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Cytotoxic and Apoptotic Effects of Para-Hydroxy Meta Methoxy Chalcone (*p*H*m*MC) on T47D Breast Cancer Cells*

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ABSTRACT
para-Hydroxy meta methoxy chalcone (pHmMC), a chalcone derivate of 3 - (4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propene-1-on, was investigated on its cytotoxicity, apoptosis induction, and Bcl-2 expression against human breast cancer-T47D cell lines. The cytotoxic effect was analysed using MTT [3-(4,5 dimethyltiazol-2-yl)-2,5-diphenyltetrazoilium bromide] assay, the apoptotic effect was determined by Annexin-V flowcytometry method, and the expression of Bcl-2 was identified using immunocytochemistry method. The research results showed that the pHmMC's IC_{50} on T47D cells was 48 □M, and it increased the apoptotic induction and decreased the Bcl-2 expression level.

Keywords: 3 - (4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propene-1-on (*p*H*m*MC), cyto-toxic effect, apoptosis induction, Bcl-2 expression, T47D breast cancer cells

INTRODUCTION

Cancer is the second most fatal disease after heart attack. Breast cancer is one type of cancer that has high prevalence and the second most common cancer worldwide which causes mortality[1]. In Indonesia, breast cancer is the second most frequent one in women after cervical cancer[2]. Current cancer therapies (surgery, radiation, immunotherapy, and chemo-therapy)[3] lead to undesired physical and psychological distress to the patients. So that researches on finding new anticancer compounds that have better therapeutic efficacy and fewer side-effects are still needed. Chalcones (derivates of trans-1, 3-diaryl-2-propen-1-on)[4], are biosynthetic products of the shikimate pathway and are precursors for biosynthesis of open chain flavonoids and isoflavonoids. These compounds are abundant in edible plants. Chalcones can be synthesized through an aldol condensation reaction between benzaldehyde and acetophenone in the presence of strong bases. There are also some reports of chalcone production through an acid-catalysed aldol condensation[5]. Chalcone and its derivates have been attracting attention due to their numerous pharmacological applications. They have displayed a broad spectrum of pharmacological activities, one of them is as anticancer[6-10]. Many chalcones and their derivates have been shown to induce apoptosis in different types of cancer cells through a wide variety of mechanisms. These compounds have a common target, the Bcl-2 protein, which can induce apoptosis in cancer cells[11-14]. A chalcone derivate compound, *p*H*m*MC or 3 - (4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propene-1-on was synthesized by reaction between vanillin and acetofenon through a cross-aldol condensation reaction under acidic conditions[15]. A study reported that this compound inhibited the cell cycle of breast cancer cell lines (T47D and MCF-7)[16] through the phase of G_2/M, and induced apoptosis in MCF-7[17]. However, there has been no research on how it affects apoptosis and Bcl-2 protein expression in T47D cells. In this study, the effects of cytotoxicity, apoptosis induction and Bcl-2 expression of *p*H*m*MC on breast cancer cell lines T47D were investigated.

MATERIAL AND METHODS

*Material: p*H*m*MC *Solution*

para-Hydroxy *meta* methoxy chalcone (*p*H*m*MC) was obtained from Prof. Indyah Sulistyo Arty. The compound was produced through a cross-aldol condensation reaction of vanillin and acetofenon in an acidic condition. The compound was used as a stock solution with concentration of 100 mg/ml in dimethylsulfoxide (DMSO). The final concentration of DMSO in the study wells was kept less than 0.1%.

T47D *Cell Culture*

Human breast cancer cells of T47D cell-line was obtained from the collection of the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada (UGM). The cells were grown in medium culture of DMEM (Dulbecco's modified Eagle's Medium) from Gibco which containing 10% FBS (Fetal Bovine Serum, Gibco) and 1% penicillin-streptomycin (Gibco), and was incubated at 37ºC in a humidified atmosphere of 5% CO_2-95% air. Trypsin-EDTA 0.025% (Gibco) solution was used to detach cells on the flask.

