

EFFECT OF VARIATIONS OF SOLVENT CONCENTRATION TO ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF BUAS-BUAS STEM (*Premna serratifolia* L.) USING DPPH (2,2-diphenyl-1 picrylhidrazyl) SCAVENGING METHOD

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Abstract. Buas-buas plant (*Premna serratifolia* L.) was one of the plants found in Indonesia and was thought to have antioxidant compounds. Buas-buas leaves were reported to contain compounds as antioxidants, in addition to leaves, stems and roots also have compounds as antioxidants. This study aims to determine the effect of ethanol concentration in the ethanolic extract of buas-buas stem as antioxidants by DPPH scavenging method. Ethanol was used as solvent with concentration of 70%, 80%, 90%, and 96%. The extraction was done by soxhlet extraction method, where the extract obtained was determined for the total phenolic content by using Folin-Ciocalteu method, and antioxidant activity by DPPH scavenging method. The results showed that the highest total phenolic content of 90% ethanol extract was 56.4% equivalent of gallic acid (EAG). Based on the % inhibition value analyzed by using SPSS 21 with Moses Extreme Reactions test obtained the p value <0.05, where the concentration of solvent can affect the antioxidant activity of ethanolic extract of buas-buas stem. While the antioxidant activity that has the highest IC₅₀ value was shown by 80% ethanol extract with a value of 63.93 mg / L.

Keywords : *Premna serratifolia* L. Stem, Antioxidant, DPPH

1. Introduction

Free radicals are atoms or molecules that are not stable. It has one or more unpaired electrons so to become stable, it will tend to take electrons from other molecules which then produce abnormal compounds and started a chain reaction that can damage the tissue[1]. Free radicals that enter the body can be from cigarette smoke, air pollution including lead from combustion of car engines, environmental pollutants, pesticides, medicines, and processed foods containing many preservatives[2].

Antioxidants are produced by the body to protect the body from radical compounds by providing electrons (electron donors) or reductants. This antioxidant compound that has a small molecular weights, has the ability to release hydrogen atoms and decrease radical reactivity[3]. Our body can produce antioxidants, but only in small amounts while free radicals were produced

every day, causing these natural antioxidants to be unable to compete with free radicals.

Extracts of medicinal plants made from simplicia can be used as starting materials, intermediates or finished product ingredients. For that the extracts made must meet the quality standards, starting from raw materials, processes until product testing. Several factors affecting the quality of extract are chemical factors such as type and amount of chemical compound, extraction method and solvent used[4].

According to research by Rajendran et al., the IC₅₀ value of the extract from stem and bark of stem of buas-buas plant using 95% ethanol solvent was 203 µg / ml by DPPH scavenging method[5]. Meanwhile, according to Muthukumaran et al. , the IC₅₀ value of the extract from skinless stem of buas-buas plants using 90% ethanol as solvent was 155 µg / ml which was smaller than previous research[6]. Differences of IC₅₀ values that

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occur from the results of the above research due to the variation of ethanol solvent concentration used. So it can be concluded that the lower the concentration of solvent used in the extraction process, The better the antioxidant inhibition ability will be.

Based on the above explanation, the aim of this research was to find out whether the concentration of solvent for extraction will affect the antioxidant activity of ethanolic extract of Buas-buas stem.

2. Methodology

The experimental research was conducted in Pharmaceutical Biology and Pharmaceutical Analysis Laboratory, Medical Faculty of Tanjungpura University.

2.1. Equipments and materials

Equipments used are a set of UV-Vis spectrometer (Shimadzu®), oven (Mettler®), waterbath (Mettler®), analytical scales (Precisa®), desiccator, glassware, test tube, tube rack, micro pipette, TLC plate, 254 nm and 366 nm UV lamp, crucible, TLC chamber, spray bottle, soxhlet tool set, evaporator set, pycnometer, micropipette, dropper pipette, stirrer bar, stainless spoon, blender, filter paper, vial.

