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Acaricidal Constituents Isolated from *Sinapis alba* L. Seeds and Structure−Activity Relationships

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Allyl isothiocyanate (AITC) and phenethyl isothiocyanate (PEITC) were isolated from *Sinapis alba* L. seeds and their effects against *Dermatophagoides farinae* and *D. pteronyssinus* were evaluated using the impregnated fabric disk method. The LD_{50} values of their compounds and derivatives were then compared with those of a commercial acaricide, benzyl benzoate. On the basis of the LD_{50} values against *D. farinae*, PEITC (0.21 $\mu g/cm^2$) was the most toxic, followed by benzyl isothiocyanate (0.55 $\mu g/cm^2$), phenyl isothiocyanate (1.09 $\mu g/cm^2$), butyl isothiocyanate (1.24 $\mu g/cm^2$), and AITC (1.36 $\mu g/cm^2$); acetyl isothiocyanate (195.01 $\mu g/cm^2$) was the least toxic. In addition, the acaricidal effects of AITC and PEITC against *D. farinae* were 7.4- and 47.8-fold greater than those of benzyl benzoate, respectively. Against *D. pteronyssinus*, PEITC was the most toxic (0.19 $\mu g/cm^2$), followed by benzyl isothiocyanate (0.77 $\mu g/cm^2$), phenyl isothiocyanate (1.37 $\mu g/cm^2$), butyl isothiocyanate (1.50 $\mu g/cm^2$), and AITC (2.88 $\mu g/cm^2$); acetyl isothiocyanate (168.82 $\mu g/cm^2$) was the least toxic. AITC and PEITC were 3.3- and 50.4-fold more active than benzyl benzoate against *D. pteronyssinus*, respectively. Taken together, these findings indicate that AITC, PEITC, and partial derivatives may be useful as preventive agents against dust mites. In addition, these results indicate that structure−activity is related to the aromatic structure, the number of carbon atoms, and the compounds hydrophobicity.

KEYWORDS: Acaricidal activity; allyl isothiocyanate; *Dermatophagoides farinae*; *D. pteronyssinus*; phenethyl isothiocyanate

INTRODUCTION

Approximately 10−15% of the general population suffers from symptoms of allergies such as asthma, perennial rhinitis, and atopic dermatitis (*1*). Allergic symptoms can occur because of pollen, pet dander, wool, food, and house dust mites (*2*). House dust mites are extremely important sources of allergens because they are perennial rather than seasonal (*3*). Almost all house dust mites found in indoors belong to the genus *Dermatophagoides*, and most of these are either *D. farinae* or *D. putrescentiae* (*3, 4*). The allergens associated with house dust mites are caused not only by house dust mites themselves but also by their excrement, and with eggs of house dust mites (*5*). It has become clear, especially after the work of Tovey et al. (*6*) that not only the dust mites, but also their deposited feces, constitute relevant sources of allergens (*7*). Upon entering the body, the allergens may respond to different elements of the immune system and trigger a release of chemical mediators, thereby causing allergy (*5*). Recently, many studies have suggested that controlling mite populations results in a decrease in the clinical symptoms of allergies to house dust mites (*8*). Accordingly, various methods to remove house dust mites have been evaluated, including environmental controls of indoor, physical methods, and the use of synthetic acaricides (*9, 10*). However, there have been many problems associated with these methods, including difficulties controlling mites in/on humans, the methods being too labor intensive, and potential toxicity (*5, 11, 12*). An alternative to these methods is the utilization of natural products produced by plants, such as essential oils (*13*). Essential oils, consisting of plant secondary metabolites, are hydrophobic liquids that contain volatile compounds and are often characterized by a strong odor (*14*). Essential oils not only are known for antiseptic, bactericidal, and virucidal effects, but play an important role as insecticides (*14*). Previous reports have shown that the essential oils of *Pelargonium graveolens* and *Thymus vulagris* are effective against house dust mites. In addition, the active constituents of these oils have been identified as monoterpenoids, such as β-citronellol, carvacrol, and thymol (*15*).

