

Cytotoxicity and Antiobesity Activity of Freeze-Dried *Malus domestica*, *Canarium sp.* and *Averrhoa bilimbi* Fruit

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ABSTRACT

Obesity has a role in the development of diseases such as diabetes, cardiovascular disease, and hyperlipidemia which is characterized by the increase of adipose tissue mass due to an imbalance of energy intake and expenditure. Freeze-dried fruits are well known to possess antiobesity activity. In this study, we have evaluated the antiobesity activity of freeze-dried fruit (*M. domestica*, *Canarium sp.*, and *A. bilimbi*) using CHOL, G6PDH, TG level, and Oil Red O assay. The viability of 3T3-L1 cell in the *Canarium sp.* freeze-dried in the concentration of 12.50 µg/ml has a higher value compared to *M. domestica* and *A. bilimbi*. The measurements of CHOL, G6PDH, TG level, and Oil Red O assay of the *M. domestica* freeze-dried in the concentration of 75 µg/ml has higher inhibitory activity compared to the *Canarium sp.* and *A. bilimbi* freeze-dried. In the CHOL assay, *M. domestica* freeze-dried has a higher value compared to *A. bilimbi* and *Canarium sp.* In the G6PDH assay, the freeze-dried of *M. domestica* has the value of 49.56%, *Canarium sp.* (45.22%), and *A. bilimbi* (47.13%), while in the Oil Red O assay, *M. domestica* has inhibition activity of 62.63%, *A. bilimbi* 50.01% and *Canarium sp.* 44.13%. The level of TG showed that *M. domestica* has higher activity with the value of 60.61%, *A. bilimbi* 57.54%, while *Canarium sp.* 55.03%. The freeze-dried of *M. domestica* in the concentration of 75 µg/ml has good inhibitory activity of lipid compared to *A. bilimbi* and *Canarium sp.*

Keywords: Antiobesity; adipogenesis; freeze-dried; *Malus domestica*; *Canarium sp.*; *Averrhoa bilimbi*

INTRODUCTION

Obesity is defined as excessive fat accumulation in adipocytes (Jou *et al.*, 2010), and triggers various diseases such as Type 2 Diabetes Melitus (T2DM), cardiovascular disease, and cancer (Lois & Kumar, 2009). When a person becomes obese and adipocytes dilatation, the molecular and cellular adipose tissue undergo changes that will affect systemic metabolism (Attie & Scherer, 2009). Various efforts are made to overcome obesity, the most popular method is a low-fat diet and consumption of various drugs.

The use of chemical drugs in the long term can cause a variety of side effects such as headaches, stomach pain, vomiting, and heart attacks (Park *et al.*, 2007). Various types of fruits containing Hydroxycinnamic Acid (HCA) and phenol compounds, flavonoids high have antiobesity activities (Pittler & Ernst, 2004; Hsu & Yen, 2008; Kim *et al.*, 2010; Kamisoyama *et al.*, 2008; Swick, 2011; Dzomba & Musekiwa, 2014). Therefore, the consumption of fruits need to be

increased, considering the side effects that could occur and the habit of eating fruits is good for health besides reducing the weight of obesity patients. The different types of fruits containing HCA, phenol, and flavonoid (Pittler & Ernst, 2004; Hsu & Yen, 2008; Kim *et al.*, 2010; Kamisoyama *et al.*, 2008; Swick, 2011; Dzomba & Musekiwa, 2014).

Canarium sp., *Malus domestica*, and *Averrhoa bilimbi* are fruits that have bioactive compounds and antiobesity properties. *Canarium sp.* contains some bioactive compounds proanthocyanidins (PAs), flavan-3-ols, prodelphinidin, epicatechin, and epigallocatechin. The results of *gas chromatography-mass spectroscopy* (GC-MS) has shown that *M. domestica* phenolic content is (+)-catechin (C) and (-)-epicatechin (EC), phloridzin (dihydrochalcone glycosides), quercetin (flavonols), cyanidin (anthocyanidins), cyanidin -3-O-galactoside (anthocyanins), chlorogenic acid (phenolic acids), and hydroxycinnamates (p-coumaric acid) (Vrhovsek *et al.*, 2004; McGhie *et al.*, 2005; Cuthberton *et al.*, 2012; Francini & Sebastiani, 2013). *A. bilimbi* contains *ascorbic acid* 36,68-60,95 mg/100 g, *oxalic acid* 8,57-9,82

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mg/100 g, amino acid, citrate acid, cyanidin -3-O-D-glucoside, phenols, flavonoids, saponins, and triterpenoids, the various of *A. bilimbi* is *Averrhoa carambola* contains apigenin -6-C-β-L-fucopyranoside, apigenin -6-C-(2"-O-α-L-rhamnopyranosyl)-β-fucopyranoside, apigenin-6-C-(2"-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside (Lima *et al.*, 2001; Kumar *et al.*, 2013; Moresco *et al.*, 2012). In this study, we evaluated the effectivity of *Canarium* sp., *M. domestica*, and *A. bilimbi* as an antiobesity agent in 3T3-L1 adipocyte cell line.

