Journal of Faculty of Sciences

A Refereed Scientific Journal
Faculty of Pure and Applied Sciences
International University of Africa

Volume No. 6 , December , 2019

ISSN: 1858 – 5

Effect of Essential Oils Combination on Chemical Composition and Biological Activities

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Abstract

Medicinal plants play an important role as a useful resource in producing new and safer drugs for the treatment of various diseases. Basil (*Ocimum basilicum)*, Lemongrass (*Cymbopogon citratus)* and Mexican mint (*Plectranthus amboinicus*) are plants which have been claimed to have many traditional uses. *O. basilicum* commonly known as “sweet basil” and *C. citratus* is a tall perennial grass. Meanwhile, *P. amboinicus* is a famous Indian herb that has been used in treating cold, cough and other diseases. In this study, the chemical composition of the essential oils from the three plants were determined and screened for their cytotoxicity using Brine Shrimp Lethality Test (BSLT). The essential oils were obtained from fresh leaves by steam distillation; and their chemical compositions determined by GC-MS. In the cytotoxicity test, seven sample solutions were prepared, A (lemongrass); B (basil); C (Mexican mint) and A+B, B+C, A+C and A+B+C. The combinations in a ratio of 1:1 and 1:1:1.All sample solutions

were screened for the cytotoxicity test. For the bioassay, four different concentrations from each oil sample were prepared (10, 25, 50, and 100 ppm).The compounds, citral, 71.79% (Trans-citral 42.64% and cis-citral29.15%), methyl cinnamate (57.60%) and thymol (75.46%) were the predominant compounds in the *C. citrates*, *O. basilicum* and *P. amboinicus*, respectively. The results obtained of the essential oils alone or in combinations showed strong cytotoxic activity against brine shrimps. The LC_{50} values were 37.5, 62.5, 37, 17.5, 81.25 and 28 ppm for A, B, A+B, B+C, A+C, and A+B+C, respectively. The mortality rate of oil C is very high, which was almost 100% from the lowest concentration to the highest concentration that have been tested. The essential oils had different toxicities due to quantitative and qualitative variations in the compositions of the essential oils. From the study, the BSLT results may be used as a preliminary indicator as an anticancer agent and/or for further cytotoxicity with specific bioassays and determination of bioactive compounds.

Keywards: *O. basilicum*; *Cymbopogon citratus; Plectranthus amboinicus*; essential oil; cytotoxicity; brine shrimp

1. Introduction

Medicinal plants have been used by humans as a source of medicines since long time ago. These medicinal plants constitute an important component of flora which is widely distributed all around the world. More than 35,000 plant species have been reported to be used in various human cultures around the world for medical purposes (Ibrahim, 2004). The chemical compounds or substances found in the medicinal plants made them to be highly potential in producing various chemicals as a defense against diseases, viruses and others. A large number of plant species have already

been tested for their potential biological, therapeutic and pharmaceutical activities (Hussain et al., 2008, Krishnaiah et al., 2009).

The huge variety of the flora with chemical diversity is one of the vital factors that make natural products an outstanding candidate for any screening practice (Jamal et al., 2011).Natural products and related drugs are used to treat 87% of all categorized human diseases including bacterial infection, cancer and immunological disorders (Uddin et al., 2009). Currently, plant derived-bioactive compounds have received considerable attention due to their therapeutic potential as antimicrobial, anti-inflammatory, anticancer and antioxidant activities (Oskoueian et al., 2011). The genus *Ocimum*in which *Ocimum basilicum* (Family: *Lamiaceae*)is a perennial crop generally known as sweet basil that is native to Asia, Africa, South America and the Mediterranean, but widely cultivated in many countries (Jayasinghe et al., 2003). Traditionally, basil has been used as a potent antiseptic and preservative, slight sedative, digestive regulator, diuretic, headaches, coughs, infections of upper respiratory tract and kidney malfunction (Beric et al., 2008).

