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## Water and ethanol extracts of tamarind (*Tamarindus indica*) suppress lipid accumulation in 3T3-L1 cells

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### Abstract

Obesity is one of the major health problems which could trigger the development of many other non-communicable diseases. Tamarind (*Tamarindus indica*) is a multi-purpose plant that provides many health benefits, including anti-obesity potentials. In Indonesia, it is often used as a traditional food component in many dishes. A recent *in vivo* study proved that tamarind might reduce the body weight of obese rats. To further investigate the anti-obesity effect of tamarind, an *in vitro* study using 3T3-L1 pre-adipocytes was conducted. During differentiation, 3T3-L1 cells synthesize and accumulate lipid in the form of triglycerides in the cytoplasm. Tamarind water and ethanol extracts were incorporated into the medium during the differentiation stage of the cells. Oil red O staining assay was used to measure the total lipid accumulated. Results showed that both tamarind water and ethanol extracts exhibited non-cytotoxicity effects on 3T3-L1 cells. In addition, tamarind water extract at 10 mg/mL decreased the total lipid accumulated significantly in 3T3-L1 cells by 20% compared with that of control, while tamarind ethanol extract showed less lipid accumulation inhibitory effect at any doses tested. This study revealed that tamarind water extract has the potential to reduce lipid accumulation, thus showing an anti-obesity effect.

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## 1. Introduction

Obesity is one of many major health problems in the world. It is caused by an abnormal accumulation of fat in the adipose tissue due to an imbalance in food intake. Moreover, many other factors could contribute to the development of obesity, such as lifestyle habits (sedentary lifestyle), physical inactivity, medical condition, and genetics. According to WHO, a total of 13% of the world's population was afflicted by obesity in 2016 (1). The prevalence of obesity amongst the adult population in Indonesia lies at 23.1% (2). Furthermore, the disease itself is often associated with the development of many other non-communicable diseases such as type 2 diabetes, hypertension, cancer, and CVD (3). Many actions could be taken to prevent the development of obesity. The WHO stated that 80% of population in developing countries have been utilizing herbs for primary healthcare (4). Moreover, utilization of herbs or other medicinal plants might be more preferable since chemical base

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drugs might possess several disadvantages and cause negative side effects on the human body (5). Increasing awareness of herbs or medicinal plants utilization has made scientists do more research on its applications to overcome many diseases or health-related problems including obesity.

Tamarind (*Tamarindus indica*) or commonly known as “Asam Jawa” by Indonesian people is a multipurpose plant that is commonly used for many occasions. Other countries such as India, Sri Lanka, Malaysia, Thailand, and also Indonesia (especially in Java) have been using tamarind in their local food recipes (6). In Indonesia, tamarind is used in *sayur asam*, *asem-asem*, *garang asem*, etc., and also incorporated in drinks “jamu” and used for confectionery products like tamarind candies. Furthermore, different part of tamarind has been consumed as a treatment to many diseases. In Africa, the fruit/ pulp of tamarind is used as a laxative and also to treat fever, the bark of tamarind is used to treat diarrhoea, and as for the leaves are often used to aid in wound healing (7). Thus, tamarind could also be considered a functional food due to its natural availability and can be consumed daily (8).

Further studies have revealed that tamarind exhibits positive effects on several diseases. These effects include anti-diabetic (9,10), anti-inflammatory (11), antioxidant (12), anticancer (13), anti-obesity, and anti-bacterial properties (14,15). Tamarind possesses many bioactive compounds or phytochemicals which include: polyphenols, anthraquinones, flavonoids, alkaloids, phlobatannins, saponin steroids, and phytosterol (16,17). Other than the aforementioned bioactive compounds, protease inhibitors in the form of trypsin inhibitor could also be found in tamarind (18). Trypsin inhibitors have been found in several studies to be involved in the control of obesity in wistar rats by affecting the satiety-inducing hormones production, lipid profile improvement, and reduction of inflammation related to obesity (19,20). Therefore, the utilization of tamarind could provide many health benefits to the body and possibly aid in reducing the risk of developing obesity.

