JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Organic Acids from Leaves, Fruits, and Rinds of *Garcinia cowa*

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Organic acids in fresh leaves, fruits, and dried rinds of *Garcinia cowa* (*G. cowa*) were determined by high-performance liquid chromatography. Fresh leaves, fruits, and dried rinds were extracted with water at 120 °C for 20−30 min under 15 lbs/in^2 pressure. Also, dried rinds were extracted with solvents (acetone and methanol) using a Soxhlet extractor at 60 °C for 8 h each. The samples were injected to HPLC under gradient elution with 0.01 M phosphoric acid and methanol with a flow rate of 0.7 mL/min using UV detection at 210 nm. The major organic acid was found to be (−)-hydroxycitric acid present in leaves, fruits, and rinds to the extent of 1.7, 2.3, and 12.7%, respectively. (−)-Hydroxycitric acid lactone, and oxalic and citric acids are present in leaves, fruits, and rinds in minor quantities. This is the first report on the composition of organic acids from *G. cowa.*

KEYWORDS: ***Garcinia cowa*; (−)-hydroxycitric acid; lactone; citric acid; HPLC**

INTRODUCTION

(−)-Hydroxycitric acid (1,2 dihydroxypropane-1,2,3-tricarboxylic acid; **Figure 1**) was encountered first in nature as the principal acid in the highly acidic fruits of *Garcinia cambogia* (*1*). So far, (−)-hydroxycitric acid (HCA) has been found in the fruits of certain species of *Garcinia*, which includes *G. cambogia*, *G. indica*, and *G. atroviridis* (*1*−*3*). The chemistry and biochemistry of HCA has been discussed recently (*4*). During extensive animal studies, HCA has been proven to effectively curb appetite, suppress food intake, increase the rates of hepatic glycogen synthesis, reduce fatty acid synthesis and lipogenesis, and decrease body-weight gain (*5*−*13*). As a dietary supplement, then, HCA is an effective addition to any weight management program. Allison et al. (*14*) has reviewed the use of HCA as one of the alternative treatments for weight loss. The derivatives of HCA have been incorporated into many pharmaceutical preparations in combination with other ingredients for the purposes of enhancing weight loss, cardioprotection, correcting the conditions of lipid abnormalities, and endurance in exercise (*15*−*17*). This increases the consumer demand for HCA. So far the fruits of *G. cambogia* and *G. indica* have been exploited as the source of HCA. The availability of these two species of *Garcinia* is limited; hence, there is a need for finding newer source(s) of HCA.

Garcinia (Family Guttiferae) is a large genus of polygamous trees or shrubs distributed in tropical Asia, Africa, and Polynesia. It consists of 180 species, of which about 30 species are found in India (*18*). The proposition made by Lewis and Neelakantan (*1*) that a survey of organic acids in a large number of fruits belonging to the *Garcinia* genus would doubtless reveal the presence of HCA in many of them besides those reported in their studies tempted us to examine the organic acids in the fruits of *G. cowa* which are found in the northeastern parts of India and the Andaman Islands. In upper Assam, *G. cowa* is often cultivated in homesteads for its acid fruits. The tree flowers during January−March, and the fruits ripen in May−June. The fruits are not palatable because of their acidic flavor. They can be made into jam or preserves. In Assam, the fruit is popularly known as kujithekera or kauthekera, and the sundried slices of the fruits are used in the treatment of dysentery. In Burma, young leaves are cooked and eaten as vegetables (*18*). In the present communication, we report the composition of organic acids in leaves, fruits, and rinds of *G. cowa* by HPLC. This is the first report on the composition of organic acids from *G. cowa*.

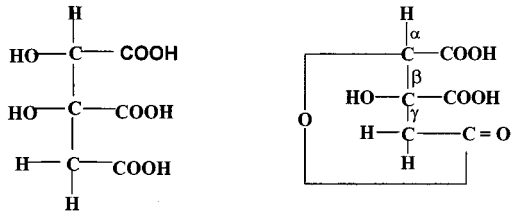

Figure 1. Structures of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone.

