Doctor's Thesis

学位論文

Allergenicity evaluation of the volatile constituents of *myoga* using Guinea-pig maximization test and local lymph node assay

(GPMT および LLNA 法によるミョウガ芳香成分の感作性評価)

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CONTENTS

Part Ⅱ Quantitative study

PREFACE

This thesis is the result of series of investigations which took place during my graduate study at Kumamoto University in Kumamoto, Japan. it is based on the work recently published in Journal of Occupational Health entitled Toxicity study of the volatile constituents of *myoga* utilizing acute dermal irritation assays and the Guinea-pig maximization test.

This thesis is divided into two parts: Part I Qualitative study and Part II Quantitative study. The first part gives a general introduction to the GPMT. The second part provides a detailed the results of the LLNA and discussion.

The purpose of this study was to assess the allergenicity of *myoga* and its major volatile components using two methods (the GPMT and the LLNA). I believe that the data presented here will lead to further understanding of the mechanisms of allergic contact dermatitis for *myoga* cultivators

SUMMARY

Introduction: Mioga is a fragrance plant which is the special product of Japan and is cultivated everywhere. Presently there is no report about Mioga's provoking allergic contact dermatitis. According to our investigation (unpublished data), the *myoga* cultivators in somewhere of Japan, 8 of 35 cultivators experienced contact dermatitis in the harvest season. The purpose of this study was to assess the allergenicity of *myoga* and its major volatile components.

Materials and Methods: we analyzed the major volatile component of *myoga* by gas chromatograph and performed toxicity study of extract components by using the Guinea-pig maximization test (GPMT) and the murine local lymph node assays in order to probe the mechanism of allergic contact dermatitis.

Results: The major volatile component of *myoga* includes α-pinene, β-pinene and limonene. In acute dermal irritation assays, α-pinene, β-pinene and limonene showed positive responses at concentrations of 4%, limonene oxide at 20% and mioga showed a positive response at a concentration of 100%. From the results of the GPMT, according to

Kligman scores, limonene oxide was identified as an extreme skin sensitizer and *myoga* as a mild skin sensitizer. In the LLNA, limonene, limonene and β-Phellandrene had a positive responses and EC3 was 35.8%, 8.22% and 10.86%, respectively.

Discussion The GPMT and LLNA recommended by OECD is a standard method for the identification of contact sensitizer. The results of the present study confirm that limonene oxide is the most important allergen amongst chemical components of Mioga. Although the non-oxidied limonene itself is weak allergenic, it easily forms the strong allergenic products due to autoxidation during handing and storage. As the result, we think it is the reason that mioga cultivators were caused allergic contact dermatitis. Of course it should be kept in mind that the actual risk for humans to develop allergy depends on many factors. Besides the concentration, the frequency, duration of exposure and the condition of the human skin are important factors. The data was derived from animals. However, further validation of that from humans is left to be researched.

Publication list

1) Qingjun WEI, Koichi HARADA, Shoko OHMORI, Keiko MINAMOTO, Changnian WEI and Atsushi UEDA. Toxicity Study of the Volatile Constituents of *Myoga* Utilizing Acute Dermal Irritation Assays and the Guinea-pig maximization Test. *J Occup Health 2006; 48: 480–486*

2) Shoko OHMORI, Koichi HARADA, Chang Nian WEI, Qingjun WEI and Atsushi UEDA. Effect of estradiol on heme biosynthetic pathway in lead-poisoned rabbits. Environ. Health Prev. Med. 11, 277-285.

3) Atsushi Ueda, Koichi Harada, Shoko Ohmori, Chang Nian Wei, Kazuhiko Imamura, Qing Jun Wei and Keiko Minamoto. Occupational allergic hazards of agricultural chemicals and its prevention. JEnviron Occup Med, Aug 2005 Vol.22 No.4 320-324

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ABBREVIATIONS

ACD Allergic contact dermatitis

DMSO Dimethyl sulfoxide

EC3 The estimated concentration of chemical required to induce an SI of 3 relative to concurrent vehicle-treated controls value

GC Gas chromatography

GPMT Guinea-pig maximization test

HCA α-Hexylcinnamaldehyde

LNC Lymph node cell

LC Langerhans cells

LLNA Local lymph node assay

SI Stimulation index

Part I Qualitative study

Chapter 1 Introduction

1.1. *myoga*

Myoga (*Zingiber Myoga Roscoe*) (Fig.1) is a perennial herb with pungently aromatic flower buds which is native to Eastern Asia[1]. In Japan, the flower buds of *myoga* have been eaten as spice or pickles from ancient times. *Myoga* is now being consumed in amounts exceeding 6,000 tons per year and is cultivated throughout Japan. In the early 1990s, the technology for cultivating *myoga* in greenhouses was established which enabled the flower buds to be supplied to markets all year round. *Myoga* belongs to the ginger family (*Zingiberacea*). Ginger (*Zingiber officinale*), galangal (*Alpinia officinarum*, *Alpinia galangal*), turmeric (*Curcuma longa*) and cardamom (*Elletaria cardamom*) belong to the same family and have been reported to cause allergic contact dermatitis[2].The volatile constituents of fresh flower buds of *myoga* have been studied; 2-isopropyl-3-methoxypyrazine, 2-sec-butylzine were found to be the aroma compounds by GC-MS.[3] Some of the biological activities of pungent principles occurring in the Zingiberaceae, such as ginger[4,5] and *alpinia galanga*[6,7] have been studied in recent years, but it is allergenicity

that no report.

1.2. α-Pinene

α-Pinene ($C_{10}H_{16}$) (Fig.2) is an organic compound of the terpene class, one of two isomers of pinene[8,9] It is an alkene and it contains a reactive four-membered ring. It is found in the oils of many species of many coniferous trees, notably the pine. Both enantiomers are known in nature; 1*S*,5*S*- or (−)-α-pinene is commoner in European pines, whereas the 1*R*,5*R*- or (+)-α-isomer is commoner in North America. The racemic mixture is present in some oils such as eucalyptus oil. α-Pinene, has been so far reported to be found in a wide-range of essential oils such as *Salvia lavandulaefolia*, *Teucrium lusitanicum* and *Stachys aleurites*, etc[10,11,12]. According to the our literature survey, no anti-inflammatory and analgesic effect of α-pinene has been so far reported while it was shown to display insecticidal, spasmolytic, antilisterial and anticholinesterase effects[13,14,15,16]. Interestingly, this compound was found to possess antistress potency, exerting an alleviative effect on stress-induced hyperthermia in rats[17].

1.3. β-pinene

β-pinene (Fig 3) is a colorless liquid, soluble in alcohol, but not water. It has a woody-green pine-like smell. It occurs naturally in rosemary, parsley, dill, basil and rose.[18,19)]

1.4. R-(+)-limonene

R-(+)-limonene (Fig 4) is a clear liquid. Limonene is a monoterpene, made up of two isoprene units. Limonene occurs in two optically active forms, l-limonene and d-limonen. Both isomers have different odours: l-limonene smells piney and turpentine like and d-limonene has a pleasing orange scent. Limonene is found in the essential oils of citrus fruits and many other plant species[20)]. Industrial limonene is produced by alkali extraction of citrus residues and steam distillation. This distillate contains more than 90% d-limonene. Studies have shown that limonene have anti-cancer effects. This monocyclic monoterpene has shown chemopreventive and therapeutic activity against a wide variety of experimental tumors, such as lung neoplasms, DMBA-induced mammary cancer, and pancreatic and prostatic tumors[21,22,23,24)]. Phase I and phase II clinical trials of D-limonene

and perillyl alcohol (D-limonene-derived terpene) were undertaken; the preliminary results showed that these agents are well tolerated in cancer patients[25,26,27,28]. The mechanism of D-limonene antitumor activity is still under study, but several potential actions have been proposed, including apoptosis induction, G1 cell cycle arrest, p21Ras isoprenylation inhibition, and overexpression of mannose 6-phosphate/IGF-II and TGF-β type II receptor genes[29,30,31,32].Limonene is also used as a solvent and cleaner. It can replace white spirit and other solvents.

Limonene is considered a skin irritant and a sensitizer in man [33,34] and it is usually included among the fragrance allergens [35,36]. It is thought to be the principal sensitizer in *Citrus* species, but the incidence of contact dermatitis from these fruits is unknown. Skin reaction among consumers are seldom seen by dermatologists since the problems disappear when the fruits are avoided [37], but many reports ascribe occupational cutaneous diseases to citrus fruits [38]. Exposure to citrus fruits is also increased in food and beverage preparation, for example among cooks, bakes and bartenders [39]. Allergic contact dermatitis from dipentene in paint thinners and honing oil has been described [40,41]

Experimental studies both in humans and in animals have been reported. A human maximization test with limonene was carried out in 25 volunteers, but without provoking sensitization [42]. In a comprehensive study by four different methods with experimental animals, limonene was one of 32 substances tested [43]. Sensitization was obtained in three of the four methods used.