METHODOLOGY

Freeze Dried Samples

M. domestica, *Canarium* sp., and *A. bilimbi* were collected from production centers, *Canarium* sp. in West Java, *M. domestica* in Malang, East Java, and *A. bilimbi* L. in West Java. The plant organs were identified by herbarium staff, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The plant organs (leaves, stem bark, and branch) (20 kg) were washed and milled respectively, and then freeze-dried to reduce the water compound in the sample. Freeze-dried *M. domestica* (FDM), freeze-dried *Canarium* sp (FDC), freeze-dried *A. bilimbi* (FDA) were stored at 20 °C (Utami *et al.*, 2019).

3T3-L1 Cell Culture and Adipocyte Differentiation Induction

The mouse pre-adipocytes cells 3T3-L1 (ATCC®CL-173) were obtained from Aretha Medika Utama. The 3T3-L1 cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 11995065) supplemented with 10% fetal calf (bovine) serum (FBS) (Gibco, 26140079) and 1% antibiotic/antimycotic (ABAM) (Gibco, 15240-062) then incubated for 24 h at 37 °C, 5% CO₂. After the confluence of the cells, the medium was discharged, and cells were seeded in a 96-well plate (3 × 10⁴ 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 ECM. The medium was replaced by an 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 a maintenance medium (DMEM supplemented with 10% FBS) and incubated for 2-4 days in a 37 °C incubator (Lahrita *et al.*, 2015; Hidayat *et al.*, 2015; Widowati *et al.*, 2018; Widowati *et al.*, 2020).

Measurement of Lipid

Differentiated cells were treated with FDM, FDC, FDA (25, 75 µl), then incubated for 24 h. The medium was discarded, washed with Phosphate Buffer Saline (PBS), then added with Oil-red O 500 µl, incubated for 15-30 minutes. Oil-red O was removed, and cells were washed with Wash Solution. Cells were observed under the inverted light Olympus microscope (Olympus *Inverted Microscope CKX41-F32FL*). Cells were extracted with Dye Extraction 500 µl, incubated in an orbital shaker for 15-30 min. Dye extraction was transferred into a 96-well plate, and absorbance was read at 490 wavelengths (Multiskan™ GO Microplate Spectrophotometer) (Hidayat *et al.*, 2015; Widowati *et al.*, 2018).

Viability Assay

Viability assay in this study is using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega; Madison, WI, USA) which is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity (Hidayat *et al.*, 2015; Widowati *et al.*, 2016). The 3T3-L1 cells were seeded in 96-well plates (5 × 10³ 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% CO₂. The medium then washed and supplemented with 99 µl new medium and 1 µl of FDM, FDC, FDA in various concentrations (10, 50, and 100 µg/ml) and incubated for 48 h at 37 °C and 5% CO₂. 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 (Hidayat *et al.*, 2015; Widowati *et al.*, 2018). The viability assay was performed to determine the safe concentrations for the next assay.

Cholesterol Assay

The cholesterol assay was determined by enzymatic photometric test or CHOD-PAP method, using DiaSys kit protocol (DiaSys 1 1300 99 10 021). The mix solution was seeded in 24 well plates (5 µL ddH₂O, 5 µl sample, and 450 µl reagent kit. ddH₂O was used as a blank sample. Briefly, mixed solutions were incubated for 10 min at 37 °C. The sample absorbance was measured in Miniskan Reader at 500 nm wavelength (Widowati *et al.*, 2018). The absorbance values were used to determine the cholesterol level in each sample using the formula below.

$$\text{Cholesterol (mmol/L)} = \frac{\text{Absorbance of ample}}{\text{Absorbance of Cal 1}} \times \text{Standard Concentration}$$

Table I. Cytotoxicity Activity of Freeze. *domestica*, *A. bilimbi*, and *Canarium sp.* in 3T3L1 Cell Line

