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Potent Cytotoxicity and Nitric Oxide Suppression of Compounds Derived from *Kaempferia elegans* Rhizomes: Molecular Modeling on EGFR Inhibition

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Abstract: A new naturally occurring diarylheptanoid, (1*E*,4*Z*,6*E*)-5-hydroxy-1,7-diphenylhepta-1,4,6-trien-3-one (**3**), was isolated from the rhizomes of *K. elegans* along with six known compounds, flavokawain B (**1**), 5,6-dehydrokawain (**2**), pinocembrin (**4**), cardamonin (**5**), alpinetin (**6**), and crotepoxide (**7**), among which compound **6** had not previously been isolated from this plant species. Two chalcones, flavokawain B (**1**) and cardamonin (**5**) were active against nitric oxide (NO) radicals released from LPS-induced RAW264.7 macrophages, resulting in 91.58% and 98.68% inhibition of NO production, respectively. Furthermore, compounds **1** and **5** showed superior cytotoxicity against MCF-7 (IC_{50} = 23.07 and 20.84 μM) and MDA-MB-231 (IC_{50} = 21.77 and 26.64 μM) cell lines, respectively. *In silico* molecular modeling studies of the most active compounds **1** and **5** against epidermal growth factor receptors (EGFR) suggested that π–π interactions with residues on the EGFR protein contributed to their anticancer properties. The results suggest that cardamonin (**5**) could be a promising candidate for further development of anti-inflammatory and anticancer agents.

Keywords: *Kaempferia elegans*; flavokawain B; cardamonin, anti-inflammation; human cancer cell; molecular modeling.

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1. Plant Source

The rhizomes of *Kaempferia elegans* (Wall.) were collected from Chiang Dao district, Chiang Mai, Thailand, in December 2019. A voucher specimen (KKU 26763), has been deposited at the Khon Kaen University (KKU) herbarium, Khon Kaen, Thailand.

2. Previous Studies

In 2018, Chawengrum and coworkers reported the isolation of two new diterpenoids (propadanes A-B), along with nine known compounds, from the *K. elegans* rhizomes collected in Kanchanaburi, Thailand [1]. Additionally, in 2021, this research group also reported the discovery of ten new diterpenoids, named elegansins A−E and elegansols A−E, together with seven known diterpenoids. It was found that abieta-8,11,13-trien-11-ol, a known compound, exhibited moderate to weak cytotoxicity against various types of cancer cell lines, including leukemia, lung carcinoma, hepatocellular carcinoma, cervical cancer, cholangiocarcinoma and breast cancer with IC_{50} values ranging from 27.2 to 137.6 μM [2]. Furthermore, flavokawain B isolated from *K. elegans* rhizomes collected from Chiang Mai, Thailand, demonstrated the most effective protection against UVA and UVB and showed potent antioxidant activities [3].

3. Present Study

The *K. elegans* rhizomes powders (3.1 kg) were dried and then gradient extraction to provide a crude hexane extract (17.3 g), a crude ethyl acetate extract (142.5 g), and a crude methanol extract (160.8 g) [4]. The isolation of crude ethyl acetate extract (35.8 g) (Scheme S1) afforded flavokawain B (**1**) (2.5 g) [5], 5,6-dehydrokawain (**2**) (391 mg) [6], (1*E*,4*Z*,6*E*)-5-hydroxy-1,7-diphenylhepta-1,4,6-trien-3-one (**3**) (148 mg) [7], pinocembrin (85 mg) [8], cardamonin (**5**) (227 mg) [8], alpinetin (**6**) (351 mg) [8], and crotepoxide (**7**) (65 mg) [9] (Figure 1). All isolated compounds were spectroscopically characterized, and their structures were determined by comparing the melting points and IR, NMR, and HRESIMS spectral data with those previously reported.

