Advances in Biological Sciences Research, volume 15
Proceedings of the 10th International Seminar and 12th Congress of Indonesian Society for Microbiology (ISISM 2019)

Antibacterial Activity and Secondary Metabolite Content of Polyploid Mutant of Artemisia cina Berg ex Poljakov

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ABSTRACT

Artemisia cina is one of the members of Compositae family that have been identified to display antimicrobial activity. This study aims to examine the ability of wild-type *A. cina* (KJT genotype) and its polyploid mutant (J genotype) in inhibiting the growth of Gram-positive and Gram-negative bacteria as well as to determine the concentration of kaemferol, quercetin, and artemisinin as secondary metabolites. Antibacterial activity was tested using Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). The test of antibacterial activity was conducted using a disk diffusion method with 25, 50, 75 and 100% extract concentrations. Kaemferol, quercetin, and artemisinin contents were measured using High-Performance Liquid Chromatography. The extracts of both KJT and J genotypes indicated an inhibition on Gram-negative and Gram-positive bacteria growth. The antibacterial activity of both polyploid mutant (J) and wild type (KJT) genotypes was showed insignificantly different. The largest bacterial activity was observed in J genotype extract at 50% concentration against B. subtilis with a diameter of inhibition of 14.3 ± 0,60 mm. J genotype extract also showed a higher kaemferol, quercetin and artemisinin concentration compared to KJT genotype.

Keywords: *Antibacterial activity, metabolite, Artemisia cina Berg ex Poljakov.*

1. INTRODUCTION

Uncontrolled and excessive usages of antibiotics often cause increasing bacterial resistance and often associated with the adverse effects on human health [1]. Because of this, the antibiotic-resistant bacteria become the focus of society and pushed researchers to discover new antibacterial compounds. Plants as a source of medicine have been identified and used from ancient times. Medicinal plants are a significant group among all plants have been supporting human health through the biologically active pharmaceuticals which they contain [2]. Nowadays, traditional medicinal plants get a lot of public attention because of the various secondary metabolite compounds that can be used for the discovery of new drugs. The research on the search for new plant-derived antibacterial agents is very important today because of that increasing prevalence of bacterial resistance. Various extracts of traditional medicinal plants have been studied for their potential as sources of new antibacterial agents.

Secondary metabolites of different medicinal plants are being the subject for many investigation studies due to their biological activities, as antibacterial, antifungal, antitumor, and antiinflammatory [3]. Among all the secondary metabolites, the group of monoterpenes, flavonoids, and sesquiterpene lactones are reported to be responsible for antibacterial activity in many cases. Those compounds are believed to have the ability to kill bacteria because of their inhibitory and bactericidal properties [1].

Artemisia is one of the members of the Compositae family and has great potential for bioprospection. The members of the *Artemisia* genus are generally small herbs or shrubs and have been widely applied to treat various types of diseases in humans and plants, and also in the pharmaceutical industry. Many species of *Artemisia* have been used since ancient times as traditional medicine. Several species of *Artemisia* are some of the medicinal plants possess antibacterial activity and also having secondary metabolites [4]. The antibacterial activity of several *Artemisia* species

has been investigated and showed promising results. The terpene compounds as an essential oil from the several *Artemisia* species has been investigated antibacterial activity against several bacteria by using different methods such as agar dilution method, diffusion disk method, and reverse petri dish method depends on the nature of the bacterial species [5]. Essential oils of various *Artemisia* species that have been studied and reported to show antibacterial activity are from *A. herba-alba* [6,7], *A. absinthium* [8,9], *Artemisia* spp. [10], *A. annua* [1], *A. arborescens* [11], *A. nilagirica* [12], *A. vulgaris* [13], and *A. cina* [14]. In addition to essential oils, the secondary metabolites such as flavonoids (kaemferol and quercetin) and terpenoid (artemisinin) also exhibited highly potential biological activities that have been attributed to antibacterial activities [3].

A. cina is another species of a member of the *Artemisia* genus which also has potential as an antibacterial [14]. Constraints on the usage of secondary metabolites of *A. cina* as a source antibacterial agents are the low content of secondary metabolites produced by wild plants. *A. cina* plants that have been mutated into polyploid is reported to have a secondary metabolite content higher than its wild plants [15]. In the present study, the ability of wild-type *A. cina* (KJT genotype) and its polyploid mutant (J genotype) in inhibiting the growth of Gram-positive and Gram-negative bacteria as well as to determine the concentration of kaemferol, quercetin, and artemisinin as secondary metabolites were investigated.

