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Chemical composition, anti-angiogenic and cytotoxicity activities of the essential oils of *Cymbopogan citratus* (lemon grass) against colorectal and breast carcinoma cell lines

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The essential oil of *Cymbopogan citratus* (lemon grass) was isolated by steam distillation method and subjected to cytotoxicity activity using two different cell lines, human colon carcinoma (HCT-116), breast carcinoma cell lines (MCF-7) and anti-angiogenic activity. The cytotoxicity activity study was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,4-tetrazolium bromide] assay and anti-angiogenic activity using rat aortic ring model. The chemical composition of the essential oil was determined by gas chromatography–mass spectrometry (GC–MS) and GC–flame ionization detection (FID). Forty-one compounds, representing 88.5% of the lemon grass oil was identified and the main components were neral (29.8%) and geranial (44.6%). The free radical scavenging activity of the essential oil showed an IC_{50} value of 156.1 μg/mL. The results showed that essential oil of lemon grass possessed potential cytotoxic effect on HCT-116 and with the IC_{50} value of 27.41 ± 4.3 μg/mL comparing to MCF-7 with IC_{50} value of 41.90 ± 1.2 μg/mL. Significant anti-angiogenic activity of the sample was observed with 99 ± 0.8% of inhibition at 100 μg/mL.

Keywords: angiogenesis; *Cymbopogan citratus*; cytotoxicity; essential oil; essential oil composition

Introduction

Recently, essential oils have gained popularity and scientific interest. Essential oils are natural, complex, multi-component systems composed mostly of terpenes with some other non-terpene components (1). The essential oils are demonstrated to show various pharmacological effects, such as spasmolytic, carminative, hepatoprotective, antiviral and anticarcinogenic effects (2, 3). *Cymbopogon citratus* (lemon grass) is a widely used herb, particularly in southeast Asia and Brazil, where it is used as a food flavoring, as a perfume, and has been found to possess medicinal properties (4) and contain 1 to 2% essential oil on a dry basis (5). Studies have shown that lemon grass has antidepressant, antioxidant, antiseptic, astringent, bactericidal, fungicidal, nervine and sedative properties (6) and the lemon grass oil is reported to have significant anticancer activity and leads to loss of tumor cell viability (7, 8). The anticancer activity of lemon grass oil was demonstrated by induction of Glutathione S-Transferase (GST). GST has been suggested to increase the host's ability to detoxify xenobiotics, including carcinogens.

Angiogenesis is a process of sprouting of new blood vessels from pre-existing vessels which is an essential feature of tissue remodeling associated with wound healing, solid tumor development, proliferative retinopathies, atherosclerosis and rheumatoid arthritis (9). Furthermore, angiogenesis is important for growth and metastasis of most solid malignancies and vascular endothelial growth factor-A (VEGF-A) which is assumed to be essential for tumor angiogenesis (10). Nicosia and Ottinetti (11) demonstrated that rat aorta rings reproducibly make micro vessel outgrowths in fibrin or collagen gels and supply a sensitive assay for the study of angiogenic agonists and antagonists in a chemically defined environment. In addition to that, tumor angiogenesis is considered to be a vital pharmacological target for cancer prevention and treatment (12–14).

Early reports also have indicated that essential oil components in lemon grass, especially monoterpenes, have multiple pharmacological effects on mevalonate metabolism which could account for the terpene–tumor suppressive activity (15). Monoterpenes have been shown to exert chemopreventive as well as chemotherapeutic activities in mammary tumor models and thus may represent a new class of therapeutic agents (16).

The aim of this study was to determine the cytotoxicity affect of essential oil extracted from locally grown lemon grass (*Cymbopogan citratus*) on colorectal (HCT-116) and breast (MCF-7) carcinoma cell lines using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,4-tetrazolium bromide] assay and angiogenic activity on rat aorta ring.

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Experimental

Plant material

The fresh plants of *Cymbopogon citratus* were obtained from Balik Pulau, Penang, Malaysia. Voucher specimens (11052) *C. citratus* was authenticated and deposited at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia.

