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Identification of Antioxidative Component from Stem Bark of *Rhus verniciflua*

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옻나무 껍질에서 분리한 항산화물질의 성분

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Abstract

An antioxidant compound was obtained from the water extract of the stem bark from *Rhus verniciflua*, which has been used in traditional folk remedies. The compound was purified by HPLC, using DEAE, CN and ODS columns. The chemical structure of the compound was identified as gallic acid (3,4,5-hydroxylbenzoic acid) by spectral data including UV, IR, EI (HR)-MS, ^{1}H-NMR, ^{13}C-NMR and elemental analyzer. This compound was found show cytotoxicity against HeLa cell (IC_{50} : 8.5μg/ml).

Key words : *Rhus verniciflua* (RV), structure, gallic acid, antioxidant.

Introduction

Plant polyphenols are well known to show biological and pharmacological activity, such as antimutagenicity, anticarcinogenicity, antiviral and antioxidative activity [1~4]. Many agents with antioxidative activity have been shown to be effective in suppressing cancer expansion in a rodent hepatocarcinogenesis model[5,6]. Antioxidants are considered to act as anticarcinogens to antimutagens by interacting with carcinogen[7~9]. Recently, a concept that agents for cancer therapy are required to act as a stimulator of cell death in cancer cells by apoptosis rather than as a growth inhibitor has been proposed and widely accepted[10]. In addition, agents with selectivity for cancer cells rather than normal cells, for an originator of cancer for a specified phase to the cell cycle must be further explored[11]. Many scientific studies provide support for the benefits of flavonoids, which abundantly exist in wine, tea and to human health by invalidating the adverse effects of active oxygen species[12,13]. As preventive and therapeutic measures to deal with diseases caused by oxidative stress become more common, the opportunities for the biochemical and clinical application of natural antioxidants increase. Consequently, numerous compounds have been used to treat diseases by reducing oxidative stress[14]. *Rhus verniciflua* (RV) is traditionally employed for both the preservation of antique furniture, as well as for herbal medicine in Korea. Recently, the various biological activities of RV were reported by a number of investigators[6~8,15,16]. The crude RV was shown to have an antioxidant effect against hydroxyl radicals, the antiproliferative activity in human cancer cell lines, and stimulating activity for the activity of cell associated detoxifying enzymes in hepatocytes[6]. interest

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has been focused on Korean Herbal medicines that are known to contain high content of polyphenol compounds. In previous study[17], we isolated an antioxidant substance from the stem bark of *Rhus verniciflua*. This paper describes the structural elucidation, which an active compound was isolated and identified.

Materials and Methods

1. Isolation of the Active Compound

Naturally grown *Rhus verniciflua* (RV) was obtained from Wonju, Kwangwon province, Korea, in June 1999. To detoxicate from preparation of RV carried out according to Kim's method[18,19]. The stem of RV was autoclaved in 15L of water at 125℃ for 3 hrs. The water extract was filtered and the supernatant was collected. The supernatant was concentrated with a rotary evaporator under reduced pressure. The isolation of an active compound was performed according to the method previously describes[17]. The crude solution was purified by HPLC with a DEAE column(8×75mm, 5μm, 120Å, YMC IES-AX, Japan), CN column (4.6×250mm, 5μm, YMC Co., Japan) and reversed phase column (hydrosphere C_{18}, 4.6×250mm, 5μm, YMC Co. Japan), consecutively.