The objective of this study was to investigate the effect of *Tamarindus indica* extract on the prevention of lipid accumulation during adipogenesis in 3T3-L1 adipocytes. The 3T3-L1 cells are widely known for its ability towards the accumulation of lipid; thus, it is playing important role in the studies of adipogenesis, lipid metabolism, and hormonal action (21). Other than that, 3T3-L1 cells were chosen because the cells are less expensive, easier to handle, can undergo a higher number of passages, and provide homogenous response towards the treatment on the cell population (22).

## 2. Materials and Methods

### 2.1. Place

Experiments were conducted at Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia, and Laboratory of Animal Cell Technology, Faculty of Agriculture, Ehime University, Matsuyama, Japan. Samples were prepared at i3L, which includes peeling, cleaning, freeze-drying and extraction. The processed extract was then brought to Japan for further analysis. The cell assay was conducted at Ehime University.

### 2.2. Reagent

Reagents used were Dulbecco’s modified Eagle’s medium (DMEM), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX) which were obtained from Sigma-Aldrich (St. Louis, MO, USA). In addition, phosphate buffered saline (PBS), trypsin, Oil red O (ORO) solution, WST-8 reagent,

and the other materials and equipment were purchased from Fujifilm Wako Chemicals (Osaka, Japan) and/or Nacalai Tesque (Kyoto, Japan).

### 2.3. Preparation of *Tamarindus indica* Extract

Fresh whole tamarinds were purchased from a local supermarket in the Jakarta area. The peels were removed from the fruits/pulp. Tamarinds were freeze-dried and stored for further analysis. Tamarind was extracted with water (TE-W) and pure ethanol (TE-E) with a ratio of 1:10 (w/v or equal to 0.1 g/mL). The dissolved tamarind samples were put into a tube rotator for 20 hours until they became homogeneous. Two-step centrifugation was done at 2,000 rpm for 10 minutes and 22,000 rpm for 20 minutes, both at 4°C to obtain the supernatant. Regarding the TE-E sample, the collected supernatant was evaporated using a rotary evaporator for 48 hours, then weighed and dissolved using a DMSO solution to make a 200 mg/mL sample stock. The dissolved sample stock was then filtered consecutively using a 0.45 µm and a 0.22 µm syringe filter. TE-E samples were then stored in vials covered with aluminum foil. Meanwhile, the pH of the collected supernatant of TE-W was adjusted to reach a neutral pH of 7.0 using 1 N NaOH. After that, the supernatant was filtered using a 3 µm, a 0.8 µm, and a 0.45 µm syringe filters, consecutively. The filtered samples were then freeze-dried for 48 hours, then weighed and dissolved in water to make 20 mg/mL and 100 mg/mL sample stocks. The pH was adjusted afterward to 7.4, then filtered using a 0.22 µm syringe filter into sample tubes. Both extracts were stored at -35°C for further use.

### 2.4. Cell Culture and Treatment

Cell culture and treatment was done in accordance to Yasunaga *et al.* (23) with a slight modification. The 3T3-L1 pre-adipocytes were cultured in a 10-cm culture dish with a 10% FBS-DMEM medium. After removal of the cell supernatant, the cells were washed with PBS. The addition of 1:1 (v/v) PBS-trypsin was done to detach the cells from the bottom of the culture dish. After 1-minute incubation, a medium containing FBS was added to stop trypsinization. After that, the cells were collected into a 15 mL centrifuge tube then centrifuged at 1000 rpm for 5 minutes. After centrifugation, the supernatant was removed and the cell concentration was adjusted to  $1.0 \times 10^5$  cells/mL before being moved to a new petri dish. Cells were then incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator. Cell maintenance was done every 2 days.

