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RESEARCH ARTICLE

Comparison of maceration and infundation towards antioxidant capacity of leaves aqueous extracts of balik angin (*Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz)

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Abstract

Background: Excessive exposure to free radicals can trigger various degenerative diseases. Therefore, external antioxidants are needed from nature. One plant with the potential as a natural antioxidant is balik angin (*Alphitonia incana* (Roxb.) Teijsm. & Binn. Ex Kurz). **Objective:** This study aimed to determine the differences in the content of secondary metabolites and the antioxidant capacity of the aqueous extract of balik angin leaves from two different extractions. **Method:** The extraction methods were maceration and infundation, followed by phytochemical screening to identify secondary metabolites. The antioxidant activity assay was carried out by UV-Vis spectrophotometry by measuring the absorbance of the sample reaction with CUPRAC and FRAP reagents. **Result:** The phytochemical screening results of balik angin leaves infusion contained phenols, flavonoids, saponins, triterpenoids, and tannins, while the macerated extract obtained triterpenoids and tannins negatively. The quantitative assay showed the balik angin leaves infusion resulted in a powerful antioxidant category with EC50 values of 17.762 µg/mL and highly antioxidant content, which was 726 mg AAE/g extract compared to macerated aqueous extract antioxidant capacity with EC50 values of 44.719 µg/mL and 635 mg AAE/g extract antioxidant content. **Conclusion:** The infused aqueous extract of balik angin leaves has the potential to be a powerful antioxidant compared to the maceration product.

Introduction

Environmental pollution, fast food that is fatty and high in cholesterol, and an unhealthy lifestyle, such as smoking, are the factors that can play an important role in the formation of free radicals in the body. Excessive exposure to free radicals will cause oxidative stress and can trigger various degenerative diseases (Elkhateeb & Alshammary, 2017). The human body does not have sufficient antioxidant reserves to overcome excessive exposure to free radicals, therefore the human body needs antioxidants that come from the outside. Based on the source of their production, antioxidants are divided into two categories: natural and synthetic antioxidants (Chaudhary *et al*., 2023). One of the plants that has the potential as a natural antioxidant is balik angin (*Alphitonia incana* (Roxb.) Teijsm. & Binn. Ex Kurz) (Forestryana *et al*., 2022). Balik angin, identical to *A. excelsa*, *A. moluccana*, and *A. philippinensis,* is an endemic plant that is efficacious as a traditional medicine on Borneo island. An ethnobotanical study states that balik angin leaves are used as traditional medicine by the people of Borneo, especially the Dayak tribe. The leaves are used daily as a natural

bath soap for skin care and to treat skin diseases (Wardah & Sundari, 2019; Ramadhan *et al*., 2023).

The main flavonoid compound proven to be contained in the balik angin plant is Alphitonin. This plant also contains quercetin and isorhamnetin derivatives (Ahmed *et al*., 2019; Al Omar *et al*., 2022). Flavonoid compounds have shown several activities, including protection against free radicals, inhibition of oxidative reactions and hydraulic enzymes, and production of anti-inflammatory properties and other bioactivities (Maiti *et al*., 2019). The leaves and other parts of the balik angin plant also contain other compounds, such as the triterpenoids group, which are Alphitolic acid, Ceanothic acid, Betulinic acid, Betulin, Alphitexolide, Lupeol, Zizyberenalic acid, Platanic acid, and Ceanothenic acid. Phytosterols group also were identified in the chloroform extract of the stem, among others β-Sitosterol and Stigmasterol. Many researchers were reported that compounds have anti-inflammatory, anti-cancer, and cytotoxic activities, and treat several degenerative diseases (Al Omar *et al*., 2022).

Previous research by Cock (2020) stated that the use of polar solvents such as methanol and water can attract more secondary metabolites than non-polar solvents, thus affecting the resulting bioactivity. This research shows that the macerated aqueous extract from balik angin leaves contains phenols, saponins, triterpenoids, flavonoids, and tannins, but the antioxidant activity is still unknown. Based on this research, exploring the antioxidant potential of the active compounds in the water extract resulting from the maceration of balik angin leaves is necessary, compared with the results of hot extraction infundation.

