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Acaricidal Activity of Active Constituent Isolated in *Chamaecyparis obtusa* Leaves against *Dermatophagoides* spp.

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Acaricidal activities of materials derived from *Chamaecyparis obtusa* leaves against *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* were examined using the dry film method and compared with that of commercial benzyl benzoate and *N*,*N*-diethyl-*m*-toluamide (DEET). The active constituent of the *C. obtusa* leaves was identified as β-thujaplicin ($C_{10}H_{12}O_2$) by spectroscopic analyses. Responses varied with dose. On the basis of a 24 h LC_{50} value, acaricidal activity against *D. farinae* was more pronounced with β-thujaplicin (72.2 mg/m^2) than benzyl benzoate (89.9 mg/m^2) and DEET (377 mg/m^2). Acaricidal activity against *D. pteronyssinus* was more pronounced in β-thujaplicin (62.1 mg/m^2) than benzyl benzoate (72.4 mg/m^2) and DEET (193 mg/m^2). These results indicate that acaricidal activity of *C. obtusa* leaves likely results from by β-thujaplicin. β-Thujaplicin merits further study as potential house dust mite control agents or lead compounds.

KEYWORDS: Natural acaricide; house dust mite; *Dermatophagoides farinae*; *Dermatophagoides pteronyssinus*; *Chamaecyparis obtusa*; β-thujaplicin

INTRODUCTION

In industrialized countries most individuals spend over 95% of their time within closed environments, where the air may contain pollutants and contaminants at higher concentrations than those found in the open air (*1*). For this reason the quality of the air in closed environments is presently considered to be at least as important as that of the open air for health in general and for atopic dermatitis, bronchial asthma, rhinitis, and conjunctivitis in particular (*1*). It has been suggested that this increase is in response to provocative factors, such as house dust mites (*2*). Toward the development of diagnostics and a therapeutic vaccine, important house dust mite allergens have been explored and now classified as major house dust mite antigens (*3*−*5*).

The most important pyroglyphid mites are *Dermatophagoides pteronyssinus* (Trouessart) and *Dermatophagoides farinae* (Hughes) for the following three reasons: (1) Their cosmopolitan occurrence and abundance; (2) They are a major source of multiple potent allergens; (3) Their causal association with sudden infant death syndrome (*6*−*9*). Living environmental changes (such as a rise in the number of apartment households with central heating, space heating, tighter windows, and wall-to-wall carpeting) have improved conditions for the growth of dust mites (*9*). Control of these mite populations has been principally through the use of chemicals such as benzyl benzoate and *N*,*N*-diethyl-*m*-toluamide (DEET) (*9*). Although effective, their repeated use has sometimes resulted in the widespread development of resistance (*9*−*11*), undesirable effects on nontarget organisms, and fostered environmental and human health concerns (*10*, *11*). These problems have highlighted the need for the development of new strategies for selective control of dust mites.

Environmental control has been considered as one useful means of controlling house dust mite populations. Washing of bedding is only effective in killing mites at temperatures greater than 70 °C, and vacuuming of carpets should not be considered equivalent to replacing carpets with vinyl as inadequate suction or leaking vacuum bags can exacerbate the problem by increasing the quantities of allergen that become airborne (*12*). Furthermore, research into plant-derived acaricides is now being intensified as it becomes evident that these materials have enormous potential for management of mites for public health and agriculture. Plant extracts or their constituents may provide an alternative to currently used acaricidal agents to control house dust mites (*13*, *14*). Because many of them are largely free from adverse effects and have excellent biological actions, they could lead to the development of new classes of possibly safer acaricides. This paper describes a laboratory study to examine the active constituent isolated from the *Chamaecyparis obtusa* leaves against house dust mites. The acaricidal activities of the *C. obtusa* leaf-derived compounds were compared with those of the commonly used benzyl benzoate and *N*,*N*-diethyl-*m*-toluamide.

