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# ANTIOXIDANT ACTIVITY OF COMBINATION OF WHITE-PURPLE *Orthosiphon aristatus* (Blume) Miq HERB EXTRACT AND *Stevia rebaudiana* Bertoni USING FRAP METHOD

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## ABSTRACT

Oxidative stress is caused by an increase or decrease in the antioxidant defense activity. *Orthosiphon aristatus* (Blume) Miq and Stevia are two plants that contain antioxidant compounds and can bind free radical compounds. The combination *of Orthosiphon aristatus* (Blume) Miq and Stevia was expected to increase antioxidant activity. This study aimed to determine the antioxidant activity of a combination of *the Orthosiphon aristatus* (Blume) Miq white-purple variety and *Stevia rebaudiana* Bertoni extract. Antioxidant activity was tested using the FRAP method with a UV-visible spectrophotometer at a wavelength of 710 nm. The results of phytochemical screening showed that both plant extracts and dry material contained secondary metabolite compounds, including flavonoids, polyphenols, saponins, quinones, steroid-triterpenoids, and monoterpenes-sesquiterpenes. A qualitative test relating to antioxidants using TLC showed positive antioxidant activity, which was proven by the presence of green or blue light on the TLC plates. This was due to electron transfer in the compound, which reduced $Fe^{3+}$ to $Fe^{2+}$. On the other hand, quantitative measurements using the FRAP method showed the antioxidant capacity values consecutively, F1 combination extract (1:1) of 43.725 mg of QE/g extract, F2 combination extract (2:1) of 57.963 mg of QE/g extract, and combination extract of F3 (3:1) of 64.849 mg of QE/g extract. The extract combination of F3 showed the best formula, with a value of 64.849 mg QE/g extract.

**Keywords**: *Orthosiphon aristatus* (Blume) Miq, Stevia, antioxidants, FRAP

## INTRODUCTION

Free radicals are the result of the homolytic splitting that occurs in covalent bonds. This splitting causes the molecule to become a free radical with an unpaired electron requiring a pair to balance its spin value. Consequently, radical molecules become unstable and easily react with other molecules to form new radicals (Fakriah *et al*., 2019). However, the body cell molecules whose electrons are taken will turn into other free radicals that are ready to attack other molecules again, and eventually a very dangerous chain reaction will form (Neha *et al*., 2019). If not stopped, it leads to a state of oxidative stress that damages DNA, proteins, carbohydrates, and fats, causing cell and tissue damage. As a result, this can lead to serious diseases such as diabetes (Prasetyaningsih *et al*., 2022).

Oxidative stress is caused by the increased or decreased activity of antioxidant defenses. The terms used for this condition are reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Oyenihi *et al*., 2015). Hyperglycemia-induced oxidative stress in diabetes mellitus is usually associated with greater endothelial cell death in vitro and in vivo. This is evidenced by several studies showing increased free radical formation and decreased

antioxidant capacity (Prawitasari, 2019). Lower glucose levels lead to oxidation and glycolysis, which produce ROS and AGEs and induce oxidative rioting of amino acid residues, resulting in carbonyl compounds (Isfandiary *et al*., 2021).

Antioxidant administration is an attempt to inhibit oxidative stress and vascular complications associated with diabetes by inhibiting intracellular free radical production or by enhancing the ability of defense enzymes against free radicals. Supplements containing antioxidants or factors that can increase nitric oxide (NO) production have the potential to improve endothelial dysfunction and mitochondrial function in cells and reduce the activity of NADPH oxidase. In patients with diabetes mellitus who have macrovascular or microvascular complications, antioxidant therapy can be beneficial if combined with treatment to control blood pressure, dyslipidemia, and optimal glucose control. Ascorbic acid (vitamin C) and tocopherol (vitamin E) are sources of antioxidants (exogenous antioxidants) that have been widely studied. These two types of vitamins, such as polyphenols, phenolic acids, and flavonoids, can function as antioxidants (Prawitasari, 2019). The *Orthosiphon aristatus* (Blume) Miq plant has the potential to reduce antioxidants.

*Orthosiphon aristatus* (Blume) Miq has long been used in traditional medicine in East India, China, Southeast Asia, and tropical Australia. *Orthosiphon aristatus* (Blume) Miq has antioxidant, antidiabetic, and diuretic effects (Faramayuda *et al*. 2021). *Orthosiphon aristatus* (Blume) Miq leaf extract contains flavonoid and saponin compounds that play a role in regulate blood glucose levels (Andriaty *et al*., 2019). Biologically, metabolite compounds of *Orthosiphon aristatus* (Blume) Miq leaf extract can reduce blood glucose levels by acting as antioxidants. This allows the metabolite compound to suppress and repair the cell damage caused by alloxan induction (Azam *et al*., 2017).

In addition to *Orthosiphon aristatus* (Blume) Miq, plants that contain antioxidants include *Stevia rebaudiana Bertoni*. Stevia also contains antioxidant compounds that can bind to free radical compounds (Hardiansyah *et al*., 2022). Stevia contains steviosides, which can act as sugars for diabetic patients to maintain blood glucose levels. Steviosides are diterpenoid glycosides that are 300 times sweeter than sucrose and exhibit several pharmacological properties such as antioxidant and antidiabetic properties (Casas-Grajales *et al*., 2019). Stevia leaves reduce blood glucose and control glucagon levels in diabetic patients by increasing the insulinogenic index (Chowdhury *et al*., 2022).

