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Original Article

Inhibitory potential of rambutan seeds extract and fractions on adipogenesis in 3T3-L1 cell line

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ABSTRACT

Objective: Type 2 diabetes is a global health problem with increasing prevalence related to several conditions; one of these is due to obesity. Rambutan (*Nephelium lappaceum* L) seeds contain various phenolic compounds. The present study was designed to evaluate the phytochemical content and the inhibitory potential of rambutan seeds extract and fractions on glucose-6-phosphate dehydrogenase (G6PDH), α-glucosidase, and triglyceride activities *ex vivo* in 3T3-L1 cell line (pre-adipocytes) for an antidiabetic and antidiapogenesis agent screening. **Materials and Methods:** Phytochemical analysis was performed using modified Farnsworth method. Cytotoxicity or cell viability of rambutan seed extracts (distillated ethanol 70%) and fractions (hexane, ethyl acetate, butanol, and water fractions) were assayed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Triglyceride (TG) level, G6PDH and α-glucosidase activity and inhibitory activities were determined by commercial assay kits. **Results:** Extract and fractions of rambutan seed contained alkaloid, terpenoid, triterpenoid, and phenol; flavonoid, tannin, saponin, and steroid were undetected. The lowest cytotoxic activity and safe substances on 3T3-L1 cell were rambutan seed extract and hexane fraction. Rambutan seed extract at the dose of 50 μg/ml was the most active to lower G6PDH and α-glucosidase as well as TG level. **Conclusion:** Rambutan seed extract and hexane fraction have the phytochemical bioactive content to possess inhibitory potential on G6PDH and α-glucosidase as well as TG level in the present experimental set of 3T3-L1 cell lines.

KEY WORDS: 3T3-L1, anti-adipogenesis, antidiabetic, *Nephelium lappaceum* L, triglyceride

INTRODUCTION

Prevalence of Type 2 diabetes mellitus (T2DM) is increasing especially in the developing countries. In Indonesia, T2DM is predicted to be as much as 21.2 million in 2030. The increasing prevalence is due to population growth, aging, urbanization, obesity, and physical inactivity. Obesity is leading the risk for death; more than 4.3 million adults die yearly as a result of being obese [1]. Obesity is defined as excessive fat accumulation in adipocytes [2], and trigger to various diseases such as T2DM, cardiovascular disease, and cancer [3]. Excessive caloric intake such as carbohydrates and fat, and sedentary lifestyles will induce hyperglycemia and increase fatty acid level; these can increase free radical production by mitochondria and lead to oxidative stress. The oxidative stress may cause several problems such as beta cell damage and altered insulin production, increase insulin resistance and endothelial dysfunction. Accumulation of fat and the differentiation of adipocytes are related to obesity development [4]. All of these can lead to T2DM and metabolic syndrome [5].

Oral antidiabetic agents have different mechanisms including increasing the insulin secretion, decreasing the glucagon secretion, increasing the insulin receptor sensitivity, decreasing the glucose absorption, and inhibiting several glucosidase enzymes [6-9]. Many plants are known as the source of potent and powerful drugs that have fewer side effects and less toxicity compared to synthetic drugs [10-13]. Many phenolics are known to possess anti-obesity properties through mechanisms that inhibit adipogenesis, stimulate lipolysis or induce apoptosis [14]. Rambutan (*Nephelium lappaceum* L), belonging to the Sapindaceae family, is a tropical fruit widely distributed in South-East Asia. Its consumption results the vast amounts of waste from the seeds and peels of the fruit [15]. Rambutan seeds contain various phenolic compounds such as ellagic acid, corilagin, and geraniin [16].

In the previous study, rambutan seeds extract and fractions possess an inhibitory effect on α-glucosidase activity *in vitro* [17]. Hypoglycemic activity plays an important role in T2DM therapy [18]. Therefore, the present study is a continuing work designed to evaluate additional parameters

including phytochemical analysis and inhibitory potential of rambutan seeds extract and fractions on Glucose-6-phospate dehydrogenase (G6PDH) and α-glucosidase activities, and TG levels *ex vivo* in the 3T3-L1 cell line for antidiabetic and anti-adipogenesis agent screening. In addition, viability assay was performed to confirm the suitable concentration for the next assays. The mouse preadipocyte cells, 3T3-L1 is a suitable *ex vivo* model for this research because it can be differentiated to become adipocyte similar as the *in vivo* process [5].