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MATERIALS AND METHODS

Chemicals. Bornyl acetate, camphene, 2-carene, β-caryophyllene, α-cedrol, *p*-cymene, limonene, myrcene, α-pinene, α-terpinene, γ-terpinene, terpinen-4-ol, and α-thujene were supplied by Sigma (St. Louis, MO). Benzyl benzoate and *N*,*N*-diethyl-*m*-toluamide (DEET) were purchased from Aldrich (Milwaukee, WI). All other chemicals were of reagent grade.

Dust Mites. Cultures of *D. farinae* and *D. pteronyssinus* were maintained in the laboratory for 5 years without exposure to any known acaricide. They were reared in plastic containers (15 cm × 12 cm × 6 cm) containing 30 g of sterilized diet (fry feed No. 1/dried yeast, 1:1 by weight) at 25 ± 1 °C and 75% relative humidity in the dark. The fry feed (Miropa) was purchased from Korea Special Feed Meal Co. Ltd., Chonju, South Korea.

Isolation and Identification. The dried *C. obtusa* leaves (5 kg), purchased as commercially available product, were finely powdered, extracted twice with methanol (10 L) at room temperature, and filtered (Tokyo filter paper No. 2, Tokyo Roshi, Japan). The combined filtrate was concentrated in vacuo at 35 °C to yield approximately 9.2%. The extract (460 g) was sequentially partitioned into *n*-hexane (115 g), chloroform (55 g), and water-soluble (290 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation (EYELA autojack NAJ-100 Japan) at 35 °C, and the water portion was freeze dried. The hexane (12 g) portion was chromatographed on a silica gel column (Merck 70−230 mesh, 600 g, 5.5 i.d. × 68 cm) and successively eluted with a gradient step of hexane:EtOAc (5:1 to 0:1, v/v), giving eight fractions (H1−H8). The bioactive H4 (3.2 g) fraction was rechromatographed on a silica gel column and successively eluted with hexane:EtOAc (7:3, v/v). The column fractions were analyzed by TLC, and fractions with similar TLC patterns were pooled. In this step the six fractions (H41−H46) were obtained and bioassayed at 80 μg/cm^2 as described below. The active H42 fraction (714 mg) was purified by preparative HPLC (Spectra System P2000, Thermo Separation Products) for separation of the biologically active constituent. The column was Asahipak ODP-50 (150 mm i.d. × 4.6 mm, Waters), using hexane:EtOAc (75:25) at a flow rate of 0.5 mL/min and detection at 240 nm. In this step the five fractions (H421−H425) were obtained and bioassayed at 80 μg/cm^2. The active H422 fraction (279 mg) was rechromatographed under the same condition. Finally, an active compound (174 mg) with a retention time of 32.5 min was isolated. The structure of the active isolate was determined by instrumental analysis. ^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform with a JNM-LA 400F7 spectrometer at 400 and 100 MHz (TMS as an internal standard), respectively, and chemical shifts are given in δ (parts per million). UV spectra were obtained in methanol with a Jasco V-550 spectrometer and EI-MS spectra on a JEOL GSX 400 spectrometer.

Gas Chromatography−Mass Spectrometry (GC-MS). The hexane fraction of *C. obtusa* leaves was analyzed on a gas chromatograph (HP6890)−mass spectrometer (JMS-600W, JEOL) (GC-MS). The GC column was a 60 m × 0.25 mm i.d. DB-WAX (0.25 μm film) fused silica capillary column (J&W Scientific, Folsom, CA). The GC conditions were as follows: injector temperature, 210 °C; column temperature, isothermal at 50 °C for 15 min, then programmed to 200 °C at 2 °C/min, and held at this temperature for 15 min; ion source temperature, 200 °C. Helium was used as the carrier gas at a rate of 0.8 mL/min. The effluent of the GC column was introduced directly into the source of the MS. Spectra were obtained in the EI mode with 70 eV of ionization energy. The sector mass analyzer was set to scan from 50 to 800 amu for 2 s. Compounds were identified by comparison with retention times, and the mass spectra were obtained with authentic standards on the GC-MS system used for analysis. When an authentic sample was not available, identification was carried out by comparison of mass spectra with those in the mass spectra library (The Wiley Registry of Mass Spectral Data, 6th ed.).

