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Antimicrobial potential and chemical constituent of *Mikania micrantha* H. B. K.

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The aerial parts of *Mikania micrantha* H. B. K. were prepared with different polarities solvents by the associated ultrasonic extracting method. The screening antimicrobial activities assays were designed against fungi (*Fusarium solani*, *Phytophthora parasitica* Destur, *Pythium aphanidermatum*, and *Rhizoctonia solani* Kühn) and bacteria (*Ralstonia dolaanacearum*, *Xanthomonas oryzae pv. Oryzae*). The results show that the leaves extracts were higher bioactivity than the stem extracts, and the chloroform extracts were observed the highest bioactivity. The antimicrobial potential of the leaves extracts of *M. micrantha* were also assessed. The results show that concentration for 50% of maximal effect (EC_{50}) values was 1313.92, 2493.63, 1718.97, and 1084.91 mg/l respectively at 48 h against the five fungi tested. Determination of Minimum Inhibitory Concentration (MIC) values were found to be 250 and 500 mg/l respectively against the two bacteria tested. The result of Gas Chromatography-Mass Spectrometer (GC-MS) analysis indicated that 13 compounds, which represented 87.03% of total, were present in the leaves extract, and 1-(4-chlorophenyl)-2- hydroxyethanone (57.44%) was the major chemical constituent.

Key words: *Mikania micrantha* H.B.K., leaves extracts, chloroform extracts, fungistasis efficacy, bacteriostatic efficacy, GC-MS analysis.

INTRODUCTION

In agriculture, the treatment of bacterial and fungal infections still remains an important and challenging problem because of factors that include emerging infectious diseases and the increasing number of multi-drug resistant microbial plant pathogens. In spite of the large number of antimicrobial for use, the emergence of old and new antimicrobial resistant microbial strains in the last decades constitutes a substantial need for new classes of antimicrobial agents. Many naturally occurring extracts from edible and medicinal plants, herbs and spices have been shown to possess antimicrobial functions and serve as a source for antimicrobial agents against plant pathogen, for example allicin garlicin (Balestra et al., 2009), caryophyllus oil (Mohammed and Al-Bayati, 2008), matrine (Guo et al., 2011), and so on.

Mikania micrantha H.B.K. (Asteraceae), commonly known as mile-a-minute weed, is an extremely fast-growing, perennial creeping weed. Its native distribution is in tropical central and South America. The weed was introduced into Indonesia as ground cover in the 1940’s where it then spread to the Pacific Islands and Southeast Asia. This weed may compete for nutrients, light and soil

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moisture with nearby plant species and then kill the plant species (Huang et al., 2000). In addition, its rampant growth characteristics and potential allelopathic effects (Ismail and Kumar, 1996) can devastate most native species populations (Ismail and Mah, 1993) and cause substantial damage to natural ecosystem and biodiversity. So *M. micrantha* is listed as one of the world's worst weeds. By consulting literatures, sesquiterpenoids, flavonoids, polyphenols, sesquiterpene lactones have been isolated from *M. micrantha* (Huang et al., 2009). The leaves of *M. micrantha*, commonly known as 'guaco', are used to make a poultice for snake bites and scorpion stings, decoction of the leaves is used to bath rashes, skin itches, athlete's foot and as wound dressings in Jamaica (Ayensu, 1981). More particularly, the extracts from leaves and stem of *M. micrantha* are known to be active against a wide variety of microorganisms, including Gram-negative bacteria and Gram-positive bacteria (Ghosh et al., 2008; Wu et al., 2007), especially, several references on the antimicrobial activity of *M. micrantha* against some plant pathogens are available in the literature Zhuang et al. (2010) and Hao et al. (2007). The antimicrobial activity of *M. micrantha* is assigned to a number of sesquiterpene lactones compounds, which also in pure form have been shown to exhibit antibacterial or antifungal activity (Bakir et al., 2004).

We report here a systematic screening of a wide range of antimicrobial with the aim of investigating their potential against four reference fungi, *Rhizoctonia solani* Kühn, *Phytophthora parasitica* Destur, *Fusarium solani, Pythium aphanidermatum* and two reference bacteria, *Ralstonia dolaanacearum*, *Xanthomonas oryzae pv. Oryzae*. In addition, the major chemical constituent about the extract of *M. micrantha* were determinate using GC-MS. This project is a springboard for future research and instrument of practical utility for plant protect providers.

