Ethanolic Extract of *Hedyotis corymbosa* and Its Combination with 5-FU Inhibit Cyclin D Expression on WiDr Colorectal Cancer Cell

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Abstract

*Hedyotis corymbosa* has been used for long time as an important component in several folklore medicine formula to clinically treat various types of cancer, including colorectal cancer (CRC). Previously, *Hedyotis corymbosa* ethanolic extract (HEE) which contain ursolic acid reported to inhibit CRC growth via induction of cancer cell apoptosis and blocked the cell cycle, preventing G1 to S progression where cyclin D highly expressed in this phase. 5-fluorouracil (5FU), the first line chemotherapy of colorectal cancer have had resistance and possessed several side effects such as neutropenia, immunosuppression, diarrhea, and also constipation. Therefore, the aim of this research is to conduct the antiproliferative effect and molecular analysis of HEE and its combination with 5FU. Molecular docking study was also done to approach the specific protein target of the compound. Antiproliferative effect was conducted by MTT assay, while cyclin D expression was examined by immunofluorescence. The proliferative effect showed that both HEE and 5-FU had cytotoxic effect with IC50 value of 65 µg/mL and 90 µM respectively, meanwhile the combination of HEE and SFU have synergism effect with CI = 0.48 on dose HEE = 22 µg/mL and SFU= 6.25 µM. Immunofluorescence assay showed HEE and its combination with SFU suppressed the expression of cyclin D. From molecular docking simulation, ursolic acid performed stable interaction with cyclin D. Our findings suggest that HEE may be an effective treatment for co-chemotherapeutic for 5-FU through inhibition of cyclin D expression.

Keywords: *Hedyotis corymbosa*, 5-fluorouracil, colorectal cancer, WiDr, cyclin D

INTRODUCTION

Cancer cells are characterized by uncontrolled proliferation (Hanahan and Weinberg, 2011), therefore inhibiting this excessive proliferation is one of the key for the development of anticancer drugs. The transition from G1 phase of the cell cycle to S phase plays crucial role for the control of eukaryotic cell proliferation, and its misregulation promotes oncogenesis. During G1 phase, growth dependent cyclin-dependent kinase (CDK) activity promotes DNA replication and initiates G1 to S phase transition (Bertoli et al., 2013).

Colorectal cancer is one cancer type which becomes the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women. The prevalence of colorectal cancer has been increased along the increasing human population (ACS, 2014). First line therapy for CRC, 5-fluorouracil (5FU) has been become resistance despite its side effect like neutropenia, immunosuppression, cardiotoxicity and diarrhea (Denise et al., 2015; Gressett et al., 2006; Sorrentino et al., 2012).

Recently, natural products have received great interest since they have relatively few side effects compared with modern chemotherapeutics and have been used clinically for thousands of years as significant alternative remedies for a variety of diseases including cancer (Kellof, 2000; Jeune et al., 2005; Won et al., 2006).

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One promising medicinal plant is *Hedyotis corymbosa* that belongs to the Rubiaceae family and is widely distributed throughout Southeast Asia. *Hedyotis corymbosa* which contain ursolic acid that has been reported to inhibit the growth of CRC through the induction apoptosis and the blocked the cell cycle on G1/S progression (Suparman, 2008). To further exploration the activity of *Hedyotis corymbosa* ethanolic extract (HEE), we investigated its molecular mechanism on the proliferation of human colon carcinoma WiDr cells through expression of cyclin D that regulated cell cycle on G1/S progression.

CO2 at 37°C for 4 h and then formazan crystals were dissolved in 100 µl of 0.01 N HCl and incubated in the dark for overnight. The absorbance of cells was measured at 595 nm by a microplate reader. Cells absorbance was converted to % cell viability. Linear regression between concentration and % cell viability giving the equation $y = Bx + A$ were used to calculate IC50 value, that is the concentration inhibiting 50% cell proliferation.

**Immunofluorescence Assay**

Cells (5 x 10^4 cells/well) were seeded on coverslip in 24 well plates until 80% confluent. Cells were treated with fresh medium contain HEE, 5FU, and the combination and incubated in 37°C for 24 h, cells were harvested and were washed using PBS and fixed it with 70% ethanol for 10 min at room temperature. After rinsed again with PBS, cells were incubated with blocking serum 1% BSA for 30 min at room temperature. Then, cells were incubated with primary antibody (anti-cyclin D) for 1 hour at room temperature. After rinsed with PBS, cells were incubated with secondary antibody conjugated by FITC for 1 hour at room temperature in the dark. Then, cell were added by DAPI solution and incubated for 10 min at room temperature in the dark. After rinsed with PBS, cell were added with mounting solution (Fluoromount), put on slide glass, and store at 4°C. The protein expressions were observed under fluorescence microscope.

