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Nitric Oxide Inhibition and Procollagen Type I Peptide Synthesis Activities of a Phenolic Amide Identified from the Stem of *Lycium chinense* Miller

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The bioactivities of boxthron fruits, a source of oriental medicine, are well known, whereas phytochemical studies of the boxthorn stem are rare. In this study, the stem extract of boxthorn (*Lycium chinense* Miller) and its subfractions were evaluated for their effects on nitric oxide (NO) inhibition and procollagen type I peptide (PIP) synthesis. A phenolic amide isolated from the stem extract was also assayed for these effects. The compound, *N-trans*-feruloyltyramine, was identified by ^{1}H, ^{13}C, and 2D-nuclear magnetic resonance analyses. In NO inhibition, the chloroform fraction (CF) exhibited the strongest inhibitory activity (MIC_{50} = 24.69 μg/ml) among the subfractions of the ethanol extract (EE). *N-trans*-feruloyltyramine isolated from the CF showed strong NO inhibitory activity, presenting with an MIC_{50} of 31.36 μg/ml. The EE, CF, and *N-trans*-feruloyltyramine shown to have NO inhibition activity were assayed for the activity of PIP synthesis. The EE and CF showed relatively high PIP values of 38.8% and 24.21% at 100 μg/ml, respectively. The PIP value for 20 μg/ml *N-trans*-feruloyltyramine showed a 36% increase compared with the non-treated control, whereas that treated with 20 μg/ml ascorbic acid as a positive control showed a 13% increase. The results suggest that the proper stem extract of boxthorn stem could be efficiently used to produce good cosmetic effects.

Keywords: Boxthorn, nitric oxide inhibition, *N-trans*-feruloyltyramine, procollagen type I peptide synthesis

Introduction

Boxthorn (*Lycium chinense* Miller) belongs to the Solanaceae family originating from China and is now widely planted in subtropical areas such as Korea, Japan, and Southeastern Asia [1, 2]. Boxthorn fruits, leaves, and root bark are used in cooking and medicine [3]. The red-colored fruits contain several volatile, steroidal, and alkaloidal compounds [4]. These compounds have medical functions such as anti-hypertensive activity, inhibition of fatty liver development, and reducing the sugar content in the blood [5]. The leaves of one boxthorn species (*L. barbarum*) are rich in flavonoids that have radical scavenging activity, and antioxidation, anti-inflammation, and anticancer effects [6-8]. The root bark contains acyclic diterpene glycosides, cyclic peptides, sesquiterpenes, spermine alkaloids, flavonoids, and cerebrosides [4, 9].

Inflammation, a body defense mechanism, is generated by leukocyte migration into damaged tissues. Migrating leukocytes destroy injurious factors, which include physical, chemical, and microbiological toxins [10]. Generally, acute inflammation induces beneficial responses such as inactivation or destruction of toxic organisms, removal of irritants, and restoration of damaged tissues, whereas chronic inflammation and infection are recognized risk factors for a variety of human cancers, skin problems,

rheumatoid arthritis, bronchitis, gastritis, and enteric disease [10, 11]. Nitric oxide (NO), which is synthesized from L-arginine oxidation by catalyzing nitric oxide synthases (NOS), is a major pathogenic substance for chronic skin inflammation [12].

In dermal anti-aging effects, there are important elements that enhance the collagen level and inhibit NO synthesis in epidermal cells. Boxthorn, which is rich in various phenolics and carotenoids, has anti-aging activity [4, 13]. However, most phytochemical studies of *Lycium* species were performed using the edible parts such as fruits and leaves, but not stems. Thus, we identified one representative compound in boxthorn stem and evaluated its anti-aging activity by conducting procollagen type I peptide synthesis (PIP) tests and measuring the NO inhibition activity.

Materials and Methods

Plant Materials

Whole boxthorn plants were purchased from a farm in Cheongyang-gun, Korea in 2014. The stems were dehydrated at room temperature and finely ground using a grinder (HMF-3000S; Hanil Electric Co., Korea). Organic solvents used in this experiment were either analytical or HPLC grade purchased from Daejung Chemical & Metals Company (Korea).