Sinapis alba L., which belongs to Cruciferae, is a perennial crop that is commonly used as a condiment and a spice due to its pungent taste and peculiar flavor. *S. alba* is also known to exert a wide variety of biological activities, including antineoplastic, antimicrobial, and insecticidal functions (*16, 17*). However, no studies evaluating the activity of *S. alba* L. seeds against house dust mites have been conducted to date. Therefore, we evaluated the acaricidal effects of the essential oils derived

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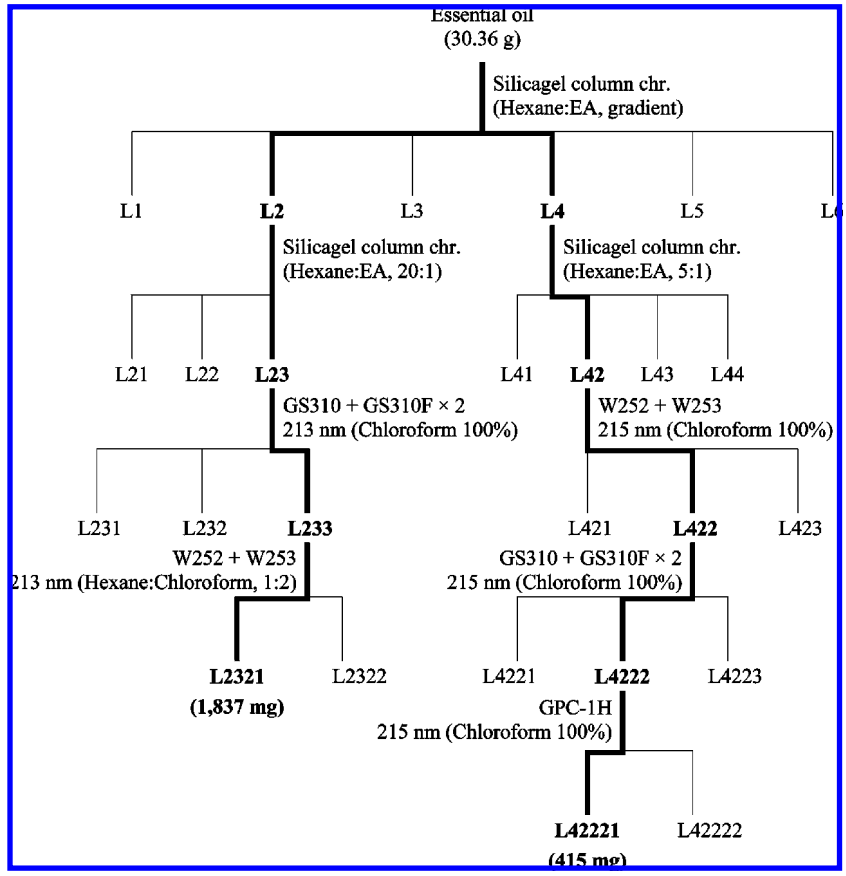

Figure 1. Isolation procedures of the acaricidal constituents from the essential oil.

from *S. alba* L. seeds to determine if treatment with these oils could alleviate allergy symptoms caused by house dust mites. Furthermore, we describe the procedure used to purify the active constituents of *S. alba* L. seeds, and then characterize these compounds while investigating their quantitative structure−activity relationships.

MATERIALS AND METHODS

Preparation. Allyl isothiocyanate, benzyl benzoate, phenethyl isothiocyanate, three aliphatic isothiocyanates (acetyl, methyl, butyl), and three aromatic isothiocyanates (benzyl, benzoyl, phenyl) were purchased from Fluka (Buchs, Switzerland) or Aldrich (Milwaukee, WI). All other chemicals used in this experiment were of reagent grade (GR). *S. alba* L. seeds were obtained from a local market in Chonju. The air-dried seeds of *S. alba* L. were ground to a fine powder and then subjected to a hydro distillation using a modified Clevenger-type apparatus to obtain the essential oil. The obtained essential oil (yield 0.1%) was then dried over anhydrous sodium sulfate and stored at 4 °C to minimize the loss of volatile compounds.

House Dust Mite Propagation. The respective cultures of *D. farinae* and *D. pteronyssinus* were maintained without exposure to any known acaricides. They were reared in plastic containers (15 cm × 12 cm × 6 cm) containing 30 g of sterilized diet (fry feed No. 1 and 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, and consisted of crude protein (44.0%), cellulose (4.0%), crude lipid (3.0%), P (2.0%), and Ca (1.8%).

