

Antibacterial Activity and Flavonoids Content of *Artemisia cina* Berg. ex Poljakov Ethyl Acetate Extracts

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Abstract. The medicinal plant-derived bioactive compounds have a potential for many biological activities, including antimicrobial activity. *Artemisia cina* is a medicinal plant from the Compositae family with the potential of having antitumor, antifungal, and antibacterial activity. This study aimed to determine the antibacterial activity and the flavonoid content of *A. cina*'s ethyl acetate extract. Plants samples were extracted by ethyl acetate maceration method. Antibacterial activity was tested against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) by a disk diffusion method using 25, 50, and 100 mg/l extract concentrations. The flavonoid contents (quercetin and kaempferol) were measured using High-Performance Liquid Chromatography. The extracts of diploid and polyploid *A. cina* displayed some antibacterial activity, with the Gram-negative bacteria being more resistant than the Gram-positive counterpart. However, no significant difference was observed between the diploid and polyploid extracts. As for the flavonoid content, the highest quercetin content (0.5501 mg/ml) was found in the polyploid *A. cina* (J), while the highest kaempferol content (0.5818 mg/ml) was observed in the diploid *A. cina* (KJT). Although *A. cina* is widely grown in Indonesia, compared to other *Artemisia* species, *A. cina* has not been widely studied, especially its antibacterial potential and in related to its flavonoid content and the use of ethyl acetate as the extraction solvent. This study reveals the potential of *A. cina* as a natural antibacterial agent.

Key words: antibacterial; *Artemisia cina*; ethyl acetate; kaempferol; quercetin

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INTRODUCTION

The extensive antibiotics use leads to an increased bacterial resistance. The uncontrolled use of antibiotics is tightly associated with adverse effects in humans (Massiha et al., 2013). The increasing incidence of bacterial resistance to certain antibiotics has caused problems in many infectious disease treatments. Therefore, it is crucial to explore new potential antibiotics. One of the promising sources of the new antibiotics is from medicinal plants. Some bioactive compounds isolated from medicinal plants have been used as antibiotic alternatives due to their antimicrobial activities. Thus, many medicinal plants received significant attention for the new antibiotic developments (Compean & Ynalvez, 2014).

Many traditional medicinal plants are known to produce bioactive compounds such as phenolics, alkaloids, flavonoids, and terpenoids (Othman et al., 2019). Flavonoid compounds have been reported to contribute to antimicrobial activity. With the presence of other bioactive compounds, flavonoids showed different antibacterial activities (Mariita et al., 2011). Flavonoids inhibit bacteria by directly killing the bacteria, synergistically activating antibiotics, and

attenuating bacterial pathogenicity. Thus, reducing the harm to the host organism (Xie et al., 2015). A large concentration of flavonoids can be found in the stem, leaves, flowers, and fruit of many species from Compositae family (Panche et al., 2016; Taleghani et al., 2020). *Artemisia* is one of the genera in the Compositae family which has the potential to be studied for its flavonoid content and antibacterial activity. *Artemisia* contains bioactive compounds such as essential oils, monoterpenes, flavonoids, sesquiterpenes lactone, α -xanthine, and artemisinin acid, which contribute to its medicinal properties (Ahameethunisa & Hopper, 2010). Those compounds were known to act as antibacterial agents (Tan et al. 1998). Several studies of antibacterial activity from various extracts of *Artemisia* species have been reported. Ahameethunisa and Hopper (2010) reported that the hexane extract of *A. nilagirica* leaves displayed an inhibition against various groups of bacteria.

The water fraction of *A. indica* showed maximum activity against *Bacillus subtilis* (Bibi et al., 2011). The methanol and ethanol extracts of *A. annua* leaves demonstrated an inhibition against *Klebsiella*

pneumonia PTCC 1053, *Shigella dysenteriae* PTCC 1188, *Salmonella enterica* PTCC 1231, *Escherichia coli* PTCC 1399, and *Staphylococcus aureus* PTCC 1431 (Tajehmiri et al., 2014). The reported studies on antibacterial activity of *Artemisia* usually related to its essential oils. The essential oils content of various *Artemisia* species and their antibacterial activity that have been studied are *A. herba-alba* (Mohamed et al., 2010; Younsi et al., 2016), *A. absinthium* (Joshi, 2013; Msaada, et al., 2015), *Artemisia* spp. (Dadasoglu et al., 2015), *A. annua* (Massiha et al., 2013), *A. arborescens* (Militello, et al., 2011), *A. nilagirica* (Ahameethunisa & Hopper, 2010), *A. vulgaris* (Addo-Mensah et al., 2015), and *A. cina* (Kristiani et al., 2015).

