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Isolation of Coumarins, Polymethoxylated Flavonoids, and Triterpenoids from *Citrus grandis* (Tubtim Siam Cultivar) Leaves and Evaluation of Their Biological Activities

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ABSTRACT: *Citrus grandis* cv. Tubtim Siam is an economically important pomelo cultivar in Thailand. Although the phytochemical constituents of its fruit tissues have received attention, information regarding the chemical composition and biological properties of its leaves remains limited. As the leaves represent a potential source of bioactive secondary metabolites, the present study aimed to isolate and identify the major constituents of *C. grandis* leaves and evaluate their biological activities. Phytochemical investigation of the leaves resulted in the isolation of six compounds: two coumarins, 7,8-dimethoxycoumarin (2) and 6,7-dimethoxycoumarin (3); two polymethoxylated flavonoids, tangeretin (4) and nobiletin (5); and two triterpenoids, friedelin (1) and limonin (6). Their structures were elucidated by NMR spectroscopic analysis and comparison with published data. The extract and selected compounds were evaluated for their antioxidant, antibacterial, and antiproliferative activities. Among the tested samples, tangeretin exhibited the strongest antioxidant activity (IC_{50} = 1.86 ± 0.42 µg/mL), while the crude extract also demonstrated notable radical scavenging activity (2.30 ± 0.08 µg/mL), suggesting that multiple constituents may collectively contribute to the observed antioxidant effect. In contrast, all samples exhibited weak antibacterial and antiproliferative activities under the experimental conditions. The results indicate that the biological relevance of *C. grandis* leaves is primarily associated with their antioxidant properties rather than antibacterial or antiproliferative effects. The phytochemical profile further suggests tissue-specific distribution of secondary metabolites in citrus species and provides chemotaxonomic insights into this cultivar. Overall, this study expands current knowledge of the phytochemical constituents of *C. grandis* leaves and supports their potential as a natural source of antioxidant agents.

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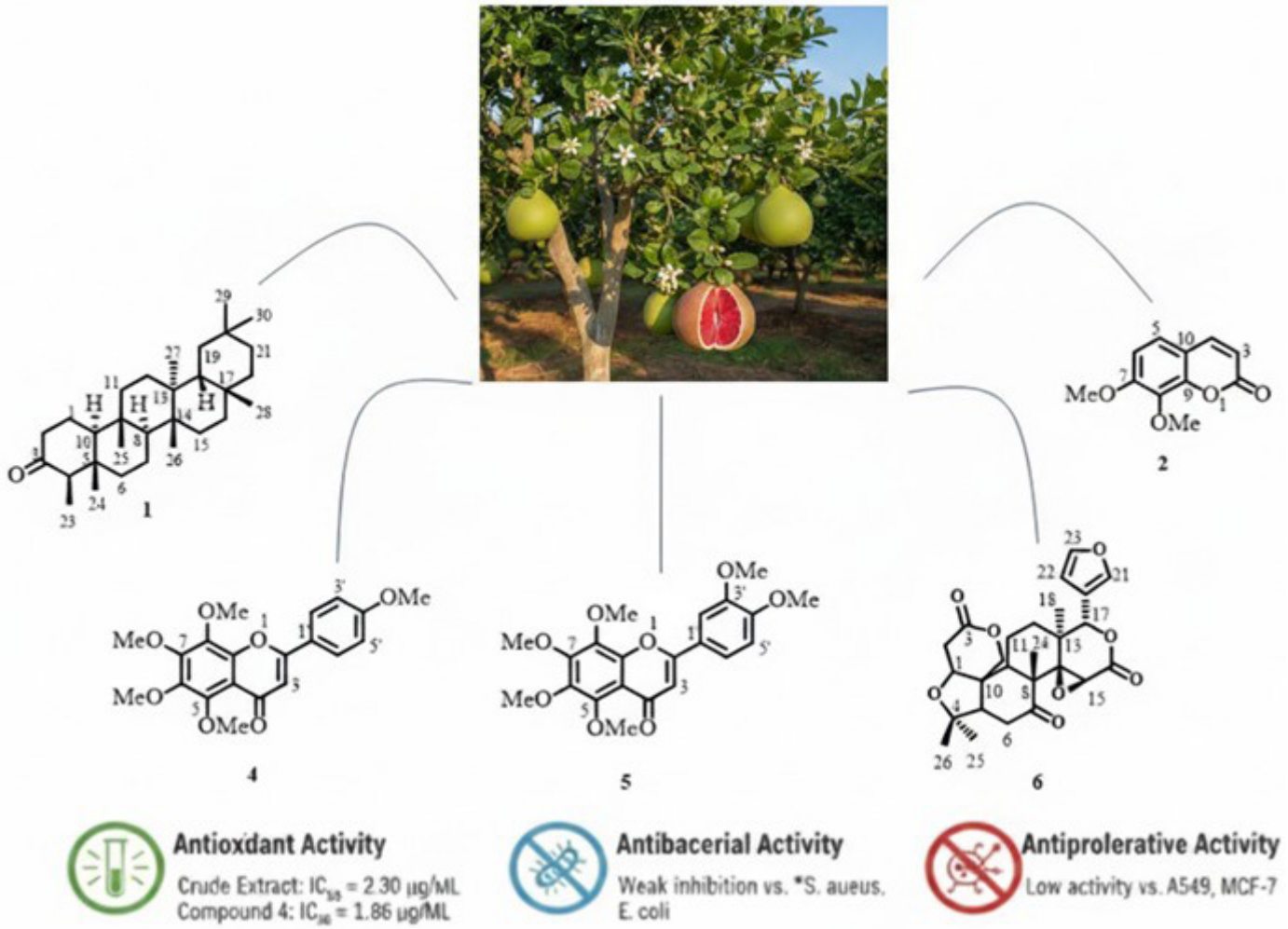
GRAPHICAL ABSTRACT



1. INTRODUCTION

Despite significant advances in modern medicine, oxidative stress and microbial infections remain major health concerns worldwide. Oxidative stress occurs when the production of reactive oxygen species (ROS) surpasses the body's capacity to neutralize them, leading to cellular damage and biomolecular disruption (Minami et al., 2025). This redox imbalance has been widely associated with the pathogenesis of numerous chronic disorders, including cancer, cardiovascular and metabolic diseases, diabetes, neurodegenerative disorders, and other age-related illnesses. In addition, infections caused by pathogenic microorganisms remain a global concern and are increasingly associated with antimicrobial resistance. These challenges have stimulated growing interest in the isolation of natural products with antioxidant and antimicrobial properties for potential applications in pharmaceutical, cosmetic, and nutraceutical applications.