The materials used are stem (*Premna serratifolia* Linn.), 2,2-diphenyl-1-picrylhydrazyl (DPPH) pa (Merck®), vitamin C (Kimia Farma®), methanol pa (Merck®), chloroform pa (Merck®), n-hexane pa (Merck®), 70%, 80%, 90%, and 96% ethanol (Merck®), magnesium powder, 2 N HCl solution, 1% FeCl₃ solution, 5% NaCl solution, gelatin salt, Lieberman-Burchard reagent, Dragendroff reagent, Mayer reagent, aquadest, Aluminium foil, filter paper, 60 F₂₅₄ silica gel TLC plate (Merck®), gallic acid.

2.2. Place and time of research

This research was conducted in Laboratory of Pharmaceutical Biology Faculty of Medicine and Chemical Laboratory at Faculty of Mathematics and Natural Sciences of Tanjungpura University for 6 (six) months.

3. Research Design

3.1. Plant Determination

Determination of buas-buas plants was done in the Biology Laboratory Faculty of Mathematics and Natural Sciences University of Tanjungpura by using whole plant sample from roots, stems, and leaves.

3.2. Collecting of samples

The samples used were buas-buas plants (*Premna serratifolia* L.) that was suspected of having antioxidant activity. The stem of buas-buas plants (*Premna serratifolia* L.) was collected from Desa Sungai Raya Dalam, Kabupaten Kubu Raya, West Borneo Province.

3.3. Simplicia Processing

There are several steps of simplicia processing such as wet sorting, washing, deforming, drying and dry sorting.

3.4. Preparation of Ethanolic Extract of Buas-buas stem

A total of 645 grams of simplicia were divided into four parts and was extracted using 70%, 80%, 90%, and 96% ethanol respectively by soxhlet extraction method. Then the extract obtained was further concentrated using a rotary evaporator to obtain a thick and viscous ethanolic extract of buas-buas stem.

3.5. Nonspecific Parameter assay

3.5.1. Lost of Drying Assay

Weighed 1 gram of extract and put into a bottle that has been preheated at 105°C for 30 minutes and has been weighted. The sample was then put into the drying chamber while the bottle was opened. The drying process was done at 105°C until it reached constant weight. Before each drying process, let the bottle cools down to room temperature inside desicator[7].

3.5.2. Determination of Water Soluble Compound

One gram of extract was macerated with 1 ml of chloroform and 9 ml of water for 24 hours, using a measuring flask while repeatedly shaken for the first 6 hours, then was shaken again after 18 hours and then filtered. The 5-ml filtrate was evaporated in a shallow, flat-bottomed dish until the solvent evaporated and formed residue, then the residue was heated at 105°C until constant weight.

3.5.3. Determination of Ethanol Soluble Compound

A 1 g of extract was macerated with 25 mL of 96% ethanol for 24 hours using a clogged flask while repeatedly shaken for the first 6 hours. Then let stand for 18 hours and filtered quickly to avoid ethanol evaporation. The 5-mL filtrate was evaporated in a shallow, flat-bottomed dish that has been weight until the solvent evaporates and formed residue, the residue was heated at a temperature of 105°C until constant weight[8].

3.6. Specific Parameter assay

3.6.1. Phytochemical Screening

3.6.1.1. Screening of Alkaloid

The extract solution was added with 0.5-1 ml of 2N sulfuric acid and shaken until two layers were formed. Pipette the acid layer (top) and put it into three test tubes. The first test tube was added two drops of Mayer reagent. In the second test tube, add two drops of Dragendorff's reagent. In the third test tube added two drops of Wagner reagent. The presence of alkaloid compounds was characterized by the formation of white precipitate in the first test tube and the reddish-brown precipitate formation in the second and third test tubes[9].

3.6.1.2. Screening of Tannin

A few drops of 5% FeCl₃ solution was added to 1 ml of the extract solution. The color change to dark blue indicates the presence of tannins in the extract[9].

3.6.1.3. Screening of Flavonoid

A 2 ml extract solution was added with 0.5 ml of concentrated HCl and several milligrams of Mg metal powder. The presence of flavonoids was characterized by red, orange and green color formation depending on the flavonoid structure contained in the sample[9].

3.6.1.4. Screening of Steroid and Triterpenoid

A 1 ml extract solution was added 3 drops of anhydrous CH₃COOH and one drop of concentrated H₂SO₄ solution. The color turns red indicating the presence of terpenoid compound and the color turns blue indicating the presence of a steroid compound[9].