The other antibacterial potential of *A. cina* which is widely grown in Indonesia has not been studied, especially related to its flavonoid content and the use of ethyl acetate as the extraction solvent. The study of the potential antimicrobial activity of ethyl acetate extract of *A. cina* can provide meaningful information as an alternative for an antibacterial agent. Therefore, this study was conducted to determine the antibacterial activity and flavonoid content of *A. cina* ethyl acetate extract.

METHODS

Plant material

A. cina samples were obtained from B2P2TOOT (The Center for Research and Development of Medicinal Plants and Traditional Medicine), Tawangmangu, Central Java, Indonesia. The *A. cina* from B2P2TO2T which were propagated directly in the greenhouse and by tissue culture were diploid plant samples and were marked as TWN and KJT, respectively. Meanwhile, two samples of polyploid *A. cina* were created by polyploidization through the shoot culture technique. Genotype labeled J was created by 3 mg/l 2,4-D and 2 mg/l IBA induction for 21 days. The M genotype was formed by 2 mg/l 2,4-D and 1.5 mg/l IBA induction for 21 days.

Plant extracts preparation

All *A. cina* leaf samples were washed thoroughly using distilled water and were then air-dried without direct sun exposure. The dried leaves samples were finely ground using a blender (Philip HR1538). The powdered plant samples of as much as 50 g were extracted using the maceration method with ethyl acetate as a solvent and stirred using the magnetic stirrer for 3 hours. The maceration process was repeated five times for each sample. The extracts were then filtered (Whatman's filter paper), and the ethyl acetate filtrates were then concentrated using a rotary evaporator (Rotavapor R114 Buchi) under

vacuum (Eyela A-1000S) at 40°C. The extracts were dried at room temperature and were stored in the refrigerator until further analysis. The dried and powdered leaves (shoots) of *A. cina* were extracted by the maceration method using ethyl acetate as shown in Figure 1.



Figure 1. (a) *A. cina* plants, (b) dry powdered samples, (c) maceration process, (d) filtrate, (e) filtrate condensation using a rotary evaporator, (f) dried extract, and (g) collected extract for antibacterial analysis.

Preparation of bacterial culture

The bacteria used in this study were two species of gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). The bacteria were obtained from the culture collection InaCC, LIPI. The test bacteria were subcultured in Nutrient Agar (NA) medium and incubated overnight at 37°C. Each isolate in the NA slope medium was taken with an inoculating loop and diluted with physiological saline solution. The turbidity of the bacterial suspension was adjusted to the density of 0.5 McFarland standards, which equals to 1.5×10^8 CFU/ml (Sutton, 2011; Yemata et al., 2019). The bacterial suspension was then taken as much as 100 μ l and evenly spread on previously prepared NA plates.

Antibacterial activity test

The antibacterial activity of the extracts was determined by the disk diffusion method (Addo-Mensah et al., 2015). The extracts of four genotypes of *A. cina* were dissolved in ethyl acetate as the solvent. Three extracts concentrations (25, 50, 100 mg/l) were tested against the four bacterial species. Each sterile paper disk (Oxoid) was placed on the previously seeded NA plates. During the application, the paper disks were pressed downward on NA plates. They were then infused with 20 μ l of each tested extract and 100 μ g/ml of Streptomycin as well as 100% ethyl acetate used as positive and negative controls, respectively. The seeded NA plates were

incubated at 37°C for 24 h, then the clear zone that was observed around the paper disk indicating the inhibition of bacterial growth. The complete inhibition zones around each of the disks were measured in mm using a ruler at the widest diameter including the disk for each of the NA plates.

