Research Article

Isolation of Arbutin from Leaves and Fruits of Buni (*Antidesma Bunius* L. Spreng) As Tyrosinase Enzym Inhibitor

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ABSTRACT

Several studies have shown that plant extractive substances have the potential as active compounds inhibiting the enzyme tyrosinase. Arbutin is an enzyme inhibitor of tyrosinase, known as a popular whitening agent used in cosmetics because of its effectiveness in overcoming skin hyperpigmentation. The purpose of this study was to conduct a qualitative and quantitative analysis of arbutin on Buni Leaves and Fruits (*Antidesma bunius* L. Spreng). The raw simplicia used are mature and young buni leaves, green, red and purple buni fruits. The extraction method is maceration using methanol as solvent. The initial screening for arbutin content was carried out using thin layer chromatography (TLC) and dichlormethan:methanol 50:50 used as mobile phase. Isolation of arbutin content was carried out using Preparative TLC with the same eluent. Qualitative and quantitative analysis were performed using *High Pressured Liquid Chromatography* (HPLC) with mobile phase of acetonitrile: water 60:40. The tyrosinase enzyme inhibition activity test was then carried out in vitro using 96-well microplate, l-tyrosine and l-dopa were used as substrate at a wavelength of 492 nm. The Rf values obtained for mature buni leaves and green buni fruits, respectively 0.61 and 0,62. The retention time of HPLC chromatogram respectively 2,784 minutes and 2,758 minutes. Arbutin levels in leaves and fruits are 7.9 mg / g and 2 mg / g. The activity of the enzyme tyrosinase of mature buni leaves on L-dopa and L-tyrosine substrate were respectively stated as IC50 values of 88.7191ppm and 101.33347 ppm. The activity of the enzyme tyrosinase of the green buni fruit on L-dopa and L-tyrosine substrate respectively stated IC50 values of 198,0293 ppm and 246,1296 ppm.

Keywords: Arbutin, Buni Leaves, Buni fruits, TLC, HPLC, Tyrosinase enzyme inhibitor.

Introduction

The market for premature antiaging cosmetic products and skin whitening in Indonesia

is very potential. This is caused by the condition of Indonesia which is located in the tropics which causes the skin of its people to

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experience premature aging and browning. Exposure to UV light from the sun in the tropics is a source of free radicals that cause skin aging (1) and increases the activity of the enzyme tyrosinase synthesizing melanin pigment so that the skin color becomes increasingly brown (2). Melanin is the main pigment that determines skin color (Baumann *et al*, 2009) which is synthesized in melanosomes, specifically organelles in melanocytes located in the basal layer of the epidermis (3). Most cosmetic products contain dangerous synthetic chemicals. Skin whitening compounds such as hydroquinone and mercury are carcinogenic and cause skin irritation, skin redness, heat, and itching (4)

Several studies have shown that plant extractive substances have the potential to be active antioxidant compounds and tyrosinase enzyme inhibitors. Natural antioxidant compounds from plant phenolic groups can inhibit skin aging (5).

Arbutin is a tyrosinase enzyme inhibitor, known as a whitening agent that is popularly used in cosmetics because of its effectiveness in overcoming skin hyperpigmentation. Arbutin is not cytotoxic in humans. Its chemical structure is similar to hydroquinone, which consists of phenol and glucose molecules which are connected in the para position. Arbutin can be used as a substitute for hydroquinone, which is cytotoxic against melanocytes in the human body (6).

Suwanpraset (2018) has successfully analyzed the presence of arbutin content in mature Mao Leaves (*Antidesma thwaitesianum*) of 10.6 mg / 100gr of fresh leaves and in green mao fruit (*Antidesma thwaitesianum*) of 1.2 mg / 100 g of fresh fruit. Mao (*Antidesma thwaitesianum*) has the same family and genus as Buni (*Antidesma bunius*). The chemotaxonomic approach states that a plant in the same taxon can have secondary metabolites that do not differ greatly (7). Hamidu *et al* (2018) reported that the buni fruit ethyl acetate extract contained a total of 7.9% flavonoids, 10.72% n-hexane extract, and 3.56% ethanol extract while Wijayanti (2016) reported the buni fruit ethanol extract contained phenolic total of 0.2794 ± 0.0048 mg equivalent of gallic acid per gram of ethanol extract of buni fruit.

Methods

Sample (Material)

Young buni leaves, mature buni leaves, green buni fruits, red buni fruits and purple buni obtained from the village of Gelonggong Bojong Gede, Bogor, West Java. Arbutin, tyrosinase, methanol, ethyl acetate, acetonitrile, silica gel F 254, dichloromethane, disodium hydrogen phosphate and sodium hydrogen phosphate.

