Berkala **PENELITIAN HAYATI**

ISSN: 08526834 | E-ISSN:2337-389X

Volume 27| No. 2| June | 2022

Original article

Increasing antioxidant activity of soursop (*Annona muricata* L.) and noni (*Morinda citrifolia* L.) leaves fermented by *Lactobacillus plantarum* BP102

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Abstract

Free radicals are constantly produced by either cell metabolism or from external sources. At high concentration, they induced a tissue damage called oxidative stress. Soursop leaf (*Annona muricata* L.) and noni leaf (*Morinda citrifolia* L.) are medicinal plants with potency as antioxidants. This study aimed to evaluate the capacity of *Lactobacillus plantarum* BP102 in elevating the antioxidant activity of soursop and noni leaves. Dried-powder and methanol extract of soursop and noni leaves were diluted with sterile distilled water 3 g/30 mL and 0.3 g/30 mL, respectively, inoculated with 1% (v/v) of *L. plantarum* BP102 inoculum. The antioxidant activity was carried out using the 2.2-diphenyl-1-picrylhydrazyl (DPPH) method. The antioxidant activity increased in dried-powder and methanol extract of soursop and noni leaves with different activity levels after being fermented using *L. plantarum* BP102 based on IC_{50}. The increasing antioxidant activity in dried-powder of soursop leaves IC_{50} 6.41±0.06 to 0.034±0.01 mg/mL (99.5%) was higher than of the methanol extract IC_{50} 2.78±0.00 to 0.11±0.01 mg/mL (96%). Unfortunately, the effect of fermentation towards noni leaves could only be observed in the form of methanol extract IC_{50} 12.8±0.01 to 0.33±0.02 mg/mL (increased by 97.4%), the dried-powder of noni leaves was suspended and produced a dark color. The probiotic *L. plantarum* BP102 was used as a fermented agent in increasing the bioactive compounds especially related to antioxidant activity.

Keywords: antioxidant, fermented plant extract, *Lactobacillus plantarum*, noni leaf, soursop leaf

Received: October 18, 2021 Revised: December 24, 2021 Accepted: January 4, 2022

Introduction

Free radicals are molecules or atoms that have unstable and having one unpaired electron in its outermost orbit (Sukweenadhi et al., 2020). The increase of free radicals should be balanced by antioxidants to prevent oxidative stress. Oxidative stress is a significant factor causing inflammation, such as asthma, arthritis, stroke, heart disease, hypertension, Parkinson's, preeclampsia, atherosclerosis, Alzheimer's, and many other disorders (Chiavaroli et al., 2011, Mahdi-Pour et al., 2012). Antioxidants are defined as substances that delay or inhibit oxidative damage.

It is already known that probiotics have health benefits to the gut microbiota, modulating the immune system (preventing allergies, enhancing the immune system), and producing antimicrobial compounds (Manhar et al., 2016, Mulaw et al., 2019). In recent years, probiotic products have become popular all over the world (Guan et al., 2020). Lactic acid bacteria as probiotic also played an important role in the fermentation of plant extracts to increase the bioactive compounds exerting antioxidant activity. *Lactobacillus plantarum* is a member of lactic acid bacteria that has potential as a probiotic. Several studies reported that Ginseng marc fermented with *L. plantarum* could increase antioxidant activity and the content of active compounds of ginseng marc in the form of phenolics and flavonoids by 32.4 and 23.3%, respectively (Eom et al., 2017). The effect of papaya juice fermented with *L. acidophilus* and *L. plantarum* experienced similar changes in pH and reduction in sugar content during the 48 h of fermentation period. However, *L. plantarum* resulted in a better antioxidant activity than of L. acidophilus after 48 h of fermentation (Chen et al., 2018). Olives fermented with *L. plantarum* also can increase antioxidant activity by 24% (Kachouri et al., 2015).