Extraction of essential oil

The fresh plant sample was collected and submitted to water distillation for 3 hours using a modified Clevenger's apparatus. The ground samples (200 g) were boiled with water (200 mL) for 3 hours in a 1 L round bottom flask fitted with a condenser (yield 0.3% v/w). The extracted essential oils were dried over anhydrous sodium sulfate and after filtration, stored at 4°C until tested and analyzed.

Essential oil analysis

The essential oil was analyzed by gas chromatography–mass spectrometry (GC–MS) and GC using HP-5 capillary columns. The identification of essential oil components was performed by comparison of retention indices on mass spectra and co-injection of authentic standards. A standard solution of C_8–C_{40}, *n*-alkanes was used to obtain the retention index (RI). Mass spectra were obtained on an Agilent model 5973 MSD mass spectrometer, coupled directly to Agilent 6890 gas chromatograph fitted with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The GC–MS was operated under the following conditions. Separation was performed on HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The injector was set at 250°C using splitless mode. The initial oven temperature was set at 50°C (hold for 2 minutes) and increased at a rate of 10°C/minute to 300°C and held for 3 minutes. The flow rate of the carrier gas (helium) was maintained at 1.1 mL/minute. Mass spectra were recorded using an electron ionization (EI) system with an ionization energy of 70 eV. Quantification was performed using an Agilent 6890 gas chromatograph equipped with a flame ionization detector (FID) under the same conditions described earlier. FID temperature was set at 300°C. Relative percentage amounts of the separated compounds were calculated from FID chromatograms and shown in Table 1. The components were identified based on the comparison of their retention indices and mass spectra with those of standards, commercially available spectra [National Institute of Standards and Technology (NIST) mass spectral search program for NIST/EPA/NIH Mass Spectral Library V2.0] and literature data (17).

Free radical scavenging activity

The hydrogen atoms or electrons donation ability of the corresponding oils were measured from the bleaching of purple colored methanol solution of DPPH. The DPPH-radical scavenging activity was determined as per method of Shimada et al. (18). Thus, 50 μL of 1 mM DPPH (Sigma-Aldrich) in methanol was added to 200 μL of various concentrations of sample in methanol (6.25–100 μg/mL range). The solutions were incubated at room temperature for 30 minutes in the dark. The absorbance of the solutions, including a blank (containing all reagents except the sample) and a positive control (BHT, *tert*-butylated hydroxytoluene) was measured at 517 nm. The percentage of DPPH radical scavenged compared to the blank was determined using the following formula:

$$I\% = (A_{blank} - \frac{A_{sample}}{A_{blank}}) \times 100$$

where A_{blank}, absorbance of blank; A_{sample}, absorbance of sample.

Extract concentration giving 50% inhibition (IC_{50}) was extrapolated from the graph of inhibition percentage versus concentration of extract. Each sample assay was carried out in triplicate and data presented as a mean of the three values.

Antiangiogenic assay

Experimental animals

Four (12–18 weeks old) Sprague Dawley male rats were obtained from the animal unit facility of Universiti Sains Malaysia. The animals were kept for one week in the transit animal unit to acclimatize with the new environment. The rats were kept in ventilated cages for 12 hour light cycles with continuous supply of food and water. After euthanizing the animals by carbon dioxide (CO_2), a midline incision was made into the abdominal and thoracic cavities and the thoracic aortas were collected. The experiment was performed according to guidelines of Universiti Sains Malaysia Animal Ethics Committee and had their approval, reference USM/Animal Ethics Approval/2011/(66) (301).

Antiangiogenic effects on rat aortic rings

Antiangiogenic effect of the essential oils was investigated *ex vivo* on rat aortic rings according to the protocol reported by Brown et al. (19). Briefly, the cleansed thoracic aortas were cross-sectioned into thin rings of about 1 mm thickness. One ring was placed in the center of each well of 48-well plate containing 500 μl of M199 basal medium supplied with fibrinogen, aprotinin and l-glutamine at 3 mg/mL, 5 μg/mL and 1% wt/v, respectively. Then, 10 μL thrombin was added to each well. After 90 minutes incubation

Table 1. Composition of the essential oil of *Cymbopogan citratus*.

