The Cytotoxicity Effect of Ethanol Extract and Alkaloid Fraction of *Mirabilis jalapa* Leaves in Hepatocarcinoma Cell Line

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Abstract. *Mirabilis jalapa* is a dry tropical plant which is well grown in Indonesia. *Betaxanthin* active compound is present in the *M. jalapa* plant, which plays a role in iron metabolism. Cytotoxicity activity is one important assay for drug development. Therefore, this study aimed to determine whether ethanol extract and alkaloid fraction of *M. jalapa* leaves had a cytotoxic effect or not in hepatocarcinoma (HepG2) cell line. *M. jalapa* plants were cultivated in Merapi Farma, Yogyakarta. Extraction and fractionation of *M. jalapa* leaves were performed in the Pharmacy Laboratory, Surakarta. HepG2 cell line was subcultured in a complete culture media. Confluent cells were then treated with extract and fraction of *M. jalapa* leaves for 24 h. To determine the toxic dose of extract and fraction of *M. jalapa* leaves, we used tetrazolium bromide (MTT) and Resazurin assays, which was indicated as 50% inhibitory concentration (IC_{50}). In the MTT assay, IC_{50} of ethanol extract was 51.69 ppm, higher than the IC_{50} of alkaloid fraction (47.79 ppm). In comparison with the Resazurin assay, the IC_{50} both ethanol and alkaloid fraction were higher than that of the MTT assay (135.87 and 149.08 ppm respectively). Furthermore, these toxic doses were still safe for HepG2 cells (>30 ppm). Ethanol extract and alkaloid fraction of *M. jalapa* leaves have low cytotoxic effect in HepG2 cell line. The next research is required to evaluate the effect of extract and fraction of *M. jalapa* leaves on iron metabolism in HepG2 cells.

Keywords: Betaxanthin · Cytotoxicity Effect · HepG2 Cells · *Mirabilis jalapa* leaves

1 Introduction

Drug development for anemia is still being pursued considering the high cases of anemia, including iron deficiency anemia (IDA) [1, 2]. The high prevalence of IDA is mainly

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caused by non-responsive to oral iron. Hepcidin is main protein that regulates iron metabolism and is responsible for oral iron non-responsive [3]. Unfortunately, hepcidin antagonists drug development that have been carried out to date has not been satisfactory [4, 5]. One of the high-throughput, effective and efficient drug development methods is the molecular docking [6]. From thousands of phytochemicals can be screened only some phytochemicals that potentially have a role in iron metabolism [7]. In previous study, we found several phytochemicals in silico using molecular docking approach that play a role in iron metabolism. *Indicaxanthin, Miraxanthin,* and *Boeravinone F*, the member of *Betaxanthin* group found in *Mirabilis jalapa* have potentially developed as antagonist hepcidin and erythropoietin agonist [8–10].

In vitro assay is the next stage in drug development after in silico study and extraction/fractionation/isolation of active substances found in natural products [11]. The cell line that is widely used for drug development studies, including its toxicity and cellular mechanism, is the hepatoma cell line. One type of hepatoma cell line that is often used is HepG2. HepG2 is a non-tumorigenic cell, high proliferation rate, epithelial-like morphology, many metabolic activities, and expresses many proteins [12].

In vitro study starts from cytotoxic assay to determine whether the drug candidate substance is toxic or not to the cells. There are many methods to identify the cytotoxicity effect of a compound, but the most commonly used are *tetrazolium bromide* (MTT) and the *Alamar blue* (Resazurin) assay. MTT assay has many limitations compared to Resazurin [13]. MTT reagents can react with non-enzymatic molecules such as ascorbic acid, glutathione and coenzyme A which can increase absorbance even in dead cells [14]. The formation of needle-like formazan crystals can also damage the integrity of the cell that causing cell lysis [15]. Resazurin can increase the sensitivity and reliability of cell viability measurements. Resazurin is permeable, easily diffuses into cells and is metabolized to resorufin emit a fluorescent-pink color in living cells only [16, 17]. The aimed of this study was to determine the cytotoxic effect of extract and fraction of *M. jalapa* leaves and to detect the cytotoxic effect of both extract and fraction using MTT and Resazurin on HepG2 cells.

2 Materials and Methods

2.1 Powder of *M. jalapa* Leaves

M. jalapa leaves used in this study came from the center of the medicinal plant nursery "Merapi Farma" Yogyakarta, Indonesia, which was used in our previous studies. Sample selection was based on plant determination, plant age, harvest age and leaf color. Samples were washed, cut into pieces and dried in the sun for 1 week. Samples were dried in the oven at 60 °C and were powdered to produce 1 kg of dry powder.

