A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Acaricidal and Allergen-Denaturing Activities of *Pinus densiflora* Needle Oil and *Hovenia dulcis* Constituents against *Dermatophagoides farinae*

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UNDER THE DIRECTION ADVISER YOUNG JOON AHN
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

House dust mites are most important allergen source of worldwide. Their body and feces composed of many allergens which induce asthma, rhinitis, of atopic dermatitis in human. The warm and humid climates are habitable for *Dermatophagoides farina*e which is the most popular and abundant in the world. Among the 20 groups of allergen, Der f 1 (25kDa, Molecular weight) and Der f 2 (14kDa) are the major antigen causing T cell reaction. It is very difficult to control of house dust mites physically because their small size, deeply habit beneath bed and pillow, prolific property demand large of efforts. Synthetic chemical controllers like Deet, benzyl benzoate and Dibutyl phthalate is very harmful not only human health but also environment. As an allergen denaturant, Tannic acid is affordable but their residual property is criticized. These problems occur necessary of develop new controller with safety and effect. *Hovenia dulcis Thunb* (Korean raisin)

has been used in Korean and Chinese traditional medicine as detoxification of ethanol, and possess hepatoprotective, antioxidative, antimicrobial and antidiabetic properties. *Pinus densiflora* essential oil (Korean Red pine needle oil) has been used for over 4,000 years in traditional oriental medicine as maintenance of a healthy body and high energy levels.

The present study aim at isolating allergen denaturing activity compound from the branches of *H. dulcis* and checking the allergen denaturing and acaricidal activities of constituents derived from *P. densiflora* against *D. farinae*. The allergen denaturing activities of a active principle from *H. dulcis* and *P. densiflora* are compared with those of the most widely used allergen denaturant, tannic acid. The activities were analyzed by measuring of intensity either Immunoblot or SDS-PAGE. Moreover, the toxicity of red pine needle hydrodistillate (RPN-HD), 19 RPN-HD constituents and 12 structurally related compounds and control efficacy of four experimental spray formulations containing RPN-HD (0.5. 1, 2 and 3% sprays) to adult *D. farinae* was evaluated.

H. dulcis show activity in SDS-PAGE toward crude *D. farinae* extracts for screening of allergen denaturing active plants. The active principle was separated using column chromatography and glass thin layer chromatography. In immunoblot using sera of asthma infected children, active principle exhibited allergenic inhibition. HPLC proved that this compound was separated with 96.09% purity. The results from the immunoblot bioassay with this principle demonstrated that its allergen denaturing activity (Relative allergenicity, 46.62%) was stronger more than that of tannic acid (45.65%). In *P. densiflora* derived compounds, γ-Terpinene showed the most potent allergen denaturing

compound, followed by *p*-cymene, 1,8-cineole, aromadendrene, geranyl acetate, and (1*S*)-(-)-α-pinene (28.6–19.2%). The activity of these compounds were comparable to that of tannic acid. Moderate or low allergen denaturing activity was obtained from seven (16.8–11.2%) and 20 compounds (9.3–2.0%), respectively.

In the acticidal activity bioassay, RPN-HD (24 h LC50, 68.33 μg cm^{-2}) was toxic. Menthol was the most toxic compound (12.69 μg cm^{-2}) and the toxicity of this compound and benzyl benzoate did not differ significantly from each other. High toxicity was also produced by α-terpineol, bornyl acetate, geranyl acetate, thymol, linalyl acetate, terpinyl acetate, citral, linalool and camphor (18.79–36.51 μg cm^{-2}). These compounds were more toxic than either deet or dibutyl phthalate. In vapour-phase mortality tests, these compounds were consistently more toxic in closed versus open containers, indicating that their mode of delivery was largely a result of vapour action. RPN-HD 3% experimental spray provided 95% mortality against adult *D. farinae*, whereas permethrin (cis:trans, 25:75) 2.5 g L^{-1} spray treatment resulted in 0% mortality.

H. dulcis branches principle and pine needle oil as well as its constituents possess allergen denaturing and acaricidal activity against house dust mites protein, respectively. They merit further study as potential agents of allergy denaturing, and potential biocide that control of *Dermatophagoides* populations through fumigants with contact action. In addition, in the light of global efforts to reduce the level of highly toxic synthetic acaricides in indoor environments, it is suggested that further investigations into the use of plant materials as acaricides and allergen denaturants are warranted.

Key words: Acaricidal activity, Allergen denaturing activity, House dust mite, *Pinus densiflora* essential oil, *Hovenia dulcis*.

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INTRODUCTION

The American house dust mite, *Dermatophagoides farinae* Hughes, and the European house dust mite, *Dermatophagoides pteronyssinus* Trouessart, are two of the most important pyroglyphid mites because of their cosmopolitan occurrence and abundance in indoor environments (Pollart et al., 1987) and also because they produce a variety of allergens causing allergic symptoms, such as asthma, atopic dermatitis, conjunctivitis, and perennial rhinitis in sensitive humans. In addition, dust mite allergens may follow various allergic reactions showing clinical symptoms with swelling, watery eyes and nose, erythema, difficulty breathing, headaches, skin rash, and itching (Arlian, 2002). Epidemiological studies revealed that mite allergy affects about 10-20% of world population (Platts-Mills, 2000). Of the diseases caused by dust mite excretion, asthma is the most important because of its high mortality. Approximately 5-10% of all adults and 10-20% of all children worldwide suffer from asthma (UK BIVDA, 2002). Most mite allergens are proteins with molecular weight range of 14-60 kDa. Of at least 32 denominated mite allergens, most allergic patients showed over 90% adverse reactions to group 1 and 2 allergens, two major mite allergens (Bard and Esch, 1992, Noli et al., 1996). Tannic acid is a currently available protein-denaturing agent since it was first recommended for reducing the allergenicity of House dust (Woodfolk et al., 1994). However, rigorous allergen reduction treatment in typical home setting is very difficult and tannic acid stains fabrics in a practical application. There was also residual tannic acid in carpet dust. These are, therefore, a critical need for the development of new

improved dust mite-protein denaturants, particularly those with acaricidal action.

Plant and their constituents have been suggested as alternative sources for control and protein-denaturing products largely because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and pose fewer risks to the environment, with minimal impacts to human and animal health, and often act at multiple and novel target sites (Isman, 2001). These potential new acaricide and protein denaturants can be applied to house dust mite habitats in the same manner as synthetic acaricide and tannic acid.

In this study, an assessment is made of the potential of red pine needle hydrodistillate (RPN-HD), 19 RPN-HD constituents, another one RPN-HD constituent and 11 structurally related organic pure compounds using fabric-circle contact + fumigant and vapor-phase mortality bioassays. It was also aimed at isolating allergen denaturing active compound from *Hovenia dulcis* branch against *D. farinae*. The toxicities of these materials to adult *D. farinae* were compared with those of the three conventional acaricides benzyl benzoate, dibutyl phthalate and *N*,*N*-diethyl-*m*-toluamide (deet). In addition, the efficacy of four experimental spray formulations containing RPN-HD (0.5, 1, 2, and 3% sprays) against adult *D. farinae* was evaluated using a spray bioassay to determine the most effective formulations and compared with that of commercially available permethnrin (*cis*:*trans*, 25:75) 2.5 g L^{-1} spray. Allergen denaturing activities of the test compounds and the active principles from *H. dulcis* branch were evaluated using sodium dodecyl sulfate-poly acrylamide electrophoresis and dot-blot immunoassay. Results were compared with those of the most widely used allergen denaturant, tannic acid.

LITERATURE REVIEW

House dust mites belong to the family Pyroglyphidae. In most temperate humid areas of the world, they are well known for induction of allergen and they also are an important decomposer, contributing to the recycling of bio-material. The Pyroglyphidae family composed of 16 genera and 46 species (Wharton, 1976; O'Connor, 1982; Hart, 1998). The most prevalent species are the American house dust mite, *Dermatohphagoides farinae* Hughes and the european house dust mite, *Dermatophagoides pteronyssinus* Trouessart (Arilian and Morgan, 2003). There are many different allergic substances in house dust. It is composed of disintegrated products of various material occurred by carpeting, mattresses, feathers, furniture, drapes, blankets and stuffed animals. These disintegrated products include human dander, insect parts, molds, bacteria, animal dander and mites (Winter., 2007). House dust mites are believed to be the primary allergen present in house dust. Human are exposed to house dust mite especially in the bedroom mattress, which provides the abundant food as well as warm and humid environment for their growth. Human sheds their skin 0.5-1.0 grams per day which is enough feed for one million house dust mites (Korsgaard J., 1998). The waste products that the mites produce are actually the main substance which allergic people react to. Although a dust mite only lives 2 to 4 months, during that time it produces about 200 times its own body weight in waste. This waste product breaks down into tiny particles, mixes with the dust, and then can be inhaled or ingested (El-Dib, 2010).

Df and Dp have similar properties in a morphologic construction. The length of the female is 370-430 μm which is larger than length of male, 300-350 μm. They are globular in shape, creamy white and have a striated cuticle. There are many wrinkles in the belly and the back, so it can be useful feature for identification between dust mite and other species. The bursa copulatrix is in the besidse of anal region of female (Ree, 2005). They reproduce sexually and the female possess the sperm in a seminal vesicle. The sperm is released into the oviduct to fertilize the egg during ovulation. The seminal vesicle and bursa copulatrix help to distinguish *Dermatophagoides species* from others (Arlian, 1989; Walzl, 1992).

House dust mite is grown through 5 stages: egg, larva, protonymph, tritonymph, and adult. The female lay the 1 to 3 egg per day, and total number of eggs laid per female is about 200 to 300 in their active reproductive life. The egg will hatched after 7 to 8 days and become the larva with three pair of leg. After having an active life for 5-6 days and 3 stop days, they become the protonymph. And as the same manner, they become the deutonymph and adult which has four pair of leg. Time for development from egg to adult is about one month and the life span of adult is about two month under the optimum humidity. House dust mite development is dependent on temperature, moisture, and an adequate food supply. The most favorable conditions for dust mites are 80-90% relative humidity and a temperature of 23-30℃ (Ree, 2005). Dust mites do not drink free water, but extract water from the unsaturated air and the amount of passive gain is proportional to the humidity of the environment (Furumizo, 1973; Ree, 2005).

Allergic diseases have been increased gradually result from that environment is becoming more polluted, change of life-style and inflow of new type of allergen. About 20-30% of people have been suffer from allergic disease of the present day (Ferguson, 2008). Meanwhile, we can suppossed that the importance of indoor envrionment is larger than ever relatively because most of living space has been moved from outdoor to indoor. It can't be denied that korean residential envronment has been changed in like manner, so the indoor allergic diseases are become important than the former days (Jeong at al., 2007). Eggleston investigated to what kinds of allergen exist in the home of urban area with children especially for the house dust mite, cockroach and cat's hair (Eggleston at al., 1998). In the allergen intracutaneous reaction, they found that allergic reaction of children is proportional to the concentration of allergen in indoor environment. There are also evidence of it is easy to induce allergic reaction who was exposed to allergen in early age (Wahn at al., 1997). So, allergen is become the one of the most important source which is affect to human health in indoor environment. Thus, the study of the indoor allergen is the basic step for the study of the allergic disease. It is also required to systematic investigation and analysis and also set up the management policies at the state level as well as simple investigation of distribution and comparison. There are noticeable indoor allergen such as house dust mite, cockroach, cat's and dog's hair and mold (Platts-Mills and Pollart Squilance, 1997).

Among these allergen, house dust mites which are belong to the arthropod phylum is recognized as important allergen source throughout all over the world (da Silva Ezequiel

at al., 2001; Kalpaklioglu at al., 2004). Many studies have been done about dust mite allergen and there's no doubt about dust mite bodies and feces are the sources of many allergens (Tovey at al., 1981; Arlian at al., 1987). The allergen may become airborne and inhaled by patients, giving occur to asthma, rhinitis, or atopic dermatitis (Van Bronswiik, 1981). 15 million disability-adjusted life years (DALYS) are caused by bronchial asthma which is the chronic inflammatory affecting 300 million individuals (Masoli at al., 2004). Many of the study which try to establish sensitization to indoor allergens over the world reveal that house dust mite is a major risk factor for asthma development and severity. It is also known allergen-specific immunotherapy is effect to reduction of allergen-specific IgE (Custovic at al., 1998).

