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Cytotoxic Activity of Major Compounds from *Phaleria macrocarpa* (Scheff.) Boerl. Fruits

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Graphical abstract

Abstract

P. macrocarpa is a well known Indonesian medicinal plant which is traditionally claimed to have anticancer properties. To date, there are numerous cytotoxic studies conducted on crude extracts of this plant. However, there are limited informations available regarding cytotoxic activity of the compounds isolated from this plant. Thus, this study investigated cytotoxic activity of two benzophenones derivatives identified as 2,6,4'-trihydroxy-4-methoxybenzophenone (1) and 6,4'-dihydroxy-4-methoxybenzophenone-2-*O*-*β*-D-glucopyranoside (2) isolated from the ethyl acetate extract. Cytotoxic activities of these compounds were performed against human cervical carcinoma cell line (HeLa) and mouse embryonic fibroblast cell line (3T3) using MTT assay. The result showed that benzophenone (1) exhibited low cytotoxic effect against HeLa and 3T3 cell lines with IC_{50} values of 132 µg/ml and 158 µg/ml, repectively while benzophenone (2) was non toxic against HeLa and 3T3 cell lines are because the IC_{50} is more than 250 µg/ml. These findings may sheds light on the actual properties of this plant.

Keywords: *P. macrocarpa*; benzophenones; cytotoxic; MTT; HeLa; 3T3

Abstrak

P. macrocarpa merupakan tumbuhan herba Indonesia yang terkenal di mana secara tradisionalnya, ia dipercayai mempunyai sifat sebagai antikanser. Sehingga hari ini, terdapat banyak kajian yang telah dijalankan mengenai sitotoksik ekstrak mentah dari tumbuhan ini. Namun, informasi mengenai sitotoksik sebatian dominan daripada tumbuhan ini adalah masih terhad. Oleh itu, kajian ini dijalankan bagi mengkaji aktiviti sitotoksik untuk dua ahli benzofenon yang berjaya diasingkan daripada ekstrak etil asetat. Struktur kedua-dua benzofenon ini telah dikenalpasti sebagai 2,6,4'-trihidroksi-4-metoksibenzofenon (1) dan 6,4'-dihidroksi-4-metoksibezofenon-2-*O*-*β*-D-glukopiranosid (2) Aktiviti sitotoksik sebatian ini telah diuji ke atas sel karsinoma servik manusia (HeLa) dan sel fibrolas embrionik tikus (3T3) dengan menggunakan asai MTT. Hasil kajian menunjukkan bahawa benzofenon (1) mempunyai kesan sitotoksik yang rendah terhadap sel HeLa dan 3T3 dengan nilai IC_{50} 132 µg/ml and 158 µg/ml masing-masing manakala benzofenon (2) tidak toksik terhadap sel HeLa dan 3T3 kerana tidak mencapai nilai IC_{50} pada kepekatan 250 µg/ml. Penemuan ini akan membawa kepada sifat sebenar tumbuhan ini.

Kata kunci: *P. macrocarpa*; benzofenon; sitotoksik; MTT; HeLa; 3T3

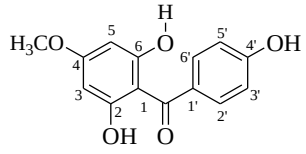
1.0 INTRODUCTION

Phaleria macrocarpa is a dense evergreen tree, growing well in tropical climate and was originally found in Irian Jaya, in the eastern part of Indonesia.[1] This plant is locally known as 'Mahkota dewa', 'God Crown' or 'Pau'. The stems, leaves and fruits of this plant are utilized as alternative medicine to treat cancers in Indonesia.[2,3]

Preliminary studies have reported that one of the fraction of methanol extract from the leaves of *P. macrocarpa* showed moderate inhibitory activity against myeloma cells (NS-1) with IC_{50} value of 81 µg/ml.[4] Further studies showed that the chloroform extract from the leaves of *P. macrocarpa* exhibited antiproliferative properties against cervical cancer cell (HeLa), melanoma skin cancer cell (HM3KO) and breast cancer cell (MCF-7) with IC_{50} value of 40.2 µg/ml, 62.9 µg/ml and 70.8 µg/ml, respectively.[5] In addition, the ethyl acetate extract from the barks of *P. macrocarpa* showed strong cytotoxic activity

against mouse leukemia cell (L1210) with IC_{50} value of 10.2 µg/ml.[6] Cytotoxicity test on ethyl acetate and methanol extracts from the leaves of *P. macrocarpa* showed that these extracts displayed low mild cytotoxic effect against human hepatoma cell line (HepG2) with IC_{50} value of 32.5 µg/ml and 40 µg/ml, respectively.[7]