Lemongrass plant belongs to the genus *Cymbopogon* of which *Cymbopogon citratus,* (Family Poaceae), and its derived products have been used for medicinal purposes since long time ago. Lemongrass essential oil with high content of citral, which is an oxygenated terpenoid (aldehyde), has been identified as a compound exhibiting antifungal properties has made the oil fungistatic and fungicidal against *A. flavus* (Paranagama et al., 2003).

Plectranthus amboinicus is a perennial plant from the family *Lamiaceae* (Senthilkumar and Venkatesu, 2010). This plant has more than 300 species that are used by tradition to cure various diseases such as treat skin, digestive and respiratory diseases (Murthy et al., 2009). Besides, *Plectranthus* leaves have been used in malarial fever, chronic asthma, hiccough, bronchitis, anthelmintic, hepatopathy, renal, vesical calculi, cough, colic and convulsions (Kaliappan and Viswanathan, 2008).

Currently, most of the industries are looking into alternative sources of anticancer, antioxidants, antimicrobials, antibiotics and pest control agent, which are environmentally friendly, safe and more natural. Therefore, the purpose of this study was to test the cytotoxic activity of essential oil from three chemotypes alone and in combination using BSLA.

2. Materials and Methods

2.1 Plant material

Commercial basil seed accession obtained from Sudan and cultivated at University Malaysia Pahang-UMP, Kuantan, Malaysia. Taxonomic identification of the plants was done by a Botanist of the School of Environmental Sciences and Natural Resources, National University of Malaysia, Selangor. While lemongrass obtained from Taman Pertanian Jubli Perak Sultan Haji Ahmad Shah Kuantan, Pahang, Malaysia and fresh leaves of *P. amboinicus* were collected from Seremban, Malaysia. Essential oils from these plants were obtained by steam distillation from fresh plant

leaves. The obtained essential oils were dried over anhydrous Na_2SO_4 and stored in a dark bottle and kept at 4°C until analysis and for further works.

2.2 GC-MS

The composition of the essential oils was determined using an Agilent 7890A Gas Chromatography – Mass Spectrometry instrument. Oxygen-free nitrogen was used as a carrier gas, and hydrogen was used for the flame. The GC conditions used were as follows: capillary column; fused silica (polydimethyl siloxane, 0.25 µm film thickness); temperature program: 70 °C (2 min-1), 70 – 230 °C (3 min-1), 230 – 240 °C (5 min-1), 270 °C (5 min-1); carrier gas, He at 5 bar, linear velocity of 20 cm min-1; injection port splitless at 250 °C; injection volume, 0.1 µL. The MS conditions were as follows: ionisation EI at 70 eV; m/z range, 30-300 °C; scan rate 1 sec-1; ionisation chamber at 180 °C; and transfer line at 280°C. The identification of the essential oil constituents was based on a comparison of their retention times, and these constituents were further identified and authenticated using their MS data compared to the NIST mass spectral library.

2.3 Preparation of artificial sea water, hatching and rearing of Brine Shrimps

Salinity of artificial sea water (25 ppt) was prepared. Brine shrimp eggs were hatched using a hatching container that was filled with artificial sea water. Optimum incubation period for the eggs was 24 hours, with constant light source and aeration to trigger the hatching mechanism. After 24 hours of incubation at room temperature (27-29°C), shells and the newly hatched shrimps (nauplii) were separated. During the harvesting, the naupliiwere fed with denatured yeast. The light source was constantly provided and aeration was provided regularly using air pump for one week before BSLA.

2.4 Test sample preparation

Test samples were prepared in four different concentrations by using DMSO to dissolve the oil in the distilled water for all seven samples (A, B, C, A+B, B+C, A+C and A+B+C), with two replicates test for each concentration. Four concentrations were used 10, 25, 50, and 100 ppm. Potassium dichromate was usedas positive control, whereas, distilled water and 5% of DMSO in distilled water were used as negative controls. The cytotoxicity test for both controls also was performed in replicate.

