

NOTE

Collagen Synthesis-Promoting Effects of Andiroba Oil and its Limonoid Constituents in Normal Human Dermal Fibroblasts

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Abstract: The seed oil of andiroba (*Carapa guianensis*, Meliaceae) was found to promote collagen synthesis in normal human dermal fibroblasts. To characterize the active constituents of this oil, the collagen synthesis-promoting activities of 10 principal limonoid constituents, gedunin (1), 6 α -acetoxygedunin (2), 7-deacetoxy-7-oxogedunin (3), 7-deacetoxy-7 α -hydroxygedunin (4), andirolide H (5), 6 α -hydroxygedunin (6), methyl angolensate (7), 17 β -hydroxyazadiradione (8), and carapanosides C (9) and R (10), were examined. Among them, 1–4, 6, 7, and 9 were found to significantly promote collagen synthesis without cytotoxicity at the effective concentrations.

Key words: andiroba oil, *Carapa guianensis*, limonoid, collagen synthesis-promoting activity, gedunin

1 INTRODUCTION

Carapa guianensis Aublet, a plant belonging to family Meliaceae, is popularly known in Brazil as andiroba^{1–5}. The seeds of this plant are the source of andiroba oil, which possesses a wide range of biological activities and ethnopharmacological uses⁶ such as antibacterial⁶, anticancer⁷, antifungal⁸, insect repellent⁹, analgesic¹⁰, antimalarial¹¹, anti-inflammatory¹², antiallergic¹³, antiplasmodial¹⁴ and antioxidative effects¹⁵. During our characterization studies on the chemical constituents of *C. guianensis*, we isolated several limonoids such as andirolides A–Y^{16–19}, carapanolides A–X^{20–24}, guianolides A and B²⁵, carapanosins A–C²⁶, guianolactones A and B²⁷, and guianofruits A and B²⁸. Regarding their biological activities, we have reported cytotoxic^{16, 18, 20, 21, 25}, antimalarial¹⁷, anti-inflammatory^{19, 22, 24, 26, 28}, triglyceride metabolism-promoting²³, and hepatoprotective activities²⁹. Continuing studies on the biological activities of *C. guianensis* revealed that its seed oil could promote collagen synthesis in normal human dermal fibroblasts (NHDFs). To characterize the active principles of andiroba oil responsible for this effect, the activities of 10 principal limonoid constituents; gedunin (1)^{17, 29}, 6 α -acetoxygedunin

(2)^{16, 29}, 7-deacetoxy-7-oxogedunin (3)^{16, 29}, 7-deacetoxy-7 α -hydroxygedunin (4)²³, andirolide H (5)¹⁷, 6 α -hydroxygedunin (6)¹⁷, methyl angolensate (7)¹⁷, 17 β -hydroxyazadiradione (8)²³, and carapanosides C (9)²¹ and R (10)²³ were also evaluated (Fig. 1).

2 MATERIALS AND METHODS

2.1 Plant material

The seed oil of *C. guianensis* Aublet (Meliaceae) was collected in Amazon, Brazil in March of 2011. Voucher specimens (CGS-01-1) were deposited at the Herbarium of the Laboratory of Medicinal Chemistry at Osaka University of Pharmaceutical Sciences as described previously^{23, 29}.

2.2 Chemicals and reagents

Limonoid constituents (1–10) were isolated from andiroba oil as described previously²⁹. Dulbecco's minimum essential medium (DMEM; glucose 1000 mg/L) was purchased from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum (FBS) was obtained from Life Technologies (Rock-