1.5. Limonene oxide

Limonene oxide ($C_{10}H_{16}O$) (Fig 5), also known as Limonene-1,2-epoxide, or Limonene Monoxide, is found in natural sources and is used as a fragrance ingredient. It is an active cycloaliphatic epoxide with low viscosity which may also be used with other epoxides in applications including metal coatings, varnishes, and printing inks. Limonene oxide is the potent skin allergen and it was suggested as the putative mechanism[44]. More recent investigations have supported the assumption that the allergenic or sensitizing potential is due to oxidation products of limonene rather than limonene itself[45].

1.6. β-phellandrene

Phellandrene ($C_{10}H_{16}$) (Fig 6) is the name for a pair of organic compounds that have a similar molecular structure and similar chemical properties. α-Phellandrene and β-phellandrene are cyclic monoterpenes and are double bond isomers. In α-phellandrene both double bonds are endocyclic and in β-phellandrene one of them is exocyclic. Both are insoluble in water, but miscible with ether. α-Phellandrene is a constituent of the essential oils of *Eucalyptus dives* and of *Eucalyptus phellandra* (hence the name). β-Phellandrene has been isolated from the oil of water fennel and Canada balsam oil. The phellandrenes are used in fragrances because of their pleasing aromas. The odor of β-phellandrene has been described as peppery-minty and slightly citrusy

1.7. Allergic contact dermatitis

Contact dermatitis is the most frequent occupational dermatosis[46)], Includes irritant contact dermatitis and allergic contact dermatitis. Irritant contact dermatitis is an inflammatory reaction to an external substance where no memory T-cell function or antigen-specific immunoglobulins are

involved. Clinical features include transient erythema, chapping, edema, inflammation, pain, and vesiculation. In severe cases, exudation, bullae formation, and tissue necrosis may be present. Immediate nonimmune contact reactions occur without prior sensitization[47]. The clinical appearance of a cutaneous reaction can occur within minutes with a strong irritant or longer with repeated exposures to weaker irritants[48]. Allergic contact dermatitis is caused by body's reaction to something that directly contacts the skin. Many different substances can cause allergic contact dermatitis, which are called 'allergens'. Usually these substances cause no trouble for most people, and may not even be noticed the first time the person is exposed. But once the skin becomes sensitive or allergic to the substance, any exposure will produce a rash. The rash usually doesn't start until a day or two later, but can start a soon as hours or as late as a week. Allergic contact dermatitis is not usually caused by things like acid, alkali, solvent, strong soap or detergent. These harsh compounds, which can produce a reaction on anyone's skin, are known as 'irritants'. Although some chemicals are both irritants and allergens, allergic contact dermatitis results from brief contact with substances that don't usually provoke a

reaction in most people. The dermatitis usually shows redness, swelling and water blisters, from tiny to large. The blisters may break, forming crusts and scales. Untreated, the skin may darken and become leathery and cracked. Allergic contact dermatitis can be difficult to distinguish from other rashes, especially after it been present for a while.

ACD is inflammation of the skin manifested by varying degrees of erythema, edema, and vesiculation. It is a delayed type of induced sensitivity (allergy) resulting from cutaneous contact with a specific allergen to which the patient has developed a specific sensitivity[49]. Jadassohn first described ACD in 1895. He developed the patch test to identify the chemicals to which the patient was allergic[50]. Sulzberger popularized patch testing in the US in the 1930s. The Finn chamber was designed in the 1970s; this is the standard method for patch testing individuals to chemicals not found in the thin-layer rapid use epicutaneous (TRUE) test, which became available in the US in the 1990s

Most chemicals able to provoke ACD have small molecules (<500 d). Approximately 3000 chemicals are well documented as specific causes of

ACD. The small chemical molecules responsible for ACD must bind to carrier proteins on Langerhans cells, which are situated within the suprabasilar layer of the epidermis. Langerhans cells are the antigen-presenting cells within the skin. Langerhans cells interact with $CD4^+$ T cells (helper T cells). Cytokines also play an important role in ACD because they regulate accessory-adhesion molecules, such as intercellular adhesion molecule 1. Interleukin 8 may be a cytokine indicating ACD, not irritant contact dermatitis. Langerhans cells can migrate from the epidermis to the regional draining lymph nodes. Sensitization to a chemical requires intact lymphatic pathways[51,52]. The initial sensitization typically takes 10-14 days from initial exposure to a strong contact allergen such as poison ivy. Some individuals develop specific sensitivity to allergens following years of chronic low-grade exposure associated with chronic irritant contact dermatitis resulting from the alkaline nature of cement. Once an individual is sensitized to a chemical, ACD develops within hours to several days of exposure.

Chapter 2 Background

Agricultural workers are exposed to various kinds and large amount of pesticides and/or other agricultural chemicals such as growth regulating hormones on their works. There have been many reports on the cases of agricultural workers developing adverse effects due to pesticides. The reported hazards are classified as acute poisoning such as poisoning to lever, respiratory, neurological system and whole organs and chronic toxic reaction such as inducing caner, mal-affection to ascendants, allergy and immune toxicity and residual intoxication such as abortion, sterility, deformity, cancer and chemical sensitivity. Among those hazards, allergy, especially allergic contact dermatitis, is one of the most serious problems. Allergy is a typical environment-related disease and then establishment of the preventive measure may lead to achievement of safety and comfortable condition of agricultural work places. Actually large numbers of suffering cases are still reported and the appropriate preventive control activities are not induced to worksite handling those agricultural chemicals[53,54,55,56].

Myoga is a fragrant plant which is a special product of Japan and it is cultivated throughout Japan. According to our earlier investigation

(unpublished data), performed in an area where about 20 households were cultivating *myoga* in greenhouses, 8 of 35 cultivators experienced contact dermatitis in the harvest season. The purpose of this study was to assess the allergenicity of *myoga* and its major volatile components.

Chapter 3 Materials and Methodology

3.1. Chemicals

α-Pinene, β-pinene, R-(+)-limonene and limonene oxide were obtained from Kanto Chemical Co. Inc (Japan). Freund's complete adjuvant was purchased from Sigma (St. Louis, USA). Dimethylsulfoxide (DMSO) was used as the vehicle for all test compounds. All the reagents used were of the highest grade available.

3.2 Synthesis of β-phellandrene

Into a pressure vessel were added β-pinene (1.21 moles), water (250 ml.), methanol (250 ml), sodium bisulfite (1.27 moles), and potassium nitrate catalyst (0.13 moles). This reaction mixture was heated at 110℃. While oxygen at 5 psig was passed into the reaction mixture. An exothermic reaction was observed within 10 minutes after commencement of the oxygen and the reaction pressure temporarily was increased by about 5-10 psig. After 4 hours reaction time, 392 grams of a white crystalline solid was recovered from the reaction mixture. This solid was recrystallized from 90% ethanol to yield 148.3 grams of recrystallized solid. This solid

analyzed as para-menth-1-ene-7-sodium sulfonate (47.5% theory yield of the monohydrate of the sodium sulfonate product based upon β-pinene fed to the process). Complete recovery of the sodium sulfonate product is difficult due to its relatively high solubility in water. Exhaustive recovery procedures were not practiced in the examples and likely all reported yields are lower than actual yields. The procedure was repeated with 3 grams of the sodium sulfonate salt and 20 grams of NaOH. The yellow oil collected (1.3 grams) analyzed by GLC to be mostly (about 85%) the desired β-Phellandrene product.[57)]

3.3 Preparation of *myoga* juice

Fresh flower buds of *myoga* (100 g) were purchased at a local market. They were cut into suitable segments and were stirred for five minutes using a homogenizer (Polytron, Kinematica GmbH, Switzeraland) on ice. The homogenized solution was filtered through a double layer of gauze. The filtered solution was used as *myoga* juice in the present study. We did not investigate agrichemical residues on the *myoga* buds, because the cultivators did not use the chemicals to prevent food contamination in the

harvest season.

3.4 Gas chromatography analyses

One milliliter of the homogenized *myoga* juice was put into headspace vials (20 ml) which were sealed and warmed to 37℃.Then, 1 ml gas produced from the vials was analyzed by GC (SHIMADZU GC-2010, Kyoto, Japan). GC was performed using a 50 m × 0.2 mm fused silica column (14%--Cyanopropylphemyl—methylpolysiloxame column; code: CBP10-M25-025, SHIMADZU, Kyoto, Japan) under the following conditions: injector and detector temperature of 250℃, oven temperature program 60℃ held for 1 min, then progressively raised to 115℃ at 2.5℃/min, and to210℃ at 10□/min, then held for 30 min. Identification of peaks was made by retention indices.

3.5 Testing materials

FINN CHAMBERS for epicutaneous testing were purchased from EPITTEST Ltd. Oy (Tuusla, Finland). The adhesive plasters for the patch tests were purchased from Torii Pharmaceutical Co. Ltd. (Tokyo, Japan).

Adhesive plasters, YUTOKUBAN, overlapping impermeable plastic adhesive tape for sealing the abdomen were purchased from YUTOKU Pharmaceutical IND Co. Ltd (Saga, Japan).