The antioxidant activity assays of balik angin leaves can use several methods, including the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) and FRAP (Ferric Ion Reducing Antioxidant Power) methods. Both of these methods have antioxidant mechanisms as metal-chelating agents. This chelating agent functions as a catalyst. The chelating process will reduce the catalytic activity of copper and iron metals to reduce the redox potential value and the formation of OH radicals (Timoshnikov *et al*., 2022).

Methods

Chemicals and instruments

All the experiments' chemicals, solvents, and reagents were analytical grade. They were purchased from Merck KGaA, excepting standard quercetin and ascorbic acid which came from Sigma Aldrich Co. All spectrophotometric measurements were made with a pair of matched quartz cuvettes using a PG Instruments-T60 UV-Vis spectrophotometer.

Sample of the plant

Balik angin leaves were collected in January 2023 and were obtained from the balik angin tree in Mount Tahura, Mandi Angin area located in Karang Intan District, Banjar Regency, South Kalimantan. The collected plant specimens were identified at the Indonesian Institute of Sciences (LIPI), Cibinong Biology Research Center, Bogor.

Preparation of leaf extraction

The mature leaves of balik angin were collected and washed thoroughly with running water, then cut crosswise and dried for approximately two weeks at room temperature. The dried samples were powdered and sifted with a size 40 mesh (Cock, 2020; Fuentes *et al.,* 2020). The extraction of the powders was conducted according to the following methods: (1) Simplicia balik angin leaves powder is extracted using the maceration method by taking two grams and dissolving it in 100 mL of sterile deionised water (containing 1% DMSO) and storing it in the refrigerator at 4^{o}C for 24 hours (Cock, 2020). (2) The Infundation method is carried out by taking ten grams of simplicial powder and then dissolving it in 100 mL in an infusion pan at a temperature of 90^{o}C for 15 minutes. The resulting extract is filtered, and water is added until a volume of 100 mL is reached (Hasim *et al*., 2016).

Phytochemical screening

Flavonoid test

A total of 1 mL of extract was put into a test tube, and 1 mL of concentrated HCl reagent, 0.1 g of Mg metal, and 1 mL of amyl alcohol were added, shaken vigorously and allowed to separate. Positive results are indicated by the formation of red, yellow, or orange colours on the amyl alcohol layer (Ramadhan *et al*., 2023).

Phenol test

A total of 1 mL of balik angin leaf extract was put into a tube, and 1 mL of 10% $FeCl_3$ was added. Positive results containing phenol are indicated by the formation of blue, green, red, purple, or blackish colour (Ramadhan *et al*., 2020).

Triterpenoid-steroid test

A total of 1 mL of balik angin leaves extract was added to 2-3 ml chloroform, ten drops of anhydrous acetic

acid, and two to three drops of concentrated H2SO4 through the tube wall. The formation of a blue-to-green colour indicates a positive result for steroids. Meanwhile, positive result for triterpenoids is indicated by the formation of a red or purple color (Ramadhan *et al*., 2020).

Tannin test

A total of 1 mL of balik angin leaves extract was put into a test tube and added with 2 mL of 1% gelatin solution containing NaCl. A positive result indicating the presence of tannin is characterised by the formation of a white precipitate (Ramadhan *et al*., 2023).

Saponin test

A total of 1 mL of balik angin leaves extract was added to 10 ml of distilled water and shaken for 10 seconds, then 1 mL of 2 N HCl was added and left for 10 minutes. Positive results occur if stable foam is formed when 2 N HCl is added (Ramadhan *et al*., 2020).

Alkaloid test

A total of 2 mL was added to 5 ml of HCl, then divided into three test tubes. Several reagents are used to precipitate alkaloids: Mayer reagent solution, which produces a white to yellowish precipitate after being added to the first tube; Dragendorff reagent solution in acid solutions which appears an orange-brownish precipitate after being added to the second tube; and Wagner reagent solution which shows a brown precipitate in acidic solutions after its addition to the third test tube suggesting the presence of alkaloids (Ramadhan *et al*., 2023).

