

RESEARCH ARTICLE

In vitro* Antioxidant and Anti-obesity Activities of Freeze-dried *Canarium* sp., *Averrhoa bilimbi* L. and *Malus domestica

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Abstract

BACKGROUND: The number of obesity cases is still increasing worldwide and has reached an epidemic scale. Plants are known to have a protection role in the development of obesity, however their antioxidant and anti-obesity activities have not widely known. This study was conducted to assess the *in vitro* antioxidant and anti-obesity activities of different types of freeze-dried fruits cultivated in Indonesia, especially *Canarium* sp., *Averrhoa bilimbi* L. and *Malus domestica*.

METHODS: Total phenolic content of freeze-dried fruits was identified by Folin-Ciocalteu method, while the total flavonoid content was measured by aluminium chloride colorimetric assay. To assessed the antioxidant activity, 2,2-diphenyl 1-picrylhydrazyl (DPPH) scavenging activity assay and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reducing activity assay were performed. The α -amylase and lipase inhibitory activity assay were performed to assess the anti-obesity activity. For comparison, hydroxycitric acid (HCA) compound was

also assessed with DPPH, ABTS, α -amylase and lipase assays.

RESULTS: *A. bilimbi* had the highest total phenol content (6.35 μg GAE/mg), meanwhile *M. domestica* had the highest total flavonoid content (2.06 μg QE/mg). *A. bilimbi* also showed the highest antioxidant activity both in DPPH and ABTS assay, with inhibitory concentration (IC_{50})=279.99 $\mu\text{g}/\text{mL}$ and 631.78 $\mu\text{g}/\text{mL}$, respectively. The freeze-dried *M. domestica* had the highest anti- α -amylase activity (IC_{50} =258.85 $\mu\text{g}/\text{mL}$), while *Canarium* sp. had the highest anti-lipase activity (IC_{50} =118.66 $\mu\text{g}/\text{mL}$).

CONCLUSION: Freeze-dried fruits demonstrate *in vitro* benefits toward obesity. *A. bilimbi* has potent antioxidant activity and is beneficial against obesity-related adverse health effect by relieving oxidative stress. *M. domestica* and *Canarium* sp. hamper the fat accumulation by reducing the carbohydrate absorption and dietary lipid.

KEYWORDS: antioxidant, anti-obesity, *Canarium* sp., *Averrhoa bilimbi* L., *Malus domestica*, hydroxycitric acid

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Introduction

Obesity is defined as excessive body fat accumulation, which increase the risk of adverse health effect. As described by World Health Organization (WHO), people with body

mass index (BMI) over 30 kg/m^2 are diagnosed as obese.(1) In Indonesia, the BMI of 22.9 kg/m^2 is considered to be the normal weight.(2)

In recent years, the number of obesity cases is still increasing worldwide up to 650 million in adults (3), and has reached the epidemic scale, especially in high-income

countries (4). Obesity is the main preventable cause of death. An obese person has a higher risk of developing various adverse health effects, including cardiovascular diseases and diabetes.(5) There are multiple risk factors ranging from an unhealthy lifestyle to genetics that contribute to the pathogenesis of obesity.(6) Oxidative stress, a condition caused by excessive production of reactive oxygen species (ROS), plays an important role in the development of obesity and its adverse health effect.(7,8)

Plants are the source of multitude phytochemical compounds that are useful in various biological activities. Previous study found that several plant extracts were able to prevent fat absorption through the inhibition of pancreatic lipase.(9) Other study demonstrated that plant extract could protect an animal model from hyperglycemia.(10) Nowadays, the use of fruits continues to be developed, considering its less side effects and toxicity.

