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Effect of Media Variation on the Induction and Phytochemical Profile of Callus in Two Varieties of Cat's Whiskers (*Orthosiphon aristatus* Blume Miq)

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ABSTRACT

The levels of rosmarinic acid and sinensetin in purple and white-purple varieties of *Orthosiphon aristatus*, cat's whiskers, can be increased using modified *in vitro* culture. This work focused on callus induction of the purple and white-purple variety of cat's whiskers grown on Gamborg (B5) and CHU (N6) with the addition of growth regulators 2,4-dichlorophenoxyacetis acid. Our observation suggested that the callus could grow within three weeks and produce rosmarinic acid and sinensetin. The level of sinensetin from various extraction methods is relatively low; in contrast, the rosmarinic acid from purple callus was detected at about 5% w/w, while the white-purple variety was around 2% w/w. The results of this study also provided new information on the basic media other than MS that can grow cat's whiskers callus while producing active compounds.

1. Introduction

The cat's whiskers plant (*Orthosiphon aristatus*) that grows in Indonesia is divided into three varieties, named white, white-purple, and purple, based on the flower color (Faramayuda *et al.* 2021a). Two compounds extracted from cat's whiskers, rosmarinic acid and sinensetin, have some pharmacological activities. For instance, rosmarinic acid potentially inhibits liver virus replication (Tsukamoto *et al.* 2018) and COVID-19 (*in silico* study, Faramayuda *et al.* 2021b; Wondmkun and Mohammed 2020). It also acts as a treatment for hand, foot, and mouth diseases caused by enterovirus 71 (EV71) (Hsieh *et al.* 2020; Lin *et al.* 2019) or metabolic disorders caused by estrogen level decrease (Zych *et al.* 2019). In addition, the second compound, sinensetin, can be developed as a drug for H1N1 influenza virus infection (Li *et al.* 2020). It also exhibits potent anticancer activity against drug-resistant human gallbladder adenocarcinoma cells (Huang *et al.* 2020) and may treat breast cancer (Rezakhani *et al.* 2020).

Based on the various extraction methods, some reports mentioned the low amount of major secondary metabolites in cat's whiskers extracts. Guo *et al.* (2019) reported the levels of rosmarinic acid were 2.826 mg/g and sinensetin at 0.057 mg/g. Furthermore, in acetone-water extract (70:30) of cat's whiskers, the sinensetin content was 0.32% w/w and methanol-water (1:1) 0.15% w/w (Hossain and Ismail 2016). Similarly, Cai *et al.* (2018) and Batubara *et al.* (2020) also mentioned the low amount of both metabolites in cat's whiskers extract.

Then, this study focuses on a purple and white-purple variety of cat's whiskers to improve the rosmarinic acid and sinensetin productions via callus culture by implementing some practical steps. Callus induction is one of the tissue culture methods by stimulating continuous cell division from certain plant parts such as leaves, roots, and stems by using growth regulators to form a cell mass (Muguerza *et al.* 2022). In a previous study, callus induction of both varieties of cat's whiskers was carried out using Murashige and Skoog (MS) + 2,4-D 0.4 ppm media resulting in a small amount of rosmarinic acid, 1.28% w/w (purple variety) and 2.22 %w/w (white-purple variety) respectively

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(Faramayuda *et al.* 2020). It is then necessary to observe callus induction of two varieties of cat's whiskers on media other than MS and then the rosmarinic acid and sinensetin productions.

2. Materials and Methods

2.1. Chemicals and Reagents

Two callus-inducing mediums, Gamborg (B5) and CHU (N6), were purchased from Phytotechlab (Lenexa, Kansas, US). Meanwhile, the Gamborg (B5) and CHU (N6) medium contained potassium nitrate as a source of nitrate and macronutrients, micronutrients, vitamins, amino acids, and carbohydrates. 2,4-dichlorophenoxyacetis acid (2,4-D) (plant growth regulator) and agar phytagel were purchased from Sigma (St. Louis, MO, USA). Then, ethanol, acetone, and ethyl acetate for solvent extraction (Merck Jakarta, Indonesia). The HPLC standards were rosmarinic acid and sinensetin (powder, Sigma, St. Louis, MO, USA). In addition, for the mobile phase, HPLC grade methanol (Merck, Jakarta, Indonesia), HPLC grade acetonitrile (Merck, Indonesia), and formic acid (Loba Chemie, India).

2.2. Plant Collection

Cat's whiskers' leaves and stems of purple and white-purple varieties were collected from Lembang, Indonesia. Moreover, the identification of plants was conducted at the School of Life Science and Technology, Bandung Institute of Technology (ITB).

2.3. Callus Induction

The surface sterilization of leaf explants was conducted by washing the leaf under running water for 15 minutes, soaking in detergent for 15 minutes, and immersing in 2% fungicide for 10 minutes. The explants were sterilized in 70% ethanol for 1 minute and immersed in a commercial bleach solution (Johnson, Jakarta, Indonesia) for 5 minutes in laminar airflow. Explant then was rinsed with sterile water three times.

The media for callus induction were Gamborg (B5) and CHU (N6) (pH 5.7-5.8), with 2.4-D (0, 0.4, 1, and 2 ppm) as a growth regulator. Media sterilization was performed using an autoclave at 121°C for 15 minutes. Aseptically, transferring explants into the media in the culture bottle. Then, culture bottles were stored in the incubator room with a 36W Philips lamp on the light/dark cycle 8/16 at 19-20°C for 21 days. Five replicates on each medium were employed for callus induction.

2.4. Callus Extraction

The callus was separated from the medium, oven dried (70°C), and crushed to obtain callus powder. Maceration of the powder was then conducted separately using three solvents: acetone, ethyl acetate, and ethanol (Table 1).

