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Short communication

Determination of organic acids in leaves and rinds of *Garcinia indica* (Desr.) by LC

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Abstract

Organic acids in leaves and rinds of *Garcinia indica* (Kokam) were determined by high-performance liquid chromatography. The major organic acid in leaves and rinds has been found to be (−)-hydroxycitric acid present to the extent of 4.1–4.6 and 10.3–12.7%, respectively, by isocratic elution with 8 mM sulfuric acid as mobile phase with a flow rate of 1.0 ml/min using UV detection at 210 nm. Hydroxycitric acid lactone and citric acid are present in leaves and rinds in minor quantities. This method has been shown to be very reproducible with the coefficient of variation ranging from 2.8 to 4.2%. This is the first report on the composition of organic acids in the leaves and rinds of *G. indica* by HPLC.

Keywords: *Garcinia indica*; (−)-Hydroxycitric acid; Antiobesity

1. Introduction

Fruit rinds of *Garcinia indica* (*G. indica*) and *Garcinia cambogia* (*G. cambogia*) contain 10–30% (−)-hydroxycitric acid (1,2 dihydroxypropane-1,2,3-tricarboxylic acid; Fig. 1) and it is valued for health benefits. The trees of both the *Garcinia* species thrive prolifically in the Indian subcontinent and Western Sri Lanka. Dried rinds of *Garcinia* are widely used all over South India for culinary purposes and 'Colombo curing' of fish [1]. The acid has been isolated as lactone from the rinds of *Garcinia* by Lewis and Neelakantan (1965) using organic solvents. (−)-Hydroxycitric acid (HCA) is susceptible to lactonisation especially during evaporation and concentration. The lactone has very low biological activity compared with free HCA, Hence, the stable derivatives of HCA viz. the ester [1], sodium and potassium salts of HCA [2] and calcium salt [3] are prepared.

A number of HCA containing products are available in the market as weight control agent [4]. HCA has been shown to suppress food intake [5,6] and decrease body weight gain in experimental animals [7]. Rao and Sakariah [8] have shown

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a dose dependent reduction of food intake, body weight, epididymal fat and serum triglyceride in Albino rats of Wister Strain which were fed lipogenic diet. Studies have also been shown that HCA derived from *Garcinia* promotes weight loss and reduction in serum triglycerides and cholesterol levels, humans fed on high carbohydrate diet [9–11].

The existing method of assay of organic acids consists of titration of extract against standard sodium hydroxide [12]. Gas chromatography method requires derivatization of the acid before analysis [13]. Some authors [14–17] have described the methods for the determination of organic acids by HPLC. In these methods reversed-phase HPLC was used which requires a lengthy ion exchange column clean up. Moreover, no reports on HPLC methods for the analysis of organic acids in rinds and leaves of *G. indica*. The present paper reports the isolation of HCA from *G. indica* leaves. This gives the additional source for HCA, because of limited availability of two known sources i.e. rinds of *G. indica* and *G. cambogia*. In the present communication, we report the HPLC method for the determination of organic acids in leaves and rinds of *G. indica* by HPLC.

2. Experimental

2.1. Materials and equipment

Leaves and rinds of *G. indica* were obtained from coastal Karnataka. *G. indica* fruits are found commonly in coastal Karnataka and Goa on a large-scale [18]. All solvents used were of AR/HPLC grade. Dowex 50WX8, mesh size 100–200 was obtained from Sigma (St. Louis, MO, USA). The high performance liquid chromatographic system consisted of a Hewlett Packard HPLC model HP 1100 Series (Hewlett–Packard, CA, USA), fitted with a Waters μ-Bondapack™ (Waters Corporation, Milford, MA, USA) C_{18} column (250 × 4.6 mm I.D.). The injection system (Rheodyne) used was 20 μl sample loop. Detection was done by a HP 1100 Series Variable Wavelength Detector at wavelength of 210 nm. The elution was carried out with 8 mM Sulfuric acid and flow rate was 1.0 ml/min under isocratic condition. The compounds were quantified using HP CHEMSTATIONS software. An IR spectrum was recorded on a Bruker-IFS 25 spectrometer using KBr discs. ^{1}H-NMR (D_2O) were recorded at 400 MHz on a Bruker AMX 400 FT instrument (Bruker, Rheinstetten, Germany). TMS was used as the internal standard.

