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The cytotoxicity of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan isolated from *Swietenia macrophylla* King seed extract on 3T3-L1 and RAW 264.7 cells

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Abstract

The exploration of natural products as anti-obesity candidates needs to be performed urgently since obesity has become a worldwide epidemic and many drugs for this health condition were withdrawn due to their serious adverse events such as psychiatric disorders and drug dependence. The 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan, a saponin isolated from Indonesian *Swietenia macrophylla* seeds extract has the potential to modulate obesity because it acts as a potent PPAR-γ agonist *in silico* and the seeds' hypolipidemic effect has been proven. The cytotoxicity study is a crucial part of the early pharmaceutical development stage, whilst, the exploration of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan to ensure the compound's safety in pre-adipocytes and macrophage cells which play a crucial role in obesity pathogenesis is still limited. This study aims to explore the cytotoxicity of saponin isolate on 3T3-L1 (pre-adipocytes) and RAW 264.7 (macrophages) cell lines to ensure its safety profile *in vitro*. Both cell lines were exposed to various concentrations of the isolate (3.125-200 µg/mL), then metformin was used as the standard drug and DMSO as solvent control for 24 hrs. The cytotoxicity test was conducted by MTT assay and IC_{50} was determined by probit analysis. Most of the 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan did not affect the viability of 3T3-L1 and RAW 264.7 with the IC_{50} 148.90±12.22 and 84.78±1.69 µg/mL, respectively. Metformin and DMSO also did not alter the viability of both cells. These results were promising as a basis for further development since the isolates' IC_{50} value was more than the cytotoxic threshold of NCI guidelines.

1. Introduction

Obesity is a major health burden and global concern since it increases the risk of developing diseases and is associated with several health conditions such as hypertension, diabetes, cardiovascular disease, and cancer (Johnson and Mincey, 2016; Chooi *et al*., 2019). The most widely used for measuring obesity is body mass index (BMI), due to a relatively inexpensive procedure and simple metric which indicates overall body fatness (Nguyen and El-Serag, 2010; Chooi *et al*., 2019) The BMI is calculated as weight in kilograms divided by height in meters squared (kg/m^2). The World Health Organization (WHO) and the National Institutes of Health categorize individuals as overweight once their BMI ranges from 25 to 29.9 kg/m^2 and as obese when more than 30.0 kg/m^2 (Nguyen and El-Serag, 2010).

Over the past 35 years, obesity prevalence has been almost 2-fold higher worldwide. In 2014, among the adult population over 18 years, the cases reached 11% in men and 15% in women. Additionally, more than 42 million children aged below 5 years were overweight in 2013 (Johnson and Mincey, 2016). Overall estimation shows that obese individuals spend 42% more on healthcare expenditures than their normal-weight counterparts (McCafferty *et al*., 2020).

Approximately 90% of type 2 diabetes (T2D) patients are included in the obesity category based on BMI criteria. Furthermore, mounting evidence proved that the increase in BMI has a linear relationship with an

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elevated risk of T2D and insulin resistance development (Selassie and Sinha, 2011). Obesity and T2D have been recognized as chronic pro-inflammatory conditions, and macrophage polarization serves as a link in their pathogenesis. In obesity, an enlargement of adipose tissue triggers monocytes' recruitment to adipocytes and other peripheral tissues. This process also creates an important influence on glucose homeostasis in the liver, skeletal muscle and pancreas. In obesity, monocytes differentiate and polarize towards proinflammatory M1 macrophages which produce cytokines and induce inflammation, cell dysfunction and insulin resistance development (Samaan, 2011; Kraakman *et al*., 2014).

There are three pivotal approaches related to lifestyle modification in the management of obesity, including dietary and physical activity interventions, as well as behaviour modification (McCafferty *et al*., 2020). Meanwhile, pharmacological intervention is needed for people with a BMI exceeding 30 kg/m^2 and obese patients with related comorbidities and BMI of more than or equal to 27 kg/m^2 (Apovian *et al*., 2015).