Flavonoid content determination

The flavonoid content as kaempferol and quercetin was determined using High-Performance Liquid Chromatography (HPLC). The modification of Tokusoglu method (2003) was used to measure quercetin and kaempferol contents. The HPLC conditions for the separation of quercetin and kaempferol were using the Chromosorb Column RP C18 (150 × 5 mm id - Knauer), H₃PO₄ 0.1%: acetonitrile (60:40) as the mobile phase, the flow rate 1 ml/min, the volume of injection of 20 µl, ambient temperature, and using UV 370 nm detector. The quantitation of the amounts of the quercetin and kaempferol in *A. cina* sample extracts was determined using a calibration standard curve of quercetin and kaempferol pure compounds.

Data analysis

The data were analyzed using one-way analysis of variance (ANOVA) using the SAS ver. 9.1.3, followed by Tukey test to compare the mean difference between samples ($P > 0.05$). All experiments were conducted in triplicate.

RESULTS AND DISCUSSION

Antibacterial activity

A. cina is one of the members of the genus *Artemisia* which is often used for medicinal purposes. In this study, the antibacterial activity potential of *A. cina* was determined by observing the growth inhibition zone on four bacteria species. Those bacteria including both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*). The clear zones formation in the disk diffusion method indicated an inhibition on bacterial growth due to the antibacterial activity (Figure 2). The antibacterial activity of *A. cina* ethyl acetate extract was compared to the standard antibiotic (streptomycin). The tested bacteria are widely used in

antibacterial tests because these species are known to infect humans (Ahameethunisa & Hopper, 2010).

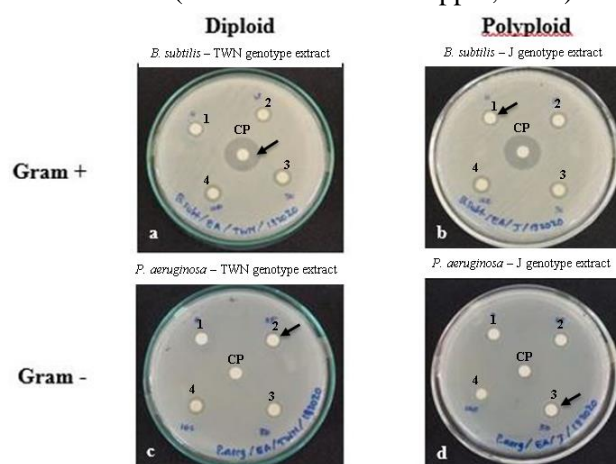


Figure 2. The antibacterial activity of *A. cina* ethyl acetate extracts tested using disk diffusion assay. The inhibition zone diameter (**arrow**) of the *A. cina* genotype represented here; (a) diploid TWN extract against Gram-positive *B. subtilis*, (b) polyploid J extract against Gram-positive *B. subtilis*, (c) diploid TWN extract against Gram-negative *P. aeruginosa*, and (d) polyploid J extract against Gram-negative bacteria *P. aeruginosa*. The four different extract concentrations used were 1. 0 µg/ml (negative control, a paper disk with solvent), 2. 25 µg/ml, 3. 50 µg/ml, and 4. 100 µg/ml. The center paper disks (CP) with no label contain positive control streptomycin at 100 µg/ml.

The result of the antibacterial activity of diploid and polyploid extracts against Gram-positive bacteria are shown in Table 1 and Table 2. It can be observed that ethyl acetate extract of *A. cina* exhibited a slight inhibition on all tested bacteria (Figure 2, Table 1 and 2). The results showed that the extracts successfully inhibited the growth of *B. subtilis* and *S. aureus*. Also, the inhibitions were still lower compared to streptomycin. However, any significant inhibition was not observed among all tested extracts on a particular bacterium.

The polyploid mutants' (J and M) activity was not significantly different from the diploid genotype (KJT and TWN). The highest activity of *A. cina* extracts against *B. subtilis* was found at 50 µg/ml for all tested genotypes. Overall, TWN and M genotypes showed a greater inhibition than KJT and J against the two Gram-positive bacteria, especially *B. subtilis*.