2.2. Preparation of pure HCA

Pure HCA lactone or free HCA is not commercially available in market. Hence, pure HCA was prepared as lactone in our laboratory according to the method of Singh et al. (1995) and its purity was analyzed by GC, acid–base titration and optical rotation. The structure of HCA lactone was further confirmed by ^{1}H-NMR spectra. The chemical shifts were matched with reported values [3]. Free HCA was prepared from lactone by treating 50 mg of lactone with 15 ml of 0.1 M sodium hydroxide and the solution was heated in a water bath for 60 min [13]. The sodium hydroxycitrate so obtained was passed through Dowex 50 [H^+]. The resin was washed to neutral pH. The washings and the supernatant from the resin were combined made up to 50 ml and filtered. Five standard HCA solutions of concentrations ranging from 0.20 to 1.0 mg/ml were prepared and stored at 4 °C until further use.

2.3. Sample preparation

2.3.1. Leaves

About 20 g of fresh leaves of *G. indica* were extracted with 250 ml of water at 15 lbs/in.^{2} pressure for 20 min and filtered. The extraction

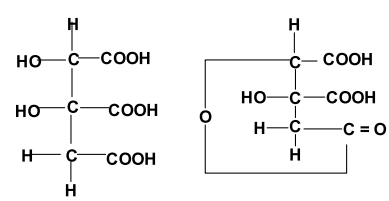

Fig. 1. Structures of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone.

and filtration was repeated twice for complete extraction of the organic acids. The extract was concentrated to 50 ml under vacuum and was treated with 200 ml of ethanol to remove pectinaceous material and centrifuged. The supernatant was concentrated under reduced pressure to 25 ml and stored at 4 °C until further use. The acid content was found to be 5.88–6.29% (w/w) as determined by acid–base titration using phenolphthalein indicator.

2.3.2. Rinds

About 10 g of *G. indica* rinds were extracted with 50 ml of water at 15 lbs/in.^{2} pressure for 20 min and filtered. The extraction and filtration was repeated twice for complete extraction of the organic acids. The extract was decolorized using activated charcoal and filtered. The decolorized extract was concentrated to 25 ml under vacuum and was treated with 100 ml of ethanol to remove pectinaceous material and centrifuged. The supernatant was concentrated under reduced pressure to 25 ml and stored at 4 °C until further use. The acid content was found to be 12.5–15.1% (w/w) with respect to weight of rinds as determined by acid–base titration.

2.4. Validation of HPLC method

2.4.1. Calibration and linearity

The linearity of the method was evaluated by analyzing a series of HCA standards. About 10 μl of each of the five working standard solutions containing 2–10 μg of free HCA was injected on to the HPLC and 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 three runs).

2.4.2. Range

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

2.4.3. Determination of the limit of quantification

The limit of quantification (LOQ) was defined as the lowest HCA concentration, which can be determined with an accuracy and precision $< 20\%$.

2.5. Quantification of organic acids in samples

A known volume of (20 μl) of the sample prepared above was injected into the HPLC and concentration of organic acids was obtained directly from the peak area and by application of the dilution factor. The organic acids in the samples were expressed as g/100 g of sample.

2.6. Recovery of HCA

The organic acids of selected samples of *G. indica* leaves and rinds were approximately doubled by spiking with known amounts of HCA. The spiked samples were prepared for acid determination as described in the sample preparation section and were analyzed.

3. Results and discussion

Pure HCA was prepared and characterized as lactone in our laboratory according to the method of Singh et al. (1995). Isolated pure HCA lactone had a melting point 182 °C and optical rotation $[\alpha]_D^{20} + 99.8°$ ($c = 1.0$; H_2O), these values matched well with those of pure HCA lactone reported values [2,3]. The structure of HCA lactone was further established by IR and ^{1}H-NMR spectroscopy. HCA lactone displayed strong IR bands at 3200, 1760 and 1680 cm^{-1}. ^{1}H-NMR spectra of HCA lactone showed two protons at γ-carbon which gives a AB quartet at δ 2.53 and 2.74 with $J = 17.1$ Hz and one proton at α-carbon showed a singlet at δ 5.15.

Major organic acid found in kokam leaves and rinds by HPLC was HCA as shown in Fig. 2. Six minor peaks are also observed of which two were identified as HCA lactone and citric acid by co-injection of standard acids. The other 3–4 minor peaks are not matched to retention times of tartaric acid, oxalic acid, lactic acid, malic acid and

