The effect of heat treatment on antioxidant activity of ethyl acetate extracts Arbila beans from Kapan, East Nusa Tenggara

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Abstract. Oxidative stress is a condition of imbalance between free radicals and antioxidants in the body. This condition is dangerous because free radicals easily react with biomolecules such as DNA and protein, causing damage. This oxidative stress can be prevented by intake of antioxidants in food or supplements. Arbila beans (Phaseolus lunatus L.) is a bean species that grows in East Timor and had been utilized by the local community from a long time ago. This research was aimed to analyze the antioxidant activity of Arbila bean ethyl acetate extract. The results of the research showed that antioxidants activity, which was expressed in IC₅₀ value, was detected on the ethyl acetate extract of Arbila bean which is not cooked or cooked. The IC₅₀ value of dry extract (LKE) and cooked extract (LRE) were 197.61 µg/mL and 207.93 µg/mL. Based on qualitative with HPLC, dry extract has 10 compounds and after given heat treatment is reduced to 6 compunds.

1. Introduction
Antioxidant is a chemical that is used to prevent oxidation by protecting cell components by neutralizing the damage effects of free radical [1]. Free radicals greatly affect regulation in the body, especially in cell, which causes imbalance in the control of signal transduction and gene expression [2]. One alternative of the problems caused by free radicals is natural antioxidant sourced from nature such as plants. Some plants are also known to have activity as safe antioxidants [3].

Arbila bean (Phaseolus lunatus Linn), found in Timor island, is a bean which grows wild and has a carbohydrate content of 201.70 mg/g and protein 68.40 mg/g [30] so as local people make it as an alternative food resource. Several studies have shown that Arbila beans have benefits as antidiabetic [4], antifungal and anti-proliferative [5], antimicrobial [6], cysteine proteinase inhibitors [7], hypercholesterolemia [8], antioxidants [9] and trypsin and chymotripsin inhibitors [10].

DPPH method is an easy antioxidant test that is used to analyze quantitative antioxidant activity. The mechanism of action of DPPH is that DPPH free radicals reacted with the appropriate reduction of the agent and produced new bonds, so that it can caused change the color of the solution. The color of the solution changes to indicate the electron has been taken by DPPH free radicals [11].

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Several studies have found that legume (Phaseolus) plants are known to have antioxidant benefits, such as Phaseolus vulgaris plants [12-17] and Phaseolus radiatus L.[18]. The purpose of this study is to evaluate the potential of ethyl acetate extract as a natural antioxidant.

![Figure 1. Phaseolus lunatus Linn](image)

**2. Experimental**

**2.1. Materials**

Dry beans (Phaseolus lunatus L.) grow in Kapan, East Timor dan harvested in August 2018.

**2.2. Sample preparation**

300 g Arbila beans cooked in 1.5 liter of distilled water (in 100°C, 3 hours) and dried in oven with temperature 55°C during 12 hours (cooked treatment). 300 g Arbila beans was dried in oven during 48 hours with temperature 40°C (dry treatment). Every treatment dry treatment and cooked treatment were powdered using blender and keep in box.

**2.3. Extractions**

Arbila beans extractions dry extract (LKE) and cooked extract (LRE) was using ethyl acetate as eluent. LKE and LRE (100g) were macerated in ethyl acetate (1:5 w/v) separately during 48 hours by mixing three times in room temperature. The filtrate was filtered using filter paper and evaporated with rotary evaporator at temperature 40°C until thick. Extract LKE and LRE first test DPPH by comparison the color in crude extract that has been reacted with solution of DPPH. The indicator color of it was purple solid showed the absence of the reaction on scavenging of free radicals by crude extract. Indicator color yellow whitish showed indicates the presence of the reaction of scavenging of free radicals.

