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Antioxidative and Antimicrobial Activity of Epicatechin Isolated from Leaves of Loquat (*Eriobotrya japonica*)

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Abstract

Methanol extracts were prepared from loquat leaves (2 kg) and successively fractionated with hexane, chloroform, ethyl acetate, butanol, and water solvents. The ethyl acetate fraction exhibited the highest antioxidative and antimicrobial activities. Therefore, the ethyl acetate fraction was purified and a chemical structure was identified by ^{1}H and ^{13}C-NMR spectra, FT-IR and EI/MS spectroscopies. The isolated antioxidative and antimicrobial substance was identified as epicatechin.

Key words: loquat, antioxidative, antimicrobial activities, epicatechin

INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), alkoxyl radical ($RO\cdot$), and peroxy radical ($ROO\cdot$) are generated from the autoxidation of lipids, as well as reactive nitrogen species (RNS) (1,2). Formations of these excess ROS and RNS by UV irradiation, smoking and drug metabolism are likely to damage several cellular components such as lipids, proteins, nucleic acid, and DNAs through oxidation and nitration processes (3). In addition, these reactive species cause inflammation or lesions on various organs and are associated with various degenerative diseases, including: cancer, aging, arteriosclerosis, and rheumatism (4-8). Plants contain a wide variety of chemicals that have potent antioxidant activity. The best known phytochemical antioxidants are traditional nutrients, such as β-carotene, ascorbic acid, and α-tocopherol. However, there is growing evidence that a significant portion of the antioxidant capacity of many food plants is due to compounds other than the traditional vitamins (9). Recently, researchers have sought to isolate powerful and nontoxic natural antioxidant from edible and medicinal plants not only to prevent these human disorders and food deterioration by autoxidation and lipid peroxidation, but also to replace synthetic antioxidants, which may be toxic to the lungs and liver (10). The loquat (Rosaceae), is a small tree native to Japan and China that is widely cultivated for its succulent fruit. Its leaves have been used as a folk medicine for treatment of chronic bronchitis, coughs, phlegm, high fever, and ulcers in Japan and other Asian countries (11). A number of triterpenoids with anti-imflammatory (12) and antiviral effects (13), triterpene (14,15), a small amount of amygdalin (12), several polyphenolic constituents (16,17) have been identified in loquat. However, little information is available about the antioxidant and antimicrobial activity of loquat leaves. Therefore, the objective of this study was to investigate the antioxidative and antimicrobial activities of extracts from loquat leaves. In addition, a compound was isolated and identified from the active ethyl acetate fraction of loquat leaves.

MATERIALS AND METHODS

Instruments

IR spectra were obtained with a Hitachi 270-50 spectrophotometer, and MS data were measured with a Jeol JMS-700 spectrometer in the EI mode. ^1H- and ^{13}C-NMR along with 2D-NMR data were obtained with a Brucker AM 500 (^1H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz) spectrometer in DMSO-d_6.

DPPH radical scavenging

The effect of epicatechin on DPPH radical scavenging was estimated according to the method of Blois (18). Epicatechin (0.975~500 μg) in 1 mL of distilled water was added to an ethanol solution of DPPH (1 mM, 4 mL). The mixture was shaken and left to stand at room temperature for 30 min; the absorbance of the resulting solution was measured spectrophotometrically at 517 nm.

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Reducing power

Epicatechin (31.25 ~ 500 μg) in 1 mL of distilled water was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%), then the mixture was incubated at 50°C for 30 min. Afterward, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. Finally, 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm (19). Increased absorbance of the reaction mixture indicated increased reducing power.

Antimicrobial activity

Food spoilage bacteria strains were obtained from KCCM (Korean Culture of Microorganism). Gram positive bacteria used were *Bacillus subtilis* (ATCC 9372), *Bacillus cereus* (ATCC 27348), *Staphylococcus aureus* (ATCC 13301), *Listeria monocytogenes* (ATCC 19111). Gram-negative bacteria used were *Escherichia coli* (ATCC 15489), *Salmonella* Typhimurium (ATCC 14038), *Vibrio parahaemolyticus* (ATCC 33844), *Pseudomonas aeruginosa* (ATCC 10490). The five strains (DCDC059, DCDC060, ACH5, KSC370, KSC109) of *E. coli* O-157:H7 were obtained from Prof. Y. H. Kim of the Department of Veterinary Medicine at Gyeongsang National University. Antimicrobial activity were determined by the disc diffusion method (20). A weighed aliquot of the dry sample was dissolved in methanol and 20 μL portion of these solution was placed on 8 mm paper discs (Adventec, 8-mm diameter and 1-mm thickness, Toyo Roshi) to give concentrations of 1, 5, 10 and 20 mg for epicatechin. The disc was then placed on agar plate seeded with microorganism and after incubation for 24 h, and the zone of inhibition was measured.

