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Ascorbic Acid and Tannins from *Emblica officinalis* Gaertn. Fruits—A Revisit

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The fruits of *Emblica officinalis* Gaertn. (Euphorbiaceae), also known as *amla* in Ayurveda, are considered to be a rich source of ascorbic acid. However, the antioxidant activities exhibited by *E. officinalis* extract are superior to those of ascorbic acid itself. Low molecular hydrolyzable tannins emblicanins A and B have been suggested in the earlier literature to be the contributory antioxidant molecules in the extract. This work finds no evidence for the presence of emblicanins A and B in the extract. In addition, the high content of ascorbic acid is also questionable due to previous nonidentification of coeluting mucic acid gallates. This paper reports a new HPLC method to detect even trace amounts of ascorbic acid in *E. officinalis* fruit juice or extract.

KEYWORDS: ***Emblica officinalis*; amla; ascorbic acid; HPLC; HPTLC; NMR; LC-MS**

INTRODUCTION

The fruits of *Emblica officinalis* Gaertn. (Euphorbiaceae), commonly known in India as *amla* (Sanskrit name *amalaki*), are consumed as fruit or in the form of food products. This fruit also forms an important constituent of many Ayurvedic preparations such as *chyvanprash* and *triphala* and is regarded as "one of the best rejuvenating herbs". It is a medium-sized deciduous tree found throughout India. The fruits are globular, fleshy, and smooth and of striated of yellowish-green color. They contain an obovate-obtusely triangular six-celled nut (*1*).

Traditionally, the fruit is useful as astringent, cardiac tonic, diuretic, laxative, livertonic, refrigerant, stomachic, restorative, alterative, antipyretic, anti-inflammatory, hair tonic, and digestive medicine (*1*). It is used for a variety of ailments such as anemia, hyperacidity, diarrhea, eye inflammation, anomalies of urine, leucorrhea, jaundice, nervine debility, liver complaints, and cough (*1*). It is reported to be effective in the treatment of peptic ulcer and dyspepsia. It is also reported to have hepatoprotective, antioxidant, antimutagenic, cytoprotective, antitumor, antifungal, antimicrobial, hypolipidemic, and antiatherosclerotic effects (*2*).

The fruits of *E. officinalis* are reported to contain hydrolyzable tannins, emblicanin A and emblicanin B, along with pedunculagin and punigluconin (*3*). These fruits were to be considered rich sources of ascorbic acid until Ghosal et al. questioned its presence in 1996 (*3*). However, in 2006, Scartezzini et al. proposed a reliable HPLC-DAD for the identification and quantification of ascorbic acid and further indicated that high antioxidant activity is due to a large percentage of the presence of ascorbic acid (*4*). Recently, Raghu et al. compared ascorbic acid content of the fruits by conventional colorimetric estimation and specific enzymatic method and as the *o*-phenylene diamine derivative of dehydroascorbic acid and found contents of 34−38 mg of vitamin C equivalent to 100 g of fresh weight (*5*).

In the present paper we have revisited the emblicanins and ascorbic acid content of the fruit juice and extract. During our investigations we observed that ascorbic acid coelutes with other compounds of similar spectral behavior. Additionally, the reported hydrolyzable tannins emblicanins A and B, as described by the detailed HPTLC patterns by Pozharitskava et al., when evaluated were found to be structurally different from the previously reported structures (*3, 6*). Herein, we report our studies on the isolation and identification of emblicanins and the detection of ascorbic acid using multiple methods such as HPTLC, HPLC-DAD, preparative HPLC, LC-MS, and NMR.

MATERIALS AND METHODS

General Procedures. NMR was recorded on a Varian spectrometer (Palo Alto, CA) with proton at 300 MHz and with ^{13}C at 75 MHz. The spectra were recorded in either D_2O or DMSO-d_6 with TMS as internal standard. LC-MS studies were done on a Thermo-Finnigan LCQ Advantage Max spectrometer (San Jose, CA) with ESI probe. The system consisted of a Thermo-Finnigan surveyor PDA detector, an LC pump, and an autosampler. The ESI probe was operated in negative mode. For preparative chromatography a Shimadzu preparative HPLC system (Kyoto, Japan) equipped with Class Vp software with a binary gradient (LC8A) pump, a UV−vis dual wavelength detector (SPD-10AVp), a system controller (SCL-10AVp), and a Rheodyne injector 7725i with a 5 mL sample loop was used. HPLC-DAD analyses were carried out on a Shimadzu HPLC system equipped with Class Vp software (ver. 6.14 SP2) with a binary gradient (LC10 ATVp) pump,

