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Antioxidant Activities and Oral Toxicity Studies of Chamaecyparis formosensis and Cymbopogon nardus Essential Oils

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Abstract

Essential oils of two native Taiwan plants, *Chamaecyparis formosensis* and *Cymbopogon nardus*, were distilled and their composition, antioxidant activity and mouse oral toxicity were examined. *C. formosensis* essential oil was composed primarily of 22.4% myrtanol, 17.34% (+)-nopinone and 13.24% δ-cadinene, while *C. nardus* essential oil consisted mainly of 42.12% citronellal and 15.15% geraniol. Antioxidant ability was evaluated by 2,2-diphenyl-1-picrylhydrazyl assay and the β-carotene bleaching test, two *in vitro* assays, as well as confirmed by reactive oxygen species assay and lipid peroxidation assay, two cell culture-based assays. *C. nardus* essential oil revealed a greater ability to activate the antioxidant enzyme superoxide dismutase. Examination of acute oral toxicity in mice showed relatively low acute oral toxicities, with an LD_{50} value over 2000 mg/kg for both essential oils. In subacute tests, only *C. nardus* essential oil exhibited mild effects on the liver and stomach weights of treated mice, as well as increased levels of serum alanine transaminase and blood urea nitrogen.

Keywords: *C. formosensis; C. nardus; essential oil; antioxidant; oral toxicity*

1. Introduction

Plant-based products have been used by humans for a very long time for purposes such as health improvement, domestic pest control, environmental hygiene and in the food industry. Investigation into and a search for natural substances are ongoing worldwide. Essential oils are volatile, aromatic oily liquid compounds formed by aromatic plants as secondary metabolites. They can be obtained in several ways, but the method of steam distillation is most commonly used for commercial production [1, 2]

Chamaecyparis formosensis (*C. formosensis*), also called Formosan Cypress or Taiwan red cypress,

grows in the central mountains at moderate to high altitudes of 1500–2150 m in Taiwan. *C. formosensis* is a long-lived plant, grows slowly, and finally grows into a huge tree, 65 m in height and 7 m in diameter on average. Previous study of *C. formosensis* essential oil from wood indicated significant antifungal activity against two typical wood fungi, *Laetiporus sulphureus* and *Trametes versicolor* [3] . A later report by the same team described insecticidal activity: *C. formosensis* heartwood essential oil completely killed the larvae of *Aedes aegypti* and *Aedes albopictus*, and also resulted in a 100% mortality of silverfish [4]. Another study showed that twig essential oil of *C. formosensis* inhibited the growth of several phytopathogenic fungi, with high antifungal indices. The two main compounds, tau-muurolol and α-cadinol, exhibited excellent activity [5]. The ethyl acetate (EA) fraction of the methanol extract of *C. formosensis* showed strong inhibition of liposaccharide (LPS)-mediated nitric oxide (NO) production in the murine macrophage-like cell line RAW264.7, and certain compounds identified only in this tree species exhibited a NO reduction effect *via* the suppression of inducible nitric oxide synthase (iNOS) [6].

Cymbopogon nardus (*C. nardus*) is a perennial grass cultivated in Southeast Asia. The essential oil of *C. nardus* is commonly used as a mosquito repellent, household fumigant, food industry and in some cosmetics. Studies have investigated the biological activities of the essential oil of *C. nardus*, and indicated its broad inhibition and killing capacity against fungi, including *Aspergillus niger* [7], *Aspergillus candidus*, *Aspergillus flavus*, *Eurotium amstelodami*, *Penicillum citrinum*, and so on [3]. Recently, a test of *C. nardus* essential oil demonstrated significant acaricide efficacy against *Anocentor nitens* and *Amblyomma cajennense* [8].

To date, in spite of the common application of *C. formosensis* and *C. nardus* essential oils and their related products, the biological activities *in vivo* have never been investigated. In this study, essential oils from two Taiwan native plants, *C. formosensis* and *C. nardus*, were prepared, and their composition, antioxidant activity, and acute and subacute oral toxicities in a mouse model were investigated.

2. Methods

2.1. Chemicals and reagents

3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), butylated hydroxytoluene (BHT), β-carotene, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and corn oil were purchased from Sigma (St. Louis, MO, USA). General chemical reagents were purchased from JT Baker (Phillipsburg, NJ, USA). Cell culture-related reagents were purchased from Life Technologies (Gaithersburg, MD, USA).

2.2. Gas chromatography mass spectrometry (GC-MS)

Gas chromatography was carried out in a fused silica capillary HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm; Agilent, Santa Clara, CA, USA). Helium was used as the carrier gas at a flow rate of 1 ml/min. The temperature program was as follows: ion source temperature at 200°C, injector at 250°C, and oven maintained at 40°C, which was then increased to 90°C at 4°C/min and to 220°C at 10°C/min, and finally held isothermally for 25 min. 1-μl hexane-diluted samples were injected manually. GC-MS analysis was conducted on an Agilent 6980 GC System Gas Chromatograph coupled to a 5973 Network Mass Selective Detector (Agilent) operating in the EI mode at 70 Ev, equipped with a split/splitless injector at 250°C. Identification of each compound was based on comparison of their retention time (RT) and mass spectra with those obtained from authentic standards. The relative percentage was calculated based upon the individual peak area of the total identified compound peak area.

