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Profiling Rosmarinic Acid and Sinensetin Content of *Orthosiphon aristatus*. from Three Different Locations with Variety Ethanol Concentration

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Abstract: *Orthosiphon aristatus* is a well-known medicinal plant acknowledged for its therapeutic effect in treating urinary tract diseases, hypertension, diabetes mellitus, and arthritis. It is widely used as an ingredient in herbal medicine and distributed throughout the world, including China, Europe, and Indonesia. Sinensetin is normally used as a chemical marker to evaluate and control the quality of *O. aristatus*. However, in 2021, the European Medicines Agency changed the marker to rosmarinic acid. To determine the levels of rosmarinic acid and sinensetin in *O. aristatus* as well as the correlation between the two compounds, this study used high-performance liquid chromatography (HPLC) with a UV detector to analyze *O. aristatus* extract from three distinct locations with four different ethanol concentrations (p.a, 75%, 50%, and 25%). The results showed that the combination of solvent concentration and growing location had a significant effect on the levels of rosmarinic acid and sinensetin, with a p-value < 0.05. The Spearman test obtained a correlation coefficient (r) of - 0.423. It can be concluded that there is no correlation between the content of rosmarinic acid compounds in *O. aristatus* leaves and the content of sinensetin compounds.

Keywords: *Orthosiphon aristatus*; rosmarinic acid; sinensetin; ethanol concentration

1. INTRODUCTION

Orthosiphon aristatus (Blume) Miq., locally known "cat's whiskers" or as "kumis kucing", is a medicinal plant from the Lamiaceae family distributed widely throughout China, Europe and Southeast Asian countries, including Indonesia. The leaves of its plant have been used empirically to treat kidney stones and urinary tract diseases [1]. Furthermore, pharmacological studies have verified that this plant is therapeutically effective in treating diabetes mellitus [2], decreasing blood pressure [3], [4], [5], treating urinary tract diseases[6], and managing arthritis due to its anti-inflammatory properties [7]. Due to its potency, *O. aristatus* is widely used as an ingredient in herbal medicine product. According to the Indonesia Food and Drug Administration (BPOM), more than three hundreds herbal medicine products have been registered for containing *O. aristatus* [8]. In addition to being an economic opportunity, the growing interest in herbal medicine is also a challenge in the field of quality assurance. Quality assurance is essential to ensure the safety and quality of the products.

The quality of herbal medicine can be assessed by a number of methods, such as fingerprinting and marker compound analysis. Although fingerprint-based systems have gained more attention, the conventional technique of using chemical makers is commonly chosen by herbal pharmacopeia in many countries [9]. Phytochemical studies discovered that the ethanol extract of *O. aristatus* leaves contained 34 compounds, and 14 of them were successfully absorbed and retained in mice's blood plasma. Among the fourteen phytochemical constituents, tanshinone IIA, salvianolic acid B, salvigenin, sinensetin, and rosmarinic acid are the compounds that are responsible for the pharmacological activity and are suggested to be the marker compounds of *O. aristatus* [10]. Sinensetin has been used as chemical marker of *Orthosiphon aristatus* by Indonesia's Herbal Pharmacopoeia (2017) and European Medicines Agency (2010) [11], [12]. However, in 2021, the European Medicines Agency updated the *O. aristatus* marker into rosmarinic acid [13]. This change is undoubtedly a challenge in the analysis field. Therefore, it is necessary to know the level of rosmarinic acid and sinensetin in *O. aristatus* along with the correlation between the two.

The content of sinensetin and rosmarinic acid in *Orthosiphon aristatus* extract is variable and can be influenced by some factors such as the origin and extraction methods. Phytochemical study reported that the different location of *O. aristatus* effect of the level of sinensetin [14]. On the other hand, the choice of solvents is important due to the targeted compound to be extracted from the plant. The most common solvents used for *O. aristatus* extraction are methanol, ethanol, and distilled water [11], [15], [16]. Although methanol gives the high yield of rosmarinic acid and sinensetin than ethanol, but the use of methanol is frequently questioned because of its toxic to humans. Thus, the industry finds ethanol more appealing for the extraction because less toxic and shares similar chemical properties with methanol[17]. According to a literature search, no one has reported the effect of the combination of ethanol concentration and growing location on the content of rosmarinic acid and sinensetin in cat whisker extract or the relationship between the two compounds. Therefore, this study aims to determine the levels and correlation of rosmarinic acid and sinensetin in *O. aristatus* from various locations and ethanol concentrations.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Plant materials