2.5. Preparation of Standard Solutions and Samples

Qualitative and quantitative analyses of the callus of two varieties of cat's whiskers were performed using HPLC instruments. The standard solutions for HPLC are diluted in methanol and have the following

Table 1. Detail of callus extraction of two varieties of *O. aristatus*

Code	Media	Sample	Solvent
CP 1	Gamborg (B5) medium	callus purple	acetone
CP 2	Gamborg (B5) medium	callus purple	ethyl acetate
CP 3	Gamborg (B5) medium	callus purple	ethanol
CP 4	CHU (N6) medium	callus purple	acetone
CP 5	CHU (N6) medium	callus purple	ethyl acetate
CP 6	CHU (N6) medium	callus purple	ethanol
CWP 1	Gamborg (B5) medium	callus white-purple	acetone
CWP 2	Gamborg (B5) medium	callus white-purple	ethyl acetate
CWP 3	Gamborg (B5) medium	callus white-purple	ethanol
CWP 4	CHU (N6) medium	callus white-purple	acetone
CWP 5	CHU (N6) medium	callus white-purple	ethyl acetate
CWP 6	CHU (N6) medium	callus white-purple	ethanol

concentrations of rosmarinic acid 1 mg/ml. The test solution was prepared by dissolving 15 mg of the extract in 1 ml of methanol (1,500 ppm) and sonicating for 45 minutes. The HPLC was conducted in three replications (3 analyses using the same sample).

2.6. Instrumentation and HPLC Conditions

The instrument is a gradient system HPLC using a reversed-phase C18 column with a conditioned temperature of 25°C. The mobile phase consisted of 0.1% formic acid solution and acetonitrile with a gradient elution system in which the ratio of formic acid was 0.1%: acetonitrile at 0 minutes (85:15), 1 minute (85:15), 12 minutes (35:65). The flow rate of the mobile phase was 1 ml/minute, while the separation time was 20 minutes (Saidan *et al.* 2015).

2.7. Data Analysis

Data processing was performed using SPSS 22 (IBM SPSS Statistics for Windows, Version 22.0. IBM Corp., Armonk, NY). Data are expressed as mean ± SD, and a P-value <0.05 was considered statistically significant.

3. Results

3.1. Morphology Characterization

Purple and white purple varieties of *O. aristatus* varieties can be distinguished based on the morphology of the flowers. The purple varieties have a purple crown; meanwhile, the white-purple varieties have white color (Figure 1).

3.2. Induction of the Callus

B5p-0.4; N6p-0.4; B5wp-0.4 and N6wp-0.4 could grow callus within 12 days (Table 2, Figure 2). The plant growth regulators 2,4-D at 0.4 ppm produced friable callus with white color until slightly brown; while 2,4-D at a concentration of 1 and 2 ppm produced a white-brown color with semi-friable texture (Table 3, Figure 3).

3.3. Qualitative and Quantitative Analysis of Compounds in Callus Extract of Purple and White-purple Varieties of Cat's Whiskers

Rosmarinic acid and sinensetin standards were detected at retention times (RT) of 6.19 and 10.97 (Figure 4). In the CP 1 dan CWP 1, rosmarinic acid was detected. (Table 4, Figure 5 and 6). In CHU (N6), medium rosmarinic acid was detected in CP 4 and CP 6 (Figure 7), while in white-purple varieties, rosmarinic acid was found in CWP 4, CWP 5, and CWP 6 (Table 4, Figure 8). Sinensetin compounds were detected in CP 6 (Figure 7).

The highest rosmarinic acid level was found in CP 4, with 4.94% w/w. However, it was still not significantly different from CP 6, with a rosmarinic acid level of 4.79% w/w. The concentration of sinensetin in the CP 6 medium was 0.43% w/w (Table 5, Figure 9).

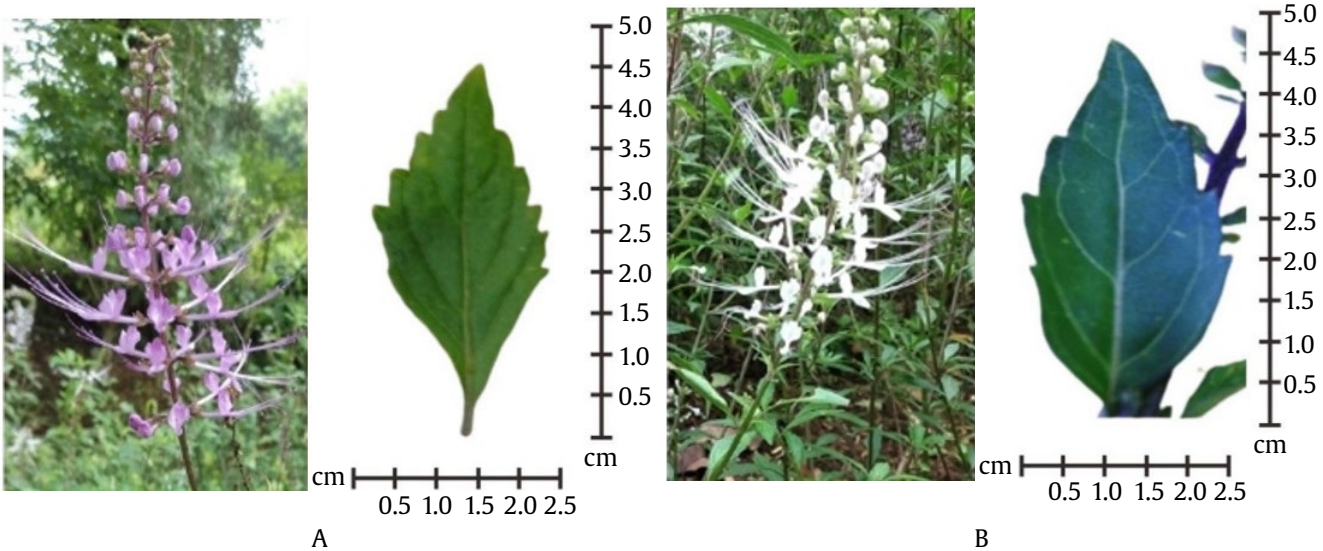


Figure 1. Morphology of the two varieties of *O. Aristatus*: purple (A) and white-purple (B) variety