2.3. Steam distillation of essential oils

C. formosensis and *C. nardus* samples were distilled by Yu-Yuan-Tang Inc. (Taidong, Taiwan) and provided by Young Living Company (Lehi, UT, USA). Wood of *C. formosensis* and partially-dried grass of *C. nardus* were harvested as is or chipped to a smaller size and placed in a stainless steel distillation vat, in which steam was passed from the base of the vat towards the top. A condenser on the top of the vat was used to cool the steam and the essential oil was condensed into liquid. The essential oil floated on top and was separated from the water and stored at -20°C. Reference samples of the essential oils were retained by Young Living Essential Oil Company (Lehi).

2.4.DPPH radical-scavenging assay

A DPPH free radical-scavenging assay was carried out for the evaluation of antioxidant activity. This test provides information regarding the ability of a compound to donate a hydrogen atom, the number of electrons a given molecule can donate, and the mechanism of the antioxidant action [9]. The essential oils were dissolved in methanol, and various concentrations of each oil were obtained. A well-known strong antioxidant compound, BHT, was included as a positive control for comparison [10].

The assayed mixture of a total volume of 1 ml consisted of 500 µl of the tested sample, 125 µl of the prepared DPPH (1 mM in methanol) and 275 µl methanol. After the solution had been left standing for 30 minutes at 25°C, a microplate reader (model 680, BioRad Laboratories, Hercules, CA, USA) was used to measure the absorbance at 540 nm. The DPPH radical-scavenging activity was estimated from the decrease in absorbance at 540 nm and is expressed as % DPPH scavenging activity.

2.5. β-carotene bleaching (BCB) test

The BCB assay principle has been described in the literature [11]. 0.5 g β-carotene was dissolved in 1 ml of chloroform and mixed with 6.25 µl of linoleic acid and 25 µl of Tween-20. The chloroform was evaporated under vacuum at 35°C, then 25 ml of distilled oxygenated water were added and the solution was stirred. 100 µl stable emulsion were transferred into a different well in a 96-well plate containing essential oil dissolved in absolute ethanol at various final concentrations. BHT was used as a synthetic reference [12, 13]. The 96-well plate was incubated at 50°C for different incubation durations, and the absorbance was measured using a Microplate Reader (Bio-Rad model 680) at 470 nm at 30 min intervals. A control blank containing ethanol instead of the sample was also included.

2.6. Cell culture

Murine macrophage-like cell line RAW 264.7 cells were suspended in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml Penicillin and 100 µg/ml Streptomycin. Mouse hepatocyte FL83B cells were cultured in Kaighn's modification of Ham's F12 (F-12K) medium with L-glutamine medium supplemented with 10% fetal bovine serum and 100 U/ml Penicillin and 100 µg/ml Streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. FL83B cells were grown as a monolayer and subcultures were obtained with trypsin.

2.7. Cellular cytotoxicity

The MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) cleavage assay, originally described by Mosmann [14], is used for measuring cell survival and/or proliferation. The MTT assay was used as a viability assay for RAW 264.7 and normal mouse hepatocyte FL83B cells. 1×10^4 and 6×10^3 cells/well respectively were seeded into 96-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The plates were then treated with varying types and concentrations of essential oils and incubated for 24, 48 and 72 h. All essential oils were dissolved in 0.04% Tween 20. The concentrations of essential oils used were 0-50 µg/ml. Each group was tested in triplicate. After incubation, MTT solution was added to each well to a final concentration of 0.5 mg/ml per well and the plates were incubated at 37°C for another 4 h. Then, the mixture containing the medium, the drug, and the unconverted MTT was removed. 100 µl DMSO were added to each well to dissolve the formazan and the absorbance was read at 540 nm. The cytotoxic concentration of the essential oil that reduced the viable cell number by 10% (IC_{10}) was determined from concentration-response curves.

2.8. Determination of intracellular reactive oxygen species (ROS)

Reactive oxygen species (ROS) measurement was performed using an OxiSelect Intracellular ROS Assay Kit (Cell Biolabs, San Diego, CA). The assay was performed according to the manufacturer's instructions. Briefly, cultured cells in a 96-well plate were pre-treated with the IC_{10} of the tested essential oils or control corn oil for 24 h. Cells were washed with PBS and treated with 1 M H_2O_2 for 1 h then incubated with the cell-permeable fluorescent probe 2',7'-dichloro-dihydro-fluorescein diacetate (DCFHDA) for 40 min at 20 µM in a 37°C incubator. The fluorescence was measured at wavelengths of 485 nm (excitation) and 527 nm (emission) using a fluorescence plate reader (model 9200-002, Turner BioSystems, Sunnyvale, CA, USA).

