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ORIGINAL PAPER

Inhibitory effects of constituents of *Morinda citrifolia* seeds on elastase and tyrosinase

Megumi Masuda · Kazuya Murata · Akiko Fukuhama · Shunsuke Naruto · Tadashi Fujita · Akemi Uwaya · Fumiyuki Isami · Hideaki Matsuda

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Abstract A 50% ethanolic extract (MCS-ext) from seeds of *Morinda citrifolia* ("noni" seeds) showed more potent in vitro inhibition of elastase and tyrosinase, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity than extracts of *M. citrifolia* leaves or flesh. Activity-guided fractionation of MCS-ext using in vitro assays led to the isolation of ursolic acid as an active constituent of elastase inhibitory activity. 3,3′-Bisdemethylpinoresinol, americanin A, and quercetin were isolated as active constituents having both tyrosinase inhibitory and radical scavenging activities. Americanin A and quercetin also showed superoxide dismutase (SOD)-like activity. These active compounds were isolated from noni seeds for the first time.

Keywords *Morinda citrifolia* · Noni · Seed · Tyrosinase · Elastase

Introduction

Tyrosinase is one of the key enzymes in the melanin biosynthetic pathway: tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA), followed by the oxidation of L-DOPA to dopaquinone, and oxidative polymerization of several dopaquinone derivatives to give rise to melanin. On the other hand, it has been reported that superoxide dismutase (SOD) is one of key factors involved in reducing melanin production caused by UV irradiation [1]. Thus tyrosinase inhibitors with SOD-like activity and/or antioxidant activity may be useful ingredients in the field of skin-whitening cosmetics. In addition, UV irradiation promotes photoageing [2] and melanogenesis [3] in human skin; chronic UV irradiation denatures collagen and elastic fiber in the dermis and induces wrinkles [2]. Neutrophils play an important role in the process of photoageing of human skin. They infiltrate the skin, releasing active enzymes such as human leukocyte elastase (HLE), and matrix metalloproteinase [collagenase (MMP-1) and gelatinase (MMP-9)] [2]. HLE cleaves the helix structure of type I collagen and then degrades elastic fiber in human skin [4]. Thus, HLE inhibitors may be useful ingredients in preparations designed to prevent skin wrinkles.

The fruits, roots, bark and leaves of the tropical tree *Morinda citrifolia* L. (Rubiaceae)—commonly known as "noni" in Hawaii and Tahiti—have long been used as a folk medicine for the treatment of many diseases, e.g., hypertension and diabetes, throughout Polynesia [5]. In the last decade, several papers have reported the chemical constituents of noni and noni extracts, revealing the presence of anthraquinones [6], iridoids [6], flavones [7], lignans [7, 8], fatty acid derivatives [9], and biological activities such as hypotensive activity [10], anticancer activity [11] and inhibition of copper-induced low-density lipoprotein oxidation [8]. Recently, in Japan, the juice of noni fruit and tea made from noni leaves have been launched on the functional food market, and are drunk in the expectation that they can help prevent lifestyle-related diseases such as diabetes mellitus, hypertension and so on.

M. Masuda · K. Murata · A. Fukuhama · S. Naruto · T. Fujita · H. Matsuda (✉)
School of Pharmacy, Kinki University,
3-4-1 Kowakae, Higashiosaka,
Osaka 577-8502, Japan
e-mail: matsuda@phar.kindai.ac.jp

A. Uwaya · F. Isami
Research and Development, Tahitian Noni Inc., 3-2-2
Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

As a part of our continuous program to find novel ingredients for functional foods and/or cosmetics from natural resources [12], the present study examined whether noni can be utilized as a novel plant resource in skin-whitening and/or wrinkle-prevention cosmetics; to date, there are no reports on the tyrosinase and/or elastase inhibitory activities of noni. First, we examined in vitro HLE inhibitory activity, mushroom tyrosinase inhibitory activity, and antioxidant activity of 50% ethanolic extracts of fruit flesh, leaves, and seeds of noni. To assess antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of the extracts were tested. Second, in order to identify active constituents of the extract, we carried out activity-guided fractionation using the above-mentioned in vitro assays.

