Cytotoxic Activity of Ethyl Acetate Fraction of Aglaia elliptica Blume Leaves Extract on HepG2 Hepatocarcinoma Cells

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Abstract

Aglaia elliptica Blume belongs to Meliaceae family which contain an active compound of rocaclamid as anticancer. The research was conducted to evaluate the biological activities of ethyl acetate fraction of Aglaia elliptica Blume leaves. The dry powder of Aglaia elliptica Blume leaves was extracted with methanol using maceration method, then was fractionated using n-hexane and ethyl acetate, guided by brine shrimp lethality test (BSLT). The most toxic fraction then processed by column chromatography and resulted in eight subfractions. Three of them showed the most toxic effect on BSLT namely FEA 3.3, FEA 3.4 and FEA 3.5 with LC50 of 40.81, 18.56 and 13.40 ppm respectively. The cytotoxic activities of those three active subfractions were assayed on HepG2 hepatocarcinoma cells using enzymatic reaction of 3-(4,5-dimethylthiazoyl-2-yl) 2,5 diphenyltetrazolium bromide (MTT), and the results showed that the IC50 were 35.10, 14.36 and 14.09 ppm respectively. The most active sub fraction (FEA 3.5) was then performed further analyzed using preparative high performance liquid chromatography (HPLC). The HPLC results indicated that there were three active compounds, which were suspected as derivatives of rocaclamid. The molecular docking simulation indicated that rocaclamid formed complex with Toll-like Receptor 4 in HepG2 hepatocarcinoma cells and affected the inhibition of proliferation of its cell.

Keywords: Aglaia elliptica Blume leaves extract, fractionation, BSLT, MTT assay, hepatocarcinoma cell lines.

INTRODUCTION

Hepatocarcinoma is the third leading cause of death in the world, especially in Asia. Hepatocarcinoma generally occurs in men two times more often than in women. Globally, Asian countries have 80% of patients with hepatocarcinoma, of which approximately 600,000 cases diagnosed every year (IARC, 2012). Nowadays the cancer management consists of the combination of drug uses, surgical for removing of cancer tissue, radiation therapy and palliative. But there are still limitations on the cancer management, so it needs further research for inhibiting the cancer growth, one

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of which is utilizing medicinal plants. Empirically, several medicinal plants have been widely used to treat a tumor or cancerous diseases. Antitumor drugs of natural contain active compounds that are toxic to certain phase of tumor cells cycle but not toxic or interfere the normal cells.

*Aglaia* sp. belongs to Meliaceae family, has potential as an anticancer. Rocaglamid is a bioactive compound contained in *Aglaia* sp. was very effective in inhibiting the growth of cancer cells. Previous research reported that various rocaglamid derivatives have been isolated and identified, and most of them showed a very strong cytotoxic potential. Two cytotoxic rocaglate derivatives, namely silvestrol and episilvestrol were isolated from the fruits and twigs of *Aglaia silvestris*, have an cytotoxic activity on KB human oral epidermoid carcinoma cell line. (Hwang, *et al.*, 2004). A compound resulted from fractionation of *Aglaia rubiginosa* twigs, exhibits potent cytotoxic activity on KB human oral epidermoid carcinoma cells (Rivero-Cruz, *et al.*, 2004) Many new compounds of silvestrol analogues were isolated from stem bark of *Aglaia foveolata* were exhibit cytotoxicity against HT-29 cells (Pan, *et al.*, 2010). Several compounds were isolated from a combination of fruits, leaves, and twigs and roots of *Aglaia perviridis*, which had cytotoxic activity on HT-29 human colon cancer cells (Pan, *et al.*, 2013).

The brine shrimp toxicity bioassay is a simple method of screening crude plant extracts for cytotoxicity (Meyer, *et al.*, 1982). This method is an attractive pre-screen for such activities as it is relatively simple and inexpensive to test large numbers of crude plant extracts in a relatively short time, therefore until now still widely used by researchers, for example as bioassay to assess the bioactivity of the medicinal plants used by eastern Nicaraguan healers in traditional medicine (Coe, *et al.*, 2010), as a biological model for the preliminary selection of pediculicidal components from a natural source (Vidotto, *et al.*, 2013), as bioassay for screening several methanol extracts of Pacific Northwest plants in search of general cytotoxic activity (Karchesy, *et al.*, 2016).

