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Food Properties of Lebui Bean Powder Extract Fermented by *Rhizopus* sp.

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Abstract

The fermentation that involves the performance of microbes is very effective for breaking the glycoside bonds that bind bioactive compounds in cells without using high temperatures and chemicals, as well as to improve the quality of nutritional components. Bioactive compounds in free form, obtained from the fermentation of black bean powder (Cajanus sp.) need to be extracted and characterized to obtain the types of bioactive compounds that have the potential to be applied in functional foods. Fermented black bean powder used in this experiment is the fermented lebui bean powder that has been fermented using the fungus *Rhizopus* sp. for two days. These lebui beans are local beans from Lombok Island, West Nusa Tenggara, Indonesia. This experiment aimed to determine the type and concentration of organic solvents, which can maintain the quality of nutritional contents and bioactive compounds of fermented lebui bean powder. The nested design was used. The main factor was the type of solvent (n-hexane or ethanol), while the solvent concentration (70% or 90%) was the second factor and nested in the main factor. The result showed that ethanol 90% was the best organic solvent to extract the bioactive compound in fermented lebui bean powder, and this extract contained protein, lipid, moisture, ash, fiber, and carbohydrates respectively 26.33%; 13.60%; 14.24%; 3.28%; 16.03%; and 26.54%. The bioactive compounds identified were phenolic, flavonoid, and anthocyanin, respectively 78.544 mgGAE/g; 217.358 mgQE/g; and 147.665 ppm.

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Keywords

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Introduction

The flavonoid, dietary fiber, terpenoid, alkaloid, phenolic saponin, include derivate bioactivesubstances belong to terpenoid and fatty acid compounds thoroughly contained in *Leguminosae*.[1,2,34] One of the *Leguminosae* that present a lot of bioactive properties isLebui Beans (Cajanussp.) native to Lombok Island, Indonesia.[5] Nevertheless, the potential usability of bioactive compounds in Lebui Beans still has been rarely

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reported. Lebui beans have some restrictions factor caused by a hard shell which may lead to low digestibility, long period of processing, and also generate impure bioactive-glycoside substances. Appropriate processing isa prerequisite to produce optimal purity of bioactive properties inefficient pathway.

Fermentation form in vitro processed which could release any bonding to be single compounds by microorganism.[6,7] Recently, *Rhizopussp* or *Saccharomycessp*.are effective as a fermentation agent to relieve bioactive substances from glycoside bonds for 2-3 days in dark conditions. Submerging Lebui Beans thoroughly reduce the shape to be powder in the previous step evident promote the fermentation result.[7,8] The agents would release enzymes as well as amylases, proteases, hemicelluloses, or phytases that could eliminate glycoside bonds sine high temperature or long periods either, intensify of bioavailability compound and more as in vitro could increase phenolic activity in cells.[9] Limitations of bioactive substances generate by fermentation are sometimes sensitive in pH, high temperature, and environment alteration, highly oxidized,and may affect damage. So, they do not have a much longer time to use after produced. An alternative way is continued further processing with offer convenient application or as functional-food resources.[10, 11]

The advanced process would involve the extraction of bioactive substances to become optimal. Optimal antioxidant activity, the stability of bioactive substances, and the availability of nutritional compounds would be achieved while organic solvent was applied in the extraction process. The appropriate solvent may also eliminate the limitation of bioactive extraction in Lebui beans in powder form. Several extraction methodshave been applied, as diverse as applying chemist solvent, pressure and chemist solvent combination, or utilize microwave, ultrasound, and supercritical method.[12] This experiment aimed to determine the type and concentration of organic solvents, which can maintain the quality of nutritional contents and bioactive compounds of fermented lebui bean powder. As mentioned before that shell characteristic of Lebui beans is too hard to process, meanwhile it contains rich bioactive. A refined method is needed to optimize the yield of bioactive compounds from Lebui Beans, by applying the fermentation process and selecting the right solvent for extraction.

Materials and Methods

Experimental Design

Nested design with the type of solvent as the main factor, whilst ethanol concentration as the second factor which is lodged in the main factor was bringing in this experiment. Nested design is one of the experimental designs that could determine the best result in different types of samples. This study applied ethanol and n-hexane (proanalyst) as solvents. Moreover, the concentrations of ethanol hexane respectively 70% and 90%. A combination of each treatment was repeated twice. The data were analyzed by Analysis of Variance (ANOVA) nested design and the best combination evaluate by the Effectiveness Index method.