Therefore, it is necessary to conduct research on the antioxidant activity of a combination of *Orthosiphon aristatus* (Blume) Miq and stevia leaves (*Stevia rebaudiana Bertoni*.). This is expected to increase the antioxidant activity. In contrast, stevia leaves have a sweet taste, so they can cover the bitter taste of *Orthosiphon aristatus* (Blume) Miq leaves.

## RESEARCH METHODS

### Equipment and Materials

Analytical balance (Shimadzu), parchment paper, microscope, blender, laboratory glassware, aluminum foil, furnace, desiccator, hot plate, ash-free filter paper, oven, spatel, infusion pot, thermometer, micropipette, micropipette tip, eppendorf tube, ice gel, ice box, 96-well plate, pH meter (Hanna HI98107), refrigerator, silica gel thin-layer plate F254 (Merck), freeze dryer (Biobase), incubator (UN30, Memmert), UV-Visible spectrophotometer (Shimadzu), *Orthosiphon aristatus* (Blume) Miq white-purple variety obtained from PT Holistic Bio Medicine located in Purwakarta Regency at an altitude of 290 masl, stevia leaves (*Stevia rebaudiana Bertoni*) obtained from Bumi Herbal Dago located in Cimenyan Bandung at an altitude of 1.200-1.350 masl, sodium hydroxide, aquadestilata (Amidis), toluene analytical grade (Smart Lab), Mg powder, hydrochloric acid analytical grade (Emsure), ammonia, chloroform, chloralhydrate, mayer reagent, dragendorff reagent, gelatin, sulfuric acid, FeCl3, citric acid, boric acid, AlCl3, amyl alcohol, ether analytical grade (Smart Lab), quercetin (Sigma), potassium ferricyanide, trichloroacetic acid, oxalic acid.

**Research Procedure**

**Plant Identification**

Taxonomic identification of the plants was performed at the Herbarium Jatinangoriense, Biosystematics and Molecular Laboratory, Department of Biology, FMIPA Padjadjaran University. The results indicate that the plants used were *Orthosiphon aristatus* (Blume) Miq and *Stevia rebaudiana Bertoni*.

**Sample Processing and Storage**

Samples of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves were cleaned to remove dirt, washed under water flow, and dried. The drying of *Orthosiphon aristatus* (Blume) Miq herb was carried out by aeration in indirect sunlight for five days. The stevia leaves were dried in a drying cabinet at a temperature of ± 70°C for 10–12 hours and then pulverized with a blender until simplisia powder was obtained. The simplisia powder obtained was stored in a tightly closed container and protected from sunlight.

**Examination of Sample Characteristics**

Characteristic examination was performed on several parameters, including macroscopic examination, microscopic examination, determination of water content, determination of loss of drying, determination of water soluble extract content, determination of ethanol soluble extract content, determination of total ash content, determination of water soluble ash content, and determination of acid insoluble ash content.

**Phytochemical Screening**

Phytochemical screening was carried out on simplisia and extracts of white-purple varieties of *Orthosiphon aristatus* (Blume) Miq and stevia leaves to determine the presence of secondary metabolites, including alkaloids, flavonoids, polyphenols, tannins, quinones, saponins, monoterpenoids, sesquiterpenoids, steroids, and triterpenoids.

**Extraction**

The extraction of the white-purple variety of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves was carried out by the infusa method using water as a solvent. A total of 100 grams of sample was placed in an infusa pot, then 1 liter of water was added, heated at 90°C in an infusa pot for 15 minutes, and then filtered. The extraction was performed twice. The water extract was then dried using a freeze dryer until a dry extract was obtained.

**Extract Combination Formula**

The combination formula of the white-purple variety of *Orthosiphon aristatus* (Blume) Miq herb extract and stevia leaf extract is shown in Table I.

**Table I. Extract Combination Formula**

| Composition | Comparison | | |
|---|---|---|---|
| | **Formula 1** | **Formula 2** | **Formula 3** |
| *Orthosiphon aristatus* (Blume) Miq white-purple variety herb extract | 1 | 2 | 3 |
| Stevia leaves extract | 1 | 1 | 1 |

**Monitoring by Thin Layer Chromatography (TLC)**

The extracts of *Orthosiphon aristatus* (Blume) Miq herb white-purple variety and stevia leaves were observed by thin layer chromatography using a silica gel 60 $F_{254}$ plate. The plate was given an upper limit (0.5 cm) and a lower limit (1 cm), and the sample solution was bottled. The TLC plate was inserted into a chamber containing the appropriate mobile phase. The chamber lid was placed in place and the mobile phase was allowed to reach the upper limit. After the elution process ended, the TLC plate was taken, dried, and observed under UV light at 254 nm and 366 nm. The samples were sprayed with a 0.8% FRAP solution and observed visually. Subsequently, the appropriate specific spray agent was continued and observed again under UV light at 254 and 365 nm.

**Antioxidant Activity Test with FRAP Method**

**Preparation of Solution**

The solution that needs to be prepared is phosphate buffer solution 0.2 M pH 6.6, 1% oxalate solution, 1% potassium ferricyanide solution, 0.1% $FeCl_3$ solution, and 10% trichloroacetic acid (TCA) solution (Maryam *et al*., 2016).