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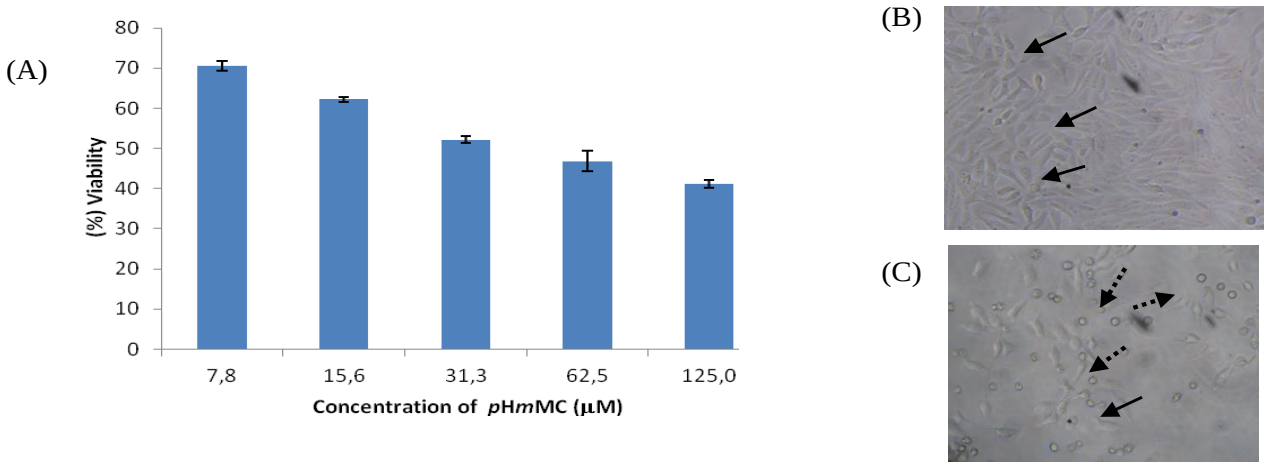


Figure 1: The cytotoxic effect of *p*H*m*MC on T47D cells was determined using MTT assay. The studies were conducted by incubating 5x10^3 cells in 96 well plates for 24 hours in DMEM medium without or with *p*H*m*MC treatments of 7.8; 15.6; 31.3; 62.5 and 125 □M. The profile of cell viability is expressed in mean ± SD from three experiments (A). The morphological changes and cell populations under the treatment of *p*H*m*MC concentrations of 0 □M or control (B) were compared to that of 31.3□M (C).

Cytotoxic Assay

Cytotoxic assay was conducted using [3-4, 5-dimethyltiazol-2-yl) - 2,5 diphenyltetra-zoilium bromide] (MTT) assay. The T47D cells were seeded and plated on a 96 tissue culture well-plate with cell density of 5 x10^3 cells per well. Each well then was added with various concentrations of *p*H*m*MC, and incubated for 24 hours. After 24 hour-incubation, the medium was removed from each well and the cells were washed with Phosphate Buffered Saline (PBS) of Sigma, and then were added with 100 □l of solution of 0.5 mg/ml MTT (Sigma) in the culture medium. The treated cells were then stayed for 4 hours to proceed the production of formazan (the indicator). A stopper reagent of 10% Sodium dodecyl sulphate (SDS) of Sigma in 0.01M HCl (Merck) was added and incubated for 12 hours or overnight at room temperature and protected from light. The absorbance of each well was measured using ELISA reader (Bio-Rad) at λ 595 nm. The absorbance was converted to a percentage of viable cells[18]. The IC_{50} value was calculated by the concentration that caused 50% inhibition of cell growth[19]. Calculations of the IC_{50} value were done using the linear regression of log concentration versus cells viability.

Apoptosis Analysis

T47D cells were seeded on a six tissue culture well-plate at 5 x 10^5 cells per well. After 24 hours incubation the cells were treated with various cincentration of *p*H*m*MC. After incubation the cells were removed using 0.25% trypsine solution and then spinned at 2000 rpm for 3 minute, and then washed twice with cold PBS. The cells were re-suspended in 500 □l of Annexin V buffer (Roche) and then treated with Annexin V and propidium iodide (PI) for 10 minutes at room temperature and protected from light. The treated cells were subjected to a FAC-Scan flow-cytometer. Bivariant analysis of FITC-fluorescence (FL-1) and PI-fluorescence (FL-3) gave different cell populations where the phenotype of FITC (-) and PI (-) were designed as viable cells, while the FITC (+) and PI (-) as early apoptotic cells; the FITC (-) and PI (+) as necrotic cells; and the FITC (+) and PI (+) as late apoptotic cells.