Toll-like receptor 4 (TLR4) is an extracellular pathogen recognition receptor (PRR) which recognizes a wide range of pathogens and damage associated molecular patterns (PAMPs and DAMPs). It can activate intracellular signaling and transcription factor which participate in transcription from either immune related or malignancy genes. Thus, TLR4 is an innate immunity receptor which plays a pathogenic role during chronic inflammation and can induce hepatocellular carcinoma (HCC) in human (Sepehri, *et al.*, 2017). TLR4 is known to influence growth and migration of hepatocellular tumors, and a TLR4 agonist Lipopolysaccharide (LPS), was found to significantly up-regulate TLR4 expression in HepG2 cells (Hsiao, *et al.*, 2015).

The research was proposed to identify the cytotoxic activities of ethyl acetate fractions of *A. elliptica* Blume leaves extract on hepatocarcinoma cancer cell lines HepG2, and molecular docking simulation to analyzed the interaction between rocaglamide with TLR4.

**MATERIALS AND METHODS**

**Extract Preparation**

The dried powder simplicia of *A. elliptica* Blume was macerated using methanol distillate with stirring, the process was repeated 3 times. then allowed to stand for 24 hours and then was filtered. The filtrate was then concentrated by vacuum rotary evaporator to obtain a methanol crude extract. Subsequently, the methanol crude extract was fractionated using n-hexane and ethyl acetate solvents, until obtained three extract phase, namely n-hexane phase, ethyl acetate phase and methanol-water phase. Each partition filtrates then were evaporated and then the results are analyzed qualitatively by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). To confirm the biological activity of extract fractions were tested using brine shrimp lethality test (BSLT).
Isolation of Active Compound

Active compounds isolation performed by column chromatography and semi-preparative HPLC, guided by TLC and analytical HPLC. From the extraction and the partitioning process resulted in three extract phases, namely n-hexane phase, ethyl acetate phase, and methanol-water phase. Subsequently, ethyl acetate phase was separated using Vacuum Liquid Chromatography (VLC) with the 15 cm column length and 4 cm column diameter. Silica gel 60 Merck was used as stationary phase, and the mobile phase was combination between dichloromethane and methanol (9.5:0.5) with the following gradient ratio:dichloromethane 100%, 20:80, 60:40, 80:20, 5:100% methanol. The resulting fractions then were analyzed by TLC using the same mobile phase mixture of dichloromethane-methanol and for the stationary phase was silica gel GF 254. and also analyzing qualitatively using HPLC. Purification extract then continued using semi preparative HPLC using the reverse phase C18 as stationary phase, and the combination of methanol-water as mobile phase. The column length was 15 cm and the diameter were 5 µm, the injection volume was 200 µL and the flow rate was 4.70 mL/min.

Brine Shrimp Lethality Test (BSLT)

Preliminary screening of biological activity of crude extract and fractions resulted from partitioning using semi preparative HPLC and VLC of A. elliptica extract were subjected to the BSLT. The stock solution sample was prepared in the 1000 µg/mL by diluted in the dimethyl sulfoxide (DMSO). Then each sample of crude extract and fractions were diluted at concentration of 100 µg/mL in marine solution, in triplicate. Briefly, these samples were solubilized in 5 mL of marine solution with 1% DMSO (v/v), and 20 nauplii of brine shrimp were added in each flask and maintained in direct contact with the sample solutions for 24 hours. A magnifying glass, a light focus and a dark background were used to conduct the observations.

