INTRODUCTION

In recent years, environmental conditions that are favorable for mite breeding have been created by widespread use of air-conditioning and consequent lack of fresh air circulation. This has led to a serious problem of dust mite infestation. House dust mite, *Dermatophagoides pteronyssinus* is one of the main source of mite allergens (Arlia, 2002). It is widely known that dust mites and their feces, eggs, and carcasses act as major allergens causing asthma and atopic dermatitis in children and the elderly (Wharton, 1976). Reducing the amount of dust and house dust mites reduces these allergic symptoms of house dust (Murray et al., 1983).

There are various issues regarding the control of mites in the indoor environment. For example, drying of bedding is not effective unless the temperature of the bedding reaches above 70°C in the process. It has also been pointed out that allergens float due to insufficient suction and leakage from vacuum bags when cleaning carpets (Pollart et al., 1987b). Synthetic acaricides such as benzyl benzoate and DEET (*N,N*-diethyl-meta-toluamide) have been used for controlling mites (Van, et al. 1971). However, there are increasing cases of developing resistance to synthetic acaricides after repeated use (Van, et al. 1971; Pollart et al., 1987a), effects on non-target organisms, and environmental and human safety concerns (Pollart et al., 1987a). To overcome these problems, the creation of new acaricides for controlling *D. pteronyssinus* is desirable. In this study, we focused on fatty acids for their acaricidal activity. Potassium salts of straight-chain fatty acid of various lengths have been shown to have antifungal effect against *Penicillium pinophilum* (Era et al., 2015), antibacterial effect against oral bacteria.
(Masuda et al., 2015), and antiamebic effect against Acanthamoeba castellanii (Tanaka et al., 2017). All these activities differed greatly, depending on the length of the carbon chain constituting the linear fatty acid salts. Potassium laurate with 12 carbons and potassium caprate with 10 carbons were reported to be highly effective (Era et al., 2015; Masuda et al., 2015; Tanaka et al., 2017). Ethyl oleate, with an ethyl group attached to a fatty acid, has typical neurotoxic symptoms, including excitation, convulsions and paralysis in the agricultural pest Tetranychus urticae (Boisduval) (Masuda et al., 2018). This is brought about by the inhibition of monoamine oxidase (MAO) of the spider mite by the ethyl oleate. It is also clear that synthetic sugar esters with fatty acids have a miticidal effect against Tetranychus urticae Koch (Puterka et al., 2003).

However, few studies have investigated the effects of fatty acids on house dust mites such as D. pteronyssinus. In this study, we have investigated the mite control by branched fatty acids with different carbon chains against D. pteronyssinus and their mechanisms of action.

**MATERIALS AND METHODS**

**Mite**

D. pteronyssinus was purchased from Earth Corporation, Japan, and cultures were maintained without exposure to any known acaricide for 2 years. They were reared in petri dishes (diameter:8.5cm, depth:5.5cm) containing sterilized diet (feed for mouse, rat and hamster/dried yeast, 1:1 by weight) at 25±1°C and 75% relative humidity in the dark. The feed for mouse, rat and hamster was purchased from Oriental Yeast Co., Ltd., Tokyo, Japan, and the dried yeast was purchased from Asahi Breweries, Ltd., Tokyo, Japan.

**Chemicals**

Three branched chain fatty acids were tested in this study (Table 1). Of these, 2-ethylhexanoic acid (iso-C8) was from KH Neochem Co., Ltd., Japan. 2-butylloctanoic acid (iso-C12) was bought from Fujifilm Wako Pure Chemical Corporation, Japan, and isopalmitic acid (iso-C16) was from Nissan Chemical Corporation, Japan. We used 100% of branched chain fatty acids (iso-C8: 6.2×10^3 mM, iso-C12: 4.0×10^3 mM, iso-C16: 3.2×10^3 mM) in the tests. Three straight chain fatty acids were tested in this study. Caprylic acid (C8) and lauric acid (C12) were obtained from Tokyo Chemical Industry Co., Ltd., Japan, while palmitic acid (C16) was purchased from Fujifilm Wako Pure Chemical Corporation, Japan. In the tests, we used straight chain fatty acids at 3.5×10^3 mM.