CUPRAC assay

Determination of maximum wavelength

To determine the maximum wavelength, the following method is carried out: 1 mL of 0.01 M $CuCl_2.2H_2O$, 1 mL of 0.0075 M Neocupproine Ethanolic, 1 mL of 1 M NH_4Ac Buffer, and 0.1 mL of distilled water are taken and put into a vial, then 1 mL of 96% ethanol is added. This solution is then incubated for 30 minutes in a dark place. After incubation, the absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 400-600 nm (Apak *et al*., 2014; Sayakti *et al*., 2022).

Blanko solution test

The blanko solution test is carried out by mixing 1 mL of 0.01 M $CuCl_2.2H_2O$ solution, 1 mL of 0.0075 M Neocupproine Ethanolic, and 1 mL of 1 M NH_4Ac Buffer in a vial, then adding 1 mL of 96% ethanol and 0.1 mL of distilled water. The mixture is incubated at room temperature and in the dark for 30 minutes. Absorbance was measured using a UV-Vis Spectrophotometer at the maximum wavelength obtained (Apak *et al*., 2014; Sayakti *et al*., 2022).

Antioxidant activity test

To test the antioxidant activity of balik angin leaves aqueous extract and quercetin (comparative standard), 1 mL of each sample solution separately with a concentration series of 15 ppm, 20 ppm, 25 ppm, 30 ppm and 35 ppm for aqueous extract and 1-5 ppm for quercetin, were taken and each put into a vial. 1 mL of 0,01 M $CuCl_2.2H_2O$ solution, 1 mL of 0,0075 M Neocupproine Ethanolic, 1 mL of 1 M NH_4Ac Buffer, and 0.1 mL of distilled water were added. All sample series were incubated in a dark room for 30 minutes. The absorbance was then measured using a UV-Vis Spectrophotometer at a predetermined maximum wavelength. This process was replicated and carried out three times. (Apak *et al*., 2014; Sayakti *et al*., 2022).

EC_{50} determination

The determination of EC_{50} was carried out after obtaining the sample absorbance value. According to Ramadhan et al. (2022a), the percent capacity value is calculated using the formula :

$$\% \text{ Capacity} = (1 - \text{Antilog of Absorbance}) \times 100\%$$

A standard curve for the relationship between %capacity and concentration is created to calculate the EC_{50} value using the line equation y = bx + an obtained from the standard curve. This equation can be used to determine the EC_{50} value of the sample by entering the y value of 50 and the x value as the concentration, which will be obtained as EC_{50}. So, the EC50 value can be calculated using the formula :

$$EC_{50} = \frac{(50 - a)}{b}$$

FRAP assay

Determination of maximum wavelength

Determination of maximum wavelength was obtained by adding 1 mL of ascorbic acid of 60 ppm (diluted with 1% oxalic acid) into a centrifuge tube mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50˚C for 20 minutes. After incubation, 1 mL of trichloroacetic acid (TCA) was added, homogenised for ten minutes, and centrifuged

at 3000 rpm for ten minutes. The top layer was taken from the solution in 1 mL and then added to 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. Absorbance was measured at 650-750 nm using a UV-Vis spectrophotometer (Suhaera *et al*., 2022).

Blanko solution test

The blanko solution test is carried out by mixing 1 mL of 1% oxalic acid with 1 mL of phosphate buffer solution (0.2 M pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$, incubated at 50˚C for 20 minutes. Then, 1 mL of 10% TCA solution was added and centrifuged for ten minutes at a speed of 3000 rpm. The supernatant of 1 mL was pipetted into a test tube, 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ were added and incubated for ten minutes. The absorbance was read at the maximum wavelength obtained previously with a UV-Vis spectrophotometer (Suhaera *et al*., 2022).

Ascorbic acid standard curve

The standard curve of ascorbic acid was made with correlated concentration series (60-100 ppm) and absorbances. Each ascorbic acid concentration was taken as 1 mL and mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50˚C for 20 minutes. After incubation, 1 mL of TCA was added and homogenised for ten minutes, then centrifuged at 3000 rpm for ten minutes. One millilitre of the top layer was taken from the solution, added with 1 mL of distilled water and 0.5 mL of 0.1% FeCl3, and incubated again for ten minutes. Absorbance was measured at the maximum wavelength obtained previously with a UV-Vis spectrophotometer (Suhaera *et al*., 2022).

