Quantification of α -Methylene- γ -butyrolactone Extracted from Different Parts of *Alstroemeria wilhelmina* and Evaluation of its Antigenicity Using the Guinea-Pig Maximization Test

Koichi HARADA¹, Shoko OHMORI¹, Chang-Nian WEI², Yoshiki ARIMATSU³ and Atsushi UEDA¹

¹Department of Hygiene, Kumamoto University School of Medicine, Kumamoto

²Kumamoto Total Institute of Fitness, Kumamoto

³Ginkyo College of Medical Science, Kumamoto

Abstract

To detect the type of contact dermatitis caused due to the handling of *Alstroemeria wilhelmina*, 1% α -methylene- γ -butyrolactone (α -MBL) dissolved in physiological saline and a five-fold diluted saline solution of original extracts of flowers, leaves and stems of the flower were applied to guinea-pigs for induction treatment, and 0.1% α -MBL physiological saline and a ten-fold diluted solution of the original extracts were applied to the animals as the challenge treatment in compliance with the guinea-pig maximization test (GMT). As a consequence, not only primary irritant dermatitis was observed, but also delayed type allergic contact dermatitis due to *Alstroemeria wilhelmina* was observed. α -MBL determined in the extracts using high-performance liquid chromatography (HPLC) was found to be the biochemical material cause of the contact dermatitis. The flower region contained α -MBL in the highest concentrations compared with those of the leaves and stems. Therefore, the quantification of α -MBL in the extracts was concluded as being a useful evaluating method for contact dermatitis due to the handling of *Alstroemeria*.

Key words: guinea-pig maximization test (GMT); α -methylene- γ -butyrolactone (α -MBL); *Alstroemeria wilhelmina*; contact dermatitis; high-performance liquid chromatography (HPLC)

Introduction

Allergic contact dermatitis caused by handling *Alstroemeria* has been widely observed in farmers and florists^{1–4}. Santucci et al. found that some workers who suffered from dermatitis due to *Alstroemeria* were all patch test-positive to 6-tuliposide A and α -methylene- γ -butyrolactone (α -MBL)⁵. Hausen also reported a strong sensitizing capacity of α -MBL in sensitized guinea-pigs by open epicutaneous exposure⁶. Kawashima et al.⁷ showed that the experimental animals became sensitized to *Alstroemeria* extracts or α -MBL using the Split-adjuvant technique⁸. *Alstroemeria* have a wide range of species from the wild-type, *Alstroemeria ligutu*, to hybrid-types, such as *Alstroemeria wilhelmina*. The allergenicity of contact dermatitis in the hybrid-type, *Alstroemeria wilhelmina*, is not clear. The guinea-pig is a useful laboratory animal for the establishment of delayed-type allergic contact sensitization^{7,9}. Magnusson and Kligman developed a useful method to identify allergic contact dermatitis in guinea-pigs, known as the guinea-pig maximization test (GMT)^{9,10}, in which Freund's complete adjuvant

was required. GMT requires many guinea-pigs and takes time to deliver the results, and therefore there have been few studies using it to investigate allergic contact dermatitis due to handling *Alstroemeria*. However, confirming allergic contact dermatitis due to *Alstroemeria wilhelmina* using GMT, is thought to be one of the most reliable experimental models to detect the dermatitis.

Alstroemeria species contain some irritating or sensitizing gradients in their plant bodies, such as Tuliposide A and α -MBL. α -MBL, formed by the hydrolysis of Tuliposide A, was suggested to combine proteins and show the allergenicity, the contact dermatitis due to *Alstroemeria*⁷. Christensen and Kristiansen isolated and quantified α -MBL in *Alstroemeria* using high performance liquid chromatography (HPLC)¹¹. Van der Mei et al. determined the prevalence of hand dermatitis in workers handling *Alstroemeria* and revealed that more than half the workers were sensitized to α -MBL¹².

The aim of the present study was to detect the type of contact dermatitis caused by handling *Alstroemeria wilhelmina* using GMT. We also tried to estimate the intensity of contact dermatitis by determining the α -MBL concentrations in the extracts, prepared from *Alstroemeria wilhelmina*, using the HPLC method.

