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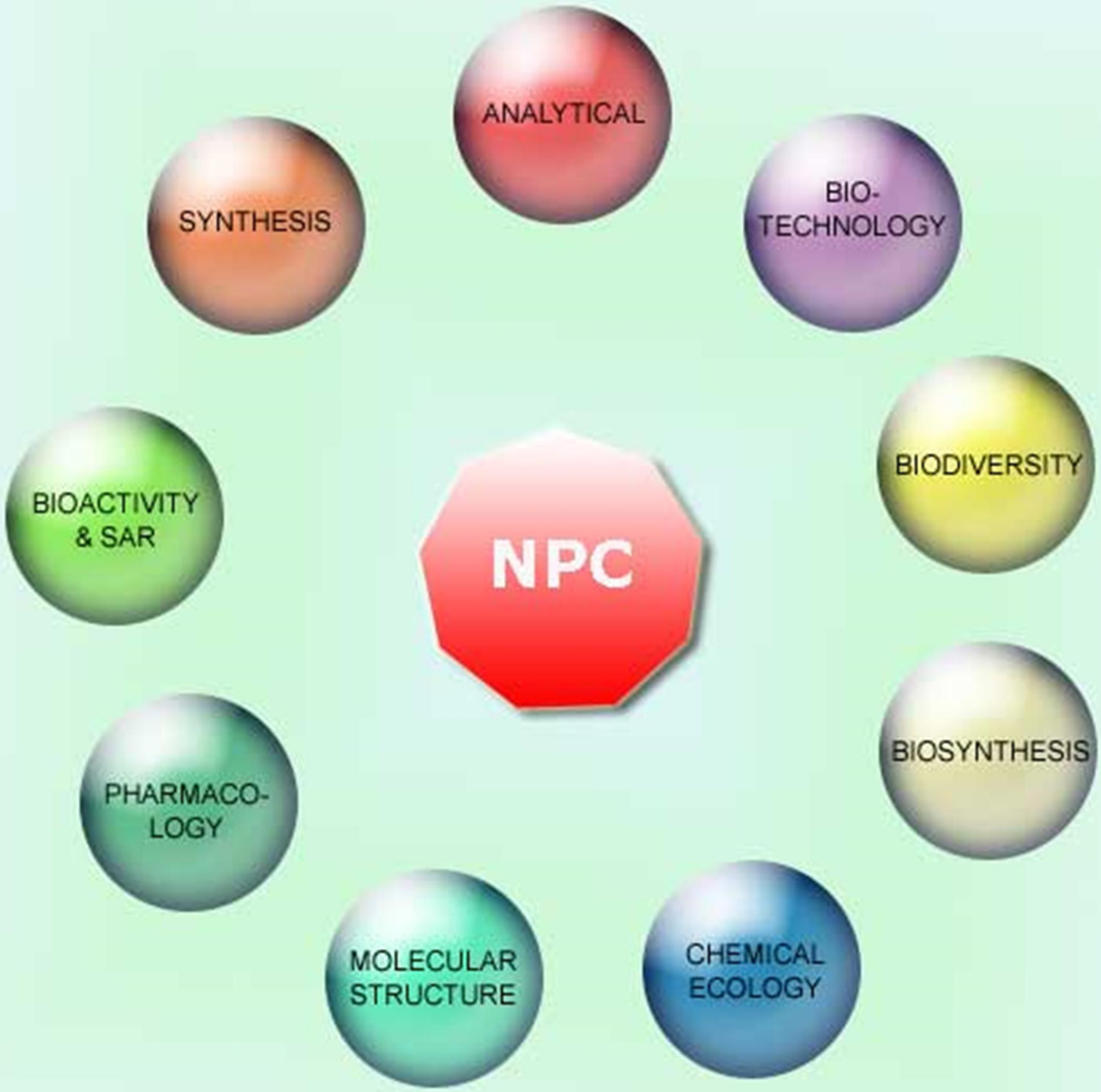

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The Composition, Anti-mildew and Anti-wood-decay Fungal Activities of the Leaf and Fruit Oils of *Juniperus formosana* from Taiwan