Boxthorn Stem Extraction and Isolation Process

Dried and ground boxthorn stems (80 g) were extracted twice in 1.6 L of 60% (v/v) ethanol (EE) at 50°C for 2 h. The extraction was filtered under the condition of reduced pressure. The solvent of the sample extraction was removed using a rotary vacuum evaporator (Eyela Co., Japan) in a 40°C water bath. From 80 g of dried stems, 7.11 g of extract was acquired, dissolved in 1 L of distilled water, and fractionated with *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and distilled water. Among the fractionated extracts, 190 mg of the chloroform fraction (CF) was acquired. The CF was loaded onto a glass column (Sephadex LH-20; GE Healthcare Bio-Sciences AB, Sweden) filled with normal silica gel 60 (KGaA 230–400 mesh ASTM; Merck, Germany) and $CHCl_3$:methanol (9:1 → 8:1 → 7:1) solvent. Extracts of EE, CF, and an isolate compound were analyzed by reverse-phase HPLC (Waters 2695 model HPLC; Waters, USA) using an octadesylsilane column (Kromasil C18, 5 μm in diameter, 4.5 × 150 mm; Akzonobel, Sweden). The mobile phase was a mixture of 0.4% formic acid in HPLC-grade water (Solvent A) and 0.4% formic acid in methanol (Solvent B). Gradient elution was conducted at a flow rate of 0.8 ml/min, initiating with 100% of solvent B at 0–40 min and 0% of solution B at 40–50 min. The injection volume of samples was 10 μl. Column and sample temperatures were 40°C and 25°C, respectively. Peaks were monitored at 318 nm with a Waters 996 photodiode array detector (Waters).

NMR Analysis of the Amide Compound

The structure of the isolated compound was determined based on NMR analysis, such as ^{1}H, ^{13}C, and 2D-NMRs (COSY, HSQC, and HMBC) (JNM-LA 400, FT-NMR; JEOL, Japan).

Nitric Oxide Inhibition Assay

RAW 264.7 cells were cultured in 96-well (2×10^4 cells/well) plates and inoculated in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, USA) containing 10% fetal bovine serum (FBS; HyClone Laboratories). The culture conditions were maintained at 37°C in a humidified incubator with 5% CO_2 overnight. The extracts from boxthorn stems (0, 10, 50, and 100 μg/ml) were added to the culture plate with or without lipopolysaccharide in fresh media. Accumulated nitrite in the cultured medium served as a detector of NO production and was measured according to the Griess test [14].

Cell Viability Assay

Cell viability was determined to identify toxicity by dose-dependent treatments of boxthorn stem extracts. The absorption of neutral red dye (in vitro cytotoxicity assay kit; Sigma-Aldrich Co., USA) by viable cells was measured according to the method of Fautz *et al*. [15].

Procollagen Type I Peptide Measurement

To evaluate the ability for procollagen synthesis, HDF-N cells were used. The cells (1.5×10^4 cells per well) were incubated in 24-well plates with DMEM containing 10% FBS and 100 unit/ml penicillin-streptomycin at 37°C overnight in a humidified incubator with 5% CO_2. At 12 h after removal of the media, 20 μl of each boxthorn stem EE, CF, and the isolated compound at 0, 5, 10, and 20 μg/ml FBM, respectively, was inoculated onto the plates. TGF-β with 10 ng/ml was used as the positive control. After a 24 h incubation, the cultured supernatants were collected by centrifugation at 13,000 ×*g* at 4°C for 20 min. The amount of PIP synthesis in the supernatants was measured using a PIP EIA kit according to the supplier's instructions (MK101; Takara Shuzo, Japan).

Statistical Analysis

Data are presented as the mean and standard error and were analyzed using SAS software (version 9.3; SAS Institute Inc., USA). The significance of differences was determined at the 5% level using Duncan's multiple range test.

Results and Discussion

Separation and Identification of Compounds

A major peak was detected at 318 nm on HPLC chromatograms of EE, CF, and the isolated compound (Fig. 1). The peak was observed from 25 to 26 min on the chromatograms. Twenty-one milligrams of the major peak was separated from the CF through silica gel chromatography.

