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Antibacterial and Antibiofilm Activity of *Daemonorops draco* Resin

Wulan Tri Wahyuni[1,2], Sri Purwanti[1], ✉Irmanida Batubara[1,2]

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[1]Department of Chemistry, Institut Pertanian Bogor, Indonesia
[2]Tropical Biopharmaca Research Center, Institut Pertanian Bogor, Indonesia

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Abstract

Daemonorops draco has been reported for its antibacterial activity and empirically used for wound healing by Anak Dalam ethnic at Jambi Province, Sumatera-Indonesia. This study was performed to evaluate antibacterial and antibiofilm activity of *D. draco* resin collected from Jambi. *D. draco* resin was extracted using *n*-hexane, ethyl acetate and methanol, respectively. Antibacterial activity of the extracts was evaluated using agar diffusion method against *Staphylococcus aureus* and *Eschericia coli,* whereas the minimum inhibitory concentration (MIC) and minimum bacteriacidal concentration (MBC) was determined by microdilution method. In addition, antibiofilm activity was evaluated by violet crystal method. The result showed that extraction yield of ethyl acetate was higher than methanol and *n*-hexane. Ethyl acetate and methanol extracts of *D. draco* exhibited stronger antimicrobial activity against *S. aureus* compare to *n*-hexane extract. MIC and MBC of methanol extract and chromatographic fraction (F5.1) of ethyl acetate extract were 0.5 and 1.0 mg/mL, respectively. In addition, antibiofilm assay revealed that all extracts were inhibit initial attachment of bacteria cell in biofilm formation. This result revealed a novel information that *D. draco* extracts was potential as inhibitor of biofilm formation. TLC bioautography of *D. draco* extracts indicated that constituent with Rf of 0.71 performed antimicrobial activity against *S. aureus*. This finding expected to strengthen the scientific backup for utilization of *D. draco* by society.

How to Cite

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✉ Correspondence Author:
Dramaga Campus, Bogor, Indonesia 16680
E-mail: ime@apps.ipb.ac.id

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INTRODUCTION

Antibiotic overuse lead to the emergence of resistance microbes which is considered as the major health problem in the world (Millar *et al.*, 2008). A new antibacterial compound was required to treat the resistance microbes. On the other hand, many health problems also happened due to the formation of biofilms. Some bacterias formed a complex matrix of microorganisms (biofilm) which is bind and attached to either biotic or abiotic surface (O'Toole *et al.*, 2000). Bacteria in biofilms are more resistance to antibiotics and other chemical agents than the bacteria in suspension (Stewart, 2002). Consequently, it is also considerably necessary to find a new antibiofilm compounds.

Exploration of antimicrobe and antibiofilm from natural resources such as medicinal plant has already reported. Sanches *et al.* reported antibacterial and antibiofilm of *Prosopis laevigata*, *Opuntia ficus-indica*, and *Gutierrezia microcephala* (Sanchez *et al.*, 2016). Teanpaisan *et al*. explored potency of Thai medicinal plant extract as antibacterial and antibiofilm against oral microorganism (Teanpaisan *et al.*, 2017). While Wahyuni *et al*. reported the activity of *Curcuma aeruginosa* essential oil as antibacterial and antibiofilm against *Streptococcus mutans* (Wahyuni *et al.*, 2017). Natural resin also reported as potential antimicrobial agent (Termentzi *et al.*, 2011).

Daemonorops draco is a plant in family Arecaceae that widely spread in tropic and subtropics area of southeast Asia. The fruit of *D. draco* produce a bright red natural resin known as "dragon's blood" resin. This resin has been considered to use in wound, headache, and fever healing, by local ethnic in Jambi Province, Indonesia (Andhika *et al.*, 2015). *D. draco,* which commercially produced by Meer corporation has been reported for its antibacterial activity (Rao *et al.*, 1982). *D. draco* resin also reported for its antiviral (Gupta & Gupta, 2011), anticancer (Yu *et al.*, 2013), and anti-inflammatory (Kuo *et al.*, 2017) activity. To the best of my knowledge, antibiofilm activity of *Daemonorops draco* resin extracts has not reported yet. This study was carried out to evaluate the antibacterial and antibiofilm activities of *D. draco* resin extracts. The findings of this work expected to strengthen the scientific backup for utilization of *D. draco* by local ethic in Jambi Province and other area in Indonesia.

