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Cytotoxicity, Anti-Platelet Aggregation Assay and Chemical Components Analysis of Thirty-Eight Kinds of Essential Oils

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ABSTRACT

Thirty-eight essential oils (EOs), including 4 products from Taiwan high-latitude trees, *Chamaecyparis formosensis*, *Taiwania cryptomerioides*, *Cryptomeria japonica*, and *Chamaecyparis obtuse*, as well as 34 commercial essential oils, were assayed on their cytotoxicity against 5 cancer cell lines and anti-platelet aggregation function.

Two EOs from *Juniperus virginiana* and *Santalum album* showed significant cytotoxicity. Moreover, 2 EOs from *Melissa officinalis* and *Piper nigrum* exhibited moderate inhibitory effects on an anti-platelet aggregation assay. Some essential oils had been reported to contain cytotoxicity. However, the cytotoxicity of EOs from *J. virginiana* and *S. album* is revealed for the first time. To the best of our knowledge, this is the first report for anti-platelet aggregation effect of EOs.

Key words:bioactivity, essential oils (EOs), *Juniperus virginiana*, *Santalum album, Melissa officinalis, Piper nigrum*

INTRODUCTION

Essential oils (EOs) are known as volatile oils (VOs), or ethereal oils of the plants from which they were extracted. EOs of herbs and their main components have many applications in folk medicine, food flavoring and preservation as well as in the fragrance and pharmaceutical industries[(1)]. Furthermore, the bioactive compounds of EOs commonly found in fruits, vegetables, herbs and various plants have been shown to have possible health benefits with antioxidative, antimicrobial, antitumoral, anticarcinogenic, anti-inflammatory, atherosclerosis, antimutagenic, and angiogenesis inhibitory activities[(2-4)].

Cancer is considered the end stage of a chronic disease process characterized by abnormal cell and tissue differentiation[(5)]. This progress of carcinogenesis may cause the final outcome of invasive and metastatic cancer. The molecular biology, along with experimental, epidemiological, and clinical trial data, has led to the development of cancer chemoprevention research in recent years[(6-9)]. Medicinal plants are sources of natural compounds, and many researchers are interested in their ability to cure various cancers[(10)].

Numerous evidence indicate that platelets contribute significantly to the pathogenesis of arterial thromboembotic diseases, such as acute coronary syndrome and ischemic stroke, which are the major causes of death in developed countries[(11,12)]. Antiplatelet drugs, such as aspirin and ticlopidine, are used to protect against myocardial infarction, stroke, cardiovascular death, and other serious vascular events in patients with a history of previous vascular events or known risk factors for cardiovascular disease[(13,14)]. However, the current antiplatelet drugs still have some restrictions in their mode of action and efficacy. Therefore, research and development of a new generation of antiplatelet drugs continue as important targets.

Medicinal plants are sources of natural compounds, and many researchers are interested in them. To the best of our knowledge, a systematic study on cytotoxicity and anti-platelet aggregation effects of essential oils has not yet been undertaken. In Taiwan, many EOs were externally used in massage and folk therapy, but the quality of EOs was hard to control. In this study, the aim is to investigate their

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cytotoxicity on a series of human cancer cell lines: Hep G2 and Hep 3B (liver), MCF-7 and MDA-MB-231 (breast), and A549 (lung) of thirty-eight commonly used essential oils. Moreover, anti-platelet aggregations of these essential oils were also evaluated.

Among these EOs, two EOs from *Juniperus virginiana* and *Santalum album* showed moderate cytotoxicity, and 2 EOs from *Melissa officinalis* and *Piper nigrum* exhibited significant inhibitory effect on an anti-platelet aggregation assay. The components of the aforementioned essential oils were further identified by GC-MS analysis. However, the component of 4 Taiwanese essential oils, including *C. formosensis*, *T. cryptomerioides*, *C. japonica*, and *C. obtuse,* were analyzed and reported before[15-18], and not analyzed in the current paper.

MATERIALS AND METHODS

I. *Materials*

The HPLC-grade solvents were from Echo Chemical Co. Ltd., Miaoli, Taiwan and Merck, Darmstadt, Germany. Thirty-four sample essential oils were provided by a company in Kaohsiung, Taiwan and 4 Taiwanese essential oils were provided by Prof. Sheng-Yang Wang. All of the commercial EOs have certificates of analysis (COA) from the manufacturers. The COAs listed the product name, botanical name, origin, Chemical Abstract No., EINECS no., FEMA No., FDA No., Product No., Lot No. Date, appearance, color, odor, special gravity at 20°C, optical rotation at 20°C, the requirement for stability and storage etc., individually. The information for the essential oils is listed in Table 1.