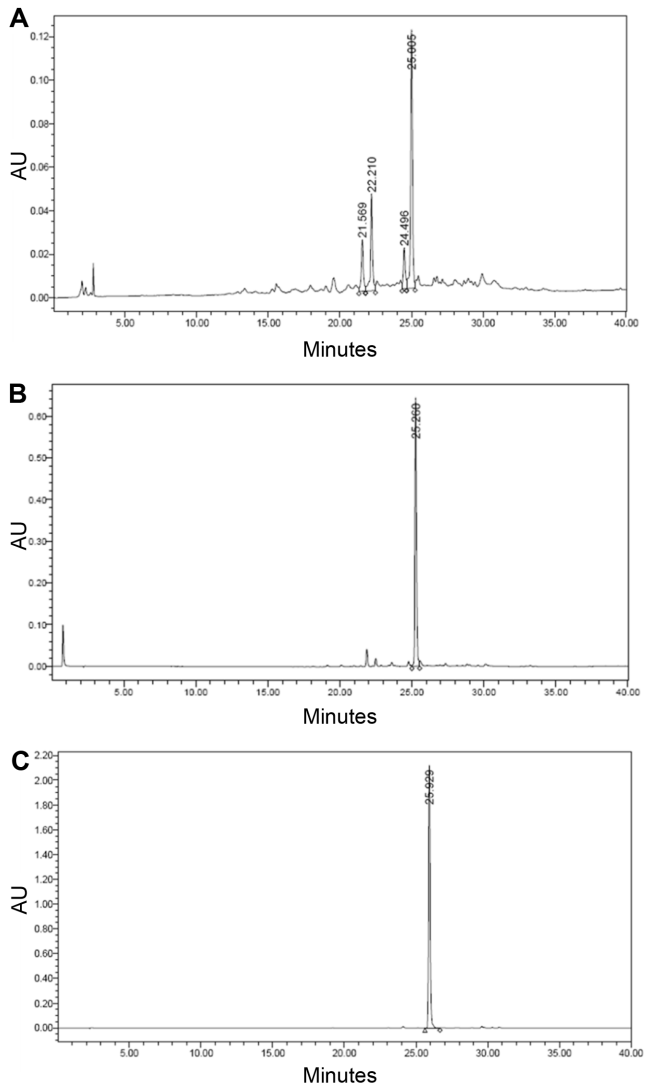

Fig. 1. HPLC chromatograms of the ethanol extract (**A**), chloroform fraction (**B**), and isolated compound (**C**).

Structural Interpretation of the Isolated Compound

^{1}H-NMR and ^{13}C-NMR were applied to the structural analysis of the isolated compound using silica gel open chromatography (Fig. 2). The spectral data are as follows:

^{1}H-NMR (400 MHz, CD_3OD) δ 2.74 (2H, t, *J* = 7.4 Hz, H-7′), 3.46 (2H, t, *J* = 7.4 Hz, H-8′), 3.86 (3H, s, OCH_3), 6.38 (1H, d, *J* = 15.5 Hz, H-8), 6.71 (2H, d, *J* = 8.4 Hz, H-3′ and H-5′), 6.79 (1H, d, *J* = 8.2 Hz, H-5), 6.99 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 7.04 (2H, d, *J* = 8.4 Hz, H-2′ and H-6′), 7.07 (1H, d, *J* = 2.0 Hz, H-2), and 7.42 (1H, d, *J* = 15.5 Hz, H-7) (Fig. 2A).

^{13}C-NMR (100 MHz, CD_3OD) δ 35.7 (C-7′), 42.4 (C-8′), 56.3 (OCH_3), 111.3 (C-2), 116.2 (H-3′ and H-5′), 116.3 (C-5), 118.6 (C-8), 123.1 (C-6), 128.1 (C-1), 130.6 (C-2′ and H-6′), 131.1 (C-1′), 141.9 (C-7), 149.1 (C-4), 149.6 (C-3), 155.6 (C-4′), and 169.0 (C-9) (Fig. 2B).