Plants are a rich source of natural compounds with diverse biological activities. They have long been used in traditional medicine and modern drug development (Chaachouay & Zidane, 2024; Elshafie et al., 2023; Nasim et al., 2022). The therapeutic potential of plants is largely attributed to the production of structurally diverse secondary metabolites. Flavonoids, coumarins, alkaloids, and terpenoids are among the most extensively studied classes of phytochemicals because of their wide range of biological activities, including antioxidant, antityrosinase, antimicrobial, anti-inflammatory, and cytotoxic effects (Dias et al., 2021; Yang et al., 2024). Therefore, phytochemical investigations of medicinal and edible plants remain an important approach for identifying biologically active compounds and exploring their potential health benefits.

The genus *Citrus* is well known for its rich phytochemical diversity and has been extensively investigated as a plant-derived source of bioactive metabolites with potential health-promoting properties (Pasdaran et al., 2023;

Šafranko et al., 2023; Saini et al., 2022). Species within this genus contain a wide range of structurally diverse secondary metabolites, including coumarins, flavonoids, triterpenoids, benzenoids, steroids, lignans, amides, and alkaloids (Alam et al., 2022; Bhowal et al., 2022; Ngolong et al., 2024). Previous phytochemical studies of *Citrus grandis* have revealed a tissue-specific distribution of bioactive secondary metabolites among different plant organs. For example, coumarins and alkaloids have been isolated from the root and stem bark (Tian-Shung et al., 1988, 1994), whereas several hepatoprotective coumarin derivatives have been reported from the fruit pericarp (Tian et al., 2019). Glycosidic compounds such as meranzin hydrate and roseoside derivatives have also been isolated from the peels (Feng et al., 2001). More recently, compounds such as zeylenol and limonin, isolated from the stem bark, have been reported to exhibit antioxidant, antimicrobial, and cytotoxic properties (Sakib et al., 2023). Previous studies have demonstrated that the peel extract of *C. grandis* contains coumarins and flavonoids with notable antioxidant and antityrosinase activities (Phetkul et al., 2024). Furthermore, phytochemical screening and bioactivity evaluation revealed that both leaf and peel extracts of *C. grandis* are rich in bioactive constituents and possess considerable antioxidant potential, with the ethanolic peel extract exhibiting the highest activity (Phetkul et al., 2025). However, the specific secondary metabolites responsible for the biological activities of the leaf extracts have not yet been fully characterized. Therefore, further phytochemical investigation of *C. grandis* leaves is warranted.

Despite extensive phytochemical studies on various parts of *C. grandis*, the leaves, particularly those of the Tubtim Siam cultivar, remain largely unexplored. In many agricultural systems, these leaves are often discarded as by-products despite their potential as a source of bioactive phytochemicals. Therefore, the present study aimed to investigate the phytochemical constituents of *C. grandis* cv. Tubtim Siam leaves collected in Thailand through the isolation and structural characterization of secondary metabolites, together with the evaluation of their antioxidant, antibacterial, and antiproliferative activities. To the best of our knowledge, this is the first report describing the phytochemical constituents isolated from the leaves of *C. grandis* cv. Tubtim Siam. These findings expand current knowledge of the phytochemical composition of this underexplored plant material and highlight its potential as a natural source of antioxidant agents.

2. MATERIALS AND METHODS

2.1. General experimental procedures

Silica gel-based chromatographic techniques were employed for compound isolation, using silica gel 60 GF254 (Merck) for quick column chromatography (QCC) and silica gel 100 (70–230 mesh ASTM, Merck) for conventional column chromatography (CC. Thin-layer chromatography analyses were conducted on silica gel 60 F254 plates (Merck). All solvents used for extraction and purification were distilled prior to use. Structural characterization of the isolated metabolites was performed using one- and two-dimensional NMR spectroscopy on Bruker Ultra Shield™ 500 MHz and Varian Unity Inova 500 MHz spectrometers, with chemical shifts referenced to tetramethylsilane (TMS).

2.2. Plant material

Leaves of *C. grandis* cv. Tubtim Siam were collected in November 2022 from cultivated plants maintained for educational and training purposes at Rajamangala University of Technology Srivijaya, Thailand (Phetkul et al., 2025). These plants exhibited characteristic morphological features, including the stem, leaves, flower, and fruit flesh, as shown in Figure 1.

2.3. Extraction and isolation

Air-dried leaves of *Citrus grandis* cv. Tubtim Siam (2.0 kg) were extracted with acetone by maceration at room temperature for 7 days. The solvent was evaporated under reduced pressure to afford a dark brown crude extract (39.88 g). The extract was subsequently fractionated by QCC over silica gel 60 using a gradient elution system of hexane, hexane–acetone, acetone, acetone–methanol, and methanol, yielding ten fractions (A–J). Fraction B (1.8745 g) underwent further purification by column chromatography (CC) using a hexane:CH_2Cl_2 (9:1, v/v) solvent system, resulting in nine subfractions (B1–B9). Subfraction B3 (145.3 mg) was subsequently subjected to CC using hexane:CH_2Cl_2:EtOAc (7:2:1, v/v) mixture, yielding compound **1** as a white solid (6.7 mg). Fraction D (1.3124 g) was further fractionated by CC using a hexane:CH_2Cl_2:Me_2CO (8:1:1, v/v) solvent system, yielding eight subfractions (D1–D8). Subfraction D4 (109.8 mg) was further purified by CC using hexane:CH_2Cl_2:Me_2CO (7:2:1, v/v), affording compound **2** as a yellow viscous oil (8.7 mg). Subfraction D6 (198.6 mg) was further fractionated by CC using hexane:Me_2CO (7:3, v/v), yielding nine subfractions (D6A–D6I). Subfraction D6A (48.1 mg) was subsequently purified by CC using hexane:Me_2CO (7:3, v/v) to afford compound **3** as a yellow solid (1.2 mg). Fraction E (1.0022 g) was subjected to CC using hexane:Me_2CO (7:3, v/v), yielding nine subfractions (E1–E9). Subfraction E3 (140.1 mg) was further fractionated by CC using hexane:Me_2CO (7:3, v/v),

