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Original Paper

The Extraction and Partial Characterization of Lunatin from Lima Bean (*Phaseolus lunatus* L.)

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Abstract—Lima Bean (Phaseolus lunatus L.) contains a lectin called lunatin which is an antinutrient with antioxidant, antifungal, and antiproliferative properties. The purpose of this study was to determine the effect of different processing of Lima bean seeds, namely crushed dry seeds, Lima bean flour, and Modified Legume Flour (MOLEF). In addition, it delved into the differences resulting from different extraction temperatures (27ºC, 40ºC, and 50ºC) on the lunatin extract obtained. The results show that the total protein in dry seeds, flour and MOLEF Lima beans are 23.18%, 24.20% and 17.12%, respectively. The molecular weight of lunatin detected is 30.25 kDa; The highest antioxidant activity, marked at 83.58% , is obtained by lunatin extract from samples of crushed dried Lima beans, with an extraction temperature of 50ºC, while the lowest activity, 45.97%, is indicated by lunatin extract from MOLEF samples of Lima beans, with extraction temperature of 40ºC. Temperature variations in general do not affect the protein bands detectable. Likewise, the antioxidant activity also does not show antifungal activity against Aspergillus niger.

Keywords— lunatin, lima bean, MOLEF, antioxidant, antifungal activity

I. INTRODUCTION

Lima bean (*Phaseolus lunatus L.*) is a legume plant that has high protein and fiber content and is low in fat. The plant is easy to cultivate [17] and has high productivity, which is around 800-900 kg/ha on dry land and about 1700 kg/ha on irrigated land. In addition, Lima bean also has a fairly high nutritional value, namely carbohydrates (60.55-74.62%), protein (19.93-21.40%), fat (0.99-1.21%), fiber (4.20-5.50%), moisture (11.58-13.83%), and ash content (3.46-3.61%) on a dry basis [3, 21, 24].

Lima bean can be modified into protein-rich flour and Modified Legume Flour (MOLEF). MOLEF Lima bean can be produced by controlling fermentation using lactic acid bacteria (LAB) culture, namely *L. plantarum* [14]. Lima bean flour and MOLEF Lima bean have been used in several food products, including as a substitute for wheat flour in seasoning flour [6], mushroom nuggets [14], and Texturized Vegetable Protein (TVP) [18, 20].

Even so, Lima bean is still understudied and has not been widely used by public because of its anti-nutritional components. Lima bean contains toxic compounds or anti-nutritional substances such as cyanide acid (HCN) which is quite high at around 14.96-26.22 mg/g and phytic acid around 8.76-19.75 mg/g [3]. Lima bean also contains a lectin called lunatin which is a carbohydrate-binding protein that has hemagglutination properties. [9, 18] have reported the toxic effects of lectins related to the digestive process and the abnormal absorption of nutrients in the mammalian digestive tract.

The content of anti-nutritional substances in Lima beans can be affected by processing method. Cyanide and phytic acid can be removed using proper processings, one of which is by immersion [4]. Lectins from Lima bean can be reduced after going through soaking, autoclaving, or roasting [1]. Heat treatment is a method often used to reduce lectin content [8]. [2] reports that fermentation is also able to reduce the content of anti-nutritional compounds and increase soluble protein in koro-koroan or grains.

Apart from being an antioxidant, lunatin also has functional properties. [16] mentioned, in general, lectins are known to agglomerate harmful cells and stimulate mitosis in lymphocytes. Lunatin also has the ability as antifungal against Pythium aphanidermatum, Fusarium soloni, Fusarium oxysporum and Botrytis cinerea; and antiproliferative ability against HepG2, HeLa, and K562 tumor cells [19]. Lunatin also shows activity as an antioxidant, antitumor, and gastroprotective [5].

Lima beans used in this study were in the form of crushed dry Lima bean seeds, Lima bean flour, and MOLEF. Lunatin from these three raw materials can be obtained using various methods, one of which is extraction using solvents. The temperature used during the extraction process can affect the total protein, including the resultant lunatin.

This study aimed to determine the chemical and functional characteristics of lunatin extracted from three processed raw materials (crushed dry seeds, flour, and MOLEF), at various extraction temperatures (27ºC, 40ºC, and 50ºC).