In recent years, several classes of obesity drugs have been available such as lipase inhibitors, selective serotonin receptor agonists and GLP-1 agonists. However, those used in clinical settings raise concern due to the risk of undesired side effects including moderate to severe gastrointestinal disturbances, specifically for lipase inhibitors and GLP-1 agonists. Moreover, selective serotonin receptor agonists have been reported to cause adverse events ranging from mild e.g. headaches and dizziness to severe types e.g. serotonin syndrome and depressant effects (McCafferty *et al*., 2020). Sibutramine (a serotonin and noradrenaline reuptake inhibitor) was an anti-obesity drug withdrawn from the market in 2012 by the US FDA due to increasing the risk of heart attack and stroke. In late 2008, rimonabant was also withdrawn from the European market because meta-analysis studies showed that it elevated risks for the development of severe side effects related to brain functions such as anxiety, depression and a high risk of suicide (Dietrich and Horvath, 2012).

Anti-obesity drugs' safety needs to be improved, and the development of alternative drugs should be investigated more intensively, hence the herbal remedies exploration currently become an attractive focus (Liu *et al*., 2017). In recent decades, one of the secondary metabolites that have been highlighted regarding the pharmacological potential as anti-obesity from natural compounds is saponin (Marrelli *et al*., 2016). Chiisanoside saponins isolated from the leaves of *Acanthopanax sessiliflorus* were proven to significantly lower serum triglyceride, inhibit dietary fat absorption, and suppress body weight gain in high-fat diet-induced obese mice (Yoshizumi *et al*., 2008).

The 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo (3,4-c) furan, a saponin isolated from Indonesian *S. macrophylla* seeds extract is expected to have biological effects in modulating obesity because the in-silico study showed its potential as a PPAR-γ agonist (Nugraha, 2012). The PPAR-γ molecule acts as a regulator to restore macrophage polarization to the M2 state. Another PPAR-γ agonist is thiazolidinediones (TZD) which suppress circulating low-density lipoprotein (LDL) and triglyceride levels. Additionally, PPAR-γ agonists create anti-inflammatory effects on adipocytes through their role in suppressing the production of pro-inflammatory mediators such as TNF-α, IL-6, and iNOS (Corzo and Griffin, 2013). This evidence underlies the possibility of the isolated saponin being a potential anti-obesity agent.

Previous in vivo evidence also showed that the alcoholic extract of *S. macrophylla* seeds possesses a hypolipidemic effect since it reduces the levels of total cholesterol, LDL-cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C) and triglycerides while increasing high-density lipoprotein cholesterol (HDL-C) in rats induced by streptozotocin (Kalpana and Pugalendi, 2011). Besides being effective in lowering blood glucose, the methanol extract of *S. macrophylla* seeds decreases cholesterol and triglycerides in T2D rat models induced by streptozotocin and nicotinamide (Maiti *et al*., 2008).

The development of the saponin isolate from *S. macrophylla* seeds as an anti-obesity candidate requires a sequential drug discovery process. The cytotoxicity study is a crucial part of the modern pharmaceutical development process at the early stage of creating the formulation. The use of appropriate cell-based assays such as in vitro cell line models for assessing the toxicity offers early identification related to the potential cytotoxicity of a compound and acts as a prediction for human-specific toxicity. Moreover, the application of in vitro toxicity tests brings benefit to preclinical studies due to its feasibility, cost-effectiveness and reproducibility (Bácskay *et al*., 2018).

On the other hand, the exploration of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan, particularly to ensure its safety profile in pre-adipocytes and macrophage cells which play a crucial role in obesity pathogenesis is still limited. This study aimed to explore the cytotoxicity of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan on 3T3-L1 and RAW 264.7 cell lines. It also serves as an initial effort to discover and develop the compound as a new candidate for obesity therapy.

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2. Materials and methods

2.1 Materials

Both 3T3-L1 and RAW 264.7 were obtained from the collection of the cell culture laboratory, Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. These cell lines were originally purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan isolate was obtained from Dr. Sri Mursiti, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang. Furthermore, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, fungizone, dimethyl sulfoxide (DMSO), and trypsin-ethylene diamine tetra acetic acid (EDTA) were obtained from Gibco (Life Technologies Corporation, United Kingdom). Meanwhile, metformin hydrochloride was purchased from Sigma-Aldrich, St. Louis, USA.