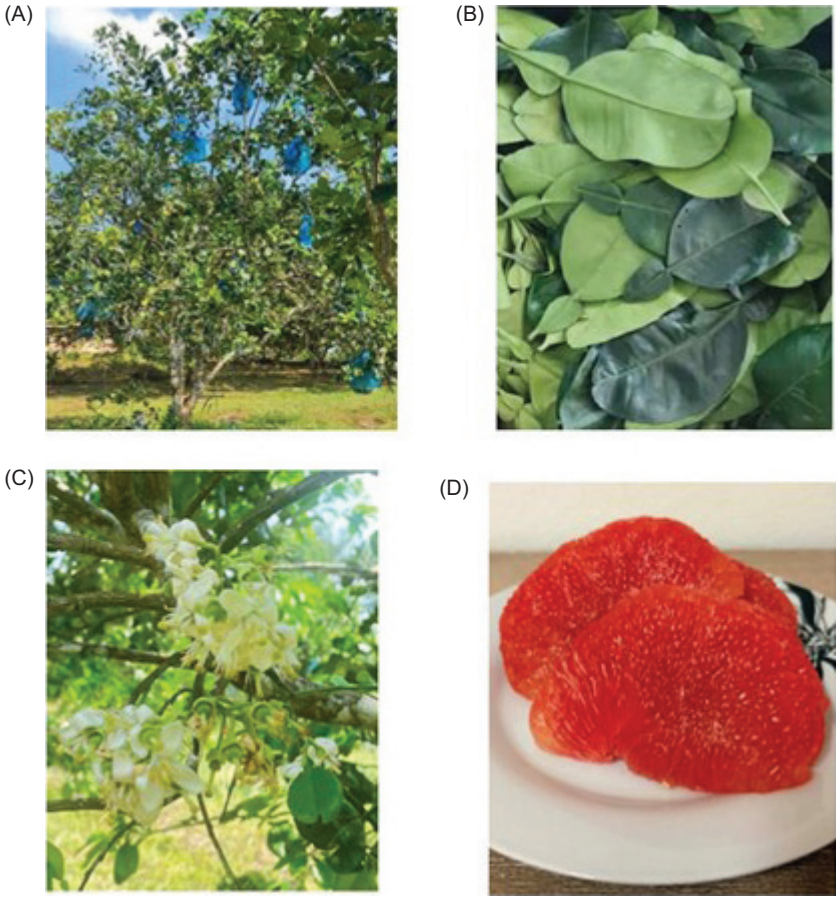


Figure 1. Morphological characteristics of *C. grandis* cultivar "Tubtim-Siam". (A) stem (B) Leaves, (C) flower, and (D) fruit flesh.

yielding six subfractions (E3A–E6F). Subfraction E3C was further purified by CC using hexane:CH_2Cl_2:EtOAc to afford compound **4** as a yellow solid (5.9 mg). Fraction F (1.5655 g) was fractionated by CC using a hexane:CH_2Cl_2:Me_2CO (6:2:2, v/v) solvent system, yielding 13 subfractions (F1–F13). Subfraction F5 (106.6 mg) was further purified by CC using hexane:Me_2CO (6:4, v/v) to afford compound **5** as a yellow solid (4.3 mg). Finally, fraction G (3.3852 g) was subjected to CC using hexane:CH_2Cl_2:Me_2CO (7:2:1, v/v), yielding 11 fractions (G1–G11). Subfraction G4 (278.9 mg) was further separated by CC using the same solvent system (7:2:1, v/v), affording 8 subfractions (G4A–G4H). Subfraction G4C (63.5 mg) was purified by CC using hexane: CH_2Cl_2:EtOAc (6:2:2, v/v), affording compound **6** as a yellow solid (13.8 mg).

Friedelin (**1**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 1.70 (*m*, H-1α), 1.96 (*m*, H-1β) , 2.28 (*ddd*, H-2α), 2.38 (*ddd*, H-2β), 2.25 (*q*, H-4), 1.27 (*m*, H-6α), 1.74 (*m*, H-6β), 1.38 (*m*, H-7α) 1.49 (*m*, H-7β), 1.40 (*m*, H-8), 1.53 (*m*, H-10), 1.25 (*m*, H-11α), 1.46 (*m*, H-11β), 1.35 (*m*, H-12), 1.23 (*m*, H-15α), 1.51 (*m*, H-15β), 1.36, (*m*, H-16α), 1.57 (*m*, H-16β), 1.56 (*m*, H-18), 1.23 (*m*, H-19α), 1.38 (*m*, H-19β), 1.32 (*m*, H-21α), 1.51 (*m*, H-21β), 0.95 (*m*, H-22α), 1.50 (*m*, H-22β), 0.88 (*s*, H-23), 0.72 (*s*, H-24), 0.87 (*s*, H-25), 1.00 (*s*, H-26), 1.04 (*s*, H-27), 1.18 (*s*, H-28), 0.95 (*s*, H-29), 0.99 (*s*, H-30)

7,8-Dimethoxycoumarin (**2**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 6.26 (*d*, 9.0, H-3), 7.65 (*d*, 9.0, H-4), 7.18 (*d*, 8.5, H-5), 6.86 (*d*, 8.5, H-6), 3.95 (s, 7-OMe), 3.98 (*s*, 8-OMe)