Materials and Methods

Chemicals

α -MBL and Freund's complete adjuvant were purchased

Received Mar. 22 2001/Accepted Jul. 11 2001

Reprint requests to: Koichi HARADA

Department of Hygiene, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860-0811, Japan

TEL: +81(96)373-5105, FAX: +81(96)373-5108

E-mail: haradako@gpo.kumamoto-u.ac.jp

from Sigma (St. Louis, USA). All the reagents used were of the highest grade available.

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

Animals

Unsensitized female Hartley guinea-pigs weighing 300–350 g were purchased from an animal breeder, Kuroda (Kumamoto, Japan). Since the susceptibility is not influenced by gender and the females are easier to treat¹⁰, they were chosen in the present study. The animals were kept at a constant temperature ($25\pm5^{\circ}\text{C}$), 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.

Extraction of the sensitizer from *Alstroemeria wilhelmina*

Extraction of the sensitizing constituents of *Alstroemeria wilhelmina* (Fig. 1) was performed by the method of Christensen and Kristiansen¹¹. Twenty grams of shredded fresh region material, flowers, leaves, or stems of the flower, were ground with 40 ml distilled water for 24 hours. The extracted sensitizer was concentrated to 1 ml at 37°C under reduced pressure using a rotary evaporator, prepared as an original extract solution and then stored

at -50°C before use. The original extracts were diluted with saline in an aliquot at 5-, 10-, and 20-fold dilution before applying to the animals.

The primary irritant dermatitis

Prior to the sensitizing study, the threshold of primary irritation was evaluated on unsensitized female guinea-pigs by the patch test using FINN CHAMBERS. Aliquots of 20 μl of the extracts containing the sensitizer or diluted α -MBL physiological saline 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.

GMT

Induction and challenge treatment were performed in compliance with the GMT method originally reported by Magnusson and Kligman¹⁰ as follows.

Induction procedure

Induction consisted of a two-stage operation. First, three pairs of injections were made simultaneously as the interdermal injection (A). Second, closed patch exposure was performed over the injection sites one week later as the topical application (B). The shoulder region was used as the injection site and the application site one week later.

A. Interdermal injection

A row of 3 injections, six in total, were made on each side as follows. (1) 0.1 ml (0.05 ml $\times$ 2) of the adjuvant without the test reagent. (2) 0.1 ml (0.05 ml $\times$ 2) of the test agent without adjuvant and (3) 0.1 ml (0.05 ml $\times$ 2) of the test substance emulsified in complete adjuvant.

B. Topical application

One week after the interdermal injection, the test reagent (150 μl) was applied to the Torii patch and the patch was put on the site. The patch was covered by an overlapping impermeable, plastic adhesive tape, YUTOKUBAN. This was firmly held in place by an elastic adhesive bandage wound around the torso of the animals. This was then left in place for 48 hours.

Challenge procedure

A series of fluid combinations were used. The guinea-pigs were challenged two weeks after the topical induction. The underfur was removed from an area of the abdomen by clipping and shaving as before. This patch was sealed to the abdomen for 24 hours using the FINN CHAMBERS, containing 20 μl of each fluid. Observation of the challenge reaction was as follows. The challenge site was evaluated at 24 hours after removal of the sealed patch. Three hours before the first reading in the morning, the test site was shaved with an electric razor, and the skin was gently cleaned with an excess of purified water. The readings were made indoors under luminescent light. Erythema constituted the minimum criterion of an allergic reaction and the rating of allergenicity was based on the percentage of animals.

Quantification of α -MBL in the plant extracts

α -MBL in the extracts was determined by HPLC. A Shimadzu HPLC (Shimadzu Inc., Kyoto, Japan) equipped with two liquid chromatograph pumps (LC-5A), an absorbance detector (UV-VIS

Fig. 1 *Alstroemeria wilhelmina*

SPD-2A) and an integrator (C-R1B chromatopac) were used. The UV detector was operated at 208 nm with an absorbance range of 0.16–1.28 AUFS. Chromatographic separations were carried out at 40°C in the column oven (CTO-2A) and on an analytical Shim-pack CLC-ODS (5 mm; 6.0 mm×15 cm) column at a flow rate of 1.0 ml/min. The mobile phase consisted of distilled water and was degassed before use. The recovery rate of α -MBL added into the assay system was 96.8±8.7% (mean±1 SD, n=10).