II. *Methods*

(I) *Cytotoxicity Assays*

Human liver (Hep G2 and Hep 3B), breast (MDA-MB-231 and MCF-7), and lung (A549) cancer cell lines were obtained from American Type Culture Collection. All cell lines were propagated in RPMI-1640 medium supplement with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell viability was measured by the MTT colorimetric method[19]. In brief, freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5,000-10,000 cells per well. The 38 EOs were dissolved in DMSO at 10 mg/mL as stock concentration, and then serially diluted with culture medium to the testing concentrations (the final concentration of DMSO in culture medium was less than 0.2%). Doxorubicin (Sigma Chemical Co., purity 98%) was used as a positive control. After 3 days in the culture, the attached cells were incubated with MTT (0.5 μg/mL, 1 h) and subsequently solubilized in DMSO. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of agents that reduced cell growth by 50% under the experimental conditions. The results represent the mean of two experiments, each performed in duplicate.

(II) *Antiplatelet Aggregation Assay*

Platelet-rich plasma preparation and antiplatelet aggregation assay were carried out in accordance with the methods described by Chia *et al.*[20,21]. Briefly, platelet pellets collected from human blood was re-suspended in Tyrode's solution consisting of NaCl (136.8 mM), KCl (2.8 mM), $NaHCO_3$ (11.9 mM), $MgCl_2$ (2.1 mM), NaH_2PO_4 (0.33 mM), $CaCl_2$ (1.0 mM), glucose (11.2 mM) and bovine serum albumin (0.35%). These platelet suspensions were incubated with DMSO (vehicle) or 38 EOs at 37°C for 3 min under stirring (1200 rpm) before the addition of inducers (0.1 U/mL thrombin and 10 μg/mL collagen). The final concentration of DMSO in platelet suspensions was fixed at 0.5%- a concentration that had no effect on platelet aggregation. By using a Lumi-aggregometer, the extent of platelet aggregation was measured as the increase of light transmission at 5 min after the addition of aggregation inducer.

(III) *Gas Chromatography-Mass Spectrometry (GC-MS)*

The components of 4 essential oils (Mellisa, Black pepper, Cedarwood, and Sandalwood) were analyzed by using a Thermo GC-MS system (Trace GC ultra, DSQ II-Mass Spectrometer, MS 2205862, Thermo, USA) equipped with an HP-5MS capillary column (5% phenyl methyl siloxane, length = 30 m, i.d. = 0.25 mm, film thickness = 0.25 μm). Electron impact ionization was carried out at energy of 70 eV. Helium was used as carrier gas at a flow rate of 1.0 mL/min. An aliquot of 10 μL essential oils (Mellisa, Black pepper, Cedarwood, and Sandalwood) dissolved in 10 mL EtOAc, respectively, and adjusted to 250, 500, and 1,000 ppm. The injection volumns of 4 essential oils were 1, 1, 1 and 3 μL, individually. The operating conditions were shown in the Table 2. The components were identified by comparing with the following database (NIST/EPA/NIH Mass spectral Library Version 2.0 d, build APR 26 2005) and reference [22-28]. The area percentages of major compounds in 4 essential oils were quantified on the basis of the peak area integrated by Thermo Xcalibur™ data analysis program.

RESULTS AND DISCUSSION

I. *Cytotoxicity Assays*

The cytotoxicity of thirty-eight essential oil samples against the proliferation of a limited panel of cancer cell lines, including HepG2, Hep3B, A549, MCF-7, and MDA-MB-231 cells, was then evaluated. The results showed that the cedar wood and sandalwood EOs exhibited significant cytotoxicity against HepG2, Hep3B, A549, MCF-7, and MDA-MB-231 cancer cell lines. The cytotoxicity of mellisa (*Melissa officinalis*), three Taiwanese EOs, and positive control

