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Antibacterials and Phytochemicals Investigations of *Chromolaena odorata* (l.f) King and Robinson (Asteraceae) from Sabah, Malaysia

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ABSTRACT

Antimicrobial properties and phytochemical constituents in leaf extract of *Chromolaena odorata* was evaluated in this study. *C. odorata* leaves were subjected to liquid-liquid extraction by using methanol, hexane, ethyl acetate, chloroform, buthanol and water. All extract partitions were tested for antibacterial activity against five Gram-positive and Gram-negative bacteria by using disc diffusion method. Crude methanolic extract (CME), ethyl acetate extract (EAE) and chloroform extract (CE) showed good antibacterial properties against the tested bacterial strains. However, only the CE was further separated using silica column chromatography. About 10 semi purified fractions was obtained and fraction 2 (F2) showed consistent inhibitory zones against all bacterial tested. Phytochemical investigations on the extract partitions and fractions showed the presence of alkaloids, flavonoids, tannins, polyphenols, saponins and triterpenoids. Fraction F2 was subjected to GC-MS analysis to characterise the bioactive compounds. The GC-MS spectral data has identified 10 major compounds which are hexachloro-ethane, n-nonylaldehyde, methyl-4-oxooctanoate, longiverbenone, 2-butenal,2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl), neophytadiene, phytol, dihydro-neoclovene, 2,6-ditert-butylquinone and aromadendrene

INTRODUCTION

Antibiotic resistance that commonly being the major problems in bacterial infections had led to the increasing demands of new antibacterial agents. Potential agents with diverse bioactive compounds involved in novel mechanisms which actively combat either new or re-emerging infections is now in urgency [1]. Current interest in biocides had led to emergence of various new studies. Phytochemicals constituents in plants had been continuously correlated with its pharmacological activities [2]. Traditional healing properties of plants had been explored and scientific research had been gradually taking place. Asteraceae family had been reported to contain various classes of secondary metabolites. This family were reported to have phototoxic compounds such as polyacetylenes and thiophenes which contribute in plant defence mechanisms [3]. Chromolaena genera in which belongs to Asteraceae family; is also one of the common medicinal plants used by Malaysian folks. The leaf of *C. odorata* is used for burns, wounds and skin infections [4]. However, scientific research on biological properties of locally collected *C. odorata* in Sabah, Malaysia is limited. Thus, this research was carried out to explore hidden potential of *C. odorata* as therapeutics plants for pharmaceuticals industry. The aim in the present study was to evaluate the antimicrobial activities and to elucidate phytochemicals compositions of *C. odorata*.

MATERIALS AND METHODS

Plant material

The plant materials were collected around Sabah, Malaysia in September 2010. The plant was authenticated and deposited in the BORNEENSIS, Institute of Tropical Biology and Conservation (ITBC), University Malaysia Sabah (BORH number 0962). Plant leaves were washed thoroughly with tap water, air dried, powdered, weighed and stored in air-tight containers and being stored at room temperature for the test.

Extraction

Powdered leaves of the sample were successively refluxed with methanol with the ratio 1:10 between the samples weight and the solvents. The extracts obtained then were subsequently referred to as crude methanolic extract (CME).

Liquid-liquid extraction

The CME then being furthered separated by using liquid-liquid extraction methods adopted from Harborne (1998) with a slight modification. Six different extracts samples with different polarity were obtained; they are Hexane Extract (HE), Ethyl Acetate Extract (EAE), Chloroform Extract (CE), Chlroform:Methanol (3:1) Extract (CME), Buthanol Extract (BE) and Aquoeus Extract (AE). All extracts were evaporated in vacuo at 450C using rotary evaporator (Heidolph, Germany). Residues were dissolved in respective solvents at 100mg/ml. Solvents (analytical grade) for extraction were obtained from Fisher, USA.