Compound **3** exhibited a molecular ion peak at *m/z* 275.1075 $[M\text{-}H]^-$ (calculated for $[C_{19}H_{16}O_2\text{-}H]^-$, 275.1078) by HRESIMS, which validated the molecular formula of $C_{19}H_{16}O_2$. The IR spectrum showed a C=O stretching of *α,β*-unsaturated ketone (1680 cm^{-1}). ^1H NMR spectra of **3** displayed the broad singlet of C-5 hydroxyl proton at δ 15.91 (*br s*) which displayed the six-membered ring intramolecular hydrogen bonding with the carbonyl group, two *trans* olefin protons at δ 7.66 (*d, J* = 15.8 Hz, H-1, H-7), diphenyl proton linked to C-1 and C-7 in the range of δ 7.39-7.55, the two olefinic protons signal at δ 6.63 (*d, J* = 15.8 Hz, H-2, H-6) and a singlet methine proton at δ 5.85 (*s*, H-4). The ^{13}C NMR data exhibited eight carbon signals, including the carbonyl carbon (at δ 183.3), four olefinic carbons (at δ 140.6 and 124.1), and aromatic carbons (at δ 128.1 to 135.0) and one signal (at δ 101.8) was identified as the methine carbon (C-4). Furthermore, the 2D NMR spectra were also recorded to confirm the assignments shown in Table S1 and Figures S7–S10. From all the spectroscopic data, the structure of **3** was identical to the previous synthetic analogue (Table S2) [7], thus identified as (1*E*,4*Z*,6*E*)-5-hydroxy-1,7-diphenylhepta-1,4,6-trien-3-one.

The anti-inflammatory properties of all isolated compounds on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells were evaluated by measuring the inhibition of NO production [10]. The NO release was effectively inhibited by pretreating with flavokawain B (**1**) (10 and 50 μM), cardamonin (**5**) (10 and 50 μM), and Bay11 as a positive control (5 μM) as shown in Figure S23A. Additionally, the cell viability was assessed to confirm that these treatments did not induce cellular toxicity in RAW264.7 cells. As shown in Figure S23B, compounds **1** and **5**, which feature a hydroxy group at the C2′ position in their chalcone structures, were not cytotoxic to the RAW264.7 macrophages at a dose of 10 μM compared with the control group.

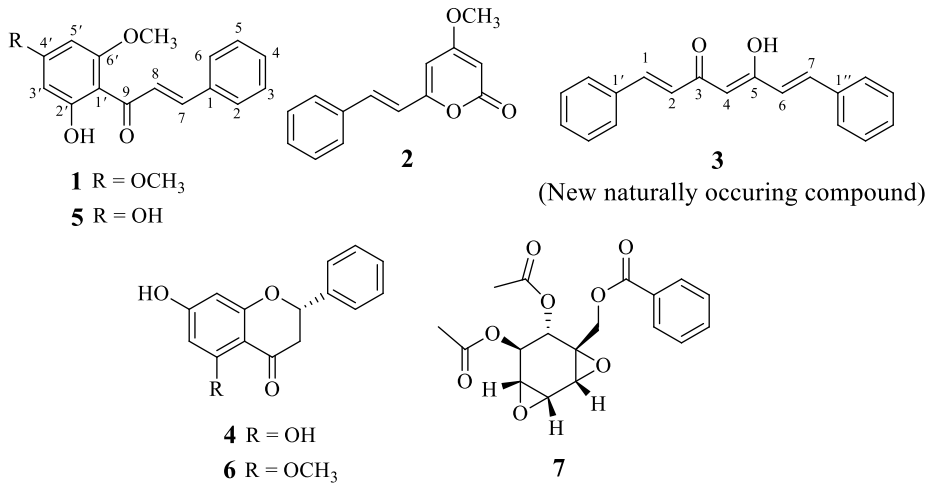

Figure 1. Structures of compounds **1–7** isolated from the rhizomes of *K. elegans*

