In vitro cytotoxicity of the Synthesized Gallic Acid Derivatives (N-Alkyl Gallamide) Against Breast MCF-7 Cancer Cells

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ABSTRACT

Gallic acid is a phenolic compound distributed in plants and fruits which has been reported to have cytotoxic effect on MCF-7 breast cancer cell line. In this research, we investigated in vitro cytotoxic effect of six synthesized compounds of gallic acid derivatives (N-alkyl gallamide), namely N-methyl gallamide (2); N-ethyl gallamide (3); N-butyl gallamide (4); N-sec-butyl gallamide (5); N-tert-butyl gallamide (6) and N-hexyl gallamide (7) against breast MCF-7 cells by MTT assay. Linear regression analysis is utilized to analyze data to regenerate IC₅₀ value. The results will be compared with gallic acid as an original compound and doxorubicin as a positive control. Among six synthesized compounds, N-tert-butyl gallamide (6) with IC₅₀ value of 2.1 µg/mL, and N-hexyl gallamide (7) with IC₅₀ value of 3.5 µg/mL, showed the stronger cytotoxicity against breast MCF-7 cells compared to gallic acid and doxorubicin. Thus, N-tert-butyl gallamide (6) and N-hexyl gallamide (7) are potential to be further developed as a promising anti-breast cancer agents.

Keywords: Cytotoxicity, Gallic acid derivative, N-alkyl gallamide, Breast MCF-7 cells, MTT assay.

INTRODUCTION

Breast cancer is a frequent diagnosed malignancy in females, and considerably the second most common human carcinoma in the world. In 2012, there were approximately 1.67 million of breast cancer cases which has increased by more than 20% since the 2008 estimates. It ranks fifth as a cause of death globally with estimated number of 522,000 deaths (13 per 100,000 population) during
Moreover, the death rate of breast cancer in women is higher in less developing areas (324,000 breast cancer deaths in 2012), and lower death rate in more developing areas (198,000 breast cancer deaths in 2012). Indonesia accounted for 17% of female breast cancer deaths within the Asia-Pacific Region in which 116,000 deaths occurred. The prognosis for female breast cancer depends on the stage. A five-year relative survival rate reaches until close to 100% for stage 0 and stage 1. This rate moderately declines to 93% and 72% for stage II and stage III consecutively. Furthermore, five-year relative survival rate for female breast cancer patient on stage IV with metastatic is sharply drop to around 22%. Treatment choices for breast cancer include surgery, chemotherapy, and radiotherapy, however these treatments inconstantly successful and could produce unexpected side effects. Therefore, in order to improve the survival rate of patient as well as to reduce the side effects of the breast cancer treatment, the search for new molecules which show a stronger therapeutic reaction with minimum side effects compared to established ones are needed. Gallic acid (1) is a phenolic acid also known as 3,4,5-trihydroxybenzoic acid that has water soluble property and various biological activities, including an inhibitory effect against human breast cancer cells. Previously, studies have revealed that gallic acid prompted apoptosis in cancer cells as an accompaniment of oxidative stresses from mitochondrial dysfunction, Reactive oxygen species (ROS), and an incline in intracellular Ca\textsuperscript{2+} level. The length of alkyl chain in ester side chain of gallic acid derivatives influenced the hydrophobicity and physicochemical properties of the derivatives. Alkyl ester gallates with longer alkyl chain would become more hydrophobic that will increase its affinity for the lipid bilayer of the cell membrane which allow the alkyl ester derivatives penetrate easier into the cell membrane of cancer cells than gallic acid. As evidence, the ester derivatives presented more favorable pharmacological properties than those for gallic acid itself due to enhancement of carbon atom length in alkyl ester side chain. However, a longer carbon chain in alkyl ester moiety causes a greater molecular weight of hydrophobic compound that less dissolved in water. Hence, molecular mechanisms underlying the action of gallic acid remain questionable. In the present study, we have done the synthesis of gallic acid derivatives as anti-hepatitis C virus agents. Recently, we also accomplished in silico docking study of gallic acid derivatives as inhibitor of malarial dihydrofolate reductase, as an inhibitors of hepatitis C virus receptor binding target NS5B, as an inhibitor of Bcl-xl anti-apoptotic protein of breast cancer, and as an inhibitor of BRAF colon cancer. In this work, as to continue research in developing gallic acid derivatives as an anticancer agent, we focused on the cytotoxicity evaluation of N-alkyl gallamide, compound 2-7 (Fig. 1) against MCF-7 cells by MTT cell proliferation assay. The synthesis of gallic acid derivatives, N-alkyl gallamide (compound 2-7) applied in this study, have been completed recently by our research team. These alkyl amide derivatives of gallic acid demonstrated a strong cytotoxic effect on colon HCT-116 cells.