Immunocytochemistry method

T47D cells were seeded on the coverslips of a 24 well-plate at 5 x 10^4 cells per well and incubated for 24 hours (or until 80% confluent). The medium in each well was then replaced by the fresh medium containing various concentrations of *p*H*m*MC and then placed in a humidified incubator at 37 ˚C within an atmosphere of 5% CO_2 and 95% air for 24 hours. The cells were then harvested and were washed with PBS and fixed with cold methanol for 10 minutes at -4°C freezer. After that, the cells in coverslips were placed each on a respective slide. The cells were washed with PBS and distilled water, then were blocked in a hydrogen peroxide blocking solution for 10 minutes at room temperature. They were again washed with PBS, and then incubated with pre-diluted blocking serum for 10 minutes at room temperature. Next, the cells were stained with primary Bcl-2 antibody for 1 hour at room temperature. After three time-washing with PBS, the secondary antibody was applied for 15-20 min, and then washed with PBS three times. The slides were incubated with streptavidin-biotin complex for 10 minutes, and then washed with PBS three times. The slides were incubated in DAB (3, 3 diaminobenzidine) solution for 3-5 minutes and washed with distilled water. Cells were counterstained with Mayer-Haematoxilin reagent for 3-4 minutes. After incubation, the coverslips were washed with distilled water and then immersed in absolute ethanol and in xylol afterward. The protein expression was assessed under a light microscope. Cells which with expression give a dark brown color in the cell membrane, while the cells with no expression will give purple color.

RESULTS AND DISCUSSION

The cytotoxic effect of pHmMC on T47D cells

The viability of T47D cells were measured using MTT assay. This assay was done to determine the inhibitory

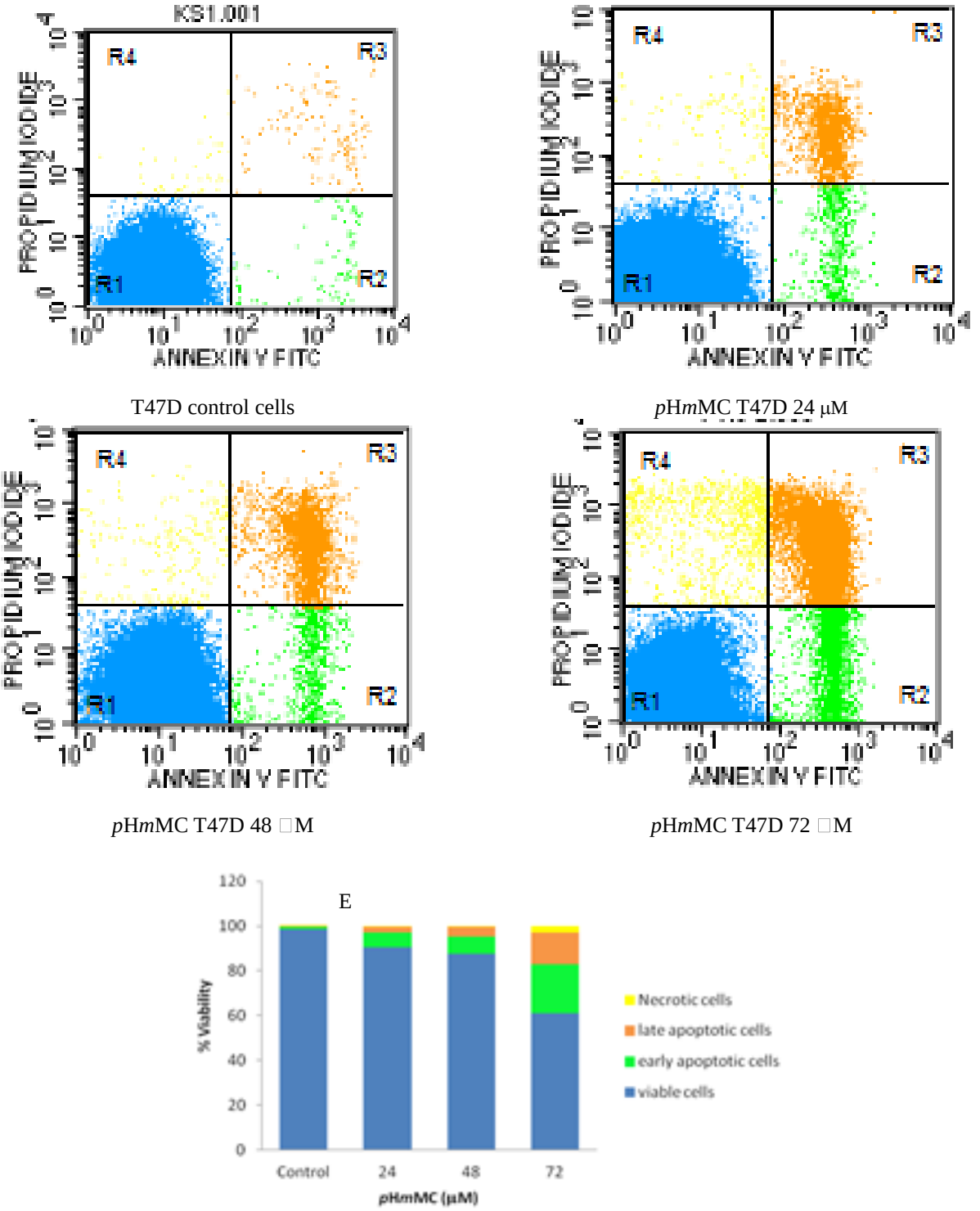
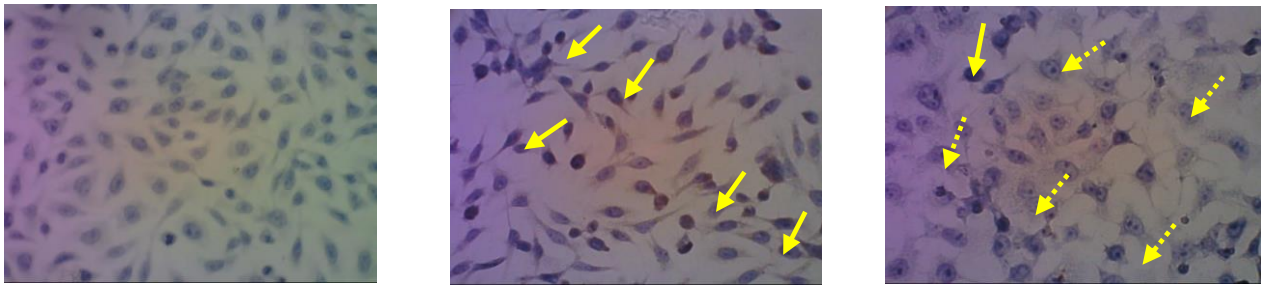