Mite allergens are divided in to specific group according to their sequence homology, molecular weight, and biochemical composition. The allergens associated with mite fecal and bodies are characterized into 19 groups. Der f 1, Der p 1, Der f 2, Der p 2 and Der f 3 are well-known allergens that arise out of the fecal pellet and bodies of house dust mite. If there are more than 2 μg(about 100 mites) of allergen in 1 mg of house dust can induce allergic reaction to sensitive patient, whereas more than 10 μg cause serious symptom (Platts-Mills et al., 1992; Peat et al,. 1996; Platts-Mills and Pollart Squilace, 1997). They may trigger attacks of asthma, hay fever of eczema (Green, 1989). There are correlation between mite allergen concentrations in mattress dust and specific IgE in the serum of atopic asthmatic subjects (Lau et al, 1992). Because house dust mite are an important source causing asthma at childhood, reduction of house dust mite allergen could be helpful to large public health in terms of asthma prevention (Peat et al,. 1996).

There are many method to reduce house dust mite population such as replacing carpets, draperies, and upholstery, vacuuming carpets, freezing soft toys and small items, air cleaning and filtration, duct cleaning, using ozone generators, using chemicals (Arlian et al., 2001). Carpet and sofa that are made of fabric is removed totally from the house. Blanket, overquilt, and pillow are covering using mite-proof encasing or washing with hot water. High temperature is important fact for improve reproductivity of house dust mite, so refrain from using damping machine and recommended to ventilate frequently (Arlian et al., 2001). It needs strenuous efforts to avoid house dust mite physically. In some ways, cleaning of bedrooms including removal of carpets, covering of mattresses, and regular washing of bedding seems to ineffective. For example, although helpful in removing surface dust including mite fecal pellets, vecuuming with a inadequate suction or leaking bag is produce more allergen that become airborne and washing of bedding is should be done at temperatures greater than 70℃ to killing mites. It can be helpful to using replacible carpets with vinyl or wooden floors rather than poor vacuuming which are inadequate suction or leaking bags (Lau-Schadendorf S et al., 1991; Dietemann et al., 1993; Vyszenski-Moher DL et al., 2000). Ambient relative humidity is important factor for house dust mite population because mite must obtain sufficient water from the air to survive. As one of the most common recommended measure, maintain realative humidity below 50% in home. Adult mite die when they are dehydrated for 5 to 11 days under relative humidity is continuously 40% or 50% and temperature is 25-34°C (Arlian, 1975; Brandt RL and Arlian, 1976). There are many

physical and chemical control methods for house dust mite (Hayden et al., 1992; Dietemann, 1993; Peat J and Biforsten B, 1998; GINA, 2009). But there are argument about their clinical effectiveness though environmental control measures exibite significant reduction in the house dust mites allergen (Carter et al., 2001; Gotzsche et al., 2001; Htut et al., 2001; Woodcock et al., 2003; Morgan et al., 2004).

Several studies have given veriety results about using and effect of chemicals in controlling house dust mites and their allergen. In some studies, some chemicals exhibit reduction in allergen concentrations. It is considered to use of chemicals indoor; safety, efficacy of the active ingredients, and the formulation of product which deliver active ingredients directly to where the mites live (Lau-Schadendorf et al., 1991; Dietemann et al., 1993; Vyszenski-Moher et al., 2000). Spraying 60 g m^{-2} of 5% benzyl benzoate to the floor and carpet. Some countries permit to sales of bedclothes cotton which is coated by benzyl benzoate. Treating 0.4% d-phenothrin to blanket, overquilt, pillow, sofa, carpet is also effect to control house dust mite population. Comprehensive and continuous treatments described above are needed for more than one year to obtain complete control effect. If there are no sensitive patients to house dust mites, whose home doesn't need to these series of control action (Ree, 2005). However, good active compounds such as benzyl benzoate, disodium octaborate tetrahydrate, sumethrin, and permethrin and also denaturants (tannic acid) are not mean that they are good mite or allergen control in many experiment with acaricides (Lau-Schadendorf et al., 1991; Dietemann et al., 1993; Vyszenski-Moher et al., 2000).

Manufacturing of imitation horn and tanning of leather are the usual application of tannic acid (TA) in the industry. Moreover, it has been known as an astringent for a conventional use and known as inhibitor to digestive enzymes. TA is abundant in many plants and in the oak species and naturally occurring found in their bark and fruit. Many multiple type of TA is exists and can be grouped according to their shape: One is the condensed group, derivatives of flavonols, and the other is hydrolyzable tinnins, esters of a sugar with one or more trihydroxybenzene carboxylic acid residues. When applied to iron and albumin, TA reacts with them to form ferric tannate and insoluble precipitates, respectively (Makkar et al., 1988; Horigorne et al., 1988; Longstaff and McNab, 1991). Environmental allergens might be neutralized by TA. TA would be a useful adjunct to stringent allergen denaturing measures. Der p 1(Cystein protease), Der p 3(trypsin) and Der p Ⅳ (amylase), and other enzymes which associated with mite faecal particles were shown the enzyme equivalent activity when they were treated with TA. There are several studies show that TA could be useful as a abolisher aganist immunochemical properties of mite allergen Der p 1 (Thompson et al., 1993). Green et al. proof that the protein-denaturing properties of TA. The house dust mite allergic patients were shown completely abolishing allergic reaction in consequence of applied 1% TA solution in the skin test. 1% TA solution also reduce allergen when applied in washing bedding and other fabrics. Further studies of Green et al. show that TA with DMS solution not only killed house dust mites but also reduced the allergenicity of house dust (Green et al., 1984). TA exhibit maximum denaturing effects when is treated with minimum concentration, 0.2-0.6 percent (w/v), while 12 homes of mite allergic

patients were entered into a double blind placebo controlled. The results suggest that single application of TA could reduce the mite allergen and the activity is durable at least 4 weeks (Thompson et al., 1993).

Because it is very difficult to completely remove the House dust mite, it need to constant and suitable prevention using both physical and chemical control (Arlian et al., 2001). Cleaning measures and humidity control with a adjuct that chemical substuances is useful to remove or prevent for house dust mite. But it can be too toxic to recommend and even ineffectve against mites. Several laboratory studies have shown that numerous acaricides such as lindane, primiphos-methyl, benzyl benzoate, dibutyl phthalate, and diethyl m-toluamid (Susan et al., 1987). There are two controversies that are whether or not they are actually safe enough for human use in the home and whether they can be applied in such a suitable way that will kill the mites in the object like mattress. Benzyl benzoate is useful material, but the products may not be effective for more than 2 months. Another alternative material is tannic acid product can be available as an effective way of denaturing mite allergen and also killing mite, but the effect isn't retained for a long tiome. It is also problems that use of chemicals to control house dust mite could be dangerous for chemically sensitive patients (Lau et al., 1991; Colloff M.J. et al., 1992; Dietemann A. et al., 1993). Most pesticides and polychlorinated biphenyls (PCB), PCP, Lindane and DDT are easy to accumulated in house dust up to specific concentration likewise other semivolatile organic compounds. These are also known as probable or possible carcinogens to humans according to IARC classification. It can be absolved to human mainly three exposure pathways like inhalation, ingestion and skin

contact which regarding the digestive and dermal pathway. So the potential for a substance to interact with humans seems to be of great importance (Harald Ertl and Werner Butte, 2012).

Because of the many advantages such as selective, produce low environmental pollution, minimal toxicity to humans and non-target organisms, and accepted relatively safe to general, biopesticides provide an alternative to synthetic pesticides (Isman, 2006; Ahn et al., 2006). Natural products of plant origin with insecticidal properties have been tried in the recent past in order to control a variety of insect pests and vectors (Liu et al., 2000). Before the discovery of synthetic organic insecticides, many herbal products have been used as natural insecticides (ICMR Bulletin, 2003). Therefore, several studies on potential acaricide or allergendenaturant have been done. Miyazaki reported that some essential oils which have volatile properties and used in culinary herbs, spices and herbal teas also shown a high level of acaricidal and allergen denaturing activities to house dust mites (Miyazaki et al., 1989). It was identified that variety of phytochemicals have some effect to insect such as flavonoids, terpenoids, lignans, polyphenolics, alkaloids, saponins and peptides (Schultes, 1978; Kim et al., 2003; Kim 2004). Thease active ingredients can be applied to dust mites nests and act in both ways, behavioural (repellence and feeding deterrence) and physiological (acute toxicity and developmental disruption) (Isman, 2006; Ahn et al., 2006).

Pine tree are widely distributed in the world, especially in Asian mountains. The leaf of *Pinus densiflora* Siebold et Zuccarini has been used as a traditional medicine, food

and dietary supplements in Korea and China (Lim et al., 1993). It is composed of 58% water, 4.5% protein, 19.6% carbohydrate, 3.9% lipid, high level of calcium and iron, and nine essential amino acids (Rural Nutrition Institute, 1991). It has been known to promote human health, cure gastrointestinal diseases and neuronal problems, and prevent aging. Moreover, it can be potential medicine for protect the aging-related chronic diseases like hypertension, atherosclerosis, and diabetes (Song, 1993, Kim and Choi, 2001). The essential oil of young pine shoots has a antimicrobial activity (Theodor et al., 1991) and also has antimicrobial effects of ethanol extracts on lactic acid bacteria (Lim et al., 2001). The steam distillated leaf has some anti-proliferative, anti-survival and/or pro-apoptotic effects on human OSCC cells (Jo et al., 2012).

Medicinal plants have a variety of phytochemical constituents as well as essential oil. It is difficult to use because of limited either by the natural occurrence or the availability of a given plant species as well as knowledge about the potential pharmaceutical application (Hänsel and Sticher, 2009). They have been act as traditional medicine over the world, in particular Asia, Far East. Each compounds from the medicinal plants have different effect because of characteristic of plant species, difference in a plant species, part, geographical location, extration and application methods (Fruno et al., 1994; Oribe and Miyazaki, 1997). For example, the stem of *Uvaria versicolor* was more effective than *U. kaineana* or *U. mocoli* against *D. pteronyssinus.* It is interesting result because some essential oil species are have similar activity toward *D. farinae* and *D. pteronyssinus* such as seven Citrus species (*C. bergamia, C. aurantium, C. paradisi, C. limonum, C. aurantifolia, C. reticulata and C. sinensis* essential oil), three Cymbopogon

species (*C. nardus, C. citrates*, and *C. martini*), and three Mentha species (*M. pulegium, M. piperita,* and *M. spicata*) (Kim, 2002).

Hovenia dulcis Thunb. which commonly known as Japanese raisin tree or Chinese rasin tree is not well arranged in textbook about there pharmacological effect because typically used in East Asia contries as a folk remedy (Hänsel and Sticher, 2009). Traditionally, it was used for the treatment of liver diseases and detoxification after alcoholic poisoning (An et al., 1999). Each part of H. dulcis has different activity as usual medicinal plant. Its fruit and peduncle has a febrifuge property and applied to treat parasitic infections (Gadelha et al., 2005). The fruit possess antispasmodic, febrifuge, laxative and diuretic properties wherease, the seed is used as a diuretic and alcohol overdosing (Gadelha et al., 2005).

CHAPTER 1. Acaricidal activity of *Pinus densiflora* needle oil and *Hovenia dulcis* branch constituents.

INTRODUCTION

Several laboratory studies have shown that numerous acaricides such as lindane, primiphos-methyl, benzyl benzoate, dibutyl phthalate, and diethyl m-toluamid (Susan et al., 1987). There is controversy whether or not they are actually safe enough for human use. Some products may not be effective for a long time (Lau et al., 1991; Colloff M.J. et al., 1992; Dietemann A. et al., 1993). Moreover, most pesticides are easy to accumulated in house dust up to specific concentration likewise other semivolatile organic compounds (Harald Ertl and Werner Butte, 2012). These problems highlighted the need for the development of alternative acaricides. Most of plant derived compounds are produce low environmental pollution, affect minimal toxicity to humans and non-target organisms, and are accepted relatively safe to general, so, biopesticides provide an alternative to synthetic pesticides (Ahn et al., 2006; Isman, 2006).

