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Anti-Inflammatory Effects of Seeds of the Tropical Fruit Camu-Camu (*Myrciaria dubia*)

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Summary The methanolic extract of seeds of the tropical fruit camu-camu was screened for its anti-inflammatory activity in carrageenan-induced paw edema model mice. The extract significantly suppressed both the formation of edema in mice by oral administration and the release of nitric oxide from macrophage-derived RAW 264.7 cells in vitro. Based on the results of a spectroscopic analysis, the active compound was identified by in vivo bioassay-guided fractionation to be 3β-hydroxy-lup-20(29)-en-28-oic acid, betulinic acid, known as an anti-inflammatory triterpenoid. These findings suggest that camu-camu seed extract is a potentially useful material as a source of betulinic acid and as a functional food for prevention of immune-related diseases.

Key Words seed of camu-camu fruit *Myrciaria dubia*, anti-inflammatory effects in mice, suppressive effect on nitric oxide release, betulinic acid

Camu-camu is a tropical fruit that is native to Peru, and is known to be a rich source of vitamin C (*1*, *2*), and anthocyanins (*3*). The squeezed fruit extract is also used in many kinds of sweets and drinks in Japan. However, other parts of the camu-camu have not yet been used. In other fruits, the seeds often contain ingredients with biological activity; for example, amygdaline in peach (*4*, 5), polyphenols in pomegranate (*6*), procyanidins in grape (*7*) and antifungal protein in passion fruit (*8*) have all been reported as bioactive compounds from seed extracts. In our studies to identify possible uses of camu-camu, we found anti-inflammatory activity in the methanol extract of the seed in carrageenan-induced edema model mice, and in LPS-treated RAW264.7 cells. After in vivo bioactive-guided fractionation from the crude extract of camu-camu seed, the main active ingredient was purified and identified as betulinic acid by spectroscopic analysis.

MATERIALS AND METHODS

Sample preparation. Fresh camu-camu seeds (92 g) were extracted with 10 volumes of MeOH at room temperature for 3 d. The pale green oil (7.0 g) yielded by filtration and evaporation of the extract was used in experiments in vivo and in vitro as a crude extract of camu-camu seeds (CCS).

Animals. Male Std:ddY mice from Japan SLC, Inc. (Hamamatsu, Japan) were used. The room was maintained at 24±1°C and 50±10% humidity under a 12-h light/dark cycle, and the animals had free access to water and standard laboratory chow, Labo MR stock (Nosan, Japan), for 1 wk to accustom them to their surroundings. Animal studies were performed according to the 2006 guidelines entitled Notification No. 88 of the Ministry of the Environment in Japan.

Carrageenan-induced paw edema in mice. The test was conducted in 9-wk-old mice according to the methods of Winter et al. (*9*) with minor modifications. Thirty minutes after the ingestion of the sample solution (1.0 mL/mouse), mice were treated with 0.05 mL of 1% carrageenan (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by subcutaneous injection into the right hind paw to induce acute inflammation. Samples were solved or suspended in distilled water or 10% DMSO. The control group received the vehicle (distilled water), and the positive control group ingested dexamethasone (1.0 mg/kg, the biochemical grade: not less than 98% of purity, Wako). At 2 and 4 h after treatment, the degree of paw edema was evaluated by a thickness gauge (Peacock, Ozaki MFG. Co., Ltd., Tokyo, Japan) and by the volume of water that overflowed from a constant vessel (common plastic tube).

LPS-induced release of inflammatory mediators in vitro. A murine macrophage cell line, RAW 264.7 (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), was used for in vitro experiments on the release of inflammatory mediators (*10*). Cells were maintained in DMEM supplemented with glutamine (1 mM) and 10%

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Abbreviations: CCS, crude extract of camu-camu seed; DMEM, Dulbecco's modified Eagle's medium; IC_{50}, concentration that gives 50% inhibition; IL-1β, interleukin 1β; iNOS, inducible NO synthase; LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tissue necrosis factor α.