MATERIALS AND METHODS

Rambutan Seed Extraction and Fractionation

Rambutan (*Nephelium lappaceum* L) was collected from Kesamben-Blitar plantation, East Java, Indonesia. The plant was identified by herbarium staff, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. Extraction was performed based on the maceration method, and the fractionation was performed by modified partition using four partitioning solvents (hexane, ethyl acetate, butanol, and water) [17,19]. Dried and milled rambutan seeds were macerated in ethanol 70%, then evaporated and yielded to rambutan seeds extract. Extract was partitioned with n-hexane and water (1:1), yielded hexane fraction; the residue was partitioned with ethyl acetate and water (1:1), yielded an ethyl acetate fraction; the residue was partitioned with butanol and water (1:1), yielded butanol fraction; and the residue was the water fraction [17,19].

Phytochemical Assay

The rambutan seed extract and four fractions were tested by phytochemical assay using modified Farnsworth method including flavonoid, phenolic, saponin, triterpenoid, steroid, terpenoid, tannin, and alkaloid assays [20].

3T3-L1 Cell Culture and Adipocyte Differentiation Induction

The mouse pre-adipocytes cells 3T3-L1 (ATCC®CL-173) were obtained from Biomolecular and CV Gamma Scientific Biolab, Malang-East Java, Indonesia, Aretha Medika Utama. The 3T3-L1 cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM; Biowest, Marcq, Belgium) supplemented with 10% fetal calf (bovine) serum (FBS; Biowest) and 100 U/ml penicillin-streptomycin (Biowest) then incubated for 24 h at 37°C humidified atmospheres and 5% CO_2. After the cells confluence, medium was discharged, and cells were seeded in 96-well plate (3×10^4 cells/well) with DMEM supplemented with 10% FBS and then incubated for 48 h. After cells reached 80% confluence, cells were induced to differentiate using Millipore ECM950 kit. Medium was replaced by initiation medium (DMEM containing FBS 10% and 1:10000 dexamethasone) and incubated for 48 h. Insulin medium was replaced with progression medium (DMEM containing FBS 10% and 1:1000 insulin) and placed in an incubator for 48 h. The medium was then replaced again with maintenance medium (DMEM supplemented with 10% FBS) and incubated for 2-4 days in 37°C incubator [21]. Cells were then observed under the inverted light microscope (Olympus) after Oil-Red-O staining (Millipore ECM950) [22].

Viability Assay

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega; Madison, WI, USA) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity [23,24]. The 3T3-L1 cells were seeded in 96-well plates (5×10^3 cells/well) in 100 μl medium (DMEM containing 10% FBS and 100 U/ml penicillin-streptomycin) for 24 h at 37°C humidified atmospheres and 5% CO_2. The medium then washed and supplemented with 99 μl new medium and 1 μl of rambutan seed extract and fractions in various concentrations (10, 50, and 100 μg/ml) and incubated for 48 h at 37°C and 5% CO_2. After 48 h medium was replaced by 20 μl MTS and incubated for 3 h at 37°C. The absorbance was measured at 490 nm [23,24]. The viability assay was performed to determine the safe concentrations for available concentrations on the next assay.

G6PDH Assay

The 3T3-L1 cells were seeded in 96-well plates (5×10^3 cells/well) in 100 μl medium (DMEM containing 10% FBS and 100 U/ml penicillin-streptomycin) for 24 h at 37°C humidified atmosphere and 5% CO_2. A commercial G6PDH kit (Abnova KA0880; Taipei City, Taiwan) was used. According to the cytotoxicity of the rambutan seeds extract and fractions on 3T3-L1, we concluded that rambutan seeds extract and hexane fraction were safe and non-toxic. 20 μl samples of medium from cell culture after rambutan seeds extract and hexane fraction treatment (10 and 50 μg/ml of concentration) and G6PDH positive kit for positive control were added into the well and then 30 μl assay buffer and 50 μl developer work were added. The assay buffer without samples was used for blank. The absorbance was measured at 450 nm. Then the samples were incubated at 37°C for 30 min in the dark room. After 30 min, the samples were measured again using at 450 nm of wavelength. G6PDH concentration was determined by following equation:

$$G6PDH = \left[\left(\frac{B}{(T2 - T1) \times V}\right)\right] \times \text{sample dilution}$$

B = blank sample,

T1 = first absorbance,

T2 = second absorbance,

V = total volume.