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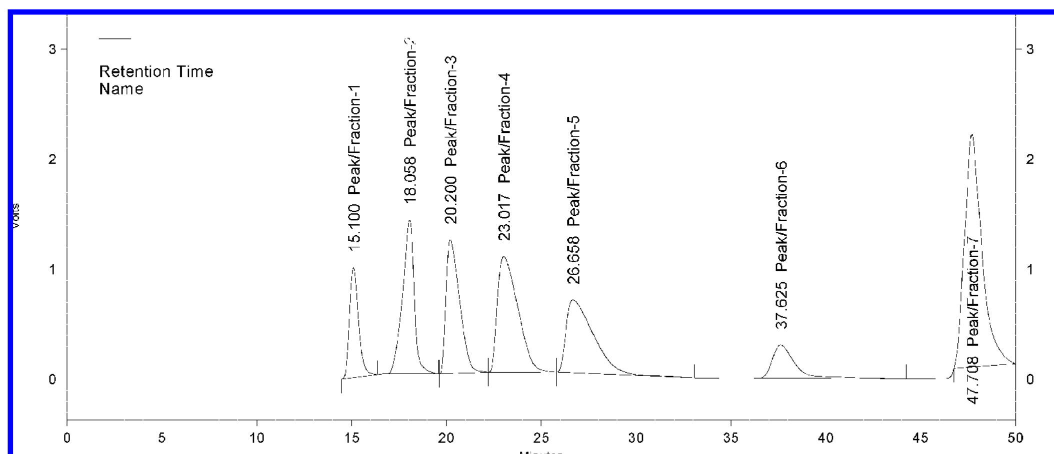

Figure 1. Preparative HPLC chromatogram (at 280 nm) of freeze-dried aqueous extract of *E. officinalis* fruits.

a DAD detector (SPD-M10AVp), a system controller (SCL-10AVp), and a Rheodyne injector with a 20 μL sample loop. For freeze-drying a 6 L Vertis freeze-dryer (Vertis Corp., Gardiner, NY) was used.

Chemicals. Ascorbic acid was purchased from Sigma (St. Louis, MO). All other chemicals and solvents were purchased locally and were of either analytical or HPLC grade.

Plant Material. The fresh fruits (10 kg) of *E. officinalis* Gaertn. (Euphorbiaceae) were procured locally in Bangalore, India. It was identified by our in-house botanist Benny Daniel, and a voucher specimen (P-80019) was deposited in the herbarium. The fruits were cut into pieces, and half were expressed to get juice and the other half cold macerated with water (6 L) at room temperature for 2 h with stirring. The filtered juice (2.1 L) and extract were freeze-dried to get 40 and 30 g of dry powder, respectively.

Isolation by Preparative HPLC. The freeze-dried extract of *E. officinalis* was separated into seven major fractions using a preparative HPLC system. Separations were performed on a Phenomenex Jupiter C18 column 300A (250 × 50 mm, 15 μm) with mobile phases (A) 0.1% formic acid and (B) methanol. A gradient solvent system was used: 0−35 min, 5% B; 35−40 min, 100% B; 40−50 min, 100% B; 50−51 min, 5% B; and 51−60 min, 5% B. The flow rate was 35 mL/min with monitoring at 240 and 280 nm. Fractions 1 (36 mL), 2 (65 mL), 3 (60 mL), 4 (85 mL), 5 (94 mL), 6 (62 mL), and 7 (72 mL) were collected and kept at −20 °C until further use. The separations were repeated, and combined fractions were lyophilized, whereupon fractions 2, 3, and 4 gave compounds **2**, **1**, and gallic acid as white amorphous powders.