6,7-Dimethoxycoumarin (**3**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 6.29 (*d*, 9.0, H-3), 7.62 (*d*, 9.0, H-4), 6.86 (*s*, H-5), 6.85 (*s*, H-8), 3.98 (*s*, 6-OMe), 3.95 (*s*, 7-OMe)

Tangeretin (**4**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 6.61 (*s*, H-3), 7.01 (*d*, 9.0, H-3›/H-5›), 7.88 (*d*, 9.0, H-2›/ H-6›), 4.02 (*s*,7-OMe), 4.11 (*s*, 8-OMe), 3.88 (*s*, 5›-OMe), 3.95 (*s*, 6-OMe/3›-OMe)

Nobiletin (**5**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 6.65 (*s*, H-3), 7.40 (*d*, 2.5, H-2›), 6.98 (*d*, 9.0, H-5›), 7.56 (*dd*, 9.0, 2.5, H-6›), 4.09 (*s*, 5-OMe), 3.95 (*s*, 6-OMe), 4.01 (*s*, 7-OMe), 3.94 (*s*, 8-OMe), 3.96 (*s*, 3›-OMe), 3.94 (*s*, 4›-OMe)

Limonin(**6**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 4.04 (*brs*, H-1), 2.23 (*dd*, 16.3, 3.0, H-2α), 2.68 (*dd*, 16.3, 3.0, H-2β), 2.46 (*dd*, 16.0, 2.8, H-5), 2.98 (*dd*, 16.0, 2.8, H-6α), 2.86 (*t*, 16.0, H-6β), 2.55 (*dd*, 12.0, 2.0, H-9), 1.90 (*m*, H-11α), 1.80 (*m*, H-11β), 1.51 (*m*, H-12α), 1.77 (*m*, H-12β), 4.04 (*s*, H-15), 5.45 (*s*, H-17), 1.17 (*s*, H-18), 4.46 (*d*, 12.0, H-19α), 4.76 (*d*, 12.0, H-19β), 7.40, (*s*, H-21), 6.34 (*brs*, H-22), 7.42 (*brs*, H-23), 1.08 (*s*, H-24), 1.29 (*s*, H-25), 1.16 (s, H-26)

2.4. Evaluation of antioxidant activity of pure compounds

To assess the antioxidant capacity of the *C. grandis* cultivar "Tubtim-Siam" extract and its purified components, a slightly modified version of the DPPH radical scavenging method described by Sae-Lim et al (2019) was employed. In this assay, 0.003 g of the extract or isolated compound was dissolved in 100 mL of 95% methanol, and 20 µL of the resulting solution was added to 180 µL of 0.1 mM DPPH solution in a 96-well microplate. The reaction mixtures were incubated in the dark at room temperature for 30 minutes to ensure completion of the reaction. Trolox was used as the positive control, while methanol served as the blank. After incubation, absorbance was measured at 517 nm using an EnSpire® microplate reader.

The percentage of DPPH radical scavenging activity was determined using the following formula:

$$\text{DPPH scavenging activity}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

$A_{control}$ and A_{sample} represent the absorbance values of the control and test sample, respectively. All measurements were conducted in triplicate.

2.5. Evaluation of antibacterial activity of pure compounds

The antibacterial activity of the leaf extract and isolated compounds from *C. grandis* cultivar "Tubtim Siam" was evaluated against two reference bacterial strains: *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The investigation was conducted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2021), with slight modifications. A 96-well broth microdilution method using twofold serial dilutions was employed to determine the minimum inhibitory concentration (MIC). Bacterial suspensions were standardized to an approximate density of 1×10^6 CFU/mL and combined with 100 µL of the test samples to obtain final concentrations ranging from 5 to 80 µg/mL. The microplates were then incubated at 37 °C for 18 hours. The MIC was defined as the lowest concentration at which no visible bacterial growth was observed, as previously described (Syukri et al., 2020). To determine the minimum bactericidal concentration (MBC), aliquots from wells lacking turbidity were streaked onto fresh agar plates. The MBC was defined as the lowest concentration that completely prevented bacterial colony formation after incubation.

2.6. Cell culture conditions and *in vitro* cytotoxicity testing

RPMI-1640 medium was used for culturing the MCF-7 human breast cancer cell line, whereas the A549 human lung cancer cell line was cultured in DMEM. Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, with the culture media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Subculturing was performed when the cells reached 80–90% confluence using 0.25% trypsin–EDTA for cell detachment. For cytotoxicity analysis, cells were seeded into 96-well plates at a density of 1×10^4 cells per well and allowed to attach during a 24-hour incubation period. Subsequently, the cells were treated with varying concentrations of the extract and selected isolated compounds (ranging from 0 to 500 µg/mL) for 72 hours. Cell viability was evaluated using the MTT assay. Briefly, MTT solution (0.5 mg/mL) was added to each well, followed by incubation at 37 °C under 5% CO_2 for 1 hour. The resulting formazan crystals were dissolved in DMSO and incubated at room temperature for 30 minutes. Absorbance was measured at 570 nm, with 650 nm as the reference wavelength, using a microplate reader. All experiments were performed in triplicate, and IC_{50} values were determined from dose–response curves using an online calculator, as described by Maungchanburi et al. (2022).

2.7. Statistical analysis

All experiments were conducted in triplicate (n = 3), and the results are expressed as mean ± SD. One-way analysis of variance (ANOVA) was performed for statistical evaluation, followed by Duncan's multiple range test to determine significant differences, with statistical significance set at $p < 0.05$.

3. RESULTS AND DISCUSSIONS

3.1. Spectroscopic analysis

Chromatographic separation of the acetone extract obtained from the leaves of *Citrus grandis* cv. Tubtim Siam

afforded six secondary metabolites (**1**–**6**) (Figure 2). Structural identification was accomplished through interpretation of 1D and 2D NMR spectroscopic data, together with comparison of the obtained data with those reported in the literature. The compounds were identified as friedelin (**1**), 7,8-dimethoxycoumarin (**2**), 6,7-dimethoxycoumarin (**3**), tangeretin (**4**), nobiletin (**5**), and limonin (**6**). The crude extract and isolated constituents were subsequently evaluated for their antioxidant, antibacterial, and antiproliferative activities.