Column chromatographic separation

Chloroform extract (CE) of *C. odorata* was gradiently eluted on silica gel column chromatography (Merck, 0.040-0.063mm, 230-400 mesh). Mobile phase chosen for *C. odorata* is absolute chloroform (99.8%, v/v). There are 10 semi purified fractions were collected and coded as F1 to F10.

Test organisms

The strains of bacteria used in this research were *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumonia* and *Salmonella typhii* which obtained from the Biotechnology programme, School of Science and Technology, University Malaysia Sabah. The bacteria were maintained by continuously subcultured on Nutrient Agar (NA) media for 1 day at 370C. Cultures then were kept at 40C.

Antibacterial susceptibility test

The antibacterial test was conducted based on the paper disc diffusion test technique [5]. Sterile Whatman paper no.3 was impregnated with approximately 20ul of sample. The dried paper discs containing the extracts were placed on the sterile NA media. The plates then were incubated for 1 day at 370C. Observations were carried out based on the diameter of the inhibition zones (mm) on the media. Ampicillin (0.25ug/ul) was used as the positive control while the extraction solvents were used as negative control. All tests were performed in triplicates, and the mean with standard deviation of the inhibition zones are recorded.

Phytochemical screening

All extracts were subjected to phytochemicals test using standard technique [6,7]. The Wagner's test was carried out for alkaloids; foaming test for saponins; Wilstatter-Sianidin test, Batesmith test and Metcalf test for flavonoids, Gelatin test and ferric chloride test were carried out for tannins and polyphenols test, and Salkowski test for triterpenoids.

GC-MS analysis

The Gas Chromatography-Mass Spectroscopy analysis was carried out on a GC-MS (Model; QP 2010 series, Shimadzu, Tokyo, Japan) equipped with a ZB-5ms fused silica capillary column (30m x 0.25mm x 0.25µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as carrier gas at constant flow rate of 1.23 ml/min. Injector temperature was maintained at 2800C; 300˚C interface temperature and 150˚C ion source temperature. The column temperature was initially held at 600C for 0.5 minute, and then the temperature was raised to 1800C at a rate of 100C per min, from 1800C to 3000C at a rate of 50C per min and held for 10 min for a total of 60 minute running time. About 2 µl of the diluted sample was injected in the split less mode; with mass scan from m/z 45-850 amu; interval of 0.5 s. Interpretation on the mass spectrum GC-MS was assigned by the comparisons of their retention indices and mass spectra fragmentation patterns with those stored on the computer library. National Institute of Standards Technology (NIST21.LIB) and Wiley Registry of Mass Spectral Data's (WILLEY229.LIB) library sources were used for matching the identified compounds from the plant extract.

RESULTS AND DISCUSSION

Antibacterial activity

Table 1 shows the antimicrobial activity of *Chromolaena odorata*. Crude methanolic extract (CME) shows activities against *P. aeruginosa, S. aureus* and *S. pneumonia* with inhibitory zones 7.67±0.58mm, 7.33±0.58mm and 8.00±1.00mm; respectively. Ethyl acetate extract (EAE) also shows potential antimicrobial activity against *P. aeruginosa* (8.33±1.53mm), *S. aureus* (8.00±.000mm) and *S. pneumonia* (8.50±1.50mm). No significant activities were observed on Hexane extract (HE), Chloroform-methanol extract (C:ME), Buthanol extract (BE) and Aqueous extract (AE).

Table 1. Antibacterial activities of extracts obtained from *C. odorata*.