METHODS

D. draco resin was collected from Sarolangun Jambi, Sumatera, Indonesia. Approximately 150 g dried resin was extracted consecutively by 300 mL *n*-hexane, ethyl acetate, and methanol. Ethyl acetate extract was then fractionated by solvent-solvent extraction using methanol and *n*-hexane 1:1 by volume. *n*-hexane fraction was collected and separated by column chromatography with silica gel $G_{60}F_{254}$ as the stationary phase and chloroform:*n*-hexane (9:1) as the mobile phase. From this separation step, 9 fraction was collected (1-9). Fraction number 5 was further separated by preparative thin layer chromatography using $G_{60}F_{254}$ silica gel plate as stationary phase and dichloromethane:n-hexane:methanol (9:1:0.1) as mobile phase generated fraction 5.1 and 5.2.

Determination of antibacterial activity

Antimicrobial activities of the resins against *S. aureus* (ATCC 12600) and *E. coli* were determined by using agar diffusion method. The bacterias were incubated at 37 °C for 24 h in nutrient broth medium. The inoculum was then added into agar medium in petri dish and solidified. Whatman paper with diameter of 6 mm was placed on the plates afterward. Samples with various concentration were then injected to the Whatman paper. Right after the incubation process at 37 °C for 24 h was done, the diameter of inhibition zones were measured (in mm). In addition, tetracycline was used as a positive control and dimethyl sulfoxide (DMSO) 20% used as negative control (Tillah *et al.*, 2017).

The antibacterial assay against *S. aureus* (ATCC 12600) was conducted using microdilution method (Batubara *et al.*, 2009). Extracts were diluted in DMSO resulting a stock solution with concentration of 10000 mg/mL. The stock solution then diluted into various concentrations of extract solutions (15.63-2000 mg/mL). Extract solutions, tryptic soy broth (TSB) medium, and bacterial inoculant were added into each well of sterile 96-well plates. The mixture was then incubated at 37 ºC for 24 h and the minimum inhibitory concentration (MIC) was determined. The minimum bacteriacidal concentration (MBC) was determined after conducting 24 hour incubation of the MIC clear zone in a new media. Tetracycline and ciprofloxacinee was used as a positive control and dimethyl sulfoxide (DMSO) 20% was used as negative control.

Inhibition of initial bacteria cell attachment

The resin extracts at the same concentration as the MIC value were evaluated for their potential inhibition of cell attachment (anti adhesion test). Each 100 µL of extracts and posi-

tive control (ciprofloxacine and tetracycline in concentration of 0.0025 mg/mL) was added into a 96-well microplate. Then, a 100 µL of bacteria culture (10^6 CFU/mL) of *S. aureus* (ATCC 12600) was added in to each well (final volume in each well was 200 µL). Precisely, 200 µL of medium was added into the well of blank without bacteria culture. The plates were wrapped loosely with parafilm and incubated at 37 °C for 8 h without any shaking treatment in order to allowed the cells to attach to the surface. After the biofilm formed, the remained contents of each well were removed afterward. Biofilm, which sticked to the wells was rinsed three times with sterile distilled water in order to remove the loosely attached cells and the non-adherent cells. This step was then validated by staining the wells with 200 µL of 1% crystal violet followed by incubation at room temperature for 15 min. The plates were then rinsed three times with sterile distilled water to remove unabsorbed stain. The wells were then destained by adding 150 µL of ethanol. Precisely, 100 µL of the destaining solution was transferred into a new plate and its absorbance was measured at 590 nm using a microplate ELISA reader. In addition, each assay was performed in triplicate. The mean of samples optical density was then determined and the absorbance of blank well was subtracted from the optical density reading and also the inhibition percentage and efficiency was determined (Bazargani *et al.*, 2016).

