

A Stabilized Flavonoid Glycoside in Heat-Treated *Cassia alata* Leaves and Its Structural Elucidation

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Heat-treated leaves of *Cassia alata* were studied for any change in chemical constituents using sun dried leaves as the reference standard. A high concentration of a constituent was observed in the heat-treated leaves. Spectroscopic studies revealed the structure of the constituent as kaempferol 3-gentiobioside, which has not yet been detected in the *Cassia* species. In a stability study disappearance of kaempferol 3-gentiobioside was noted in the sun dried leaves while there was little or no change in the kaempferol 3-gentiobioside concentration in the heat-treated leaves when incubated in an aqueous solution, suggesting a possible presence of enzymatic activities in the sun dried leaves. Therefore, heat-treatment may be a good method to stabilize kaempferol 3-gentiobioside in *Cassia alata* leaves.

Key words—*Cassia alata*; heat-treatment; flavonoid glycoside; Leguminosae; HPLC; NMR; kaempferol 3-gentiobioside

INTRODUCTION

Cassia alata L. (Leguminosae) is a native of tropical America, but now widely grown in the tropics, including Indonesia.¹⁾ It has been reported to contain anthraquinones^{2–6)} and flavonoids⁷⁾ from different parts of *Cassia alata*. However, little attention has been paid to changes of the chemical constituents of heat-treated *Cassia alata* leaves while heat-treatment has been known in China for plant medicines as part of the pretreatments for modifying various activities of the plant medicines.⁸⁾ In this paper, we report a change in a chemical constituent in the extract of heat-treated *Cassia alata* leaves using that of sun dried leaves as the reference standard.

MATERIALS AND METHODS

1. Plant Materials Both sun dried and heat-treated *Cassia alata* leaves were obtained from PT Haldin Pacific Semesta (Jakarta, Indonesia). *Cassia alata* was grown at a region 80 km southeast of Jakarta. Leaflets with sizes ranging 5–9 cm in width and 10–16 cm in length were harvested in January 1999, September 1999 and January 2000. For heat-treatment, fresh leaves harvested in January 2000 were placed in hot water at 85°C for 4 min, cut and further heated at about 40°C for 4 h under high humidity. The leaves were, then, subjected to sun drying. Production yields for the heat-treated and sun dried leaves from fresh leaves harvested in January 2000

were about 17.0% and 20.5% with the moisture contents of about 8.5% and 9.5%, respectively. The moisture contents of the other sun dried leaves with the different harvest dates were also about 9.5%. They were kept in plastic bags at room temperature until use.

2. Chemicals Unless otherwise stated, all chemicals are of reagent grade. Acetonitrile is of HPLC grade. Gentiobiose was obtained from Sigma (U.S.A.). Kaempferol was of Wako Pure Chem Ind. (Japan).

3. HPLC Analyses for the Constituent 500 mg of pulverized sun dried leaves with the aforementioned harvest dates and heat-treated leaves were incubated in 50 ml distilled water at about 85°C for 1 h. Then, each of the extracts was made up to the volume in a 100-ml volumetric flask with distilled water and filtered for HPLC analyses. The HPLC system is as follows: HPLC system/S-8030 (Soma, Japan), pump: Intelligent Pump S-400 (Soma), column: YMC-Pack ODS-A, 150×4.6 mm I.D., 5 μm, detector: S-310A Model II (Soma) at 340 nm, recorder: C-R8A CHROMATOPACK (Shimadzu, Japan), mobile phases: diluted glacial acetic acid (80-fold with distilled deionized water): acetonitrile (4:1), sample size: 10 μl, column temperature: 25°C, and flow rate: 1 ml/min.

4. Isolation of the Constituent 5.0 g of the sun dried and 10.0 g heat-treated leaves were extracted with distilled water at 85°C for 1 h. The extracts

were concentrated under reduced pressure. The concentrates were repeatedly separated by using preparatory column (YMC-Pack ODS-AQ, 250×20 mm I.D., 10 μm) with the aforementioned mobile phase. (See "3. HPLC Analyses for the Constituent".) The collected fractions rich in the constituent were combined and concentrated under reduced pressure to dryness and then recrystallized from water, obtaining 29 mg and 90 mg of the constituent from the sun dried and heat-treated leaves, respectively.

