Separation of *Leucas aspera*, a Medicinal Plant of Bangladesh, Guided by Prostaglandin Inhibitory and Antioxidant Activities

Samir Kumar SADHU, Emi OKUYAMA,* Haruhiro FUJIMOTO, and Masami ISHIBASHI

Graduate School of Pharmaceutical Sciences, Chiba University; 1–33 Yayoi-cho, Inage-ku, Chiba 263–8522, Japan.
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According to the traditional usage of the plant for antiinflammation and analgesia, *Leucas aspera* was tested for its prostaglandin (PG) inhibitory and antioxidant activities. The extract showed both activities, *i.e.*, inhibition at 3×10^{-4} g/ml against PGE_1- and PGE_2-induced contractions in guinea pig ileum and a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect. The separation guided by the activities in these dual assay methods provided eight lignans and four flavonoids, LA-1—-12, among which LA-1—-7 and LA-10—-12 were identified as nectandrin B, *meso*-dihydroguaiaretic acid, macelignan, acacetin, apigenin 7-*O*-[6″-*O*-(*p*-coumaroyl)-β-D-glucoside], chrysoeriol, apigenin, *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol, myristargenol B, and machilin C, respectively. LA-8 was determined to be (−)-chicanine, the new antipode of the (+) compound, by spectroscopic methods including CD and ORD. Chiral-HPLC analysis of LA-9 showed that it was a mixture of two enantiomers, (7*R*,8*R*)- and (7*S*,8*S*)-licarin A. All of these components were first isolated from *L. aspera*. PG inhibition was observed in LA-1, LA-2, and LA-5, and antioxidant activity in LA-1—-3 and LA-8—-12.

Key words *Leucas aspera*; prostaglandin inhibition; antioxidant activity; lignan; (−)-chicanine

Leucas aspera LINK (Labiatae) (darkolos or dandokolos in Bangladesh) is a common aromatic herb and grows abundantly in Bangladesh and also in the wide area of South Asia. Traditionally, the decoction of the whole plant is taken orally for analgesic-antipyretic, antirheumatic, antiinflammatory, and antibacterial treatment, *etc.*, and its paste is applied topically to inflamed areas.[1] Some reports have been published on the chemical constituents such as sterols, fatty acids, lactones, long-chain compounds, aliphatic ketols, and phenols.[2—6] However, the biological activities of this plant have not been studied, except for antifungal effects.[7] In our continuous research on traditional medicines concerning their herbal usage,[8,9] the extract of *L. aspera* indicated prostaglandin (PG) inhibition in the Magnus method and antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The paper deals with the activity-oriented isolation of the extract using dual assay guides to identify the bioactive components of this plant.

The MeOH extract of *L. aspera* 3×10^{-4} g/ml showed inhibition against both PGE_1- and PGE_2-induced contractions in guinea pig ileum. It also showed positive (discolored) spots with a reddish purple background on TLC using DPPH as a spray reagent. After removal of chlorophylls by Diaion HP 20 column chromatography, the extract was evaluated for antioxidant activity (IC_{50} *ca.* 100 μg/ml) using a microplate reader with DPPH reagent. It was then partitioned with *n*-hexane, *n*-BuOH, and water, and the PG inhibitory activity was concentrated in the *n*-BuOH fraction. The DPPH radical scavenging effect was mostly observed in the *n*-hexane and *n*-BuOH fractions. Based on these results, the *n*-BuOH fraction was further separated by Sephadex LH-20 column chromatography to obtain the fractions with PG inhibitory activity, fr. 1-B—1-E. Among them, fr. 1C and fr. 1D also showed clear DPPH-positive spots on TLC. Both fractions were then separated independently by repeated column chromatography by targeting DPPH-positive spots on TLC. Three major components, **LA-1**—**-3**, together with five minor ones, **LA-8**—**-12**, were obtained (Fig. 1). **LA-1**—**-3** and **LA-10**—**-12** were identified as nectandrin B,[10,11] *meso*-dihydroguaiaretic acid,[12] macelignan,[13] *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol,[14,15] myristargenol B,[12] and machilin C,[16] respectively, by comparison with the published data.