The *Ortoshipon aristatus* leaves were collected from 3 different locations (Sleman, Bantul, Klaten) on March 2024. The third to eights leaves from the shoots were picked by hand. The leaves are green, with a lanceolate leaf blade and a serrate margin. The venation is reticulate-pinnate, the petiole is reddish purple in colour, and the leaves range in size from 7 to 11 cm (Figure 1). The leaves were dried in an oven at 45 ^{O}C for 24 hours. Afterward, the dried leaves were ground and sifted with a 40-mesh sieve to obtain brownish green powder of *O. aristatus.*

2.1.2 Reagent

Rrosmarinic acid (Markherb, purity ≥ 95%), sinensetin (Chemfaces, purity ≥ 98%), ethanol p.a (Merck, Germany), acetonitril HPLC grade (Merck, Germany), trifluoroacetic acid HPLC grade (Sigma Aldrich), and Milli-Q water was prepared in the laboratory.

2.2 Methods

2.2.1. Sample extraction

The preparation of extract was modified according to previous report [14]. Approximately 500 mg of *O. aristatus* leaves powder was added with 5 mL of ethanol (p.a., 75%, 50% and 25%), then extracted using *ultrasound-assisted extraction method* for 15 minutes at room temperature and filtered through a 0.45 PVDF syringe filter. After that, each *O. aristatus* extract solution was dissolved in ethanol to obtain a concentration 10 mg/ml. The sample solution was filtered through a 0.45 PTVE syringe filter, and 10 μL was injected into HPLC.

2.2.2 Preparation of The Standard Solutions

A standard solution was prepared for rosmarinic acid and sinensetin. Each compound was made into a stock solution with concentration of 500 μg/ml in methanol. The combined working solution was obtained by mixing the stock solution of rosmarinic acid and sinensetin for 120 μL and 20 μL, respectively. The combined working solution was further diluted to five series concentration 3-60 μg/ml (rosmarinic acid) and 0.5-10 μg/ml (sinensetin).

2.2.3 HPLC Conditions

The analysis conditions for the quantification of rosmarinic acid and sinensetin compound in *O. aristatus* leave were conducted according to Ramadhani et al [18]. A Shimadzu prominence-high performance liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) equipped with ultraviolet (SPD-20A/20AV) detector was used for the analysis. Separation was archived on cosmosil C18-MS-II column (4.6 i.d x 250 mm, 5.0 μm) using a 0.1% TFA in water (A) and acetonitrile (B) as a mobile phase

2.2.4 Data analysis

A nested analysis of variance (nested-ANOVA) was performed to determine the significant effect of the ethanol concentration on sinensetin and rosmarinic acid from three different locations of O. aristatus. Then, a spearman correlation analysis using Minitab 21.4.1 software was performed to determine the association between rosmarinic acid and sinensetin levels in O. aristatus at different ethanol concentrations and locations.

3. RESULTS AND DISCUSSION

In this study, extraction was performed using four different ethanol concentrations (p.a, 75%, 50%, and 25%) and three growing locations (Sleman, Bantul, and Klaten). Table 1 provides a detailed description of the sample's location. The results showed that different ethanol concentrations affected the present of the bioactive compound in *O. aristatus* extract. Figure 1 displays each extract's chromatogram at various concentrations and locations.

Table 1. A details of the *O. aristatus* locations

Origins	Latitude, Longitude	Elevation (meter)
Sleman	7°37'42.0"S and 110°25'34.8"E	619
Bantul	7°49'29.7"S and 110°21'15.8"E	82
Klaten	7°43'31.6"S and 110°30'12.1"E	194

The fact that some peaks are absent in the beginning retention times and present in the end retention time indicates that the use of ethanol p.a tends to extract non-polar compounds rather than

polar compounds. Meanwhile, the use of binary solvents (ethanol and water) significantly extracted polar compounds, as evidenced by the appearance of many peaks at the initial retention time. This may indicate that a binary solvent is suitable for optimizing the extraction of polar compounds.