2.2 Characterization of Extract and Fraction of *M. jalapa* Leaves

Maceration was used for *M. jalapa* leaves extraction using 96% ethanol as solvent and was modified from study of Maulina and co-workers [18]. Fractionation of the ethanolic extract to obtain the alkaloid fraction was carried out by a modified acid-base

method from study of dos Santos and co-workers [19]. Qualitative alkaloid fraction was determined by Thin Layer Chromatography (TLC) and was followed by measurement of *Betaxanthin* levels using UV-Vis spectrophotometry (U-2900 Shimadzu®).

2.3 HepG2 Cell Culture

HepG2 cells from Parasitology Laboratory of Medical Faculty of Gajah Mada University were maintained in DMEM medium with 2 mM L-glutamine (Gibco®, US), 200 U/mL penicillin (Gibco®, US), 200 mg/mL streptomycin (Gibco®, US), 1 mM sodium pyruvate (Gibco®, US), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco®, US) incubated at 37 °C 95% humidity and 5% CO_2. Experimental HepG2 cells were subcultured in the culture flasks 75. Cell culture medium was changed every 2 days until sufficient number of cells was obtained for cytotoxic assay.

2.4 Cytotoxic Assay

MTT and Resazurin assay to determine cytotoxic effect of the extract and fraction of *M. jalapa* leaves were conducted according to the standardized protocol. Extract and fraction were dissolved in *dimethyl sulfoxide* (DMSO) (Sigma®, USA) to reach 5 mg/mL stock solution. Serial doses of extracts and alkaloid fractions were started at 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 ppm. 10^4 cells were grown in each well on a plate of 96 wells for 24 h to reach 80% confluent. Plate 1 was used for the MTT and plate 2 was used for the Resazurin assay. Confluent cells were treated with extracts and alkaloid fractions with 10 serial doses. Observation of cell viability after 24 h for MTT and every 6 h up to 24 h for Resazurin assay.

2.5 Data Analysis

Cells viability was measured by spectrophotometry at OD_{595} followed by 50% Inhibitory Concentration (IC_{50}) calculation. The effect of all treatments on HepG2 cell proliferation was expressed as % cells viability, using the following formula:

$$\%\ \text{Cells viability} = \frac{\text{Absorbance treated cell} - \text{absorbance medium}}{\text{Absorbance control cell} - \text{absorbance medium}} \times 100\% \quad (1)$$

The IC_{50} was determined by plotting the percentage of cells viability vs serial doses using probit analysis.

3 Results and Discussion

3.1 MTT Assay

IC_{50} ethanol extract in the MTT assay was 51.69 ppm while the alkaloid fraction was 47.79 ppm (Fig. 1). IC_{50} obtained from MTT assay is low indicating that many cells die at doses above 50 ppm. However, this dose is still a non-toxic dose for HepG2 considering

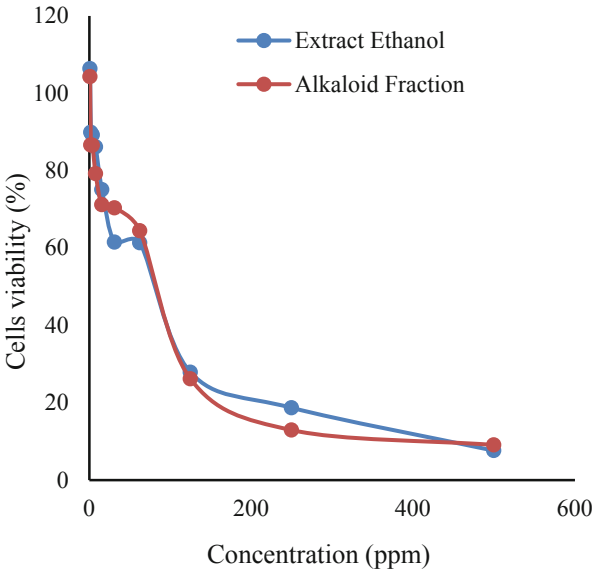

Fig. 1. The cytotoxic effect of extract and fraction of *M. jalapa* leaves on HepG2 cell lines using MTT assay.

that the toxic dose of HepG2 is <30 ppm according to the National Cancer Institute [20]. Factors that need to be considered in the MTT assay is the optimal time for treatment cannot be observed in real time. It could be that 24 h is not the optimal time for treatment so that the cells already die due to too long exposure and not because of the toxic nature of the compound. Another factor of concern is the limitations of the MTT assay related to the possibility of false negatives or false positives due to cell damage by formazan or reactions with non-enzymatic molecules respectively [13–15].