Spectroscopic analysis including 2D-NMR of **LA-8** indicated a plane structure that was identical with that of (+)-chicanine, although the CD and ORD spectra were antipodal to the published data.[17,18] Therefore, **LA-8** was determined to be a new compound, (−)-chicanine.

The ^{1}H- and ^{13}C-NMR data of **LA-9** were superimposable on those of licarin A.[19,20] Although **LA-9** showed a positive Cotton effect at 268 nm in the CD spectrum, the $\Delta\varepsilon$ value seemed to be very low compared with the published data of a (7*R*,8*R*)-isomer ($\Delta\varepsilon_{275}$ +5.06, $\Delta\varepsilon_{222}$ −4.04).[21] **LA-9** was subjected to chiral-HPLC analysis, which resulted in two separable peaks with an approximately 3 : 2 ratio, as shown in Fig. 2. **LA-9** should be a 3 : 2 mixture of (7*R*,8*R*)- and (7*S*,8*S*)-licarin A, respectively. Chiral HPLC analysis of **LA-3** and **LA-10**, however, did not show any separated peaks under the conditions used.

From fr. 1E, the other PG inhibitory fraction with no DPPH activity, **LA-4**—**-7** were isolated, which were identified as acacetin, apigenin 7-*O*-[6″-*O*-(*p*-coumaroyl)-β-D-glucoside],[22] chrysoeriol,[23] and apigenin,[24] respectively, by comparison with the authentic sample and/or the published data.

The PG inhibitory activities of **LA-1**—**-7** were evaluated by the method using PG-induced contraction in guinea pig ileum. **LA-1** exhibited inhibition at concentrations of 9×10^{-7} g/ml (2.6 μM) and 3×10^{-6} g/ml (8.7 μM) against PGE_1- and PGE_2-induced contractions, respectively, and **LA-2** was inhibitory at a concentration of 3×10^{-6} g/ml (9.1 μM) for both contractions. **LA-3** was found to be inactive at concentrations up to 9×10^{-7} g/ml (270 μM), although its structure was very similar to that of **LA-2**, except for a methylenedioxy substituent in the A ring. Among the isolated flavonoids, **LA-5** only caused inhibition at 3×10^{-6} g/ml

* To whom correspondence should be addressed. e-mail: emi@p.chiba-u.ac.jp © 2003 Pharmaceutical Society of Japan

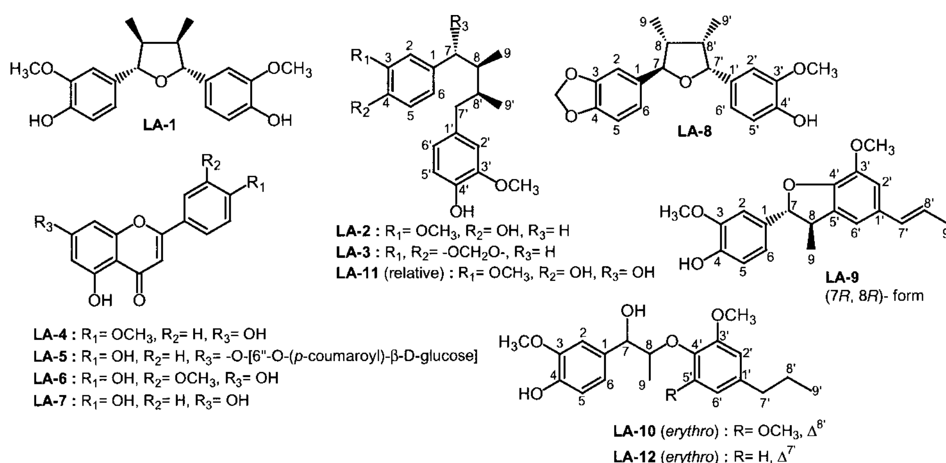

Fig. 1. Structures of the Isolated Compounds of *Leucas aspera*

(5.2 μM) against PGE_1-induced contraction, but was inactive against PGE_2 at the same concentration.