**Antioxidant Activity Test**

**Determination of Maximum Wavelength**

Quercetin solution at a concentration of 20 ppm was pipetted to a volume of 1 mL, then 1 mL of 0.2 M phosphate-buffered saline (pH 6.6) and 2 mL of potassium ferricyanide 1% was put into a test tube and incubated for 20 minutes at 50 °C. after incubation the solution was added 1 mL of TCA, the solution was centrifuged at 3000 rpm for 10 minutes, then 1 mL of the top layer was taken then added 1 mL of distilled water and 0.5 mL of $FeCl_3$, enough with methanol p.a until the limit mark. The absorbance was measured with a UV-visible spectrophotometer that set the wavelength from 400 to 800 nm until the maximum wavelength was obtained (Rahayu *et al*., 2021).

**Determination of Operating Time**

The results of the maximum wavelength were continued with operating time testing to determine the time at which the reaction was most stable, and the absorbance was read at minutes 1 to 10 (Rahayu *et al*., 2021).

**Preparation of Blank Solution**

A total of 2 mL of phosphate buffer pH 6.6 and 2 mL of potassium ferricyanide were pipetted into a 10 mL volumetric flask and incubated for 20 minutes at 50°C. After incubation, 2 mL of TCA was added, and the mixture was sonicated for 10 minutes, after which 2 mL of the solution was pipetted and placed into a 10 mL volumetric flask, 2 mL of distilled water and 0.4 $FeCl_3$ were added, and the mixture was allowed to stand for 30 minutes. Absorbance was measured at the maximum wavelength (Muslikha, 2020).

**Preparation of Quercetin Solution of Various Concentrations by FRAP Method**

Weighed carefully 5 mg of quercetin and dissolved with methanol p.a in a 50 mL volumetric flask to obtain 100 ppm mother solution. Furthermore, 0.5 mL, 1 mL, 1.5 mL, 2 mL, 2.5 mL and 3 mL were pipetted respectively in a 10 mL volumetric flask to obtain concentrations of 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, and 30 ppm then from each concentration pipetted 1 mL, then added 1 mL of phosphate buffer pH 6.6 and 1 mL of 1% $K_3Fe(CN)_6$ solution was pipetted into a 10 mL volumetric flask then incubated at 50°C for 20 minutes the solution was added with 1 mL of TCA then if two phases were formed the solution was centrifuged at 3000 rpm for 10 minutes, after the centrifugation process was complete the top layer was pipetted as much as 1 mL into a 10 mL volumetric flask then allowed to stand again for 10 minutes then 1 mL of aqudes and 0.5 mL of $FeCl_3$ was added and sufficed with methanol p.a to the limit, then measured the sera of the concentration of 5 ppm, 10 ppm, 15 ppm, 25 ppm, and 30 ppm. a to the limit mark, and then measured the absorption at the maximum wavelength.

**Sample Absorption Measurement**

In the comparison of *Orthosiphon aristatus* (Blume) Miq herb extract and stevia leaves (1:1), (2:1), and (3:1), optimization was carried out first to obtain 6 concentration variations. Optimization was carried out with 3 concentrations: 10 ppm, 100 ppm, and 250 ppm. Next, 1 mL of sample solution was pipetted, 1 mL of 0.2 M phosphate buffer (pH 6.6), and 1 mL of $K_3Fe(CN)_6$ (1 %) were added, followed by incubation for 20 minutes at 50 °C. After incubation, 1 mL TCA was added, and if two phases were formed, the solution was centrifuged at 3000 rpm for 10 minutes. After centrifugation, 1 mL of the upper layer was pipetted into a test tube, and 1 mL of distilled water and 0.5 mL of $FeCl_3$ 0.1% were added. The solution was allowed to stand for 10 minutes and the absorbance was measured at the maximum wavelength. A mixture of phosphate buffer, potassium ferricyanide solution, and TCA solution was used as a blank. Calibration curves were prepared using various concentrations of quercetin solutions. The FRAP antioxidant capacity value is expressed as the *Quercetin Equivalent Antioxidant Capacity* (QEAC) value based on absorbance and

concentration data from the equation y = a + bx, and the antioxidant capacity value can be calculated using the following formula (Utami, 2020):

$$\text{Capacity Antioxidant} = \frac{C \times V \times df \times 10^{-3}}{\text{initial sample weight}}$$

Notes:
C = sample concentration or x value (mg QE/L)
V = volume of extract used (mL)
df = dilution factor

**Data Analysis**

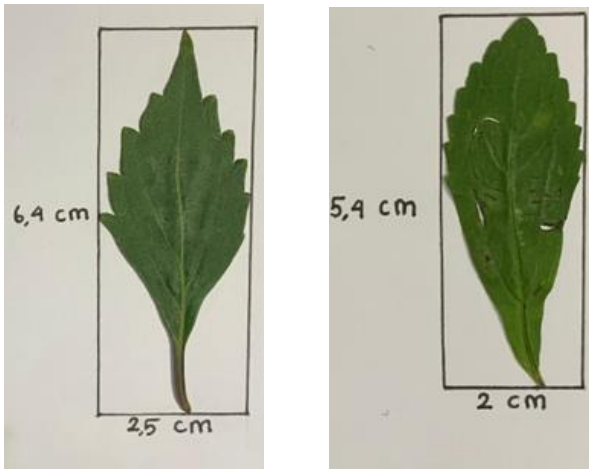
The antioxidant measurement data were analyzed using SPSS version 29.0.2.0 application to determine whether there were differences in the antioxidant capacity values produced in each combination formula of *Orthosiphon aristatus* (Blume) Miq herb extract and stevia leaf extract using one-way ANOVA. From the statistical analysis of the one-way ANOVA of the three combinations of extracts with formulas 1:1, 2:1, and 3:1. If the data showed significant differences with the results of p-values < 0.05, namely 0.001 (p <0.05). The data differences were then clarified using Tukey's test.