MATERIALS AND METHODS

Material

Plant material: The leaves of *M. micrantha* were collected in April 2011, in Dongguan, Guangdong Province of China. The sample was identified by Professor Xiao-yi Wei. An authenticated voucher specimen (No. 020701) was deposited at the herbarium of South China Institute of Botany, Chinese Academy of Sciences, Guangzhou, and People's Republic of China.

Four reference fungi and two reference bacteria, *Rhizoctonia solani* Kühn, *Phytophthora parasitica* Destur, *Fusarium solani, Pythium aphanidermatum*, and *Ralstonia dolaanacearum* and *Xanthomonas oryzae* pv. *Oryzae*, were used during the study. The tested strains were obtained from Institute of Plant Protection, Chinese Academy of Agricultural Sciences China. The fungi were incubated for 72h at 28°C in Potato Dextrose Agar (PDA) and stored in plate at 4°C. The tested bacteria strains were cultured in beef extract peptone agar medium (PBA) at 27°C and stored in nutrient agar slants at 4°C.

Fungistasis assay method

Fungistasis of the extracts was tested using the inhibit hypha growth method. The extracts were prepared with different concentrations using dimethyl sulfoxide (DMSO) and diluted by Potato Dextrose Agar (PDA) medium as needed. The PDA mediums were dumped separately into Petri dishes (9 cm diameter) as plating. An agar plug of fungal inoculums (6 mm in diameter) was removed from a previous culture of all the fungal strains tested and placed upside down in the center of the Petri dishes. The same amount of DMSO and distilled water which were used to replace the extracts were added respectively; the platings as the control and the blank test. Each treatment was done with three replicates. All materials were subjected to autoclaving at 121°C for 30 min. The means diameter of fungal colony was measured by criss-cross method with calipers by incubated at 28°C and the growth inhibition ratio was calculated according to the following formula in equation [1]. Regression equation of toxicity, concentration for 50% of maximal effect (EC_{50}), confidence interval of 95% was calculated using probability value analysis method.

$$\text{Inhibition ratio (\%)} = \frac{\text{Control } (\geq \text{diam}) - \text{Treated } (\geq \text{diam})}{\text{Control } (\geq \text{diam})} \times 100\ \% \quad (1)$$

Antibacterial assay method

Bacteriostatic effect of the extracts was determined using disc method (Liu et al., 2006). For this, the tested strains were inoculated on BPA medium plates for 24 h at 37°C. Colonies were suspended in 2 ml sterile water, then shake for a few minutes on vortex mixer, for uniform distribution. The suspensions were adjusted to approximately 10^7 CFU/ml (colony forming units per ml) (OD_{610}=1.0) using a UV spectrometer and confirmed by bacteria counting apparatus. The suspensions were diluted 100 times with 50°C of the medium (BPA). Five milliliter (5 ml) of diluted suspensions (10^5 CFU/ml) were added to surface of 15 ml solidified BPA medium in Petri dishes (9 cm diameter). The extracts were prepared with different concentrations using DMSO and diluted by distilled water as needed. Sterilized Oxford cups (φ5 mm) were placed on the medium and were filled with 200 µl of the extracts. Equivalent amounts of DMSO and distilled water were used as the control and blank. Each plate was incubated at 37°C for 12 h. Each treatment was done with three replicates. All material of the experiment was subjected to autoclaving at 121°C for 30 min. The antimicrobial activity was evaluated by measuring the diameter of transparent inhibition zone against strains tested. Results were measure in means ± standard deviations from three replicates of independent experiments of the same treatment.

Determination of minimum inhibitory concentration (MIC) method

MIC of the extract was assessed using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997, 1999). An inoculum of the microorganism was prepared from 24 h Mueller-Hinton Broth (MHB) cultures and suspensions were adjusted with turbidity equivalent to that of a 0.5 McFarland standard. Suspensions were further diluted 1:10 in sterile MHB to obtain a final inoculum of 5×10^5 CFU/ml. The 96-well round bottom sterile plates were prepared by dispensing 180 µl of the inoculated broth into each well. A 20 µl aliquot of the extracts was added. The concentrations of the extract tested were 50.0, 100.0, 250.0, 500.0, and 1000.0 mg/ml. Dilutions of ampicillin

Table 1. Screening results of fungistatic activity of the extracts of *M. micrantha*.