Cytotoxicity Assay Using MTT Method

The HepG2 human hepatocarcinoma cells was maintained as monolayer cultures in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with antibiotics 100 IU/mL penicillin and 100 µg/mL streptomycin and 10% fetal bovine serum in a humidified incubator containing 5% CO2 at 37°C. Subcultures were obtained by trypsin treatment of confluent cultures. The cells were plated in 100 µL of medium into 96 micro well plates at a density of 5x10³ cells/well, and incubated at 37°C and 5% CO2 incubator. One day later the cell culture medium in each wells was discarded and added with 100 µL of fresh medium containing the indicated concentrations (0, 5, 10, 20, 40, 60 and 80 µg/mL) of extract in triplicate. After 24 hours of treatment, the cell culture medium was removed and replaced with fresh medium containing of 0.5 mg/ml of MTT and maintained at 37ºC in 5% CO2 incubator for 4 hours to allow MTT to be converted to formazan crystals by reacting it with metabolically active cells. The reaction was stopped by added sodium dodecyl sulfate (SDS) 10%, and the absorbance was measured at 570 nm using an ELISA plate reader (Wang, et al., 2000).

RESULTS

Extract Preparation

The extraction of 1.8 kg A. elliptica Blume dried leaf powder using methanol solvent resulted in 15.52% yield of crude methanol extract as much as 279.36 g. The partitioning process of the crude methanol extract obtained the following extract phase, namely n-hexane phase, ethyl acetate phase and methanol-water phase. The biological activities of those crude extract and extract phases on
BSLT at 100 µg/mL resulted the percent mortality about 60-86.67% (Table 1). Then all of the extract and phase extracts were analyzed qualitatively by TLC which were detected on 366 nm of UV (Figure 1A).

<table>
<thead>
<tr>
<th>Extract / phase</th>
<th>Yield (g)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude methanol extract</td>
<td>279.36</td>
<td>80</td>
</tr>
<tr>
<td>n-hexane phase</td>
<td>24.70</td>
<td>63.33</td>
</tr>
<tr>
<td>Ethyl acetate phase</td>
<td>23.38</td>
<td>86.67</td>
</tr>
<tr>
<td>Methanol-water phase</td>
<td>54.04</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 1. Yield of *A. elliptica* leaves crude extract and phase extracts, and their toxicities on BSLT.

Isolation of Active Compound

Six fractions were resulted from 20 g ethyl acetate phase extract using VLC. Each fraction was then analyzed using TLC (Figure 1B) and also determined for their biological activities by BSLT at 50 µg/mL. The toxicity tests with BSLT indicated that the fraction F.EA.2 was most toxic among other fractions with a mortality score of 76.67% followed by the F.EA.3 with a mortality score 70% (Table 2). Whereas the yield of F.EA.3 (4.588 g) was much higher than F.EA.2 fraction (2.075 g). Therefore F.EA.3 fraction would be proceeded to the next purification process using column chromatography.

<table>
<thead>
<tr>
<th>Extract Fractions</th>
<th>Gradients Eluent</th>
<th>Fraction</th>
<th>Yield (g)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.EA.1</td>
<td>Dichloromethane :</td>
<td></td>
<td>0.6369</td>
<td>50</td>
</tr>
<tr>
<td>F.EA.2</td>
<td>Methanol 100 : 0</td>
<td></td>
<td>2.0754</td>
<td>76.67</td>
</tr>
<tr>
<td>F.EA.3</td>
<td>80 : 20</td>
<td></td>
<td>4.5882</td>
<td>70</td>
</tr>
<tr>
<td>F.EA.4</td>
<td>60 : 40</td>
<td></td>
<td>2.8228</td>
<td>58.3</td>
</tr>
<tr>
<td>F.EA.5</td>
<td>40 : 60</td>
<td></td>
<td>1.239</td>
<td>53.33</td>
</tr>
<tr>
<td>F.EA.6</td>
<td>20 : 80</td>
<td></td>
<td>0.2093</td>
<td>51.67</td>
</tr>
</tbody>
</table>

Table 2. The yield of extract fractions of *A. elliptica* Blume resulted from VLC fractionations and their toxicities on BSLT.
Fractionation of the F.EA.3 using column chromatography resulted in 8 subfractions. Then the biological activities of each subfraction were tested on BSLT 50 µg/mL and to identify the active compound contained in the subfraction were analyzed using TLC (Figure 1C). The yield of each subfraction, and biological activities on BSLT could be seen in Table 3.