Samples	Cells viability (%)		
	FDM	FDC	FDA
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
100.00 µg/ml	86.24 ± 5.46	88.85 ± 4.33	72.18 ± 3.33
50.00 µg/ml	103.90 ± 2.73	108.16 ± 7.03	104.01 ± 3.16
25.00 µg/ml	106.34 ± 1.87	116.81 ± 2.61	103.56 ± 2.11
12.50 µg/ml	114.26 ± 7.38	122.78 ± 8.58	102.18 ± 4.19

Triglyceride Assay

The total triglyceride level in cells was measured according to colorimetric enzymatic tests using glycerol-3-phosphate-oxidase (GPO) using a kit (DiaSys, 1 5760 99 10 021). A 500 µL mixed reaction containing 450 µL reagent with 5 µL sample (cell lysate after treatment in the concentration of 10 and 50 µg/ml) was incubated in 37 °C for 5 minutes. ddH₂O was used for blank well and standard reagent was used for standard well. Standard reaction was prepared in seven different concentrations us serial dilution (2.180; 1.090; 0.545; 0.273; 0.068 and 0.034 mmol/L). The absorbance was measured at 500 nm of wavelength (Widowati *et al.*, 2018). Triglyceride concentration was calculated using the formula:

$$\text{Triglyceride level} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{standard concentration}$$

Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

The 3T3-L1 cells were seeded in 96 well plates (5 x 10³ cells/ well) in 100 µL medium (DME containing 10% calf serum and Abam) for 24 hours at 37 °C the humidified atmosphere and 5% CO₂. The glucose-6-phosphate dehydrogenase (G6PDH) assay is based on the G6PDH kit (Abcam, ab176722). Sample (20 µL) of medium from cell culture and G6PD positive kit for positive control was 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 dark room for 30 min. And then, the samples were measured again using 450 nm of wavelength (Lahrita *et al.*, 2015; Widowati *et al.*, 2018). G6PDH concentration was determined by the formula:

$$\text{G6PDH} = \left[\left(\frac{B}{(T_2 - T_1) \times V} \right) \right] \times \text{sample dilution}$$

B= blank sample; T1= first absorbance; T2= second absorbance; V= Total volume

Statistical analysis

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) statistics version 17.0 software. One-way analysis of variance (ANOVA) was conducted, followed by Duncan post-hoc test, and p<0.05 was considered to be significant. Data are presented as mean ± SD.

RESULT AND DISCUSSION

The cytotoxicity activity of FDM, FDC, FDA. were determined in Table I, which shows that FDC in the lowest concentration (12.50 µg/ml) has the highest cytotoxicity activity (122.78%) compared to FDM and FDA with value 114.26% and 102.18% respectively. The FDM, FDC, FDA. in concentration 50.00 µg/ml has viability >100%, while in concentration of 100.00 µg/ml show viability <100%. So, the appropriate concentration for the future study in FDM, FDC, FDA is using concentrations 75.00 µg/ml and 25.00 µg/ml.

The total cholesterol level of freeze-dried fruit in the 3T3L1 cell line was shown in Table II. Based on Table II. the total cholesterol of FDM in concentration 75 µg/ml has the lowest level compared with FDC and FDA compared to positive control but not lower than the negative control. Based on statistical analysis (*Duncan Post Hoc Test*), FDM was significantly in total cholesterol inhibition activity in concentration 75.00 µg/ml compared to FDC and FDA. This indicated FDM in concentration 75.00 µg/ml more active to reduce total cholesterol compared to FDC and FDA.

Triglyceride level of FDM, FDC, FDA in 3T3L1 cell line show in Table III. FDC in concentration 25 µg/ml has the most significant result in total triglyceride (p<0.05) (Table 3) compared to FDM and FDA, but not higher than the positive control. In triglyceride inhibitory activity, FDM (75 µg/ml) show the highest value compared to FDA and FDC. FDM has highest in decrease of triglyceride level compared to FDC and FDA in concentration 75 µg/ml.