2. Instrumental Analysis

The ultraviolet spectrum was monitored by a UV-160A UV-spectrophotometer(Shimadzu, Japan). The infrared spectrum was recorded on the Shimadzu IR 435 spectrophotometer(Shimadzu, Japan), Nuclear magnetic resonance(400 MHz for ^{1}H-NMR, 100 MHz for ^{13}C-NMR) spectra were investigated by a Bruker DPX-600 spectrophotometer(Bruker, Germany) using tetramethylsilane as the internal standard. Elemental analyzer are used the CE Model Flash EA 1112(CE Instruments, Italy) and mass spectra were obtained by a Jeol JMS-700 high resolution mass spectrometer, provided by Korea Basic Science Institute(Seoul, Korea). The analytical silica gel TLC (Merck, Kiesel gel 60 F_{254}, 0.25mm) plates were used without activation. The HPLC (Gilson, France) was performed on a DEAE, CN and ODS column (YMC co, Japan) by monitoring with a UV detector.

3. Cytotoxicity Assay

0.1ml of cell suspension at the concentration of 3×10^{4} ~ 1.5×10^{5} cells/ml was incubated in a 96 multi-well plate and cultured for 24 hours. After washing the cells with PBS (phosphate buffer saline), 0.1ml of medium containing purified compound at appropriate concentrations was added and the mixture was discarded and the remaining sample was removed by through washing with fresh medium. The surviving cell number was determined by the MTT method[20]. In the case of HL-60RG cells, cytotoxicity was determined by the trypan blue dye exclusion method.

Results

1. Structure Determination

Physico-chemical properties of the purified compound from RV are summarized in Table 1. The ultraviolet absorption spectrum of the compound dissolved in methanol showed two maximum absorption peaks at 210nm and 270nm. Positive result was obtained in 1% $FeCl_3$ test. The Infrared spectrum showed a strong aromatic C-H asymmetric stretching absorption at 3044~3065cm $^{-1}$. A broad aromatic −OH stretching (3044~3366cm $^{-1}$) band and C=C stretching vibration at 1469cm $^{-1}$ were also present. The character C=O absorption band of the carboxyl group appeared at 1703cm $^{-1}$. The ^{1}H spectrum revealed the compound contained an aromatic proton (δ 6.92 ppm). The ^{13}C-NMR showed that there was four aromatic quaternary carbon (δ 146,21 to 109.54 ppm) and a carbonyl group (δ 168.25 ppm) (Fig. 1).

Table 1. Physico-chemical properties of purified compound

Property	Purified compound
Appearance	White needle power
EI-MS (m/z)	170
Molecular	$C_7H_6O_5$
UV λmax (H_2O)	210, 270
IR (KBr) cm^{-1}	3065, 3044, 2657, 1551, 1544, 1469, 1450,
Color reaction	$FeCl_3$
Rf values*	0.54
Solubility	
soluble	H_2O, MeOH, EtOH
insoluble	Benzene, Hexane

* n-butanol : methanol : H_2O (4:1:2)

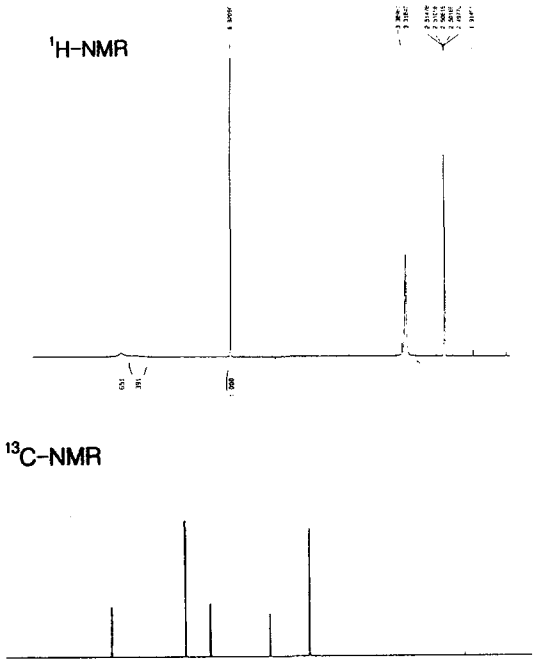

Fig. 1. ^{1}H, ^{13}C-NMR spectrum.