Isolation and Identification. The essential oil (5 g) was loaded onto a silica gel (70−230 mesh, Merck) column (550 mm i.d. × 700 mm) and then eluted stepwise with hexane and mixtures of hexane:ethyl acetate (50:1, 20:1, 5:1, 1:1) to give six fractions (L1 to L6) (**Figure 1**). The L2 and L4 fractions were bioassayed at 80 μg/cm^2 and exhibited strong activity against *D. farinae* and *D. pteronyssinus.* Therefore, two active fractions (L2, L4) were, respectively, rechromatographed on a 300 mm i.d × 800 mm column. During this step, the L2 fraction was eluted with hexane−ethyl acetate (20:1), and the L23 fraction (1.14 g) was found to be active. In addition, the L42 fraction (0.55 g) of the L4 fraction, which was eluted using a mixture of hexane−ethyl acetate (5:1), was also found to be active. Next, the active fractions were purified by preparative HPLC (Japan Analytical Industry Co., Ltd.). The L23 was separated into three fractions (L231 to L233) using a JAI gel GS Series HPLC under the following conditions: column, GS 310 500 mm + GS 310F 300 mm × 2; mobile phase, chloroform; flow rate, 3 mL/min; detector, UV (213 nm). The L233 fraction exhibited strong activity, and therefore it was purified by applying it to a JAI gel W Series column (W-252 500 mm + W-253 500 mm) and then eluted in hexane:chloroform (1:1) under the same conditions as described for the L23 fraction. The L42 fraction was purified using a JAI gel W Series column (W-252 500 mm + W-253 500 mm) using chloroform at a flow rate of 3 mL/min as the mobile phase and detected at 215 nm to separate the biologically active constituents. The active L422 fraction was further rechromatographed using a JAI gel GS Series Column (W-253 500 mm + W-252 500 mm) and a mixture of hexane−chloroform (1:2) under the same conditions. All fractions were analyzed by TLC (thin layer chromatography, hexane−ethyl acetate, 10:1) and fractions with similar patterns were combined and bioassayed at 80 μg/cm^2. Finally, two potent active compounds, L2321 (1837 mg) and L4222 (415 mg), were isolated. The structures of these active principles were then determined by spectral analyses. Specifically, the ^{1}H and ^{13}C NMR spectra were obtained using a JNM-ECA600 spectrometer (JEOL Ltd., Japan; ^{1}H-600 MHz; ^{13}C-150 MHz) with $CDCl_3$ being used as an internal standard: (1) allyl isothiocyanate (AITC, C_4H_5NS, MW: 99); EI-MS (70 eV) *m/z* (% relative intensity) M^+ 99, 96, 93, 86, 84, 81, 76, 72, 67, 64, 61, 58, 54, 51; ^{1}H NMR ($CDCl_3$, 600 MHz) δ = 5.82−5.88 (1H, *m*), 5.28−5.41 (2H, *m*) and 4.16−4.42 ppm (2H, *m*); ^{13}C NMR ($CDCl_3$, 150 MHz) δ = 130.4, 130.2, 117.5 and 46.9 ppm; and (2) phenethyl isothiocyanate (PEITC, C_9H_9NS, MW: 163); EI-MS (70 eV) *m/z* (% relative intensity) M^+ 163, 147, 135, 116, 105, 91, 77, 65, 51; ^{1}H NMR ($CDCl_3$, 600 MHz) δ = 7.31−7.34 (2H, *t*), 7.25−7.27 (2H, *t*), 7.19−7.22 (1H, *t*), 3.67−3.69 (2H, *t*) and 2.94−2.97 ppm (2H, *t*); ^{13}C NMR ($CDCl_3$, 150 MHz) δ =

Table 1. Acaricidal Activities of *S. alba* Oil, Isolated Constituents, Isothiocyanate Derivatives and Commercial Acaricide against *D. farinae* and *D. pteronyssinus*, As Determined Using the Impregnated Fabric Disk Method