**Bioassay**

**Contact mortality bioassay**

A contact mortality bioassay was carried out using modified Oh’s method (Oh, 2011). For this, the fatty acid samples were diluted with ethanol (100%, 50%, 25%, 13%, 6.3%, 3.2%, 1.6%, 0.8%, and 0.4%). A piece of black cloth (45mm×45mm) (100% cotton) was pasted in a petri dish (diameter: 9.5cm) with double-sided tape. The growth medium placed on medicine paper for 5min. The medium was returned, and 30 adult mites (no food attached) remaining on the medicine paper were placed on the black cloth using a brush so as not to be damaged. Then, 100μL of branched chain fatty acid (iso-C8, iso-C12, iso-C16) or a straight chain fatty acid (C8, C12, C16) was applied using a micro pipettor. Each compound was applied at the appropriate dilution. 100% ethanol served as a control. Treated and control mites were maintained at 25±1°C and 75%RH in the dark. Mortalities were determined 24h after treatment, under an upright microscope (Model YS100, Nikon Instech Co., Ltd., Japan). The results were based on the method of Faddy et al., (2010) and the observed mortality data were corrected for control mortality using Abbott’s formula (Abbott, 1925). All treatments were replicated three times.

**Fumigant mortality bioassay**

A fumigant mortality bioassay was modified from the method described by Kim et al. (2003). A piece of black cloth (45mm×45mm) (100% cotton) was pasted in the bottom of a petri dish with double-sided tape and 30 adult mites were placed on it. The growth medium placed on medicine paper for 5min. The medium was returned, and 30 adult mites (no food attached) remaining on the medicine paper were placed on the black cloth using a brush so as not to be damaged. Another piece of black cloth (45mm×45mm), treated with 100μL of the undiluted compound by micropipette, was pasted on the inside of a petri dish lid with double-sided tape. The petri dish was closed and sealed by wrapping in several layers of Parafilm®, and incubated at 25±1°C and 75%RH in the dark. Mortalities were determined 24h after treatment under an upright microscope and the data were processed as described before. Non-treatment served as a control. All treatments were replicated three times.

**Repellent bioassay**

A repellent bioassay was carried out using modified Oh’s method (Oh, 2011). For this test, 0.2% and 0.1% dilutions of the fatty acids were also used, in addition to the previously described doses. A piece of black cloth (45mm×45mm) (100% cotton), treated with 100μL of compounds in a quadrant (22.5mm×22.5mm) by
Micropipette, was secured in a petri dish (diameter: 9.5cm) with double-sided tape. The growth medium placed on medicine paper for 5min. The medium was returned, and 30 adult mites (no food attached) remaining on the medicine paper were placed on the compound-treated area using a brush so as not to be damaged. 100% ethanol was used as a control. Treated and control mites were held at 25±1°C and 75%RH in the dark. The case where mites escaped from the chemical treatment area was determined to be avoidance. Referring to Oh’s method (Oh, 2011), repellent rates were determined 3h as described earlier. All treatments were replicated three times.

Observation of mite epidermis

By observation with scanning electron microscope, an adhesion layer was observed on the body surface of T. cinnabarinus after application of the surfactant (Otsuji, 1985). Based on this, we assumed that the same phenomenon could be observed in this test.

A piece of black cloth (45mm×45mm) (100% cotton) was pasted on a petri dish (diameter: 9.5cm) with double-sided tape and 30 adult mites were placed on it. Then, 100 µL of branched fatty acids (100%) were added. Treated and control mites were maintained at 25±1°C and 75%RH in the dark for 24h. After lyophilization with the freeze dryer (Eyela FDU-1100, Tokyo Rikakikai Co., Ltd., Japan) for 24h, the mite epidermis was observed with the scanning electron microscope (S-3000N, Hitachi High-Tech Fielding Corporation, Japan).

