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Synergism of Antioxidant Activity Combination of Buas-Buas (*Premnaserratifolia* Linn.), Meniran (*Phyllanthusniruri* L.), Secang (*Caesalpiniasappan*) and Roselle (*Hibiscus sabdarifa*) Extracts

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ABSTRACT

Buas-buas, meniran, secang, and rosella have biological and pharmacological activities as antioxidants. The combination of the four plants is expected to provide a more potent synergistic effect on antioxidant activity. The purpose of this study was to analyze the total phenol content, total flavonoids, and antioxidant effects before and after combination. The combination of extracts, buas-buas, meniran, secang, and rosella which are used in sequence is (1: 1: 1/2: 1/2), (1: 2: 1/2: 1/2), and (2: 1 : 1/2: 1/2). Plants used in the form of simplicia was extracted by maceration method. Radical capture activity uses DPPH and IC_{50} values are determined. Determination of total phenol is expressed equivalent to gallic acid. Total flavonoids are expressed as quercetin equivalents. The phenol and flavonoid content obtained are then correlated with antiradical activity. The results showed that the best IC_{50} values were in the combination of ratios (1: 1: 1/2: 1/2) that is (11.0 µg / mL), then (1: 2: 1/2: 1/2) which was 13.3 µg / mL, and (2: 1: 1/2: 1/2) which is 19.4 µg / mL. The highest total phenolic and flavonoid content in the ratio (1: 2: 1/2: 1/2) is 33.57% w/w EAG and 74.00% w/w EQ. Correlation analysis between IC_{50} values with total phenolic and flavonoid contents showed a positive correlation with R^2 values of 0.8236 and 0.0102 with positive slope. Thus, it can be concluded that the total phenol content influences free radical scavenging activity by 82.36%, while the effect of total flavonoid content was only 1.02%.

Key words: antioxidant; DPPH; buas-buas; meniran; secang; rosella

INTRODUCTION

Utilization of medicinal plants that have natural antioxidant activity has been widely developed. Karthikeyan and Deepa (2011) reported that in India of buas-buas, used in Ayurveda as a carminative, galactagogic, flatulence, cough, fever, dyspepsia, colic, hemorrhoids, neuralgia, and tumor. In Indonesia and Malaysia, young leaves buas-buas are eaten as vegetables after boiling (de Kok, 2013). A mixture of leaves and roots to treat fever and difficulty breathing, in nursing women to increase milk production in Indonesia, besides that it also makes a fence because it is easily propagated through cuttings (de Kok, 2013). Malaysians, root plants that are boiled to treat stomach pain and diarrhea (Bramley *et al.,* 2011). Other research data indicate buas-buas has antioxidant activity (Isnindar *et al.,* 2016; Chua *et al.,* 2015; Aiswarya 2013; Ferreira *et al.,* 2007; Rodrigo and Bosco 2006). Unander *et al.,* (1993) Meniran is also used in the Ayurveda treatment system which is useful as a medicine for stomach, liver, kidney, spleen, antitussive, antispasmodic, bleeding, urinary monitoring, antiviral and gallstones. Other data indicate meniran have antioxidant activity (Mrinal *et al.,* 2010; Bagalkotkar *et al.,* 2007; Grewal *et al.,* 1984). Sari Ramdana and Suhartati (2016) state that secang water is a favorite drink for most people in South Sulawesi, including the Bugis-Soppeng tribe, in addition to that other data also shows secang wood has antioxidant content (Harjit *et al.,* 2016). Sipahli (2016) describes the anthocyanin content in roselle which regulates natural dyes that give red, orange, blue, and purple colors, other data roselle has antioxidant activity (Inggrid *et al.,* 2018, Awwalina *et al.,* 2016). Search results, literature studies, other articles about plants that have antioxidant activity, contain active phytochemicals, such as polyphenols or flavonoids.

