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ANALYSIS OF MAJOR SECONDARY METABOLITES IN TWO VARIETIES OF MEDICINAL PLANT (*Orthosiphon aristatus* BLUME MIQ)

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ABSTRACT

Cat's Whiskers (Orthosiphon aristatus) is one of the well-known medicinal plants that have been utilized for medical purposes. The flower morphology of *O. aristatus* plants generally reveals their diversity. There are no reports of secondary metabolite differences between the purple and white-purple varieties of *O. aristatus*. To maximize the use of the two varieties of *O. aristatus* as herbal medicines, qualitative and quantitative analyses of secondary metabolites were performed on both varieties. Analyses of phytochemical profiles were conducted using high-performance liquid chromatography (HPLC) with a mobile phase (0.1% formic acid-acetonitrile), the concentrations of active compounds such as rosmarinic acid, sinensetin, and eupatorin were determined. The purple variety's ethanolic extract of its leaves contained rosmarinic acid. Sinensetin concentrations in ethyl acetate and ethanol extracts of purple variety leaves were 2.13 and 3.07% w/w, respectively. In comparison, both white-purple leaf extracts contained up to 0.95 and 1.82 percent sinensetin by weight, respectively. The purple variety leaves' ethanol extract contained the highest concentration of rosmarinic acid at 2.21 % w/w, while the white-purple variety leaves' ethyl acetate extract contained the highest concentration of eupatorin at 3.70 % w/w. Despite belonging to the same species, the composition of the two varieties of *O. aristatus* differed significantly. The concentration of each active compound, such as sinensetin, rosmarinic acid, and eupatorine, must be considered when *O. aristatus* is used to prepare herbal medicines.

Keywords: *Orthosiphon aristatus*, Purple Varieties, White-Purple Varieties, Rosmarinic Acid, Sinensetin, Eupatorin.
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INTRODUCTION

Othosiphon aristatus (Blume) Miq. (Family Lamiaceae) the plant was spread from India, Indonesia, and Thailand, passing through Malaysia to Australia's tropics. As a wild plant, it can grow throughout Malaysia, but its presence is rare in Kalimantan, Sulawesi, and Maluku.[1] Some of the pharmacological activities of *O. aristatus* that have been reported are diuretics[2,3,4], treatment of overcome gastric disorders[5,6], analgesic, and antipyretic[7], antidiabetic[8,9], antihypertensive[10], hepatoprotective effect[11,12], antimicrobial activity[13-15], antioxidants[16,17], enhancing memory[18], anti-epilepsy[19], treating cardiovascular disorders[20], rheumatoid treatment, and osteoarthritis arthritis[21], prevention, and treatment of cancer[22,23], antiviral.[24] Clinical studies of *O. aristatus* have been carried out. Clinical studies of *O. aristatus*.[25] The varieties of *O. aristatus* that grow in Indonesia are purple-flowered, white-flowered, and white-purple-colored *O. aristatus*.[26] *O. aristatus* contains several pharmacologically active compounds; the most important compounds are the phenol group. The main flavonoid isolated from *O. aristatus* leaf extract were sinensetin, eupatorin, and 3'-hydroxy-5,6,7,4'- trimethoxy flavones.[27] Sinensetin is a bioactive substance with multiple biological properties.[28] Eupatorin has anticancer properties.[29] Similarly, rosmarinic acid is appealing to the pharmaceutical, food, and cosmetics industries.[30]

The quantitative analysis determined the concentrations of five secondary metabolites in *O. aristatus* samples from different batches. The highest average rosmarinic acid content is 2.83 mg/g.[31] The qualitative and quantitative secondary metabolite analysis of the purple and white-purple *O. aristatus* varieties has not been reported. This study aimed to provide new information regarding the comparison of secondary content

metabolites in the purple and white-purple varieties and to maximize the potential medicinal use of the white-purple variety, which is seldom used in traditional medicine.