Observation of toxic symptoms

Toxic symptoms were observed using modified Oh’s method (Oh, 2011). A piece of black cloth (45mm×45mm) (100% cotton) was pasted on a petri dish (diameter: 9.5cm) with double-sided tape and 30 adult mites were placed on it. Then, 100 µL of undiluted branched chain fatty acids were applied. Treated and control mites were held at 25±1°C and 75%RH in the dark. Treated mites were evaluated for toxic symptoms over time (5, 10, 20, 30, 60, 90, 120 and 180min) in contact with compounds without transferring to another filter paper or cloth. Toxic symptoms were determined as described by Hashimoto et al. (2000). The mites that turned over or lay on their side, were counted as knocked down mites. The standing mites, without moving were counted as immobilized mites. In addition, the corrected mortality rate for each elapsed time was examined in the same method as contact mortality bioassay. After lethality confirmation, mites were returned to the middle of the black cloth.

RESULTS AND DISCUSSION

Miticidal activities of branched chain fatty acids (contact mortality)

Fig. 1 to 3 show the miticidal effects of the 3 branched chain fatty acids on D. pteronyssinus 24h after contact. Ethanol was used as a diluent. Since there were no dead mites after ethanol treatment, this effect was not because of ethanol but specifically due to the fatty acids used for testing.

Iso-C8 showed the corrected mortality rates of over 85% at a minimum dose of 1.6% (99 mM). Using probit method (Fukaya et al., 1960), the LC50 of iso-C8 was calculated to be 0.82% (51 mM). For iso-C12, mortality rates of over 75% was achieved at 0.80% (32 mM) dose and the LC50 was 0.32% (13 mM), while iso-C16 exerted the corrected mortality rate of more than 50% with a minimum dose of 3.2% (1.0×103 mM). The LC50 of iso-C16 was 1.2% (39 mM).

In the results of iso-C12, there was a slight difference between LC50 and experimental values. This is thought to be due to the fitting by the probit method. On the other hand, as a result of examining each straight-chain fatty acid (C8, C12, C16), the corrected mortality rate of each compound was less than 50% at a concentration of 350mM. The corrected mortality rate for C8 (350mM) was 42%, for C12 (350mM) it was 34%, and for C16 (350mM) it was mere 13%. Results of this study show that the branched chain fatty acids brought about higher mortality rates than the straight-chain fatty acids, irrespective of the length of their carbon chains (8, 12, and 16 carbons).

The miticidal effect is thought to involve hydrophobicity

| TABLE 1. Structures of branched chain fatty acids |
|-----------------------------------------------|
| Branched chain fatty acid | Structural formula |
| 2-ethyl/hexanoic acid (iso-C8) | CH-COOH |
| 2-butyloctanoic acid (iso-C12) | CH-COOH |
| isopalmitic acid (iso-C16) | CH-COOH |
and hydrophilicity balance (HLB) (Otsuji, 1985). In terms of physical properties, as the carbon number of the alkyl group increases, the lipophilicity increases and HLB decreases. Otsuji et al. (1985) showed that lower the HLB of the surfactant, higher the insecticidal and ovicidal effects against the spider mite. It was reported that the maximum mite control effect was seen with HLB 6-8, and the ovicidal effect was greater at lower HLB levels (Otsuji, 1985). These results suggest that HLB is an important factor in the miticidal effect. The HLB of the branched chain fatty acids used in this study was calculated by the Griffin method (Griffin, 1949). HLB of iso-C8 was 6.2, for iso-C16, it was 3.5, while for iso-C12, with the highest miticidal activity, it was 4.5. From the results of this study, unlike the reported findings, it appears that, the effect tends to be the highest, when the HLB value is around 4.5. Miyamoto et al. (1994) showed that greater the hydrophilicity, easier the transport of the compound from the skin to the body fluid. This suggests that a small HLB value alone is not enough to be effective, but also requires optimum hydrophilicity. Present results suggest that HLB of iso-C12 was the most effective, and this suggestion was substantiated by its highest miticidal effect in this study. However, it is considered that the miticidal effect involves not only the HLB value but also the structure of the compound. The HLB values of straight chain fatty acids and branched chain fatty acids were the same as calculated by the Griffin method, but branched chain fatty acids showed a higher miticidal effect. From this, it seems that the branched structure is also a factor that enhances the acaricidal effect.