**EXPERIMENTAL**

**Synthesis of derivative 2-7**

As reported previously, six gallic acid derivatives, compound 2-7 (Fig. 1) were designed by replacing carboxyl group of gallic acid with a series of corresponding alkylamines to generate N-alkyl gallamide 2-7. Four compounds, namely derivative 2, 3, 4 and 7, were designed to have a linear aliphatic carbon chain in N-alkyl benzamide group, whereas compound 5 and compound 6 were designed to have a branched alphatic carbon chain. As summarized in Fig. 2, amidation of gallic acid (1) with methylamine, ethylamine, butylamine, sec-butylamine, tert-butyl amine, and hexylamine using a combination of WSCD.HCl/HOBt in the presence of basic NMM afforded the corresponding N-alkyl gallamides, which are N-methylgallamide (2), N-ethyl gallamide (3), N-butyl gallamide (4), N-sec-butyl gallamide (5), N-tert-butyl gallamide (6) and N-hexyl gallamide (7) with a yield ranging from 18% to 84%.
MTT Assay in Determining the Cytotoxicity

For the MTT assay, 1 x 10⁶ cells/well were seeded into a 96-well cell plate in a total volume of 100 µL for 24 hours. The medium was then removed, and the cells were treated with the following N-alkyl gallamides: N-methyl, N-ethyl, N-butyl, N-sec-butyl, N-tert-butyl, N-hexyl gallamides at concentrations ranging from 1.5 to 200 µg/mL. After 24 h incubation, the medium was removed while 100 µL of MTT was added to each well, followed by a 2- to 4- h incubation period. MTT solution was then discarded and added with 100 µL DMSO. The absorbance was then measured at 630 nm using ELISA reader. Data of cell growth inhibition were provided as the average ± standard deviation, whereas the Inhibition rate (Ir) can be calculated using formula:

\[ Ir = 1 - \left( \frac{\text{absorbance of treatment group}}{\text{absorbance of control group}} \right) \times 100\%
\]

This procedure was triplicated for each plate. The data obtained will be processed using Microsoft Excel 2016 by extrapolating the concentration of N-alkyl gallamide (in x-axis) versus the percentage of breast cancer cell inhibition (in y-axis), where a linear regression was then created. IC₅₀ value was determined using probit method by plotting the concentration of the sample against percentage of Inhibition.

RESULT AND DISCUSSION

In vitro cytotoxicities of 3,4,5-trihydroxy-N-alkyl-benzamides against breast MCF-7 cancer cell lines displayed in Table 1. Cytotoxicities expressed by half maximal inhibitory concentration (IC₅₀) value. The lower IC₅₀ value the stronger cytotoxic activity. Atjanasuppat et al., reported that cytotoxicity of compound were categorized into four groups based on IC₅₀ value. IC₅₀ value below or equal 20 µg/mL, strong cytotoxic activity; IC₅₀ value in range of 20 to 100 µg/mL, moderate cytotoxic activity; IC₅₀ value ranging from 100 to 1000 µg/mL, weak cytotoxic activity; and IC₅₀ value over than 1000...
µg/mL, shows no cytotoxic activity.\textsuperscript{14} From the result of this experiment, as shown in Table 1, all N-alkyl gallamides (compound 2-7) exhibited a strong cytotoxic activity against breast MCF-7 cancer cells as all of the IC\textsubscript{50} values were below 20 µg/mL.

