

PHYTOCHEMICAL ANALYSIS OF CALLUS TWO VARIETIES *ORTHOSIPHON ARISTATUS* (BLUME) MIQ ON MURASHIGE AND SKOOG MEDIA: A STRATEGIC STEP OF SECONDARY METABOLITE PRODUCTION

FAHRAUK FARAMAYUDA^{1,2}, TOTIK SRI MARIANI³, ELFAHMI^{1,4}, SUKRASNO^{1*}

¹School of Pharmacy, Institut Teknologi Bandung (ITB), Bandung, West Java, Indonesia, 40132, ²Faculty of Pharmacy Universitas Jenderal Achmad Yani (UNJANI), Cimahi, West Java, Indonesia, 40532, ³School of Life Sciences and Technology, Institut Teknologi Bandung (ITB), Bandung, West Java, Indonesia, 40132, ⁴Biosciences and Biotechnology Research Center, Institut Teknologi Bandung (ITB), Bandung, West Java, Indonesia, 40132
*Email: sukras@fa.itb.ac.id

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ABSTRACT

Objective: The research aimed to provide new information regarding the secondary metabolites content of purple and white-purple *Orthosiphon aristatus* (Blume) Miq. callus, which can then be used as a basis for developing towards cell suspension and ultimately producing secondary metabolites using bioreactors.

Methods: Callus induction of two varieties of *O. aristatus* were performed by inoculating sterile leaf explants grown on Murashige and Skoog basal media supplemented with 2,4-dichlorophenoxyacetic acid 0.4 ppm. The secondary metabolites were analysed and quantified using high-performance liquid chromatography with gradient elution.

Results: The results showed the growth of callus two varieties of *O. aristatus* in growth media MS with 2,4-D 0.4 ppm. Rosmarinic acid content in the acetone extract of the purple variety callus was 1.28% w/w, and the white-purple variety was 2.22% w/w.

Conclusion: This study could form the basis for the development of rosmarinic acid production by *In vitro* culture modification.

Keywords: Purple variety *O. aristatus*, White-purple variety *O. aristatus*, Modification of *in vitro* culture, Growth media, Phytochemical profiling of callus

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INTRODUCTION

Orthosiphon aristatus is distributed in China, Africa, Malaysia, India, and Indonesia [1]. Bogor area is one of the areas where cat whiskers are cultivated in Indonesia [2]. The meaning of *Orthosiphon* is orthos (straight) and siphon (cylinder). [3]. The leaves of *O. aristatus* have a rhombic shape [4]. Based on the color of flowers and petals, there are *O. aristatus* purple, white and white-purple varieties. The secondary metabolite content in purple varieties is higher than in white varieties [5].

The *O. aristatus* by local communities in Indonesia has been used as a diuretic or urinary remedy to cure diabetes mellitus [6]. The plant used alternative medicine in Malaysia and has also been sold as a dietary supplement in recent years [7]. In many European countries, *O. aristatus* is consumed as a herbal tea to promote health due to its high antioxidant properties [8]. Japan uses *O. aristatus* as an essential ingredient in health teas [9-11].

The content of metabolites, the main secondary to *O. aristatus* is sinensetin, rosmarinic acid, and eupatorin. In 2018 Cai reported that sinensetin levels in *O. aristatus* leave, 2.719 mg/g rosmarinic acid levels in leaves of 19,861 mg/g and eupatorin levels in leaves 4.731 mg/g [12]. The levels of secondary metabolites in *O. aristatus* are still low, so it is necessary to increase the levels, one of which is by plant tissue culture techniques [13].

O. aristatus callus production was obtained when leaf explants were cultured in MS, added with by 1.0 mg/l 2,4-D, and 1.0 mg/l NAA as growth regulators. A callus is used to initiate cell suspension culture, 0.75 g callus cells in the liquid medium MS 20 ml is added with 1.0 mg/l 2,4-D was the best conditions for the culture of *O. aristatus* cell suspension. Optimal cell culture growth is maintained by subculturing every two weeks [14]. Previous research reported that leaf explants of two varieties of *O. aristatus* grown on MS and 2,4-D 0.4 ppm media produced good weight, callus texture within two weeks [15]. Research on the identification and determination of secondary metabolite levels in callus of two varieties of *O. aristatus*

has not been reported. The results of this study are expected to provide new information regarding the secondary metabolites content of purple and white-purple *O. aristatus* callus, which can then be used as a basis for developing towards cell suspension and ultimately producing secondary metabolites using bioreactors.