Buas-buas active phytochemicals namely phenols, flavonoids (Ferreira *et al.,* 2007; Rodrigo and Bosco 2006), meniran namely flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarin and saponins (Bagalkotkar *et al.,* 2007; Calixto *et al.,* 1998; Meixa *et al.,* 1995). Roselle, namely anthocyanin, flavonoids, polyphenols and ascorbic acid (Mardiah *et al.,* 2015; Sarbini, 2007). Secang wood, namely phenols, flavonoids and terpenoids (Harjit *et al.,* 2016; Sari and Suhartati, 2016). To prove the results, empirical study literature also about plants that have antioxidants and phenolic and flavonoid

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content above, the researchers tried to focus research on these plants by combining them to increase the use of synergism against antioxidant activity.

METHODOLOGY

Materials

The materials used in this study were herbaceous meniran (*Phyllanthusniruri* L.), buas-buasleaves (*Premnaserratifolia* Linn.), Secang wood (*Caesalpiniasappan*), roselle flower (*Hibiscus sabdarifa*), distilled water, demineralized water, gallic acid, quercetin, folinciocalteu, sodium carbonate, $NaNo_2$, NaOH, $AlCl_3$, ethanol (pro analysis and technical), 2,2-diphenyl-1-picrylhydrazyl crystal (DPPH).

Method

Sample preparation

Plant samples consisted of simplicia powders obtained from CV. Herbal AnugerahAlam Yogyakarta. Then the sample was weighed for maceration extraction.

Extraction

Extraction has been done using maceration method. Simplisia powder was put into each maceration vessel and 96% ethanol was added until the powder was completely submerged, and then left in the maceration vessel for 3 days. It is stirred and filteredeveryday, liquid was taken from the pulp and stored in a storage container. Then the pulp was extracted again in the same way and repeated several times. The liquid extract obtained was collected into one and concentrated using a rotary evaporator at 50°C, then concentrated using a waterbath until thick extracts were obtained. Each single extract and a combination of total phenols, total flavonoids and their antioxidant activity.

Determination of Total Phenol and Total Flavonoid Levels

Making of Raw Gallic Acid Curves

Preparation of a standard curve weigh the standard gallic acid standard 10.0 mg add 0.5 ml of the folin-ciocalteu reagent and 7.5 ml of demineralized water. The mixture was left for 10 minutes at room temperature, then add 1.5 ml of 20% sodium carbonate. The mixture was then heated in a water bath at 40°C for 20 minutes and immediately cooled in liquid ice. Dilute with demineralized water to a volume of 10 ml. Move it into the cuvette, keep it absorbing at a wavelength of 760 nm.

Measurement of Total Sample Phenol Levels

Take ± 0.02 g of the test sample carefully, add 0.5 ml of the folin-ciocalteu reagent and 7.5 ml of demineralized water. The mixture was left for 10 minutes at room temperature, then add 1.5 ml of 20% sodium carbonate. Add demineralized water to a volume of 10 ml. Dilute 10x. Move it into the cuvette, keep it absorbing at a wavelength of 760 nm.

$$\text{Total Phenol (\%w/w)} = \frac{reading\ results\ (ppm) x Vol.\ add\ end\ (mL)}{sample\ weight\ (g)} xdf/10000$$

Making of the Quercetin Raw Curve

Weigh the standard Quercetin standard 10.0 mg add 0.3 ml of sodium nitrite 5%. After 5 minutes add 0.6 ml of aluminum chloride 10%, wait 5 minutes, add 2 ml of 1 M sodium hydroxide. Add with distilled water to 10 ml with measuring flask. Move it into the cuvette, keeping it absorbed at a wavelength of 510 nm.

Measurement of Total Sample Flavonoid Levels

Determination of the total flavonoid test sample take 20 mg of the sample, put it in a 10 ml test tube. Add 0.3 ml of 5% sodium nitrite. After 5 minutes add 0.6 ml of 10% aluminum chloride, wait 5 minutes, add 2 ml of 1 M sodium hydroxide. Add the distilled water to 10 ml with a measuring flask. Dilute as needed. Move it into the cuvette, keeping it absorbed at a wavelength of 510 nm.

$$\text{Total Flavonoids (\%w/w)} = \frac{reading\ results\ (ppm) x Vol.\ add\ end\ (mL)}{sample\ weight\ (g)} xdf/10000$$

Determination of Antioxidant Activity with UV-Vis Spectrophotometer

Making DPPH Solution

The DPPH stock solution was prepared by weighing as much as 15.7 mg of DPPH powder and then placed in a 100 mL volumetric flask and adding ethanol p.a. to the limit mark. to obtain a solution with a concentration of 0.4 mM.

