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Toxicity of Atractylon and Atractylenolide III Identified in *Atractylodes ovata* Rhizome to *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*

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The acaricidal activity of materials derived from rhizome of *Atractylodes ovata* (*Atractylodes macrocephala*) toward adult *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* was examined using fabric-circle residual contact and vapor-phase toxicity bioassays. Results were compared with those of the currently used acaricides: benzyl benzoate, dibutyl phthalate, and *N*,*N*-diethyl-*m*-toluamide (Deet). The active principles of *A. ovata* rhizome were identified as the sesquiterpenoids, atractylenolide III (**1**) and atractylon (**2**), by spectroscopic analysis. In fabric-circle residual contact bioassays with adult *D. farinae*, atractylenolide III (LD_{50}, 103.3 mg/m^2) and atractylon (136.2 mg/m^2) were five and four times more toxic than Deet and 1.7- and 1.3-fold more active than dibutyl phthalate, respectively, based on 24 h LD_{50} values. These compounds were less toxic than benzyl benzoate (LD_{50}, 45.8 mg/m^2). Against adult *D. pteronyssinus*, atractylenolide III (LD_{50}, 73.8 mg/m^2) and atractylon (72.1 mg/m^2) were eight times more active than Deet and 2.5-fold more toxic than dibutyl phthalate. These compounds were slightly less effective than benzyl benzoate (LD_{50}, 46.0 mg/m^2). In vapor-phase toxicity tests with both mite species, atractylenolide III and atractylon were effective in closed but not in open containers. These results indicate that the effect of these sesquiterpenoids was largely a result of action in the vapor phase. Naturally occurring atractylenolide III and atractylon merit further study as potential house dust mite control agents or leads because of their great activity as a fumigant.

KEYWORDS: Botanical acaricide; natural fumigant; *Dermatophagoides farinae*; *Dermatophagoides pteronyssinus*; *Atractylodes ovata*; atractylenolide III; atractylon

INTRODUCTION

The American house dust mite, *Dermatophagoides farinae* Hughes, and the European house dust mite, *Dermatophagoides pteronyssinus* Trouessart, are the most serious pyroglyphid mites because of their cosmopolitan occurrence and abundance in homes (*1*) and because of a major source of multiple potent allergens causing allergic symptoms to sensitive humans such as atopic dermatitis, asthma, rhinitis, and conjunctivitis (*2*−*4*). They are causally associated with sudden infant death syndrome (*5*). Control of these mite populations worldwide has been provided principally by the use of various contact and residual insecticides such as benzyl benzoate, diethyl-*m*-toluamide (Deet), dibutyl phthalate, pirimiphos-methyl, and pyrethroids (*1*, *6*). Although effective, their repeated use has often resulted in the development of resistance (*7*) and raises environmental and human health concerns (*1*, *6*, *8*). Additionally, some commonly used organophosphates and carbamates will be phased out in the near future in the United States by the U.S. Environmental Protection Agency (EPA) under the 1996 Food Quality and Protection Act (*9*). These problems highlight the need for selective control alternatives for house dust mites.

Plants have been suggested as alternative sources for dust mite control products because they constitute a range of bioactive chemicals (*10*) and because some are selective and often biodegrade to nontoxic products (*11*, *12*). They can be applied to dust mite nests such as mattresses, carpets, furniture, and sofas in the same manner as the acaricides currently used. Because certain plant preparations and their constituents meet the criteria of reduced risk pesticides (*13*), much effort has been focused on them as potential sources of commercial acaricidal products. In a preliminary experiment, a methanolic extract of the rhizome from *Atractylodes ovata* DC (*Atractylodes macrocephala* Koidzumi; Asteraceae, formerly Compositae) was shown to have potent acaricidal activity toward adult *D. farinae* and *D. pteronyssinus*.

This study was aimed at isolating acaricidal principles from the rhizome of *A. ovata* active toward adult *D. farinae* and *D. pteronyssinus* and determining their acaricidal mode of delivery. Also, the acaricidal activity of *A. ovata* rhizome-derived compounds was compared with that of the commonly used acaricides: benzyl benzoate, Deet, and dibutyl phthalate.

