SUPPLEMENTARY MATERIAL

Phytochemical analysis and antioxidant potential of the leaves of *Garcinia travancorica* Bedd.

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Phytochemical analysis of the leaves of *Garcinia travancorica*, a hitherto uninvestigated endemic species to the Western Ghats of south India, resulted in isolation and characterisation of the polyisoprenylated benzophenones 7-epi-nemorosone (**1**) and garcinol (**2**) along with the biflavonoids GB-1a (**3**), GB-1 (**4**), GB-2 (**5**), morelloflavone (**6**) and morelloflavone-7''-O- β -D-glycoside or fukugiside (**7**). The compounds were identified using various spectroscopic techniques, mainly through NMR and MS. The methanol extract and the biflavonoids **3**, **4**, **5** and **7** showed potential *in vitro* antioxidant activities. The IC₅₀ value of DPPH radical scavenging activity of compound **7** was 8.34 $\pm$ 2.12 μ g/ml, comparable to standard ascorbic acid (3.2 $\pm$ 0.50 μ g/ml). In superoxide radical scavenging assay, compound **7** gave IC₅₀ value of 6.95 $\pm$ 1.33 μ g/ml close to standard ascorbic acid with IC₅₀ value of 5.8 $\pm$ 0.25 μ g/ml. Validated HPTLC estimation revealed *G. travancorica* as a rich source of morelloflavone-7''-O- β -D-glycoside (7.12% dry wt. leaves).

Key words: *Garcinia travancorica*, fukugiside, garcinol, HPTLC, antioxidant

Experimental

General experimental procedures

UV spectra were recorded on a Shimadzu spectrophotometer -UV 1800, Japan. IR spectra were taken with Alpha FT-IR, Bruker Optics. ¹H and ¹³C NMR spectra were recorded on a Bruker-Avance 400 MHz FT-NMR spectrometer operating at 400 MHz for ¹H NMR and 100MHz for ¹³C NMR. The chemical shifts were expressed as δ (ppm, parts per million) referring to internal standard, tetramethyl- silane (Me₄Si). Mass spectra were recorded using JEOL JMS 600 H mass spectrometer. LC-MS/MS analysis was carried out with Acquity H class (Waters) UPLC; Xevo G2 (Waters) Q-ToF.

Plant Material

Fresh leaves of the tree were collected from Chemunji forest area of South Western Ghats, Thiruvananthapuram district, Kerala, India during March 2011 and a voucher specimen (No. 66417) was deposited at the JNTBGRI Herbarium (TBGT).

Extraction and isolation of chemical constituents

Dried and finely powdered leaves of *G. travancorica* (1.0 kg) were extracted with hexane followed by methanol using Soxhlet apparatus. Removal of the solvent *in vacuo* at 45°C yielded 15g hexane extract and 67g methanol extract respectively. The hexane extract (8g), subjected to column chromatography over silica gel (60-120 mesh, 250 g) using gradient elution with hexane, ethylacetate and methanol, yielded the compounds **1** and **2**. Compound **1** was further purified using the isocratic solvent system hexane: ethylacetate (9:1) through silica gel (100-200 mesh) column chromatography (yield: 45 mg). Final purification of compound **2** was achieved by preparative TLC using the solvent system hexane: ethylacetate (7:3) to yield 35 mg of pure compound. The methanol extract (20 g) was subjected to column chromatography over silica gel (60-120 mesh, 750 g) using gradient elution with hexane, ethylacetate and methanol. Hexane: ethylacetate (3:7) gave mixture of three compounds, and further purification using column chromatography with isocratic elution using hexane: ethylacetate (3:7) yielded compound **3** (80 mg), compound **4** (220 mg) and compound **5** (150 mg) in pure form. Isolation at 70% ethyl acetate yielded compound **6** and 100% ethylacetate gave compound **7** (800 mg).