In this chapter, acaricidal constituents of pine needles oil toward adults of *D. farinae* were identified. The acaricidal activities of the active principles were compared with those of the most commonly used acaricides: benzyl benzoate, deet, and dibutyl phthalate.

MATERIALS AND METHODS

1. Test mite

The stock cultures of *D. farina* (Kim et al., 2007) have been maintained in a temperature-controlled incubator without exposure to any known acaricide for more than 12 years. Mites were reared in plastic containers (12.5 × 10.5 × 5.0 cm) containing 40 g of sterilized diet (fry feed no. 1 + dried yeast, 1 + 1 by weight). The containers were incubated at 25 ± 1°C and 70–80% relative humidity in darkness. The fry feed and dried yeast were purchased from Korea Special Feed Meal (Inchon, South Korea) and Daeheung Pharmaceutical (Seoul, South Korea), respectively.

2. Chemicals

The thirty-one commercially-available organic pure compounds examined in this study are listed in Table 1, along with their sources. Benzyl benzoate, dibutyl phthalate, and deet were purchased from Sigma-Aldrich (St. Louis, MO). A commercially available insecticide examined in this study was Dongsung Pharmaceuticals permethnrin (*cis*:*trans*, 25:75) 2.5 g L^{-1} spray (Asan, Chungnam Province, South Korea). Ethoxylated castor oil, an emulsifier, was a gift from Yoo Sung Chem R&T Co. Ltd. (Daejeon, South Korea). All of the other chemicals used in this study were of reagent-grade quality and available commercially.

Table 1. The thirty-one compounds examined in this study

Compound	Source	Compound	Source
Aromadendrene	S-A[a]	Linalyl acetate	WK[b]
Borneol	S-A	Menthol	S-A
Bornyl acetate	S-A	β-Myrcene	TCI
Camphene	S-A	α-Phellandrene	TCI
Camphor	S-A	(1*R*)-(+)-α-Pinene	TCI
δ-3-Carene	S-A	(1*S*)-(-)-α-Pinene	S-A
β-Caryophyllene	TCI[c]	(1*R*)-(+)-β-Pinene	S-A
Caryophyllene oxide	S-A	(1*S*)-(-)-β-Pinene	S-A
1,8-Cineole	WK	α-Terpinene	S-A
Citral	S-A	γ-Terpinene	S-A
p-Cymene	S-A	Terpinen-4-ol	TCI
Geraniol	S-A	α-Terpineol	S-A
Geranyl acetate	WK	α-Terpinolene	TCI
Limonene	TCI	Terpinyl acetate	TCI
α-Humulene	S-A	Thymol	S-A
Linalool	S-A		

[a] Purchased from Sigma-Aldrich (St. Louis, MO).

[b] Purchased from Wako (Osaka, Japan).

[c] Purchased from Tokyo Chemical Industry (Tokyo, Japan).

3. Plant

The fresh needles of the red pine were collected from the Mt. Gwanak (Seoul) in early November 2012 because the medicinal effects of the needles are strongest in winter (Korea Food & Drug Administration, 1997).

4. Extraction of essential oil from *P. densiflora*

Fresh needles (250 g) of the red pine were finely ground and subjected to hydrodistillation at 100°C for 5 h using a Clevenger-type apparatus. The volatile oil was dried over anhydrous sodium sulfate and stored in a sealed vial at 4°C until use. The yield of the hydrodistillate from the pine needles was 0.21% (w/w).

5. Experimental spray formulations

Four experimental spray formulations containing RPN-HD in 5 mL glass container with a polypropylene pump spray nozzle (Homewell, Seoul) were prepared to determine the effective acaricidal products (Table 2). A single spray application of 0.5, 1, 2, and 3% concentrations of the RPN-HD preparations delivered ca 1.28, 2.56, 5.12, and 7.68 μg cm^{-2} of total material to a 5 cm diameter black cotton-fabric circle, respectively.

Table 2. Four experimental spray formulations containing red pine needle hydrodistillate

Spray formulation[a]	Per cent content			
	Hydrodistillate	Castor oil[b]	Ethanol	DW[c]
RPN-HD-0.5	0.5	0.25	30	69.25
RPN-HD-1	1	0.25	30	68.75
RPN-HD-2	2	0.25	30	67.75
RPN-HD-3	3	0.25	30	66.75

[a] Red pine needle hydrodistillate 0.5, 1, 2, and 3%.

[b] Ethoxylated castor oil.

[c] Distilled water.

6. Contact and fumigant mortality bioassay

A fabric-circle contact + fumigant mortality bioassay (Yun et al., 2012) was used to evaluate the toxicity of RPN-HD, and all compounds of *P. densiflora* against adult *D. farinae*. Based on the preliminary test results, four to five concentrations of each test compound in 100 μL of ethanol were applied to 5 cm diameter black cotton-fabric circles. After drying in a fume hood for 90 s, each fabric circle was placed onto the bottom section of a disposable Petri dish (5 cm diameter × 1 cm). Groups of 40–50 adult mites (including both sexes, 7–10 days old) were separately placed onto the treated fabric circles. Each Petri dish was then sealed with the original tight-fitting lid and wrapped with Parafilm. Benzyl benzoate, deet, and dibutyl phthalate served as positive controls and were similarly prepared. Control fabric circles (i.e., no test material or acaricide) received 100 μL of ethanol only.

Treated and control (ethanol only) mites were held under the same conditions as those used for colony maintenance. Mortalities were determined 24 h post-treatment under a dissecting microscope (×20). A mite was considered dead if its body and appendages did not move when prodded with a fine wooden dowel (Yun et al., 2012). Because not all bioassays could be conducted at the same time, treatments were blocked over time with a separate control treatment included in each block (Robertson and Preisler, 1992) Freshly prepared solutions were used for each block of bioassays. All treatments were replicated 3 times using 40–50 adult mites per replicate.

7. Vapor-phase mortality bioassay

The closed and open container treatment described by Kim *et al* (2007) was used to determine whether the lethality of 11 selected compounds against adult *D. farinae* was attributable to contact or fumigant action. Groups of 40–50 adult mites (both sexes, 7–10 days old) were placed separately onto untreated black cotton-fabric circles on the bottom section of polystyrene container (5 cm diameter × 2 cm), and each container was sealed with the original tight-fitting lid that had a fine wire screen covering a 3 cm diameter central hole. Approximately twofold quantities of the contact + fumigant LC_{50} values of each test compound were applied to 5 cm diameter filter papers as stated in Section 6. Each treated filter paper was placed on top of the wire screen, which prevented direct contact of adult mites with the test compound. Each container was sealed with either another solid lid (closed container treatment method) or sealed with another lid with a 4 cm diameter central hole (open container treatment method) to

investigate the potential vapor-phase toxicity of the test compounds. Control filter papers received 100 µL of ethanol only. Mortalities were determined 24 h post-treatment as stated in Section 6. All bioassays were replicated 3 times using 40–50 adult mites per replicate.

8. Spray bioassay

A spray bioassay was used to evaluate the efficacy of the four experimental spray formulations against adult *D. farinae*. Each test solution was sprayed 2 times successively at 10 cm upwards onto the black cotton-fabric circle (5 cm diameter). After drying for 5 min, each fabric circle was placed onto the bottom section of a disposable Petri dish (5 cm diameter × 1 cm). Groups of 40–50 adult mites (both sexes, 7–10 days old) were placed separately onto the treated fabric circles, and each container was sealed with the original tight-fitting lid. Permethnrin (*cis*:*trans*, 25:75) 2.5 g L^{-1} spray served as positive controls. Negative controls consisted of the ethanol-ethoxylated castor oil solution in distilled water or water. Mortalities were recorded as stated in Section 6. Each treatment was replicated three times using 40–50 adults per replicate.

9. Chromatographic analysis

A PerkinElmer Clarus 680 gas chromatograph (Fort Belvoir, VA), equipped with a split injector and a flame ionisation detection system, was used to separate and detect the constituents of RPD-HD. Analytes were separated with an Agilent 60 m × 0.25 mm ID (d_f = 0.25 µm) DB-5MS capillary column (Folsom, CA). The flow velocity of the helium carrier gas was 1.0 mL min^{-1}. The oven temperature was kept at 50°C (5 min

isothermal) and programmed to 300 °C at a rate of 2 °C min^{-1}, then isothermal at 300 °C for 20 min. The injector temperature was 280°C. The linear velocity of the helium carrier gas was 24.4 cm s^{-1} (30°C) at a split ratio of 1:50. Chemical constituents were identified by co-elution of authenticated samples following co-injection.

Gas chromatography-mass spectrometry (GC-MS) analysis was performed using a PerkinElmer Clarus 680T gas chromatograph-mass spectrometer. The capillary column and temperature conditions for the GC-MS analysis were the same as described above for GC analysis. The ion source temperature was 250°C. The interface temperature was kept at 260°C, and mass spectra were obtained at 70 eV. The sector mass analyser was set to scan from 35 to 550 amu every 0.2 s. Chemical constituents were identified by comparison of mass spectra of each peak with those of authentic samples in a mass spectrum library (The NIST Mass spectral search program, Columbia, MO)

10. Data analysis

Data were corrected for control mortality using Abbott's formula (Abbott, 1925). Mortality percentages were transformed to arcsine square root values for analysis of variance. Student's *t*-test was used to test for significant differences between two treatment methods (SAS OnlineDoc®, 2004). Means (± SE) of untransformed data are reported. Concentration–mortality data were subjected to probit analysis (SAS OnlineDoc®, 2004). The LC_{50} values of their treatments were considered to be significantly different from one another when 95% confidence limits failed to overlap. Compounds having LC_{50} >500 μg cm^{-2} were considered to be ineffective.

RESULTS

1. Chemical composition of red pine needle hydrodistillate

The RPN-HD was composed of 10 major (≥3.0%) and 38 minor constituents by comparison of mass spectral data and co-elution of authenticated samples following co-injection (Figure 1, Table 3). The 10 major constituents were α-pinene, β-caryophyllene, germacrene D, δ-cadinene, bornyl acetate, γ-muurolene, camphene, γ-cadinene, limonene and α-terpinolene, and comprised 14.1, 12.3, 9.5, 9.5, 6.9, 5.2, 4.2, 4.2, 3.2, and 3.0% of the hydrodistillate, respectively.

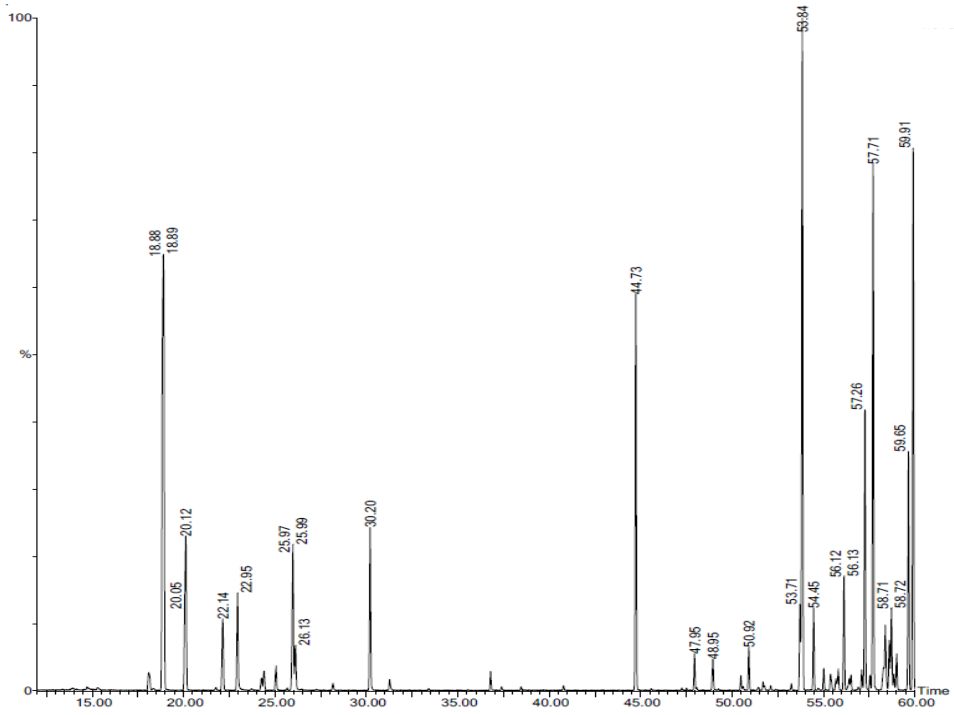

Figure 1. The spectrum of red pine needle hydrodistillate constituents identified by by gas chromatography and gas chromatography-mass spectrometry (GC-MS).

