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NEW MUCIC ACID GALLATES FROM *Phyllanthus emblica*

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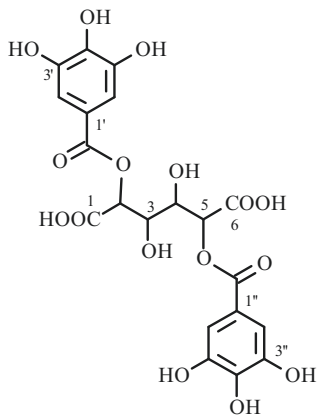
*Two new compounds that were identified as mucic acid 2,5-di-*O*-gallate (**1**) and mucic acid 2,5-di-*O*-gallate 1,4-lactone (**2**) and 14 known compounds, including for the first time in a plant 3,4,6-tri-*O*-galloyl-β-D-glucose, were identified by a phytochemical investigation of* Phyllanthus emblica *(Phyllanthaceae) fruit. Compounds **1** and **2** exhibited pronounced antioxidant activity.*

Keywords: *Phyllanthus emblica*, mucic acid 2,5-di-*O*-gallate, mucic acid 2,5-di-*O*-gallate 1,4-lactone, antioxidant activity.

Phyllanthus emblica L. (*Emblica officinalis* Gaertn.; Indian gooseberry, amla) is a medicinal plant of the family Phyllanthaceae. Its fruit is widely used in traditional Asian medicine (China, India, Japan) as an anti-inflammatory and antipyretic agent [1]. A mixture of fruits from *P. emblica*, *Terminalia chebula* Retz., and *T. bellirica* (Gaertn.) Roxb. (Tibetan bras gsum; Indian, triphala) used individually or in combination with other plants is one of the most frequently used preparations in Tibetan and Ayurvedic medical practice [2]. Ellagotannins, gallotannins, mucic acid derivatives, nor-sesquiterpenoids, flavonoids, and essential oil occur in fruit of *P. emblica* according to reports of its chemical composition [3]. Gallates of galactaric (mucic) acid and its 1,4-lactone were isolated from *P. emblica* [4] and represent a unique group of highly hydrophilic phenolic compounds with antioxidant and antiproliferative activity [5]. We isolated 14 known compounds (**3**–**16**) and 2 new mucic acid derivatives (**1** and **2**) during a study of phenolic constituents from *P. emblica* fruit and studied the antioxidant activity of the new compounds.



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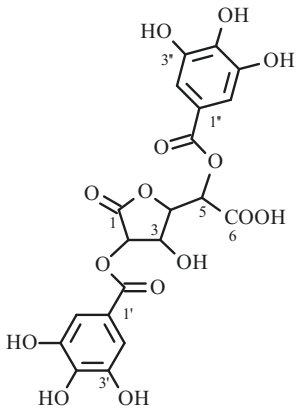

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Solvent extraction of the Me_2CO (60%) extract of *P. emblica* fruit followed by chromatographic separation of the obtained fractions over Amberlite® XAD1180N, Superlite™ DAX-8, Sepabeads® SP-20SS, Sephadex LH-20, Toyopearl® HW-75F, and RP-SiO_2 using preparative HPLC isolated 16 compounds. A comparison of the physicochemical and spectral data with those of standards and the literature identified the known compounds gallic acid (**3**), glucogallin (**4**), 1,6-di-*O*-galloyl-*β*-D-glucose (**5**), 3,4,6-tri-*O*-galloyl-*β*-D-glucose (**6**), mucic acid 2-*O*-gallate (**7**), mucic acid 2-*O*-gallate 1,4-lactone (**8**), mucic acid 5-*O*-gallate 1,4-lactone (**9**), chebulic acid (**10**), chebulanin (**11**), chebulagic acid (**12**), chebulinic acid (**13**), corilagin (**14**), ellagic acid (**15**), and isoquercitrin (**16**). Compounds **3**–**5** and **7**–**16** were detected earlier in *P. emblica* [3] whereas **6** was isolated for the first time from this species.

