Research Journal of
Phytochemistry

ISSN 1819-3471

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Phytochemistry 8 (3): 64-73, 2014
ISSN 1819-3471 / DOI: 10.3923/rjphyto.2014.64.73

Biofilm Killing Effects of *Chromolaena odorata* Extracts against *Pseudomonas aeruginosa*

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ABSTRACT

Chromolaena odorata is known to possess antimicrobial effects against a wide range of microorganisms including *Pseudomonas aeruginosa*. However, the inhibitory effects of *Chromolaena odorata* extracts against the biofilm growth mode of *Pseudomonas aeruginosa* remain uncertain. Therefore, this study was carried out to determine the antibiofilm activity of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* under aerobic and anaerobic conditions. Phytochemical screening using gas chromatography mass spectrometry revealed the major constituents in both *Chromolaena odorata* chloroform and ethanolic extracts, namely germacrene D. All microbial tests were carried out under aerobic and anaerobic conditions. Based on microbroth dilution assay performed, oxygen level did not show any effect towards the minimum inhibitory concentration and minimum bactericidal concentration of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa*. However, antibacterial susceptibility test showed that the size of inhibition zones of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* were slightly different between the aerobic and anaerobic conditions. Colony forming unit counting of biofilm cells demonstrated that *Chromolaena odorata* chloroform extract had greater antibiofilm activity against *Pseudomonas aeruginosa* as compared to *Chromolaena odorata* ethanolic extract under aerobic condition. In contrast,*Chromolaena odorata* ethanolic extract showed greater antibiofilm activity against *Pseudomonas aeruginosa* biofilm than *Chromolaena odorata* chloroform extract in the absence of oxygen. Furthermore, treatment of both *Chromolaena odorata* chloroform and ethanolic extracts resulted in changes in biochemical composition of *Pseudomonas aeruginosa* biofilm extracellular matrixes under both experimental conditions, as indicated by variation in the infrared spectra in the region between 1700 and 900 cm^{-1}. We conclude that the antibiofilm activities of *Chromolaena odorata* extracts depend on solvent extraction method and oxygen level. The findings from this study would improve the existing antimicrobial treatment plan to combat facultative anaerobic *Pseudomonas aeruginosa* biofilm.

Key words: Antibiofilm activity, *Chromolaena odorata, Pseudomonas aeruginosa*

INTRODUCTION

Biofilms are defined as microbial cells attached to a surface and protected from the attack of host immune system by Extracellular Kolymeric Substance (EPS). According to Lewis (2005), difficulty in combating biofilm-related diseases is normally attributed to the presence of persisters, which are the microbial cells that neither grow nor die in the presence of bactericidal agents. The formation of persisters is believed to be associated with overexpression of HipA gene product of

Toxin/Antitioxin (TA) module which shuts down many cellular functions thereby causing dormancy of persisters and protecting the cells from drug attack. One of the important biofilm producing bacteria is *Pseudomonas aeruginosa*. Infections produced by *Pseudomonas* include bacteremia, respiratory infection, urinary tract infection, osteomyelitis and infections of burns and soft tissues (Pierce, 2005). These infections are difficult to treat due to the high resistance of biofilms to antibiotic intervention (Lewis, 2001). Imipenem and piperacilllin treatments resulted in the expression of **β**-Galactosidease in *Pseudomonas aeruginosa* biofilm (Giwercman *et al*., 1999) which was possibly associated with over expression of multidrug resistance (MDR) protein pumps (Brooun *et al*., 2000), a group of integral protein pumps responsible to protect the cells by ejecting a variety of unwanted molecules. For many decades, there have been many medicinal plants tested to control *Pseudomonas aeruginosa* infections including *Chromoalaena odorata* (Asteraceae).

Chromolaena odorata (L.) R. M. King and H. Rob. is a topical flowering shrub. It is native to North America and has been introduced to South East Asia, West Africa and parts of Australia. The leaves are triangular and petioles are 1-4 cm long. In traditional medicine, leaf extract of *Chromolaena odorata* is used to treat cough and malaria infection in combination with lemon grass and guava leaves (Doss *et al*., 2011).*Chromolaena odorata* leaf extract also has antibacterial (Lavanya and Brahmaprakash, 2011) anti-inflammatory (Matsui *et al*., 2009) and wound healing activities (Kilani, 2006). Considering the facts that *Chromolaena odorata* extract possesses antibacterial activity and its inhibitory effects against microbial biofilms remains unknown, so there is a need to determine whether it can inhibit the *Pseudomonas aeruginosa* biofilm. Therefore, this study was conducted to determine the antibiofilm activity of chloroform and ethanolic extracts of *Chromolaena odorata* leaf on *Pseudomonas aeruginosa* biofilm under aerobic and anaerobic conditions.