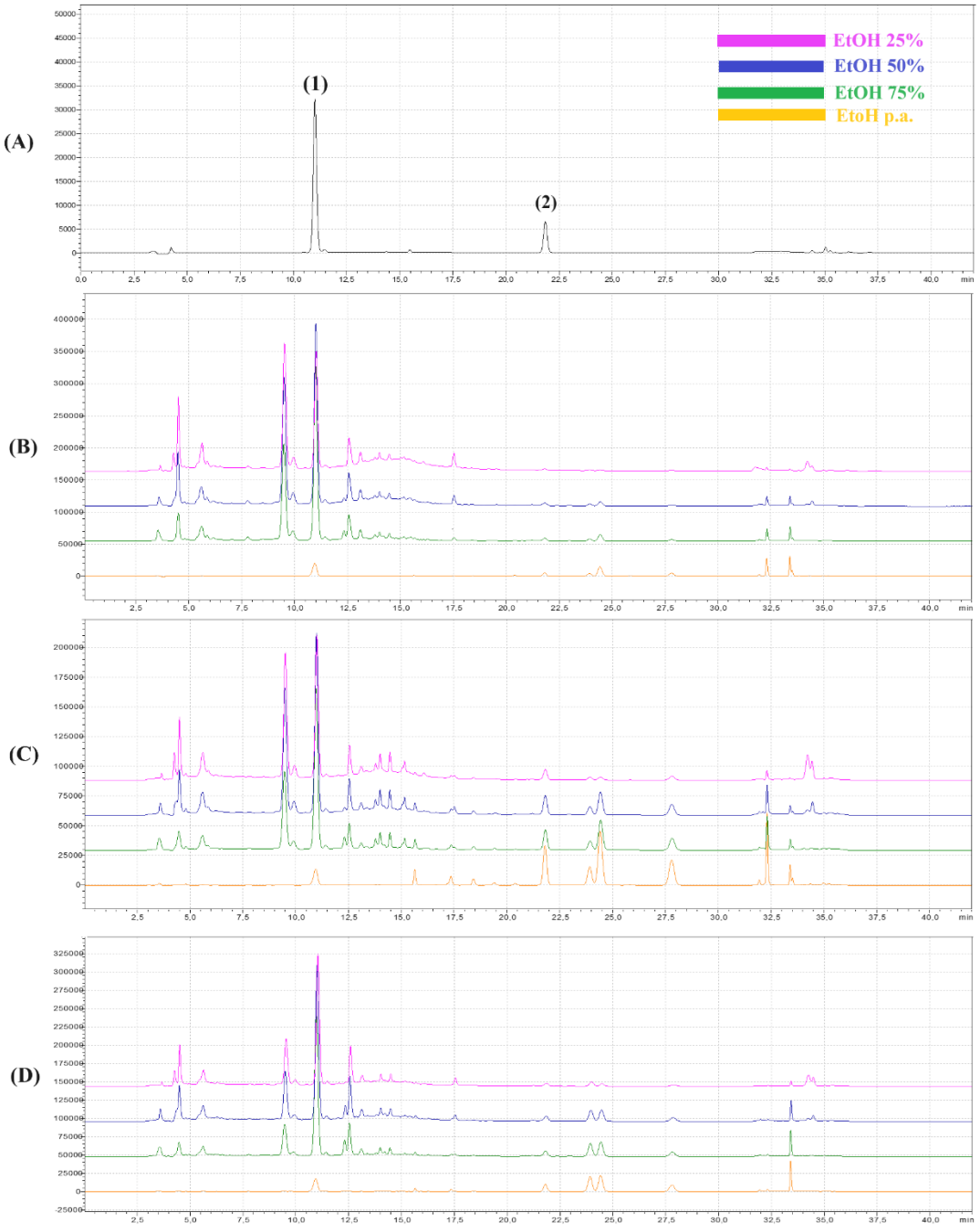

Figure 1. (A) Rosmarinic acid (1) and sinensetin (2) chromatogram, (B) HPLC chromatogram of *O. aristatus* extract from Sleman, (C) HPLC chromatogram of extract from Klaten, (D) HPLC chromatogram of *O. aristatus* extract from Bantul

Sinensetin is a flavone group compound with five methoxy groups bound to the benzene ring (Figure 2). The methoxy groups cause sinensetin to be relatively less polar than rosmarinic acid, which has four hydroxyl groups. The use of a C18 reversed phase column and acetonitrile as a solvent

causes less polar compounds bond to the stationary phase more strongly and move more slowly through the column, so the peak could be seen at the end of the retention time. The chromatogram showed that the peak for sinensetin was visible at a retention time of 21.827, whereas the peak for rosmarinic acid was apparent at 10.995.

