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Communication

Antiglycation and antioxidant activities of oxyresveratrol extracted from the heartwood of *Artocarpus lakoocha* Roxb.

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Abstract: From the heartwood of *Artocarpus lakoocha,* oxyresveratrol was isolated with a yield of 10%. The isolated oxyresveratrol showed strong antiglycation and antioxidant activities. The IC_{50} value for antiglycation was 2.0±0.03 μg/ml (five times higher than that of aminoguanidine), and the IC_{50} values for antioxidation were 0.1±0.01 mg/ml (DPPH method) and 0.43±0.03 mg/ml (TBARS method), which were nearly twice as strong as those of resveratrol.

Keywords: *Artocarpus lakoocha,* antioxidant, antiglycation, oxyresveratrol

INTRODUCTION

The *Artocarpus* species are rich in phenolic compounds including flavonoids, stilbenoids and arylbenzofurons [1]. *Artocarpus lakoocha* Roxb. has been known for its high content of stilbenoids such as pinostilbene, desoxyrhapontigenin, pterostilbene, resveratrol and oxyresveratrol [2-10].

In our previous study [11], fifteen medicinal plant extracts were investigated for total phenolic content, free radical scavenging activities by DPPH and ABTS methods, anti-lipid peroxidation activity by TBARS, and antiglycation activity. We found that the ethanolic extract of Thai *A. lakoocha* shows high antiglycation and antioxidant activities. Glycation is a reaction that takes place when the reducing monosaccharides such as fructose or glucose bind to the proteins or lipids without the involvement of an enzyme and result in the formation of rogue molecules known as advanced glycation endproducts (AGEs).

A. lakoocha, an indigenous plant known in Thai as 'Mahaad', has been used in Thai traditional medicine for centuries. However, there has been no report dealing with antigycation compounds in *A.*

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lakoocha. This fact has inspired us to undertake this study, which has been designed to isolate and characterise the constituent(s) which shows both the antiglycation and radical scavenging activities in *A. lakoocha*.

MATERIALS AND METHODS

Plant Material

The heartwood of *A. lakoocha* was bought from Thai Lanna Herbal Industry Company Ltd. (52/2 Tambol Maehorphra, Maetang District, Chiang Mai) in August, 2006. The pieces of the sample were compared under microscope with the specimens of *A.lakoocha* from the herbarium of the Faculty of Pharmacy, Chiang Mai University and the authenticity of the plant was established.

Chemicals

The highest grades of all chemicals were used. Absolute ethanol, acetic acid and methanol were purchased from Lab-scan Ltd., Ireland. Aminoguanidine, bovine serum albumin (BSA), acetone, 1-butanol, 2,2-diphenyl-1-picryl hydrazyl (DPPH), D-glucose, ethyl acetate , n-hexane, 2-thiobarbituric acid (TBA), t-octylphenoxypolyethoxyethanol (Triton X-100), sodium sulphate anhydrous and quercetin were purchased from Sigma-Aldrich Chemical Co. Ltd, USA. Chlorofrom, D-fructose and sulfuric acid were purchased from Merck, Germany. 2,2'-Azobis-2-methyl-propanimidamide dihydrochloride (AAPH), butylated hydroxytoluene (BHT) and cholesterol (from lanolin) were purchased from Wako Pure Chemical Industries, Japan, Parchem Trading Ltd., USA, and Fluka Chemie GmbH., Japan respectively. OxyResvenox (oxyresveratrol), resveratrol and phosphatidylcholine were purchased from Sabinsa Co. Ltd, Germany, Calbiochem, Darmstadt, Germany and Epikulon 200, Degussa, Germany respectively.