MATERIALS AND METHODS

Preparation of extracts: Fresh leaves of *Chromolaena odorata* (L.) R. M. King and H. Rob. were collected from Puncak Alam, Selangor, Malaysia (Coordinates: 3°13' 42.30"N 101°25' 41.65"E). The taxonomic identity of the plant was authenticated by Dr Shamsul Khamis from Universiti Putra Malaysia. The leaves were washed with water and dried in oven for 48 h at 60°C. The dried leaves were ground into fine powder and soaked into absolute ethanol or absolute chloroform in a ratio of 1:10 (w v^{-1}) in water bath at 50°C for 72 h. The impurities were then filtered using muslin cloth. Rotary evaporator was used to remove excess solvents.

Phytochemical screening of plant extract: The plant extracts were analyzed using a Clarus 600 GC-MS system (Perkin Elmer, USA). The compounds were separated on 30 m×0.25 mm×0.25 μm Elite-5MS column and the column temperature was set from 40-220°C at a rate of 4°C min^{-1}; injector temperature, 250°C; injection volume, 1 μL; transfer temperature, 280°C. The peaks in chromatogram were identified based on library search using NIST and Wiley Registry 8 Edition.

Preparation of test microorganism: *Pseudomonas aeruginosa* ATCC 10145 was grown in nutrient broth for 24 h. The purity of bacterial culture was determined with light microscopy whilst bacterial growth pattern was determined spectrophotometrically at 600 nm. A 0.5 McFarland standard was used to prepare inoculum density of 1.5×10^8 CFU mL^{-1} in Phosphate Buffered Saline (PBS) pH 7.4 for all assays.

Determination of Minimum Inhibitory Concentration (MIC): Determination of MIC was performed by observing turbidity in the test samples. A volume of 100 µL of fresh *Pseudomonas aeruginosa* inoculum was loaded into five wells of 96-microplate in triplicates. A volume of 100 µL extracts was then inoculated into the wells of triplicates. Four-fold serial dilutions were started at 200 mg mL^{-1} of extract. Negative control well was filled with 100 of extracts and 100 µL of fresh broth. The procedures were performed in two different 96-microplates for two experimental conditions. The two 96-microtiter plates were incubated separately into aerobic and anaerobic conditions for 48 h at 37°C. The values of MIC were recorded at the lowest concentration which showed sign of turbidity.

Determination of Minimum Bactericidal Concentration (MBC): The bacterial suspension of three test concentrations greater than equal MIC were streaked on Mueller Hinton Agar (MHA) plates using sterile wire loop and incubated overnight at 37°C. The lowest concentration of the plant extracts which showed no growth of organism on plates after 24 h of incubation was considered as MBC.

Antimicrobial susceptibility test: Kirby Bauer Disc Diffusion method was used in this procedure. The turbidity of overnight *Pseudomonas aeruginosa* ATCC 10145 culture was standardized to 0.5 McFarland standards. A volume of 10 µL of *P. aeruginosa* culture was streaked evenly on MHA plates using hockey stick spreader. The plates were allowed to dry slightly. Using sterile forceps, sterile 6 mm discs were submerged in 20 µL of freshly prepared test concentration of 200, 50, 12.5 and 3.13 mg mL^{-1}. Ciprofloxacin (10 µg) and DMSO were used as positive and negative control respectively. The discs were laid and pressed gently on the plates. The plates were allowed to stand about 30 min to allow diffusion of extract. The plates were then incubated separately into aerobic condition and anaerobic condition for 24 h at 37°C. Anaerobic jar was used to simulate anaerobic condition. The antimicrobial activity of the plant extracts was determined by measuring diameter of inhibition zone around the respective discs.