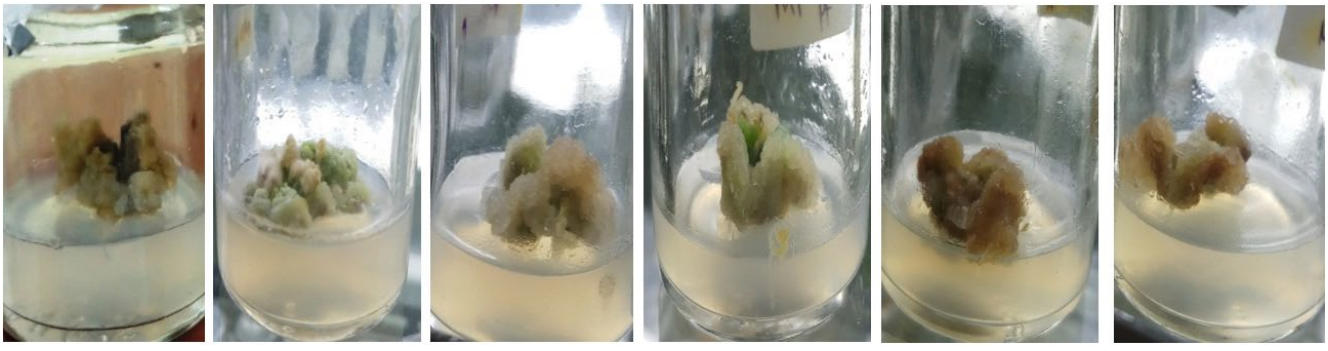
Table 2. Callus induction of the purple variety of *O. aristatus* callus in various media

Media + growth regulator	Time to form callus (days) n = 3±SD	Texture (21 days)	Color (21 days)
Purple variety			
Gamborg (B5) + 2,4 D 0.4 ppm (B5p-0.4)	12±0.01	friable	white–less brown
Gamborg (B5) + 2,4 D 1 ppm (B5p-1)	16±0.01	semi friable	white–brown
Gamborg (B5) + 2,4 D 2 ppm (B5p-2)	18±0.01	semi friable	white–brown
	-	-	-
CHU (N6) + 2,4 D 0,4 ppm (N6p-0.4)	12±0.01	friable	white–less brown
CHU (N6) + 2,4 D 1 ppm (N6p-1)	13±0.00	semi friable	white–brown
CHU (N6) + 2,4 D 2 ppm (N6p-2)	17±0.01	semi friable	white–brown
	-	-	-

(-) = no callus formed

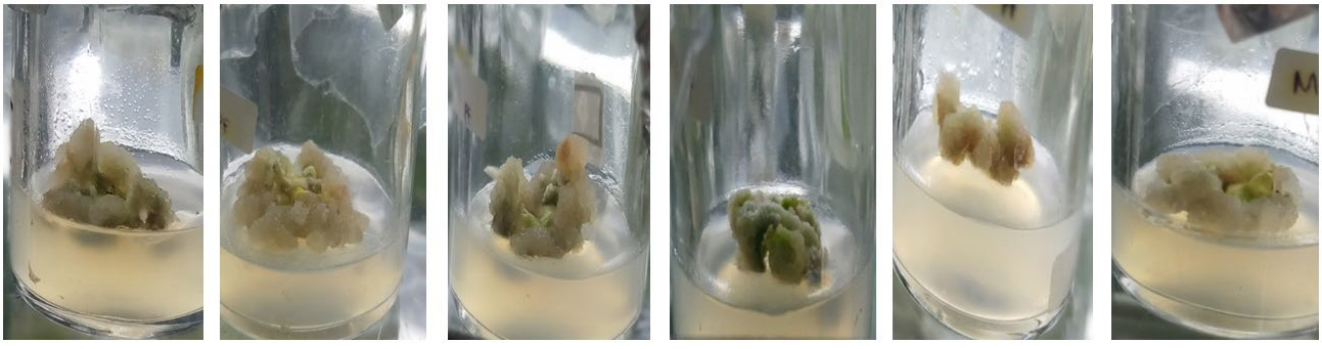
Table 3. Induction of the white-purple variety of *O. aristatus* callus in various media

Media + growth regulator	Time to form callus (days) n = 3±SD	Texture (21 days)	Color (21 days)
White-purple variety			
Gamborg (B5) + 2,4 D 0.4 ppm (B5wp-0.4)	12±0.00	friable	white–less brown
Gamborg (B5)+ 2,4 D 1 ppm (B5wp-1)	12±0.01	Semi friable	white-brown
Gamborg (B5)+ 2,4 D 2 ppm (B5wp-2)	16±0.00	Semi friable	white-brown
	-	-	-
CHU (N6) + 2,4 D 0.4 ppm (N6wp-0.4)	12±0.01	friable	white–less brown
CHU (N6) + 2,4 D 1 ppm (N6wp-1)	12±0.01	Semi friable	white-brown
CHU (N6) + 2,4 D 2 ppm (N6wp-2)	14±0.00	Semi friable	white-brown
	-	-	-