compound[a]	species	slope (±SE)	LD_{50} ($\mu g/cm^2$)	95% confidence limit	RT[b]
oil	*D. farinae*	5.56 (±0.32)	0.91	0.87−0.95	11.0
	D. pteronyssinus	4.61 (±0.30)	0.88	0.81−0.93	10.9
AITC	*D. farinae*	4.05 (±0.38)	1.36	1.24−1.48	7.4
	D. pteronyssinus	3.41 (±0.49)	2.88	2.80−3.00	3.3
acetyl isothiocyanate	*D. farinae*	7.88 (±0.79)	195.01	194.93−195.09	0.1
	D. pteronyssinus	8.96 (±0.98)	168.82	168.70−168.95	0.06
methyl isothiocyanate	*D. farinae*	5.97 (±0.67)	2.92	2.85−2.99	3.4
	D. pteronyssinus	4.44 (±0.52)	3.17	3.05−3.29	3.0
butyl isothiocyanate	*D. farinae*	3.48 (±0.72)	1.24	1.16−1.32	8.1
	D. pteronyssinus	2.53 (±0.47)	1.50	1.42−1.58	6.4
PEITC	*D. farinae*	3.02 (±0.28)	0.21	0.11−0.31	47.8
	D. pteronyssinus	2.79 (±0.31)	0.19	0.13−0.25	50.4
benzyl isothiocyanate	*D. farinae*	3.97 (±0.49)	0.55	0.48−0.62	18.3
	D. pteronyssinus	4.47 (±0.58)	0.77	0.71−0.83	12.4
benzoyl isothiocyanate	*D. farinae*	6.89 (±0.52)	13.58	13.52−13.64	0.7
	D. pteronyssinus	5.48 (±0.79)	7.63	7.56−7.70	1.3
phenyl isothiocyanate	*D. farinae*	2.99 (±0.62)	1.09	1.01−1.17	9.2
	D. pteronyssinus	3.65 (±0.47)	1.37	1.30−1.45	7.0
benzyl benzoate	*D. farinae*	4.99 (±0.63)	10.03	9.94−10.12	1.0
	D. pteronyssinus	6.09 (±0.68)	9.58	9.44−9.72	1.0

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

136.9, 130.6, 128.7, 127.1, 46.2 and 36.4 ppm. In addition, a UV−visible absorption spectrum was obtained using a UV spectrometer (DR/4000 spectrophotometer, HACH, Korea).

Experimental Treatments. An impregnated fabric disk method was used to determine the acaricidal activities of *S. alba* L. oil, the active compounds, and their derivatives. This method was slightly modified from the method described by Jeong et al. (*15*). Eight concentrations of test samples were used (80, 60, 40, 20, 10, 2.0, 2.5, and 1.25 $\mu g/cm^2$). Acetone was the carrier solution, and 20 μL of the test sample was applied to paper disks (Advantec, 8 mm diameter, 1 mm thickness, Toyo Roshi). In addition, acetone was applied to a disk at the same doses as a control. The paper disks were then dried under a fume hood (19 °C) for 30 s, after which disks were individually placed in the cap of a microtube (2 mL, Greiner bio-one Gm bH, Germany). After preparing the bioassay, groups of 25−30 adult mites (7−10 days old) were placed in microtubes that were then sealed using the caps containing the treated paper disks. Treated and control mites were then maintained at 25 ± 1 °C and 75% RH in darkness. Mortality rates were determined 24 h after treatment by observing the mites under a binocular microscope (×20, Olympus, Tokyo, Japan). Mites were considered dead if the appendages did not move when prodded with a pin. All treatments were replicated three times.

Statistical Analysis. LD_{50} values were calculated by probit analysis (*18*). Relative toxicity (RT) was determined as the ratio of synthetic acaricide LD_{50}/test chemical LD_{50} as previously described (*7, 15*).

RESULTS AND DISCUSSION

The steam distillate of *S. alba* L. seeds was evaluated using the impregnated fabric disk method, which revealed that the distillate exerted acaricidal activity against *D. farinae* and *D. pteronyssinus*. The essential oil produced 100% mortality against *D. farinae* and *D. pteronyssinus* at a concentration of 2.4 $\mu g/cm^2$ (**Table 1**). In addition, treatment with 1.2 $\mu g/cm^2$ of the essential oil resulted in 94.8% mortality against *D. farinae* and 97.3% mortality against *D. pteronyssinus*. The LD_{50} values of the essential oil were 0.91 and 0.88 $\mu g/cm^2$ against *D. farinae* and *D. pteronyssinus*, respectively. This result indicated that the two mite species are equally susceptible to the essential oil of *S. alba* seeds. Furthermore, this study is the first to report the acaricidal function of *S. alba* oil against dust mites.