Alpha-glucosidase Activity Assay

Alpha-glucosidase activity assay was performed using Abnova KA1608 assay kit. This assay is based on a kinetic reaction;

working reagent 4-nitrophenyl-α-d-galactopyranoside (α-NPG) was prepared by dissolving 8 μl α-NPG with 200 μl assay buffer. 20 μl of samples (medium from 3T3-L1 cell culture after rambutan seeds extract and hexane fraction treatment in concentration of 10 and 50 μg/ml) were dissolved in working reagent. The absorbance was measured in 450 nm of wavelength (T1); after 20 min incubation, the absorbance was measured again in 450 nm of wavelength (T2). 20 μl of distilled water (dH_2O) was dissolved in 200 μl H_2O as blank (OD H_2O), and 20 μl dH_2O was dissolved in 200 μl calibrator as a standard solution (OD Cal). The absorbance of blank and standard solution was measured in 450 nm of wavelength. The glucose concentration was determined by the formula given below:

$$\text{Glucose content}\left(\frac{\text{U}}{\text{ml}}\right)=\left(\frac{\text{T2-T1}}{\text{OD Cal1-OD H}_2\text{O}}\right)\times 250$$

T1 = first absorbance,

T2 = second absorbance,

OD (optical density) Cal = calibrator absorbance,

OD H_2O = H_2O absorbance.

Triglyceride Assay

The triglyceride level was measured using Randox TR210 (Antrim, United Kingdom) assay kit. 500 μl mix reaction containing 450 μl reagent with 5 μl sample (cell lysate after rambutan seeds extract and hexane fraction treatment in concentrations of 10 and 50 μg/ml) was incubated in 37°C for 5 min. Double-distilled water (ddH_2O) was used for a blank well, and standard reagent was used for standard well. Standard reaction was prepared in seven different concentrations using serial dilution (2.18, 1.09, 0.545, 0.273, 0.136, 0.068, and 0.034 mmol/l). The absorbance was measured in 500 nm of wavelength. Triglyceride concentration was calculated using the equation:

$$\text{Triglyceride level}=\frac{\text{absorbance of sample}}{\text{absorbance of standard}}\times\text{standard concentration}\left(\frac{\text{mM}}{\text{l}}\right)$$

RESULTS

Phytochemical Content of Rambutan Seed Extract

The phytochemical assay showed that ethanol 70% rambutan seeds extract contained the highest terpenoids. Seeds extract and all fractions contained the lowest alkaloids. Tannin, steroid, saponin, and flavonoid were undetected in the rambutan seeds extract and fractions. The hexane, ethyl acetate, and water fractions contained moderate level of triterpenoid. The extract, hexane, ethyl acetate, and butanol fractions also contained moderate level of phenol [Table 1].

Adipocyte Differentiation and Viability

3T3-L1 cells, in normal conditions, can propagate into fibroblast cells. 5 days insulin induction accumulated intracellular lipid and transformed lipid droplets in the cell. Figure 1 shows the normal cells and adipocyte differentiation induced cells with red lipid droplets.

Cytotoxic or viability activity was assayed using the MTS assay. The percent of cells viability was determined by comparing the cells' viability value of samples (extract or fractions) to the control. As result viability of samples were found to be in concentration-dependent manner [Table 2]; rambutan seeds extract and hexane fraction in 10 and 50 μg/ml concentrations were more safe compared to the other fractions, so it can be used for the next assays in 3T3-L1 cells.

The Inhibition of Alpha-glucosidase Activity

Alpha-glucosidase is the enzyme involved in the complex carbohydrate dissociation in the small intestine. Table 3 shows the α-glucosidase concentration and α-glucosidase inhibitory activity. The most effective α-glucosidase inhibitory activity was shown by rambutan seeds extract at the dose of 50 μg/ml. In individuals with T2DM, the inhibition of α-glucosidase activity can reduce HbA_{1C} and postprandial insulin levels [25].

The Inhibition of Glucose-6-Phospate Dehydrogenase Activity

Extract and hexane fraction of rambutan seeds were used to determine G6PDH inhibition activity in 3T3-L1 cells. Table 4 shows the concentration of 50 μg/ml presented the highest inhibitory activity for both rambutan seeds extract and hexane fraction. G6PDH is a cytosolic enzyme involved in the pentose phosphate pathway. The high activity of G6PDH in adipocytes can cause lipid metabolism dysregulation and insulin resistance [26].