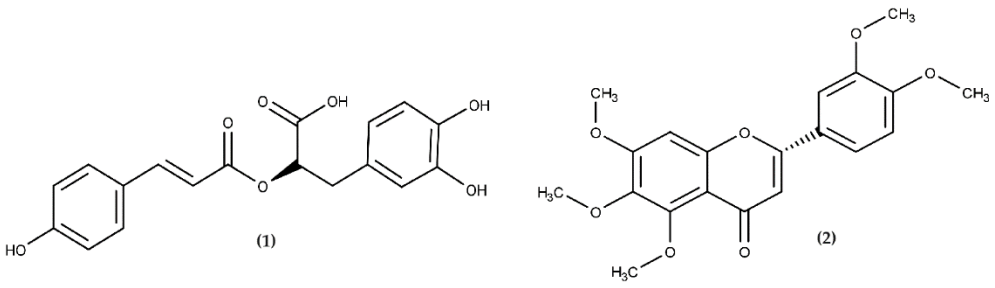

Figure 2. Structure of Rosmarinic Acid (A) and Sinensetin (B)

The comparation of rosmarinic acid and sinensetin content in *O. aristatus* from three different location with varying ethanol concentration are presented in Figure 3 (A) and (B). A nested ANOVA revealed that the solvent-nested growth location variable had a *p-value* less than 0.05. These results indicate that the ethanol concentration and growing location had a significant effect on the rosmarinic acid and sinensetin content.

Among all location, hydro-ethanol achieved a significantly higher rosmarinic acid content than ethanol p.a. The results of this study are similar to those of Suhaimi [19], who found that variation in the concentration of ethanol had a significant effect on the levels of rosmarinic acid in the *O. aristatus* extract. The content of rosmarinic acid increases as the ratio of water to ethanol increase. However, when the ethanol percentage was less than 70%, rosmarinic acid levels decreased. The presence of water in the extraction acts as a swelling agent, which can increase extraction efficiency by increasing the contact surface area of the plant material and solvent [20]. The high presence of water in the solvent reduces the rosmarinic acid content because rosmarinic acid is a relatively polar compound but insoluble in water [21]. The Hildebrand solubility value (δ) of 21.18 tends to make rosmarinic acid more soluble in ethanol (δ = 26.6) than in water (δ = 47.8) [22]. Taken together, in this study, we conclude that binary ethanol (50-75%) was the most efficient solvent to extract rosmarinic acid.

Among all locations, *O. aristatus* from Klaten had a higher sinensetin content compared to others. A nested-ANOVA revealed that the growing locations variable had the most significant effect on sinensetin content. Therefore, it can be concluded that the growing location influences the level of sinensetin in O*. aristatus.* These results are similar to the previous study, which showed that the level of sinensetin in *O. aristatus* varies depending on the growing location [14], [16], [23]. The amounts of sinensetin found in this study are similar to those found by Kartini et al. (2022), who analysed *O. aristatus* samples from 14 different areas in Indonesia and found levels ranging from 0.0238 to 0.1533 mg/g.

