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Cytotoxicity Activity of Biotransformed Ethyl *p*-methoxycinnamate by *Aspergillus niger*

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ABSTRACT

The extraction of *Kaempferia galanga* rhizome using steam distillation and supercritical fluid extraction (SFE) was carried out. After fractionation, the major compound of the *K. galanga* extract, ethyl *p*-methoxycinnamate (EPMC) was transformed using *Aspergillus niger* into ethyl p-hydroxycinnamate (EPHC). The biological anticancer activity of EPMC and its biotransformed product (EPHC) was established by cytotoxicity activity on the human breast cancer (MCF-7) cell line using MTT assay. Ethyl p-hydroxycinnamate (EPHC) was most cytotoxic against MCF-7 at 1000 µg/mL where percentage of cell viability was 9.87 %, while IC_{50} was 340 µg/mL. EPHC showed slightly higher cytotoxicity activity compared to EPMC. The results of this study show that the biotransformation process was able to produce a metabolite (EPHC) with higher cytotoxicity activity compared to its parent compound (EPMC).

Keywords: *Kaempferia galanga,* ethyl *p*-methoxycinnamate, biotransformation, cytotoxic.

INTRODUCTION

Microbial transformation has been extensively used to create new metabolites from natural product constituents. This transformation process can be used as an alternative to chemical synthesis for the preparation of pharmacologically active compounds[1-4]. Biotransformation using *Aspergillus niger* has been used to transform asiaticoside to produce a product with excellent wound healing properties[5]. Other studies have reported that the biotransformed product of ethyl *p*-methoxycinnamate exhibited antimicrobial properties against selected bacteria and fungus[6,7].

Malaysian *Zingiberaceae* plants have been studied extensively due to their pharmaceutical properties. These include plant species from *Alpinia*[8], *Zingiber*[9,10], *Galanga*[10] and *Kaempferia*[6,7]. Cytotoxicity studies show that *Kaempferia galanga*

extracts inhibited the proliferation of human cervical cancer C33A cell line[11]. In another study, the methanolic extract of *K. galanga* rhizomes reportedly contained ethyl-*p*-methoxycinnamate, which is highly cytotoxic to HeLa cells[12]. Ethyl p-methoxycinnamate has been reported to possess many biological properties such as anticancer[13] and anti-monoamine oxidase activities[14]. Recently, Jagadish and his co-researchers reported that successive ethyl acetate extract of *K. galanga* showed selective toxicity against four types of cancer cells[15].

Thus, this study aims to evaluate the *in vitro* cytotoxicity of ethyl *p*-methoxycinnamate and ethyl *p*-hydroxycinnamate against the human breast cancer (MCF-7) cell line in order to screen their potential as anti-cancer agents.

MATERIALS AND METHOD

Chemicals for Cell Culture

Human breast cancer cell line (MCF-7) was obtained from the Kulliyyah of Pharmacy, IIUM Kuantan, Malaysia**.** Phosphate buffer saline (PBS, GIBCO), Dulbecco's modified eagle medium (DMEM, GIBCO) and trypsin solution (GIBCO) were obtained from Fisher Scientific Shah Alam Malaysia, while fetal bovine serum (FBS), Thiazolyl blue tetrazolium bromide (MTT) stock solution and 90% methanol were obtained from Sigma-Aldrich Subang Jaya Malaysia.

Plant Materials

K. galanga rhizomes were obtained from Taman Pertanian Jubli Perak Sultan Haji Ahmad Shah Kuantan Malaysia. The rhizomes were washed and sliced before drying in the vacuum oven (Memmert, Manchester) at 45°C for 5 days until the samples were completely dry. Then, the samples were ground using a blender and stored at -4°C prior to further analyses.