2.9. Lipid peroxidation

Lipid peroxidation was evaluated in terms of the malondialdehyde (MDA) content by a thiobarbituric acid (TBA) reactive substance (TBARS) assay with some modifications [15]. RAW264.7 and FL83B cells were treated with the IC_{10} concentrations of the essential oils for 24 h, then washed with cold PBS, scraped into 2.8% trichloroacetic acid (TCA) and sonicated. An equal amount of cellular homogenous lysate was mixed with SDS, acetic acid and TBA. The mixture was vigorously mixed and incubated in a water bath at 95°C for 30 min and centrifuged at 1200 ×*g* for 10 min. The TBA-reactive substance containing the supernatant had a pink color, and the absorbance at 532 nm was measured. Lipid peroxidation was calculated as nM MDA per mg

protein by interpolation of a standard curve and expressed as the inhibition percentage against corn oil-treated cells.

2.10 Measurement of antioxidant enzyme activity

RAW264.7 and FL83B cells cultured in a 6-cm dish were incubated with corn oil or the tested essential oils for 24 h at the non-toxic IC_{10}. A superoxide dismutase (SOD) activity assay kit was purchased from BioVision (Mountain View, CA, USA). The assay was performed according to the instruction manual provided with the kit. Briefly, treated cells were collected, washed and lysed with cold lysis buffer (100 mM Tris, pH 7.5, 0.5% Triton X-100, 5 mM β-mercaptoethanol, 0.1mg/ml PMSF). The supernatant contained the total SOD activity from cytosol and mitochondria. Samples were mixed with a prepared xanthine and chromogen solution reagent, which was provided with the kit, and the 96-well plate was incubated at 37°C for 30 mins. The absorbance was read at 450 nm. The SOD activity (inhibition rate %) was calculated using the following equation: SOD Activity (inhibition rate %) = $[(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})]/(A_{blank1} - A_{blank3})$, where blank1-3 were prepared according to the instructions provided with the kit.

2.11. Acute oral toxicity in mice

Determination of the LD_{50} values of *C. formosensis* and *C. nardus* essential oils was conducted using 28 BALB/c mice of both sexes of 28-30 g in weight. They were divided into 7 groups and three different doses of 1000, 3000 and 5000 mg/kg of the two essential oils were administered. One group was fed with corn oil as a negative control. The total volume of the sample was 150 µl, administered *via* the oral route. The experimental procedure used for the toxicity studies was according to the Organization for Economic Co-operation and Development (OECD) guidelines no. 401 and 425 [16]. Over 24 hours, the behavior of the mice was observed, including convulsions, disorientation, weakness, hyperventilation, food intake, and salivation. The toxicological effect was assessed based on mortality, which was expressed as the LD_{50}. The percentage of animals that had died at each dose was transformed to a probit value, and the probit values obtained were plotted against the log of the dose, calculated according to the Miller and Tainer method described in Randhawa [17] and in the literature [18]. Serum samples were collected from 5000 mg/kg-treated dead mice and corn oil-treated control mice, and were then subjected to biochemical analysis.

2.12. Subacute oral toxicity in mice

20 BALB/c mice of both sexes weighing 28-30 g were used in this study. 5 groups were designated, group 1 mice being corn oil-treated controls. Essential oils were administered to the mice daily by gavage for 28 days at doses of 300 and 600 mg/kg body weight on the basis of the LD_{50} value. The behavior, food and water intake of the mice, as well as the body weight, were observed and recorded every day. At the end of the experiment, serum samples were collected by centrifugation and subjected to biochemical assays. All animals were also subjected to full, gross necropsy of the external surface of the body, all orifices, and body cavities. The organs were dissected out and their size and weight recorded. The external appearance of the animals and the appearance of the solid organs were carefully examined, and any significant differences from the control mice were recorded [19]. The significant differences were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test as a post-analysis. Statistical analysis was carried out using GraphPad Prism Software version 6.00 for Windows (San Diego, CA, USA).

2.13. Biochemical studies

Four parameters were evaluated to compare the liver and renal functions between the control mice and the essential oil-treated mice. Concentration measurements of aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine (CRE) in the serum were obtained by SPOTCHEM EZ sp-4430 (ARKRAY, Japan) using SPOTCHEM II AST, ALT, BUN, and CRE Reagent Strips, respectively. The rationales of each assay and procedures were described in our previous study [20]. Significant differences were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test as a post-analysis. Statistical analysis was carried out using GraphPad Prism Software version 6.00 for Windows.

