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Research Article

ANALYSIS OF ACTIVE COMPOUNDS in BLOOD PLASMA OF MICE AFTER INHALATION OF CAJUPUT ESSENTIAL OIL (*Melaleuca leucadendron* L.)

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ABSTRACT

The research is intended for the determination of volatile compounds of cajuput oil (*Melaleuca leucadendron* L.) in a group of mice (3 mice) after inhaling 1mL cajuput oil for 1/2, 1 and 2h(s). The blood plasma of three mice was collected in heparin tube, and the volatile compounds were isolated and concentrated using C-18 Solid Phase Extraction (SPE) column with methanol and bidistilled water mixture (60:40) as the solvent. The analysis was carried out by GC/MS. The results showed that there were 52 compounds in the cajuput oil, especially 1,8-cineole,α-terpineol, β-pinene, α-pinene, β-myrcene, 4-terpineol, linalool, γ-terpinene, and terpinolene. The major compound identified in blood plasma of mice was 1,8-cineole. Half an hour after 1mL inhalation of cajuput oil per mouse, the concentration of 1,8-cineole identified was 41.5μg/mL, while 1 and 2h after 1mL inhalation of cajuput oil, the level of 1,8-cineole in blood plasma of 3 mice was 646.9μg/mL and 138.0μg/mL in blood plasma of each mouse. Other compounds identified were α-terpineol, 4-terpineol linalool, terpenyl acetate and hydrocarbon terpenes. Concentrations of target compounds in the blood plasma were relatively in low level (μg/mL). In conclusion, the volatile compounds of 4-terpineol, linalool, α-terpineol and 1,8-cineol that identified in blood plasma of mice after inhalation of the cajuput oils might correlate with depressive characteristics of essential oil given by inhalation.

Key words: Cajuput, Essential Oils, Inhalation, SPE, GC-MS

INTRODUCTION

Essential oil has been utilized for perfumes, cosmetics, food additives and drugs (Buchbauer, 2000). It has been proven that aromatic compounds or fragrance of essential oil of a plant material can affect locomotor activity (Buchbauer *et al.,* 1991; Buckle, 1999; Diego *et al.,* 1998; Martin, 1998). The research on essential oil that has an effect on locomotor activity is initiated by Kovar *et al.* (1987) reporting that isolated compound of 1,8 cineol from essential oil in rosemary can increase locomotor activity of mice. According to Buchbaurer, *et al.* (1991), volatile compounds identified in blood plasma are expected to be active components that are responsible for pharmacological activity in affecting the central nervous system (CNS). It is due to compounds that stimulate CNS work on Blood-Brain Barrier (BBB) or cross the blood-brain (Buchbauer 2004).

Ethnopharmacology study regarding aromatherapy plants showed that Indonesia has 12 types of aromatic plants used empirically which have refreshing and relaxing effects (anxiolytic) for physical healthy (Sangat and Roematyo 1996). Cajuput (*Melaleuca leucadendron* L.) is one of Indonesian aromatic plants, especially in Java (Heyne 1987). Cajuput contains essential oil about 0.80-1.00%$^{v}/_{b}$, with a density ranging from 0.910-0.923g/cm^3 (FI III, 1979).

Applying cajuput oil orally (Wiendah, *et al.,* 1999) and inhalation (Muchtaridi, *et al.,* 2011), in fact, can reduce locomotor activity of mice, although 1,8-cineol is the dominant component in cajuput oil. In this study, the application of SPE has been used for chemical analysis of essential oil compounds in the blood of mice after the inhalation of cajuput essential oil (*Melaleuca leucadendron* L.). The findings from this study suggest that the 4-terpineol,

linalool, α-terpineol and 1,8-cineol might correlate with the locomotor activity of mice.

1,8-cineol compound is the main component in cajuput essential oil, followed by α-terpineol, linalool and other terpens from cyclic and acyclic groups (Muchtaridi *et al.,* 2011). According to Koyar (Kovar *et al.,* 1987) and Balchin (Lis-Balchin, 1999), 1,8-cineol can boost locomotor activity of mice and rats, while α-terpineol and linalool reduce the locomotor activity (Buchbauer and Jirovetz, 1994).

The aim of this research is to identify active compounds in cajuput oil that are responsible for reducing locomotor activity.

MATERIAL AND METHODS

Eucalyptus leaves (*Meulaleuca leucadendron* Linn) were taken from Kuningan, West Java. Specimens are identified by the Herbarium Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia and voucher specimens are stored in the Herbarium of Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran.