In the case of antioxidant activity on TLC sprayed with DPPH reagent, all the lignans and neolignans **LA-1**—**-3** and **LA-8**—**-12** showed positive spots on TLC, whereas the flavonoids **LA-4**—**-7** were negative. The IC_{50} values of **LA-1**—**-3** and **LA-5** and quercetin as a positive control recorded on a microplate reader with DPPH were 60, 28, 50, >500, and 30 μM, respectively. The antioxidant activity of nectandrin B (**LA-1**), *meso*-dihydroguaiaretic acid (**LA-2**), and macelignan (**LA-3**) have been already reported to have IC_{50} values of 74, 35, and 69 μM, respectively, using ESR spectroscopy.[25] It was reported that *meso*-dihydroguaiaretic acid (**LA-2**) significantly preserved the levels and activities of glutathione, superoxide dismutase, glutathione oxidase, and catalase, and ameliorated lipid peroxidation.[26] Acacetin (**LA-4**), chrysoeriol (**LA-6**), and apigenin (**LA-7**) were reported to have IC_{50} values of >500 μM as a result of DPPH assay.[27]

L. aspera has been used traditionally for its analgesic, anti-inflammatory, and antirheumatic properties. To find out the components responsible for the efficacy of this plant, we used dual assay methods, PG inhibitory and radical scavenging activities, since PGs and reactive oxygen and nitrogen species are closely related to inflammation and rheumatoid arthritis.[28,29] From this point of view and our results, the major active components, **LA-1**—**-3** and **LA-5**, together with other minor ones, may contribute to the efficacy through inhibition of the inflammatory process. Some flavonoids, such as acacetin (**LA-4**), chrysoeriol (**LA-6**), and apigenin (**LA-7**), did not indicate any activity in this experiment. The following reports, however, suggested their contribution to the anti-inflammatory effect as well: acacetin for inhibition of COX and 5-LOX; apigenin for the inhibitory effect on NO production and PGE_2 release; a reduction of iNOS and COX-2 expression; suppression of the LPS-induced activation of NF-*k*B; and an inhibitory effect on some other inflammatory mediators.[30—34] *In vivo* effects of chrysoeriol and apigenin were reported using TPA-induced mouse ear edema and carrageenan-induced rat paw edema, respectively.[33,34]

Compounds **LA-1**—**-12** were first isolated from *L. aspera*. The separation of the remaining PG inhibitory fraction is continuing.

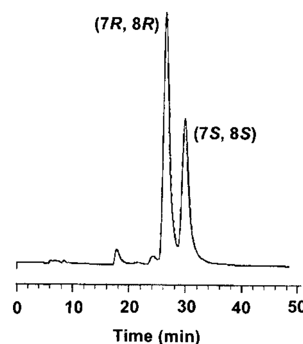

Fig. 2. Chiral-HPLC Analysis of **LA-9**

The chromatogram was obtained using a SHISEIDO CD-Ph (4.6×250) column, a mobile phase of ethanol : *n*-hexane 1/9 at a flow rate of 0.5 ml/min, and a UV detector (254 nm). The first and second eluted enantiomers corresponded to (7*R*,8*R*)- and (7*S*,8*S*)-licarin A with the peak area of approximately 3 : 2 ratio, respectively.

Experimental

General Procedures Specific rotations were measured with a JASCO DIP-140 digital polarimeter and a JASCO P-1020 polarimeter, and ORD and CD with a JASCO J-720WI spectropolarimeter, EI-MS by JEOL JMS-AUTOMASS 20 or JEOL GC-Mate, and FAB-MS by JEOL HX-110A, and ^{1}H- and ^{13}C-NMR by JEOL E 600, A 500, and A 400 spectrometers with a deuterated solvent as an internal standard.

Materials PGE_1 and PGE_2 (Cayman Chemical, U.S.A.) and SC-51089 (BIOMOL Research Laboratories, U.S.A.) were dissolved in DMSO, and further diluted with Dulbecco's phosphate buffered saline (−) (Nacalai Tesque, Japan). Acacetin purchased from Sigma Chemical (U.S.A.), quercetin dihydrate (98%) from Kanto Chemical (Japan), and DPPH (purity checked by NMR) from Wako Pure Chemical Industries (Japan) were used. All other chemicals used in the experiment were of analytical grade.

