Research Article

Chemical Compound Identification of Two Varieties Cat of Whiskers (*Orthosiphon aristatus* Blume Miq) from *In Vitro* Culture

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Abstract | The cat's whiskers plant (*Orthosiphon aristatus* Blume Miq) is empirically used in Indonesia, Malaysia, Australia, and Southern Asia as an antibacterial, antidiabetic, antihypertensive anti-inflammatory. The country of Indonesia has three varieties of *O. aristatus*, namely purple, white-purple and white. The purple and white-purple population has started to decline. Efforts are needed to maintain the two varieties' population, one of which is plant tissue culture. It is hoped that *in vitro* cultured plants contain the same secondary metabolites as the wild type. This study aims to identify the chemical content of two varieties of *O. aristatus* resulting from *in vitro* culture. The five-month-old variety of *O. aristatus* purple and white purple and wild type was extracted using two solvents with different polarity levels, namely ethanol and ethyl acetate. The concentrated extract was identified for its chemical content using gradient system HPLC 0.1% formic acid: acetonitrile. The qualitative analysis results showed that the two extracts of *O. aristatus* resulted from *in vitro* culture containing rosmarinic acid and sinensetin. When compared to the wild type, the chromatogram had a larger area than the wild type. This study provides new information regarding the secondary metabolite content of two *O. aristatus* from *in vitro* culture.

Received | February 27, 2021; **Accepted** | June 03, 2021; **Published** | September 18, 2021
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Citation | Faramayuda, F., T.S. Mariani, Elfahmi and Sukrasno. 2021. Chemical compound identification of two varieties cat whiskers (*Orthosiphon aristatus* Blume Miq) from *in vitro* culture. *Sarhad Journal of Agriculture,* 37(4): 1355-1363.
DOI | https://dx.doi.org/10.17582/journal.sja/2021/37.4.1355.1363
Keywords | *O. aristatus, In-vitro* culture, Wild type, Secondary metabolite, Qualitative analysis

Introduction

Since 2019, there was a pandemic due to COVID -19, the search for sources or medicinal plants that can act as antiviral and immunomodulators. One of the plants that have the potential for this activity is *O. aristatus*. Several studies have reported the potential of the extract and some chemical properties of the *O. aristatus* plant as an antiviral (Faramayuda *et al*., 2021b). Water extract of leaves, flowers, and all the plants in addition to the root of the *O. aristatus* (0.39 mg/ mL) had high antiviral activity observed after normal cells (Vero cells) were inoculated with herpes simplex virus type 1 (HSV-1) (post-treatment) with a 100% reduction in HSV-1 plaque. In the pre-treatment test, leaf water extract, flowers, and all parts of plants other than roots showed HSV-1 plaque reduction activity was 79%, 84%, and 97% using the same concentration (Ripim *et al*., 2018). The chemical content in *O. aristatus* that has the potential to be antiherpetic is caffeic acid, eugenol, N-transferulolyl tyramine, limonene, β-caryophyllene, beta-pinene, p-cymene (Ikeda *et al*., 2011; Bourne *et al*., 1999; Benencia and Courreges,

2000; Medini *et al*., 2016; Astani and Schnitzler, 2014; Astani *et al*., 2011; Sharifi-Rad *et al*., 2018). Rosmarinic acid compound, the main ingredient of *O. aristatus* based on in-silico studies, can inhibit COVID-19 (Sarkar and Das, 2020; Wondmkun and Mohammed, 2020; Sampangi-crowdedah *et al*., 2019). The potential of other compounds in *O. aristatus* as an inhibitor of COVID-19 is sinensetin, cirsimaritin, 1,8-cineole, sagerinic acid, caffeic acid derivatives, and β-caryophyllene (Rowaiye *et al*., 2020; Sekiou *et al*., 2020; Sharma and Kaur, 2020; Dahab *et al*., 2020; Adem *et al*., 2020; Narkhede *et al*., 2020). Other studies have reported the effects of sinensetin, caffeic acid, limonene, 1,8-cineol, eugenol, and aurantiamide compounds as anti-influenza viruses (Shin *et al*., 2012; Li *et al*., 2020; Utsunomiya *et al*., 2014; Nagy *et al*., 2018; Li *et al*., 2016; Choi, 2018; Dai *et al*., 2013; Zhou *et al*., 2017). Research conducted by Harun *et al*. (2015) explains that the methanol extract of *O. aristatus* has the potential as an immunomodulator. The compound content in *O. aristatus* with the potential as an immunomodulator is rosmarinic acid (Harun *et al*., 2015).