Despite its many benefits, fruits are easily spoiled. Thus, diverse methods are found to preserve fruits, one of which is the freeze-drying technique.(11) Freeze-drying technique is known to have many advantages to maintain fruits and its phytochemicals quality. By using the technique, the colour stability and anthocyanin content in fruit are able to be preserved.(12)

Unfortunately, the antioxidant and anti-obesity activities of several common fruits found in Indonesia, such as *Canarium* sp. (galip nut), *Averrhoa bilimbi* L. (star fruit) and *Malus domestica* (apple), especially when preserved with freeze-drying technique, still has not widely known. To address this, the freeze-dried *Canarium* sp., *A. bilimbi* and *M. domestica* fruit samples were assessed by antioxidant and anti-obesity assays. This study aimed to describe the possible benefit of freeze-dried fruits cultivated in Indonesia against obesity.

Methods

Freeze-dried Samples Material

The *Canarium* sp., *A. bilimbi* and *M. domestica* samples were obtained from the plantations located in Jawa Barat and Jawa Timur, Indonesia. The plants were identified at the School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, Indonesia. As much as 1.35 kg *Canarium* sp., 1.6 kg *A. bilimbi* and 2 kg of *M. domestica* were washed and milled, before being freeze-dried using the freeze drier (Nanbei, Henan, China), which resulted in 28.22 gram freeze-dried *Canarium* sp., 9.98 gram freeze-dried *A. bilimbi* and 57.6 gram freeze-dried *M. domestica*.

For comparison, hydroxycitric acid (HCA) compound (Cat #59847-5G, Sigma Aldrich, Missouri, USA), a derivative of citric acid which is found in some tropical plants, was also prepared and further analysed.

The Identification of Total Phenolic Content

The total phenolic content in freeze-dried fruit samples was evaluated using the modified colorimetric method. The method was marked by the reduction of Folin-Ciocalteu reagent (Cat #1.09001.0500, Merck, Darmstadt, Germany) by phenolic compounds with a concomitant formation of a blue complex. Each freeze-dried fruit samples and 100 μ L of gallic acid standard was mixed with 75 μ L of 10% Folin-Ciocalteu reagent and 60 μ L of 7.5% Na_2CO_3 in the microplate, and then was incubated at the temperature of 45-50°C for 10 minutes. The absorbance was measured in 750 nm of wavelengths using the microplate reader.(13,14)

The Identification of Total Flavonoid Content

The total flavonoid content of freeze-dried fruits was measured by aluminium chloride colorimetric assay. Briefly, each freeze-dried fruit samples and 250 μ L of standard was mixed with 75 μ L of 5% NaNO_2 . After 6 minutes, 150 μ L of 10% $\text{AlCl}_3 \cdot \text{H}_2\text{O}$ solution was added. After another 5 minutes, 0.5 mL of 1M NaOH solution was added to the mixture. The final mixture was measured using the microplate reader absorbance at 510 nm of wavelength.(15)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity Assay

To evaluate the antioxidant activity, the DPPH assay was conducted. Fifty μ L of freeze-dried fruit samples and HCA at various concentrations were added to the 96-well microplate. Two-hundred μ L of DPPH solution (Cat #D913-2, Sigma Aldrich) was then added into each well, and was incubated at 20-25°C in the darkroom for 30 minutes. The absorbance was measured at 517 nm of wavelengths using Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Massachusetts, USA). A 250 μ L of DPPH solution was used as the negative control, while 250 μ L of methanol was used as the blank control. The scavenging activity was measured based on the sample DPPH absorption rate in comparison to negative control.(14,16)

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS)-reducing Activity Assay

Two μ L of each freeze-dried fruit samples and HCA were added to the sample well, then 198 μ L of ABTS solution (Cat #A1888-2G, Sigma Aldrich) was added to each well,

and was incubated at 30°C for 6 minutes. The absorbance was measured every 1 minute at 420 nm of wavelength using the Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific) for 8 minutes.(14,16-18) This method was done to evaluate the antioxidant activity.