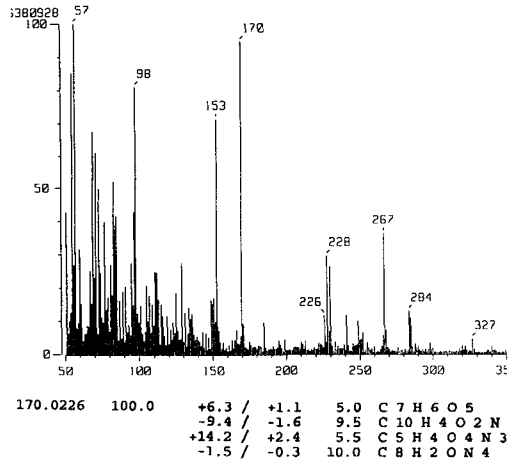

Fig. 2. EI (+, HR) MS chromatograms.

The results of a carbon, hydrogen and oxygen elements analysis have shown to contain no elements other than C(41.43999%), H(6.33241%) and O(48.05046 %). Fig. 2 shows the HR-EI positive(high resolution- electron impact) mass spectrum(70 eV) of this molecule. Major ions appeared at m/z 170 and 153. These corresponded to a molecular ion(M^+) sequential loss of −OH group. The elemental composition of the HR-EI mass spectrum at m/z 170 was estimated the empirical formula as

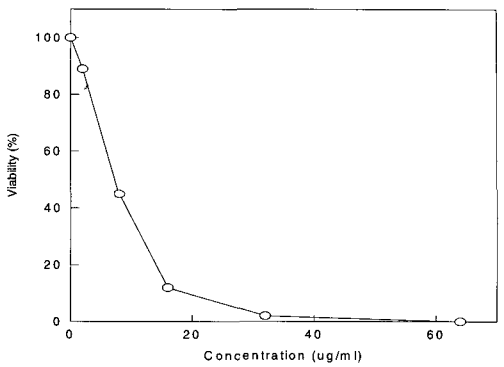

Fig. 3. Cytotoxicity of a purified compound against cancer cell.

Cell was incubated with a purified compound at various concentrations for 48 hr. After removing a compound by washing with fresh medium, the viable number was determination by MTT method.

$C_7H_6O_5$, $C_{10}H_4O_2N_1$, $C_5H_4O_4N_3$ and $C_8H_2O_1N_4$. Based on the EI positive mass spectrum(high resolution) in combination with ^{1}H and ^{13}C-NMR spectral date, the molecular weight and formula of the isolated compound were found to be 170 and $C_7H_6O_5$, respectively.

This compound was proved to be gallic acid (3,4,5-hydroxylbenzoic acid) by comparison of the spectral data with published data[21,22].

2. Cytotoxicity

In our phytochemical study, we isolated gallic acid as a component with anti-cancer activity demonstrated by cytotoxicity from Rhus stem (*Rhus verniciflua*), which has been used as an ingredient in traditional Korea medicine for the therapy of gastrointestinal trouble. Various anticancer agents are known to show cytotoxicity or cell growth inhibitory activity against cultured cells *in vitro*. we examined the cytotoxicity of the purified compound to cancer cell(HL-60RG, 6×10^4 cells/ml), The IC_{50} for HeLa was 8.5μg/ml(Fig. 3).

Discussion

Recently, the biological, pharmacological and medical properties of plant polyphenols have extensively researched[1,3,5]. They have been reported to possess various biological activities, such as antitumor, antibacterial,

enzyme inhibitory and antimutagenic properties[5,15]. In addition, consumption of tea and wine rich in polyphenols has been found to be protective against cardiovascular diseases, oxidation of low-density lipoprotein, and certain forms of cancer[23~25]. These protective effects have been believed to be due to the antioxidant activity of polyphenols because, in general, oxidative damage to the living body is mainly induced by reactive oxygen species and causes various diseases[26]. Thus the efficiency of polyphenols as antioxidants greatly depends on their chemical structure, mainly phenolic hydroxyl substitutions.