This compound contained two benzene rings, both with ortho coupling. One was a 1,3,4-trisubstituted benzene ring at δ 6.79 (d, *J* = 8.2 Hz), 6.99 (dd, *J* = 8.2, 2.0 Hz), and 7.07 (d, *J* = 2.0 Hz). The other was a 1,4-disubstituted benzene ring with a coupling constant (*J* = 8.4 Hz) at δ 6.71 and 7.04. Triplets at δ 2.74 and 3.46 suggested the presence of two coupled methylene protons. In the results of heteronuclear multiple bond connection (HMBC) analysis (data not presented), there were long-range couplings between methine protons at δ_H 6.38 (H-8) and 7.42 (H-7) and carbonyl at δ_C 169.0 (C-9). Methoxy protons at δ_H 3.86 and δ_C 149.1 (C-4) indicated the presence of a ferulic acid moiety substructure. A tyramine moiety, another substructure, suggested a nitrogen connection at δ_H 3.46 and δ_C 42.4. There were two long-range couplings from the methylene proton at δ_H 2.74 (H-7′) to carbon at δ_C 131.1 (C-1′) and at 130.6 (C-2′ and H-6′). *N-trans*-feruloyltyramine was identified from the NMR and HMBC data published by Yoshihara *et al*. [16], Chen *et al*. [17], and Kim *et al*. [18] (Fig. 3). This phenolic amide compound has been previously isolated from the CF layer of various plant-part extracts, such as those from *Solanum melongena* and *sordidum* and *Enicosanthum cupulare* plants [16, 19].

Effects of Boxthorn Stem Extracts on NO Inhibition Activity

Fig. 4 shows the NO inhibitory activity of boxthorn stem extracts as measured by NO production in an in vitro cell assay. The NO content decreased sharply after treatment with increasing concentration of boxthorn stem extracts, except for the EE. According to the obtained results, the EE was too weak to inhibit NO production, with values of NO inhibition at 4.5% (10 μg/ml), 19.1% (50 μg/ml), and 41.7% (100 μg/ml). There was no cytotoxicity of the treatments over the tested concentration range (data not shown). The NO inhibition of *N-trans*-feruloyltyramine was weaker than that of the CF, which contains other NO suppression components. The NO inhibitory activity of *N-trans*-feruloyltyramine was 84% at 100 μg/ml, whereas the activity of the CF was 98%. Other subfractions of EE, including *n*-hexanol, ethyl acetate, *n*-butanol, and aqueous fractions, showed relatively low activities of NO inhibition (Fig. 4). According to previously published papers, mostly nonpolar phytochemicals such as quercetin [21], saponin [22, 23], and phenolic compounds have anti-inflammatory effects. In certain cases, it was reported that a butanol extract was more efficient than a methanol extract from *Balanites aegyptiaca* roots [23]. Kim *et al*. [24] reported that some phenolic