1-*O*-Galloyl-β-D-glucose (β-Glucogallin) (**1**): amorphous white powder; $[\alpha]_D^{20}$ −15.8 (*c* 0.038, H_2O); UV λ_{max} 278 nm (methanol); ESI-MS (negative mode) *m/z* 331 [M − H]; ^{1}H NMR (DMSO-d_6) galloyl moiety, δ 7.20 (2H, s); glucose moiety, δ 5.67 (1H, d, J = 7.8 Hz), 3.89, 3.48−3.76; ^{13}C NMR (DMSO-d_6) δ 166.1, 145.2, 139.2, 119.3, 109.8, 94.8, 77.2, 76.1, 72.4, 69.6, 61.0.

Mucic acid 1,4-lactone 5-*O*-gallate (**2**): amorphous white powder; $[\alpha]_D^{20}$ −30.8 (*c* 0.052, methanol); UV λ_{max} 280 nm; ESI-MS (negative mode) *m/z* 343 [M − H]; ^{1}H NMR (DMSO-d_6) galloyl moiety, δ 6.97 (2H, s); mucic acid lactone moiety, δ 5.29 (1H, s), 4.61 (1H, d, J = 8.1 Hz), 4.43 (1H, d, J = 8.1 Hz), 4.04 (1H, t, J = 8.4 Hz); ^{13}C NMR (DMSO-d_6) δ 174.3, 169.1, 165.6, 146.3, 139.8, 118.8, 109.4, 78.9, 73.8, 73.3, 69.0.

High-Performance Liquid Chromatography (HPLC) Analysis. Separations were performed on a Zorbax C18 column (250 × 4.6 mm, 5 μm) using one of the following methods. *Method a*: mobile phases A, water (0.1% formic acid), and B, methanol. A gradient was used as 0−25 min, 5% B; 25−30 min, 100% B; 30−40 min, 100% B; 40−41 min, 5% B; and 40−50 min, 5% B. The flow rate was 0.7 mL/min and monitoring was performed at 240 and 280 nm. *Method b*: 100% water with a flow rate of 0.5 mL/min and monitoring at 240 and 280 nm.

HPTLC Analysis. For the analysis silica gel 60 F_{254} (10 × 10 cm) precoated glass plates (Merck KGaA, Darmstadt, Germany) were used. Samples were applied with a 100 μL Linomat syringe using a semiautomatic Linomat V applicator (Camag, Muttenz, Switzerland). Samples consisting of 5 μL of solution each were applied as 8 mm bands with a 11.6 mm distance between two spots. Plates were developed using an ADC2 automated development chamber (Camag). The conditions used for developing TLC plates were temperature, 25.4 °C; relative humidity, 71.1%; and final migration, 70.8 mm. The developing solvent was a combination of ethyl acetate/formic acid/glacial acetic acid/water (10:1:1:2), as reported earlier (*6*). Using scanner 3 (Camag) the plate was scanned at 241 and 280 nm with deuterium illumination. For scanning the slit dimension was 6 × 0.20 mm with a scanning speed of 20 mm/s and data resolution of 100 μm/step. The images were captured on Camag reprostar 3 with winCATS software (ver. 1.4.3.6336).

RESULTS AND DISCUSSION

In 1996, Ghosal et al. reported emblicanins A and B and reported their antioxidant effect (*3*). In our attempts to isolate these molecules, we processed *E. officinalis* extract on preparative reverse phase column chromatography, and a total of seven fractions obtained were lyophilized (**Figure 1**).

On spotting these fractions with ascorbic acid on a HPTLC plate, it was observed that fraction 3 has the same R_f as ascorbic acid (**Figure 2**). However, HPLC analysis of fraction 3 using method a showed that it has a RT of 12.6 min as compared to the 4.56 min of an authentic sample of ascorbic acid, indicating that it is not ascorbic acid (**Figure 3**). In 1996, Ghosal et al. reported emblicanin A to have the same the R_f as ascorbic acid in the given solvent system. This suggested that fraction 3 contains emblicanin A and not ascorbic acid. The HPTLC profile of fraction 3 corroborated that it contained emblicanin A from the previously reported HPTLC-DPPH studies on *E. officinalis* extract (**Figure 4**) (*6*). This indicated the compound from fraction 3 to be emblicanin A.