II. MATERIALS AND METHODS

A. Materials

The seeds of Lima bean (*Phaseolus lunatus L.*) (kidney-shaped, white in color, and weighing about 30-45.3 g/100 seeds) were obtained from the Indonesian Community Forest Farmers Association (ATHRI). Materials for the production process of Modified Legume Flour (MOLEF) such as NaCl (Kapal brand), refined sugar (Gulus), skim milk (Prolac), and citric acid (Gajah brand) are obtained from traditional markets.

Inoculums of Lactobacillus plantarum and Aspergillus niger were obtained from the Laboratory of Microbiology of Food and Agricultural Products, Faculty of Agricultural Technology, University of Jember.

This study also used other materials such as *de Mann Rigorosa Sharpe Broth* (MRSB), *Potato Dextrose Agar* (PDA),CH_3COONa, $(NH_4)_2SO_4$, H_3PO_4 (*Merck*) obtained from Ina Lab; DPPH (2,2-diphenyl-1-picrylhydrazyl) from Sigma-Aldrich, St.Louis, USA; sodium citrate, ethanol, and hippuric acid buffer from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan; e-PAGEL mini gel ET10L, EzProtein Ladder WSE-7020, EzApply AE-1430, EzRun AE-1410, and CBB *Staining Reagent* Ez Stain Aqua AE-1340 from ATTO Corporation, Osaka, Japan.

B. Methods

1. This study involved two factors, which consisted of factor A (processed raw materials) and factor B (extraction temperatures). The methods for processing raw materials involved crushed dried Lima bean seeds, Lima bean flour, and MOLEF Lima bean. Meanwhile, the extraction temperatures were set at 27ºC, 40ºC, and 50ºC. Each treatment was repeated three times. This research was carried out in two stages, namely processing Lima Bean and extracting protein from Lima Bean.

2. The Preparation of Crushed Dry Lima Bean

Lima bean was dried under the sunlight for 6 hours then followed by drying using an oven for 20 hours at a temperature of 60ºC. The dried Lima beans were then crushed using a blender to facilitate the extraction process.

The Production of Lima Bean Flour [6]

Lima beans sorted were then washed using water and soaked for 24 hours. The soaked materials were then drained and separated from the skin. After that, it was dried in the sun for 6 hours and continued by oven drying at 60ºC for 20 hours. Eventually, the beans were then ground and sieved through an 80 mesh sieve.

The Production of MOLEF Lima Bean Using Spotaneous Fermentation [14]

Lima beans were soaked for 16 hours at pH 5 with a ratio of 3:1 (w/v), and then washed as well as soaked in 10% NaCl solution with a ratio of 3:1 for 15 minutes. After going through the immersion, the beans were washed and boiled for 30 minutes. The beans which had been boiled were sliced into 2-3 mm thick, dried in the sun for 1 hour, and put in oven drying at 60ºC for 24 hours. The resultatnt beans were ground, and sieved through an 80 mesh sieve.

The Culture Preparation of MOLEF Lima Bean [14]

Preparation of MOLEF culture was carried out by rejuvenating *L. plantarum* culture on 37ºC MRSB medium for 24 hours. 2% v/v rejuvenated cultures were inoculated in 10% w/v sterile artificial media solution consisting of 5% spontaneous Modified Legume Flour (MOLEF), 3% sugar, and 2% skim milk. The artificial media was incubated at 37ºC for 24 hours and was referred to as the parent culture. Two percent of the parent culture was inoculated into a sterile artificial media solution to serve as an intermediate culture. Subsequently, 2% v/v of the intermediate culture was reinoculated as a working culture.

The Production of MOLEF Lima Bean through Controlled Fermentation [14]

Lima beans were immersed in a solution of citric acid with a pH of 5 and given UV light for 30 minutes. After that, 10% LAB culture was inoculated and incubated at 37ºC for 24 hours. The fermented Lima beans were then washed and soaked in a 10% NaCl solution with a ratio of 3:1 for 15 minutes. The beans was then washed twice and drained. After that these were ground using blender, dried in the sun for 1 hour followed by oven drying at 24ºC for 24 hours, and sieved through an 80-mesh sieve.