**2.4. Determination of phenolic and flavonoid**

Determination of total phenolic and total flavonoid content refers to Li [19] and Kiyani-Sam [20]. Determination of total phenolic made by taking 0.5 mL of extract LKE and LRE then dissolved in 7.5 mL of distilled water and then added with 0.5 mL of Folin-Ciocalteu reagent 10% (v/v). After 1 minute added 1.5 mL of sodium carbonate 20% (w/v). The solution was shaken and incubated in a dark room (25°C during 2 hours). The absorbance of the sample was measured on a spectrophotometer UV-Vis Double Beam Shimadzu 1601 with a wavelength of 750 nm. Gallic acid (1-19 µg/mL) was used as a standard. The result was expressed together with Gallic acid (mgGAE/g dry weight). Determination of total flavonoids was determined based on the formation of flavonoid-aluminum. 1 mL samples of LKE and LRE are mixed with 3 mL of methanol and 0.2 mL of a solution of aluminum chloride 10% (w/v). Then added with 0.2 mL of 1M potassium acetate and 5.6 mL of distilled water then incubated for 30 min at room temperature. The samples was measured the length absorbance at 415 nm using UV spectrophotometer. The standards used for the calibration curve is Rutin (2.5-20 µg/mL). Total flavonoid was the same with the Rutin (RE mg/g dry weight).
2.5. DPPH antioxidant test
Antioxidant test using DPPH as method to see radical scavenging ability from sample [21-22]. 200 mL of sample LKE and LRE were mixed to 3 mL of DPPH 0.1 mM and incubated for 30 minutes at room temperature in the dark. The samples were measured absorbance at 517 nm. All treatment were repeated three times. Radical scavenging activity was calculated by using formula = % Inhibition = (Abs DPPH-Abs sampel )/(Abs DPPH) x 100%. Radical scavenging activity by sample using DPPH reagent was indicated by change of the color from dark purple to yellow white which is measured the absorbance and compared with control that was methanol.

![Figure 2. Seed core of Phaseolus lunatus Linn](image)

3. Result and Discussion

3.1. Total Phenolic and total flavonoid

Total phenolic and flavonoid

Arbila bean seeds used are whole seeds consisting of seed coat and seed core. The content of phytochemical compounds is more contained in the seed coat. In general, beans with a seed coat that has a bright color show lower phenol levels compared to black beans [23]. In this study, Arbila beans have a deep black seed coat. The total phenolic content that is contained in the ethyl acetate extract with raw (LKE) and cooked (LRE) treatments ranged from 118.46 mg GAE/gr to 169.44 mg GAE/gr with gallic acid as standard (Table 1). The results of this study are the same as the results of research conducted by Silva [24] The content of phenolic compounds after cooking is reduced by 50.98 mg GAE/gr. The highest concentration is in the LKE sample. Although several studies such as Huber [25] prove that treatment cooked with maceration increases antioxidant activity with high concentrations of kaemferol and catechin. Anna [26] also proved that there was an increase in total phenolic after heat treatment. The results of research conducted by [15] stated that total phenolic experienced a significant increase when given heat treatment and given additional NaHCO₃. This contrasts with Granito [27] saying that processing legumes such as cooking might increase the degradation between the aromatic rings of phenolic compounds which can be structurally damaging and cause a phenolic decrease than the cooked beans. Huber [25] also added that providing cooked treatment can cause the loss of certain bioactive molecules. The results of research conducted by Huber [25] also prove that flavonoid levels are higher when cooked, but the results of this study decreased total flavonoids. Some studies show a decrease in total phenolic if given heat treatment [29-30]. Processing a food by providing heat can provide significant changes to the chemical compounds contained either increasing or decreasing in composition [29]. When cooking for longer can cause the diffusion of phenols from the seed coat to the cooking water even though it can provide activities similar to extracts without thermal treatment even though the phenol content is 80% lower [30]. This also happened in this study, namely the
decrease in total phenolic when given heat treatment (cooked). Flavonoids are a group of compounds that give fruit and vegetables flavor and color. The flavonoid content in Arbila beans after boiling is reduced, because when given heat (cooked) some structures become degradation and have decomposition of thermolabile and are left behind in the solvent used to boil. Flavonoid compounds are included in one of the most compound groups in the phenol compound group. Warming can cause cell wall adhesion so that it can induce the release of phenolic compounds such as flavonoids that accumulate in the vacoule [15]. Flavonoids are mostly located in the seed coat, non-flavonoid phenolic compounds such as hydroxybenzoate and hidrosikanat located in cotyledons. Hydrosisinamic derivatives form the main phenolic component of peanuts. The longer the heating time with higher temperatures will cause the antioxidant compounds to weaken. Warming at a temperature of 100°C with a time of 3 hours can be a cause of a decrease in total flavonoids contained in LKE samples.