Aerial part	Tested Fungi strain	Time (h)	Inhibition ratio （%）				
			Methanol	Ethyl acetate	Acetone	Chloroform	Petroleum ether
Leave extract	*Rhizoctonia solani* Kühn	48	27.2358±0.7937	56.0976±0.2778	71.1382±0.2438	72.4390±0.2252	6.300±0.7937
		72	0	19.6970±1.9048	57.7178±0.7852	78.6458±0.8718	0
		96	0	0	37.6849±0.7330	78.9672±1.1894f	0
	Phytophthora parasitica Destur	48	0	69.3237±0.0260	73.7923±0.2264	65.5193±0.1333	0
		72	0	66.5645±0.6586	73.9190±0.5341	52.826±0.3629	0
		96	0	74.0798±0.3069	77.2810±1.0537	61.9983±0.2990	0
	Fusarium solani	48	15.1211±0.5460	83.7510±0.0285	83.9181±0.2836	89.3484±0.3153	4.7201±1.0388
		72	10.8934±0.5321	49.9883±0.0523	76.4404±0.5412	70.9354±0.9862	0
		96	18.1894±0.1667	51.1883±0.5391	71.9814±0.9411	70.1182±0.1701	0
	Pythium aphanidermatum	48	35.2120±0.1894	24.1546±0.1270	69.5652±0.4105	77.5094±0.3967	2.0397±0.4957
		72	11.8685±0.7411	45.3845±0.7218	66.7681±0.4216	80.5640±0.5290	3.5942±0.1637
		96	15.1257±0.7610	49.9106±0.9447	65.5412±0.5654	76.8907±1.1264	8.6568±0.9447
Stem extract	*Rhizoctonia solani* Kühn	48	21.3008±0.8251	17.1545±0.7988	35.6098±0.2851	19.6748±0.7201	0
		72	0	0	0	0	0
		96	0	0	0	0	0
	Phytophthora parasitica Destur	48	0	0	0	0	0
		72	0	0	0	0	0
		96	0	0	0	0	0
	Fusarium solani	48	1.0860±0.6744	76.1487±0.1785	14.3693±0.4410	31.3701±0.5299	0
		72	0	36.7856±0.2149	21.9034±0.3547	11.1966±0.9647	0
		96	0	31.8046±0.7965	35.2323±0.2969	19.2404±0.3239	0
	Pythium aphanidermatum	48	7.9442±0.3175	11.3258±0.2714	11.0038±0.0033	0	20.9340±0.1572
		72	5.4372±0.2022	15.6421±0.3387	8.1355±0.7639	0	5.1532±0.2862
		96	8.1203±0.7233	17.0716±0.5220	11.4758±0.0736	0	9.0986±0.8643

The concentrations were 3000 mg/l.

trihydrate served as positive control, while broth with 20 µl of DMSO was used as negative control. Plates were covered and incubated for 12 h at 37°C. After incubation, minimum inhibitory concentrations (MIC) were read visually.

Gas Chromatography and Mass Spectroscopy.

GC-MS was performed on a Finnigan TM PolarisQ. The column was an AB-50 ms, length of 30 m, 0.25 mm internal diameter, film thickness 0.25 µm, produced by Abel Industries. The carrier gas was helium at a flow rate of 1.0 ml min-1. Ion source-heating at 250°C, EI-mode was 70 eV, and the scan-range was 50-500 amu. The temperature program was: 50°C (1 µl injection volume), hold for 1 min, then 5 °C /min to 280°C, hold for 10min.

Mass spectra correlations were made using Wiley/NIST, and to determined the molecular formula and chemical name of the compounds. The relative mass fraction of chemical constituent of the leaves extract was determined according to peak area normalization method for quantitative analysis.

Preparation of plant extract

The leaves and stem of *M. micrantha* were air-dried at room temperature under shade, and ground into powder using an electric mill. The powder was packaged and stored in dry and well-ventilated room until use. Petroleum ether, chloroform, ethyl acetate, acetone, and methanol extracts with different polarities were obtained by associated ultrasonic extraction method at ambient temperature. Active carbon was added to the extract to remove chlorophyll, then filtered in order to get rid of active carbon and undissolved substance. The extracts were concentrated using rotary evaporator to give crude residue.