Compound **1** had the formula $C_{20}H_{18}O_{16}$ according to FAB-MS (*m*/*z* 513, $[M - H]^-$) and ^{13}C NMR spectroscopy. Gallic acid was identified in the hydrolysate after hydrolysis of **1** by tannase. Subsequent methylation of the product mixture by methyl iodide formed mucic acid dimethyl ester, which was identified using PMR and ^{13}C NMR data compared with those obtained after methylation of galactaric (mucic) acid. PMR and ^{13}C NMR data showed that **1** contained four carboxylic C atoms (δ 166.4, 167.0, 170.3, 170.8), four *O*-containing methines [δ_C 71.7, 72.1, 72.9, 74.5 and the corresponding resonances δ_H 4.81 (1H, dd, J = 2.0, 9.7), 4.39 (1H, dd, J = 2.1, 9.7), 5.60 (1H, d, J = 2.1), 5.87 (1H, d, J = 2.0], and two galloyl groups [δ_H 7.15 (2H, s), 7.27 (2H, s)]. The fragmentation pathway under FAB-MS conditions also confirmed that **1** contained two galloyl groups {*m*/*z* 361 $[(M - \text{galloyl}) - H]^-$ and 209 $[(M - \text{galloyl} \times 2) - H]^-$}. The HMBC spectrum exhibited correlations between methine proton doublets at δ_H 5.87 (C-2) and 5.60 (C-5) with galloyl carboxylic C resonances at δ_C 167.0 and 166.4, respectively. Thus, the results indicated that **1** had the structure mucic acid 2,5-di-*O*-gallate.

Compound **2** had the formula $C_{20}H_{16}O_{15}$ according to FAB-MS (*m*/*z* 495, $[M - H]^-$) and ^{13}C NMR spectroscopy. An analysis of the PMR and ^{13}C NMR spectra of **2** indicated that it contained the same functional groups as **1**, i.e., four carboxylic C atoms (δ 166.5, 167.1, 169.8, 171.5), four O-containing methines [δ_C 72.4, 73.4, 75.9, 79.5 and the corresponding resonances δ_H 4.96 (1H, dd, J = 9.0, 8.4), 5.69 (1H, d, J = 2.0), 6.02 (1H, d, J = 9.0), 5.03 (1H, dd, J = 2.0, 8.4)], and two galloyl groups [δ_H 7.19 (2H, s) and 7.32 (2H, s)]. The molecular ion in the FAB-MS of **2** gave a peak at *m*/*z* 495, i.e., 18 amu less than in **1**, despite the similarity of their compositions. This phenomenon could be explained by the fact that **2** was a lactone of **1**. Lactonization of hexanoic acids is known to be possible at the *γ* (1,4-lactone) and δ (1,5-lactone) positions [4]. According to HMBC spectra, the methine proton resonances at δ_H 6.02 (C-2) and 5.69 (C-5) correlated with galloyl carboxylic C resonances at δ_C 167.1 and 166.45, respectively. Thus, the C-5 position was occupied by galloyl, which indicated that the only possible configuration for the lactone ring was a 1,4-lactone. Moreover, PMR and ^{13}C NMR spectra displayed a strong weak-field shift for the H-4 and C-4 resonances relative to those in the spectrum of **1** ($\delta_{H\text{-}4}$ 4.39→5.03; $\delta_{C\text{-}4}$ 72.1→79.5), which was a consequence of forming the 1,4-lactone bond. The studies characterized the structure of **2** as mucic acid 2,5-di-*O*-gallate 1,4-lactone.

The compounds mucic acid 2-*O*-gallate; 2-*O*-, 3-*O*-, and 5-*O*-gallates 1,4-lactone; and 3,5-di-*O*-gallate 1,4-lactone were isolated earlier from *P. emblica* fruit [4]. Compounds **1** and **2** were new natural compounds.