Isolation of compound **1** yielded a white solid. The ^{1}H NMR spectrum displayed eight methyl signals at δ 0.88 (s, H-23), 0.72 (s, H-24), 0.87 (s, H-25), 1.00 (s, H-26), 1.04 (s, H-27), 1.18 (s, H-28), 0.95 (s, H-29), and 0.99 (s, H-30), which are characteristic of protolimonoid-type triterpenoids (Figure 2). The signals observed in the δ 1.20–2.38 range were attributed to methylene and methine protons. The ^{13}C NMR spectrum revealed a carbonyl carbon signal at δ 213.5 (C-3), together with four methine carbon signals at δ 59.6 (C-5), 58.4 (C-9), 53.3 (C-8), and 43.2 (C-17). The DEPT-135 spectrum revealed eleven methylene carbons at δ 41.6 (C-22), 41.3 (C-24), 39.3 (C-1), 36.1 (C-12), 35.8 (C-2), 35.4 (C-15), 32.7 (C-23), 32.4 (C-16), 30.6 (C-7), 22.3 (C-6), and 18.3 (C-11). Several quaternary carbons were also observed at δ 42.3, 39.9, 38.5, 37.6, 32.6, and 30.2. Detailed interpretation of the 1D and 2D NMR spectra allowed the structure of compound **1** to be elucidated. The NMR results were compared with previously published data for friedelin (Mujawah et al., 2023).

Compounds **2** and **3** were identified as methoxylated coumarin derivatives based on comprehensive spectroscopic analysis and comparison with reported literature data. Compound **2** was obtained as a yellow solid. The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) showed characteristic signals of an α, β-unsaturated coumarin system, including signals at δ 6.26 (*d*, 9.0, H-3) and δ 7.65 (*d*, 9.0, H-4) (Figure 2). In addition, two ortho-coupled aromatic proton signals appeared at δ 7.18 (*d*, 8.5, H-5) and δ 6.86 (*d*, 8.5, H-6), along with two methoxy signals at δ 3.95 (7-OMe) and δ 3.98 (8-OMe). The spectroscopic data were consistent with previously reported data, indicating that compound **2** was 7,8-dimethoxycoumarin (Shimomura et al., 1979). Compound **3** showed spectroscopic features similar to those of compound **2**, particularly the characteristic signals of the α, β-unsaturated coumarin nucleus at δ 6.26 (H-3) and δ 7.62 (H-4). However, the aromatic proton pattern differed from that of compound **2**. Instead of the ortho-coupled aromatic proton signals observed in compound **2**, compound **3** displayed two aromatic singlets at δ 6.86 (H-5) and δ 6.85 (H-8), indicating a different methoxy substitution pattern on the benzene ring. Together with the two methoxy signals at δ 3.95 and 3.98, these data were consistent with scoparone (6,7-dimethoxycoumarin) (Ma et al., 2006). The presence of compounds 2 and 3 is consistent with previous studies on the genus *Citrus*, in which coumarins have frequently been reported as characteristic secondary metabolites (Russo et al., 2021). These compounds are considered valuable chemotaxonomic markers within the genus due to their characteristic distribution patterns. Furthermore, the identification of these compounds supports earlier reports describing coumarins as important contributors to the biological activities of citrus-derived extracts. The presence of 7,8-dimethoxycoumarin and scoparone in the investigated extract may therefore contribute, at least in part, to the observed bioactivities and further highlights the potential of *Citrus* species as promising sources of biologically active natural products.

Compounds **4** and **5** were identified as polymethoxylated flavonoids (PMFs), namely tangeretin and nobiletin, respectively. The spectroscopic characteristics of both compounds, including multiple methoxy signals and characteristic flavone proton patterns, were consistent with previously reported data (Han et al., 2010). Isolation of compound **4** yielded a yellow solid. The ^{1}H NMR spectrum (Figure 2) exhibited signals corresponding to a para-substituted B ring at δ 7.88 (*d*, 9.0 Hz, H-2′/H-6′) and δ 7.01 (*d*, 9.0 Hz, H-3′/H-5′). In addition, four methoxy signals corresponding to five methoxy groups were observed at δ 4.02 (*s*, 7-OMe), 4.11 (*s*, 8-OMe), 3.88 (*s*, 5′-OMe), and 3.95 (*s*, 6-OMe/3′-OMe). A singlet at δ 6.61 was assigned to H-3 of the flavone skeleton. These spectroscopic data were consistent with those reported for tangeretin (5,6,7,8,4′-pentamethoxyflavone). Compound **5** was isolated as a yellow solid. Its spectroscopic features were generally similar to those of compound **4**, particularly the presence of multiple methoxy groups and the characteristic flavone proton signal at δ 6.65 (*s*, H-3). However, unlike compound **4**, which exhibited a para-substituted B-ring pattern, compound **5** showed an ABX aromatic system corresponding to H-2′ (δ 7.40, *d*, 2.5 Hz), H-5′ (δ 6.98, *d*, 9.0 Hz), and H-6′ (δ 7.56, *dd*, 9.0, 2.5 Hz), indicating a different substitution pattern on the B ring. In addition, six methoxy signals were observed, suggesting a higher degree of methoxylation than that of compound **4**. Comparison with previously reported spectroscopic data identified compound **5** as nobiletin (Han et al., 2010). PMFs are major secondary metabolites commonly found in species of the genus *Citrus* and have been extensively studied due to their broad range of biological activities (Fontana et al., 2022; Wang et al., 2018). The identification of tangeretin and nobiletin in the leaves of the "Tubtim Siam" cultivar further supports the abundance of PMFs in *Citrus* species and suggests that citrus leaves may serve as a valuable source of bioactive flavonoids.