3.6 The acute dermal irritation assay

Prior to the sensitizing study, the threshold of primary irritation was evaluated on unsensitized female guinea-pigs by the patch test using FINN CHAMBERS. All samples were diluted by vehicle to 4 concentrations: 100%, 20%, 4% and 0.8%. Aliquots of 20 $\mu\ell$ of different concentrations of *myoga* juice and test chemicals were applied to the abdominal skin of the animals for 24 hours. The erythema on the skin was observed to find the minimum criterion of the primary irritation.

3.7 GPMT[58)]

According to the method of Magnusson and Klingman six guinea-pigs were used for each sensitization group. Induction and challenge concentrations were first determined for every compound as follows: the smallest concentrations producing a mild irritation were used for induction

and the maximal non-irritating concentration was used for challenge. For induction, 6 intradermal injections of 0.1 ml of compound and Freund's adjuvant were administered in the shaved scapular region. After 7 days, an occluded patch of 0.15 ml of compound was placed on the injection site for 48 h. In the control guinea pigs, the compound was replaced by the solvent. After 14 days, all the guinea pigs (including controls) were exposed to a challenge dose on the shaved flank for 24 h. Skin reactions were observed and scored according to a grading scale for the evaluation of challenge patch test reactions.[58)]

Chapter 4 Results

The major volatile constituents of *myoga* were analyzed by GC (Fig. 8). They included α-pinene, β-pinene and R-(+)-limonene. The retention times of the three major peaks for *myoga* juice were 2.33 min, 2.77 min and 3.36 min. Standard samples of α-pinene, β-pinene and R-(+)-limonene were found to elute at 2.37, 2.80 and 3.28 min. The results of retention time were comparable, allowing identification of the three major compounds.

The results of the acute dermal irritation assay in the guinea-pig are summarized in Table 1, and the skin erythema found on guinea-pigs in the acute dermal irritation assay is show in Fig. 9. α-Pinene, β-pinene, R-(+)-limonene, limonene oxide and *myoga* juice at concentrations of 0.8% did not provoke any erythema on the abdominal skin of the guinea-pigs. Limonene oxide and *myoga* juice at concentrations of 4% did not show erythema but α-pinene, β-pinene and R-(+)-limonene at the concentrations of 4%, 20% and 100%, limonene oxide at concentrations of 20% and 100%, and *myoga* juice at the concentration of 100% caused erythema on the abdominal skin (Table 1).

An intradermal induction concentration of R-(+)-limonene at 4%, limonene oxide at 20% and *myoga* juice at 100% and a topical induction dose of R-(+)-limonene at 0.8%, limonene oxide at 4% and *myoga* juice at 20% were selected as the maximum tolerable doses for each step of the GPMT (Table 1). The onset of allergenicity was observed at the challenge treatment sites on the guinea-pigs' abdomens sensitized by 0.8% R-(+)-limonene, 4% limonene oxide and 20% *myoga* juice on their dorsal skin. The results of GPMT for α-pinene, β-pinene, R-(+)-limonene, limonene oxide and *myoga* juice are summarized in Table 2. The results of GPMT for α-pinene and β-pinene were negative. Erythema (Fig.10) clearly developed at the abdominal site when the applied materials were diluted with DMSO. In contrast, the control animals exposed to the DMSO did not develop any erythema

Allergenicity rating was made after 48 hours on the challenge sites of the guinea-pigs abdomens applied with the 0.8% R-(+)-limonene, 4% limonene oxide or 20% *myoga* juice prepared in compliance with GPMT (Table 3). R-(+)-limonene induced moderate a reaction. Limonene oxide induced extreme reactions. *Myoga* juice induced a mild reaction.

Chapter 5 Discussion

Agricultural workers are exposed to large quantities and various kinds of plants. There have been many reports of cases of agricultural workers developing health effects due to plants [59,60,61]. Among the effects, respiratory and skin allergies, especially allergic contact dermatitis, are the most serious problems because of the large number of suffering patients and difficulty in conducting appropriate preventive control activities. It is necessary to improve the quality of life of agricultural workers by decreasing the incidence and severity of allergic contact dermatitis. So it is important to analyze the chemical components of plants and to evaluate the sensitization potencies of chemicals by sensitization testing.

GC is a technique used to identify of volatile liquid samples[62]. The Guinea-pig maximization test (GPMT) is a standard method for the identification of contact sensitizers [63]. Its purpose is to establish whether a chemical is a skin sensitizer using a single dose equal to the tolerable maximum dose through induction and challenge. Accordingly, the result is qualitative and is not suitable for quantitative assessment of allergenic

potency [58]. The GPMT is a valuable tool for the measurement of intrinsic sensitization potential, as well as relative sensitization potency, and is a conventional method recommended by the European Community for dangerous substances and preparations, as well as for predicting any delayed contact hypersensitivity induced by the test substances according to the Organization for Economic Cooperation and Development (OECD) Guideline for Testing of Chemicals [64]. The purpose of this study was to assess the allergenicity of *myoga* and it is major chemical components by GPMT.

We confirmed primary irritant dermatitis due to *myoga* juice and its volatile constituents in guinea pigs (Table 1). This suggests that contact dermatitis due to *myoga* in humans is partially primary irritant dermatitis. Delayed-type allergic contact dermatitis [65] due to *myoga*, its compounds and an oxidized substance of one of the compounds were also confirmed by GPMT. The results also suggest that the contact dermatitis caused by *myoga* is a combination of primary irritant dermatitis and delayed-type sensitized allergic contact dermatitis.

In our study, the major volatile components of *myoga* were α-pinene,

β-pinene and R-(+)-limonene. α-Pinene is a monoterpene and is a derivative of turpentine, an oleoresin that is exuded from many species of pine trees. It is widely distributed, and is one of the commonest constituents of essential oils from leaves, fruits, seeds, barks and woods of many plants[66]. Dharmagunardena *et.al* reported that α-pinene is a key allergen and appears to be a low-level potential sensitizer[63]. In our study, however, α-pinene was negative in the GPMT. R-(+)-limonene is the main constituent of oil from several citrus fruits, and is also found in caraway, dill and celery. R-(+)-limonene is ubiquitous allergen in our environment. It is a fragrant material not only used in fine fragrances but also most often incorporated in domestic and occupational products [67]. It is also used as a solvent, an insecticide and a flavoring agent. This was the first substance classified with R-43 in Europe as a skin sensitizer [68]. Experimental studies on the sensitizing potential of limonene both in humans and in animals have been reported, but the results are contradictory [69,70,71]. Our study showed that R-(+)-limonene induced a moderate reaction in the GPMT. This different from other studies and the difference may be due to interspecies variation and /or to sensitization to impurities such as

oxidation products and not to R-(+)-limonene itself.

We tried to detect limonene oxide in the *myoga* juice, but we did not detect a peak for limonene oxide in GC. The analytical conditions of the present study were not suitable for detecting limonene oxide in *myoga* juice as well as pure limonene oxide. Oxidation products of R-(+)-limonene, identified as potent allergens, were found after prolonged air exposure of R-(+)-limonene[68]. In a recent study [72], the sensitizing potential of R-(+)-limonene itself was shown to be very low. However, the sensitizing capacity was highly increased after exposure to air for 8 weeks at room temperature. R-(+)-limonene oxidizes readily in air and forms products with strong allergenic activity. Amongst the oxidation compounds, the major allergens identified are R-(+)-limonene hydroperoxides, limonene oxide and carvone. Matura *et al.* have already reported clinical data showing R-(+)-limonene to be a skin sensitizer in humans[68]. According to our data, the major fragrance compounds of *myoga* juice are α-pinene, β-pinene and R-(+)-limonene. α-Pinene, β-pinene and R-(+)-limonene at concentrations of 4%, 20%, 100%, limonene oxide at concentrations of 20%, 100% and *myoga* juice at a concentration of 100% all showed

positive reactions in the challenge test. Based on data from GPMT, R-(+)-limonene induced a moderate reaction, limonene oxide induced an extreme reaction and *myoga* juice induced a reaction of mild intensity. Therefore, the results of the present study suggest that R-(+)-limonene is the most important allergen amongst the fragrant components of *myoga*. Although, R-(+)-limonene showed only a moderate reaction, it easily forms allergenic products due to autoxidation during handing and storage. In the harvest season every year, if there is no protection when *myoga* cultivators pick the *myoga*, it is possible for the skin to be in contact with the fragrant components of *myoga* for a long time, especially with the irradiation of the ultraviolet. Limonene, which is one of the fragrant compounds of *myoga*, is easily oxidated in air and produces new chemicals such as limonene oxide, which would stimulate the skin and cause dermatitis. We think this is one of the reasons that the cultivators experience contact dermatitis. Of course it should be kept in mind that the actual risk for humans to develop an allergy depends on many factors. Besides the concentration, the frequency, duration of exposure and the condition of the human skin are important factors. Our data were derived from animals and further validation based

on human testing needs to be researched. In addition, GPMT is used to identify whether a chemical substance is allergenic or not, but cannot identify the degree of allergenicity, therefore, we need to carry out quantitive study about the degree of allergenic potency of *myoga* fragrances.

