The Effect of Asiatic Acid and Metformin on The Viability Percentage of Mouse Macrophage Cell Lines RAW264.7 and Mouse Fibroblast Cell Lines NIH3T3

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Abstract. Introduction: Diabetes can be caused by inflammatory regulation disorders. Metformin has been reported that inhibits the physiological function of normal cells. Asiatic acid, a bioactive compound from Centella asiatica, has the potential to be developed as a therapeutic agent for diabetes, but little is known about its toxic effects on macrophage cells and fibroblast cells. Objective: The study aims to evaluate the toxic effects of Asiatic acid and metformin on the viability of RAW264.7 macrophage cell lines and NIH3T3 fibroblast cells. Method: Asiatic acid and metformin with seven concentrations were given to RAW264.7 macrophage cell lines and NIH3T3 fibroblast cell lines. Viability percentage is calculated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, and the absorbance is measured at 595nm. Results: Results have shown that Asiatic acid with concentrations > 12.5 μg/mL decreases the viability of RAW264.7 and NIH3T3 cells drastically. RAW264.7 and NIH3T3 cells that had been given metformin concentrations of 11.6 μg/mL to 370 μg/mL still showed a large percentage of cell viability. Conclusion: Asiatic acid has shown that the cytotoxic effect is greater than metformin, so it is necessary to pay attention to the concentration of the treatment.

1. Introduction

Macrophage cells are the main component that regulates the inflammatory process. Disruption of inflammatory regulation causes prolonged inflammation-causing cellular dysfunction [1]. Fibroblast cells play an important role in the formation of connective tissue through extracellular matrix secretion [2]. Diabetes, one of the metabolic syndromes, is caused by inflammatory regulation disorders. Prolonged inflammation and hyperglycemia cause macrophage cells to secrete mediators such as IL-6, TNF-α, IL-1β [3]. The mediator induces tissue damage, including fibrosis, which is played by fibroblast cells [4]. Metformin has been widely used as a diabetes therapy agent and increases survival rates in
diabetic patients with cancer comorbid [5]. However, the toxic effect of long-term use of metformin still controversial because metformin has been reported to inhibit the differentiation of macrophage cells [6].

Centella asiatica has the potential to be developed as a pharmacological therapy agent. Triterpenoid compounds have been reported to have important roles in pharmacological effects, including Asiatic acid compounds, madecassic acid, madecassoside, and asiaticoside [7]. The previous study by Shou-yan et al. (2018) has been reported that the administration of Asiatic acid with concentrations of 20, 40, and 80 μM inhibits the secretion of cytokines IL-6, TNF-α, IL-1β, NO, and PGE2 in endometrial epithelial cells in mice [8]. In addition, goto-kakizaki rats given Asiatic acid 25 mg/kgBB have shown a decrease in fibrosis markers [9].

Little is known about the cytotoxic effect of the use of metformin and Asiatic acid on the viability of macrophage cells and fibroblast cells. Evaluation of cytotoxic effects is needed to develop Asiatic acid as a diabetes therapeutic agent that does not cause toxic effects. Therefore, this study was presented to evaluate the cytotoxic effects of in vitro metformin and Asiatic acid in RAW264.7 macrophage cells and NIH3T3 fibroblast cells.

2. Material and methods

2.1 Reagents and materials
Fetal bovine serum-qualified (Gibco® 26140-079), Dulbecco’s Modified Eagle (Gibco® 12800-058), Asiatic acid (ACE Biolabs CAS.464-92-6), 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide or MTT (SIGMA), inverted microscope, microplate reader (Biorad®), Biosafety Cabinet Class II (Airstream), incubator (Thermofisher®), and other research materials obtained from the Department of Pharmacy, Islamic University of Indonesia.

2.2 Cell line
Mouse macrophage cell lines RAW264.7 (ATCC® TIB-71) obtained from Department Food and Science, National Pingtung University Science and Technology (NPUST), and mouse embryonic fibroblast cell line NIH3T3 (ATCC® CRL-1658) obtained from Department Biology and Science Technology, National Pingtung University Science and Technology (NPUST). The cells were cultured in a complete medium of DMEM media supplemented with 10% of FBS and 2% Penicillin-streptomycin and fungizone 0.5%. The cells were incubated in the incubator set at 5% CO2 and 37°C.

2.3 Preparation of sample
Asiatic acid and metformin compounds have been dissolved using dimethyl sulfoxide (DMSO) as a stock solution. The working solutions were prepared using complete media with a final concentration of DMSO has adjusted to less than 0.5%.