EXPERIMENTAL

Plant material and Chemicals

Purple and white-purple *O. aristatus* leaves and stems were collected in West Bandung, Indonesia.

Chemicals and Reagents

Sinensetin (Sigma-Aldrich® Singapore), rosmarinic acid (Sigma-Aldrich® Singapore), eupatorin ((Sigma-Aldrich® Singapore), formic acid (Loba Chemie® India), acetonitrile HPLC grade (Merck®), methanol HPLC grade (Merck® Germany) and aquadest

Extraction of Plant Material

Two varieties of *O. aristatus* containing 100 g of leaves and stems were transferred to macerators and macerated for 24 hours with 1.5 L of the appropriate solvent. The filtrate was collected, evaporated using a rotary evaporator, and concentrated using water baths to produce a thick extract. The extract was then weighed and the yield of concentrated extract was calculated as a percentage.

HPLC Analysis of Extracts

Preparation of the Standard and Samples Solutions

Stock solutions (1 mg/mL) of rosmarinic acid, sinensetin, and eupatorine were prepared in HPLC-grade methanol. The stock solution was diluted with methanol into five concentrations ranging from 60 to 100 g/mL to produce standard working solutions. The acetone, ethyl acetate, and ethanol extracts of two types of *O. aristatus* were prepared as sample solutions by dissolving 15 mg of each extract in 1 mL of methanol and sonicating for 45 minutes. Following filtration with syringe filters, the sample solutions were transferred to HPLC vials.

Instrumentation and Chromatographic Conditions

The instrument utilized was an HP-Agilent HPLC equipped with a gradient. A chromatographic analysis was executed using a C18 reverse-phase column. The temperature of the column was maintained at 25 degrees Celsius. As shown in Table-1, the mobile phase consisted of 0.1% formic acid solution and acetonitrile with a radiant elution system and 1 ml/min flow rate. The separation time was twenty minutes, and the injection volume was twenty liters. The method described by Saidan *et al.* (2015) for determining the concentration of secondary metabolites, with a maximum wavelength of 340.6 nm. Table-1 HPLC gradient mobile phase system for separation of secondary metabolites in two Orthosiphon aristatus varieties.[32]

Table-1: Gradient Mobile Phase HPLC System

Time	Ratio Solvent		Flow Rate
	Formic Acid 0,1 %	Acetonitrile	
0	85	15	1ml/min
1	85	15	1ml/min
12	35	65	1ml/min
15	85	15	1ml/min
18	85	15	1ml/min

Data Analysis

For the purpose of determining the concentrations of the primary and secondary metabolites using HPLC, each test sample was produced in triplicate. The data are presented as the mean and standard deviation. Using the SPSS 22 program, a one-way ANOVA and Duncan's multiple range test were performed on the data. Statistics were considered significant for P values under 0.05.

RESULTS AND DISCUSSION

Determination and Morphology

The determination identifies two varieties of O. aristatus: purple *O. aristatus* and white-purple *O. aristatus*. The components of the flowers show how the two species differ from one another (Fig.-1). While the petals

of the white-purple variety are green, the crown is white, the pistil stalk is tinged purple, and the stamens are tinged white with purple, the petals of the purple variety are green-purple, the crown is white, the pistil stalk is tinge purple, and the stamens are purple.

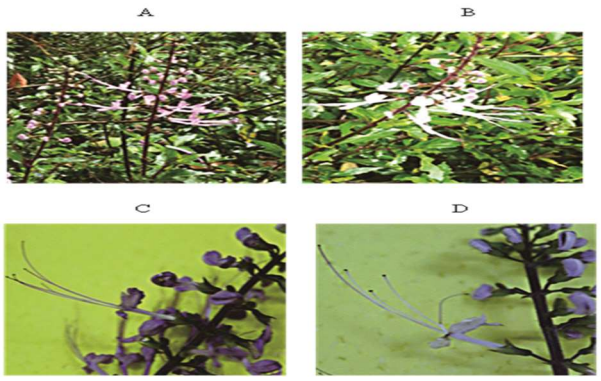

Fig.-1. Plant *O. aristatus* Purple Variety (a), White-Purple Variety (b), the Flower of Purple Varieties (c), and Flower of White-Purple Variety (d)