Natural antioxidants are generally found in medicinal plants, such as soursop leaf (*Annona muricata* L.) and noni leaf (*Morinda citrifolia* L.). Noni leaf and soursop leaf have been a popular herbal medicine and have antioxidant activity (Serafini et al., 2011, Sari, 2015). Several studies reported that the ethanolic extract of soursop leaves contains steroids, alkaloids, flavonoids, and saponins (Hasmila et al., 2019). Noni leaves extract contains active compounds, namely lignans, organic acids, alkaloids, flavonoids, triterpenoids, sterols, iridoids, chlorophyll derivatives, coumarin derivatives, and are potential sources of antioxidants and phenols (Kovendana et al,. 2014, Krishnaiah et al., 2015).

The preliminary test results of LC-MS analysis of dried-powder of soursop leaves identified 166 active compounds, while the methanol extract of soursop leaves identified 191 active compounds. On the other hand, the results of LC-MS analysis of dried-powder of noni leaves identified 108 active compounds. Meanwhile, methanol extract of noni leaves identified 120 active compounds. Therefore, it is essential to compare the antioxidant activity of dried-powder and methanol extract of soursop and noni leaves fermented by *L. plantarum*

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http://dx.doi.org/10.23869/bphjbr.27.2.20223
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BP102, thus a promising alternative to boosting antioxidants. *L. plantarum* BP102 isolates from garlic bulb was chosen because resistance to low pH, bile salt, has the best probiotic characteristics, and has the potential as a starter culture for fermenting medicinal plants (Wardhani, 2019). This study aimed to determine the difference in antioxidant activity of dried-powder and methanol extract of soursop and noni leaves using the DPPH method (2.2-diphenyl-1-picrylhydrazyl) based on the IC_{50} value before and after fermentation by *L. plantarum* BP102.

Methods

Materials and bacterial culture

Soursop and noni leaves were obtained from the UPT Laboratorium Herbal Materia Medica, Batu, East Java. The mature soursop and noni leaves were selected from the middle between the base and the tip of the twig. *L. plantarum* BP102 is endophytic LAB isolated from garlic bulb were obtained from the collection of the Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang. The probiotic properties were cultured in MRS media (Merck, Darmstadt, Germany). One loop-full of *L. plantarum* BP102 and was transferred to 9 mL of sterile MRS Broth and incubated at 37ºC for 24 h to achieve a cell density of 10^8 cell/mL calculated using Hemocytometer as starter culture for fermentation of plant extract (Wen et al., 2013). The solvents for extraction and radical scavenging activity assays used methanol. The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Fermentation of plant and extract

The soursop and noni leaves were air dried without sunlight at room temperature (27-32ºC) for one week, then baked in the oven at 60ºC until dry and ground using a blender to form a dried-powder size 90 mesh. The processing methods of soursop and noni leaves were conducted using two treatments. The first treatment was a dried-powder form (without extraction). The second treatment was extract methanol of soursop and noni leaves was prepared by maceration using methanol (1:10, w/v), at room temperature for 72 h and stirred every 6 h. The extract was then filtered using Whatman Filter paper No. 1 and evaporated in a rotary evaporator at 40ºC, pressure of 175 bar, for approximately 3 h. The extract was weighed to calculate the yield and wrapped in black plastic was stored at 4ºC until used (Ahmed et al., 2015, Almoulah et al., 2017).

The dried-powder (3 g) of soursop and noni leaves were dissolved in 30 mL of sterile distilled water. The methanol extract (0.3 g) of soursop and noni leaves added 0.5% of tween 20 to increase its solubility and dissolved in 30 mL of sterile distilled water. The dried-powder and methanol extract of soursop and noni leaves are fermented separately. Starter culture of *L. plantarum* BP102 1% (v/v) was inoculated into the suspension of dried-powder and methanol extract, and then incubated at 37ºC for 24 h under stirring conditions. Finally, the fermented products were centrifuged at 4.000 rpm for 20 min at 4ºC to obtain a cell-free supernatant (CFS) (Eom et al., 2017).

