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Antibacterial and Antifungal Activities of *Dumortiera hirsuta* Active Fractions

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ABSTRACT

Dumortiera hirsuta is an endogenous liverwort growing in Indonesia. Previous study showed that the chloroform extract of *Dumortiera hirsuta* had antibacterial and antifungal activites. This studies reported the antifungal and antibacterial activities of the fractions of the *D. hirsuta* chloroform extract. Chloroform extract was fractionated using hexane and ethyl acetate. Antimicrobial activity of fractions was tested by using disc diffusion method and it was then continued by the tube dilution method to obtain the Minimal Inhibitory Concentration (MIC) value. The result showed that fraction II (hexane:ethyl acetate = 90:10) had the highest inhibition on *Candida albicans* ATCC 10231 , while the fraction VI (hexane: ethyl acetate = 40:60; 30:70; 20:80) had the highest inhibition on *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922
KEY WORDS: antibacterial activity, antifungal activity, *Dumortiera hirsuta*

INTRODUCTION

Chemical antibiotics are quite effective as a curative and preventive agent against infectious disease. However, the use of antibiotics, in the long time, causes resistance of microbial pathogens. In addition, hypersensitivity, allergic reaction, or immune system disorder may appear as side effects (18). Therefore the efforts to continuously find new sources of new drugs to treat microbial pathogen should be done.

According to World Health Organization (WHO) (2008), more than 80% of the world population believe that herbal medicines are able to maintain their health (9) and it makes a rapidly increasing demand of antimicrobial herbal remedies (3,4). Researches on variety of plant extracts to studies the antimicrobial activities have been done in various countries in Asia, Africa and Europe (1,10,13,20).

Bryophytes have been known to have antimicrobial activity, nevertheless, only few scientific studies are reported in Indonesia. Extract of Bryophytes contains isoflavonoid, flavonoid, biflavonoid and terpenoids. Those compounds have been reported to have antimicrobial activity (2,15,21).

Liverwort is one of the classes of Bryophytes which have several active compounds having a potential as an antimicrobial agent. Ethanol extracts of *Sphagnum magellanicum* have potential as antibacterial agent on *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhii*, and *Staphylococcus aureus* (12). Extracts of *Targionia, Marchantia, Plagiochasma, Rhodobryum*, and Plagiomnium inhibit the growth of *Biden biternata* (14). Flavonoids isolated from *Marchantia convoluta* extracts could inhibit the growth of *Staphylococcus aureus*, *Bacillus enteridis*, *Streptococcus hemolitic* type B and *Diplococcus pneumoniae* (21). Liverwort *Pallavicinia lyelli* containing steroid is able to inhibit the growth of *Aspergillus fumigatus* (19). *Astrella angusta* contains antifungal compound of dibenzofuran bis (bibenzil), the same compound is also found in liverwort *Lepidozia incuruata* from Germany (14,17).

D. hirsuta is an endogenous liverwort growing in the forest in Cangar, Batu, Malang, East Java Indonesia. Our previous studies showed that crude chloroform extract of *D. hirsuta* possessed antibacterial and antifungal activities. This study reported the antibacterial and antifungal activity of the chloroform extract fraction of *D. hirsuta* on *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10231.

MATERIALS AND METHODS

A. Material

The whole plants of *D. hirsuta* were collected from the forest in Cangar, Batu, Malang, East Java, Indonesia.in April 2010. Fresh gametophytes samples of *D. hirsuta* were cleaned then soaked in 0.8% Tween 80 (8), followed by rinsed and dried. Finally, they were cut into small pieces into the form of powder.

B. Extract preparation and monitoring the bioactive

Ten grams of crushed liverwort was placed into the soxhlet tube. The continuous extraction was performed by adding 150 ml of chloroform into the apparatus and it was extracted for six hours until the

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solution was clear. The results were chloroform extract and residue. Chloroform extract was dried until the chloroform had fully evaporated. Bioactive contents of chloroform extract were monitored by Thin Layer Chromatography (TLC) using a stationary phase silica gel GF 254 and a mobile phase of hexane: ethyl acetate = 1: 1. They were observed under UV light at λ 254 nm and 366 nm and they were sprayed with Cerium (IV) sulfate.

C. Chloroform Extract Fractionation

The extract having the best potential were fractionated by Vacuum Liquid Chromatography (VLC) with the stationary phase silica gel 60 GF254 powder and various kinds of mobile phase eluent. Silica gel powder was added into chloroform extract little by little while stirred in a mortar to obtain a homogeneous and dry mixture (extract powder). The column was made by adding a silica gel into the scintered glass while it was vacuumed to obtain a compact and solid of stationary phase mass (as high as 2 cm). The extract powder was transferred into the scintered glass and the top was closed using filter paper.