MATERIALS AND METHODS

Chemicals and reagents

Media Murashige and Skoogs (MS) was purchased from Phytotechlab (KS, US). 2,4-dichlorophenoxyacetic acid (2,4-D), agar phytigel, rosmarinic acid, and sinensetin were purchased from Sigma (St. Louis, MO, USA). NaOCl Bayclin was purchased from SC Johnson (Jakarta, Indonesia). tween 80 (Labchem). Ethanol, methanol, ethyl acetate, acetone, toluene, acetonitrile HPLC grade, and methanol HPLC grade were purchased from Merck (Jakarta, Indonesia). Formic acid was purchased from Loba Chemie (Mumbai, India).

Instrumentation

HPLC Gradient (HP-agilent, Germany), autoclave (PBI international Italy), laminar airflow cabinet (Holten, UK), balance sheet (Mettler Toledo, Hong Kong), pH meter (Mettler Toledo, Hong Kong), analytical scales (Shimadzu, Japan), ovens (Memert, Germany), rotary evaporators (Heidolp, Germany).

Material collection and processing

The parts of the plants used as explants in this study were purple and white-purple varieties of *O. aristatus*, obtained from the Manoko medicinal plant garden, Lembang, Bandung, West Java. The leaves of the *O. aristatus* from the two varieties that have been obtained were carried out wet sorting, then washed with running water. Determination of plants at the School of Life Sciences and Technology, Institut Teknologi Bandung, with letter number 6115/I1. CO2.2/PL/2019 shows that the plant specimens examined were purple and white-purple *O. aristatus*.

Media and equipment sterilization

The equipments used were glassware and culture bottles washed before use, then wrapped in paper and sterilized using autoclave at a temperature of 121 °C for 15 min. Other equipment such as tweezers, scalpels, culture knives, sterilized by direct heat from a bunsen fire. The MS media made were sterilized together with the equipments that will be used in the culture process.

Explants sterilization

The explants of the *O. aristatus* were washed in running water using detergent, soaked in a 2% fungicide solution for 10 min, cleaned using running water, the explants were put in an erlenmeyer flask, added with 70% alcohol solution for 1 minute, the remaining alcohol of sterilization was discarded then added to the solution mixture Na hypochlorite, and Tween 80 were carried out for 5 min. After that, the solution mixture was discarded and the explants rinsed with sterile water three times.

Explants inoculation preparation

The explants that have been washed were then taken to the culture room. Before starting, make sure the UV lamp on the Laminar Air Flow (LAF) had been turned on for 60 min. Spray the entire surface of the LAF using 70% alcohol and wipe with a tissue. The equipments culture process and the basic media were sprayed with 70% alcohol before being transferred to the LAF.

Inoculation of explants on tissue culture media

The leaf explants of the *O. aristatus* are cut into small square pieces 1 cm in length and 2 cm wide in a petri dish and grown on MS medium plus 2,4-D 0.4 ppm.

Callus growth observations

Observations were made by recording every change in explants for three days until a change was seen in the form of explants that turned into a callus. Then the percentage of successful callus growth was calculated. The number of explants that succeeded in forming the callus was divided by the number of explants planted.

Callus drying

The freshly taken callus was dried in an oven at 50 °C and weighed every 1 h until the difference between the first weighing and the next is not more than 0.5 mg; this is the dry weight of the sample.

Callus extraction

The dry callus was then mashed and extracted by maceration using three solvents with different polarities, namely acetone, ethyl acetate, and ethanol. A total of 1g of dry callus of two varieties of *O. aristatus* put in a 25 ml vial. Each added solvent is 15 ml. Callus that had been soaked in a solvent was let to stand for 24 h while stirring in an orbital shaker at high speed. The macerate is separated by filtration. The extraction process is repeated at least twice with the same type and amount of solvent. All macerate is collected, then concentrated by evaporating using a rotary evaporator, followed by evaporation of the extract on a water bath until a thick extract was obtained.