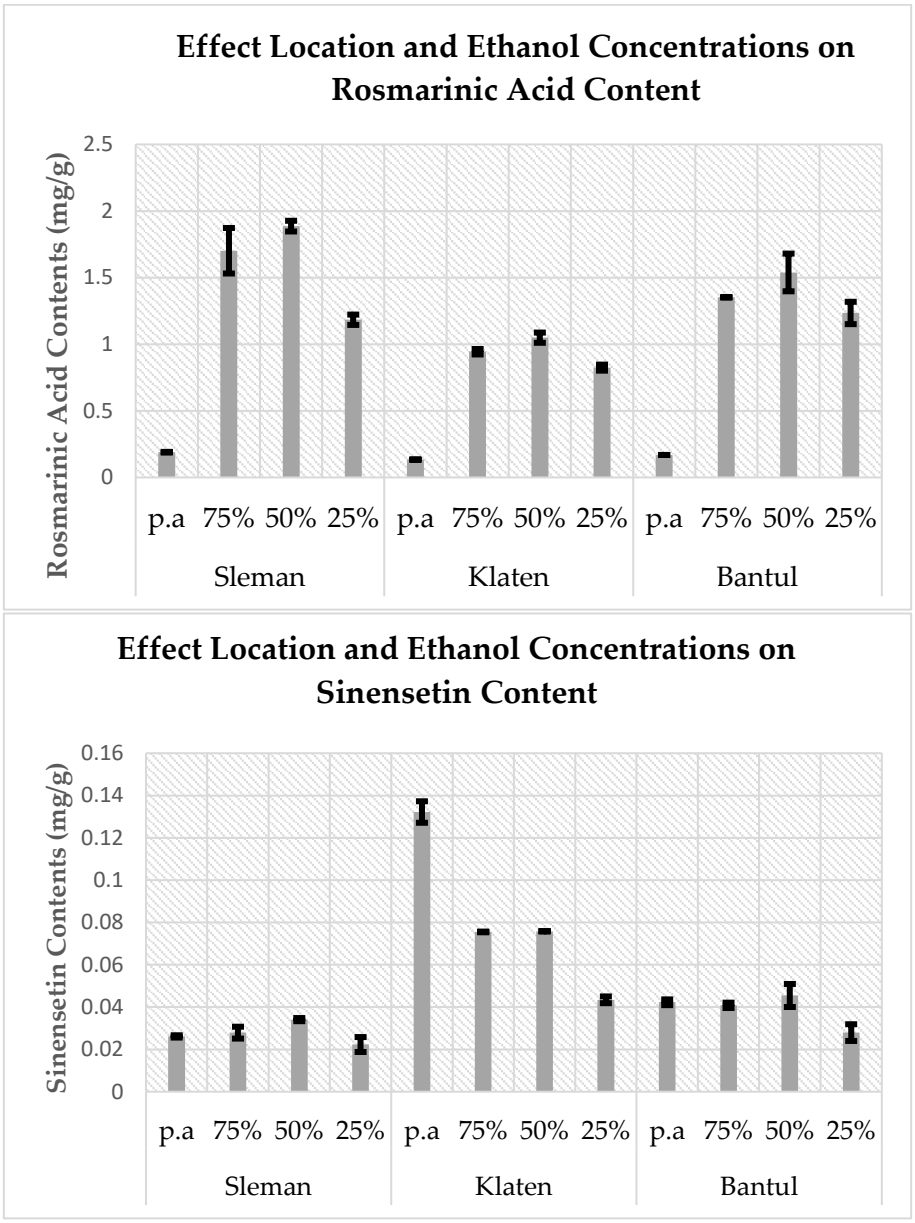

Figure 3. Effect of ethanol concentrations and locations on rosmarinic acid content (A) and sinensetin content (B)

This study evaluated the correlation or relationship between rosmarinic acid and sinensetin levels by combining the effects of growing location and ethanol concentration. According to the Spearman correlation test, the correlation coefficient between rosmarinic acid and sinensetin levels was -0.403 (Figure). The negative correlation value indicates that the two variables have an inverse relationship. However, the closeness of the relationship between the two compounds is weak, as evidenced by a coefficient value close to 0. The results of this analysis indicate that the sinensetin sinensetin levels had no correlation with rosmarinic acid levels in *O. aristatus* extract.

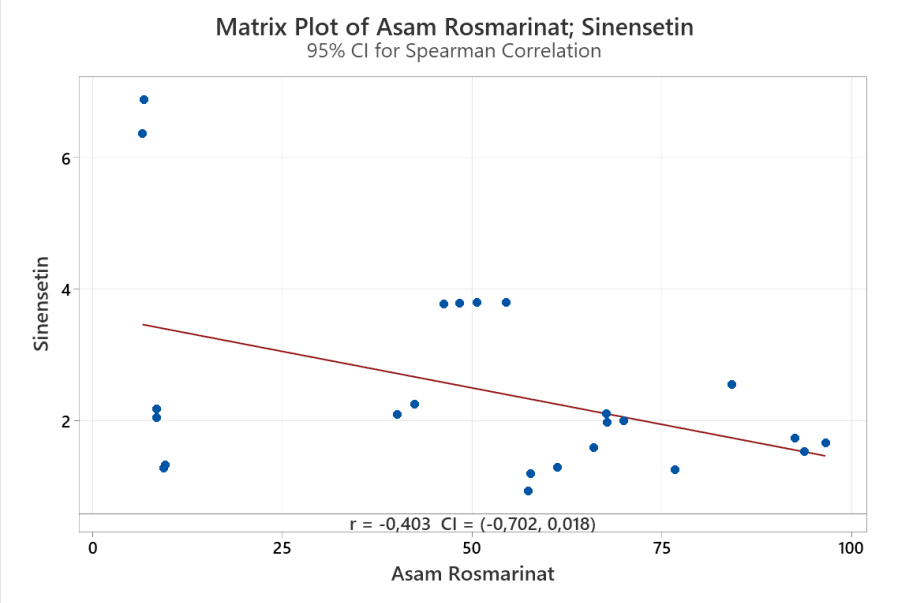

Figure 4. Spearman correlation test results of rosmarinic acid and sinensetin in the extract of *O. aristatus*

4. CONCLUSION

Variations in ethanol content and growing location significantly influenced the levels of rosmarinic acid and sinensetin in cat's whisker extract. The concentration of ethanol is the primary factor that has a significant impact on rosmarinic acid extraction. Binary ethanol (50-75%) was the most efficient solvent to extract rosmarinic acid. Meanwhile, the growing location variable had the most significant effect on sinensetin content. In addition, this study discovered that sinensetin levels had no correlation with rosmarinic acid levels (r = - 0,423). To verify current results, studies for additional places should be carried out in future research.

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Conflicts of interest: The authors declare no conflict of interest.

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