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ESTIMATION OF (-)-HYDROXYCITRIC ACID (HCA) IN *GARCINIA LANCEAEFOLIA* ROXB. USING NOVEL HPLC METHODOLOGY

Ankur Gogoi [1*], Nabajyoti Gogoi [1] and Bijoy Neog [2]

Centre for Studies in Biotechnology [1], Dibrugarh University, Assam, India.
Department of Life Sciences [2], Dibrugarh University, Assam, India.

Keywords:

(-)-Hydroxycitric acid, (-)-HCA, *Garcinia lanceaefolia*, anti-obesity, lipogenesis, HPLC, paper chromatography, endemic species, Assam, NE India

Correspondence to Author:
Ankur Gogoi

Ph.D. Scholar,
Centre for Studies in Biotechnology,
Dibrugarh University,
Dibrugarh- 786004, Assam, India.

E-mail: agogoibiotech@dibru.ac.in

ABSTRACT: (-)-Hydroxycitric acid (HCA) has been found to be the principal acid in genus *Garcinia*, which is a potential metabolic regulator of anti-obesity activity and inhibitor of lipogenesis. Presence of (-)-HCA as the principal acid has added importance to the genus *Garcinia*. The present study deals with extraction and estimation of (-)-HCA from *Garcinia lanceaefolia*, collected from four administrative districts of Assam, NE India. An improved HPLC methodology has been developed in the present study for estimation of (-)-HCA. Detection of (-)-HCA was done by observing the orange spot on paper chromatography. The R_f values were observed at 0.34 and 0.45 for free HCA and HCA lactone, respectively. Estimation of (-)-HCA was done by HPLC analysis, using water as mobile phase by taking UV detection at 210 nm and has been found to be 4.56 ± 0.13gm/10gm of dried sample. This is the first report of its kind on *Garcinia lanceaefolia* Roxb.

INTRODUCTION: (-)-Hydroxycitric acid (HCA) is a derivative of citric acid and has been found to be a potential metabolic regulator of anti-obesity activity and inhibitor of lipogenesis [1-13]. Lewis and Neelakantan (1965), for the first time reported about Hydroxycitric acid (HCA) in *Garcinia cambogia* and *Hibiscus sabdariffa* and did isolation and its identification as (-)-HCA on the basis of chemical and spectroscopic studies [14]. In recent past (-)-HCA has been successfully isolated from other species of genus *Garcinia.* As Hydroxycitric acid (1, 2-dihydroxypropane-1, 2, 3-tricarboxylic acid) has two asymmetric centers, thereby two pairs of diastereoisomers or four different isomers (I, II, III and IV) are possible [15](**Figure 1**).

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Being a γ-hydroxy acid, it cyclizes readily to the corresponding lactone (**Figure 2**). The determination of absolute configuration was done from Hudson's lactone rule, circular dichrosim curves, optical rotator dispersion curves and calculation of partial molar rotations [16].

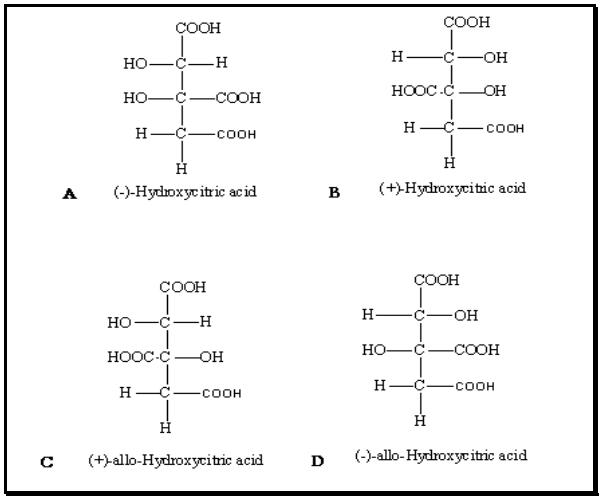


FIGURE 1: A, B, C AND D ARE FOUR DIFFERENT ISOMERS OF HYDROXYCITRIC ACID.