Preparation of marker and sample solutions

Qualitative and quantitative analysis of callus using HPLC has been done by preparation of marker and sample solutions. Rosmarinic acid was weighed as much as 1 mg and dissolved in 1 ml methanol grade HPLC. The stock solution was diluted with methanol to five concentrations ranging from 60 to 100 µg/ml. 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 a syringe filter.

HPLC instrumentation and conditions

The HPLC used is a gradient system using a reversed-phase C18 column. Column temperature 25 °C. The mobile phase consisted of 0.1% formic acid solution and acetonitrile with a gradient elution system where the ratio of formic acid 0.1%: acetonitrile at 0 min (85:15), 1 min (85:15), 12 min (35:65), the 15th minute (85:15), the

18th minute (85:15). Flow rate of 1 ml/minute. The separation time was 20 min. The method of determining the levels of secondary metabolites of *O. aristatus* refers to Saidan *et al.* (2015) with modifications to the maximum wavelength used of 340.6 nm [16].

Data analysis

HPLC analysis was prepared in three replications. Callus induction at each concentration of growth regulators was made five repetitions. Data are expressed as mean±SD. Data processing was performed by one-way ANOVA, followed by Duncan's multiple range test using SPSS 22 software. P values<0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Plant determination

The results of the two varieties of *O. aristatus* determination at the School of Life Science and Technology, Bandung Institute of Technology showed that the plant was *Orthosiphon aristatus* (Blume) Miq. purple flowers and white-purple flowers. Observation of the morphology of the two varieties of *O. aristatus* showed that the leaves of the two varieties had differences in the leaf veins purple varieties have purple leaf veins and white-purple varieties in green. The stems of the purple variety are purple and the stems of the white-purple varieties have a green color. The crown color of the purple variety is purple, while the white-purple variety is white with a purple tinge (fig. 1).

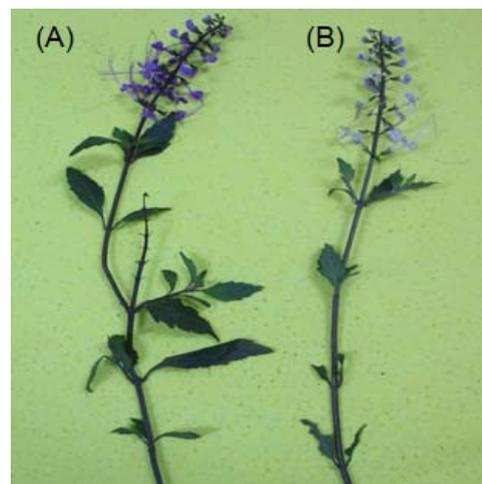


Fig. 1: Observation of the morphology of two varieties of *O. aristatus*. (A) Purple variety; (B) white-purple variety

O. aristatus are divided into three varieties white, white-purple and purple. Characterization of *O. aristatus* morphology is important to distinguish the three varieties because according to several previous studies reported that the sinensetin content in purple variety was more significant than white variety [5]. However, there were no reports on the differences in the secondary metabolite content between purple and white-purple varieties. The difference between several varieties of *O. aristatus* can be seen from the morphology of flowers [17]. In this study, the differences in the morphology of flowers from the purple and white-purple varieties, were the purple crown for the purple variety and white purple crown with purple tinge for the white-purple variety. The leaf color for two varieties was green. This study are in line with that reported by Almatar *et al.* (2013) [18].

Callus growth and drying

MS medium with the addition of growth regulators 2,4-D 0.4 ppm can grow callus from leaf explants of two varieties of *O. aristatus* within two weeks with 100% callus response and white-green color. On the 6th day observation, white-purple callus started to grow, and for the purple varieties on the 10th day, the callus started to grow. The dry weight of callus white-purple varieties on day 14 was greater than that of purple varieties (table 1, fig. 2).