According to Puterka et al. (2003), xylitol showed 15.9% insecticidal activity against Cacopsylla pyricola (A kind of Psyllidae), when octanoic acid (C8) was substituted, and 73.1% insecticidal activity when decanoic acid (C10) was substituted. When dodecanoic acid (C12) was substituted, 34.4% of insecticidal activity was exhibited. This revealed that short-chain fatty acids and long-chain fatty acids showed low insecticidal, while medium-chain fatty acids showed high activity. These observations suggest that the miticidal
effect may be changed by changing the branched fatty acid in terms of the number of carbons, the number of branches, and the three dimensional structure.

Interestingly, when *D. pteronyssinus* were treated with an undiluted branched chain fatty acid, the coat color changed from colorless to yellowish-brown after 6h compared to raw and low temperature treated mites. Fig.4 shows color photographs of the coats after 24h. These reactions could be caused by water evaporation after death and polyphenol oxidase (PPO) and tyrosinase.

PPO is present in the form of prophenol oxidase in both insects and house dust mites, and is involved in immunity and self-recognition (Lee et al., 2008). Tyrosinase is an oxidase that regulates melanin production in plants and animals (Lee et al., 2008). In phenol metabolism, polyphenol oxidase catalyzes two basic reactions; hydroxylation of the phenolic substrate at the o position, adjacent to the existing hydroxyl group (monophenol oxidase activity) and oxidation of diphenol to quinonoid (diphenol oxidase activity) (Lee et al., 2008). Diphenol oxidase is associated with high catalytic rate and quinonoid formation, leading to the production of dark brown pigment melanin (Lee et al., 2008; Jin et al., 2003). Furthermore, tyrosinase is mainly responsible for animal melanin biosynthesis (melanin production) and plant enzymatic browning (melanosis) (Jo et al., 2003).

**Miticidal activities of branched chain fatty acids (fumigant action)**

To determine whether the acaricidal activities of branched chain fatty acids against *D. pteronyssinus* were attributable to contact or fumigant action, fumigant mortality bioassay was performed (Fig. 5). From the results of this assay, undiluted iso-C8 showed a corrected mortality rate of 71%, while it was 15% for undiluted iso-C12 and 5.6% for undiluted iso-C16. All branched chain fatty acids showed lower corrected mortality rate in fumigant mortality bioassay, compared to direct contact assay. From these results, it seems that the branched chain fatty acids show higher miticidal effect in direct contact than in the gas phase.

**Repellent effect of branched fatty acids**

Fig. 6 to 8 show the results of the repellent effect of each branched chain fatty acid on *D. pteronyssinus* 3h after treatment. Ethanol was used as a diluent. Since there were no dead mites after ethanol treatment, this effect was not due to ethanol but due to the fatty acids. A maximum of 19 mites escaped when the concentration of iso-C8 was 6.3% (3.9×10⁻³ mM), and more than
80% of the mites were alive when exposed to 3.2% (1.9×10⁻²mM). At 25% (8.0×10⁻³mM) of iso-C16, more than half of the mites were alive when exposed to 6.3% (2.0×10⁻²mM). Similar to the miticidal effect, iso-C12 had the highest repellent effect. At 0.2% (8.0mM) of iso-C12, 16 mites escaped, while more than 60% of the mites were alive at 0.1% (4.0mM) concentration.

