

Characteristics of Allergy Inducing Materials Isolated from *Rhus verniciflua* Stokes on BALB/c Mice

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We purified crude urushiol from natural lacquer produced in Korea, China, and Japan and then isolated several urushiol congeners known to induce allergic reactions. The 3 major kinds of urushiol congeners that were isolated and purified were RV-1 ($C_{21}H_{30}O_2$, M.W. 314.462), RV-2 ($C_{21}H_{32}O_2$, M.W. 316.240) and RV-3 ($C_{23}H_{34}O_2$, M.W. 342.515), occupying 80% of total crude urushiol. The content of RV-1 was the highest in natural lacquer from China at 70.07%, and was the lowest in that from Japan at 62.38%. However, the content of RV-2 in natural lacquer from Japan was 9.25%, 2~3 times higher than those from Korea (4.28%) and China (3.09%). As an allergy inducing character, RV-1 had strong inducing power and durability in the primary stage, showing slow recovery. RV-2 had weak power in the primary stage and also showed slow recovery. Although RV-3 had comparatively weak power at the primary stage, it induced the strongest allergy contact dermatitis after 48 hr. However, it recovered to nearly the same level as control group 72 hr after sensitization time. Accordingly, we found out that RV-1 is the most influential of urushiol congeners in inducing allergic reactions, natural lacquer from China having the most inducible strength and slowest recovery compared to those from Korea and Japan.

Key words : *Rhus verniciflua* Stokes, urushiol congeners, allergy, contact dermatitis

Introduction

Due to overflow of chemicals and environmental wastes, many immuno-sensitive diseases have arisen recently. The most common dermal disease from this cause is contact dermatitis, induced by chemicals, drugs, food, plants, and other stimulants [18].

Some plants that cause contact dermatitis include *Rhus verniciflua* Stokes, *Rhus chinensis*, *Rhus tricarpa*, *Rhus succedanea*, *Rhus ambigua*, and *Rhus sylvestris* [3,7]. The lacquer trees are cultivated in Korea, China, India, Vietnam, Japan, Thailand, and Myanmar, which makes them exclusive to Asia.

Resin, the main content of lacquer, contains 50~80% urushiol. It is a clear substance, but when it contacts air, it turns black and becomes urushi lacquer, due to the laccase effect [23]. Allergy-inducing materials that exist in natural lacquer are several kinds of urushiol congeners of similar structures. Generally, their molecular weight ranges from 300 to 500 [8]. The urushiol is toxic and causes blisters and dermatitis [21,24], but develops a durable and beautiful luster

when it is dried. Also, urushi lacquer retains its qualities and color for a long period of time and does not react with acids, alkali, or heat (70 degrees and above). These characteristics make urushi lacquer useful as a varnish for wooden ware, mixed with other colorings [7].

The *Rhus verniciflua* Stokes is effective against cancer, and is also used as a digestive, blood-purifier, insectifuge, and pain relief [2,15,16,20,22]. There are also other functional effects to our body [1,6,9,12,14,18], but it is abstained for its allergy-inducing characteristic [19].

Recently, there has been a significant increase in the number of patients suffering from atopic dermatitis and respiratory disease, due to volatile organic compounds (VOC) produced from synthetic chemical varnish used in apartments and public buildings [5,11,13]. Urushi lacquer is possibly the most suitable and natural alternation. Therefore, this research aims to provide the groundwork in breeding the low-allergenic *Rhus verniciflua* Stokes and in developing urushi lacquer. In this research, lacquer from China, Japan and Korea were used in comparison. We isolated allergy-inducing materials from each sample and attempted to find our allergy-inducing characteristics by experimenting on BALB/c mice.

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Materials and Methods

Materials

The lacquers used in this research, Korea (Weonju products), China (Sumseo products), and Japan (Iwate products), were provided from Jinju National University's Department of Interior Materials Engineering. Particularly, the Weonju products were used to isolate the allergy inducing materials.