Due to the acaricidal activity of the essential oil, purification of the biologically active compounds was conducted using a silica gel column and prep HPLC. This resulted in isolation of two potent compounds, L2321 and L4222, from the essential oil of *S. alba* L. seeds (**Figure 1**). The structures of these compounds were then characterized by instrumental analysis including UV, EI-MS, ^1H NMR, and ^{13}C NMR spectroscopic methods, after which the values were compared to those of authentic reference compounds. On the basis of the following evidence, the bioactive components were identified as allyl isothiocyanate (L2321) and phenethyl isothiocyanate (L4222). The acaricidal effects of the two compounds isolated from *S. alba* seeds against *D. farinae* and *D. pteronyssinus* were then examined. LD_{50} values for AITC were 1.36 and 2.88 $\mu g/cm^2$ against *D. farinae* and *D. pteronyssinus*, respectively (**Table 1**). In the case of PEITC, the LD_{50} values against *D. farinae* and *D. pteronyssinus* were 0.21 and 0.19 $\mu g/cm^2$. Furthermore, we found that acaricidal effects of the essential oil of *S. alba* L. seeds against *D. farinae* and *D. pteronyssinus* were primarily attributed to AITC and PEITC. When compared to the commonly used benzyl benzoate as positive control, AITC and PEITC were found to be 7.4- and 47.8-fold more toxic than benzyl benzoate against *D. farina* and 3.3- and 50.4-fold more toxic than benzyl benzoate against *D. pteronyssinus*, respectively.

The acaricidal activities of the derivatives of AITC and PEITC against *D. farinae* and *D. pteronyssinus* were examined by comparing the LD_{50} values generated using an impregnated fabric disk method (**Figure 2**). On the basis of the LD_{50} values, the most toxic effect against *D. farinae* was exerted by benzyl isothiocyanate (LD_{50} = 0.55 $\mu g/cm^2$) followed by phenyl isothiocyanate (1.09 $\mu g/cm^2$), butyl isothiocyanate (1.24 $\mu g/cm^2$), methyl isothiocyanate (2.92 $\mu g/cm^2$), benzoyl isothiocyanate (13.58 $\mu g/cm^2$), and acetyl isothiocyanate (195.01 $\mu g/cm^2$). When compared with the commercial acaricide, benzyl isothiocyanate, phenyl isothiocyanate, butyl isothiocyanate, and methyl isothiocyanate were 18.3-, 9.2-, 8.1-, and 3.4-fold more toxic than benzyl benzoate, respectively. However, acetyl isothiocyanate and benzoyl isothiocyanate were less toxic than benzyl benzoate. When the effects against *D. pteronyssinus* were evaluated, the most toxicity was exerted by benzyl isothiocyanate (LD_{50} = 0.77 $\mu g/cm^2$), followed by phenyl isothiocyanate (1.37 $\mu g/cm^2$), butyl isothiocyanate (1.50 $\mu g/cm^2$), methyl isothiocyanate (3.17 $\mu g/cm^2$), benzoyl isothiocyanate (7.63 $\mu g/cm^2$), and acetyl isothiocyanate (168.82 $\mu g/cm^2$). In addition,