Plant Material The whole plant of *L. aspera* was collected from Khulna, Bangladesh, in March 2000. After shade drying for 15 d, most of the leaves were separated. The remaining plant parts were cut into pieces and dried in an oven at 40 °C for 3 h before grinding. A voucher specimen (No. LNP 20010-01) was deposited in the Laboratory of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Japan.

Extraction and Isolation *L. aspera* (1.55 kg) was extracted twice with MeOH (20 l total) at room temperature to give the extract (81.4 g). The extract showed inhibitory activity against both PGE_1- and PGE_2-induced contractions and also indicated a DPPH radical scavenging effect on TLC. Using these dual assay guides, the activity-oriented isolation of the extract was carried out. After removal of chlorophylls from 65.0 g of the extract by Diaion HP 20 column chromatography, the fraction (47.0 g) was partitioned successively with *n*-hexane, *n*-BuOH, and water. The PG inhibitory activity was concentrated in the *n*-BuOH fraction, and the DPPH radical scavenging effect was mainly observed in the *n*-hexane and *n*-BuOH fractions. Separa-

tion of the *n*-BuOH fraction (11.0 g) by Sephadex LH-20 column chromatography eluted with MeOH afforded five fractions. All of the fractions except for fr. 1A inhibited both PGE_1- and PGE_2-induced contractions at 9×10^{-5} g/ml. DPPH-positive spots on TLC were clearly observed in fr. 1C and fr. 1D.

Fr. 1C (1.73 g) was separated into five fractions by ODS flash-column chromatography with graduated eluents of acetone : water 1/5—1/0. The 1/1 eluates, fr. 2C (309 mg) and fr. 2D (204 mg), showed DPPH-positive spots on TLC, which were separated by repeated silica gel flash-column chromatography with *n*-hexane : acetone eluents and then purified by ODS-HPLC with MeOH : water 2/1 or Sephadex LH-20 with MeOH. Two major components, **LA-1** (182 mg) and **LA-2** (76 mg), together with three minor ones, **LA-10** (4.1 mg), **LA-11** (1.6 mg), and **LA-12** (1.4 mg), were obtained.

The acetone-soluble part (722 mg) of fr. 1D (1.91 g) was fractioned by silica gel flash-column chromatography with *n*-hexane : acetone 7/1—0/1. The 5/1 eluate (22 mg) having PG inhibition yielded **LA-8** (1.8 mg) and **LA-9** (1.9 mg) after purification on Sephadex LH-20 with MeOH and ODS-HPLC with MeOH : water 3/1. The other DPPH-positive fractions were also separated by repeated chromatography, and **LA-3** (166 mg) was obtained together with additional **LA-1** (13 mg) and **LA-2** (38 mg).

The other PG inhibitory fraction, fr. 1E (0.74 g), was treated with acetone to separate the soluble and insoluble parts. The latter was then suspended in MeOH to obtain the soluble part (463 mg), which showed inhibitory activity on PGE_1- and PGE_2-induced contractions. From the active fraction, **LA-5** (92 mg) was isolated by silica gel flash-column chromatography with *n*-hexane : acetone 1/2—0/1. Through repeated chromatography, the acetone-soluble part (187 mg) afforded **LA-4** (12 mg), **LA-6** (10 mg), and **LA-7** (5 mg) together with additional crude **LA-5** (43 mg).

Enantiomeric separations of **LA-3** and **LA-10** were carried out under the following conditions: column, SHISEIDO CD-Ph (4.6×250); mobile phase, ethanol : *n*-hexane 4/6 and MeOH : water 9/1 or 4/1; flow rate, 0.5 ml/min; UV detection, 254 nm.