Chemicals and instruments

The organic solvents used for extraction of urushiol and isolation of effective substances from *R. verniciflua*, such as acetone, dichloromethane, chloroform, methanol, n-hexane and acetonitrile etc. were of special quality grade. For column chromatography, silica gel 60 (70~230, 230~400 mesh ASTM, YMC co.) and gel ODS-A (12 nm, s-150 μ m, YMC co.) were YMC's products. The precoated silica gel 60 GF₂₅₄ TLC plate (20×20 cm, 0.25 mm glass) and Prep-TLC plate (20×20 cm, 2 mm glass) used for thin layer chromatography (TLC) were Merck's products. The CDCl₃ and CD₃OD for NMR analysis, were Aldrich's products, and DNCB (2,4-dinitrochlorobenzene) of Sigma products was used as allergen of hypersensitive reaction against mouse. Used instruments for this study are as follows; rotary vacuum evaporator (Büchi, Germany) and fraction collector (Eyela, Japan) were used for isolation and purification, and HPLC (Shimadzu LC-10AD, Japan) for determining urushiol congeners contents. NMR (Bruker, Germany) and EI/Mass (JMS, DX300, Japan) were used to identify chemical structure of allergy inducing materials.

Isolation of crude urushiol

Allergy-inducing materials that exist in natural lacquer are several kinds of urushiol congeners of similar structures. Generally their molecular weight ranges from 300 to 500. They are monomers in natural lacquer, but they easily polymerize to oligomer or polymer by laccase in the air. Therefore we removed laccase to prevent polymerization, and then isolated and analyzed urushiol congeners in monomer form [8]. In this study, we isolated the urushiol congeners after removing laccase as shown in Fig. 1.

Isolation of urushiol congeners

We isolated and purified 3 kinds of estimated urushiol congeners (RV-1,2,3) from crude urushiol as shown in Fig. 2, to separate urushiol congeners. In other words, first, we

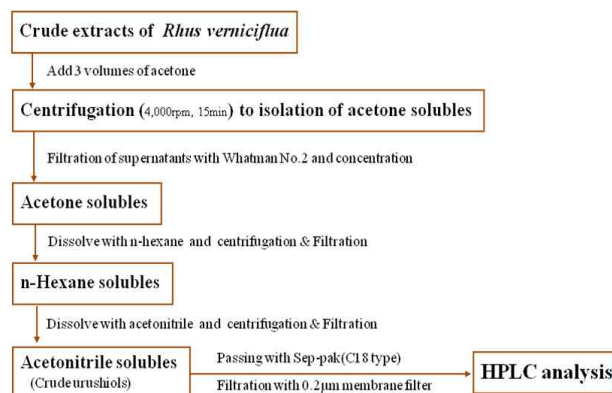


Fig. 1. Isolation procedure of crude urushiol from *Rhus verniciflua* Stokes.

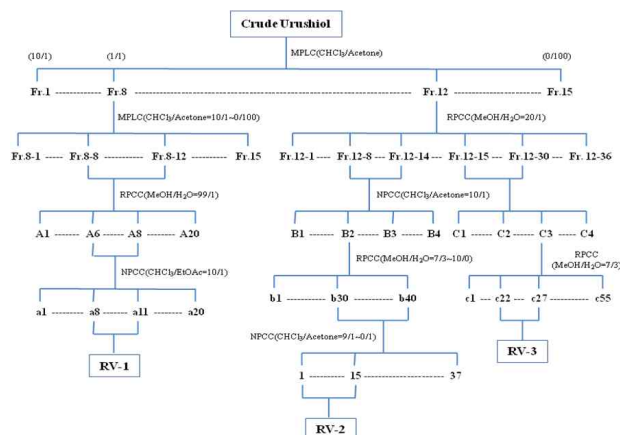


Fig. 2. Isolation and purification procedure of urushiol congeners from *Rhus verniciflua* Stokes. MPLC; medium pressure liquid chromatography, NPCC; normal phase column chromatography, RPCC; reverse phase column chromatography.

obtained 15 fractions (Fr.1~Fr.15) from crude urushiol by middle pressure liquid chromatography (MPLC), and started purification from those fractions to isolate urushiol congeners.

Depending on each situation, isolation process was performed with methods of middle pressure liquid chromatography (MPLC), normal phase column chromatography (NPCC), reverse phase column chromatography (RPCC), thin layer chromatography (TLC), and preparative thin layer chromatography (Prep-TLC).

Determination of urushiol congeners by HPLC

HPLC analytical conditions for determining the contents of urushiol congeners in natural lacquer produced in each country are as follows. That is, analytical column, mobile phase, flow rate and wavelength of UV detector were C18

ODS ($\Phi 3.9 \times 300$ mm), acetonitrile / 0.002% (v/v) acetic acid (80/20), 1 ml/min and UV 254 nm.