2. MATERIALS AND METHODS

2.1. Material

The plants of *A. cina* were obtained from The Center for Research and Development of Medicinal Plants and Traditional Medicine, Central Java, Indonesia. KJT as wild type genotype was *A. cina* plants were propagated through shoots culture in Laboratory of Plant Tissue Culture, Agriculture and Bussines Faculty, Universitas Kristen Satya Wacana, Salatiga, Central Java. J genotype is a polyploid mutant obtained by inducing *A. cina* shoot culture with 100 mg/l colchicine for 48 hours. The whole aerial parts plant (stems and leaves) of *A. cina* KJT and J genotype were collected at the vegetative stage. The plant material was washed with water, dried at the room temperature, then dried in an oven at 40^{o}C. The identification of the plant species was confirmed by a botanist expert from Herbarium Bogoriense, Bogor. The samples were ground to powder using a mechanical grinder. The powdered samples of plants were stored in an airtight container and maintained at a low temperature until use.

The bacteria used as an indicator for the inhibition assays were obtained from the culture collection of the Divisi Food & Nutrition Culture Collection, Gadjah Mada University, Yogyakarta. Two species of Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) were used as test bacteria. All chemical reagents made by E-Merck, Jerman: toluene, ethyl acetate, ethanol, artemisinin, quercetin, kaempferol. Nutrient Agar (Oxoid), tetracycline (Oxoid) and paper disk (Whatman).

2.2. Method

2.2.1. Preparation of plant extracts

The powdered plant samples of each genotype were macerated with ethanol absolute pro analysis. Fifty grams of powder was extracted with 100 ml of ethanol absolute pro analysis at room temperature for 24h. The extract was filtrated and stored in the refrigerator until use.

2.2.2. Preparation of bacteria test

The test bacteria were subcultured in Nutrient Agar (NA) medium and incubated overnight at 37^{o}C. Each isolate of four bacteria test from the nutrient agar slope medium was taken with an inoculating loop and diluted with normal saline solution. Turbidity of the bacterial suspension absorbance was adjusted to the value of absorbance 0.08 – 0.1 with a spectrophotometer at 600 nm. The value of absorbance 0.08 – 0.1 equivalent to the density of 0.5 McFarland standards [16]. The bacterial population at 0.5 McFarland standards is approximately equal to 1.5×10^{8} CFU/ml (Yemata et al., 2019). The bacterial suspension that has been adjusted their turbidity was taken as much as 100 microliters and evenly inoculated on previously prepared NA plates.

2.2.3. Antibacterial activity test of plant crude extracts

The disk diffusion method was used to evaluate the antibacterial activity of the plant crude extracts [13]. The crude extracts of plant genotype were dissolved in ethanol as the solvent. Five extracts concentration was tested their antibacterial activity to four species bacteria (*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis,* and *Staphylococcus aureus*). Whatman's filter paper was used to prepare a disc with paper borer. Each paper disc was sterilized and infused with 20 µl of each tested crude extracts

concentrations. The disc papers soaked in 30 mg/ml of tetracycline and 20 µl of 100% ethanol used as positive and negative controls, respectively. All disc papers were dried before application. The dried disc papers were applied to the inoculated NA plates within 15 minutes of inoculation. During application, the disc papers were pressed downward on NA plates and kept in a normal position until the disc papers get wet. The NA plates were incubated at 37ºC for 24 h, then observed for the presence of inhibition of bacterial growth. The complete inhibition zones around each of the disc were measured in mm using a ruler at the widest diameter including the disc for each of the NA plates.

The relative percentage inhibition of the plant crude extract was determined with respect to the same concentration of the positive control (tetracycline). Relative percentage inhibition was calculated by using the formula described in [17] = *[(X-Y)/(Z-Y)] x 100*, where X: total area of inhibition of the test extract, Y: total area of inhibition of the solvent, and Z: total area of inhibition of the standard antibiotic.