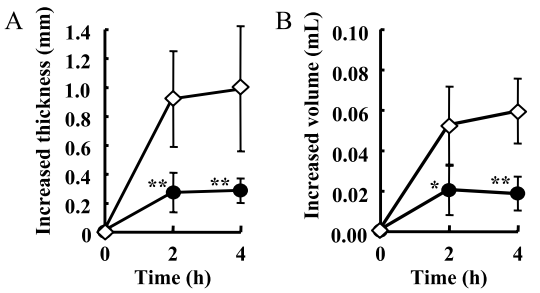

Fig. 1. Effects of pre-treatment with oral CCS (2,000 mg/kg, closed circles) on paw edema induced by 1% carrageenan (0.05 mL/paw) in mice. Edema was evaluated in terms of the increase in both the thickness (A) and volume (B) of the paw after induction. Control mice were pre-treated with distilled water (open diamonds). Data are expressed as the mean±SE for 6 animals per group. *$p<0.05$ and **$p<0.01$ compared to control.

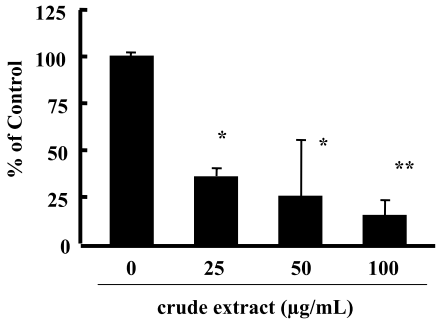

Fig. 2. Effects of CCS on LPS-induced NO production in RAW 264.7 cells. After stimulation with LPS (500 ng/mL) for 24 h, the culture supernatants were isolated and analyzed for nitrate levels, which are expressed as a% of the control without CCS. Values are the means±SE of duplicate determinations from three separate experiments. *$p<0.05$ and **$p<0.01$ compared to control.

fetal bovine serum (FBS) at 37˚C in an atmosphere of 5% CO_2. Samples were dissolved in MeOH and diluted with medium in 100 μL each on a 96-well plate, and then the cell solution (final 10^6 cells/well) was added. Finally, cells were stimulated with LPS solution (final 500 ng/mL) and incubated for 24 h. After stimulation in the presence or absence of samples, mediators that were released into the culture supernatant were evaluated as described below.

NO synthesis in cell cultures was measured by a microplate assay method after treatment with Griess reagent. Other inflammatory mediators were measured by ELISA with the respective antibody using commercially available kits from Cosmo Bio Co. Ltd. (Tokyo, Japan) for TNF-α and IL-1β. In the same experiment, cell viability was checked with MTT reagent, to confirm that the change in the NO level did not depend on the number of cells in each experiment.

Bioassay-guided fractionation and structure determination. The active component of CCS was fractionated by partition and column chromatography techniques based on inhibitory activity against NO release in vitro. The structure of the purified compound was then determined based on spectral data by NMR experiments. NMR spectra in $CDCl_3$ were obtained on a Bruker AV-400 spectrometer (Bruker, Tokyo, Japan). FAB-MS was recorded on a JMS-700 (JEOL, Tokyo, Japan).

Statistical analysis. One-way analysis of variance (ANOVA) followed by a post-hoc Tukey-Kramer test for multiple comparisons was performed to determine the significance of differences in the data among groups. p values less than 0.05 were considered significant. The values are expressed as mean±SE.

RESULTS AND DISCUSSION

Anti-inflammatory effects of the crude extract of camu-camu seeds (CCS)

The mouse paws became edematous after the injection of carrageenan, and edema reached its peak at 4 h in the control group. The increase of paw edema was perfectly suppressed to less than 0.1% by oral treatment of dexamethasone (1.0 mg/kg). On the other hand, pretreatment of mice with CCS (2,000 mg/kg) significantly reduced edema formation with respect to both size (Fig. 1A) and volume (Fig. 1B) at 2 and 4 h after carrageenan treatment. The inhibitory effects of CCS were demonstrated in four independent experiments. The averaged inhibitory ratio was calculated as 35.7±6.7% at 2,000 mg/kg, 63.8±7.3% at 1,000 mg/kg and 85.1±10.3% at 500 mg/kg against the control value of paw thickness at 2 h in carrageenan-treated mice. It has been shown that carrageenan induces edema via a completely localized inflammatory response in rodents (*11*). Our results revealed that oral pretreatment with CCS may suppress paw edema formation caused by the inhibition of localized inflammation in a dose-dependent manner in mice.