Antioxidant activity of the mucic acid gallates and its 1,4-lactone was studied against the free radicals $DPPH^•$, $ABTS^{•+}$, and superoxide radical ($O_2^{•-}$) and also a model for protecting *β*-carotene from peroxidation (CBA). The study showed that the compounds were pronounced antioxidants (Table 1). The activities of monogallates **7**, **8**, and **9** were less than that of the reference compound gallic acid. The activities of **1** and **2** were similar ($O_2^{•-}$) or greater than that of gallic acid ($DPPH^•$, $ABTS^{•+}$, CBA).

According to HPLC, the contents of mucic acid derivatives in *P. emblica* fruit were 44.64–53.53 mg/g (Table 2). The dominant constituent was mucic acid 2-*O*-gallate (**7**), the fraction of which reached 82–89% of the total of all mucic acid derivatives. The studied raw material was characterized by high concentrations of gallic acid derivatives (42.77–70.24 mg/g), ellagotannins (31.95–42.75 mg/g), and ellagic acid (8.17–12.78 mg/g).

The high content of ascorbic acid in *P. emblica* fruit that was claimed earlier [6] was not confirmed by us. Chromatographic analysis for the concentration of this compound in *P. emblica* found only traces (<0.001 mg/g). Apparently, the cause of the erroneous claim was the similarity of the retention times for ascorbic acid and mucic acid gallates. Furthermore, we could not isolate 2-ketogluconolactone hexahydroxydiphenoyl derivatives emblicanins A and B, which were determined earlier as the dominant constituents in *P. emblica* ellagotannins [7]. Only chebuloyl ellagotannins **10**–**13** and corilagin (**14**) were detected in them. Therefore, we agree with the previous suggestion that the identification of emblicanins A and B was erroneous and that they were probably glucogallin (**4**) and one of the mucic acid gallates (**7** or **9**) [8].

TABLE 1. Antioxidant Activity of Mucic Acid Gallates and Its 1,4-Lactone, IC_{50}, μM (±SD)

Compound	DPPH$^{\cdot}$	ABTS$^{\cdot+}$	$O_2^{\cdot-}$	CBA
1	4.22 ± 0.10	0.94 ± 0.02	7.03 ± 0.25	14.73 ± 0.53
2	5.63 ± 0.16	1.12 ± 0.03	6.57 ± 0.22	16.67 ± 0.58
3*	7.47 ± 0.19	1.41 ± 0.04	6.97 ± 0.24	26.35 ± 0.89
7	10.16 ± 0.36	6.37 ± 0.19	22.67 ± 0.81	31.16 ± 1.06
8	12.67 ± 0.45	7.53 ± 0.22	18.37 ± 0.64	31.74 ± 1.07
9	12.84 ± 0.48	7.93 ± 0.25	18.50 ± 0.67	31.87 ± 1.09

*Reference compound.

TABLE 2. Quantitative Contents of Phenolic Compounds (PC) in *P. emblica* Fruit, mg/g (±SD)

Compound	Sample		
	PE-01	PE-02	PE-03
	Mucic acid derivatives (MAD)		
1	0.63 ± 0.02	0.60 ± 0.02	1.57 ± 0.03
2	0.38 ± 0.01	0.12 ± 0.01	0.42 ± 0.01
7	43.85 ± 0.96	38.12 ± 0.87	45.62 ± 1.14
8	3.62 ± 0.08	3.73 ± 0.10	2.67 ± 0.06
9	5.05 ± 0.12	2.07 ± 0.04	1.17 ± 0.03
	Gallic acid derivatives (GAD)		
3	43.84 ± 0.88	30.74 ± 0.76	40.78 ± 1.01
4	19.63 ± 0.37	8.63 ± 0.20	25.37 ± 0.53
5	Tr.*	0.57 ± 0.01	1.36 ± 0.03
6	3.67 ± 0.08	2.83 ± 0.05	2.73 ± 0.06
	Ellagotannins (EL)		
10	Tr.	Tr.	0.91 ± 0.02
11	12.67 ± 0.29	10.53 ± 0.25	14.52 ± 0.37
12	14.36 ± 0.32	9.67 ± 0.24	12.60 ± 0.35
13	2.75 ± 0.07	1.22 ± 0.03	3.15 ± 0.07
14	12.97 ± 0.31	10.53 ± 0.24	9.62 ± 0.23
	Other classes		
15	8.17 ± 0.22	12.78 ± 0.31	10.82 ± 0.27
Σ_{PC}	171.59	132.14	173.31
Σ_{MAD}	53.53	44.64	51.45
Σ_{GAD}	67.14	42.77	70.24
Σ_{EL}	42.75	31.95	40.80
Σother classes	8.17	12.78	10.82