No.	Compound[a]	RI[b]	RI[c]	Percent[d]	[e]Identification of component[e]
1	α-thujene	931	925	0.1	MS,RI
2	α- pinene	939	935	0.1	MS,RI
3	camphene	946	949	0.1	MS,RI
4	2-octanone	986	983	1.1	MS,RI
5	myrcene	991	989	0.7	MS,RI
7	limonene	1029	1027	0.1	GC,MS,RI
8	eucalyptol	1032	1028	0.1	MS,RI
9	*trans*-β-ocimene	1037	1038	0.1	MS,RI
10	*trans*-linalool oxide	1073	1069	0.1	MS,RI
11	*cis*-linalool oxide	1087	1085	0.1	MS,RI
12	linalool	1097	1099	0.1	MS,RI
13	citronellal	1153	1149	0.7	MS,RI
14	rosefuran epoxide	1170	1172	0.8	MS,RI
15	citronellol	1226	1224	0.8	MS,RI
16	neral	1230	1236	29.8	GC,MS,RI
17	geraniol	1271	1267	2.7	MS,RI
18	geranial	1264	1266	44.6	GC,MS,RI
19	citronellyl acetate	1353	1353	0.2	MS,RI
20	geranyl acetate	1379	1382	1.9	MS,RI
21	β-elemene	1391	1397	0.2	MS,RI
22	α-gurjunene	1410	1408	0.7	MS,RI
23	β-caryophyllene	1417	1419	0.2	MS,RI
24	β-gurjunene	1409	1403	0.1	MS,RI
25	α-bergamotene	1434	1432	0.3	MS,RI
26	β-humulene	1440	1435	0.1	MS,RI
27	γ-gurjunene	1473	1478	0.1	MS,RI
28	α-amorphene	1512	1510	0.2	MS,RI
29	β-selinene	1490	1489	0.1	MS,RI
30	δ-selinene	1495	1493	0.1	MS,RI
31	α-muurolene	1500	1501	0.2	MS,RI
32	β-bisabolene	1505	1499	0.2	MS,RI
33	γ-cadinene	1513	1511	0.1	MS,RI
34	δ-cadinene	1524	1522	0.2	MS,RI
35	elemol	1549	1554	0.1	MS,RI
36	caryophyllene oxide	1581	1583	0.5	GC,MS,RI
37	ledol	1565	1569	0.1	MS,RI
38	humulene epoxide	1606	1611	0.1	MS,RI
39	*tert*-muurolol	1640	1643	0.4	MS,RI
40	*trans*-farnesol	1722	1728	0.1	MS,RI
41	*cis*-farnesal	1696	1691	0.2	MS,RI
	Percent of identification			88.5	
	Monoterpene hydrocarbons			3.1	
	Oxygenated monoterpenes			81.0	
	Sesquiterpene hydrocarbons			3.2	
	Oxygenated sesquiterpenes			1.2	

Notes: [a]Compounds listed in order of elution from a DB-5 column. [b]Retention index (RI) in reference to the literature (17). [c]Retention index (RI) in reference to *n*-alkanes on DB-5 column. [d]Relative percentages of the compounds are the mean of three runs and were obtained electronically from FID area percent data. [e]GC, co-injection with standards; MS, tentatively identified based on computer matching of the mass spectra of peaks with NIST/EPA/NIH library data; RI, identification based on comparison of retention index with those of published data (17).

at 37°C, another 500 µL M199 medium supplied with; fetal bovine serum (FBS) at 20% v/v, l-glutamine at 2 mM, aminocaproic acid at 1 mg/mL, amphotericin-B at 2.5 µg/mL and gentamicin at 60 µg/mL were added on top of the solidified bottom layer. Essential oils (6.25–100 µg/mL range) were also included in the top layer medium. The top layer medium was replaced with a fresh one containing the extracts after four days of incubation at 37°C in 5% CO_2. The magnitude of the growth of sprouting microvessels was quantified on day five according to Nicosia et al. (20). Briefly, the distance of growth of at least twenty points was measured for each ring. The results were presented as mean percent inhibition ± standard deviation (SD) (n = 6). Suramine was used as a positive control. The following formula was used to calculate the percentage of blood vessel inhibition:

$$\text{Percentage of blood vessel inhabition}(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{sample}, distance of blood vessel growth from the aorta ring in the sample; A_{control}, distance of blood vessel growth from the aorta ring in the control.