Observation of plant morphology or sources of traditional medicinal ingredients is essential to ensure the plants' correctness and authenticity. Morphological observations in this study are essential to distinguish purple and white-purple varieties because there is a possibility of differences in the secondary metabolites of the two varieties. Research comparing the morphology of purple, white-purple, and white varieties has also been done by Febijslami *et al.* (2019), Keng and Siong (2016), and Almater *et al*. (2013).[32-35] According to another study, secondary metabolites in the purple variety of *O. aristatus* are more significant than those in the white variety. However, secondary metabolite levels in purple and white-purple varieties have not been compared. The purple and white-purple varieties had rhombic-shaped leaves, consistent with what Keng and Siong reported in 2006.[34] The leaf color of the two *O. aristatus* varieties did not differ from what Almater *et al.* (2013)[35] reported. The color of purple flower petals is identical to what Keng and Siong (2016) reported, whereas the white-purple variety is white. The purple variety has a purple crown, while the white-purple variety has a white crown. The flower morphology is the most fundamental difference between these two varieties. The morphology of stems, leaves, and flowers of Lamiacea species exhibits both differences and similarities.

The Percentage Yield of the Extracts

A total of 1000 grams of fresh ingredients of purple and white varieties of *O. aristatus* and purple varieties were taken and dried in a drying cabinet at a temperature of 60ºC. The yield of raw material for fresh ingredients is shown in Table-2.

Table-2: The Yield of Raw Material on Fresh Ingredients of Two Varieties of *O. aristatus*.

No	Sample	Fresh Weight (g)	Weight of Raw Material (g)	% Yields (b/b)
1	Purple variety of *O. aristatus* leaves	1000	325	32.5
2	Purple variety of *O. aristatus* stem	1000	234	23.4
3	White-purple varieties of *O. aristatus* leaves	1000	272	27.2
4	White-purple variety of *O. aristatus* stem	1000	205	20.5

In the purple variety, LP3 gave the highest percentage of yield (27.2 % w/w) followed by LP2 (25.6 % w/w) and LP1 (18.5 % w/w) while in the white-purple varieties leaves the LWP3 (27.3 % w/w) followed by LWP2 (19.7 % w/w) and LWP1 (17.5 % w/w). SP3 gave the highest percentage of yield (16.2 % w/w) in the purple varieties of *O. aristatus*, followed by SP2 (14.9 % w/w) and SP1 (12.8 % w/w). The percent

yield of the white-purple variety *O. aristatus* is SWP3 (16.9 % w/w), SWP2 (11.7 % w/w), and SWP1 (10.7 % w/w). Ethanol solvent gives the highest yield, followed by ethyl acetate and acetone. Leaves give a higher yield than stems. The yield of ethanol extract from white leaves is the same as the purple. The percentage yield of the leaves of *O. aristatus* extract was presented in Table-3.

Table-3: Percentage yield of the *O. aristatus* extract

Extract	Mass (g)	Yield (% w / w)
LP1	18.5	18.5
LP2	25.6	25.6
LP3	27.2	27.2
LWP1	17.5	17.5
LWP2	19.7	19.7
LWP3	27.3	27.3
SP1	12.8	12.8
SP2	14.9	14.9
SP3	16.2	16.2
SWP1	10.7	10.7
SWP2	11.7	11.7
SWP3	16.9	16.9

LP1: leaves acetone, LP2: leaves ethyl acetate extract purple, LP3: leaves ethanol extract purple, LWP1: leaves acetone extract white-purple, LWP2: leaves ethyl acetate extract white-purple, LWP3: leaves ethanol extract white-purple, SP1: stem acetone extract purple, SP2: stem ethyl acetate extract purple, SP3: stem ethanol extract purple, SWP1: stem acetone extract white-purple, SWP2: stem ethyl acetate extract white-purple, SWP3: stem ethanol extract white-purple.