#### 2.4.1. Cell Viability Assay (WST-8 Assay)

Cell viability was examined using a Cell Count Reagent (Nacalai Tesque) based on WST-8 according to the manufacturer's instructions and as described by Santoso *et al.* (24) and Nishi *et al.* (25). The prepared cells were seeded into a 96-well culture plate for the cytotoxicity assay. The concentration of the cells was adjusted to  $1.0 \times 10^4$  cells/mL for each well and then incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator overnight. On the following day, mediums were changed with a fresh new medium containing samples at different concentrations added to each well, respectively. The sample concentrations of TE-W in culture media were as follows: 10 mg/mL, 2.5 mg/mL, 0.63 mg/mL, 0.16 mg/mL, 0.04 mg/mL, and 0.01 mg/mL, while final sample concentrations of TE-E in culture media were as follows: 1.0 mg/mL, 0.25 mg/mL, 0.063 mg/mL, 0.016 mg/mL, 0.004 mg/mL, and 0.001 mg/mL. A mixture of medium and water or ethanol was used as control, while the blanks used were medium supplemented with water or ethanol or samples without cells. Furthermore, after 48

hours of incubation, the culture media was aspirated and washed with a DMEM medium. After aspirating the medium, 10% of WST-8 solution (relative to the final volume of the culture medium) was added to each well and further incubated for 30 minutes. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, CA, USA). The cell viability was calculated with the following equation:

$$\text{Relative cell viability (\%)} = 100\% \times \frac{(\text{Abs of sample}) - (\text{Abs of sample blank})}{(\text{Abs of control}) - (\text{Abs of control})} \quad (1)$$

#### 2.4.2. Oil Red O Staining (ORO)

Oil red O staining assay was done in accordance to Kanda *et al.* (26) with slight modification. The previously prepared cells were seeded into a 24-well collagen-coated culture plate to be differentiated for the ORO staining assay. The concentration of the cells used for each well was adjusted to  $1.0 \times 10^5$  cells/mL. Cells were then incubated for 48 hours at 37°C in a humidified 5% CO<sub>2</sub> to reach confluency. The medium of the cells was changed with a fresh medium for the next 48 hours. After that, the medium was aspirated and the cells were treated with 10% FBS-DMEM containing MDI (insulin, dexamethasone, and IBMX mixture) and samples with different concentrations to each well, respectively. The samples used for the ORO staining assay were chosen based on the result of the cell viability assay. On day 8, culture medium was aspirated and the cells were washed using PBS. After removal of PBS, 4% PFA was added to each well and the plate was incubated for 20 minutes at room temperature for cell fixation. After fixation, the cells were washed with water, and ORO solution was added to each well. After 15 minutes of incubation, the cells were washed three times using water and the stained lipid droplets were photographed using a CCD camera attached to a microscope. Furthermore, the cells were left to dry at room temperature and after that, isopropanol was added to extract the cells followed by 1-hour incubation at room temperature. The extracted cells were then quantified by measuring the absorbance at 540 nm using a microplate reader. The total lipid accumulated within the cells was calculated with the following equation:

$$\text{Total lipid accumulation (\%)} = 100\% \times \frac{\text{Abs of sample}}{\text{Abs of control}} \quad (2)$$

#### 2.5. Statistical Analysis

The data obtained were expressed as mean  $\pm$  SD. Significant differences of every group were measured using one-way ANOVA followed by Tukey's HSD (honestly significant difference) as a Post Hoc test. A *p*-value of less than 0.05 is considered to be significant.

### 3. Results and Discussion

#### 3.1. Cell Viability

Cell viability assay was done to evaluate the viability/survivability of the 3T3-L1 cells after being treated with tamarind extracts. WST-8 assay reagent was used in this experiment. The basic principle of the assay is that the WST-8 reagent (water-soluble tetrazolium salt reagent) will be reduced by NADPH produced by the living cells. This reaction will produce a formazan dye with strong orange color (27).

As shown in Figure 1a and 1b, cell viability (%) values were quite high (more than 85% of cells are viable) indicating that samples showed no cytotoxic effects on 3T3-L1 cells at all

concentrations tested. Furthermore, two of the highest concentrations from both TE-W (10 mg/mL and 2.5 mg/mL) and TE-E (1.0 mg/mL and 0.25 mg/mL) samples were chosen as the concentrations of the samples to be used for further analysis. However, sample concentrations of TE-E samples were made lower compared to TE-W because of DMSO solution used as a solvent to dissolve the samples. DMSO solution could be considered toxic towards the cells at a certain concentration, hence the maximum concentration of DMSO should be limited to around 0.1 to 0.5% of DMSO solution within the samples (28).