HPTLC estimation

Silica gel HPTLC plates (Kieselgel 60 F 254, 20 cm × 20 cm, 0.2 mm thickness, Merck, Germany) were washed with methanol before use and kept at 60°C for 30 min for the analysis. Compounds **5**, **7** and *G. travancorica* leaf extract stock solutions were made in methanol. The samples at different concentration were spotted by means of Camag Linomat V fitted with a Hamilton microlitre syringe. The plates were developed using hexane-ethyl acetate solvent system (30:70 v/v) for compound **5** and ethylacetate- methanol-formic acid (80:17.5:2.5) for compound **7** in the CAMAG twin-trough glass chamber, previously saturated with the solvent for 30 minutes. The mobile phase compositions were chosen after testing different solvent systems of varying polarity. After development, the plates were dried in an air oven at 50°C and scanned using a TLC Scanner 3 (Camag) in absorbance-reflectance mode. Compound **5** (R_f 0.30) was scanned at 254 nm and compound **7** (R_f 0.35) at 580nm. Various concentrations of isolated compounds and extracts were analyzed in triplicate. Amounts of **5** and **7** were determined by means of calibration plots obtained through concentrations against peak area. Data processing was performed with WinCATS planar chromatography manager software (version 1.43).

Total phenolic content

Modified Folins Ciocalteu's reagent colorimetric method was used for the determination of total phenolics (Mc Donald et al. 2001). 0.5 ml methanol extracts of *G. travancorica* was mixed with 5ml Folins Ciocalteu's reagent (1:10 dilution with distilled water). After 5 min, 4 ml of 20% sodium carbonate solution was added. The mixture was allowed to stand for 15 minutes and the absorbance was measured at 765nm. Total phenolics were estimated as gallic acid equivalent (GAE) mg/g dry weight from the calibration curve using standard gallic acid (10 µg-50 µg) in methanol. Experiments were done in triplicate and results were expressed as Mean±SD.

Total flavonoid content

Aluminium chloride colorimetric method was used for total flavonoids determination (Chang et al. 2002). 0.5 ml of methanol extract of *G. travancorica* was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and left at room temperature for 30 minutes. Absorbance of the reaction mixture was measured at 415 nm. Total flavonoids were estimated from the calibration curve using standard quercetin (10 µg-50 µg) in methanol and the results are expressed as quercetin equivalent. Experiments were done in triplicate and results were expressed as Mean±SD.

Antioxidant activity studies

DPPH radical scavenging assay

Radical scavenging activity was determined by spectrophotometric method based on the reduction of the stable free radical 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) (Kabouche . A et al, 2007). Different concentrations of leaf methanolic extract and isolated compounds were added at an equal volume to a methanolic solution of DPPH (200µM DPPH). After 15 min at room temperature, the absorbance was recorded at 517 nm. The activity was measured as percentage, calculated using the formula: % scavenging = $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$. The activity was reported as IC₅₀ value; the concentration of sample required scavenging 50% of DPPH radical, relative to the control (DPPH without antioxidant agents added). Ascorbic acid was used as positive control. Experiments were done in triplicate and the results were expressed as mean value with standard deviation.

Superoxide scavenging assay

The method depends on the light induced superoxide generation by riboflavin and the corresponding reduction of nitroblue tetrazolium (McCord 1969). The reaction mixture contained 200 µl ethylenediamine tetraacetic acid (EDTA; 6 µM, containing 1.5 mg NaCN/100 ml), 50 µl riboflavin (2 µM), 100 µl nitroblue tetrazolium (50 µM), 100 µl various concentrations of the extract and isolated compounds and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The reaction mixture was uniformly illuminated with an incandescent visible light for 15 minutes. The absorbance was measured at 560 nm. Reagents omitting the test sample were used as a control and the blank contained all the reagents except riboflavin. The activity was measured as percentage scavenging of superoxide radical using the formula: $[(A_{\text{control}} - (A_{\text{sample}} - A_{\text{sample blank}})) / A_{\text{control}}] \times 100$. The activity was reported as IC₅₀ value, the concentration of sample that produced 50% inhibition of superoxide radical. Ascorbic acid was used as positive control. Experiments were done in triplicate and the results were expressed as mean value with standard deviation.

Reducing power Assay

Reducing power was determined according to the method previously described by Oyaizu (Oyaizu, 1986). The presence of antioxidants causes the reduction of the yellow coloured Fe³⁺/ferricyanide complex to blue coloured ferrous form and the absorbance at 700 nm can monitor the Fe²⁺ concentration. Various concentrations of the leaf methanol extract and isolated compounds in methanol (1.0 ml) were mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide (1 ml) and incubated at 50°C for 20 min. Aliquots of 10% trichloroacetic acid (1ml) were added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the centrifuged solution (1 ml) was mixed with distilled water (1 ml) and freshly prepared 0.1% ferric chloride solution (0.25 ml) and the absorbance was measured at 700 nm. A blank was prepared without adding extract. Ascorbic acid was used as standard. Increased absorbance of the reaction mixture at 700 nm indicates increase in reducing power. The assays were done in triplicate and the results were expressed as mean value with standard deviation. Experiments were done in triplicate and the results were expressed as mean value with standard deviation.