2.5 Brine shrimp lethality assay (BSLA)

The test was conducted using four different concentrations (10, 25, 50, and 100ppm). Exactly, 10 shrimps were placed in each test tube containing the sample. The toxicity was determined after 0.5 h, 1 h, 2 h, 3 h,

6 h, 9 h, 12 h and 24 h of exposure. The numbers of survivors were counted and percentages of deaths were calculated. A larva is considered dead if it does not exhibit any internal or external movement during several seconds of observation. The mean percentage mortality was plotted against the concentrations for all seven samples and the concentration killing fifty percent of the larvae (LC_{50}) were determined from the graph.

3. Results and Discussion

3.1 Oil content and chemical composition of fresh leaves essential oils of basil, lemongrass and Mexican mint

Three plants were used in this research, which are *C. citratus* (Lemongrass), *O. basilicum* (Basil) and *P. amboinicus* (Mexican mint). Essential oils of these plants were studied for its chemical composition and cytotoxicity activity against brine shrimps. The chemical compositions of the essential oil were analyzed by using GC-MS.

Table 1 and 2 shows the chemical composition of essential oil alone and in combinationsanalyzed by GC-MS. The leaves of sample A (Lemongrass) yielded 0.7% (w/w) of essential oil with light yellow in color. The major chemical compound was citral (71.79%). An earlier report mentioned this essential oil consist of high content of citral (>70%) (Paranagama et al.,2003). It has almost the same content as the present research. Tchoumbougnang et al., (2005) reported that, the oil of *C. citratu*s also contained geranial (32.8%), neral (29.0%), myrcene (16.2%) and β-pinene (10.5%).

As forsample B(Basil), it yielded 0.5% (w/w) of essential oil with very light yellow in color. Mainly seven compounds (>1%) were identified and the major chemical compound was methyl cinnamate (57.60%). Previous studies reported that this essential oil consisted of linalool as the most abundant component (56.7-60.6%), followed by epi-a-cadinol (8.6-11.4%), a-bergamotene (7.4-9.2%) and c-cadinene (3.2-5.4%) (Hussain et al., 2008).

For sample C (Mexican mint), it yielded 0.3% (w/w) of essential oil with light brown color. Mainly eight compounds (>1%) were identified and the major chemical compound was thymol (75.46%). Previous studies show the major chemical compounds were carvacrol (28.65%) followed by thymol (21.66%), α-humulene (9.67%), undecanal (8.29%), γ-terpinene (7.76%), ρ-cymene (6.46%), caryophyllene oxide (5.85%), α-terpineol (3.28%) and β-selinene (2.01%) (Senthilkumar and Venkatesu, 2010).

As for sample A+B which is a combination between two different essential oils which were lemongrass and basil in a ratio of 1:1, mainly ten compounds (>1%) were identified. The major chemical compounds were linalool (20.79%), cis-citral and trans-citral (25.04%) and geraniol (10.65%). For sample B+C which is the combination of basil and Mexican mint, mainly 13 compounds (>1%) were identified. Some of the major compounds were thymol (35.96%), linalool (13.74%) and geraniol (10.52%). For sample A+C which isa combination of lemongrass and Mexican mint, the major chemical compound was thymol (40.39%) as well.

Some other major compounds from all 12 compounds that have been identified were cis-citral (10.71%) and trans-citral (14.60%).

Lastly, For sample A+B+C which is combination between three different essential oils which were lemongrass, basil and Mexican mint in a ratio 1:1:1, mainly 12 compounds (>1%) were identified. The major chemical compounds werethymol (31.49%), trans-citral (14.93%) and linalool (10.59%).

Table 1. Chemical composition of essential oilsfrom fresh leaf of lemongrass (A), basil (B) and mexican mint (C). Components <1% not included.