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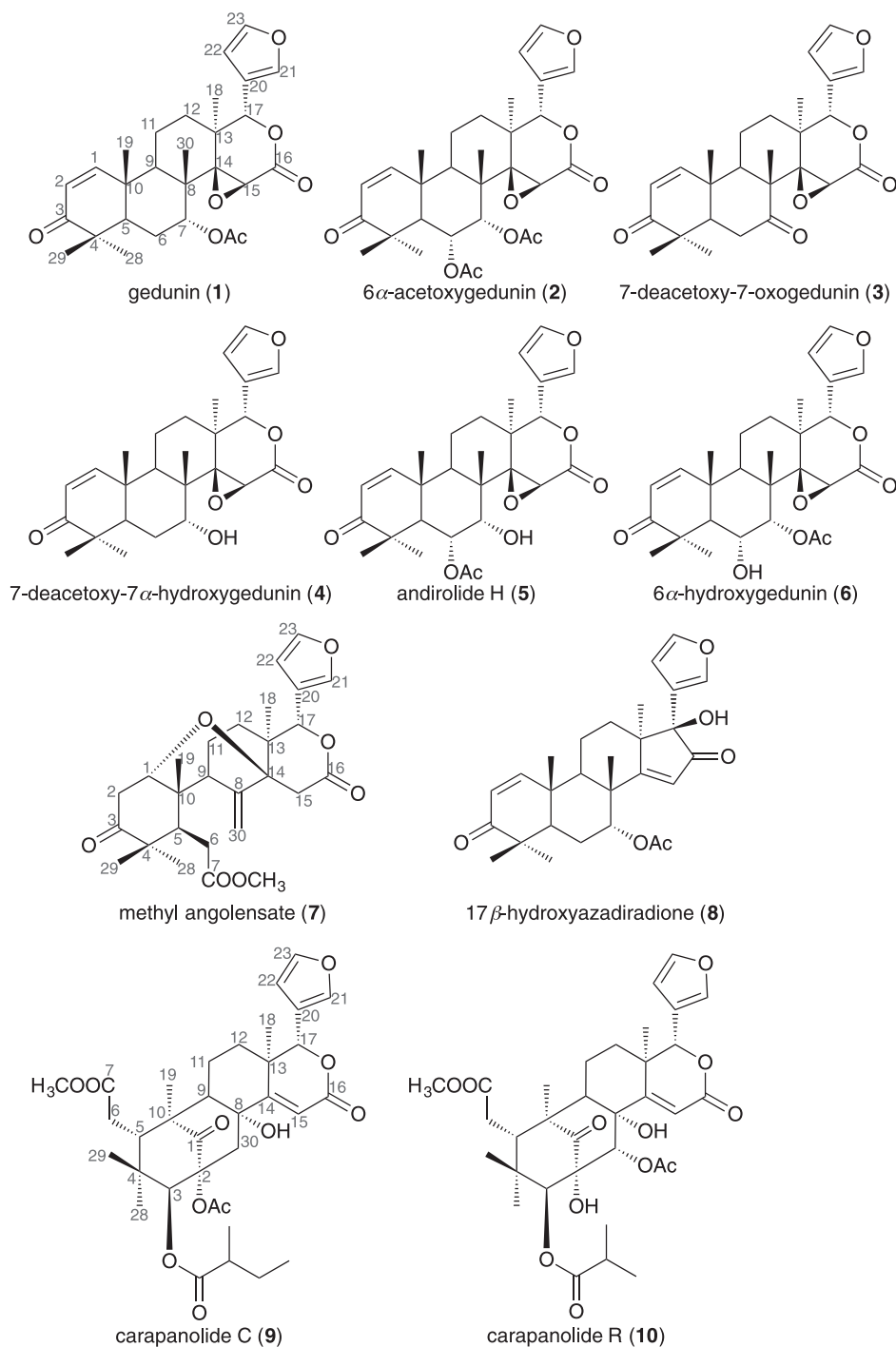


Fig. 1 Limonoid constituents (1–10) from andiroba oil.

ville, MD, USA), and other chemicals were obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). The 96-well microplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

2.3 Cell culture

Normal human dermal fibroblasts (NHDFs), derived from human fetal foreskin fibroblasts, were purchased from

Kurabo Industries Ltd. (Osaka, Japan), and cultured in Kurabo's modified medium 106S supplemented with 2% FBS and human skin fibroblast growth factor (Kurabo Industries Ltd.) at 37°C in a humidified atmosphere containing 5% CO₂.

