The Activity of Liposome-Parijoto Formula Through p53 Expression in HepG2 Cell Line

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Abstract

Parijoto, one of Melastomaceae family, has been known to have cytotoxic activity in HepG2, a hepatocellular cancer cell line, but with low activity. However, the ethyl acetate fraction of Parijoto gave the highest antioxidant and cytotoxic activity in 4T1. Then, purification and liposome formulation need to be carried out to increase the cytotoxic activity of Parijoto extract. Objective: This research aimed to study the cytotoxic activity and p53 gene expression of LEA (Liposom-Ethyl Acetate of Parijoto Fraction) in HepG2.

Method: Extraction has been done by maceration, followed by partition using n-hexane, ethyl acetate, and methanol. LEA formulation was carried out by thin-layer hydration with modification and the formula was sized using a bath sonicator. Cytotoxic activity test of LEA and extract was performed in five serial concentrations (3.9 µg/mL–250 µg/mL), while the positive control doxorubicin performed in 3,9–250 µg/mL by MTT assay. P53 gene expression was analyzed by using PCR-electrophoresis.

Result: Results showed that LEA increased the cytotoxic activity (IC50 = 28.40 µg/ml). Furthermore, based on the electrophoresis study, LEA induced the p53 expression while the extract only did not.

Conclusion: Liposome formula from ethyl acetate fraction of Parijoto extract (LEA) was able to increase cytotoxic activity and p53 gene expression was possible through the apoptotic mechanism. This shows that this formula is a promising strategy to improve the bioavailability of herbal medicines as cytotoxic agents.

Keywords: Liposome; Parijoto; p53; HepG2

INTRODUCTION

Hepatocellular carcinoma (HCC) is primary and mortality liver cancer that causes the second death in worldwide1. Pathogenesis of HCC is closely associated with chronic hepatitis that is caused by several factors such as infection with a virus (Hepatitis B or C), chemical exposure (Aflatoxin), alcoholic lifestyle, and other conditions (diabetic disease, obesity). Those factors will increase the risk of liver cirrhosis which will develop to be liver cancer1. Nowadays, treatment of HCC has been done by curative methods (orthotopic liver transplantation, surgical resection, and local destruction) and palliative methods (trans arterial chemo-embolization, systemic chemotherapy, interferon, and hormonotherapy). Those treatments still have limitations such as the impact on the long-term survival of patients who need the adjuvant after/before curative treatment2. Therefore, there is a need to look for new active ingredients and strategies to improve cancer treatment with low side effects.

A previous study demonstrated that Parijoto extract (Medinilla speciosa) is known to have low cytotoxic activity on the HepG2 cell line, which means it has low bioavailability3. Moreover, the ethyl acetate fraction from Parijoto extract showed the highest antioxidant activity that correlated with cytotoxic effect in 4T1 cell line4.

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Hence, the extract bioavailability will be increased by purification and lipid-based encapsulation of ethyl acetate fraction to form liposome-ethyl acetate (LEA). The liposome is a vesicle enclosed by phospholipids which is an analog to the cell membrane. This vesicle can improve the bioavailability and solubility of water-insoluble drugs which is poorly-permeable. However, liposome is more promising to deliver hydrophobic drugs than peptide and protein drugs for oral drugs, especially for phenolic compounds. Melastomaceae family has been known has several phenolic compounds, such as ellagitannin (Medinillin A, Medinillin B, ellagic acid) and flavonoids which influence their activity as antioxidant and cytotoxic. Therefore, the active compounds will be able to be encapsulated into liposomes that are expected to increase their bioavailability.

A molecular study of HCC pathogenesis revealed that there is a genetic change in a signaling pathway which is mediated by p53, Ras/ERK, PI3K/AKT, and wnt/B-catenin. Another study on p53 showed that its role was in the regulation of the cell cycle, apoptosis, and genomic stability. However, p53 was mutated and plays a key role in the signaling pathway of PI3K/AKT, TGF-B and B-catenin to metastasis in HCC cells. Hence, it is necessary to investigate the cytotoxic activity of liposome-Parijoto and its molecular mechanism through the p53 gene expression

**MATERIALS AND METHOD**

**Extraction and purification**

Extraction of Parijoto (Medinilla speciosa) fruit was carried out by three days of maceration using ethanol 70% and then evaporated using rotary evaporation. The macerate was separated using n-hexane, ethyl acetate, and methanol. Each fraction was evaporated and the ethyl acetate fraction was encapsulated to form the LEA.

![Figure 1. Optical microscope image of LEA formula (400x resolution)](image)

**LEA formulation and characterization**

LEA was made using the thin-layer hydration method. Lipids were prepared by dissolving 1:1 b/b soya lecithin: cholesterol in chloroform, then evaporated using a dehydrator until the thin layer was performed. Hydration was carried out by mixing the ethyl acetate fraction solution in 1% methanol followed by water addition using a magnetic stirrer for 40 minutes. Then, sizing was done by bath sonicator for 10 minutes. The characterization of LEA was carried out by the microscopic study.

**Cytotoxic activity**

Cell lines (1x10^5 cells/well) were cultured into a 48-well plate and incubated overnight. The serial concentration of LEA samples was in the range of 3.9 µg/mL to 250 µg/mL and the positive control doxorubicin was 3.9 µg/mL – 250 µg/mL were put onto cells and incubated overnight. A 100 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) on PBS (Phosphate Buffer Saline) 5% was put onto cell solution followed by incubation for 4 hours until the formazan formed. The reaction has been stopped by adding DMSO (Dimethyl sulfoxide) 10-20% in a protected light for 5 minutes and incubated. The absorbance was read using a microplate reader in 595 nm. The % of inhibition was calculated according to this formula:

\[
\frac{(\text{sample abs-medium abs})}{(\text{cell control abs - medium abs})} \times 100\%
\]

The IC50 was calculated based on linear regression of log concentration vs %inhibition.

