinesterase, sterol C-24 reductase and N-myristoyltransferase (Parente Rocha et al., 2025). These effects may impair parasite growth and highlight the potential of hesperetin as a promising antiparasitic candidate. However, current evidence remains largely limited to *L. donovani*, and the underlying mechanisms of action have not yet been elucidated in *T. gondii*.

Although our study provides evidence supporting the activity of hesperetin against *T. gondii*, it is important to note that the findings are based solely on *in vitro* experiments and lack detailed mechanistic validation. Therefore, further studies, including comprehensive mechanistic investigations and *in vivo* evaluations, are necessary to confirm the efficacy and safety of hesperetin before its potential clinical application. Conversely, although naringin did not exhibit inhibitory activity against *T. gondii* in our study, it has been reported to demonstrate antiparasitic effects in other systems, including antimalarial activity, particularly when used in combination with the standard drug chloroquine (Bhatt et al., 2024). This observation suggests that naringin may have potential as an adjunctive agent rather than as a standalone therapy. The difference in activity between hesperetin and naringin may be attributable to their structural differences. Hesperetin, an aglycone, is more readily absorbed and can penetrate cell membranes efficiently, whereas naringin, a glycoside, requires microbial hydrolysis before absorption, resulting in slower and less efficient bioavailability (Baeza-Jiménez et al., 2026). This characteristic may limit its ability to access intracellular parasites such as *T. gondii* under *in vitro* conditions (Manach et al., 2005; Zhang et al., 2020). However, the relevance of this mechanism to *T. gondii* infection has not been clearly established and requires further investigation. Although the findings of this study demonstrate the potential of hesperetin as an anti-*T. gondii* agent, an important limitation should be acknowledged. The compound was evaluated at only a single concentration (100 µg/mL), preventing assessment of a dose–response relationship. Further investigations, particularly dose–response studies of *T. gondii* inhibition and detailed analyses of the underlying molecular mechanisms, are required to comprehensively validate the potential of hesperetin for the development of effective anti-*T. gondii* therapies.

CONCLUSIONS

This study demonstrated the *in vitro* anti-*T. gondii* activity of citrus-derived flavonoids. Hesperetin exhibited strong inhibitory effects against *T. gondii*, whereas naringin showed no significant activity under the same experimental conditions. Both compounds displayed low cytotoxicity toward HFF cells, indicating a favorable safety profile. Although hesperetin and naringin belong to the same flavonoid class, they exhibited markedly different antiparasitic activities. These findings suggest that hesperetin may represent a promising candidate for further investigation as a potential anti-*T. gondii* agent.

AUTHOR CONTRIBUTIONS

Preeyakamon Junpor: Conceptualization (lead); Methodology (lead); Investigation (lead); Data curation (lead); Writing – original draft (lead).

Tachin Khulmanee: Investigation (supporting); Formal analysis (supporting).

Ruenruetai Udonsom: Investigation (supporting); Project administration (supporting).

Supaluk Popruk: Writing – review & editing (supporting).

Aongart Mahittikorn: Writing – review & editing (supporting); Language editing (supporting).

Oranit Deesithivech: Formal analysis (supporting).

Onrapak Reamtong: Resources (supporting); Materials provision (supporting).

Tipparat Thiangtrongjit: Resources (supporting); Materials provision (supporting).

Khuanchai Koompapong: Conceptualization (lead); Methodology (lead); Investigation (lead); Writing – review & editing (lead).

Kruawan Chotelersak: Supervision (lead); Project administration (lead); Funding acquisition (lead).

CONFLICT OF INTEREST

There is no conflict of interest with the publication of this article. The authors declare that Artificial Intelligence (AI) tools, specifically ChatGPT by OpenAI, were used for translating text between languages and improving grammar, clarity, and readability. The authors confirm that all outputs were critically reviewed and that they take full responsibility for the integrity and accuracy of the content.

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