Procedure

Extraction of Buni Leaves

Fresh samples of light green leaves, dark green leaves, green buni fruits, red buni fruits and purple buni fruits weighed 1500 g each and then dried at 50 ° C for 6 hours and then chopped into small pieces, each sample was immersed in 1500 ml of methanol for ± 3 days at room temperature. Then the solvent is removed by evaporation using a rotary evaporator (40-50 ° C) until a thick extract is obtained. The two extracts were then weighed and stored for further analysis (8).

Thin-layer chromatography (TLC)

Each extract with a weight of about 0.01 g was dissolved in methanol 1 ml and then poured on the TLC plate with arbutin as standard. TLC plates that have been dented are placed into chambers which already contain Diklormetan: methanol at a ratio of 30:70 as the mobile phase. and is allowed to stand on the plate until the solvent permeates the sample to separate the arbutin, then waits until the mobile phase rises to the limit mark, after reaching the boundary mark then the plate is lifted, then all stains are detected using a UV light source with a wavelength of 254 nm and 366 nm to compare moving sample rates to standard arbutin levels. The stains from the extract had the same level as the arbutin standard in scrapings of all TLC plates and collected. The arbutin is isolated from silica particles using methanol. The solvent is separated from the silica particles by centrifugation (8).

Qualitative Analysis of arbutin

After centrifugation of the separate extraction solvent was removed and concentrated

again using waterbath, then 25 mg of the isolated sample was weighed and dissolved in 5 ml of methanol for chromatographic analysis. Analysis was carried out using the zorbax eclipse c-18 column at 224 nm wavelength. The column temperature is set at 35 ° C with an injection volume of 20 µl then elution is carried out at a flow rate of 1 ml / min under an isocratic system with an acetonitrile: water ratio, 40: 60 v / v (8)

Quantitative Analysis of Arbutin

The quantification in five samples was carried out using the single standard curve method. Analysis was performed using zorbax eclipse c-18 columns and observed at 224 nm wavelength. The column temperature is set at 35 ° C with an injection volume of 20 µl then elution is carried out at a flow rate of 1ml / min under an isocratic system with an acetonitrile: water ratio, 40: 60 v / v. Arbutin concentration calculated is expressed in mg / g sample (8)

Tyrosinase Enzyme Inhibition Activity

The activity of tyrosinase enzyme is tested based on the method that has been done by Coal et al (2010). Extract solutions was made into various concentrations by dissolving 20 mg of sample using 10 ml of water until the concentration of 2000 ppm can be obtained. This solution is a stock that will be diluted later in the sodium phosphate buffer 150 mm and pH 6.5. The extract concentration used in this test was 31.25-2000 ppm, arbutin was used as a positive control and tested at a concentration of 7.8125-500 ppm. This test uses 96 microplate wells in the wellbore. Samples are put into various concentrations of 70 µl and then added with 30 µl tyrosinase, each concentration is tested with three replications, then the substrate is added (12 mm L-DOPA and L- TI-ROSIN) as much as 110 µl into each well, then the plates were incubated for 30 minutes. Then absorbance measurements are carried out, absorbance measurements are carried out using a multiwell-plate reader with a wavelength of 510 nm and then the concentration of each ex-

tract that can inhibit half of the tyrosinase activity (IC₅₀) is determined by comparing the absorbance of the sample without the addition of extract with the addition of extract at a wavelength of 510 nm (9).

$$\% \text{ Tyrosinase inhibition} = \frac{[(B-KB)-(S-KS)]}{(B-KB)} \times 100\%$$

Remarks:

B : Blank

KB : Control

S : Sample

KS : Control sample

Then IC₅₀ is calculated based on the intersection (plot) between the concentration and% inhibition of the tyrosinase enzyme.

Results and Discussion

Results of extraction of *Buni* leaves and *Buni* fruits

The extraction of samples begins with the preparation of *Buni* leaves and *Buni* fruits simplicia. *Buni* Leaves used in this study are Dark green leaves and Light green leaves. Whereas the *Buni* Fruits used in this study were green buni, red buni and purple buni. Sampling was carried out in the village of Gelonggong Bojong Gede. *Buni* leaves are collected and then sorted wet to remove impurities that stick to the simplicia and then wash using running water. Then a chopping is done to simplify the drying process. The drying process is carried out by aerated without direct sunlight for about one week, after which the dried simplicia is mashed using a blender and then sifted in order to expand the contact surface between the particles with the liquid of the extractor so that the extraction results are more optimal. The dried powder obtained was then extracted by maceration method using methanol as a solvent for 3 days then remastered as many as 5 repetitions with the aim to dissolve the compound that has not been extracted completely in the maceration process, after which the extract was concentrated using a rotary evaporator at a temperature of 50° C. the obtained extract is then stored for further testing.