5. Spectroscopic Studies a) UV-Visible Recording Spectrophotometer UV-250 (Shimadzu, Japan) was used for UV spectrum. b) FAB-MASS was performed on a JMS SX102 spectrometer (JEOL, Japan). c) ¹H-NMR and ¹³C-NMR spectra were measured by 270 MHz (JNM-LA270) and 500 MHz (JNM-LA500) spectrometers (JEOL, Japan) using DMSO-d₆, respectively.

6. Properties of the Constituent The constituent, yellow needles from water, $[\alpha]_D^{24}$ -43.9° (c = 0.70, pyridine), mp 196–202°C, C₂₇H₃₀O₁₆, negative FAB-MS at m/z 609 (M-H)⁻, UV λ max nm: 266, 299 sh, 349; (+NaOMe) 276, 326, 401; (+NaOAc) 276, 304, 380; (+AlCl₃) 276, 305, 351, 401. ¹H-NMR (DMSO-d₆) δ: 8.10 (2H, d, J=9.0 Hz, H-2', 6'), 6.89 (2H, d, J=9.0 Hz, H-3', 5'), 6.41 (1H, d, J=2.0 Hz, H-8), 6.21 (1H, d, J=2.0 Hz, H-6), 5.22 (1H, d, J=7.5 Hz, H-1'), 4.14 (1H, d, J=8.0 Hz, H-1''), ¹³C-NMR (DMSO-d₆) δ: 156.48 (C-2), 133.18 (C-3), 177.32 (C-4), 161.13 (C-5), 98.60 (C-6), 164.03 (C-7), 93.62 (C-8), 156.35 (C-8a), 104.00 (C-4a), 120.78 (C-1'), 130.85 (C-2'), 115.01 (C-3'), 159.85 (C-4'), 115.01 (C-5'), 130.85 (C-6'), 101.01 (C-1''), 73.32 (C-2''), 76.44 (C-3''), 69.61 (C-4''), 78.86 (C-5''), 67.96 (C-6''), 103.09 (C-1'''), 74.00 (C-2'''), 76.49 (C-3'''), 69.66 (C-4'''), 76.18 (C-5'''), 60.73 (C-6''').

7. Acid Hydrolysis A solution of the flavonoid in 1% sulfuric acid was heated at 95°C for 1 h. After cooling, the hydrolysates were precipitated and filtered for aglycone, which was identified on a TLC plate Silicagel 60 F254 (Merck, U.S.A.) with the solvent system of hexane: EtOAc: glacial acetic acid (40 : 40 : 1). The plate was sprayed with 10% sulfuric acid solution and heated at 100°C for 5 min. The R_f values were compared with that of the authentic sample of kaempferol. The aqueous layer was neutralized and concentrated to obtain saccharides identified on a TLC plate Silicagel 60 F254 (Merck) with the solvent

system of EtOAc: glacial acetic acid: methanol: water (60 : 15 : 15 : 10). The plate was sprayed with naphthoresorcinol in 10% sulfuric acid solution and heated at 100°C for 5 min. The R_f values were compared with those of the authentic samples of glucose and gentiobiose. Separation for gentiobiose from glucose was achieved by a Sephadex LH-20 column with distilled water. Then, ¹³C NMR spectroscopy was performed for gentiobiose.

8. Stability Studies 1.0 g of the pulverized *Cassia alata* sun dried or heat-treated leaves was incubated with 100 ml of distilled water in a beaker with a magnetic stirrer at 37°C. Not more than 0.2 ml of the extracts incubated at the predetermined times was taken and filtered for HPLC determinations. For the test samples without the presence of the leaves, the extracts withdrawn at 0 min were used and incubated under the aforementioned conditions. 0 min is defined as the time when the extract was taken after combining leaves and distilled water and stirring them for 30 s, thereby already containing some kaempferol 3-gentiobioside as the 0 time concentration. Relative absorbance was calculated as the absorbance at 340 nm for kaempferol 3-gentiobioside at the predetermined incubation times/the absorbance at 340 nm for kaempferol 3-gentiobioside at the 0 min incubation time.