Compound	**Percentage (%)**		
	A	**B**	**C**
Eucalyptol	-	1.14	-
Linalool	1.17	13.85	-
L-4-Terpineol	-	-	1.91
Cis-Citral	29.15	-	-
Trans-citral	42.64	-	-
Cis-Geraniol	1.31	-	-
Methyl cinnamate	-	57.60	-
(+)-Epi-bicyclosesquiphellandrene	-	5.03	-
Thymol			
m-cymene	-	-	75.46
gamma-terpinene	-	-	5.16
Caryophyllene	-	-	5.03
2-norpinene	-	-	4.23
α- Caryophyllene	-	-	2.90
Caryophyllene oxide	-	-	1.42
β –myrcene	-	-	1.47
α- longipinene	1.93	-	-
germacrene A	-	2.14	-
guaienes	-	2.58	-
	-	2.12	-

Samples: A= Lemongrass; B = Basil; C= Mexican mint

Table 2. Chemical composition of combinations essential oils from A+B; B+C, A+C and A+B+C. Components <1% not included

Compound	**Percentage (%)**			
	A+B	**B+C**	**A+C**	**A+B+C**
β –Myrcene	2.46	-	2.04	1.55
Eucalyptol	4.94	4.15	-	3.18
Fenchone	1.68	1.13	-	-
Linalool	20.79	13.74	-	10.59
Terpineol	1.53	-	-	-
Cis-Citral	11.72	-	10.71	6.92
Trans-citral	13.32	-	14.60	14.93
Methyl cinnamate	3.58	6.84		5.49
(+)-Epi-bicyclosesquiphellandrene	1.56	1.14	-	-
Geraniol	10.65	10.52	-	-
m-cymene	-	4.45	-	3.85
Gamma-terpinene	-	4.66	5.28	4.11
L-4-terpineol	-	1.29	1.42	1.12
Thymol	-	35.96	40.39	31.49
Caryophyllene	-	3.67	3.85	2.74
(Z,E)- α – Farnesene	-	3.33	-	-
α- Caryophyllene	-	1.10	1.31	-
(+)-4-carene	-	-	1.03	-
prehnitene	-	-	5.87	-
2-norpinene	-	-	2.93	2.74
Caryophyllene oxide	-	-	1.03	-

3.2 Brine shrimp lethality test (BSLT)

Essential oils of all three plantsamples were used to prepare seven samples and were studied for theircytotoxicity activity against brine shrimps. For each sample, 4 different concentrations of 10, 25, 50, and 100 ppm (0.01%, 0.025%, 0.05%, and 0.1%) were prepared.The result obtained shows all samples of essential oils have strong cytotoxic activity against

brine shrimp. The LC_{50} valuesafter 0.5 h were 37.5, 62.5, 37, 17.5, 81.25 and 28 ppm for A, B, A+B, B+C, A+C, and A+B+C respectively. Meanwhile for sample C the mortality rate is very high, which was almost 100% from the lowest concentration to the highest concentration that have been tested.

The cytotoxicactivity of all the samples was shown in Figures 1, 2, 3, 4, 5, 6 and 7 respectively. For all concentrations, after 24 h of nauplii exposure to the essential oil shows 100% of mortality. Mortality is increased with an oil dose increased and the different concentrations couldachievethe 100% mortality in different times. The highest concentration (100 ppm) produces this mortality (100%) after 0.5 h by sample A, C and B+C whereas samples B, A+B, A+C and A+B+C achieved after 1 h of exposure.

At the highest dose (100 ppm) sample A, C, and B+C are better than B, A+B, A+C and A+B+C in terms of achieving complete mortality in a short time which is 0.5 h (Fig 1, 3 and 5). While at 50 ppm, all can achieve 100% mortality after 2 h except for A+C. Whereas, at 25 ppm, all samples showed better results except for sample B (basil) and A+C; and all other five samples achieved the compete mortality of larvae after 3 h of exposures. At the lowest concentration (10 ppm) sample C the best among others and achieved complete mortality after 1 h, while A, B+C and A+B+C achieved the complete mortality after 6 h of exposures (Figs 1, 5 & 7). Meanwhile sample B and A+C achieved the complete mortality only after 24 h of exposure. Table 3 shows mortality of essential oil samples against shrimps.

Table 3. Mortality of brine shrimp.