Inhibition of biofilm formation and growth

Biofilm formation was conducted 4 hours before the addition of resin extracts. The extracts which exhibited at least 50% inhibition in bacteria cell attachment were evaluated by biofilm formation inhibitory assay. In brief, 100 µL of *S. Aureus* (ATCC 12600) bacteria culture (10^6 CFU/mL) was added to each well of a 96-well microtiter plate, then incubated for 4 h at 37 °C to allow the attachment of cell and the biofilm formation. The following incubation was conducted by adding 100 µL of each resin extracts in order to obtained a final concentration (MIC value) in the wells. The equal volume of ciprofloxacine and tetracycline in concentration of 0.0025 mg/mL was added as a positive control, while the negative control was 100 µl of medium without extract and 200 µL of medium was used as a blank. The plates were then incubated for 24 h. Finally, the inhibition of biofilm formation and growth was determined by crystal violet staining assay, and the inhibition percentage was then calculated. Each assay was performed in triplicate (Bazargani *et al.*, 2016).

Biofilm degradation

A complete biofilm formation was conducted 24 hours before the addition of the extracts. To the 96-well microtiter plate was added 100 µL of *S. aureus* (ATCC 12600) bacteria culture (10^6 CFU/mL), then incubated for 24 h at 37 °C. Once biofilm formed, the remained medium was removed. A 100 µL of each resin extract was then added into the wells in order to obtained a final concentration (MIC value). Ciprofloxacine and tetracycline in concentration of 0.0025 mg/mL was added as a positive control, while the negative control was 100 µl of medium without extract and 200 µL of medium was used as a blank. The plates were then incubated for 24 h at 37 °C. Lastly, the inhibition of biofilm formation and growth was determined by crystal violet staining assay, and the inhibition percentage was calculated. Each assay was performed in triplicate (Batubara *et al.*, 2016).

Biofilm microscopic visualization

Biofilm formation and degradation assay was conducted as described above. Furthermore, before destaining the 96-well microtiter plate with ethanol, biofilms were evaluated and confirmed by light microscopy at 10x magnification beforehand (Bazargani *et al.*, 2016).

TLC- Bioautography

Briefly, 10 µL of resin extracts (2 g in ethanol) was applied to the TLC Silica gel 60 F254. Chromatography method was conducted using chloroform:methanol:water (9:1:0.1) as mobile phase. After the elution finished, the TLC plate was dried at room temperature to complete a solvent removal, then transferred into a petri dish and added by agar medium, which was spreaded together with the inoculum of *S. aureus*. After incubation at 37 °C for 24 h, the 2,3,5-triphenyltetrazolium chloride (TTC) (20 mg/mL) was then sprayed on to TLC plate. The clear zone indicated antimicrobial activity was observed against the pink background (Rossi *et al.*, 2011).

RESULTS AND DISCUSSION

Extracts and Chromatographic fraction of *D. draco* and Antibacterial Activity

The result of extraction process exhibited that ethyl acetate provided highest extraction yield of *D. draco* compare to methanol and *n*-hexane (Table 1). Yield of ethyl acetate was eleven times higher than methanol and hundreds time higher than *n*-hexane. This result indicated that

the major component in *D. draco* resin was soluble in moderate polarity solvent such as ethyl acetate. Previous report pointed out that some semipolar and polar constituent has been isolated from *D. draco*. Dracorhodin, dracorubin, and nordracorubin were isolated from chloroform-methanol extract [9], dracoflavan B1, B2, C1, C2, D1 and D2 were isolated from ethyl acetate extract (Arnone *et al.*, 1997), daemonorol group (A-F) were isolated from acetone extract (Nakashima *et al.*, 2009) and dimethoxyflavan group were isolated from chloroform extract (Hao *et al.*, 2015).