2.4 Effects of andiroba oil and its limonoid constituents on collagen synthesis in NHDFs

Collagen synthesis was evaluated according to the method described previously with slight modifications^{30,31}. Briefly, a cell suspension of 2.5×10^4 NHDFs in 100 μ L DMEM containing FBS (10%), penicillin G (100 U/mL), and streptomycin (100 μ g/mL) was inoculated in a 96-well microplate and pre-incubated for 24 h at 37°C in 5% CO₂. Then, the medium was replaced with 100 μ L of fresh medium [FBS(-)] with or without the test compound. After incubation for 48 h, type I collagen content in the supernatant was determined using a commercial kit [Procollagen Type I C-peptide (PIP) EIA Kit (Takara Bio Inc., Shiga, Japan)]³². Data were expressed as % of control collagen content.

2.5 Effects of andiroba oil and its limonoid constituents on cell viability in NHDFs

Cell proliferation was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay³⁰. The medium was replaced with 100 μ L of fresh medium, and 10 μ L of MTT (5 mg/mL in phosphate-buffered saline [PBS(-)] solution) was added. After incubation for 4 h, the medium was removed, and isopropyl alcohol (100 μ L containing 0.04 M HCl) was added to dissolve formazan produced by the cells. The optical density of the formazan solution was measured by a microplate reader at 570 nm (reference: 655 nm). Each test compound was dissolved in dimethylsulfoxide (DMSO), and the solution was added to the medium to reach a final DMSO concentration of 0.5%. Human recombinant transforming growth factor (TGF)- β 1³⁰, asiaticoside^{30,33,34}, and madecassoside^{30,33,34} were used as reference compounds.

2.6 Effects of andiroba oil and its limonoid constituents on collagenase activity

The experiments were performed according to the method described previously with slight modifications^{35,36}. A test compound solution was prepared by dissolving the test compound in DMSO and diluting the solution with 0.1% (w/v) FBS containing PBS(-) to reach a DMSO concentration of 2%. A substrate solution [50 μ L of PBS(-) containing 2.5 μ M of MOCAC-Pro-Leu-Gly-Leu-A₂Pr (Dnp)-Ala-Arg-NH₂ (Peptide Institute Inc., Osaka, Japan)] and the test compound solution (50 μ L) were mixed into a 96-well half area black microplate (Greiner Bio-One, Frickenhausen, Germany) at 37°C for 10 min. Then, 100 μ L of an enzyme solution containing 20 μ g/mL of collagenase B (from *Clostridium histolyticum*, Sigma-Aldrich) was added and the reaction mixture was incubated at 37°C for 30 min to induce the fluorescent signals. Fluorescence was measured using a fluorescence microplate reader (SH-9000, CORONA) at an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Experiments were per-

formed in triplicate, and IC₅₀ values were determined graphically. The collagenase inhibitor caffeic acid (IC₅₀ = 75.6 μ M) was used as a reference compound.

2.7 Statistical analysis

Values were expressed as the mean \pm standard error of the mean (S.E.M.). Results were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability (*p*) values less than 0.05 were considered statistically significant.

3 RESULTS and DISCUSSION

3.1 Effect of andiroba oil on collagen synthesis in NHDFs

One of the main causes of skin aging is the decrease in type I collagen, a primary component of the dermal layer of the skin³⁷. Thus, compounds that can maintain type I collagen levels may be able to prevent skin aging³². Collagen, a fibrous extracellular matrix protein, is a major component in the connective tissue of the human body. Collagen constitutes approximately 3–6% of the total tissue protein in the body. The functional properties of the skin depend on the integrity of collagen in the dermis. Collagen deposition is finely controlled, and is dependent on the physiological status of the body. Changes in the rate of collagen deposition occur during wound healing, development of new bone, and aging. Thus, the control of collagen metabolism may be useful for various therapeutic and cosmetic applications³¹.

