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ANTITERMITIC AND ANTIFUNGAL ACTIVITIES OF ESSENTIAL OIL OF *Calocedrus formosana* LEAF AND ITS COMPOSITION

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Abstract—*Calocedrus formosana* Florin (Cupressaceae) is an endemic tree species in Taiwan; its timber is recognized for natural decay resistance. To examine the antitermitic and antifungal activities of leaf essential oil and its main constituents, *C. formosana* leaves were extracted and the essential oils analyzed by GC-MS. Bioactivity tests against the termite *Coptotermes formosanus* demonstrate that the LC_{50} value of leaf essential oil is 27.6 mg/g. Furthermore, exposure to T-muurolol caused 100% mortality at a dosage of 5 mg/g after 14 d. Leaf oil constituents displayed activity against four fungi, *Lenzites betulina*, *Pycnoporus coccineus*, *Trametes versicolor*, and *Laetiporus sulphureus*. Two compounds, α-cadinol and T-muurolol, exhibited the strongest antifungal activity. The LC_{50} values of α-cadinol against *L. sulphureus*, *L. betulina*, and *T. versicolor* are 9.9, 28.6, and 30.4 μg/ml, respectively.

Key Words—*Calocedrus formosana*, leaf, essential oil, GC-MS, *Coptotermes formosanus*, antitermitic activity, antifungal activity, α-cadinol, T-muurolol.

INTRODUCTION

Wood, a naturally occurring polymer composite, is mainly composed of cellulose, hemicelluloses, lignin, and extractives. Due to its biological nature, unprotected wood is susceptible to discoloration and biological deterioration, which reduce its mechanical and physical properties (Chang et al., 2002). Developing methods that prolong the service life of wood has always been the interest of wood researchers. From an environmental perspective, finding naturally occurring constituents in

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highly durable tree species and understanding their mechanisms are the most appropriate approaches to achieving wood protection while preserving the environment (Chang et al., 2000). In recent years, many studies have investigated the relationship between wood properties and wood essential oils (Kinjo and Yata, 1986; Kondo and Imamura, 1986; Asada et al., 1989; Yoneyama et al., 1990; Nabeta et al., 1992; Morita et al., 1997; Chang et al., 2001a,b; Chang and Cheng, 2002). Therefore, extraction of natural compounds having specific bioactivies and/or medicinal properties from plants is an important application of natural product research.

Due to the unique ecosystem, there are many valuable tree species endemic to Taiwan. In the past few years, we have studied the relationship between wood properties and chemical constituents of endemic trees in Taiwan. In previous studies, we found that *Taiwania cryptomerioides*, *Cinnamomum osmophloeum*, and C*ryptomeria japonica* possess significant antifungal and antitermitic activity (Chang et al., 1998, 1999, 2001a,b; Chang and Cheng, 2002; Cheng and Chang, 2002).

Calocedrus formosana Florin (*Cupressaceae*) is an indigenous tree that grows at elevations of 800–1500 m in the northern part of the central mountain region of Taiwan and is one of the five useful conifer trees in Taiwan. *Calocedrus* is named for its beauty and resin. The wood is pale yellow in color. The physical–chemical properties of the timbers of this plant are recognized for their decay resistance, durability, and an incense-like smell. Its essential oil, of which the acidic constituents (shonanic acid) in particular, was investigated in 1932 by Ichikawa (Lo and Lin, 1956). More than 50 compounds have been isolated including monoterpenes, diterpenes, lignans, and steroids (Lin et al., 1956; Cheng et al., 1961; Fang et al., 1985, 1987, 1989a,b). *C. formosana* leaves have been found to be a suitable cell culture material for hinokitiol (a strong antimicrobial compound) and have the highest hinokitiol production among tested members of the Cupressaceae family (Mikage et al., 1988; Ono et al., 1998). However, there has been little research into the relationship between its wood properties and various extractives. For this reason, the essential oil of *C. formosana* leaves was distilled and its constituents analyzed by GC-MS. The antitermitic activities of the essential oil and isolated constituents against the termite *Coptotermes formosanus* were investigated using direct contact application. In addition, the antifungal activities of these constituents against four wood-rot fungi—*Laetiporus sulphureus*, *Pycnoporus coccineus*, *Lenzites betulina*, and *Trametes versicolor*—were also examined.