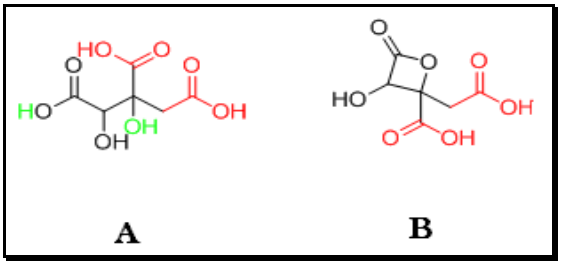


FIGURE 2: STRUCTURAL REPRESENTATION OF (A) HYDROXYCITRIC ACID AND (B) HYDROXYCITRIC ACID LACTONE.

Presence of (-)-HCA as the principal acid has added importance to the genus *Garcinia.* The genus *Garcinia* Linn., belonging to the Kingdom Plantae, Order Malpighiales, family Clusiaceae (=Guttiferae). *Garcinia* is a large genus of evergreen trees or shrubs. About 200 species of *Garcinia* are distributed in tropical states of Asia, Africa, and Polynesia; of which about 30 species are found in India [17]. Among the 30 species found in India, 17 are endemic. Of these, seven are endemic to the Western Ghats, six in the Andaman and Nicobar Islands and five in the north-eastern region of India.

Garcinia lanceaefolia reported to be endemic to N.E. India. The fruits of *Garcinia* species are sour in taste. The dried rind of the fruit of *Garcinia* species popularly known as "Thekera" in Assamese is extensively used in traditional dishes in Assam. *Garcinia lanceaefolia*, distributed in the States of Assam, Meghalaya and Nagaland is a beautiful glabrous evergreen tree with dark-brown bark. Stem decussately branched.

Leaves dark glossy-green (4.5-6x2.5-3.5) cm, lanceolate, long-acuminate. Flowers hermaphrodite, polygamous, red, terminal of axillary. Sepals 4, petals 4, Stamens in 4 bundles of 4-5 each; anthers 4 celled. Ovary obovoid; stigma 6-8 lobed, glandular-tubercled. Fruit obovoid, about 4-5cm diam, fruits green in colour and turn bright orange red when ripe, 6-8 seeded. It flowers from March to May and fruiting started from October to February [18, 19].

MATERIALS:

Collection and sample preparation: Fresh fruits of *Garcinia lanceaefolia* were collected from four administrative districts of Upper Brahmaputra valley of Assam. Fresh weights were recorded and sliced fruit rinds were oven dried at 60^0C to maintain the uniformity and water content was calculated. Commercially available samples avoided as they contains added salt.

Chemicals and equipments: All the reagents and chemicals were of HPLC, GR, AR grade purchased from Merck. Dowex ® 50WX8 Hydrogen form (mesh size 100-200), (-)-Garcinia Acid ($C_6H_6O_7$) and Potassium hydroxycitrate tribasic monohydrate ($C_6H_5K_3O_8.H_2O$) from Sigma Aldrich, HPLC system (Agilent, 1260 Infinity Series), HPLC pump (1260 Quat Pump), C_{18} RP Column 4.6x150 mm, Dual λ absorbance detector (1260 MWDVL), The system consists of a Rheodyne injector with sample loop of 20 µL, OpenLab CDS (Chem station edition) software. Glasswares from Merck and plasticwares from eppendorf.

Methodology

Extraction and preparation of extracts: In the present study, method of assay of organic acid comprises of HPLC and titration of extract against standard KOH for validation of HPLC method.

