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ORIGINAL ARTICLE

Antioxidant, anti-hyaluronidase and antifungal activities of *Melaleuca leucadendron* Linn. leaf oils

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Abstract The present study examined the chemical composition, in vitro antioxidant, anti-hyaluronidase and antifungal activities of essential oils of *Melaleuca leucadendron* Linn. from Gundih-Central Java, Indonesia in different plant ages of 5, 10 and 15 years old. The Chemical composition of essential oils were analyzed by GC/MS. Twenty-six components were identified, of which 1,8-cineole (49.22–55.04 %), α-terpineol (8.79–10.70 %), *d*-limonene (5.58–6.39 %), and β-caryophyllene (5.03–7.64 %) were the main compounds in these oils. The antioxidant assay and anti-hyaluronidase assay showed that *M. leucadendron* leaf oils possess mild antioxidant activity with IC_{50} between 7.21 and 9.23 mg/ml and anti-hyaluronidase activity with IC_{50} between 1.94 and 3.03 mg/ml. The antifungal assay showed the effectiveness of these essential oils against *Fomitopsis palustris* (IC_{50} 0.12–3.16 mg/ml), *Trametes versicolor* (IC_{50} 0.01–0.06 mg/ml), *Cladosporium cladosporioides* (IC_{50} 0.03–0.49 mg/ml), and *Chaetomium globosum* (IC_{50} 0.06–0.15 mg/ml).

Keywords *Melaleuca leucadendron* Linn. · Essential oil · Antioxidant · Anti-hyaluronidase · Antifungal

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Introduction

Essential oils and their components have recently been of great interest because of their relatively safe status, wide acceptance by consumer and the possibility of their exploitation for potential multi-purpose functional uses [1]. Many researchers have reported utilization of *Melaleuca* essential oils, mainly used in the manufacture of cosmetics, germicides, as clinical efficacy and medicinal. These essential oils are also used as antiseptic, antibacterial, antiviral, antifungal, antiprotozoal, antioxidant, anti-inflammatory agents and oral cleaner [2–5]. Genus *Melaleuca* is currently represented by approximately 250 species, one species that is found in Indonesia and can produce commercial essential oil is *Melaleuca leucadenron* Linn. This species is one of the most important essential oil producing species. Leaves and stems of this species produce strongly scented essential oils, some of which have useful medicinal properties [6]. Essential oil from this species is widely used in Indonesia as an expectorant for throat preparations and as ointments for stomach upsets and mosquito bites. However, there is no sufficient scientific information about bioactivities of *M. leucadendron* leaf oil from Indonesia.

In the previous study [7], we investigated the chemical components of *M. leucadendron* Linn. leaf oils from Java, Indonesia. The results showed that leaf essential oils of *M. leucadendron* from Java, Indonesia contained 26 compounds which were mostly monoterpenes, sesquiterpenes and related alcohols. GC/MS analyses have shown the presence of 1,8-cineole, α-terpineol, *d*-limonene, and β-caryophyllene as major compounds in these oils. The aforementioned study indicates that some important compounds which can be considered as bioactive agents are contained in these oils.

The antioxidant activity of plant extracts has been recently highlighted [8, 9] due to the fact that free radicals such as reactive oxygen species (ROS) are responsible for various diseases and salinity [10]. The human health promoting effects of plants were elucidated to be due to some of bioactive substances such as essential oils having antioxidant activity [11]. Several chemical constituents from essential oils also have been reported to inhibit hyaluronidase activity and display anti-inflammatory effects; they also have significant ecological functions for the protection against fungal infections [12].

Therefore, this present study was carried out to investigate *M. leucadendron* leaf oils from Gundih, Central Java, Indonesia at 3 plant ages (5, 10 and 15 years) and its major components in order to evaluate their effectiveness as antioxidant, anti-hyaluronidase and antifungal agents. Antioxidant activities in this study were investigated in vitro by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, anti-hyaluronidase activities by using hyaluronidase enzyme and antifungal activities by agar diffusion method against six fungal species.