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MATERIALS AND METHODS

Apparatus. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on an AVANCE 600 spectrometer (Bruker, Karlsruhe, Germany), using tetramethylsilane as an internal standard, and chemical shifts are given in δ (ppm). Heteronuclear multiple-bond correlation, heteronuclear multiple-quantum coherence, and correlation spectroscopy spectra were acquired using the standard Bruker software. Fourier transform infrared spectra were obtained on a Magna 550 series II spectrophotometer (Nicolet, Midac, Irvine, CA), and mass spectra were obtained on a GSX 400 spectrometer (JEOL, Tokyo, Japan). Optical rotation was measured with an Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ). Silica gel (0.063−0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Precoated silica gel plates (Kieselgel 60 F_{254}) (Merck) were used for analytical thin-layer chromatography (TLC). A 1200 series high-performance liquid chromatograph (HPLC) (Agilent Technologies, Santa Clara, CA) was used for isolation of active principles.

Chemicals. Benzyl benzoate (≥99.0% purity) and dibutyl phthalate were purchased from Sigma-Aldrich (St. Louis, MO). Deet (97% purity) was supplied by Aldrich (Milwaukee, WI). All other chemicals were of reagent grade and available commercially.

Dust Mites. The stock cultures of *D. farinae* and *D. pteronyssinus*, originally obtained from I. Y. Lee (Department of Parasitology, College of Medicine, Yonsei University, Seoul) in 1999, were separately maintained in the laboratory without exposure to any known acaricide. Mites were reared in a plastic container (12.5 cm × 10.5 cm × 5.0 cm) containing 40 g of sterilized diet (fry feed no. 1/dried yeast, 1:1 by weight). Plastic containers were incubated at 25 ± 1 °C and 75% relative humidity in darkness. The fry feed was purchased from Korea Special Feed Meal Co. (Inchon, Korea). The feed consisted of protein (44.0%), lipid (3.0%), cellulose (4.0%), Ca (1.0%), and P (1.8%). Dried yeast was supplied by Daeheung Pharm. Co. (Seoul).

Plant Material. The rhizomes of *A. ovata*, originally cultivated in a local farm in Kwangwon Province, were purchased from Boeun medicinal herb shop, Kyoungdong Market (Seoul, Korea), and identified by Dr. Sang-Cheol Shin, Department of Forest Environment, Korea Forest Research Institute (Seoul). A voucher specimen (KNMP-12) was deposited in the Research Institute for Agriculture and Life Sciences, College of Agricultural and Life Sciences, Seoul National University (Seoul, Korea).

Extraction and Isolation of Active Principles. Air-dried rhizomes (600 g) of *A. ovata* were pulverized, extracted with 3 L of methanol twice at room temperature for 2 days, and filtered. The combined filtrate was concentrated under vacuum at 40 °C to yield ~54.6 g of an extract. The extract was sequentially partitioned into hexane (38.2 g), chloroform (13.9 g), and water-soluble (2.5 g) portions. The organic solvent-soluble portions were concentrated to dryness by rotary evaporation at 40 °C, and the water-soluble portion was concentrated at 50 °C. For isolation of active principles, 50.96 μg/cm^2 (1 mg/5 cm diameter fabric circle) of each *A. ovata* rhizome-derived material in ethanol was applied to a fabric-circle residual contact bioassay. This quantity was found to be an appropriate dose for a screening of acaricidal activity of plant preparations (*14*−*16*).

The most active hexane-soluble fraction (12 g) was chromatographed on a 70 cm × 5.5 cm i.d. silica gel column (600 g) and successively eluted with a gradient of hexane and ethyl acetate (100:0, 98:2, 95:5, 90:10, 80:20, 70:30, 60:40, and 0:100 by volume) and finally with methanol. Column fractions were monitored by TLC on silica gel plates with hexane/ethyl acetate (7:3 by volume). Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 30% H_2SO_4 and then heating on a hot plate. Of the seven fractions, two bioactive H1 (2.12 g) and H2 (2.25 g) fractions were obtained. The active H1 fraction afforded compound **1** (0.44 g) by repeated recrystallization in hexane. The H2 fraction was rechromatographed on a silica gel column, using hexane, to give four fractions. The active H23 fraction (1.86 g) was dissolved in hexane. The bioactive hexane-soluble (HS) fraction (1.37 g) was obtained. For further separation of the constituents from the HS fraction, a HPLC was used. The column was a 150 mm × 4.6 mm i.d. Zorbax Eclipse (Agilent Technologies) using a mobile phase of ethanol and water (9:1 by volume) at a flow rate of 1 mL/min. Chromatographic separations were monitored using a UV detector at 237 nm. Finally, a potent active compound **2** (0.87 g) was isolated at a retention time of 3.3 min.