**RESULTS AND DISCUSSIONS**

*Hedyotis corymbosa, 5-FU, and their combination Inhibited the proliferation of WiDr Cells*

Cell viability assay was done to determine the inhibitory effect of HEE and 5-FU single treatment on WiDr cells. The single treatment of 5-FU for 48 h with range of concentration up to 90 µM revealed to
possess cytotoxic effect in dose dependent manner. The cytotoxic effect was proven by morphological change on cells (shrinkage and fragmentation). Based on this study, 5-FU had cytotoxic effect by giving IC₅₀ value of 90 μM. Interestingly, the treatment of single 5-FU in 24 h did not show significant inhibition on cells proliferation (data not shown), probably because of low sensitivity of WiDr cells toward 5-FU and high expression of timidilat sintetase (TS) on cells (Meyerhardt and Mayer, 2005; Giovannetti et al., 2007). Moreover, 5-FU was also reported to induce NF-κB expression which regulated anti-apoptosis transcription, causing cells were able to escape the apoptosis (Peters et al., 2002).

![Figure 1. Cytotoxic effect of 5-FU on WiDr cells. Briefly 8x10⁴ WiDr cells/well were seeded in 96 well-plate and incubated for 24 h before treated with 5-FU for 48 h as described in methods. Cells viability was determined by using MTT assay as described in the methods. (A) Cell viability profile after single treatment of 5-FU for 48 h on WiDr cells. The cells morphological were shown above as (B) cell control, (C) 10 μM of 5-FU, (D) 40 μM of 5-FU. The cells were observe under inverted microscope with 100x magnification. Normal cells were marked as meanwhile irregular cells were marked as .](image)

The cytotoxic effect of Hedyotis corymbosa ethanolic extract (HEE) was also carried out for 48 h, based on several previous studies reported that the bioactive compound in HEE, ursolic acid revealed to give optimum cytotoxic effect after 48 h of treatment (Dong-Kyoo et al., 2000; Harmand et al., 2005; Li et al, 2010; Shisodia et al., 2003). Similar to 5-FU, the treatment with HEE showed inhibitory effect in dose dependent manner; given IC₅₀ value of 65 μg/mL. The morphological changes on cells was seen in higher concentration of HEE, indicated that HEE possessed cytotoxic effect toward WiDr cancer cells.
Figure 2. Cytotoxic effect of HEE on WiDr cells. Briefly, 8x10³ WiDr cells/well were seeded in 96 well-plate and incubated for 24 h before treated with HEE for 48 h as described in methods. Cells viability was determined by using MTT assay as described in the methods. (A) Cell viability profile after single treatment of HEE for 48 h on WiDr cells. The cells morphological were shown above as (B) cell control, (C) 10 μg/mL of HEE, (D) 40 μM of HEE. The cells were observe under inverted microscope with 100x magnification.

Furthermore, the combination between HEE and 5-FU was carried out on WiDr cells to determine its combination effect on cells proliferation. Based on this study, the treatment of 5-FU and HEE exhibited synergistic effect on WiDr cells, given CI value less than 1, with lowest CI value was showed in combination of 10 μg/ml HEE and 1,25 μM 5-FU with CI value of 0.33. Based on MTT assay, it was demonstrated that the treatment of HEE in combination with 5-FU inhibited cells proliferation significantly, as the single treatment with 5-FU only inhibit cells proliferation up to 40%, when in combination with 40 μg/ml HEE could decreased cells viability up to 65%. Thus, HEE could be applied as co-chemotherapeutic agent to increase 5-FU sensitivity on colon cancer.
Figure 3. The combination treatment of HEE and 5-FU after 48 h on WiDr cells. Briefly, 10^4 WiDr cells/well were seeded in 96-wellplate before treated with HEE and 5-FU and incubated for next 48 h as described in methods. The effect of combination treatment was determined as % of cells viability (n=3, p<0.05).

Table 1. Combination Index (CI) value from combination treatment of HEE and 5-FU on WiDr cells for 48 h

<table>
<thead>
<tr>
<th>HEE (µg/ml)</th>
<th>1.25</th>
<th>2.5</th>
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<td>0.61</td>
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<td>0.53</td>
<td>0.50</td>
<td>0.67</td>
</tr>
<tr>
<td>40</td>
<td>0.43</td>
<td>0.67</td>
<td>0.51</td>
<td>0.65</td>
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</tbody>
</table>

Previous study had been reported that HEE caused cell cycle arrest on G1/S phase. Further mechanism has been conducted to evaluate cyclin D expression which regulated cell cycle in G1 to S transition by using immunofluorescence assay.

Figure 4. Effect of 5-FU and HEE on cyclin D expression on WiDr Cells. Cells were grown on coverslips and treated with compounds as indicated in methods, then subjected to immunofluorescent staining with cyclin D and DAPI as described in materials and methods. Visualizations were done under microscope fluorescent with 400x magnification.

This research suggests that HEE perform promising potency to be developed as co-chemotherapeutic agent for CRC.

The results demonstrated that single treatment of 5-FU decreased cyclin D expression significantly compared to control.
group, meanwhile HEE also decreased cyclin D expression, indicated that the inhibition in G1/S phase was possibly happened through inhibition of cyclin D which regulated the transition. The combination, however did not give significantly decreasing level of cyclin D compared to 5-FU solely. Based on this study, HEE had potential to be applied as an effective co-chemotherapeutic for 5-FU in colon cancer.

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