| Table 1. Total phenolic content, total flavonoid content and antioxidant activity (IC_{50} value) |
|-------------------------------------------------|-------------------------------------------------|---------------------------------|-------------------------------|
| Total phenolic (mg GAE/gr) | Total flavonoid (mg RE/gr) | DPPH Antioxidant activity (IC_{50} value) |
|--------------------------|--------------------------|---------------------------------|-------------------------------|
| LKE                      | 169.44 ± 5.43            | 33.27 ± 3.10                    | 197.61 µg/mL                  |
| LRE                      | 118.46 ± 5.43            | 29.70 ± 3.10                    | 207.93 µg/mL                  |

Each value represents the mean value ± SD; GAE = Gallic Acid Equivalent; RE = Routine equivalent. LKE was dry extract; LRE was cooked extract

3.2. The DPPH antioxidant activity
The DPPH method is an antioxidant method that is often used. LKE and LRE samples showed antioxidant activity with the appearance of an antidote to DPPH free radicals. Methanol was blank to measured absorbance and abs DPPH that was measured is 0.945. The value of free radical inhibitory concentration was calculated by the IC_{50} value. Antioxidants detected by the DPPH method is classified according to Molyneaux [31] that LKE is classified as a weak antioxidant, and LRE is classified as a very weak antioxidant. Molyneaux classifies antioxidants in the level of strong and weak inhibit free radicals that are <50µg/mL (very strong), 50-100µg/mL (strong), 100-150µg/mL (moderate),150-200µg/mL (weak) and >200µg/mL (very weak). Some studies also show that heat processing can increase antioxidant capacity [34], and at temperatures above 120°C the antioxidant activity decreases significantly [30]. The appeal of BHT is used as a synthetic antioxidant. IC_{50} of BHT value is 166.99 µg/mL which is included in the group of weak antioxidants. On heating the sample at a temperature of 70°C-90°C causes the degradation of some stronger antioxidant compound that the difference in antioxidant activity is higher than without heat processing. Thermal treatment can affect permanent changes in nutrition depending on the level of the process and is able to change the total antioxidant capacity significantly higher than the total phenol raw seeds, DPPH free radical antioxidant activity [32]. The relationship between total phenolic and total flavonoids is directly proportional to the antioxidant activity of IC_{50} value. From this research, there is antioxidant bioactivity even though the range is weak or even very weak. This is due to the selection of the sample preparation that will be used should only be part of the seed coat only, not with the seed core. Akond [12] said that arbila beans had a carbohydrate content of 201.70 mg/g and protein 68.40 mg/g, so it could be concluded that the antioxidant activity was low because almost 70% of the extracted seeds were part of the seed core. Dewanto [32] also explained that most of the phenols were in the skin of the seeds and only a few were located in the core of the seeds. This is also proven by research conducted by Anton [33] that Phaseolus vulgaris L. beans with a brighter skin color that (red) has high antioxidant activity and also a high total phenolic as well.
In the qualitative determination results by identifying the compounds present in the LKE extract and LRE were done by HPLC (High Performance Liquid Chromatography), in the LKE results there were 10 compounds identified, but after boiling (LRE) there were only 6 compounds indicated by the emergence of peaks at the time only certain retention. So it can be concluded that when given heat treatment is able to eliminate some of the compounds that previously existed (without treatment) or the missing compounds was thermolable compounds.

4. Conclusion
This study concludes that the antioxidant activity of ethyl acetate extract especially LK (dry extract) without given heat treatment has the potential as a natural antioxidant even though with a group of weak antioxidant activity.

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