In addition, for all branched fatty acids, the number of deaths tended to decrease and the number of escapes tended to increase as the sample concentration decreased. This suggests that these branched chain fatty acids have a higher repellent effect than the acaricidal effect as their concentrations decrease.
Observation of mite body epidermis by scanning electron microscope

Otsuji (1985) observed the body surface and the bristle base. Therefore, we focused on them of the part shown in Fig. 9(a). No significant changes were observed in the epidermis of mites from any of the treatment groups (iso-C8, iso-C12, and iso-C16) as compared to the untreated controls (Fig. 9) under electron microscope. Otsuji (1985) showed that higher the adhesion of the surfactant, higher the control of T. cinnabarinus. Furthermore, as a result of SEM observation of the surface of the spider mite after the surfactant treatment, an adhesion layer was observed. From this, they considered that gas exchange through the body surface of spider mites was blocked. However, in the present study, no effects of branched chain fatty acids with high miticide effects were observed on the mite skin. From these results, it seems that the possibility of inhibition of respiration due to the attachment of branched chain fatty acids to the skin is low, and that there are other major mechanisms of miticidal action.

Toxic symptoms and immediate effects after drug contact

From Hashimoto et al. (2000), symptoms of poisoning caused by acaricides on Dermatophagoides farina and Tyrophagus putrescentiae were classified into knock-down (KD) type and immobilizer (IM) type. KD type causes symptoms such as paralysis and convulsions, and IM type leads to quiet death. After treatment with undiluted iso-C8, 22 mites showed IM type symptoms after 20min and corrected mortality rate was 87% (Fig. 10). After 60min, almost all mites showed IM type symptoms. After iso-C12 treatment, 23 mites showed IM type symptoms after 20min and almost all mites had them after 60min (Fig. 11). After iso-C16 treatment, 11
mites showed IM type symptoms after 60min and almost all mites had them after 180min (Fig. 12). All branched chain fatty acids showed about 100% mortality after 180min. However, mortality did not reach 100% after 24h of compound treatment in contact mortality bioassay. This result is thought to be caused by returning mites to the middle of the black cloth after confirming the lethality.

KD-type features, like those brought about with organophosphates and pyrethroids for *D. farina* and *T. putrescentiae*, impair the mechanisms of neuro-transmission and cause symptoms such as abnormal excitability and convulsions (Hashimoto et al., 2000). In addition, IM type of symptoms, such as those due to benzyl benzoate and DEET for *D. farina* and *T. putrescentiae*, cause the immobilization and subsequent death and suggested a possible interference with the respiratory system (Fukami, 1976; Hashimoto et al., 2000). From the results of the present study, it seems that branched chain fatty acids target the respiratory system as IM symptoms set in within a short time. In this study, we studied high concentrations, but the mechanism of miticidal action might change at low concentrations. Therefore, it is considered that further experiments are necessary.

In conclusion, this study showed that branched chain fatty acids, iso-C8, iso-C12, iso-C16 showed a strong acaricidal effect on *D. pteronyssinus*. Among these, iso-C12 had the highest effect, with LC50 at 13mM. Body color of the mites after the treatment with branched fatty acids changed to yellowish-brown. In the fumigation test, all branched chain fatty acids showed a higher miticidal effect, when the compounds were indirect contact with the body of the mite. In the repellent test, at high concentrations of all branched chain fatty acids, significantly higher number of deaths were observed. In addition, as the sample concentration decreased, the number of deaths decreased and the number of escapes increased. Iso-C12, which had the highest miticidal effect, had a maximum number of escapes (16) at a lower concentration of 0.2% (7.8 mM). Mite skin showed no significant changes due to contact with branched chain fatty acids, as compared to the untreated skin. This led to an inference that the mechanism of acaricidal action of the branched chain
fatty acids was not through an inhibition of skin respiration through coating the body. All branched chain fatty acids immobilized more than half of the mites within 90 min of exposure. These results were consistent with the tendency of IM type miticides targeting the respiratory system.

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