The α -amylase Inhibitory Activity Assay

To assess the anti-obesity activity of each freeze-dried fruits and HCA, α -amylase inhibitory activity assay were performed. Thirty μ L of each freeze-dried fruit samples and HCA were added into the sample and negative control well, while 30 μ L of dimethyl sulfoxide (DMSO) was served as the blank control. Ten μ L of α -amylase enzyme (Cat #7595-50ML, Sigma Aldrich) was added into each well, except for the blank control well. After incubated at 37°C for 10 minutes, 40 μ L starch solution was added to each well except for the negative control well. Forty μ L of phosphate buffer was then added to the negative control well, and was incubated at 37°C for 15 minutes. The enzymatic reaction was terminated by the addition of 100 μ L of acidic iodine solution in each well. The absorbance was measured at 565 nm of wavelength using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific).(16,19,20)

The Lipase Inhibitory Activity Assay

Lipase inhibitory activity assay to evaluate the anti-obesity activity was performed based on following procedures. Twenty mL of each freeze-dried fruit samples and HCA, 20 mL of porcine pancreas lipase (PPL) enzyme (Cat #L3126-100G, Sigma Aldrich), and 100 mL of potassium phosphate buffer were added to the 96-well microplate, and then was incubated at 37°C for 1 hour. Furthermore, 10 mL of nitrophenyl butyrate (NPB) (Cat #N9876-1G, Sigma Aldrich) substrate was added, and was incubated at 37°C for 5 minutes. The absorbance was measured at 405 nm of wavelength.(19,21)

Results

Total Phenolic Content

Total phenolic content of each freeze-dried fruits was evaluated. The result was expressed in μ g gallic acid equivalent (GAE)/mg. Freeze-dried *A. bilimbi* had the highest level of phenolic content compared to the other two freeze-dried fruits, with the concentration of 6.35 ± 0.95 μ g GAE/mg. Meanwhile, the lowest concentration of phenol was found in the freeze-dried *M. domestica* 2.46 ± 0.93 μ g GAE/mg (Table 1).

Total Flavonoid Content

The flavonoid content of freeze-dried fruits was also evaluated. The result was expressed in μ g quercetin equivalent (QE)/mg. Freeze-dried *M. domestica* had the highest level of flavonoid content compared to the other freeze-dried fruits, with concentration of 2.06 ± 0.46 μ g QE/mg, while the lowest was the freeze-dried *Canarium* sp., with the concentration of 0.04 ± 0.32 μ g QE/mg (Table 1).

Table 1. Total phenolic and total flavonoid content of the freeze-dried fruits.

Freeze-dried Fruit Sample	Total Phenolic Content (μ g GAE/mg)	Total Flavonoid Content (μ g QE/mg)
<i>Canarium</i> sp.	6.07 ± 0.50	0.04 ± 0.32
<i>Averrhoa bilimbi</i> L.	6.35 ± 0.95	0.29 ± 0.48
<i>Malus domestica</i>	2.46 ± 0.93	2.06 ± 0.46

*The data was presented as mean \pm standard deviation.

DPPH Scavenging Activity

The antioxidant activity of each freeze-dried fruits and HCA based on DPPH scavenging assay were evaluated. Based on the result, freeze-dried *A. bilimbi* had the highest DPPH scavenging activity (49.73%) when compared to the freeze-dried *Canarium* sp., freeze-dried *M. domestica* and HCA (Figure 1A). Based on the inhibitory concentration (IC₅₀) values, freeze-dried *A. bilimbi* also showed the highest scavenging activity (279.99 μ g/mL), compared to the other three compounds (Table 2).

ABTS-reducing Activity

The antioxidant activities of each freeze-dried fruits and HCA were also evaluated based on the ABTS assay. The freeze-dried *A. bilimbi* had the highest percentage in ABTS-reducing activity (12.46%) compared to freeze-dried *Canarium* sp., freeze-dried *M. domestica* and HCA (Figure 1B). The IC₅₀ value was calculated, HCA had the highest ABTS-reducing activity (166.23 μ g/mL), and followed by freeze-dried *A. bilimbi* (634.37 μ g/mL) which had the highest value among the three freeze-dried fruits, whereas the freeze-dried *M. domestica* had the lowest ABTS-reducing activity (3183.41 μ g/mL) (Table 2).