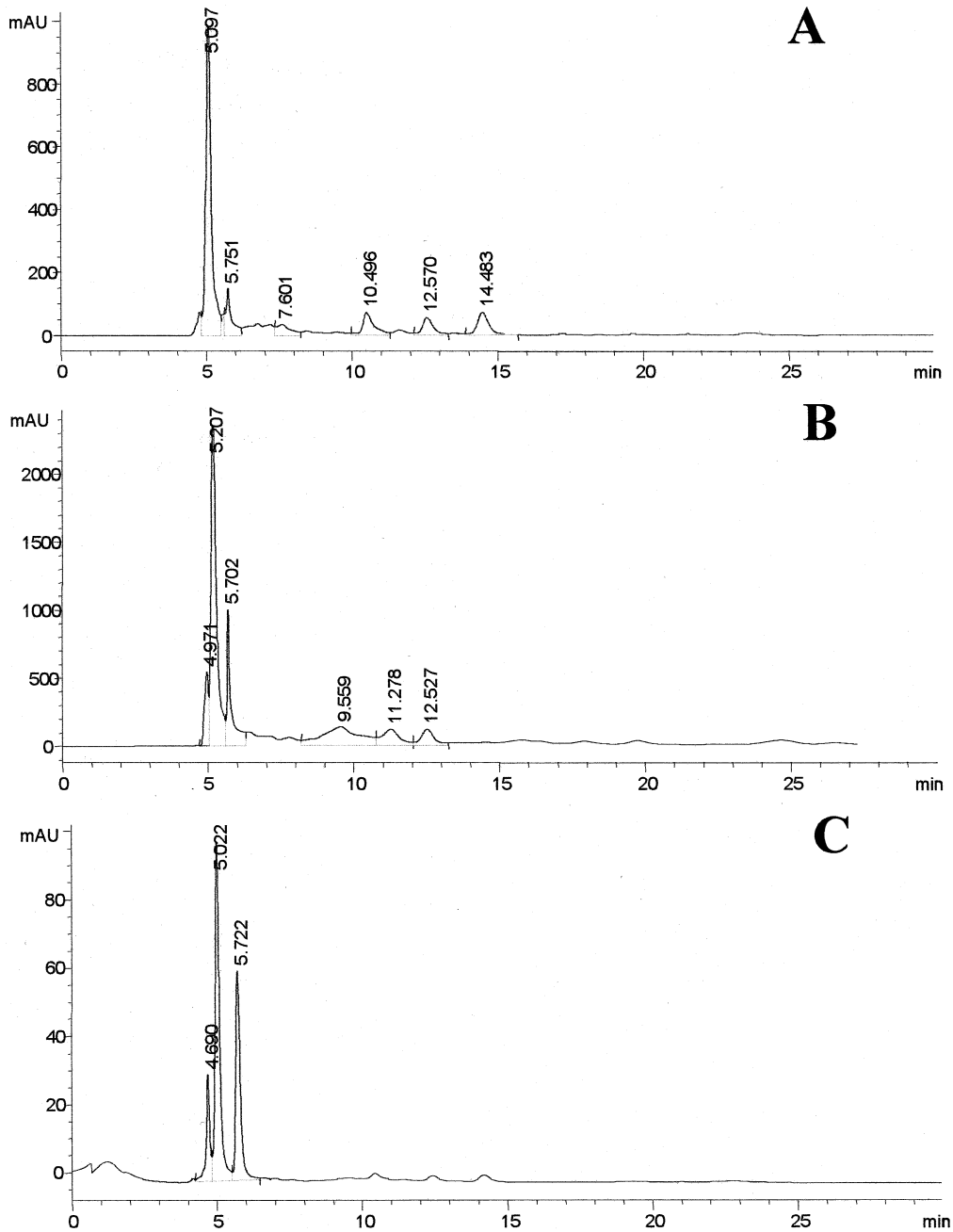

Fig. 2. HPLC chromatograms of (A) rinds; (B) leaves; and (C) standard HCA lactone, HCA and citric acid.

succinic acid. Table 1 gives the HCA, HCA lactone and citric acid content by HPLC method and acid–base titration method in three different samples of kokam rinds and leaves. As is to be expected, the acid–base titration gives slightly higher values for HCA, which is due to the presence of other acids. The high S.D. value of Table 1 in case acid–base titration may be due to less

Table 1
Comparison of organic acids in three samples of Kokam rinds and leaves by HPLC and titration method*

Samples	Organic acids by HPLC method (g/100 g)			Acid–Base titration (g/100 g) mean ± S.D.
	HCA mean ± S.D.	HCA lactone mean ± S.D.	Citric acid mean ± S.D.	
Rinds-I	10.27 ± 0.18	0.85 ± 0.015	0.203 ± 0.016	12.48 ± 0.68
Rinds-II	10.50 ± 0.30	1.77 ± 0.028	0.313 ± 0.021	13.88 ± 0.85
Rinds-III	12.74 ± 0.21	1.51 ± 0.030	0.227 ± 0.025	15.10 ± 0.70
Leaves-I	4.11 ± 0.08	0.62 ± 0.032	0.83 ± 0.012	5.90 ± 0.20
Leaves-II	4.64 ± 0.11	0.61 ± 0.021	0.95 ± 0.016	6.29 ± 0.25
Leaves-III	4.10 ± 0.06	0.61 ± 0.024	0.82 ± 0.015	5.88 ± 0.32

*$n = 4$.

sensitivity of the end point compare with HPLC detection and interference of other acids present in the extracts.