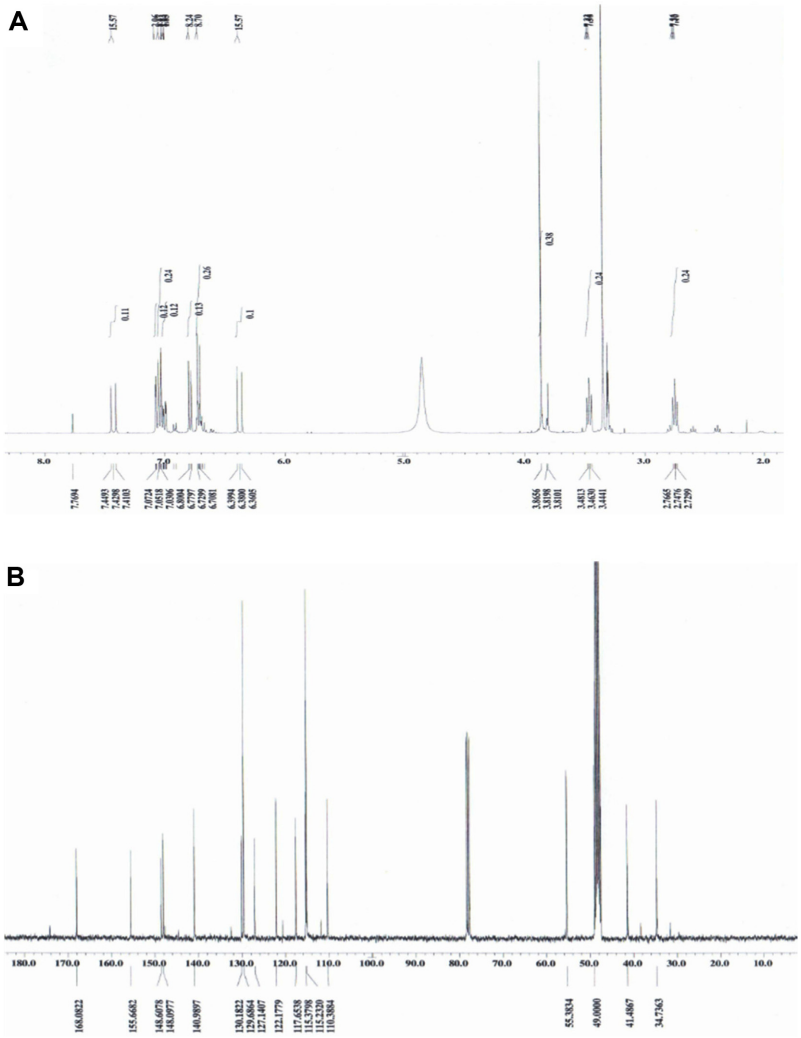

Fig. 2. ^{1}H-NMR and ^{13}C-NMR chromatograms of *N-trans*-feruloyltyramine.

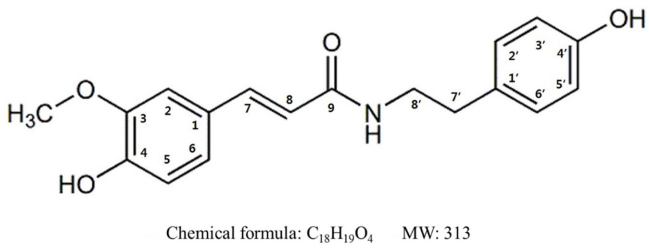

Fig. 3. Structure of *N-trans*-feruloyltyramine.

amides containing *N-trans*-feruloyltyramine suppressed NO production in RAW 264.7 cells.

Effects of Boxthorn Stem Extracts on Procollagen Type I Peptide Synthesis

The assays of PIP synthesis were conducted with the EE,

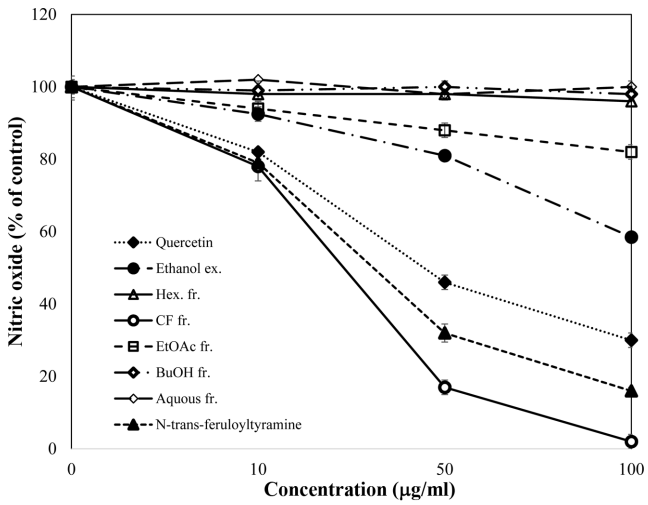

Fig. 4. Nitric oxide inhibition by boxthorn stem extracts.

Table 1. Percentage of procollagen type I peptide (PIP) relative to the control using the 60% ethanolic extract, and a chloroform-partitioned layer of boxthorn stems.