Compound **6** was obtained as a yellow solid and identified as a limonoid derivative. The ^{13}C NMR spectrum showed

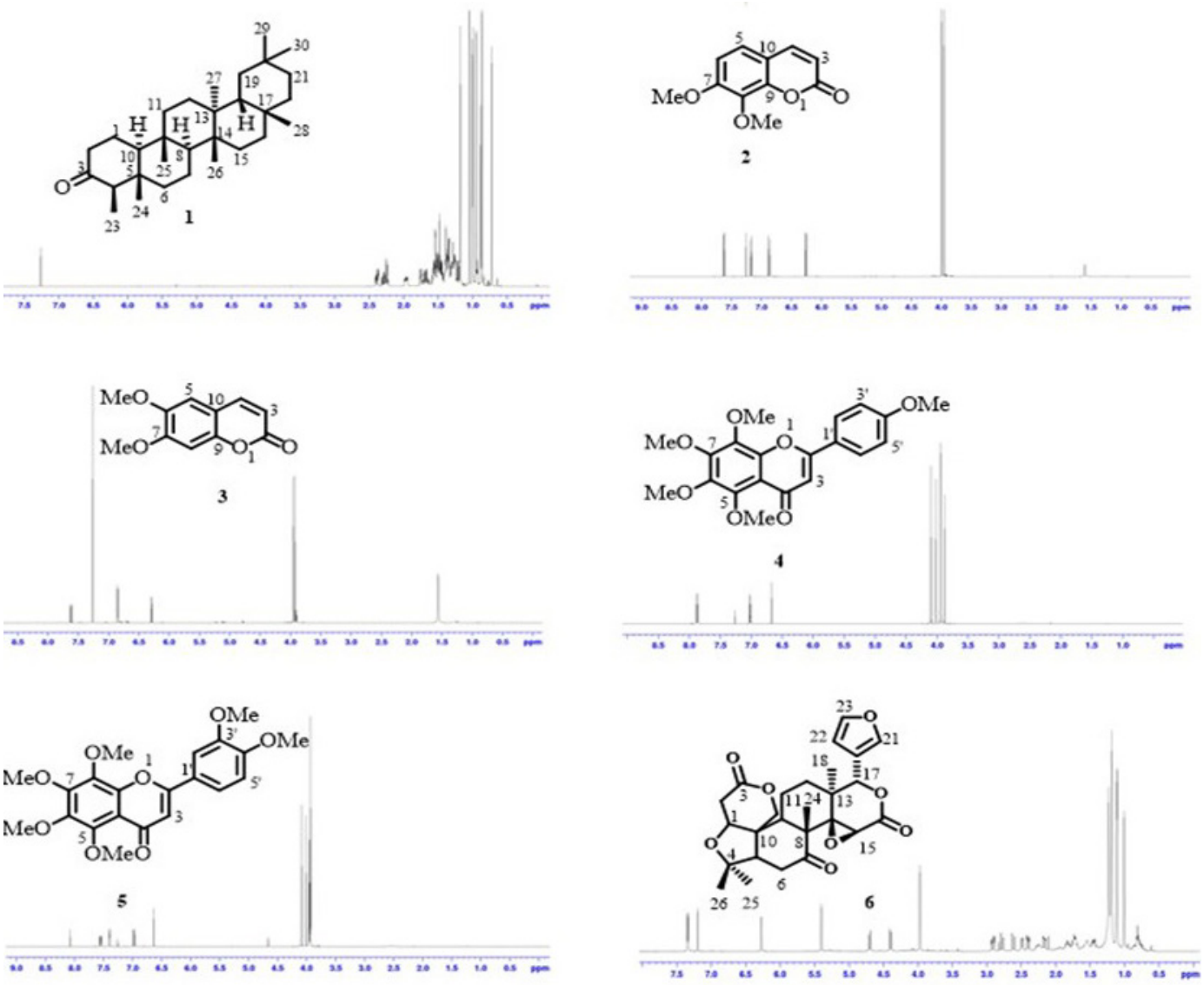


Figure 2. ^{1}H NMR spectrum (500 MHz) of the compounds (**1**-**6**).

characteristic signals of an epoxy-lactone moiety, including a carbonyl carbon at δ 171.7 (C-16) and oxygenated carbons at δ 83.9 (C-17), 58.7 (C-15), and 70.8 (C-14). The ^{1}H NMR spectrum (Figure 2) revealed signals corresponding to a substituted furan ring at δ 7.40 (*s*, H-21), 6.34 (*d*, 1.5 Hz, H-22), and 7.42 (*d*, 1.5 Hz, H-23), along with four methyl signals at δ 1.17 (H-18), 1.08 (H-24), 1.29 (H-25), and 1.16 (H-26). Additional signals corresponding to the lactone moiety and oxygenated methylene protons were also observed. Detailed interpretation of the 1D and 2D NMR spectra (^{1}H, ^{13}C, COSY, HSQC, and HMBC), together with comparison with reported data, confirmed the structure of compound **6** as limonin (Khalil et al., 2003). Limonoids are highly oxygenated triterpenoids widely distributed in citrus plants and are frequently associated with defense-related biological functions.

Several compounds identified in the present study, including friedelin (**1**), tangeretin (**4**), nobiletin (**5**), and limonin (**6**), have previously been reported from other cultivars or plant parts of *C. grandis* (Bhowal et al., 2022; Chinapongtitiwat et al., 2013; Mizuno et al., 1991; Ngolong et al., 2024). However, this study differs from previous investigations by focusing on the leaves of the *C. grandis* cultivar "Tubtim Siam" and by identifying dimethoxylated coumarin derivatives, particularly compounds **2** and **3**. Interestingly, comparison with previous phytochemical studies of *C. grandis* peels revealed clear tissue-specific patterns of metabolite distribution, with tangeretin being the only compound detected

in both peels and leaves (Phetkul et al., 2024). Such variation may reflect differences in biosynthetic pathways, ecological functions, and metabolite accumulation among plant tissues. To the best of our knowledge, all isolated compounds are reported here for the first time from the leaves of *C. grandis* cultivar "Tubtim Siam." These findings expand the current phytochemical knowledge of this economically important citrus cultivar and provide additional chemotaxonomic information on the distribution of secondary metabolites in citrus plants.