Extracts	Inhibition zones (mm) Gram Negative Bacteria			Gram Positive Bacteria	
	E. coli	*P. aeruginosa*	*S. typhii*	*S. aureus*	*S. pneumonia*
Control positive					
Ampicillin	13.21±0.81	10.43±0.50	16.71±0.70	28.25±1.4	32.67±6.43
CME	-	7.67±0.58	-	7.33±0.58	8.00±1.00
HE	-	-	-	-	-
EAE	-	8.33±1.53	-	8.00±.000	8.50±1.50
CE	-	-	-	7.00±0.00	8.00±0.00
F1	-	7.00±0.00	-	7.50±0.50	7.00±0.00
F2	8.00±0.00	10.67±0.58	8.00±0.00	10.33±0.58	11.67±2.08
F3	-	7.67±1.15	-	7.67±1.15	7.33±0.58
F4	-	9.00±1.00	-	8.00±0.00	8.67±1.15
F5	-	10.00±0.00	-	8.00±0.00	10.00±0.00
F6	-	8.00±0.00	-	7.67±0.58	8.00±1.00
F7	-	8.00±0.00	-	7.67±0.58	8.00±1.00
F8	-	7.00±0.00	-	7.50±0.50	8.00±0.00
F9	-	7.50±0.50	-	7.50±0.50	9.00±0.00
F10	-	8.00±0.00	-	9.00±0.00	7.00±0.00
C:ME	-	-	-	-	-
BE	-	-	-	-	-
AE	-	-	-	-	-

Notes: ME=Crude methanolic extract, HE=Hexane extract, EAE=Etnylacetate extract, CE=Chloroform extract, CME=(chloroform:methanol) extract, BE=Buthanol extract, AE=Aqueous extract, F1-F10=CC fractions of C. odorata
(-) = No activity

Chloroform extract (CE) however only showed weak inhibitory zones against gram positive bacteria *S. aureus* (7.00±.000mm) and *S. pneumonia* (7.00±.000mm). However, CE fractions (F1-F10) exhibited inhibitory zones against *P. aeruginosa*, *S. aureus* and *S. pneumonia* with the biggest inhibitions' zones were observed from F2 (10.67±0.58mm, 10.33±0.58mm and 11.67±2.08mm respectively). In addition, F2 also found to be weakly inhibited both E. coli and S. typhii (8.00±0.00mm). Column chromatography fraction of *C. odorata* (F2) was able to constantly inhibit all gram positive and Gram negative bacteria. This finding is in agreement with previous research by Sukanya et al. [1] and Vital and Rivera, [8].

Phytochemicals analysis

Crude methanolic extracts (CME) show positive result in Wagner test, Gelatin and $FeCl_3$ test, Foam test and Salkowski test. Furthermore, both Hexane extract (HE) and Chloroform extract (CE) found out to contain tannin, polyphenol, saponin and triterpenoids. Ethyl acetate extract (EAE) only positive during FeCl3 test, Foam test and Salkowski test. However, Chloroform-methanol extract (C:ME) and Butanol extract (BE) were found to have saponin while the Aqueous extract (AE) were contained

alkaloid and saponin. CE fractions (F1 to F10) were found to be positive for foam test. Other than that, tannin was also found in F2, F3, F4 and F5; polyphenol in F2 and F10, and triterpenoids in F2, F3, F4, F5 and F6 (**Table 2**). Unexpectedly, flavonoid was detected in F10 may due to the enough concentration to be detected qualitatively. *C. odorata* has reported rich with phytochemicals such as terpenoids, alkaloids, tannins and other phenolic compounds [9,10] but little content with flavonoid [11] which is obviously matched with the results obtained in this study.

Table 2. Phytochemicals constituents of *C. odorata*.