Materials and methods

Essential oils and GC/MS analysis

Fresh samples of *M. leucadendron* leaves were collected from plantations of *M. leucadendron* in Gundih, Central Java Province, Indonesia in different plant ages of 5 (A1), 10 (A2) and 15 (A3) years old. The 5–7 kg of fresh leaves from each sample was extracted by hydrodistillation method for 5 h. The oils produced were kept in a labeled bottle at approximately 0 °C until used.

The chemical analysis of the oils was conducted by a GC-17A gas chromatograph (GC) coupled to a QP5050A mass spectrometer (Shimadzu Co. Ltd., Kyoto, Japan) using a fused-silica capillary column TC-1701 (0.25 mm i.d. × 15 m, 0.25 μm film thickness; GL Sciences). GC/MS was performed using the following conditions: carrier gas He; flow rate 20.6 ml/min; splitless injection; injection volume 1.0 μl; injection temperature 230 °C; oven temperature programmed from 30 °C (5 min hold) to 100 °C at 10°C/min (5 min hold), and from 100 to 230 °C at 15°C/min (5 min hold); interface temperature 230 °C; and electron-impact ionization at 70 eV. The identification of oil components was confirmed by comparison of retention time with those of authentic samples (α-terpineol, *d*-limonene, 1,8-cineole, *β*-caryophyllene, eugenol), and with National Institute of Standards and Technology (NIST) database library, and Kovats retention index (RI) [13] which was calculated from the retention times of authentic aliphatic saturated hydrocarbons (C_8–C_{22}, Wako Chemicals Co.) and compared with literature data [14].

Antioxidant assay

The antioxidant activities of *M. leucadendron* leaf oils were evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. An ethanol solution (1 ml) of sample was added to a solution (10 ml) of 0.25 mM DPPH in ethanol. Absorbance of blank sample containing the same amount of ethanol and DPPH was measured as a control. The solution was rapidly mixed and was kept in water bath at 30 °C for 30 min and absorbance was measured at 515 nm by using U-2810 spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). The percent of inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [(A_c - A_s)/A_c] \times 100,$$

where A_c is absorbance of the control and A_s is the absorbance of the sample tested. IC_{50} values, which represent the concentrations of the essential oils that cause 50 % inhibition is determined by linear regression analysis.

Anti-hyaluronidase assay

Anti-hyaluronidase activity was examined by the Lee and Choi [15] method with slight modifications. Sample solutions (1, 2 and 4 mg/ml) were prepared by using *M. leucadendron* leaf oils or authentic compounds dissolved in mixed solvent (5 % DMSO in ethanol). Fifty μl of ovine hyaluronidase (7900 U/ml) dissolved in 0.1 M acetate buffer (pH 3.5) was mixed with 100 μl of each sample solution, and then incubated for 20 min in a water bath at 37 °C. One hundred μl of 12.5 mM calcium chloride was added to the reaction mixture, and then the mixture was incubated for 20 min in a water bath at 37 °C. The hyaluronidase activated by Ca^{2+} was reacted with 250 μl of sodium hyaluronate (1.2 mg/ml) dissolved in 0.1 M acetate buffer (pH 3.5), and then incubated in a water bath at 37 °C for 40 min. 100 ml of 0.4 N sodium hydroxide and 100 μl of 0.4 M potassium borate were added to the reaction mixture, and then incubated in a boiling water bath for 3 min. After cooling to room temperature, 1.5 ml of dimethylaminobenzaldehyde (PDMAB) was added to the reaction mixture, and then incubated in a water bath at 37 °C for 20 min. Optical density (OD) at 585 nm of the reaction mixture was measured by using U-2810 spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). The percentage of inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [(OD_c - OD_s)/OD_c] \times 100,$$

where OD_c is optical density of control and OD_s is optical density of the sample tested. IC_{50} values, which represent the concentrations of the essential oils that cause 50 % inhibition is determined by linear regression analysis.

