COMPOSITION AND ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS AND ESSENTIAL OIL OF *Callistemon viminalis* LEAVES

ERNAWITA [(1)(2)*], SHAIDA FARIZA SULAIMAN [(2)]

[(1)]Biology Department of Educational Faculty, Almuslim University,Bireun, Aceh

[(2)] School of Biological Sciences, Universiti Sains Malaysia, 11800, Penang, Malaysia.

Abstract

Antimicrobial activity of crude extracts and essential oil of *Callistemon viminalis* against several pathogenic bacteria and one yeast (*Candida albicans*) were assessed using sensitivity test and MIC determination test. Hexane extract showed the strongest antibacterial activity compared with other crude extracts. While essential oil of *C. viminalis* in particular showed strong anticandidal activity. GC-MS analysis of crude hexane extract and essential oil have identified the presence of eucalyptol, α-pinene, α-phellandrene and D-limonene as active antimicrobial compounds in all analyzed samples.

Keywords: Antimicrobial activity; *Callistemon viminalis*, Myrtaceae, Terpenoids, GC-MS

INTRODUCTION

Microorganisms daily caused 50 thousands of premature death (Carey *et al*., 2004; Esterhuizen *et a*l., 2006). And eventhough there is rapid progress in understanding the growth and control of many pathogens, bacterial infections still primary leading death caused especially in developing countries (Kamatou *et al*., 2007). Thus, the control of microorganisms is critical in prevention and curing of diseases caused by their actions. The emergence of bacterial resistance then raised the demand of new antimicrobial agents. Traditional therapeutic value of plants to treat bacterial infections has long been recognized in many different communities. Researches have also been conducted to validate traditional therapeutic value of the plants, as well as to discover its antimicrobial activity, such as Wiart *et al.,* (2004), Ibrahim and Osman (1995) and Grosvenor *et al.,* (1995).

Callistemon. viminalis (Gaertn.) G. Don., also known as bottle brush is plant native to Australia, but now widely spread to Asia, Africa, America and Europe. This plant is most notable for its beautiful scarlet blossoms during flowering time. The flower rich with nectar and much sought after by birds and bees (Srivastava *et al.,* 2003). Eventhough considered as the most widely cultivated species of *Callistemon*, yet very limited information available regarding its biological activity. Strong anti-quorum sensing activity reported from ethanolic extract of inflorescence of *C. viminalis* (Adonizo *et al*., 2006). While antihelminthic activity of this plant previously reported by Garg and Kasera (1982). Antifumigant activity of this species also previously reported by Lee *et al*., (2004).

Taking into account previous research conducted, *C. viminalis* showed antimicrobial activity and worth for further research to validate this assumption.

RESEARCH METHOD

Plant material

The plant specimen was collected in the main campus of Universiti Sains Malaysia (USM), Penang. The specimen was then washed and left until all the remaining water evaporated at room temperature. Voucher specimen was deposited at Universiti Sains Malaysia herbarium collection number 11012.

Preparation of crude extracts and essential oil

Fresh leaves of *C. viminalis* were ground and extracted with hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and 80% MeOH for 48 hours consecutively. The solvents were then evaporated to yield the crude extracts. These extracts were then subjected to sensitivity test and MIC determination test. Essential oil of *C. viminalis* leaves was prepared using steam-distillation procedure. Petroleum-ether was used as the oil-trapper. The trace of water in essential oil was removed by addition of sodium sulphate anhydrate. The essential oil was tightly sealed with parafilm to avoid evaporation and stored at 4^{o}C until tested.

Microorganisms
The test organisms used were clinical isolates of *Bacillus licheniformis, Staphylococcus aureus, Staphylococcus* coagulase (-), *Escherichia coli, Pseudomonas aeruginosa* and yeast *Candida albicans* obtained from Microbiology Laboratory, school of Medical Sciences, USM and *Acinetobacter anitratus, Acinetobacter calcoaceticus,* and *Klebsiella pneumoniae* that obtained from stock culture of Microbiology Laboratory, School of Biological Sciences, USM. Culture stocks of bacteria were kept in NB medium with 20% glycerol at -80ºC.