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In this study, anti-mildew and anti-wood-decay fungal activities of the leaf and fruits essential oil and its constituents from *Juniperus formosana* were evaluated *in vitro* against seven mildew fungi and four wood decay fungi, respectively. The main compounds responsible for the anti-mildew and anti-wood-decay fungal activities were also identified. The essential oil from the fresh leaves and fruits of *J. formosana* were isolated using hydrodistillation in a Clevenger-type apparatus, and characterized by GC–FID and GC–MS, respectively. The leaf oil mainly consisted of α-pinene (41.0%), limonene (11.5%), α-cadinol (11.0%), elemol (6.3%), and β-myrcene (5.8%); the fruit oil was mostly α-pinene (40.9%), β-myrcene (32.4%), α-thujene (5.9%) and limonene (5.9%). Comparing the anti-mildew and anti-wood-decay fungal activities of the oils suggested that the leaf oil was the most effective. For the anti-mildew and anti-wood-decay fungal activities of the leaf oil, the active source compounds were determined to be α-cadinol and elemol.

Keywords: *Juniperus formosana*, Essential oil, Antimildew activity, Anti-wood-decay fungal activity, α-Cadinol, Elemol.

Juniperus formosana Hayata (Cupressaceae) is a large tree mainly distributed in Taiwan, and China [1]. However, only three references were found regarding the chemical compositions of this species from China [2-4]. In Taiwan, there is no report of the essential oil composition and bioactivities for *J. formosana*. Therefore, in this study, the essential oil from the leaves and fruits was first isolated using hydrodistillation, and then analyzed. In addition, the climate of Taiwan is warm and humid, and thus conducive to the growth of mildew and wood decay fungi. Mildew growth causes problems in the preservation of cultivated crops as well as inducing allergies, asthma, bronchitis, onychomycosis, cerebral infections, pneumonia, peritonitis, and immune-deficiency syndrome [5]. The wood decay fungi can easily cause damage to wooden products. Therefore, we also applied the essential oils to seven strains of mold fungi and four of wood decay fungi to examine their interdiction efficacies, respectively. The second part of the study examined the anti-mildew and anti-wood-decay fungal activities of the leaf and fruit oils. The purpose of this study was to establish a chemical basis for the effective multipurpose utilization of the species.

Hydrodistillation of *J. formosana* leaves and fruits produced yellow-colored oils with yields (v/w), on a moisture free basis, of 1.51 ± 0.06 and 1.86 ± 0.05, v/w, respectively. All compounds are listed in order of their elution from the DB-5 column (Table 1). A total of 49 compounds were identified from the hydrodistilled leaf oil of *J. formosana*. Monoterpene hydrocarbons were predominant (69.2%), followed by oxygenated sesquiterpenes (20.5%), sesquiterpene hydrocarbons (5.4%), oxygenated monoterpenes (3.6%), and non-terpenoids (1.2%). Of the monoterpene hydrocarbons, α-pinene (41.0%), limonene (11.5%) and β-myrcene (5.8%) were the major compounds. α-Cadinol (11.0%) and elemol (6.3%) were the chief sesquiterpene hydrocarbons. In *J. formosana* leaf oil, Yu *et al*. [2] found 55 compounds, mainly α-pinene (9.6%), bornyl acetate (5.2%), limonene (4.3%), and myrcene (4.1%). Adams *et al*. [3] found 70 compounds, mainly α-pinene (47.7%), myrcene (7.2%), limonene (4.0%), β-pinene (2.9%), γ-cadinene (2.4%), and germacrene D (2.3%). Our results differed from the above papers with α-pinene, limonene, α-cadinol, elemol, and β-myrcene as the major compounds. This is the first presentation of these compounds in *J. formosana* leaf oil.