Next, all isolated compounds from *K. elegans* were tested for their cytotoxic activity against breast cancer (MCF-7 and MDA-MB-231) and hepatocellular carcinoma (Huh-7 and SNU449) cell lines [11]. As summarized in Table 2, flavokawain B (**1**) and cardamonin (**5**) exhibited potent cytotoxic activities against both MCF-7 and MDA-MB-231 with IC_{50} values of 23.07 and 21.77 μM and 20.84 and 26.64 μM, respectively. These IC_{50} values are two times higher than those of cisplatin, which exhibited IC_{50} values of 36.53 and 44.70 μM against MCF-7 and MDA-MB-231, respectively. This suggested that flavokawain B (**1**) and cardamonin (**5**) could be promising cytotoxic agents for breast cancer treatment, which have not yet been reported from this plant. Moreover, (1*E*,4*Z*,6*E*)-5-hydroxy-1,7-diphenylhepta-1,4,6-trien-3-one (**3**) exhibited an IC_{50} value of 80.88 μM against MCF-7, demonstrating lower potency than curcumin, which showed an IC_{50} value of 25.30 μM for 48 hours. The key difference in cytotoxic efficacy may be attributed to the structure of compound **3**, which lacks methoxy and hydroxy groups on the benzene ring. These functional groups are known to enhance the potency against MCF-7 cells [12].

Table 2. Cytotoxicity of compounds **1–7** isolated from *K. elegans* against breast cancer, hepatocellular carcinoma, and normal cell lines.

Compound	IC_{50} (μM)				
	MCF-7	**MDA-MB-231**	**Huh-7**	**SNU449**	**HEK293T**
1	23.07 ± 4.96^{a}	21.77 ± 4.82^{a}	22.29 ± 4.85^{b}	27.81 ± 5.45^{b}	6.85 ± 1.76^{a}
2	inactive	inactive	96.65 ± 10.16^{d}	inactive	47.84 ± 7.89^{c}
3	80.88 ± 8.47^{c}	inactive	58.44 ± 7.56^{c}	inactive	inactive
4	inactive	inactive	inactive	inactive	11.51 ± 3.16^{a}
5	20.84 ± 4.81^{a}	26.64 ± 5.48^{a}	32.14 ± 5.99^{b}	46.47 ± 7.03^{c}	38.03 ± 6.47^{b}
6	inactive	inactive	92.94 ± 8.95^{d}	inactive	inactive
7	inactive	inactive	inactive	inactive	inactive
Cisplatin*	36.53 ± 0.55^{b}	44.70 ± 9.78^{b}	5.28 ± 0.97^{a}	2.80 ± 0.18^{a}	11.54 ± 1.84^{a}

*Positive control; Inactive at concentration of >100 μM

One-way ANOVA was used to compare the mean of IC_{50} values in each cell line, and different letters within a column indicate the significant difference ($p < 0.05$).

All isolated compounds were tested against HEK293T as a normal cell line. The results showed that cardamonin (**5**) exhibited approximately three-fold lower toxicity than cisplatin against normal cells, with IC_{50} values of 38.03 and 11.54 μM, respectively. Conversely, flavokawain B (**1**) showed two-fold higher toxicity toward HEK293T cells (IC_{50} = 6.85 μM) than cisplatin.

Considering that chalcones and flavonoids are potent inhibitors of EGFRs [13-16] and that EGFR is overexpressed in MCF-7 [16] and promotes migration of triple-negative MDA-MB-231 cell

lines, [17] binding studies were conducted using molecular docking simulations to investigate the inhibitory potential of the isolated compounds against EGFR.

The cocrystallized lapatinib ligand was removed from the EGFR crystal structure, energy-minimized, and docked back into the EGFR binding site. The lowest energy conformer was superimposed onto the cocrystallized lapatinib ligand, and the root-mean square deviation (RMSD) was calculated for the heavy atoms using DockRMSD [18] (Figure S24). The calculated RMSD value for heavy atoms was 3.58 Å. This large RMSD value could stem from the lack of side chain overlap extending from the furan ring. Visual inspection showed that the sulfone side chain extended outward to the aqueous environment, which is in line with its role as a solubilizing group for drug distribution in the blood plasma, i.e., the sulfone chain does not bind strongly with EGFR. Therefore, the large RMSD value suggests that the docking protocol is reliable.