METHODS AND MATERIALS

Termite. *Coptotermes formosanus* Shiraki, was collected from Tainan in southern Taiwan. The colony was reared in an incubator at 26.5°C and 80% RH for more than 1 year. Water and newspapers were used as food sources.

Fungi. Three white-rot fungi [*Lenzites betulina* (CCRC 35296), *Pycnoporus coccineus*, and *Trametes versicolor* (CCRC 35253)] and one brown-rot fungus [*Laetiporus sulphureus* (CCRC 35305)], were used in these experiments. *L. betulina*, *T. versicolor*, and *L. sulphureus* were obtained from the culture collection and research center of the Food Industry Research and Development Institute in Taiwan. *P. coccineus* was a gift provided by Dr. Tun-Tschu Chang (Taiwan Forestry Research Institute).

Essential Oil Distillation. Leaves of 41-year-old *C. formosana* were collected from the Experimental Forest of the National Taiwan University in central Taiwan. Leaf essential oils were extracted by water distillation (8 hr).

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. The chemical composition of the essential oil was analyzed using GC-MS. An Agilent Technologies HP 5973N mass selective detector in the electron impact (EI) ionization mode (70 eV) was used in conjunction with a Hewlett-Packard 6890 gas chromatograph. Leaf oil constituents were separated under the following conditions: capillary column, HP-1MS (30 m $\times$ 0.25 mm; film thickness 0.25 μm); temperature program, 50°C (held for 5 min) raised to 120°C at a rate of 2°C/min and from 120°C to 220°C at a rate of 5°C/min held for 5 min; injector temperature, 270°C; and carrier gas, helium, at a flow rate of 1 ml/min. Split ratio was 1:10. Identification of the components of *C. formosana* leaf oil was confirmed by comparison with standards as well as by spiking. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

Essential Oil Constituents. The following essential oil constituents were purchased from Acros (Belgium): α-pinene, β-pinene, β-myrcene, limonene, β-caryophyllene, and caryophyllene oxide. α-Cadinol and T-muurolol were isolated from leaf essential oil.

Antitermitic Activity. The no-choice bioassay method of Kang et al. (1990) was used to evaluate antitermitic activity. Samples of 10, 25, and 50 mg of leaf essential oil as well as 1 and 5 mg of each individual compound dissolved in 600 μl of acetone were applied to 1 g filter paper samples (Whatman #3, 8.5 cm in diam). A piece of filter paper treated with solvent only was used as control. After air-drying at room temperature, 50 active termites (45 workers and 5 soldiers) above the third instar were placed onto each filter paper impregnated with the test materials in a Petri dish (9 cm diam $\times$ 1.5 cm height). The test dishes with covers were then placed into an incubator maintained at 26.5°C and 80% RH. A few drops of water were periodically added to the bottom edge of each Petri dish. Three replicates were prepared for each test sample, and the mortality of the termites was counted daily for 14 d.

Antifungal Assay. The method of Chang et al. (1999) was employed for antifungal evaluation of both the essential oil and its main constituents, which were tested at 1000, 200, 100, and 50 μg/ml concentrations against *L. betulina*, *P. coccineus*, *T. versicolor*, and *L. sulphureus* in 9 cm Petri dishes. Antifungal assays were performed three times. After fungal mycelia reached the edges of control plates (without adding essential oils or compounds) by incubating at 26°C for ca. 10 d, the antifungal index was calculated as follows: Antifungal index (%) = (1−Da/Db) × 100 where, Da= the diameter of growth zone in the experimental plate (cm), Db= the diameter of growth zone in the control plate (cm).

Statistical Analyses. All results were obtained from three independent experiments and expressed as mean ± SD. Significant differences ($P < 0.05$) were determined by using the Scheffe test.