Determination of Maximum Wavelength (λmax)

Determination of the maximum wavelength (λmax) was carried out in a DPPH solution with a concentration of 0.4 mM. Then scanning the maximum wavelength from 450 nm - 550 nm. DPPH absorbance is at a wavelength of 514.5 nm.

Making Sample Solutions

The thick extract was made by weighing each extract as much as ± 100 mg and dissolving it

with ethanol p.a. 10 mL. Dissolution was carried out using ultrasonic and centrifugation, then dilution factors were made 10 times to obtain a final concentration of 1 mg / mL. A stock solution was pipetted and each solvent was added to the boundary mark so that variations in sample concentration (depending on IC_{50} of plants against DPPH) are obtained.

Measurement of Radical Scavenging Absorbance

A sample solution with a certain concentration that has been made was then added 0.4 mM DPPH solution. The mixture was then shaken and incubated at room temperature for 30 minutes in a dark place. Then the absorbance was measured at a wavelength of 514.5 nm and calculated the percent of antioxidant activity (% inhibition) in each sample. The blank concentration used was DPPH solution and 0.881 absorbance was obtained.

$$\% \text{ Inhibition} = \frac{A\ control - A\ extract}{A\ control}\ x\ 100\%$$

Information: A control = Absorbance of DPPH solution without extract; A extract = Absorption of extract was tested.

RESULT AND DISCUSSION

Making simplicia

Samples obtained in the form of simplicia powder from CV. Herbal AnugerahAlam Yogyakarta. Then the sample is weighed for maceration extraction. The simplicia powder drying process in an oven temperature of 40^0C.

Making ethanol extract

Each sample was immersed with solvent in a vessel for 3x24 hours while stirring occasionally. This method was chosen because it requires a relatively small amount of mobile phase and is easy to do. In addition, because it does not use heating, the active substance which cannot stand the heat remains stable in the material. Maceration is done by avoiding sun exposure and using closed vessels. Exposure to sunlight can cause the decomposition of chemical compounds in the material as well as the occurrence of unwanted reactions. Closed vessels are used so that the mobile phase used does not evaporate and contaminants from outside cannot enter. The mobile phase used is ethanol 96%. Ethanol is a polar solvent that is able to attract secondary polar metabolites such as phenols and flavonoids.

Testing antioxidant activity

Determination of antioxidant activity is carried out by the DPPH radical scavenging method (Isnindar *et al.,* 2016). A stock solution was made for maceration extraction by weighing 100 mg of the sample dissolved in 10 ml of the solvent, then a dilution factor of 10 times was made with the final result of 1 mg / mL. Next, five concentration series were made and 1 ml of DPPH was added to each concentration, then the mixture was vortex and left for 30 minutes and the absorbance measured at a wavelength of 514.5 nm. The DPPH method was chosen because it is simple, easy, fast, sensitive, accurate and only requires a small sample. For testing the antioxidant activity, the extract was dissolved in ethanol according to DPPH solubility. Antioxidant compounds will react with DPPH radicals through a hydrogen atom donation mechanism and cause DPPH color decay from purple to yellow measured at wavelength 517. This method is based on measuring absorbance of DPPH that does not react with antioxidant compounds.

Determination of maximum wavelength and determination of operating time is done to minimize errors when absorbing readings. Operating time is needed to give the radical capture compound time to react to DPPH perfectly. Absorbance reading at operating time is aimed at keeping the reading steady. Antioxidant activity was determined by IC_{50} antioxidant compounds. IC_{50} is the ability of antioxidant compounds to capture 50% of DPPH free radicals during operating time. IC_{50} values were obtained from plotting to the linear regression equation with (x) as the sample concentration and (y) is the percent of antioxidant activity. The smaller the IC_{50} value, the more potent the antioxidant activity of the compound. The linear regression curve for the extraction of maceration, meniran, buas-buas, secang, roselle, and its combination is made in a ratio (1: 1: 1/2: 1/2); (2: 1: 1/2: 1/2); (1: 2: 1/2: 1/2).