Cytotoxicity Assay

The cytotoxicity assay was conducted to the three subfractions which exhibited most toxic effect on the BSLT, namely F.EA.3.3; F.EA.3.4 and F.EA.3.5 subfractions. The cytotoxic activities of those subfractions were analyzed of against HepG2 human hepatocarcinoma cells using MTT method. The effect of each subfractions on hepatocarcinoma cell viability could be seen in Table 4 and Figure 2.

Table 3. The subfractions of *A. elliptica* Blume resulted from column chromatography fractionations and their biological activities on BSLT.

<table>
<thead>
<tr>
<th>Subfractions</th>
<th>Yield (mg)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.EA.3.1</td>
<td>134.2</td>
<td>41.67</td>
</tr>
<tr>
<td>F.EA.3.2</td>
<td>479</td>
<td>48.33</td>
</tr>
<tr>
<td>F.EA.3.3</td>
<td>366.8</td>
<td>50</td>
</tr>
<tr>
<td>F.EA.3.4</td>
<td>168</td>
<td>58.33</td>
</tr>
<tr>
<td>F.EA.3.5</td>
<td>122.4</td>
<td>68.33</td>
</tr>
<tr>
<td>F.EA.3.6</td>
<td>23.1</td>
<td>48.33</td>
</tr>
<tr>
<td>F.EA.3.7</td>
<td>14.9</td>
<td>48.33</td>
</tr>
<tr>
<td>F.EA.3.8</td>
<td>97.4</td>
<td>31.67</td>
</tr>
</tbody>
</table>

Table 4. The subfractions of *A. elliptica* Blume resulted from column chromatography fractionations and their toxicity on BSLT (LC₅₀) and cytotoxic activities on HepG2 hepatocarcinoma cells (IC₅₀).

<table>
<thead>
<tr>
<th>Subfractions</th>
<th>LC₅₀ (µg/mL)</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.EA.3.3</td>
<td>40.81</td>
<td>35.1</td>
</tr>
<tr>
<td>F.EA.3.4</td>
<td>18.56</td>
<td>14.96</td>
</tr>
<tr>
<td>F.EA.3.5</td>
<td>13.4</td>
<td>14.09</td>
</tr>
</tbody>
</table>

Semi-preparative HPLC Separation

The semi-preparative HPLC separation of FEA 3.5 fraction was conducted and 10 isolates were obtained, which were accommodated at
minute of 11, 12, 13, 14, 17, 19, 21, 23 and 24. The isolates were then analyzed by analytical HPLC. Three isolates namely 4, 5 and 6 were identified on analytical HPLC and predicted as rocaglamid derivative, the chromatogram of those isolates could be seen on Figure 3 -5.

![Figure 3. The chromatogram of analytical HPLC (A) and UV spectrum (B) of isolate 4 of FEA 3.5 sub-fraction at the 12th and 14th minutes.](image)

**Figure 3. The chromatogram of analytical HPLC (A) and UV spectrum (B) of isolate 4 of FEA 3.5 sub-fraction at the 12th and 14th minutes.**

![Figure 4. The chromatogram of analytical HPLC (A) and UV spectrum (B) of isolate 5 of FEA 3.5.5 subfraction at the 14th and 16th minutes](image)

**Figure 4. The chromatogram of analytical HPLC (A) and UV spectrum (B) of isolate 5 of FEA 3.5.5 subfraction at the 14th and 16th minutes.**

**Interaction between Rocaglamide with TLR4**

Molecular docking simulation of rocaglamide into TLR4 was performed to predict the cytotoxic mechanism. The 3D structure of TLR4 was downloaded from Protein Data Bank (PDB ID: 4G8A) which formed complex with LPS (Figure 6). Rocaglamide as ligand was docked at the replacement of LPS position. Rocaglamide formed complex with TRL4 with affinity energy binding prediction about -63 kcal/mol. It interacted with amino acid residue Ser 120 chain C and Lys133 Chain D of TLR4 (Figure 7). These interactions will affect to inhibiting of the TRL4 activity in HepG2 cells proliferation.