RESULTS

Fungistasis assay

The screening results of fungistasis assay is shown in Table 1. The leaves extracts observed remarkably higher fungistasis activity than the stem extracts. The chloroform extracts showed the highest fungistasis activity. Then, the results of fungistasis efficacy assay showed that the chloroform extract of leaves of *M. micrantha* exhibited high fungistasis efficacy against all the plant pathogens tested. As shown in Table 2, *R. solani* Kühn was been

Table 2. Fungistatic efficacy of the leaves extract of *M. micrantha*.

Tested fungus	Time (h)	Regression equation of toxicity	EC_{50} (mg/l)	Confidence interval of 95%
Fusarium solani	48	y=0.8298+1.3372x	1313.92	1028.6342--1678.399
	72	y=0.9432+1.2485x	1778.66	1382.7614--2287.904
	96	y=0.9266+1.2075x	2362.79	1882.2242--2966.508
	120	y=1.9797+0.8666x	3057.23	2771.258--3372.714
Phytophthora parasitica Destur	48	y=1.6770+0.9098x	2493.63	3241.8494--6228.7728
	72	y=0.7377+1.2616x	2390.4	1836.7939-3110.8539
	96	y=0.1034+1.5076x	2529.29	2322.2502-2754.7956
	120	y=0.3428+1.2983x	3864.85	2075.0367-7198.4755
Pythium aphanidermatum	48	y=1.0141+1.2320x	1718.97	1617.4164-1826.899
	72	y=0.5681+1.3448x	1974.54	1592.0599-2448.9061
	96	y=0.0511+1.4870x	2128.42	1583.9644-2860.0332
	120	y=0.4633+1.3395x	2436.91	1923.3645-3087.5728
Rhizoctonia solani Kühn	48	y=1.2808+1.2253x	1084.91	928.0855-1268.2365
	72	y=0.4311+1.4706x	1278.82	1278.8182-1620.5602
	96	y=1.2805+1.2245x	1090.47	966.0789-1230.8672
	120	y=1.1943+1.2262	1269.52	1132.5054-1423.1053

Table 3. Screening results of bacteriostatic activity of *M. micranth.*

Aerial part	Tested Bacteria strain	The diameter of inhibition zone (mm)				
		Methanol	Ethyl acetate	Acetone	chloroform	Petroleum ether
Leaf extraxt	*Ralstonia dolaanacearum*	13.6367±1.0883	14.9867±0.7396	16.3833±0.7121	19.3267±0.2785	-
	Xanthomonas oryzae pv. aryzae	6.29±0.8208	8.82±0.6894	9.0733±0.3484	12.4300±0.1504	1.0267±0.2689
Stem extract	*Ralstonia dolaanacearum*	4.08±0.1801	9.9133±0.7803	4.08±0.1801	9.3267±0.4160	7.1333±0.1770
	Xanthomonas oryzae pv. aryzae	6.29±0.8208	10.4667±1.1319	-	3.7500±0.942	-

The concentrations were 3000 mg/l.

found to be the most susceptible fungal pathogens for the leaves extract. EC_{50} values ranged from 1084.91 to 1269.52 mg/l after various time treatments. Thus, the data of bioassay demonstrated the order of susceptibility about fungal pathogens tested as *R. solani Kühn* > *P. aphanidermatum* > *F. solani* > *P. parasitica Destur*.

Bacteriostatic assay

The screening results of bacteriostatic assay is shown in Table 3. As seen, the results were similar to that of fungistasis assay; bacteriostatic activities of the leaves extracts were higher than the stem extracts. As parallel, the chloroform extracts observes the highest bacteriostatic activity. The results of bacteriostatic efficacy assay of the chloroform extract of leaves of *M. micrantha* are given in Table 4. It can be seen that the leaves extract possessed remarkable bacteriostatic activities against the bacteria tested. *Ralstonia dolaanacearum* showed more susceptibility than *Xanthomonas oryzae pv. Oryzae.* With 5000 mg/l of concentration, mean of diameters of inhibition zone were 19.3267 mm and 17.2833 mm respectively. But at 1250, 625 and 312 mg/l of concentration, the bacteriostatic efficacy reversed. Mean of diameters of inhibition zone of *X. oryzae pv. Oryzae* were 9.3533, 8.5000 and 3.5833 mm respectively. Mean of diameters of inhibition zone of *R. dolaanacearum* were 8.3867, 3.4000 and 0.5033 mm respectively. Further study is therefore needed to determine MIC of the leaves extract against the bacteria tested.