Twenty-five components were identified from the fruit oil. Among them, monoterpene hydrocarbons were the most dominant (93.4%), followed by sesquiterpene hydrocarbons (2.5%), oxygenated sesquiterpenes (2.2%), and oxygenated monoterpenes (1.9%). α-Pinene (40.9%), β-myrcene (32.4%), α-thujene (5.9%) and limonene (5.9%) were the major monoterpene hydrocarbons. In *J. formosana* fruit oil, Yu and Xie [4] found 47 compounds mainly myrcene (27.1%), α-pinene (26.1%), γ-terpinene (10.7%), and limonene (6.0%). Our results differed from the above paper with α-pinene, β-myrcene, α-thujene and limonene as the main compounds. This is the first presentation of these compounds for *J. formosana* fruit oil.

The leaf and fruit oils of *J. formosana* were tested against seven mildew fungi (*Aspergillus clavatus* (*A. c.*), *A. niger* (*A. n.*), *Chaetomium globosum* (*Ch. g.*), *Cladosporium cladosporioides* (*Cl. c.*), *Myrothecium verrucaria* (*M. v.*), *Penicillium citrinum* (*P. c.*), and *Trichoderma viride* (*T. v.*). The antifungal indexes demonstrated clearly that the leaf oil had antifungal activities superior to those of the fruit oil (Fig. 1). Among the fungi tested, the leaf oil was totally inhibitory of mycelial growth of *A. clavatus, Cl. cladosporioides*, *Ch. globosum*, and *M. verrucaria* at a 1 mg/mL concentration. The leaf oil was superior to the anti-mildew fungal activities of the essential oils from *Eucalyptus urophylla*, *E. grandis*, *E. camaldulensis*, *E. citriodora* [5], *Litsea cubeba* [6], *L. coreana* [7], and *Neolitsea parvigemma* [8]. The results verified that *J. formosana* leaf oil has notable antifungal activities.

However, to ascertain the source compounds responsible for *J. formosana* antifungal activities, the main components were

Table 1: Chemical composition of the leaf and fruit oils of *J. formosana*.

Consituents	K.I. [a)]	Concentration (%)		Identification [b)]
		Leaf	Fruit	
Tricyclene	927	0.1	- [c)]	MS, KI, ST
α-Thujene	930	0.1	5.9	MS, KI, ST
α-Pinene	939	41.0	40.9	MS, KI, ST
Camphene	954	0.6	0.5	MS, KI, ST
Verbene	968	0.5	0.4	MS, KI
β-Pinene	979	4.1	3.7	MS, KI, ST
β-Myrcene	991	5.8	32.4	MS, KI, ST
δ-2-Carene	1002	3.8	0.7	MS, KI
α-Phellandrene	1003	0.5	0.7	MS, KI, ST
p-Cymene	1025	0.8	0.3	MS, KI, ST
Limonene	1029	11.5	5.9	MS, KI, ST
Terpinolene	1089	0.4	1.9	MS, KI, ST
p-Cymenene	1091	0.2	-	MS, KI, ST
Linalool	1097	-	0.2	MS, KI, ST
allo-Ocimene	1132	-	0.2	MS, KI
trans-Pinocarveol	1139	0.2	-	MS, KI
cis-Verbenol	1141	0.2	-	MS, KI
Borneol	1169	0.3	-	MS, KI, ST
cis-Pinocamphone	1175	0.3	-	MS, KI
4-Terpineol	1177	0.4	0.8	MS, KI, ST
α-Terpineol	1189	-	0.6	MS, KI, ST
Citronellol	1226	0.5	-	MS, KI, ST
trans-Chrysanthenyl acetate	1238	0.1	-	MS, KI
Piperitone	1253	0.5	-	MS, KI
Bornyl acetate	1289	1.1	0.4	MS, KI, ST
Thymol	1290	0.1	-	MS, KI, ST
2-Undecanone	1294	0.4	-	MS, KI
Citronellic acid	1313	0.5	-	MS, KI, ST
α-Terpinyl acetate	1349	0.2	-	MS, KI, ST
α-Cubebene	1351	0.2	-	MS, KI, ST
α-Copaene	1377	0.4	-	MS, KI, ST
β-Bourbonene	1388	0.1	-	MS, KI
β-Caryophyllene	1419	0.4	0.9	MS, KI, ST
(*Z*)-β-Farnesene	1443	0.1	-	MS, KI, ST
α-Caryophyllene	1455	0.2	0.8	MS, KI, ST
(*E*)-β-Farnesene	1457	0.2	-	MS, KI
γ-Muurolene	1480	0.2	-	MS, KI, ST
β-Selinene	1490	0.3	-	MS, KI
α-Muurolene	1500	0.4	0.2	MS, KI, ST
β-Bisabolene	1506	-	0.3	MS, KI
γ-Ccadinene	1514	-	0.2	MS, KI
Cubebol	1515	0.4	0.7	MS, KI
δ-Cadinene	1523	2.0	-	MS, KI, ST
trans-Calamenene	1529	0.5	-	MS, KI
Elemol	1550	6.3	-	MS, KI, ST
Germacrene B	1561	0.5	-	MS, KI, ST
Dodecanoic acid	1567	0.2	-	MS, KI, ST
Caryophyllene oxide	1583	0.1	-	MS, KI, ST
Cedrol	1601	0.5	-	MS, KI, ST
γ-Eudesmol	1632	0.2	-	MS, KI
α-Muurolol	1646	0.9	0.9	MS, KI, ST
α-Eudesmol	1654	0.7	-	MS, KI, ST
α-Cadinol	1654	11.0	0.3	MS, KI, ST
α-Bisabolol	1686	0.3	0.4	MS, KI
Monoterpene hydrocarbons (%)		69.2	93.4	
Oxygenated monoterpenes (%)		3.6	1.9	
Sesquiterpene hydrocarbons (%)		5.4	2.5	
Oxygenated sesquiterpenes (%)		20.5	2.2	
Others (%)		1.2	-	
Oil Yield (mL/100 g)		1.51 ± 0.06	1.86 ± 0.05	