Residual Contact Toxicity Bioassay. A fabric-circle residual contact bioassay (*14*) was used to evaluate the toxicity of *A. ovata* rhizome-derived materials to adult *D. farinae* and *D. pteronyssinus* (7−10 days old). Four to six concentrations of each test material in 20 μL of ethanol were applied to black cotton fabric circles (5 cm diameter). Control fabric circles received 20 μL of ethanol. After they were dried in a fume hood for 20 s, each fabric circle was placed on the bottom of a Petri dish (5 cm × 1 cm). Groups of 25 adult mites were separately placed on the fabric circles, and each Petri dish was then sealed with a solid lid. Benzyl benzoate, Deet, and dibutyl phthalate served as standards for comparison in direct contact toxicity tests.

Treated and control (ethanol only) mites were held at the same conditions as used for colony maintenance. Mortalities were determined 24 h posttreatment under a binocular microscope (20×). Mites were considered to be dead if appendages did not move when they were prodded with a fine wooden dowel. All treatments were replicated 4−6 times.

Vapor-Phase Toxicity Bioassay. The closed and open container treatment methods (*14*) were used to determine whether the lethal activity of *A. ovata* rhizome-derived compounds toward adult *D. farinae* and *D. pteronyssinus* (7−10 days old) was attributed to contact toxicity or fumigant action. Groups of 25 adult mites were placed on the bottom of a Petri dish (5 cm × 1 cm) and sealed with a lid that had a fine wire screen (200 mesh; 4 cm diameter) covering a central hole (3 cm diameter). Black cotton fabric circles (5 cm diameter) were treated with 50.96 μg/cm^3 of each test compound in 20 μL of ethanol. After they were dried in a fume hood for 20 s, each treated fabric circle was placed on top of the wire screen, which prevented direct contact of mites with test compound. Petri dishes were either sealed with a solid lid (closed container treatment method) to investigate the potential vapor-phase toxicity of the test compounds or left unsealed (open container treatment method). Treated and control (ethanol only) mites were held at the same conditions as used for colony maintenance. Mortalities were determined 24 h posttreatment. All bioassays were replicated four times.

Data Analysis. Mortality percentages were transformed to arcsine square root values for analysis of variance (ANOVA). The Bonferroni multiple-comparison method was used to test for significant differences among the test materials (*17*). A paired *t* test was used to test for significant differences between two treatment methods (*17*). Means ± standard errors (SE) of untransformed data are reported. LD_{50} values were calculated by probit analysis (*17*). Acaricidal activity was considered to be significantly different when 95% confidence limits of the LD_{50} values failed to overlap. The susceptibility ratio (SR) was calculated as the ratio of *D. farinae* LD_{50}/*D. pteronyssinus* LD_{50}.

RESULTS

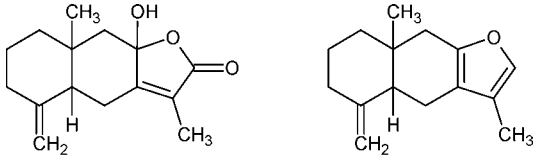
Bioassay-Guided Fractionation and Isolation of Active Principles. Fractions obtained from the methanolic extract of *A. ovata* rhizome were bioassayed by residual contact application (**Table 1**). Significant differences in toxicity in fractions of the extract were observed, and they were used to identify peak activity fractions for the next step in the purification. At a dose of 50.96 μg/cm^2, the hexane fraction exhibited more than 99% mortality against adult *D. farinae* and *D. pteronyssinus* 24 h posttreatment, whereas little or no lethal activity was observed in the chloroform and water fractions. There was no mortality in the ethanol-treated controls.

