SUPPLEMENTARY MATERIAL

5,6-dehydrokawain from the rhizome of *Alpinia mutica* Roxb. induced proangiogenic tumour-derived VEGF of HT-29 colorectal cancer

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**Abstract:** Vascular endothelial growth factor (VEGF) is a glycoprotein vital to the regulation of vascular endothelial cells proliferation, migration and angiogenesis. The expression of VEGF is required for the formation of new blood vessels critical in supplying oxygen and nutrition in the course of tumorigenesis. The present study investigated the effect of 5, 6-dehydrokawain isolated from the rhizomes of *Alpinia mutica* on VEGF expression *in vitro* using HT-29 cell line. The results revealed that 5, 6-dehydrokawain induced the expression of proangiogenic tumour-derived VEGF of HT-29 cells, which may explain the inability of 5, 6-dehydrokawain in suppressing cancer cells proliferation.

**Keywords:** 5, 6-dehydrokawain; VEGF; angiogenesis, HT-29 cells

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**Figure S1.** Cytotoxicity studies of HT-29 cells treated at different concentrations with either 5, 6-dehydrokawain, 5-flourouracil or curcumin. 56DKW: 5, 6-dehydrokawain; 5FU: 5-flourouracil
Experimental

Plant Material

A dry rhizome of A. mutica (Voucher No. SK 3095/16) weighing about 5 kg was obtained from the Institute of Bioscience and authenticated by Dr. Mohd Firdaus Ismail of the same Institute, Universiti Putra Malaysia, Malaysia.

Extraction, isolation and purification of 5, 6-dehydrokawain

The powdered rhizome (628 g) was sequentially extracted with 3.2 L each of the solvent in order of increasing polarity viz: Hexane, chloroform, ethyl acetate, and finally methanol as we reported elsewhere (Malami et al., 2016). Each of the filtered solution was concentrated in a reduced pressured rotatory evaporator and further allowed to dry in the fume hood. The total weight of the final crude chloroform extract after complete dryness was recorded as 24 g and obtained as dark-brown. The crude chloroform extract was profiled using TLC and determined the right solvent system used in the isolation. The crude extract was mounted into the column chromatography containing packed silica gel 60 F_{254}, whilst mixture of petroleum
ether and ethyl acetate in different percentage was used as mobile phase. All resulting fractions from the crude chloroform were constantly monitored under UV spotted on TLC, whilst non-UV absorbance were identified after spraying with 10% sulfuric acid (H₂SO₄) followed by heating. Subsequently, repeated washing of resulting fraction with petroleum ether afforded 5, 6-dehydrokawain (2.184 g, 9.1%). The compound was analysed by spectroscopic techniques particularly, ¹H NMR ¹³C NMR, and DIMS.

3.2.1. 5, 6-dehydrokawain

Pale yellow amorphous solid, mp 132-133 °C (Mustahil, 2013; 135-136 °C). DIMS m/z (%): 228 ([M⁺], 100), 200 (27), 157 (39), 77 (37), 69 (59). ¹H NMR (500 MHz, CDCl₃): δ 7.51 – 7.50 (2H, m, H-10 and H-14), 7.48 (1H, d, J = 16.0 Hz, H-8), 7.40 – 7.30 (3H, m, H-11, H-12 and H-13), 6.57 (1H, d, J = 16.0 Hz, H-7), 5.94 (1H, s, H-5) 5.49 (1H, d, J = 2.0 Hz, H-3), 3.82 (3H, s, OMe). ¹³C NMR (125 MHz, CDCl₃): δ 127.4 (C-14), 128.9 (C-13), 129.4 (C-12), 128.9 (C-11), 127.4 (C-10), 135.2 (C-9), 135.8 (C-8), 118.6 (C-7), 158.6 (C-6), 101.4 (C-6), 56.0 (C-OMe), 171.1 (C-4), 88.8 (C-3), 164.3 (C-2).

3.3. Cell culture

Colorectal cancer cell line (HT-29, ATCC) employed in this study was obtained from the cell lines storage of UPM MAKNA Cancer Research Laboratory of the Institute of Bioscience, Universiti Putra Malaysia. The cell line was supported with Dulbecco’s modified eagle’s medium (DMEM) media (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (PAA, Freibug, Germany) and antibiotic comprising 1% 100 IU penicillin and 100 µg.mL⁻¹ streptomycin (Sigma, USA). Cultures were maintained at 37 °C humidified incubator with constant supply of 5% CO₂.

3.4. Cell viability assay

The MTT assay was used to examine the effect of 5, 6-dehydrokawain on HT-29 cells. Briefly, cells at the concentration of 0.5 × 10⁴ cells/mL were cultured in 96-well microplate. Following overnight incubation, cells were serial diluted with either 5, 6-dehydrokawain, curcumin (Sigma-Aldrich, St. Louis, MO, USA, purity 97.35%) or 5-fluorouracil (400, 100, 50, 25, 12.5, 6.25 µM) for 72 h, whilst DMSO (0.1%, v/v) was used as negative control. On the third day, 25 µL from stock solution containing MTT (5 mg/mL) (Sigma-Aldrich, St.
Louis, MO, USA) was added to each well and allowed incubation in the dark for 4 h. DMSO (100 µL) was added to each well and immediately measured at 570 nm using ELISA microplate reader (Beckman, Brea, CA, USA). The percentage amount of viable cells to that of the total cell population was expressed as cell viability and 50% growth inhibition induced by the drugs was expressed as IC$_{50}$. Three different experiments were performed independently.