Figure 2: The effect of *p*H*m*MC on the apoptosis induction of T47D cells. The cells were seeded at $5x10^5$ cells/well on six wells tissue culture plate, then treated with *p*H*m*MC concentrations of 0, ½ IC_{50}, IC_{50}, and 1½ IC_{50}. After 24 hours incubation, cell were harvested as described in the methodology, added with AnnexinV and PI reagents, were then subjected to FACS flow-cytometer. The flowcytometric profiles of cells were treated respectively with 0 µM or control (A), 24 µM (B), 48 µM (C), and 72 µM (D).

concentration (IC_{50}) of the *p*H*m*MC treatment. The result showed that all treatments of the *p*H*m*MC inhibited the cell growths (Fig.1). Based on the *p*H*m*MC treatments and the cell viabilities affected, there was a linear correlation between the *p*H*m*MC treatment - concentrations of 7.8; 15.6; 31.3; 62.5 and 125 µM versus their toxicity effects; with the IC_{50} of 48 µM ($P<0.05$). The *p*H*m*MC treatment led to T47D cell morphological changing. The cytotoxic effect could be related to an apoptotic induction effect.



A B C

Figure 3: The expression of Bcl-2 on T47D cells was determined using immunocytochemistry method. (A) is the control cells without antibody, (B) control cell with Bcl-2 Ab, and (C) *p*H*m*MC 24 □M.

The pHmMC effect on apoptosis induction

The apoptotic induction effect of *p*H*m*MC treatment on T47D cells was determined using flow-cytometric method. The method detects the translocations of phosphatidyl serine-residues which are in the internal phospholipid layer (in normal cell) and at the external layer (in apoptotic cells). The result showed that *p*H*m*MC induced the T47D cell apoptosis at early and late phases of cell apoptosis with rates as shown in Fig.2. The percentage of apoptotic cells increased after treatment with *p*H*m*MC. Increasing in concentration of *p*H*m*MC treatment causes more T47D cells undergo apoptosis.