Table 3 and Table 4 displayed the inhibition activity of the extracts against Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Similarly, as previously demonstrated on Gram-positive, all four extracts' antibacterial activity against Gram-negative bacteria did not show significant differences between the genotypes. From the relatively small inhibition zone being displayed, it indicated that both *E. coli* and *P. aeruginosa* were more resistant against the extracts. The inhibition zones observed on the Gram-negative test plates were generally smaller compared to that of the Gram-positive. The susceptibility of bacteria to antibiotics is influenced by many factors, including phenotype and genotype variations in exposed bacterial populations. Bacterial resistance can develop either through the unexpressed expression of the previous resistant phenotype or as the result of an adaptation (Othman et al., 2019).

Previous studies also showed a similar pattern, the Gram-positive were more sensitive against the ethanol extract of *A. cina* than the Gram-negative bacteria. The essential oils from various *Artemisia* species exhibited a higher antibacterial activity on Gram-positive than on Gram-negative bacteria, such

as *A. annua* (Massiha et al, 2013), *A. arborescens* (Militello et al, 2011), *A. cina* (Kristiani et al., 2015), *A. absinthium*, and *A. vulgaris* (Poiata et al., 2009). Al-Wahaibi et al. (2020) reported that essential oils from two species of *A. judaica* and *A. herba-alba* had high antimicrobial activity against all test microorganisms, except for Gram-negative bacteria *P. aeruginosa*. The results of research conducted by Prastiyanto et al. (2020) report that extract of bilimbi (*Averrhoa bilimbi*) fruit with various solvents (methanol, ethanol, chloroform, n-hexane, and water) showed inhibition of growth to multi-drug resistance (MDR) bacteria. The extracts from the various solvents showed highly varying MBC (Minimum bactericidal concentration) values against MDR bacteria, but the lowest MBC value was found to water extract (12.5 mg/mL) against ES β L + CR *P. aeruginosa*. Wahyuni et al. (2018) reported that the ethyl acetate extraction yield of *Daemonorops draco* was higher than methanol and n-hexane, and the ethyl acetate and methanol extracts showed stronger antimicrobial activity against *S. aureus* compared to n-hexane extract.

Table 1. The antibacterial activity of the *A. cina* diploid (KJT and TWN) and polyploid (M and J) genotype extracts against gram-positive bacteria *Bacillus subtilis*

Ethyl acetate extract concentration (mg/l)	Diameter zone of inhibition (mm)			
	Diploid (KJT)	Diploid (TWN)	Polyploid (M)	Polyploid (J)
0	8.17 \pm 1.04 cde	7.33 \pm 0.58 e	7.83 \pm 0.29 de	7.83 \pm 0.58 de
25	9.33 \pm 0.58 cd	8.67 \pm 0.58 cde	8.83 \pm 0.76 cde	8.67 \pm 1.15 cde
50	9.50 \pm 0.50 cd	9.67 \pm 1.15 c	9.67 \pm 0.58 c	9.33 \pm 1.15 cd
100	8.83 \pm 1.04 cde	9.33 \pm 1.15 cd	8.50 \pm 0.50 cde	8.83 \pm 1.04 cde
Streptomycin	19.67 \pm 0.58 a	16.67 \pm 0.58 b	18.00 \pm 2.00 b	18.00 \pm 0.50 b

Results are the mean \pm standard deviation (SD) of three replications. Mean values in the same column and row with different letters are significantly different ($p > 0.05$).

Table 2. The antibacterial activity of the *A. cina* diploid (KJT and TWN) and polyploid (M and J) genotype extracts against Gram-positive bacteria *Staphylococcus aureus*