Structural elucidation of secondary metabolites

7-epi-nemorosone (1), yellow oil. UV (CH₃Cl, 0.1%) λ_{max}/nm: 281, 265. HRMS m/z- 501.3018 (M-H)⁻ for C₃₃H₄₁O₄ (calcd. 501.3005); MSⁿ experiment (m/z) 501.3, 432.2, 417.2, 363.2, 309.1, 242.0, 145.0. ¹H NMR (CDCl₃, 400 MHz, δ ppm) δ 2.09 (H-6a, m); 2.11 (H-

6b, m); 1.52 (H-7, m); 7.55 (H-12, dd, $J = 7.6$ and 1); 7.38 (H-13, t, $J = 7.6$); 7.39 (H-14, t, $J = 7.6$); 7.37 (H-15, t, $J = 7.6$); 7.54 (H-16, d, $J = 7.6$); 2.72 (H-17a, overlapped); 2.72 (H-17b, overlapped); 5.01 (H-18, m); 1.70 (3H, s, $\text{CH}_3 = 20$); 1.70 (3H, s, $\text{CH}_3 = 21$); 2.54 (H-22a, m); 2.55 (H-22b, m); 5.04 (H-23, m); 1.54 (3H, s, $\text{CH}_3 = 25$); 1.99 (H-27a, m); 2.16 (H-27b, m); 4.90 (H-28, m); 1.60 (3H, overlapped, $\text{CH}_3 = 30$); 1.64 (3H, overlapped, $\text{CH}_3 = 31$); 1.51 (3H, s, $\text{CH}_3 = 32$); 1.25 (3H, s, $\text{CH}_3 = 33$). ^{13}C NMR (100 MHz, δ ppm) δ 73.0 (C1); 192.6 (C2); 120.4 (C3); 193.9 (C4); 64.6 (C5); 41.5 (C6); 47.6 (C7); 48.6 (C8); 207.5 (C9); 197.5 (C10); 137.3 (C-11); 128.9 (C-12), 127.8 (C-13); 132.5 (C-14); 127.7 (C-15); 128.8 (C-16); 23.7 (C-17); 120.4 (C-18); 134.5 (C-19); 17.9 (C-20); 25.8 (C-21); 30.2 (C-22); 119.9 (C-23); 133.3 (C-24); 18.1 (C-25); 25.6 (C-26); 29.7 (C-27); 123.3 (C-28); 132.5 (C-29); 18.1 (C-30); 26.1 (C-31); 26.7 (C-32); 23.7 (C-33).

Garcinol (2), pale yellow crystals. UV (CH_3Cl , 0.1%) λ_{max} (nm) 306, 244. IR 3200–3400, 1727, 1562 cm^{-1} , HRMS (m/z) 603.3681 ($\text{M} + \text{H}$) $^+$ for $\text{C}_{38}\text{H}_{51}\text{O}_6$ (calcd. 603.3686); MS^n experiment (m/z) 603.3, 467.2, 411.1, 343.1, 287.0, 233.0, 177.0, 137.1, 95.0; ^1H NMR (400 MHz, MeOD, δ ppm) ^1H NMR spectrum of compound **2** in MeOD showed the presence of three aromatic protons characteristic of 3,4-dihydroxybenzoyl group at δ 7.05, 6.71, 6.69 (d; $J = 8$ Hz), three isopropylidene groups (a broad triplet of 3H at 4.9 and 6 Me groups at 1.58 and 1.68), one isopropenyl group (a broad singlet of 2H at 4.51 together with a Me signal around 1.68), and two methyl groups on a saturated carbon (two singlets of 3H each at 0.97 and 1.17), in addition to methylene and methine protons (a complex multiplet in the regions 1.4 to 2.7). ^{13}C NMR (100 MHz, δ ppm) spectrum of compound **2** showed the presence of three methine carbons of trisubstituted olefinic groups at δ 124.4, 124.6 and 122.6 and a terminal methylene carbon at δ 112.0. Other ^{13}C assignments were δ 206.2 (C-9, C=O), 194.0 (C-2, C=O), 195.1 (C-4, C-OH), 199.0 (C-15, C=O); 131.5 (C-12, CMe_2), 132.3 (C-34, CMe_2), 134.0 (C-26, CMe_2); 149.8 (C-28, C (Me) = CH_2), δ 116.6 (C-17, Ar-CH), 149.8 (C-20, Ar-CH), 122.5 (C-21, Ar-CH); 145.2 (C-18, Ar-C-OH), 132.5 (C-19, Ar-C-OH); 126.3 (C-16, Ar-C-C=O); 116.9 (C-3), 68.6 (C-1), 48.8 (C-8), 47.9 (C-7), 59.9 (C-5), 43.0 (C-6, 23); 26.8, 27.4, 32.9, 37.4, 43.0 (5 CH_2); 18.1, 18.3, 18.7, 25.9, 26.3 (6 Me, C = CMe); 23.3 (C(Me) = CH_2); 17.6 and 26.7 (ring CMe_2).

