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Quantification of coumarin derivatives in Noni (*Morinda citrifolia*) and their contribution of quenching effect on reactive oxygen species

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ABSTRACT

The quantification of coumarin derivatives such as scopoletin, 7-hydroxycoumarin (7-HC) and 4-hydroxycoumarin (4-HC) in Noni (*Morinda citrifolia*) was described. The coumarin derivatives were determined by HPLC-UV or -fluorescence detection. More than 95% of peak purity for coumarin derivatives in Noni sample was confirmed by a multi-wavelength fluorescence detector. Amounts of scopoletin and 7-HC in Noni juices (A–H) were ranging 5.1–231 μg/ml and 0.04–0.45 μg/ml, respectively (n = 12). No 4-HC was detected in any Noni samples examined.

Furthermore, the quenching effects of Noni products and coumarin derivatives on reactive oxygen species (ROS) were evaluated by a luminol chemiluminescent assay. Both Noni samples and coumarin derivatives dose-dependently quenched ROS such as superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (·OH) and peroxynitrite ($ONOO^-$). The EC_{50} of scopoletin for O_2^-, $^1O_2$, ·OH and $ONOO^-$ were 1.27 ± 0.22 mg/ml, 0.68 ± 0.04 mg/ml, >4.00 mg/ml, and 0.042±0.002 mg/ml, respectively. The contribution ratio of scopoletin for ROS in Noni juices was also evaluated.

1. Introduction

Morinda citrifolia commonly known as Noni has a long history of wide use as a food in tropical regions from Indonesia to Hawaiian island, and is used herbal remedies for different diseases. Recently, Noni fruit juice is in high demand as an alternative medicine due to its possibilities for anti-microbial, anticancer, anti-inflammatory, antioxidant effects (Wang et al., 2002). However, scientific evidence for the benefits of the Noni fruit juice is still limited. The increasing use of Noni products as dietary supplements suggested an urgent requirement to check their advocated effect for quality control purposes.

Several classes of compounds as ingredients have been isolated from Noni including amino acids, anthraquinones, coumarins, fatty acids, flavonoids, iridoids, lignans and polysaccharides (Chan-Blanco et al., 2006). Among these scopoletin, a coumarin derivative, is one of the representative ingredients in Noni. Its contribution for anti-microbial and anti-inflammatory activities with antioxidative activity has been well-elucidated (Deng et al., 2007). Samoylenko et al. (2006) recommended scopoletin as a maker constituent for quality control of Noni. This compound was ubiquitously found in Noni collected from Atlantic and Pacific regions and all fruit squeezed juices examined. Determination of scopoletin in Noni might be help for quality control of Noni products. However, there is no report to evaluate antioxidative activity of scopoletin and other coumarin derivatives in Noni.

Many reports on antioxidative activity of Noni itself have been published. Free-radical-scavenging activity with 1,1-diphenyl-2-picrylhydrazyl (Su et al., 2005; Yang, Paulino, Janke-Stedronsky, & Abawi, 2007), inhibition of copper-induced low-density lipoprotein oxidation (Kamiya, Tanaka, Endang, Umar, & Satake, 2004), nitric oxide scavenging activity (Basu & Hazra, 2006) and quenching of H_2O_2 (Chong, Abdullah, Fadzillah, Lai, & Lajis, 2004; Jeffers, Kerins, Baker, & Kieran, 2007) were carried out to evaluate antioxidative effects of Noni and its products. Polyphenols, reducing glycosides (Calzuola, Gianfranceschi, & Marsili, 2006), lignan derivatives (Su et al., 2005) and anthraquinones (Chong et al., 2004) were suggested as sources of antioxidative activity in Noni. However, there is little available information to evaluate the antioxidative activity with quantitative data of ingredient. Recently, a correlation between total phenol and free-radical-scavenging activity was reported (r = 0.41) (Yang et al., 2007).