**RESULTS AND DISCUSSION**

An examination of the simplisia characteristics was performed to determine the specific and non-specific criteria of the material used. The specific parameters were examined macroscopically and microscopically. Non-specific parameters include determining the total ash content, water-soluble ash content, acid-insoluble ash content, water-soluble extract content, ethanol-soluble extract content, water content, and loss on drying. Macroscopic examination was carried out on fresh plants and simplisia from *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves, including shape, color, odor, and taste. Macroscopic examination of *Orthosiphon aristatus* (Blume) Miq leaves was performed according to (Depkes RI, 2017). In a previous study, the stevia leaves were oval-shaped, the edges of the leaves were finely serrated, and the leaves were pinnate (Ratnani & Anggraeni, 1985). The results of the macroscopic examination are presented in Table II.

**Table II. Results of Macroscopic Examination of Fresh Plants and Simplisia of *Orthosiphon aristatus* (Blume) Miq and Stevia Leaves**

| Check | Sample | Description | |
|---|---|---|---|
| | | ***Orthosiphon aristatus* (Blume) Miq** | **Stevia Leaves** |
| Shape | Fresh | The leaves blade is ovoid, pinnate, leaves surface is hairy, long ±6.1 cm and wide ±2.3 cm | The leaves blade is ovoid, pinnate, the leaves surface is downy, the length is long ±5.2 cm and wide ±2.1 cm |
| | Simplisia | Fine powder | Fine powder |
| Color | Fresh | Purple, white, dark green | Dark green |
| | Simplisia | Dark green | Dark green |
| Odor | Fresh | Odorless | Odorless |
| | Simplisia | Odorless | Odorless |
| Taste | Fresh | Bitter | Sweet |
| | Simplisia | Bitter | Sweet |




(a) (b)

**Figure 1. Macroscopic examination results of *Orthosiphon aristatus* (Blume) Miq leaves (a) and Stevia leaves (b)**

Microscopic examination was performed on fresh plants and simplisia from *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves to identify fragments in the sample. This was performed using a light microscope with the addition of chloralhydrate, which aims to purify the cell contents so that the identifying fragments are more clearly visible when observed by a microscope. Observations were made at 100x magnification. The results of the microscopic examination of fresh plants from *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves are shown in Figure 2.

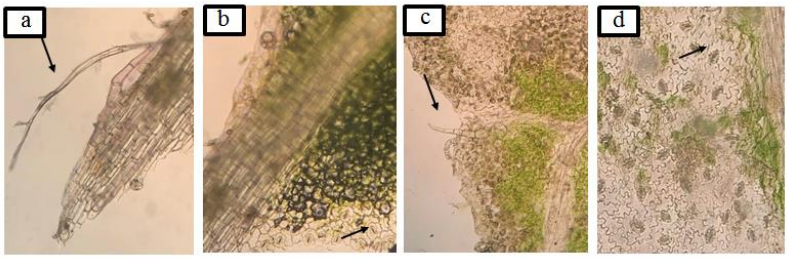



**Figure 2. Microscopic examination results of fresh plants of *Orthosiphon aristatus* (Blume) Miq herb and Stevia leaves** (a) multicellular-type covering hairs of *Orthosiphon aristatus* (Blume) Miq herb (b) upper epidermis with *Orthosiphon aristatus* (Blume) Miq herb diasitic-type stomata (c) multicellular-type covering hairs of Stevia leaves (d) anomocytic-type stomata of Stevia leaves

One of the abilities of plants is photosynthesis. Plant photosynthesis by reducing $CO_2$ and increasing $O_2$ levels. The ability of plants to absorb carbon dioxide and produce oxygen is highly dependent on their stomata. Stomata are a derivate of the epidermis that functions as a place of oxygen exchange (Kirana *et al*. 2022). Stomata found in a plant have certain types of stomata. *Orthosiphon aristatus* (Blume) Miq is a diasitic type. Diasitic stomata are two neighboring cells that are perpendicular to the guard cell. In stevia leaves, the stomata are anomocytic. The anomocytic type of covering cell is surrounded by several cells that do not differ in size or shape from other epidermal cells (Salira & Chatri 2021). The microscopic results for fresh simplisia from *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves are shown in Figure 3.