The Calculation of Total Protein in the Materials [15]

The total protein was calculated using a tool in the form of a C-N Corder to determine the carbon and nitrogen content in the material. In the process, 50 mg of sample and controls in the form of hippuric acid weighing 10 mg, 20 mg, 30 mg, and 40 mg were put into the C-N Corder for 3 hours. Afterward, the percentage of carbon and nitrogen in the material was measured. Protein content was calculated using the formula below:

$$\% \text{ Protein} = \%N \times \text{Correction factor } (6{,}25)$$

Lunatin Extraction

The materials weighing 50 g (crushed dry Lima bean seeds, Lima bean flour, and MOLEF Lima bean) were homogenized using 0.02 M sodium acetate buffer at pH 5.4 for 15 hours with temperature variations of 27ºC, 40ºC, and 50ºC. The homogenate obtained was centrifuged at 12,000 rpm for 30 minutes at 4ºC. The supernatant obtained was then referred to as the initial extract [19].

From the initial extract, 50 ml extract was taken and saturated to a saturation level of 40% with the addition of $(NH_4)_2SO_4$. This was then stored at 4ºC for 12 hours. After that, the solution was centrifuged at 12,000 rpm for 30 minutes. The supernatant obtained was then re-saturated to a saturation level of 80% with the addition of $(NH_4)_2SO_4$ and stored for 16 hours at 4ºC. The next day, an increase in volume was identified. The suspension was then centrifuged at 12,000 rpm for 30 minutes. The precipitate obtained was then dissolved in 500 mL of 0.02 M sodium acetate buffer with a pH of 5.4 and dialyzed for 24 hours at 4ºC. The sample was centrifuged at 12,000 rpm for 30 minutes at 4ºC. Subsequently, the supernatant obtained was stored at 4ºC [12, 22].

The Calculation of Molecular Weight of Protein Bands [11]

The electrode buffer was filled into the mini electrophoresis system device. Then 10% precast gel was put in it. Each sample and molecular weight marker was reacted with reagent for sample preparation in a ratio of 1:1. The sample was then centrifuged at 6200 rpm, heated for 5 minutes at 100ºC, and then dripped into the precast gel well. The energy source used was an electric current of 20 mA.

The coloring reagent was poured into a microwave oven-proof container of about 50 mL. The gel was removed from the

safety glass and immersed in it, covered with plastic wrap with several holes, and put in a microwave oven for 50 seconds with an energy setting of 600 W. After the removal, the solution was agitated at 50 rpm for 60 minutes. The dye reagent was then rinsed with distilled water with the same treatment as during the coloring process. This step was repeated several times until the color of the gel was clearly visible. Protein bands were observed on the gel doc to determine the molecular weight according to the RF (Retention Factor) calculation.

Testing Antioxidant Activity [13]

Samples were centrifuged at 3000 rpm for 10 minutes at room temperature. It was then filtered using a 0.22 m filter, and diluted 5x and 10x. 400 L of the sample was reacted with 640 L of sodium citrate buffer, 1 mL of absolute ethanol, and 560 L of DPPH (in absolute ethanol). The mixture obtained was homogenized for 80 minutes at 100 rpm in a dark area. The absorbance was calculated at a wavelength of 517 nm. The calculated antioxidant activity in the sample was compared with the standard (60% ethanol) and ascorbic acid. Antioxidant activity was calculated by the following formula:

$$\text{Antioxidant Activity (\%)} = \frac{(A_{standard} - A_{sample})}{A_{standard}} \times 100\%$$

Testing Antifungal Activity [19]

3. Fungi were inoculated in Potato Dextrose Agar (PDA) on a petri dish with a diameter of 100 mm and a height of 15 mm. Three sterile paper disks with a diameter of 0.5 cm were immersed in the purified sample and one paper disk as a control was immersed in the lectin-free buffer. The four were then placed about 4 cm apart from each other around the previously inoculated fungi. The Petri dish was then re-incubated at 37ºC for 48 hours until mycelia growth developed.

4. Data Analysis

Obtained data were processed using ANOVA with $\alpha \leq 5\%$. In addition, analysis of difference was carried out using Tukey.

III. RESULTS AND DISCUSSION

A. Total Protein in Processed Materials

The total protein in the raw materials showed that crushed dried Lima beans, Lima bean flour, and MOLEF Lima beans had total protein ranging from 17.12% to 24.20% of the dry weight. Total protein data is shown in Figure 1.