DPPH antioxidant activity analysis

The dried-powder (0.25 g) and methanol extract (0.1 g) samples either before or after fermentation were dissolved in 10 mL of methanol. The samples were made in various series concentrations namely (25, 50, 75, 100, and 125 mg/mL). Each concentration was added with 140 µL of DPPH ($0.6x10^{-6}$ mol/L). After being incubated for 30 min at room temperature in the dark condition, samples absorbance were measured at a wavelength of 516 nm using UV-vis spectrophotometer (Shimadzu, Japan) (Almoulah et al., 2017, Hidayat et al., 2017). Methanol was use as blank, while the control was DPPH reagent and methanol (without samples). Ascorbic acid (200 µL) was also used as the comparison control. The experiment was conducted in triplicates. The percentage of inhibitory activity of antioxidant was calculated by the following equation (Elfahri et al., 2016, Hrichi et al., 2020):

$$\%\ Inhibitory\ activity\ of\ antioxidant = \frac{Abs\ control - Abs\ sample}{Abs\ control}\ x\ 100\%$$

The IC_{50} value was determined using the regression equation, sample concentration on X-axis and percentage of inhibitory activity of antioxidants on Y-axis. The smaller the IC_{50} (Inhibitory Concentration) value, the greater the antioxidant activity. A compound was categorized as a very strong antioxidant if the IC_{50} value was less than 0.05 mg/mL, a strong antioxidant has IC50 value of 0.05-0.1 mg/mL, a medium antioxidant has IC_{50} of 0.1-1.5 mg/mL, and a weak antioxidant has IC_{50} values of 1.51-0.2 mg/mL (Molyneux, 2004).

Results

The yield of soursop leaf extract was 20.58%, while the noni leaf extract was 9.93%. The antioxidant test results showed an effect in antioxidant activity after fermentation with *L. plantarum* BP102 were shown in Table 1. The antioxidant activity of dried-powder and methanol extract of soursop leaves increased after fermentation with IC_{50} value of (0.034 mg/mL and 0.11 mg/mL), respectively, if it was compared to before fermentation (6.41 mg/mL and 2.78 mg/mL, respectively). The dried-powder noni leaves exhibited an IC_{50} value of 58.05 mg/mL. Unfortunately, fermented dried-powder of noni leaves failed to get IC_{50} value because the sample was suspended and produced a dark color. The IC_{50} value of methanol extract of noni leaves after being fermented was increased by 97.4%

The highest antioxidant activity in the samples before being fermented based on the IC_{50} value was soursop leaf extract and noni leaf extract, namely 2.78 mg/mL and 12.8 mg/mL, respectively. The extraction process has succeeded in acquiring antioxidant compounds thoroughly. The antioxidant category in the non-fermented samples was classified as very weak (>0.2 mg/mL), possibly due to the low antioxidant compounds in the samples. Meanwhile, in the fermented samples, the antioxidant category of soursop leaf powder was classified as very

strong (<0.05 mg/mL), while soursop leaf extract was categorized as medium (0.1-1.5 mg/mL).

Table 1. The IC_{50} value of soursop and noni leaves before and after fermentation.

Samples		Before Fermentation		After Fermentation		Increasing Antioxidant Activity (%)
		IC_{50} (mg/mL)	Antioxidant Level	IC_{50} (mg/mL)	Antioxidant Level	
Soursop leaves	**dried-powder**	6.41±0.06	very weak	0.034±0.01	very strong	99.5
	methanol extract	2.78±0.00	very weak	0.11±0.01	medium	96
Noni leaves	**dried-powder**	58.05±0.67	very weak	n.a*	n.a*	n.a*
	methanol extract	12.8±0.01	very weak	0.33±0.02	medium	97.4
Control	**Ascorbic acid**		0.002±0.00		very strong	-

n.a*: sample is not sufficient for analysis

Discussion

Antioxidants are essential substances because they can protect from the oxidative stress due to free radicals (Mahdi-Pour et al., 2012). The determination of the value of antioxidant activity in this study using the DPPH method (2.2-diphenyl-1-picrylhydrazyl) was fast, easy, and required a small number of samples to evaluate the antioxidant activity. The DPPH assay has been widely used to examine the ability of compounds that act as antioxidants electron donors (Zhang et al., 2017).