Bioassay
All bacteria were maintained in NA agar slant and prior to susceptibility test, each isolate was inoculated on NA agar to ensure optimal growth characteristics and purity. Yeast *Candida albicans* was maintained in PDA agar slant and freshly inoculated on PDA agar prior to susceptibility test.

Preliminary screening using disk diffusion method
Whatman filter paper disc 6 mm in diameter were impregnated with 20 µl of 50 mg/ml extracts diluted in DMSO to give final concentration 1 mg/ml per disc. Amoxycillin was prepared as above and served as positive control and 20 µl of DMSO served as negative control. Duplicate of discs prepared for each sample. Bacterial suspension of about 1.5×10^6 CFU/ml was prepared by adjusting the turbidity to standard McFarland 0.5. Sterile cotton buds were used to swab the bacterial onto the agar surface. The impregnated discs were then placed onto the agar surface. The plates were then incubated at 37ºC for 21-24 hours.

Minimum Inhibitory Concentration
Minimum inhibitory concentration (MIC) was determined using broth macrodilution method according to NCCLS. A serial doubling dilution was prepared over the range of 1.25 – 500 µg/ml in Nutrient Broth. Cell suspension adjusted with McFarland 0.5. The medium then inoculated and incubated at 37ºC for 24 hours. The bacterial growth was indicated by the turbidity of test tubes compared with controls. MIC was defined as the least concentration that showed no visible growth compared with bacteria-free control.

Minimum Inhibitory Concentration of essential oil
MIC determination test of essential oil was conducted using disc diffusion method according to method as proposed by Palazzo *et al.,* (2007). Briefly, a serial doubling of essential oil was prepared over the range of 0.63-20 µl/ml in DMSO to give the range of essential oil in 3.15% to 100% purity percentage of essential oil and then pipetted to the surface of sterile disc. Cell suspension adjusted with McFarland 0.5 and then aseptically inoculated onto the surface of MHA medium using sterile swab. The disc then placed onto the surface of agar. The medium then inoculated and incubated at 37ºC for 48 hours. The MIC value was defined as the least concentration that inhibits the growth of *C. albicans.*

Identification of active compounds using Gas chromatographic-mass spectral (GC-MS) analysis
Crude hexane extract and essential oil were submitted to GC-MS analysis. The analysis were performed on Hewlett-Packard 6890N Network GC system gas chromatography instrument with Hewlett –Packard 5973 inert mass selective detector mass spectrometer. High polarity HP-Innowax columns were used (30 m, 0.25 mm i.d, 0.25 µm thick). The carrier gas was Helium. The initial temperature was 70ºC then 300ºC for 78 minutes. Injector temperature was 250ºC and detector temperature was 300ºC. Injection volume was 1µl. Identification was made based on the computer matching of the mass spectra of the major compounds against the NIST library built up in pure compounds. Matching percentage of more than 80% identical was considered as matched with the suggested compounds from the spectra library. The components from gas chromatographic-mass spectral analysis are listed in Table 4.

RESULT AND DISCUSSION

Sensitivity test results of crude extracts and essential oil
Sensitivity test results of crude extracts and essential oil of *C. viminalis* as showed in Table 1 revealed broad spectrum activity of samples tested. Least polar extracts with hexane extract in particular showed strong antibacterial activity against Gram positive bacteria. Table 1 also pointed out strong anti-candidal activity of essential oil of *C.*

viminalis. MIC determination test was then further conducted to determine definite inhibition concentration of active samples as presented in Table 2.

Table 1 Antimicrobial activities of crude extracts and essential oil of *C. viminalis* leaves