Table 1. Information on thirty-eight essential oils

NO.	Name	Scientific name	Origin	Extraction method	Extraction part
1	Bergamot	*Citrus bergamia*	Italy	Expression	fruit rind
2	Cypress	*Cupressus sempervirens*	France	Distillation	leaves
3	Eucalyptus	*Eucalyptus globulus*	Australia	Distillation	leaves
4	Fennel	*Fonneculum vulgare*	Spain	Distillation	seeds
5	Fankincense	*Boswellia carterii*	Somalia	Distillation	gum
6	Juniper-Berry	*Juniperus communis*	Nepal	Distillation	fruit
7	Lavender	*Lavandula officinalis*	France	Distillation	flowers
8	Lemon	*Citrus limonum*	Spain	Expression	fruit rind
9	Peppermint	*Mentha piperita*	USA	Distillation	leaves
10	Marjoram	*Origanom marjorana*	Egypt	Distillation	leaves
11	Mellisa	*Melissa officinalis*	France	Distillation	–[a]
12	Niaouli Nerolidol	*Melaleuca quinquenervia*	Australia	Distillation	–
13	Orange	*Citrus sinensis*	USA	Expression	fruit rind
14	Rosemary	*Rosmarinus officinalis*	Tunisia	Distillation	leaves and flowers
15	Spanish-Sage	*Salvia lavandulifolia*	Spain	Distillation	leaves and flowers
16	Tea Tree	*Melaleuca alternifolia*	Australia	Distillation	leaves
17	Black pepper	*Piper nigrum*	India	Distillation	–
18	Geranium-Rose	*Pelargonium roseum*	Madagascar	Distillation	leaves
19	Petitgrain	*Citrus aurantium*	–	–	–
20	Rose	*Rosa damascena*	Bulgaria	Distillation	flowers
21	Chamomile-Roman	*Anthemis nobilis*	–	–	flowers
22	Ravensara	*Ravensara aromatica*	–	–	leaves
23	Rosewood	*Aniba rosaeodora*	–	–	wood
24	Cedarwood	*Juniperus virginiana*	USA	Distillation	wood
25	Pine	*Abies balsamea*	Canada	Distillation	leaves
26	Basil	*Ocimum basilium*	–	–	leaves and flowers
27	Ylang Ylang	*Cananga odorata*	Indonesia	Distillation	flowers
28	Thyme	*Thymus vulgaris*	Spain	Distillation	leaves and flowers
29	Cajeput	*Melaleuca leucadendron*	–	–	–
30	Vetivert	*Vetiveria zizanoides*	–	–	roots
31	Myrrh	*Commiphora myrrha*	–	–	gum
32	Patchouli	*Pogostemon cablin*	–	–	leaves
33	Mandarin	*Citrus reticulata*	Italy	Expression	fruit rind
34	Sandalwood	*Santalum album*	West Australia	Distillation	wood
35	Formosan Cypress	*Chamaecyparis formosensis*	Taiwan	Distillation	wood
36	Taiwania Fir	*Taiwania cryptomerioides*	Taiwan	Distillation	wood
37	Cryptomeria	*Cryptomeria japonica*	Taiwan	Distillation	leaves
38	Taiwan Cypress	*Chamaecyparis obtusa var. formosana*	Taiwan	–	wood

[a] unknown

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Table 2. The operating conditions of GC-MS

NO.	Name	injection volumn (uL)	Injector (°C)	ion source (°C)	MS transfer line (°C)	Mass range	split ratio	oven program		
								Rate (°C/min)	temp. (°C)	hold time (min)
11	Mellisa	1	200	250	200	38-350	10:1		60	5
								2	120	2
								35	250	2
17	Black pepper	1	230	280	250	35-350	10:1		60	15
								1	70	2
								10	110	1
								3	210	1
								30	275	2
24	Cedarwood	1	250	250	250	50-350	100:1		60	5
								25	118	5
								0.5	125	2
								1	170	2
								30	280	2
34	Sandalwood	3	250	200	200	35-350	splitless		40	5
								20	110	1
								1	140	20
								10	220	2

Table 3. Cytotoxicity assays and antiplatelet aggregation assay of the mellisa, black pepper, cedarwood, and sandalwood essential oils

NO.	Name	IC_{50} (μg/mL)[a]						
		HepG2	Hep3B	A549	MCF-7	MDA-MB-231	Thrombin	Collagen
11	Mellisa	NA b	NA	NA	18.62 ± 1.09	NA	3.98 ± 0.20	3.26 ± 0.15
17	Black pepper	NA	NA	NA	NA	NA	3.81 ± 0.22	2.36 ± 0.07
24	Cedarwood	11.18 ± 0.71	3.02 ± 0.03	1.79 ± 0.07	3.99 ± 0.13	4.32 ± 0.05	*c	*
34	Sandalwood	7.92 ± 0.46	5.28 ± 0.18	11.37 ± 1.21	5.67 ± 0.07	10.73 ± 0.42	*	*
35	Formosan Cypress	114.03 ± 0.31	94.21 ± 5.79	68.66 ± 2.94	79.89 ± 0.33	88.00 ± 12.00	NA	NA
36	Taiwania Fir	43.23 ± 0.68	28.15 ± 0.33	53.15 ± 3.66	68.79 ± 1.66	72.92 ± 1.23	NA	NA
38	Taiwan Cypress	62.38 ± 0.15	NA	130.27 ± 1.80	145.01 ± 4.82	142.61 ± 8.85	NA	NA
	Doxorubicin [d]	0.23 ± 0.04	0.49 ± 0.00	0.69 ± 0.04	0.73 ± 0.03	0.71 ± 0.01	-	-
	Aspirin e	–	–	–	–	–	15.9 ± 0.30 [e]	>100 [e]

[a] Data expressed as mean ± SD (n = 2)
[b] NA (not active): The IC_{50} concentration was greater than 200 μg/mL.
[c] *: The tested samples lead to platelet aggregation effect.
[d] The positive control in cytotoxicity assay.
[e] The positive control in antiplatelet aggregation assay.