α -amylase Inhibitory Activity

To assess the anti-obesity of each freeze-dried fruits and HCA, the α -amylase inhibitory activity was evaluated. Figure 2A showed that freeze-dried *M. domestica* had the

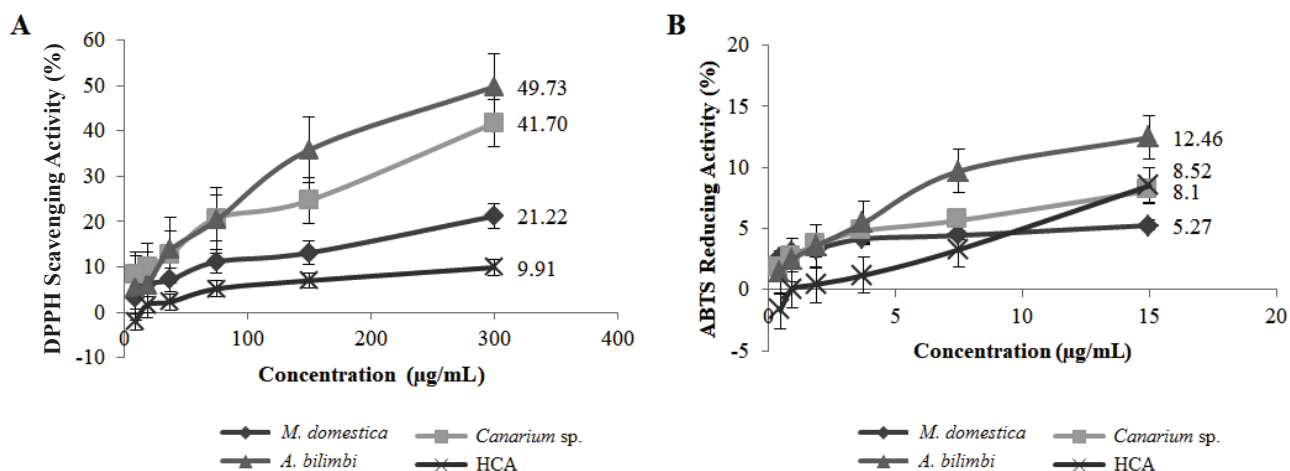


Figure 1. Concentration of freeze-dried *Canarium sp.*, *Averrhoa bilimbi* L., *Malus domestica*, and HCA based on DPPH scavenging activity assay (A) and ABTS-reducing activity assay (B).

highest α -amylase inhibitory activity (18.18%), compared to freeze-dried *Canarium sp.*, freeze-dried *A. bilimbi* and HCA. While based on the IC_{50} values, HCA had the highest α -amylase inhibitory activity (196.16 $\mu\text{g/mL}$), followed by freeze-dried *M. domestica* (258.85 $\mu\text{g/mL}$). While freeze-dried *Canarium sp.* had the lowest α -amylase inhibitory activity (16496.93 $\mu\text{g/mL}$) (Table 3).

Lipase Inhibitory Activity

The lipase inhibitory activity of freeze-dried fruits and HCA were evaluated to assess the anti-obesity activity. Freeze-dried *Canarium sp.* had the highest lipase inhibitory activity (77.89%) at the highest concentration tested, while *A. bilimbi* had the lowest among the three freeze-dried fruits (Figure 2B). Based on the calculated IC_{50} values, HCA had the best value for inhibiting the enzyme lipase (31.22 $\mu\text{g/mL}$), followed by freeze-dried *Canarium sp.* (118.66 $\mu\text{g/mL}$).