Cytotoxicity activity

Cell culture

Human colorectal carcinoma cell lines HCT-116 and human breast cancer cell lines MCF-7 were purchased from American Type Culture Collection (ATCC, USA). These cells were grown and maintained in a humidified incubator at 37°C, 5% CO_2 atmosphere. RPMI-1640 cell culture medium supplemented with 10% FBS and 1% Penicillin/Streptomycin (PS) was used as the growth medium of HCT-116. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% PS was used as the growth medium of MCF-7.

In vitro assay for cytotoxic activity (MTT assay)

The cytotoxicity was measured using MTT assay developed by Mosmann (21) with slight modifications. Briefly, after being harvested from culture flasks, the cells were seeded at 1.5×10^4 cells in each well of 96-well plate containing 100 µL of fresh growth medium per well and cells were permitted to adhere for 24 hours. The cells were treated with the lemon grass essential oil which were serially diluted with growth medium to obtain various concentration (100, 50, 25, 12.5, 6.25, 3.125 µg/mL). Then, 100 µl of each concentration was added to each well. After 48 hours of treatment the medium was aspirated and the cells were washed once with sterile phosphate buffered saline (PBS). To each well 5 mg/mL of MTT in PBS was added at 10% v/v and the plate was incubated at 37°C in 5% CO_2 for 3 hours. The medium was discarded and 200 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the dark blue crystals of formazan salt. After incubation at 37°C for 10 minutes, the absorbance was measured at a primary wavelength of 570 nm and a reference wavelength of 650 nm, using a Multiskan Ascent microplate reader. Each plate contained the essential oils, a negative control (0.1% DMSO) and a blank. Cytotoxicity is expressed as the percentage of inhibition of cell growth ± SD. All tests and analyses were run in triplicate.

Statistical analysis

The results were stated in mean ± SD. The differences between the cell lines were analyzed using one-way and two-way analysis of variance (ANOVA) with $p < 0.05$. This statistical analysis was carried out using the GraphPad Prism 5.

Results and discussion

The steam distillation of lemon grass yields 0.3% (w/w) on wet weight basis. Forty-one compounds, representing 88.5% of the lemon grass oil were identified. The qualitative analytical results are shown in Table 1. GC–MS and GC–FID analysis revealed that the constituents of lemon grass oil have been grouped into four sub-classes with monoterpene hydrocarbons 3.1%, oxygenated monoterpene 81.0%, sesquiterpene hydrocarbons 3.2%, and oxygenated sesquiterpene 1.2%. The major compounds in the oil were neral (29.8%) and geranial (44.6%) which isomerically forms citral and geranyl acetate (1.9%). The chemical composition of the studied lemon grass essential oil showed similarities to the previously reported studies (4, 5, 22).

The antioxidant potential of an essential oil can be mainly attributed to its reducing power, radical scavenging ability and singlet oxygen quenching ability. The effect of antioxidants of essential oils on the DPPH radical scavenging was determined by their hydrogen donating ability. $DPPH^{\cdot}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (23). The scavenging effects of lemon grass oil on DPPH were examined at different concentrations (6.25–100 µg/mL) and the IC_{50} value was determined and shown in Table 2. A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value. The antioxidant activity of the essential oil of lemon grass showed an IC_{50} value of 156.1 µg/mL. The antioxidant activities were compared with the standard commercial synthetic antioxidant, butylated hydroxytoluene (BHT). The IC_{50} of BHT was evaluated at 18.46 µg/mL.

Table 2. Antioxidant activity of the essential oil of *Cymbopogan citratus* and BHT in DPPH free radical-scavenging activity.