Qualitative Analysis Using HPLC

In this HPLC condition, rosmarinic acid has a retention time of 6 min, sinensetin around 10 min, and eupatorin around 13 min. Chromatogram of acetone, ethyl acetate, and ethanol extracts from leaves and stems of two varieties of *O. aristatus* can be seen in Fig.-3, in this LWP1, LP1, LWP2, LP2 sinensetin were detected. Rosmarinic acid was detected in LP1, LP2.LP3, LWP3. Eupatorin was detected on LWP1, LWP2, and LWP3 (Fig-3).

Quantitative Analysis Using HPLC

Ethyl acetate and ethanol leaf extracts of the purple variety contained sinensetin at 2.13 % w/w and 3.07 % w/w, respectively, whereas in leaves of the white-purple variety 1.95 % w/w and 1.82 % w/w. The highest levels of rosmarinic acid with a value of 2.21 % w / w were found in the ethanol extract of the leaves of the purple varieties, and the highest eupatorine content with a value of 3.70 % w/w was found in the ethyl acetate extract of the leaves in the white-purple variety (Table-4 and Fig.-2).

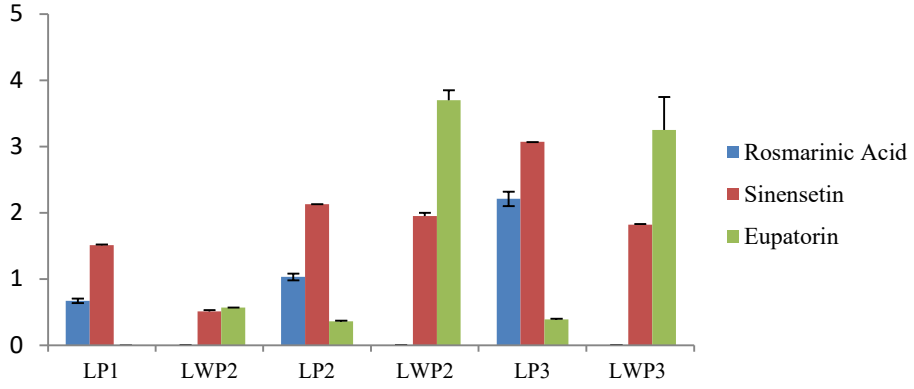

Fig.-2: Comparison of Rosmarinic Acid, Sinensetin, and Eupatorin Acid Extracts of Two Varieties of *O. aristatus*

Table-4: Levels of Rosmarinic Acid, Sinensetin, and Eupatorin from Acetone, Ethyl Acetate, and Ethanol Extracts of Two Varieties of *O. aristatus*

Sample	Rosmarinic Acid (% w/w) ±SD (n=3)	Sinensetin (% w/w) ±SD (n=3)	Eupatorin (% w/w) ±SD (n=3)
LP1	0,67 ± 0,02[a]	1,01 ± 0,01[a]	ND
LWP1	ND	0,51 ± 0,02[b]	0,57 ± 0,02[a]
LP2	1,03 ± 0,21[b]	2,13 ± 0,00[c]	0,36 ± 0,01[b]
LWP2	ND	1,95 ± 0,05[d]	3,70 ± 0,15[c]
LP3	2,21 ± 0,00[c]	3,07 ± 0,08[e]	0,39 ± 0,01[b]
LWP3	ND	1,82 ± 0,01[f]	3,25 ± 0,50[c]