Ethyl acetate extract concentration (mg/l)	Diameter zone of inhibition (mm)			
	Diploid (KJT)	Diploid (TWN)	Polyploid (M)	Polyploid (J)
0	7.83 \pm 0.29 b	7.00 \pm 1.00 bc	7.33 \pm 0.58 bc	5.00 \pm 0.29 c
25	8.67 \pm 1.15 b	7.67 \pm 0.58 b	8.50 \pm 0.87 b	7.67 \pm 0.58 b
50	7.83 \pm 0.29 b	7.67 \pm 2.08 b	8.83 \pm 0.76 b	8.00 \pm 1.00 b
100	8.50 \pm 0.50 b	7.67 \pm 0.58 b	8.50 \pm 0.50 b	8.00 \pm 1.73 b
Streptomycin	16.67 \pm 0.58 a	15.00 \pm 0.00 a	16.67 \pm 1.15 a	14.67 \pm 1.53 a

Results are the mean \pm standard deviation (SD) of three replications. Mean values in the same column and row with different letters are significantly different ($p > 0.05$).

Table 3. The antibacterial activity of the *A. cina* diploid (KJT and TWN) and polyploid (M and J) genotype extracts against Gram-negative bacteria *Escherichia coli*

Ethyl acetate extract concentration (mg/l)	Diameter zone of inhibition (mm)			
	Diploid (KJT)	Diploid (TWN)	Polyploid (M)	Polyploid (J)
0	8.00 ± 0.00 c	7.17 ± 0.29 c	7.17 ± 0.29 c	8.00 ± 0.00 c
25	8.17 ± 0.29 c	7.33 ± 0.76 c	8.17 ± 0.29 c	8.00 ± 0.00 c
50	8.17 ± 1.04 c	7.00 ± 0.87 c	8.33 ± 0.58 c	8.00 ± 0.00 c
100	7.67 ± 0.76 c	8.17 ± 0.76 c	7.67 ± 1.15 c	8.00 ± 0.00 c
Streptomycin	14.00 ± 1.00 ab	13.33 ± 0.58 b	15.00 ± 2.65 a	14.67 ± 0.58 ab

Results are the mean ± standard deviation (SD) of three replications. Mean values in the same column and row with different letters are significantly different ($p > 0.05$).

Table 4. The antibacterial activity of the *A. cina* diploid (KJT and TWN) and polyploid (M and J) genotype extracts against gram-negative bacteria *Pseudomonas aeruginosa*

Ethyl acetate extract concentration (mg/l)	Diameter zone of inhibition (mm)			
	Diploid (KJT)	Diploid (TWN)	Polyploid (M)	Polyploid (J)
0	6.67 ± 0.29 abc	8.00 ± 0.00 abc	7.83 ± 1.26 abc	7.00 ± 0.00 abc
25	7.00 ± 0.00 abc	8.00 ± 1.00 abc	8.67 ± 0.58 ab	8.00 ± 0.00 abc
50	5.33 ± 0.29 c	8.33 ± 0.58 abc	5.67 ± 0.50 bc	8.33 ± 0.58 abc
100	8.33 ± 0.58 abc	9.00 ± 0.00 a	8.17 ± 1.44 abc	7.00 ± 1.00 abc
Streptomycin	6.50 ± 0.00 abc	8.33 ± 0.58 abc	6.50 ± 0.00 abc	7.67 ± 0.58 abc

Results are the mean ± standard deviation (SD) of three replications. Mean values in the same column and row with different letters are significantly different ($p > 0.05$).

The Gram-positive bacteria are more sensitive against many plant extracts compared to the Gram-negative bacteria. Gram-positive bacteria's sensitivity can be related to the cell envelope constituents. It comprises an outer peptidoglycan layer that is relatively ineffective as a permeability barrier. Meanwhile, Gram-negative bacteria have lipopolysaccharide as their outer membrane envelope. This structure is a more resistant and more effective impermeable barrier (Al-Wahaibi et al., 2020; Baba & Malik, 2014).

Flavonoid content

The development of natural antibiotics generally comes from medicinal plants. The medicinal plants usually produce important secondary metabolites such as alkaloids, terpenoids, essential oils, and phenolics (Othman et al., 2019). The antibacterial properties of *A. cina* might be attributed to the flavonoid contents such as kaempferol and quercetin. Therefore, their concentrations in the ethyl acetate extracts were analyzed by the HPLC method. The measurement results of flavonoid contents are displayed in Figure 3A (quercetin content) and 3B (kaempferol content).