Discussion

Phytochemical has various biological activities underlying the utilization of plant extract as traditional medicine.(22) Some phytochemical contents, especially the phenolic and polyphenolic compound, are well known for its antioxidant capacity, since its structures are able to turn into its less-reactive form.(23,24) The consumption of high phenolic content beverages, such as wine and tea, is also found to increase the serum antioxidant capacity as measured with antioxidant assay.(25,26) The phenolic and polyphenolic content in fruits are able to be metabolized and secreted

by the body over a 24 h period after ingestion, thus further increase the safety of its consumption.(27,28)

Freeze-drying is one solution to keep nutritional value, antioxidant activity and phytochemical content of fruits from changing.(12,29) In present study, the phenolic and polyphenolic content of freeze-dried fruits were measured, and it was found that freeze-dried *A. bilimbi* had the highest phenolic content compare to the other freeze-dried fruits. However, the result of this study showed that the freeze-dried *A. bilimbi* and freeze-dried *Canarium sp.* had lower phenolic content compared to previous studies.(30,31) Meanwhile, the freeze-dried *M. domestica*, which contained the least phenolic content, showed similar amount of phenolic content ($\pm 3 \mu\text{g/mg}$) compared to another study.(32)

Total flavonoid assay suggested that freeze-dried *M. domestica* had the highest flavonoid content, while freeze-dried *Canarium sp.* had the lowest. Flavonoid content of freeze-dried *M. domestica* found in this study was slightly

Table 2. The IC_{50} value of freeze-dried *Canarium sp.*, *Averrhoa bilimbi* L., *Malus domestica*, and HCA based on DPPH scavenging activity assay and ABTS-reducing activity assay.

Freeze-dried Fruit Sample	IC_{50} ($\mu\text{g/mL}$)	
	DPPH	ABTS
<i>Canarium sp.</i>	370.84	1210.93
<i>Averrhoa bilimbi</i> L.	279.99	631.78
<i>Malus domestica</i>	805.11	3183.41
Hydroxycitric acid	349	166.23

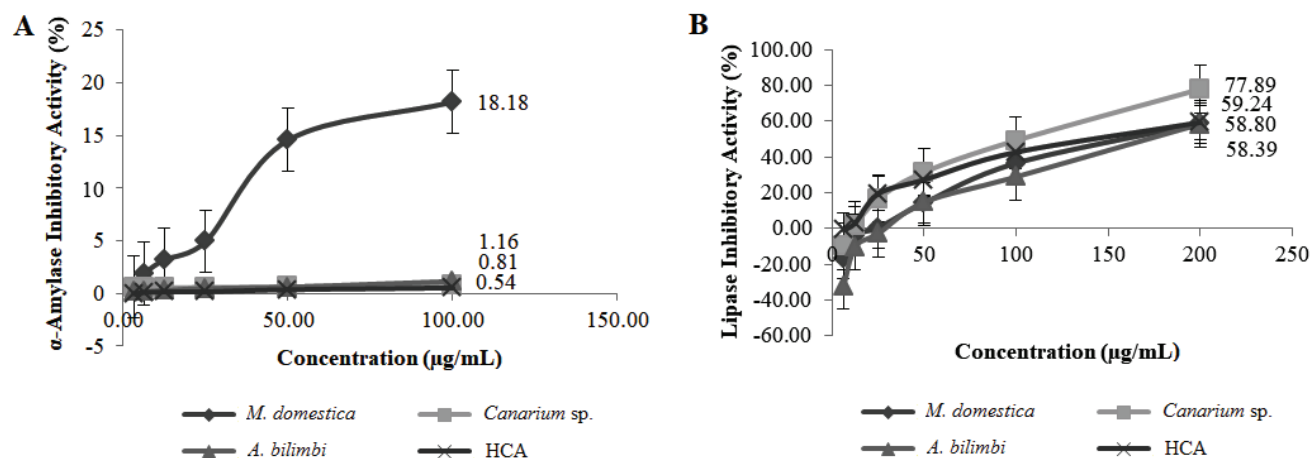


Figure 2. Concentration of freeze-dried *Canarium sp.*, *Averrhoa bilimbi L.*, *Malus domestica*, and HCA based on α -amylase inhibitory activity assay (A) and lipase inhibitory activity assay (B).