Antifungal assay

Antifungal activity was examined by Wang et al. [16] method with slight modifications against wood rot fungi of *Fomitopsis palustris* NBRC 30339 and *Tramestes versicolor* NBRC 4937, and household molds of *Aspergillus niger* NBRC 6342, *Cladosporium cladosporioides* NBRC 6348, *Chaetomium globosum* NBRC 6347, and *Penicillium citrinum* NBRC 6352. PDA (Potato Dextrose Agar, Difco) plates were prepared for fungal media using Petri dishes (9 cm diameter). The different concentration of the oil samples and major compounds were serially diluted with methanol and added to 20 ml of PDA. Petri dishes are kept in clean bench at room temperature for a day and methanol is removed by vaporization. Each agar mycelium plug was inoculated at the center of the Petri dish and incubated in dark at 25 °C. Colony growth diameter was measured every day for 14 days or while the fungal growth in the control treatment had completely covered the media in the Petri dishes. PDA plates used as a control contain only methanol without essential oil or authentic compound solutions. Growth inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [1 - (S_a/S_b)] \times 100,$$

where S_a is surface area of mycelium growth of treatment (cm^2) and S_b is surface area of mycelium growth of control (cm^2). The IC_{50} value of antifungal activity for each sample was graphically estimated using logarithmic or linear regression analysis by extrapolation of percent inhibition versus concentration.

Statistical analysis

Data were analyzed using SPSS (IBM) and Excel program. All tests and analyses were run in triplicate and averaged. Data were compared using Scheffe's test and expressed as mean ± SD. Results with $p < 0.05$ were considered to be statistically significant.

Results and discussion

GC/MS analysis of essential oil

Our previous study of *M. leucadendron* leaf oils from Java, Indonesia showed 26 compounds in these essential oils identified by GC/MS analysis [7]. This study showed that the components of essential oils from Gundih, in different plant ages are similar to those in previous ones with a few differences in percentages of the contents. These oils were composed of hydrocarbons and alcohols, mainly monoterpenes and sesquiterpenes. Table 1 shows that *M. leucadenron* leaf oils from plant ages 5 (A1) and 10 years (A2) contained 26 compounds and from plant age 15 years (A3) contained 24 compounds. The oils were rich in 1,8-cineole (49.22–55.04 %), α-terpineol (8.79–10.70 %), *d*-limonene (5.58–6.39 %), and *β*-caryophyllene (5.03–7.64 %). The increase of tree age results in decrease of 1,8-cineole content, adversely increase of *β*-caryophyllene content. The highest quantities of α-terpineol and *d*-limonene were obtained from A2 and A1, respectively.

Table 1 Chemical compounds (%) of *M. leucadendron* leaf oils

RI[a]	Compound	Percentage (%)		
		A1	A2	A3
944	α-Thujene[c]	0.28	0.31	0.29
948	α-Pinene[b]	2.12	1.82	2.18
989	2-Pentanone[c]	1.74	1.76	1.65
1001	*β*-Pinene[c]	1.72	1.37	1.64
1017	*β*-Myrcene[d]	0.68	0.74	0.67
1037	Carene[b]	0.36	0.63	0.71
1044	*d*-Limonene[b]	6.39	5.58	5.92
1062	1,8-Cineole[b]	55.04	51.32	49.22
1073	*γ*-Terpinene[c]	2.39	2.76	3.15
1101	Terpinolene[b]	1.16	1.56	1.68
1199	Linalool[b]	0.16	0.08	0.17
1298	Terpinene-4-ol[b]	0.63	0.67	0.71
1314	Ocimenol[c]	0.10	0.11	0.11
1330	α-Terpineol[b]	8.79	10.70	10.42
1400	Cedrene[b]	0.14	0.19	–
1443	*γ*-Terpineol[c]	1.57	1.13	1.16
1485	*β*-Caryophyllene[b]	5.03	7.33	7.64
1529	Eugenol[b]	3.27	4.55	4.35
1547	Humulene[c]	0.53	0.75	0.84
1556	*β*-Eudesmene[c]	2.30	2.18	2.23
1560	Patchoulene[c]	2.80	2.09	2.91
1576	Germacrene D[c]	0.17	0.24	0.33
1637	Aromadendren[c]	0.16	0.12	–
1738	Globulol[b]	1.28	1.37	1.61
1806	Viridiflorol[c]	0.16	0.29	0.25
1814	Cubenol[c]	1.00	0.17	0.18
	Total (%)	99.97	99.82	100.00