No	Bacteria	Diameter of inhibition zone (mm)					
		80% MeOH	EtOAc	$CHCl_3$	Hexane	Essential Oil	Amoxycillin
	Gram positive						
1	*B. licheniformis*	12 ± 0	14 ± 0	20 ± 0	21 ± 0	7 ± 0	20.50 ± 0.71
2	*S. aureus*	14.50 ± 0.70	14.50 ± 0.70	19.50 ± 0.70	21.50 ± 0.70	9.50 ± 0.70	20.50 ± 0.70
3	*Staphylococcus* coagulase (-)	12.50 ± 0.70	16 ± 0	22 ± 0	19.50 ± 0.70	9 ± 1.40	25 ± 0
	Gram negative						
4	*A. anitratus*	11.50 ± 0.70	10.50 ± 0.50	16 ± 0	15 ± 0	8 ± 0	38.50 ± 0.71
5	*A. calcoaceticus*	16 ± 0	13 ± 0	12 ± 0	12 ± 0	12.50 ± 1.50	-
6	*E. coli*	-	-	-	-	8 ± 0	32 ± 1.40
7	*K. pneumoniae*	10.50 ± 0.50	-	-	10.50 ± 0.50	8 ± 0	10 ± 0
8	*P. aeruginosa*	-	-	-	-	-	10.50 ± 0.70
	Yeast						
9	*C. albicans*	14.50 ± 0.71	11 ± 1.41	8 ± 0	-	18 ± 0	-

Table 2 Minimum inhibitory concentration of crude extracts of *C. viminalis* against selected bacteria

No	Samples	Minimum Inhibitory Concentration (µg/ml)			
		80% MeOH	EtOAc	$CHCl_3$	Hexane
Gram positive					
1	*B. licheniformis*	ND	ND	2.50	1.25
2	*S. aureus*	62.50	5	5	5
3	*Staphylococcus* coagulase *(-)*	62.50	10	10	5
Gram negative					
4	*A. anitratus*	ND	ND	31.25	62.50
5	*A. calcoaceticus*	62.50	ND	ND	ND

ND = Not Determined

While MIC value of essential oil of *C. viminalis* against *C. albicans* was at 25% purity of essential oil (Table 3).

Table 3 MIC value (µl/µl) of essential oil of *C. viminalis* against *C. albicans*

Essential oil percentage (%)	Inhibition zone
100	+
50	+
25	+
12.50	-
6.25	-
3.13	-

GC-MS analysis of hexane extract and essential oil identified 13 compounds from essential oil, 10 compounds from hexane extract. Six compounds identified from hexane extract were also found in essential oil of *C. viminalis.*

Table 4 Main components from essential oil, hexane extract and refraction df6 (%) of the leaves of *C. viminalis*

Peak	Compounds	Sample	
		Hexane extract	Essential oil
1	α-Pinene	1.07	12.49
2	*Iso*-Butyric acid	-	0.39
3	β-Pinene	-	0.60
4	*o*-Cymene	-	-
5	α-Phellandrene	0.32	2.31
6	D-Limonene	0.70	
7	Eucalyptol	4.17	47.83
8	α–Terpinene	-	0.54
9	*p*-Cymene	0.41	3.51
10	α-Terpinolene	-	0.51
11	Linalool	-	0.68
12	Terpinen-4-ol	-	0.79
13	α-Terpineol	0.31	10.01
14	Spathulenol	-	0.73
15	Hexahydrofarnesyl acetone	50.46	-
16	1-Octadecanol	0.57	-
17	1-Eicosene	0.78	-
18	n-Hexadecanoic acid	1.30	-

DISCUSSION

Hexane extract showed the strongest antibacterial activity. Dupont *et al.*, (2006) previously reported antibacterial activity of hexane extracts. According to Esterhuizen *et al*. (2006), the lipophilic characteristic of the non-polar compounds aids the passive diffusion of the compounds over the cell wall of bacteria and can be the reason for the higher antibacterial potency against Gram-positive bacteria. MIC determination test conducted further supporting the antibacterial potency of the crude extracts. Rios *et al.* (1988) proposed that plant extracts that display antimicrobial activity at concentrations lower than 100 µg/ml could be considered as potential antimicrobial agent, since there is a chance that the active compounds obtained will active at lower concentrations. MIC values of crude extracts are within the range of 1.25 µg/ml - 61.25 µg/ml, indicating that crude extracts of *C. viminalis* were potential antimicrobial agents. Hexane extract showed the foremost antimicrobial potential agent with the lowest MIC value against all tested bacteria except for *A. anitratus*. Almeida *et al.* (2008) also encountered high antimicrobial activity of hexane extract of peel and seed of *Rheedia brasiliensis* against *Streptococcus mutans* with MIC value obtained were within the

