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Purification and Characterization of Antioxidant Substance from the Stem Bark of *Rhus verniciflua*

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옻나무 껍질에서 항산화물질의 정제와 특성

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Abstract

In order to isolate antioxidant substances from *Rhus verniciflua* (RV), the dried stem bark was extracted with water. The crude water extracts was purified by using HPLC method with a DEAE (anionic type), CN and ODS column. The purified compound remained stable at pH 3.0~6.0. but unstable above pH 6.5. It was stable at 100℃ for 4 hours, but still had about 80% of residual activity after treatment at 100℃ for 5 hours. In antimicrobial test, no inhibition was observed against Gram-positive and negative bacteria. This compound was stronger than that of commercial antioxidant showed that DPPH test, such as BHT, BHC at the same concentration (20 μg/ml).

Key words : *Rhus verniciflua* (RV), antioxidant substance, purification.

Introduction

The sap of *Rhus verniciflua*, an Anacardiaceae plant family, has been used as a surface coating material for wood, porcelain and metalwares for thousands of year in Korea, China and Japan, because it forms a brilliant film. These saps are rates composed of urushiol (60~65%), water (20~25%), plant gum (5~7%), glycoprotein (2~5%) and laccase enzyme (less than 1%)[1,2]. The natural antioxidants discovered recently have been expected to replace the synthetic antioxidants which are widely used at present time[3,4]. There is currently interest in free radical mediated damage associated with many diseases, and much effort has gone into the study of antioxidative substances especially of plant origin (vegetables, fruites, spices, herbs, etc) detected in foods, and of therapeutic effect against various diseases which are caused by *in vivo* radical reactions by ingestion in raw or after processing (heating, mixing, fermentation, etc)[5-9]. The Rhus verniciflua contains alkly(en)-catechol type allergens with a saturated or unsaturated alkly chain of 15 or 17 carbon atoms[10]. They have been used for preservation as an antiseptic or paint and as an agent for medical purposes. Korean have traditionally used the herb with chicken in a boiled form for folk medicinal purposes. But it has been recognized as an extremely active allergen causing skin reactions similar to poison ivy[11]. The allergic contact dermatitis induced by the urushiol is known to be mediated by T lymphocytes which specifically recognize the hapten urushiol[12]. Therefore, direct use of this plant as a medicinal purpose might imply

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a considerable hazard in Korea. In this paper, using the established method for the detoxification from the stem bark of *Rhus verniciflua*, an strong anti-oxidant substance was isolated and characterized.

Materials and Methods

1. Chemical and Instrument

Reagent grade butylated hydroxy anisole (BHA), butylated hydroxytoluene (BHC) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (USA). The purity of each reagent was more than 98%. The other reagents were of analytical grade, and all solvent used were of HPLC grade (Fisher, USA). The Mass spectra were measured the molecular weights of compound with positive Electron Spray Impact (+ ESI) Mass Spectrum (MS) at 70eV (VG Biotech. Co., USA, Korea Basic Science Institute, Daejeon, Korea). An UV-visible absorbtion spectrum in H_2O was recorded on a spectrophotometer (UV 120A, Shimazu Co., Japan). HPLC was performed on a Gilson Model 505 (France) with photodiode array UV-VIS detector.

2. Sample Preparation

Naturally grown *Rhus verniciflua* (RV) was obtained from Wonju, Kwangwon province in June 1999. To detoxicate from preparation of sample (RV) was carried out according to the method of Kim[13]. The stem bark of RV (150g) were soaked and autoclaved in 15L of water at 125℃ for 3 hrs. The water extract was filtered by detoxification extract method[14] with glass fiber filter (extra thick, Gelman, USA) and the supernatant was collected. The supernatant obtained was concentrated with a rotary evaporator under reduced pressure. Urushiol, gum and enzyme was removed from the concentrate by filtration and this filtrated solution was adjusted to pH 5.0 with 30% acetic acid.