Antioxidant activity test

The test of the antioxidant activity of balik angin leaves aqueous extract was carried out by making a concentration of 100 ppm of each extract and pipetted 1 mL to added 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$. The solution was incubated for 20 minutes at 50˚C. After incubation, 1 mL of 10% TCA solution was added and homogenised for ten minutes, then centrifuged at 3000 rpm for ten minutes. The supernatant of 1 mL was pipetted into a test tube, 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ were added, and incubated for ten minutes. The absorbance of the solution was measured at the maximum wavelength previously obtained using a UV-Vis spectrophotometer (Rahayu *et al*., 2021).

Antioxidant power determination

Determination of antioxidant power was calculated after obtaining the linear regression equation from the ascorbic acid standard curve with the following formula:

$$\text{Sample Concentration (x)} = \frac{y + a}{b}$$

Ahmed et al. (2019) stated that the antioxidant power value is shown as milligram Ascorbic Acid Equivalent per gram (mg AAE/g) extract and was calculated using the following formula:

$$\text{AAE} = \frac{\text{Sample Concentration x Volume x Dilution Factor}}{\text{Sample Weight}}$$

Statistical analysis

Statistical analysis to determine differences in antioxidant activity was tested using SPSS Version 25. The normality test of the data was tested using Levene's Test. If the data is met, it is continued with One Way Anova analysis. If the normality and homogeneity tests are not met, then the data is analysed using the Kruskal Wallis test (Ramadhan *et al*., 2022b).

Results

The results of this study showed that the infusion aqueous extract has a higher number of secondary metabolites group than the macerated aqueous extract based on phytochemical screening in Table I. Both extracts contain phenolics, flavonoids, and saponin, but the infusion contains tannins and triterpenoids, which are more than macerated extract.

Table I: Phytochemical screening of balik angin leaves

Secondary metabolites	Aqueous extraction method	
	Maceration	Infundation
Phenols	+	+
Flavanoids	+	+
Alkaloids	-	-
Tannins	-	+
Saponins	+	+
Steroids	-	-
Triterpenoids	-	+

The measurement curve of antioxidant activity on the leaves of balik angin consists of three treatment groups (macerated extract, infusion, and quercetin as positive control), as shown below in Figure 1.

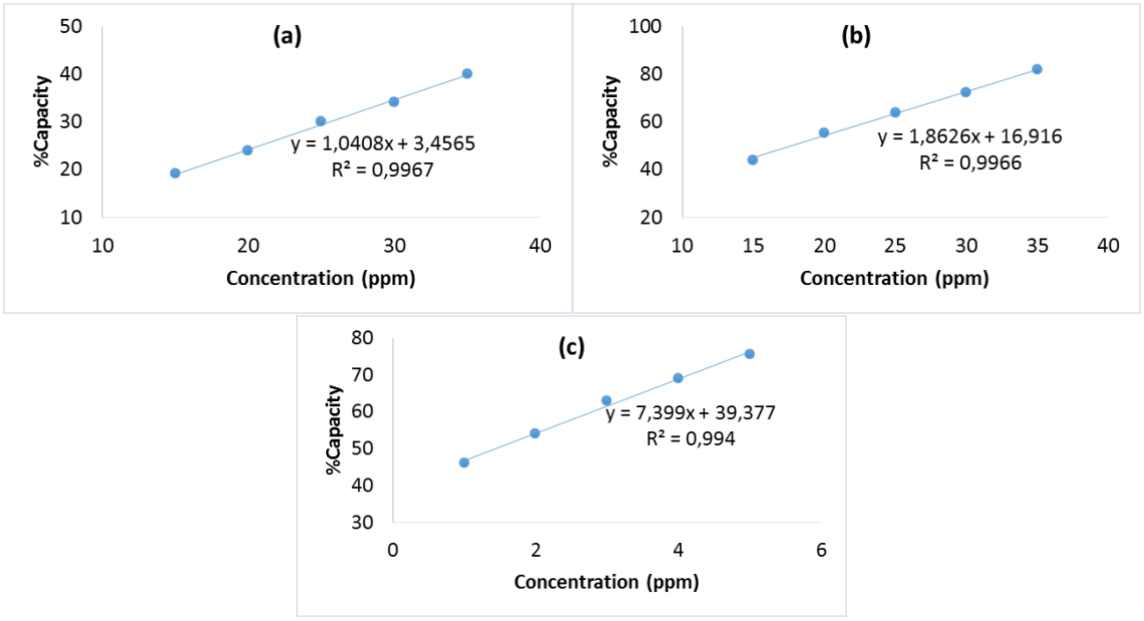

Figure 1: Correlation between concentration and % capacity of (a) macerated aqueous extract of balik angin leaves, (b) infusion of balik angin leaves, and (c) quercetin.