3.6.1.5. Screening of Saponin

Add 50 mg of the extract into the test tube, then add 20 ml of water and shake it firmly for 15 minutes. A positive result was shown by the formation of a foam with a height of 2 cm[9].

3.7. Determination of Total Phenolic Content

Determination of total phenolic content was done by using Folin Ciocalteu method. Standard solution was made by weighing 1 gram of gallic acid in 10 mL of methanol. Variation of concentration used was 800, 900, 1000, 1100, 1200, and 1300 ppm. Then 0.5 mL of Folin-Ciocalteu (1:10) reagent was added into 0.5 mL of standard gallic acid solution and was stirred until homogeneous. After 2 min, 2 mL of 7.5% sodium carbonate was added and aquadest was added until 10 mL volume after that the mixture was allowed to stand for 60 min. The absorbance was measured at a wavelength of 765 nm using UV-Vis spectroscopy. A

total of 50 mg of extract was dissolved with 50% methanol until 5 mL volume, treated equally with standard solution treatment above and then analyzed by UV-Vis spectroscopy[10].

3.8. TLC assay

TLC plate was cut with size 1 cm x 10 cm, with upper limit of 1 cm and lower limit 1cm. Capillary tube was used to drip samples onto the TLC plate. The TLC plate was then put into the chamber that has been filled with the mobile phase. After the stain reaches the upper limit, the plates are lifted and cooled[11].

The spots obtained were detected by spraying using particular reactant until it becomes apparent.

3.9. Antioxidant Activity assay

3.9.1. Qualitative assay

3.9.1.1. Preparation of 0.2 % DPPH solution

The DPPH crystals were weighed as much as 0.02 g then put into a 10 ml volumetric flask and methanol solvent was added. Precise 0.2% DPPH solution must be immediately used and maintained at low temperatures (in refrigerators) and protected from light[12].

3.9.1.2. Preliminary Antioxidant Activity assay by TLC

Preliminary antioxidant activity assay was performed according to a study conducted by Isnindar (2011) with slight modification using thin layer chromatography (TLC) sprayed with 0.2% DPPH solution in methanol solvent.⁽¹³⁾

TLC plate was heated at 105°C for 10 minutes before being used. The extract solution was plotted onto a 60 F₂₅₄ silica gel TLC plate using a capillary pipe, at a distance of approximately 1 cm from the bottom. Plotting process was done 2-3 times and left to dry. The plates were eluted inside a TLC eluting chamber by using an appropriate mobile phase. Then the TLC plate was observed in different UV wavelength ie UV 254 nm and UV 366 nm. After that the TLC plate was sprayed with a 0.2% DPPH solution. After 30 minutes of spraying, the spots will become pale yellow with a purple background[12].

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3.9.2. Quantitative assay

3.9.2.1. Preparation of 0.1 mM DPPH solution

The DPPH crystal weighed 0.985 mg and then put into a 25 ml volumetric flask and methanol solvent was added then homogenized. DPPH solution that has been made must be immediately used and must be kept protected from light[12].

3.9.2.2. Maximum Wavelength Optimization of Solution

Antioxidant activity assay of ethanolic extract was begin with determination of maximum wavelength (λ max) of DPPH 0,1 mM solution using UV-Vis spectrophotometry. The maximum wavelength of DPPH solution was scanned at a wavelength of 450-700 nm[14].

3.9.2.3. Making of stock solution

A total of 100 mg of test sample (ethanolic extract) was weighed, put into a 10 ml volumetric flask and methanol solvent was added until the etched line (concentration 10.000 μ g / ml). Then it was further diluted into several concentration[12].

3.9.2.4. Preparation of Vitamin C Solution

A total of 25 mg of vitamin C powder was weighed, put into a 25 ml volumetric flask and methanol solvent was added until the etched line (concentration of 1000 μ g / ml). Then a series of concentrations of 2 μ g / ml, 3 μ g / ml, 4 μ g / ml, 5 μ g / ml were prepared[12].