A B C D E F

Figure 2. Callus *O. aristatus* purple varieties. (A) Gamborg (B5) + 2,4D 0.4 ppm, (B) Gamborg (B5) + 2,4D 1.0 ppm, Gamborg (B5) + 2,4D 2.0 ppm, CHU (N6) + 2,4D 0.4 ppm, CHU (N6) + 2,4D 1.0 ppm, CHU (N6) + 2,4D 2.0 ppm



A B C D E F

Figure 3. Callus *O. aristatus* white-purple varieties. (A) Gamborg (B5) + 2,4D 0.4 ppm, (B) Gamborg (B5) + 2,4D 1.0 ppm, Gamborg (B5) + 2,4D 2.0 ppm, CHU (N6) + 2,4D 0.4 ppm, CHU (N6) + 2,4D 1.0 ppm, CHU (N6) + 2,4D 2.0 ppm

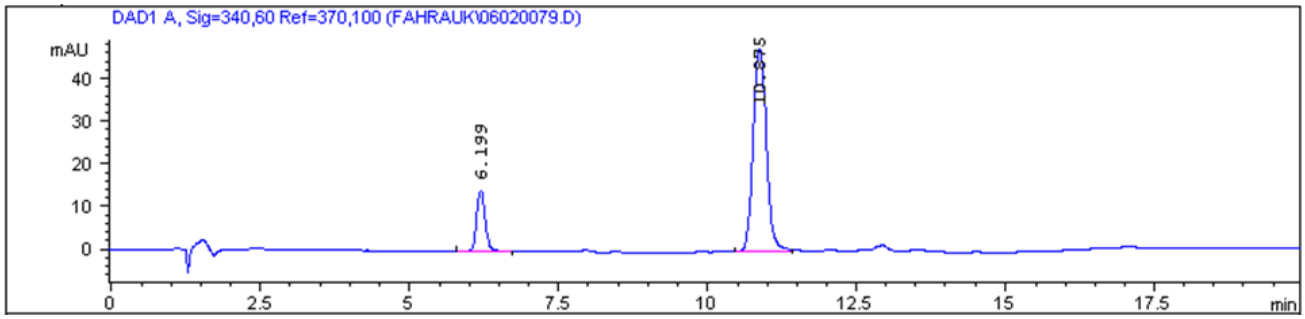


Figure 4. The chromatogram of a standard mixture of rosmarinic acid (RA) and sinensetin (S)

Table 4. Data on retention time and peak area of callus extracts of two varieties of *O. aristatus*

Sample	Signal 340.6 nm		Signal 254.24 nm	
	RT (minute)	Area (mAU*s) $\bar{x}$ (n = 3)	RT (minute)	Area (mAU*s) $\bar{x}$ (n = 3)
Gamborg (B5) medium				
Purple variety callus acetone extract (CP 1)	3.7	698.81	3.7	698.81
	6.19	2477.60	6.19	2477.60
	11.1	1342.25	11.1	1342.25
Purple variety of callus ethyl acetate extract (CP 2)	8.8	935.53	3.41	1886.08
			6.35	973.46
			9,07	160014.6
			12.29	1903.85
			13.70	1056.51
Purple variety callus ethanol extract (CP 3)	-	-	-	-
White-purple variety of callus acetone extract (CWP 1)	3.83	415.78	7.09	5633.04
	6.19	3436.54	13.87	362404.00
	7.09	708.89		
	8.86	601.475		
White-purple variety of callus ethyl acetate extract (CWP 2)		ND	3.76	698.81
			6.76	2477.60
			11.10	1342.25
White-purple variety callus ethanol extract (CWP 3)	ND	ND	ND	ND
CHU (N6) medium				
Purple variety callus acetone extract (CP 4)	3.75	512.89	7.03	665321
	5.31	495.78	9.14	5724.87
	6.19	126488.5	10.83	898.51
	7.09	1596.41	12.00	963.50
	8.80	1196.9	13.77	2443.03
	10.78	638.39		
	12.02	1434.66		
	12.7	1084.18		
Purple variety of callus ethyl acetate extract (CP 5)	3.30	615.00	5.07	1886.08
	8.70	1279.27	6.50	972.41
	10.80	865.24	7.30	170011.5
			8.30	1800.85
			10.40	1016.51
			13.72	6100.28
Purple variety callus ethanol extract (CP 6)	3.79	811.88	6.50	2571.49
	5.30	1101.79	7.20	4786.46
	6.19	120684	8.30	148078
	8.98	3366.73	11.40	5053.48
	10.80	1200.75	13.44	5582.52

Table 4. Continued

Sample	Signal 340.6 nm		Signal 254.24 nm	
	RT (minute)	Area (mAU*s) $\bar{x}$ (n = 3)	RT (minute)	Area (mAU*s) $\bar{x}$ (n = 3)
CHU (N6) medium				
White-purple variety callus acetone extract (CWP 4)	6.19	4193.4	7.01	353662.5
	8.79	4443.5	9.10	5793.9
	12.67	957.8	11.96	2143.67
			13.75	2325.22
White-purple variety callus ethyl acetate extract (CWP 5)	3.85	1140.2	3.39	1840.9
	6.19	2591.5	9.10	4992.09
			11.14	776.26
			12.33	1470.05
			13.75	2061.39
White-purple variety callus ethanol extract (CWP 6)	3.77	1222.71	5.05	7454.75
	6.19	5655.66	11.82	2572.33
	8.97	1503.89	13.46	2489.03
	12.65	957.93		