Part II Quantitative study

Chapter 1 Introduction

The local lymph node assay (LLNA) is recognized as a stand-alone method for determining the skin sensitizing potential of chemicals. It has been incorporated into official test guidelines, among those published by the Organization of Economic Cooperation and Development (OECD, 2002)[73], and by the United States Environment Protection Agency (US-EPA, 2003)[74]. The method identifies chemicals that have skin sensitizing potential as a function of their ability to stimulate lymphocyte proliferative responses in lymph nodes draining the site of repeated topical applications; chemicals inducing a 3-fold or greater stimulation index (SI) being classified as contact allergens[75,76,77]

Chemical allergens must conjugate, either directly or indirectly, with free or cell-associated protein to form an immunogenic complex capable of stimulating a T lymphocyte response. Epidermal Langerhans cells (LC) play a pivotal role in transporting antigen from the skin to draining lymph nodes, where cutaneous immune responses are initiated. In response to topical encounter with a contact allergen (or other events in the skin), LC

are mobilized and move from the epidermis, via the afferent lymphatics, to skin-draining lymph nodes where they localize within the paracortical (T lymphocyte) regions. While in transit from the skin, LC is subject to a functional maturation such that they are able in the lymph nodes to present transported antigen effectively to responsive T lymphocytes. The activation of LC during skin sensitization, and their mobilization, migration, and accumulation and localization within peripheral lymph nodes are processes tightly controlled by chemokines, epidermal cytokines and other factors [78,79,80,81,82]. The end result is that the chemical allergen is presented in an appropriate form to T lymphocytes in the draining lymph node. Responsive cells are activated and induced to divide and differentiate; cell division resulting in selective clonal expansion and a quantitative increase in the frequency of T lymphocytes able to recognize and respond to the inducing antigen. It is this increase in the number of specific T lymphocytes that represents the cellular basis of immunological memory and is the seminal event in the acquisition of skin sensitization. From first principles, and on the basis of experimental observation [83], it can be concluded that the magnitude of this response correlates with the extent to which sensitization

is established. It is the induction of lymphocyte proliferative responses that forms the basis of the LLNA.

Chapter 2 Materials and Methodology

2.1 Chemicals

α-pinene, β-pinene, R-(+)-limonene and limonene oxide were obtained from Kandon (Japan). Freund's complete adjuvant was purchased from Sigma (St. Louis, USA). All the reagents used were of the highest grade available. Working concentrations of all chemicals were prepared immediately prior to dosing. Acetone: olive oil (AOO v/v 4:1) was used as the vehicle for all test compounds. All compounds were completely soluble in the appropriate vehicle at all concentrations tested.

2.2 Animals

Young adult (8-12 weeks old) CBA/N strain female mice (Japan SLC, Inc). Since the susceptibility is not influenced by gender and females are easier to treat, only female were used in the present study. The animals were kept at a constant temperature (25±5□), relative humidity (50-70%), and a 12/12-hour dark/light schedule. The animals received care in compliance with the Guide for the Care and Use of Laboratory Animal

Research Center, Kumamoto University School of Medicine. All animals had access to Certified Rodent Diet (GB-1, hunebasi. Co .Ltd, Japan) and water were available ad libitum.

2.3 Procedure of LLNA

The LLNA was performed according to the standard protocol described previously (Fig.11) [73]. Groups of male CBA/N strain mice (n = 4) were exposed topically on the dorsum of both ears to 25 ml of various concentrations of chemical, or to the same volume of vehicle alone, daily for 3 consecutive days. 5 days after the initiation of exposure, all mice were injected intravenously via the tail vein with 20 mCi of [3H]methyl thymidine (3H-TdR; specific activity 2 Ci/mmol; Amersham International, Amersham, UK) in 250 ml of phosphate-buffered saline (PBS). 5 hr later, mice were killed, and the draining auricular lymph nodes were excised and pooled for each experimental group. A single-cell suspension of LNCs was prepared by gentle mechanical disaggregation through 200-mesh stainless-steel gauze. Cells were washed twice with an excess of PBS and precipitated in 5% trichloroacetic acid (TCA) at 4_C. Approximately 12 hr

later, pellets were resuspended in 1 ml of 5% TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Hisafe3'; Wallac, Turku, Finland). Incorporation of 3HTdR was measured by b-scintillation counting as disintegrations per minute (dpm) per node for each experimental group. In each case, a stimulation index (SI) relative to the concurrent vehicle-treated control value was derived.

2.4 Mathematical analysis

The estimated concentration of chemical required to induce an SI of 3 relative to concurrent vehicle-treated controls (EC3) value, was derived by linear interpolation of dose–response data as described previously (17). The EC3 value was calculated by interpolating between 2 points on the SI axis, 1 immediately above, and the other immediately below, the SI value of 3. The vehicle-treated control (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the co-ordinates (a, b) and (c, d), respectively, then the EC3 value may be calculated using the following equation:

$$EC3 = c + [(3 - d)/(b - d)](a - c)$$

Chapter 3 Results

The results were summarized in Table 4. Details of the chemicals, test concentrations and vehicles used are displayed in Table 4. The results obtained are shown as the incorporation of 3HTdR into the lymph nodes draining the site of topical exposure (dpm/node) and summarized in detail in Table 4. They are also displayed in the form of stimulation indices (SI) (Table 4). Where possible the estimated concentration required to induce a threefold increase in proliferation compared with concurrent vehicle-treated controls (EC3 value) was derived by linear interpolation for each compound and these are shown in Table 4.

Little variation was observed in the levels of thymidine incorporation in LNC derived from AOO vehicle-treated animals with levels extending over a relatively narrow range from 37dpm/node to 146dpm/node (Table 4). These data demonstrate a high degree of inter-experimental consistency between the local lymph node assays conducted during these investigations and those reported previously[84)]. A dose-dependent induction of LNC proliferation was observed in response to β-phellandrene with very vigorous local lymph node assay responses being provoked at test

concentrations of 1% or greater. Levels of thymidine incorporation of 353.5 and 655 dpm/node and corresponding stimulation indices of 4.71 and 8.73 were achieved at 1 and 5%, respectively. For Limonene oxide at test concentration of 25%. Level of thymidine incorporation of 950 dpm/node and corresponding SI was 7.85. Although a dose-dependent induction of LNC proliferation was observed in response to limonene, proliferative responses were substantially weaker when compared with those described previously for β-phellandrene and limonene oxide. Topical exposure of mice to limonene gave a positive response at the highest concentration (100%) only, with a SI of 7.09 being recorded.

Both α-pinene and β-pinene failed at all test concentrations to induce a positive response in the LLNA with relatively low levels of thymidine incorporation being recorded at all concentrations tested. The maximal SI for α-pinene and β-pinene were 2.59 and 2.55 at the highest concentration (100%) in each case.

Finally, the estimated concentration of chemical required to induce a threefold increase in proliferation compared with concurrent vehicle-treated controls (EC3 value) was derived by linear interpolation (Table 4). EC3

values were calculated for the three materials which provoked positive responses in the local lymph node assay. These were 0.54% for β-phellandrene, 8.22% for limonene oxide and 10.15% for HCA and 35.8% for limonene. For the remaining two chemicals, α-pinene and β-pinene, which failed to stimulate a positive response at any concentration tested, EC3 values of >100%, respectively, were assigned, according to the maximum concentrations tested in each case.

Chapter 4 Discussion

The accurate identification of skin-sensitization hazard is a necessary first step in the overall toxicological evaluation of likely human health risks. For the development of effective risk assessments, it is necessary (in addition to a consideration of likely conditions of exposure) to appreciate relative potency. In the case of contact allergy this may be best defined operationally as the amount of chemical required to induce skin sensitization in a previously unsensitized subject. The importance of this is best illustrated by the observations that contact allergens appear to differ by four or more orders of magnitude with respect to their relative ability to cause the acquisition of skin sensitization[85,86,87]. For this purpose, a standard LLNA was performed with the test chemical of the major volatile components of *myoga* in AOO vehicle. The murine local lymph node assay (LLNA) was originally developed as an alternative to the guinea pig prediction tests for the sensitization potential of chemicals (Basketter and Scholes, 1992; Basketter *et al.*, 1996; Kimber *et al.*, 1994), such as the Guinea-pig maximization test (Magnusson and Kligman, 1969)

and the occlusive patch test of Buehler (Buehler, 1995). The LLNA has the advantages of requiring a short test period and a low operation cost, contributing to animal welfare, and providing a quantitative endpoint (Kimber, 2002). Recently, the standard LLNA using radioisotopes has been recognized as a stand-alone sensitization test and has been incorporated into the official test guidelines published by the United States Environmental Protection Agency (US-EPA, 2003) and the Organization for Economic Cooperation and Development (OECD, 2002). Furthermore, OECD test guideline No. 429 mentions that other endpoints for assessing proliferation in the LLNA may be employed, provided there is justification and appropriate scientific support, including full citations and description of the methodology