Extraction and fractionation of ethyl p-methoxycinnamate (EPMC)

The powdered rhizomes of *K. galanga* were extracted using steam distillation and supercritical fluid extraction (SFE) according to previously reported methods[6,7,9]. For fractionation of ethyl *p*-methoxycinnamate (EPMC), the essential oil was stirred with boiling water and then recrystallized at cold temperature (-4°C). After crystallization, the mixture was filtered and the crystal was kept in the desiccator for 24 hours prior to further analysis.

Fungus culture preparation and biotransformation procedure

The culture preparation and biotransformation was carried out according to methods previously reported[6,7]. The fungus *A. niger* was streaked on SDA at 30°C for a week and stored at 4°C. After cultivation, the well grown mycelia were placed in a conical flask (250 mL) and inoculated with 10.0 mL of sterilized medium broth containing glucose, glycerol, peptone, yeast extract, KH_2PO_4, NaCl and distilled H_2O. The flask was incubated at 30°C for 48 hr at 120 rpm. Ethyl *p*-methoxycinnamate, EPMC (480 mg) was dissolved in dimethyl sulfoxide (DMSO) (24 mL) and distributed among 48 flasks containing 48 h stage culture media and continuously shaken for 24 h using a rotary shaker (120 rpm) at 30°C. After incubation, the culture media and mycelium were separated using cotton in a funnel. Then, mycelium was washed with ethyl acetate (1.5 L) while the culture media was extracted 3 times with ethyl acetate (1.5 L). The biotransformed products were isolated by column chromatography using silica gel column (200 - 300 mesh, Merck Ltd.) with hexane : ethyl acetate as solvent[7].

MTT assay

The human breast cancer cell line (MCF-7) was maintained in DMEM containing 2 % FBS and grown in 6 cm^2 tissue culture dishes until confluent[11,15]. After confluence, the cells were washed using PBS to remove the FBS. Then, after the addition of trypsin (1 mL), the dish was shaken and incubated at 37°C under 5 % CO_2 for 3 min to detach the cells from the flask surface. After adding 4 mL of DMEM, the dish was shaken and the cells were divided equally into two new 60-mm culture dishes assigned to the EPMC and EPHC assays. The volumes of the new petri dishes were made up to 5 mL using DMEM and the cell was incubated for 48 hr at 37°C under 5 % CO_2. Finally, the cell mixture (100 µL) was added into each well of a 96-well plate and incubated for 24 hr at 37 °C under 5 % of CO_2.

The cytotoxic assay was carried out using the two-fold broth microdilution method and

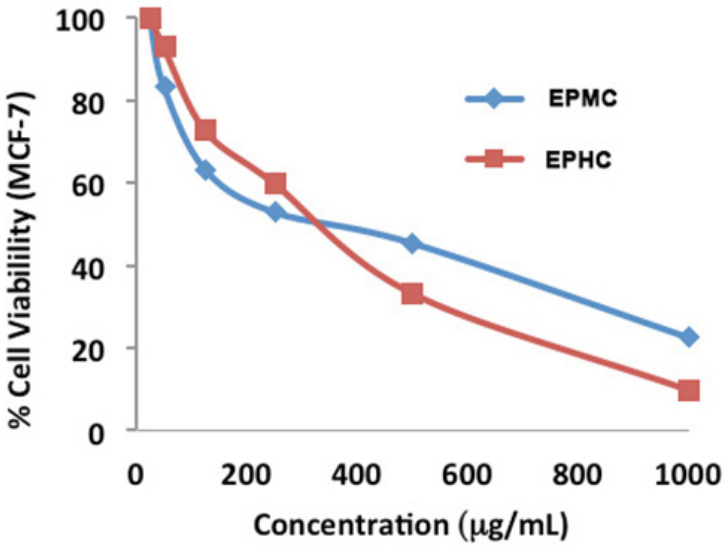

Fig. 1: Cell viability of the human breast cancer (MCF-7) cell line at different concentrations of EPMC and EPHC using MTT assay