MATERIALS AND METHODS

Materials and Equipment. Fresh leaves, fruits, and rinds of *Garcinia cowa* were obtained from Assam. All solvents and other chemicals used were of AR/HPLC grade and were obtained from E-Merck, Mumbai, India. Dowex 50WX8, mesh size 100−200 was obtained from Sigma Chemical Co. (St. Louis, MO). (−)-Calcium threo-hydroxycitrate tribasic hydrate was obtained from Fluka (M/S Fluka Chemie Gmbh, Switzerland). Double-distilled water was used for the extraction of organic acids, and triple-distilled water was used for HPLC analysis. A Millipore Swinnex-type filter (pore size 0.45 μm) was obtained from Millipore (Bangalore, India). ^{1}H NMR spectra (D_2O) were recorded at 400 MHz on a Bruker AMX 400 FT instrument (Bruker, Rheinstetten, Germany). TMS was used as the internal standard.

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10.1021/jf011627j CCC: $22.00
Published on Web 05/04/2002

Isolation of HCA Lactone. Standard HCA lactone is not commercially available. Hence, pure HCA was isolated from the fruits of *Garcinia cambogia* by the method reported earlier and its purity was analyzed by GLC and optical rotation (*19*). The structure of HCA lactone was further confirmed by ^{1}H NMR spectra. The chemical shifts were matched with reported values (*19*). The isolated pure HCA lactone was dissolved in triple-distilled water at concentrations from 0.2 to 1.0 mg/mL and used for HPLC analysis.

Regeneration/Activation of Dowex 50. Four resin volumes of 5% HCl was percolated in the Dowex 50 resin for 45 min, then it was displaced with 4 resin volumes of distilled water for 45 min. Finally, the resin was rinsed with distilled water to get neutral pH and the Dowex 50 was thus in the form of [H^+].

Preparation of Free HCA. Calcium threo-hydroxycitrate tribasic hydrate (50 mg) was suspended in a 50-mL beaker containing 5.0 mL of water, and it was treated with 500 mg of Dowex 50 [H^+]. The mixture was stirred using a magnetic stirrer for 10 min. The supernatant was decanted, and the resin was washed with water to neutral pH. The washings and the supernatants were combined and made up to 25 mL, mixed, and filtered using Whatman filter paper 1. Five standard HCA solutions of concentrations ranging from 0.20 mg/mL to 1.0 mg/mL were prepared.

Preparation of Standard Solution of Oxalic and Citric Acids for HPLC. Standard solutions of oxalic and citric acids were prepared separately at concentrations of 0.2 to 1.0 mg/mL using triple-distilled water.

Sample Preparation. *Fresh Leaves and Fruits.* Leaves and fruits (10 g each) of *G. cowa* were cut into small pieces and extracted separately with 100 mL of water at 15 lbs/in^2 pressure for 20 min using an autoclave, and then filtered through muslin cloth. The extraction and filtration was repeated once more for complete extraction of the organic acids. The extracts were combined and treated with 4 g of activated charcoal and heated on a water bath for 30 min. The extracts were filtered using Whatman filter paper 41, and the charcoal was washed twice with 15 mL of water and the washwater was filtered. The combined extracts were concentrated to 20 mL under vacuum, treated with 100 mL of ethanol with continuous stirring, left for 15 min to precipitate the pectinaceous material, and then the whole content was centrifuged at 1000*g* for 10 min. The supernatant was collected, and the precipitate was washed two times with 20 mL of ethanol to recover the adhering organic acids and centrifuged. Both the supernatants were pooled and concentrated under reduced pressure to 25 mL and stored at 4 °C until further use. The acid contents of leaves and fruits were found to be 4.46 and 5.92% (w/w), respectively, as determined by acid−base titration using 0.1 N sodium hydroxide and phenolphthalein indicator.

Dried Rinds: Method 1. Rinds (10 g) of *G. cowa* were extracted with 150 mL of water at 15 lbs/in^2 pressure for 30 min using an autoclave and filtered using muslin cloth. The extraction and filtration was repeated twice for complete extraction of the organic acids. The combined extracts were treated with 4.0 g of activated charcoal, heated on a water bath for 30 min, and filtered using Whatman filter paper 41. The charcoal was washed twice with 15 mL of water and the washwater was filtered. The extracts were combined and concentrated to 30 mL under vacuum. The extract was treated with 120 mL of ethanol with continuous stirring, left for 15 min to precipitate the pectinaceous material, and centrifuged at 1000*g* for 10 min. The supernatant was collected, the precipitate was washed two times with 20 mL of ethanol to recover the adhering organic acids, and the washwater was centrifuged. Both supernatants were pooled and concentrated under reduced pressure to 25 mL and stored at 4 °C until further use. The acid content was found to be 27.1% (w/w) with respect to weight of rinds as determined by acid−base titration using 0.1 N sodium hydroxide and phenolphthalein indicator.