2.2 Cell culture

The two cell lines were grown and maintained in a DMEM medium supplemented with 10% FBS, HEPES [4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid] 2 g/L and $NaHCO_3$ (sodium bicarbonate) 2 g/L. To minimize contamination risk, 1% penicillin-streptomycin and 0.5% fungizone were added to the culture medium. The cells were incubated at 37°C in humidified 5% CO_2 atmosphere until they reached confluence, and then harvesting was performed by trypsinization procedure for further cytotoxicity testing.

2.3 Cell cytotoxicity assay

The cytotoxicity measurements were conducted on 3T3-L1 and RAW 264.7 using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. This was based on the principle that tetrazolium, an MTT substrate was converted into purple formazan by viable cells with an active metabolism state. The formazan accumulated as an insoluble precipitate. Hence, it was necessary to add a solubilizing solution such as a mixture of detergent and organic solvent to promote absorbance detection on a spectrophotometer and activity as a marker of viable cells (Riss *et al*., 2004).

The 3T3-L1 and RAW 264.7 were added into 96 well microplates at a density of 10^4 cells per well and incubated at 37°C overnight to permit their attachment. The cells were exposed to various concentrations of the isolate (3.125-200 µg/mL), for 24 hrs at 37°C in humidified 5% CO_2 atmosphere. Additionally, other groups were administrated with metformin as a standard drug or DMSO as a solvent control at the range of 7.81-500 µg/mL and 3.125×10^{-5}- 2×10^{-3} v/v, respectively.

A day later, the media was discarded from the microplate and 100 mL of MTT solution was added to each well. Following the incubation for 4 hrs at 37°C with 5% CO_2 humidity was the addition of 100 µL of SDS and NaOH mixture. The microplate was wrapped immediately using aluminium foil and then incubated overnight at room temperature.

A microplate reader (Bio-RAD iMark^{TM}) at 595 nm wavelength was used for absorbance assessment, and the cell viability in each concentration was calculated by the following formula (Intarasam *et al*., 2020):

$$\text{Cell Proliferation}(\%) = \frac{\text{Absorbance of treated cell} - \text{Absorbance of Medium(Blank)}}{\text{Absorbance of control cell} - \text{Absorbance of Medium(Blank)}} \times 100\%$$

2.4 Statistical analysis

The data of 3T3-L1 and RAW 264.7 cell viability toward *S. macrophylla* seed isolate, metformin, and DMSO were expressed as IC_{50} (half-maximal inhibitory concentration) that was evaluated from probit analysis, then the statistical analysis was conducted using SPSS software 23 version.

3. Results

3.1 The cytotoxicity of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan on 3T3-L1 and RAW 264.7 cell lines

To explore and initiate the drug discovery and development pipelines of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan as an anti-obesity candidate, a cytotoxicity study was conducted on 3T3-L1 and RAW 264.7. These cell lines represented adipocytes and macrophages associated with obesity pathogenesis.

Figure 1 shows that 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan at the most given range of concentrations did not affect the viability of 3T3-L1 cells, except at 100-200 µg/mL with an IC_{50} value of 148.90±12.22 µg/mL. Meanwhile, Figure 2 demonstrates the lower IC_{50} value of the isolate for RAW 267.4 cells (84.78±1.69 µg/mL) compared to 3T3-L1.

3.2 The cytotoxicity of metformin as positive control on 3T3-L1 and RAW 264.7 cell lines

A positive control is used as a reference to assess the expected response of a test. Its selection should be initiated promptly in an in vitro-based development study. The positive control is necessary to run concurrently with the test item whenever an in vitro method is performed (OECD, 2018). Therefore, the cytotoxicity of metformin as a positive control of obesity