Initial experiments were conducted to determine that the major volatile components of *myoga* were α-pinene, β-pinene and R-(+)-limonene. Kurobayashi et al[3)] reported β-phellandrene is one of the major volatile components of *myoga*. So we evaluate the allergenicity of these chemical to find the reason that *myoga* provoke ACD. There is an increasing appreciation that the inherent skin-sensitization potential of chemicals is

dependent upon a variety of factors, included among which are the ability to gain access to the viable epidermis across the stratum corneum, to provoke the expression or increased expression of skin cytokines that are required for the initiation of a cutaneous immune response, to form stable associations with proteins, and to trigger the activation of specific T lymphocytes in regional lymph nodes [88,89,90]. Collectively, these biological processes permit the delivery of the allergenic stimulus in an immunogenic form to regional (skin draining) lymph nodes where specific T lymphocytes become activated and are induced to divide and differentiate. It is the proliferation of LNC within draining lymph nodes that results in the selective clonal expansion of allergen- specific T lymphocytes. This is the cellular basis for skin sensitization and the acquisition of immunological memory, with the expanded pool of allergen-reactive T lymphocytes permitting accelerated and more aggressive responses to be provoked following subsequent encounter with the same contact allergen. There is therefore a good reason, from a theoretical perspective, and from experimental evidence, to believe that the vigour of lymphocyte proliferative responses induced in draining lymph nodes by topical

exposure to chemical allergens provides an appropriate basis on which to base judgements of relative potency, and this is the approach employed in estimating potency from derived EC3 values using the LLNA. Information available to date indicates that such EC3 values correlate closely with what is known from clinical experience of the relative skin-sensitizing potency of contact allergens among human populations, and one view is that this represents the most reliable approach to estimating relative potency. It is this strategy that we have adopted here to examine in detail the skin-sensitizing potency of the major volatile components of *myoga*.

The standard LLNA use the EC3 value as a parameter for evaluating the sensitization potency of chemical. α-Pinene and β-pinene is a monoterpene and is a derivative of turpentine, an oleoresin that is exuded from many species of pine trees. It is widely distributed, and is one of the commonest constituents of essential oils from leaves, fruits, seeds, barks and woods of many plants[66]. Dharmagunardena *et.al* reported that α-pinene is a key allergen and appears to be a low-level potential sensitizer[63]. In our study, however, α-pinene and β-pinene was negative in the LLNA, either fail to induce positive responses in the LLNA. The EC3 values for α-pinene

and β-pinene was more than 100% (Fig12, Fig13).

R-(+)-limonene is ubiquitous allergen in our environment. It is a fragrant material not only used in fine fragrances but also most often incorporated in domestic and occupational products [67]. It is also used as a solvent, an insecticide and a flavoring agent. This was the first substance classified with R-43 in Europe as a skin sensitizer [68]. Experimental studies on the sensitizing potential of limonene both in humans and in animals have been reported, but the results are contradictory [69,70,71]. In our experiment, the EC3 values for limonene was reported to be 35.8%, provoke moderate responses in the standard LLNA (Fig14). Oxidation products of R-(+)-limonene, identified as potent allergens, were found after prolonged air exposure of R-(+)-limonene[68]. R-(+)-limonene oxidizes readily in air and forms products with strong allergenic activity. Amongst the oxidation compounds, the major allergens identified are R-(+)-limonene hydroperoxides, limonene oxide and carvone. According to our datas, limonene oxide was induces strong responses and EC3 values was 8.22% (Fig15). α-Phellandrene is a constituent of the essential oils of *Eucalyptus dives* and of *Eucalyptus phellandra* (hence the name). Phellandrene

($C_{10}H_{16}$) (Fig 6) is the name for a pair of organic compounds that have a similar molecular structure and similar chemical properties. β-Phellandrene has been isolated from the oil of water fennel and Canada balsam oil. The phellandrenes are used in fragrances because of their pleasing aromas. The odor of β-phellandrene has been described as peppery-minty and slightly citrusy. Some of the biological activities of β-Phellandrene have been studied in recent years[57], but no toxicity study on β-Phellandrene has been reported to our knowledge. The results of this experiment showed that β-Phellandrene induces extreme responses in the LLNA, and the EC3 values reported to be 0.54% (Fig16). HCA was examined in the experiments as a control test chemical. It is induces a mild responses in the LLNA and EC3 values was 10.86% (Fig17).

Although it must be acknowledged that this is a far from extensive or exhaustive analysis, the results nevertheless serve to emphasize that the relationships between the test chemical of the major volatile components of *myoga* and ACD of the mioga cultivators. According to our data, R-(+)-limonene, limonene oxide and β-Phellandrene were determined to sensitization potency for skin of mice. Although, R-(+)-limonene showed

only a moderate reaction, it easily forms allergenic products due to autoxidation during handing and storage. In the harvest season every year, if there is no protection when *myoga* cultivators pick the *myoga*, it is possible for the skin to be in contact with the fragrant components of *myoga* for a long time, especially with the irradiation of the ultraviolet. Limonene, which is one of the fragrant compounds of *myoga*, is easily oxidated in air and produces new chemicals such as limonene oxide, which would stimulate the skin and cause dermatitis. We think this is one of the reasons that the cultivators experience contact dermatitis. Of course it should be kept in mind that the actual risk for humans to develop an allergy depends on many factors. Besides the concentration, the frequency, duration of exposure and the condition of the human skin are important factors. Our data were derived from animals and further validation based on human testing needs to be researched. The assays reported here indicate that the LLNA may be of some value in investigating the overall sensitizing capacity of chemical of the major volatile components of *myoga* . As suggested, further work is necessary to determine the relevance of such data to the human situation. Additional studies in humans on

essential oil of *myoga* are needed to a refine for induction and aid in future risk assessment.

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Figure.1 *Myoga* flower buds

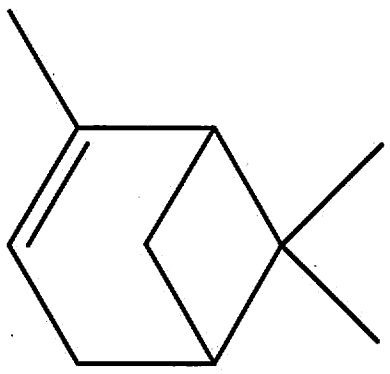

Figure.2 α-pinene
(2,6,6-trimethylbicyclo[3.1.1]hept-2-ene)

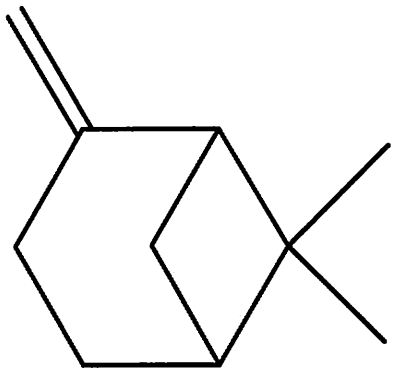

Figure.3 β-pinene
(6,6-dimethyl-2-methylene-bicyclo[3.1.1]heptane)

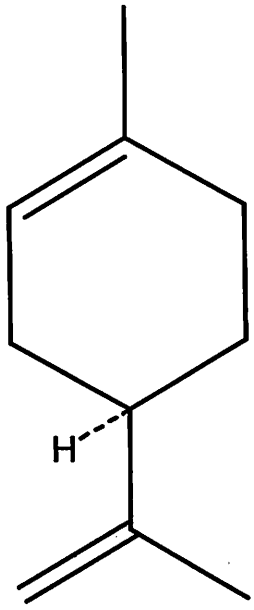

Figure.4. R-(+)-limonene
(Methyl-4-isopropenyl cyclohexene)

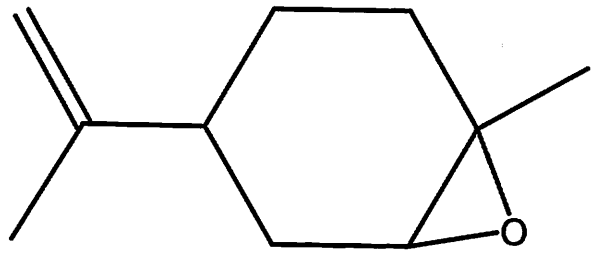

Figure.5 limonene oxide
(1,2-epoxy-p-menth-8-ene)

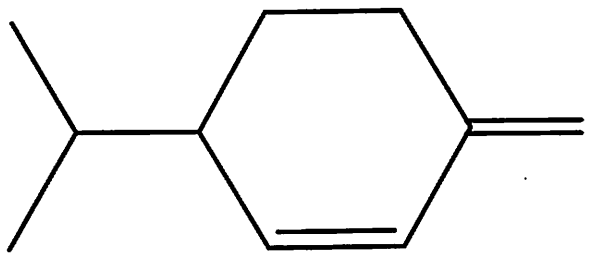

Figure.6 β-phellandrene
(4-Isopropyl-1-methylene-2-cyclohexene)