Based on the leaf morphology and flower color of the *O. aristatus*, it is divided into three varieties, namely purple, white, white-purple (Faramayuda *et al*., 2020, 2021a). The research report by Febjislami (2019) explains that the *O. aristatus* widely grown in Indonesia are white varieties, while white-purple varieties, mostly purple, have a small distribution. Sinensetin levels in purple varieties are more significant than other varieties (Febjislami *et al*., 2017). To maintain the population of white-purple and purple varieties of *O. aristatus* and their potential as antiviral and immunomodulatory herbal agents, necessary to make propagation efforts in the two varieties. One of the strategies is *in vitro* culture modification based on plant tissue culture. Several research reports on micropropagation efforts from *O. aristatus* have been carried out, including nodal explants derived from MS medium added with the growth regulator BAP 6.7 µM were able to grow shoots of one variety of *O. aristatus* (Lee and Chan, 2004). Another report states that the shoots of one variety of *O. aristatus* were successfully formed within *in vitro* modification of the culture, where the explants came from petiole and axillary buds (Zainuddin and Kamil, 2019). The chemical content profile of two varieties of *O. aristatus* modified by *in vitro* culture has never been carried out, so this study is expected to provide new information. Then it can be developed into the production stage of *O. aristatus* with better quality than the wild type.

Materials and Methods

Chemicals and reagents

Rosmarinic acid and sinensetin were purchased from Sigma (St. Louis, MO, USA). Ethanol, ethyl acetate, acetonitrile HPLC grade, and methanol HPLC grade were purchased from Merck (Jakarta, Indonesia). Formic acid was purchased from Loba Chemie (Mumbai, India).

Instrumentation

HPLC (Shimadzu Serial No L201354 Japan), ovens (Memert, Germany), rotary evaporators (Heidolp, Germany), balance sheet (Mettler Toledo, Hong Kong), analytical scales (Shimadzu, Japan).

Collection of plants

O. aristatus (wild type), five months old, was collected from the medicinal plant garden, Faculty of Pharmacy, Universitas Jenderal Achmad Yani. The plants were taxonomically identified School of Life Science and Technology, Institut Teknologi Bandung (ITB). The purple and white-purple varieties of *O. aristatus* modified by in-vitro culture aged three months were obtained from the Plant Tissue Culture Laboratory of the Center for Research and Innovation ITB and then grown on the same land as the wild type until five months old. The research was conducted from October 2020 to January 2021.

Geographical data where plants are grown

The city of Cimahi is a place to grow, it has an altitude from sea level 685 meters and an average rainfall of 2000-5000 mm/year. Cimahi City is located between 107 ° 30'30 " East Longitude -107 ° 34'30 'and 6 ° 50'00' '- 6 ° 56'00'. The optimal location for growing *O. aristatus* is at an altitude of 500 -1200 above sea level with 3000 mm/year (Syukur, 2008).

Morphological characterization of in vitro culture and wild type O. aristatus

Morphological observations on purple and white-purple varieties of *O. aristatus* resulting from *in vitro* and wild type cultures include leaf shape, venation, petal color, crown, pistil, and stamens.

Extraction of two varieties of O. aristatus

A total of 200 grams of fresh plant *O. aristatus* was cleaned with running water and dried in an oven at 60°C. The dry material is then mashed. As much as 50 g of plant material powder is extracted separately by

maceration using 750 mL of ethanol and ethyl acetate as solvent. Extract concentration was performed using a rotary evaporator and thickening using a water bath.

Preparation of marker and sample solutions

Qualitative analysis of *in vitro* cultured and wild-type plants using HPLC was performed by preparing marker and sample solutions. Rosmarinic acid and sinensetin were weighed as much as 1 mg and dissolved in 1 mL of HPLC grade methanol. The stock solution was diluted to 100 µg/mL with methanol. The test solution was prepared by dissolving 15 mg of the extract in 1 mL of methanol and sonicated for 45 min. The test solution is then filtered through the filter of the syringe.