3.2 Resazurin Assay

There are variations in curve in the Resazurin assay every 6 until 24 h. An exponential decrease of cells viability occurred in the 18 h treatment. The most optimal curve was obtained when the ethanol extract and alkaloid fraction were administered for 18 h (Figs. 2 and 3). IC_{50} in HepG2 cells with ethanol extract treatment for 18 h was 135.87 ppm while the alkaloid fraction was 149.08 ppm.

Resazurin is widely chosen as a cytotoxic assay of a compound against cells. This assay has many advantages including non-toxic to cells, easy to diffuse into cells, does not form compounds that damage cell integrity, can be used for little cells, real time observations because it does not lysis cells and high measurement reliability [14–16].

The IC_{50} in the ethanol extract and the alkaloid fraction obtained from the Resazurin was much higher than the MTT assay. This indicates that the optimal treatment time was reached at 18 h. The IC_{50} of Resazurin assay is more valid than the MTT so that it can be used as a reference for further *in vitro* assay. The IC_{50} of extract and fraction of *M. jalapa* leaves was higher than the toxic dose for HepG2 cells.

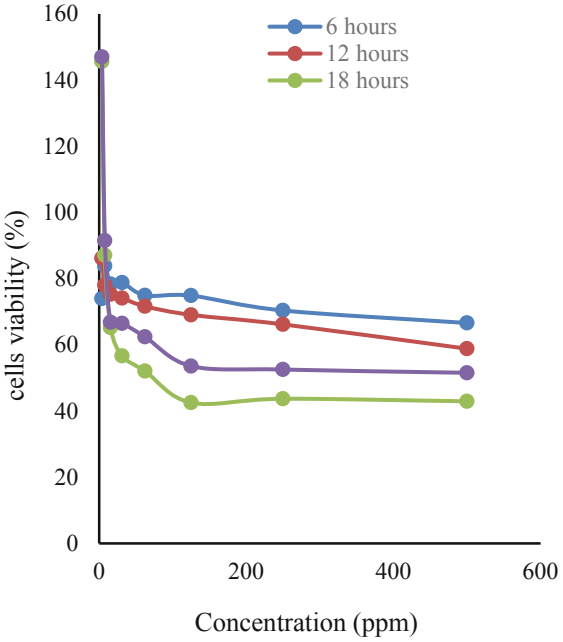

Fig. 2. The cytotoxic effect of extract of *M. jalapa* leaves on HepG2 cells using Resazurin assay.

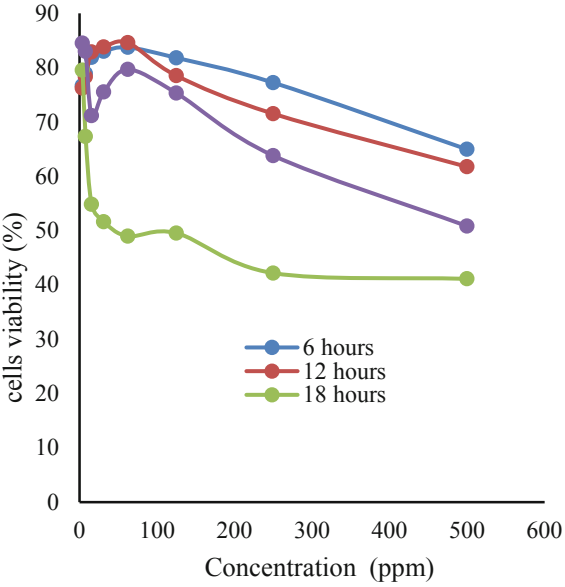

Fig. 3. The cytotoxic effect of alkaloid fraction of *M. jalapa* leaves on HepG2 cells using Resazurin assay.

This is still a preliminary study so it needs to be proven by a triplicate assay to confirm the comparison between IC_{50} using the MTT and Resazurin assay statistically.

Ethanol extract and alkaloid fraction of *M. jalapa* leaves have low cytotoxic effect in HepG2 cell line. Resazurin was more valid and reliable cytotoxic assay than MTT. The next research is required to evaluate the effect of extract and alkaloid fraction of *M. jalapa* leaves on iron metabolism in HepG2 cells.

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