The cytotoxic activity of pericarp, mesocarp and seed of the fruits of *P. macrocarpa* has been conducted against human colon cell line (HT-29), human breast cancer cell line (MCF-7), human cervical cell line (HeLa) and human hepatocytes cell lines (Chang liver). The results indicated that all parts of the fruits exhibited strong cytotoxic properties against MCF-7 and HeLa with IC_{50} value between 20 - 40 µg/ml.[8] However, no study conducted to determine the specific compound that contribute to these significant anticancer effect. The isolation of two major compounds, identified as 2,6,4'-trihydroxy-4-methoxybenzophenone (**1**) and 6,4'-dihydroxy-4-methoxybenzophenone-2-*O*-*β*-D-glucopyranoside (**2**) from *P. macrocarpa* fruits and their cytotoxicity against MCF-7 cell line has been investigated before.[9] The results prove that these compounds are not involved in the strong cytotoxic properties of the extracts since the IC_{50} values obtained were exceed 100 µg/ml. Therefore, this study was proposed specifically to evaluate the cytotoxic activity of these major compounds against HeLa and 3T3 cell line.



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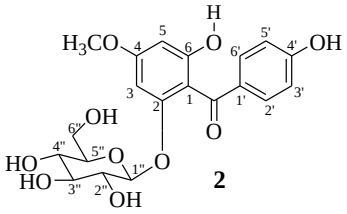

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Figure 1 Chemical structure of two major compounds

2.0 EXPERIMENTAL

2.1 Plant Materials

Fruits of *P. macrocarpa* were collected from Johor, Malaysia in May 2010. The fruits were dried at room temperature for 2 weeks and ground into a small pieces (cotton-like) using grinding machine.

2.2 Extraction and Isolation

The dried fruits (500 g) were extracted with ethanol at room temperature for 48h. The residue was extracted twice and the extract was filtered using Whatman No. 1 filter paper. The filtrate was then evaporated to dryness using vacuum distillation and rotary evaporator at 50°C. The ethanol crude extract was partitioned separately with chloroform-ethyl acetate and evaporated to give chloroform crude extract (7.55 g) and ethyl acetate crude extracts (2.40 g). The ethyl acetate crude extract (1.00 g) was then subjected into column chromatography on silica gel using gradient elution petroleum ether and ethyl acetate to give 13 fractions (Fr. 1- Fr. 13). Benzophenone (1) (50 mg) was obtained from Fr. 10 at eluent system of petroleum ether: ethyl acetate (85:15) while benzophenone (2) (70 mg) was obtained from purification of Fr. 11 (0.31 g) using gradient elution of chloroform and methanol (90:10). From the analysis of spectroscopic data and literature data, the structure of benzophenone (1) and (2) were deduced as 2,6,4'-trihydroxy -4-methoxybenzophenone and 6,4'-dihydroxy-4-methoxybenzophenone-2-*O*-*β*-D-glucopyranoside, respectively (Figure 1).[6,10]

2.3 Cell Cultures

The human cervical carcinoma cell line (HeLa) and mouse embryonic fibroblast cell line (3T3) were obtained from American Type Culture Collection (ATCC) and were a generous gift from Dr Zainah Adam from Agensi Nuklear Malaysia, Kajang, Selangor. 3T3 cell line was cultured in Dulbeccos Modified Eagles Medium and HeLa cell line was cultured in Roswell Park Media Institute (RPMI) 1640); suplemented with 10% of FBS, 100 IU/ml of penicillin and 100 µg/ml of streptomycin using 25-cm^2 flask, in humidified incubator with 5% CO_2 and 95 % air at 37°C. All media and supplement were purchased from Biowest, Medigene, Puchong, Selangor, Malaysia.

2.4 MTT Assay

Cells were seeded in 96-well plates at density of 5×10^4 cells/well in 100 µl of medium and incubated 24h to allow the cells to attach to the well. The medium was discarded and the cells were feed with 100 µl fresh medium. Then various concentrations of the isolated benzophenones (ranging from 1.95 to 250 µg/ml) were added to the cells in 100 µl medium while the control group were only cells cultured in 200 µl medium. Cells were incubated for 72h and each concentration was tested in four replicates.

