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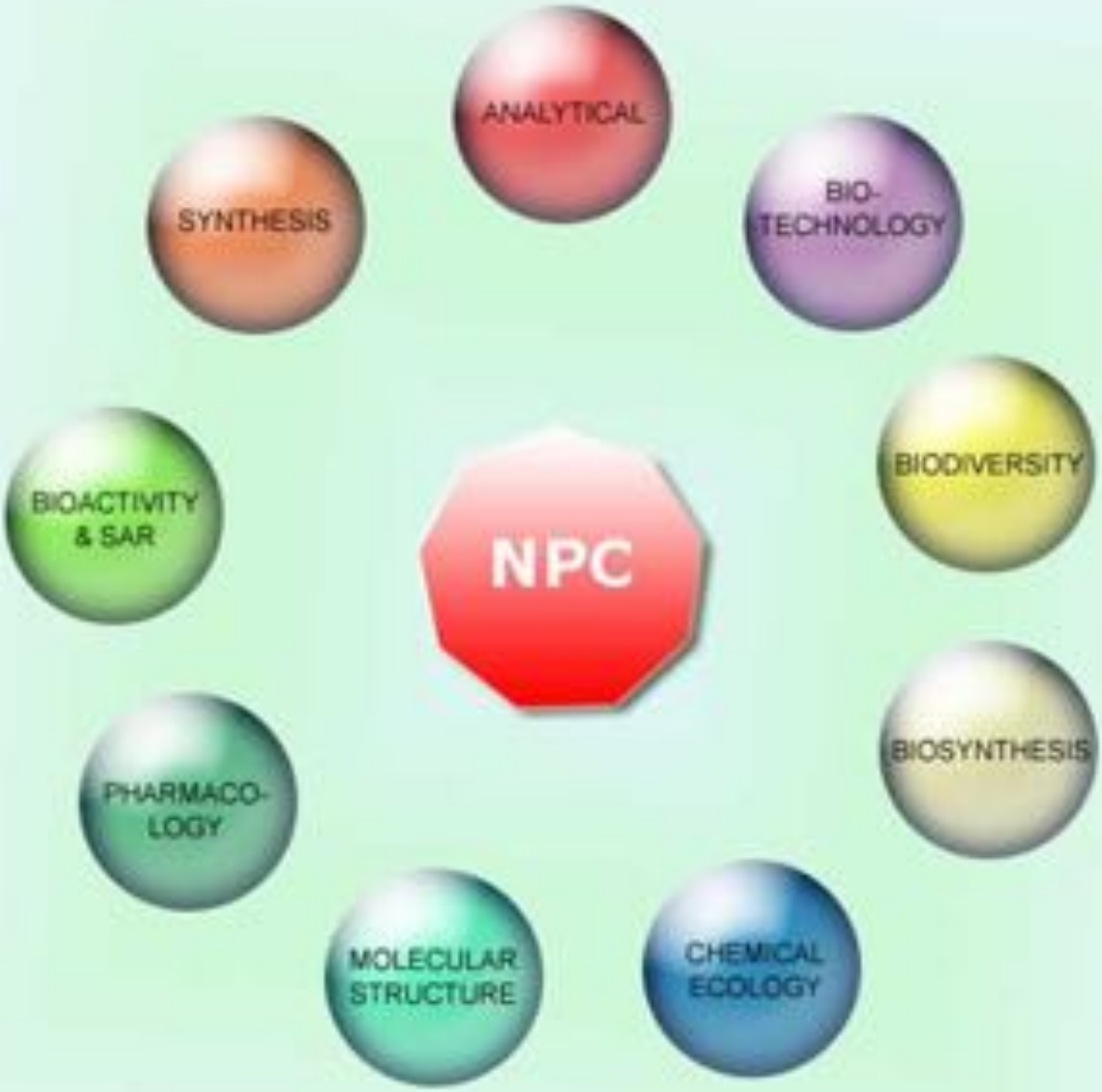