The sample herbs dried (500g) were water-distilled at Balitro, Monaco Lembang for 3h. The oil was stored at -20°C after addition of sodium sulphate (Sigma, USA). Essential oil of eucalyptus leaves obtained was 2.31 %.

Animals-Male mice weighing 25 to 30g and 2 to 3 months old were used. The mice were adapted for one week to the laboratory in which locomotor activity experiments were conducted and were selected for wheel rotations of between 150 to 300 before the experiments were started. All experimental procedures for animal use have been approved by the Ethical Committee of Experimental Animals, Universitas Padjadjaran.

Chemicals-Methanol pro.analyse (Merck, Germany) was used as a mobile phase for SPE. Heparin tubes (Boehringer, Germany) were used for blood collection. Pure lavender (*Lavandula officinalis*) oils were obtained from Martina Bertho (Indonesia). Alkane standards C_8-C_{20}, C_{21}-C_{40}, 1,4-dichlorobenzene from Sigma (USA), and 4-terpineol, linalool, α-terpineol dan 1,8-cineol were obtained from Dragoco (France).

Identifying compounds of blood plasma

Collecting blood plasma of mice is based on the method performed by Jirovetz *et al.* (1992), Kovar *et al*., 1987, and Muchtaridi *et al.,* 2011). Please see the instruction for authors for reference style.

Isolation of essential oils

Dried samples of each plant (500g) were submitted to water-distillation in Monaco Lembang, West Java, for 3h to isolate the essential oils. The oils were stored at -20°C after the addition of sodium sulphate (Muchtaridi *et al.,* 2014).

Mouse locomotor activity tests

Locomotor activity of mice was measured using a wheel cage, in which mice ran and the number of rotations was recorded by a meter. Cage inhalators contained a glass fiber (20cm × 20cm × 30cm) and were equipped with an electric fan for the evaporation and distribution of volatile compounds. The mice were selected by weight (25 to 30g) and by their ability to rotate the wheel cage up to 300 times in 30min; eligible mice were then divided into three groups: a control group, a lavender oil group as positive control group (using 0.1, 0.3 and 0.5mL/cage), and a essential oils tested as treatment group (using 0.1, 0.3, and 0.5mL/cage). The application of the doses were based on the preliminary examination in which those doses were reasonable to be used and based on Kovar *et al*., (Kovar *et al.,* 1987). Each group consisting of five mice was tested three separate times. After 30min of inhalation, the mice were placed into the wheel cage and after 5min; the number of rotations were recorded for 75min in 15min intervals.

GC/MS analysis

Analyses have been done according to a previously published method. Measurements were performed using a Shimadzu QP-5050A gas chromatograph coupled to a VG Autospec Mass Spectrometer at 70eV, 40-550amu with a fused silica capillary column (DB-5MS, 30m×0.25mm) using helium as a carrier gas and with temperature programming from 60°C/5 min to 300°C/min (10°C/min) for blood plasma and 60°C/5min to 300°C/2min

(10°C/min) for essential oils. The MS was operated using an interface temperature of 240°C, and an electron impact ionization of 70eV with a scan mass range of 40-350m/z (sampling rate of 1.0scan/s).

Qualitative analysis

Identification of the compounds was conducted by comparing their linear retention indices (LRI) with literature values and their mass spectral data with those from the MS data system (Wiley-229 lib, Nist-62 library and Nist-12 library) (Adams 1995). Linear retention indices were calculated using GC data of a homologous series of saturated aliphatic hydrocarbons (C_8 to C_{40}) separated on the same column using the same conditions as for GC analysis of the essential oils and the blood samples. The blood samples were collected from the corner parts of the eyes using capillary tubes and placed in a heparin tube. Blood samples were collected immediately after the mice inhaled the essential oil for a period of ½, 1 or 2h.

Quantitative analysis

Detailed analysis using methods were performed in Kovar *et al.,* 1987; Jirovetz *et al.,* 1992; and Jirovetz *et al.,* 1991 with modification. The blood samples (500 to 600µL), obtained according to Jirovetz (Jirovetz *et al.,* 1992), were centrifuged (1.800rpm/10min) at room temperature and concentrated on a C_{18}-column (100mg). Volatile compounds were separated using a mobile phase of the mixture of methanol-bidistilled water (60:40). Five micro-litres re-injected into the GC-MS. Quanti-fication of the volatile compounds in the blood samples was accomplished using 1,4-dichloro-benzene 0.5% (500µL) as an internal standard as according to the following equation:

$$[C] = \frac{A}{IS} \times \frac{IS\ weight\,(g)}{100\ mL} \times \%\ EO \times IS\ volume \times 10^6 \quad \text{.....(1)}$$

where: C = concentration (g/g); IS = GC peak area of Internal Standard; A = GC peak area of compounds of essential oils; % EO = yield of essential oils.