Organoleptic Test Results**Table 1. Organoleptic Characteristics of Buni Leaves and Buni Fruits Extract**

No	Sample	Characteristics	
1.	Mature Green Leaf Extract	Consistency	: Thick extract
		Color	: Dark Green
		Odor	: Typical leaves
2.	Young Green Leaf Extract	Consistency	: Thick extract
		Color	: Dark Green
		Odor	: Typical leaves
3.	Green Fruits Extract	Consistency	: Thick extract
		Color	: Brownish Green
		Odor	: Typical fruits
4	Red Fruits Extract	Consistency	: Thick extract
		Color	: Brownish Green
		Odor	: Typical fruits
5	Purple Fruits Extract	Consistency	: Thick extract
		Color	: Dark purple
		Odor	: Typical fruits

Rendemen Test Results**Table 2. Rendemen value of Buni Leaves and Buni Fruits**

No	Sample	Rendemen Value
1	Mature Green Leaf Extract	7,6 %
2	Young Green Leaf Extract	4,82%
3	Green Fruits Extract	16,66 %
4	Red Fruits Extract	16,09 %
5	Purple Fruits Extract	27,47 %

The rendemen value shows how much the amount of content that can be extracted by the solvent. In this study yields were obtained namely 7.6% and 4.82% for each extract. The yield value obtained is relatively small, this is probably due to the small amount of the compound contained by methanol as a solvent. The results obtained are in line with the yield obtained by Puspitasari and Evi (2009) who extracted Buni fruit using methanol as a solvent of 4.084% and lower than the yield obtained by Wijayanti (2016) which is 13.841% of Buni fruit ethanol extract.

The yield produced is 16.09% to 27.47%. The greater the yield produced, the more effi-

cient the extraction process is applied (De-watisari *et al*, 2017). The yield is high enough to show that the chemical compounds that can be found in the extract are also quite large. This is possible because of the large number of chemical compounds present in simplicia (Kartikasari *et al*, 2014). So the difference in yield obtained may be caused by differences in the content of active compounds in each simplicia. The yield obtained is greater than Puspitasari & Evi's research (2009) to get a rendition of buni fruit methanol extract of 4,084% and not much different from the yield of 96% ethanol extract of buni fruit conducted by Pangestuty (2016) that is equal to 13,841%.

Thin Layer Chromatography Results (TLC)

Table 3. Thin Layer Chromatography Results (TLC)

No	Sample	Rf Value	Color stain		
			No	UV 254 nm	UV 366 nm
1	Arbutin	0,62	Colorless	White Fluorescent	White
2	Mature Green Leaf Extract	0,61	Brownish green	Faded brown	Brown fluorescent
3	Young Green Leaf Extract	0,63	Brownish green	Faded brown	Brown fluorescent
4	Green Fruits Extract	0,62	Light green	White spots	White spots
5	Red Fruits Extract	0,61	Light green	White spots	White spots
6	Purple Fruits Extract	0,61	Light green	Red spots	Red spots

The TLC test was carried out as a preliminary identification of the arbutin content in the extracts of Buni leaves and Buni Fruits and aimed to isolate the suspected arbutin compounds for further qualitative and quantitative arbutin testing. The eluent used was dicloromethane: methanol in a ratio of 30:70 while the stationary phase used silica gel 60 GF₂₅₄. The plate used was previously heated first in order to eliminate the water content contained in plate (10). Then the extract is bottled using a capillary tube at the bottom edge of the plate. Before eluting the eluent in the vessel, it is saturated first so that the eluent mixture can conduct the extract well. The bottled palate is put into a vessel and eluted to the elution limit of the solvent. After that the plate was observed under UV light at wavelengths of 254 nm and 366 nm this is because some organic compounds including arbutin shine and fluoresce at that wavelength so that the stain on the sample will easily appeared. The appearance of color at this wavelength is caused by the interaction between UV light and chromophore groups which are bound by ausochromes on the stain. Visible light fluorescence is the emission of light emitted by these components when electrons are extracted from the basic energy level to a higher energy level and then returns while releasing energy (Zahrul, 2011). After thin layer chromatography (TLC) tests, the arbutin rf value was 0.62 and the rf extract of mature green leaves, young green leaves, Green buni fruits, Red buni fruits and Purple buni fruits respectively 0.61 and 0.63, 0.62, 0.61, 0.61. The results obtained for the arbutin standard are lower than the results of previous studies

where Swanpraset, (2017) uses the eluent in diklormetan: 50:50 methanol has an arbutin rf value of 0.63 and is greater than the arbutin rf value obtained by Alam *et al* (2011) that is, 0.32 using methanol; chlorofrom in a ratio of 3: 7. The difference in rf value is probably due to the different eluents used so that the separation is done at different polarity levels. Different polarity causes the sample to elute at different distances so that different rf values are obtained (11).