Concentration[1]	Percentage of PIP to control (%)	
	60% ethanol extract	Chloroform layer
10	104.8a	102.6a
50	124.2a	113.4b
100	138.8a	124.2a

[1]Concentration (μg/ml) in test medium.
Different letters within rows indicate statistical differences by Duncan's multiple range test at the 5% level.

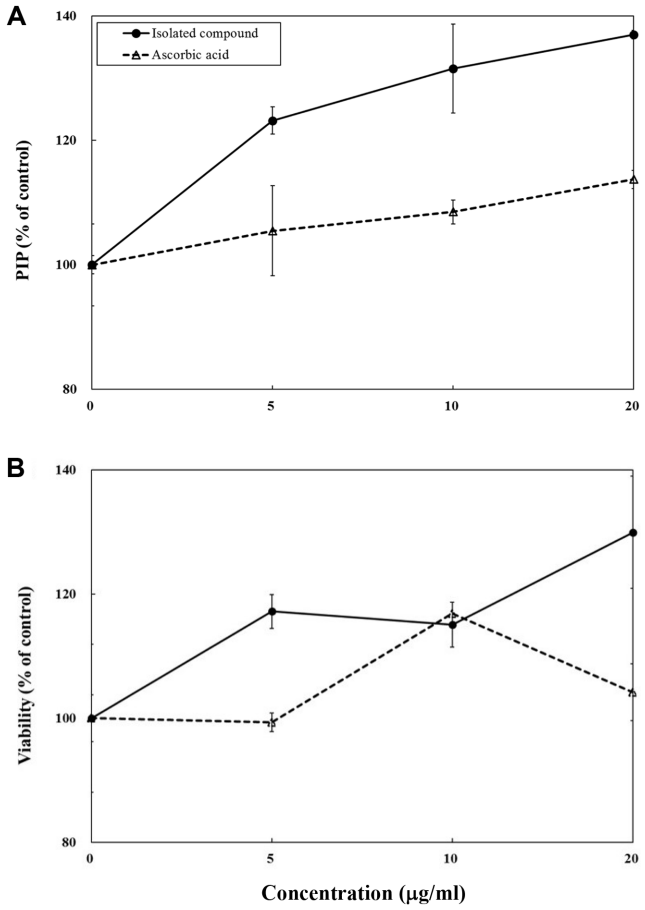

Fig. 5. Effects of *N-trans*-feruloyltyramine on procollagen peptide I (PIP) synthesis (**A**) and cell viability (**B**).

CF, and *N-trans*-feruloyltyramine because the samples exhibited efficient NO inhibition. The ratio of PIP to control from the boxthorn stem extracts at different concentrations was assessed (Table 1). The highest value (38.8%) was from EE at a 100 μg/ml concentration, whereas the CF extract at 10 μg/ml had the lowest PIP value (2.6%). For the isolated compound, *N-trans*-feruloyltyramine, from boxthorn stem, the percentage of PIP to control was evaluated at 5, 10, and 20 μg/ml concentrations (Fig. 5A). Different concentrations of each extract, such as 5 μg/ml of isolated compound (23.2%), 50 μg/ml of EE (24.2%), and 100 μg/ml of CF (24.2%), were needed to achieve comparable PIP values. The highest PIP value was 37.0%, obtained at a 20 μg/ml concentration of *N-trans*-feruloyltyramine, which is comparable to the PIP value (36.0%) of *Emblica officinalis* extracts at the same concentration [20].

In conclusion, boxthorn has been used for medicinal purposes because of its anti-aging activities, although its medicinal use has been limited to its fruits. Using HPLC and NMR analyses, the amide compound *N-trans*-feruloyltyramine was isolated from stem extracts (Figs. 1 and 2). *N-trans*-feruloyltyramine suppressed NO production and enhanced PIP synthesis. Moreover, several studies have reported that the compound suppresses melanogenesis by inhibiting the expression of the tyrosinase protein in murine B16 melanoma cells [25, 26]. The CF, a subfraction of the EE, showed stronger NO inhibition and PIP synthesis than the other extracts, which indicates that boxthorn stem contains additional active compounds. Therefore, boxthorn stem extracts can be applied as a cosmetic material.

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