3.2. Biological activities

Five isolated compounds (**1**, **2**, and **4**–**6**) were subjected to biological evaluation for their antioxidant, antibacterial, and antiproliferative activities. Compound **3** was not evaluated because the amount obtained was insufficient for further bioassays.

3.2.1. Antioxidant activity

Antioxidant activity was assessed using the DPPH radical scavenging assay on the crude extract and isolated compounds from C. *grandis* leaves, as shown in Table 1. The concentration required to scavenge 50% of DPPH radicals (IC_{50}) was used to evaluate antioxidant activity, with lower IC_{50} values indicating greater antioxidant potential. Among the tested samples, compound **4** exhibited the strongest antioxidant activity, with an IC_{50} value of 1.86 ± 0.42 µg/mL, followed by the crude extract (2.30 ± 0.08 µg/mL) and compound 1 (3.59 ± 1.35 µg/mL). Compound **6** showed moderate radical scavenging activity, whereas compounds **2** and **5** demonstrated comparatively weak effects.

Interestingly, the crude extract demonstrated stronger antioxidant activity than several isolated compounds, suggesting possible synergistic interactions among multiple phytochemical constituents. Similar synergistic effects have frequently been observed in plant extracts containing complex mixtures of flavonoids, phenolics, and terpenoids (Xu et al., 2014). Such interactions may enhance antioxidant potential through complementary mechanisms, including hydrogen atom transfer, electron donation, and stabilization of radical intermediates (Oktiansyah et al., 2024). The strong antioxidant activity observed for tangeretin (**4**) is consistent with previous reports describing the biological properties of citrus polymethoxylated flavonoids. Flavonoids are known to neutralize free radicals through electron transfer and hydrogen atom donation mechanisms, and their antioxidant efficiency is strongly influenced by structural features such as aromatic conjugation and substituent patterns (Wang et al., 2018). Although polymethoxylated flavonoids generally exhibit lower antioxidant activity than hydroxylated flavonoids because of their reduced hydrogen-donating ability, tangeretin demonstrated remarkable activity under the present experimental conditions. This observation may be attributed to enhanced electron delocalization within the conjugated flavone system and to assay-dependent radical-stabilization mechanisms. In contrast, nobiletin (**5**), which contains a higher degree of methoxylation, exhibited weaker antioxidant activity. This finding is consistent with previously reported structure–activity relationships indicating that excessive *O*-methylation, particularly on the B ring, may reduce antioxidant efficiency by decreasing molecular planarity and limiting hydrogen atom transfer capacity (Heim et al., 2002). The present results therefore support the importance of substitution patterns in determining the antioxidant behavior of citrus flavonoids.

Friedelin (**1**) and limonin (**6**), both non-phenolic terpenoid compounds, also demonstrated measurable antioxidant activity despite lacking free phenolic hydroxyl groups. Their radical scavenging effects may be attributed to alternative stabilization mechanisms involving carbonyl functionalities and conjugated structural elements (Nguelefack et al., 2011). Notably, both compounds exhibited stronger antioxidant activity than Trolox under the same assay conditions, highlighting the potential contribution of non-phenolic metabolites to the antioxidant properties of *C. grandis* leaves. Overall, the antioxidant results suggest that the leaves of *C. grandis* cultivar "Tubtim Siam" represent a promising source of antioxidant phytochemicals, particularly polymethoxylated flavonoids.

Table 1
DPPH radical scavenging activity (IC_{50}, µg/mL) of extract and isolated compounds

Sample	IC_{50} (µg/mL)
1	3.59±1.35[a]
2	37.23±8.86[b]
4	1.86±0.42[a]
5	28.30±7.42[b]
6	9.23±5.52[a]
Extract	2.30±0.08[a]
Trolox	34.70±1.7[b]

3.2.2. Antibacterial activity

Antibacterial activity of the crude extract and isolated compounds was assessed against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, and the results are summarized in Table 2. Overall, the tested samples exhibited weak antibacterial activity, with MIC values ranging from 512 to >1024 µg/mL and no detectable bactericidal effects within the tested

Table 2
MIC and MBC values (μg/mL) of samples and Extract Against Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922

Sample	MIC/MBC (μg/mL)	
	S. aureus ATCC 25923	*E. coli* ATCC 25922
1	>512/-	>512/-
2	>512/-	>512/-
4	>1024/-	>1024/-
5	>512/-	>512/-
6	>1024/-	1024/>1024
Extract	>1024/-	>1024/-
Vancomycin	0.5/1	–
Gentamicin	–	0.25/0.5

concentration range. Compounds **1**, **2**, and **5** showed moderate inhibitory effects, with MIC values of 512 μg/mL against both bacterial strains, whereas compounds **4** and the crude extract exhibited MIC values greater than 1024 μg/mL. Compound **6** exhibited slightly stronger activity against *E. coli* than the other samples; however, the activity remained weak relative to the reference antibiotic gentamicin. The structural characteristics of the isolated metabolites may account for the generally weak antibacterial activity observed in this study. In particular, polymethoxylated flavonoids and methoxylated coumarins exhibit increased lipophilicity due to extensive *O*-methylation, which may enhance interactions with bacterial membranes but simultaneously reduce hydrogen-bonding capacity and limit effective binding to intracellular targets. In addition, these structural features may affect diffusion across bacterial cell walls, especially in Gram-negative bacteria. These structure–activity relationships are consistent with previous reports highlighting that the antibacterial effects of flavonoids largely depend on their interactions with microbial proteins and membrane-associated systems (Donadio et al., 2021; Sharma et al., 2025).

The antibacterial activity of friedelin reported here is consistent with previous studies demonstrating weak to moderate inhibitory effects against bacterial strains (Kamdem et al., 2022). Similarly, previous reports on tangeretin and nobiletin have demonstrated limited antimicrobial activity but suggested that these compounds may interfere with membrane permeability and biofilm formation rather than exerting direct bactericidal effects (Ortuño et al., 2006; Yao et al., 2012). Therefore, although the isolated compounds exhibited limited antibacterial potency in the present study, they may still possess biological relevance through alternative antimicrobial mechanisms that were not evaluated in the current assay system.