RESULTS AND DISCUSSION

In an attempt to find any change in chemical constituents of the extract of heat-treated *Cassia alata* leaves compared with those of sun dried leaves, we observed the significant difference in the chemical constituent by HPLC analyses. Figure 1 shows chromatograms of the extracts of *Cassia alata* sun dried leaves with different harvest dates and of the heat-treated leaves. It is apparent that the concentration of an unknown constituent was low in the leaves with the old harvest dates; however, the concentration of the constituent was high in heat-treated leaves, suggesting that some degradation might have occurred in sun dried leaves after harvesting and during drying or storing.

The constituent exhibited the absorption maxima at 266, 299 sh, and 349 in the UV spectrum, which is in agreement with the previously reported UV spectrum data for kaempferol 3-gentiobioside.⁹⁾ The UV spectrum shifts of the constituent on addition of sodium methoxide, sodium acetate or aluminum hydro-

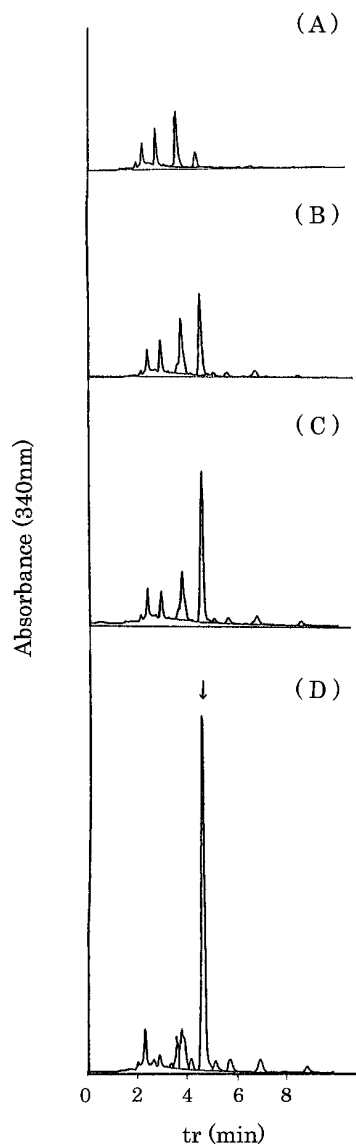


Fig. 1. HPLC Chromatograms of the Extracts of *Cassia alata* Leaves with Varying Harvest Dates

(A) Harvested in January 1999, (B) Harvested in September 1999, (C) Harvested in January 2000 and (D) Heat-treated leaves harvested in January 2000. Arrow shows the constituent peak (See "MATERIALS AND METHODS").

xide showed sugar was linked to kaempferol at C-3.^{10,11)} In the NMR spectrum the signal of the β -anomeric proton at 5.22 ppm could be assigned to the anomeric proton of sugar linked to C-3 of kaempferol. On partial acid hydrolysis, the constituent yielded an aglycone, which was identified as kaempferol using TLC, also confirming the data of ¹H-NMR in comparison with the authentic sample. The sugar components were found to be gentiobiose and glucose on TLC (R_f 0.20 and R_f 0.30, respectively) by comparing with the authentic samples. Fur-