For further structural studies, fraction 3 was freeze-dried to give compound **1** as an amorphous white powder. Spectral characterization of this compound gave a pseudomolecular ion peak (M − H) of *m/z* 331. The ^{1}H NMR showed a singlet appearing at δ 7.20 for two galloyl protons and a doublet at δ 5.67 with a coupling constant of 7.8 Hz, indicating a β-linked anomeric proton. Further ^{13}C NMR showed a total of 11 carbon signals typical of sugar and a phenolic moiety. Using these data and comparing them with the reported data, compound **1** was characterized as β-glucogallin (**Figure 5**) (*7*). This suggests that the emblicanin A originally reported is β-glucogallin and not 2,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-lactone (*3*).

Using the same HPTLC profile (**Figure 4**) it was observed that peak 2 corresponds to emblicanin B on TLC and was freeze-

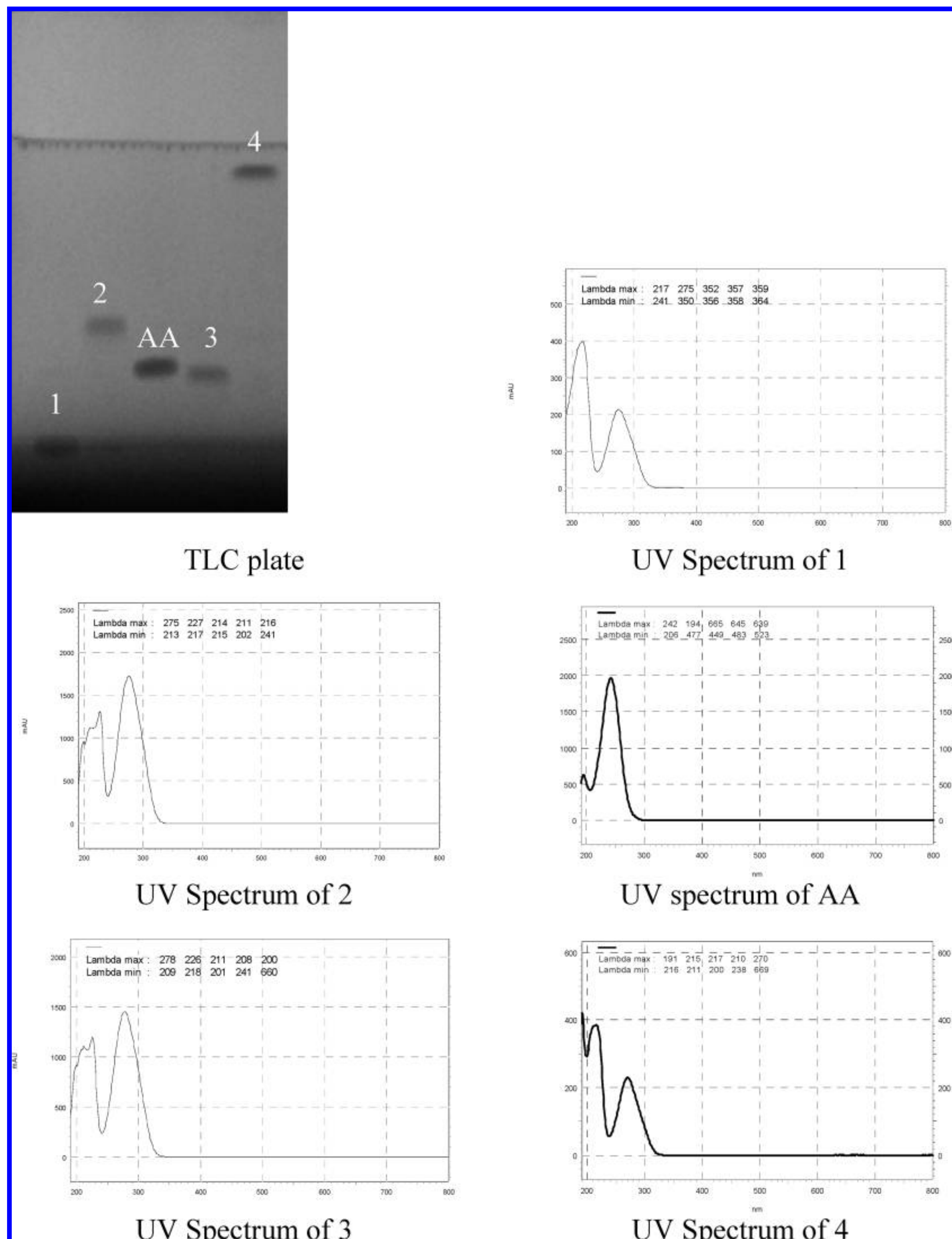

Figure 2. UV spectrum and TLC of isolated peaks with ascorbic acid (spots from left to right: 1, fraction 1; 2, fraction 2; AA, ascorbic acid; 3, fraction 3; 4, fraction 4).