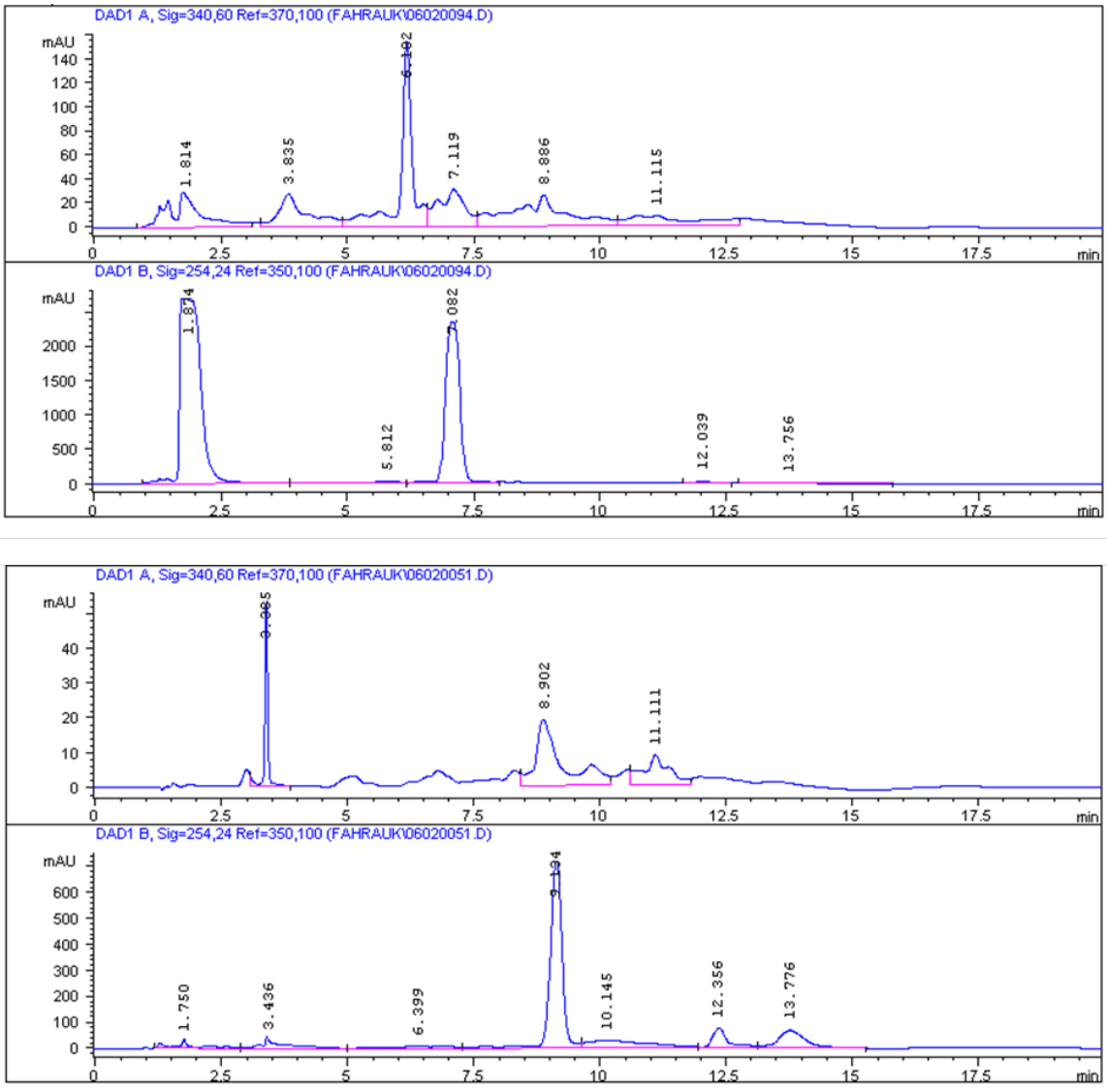


Figure 5. Chromatogram of callus extract *O. aristatus* purple varieties (Gamborg (B5) media). CP 1: purple variety callus acetone extract, CP 2: purple variety callus ethyl acetate extract

