Korean J. Medicinal Crop Sci. 10(4) : 288-293(2002)

Anticancer and Antioxidant Activity of Allergen-Removed Extract in *Rhus verniciflua* Stokes

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ABSTRACT : Allergen-removed-extract was produced from *Rush verniciflua* by two phase methods. Phase one was high temperature treatment of *Rush verniciflua* tree to get allergen-removed-extract. Phase two was extraction of solution from phase one product using water or organic solvents. The solutions from above method show high antioxidant activity, anticancer activity, and improvement in lung function, but did not contain urushiol family compounds.

Key words : *Rhus verniciflua*, antioxidant, anticancer

INTRODUCTION

For thousands of years(Onishi, 1995; Tyman, 1979) the sap of oriental *Rhus verniciflua* has been used as an excellent preservative surface coating material for wood, porcelain, and metallic wares in Asian countries. Contact with poison ivy plant and related plant species including *Rhus verniciflua* causes irritation, inflammation, and blistering of the skin. For this reason, there has been great interested in determining the chemical structure of irritating principles. After many investigations on the active principles that induce irritation, Majima(1922) reported that a mixture of olefinic catechols having an n-C15 alkyl side chain, commonly referred to as urushiol, were the main irritating component of sap constituents of *Rhus verniciflua*. There are many reports on isolation of urushiol derivatives in the irritation ivy and related plants(Markiewitz et al., 1965; Hill et al., 1934; Adawadkar et al., 1983). It is interesting to see the old wares coated with *Rhus verniciflua* sap might contain the strong antioxidants. The mechanism of the oxidative polymerization and cross linking of Japanese *Rhus verniciflua* has been studied(Tyman, 1979).

The oriental *Rhus verniciflua* was registered in a medicinal books and pharmacopoeia of the oriental three Nations and permitted to be used as the original form of medicinal material. By above medicinal books, the typical prescription of anticancer in *Rhus verniciflua* was Daehwangjachunghwan. At present, the original form of materials was used in Korea, China, and Japan. When the material of *Rhus verniciflua* was mixed with other kinds of medicinal materials, there was no report about adverse reaction of medicine such as skin irritation and urtication. When only single material of *Rhus verniciflua* without other added materials was used for medicine, there was rare skin irritation such as urtication and eruption, but the frequency of skin irritation induction was below about 0.01%, only.

However, there was no report on the antioxidative

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Received 10 October, 2002 / Accepted 30 October, 2002