LA-3 (Macelignan): Colorless needles. $[\alpha]_D^{24}$ *ca.* +5° (*c*=0.96, $CHCl_3$), {lit.,[13] $[\alpha]_D^{24}$ +5.28° ($CHCl_3$)}. In the ^{1}H-NMR spectrum, the published data at the position of 5-H and 5′-H should be reversed.[13]

LA-5 {Apigenin 7-*O*-[6″-*O*-(*p*-coumaroyl)-*β*-D-glucoside]}: Yellowish powder. FAB-MS (NBA) *m/z*: 579 $(M+H)^+$, 271. ^{1}H-NMR (DMSO-d_6) δ: 3.26 (1H, dd, *J*=9.6, 8.8, 4″-H), 3.36[b] (1H, dd, *J*=8.8, 7.4, 2″-H), 3.40[b] (1H, dd, *J*=8.8, 8.8, 3″-H), 3.83 (1H, ddd, *J*=9.6, 7.2, 1.9, 5″-H), 4.17 (1H, dd, *J*=11.9, 7.2, 6″a-H), 4.47 (1H, dd, *J*=11.9, 1.9, 6″b-H), 5.16 (1H, d, *J*=7.4, 1″-H), 5.24[a] (1H, br s, 2″-OH), 5.35[a] (1H, br s, 3″-OH), 5.48 (1H, br s, 4″-OH), 6.32 (1H, d, *J*=15.9, 8‴-H), 6.48 (1H, d, *J*=1.9, 6-H), 6.66—6.68 (2H, m, 3‴ and 5‴-H), 6.81 (1H, s, 3-H), 6.81 (1H, d, *J*=1.9, 8-H), 6.89—6.94 (2H, m, 3′ and 5′-H), 7.35—7.37 (2H, m, 2‴ and 6‴-H), 7.49 (1H, d, *J*=15.9, 7‴-H), 7.93—7.94 (2H, m, 2′ and 6′-H), 10.16 (1H, br s, 4′-OH), 10.16 (1H, br s, 4‴-OH), 12.96 (1H, br s, 5-OH); [a] interchangeable, [b] signals observed at 70 °C.

LA-8 [(−)-Chicanine]: Colorless amorphous. HR-FAB-MS (NBA/PEG) *m/z*: 342.1408 (Calcd for $C_{20}H_{22}O_5$: 342.1467). EI-MS *m/z* (%): 342 (M^+, 82), 218 (2), 192 (63), 190 (100), 180 (27), 177 (17), 175 (66), 164 (14), 137 (9), 135 (21). ORD (*c*=0.002 mol/l, MeOH) $[\phi]^{22}$ (nm): −5000 (296, tr), −16000 (243, tr), {lit.[17] for (+)-chicanine, ORD (MeOH) $[\phi]$ (nm): +5521, (296, pk), +19000, (245, pk)}. CD (*c*=0.001 mol/l, MeOH) $[\phi]^{22}$ (nm): −9000 (221, max), −24000 (236, min), −700 (258, max), −4900 (288, min), 0 (308), {lit.[18] for (+)-chicanine, CD (MeOH) $[\theta]$ (nm): +9670 (221, tr), +24665 (236, pk), −467 (256, tr), +4858 (285, pk), 0 (300)}. ^{1}H-NMR ($CDCl_3$) δ: 0.59 (3H, d, *J*=7.0, 9′-H), 0.98 (3H, d, *J*=6.7, 9-H), 2.37—2.43 (2H, m, 8, 8′-H), 3.87 (3H, s, 3′-OCH_3), 4.60 (1H, d, *J*=9.2, 7-H), 5.40 (1H, d, *J*=4.6, 7′-H), 5.49 (1H, s, 4′-OH), 5.92 and 5.93 (each 1H, d, *J*=1.5, –OCH_2O–), 6.75 (1H, dd, *J*=7.9, 1.5, 6′-H), 6.76 (1H, d, *J*=7.9, 5-H), 6.81 (1H, dd, *J*=7.9, 1.8, 6-H), 6.86 (1H, d, *J*=7.9, 5′-H), 6.90 (1H, d, *J*=1.5, 2′-H), 6.91 (1H, d, *J*=1.8, 2-H). ^{13}C-NMR ($CDCl_3$) δ: 9.4 (C-9′), 11.8 (C-9), 43.4[a] (C-8′), 47.6[a] (C-8), 56.0 (–OCH_3), 84.8 (C-7′), 85.7 (C-7), 100.9 (–OCH_2O–), 106.4 (C-2), 108.0 (C-5), 108.7 (C-2′), 113.9 (C-5′), 118.8 (C-6′), 119.5 (C-6), 132.5 (C-1′), 137.2 (C-1), 144.3 (C-4′), 146.2 (C-3′), 146.9 (C-4), 147.8 (C-3); [a] interchangeable.