BALB/c mouse for characteristics of allergy reaction test

BALB/c mice, four-weeks of age (20 ± 2 g), were purchased from Hyochang science co. and were used in this research after adaptation in the breeding room for two-weeks. We supplied water freely and conditions of breeding room were kept at $22 \pm 2^\circ\text{C}$ with controlled 12-hr-light / 12-hr-dark cycle during the all experimental period. The mice were divided into eight groups. 8 test groups were made as follows: control, treated DNCB, treated natural lacquer (Korea, China and Japan) and treated urushiol congeners (RV-1, 2 and 3). The ear thickness and weight of each mouse were measured after 24, 48 and 72 hr.

Ear swelling effect by DNCB and natural lacquer

In order to prepare allergens of hypersensitive reaction, DNCB and natural lacquer were dissolved in the solution of acetone and olive oil (1:1, v/v) to result 5% and 2.5% concentration. The 1st sensitization were performed by spreading the 5% allergens at hairless abdominal site as the diameter of 1 cm. When 7 days had passed from 1st sensitization, 2nd sensitization were performed by spreading 4 μl of 2.5% allergens at right auricle of mice. After 24, 48 and 72 hr from 2nd sensitization, the thickness of right ear was determined with vernical calipers (Mitutoyo, Japan), and the weight of right ear resected as same sizes were determined with accurate electric balance. The average of all determined values was compared with each test groups.

Result and Discussions

Identification of urushiol congeners

The chemical structure of urushiol consists of a benzene ring with 2 hydroxyl group at 1st and 2nd carbon and a side chain containing 15~17 carbon at 3rd carbon. It was reported that the kind of urushiol congeners are decided according to location and number of double bonds belonging to the side chain, and strength of allergy induction may differentiate as well [17,25].

The chemical structures of three kinds of purified compounds isolated from natural lacquer, were analyzed with nuclear magnetic resonance (NMR) spectrometer, mass spectrometer and element analyzer etc, and they were confirmed

as urushiol congeners.

Spectrophotometric data of the three kinds of urushiol congeners (RV-1; $\text{C}_{21}\text{H}_{30}\text{O}_2$, MW 314.462, C;80.21%, H;9.62%, O;10.18%, RV-2; $\text{C}_{21}\text{H}_{32}\text{O}_2$, MW 316.240, C;79.70%, H;10.19%, O;10.11%, RV-3; $\text{C}_{23}\text{H}_{34}\text{O}_2$, MW 342.515, C;80.65%, H;10.01%, O;9.34%) are as follows.

RV-1: EI/MS (70 eV) m/z 314; $^1\text{H-NMR}$ (500 MHz, CD_3COCD_3) δ : 6.67 (1H, dd, $J=2.13, 7.3$ Hz), 6.60 (1H, dd, $J=2.13, 7.6$ Hz), 6.58 (1H, dd, $J=7.5, 7.5$ Hz), 6.38 (1H, qdd, $J=1.4, 2.8, 10.9, 15.0$ Hz), 5.96 (1H, ddd, $J=1.4, 10.9, 10.9$ Hz), 5.63 (1H, m), 5.35-5.47 (3H, m), 2.73-2.87 (2H, m), 2.61 (2H, t, $J=7.7$ Hz), 1.70 (2H, dd, $J=1.6, 7.1$ Hz), 1.60 (2H, q, $J=7.1$ Hz), 1.30-1.41 (11H, m); $^{13}\text{C-NMR}$ (125 MHz, CD_3COCD_3) δ : 145.6, 144.4, 133.4, 132.0, 130.8, 130.5, 128.1, 126.8, 124.9, 122.3, 120.2, 113.9, 31.5, 31.1, 29.7, 28.1, 13.7

RV-2: EI/MS (70 eV) m/z 316; $^1\text{H-NMR}$ (500 MHz, CD_3COCD_3) δ : 7.65 (2-OH), 6.67 (1H, dd, $J=2.06, 7.3$ Hz), 6.60 (1H, dd, $J=2.05, 7.6$ Hz), 6.57 (1H, dd, $J=7.6, 7.6$ Hz), 6.37 (1H, ddd, $J=1.2, 10.9, 13.6$ Hz), 5.94 (1H, ddd, $J=1.2, 10.9, 10.9$ Hz), 5.68 (1H, m), 5.33 (1H, m), 2.61 (2H, t, $J=7.7$ Hz), 2.2 (2H, m), 1.70 (2H, dd, $J=1.6, 7.1$ Hz), 1.60 (2H, q, $J=7.3$ Hz), 1.29-1.49 (15H, m); $^{13}\text{C-NMR}$ (125 MHz, CD_3COCD_3) δ : 145.2, 144.0, 135.2, 130.6, 130.1, 126.3, 124.1, 121.9, 119.8, 113.5, 28.5, 38.2, 30.8, 30.7, 30.4, 29.3, 26.5, 13.3