High-performance liquid chromatography (HPLC) instrumentation and conditions

The HPLC used is a gradient elution using reversephase C18 column of the reversed process. The temperature of the column is 25°C. The mobile phase consisted of 0.1% formic acid solution and acetonitrile with a gradient elution system where the ratio was 0.1% formic acid: acetonitrile at 0 min (85:15), 1 minute (85:15), 12 minutes (35:65), 15 minutes (85:15), and 18 minutes (85:15). The flow rate was 1 mL per minute. The time of separation was 20 min. The method used to assess the levels of secondary metabolites *O. aristatus* refers to Saidan *et al.* (2015) with improvements to the maximum wavelength used at 340.6 nm.

Data analysis

Data analysis included retention time and area under the curve from *in vitro* and wild-type plant extract chromatograms. The test on each sample was carried out in three replicates.

Results and Discussion

Identification and characterization of O. aristatus morphology from in vitro culture and wild type

The purple and white-purple varieties of *O. aristatus* resulting from *in vitro* culture at the age of 2 months were obtained from the ITB research and innovation center's plant tissue culture laboratory. The *in vitro* culture's shoots came from internode explants grown on media MS + Zeatin 3 µg/mL + 2.4 D 2 µg/mL from the information obtained. The shoot induction was carried out on MS medium + IBA 0.75 µg/mL. Acclimatization is carried out on soil media by watering it with water twice a day. After the plants reach two months of age, the plantlets are transferred to the medicinal plant garden of the Faculty of Pharmacy Universitas Jenderal Achmad Yani, Cimahi City, until they are five months old.

In vitro and wild-type, *O. aristatus* were grown in the same place, namely the Faculty of Pharmacy's medicinal plant garden, Universitas Jenderal Achmad Yani. The age of the plants used in this study was five months. The similarity in place of growth and age of the plants can maintain the comparisons between *in vitro* and wild-type cultivars.

The identification process uses fresh ingredients which cover all parts of the plant. The results of plant identification issued by the School of Life Sciences and Technology, Institut Teknologi Bandung, show that the two plant samples are purple and white-purple varieties of *O. aristatus* with voucher number 6115 / I1.CO2.2 / PL / 2019. The observation showed no difference in leaf morphology between the two *O. aristatus* cultured *in vitro* and wild type. In purple varieties, both the *in vitro* and wild-type leaves are rhombus shaped with purple leaf venation (Figure 1). The leaf morphology of the white-purple varieties of *O. aristatus* resulting from *in vitro* culture and wild type is rhombus shaped with green venation (Figure 2). According to Lai and Siong (2006) and Almatar *et al.* (2013), morphologically, the *O. aristatus* plant varieties can be distinguished by color on the flower and leaf components. The results of plant morphology observations from *in vitro* culture and wild type two varieties of *O. aristatus* are in line with what was reported by Faramayuda *et al.* (2020, 2021a).

Analysis of chemical component content of O. aristatus from in vitro culture and wild type

The results of standard chromatogram observations, both in the single and mixed form at a wavelength of 340.6 nm, showed rosmarinic acid appeared at 7.650 minutes and sinensetin at 12.640 minutes (Figure 3). This standard mixture chromatogram is a reference for determining rosmarinic acid and sinensetin in *O. aristatus in vitro* and wild types.

The observation of the chromatogram pattern of the ethanol and ethyl acetate extract samples of purple and white-purple varieties *O. aristatus* from *in vitro* culture showed the presence of rosmarinic acid and sinensetin in both shown by the presence of peaks at 7,650 and 12,640 minutes (Figures 4, 5, 6, 7). Likewise, with the ethanol and ethyl acetate extracts of wild type *O. aristatus* (wild type), it was identified that there were compounds of Rosmarinic acid and

sinensetin (Figures 4, 5, 6, 7). In the two varieties of *O. aristatus* extract (*in vitro* culture), the area of rosmarinic acid was greater than that of the wild type (Table 1). The area of the sinensetin peak in the ethyl acetate extract of purple varieties (*in vitro* culture) was greater than that of the wild type (Table 1). Observation of rosmarinic acid peak areas in white-purple varieties of *O. aristatus* (*in vitro* culture) showed a greater value than wild type. The area of sinensetin compounds in white-purple varieties is more extensive than in wild types (Table 1).