3. Purification

The filtrated crude solution was loaded by HPLC with a DEAE column (8×75mm, 5 μm, 120 Å, YMC IES-AX, Japan) previously equilibrated with 20mM sodium acetate buffer (pH 5.0), and was washed with 20ml the same buffer solution. The active fraction was eluted with a linear gradient of NaCl from 0 to 0.5M in the same buffer at a flow rate of 1.0ml/min and the fractions monitored by HPLC. The active fractions were pooled and concentrated under reduced pressure by speed vac (Hanil, Korea) was dissolved in a small amount of 1mM acetic acid. A CN column (4.6×250mm, 5 μm, YMC Co., Japan) was used for reversed phase chromatography and was eluted with 1mM acetic acid at a flow rate 1 ml/min. The active elute was concentrated in vacuo and HPLC was performed using reversed phase column (hydrosphere C^{18}, 4.6×250mm, 5 μm, YMC Co. Japan) with 30% acetonitrile containing 1mM acetic acid as elution solvent. Active fractions were collected and lyophilized to obtain white powder.

4. DPPH Radical Scavenging Activity

DPPH (diphenypricryl hydrazyl) assay[15] measures hydrogen atom-donating activity and hence provides a measure of free radical scavenging anti-oxidant activity. DPPH, a purple-colored stable free radical, is reduced to yellow-colored diphenylpicryl hydrazine by antioxidants to deducing agents. Anti-oxidative effects of the water extract from RV were measured by DPPH assay. Twenty microliters of the extract was added to 1ml of 100mM DPPH solution in ethanol. The mixture was shaken and left to stand for 10min at room temperature. The amount of residual DPPH was determined through absorbance at 515nm. Control was made by adding 20 μl of distilled water instead of the sample solution. The DPPH radical scavenging activity of the purified compound was compared with BHA (butylate hydoxyl anisole) in ethanol and the electron donation ability (EDA), according to the following equation[3].

$$EDA\ (\%) = [\,1 - (A / B)\,] \times 100$$

A : Absorbance of sample

B : Absorbance of control

5. TLC

The homogeneity of the purified compound was identified by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Precoated TLC-plate (F_{254}, Merck) spotted with sample: n-butanol : methanol : H_2O (4:1:2), n-butanol : acetic acid : H_2O (3:1:1), ethanol : ether (1:1). After the development, the TLC plate was dried and exposed at 254m under uv light (Model CN-15LC, Vilber, Lourmat, France) to confirm the separation or by spraying with a 10% $FeCl_3$ in EtOH.

Results and Discussion

1. Isolation of the Active Compound from *Rhus verniciflua* (RV)

The crude water extract (detoxification) was purified by the procedure summarized in Fig. 1. An ion exchange chromatography was performed by HPLC with a DEAE column (8.0×75mm). The crude active fraction was charged, washed with buffer for 5 min and eluted with a linear gradient of NaCl from 0 to 0.5M NaCl for 30min. The elution time was 19.10 min (flow rate: 1 ml/min). The active dark brown fraction was concentration and was performed by HPLC with a CN column (4.6×250mm) at a flow rate of 1 ml/min for 15 min. The active fraction was eluted at 8.05 min in CN HPLC. The pooled active elute was concentrated *in vacuo* and the residue was dissolved in a small amount of in 1mM acetic acid, and further purified by HPLC on hydrosphere C^{18} column using 30% acetonitrile in 1mM aqueous acetic acid as a mobile phase at a flow rate of 1 ml/min for 13min. The elution time was 8.70 min in reversed phase HPLC (Fig. 2.). Finally, an active compound was obtained as a white needle shape powder (31mg).

Rhus verniciflua (150g, stem bark of RV)
↓← extracted with H_2O
↓← concentrate in vacuo
Crude filtrate
↓
DEAE HPLC
↓← gradient 0~0.5M NaCl in 20mM sod. citrate buffer (pH 5.0)
CN HPLC
↓← eluated with 1mM aqueous acetic acid
ODS HPLC
↓← eluated with 30% acetonitrile in 1mM aqueous acetic acid
white needle powder

Fig. 1. Purification scheme of the antioxidative compound from *Rhus verniciflua*.