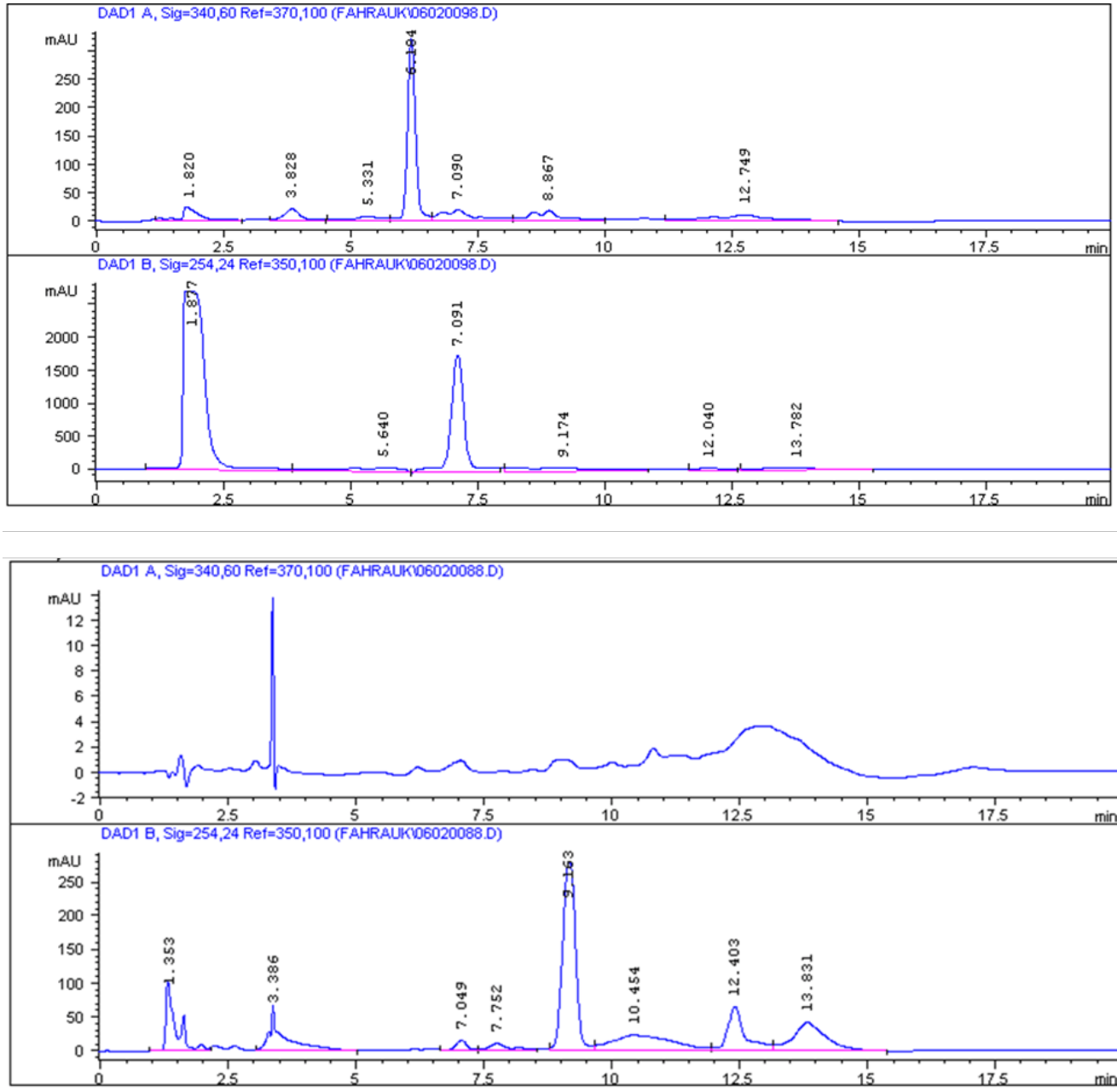


Figure 6. Chromatogram of callus extract *O. aristatus* white-purple varieties (Gamborg (B5) media). CWP 1: white-purple variety of callus acetone extract, CWP 2: White-purple variety of callus ethyl acetate extract

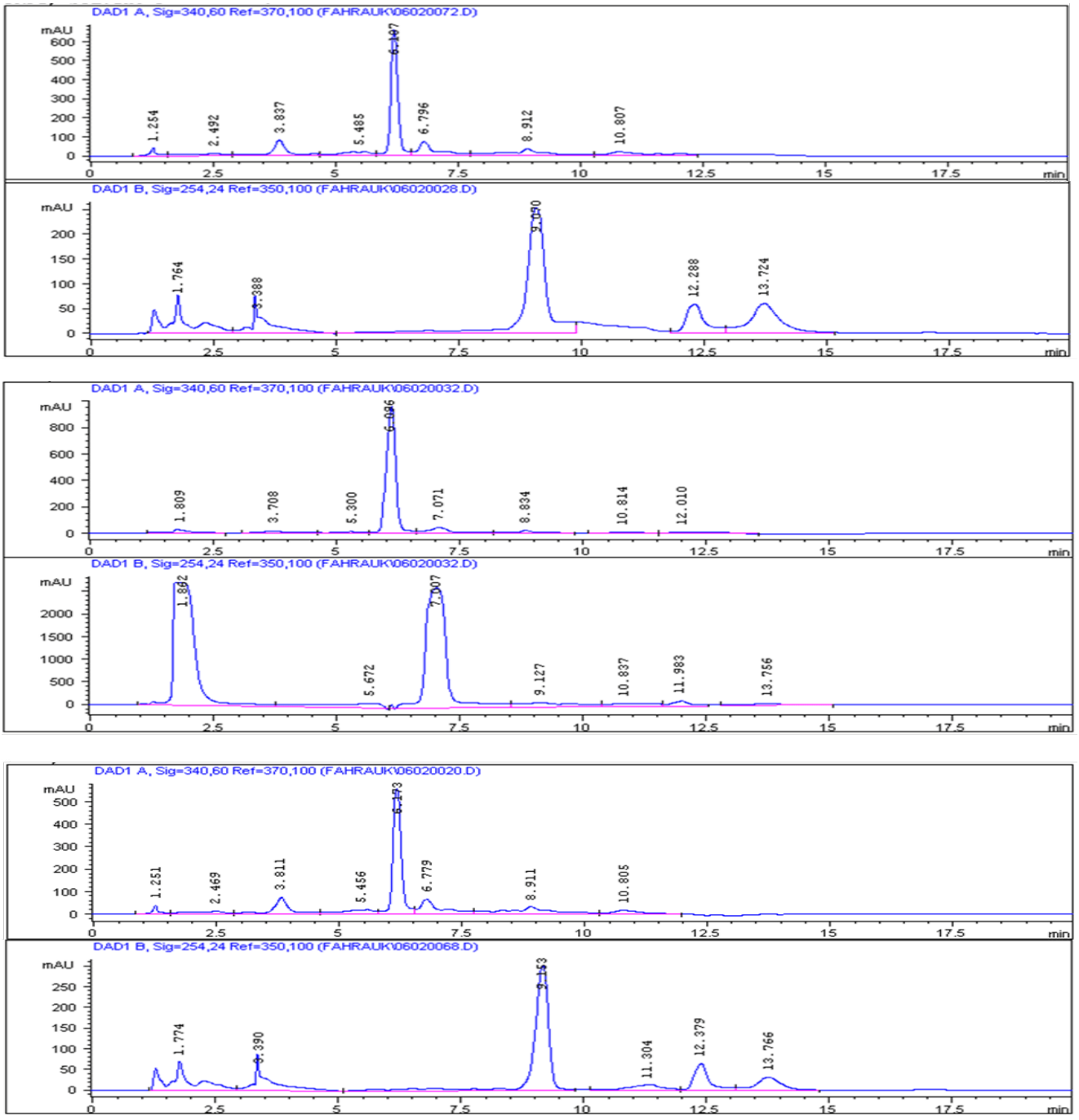


Figure 7. Chromatogram of callus extract *O. aristatus* purple variety CHU (N6) medium. CP 4: purple variety callus acetone extract, CP 5: purple variety of callus ethyl acetate extract, CP 6: purple variety callus ethanol extract (CP 6)