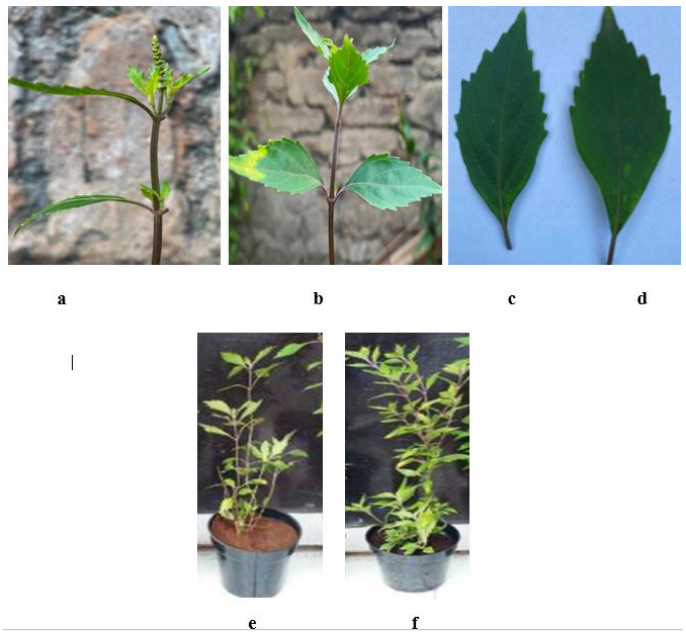

Figure 1: *Morphology of leaves and stem of purple varieties of O. aristatus from in vitro cultures and wild type. a: purple stems (in vitro culture), b: purple stems (wild type), c: purple leaves (in vitro culture), d: purple leaves (wild type), e: purple varieties 5 months old (in vitro culture), f: purple varieties 5 months old (wild type).*

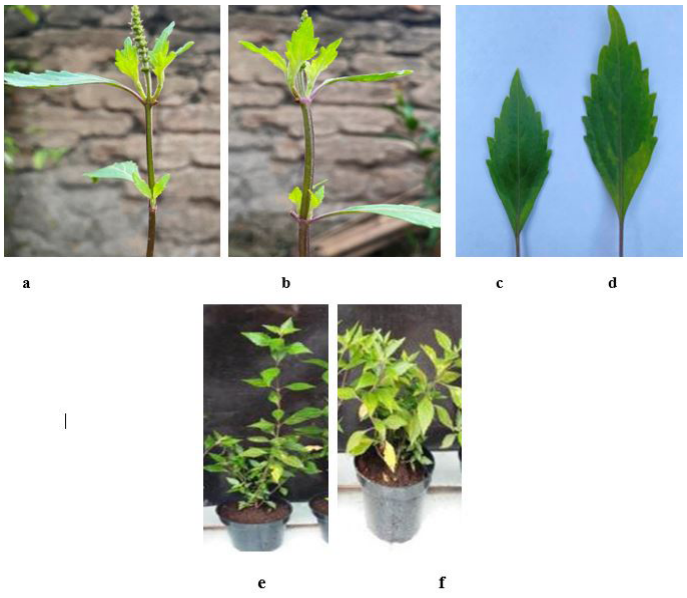

Figure 2: *Morphology of leaves and stem of white-purple varieties of O. aristatus from in vitro cultures and wild type. a: white-purple stems (in vitro culture), b: white-purple stems (wild type), c: white-purple leaves (in vitro culture), d: white-purple leaves (wild type), e: white-purple varieties 5 months old (in vitro culture), f: white-purple varieties 5 months old (wild type)*