Effect of chain length in cytotoxic property

As shown in Table 1, generally the cytotoxicity of derivatives that have a linear aliphatic carbon chain in N-alkyl gallamides greatly improved by addition of carbon chain in N-alkyl moiety. It can be observed from IC\textsubscript{50} value of N-methyl gallamide 2 (IC\textsubscript{50}: 12.6 µg/mL), -N-ethyl gallamide 3 (IC\textsubscript{50}: 11.3 µg/mL), -N-butyl gallamide 4 (IC\textsubscript{50}: 10.3 µg/mL) and N-hexyl-gallamide 7 (IC\textsubscript{50}: 3.5 µg/mL) consecutively, which are decreasing by addition of carbon chain in N-alkyl-benzamide group. This suggested that as the carbon chain is longer, the derivative become more non-polar, its permeability towards cell membrane was greatly increased, which lead to the increases of its cytotoxicity against MCF-7 cancer cells.

Effect of chain branching in cytotoxic property

Derivatives with a branched carbon chain in N-alkyl benzamide group shows a greater cytotoxicity compared to derivatives with a linear carbon chain. It can be observed on the branched-chain four-carbon of N-sec-butyl gallamide 5 with IC\textsubscript{50} value of 8.4 µg/mL, and N-tert-butyl gallamide 6 with IC\textsubscript{50} value of 2.1 µg/mL, which have a greater cytotoxicity than N-butyl gallamide 4 (IC\textsubscript{50}: 10.3 µg/mL) which also has four-carbon chain, but with a linear structure.

Another study that gave similar results was a study conducted by Frey \textit{et al.}, which proved that isobutylylgallate had lower IC\textsubscript{50} value compared with butyl gallate against sarcoma 786A,TA3 and TA3-MTX-R tumor cells. Correspondingly, cytotoxic activity of isoamyl and isopropyl gallate were stronger than the cytotoxicity of amyl and propyl gallate.\textsuperscript{15}

The addition of branch into the derivative of gallic acid will change the solubility. Chain branching allows the compound to be more lipophilic owing to the decreasing the polarity, thus making it easier to penetrate the lipid bilayer.\textsuperscript{16}

Among the six synthesized derivatives, N-tert-butyl gallamide 6 and N-hexyl gallamide 7 showed a greater cytotoxicity against breast MCF-7 cells than gallic acid (IC\textsubscript{50}: 7.5 µM) and doxorubicin (IC\textsubscript{50}: 10.6 µM) as a positive control. These results indicated that modifying the carboxyl group of gallic acid become a branched-alkyl chain with four carbon atom in N-tert-butyl gallamide 6, and become a linear-alkyl chain with six carbon atom in N-hexyl gallamide 7, have successfully improved its cytotoxicity against breast MCF-7 cancer cells. Thus, N-tert-butyl gallamide 6 and N-hexyl gallamide 7 are potential to be further developed as anti-breast cancer drugs.

Table 1: Cytotoxicities (IC\textsubscript{50} in µg/mL) of N-alkyl gallamides 2-7, gallic acid (1), and doxorubicin against breast MCF-7 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxicity (IC\textsubscript{50}, µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid (1)</td>
<td>7.5</td>
</tr>
<tr>
<td>N-methyl gallamide (2)</td>
<td>12.6</td>
</tr>
<tr>
<td>N-ethyl gallamide (3)</td>
<td>11.3</td>
</tr>
<tr>
<td>N-butyl gallamide (4)</td>
<td>10.3</td>
</tr>
<tr>
<td>N-sec-butyl gallamide (5)</td>
<td>8.4</td>
</tr>
<tr>
<td>N-tert-butyl gallamide (6)</td>
<td>2.1</td>
</tr>
<tr>
<td>N-hexyl gallamide (7)</td>
<td>3.5</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>10.6</td>
</tr>
</tbody>
</table>

CONCLUSION

Six synthesized gallic acid derivatives of N-alkyl gallamide have been evaluated on their basis of cytotoxicity against breast MCF-7 cells. Among six derivatives, N-tert-butyl gallamide (6) and N-hexyl gallamide (7) showed a greater cytotoxicity against breast MCF-7 cells.

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