GB-1a (3), yellow crystalline solid. UV (MeOH, 0.1%) λ_{max} /nm: 289, 207. IR: 3227, 1598, 1515, 1158, 1084, 830 cm^{-1} . HRMS (m/z) 543.1264 [$\text{M} + \text{H}$] $^+$ for $\text{C}_{30}\text{H}_{23}\text{O}_{10}$ (calcd. 543.1291). ^1H NMR (MeOD, 400 MHz, δ -ppm): δ 5.42 (1H, d, $J = 11.2$ Hz, H-2), 5.2 (1H, d, $J = 12$ Hz, H-3), 5.91 (1H, d, $J = 2$ Hz, H-6), 5.72 (1H, d, $J = 2$ Hz, H-8), 7.05 (2H, d, $J = 8.4$ Hz,

H-2',6'), 6.61 (2H, d, J=8.4 Hz, H-3',5'), 5.32 (1H, d, J=12 Hz, H-2''), 2.67 (2H, m, H-3''), 5.76 (1H, s, H-6''), 7.07 (2H, d, J=8.4 Hz, H-2''',6'''), 6.62 (2H, d, J=8.4 Hz, H-3''',5'''). ¹³C NMR (100 MHz, δ-ppm): δ 80.5 (C-2), 48.4 (C-3), 197.0 (C-4), 163.0 (C-5), 96.6 (C-6), 164.8 (C-7), 96.2 (C-8), 165.6 (C-9), 103.2 (C-10), 129.0 (C-1'), 127.9 (C-2'/6'), 115.7 (C-3'/5'), 158.7 (C-4'), 83.7 (C-2''), 44.0 (C-3''), 197.0 (C-4''), 164.8 (C-5''), 97.3 (C-6''), 168.0 (C-7''), 102.3 (C-8), 165.6 (C-9), 102.3 (C-10''), 83.7 (C-1'''), 129.8 (C-2'''/6'''), 116.3 (C-3'''/5'''), 158.7 (C-4''').

GB-1(4), Yellow crystalline solid. UV (MeOH, 0.1%) λ_{max}/nm: 290, 211. IR: 3200, 1595, 1515, 1155, 1083, 828 cm⁻¹. HRMS (m/z) 559.1221 [M + H]⁺ for C₃₀H₂₃O₁₁ (Calcd. 559.1240) and 581.1043 [M + Na]⁺. ¹H NMR (MeOD, 400 MHz, δ-ppm): δ 5.66 (1H, d, J=12 Hz, H-2), 3.31 (1H, s, H-3), 5.90 (1H, d, J=2 Hz, H-6), 5.97 (1H, m, H-8), 7.15 (2H, d, J=8 Hz, H-2',6'), 6.61 (2H, d, J=8 Hz, H-3',5'), 4.50 (1H, m, H-2''), 4.07 (2H, m, H-3''), 6.04 (1H, s, H-6''), 7.17 (2H, d, J=8 Hz, H-2''',6'''), 6.67 (2H, m, H-3''',5'''). ¹³C NMR (100 MHz, δ-ppm): δ 79.5 (C-2), 49.1 (C-3), 196.0 (C-4), 164.9 (C-5), 97.2 (C-6), 165.1 (C-7), 98.4 (C-8), 105.7 (C-9), 103.2 (C-10), 129.4 (C-1'), 124.0 (C-2'/6'), 115.7 (C-3'/5'), 158.7 (C-4'), 82.8 (C-2''), 71.0 (C-3''), 196.0 (C-4''), 165.7 (C-5''), 98.9 (C-6''), 165.8 (C-7''), 102.0 (C-8''), 168.8 (C-9''), 103.3 (C-10'') 129.9 (C-1'''), 129.9 (C-2'''/6'''), 116.1 (C-3'''/5'''), 158.7 (C-4''').