Table 3. Chemical constituents of red pine needle hydrodistillate identified by gas chromatography and gas chromatography-mass spectrometry (GC-MS)

Peak no.	Compound	RT[a] (min)	% Area
1	Tricyclene	18.09	0.5
2	α-Pinene[b,*]	18.89	14.1
3	Camphene[b,*]	20.12	4.2
4	β-Pinene[b]	22.14	1.7
5	β-Myrcene[b]	22.95	2.1
6	α-Phellandrene[b]	24.27	0.2
7	δ-3-Carene[b]	24.40	0.4
8	α-Terpinene[b]	25.06	0.5
9	Limonene[b,*]	25.99	3.2
10	β-Thujene	26.13	1
11	γ-Terpinene[b]	28.18	0.1
12	α-Terpinolene[b,*]	30.20	3
13	Linalool[b]	31.28	0.2
14	Borneol[b]	36.79	0.3
15	Bornyl acetate[b,*]	44.73	6.9
16	β-Elemene	47.95	0.6
17	α-Cubebene	48.95	0.6
18	α-Ylangene	50.48	0.2
19	α-Copaene[b]	50.92	0.7
20	β–Cubebene	53.71	1.5
21	β-Caryophyllene[b,*]	53.84	12.3
22	Unknown	54.45	1.5
23	Aromadendrene[b]	55.01	0.4
24	Unknown	55.40	0.4
25	Unknown	55.69	0.2

26	Unknown	55.81	0.4
27	α-Humulene[b]	56.13	2
28	Unknown	56.4	0.2
29	Unknown	56.51	0.3
30	α-Cadinene	57.09	0.3
31	γ-Muurolene[*]	57.27	5.2
32	Unknown	57.54	0.2
33	Germacrene D[*]	57.71	9.5
34	Unknown	58.38	1.8
35	Unknown	58.60	0.8
36	α-Muurolene	58.72	1.4
37	Unknown	58.85	0.2
38	β-Cadinene	59.01	0.6
39	γ-Cadinene[*]	59.64	4.2
40	δ-Cadinene[*]	59.91	9.5
41	Unknown	60.23	0.3
42	Cubinene	60.80	0.4
43	Unknown	61.04	0.4
44	Veridiflorol	64.07	0.1
45	Caryophyllene oxide[b]	66.06	0.3
46	Cubenol	66.44	0.3
47	T-Cadinol	67.26	0.9
48	α-Cadinol	67.36	1.2

[a] Retention time.

[b] Compounds identified by GC with authentic sample co-injection. The other constituents were identified by GC-MS without authentic sample co-injection

[*] Major constituent.

2. Toxicity of test materials

The toxicity of RPN-HD, all compounds, and three acaricides to adult *D. farinae* was evaluated using a fabric-circle contact + fumigant mortality bioassay (Table 4). As judged by 24 h LC_{50} values, RPN-HD, deet, and dibutyl phthalate did not differ significantly in toxicity from each other. The RPN-HD was ca 6 times less toxic than benzyl benzoate.

The toxic effects of 31 commercial organic pure compounds on adult *D. farinae* were likewise compared (Table 4). Based on 24 h LC_{50} values, menthol was the most toxic compound (12.69 μg cm^{-2}) and the toxicity of this compound and benzyl benzoate (11.06 μg cm^{-2}) did not differ significantly from each other. The toxicities of α-terpineol, bornyl acetate, geranyl acetate, and thymol (LC_{50}, 18.79–23.82 μg cm^{-2}) were significantly higher than those of linalyl acetate, terpinyl acetate, citral, linalool, and camphor (LC_{50}, 33.79–36.51 μg cm^{-2}). These compounds were more toxic than either deet or dibutyl phthalate. Low to no toxicity was obtained from the other 21 compounds and three medicinal plants.

Table 4. Toxicity of red pine needle hydrodistillate (RPN-HD), 19 RPN-HD constituents, another 12 structurally related organic pure compounds, and three acaricides to adult *Dermatophagoides farinae* using contact + fumigant mortality bioassay during a 24 h exposure

Material	LC_{50} (μg cm^{-2}) (95% CL[a])	Slope (±SE)	χ^{2b}	*P*-value
RPN-HD	68.33 (63.82–73.43)	5.8 (±0.65)	3.8	0.803
Menthol[c]	12.69 (11.99–13.37)	7.0 (±0.69)	3.4	0.846
α-Terpineol[d]	18.79 (17.87–19.67)	7.5 (±0.87)	1.47	0.983
Bornyl acetate[d,e,f]	22.81 (21.84–23.68)	9.5 (±1.10)	1.15	0.992
Geranyl acetate[c]	23.01 (22.04–23.87)	9.3 (±1.08)	1.79	0.971
Thymol[c]	23.82 (22.85–24.72)	9.5 (±1.15)	2.79	0.903
Linalyl acetate[c]	33.79 (31.64–35.55)	7.5 (±0.95)	4.86	0.677
Terpinyl acetate[c]	34.88 (33.04–36.48)	8.2 (±0.94)	4.14	0.763
Citral[c]	35.23 (34.02–36.70)	8.5 (±0.87)	4.92	0.896
Linalool[d,e]	36.13 (34.58–37.59)	9.2 (±0.98)	3	0.884
Camphor[c]	36.51 (34.70–38.51)	6.0 (±0.62)	2.94	0.983
Terpinen-4-ol[c]	54.72 (50.20–60.83)	3.3 (±0.34)	4.33	0.987
Geraniol[c]	58.67 (57.09–60.15)	10.5 (±1.11)	3.07	0.998
Borneol[d,e,f]	71.46 (62.05–82.51)	2.0 (±0.20)	5.04	0.888
Aromadendrene[e,f]	82.99 (79.92–86.30)	7.0 (±0.75)	1.48	0.999
α-Humulene[e,f]	83.60 (80.53–86.95)	7.0 (±0.76)	0.86	0.948
Caryopyllene oxide[e]	85.05 (75.92–93.75)	3.2 (±0.31)	4.08	0.944
β-Caryopyllene[e,f]	120.47 (112.50–127.08)	5.9 (±0.75)	4.37	0.929

Terpinolene[d,e,f]	133.28 (128.87–137.92)	8.7 (±0.77)	6.06	0.81
1,8-Cineole[c]	166.27 (161.64–170.95)	9.6 (±0.76)	5.05	0.993
p-Cymene[c]	188.07 (180.47–195.54)	8.8 (±0.90)	3.88	0.796
γ-Terpinene[d,e]	239.95 (232.63–246.65)	9.7 (±1.00)	6.95	0.905
Limonene[d,e,f]	254.60 (243.95–263.93)	9.4 (±0.97)	1.47	0.999
α-Terpinene[d,e]	277.80 (260.79–294.84)	6.1 (±0.68)	5.43	0.607
δ-3-Carene[d,e]	424.81 (393.15–458.50)	4.6 (±0.48)	4.25	0.75
β-Myrcene[d,e,f]	>500			
Camphene[d,e]	>500			
α-Phellandrene[d,e,f]	>500			
(1*R*)-(+)-α-Pinene[d,e,f]	>500			
(1*S*)-(-)-α-Pinene[d,e,f]	>500			
(1*R*)-(+)-β-Pinene[d,e,f]	>500			
(1*R*)-(-)-β-Pinene[d,e,f]	>500			
Benzyl benzoate	11.06 (9.50–12.72)	2.6 (±0.28)	0.89	0.996
Deet	70.76 (66.94–75.14)	5.5 (±0.64)	2.85	0.985
Dibutyl phthalate	75.49 (69.21–82.14)	4.0 (±0.47)	0.86	0.997

[a] CL denotes confidence limit.

[b] Pearson χ^2, goodness-of-fit test.

[c] Compounds structurally related to red pine needle hydrodistillate constituents.

[d] Constituents of red pine needle steam distillate reported by Park and Lee (2011).

[e] Constituents identified in this study.

[f] Constituents of red pine needle steamdistillate reported by Kim and Shin (2005).

3. Acaricide mode of delivery

The fumigant toxicity of 11 selected compounds to adult *D. farinae* was examined using the vapor-phase mortality bioassay in two formats (Table 5). After 24 h of exposure to 36.51 mg cm^{-3} camphor, there was a significant difference (P<0.0001) in lethality between exposure in a closed container, which resulted in 96% mortality, and exposure in an open container, which resulted in 8% mortality against adult *D. farinae*. Similar differences in the response of adult *D. farina* to citral, geraniol, geranyl acetate, linalool, linalyl acetate, menthol, terpinen-4-ol, α-terpineol, terpinyl acetate, and thymol in closed versus open container treatments were also observed.

Table 5. Fumigant toxicity of 11 selected compounds to adult *Dermatophagoides farinae* using vapor-phase mortality bioassay during a 24 h exposure

Compound	Conc. (μg cm^{-3})	Mortality (%) (± SE)		*P*-value[a]
		Vapor in closed container	Vapor in open container	
Camphor	36.51	96 ± 1.0	8 ± 1.2	0.0001
Citral	35.55	100	2 ± 1.1	0.0001
Geraniol	58.09	90 ± 1.6	3 ± 1.4	0.0001
Geranyl acetate	23.01	88 ± 2.1	2 ± 0.9	0.0001
Linalool	35.95	100	9 ± 0.8	0.0001
Linalyl acetate	32.37	83 ± 2.3	2 ± 1.1	0.0001
Menthol	12.63	96 ± 0.5	9 ± 0.1	0.0001
Terpinen-4-ol	54.73	100	12 ± 0.9	0.0001
Terpineol	18.79	100	8 ± 1.5	0.0001
Terpinyl acetate	34.78	88 ± 1.3	6 ± 1.2	0.0001
Thymol	23.82	100	0	0.0001

[a] According to Student's *t*-test.

4. Efficacy of experimental spray formulations

The control efficacy of four experimental spray formulations containing RPN-HD and a commercial permethrin (*cis*:*trans* = 25:75) 2.5 g L^{-1} spray against adult *D. farinae* was evaluated using a spray bioassay (Table 6). Red pine needle applied as 3% spray (RPN-HD-3) provided 95% mortality, whereas the 2% spray (RPN-HD-2) resulted in 58% mortality. The lethalities of the 1 (RPN-HD-1) and 0.5% (RPN-HD-0.5) sprays were 29 and 12%, respectively. Permethrin spray treatment resulted in 0% mortality against adult *D. farinae*. There was no mortality for the emulsifier-ethanol-water-treated or water-treated dust mites in the spray bioassay.

Table 6. Effectiveness of four experimental spray formulations containing red pine needle hydrodistillate(RPN-HD) and commercial permethrin against adult *Dermatophagoides farinae* using spray bioassay during a 24 h exposure

Spray treatment	Mortality[a] (%) (±SE)
RPN-HD-0.5[b]	12 ± 1.0d
RPN-HD-1[b]	29 ± 2.4c
RPN-HD-2[b]	58 ± 1.5b
RPN-HD-3[b]	95 ± 1.3a
Permethrin 2.5 g L^{-1} spray[c]	0e
Control[d]	0e

[a] Means within a column followed by the same letter are not significantly different at $P = 0.05$ (Bonferroni test).

[b] Red pine needle hydrodistillate 0.5, 1, 2 and 3%.