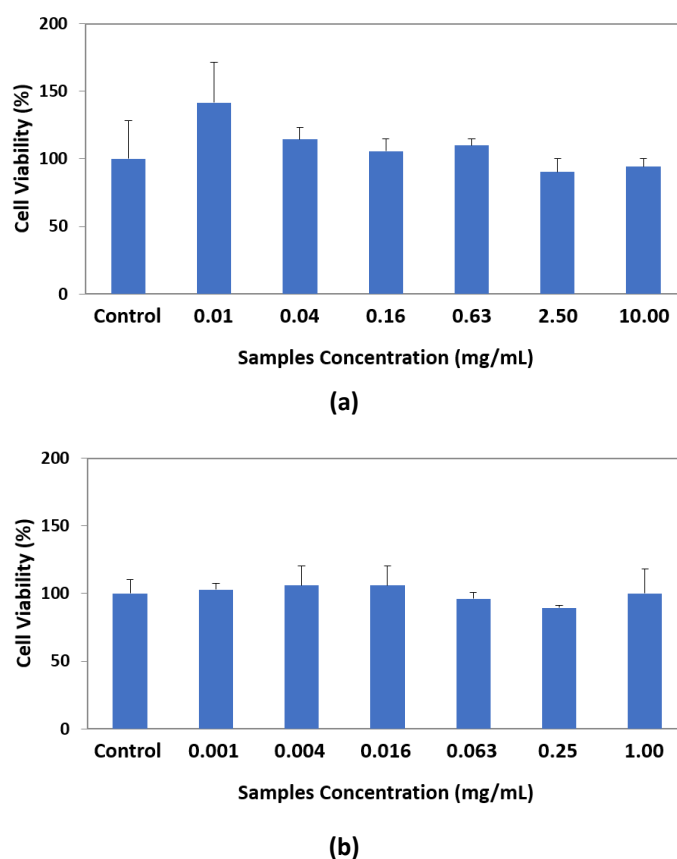


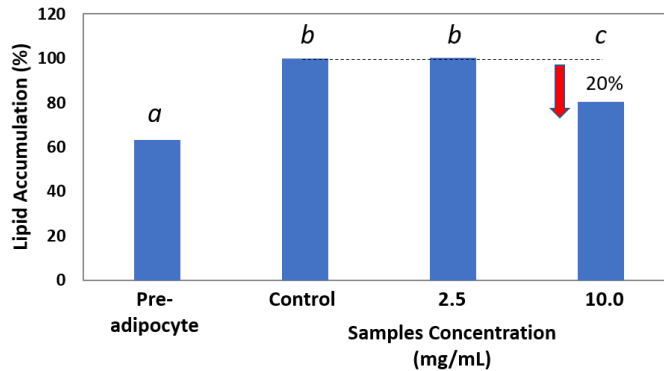
Figure 1. Viability (WST-8 assay) of 3T3-L1 cells treated with (a) TE-W and (b) TE-E.

### 3.2. Oil Red O Staining

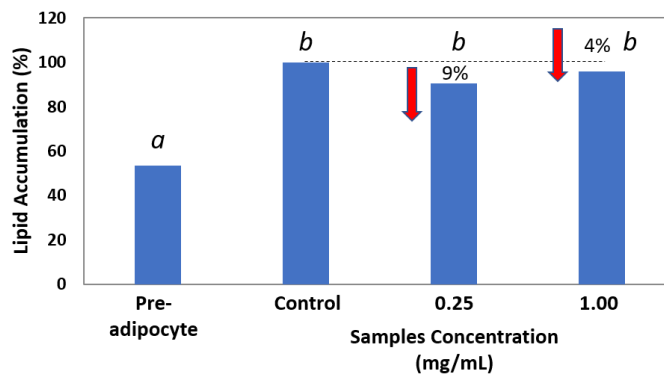
Oil red O (ORO) assay was done to measure the amount of lipid accumulated within the cells. ORO dye is a lipid-soluble dye that will strongly stain the lipids (29). The assay will be followed by quantitative measurement by utilizing isopropanol to elute the dye from the cells for further reading of the absorbance in a microplate reader. Before the assay, cell differentiation was initiated. The cells were subjected to media differentiation inducers (MDI) for 8 days until the cells were fully differentiated. During differentiation, samples with previously chosen concentrations were added to observe the difference in the total lipid accumulated in the differentiated cells.