**P53 gene expression study**

Cell lines were cultured in 48-cell wells and incubated overnight. The IC50 concentration of LEA was put into the well and incubated overnight. RNA isolation was carried out according to the Invitrogen™ TRIZOL™ Plus RNA Purification Kit (Roche, Swiss) protocol for the cells. cDNA synthesis was done using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Swiss). PCR reaction was used according to the FastStart High Fidelity PCR System Kit (Roche, Swiss) condition (Pre-denaturation 95°C, 1 minute; Denaturation 95°C, 15 seconds; Annealing 58°C, 15 second; Elongation 72°C, 30 second; 35 cycles). The primer used in this research can be seen in Supplementary File 1. The visualization was done by using electrophoresis in 2% agarose

**RESULTS**

**LEA characterization**

LEA formula showed Giant Unilamellar Vesicles (GUVs) (Figure 1) with one compartment/lamellarity after sizing with a bath sonicator. Furthermore, the average particle sizes ranged between 3,73 to 16,64 µm.

**Cytotoxic activity**

Results obtained the LEA formula has a potent cytotoxic activity when compared with the extract. Moreover, it gave a lower concentration to inhibit cell proliferation in HepG2 from IC50 value (Table 1). However, this finding suggested that the cytotoxic activity in the HepG2 cell line was greatly improved by lipid encapsulated in liposome formula

**p53 gene expression**

p53 gene expression could be seen in Figure 2. Our results showed that the LEA formula induced the p53 gene expression while the extract did not. This finding demonstrated that the LEA formula influenced cell proliferation by inducing p53 gene expression which influenced the apoptotic program.
Table 1. The IC50 value of the samples in HepG2

<table>
<thead>
<tr>
<th>IC50 value (µg/mL)</th>
<th>Doxorubicin</th>
<th>LEA</th>
<th>Extract</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.23</td>
<td>28.4</td>
<td>&gt;250*</td>
<td>11.12</td>
</tr>
</tbody>
</table>

*The extract did not show 50% inhibition of HepG2 until 250 µg/mL³.

Figure 2. The expression of p53 in HepG2. A.GADPH (150 bp). B.p53 (300 bp), left to right: untreated cell, positive control (Doxorubicin), LEA; extract. Treated cell was induction according to IC50 value of LEA. There was no expression of GADH and p53 when the cell treatment to 28.4 µg/mL extract.

DISCUSSION
Mutations in the p53 gene cause cell malignancy and unrestricted DNA replication, resulting in uncontrolled cell proliferation and cancerous tumors. p53 activity is impaired by multiple mechanisms in HCC, hence contributing to HCC genesis. The HCC-inducing extrinsic factors which etiologically associated with p53 are AFB1 (Aflatoxin B1), vinyl Chloride, NAFLD (Non-Alcoholic Fatty Liver Disease), Iron, HBV (Hepatitis B Virus), and HCV (Hepatitis C Virus) infection⁹. The results of our study showed that the IC50 of the LEA formula increased sixty times higher than the extract. It indicated that the fractionation and encapsulation of the LEA formula could increase the bioavailability of the extracts. Similar results were shown in the research of Mabrok, 2002 which states that Apigenin (flavonoid from Apium graveolens) that is encapsulated into chitosan and albumin-folic acid can improve its hydrophilicity, stability, and bioavailability to target the cancer cells. The treated HePG-2 cells with Ap-CH-BSA-FANPs demonstrated the induction of apoptosis by increasing p53 gene expression, arresting the cell cycle, increasing caspase-9 levels, and decreasing both the MMP9 gene and Bcl-2 protein expression levels¹⁰.

Parijoto, the Melastomataceae family, contains flavonoids¹¹ and some ellagitannin¹². Furthermore, these flavonoids and ellagitannins can exert cytotoxic activity in various cancer cell lines. Granado-Serrano et al., in 2006 reported quercetin-induced apoptosis in the HepG2 cell line and evaluated the modulation and expression of Bcl-x and Bax. Bcl-xL has been identified as a caspase substrate and the product of Bcl-xL cleavage, Bcl-xS, has a pro-apoptotic function¹². Moreover, hydroxygenkwanin along with kaempferol showed both cytotoxic and antioxidative potential against HepG2 cell lines¹³.

Yohida et, al, reported that Medinilla magnifica, which has the same genus as Medinilla speciosa, was composed by medinillin A and B, the ellagitannin compounds. These compounds give cytotoxic activity through a p53-dependent pathway. Punicalagin, one of ellagitannin from Punica granatum, has been shown to impact the prosapoptotic protein such as Bax, caspace 3 and 9, and the tumor suppressor p53 in human cervical cancer cell lines¹⁴. Furthermore, the other ellagitannin such as 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose (PGG), showed its antiproliferative activity in humanSK-HEP-1 hepatocellular carcinoma cells which caused by the suppression of NF-kB activation and G0/G1-phase arrest via an IκB-mediated mechanism. Moreover, PGG was reported to induce atypical senescence-like S-phase arrest in HepG2 and Huh-7 human hepatocarcinoma cells. Furthermore, it also induced the senescence-associated β-galactosidase activity, inhibited proliferative capacity, and influenced the autophagy process by activating the MAPKs/9/10 on two model studies (in vitro and in vivo) of human HepG2 liver cancer¹⁵.

CONCLUSION
The fractionation and lipid-based encapsulated formula influenced the increasing cytotoxic activity of Parijoto extract. The induced p53 expression was a key role in the apoptotic program. This formula was a promising strategy to improve the bioavailability of herbal extracts as cytotoxic agents.

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