Materials and methods

Plant materials and reagents

Fruits and leaves of *M. citrifolia* were collected in French Polynesia during 2004–2006. Samples were identified by Tropical Resources (Provo, UT). The fruits were separated into flesh and seeds by hand. The flesh of the fruits was freeze-dried, and the leaves and seeds were air-dried. Voucher specimens of dried flesh of fruits (Noni: Lot No. 54288 Batch # 23401), leaves (Noni Leaf: Lot No. L06-017-MIT 75), and seeds (Noni Seed: 004-239A-0014) are deposited in Kinki University (Osaka, Japan). Chemical and biochemical reagents were reagent grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

HLE inhibitory activity

HLE activity was measured according to the method of Cainelli et al. [13]. As a positive reference, phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO). The test samples were dissolved in a solution of Hepes buffer (0.1 M Hepes, 0.5 M NaCl, 10% DMSO, pH 7.8)/DMSO (1:4, v/v). Mixtures of 90 µl HLE (Sigma-Aldrich, 250 U/ml in Hepes buffer) and 5 µl test sample solution were incubated at 4°C for 15 min in a 96-well plate. Substrate [*N*-methoxysuccinyl-ala-ala-pro-val *p*-nitroanilide (Sigma-Aldrich); 5µl of an 8 mM solution in DMSO] was then added. The mixture was incubated at 37°C for 20 min. A solution of Hepes buffer/DMSO (1:4, v/v) was used as a control (A) for the assay of extracts. DMSO was used as a control (B) for the assay of PMSF. Hepes buffer was used as a blank solution for HLE. The optical density (OD) at 405 nm of the resulting solution was measured with a microplate reader (Sunrise Rainbow Thermo; Tecan Japan, Kanagawa, Japan). The inhibitory percentage of HLE was calculated as follows:

$$\% \text{ inhibition} = [(C - D) - (E - F)]/(C - D) \times 100$$

where C is OD at 405 nm with enzyme, but without test sample, D is OD at 405 nm without test sample and enzyme, E is OD at 405 nm with test sample and enzyme, F is OD at 405 nm with test sample, but without enzyme.

Tyrosinase inhibitory activity

Tyrosinase activity was measured according to the method of Mason and Peterson [14] as described previously [15].

SOD-like activity

SOD-like activity was measured according to the method of Oyanagui [16] with minor modifications. The test sample was dissolved in DMSO and diluted with 0.5 mM disodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA)-phosphate-buffered saline (PBS) buffer (pH 8.2) to a final DMSO concentration of 1% v/v. As a reference, SOD from bovine erythrocytes (Sigma-Aldrich) was dissolved in 0.5 mM EDTA-PBS buffer (pH 8.2). A mixture of 0.5 mM EDTA-PBS buffer (pH 8.2; 0.2 ml), 0.5 mM hypoxanthine in EDTA-PBS buffer (pH 8.2; 0.2 ml), reagent A solution (10 mM hydroxylamine hydrochloride and 1 mg/ml hydroxylamine-*O*-sulfonic acid in water; 0.1 ml), water (0.2 ml) and the sample solution (0.1 ml) was preincubated at 37°C for 10 min (solution A). A solution of xanthine oxidase from cow milk (Roche Diagnostics, Mannheim, Germany) was prepared at 5 mU/ml with 0.5 mM EDTA-PBS buffer (pH 8.2). Solution A and xanthine oxidase solution (0.2 ml) were mixed and incubated at 37°C for 30 min. Reagent B solution (30 µM *N*-1-naphthylethylenediamine 2 HCl, 3 mM sulfanilic acid and 25% acetic acid in water; 2 ml) was added to the reaction mixture. The resulting mixture was allowed to stand for 30 min at room temperature, and then the OD was measured at 550 nm with a spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan). The SOD-like activity of each sample was expressed as the percentage decrease in OD vs that of control A (1% DMSO in 0.5 mM EDTA-PBS buffer) or control B (0.5 mM EDTA-PBS buffer). IC_{50} values represent the concentration of sample required to scavenge 50% of superoxide anion produced by hypoxanthine–xanthine oxidase system.