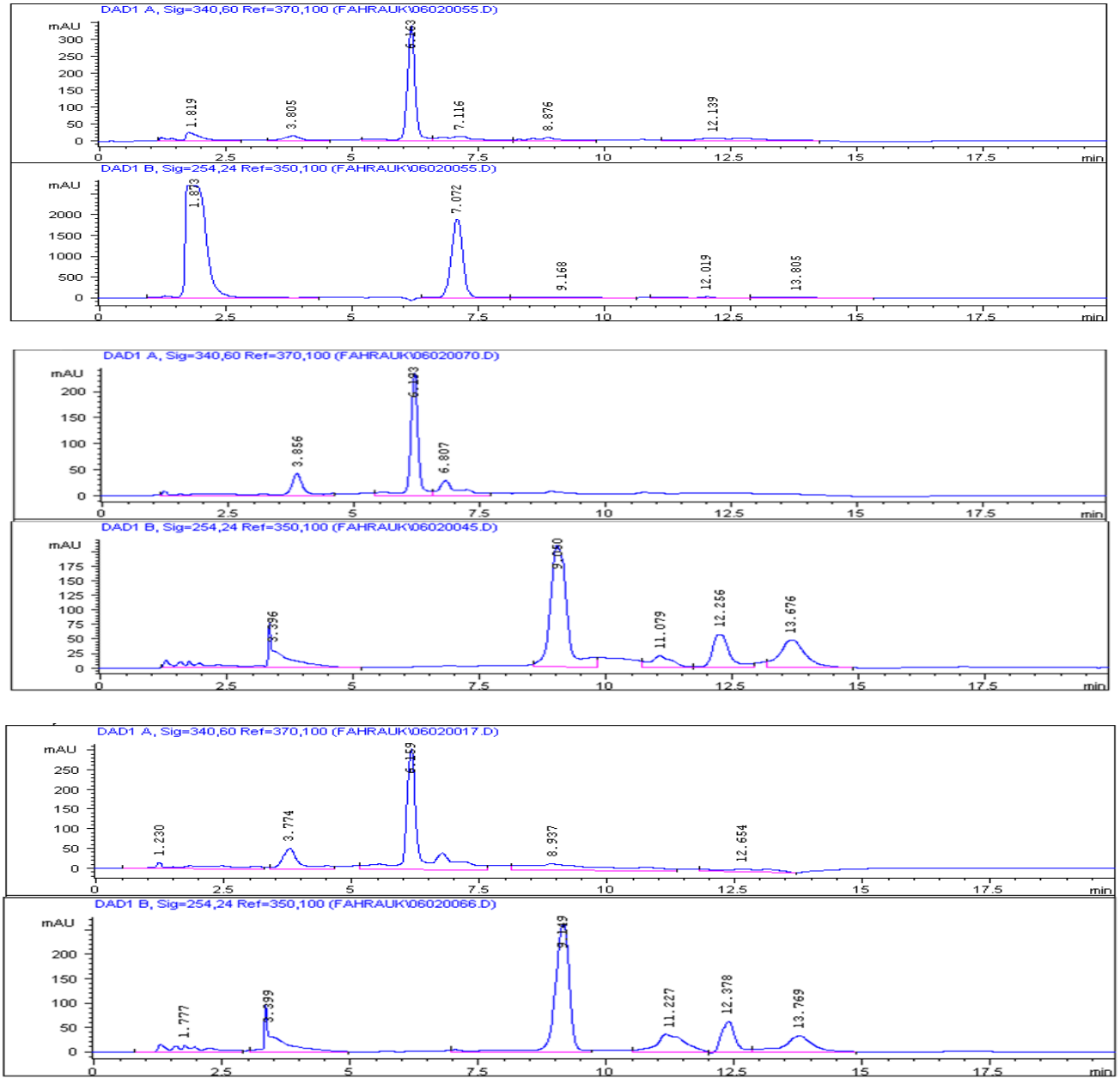


Figure 8. Chromatogram of callus extract *O. aristatus* white-purple varieties CHU (N6) medium. CWP 4: white-purple variety callus acetone extract, CWP 5: white-purple variety callus ethyl acetate extract, CWP 6: white-purple variety callus ethanol extract