range of 12.50-25 µg/ml. While essential oil showed prominent anticandidal activity. Plants essential oils also have been reported to inhibit the growth of *C. albicans* (Hayes and Markovic, 2002; Matasyoh *et al*., 2007; Souza *et al*., 2007). Strong antibacterial activity of hexane extract might be attributed by the presence of terpenoid compounds identified from crude hexane extract (data are not shown). Terpenoids are suggested as responsible compounds with antimicrobial activity from hexane extract. Antibacterial activities of terpenoids are well recorded. Terpenoids mechanisms of actions including attacking of the cell wall and cell membranes, thereby affecting cell permeability and release of intracellular constituents. Terpenoids action in the cell wall also affecting membrane function, such as electron transport, nutrient uptake, protein and nucleic acid synthesis and enzyme activity (Xianfei *et al*., 2007). Presumably, terpenoids compounds in hexane extracts and essential oil were diffused through the cell membrane, followed with releasing of the intracellular matrices to the surrounding environment.

Crude hexane extract of *C. viminalis* showed broad antibacterial activity (Table 1). GC-MS analysis identified α-pinene, α-phellandrene, D-limonene and eucalyptol as main components are believed to be responsible for antibacterial activity of the samples. Moderate antibacterial activity of eucalyptol were previously reported by Candan *et al*., (2003), Hwang *et al*., (2004), Chung *et al.,* (2006) and Sivropoulou *et al.,* (2007). While antibacterial activity of α-pinene previously reported by Magiatis *et al.,* (1999) and Matasyoh *et al.,* (2007) to be comparable with chloramphenicol against *E. coli* and *S. aureus*. D-limonene was reported to inhibit the growth of *Bacillus* sp., *C. albicans, E.coli* and *S. aureus* (Perez *et al.,* 1999; Costa *et al.,* 2000) with the mechanisms suggested are the alkyl-groups in limonene influenced the Gram-reaction sensitivity of the bacteria (Dorman and Deans, 2000). α-Phellandrene was reported to inhibit the growth of *S. aureus, S. epidermidis, P. aeruginosa* and *C. albicans* (Perez *et al.,* 1999 and Demirci *et al.,* 2007). The most abundant components identified from essential oil of *C. viminalis* were eucalyptol, α-pinene, α-terpineol, D-limonene, *p*-cymene and α-phellandrene. However, the presence of iso-butyric acid, α-phellandrene and terpinen-4-ol were not reported by Srivastava *et al.,* (2003) that worked with the same species. Several factors might affect the composition of any plant essential oil studied, such as locality, climatic, seasonal and experimental conditions (Daferera *et al.,* 2000).

On the other hand, essential oil of *C. viminalis* showed strong anticandidal activity. It might be attributed by its main components such as eucalyptol, α-pinene, α-terpineol, D-limonene, α-phellandrene and *p*-cymene as previously reported by Unlu *et al.,* (2002); Candan *et al.,* (2003); Tabanca *et al.,* (2007); Sibanda *et al.,* (2004), Filipowicz *et al.,* (2003) and Magwa *et al.,* (2006). More prominent antifungal activity displayed by essential oil rather than crude hexane extract or refraction might be contributed by the more complex mixture of terpenoids compounds found in essential oil than in crude extracts as indicates by GC-MS analysis. Mechanisms of action involved including the leakage of K+ and H+ from yeast cells by the action of these compounds, hence affecting yeast respiration (Griffin *et al.,* 1999) and also disruption of fungal membrane integrity (Magwa *et al.,* 2006). Synergistic activity between major and minor components must also be considered when dealing with essential oil (Kelen and Tepe, 2008).

REFERENCES

Adonizo, A. L., Downum, K., Bennet, B. C. & Mathee, K. (2006) Anti-quorum sensing activity of medicinal plants in southern Florida. *Journal of Ethnopharmacology,* 105**,** 427 - 435.

Almeida, L. B., Murata, R. M., Yatsuda, R., Santos, M. H. D., Nagem, T. J., Alencar, S. M., Koo, H. & Rosalen, P. L. (2008) Antimicrobial activity of *Rheedia brasiliensis* and 7-epiclusianone against *Streptococcus mutans*. *Phytomedicine*, Article in Press.