Concentration of the sample (ppm)		Percentage of mortality of larvae (%)								LC_{50} (after 0.5 hr)
		0.5 hr	1 hr	2 hr	3 hr	6 hr	9 hr	12 hr	24 hr	
	A	25	40	65	95	100	100	100	100	
	B	10	15	15	15	25	30	45	100	
	C	95	100	100	100	100	100	100	100	**A** 37.5 ppm
10	**A+B**	0	25	45	45	45	55	80	100	
	B+C	15	35	60	70	100	100	100	100	
	A+C	0	0	5	5	15	25	45	100	
	A+B+C	35	40	50	70	100	100	100	100	
										B 62.5 ppm
	A	35	65	85	100	100	100	100	100	
	B	15	25	65	80	100	100	100	100	
	C	100	100	100	100	100	100	100	100	
25	**A+B**	45	50	65	100	100	100	100	100	**C**
	B+C	85	100	100	100	100	100	100	100	Not available
	A+C	15	20	45	70	100	100	100	100	
	A+B+C	45	55	80	100	100	100	100	100	
										A+B
	A	65	85	100	100	100	100	100	100	37.0 ppm
	B	40	70	100	100	100	100	100	100	
	C	100	100	100	100	100	100	100	100	
50	**A+B**	60	75	100	100	100	100	100	100	**B+C**

	B+C	100	100	100	100	100	100	100	100	17.5 ppm
	A+C	40	45	55	85	100	100	100	100	
	A+B+C	80	85	100	100	100	100	100	100	
										A+C
	A	100	100	100	100	100	100	100	100	81.25 ppm
	B	75	100	100	100	100	100	100	100	
	C	100	100	100	100	100	100	100	100	
100	**A+B**	95	100	100	100	100	100	100	100	**A+B+C**
	B+C	100	100	100	100	100	100	100	100	28 ppm
	A+C	55	55	75	100	100	100	100	100	
	A+B+C	85	100	100	100	100	100	100	100	

From obtained results, it can explain that sample A is better than sample B.But the LC_{50} of sample A+B was 37.0 ppm and this value is lower than that for both A (37.5) and B (62.5). This means, the cytotoxicity and activity of the oils increase when combined and could work better than in alone. The phenomenon can explain as the activity increased because of synergistic combination. In general it can be summarized that the cytotoxic activity against brine shrimp may be attributed by the presence of the major, quality and quantity of bioactive compounds of the essential oil. Therefore, might be one of the options and ways to increase the efficiency and reduce the amount of oils by synergistic combinations of oils.