Radical scavenging activity

Radical scavenging activity was measured according to the method of Blois [17] with minor modifications. Test

samples were dissolved in DMSO and diluted with 0.5 M acetate buffer (pH 5.5) to a final DMSO concentration of 1% v/v. A mixture of test sample solution (2 ml), ethanol (EtOH, 1.6 ml), 0.5 M acetate buffer (pH 5.5) (0.4 ml) and 0.5 mM DPPH/EtOH solution (1.0 ml) was allowed to stand for 30 min at room temperature. The OD at 520 nm of the resulting mixture was measured with a spectrophotometer (UV-2450; Shimadzu). L-Ascorbic acid was used as a reference agent. The scavenging activity of each sample was expressed as the percentage decrease in OD vs that of the control DPPH solution. IC_{50} values represent the concentration of sample required to scavenge 50% of DPPH free radical.

Extraction and fractionation

Dried noni seed (200 g) was extracted with 50% EtOH (2 × 2 l) for 2 h under reflux. The extract was evaporated under reduced pressure, and then lyophilized to give a 50% EtOH extract (MCS-ext) of the seeds at 5% yield. Similarly, 50% EtOH extracts of dried flesh (MCF-ext) and dried leaves (MCL-ext) were obtained at 33% and 36% yield, respectively.

A suspension of MCS-ext (4 g) in water (40 ml) was extracted with hexane (40 × 2 ml) followed by ethyl acetate (40 × 5 ml). Evaporation of solvent from each of the combined extracts gave a hexane soluble fraction (0.05 g) and an ethyl acetate soluble fraction (1.0 g). The aqueous layer was evaporated, followed by lyophilization to give a water soluble fraction (2.5 g). The HLE inhibitory, tyrosinase inhibitory, and DPPH radical scavenging activities of each fraction were assayed.

A portion of the ethyl acetate soluble fraction (0.95 g) was submitted to column chromatography over 100 g silica gel (Merck No. 1.07734 silica gel 60, 3.6 × 23 cm, Merck, Darmstadt, Germany). Elution with chloroform ($CHCl_3$)