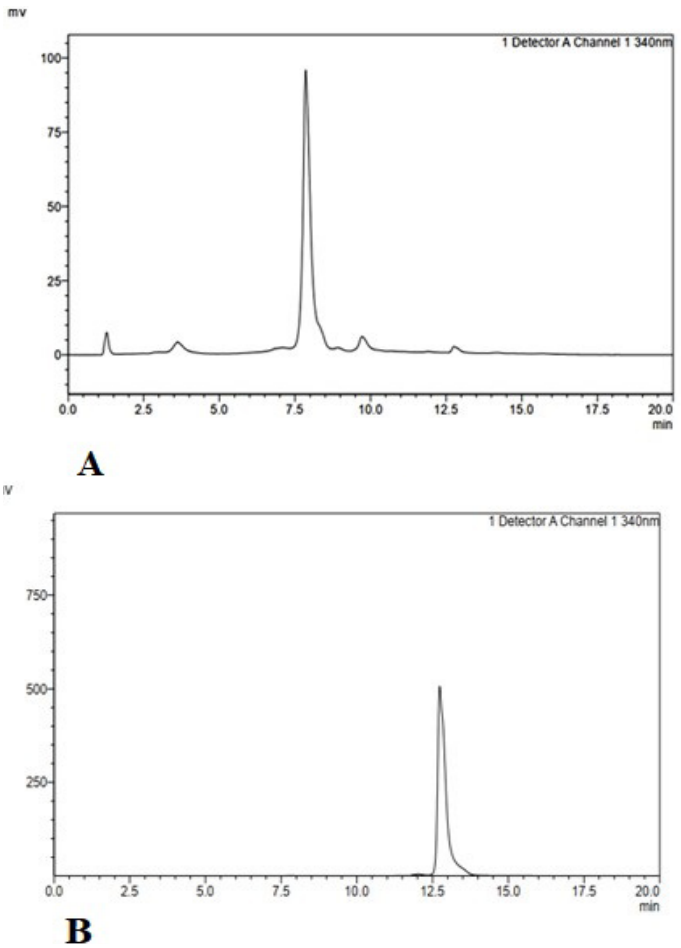

Figure 3: *Chromatogram of rosmarinic acid and sinensetin standard at 340.60 nm. A, rosmarinic acid and B, sinensetin.*

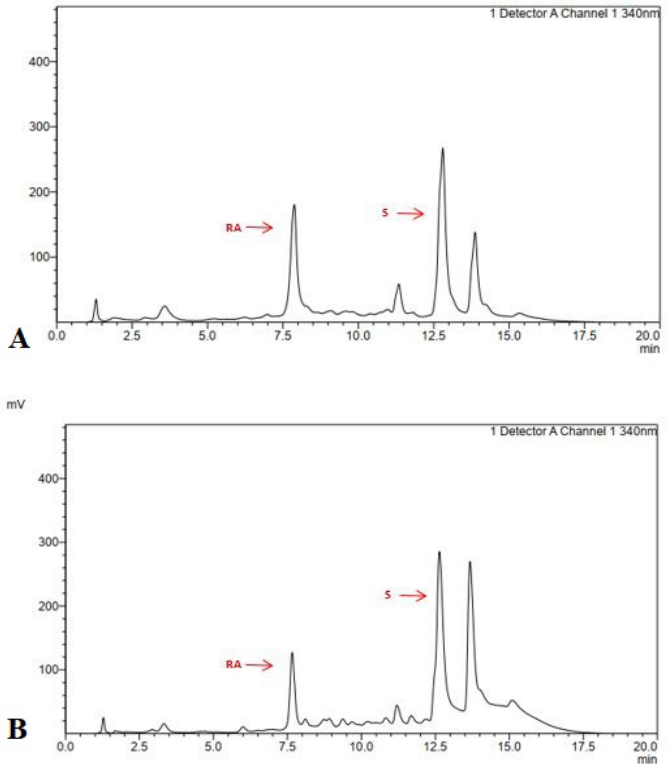

Figure 4: *Chromatogram of ethanol extract of purple variety at 340.60 nm. a = in vitro culture b = wild type.*

Table 1: *Data retention time and area of peak extract of two varieties of O. aristatus (in vitro culture and wild type).*

Sample	*In Vitro* Culture		Wild Type	
	RT (minute)	Area X (n=3)	RT (minute)	Area X (n=3)
Purple variety ethanol extract	7.650	5380136	7.650	1747728
	11.343	996280	12.640	7163977
	12.640	5534391	13.676	7112459
	13.870	3154941		
Purple variety ethyl acetate extract	7.650	5641583	7.651	664997
	11.643	177876	12.642	45583689
	12.654	64350937	13.664	32891426
	13.994	6350607		
White-Purple variety ethanol extract	7.650	4246492	7.650	2539234
	11.356	915628	12.614	7755333
	12.640	4841599	13.657	3013235
	13.888	3008935	14.033	4854084
White-Purple variety ethyl acetate extract	7.650	6153797	7.650	1583932
	11.210	2248920	12.640	39964971
	12.640	13628811	13.620	11963900
	13.692	8362047	13.990	17101929
			15.038	12788864