–, no compound

[a] Retention indices on the column relative to C_8–C_{22} *n*-alkanes

[b] Compounds were identified by comparison with authentic compounds

[c] Compounds were identified by comparison with National Institute of Standards and Technology (NIST) database library

Table 2 Antioxidant activities and IC $_{50}$ of *M. leucadendron* leaf oils and authentic compounds by DPPH method

Sample	Concentration (mg/ml)	Inhibition[A] (%)	IC_{50} (mg/ml)
A1	5	23.77 ± 0.23b	9.23
	7.5	39.58 ± 0.09c	
	10	54.59 ± 0.45e	
A2	5	29.41 ± 0.49b	7.21
	7.5	50.53 ± 0.34de	
	10	78.96 ± 1.09g	
A3	5	27.10 ± 0.15b	7.71
	7.5	44.33 ± 0.34d	
	10	72.79 ± 0.90g	
1,8-cineole	2.5	23.29 ± 0.18b	4.92
	5	52.64 ± 0.15e	
	7.5	76.60 ± 0.08g	
d-limonene	2.5	28.49 ± 0.25b	4.58
	4	44.31 ± 0.02d	
	5	54.17 ± 0.09e	
β-caryophyllene	1	21.73 ± 0.28b	3.68
	3	44.67 ± 0.04d	
	5	60.76 ± 0.13e	
α-terpineol	2.5	29.06 ± 0.23b	4.59
	4	44.41 ± 0.04b	
	5	53.88 ± 0.06e	
BHA (butylated hydroxyanisole)	20×10^{-3}	43.47 ± 0.41d	25.68×10^{-3}
	50×10^{-3}	71.41 ± 0.14g	
	80×10^{-3}	82.09 ± 0.23g	
Eugenol	3×10^{-4}	8.29 ± 0.91a	3.25×10^{-3}
	6×10^{-4}	25.79 ± 0.07b	
	6×10^{-3}	82.08 ± 0.22g	

[A] The value in the column followed by the same letter is not significantly different at $P < 0.05$

Table 3 Anti-hyaluronidase activities and IC_{50} of anti-hyaluronidase of *M. leucadendron* leaf oils and some authentic compounds

Sample	Sample solution (mg/ml)	Inhibition[A] (%)	IC_{50} (mg/ml)
A1	1	0.00 ± 0.00a	3.03
	2	27.08 ± 2.09b	
	4	72.55 ± 0.05d	
A2	1	6.25 ± 0.25a	2.67
	2	45.83 ± 0.03c	
	4	75.49 ± 0.99d	
A3	1	31.25 ± 6.23b	1.94
	2	58.33 ± 0.03c	
	4	77.45 ± 2.94d	
1,8-cineole	1	44.93 ± 0.10c	1.17
	2	65.60 ± 0.55d	
	4	87.45 ± 1.16e	
d-limonene	1	ne	–
	2	ne	
	4	ne	
	d-limonene only[B]	ne	
β-caryophyllene	2×10^{-3}	22.31 ± 0.02b	4.16×10^{-3}
	4×10^{-3}	47.63 ± 0.03c	
	6×10^{-3}	73.85 ± 0.05d	
α-terpineol	1	ne	–
	2	ne	
	4	ne	
	α-terpineol only[B]	ne	

ne no effect of authentic compounds as anti-hyaluronidase, – no IC_{50}

[A] The value in the column followed by the same letter is not significantly different at $P < 0.05$