Pre-Treatment Procedure and Fermentation

At the first step of this experiment, the beans were collected from Lombok, West Nusa Tenggara, Indonesia. These lebui beans were collected and dried by sun drying for 2-3 days. This experiment used the beans with0.5-0.8 cm diameter, then sorted, and cleaned by running water. Let its air-dried then finely grounded by Grinder (Phillips Grinder, HR2223 2 L) to be powder and sifted by 60 mesh size of Sieve BSS. Lebui bean powdered was heated at 40^{o}C for 5 minutes, then mixed thoroughly with distilled water 125 ml/100 grams. *Rhizopus* sp. culture with biomass in approximately 2% was added on lebui beans powder and incubated for 2 days at room temperature and in the dark. Lebui beans powder fermented result was taken and fore dried at 40^{o}C for 5 hours using an oven (Memmerth Oven 10L), henceforth terminated by grounded and sifted by a 60 mesh size11.

Extraction Procedure

The first step is made the fermented lebui beans to be powder, then extracted by maceration-percolation combination method.[6] This method was conducted with a stirrer in the whole process. Extraction of fermented lebui beans powder was carried out four times using ethanol and n-hexane (proanalyst) as solvents with 70% and 90% respectively (ratio 1:8 (w/v)), at 25^{o}C for 24 hours. Finally, the extracts were purified by rotary evaporator vacuum RE300 and the shaker Barnstead SHKE2000. Purification in the rotary evaporator was used to obtain extracts

that more concentrated and free from solvents. The temperature used in the rotary evaporator was 40°C and the solvent was removed by blowing with nitrogen gas.

Results

Table 1: Nutritional content of lebui bean, lebui bean powder, fermented lebui bean powder, and extract of fermented lebui bean powder

Sample	Nutritional Content					
	Protein	**Lipid**	**Moisture**	**Ash**	**Carbohydrate**	**Fiber**
Lebui bean[5] (% d.b.)	18.49	0.97	8.79	3.37	61.96	7.88
Lebui bean powder (soaking-pre treatment) (% d.b.)	18.53	0.90	8.21	3.45	68.91	7.89
Lebui bean powder (non-soaking-pre treatment) (% d.b.)	18.50	0.88	8.18	3.35	69.02	7.93
Fermented lebui bean powder[6] (% d.b.) (*Rhizopussp.*, soaking-pre treatment) (% d.b.)	22.70	0.50	6.80	3.20	66.80	8.10
Fermented lebui bean powder (*Rhizopussp.*, non -soaking-pre treatment) (% d.b.)	22.39	0.41	6.73	3.18	67.29	8.23
Extract of fermented lebui bean powder[6] (*Rhizopussp.*, soaking-pre treatment) (% w.b.)						
• n-hexane 70%	3.31	53.14	1.50	24.51	16.28	1.27
• n-hexane 90%	3.30	53.28	0.60	24.52	17.46	0.85
• etanol 70%	19.28	12.49	13.49	3.37	33.50	17.88
• etanol 90%	19.37	12.53	13.50	3.38	33.60	17.95
Extract of fermented lebui bean powder (*Rhizopussp.*, non-soaking-pre treatment) (% w.b.)						
• n-hexane 70%	23.36a	53.29a	1.28b	4.54a	16.28a	1.27b
• n-hexane 90%	23.28a	53.24a	0.70a	4.53a	17.28b	0.9 a
• average of n-hexane fraction	23.32a	53.26b	0.99a	4.53b	16.78a	1.12a
• etanol 70%	25.60a	13.59a	15.46b	3.27a	26.33a	15.77a
• etanol 90%	26.33b	13.60a	14.24a	3.28a	26.54b	16.0 b
• average of etanol fraction	25.96b	13.59a	14.85b	3.27a	26.43b	15.89b

Different letters in the same column showed significance ($P < 0.05$)

Results

Nutritional and Bioactive Content Analysis

Nutritional content as well as protein, ash, water, fat, carbohydrates, and total fiber, whereas total phenolic and total flavonoids were based on AOAC method[13] in Airlangga University Testing Laboratory (ULP) (2015).

Discussion

Nutritional Content

The analysis showed that in each parameter observed nutrient levels including protein, fat, water, ash, carbohydrates, and fiber showed a significant difference between n-hexane and ethanol distinguish in highest average in ethanol fraction as shown in Table 1.