RV-3: EI/MS (70 eV) m/z 342; $^1\text{H-NMR}$ (500 MHz, CD_3COCD_3) δ : 6.68 (1H, dd, $J=2.1, 7.3$ Hz), 6.60 (1H, dd, $J=2.1, 7.6$ Hz), 6.57 (1H, dd, $J=7.5, 7.5$ Hz), 6.39 (1H, ddd, $J=1.2, 10.8, 13.6$ Hz), 5.96 (1H, ddd, $J=1.1, 10.8, 10.8$ Hz), 5.63 (1H, m), 5.33-5.46 (3H, m), 2.80-2.87 (2H, m), 2.61 (2H, t, $J=7.7$ Hz), 1.70 (2H, dd, $J=1.6, 7.1$ Hz), 1.60 (2H, q,

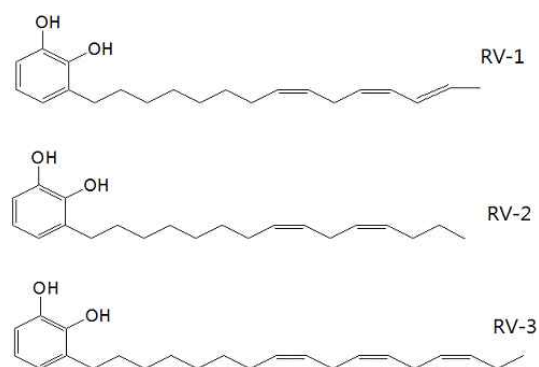


Fig. 3. Chemical structures of isolated and purified urushiol congeners from *Rhus verniciflua* Stokes. RV-1; $\text{C}_{21}\text{H}_{30}\text{O}_2$, Mol. Wt., 314.462, C;80.21%, H;9.62%, O;10.18%, RV-2; $\text{C}_{21}\text{H}_{32}\text{O}_2$, Mol. Wt., 316.240, C;79.70%, H;10.19%, O;10.11%, RV-3; $\text{C}_{23}\text{H}_{34}\text{O}_2$, Mol. Wt., 342.515, C;80.65%, H;10.01%, O;9.34%.

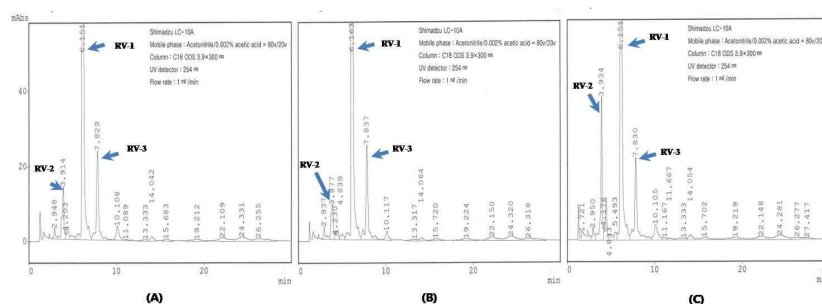


Fig. 4. The content of urushiol congeners in natural lacquer of Korea, China and Japan. (A) Korea (Weonju), (B) China (Sumseo), (C) Japan (Iwate).

$J = 7.2$ Hz), 1.29-1.46 (15H, m); $^{13}\text{C-NMR}$ (125 MHz, CD_3COCD_3) δ : 145.2, 144.0, 133.0, 131.6, 130.4, 130.1, 127.7, 126.4, 124.6, 121.9, 119.8, 113.5, 31.2, 30.7, 30.6, 29.3, 27.7, 13.3

Contents of urushiol congeners

The contents of urushiol congeners from lacquer extracts according to its production region are shown in Fig. 4 and Table 1. The major urushiol that induces allergy is thought to be RV-1, 2, and 3, and urushiol congeners of these three kinds comprise 80% of total congener content. Among the three, RV-1 has the most amount of urushiol congeners and its molecular weight is 314. RV-1 has 3 double bonds with 15 carbons on its side chain and has the smallest molecular weight. The contents of urushiol congeners were the highest in that of China as 70.07%, 66.82% of Korea, and 62.38% of Japan.