Candan, F., Unlu, M., Tepe, B., Daferera, D., Polissiou, M., Sokmen, A. & Akpulat, H. A. (2003) Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae). *Journal of Ethnopharmacology,* 87**,** 215-220.

Carey, J. (2004) Fighting superbugs with superdrugs. *Drug Discovery Today,* 9**,** 637 - 640.

Chung, J. Y., Choo, J. H., Lee, M. H. & Hwang, J. K. (2006) Anticariogenic activity of macelignan isolated from *Myristica fragrans* (nutmeg) against *Streptococcus mutans*. *Phytomedicine,* 13**,** 261-266.

Costa, T. R., Fernandes, O. F. L., Santos, S. C., Oliveira, C. M. A., Liao, L. M., Ferri, P. H., Paula, J. R., Ferreira, H. D., Sales, B. H. N. & Silva, M. D. R. R. (2000) Antifungal activity of volatile constituents of *Eugenia dysenterica* leaf oil. *Journal of Ethnopharmacology,* 72**,** 111-117.

Daferera, D. J., Ziogas, B. N. & Polissiou, M. G. (2000) GC - MS analysis of essential oils from Greek aromatic plants and their fungitoxicity on *Penicillum digitatum Journal of Agricultural and Food Chemistry,* 48**,** 2576 - 2581.

Demirci, B., Kosar, M., Demirci, F., Dinc, M. & Baser, K. (2007) Antimicrobial and antioxidant activities of the essential oil of *Chaerophyllum libanoticum* Boiss. *et* Kotsky. *Food Chemistry*.

Dorman, H. J. D. & Deans, S. G. (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology,* 88**,** 308-316.

Dupont, S., Caffin, N., Bhandari, B. & Dykes, G. A. (2006) In vitro antibacterial activity of Australian native herb extracts against food-related bacteria. *Food Control,* 17**,** 929-932.

Esterhuizen, L. L., Meyer, R. & Dubery, I. A. (2006) Antimicrobial compounds from *Coleonema album* (Rutaceae). *Z Natursforch,* 61 c**,** 489 - 498.

Filipowicz, N., Kaminski, M., Kurlenda, J. & Asztemborska, M. (2003) Antibacterial and antifungal activity of Juniper Berry oil and its selected components. *Phytotherapy Research,* 17**,** 227 - 231.

Garg, S. C. & Kasera, H. L. (1982) Antihelminthic activity of the essential oil of *Callistemon viminalis Fitoterapia,* 53**,** 179 - 181.

Griffin, S. G., Wyllie, S. G., Markham, J. L. & Leach, D. N. (1999) The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. *Flavour and Fragrance Journal,* 14**,** 322-332.

Grosvenor, P. W., Supriono, A. & Gray, D. O. (1995) Medicinal plants from Riau Province, Sumatra, Indonesia. Part 2: antibacterial and antifungal activity. *Journal of Ethnopharmacology,* 45**,** 97 - 111.

Hayes, A. J. & Markovic, B. (2002) Toxicity of Australian essential oil *Backhousioa citriodora* (Lemon myrtle). Part 1. Antimicrobial activity and in vitro cytotoxicity. *Food and Chemical Toxicology,* 40**,** 535 - 543.

Hwang, J. K., Shim, J. S. & Chung, J. Y. (2004) Anticariogenic activity of some tropical medicinal plants against *Streptococcus mutans*. *Fitoterapia,* 75**,** 596 - 598.

Ibrahim, D. & Osman, H. (1995) Antimicrobial activity of *Cassia alata* from Malaysia. *Journal of Ethnopharmacology,* 45**,** 151 - 156.

Kamatou, G. P. P., Vuuren, S. F. V., Heerden, F. R. V., Seaman, T. & Viljoen, A. M. (2007) Antibacterial and antimycobacterial activities of South African *Salvia* species and isolated compounds from *S. chamelaeagnea*. *South African Journal of Botany,* 73**,** 552 - 557.

Kelen, M. & Tepe, B. (2008) Chemical composition, antioxidant and antimicrobial properties of the essential oil of three *Salvia* species from Turkish flora. *Bioresource Technology,* 99**,** 4096 - 4014.