Microplate biofilm formation assay: A volume of 100 µL of fresh *Pseudomonas aeruginosa* inoculum was loaded into five wells of 96-microtiter plate in triplicates. A volume of 100 µL extracts was then inoculated into the wells in triplicates. The chloroform and ethanolic extracts were tested at 200, 50, 12.5 and 3.13 mg mL^{-1}. Wells of control contained 200 µL of fresh broth. The procedures were performed in two different 96-microplates for two experimental conditions. The two 96-microtiter plates were incubated separately into aerobic and anaerobic conditions for 48 h at 37°C. At 48 h post inoculation, the media containing planktonic cells in each well was discarded. All wells were then rinsed 2x with 100 µL of PBS pH 7.4. Biofilm cells were detached by pipetting action and suspended in the PBS buffer. The biofilm suspension was then used for Colony Forming Unit (CFU) counting and attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR).

Colony Forming Unit (CFU) counting: The biofilm suspension was obtained from the microplate biofilm formation assay. Dilution of biofilm fraction was made at a ratio of $1:10^6$. A volume of 10 µL of the biofilm suspension was streaked on the sterile Muller Hilton agar. The plates were then incubated for 24 h at 37°C. The viable cells were counted to obtain CFU mL^{-1}.

Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR): ATR-FTIR analyses were performed using Thermo Scientific NICOLET 6700 FT-IR. The biofilm

suspension was obtained from the microplate biofilm formation assay and placed in direct contact with horizontal attenuated total reflectance (ZnSe crystal) at controlled ambient temperature. The infrared (IR) spectra were scanned in the region between 4000-400 cm^{-1}.

RESULTS

Phytochemical constituents: The phytochemical constituents in *Chromolaena odorata* extract were separated according to their chemical properties which were then ionized, fragmented and detected by mass spectrometer based on their mass to charge ratio. The phytochemical constituents identified in *Chromolaena odorata* chloroform and ethanolic extracts are listed in Table 1 and 2, respectively. There were 7 and 10 phytochemical constituents identified in *Chromolaena odorata* chloroform and ethanolic extracts, respectively. The retention time for *Chromolaena odorata* chloroform extract constituents ranged from 20.83 to 26.89 whilst the retention time for *Chromolaena odorata* ethanolic extract constituents ranged from 22.3 to 42.73. The major constituent for both *Chromolaena odorata* chloroform and ethanolic extracts was germacrene D. In comparison between *Chromolaena odorata* chloroform and ethanolic extracts, it seems that the choice of solvent in the extraction may have an effect on the phytochemical profile of *Chromolaena odorata* extracts. This could be seen on the variation in type and abundance of phytochemical consitutents.

Test concentrations and zone of inhibition: The determination of MIC and MBC of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* was performed under aerobic and anaerobic conditions. Consideration of two conditions with varying oxygen level was made because *Pseudomonas aeruginosa* is a facultative anaerob which can survive metabolically in the presence and absence of oxygen. Table 3 denotes the MIC and MBC values which were recorded from the microbroth dilution assay. Both *Chromolaena odorata* chloroform and ethanolic extracts exhibited antibacterial activities against *Pseudomonas aeruginosa* under aerobic and anaerobic conditions. The MIC value for both *Chromolaena odorata* chloroform and ethanolic extracts was

Table 1: Phytochemical constituents of *Chromolaena odorata* chloroform extract as identified by GC-MS

RT	Area (%)	Compound	Quality value	Common name
23.69	8.37	Caryophyllene	98	β-Caryophellene
25.61	25.95	1,6-cyclodecadiene, 1-methyl-5-methlene-8-(1-methyethyl)	99	Germacrene D
26.89	7.43	Naphthalene, 1, 2, 4a, 5, 8, 8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl)	90	β-Cadinene

Table 2: Phytochemical constituents of *Chromolaena odorata* ethanolic extract as identified by GC-MS