Fabric-circle residual contact bioassay-guided fractionation of *A. ovata* rhizome extract afforded two active principles identified by spectroscopic analysis, including MS and NMR. The two active principles were the sesquiterpenoids, atractylenolide III (**1**) and atractylon (**2**) (**Figure 1**). Atractylenolide III (**1**): white powder; $[\alpha]_D^{20}$, +185.7° (*c* 3.6, $CHCl_3$). EI-MS (70 ev), *m*/*z*: 248 $[M]^+$, 147 (100, base peak). IR ν_{max} cm^{-1}: 3345, 1743, 890. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.04 (3H, s), 1.20

Table 1. Lethal Activity of Each Solvent Fraction of Hexane Fraction Derived from Methanolic Extract of *A. ovata* Rhizome toward Two *Dermatophagoides* spp., Using the Residual Contact Toxicity Bioassay[a]

material	mortality[b] (%) (mean ± SE)	
	D. farinae	*D. pteronyssinus*
methanol extract	100 ± 0.0 a	100 ± 0.0 a
hexane fraction	99 ± 1.0 a	100 ± 0.0 a
chloroform fraction	3 ± 1.0 b	2 ± 1.2 b
water fraction	3 ± 1.0 b	4 ± 2.8 b

[a] Exposed for 24 h at a dose of 50.96 μg/cm^2, which was equivalent to 1 mg/5 cm diameter fabric circle. [b] Means within a column followed by the same letter are not significantly different ($P = 0.05$, Bonferroni method) (25 adult mites per replicate; four replicates per treatment).



(**1**) (**2**)

Figure 1. Structures of the sesquiterpenoids, atractylenolide III (**1**) and atractylon (**2**).

(1H, m), 1.54 (1H, d, $J = 14$ Hz), 1.63 (2H, dd, $J = 11$, 4 Hz), 1.80 (3H, d, $J = 1$ Hz), 1.96 (1H, 10 Hz), 2.29 (1H, d, $J = 14$ Hz), 2.37(1H, m), 2.43 (1H, dd, $J = 13$, 1 Hz), 2.62 (1H, dd, $J = 13$, 3 Hz), 4.60 (1H, d, $J = 1$ Hz), 4.86 (1H, d, $J = 1$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 8.2 q, 16.6 q, 22.3 t, 24.6 t, 36.1 t, 36.7 s, 41.3 t, 51.2 d, 51.7 t, 103.8 s, 106.8 t, 122.0 s, 148.6 s, 161.2 s, 172.7 s. Atractylon (**2**): colorless needles; $[\alpha]_D^{20}$, +44° (*c* 0.5, $CHCl_3$). EI-MS (70 ev), *m/z*: 216 $[M]^+$, 108 (100, base peak). IR ν_{max} cm^{-1}: 3080, 1646, 890. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 0.77 (3H, s), 1.4−1.8 (5H, m), 1.96 (3H, s), 2.0−2.5 (6H, m), 4.71 (1H, s), 4.87 (1H, s), 7.06 (1H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 8.3 q, 17.7 q, 21.0 t, 23.5 t, 36.8 t, 37.5 s, 39.4 t, 42.1 t, 45.9 d, 107.4 t, 116.3 s, 119.7 s, 137.1 d, 149.5 s, 150.0 s.

Toxicity of *A. ovata* Rhizome Sesquiterpenoids. The acaricidal activity of atractylenolide III (**1**) and atractylon (**2**) was evaluated by comparing the LD_{50} values estimated from the residual contact toxicity bioassay toward adult *D. farinae* (**Table 2**). As judged by 24 h LD_{50} values, atractylenolide III (LD_{50}, 103.3 mg/m^2) and atractylon (136.2 mg/m^2) were 5.2 and 4.0 times more toxic than Deet and 1.7- and 1.3-fold more active than dibutyl phthalate against adult *D. farinae*, respectively. These compounds were less effective than benzyl benzoate (LD_{50}, 45.8 mg/m^2). No mortality was observed in the ethanol-treated controls.

Toxic effects on adult *D. pteronyssinus* in the residual contact toxicity bioassay of *A. ovata* rhizome sesquiterpenoids were compared with those of three acaricides tested (**Table 2**). On the basis of 24 h LD_{50} values, atractylenolide III (LD_{50}, 73.8 mg/m^2) and atractylon (72.1 mg/m^2) were approximately eight times more active than Deet and 2.5-fold more toxic than dibutyl phthalate against adult *D. pteronyssinus*. These compounds were less effective than benzyl benzoate (LD_{50}, 46.0 mg/m^2).