Protein Glycation Assay

Protein glycation was assayed according to the method of Kim and Kim [12]. The test substance dissolved in 95% ethanol to make up an appropriate concentration was added to a solution consisting of bovine serum albumin (20 mg/ml), D-fructose (235 mM) and D-glucose (235 mM) in potassium phosphate buffer (200 mM, pH 7.4). The mixture was filtered through Whatman no.4 filter paper and incubated at 60^{o}C for 30 hr. The fluorescence intensity was measured by a multimode detector spectrofluorometer (Model DTX 880, Beckman Coulter, Inc., USA) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. Aminoguanidine dissolved in 95% ethanol was used as a standard inhibitor [13].

Radical Scavenging Activity (DPPH Method)

The experiment was carried out according to the method of Liu et al. [14] with slight modification. Briefly, 20 µl of the test substance solution in 95% ethanol was mixed with 180 µl of 167 µM DPPH in methanol. The mixture was incubated at 37°C for 30 min and the absorbance was measured at 540 nm against reagent blank. The percentage of DPPH radical scavenging activity of the

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test sample was determined at five sample concentrations within the range of 10–90% reduction in absorbance and calculated as follows:

% DPPH radical-scavenging activity =

[(absorbance of control − absorbance of sample) × 100]/ absorbance of control,

where 150.3 μM DPPH solution in 95% ethanol was used as control.

Lipid Peroxidation Inhibitory Activity (TBARS Method)

Lipid peroxidation assay was carried out according to thiobarbituric acid-reactive substance (TBARS) method reported by Chung et al. [15] with slight modification. A solution of soybean phosphatidylcholine (247.36 mg) and cholesterol (30.92 mg) in chloroform was dried under reduced pressure in a rotary evaporator below 50°C to give a thin homogenous film, which was placed in a desiccator for 24 hr. The film was then dispersed in phosphate buffer saline (PBS, 0.2 M, pH 7.2, 20 ml) in a water bath at 50°C. The mixture was sonicated to give a homogeneous suspension of liposome and then the test sample solution was added to the liposome suspension. In the following steps, AAPH, TBA, Triton X-100 and BHT dissolved in 95% ethanol were used. Lipid peroxidation was initiated by adding 60 μl AAPH solution (0.07 M) to the liposome in PBS solution (600 μl). The mixture was incubated at 50°C for 24 hr. After incubation, 250 μl of TBA solution (0.6% w/v) and 100 μl of Triton X-100 (3% v/v) were added, and 500 μl of BHT solution (20% v/v) was then added to terminate the reaction. The samples were then heated at 90°C for 30 min, cooled and the absorbance of the upper organic layer was measured against reagent blank at 540 mm.

Thin-Layer Chromatography (TLC)

Silica gel 60 F_{254} plates (Merck) were used. The developing solvent was hexane/ethyl acetate/acetone (2:2:1 v/v/v). The spots were detected by UV irradiation (256 and 365 nm) and by heating after spraying with 20% sulfuric acid solution in 70% aqueous ethanol.

Isolation of Oxyresveratrol

The heartwood of *A. lakoocha* (2 kg.), which had been dried at 50°C in an air oven for 24 hr, was ground to powder and macerated for 6 hr in 95% ethanol three times. The alcoholic extracts were pooled and evaporated under reduced pressure below 45°C, whereupon 538 g of a crude extract was obtained. The crude extract (15.1 g) was suspended in 20% methanol (150 ml) and extracted with chloroform (300 ml). The aqueous layer was further extracted with ethyl acetate (3x300 ml). The ethyl acetate layer was evaporated to dryness below 45°C, whereupon 12.8 g of a powder was obtained. The powder (8 g) was dissolved in a small portion of ethyl acetate, silica gel (20g) was added and the mixture was dried under reduced pressure. The dried mixture was loaded onto a dry-packed silica gel column (7.5 cm i.d.×5.5cm) and developed successively using a solvent system of n-hexane/ethyl acetate/methanol (50:50:1.25 ; 48.75:48.75:2.5 ; 48.25:48.25:3.5 ; 46:46:8 ; 44:44:12). All the fractions were monitored by TLC and the fractions which showed similar TLC patterns were combined. A small portion of each combined fraction was tested for antiglycation and antioxidant activities. The fraction which showed highest activities was evaporated to dryness, whereupon 5.2 g of a powder was obtained. It was further purified by a silica gel column (4.5 cm i.d.×15 cm) using a solvent system of hexane/ethyl

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acetate/methanol (2:2:0.05 v/v/v), whereupon a pure solid (AE5-3) was obtained (3.6 g, 10.3% overall from heartwood).