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8-Hydroxycudraxanthone G Suppresses IL-8 Production in SP-C1 Tongue Cancer Cells

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Production of IL-8 primarily promotes angiogenic responses in cancer cells, which lead to favorable disease progression. Suppressing this production may, therefore, be a significant therapeutic intervention in targeting tumor angiogenesis. This study aimed to evaluate the reduction effects of xanthones in cancer cell lines. Nine known prenylated xanthones (**1-9**), isolated from the pericarp of *Garcinia mangostana* Linn (GML), were tested for their ability to suppress IL-8 (interleukin-8) of the SP-C1 (Supri's Clone 1) tongue cancer cell line. Of these compounds, 8-hydroxycudraxanthone-G (**4**) suppressed IL-8 within 48 hours. This is the first report of 8-hydroxycudraxanthone G suppressing the production of IL-8 (45% at 15.7 μg/mL in 48 hours). These results suggest that the prolonged suppression of IL-8 production by cancer cell lines is concerned in the anti-cancer activity of 8-hydroxycudraxanthone.

Keywords: IL-8 production, *Garcinia mangostana* Linn (GML), Prenylated xanthones, 8-Hydroxycudraxanthone G, SP-C1 tongue cancer cell lines.

Garcinia mangostana Linn. (GML), family Guttiferae, has a long history of use as a medical plant, mostly in Southeast Asia [1]. People in these countries have used the pericarp (peel, rind, hull or ripe) of GML as a traditional medicine for the treatment of abdominal pain, diarrhea, dysentery, infected wounds, suppuration, and chronic ulcer [2]. The pericarp of GML is a source of xanthones and other bioactive substances [1]. Prenylated xanthones isolated from GML have been extensively studied; some of these compounds possess anti-oxidant, anti-tumor, anti-allergic, anti-inflammatory, anti-bacterial, anti-fungal and anti-viral properties [2]. Although, the anti-cancer activity of xanthone derivative has been reported,the anti-angiogenic effect against Supri's Clone (SP-C1) tongue cancer cells is still unknown. We now report the anti-angiogenic effect of prenylated xanthones, isolated from the pericarp of *G. mangostana*, against Supri's Clone (SP-C1) tongue cancer cells.

Generally, the process of growth and development in cancer cells is affected by the supply of oxygen, nutrient, growth hormone, proteolysis enzyme, and the dissemination of tumor cells, facilitated by blood vessels, to the dispersive area. When the tumor mass develops, the existed vessels become insufficient, leading to a hypoxic condition [3]. Oxidative stress in cancer cell growth, besides leading to hypoxia, may also result in prolonged inflammation. Several pro-inflammatory gene products have been identified to mediate the important role in suppressing apoptosis, proliferation, angiogenesis, invasion, and metastasis [4]. Numbers of cytokine, chemokine, and pro-inflammatory factors are involved in this process, one of which is interleukin-8 (IL-8) [3].

Interleukin-8 (IL-8) is a proinflamatory chemokine, known alternatively as CXCL8. Production of IL-8 is primarily regulated by activator protein and nuclear factor-**κ**B-mediated transcriptional activity. Accordingly, production of IL-8 has been shown to be regulated by a number of different stimuli, including inflammatory signal and chemical/environmental stresses (eg. exposure to chemotherapy agents and hypoxia) [5].

Intra tumor production of IL-8 is proposed to be a key regulator of infiltrating neutrophil recruitment into the tumor microenvironment, which later promotes metastasis. In addition, other studies indicate that intra tumor levels of IL-8 may also enhance the colonization of metastatic lesions. Induction of IL-8 production seems to be an adaptive response of cancer cells that is used to with stand environmental and chemical stresses [5]. Exposure to a number of chemotherapy agents (for example, 5-fluorouracil, adriamycin, dacarbazine, paclitaxel) has been shown to induce IL- 8 production and secretion in cancer cells [6].