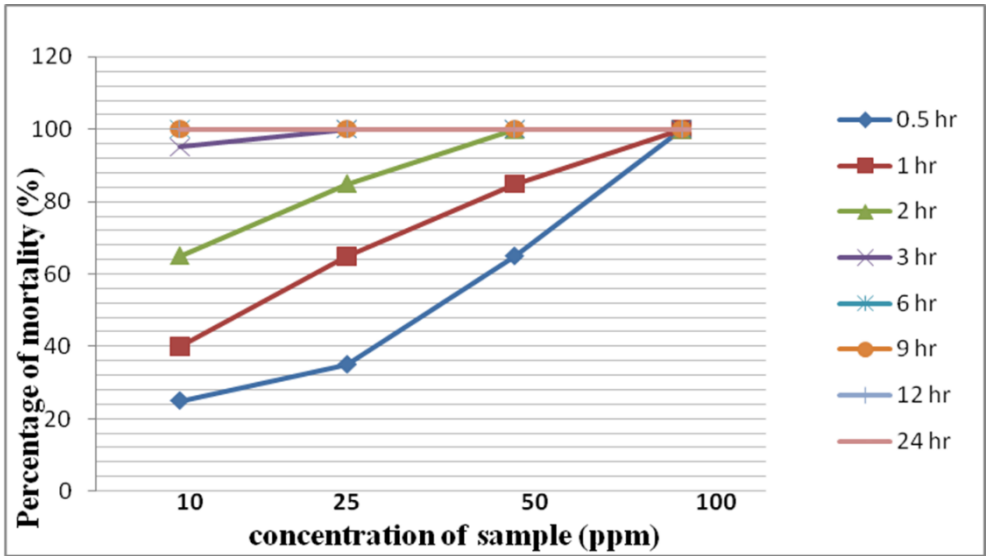

Figure 1. Cytotoxicity activity of sample A(lemongrass) essential oil

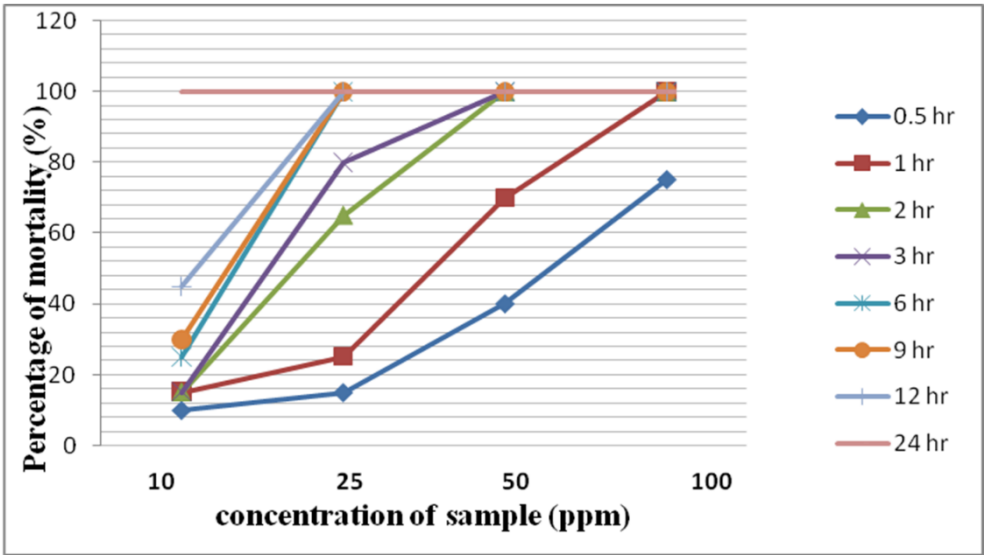

Figure 2. Cytotoxicity activity of sample B (basil) essential oil

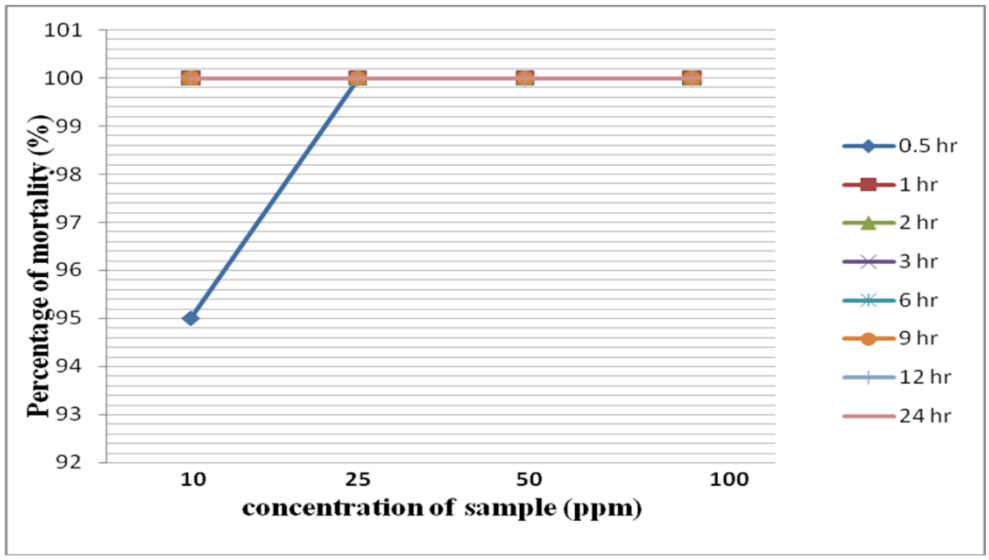

Figure 3. Cytotoxicity activity of sample C (Mexican mint) of essential oil

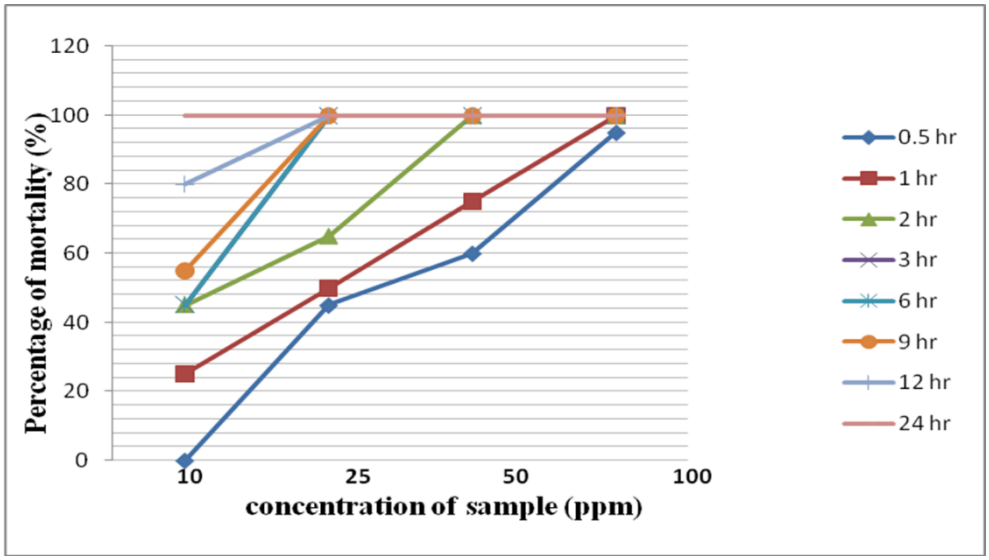