Determining of LRI and Concentration

Confirming identity of identification result has been carried out by determining (Linear Retention Index) LRI value. This value is calculated based on standard retention time of alkanes (C_8-C_{40}) which is injected in GC-MS with the same condition and column.

Five microliters were injected into the GC-MS. Quantification of the volatile compounds in the blood samples was accomplished using 1,4-dichlorobenzene 0.5% (5mg in 1mL blood plasma) as an internal standard according to the following equation (1)(Muchtaridi *et al.,* 2010):

$$[C] = \frac{A}{IS} \times \frac{IS\ weight\,(g)}{100\ mL} \times \%\ EO \times IS\ volume \times 10^6 \quad \text{.....(1)}$$

C = concentration (g/g); IS = GC peak area of Internal Standard; A = GC peak area of compounds of essential oils; % EO = yield of essential oils.

Recovery is calculated based on the ratio between the concentration of 1,4-dichlorobenzene in blood plasma and the concentration of 1,4-dichlorobenzene in methanol (blank) by repeating it twice.

RESULT AND DISCUSSION

Analysis of cajuput essential oil components

Cajuput essential oil (*Melaleuca leucadendron* Linn.) obtained from 1.3kg of dried cajuput leaves was 30mL. Cajuput essential oil yield was 2.31% v/b. Essential oil obtained had yellowish translucent colour and typical smell. The compound of 1.8-cineole dominated the composition of cajuput essential oil leaves about 22.45%. That level was smaller as compared to the data in Proscea (30-50%) (Oyen Nguyen, 1999). According to its great level, the following components are α-terpineol (12.45%), caryophillene (6.95%), β-pinene (5.74%), α-humulene (4.70%), β-selinene (3.82%), β-myrcene (3.58%), α-selinene (2.9%), and α-terpenyl acetate (2.74%). To be specific, it can be seen (Table III).

Analysis of essential compounds in blood plasma of mice after the inhalation of cajuput oil

To know the effects of cajuput essential oil on locomotion, this oil was administered to mice via inhalation. Doses of 0.1, 0.3, and 0.5mL/cage could decrease on locomotor activity by 46.81%, 69.47%, and 62.04%, respectively (Table III).

Table I. Essential compounds identified in blood plasma of mice after the inhalation of nutmeg seed oil with SPE C-18 preparation, analyzed by GC-MS.

Peak No [d]	Compounds	*Duration of Inhalation*						*LRI*
		½ hours (R[c] = 81 %)		*1 hours* (R[c] = 91 %)		*2 hours* (R[c] = 88 %)		
		LRI Exp[b]	Concentration µg/mL	LRI Exp[b]	Concentration µg/mL	LRI Exp[b]	Concentration µg/mL	Ref[a]
1.	α-Pinene	nd	nd	936	24.9	nd	nd	939
2.	Benzaldehyde	nd	nd	969	11.4	nd	nd	961
3.	β-Pinene	nd	nd	989	23.3	nd	nd	980
4.	Limonene	nd	nd	1034	53.7	1032	13,4	1031
5.	1,8-Cineole	1037	41.5	1037	646.9	1035	138.0	1033
6.	Δ-Carene	nd	nd	1057	15.9	nd	nd	1011
7.	α Terpinolene	nd	nd	1080	9.1	nd	nd	1088
8.	Linalool	nd	nd	1090	17.6	nd	nd	1098
9.	4-Terpineol	nd	nd	1165	17.6	1170	2.6	1177
10.	α-Terpineol	1177	13.3	1177	208.7	1179	35.5	1189
11.	Linalyl acetate	nd	nd	1281	*td*	1260	5.2	1257
12.	α-Terpenyl acetate	nd	nd	1283	24.9	nd	nd	1350
13.	(E)-Cariophyllen	nd	nd	1417	9.4	nd	nd	1418

nd = no detection, a: LRI *reference* in Adams (1995) with DB5-MS column, b : LRI experiment with DB5-MS column, c: *Recovery*, d: Number of peak in line with number of the peak on GC chromatogram (Figure 1)

Table II. Average number of mice wheel cage rotations within 90min of inhalation of nutmeg and lavender essential oils.