Fractionation was performed by pouring eluent slowly on the surface of filter paper (Whatman No. 4 filter paper). The extract was fractionation with eluent from non polar until polar (Table 1). Fractions were collected in porcelain cup and they were then dried. The monitoring of chromatogram profile of VLC results was done by TLC stationary phase silica gel GF 254 and a mobile phase of hexane: ethyl acetate = 1: 1. It was observed under UV light λ 254 nm and 366 nm and it was detected with a spray reagent Cerium (IV) sulfate. Fractions that have similar chromatogram patterns were combined to simplify the test. The mass of combined fraction was weighed and transferred to the flask. Each combined fraction was tested for its antibacterial and antifungal activities. The obtained data were diameter of inhibition zone and MIC values were analyzed descriptively.

D. Determination of Antimicrobial Activity

The liverwort extracts were tested for antibacterial and antifungal activities by the disc diffusion method. The medium in this test was sterile Mueller Hinton Agar (MHA). Microbial suspension was made in accordance with the standard 0.5 Mc Farland. To meet the standard, the turbidity of microbial suspensions of 0.1 at absorbance of 625 nm for bacteria and 600 nm for fungi should be determined. One ml suspension of microbes was inserted into a sterile Petri dish then 15 ml of MHA media was added into the petri dish to be homogenized, the agar media was left to cool and solidify. Fraction of liverwort as much as 25 ml was injected onto a paper disc with at concentration of 0, 0.0025, 0.00625, 0.0125, 0.01875, 0025 mg / disc. Three pieces of sterile paper disc, a diameter of 6 mm and the same thickness were placed on the agar surface at 5 cm distance. They were placed not too close to the edge of the Petri dish with triangular-shaped position. Subsequently, they were incubated for 24 hours for bacteria and 48 hours for yeast at a room temperature. The inhibition of growth was indicated by the formation of a clear inhibition zone (halo) around the paper disc. The inhibition zone diameter was measured using a shove.

E. Tube Dilution Method

Tube dilution method was used to determine the ability of an antimicrobial substance more accurately. It is generally used to obtain MIC values. This method was started by making a suspension of microbial tests on the physiological water in order to obtain suspension turbidity of 0.5 at 600 nm absorbance. The concentration range of antimicrobial substances that would be tested should be dense in order to emphasize the MIC values.

One ml of extract solution in sterile aquadest at various concentrations were inserted into the tube and 1 ml of tested microbes were grown in it. Hence, the concentration of the obtained extract was in accordance with the tested concentration. Culture was homogenized and incubated for 24 hours. Antibacterial activity could be seen if there were a decrease of turbidity in the culture. This activity showed the MIC values. If there was no microbial growth, the concentration would be the MBC / MFC

RESULTS AND DISCUSSION

In this study, fractionation was done using VLC because this method was relatively quick in separating the extract into its fractions. Eluent selection was based on the characteristic of compounds from the separation contained in the extract and it was then sorted from non polar eluent to more polar eluent. Therefore, fractions with a more specific chemical content could be obtained. Chloroform extract was fractionated into 11 different fractions (Table 1).

Table 1.Eluents for fractionation of chloroform extract

Fraction	Eluent	Volume (ml)	Mass (gram)
1	Hexan (Hx)	100	0.04
2	Hx:EA	90:10	0.43
3	Hx:EA	80:20	0.52
4	Hx:EA	70:30	0.44
5	Hx:EA	60:40	0.19
6	Hx:EA	50:50	0.13
7	Hx:EA	40:60	0.08
8	Hx:EA	30:70	0.1
9	Hx:EA	20:80	0.2
10	Hx:EA	10:90	0.07
11	Ethyl acetate (EA)	100	0.11

All fractions were monitored using TLC to see the profile of the compound content (Figure 1). Fractions having a similar chromatogram profiles were combined to simplify the number of fractions. Chloroform extract fraction was simplified into 7 combined fractions. The result of the combined fraction can be seen in Table 2.