Extract		Alkaloid test	Flavonoid test			Tannin and polyphenol test		Saponin test	Triterp-enoids test
		Wagner test (formation of cloudy sediment)	Wilstatter-Sianidin test	Bates-mith test	Metcalf test	Gelatin test	$FeCl_3$ test	Foam test (format-ion of bee dane)	Salko-wski test
CME		(+++)	-	-	-	(++)	(++++)	(+++)	(+)
HE		-	-	-	-	(++)	(+)	(++)	(++)
EAE		-	-	-	-	-	(++++)	(++++)	(++)
CE		-	-	-	-	(++)	(+++)	(+++++)	(+)
C:ME		-	-	-	-	-	-	(+)	-
BE		-	-	-	-	-	-	(+++++)	-
AE		(++)	-	-	-	-	-	(+++)	-
CE	F1	-	-	-	-	(+++)	(++++)	(++)	(++)
	F2	-	-	-	-	(++)	-	(+++++)	(++)
	F3	-	-	-	-	(++++)	-	(+++)	(++)
	F4	-	-	-	-	(+)	-	(+++++)	(++)
	F5	-	-	-	-	-	-	(+++++)	(++)
	F6	-	-	-	-	-	-	(++)	(++)
	F7	-	-	-	-	-	-	(+++)	-
	F8	-	-	-	-	-	-	(+++++)	-
	F9	-	-	-	-	-	(++)	(++++)	-
	F10	-	(+++)	-	-	-	-	(++)	-

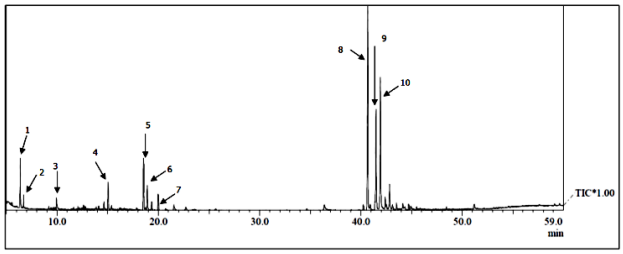
Notes: CME=Crude Methanolic Extract, HE=Hexane Extract, EAE=EtnylAcetate Extract, CE=Chloroform Extract, CME=(Chloroform:Methanol) Extract, BE=Buthanol Extract, AE=Aqueous Extract. Score: (+++++)= copiously present, (++++)= present, (+++)= moderately present, (++)=present, (+)=weekly present, (-)= No activity. UMS71= *Chromolaena odorata,* UMS91= *Mikania micarantha*

GC-MS analysis

The GC-MS analysis of fraction F2 (**Fig. 1**) revealed the presence of 10 major phytocompounds; hexachloro-ethane, n-nonylaldehyde, methyl-4-oxooctanoate, longiverbenone, 2-butenal,2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl), neophytadiene, phytol, dihydro-neoclovene, aromadendrene and 2,6-ditert-butylquinone (**Table 3, Fig. 2**). Majority of the compounds present were terpenes. Aromandendrene is a sesquiterpenoid hydrocarbon with molecular formula C15H24. Phytol or (2E, 7R, 11R)-3,7,11,15-tetramethyl-2-hexadecen-1-ol is an acyclic diterpene alcohol with molecular formula of $C_{20}H_{40}O$. Phytol is commonly seen as clear to slightly yellow liquid. Instead of that, longiverbenone which is a naturally occurring sesquiterpene also present with molecular formula $C_{15}H_{22}O$. Neophytadiene or 2,6,10-Trimethyl,14-ethylene-14-pentadecne is a compound with molecular formula $C_{20}H_{38}$. All phytocompounds were known compounds as they had been successfully isolated from various plant species. Their potential biological activities had also been reported. For instance, aromadendrene was recently found as predominant constituents in *Scapania verrucosa* (Scapaniaceae), *Ficus religiosa* (Moraceae) and *Eucalyptus globules* (Myrtaceae) [12,13].

Extracts containing aromadendrene was reported to exert antimicrobial and antitumor activity [12]. Instead of that, phytol is well known to possess antimicrobial, anti-inflammatory and anticancer diuretic activity [14,15]. Recent research showed the presence of longiverbenone on eaglewood tree, *Aquilaria agallocha* (Thymelaeaceae)[16]. Rahman and Anwar, [17], had studied the antibacterial activity of longiverbenone and found the effectiveness of this compound as antibacterial agents.