3.2.3. Antiproliferative activity

Antiproliferative activity against A549 and MCF-7 cell lines was assessed for the crude extract and isolated compounds, and the results are presented in Table 3. All tested samples exhibited weak cytotoxic activity, with IC_{50} values exceeding 500 μg/mL. Among the tested compounds, compound **2** demonstrated the highest activity, with IC_{50} values of 663.3 ± 66.5 μg/mL against A549 cells and 572.6 ± 8.20 μg/mL against MCF-7 cells. Compounds **5** and **6** showed slightly lower activity, whereas friedelin (**1**) exhibited the weakest inhibitory effect. The crude extract displayed particularly weak cytotoxicity, suggesting either low concentrations of active constituents or antagonistic interactions among metabolites in the crude mixture. According to the National Cancer Institute criteria, compounds with IC_{50} values greater than 20 μg/mL are generally considered to possess low cytotoxic activity *in vitro* (Boik, 2001; Suffness & Pezzuto, 1990). Therefore, the isolated compounds investigated in this study should be regarded as weak antiproliferative agents under the present experimental conditions. The weak activity observed for friedelin is consistent with previous reports indicating negligible cytotoxic effects against multiple cancer cell lines (Nguyen et al., 2020; Ragasa et al., 2012). Similarly, previous studies on methoxylated coumarins, such as 7,8-dimethoxycoumarin, have demonstrated limited antiproliferative activity (Misra et al., 2013). These findings support the notion that methoxylated coumarin derivatives generally possess low direct cytotoxic potential. In contrast, several previous investigations have reported stronger antiproliferative activities for tangeretin and nobiletin, often in the low micromolar range (Du & Chen, 2010; Manthey & Guthrie, 2002). The substantially weaker activity observed in the present study may arise from differences in experimental conditions, including cell line characteristics, passage number, compound purity, exposure duration, and assay methodology (Femina et al., 2023; Garai et al., 2026). Similar variability has frequently

Table 3
In Vitro Antiproliferative Activity (IC_{50}, μg/mL) of Samples Against A549 and MCF-7 Cancer Cell Lines.

Sample	IC_{50} (μg/mL)	
	A549	MCF-7
1	992.0+4.24	965.85+5.87
2	663.3+66.5	572.6+8.20
4	788.6+9.26	709.95+2.89
5	621.5+6.43	691.0+8.34
6	718.2+7.64	732.7+6.36
Extract	2,591+5.66	2,633.5+10.61
Doxorubicin	0.918+0.006	0.657+0.011

been reported in natural product cytotoxicity studies. Limonin has also been reported previously to inhibit proliferation in breast and lung cancer cell lines through apoptosis-related mechanisms (Bai et al., 2014; Kim et al., 2013; Wu et al., 2007). However, the present study demonstrated only weak activity. Such discrepancies further emphasize the influence of experimental conditions on biological evaluation outcomes. Although the isolated compounds displayed limited antiproliferative activity, the present study remains significant from phytochemical and chemotaxonomic perspectives. The findings suggest that the biological relevance of *C. grandis* cultivar "Tubtim Siam" leaves may be more closely associated with antioxidant-related applications rather than direct cytotoxic effects. Moreover, the identification of polymethoxylated flavonoids and limonoids provides valuable information regarding the secondary metabolite composition of this citrus cultivar.

Despite the valuable phytochemical and biological findings obtained in the present study, this work has several limitations that should be considered. The biological activities were evaluated only using *in vitro* assays against selected targets and therefore may not fully reflect pharmacological effects under physiological conditions. In addition, the mechanisms underlying the observed antioxidant and antiproliferative activities were not investigated in detail. Further studies involving quantitative phytochemical analysis, mechanistic investigations, synergistic interaction studies, and *in vivo* evaluation are necessary to better clarify the biological significance and therapeutic potential of the isolated compounds and crude extract.

4. CONCLUSION

This study represents the first phytochemical investigation of the leaves of *C. grandis* cultivar "Tubtim Siam," resulting in the isolation and identification of six compounds, namely friedelin (**1**), 7,8-dimethoxycoumarin (**2**), 6,7-dimethoxycoumarin (**3**), tangeretin (**4**), nobiletin (**5**), and limonin (**6**). The occurrence of these metabolites, particularly polymethoxylated flavonoids and methoxylated coumarins, expands the current phytochemical knowledge of this cultivar and highlights tissue-specific differences in secondary metabolite distribution between leaves and previously investigated plant parts. Among the tested samples, tangeretin (**4**) and the crude extract exhibited strong antioxidant activity in the DPPH assay, suggesting that the leaves of *C. grandis* cultivar "Tubtim Siam" may serve as a promising source of natural antioxidant constituents. The stronger activity observed in the crude extract compared with several isolated compounds further indicates the possible contribution of synergistic interactions among multiple phytochemicals. In contrast, the antibacterial and antiproliferative activities of the isolated compounds were generally weak under the experimental conditions used, indicating that the biological potential of this plant may be more closely associated with antioxidant-related applications rather than cytotoxic or antibacterial effects. Overall, the present findings provide new chemotaxonomic and biological information regarding *C. grandis* cultivar "Tubtim Siam" and support the importance of citrus species as valuable sources of structurally diverse bioactive metabolites. Further studies involving quantitative phytochemical analysis, mechanistic antioxidant investigations, synergistic interaction studies, and *in vivo* biological evaluation are warranted to better clarify the pharmacological potential and possible applications of these compounds.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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ETHICS STATEMENT

Not applicable.

AUTHOR CONTRIBUTIONS

Uraiwan Phetkul: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing original draft and Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. Kewalee Chaichan: Resources. Supakit Paosen: Methodology, Formal analysis, Investigation, Writing—original draft. Saowanee Maungchanburi: Methodology, Formal analysis, Investigation, Writing—original draft. Thitikorn Prombanchong: Validation, Formal analysis, Writing—original draft.

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