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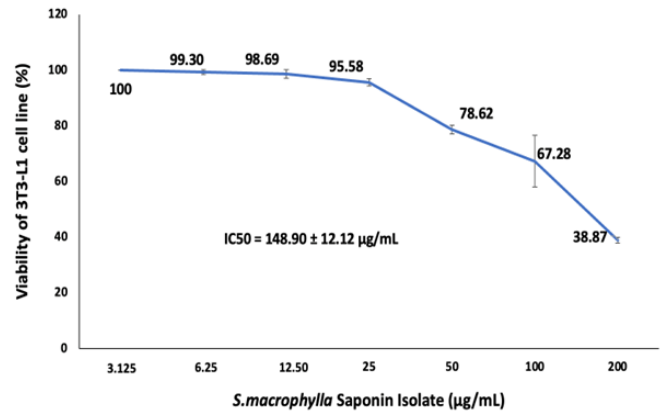

Figure 1. The percentage of cell viability and IC_{50} in 3T3-L1 cell line after 24 hrs incubation with various concentrations of saponin isolate of *S. macrophylla* seeds extract. Measured using MTT assay. The values represent as mean ± standard deviation from two independent experiments.

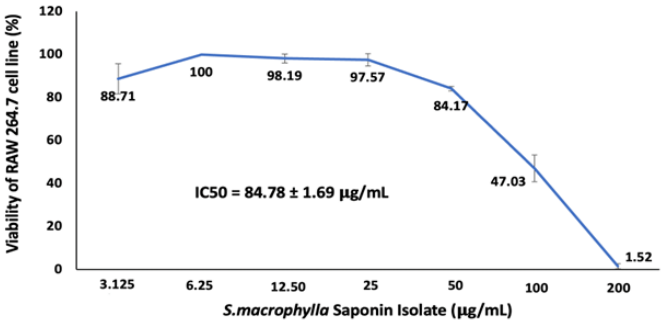

Figure 2. The percentage of cell viability and IC_{50} in RAW 264.7 cell line after 24 hrs incubation with various concentrations of saponin isolate of *S. macrophylla* seed extract. Measured using MTT assay. The values represent as mean ± standard deviation from two independent experiments.

therapy was evaluated against 3T3-L1 and RAW 264.7 cell lines.

According to Figures 3 and 4, metformin at all concentrations given (7.81-500 µg/mL) did not have negative effects on the viability of 3T3-L1 and RAW 264.7. The viability of both cell lines at various concentrations of metformin was above 88%.

3.3 The cytotoxicity of DMSO as control solvent on 3T3-L1 and RAW 264.7 cell lines

Applied solvents should not affect the health or phenotypes of cells used in in vitro methods. When diluted in media, the solvent concentration should be kept as low as possible. The common solvent concentration for DMSO and ethanol is less than 1% (OECD, 2018). The potential toxic effect on the test system should be assessed by comparing the groups with and without solvent exposure. To ensure the cytotoxicity results of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-C) furan on 3T3-L1 and RAW 264.7 were not affected by the solvent, DMSO cytotoxicity was also evaluated in both cell lines.

Figures 5 and 6 demonstrate that the various concentrations of DMSO (3.125×10^{-5} - 2×10^{-3} v/v) did not affect 3T3-L1 and RAW 264.7 viability. The cell viability was above 94% at all concentrations of DMSO.

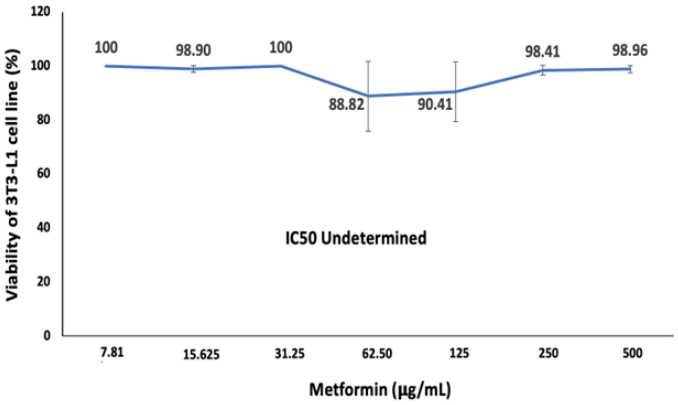

Figure 3. The percentage of cell viability and IC_{50} in 3T3-L1 cell line after 24 hrs incubation with various concentration of metformin as positive control. Measured using MTT assay. The values represent as mean ± standard deviation from two independent experiments.