3. Results

3.1. Chemical constituent identification

There were 15 compounds representing 86.61% of the *C. formosensis* essential oil, while several compounds were unidentified. GC and GC/MS analysis revealed that the most abundant compounds were myrtanol (22.4%), (+)-nopinone

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(17.34%), δ-cadinene (13.24%) and myrtenal (4.97%), which constituted more than 50% of the composition of the oil. Regarding *C. nardus* oil, 19 compounds were identified, which represented more than 90% of the content. Among these, citronellal (42.12%) was the most abundant constituent, followed by geraniol (15.15%) and citronellol (10.27%). The other components were present as minor constituents or at trace level. The compounds are listed in Table 1.

Table 1. Chemical compositions of *C. formosensis* and *C. nardus.*

No.	Compound	Retention time (min)	Relative amount %	
			C. formosensis	*C. nardus*
1	Limonene	17.695	1.66	
2	2-Fenchanol	23.453	1.17	
3	Nopinone	24.948	17.34	
4	Borneol	27.015	2.46	
5	α-Terpineol	28.875	3.17	
6	Myrtenal	29.098	4.97	
7	Myrtanol	33.448	22.4	
8	β-Elemene	42.133	2.38	
9	Isoledene	42.622	2.51	
10	γ-Muurolene	47.253	1.89	
11	β-elemene	47.669	3.49	
12	α-elemene	48.238	2.50	
13	α-Muurolene	48.706	3.16	
14	γ-Cadinene	49.437	4.27	
15	δ-Cadinene	50.090	13.24	
16	Limonene	17.656		3.99
17	Linalool	22.852		0.47
18	Citronellal	26.551		42.12
19	Citronellol	31.795		10.27
20	Geraniol	33.533		15.15
21	Citral	34.503		0.48
22	Citronellyl acetate	40.053		2.93
23	Geranyl acetate	41.995		1.93
24	β-elemene	42.122		2.25
25	Germacrene D	47.407		0.70
26	α-Muurolene	48.722		0.41
27	γ-Muurolene	49.434		0.65
28	δ-Cadinene	50.083		2.36
29	Elemol	51.552		5.88
30	Fenchone	52.924		1.90
31	β-eudesmol	56.082		0.52
32	τ-muurolol	56.687		1.08
33	α-Eudesmol	57.186		0.49
34	β-eudesmene	57.958		0.75

3.2. Evaluation of antioxidant activity

Essential oil or plant extracts can act as antioxidants to provide a protective role in animal tissues [21]. The antioxidant potentials of the two essential oils were examined using two independent complementary experiments, a DPPH assay and BCB tests. The method of scavenging DPPH free radicals can be used to evaluate the antioxidant capacity in a short time. As shown in Fig. 1A, 100 μg/ml BHT reduced the level of free radicals by about 50% in our system. *C. formosensis* essential oil showed a low to moderate DPPH scavenging activity; however, essential oil of *C. nardus* revealed an excellent DPPH scavenging activity, a BHT-comparable activity at a concentration of 10 μg/ml, and greater than 80% free radical scavenging at 50 μg/ml. The reactions of the two essential oils followed a dose-dependent pattern. In a linoleic acid system, *C. nardus* essential oil suppressed the oxidation of β-carotene and linoleic acid more efficiently than *C. formosensis* essential oil. At a 50 μl/ml dose, *C. nardus* essential oil revealed an antioxidant ability comparable to that of the positive control BHT (Fig. 1B).

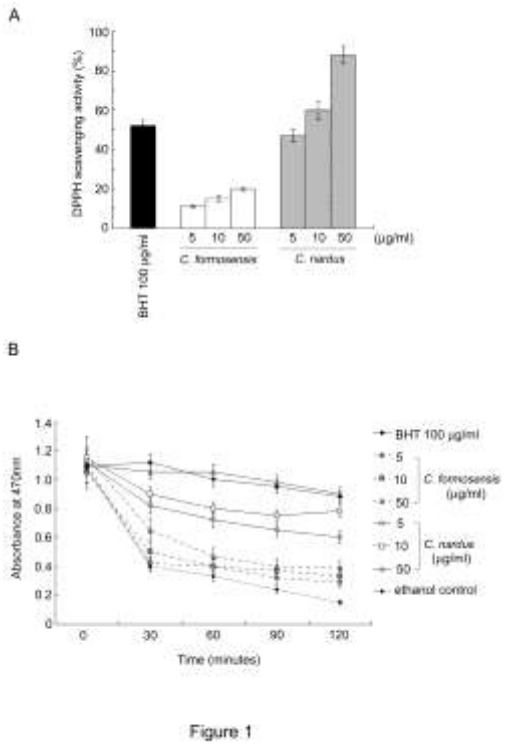

Figure 1

Figure 1. *In vitro* antioxidant effects of *C. formosensis* and *C. nardus* essential oils.
(A) DPPH assay. The y-axis indicates the free radical scavenging percentage, and the x-axis

illustrates each reaction condition. DPPH scavenging activity (%) = (Abs $_{control}$-Abs $_{sample}$)/ Abs $_{control}$ × 100 %, where Abs $_{control}$ is the absorbance of the control reaction (containing all reagents except the tested sample) and Abs $_{sample}$ is the absorbance of the tested samples. The experimental results are expressed as means ± SD. All measurements were replicated at least three times.