Table 4. Bacteriostatic efficacy of the leaves extract of *M. micrantha*.

Tested bacteria	Concentration (mg/l)	Diameter of inhibition zone (mm)	Confidence interval of 95%
Ralstonia dolaanacearum	5000	19.3267 ± 0.2785 a	18.1285-20.5248
	2500	12.4300 ± 0.1504 b	11.7827-13.0773
	1250	8.3867 ± 0.3835 c	6.7366-10.0368
	625	3.4000 ± 0.5853 d	0.8814-5.9168
Xanthomonas oryzae pv. *Oryzae*	5000	17.2833 ± 0.3269 a	15.8769-18.6897
	2500	12.3067 ± 0.6181 b	9.6472-14.9661
	1250	9.3533 ± 0.5139 c	8.6913-10.0154
	625	8.5000 ± 0.6650 c	5.6387-11.3613

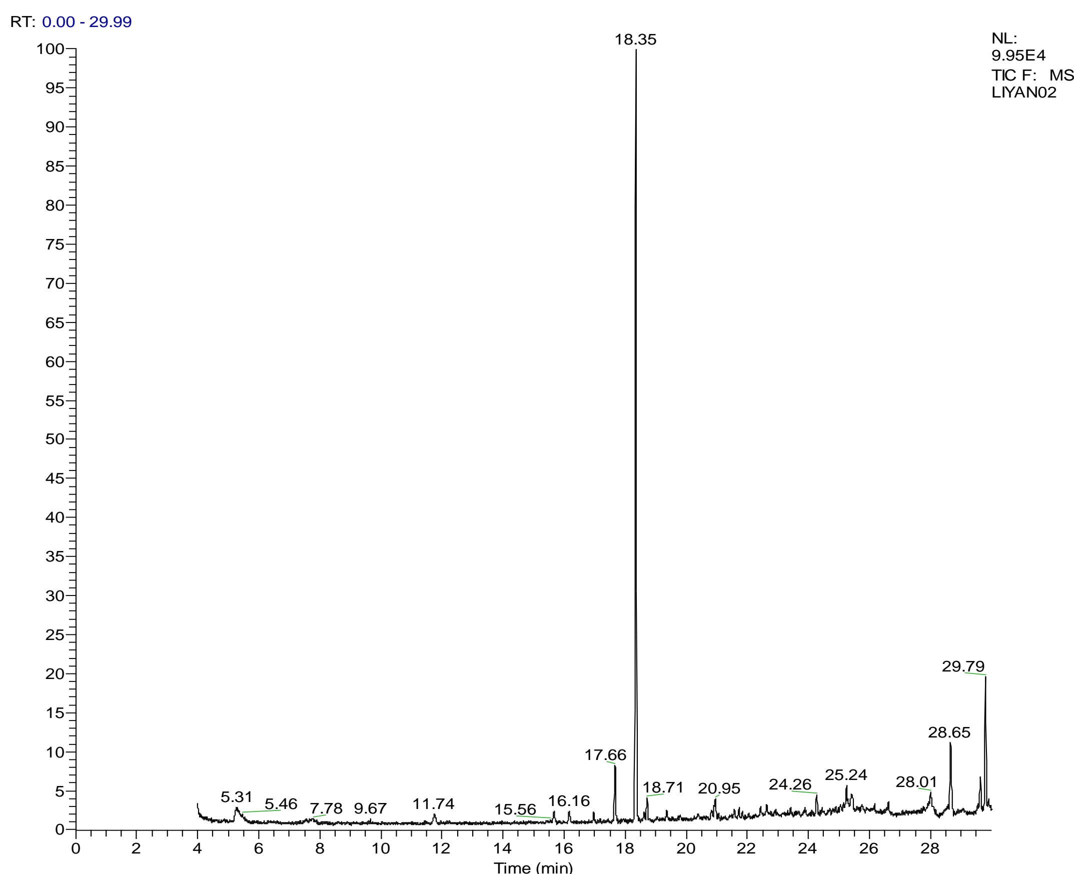

Figure 1. Total ion chromatogram of the leaves extract from *Mikania micrantha* at 100 µg/ml.