Involving microbial proteolytic enzymes in fermentation can hydrolyze macro-protein molecules into amino acids and their peptides to then become the smallest unit, namely amino nitrogen so that the total nitrogen produced during the fermentation process is extracted, and shown higher protein content form than Lebui Bean without fermentation. Mostly, protein content by ethanol fraction extract was raised higher than the hexane. As known, the solubility of a protein in a particular solvent caused by open-close of protein bonds, while the fold is open it will increase α-helical structure that can contact with ethanol and this structure will be stable in a solution containing ethanol. Protein stability in ethanol solutions is determined by certain groups of peptides which are more polar naturally.[14,15]

Different results appear fat content, which tends to decrease, caused by the activity of lipolytic enzymes produced by microbes during fermentation to support their growth.[14] The fat content is quite low and does not change much due to the process, reflecting the chemical characteristics of the fat owned by the beans from Pulau Lombok, Nusa Tenggara Barat, and continually that for the process of making lebui bean powder does not affect its fat content. Lebui beans throughout their extracts have lower crudefat than Pigeon pea which ranges from 9.8-13% w/w.[15]

The characteristic and polarity level of the n-hexane solvent made the fat or fatty acids in extracted Lebui Beans fermented-powder higher than the ethanol solvent. Polar solvents would dissolve the fats or other compounds in non-polar solvents. The average fat content in the Pigoan peas classified as *Leguminosae* is 9.8 – 13% and this level is still lower when compared to the fat content in the Lebui bean extract.[16] However, its information is still rarely reported and needs to be further investigated regarding the composition of fatty acids contained in the n-hexane fraction.

The process may affect in ash content of material on different levels.[17] It appears during the extraction step of lebui beans. However, the ash content of lebui beans and extracts obtained with ethanol solvents are within middle limits as raw materials in food processing, reminders that the ash content of lebui beans is still lower than 3.5%. Leguminous Seeds. The highest contribution of α-linolenic acid (ALA, 18:3, n-3) in total fatty acids was noted in the lentil (13.8 in 100 g^{-1} fat), common bean (11.9 in 100 g^{-1} fat), and pea seeds (10.4 in 100 g^{-1} fat). Carbohydrate levels have also exsiccated, this occurs in all the fermentation of various seeds and beans during the 24 hour fermentation period. The decrease in total carbohydrates is triggered by an increase in the number of microbes that secrete the amylase enzyme18-23 Carbohydrate levels in the study were also determined by the percentage of non-carbohydrate components, where the carbohydrate content was calculated based on the total carbohydrate by difference method. This method calculates carbohydrate content based on the results of a 100% reduction with a total component of ingredients other than carbohydrate, namely the percentage w, ash, fat, and protein. In case the percentage of the total components of non-carbohydrate ingredients escalates, precisely for the percentage of carbohydrates will degenerate caused by microbes utilization. The fermentation process also results in the breakdown of the glycoside bonds that exist in the cell wall, so that it will increase the levels of fermented powdered fibers and bean extracts. An increase of solvent concentration, in this case, is most influential in increasing carbohydrate content in the results of the lebui bean extract. This tendency shows that the increasing concentration of the solvent will increase the ability of the solvent to attract or dissolve a component when the polarity is the same. An extract obtained from extraction using polar solvents such as ethanol and water will contain more polar and non-phenol components including carbohydrates and terpenoids in higher concentrations when compared to methanol and acetonesolvents. The fiber content in lebui beans is classified as well as higher than the type of kidney bean (*Phaseolus vulgaris* L.) within an average of 5.5-6.1% w / w.[24]

Total Flavonoids

Based on Table 2, it is seen that the total flavonoids in successive fraction factors for n-hexane and ethanol show significant differences. Flavonoid levels in extraction using ethanol fraction had higher concentrations than using n-hexane fraction, both with and without immersion treatment for the pre-fermentation stage.[5,7] The fermentation process is proven to be able to increase the levels of flavonoids compared to raw materials without treatment. During fermentation, microbes produce various types of enzymes to hydrolyze complex molecules of materials used to support their growth. Enzymes resulting from microbial secretion during fermentation take place including the α-glucosidase enzyme which plays a role in hydrolyzing glycoside bonds that bind to bioactive compounds in cells,[25] as well as proteolytic enzymes that play a role in the hydrolysis of phenolic complex compounds to free form and easily soluble aglycones in polar solvents (polar solvents).[26]

Total Phenolics

Mold microorganisms can produce several types of enzymes which are higher effectiveness in breaking down bonds of bioactive compounds when compared to microbial types during fermentation. The results of previous studies indicate that fermentation using *Rhizopus* sp. can produce lebui bean powder and

extract containing higher phenolic and flavonoid concentrations compared to fermentation using bacteria.[5,7]