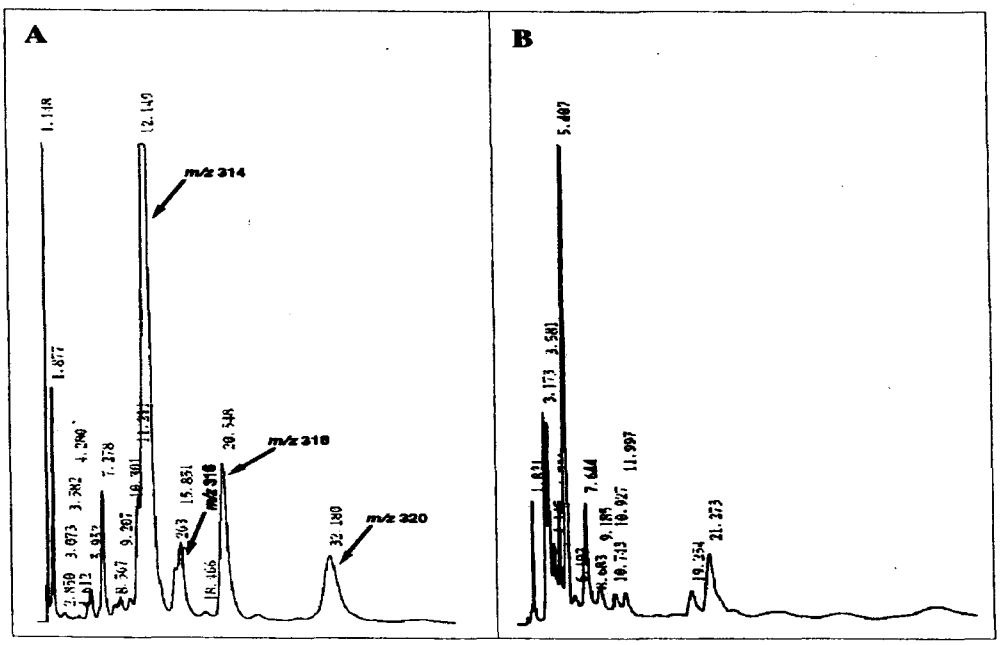

Fig. 1. HPLC profiles of urushiol mixture seperated from the sap and allergen-removed extract of *Rhus verniciflua*. The urushiol mixture was loaded onto an ODS(3.9 300mm, μ-bondpack C-18, Waters) eluting with a isocratic of 85% methanol of 45min at a flow rate of 1.0 *ml*/min. The urushiol derivatives was detected at 285 nm. A : HPLC profile of fresh sap. B : HPLC profile of allergen-removed extract of *Rhus verniciflua*.

activity in the sap of *Rhus verniciflua*. Therefore, we tried to have allergen-removed-extract from Korean *R. verniciflua* by two phase methods. This study was conducted to determine the antioxidant and anticancer activity of extract of *Rhus verniciflua* which removed the urushiol completely(Fig. 1). We confirmed that the ARE of *Rhus verniciflua* used in this study have no any urushiol by HPLC analysis (Fig. 1; allergen-removed-extract of *R. verniciflua*, Patent application no. 215390) As a result, in this paper, we described the antioxidant and anticancer activities of allergen-removed-extract named as ARE. The LD_{50} of allergen-removed extracts was above 2,000mg/kg in LD_{50}, showing the low cytotoxicity(Table 4).

MATERIALS AND METHODS

Extraction of allergen-removed-extract

Allergen-removed-extract was produced from Rush verniciflua by two phases methods. Phase one was high temperature treatment of Rush verniciflua tree to get allergen-removed-extract. Phase two was extraction of solution from phase one product using water or organic solvents. The above pre-treated materials from Rush trees should indirectly be roasted in a iron pot at the temperature of 100 to 240℃ for 10 to 50 minutes. More desirable condition is to roast pre-treated Rush trees at 180℃ for 30 minutes. The Rush trees were extracted with water or alcohol, and then the extracts were fractionated with n-hexane, EtOAc, of BuOH.

DPPH radical scavenging activity

The 1,1-diphenyl-2-picryhydrazyl(DPPH) free radical scavenging activity was measured according to Xiong et al. One*ml* if 0.15mM DPPH in ethanol was added to sample solution containing 4*ml* of methanol and allowed to react for 30min at room temperature, then the optical density was measured at 517nm. For the blank, ethanol was used instead of DPPH solution, and for control, methanol was used instead of the sample solution. The IC_{50} values were calculated from the regression lines where the abscissa represented the concentration of tested compound and the ordinate the

average percent reduction of DPPH radical from three separate tests.

Inhibitory activity on lipid peroxidation in rat liver microsomes

The inhibitory activity on lipid peroxidation in rat liver microsomes was measured by the thiobarbituric acid (TBA) method(Kim et al., 1996). Rat liver microsomes prepared according to the method of Ohkawa et al.(Ohkawa te al., 1979) with some modification were suspended in 100mM Tris-HCl buffer (pH 7.4). Reaction was initiated by the addition of 100μM $FeSO_4 \cdot H_2O$. After 30min at 37℃ under reciprocal agitation, the reaction was stopped by the addtion of 3M trichloroacetic acid in 2.5N HCl. Lipid peroxidation were assessed by measuring TBA reactive products. Percent inhibition was calculated as follows : (1-T-B)/(C-B))×100(%), in which T, C, and B are absorbance values at 530nm of the compound treatment, the control (peroxidation without compound) and the 0 time control (no peroxidation), respectively. Reagents including DPPH were purchased from Sigma.