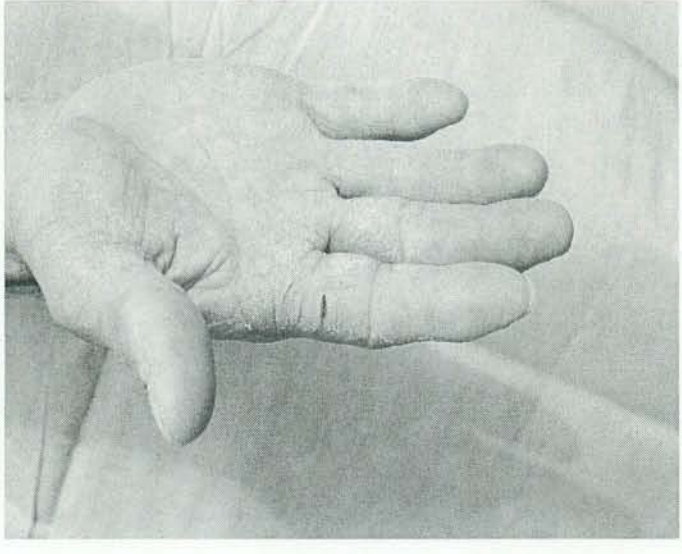

Figure 7. Symptoms on the hand of a *myoga* cultivator

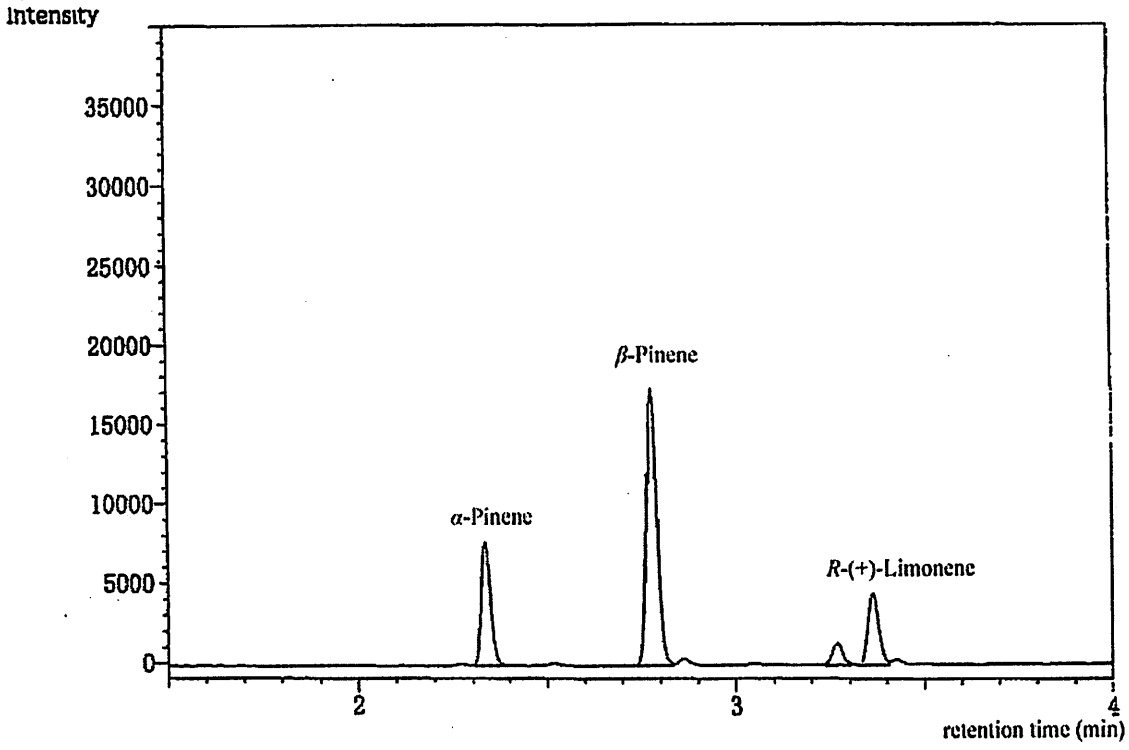

Figure8. Gas chromatogram of fragrant components of *myoga*

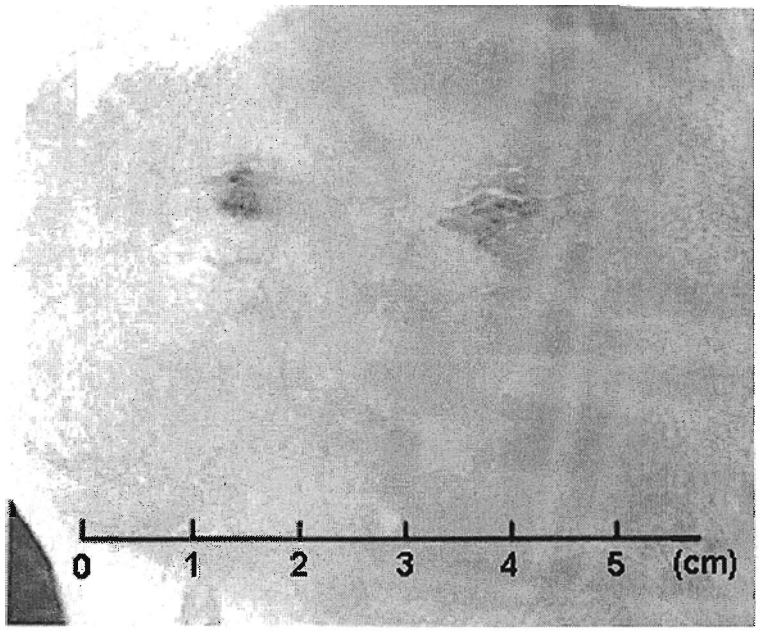

Figure.9 Skin erythema on a guinea-pig in the acute dermal irritation assay (sample: limonene oxide)

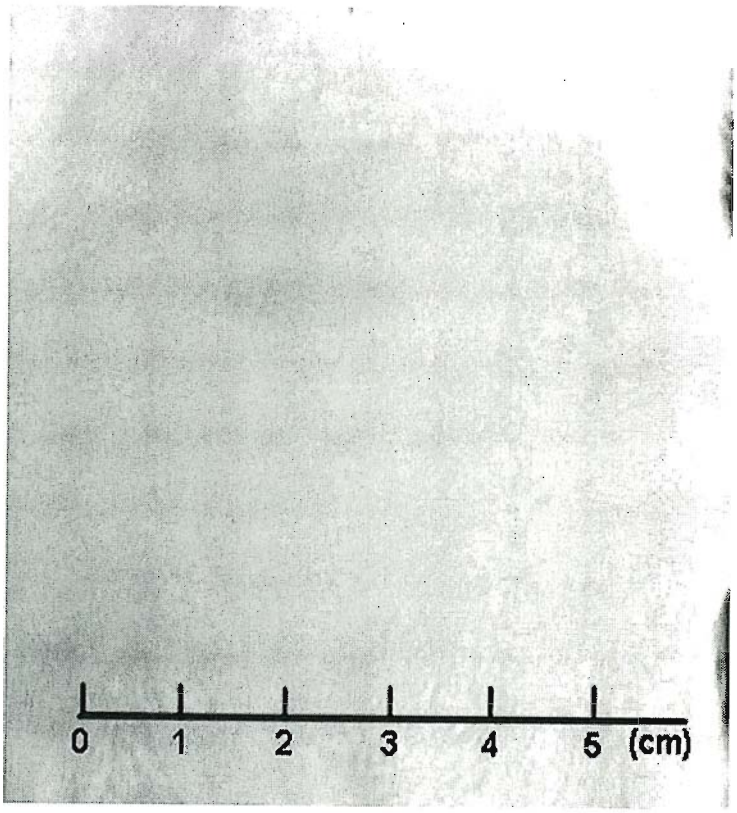

Figure10. Typical skin erythema on a guinea-pig in GPMT challenged by limonene oxide