dried to get compound **2** as a hygroscopic white powder (*6*). This compound was unstable in water and was quickly analyzed once in solution. It gave a pseudomolecular ion peak (M − H) of *m*/*z* 343. Direct comparison of NMR data with the literature led to identification of compound **2** as mucic acid 1,4-lactone 5-*O*-gallate (**Figure 3**) (*8*). This indicated that the emblicanin B reported earlier is not 2,3,4,6-bis-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-lactone (*3*). Spectral analysis of lyophilized fractions 4 and 5 revealed them to be gallic acid and mucic acid 6-methyl ester 2-*O*-gallate, respectively (*8*).

Previously, the fruits of *E. officinalis* have been known to be rich in ascorbic acid. However, Ghosal et al. questioned the presence of ascorbic acid and reported tannins (*3*). Recently, Scartezzini et al. not only reported the presence of ascorbic acid but quantified it in the extract and in an Ayurvedic formulation, thereby making a case for the ascorbic acid as a bioactive constituent of *E. officinalis* fruits (*4*). Also, Raghu et al. contradicted the claim of the absence of ascorbic acid in amla fruits using a combination of different methods (*5*). The HPLC analysis using method a showed that ascorbic acid eluting at 4.56 min (**Figure 3**) corresponds to peak 1, which on HPTLC studies (**Figure 2**) showed an R_f value different from that of ascorbic acid, indicating the absence of ascorbic acid. To study the identity of fraction 1 and ascorbic acid content beyond visual or HPTLC densitometric determination, fraction 1 was further evaluated on analytical HPLC.

During HPLC analysis it was found that peak 1 has the same retention as ascorbic acid (**Figure 6**). Furthermore, the DAD

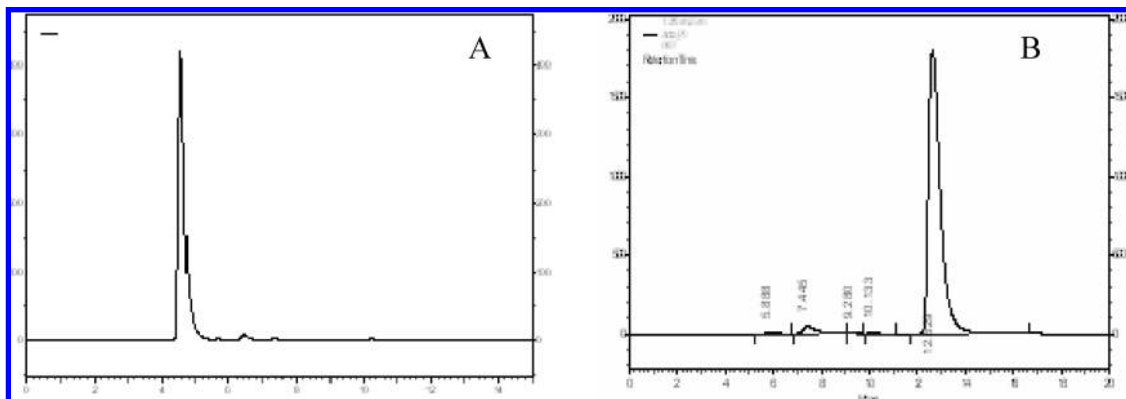

Figure 3. HPLC chromatogram of **(A)** ascorbic acid (RT 4.56 min) and (**B**) **1** (RT 12.6 min).