(B) BCB test. The y-axis represents the O.D. value at 470 nm. The x-axis shows the incubation duration for each condition. Experiments were conducted three times independently, and the test under each condition was performed in duplicate. The data shown are the means ± SD.

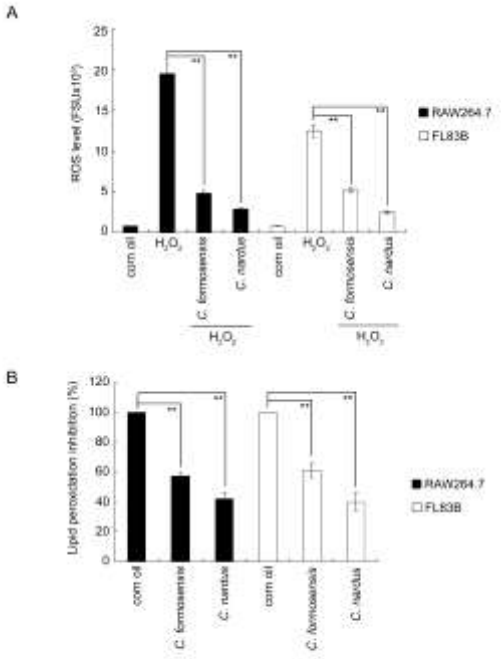

Figure 2

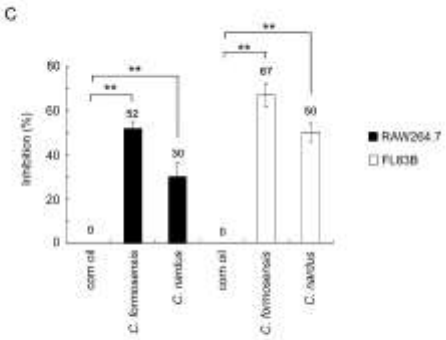

Figure 2

Figure 2. Antioxidant effects of *C. formosensis* and *C. nardus* essential oils in cultured cells.

(A) ROS assay. The arbitrary value of fluorescence standard units (FSU) is shown on the y-axis. The x-axis indicates the treatment, i.e., with corn oil, H_2O_2 only, or combined treatment.
(B) Lipid peroxidation analysis. The y-axis shows the inhibition percentage against corn oil-treated cells.
(C) SOD activity. The y-axis indicates the superoxide anion reduction percentage. The x-axis represents the treatment condition, i.e., corn oil or IC_{10} concentrations of the essential oils. The experimental results are expressed as means ± SD. All measurements were replicated at least three times. The level of significance was expressed using one-way analysis of variance (ANOVA) followed by Student's *t*-test. $*P < 0.05$ and $**P < 0.01$ as indicated. The average values are shown.

We next further extended our examination of the antioxidant activity and confirmed it using a cell culture-based system. This was the first time that the antioxidant activities of these two essential oils were examined using cultured cells. Two cell types were used, a macrophage-like RAW264.7 cell line that belongs to the category of immune cells, and a normal hepatocyte FL83B mouse cell line. RAW264.7 has been reported to be a suitable cell model to assay the cellular antioxidant activity [22]. As the liver is a key organ that is prone to injury due to chronic exposure to environmental toxicants, drugs and other chemicals, we therefore chose FL83B, a normal mouse non-cancer hepatocyte cell line, as the *in vitro* model for this study. To determine the non-toxic concentrations of the cells, a MTT assay was employed. Treatment with essential oils at various concentrations was administered for 24, 48 and 72 h, and the IC_{10} values were calculated, as shown in Table 2. The cells revealed different cell toxicity responses, FL83B being more sensitive in general than RAW264.7 to a certain degree, while during 72-h treatment with *C. formosensis* essential oil, FL83B was found to be more resistant than RAW264.7. Thus, the subsequent functional experiments used the IC_{10} as the working concentration to ensure that at least 90% of the cells were viable. To investigate the antioxidant protective effects of the essential oils, the intracellular ROS level was measured after hydrogen peroxide (H_2O_2) treatment [23, 24]. Negative control corn oil treatment did not alter the ROS level. In response to H_2O_2, DCF fluorescence increased in both cell types, although the increase was greater in the RAW264.7 cells (Fig. 2A). Pretreatment with the two tested essential oils at the IC_{10} concentrations significantly reduced ROS generation, a more dramatic effect being observed following *C. nardus* treatment (Fig. 2A). The results suggested that both essential oils are good ROS scavengers. Free radicals and the ROS-mediated process are markedly associated with lipid peroxidation of biological membranes, and malondialdehyde (MDA) is a major reactive aldehyde used for the estimation of lipid peroxidation [25]. As indicated in Fig. 2B, the lipid peroxidation inhibition abilities of the two tested essential oils at the IC_{10} concentration were significant for both cell types, essential oil of *C. nardus* showing the better lipid peroxidation activity. Having confirmed the antioxidant functions of the essential oils, the antioxidant enzyme activity was examined to provide further evidence to support our findings. SOD catalyzes the dismutation reaction of the superoxide anion into hydrogen peroxide and molecular oxygen, and is one of the most critical antioxidative enzymes [26]. The assay generated superoxide anions (O_2^-) by a Xanthine/Xanthine Oxidase (XOD) system, and detection was then performed using a chromagen solution. In the presence of SOD, the superoxide anion concentration is reduced, producing a lesser colorimetric signal. The inhibition percentage level was significant upon *C. formosensis* and *C. nardus* essential oil pretreatment (Fig. 2C). In conclusion, by utilization of a cell culture-based assay, we demonstrated the antioxidant activities of *C. formosensis* and *C. nardus* essential oils, and showed that *C. nardus* essential oil had a better antioxidant efficacy.