2.2.4. Determination of secondary metabolite content

The secondary metabolite content as kaempferol, quercetin, and artemisinin was analyzed using High-Performance Liquid Chromatography (HPLC). Tokusoglu [18] method with modification was used to measured quercetin and kaempferol contents. In the sample of 2,5 g plant powder was added 25 ml methanol contained HCl 1% and sonicated using Sonicator Krisbow DSA50-GL2-2,5L for 30 minutes. The samples were filtered and added with solvents up to 25 ml and 5 ml 1.2 M HCl, then refluxed for 2 hours. The obtained extract was cooled to room temperature, then sonicated for 3 minutes and filtered using a 0.45 µm filter membrane. The filtrate as much as 20 µl was injected into HPLC. The HPLC conditions for separation of quercetin and kaempferol were using the Chromosorb Column RP C18 (150 x 5 mm id - Knauer), H_3PO_4 0.1%: acetonitrile (60:40) as the mobile phase, the flow rate 1 ml/min, the volume of injection 20 µl, ambient temperature, and using UV 370 nm detector. The quantitation of the amounts of the quercetin and kaempferol in *A. cina* samples extracts was determined using a calibration standard curve of quercetin and kaempferol pure compounds.

The artemisinin content was measured using HPLC according to [19] method with modification. The granular quartz as much as 100 mg and 2 ml toluene was added to 100 mg the powdered of plant, crushed using mortar, filtered and the filtrate was stored in a container. The filtrate as much as 500 µl was evaporated using a rotary evaporator and then dissolved again in 200 µl methanol and 800 µl NaOH 0.2 % (w/v). The sample solution was agitated using a vortex mixer (Scilogex Type MX-S), heated in a water bath (Memert) at 50ºC for 30 minutes, cooled, then homogenized with 200 µl methanol and 800 µl acetic acid and filtered using a 0.45 µm filter membrane. The filtrate was ready to be injected into HPLC. The condition of HPLC was modified in using the Chromosorb column RP C18 (150 x 5 mm id – Knauer), buffer Phosphate 0,01 M pH 7: methanol (55:45) as the mobile phase, the volume of injection as much as 20 µl, ambient temperature, and UV 260 nm as detector. The quantitation of artemisinin in *A. cina* samples extracts was determined using a calibration standard curve of pure artemisinin compounds.

2.2.5. Data analysis

All experiments were carried out in three replications. The data were expressed as mean ± standard deviation. The data were statistically analyzed using one-way analysis of variance (ANOVA) using SAS program (version 9.1.3). The one-way analysis of variance (ANOVA) followed by Duncan multiple range tests was employed to compare the mean difference between samples, values were considered significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

The ethanolic crude extract of polyploid mutant (J) and wild type (KJT) genotypes *A. cina* exhibited antibacterial activity against all Gram-negative (**Table 1**) and Gram-positive (**Table 2**) test bacterial. The antibacterial activity of plant extracts is determined based on the formation of bacterial growth inhibition zone diameter (a clear zone) using the disc diffusion method (**Figure 1**). The results showed that the inhibition zone diameter is not affected by the plant genotypes, but significantly affected by the test extract concentration. The highest antibacterial activity against Gram-negative bacteria was observed to *E. coli and P. aeruginosa* at 25% extract concentration in KJT genotypes with inhibition zone diameter of 10,0 ± 1,20 and 9,3 ± 0,60 mm, respectively. The evaluated crude extracts of the two *A. cina* genotypes against Gram-positive bacteria showed that the antibacterial activity of the highest was observed to polyploid mutant (J genotype). The highest was found at a 50% extract against *B. subtilis* with inhibition zone diameter of 14.3 ± 0,60 mm. The crude extract of two genotype *A. cina* showed an insignificant difference of inhibition zone diameter against *S. aureus*.

Table 1. The antibacterial activity of the *A. cina* genotype (KJT and J) extracts to Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*).