(Doxorubicin) was also recorded (Table 3). The other EOs were not cytotoxic at the concentration of 200 μg/mL.

II. *Antiplatelet Aggregation Assay*

Thirty-eight essential oils are tested on antiplatelet aggregation effect induced by thrombin and collagen. Melissa and black pepper EOs displayed significant antiplatelet aggregation induced not only by thrombin but also by collagen. Their IC_{50} values were listed in Table 3. Besides the data for antiplatelet aggregation shown in Table 3, the other 31 EOs led to platelet aggregation effect at the concentration of 100 μg/mL.

III. *Chemical Composition*

The compositions of active EOs were further investigated. We used GC-MS to analyze the chemical compositions

Table 4. Chemical compositions of mellisa, black pepper, cedarwood, and sandalwood essential oils

Name	Compound	RT (min)	Area (%)
Mellisa	Limonene	12.08	16.33
	Citronellal	19.80	34.87
	Neral (Z-citral)	25.38	13.34
	Geranial (E-citral)	27.39	18.84
Black pepper	α-Pinene	10.27	10.68
	Limonene	14.88	9.48
	β-Caryophyllene	40.28	62.60
Cedar wood	α-Cedrene	19.17	32.84
	β-Cedrene	19.58	7.19
	Thujopsene	20.27	13.87
Sandalwood	Santalol (Z-α and Z-β)	44.16	87.85

of cedarwood and sandalwood EOs, which were toxic toward cancer cells, along with black pepper and melissa EOs, which were active in inhibition of platelet aggregation. The results are shown in the Table 4. The main components of cedarwood are cedrene (32.84%), thujopsene (13.87%) and *β*-cedrene (7.19%)[22]. In previous literature, cedrene was found to have cytotoxic activity (IC_{50} = 4.07 μg/mL) on human leukemia cells[23]. It suggested that the main active component of the cedarwood EO should be cedrene. The main component of sandalwood is santalol (the mixtures of Z-*a* and Z-*β* forms)[24], accounting for 87.85% of the total content. In previous literature, santalol was found to cause apoptosis of human epidermoid carcinoma A431 cells[25,26] and inhibited UVB-induced skin cancer[27]. We suggested that the cytotoxic effect of sandalwood EO may be due to the main component, santalol.

In the part of active EOs in anti-platelet aggregation, the main components of mellisa (lemon balm) are citronellal (34.87%), limonene (16.33%), geranial (18.84%) and neral (13.34%)[28,29]. In previous literature, lemon balm essential oil has anti-microbial, anti-oxidation[28] and anti-herpes virus activity[29]. The main components of black pepper are *β*-caryophyllene (62.60%), *a*-pinene (10.68%) and limonene (9.48%)[30,31,32]. According to previous literature, *Salvia fir* EO was also reported to have large amount of *β*-caryophyllene and *a*-Pinene[33], which had significant antioxidant activity. *Salvia leriifolia* EO had large amount of *a*-Pinene, which was found to have significant anti-inflammatory activity[34]. However, no EO has been tested for its anti-platelet aggregation activity before.

In 2006, Tipton *et al*. found that the NF-kappa B (NF-κB) transcription factor regulates many genes that permit cells to respond to infection and inflammation. The inflammatory protein NF-κB product expression is inhibited by sesquiterpenes, compounds that are commonly present in essential oils[35]. Of the 38 essential oils that showed the strongest effects of cytotoxicity in the tumor cells were cedarwood and sandalwood, perhaps they had a higher sesquiterpene (santalol, cedrene, etc.) compositions. Moreover, 3 EOs from *C. formosensis*, *T. cryptomerioides*, and *C. obtuse* showed cytotoxic activity. Coincidently, *S. album*, *J. virginiana*, *C. formosensis, T. cryptomerioides, and C. obtuse* are all alpine and long-living plants, probably because they produce EOs that has cytotoxic effects.

In our experiments, we found that black pepper and mellisa EOs has very good anti-platelet aggregation activity. It should be further investigated if the main components of these 2 plants' EOs, citronellal and *β*-caryophyllene, have anti-platelet aggregation activity.

The composition and function of EOs are very complex. Some EOs are cytotoxic and platelet aggregation inhibition that may propose further work for potential uses in anti-cancer and cardiovascular diseases. Moreover, the functions and safety of massage oils on the market is needed in future research.

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