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THE VIABILITY TEST OF SAPPAN WOOD (*CAESALPINIA SAPPAN* L.) ETHANOL EXTRACT IN THE H9C2 CELL LINE

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ABSTRACT

Objective: In this study, the embryonic rat cardiomyocyte cell line H9C2 was used to investigate the cardiotoxicity effect of sappan wood ethanol extract (SWEE).

Methods: Sappan wood was extracted in 96% ethanol and divided into dose concentrations of 2.5, 5, 10, 50, 100, and 150 µg/ml, with deferiprone used as a control. Cell viability was assessed using the PrestoBlue Cell Viability Reagent, according to manufacturer protocols.

Results: Microscopic examination showed that the cell viability of H9C2 was preserved by SWEE treatments at a dose of 10 µg/ml and suggested dose concentrations of 50 µg/ml of SWEE. The percentage of viable cells was greater than 95% with a dose concentration of 10 µg/ml of SWEE, but it was significantly reduced with a dose concentration of 50 µg/ml of SWEE ($p<0.05$).

Conclusion: The optimal dose concentration of SWEE to reach 95% cell viability was 10 µg/ml.

Keywords: H9C2 cell line, iron overload, Sappan wood ethanol extract

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INTRODUCTION

Blood transfusion plays an important therapeutic role in patients with a history of diseases such as β-thalassemia, anemia, and myelodysplastic syndromes (MDS) [1–3]. Blood transfusion therapy has the risk of long-term side effects, causing an iron overload, where excess iron in the body accumulates in several organs, such as the liver, endocrine system, and heart. The heart is one of the main sites where excess iron accumulates [1]. The heart is an organ with high oxygen consumption, and it is rich in unsaturated fatty acids that can produce reactive oxygen species (ROS) [4]. Excessive iron accumulation in the heart causes cardiomyopathy, a disease which can lead to heart failure [1]. Cardiomyopathy is caused by cell death that begins with an increase in oxidative stress that causes apoptosis. When iron levels exceed the capacity limit of ferritin, cell death and organ damage will occur due to the presence of ROS and their consequent mutations [5]. Based on this reaction, it is clear that iron chelation is needed to control the amount of iron in the body, especially in iron overload conditions.

Iron chelators are categorized into several types, such as synthetic chelators, siderophores, and phytochemical chelators. Iron chelators that are widely used in therapy derive from synthetic chelators, for example, deferiprone [6]. However, long-term use of deferiprone can cause side effects. Iron chelator agents were revealed to be ineffective and toxic in most patients and have side effects such as diabetes mellitus (DM) in non-DM patients [7]. At this time, much research has focused on phytochemical chelators, such as sappan wood (*Caesalpinia sappan* L.). Sappan wood is a good candidate for iron chelation because it contains high flavonoid compounds and brazilin. Our previous studies showed that flavonoid and brazilin could decrease the ferritin level and increase transferrin with almost the same results as deferiprone, an iron-chelating agent, as a control. The decrease in ferritin indicated that sappan wood extract has the ability to chelate the iron. Research has tested the use of sappan wood ethanol extract (SWEE) as an iron chelator *in vivo* using rats, where SWEE reduced iron in the blood and improved the function of rat livers with excessive iron accumulation [8–10]. However, further investigation is necessary to investigate the toxicity of SWEE in the target organ. In this study, we tested the viability of SWEE *in vitro* using the H9C2 rat cardiomyocytes cell line.

MATERIALS AND METHODS

Preparation of SWEE

Sappan wood was dried in the open air and extracted with a 96% ethanol solvent using the maceration method. The maceration was concentrated on a rotary evaporator at 40°C [10]. The dried extract of Sappan wood was dissolved in dimethyl sulfoxide (DMSO) for further testing.

Cell cultures and treatment

The H9C2 cell line was obtained from Central Laboratory in our university and the test was carried out from July to August 2019. The cell culture and treatment was modelled after Hanif *et al.* (2019). The H9C2 cell line was plated in a Roswell Park Memorial Institute (RPMI) medium with 10% Fetal Bovine Serum (FBS) and with antibiotics at 50 µl/50 ml on 96 well plates [12]. After reaching confluence, cells were treated with SWEE in different concentrations, i.e. 150, 100, 50, 10, 5, 2.5 µg/ml. Deferiprone was used as a positive control in various concentrations, i.e. 100 mmol, 10 mmol, 1 mmol, 100 µM, 10 µM, 1 µM. Cells were incubated at 37°C for 24 h in an incubator with 5% CO_2 levels.