Cytotoxic sulforhodamine B (SRB) assay

Six kinds of human cancer cell lines were used and cultured with RPMI 1640 containing 10% fetal calf serum(FCS). For SRB assay, cells were cultured performed by previous method (Kim et al., 1996). Cell suspension (3~40,000 cells/ml) was made in culture medium and inoculated to each well of 96-well micro titer plate. One day after plating, time zero control plate was made, compounds (1~4)were directly treated, and cells were incubated for further 48 hrs in a CO_2 incubator. Cells were fixed with 50μl of 50% trichloroacetic acid solution for 1h at 4℃ and plates were washed 5times with tap water and air-dried. 100μl of SRB solution (0.4% in 1% acetic acid) was added and staining was done at room temperature for 30 min. Residual dye was washed out with 1% acetic acid and air-dried. To each well, Tris solution (10 mM, pH 10.5) was added. Optical density (OD) was measured with microtiter plate reader at 540nm. Growth inhibition was calculated according to the previous method. Briefly, OD of treated well was subtracted PD at time-zero(Tz) plate and divided by calculated value of untreated control. Growth inhibition of 50%(GI_{50}) was calculated by Probit method (Wu et al., 1992).

RESULTS AND DISCUSSION

Analysis of antioxidant activity

A free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to analyze the antioxidant activity of the extracts from Rush trees. Allergen-removed-extract has high antioxidant activity. Ethylacetate and butanol fractionation of allergen-removed-extract have the stronger antioxidant activity than α-tocopherol, and water fractionation and the extracts from Rush trees also shows high antioxidant activity similar to α-tocopherol in control(Table 1).

The sap of Korean *Rhus vernicifera* was partitioned between n-hexane and water. The hexane soluble fraction with a free radical scavenging activity was subjected to silica and ODS gel column chromatography. The antioxidative compounds were identified as urushiol derivatives(Kim et al., 1997). The activities of four urushiol derivatives differed depending on each compound. However, even though urushiol derivatives have strong antioxidant activities , these compounds caused the allergy. Therefore, allergen-removed-extract could be developed for a useful antioxidant for various application.

Table 1. Antioxidant activity of each solvent fractionation

Fraction/ Standard compounds	Antioxidant activity (RC_{50} : $\mu g/ml$) (DPPH removal activity)
Allergy removed extract	12
Hexane fraction	) 50
EtOAc fraction	4
BuOH fraction	6
H_2O fraction	19
α-tocopherol	12

Analysis of antilipidperoxidative activity

All the absorbance reading was at 530nm in

inhibitory activity on lipid peroxidation of allergen-removed-extracts from Rush verniciflua. The absorbance at 530nm was compared to anti-lipid-peroxidative activity in control group using α-tocopherol. Ethylacetate fraction showed strong anti-lipid-peroxidative activity as much as in α-tocoperol control, and in butanol fraction positive anti-lipid-peroxidative activity was detected (Table 2).

Contrary to this study, there was a report that urushiol has strong inhibitory activities on lipid peroxidation (Kim.et al., 1997). Four urushiol derivatives isolated from hexane extracts showed high scavenging activities on DPPH radical (Kim et al., 1997) Four compounds showed slightly different activities depending on the number of double bonds in side chain. Particularly, 4 showed the highest activity (IC_{50} : 1.2 μg/ml) which was about two times higher than those of a synthetic antioxidant, 3-tert-butyl-4-hydroxyanisole (BHA) and α-tocopherol. In this study, allergen-removed-extract also showed strong antilipid peroxidation activity. Thus, allergen-removed-extract could be the better sources than urushiol derivatives having allergy for industrial applications such as foods and medicine.

Table 2. Anti-lipid-peroxidative activity in each solvent fractions

Fraction/ Standard compounds	Anti-lipid-peroxidative activity (RC_{50};μg/ml)
Allergy removed extract	〉100
Hexane fraction	〉100
EA fraction	5
BuOH fraction	49
H_2O fraction	100
α-tocopherol	3.1

Analysis of anticancer activity

In general, urushiol derivatives have been officially recognized as allergy induction components by many studies in the world(Kim et al., 1997; Jung et al., 1990; Kenneth et al., 1964; James et al., 1983). Even though Rush trees have some skin irritation induction, Rush verniciflua has been used as an excellent medicine in Oriental countries for about 1,000 years because of it's outstanding anticancer activities. Rush tree inducing some allergies has very weaker adverse effect of medicine, compared with anticancer chemicals which have been already used. It has been known that the materials of *Rhus verniciflua* mixed with other different kinds of medicinal materials did not have the allergies.