Figure 4. Cytotoxicity activity of combination (A+B) of essential oils

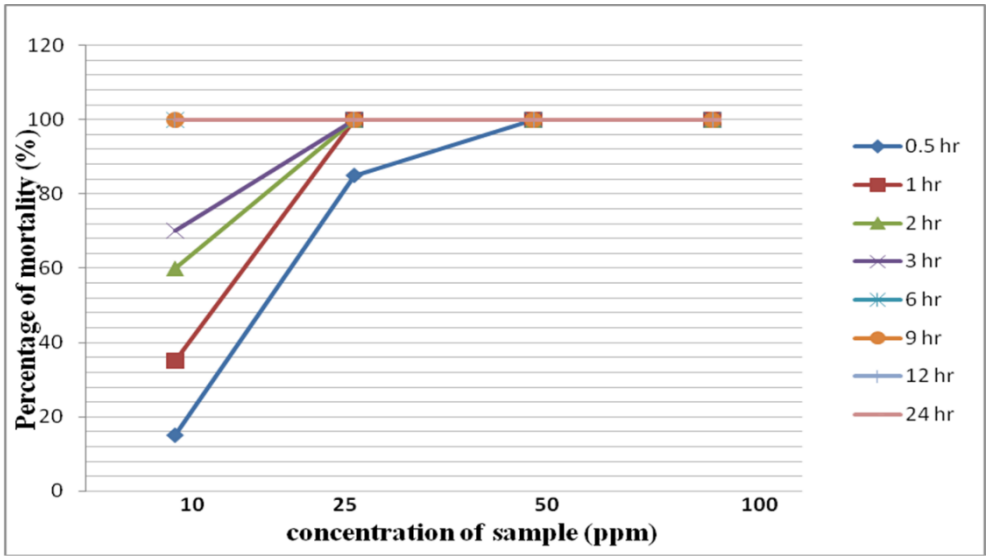

Figure 5. Cytotoxicity activity of combination (B+C) of essential oils

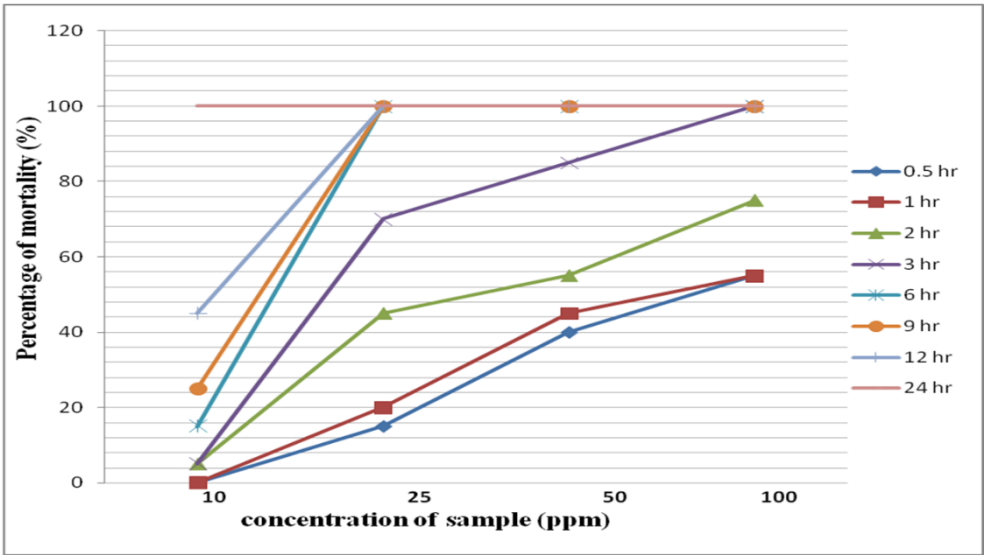

Figure 6. Cytotoxicity activity of combination (A+C) of essential oils

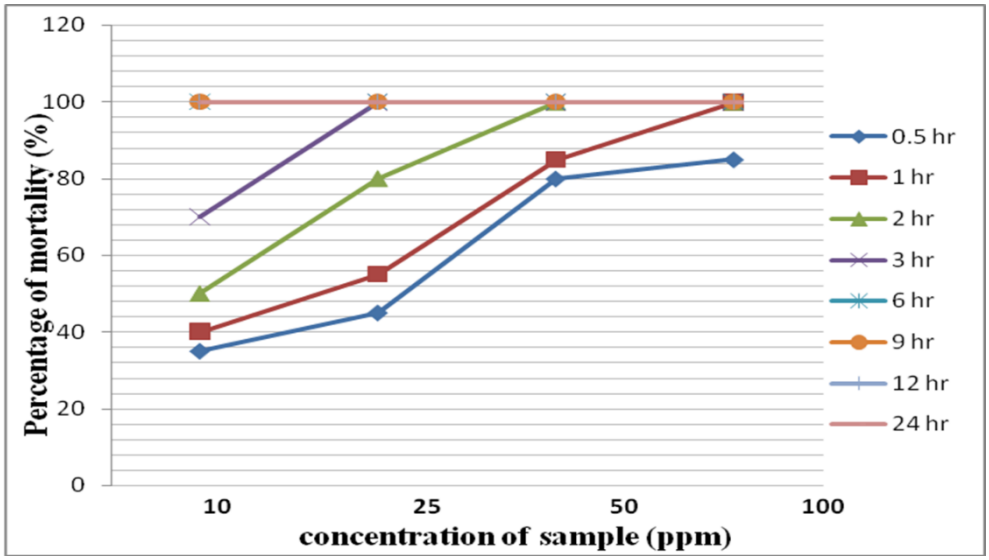

Figure 7. Cytotoxicity activity of combination (A+B+C) of essential oils

Acknowledgements

The authors thank Botanist of the School of Environmental Science and National Resources, National University of Malaysia; and Taman PertanianJubli Perak Sultan Haji Ahmad Shah Kuantan, Pahang, Malaysia for basil plant identification and lemongrass supplying resp.

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