2. Stability of pH and Temperature

The effects of temperature and pH on the purified compound were investigated. To measure the pH stability of the compound, antioxidative activity was

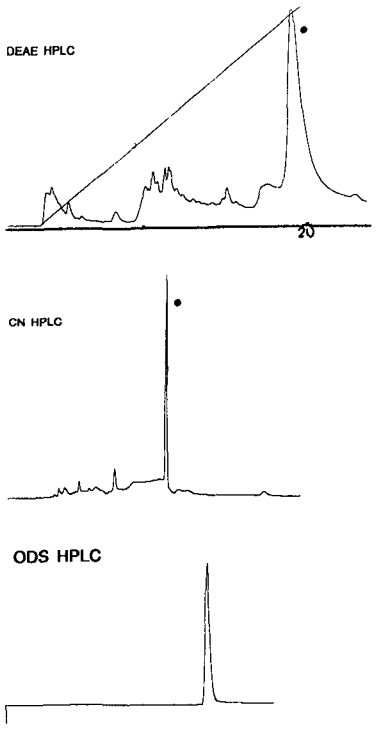

Fig. 2. HPLC chromatograms of the purified compound. DEAE HPLC : DEAE column (8×75mm, 5 μm, 120Å, YMC IES-AX, Japan), Elution solvent: 0~0.5M NaCl gradient system at pH 5.0, flow rate: 1ml/min, Elution time: 30 min, Detection : 280nm, CN HPLC: CN column (4.6×250mm, 5 μm, YMC Co., Japan), Elution solvent: 1mM aqueous acetic acid at pH 5.0, flow rate: 1ml/min, Elution time: 15min, Detection: 280nm, ODS HPLC: ODS column (hydrosphere C^{18}, 4.6×250mm, 5 μm, YMC Co. Japan), Elution solvent: 30% acetonitrile in 1 mM aqueous acetic acid solution flow rate: 1ml /min, Elution time: 15min, Detection: 280nm.

determined at different pH range using 20mM buffer solution (Britton and Robinson buffer, range pH 2.5 ~11.0). This compound remained stable at pH 3.0~6.0, but it was unstable above pH 6.5 (Fig. 3). It was heat stable at 100℃ for 4 hours, but it had about 80% residual activity after treatment for 5 hours at 100℃ (Fig. 4).

3. Characteristics

The purified compound was detected as one spot on the TLC (F_{254}, Merck) with various solvent system. The R_f of the compound on the thin layer chromatography under uv light (at 254nm) were 0.54 in n-butanol : methanol : H_2O (4:1:2), 0.67 in n-butanol : acetic acid : H_2O (3:1:1), 0.70 in ethanol : ether (1:1). Positive result was obtained in

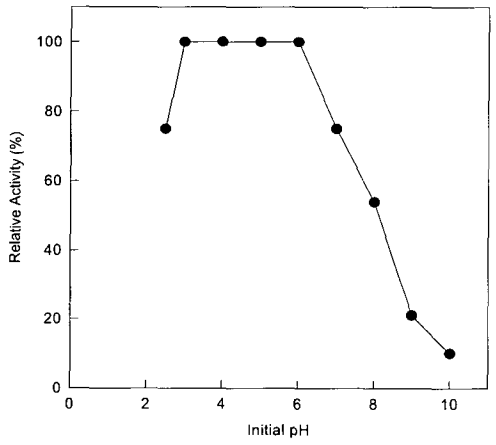

Fig. 3. pH stability of the purified compound.