Table 2. IC_{10} values of *C. formosensis* and *C. nardus* essential oils in relation to cytotoxicity in cultured cells

Essential oil (μg/ml)	*IC_{10} of RAW 264.7			*IC_{10} of FL83B		
	24 h	48 h	72 h	24 h	48 h	72 h
C. formosensis	8.49	4.19	1.04	2.33	2.45	2.03
C. nardus	9.03	6.88	4.58	1.79	1.85	1.88

*Experiments were performed at least three times independently, and for each condition in duplicate. Based on the dose-response data, the IC_{10} was obtained using a four-parameter logistic function

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3.3. Acute and subacute oral toxicity

With the aim of future application of essential oils for human health care, knowledge of *in vivo* oral toxicity is required. To establish the safety of these two essential oils, the LD_{50} in a mouse model needed to be defined. No significant clinical toxic signs and no deaths in the control group or the 1000 mg/kg essential oils oral administration groups were noted within 24 h. Nevertheless, at dosages of 3000 and 5000 mg/kg, significant mouse death and toxic signs within 24 h were clearly observed. The calculated LD_{50} was 2290.87 ± 684.28 and 2511.89 ± 791.51 for *C. formosensis* and *C. nardus* essential oils, respectively, with 95% confidence intervals. The 24-h observation record is shown in Table 3. In order to clearly understand the relationship between organ damage and mice survival, serum samples from the controls and dead mice were collected and subjected to biochemical parameters analysis.

Table 3. Symptoms and survival durations of mice treated with essential oils at various doses.

Treatment	Dose (mg/kg)	Death/ Total	Symptoms	Survival duration
Corn oil		0/4	None	7 days
C. formosensis	1000	0/4	Sedation, piloerection	7 days
C. formosensis	3000	04-Mar	Sedation, convulsion, decreased respiration	2h-7 days
C. formosensis	5000	04-Apr	Sedation, convulsion, darkened skin color, drastically decreased respiration, weakness, decreased food and water intake	2-22 h
C. nardus	1000	0/4	Sedation	7 days
C. nardus	3000	04-Feb	Sedation, piloerection	2h-7 days
C. nardus	5000	04-Apr	Sedation, darkened skin color, decreased respiration, weakness, drastically decreased food and water intake	4-21 h

The dead mice exhibited significantly elevated AST, ALT, BUN and CRE levels as compared with the control mice (Table 4). Thus, these results conclusively indicated clear toxicity and organ damage at higher dosages *via* the oral route.

Table 4. Biochemical profiles of the dead mice treated with *C. formosensis* and *C. nardus* and the corn oil-treated mice

	Corn oil-treated	*C. formosensis*	*C. nardus*
AST (U/L)	191.5 ± 8.6	*>1000	>1000*
ALT (U/L)	67.6 ± 5.4	*436.4 ± 56.3	*656 ± 85.4
BUN (mg/dL)	23.9 ± 2.3	*39.1 ± 5.8	*46.4 ± 7.2
CRE (mg/dL)	0.3 ± 0.1	*0.7 ± 0.2	*1.8 ± 0.2

The data are the average values of the collected samples. 4 mice were in the corn oil-treated group, 7 dead mice received *C. formosensis* and 6 dead mice received *C. nardus* essential oil. * represents a significant difference as compared with the control ($P < 0.05$).