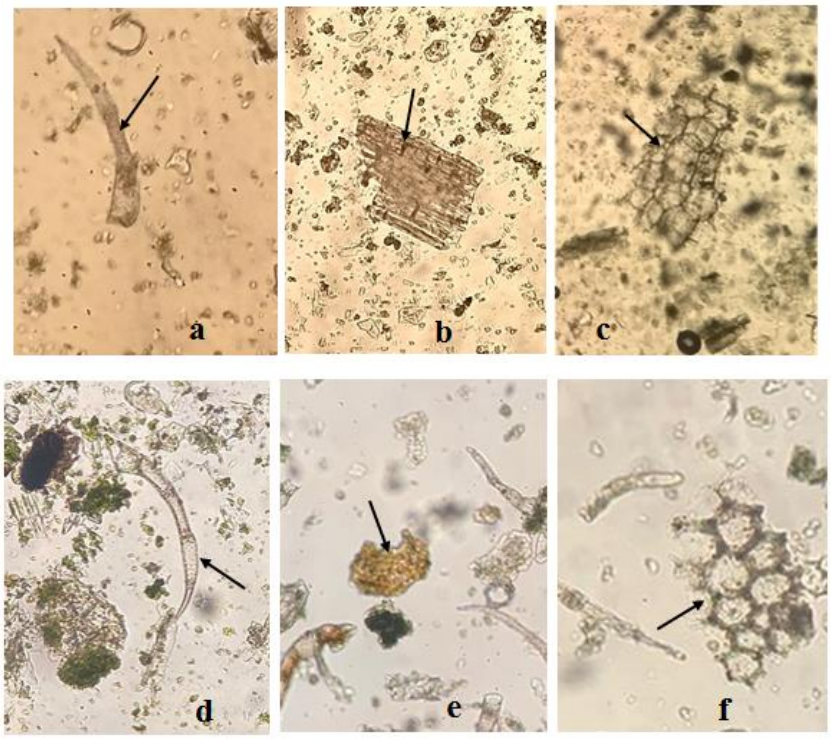



**Figure 3. Microscopic examination results of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves simplisia** (a) *Orthosiphon aristatus* (Blume) Miq herbaceous covering hairs , (b) parenchymal tissue of *Orthosiphon aristatus* (Blume) Miq herb, (c) polygonal epidermal tissue of *Orthosiphon aristatus* (Blume) Miq herb, (d) hair covering of stevia leaves, (e) Stevia leaves oil cells, dan (f) polygonal epidermal tissue of stevia leaves

Standardization of *Orthosiphon aristatus* (Blume) Miq herb and Stevia leaves was carried out to ensure the quality and safety of simplisia as a research raw material. Standardization of simplisia includes the total ash content, water soluble ash content, acid insoluble ash content, water soluble extract content, ethanol soluble extract content, water content, and loss during drying. The results of the standardization are shown in Table III.

**Table III. Simplisia Standardization Results of *Orthosiphon aristatus* (Blume) Miq Herb and Stevia Leaves**

| Parameters | *Orthosiphon aristatus* (Blume) Miq Herb | | Stevia Leaves |
|---|---|---|---|
| | Result | Literature (FHI) (Depkes RI., 2017) | Result |
| Total ash content (%w/w) | 12.34±0.13 | ≤10.2 | 9.25±0.18 |
| Water soluble ash content (%w/w) | 7.63±0.61 | - | 4.36±0.07 |
| Acid insoluble ash content (%w/w) | 4.10±0.20 | ≤3.4 | 2.75±0.07 |
| Water soluble extract content (%w/w) | 28.41±0.65 | ≥10.2 | 39.41±0.30 |
| Ethanol soluble extract content (%w/w) | 5.08±0.10 | ≥7.2 | 33.45±0.50 |
| Water content (%v/w) | 3.79±0.52 | ≤10 | 0.83±0.28 |
| Loss on drying (%w/w) | 4.90±0.006 | ≤10 | 3.40±0.58 |

The total ash content was examined to determine the remaining non-vaporized ash from the simplisia during combustion. In determining the total ash content, ash can come from parts of the plant tissue itself or from other impurities, such as sand or soil. The higher the ash content, the lower the quality (Name *et al*., 2021). water soluble ash content was determined to determine the content of internal minerals, such as magnesium, sodium, and calcium. The acid-soluble ash content was determined to determine the content of internal minerals in the simplisia such as magnesium, sodium, and calcium. The acid insoluble ash content was determined to determine the content of external minerals in simplisia, such as mercury, lead, and other impurities.

Determination of water soluble extract content and ethanol soluble extract content aims to determine the amount of compound content in simplisia that can be attracted by water and ethanol. From the results of the study that the water soluble extract content was greater than the ethanol soluble extract content, it is suspected that the secondary metabolite compounds in *Orthosiphon aristatus* (Blume) Miq herb simplisia and stevia leaves simplisia are more soluble in water.

Water content determination aims to provide an overview of the water content of simplisia. The water content was determined by azeotrop distillation using a toluene reagent that had previously been saturated with water for 24 hours. The purpose of this saturation process is to ensure that the water content contained in simplisia is not attracted by toluene, so that the resulting water content is the actual content contained in simplisia.

Determining the loss of drying aims to determine the amount of compounds lost during the drying process. Simplisia that experiences high drying shrinkage indicates that it contains excessive and unstable water (Sinaga, 2021).

Simplisia was extracted using the infusa method. Infusa is an extraction method that uses water as the polar solvent. The same compound will be more easily dissolved in solvents that have the same level of polarity, so infusa on *Orthosiphon aristatus* (Blume) Miq and stevia is an effective way to obtain compounds such as flavonoids because these compounds can dissolve in water solvents. The extract was freeze dried. The dry extract of *Orthosiphon aristatus* (Blume) Miq herb was obtained in as much as 100 grams with a percentage yield of 9.68%, and the dry extract of stevia leaves was obtained in as much as 100 grams with a percentage yield of 10.61%.

The results of measuring the specific gravity of the extract showed that the specific gravity of *Orthosiphon aristatus* (Blume) Miq herb extract and stevia leaves were 1.003 ± 0.0001 g/mL and 1.005 ± 0.0005 g/mL, respectively. A specific gravity test of this extract was carried out to determine the limit of mass per unit volume, which is a special parameter of concentrated extracts that can still be poured (Depkes RI, 2017).

Phytochemical screening of simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves aims to provide an overview of the class of secondary metabolite compounds contained in simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves. The results of phytochemical screening are shown in Table IV.