RESULTS AND DISCUSSION

Yields and Chemical Constituents of Essential Oil. The leaf essential oil of *C. formosana* was obtained in a yield of 0.3% (or 3.4 ml/kg) dry weight. GC-MS analysis of the essential oil is shown in Table 1, where components are listed in order of their elution from the HP-1MS column. Nineteen constituents, accounting for more than 94.0% of the total oil composition, were identified. Nine monoterpenes (78.0%) and ten sesquiterpenes (16.5%) were identified in the essential oil. The main components of the leaf essential oil were α-pinene (44.2%),

TABLE 1. CONSTITUENTS OF ESSENTIAL OIL FROM *Calocedrus formosana* LEAVES

Compounds	R_t (min)[a]	Formula	% RA[b]
Tricyclene	6.39	$C_{10}H_{16}$	0.25
α-Pinene	7.29	$C_{10}H_{16}$	44.23
Camphene	7.86	$C_{10}H_{16}$	0.42
β-Phellandrene	9.52	$C_{10}H_{16}$	0.39
β-Pinene	9.64	$C_{10}H_{16}$	1.20
β-Myrcene	11.09	$C_{10}H_{16}$	8.92
Limonene	13.58	$C_{10}H_{16}$	21.57
4-Carene	17.71	$C_{10}H_{16}$	0.55
4-Terpineol	23.47	$C_{10}H_{18}O$	0.48
β-Caryophyllene	40.09	$C_{15}H_{24}$	8.23
α-Caryophyllene	41.83	$C_{15}H_{24}$	0.82
α-Cadinene	44.06	$C_{15}H_{24}$	0.30
γ-Cadinene	44.48	$C_{15}H_{24}$	0.24
δ-Cadinene	44.91	$C_{15}H_{24}$	0.87
Elemol	45.61	$C_{15}H_{26}O$	0.68
Caryophyllene oxide	46.56	$C_{15}H_{24}O$	2.44
γ-Eudesmol	48.22	$C_{15}H_{26}O$	0.25
T-Muurolol	48.48	$C_{15}H_{26}O$	1.09
α-Cadinol	48.81	$C_{15}H_{26}O$	1.56

[a] R_t: Retention time.
[b] RA: Relative area (peak area relative to total peak area).

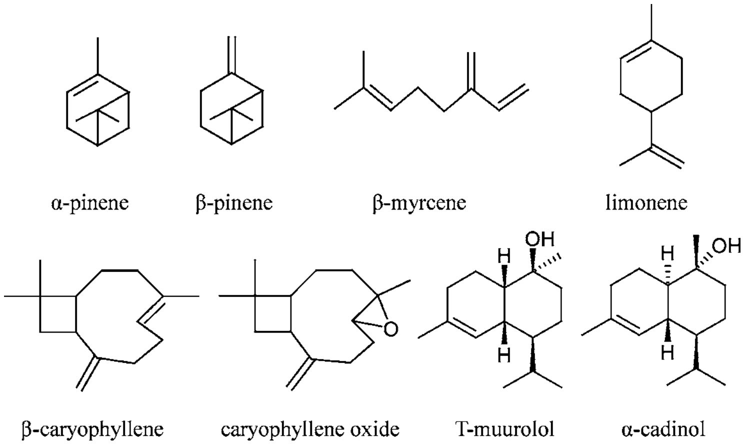

FIG. 1. Structures of terpenoids from *Calocedrus formosana* leaf essential oil.

limonene (21.6%), β-myrcene (8.9%), β-caryophyllene (8.2%), caryophyllene oxide (2.4%), α-cadinol (1.6%), β-pinene (1.2%), and T-muurolol (1.1%). The chemical structures of these compounds are shown in Figure 1.

Antitermitic Activity. The antitermitic activity of *C. formosana* essential oil and various isolated constituents are shown in Figure 2. At a dosage of 10 mg/g, the leaf essential oil killed 26.7% of the termites after 14 d. Termite mortality increased to 76.7% when dosage was increased to 50 mg/g. The LC_{50} value of leaf essential oil against *C. formosanus* was 27.6 mg/g.

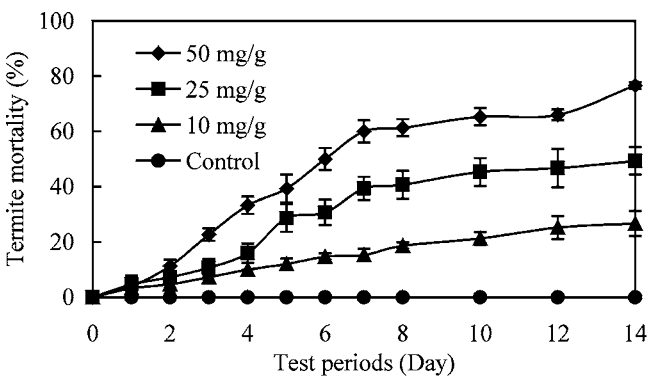

FIG. 2. Antitermitic activity of leaf essential oil from *Calocedrus formosana* against *Coptotermes formosanus*. Means ($N = 3$) using 50 termites per replicate.