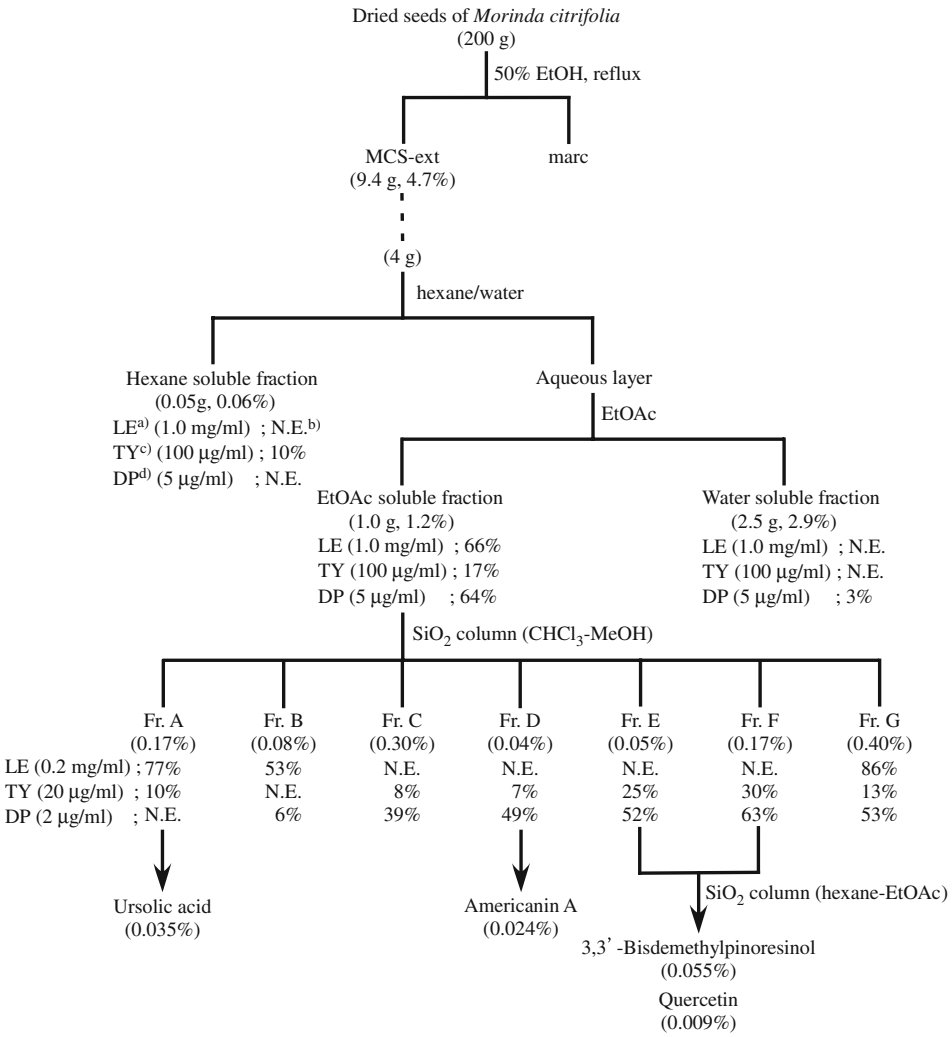

Fig. 1 Fractionation of 50% ethanolic extract (MCS-ext) from seeds of *Morinda citrifolia*

a); LE; HLE inhibition (%) at the cited concentration, b): N.E.; no effect. c): TY; tyrosinase inhibition (%) at the cited concentration, d): DP; DPPH radical scavenging activity (%) at the cited concentration.

Table 1 Inhibition of human leukocyte elastase by 50% ethanolic extracts of flesh of fruit (MCF-ext), leaves (MCL-ext), and seeds (MCS-ext) of *Morinda citrifolia* and phenylmethanesulfonyl fluoride (PMSF). Each value represents the mean ± SE of 3 experiments. *OD* Optical density

Sample	Concentration	OD (×1,000) at 405 nm	Inhibition (%)	IC_{50} value
Control A[a]		946 ± 23	0	
MCF-ext	0.1 (mg/ml)	994 ± 38*	−8	
	0.5	1,014 ± 9*	−10	
	1.0	1,039 ± 9*	−12	
MCL-ext	0.1 (mg/ml)	1,014 ± 16*	−10	
	0.5	1,023 ± 18*	−11	
	1.0	1,042 ± 36*	−13	
MCS-ext	0.1 (mg/ml)	1,052 ± 20*	−14	1.0 (mg/ml)
	0.5	722 ± 21*	22	
	1.0	467 ± 30*	50	
Control B[b]		925 ± 9	0	
PMSF	0.08 (mM)	676 ± 7**	29	0.14 (mM)
	0.15	383 ± 14**	60	
	0.50	100 ± 1**	90	

*Significantly different from control A group, $P < 0.01$

**Significantly different from control B group, $P < 0.01$

[a] Control for extracts; a solution of Hepes buffer/DMSO (1:4, v/v)

[b] Control for PMSF; DMSO

and methanol (MeOH) in increasing proportions monitored with TLC [Merck No. 1.05735 silica gel 60 F254, $CHCl_3$–MeOH (5:1, v/v), detection; UV and 10% H_2SO_4 followed with heating] gave 88 chromatographic fractions of 14 ml each. TLC analysis of the collected fractions allowed us to assemble them into seven fractions (Fr. A–G) shown in Fig. 1. HLE inhibitory, tyrosinase inhibitory, and radical scavenging activities of each fraction were assayed. Fr. A (135 mg) was purified by silica gel column chromatography eluted with $CHCl_3$ to give ursolic acid (28 mg, isolation yield, 0.035%). Fr. D (33 mg) was purified by silica gel column chromatography eluted with $CHCl_3$–MeOH (9:1, v/v) to give americanin A (20 mg, 0.024%). Americanin A gave a triacetate [18] by usual acetylation with acetic anhydride-pyridine. A mixture of Frs. E and F was purified by silica gel column chromatography eluted with hexane–ethyl acetate (1:1, v/v) to give quercetin (5 mg, 0.009%) and 3,3′-bisdemethylpinoresinol (30 mg, 0.055%). The identification of ursolic acid and quercetin was confirmed by direct comparison with authentic samples. The structures of americanin A, americanin A triacetate, and 3,3′-bisdemethylpinoresinol were identified by comparison of physico–chemical data, e.g., ^{1}H- and ^{13}C-NMR spectral data and optical rotation data, with reported data [8, 18].