Tratment	The Average number of mice wheel cage rotations **(Averages ± SD)**	**Decreasing of Activity (%)****
Normal Control	285.63±32.22	0
Lavender 0.1mL	214.67±32.19*	24.84
Lavender 0.2mL	171.47±17.50*	39.97
Lavender 0.3mL	151.43±15.16*	46.91
Cajuput 0.1mL	151.93±46.16*	46.81
Cajuput 0.2mL	87.20±33.95*	69.47
Cajuput 0.3mL	108.43±45.53*	62.04

* = There were significantly different with normal (α=5%)
** = The decreasing score was compared than normal control (0%)

Thus, the effect of cajuput oil on locomotion was dose-dependent. A strong inhibitory effect of the cajuput oil on locomotion was observed;

As the previous study, we compared the activity of cajuput oil to that of lavender oil as a positive control, because lavender oil has been proven to decrease locomotion of female and male laboratory animals (Muchtaridi *et al.,* 2010). As shown in table I, cajuput oil afforded a greater inhibitory effect than lavender oil did. The inhalation of cajuput oil reduced the locomotor activity of mice up to 69.47% (Table II).

Compounds of 1.8-cineole, α-terpineol and 4-terpineol were the three dominant compounds in blood plasma of mice after inhaling cajuput leaves. The result of identifying essential compounds in blood plasma of mice after inhaling cajuput leaves (Figure 1).

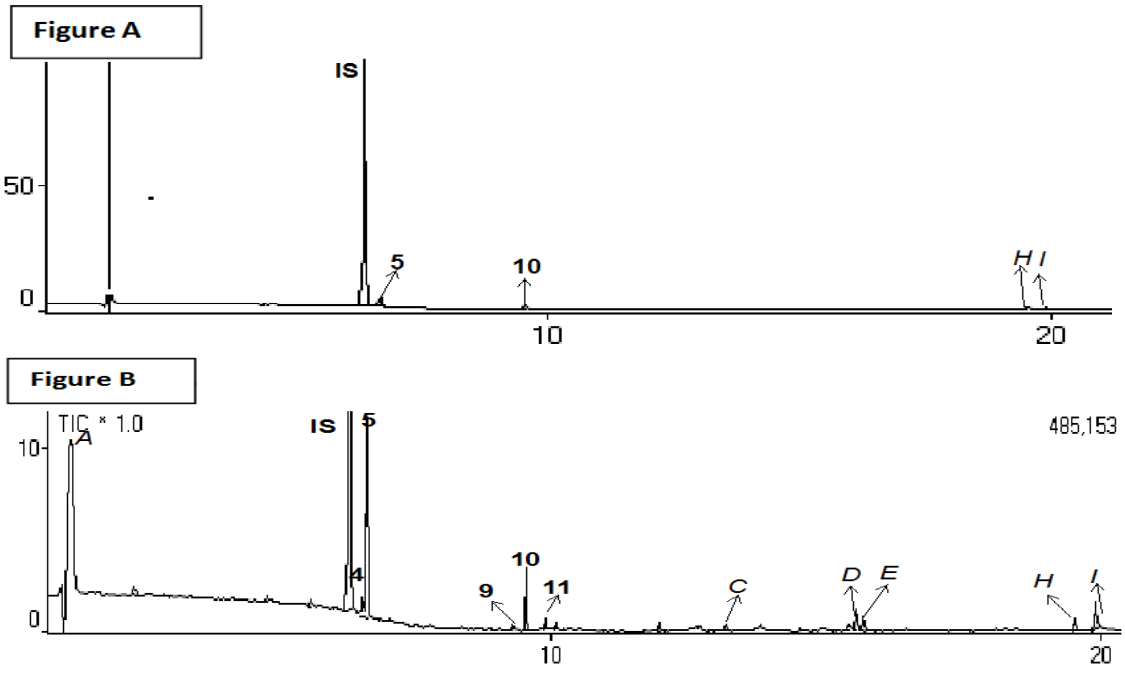
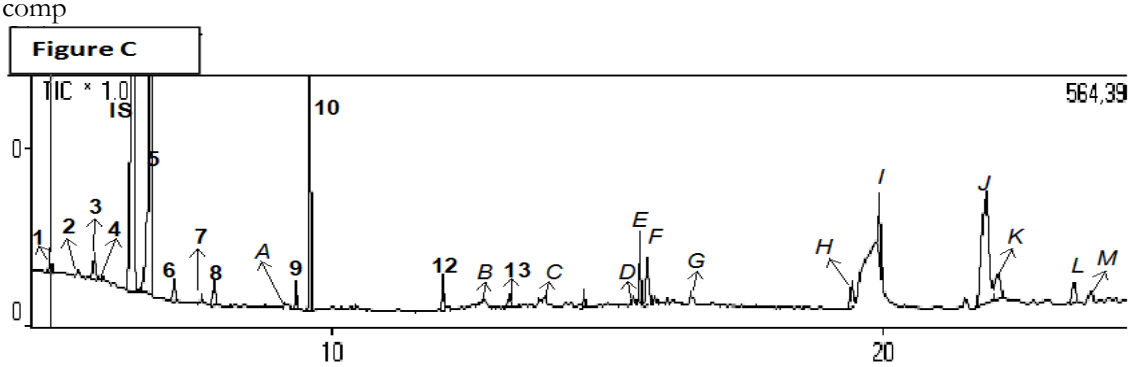