A series of 10 grams of the dried samples were weighed and autoclaved at 15 lbs/in^2 for 15 minutes with 30 ml of double distilled water in a flat bottomed boiling flask. The procedure was repeated twice with final cold water rinse and filtration through qualitative filter paper grade no. 1 for complete extraction of the desired acid. Extracts were treated with activated charcoal for decolourisation and filtered. The collected extracts were then oven dried to reduce the volume and treated with ethanol to remove the pectin. Neutralization of extract pH with KOH results in the precipitation of the potassium salt as a thick syrup like heavy liquid at the bottom of the beaker, which were freed of impurities by repeated washing with ethanol to obtain a pale yellow coloured highly hygroscopic alkali salt of the acid. Crystal develops upon evaporation. For complete recovery of the free acid aqueous solution of the alkali salt were treated with Dowex ® 50WX8 Hydrogen form resin. Evaporation of the aqueous solution of the acid yields the lactone.

Paper chromatography yields two spots. These were eluted, concentrated and then run again on the paper chromatography and the result gave two

original spots. This was supported by titration with alkali, using phenolphthalein as indicator generated two different end points. Only single spot developed after passing the extract through the Dowex ® 50WX8 Hydrogen form resin. That implies that two spots developed on the chromatograms are of free Hydroxycitric acid and its lactone (**Figure 3 and 4**).

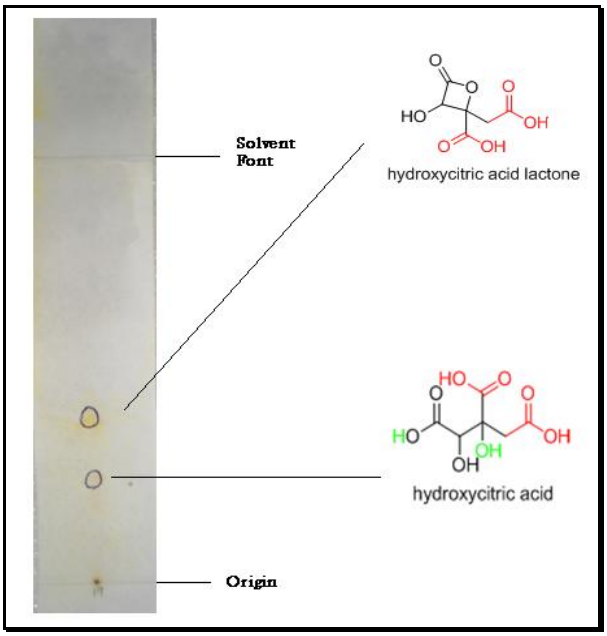


FIGURE 3: PAPER CHROMATOGRAM WITH TWO SPOTS OF FREE HCA AND ITS LACTONE RESPECTIVELY.

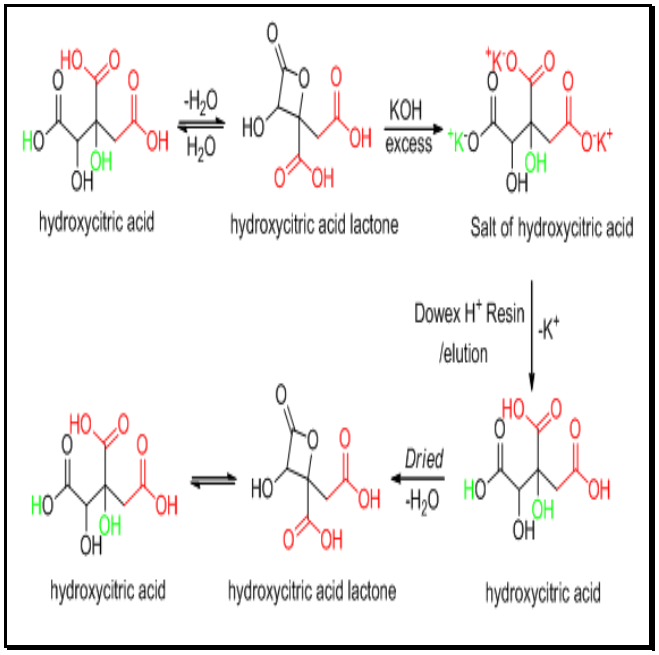


FIGURE 4: REPRESENTATION OF STRUCTURE OF HCA, ISOLATION AND ITS SUBSEQUENT CONVERSION TO CORRESPONDING LACTONE.