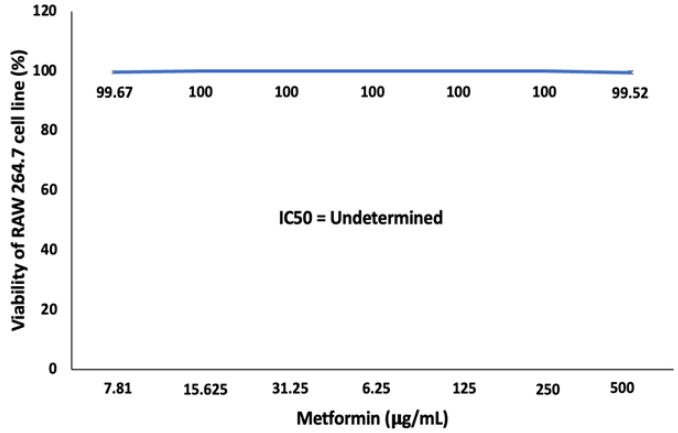

Figure 4. The percentage of cell viability and IC_{50} in RAW 264.7 cell line after 24 hrs incubation with various concentrations of metformin as positive control. Measured using MTT assay. The values represent as mean ± standard deviation from two independent experiments.

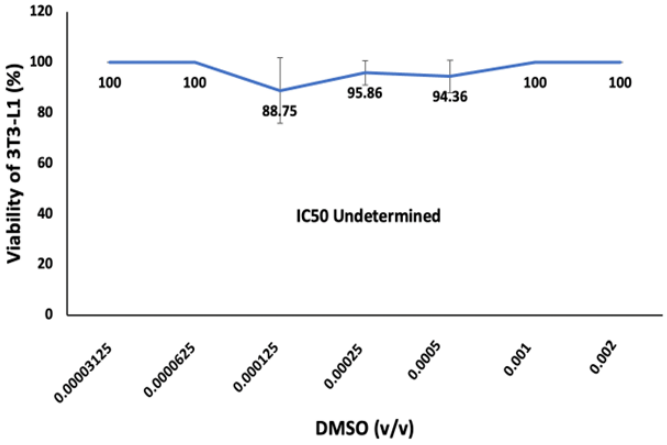

Figure 5. The percentage of cell viability and IC_{50} in 3T3-L1 cell line after 24 hrs incubation with various concentration of DMSO as solvent control. Measured using MTT assay. The values represent as mean ± standard deviation from two independent experiments.

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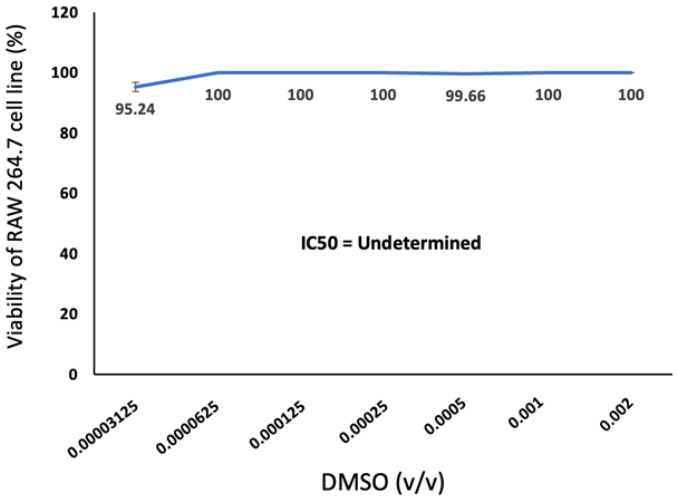

Figure 6. The percentage of cell viability and IC_{50} in RAW 264.7 cell line after 24 hrs incubation with various concentrations of DMSO as solvent control. Measured using MTT assay. The values represent as mean ± standard deviation from two independent experiments.