RT	Area (%)	Compound	Quality value	Common name
22.30	6.44	Copaene	98	Copaene
23.68	17.30	Caryophyllene	99	Caryophyllene
24.75	4.22	1, 4, 7, -Cycloundecatriene, 1, 5, 9, 9-tetramethyl-, z, z, z	98	-
25.47	2.96	Naphthalene, 1, 2, 3, 4, 4a, 5, 6, 8a-octahydro-7-methyl-4-methylene-1-1 (1-methylethyl)	99	γ-Cadinene
25.61	41.19	1,6-Cyclodecadiene, 1-methyl-5 methylene-8-(1-methylethyl)	96	Germacrene D
26.07	3.99	1-Isopropenyl-2-methylcyclohexane	86	o-Menth-8-ene
26.20	0.52	Naphthalene, 1, 2, 4a, 5, 8, 8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl)	93	β-Cadinene
26.89	14.47	Naphthalene, 1, 2, 3, 5, 6, 8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl)	95	δ-Cadinene
38.64	4.72	n-Hexadecanoic acid	87	Palmitic acid
42.73	4.32	9, 12, 15-octadecatriene-1-ol (z, z, z)	91	-

Table 3: Minimum inhibitory concentration and minimum bactericidal concentration of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa*

Parameters	Conditions	Chloroform extract (mg mL^{-1})	Ethanolic extract (mg mL^{-1})
MIC	Aerobic	3.13	3.13
	Anaerobic	3.13	3.13
MBC	Aerobic	6.25	6.25
	Anaerobic	6.25	6.25

Table 4: Antibacterial activity of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* under aerobic condition, n = 5

	Inhibition zone (mm)	
Concentration (mg mL^{-1})	Chloroform	Ethanol
200	10.00±0.7	10.00±1.0
50	9.67±1.2	9.70±0.6
12.5	10.67±0.6	6.80±0.3
3.13	7.00±1.0	6.50±0.5
Positive	33.67±0.3	23.00±1.0
Negative	0.00	0.00

Table 5: Antibacterial activity of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* under anaerobic condition, n = 5

	Inhibition zone (mm)	
Concentration (mg mL^{-1})	Chloroform	Ethanol
200	13.00±0.7	9.30±0.6
50	10.33±0.3	7.50±1.3
12.5	10.33±0.5	6.70±0.3
3.13	10.33±0.4	6.50±0.5
Positive	34.43±0.5	24.70±0.6
Negative	0.00	0.00

Table 6: Antibiofilm activity of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* biofilm under aerobic condition, n = 3

	CFU mL^{-1}	
Concentration (mg mL^{-1})	Chloroform	Ethanol
200	73.3±5.51×10^{8}	0.00
50	124±4.93×10^{8}	246±5.51×10^{8}
12.5	162±5.03×10^{8}	376±16.65×10^{8}
3.13	257±8.88×10^{8}	458±21.38×10^{8}
Control	281±6.08×10^{8}	502±13.01×10^{8}

Table 7: Antibiofilm activity of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* biofilm under anaerobic condition, n =3

	CFU mL^{-1}	
Concentration (mg mL^{-1})	Chloroform	Ethanol
200	74.6±5.68×10^{8}	10±1.15×10^{8}
50	135±7.64×10^{8}	188±5.51×10^{8}
12.5	193±7.02×10^{8}	278±16.65×10^{8}
0.13	230±8.54×10^{8}	389±21.38×10^{8}
Control	266±5.86×10^{8}	529±13.01×10^{8}

Table 8: ATR-FTIR analysis of *Pseudomonas aeruginosa* biofilm extracellular matrix under aerobic condition

			Chloroform extract (mg mL^{-1})				Ethanolic extract (mg mL^{-1})			
Biomolecules	Wave number (cm^{-1})	Functional groups	200	50	12.5	3.13	200	50	12.5	3.13
Polysaccharide	1300-900	C-O stretch (1067 cm^{-1})	+	+	+	+	+	+	+	+
Nucleic acids	1300-900	Phosphodiester linkage (1185 cm^{-1})	+	+	+	+	+	+	+	+
Proteins	1700-1500	Amide I (1642 cm^{-1}); Amide II (1544 cm^{-1})	+	-	+	+	+	+	+	+

+,-: Altered and unaltered IR spectra, respectively in comparision to *Chromolaena odorata*-treated and untreated biofilm. The changes in spectral data are indicated by distinct No. and intensity of absorbance peaks in the IR spectra

Table 9: ATR-FTIR analysis of *Pseudomonas aeruginosa* biofilm extracellular matrix under anaerobic condition