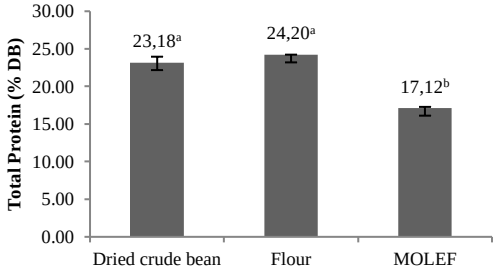


Figure 1. Total protein of dried crushed Lima bean, Lima bean flour and Lima bean MOLEF

Figure 1 shows that the highest total protein is in Lima bean flour at 24.20%, followed by crushed dried Lima beans (23.18%), and MOLEF Lima beans (17.12%). This was presumably because the crushed dry seed samples only underwent a short processing process, resulting in only a slight degradation of the protein components. While MOLEF has the lowest total protein because during the fermentation dissolved protein levels can increase due to microbial activity which hydrolyzes complex proteins into free amino acids/simple peptides [2, 16, 26]. The dissolved protein in this study can be dissolved with the citric acid solution after the soaking process is complete or after the salt solution soaking process[25]. [10] in his research report that an increase in fermentation time causes more proteinase enzymes to be produced, so that protein is dissolved in water and wasted in flouring process. This leads to lower protein content of modified Lima beans in the study.

B. Molecular Weight of Protein Bands

The molecular weight of protein bands was calculated using SDS-PAGE (Sodium Dedocyl Sulphate Polyacrylamide Gel Electrophoresis) as in the research method [11]. This calculation aimed to determine the molecular weight of the protein obtained from this study, which would be compared with previous studies. The calculation was done by measuring the total distance traveled from the starting point to each protein band formed and analyzing the retardation factor (Rf) of the protein bands to produce a linear gel regression curve equation.

The gel used in this study was a gel with a concentration of 10% which had 14 wells. The first and last wells were used markers, and the nine types of treatment are placed between the two. Gel with a concentration of 10% was used because it was able to detect a molecular weight range between 21 kDa to 200 kDa, as corroboated by previous studies regarding the molecular weight of lunatin. The results of electrophoresis are presented in Figure 2.

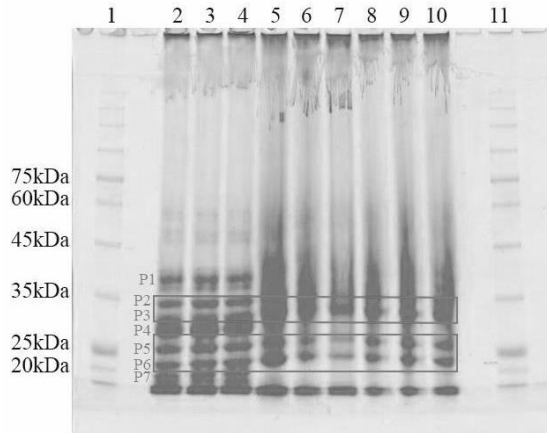


Figure 2. Electrophoresis results (1 and 11 = marker; 2 = A1B1; 3 = A1B2; 4 = A1B3;5 = A2B1; 6 = A2B2; 7 = A2B3; 8 = A3B1;9 = A3B2; 10 = A3B3; P1 = Protein band 1;P2 = Protein band 2; P3 = Protein band 3; P4 = Protein band 4; P5 =Protein band 5;P6 = Protein band 6; P7 = Protein band 7)

The results of electrophoresis in Figure 2 show that treatments A1B1, A1B2, and A1B3 derived from crushed dried Lima bean raw materials have seven protein bands detected, namely P1, P2, P3, P4, P5, P6, and P7. The samples A2B1, A2B2, and A2B3 originating from the raw material of Lima bean flour; and A3B1, A3B2, and A3B3 derived from MOLEF Lima beans only have three types of protein bands detected, namely P3, P5, and P6.

The analysis of the molecular weight for each sample was based on the available markers. Based on Figure 2, the total distance from each detected protein band is measurable. The retardation factor (Rf) is estimated from these results to mark a linear regression curve corresponding to the gel. The linear regression curve of this gel is presented in Figure 3.