4. Discussion

Exploration of natural products as a new strategy for obesity therapy needs to be urgently performed. This is because the trend of obesity continues to rise and has become a worldwide epidemic (Liu *et al*., 2017). On the other hand, many anti-obesity drugs have been withdrawn from the market. Onakpoya *et al*. (2016) reported the withdrawal of 25 anti-obesity drugs from 1964 to 2009, mainly monoamine neurotransmitters class because they caused serious adverse events such as psychiatric disorders, cardiotoxicity, abuse and drug dependence (Onakpoya *et al*., 2016).

Obesity occurs due to an imbalance between energy intake and expenditure. It is characterized by dysfunction of white adipose tissues which are unable to expand adequately to store excess energy. This process leads to lipotoxicity because of ectopic fat accumulation (Longo *et al*., 2019). Adipose tissue plays an important role in obesity and metabolic complication. The ectopic fat accumulation, specifically in the liver and skeletal muscles is one of the prone mechanisms which describe the link between obesity, insulin resistance and T2D development (Bano, 2013). During obesity, there is recruitment and differentiation of monocytes into macrophages, followed by their polarization toward M1 macrophages. The M1 macrophages trigger a pro-inflammatory state, whilst M2 secretes anti-inflammatory cytokines for restoring adipose tissue homeostasis (Castoldi *et al*., 2016).

The 3T3-L1 is a well-established pre-adipose cell line which was derived from Swiss 3T3 mouse embryos at the age of 17 to 19 days. The morphology of this cell line is similar to fibroblasts which can be differentiated to produce a mature adipocyte-like phenotype when cultured under appropriate conditions. Nearly one-third of studies focusing on the adipogenesis and obesity-related characteristics published in the last 5 years used 3T3-L1 as a subject (Ruiz-Ojeda *et al*., 2016). The 3T3-L1 are easier to culture at a more affordable cost compared to freshly isolated cells. This cell line is quite useful in identifying the crucial molecular markers, transcription factors and various pathways related to preadipocyte differentiation (Poulos *et al*., 2010). Therefore, 3T3-L1 cells have been used massively for the exploration of compounds or nutrients' role in the adipogenesis process as well as to study their molecular mechanisms to evaluate the potential of these various agents as anti-obesity candidates (Poulos *et al*., 2010; Ruiz-Ojeda *et al*., 2016).

The RAW 264.7 is a monocyte/macrophage-like cell of the Abelson leukaemia virus transformation cell line which originated from the BALB/c mice. This cell line is often used for in vitro studies because it is described as a suitable model for macrophage cells (Taciak *et al*., 2018). Due to the important role of 3T3-L1 and RAW 264.7 in obesity pathogenesis, 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan should be basically considered as a non-toxic compound on both cell lines for further development.

4.1 The viability of 3T3-L1 and RAW 264.7 cell lines toward saponin isolate of Swietenia macrophylla seeds extract

The cytotoxicity results in Figures 1 and 2 showed that the isolate at the most given range of concentrations did not affect 3T3-L1 viability, except at 100-200 µg/mL with an IC_{50} value of 148.90±12.22 µg/mL. On the RAW 267.4 macrophages, a lower IC_{50} value (84.78±1.69 µg/mL) was discovered compared to 3T3-L1. Based on the US National Cancer Institute (NCI) guidelines, the isolate was not toxic to all of the cell lines in this study. A crude extract is considered a highly cytotoxic agent provided it has an IC_{50} value of ≤ 20 µg/mL, whilst the cut-off IC_{50} value for a pure compound is ≤ 4 µg/mL following the incubation periods of 48 and 72 hrs in vitro (Ramasamy *et al*., 2012; Graidist *et al*., 2015; Alabsi *et al*., 2016).

4.2 The viability of 3T3-L1 and RAW 264.7 cell lines toward metformin

Metformin was used as a positive control because it is an oral antihyperglycemic agent that has been approved and is widely known for T2D treatment. Recently, metformin is the main focus of many studies as additional therapy in pediatric obesity management

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(Lentferink *et al*., 2018). A meta-analysis by Hui *et al*. (2019) on 34 trials involving a total of 8461 participants revealed metformin significantly decreases the body weight as well as BMI index. This also showed that the most appropriate dose for adolescents is 1000 mg/day for 3 months and adults are to consume 3000 mg/day for 6 months (Hui *et al*., 2019).