			Chloroform extract (mg mL^{-1})				Ethanolic extract (mg mL^{-1})			
Biomolecules	Wave number (cm^{-1})	Functional groups	200	50	12.5	3.13	200	50	12.5	3.13
Polysaccharide	1300-900	C-O stretch (1067 cm^{-1})	+	-	-	+	+	+	+	+
Nucleic acids	1300-900	Phosphodiester linkage (1185 cm^{-1})	+	-	-	+	+	+	+	+
Proteins	1700-1500	Amide I (1642 cm^{-1}); Amide II (1544 cm^{-1})	-	+	+	+	+	+	+	+

+,-: Altered and unaltered IR spectra, respectively in comparision to *Chromolaena odorata*-treated and untreated biofilm. The changes in spectral data are indicated by distinct No. and intensity of absorbance peaks in the IR spectra

3.13 mg mL^{-1} regardless of the oxygen level. Meanwhile, the MBC value for both *Chromolaena odorata* chloroform and ethanolic extracts was 6.25 mg mL^{-1} under both experimental conditions. Collectively, we suggest that both MIC and MBC values of *Chromolaena odorata* extracts are not dependent on oxygen level and the choice of organic solvent.

The antibacterial activity of *Chromolaena odorata* extracts were also tested using disc diffusion assay at several test concentrations ranging from 3.13-200 mg mL^{-1}. The diameter of inhibition zone (mm) measured from the disc diffusion assay under aerobic and anaerobic conditions are presented in Table 4 and 5, respectively. It was observed that both *Chromolaena odorata* chloroform and ethanolic extracts exhibited moderate inhibitory effect against *Pseudomonas aeruginosa* at several test concentrations. Under aerobic conditions, the inhibition zone of *Chromolaena odorata* chloroform extract ranged from 7.0±1.0 mm to 10.67±0.6 mm whilst inhibition zone of *Chromolaena odorata* ethanolic extract ranged from 6.50±0.5 mm to 10.00±1.0 mm. This indicates that there is no apparent disparity of antibacterial potential between *Chromolaena odorata* chloroform and ethanolic extracts under aerobic condition. Meanwhile, anaerobic antibacterial test revealed that the inhibition zone of *Chromolaena odorata* chloroform extract ranged from 10.33±0.4 mm to 13.00±0.7 mm whilst the inhibition zone of *Chromolaena odorata* ethanolic extract ranged from 6.50±0.5 mm to 9.30±0.6 mm. It is noted that *Chromolaena odorata* chloroform extract had higher antibacterial potential than Chromolaena odorata ethanolic extract under anaerobic condition. Furthermore,*Chromolaena odorata* chloroform extract was also found to be more effective under anaerobic condition. Taken together, the inhibition zone of *Chromolaena odorata* chloroform extract is likely to be dependent on oxygen level while *Chromolaena odorata* ethanolic extract is not.

Viable biofilm cells: The inhibitory effect of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* biofilm was evaluated using microplate biofilm assay system. Similar to

disc diffusion assay, this assay system was also carried out under both aerobic and anaerobic conditions. Table 6 and 7 shows antibiofilm activity of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* biofilm under aerobic and anaerobic, respectively. It was revealed that both *Chromolaena odorata* chloroform and ethanolic extracts exhibited strong inhibitory effect against *Pseudomonas aeruginosa* biofilm at several test concentrations. Under aerobic condition, all test concentrations of *Chromolaena odorata* chloroform extract (from 73.3±5.51×10^8 to 257±8.88×10^8) displayed lower CFU count than that of control (281±6.08×10^8). All test concentrations (from 0 to 458±21.38×10^8) of *Chromolaena odorata* ethanolic extract also exhibited lower CFU count than that of control (502±13.01×10^8). In particular, the test concentrations at 50 and 200 mg mL^{-1} of both *Chromolaena odorata* extracts are likely to be very effective against *Pseudomonas aeruginosa* biofilm since more than 50% of biofilm inhibition is observed. Higher concentration of *Chromolaena odorata* extracts results in greater biofilm inhibition indicating that the antibiofilm effect of *Chromolaena odorata* extracts under aerobic condition is concentration-dependent. Based on the antibiofilm assay under anaerobic condition, all test concentrations of *Chromolaena odorata* chloroform (from 74.6±5.68×10^8 to 230±8.54×10^8) and ethanolic (from 10±1.15×10^8 to 389±21.38×10^8) extracts displayed lower CFU count than their control (266±5.86×10^8 and 529±13.01×10^8 respectively). In the absence of oxygen, higher concentration of *Chromolaena odorata* extracts also resulted in greater biofilm inhibition. Based on all findings from the microplate biofilm assay performed, it could be inferred that the concentration-dependent antibiofilm activity of *Chromolaena odorata* extracts is constant under both aerobic and anaerobic conditions.