Method 2. Rinds (25 g) of *G. cowa* were extracted in a Soxhlet extractor with 100 mL each of acetone and methanol for 8 h each at 60 °C. The extracts were filtered using Whatman filter paper 1 and concentrated under vacuum. The acetone and methanol extracts were suspended in 20 mL of water and 4.0 g of activated charcoal separately. Both the extracts were heated on a boiling water bath for 30 min and filtered using Whatman filter paper 41. The charcoal was washed twice with 10 mL of water, and the washwater was made up to 50 mL. The acid contents of acetone and methanol extracts were found to be 23.6 and 25.4% (w/w), respectively, as determined by acid−base titration using 0.1 N sodium hydroxide and phenolphthalein indicator.

HPLC Analysis. The high-performance liquid chromatographic system used in the present study consisted of a Hewlett-Packard HPLC model HP 1100 Series (Palo Alto, CA) equipped with an quaternary HPLC pump, and fitted with a Zorbax C_{18} (Hewlett-Packard) analytical column (25 cm × 4.6 mm i.d., 5-micron particle size). The injection system (Rheodyne) used was a 20-μL sample loop. Detection was done by a HP 1100 Series variable wavelength detector at a wavelength of 210 nm. The gradient mobile phase consisted of (A) MeOH, and (B) 0.01 M phosphoric acid, with a flow rate of 0.7 mL/min. The elution program involved a linear gradient from 10 to 30% A in B for 0−25 min, 90% A in B for 30 min, followed by 5 min of equilibrium with 90% A. All standards and samples were filtered through 0.45-μm Millipore filter and injected to HPLC. The compounds were quantified using HP CHEMSTATIONS software.

Calibration and Linearity. The linearity of the method was evaluated by analyzing a series of HCA standards. Aliquots of 10 μL of each of the five working standard solutions containing 2−10 μg of free HCA were injected on to the HPLC, elution was carried out as discussed above, and peak area responses were obtained. The calibration curve for HCA was prepared by plotting concentration of HCA versus peak area (average of 3 runs). Similarly, standards of HCA lactone, and oxalic and citric acids were injected to HPLC, elution was carried out as discussed above, and peak area responses were obtained.

Range. The calibration range was established through consideration of the practical range necessary according to the HCA concentrations present in the samples. This range includes concentrations from the lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ).

Determination of the Limit of Quantification. The limit of quantification (LOQ) was defined as the lowest HCA concentration which can be determined with accuracy and precision <20%.

Quantification of Organic Acids in Samples. The prepared samples were diluted to 1:9 ratio with triple-distilled water, except the extract of fresh fruits for which the dilution was 1:2. A known volume (20 μL) of each sample was injected to the HPLC, and the concentrations of HCA lactone, oxalic acid, and citric acid were obtained directly from the peak area and by application of the dilution factor. But concentration of HCA has been calculated using a calibration curve. The concentrations of HCA, HCA lactone, oxalic acid, and citric acid in the sample were expressed as g/100 g of sample.

RESULTS AND DISCUSSION

Standard HCA lactone is not commercially available. Hence, it was isolated from the fruits of *Gacinia cambogia* by the method reported earlier, and its purity was analyzed by GLC and optical rotation (*19*). HCA lactone had a melting point of 182 °C and optical rotation of $[\alpha]^{20}_D$ + 99.7 °(c = 1.0; H_2O); these values matched well with those of pure HCA lactone reported values (*3, 19*). The purity of isolated HCA lactone was found to be 99%. The structure of HCA lactone was further established by ^{1}H NMR spectroscopy. ^{1}H NMR spectra of HCA lactone showed two protons at γ-carbon which gives an AB quartet at δ 2.53 and δ 2.75 with J = 17.0 Hz, and one proton at α-carbon showed a singlet at δ 5.15.