### 2.5. Preparation of cell culture supernates

HT-29 cells were seeded at a concentration of $1 \times 10^5$ ml in a 6-well plate and allowed overnight incubation. Cell culture media was collected into a 15 mL centrifuge tube after 72 h treatment with either 5, 6-dehydrokawain or curcumin at different concentration. The media was centrifuge at 2000 rpm for 10 min at 4 °C. The supernatant was carefully aliquoted and refrigerated at -20 °C until use.

### 3.6. ELISA quantification of human VEGF

Human VEGF immunoassay was employed for the quantification of VEGF protein expression using Quantikine ELISA kits (R&D Systems, Minneapolis, MN, US) according to manufacturer’s protocol. Briefly, 200 µL of either standard, sample or control was added into each microplate strip containing 50 µL of assay diluent and incubated at room temperature for 2 h. Each well was aspirated and washed with 400 µL wash buffer for 3 consecutive times. Subsequently, 200 µL of human VEGF conjugate was added into each well and incubated at room temperature for another 2 h. Each well was aspirated and washed with 400 µL wash buffer for 3 consecutive times. A substrate solution (200 µL) was added into each well and allowed incubation at room temperature in the dark for 20 mins. Subsequently, 50 µL of stop solution was added into each well containing substrate solution and immediately measured with ELISA microplate reader at 450/540 nm. All experiment were performed in triplicate.
Figure S3: DIMS spectrum of 5, 6-dehydrokawain
Figure S4: $^1$HNMR spectrum of 5, 6-dehydrokawain
Figure S5: $^{13}$CNMR spectrum of 5, 6-dehydrokawain
**Figure S6:** Raw data from VEGF expression (pg/mL)

|       | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   |
|-------|------|------|------|------|------|------|------|------|------|------|------|------|
| A     | 0    | 0    | 0    | 0    | 0    | 0    | 1.92 | 1.9  | 1.91 | 1.426| 1.473| 440  |
| B     | 0    | 0    | 0    | 0    | 0    | 0    | 1.92 | 1.89 | 1.9  | 0.878| 0.794| 440  |
| C     | 0    | 0    | 0    | 0    | 0    | 0    | 1.886| 1.886| 1.879| 0.536| 0.564| 440  |
| D     | 0    | 0    | 0    | 0    | 0    | 0    | 1.791| 1.826| 1.737| 0.454| 0.337| 440  |
| E     | 0    | 0    | 0    | 0    | 0    | 0    | 1.383| 1.463| 1.527| 0.338| 0.293| 440  |
| F     | 0    | 0    | 0    | 0    | 0    | 0    | 1.268| 1.399| 1.313| 0.266| 0.268| 440  |
| G     | 0    | 0    | 0    | 0    | 0    | 0    | 1.263| 1.179| 1.48 | 0.252| 0.24 | 440  |
| H     | 0    | 0    | 0    | 0    | 0    | 0    | 0.486| 0.408| 0.406| 0.45 | 0.367| 440  |

|       | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   |
|-------|------|------|------|------|------|------|------|------|------|------|------|------|
| A     | 0    | 0    | 0    | 0    | 0    | 0    | 0.061| 0.062| 0.082| 0.13 | 0.084| 340  |
| B     | 0    | 0    | 0    | 0    | 0    | 0    | 0.071| 0.068| 0.076| 0.11 | 0.09 | 340  |
| C     | 0    | 0    | 0    | 0    | 0    | 0    | 0.073| 0.069| 0.08 | 0.078| 0.08 | 340  |
| D     | 0    | 0    | 0    | 0    | 0    | 0    | 0.04 | 0.039| 0.067| 0.052| 0.076| 340  |
| E     | 0    | 0    | 0    | 0    | 0    | 0    | 0.031| 0.032| 0.061| 0.054| 0.072| 340  |
| F     | 0    | 0    | 0    | 0    | 0    | 0    | 0.033| 0.031| 0.03 | 0.074| 0.078| 340  |
| G     | 0    | 0    | 0    | 0    | 0    | 0    | 0.031| 0.028| 0.033| 0.068| 0.076| 340  |
| H     | 0    | 0    | 0    | 0    | 0    | 0    | 0.033| 0.033| 0.036| 0.033| 0.033| 340  |

**56DKW (μM)**

| Concentration (μM) | 12.5 | 25 | 50 | 0.1% |
|-------------------|------|----|----|------|
| 569.0479          | 608.6521 | 609.6393 | 557.2392 | 417.7377 | 365.7556 | 357.6726 |

**FINAL RESULTS**

| Treatment          | Mean (pg/mL) |
|--------------------|--------------|
| 56DKW              | 2.852        |
| 66DKW              | 1.498        |
| 56DKW              | 1.048        |
| 1.67 DC            | 0.598        |
| 1.466 CU           | 0.514        |
| 1.283 CU           | 0.458        |
| 1.447 CU           | 0.404        |
| Blank              | 0.334        |

**Calculation of Results (pg/mL)**

| Treatment          | Mean (pg/mL) |
|--------------------|--------------|
| 1.482 56DKW        | 2.4165       |
| 1.475333 56DKW     | 1.1965       |
| 1.456667 56DKW     | 0.6455       |
| 1.356667 DC        | 0.3655       |
| 1.023 CU           | 0.1725       |
| 0.898667 CU        | 0.1025       |
| 0.879333 CU        | 0.0445       |
| Blank              | 0            |
| Conc (pg/mL) | OD    |
|-------------|-------|
| 1000        | 2.417 |
| 500         | 1.197 |
| 250         | 0.646 |
| 125         | 0.357 |
| 62.5        | 0.173 |
| 31.25       | 0.103 |
| 15.625      | 0.045 |
| 0           | 0     |

**Figure S7**: Standard curve of VEGF expression (pg/mL)