High phenolic concentration in this study shows that fermentation is more effective compared with Lebui beans without fermentation[5] because it functions in breaking down cell walls and outlining the glycoside bonds that bind bioactive compounds in lebui beans including the phenolic component.[5,7,27] Total phenolic content in the extract has a higher value compared to some peanutsor beans extracts from the *Leguminosae*, including Ox-eye bean (*Mucunagigantea* W) extract with levels of 148 ± 12.8 mgEAG/g (d.b.) and 2.406 ± 0.055- 3.21 ± 0.07 mgEAG/g contained in 80/20 (v/v) methanol/water fraction extract.[28] Differences in extraction methods, differences in species and varieties, genotypes, geographical conditions, as well as the type of solvent and conditions at extraction may affect the differences in phenolic concentrations.[29,30]

Table 2: Total flavonoids, total phenolics, and anthocyanins levels of fermented lebui bean powder and extracts

Sample	Total flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Anthocyanin levels (ppm)
Lebui bean (d.b.)	104.348	30.580	152.370
Non-fermented lebui bean powder (d.b.)	102.910	30.501	150.270
Fermented lebui bean powder (*Rhizopussp*., soaking-pre treatment) (d.b.)	163.537	53.266	134.70
Fermented lebui bean powder (*Rhizopussp*., non-soaking-pre treatment) (d.b.)	175.460	57.150	144.520
Extract of non-fermented lebui bean powder (d.b.)			
n-hexane 70%	2.450	0.829	1.9064
n-hexane 90%	2.230	0.755	0.0017
etanol 70%	162.960	55.186	126.800
etanol 90%	168.610	57.100	131.200
Extract of fermented lebui bean powder (*Rhizopussp*., soaking-pre treatment) (d.b.)			
n-hexane 70%	4.5147	0.0841	0.120
n-hexane 90%	1.9273	0.0881	0.140
etanol 70%	313.2172	78.1760	77.230
etanol 90%	317.3583	78.5443	77.670
Extract of fermented lebui bean powder (*Rhizopussp*., non-soaking-pre treatment) (d.b.)			
n-hexane 70%	4.515 a	2.042a	1.115a
n-hexane 90%	4.577a	2.088b	1.135a
Average of n-hexane fraction	4.546a	2.065a	1.125 a
etanol 70%	213.217a	78.176a	147.225a
etanol 90%	217.358a	78.544b	147.665b
Average of etanol fraction	215.288b	78.360b	147.665b

Different letters in the same column showed significance ($P < 0.05$)

Anthocyanin Levels

The highest anthocyanin levels were obtained from extracts using an ethanol fraction of 147,445 ppm while the lowest value was produced in the n-hexane fraction of 1,125 ppm. Analysis of the test of the effect of solvent fractions (n-hexane and ethanol) with a concentration factor (70% and 90%) nested in the fraction of the anthocyanin level was carried out by the nested ANOVA method. There is a very significant average difference between

treatments used for anthocyanin levels measured with significance values less than α (0.05 and 0.01). Further test results, the fraction factor showed a significant difference between the fraction of n-hexane and ethanol with the highest average in the n-hexane fraction (Table 2). The n-hexane fraction showed no significant difference between 70% and 90% concentrations, while the ethanol fraction showed a significant difference between 70% and 90% concentrations with an average higher at 90% concentration.

High levels of anthocyanin in ethanol fraction extract, when compared with n-hexane fraction extract, may be caused by polar properties that require anthocyanin composition.[5] In previous studies mentioned that anthocyanins are a group of secondary metabolites that support the formation of color components and are easily soluble in water, and their concentrations can be more distinguished in each type of plant that depends on growth and biosynthetic processes. Anthocyanin levels of lebui beans and lebui beans powder that has been fermented and their extracts at fractions have a much higher value than the initial content of the raw material so that it can be used for the extraction process according to the right conditions and would not ruin the anthocyanin in the extract. Application of temperature <40°C in the extraction, storage of extracts in closed and dark glassware are an important variable to produce extracts with anthocyanin levels which cannot increase the revenue from raw materials.[31,32]

Conclusion

The highest extract yield carried out by 90% ethanol solvent given protein, lipid, moisture, ash, fiber, and carbohydrates respectively were 26.33%; 13.60%; 14.24%; 3.28%; 16.03%; and 26.54%. The bioactive compounds identified were phenolic, flavonoid, and anthocyanin, respectively 78.544 mgGAE/g; 217.358 mgQE/g; and 147.665 ppm.

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Conflict of Interest

The authors do not have any conflict of interest.

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