Statistical analysis

The experimental data were tested for statistical significance using Bonferroni/Dunn's multiple range test method.

Table 2 Tyrosinase inhibitory activities of MCF-ext, MCL-ext, and MCS-ext and kojic acid. Each value represents the mean ± SE of 3 experiments. *OD* Optical density

Sample	Concentration	OD (×1,000) at 475 nm	Inhibition (%)
Control		472 ± 4	0
MCF-ext	20 (μg/ml)	471 ± 3	0
	100	451 ± 2	5
	500	419 ± 9*	11
MCL-ext	20 (μg/ml)	471 ± 2	0
	100	460 ± 2	3
	500	442 ± 1*	6
MCS-ext	20 (μg/ml)	449 ± 5*	5
	100	394 ± 4*	17
	500	365 ± 4*	23
Kojic acid	10 (μM)	207 ± 3*	56
	50	77 ± 3*	84

*Significantly different from control group, $P < 0.01$

Results and discussion

Assay results of 50% ethanolic extracts of flesh of fruit (MCF-ext), leaves (MCL-ext), and seeds (MCS-ext) of noni are presented in Tables 1, 2, and 3. The in vitro HLE inhibitory, tyrosinase inhibitory, and DPPH radical scavenging activities of MCS-ext were more potent than those of MCF-ext and MCL-ext.

Table 3 Radical scavenging activities of MCF-ext, MCL-ext, MCS-ext and ascorbic acid. Each value represents the mean ± SE of 3 experiments. *OD* Optical density

Sample	Concentration	OD (×1,000) at 520 nm	Inhibition (%)	IC_{50} value[a]
Control		974 ± 21	0	
MCF-ext	100 (μg/ml)	759 ± 3*	22	240 (μg/ml)
	200	536 ± 5*	45	
	400	188 ± 3*	81	
MCL-ext	50 (μg/ml)	765 ± 4*	22	113 (μg/ml)
	100	570 ± 3*	42	
	200	301 ± 6*	69	
MCS-ext	5 (μg/ml)	771 ± 2*	21	12 (μg/ml)
	10	537 ± 7*	45	
	20	121 ± 5*	88	
Ascorbic acid	20 (μM)	570 ± 4*	41	23 (μM)
	50	79 ± 2*	92	

*Significantly different from control group, $P < 0.01$

[a] IC_{50} value represents the concentration of sample required to scavenge 50% of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical

Since noni fruit contains a lot of seed in the flesh, these seeds are removed and discarded during the production process of noni fruit juice. A limited amount of the seeds are used as a resource in the form of noni oil as an ingredient in cosmetics because the seeds contain oily fatty acid derivatives [9]. However, the seed marc remaining after extraction of noni oil is also discarded. Therefore, it was suggested that a large amount of noni seed is not fully utilized in comparison with the leaf and flesh. Moreover, with the exception of fatty acid derivatives, the chemical constituents of noni seed have not been studied hitherto. Thus, we focused our attention on the utility value of noni seed, and investigated active constituents of MCS-ext by activity-guided fractionation using in vitro assays.

As shown in Fig. 1, MCS-ext was fractionated by solvent extraction to give a hexane soluble fraction, an ethyl acetate soluble fraction, and a water soluble fraction. Of these, the ethyl acetate soluble fraction showed the most potent activity (Fig. 1). Activity-guided chromatography of the active ethyl acetate soluble fraction using in vitro assays led to the isolation of ursolic acid, americanin A [8], 3,3′-bisdemethylpinoresinol [8, 19] and quercetin [7] as depicted in Fig. 1. Ursolic acid was isolated as an active constituent from Fr. A, which had potent HLE inhibitory activity. As a positive reference, phenylmethanesulfonyl fluoride (PMSF) inhibited HLE at IC_{50} 0.14 mM; the IC_{50} value of ursolic acid was 0.07 mM (Table 4), whereas americanin A, 3,3′-bisdemethylpinoresinol and quercetin were inactive. HLE inhibitory activity of ursolic acid has been reported previously [21] but, although ursolic acid has been isolated from leaves of *M. citrifolia* [20], isolation of ursolic acid from noni seed is not known hitherto.