HPLC analysis: As free (-)-HCA ($C_6H_8O_8$) is not available commercially, (-)-Garcinia Acid ($C_6H_6O_7$) and Potassium hydroxycitrate tribasic monohydrate ($C_6H_5K_3O_8.H_2O$) were taken as reference standard. The high-performance liquid chromatography system used in the study was from Agilent (1260 Infinity Series, 1260 Quat Pump) with C_{18} RP Column 4.6x150 mm. The system consists of a Rheodyne injector with sample loop of 20 µL, OpenLab CDS (Chem station edition) software. In the present study, attempts were made to develop an improved HPLC methodology from the existing protocols [20-28].

The mobile phase used was HPLC grade (Millipore) water and the HPLC method was carried out taking a series of different (-)-HCA concentrations with final preeminent flow rate of 0.5 ml/min. Detection was done by Dual λ absorbance detector (1260 MWDVL) at a wavelength of 210 nm and sample eluted was 20 µl for 10 minutes at 58.8 bar Quart pump and 25^{0}C Column comp. (Figure 5). Prior to injection to the HPLC system, all the samples and the standards were filtered through membrane filters of pore size 0.45 µm. As water has been used in the study, to maintain the efficiency and column life after completion of the experiment it is purged several times with methanol.

Calculation: The followed calculation method has been used for estimation of HCA. To determine unknown concentration of the sample from the HPLC results;

$$\text{Response Factor (RF)} = \frac{\text{Peak area (mAU) of the standard}}{\text{Conc. of the Standard (mg)}}$$

$$\text{Amount of unknown sample (mg)} = \frac{\text{Peak area (mAU) of the unknown sample}}{\text{RF}}$$

$$\text{Area \% of sample} = \frac{\text{Area of the sample (mAU)}}{\sum \text{Area of each peak (mAU)}}$$

Taking these two formulae as base, calculations were done for estimation of (-)-HCA.

RESULT AND DISCUSSION:

Preliminary investigation and identification of HCA was done by observing the orange spot on paper chromatography using 1% Sodium metavanadate reagent spray on it and the R_f values

were observed at 0.34 ± 0.032 and 0.45 ± 0.037 for free HCA and HCA lactone, respectively. Estimation of (-)-HCA was done using water as a mobile phase, by HPLC with C_{18} RP Column has been found to be 4.56 ± 0.13gm/10gm or 45.68 ± 1.30 % of dried sample by taking UV detection at 210 nm (**Figure 5**).

Different wavelengths and sample run time were attempted before the concluded protocol. The acid-base titration values are of the total acid content of the extracts; even then they were comparable to the HPLC generated results, thereby suggesting that the extraction procedure was of high-quality and acceptable. Average water content of the fruit and dissolved pectin content in the extracts were found to be 92.43% and 0.308 gm out of 10 gm, respectively. The retention time of (-)-HCA in the standard and all samples were found to be 2.569 ± 0.040 and 2.553 ± 0.124 respectively.