Biochemical composition of biofilm extracellular matrix: In this study, the biofilm suspension was prepared from the microplate assay system by removal of media-containing planktonic cells, pipetting and centrifugation. The biofilm suspension was then subjected to ATR-FTIR analysis. The composition of biomolecules in the biofilm extracellular matrix such as polysaccharide, nucleic acids and proteins were determined based on absorbance peak in the infrared (IR) spectra ranging from 1700 to 900 cm^{-1} (Jiao *et al*., 2010). The spectral region between 1300 and 900 cm^{-1} includes C-O stretch and phophodiester linkages while the spectral region between 1700 and 1500 cm^{-1} includes amide I and amide II. The effect of *Chromolaena odorata* extracts on the IR spectra of *Pseudomonas aeruginosa* biofilm extracellular matrix under aerobic and anaerobic conditions is shown in Table 8 and 9, respectively. Under aerobic condition, changes in biochemical composition of *Pseudomonas aeruginosa* biofilm extracellular matrix following treatment of *Chromolaena odorata* extracts were observed. The treatment of *Chromolaena odorata* chloroform extract altered the IR spectra of biofilm extracellular matrix at all test concentrations except 50 mg mL^{-1} while treatment of *Chromolaena odorata* ethanolic extract resulted in changes of IR spectra of biofilm extracellular matrix at all test concentrations. Moreover, the effect of *Chromolaena odorata* chloroform extract on the biochemical composition was found to be not consistent under anaerobic condition. In particular, at test concentrations of 200, 12.5 and 3.13 mg mL^{-1}, the treatment of *Chromolaena odorata* chloroform extract did not alter IR spectra of biofilm extracellular matrix with respect to composition of protein, polysaccharide and nucleic acids. In addition, treatment of *Chromolaena odorata* ethanolic extract altered the IR spectra of biofilm extracellular matrix at all test concentrations under anaerobic condition. In comparison between all the IR spectra of treated biofilm extracellular matrixes, we propose that *Chromolaena*

odorata ethanolic extract has produced greater effect on the biochemical composition of *Pseudomonas aeruginosa* biofilm extracellular matrix than *Chromolaena odorata* chloroform extract.

DISCUSSION

Felicien *et al.* (2012) studied the chemical composition and biological activity of essential oil from *Chromolaena odorata* leaves using GCMS and disc diffusion assay, respectively. They revealed the presence of germacrene D 9.7, δ-cadinene 0.6 and δ-cadinene 3.7% in the essential oil while its major constituent was á-pinene (20.7%). The MIC values of the *Chromolaena odorata* essential oil against some microorganisms were lower than 6 mg mL^{-1}. Chemically, both germacrene D and cadinine are sesquiterpene compounds possessing antimicrobial activities as previously reported (Kundu *et al.*, 2013; Jovanovic *et al.*, 2005). Hence, based on the GCMS data, we strongly believe that germacrene D is the major antimicrobial constituent in *Chromolaena odorata* extracts. Meanwhile, the properties of solvent significantly affected the total phytochemical consitutents (approximately 25% variation) and biological activity (up to 30% variation) (Michiels *et al.*, 2012). Taken together, we suggest that the disparities in the contexts as follows: (a) Comparison between chloroform and ethanolic extracts of *Chromolaena odorata* (b) Comparison between solvent extracts and hydrodistillated essential oil of *Chromolaena odorata* and (c) Different localities (Puncak Alam, Malaysia and Godomey, Benin) to collect *Chromolaena odorata* leaves, contribute to the variation observed in the phytochemical profiles of *Chromolaena odorata*.