In previous studies, asiaticoside and madecassoside, ursane-type triterpene 28-O-oligoglycosyl esters, were isolated from *Centella asiatica*, a herb used in Sri Lanka and Indian Ayurvedic traditional medicine^{33,34}. These triterpene saponins were reported to promote the synthesis of human type I collagen^{32,38–41}. We have previously reported that the saponin constituents isolated from the flowers of *Bellis perennis* could significantly promote collagen synthesis without cytotoxicity at the effective concentrations. For example, perennisosides I, II, VII, IX, XI, and XVIII increased collagen content to 120.6% \pm 3.4, 131.7% \pm 3.1, 132.0% \pm 5.0, 126.1% \pm 6.0, 124.8% \pm 4.6, and 113.1% \pm 1.1 at 3, 30, 10, 10, 10, and 30 μ M, respectively. In addition, asterbatanoside D, bernardioside B₂, bellissaponin BS5, and bellissaponin BS9 increased collagen content to 121.7% \pm 2.8, 115.4% \pm 1.9, 127.0% \pm 2.7, and 127.6% \pm 2.9 at 10, 30, 10, and 30 μ M, respectively³⁰.

In the present study, we investigated the collagen synthesis-promoting effects of andiroba oil in NHDFs at concentrations of 10–100 μ g/mL (Table 1).

3.2 Effect of the principal limonoids (1–10) on collagen synthesis in NHDFs

To identify the active constituents of andiroba oil, the

Table 1 Effects of andiroba oil and TGF- β 1 on collagen synthesis in NHDFs.

	Inhibition (%)				
	0 μ g/mL	3 μ g/mL	10 μ g/mL	30 μ g/mL	100 μ g/mL
Andiroba oil	100.0 \pm 4.3 (100.0 \pm 1.0)	111.3 \pm 4.1 (107.8 \pm 2.7)	121.6 \pm 1.8** (98.4 \pm 4.0)	115.2 \pm 2.4* (88.8 \pm 0.7)	128.4 \pm 4.3** (85.8 \pm 1.3)
TGF- β 1 ³⁰⁾	0 ng/mL	1 ng/mL	3 ng/mL	10 ng/mL	30 ng/mL
	100.0 \pm 1.8 (100.0 \pm 2.4)	196.5 \pm 5.7** (85.2 \pm 1.2)	240.0 \pm 5.7** (74.1 \pm 3.1 [#])	255.9 \pm 10.1** (71.0 \pm 2.6 [#])	261.0 \pm 2.0** (70.5 \pm 2.0 [#])

Each value represents the mean \pm S.E.M. ($N = 4$); asterisks denote significant differences from the control group, * $p < 0.05$, ** $p < 0.01$; [#]cytotoxic effects were observed, and values in parentheses indicate cell viability (%) in MTT assay.

Table 2 Effects of limonoid constituents (1–10) from andiroba oil, asiaticoside, and madecassoside on collagen synthesis in NHDFs.