The data in Figures 3 and 4 showed that the IC_{50} of metformin for 3T3-L1 and RAW 264.7 could not be determined by probit analysis because the cells' viability at all concentrations was above 88%. Compared to the IC_{50} values of metformin which were undetermined in all tested cell lines (Figures 3 and 4), the IC_{50} of 1,4-bis (3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan was lower and at risk for further development. Meanwhile, the isolate's safety profile is still promising concerning the IC_{50} cut-off value from NCI and considering the results from cytotoxicity screening of other natural compounds which was conducted on the 3T3L1 cell line.

Several traditional remedies from the Newfoundland region of Canada were reported as potential candidates in T2D and obesity management. They have activities in inhibiting a-amylase and a-glucosidase enzymes as well as decreasing lipid droplet and intracellular lipid accumulation in the 3T3-L1 cell line observed by Oil-red O staining. Moreover, 3T3-L1 viability was more than 80% in all of the treatment compounds at a concentration of 50 µg/mL (Sekhon-Loodu and Rupasinghe, 2019). Hasan *et al*. (2017) suggested that a compound concentration is considered safe provided the tested cell's viability exceeds 50%. The ethanol extract of *Tetracera indica* stems at the highest concentration (100 µg/mL) was documented to be safe against 3T3-L1 adipocytes since it only causes 18.40% inhibition or the cell viability still reaches 81.60% (Hasan *et al.*, 2017).

The isolate concentrations estimated to be safe for 3T3-L1 and RAW 264.7 cell lines were 148.90±12.22 and 84.78±1.69 µg/mL, respectively since at those concentrations the cells' viability exceeded 50%. This present study provides evidence in determining the proper concentration for further in vitro-based studies to obtain the isolate's efficacy, specifically by exploring its potency as an anti-obesity candidate.

4.3 The viability of 3T3-L1 and RAW 264.7 cell lines toward DMSO

Figures 5 and 6 indicate that DMSO was not toxic to 3T3-L1 and RAW 267.4 because the cells' viability exceeded 94% at all its concentrations. This result indicates the used of DMSO as a solvent in this present study can be tolerated due to not affecting the cytotoxicity results of the tested isolates.

These results are in agreement with the reports of Hebling *et al.* (2015) who demonstrated that DMSO 0.0004-0.008% v/v administered to the odontoblast-like MDPC-23 cell line did not reduce the number of adherent cells or induce cellular necrosis. Although the DMSO concentrations used were relatively higher (0.003 -0.2% v/v) than previous study, they also had no effect on 3T3-L1 and RAW 264.7 viability.

The DMSO concentration mentioned in this current study can still be tolerated because it is supported by another study which showed DMSO at 0.5% v/v did not influence lymphocyte cell proliferation. The lymphocyte cell proliferation began to decrease with exposure to concentrations of 1% and 2% v/v (De Abreu Costa *et al*., 2017).

The results showed that the isolate was not toxic to the 3T3-L1 and RAW 267.4 cell lines. These were promising since the toxic effect of a drug candidate is the most important cause of the termination in the development project of a new drug (Emmerich *et al*., 2021). The in vitro safety profile of the isolate supports its further development as a candidate for future obesity management.

4. Conclusion

It was indicated that 1,4-bis(3,4,5-trimethoxyphenyl) -tetrahydro-furo(3,4-c) furan, a saponin isolated from Indonesian *S. macrophylla* seed extract at certain concentrations did not affect the viability of 3T3-L1 and RAW 267.4 cell lines with IC_{50} values more than the cut-off value from NCI guidelines. These results were promising as a basis for further development of the compound, considering that the two cell lines tested play a significant role in obesity pathogenesis.

As a preliminary study, this can also be used as a guide for determining the appropriate concentration of 1,4-bis(3,4,5-trimethoxyphenyl)-tetrahydro-furo(3,4-c) furan for further in-depth in vitro and in vivo studies to explore its efficacy and might lead to the discovery of a new safe candidate for obesity management.

Conflict of interest

The authors declare no conflict of interest.

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