*Tr.: traces (<0.05 mg/g).

EXPERIMENTAL

Plant Material. *P. emblica* fruit were acquired from commercial producers Bazaar of India, Herbal Remedies USA, LLC (USA, No. PE-01); Bliss Ayurveda (I) Pvt. Ltd. (India, No. PE-02); and NHCL Ltd. (India, No. PE-03).

Column chromatography (CC) used Amberlite® XAD1180N (Fluka), Superlite™ DAX-8 (Supelco), Sepabeads® SP-20SS (Supelco), Sephadex LH-20 (Pharmacia), Toyopearl® HW-75F (Supelco), and RP-SiO_2 (Sigma). Spectrophotometry was performed on an SF-2000 spectrophotometer (spectrum) and a Uniplan microplate spectrophotometer (Pikon). MS analysis used an MAT 8200 high-resolution mass spectrometer (Finnigan). NMR spectra were recorded on a VXR 500S NMR spectrometer (Varian).

Extraction and Fractionation. Ground *P. emblica* fruit (sample PE-01, 1 kg) was extracted with Me_2CO (60%, 1:15, 2×) in an ultrasonic bath at 50°C for 60 min. The resulting extract was concentrated in vacuo at 40°C and dried to afford

dry extract (462 g), a portion (400 g) of which was suspended in H_2O and fractionated using solvent extraction by hexane and *n*-BuOH. The BuOH fraction was concentrated to dryness (215 g), placed on a column with Amberlite® XAD1180N (800 g), and eluted with H_2O and EtOH (40 and 90%) to afford fractions PEXAD-01 (142 g), PEXAD-02 (48 g), and PEXAD-03 (19 g), respectively. Fraction PEXAD-01 (140 g) was placed on a column with Superlite™ DAX-8 (600 g) and eluted with H_2O to afford fraction PEXAD-01-1 (92 g), which was separated over a column of Sepabeads® SP-20SS (500 g) using an H_2O–Me_2CO gradient (100:0→40:60). Fractions eluted by H_2O and Me_2CO (10%) were placed on Sephadex LH-20 (CC, 2 × 90 cm, H_2O–Me_2CO eluent, 100:0→30:70) and on Toyopearl® HW-75F (CC, 500 mL, H_2O–Me_2CO eluent, 100:0→50:50) to isolate **1** (93 mg), **2** (54 mg), mucic acid 2-*O*-gallate (**7**, 437 mg), mucic acid 2-*O*-gallate 1,4-lactone (**8**, 118 mg), mucic acid 5-*O*-gallate 1,4-lactone (**9**, 127 mg) [4], gallic acid (**3**, 10.47 g) [9], glucogallin (**4**, 1-*O*-galloyl-*β*-D-glucose, 1.64 g), 1,6-di-*O*-galloyl-*β*-D-glucose (**5**, 14 mg), and 3,4,6-tri-*O*-galloyl-*β*-D-glucose (**6**, 52 mg) [10]. Fraction PEXAD-02 (40 g) was separated over Sephadex LH-20 (CC, 2 × 50 cm, H_2O–Me_2CO eluent, 100:0→30:70) and RP-SiO_2 (CC, 2 × 40 cm, H_2O–MeCN eluent, 100:0→0:100) and by preparative HPLC (conditions 1) to isolate chebulic acid (**10**, 29 mg), chebulanin (**11**, 118 mg), chebulagic acid (**12**, 153 mg), chebulinic acid (**13**, 34 mg) [11], corilagin (**14**, 37 mg) [12], ellagic acid (**15**, 573 mg) [13], and isoquercitrin (**16**, 9 mg) [14]. Fraction PEXAD-03 contained polymeric phenolic constituents (probably procyanidins) and was not investigated further.