Bioassay. Dry film method was used to access acaricidal activity of test materials. Amounts (800, 500, 400, 300, 250, 200, 150, 100, 90, 80, 70, 60, 50, 40, 30, and 20 mg/m^2) of each test material dissolved in 100 μL of ethanol were applied to disks of black cotton fabric (0.5 g, 5 cm diameter: 700 mesh). Control fabric disks received 20 μL of ethanol. After the disks were dried in a fume hood (19 °C) for 30 s, each disk was placed in the bottom of a Petri dish (5 cm diameter × 1.2 cm). Then 30 individuals of *D. farinae* (7−10 day-old adults) or *D. pteronyssinus* (7−10 days old) were placed in each Petri dish and covered with a lid. Treated and control mites were held at 25 ± 1 °C and 75% relative humidity in the dark. Mortalities were determined 24 h after treatment under a binocular microscope (20×). Mites were considered to be dead if appendages did not move when prodded with a pin. All treatments were replicated three times. LC_{50} values were calculated by probit analysis (*15*). The percentage mortality was determined and transformed to arcsine square-root values for analysis of variance (ANOVA). Treatment means were compared and separated by Scheffe's test at $P = 0.05$ (SAS Institute) (*16*).

Table 1. Acaricidal Activity of *C. obtusa* Leaf Extract, β-Thujaplicin, Commercial Constituents Derived from *C. obtusa* Leaves, and Synthetic Acaricide against *D. farinae* and *D. pteronyssinus*[a] Using the Dry Film Method

compound	mite species	LC_{50} mg/m^2	95% confidence limit	RT[b]
methanol extract	*D. farinae*	231	225.2–236.4	0.4
	D. pteronyssinus	209	205.1–214.3	0.4
hexane extract	*D. farinae*	192	187.2–198.1	0.5
	D. pteronyssinus	171	166.3–176.3	0.4
β-thujaplicin	*D. farinae*	72.2	68.1–76.9	1.3
	D. pteronyssinus	62.1	58.2–65.9	1.2
2-carene	*D. farinae*	397	391.6–402.1	0.2
	D. pteronyssinus	375	370.2–380.3	0.2
α-cedrol	*D. farinae*	98.8	93.6–103.1	0.9
	D. pteronyssinus	84.9	80.2–88.4	0.9
benzyl benzoate	*D. farinae*	89.9	85.6–93.8	1.0
	D. pteronyssinus	72.4	67.7–76.1	1.0
DEET	*D. farinae*	377	371.9–383.2	0.2
	D. pteronyssinus	193	187.3–197.9	0.4

[a] Exposed for 24 h. [b] Relative toxicity = LC_{50} value of benzyl benzoate/LC_{50} value of each chemical.

RESULTS AND DISCUSSION

When the methanolic extract of *C. obtusa* leaves was bioassayed by the dry film method acaricidal activity was observed against *D. farinae* and *D. pteronyssinus* (**Table 1**). The dust mite species were equally susceptible, and the extract of *C. obtusa* leaves showed a clear dose−response relationship for both species. Concentrations of 800 mg/m^2 or greater caused complete mortality in both species. The acaricidal activity of the methanolic extract was compared with those of benzyl benzoate and DEET against *D. farinae* and *D. pteronyssinus* adults (**Table 1**). The commonly used synthetic acaricides, benzyl benzoate and DEET, served as positive controls for acaricidal activity. LC_{50} values of the methanolic extract and hexane fraction of *C. obtusa* leaves were 231 and 192 mg/m^2 against *D. farinae* adults and 209 and 171 mg/m^2 against *D. pteronyssinus* adults, respectively. On the basis of LC_{50} values the hexane fraction derived from the methanolic extract of *C. obtusa* leaves was comparable to that of DEET and about 0.5 and 0.4 times less toxic than benzyl benzoate against *D. farinae* and *D. pteronyssinus* adults, respectively. There was no mortality in the untreated controls. This study is the first to report the acaricidal function of *C. obtusa* leaf-derived materials against *D. farinae* and *D. pteronyssinus* adults. Very little work has been done with respect to managing arthropod pests including house dust mite.