Targeting IL-8 production within the cancer cell may also assist in sensitizing cancer cells to conventional chemotherapy and novel treatment strategies. The sensitization of cancer cells to undergo apoptosis on inhibiting IL-8 production suggests its therapeutic relevance to modulate the overall tumor response to conventional and novel therapies [5].

A similar process as in other cancer cells, oral squamous cell carcinoma, which is the most common oral cancer, also pursues the angiogenesis process in order to grow and develop. Supri's Clone (SP-C1) cells are a tongue cancer isolated from the cervical lymph node of an oral squamous cell carcinoma patient [7]. The original SP-C1 cells were a moderately differentiated squamous cell carcinoma of the tongue, and were not invasive into the muscle layer. SP-C1 cell cultures are characterized by rapid growth, and high invasion and metastasis ability, because the cells come from a phenotypic refractory carcinoma of the tongue, recurrence of which is very high, despite radical surgery and the mean duration of survival is short [8, 9]. The *in vitro* characteristics of SP-C1 cells make them an ideal model for studying apoptosis and angiogenesis [10].

Anti-angiogenic evaluation was made of fractions obtained by liquid-liquid partitioning of the EtOH extract of dried pericarps of GML. The compounds were tested for Supri's Clone (SP-C1) tongue cancer cells activity and exhibited 25% inhibition at 100 µg/mL [11]. Chromatographic procedures on this fraction yielded nine known compounds, which were identified by NMR spectroscopy and by comparison with published data as γ-mangostin (**1**) [12], α-mangostin (**2**) [12], β-mangostin (**3**) [13], 8-hydroxycudraxanthone G (**4**) [14], cudraxanthone G (**5**) [15], garcinone D (**6**) [16], mangostanin (**7**) [17], garcinone C (**8**) [18], and 1-mangostanin (**9**) [19].

Table 1: IC_{50} of prenylated xanthones (**1-9**).

xanthones	IC_{50} (µg/mL)
γ-Mangostin (**1**)	352.1
α-Mangostin (**2**)	14.4
β-Mangostin (**3**)	291.4
8-Hydroxycudraxanthone G (**4**)	64.0
Cudraxanthone G (**5**)	36.9
Garcinone D (**6**)	72.4
Mangostanin (**7**)	335.1
Garcinone C (**8**)	76.0
1-Isomangostanin (**9**)	146.7

α-Mangostin was the most active compound with an IC_{50} against SP-C1 cells of 14.4 µg/mL, while the least active was γ-mangostin (IC_{50} 352.1 µg/mL) (Table 1), which indicated that the presence of the 7-methoxyl group increases the cytotoxic activity against Supri's Clone (SP-C1) tongue cancer cells. Ahmat *et al,* also showed α-mangostin to be the most potent compound in inhibiting cancer cell growth (IC_{50} 10.5 µg/mL) [20]. A pure compound is classified as active and a potential anti-cancer agent if its IC_{50} value is below 100 µg/mL [20]. Five of the prenylated compounds tested had values below 100 µg/mL (Table 1), and these were further tested for their effects on IL-8 production using ELISA quantitative technique based separately on interpolation to the standard curve. Data were recorded using ELISA reader with λ_{maks} 450 nm and gives an Optical Density (OD) data. Suppression of IL-8 production occurs within 24 hours only in 8-hydroxycudraxanthone G (35%) and garcinone C (30%) at the concentration of 15.7 µg/mL. In 48 hours period, suppression of IL-8 production occurs in all five prenylated xanthones, the highest IL-8 suppression seen in 8-hydroxy-cudraxanthone G (45%) and the lowest is α-mangostin (15%).

Production of IL-8 from cancer cells can enhance the proliferation and survival of these cells through autocrine signaling pathways. In addition, tumor-derived IL-8 will activate endothelial cells in the tumor vasculature to promote angiogenesis and induce a chemotactic infiltration of neutrophils into the tumor site. Although IL-8 can promote cell invasion and migration, the capacity of IL-8 to induce tumor-associated macrophages to secrete additional growth factors will further increase the rate of cell proliferation and cancer cell invasion at the tumor site [3].