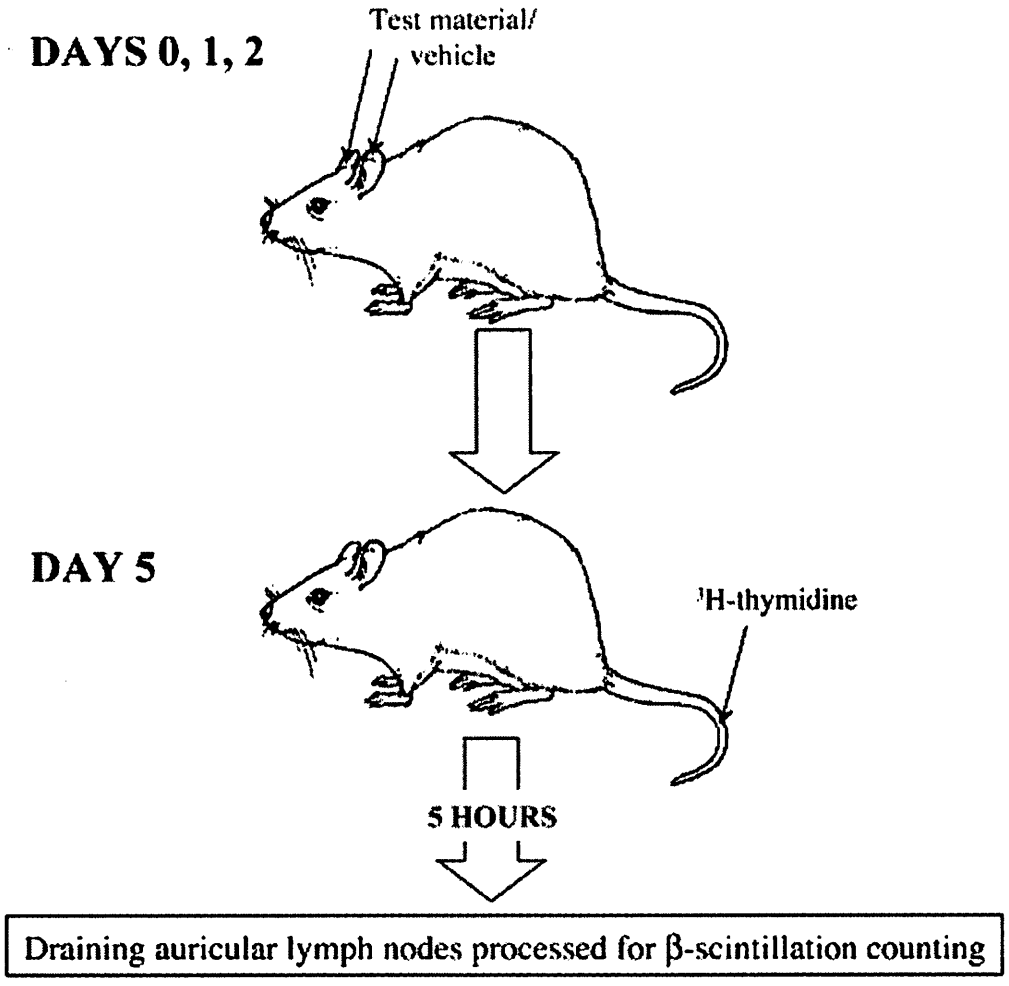

Fig11. Standard local lymph node assay. A scheme of the standard local lymph node assay is shown. Mice (CBA strain) receive 3 consecutive daily applications of 25 μL of various concentrations of the test material, or of the relevant vehicle alone, to the dorsum of both ears. 5 days after the initiation of exposure, mice receive an intravenous injection, via the tail vein, of 20 μCi of ^{3}H-thymidine in 250 μL of phosphate-buffered saline. 5 h later, draining auricular lymph nodes are excised and pooled for each experimental group, or on an experimental animal basis, and are processed for β-scintillation counting.