Mucic Acid 2,5-Di-*O*-gallate (1). $C_{20}H_{18}O_{16}$. UV spectrum (MeOH, λ_{max}, nm): 209, 275. FAB-MC, *m*/*z*: 513 $[M - H]^-$, 361 $[(M - galloyl) - H]^-$, 209 $[(M - galloyl \times 2) - H]^-$, 191 $[(M - galloyl \times 2 - H_2O) - H]^-$. ^{1}H NMR spectrum (500 MHz, MeOH-d_4, δ, ppm, J/Hz): mucoyl: 4.39 (1H, dd, J = 2.1, 9.7, H-4), 4.81 (1H, dd, J = 2.0, 9.7, H-3), 5.60 (1H, d, J = 2.1, H-5), 5.87 (1H, d, J = 2.0, H-2); 2-*O*-galloyl: 7.15 (2H, s, H-2′, 6′); 5-*O*-galloyl: 7.27 (2H, s, H-2″, 6″). ^{13}C NMR spectrum (125 MHz, MeOH-d_4, δ, ppm): mucoyl: 71.7 (C-3), 72.1 (C-4), 72.9 (C-5), 74.5 (C-2), 170.3 (C-6), 170.8 (C-1); 2-*O*-galloyl: 110.2 (C-2′, 6′), 121.0 (C-1′), 139.7 (C-4′), 146.3 (C-3′, 5′), 167.0 (C-7′); 5-*O*-galloyl: 110.2 (C-2″, 6″), 121.5 (C-1″), 139.3 (C-4″), 146.0 (C-3″, 5″), 166.4 (C-7″).

Hydrolysis of 1. A solution of **1** (10 mg) in H_2O (1 mL) was incubated with tannase (10 U, from *Aspergillus ficuum*, Sigma, 150 U/g) at 37°C for 10 h. The resulting mixture was concentrated to dryness in vacuo, dissolved in MeOH (1 mL), and analyzed by HPLC (conditions 1). Gallic acid (t_R 3.12 min) was detected in the hydrolysate. Then, the solution was treated with MeI—Na_2CO_3 (0.5 mL/200 μg) at 25°C for 30 min. The reaction mixture was placed on an SiO_2 column (1 × 15 cm) and eluted by $CHCl_3$–MeOH (100:0→50:50) to afford mucic acid dimethyl ester (2.5 mg) that was identified by PMR and ^{13}C NMR data and comparison with a sample prepared by methylation of mucic acid (Aldrich) in an analogous manner.

Mucic Acid Dimethyl Ester. ^{1}H NMR spectrum (500 MHz, Py-d_5, δ, ppm): 3.61 (6H, s, OC$\underline{H}_3$), 5.12 (2H, s, H-2, 5), 5.44 (2H, s, H-3, 4). ^{13}C NMR spectrum (125 MHz, Py-d_5, δ, ppm): 49.6 (O$\underline{C}H_3$), 70.4 (C-2, 5), 71.9 (C-3, 4).