In addition, a compound that inhibits angiogenesis shows in the suppression of proangiogenic factor, one of which is IL-8. Decreasing this proangiogenic factor production will result in the accumulation of intercellular ROS, which results in new blood vessel failure [20]. Angiogenesis is considered as an effective therapeutic strategy related to metastasis in the primary tumor that will not duplicate itself until new blood vessels are available. Angiogenesis inhibition has the potential of inhibiting tumor growth, as well as stopping the spread to other parts of the human body [8]. Figure 1 shows the suppression of IL-8 production by 8-hydroxycudraxanthone G (**4**) over 48 hours of testing. This might be useful for the inhibition of the angiogenesis process. Among the other compounds, α-mangostin (**2**) and cudraxhantone G (**5**) showed cytotoxic activity, and 8-hydroxycudraxanthone G (**4**) showed suppression of IL-8 production. In addition, 8-hydroxy-cudraxanthone G (**4**) and garcinone C (**8**) exhibited a suppression effect in that system, but their analogous compounds, α-mangostin (**2**) and cudraxanthone G (**5**) showed a promotion effect, indicated that the prenylated xanthones had an opposite effect on cytotoxic activity and delaying IL-8 production.

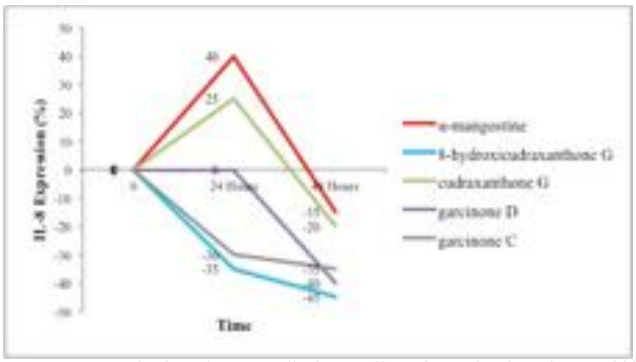

Figure 1: IL-8 production of SP-C1 cells due to effect of prenylated xanthones within 48 hours at a concentration of 15.7 µg/mL.

Experimental

General: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured on a JEOL JNM A-500 instrument, and chemical shifts were given in δ (ppm) values using TMS as an internal standard. Chromatographic separations were carried out on silica gel 60 (Merck) and silica gel GF_{254} for preparativeTLC. TLC plates were precoated with silica gel GF_{254} (Merck, 0.25 mm) and detection was achieved using UV light at wave lengths of 254 and 367 nm.

Plant materials: The pericarp of *Garcinia mangostana* (GML) was collected in Puspahiang Plantation center, Tasikmalaya, West Java, Indonesia in April 2011 and identified by Mr Joko Kusmoro (Universitas Padjadjaran). A voucher specimen (No. 011/HB-07/2011) has been deposited at the Herbarium of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor, Sumedang, Indonesia.

Extraction and isolation: The dried pericarp (538.4 g) of GML was extracted with ethanol exhaustively (4L x 3) at room temperature for 3 days. The extract was filtered and concentrated in a rotary evaporator to yield a reddish-brown extract (92.4 g). The extract (90 g) was subjected to silica gel vacuum liquid cromatography with a *n*-hexane-ethyl acetate-methanol gradient to obtain 12 fractions, which were combined according to TLC results.

The 10% ethyl acetate eluate was rechromatographed through a silica gel column with *n*-hexane:ethyl acetate (7:3) to obtain 12 fractions (A1-A12). Subfraction A6 was further separated by preparative TLC on silica gel GF_{254} with *n*-hexane:ethyl acetate (8:2) to yield compounds **1** (60 mg) and **2** (42 mg).

The 20% ethyl acetate eluate was chromatographed on silica gel, eluted successively with a gradient of *n*-hexane: ethyl acetate (20:1 to 1:2), to give 7 sub-fractions (B1-B7). Sub-fraction B3 was chromatographed on a column of silica gel, eluted with *n*-hexane: acetone (1:1), to give compound **3** (50.8 mg).