The SR was dependent on the compound tested (**Table 2**). Adult *D. farinae* was 1.4 and 1.9 times less susceptible than adult *D. pteronyssinus* to atractylenolide III and atractylon, respectively. There was no great difference in toxicity of three test acaricides between adult *D. farinae* and *D. pteronyssinus*.

Acaricidal Route of Action. The fumigant toxicity of atractylenolide III and atractylon to adult *D. farinae* was investigated using the vapor-phase toxicity bioassay in two formats (**Table 3**). After 24 h of exposure to 50.96 μg/cm^3, there was a significant difference ($P < 0.0001$) in lethal activity of atractylenolide III between exposure in a closed container, which resulted in 98% mortality, and exposure in an open container, which resulted in 7% mortality toward adult *D. farinae*. Similar differences in the response of adult *D. farinae* to atractylon in closed and open treatments were likewise observed. There was no mortality in the ethanol-treated controls.

Toxic effects of the vapors of the test compounds on adult *D. pteronyssinus* were examined at 50.96 μg/cm^3 (**Table 3**). There was a significant difference in lethal activity of atractylenolide III in closed (99% mortality) vs open containers (11% mortality) toward adult *D. pteronyssinus*. Similar differences in the response of adult *D. pteronyssinus* to atractylon in two treatments were likewise observed.

DISCUSSION

In the Chinese Pharmacopoeia, the rhizome of *A. ovata* has long been considered to have medicinal properties such as digestive, diuretic, and antihidrotic properties (*18*). It contains sesquiterpenoids (1.5−3.0% by weight), such as atractylenolide I, II, and III, atractylon, and 3β-acetoxyatractylon, as well as polyacetylene compounds, such as (6*E*,12*E*)-tetradecadiene-8,-10-diyne-1,3-diol mono acetate, (6*E*,12*E*)-tetradecadiene-8,10-diyne-1,3-diol, and 6-methyl-2-geranyl-*p*-benzoquinone (*18*−*21*). Very little information exists with respect to managing house dust mites with *A. ovata* despite its excellent pharmacological actions (*18*−*22*). In the present study, *A. ovata* rhizome-derived materials exhibited potent acaricidal activity toward adult *D. farinae* and *D. pteronyssinus*.

Various compounds such as alkaloids, phenolics, and terpenoids exist in plants, and jointly or independently, they contribute to behavioral efficacy such as repellence and feeding deterrence and physiological efficacy such as acute toxicity and developmental disruption against various arthropod species (*11*, *12*). Many plant preparations and their constituents manifest acaricidal activity toward *D. farinae* and *D. pteronyssinus* (*23*). Much effort has been focused on determining the distribution, nature, and practical use of plant-derived chemicals that have acaricidal activity. Naturally occurring acaricidal compounds, such as alkaloids, aldehydes, ketones, benzofuranoids, benzoic acids, benzpyranoids, diterpenoids, monoterpenoids, phenylpropanoids, polyketides, and sesquiterpenoids, against *D. farinae* and *D. pteronyssinus* have been well-described by Ahn et al. (*23*). Additionally, it has been reported that susceptibility to six Lauraceae plant leaf oils was greater in adult *D. farinae* than adult *D. pteronyssinus* (*24*). However, adult *D. farinae* is found to be more tolerant to eugenol, isoeugenol, and methyleugenol than adult *D. pteronyssinus* (*25*). No significant differences in toxicity of either butylidenephthalide (*14*) or paeonol and benzoic acid (*15*) between *D. farinae* and *D. pteronyssinus* were reported.