**Table IV. Phytochemical Screening Results of *Orthosiphon aristatus* (Blume) Miq Herb and Stevia Leaves**

| Compound | Reagents | *Orthosiphon aristatus* (Blume) Miq Herb | | Stevia Leaves | |
|---|---|---|---|---|---|
| | | Simplisia | Extract | Simplisia | Extract |
| Alkaloids | Mayer | - | - | - | - |
| | Dragendorf | - | - | - | - |
| Flavonoids | Mg powder, HCl 2N, Amyl Alcohol | + | + | + | + |
| Tannins | Gelatin | - | - | - | - |

| Compound | Reagents | *Orthosiphon aristatus* (Blume) Miq Herb | | Stevia Leaves | |
|---|---|---|---|---|---|
| | | Simplisia | Extract | Simplisia | Extract |
| | Steasny | - | - | - | - |
| Polyphenols | $FeCl_3$ | + | + | + | + |
| Saponins | Dilute HCl | + | + | + | + |
| Quinones | KOH 5% | + | + | + | + |
| Steroid-triterpenoids | Liebermann-Bourchard | + | + | + | + |
| Monoterpenes-seskuiterpenes | Vanilin-Sulfate | + | + | + | + |

Notes:
(+) The presence of a class of secondary metabolite compounds is detected
(-) The presence of secondary metabolite compounds was not detected

The flavonoid group tested positive for the formation of a red-orange ring in the amyl alcohol layer after reaction with Mg powder and 2N HCl. The addition of Mg powder and concentrated HCl reduced the benzopyrone core contained in the flavonoid structure, and Mg was used to activate flavonoids in the extract. Flavonoids react with Mg to form flavilium salts that are orange or red in color (Suhendar & Sogandi, 2019). Previous studies have reported that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herbs and stevia leaves contain flavonoids (Faramayuda *et al*., 2021; Irdina & Santoso, 2018). The current study shows that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq and stevia leaves contain flavonoids.

In the polyphenol test, the $FeCl_3$ reagent was added to the samples. A positive reaction is characterized by the formation of green, blue, and black colors. Previous studies have reported that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves contain polyphenols (Faramayuda *et al*., 2021; Irdina & Santoso, 2018). The current study shows that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq and stevia leaves contain polyphenols.

In the saponin test, foam or froth was formed after shaking and adding 2N hydrochloric acid. The formation of foam is due to the presence of glycosides that have the ability to form foam in water, which is hydrolyzed into glucose and other compounds. Hydrolysis occurs due to the addition of HCl, which breaks the glycoside group in *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves (Mien *et al*., 2015). Previous studies have reported that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves contain saponins (Faramayuda *et al*., 2021; Irdina & Santoso, 2018). The current study shows that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herbs and stevia leaves contain saponins.

Examination of quinone compounds using a 5% KOH reagent showed positive results because of the yellow to reddish color change. KOH reagent was used because it can bind to the phenol groups of quinones to form phenolate ions. Previous studies have reported that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herb and stevia leaves contain quinones (Faramayuda *et al*., 2021; Irdina & Santoso, 2018). The current study shows that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq and stevia leaves contain quinones.

In the steroid-triterpenoid test, the presence of these compounds is indicated by the appearance of a bluish green color with Liebermann-Bouchard reagent for steroids and purple color for triterpenoids. Previous studies have reported that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herbs and stevia leaves contain steroids and triterpenoids (Faramayuda *et al*., 2021; Irdina & Santoso, 2018). The current study shows that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herbs and stevia leaves contain steroids and triterpenoids.

In the monoterpenoid-sesquiterpenoid test, vanillin sulfate reagent was used, which was detected through color. Previous studies have reported that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herbs and stevia leaves contain monoterpenoids and sesquiterpenoids (Faramayuda *et al.*, 2021; Irdina & Santoso, 2018). The current study shows that simplisia and extracts of *Orthosiphon aristatus* (Blume) Miq herbs and stevia leaves contain monoterpenoids and sesquiterpenoids.

Furthermore, qualitative antioxidant testing was performed as an initial detection of the antioxidant activity of the extract to determine the presence of antioxidant activity. Thin-layer chromatography (TLC) was used as a qualitative method. In the presence of fluorescence and retention factor (Rf) values, this method can help identify the class of compounds that are likely to release antioxidant activity. The Rf value obtained is the ratio of the elution distance between the sample and mobile phase on TLC. The Rf values were measured to compare the specific types of compounds detected in the samples. The stationary phase used was silica gel 60 F254 with a mobile phase of chloroform-ethyl acetate in a ratio of 15:3:2. The mobile phases were chosen because they have different polarities, which allows them to separate and identify the compounds contained in the extract.

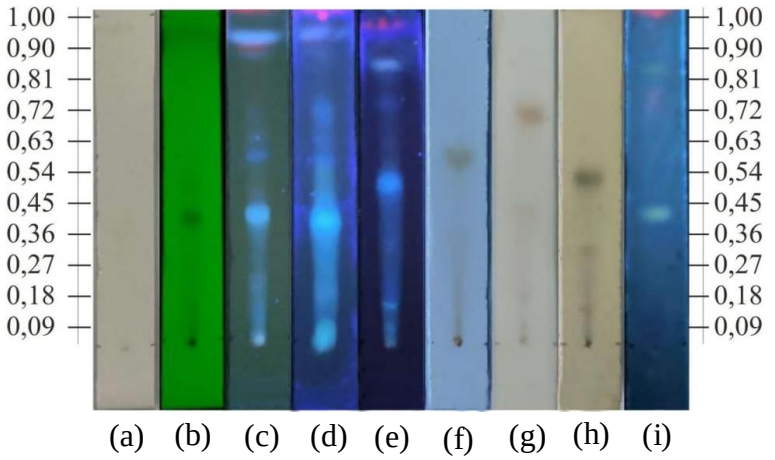



**Figure 4. TLC pattern of *Orthosiphon aristatus* (Blume) Miq herb extract with silica gel 60 $F_{254}$ and ethyl acetate-methanol-water mobile phase (15:3:2)**