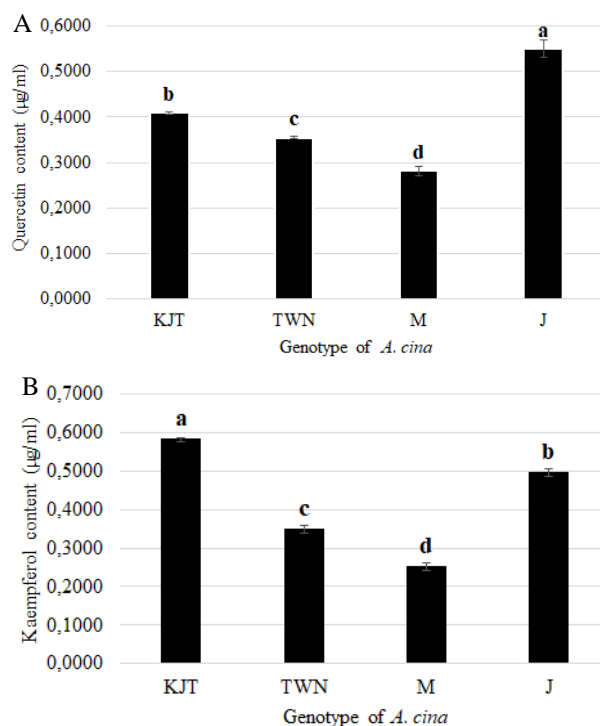


Figure 3. Quercetin (A) and kaempferol (B) contents of ethyl acetate extracts of diploid and polyploid *A. cina* plant. KJT and TWN genotype are the *A. cina* plant from B2P2TO2T which were propagated directly in the greenhouse and by tissue culture. M and J genotypes are *A. cina* were created by polyploidization through shoot culture technique.

The flavonoid content of both quercetin and kaempferol showed a significant difference between the ethyl acetate extract of the genotype *A. cina* polyploid and diploid. The highest quercetin content at 0.5501 mg/ml was found in the genotype *A. cina* polyploid (J), while the highest kaempferol content was in the diploid genotype (KJT) at 0.5818 mg ml. The lowest quercetin and kaempferol content were found in the polyploid (M) genotype. The high content of flavonoids in *A. cina* extracts was thought to be closely correlated with the higher and more effective antibacterial activity (Xie et al., 2015). Several flavonoid compounds exhibit antimicrobial actions such as quercetin, apigenin, myricetin, robinetin, luteolin. Quercetin exhibits antibacterial action by blocking ATPase activity in bacteria through protein binding (Othman et al., 2019; Shin et al., 2018). Many plant extracts with high flavonoid and terpenes content were reported to exhibit antibacterial activity, such as *Zapoteca portoricensis* (Agbafor et al., 2011), *Ocimum gratissimum* (Munyendo et al., 2011) *Morinda citrifolia* (Sibi et al., 2012), *Phoenix dactylifera* (Selim et al., 2012).

Studies on the use of *Artemisia* as a source of natural ingredients for the treatment of various diseases have been reported, including as an antimicrobial. The various species of *Artemisia* have been investigated for their antimicrobial activity. However, there is not much information about the biological activity and secondary metabolite content of polyploid *Artemisia*, particularly *A. cina*. The results of this study are expected to provide new information, especially regarding the antibacterial activity and flavonoid content of *A. cina* polyploid, as well as adding information about *Artemisia*. Apart from that, it can also support the utilization of *A. cina* polyploid as a natural antibacterial source.

CONCLUSION

The ethyl acetate extract of *A. cina* from both diploid and polyploid genotype showed inhibition against the tested Gram-positive bacteria. *A. cina* extracts from all tested genotypes inhibit *B. subtilis* the most at a concentration of 50 mg/l. A greater antibacterial activity was demonstrated by the diploid TWN and polyploid M genotypes against the Gram-positive *B. subtilis*. The highest quercetin content (0.5501 mg/ml) was found in the polyploid J genotype, while the highest kaempferol content (0.5818 mg/ml) was in the diploid KJT genotype. The lowest quercetin and kaempferol content were found in the polyploid (M) genotype.

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