

Toxicity Study of the Volatile Constituents of *Myoga* Utilizing Acute Dermal Irritation Assays and the Guinea-Pig Maximization Test

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Abstract: Toxicity Study of the Volatile Constituents of *Myoga* Utilizing Acute Dermal Irritation Assays and the Guinea-Pig Maximization Test: Qingjun WEI, *et al.* Department of Preventive and Environmental Medicine, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University—*Myoga* is a fragrant plant which is the special product of Japan and is cultivated throughout Japan. According to our earlier investigation (unpublished data) of *myoga* cultivators in Japan, 8 of 35 cultivators experienced contact dermatitis in the harvest season.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. The volatile components of *myoga* were analyzed by gas chromatograph (GC). They included α -pinene, β -pinene and R-(+)-limonene. We performed a toxicity study of each of the major fragrant components of *myoga* using acute dermal irritation assays and the Guinea-Pig Maximization test (GPMT) in order to probe the mechanism of allergic contact dermatitis. In acute dermal irritation assays, α -pinene, β -pinene and limonene showed positive responses at concentrations of 4%; limonene oxide at 20% and *myoga* showed a positive response at concentrations 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. The results of the present study show that R-(+)-limonene is the most important allergen amongst the chemical components of *myoga*, and we consider it to be the reason why *myoga* cultivators experience allergic contact dermatitis.

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Key words: *Myoga* (*Zingiber Myoga Roscoe*), Gas chromatograph (GC), Acute dermal irritation assays, The Guinea-Pig Maximization test (GPMT), Allergic contact dermatitis

Myoga (*Zingiber Myoga Roscoe*) (Fig.1) is a perennial herb with pungently aromatic flower buds which is native to Eastern Asia¹. 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. The number of *myoga* greenhouses has increased in some areas in Japan and hand dermatitis suspected to be due to allergy to *myoga* has been recognized in *myoga* greenhouse cultivators.

Myoga belongs to the ginger family (*Zingiberaceae*). 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 dermatitis². Minamoto *et al.* firstly reported a case of contact dermatitis from *myoga*³. According to our earlier investigation (unpublished data) of the *myoga* cultivators in Japan, 8 of 35 cultivators experienced contact dermatitis in the harvest season. Typical contact dermatitis was present on the hand of cultivators (Fig. 2).

The volatile constituents of fresh flower buds of *myoga* have been studied by gas chromatography (GC)⁴. Although the volatile constituents were detected by GC, they originally existed in *myoga* juice as soluble constituents. Some of the biological activities of the principle fragrances found in *myoga* have been studied

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Fig. 1. *Myoga* flower buds.



Fig. 2. Symptoms on the hand of a *myoga* cultivator.

in recent years⁵⁾, but no toxicity study on *myoga* has been reported to our knowledge. In order to elucidate the mechanism of allergic contact dermatitis from *myoga*, we performed an aromatic component analysis of fresh flower buds of *myoga* and a toxicity study of the major volatile compounds of *myoga* using acute dermal irritation assays and the Guinea-Pig Maximization test (GPMT).

Material and Methods

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.

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, Switzerland) 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.

Gas chromatography analyses

One milliliter of the homogenized *myoga* juice was put into headspace vials (20 ml) which were sealed and warmed to 37°C. Then, 1 ml gas produced from the vials was analyzed by GC (SHIMADZU GC-2010, Kyoto, Japan). GC was performed using a 50 m $\times$ 0.2 mm fused

silica column (14%—Cyanopropylphenyl—methylpolysiloxane column; code: CBP10-M25-025, SHIMADZU, Kyoto, Japan) under the following conditions: injector and detector temperature of 250°C, oven temperature program 60°C held for 1 min, then progressively raised to 115°C at 2.5°C/min, and to 210°C at 10°C/min, then held for 30 min. Identification of peaks was made by retention indices.

Testing materials

FINN CHAMBERS for epicutaneous testing were purchased from EPITEST Ltd. Oy (Tuusula, 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).