[B] Sample solution used for testing was the authentic compound without diluting solvents

Antioxidant assay

The DPPH radical scavenging activities of various concentrations of *M. leucadendron* leaf oils are shown in Table 2. The in vitro antioxidant activities of the oils were also compared with those of BHA (butylated hydroxyanisole) and eugenol.

Table 2 shows that the radical scavenging activities of A1 (IC_{50} 9.23 mg/ml), A2 (IC_{50} 7.21 mg/ml), A3 (IC_{50} 7.71 mg/ml) and four major compounds of 1,8-cineole (IC_{50} 4.92 mg/ml), *d*-limonene (IC_{50} 4.58 mg/ml), *β*-caryophyllene (IC_{50} 3.68 mg/ml), and α-terpineol (IC_{50} 4.59 mg/ml) were relatively low if compared with the single compounds like BHA (IC_{50} 25.68×10^{-3} mg/ml) and eugenol (IC_{50} 3.25×10^{-3}). BHA and eugenol, usually utilized as commercial antioxidant agents have strong radical scavenging activity. The essential oil of A1 had lower antioxidant activity than A2 and A3 (Table 2). This is probably due to the presence of phenolic compounds such as eugenol, A1 has lower eugenol content (3.27 %) than A3 (4.35 %) and A2 (4.55 %). Several studies also reported the phenolic compounds like eugenol exhibited high antioxidant activity [17, 18]. Previous studies on antioxidant properties of *Eucalyptus camaldulensis* leaf oils [19], *Aframomum corrorima* essential oil [20], Italian large leaf, purple ruffles, cinnamon, and lemon basil oils [21], show that the antioxidant activity of the essential oils are usually lower than those of commercial antioxidants, but the content of phenolic compounds like eugenol, thymol etc., largely influences this ability of essential oils. In this study *M. leucadendron* leaf oils possess variable radical scavenging activities, probably due to the different quantity of eugenol in these essential oils. Their mild antioxidant activity is assumed to be moderate for several purposes, such as ointments, plasters, etc.

Table 4 Antifungal indices of *M. leucadendron* Linn. leaf oils

Fungi	Conc. (mg/ml)	Antifungal indices (%)		
		A1	A2	A3
F. palustris	2.5	34.01 ± 6.43de	68.78 ± 7.49bcd	38.58 ± 10.92cde
	5	81.55 ± 17.94ab	87.75 ± 3.10ab	72.49 ± 2.90abc
	10	95.36 ± 0.64a	100.00 ± 0.00a	100.00 ± 0.00a
T. versicolor	0.05	25.90 ± 4.80ef	34.10 ± 4.49def	57.16 ± 2.38bcd
	0.1	85.05 ± 4.15abc	96.41 ± 0.98a	90.52 ± 3.34a
	1	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a
A. niger	5	18.20 ± 6.51e	22.63 ± 3.37de	27.13 ± 5.54de
	10	27.83 ± 0.80de	32.48 ± 2.30de	30.03 ± 2.93de
	15	55.81 ± 3.44b	60.00 ± 2.50b	57.87 ± 2.45b
	20	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a
C. cladosporioides	1	79.61 ± 1.85b	69.05 ± 2.66bc	61.88 ± 5.73c
	5	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a
C. globosum	0.1	39.60 ± 14.20bc	58.05 ± 6.53b	52.78 ± 1.13bc
	1	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a
P. citrinum	5	33.66 ± 11.85cd	50.04 ± 8.41bcd	49.37 ± 8.30bcd
	10	55.10 ± 10.35bcd	79.90 ± 1.22a	78.58 ± 1.83ab