3.9.2.5. Radical Scavenging Measurement

Several variations of the concentration of the stock solution was pipetted and added into 2 ml DPPH solution. The mixture was further shaken and left for 30 minutes at room temperature in darkness. This solution was then measured for absorbance at a maximum wavelength (λ max) of 0.1 mM DPPH in methanol using a UV-Vis spectrophotometer. The absorbance of blank and vitamin C solution was also done by using UV-VIS spectrophotometer[12].

3.10. Analysis of Results

3.10.1. Preliminary Test of Antioxidant Activity by TLC

A compound can be said to have antioxidant activity if on a TLC plate that has been plotted with the test extract solution and has been eluted shows pale yellow spots with purple background.

3.10.2. Radical Scavenging Measurement Using UV-Vis Spectrophotometer

The IC₅₀ calculation was performed by linear regression line equation expressing the relationship between the concentration of the test compound (sample) (X) and the average radical scavenging activity (Y) of the replication series. The antioxidant activity of the extract was compared with vitamin C as a positive control. The smaller the value of the IC₅₀, the better the radical scavenging ability will be[15].

3.11. Data Analysis

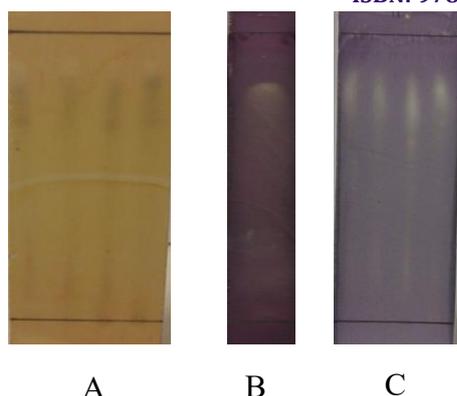
The data were analyzed by using SPSS 21 software. The data were first tested by the distribution and homogeneity of the variant ($P > 0.05$), if normally distributed and homogeneous variance, the next step was one-way ANOVA test with 95% confidence level ($P < 0.05$). If the result of the analysis shows a significant difference, post hoc test carried out. If the parametric test requirements are not met, then the analysis was performed by comparable nonparametric Kruskal-Wallis test, followed by Mann-Whitney test if there is a significant difference[16].

4. Results and Discussion

The result of the plant determination showed that the sample used was *Premna serratifolia* L. species and has a synonym of *Premna obtusifolia* R.Br. from the family Lamiaceae, and local name Buas-buas plant. The simplicia was extracted using four types of solvents, that is 70%, 80%, 90%, and 96% ethanol concentration.

The results of phytochemical screening showed the presence of tannin, flavonoid, saponin and phenol. The total phenolic content determination in the sample was performed using gallic acid as the standard solution. Based on these results, it can be concluded that the highest total phenolic content was extract using 90% ethanol concentration as solvent. There are several things that can affect the total phenolic content in plants, and one of them is the type of solvent. The type of solvent used has the biggest influence on the total amount of total phenol obtained.^(12, 13)

TLC assay was conducted to determine the content of phenol compounds contained in the extracts of buas-buas stem. The identification of the four samples showed the presence of phenolic compounds with the presence of black spots after spraying with FeCl₃ reagents. The mobile phase used was methanol and ethyl acetate (8:7) with silica gel 60 F₂₅₄ plate as stationary phase.⁽¹⁷⁾ The results showed that all four samples had the same pattern on separation of each sample on TLC plate and spraying result using DPPH 2 % reagent.



Description : mobile phase methanol : ethyl acetate (8:7), a) extract plate that sprayed using FeCl₃ 3% reagent, b) control galic acid plate sprayed using DPPH 2% reagent, c) extract plate sprayed using DPPH 2% reagent.

Fig 1. Results of TLC Assay

The antioxidant activity assay by DPPH scavenging method in the sample can be seen from the parameter of percentage of free radical inhibition (% inhibition) and based on IC₅₀ value. Value of the % inhibition was obtained by dividing the absorbance of the DPPH blank by the difference between sample absorbance and DPPH absorbance. The absorbance of DPPH blank solution was 0.37253.

Based on the results obtained it can be seen that the highest % inhibition was shown by samples with 80% ethanol concentration as solvent, followed by ethanol concentrations of 70%, 96%, and 90% respectively. This proves that the content of secondary metabolites that has antioxidant activity on ethanol extract 80% was higher than ethanol extract 70%, 90%, and 96%. The difference in secondary metabolite content in all four extracts was due to the difference in polarity between the four solvents used.