Figure 1. Total ion chromatogram of essential oil compounds in blood plasma of mice after inhaling essential oil of cajuput leaves. Figure **A** shows the chromatogram of essential compound after ½h inhalation, while figure **B** shows the chromatogram of essential comp



ounds after 1h inhalation, and figure **C** shows the chromatogram of essential compounds after 2h inhalation. **IS**: Internal Standard. The peak which is indicated by A to M is such compounds other than essential oil (Table II).

In figure 1, the peak no.5 (*1,8-cineole*) always seems to appear with the largest concentration, followed by the peak no.10 (*α-terpineol*). On the other hand, the peak no.9 (*4-terpineol*) appears in figure A (blood plasma of mice after ½h inhalation of cajuput oil) and B (blood plasma of mice after 1h inhalation of cajuput oil). These compounds are allegedly considered as active compounds that are responsible for inhibiting locomotor activity. It is proved by the previous research (Kovar, *et al.,* 1987; Aoshima, *et al.,* 2001). Active compounds in essential oil are responsible for the decrease of locomotor activity for each different essential oil, depending on the composition of the essential oil. Identified compounds in blood with the high bioavailability at 30min to 2h is allegedly expected to be responsible for the pharmacological activity of essential oil, or called as the lead compound (Buchbauer *et al.,* 1993).

Table III. Chemical composition of the essential oil of Cajuput (*Meulaleuca lecadendron* Linn.)

Peak No	Retention Time	LRI Exp[a]	LRI Ref[b]	Compounds	% Area
2	6.542	940	939	α-Pinene	5.25
3	6.914	953	953	Camphene	0.04
4	7.775	984	980	β-Pinene	5.74
5	8.035	993	991	β- myrcene	3.58
6	8.392	1007	1031	L-Limonene	0.14
7	9.168	1038	1033	1.8-Cineole	22.45
10	9.81	1063	1062	γ -Terpinene	1.54
11	10.343	1085	1088	Terpinolene	0.98
13	10.66	1097	1098	Linalool	1.71
14	10.833	1106	1111	Rose oksida	0.04
17	11.63	1148	1146	Isopulegol	0.13
19	12.074	1172	1177	4-Terpineol	1.71
20	12.31	1185	1189	α -Terpineol	12.45
23	13.326	1243	1240	Neral	0.24
24	13.489	1253	1255	Geraniol	0.13
25	13.811	1272	1270	Geranial	0.31
28	14.582	1318	1350	α-Terpenyl acetate	0.14
30	14.964	1342	1339	Δ-Elemene	0.23
31	15.18	1356	1350	α -Terpenyl acetate	2.75
33	15.557	1380	1372	α-Ylangene	0.37
34	15.653	1386	1351	α-Cubebene	0.36
36	15.856	1398	1391	β-Elenene	1.01
37	16.156	1419	1409	α-Gurjunene	0.16
38	16.489	1441	1418	(E)-Caryophillen	6.95
39	16.67	1453	1439	Aromadendrene	0.45
41	17.004	1476	1454	α -Humulene	4.7
42	17.146	1486	1485	γ -Selinene	0.95
44	17.483	1509	1485	β -Selinene	3.82
45	17.573	1516	1494	α -Selinene	2.9
46	17.762	1529	1524	Δ-Cadinena	0.98
51	18.286	1567	1581	Caryophillen oxide	0.21
52	18.414	1577	1556	Germacrene B	0.97
55	18.797	1605	1583	Globulol	2.94
56	18.97	1618	1590	Viridiflorol	3.52
61	19.395	1651	1630	γ -Eudesmol	1.81
64	19.761	1679	1649	β-Eudesmol	3.63

a LRI reference in Adams (1995) with DB5 column
b LRI experiment with DB5-MS column