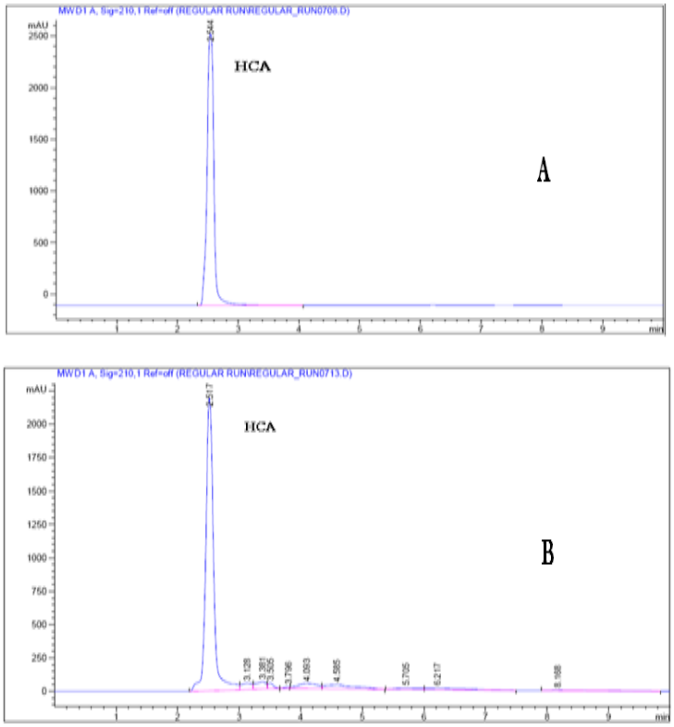
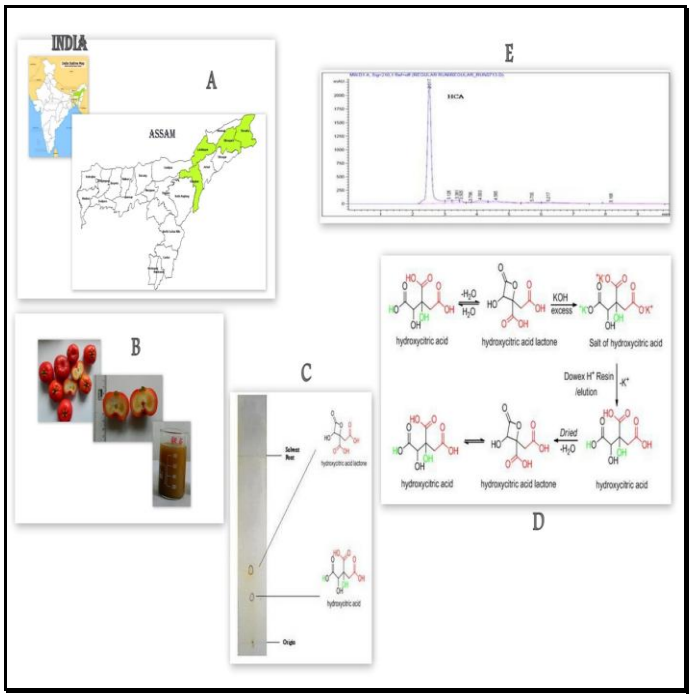


FIGURE 5: HPLC CHROMATOGRAMS OF (A) STANDARD AND (B) SAMPLE AT 10 MINUTES RUN TIME.

CONCLUSION: The methodology developed in the present study is an improved and simple with less expensive and faster analysis time for extraction and estimation of (-)-HCA from the fruit rinds of *Garcinia lanceaefolia.* Methodology has been validated in terms of accuracy, repeatability and other validation parameters. The values found in the HPLC and titration procedures were comparable and this protocol can be well followed for large scale analysis of samples of *Garcinia lanceaefolia* and other species of genus *Garcinia* as well. This is the first report of its kind on *Garcinia lanceaefolia.*



COLLECTION AREAS (A), SAMPLE PREPARATION (B), PRELIMINARY DETECTION OF ACID BY PAPER CHROMATOGRAM (C), MECHANISM OF HCA ISOLATION (D) AND HPL CHROMATOGRAM OF EXTRACTED HCA FROM *GARCINIA LANCEAEFOLIA* (E).

BRIEF SUMMARY:
Fruits of *Garcinia lanceaefolia* were collected from four administrative districts of Assam, NE India; sample preparation was done in the laboratory. Preliminary investigation of presence of HCA was done with paper chromatography. Conversion of HCA to corresponding lactone and isolation of HCA, estimation of HCA in the study samples have been studied.

CONFLICT OF INTEREST: The authors hereby declare no competing financial conflict of interest.

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