Glucose-6-phosphate dehydrogenase (G6PDH) is the first reaction catalyzing enzyme of the

Table II. Total Cholesterol of Freeze Dried of *M. domestica*, *A. bilimbi*, and *Canarium sp.* in 3T3L1 Cell Line

Sample	Total Cholesterol (mg/dL)	Inhibition Activity (%)
Negative Control	178.02 ± 30.46 ^a	0.00 ± 16.82 ^a
Positive Control	267.23 ± 44.94 ^d	33.38 ± 11.40 ^d
FDM 75.00 µg/ml	191.62±13.81 ^{ab}	28.29±5.17 ^{cd}
FDM 25.00 µg/ml	227.23±21.55 ^{bcd}	14.97±8.06 ^{abc}
FDC 75.00 µg/ml	210.03±22.80 ^{abc}	21.41±8.53 ^{bcd}
FDC 25.00 µg/ml	250.83±3.60 ^{cd}	6.14±1.35 ^{ab}
FDA 75.00 µg/ml	202.02±20.31 ^{ab}	24.40±7.60 ^{cd}
FDA 25 µg/ml	224.43±20.05 ^{abcd}	16.02±7.50 ^{abcd}

Data was presented as mean ± SD from 3 replications. Superscript letter (^{a-d}) in each column indicates significance different among concentration based on post hoc Duncan test with $p < 0.05$ is considered as significantly different. FDM : freeze dried *M. domestica*; FDC : freeze dried *Canarium sp.*; FDA : freeze dried *A. bilimbi*

Table III. Total Triglyceride of Freeze Dried of *M. domestica*, *A. bilimbi*, and *Canarium sp.* in 3T3L1 Cell Line

Sample	Total Triglyceride (mg/dL)	Inhibition Activity (%)
Negative Control	191.66±27.52 ^a	61.73±5.50 ^e
Positive Control	500.84±39.17 ^e	0.00±7.82 ^a
FDM 75.00 µg/ml	197.26±19.23 ^{ab}	60.61±3.84 ^{de}
FDM 25.00 µg/ml	278.40±66.55 ^{cd}	44.41±13.29 ^{bc}
FDC 75.00 µg/ml	225.24±33.43 ^{abc}	55.03±6.67 ^{cde}
FDC 25.00 µg/ml	312.44±24.88 ^d	37.62±4.97 ^b
FDA 75.00 µg/ml	212.65±19.59 ^{abc}	57.54±3.91 ^{cde}
FDA 25 µg/ml	263.94±43.26 ^{bcd}	47.30±8.64 ^{bcd}

Data was presented as mean ± SD from 3 replications. Superscript letter (^{a-d}) in each column indicates significance different among concentration based on post hoc Duncan test with $p < 0.05$ is considered as significantly different. FDM : freeze dried *M. domestica*; FDC : freeze dried *Canarium sp.*; FDA : freeze-dried *A. bilimbi*

phosphate pentose pathway and provides a reduction effect on all cells in the form of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate). The following can be seen for the total G6PDH assay in Table IV. FDM has the lowest total G6PDH compared to FDC and FDA in concentration 75 µg/ml, this result in line with lipid inhibitory activity, FDM has the highest inhibition activity compared to FDC. and FDA Based on statistical analysis not show a significant difference. However, FDM in concentration 75 µg/ml has the highest G6PDH inhibitory activity compared to FDA and FDC.

Based on Table IV, FDM in concentration 75 µg/ml has the lowest absorbance compared to FDA and FDC., but FDM shows not lower than control. The lipid inhibitory activity, FDM (in concentration of 75 µg/ml) has the highest activity compared to FDC and FDA, but not higher compared to control. Based on statistical analysis (*Duncan Post Hoc Test*), FDM (75 µg/ml) shows the most significant result compared to others ($p < 0.05$). However, FDM

in concentration 75µg/ml has the highest lipid inhibitory activity compared to FDC and FDA.

Obesity may be reduced by preventing immature fat cells from developing into mature cells, by inhibiting population growth and apoptotic induction of programmed cell death in 3T3-L1 preadipocyte cells (Soeng *et al.*, 2015). This study aims to examine the cytotoxicity and antiobesity activity of freeze-dried FDM, FDC., and FDA to 3T3 L1 cell line, the variables of this study are TG, CHOL, GPDH. The various fruits such as *M. domestica*, *Canarium sp.* and *A. bilimbi* contains phenol compounds. In the present study, appropriate concentration for the future study in freeze-dried *M. domestica*, *A. bilimbi*, and *Canarium sp.* using concentration 75.00 µg/ml and 25.00 µg/ml based on viability assay in 3T3L1 adipocyte cells. Methyl gallate isolated from herbs is phenol compounds that may be capable of inhibiting form lipid through the staining test of Oil Red O and decreasing TG levels and also be increasing the release of glycerol levels in 3T3L1 adipocyte cells