Figure 2 showed that the cells that were treated with TE-W at 10 mg/mL concentration showed a significant decrease ( $p < 0.05$ ) by 20% of total accumulated lipid compared to the control. However, TE-W at the concentration of 2.5 mg/mL showed no effect on the total lipid accumulation during the cell differentiation. The same results were shown on the TE-E treated

cells in which at both concentrations, the samples only showed a slight reduction of 4-9% in the total lipid accumulated within the cells.



(a)



(b)

Figure 2. Lipid accumulation in 3T3-L1 cells treated with (a) TE-W and (b) TE-E. The percentage of accumulated lipid was calculated using the equation mentioned above. Statistical calculations were done by comparing the absorbance values of each treatment (n=4) using Tukey's HSD in IBM SPSS Statistics 20. Total lipid accumulated without a common superscript (*a*, *b*, *c*) differs significantly between samples ( $p < 0.05$ ).

Results indicated that TE-W might have the ability to significantly suppress the lipid accumulation during the cell differentiation stage (Figure 2a), while TE-E might have the ability but not significantly (Figure 2b). These results were supported by the photographic images of the cells after being stained by ORO solution as seen in Figure 3 below.



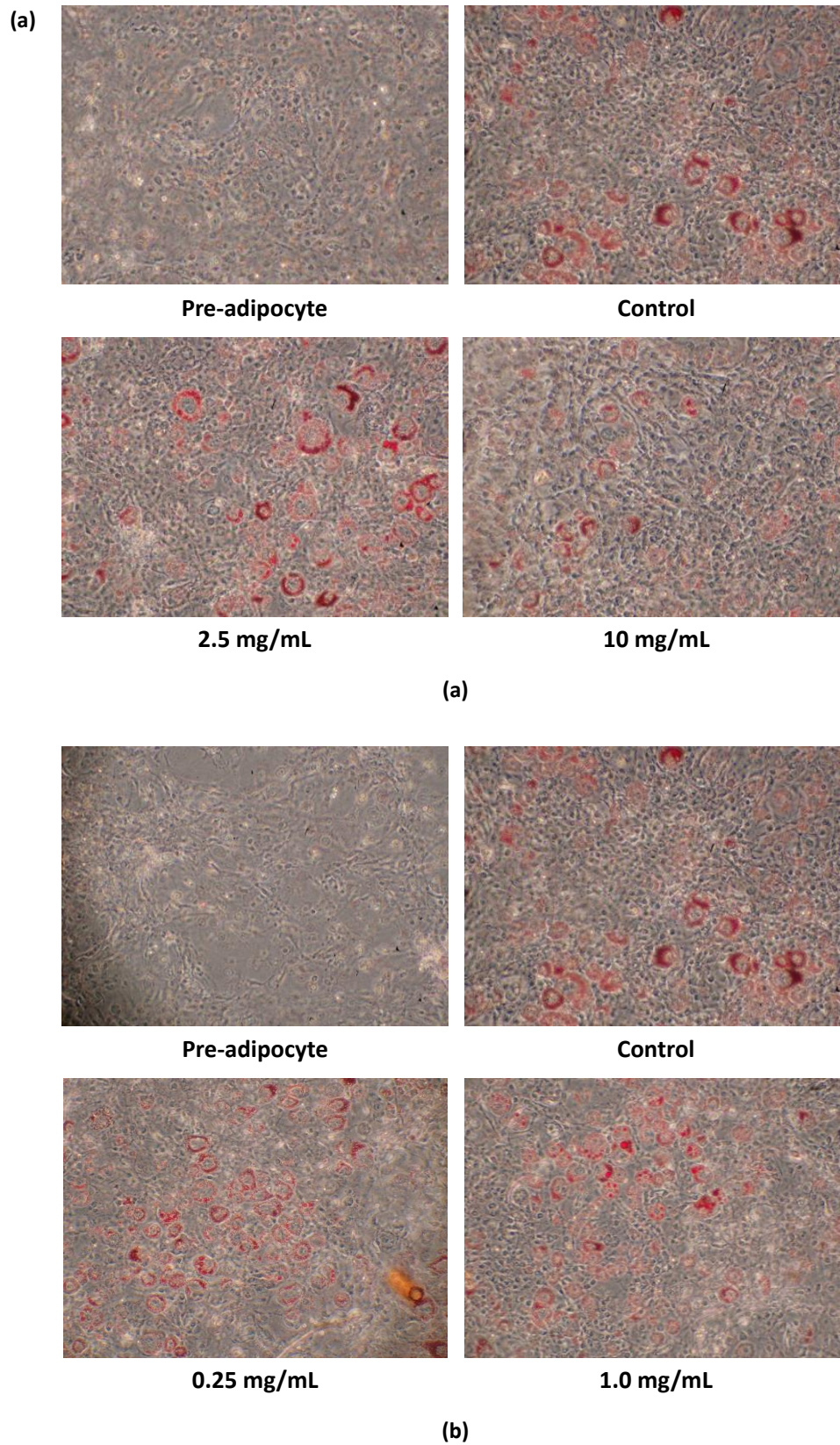


Figure 3. Oil red O staining on 3T3-L1 cells treated with (a) TE-W samples and (b) TE-E samples with  $\times 400$  magnification. Accumulated lipid is stained in red color.

According to the photographic images, it is clear that TE-W at 10 mg/mL concentration (Figure 3a) showed to have less accumulated lipid in 3T3-L1 adipocytes compared to those of control and other concentration tested. Unfortunately, TE-E did not show a strong inhibition effect on lipid accumulation in 3T3-L1 adipocytes (Figure 3b).