Lee, B.-H., Annis, P. C., Tumaalii, F. A. & Choi, W.-S. (2004) Fumigant toxicity

of essential oils from the Myrtaceae family and 1,8-cineole against 3 major stored-grain insects. *Journal of Stored Products Research,* 40**,** 553 - 564.

Magiatis, P., Melliou, E., Skattsounis, A. L., Chinou, I. & Mitaku, S. (1999) Composition and antimicrobial activity of the essential oils of *Pistachia lentiscus* var chia. *Book of abstracts: 2000 years of natural products research - past present and future.* Amsterdam, Leiden University.

Magwa, M. L., Gundidza, M., Gweru, N. & Humphrey, G. (2006) Chemical composition and biological activities of essential oil from the leaves of *Sesuvium portulacastrum*. *Journal of Ethnopharmacology,* 103**,** 85-89.

Matasyoh, J. C., Kiplimo, J. J., Karubiu, N. M. & Hailstorks, T. P. (2007) Chemical composition and antimicrobial activity of essential oil of *Tarchonanthus camphoratus Food Chemistry,* 101**,** 1183-1187.

Palazzo, I. C. V., Rehder, A. & Darini, A. L. C. (2007) Quantitative disk diffusion as a convenient method for determining minimum inhibitory concentrations of oxacillin for staphylococci strains. *Journal of Microbiological Methods,* 71**,** 186-190.

Perez, C., Agnese, A. M. & Cabrera, J. L. (1999) The essential oil of *Senecio graveolens (Compositae)*: chemical composition and antimicrobial activity tests. *Journal of Ethnopharmacology,* 66**,** 91 - 96.

Rios, J. L., Recio, M. C. & Villar, A. (1988) Screening methods for natural products with antimicrobial activity: A review of the literature. *Journal of Ethnopharmacology,* 23**,** 127 - 149.

Sibanda, S., Chigwada, G., Poole, M., Gwebu, E. T., Noletto, J. A., Schmidt, J. M., Rea, A. I. & Setzer, W. N. (2004) Composition and bioactivity of the leaf essential oil of *Heteropyxis dehniae* from Zimbabwe. *Journal of Ethnopharmacology,* 92**,** 107 - 111.

Sivropoulou, A., Nikobu, C., Papanikolau, E., Kokkini, S., Lanaras, T. & Arsenakis, M. (2007) Antimicrobial, cytotoxic, and antiviral activities of *Salvia fruticosa* essential oil. *Journal of Agricultural and Food Chemistry,* 45**,** 3197-3201.

Souza, E. L., Stamford, T. L. M., Lima, E. O. & Trajano, V. N. (2007) Effectiveness of *Origanum vulgare* L. essential oil to inhibit the growth of food spoiling yeasts. *Food Control,* 18**,** 409 - 413.

Srivastava, S. K., Ahmad, A., Syamsunder, K. V., Aggarwal, K. K. & Khanuja, S. P. S. (2003) Essential oil composition of *Callistemon viminalis* leaves from India. *Flavour and Fragrance Journal,* 18**,** 361 - 363.

Tabanca, N., Demirci, F., Demirci, B., Wedge, D. E. & Baser, K. H. C. (2007) Composition, enantiomeric distribution, and antimicrobial activity of *Tanacetum argentum* subsp. *flabellifolium* essential oil. *Journal of Pharmaceutical and Biomedical Analysis,* 45**,** 714 - 719.

Unlu, M., Daferera, D., Donmez, E., Polissiou, M., Tepe, B. & Sokmen, A. (2002) Compositions and the in vitro antimicrobial activities of the essential oils of *Achillea setacea* and *Achillea teretifolia* (Compositae). *Journal of Ethnopharmacology,* 83**,** 117-121.

Wiart, C., Mogana, S., Khalifah, S., Mahan, M., Ismail, S., Buckle, M., Narayana, A. K. & Sulaiman, M. (2004) Antimicrobial screening of plants for traditional medicine in the state of Perak, Peninsular Malaysia. *Fitoterapia,* 75**,** 68 - 73.

Xianfei, X., Xiaoqiang, C., Shunying, Z. & Guolin, Z. (2007) Chemical composition and antimicrobial activity of essential oils of *Chaenomeles speciosa* from China. *Food Chemistry,* 100**,** 1312 – 1315.