The acid−base titration method has been used for the determination of organic acids in the extracts, which gives the total acidity of extracts (*20*). But in this method the concentrations of HCA, HCA lactone, and citric and oxalic acids cannot be estimated separately. Generally, GC estimation involves the conversion of acid to volatile silyl derivatives. For silylation the sample should be dried completely. But HCA has the tendency for lactonization during drying (*2*), and because of the highly hygroscopic nature of HCA it is rather difficult to dry the sample. Recently, Jayaprakasha and Sakariah (*17, 21, 22*) have developed isocratic HPLC methods for the determi-

Table 1. Comparison of Organic Acids in Fresh Leaves, Fresh Fruits, and Dried Rinds of *G. cowa* by HPLC and Titration Methods[a]

		organic acids by HPLC (g/100 g)				
sample	extraction solvent	oxalic acid mean ± SD	HCA lactone mean ± SD	HCA mean ± SD	citric acid mean ± SD	acid–base titration (g/100 g)
fresh leaves	water	0.127 ± 0.004	0.817 ± 0.008	1.676 ± 0.072	1.469 ± 0.082	4.457 ± 0.191
fresh fruit	water	0.030 ± 0.017	0.821 ± 0.001	2.856 ± 0.057	1.091 ± 0.110	5.92 ± 0.099
dried rinds	water	0.576 ± 0.074	5.650 ± 1.076	12.695 ± 0.954	6.918 ± 0.277	27.1 ± 0.760
dried rinds	acetone	0.228 ± 0.045	4.553 ± 0.298	10.209 ± 0.361	6.066 ± 0.541	23.6 ± 0.890
dried rinds	methanol	0.417 ± 0.083	4.595 ± 0.716	12.260 ± 1.201	6.593 ± 0.960	25.4 ± 0.921

[a] Average of three replications.