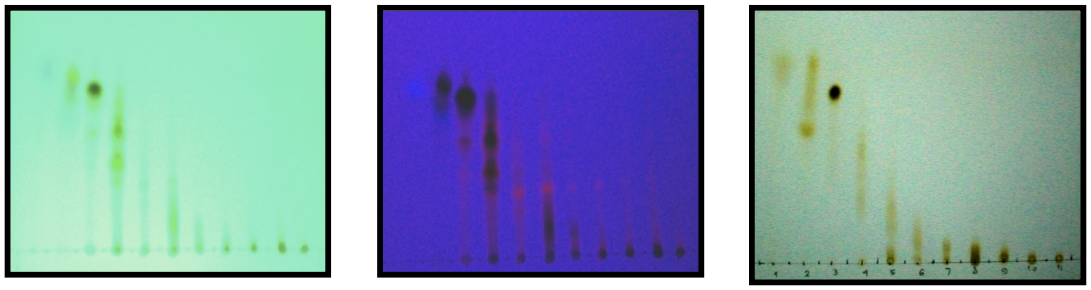

Figure 1. Profile of fraction chromatogram from *D. hirsuta* chloroform extract (a) UV λ 254 nm (b) λ 366 nm, (c) Cerium (IV) sulfate

Table 2. Combined fraction of chloroform extract

Combine fraction	Initiation fraction	Mass (gram)
I	1	0.04
II	2	0.43
III	3	0.52
IV	4	0.44
V	5,6	0.32
VI	7,8,9	0.30
VII	**10,11**	**0.18**

Inhibitory activities of all combined fractions on the three types of microbial tests showed positive results. It was shown that the formation of inhibition zone (halo) around the paper disc appeared at 24 hours incubation for bacteria and 48 hours for yeast. All combined fractions inhibited the growth on *S. aureus*, *E. coli*, and *C. albicans.* This was caused by the fact that each type of fraction contained different active compounds in inhibiting the three microbes. Based on the observations, the higher the concentration the higher the diameter of the produced inhibition.

Antimicrobial activities of all combined fractions showed that the fraction VI (hexane: ethyl acetate = 40:60; 30:70; 20:80) had the highest inhibitory activity against *S. aureus* at a concentration of 0.0025 mg/disc (Table 3), this was higher than amoxicillin, ciprofloxacin, consalcetine, streptomycin and tetracycline antibiotics at the same concentration (Table 4), and the inhibitory activity of fraction VI at a concentration of 0.01875 mg / disc was higher than the ciprofloxacin, consalcetine, and streptomycin at a concentration of 0,02 mg /disc.

Table 3. Inhibition zone diameter (mm) of combined fraction on *S. aureus*, *E. coli*, and *C.albicans*

Combine fraction	*S. aureus ATCC 25923*						*E. coli ATCC 25922*						*C. albicans ATCC 10231*					
	Concentration (mg/disc)						Concentration (mg/disc)						Concentration (mg/disc)					
	0	0.0025	0.00625	0.0125	0.01875	0.025	0	0.0025	0.00625	0.0125	0.01875	0.025	0	0.0025	0.00625	0.0125	0.01875	0.025
I	0	8.3	8.3	8.6	8.6	8.9	0	8.6	9.2	9.4	9.5	9.8	0	8.3	8.4	8.8	8.8	8.9
II	0	8.3	8.8	8.6	9.2	9.2	0	8.8	9.0	9.50	9.9	11.2	0	10.5	11.5	13.4	14.1	15.1
III	0	8.8	9.4	9.3	9.7	10.0	0	8.9	9.1	9.4	9.4	9.5	0	0	0	8.4	8.7	8.7
IV	0	8.5	9.1	9.0	9.4	9.4	0	8.4	8.6	9.0	9.2	9.2	0	8.0	8.9	8.9	9.0	9.1
V	0	9.3	9.5	9.6	9.8	9.8	0	8.8	8.8	9.0	9.3	9.9	0	0	8.1	8.3	8.7	8.7
VI	0	10.0	10.0	10.3	11.5	13.2	0	10.4	10.8	11.3	12.4	13.2	0	0	8.3	8.6	9.3	9.4
VII	0	8.5	8.6	8.7	8.7	9.2	0	9.5	9.5	9.7	9.9	10.1	0	0	8.6	8.7	8.9	9.3

Similar to *S. aureus*, the highest inhibitory activity against *E. coli* was also found at fraction VI. At a concentration of 0.0025 mg / disc, the activity was equivalent to erythromycine and tetracycline and it was higher than amoxicillin, consalcetine, rifampicin, and streptomycin antibiotics (Table 3 and 4). At a concentration of 0.01875 mg / disc, the activity was higher than antibiotics except for ciprofloxacin at a concentration of 0:02 mg / disc.

All the fractions led to antimicrobial activity against *C. albicans* at a concentration of 0.0025 mg / disc except fraction III, V, and VI (Table 3). If it was compared with variation of antibiotics tested (9), at a concentration of 0.0025 mg / disc, the activity was equivalent to the amoxicillin antibiotic activity and was higher compared to the ciprofloxacin, consalcetine, erythromycin, rifampicin and tetracycline antibiotics. While, at a concentration of 0.01875 mg / disc, the activity was higher than the rifampicin, streptomycin and tetracycline antibiotics (Table 4).