Sample	IC_{50} of DPPH radical-quenching activity (µg/mL)[a]
Cymbopogan citratus	156.1
BHT[b]	18.46

Notes: [a]IC_{50} values of DPPH assay (as lg/ml). [b]Butylated hydroxyl toluene.

Table 3. Anti-angiogenic activity of essential oil of *Cymbopogan citratus* and suramine on rat aortic ring assay.

Samples	Concentration (μg/mL)	Percentage of inhibition (%) ± SD
Cymbopogan citratus	100	99 ± 0.8*
	50	42 ± 1.5*
	25	21 ± 1.8
	12.5	18 ± 1.9*
	6.25	6 ± 1.6
Suramine[a]	10	39 ± 2.7

Notes: [a]Suramine as positive control. *$p < 0.05$, evaluated by one-way analysis of variance (ANOVA).

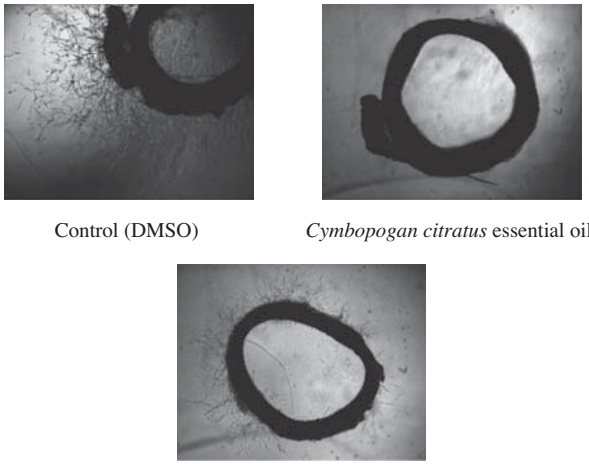

Figure 1. Effect of essential oil of *Cymbopogan citratus* on sprouting vessel of rat aortic ring. Vascular vessels sprouting from each ring was photographed at a 100× magnification using a Leica inverted phase-contrast microscope equipped with the quick imaging system. Representative photographs were indicated from six replicates.

The rat aortic assay is regarded to mimic the *in vivo* environment because it involves non-endothelial cells besides the endothelial cells and the endothelial cells themselves are not preselected by sub-culturing and thus are not in the proliferation state at the time of explanation and more representative of real life (24). The effects of the lemon grass essential oil are illustrated in Figure 1 and the percentage of inhibition of the growing blood vessels from the rat aortic ring by the essential oil (Figure 2) is shown in Table 3. The lemon grass oil showed significant anti-angiogenic activity with percentage of inhibition of 99 ± 0.8% at 100 μg/mL while suramine showed 39 ± 2.7% inhibition at 10 μg/mL. IC_{50} of lemon grass oil was calculated at 52.3 μg/mL ($y = 0.962x - 0.2958$, $R^2 = 0.9839$).

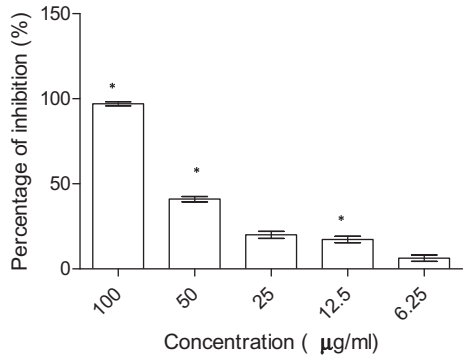

Figure 2. Inhibition of growth of new blood vessel by essential oil of lemon grass using various concentrations (12.5–100 μg/mL). Data are expressed as means ± SD based on six replicates. [*$p < 0.05$, evaluated by one-way analysis of variance (ANOVA).]