performed using sterile 96-well flat bottom plates. 7 µg of EPMC (or EPHC) was added to 1393 µL of DMEM in the first well to a concentration of 0.5 %. Then, the samples were diluted using two-fold serial dilution to a final concentration of 1000, 500, 250 and 125 µg/mL. The diluted samples were transferred to 96-well plates containing MCF-7 cells and incubated at 37°C for 24 hours. Colorimetric MTT assay was carried out as described by Mosmann[16]. After 24 h, 30 µL of MTT solution was added to the wells and left in the incubator at 37°C for 3-4 hr. This was followed by the addition of 150 µL of DMSO into each well to stop the reaction. The plate was then read using a 96-well micro plate reader at a wavelength of 570 nm within 1 h after the addition of DMSO.

RESULTS AND DISCUSSION

MTT Assay and minimum inhibitory concentration

Figure 1 shows the percentage of cell viability using MTT assay and the half maximal inhibitory concentration (IC_{50}) of EPMC and EPHC against the human breast cancer (MCF-7) cell line. The IC_{50} value is the concentration of the sample where the cell viability was at 50 %. Based on the results shown in Figure 1, the IC_{50} of EPMC was 360 µg/mL, and in EPHC was 340 µg/mL. The ability of microorganisms to modify natural products into other more bioactive compounds that exhibit higher activity compared to their parental compound has attracted a great deal of attention in recent years. Several reports of biotransformed products screened for their activity and compared against the parental compound are present in literature. For example, the biotransformed product of asiaticoside was reported to have better wound healing activity compared to its parental compound[5], and several biotransformed products showed better antimicrobial properties than their parental compound[6]. In this study, the EPHC as the biotransformed product was compared against its parent compound EPMC for activity. MTT was carried out to determine the cytotoxicity of ethyl *p*-methoxycinnamate (EPMC) and its biotransformed product ethyl *p*-hydroxycinnamate (EPHC) against the MCF-7 human breast cancer cell line. The assay was carried out to identify the minimum concentration of compound that could inhibit cell growth or in this case cause cell viability to decrease. Ethyl *p*-hydroxycinnamate (EPHC) showed higher cytotoxicity against the MCF-7 cell line since it lowered percentage of cell viability to 9.87 % compared to ethyl *p*-methoxycinnamate (EPMC), where cell viability was 22.58 % at the highest concentration (1000 µg/ml). However, both compounds showed cytotoxic activity against the MCF-7 cell line at all concentrations used in the assay.

The half maximal inhibitory concentration (IC_{50}) which is the measure of the effectiveness of a compound in inhibiting biological or biochemical functions was determined. The readings were taken by measuring the concentration of the sample when the cell viability was at 50 %. By extrapolating the results obtained in Figure 1, it can be said that EPHC is more cytotoxic than EPMC as its IC_{50} value against MCF-7 was 340 µg/mL, while the IC_{50} of EPMC was higher at 360 µg/mL. Therefore, from the results obtained, it can be concluded that Ethyl *p*-hydroxycinnamate (EPHC) is the more potent cytotoxic agent since a lower concentration is required to inhibit at least 50 % growth of the MCF-7 cancer cell line.

CONCLUSION

Both compounds (EPMC and EPHC) were active and exhibited good inhibition potential against MCF-7 cell lines. Ethyl *p*-hydroxycinnamate (EPHC) resulted in lower cell viability against the MCF-7 cell line at high concentrations of 1000 µg/mL compared to ethyl *p*-methoxycinnamate (EPMC). The IC_{50} value of EPHC was 340 µg/mL against the MCF-7 cell line. Therefore, the results of cytotoxicity studies and the IC_{50} values demonstrate the potent selective toxicity property of ethyl *p*-hydroxycinnamate against the breast cancer cell line. In conclusion, the biotransformed product, ethyl *p*-hydroxycinnamate (EPHC) has good potential as an anti-cancer agent indicated by higher positive results compared to its parental compound, ethyl *p*-methoxycinnamate.

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