The effects of CCS on nitric oxide (NO) synthesis were examined in RAW264.7 cells. Whereas LPS-stimulated cells showed significantly increased NO levels compared to normal cells without LPS, this stimulation was inhibited in the presence of CCS in a dose-dependent manner (Fig. 2). The IC_{50} value of CCS is 6.4 μg/mL under these conditions. On the other hand, cell viability was not affected by CCS treatment as measured by MTT assay in the same range of sample concentrations (data not shown).

These results in vivo and in vitro suggest that CCS suppresses the formation of paw edema by inhibiting localized NO production in carrageenan-treated mice.

Bioassay-guided fractionation

Based on the inhibitory activity toward NO production as an index, the main ingredient in CCS was separated as shown in Fig. 3A. The crude extract (CCS, 5.0 g) was fractionated by solvent partition between ethyl acetate and water (3 times of 500 mL each). The ethyl acetate-soluble fraction (2.0 g) was dried and the hexane-soluble and 60% MeOH-soluble fractions were removed by solvent partition (3 times each of 200 mL each) to give an active fraction (CH_2Cl_2-soluble fraction, 0.45 g). Then the active fraction was separated on a sil-

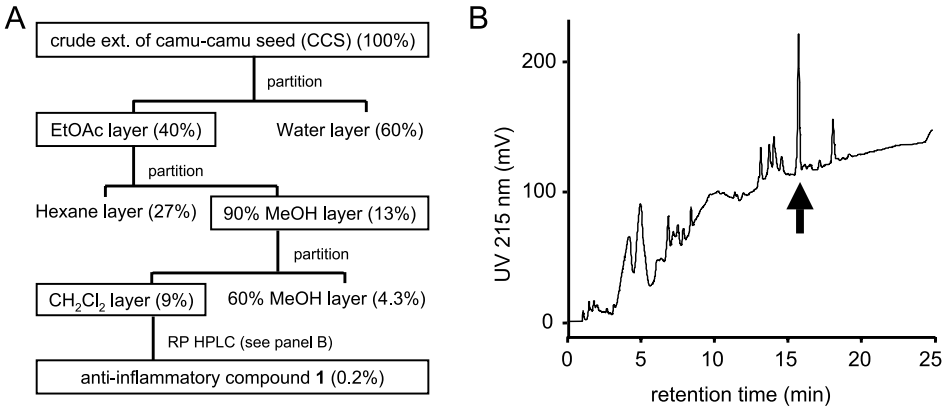

Fig. 3. Purification processes (A) and HPLC pattern (B) of anti-inflammatory compound from crude extract of camu-camu seed (CCS). A: The numbers in parentheses mean recovery rates from crude extract (100%). Framed fractions showed anti-inflammatory activity in mice. B: Dichloromethane soluble fraction was separated on a reversed phase column, Develosil ODS-HG-5 (20×250 mm) with a linear gradient (40% MeOH-H_2O→MeOH, 5.0 mL/min) for 15 min by detecting 215 nm using an HPLC system (JASCO, Tokyo, Japan).

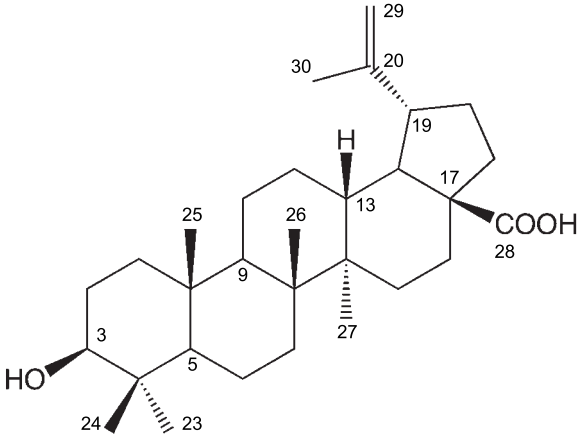

Fig. 4. Chemical structure of betulinic acid (**1**) isolated from camu-camu seed.