[c] Mixture (*cis*:*trans*, 25:75).

[d] Ethoxylated castor oil-ethanol-water-treated or water-treated control.

DISCUSSION

House dust mites is the most important allergy source that abundance in homes (Pollart et al., 1987; International Workshop Report, 1992). It is known that there are nineteen groups of house dust mite allergen and all of its body, dead body fragment and feces become allergen. These allergens are broken down and invade to human body causing allergic symptom such as atopic dermatitis, asthma, rhinitis, and conjunctivitis (Arilian, 2002). There are correlation between mite allergen concentrations in mattress dust and specific IgE in the serum of atopic asthmatic subjects (Lau et al, 1992). More than 20% of children is affected by atopic dermatitis (Illi et al., 2004) and exposed to a risk of subsequent development of bronchial asthma and allergic rihinitis (International Workshop Report, 1992). If human are exposed 2 μg of gruoup I allergen (25 kDa, molecular weight), sensitization and bronchial hyper-reactivity could be increased (Boulet et al., 1997).

There exist about 100 species of the genus *Pinus* widely found in the world, with 21 species being distributed in South Korea (Lee Y.N., 2002). The red pine trees are widely distributed in Japan, Korea, northeastern China and the extreme southeast of Russia. Historically red pine needles have been widely used for promoting health as a traditional medicine or as food (Lim et al., 1997). In the present study, the RPN-HD exhibited good acaricidal activity against adult *D. farinae*.

Certain essential oils and their constituents can be developed into products suitable for integrated pest management because they can be selective, have few harmful effects

on non-target organisms and are environmentally non-persistent (Ahn et al., 2006; Isman, 2006). They also can be used in conjunction with biological control (Isman, 2006). Essential oils consist of highly complex mixtures of hydrocarbons (usually terpenoids) and oxygenated compounds (alcohols, aldehydes, esters, ketones, oxides, and phenols) (Lawless, 2002). They act in many ways and have both behavioural (repellence and feeding deterrence) and physiological (acute toxicity and developmental disruption) efficacy against various types of pest complex (Ahn et al., 2006; Isman, 2006). They can be also applied to dust mite nests such as mattresses, pillows, carpets, rugs, and upholstered furniture in the same manner as other conventional acaricides (Ahn et al., 2006). Naturally occurring acaricidal compounds against house dust mites have been well described by Ahn *et al*. (2006). In the present study, the acaricidal principles of RPN-HD was identified as the monoterpenoids bornyl acetate and linalool. Of the compounds examined, high toxicity was obtained from menthol, α-terpineol, geranyl acetate, bornyl acetate, thymol, linalyl acetate, linalool, citral, terpinyl acetate, and camphor against adult *D. farinae*. The toxicity of menthol is comparable to that of benzyl benzoate. The other compounds were more toxic than either deet or dibutyl phthalate and were less toxic than benzyl benzoate. In addition, the 3% spray containing the RPN-HD resulted in good control efficacy compared with permethrin (*cis*:*trans* = 25:75) 2.5 g L^{-1} spray. This original finding indicates that the RPN-HD and the compounds described may hold promise for the development of novel and effective products for the control of house dust mites.

Investigations on the modes of action and delivery of naturally-occurring biocides

provide important practical information for dust mite control, such as the most appropriate formulations and delivery means to be adopted for their future commercialisation and for future resistance management (Ahn et al., 2006; Kim et al., 2006). Essential oil volatile constituents, such as alcohols, aldehydes, esters, ketones, oxides, phenols and terpenoids (particularly monoterpenoids) (lawless, 2002), primarily act as fumigants with additional contact action (Ahn et al., 2006). Fumigant toxicity to adult *D. farinae* and *D. pteronyssinus* has been reported for atractylon and atractylenolide III (Kim et al., 2007); ally isothiocyanate (Yun et al., 2012); butylidenephthalide (Kwon and Ahn, 2002); and paeonol (Kang et al., 2006). In a separate study, bisabolangelone was found to be largely toxic through contact action (Kang et al., 2006).

In the present study, the 11 selected compounds described were more effective in closed but not open containers. These results indicate that the route of acaricidal action of these compounds was largely a result of vapour action. The dual contact + fumigant action of the RPN-HD and test compounds, as demonstrated through our contact + fumigant, vapor-phase mortality and spray bioassays, is of practical importance because volatile compounds can easily reach deep harbourages in closed space such as a building or wardrobe, resulting in good control. This system has advantages because exposure to volatile compounds can be easily controlled. Because of high volatility of the RPN-HD-derived compounds and the test compounds described, the binary mixtures of these compounds and compounds with contact action (e.g. bisabolangelone (Kang et al., 2006)) could be useful in a space where a window is open. Detailed tests are needed to understand the mode of action of these compounds, although the octopaminergic and γ-

aminobutyric acid receptors have been suggested as novel target sites for some compounds by Kostyukovsky et al. (2002) and Priestley et al. (2003), respectively. In addition, it has been suggested that isothiocyanates are capable of reacting and forming a covalent bond with thiol groups of essential enzymes, leading a change in protein conformation and thus loss of activity (Wink, 2006).

In conclusion, biocides derived from RPN-HD containing bornyl acetate and linalool and other compounds described could be useful as contact-action fumigants for protection from humans from various allergic diseases caused by house dust mites. For the practical use of these materials as novel acaricides to proceed, further research is needed to establish their human and animal safety, although incorporating red pine needle-based products into the diet because of their health-promoting properties are in the marketplace in South Korea (Kim and Chung, 2000). In addition, formulations (aerosol, smoking agent, spray, or fumigant) for improving acaricidal potency and stability need to be developed.

CHAPTER 2. Allergen denaturing activity of *Pinus densiflora* needle oil and *Hovenia dulcis* branch constituents.

INTRODUCTION

Dust mites are microscopic, spider-like insects that can be found in most kinds of natural and synthetic fibers (Furumizo, 1973; O'Connor, 1982; Arlian, 1989). During the lifetime it produces about 200 times its own body weight in waste. This waste product breaks down into tiny particles, mixes with the dust, and then can be inhaled or ingested (El-Dib, 2010). These allergens are become the one of the most important source which is affect to human health in indoor environment. About 20-30% of people have been suffer from allergic disease of the present day (Ferguson, 2008). The allergens associated with mite fecal and bodies are characterized into 19 groups. Der f 1, Der p 1, Der f 2, Der p 2 and Der f 3 are well-known allergens that arise out of the fecal pellet and bodies of house dust mite (Platts-Mills et al., 1992; Platts-Mills and Pollart Squilace, 1997). Because house dust mite are an important source causing asthma at childhood, reduction of house dust mite allergen could be helpful to large public health in terms of asthma prevention (Peat et al,. 1996). There are several studies show that tannic acid could be useful as a abolisher aganist immunochemical properties of mite allergen (Thompson et al., 1993). However, rigorous allergen reduction treatment in typical home setting is very difficult. In aspect of practical application, tannic acid also has a

problem that stains fabrics. These problems have highlighted the new alternatives product.

In this chapter, allergen denaturing activity of pine needle oil and *H. dulcis* branch constituents against *D. farinae.*

MATERIALS AND METHODS

1. Materials

The thirty-one chemicals used in this study are listed in Table 1. Red pine needle hydrodistillate was obtained, as discussed in Chapter 1. Bradford reagents, Coomassie brilliant blue, (–)-α-copaene, sodium dedocyl sulfate (SDS), Tannic acid, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS) and skim milk were purchased from Sigma-Aldrich and BD Difco (Becton Drive, NJ), respectively. Precision Plus Protein Standards was supplied by Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin was supplied by Sigma-Aldrich. Goat anti-human IgG (H+L)-HRP and IgE-HRP conjugate were purchased from Zymed Laboratories (San Francisco, CA). ECL Western blotting detection reagent and nitrocellulose membrane strips were purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). All of the other chemicals and reagents were of reagent-grade quality and available commercially.

2. Plant

Air-deried branch of *H. dulcis* was purchased from Boeun medicinal herb shop (Kyoungdong market, Seoul) and used for extraction. A voucher specimen (HDB–01) was deposited in the Research Institute for Agriculture and Life Science, Seoul National University.

3. Dust mite extracts

Fresh *D. farinae* adults were extracted as described previously (Tang et al., 1990). Whole mites (7-10 days old, 10 mg fresh weight/ 200 μL PBS) were homogenized in 0.1 M cold PBS (pH 7.4). The homogenate was centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant was filtered through a 0.2 μm syringe filter. The filtered mite extract was stored at –70°C until use. The protein content was determined using a Bradford assay kit. BSA was used as a protein standard. The filtered mite solution (5 μL) was mixed with 200 μL Bradford solution and 795 μL PBS. Control was composed of 200 μL Bradford reagent and 800 μL PBS. Each mixture was incubated at room temperature for 4 min. Control and filtered mite solutions were measured by an Optizen Pop spectrophotometer (Daejeon, South Korea).

4. Human serum

The human serum was supplied by child patient who was attending the allergy clinics at Seoul National University Hospital. The allergic child is sensitive to both house dust mites species, *D. farinae* and *D. pteronyssinus.* The allergic reaction was certified by skin and RAST tests. Total IgE value is 3220 which means that allergic reaction value. *D. farinae* Specific IgE is 3.98 which means that species specific immune response. If the total IgE value and specific IgE is over than 200 and 2, respectively, it can be considered as allergic disease to specific target. Sera from healthy children did not show IgE-binding reactivity to mite extracts.

5. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed as described by Laemmli (1970) in 12% (w/v) poly acrylamide gels by using Bio-Rad mini-protean 3 electrophoresis cell (Hercules, CA). Mixtures of *D. farinae* extract (20 μg) and each test compound (1, 5, 10, 100, 500 each) dissolved in 5 μL of dimethyl sulfoxide (DMSO) were incubated for 1 h at 25 ± 1°C. Samples were mixed with 5 x sample buffer (4% SDS, 4% mercaptoethanol, 100 mM Tris-HCl, pH 8.0). After boiling for 10 min, each sample was loaded onto gel. Gels were run at 120 V for 3 h, and the proteins were visualized by staining Coomassie brilliant blue. The intensity of the gel was determined using a Bio-Rad Gel Dox XR image analyzer (Herculex, CA) with a Bio-Rad Quantity One software according to the manufacturerer's instructions. Negative controls consisted of 5 μL of DMSO. Tannic acid served as a positive control and was similarly prepared on the test compounds. All experiments were performed three times.

6. Immunoblot assay

A dot-blot immunoassay for human-specific IgE-binding reactivity were performed as described previously (Tsai et al., 2000). Each concentration of the test sample was dissolved in 5 μL of DMSO and protein soution which conatine 20 μg of *D. farinae* protein was mixed together. This mixture was incubated for 1 h in room temperature. After incubation, the mixture was socked into the nitro celluose membrane strips using a Bio-Rad Dot Blot Apparatus (San Francisco, CA). Adding about 35 μL of extra PBS and dried for a few minute. After washing the membran with PBS for 10 s, it was blocked with 5% skim milk-PBS solution with shaking in the rotary shaker for 1 h. And then it was incubated for 3 h with human serum which diluted in 1:500 ratio with 5% skim milk-PBS solution. The membrane was moved into the 0.5% PBS-Tween 20 (PBS-T) and washed 3 times, each time for 10 min. And then, the membrane was bounded for 1 h with goat anti-human IgG (H+L)-HRP and IgE-HRP conjugate antibody which diluted in 1:20,000 ratio with 5% skim milk-PBS solution on the rotary shaker. The membrane was washed with 0.5% PBS-T for 10 min, 3 times. The antibody was reacted with ECL Western blotting detection reagent for 1 min and covered by OHP flim and X-ray film. It was exposed for 30 s or 1 min in darkness. The denaturing activities (DA) of each test sample were calculated by Bio-Rad Quantity one software (Hercules, CA) and Gel Doc XR image analyzer (Bio-Rad, Hercules, CA). The value of DA was calulated by following formula: $DA = [(C-T)\ C^{-1}] \times 100$, where C is intensity in control, and T is intensity of the treated.