From the regression curve data produced by maceration and quercetin extraction as a positive control showed that there was a close relationship between concentration andpercent antioxidant activity (% inhibition). This is shown by the value of R^2 (correlation coefficient) above 0.99. The value of R^2 states that there is a correlation between the concentration of the sample and the percent inhibition observed with the degree of closeness in the extraction of maceration meniran, buas-buas, secang, roselle, and combinations in comparison (1: 1: 1/2: 1/2); (2: 1: 1/2: 1/2); (1: 2: 1/2: 1/2) in a row that is 0.9973; 0.9898; 0.99; .9918; 0.9961; 0.9942; and 0.9923. Quercetin as a positive control is 0.9999. This shows that more than 99% of the degree of inhibition is influenced by the concentration of the material, whereas less than 1% is influenced by other factors.

Table I. Comparison of IC_{50} values of meniran, buas-buas,secang wood, roselle, and maceration extraction combinations with comparison

No.	Plant	Graph Equation	IC_{50}Value (µg/ml)
1.	Herbs Meniran (HM)	Y = 1720x + 0.3557 R2 = 0.9973	28.9
2.	Buas Buas leaves (DBB)	Y = 1634.1x + 13.852 R2 = 0.9898	22.1
3.	Sappan Wood (KS)	Y = 7315.6x + 5.891 R2 = 0.99	6.0
4.	Roselle Flower (BR)	Y = 224.74x + 16.012 R2 = 0.9918	151.3
5.	HM : DBB : KS : BR (1 : 1 : ½ : ½)	Y = 1710.8x + 31.279 R2 = 0.9961	11.0
6.	HM : DBB : KS : BR (2 : 1 : ½ : ½)	Y = 1485x + 30.291 R2 = 0.9942	13.3
7.	HM : DBB : KS : BR (1 : 2 : ½ : ½)	Y = 1992.1x + 11.381 R2 = 0.9923	19.4
8.	Quercetin	Y= 11.406x + 31.264 R2 = 0.9999	1.644

Table II. Relationship of total gallol equivalent phenol equivalents, total quercetin equivalent flavonoids, and maceration extraction antioxidant activity

No.	Plant	Total Phenol (%w/w EAG)	Total Flavonoids (%w/w EQ)	IC_{50} (µg/mL)
1.	Herbs Meniran (HM)	30.60	14.78	28.9
2.	Buas Buas leaves (DBB)	9.82	54.17	22.1
3.	Sappan Wood (KS)	11.10	139.76	6.0
4.	Roselle Flower (BR)	26.15	4.96	151.3
5.	HM : DBB : KS : BR (1 : 1 : ½ : ½)	28.99	44.73	11.0
6.	HM : DBB : KS : BR (2 : 1 : ½ : ½)	33.57	74.00	13.3
7.	HM : DBB : KS : BR (1 : 2 : ½ : ½)	7.58	55.09	19.4

The test results show that the higher the concentration of the sample, the higher the percentage of inhibition, this is due to the more samples, the higher the antioxidant content so that it also affects the level of inhibition of free radicals carried out by antioxidant compounds. Quercetin in this study is used as a positive control to prove that the method used to test antioxidant activity is correct. Quercetin is used because it belongs to an antioxidant flavonoid class that is able to ward off various free radicals.

Data (Table I) shows that to capture 50% DPPH radicals, meniran, buas-buas, secang, roselle extracts and combination extracts are 28.9 µg / mL, 22.1 µg / mL, 6.00 µg / mL, 151.3 µg / mL respectively mL, 11.0 µg / mL, 13.3 µg / mL, 19.4 µg / mL and quercetin of 1,644 µg / mL. From the results of the data show that in the form of a combination of synergistic effects or mutually reinforcing antioxidant activity is characterized by the results of the IC_{50} value of the combination that is getting smaller when compared in a single form.