Ethanol extract concentration (%)	Zone of inhibition (diameter mm) of *A. cina* genotype to Gram-negative bacteria			
	Escherichia coli		*Pseudomonas aeruginosa*	
	Diploid (KJT)	Polyploid (J)	Diploid (KJT)	Polyploid (J)
0	0.0 ± 0.00**e**	0.0 ± 0.00**e**	0.0 ± 0.00**e**	0.0 ± 0.00**e**
25	10.0 ± 1.20**b**	7.7 ± 0.60**c**	9.3 ± 0.60**b**	7.0 ± 1.00**cd**
50	6.0 ± 0.00**d**	6.0 ± 0.00**d**	6.0 ± 0.00**d**	6.0 ± 0.00**d**
75	7.0 ± 1.00**cd**	7.3 ± 1.50**cd**	7.0 ± 0.00**cd**	7.0 ± 0.00**cd**
100	7.7 ± 0.60**c**	6.7 ± 0.60**cd**	7.3 ± 1.50**c**	7.0 ± 0.00**cd**
Tetracycline	32.0 ± 1.00**a**	32.0 ± 1.00**a**	23.7 ± 1.20**a**	23.7 ± 1.20**a**

Results are the mean ± standard deviation (SD) of three replications. Values with the same letter in a column means not significantly different ($p > 0.05$).

The results showed that both artemisia extracts (KJT and J genotypes) were more potential to inhibit the growth of Gram-positive bacteria compared to Gram-negative. The similarly results reported by researchers that essential oils from various species of artemisia have higher antimicrobial activity against gram-positive bacteria compared to gram-negative bacteria, such as *A. annua* [1], *A. arborescens* [11], *A. cina* [14], *A. absinthium* and *A. vulgaris* [20], *A. judaica* and *A. herba-alba* [21]. Al-Wahaibi [21] reported essential oils from two species of *A. judaica* and *A. herba-alba* to have high antimicrobial activity against all microorganisms test, except against Gram-negative bacteria (*P. aeruginosa*).

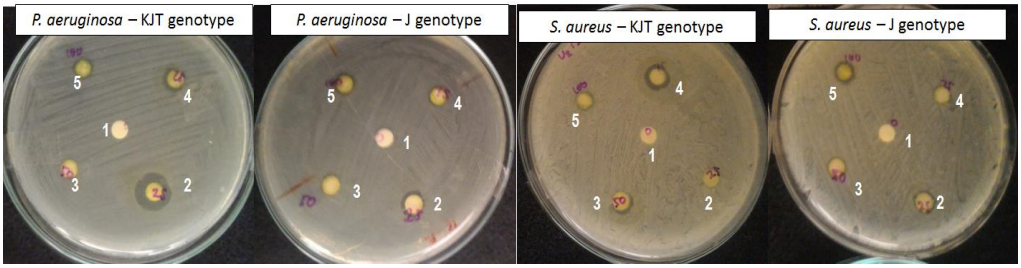

Figure 1. The inhibition zone diameter of the *A. cina* genotype (KJT and J) extracts against *P. aeruginosa* and *S. aureus*. 1. A negative control, a paper disc with solvent, 2. 25%, 3. 50%, 4. 75%, and 5. 100% of extract concentration.

The Gram-positive bacteria are highly sensitive against plant extract if compare to Gram-negative bacteria that was completely resistant. The sensitivity of Gram-positive bacteria to plants extract can be related to the cell membrane constituents that composed of an outer peptidoglycan layer as an ineffective permeability barrier. Meanwhile, Gram-negative bacteria have lipopolysaccharide in their outer membrane, that are more resistant and more effective as a strong impermeable barrier than peptidoglycan in the outer membrane of Gram-positive bacteria [21,22,23].

The antibacterial activity of plant ethanolic extract compared with standard antibiotic of tetracycline. The results of the antibacterial activity of the crude extracts of two genotypes *A.cina* (polyploid mutant and wild type) were compared with the tetracycline standard antibiotic to evaluate their relative percentage inhibition (**Figure 2**). At all concentration test, the relative percentage inhibition of the extract of two genotypes against *S. aureus* was higher than against other three bacteria tested. At 75% extract concentration test, the wild type (KJT genotype) of *A. cina* exhibited the highest relative percentage inhibition against *S. aureus* (70,7%), followed by mutant polyploid (J genotype) (65%).

Table 2. The antibacterial activity of the *A. cina* genotype (KJT and J) extracts to Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*).