7. Extraction and isolation of active constituents from *H.dulcis*

The dried branches (600 g) from *H. dulcis* were pulverized and extracted with methanol (3 L) three times at room temperature and filtered using Whatman no. 2 filter paper (Maidstone, UK). The combined filtrate was concentrated under vacuum at 42℃ to yield approximately 3.75% on the dark brounish tar (based on the weight of the dried branch). The extract (22.5 g) was subsequently partitioned into hexane- (1.23 g), chloroform- (4.65 g), ethyl acetate- (1.05 g), butanol- (2.69 g), and water-soluble portions (12.89 g) for subsequent bioassay (Figure 2). The organic solvent-soluble fractions were concentrated to dryness by rotary evaporation at 42℃, and the water-soluble fraction was concentrated at 48℃.

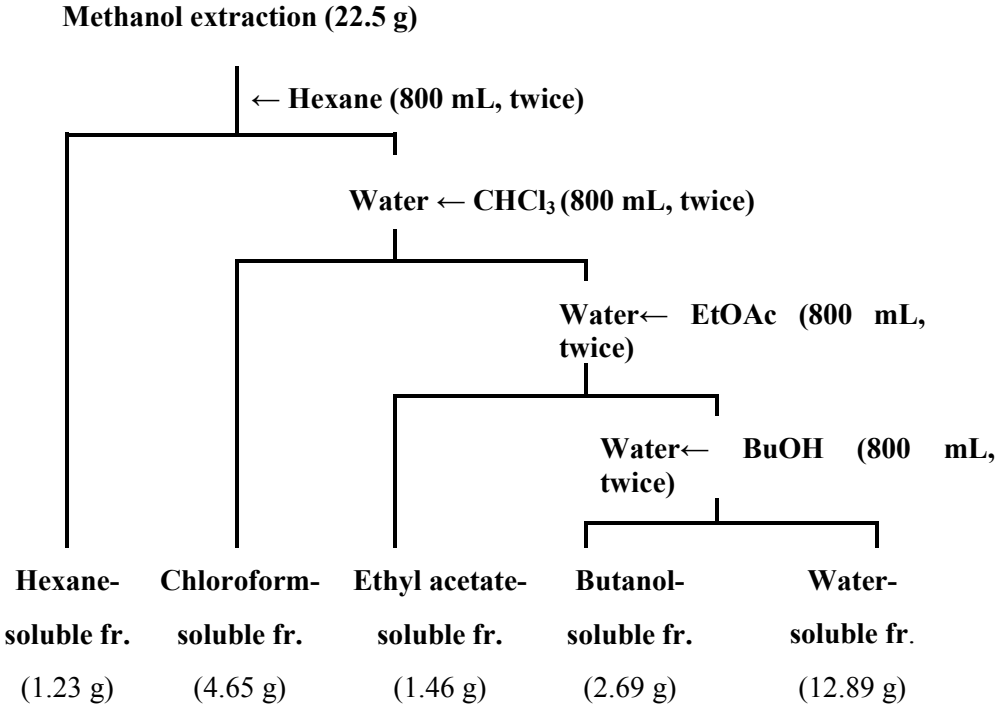

Figure 2. Solvent fraction procedures of methanol extract from *H. dulcis.*

Due to its potent allergen denaturing activity toward *D. farinae* and, the 4.65 g of chloroform-soluble fraction was chromatographed by MPLC (Biotage, Isolera One, Sweden) with SNAP 340 g silica (Biotage) column and eluted with a gradient of chloroform and methanol [100:0 (0.5 L), 99:01 (0.5 L), 98:02 (1 L), 95:05 (3 L), 90:10 (2 L), 75:25 (3 L) by volume] and finally with methanol (1 L) to provide 46 fractions (each about 240 mL). Column fractions were monitored by TLC on silica gel plates with chloroform and methanol (95:5 by volume). Fractions with similar R_f values on the TLC

plates were pooled. Spots were detected by spraying with 2% H_2SO_4 and then heating on a hot plate.

As a results of TLC pattern totally six fractions were received which named as C1 to C6. The active fraction C3 (1.2 g) was pooled and separated by MPLC with SNAP KP-C18-HS (120 g) reverse column and eluted with a gradient of water and methanol [100:0 (0.5 L), 99:01 (0.5 L), 98:02 (1 L), 95:05 (1 L), 90:10 (1 L), 75:25 (1 L), 50:50 (1 L) by volume] and finally with methanol (1 L) to provide 25 fractions (each about 240 mL). Column fractions were monitored by TLC (RP-18 $F_{254}S$, Merck) on silica gel plates with water and methanol (6:4 by volume). Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 2% H_2SO_4 and then heating on a hot plate. The active fraction C343 (106 mg) were pooled and purified by preparative TLC [chloroform: methanol (95:5) by volume] to yield compound **1** (24 mg, R_f = 0.21). Compounds **1** (Figure 4) was purified by HPLC (Agilent 1200 Series) with C18 column (7.8 mm i.d. × 300 mm Waters μBondapak C18 (Milford, MA) using a mobile phase of methanol and water (7:3 by volume), at 310 nm in flowrate 1 mL/min.

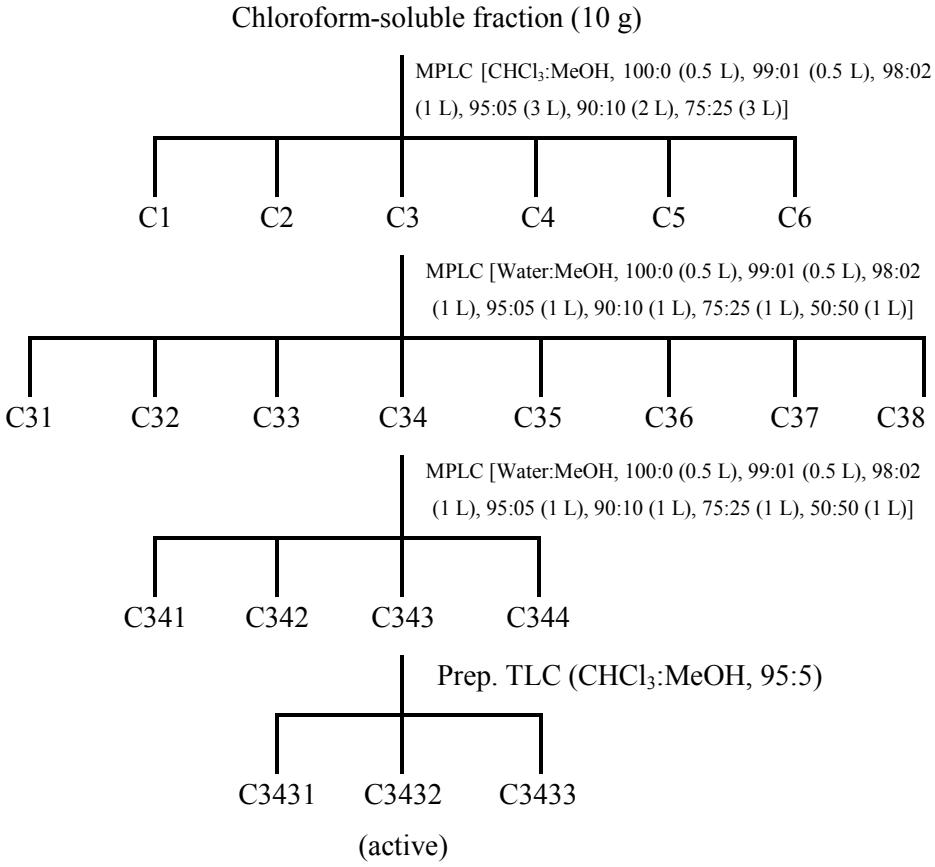

Figure 3. Isolation procedures of *H.dulcis* branch derived compound.

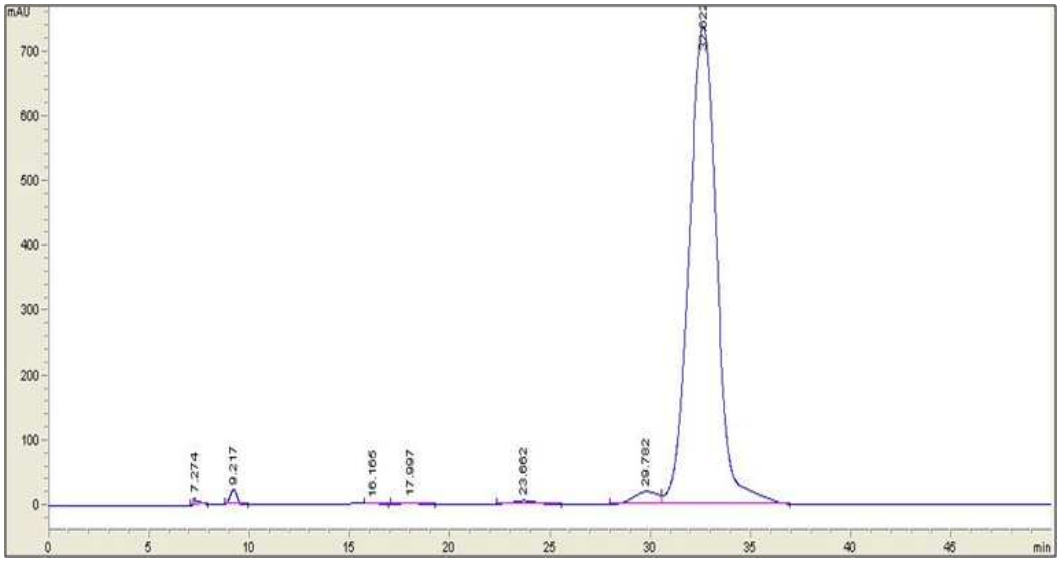

Figure 4. HPLC chromatogram of compound 1.

RESULTS

1. Allergen denaturing activity of *P. densiflora* constituents

The IgE-binding reactivity to crude *D. farinae* extracts of an asthmatic patient's serum by RPN-HD, 32 test compounds, and tannic acid were investigated using the immunoblot (Figure 5, Table 7). Responses varied according to test compound. γ-Terpinene showed the most potent allergen denaturing compound, followed by *p*-cymene, 1,8-cineole, aromadendrene, geranyl acetate, and (1*S*)-(-)-α-pinene (28.6–19.2%). The activity of these compounds were comparable to that of tannic acid. Moderate or low allergen denaturing activity was obtained from seven (16.8–11.2%) and 20 compounds (9.3–2.0%), respectively.

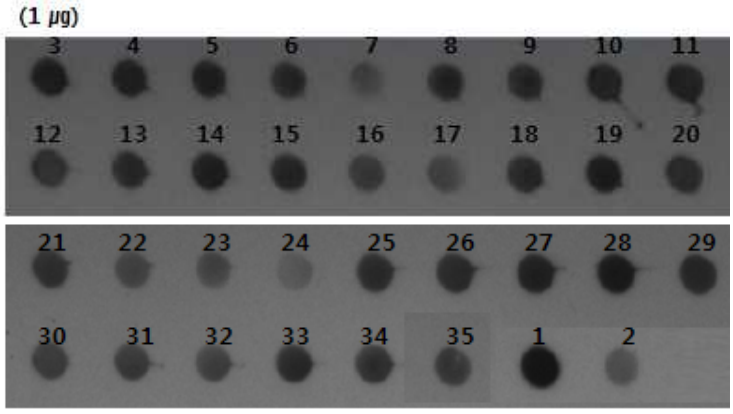

Figure 5. Immunoblot analysis of IgE-binding reactivity to crude D. farinae extracts by red pine needle hydrodistillate (RPN–HD), 32 test compounds, and tannic acid. The number is same as in Table 6.