Rhus verniciflua belongs to Anacardiaceae family is a plant indigenous to Korea and used traditionally in herbal medicines[2,16]. The lacquer tree RV is the common source for lacquer. The stem bark of lacquer trees grown for several years is removed, the trunk is cut, and the sap that exudes is collected. The sap is a water/oil emulsion, and the "oily" portion consists of about 60~65% of urushiol. The sap also consists of about 20~25% of water and about 10% of water soluble plant gums, mono-, oligo- and polysaccharides and small amounts of enzymes such as stellacyanin and peroxidase, but most importantly, the enzyme laccase[27]. Laccase is the most important enzyme for polymerization of urushiol[28]. It is a copper-glycoprotein enzyme, has a highly specific activity, and causes the oxidation of *ortho* and *para*-dehydroxybenzene derivatives, the active components of urushiol. Urushiol from RV consists of about 80% of a mixture of catechol derivatives substituted in 3-position with C_{15} hydrocarbon carbon chains: about 70% are trienes and 20% are monoenes with some dienes also present[29]. The composition of the urushiol may vary depending on growing conditions of the *Rhus verniciflua* tree and on the season. The components with an unsaturated side chain are important contributors to the industrially useful polymerization properties of these natural products.

In Korea, for a long time RV has traditionally been used as an herbal medicines plant. It is known to contain various biological activities[30]. However, the most common way of the Rhus tree ingestion is "*Rhus* chicken" which has been used as a folk remedy for those who have functional gastrointestinal trouble such as indigestion, loose stool and bloatedness. Some Korean still enjoys eating the early shoots of the *Rhus* tree in the spring. But there are many cases with systemic contact dermatitis (SCD) due to "*Rhus* chicken". In SCD, an orally administered contact allergen may reach the skin through the blood circulation. The allergic contact dermatitis induced by the urushiol is known to be mediated by T lymphocytes that specifically recognize as a hapten[31].

Recently, the various biological activities of RV were reported that phenolic compounds from organic solvent extract of RV has a strong antioxidant, major components being gallic acid, butin, butein, fisetin, fustin, quercetin, sulfuretin[21,22,30]. Gallic acid is naturally occurring plant phenol, which was been reported to show a number of biological activities[10]. Although gallic acid has three hydroxyl groups and one carboxyl group, structure-activity relationship studies reveal that methylation of the phenolic hydroxyl group and esterification of the carboxyl group markedly reduces cytotoxic activity[11,12]. A recent study has suggested that apoptosis inducing activity might be paralleled by the intensity of both the gallate radical and oxidation potential, although such radicals were determined at pH 9.0, but not under physiological conditions[5]. Furthermore, the distinctive characteristic of gallic acid-induced cell death is that normal cells such as primary cultured rat hepatocures and macrophages are resistant to gallic acid, while endothelial cells and fibroblasts are less sensitive to gallic acid than cancer cells[12,32].

Further mechanistic and clinical studies will focus in isolating the mechanisms underlying the biochemical and biomolecular roles of various active "*Rhus verniciflua*" compounds.

요 약

옻나무(*Rhus verniciflua*)는 한국에서 위장병, 변비, 어혈, 구충 등에 효과가 있다고 알려져 있어 민간요법과 보양식품으로 옻닭으로 식용하고 있으나 독성(allergy, 옻 오름)이 심각하여 주의를 요하는 식품이다. 옻의 독성 제거방법을 사용하여 옻나무 껍질을 물로 추출한 후 항산화 작용을 갖는 수용성 물질을 분리하여 물리, 화학적 특성과 기기 분석을 통하여 (UV, IR, EI (HR)-MS, ^{1}H-NMR, ^{13}C-NMR, elemental analyzer) 구조 분석한 결과 gallic acid (3,4,5-hydroxylbenzoic acid) 임을 확인하였다.

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