After that, preparative TLC was carried out aimed at isolating the compound suspected of arbutin. Isolation was carried out by re-extracting the extract sample by TLC, then staining was formed under UV light of 254 nm and 366 nm. The stains obtained are then calculated the rf value and then the silica plate with the same rf value as the rf value in the analytical TLC is dredged, dissolved in methanol, then centrifuged to separate arbutin from silica is at the bottom while the arbutin will be at the top (12). The supernatant is then concentrated again to obtain isolation results in the form of compounds suspected to be arbutin.

Results of Qualitative Analysis of Arbutin

HPLC (*High Pressure Liquid Chromatography*) is a method of detection and quantification of very high sensitivity for various chemical compounds in a particular sample using UV-Vis absorbance (13). A total of 20 µL of a liquid sample of isolates that had been separated before using preparative TLC, was injected into the HPLC for analysis. The sample was eluted at a wavelength of 224 nm. As a mobile phase, acetonitrile: water 40:60 is used, which is the

result of optimization of previous studies. Based on the results of HPLC analysis conducted on both isolate samples and arbutin standards as a comparison *retention time* for the arbutin standard is at 2.754 minutes. Arbutin detection in both samples of arbutin isolates, dark green leaves, light green, green buni, red buni, purple buni, can be observed with the emergence of *peaks* respectively at 2,784, 2,760, 2,758, 2,749 and 2,733. In both isolate sample chromatograms, there were several

peaks that could be observed. This showed that the separation of the compound was not optimal. *The peak* first that was detected in both isolates was thought to be the absorption of the compound from the methanol solvent used because its retention time was the same as the retention time of the methanol injection result. The uptake is thought to be an uptake of impurities or compound components contained in the methanol solvent.

Figure 1. Arbutin standard HPLC chromatogram results with Rt 2.754 minutes

Figure 2. HPLC chromatogram results Dark green leaves with Rt 2.784 Minutes

Figure3. Chromatography Results of Buni Green Fruits with Rt 2,758 Minutes

Results of Quantitative analysis of Arbutin

The quantitative arbutin analysis results in the two samples are as follows,

Figure 4. Arbutin Quantitative analysis results on Buni leaves

Based on the quantitative arbutin analysis results contained in the sample, the obtained amount of arbutin in the young green leaf sample is smaller namely 5.8 mg / gram sample and mature green leaves which is 7.9 mg / gram sample. The variation of arbutin content in buni leaves is probably due to differences in age of leaf development that occurs in different leaf locations. Mature leaves have the optimal

ability to produce secondary metabolites, so that phenolic and flavonoid levels are large. In light green leaves, secondary metabolites are still not produced in large quantities, while the secondary metabolite content of mature leaves generally decreases. In addition, seasonal, genetic, and agronomic factors also influence phenolate levels and flavonoid content (14).

Figure 5. Results of Quantitative Arbutin Analysis on Buni Fruits

While in Buni fruit, it was found that the arbutin content in each extract of Buni fruit from the highest to the lowest respectively were green, red and purple Buni fruits. The difference in arbutin levels is probably due to the maturity factor of the buni fruit. Where the highest

arbutin content is the green Buni fruit. The results of this study are in line with the results of the study of Dabbouet *al* (2016) which states that the phenol content has decreased in line with the level of fruit maturity.

Results of Tyrosinase Enzyme Inhibition Activity

Figure 6. Results of tyrosinase enzyme inhibition activity in mature Buni Leaves

This study uses a *Microplate reader*. The purpose of using a *microplate reader* is that it has the advantage of being relatively fast, easy and inexpensive. Tyrosinase inhibition activity testing was carried out on mature green leaf samples because it has the highest arbutin content of the two samples. The purpose of this tyrosinase inhibition activity test is to determine the best inhibitory power of the tyrosinase enzyme. The parameter used is IC50 (*inhibition concentration*) which is a concentration that can inhibit 50 percent of the activity of the tyrosinase enzyme in this study. As a substrate used 2 types of substrate, namely L-tyrosine and L-dopa. Using two substrates because both of them with the enzyme tyrosinase will produce dopakrom which can then form melanin. If the sample contains tyrosinase inhibitor compounds, then the activity of the tyrosinase enzyme will be inhibited in producing melanin.