The value in the column followed by the same letter is not significantly different at $p < 0.05$

Anti-hyaluronidase assay

Hyaluronidase is an enzyme that depolymerizes the hyaluronic acid (HA) in the extracellular matrix of connective tissue, it is found both in organs (testis, spleen, skin, eye, liver, kidney, uterus and placenta) and in body fluids (tears, blood and sperm) [22, 23]. The enzyme is known to be involved in allergic effects [24], migration of cancer [25], inflammation, petechial hemorrhages following its injection in mesentery preparations, and also an increase in the permeability of the vascular system [26]. High molecular weight HA has an important role in the regulation of scarless repair in fatal wound healing by markedly diminishing the inflammatory response. Enzyme hyaluronidase degrades HA by lowering its viscosity and increasing the permeability. Degradation products of HA lead to increase inflammation, angiogenesis, fibrosis, and collagen deposition in wound healing [27].

The effect of *M. leucadendron* leaf oils as anti-hyaluronidase was investigated in this study. The IC_{50} values of anti-hyaluronidase assays of *M. leucadendron* leaf oils were from 1.94 to 3.03 mg/ml. The result showed that A1 (IC_{50} 3.03 mg/ml) had lower anti-hyaluronidase activity than A3 (IC_{50} 1.94 mg/ml) and A2 (IC_{50} 2.67 mg/ml). Four major compounds of *M. leucadendron* leaf oil were also tested for their anti-hyaluronidase activity. The results showed that *β*-caryophyllene had high anti-hyaluronidase activity (IC_{50} 4.16×10^{-3} mg/ml), 1,8-cineole possess low anti-hyaluronidase activity (IC_{50} 1.17 mg/ml), and *d*-limonene and α-terpineol showed no anti-hyaluronidase activity (Table 3). Probably the content of *β*-caryophyllene in the oils affects the anti-hyaluronidase activity of A1 lower than those of A2 and A3. Several studies show that sesquiterpenes have a calming effect as well as inhibition of hyaluronidase (anti-inflammatory) and anti-infectious effects. *β*-Caryophyllene is a sesquiterpene widely distributed in essential oils of various plants and known to possess anti-inflammatory effect [28]. Some studies also show phenolic compounds have high anti-hyaluronidase. [29–31] This study indicates that *M. leucadendron* leaf oils possess moderate anti-hyaluronidase activity caused from *β*-caryophyllene and slightly 1,8-cineole. Although anti-hyaluronidase activity of *M. leucadendron* leaf oil is not so large, it seems to increase in proportion to the content of *β*-caryophyllene.

Antifungal assay

The antifungal indices of the oil samples and the major compounds against 6 fungi are shown in Tables 4 and 5, respectively. The IC_{50} values of oils and major compounds were determined using various concentrations of these substances encompassed their values.

The antifungal activities of the *M. leucadendron* leaf oils against wood rot fungi are shown in Table 4, using the oil concentration of 2.5, 5 and 10 mg/ml for *F. palustris* and the oil concentration of 0.05, 0.1, 1 mg/ml for *T. versicolor*. Essential oils of *M. leucadendron* showed higher inhibitory effects against *T. versicolor* than against *F. palustris*, complete inhibition against *T. versicolor* was achieved at concentration of 1 mg/ml. Antifungal indices of major compounds showed α-terpineol was the highest inhibitory effect against *F. palustris* and *T. versicolor,* followed by 1,8-cineole, *β*-caryophyllene, and *d*-limonene.