In case of RV-2, the urushiol congener contents were 4.28% (Korea) and 3.09% (China), but that of Japan contained twice as much as the other two kinds (9.25%). RV-3 contained 9.83% (China), 8.34% (Korea), and 8.60% (Japan) respectively.

Inducing character of allergic contact dermatitis

DNCB-induced allergic contact dermatitis is known to be generated from increased blood vessel permeability due to

secretion of inflammatory substance from mast cells. That is, the substance which becomes the antigen of allergic contact dermatitis penetrates the skin and combines with protein. As it comes into contact with the Langerhans cells, it becomes a primed cell and when this contacts the allergen once again, dermatitis is generated [4,10,21,24].

As antigen to contact dermatitis, DNCB and lacquer was each dissolved in a mixed solution of acetone and olive oil. This antigen intentionally induced hypersensitiveness, twice on each mouse. Table 2 and 3 shows the measurement of swelling and weight of the right ear of each mouse, and therefore shows contact sensitivity.

The ear swelling effect was observed by measuring the thickness of the right ear. For the control group, which had not been applied with antigen, the change in the thickness of the ear was insignificant. The ear of the group that had been applied with DNCB antigen swelled twice its normal size after 24 hr as 0.40 ± 0.05 mm, and was the thickest after 48 hr as 0.45 ± 0.1 mm. However, the thickness diminished after 72 hr as 0.25 ± 0.0 mm.

When treated with antigen made of lacquer, the ear swelling effect differed depending on the lacquer's origin. After 24 hr, the thickness of the right ear were 0.37 ± 0.03 (Korea), 0.37 ± 0.015 (China), and 0.28 ± 0.01 mm (Japan), showing notable increase in its thickness compared to the control group. After 48 hr, thickness of the ears decreased significantly, as 0.21 ± 0.01 (Korea), 0.25 ± 0.006 (China), and 0.24 ± 0.02 mm (Japan). As a result, allergy inducing power of lacquer from Korea was strong in the beginning but returned to the state of control group after 48 hr. The Japanese lacquer showed relatively weak allergy inducing power in the beginning, but recovery to its primary state was slower than Korean lacquer. In case of Chinese lacquers, the allergy inducing power was strong from the beginning and the swelling could not recover fully even after 72 hr.

Table 1. The contents of urushiol congeners of *Rhus verniciflua* Stokes on different produced country

Urushiol congeners	Contents of urushiol congeners(%)			Remarks
	Korea	China	Japan	
RV-1 ¹⁾	66.82	70.07	62.38	
RV-2	4.28	3.09	9.25	
RV-3	8.60	9.83	8.34	
The others	20.30	17.01	20.03	

¹⁾ RV-1, -2 and -3 are purified urushiol congeners from *Rhus verniciflua* Stokes

Table 2. The ear swelling effect of the crude extracts and purified urushiol congeners

(units; mm)

Groups	Initation		After 24 hr		After 48 hr		After 72 hr	
	Right	Left	Right	Left	Right	Left	Right	Left
Control	0.16±0.006 ⁴⁾	0.16±0.006	0.20±0.006	0.18±0.006	0.21±0.006	0.21±0.006	0.20±0.050	0.18±0.031
DNCB ¹⁾	0.16±0.008	0.16±0.006	0.40±0.050	0.19±0.017	0.45±0.100	0.25±0.000	0.25±0.000	0.19±0.045
Korea ²⁾	0.15±0.006	0.15±0.006	0.37±0.015	0.16±0.006	0.21±0.010	0.15±0.006	0.22±0.026	0.15±0.003
China	0.14±0.006	0.14±0.006	0.37±0.026	0.15±0.006	0.25±0.006	0.16±0.010	0.24±0.006	0.15±0.006
Japan	0.14±0.006	0.14±0.006	0.28±0.010	0.15±0.006	0.24±0.015	0.16±0.006	0.20±0.006	0.15±0.006
RV-1 ³⁾	0.15±0.006	0.16±0.006	0.31±0.200	0.17±0.006	0.28±0.025	0.18±0.025	0.27±0.021	0.22±0.064
RV-2	0.15±0.006	0.16±0.006	0.23±0.006	0.18±0.010	0.24±0.006	0.22±0.029	0.23±0.017	0.15±0.006
RV-3	0.16±0.006	0.14±0.006	0.22±0.010	0.17±0.006	0.28±0.023	0.23±0.035	0.20±0.006	0.16±0.035

¹⁾DNCB (2,4-dinitrochlorobenzene) was used as allergen of hypersensitive reaction against mouse.²⁾Crude extracts isolated from *Rhus verniciflua* Stokes on different production country.³⁾RV-1, -2 and -3 are purified urushiol congeners from *Rhus verniciflua* Stokes.⁴⁾Values are given as mean±SD (n=8).