2.4 MTT Assay
The RAW264.7 and NIH3T3 cells were seeded in a 96-well microplate in complete medium supplemented with 10% FBS until 24 hours. Culture media are discarded and replaced with complete media containing Asiatic acid and metformin with seven series of concentrations. metformin (740.7; 370; 185; 92.6; 463; 231; 11.6 ug/mL), Asiatic acid (200; 100; 50; 25; 12.5; 6.25; 3.125 ug/mL) while the high glucose treatment has prepared in 4 concentrations (25; 30; 35; and 40 mM). Cells were incubated in Asiatic acid and metformin treatment solutions for 24 hours, while 48 hours in the treatment of high glucose culture media. The cells were incubated in culture media containing 0.5% MTT reagents for 4 hours and then added 10% sodium dodecyl sulfate to dissolve formazan crystals. Incubate at room temperature overnight. The optical densities (OD) were measured by microplate readers at 595 nm. The viability percentage of cells was calculated using this formula:

\[ \text{Viability percentage (\%)} = \frac{OD\text{ treated cells} - OD\text{ medium control}}{OD\text{ untreated cells}} \times 100 \]  

(e.q 1)
3. Result
The viability percentage of macrophage cell and fibroblast cell decreases dose-dependent after Asiatic acid and metformin treatment (figure 1 and 2). The cell viability is different after exposure that macrophage cells are more susceptible to death because of the toxic effects of Asiatic acid and metformin compared to fibroblast cells. In figure 1, the greatest decrease in viability occurs at the concentration of Asiatic acid 25 μg/mL to 200 μg/mL. The concentration of Asiatic acid 3.125 μg/mL to 12.5 μg/mL shows the viability of macrophage cells and fibroblast cells of more than 80%. Based on these data, the inhibitory concentration value of 50% (IC50) of Asiatic acid in macrophage cells is 15.21 ± 5.22μg/mL (mean ± SEM), whereas in fibroblast cells is 18.95μg/mL.

![Figure 1](image1.png)

**Figure 1.** Percentage of viability (mean ± SEM) of macrophage cell lines RAW264.7 and fibroblasts cell lines NIH3T3 after treatment with Asiatic acid for 24 hours

In figure 2, metformin administration has shown a dose-dependent reduction in cell viability. Macrophage cells showed the lowest viability after high-dose metformin treatment (740.7 μg/mL). Metformin with concentrations of 11.6 μg/mL up to 370.4 has shown that cell viability is more than 80%. The IC50 value of metformin in macrophage cells was 1078.46 ± 344.8 μg/mL (mean ± SEM) and in fibroblast cells 1592.20 ± 178.8 μg/mL. It is clearly known that the IC50 value of Asiatic acid is smaller than that of metformin.

![Figure 2](image2.png)

**Figure 2.** Percentage of viability (mean ± SEM) of macrophage cell lines RAW264.7 and fibroblasts cell lines NIH3T3 after treatment with metformin for 24 hours
4. Discussion
In this study, we have investigated the feasibility of RAW264.7 macrophage cells and NIH3T3 fibroblast cells after Asiatic acid, metformin, and high glucose treatment, to obtain concentrations that do not show toxic effects on the development of diabetes drugs in vitro studies. Our research clearly indicates that Asiatic acid has a higher toxic effect compared to metformin. Therefore the development of Asiatic acid into a drug therapy agent needs to pay attention to the toxic effects that will appear at the molecular or cellular level.

Macrophage cells are a component of the immune system regulator that has the role of maintaining tissue homeostasis. Mediators secreted by macrophages can influence cellular responses [10], whereas fibroblast cells maintain structural protein balance through the extracellular matrix, such as matrix metalloproteinase (MMP), collagen secretion, fibronectin and adhesion factors [2]. Therefore, the administration of therapeutic agents that have toxic effects can interfere with the physiological functions of these cells.

Asiatic acid is a single triterpenoid compound which can be found one of them in Centella asiatica. Triterpenoid compounds, including asiaticoside, madecassoside, madecassic acid, and Asiatic acid contained in ethanol extracts have been reported to show pharmacological effects as anti-inflammatory [11]. Asiatic acid has a stronger potential pharmacological effect than other triterpenoid compounds [12]. Asiatic acid induces cell apoptosis through various pathways. In a previous study conducted by Soyingbe et al. (2018) it has been reported that Centella Asiatica extract (methanol, ethyl acetate, acetone, methanol extract) show toxic effects with IC50 <100 ug/mL on MCF-7 cells, Hela cells, Caco-2 cells, and A549 cells [13]. Several studies have been widely studied as Asiatic acid as a cancer therapy agent. It has been reported that Asiatic acid concentrations of 5 ug/mL induce apoptosis of MCF-7 and MDA-MB human breast cancer cell lines through the Bax, Bcl-2, and Bcl-xL protein pathways [14]. In addition, Asiatic acid treatment with the concentration of 15 ug/mL and 25 ug/mL to human colon cancer cells SW480 and HCT116 also activates the PI3K/Akt/mTOR protein which is a cell apoptosis pathway [15].

Metformin, an antidiabetic therapy agent, also has the activity to inhibit tumor cells. Research conducted by Li et al. (2018). It has been reported that metformin has IC50 over 4000 ug/mL in MCF-7 cells. The cytotoxic mechanism of metformin has been reported by gradually inhibit the physiological functions of cells such as inhibition of mitochondrial function and induce dose-dependent acetylation. Metformin also inhibits ABC transporters and expression of HIF-1α proteins, which might influence cell proliferation [16]. Therefore, the cytotoxic mechanism of metformin is time-dependent and dose-dependent [17][18]. Asiatic acid and metformin have different mechanisms that cause toxic effects. Therefore, the two compounds have different values of cell viability to the test material.

5. Conclusion
Asiatic acid treatment with concentrations of 3.125 ug/mL to 12.5 ug/mL and metformin concentrations of 11.6 ug/mL to 370 ug/mL did not show cytotoxic signs in RAW264.7 macrophage cells and NIH3T3 fibroblast cells.

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