[a] Kovats index on a DB-5 column with reference to *n*-alkanes [9]. [b] MS, NIST and Wiley library spectra and the literature; KI, Kovats index; ST, authentic standard compounds. [c] Not detected.

individually tested for their antifungal activities (Fig. 2). As for α-pinene, β-myrcene and limonene, very low levels of activity were found against the seven mold fungi; none of the antifungal indices exceeded 30%. However, the sesquiterpenoids, elemol and α-cadinol exhibited better activities. Elemol and α-cadinol exhibited activity against *A. clavatus*, *Cl. cladosporioides, Ch. globosum*, *M. verrucaria* and *T. viride*, with the highest antifungal indexes ranging from 80% to 100% at 100 μg/mL. The IC_{50} values for α-cadinol against these five fungi were 20.8, 12.8, 33.8, 20.2, and

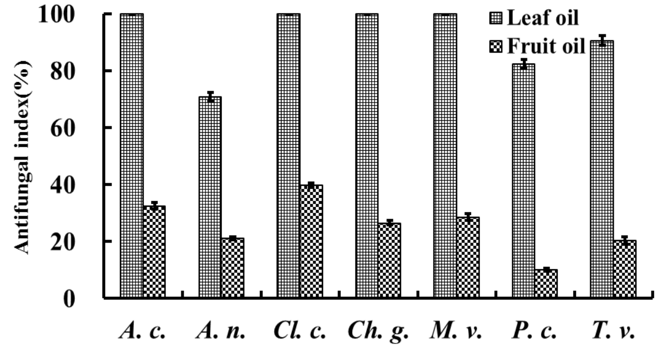

Figure 1: Antifungal activities of the leaf and fruit oils (1 mg/mL) from *J. formosana* against: *A. c.*: *Aspergillus clavatus*; *A. n.*: *A. niger*; *Cl. c.*: *Cladosporium cladosporioides*; *Ch. g.*: *Chaetomium globosum*; *M. v.*: *Myrothecium verrucaria*; *P. c.*: *Penicillium citrinum*; *T. v.*: *Trichoderma viride*