The highest level of 1,8-cineole in the inhalation of cajuput essential oil was in 1h inhalation, but rapidly declined in 3h inhalation. It means that in 2h after the essential components reached circulatory system, the components are immediately distributed into tissues and metabolized or have been eliminated either entirely or partially through

urine, lungs or other elimination routes, thus the concentration in blood is very small and cannot be identified. After 1,8-cineole began to secrete for 2h, either through urine or sweat, 1,8-cineole gives a strong spasmolytic effect by inducing acetyl choline (Buchbauer *et al.,* 1993). Single compound of 1,8-cineole boosts locomotor activity or stimulation to CNS, but when it interact in essential oil, this compound will synergize with alcohol terpen as well as *4-terpineol* that reduces locomotor activity (Aoshima *et al*., 2001; Buchbauer *et al*., 1993b) and synergizes with α-pinen by reducing smooth muscle strain (spasmodic).

α*-terpineol* and 4-*terpineol* compounds give the same effect on GABA system. Through these compounds, $GABA_A$ receptor potentiality is very strong in small concentration. 4-*Terpineol* gives stronger activity as compared to α-*terpineol* (Aoshima, *et al.,* 2001).

Recovery analysis using SPE

The effectiveness of SPE in the analysis of volatile compounds in blood after the inhalation of essential oil has been explained in the previous publication (Muchtaridi and Musfiroh 2012). The recovery on the analysis of 1.8-cineole in blood plasma of mice after inhaling nutmeg seed oil using C_{18} (Sep Pak Waters) reaches 90% in comparison to untreated SPE. In addition, other volatile compounds are greatly detected (Table I).

The use of SPE in the research is not able yet to reduce all impurities. Figure 1, the peak A to M is compounds other than essential compounds. However, the origin of the non-essential compounds has not been clearly explained in the research. As well as the non-essential compounds in blood plasma of mice after the inhalation of basil oil, contaminant compounds detected in blood plasma of mice possibly come from contamination of plastic container (peak D, H and I). In addition, there are also no alcohol sesquiterpen compounds in cajuput essential oil, such as *viridflorol* and *palustrol*. It is expected that the compounds probably come from the previous injection sample in GC-MS (Table III).

Locomotor activity of mice in regard to single compound identified

The locomotor activity of mice in regard to single compound identified in blood after the inhalation of cajuput leaves oil was tested to ensure that identified compounds influence on the locomotor activity of mice. Linalool, α-terpineol and 1,8-cineol compounds reduced locomotor activity of mice as compared to nomal control (100%) (figure 1).

Essential oil of cajuput leaves inhalation provides a greater decline than a single compound of 4-terpineol, linalool, α-terpineol and 1,8-cineol. However, all single compounds generally reduce locomotors activity towards normal. Only 1,8-cineol compound has insignificant activity in comparison to normal control. Moreover, 1,8-cineol with 0.5mL dose gives stimulant effect towards central nervous system of mice (CNS). These results indicated that 4-terpineol, linalool, α-terpineol and 1,8-cineol that identified in blood plasma of mice after inhalation of the cajuput oils might correlate with depressive characteristics of essential oil given by inhalation.

According to Kovar, *et al*., (1987), 1,8-cineole gives CNS stimulant activity of mice. The greater dose given will remain the greater stimulant effect produced. Besides, these findings are in line with the research conducted by Aoshima *et al.* (2001) and Buchabuer *et al.* (1993) who state that 4-terpineol, linalool and α-terpineol boost the affinity of GABA work system.

CONCLUSION

Essential oil compounds detected in blood with a dominant duration were 4-terpineol, linalool, α-terpineol, and 1,8-cineole. This study reported that the volatile compounds detected in blood samples such as 4-terpineol, linalool, α-terpineol and 1,8-cineol were related with inhibition of locomotor activity in mice. It is suggested that the locomotor inhibition by cajuput essential oil is due, at least in part, to the direct pharmacological action of one or more of its constituents.

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