In this study, the formation of dopakrom products is characterized by the appearance of chocolate color which can cause substrate-tyrosinase reactions to be seen resulting in reduced dopakrom production which is characterized by a decrease in the intensity of the brown color (15). The whole process involves an enzymatic reaction in which all reactions run at optimum conditions so incubation is carried out at 37 °C because it is the optimum temperature for the work of the tyrosinase enzyme. Then a phosphate buffer solution of pH 6.5 is used. Before tyrosinase resistance analysis is carried out, the pH can be checked using a pH meter. The optimum pH of the tyrosinase catalysis reaction was in the range of 6.5-7.0, so that in this study the tyrosinase catalysis reaction took place under conditions of temperature and pH that were in accordance with the literature.

Figure 7. The results of tyrosinase inhibition activity in Green Buni Fruits

The tyrosinase inhibitor activity refers to the method that has been carried out by Coal et al. (2010). This test uses a *plate* with 96 wells, in which the wells are extracted from various concentrations of 70 μ L and then added with 30 μ L tyrosinase, each concentration is carried out with three replications. After that, the plates were stored in a temperature incubation room (37°C) for 5 minutes. Furthermore, substrate (2 mM L-tyrosine and 12 mM L-DOPA) was added as much as 110 μ L into each wellbore, then the plate was stored back into the incubation chamber for 30 minutes. The optical length of each well was then determined using an Elisa reader at a wavelength of 492 nm. Furthermore, the concentration of each sample that could inhibit half of the tyrosinase (IC50) activity was determined by comparing the absorbance of the sample without adding samples by adding samples at a wavelength of 492 nm. The sample used in this activity test is the sample of the results of isolation of the green buni fruit, the sample is used because the sample contains the highest arbutin than the other three samples.

The calculation result of IC50 value of green buni fruit with standard arbutin as a positive control shows the result that the IC50 value of arbutin standard is smaller than IC50 value of green buni fruit both on L-tyrosine and L-Dopa

substrates. IC50 values of standard arbutin and green buni fruits were greater for L-Dopa substrate compared to L-tyrosine substrate. This result is in line with the results of Kurniasari's research (2018) which determined the potential of cocoa seed extract as a tyrosinase inhibitor in which the IC50 values obtained were 352.05 ppm and 836.20 ppm. IC50 value of green buni obtained in this study is smaller than the IC50 value of similar studies, this is likely due to the fact that in this study the sample compound used has been separated from other component compounds so that it has a smaller IC50 value compared to other studies using extract as a sample (15)

Between the tyrosinase enzyme and the two substrates used will produce different reactions, namely the reaction of monophenolase and diphenolase. Monophenolase is a reaction between an enzyme and an L-tyrosine substrate, where the enzyme can convert L-tyrosine to L-DOPA, then the enzyme converts L-DOPA into dopakuinone called the diphenolase reaction. After that, dopakuinone will form melanin which triggers brown skin (14). The reaction that occurs between the tyrosinase enzyme and the L-tyrosine or L-DOPA substrate in this study can be seen in the presence of a brown color.

Conclusion

1. Based on the results of this study, it can be concluded that the leaves and fruits of Buni contain arbutin compounds. TLC results showed the Rf value of mature green leaves (0.61) and green buni fruits (0.62),
2. The results of HPLC analysis showed Rt of mature green leaves at 2,784 minutes and green buni fruits at 2,758 minutes. With arbutin levels respectively were mature green leaves of 7.9 mg / g and green buni fruits of 2 mg / g.
3. Based on the results of the study it can be concluded that the results of the isolation of mature green leaves have an inhibitory activity of tyrosinase enzymes with moderate categories of L-tyrosine and L-dopa with IC50 values of 88.7191ppm and 101.33347 ppm, respectively. This IC50 value is lower than the IC50 standard arbutin against L-tyrosine by 15.48521 ppm and the arbutin standard against L-dopa by 24.78643 ppm which has inhibitory activity of tyrosinase enzymes with high category.
4. Based on the results of this study it can be concluded that the results of the isolation of green buni (*Antidesma bunius* L. Spreng) have an inhibitory effect of tyrosinase enzymes in the medium category against L.tirosin with IC50 of 198.0293 ppm and L. dopa with IC50 of 246.1296 ppm. This IC50 value is lower than the standard IC50 of arbutin against L-tyrosine by 15.48521 ppm and the standard arbutin against L-Dopa by 24.78643 ppm which has high activity of tyrosinase enzyme activity.

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