The extracts of Rush tree used in this study were removed urushiol derivatives completely, and named as ARE. The components of this extracts were 12.5% fustin, 1.5% gallic acid, 0.1% 3′,4′,7-trihydroxyflavone, and 0.18% fisetin. To analyze the effects of the extracts from Rush trees on the anti-cancer activity, the extracts were used on the human cancer cells using the SRB method (Skehan et al., 1989). In this experiment, PC-3, colon cancer cell line HCT-5 and SW-620, renal cancer line ACHN, lung cancer cell line A459, and leukemia cancer line MOLT-4F were cultured using RPMI 1640 media. Hexane fractionation showed the strong inhibition of the growth of four cancer cell lines. Especially the extracts of Rush trees show high inhibition of the growth of leukemia cancer cell line MOLT-4F(Table 3). The cytotoxicity of allergen-removed extracts was above 2,000mg/kg in LD_{50}. The value of cytotoxicity was very low(Table 4), and means that there were no harmful effects on human such as death or any kinds of adverse medicinal effects. Recently, the clinical demonstrations in Kwanghyeowon Medical Foundation Cancer Center have been conducting very successfully, using ARE. There was no any allergy adverse reactions in clinical demonstrations of Kwanghyeowon Medical Foundation Cancer Center, Korea for 4 years. There was no even one adverse reaction such as allergy induction. Allergen-removed extract used in this study have strong antioxidant , anticancer activities and low cytotoxicity. There was no any study, using allergen-removed extract of Rush tree on antioxidant and anticancer activities. Therefore, the results in this study would be very useful and valuable for anticancer and antioxidant. Also, they still show promise as an immunochemotherapeutic drug owing to their bifunctional activities on cancer.

Table 3. Effects of each solvent fractionation on the inhibition of the growth of cancer cell lines

Fraction/ Standard compound	Anti-cancer activity(GI_{50} : $\mu g/ml$)						
	PC-3	HCT-15	SW-620	ACHN	A549	MOLT-4F	Average
Allergy removed extract	〉30	〉30	〉30	〉30	〉30	〉30	〉30
Hexane fraction	〉30	〉30	16.19	19.31	20.54	13.11	21.53
EtOAc fraction	〉30	〉30	〉30	〉30	〉30	〉30	〉30
BuOH fraction	〉30	〉30	〉30	〉30	〉30	〉30	〉30
H_2O fraction	〉30	〉30	〉30	〉30	〉30	〉30	〉30
Adriamycin	0.16	0.19	〈0.03	0.14	〈0.03	0.07	0.10

Table 4. Two week DRS(dose range study) of allergen-removed extract of *Rhus verniciflua* in rats

Item	Group	Control	Low	Medium	High
	mg/kg	0	100	900	2000
Mortality		0/6	0/6	0/6	0/6
			normal		
Clinical Sign		0/6	0/6	0/6	0/6
			normal		
Body weight	day1	193.4	193.4	196.4	194.7
	day3	211.2	219.2	206.8	202.3
	day7	224.2	236.2	215.7	203.8
	day10	240.6	251.6	232.9	210.3
	day13		278.4	240.0	224.7
			normal		
Body weight Increment	~day1	2.5	6.7	4.0	4.8
	~day3	20.3	32.5	14.4	12.4
	~day7	33.3	49.5	23.3	40.5
	~day10	49.7	65.0	13.9	20.4
	~day13	71.9	123.7	47.7	34.8
			normal		
Food Intake	before medication	16.7	15.2	16.7	16.9
	one week	19.7	20.5	17.7	15.5
	two week	20.6	22.1	19.6	15.3
			normal		
Water Intake	before medication	34.2	30.8	35.8	30.8
	one week	31.7	32.6	28.6	28.9
	two week	33.9	29.3	33.5	27.8
			normal		
Organ weight			normal		
Urine analysis			normal		
Blood biochemistry			normal		
Hematalogy			normal		

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