Table 7. The allergenicity inhibition activities of RPN-HD, 32 compounds toward 20 μg of *D. farinae* protein

No.	Compounds	Inhibition of allergenicity activity (%)
		1 μg of smaple
1	Control	0
2	Tannic acid	24.9
3	RPN-HD	3.3
4	Menthol	4.2
5	α-Terpineol	4.7
6	Bornyl acetate	8.1
7	Geranyl acetate	19.3
8	Thymol	6.4
9	Linalyl acetate	7.9
10	Terpinyl acetate	5.8
11	Citral	5.3
12	Linalool	11.8
13	Camphor	8.5
14	Terpinen-4-ol	4.3
15	Geraniol	7.1
16	Borneol	14.5
17	Aromadendrene	19.3
18	α-Humulene	8.3
19	Caryopyllene oxide	3.4
20	β-Caryopyllene	9.0
21	Terpinolene	11.2
22	1,8-Cineole	19.4

23	*p*-Cymene	21.2
24	γ-Terpinene	28.6
25	Limonene	9.3
26	α-Terpinene	9.2
27	δ-3-Carene	4.9
28	β-Myrcene	2.0
29	Camphene	7.3
30	α-Phellandrene	16.8
31	(1*R*)-(+)-α-Pinene	15.9
32	(1*S*)-(-)-α-Pinene	19.2
33	(1*R*)-(+)-β-Pinene	9.5
34	(1*R*)-(-)-β-Pinene	12.2
35	(-)-α-Copaene	14.8

2. Allergen denaturing activity of three medicinal plants

A Coomassie brilliant blue-stained SDS-PAGE showed that, at 500 and 250 μg of *H. dulcis* branch, all *D. farinae* protein bands completely disappeared (Figure 6). At 500 and 250 μg of *Lycium chinense* fruit and *Leonurus japonicus* whole plant, the protein band nearly disappeared. The band intensity of the gels treated with *H. dulcis* branch was comparable to that of the gels treated with tannic acid.

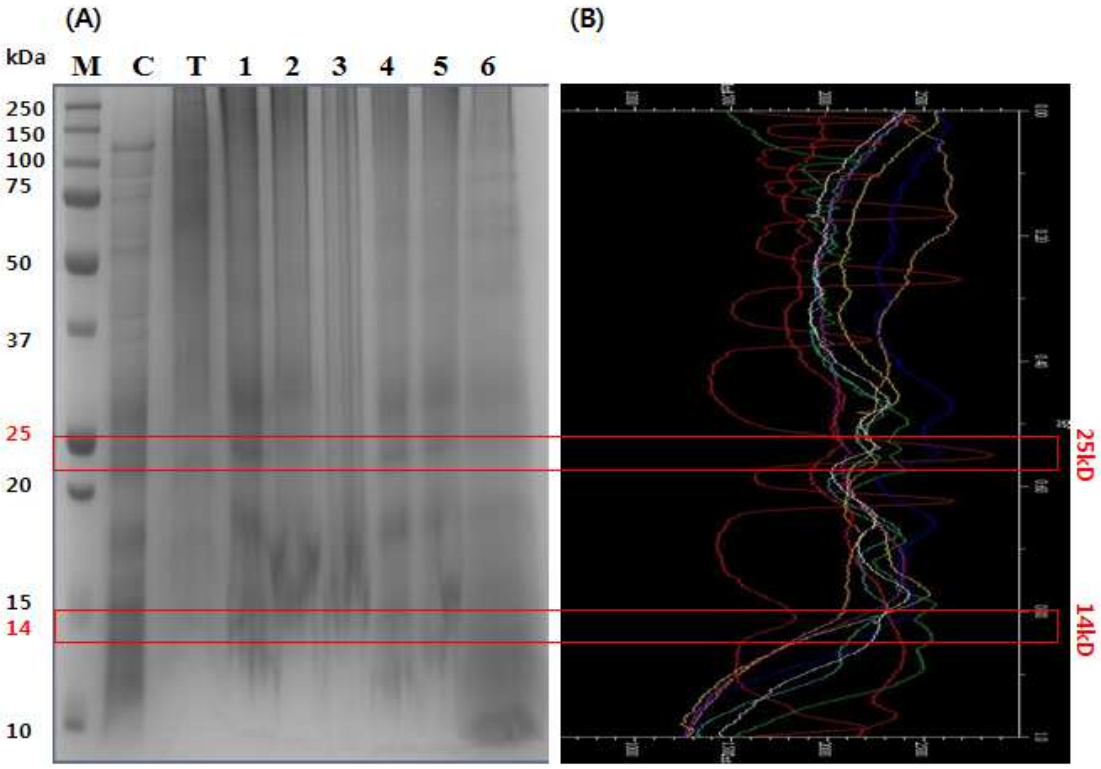

Figure 6. SDS-PAGE profile (A) and band intensity (B) of crude *D. farinae* extracts treated with or without test materials. Proteins were separated under reducing condition on 12% SDS-PAGE. Separated proteins of whole mite extract (20 μg) were visualized by staining with Coomassie brilliant blue. Lanes : M, protein marker; C, D. farinae extract (20 μg); T, Tannic acid; 500 μg; 1, *Lycium chinense* fruit ext., 500 μg; 2, *Leonurus japonicus* whole plant ext., 500 μg; 3, *Hovenia dulcis* branch ext., 500 μg; 4, *L. chinense* fruit ext., 250 μg; 5, *L. japonicus* whole plant ext., 250 μg; 6, *H.dulcis* branch ext., 250 μg.

3. Allergen denaturing activity of *H. dulcis* branch-derived materials

The IgE-binding reactivity to crude *D. farinae* extracts of an asthmatic patient's serum by fractions obtained from the solvent hydrolysable of the methanol extract of *H. dulcis* branch was examined using the immunodot (Figure 7, Table 8). The chloroform-ethyl acetate-, and butanol-soluble (liquid) fractions showed potent allergen denaturing activity. The chloroform-soluble fraction was used to identify peak activate fractions for the next step in the purification.

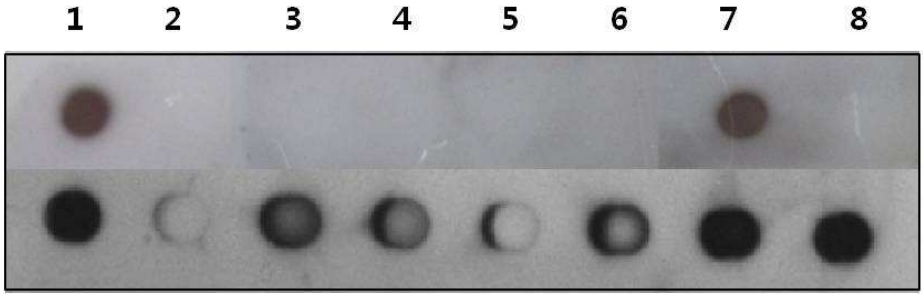

Figure 7. Immunoblot analysis of IgE-binding reactivity to 20 μg of crude *D. farinae* extracts by fractions obtained from the solvent hydrolysable of the methanol extract of *H. dulcis* branch.

Table 8. The allergenicity inhibition of fractions obtained from the solvent hydrolysable of the methanol extract of *H. dulcis* branch toward 20 μg of crude *D. farinae* extract

No.	Fraction	Inhibition of allergenicity activity (%)	
		500 μg of sample	100 μg of sample
1	Control	0	0
2	Tannic acid	63.4	50.5
3	Hexane-soluble fr.	70.1	16.4
4	Chloroform-soluble fr.	71.2	29.6
5	Ethyl acetate-soluble fr.	71.7	46.2
6	Butanol-soluble fr. (liquid)	73.2	25.5
7	Butanol-soluble fr. (solid)	10.4	1.9
8	Water-soluble fr.	59.9	1.1

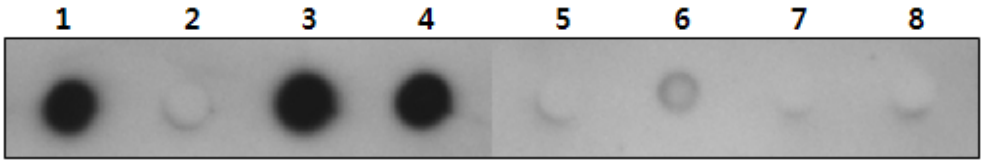

Figure 8. Immunoblot analysis of IgE-binding reactivity to 20 μg of crude *D. farinae* extracts by chloroform-soluble subfractions derived from *H. dulcis* branch.

Table 9. The allergenicity inhibition of chloroform-soluble subfractions derived from *H. dulcis* branch toward 20 μg of crude *D. farinae* extract

No.	Fraction	Inhibition of allergenicity activity (%)
		100 μg of sample
1	Control	0
2	Tannic acid	57.4
3	C1	0
4	C2	0
5	C3	63.0
6	C4	57.9
7	C5	66.0
8	C6	63.6

Bioassay-guided fractionation of *H. dulcis* branch extract (Figure 8 and Table 9, Figure 9 and Table 10, and Figure 10 and Table 11) afforded a potent allergen denaturing principle C3432 (Figure 4, Figure 11 and Table 12). The activity of the principle was slightly lower than that of tannic acid.

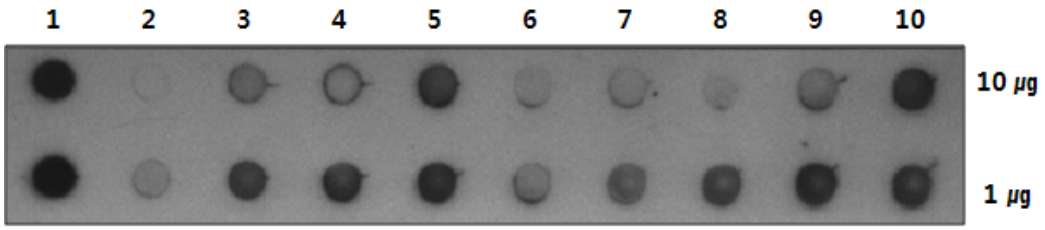

Figure 9. Immunoblot analysis of IgE-binding reactivity to 20 μg of crude *D. farinae* extracts by C3 subfractions derived from *H. dulcis* branch.

Table 10. The allergenicity inhibition of C3 subfractions derived from *H. dulcis* branch toward 20 μg of crude *D. farinae* extract

No.	Fraction	Inhibition of allergenicity activity (%)	
		10 μg of sample	1 μg of sample
1	Control	0	0
2	Tannic acid	42.3	39.4
3	C31	27.0	14.0
4	C32	32.6	12.6
5	C33	11.0	8.4
6	C34	38.3	34.4
7	C35	36.3	25.6
8	C36	38.4	17.3
9	C37	27.8	8.4
10	C38	6.2	11.2

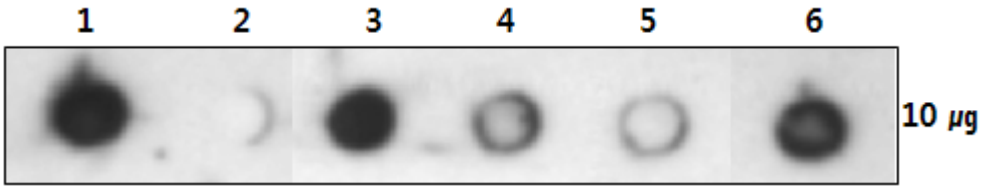

Figure 10. Immunoblot analysis of IgE-binding reactivity to 20 µg of crude *D. farinae* extracts by C34 subfractions derived from *H. dulcis* branch.

Table 11. The allergenicity inhibition of C34 subfractions derived from *H. dulcis* branch toward 20 µg of crude *D. farinae* extract

No.	Fraction	Inhibition of allergenicity activity (%)
		10 µg of sample
1	Control	-
2	Tannic acid	88.2
3	C341	7.1
4	C342	47.3
5	C343	72.0
6	C344	10.9

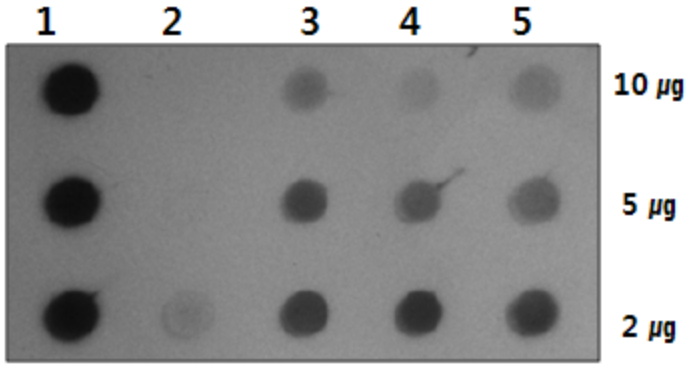

Figure 11. Immunoblot analysis of IgE-binding reactivity to 20 μg of crude *D. farinae* extracts by C343 subfractions derived from *H. dulcis* branch.