The regression equation obtained from Figure 1 was used to calculate the EC_{50} value. The quercetin antioxidant activity test results are included in the strong antioxidant activity group with an EC_{50} value of 1.4357 ppm. Macerated extract and infusion antioxidant capacities are shown in Table II and included in the strong antioxidant category. The antioxidant power of FRAP assay was calculated using the regression equation of the ascorbic acid standard which was y = 0.013x – 0.5529 which continued with the determination of mg AAE/g extract resulting in the value of antioxidant powers in Table II.

Table II: The result of CUPRAC and FRAP assay on balik angin leaves

Extraction method	EC_{50} (ppm)	Antioxidant category	Antioxidant power (mg AAE/g extract)
Maceration	44.71	Very strong	635 ± 0.5
Infundation	17.76	Very strong	726 ± 0.8

Discussion

The extraction of balik angin leaves was carried out by maceration and infundation. The extraction method used greatly affects the extraction results. Due to some simplicia's instability and thermolabile nature (which can be damaged by heating), the extraction method can affect its concentration or therapeutic effect. This research uses maceration and infundation methods, the most common methods used to extract natural resources. The temperature difference between maceration and infundation can affect the solubility of flavonoids and phenols contained in the leaves of the balik angin, where high temperatures will increase the solubility of phenol using the solvent. An increase in temperature can cause a greater diffusion process. It is proven that the infusion of aqueous extract has a higher number of secondary metabolite groups than the macerated aqueous extract based on phytochemical screening (Hasnaeni *et al*., 2019).

The research that has been done shows that infusion extraction can attract compounds more optimally in the balik angin leaves than the maceration method. Qualitative test results from the phytochemical screening of aqueous extract of balik angin leaves resulting from maceration extraction showed positive results for phenols, flavonoids, and saponins. Meanwhile, the results of infundation extraction obtained positive results for phenols, flavonoids, tannins, saponins and triterpenoids. Polyphenolic phytochemicals are non-nutritional plant compounds used to prevent and treat diseases triggered by oxidative stress (Abu Bakar *et al*., 2015). Phenols are a class or large group of secondary metabolites commonly found in plants. A distinguishing feature of

the phenolic compounds is that they possess an aromatic ring containing at least one hydroxyl group. This group of compounds consists of several classes, such as phenolic acids, flavonoids, and tannins (Tamsin *et al*., 2023).

Flavonoids are the most common and widely distributed group of plant phenolic compounds that act as natural antioxidants (Kumar & Pandey, 2013; Hasibuan & Mardiana, 2018). The mechanisms of flavonoids, tannins, and saponins can donate hydrogen atoms to free radical compounds. The results of the infundation extraction were positive, containing terpenoids, while the results of the maceration extraction were not and did not contain terpenoids. Terpenoids are the largest class of natural products, mostly derived from plants but found in all living organisms, serving as energy sources, biological building blocks, and/or signalling molecules. The terpenoids are grouped into three classes: sesquiterpenoids, diterpenoids, and triterpenoids (Ling *et al*., 2022). Terpenoid compounds are active compounds included in the lipophilic antioxidant type, these compounds can capture oxygen radicals which inhibit the initiation and propagation processes in oxidative chain reactions in hydrophobic systems (Muhammad *et al*., 2014).