Quercetin and two lignans (americanin A and 3,3′-bisdemethylpinoresinol) exhibiting both DPPH radical scavenging and tyrosinase inhibitory activities were isolated from Frs. D, E and F. As shown in Table 4, quercetin (IC_{50}, 0.1 mM) exhibited potent tyrosinase inhibitory activity, in accordance with reported data [22], with americanin A (IC_{50}, 2.7 mM) and 3,3′-bisdemethylpinoresinol (IC_{50}, 0.3 mM) showing significant activity. Kojic acid inhibited tyrosinase at IC_{50} 0.03 mM, whereas arbutin was only weakly inhibitory (IC_{50}, 83.3 mM).

The DPPH method has been used widely to measure the radical scavenging ability of plant extracts and their constituents, and the IC_{50} value indicates the concentration of sample required to scavenge 50% DPPH free radical [23]. As well as americanin A (IC_{50}, 11 μM), 3,3′-bisdemethylpinoresinol (IC_{50}, 4 μM) and quercetin (IC_{50}, 6 μM) exhibited potent activities, and the IC_{50} value of ascorbic acid, a positive control, was 23 μM (Table 4). Since tyrosinase inhibitors with SOD-like activity may be useful as a component of skin-whitening cosmetics, the SOD-like activity of quercetin and the two lignans was examined using SOD (IC_{50}, 0.3 U/ml) as a positive agent. In this experiment, the IC_{50} value represents the concentration of sample required to scavenge 50% of the superoxide anion produced by a hypoxanthine–xanthine oxidase system. As shown in Table 4, both quercetin (IC_{50}, 30 μM) and americanin A (IC_{50}, 170 μM) exhibited a potent SOD-like activity. Recently, the isolation of quercetin from noni fruit [7] as well as the isolation of americanin A and 3,3′-bisdemethylpinoresinol as antioxidant constituents of noni fruit [8] have been reported; however, this is the first report of the isolation of quercetin

Table 4 IC_{50} values of ursolic acid, americanin A, 3,3′-bisdemethylpinoresinol, quercetin and reference compounds

Sample	Human leukocyte elastase inhibitory activity (mM)	Tyrosinase inhibitory activity (mM)	SOD-like activity (μM or U/ml)[a]	Radical scavenging activity (μM)[b]
Ursolic acid	0.07	ND	ND[c]	ND
Americanin A	NE[d]	2.7	170 (μM)	11
3,3′-Bisdemethylpinoresinol	NE	0.3	NE	4
Quercetin	NE	0.1	30 (μM)	6
Phenylmethanesulfonyl fluoride	0.14	ND	ND	ND
Kojic acid	ND	0.03	ND	ND
Arbutin	ND	83.3	ND	ND
Superoxide dismutase (SOD)	ND	ND	0.3 (U/ml)	ND
Ascorbic acid	ND	ND	ND	23

[a] IC_{50} value represents the concentration of sample required to scavenge 50% of superoxide anion produced by hypoxanthine-xanthine oxidase system

[b] IC_{50} value represents the concentration of sample required to scavenge 50% of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical

[c] Not determined

[d] No effect

and these two lignans from noni seed. Although Fr. G showed both potent HLE inhibitory activity and DPPH radical scavenging activity, our attempts to identify its active constituents have been unsuccessful so far. Thus, the potent DPPH radical scavenging activity of MCS-ext (IC_{50}, 12 μg/ml) may be attributable to two lignans and quercetin.

In conclusion, MCS-ext was found to contain HLE inhibitory, tyrosinase inhibitory, and antioxidant active constituents, namely ursolic acid, 3,3′-bisdemethylpinoresinol, americanin A and quercetin. Hitherto, noni seed was deemed worthless and was discarded during the production process of noni fruit juice and noni oil. The findings presented here suggest that noni seed may have a role as a useful ingredient in cosmetics for skin-whitening and/or wrinkle-prevention.

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