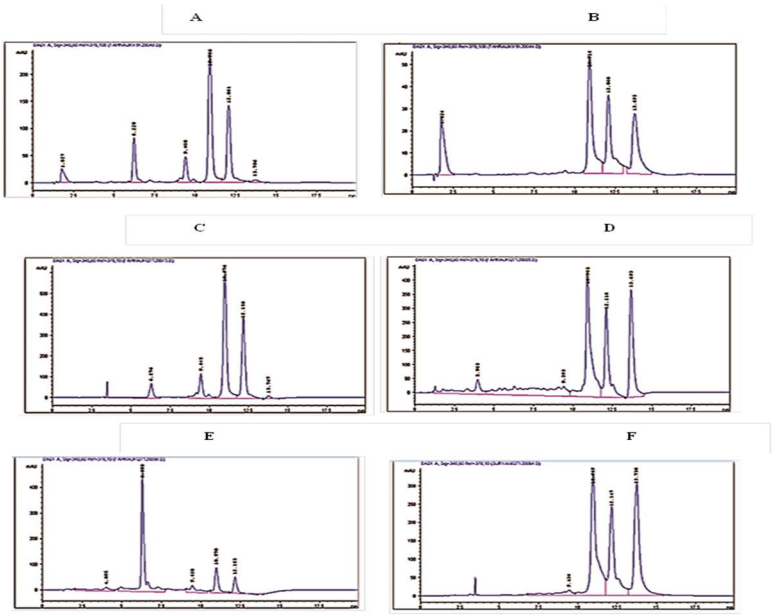

Fig.-3: Acetone, Ethyl Acetate, and Ethanol Extract Chromatograms of Two Types of *O. aristatus*: LP1 (A), LWP1 (B), LP2 (C), LWP2 (D), LP3 (E), LWP3 (F)

Qualitative and quantitative testing of primary, and secondary metabolites in *O. aristatus* (rosmarinic acid, sinensetin, eupatorin) was also carried out using HPLC. The method used was that of Saidan *et al.* (2015) with slight modification on wavelength detection. Gradient solvent system, as shown in (Table-1), columns LichroCAR T ^{R} C-18 (4.0 mm, 150 mm, 10 m), column temperature 25 °C, the flow rate of 1 mL/min, and detection wavelength 340, 6 nm were used in these experiments. The HPLC gradient is essential for the effective elution of all sample components and optimal resolution.[32] After determining the optimal HPLC separation conditions for rosmarinic acid, sinensetin, and eupatorin, linearity was determined by injecting a standard solution of rosmarinic acid, sinensetin, and eupatorin. The linearity test demonstrated the existence of a linear relationship between analysis concentration and instrument response by producing a linear line equation with a correlation coefficient above 0.990. The evaluation can be based on criteria R2 values greater than or equal to 0.9938. The results of this study are in line with Cai *et al.* (2018) report on the content of the main secondary metabolites in the variety of *O. aristatus* plants.[36] Leaves had the highest content of sinensetin, eupatorin, and rosmarinic acid compared to the stem and root. These three compounds have good antioxidant activity.[17] Methoxylated flavonoids (sinensetin and eupatorin), which are lipophilic, are more stable in low-polar solvents such as isopropanol and chloroform.[37] Rosmarinic acid is more easily dissolved in water and methanol than isopropanol. A mixture of water and alcohol solvents attracts more flavonoids and phenolic compounds in *O. Aristatus.*[37] Sinensetin concentrations are lower in non-polar solvents such as hexane compared to ethyl acetate solvents.[38] Rosmarinic acid contains four hydroxyl groups and one carboxylic group and is more soluble in polar solvents (water). Carboxylic acids and ester groups in acids can react with H_2O and ethanol.[39] Rosmarinic acid compounds are more attracted to ethanol solvents than using ethyl acetate solvent.[38] In non-polar solvents such as hexane, rosmarinic acid is low.[38]

CONCLUSION

Based on TLC and HPLC data, sinensetin and rosmarinic acid are better extracted from the leaves of purple types of *O. aristatus* by maceration and ethanol whereas eupatorin is from the same material with ethyl acetate as the solvent.

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AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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