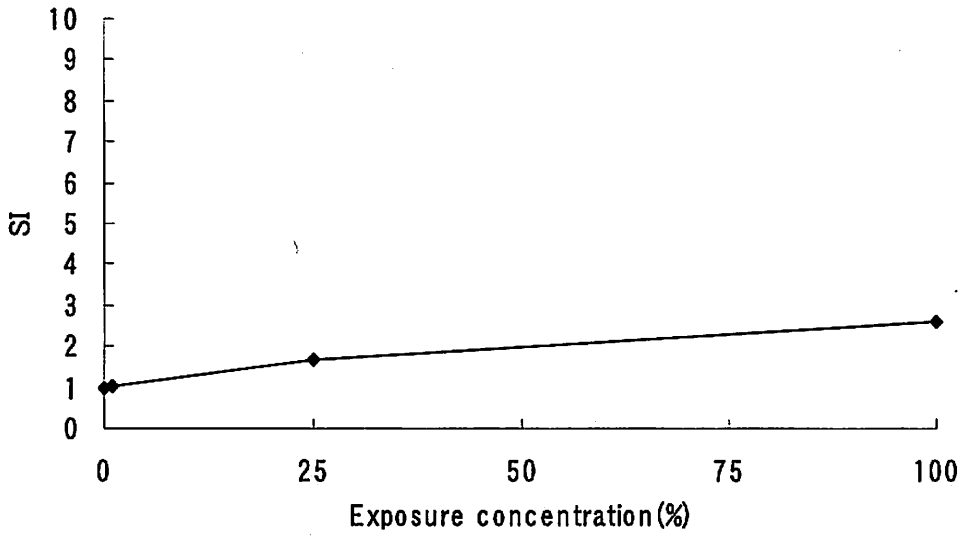

Figure12. LLNA dose-responses to α-pinene

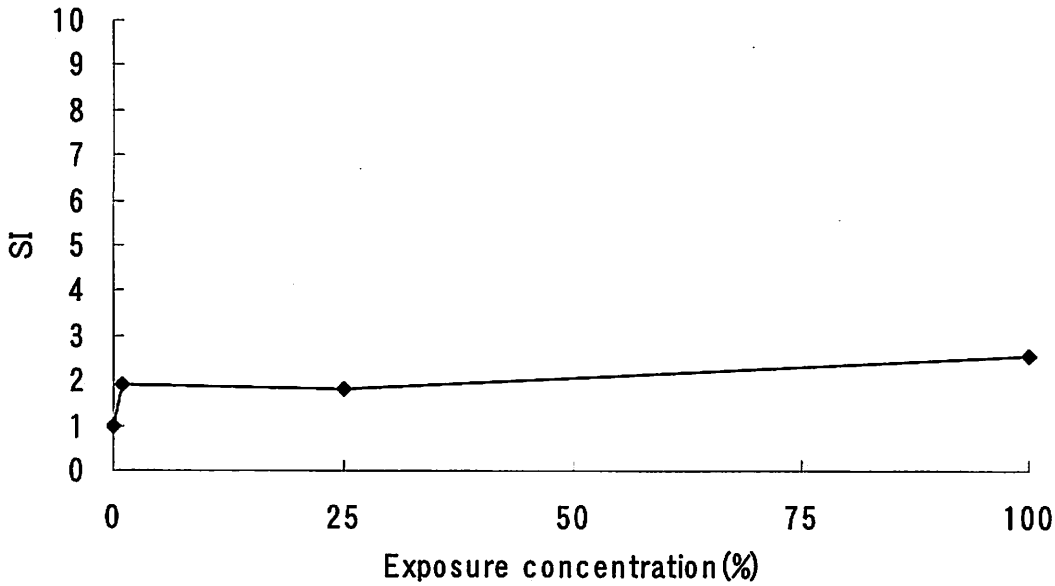

Figure13. LLNA dose-responses to β-pinene

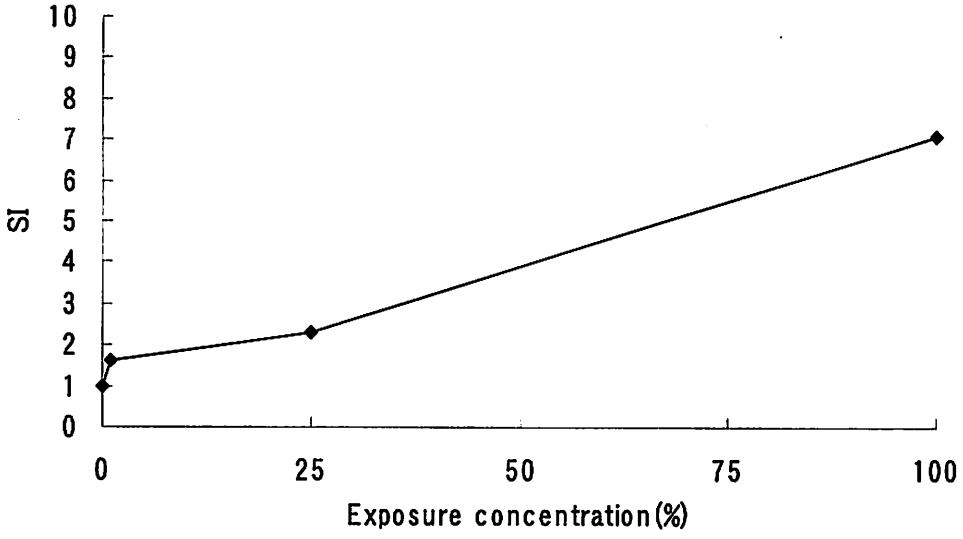

Figure14. LLNA dose-responses to R-(+)-limonene

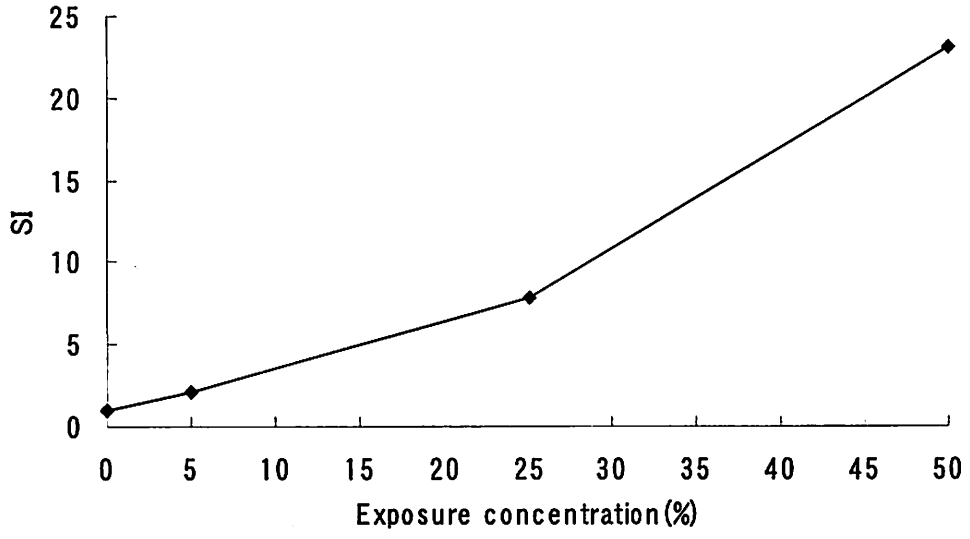

Figure15. LLNA dose-responses to limonene oxide

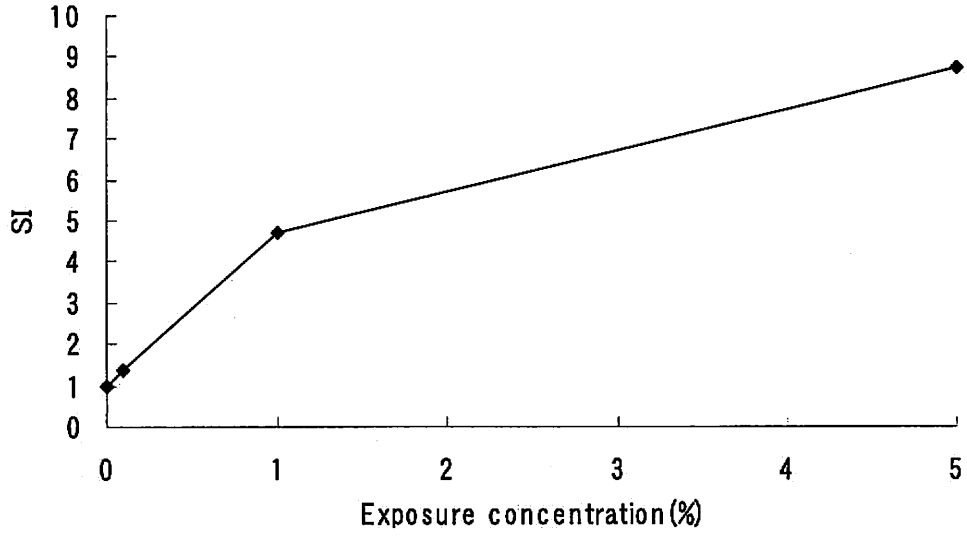

Figure16. LLNA dose-responses to β-phellandrene

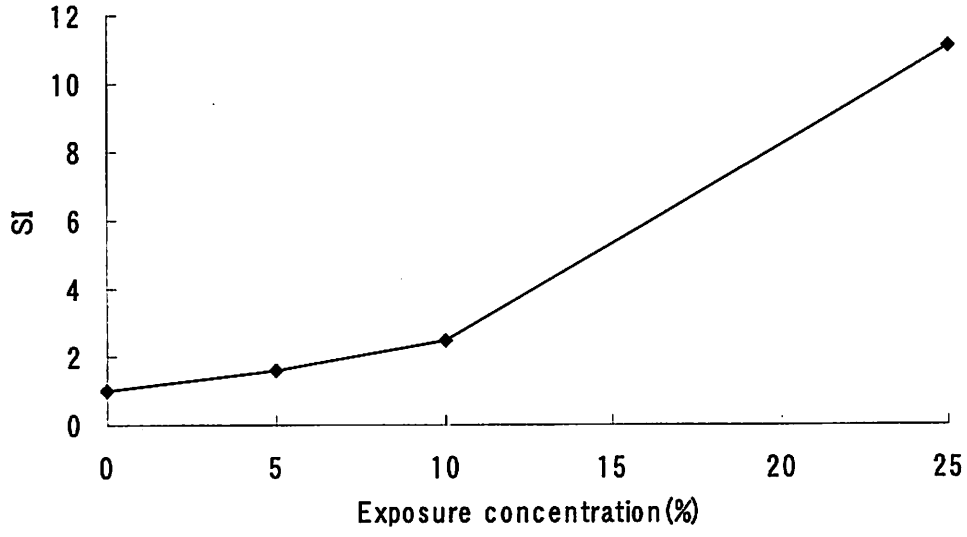

Figure17. LLNA dose-responses to HCA

Table1. The acute dermal irritation assay by α-pinene, β-pinene, R-(+)-limonene, limonene oxide and *myoga* in guinea-pig

Material	Concentration	Guinea-pig; 24hr*
α-pinene	0.8%**	0/6***
	4%	1/6
	20%	6/6
	100%	6/6
β-pinene	0.8%	0/6
	4%	5/6
	20%	6/6
	100%	6/6
R-(+)-limonene	0.8%	0/6
	4%	4/6
	20%	6/6
	100%	5/6
Limonene oxide	0.8%	0/6
	4%	0/6
	20%	6/6
	100%	6/6
Myoga juice	0.8%	0/6
	4%	0/6
	20%	0/6
	100%	4/6
Dimethyl sulfoxide	100%	0/6

* Guinea-pigs were exposed to the materials on their abdomens, then evaluated after 24 hours application.

** The applied materials were diluted with dimethyl sulfoxide

*** Denominator: number of guinea pigs used; numerator: number of guinea pigs developing erythema on their abdomens at 24 hours after application.

Table 2. Allergenicity of α-pinene, β-pinene, R-(+)-limonene, limonene oxide and *myoga* in the GPMT*

Induction material**	Challenge material**	Challenge results
4% α-pinene	Dimethyl sulfoxide	0/6***
Dimethyl sulfoxide	Dimethyl sulfoxide	0/6
4% α-pinene	0.8% α-pinene	0/6
Dimethyl sulfoxide	0.8% α-pinene	0/6
4% β-pinene	Dimethyl sulfoxide	0/6
Dimethyl sulfoxide	Dimethyl sulfoxide	0/6
4% β-pinene	0.8% β-pinene	0/6
Dimethyl sulfoxide	0.8% β-pinene	0/6
4% R-(+)-limonene	Dimethyl sulfoxide	0/6
Dimethyl sulfoxide	Dimethyl sulfoxide	0/6
4% R-(+)-limonene	0.8% R-(+)-limonene	2/6
Dimethyl sulfoxide	0.8% R-(+)-limonene	0/6
20% limonene oxide	Dimethyl sulfoxide	0/6
Dimethyl sulfoxide	Dimethyl sulfoxide	0/6
20% limonene oxide	4% limonene oxide	6/6
Dimethyl sulfoxide	4% limonene oxide	0/6
100% *myoga* juice	Dimethyl sulfoxide	0/6
Dimethyl sulfoxide	Dimethyl sulfoxide	0/6
100% *myoga* juice	20% *myoga* juice	1/6
Dimethyl sulfoxide	20% *myoga* juice	0/6

* Experimental procedure according to the GPMT originally reported by Magnusson and Kligman[58)].

** The applied materials were diluted with dimethyl sulfoxide.

*** Denominator: number of guinea pigs used; numerator: number of guinea pigs developing erythema on their abdomens at 48 hours after application.

Table 3. Allergenicity rating with R-(+)-limonene, limonene oxide and *myoga*

Material	Sensitization rate	Grade*	Classification*
R-(+)-limonene	33%	□	Moderate
limonene oxide	100%	□	Extreme
myoga	17%	□	Mild

*Grading and classification were performed in compliance with the GPMT originally reported by Magnusson and Kligman [58].

Table 4 Local lymph node assay responses to test chemical

Chemical	Vehicle	Exposure concentration (%)	dpm/node	SI**	EC3(%)
α-pinene	AOO*	0	37	1	
		1	38	1.03	
		25	61	1.65	
		100	96	2.59	
β-pinene	AOO	0	89	1	
		1	168	1.89	
		25	162	1.82	
		100	227	2.55	
R-(+)-limonene	AOO	0	146	1	
		1	234	1.60	
		25	337	2.31	
		100	1035	7.09	35.80
Limonene oxide	AOO	0	121	1	
		5	251	2.07	
		25	950	7.85	
		50	2787	23.04	8.22
β-phellandrene	AOO	0	75	1	
		0.1	101	1.35	
		1	353.50	4.71	
		5	655	8.73	0.54
HCA	AOO	0	124	1	
		5	196	1.58	
		10	427.80	2.45	
		25	1373.90	11.08	10.86

*AOO: Acetone:Olive oil,4:1

**SI: the mean dpm/mouse (treatment group)÷the mean dpm/mouse (vehicle treated group)

SI>3 the test substance is regarded as a skin sensitizer