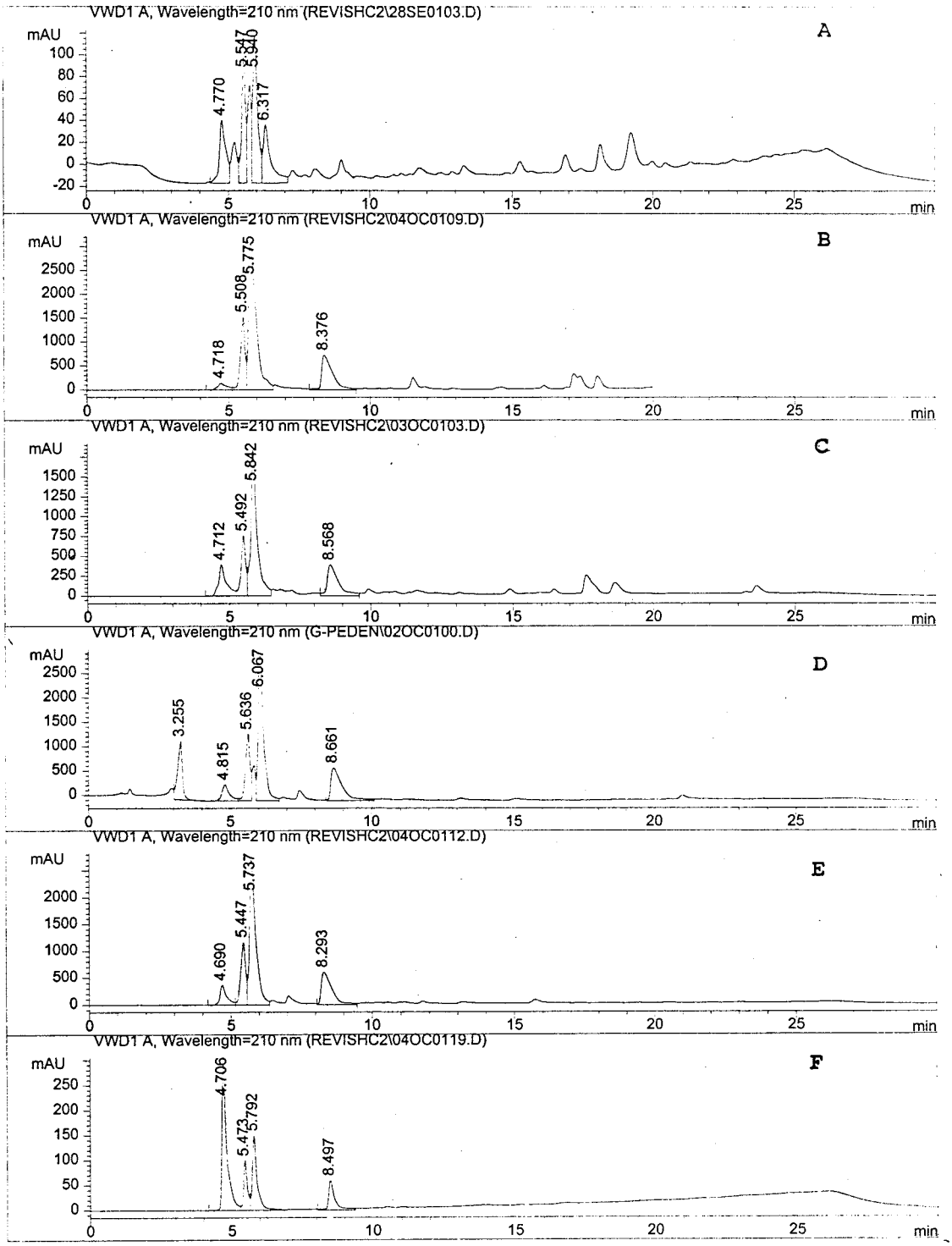

Figure 2. HPLC chromatograms of (A) leaves, (B) fresh fruits, (C) rinds water extract, (D) rinds acetone extract, (E) rinds methanol extract, and (F) standard oxalic acid, HCA lactone, HCA, and citric acid.

nation of HCA, as well as other organic acids, in fruits of *G. cambogia*, commercial samples of *G. cambogia* extracts, and leaves and dried rinds of *G. indica*. In these HPLC methods, dilute extracts can be quantified for HCA and other organic acids without concentration, drying, and derivatization. Furthermore, the advantage of these methods is that the HCA and its lactone can be quantified separately.

The organic acids composition of fresh leaves, fruits, and rinds of *G. cowa* as determined by HPLC and acid−base titration methods are presented in **Table 1**. It is observed that the acid−base titration method gives higher values due to the presence of other organic acids. The values obtained by the HPLC method accounted for only HCA, because the values correspond to the area of HCA peak. The major organic acid found in leaves, fruits, and rinds of *G. cowa* by HPLC is HCA, as shown in the HPLC chromatogram in **Figure 2**. Three of the minor peaks were identified as HCA lactone, oxalic acid, and citric acid by co-injection of standard acids. HCA was almost resolved as a single peak in all samples analyzed, except the leaves extract as shown in chromatogram A in **Figure 2**. The identity of the HCA peak was confirmed by determination of relative retention time and by spiking with standard HCA. The retention times of the oxalic acid, HCA lactone, HCA, and citric acid in all samples were found to be 4.7 ± 0.1, 5.4 ± 0.1, 5.7 ± 0.1, and 8.4 ± 0.2 min, respectively.

A calibration curve was derived from three injections of five concentrations of HCA. Linearity was found in the 1.5−9.0 μg concentration range and it has a good reproducibility and accuracy. The following regression equation was obtained: $y = 1573.5x - 90.362$, where y is the peak area and x is the concentration of HCA. The correlation coefficient of the calibration graph was ≥0.9967. The estimated LOQ in this study was found to be 1.4 μg.

The extraction/recovery of HCA from the rinds, fruits, and leaves are important. During the treatment with charcoal, traces of HCA may be adhering to the charcoal. This was recovered by washing the charcoal with excess of water 2 times. These steps were standardized in our earlier methods (*21*, *22*). The recovery of HCA through Dowex during the preparation of HCA from calcium hydroxycitrate the resin was washed up to neutral pH. Moreover, in our earlier work (*17*) we have seen that the recovery of HCA from the sample/standard through Dowex-50 was more than 99%, even 100% in some cases.

It was found that the composition of organic acids is similar in leaves, fresh fruits, and dried rinds of *G. cowa*. It can be seen from **Table 1** that aqueous extract was found to be the preferable solvent for complete extraction of organic acids rather than methanol and acetone. The contents of organic acids in dried rinds are comparable with those in *G. cambogia* and *G. indica* (*21*, *22*). However, HCA was shown to be the principal acid in the fruit and dried rinds of *G. cowa* (HCA > citric acid > HCA lactone > oxalic acid); but the leaves contain almost the same quantity of HCA and citric acid. This is the first report that fruits of *G. cowa* contain a substantial amount of HCA. Hence, this study reveals that the fruits of *G. cowa* can also be used as a source of HCA so that the limit of the source is extended.

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Received for review December 10, 2001. Revised manuscript received March 15, 2002. Accepted March 18, 2002.

JF011627J