Table 1. Total Phenolic Content

| Sample groups | Phenol solvent volume | Extract volume | Dilution factor | Weight of sample | Total phenolic content (mg GAE/g) |
|---------------|-----------------------|----------------|-----------------|------------------|-----------------------------------|
| Ethanol 70% | 0.534 mg/ml | 0.5 ml | 10 | 0.05 g | 53.443 % |
| Ethanol 80% | 0.528 mg/ml | 0.5 ml | 10 | 0.05 g | 52.8 % |
| Ethanol 90% | 0.564 mg/ml | 0.5 ml | 10 | 0.05 g | 56.4 % |
| Ethanol 96% | 0.508 mg/ml | 0.5 ml | 10 | 0.05 g | 50.8 % |

IC₅₀ value can be used to show the level of antioxidant activity based on free radical inhibition by 50%. Extracts with 70%, 80%, 90%, and 96% ethanol concentrations

was categorized as active level of antioxidant activity while vitamin C was categorized as very active antioxidant activity.

Table 2. % inhibition of sample and Vitamin C

| % inhibition | Ethanol 70% extract | Ethanol 80% extract | Ethanol 90% extract | Ethanol 96% extract | Vitamin C (4 ppm) |
|--------------|---------------------|---------------------|---------------------|---------------------|-------------------|
| 1 | 39.2478% | 41.1779% | 32.7437% | 36.6762% | 34.1234% |
| 2 | 39.4438% | 41.2262% | 33.5167% | 36.9903% | 35.3904% |
| 3 | 39.5270% | 41.2396% | 33.4604% | 38.2358% | 32.4296% |
| Mean ± SD | 39.4062% ±0.1433 | 41.3114% ±0.1804 | 33.2403% ±0.4310 | 37.3007% ±0.8248 | 33.9811% ±1.4855 |

Based on the results obtained, there was a difference between the value of IC₅₀ with total phenolic content. The highest total phenolic content was found in extracts with 90% ethanol concentration, followed by ethanol concentrations of 70%, 80%, and 96%. While the lowest value of IC₅₀ or inhibition of free radical was shown by

extract with ethanol concentration 80% followed by ethanol concentration 90%, 70%, and 96%. Antioxidant activity was due to presence of phenolic compounds in the extract.

Examination using Moses Extreme Reactions method has been done by comparing the two groups of each sample, and obtained a significance value of 0.000. From these results it can be said that each sample has a significant difference, or in other words that the concentration of ethanol solvent used can affect the antioxidant activity.

Table 3. IC₅₀ value of sample and Vitamin C

| Sample | IC ₅₀ Value (mg/L) |
|-----------|-------------------------------|
| 70% | 74.3 |
| 80% | 63.93 |
| 90% | 70.43 |
| 96% | 77.01 |
| Vitamin C | 1.5976 |

Based on the results of the study, it was found that the highest % inhibition value in the buas-buas stem ethanol extract at 80% solvent concentration, in contrast to the highest total phenol content at 90% solvent concentration. Antioxidant activity is not affected only from phenol compounds but also from several other compounds contained in buas-buas stem extracts of ethanol in reducing free radicals. Other antioxidant compounds besides phenolics, such as vitamin C, vitamin E, beta carotene, and others. In a study by Matsushige et al. (2012) on soursop leaves where alkaloid compounds contained in soursop leaves have the ability as antioxidants. Alkaloid compounds such as the indol group have the ability to reduce free radicals, where radicals from amine derivatives compounds have long termination stage. Another compound such as flavonoid have antioxidant activity from the ability of these compounds to transfer an electron to free radical compounds.

5. Conclusion

Based on the analysis results of % inhibition test with SPSS 21 program, different concentrations of the ethanol can affect the antioxidant activity of ethanolic extracts of Buas-buas stem. The highest antioxidant activity was shown by ethanol extract of 80% with IC₅₀ value 63.93 mg / L, followed by ethanol extract 90%, 70%, and 96% with the respective values of 70.43 mg / L, 74.3 mg / L, and 77.01 mg / L, and can be categorized as active antioxidant level.

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