HPLC method was carried out using different mobile phases i.e. 6, 8, 10, 12 mM H_2SO_4. It was found that is the best solvent for the separation of all peaks from *G. indica* samples. Before actual extraction solvent was chosen, preliminary studies were performed with different solvents like acetone and methanol [19]. It was concluded that aqueous extraction yielded maximum yield of organic acids.

HCA was resolved as single peak in all samples analyzed with no interference from other compounds. The identity of the HCA peak was confirmed by determination of retention time and by spiking with standard HCA. The retention times of the HCA lactone, HCA and citric acid in all samples were found to be 4.69 ± 0.28, 5.02 ± 0.19 and 5.75 ± 0.03 min, respectively.

A calibration curve was derived from three injections of six concentrations of HCA. Linearity was found in the 2–9 μg concentration range and it has a good reproducibility and accuracy (Fig. 3). The following regression equation was obtained $y = 1571.9x - 78.784$, where y is the peak area and x is the concentration of HCA. The correlation coefficient of the calibration graph was ≥ 0.9966. The estimated LOQ in this study was found to be 1.4 μg.

To determine the recovery and to ensure the validity and reproducibility of the proposed method, repeated injections of the same samples of each of the studied *G. indica* rinds and leaves were used. These samples were prepared by addition of known amount of standard HCA to exact weights of previously assayed rinds and leaves. The obtained results indicated the HCA was almost quantitatively recovered from the three studied samples. The recoveries of HCA from different samples are presented in Table 2. They ranged from 93.5 ± 3.9 to 96.9 ± 2.7% emphasizing the accuracy of the method. The coefficients of variation resulting from three determinations were 2.8–4.2% indicating the precision of the method.

The existing methods for the determination of organic acids involve, an acid–base titration, which gives the total acidity of extracts. Determination by means of GLC is lengthy in that the

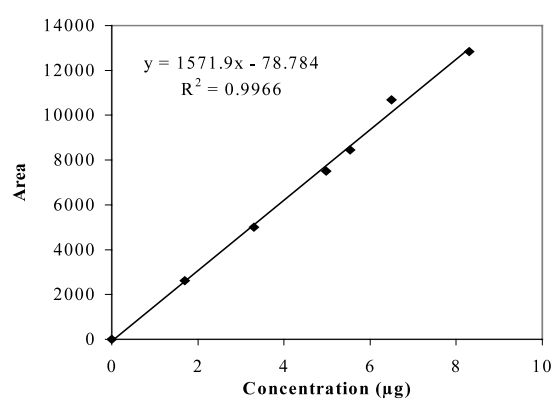

Fig. 3. The linear relationship between the area and concentration of HCA.

Table 2
Recovery of HCA from Kokam rinds and leaves*

Samples	% Recovery	% CV
Rinds-I	95.0 ± 3.4	3.6
Rinds-II	93.9 ± 3.2	3.4
Rinds-III	93.5 ± 3.9	4.2
Leaves-I	95.9 ± 2.7	3.0
Leaves-II	96.9 ± 2.7	2.8
Leaves-III	93.6 ± 3.4	3.6

CV, coefficient of variation; $*n = 3$.

organic acids must be derivatised to volatile silyl derivatives. For silylation the sample should be dried completely and the HCA has the tendency to undergo cyclization of the γ-lactone during drying [13]. Due to the highly hygroscopic nature of HCA it is rather difficult to dry the sample completely. Hence, free HCA can not be estimated by GLC. The above two methods have its own merits and demerits in respect of accuracy and convenience. But, in liquid chromatography free HCA and HCA lactone can be quantified without concentrating, drying and derivatiztion. This is the first report on the chemical composition of organic acids in leaves and rinds of *G. indica*.

4. Conclusion

The present method is simple and accurate for the determination of organic acids in leaves and rinds of *G. indica* by HPLC. Values found by the titration and HPLC methods were comparable but were 0.1–1.3% higher than the total acid by HPLC. The coefficients of variation were 2.8–4.2% indicating the precision of the method. Finally this method can be used an excellent alternate to GLC and titration method for the estimation of HCA in dilute extracts. Reproducibility, accuracy and sensitivity of the method are satisfactory. This method may be considered for routine analysis of large number of samples of *G. indica*.

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