Ethanol extract concentration (%)	Zone of inhibition (diameter mm) of *A. cina* genotype to Gram-positive bacteria			
	Bacillus subtilis		*Staphylococcus aureus*	
	Diploid (KJT)	Polyploid (J)	Diploid (KJT)	Polyploid (J)
0	0.0 ± 0.00**e**	0.0 ± 0.00**e**	0.0 ± 0.00**d**	0.0 ± 0.00**d**
25	9.3 ± 0.60**c**	9.7 ± 1.20**c**	7.0 ± 1.00**c**	7.7 ± 0.60**bc**
50	9.7 ± 1.50**c**	14.3 ± 0.60**b**	6.7 ± 0.60**c**	6.7 ± 0.60**c**
75	10.3 ± 1.20**c**	7.3 ± 0.60**d**	8.7 ± 1.50**b**	8.0 ± 0.00**bc**
100	7.0 ± 0.00**d**	7.0 ± 0.60**d**	7.7 ± 0.60**bc**	8.0 ± 1.00**bc**
Tetracycline	34.7 ± 1.00**a**	34.7 ± 1.00**a**	12.3 ± 1.50**a**	12.3 ± 1.50**a**

Results are the mean ± standard deviation (SD) of three replications. Values with the same letter in a column means were not significantly different ($p > 0.05$)

The result showed that crude extracts of polyploid mutant and wild type genotypes of *A. cina* had potency as compared to the tetracycline. The wide variety of secondary metabolites in the crude extract of the medicinal plant could be used as a potential for antimicrobial and resistance modifiers. The complex mixture of secondary metabolites in plant crude extract is attributed to the role as effective modulators of related cellular processes viz immune response, mitosis, apoptosis, signal transduction, and key events affect in the pathogenic process [24,25].

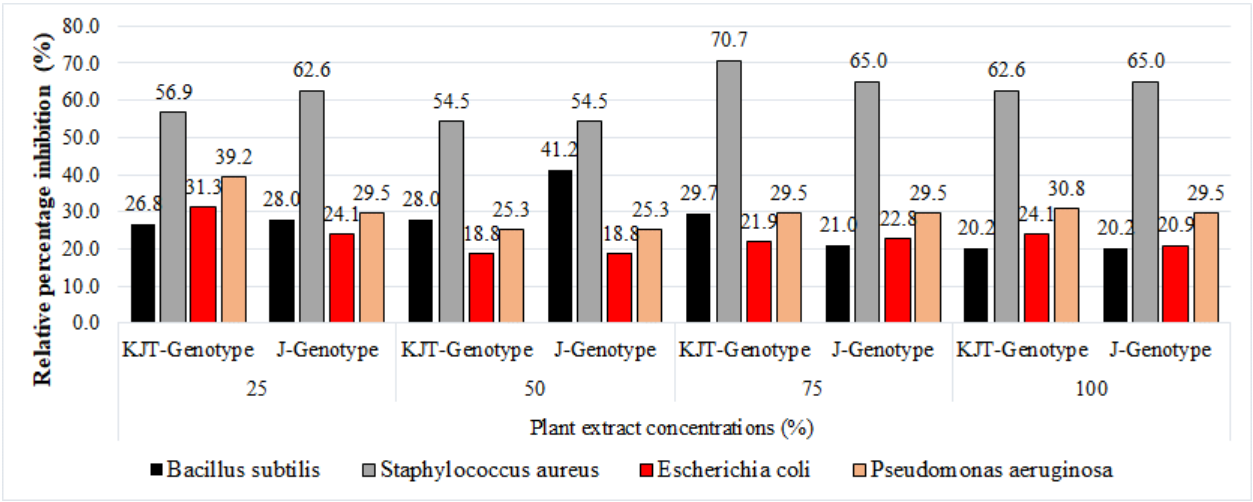

Figure 2. Relative percentage inhibition of *A. cina* genotype (KJT and J) extracts calculated against the standard antibiotic; tetracycline at 30 mg/ml concentration

Plants have a great ability to produce secondary metabolites such as phenolics, alkaloids, terpenoids, essential oils, lectins, and others [26]. In this study, the antibacterial activity of *A. cina* (KJT and J genotypes) extracts might be attributed to the contents of secondary metabolites as a flavonoid (kaempferol and quercetin) and terpene (artemisinin). The content of artemisinin, kaempferol, and quercetin of polyploid mutant (genotype J) showed higher values of 0.003%, 0.026%, and 0.104%, compared to wild type (KJT genotype) with values of 0.002%, 0.023%, and 0.037% respectively. The high content of secondary metabolites in *A. cina* polyploid mutants was thought to be closely correlated with the higher and more effective antibacterial activity of these extracts against test bacteria especially Gram-negative bacteria (*B. subtilis* and *S. aureus*).