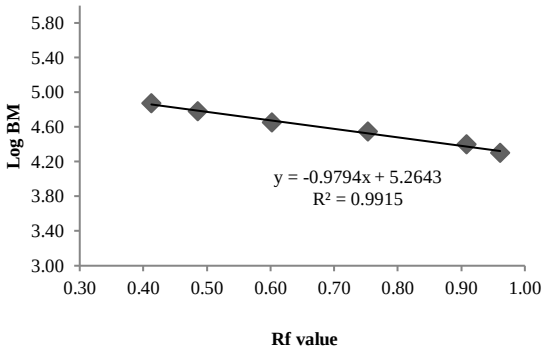


Figure 3. Linier regression curve of the gel

The linear regression curve was used to determine the molecular weight of the protein bands formed in each treatment, as shown in Table I.

TABLE I Molecular Weight of the Protein Bands

Protein Band	Distance (r)	Molecular Weight (kDa)
P1	7,20	39,15
P2	7,95	33,32
P3	8,40	30,25
P4	8,70	28,37
P5	9,30	24,94
P6	9,75	22,64
P7	10,1	21,00

The data in Figure 2 and Table 1 show that the protein (which can be lunatin) may have a molecular weight of about 30.25 kDa (P3), 28.37 kDa (P5), or 22.64 kDa (P6) because the three molecular weights are detected in all tested samples. This is in accordance with the study by [7] which isolated two agglutinating factors from *P. lunatus*. Both agglutination factors have a molecular weight of 62 kDa, but when a reducing reagent in the form of DTT is added, a new protein band appears with a molecular weight of 31 kDa. The agglutination factor in the study [5] is expressed as a single lectin at two different stages of aggregation. The lectin in *P. lunatus* in the study [19] is hereinafter referred to as lunatin. As previously explained, molecular weight testing in this study also used DTT as a reducing reagent, so the protein band detected in P3 originating from Lima bean (30.25 kDa) possibly had the most substantial molecular weight because it was detected in all samples and was close to lunatin molecule found in a research by [7], which was 31 kDa.

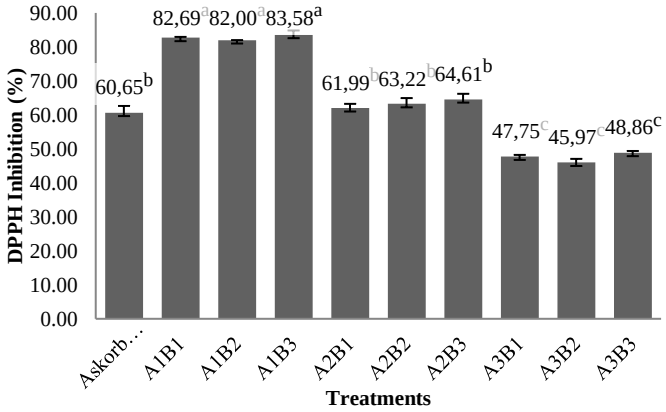


Figure 4. DPPH inhibition (A1B1 = dried crushed bean lunatin extract, 27ºC extraction;A1B2 = dried crushed bean extract, 40ºC extraction; A1B3 = dried crushed bean extract, 50ºC extraction;A2B1 = flour lunatin extract, 27ºC extraction; A2B2 = flour lunatin extract, 40ºC extraction; A2B3 = flour lunatin extract, 50ºC extraction; A3B1 = MOLEF lunatin extract, 27ºC extraction;A3B2 = MOLEF lunatin extract, 40ºC extraction; A3B3 = MOLEF lunatin extract, 50ºC extraction)

C. Antioxidant Activity

The antioxidant activity of DPPH radicals (2,2-diphenyl-1-picrylhydrazyl) was estimated using the method of [13, 23]. The absorbance was calculated at a wavelength of 517 nm. The results corresponded to the antioxidant activity of the samples compared with the standard (60% ethanol) and ascorbic acid.

The antioxidant activity of lunatin extract as indicated by the percentage of DPPH inhibition is presented in Figure 4. Based on the results, each sample has a percentage of DPPH inhibition ranging from 45.97% to 83.58%. The process generated a significant effect on the percentage of DPPH inhibition, while the extraction temperature used in this study had no significant effect on the percentage of DPPH inhibition.

These results are in congruence with research by [1] which shows potentially serving as antioxidants in Lima bean can be reduced through as soaking, roasting and autoclaving. Meanwhile, concerning the effect of extraction temperature, lectins are found stable at temperatures up to 50ºC [5] so the extraction temperatures involved in this study (27ºC, 40ºC, and 50ºC) did not significantly affect the lectin content in the sample.