Prestoblue cell viability assay

Cell viability was measured using the PrestoBlue Cell Viability assay. PrestoBlue reagents were added to the media at a ratio of 9:1. The mixture of reagents and media (100 µl) was added to treatment cells and incubated at 37 °C for 2 h. Absorption was measured for cell viability with a wavelength of 570 nm (reference: 600 nm) using a multi-mode plate reader.

Microscopy examination

Microscopic examination of H9C2 cardiomyocytes was performed by using EVOS XL CORE microscope with magnification 20×.

Statistical analysis

Data were analyzed using GraphPad Prism. One-way analysis of variance (ANOVA) was conducted for three samples, and the Tukey post-hoc multiple comparison test was performed to evaluate differences. A p-value of<0.05 was considered statistically significant.

RESULTS

Cytotoxic activity of SWEE on the H9C2 cell line

A cytotoxic examination of SWEE with concentrated variants of the H9C2 cell line showed a change of color after the PrestoBlue reagent was applied: the cell line turned purple and pink on several wells (fig. 1). The pink color indicated that cells were still growing, and the purple color indicated cell death. The multi-mode plate reader showed an increase in the percentage of cell viability from high to low concentrations, or concentrations of 150 µg/ml to 2.5 µg/ml (fig. 2). The percentage of cell viability was more than 95% with a dose concentration of 10 µg/ml of SWEE, and the number was reduced with a dose concentration of 50 µg/ml of SWEE ($p<0.05$).

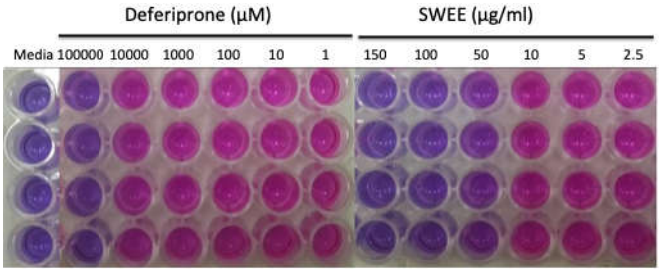

Fig. 1: Presto blue cell viability assay with deferiprone and SWEE

Deferiprone was used as a positive control because it is frequently used in therapy to reduce iron overload conditions in patients with blood disorders who need long-term blood transfusions (thalassemia mostly). The results showed that well colour shifted to purple only at a concentration of 100 mM of deferiprone (fig. 1), indicating cell death at that concentration. The multi-mode plate reader results showed that the percentage of cell viability decreased dramatically at a concentration of 100 mM deferiprone (fig. 2). Cell death in deferiprone was only seen at its highest concentration.

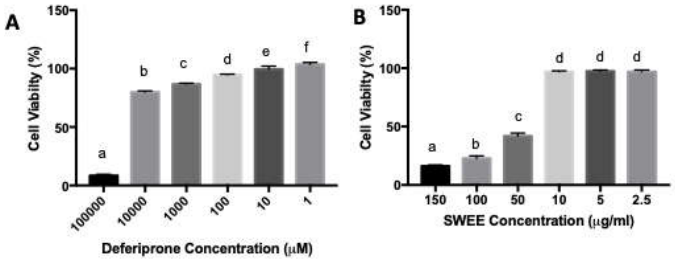

Fig. 2: Effects of deferiprone (A) and SWEE (B) Toxicity on H9C2 cell line viability from highest to lowest concentrations. The different letter indicated statistically significant ($p<0.05$)