Table 1: Callus induction of two varieties of *O. aristatus*

Variety	Growth regulator	Time formed callus (days)	Texture (14 d)	Color (14 d)	Callus response (%)	Dry weight (g)
Purple variety	MS and 2,4-D 0.4 ppm	10 ^(a) ±0.00	friable	White-green	100	2.10±0.06 ^(a)
White-purple variety	MS and 2,4-D 0.4 ppm	6 ^(b) ±0.00	friable	White-green	100	2.48±0.01 ^(a)

The data was given in mean±SD, n= 5. The mean value was significantly different (p<0.05)

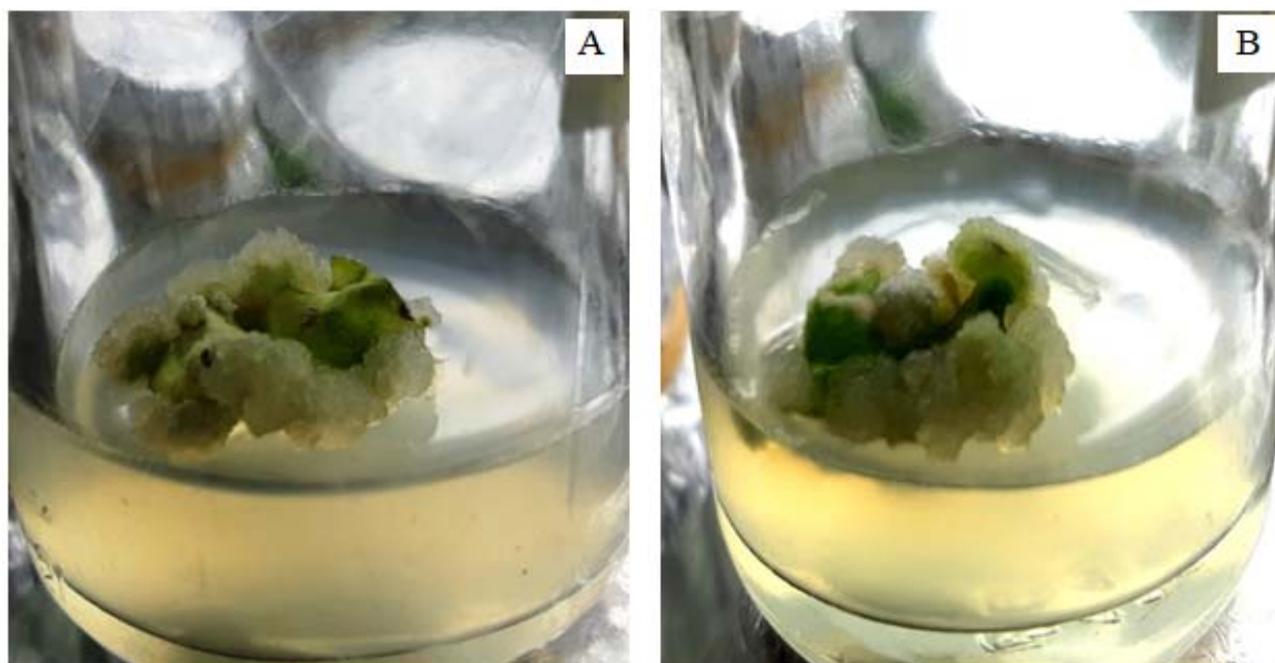


Fig. 2: Callus of two varieties of *O. aristatus*: (A) purple callus with 2,4-D 0.4 ppm; (B) white-purple callus with 2,4-D 0.4 ppm

Callus culture is an *In vitro* culture technique that is widely used to produce disease-free plant seeds. There are many advantages to the use of callus culture, including it can be produced in large quantities under controlled environmental conditions, does not require a large area of land, and can produce higher metabolites than the original plant. The optimal callus induction depends on the selection and concentration of growth regulators [19-22].