Animals

Unsensitized female Hartley guinea-pigs weighting 300–350 g were purchased from an animal breeder, Kyudo (Kumamoto, Japan). Since the susceptibility is not influenced by gender and females are easier to treat, only females were used in the present study. The animals were kept at constant temperature ($25 \pm 5^\circ\text{C}$) and relative humidity (50–70%), on a 12/12-h 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, Funabashi. Co.Ltd, Japan) or equivalent *ad libitum*.

The acute dermal irritation assay

Prior to the sensitizing study, the threshold of primary irritation was evaluated on unsensitized female guinea-

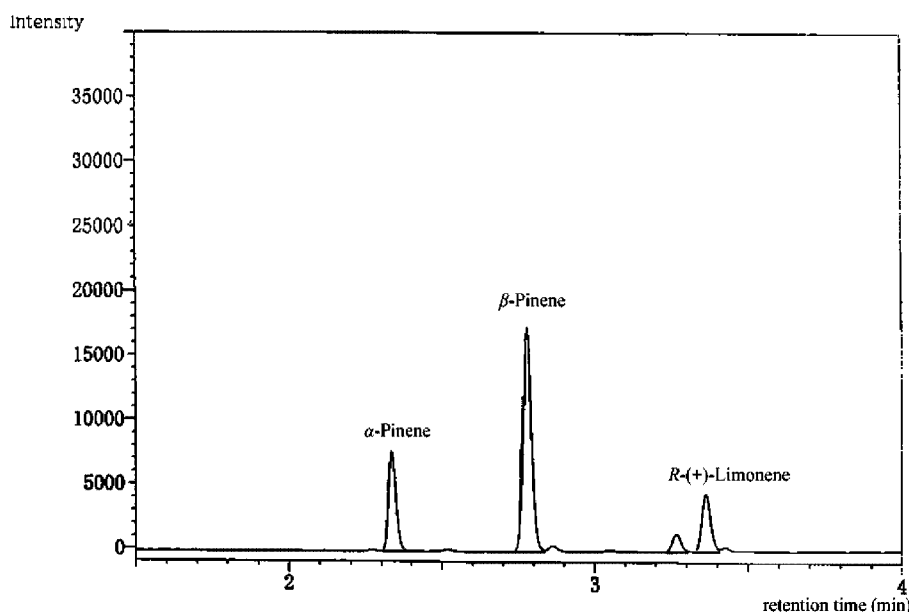


Fig. 3. Gas chromatogram of fragrant components of *myoga*.

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 μ l of different concentrations of *myoga* juice and test chemicals were applied to the abdominal skin of the animals for 24 h. The erythema on the skin was observed to find the minimum criterion of the primary irritation.

GPMT⁶⁾

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 d, 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 d, 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 reactions⁶⁾.

Results

The major volatile constituents of *myoga* were analyzed by GC (Fig. 3). 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.

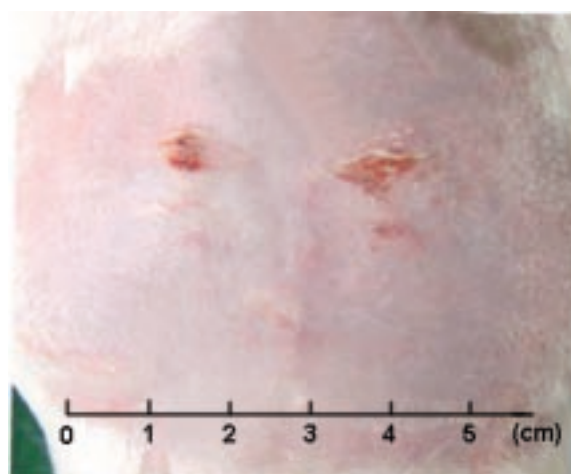
The results of the acute dermal irritation assay in the guinea-pig are summarized in Table 1, and the skin erythema found on guinea-pigs in the acute dermal irritation assay is shown in Fig. 4. α -Pinene, β -pinene, R-(+)-limonene, limonene oxide and *myoga* juice at concentrations of 0.8% did not provoke 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. 5) 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

Table 1. The acute dermal irritation assay by α -pinene, β -pinene, R-(+)-limonene, limonene oxide and *myoga* in guinea-pig

Material	Concentration	Guinea-pig; 24 h*
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
<i>Myoga</i> 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 h 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 h after application.