TABLE 2. ANTITERMITIC ACTIVITY OF EIGHT ESSENTIAL OIL CONSTITUENTS FROM *Calocedrus formosana* LEAVES AT 1 mg/g DOSAGE

Compounds	Termite mortality[a] (%) 7 d	14 d
Limonene	1.3 ± 2.3c	1.3 ± 2.3e
β-Caryophyllene	19.3 ± 2.3a	25.3 ± 2.3a
α-Pinene	2.7 ± 1.2c	5.3 ± 2.3c,d,e
β-Pinene	4.0 ± 2.0c	7.3 ± 3.1c,d,e
β-Myrcene	7.3 ± 3.1b,c	11.3 ± 4.6b,c,d
Caryophyllene oxide	13.3 ± 4.6a,b	17.3 ± 1.2a,b
T-Muurolol	2.0 ± 0.0c	2.0 ± 0.0d,e
α-Cadinol	5.3 ± 3.1b,c	14.0 ± 4.0b,c
Control	0.0 ± 0.0e	0.0 ± 0.0e

[a] Means ($N = 3$) using 50 termites per replicate. Numbers followed by different letters (a–e) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

Antitermitic Activity of Isolated Constituents. To understand the relationship between the main constituents of *C. formosana* essential oil and antitermitic activity, eight constituents were tested for antitermitic activity. Table 2 shows the activity of these constituents at a dose of 1 mg/g. β-Caryophyllene (19.3%) and caryophyllene oxide (13.3%) caused the highest termite mortality after 7 d, followed by β-myrcene (7.3%), α-cadinol (5.3%), β-pinene (4.0%), α-pinene (2.7%), T-muurolol (2.0%), and limonene (1.3%). When the test was extended to 14 d, termite mortality increased slightly (Table 2).

The antitermitic activities of eight isolated oil constituents at 5 mg/g dosages are presented in Figure 3. The order of antitermitic activity was T-muurolol, followed by β-caryophyllene, caryophyllene oxide, α-cadinol, β-myrcene, β-pinene, α-pinene, and then limonene. The respective termite mortalities (at 5 mg/g dosage after 14 d) were 100.0%, 44.0%, 35.3%, 30.0%, 18.7%, 14.0%, 10.7%, and 8.0%. These results agree with those of Ohtani et al. (1997), who also reported the antitermitic activity of α-cadinol and T-muurolol isolated from *Chamaecyparis obtusa* heartwood.

Antifungal Activity of Essential Oil. Figure 4 shows the antifungal index of *C. formosana* leaf essential oil. The constituents were effective in reducing the growth of *L. betulina*, *P. coccineus, T. versicolor,* and *L. sulphureus* at 1000 μg/ml compared with the untreated control. The antifungal indices of the leaf essential oil were 18.9, 67.7, 10.9, and 27.6% against *L. betulina*, *P. coccineus*, *T. versicolor,* and *L. sulphureus*, respectively.

Antifungal Activity of Isolated Constituents. The antifungal indices of the eight compounds at a concentration of 100 μg/ml against the four fungi are