The viability of cells was determined by MTT assay which measure the changes in color. This assay was done to determine the enzyme activity that reduces the MTT yellow colour (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, giving a purple colour.[11] After 72h of incubation, 20 µl of MTT solution (a stock solution of 5 mg/ml in PBS) (Sigma, Subang Jaya, Selangor, Malaysia) was added to each well and incubated for 4 hours. The medium with tested extract was discarded and replaced with 225µl of buffer solution (1M HCL in 100 mM isopropanol) to each wells to solubilize the formazan crystals. The spectrophotometrical absorbance of cell extract was measured using ELISA reader (BioRad, Tokyo, Japan) at wavelength of 575 nm. The results were recorded as IC_{50} value which is the concentration of sample that inhibits cancer cells growth using the formula given below:

$$\% \text{ Cell viability} = \frac{\text{A sample (mean)}}{\text{A control (mean)}} \times 100\%$$

2.5 Statistical Analysis

SPSS16.0 software (SPSS 16.0 for Windows Evaluation Version software, SPSS Inc., USA) was used to analyze the data. The normality of data was determined using Shapiro-Wilk test. The Independent t-test was used to analyze the normal data while Mann-Whitney test was used for non-normal data.[12] The data was considered to be significant if the probability $p < 0.05$.

3.0 RESULTS AND DISCUSSION

In this study, cytotoxicity of the major compounds from ethyl acetate extract of *P. macrocarpa* were tested against human cervical carcinoma cell line (HeLa) and mouse normal embryonic fibroblast cell line (3T3) as a positive control. The IC_{50} values obtained from cell viability graph was used as parameter for cytotoxicity where it refers to 50% of cell inhibited by compounds.[13]

The cytotoxicity of benzophenone (1) and (2) against HeLa and 3T3 cell lines at eight differents concentration were tabulated in Table 1 and Table 2, respectively. From the results obtained, the cell viabilty graph were plotted against different concentration of compounds. Benzophenone (1) showed cytotoxic effect against HeLa and 3T3 cell lines with significant IC_{50} value of 132 µg/ml and 158 µg/ml, respectively (Figure 2 and 3). Previous study has reported that the IC_{50} value for isolated compound to exhibit good anticancer agent is ≤ 4 µg/ml.[14] Thus, the IC_{50} values of benzophenone (1) were considered too high and it can be said that this compound is not involved in the strong cytotoxicity of extracts from *P. macrocarpa* fruits.[8] However, previous study on antioxidant activity of benzophenone (1) has prove that this compound exhibited potent antioxidant agent with IC_{50} value of 10.57 µg/ml.[14] Thus, the strong antioxidant properties and low cytotoxic properties of benzophenone (1) can be used for future development of drugs as antioxidant since the compound is not toxic to normal cells (3T3) in low dose.

Table 1 Cytotoxic activity of major compounds from *P. macrocarpa* against HeLa cell line at eight differents concentration

% Cells viability	Concentration of compounds (µg/ml)							
	1.95	**3.9**	**7.8**	**15.6**	**31.2**	**62.5**	**125**	**250**
(**1**)	90.8 ± 0.040	* 89.6 ± 0.019	* 89.4 ± 0.026	** 88.9 ± 0.029	** 90.3 ± 0.014	** 85.2 ± 0.016	*** 52.7 ± 0.009	*** 1.1 ± 0.000
(**2**)	* 89.1 ± 0.027	* 91.0 ± 0.026	* 90.5 ± 0.021	*** 89.1 ± 0.019	** 90.2 ± 0.023	** 88.2 ± 0.030	** 87.3 ± 0.021	* 78.7 ± 0.009

Values are mean ± STDEV for 3 replicates experiment; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control

Table 2 Cytotoxic activity of major compounds from *P. macrocarpa* against 3T3 cell line at eight differents concentration