Therefore, in this study, we focused on quantification of coumarin derivatives in Noni and their contribution to antioxidative ability on ROS as follows:

(1) Coumarin derivatives such as scopoletin, 7-hydroxycoumarin (7-HC) and 4-hydroxycoumarin (4-HC) in Noni and its products were determined by HPLC-UV or -fluorescence (FL) detection. Peak purities of coumarin derivatives in Noni were confirmed with a multi-wavelength FL detector.

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(2) The quenching effects of coumarin derivatives and Noni products on reactive oxygen species (ROS) such as superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$) and peroxynitrite ($ONOO^-$) were evaluated by a luminol chemiluminescent assay. In our previous reports, the quenching effects of medicines such as flavastain and its metabolites (Nakashima et al., 2001), non-water soluble and water soluble rosemary extracts (Wada et al., 2004), grape seed extracts (Wada et al., 2006) and natural colourants (Wada et al., 2007) were examined.

(3) Contribution ratio of ROS quenching effect for scopoletin in Noni products was estimated. This is the first report to evaluate the quenching effect of coumarin derivatives in Noni on ROS.

2. Materials and methods

2.1. Noni samples

Root and stem extracts (80% EtOH) of Noni were from Philippine. Noni fruit juice samples were from Hawaii (A), Samoa (B), Polynesia (C), Indonesia (D–G), and Tahiti (H). Fruit paste (I), powder (J,K) and leaf powder (L) were from Indonesia. Samples were stored in sealed glass bottles and kept in a refrigerator until the bottles were opened for analysis. For measurement of quenching effects on ROS, Noni samples were appropriately diluted with water.

2.2. Materials and chemicals

Scopletin, 7-HC, 4-HC, ascorbic acid (ASA), xanthine oxidase (XOD) from buttermilk, H_2O_2 (30%), $FeCl_2$ and $NaNO_2$ solution were purchased from Wako Pure Chemicals (Osaka, Japan). Luminol, hypoxanthine (HX), NaBr, diethylentriaminepentaacetic acid (DETAPAC) and lactoperoxidase (LPO) from Sigma Chemical Corporation (MO, USA) were used. Water was deionized and distilled by an Aquarius GSR-500 automatic water distillation apparatus (Advantec, Tokyo, Japan). One milligram of coumarin derivatives was dissolved in EtOH to prepare 1 mg/ml each of stock solution. Working curves for determination of coumarin derivatives were prepared by further dilution with 50% EtOH. For measurement of quenching effect on ROS, coumarins were dissolved in dimethyl sulfoxide (DMSO) to prepare appropriate concentrations. However, for the measurement of quenching effect against $^{\cdot}OH$, these compounds were dissolved in dimethylformamide (DMF).

2.3. Sample treatment for determination of coumarin derivatives in Noni samples

To fifty milligram of powder or paste sample of Noni product, proper volume of 50% EtOH aqueous solution was added and sonicated for 30 min. After diluting with 50% EtOH up to 5 ml, the solution was centrifuged at 1000*g* for 10 min and filtered through a 0.45 μm membrane filter. Instead of the solution, 1 ml of Noni juice or 5 ml of Noni extract (stem or root) was used for each determination.

2.4. HPLC conditions for determination and identification of coumarin derivatives in Noni samples

An HPLC system for determination of coumarin derivatives in Noni samples was consisted of a LC-6A chromatographic pump (Shimadzu, Kyoto, Japan), a 7125 injector with a 20-μl of sample loop (Rheodyne, CA, USA), an SCL-6A system controller (Shimadzu), a Daisopak-SP-120-5-ODS-BP (250 × 4.6 mm, i.d., Daiso, Osaka), an UV-8000 detector (Tosoh, Tokyo) or an RF-10A_{XL} (Shimadzu). The eluent was monitored at 286 (for 4-HC) and 322 nm (for scopoletin and 7-HC) of UV detection wavelengths, and at 372 nm with 290 nm of excitation (for 4-HC) and 450 nm with 320 nm of excitation (for scopoletin and 7-HC) of FL detection. The separations of 4-HC and scopoletin, and scopoletin and 7-HC were carried on with 50 mM phosphate buffer (pH 3.0)/MeOH (65:35, v/v) and 50 mM phosphate buffer (pH 5)/MeOH (74:26, v/v) as a mobile phase, respectively. Data was presented as a mean of duplicate measurement of each sample. Estimation of peak purity of coumarin derivatives in Noni sample was performed by a series 1100 multi-wavelength FL detector with software of HP Chemstation (Hewlett Packard, CA).