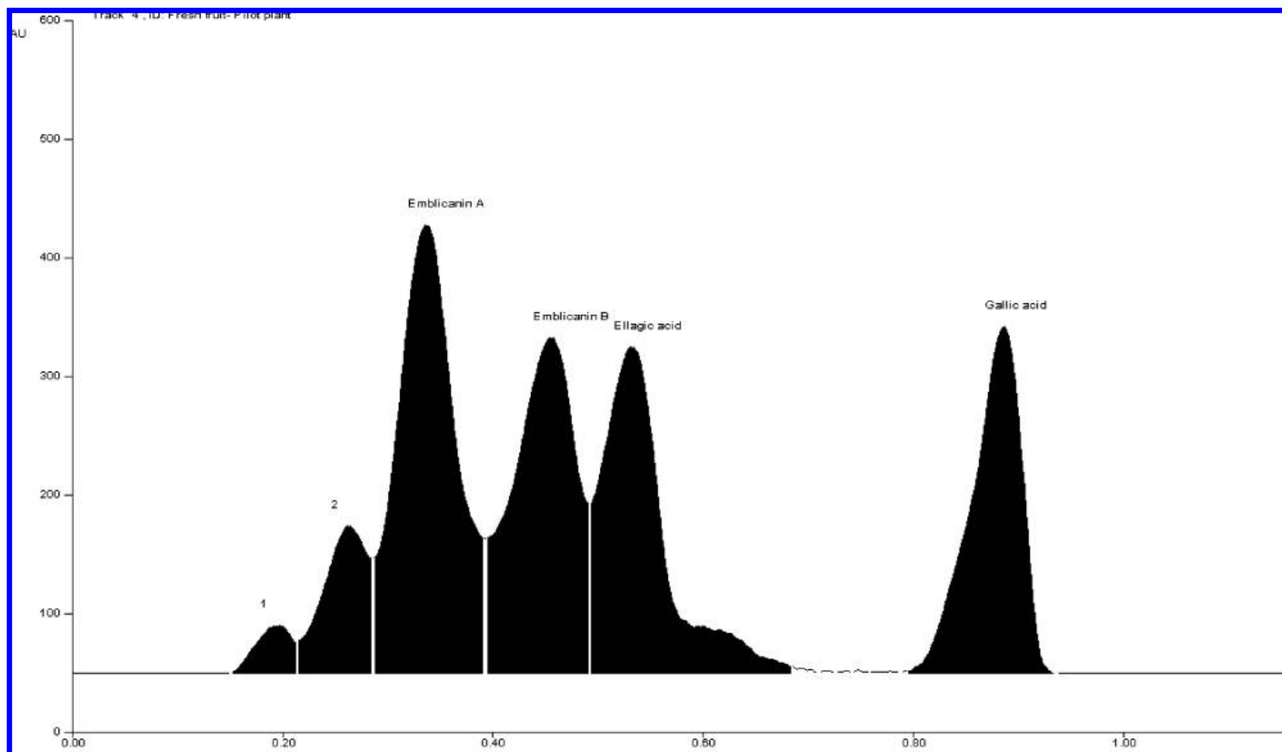

Figure 4. HPTLC profile of *E. officinalis* fruit extract.

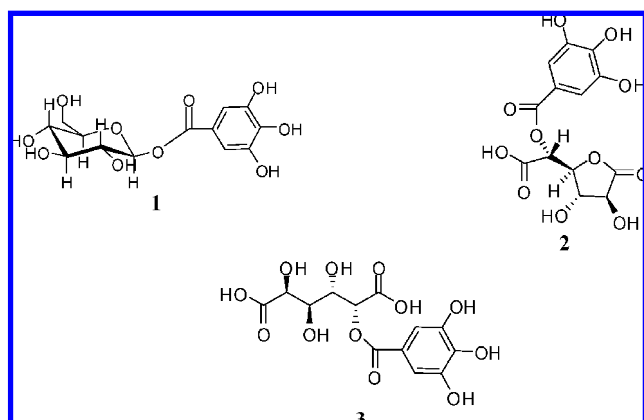

Figure 5. Compounds from *E. officinalis* fruit extract: **1**, β-glucogallin; **2**, mucic acid 1,4-lactone 5-*O*-gallate; **3**, mucic acid 2-*O*-gallate.

spectral scan of peak 1 together with ascorbic acid differed slightly from that of peak 1 alone, making it difficult to ascertain their coexistence in the extract. Hence, we tried to verify the presence of ascorbic acid by NMR. The ^{1}H NMR of fraction 1 was not directly comparable to that of standard ascorbic acid