In vitro culture modification that was carried out using a growth regulator 2,4-D 0.4 ppm was able to grow callus from two varieties of *O. aristatus*; there was a statistically significant difference in terms of the time of callus formation in white-purple varieties compared to purple varieties, callus was formed faster in white-purple varieties. The dry weight of callus varieties white-purple was higher than that of purple varieties, but statistically, there was no significant difference. Faramayuda *et al.* In 2019 reported that the growth regulators 2,4-D 0.4, 0.8, and 1.2 ppm could induce callus growth of two varieties of *O. aristatus*, where 2,4-D 0.4 ppm grew faster and produce callus weights that were higher greater than 2,4-D 0.8 and 1.2 ppm [15]. The results of other studies reported that *O. aristatus* callus was formed on MS medium supplemented with growth regulators NAA 1 ppm+2,4-D 1 ppm [14]. Colored callus was formed on MS medium with the growth regulator Kinetin 1.0 ppm+IAA 1.0 ppm, Kinetin 1.5 ppm+IAA 1.5 ppm, and kinetin 2.0 ppm [23]. *O. aristatus* leaf explants were grown on MS medium, and growth regulator 2,4-D 2 ppm was able to induce the growth of callus. Research on callus induction of two varieties of *O. aristatus* with growth regulators 2,4-D 0.4 ppm has not been reported. The response change in explant size occurs because of the interaction between the explants and the growth environment and growth regulators through nutrient absorption by the explants. The addition

of 2,4-D in this study was able to provide a swelling response. In another research report, the addition of 2,4-D 1 ppm to liquid P-4 medium can induce the growth of callus *Triticum aestivum* L [24]. Young leaf explants of *Clinacanthus nutans* grown on MS medium and 2,4-D 0.50 ppm were able to induce callus with high frequency, and on old leaves, the callus grew well at 2,4-D 0.25 ppm Induction [25]. MS medium supplemented with 2,4-D 0.25 ppm can induce somatic embryos from callus *Salicornia brachiata* Roxb [26]. *Gymnema sylvestre* leaf explants grown in MS medium supplemented with 2,4-D 0.50 ppm increased callus growth [27]. 2,4-D 2.50 ppm and 3.00 ppm can form embryogenic callus *Saccharum officinarum* [28].

Qualitative analysis of callus

In the qualitative and quantitative analysis of secondary metabolites using HPLC. The marker chromatogram pattern of rosmarinic acid and sinensetin was observed. Rosmarinic acid chromatogram appeared at 6.19 min and sinensetin at 10.87 min. The standard mixed chromatogram of rosmarinic acid and sinensetin showed the same retention time as the individual chromatogram (fig. 3).

Qualitative analysis of callus in acetone, ethyl acetate, and ethanol extracts of two varieties of *O. aristatus* using HPLC was observed at two wavelengths of 340.6 nm and 254 nm. Rosmarinic acid was detected in the acetone callus extract of purple and white-purple varieties, but for the sinensetin compound, it was not detected because there were no peaks that appeared at Rt 10.87. In observations at two wavelengths, the peaks that appeared in the acetone callus extract of white-purple varieties were more compared to other extracts (table 2; fig. 4; fig. 5).