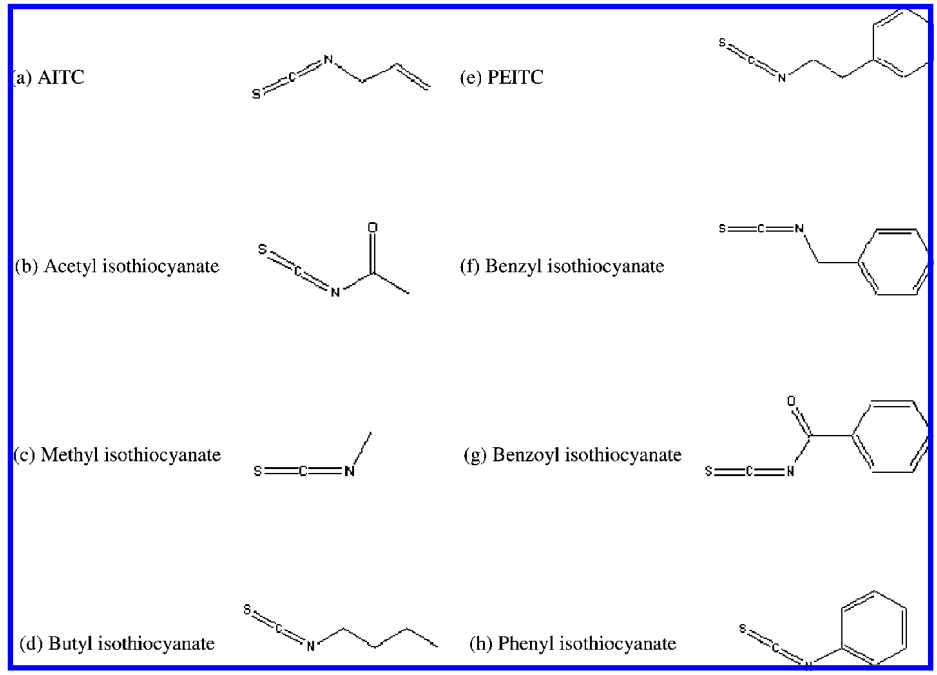

Figure 2. Structures of AITC, PEITC, and their derivatives.

benzyl isothiocyanate, phenyl isothiocyanate, butyl isothiocyanate, and methyl isothiocyanate were 12.4-, 7.0-, 6.4-, and 3.0-fold more toxic than benzyl benzoate, respectively (**Table 1**). Taken together, these results indicate that AITC, PEITC, and their partial derivatives are more effective than the currently available synthetic acaricide.

Many studies have found that various products produced by cruciferous vegetables, including isothiocyanates containing sulfur, have anticancer, anticoagulant, anti-inflammatory, and insecticidal properties (*19, 20*). The toxicity of isothiocyanate compounds occurs through interaction with reactive compounds such as nucleophilic compounds to form dithiocarbamic esters with −SH groups, thiourea derivatives with $-NH_2$ groups, and *N*-monosubstitued thiocarbamic esters with −OH groups (*21*). Isothiocyanates also have electrophilic characteristics and can therefore react and form covalent bonds with −SH, $-NH_2$, and −OH groups on enzymes and biological macromolecules. This process then results in enough biochemical damage to cause death (*22*). It has also been reported that the active molecules can intercalate into the intermolecular space of lipoproteins, thereby disrupting the lipid−protein interaction (*23*).

Interestingly, the acaricidal effect of isothiocyanates against *Dermatophagoides* spp. was found to be related to its structural characteristics. Aromatic isothiocyanates are relatively more toxic than aliphatic analogues. In this study, the acaricidal activities increased steadily as the number of carbon atoms increased. Various studies evaluating the insecticidal effects of methyl, allyl, benzyl, and phenethyl groups have also been conducted (*19*). For example, allyl isothiocyanate is much more toxic against wireworms than the ethyl group when these compounds are applied as fumigants (*24*). Furthermore, the results of the present study revealed that the presence of an aromatic ring in a molecule is essential for increased activity and that isothiocyanate toxicity increases with molecular weight. In quantitative structure−activity relationships (QSAR) analyses, the hydrophobic group has been shown to be related to biological activity, and samples with more hydrophobic groups have the highest toxicities (*25*). Accordingly, most pest control agents currently in use have a structurally uncomplicated hydrophobic character (*26*). Based on these prior studies, the results of this study are not surprising because the acetyl and benzoyl isothiocyanates were found to be remarkably less toxic than the other derivatives. These compounds contain a carbonyl group as C═O, and the difference between the electronegativities of the carbon and oxygen is large enough to cause the compound to be a hydrophile (*25*). Previously, the oral LD_{50} values of AITC and PEITC for mice were reported to be 308 and 700 mg/kg respectively, which suggest that these compounds have a relatively low acute toxicity toward mammals (*27*). In addition, AITC is often used as a natural food preservative in place of synthetic preservatives and is on the Generally Recognized as Safe (GRAS) list provided by the Food and Drug Administration (FDA) (*28, 29*).

Taken together, the results of this study and those of previously reported studies suggest that AITC, PEITC, and some derivatives (methyl, butyl, benzyl, and phenyl isothiocyanate) may be especially effective at reducing typical house dust mites in dwellings, such as *D. farinae* and *D. pteronyssinus*. However, further studies should be conducted to determine the human health and safety issues associated with these molecules and to develop effective formulations that provide acaricidal potency and stability.

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