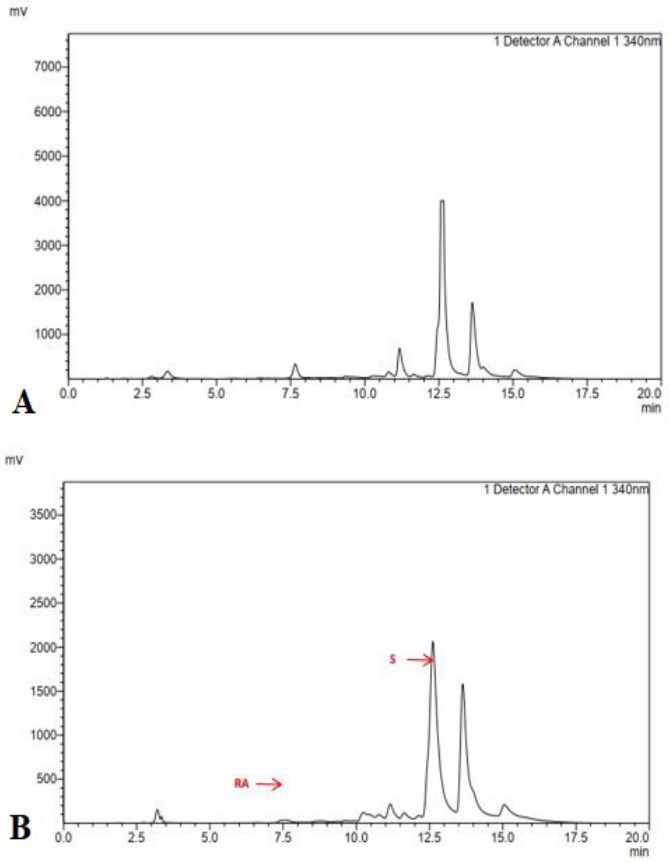

Figure 5: *Chromatogram of ethyl acetate extract of purple variety at 340.60 nm. a = in vitro culture b = wild type*

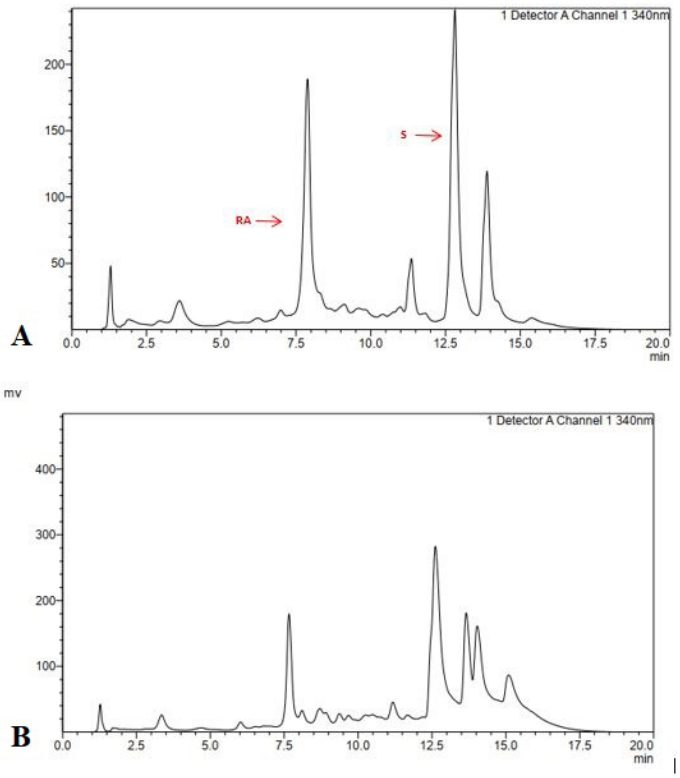

Figure 6: *Chromatogram of ethanol extract of white-purple variety at 340.60 nm. a = in vitro culture b = wild type.*