Antibacterial activity of *D. draco* extracts was determined by using agar diffusion disc method against *S. aureus* and *E. coli.* Antimicrobial activity of *D. draco* resin extracts against *S. aureus* were stronger compare to its activity against *E. coli* (Table 1). *D. draco* resin extracts were not inhibited *E. coli* growth at all. This may be because antibacterial agent in *D. draco* resin were unable to penetrate the lipopolysaccharide layer in *E. coli* bacterial cell wall. In general, ethyl acetate extract and methanol exhibited stronger antimicrobial activities against *S. aureus* than *n*-hexane extract. The inhibition zone of *D. draco* extracts against *S. aureus* was comparable with inhibition zone of *P. laevigate, O. ficus-indica*, and *G. microcephala* as reported by Sanchez *et al.* (2016).

Bioautography TLC chromatogram of antibacterial assay showed that *D. draco* extracts have antibacterial activity against *S. aureus* which indicated by a clear zone on pink background. This zone was come from the cleavege of tetrazolium chloride by dehydrogenase from the bacterials. TLC bioautography of *D. draco* extracts indicated that constituent with Rf of 0.71 performed antimicrobial activity against *S. aureus*. This active bands (indicated with red arrow) were detected in *n*-hexane, ethyl acetate, and methanol extracts (Figure 1A-C). The constituent with Rf 0.71 predicted to be moderate to nonpolar constituent since its interaction with stationary phase was weak and it was eluted by chloroform:methanol:water (9:1:0.1).

Further separation of ethyl acetate extract using *n*-hexane and methanol yield 24.46% of *n*-hexane fraction. This separation was conducted to obtain nonpolar constituent from ethyl acetate extract and evaluate its potency as antibacterial agent. *n*-hexane fraction was then further fractionated by column chromatography using silica gel as stationary phase and *n*-hexane:chloroform (9:1) as mobile phase. This fractionation provided 9 fractions. Furthermore, fraction number 4 and 5 was separated using thin layer chromatography (TLC). The TLC profile of fraction number 4 and 5 (Figure 1D) showed that retardation factor (Rf) of components in fraction 4 and 5 were 0.28, 0.53, and 0.73 respectively. Based on retardation factors, it could be predicted that fraction number 4 and 5 contain almost similar constituents. The active constituent as antimicrobe (with Rf 0.71) was also detected in F4 and F5.

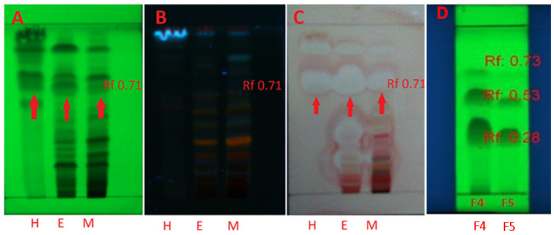


Figure 1. TLC chromatogram of *n*-hexane extract (H), ethyl acetate extract (E) and methanol extract (M) of *D.draco* monitored at 254 nm (A), 366 nm (B), visible light of antibacterial bioautography againts *S. aureus* (C), and TLC profile of F4 and F5 (D).

Antibacterial activity against *S. aureus* was also evaluated by determining the MIC and MBC value. Methanol extract and fraction number F.5.1 (band number 1 from TLC separation of F5) exhibited lowest MIC value (0.5 mg/mL) against *S. aureus*. This result indicated that methanol extract and F.5.1 owned stronger antibacterial activities compare to other extracts and fractions (Table 2). Otherwise, the results showed that *D. draco* extracts and fractions performed much lower antimicrobial activity compare to tetracycline and ciprofloxacine. Although antimicrobial activities

Table 1. Extraction yield and antibacterial activity of *D. draco* extracts

Sample	Yield (%)	Inhibition zone (mm) at concentration of extract (µg/mL)									
		S. aureus					*E.coli*				
		200	20	10	5	0.2	200	20	10	5	0.2
n-Hexane extract	0.22	10.57	-	-	-	-	-	-	-	-	-
EtOAc extract	73.31	15.05	12.55	10.89	10.01	-	-	-	-	-	-
MeOH extract	6.38	13.40	11.19	10.01	10.01	-	-	-	-	-	-
Tetracycline		*	*	*	*	19.10	*	*	*	*	13.19

(-) not detected; (*) not tested

were lower than commercial antibiotic, the result opened the possibilities for the discovery of other components in *D. draco* resin with antibacterial activity against *S. aureus*.