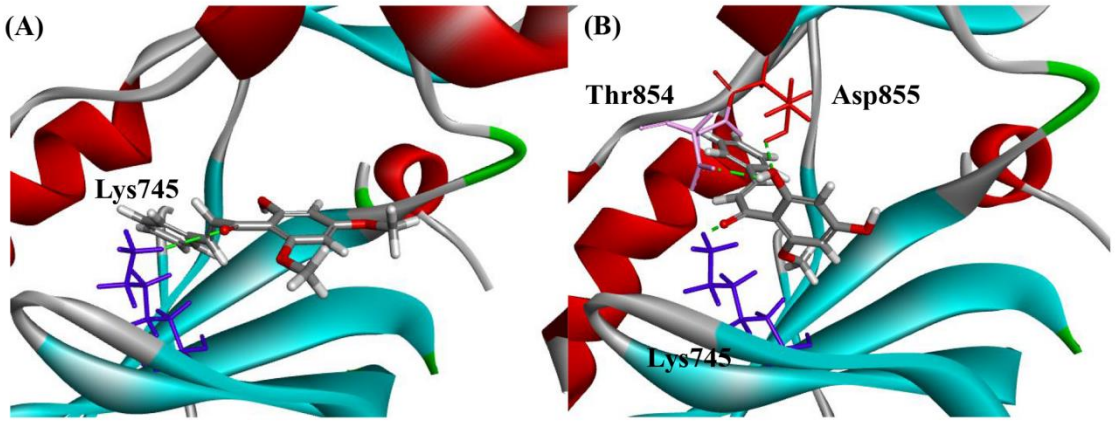

Figure 2. Predicted binding modes of **(A)** flavokawain B (**1**) and **(B)** cardamonin (**5**). Hydrogen bonds are represented as green dotted lines. Amino acid residues predicted to form hydrogen bonds are depicted: Lys745 (blue), Thr854 (pink), and Asp855 (red).

The most active molecules flavokawain B (**1**) and cardamonin (**5**) were docked to the EGFR binding site with calculated scores of 57.02 and 61.42, respectively. The two molecules have similar binding modes owing to their similar core structures, which differ only in the C4′ substitution. The aromatic phenyl groups provide compounds with substantial hydrophobic properties, and the oxygen-containing functionalities can form hydrogen bonding interactions with the EGFR protein (Figure 2). Molecular docking predicted that compound **1** inhibits EGFR by forming a hydrogen bond interaction between the carbonyl oxygen and the Lys745 side chain (Figure 2A), and the unsubstituted phenyl ring was predicted to occupy a hydrophobic pocket and form hydrophobic interactions with Ile789 and clusters of leucine residues including Leu788, Leu777, and Leu858 (Figure S25). The substituted phenyl ring was also predicted to form hydrophobic interactions with hydrophobic residues including Leu718, Gly796, Leu792, Leu844, Val726, and Ala743. Inhibition of EGFR by compound **5** was predicted from the intermolecular interactions observed in the simulation, namely, hydrogen bonds between the carbonyl functionality and Lys745 and between the hydroxyl group and the Asp855 and Thr854 residues (Figure 2B). The unsubstituted phenyl ring was found to occupy the hydrophobic pocket, similar to compound **1**, and was predicted to form hydrophobic interactions with several hydrophobic residues such as Leu777, Met766, and Phe856. Meanwhile, the substituted phenyl ring was predicted to form hydrophobic interactions with Ile744, Ile789, Leu844, and Leu718. These interactions have been reported to be related to the pharmacological activities of known EGFR inhibitors [18-20].

The molecular docking results indicate that the aromatic rings of both compounds **1** and **5** engage in hydrophobic interactions with specific regions of the EGFR protein. Additionally, these aromatic rings form π–π interactions with residues on the EGFR protein, thus stabilizing the binding between the compounds and the receptor. It should be noted that these compounds were specifically isolated from *K. elegans* collected from Chiang Dao district, Chiang Mai, Thailand. No reports on the isolation of both compounds with anticancer properties from other areas are available [1, 2].

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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