2.5. FIA conditions for measurement of quenching effects of coumarin derivatives and Noni samples on ROS

The quenching effect of coumarin derivatives and Noni samples on ROS was measured by a flow injection analysis (FIA) system according to our previous method (Wada et al., 2006). The FIA consisted of two LC-6A chromatographic pumps (Shimadzu), a 7125 injector with a 100-μl of sample loop (Rheodyne), an 825-CL chemiluminescence detector (Jasco, Tokyo) and an R-61 recorder (Rikadenki, Tokyo). Carrier and reagent solutions triggered the generation reaction of ROS are summarised in Table 1. The reaction coil lengths of Teflon® tube (0.25 mm, i.d.) after mixing of carrier and reagent solutions were 230 mm for O_2^-, $^1O_2$ and $ONOO^-$, and 120 mm for $^{\cdot}OH$. Sample solutions injected into the FIA system were prepared as follows: [O_2^-] To 5 μl of sample in a test tube (∅ 12 × 75 mm), 500 μl of 0.05 U/ml of XOD in 100 mM phosphate buffer (pH 8.3) and 500 μl of 1.2 mM luminol in phosphate buffer were successively added. [$^1O_2$] To 6 μl of sample, 400 μl of 0.1% H_2O_2 in 100 mM acetate buffer (pH 4.5), 400 μl of 80 mM NaBr in acetate buffer and 0.1 mM luminol in acetate buffer were added. The mixture was incubated at 37 °C for 10 min. [$^{\cdot}OH$] To 5 μl of sample, 500 μl of 0.1% H_2O_2 in 100 mM phosphate buffer (pH 8.3), and 500 μl of 1.2 mM luminol in phosphate buffer were successively added. [$ONOO^-$] 500 μl of 1.2 mM luminol in 100 mM phosphate buffer (pH 8.3) and 500 μl of 0.1% H_2O_2 in 100 mM phosphate buffer were added to 5 μl of sample. All of the mixtures were incubated at 37 °C for 10 min followed by immediate measurement of the CL intensity. The concentrations of radicals generated could not be estimated. The percentage of quenching effect against each ROS was calculated from the following equation:

$$\text{Quenching effect}\% = \{(CI_0 - CI)/CI_0\} \times 100$$

where CI_0 is CL intensity generated from blank (DMSO, DMF or H_2O) and RCI is CL intensity generated from the sample. The increased value indicates increase of quenching effect.

The sample concentration giving 50% quenching (EC_{50}) on ROS was calculated by triplicate measurements. The data were expressed as the mean ± SD (n = 3). The contribution ratio of scopoletin in quenching effect of Noni juice was calculated by EC_{50} and concentration of scopoletin in Noni juice and quenching effect of Noni juice samples. Data processing was performed by using Microsoft® Office Excel 2003.

Table 1
FIA conditions for carrier and reagent solutions

ROS	Carrier solution	Reagent solution
O_2^-	100 mM phosphate buffer (pH 8.3), flow rate: 0.5 ml/min	1 mM HX/100 mM phosphate buffer (pH 7.4), flow rate: 0.1 ml/min
$^1O_2$	100 mM acetate buffer (pH 4.5), flow rate: 0.5 ml/min	10 μg/ml LPO/100 mM acetate buffer (pH 4.5), flow rate: 0.1 ml/min
$^{\cdot}OH$	100 mM phosphate buffer (pH 7.4), flow rate: 0.5 ml/min	100 μM $FeCl_2$ and 4 mM DETAPAC/100 mM phosphate buffer (pH 7.4), flow rate: 0.3 ml/min
$ONOO^-$	100 mM phosphate buffer (pH 8.3), flow rate: 0.4 ml/min	2 mM $NaNO_2$/100 mM phosphate buffer (pH 8.3), flow rate: 0.1 ml/min