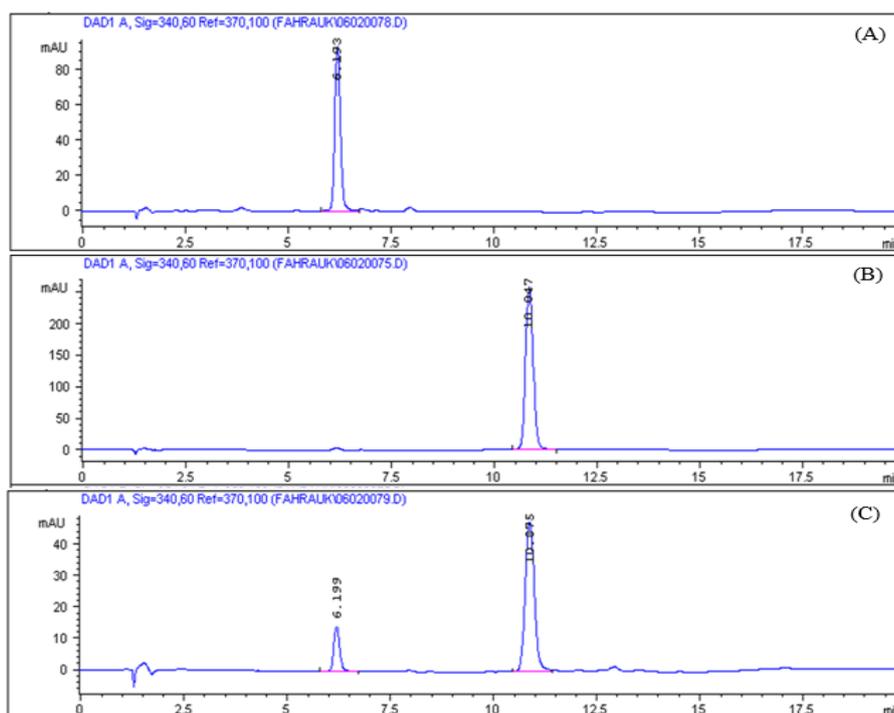


Fig. 3: High performance liquid chromatography chromatogram of rosmarinic acid and sinensetin markers at 340.60 nm: (A) rosmarinic acid; (B) sinensetin; (C) marker mixture of rosmarinic acid and sinensetin

Table 2: Data retention time and area of peak callus extract of two varieties of *O. aristatus*

Sample	Signal 340.6 nm		Signal 254.24 nm	
	RT (min)	Area (mAU*s)	RT (min)	Area (mAU*s)
Callus acetone extract (purple)	3.77	921.47	5.75	1796.26
	6.19	2609.29	7.04	459426.67
	7.08	1070.64		
	8.85	1795.5		
Callus ethyl acetate extract (purple)	11.06	154.95	3.42	154.95
			9.06	8720.01
			12.27	1668.05
			13.69	1937.97
Callus ethanol extract (purple)	6.78	1141.62	13.79	1182.14
	12.79	1217.32		
Callus acetone extract (white-purple)	3.72	1525.75	5.63	5633.04
	6.19	5123.94	7.08	362404.00
	7.13	1245.52	8.27	2509.64
	8.86	4101.92	8.88	3809.01
	11.14	1660.54	12.01	1799.15
Callus ethyl acetate extract (white purple)	8.85	704.40	13.74	2433.08
	11.09	638.92	3.45	1140.36
			9.05	5704.06
			12.26	1324.79
Callus ethanol extract (white-purple)	ND	ND	ND	ND

Note: The data were given in mean (n=3), ND = Not Detected

In the qualitative analysis using HPLC at wavelengths of 340.60 nm and 254.24 nm, several peaks were identified in the extract of acetone, ethyl acetate, and ethanol callus of two varieties of *O. aristatus*. The purple and white-purple varieties of acetone callus *O. aristatus* produced quite a lot of peaks. Both extracts also identified the presence of rosmarinic acid, which was indicated by the appearance of the peak at Rt 6.19. Observations at 254.24 nm signal on purple variety acetone callus extract showed a peak (Rt 7.02) with a considerable area value of 459426.67 mAU*s, and on Rt 7.08 callus acetone extract, white-purple varieties also identified peaks with a large area of 362404.00 mAU*s. The two peaks need to be developed and identified further because they have a large area.

Quantitative analysis of callus

Quantitative analysis of the callus of two varieties of *O. aristatus* was carried out at the peak observed at the 340.60 nm signal. In the callus acetone extract of purple and white-purple varieties, rosmarinic acid was detected because the peak appeared with a retention time (RT) of 6.19, where the RT value was the same as the value of rosmarinic acid marker. For the two extracts, rosmarinic acid levels were determined with a linearity range of 60-100 ppm, regression equation $y = 17.886x - 827.14$ and $R^2 = 0.9954$ (n = 3). The rosmarinic acid content in the callus extract of the purple variety was 1.28% w/w, and the white-purple variety was 2.22% w/w (table 3).