LA-10 [*erythro*-1-(4-Hydroxy-3-methoxyphenyl)-2-(4-allyl-2,6-dimethoxyphenoxy)propan-1-ol]: Colorless amorphous. $[\alpha]_D^{24}$ *ca.* +3° (*c*=0.05, $CHCl_3$), {lit.[15] for (7*S*,8*R*)-isomer: $[\alpha]_D^{20}$ +25.28° ($CHCl_3$)}. ^{13}C-NMR ($CDCl_3$) δ: 12.8 (C-9), 40.6 (C-7′), 56.0 (3-OCH_3), 56.1 (3′,5′-OCH_3), 72.8 (C-7), 82.3 (C-8), 105.6 (C-2′, 6′), 108.6 (C-2), 113.9 (C-5), 116.2 (C-9′), 118.8 (C-6), 132.1 (C-1), 133.1 (C-1′), 136.1 (C-4′), 137.1 (C-8′), 144.5 (C-4), 146.5 (C-3), 153.5 (C-3′, 5′).

LA-11 (Myristargenol B): Colorless amorphous. $[\alpha]_D^{24}$ *ca.* +10° (*c*=0.09, $CHCl_3$), {lit.[12] $[\alpha]_D^{24}$ +14.2° ($CHCl_3$)}.

LA-12 (Machilin C): Colorless amorphous. $[\alpha]_D^{23}$ *ca.* −30° (*c*=0.07, $CHCl_3$), {lit.[16] $[\alpha]_D^{24}$ −16.5° ($CHCl_3$)}.

PG Inhibitory Assay The PG inhibitory activity was evaluated by Magnus assay using Hartley male guinea pig ileum (350—550 g, 4—6 weeks, Japan SLC). Animals were conditioned at least one week in a 12 h light/dark-cycle room with controlled temperature and humidity and were in accordance with the experimental animal welfare guidelines of Chiba University. After being sacrificed, the ileum was maintained at room temperature in Kreb's solution (11.8 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10.0 mM glucose) bubbled with a gas mixture of 95% O_2 and 5% CO_2. A resting tension of 1.0 g was applied to each *ca.* 1 cm-ileum preparation, which was then equilibrated in 5 ml of organ bath solution bubbled with the gas mixture at 28 °C. PGE_1 or PGE_2 was added at concentrations of 3×10^{-7} M and 1×10^{-7} M, respectively. When the muscle contraction became stable, each sample dissolved or suspended in 5% DMSO-water solution was used in the experiments. A force-displacement transducer (TB-611T, NIHON KOHDEN, Japan) coupled to an amplifier (AP-601G, NIHON KOHDEN, Japan) was used for the measurement of isometric contractions, which were recorded on a chart recorder (TI-102, TOKAI IRIKA, Japan). Positive activity was evaluated if the sample showed >50% inhibition in duplicate. SC-51089, a PGE_2 (EP_1 receptor) antagonist, was used as a positive control at a concentration of 3 μM.

Antioxidant Assay The antioxidant activity was evaluated based on the DPPH radical scavenging effect. For the qualitative assay on TLC, activity was detected as in our previous reports, and ascorbic acid was used as a positive control.[8,9] Spectrophotometric assay was performed by modification of the reported microplate method to determine IC_{50} values.[27] To 10 μl of sample-DMSO solution in each microwell, 190 μl of DPPH-MeOH solution was added (final concentration of DPPH was 200 μM). After mixing in a microplate mixer for 30 min at room temperature, the absorbance was determined at 540 nm using a microplate reader (BIO-RAD Model 550). Each sample was measured in triplicate, and the mean result was taken. The antioxidant activity was expressed in terms of IC_{50} (μM and/or μg/ml, concentration required to inhibit DPPH radical formation by 50%), calculated from the log-dose inhibition curve. Quercetin was used as a positive control.

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