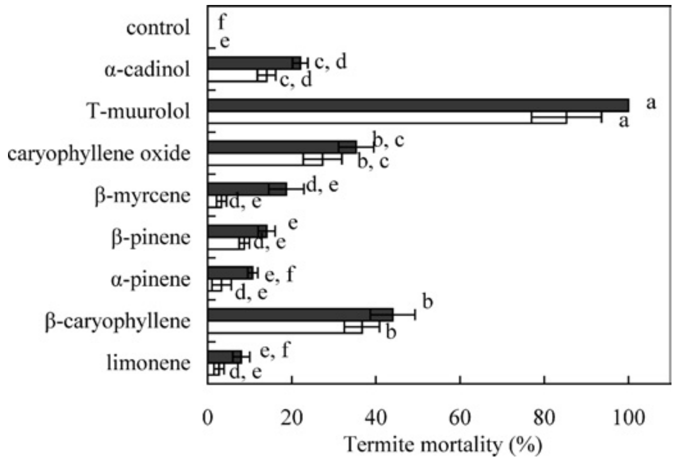

FIG. 3. Antitermitic activity of eight constituents from *Calocedrus formosana* leaf essential oil at 5 mg/g dosages against *Coptotermes formosanus* (white bar, after 7 d; black bar, after 14 d). Means ($N = 3$) using 50 termites per replicate. Numbers followed by different letters (a–f) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

presented in Table 3. The brown-rot fungus was more sensitive to the compounds than the white-rot fungi. In addition, two sesquiterpenes (α-cadinol and T-muurolol) were more effective against the four assay fungi than the monoterpenes. The order of antifungal indices of the eight compounds for *L. sulphureus* was α-cadinol > T-muurolol > caryophyllene oxide > β-caryophyllene > β-myrcene

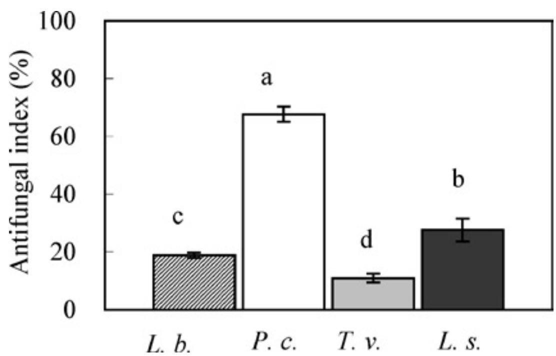

FIG. 4. Antifungal activity of leaf essential oil from *Calocedrus formosana* against fungi (at 1000 μg/ml). Each experiment was performed $\times 3$ and the data averaged ($N = 3$). Numbers followed by different letters (a–d) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

TABLE 3. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL CONSTITUENTS FROM *Calocedrus formosana* LEAVES AT 100 μg/ml[a]

Constituents	*L. betulina*	*P. coccineus*	*T. versicolor*	*L. sulphureus*
Limonene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	21.7 ± 1.2d, e
α-Pinene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	19.0 ± 2.6e, f
β-Pinene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	17.3 ± 0.8f
β-Myrcene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	20.4 ± 3.2e, f
β-Caryophyllene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	24.8 ± 2.4d
Caryophyllene oxide	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	32.3 ± 2.0c
T-Muurolol	48.0 ± 0.0b	38.1 ± 1.0b	48.0 ± 5.8b	82.0 ± 0.0b
α-Cadinol	100.0 ± 0.0a	65.1 ± 1.4a	100.0 ± 0.0a	100.0 ± 0.0a

[a]Each experiment was performed three times, and the data averaged ($N = 3$). Numbers followed by different letters (a–f) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

> α-pinene > limonene > β-pinene. Among them, α-cadinol and T-muurolol exhibited a higher antifungal activity.

The antifungal effectiveness of α-cadinol and T-muurolol were examined in greater detail. Figure 5 shows the antifungal indices of α-cadinol at serial concentrations. It appears that α-cadinol inhibited completely the growth of *L. betulina, P. coccineus, T. versicolor,* and *L. sulphureus* at a level as low as 200 μg/ml.

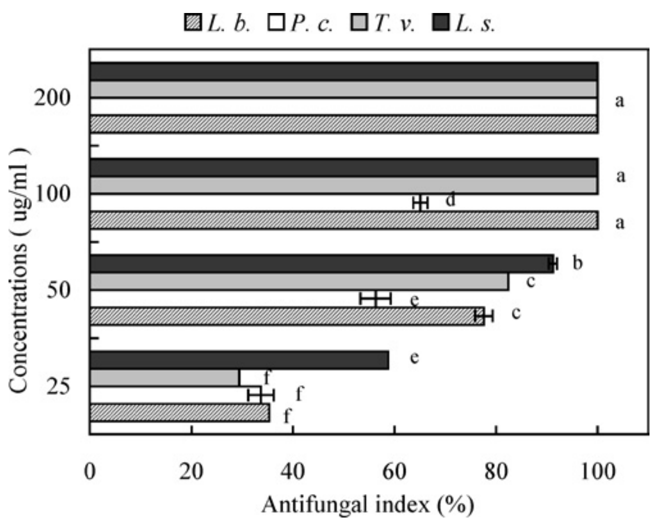

FIG. 5. Antifungal activity of α-cadinol at serial concentrations against fungi. Each experiment was performed ×3 and the data averaged ($N = 3$). Numbers followed by different letters (a–f) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