Table IV. Total G6PDH Freeze Dried of *M. domestica*, *A. bilimbi*, and *Canarium sp.* in 3T3L1 Cell Line

Samples	Total G6PDH (nmol/min/ml)	Inhibition Activity (%)
Control	0.31±0.02 ^a	61.20±2.84 ^d
Positive Control	0.80±0.04 ^d	0.00±4.56 ^a
FDM 75.00 µg/ml	0.40±0.01 ^b	49.56±1.74 ^c
FDM 25.00 µg/ml	0.56±0.03 ^c	29.63±3.40 ^b
FDC 75.00 µg/ml	0.44±0.02 ^b	45.22±2.85 ^c
FDC 25.00 µg/ml	0.57±0.09 ^c	29.08±11.26 ^b
FDA 75.00 µg/ml	0.42±0.01 ^b	47.13±0.97 ^c
FDA 25 µg/ml	0.63±0.04 ^c	21.06±4.95 ^b

Data was presented as mean ± SD from 3 replications. Superscript letter (^{a-d}) in each column indicates significance different among concentration based on post hoc Duncan test with $p < 0.05$ is considered as significantly different. FDM : freeze dried *M. domestica*; FDC : freeze dried *Canarium sp.*; FDA : freeze dried *A. bilimbi*

Table V. Oil Red O Assay Freeze Dried of *M. domestica*, *A. bilimbi*, and *Canarium sp.* in 3T3L1 Cell Line

Sample	Oil Red O (Absorbance)	Inhibition Activity (%)
Control	0.1338±0.0180 ^a	83.66±2.19 ^d
Positive Control	0.8186±0.0672 ^d	0.00±8.21 ^a
FDM 75.00 µg/ml	0.3060±0.0056 ^{ab}	62.63±0.68 ^{cd}
FDM 25.00 µg/ml	0.3808±0.0121 ^b	53.49±1.48 ^c
FDC 75.00 µg/ml	0.4574±0.0969 ^b	44.13±11.84 ^c
FDC 25.00 µg/ml	0.6807±0.0120 ^{cd}	16.85±1.46 ^{ab}
FDA 75.00 µg/ml	0.4093±0.1372 ^b	50.01±16.77 ^c
FDA 25 µg/ml	0.5192±0.1636 ^{bc}	36.57±19.99 ^{bc}

Data was presented as mean ± SD from 3 replications. Superscript letter (^{a-d}) in each column indicates significance different among concentration based on post hoc Duncan test with $p < 0.05$ is considered as significantly different. FDM : freeze dried *M. domestica*; FDC : freeze dried *Canarium sp.*; FDA : freeze dried *A. bilimbi*

so methyl gallate has antiobesity properties (Hsu & Yen, 2006).

FDM has the highest in all cholesterol assay (TG, CHOL, and G6PDH) and Oil Red O in concentration 75.00 µg/ml due to lipid inhibitory activity compared to *Canarium sp.* and *A. bilimbi*. Polyphenol in *M. domestica* has antiobesity properties (Roh *et al.*, 2012; Jelodarian *et al.*, 2012). Therefore, the addition of fruit to the daily diet reduces overall energy consumption and improves energy disequilibrium. Thus the continuous intake of fruit may restrict weight gains, reduce fat mass, and control obesity (Singh, 2014).

Obesity activity in 3T3-L1 cells, among others, can measure antiadipogenesis activity that prevents adipose deposits that will be decomposed into fat and energy. The antiadipogenesis test on 3T3-L1 cells is stimulated by IBMX, dexamethasone, triglycerides so that it undergoes differentiation into adipocytes. IBMX will increase intracellular cAMP, dexamethasone binding to

glucocorticoid receptors, and insulin binding to insulin receptors. Three stimulation pathways will activate the PPAR γ and C/EBP α genes. Adipocytes containing PPAR γ and C / EBP α will activate adipocyte-specific genes that encode secretory factors such as insulin receptors, proteins involved in the synthesis of fatty acids, fatty acid-binding proteins. Substances that can inhibit PPAR γ have antiadipogenesis activity (Sharma *et al.*, 2016). Fatty acid compositions of lipid droplets that can be stained using Oil Red O.

CONCLUSION

Freeze-dried of *M. domestica*, *Canarium sp.* and *A. bilimbi* have antiobesity potential. *M. domestica* shows the highest antiobesity activity compared to *A. bilimbi* and *Canarium sp.* via decreasing of cholesterol, triglyceride, G6PDH.

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