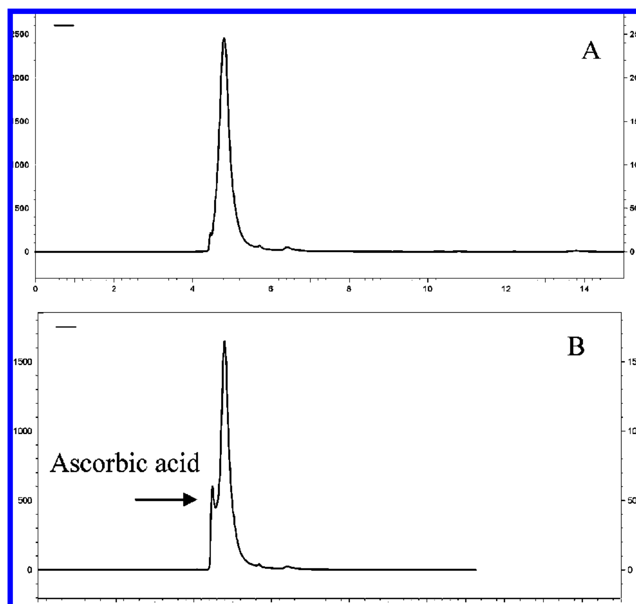

Figure 6. HPLC chromatogram of (**A**) fraction 1 and (**B**) fraction 1 spiked with ascorbic acid.