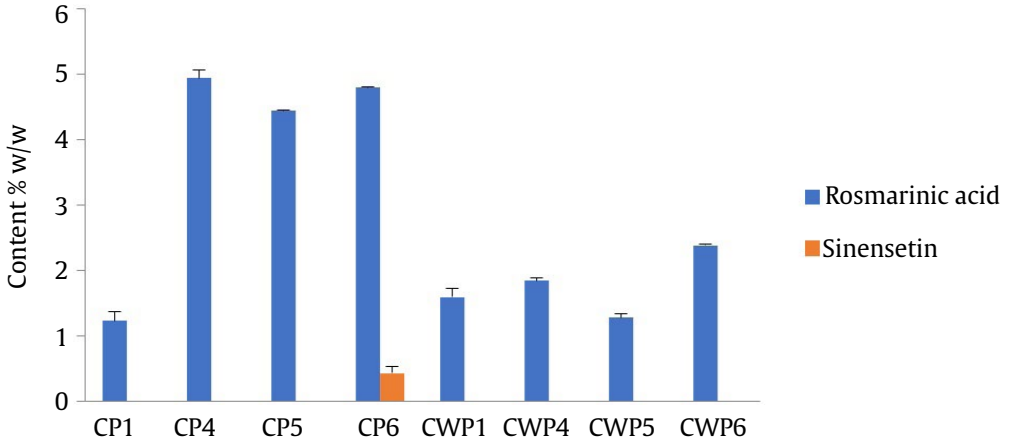


Figure 9. Comparison chart of the main secondary metabolite levels of callus extract of two varieties of *O. aristatus*. CP 1: purple variety callus acetone extract, CP 4: purple variety callus acetone extract, CP 5: purple variety of callus ethyl acetate extract, CP 6: purple variety callus ethanol extract (CP 6), CWP 1: white-purple variety of callus acetone extract, CWP 4: white-purple variety callus acetone extract, CWP 5: white-purple variety callus ethyl acetate extract, CWP 6: white-purple variety callus ethanol extract

Table 5. Rosmarinic acid levels from acetone extract of two varieties of *O. aristatus* in Gamborg (B5) and CHU (N6) medium

Sample	rosmarinic acid (% w/w) ± SD (n = 3)	Sinensetin (% w/w) ± SD (n = 3)
Media gamborg (B5)		
Purple variety callus acetone extract (CP 1)	1.23±0.14[a]	-
White-purple varieties of callus acetone extract (CWP 1)	1.59±0.14[a]	-
Media CHU (N6)		
Purple variety callus acetone extract (CP 4)	4.94±0.12[b]	-
Purple variety of callus ethyl acetate extract (CP 5)	4.44±0.01[b]	-
Purple variety callus ethanol extract (CP 6)	4.79±0.01[b]	0.43±0.10
White-purple variety callus acetone extract (CWP 4)	1.84±0.04[c]	-
White-purple variety callus ethyl acetate extract (CWP 5)	1.28±0.05[a]	-
White-purple variety callus ethanol extract (CWP 6)	2.38±0.02[d]	-

4. Discussion

The population of *O. aristatus* in Indonesia is mainly dominated by white varieties, while purple and white-purple varieties are decreasing in number (Batubara *et al.* 2020; Faramayuda *et al.* 2021c; Febjislami *et al.* 2019). Our study shows that the leaf shape of the purple variety in Indonesia is rhombic, in contrast to the report of Keng and Siong (2006) that mentioned the purple varieties in Malaysia has oval leaves with purple leaf venation. Additionally, the white varieties in Malaysia have rhombic leaves and light green leaf venation. This result is similar to Almatar *et al.* (2013), stating that the shape of the *O. aristatus* leaf was a rhombus. Another research report states that the leaf shape of *O. aristatus* was elliptic (Febjislami *et al.* 2019).

For the first time, this experiment successfully induced callus growth within three weeks by using B5 and N6. The concentration of plant growth regulators in this work is based on Faramayuda *et al.* (2020) study that reports 0.4 ppm 2,4-D is the optimal concentration for growing callus of two varieties *O. aristatus*. Several research reports on callus induction of *O. aristatus* plants explained that MS medium added with 2,4-D 1 mg/L could grow callus *O. aristatus* within six weeks (Bordbar *et al.* 2015; Wai-leng and Lai-keng 2004).

The media used in this study were CHU (N6) and Gamborg (B5), which differ in the nitrogen sources. The differences in NH_4+ and NO_3- concentration also affect many *in vitro* responses, including the development of somatic embryo (Haq and Yusuf 2004).

In addition, a qualitative analysis of the callus of two varieties of *O. aristatus* that grew on MS + 2,4-D 0.4 ppm media was previously reported by Faramayuda (2020), where the callus seems to produce rosmarinic acid and sinensetin compounds. Another qualitative and quantitative analysis of *O. aristatus* grew on MS media reported that the rosmarinic acid levels in purple callus varieties were 1.28% w/w and on white-purple varieties 2.22% w/w (Faramayuda *et al.* 2021d, 2021e).

Acetone extract of callus of purple variety produced rosmarinic acid 4.94% w/w, which was higher than the Guo *et al.* (2019) and Cai *et al.* (2018) reports. Rosmarinic acid levels in the CHU (N6) medium were still higher than Faramayuda *et al.* (2021e) reported. The sinensetin level from the ethanol extract of purple variety callus was still low, which could be due to the low levels of enzymes related to polymethoxy flavone biosynthesis in callus. The administration of abiotic elicitors, cinnamic acid, and caffeic acid precursors was predicted to increase sinensetin levels.

Growth regulators added to plant tissue culture media can modify biosynthetic pathways, enabling the detection of secondary metabolites callus (Efferth 2019; Sarfaraj *et al.* 2012). The growth regulator in this study was 2,4-D, an auxin group that affects an increase in gene expression related to the macronutrient transport process to produce secondary metabolites *in vitro* (Shilpashree and Vittal 2009). Auxins also regulate gene expression that can increase plant defense against environmental influences from pathogens (Naseem *et al.* 2015). Based on the above findings, our method in this research can be a new alternative for producing active compounds from cat's whiskers by *in vitro* culture.

In conclusion, the high levels of rosmarinic acid were found in the acetone and ethanol extract of callus in the purple varieties of *O. aristatus* that grow on CHU (N6) media. This research can be developed more by producing secondary metabolites with cell suspension culture, which a bioreactor can reproduce.

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