In the context of antibacterial activity of plant species under both aerobic and anaerobic conditions, Yahya *et al.* (2013) reported the effects of oxygen level on the antibacterial activity of Zingiber officinale extract against *Pseudomonas aeruginosa*. The pivotal role of oxygen in antibacterial action has also been shown by Suller and Lloyd (2002). Based on flow cytometric data and CFU number, the authors reported that the inhibitory effect of vancomycin antibiotics against *Staphylococcus aereus* under aerobic condition was greater than that of anaerobic condition although its MIC value was similar in both environments. In relation to these, we strongly believe that the altered oxygen level has resulted in changes in bacterial physiology thereby affecting susceptibility towards treatment by *Chromolaena odorata* extracts. Moreover, the oxygen-dependent antibacterial potential may vary between one plant species to another.

In comparison with our findings from the disc diffusion assay,*Chromolaena odorata* extracts seems to be more effective against *Pseudomonas aeruginosa* biofilm cells than their planktonic counterparts, which contradicts the general assumption that the planktonic cells are more susceptible to the antimicrobial treatments. Our finding is in agreement with Spoering and Lewis (2001) which reported the greater inhibitory effect of antibiotic against biofilm than stationary planktonic cells. They demonstrated that carbenicillin killed only rapidly growing cells whilst both ofloxacin and tobramycin were effective against the non-growing cells from the stationary planktonic and biofilm growth modes. Surprisingly, the stationary planktonic cells appeared to produce more persisters and were relatively more resistant than the biofilm cells leading to a conclusion that, the higher density of stationary planktonic cells than biofilm cells promoted greater persister formation. In conjunction with that, it is likely that the bacterial inoculums used for the disc diffusion assay herein are planktonic cells derived from the stationary phase which exhibit greater resistance towards *Chromolaena odorata* extracts and influenced the antimicrobial property measured by the disc diffusion assay.

Recently, Namasivayam and Roy (2013) investigated the antibiofilm potential of several medicinal plants from India. In that study, the extracts from *Azadirachta indica*, *Vitex negundu*, *Tridax procumbens* and *Ocimum tenuiflorumi* were coated with biocompatible polymer chitosan. By using the standard calorimetric methods, it was revealed that both free and chitosan coated plant extracts inhibited biofilm formation and also reduced total carbohydrates and total protein of biofilm extracellular matrix. This indicates that the changes in our spectral data resulting from treatment of *Chromolaena odorata* extracts are in agreement with Namasivayam and Roy (2013). According to Baillie and Douglas (2000), reduction in the biochemical composition of the biofilm extracellular matrix leads to weakening of the biofilm thus facilitating the entry of the drugs. Therefore, the biochemical composition of biofilm extracellular matrix can also be used as an index of inhibition. Moreover, the extracellular matrix of *Pseudomonas aeruginosa* biofilm has also been extensively investigated by Nivens *et al.* (2001) using ATR-FTIR in order to understand the role of alginate and its O-acetylation in the formation of microcolonies and biofilms. They revealed that the biofilms absorbed IR radiation near 1,650 cm^{-1} (amide I), 1,550 cm^{-1} (amide II), 1,240 cm^{-1} (PAO stretching, COOOC stretching and/or amide III vibrations), 1,100 to 1,000 cm^{-1} (COOH and POO stretching), 1,060 cm^{-1} (COOH stretching of alginate) and 1,250 cm^{-1} (COO stretching of the O-acetyl group in alginate). Their findings demonstrated that, alginate, although not required for *Pseudomonas aeruginosa* biofilm development, plays a role in the biofilm structure and may act as intercellular material, required for formation of thicker three-dimensional biofilms. Based on the collective findings, we suggest that the treatment of *Chromolaena odorata* extracts has caused the alterations in biochemical composition of *Pseudomonas aeruginosa* biofilm extracellular matrix which lead to the killing of biofilm cells.

CONCLUSION

The major phytochemical constituent in *Chromolaena odorata* extracts is germacrene D. Its abundance is proposed to be important for antibiofilm activity of *Chromolaena odorata* extracts. In parallel with several previous studies, we also recommend that both CFU count and IR spectra could be useful biofilm inhibition indexes for antibiofilm study of medicinal plants. The findings from this study have led us to conclude that, the antibiofilm activity of *Chromolaena odorata* extracts against *Pseudomonas aeruginosa* is dependent on solvent extraction method and oxygen level.

ACKNOWLEDGEMENT

This study was supported by Research Management Institute, UiTM and Malaysian Ministry of Higher Education under research grants, 600-RMI/DANA 5/3/RIF (54/2012) and 600-RMI/RAGS 5/3 (11/2012), respectively.

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