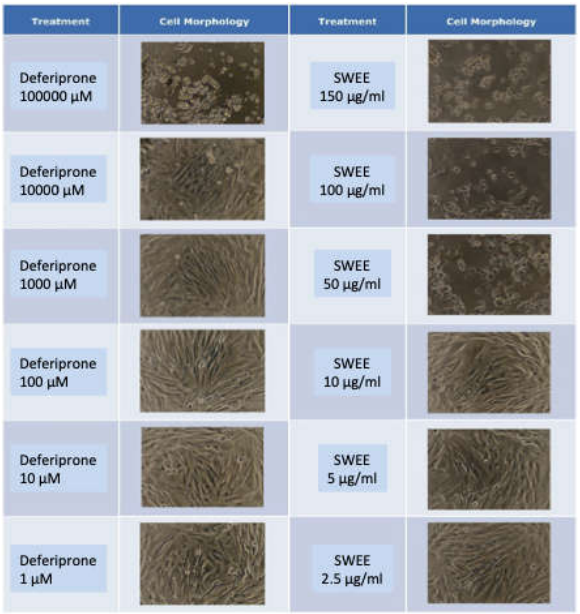

Fig. 3: The morphological appearance of the H9C2 cell line after deferiprone and SWEE treatment at several variants of concentration over 24h of incubation

Microscopic examination of cellular morphology

Microscopic examination was used to assess cellular morphology. Treating the H9C2 cell line with SWEE for 24 h showed signs of morphological toxicity. Microscopic examination results showed that cell damage began at a concentration of 50 µg/ml to 150 µg/ml of SWEE, which was marked by membrane blebbing, loss of original cell shape to loss of cell viability, and the formation of cell debris, as shown in fig. 3. Results from this microscopic examination supported the results of the multi-mode plate reader and PrestoBlue Cell Viability assays, which showed signs of cell death and decreased cell viability at concentrations of 50 µg/ml to 150 µg/ml. Different results were obtained when SWEE concentrations of 2.5 µg/ml to 10 µg/ml were added to cells, showing no signs of cell death. In these cases, cell viability also remained above 95%. When compared with the deferiprone as control, the signs of toxicity appeared in several cells at a dose of 100 mM (fig. 3).

DISCUSSION

In vitro experiments using the H9C2 cell lines were used to investigate the effects of SWEE on cardiomyocytes. SWEE had toxic effects at doses above 50 µg/ml, along with significantly decreased cell viability. Previous SWEE *in vivo* toxicity tests using Wistar rats showed no toxic effects or allergic reactions, and no rats died until the SWEE dose reached 5000 mg/kg [11]. Furthermore, SWEE toxicity testing was also carried out *in vitro* on several cancer cells to test their toxicity on the cancer cells themselves, for example, in breast, cervical, and colon cancer cells [12, 13]. In contrast with these previous studies, our study used the H9C2 rat cardiomyocytes cell line because we wanted to test the toxicity of SWEE in normal cells, including cardiomyocytes.

Blood transfusions can cause excess iron in the body [14]. The excess iron in the body then accumulates in several organs, one of which is the heart [1–3]. Excess iron in cells can increase reactive oxygen species (ROS) and result in ferroptosis. Increasing ROS can induce decreasing of glutathione peroxidase 4 (GPx4), so that can induce lipid peroxidation until ferroptosis occurred [15]. Excess cell death in the heart can cause heart failure. Heart failure is the final stage of cardiovascular disease, characterized by a loss of myocytes due to cell death [16].

Sappan wood is a plant that has high flavonoid content and contains five antioxidant compounds, including brazilin. Flavonoids and brazilin make Sappan wood a good candidate as an iron-chelating agent. Based on *in vivo* testing, SWEE was able to reduce iron in the blood and improve the function of rat livers with excessive iron [8–10]. This study performed a cell viability test using SWEE, but further testing is needed to determine the ability of SWEE to act as an iron chelator *in vitro*. The morphology of cell death from the H9C2 cell line shows the characteristics of cell death through apoptosis. The characteristics of apoptosis are marked by the presence of chromosome condensation, in contrast to necrosis and autophagy, which possess the characteristics of a destroyed plasma membrane (necrosis) and double-layered membrane vacuole (autophagy) [15].

CONCLUSION

The optimal dose concentration of SWEE to reach 95% cell viability is 10 µg/ml.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally.

CONFLICT OF INTERESTS

All authors declare there are no competing interests in this study.

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