In the current study, the acaricidal principles of *A. ovata* rhizome were identified as the sesquiterpenoids, atractylenolide III (**1**) and atractylon (**2**). The interpretations of proton and carbon signals of these sesquiterpenoids were largely consistent with those of Zhao and He (*26*) and Lee et al. (*27*). This is the first report on the acaricidal activity of atractylenolide III and atractylon. Atractylon was slightly more active than atractylenolide III. These sesquiterpenoids were much more toxic toward adult *D. farinae* and *D. pteronyssinus* than either Deet or dibutyl

Table 2. Toxicity of Atractylenolide III and Atractylon to Adult *D. farinae* and *D. pteronyssinus*, Using the Residual Contact Toxicity Bioassay during a 24 h Exposure

	D. farinae			*D. pteronyssinus*			
compound	slope ± SE	LD_{50} (mg/m^2)	95% CL[a]	slope ± SE	LD_{50} (mg/m^2)	95% CL[a]	SR[b]
atractylenolide III	3.0 ± 0.26	103.3	91.4–118.1	2.8 ± 0.27	73.8	65.1–83.8	1.40
atractylon	2.2 ± 0.19	136.2	116.6–160.9	2.9 ± 0.35	72.1	63.8–82.2	1.89
benzyl benzoate	5.7 ± 0.71	45.8	42.0–49.4	5.8 ± 0.59	46.0	42.0–49.2	1.00
Deet	2.6 ± 0.25	539.7	472.8–609.2	2.3 ± 0.24	597.1	517.1–683.4	0.90
dibutyl phthalate	3.6 ± 0.37	173.9	159.0–191.4	4.0 ± 0.42	188.1	173.3–206.2	0.92

[a] CL denotes confidence limit. [b] SR, *D. farinae* LD_{50}/*D. pteronyssinus* LD_{50}.

Table 3. Fumigant Toxicity of Atractylenolide III and Atractylon to Adult *D. farinae* and *D. pteronyssinus*, Using the Vapor-Phase Toxicity Bioassay, Exposed for 24 h at 50.96 $\mu g/cm^3$

		mortality (%) (mean ± SE)	
compound	mite species	vapor in closed container	vapor in open container
atractylenolide III	*D. farinae*	98 ± 1.2	7 ± 1.0[a]
	D. pteronyssinus	99 ± 1.0	11 ± 1.9[a]
atractylon	*D. farinae*	99 ± 1.0	9 ± 1.9[a]
	D. pteronyssinus	100 ± 1.0	9 ± 1.0[a]

[a] Significant at $P < 0.0001$, according to a paired *t* test (25 adults per replicate; four replicates per treatment).

phthalate but slightly less active than benzyl benzoate. The slopes of benzyl benzoate and dibutyl phthalate were almost similar to those reported by Heller-Haupt and Busvine (*28*), but that of Deet was lower than that reported by them. The LD_{50} values of Deet and dibutyl phthalate were higher than those of reported by Heller-Haupt and Busvine (*28*). The difference between our present and previous other studies (*28*) might be attributed to the difference in the rearing foods (fry feed vs dog food) or treatment methods (fabric circle vs cloth envelop). Additionally, adult *D. farinae* was slightly more tolerant than adult *D. pteronyssinus* to these sesquiterpenoids. There was no difference in susceptibility between adult *D. farinae* and *D. pteronyssinus* to atractylenolide III and atractylon. These sesquiterpenoids might be good candidates for naturally occurring dust mite control agents.

Investigation on the mode of action of natural acaricidal products and acaricides is of practical importance for house dust mite control because it may give useful information on the most appropriate formulations and delivery means. The fumigant activity of plant compounds, such as alkanes, alcohols, aldehydes, and terpenoids, particularly monoterpenoids, toward adult *D. farinae* and *D. pteronyssinus* has been reported for butylidenephthalide (*14*); paeonol (*15*); and eugenol, isoeugenol, and methyleugenol (*25*). However, bisabolangelone was found to be largely toxic through contact action (*16*). In this study, atractylenolide III and atractylon were much more effective in closed but not in open containers. These results indicate that the effect of the sesquiterpenoids was largely a result of action in the vapor phase. However, the exact acaricide mode of action remains to be proven.

Results of the present study indicate that atractylenolide III and atractylon could be useful as house dust mite control fumigants for *D. farinae* and *D. pteronyssinus* for a relatively long time, provided that a carrier producing a slow-release effect can be selected or developed. For practical use of these sesquiterpenoids as novel acaricides to proceed, further research is necessary on their safety issues on human health. Other areas requiring attention are acaricide mode of action and formulations for improving the acaricidal potency and stability and for reducing cost.

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