Determination of Minimum Inhibitory Concentration (MIC)

The chloroform extract of leaves of *M. micrantha* were subjected to quantitative bioassays for the determination of MIC. According to the results, MICs were found to be 250, and 500 mg/l, respectively. *R. dolaanacearum* was found to be more susceptible bacteric pathogens to the leaves extract. As the control and blank, the solvent and the sterile water did not affect the growth of the bacteria tested in this study. MICs for positive control were 250, and 250 mg/l, respectively. The result suggested that *M. micrantha* could be applied as antimicrobial agents.

GC-MS analysis of the extract

GC-MS picture of the chloroform extract of leaves of *M. micrantha* is shown in Figure 1. As a result of GC–MS analyses, 13 compounds were identified from the leaves extract, representing 87.03% of the total. The identified

Table 5. Chemical constituent analysis of the leaves extract of *M. micrantha*.

Retention time (min)	Chemical Composition	Chemical formulation	Relative content (area%)
5.31	2-butanamine	$C_4H_{11}N$	0.93
11.74	β-terpinene	$C_{10}H_{16}$	0.57
15.56	β-caryophylene	$C_{15}H_{24}$	0.69
16.16	Curcumene	$C_{15}H_{24}$	0.82
17.66	(1Z,6Z)-8-isopropyl-1-methylenecyclodeca-1, 6-diene	$C_{15}H_{24}$	4.82
18.35	1-(4-chlorophenyl)-2-hydroxyethanone	$C_8H_7ClO_2$	57.44
18.71	Benzoic acid, 4-chloro, methylester	$C_8H_7ClO_2$	1.52
20.95	Didodecyl phthalate	$C_{32}H_{54}O_4$	2.03
24.26	(3α',5Z,7E)-9,10-secocholesta-5,7,10 (19)-triene-3,24,25-triol	$C_{27}H_{44}O_3$	1.49
25.41	Trans-9-octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester	$C_{28}H_{44}O_4$	2.2
28.01	Cyclopropanetetradecanoic acid, 2-octyl-ester	$C_{26}H_{50}O_2$	3.66
28.65	2-(3-acetoxy-4,4,14-trime-thylandrost-8-en-17-yl)-propanoic acid	$C_{27}H_{42}O_4$	7.78
29.63	Stearic acid,3-(octadecyloxy) propyl ester	$C_{39}H_{78}O_3$	3.08

compounds are listed in Table 5 according to their elution order on the capillary column. The leaves extract contained a complex mixture consisting of halohydrocarbon (57.44%), sesquiterpene (6.9%), amine (0.93%), sterol (1.49%), esters (12.74%), carboxylic acid (7.78%). The major chemical constituent in the leaves extract were 1-(4-chlorophenyl)-2- hydroxyethanone (57.44%). Some studies (Yu et al., 2006; Hao et al., 2007; Zhuang et al., 2010) reported that the major chemical constituent of essential oil of *M. micrantha* were sesquiterpene and esters. No literature showed that the extract of *M. micrantha* contained halohydrocarbon as major compound. In addition, some peaks present in the chromatogram could not be identified in this study. It is not determine that 1-(4-chlorop-henyl)-2-hydroxyethan possess antimicrobial activity and nematicide activity. Further study will practice about the active ingredient of *M. micrantha*.

DISCUSSION

M. micrantha displayed the broadest range of antimicrobial activity against some plant pathogen (Zhuang et al., 2010; Hao et al., 2007). In this study, antimicrobial properties against some unreported some soil-borne plant pathogens and *Xanthomonas oryzae pv. Oryzae* were tested. The results further demonstrated the broadest range of antimicrobial properties of *M. micrantha*. In view of natural products with lower bioactivity compared with synthetics and the crude residue as experimental material, the values of EC_{50} were considered as rational datum. In addition, active compounds could not be identified in this study. With active compounds that will be isolating by bioactivity-guided fractionation, it is supposed that remarkable antimicrobial activity may be observed. Mikanolide has attracted the attention of natural products chemists because of its antimicrobial, antitumor, cytotoxic and phytotoxic activities (Ahmed et al., 2001; Aguinaldo et al., 1995; Valdes et al., 1998). It can be predicted that mikanolide will be isolated by bioactivity-guided fractionation against the plant pathogens tested in further study. The results suggested that *M. micrantha* could be applied as antimicrobial agents. In light of the result of this study for development of new products, further works is required to be done in order to evaluate the antimicrobial efficacy against various plant pathogens. If possible from collected specifically for controlling plant pathogen agent use, in order to manage indirectly and reduce adverse impact about *M. micrantha*.

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