When compared with ascorbic acid, the crushed dried Lima beans were found to have higher antioxidant activity than ascorbic acid. The samples using Lima bean flour were characterized by the abilities parallel to ascorbic acid, and samples using MOLEF Lima bean had lower antioxidant activity than ascorbic acid. The test results were in line with the previous total protein test which showed that the total protein of Lima beans and Lima bean flour was higher than the total

protein of MOLEF Lima beans. This is because lunatin which has antioxidant activity in Lima beans constitutes a lectin classified as a carbohydrate binding protein [19], which means higher total protein results in higher antioxidant activity.

D. Antifungal Activity

The antifungal activity test in the present study followed the method in the study [19]. The results of fungal mycelia growth in the antifungal activity test can be seen in Figure 5 and Figure 6. The results obtained from the antifungal activity test against *Aspergillus niger* on the nine samples showed that the protein extract tested were not satisfactory to inhibit the growth of *Aspergillus niger*. This is indicated by the fertile growth of *Aspergillus niger mycelia* on almost the entire surface of Potato Dextrose Agar (PDA) in a petri dish, as portrayed in Figure 5.

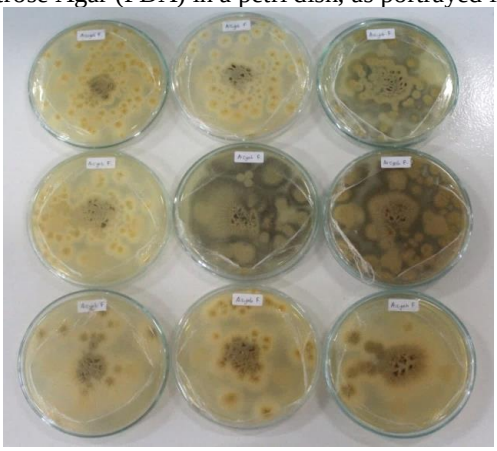
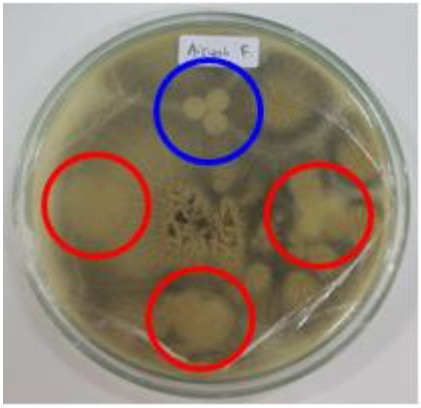


Figure 5. Antifungal activity of the nine samples towards *Aspergillus niger*

Previous research by [19] reported that lectins isolated from Lima beans were able to inhibit the growth of colonies of *Pythium aphanidermatum*, *Fusarium solani*, *Fusarium oxysporum*, and *Botrytis cinerea*. The different species of fungi used in this study may be associated with the different results. To that end, [19] point out that the ability of lectins to inhibit fungal growth may vary in relation to different fungal species [22].



(b)

Figure 6. Antifungal activity of sample A2B2 towards *Aspergillus niger*; blue circle: paper discs with control; red circle: paper discs with protein extract

Although no growth inhibitory activity of *Aspergillus niger* colonies was seen, in Figure 6 it is obvious that the disc paper containing the protein extract sample (red circle) shows a clear zone around the disc while the disc paper containing the buffer solution without the sample (blue circle) does not form a clear zone at all. This may indicate that the tested sample actually has antifungal activity, but it is insufficient to inhibit the growth of fungal colonies with *Aspergillus niger*.

IV. CONCLUSSION

The total protein in crushed dried Lima beans, Lima bean flour, and MOLEF Lima beans is 23.18%, 24.20%, and 17.12% respectively. Temperature variations do not result in any detectable differences in protein bands.

The difference in the extraction method affects the functional characteristics, but the temperature difference does not have a significant effect. The highest antioxidant activity is found in lunatin extract from dried Lima beans which are crushed and extracted at 50ºC (83.58%). By contrast, the lowest activity is identified in lunatin extract from MOLEF Lima beans extracted at 40ºC (45.97%). Lunatin extract from crushed dried Lima beans demonstrate the highest antioxidant activity.

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