Table 5 Antifungal indices of authentic compounds against 6 fungi

Fungi	Conc. (mg/ml)	Antifungal indices (%)			
		1,8-Cineole	*d*-Limonene	*β*-Caryophyllene	α-Terpineol
F. palustris	0.1	0.00 ± 0.00e	ne	–	0.00 ± 0.00e
	1	44.33 ± 10.07cd	ne	0.00 ± 0.00e	57.21 ± 2.95bc
	2.5	77.60 ± 9.38ab	ne	–	100.00 ± 0.00a
	5	100.00 ± 0.00a	ne	26.45 ± 7.73d	–
	10	–	ne	32.60 ± 10.42cd	–
	15	–	–	54.11 ± 6.10bc	–
T. versicolor	0.1	13.96 ± 5.90f	24.76 ± 7.89ef	29.86 ± 9.12def	41.66 ± 3.93cde
	1	25.81 ± 7.92ef	42.57 ± 4.18cde	46.22 ± 1.55cde	–
	5	100.00 ± 0.00a	61.50 ± 8.27bc	53.73 ± 7.44cd	100.00 ± 0.00a
	10	–	84.82 ± 3.59ab	92.68 ± 3.06a	–
A. niger	0.1	0.00 ± 0.00i	17.37 ± 7.63gh	13.68 ± 0.92hi	37.99 ± 2.86def
	0.5	–	–	–	40.94 ± 7.83cd
	1	0.71 ± 0.00i	40.66 ± 4.35cd	16.29 ± 1.03gh	100.00 ± 0.00a
	2.5	12.30 ± 1.64hi	45.58 ± 2.21cd	24.59 ± 0.47fgh	–
	5	25.71 ± 1.58fgh	52.19 ± 2.20c	31.46 ± 1.02def	–
	10	44.28 ± 1.48cd	74.33 ± 2.30b	38.34 ± 4.34def	–
C. cladosporioides	0.1	51.21 ± 7.54cde	76.67 ± 6.12bc	41.20 ± 7.62de	34.76 ± 10.92e
	0.5	–	–	–	93.41 ± 1.01ab
	1	61.46 ± 14.97cd	91.28 ± 0.78ab	71.05 ± 6.70bc	100.00 ± 0.00a
	2.5	94.70 ± 1.33ab	91.95 ± 0.75ab	76.33 ± 3.95bc	–
	5	96.83 ± 1.40a	93.41 ± 1.06ab	87.29 ± 4.08ab	–
	10	100.00 ± 0.00a	95.04 ± 1.41ab	97.46 ± 1.27a	–
C. globosum	0.1	50.30 ± 3.09c	53.76 ± 12.78c	52.90 ± 7.57c	53.17 ± 11.22c
	1	58.40 ± 1.73bc	69.29 ± 1.29bc	53.37 ± 3.88c	–
	5	100.00 ± 0.00a	69.62 ± 4.37bc	60.33 ± 0.30bc	100.00 ± 0.00a
	10	–	77.78 ± 1.72ab	63.71 ± 3.89bc	–
P. citrinum	0.1	30.20 ± 2.58ghi	3.82 ± 4.29j	12.93 ± 3.12ij	55.43 ± 9.39def
	1	42.97 ± 3.82fgh	24.68 ± 6.56hi	20.15 ± 3.13ij	–
	2.5	60.10 ± 3.86cde	57.96 ± 4.29def	40.38 ± 7.09fgh	–
	5	70.57 ± 0.67bc	65.78 ± 3.11bcd	48.45 ± 2.45efg	100.00 ± 0.00a
	10	81.60 ± 1.21ab	80.54 ± 1.84b	57.22 ± 5.37def	–

The value in the column followed by the same letter is not significantly different at $p < 0.05$

ne no effect of authentic compounds as antifungal, – sample was not analyzed

The result showed that α-terpineol completely inhibited the growth of *F. palustris* at concentration of 2.5 mg/ml and that of *T. versicolor* at 5 mg/ml, however, *d*-limonene had no effect on *F. palustris* (Table 5).