Notes:
(a) Visual observation
(b) Observation under UV 254 nm
(c) Observation under UV 366 nm
(d) Observation with sitroborate spotting agent after heating under UV 366 nm
(e) Observation with $AlCl_3$ spotting agent after heating under UV 366 nm
(f) Observation with KOH spotting agent after heating visually
(g) Observation with $H_2SO_4$ spotting agent after heating visually
(h) Observation with $FeCl_3$ spotting agent after heating visually
(i) Observation with 0.8% FRAP spotting agent after heating under UV 366 nm

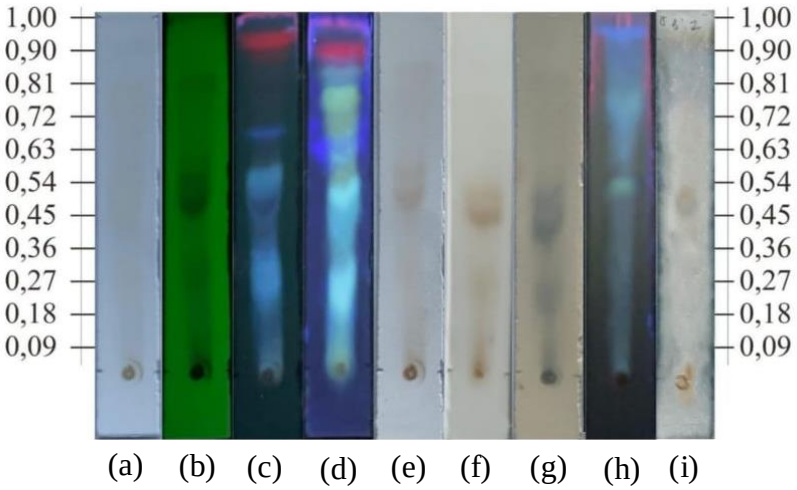



**Figure 5. TLC pattern of Stevia leaves extract with silica gel 60 $F_{254}$ and ethyl acetate-methanol-water mobile phase(15:3:2)**

Notes:
(a) Visual observation
(b) Observation under UV 254 nm
(c) Observation under UV 366 nm
(d) Observation with $AlCl_3$ spotting agent after heating under UV 366 nm
(e) Observation with KOH spotting agent after heating visually
(f) Observation with $H_2SO_4$ spotting agent after heating visually
(g) Observation with $FeCl_3$ spotting agent after heating visually
(h) Observation with 0.8% FRAP spotting agent after heating under UV 366 nm
(i) Observation with Vanillin Sulfate spotting pen after heating visually

On monitoring with TLC can be seen in Figure 4 there are flavonoid compounds marked on the spot of FRAP 0.8% with a blue spot under UV lamp 366 nm with an Rf value of 0.43 which is parallel to the cytroborate spot. This indicates that there are flavonoid compounds that are thought to play a role in antioxidant activity.

Furthermore, by monitoring with TLC, as shown in Figure 5, there are flavonoid and polyphenol compounds characterized by a 0.8% FRAP spot with a light blue spot under a UV lamp of 366 nm with an Rf value of 0.54, which is parallel to that of $AlCl_3$ and $FeCl_3$. This demonstrates that there are flavonoid and polyphenol compounds that are thought to play a role in antioxidant activity.

In addition, in Figure 5, there are compounds of the terpenoid group, which are indicated by the presence of orange spots at Rf 0.53. This shows that the secondary metabolite compound content of the terpenoid group, which is thought to play a role in antioxidant activity, is a compound of stevioside that is included in the diterpene glycoside group.

From the results of TLC monitoring of *Orthosiphon aristatus* (Blume) Miq herb extracts and stevia leaves, it can be seen from the two figures that the distribution of metabolites detected in each mobile phase, both flavonoids and polyphenols, are thought to be antioxidants. Therefore, it is believed that the three samples qualitatively provide positive results.

After confirming that both samples showed antioxidant activity qualitatively, quantitative antioxidant activity was tested using a UV Visible spectrophotometer with the Ferric Reducing Antioxidant Power (FRAP) method from both samples combined with the results shown in Table V.

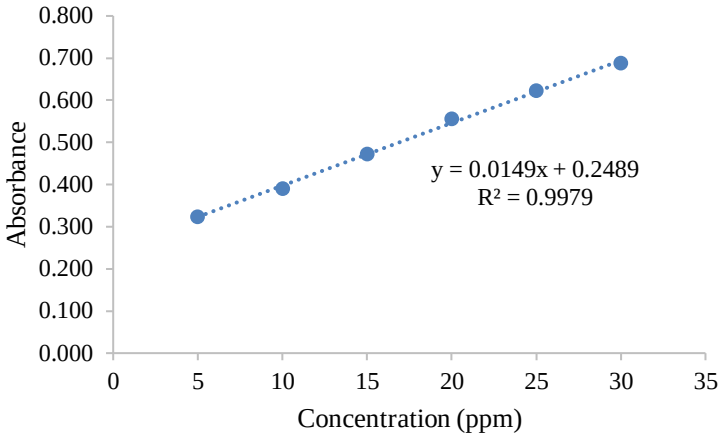



**Figure 6. Quercetin standard curve**

**Table V. Results of Quantitative Testing of Antioxidant Activity**

| Sample | Mean Antioxidant Capacity±SD (mg QE/g ekstrak) |
|---|---|
| Quercetin | 397.002±13.44* |
| *Orthosiphon aristatus* (Blume) Miq : Stevia Leaves (1:1) | 43.725±5.32* |
| *Orthosiphon aristatus* (Blume) Miq : Stevia Leaves (2:1) | 57.963±0.52* |
| *Orthosiphon aristatus* (Blume) Miq : Stevia Leaves (3:1) | 64.849±1.77* |