Determination of total phenols was expressed equivalent to gallic acid and total flavonoids were expressed equivalent to quercetin (Table II). The results showed that the best IC_{50} value in the combination ratio (1: 1: 1/2: 1/2) is (11.0 µg / mL), then successively (1: 2: 1/2: 1/2) is 13.3 µg / mL, and (2: 1: 1/2: 1/2) which is 19.4 µg / mL (Table II). The highest total phenolic and flavonoid content in combination with a ratio (2: 1: 1/2: 1/2) is 33.57% w/w EAG and 74.00% w/w EQ (Table II). Correlation analysis between IC_{50} values with phenolic content and total flavonoids (Figure 1) shows a positive correlation with R_2 values of 0.8236 and 0.0102 with a positive slope. Thus it can be concluded that the total phenol content influences free radical scavenging activity by 82.36%, while the effect of total flavonoid content is only 1.02%.

This phenomenon can be explained by the various types of phenolic and flavonoids in the sample that can provide different activities of each compound and as an additional effect of several

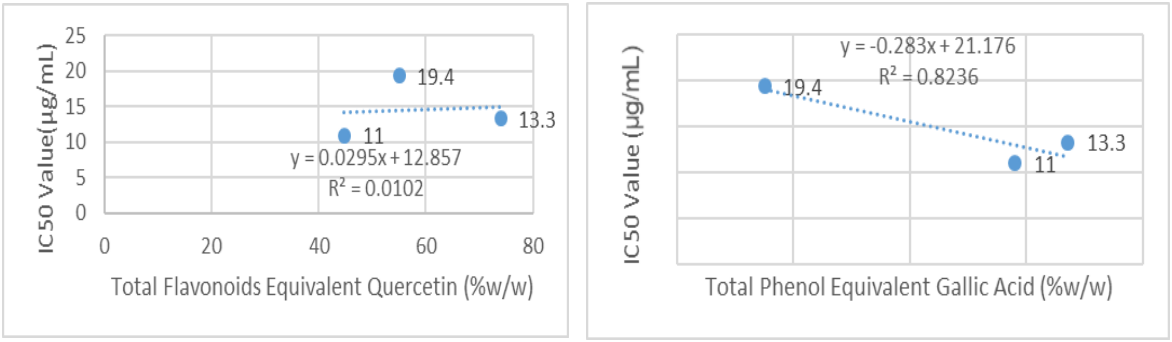

Figure 1. Linear regression curve between total phenols (Graph 1), total flavonoids (Graph 2) and percent antioxidant activity from maceration extraction

compounds. Several studies have written that the content of polyphenols and flavonoids has a positive correlation with antioxidant activity.

The reaction of phenolic compounds in inhibiting the process of autoxidation is caused because phenolic compounds function as hydrogen donors to radicals in the form of ($R^{\bullet}$) to produce RH and phenolic compounds that turn into free radicals that can be stabilized by their aromatic structures. The auto-oxidation reaction cannot be prevented but can only be slowed down and the duration of slowing down depends on the antioxidant activity in question, the concentration of antioxidants and other factors such as heat, light, metals, and other pro-oxidants present in the system. The phenolic group on antioxidants also acts as a deterrent to the formation of free radicals in oxidized fats by providing $H^{\bullet}$ to form stable non-radical products, which can stop chain reactions. (Aquino *et al.,* 2002) In addition to the phenol group, the flavonoid group can also inhibit lipid peroxidation by reducing peroxyl radicals which simultaneously terminate the radical reaction and extinguish single O_2. There are several classes of flavonoids that have antioxidant activity, namely flavones, flavanones, isoflavones, flavonols, flavan-3 ol and anthocyanins.

CONCLUSION

The results showed that the best IC_{50} values were in the combination of ratios (1: 1: 1/2: 1/2) that is (11.0 µg / mL), then (1: 2: 1/2: 1/2) which was 13.3 µg / mL, and (2: 1: 1/2: 1/2) which is 19.4 µg / mL. The highest total phenolic and flavonoid content in the ratio (1: 2: 1/2: 1/2) is 33.57% w/w EAG and 74.00% w/w EQ. Correlation analysis between IC_{50} values with total phenolic and flavonoid contents showed a positive correlation with R^2 values of 0.8236 and 0.0102 with positive slope. Thus it can be concluded that the total phenol content influences free radical scavenging activity by 82.36%, while the effect of total flavonoid content is only 1.02%.

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