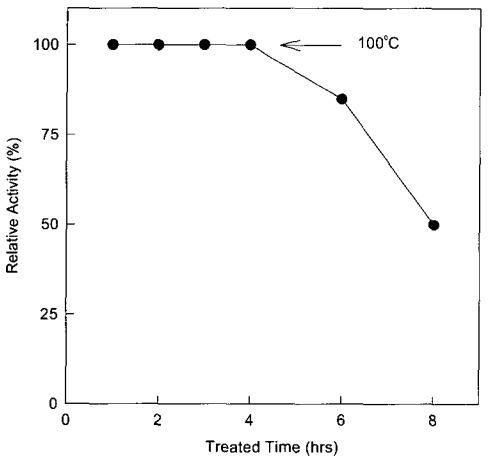

Fig. 4. Heat stability of the purified compound.

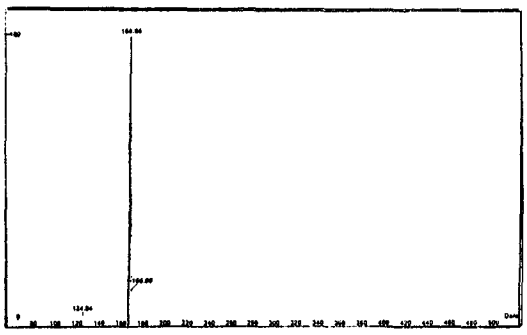

Fig. 5. ESI (M-H-) mass spectrum.

Table 1. Electron donating ability (EDA) of the purified compound isolated from *Rhus verniciflua*

Sample	EDA (%, 20 μg/ml) *
BHT	39.3
BHA	48.7
Ascorbic acid	62.7
Purified compound	58.5

* Each samples were dissolved in ethanol solvent. EDA assay was performed by DPPH method

$FeCl_3$ test. This compound was soluble in H_2O, methanol, acetonitrile and ethanol, but insoluble in chloroform and n-hexane. The uv-vis spectra of the compound showed two absorption in the ranges of 200~800nm. The molecular weights of the purified compound were determined from the ESI (electronic spray impact) positive(+) mass spectrometery as 168.95 m/z ($M-H^-$) (Fig. 5). The purified compound (32 μg/ml) was tested for their antimicrobial activity against various Gram-positive (*Bacillus cereus*, *Staphylococcus aureus* ATCC 25923) and Gram-negative bacteria (*Entrobacter aerogenes*, *Shigella dysenteriae*, *Escherichia coli* ATCC 25922, *Pseudomonas auroginosa*, *Salmonella typhi* 19430, *Klebsiella pneumoniae*) by the paper disc method[16]. No inhibition was observed against Gram-positive and negative bacteria.

4. Antioxidant Activity

For the determination of the electron donation ability (EDA), each samples containing 20 μg/ml in ethanol was carried out according to the DPPH method. The EDA of the compound from RV was

compared to BHT, BHA, ascorbic acid. The EDA of these four pure compounds are shown in Table 2. The EDA of the purified compound showed stronger activity than BHA, BHC which are currently widely used to retard the onset of rancidity by preventing oxidative degradation of lipids, but it did not exhibit higher antioxidative activity than ascorbic acid, which is known as a strong water soluble antioxidant.

요 약

옻나무는 독성이 있는 금기물질임에도 불구하고 한국에서는 민간요법으로 옻닭 등의 가공식품 형태로 사용하여 왔다. 항산화 물질은 옻나무 껍질에 물을 가하여 추출하여 분리하였다. 물 추출물은 DEAE, CN, ODS 컬럼을 사용하여 HPLC로 정제하였다. 정제된 순수물질 안정성은 pH 3.0~6.0의 산성영역에서는 안정하였으나, pH 6.5 이상에서는 불안정하였으며, 100 ℃에서 5시간 열처리하여도 80%의 활성을 보였다. 항균력 실험에서는 그람양성, 음성 균주에 대하여 항균활성이 없었다. 항산화력은 DPPH 방법으로 조사한 결과 동일한 농도에서 (20 μg/ml) BHT, BHC 보다 좋았으나, ascorbic acid 보다 낮았다.

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