% Cells viability	Concentration of compounds (µg/ml)							
	1.95	**3.9**	**7.8**	**15.6**	**31.2**	**62.5**	**125**	**250**
(**1**)	** 69.0 ± 0.028	* 69.9 ± 0.027	** 70.8 ± 0.040	*** 71.1 ± 0.007	** 70.1 ± 0.033	** 70.0 ± 0.03	** 64.2 ± 0.031	*** 3.0 ± 0.002
(**2**)	*** 67.8 ± 0.026	** 66.4 ± 0.053	*** 65.0 ± 0.015	** 67.0 ± 0.031	*** 67.5 ± 0.006	*** 67.9 ± 0.011	*** 66.9 ± 0.020	*** 67.7 ± 0.018

Values are mean ± STDEV for 3 replicates experiment; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control

Figure 2 and 3 showed that benzophenone (2) exhibited non-cytotoxic effect since the IC_{50} value was not determined. These non-toxic effect of benzophenone (2) was associated with Mahkoside A, a compound that have similar molecular structure with benzophenone (2).[15] This Mahkoside A exhibited very low cytotoxic effect against esophagel cancer cell line (EC109 and EC9706), stomach cancer cell line (MGC-803) and prostate cancer cell line (PC-3) with all IC_{50} values exceed 100 µg/ml. In addition, study on cytotoxic activity of other benzophenone glucoside, phalerin from *P. macrocarpa* leaves showed that this compound also non-toxic towards myeloma cell line (NS-1) although its methanol extract exhibited strong cytotoxic properties.[16] The lower cytotoxic effect of benzophenone (**2**) might due to the presence of glucose moiety since many bioactivities studies of benzophenone derivatives with significant results has been reported, but those reported compounds are aglycon which is not glucoside compounds.[17]

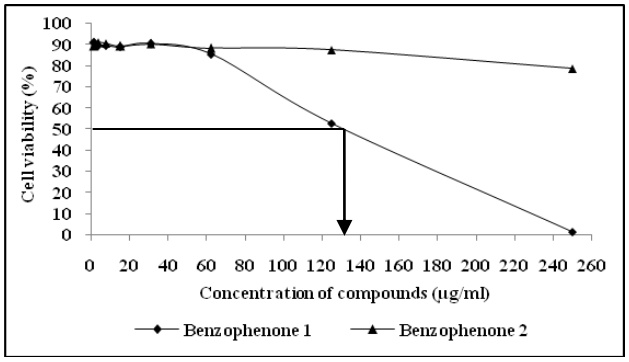

Figure 2 Percentange of cell viability of HeLa cell line against different concentrations of isolated benzophenones from *P. macrocarpa* fruits

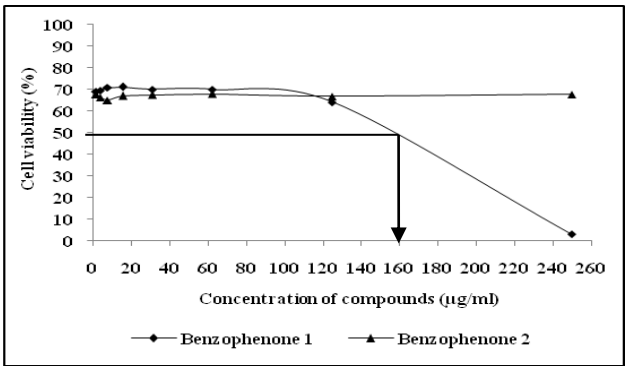

Figure 3 Percentange of cell viability of 3T3 cell line against different concentrations of isolated benzophenones from *P. macrocarpa* fruits

In overall, the results showed that both benzophenones were not directly involved in the good cytotoxic properties of extracts from *P. macrocarpa* fruits since the IC_{50} value obtained were too high. However, the cytotoxic activity of these compounds can be enhaced by doing some structural modification through substitution of amino, alkoxy, azotic alkyl groups and piperidine circle.[6,15]

4.0 CONCLUSION

In conclusion, benzophenone (**1**) (2,6,4'-trihydroxy-4-methoxy-benzophenone) was found to have weak cytotoxic effect against HeLa and 3T3 cell line while benzophenone (**2**) (6,4'-dihydroxy-4-methoxybenzophenone-2-*O*-*β*-D-glucopyranoside) non-toxic against the tested cell lines. The results indicated that these compounds are not directly involved in the strong cytotoxicity of extracts from *P. macrocarpa* fruits. Further investigation on the isolation and cytotoxicity of new compounds from *P. macrocarpa* fruits can be conducted in order to find the specific anticancer agent that contribute to the good cytotoxicity of extracts from this plant.

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