The substances identified by GC-MS in the hexane fraction derived from the *C. obtusa* leaves are presented. Analysis led to identification of 16 volatiles from the hexane fraction of the *C. obtusa* leaves. The main constituents were α-thujene (relative percent = 1.4%), α-pinene (3.7%), β-phelladrene (21.8%),

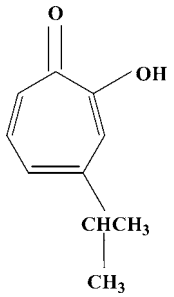

Figure 1. Structure of β-thujaplicin isolated from *Chamaecyparis obtusa* leaves against house dust mites.

myrcene (9.0%), α-terpinene (4.3%), limonene (4.2%), γ-terpinene (7.9%), 2-carene (4.3%), terpinen-4-ol (17.8%), bornyl acetate (3.6%), camphene (3.7%), β-caryophyllene (2.9%), β-thujaplicin (6.7%), germacrene D (0.9%), elemol (1.4%), and α-cedrol (2.3%). Together, β-phelladrene, myrcene, γ-terpinene, terpinen-4-ol, and β-thujaplicin made up 63.2% of the oil. The native *C. obtusa*, one of the Cupressaceae, which has been generally recognized as kiso-Hinoki, has been preserved as a valuable wood for building materials of such nationally important buildings as the empire palace and famous shrines and temples and for general use in hygienic woodenware, such as counter- or tabletops in sushi bars (*17*). Hong et al. previously reported the main constituents of *C. obtusa* as Bonyl acetate, β-caryophyllene, *p*-cymene, elemol, limonene, myrcene, α-thujene, γ-terpinene, α-terpineol, and β-thujaplicin (*18*).

Because of the strong activity of the hexane fraction, isolation of the biologically active component was pursued. Dry film method-guided fractionation of *C. obtusa* afforded an active constituent identified by spectroscopic analyses, including MS and NMR, and by direct comparison with authentic compound. The biologically active constituent was characterized as β-thujaplicin (**Figure 1**). This compound was identified on the basis of the following evidence. β-Thujaplicin (hinokitiol, 2-hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one): ($C_{10}H_{12}O_2$, MW = 164.2); EI-MS (70 eV) *m/z* (% relative intensity) M^+ 164 (76), 149 (4), 136 (12), 121 (100), 103 (11), 91 (15), 77 (17), 65 (8), 51 (5); ^{1}H NMR (CD_3OD) δ 7.44 (1H, *t*, *J* = 21.2 Hz), 7.31 (1H, *d*, *J* = 1.7 Hz), 7.22 (1H, *t*, *J* = 10.7 Hz), 7.08 (1H, *m*, *J* = 11.7 Hz), 2.96 (1H, *m*, *J* = 41.2 Hz), 1.26 (3H, *d*, *J* = 3.4 Hz), 1.26 (3H, *d*, *J* = 3.4 Hz); ^{13}C NMR (CD_3OD) δ 173.0, 172.6, 161.8, 138.9, 129.1, 125.0, 124.1, 40.0, 23.6, 23.6.

The acaricidal activity of *C. obtusa* leaf-derived compounds against *D. farinae* and *D. pteronyssinus* adults was examined (**Table 1**). Responses varied according to compound and dose. On the basis of LC_{50} values the compound most toxic against *D. farinae* was β-thujaplicin (72.2 mg/m^2) followed by benzyl benzoate (89.9 mg/m^2), α-cedrol (98.8 mg/m^2), DEET (377 mg/m^2), and 2-carene (397 mg/m^2). Against *D. pteronyssinus*, β-thujaplicin (62.1 mg/m^2) was more effective than benzyl benzoate (72.4 mg/m^2), α-cedrol (85.9 mg/m^2), DEET (193 mg/m^2), and 2-carene (375 mg/m^2). Additionally, there was no significant difference in toxicity of β-thujaplicin between *D. farine* and *D. pteronyssinus*. However, no activity was observed for bornyl acetate, camphene, β-caryophyllene, *p*-cymene, limonene, myrcene, α-pinene, α-terpinene, γ-terpinene, terpinen-4-ol, or α-thujene at 800 mg/m^2 (not shown). These results indicate that the acaricidal activity of the oil of *C. obtusa* leaves can be mostly attributed to β-thujaplicin. β-Thujaplicin was about 1.3 and 1.2 times more toxic than benzyl benzoate and 5.2 and 3.1 times more toxic than DEET against *D. farinae* and *D. pteronyssinus*, respectively. β-Thujaplicin merits further study as a potential dust mite control agent or as lead compound.