Based on the LD_{50} obtained in the acute study, two sublethal doses of 300 and 600 mg/kg were used for both oils, and the mice were fed once every 24 h for 28 days. At the end of the observation period, all mice were alive, with the exception of one animal that died in the *C. nardus* 600 mg/kg treatment group on the 22nd day of administration (Table 5). No significant differences between the control and treated mice were noted in terms of body appearance, behaviors, food and water consumption and general response, with the exception of the mice treated with *C. nardus* at a 600 mg/kg dosage (Table 5). The ratios of organs/body weight revealed significant increases in the liver and stomach weights in mice that received 600 mg/kg *C. nardus* essential oil, suggesting that the two organs were enlarged (Table 6). Finally, serum biomarkers for liver and kidney function revealed elevated ASL, ALT and BUN levels in mice orally administered 600 mg/kg of *C. nardus* (Table 7). Overall, the repeated dose toxicity data suggested that the 300 mg/kg dose is safe, whereas a higher and longer exposure will elicit more damage, and mice that received *C. nardus* revealed more adverse or hazardous effects and exhibited an impact on health in the current model.

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Table 5. Symptoms and survival durations observed in a subacute study in which mice were treated with *C. formosensis* or *C. nardus* essential oils for 28 consecutive days.

Treatment	Dose (mg/kg)	%age survival	Symptoms	Survival duration
Corn oil		100 %	None	28 days
C. formosensis	300	100 %	None	28 days
C. formosensis	600	100 %	Sedation, piloerection, Decreased body weight	28 days
C. nardus	300	100 %	Sedation	28 days
C. nardus	600	75%	Sedation, piloerection, decreased respiration, drastically decreased food and water intake	22 days

Table 6. Ratios of organs/body weight of mice treated with *C. formosensis* and *C. nardus* essential oils for 28 days.

		C. formosensis		*C. nardus*	
	Control	300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg
Heart	0.49 ±0.02	0.77 ± 0.19	0.58 ± 0.06	0.68 ± 0.04	0.51 ± 0.05
Lung	0.84 ±0.08	0.66 ± 0.04	0.67 ± 0.17	0.69 ± 0.15	0.98 ± 0.18
Spleen	0.38 ±0.02	0.50 ± 0.05	0.50 ± 0.06	0.74 ± 0.49	0.60 ± 0.20
Liver	4.88 ±0.06	5.15 ± 0.82	4.90 ± 0.87	5.16 ± 0.30	*7.80 ± 0.80
Kidney	1.63 ±0.03	1.76 ± 0.31	1.22 ± 0.10	1.60 ± 0.02	1.84 ± 0.34
Stomach	1.01 ±0.01	1.96 ± 1.49	2.39 ± 0.24	2.10 ± 0.66	*4.98 ± 2.16

The original values of organ weight divided by body weight multiplied by 100 are shown. The data are representative, shown as the means ± SD; *P <0.05 compared with the control.

Table 7. Blood chemistry of mice treated with *C. formosensis* and *C. nardus* essential oils for 28 days.

		C. formosensis		*C. nardus*	
Parameters	Corn oil	300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg
AST (U/L)	42.50 ± 2.12	43.50 ± 0.71	46.00 ± 0.00	37.50 ± 2.12	*62.50 ± 14.85
ALT (U/L)	18.33 ± 2.31	15.00 ± 1.73	20.67 ± 0.58	16.67 ± 3.79	*23.50± 3.54
BUN (mg/dL)	13.15 ± 0.28	17.10 ± 0.57	*23.85 ± 0.07	16.05 ± 4.03	*22.95 ± 0.35
CRE (mg/dl)	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00

Data are representative, shown as the means ± SD. *P <0.05 compared with the control

4. Discussion

The current study extracted the essential oils from two native Taiwan plants. The results clearly demonstrated a better antioxidant activity of *C. nardus* than *C. formosensis* essential oil. Furthermore, this was the first report to describe the acute and subacute oral toxicities of these two essential oils using a mouse model. Our observations indicated the LD_{50} values of these two essential oils, and the effects on liver and renal function serum markers. Severe toxicity was observed in the dead mice, and *C. nardus* essential oil exhibited a more evident impact on health.

Two recent reports analyzed the composition of *C. formosensis* essential oil. According to a study carried out by Ho et al. [5], the main components of fresh twig oil were β-eudesmol (25.1%), τ-muurolol (21.6%), elemol (15.0%), totarol (14.9%), and α-cadinol (12.4%). The second paper described the composition of essential oil extracted from 80-year-old heartwood of *C. formosensis*, indicating 32 constituents, α-eudesmol (18.06%), β-guaiene (8.0%), (–)-β-cadinene (7.89%) and γ-costal (7.03%) being the most abundant components [3]. An earlier study by Kafuka and Ichikawa in 1931 examined the violate compound of the *C. formosensis* leaf and showed that α-pinene was the major constituent, making up 85% of the total essential oil. A later study also confirmed α-pinene to be the predominant compound of the essential oil of *C. formosensis* leaf [27]. Four new diterpenoids have been identified from the pericarps of *C. formosensis* [28]. Kuo et al. reported the major compositions of heart wood essential oil from *C. formosensis* to be myrtenol (48.89%) and myrtenal (13.17%) [4]; critically, these