Trygliceride Level

Table 5 shows extract and hexane fraction of rambutan seeds could reduce TG level in 3T3-L1 cells. Extract of rambutan seeds was the most active to inhibit TG at both concentrations while the hexane fraction had the lowest TG inhibition

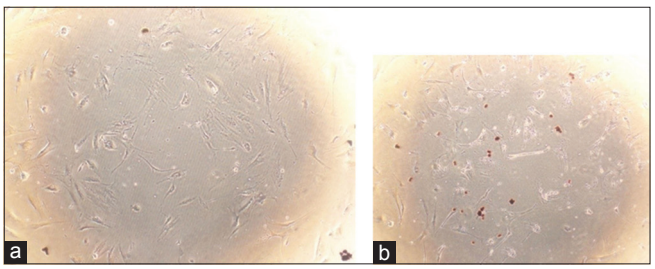

Figure 1: 3T3-L1 cell culture: (a) cell without the adipogenesis induction; (b) adipogenesis induction cells with red lipid droplets. The figure was obtained using inverted Olympus microscope (×100 magnification)

Table 1: Phytochemical assay of rambutan seeds extract and fractions

Samples	Tannin	Triterpenoid	Steroid	Terpenoid	Saponin	Alkaloid	Flavonoid	Phenol
Ethanol extract	-	+	-	++	-	+	-	++
Hexane fraction	-	++	-	+	-	+	-	++
Ethyl acetate	-	++	-	+	-	+	-	++
Butanol fraction	-	+	-	+	-	+	-	++
Water fraction	-	++	-	+	-	+	-	-

++: Moderate content, +: Low content, -: Undetected

Table 2: 3T3-L1 cell's viability of rambutan seeds extract and fractions

Samples	100 μg/ml	50 μg/ml	10 μg/ml
Control	100[a]	100[a]	100[a]
Extract	80.46±1.05[a]	90.16±0.91[b]	94.64±1.1[c]
Hexane fraction	87.27±1.57[a]	88.5±0.66[a]	91.12±2.83[a]
Ethyl acetate	75.63±1.12[a]	80.47±0.59[b]	83.98±0.1[c]
Butanol fraction	81.07±0.1[a]	83.97±0.41[b]	87.93±1.59[c]
Water fraction	71.95±1.44[a]	78.77±1.23[b]	82.32±1.39[c]

Data are presented as mean±SD. Different letters in the same row are significant (among concentrations) at $P<0.05$ (Tukey's HSD *post hoc* test, measured in triplicate), SD: Standard deviation

Table 3: α-glucosidase concentration and inhibition of extract and fractions in 3T3-L1 cells

Samples	Glucosidase activity (U/ml)	Glucosidase inhibition (%)
Extract 10 μg/ml	245.07±0.37[b]	20.7±0.12[b]
Extract 50 μg/ml	183.04±11.72[a]	40.77±3.79[c]
Hexane fraction 10 μg/ml	250.4±11.76[b]	18.97±3.8[b]
Hexane fraction 50 μg/ml	232.09±3.05[b]	24.9±0.99[b]
Positive control	316.92±8.61[d]	−2.56±2.79[a]

Data are presented as mean±SD. Different letters in the same column are significant (among samples) at $P<0.05$ (Tukey's HSD *post hoc* test, measured in triplicate), SD: Standard deviation

Table 4: G6PDH activity and G6PDH inhibition activity of extract and fraction in 3T3-L1 cells

Samples	G6PDH activity (nmol/min/ml)	G6PDH inhibition (%)
Extract 10 μg/ml	0.24±0.02[b]	10.00±6.74[b]
Extract 50 μg/ml	0.17±0.06[a]	35.54±2.29[c]
Hexane fraction 10 μg/ml	0.29±0.01[c]	−4.92±5.41[a]
Hexane fraction 50 μg/ml	0.19±0.02[a]	30.49±4.61[c]
Positive control	0.27±0.02[bc]	0.00±4.93[ab]

Data are presented as mean±SD. Different letters in the same column are significant (among samples) at $P<0.05$ (Tukey's HSD *post hoc* test, measured in triplicate), SD: Standard deviation, G6PDH: Glucose-6-phosphate dehydrogenase

Table 5: TG level and TG inhibition activity of extract and fractions in 3T3-L1 cells

Samples	Parameters		
	TG level (mol/l)	TG level (mg/dl)	TG inhibition (%)
Extract 10 μg/ml	1.05±0.02[b]	93.12±2.25[b]	25.85±1.79[d]
Extract 50 μg/ml	0.90±0.06[a]	79.65±0.46[a]	36.58±0.36[e]
Hexane fraction 10 μg/ml	1.32±0.03[d]	116.87±2.50[d]	6.94±1.99[b]
Hexane fraction 50 μg/ml	1.23±0.01[c]	109.03±0.86[c]	13.18±0.68[c]
Positive control	1.42±0.04[e]	125.65±3.26[e]	0.00±0.00[a]

Data are presented as mean±SD. Different letters in the same column are significant (among samples) at $P<0.05$ (Tukey's HSD *post hoc* test, measured in triplicate), SD: Standard deviation, TG: Triglyceride

activity. Triglyceride is one kind of molecule that affects insulin sensitivity; the higher the TG level exhibited, the lower the insulin sensitivity [27].