	Inhibition (%)					
	0 μ M	1 μ M	3 μ M	10 μ M	30 μ M	100 μ M
Gedunin (1)	100.0 \pm 3.8 (100.0 \pm 1.9)	110.0 \pm 7.1 (96.2 \pm 0.6)	133.3 \pm 3.6** (98.5 \pm 0.9)	134.7 \pm 3.6** (105.6 \pm 2.0)	136.8 \pm 3.5** (93.1 \pm 2.0)	– (58.9 \pm 1.6 [#])
6 α -Acetoxygedunin (2)	100.0 \pm 3.9 (100.0 \pm 1.9)		114.9 \pm 1.5 (95.3 \pm 1.3)	111.9 \pm 3.5 (96.8 \pm 1.1)	111.9 \pm 4.2 (103.1 \pm 0.9)	152.8 \pm 6.8** (97.4 \pm 1.0)
7-Deacetoxy-7-oxogedunin (3)	100.0 \pm 4.8 (100.0 \pm 1.4)		111.6 \pm 2.5 (98.6 \pm 1.0)	113.8 \pm 2.0 (99.2 \pm 0.2)	129.5 \pm 3.0** (99.6 \pm 0.8)	143.5 \pm 5.8** (85.7 \pm 1.0)
7-Deacetoxy-7 α -hydroxygedunin (4)	100.0 \pm 3.0 (100.0 \pm 3.3)	107.0 \pm 2.2 (119.6 \pm 1.2)	119.4 \pm 3.1** (114.6 \pm 3.5)	122.1 \pm 2.9** (110.4 \pm 4.9)	130.3 \pm 4.5** (81.5 \pm 0.9)	– (65.7 \pm 1.0 [#])
Andirolide H (5)	100.0 \pm 3.9 (100.0 \pm 1.3)		107.8 \pm 3.3 (97.3 \pm 1.8)	107.8 \pm 2.6 (108.9 \pm 1.2)	102.0 \pm 2.4 (103.8 \pm 2.6)	97.8 \pm 3.4 (57.6 \pm 1.6 [#])
6 α -Hydroxygedunin (6)	100.0 \pm 7.5 (100.0 \pm 1.1)		110.5 \pm 4.9 (93.1 \pm 0.5)	115.3 \pm 3.4 (95.1 \pm 0.6)	121.4 \pm 2.7* (103.3 \pm 1.7)	137.7 \pm 2.8** (108.8 \pm 1.4)
Methyl angolensate (7)	100.0 \pm 2.7 (100.0 \pm 1.2)		105.5 \pm 2.0 (96.2 \pm 1.0)	107.6 \pm 2.1 (100.1 \pm 1.0)	114.5 \pm 2.9** (107.5 \pm 0.9)	115.1 \pm 1.4** (115.1 \pm 3.2)
17 β -Hydroxyazadiradione (8)	100.0 \pm 4.4 (100.0 \pm 1.3)		97.5 \pm 4.3 (90.1 \pm 1.0)	90.0 \pm 3.6 (94.7 \pm 2.6)	91.3 \pm 3.9 (97.7 \pm 2.8)	– (54.0 \pm 1.4 [#])
Carapanoside C (9)	100.0 \pm 4.6 (100.0 \pm 4.1)		112.9 \pm 4.7 (107.2 \pm 4.2)	123.0 \pm 3.5** (92.7 \pm 2.0)	137.7 \pm 3.3** (96.7 \pm 1.2)	132.5 \pm 1.5** (85.9 \pm 0.2)
Carapanoside R (10)	100.0 \pm 2.2 (100.0 \pm 0.7)		98.3 \pm 4.3 (91.3 \pm 1.9)	94.0 \pm 3.0 (86.8 \pm 1.0)	95.0 \pm 1.8 (98.3 \pm 3.7)	– (35.0 \pm 1.6 [#])
Asiaticoside ³⁰⁾	100.0 \pm 3.1 (100.0 \pm 0.9)			105.5 \pm 2.7 (99.8 \pm 0.1)	109.4 \pm 4.1 (102.3 \pm 0.9)	138.1 \pm 2.3** (94.7 \pm 0.8)
Madecassoside ³⁰⁾	100.0 \pm 2.0 (100.0 \pm 4.2)			104.8 \pm 1.4 (97.1 \pm 1.1)	102.7 \pm 1.3 (94.1 \pm 2.1)	113.5 \pm 1.9** (91.9 \pm 0.6)

Each value represents the mean \pm S.E.M. ($N = 4$); asterisks denote significant differences from the control group, * $p < 0.05$, ** $p < 0.01$; [#]cytotoxic effects were observed, and values in parentheses indicate cell viability (%) in MTT assay.