Commercial *D. draco* from Meer corporation which was extracted by chloroform-methanol provide higher MIC (1.0 mg/mL) against *S. aureus* ATCC 13709. The antimicrobial activity of *D. draco* resin may be due to the presence of dracorhodin and dracorubin compounds isolated from chloroform-methanol extract [9]. These compounds were found to be active against *S. aureus* ATCC 13709, *Klebsiella pneumoniae* ATCC 10031, *Mycobacterium smegmafis* ATCC 607 and *Candida albicans* ATCC 10231 [9]. In addition, methanol extract of *D. draco* and fraction number 5.1 provide lower MBC value compare to *P. laevigate, O. ficus-indica*, and *G. microcephala* extracts as reported by Sanchez *et al*. (2016).

Antibiofilm activity

Biofilms are communities of microorganisms attached to a surface. The studies indicate that biofilms are in a stable state in a biological cycle that includes initiation, maturation, maintenance, and dissolution (Pratiwi *et al*., 2015). Since the antibiofilm of *D. draco* extracts was consider insufficiently investigated, the inhibition activity of *D. draco* extracts in initial attachment of bacteria cell, biofilm formation and growth, and degradation of formed biofilm was investigated in this research. Investigation was performed against *S. aureus* since *D. draco* extracts exhibited antibacterial activity against *S. aureus*. It is already well known that *S. aureus* biofilm induce some dieases such as osteomyelitis, periodontitis and peri-implantitis, chronic wound infection, chronic rhinosinusitis, endocarditis, ocular infection and polymicrobial biofilm infection (Archer *et al*., 2011). *D. draco* extracts at MIC value were used for this investigation.

D. draco extracts revealed a strong inhibition towards initial bacteria cell attachment. The result showed that the initial bacteria cell attachment was almost completely inhibited by all *D. draco* extracts and positive control. The formation and growth of biofilm was inhibited at the level of 61.23±1.91% and 77.79±2.13% by ethyl acetate and methanol extracts, respectively. Otherwise, Figure 2 revealed that biofilm degradation activity of *D. draco* extracts lower compare to positive control (in the range of 22.84-43.31%). This result indicated that concentration of plant extract required for biofilm degradation was higher than those for inhibition of biofilm initial attachment. It is also reported for another plant extracts. Essential oil from *Curcuma aeruginosa* stem with MIC of 0.125 mg/mL against *Streptococcus mutans* could only degradated 50% of *S. mutans* biofilm at the concentration of 1.347 mg/mL (Wahyuni *et al*., 2017), which means that biofilm is more resistance to the antimicrobial agents compared to the free floating cells.

The mechanisms of inhibition and degradation of biofilm are predicted due to a disturbance in quorum sensing, bacterial communication systems to ensure sufficient cell numbers to form bacterial populations (Pratiwi *et al*., 2015). Another possible mechanism is by by killing the bacteria in biofilm, particularly during the early stage of biofilm formation (Phuong *et al*., 2017). Further study is needed to determine the actual anti-biofilm mechanisms of the extract of *D. draco* resin.

The result indicated that *D. draco* extracts was effective for the prevention of bacteria cell attachment and biofilm formation and growth. Otherwise, its activity in biofilm degradation was not as effective as ciprofloxacine and tetracycline. The result was then confirmed by microscopic visualization. The treatment with ethyl acetate and methanol extract reduced the bacterial amount in comparison to the negative control. However, the reduction of bacterial colony by the treatment of extracts was lower, compared to the ciprofloxacine and tetracycline treatments (Figure 3).