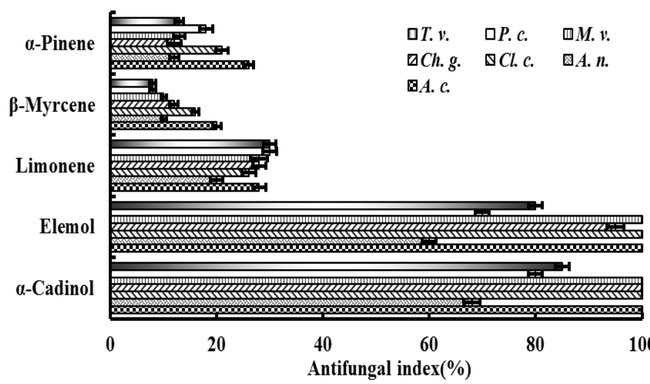

Figure 2: Anti-mildew fungal indices of the five main compounds (50 μg/mL) of the leaf essential oil of *J. formosana*.
1. α-Pinene (98.5%), 2. β-Myrcene (98.5%), 3. Limonene (98.5%), 4. Elemol (98.5%), 5.α-Cadinol (100%). Compounds 1 to 3 were purchased from the Fluka Co., compound 4 from the Wako Co. (Tokyo, Japan) and compound 5 was from an isolate of Ho *et al*'s study on *Machilus philippinenesis* essential oil [13].
Note: DDAC (didecyl dimethyl ammonium chloride) (50 μg/mL) is a wood preservative for wood decay fungi and is used as a positive control.

39.8 μg/mL, respectively. The IC_{50} values for elemol were 29.8, 20.8, 50.9, 36.8, and 48.9 μg/mL, respectively. The results indicated that the active source compounds were α-cadinol and elemol. Previous studies support the contention that these compounds have significant activity for suppressing microbial growth [8,10].

The leaf and fruit oils of *J. formosana* were tested against two white rot fungi (*Trametes versicolor, Phanerochaete chrysosporium*) and two brown rot fungi (*Phaeolus schweinitzii, Lenzites sulphureu*). The anti-wood-decay fungal indices presented in Table 2 clearly demonstrate the excellent anti-wood-decay fungal activities of the leaf oil. Growth of *T. versicolor, Phane. chrysosporium, Phaeo. schweintizii* and *L. sulphureu* was completely inhibited at concentrations of 25, 50, 12.5, and 12.5 μg/mL, respectively. The anti-wood-decay fungal activities of the leaf oil were superior to those of the essential oils from *L. coreana* [7], *Neolitsea parvigemma* [8], *Chamaecyparis formosensis* [11], *Machilus pseudolongifolia* [12], *M. philippinensis* [13], *Cinnamomum camphora* [14], *C. osmophloeum* [15], *L. mushaensis*, *L. linii* [16], and *L. acuminata* [17].

This study also tested the anti-wood-decay fungal activities of the major components of *J. formosana* leaf oil to ascertain its source compounds. Results indicated that the anti-wood-decay fungal activities were due to α-cadinol and elemol. At a concentration of 50 μg/mL, α-cadinol and elemol showed total growth inhibition

Table 2: Anti-wood-decay fungal indices of leaf and fruit essential oils of *J. formosana*.

Essential oil	Dosage (μg/mL)	Antifungal index (%)			
		Trametes versicolor	*Phaneochaete chrysosporium*	*Phaeolus schweintizii*	*Lenzites sulphureu*
Leaf	12.5	89 ± 3.3	68 ± 6.6	100 ± 0	100 ± 0
	25	100 ± 0	86 ± 3.3	100 ± 0	100 ± 0
	50	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	75	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	100	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Fruit	12.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	25	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	50	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	75	0 ± 0	0 ± 0	0 ± 0	38 ± 3.3
	100	25 ± 3.3	18 ± 3.3	39 ± 3.3	56 ± 3.3