Mucic Acid 2,5-Di-*O*-gallate 1,4-Lactone (2). $C_{20}H_{16}O_{15}$. UV spectrum (MeOH, λ_{max}, nm): 210, 274. FAB-MC, *m*/*z*: 495 $[M - H]^-$, 343 $[(M - galloyl) - H]^-$. ^{1}H NMR spectrum (500 MHz, MeOH-d_4, δ, ppm, J/Hz): mucoyl: 4.96 (1H, dd, J = 9.0, 8.4, H-3), 5.03 (1H, dd, J = 2.0, 8.4, H-4), 5.69 (1H, d, J = 2.0, H-5), 6.02 (1H, d, J = 9.0, H-2); 2-*O*-galloyl: 7.19 (2H, s, H-2′, 6′); 5-*O*-galloyl: 7.32 (2H, s, H-2″, 6″). ^{13}C NMR spectrum (125 MHz, MeOH-d_4, δ, ppm): mucoyl: 72.4 (C-3), 73.4 (C-5), 75.9 (C-2), 79.5 (C-4), 169.8 (C-6), 171.5 (C-1); 2-*O*-galloyl: 110.4 (C-2′, 6′), 121.0 (C-1′), 139.5 (C-4′), 146.5 (C-3′, 5′), 167.1 (C-7′); 5-*O*-galloyl: 110.4 (C-2″, 6″), 121.4 (C-1″), 139.1 (C-4″), 146.2 (C-3″, 5″), 166.5 (C-7″).

HPLC. Conditions 1: Summit liquid chromatograph (Dionex), LiChrospher PR-18 column (10 × 250 mm, ∅ 10 μm, Merck), mobile phase H_2O (A) and MeCN (B), gradient mode (% B): 0–100 min, 0–70%; flow rate 2 mL/min, column temperature 30°C, UV detector at λ 280 nm. Conditions 2: A-02 Milikhrom microcolumn liquid chromatograph (Ekonova), ProntoSIL-120-5-C18AQ column (2 × 75 mm, ∅ 5 μm, Metrohm AG), mobile phase $LiClO_4$ (0.2 M) in $HClO_4$ (0.01 M) (A) and MeCN (B), gradient mode (% B): 0–10 min, 5–15, flow rate 150 μL/min, column temperature 35°C, UV detector at λ 274 nm.

Plant material was quantitatively analyzed on an HPLC-UV microcolumn. For this, plant material (40 mg) was transferred to an Eppendorf tube (2 mL), treated with EtOH (60%, 1 mL), sonicated (50 kHz, 30 min, 40°C), and centrifuged (6,000 g, 20 min). The resulting extract was filtered through a membrane filter (0.45 μm) and used for the analysis (1 μL). Conditions: A-02 Milikhrom microcolumn liquid chromatograph (Ekonova), ProntoSIL-120-5-C18AQ column (2 × 75 mm, ∅ 5 μm, Metrohm AG), mobile phase $LiClO_4$ (0.2 M) in $HClO_4$ (0.006 M) (A) and MeCN (B), gradient mode (% B): (0–5 min, 2–5; 5–15 min, 5–7; 15–20 min, 7–15; 20–30 min, 15–30; 30–35 min, 30–50), flow rate 150 μL/min, column temperature 35°C, UV detector at λ 254 nm.

The contents of pure constituents were calculated from calibration curves that were constructed using commercial standards (gallic acid, ellagic acid, corilagin, all Sigma-Aldrich), isolated compounds ≥95% pure (glucogallin, 1,6-di-*O*-galloyl-*β*-D-glucose, 3,4,6-tri-*O*-galloyl-*β*-D-glucose, chebulic acid, chebulanin, chebulagic acid, chebulinic acid, mucic acid

2-*O*-gallate, mucic acid 5-*O*-gallate 1,4-lactone), and external reference samples taking into account the differences in the molecular weights (mucic acid 2,5-di-*O*-gallate for mucic acid 2-*O*-gallate and mucic acid 2-*O*-gallate 1,4-lactone and mucic acid 2,5-di-*O*-gallate 1,4-lactone for mucic acid 5-*O*-gallate 1,4-lactone).

Antioxidant Activity. Antiradical activity against the radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and the cation radical 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was determined using a microplate spectrophotometric method [15]. Superoxide radical ($O_2^{\bullet-}$) binding was studied by spectrophotometry using phenazine methosulfate:NAD:nitroblue tetrazolium [16]. The effect of the compounds on peroxidation of β-carotene (CBA) in DMSO:H_2O_2:linoleic acid was determined by the literature method [9].

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