Plants are potential products for dust mite control because many of them are selective to pests, have no or little harmful effects on nontarget organisms and the environment (*12*−*14*), and may be applied to dust mite nests such as beds, carpeted floors, furniture, and sofas in the same way as other conventional acaricides (*3*). Many plant extracts and phytochemicals are known to possess acaricidal activity against house dust mites (*13*, *14*). The reported naturally occurring acaricidal compounds against dust mites include fenchone, estragol, and thymol derived from *Foeniculum vulgare* (*13*), anisaldehyde and 3-carene from the leaves from *Pimpinella anisum* seed (*14*), and cinamaldehyde, cinnamyl alcohol, and salicylaldehyde from *Cinnamomum cassia* (*19*). It has been reported that susceptibility to some plants such as bitter almond, caraway, and perilla was greater in *D. farinae* adults than in *D. pteronyssinus* adults (*19*). However, *D. farinae* adults are found to be more tolerant to the wood of *Thuga heteropylla* and *Cryptomeria japonica* than *D. pteronyssinus* adults (*20*). However, no significant difference in acaricidal activity of cinnamaldehyde, cinnamyl alcohol, or salicylaldehyde between *D. farinae* and *D. pteronyssinus* has been noted (*19*). Our results are similar with cinnamaldehyde, cinnamyl alcohol, and salicylaldehyde derived from *Cinnamomum cassia* bark.

Typical poisoning symptoms in both *Dermatophagoides* mites to β-thujaplicin were compared with those from benzyl benzoate and DEET using the dry film method. All test compounds caused death without knockdown, but there were no distinguishable symptoms between *D. farine* and *D. pteronssinus*. β-Thujaplicin resulted in lethargy of treated mites, leading to death. Benzyl benzoate and DEET caused death following uncoordinated behavior, and in death the forelegs were extended forward in parallel. Observation of poisoning symptoms of chemicals is of practical importance for dust mite control. Five types of poisoning symptoms of chemicals against mites have been reported: a knockdown-type death caused by *Neolitsea sericea* leaf oil in adults of *D. farine* and *D. pteronyssinus* (*21*); death related with uncoordinated behavior by (*E*)-cinnamaldehye, salicycylaldehyde, benzyl benzoate, and DEET in adults of *D. farine* and *D. pteronyssinus* (*19*); death associated with desiccation by several monoterpenes such as fenchon, linalool, menthone, and pulegone in *Tyrophagus putrescentiae* (Scarank) adults (*22*); death related with a characteristic depression of the dorsal surface of the idiosoma by tricalcium phosphate and ferric phosphate in *T. purtrescentiae* adults (*23*); and death associated with lethargy by isoeugenol and methyleugenol in adults of *D. farine*, *D. pteronyssinus*, and *T. purtrescentiae.* Compounds such as cinnamaldehyde, cinnamyl alcohol, salicylaldehyde, benzyl benzoate, and DEET are found to result in a similar death symptom of the forelegs extended forward together (*19*). In our study *C. obtusa*-derived β-thujaplicin resulted in lethargy of treated *Dermatophagoides* mites, directly connected to death without knockdown and with the forelegs extended forward together.

Results of this and earlier studies indicate that *C. obtusa*-derived β-thujaplicin could be of practical use as a fumigant for *D. farine* and *D. pteronyssinus* provided that a carrier producing a slow-release effect can be selected or developed. Further research should be done on safety issues of β-thujaplicin for human health, its acaricidal mode of action, and formulations improving the acaricidal potency and stability.

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