Statistical analysis

Among the three groups (three exposed subgroups) the significance was tested by a one-way analysis of variance (ANOVA); where the ANOVA showed a significance of $P<0.05$, the Ryan's multiple comparison test^{13,14} was used to identify which subgroup or group was significantly different from the other subgroup or group.

Results

Table 1 shows the thresholds for primary irritation by the diluted α -MBL and the water extracts of *Alstroemeria wilhelmina* prepared by the method of Christensen and Kristiansen¹¹. α -MBL at 0.01, 0.05, and 0.1% in physiological saline did not provoke any erythema on the abdominal skin of the guinea-pigs. The 10- and 20-fold diluted original extract solutions from the flowers and leaves also did not induce any erythema on the abdominal skin. α -MBL at 0.5 and 1.0% in physiological saline, however, showed erythema on the abdominal skin. The original extracts and the 5-fold diluted original extract solution from the flowers and leaves also showed erythema on the abdominal skin. As for the stems, this part of the plant had a similar concentration of α -MBL, and therefore the erythema was expected to show in the application of the original extracts and the 5-fold diluted original extract solution from the stems. These erythema, which developed within the first 24 hours after applying the FINN CHAMBERS, contained the

extracted materials and were attributed to the primary irritation. The animals, therefore, were sensitized by 1.0% α -MBL in physiological saline or a 5-fold diluted original extract solution to provide the secure allergenicity as described above.

Figure 2 shows the erythema on the abdominal skin of the guinea-pigs. The animals were sensitized to a 5-fold diluted extract solution from the leaves as the induction treatment and were exposed to 5-, 10-, and 20-fold diluted extract solutions from the leaves, and to physiological saline as the challenge treatment. In Figure 2x, sites a, b and c show erythema following the challenge treatment with the 5-, 10- and 20-fold diluted extract solutions, respectively. At site b, treated with the 10-fold diluted extract solution, the erythema was induced dose-dependently. No erythema was induced at site d following the challenge treatment with physiological saline. The observed erythema at site b evaluated the sensitizing reaction in the GMT in the present study. Figure 2y shows the lack of erythema on the guinea-pig abdominal skin in the control group exposed to physiological saline as the induction treatment, followed by exposure to 5-, 10-, and 20-fold diluted extract solutions and physiological saline as the challenge treatment as described above.

Table 2 shows the allergenicity in the GMT¹⁰ of the water extracts of *Alstroemeria wilhelmina*. The onset of allergenicity was observed dose-dependently at the challenge treatment sites on the guinea-pigs' abdomens sensitized by 1.0% α -MBL saline or the 5-fold diluted plant extract solution on their backskin. The erythema clearly developed at the abdominal site; site b, applied with the 10-fold diluted solution of the original extracts as the challenge treatment when compared to the erythema provoked at the site; site c, applied with the 20-fold diluted extract saline as the challenge treatment. In contrast, the control animals exposed to the physiological saline did not develop any erythema.

Maximization grading was performed after 24 hours on the challenge sites of the guinea-pigs' abdomens applied with the 0.1% α -MBL physiological saline or the 10-fold diluted extract

Table 1 Primary irritation by α -MBL and extracts of *Alstroemeria wilhelmina* in guinea-pigs

Material	Concentration	Guinea-pig; 24 hr*
α -MBL	0%**	0/6***
	0.01%	0/6
	0.05%	0/6
	0.1%	0/6
	0.5%	5/6
	1.0%	6/6
flowers	20-fold dilution**	0/6
	10-fold dilution	0/6
	5-fold dilution	1/6
	original extract	3/6
leaves	20-fold dilution**	0/6
	10-fold dilution	0/6
	5-fold dilution	2/6
	original extract	4/6
saline	physiological saline	0/6

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

** The applied materials were diluted with physiological saline.

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

Fig. 2 Erythema on the abdominal skin in the guinea pigs in the challenge test.

In Fig. 2x, sites a, b and c show erythema following treatment with 5-, 10-fold, and 20-fold diluted extracts, respectively. Site a was to reconfirm the primary irritant dermatitis. Site c was prepared to compare with site b at a lower concentration of the extracts. Site b was evaluated for the allergenicity. Site d shows no erythema following treatment with physiological saline. Fig. 2y shows the control abdominal skin.