Table 12. The allergenicity inhibition of C343 subfractions derived from *H. dulcis* branch toward 20 μg of crude *D. farinae* extract

NO.	Fraction	Inhibition of allergenicity activity (%)	
		10 μg of sample	5 μg of sample
1	Control	0	0
2	Tannic acid	45.7	47.6
3	C3431	38.2	26.3
4	C3432	46.6	32.7
5	C3433	42.5	35.7

DISCUSSION

Mite-allergen denaturants have been suggested as a useful tool of rendering mite protein or residual protein immunologically inactive because dead mites and mite fecal pellets containing allergens persist months after eradication of live mites (Sears et al. 1989). Tannic acid is a naturally occurring substance found in the bark and fruit of many plants and gives insoluble precipitates with albumin and produces a blush black color with ferric salts (Woodfolk et al. 1994). This compound is used to denature mite allergens, which results in reducing the allergenicity of house dust (Green 1984, Ehnert et al. 1992, Woodfolk et al. 1994, 1995). The denaturing action of tannic acid is not protein specific. A 3% tannic acid solution (wt/vol) denatures group 1 allergens (Der p 1 and Der f 1) but is somewhat less effective for group 2 allergens (Der p 2 and Der f 2). Application of 3 % tannic acid to mattress casings and carpets reduces bronchial hyperreactivity in patients at 8 months after treatment, but this reducing effect was not shown 4 or 12 months posttreatment (Ehnert 1992). No information, however, is available concerning the potential of plant-derived materials for denaturing house dust mite allergens.

In this study, SDS-PAGE clearly indicates that *H. dulcis* branch extract caused dissapperance of *D. farinae* protein bands. In the dot-blot immunoassay, γ-terpinene, geranyl acetate, aromadendren, 1,8-cineole, *p*-cymene, α-phellandrene, (1R)-(+)-α-pinene, and (1S)-(-)-α-pinene strongly inhibited the IgE-binding reactivity to crude *D. farinae* extract of the asthmatic patient's serum. This original finding indicates that the

red pine needle essential oils may hold promise for the development of novel and effective natural mite-allergen denaturants, although the denaturing activity of these essential oils was lower than that of tannic acid. In addition, the fumigant toxicity of red pine needle essential oils against adult *D. farinae* has been revealded in Chapter 1. Unlike tannic acid, the dual acaricidal plus protein-denaturing action of test essential oils is of practical importance because a single application of the essential oils is cost effective. Detailed tests are needed to fully understand the modes of action of red pine needle essential oil

In conclusion, *H. dulcis*- and red pine needle essential oil-derived products with technical oils could be useful as naturally occurring mite-allergen denaturants for protection from humans from various diseases caused by house dust mites. In addiation, applications of diluted solutions of *H. dulcis* derived constituent or red pine needle essential oil are potentially an effective, acceptable, and inexpensive method for controlling house dust mites and their allergens, on the basis that plant essential oils are widely available with some being relatively inexpensive compared with plant extracts (Isman 2006). For practical use of the medicinal plant- and essential oil-derived products as novel protein denaturants to proceed, further research is needed to establish their human safety. Historically, *Hovenia dulcis*, so called ‘Raisin tree’ has been known for detoxification of alcohol in drunken people. It has a long history as a food supplement and traditional medicine in East Asia. Although there are antispasmodic, febrifuge, laxative and diuretic properties, the molecular mechanisms are not fully understood (Hyun et al., 2010). *P. densiflora* is not only important as a spice but is also

used medicinally for health promote effect such as cure gastrointestinal diseases and neuronal problems, and prevent aging (Song, 1993). Also, formulations (i.e. aerosol, spray, and odorant) for improving mite protein-denaturing potency and stability, thereby reducing costs, need to be developed.

CONCLUSION

House dust mites is the most important allergy source that abundance in homes (International Workshop Report, 1992; Pollart et al., 1987). It is known that there are nineteen groups of house dust mite allergen and all of its body, dead body fragment and feces become allergen. These allergens are broken down and invade to human body causing allergic symptom such as atopic dermatitis, asthma, rhinitis, and conjunctivitis (Arilian, 2002). There are correlation between mite allergen concentrations in mattress dust and specific IgE in the serum of atopic asthmatic subjects (Lau et al, 1992). More than 20% of children is affected by atopic dermatitis (Illi et al., 2004) and exposed to a risk of subsequent development of bronchial asthma and allergic rihinitis (International Workshop Report, 1992). If human are exposed 2 μg of gruoup I allergen (25 kDa, molecular weight), sensitization and bronchial hyper-reactivity could be increased (Boulet et al., 1997).

To control the house dust mites and remove allergens, chemical and physical methods have been suggested. Physical measure recommended such as washing bed in hot water, encasing mattresses and pillows, vacuuming or removing carpet and even reducing upholstered furnishings (McDonald and Tovey, 1992; Walshaw and Evans, 1986; Howarth et al., 1992; Hughes and Maunsell, 1973). Chemical measures such as benzyl benzoate, dibutyl phtalated, piriiphos-methyl, lindane, and tannic acid are also effective when combine with physical methods. However, these existing methods have some arguments about safety, application form, durability and resistance problems and even

they are not quit practical to apply in house (Dietemann et al., 1993; Lau-Schadendorf et al., 1991; Vyszenski-Moher et al., 2000). For examples, though tannic acid is powerful allergen denaturing agent (Thompson et al., 1993), it originally has staining properties so not suitable for apply to fabric. These problems facilitate development of practical and effective acaricidal and allergen denaturing materials.

In conclusion, biocides derived from RPN-HD containing bornyl acetate and linalool and other compounds described could be useful as contact-action fumigants for protection from humans from various allergic diseases caused by house dust mites. Moreover, allergen denaturing agent derived from RPN-HD and *H. dulcis* containing γ-Terpinene and *H. dulcis* derived compound described could be useful as allergen denaturant caused by house dust mites. For the practical use of these materials as novel acaricides and allergen denaturant to proceed, further research is needed to establish their human and animal safety, although incorporating red pine needle- and *H. dulcis*-based products into the diet because of their health-promoting properties are in the marketplace in South Korea. In addition, formulations (aerosol, smoking agent, medicine, spray, or fumigant) for improving acaricidal and allergen denaturing potency and stability need to be developed.

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집먼지진드기 (*Dermatophagoides farina*)에 대한 솔잎 (*Pinus densiflora*) 정유와 헛개나무 (*Hovenia dulcis*) 유래 물질의 살비 및 알러젠 중화 활성

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이 주 희

초 록

집먼지진드기는 따뜻하고 습한 온대, 아열대성 지방에서 많이 발견되는데, 지역적 차이가 있으나 큰다리먼지진드기(*Dermatophagoides farinae*)와 세로무늬먼지진드기(*Dermatophagoides pteronyssinus*) 종이 가장 높은 빈도로 발생한다. 이들은 생체 뿐만 아니라 사체, 탈피각, 배설물 등이 모두 천식, 비염, 아토피성 피부염 등을 일으키는 중요한 알러지 유발원으로 알려져 있다. 현대에 들어 생활양식이 실외에서 실내로 변화하고, 중앙난방시스템, 냉난방 조절장치로

인해 온도와 상대습도가 일정하게 유지됨에 따라 집먼지진드기의 번식에 적합한 환경이 조성되었다. 이에 따라 집먼지진드기의 방제를 위한 물리적, 화학적 노력들이 이루어지고 있는데, 집먼지진드기가 서식하는 베게, 이불, 카펫 등을 끓는 물에 자주 세탁하고 세척하며 창문을 열고 자주 환기를 시키는 등의 조치는 많은 시간과 노동력을 요하며 benzyl benzoate, dibutyl phthalate 등과 같은 화학합성살비제에 의한 화학적 방제는 약효의 유효성, 지속성이 집먼지진드기의 약제에 대한 저항성으로 인해 감소하고 환경과 인체에 대한 유해성 문제도 끊임 없이 제기되고 있다. 또한 탄닌산을 이용한 알러젠 중화요법은 좋은 효과를 보이고 있으나, 사용 후 잔존하는 탄닌산 처리가 시급한 실정이다. 이에 따라 안전하고 효과가 좋은 집먼지진드기 방제 및 알러젠 중화활성물질을 천연물에서 찾는 노력이 이어지고 있다.

본 연구는 집먼지진드기 자체의 방제 뿐만 아니라 알레르기원의 제거를 위하여 큰다리먼지진드기에 대하여 정유 및 식물체, 식물체유래 화합물을 이용한 살비 및 중화활성을 연구하였다. 살비 활성 생물 검정을 통해 솔잎 정유(24 h LC50, 68.33 μg/cm)가 활성을 보임에 따라 솔잎 정유의 구성성분 19종의 화합물과 구조적으로 유사한 12 종의 관련 화합물에 대해 생물 검정이 이루어졌다. 그 중 menthol (12.69 μg/cm)이 가장 높은 활성을 보였고 이는 양성대조군인 benzyl benzoate와 비슷한 수준이었다. 뒤이어 α-terpineol, bornyl acetate, geranyl acetate, thymol, linalyl acetate, terpinyl acetate, citral, linalool, camphor (18.79–36.51 μg/cm)이 높은 활성을 보였는데 양성대조군인 dibutyl phthalate

보다 효과적이었다. 훈증 활성 생물 검정 결과 위 화합물들은 개방형 용기에서 보다 밀폐형 용기에서 높은 활성을 보였다. 따라서 접촉 보다는 훈증 방식으로 집먼지진드기를 방제하는 것이라 사료된다. 또한 솔잎 정유와 물, 에탄올, 유화제를 혼합하여 분무 형태의 제형을 각각 0.5, 1, 2 및 3% 농도로 제조하여 방제 효과를 확인한 결과 3% 제형에서 95% 이상의 살비율을 확인하였다. 반면 양성대조군인 permethrin (*cis:trans*, 25:75) 2.5 g L–1제형은 0%의 살비율을 보였다.

집먼지진드기 알러젠 중화활성 검정을 위해 단백질젤전기영동 간이 검정법과 점블럿검정 방법이 이용되었으며 양성대조군으로 탄닌산를 사용해 그 결과를 비교하였다. 솔잎 유래 화합물에 대한 점블럿검정 결과, γ-Terpinene, *p*-cymene, 1,8-cineole, aromadendrene, geranyl acetate, 그리고 (1S)-(-)-α-pinene (28.6–19.2%) 에서 탄닌산과 견줄만한 높은 알러젠 중화활성을 보였다. 한방 식물체 3종의 단백질젤전기영동 간이 검정 결과 헛개나무 추출물에서 활성을 보였으며 헥산, 클로로포름, 에틸아세테이트, 부탄올, 물로 분획 후 점블럿검정으로 확인한 결과 클로로포름 분획층에서 높은 중화활성을 보였다. 이후 중압액체크로마토그래피, 박층크로마토그래피 및 고속액체크로마토그래피로 분리하며 각각의 분획층으로 점블럿검정을 수행한 결과 최종적으로 높은 활성을 보이는 C3432(46.6%) 단일 물질을 분리하였으며, 이 물질은 탄닌산(45.6%)과 비슷한 수준의 활성을 나타내었다.

이상의 결과를 바탕으로, 본 논문의 연구 성과들은 학문적으로 식물체에 함

유된 미지의 활성본체를 밝혀냈다는데 그 의의가 있고, 활성이 확인된 이들 식물체 정유, 관련 화합물들을 함유한 집먼지진드기 살비제, 알러젠 중화 활성제의 개발 등 산업적으로도 그 활용 가능성이 높다고 판단 되며 추가적인 연구가 요구된다.

검색어: 살비활성, 중화활성, 집먼지진드기, 솔잎 정유, 헛개나무

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