TABLE 4. MEDIAN LETHAL CONCENTRATION (LC_{50}, μg/ml) OF TWO MAIN CONSTITUENTS FROM THE ESSENTIAL OIL OF *Calocedrus formosana* LEAVES AGAINST FUNGI

Constituents	*L. betulina*	*P. coccineus*	*T. versicolor*	*L. sulphureus*
α-Cadinol	28.6	108.4	30.4	9.9
T-Muurolol	93.3	74.1	81.0	57.3

Similar results were obtained with T-muurolol. Table 4 shows the LC_{50} values of these compounds. It is noteworthy that α-cadinol exhibited the highest antifungal index for *L. betulina*, *P. coccineus*, *T. versicolor,* and *L. sulphureus*, followed by T-muurolol. The LC_{50} values of α-cadinol against *L. betulina*, *P. coccineus*, *T. versicolor*, and *L. sulphureus* are 28.6, 108.4, 30.4 and 9.9 μg/ml, respectively. Further, the LC_{50} values of T-muurolol against four fungi are all below 93.3 μg/ml. These results demonstrate that α-cadinol and T-muurolol showed the significant growth inhibitory effect on all test fungi. In our previous studies on the antifungal performance of *T. cryptomerioides* extractives, α-cadinol exhibited the highest antifungal activity for both *T. versicolor* and *L. sulphureus*, followed by T-cadinol, T-muurolol, ferruginol, and taiwanin C. There have been several reports that α-cadinol exhibits significant effectiveness in durable tree species against fungi or termites (Kondo and Imamura, 1986; Kinjo et al., 1988; Chang et al., 1998, 1999, 2000). The antifungal activity of leaf essential oil isolated from *C. formosana* was attributed to α-cadinol and T-muurolol, or their synergistic effect.

In addition, Table 4 also showed that the antifungal activity of two compounds were higher against brown-rot fungi than against white-rot fungi. Brown- and white-rot fungi decay wood by distinctly different mechanisms. The degradation of wood by white-rot fungi is carried out by enzymes such as cellulase or laccase. The initial stages of brown-rot fungal decay involve oxidative degradation (Highley and Dashek, 1998). DPPH is a stable radical that has been commonly used to evaluate the antioxidant activity of plant and microbial extracts (Gyamfi et al., 1999; Chang et al., 2001c; Wang et al., 2001). Results from our preliminary study showed that both α-cadinol and T-muurolol have inhibitory activity against the DPPH radical (data not shown here), suggesting that they possess some kind of antioxidant activity to prevent the deterioration induced by brown-rot fungi. The significance of antioxidant activities for the cadinane compounds is under investigation and will be addressed in the near future.

In this study, we investigated antitermitic and antifungal activities of leaf essential oil from *C. formosana* against termite and wood decay fungi. Antitermitic tests demonstrated that *C. formosana* leaf essential oil exhibited antitermitic activity. The LC_{50} value of leaf essential oil against *C. formosanus* was 27.6 mg/g after 14 d. Major constituents of *C. formosana* leaf essential oil were identified by

GC-MS. According to antitermitic activity, T-muurolol showed 100% mortality at 5 mg/g after 14 d. Its antitermitic effectiveness is much higher than that using *C. formosana* leaf essential oil. In addition, comparisons of the antifungal effectiveness of these compounds revealed that, among the eight compounds tested, α-cadinol, T-muurolol, and caryophyllene oxide possessed stronger antifungal activity. α-Cadinol exhibited the highest antifungal index for *L. betulina, T. versicolor*, and *L. sulphureus*, it inhibited completely the growth of fungi at levels as low as 100 μg/ml. These results show that α-cadinol and T-muurolol are potential compounds for the development of fungicides or termiticides in the near future.

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