High Performance Liquid Chromatography (HPLC)

A reverse phase HPLC of the crude extract, the final pure solid (AE5-3) and standard oxyresveratrol was performed on HP HPLC series 1100 (Hewlett Packard, Waldbronn, Germany) equipped with Chem Station software, a degasser G1322A, a binary detection system G1314A, an Alltech Altima C18 column (250×4.6 mm i.d., 5 μm) and a C_{18} guard column (Phenomenex 40×3.0 mm). The chromatographic separation was carried out using a linear gradient of mobile phase A (water) and mobile phase B (1% acetic acid in 35% MeOH). The elution profile was used as follows: 10%-55% B (50 min), 55%-100% B (10 min), and 100%-10% B (5 min). The flow rate was 1.0 ml/min. The eluate was on-line detected at 280 nm and monitored for 65 min. The column temperature was controlled at 25°C throughout the experiment.

Spectroscopic Studies

IR spectra (KBr tablet) were recorded on a Nicolet Nexus 470 FT-IR instrument (International Equipment Trading Ltd., USA). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Brucker Avance using TMS (for ^{1}H-NMR) and acetone-d_6 (for ^{13}C-NMR) as an internal standards at 25°C. The studied sample was dissolved in acetone-d_6.

RESULTS AND DISCUSSION

Characterisation and Quantitation of Product Isolated from *A. lakoocha*

As shown in Figure 1, the ethanolic crude extract of *A. lakoocha*, the final pure solid isolated (AE5-3), and standard oxyresveratrol showed the same retention time at 3.5 min, suggesting that AE5-3 was oxyresveratrol. The IR spectrum of AE5-3 also matched that of the authentic oxyresveratrol. The ^{1}H-NMR and ^{13}C-NMR spectral patterns of AE5-3 were in good agreement with those of standard oxyresveratrol (Figure 2) and also with those reported in the literature [16-17]. The signals of ^{1}H-NMR and ^{13}C-NMR of AE5-3 were assigned as follows: δ (ppm) 7.38 (1H, d, J=8.5Hz, H-6), 7.31 (1H, d, J=16.5Hz, H-α), 6.86 (1H, d, J=16.5Hz, H-β), 6.50 (2H, s, H-2', H-6'), 6.40 (1H, d, J=2.4Hz, H-3), 6.38 (1H, dd, J=8.5, 2.4Hz, H-5), 6.22 (1H, s, H-4'). 159.0 (C-5' or C-3'), 158.9 (C-5' or C-3'), 158.6 (C-4), 156.4 (C-2), 141.1 (C-1'), 127.8 (C-6), 125.8 (olefinic C-β), 124.0 (olefinic C-α), 116.8 (C-1), 107.5 (C-5), 105.0 (C-6' or C-2'), 105.0 (C-6' or C-2'), 103.1 (C-3), 101.8 (C-4').

From the yield of the crude product and content of oxyresveratrol (AE5-3) in the crude product as determined by HPLC and a calibration curve, the oxyresveratrol content in the heartwood of *A. lakoocha* used in this study was found to be approximately 15% w/w.