Table 2 Allergenicity of water extracts of *Alstroemeria wilhelmina* in the GMT*

Induction material**	Challenge material**	Challenge results		
		24 hr	48 hr	72 hr
1% α -MBL	saline	0/12	0/12	0/12***
saline	saline	0/12	0/12	0/12
1% α -MBL	0.1% α -MBL	12/12	11/12	8/12
saline	0.1% α -MBL	0/12	0/12	0/12
1% α -MBL	0.5% α -MBL	12/12	12/12	12/12
saline	0.5% α -MBL	6/12	7/12	6/12
5-fold dil. flowers	saline	0/12	0/12	0/12
saline	saline	0/12	0/12	0/12
5-fold dil. flowers	20-fold dil. flowers	5/12	3/12	3/12
saline	20-fold dil. flowers	0/12	0/12	0/12
5-fold dil. flowers	10-fold dil. flowers	10/12	8/12	5/12
saline	10-fold dil. flowers	0/12	0/12	0/12
5-fold dil. flowers	5-fold dil. flowers	11/12	9/12	6/12
saline	5-fold dil. flowers	0/12	0/12	0/12
5-fold dil. leaves	saline	0/12	0/12	0/12
saline	saline	0/12	0/12	0/12
5-fold dil. leaves	20-fold dil. leaves	7/12	3/12	2/12
saline	20-fold dil. leaves	0/12	0/12	0/12
5-fold dil. leaves	10-fold dil. leaves	8/12	3/12	3/12
saline	10-fold dil. leaves	0/12	0/12	0/12
5-fold dil. leaves	5-fold dil. leaves	9/12	4/12	4/12
saline	5-fold dil. leaves	1/12	1/12	0/12
5-fold dil. stems	saline	0/12	0/12	0/12
saline	saline	0/12	0/12	0/12
5-fold dil. stems	20-fold dil. stems	0/12	0/12	0/12
saline	20-fold dil. stems	0/12	0/12	0/12
5-fold dil. stems	10-fold dil. stems	9/12	5/12	2/12
saline	10-fold dil. stems	0/12	0/12	0/12
5-fold dil. stems	5-fold dil. stems	9/12	9/12	4/12
saline	5-fold dil. stems	0/12	0/12	0/12

* The paradigm of the experimental procedure according to the GMT originally reported by Magnusson and Kligman¹⁰.

** The applied materials were diluted with physiological saline.

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

Table 3 Maximization grading at 24 hours after challenge exposure in the GMT at sites challenged by 10-fold diluted plant extracts

Material	Sensitization rate %	Grade*	Classification*
α -MBL	100	V	extreme
flowers	83	V	extreme
leaves	67	IV	strong
stems	75	IV	strong

* Grading and classification were performed in compliance with the GMT originally reported by Magnusson and Kligman¹⁰.

solution of the original extracts of the flowers prepared in compliance with GMT. Both the α -MBL physiological saline and the diluted original extract solution from the flowers induced extreme reactions (Table 3). The extracts of the leaves and stems induced allergic contact dermatitis of a similarly 'strong' intensity.

Figure 3 shows the HPLC chromatogram applied to the *Alstroemeria* extracts and the authentic α -MBL. The peak of the α -MBL appeared as the second peak between the two higher peaks on the HPLC chromatograms using the Shim-pack CLC-ODS separation column. The first and third peaks were attributed to tuliposide A when compared to the HPLC chromatogram reported previously¹¹.

Table 4 shows the contents of the α -MBL in the original

extract solution from the flowers, leaves and stems of the prepared *Alstroemeria wilhelmina*. The flowers showed significantly higher concentrations, while the leaves and stems showed lower concentrations, but similar concentrations of α -MBL in the original extract solution.

Discussion

We confirmed primary irritant dermatitis⁴ due to the extracts of *Alstroemeria wilhelmina* in guinea-pigs (Table 1). This suggests that contact dermatitis due to *Alstroemeria wilhelmina* in humans is partially primary irritant dermatitis. Delayed-type allergic contact dermatitis⁴ due to the extracted antigens from *Alstroemeria wilhelmina* was also confirmed by the GMT. The results also suggested that the contact dermatitis due to *Alstroemerias* consists of primary irritant dermatitis and delayed-type sensitized allergic contact dermatitis. Manda et al. also reported that skin lesions due to okra (*Hibiscus esculentus* L.) might be responsible for allergic contact dermatitis, in addition to irritant contact dermatitis¹⁵ using a similar method to the GMT. Although the GMT requires many animals and is time consuming to evaluate the allergic contact dermatitis due to *Alstroemeria wilhelmina*, this consecutive study strongly reconfirmed the usefulness of the GMT to investigate the

Fig. 3 Analytical HPLC of (a) α -MBL, (b) aqueous flower extracts of -, (c) aqueous leaf extracts of -, (d) aqueous stem extracts of *Alstroemeria wilhelmina*. Detection by UV absorbance at 208 nm; the mobile phase was distilled water and the flow rate was 1.0 ml/min.