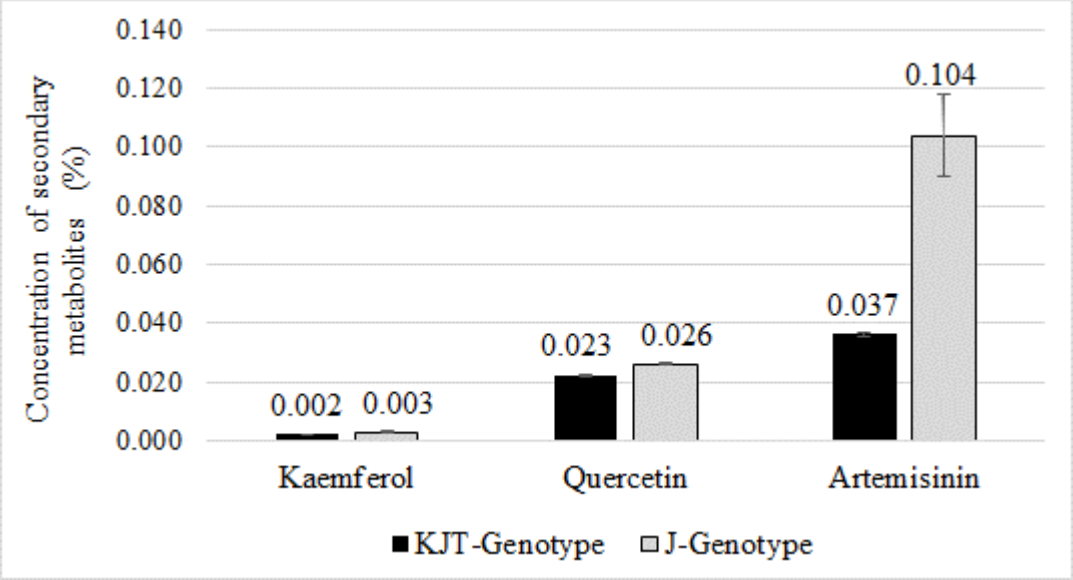

Figure 3. Artemisinin, quercetin and kaempferol content of *A. cina* genotype (KJT and J) extracts

Many plant extracts that have high concentration flavonoid and terpenes compounds reported exhibiting antibacterial activity, such as *Zapoteca portorincensis* [27], *Ocimum gratissimum* [28], *Morinda citrifolia* [29], *Phoenix dactylifera* [30]. Some of these secondary metabolites can act synergistically with classical antibiotics, other than act as antimicrobials [24]. Several flavonoid compounds exhibit antimicrobial actions such as quercetin, epigenin, myricetin, robinetin, luteolin. The flavonoid compounds as quercetin exhibit antibacterial action by blocking ATPase activity in bacteria by binding protein [26,31]. Terpene compounds like artemisinin are antibacterial in nature. The mode of antimicrobial action of terpenoids is ascribed to disruption of the membrane in bacteria [32].

4. CONCLUSION

The ethanolic extracts of both wild type and polyploid mutant of *A. cina* exhibited the antibacterial activity against Gram-negative and Gram-positive bacteria. The antibacterial activity of both polyploid mutant and wild type genotypes was showed insignificantly different. Both of *A. cina* genotypes were more potential to inhibit the growth of Gram-positive bacteria compared to Gram-negative. The largest bacterial activity was observed in polyploid mutant genotype extract at 50% concentration against *B. subtilis* with a diameter of inhibition of 14.3 ± 0,60 mm. The content of artemisinin, kaempferol, and quercetin of polyploid mutant genotype showed higher values of 0.003%, 0.026%, and 0.104%, compared to wild type genotype with values of 0.002%, 0.023%, and 0.037% respectively

ACKNOWLEDGMENTS

This research is funded by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia through the Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) Scheme Desentralisasi Program in 2019. The authors are greatly thankful for the funder.

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