Antioxidative and Antiglycation Activities

The antioxidant activity of AE5-3 was compared to those of quercetin and resveratrol by DPPH and TBARS methods. As shown in Figure 3, AE5-3 showed a strong DPPH radical scavenging activity (IC_{50} = 0.10 ± 0.01 mg/ml) and lipid peroxydation inhibitory activity (IC_{50} = 0.43 ± 0.03 mg/ml).These

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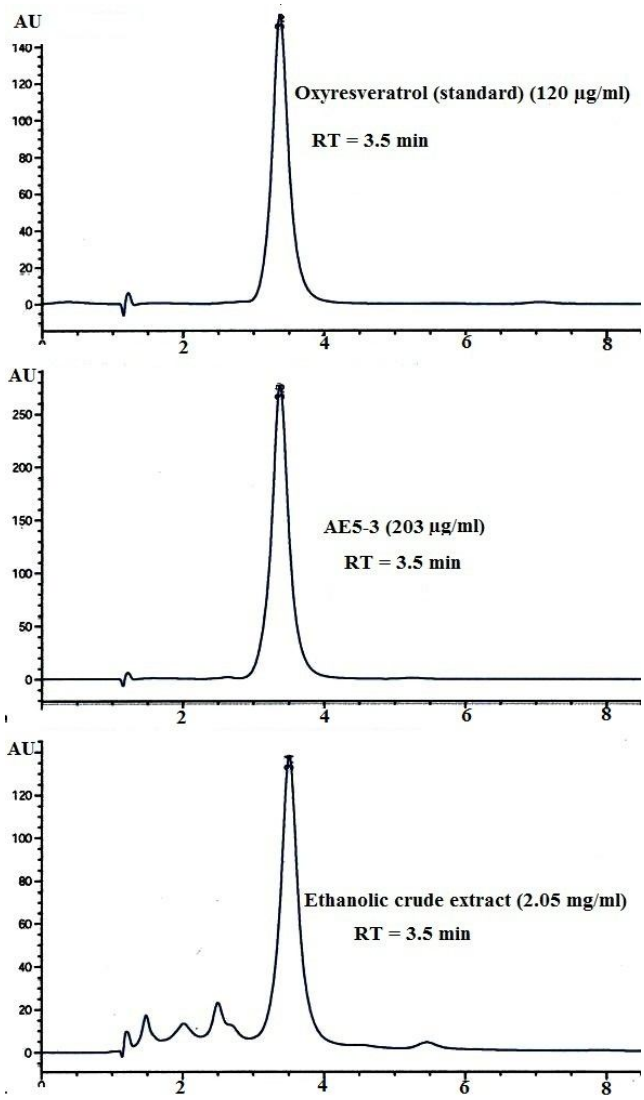

Figure 1. HPLC profile of oxyresveratrol, AE5-3 and ethanolic crude extract of *A. lakoocha*

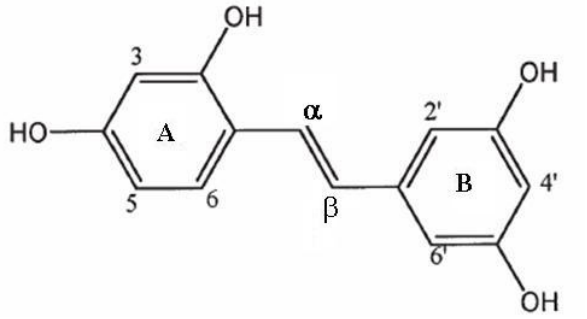

Figure 2. Chemical structure of oxyresveratrol

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activities were comparable with those of quercetin. However, they were nearly twice as high as those of resveratrol ($p < 0.05$).

As shown in Figure 4, AE5-3 showed a strong antiglycation activity (IC_{50} = 2.0±0.03 µg/ml), which was 5-fold stronger than that of aminoguanidine, a hydrazine-like compound that blocks the formation of advanced glycation end products (AGEs) by interacting with amadori-derived products [13]. It is a prototype compound for the prevention of AGEs formation [18].