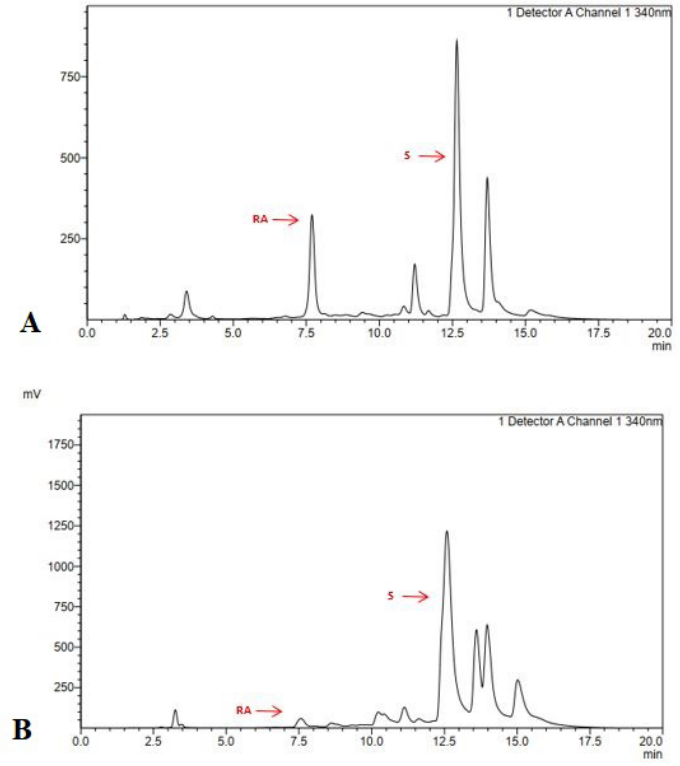

Figure 7: *Chromatogram of ethyl acetate extract of white-purple variety at 340.60 nm. a = in vitro culture b = wild type.*

Previous studies reported that sinensetin and rosmarinic acid were the main secondary metabolite components in *O. aristatus* (Guo *et al.*, 2019). Among these two compounds, rosmarinic acid has higher levels than sinensetin, especially in the leaves

(Cai *et al*., 2018). Previous research which reported the results of a qualitative analysis of plant tissue culture products of two varieties of *O. aristatus*, including Faramayuda *et al*. (2020), reported based on qualitative analysis using TLC extract of acetone callus *O. aristatus* varieties purple and white - purple showed the presence of rosmarinic acid and sinensetin compounds. Qualitative analysis using HPLC on extracts of acetone, ethyl acetate, and ethanol callus *O. aristatus* varieties purple and white-purple the presence of compounds rosmarinic acid (Faramayuda *et al*., 2021a).

Micropropagation efforts from *O. aristatus* have been made by Rashid (2012). MS medium added with BAP 1.0 mg/ L can induce shootd *O. aristatus*, and IBA 6 mg/ L produces optimal root growth in these shoots. MS media added with 0.2 ppm BAP can induce *O. aristatus* shoots, and the addition of 0.5 ppm gibberellic acid can prolong shoots, and 0.2 ppm NAA can induce roots (Zainuddin and Kamil, 2019), Petiole explants were grown on MS + BAP 1 ppm + NAA 0.2 ppm can induce the growth of roots and shoots of *O. aristatus* (Nawi and Samad, 2012). From these studies, no one has reported comparing the chemical content of *O. aristatus* from *in vitro* culture and wild type.

Shoot culture has been used to produce secondary metabolites and maintain plant populations (Nogueira and Romano, 2002; Smith *et al*., 2002; Karuppusamy, 2010; Khanam *et al*., 2012). Several other compounds have also been identified in shoots from *in vitro* culture, including isoflavones in *Psoralea cordifolia* (Shinde *et al*., 2009), vasine from *Adhatoda vasica* (Dinesh and Parameswaran, 2009), podophyllotaxin (*Podophyllum hexandrum*) (Li *et al*., 2009), myristin (*Myristica fragrans*) (Iyer *et al*., 2009).

Conclusions and Recommendations

The purple and white-purple varieties of *O. aristatus* (*in vitro* culture) contain rosmarinic acid and sinensetin compounds. This study's results are expected to be an alternative for raw material production for *O. aristatus*, which is superior to the original plant and preserves traditional medicinal plants' old resources.

Acknowledgments

This research was funded by the Ministry of Research and Technology / National Agency for Research and Innovation through "Penelitian Disertasi Doktor" with contract number 2/E1/KP.PTNBH/2020.

Novelty Statement

This study provides new information regarding the content of secondary metabolites of two varieties of *O. aristatus* (*in vitro* culture).

Author's Contribution

Fahrauk Faramayuda experimented and wrote the manuscript with support and supervision from Prof. Sukrasno, Dr. Elfahmi, and Dr. Totik Sri Mariani.

Conflict of interest

The authors have declared no conflict of interest.

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