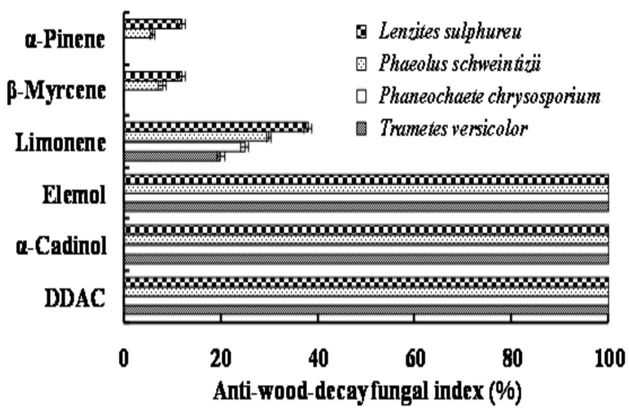

Figure 3: Anti-wood-decay fungal indices of the eight main compounds (50 μg/mL) of the leaf essential oil of *J. formosana*.
Note. DDAC (didecyl dimethyl ammonium chloride) (50 μg/mL) is a wood preservative for wood decay fungi and is used as a positive control.

against all the white-rot and brown-rot fungi tested. The presence of α-cadinol and elemol significantly contributed to the wood-decay fungi suppression activities of *J. formosana* leaf oil.

Experimental

Plant materials: Fresh leaves and fruits of *J. formosana* were collected in March 2012 from Yuanfeng (Nantou County, central Taiwan, elevation 2680 m, N 24º 07´ 58″, E 121º 14´ 68″). The samples were compared with specimen no. ou8958 from the Herbarium of the National Chung-Hsing University and positively identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-029) was deposited in the NCHU herbarium. Leaves and twigs of the species were collected for subsequent extraction and analysis.

Isolation of leaf and fruit essential oils: The essential oils of the air-dried leaves and fruits (1 kg) were hydrodistilled for 3 h using a Clevenger-type apparatus. After distillation, the volume of oils obtained was measured, and the essential oils were stored in glass containers, hermetically sealed with rubber lids, covered with aluminum foil to protect the contents from light, and kept refrigerated at < 4ºC until used. The oil yields and all test data are the average of triplicate analyses.

Essential oil analysis: A Hewlett-Packard HP 6890 gas chromatograph equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness, J&W Scientific) and a FID detector was used for the quantitative determination of oil components. Oven temperature was programmed as follows: 50ºC for 2 min, rising to 250ºC at 5ºC/min. Injector temperature: 270ºC. Carrier gas: He with a flow rate of 1 mL/min. Detector temperature: 250ºC, split ratio: 1:10. Diluted samples (1.0 μL, 1/100, v/v, in ethyl acetate) were injected manually in the split mode. Identification of the oil components was based on their retention indices and mass spectra, obtained from GC/MS analysis on a Hewlett-Packard HP 6890/HP5973 equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness, J&W Scientific). The GC analysis parameters listed above and the MS were obtained (full scan mode: scan time: 0.3 s, mass range was m/z 30-500) in the EI mode at 70 eV. All data were the average of triplicate analyses.

Component identification: Identification of the leaf essential oil constituents was based on comparisons of retention index (RI) [6], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [9,18].

Antifungal assays: The method of Su *et al.* [5] was adopted. Mold and wood decay fungi were obtained from the Culture Collection and Research Center of the Food Industry Research and Development Institute, Hsinchu City, Taiwan. References of ASTM G21, JIS Z 2911 and AATCC test method 30 were consulted for the mold fungal strains; 7 strains {*A. clavatus* (ATCC 1007), *A. niger* (ATCC 6275), *Ch. globosum* (ATCC 6205), *Cl. cladosporioides* (ATCC 13276), *M. verrucaria* (ATCC 9095), *P. citrinum* (ATCC 9849) and *T. viride* (ATCC8678)} were tested. The wood decay fungi used were *T. versicolor* (BCRC 35253), *Phane. chrysosporium* (BCRC 36200), *Phaeo. schweinitzii* (BCRC 35365) and *L. sulphureus* (BCRC 35305). Antifungal assays were carried out in triplicate and data were averaged. Different concentrations of the essential oils (12.5-1000 μg/mL) were added to sterilized potato dextrose agar (PDA). The test plates were incubated at 27ºC. When the mycelium of fungi reached the edge of the control plate, the antifungal index was calculated as follows:

Anti-fungal index (%)= (1–Da/Db) X 100, where Da is the diameter of the growth zone in the experimental dish (cm) and Db is the diameter of the growth zone in the control dish (cm).

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Meeting/Report

Review/Account

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