The principle of this method is the measurement of DPPH radical capture by compounds that have antioxidant activity using UV-Vis spectrophotometry so that the value of free radical scavenging activity is known, which is expressed by the IC_{50} value. The IC_{50} value is defined as the concentration of the sample that reduces free radicals by 50%. Thus, the smaller the IC_{50} value, the higher the free radical scavenging activity (Batubara et al., 2020).

The use of methanol as the solvent for extraction because flavonoids compounds were soluble in polar solvents. It was carried out to obtain a high antioxidant content and prevented damage to active compounds due to heating (Ukwubile, 2013, Gavamukulya et al., 2014, Sivasamugham et al., 2021). Another study stated that *Syzygium polyanthum* leaves extracted by the maceration method had a total phenolic content of 338.62±21.3 mgGAE/g and antioxidants with IC_{50} of 17.53±0.11 g/mL higher than using the soxhlet and infusion methods (Luliana et al., 2019). According to Almoulah et al. (2017) the extraction process is a mass transfer process from the solid components in the sample into the organic solvent used. Organic solvents will penetrate the cell wall and then enter the plant cells' cavity containing bioactive substances. Then the substances will be dissolved in an organic solvent outside the cell to further diffuse into the solvent, thereby attracting all the active substances and chemical components in the sample.

Fermentation is the process of decomposition of organic compounds to produce energy and the conversion of substrates into new products by microbes. Thus changing the appearance, taste, function, nutritional composition, color, and texture. The fermentation process produces a beneficial effect, namely the production of metabolites and other complex compounds beneficial to health (Kuria et al., 2021). When the acidity of the samples increased, this indicated that *L. plantarum* grew and utilized sugar, carbohydrates, and fiber from soursop and noni leaves to produce organic acids, especially lactic acid, and other by-products. Of most acidity that can be titrated, lactic acid is the primary organic acid produced by the genus Lactobacillus (Di Cagno et al., 2013, Nguyen et al., 2019). The effect of fermentation on increasing antioxidant activity was caused by an increase in the number of phenolic compounds and flavonoids during fermentation, resulting from microbial hydrolysis reactions. In addition, fermentation induces structural damage to plant cell walls, which leads to the synthesis of various antioxidant compounds. The antioxidant compounds can act as free radical terminators, metal chelators, singlet oxygen quenchers, or hydrogen donors for radicals. In addition, the production of proteases, amylase, and some other enzymes can be affected by fermentation which may have metal ion chelation activity (Hur et al., 2014, Maryati et al., 2020).

Antioxidant activity was influenced by the method, solvent and the extraction time factor. The extraction time that was too short will cause the bioactive components extracted from the material to be not optimal. On the other hand, if the extraction time is extended, the solvent will be saturated so that the results of the antioxidant activity were low (Yuliantari, 2017). This research is relevant to Xiao et al. (2015) study, which stated that fermentation on soy whey using *L. plantarum* B1-6 increases the antioxidant activity of 92,85 ± 0,24%. Previous research stated phytochemical composition and antioxidant activity *Moringa oleifera* leaves powder fermented by *Lactobacillus plantarum* (DSM 2601) and *Weissella cibaria* (27A). The fermentation by *Lactobacillus plantarum* (DMS 2601) showed there was an effect of the fermentation time on the content of phenolic compounds (Nirina et al., 2017). However, no study reported the antioxidant analysis on leaves extract fermented by *L. plantarum*. This study concludes that

fermentation using *L. plantarum* BP102 can increase antioxidant activity as indicated by very strong antioxidant levels in dried-powder of soursop leaves. Meanwhile, the fermented soursop and noni leaves extracts were categorized as medium antioxidant activity level. The changes of the bioactive compounds related to antioxidant activity after being fermented is warrant to be investigated further.

Acknowledgement

The author would like to thank Ms. Nanik Dwirahayu for her laboratory technical assistance in the Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang.

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