The 40% ethyl acetate eluate was chromatographed on silica gel, eluted successively with *n*-hexane: acetone (10:1 to 1:1), to give 10 sub-fractions (C1-C10). Sub-fraction C5 was subjected to preparative TLC on silica gel GF_{254} eluted with *n*-hexane: EtOAc-acetone (3:2.5:0.5), to give compounds **4** (7 mg) and **5** (12 mg).

The 50% and 60% ethyl acetate eluates were chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane: EtOAc (10:1 to 7:3), to give 20 sub-fractions (D1-D20). Sub-fraction D14 was subjected to preparative TLC silica gel GF_{254}, eluted with *n*-hexane:acetone (3:2), to give compound **6** (8.5 mg).

The 70% and 80% ethyl acetate eluates were fractionated by CC on silica gel 60 using a gradient of *n*-hexane and EtOAc to give 6 fractions (E1-E6), combined according to TLC results. Fraction E2 was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane: acetone (20:1 to 1:2), to give 7 sub-fractions (E2.1-E.2.7). Sub-fraction E.2.3 was chromatographed on a column of silica gel, eluted with *n*-hexane: acetone (1:1), to compound **8** (50.8 mg).

The EtOAc eluate was fractionated by CC on silica gel 60 using *n*-hexane and EtOAc as a solvent gradient to give 8 fractions (F1-F8), combined according to TLC results. Fraction F5 was chromatographed on a column of silica gel, eluted successively with a gradient of $CHCl_3$: MeOH (20:1 to 1:2), to give 6 fractions (F5.1-F5.6), combined according to TLC results. Sub-fractions F5.2 and F5.3 were subjected to preparative TLC on silica gel GF_{254} eluted with $CHCl_3$-MeOH(9.5:0.5) to yield compounds **7** (12 mg) and **9** (10.4 mg).

In vitro *assay for cytotoxic activity*

Cell lines: SP-C1 tongue cancer cell lines were maintained in DMEM (Dulbecco's modified eagle medium, Sigma, St Louis, MO, USA), supplemented with 10% FCS (fetal calf serum, Moregate BioTech, Bulimba, Australia), 100 µg/mL streptomycin, and 100 units/mL penicillin (Invitrogen Corp., Carlsbad, CA, USA), then cultured at 37°C in a humidified atmosphere of 95% air-5% CO_2 [10].

MTT assay: MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was used to assess anti-proliferative activity of extract on cancer cells. The surviving cells, after exposure, could reduce the yellow MTT tetrazoloium salt to insoluble purple formazan crystals by succinate dehydrogenase [15]. SP-C1 cells (1.5×10^4 cells/100 µL) were seeded into each well and incubated for 24 h. Medium was removed and replaced with extract and incubated for the indicated time. After exposure, the medium was discarded and incubated with MTT solution (1 mg/mL, 50 µL/well) for 1 h. Isopropanol was used to dissolve the formazan crystals and plates were gently shaken for 5 min. Surviving cells were proportional to the intensity of purple formazan determined by a micro plate reader at 590 nm ($A_{590\ nm}$ = Absorbance at 590 nm). Surviving cells (%) = ($A_{590\ nm}$of treated cells / A_{590} nm untreated cells) x 100 [9].

IL-8 production by ELISA assay: The Quantikine Human IL-8 Immunoassay (R&D System, USA) is a 3.5 hour solid phase ELISA procedure designed to measure human IL-8 in cell culture supernatants, serum, and plasma. It is based on antibodies raised against the 72 aa variant of human IL-8 derived from *Escherichia coli*. It is calibrated with the same recombinant factor. This immune assay accurately quantifies recombinant human IL-8. Measurement of natural human IL-8 or the 77 aa variant of human IL-8 give results parallel to the standard curves obtained using the *E. coli*-expressed Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IL-8 [21].

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