Table 3. The ear weight increasing effect of the crude extracts and purified urushiol congeners

(units; mg)

Groups	Ear weight after 72 hrs	
	Right	Left
Control	7.15±1.286 ⁴⁾	7.18±1.060
DNCB ¹⁾	8.60±0.200	7.77±0.929
Korea ²⁾	7.42±0.252	6.98±0.116
China	7.88±0.252	7.03±0.723
Japan	7.14±0.153	6.87±0.058
RV-1 ³⁾	8.45±0.794	7.03±0.839
RV-2	8.26±0.306	7.23±0.252
RV-3	7.36±1.159	7.13±0.651

¹⁾DNCB (2,4-dinitrochlorobenzene) was used as allergen of hypersensitive reaction against mouse.²⁾Crude extracts isolated from *Rhus verniciflua* Stokes on different production country.³⁾RV-1, -2 and -3 are purified urushiol congeners from *Rhus verniciflua* Stokes.⁴⁾Values are given as mean±SD (n=8).

When RV-1 was treated as antigen, the ear thickness was 0.31±0.2, 0.28±0.025 and 0.27±0.021 mm after 24, 48 and 72 hr, respectively. It also showed strong allergy inducing power in the beginning and did not recover fully after 72 hr. In case of RV-2, the thickness was 0.23±0.01, 0.24±0.01, 0.23±0.017 mm after 24, 48, and 72 hr, showing weak inducing power in the beginning and also showing slow recovery. On the other hand, in case of RV-3, the ear thickness was 0.22±0.01, 0.28±0.023, and 0.20±0.006 mm after 24, 48 and 72 hr respectively. Its inducing power was the strongest after 48 hr, and it also showed fast recovery as the thickness was recovered almost to the size of the control group after 72 hr.

The external condition of the skin of control group re-

mained normal throughout the experiment. The skin that had been applied with DNCB antigen and lacquer antigen showed rash due to allergy, and also festered and swelled. This condition led to a skin damage that reddened the skin after 24 hr, and after 48 hr, the damaged area had expanded but had also stabilized. After 72 hr, the skin damage had weakened compared to the condition after 48 hr.

The weight of the ear that had been treated with lacquer antigen after 72 hr was 7.42±0.25 (Korea), 7.88±0.25 (China) and 7.14±0.15 mg (Japan). When treated with RV-1, 2, and 3, the weights were 8.45±0.794, 8.26±0.306 and 7.36±1.159 respectively after 72 hr, indicating that RV-1 has the slowest recovery rate.

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초록 : 옷(*Rhus verniciflua* Stokes)으로 부터 분리한 알레르기 유발물질의 BALB/c mice에 대한 특성

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옷 allergy 유발물질을 분리하고 그 특성을 연구하기 위하여 국내산, 중국산 및 일본산 생 옷을 사용하였다. 옷의 crude extracts로부터 crude urushiol을 정제하고, 다시 allergy 유발물질로 알려져 있는 3종의 urushiol congeners를 분리하였다. 분리한 urushiol congeners는 RV-1 (C₂₁H₃₀O₂, MW 314.462), RV-2 (C₂₁H₃₂O₂, MW 316.240) 및 RV-3 (C₂₃H₃₄O₂, MW 342.515)였으며, 전체의 80%에 해당하였다. RV-1의 함량은 중국산에서 70.07%로 가장 많았으며, 일본산에서 62.38%로 가장 적었다. 반면에 RV-2는 일본산은 9.25%로서 국내산과 중국산의 4.28 및 3.09%에 비하여 약 2~3배 정도 많았다. Allergy 유발특성을 보면, RV-1은 초기유발강도가 강하고 지속적이어서 회복속도도 늦었다. RV-2는 초기유발강도는 약하지만 회복속도는 늦었다. 반면에 RV-3는 초기유발강도는 비교적 약했지만, 감작 후 48시간째에 가장 강하게 유발되었고, 회복속도가 빨라서 72시간이 경과하였을 때에는 거의 대조군 수준으로 회복되었다. 따라서 allergy 유발에 가장 큰 영향을 미치는 urushiol은 RV-1이었으며, RV-1의 함량이 가장 많은 중국산이 allergy의 유발강도가 가장 강하였고 회복속도도 늦음을 알 수 있었다.