DISCUSSION

Different parts of medicinal plants like stem, flower, seed, root, fruit, *etc.*, are used to obtain pharmacologically active metabolites [11]. In this *ex vivo* study, we evaluated the antidiabetic and anti-adipogenesis potential of rambutan seeds extract and fractions. 3T3-L1 pre-adipocytes, which can be induced to differentiate into adipocytes according to the coordinated program, are one of the most useful and established cell lines for researching the adipogenesis process [28]. Our results demonstrated that rambutan seeds extract and its hexane fraction exhibited the potency to inhibit α-glucosidase and G6PDH activities, and lower the TG level to prevent obesity-associated complications including T2DM, cardiovascular or other disease. Rambutan seeds extract and hexane fraction exhibited positive result as anti-adipogenesis and antidiabetic and are non-toxic to the 3T3-L1 cells. Viability test is an important aspect to test the bioactive toxicity or the adverse effect of bioactive substances on living organisms prior to the use as drug or chemical for clinical reasons [29-31].

High plasma TG level is associated with obesity [27]. In the present study extract and hexane fraction of rambutan seeds could lower TG level. Rambutan seeds extract and hexane fraction contain alkaloid which possess anti-obesity activity through decrease of TG levels; this result was validated with previous research that alkaloid of *Nelumbo nucifera* could inhibit 3T3-L1 pre-adipocyte differentiation and improves high-fat diet-induced obesity [32]. Triglyceride metabolism is activated by expression of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and adipocyte-specific fatty acid binding protein 2 (aP2) genes. Extract of fruit which is rich in phenolic compound such as blueberry, decrease FAS, and aP2 gene expression in 3T3-L1 cells. The reduction of FAS and aP2 expression due to due to Cytosine-Cytosine-Adenosine-Adenosine-Thymidine (CCAAT)-enhancer binding protein β and peripapillary atrophy beta family down regulation which not only slow down TG synthesis but also inhibit pre-adipocyte differentiation [33].

G6PDH can accelerate adipogenesis through generating ligand peroxisome proliferator-activated receptor γ which is a transcription factor involved in adipogenesis [26,34,35]. In the present study, extract and hexane fraction of rambutan seeds showed an ability to inhibit G6PDH in a concentration-dependent manner. Phenol as one of the compounds found in rambutan seeds extract and hexane fraction can reduce glucose-

6-phosphate by G6PDH inhibition; this result was consistent with the previous study showing phenolic compounds as antidiabetic agents [36]. The inhibition of G6PDH by *Rhodiola* likely prevented prolin oxidation required for critical ATP generation that is coupled to antioxidant enzyme reaction via pentose phosphate pathway, consequently leading to inhibition of adipogenesis [37]. These results suggest that extract and hexane fraction of rambutan seeds have anti-adipogenesis effect.

Rambutan seeds extract and hexane fraction showed a high glucose inhibition value in differentiated 3T3-L1 adipocytes; this result was confirmed with the previous research that rambutan rind extract contained high levels of phenolic compounds which had α-amylase and α-glucosidase inhibitory activities [36]. Phenolic compound and geraniin from the rambutan rind extract were also reported to have α-amylase and α-glucosidase inhibitory activities [16]. In the previous study, rambutan seeds extract and fractions were more active as α-glucosidase inhibitor compared to acarbose and glucobay [17]. Alpha-glucosidase plays an important role in oligosaccharides and disaccharides hydrolysis in the small intestine to become the blood glucose [18,37,38]. Decreasing of glucose levels in plasma has been reported as one of the most effective therapeutic approaches for T2DM [18].

Triterpenoid, terpenoid, alkaloid, and phenol compounds were found in the rambutan seeds extract and fractions; although in moderate and low concentration, those bioactive compounds have hypoglycemic effects and antidiabetic potential [36,39,40]. Rambutan seeds extract and fractions also contained furanone, corilagin and geraniin that possess antidiabetic effects through their antioxidant, free radical scavenging and *in vitro* hypoglycemic activity [16,40,41]. Based on our *ex vivo* study, we recommend that rambutan seeds extract and its hexane fraction may have beneficial effects as potential anti-adipogenesis and antidiabetic agent. However, further mode of action tests as well as preclinical and clinical studies should be pursued before pharmaceutical applications.

In conclusion, rambutan seeds extract and its hexane fraction contain phytochemical bioactive content that possess inhibitory potential on G6PDH and α-glucosidase activity, and TG levels *ex vivo* in 3T3-L1 cell line. As suggestion, an animal model such as the *in vivo* test in mice is still needed to confirm the antidiabetic and anti-adipogenesis activity of the rambutan.

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