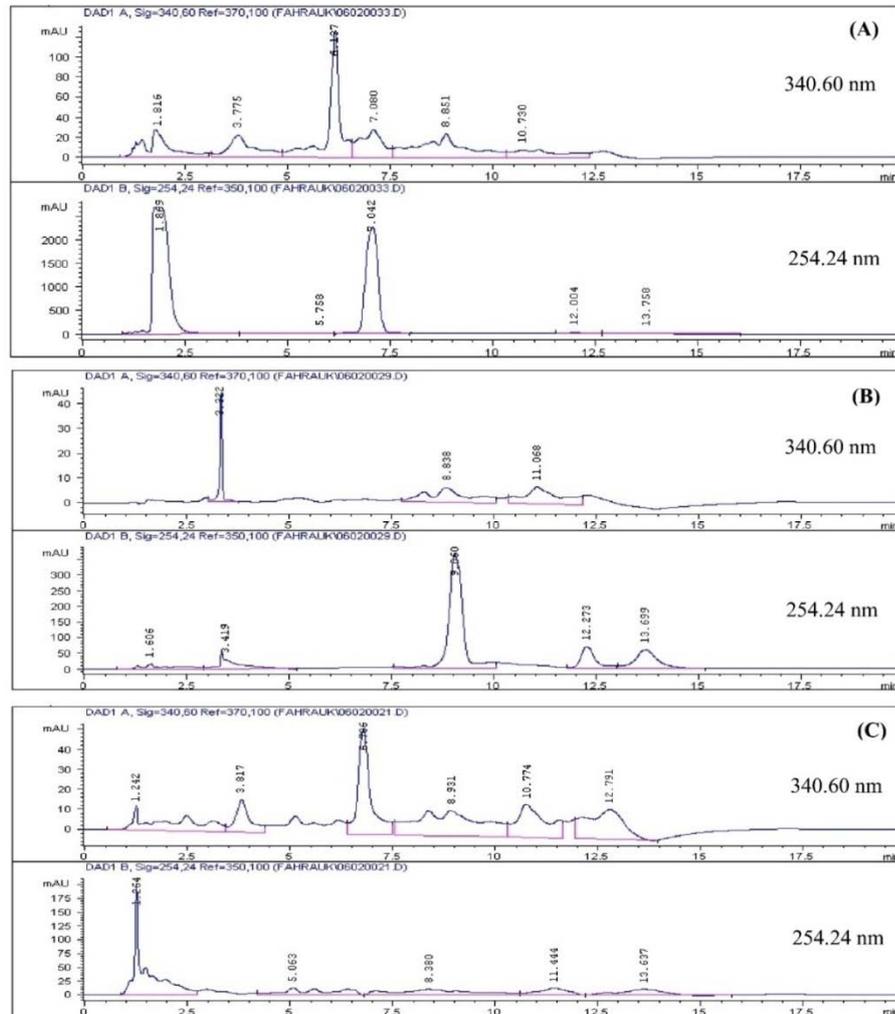


Fig. 4: High-performance liquid chromatography chromatogram extract of the purple variety callus at 340.60 nm and 254.24 nm. A = purple variety callus acetone extract, B = purple variety callus ethyl acetate extract, C = purple variety callus ethanol extract

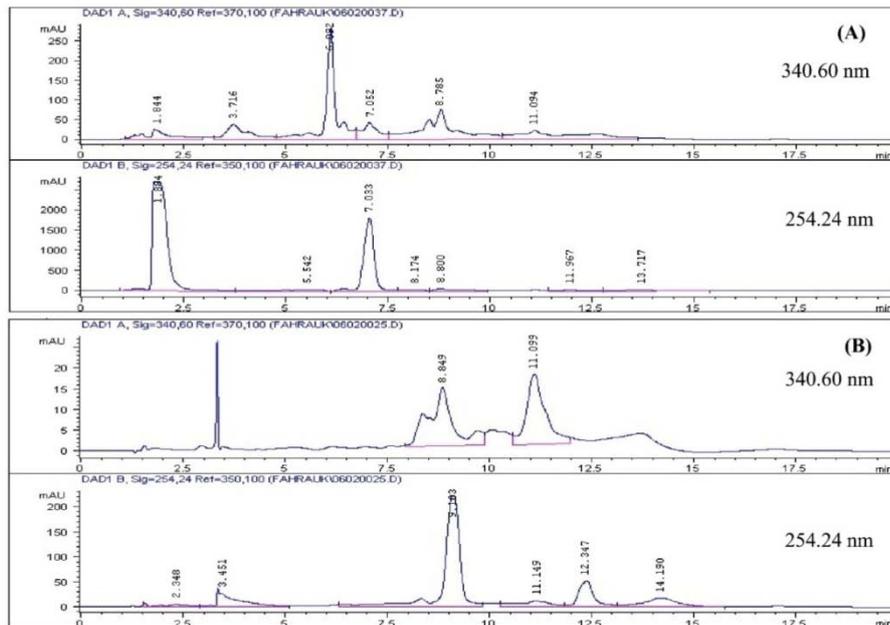


Fig. 5: High-performance liquid chromatography chromatogram extract of the white-purple variety callus at 340.60 nm and 254.24 nm. A = white-purple variety callus acetone extract and B = white-purple variety callus ethyl acetate extract