Extraction and isolation

Loquat (*Eriobotrya japonica*) leaves (2 kg) belonging to the family Rosaceae, were collected from a plantation at Geoje, Korea in june 2002. They were ground in a blender, extracted three times with methanol (5 L) each for 3 days at room temperature. The combined methanol solution was concentrated and dried under reduced pressure at a temperature no higher than 45°C. The methanol extract (30 g) was sequentially extracted with hexane (1.68 g), chloroform (0.82 g), ethyl acetate (1.06 g), butanol (11.44 g) and water extracts (15.0 g) for a subsequent bioassay. The organic solvent extracts were concentrated to dryness *in vacuo* at 45°C, and the water extract was freeze dried. The ethyl acetate fraction (1.06 g) was chromatographed on a silica gel column (Merck 70 ~ 230 mesh, 500 g, 70 cm × 5.5 I.D.) and successively eluted with a stepwise gradient of chloroform/methanol (99/1→0/1). Active fraction F13 (0.39 g) was combined and applied to a silica gel column, eluting with a dichloromethane-ethanol mixture of increasing polarity (49/1 →1/1), to give six major subfraction (F13-1 through F13-6), based on a comparison of the TLC profiles after examination with shortwave UV light (254 nm) and by spraying with 10% v/v sulfuric acid in water. F13-4 was purified on a Sephadex LH-20 resin column chromatograph using methanol. For further separation of the biologically active substance, a Waters Delta Prep 4000 HPLC was used. The column [300 mm × 3.9 mm PREP-ODS column (Hewlett Packard)] was eluted with 30% methanol at a flow rate of 5 mL/min, and the eluate was measured at 254 nm.

Compound 1 (35 mg) was isolated by recrystallization with MeOH. The homogeneity of compound 1 was demonstrated by TLC in a developing solvent system of $CHCl_3$-MeOH (3:1) (Rf=0.39).

Compound 1 (epicatechin). IR ν_{max} (KBr) cm^{-1}: 3411, 1625, 1521, 1469; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 2.50 (1H, dd, J=4.54, 16.42 Hz, H-4a), 2.70 (1H, dd, J=8.18, 11.71 Hz, H-4b), 4.03 (1H, S, H-3), 4.75 (1H, S, H-2), 5.74 (1H, d, J=2.18 Hz, H-8), 5.91 (1H, d, J=2.23 Hz, H-6), 6.68 (1H, m, H-2'), 6.69 (1H, m, H-6'), 6.91 (1H, S, H-3'); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 28.51 (C-4), 65.33 (C-3), 78.45 (C-2), 94.53 (C-8), 95.56 (C-6), 98.93 (C-10), 115.17 (C-2'), 115.30 (C-5'), 118.35 (C-6'), 131.00 (C-1'), 144.81 (C-3'), 144.87 (C-4'), 156.13 (C-5), 156.62 (C-7), 156.87 (C-9).

RESULTS AND DISCUSSION

During the preliminary screening for antioxidative and antimicrobial activities, we observed that the methanol extract of loquat leaves showed significant antioxidative and antimicrobial activities. In fractionation guided by antioxidative and antimicrobial activities, the ethyl acetate fraction was purified by silica gel column chromatography and the isolated was bioassayed. Finally, an active compound was isolated. The chemical structure was elucidated as epicatechin through spectroscopic analysis including IR and NMR.

DPPH radical scavenging

The profile of scavenging activity of epicatechin on the DPPH is shown in Fig. 1. The radical scavenging activity of epicatechin on DPPH increased with increasing concentrations of epicatechin. Epicatechin exhibited 56.21 and 83.66% radical scavenging activity at a concentration of 3.125 and 6.25 μg/mL, respectively (IC_{50}= 2.94 μg/mL). However, BHA (IC_{50}=2.42 μg/mL) and α-tocopherol (IC_{50}=2.81 μg/mL) showed 96% radical scavenging activity at a concentration of 5.24 and 12.57 μg/mL,