The difference in the results of the secondary metabolite group contained from maceration and infundation is linear to differences in resulting antioxidant activity. Table II showed that extraction of balik angin leaves through infusion produced stronger antioxidant activity than maceration using an aqueous solvent. Balik angin leaves aqueous extract extracted by infundation showed antioxidant activity with EC_{50} values and antioxidant power of 17.76 ppm and 726 ± 0.8 mg AAE/g extract respectively compared to that extracted by maceration with values of 44.71 ppm and 635 ± 0.5 mg AAE/g extract. These antioxidant activities were contributed by the secondary metabolite content, which is quite high in the infusion water extract compared to macerated aqueous extract. Statistical analysis begins with a normality test (Shapiro-Wilk test) and homogeneity (Levene's test) of each antioxidant value in each test with a confidence level of 95%. Statistical results from the CUPRAC assay show normal and homogeneous data (significance value > 0.05), so statistical analysis uses a parametric test, one-way ANOVA. The statistical analysis results show that the antioxidant activity produced by the aqueous extract of balik angin leaves resulting from maceration and infundation has a significant difference with a significance value of 0.002 ($p < 0.05$). Meanwhile, in the FRAP assay results, statistical data is not normally distributed but homogeneous, so the non-parametric test is continued. The statistical analysis also shows a significant difference between the aqueous extract of balik angin leaves from maceration and infundation in producing antioxidant activity with a significance value of 0.046 ($p < 0.05$).

The aqueous extract of balik angin leaves produces a very strong EC_{50} value because it is influenced by several factors, one of which is that balik angin leaves contain several groups of polyphenolic compounds that function as antioxidants, including flavonoids and tannins. Previous research by Rosyada *et al*. (2022) tested the methanol extract of balik angin leaves resulting from cold (maceration) and hot (soxhlet) extraction using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method which showed that the IC_{50} values were respectively 13.703 ppm (very strong category) and 9.983 ppm (category very strong). These results support the assumption that balik angin leaves have strong antioxidant potential. Even though they are extracted using a water solvent with or without high heating, the extracts produce strong antioxidant activity.

The results of the FRAP assay also prove that the infundation method can attract more antioxidant compounds in balik angin leaves because they have higher antioxidant power compared to the macerated aqueous extract of balik angin leaves. Based on these results, the antioxidant activity of balik angin (*Alphitonia incana* (Roxb.) Teijsm. & Binn. Ex Kurz) leaves aqueous extract from the maceration and infundation method, has a higher Ascorbic Acid Equivalent (AAE) value compared to previous research on the *Alphitonia philippinensis* which was extracted using methanol solvent using the soxhlet method. The results of the FRAP test on *Alphitonia philippinensis* only obtained 9.36 ± 0.05 mg AAE/g extract (Ahmed *et al*., 2019). This difference in results could be caused by ascorbic acid compounds, which tend to be polar and dissolve in water, are more effectively extracted when using water as a solvent (Krakowska-Sieprawska *et al*., 2022).

The principle of the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) method is to measure the ability of an antioxidant to reduce Cu^{2+} to $Cu^{+,}$ which is indicated by a change in colour from blue to yellow. Meanwhile, the FRAP method shows the ability of antioxidant to reduce Fe^{3+} and becomes Fe^{2+} with a change in colour from green to bluish-green. Both methods were used to measure and show that the antioxidant ability is analogous to the reducing ability of a compound (Munteanu & Apetrei, 2021). The CUPRAC method uses comparison samples of quercetin because it has very strong antioxidant activity, four to five times the effectiveness compared to vitamins C and E and twice higher than other flavonoid derivatives (Banjarnahor

& Artanti, 2014). It is proven that quercetin has a high antioxidant capacity in reducing cupric ions and includes a very strong category (EC_{50} of 1.4357 ppm). Likewise, ascorbic acid is an effective antioxidant in inhibiting free radicals and has water-soluble properties. It correlates to the solvent used in this extraction, which is water. Quercetin and ascorbic acid play a role in redox reactions as oxidants that can chelate metal ions (Mendoza-Wilson *et al*., 2022). The chelation process will reduce metal ions' catalytic activity to reduce OH radicals' formation. It will automatically reduce the process of DNA damage and the process of fat peroxidation (He *et al*., 2017).

Conclusion

It can be concluded from this study that infusion extraction of balik angin leaves aqueous extract produces better antioxidant activity which includes a very strong category of antioxidants compared to maceration product, and correlates with differences in the number of secondary metabolites contained. The results of statistical analysis show that the antioxidant activity produced by the aqueous extract of balik angin leaves resulting from maceration and infundation has a significant difference with the significance value in the one-way ANOVA test of 0.002 in the CUPRAC method and 0.046 in the Kruskal-Wallis test in the FRAP method.

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