**DISCUSSION**

The biological activity of ethyl acetate fraction of *A. elliptica* leaf extract was evaluation. Aglaia leaf dry powder was extracted with methanol by maceration, then partitioned using hexane and ethyl acetate solvents with guided by BSLT. The resultant partition was obtained by the most toxic phase of ethyl
Figure 5. The chromatogram of analytical HPLC (A) and UV spectrum (B) of isolate 6 of FEA 3.5.6 subfraction at the 18th minutes.

Figure 6. Docking rocaglamide into TLR4 as a receptor target for cytotoxic of HepG2 hepatocarcinoma cells.

acetate in the BSLT test. Further, the ethyl acetate phase was fractionated by column chromatography and 8 fractions were obtained, of which 3 fractions showed the highest activity in the BSLT. The result of sub-fractions of *A. elliptica* Blume resulted from column chromatography fractionations on BSLT indicated the strong activities whereas the LC\textsubscript{50} were 13.40-40.81 µg/mL because in the BSLT the sample was included strong toxicity when the LC\textsubscript{50}<100 µg/mL and highest activity if the LC\textsubscript{50}<10 µg/mL (Karchesy, *et al*., 2016).

Furthermore, three subfractions from column chromatography that were active on BSLT were continued with the cytotoxicity test on HepG2 hepatocarcinoma cells by MTT method. In the cytotoxic test it was known that the three fractions of FEA 3.3, FEA 3.4 and FEA 3.5 showed IC\textsubscript{50} values of 35.10, 14.36 and 14.09 µg/mL, respectively. The effect of each subfractions on hepatocarcinoma cell viability was dose dependent manner, increasing the sample concentration would decreased the cell viability. Among the three fractions, the F.EA.3.5 showed most cytotoxic activity than others, indicated with smallest IC\textsubscript{50} value that was about 14.9 µg/mL. The result of cytotoxicity test was positive correlation with BSLT, whereas the most toxic on BSLT also most toxic on HepG2 cell, and
conversely the less toxic on BSLT was also exhibited less cytotoxic on HepG2 cells. Previous research using various rocaslate derivatives from *Aglaia perviridis* leaves extract against HepG2 cells showed that the cytotoxic activities of those compounds with IC\textsubscript{50} values varied between 0.014 - more than 50 μM (An, et al., 2016).

After it was suspected that rocaaglamid is the active fraction of *A. elliptica* extract in inhibiting HepG2 cell proliferation, then a docking simulation was performed to predict the interaction between rocaaglamid and TLR4, whereas rocaaglamide as ligand was docked at the replacement of Lipopolysaccharide position. Downregulation of TLR4 induces suppressive effects on hepatitis B virus-related hepatocellular carcinoma, proven by the mRNA and protein levels of TLR4 were significantly increased in HepG2.2.15 cells. Downregulation of TLR4 significantly decreased the proliferation and induced apoptosis in those cells. In addition, TLR4 showed a physical interaction with HBx, which plays a tumor-promoting role in HBV-related HCC cells, therefore TLR4 may be a potential therapeutic target for HBV-related HCC (Wang, et al., 2015).

**CONCLUSION**

An active subfraction was obtained from chromatography separations of *A. elliptica* Blume leaves ethyl acetate fraction which exhibited strong activities against *A. salina* in the BSLT and also significantly reduced the viability of HepG2 hepatocarcinoma cells. This activity due the rocaaglamid contained in the its active subfraction, where the molecular docking simulation indicated that rocaaglamide formed complex with TLR4 in HepG2 hepatocarcinoma cells and affected the inhibition of proliferation of its cell.

**REFERENCES**


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