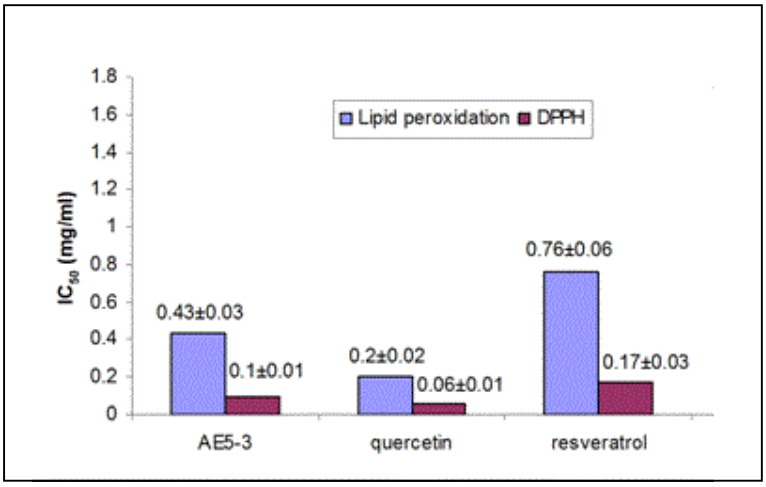

Figure 3. Antioxidant activity of AE5-3 and other antioxidant compounds. Figures in the graph were IC_{50} values (mean ± SD).

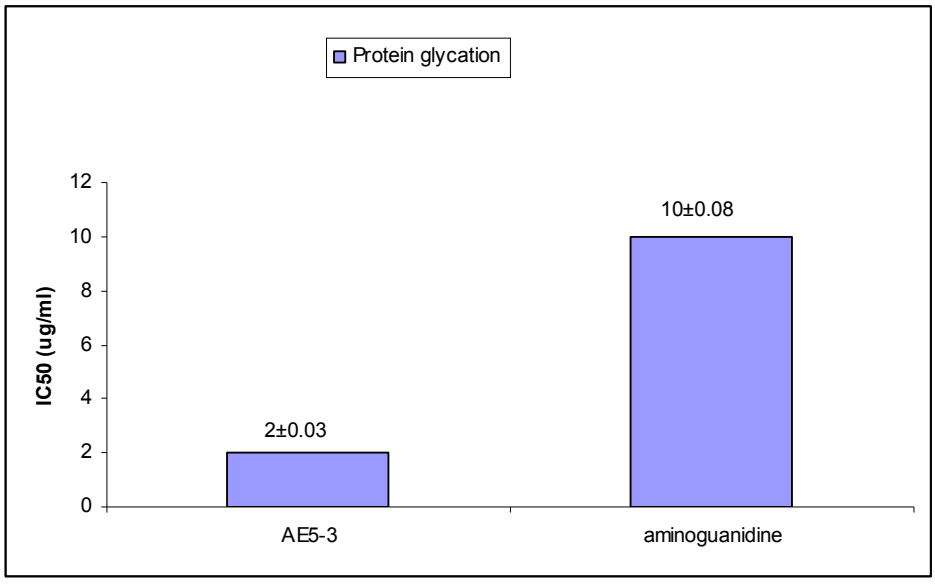

Figure 4. Antiglycation activity of AE5-3 and aminoguanidine. Figures in the graph were IC_{50} values (mean ± SD).

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Thus, in the present study, we have shown that oxyresveratrol, a compound present in a considerably large quantity in the heartwood of *Artocarpus lakoocha*, accounts for the strong antiglycation activity of its crude extract [11]. Protein glycation and oxidative stress are regarded as one of the critical factors that cause diabetes mellitus. Therefore, oxyresveratrol might prove to be another useful antidiabetic agent. Besides, this study also shows that the antioxidant activities of oxyresveratrol are about twice as strong as those of resveratrol. Several studies have shown that resveratrol can help in preventing cardiovascular diseases, cancer, inflammation and Alzheimer [19-22]. Thus, *Artocarpus lakoocha* with a high amount of oxyresveratrol may be expected to be another promising medicinal herb for these ailments.

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