IC_{50} of *M. leucadendron* leaf oils against *F. palustris* ranged from 0.12 to 3.16 mg/ml and against *T. versicolor* ranged from 0.01 to 0.06 mg/ml. IC_{50} values of 1,8-cineole, *β*-caryophyllene, and *d*-limonene against *T. versicolor* and *F. palustris* are mostly higher than those of the leaf oils (Table 6), it means that the leaf oils may have synergic effects against *T. versicolor* and *F. palustris*.

The essential oils of *M. leucadendron* leaves also showed inhibitory effects against some molds. The essential oils showed strong activity against *C. cladosporioides* and *C. globosum* with complete inhibition at concentration of 5 and 1 mg/ml, respectively. The essential oils at concentration of 10 mg/ml revealed the values for *P. citrinum* ranging between 39.86 and 79.90 %. The perfect inhibition against *A. niger* was obtained at concentration of 20 mg/ml (Table 4). The other researchers also reported low antifungal activity of essential oils against *A. niger* [32, 33]. Among major compounds, α-terpineol had the antifungal indices against *A. niger, C. cladosporioides, C. globosum* and *P. citrinum* higher than 1,8-cineole, *β*-caryophyllene and *d*-limonene; the fungal growths were completely inhibited at concentrations

Table 6 IC_{50} of antifungal indices of *M. leucadendron* leaf oils and authentic compounds

Sample	IC_{50} (mg/ml)					
	F. palustris	*T. versicolor*	*A. niger*	*C. cladosporioides*	*C. globosum*	*P. citrinum*
A1	3.16	0.06	10.97	0.03	0.15	8.70
A2	0.12	0.04	10.29	0.24	0.06	5.84
A3	3.16	0.01	10.24	0.49	0.08	5.94
1,8-Cineole	0.91	0.93	12.48	0.12	0.16	0.88
d-Limonene	ne	1.13	4.54	0.02	0.03	2.03
β-Caryophyllene	13.87	3.04	13.80	0.21	0.06	6.92
α-Terpineol	0.58	0.17	0.40	0.15	0.08	0.06

ne no effect of authentic compounds as antifungal

of 1 mg/ml on *A. niger* and *C. cladosporioides* and 5 mg/ml on *C. globosum* and *P. citrinum*, respectively (Table 5). IC_{50} values of *M. leucadendron* leaf oils against *A. niger, C. cladosprioides, C. globosum* and *P. citrinum* range from 10.24 to 10.97 mg/ml, 0.03 to 0.49 mg/ml, 0.06 to 0.15 mg/ml, and 5.84 to 8.70 mg/ml, respectively. IC_{50} value of each compound indicates that α-terpineol shows the best inhibitory effect on *A. niger* and *P. citrinum*, and *d*-limonene shows the best inhibitory effect on *C. cladosporioides* and *C. globosum*, but no effect against *F. palustris* (Table 6).

This study shows that essential oils of *M. leucadendron* are effective against *F. palustris, T. versicolor*, *C. cladosporioides* and *C. globosum*, but they are low effective against *A. niger* and *P. citrinum*. The antifungal effectiveness of the essential oils is probably due to the existence of α-terpineol. Several studies show that essential oil contained α-terpineol as major compound is effective as an antifungal agent [34, 35].

In conclusion, *M. leucadendron* leaf oils possess multi-effective properties, such as antioxidant, anti-hyaluronidase and antifungal activities. Each compound in *M. leucadendron* has different properties and biological activities. The effective components for each property are different, i.e., eugenol for antioxidant, *β*-caryophyllene for anti-hyaluronidase, α-terpineol for antifungal. Each property of the oils is not so strong compared to those of the individual commercial agents, but it is rather moderate that may be why these essential oils are widely utilized in many countries for expectorants, ointments and so on. The synergic effects for each biological activity might be found among several compounds, but they were not investigated this time. Combination of several compounds probably provides the other natural activities such as antiseptic, antifeedant, insecticide, sedative etc. Therefore, further studies are necessary to obtain the detailed information on the practical effectiveness of this essential oil for natural multi-purpose uses.

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