principal limonoids (1–10) were examined for their promoting effects on collagen synthesis in NHDFs. As shown in **Table 2**, gedunin (1), 6 α -acetoxygedunin (2), 7-deacetoxy-7-oxogedunin (3), 7-deacetoxy-7 α -hydroxygedunin (4), 6 α -hydroxygedunin (6), methyl angolensate (7), and carapanoside C (9) significantly promoted collagen synthesis in NHDFs to 133.3% \pm 3.6, 152.8% \pm 6.8, 129.5% \pm 3.0,

119.4% \pm 3.1, 121.4% \pm 2.7, 114.5% \pm 2.9, and 123.0% \pm 3.5 at 3, 100, 30, 3, 30, 30, and 10 μ M, respectively, without notable cytotoxic effects at the effective concentrations. Among them, 1 and 4 showed relatively strong activities, which were comparable to that of our previously reported perennisoside I.

As for the structural requirements of gedunin-type li-

monoids were assessed; (i) 6 α -acetoxy and 6 α -hydroxy moieties reduced the activity [gedunin (1, 133.3% \pm 3.6) > 6 α -acetoxygedunin (2, 114.9% \pm 1.5) and 6 α -hydroxygedunin (6, 110.5% \pm 2.0) at 3 μ M]; (ii) compounds with 7 α -acetoxy group exhibited higher activity than that with 7 α -hydroxy or 7-keto groups [1 > 7-deacetoxy-7-oxogedunin (3, 111.6% \pm 2.5) and 7-deacetoxy-7 α -hydroxygedunin (4, 119.4% \pm 3.1); 2 > andirolide H (5, 107.8% \pm 3.3) at 3 μ M]; (iii) compounds with an α,β -epoxy- γ -lactone moiety in the D-ring exhibited higher activity than that with an α,β -unsaturated cyclopentanone moiety [1 > 17 β -hydroxyazadiradione (8, 97.5% \pm 4.3) at 3 μ M].

3.3 Effects on collagenase activity

The skin elasticity largely depends on the presence of collagen, which is decomposed by collagenase enzyme. Thus, the discovery and development of compounds with collagenase inhibitory activity can help maintain skin elasticity and thus, provide an effective approach for preventing skin aging^{35, 36}. In order to characterize one of the mechanisms of action by which some compounds could increase collagen levels in NHDFs, we examined the collagenase inhibitory activity of the limonoid constituents. However, they did not demonstrate collagenase inhibitory activity (data not shown), which suggests that collagenase inhibition is barely involved in the mechanisms by which they promote collagen synthesis.

4 CONCLUSION

In conclusion, andiroba oil can significantly promote collagen synthesis in NHDFs at concentrations of 10–100 μ g/mL. Several limonoids such as gedunin (1), 6 α -acetoxygedunin (2), 7-deacetoxy-7-oxogedunin (3), 7-deacetoxy-7 α -hydroxygedunin (4), 6 α -hydroxygedunin (6), methyl angolensate (7), and carapanoside C (9) were investigated as the active constituents and were found to promote collagen synthesis by 133.3% \pm 3.6, 152.8% \pm 6.8, 129.5% \pm 3.0, 119.4% \pm 3.1, 121.4% \pm 2.7, 114.5% \pm 2.9, and 123.0% \pm 3.5 at 3, 100, 30, 3, 30, 30, and 10 μ M, respectively, without causing cytotoxicity. In particular, 1 and 4 showed relatively potent activities, which were comparable to that of our previously reported perennisoside I. In addition, the structural requirements of gedunin-type limonoids with regard to the collagen synthesis-promoting activity in NHDFs were found to show as followings; (i) 6 α -acetoxy and 6 α -hydroxy moieties reduced the activity; (ii) compounds with 7 α -acetoxy group exhibited higher activity than that with 7 α -hydroxy or 7-keto groups; (iii) compounds with an α,β -epoxy- γ -lactone moiety in the D-ring exhibited higher activity than that with an α,β -unsaturated cyclopentanone moiety. Further studies are required to elucidate the detailed mechanisms of action of these li-

monoids.

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