Table 2. Antimicrobial activity of *D. draco* extracts against *S. Aureus*

Sample	MIC (mg/mL)	MBC (mg/mL)
Ethyl acetate extract	1.0	1.0
Methanol extract	0.5	1.0
F.4	1.0	2.0
F.5	1.0	2.0
F.5.1	0.5	1.0
F.5.2	1.0	2.0
Tetracycline	0.0025	0.0025
Ciprofloxacine	0.0025	0.0025

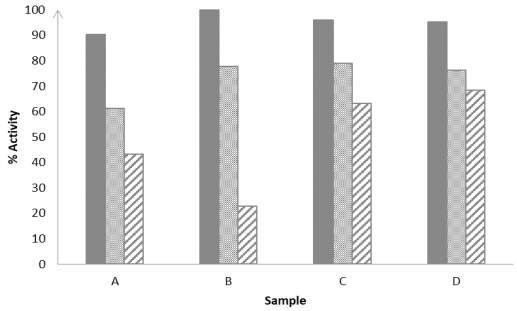


Figure 2. Antibiofilm activity of *D. draco,* % inhibition of initial *S.aureus* bacteria cell attachment (), % inhibition of *S. aureus* biofilm formation and growth (), % degadration of *S. aureus* biofilm (), ethyl acetate extract 1 mg/mL (A), methanol extract 0.5 mg/mL (B), ciprofloxacine 0.0025 mg/mL (C), tetracycline 0.0025 mg/mL (D)

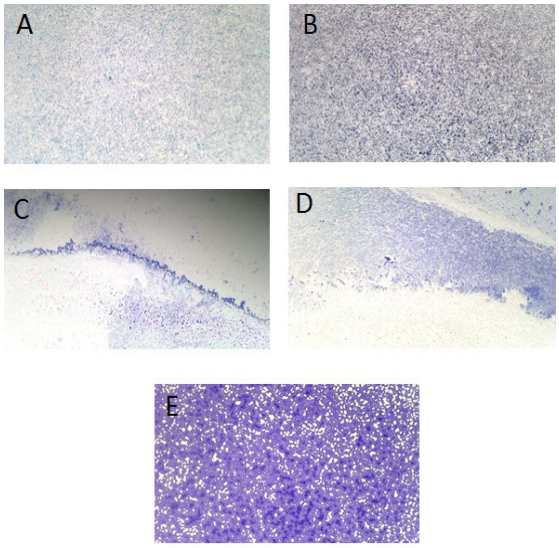


Figure 3. Visualization of *S.aureus* biofilm degradation by ethyl acetate extract 1 mg/mL (A), Methanol extract 0.5 mg/mL (B), Ciprofloxacine 0.0025 mg/mL (C), Tetracycline 0.0025 mg/mL (D), negative control (E)

As discussed above, *D. draco* extracts was potential for its utilization as antimicrobe and antibiofilm. Activity of *D. draco* resin as inhibitor of biofilm formation is a novel information which expected could increase the utilization of *D. draco* resin by the society.. Furthermore, findings of this study expected to provide scientific backup for utilization of *D. draco* by local ethic in Jambi Province and other area and increase the added value of *D. draco* utilization by the society.

CONCLUSION

Antibacterial and antibiofilm activity of *D. draco* extracts was successfully investigated. Ethyl acetate and methanol extracts of *D. draco* exhibited stronger antimicrobial activity against *S. aureus* compare to *n*-hexane extract. Furthermore, methanol extract of *D. draco* performed lowest MIC value against *S. aureus*. TLC bioautography of *D. draco* extracts indicated that constituent with Rf of 0.71 performed antimicrobial activity against *S. aureus*. On the other hand, antibiofilm assay revealed that n-hexane, ethyl acetate, and methanol extracts of *D. draco* were inhibited initial attachment of bacteria cell in biofilm formation. Indicated that *D. draco* extracts was potential as antibacterial agent and inhibitor of biofilm formation.

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