two compounds were also present in our sample at significant levels. Therefore, the fact that the chemical compounds of *C. formosensis* have been found in different compositions might be due to essential oil extraction from different parts of the plant. Regarding composition analysis of *C. nardus*, one report by Koba et al. [29] indicated citronellal (35.5%), geraniol (27.9%) and citronellol (10.7%) to be the major compounds, and another study also reported a similar composition [30], while a study by Nakahara et al. detected a much lower level of citronellal (5.8%) in *C. nardus* essential oil [31]. Our data were consistent with several previous studies reporting that citronellal, geraniol and citronellol are the most abundant three compounds. Citronella and citronellol exhibited significant anti-bacterial and anti-fungal properties, as well as a high repellent effectiveness [32]. Additionally, geraniol demonstrated inhibition of pancreatic cancer growth [33] and interfered with membrane functions of Candida and Saccharomyces [34]. The composition differences in the essential oil might arise from differing environmental conditions, such as climatic, seasonal and geographical factors, and may also be affected by the plant genetic background [35].

Numerous studies have investigated the antioxidant activities of pure or mixed natural extracts, largely based on assays performed in test tubes, such as the DPPH assay, ferric ion reducing antioxidant power (FRAP) analysis, and hydrogen peroxide-scavenging activity (TEAC). Results obtained using a single system can provide only reductive suggestions regarding the antioxidant capacities of tested samples. Furthermore, the high chemical complexity of essential oils, which contain dozens of compounds, might lead to scattered results that depend on the plant culture environment, the essential oil preparation method and conditions, and the test employed. Therefore, in addition to a DPPH assay for determination of the antioxidant activity of a hydrophilic species, the BCB test for determination of the antioxidant activity of lipophilic components was also performed in this study. We further extended the antioxidant property evaluation by using cell culture-based assays to assess the bioactivities of antioxidants. From our results, the antioxidant activity was found to be concentration-dependent and consistent between test tube assays and the cell culture system analysis. *In vitro* assays showed that the essential oil of *C. nardus* exhibited a significantly higher antioxidant activity than *C. formosensis*, while in two tested cell lines, ROS assay, lipid peroxidation and SOD activity revealed a comparable and significant antioxidant activity . Basically, the two essential oils tested in this study were found to possess the potential to be used as antioxidants in functional products. Importantly, various kinds of compounds related to antioxidant activity was identified in these two essential oils. Toxicology is the most critical aspect of pharmacology. It considers the possible effects of bioactive substances on living organisms. *In vivo* toxicity experiments in a mouse model were used to obtain the LD_{50} values and perform behavior observation during repeated administration, as well as evaluate liver and kidney function, providing valuable reference information for further clinical application. AST and ALT are good serum markers used to evaluate hepatocyte necrosis, while CRE and BUN are good biomarkers used to evaluate kidney damage [36, 37]. Severe changes were observed in the dead mice in the acute toxicity study, and mild effects were seen in the subacute study in terms of these serum markers. It was also observed that the weights of the liver and stomach increased significantly only in the mice that received a higher dose of *C. nardus* in the subacute assay.

Because of the large number of constituents, various different mechanisms might be involved in cytotoxic effects of essential oils or their specific components. Based on the lipophilic nature of essential oil, they penetrate the cytoplasmic membrane and disrupt the membrane structure. Additionally, the most common mechanisms utilized by essential oils include induction of apoptosis, necrosis, cell cycle arrest, and provoke depolarization of the mitochondrial membranes [38]. The liver is a target organ that is prone to injury due to chronic exposure to environmental toxicants and other chemicals. It also plays a major role in detoxification, in which toxic compounds are transformed to reduce toxicity. According to the results of our subacute study in mice treated with *C. formosensis* or *C. nardus* essential oils, significantly increased ALT and AST levels indicated early liver damage, and an elevated BUN level indicated renal toxicity in mice treated with *C. nardus* at 600 mg/kg; an elevated BUN level also suggested that *C. formosensis* results in renal toxicity at the higher dose of 600 mg/kg. High doses of *C. nardus* and *C. formosensis* resulted in symptoms (Table 5) that may be associated with the increased liver weight in mice treated with *C. nardus* (Table 6), and elevated ALT and AST levels were observed, which further support the presence of liver damage. An increased BUN level implied that higher doses of *C. nardus* and *C. formosensis* may cause kidney dysfunction. Hence, valuable information regarding the method of use of essential oils was obtained, and it was apparent that dosage control is critical. Subchronic and chronic long-term toxicity studies are needed in order to understand the possible adverse effects. Based on the toxicology data, identification of the sublethal dosage during short- or long-term administration will

assist in further examination of the antioxidant protective functions of essential oils in a mouse model.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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