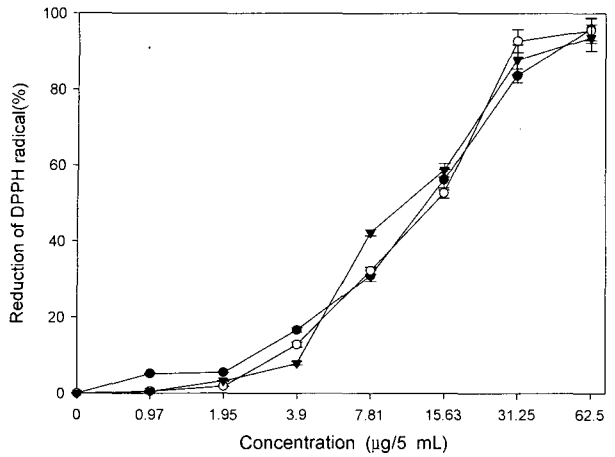

Fig. 1. DPPH free radical scavenging activity of epicatechin (●) isolated from loquat leaves, BHA (○) and α-tocopherol (▼).

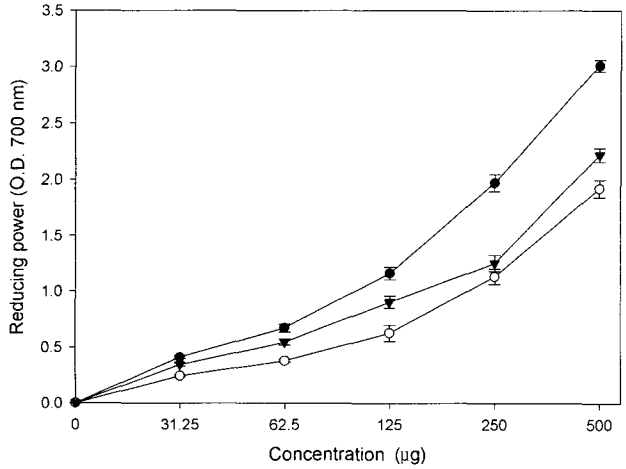

Fig. 2. Reducing power of epicatechin (●) isolated from loquat leaves, BHA (○) and α-tocopherol (▼).

respectively. This result shows that epicatechin is also a free radical inhibitor, particularly of the peroxy radical, which is the major propagator of the fatty acid autoxidation cascade, thus terminating the chain reaction. The antioxidant epicatechin was also isolated from loquat leaves. Epicatechin, a well-known antioxidant, has been found in many edible plants, such as green tea (21,22).

Reducing power

Like antioxidant activity, the reducing power of the epicatechin was also concentration-dependent. Hence, the reducing power of epicatechin increased as the amount of epicatechin increased (Fig. 2). Even in the presence of 50 μg of epicatechin, the reducing power was significantly higher than it was for the control in which there was no epicatechin. The reducing power at 300 μg of epicatechin was significantly higher than at 50, 100 and 200 μg of epicatechin, but there was no significant difference between 300 μg and 400 μg of epicatechin.

Table 1. Antimicrobial activity (zone of inhibition)[1] of epicatechin against food spoilage bacteria *in vitro*

Bacteria	1 mg	5 mg	10 mg	20 mg
Bacillus cereus	-[2]	13	15	18
Bacillus subtilis	-	10	11	14
Staphylococcus aureus	-	13	15	17
Listeria monocytogenes	-	12	14	17
E. coli	-	10	11	13
E. coli O-157 (LCDC 059)	-	10	12	14
E. coli O-157 (LCDC 060)	-	10	11	13
E. coli O-157 (ACH 5)	-	10	11	13
E. coli O-157 (KSC 370)	-	10	11	13
E. coli O-157 (KSC 109)	-	10	11	13
Salmonella Typhimurium	-	10	11	13
Pseudomonas aeruginosa	-	11	13	15
Vibrio parahaemolyticus	-	10	12	14

[1]Use 1, 5, 10, and 20 mg/paper disk for each compounds.
[2]No inhibition.

Antimicrobial activity

Four gram-positive and nine gram-negative bacteria were used for testing antimicrobial activity of epicatechin from loquat leaves (Table 1).

The epicatechin was demonstrated to be effective against all the tested microorganisms. Epicatechin was especially effective against three bacterial strains: *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*. Lee et al. (23) studied the antimicrobial activity of epicatechin in *Ulmus davidiana*. The results of this study show that the loquat extract and epicatechin can be used as an easily accessible source of natural antioxidants and as a possible food supplement or in the pharmaceutical industry.

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