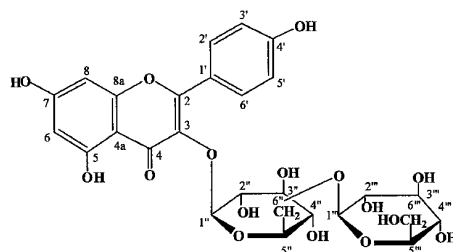


Fig. 2. Structure of the Constituent

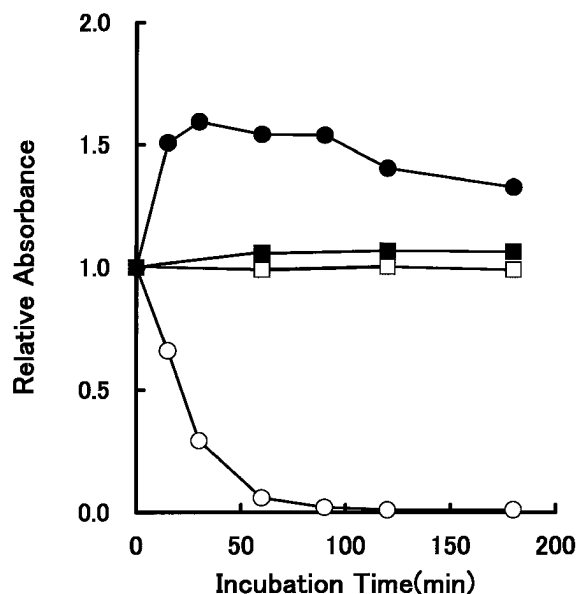


Fig. 3. Stabilities of the Constituent in Sun Dried Leaves and Heat-Treated Leaves in were Studied in an Aqueous Solution at 37°C, Demonstrating Changes in Absorbance as the Concentration of the Constituent with the Incubation Times

(○) incubation with sun dried leaves, (●) with heat-treated leaves, (□) incubation without sun dried leaves, and (■) without heat-treated leaves. $n=3$ experiments (See "MATERIALS AND METHODS").

thermore, gentiobiose was confirmed with the ¹³C-NMR data in comparison with that of the authentic sample. Based on the above data, the constituent was kaempferol 3-gentiobioside (kaempferol 3-*O*-[β -D-glucopyranosyl-(1→6)- β -D-glucopyranoside]) as shown in Fig. 2.

We further conducted stability studies of kaempferol 3-gentiobioside in an aqueous solution with and without the presence of leaves. As seen in Fig. 3, the incubation with sun dried leaves led to a rapid decrease of the constituent within 60 min; however, no change in the concentration was noted for the incubation without the presence of the leaves. For the heat-treated leaves, an increase in the concentration of kaempferol 3-gentiobioside in the solution was ob-

served after incubating for 15 min. This initial increase is presumably due to a further removal of kaempferol 3-gentibioside from the leaves, then giving a steady concentration of kaempferol 3-gentibioside in the solution. The findings suggest a possible presence of enzymatic activities in the sun dried leaves, degrading kaempferol 3-gentibioside while little or no such activities was found in the heat-treated leaves and also in the solution without the presence of the sun dried leaves. Heat-treatment might have inhibited the enzymatic activities and, consequently, stabilized kaempferol 3-gentibioside from degradation in *Cassia alata* leaves. Also, it is noteworthy that kaempferol 3-gentibioside was stable during the process of isolation.

Although the fate of kaempferol 3-gentibioside in the solution of the sun dried leaves is presently under investigation, we have observed a notable peak on a HPLC chromatogram for the extract of the sun dried leaves incubated in the aqueous solution for 180 min, but little or none detected in the solution of the heat-treated leaves.

Kaempferol 3-gentibioside has been previously studied^{9,12} and reported from *Primula sinensis*,⁹ but it has not yet been detected in the *Cassia* species. A similar structural constituent, kaempferol 3-O-sophoroside, has been reported from *Cassia alata* and it was found to possess anti-inflammatory¹³ and analgesic activities.¹⁴ Although biological studies such as anti-allergic activities using kaempferol 3-gentibioside and extracts of sun dried and heat-treated leaves of *Cassia alata* are in progress, it is interesting to perform some of the biological studies to compare kaempferol 3-gentibioside with kaempferol 3-O-sophoroside, observing the differences in activities and also stabilities due to the difference in the glycosidic linkage.

Because of degradation of kaempferol 3-gentibioside in *Cassia alata* leaves as reported in this paper, it may be meaningful to study changes of the concentration of the constituent at various drying temperatures using differed methods such as shade, heat and freeze dryings and also changes under differ-

ent storing conditions in temperature and humidity. It is also of interest to pursue studies on stabilizing other flavonoids or anthraquinone derivatives in heat-treated leaves.

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