Aromadendrene, phytol and neophytadiene were found ubiquitously in Asteraceae family such as from *Stevia rebaudiana, Blumea balsamifera* and *Lantana camara* [16,18,19]. Erigeron sumatrensis extracts containing neophytadiene had been reported to exhibit both antifungal and antibacterial activities [20]. In *Chromolaena odorata* (Asteraceae), latest report revealed the presence of aromadendrin-4-methyl-ether from the ethanolic leaves extract [21]. In fact, several studies had been conducted upon compound isolation of *C. odorata* and their potential biological activities [21,22].



Notes:
1= hexachloro-ethane
2= n-nonylaldehyde
3= methyl-4-oxooctanoate
4= longiverbenone
5= 2-butenal,2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)
6= neophytadiene
7= phytol
8= dihydro-neoclovene (II)
9= 2,6-di-tert-butylquinone
10= aromadendrene

Fig. 1. GC-MS chromatogram of fraction F2.

Table 3. List of phytocomponents identified in fraction F2.

NO.	RT (MIN)	Constituents	Molecular formula	Molecular weight
01.	6.367	Hexachloro-ethane	C_2CL_{16}	234
02.	6.708	N-nonylaldehyde	$C_9H_{18}O$	142
03.	9.958	Methyl-4-Oxooctanoate	$C_9H_{16}O_3$	172
04.	15.050	Longiverbenone	$C_{15}H_{22}O$	218
05.	18.917	2-Butenal,2-Methyl-4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)	$C_{14}H_{22}O$	206
06.	19.350	Neophytadiene	$C_{20}H_{38}$	278
07.	19.992	Phytol	$C_{20}H_{40}O$	296
08.	40.692	Dihydro-Neoclovene	$C_{15}H_{26}$	206
09.	42.842	2,6-Ditert-Butylquinone	$C_{14}H_{20}O_2$	220
10.	41.942	Aromadendrene	$C_{15}H_{24}$	204

RT= Retention Time

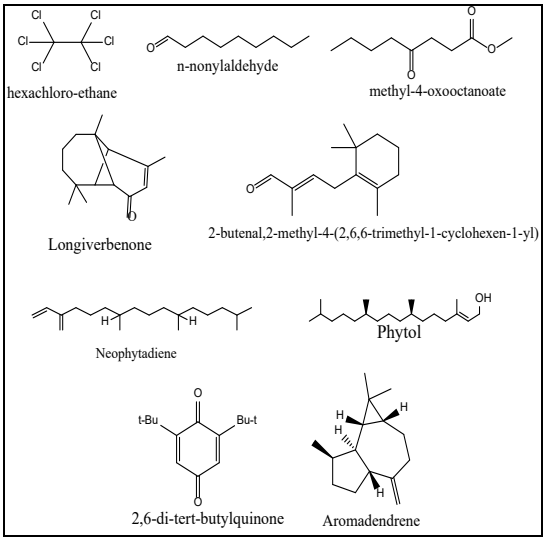


Fig. 2. Chemical structures of 10 predominant bioactive compounds of fraction F2.

CONCLUSION

The CME and EAE extracts partitions are potentially active against *P. aeruginosa, S. aureus* and *S. pneumonia,* while CE extracts partition inhibit *S. aureus* (7.00±.000mm) and *S. pneumonia* (7.00±.000mm). Fractionation of CE extracts partition has exhibited inhibitory zones against *P. aeruginosa, S. aureus* and *S. pneumonia. F2 fraction shows the most active against all bacteria strain*s with highest inhibition zones detected against *S. pneumonia* (11.67±2.08mm). GC-MS analysis of F2 fraction has revealed 10 bioactives as major constituents which are hexachloro-ethane, n-nonylaldehyde, methyl-4-oxooctanoate, longiverbenone, 2-butenal,2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl), neophytadiene, phytol, dihydro-neoclovene, aromadendrene and 2,6-ditert-butylquinone. Qualitative analysis on the presence of phytochemicals of all extract has shown the presences of alkaloids, flavonoids, tannins, saponins and triterpenoids.

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