lower compare to previous research.(33) The difference on both phenolic and flavonoid content exhibited in present study may be caused by numerous factors, including different cultivars, solvents polarity, and the extraction method used. (31) In addition, the fruit phytochemical content is also affected by different environmental conditions, ranging from the nutritional intake to the climate changes.(34)

Obesity pathogenesis was a complex process triggered by excessive fat consumption and hypoxic condition of the adipose tissue. When combined, both conditions trigger the adipocyte to produce ROS and further cause a condition known as oxidative stress. Oxidative stress stimulates the fat accumulation in adipose tissue and the differentiation of the adipocyte.(35,36) Antioxidant attenuated obesity-related health effect on mice.(37) Based on both DPPH and ABTS assay, the results of this study showed that freeze-dried *A. bilimbi* had the highest antioxidant activity, which is likely due to the high phenolic content found in freeze-dried *A. bilimbi*. Thus, freeze-dried *A. bilimbi* has a good potential

to counteract the free radicals. Antioxidant plant extract was also able to protect obese animal model from obesity related adverse health effect.(38) As evidence suggest, *A. bilimbi* may either prevent obesity and/or its adverse health effects through antioxidant activity by relieving the oxidative stress.

In addition to oxidative stress, inhibition of carbohydrate absorption and dietary lipid are also known to be beneficial in treating obesity. After consumption of food rich in carbohydrate, glucose concentration in plasma increased rapidly. The increased blood-sugar induces the body to store monosaccharide either as glycogen in the liver or fat in adipose tissue. Recent clinical trials conclude that the inhibition of α -amylase promoted weight loss.(39) Previous research reported that food rich in polyphenol had potential inhibitory properties against α -amylase and α -glucosidase.(40) As carbohydrate digestion and obesity were linked, inhibition of enzyme may provide benefits for treating this condition. The present study found that freeze-dried *M. domestica* has potential α -amylase inhibition. This activity may be related to the high polyphenolic content found in freeze-dried *M. domestica*, in this study showed by the high flavonoid content in freeze-dried *M. domestica*. This result also supported by previous research stating that *M. domestica* consumption may also limit the absorption of carbohydrate, preventing obesity.

Up until now, the inhibition of pancreatic lipase is the most widely studied mechanism for the evaluation of anti-obesity agents since inhibition of this enzyme hampers the absorption of fatty acid by the gut.(41,42) Polyphenols extracted from black tea were able to inhibit the fat absorption in the gut, which occurs due to the inhibition

Table 3. The IC_{50} value of freeze-dried *Canarium sp.*, *Averrhoa bilimbi L.*, *Malus domestica*, and HCA based on α -amylase inhibitory activity assay and lipase inhibitory activity assay.

Freeze-dried Fruit Sample	IC_{50} (µg/mL)	
	α -Amylase	Lipase
<i>Canarium sp.</i>	16496.93	118.66
<i>Averrhoa bilimbi L.</i>	4984.57	165.33
<i>Malus domestica</i>	258.85	161.01
Hydroxycitric acid	196.16	31.22

of lipase enzyme.(43) The result of present study showed that the freeze-dried *Canarium* sp. was able to inhibit lipase activity, as having the highest lipase inhibitory activity. Freeze-dried *Canarium* sp. may help to prevent obesity by reducing gut fat absorption.

This study showed that freeze-dried fruit demonstrates *in vitro* benefit toward obesity while extending its quality and storage capability. However, further *in vivo* study is required to conclude the amount of freeze-dried fruit required, since the sole purpose of this study is to investigate the possible antioxidant and anti-obesity potential of freeze-dried fruit, not to determine the dosage.

Conclusion

In summary, freeze-dried fruits demonstrate *in vitro* benefits toward obesity. Freeze-dried *A. bilimbi* has potent antioxidant activity and may be beneficial against obesity-related adverse health effect by relieving oxidative stress. While freeze-dried *M. domestica* and freeze-dried *Canarium* sp. hamper the fat accumulation by reducing the carbohydrate absorption and dietary lipid in the digestive tract.

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