**Fig. 4.** Skin erythema on a guinea-pig in the acute dermal irritation assay (sample: limonene oxide).

any erythema.

Allergenicity rating was made after 48 h 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.

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 plants⁷⁻⁹). 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 samples¹⁰). The guinea pig maximization test (GPMT) is a standard method for the identification of contact sensitizers¹¹). 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 potency⁶). 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 Chemicals¹²). The purpose of this study was to assess the allergenicity of *myoga* and its major chemical components by GPMT.

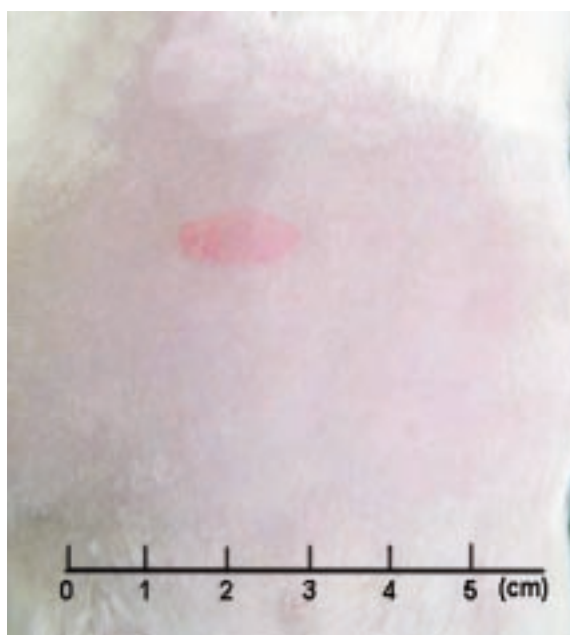
We confirmed primary irritant dermatitis due to *myoga* juice and its volatile constituents in guinea pigs (Table 1). This suggests that contact dermatitis due to *myoga* in humans is partially primary irritant dermatitis. Delayed-type allergic contact dermatitis¹³) due to *myoga*, its compounds and 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

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% <i>myoga</i> juice	Dimethyl sulfoxide	0/6
Dimethyl sulfoxide	Dimethyl sulfoxide	0/6
100% <i>myoga</i> juice	20% <i>myoga</i> juice	1/6
Dimethyl sulfoxide	20% <i>myoga</i> juice	0/6

*Experimental procedure according to the GPMT originally reported by Magnusson and Kligman⁵). **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.

**Fig. 5.** Typical skin erythema on a guinea-pig in GPMT challenged by limonene oxide.**Table 3.** Allergenicity rating with R-(+)-limonene, limonene oxide and *myoga*

Material	Sensitization rate	Grade*	Classification*
R-(+)-limonene	33%	III	Moderate
Limonene oxide	100%	V	Extreme
<i>Myoga</i>	17%	II	Mild

*Grading and classification were performed in compliance with the GPMT originally reported by Magnusson and Kligman⁵).

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 plants¹⁴). Dharmagunardena *et al.* reported that α -pinene is a key allergen and appears to be a low-level potential sensitizer¹⁰). 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 products¹⁴). 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 sensitizer¹⁶). Experimental studies on the sensitizing potential of limonene both in humans and in animals have been reported, but the results are contradictory^{17–19}). 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-(+)-limonene¹⁶). In a recent study²⁰), 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 wk 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 humans¹⁶). 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 autooxidation during handling 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 quantitative studies about the degree of allergenic potency of *myoga* fragrances.

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