Table 3: Levels of rosmarinic acid from acetone extracts of two varieties of *O. aristatus*

Sample	Rosmarinic acid (% w/w)
Purple variety callus acetone extract	1.28±0.14 ^a
White-purple variety callus acetone extract	2.22±0.14 ^b

The data were given in mean±SD (n=3). The mean value was significantly different (p<0.05)

Quantitative analysis was carried out on rosmarinic acid identified in callus acetone extract using gradient system HPLC following the validated method of Saidan and team in 2015 [16]. The rosmarinic acid content of purple callus acetone extract at a concentration of 15 mg/ml was 1.28%, and the white-purple variety was 2.22%. Previous research reports stated that in the original plant, *O. aristatus* that purple varieties had higher secondary metabolite content than white varieties [5]. However, in this study, the rosmarinic acid levels in the callus of white-purple varieties were higher than in purple varieties. This could be due to the effect of *In vitro* culture modification on the levels of rosmarinic acid, especially in white-purple varieties. Another report stated that the sinensetin content is higher in the purple variety of *O. aristatus* than other varieties [29]. Saidan et al. in 2015 determined the levels of the main secondary metabolites in one variety of *O. aristatus*, and the results were in the ethanol extract 10 mg/ml sinensetin levels were 1.42% w/w, rosmarinic acid 1.61% w/w, eupatorin 5.27% w/w and the highest levels of rosmarinic acid was found in methanol: water (1: 1) extract with a value of 4.78% [16]. Secondary metabolite levels of chloroform fraction of white *O. aristatus* (1 mg/ml) with HPLC using a non-gradient mobile phase acetonitrile: water (40:60) system were reported by Yam et. al in 2010, where the levels of sinensetin were 2.86% w/w, eupatorin 5.05% w/w and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone 1.10% w/w [30].

The effort to produce rosmarinic acid from *O. aristatus* cell suspension cultures has been carried out by Bordbar et al. (2015) where cell suspension cultures given the abiotic sucrose elicitor at pH 5.70 can produce rosmarinic acid 25.93 mg/g dried cell (2.59% w/w) [31], the rosmarinic acid content was not much different from the rosmarinic acid levels in the white-purple variety of acetone extract (2.22% w/w). Lim et al. in 2013 reported that the addition elicitor of 45 g/l sucrose and 1.5 g/l chitosan in liquid proliferation cell medium (MS+1 mg/l 2,4-D and 1 mg/l NAA) could increase total phenolic levels. in cell suspension culture, while the addition of NaCl elicitor 1-3 g/l, Casein hydrolysate 0.3-3.0 g/l, and yeast extract 0.25-0.75 g/l did not increase the total phenolic content [32]. The results of this study could be the basis for the production of rosmarinic acid from suspension cultures of *O. aristatus*. The production of rosmarinic acid compounds needs to be increased because these compounds have good pharmacological activities, including antioxidant, anti-inflammatory, antimutagenic, antigenotoxic, cytotoxic, antimetastatic, antiangiogenic, antimicrobe, and immunomodulatory [33]. *In silico* studies suggest that rosmarinic acid has antidiabetic activity [34]. Rosmarinic acid compound is thought to have activity as an inhibitor of the α -glucosidase enzyme activity in *Ocimum canum* [35].

CONCLUSION

The extract of acetone callus from two varieties of *O. aristatus* contains rosmarinic acid. The levels of rosmarinic acid in the callus of purple varieties were 1.28% w/w, and white-purple varieties were 2.22% w/w. The callus of two varieties of *O. aristatus*, which was modified by *in vitro* culture on MS media with added growth regulator 2,4-D 0.4 ppm, has the potential to be developed in the culture suspension stage for the production of rosmarinic acid compounds.

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AUTHORS CONTRIBUTIONS

Fahrauk Faramayuda carried out the experiment and wrote the manuscript with support and supervise from Prof. Sukrasno, Dr. Elfahmi and Dr. Totik Sri Mariani.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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