Notes:
*significantly different at *p value* <0.05, n=3

Quantitative testing of antioxidants in the combination extracts was carried out at a concentration of 500 ppm. The comparator used for the analysis of free radical capture was quercetin; therefore, the results of the study were calculated to be equivalent to quercetin as a comparator. Before testing the antioxidant activity of the sample, the maximum wavelength of FRAP and the operating time were determined. In a previous study, the maximum wavelength was 700 nm with an absorbance of 0.380 nm (Maesaroh *et al.*, 2018). In the current study, antioxidant activity testing against quercetin at a concentration of 20 ppm yielded a maximum wavelength of 710 nm, with an absorbance of 0.584. Different wavelength results can be caused by different tool conditions, tool differences, sample quality, reagent purity, and type of UV-Visible spectrophotometer used (Rahayu *et al.*, 2021). The operating time was determined at minutes 0–30; then, the absorbance was calculated every 1 minute from the results of the operating time measurements; starting from minute 0 to minute 10, the absorbance remained stable. The selected absorbance was measured for 1 minute.

In the measurement of quercetin comparison solutions at concentrations of 5, 10, 15, 20, 25, and 30 ppm, a linear regression equation was obtained, namely y = 0.0149x + 0.2489 with an R value of 0.9979. The results of the quercetin standard curve determination showed that the concentration correlated with the absorbance value. The higher the concentration of the quercetin standard solution, the greater the absorbance value. To determine the antioxidant activity of the combined extract using the FRAP method, the antioxidant capacity value was calculated. The FRAP value was expressed as mg quercetin equivalent/gram extract (QE). The antioxidant content of each replicate was expressed as quercetin equivalent (QE).

In a previous study, the results of antioxidant activity of a single methanol extract with the FRAP method on *Orthosiphon aristatus* (Blume) Miq with a value of 56.37 mg TE/g DW were obtained (Febriyanto *et al*., 2024). The single water extract in stevia leaves amounted to 38.24 mg TE/g DW (Ameer *et al*., 2020). After the research was conducted, the results of the antioxidant capacity of the combined extracts of *Orthosiphon aristatus* (Blume) Miq and stevia leaves, which gave the amount of antioxidant capacity from the highest to the lowest in a row, namely the combination extract F3 (3:1) of 64.849 mg QE/g extract, F2 (2:1) of 57.963 mg QE/g extract, and combination extract F1 (1:1) of 43.725 mg QE/g extract. In this study, the best activity was observed in the combination (3:1), showing that the higher the content of *Orthosiphon aristatus* (Blume) Miq herb extract in combination, the better the antioxidant activity.

Based on a literature search, it was found that antioxidant activity was correlated with the content of phenolic compounds in the sample. The higher the total phenolic value contained in the sample, the higher the antioxidant activity value in the sample (Kamarudin *et al*., 2016). The stems and leaves of *Orthosiphon aristatus* (Blume) Miq with water extract have a total phenol value of 7.82 mg GAE/g and 26.43 mg GAE/g, respectively (Arif *et al*., 2022) while the stevia leaves plant with water extract has a total phenol value of 6.50 mg GAE/g (Arumsari *et al*., 2019). In previous tests, antioxidant activity has been tested using the FRAP method using a combination of grape (*Vitis vinifera L*.) and pomegranate (*Punica granatum*) extracts (Chayaningtyas, 2021). The best antioxidant activity research results conducted by Chayaningtyas (2021) have a value of 166.242 mgAAE/g sample while the highest total phenol results have a value of 52.59 mg GAE/g extract. It can be concluded that the antioxidant activity value obtained in this study is directly proportional to the total phenolic test in existing studies.

The results of the antioxidant capacity value can be analyzed statistically using SPSS to determine if there is a significant difference in the antioxidant capacity value in each formulation of the combination of *Orthosiphon aristatus* (Blume) Miq herb extract and stevia leaves extract. The results of the one-way ANOVA test showed a significant difference with a p value smaller than 0.05, which was 0.001 ($p<0.05$). Furthermore, the tukey test was conducted to determine the existence of significant differences in each formula. The results show that there are significant differences in each formula 1:1, 2:1, 3:1 because each sample is located in a different subset, so it can be concluded that each sample or formula has a significantly different antioxidant capacity value or there is a significant effect on the combination treatment of *Orthosiphon aristatus* (Blume) Miq herb extract and stevia leaves extract on antioxidant activity.

## CONCLUSION

Based on the research that has been done, it shows that the water extract of *Orthosiphon aristatus* (Blume) Miq herb white-purple variety and *Stevia rebaudiana Bertoni* leaves extract produce a synergistic effect. The best antioxidant activity was found in the combination of F3 (3:1) at 64.849 mg QE/g extract, this shows that the higher the content of *Orthosiphon aristatus* (Blume) Miq herb extract in the combination, the better the antioxidant activity. Antioxidant activity in *Orthosiphon aristatus* (Blume) Miq herb extract and stevia leaves extract is thought to contain secondary metabolites flavonoids and polyphenols.

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