Our study could be considered new since there was no previous study reporting the anti-obesity effect of *Tamarindus indica* extract utilizing 3T3-L1 pre-adipocytes. Furthermore, our results showed a positive effect of tamarind extract on the suppression of lipid accumulation in 3T3-L1 cells, thus showing that tamarind potentially exhibits anti-obesity properties. Another study has been proving that tamarind may reduce the risk of obesity by lowering the bodyweight of rats and decreasing its serum cholesterol and LDL level after oral transmissions of tamarind extract for a certain period, hence, these findings also indicate that tamarind might be potentially used for future treatment of CVD (14).

#### 4. Conclusions

The lipid accumulation suppression effect of *Tamarindus indica* was evaluated using WST-8 and Oil red O staining assays. Ethanol extracted tamarind (TE-E) showed no cytotoxicity at all concentrations tested, which were: 1.0 mg/mL, 0.25 mg/mL, 0.063 mg/mL, 0.016 mg/mL, 0.004 mg/mL, and 0.001 mg/mL. In addition, TE-E treatments showed low suppression effect on lipid accumulation in 3T3-L1 adipocytes. However, tamarind water extract (TE-W) at 10 mg/mL exhibited a significant reduction ( $p < 0.05$ ) of the accumulated lipid in 3T3-L1 cells without any cytotoxicity.

It could be concluded from this study that tamarind at a certain concentration exhibits anti-obesity effect by suppressing lipid accumulation in 3T3-L1 cells. This lipid accumulation lowering activities make tamarind as potential functional foods. In addition, tamarind possess a lot more benefits upon consumption not only towards obesity, but also have positive effects including anti-diabetic, anti-inflammatory, antioxidant, anticancer, and anti-bacterial properties as reported elsewhere.

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#### Author Contributions

K.R.H. conceptualization, methodology, investigation, writing - original draft, data curation; M.I. supervision, data curation; K.N. supervision, validation, writing - review and editing; T.S. supervision, methodology, writing - review and editing; A.B.N.P. supervision, conceptualization, methodology, writing - review and editing.

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## Data Availability Statement

Not applicable.

## Conflicts of Interest

The authors declare no conflict of interest.

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