The effect of pHmMC on Bcl-2 expression

To confirm the mechanism of *p*H*m*MC's induced apoptosis, a study on the effect of *p*H*m*MC on the expression of Bcl-2 was conducted using an immunocytochemistry method. Bcl-2 is a protein which suppresses cell apoptosis. Interestingly, the expression of Bcl-2 of the *p*H*m*MC treated cells was lower compared to that of the control cells (Fig.3). These data showed that *p*H*m*MC has potency to induce cell apoptosis by suppressing the Bcl-2 expression. The research results showed that compound *p*H*m*MC has anticancer potency, especially through its inhibition on the cell proliferation. It has cytotoxic effect to human breast cancer cell-lines T47D with IC_{50} of 48 □M. Prayong[20] mentioned that compound with IC_{50} less than 100 µM is potentially to be developed as anticancer agent. The cytotoxic effect of *p*H*m*MC could be related with its ability to accelerate the cell apoptosis, as shown by the data that *p*H*m*MC induced apoptosis in T47D cells. Apoptosis is a programmed-cell death which is characterized by changes on cell morphology, membrane blabbing and chromatine[21]. In this study, the characteristic changes of cell morphology, such as cell shrinking, nuclear fragmentation, and cell apoptosis were more extensive after the treatment with *p*H*m*MC (Fig.3). The flow-cytometry analysis showed that treatment with *p*H*m*MC at 48 □M increased the cell death from 1.26% to 11.28%, and increased up to 36.1% after treatment with 72 □M *p*H*m*MC. These data indicated that *p*H*m*MC compound is able to induce apoptosis. Ability in inducing apoptosis on cancer cells is an important property for a prospective anticancer drug because cancer cells generally are capable to evade apoptosis. Apoptosis process occurs via various mechanisms. NF-□B protein inhibits apoptosis through increasing transcription of Bcl-2 that prevents the release of cytochrome c by Bax in mitochondria[22]. The inhibition of NF-□B also causes decreasing of Bcl-2 and Bcl-XL expressions, two main anti apoptotic proteins. The study showed that the *p*H*m*MC treatment decreased the level of Bcl-2 which in turn induced the T47D cell's apoptosis. Some references stated that the main structure of chalcones, that is 1,3-diphenyl-2-propenone, has been proven to have a chemopreventive effect on human breast cancer cell lines of MCF-7 and MDA-MB-231[23] and human bladder cancer cell lines of T24 and HT-1376[24]. It was showed to inhibit the proliferation of T24 and HT-1376 cells by inducing apoptosis and blocking cell cycle progress at G2/M phase. It significantly increased the expression of p21 and p27 proteins, and decreased the levels of cyclin B1, cyclin A and Cdc2, which contribute the cell cycle arrest. Moreover it increased the expression of Bax and Bak, and decreased the levels of Bcl-2 and Bcl-XL which subsequently triggered mitochondrial apoptotic pathway via releasing cytochrome c and activating caspase-9 and caspase-3. The bold arrow indicates normal living cells, whereas the dashed arrows indicate the cell morphology changes. The cell morphology was observed using inverted microscope with 100x magnification. There are four quadrants: the lower left (R1), marked in blue, indicates viable cells, the lower right (R2), marked in green, indicates early apoptotic cells, the upper right (R3), marked in orange, indicates late apoptotic cells, and the upper left (R4), marked in yellow, indicates necrotic cells. The graph (E) showed that *p*H*m*MC induced apoptosis. The experiment used DAB as the chromogen. Dark brown staining in cell's cytoplasm and mitochondria membrane indicated the positive expression of Bcl-2. Cells which showed purple colour after counterstained with Mayer-Haematoxilin indicated negative expression of Bcl-2. The bold arrow indicates the positive expression, whereas the dashed arrows indicate the negative expression of the protein. Chalcone has also an inhibitor activity to Nuclear factor kappa B (NF-κB) at concentration of 50 □M. NF-κB is a transcription factor that plays a major role in development and progression of cancer because it regulates more than 400 genes involved in inflammation, cell survival, cell proliferation, invasion, angiogenesis, apoptosis, cell cycle and metastasis[25,26]. The study's compound, *p*H*m*MC, is a chalcone derivate that has -3'

methoxy and -4’ hydroxyl phenyl moieties. Base on the references, those structure modifications still retain the cytotoxic activity of chalcones. It was showed that increased apoptosis induction via suppressing Bcl-2 level in T47D cells. So that *p*H*m*MC may have potential effects on the NF-κB survival system which play important roles in the anti-proliferative activity on the T47D cells. The detail molecular mechanism of the apoptotic induction is still needs to be explored.

CONCLUSION

para-Hydroxy *meta* methoxy chalcone (*p*H*m*MC) has properties of suppressing on the expression of Bcl-2 and inducing apoptosis of T47D cells. It is a potential compound to be explored and developed as a chemopreventive agent for breast cancer.

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