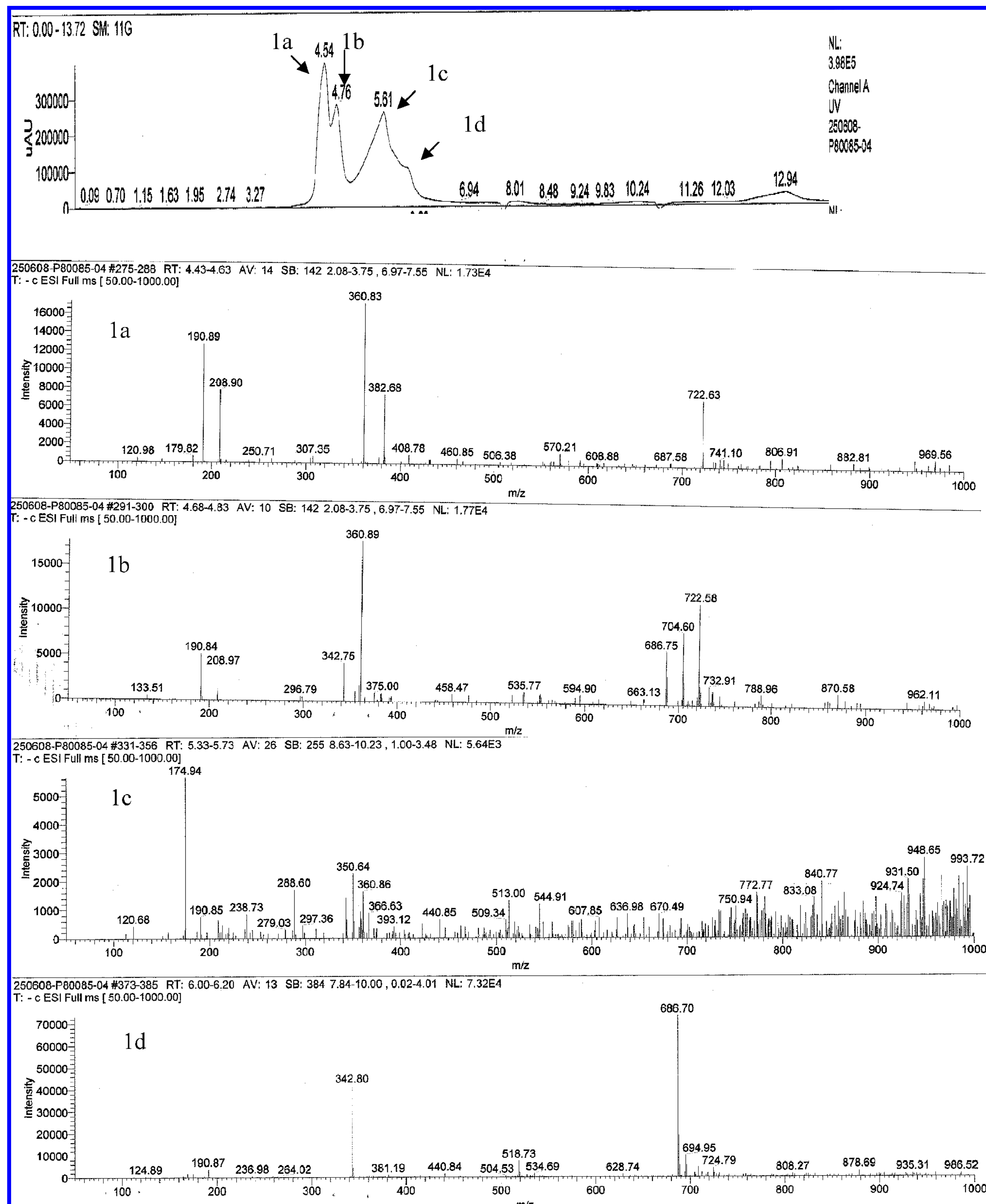

Figure 7. LC-ESIMS of *E. officinalis* extract.

in the same solvent, because of multiple peaks. However, the ^{13}C NMR of peak 1 showed a pattern similar to that of compound **2**, where characteristic signals for the quaternary carbons of ascorbic acid were absent. Also, careful scrutiny of the NMR revealed that it contained two major isomeric constituents. Hence, the mobile phase of the HPLC system was further optimized to resolve fraction 1, leading to the development of method b, in which the mixture of components was resolved into four peaks: 1a, 1b, 1c, and 1d (**Figure 7**). Analysis using method b indicated peak 1c and ascorbic acid to have the same RT. However, peak 1c was not a sharp peak but showed peak 1d at the tail. To ascertain whether peak 1c is ascorbic acid (MW 176), extract was analyzed by LC-ESIMS in negative mode. It showed a pseudomolecular ion peak (M − H) at *m/z*

175 (**Figure 7**). This indicated that ascorbic acid is present in the extract along with three more compounds undetected in previous HPLC analyses. Preparative isolation of 1a, 1b, 1c, and 1d could not be achieved due to the highly polar nature of the compounds. Peak 1a and 1b gave similar UV spectra and were concluded to have the same masses because of identical pseudomolecular ion peaks (M − H) at *m/z* 361. The masses of 1a and 1b indicated that they differ from **2** (MW 344) by 18 amu, which is due lactonization of the latter with loss of a water molecule (*8*). Taking into account the masses and using 1D NMR, it was predicted that 1a and 1b are an isomeric pair of **3**, mucic acid 2-*O*-gallate, previously reported from the juice of *E. officinalis* (*8*). Peak 1d exhibited a pseudomolecular ion peak (M − H) of *m/z* 343, indicating the isomeric mucic acid lactone of **2** previously reported from fruit juice by Zhang et al. (*8*).

The studies, in conclusion, indicate that earlier methods of estimations of ascorbic acid did not resolve coeluting mucic acid gallates. For several decades this fruit has been claimed to be a rich source of ascorbic acid. Therefore, the previous claim that amla fruits are a "good source of vitamin C" is questionable (*5*). During our attempts to quantify the ascorbic acid with newly developed methods, we found that ascorbic acid was present below the quantifiable limits of HPLC-DAD (LOD = 1 ppm and LOQ = 3 ppm). The new HPLC method (method b) is undoubtedly, however, a very reliable method for the accurate detection of free ascorbic acid in *E. officinalis* fruit extract or juice. Moreover, the earlier reported antioxidant hydrolyzable tannins, emblicanins A and B, correspond to β-glucogallin (**1**) and mucic acid 1,4-lactone 5-*O*-gallate (**2**), respectively, as established in this work. The trace amount of free ascorbic acid in *E. officinalis* fruit extract suggests that the antioxidant effects exhibited by the *E. officinalis* fruit are due to gallic acid esters. Furthermore, it can be suggested that mucic acid lactone and ascorbic acid may be related biosynthetically in *E. officinalis* fruits.

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