The anti-proliferative effects of the essential oils on human cancer cell lines were examined by MTT assay. Two human cancer cell lines, colorectal carcinoma cell line (HCT-116) and breast carcinoma cell line (MCF-7) were tested in a dose-dependent manner of lemon grass essential oil. Table 4 shows the percentage of growth inhibition by the lemon grass oil on both cell lines. The oil showed 13.4 ± 16.7% inhibition on MCF-7 while 43.0 ± 4.7% inhibition on HCT-116 at a concentration of 25 μg/mL. The results showed significant difference ($p < 0.01$) of inhibition by the oil against two cell lines (Figure 3). Between two cell lines, the HCT-116 cell line was more sensitive to the lemon grass essential oil with an IC_{50} of 32.13 μg/mL and 47.31 μg/mL against MCF-7. The betulinic acid as a positive control showed 18.0 ± 1.2% inhibition on MCF-7 and 77.1 ± 1.0% inhibition on HCT-116 at a concentration of 10 μg/mL.

The cytotoxic activity of lemon grass essential oil on both cell lines and anti-angiogenic activity are more potent due to the presence of some potential anti-cancer and anti-angiogenic compounds such as citral, limonene, geranial, and myrcene. In addition to that, monoterpenes, which are one of the major classes in lemon grass essential oil have been shown to show chemopreventive as well as chemotherapeutic activities in mammary tumor models and thus may represent a new class of therapeutic agents (1). The monoterpenes also have been reported to show multiple pharmacological effects on mevalonate metabolism which could account for the terpene–tumor suppressive activity (14). Citral, which is a major compound in lemon grass essential oil as well as many other essential oils, is a novel monoterpene inducer of GST class I, (GSTP1). This isozyme is accountable for the increase in the total GST activity of the citral-treated rat hepatocyte cells (25). It is found that the *trans*-citral (geranial) was the main contributor

Table 4. Cytotoxicity of the essential oil from *Cymbopogan citratus* on two human cancer cell lines.

Sample	Concentration (μg/mL)	Percentage of inhibition (%) ± SD[a]	
		MCF-7	HCT-116
Cymbopogon citratus	100	99.8 ± 0.5	99.4 ± 0.3
	50	72.2 ± 0.7	86.4 ± 0.4
	25	13.4 ± 16.7*	43.0 ± 4.7*
	12.5	7.4 ± 4.2*	21.9 ± 6.3*
[a]5-FU[b]	10	18.0 ± 1.2	77.1 ± 1.0

Note: [a]Data are mean ± standard deviation (SD). [b]5-FU, fluorouracil as a positive control. *$p < 0.05$, evaluated by two-way analysis of variance (ANOVA).

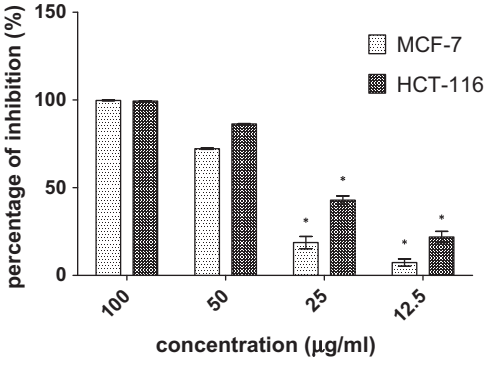

Figure 3. Inhibition of breast carcinoma cell line (MCF-7) and colorectal carcinoma cell line (HCT-116) by essential oil of lemon grass using various concentrations (12.5–100 μg/mL). Data are expressed as means ± SD based on triplicates. [*$p < 0.05$, evaluated by two-way analysis of variance (ANOVA).]

for the induction of GSTP1. The citral is also studied as a cancer chemopreventive agent targeted towards inflammation-related carcinogenesis such as skin cancer (26) and colon cancer (27). d-Limonene showed anti-angiogenic and proapoptotic effects on human gastric cancer implanted in nude mice, thus inhibiting tumor growth, metastasis (28) and chemopreventive efficacy in preclinical hepatocellular carcinoma models (29–31). Geraniol elicited a drastic reduction in the amounts of thymidylate synthase (TS) and thymidine kinase (TK) expression in colon cancer cells (32).

Conclusion

The studies on the lemon grass essential oil showed promising activity on the cytotoxicity against colorectal carcinoma cell line. Thus, bioassay guided fractionation will be conducted to determine the active compound against the colorectal cancer cell line growth.

Conflict of interest

The authors declare that there are no conflicts of interest.

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