ica gel column (60 g) into 20 mL of fractions and further separated in every detectable peak by HPLC using an ODS column (20×250 mm, Develosil ODS HG-5, Nomura Chem. Indus, Nagoya, Japan) eluted with a linear gradient from 40 to 98% of aqueous MeOH for 30 min (flow rate, 5 mL/min; detection, UV 215 nm) to give compound **1** (retention time: 15.7 min) with inhibitory activity toward NO production. Bioassay-guided fractionation eventually yielded white crystals (**1**, 10.5 mg, 0.015% of seeds) that showed a single peak in HPLC analysis (Fig. 3B).

Identification of the active component

Compound **1** showed a positive FABMS molecular ion peak at *m/z* 457 $[M+H]^+$, and a molecular formula of $C_{30}H_{48}O_3$ was surmised based on spectroscopic data of ^1H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz). Assignments for closed proton signals and multiplicity of carbons were based on 2D NMR spectra. These spectral data supported **1** as a lupen-type triterpene.

The relative stereochemistry of the hydroxyl group at C-3 was determined to be a beta position based on NOESY correlations 24-Me/25-Me, 23-Me/H-3, H-3/H-5 and H-5/H-9 and coupling constants of H2a/H3 (J=11.2 Hz) and H2b/H3 (J=4.9 Hz). Therefore, **1** was identified to be 3β-hydroxy-lup-20(29)-en-28-oic acid, betulinic acid (Fig. 4). These spectral data for **1** corresponded to those reported in the literature (*12–15*). The lupen-type triterpene, was found in the stem bark of the plant *Zizyphus mauritiana* as a melanoma-specific cytotoxic compound (*16*). It also shows anti-inflammatory activities in carrageenan-treated mice and LPS-treated RAW264.7 cells (*17*, *18*) and antitumor activities as indicated by the inhibition of NF-κB activation due to its inhibitory effect on IκB phosphorylation (*16*, *19*). In our experiment, 20 μg/mL of 1 suppressed TNF-α (1,260.0±215.0 pg/mL for control) and IL-1β (150.5±27.3 pg/mL for control) to 39.5±7.2% and 48.4±1.5%, respectively. These results on the release of inflammatory mediators were consistent with the results of previous studies on betulinic acid (*17*, *20*).

Betulinic acid has been found in various plants, such as Chilean guava *Ugni molinae* leaves (*15*), the traditional Chinese herb *Zizyphi spinosi* semen (*17*), and Cortex Acanthopanasis *Acanthopanax gracilistylus* root bark (*21*). However, this is the first report of betulinic acid as a natural constituent of the Myrciaria, including camu-camu. Since the anti-inflammatory effect of orally administrated CCS in mice should be the result of betulinic acid, the camu-camu seed is expected to be a food material with an anti-inflammatory effect. In this report, we have demonstrated that the oral administration of CCS has an anti-inflammatory effect in mice (1,000 and 2,000 mg/kg). However, the fruit pulp extract of camu-camu (1,000 mg/mL) did not have anti-inflammatory activity in mice (unpublished data). Our observations revealed that betulinic acid is localized in the seeds and is not found in the fruit pulp. On the other hand, the pulp extract contains ellagic acid, the derivatives of which have been shown to have inhibitory activity against aldose reductase in vitro (*22*). In a clinical trial with camu-camu juice for 7 d, improvements in oxidative and inflammatory parameters were

observed (*23*). These effects were considered to be due to the function of rich vitamin C and/or other unidentified ingredients in the extract. Camu-camu fruit has attracted attention as a herbal plant. However, not enough information is available on many of its functional ingredients. To better understand the various bioactivities of this health food, further investigations are needed regarding its functional ingredients.

In the present study, we found that an extract of camu-camu seeds contained betulinic acid by bioassay-guided fractionation and spectroscopic analysis, and that oral administration of the extract suppressed experimental edema formation in mice. These results suggest that the camu-camu seed may be an important source of health food.

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