Table 4 Contents of α -MBL in the original extracts and in the regions of plant body prepared from *Alstroemeria wilhelmina*

Site	α -MBL*		
	n	mg/ml	ng/g-wet
flowers	(n=5)	3.36 $\pm$ 0.15**	168 $\pm$ 7.5**
leaves	(n=5)	1.31 $\pm$ 0.34	65.3 $\pm$ 17.1
stems	(n=5)	1.37 $\pm$ 0.07	68.4 $\pm$ 3.6

* mean $\pm$ 1 SD

** P<0.05

The extraction was performed following the method of Christensen and Kristiansen¹¹.

sensitization ability of antigens, such as the extracts from *Alstroemeria wilhelmina* focused on here. Some other studies have also reported that allergic contact dermatitis is caused by *Alstroemeria ligutu*^{16,17}, suggested to be an original *Alstroemeria* species. The prepared antigens from *Alstroemeria wilhelmina* revealed the strongest allergenicity. Therefore, caution should be taken when touching the flowers directly^{18–20}.

The quantifying of the α -MBL in the extracts was concluded as being one of the useful methods to evaluate the extent of contact dermatitis caused by *Alstroemeria* species^{11,21}. α -MBL was recognized as one of the irritant chemicals to the skin²⁰. Tuliposide A hydrolyses easily into glucose and unsaturated lactone: α -MBL (Fig. 4). The α -MBL is present in most parts of the plant and can be easily extracted with water²². Slob et al.

Fig. 4 Formation of α -methylene- γ -butyrolactone (α -MBL) from Tuliposide A. α -MBL is identical to Tulipalin A.

investigated the presence of tuliposide A and α -MBL in *Alstroemeria* by the sensitive method of gas chromatography, and reported that tuliposide A causes allergic contact dermatitis^{23,24}. In the present study, the primary irritation on the sites applied with the 5-fold diluted solution and the erythema on the sites applied with the 20-fold diluted solution did not appear to be dose-dependent with the content of α -MBL. Therefore, it is necessary to determine not only the α -MBL but also the tuliposide A content in extracts of *Alstroemeria* in future studies. Further studies should attempt to clarify the combining effect of the gradients, such as tuliposide A, α -MBL and others, on the dermatitis. The α -MBL content was highest in the flower extracts and lowest in the leaf extracts. Those working with *Alstroemeria* do not touch the fragile flowers directly during the handling process, so as not to damage the shape and thus lower the value of the flowers. However, they have to touch the leaves and stems containing the lower concentration of α -MBL. This results in contact dermatitis on their fingers and hands which may in part be due to primary irritant dermatitis, and may also partially consist of delayed-type allergic contact dermatitis.

Alstroemeria wilhelmina is one of the hybrids of *Alstroemeria*^{25–27}. There are many hybrids of *Alstroemeria* and the flower companies produce new hybrids of *Alstroemeria* every year. Cultivating, trading, and arranging *Alstroemeria* species are becoming more common in the field of horticulture and flower markets. This means that the handlers of *Alstroemeria* must be aware of and take care of not only primary irritant dermatitis, but also the delayed-type allergic contact dermatitis reported here.

In conclusion, we showed not only primary irritant dermatitis, but also delayed-type allergic contact dermatitis on the skin of guinea-pigs exposed to extracts from *Alstroemeria wilhelmina* using the bioassay method of GMT. The determination of α -MBL in plants was suggested to be a useful tool to predict the extent of contact dermatitis due to *Alstroemeria*.

Acknowledgements

The present study was supported in part by Grants-in-Aid (07457097 and 12470087) for Scientific Research from the Ministry of Education, Science and Culture of Japan. The authors thank Mr. Noda for providing *Alstroemeria wilhelmina* cultivated in his garden to promote this study.

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