Table 4. Antimicrobial activity of antibiotics against test microorganism (inhibition is zone in mm) (9)

Antibiotics	*S. aureus ATCC 25923*					*E. coli ATCC 25922*					*C. albicans ATCC 10231*				
	Concentration (mg/disc)					Concentration (mg/disc)					Concentration (mg/disc)				
	0.0025	0.005	0.01	0.02	0.04	0.0025	0.005	0.01	0.02	0.04	0.0025	0.005	0.01	0.02	0.04
Amoxicillin	0	15.9	20.3	25.6	25.5	8.5	11.6	11.3	9.7	7.8	10.3	21.2	24.2	26.5	29.1
Ciprofloxacin	0	13.4	0	8.7	11.6	12.9	28.8	32.4	38.7	42.2	9.2	16.5	21.2	29.6	34.0
Consalcetine	0	0	0	8.9	13.7	8.9	14.1	9.6	10.1	10.8	9.1	8.4	12.5	19.1	25.7
Erythromycin	11.3	11.4	24.7	24.3	30.0	10.2	12.6	11.3	11.8	11.9	8.8	9.9	11.3	20.8	18.4
Rifampicin	17.2	31.0	33.1	36.0	35.8	0	9.6	0	0	9.5	0	0	0	0	0
Streptomycin	0	0	0	0	0	9.6	11.6	10.5	11.8	12.4	11.6	0	0	0	28.0
Tetracyclin	0	15.85	14.5	17.6	19.2	10.8	10.4	10.8	10.0	10.8	0	0	10.6	10.9	9.8

Tube dilution method was used to determine MIC and MBC values. Based on the MIC and MBC value of seven combined fractions, fraction II (hexane:ethyl acetate = 90:10) was the best against *C. albicans* and fraction VI (hexane: ethyl acetate = 40:60; 30:70; 20:80) was the best against *S. aureus* and *E. coli* (Table 5).

Table 5. Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC)/ Minimal Fungicidal Concentrations (MFC) of combined fractions of *D. hirsuta*

Combined Fractions	*S. aureus* ATCC 25923		*E. coli* ATCC 25922		*C. albicans* ATCC 10231	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
I	0.1	>0.1	0.1	>0.1	0.1	>0.1
II	0.1	>0.1	0.1	>0.1	0.075	0.1
III	0.1	>0.1	0.1	>0.1	0.5	>0.5
IV	0.1	>0.1	0.1	>0.1	0.1	>0.1
V	0.075	0.1	0.1	>0.1	0.25	>0.25
VI	0.05	0.75	0.05	0.075	0.25	>0.25
VII	0.1	>0.1	0.1	>0.1	0.1	>0.1

The antimicrobial activity shown by this fractions indicated that there was possibility that the fraction contained secondary metabolites terpenoid, steroids, flavonoids and alkaloid that potential as antimicrobial compounds. Antibacterial mechanisms of flavonoids were to inhibit nucleic acid and cytoplasmic membrane synthesis, and also inhibit energy metabolism. While, antifungal activity of flavonoids was to inhibit spore germination (5). Sensitivity of Gram-negative bacteria against alkaloids was caused by the interaction between the alkaloids and some cell wall components so that it caused cytotoxic damage in this bacterial group (7). The antimicrobial inhibitory mechanism by steroid occurred by blocking the process of germination of spores (19). Terpenoid group caused damage to the cell membrane and as a result the cell lysis (6).

The antimicrobial test results showed that there were differences in the activity of each fraction in inhibiting the growth of tested microbes. Differences in the ability of inhibitory activity of each fraction were influenced by the type of active compounds, the concentration of active antimicrobial compounds, the diffusion ability of the antimicrobial active compounds on agar medium, the type of microbes used, the inhibition mechanism, and the sensitivity of the tested microbial resistance to antimicrobial attack. The result was expected to be able to open opportunities for the discovery of new antimicrobial compounds from the liverwort *D. hirsuta* Indonesia.

CONCLUSION

It could be concluded that different active fraction of *Dumortiera hirsuta* have a different antimicrobial and antifungal activities.The result showed that fraction II (hexane:ethyl acetate = 90:10) had the highest inhibition on *Candida albicans* ATCC 10231 , while the fraction VI (hexane: ethyl acetate = 40:60; 30:70; 20:80) had the highest inhibition on *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922

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