GB-2 (5), Yellow crystalline solid. UV (MeOH, 0.1%) λ_{max}/nm: 291, 207. IR: 3226, 1736, 1633, 1516, 1159, 1083, 830 cm⁻¹. HRMS (m/z) 575.1175 [M + H]⁺ for C₃₀H₂₃O₁₂ (calcd. 575.1189) and 597.0993 [M + Na]⁺. ¹H NMR (DMSO-d₆, 400 MHz, δ-ppm): δ 5.35 (1H, d, J=12 Hz, H-2), 4.48 (1H, d, J=12 Hz, H-3), 5.89 (1H, d, J=2 Hz, H-6), 5.77 (1H, d, J=2, H-8), 7.11 (2H, d, J=2 Hz, H-2',6'), 6.65 (2H, d, J=8 Hz, H-3',5'), 12.14 (1H, s, Chelated OH), 4.67 (1H, d, J=12, H-2''), 3.97 (2H, d, J=11, H-3''), 5.93 (1H, s, H-6''), 6.85 (1H, s, H-2'''), 6.81 (2H, d, J=8, H-5'''), 6.79 (1H, d, J=8, H-6'''), 11.7 (1H, s, Chelated OH). ¹³C NMR (100 MHz, δ-ppm): δ 79.1 (C-2), 47.0 (C-3), 196.4 (C-4), 160.1 (C-5), 94.9 (C-6), 160.7 (C-7), 96.0 (C-8), 162.7 (C-9), 100.9 (C-10), 127.8 (C-1'), 128.0 (C-2'/6'), 115.3 (C-3'/5'), 157.7 (C-4'), 82.7 (C-2''), 71.9 (C-3''), 197.5 (C-4''), 162.0(C-5''), 96.0 (C-6''), 166.3 (C-7''), 101.2 (C-8''), 163.5 (C-9''), 106.0 (C-10'') 127.8 (C-1'''), 118.4 (C-2'''/5'''), 144.9 (C-3'''), 145.0 (C-4'''), 128.2 (C-6''').

Morelloflavone (6), Yellow crystalline solid. UV (MeOH, 0.1%) λ_{max}/nm: 376, 288. IR: 3348, 1557, 1410, 1269, 1167, 619 cm⁻¹. ¹H NMR (MEOD, 400 MHz, δ-ppm): δ 5.35 (1H, d,

J=12 Hz, H-2), 4.48 (1H, d, J=12 Hz, H-3), 5.89 (1H, d, J=2 Hz, H-6), 5.77 (1H, d, J=2, H-8), 7.11 (2H, d, J=2 Hz, H-2',6'), 6.65 (2H, d, J=8 Hz, H-3',5'), 4.67 (1H, d, J=12, H-2''), 3.97 (2H, d, J=11, H-3''), 5.93 (1H, s, H-6''), 6.85 (1H, s, H-2'''), 6.81 (2H, d, J=8, H-5'''), 6.79 (1H, d, J=8, H-6'''). ¹³C NMR (100 MHz, δ-ppm): δ 80.9 (C-2), 49.9 (C-3), 196.3 (C-4), 163.7 (C-5), 96.2 (C-6), 166.4 (C-7), 95.2 (C-8), 162.1 (C-9), 101.5 (C-10), 128.0 (C-1'), 128.4 (C-2'), 114.4 (C-3'), 157.2 (C-4'), 114.4 (C-5'), 128.4 (C-6'), 162.8 (C-2''), 102.4 (C-3''), 179.5 (C-4''), 159.7 (C-5''), 97.9 (C-6''), 161.3 (C-7''), 100.0 (C-8''), 154.0 (C-9''), 103.0 (C-10''), 121.6 (C-1'''), 114.6 (C-2'''), 145.0 (C-3'''), 147.6 (C-4'''), 116.2 (C-5'''), 120.3 (C-6''').

Morelloflavone-7''-O-β-D-glycoside (7), Yellow crystalline solid, $\alpha_D^{29} + 46.49$ (c. 1% MeOH), UV (MeOH, 0.1%) $\lambda_{\text{max/nm}}$: 377, 288. IR: 3252, 1738, 1593, 1364, 1069, 1083, 824 cm⁻¹. ¹H NMR (DMSO- d₆, 400 MHz, δ-ppm): δ 5.80 (1H, d, J=12 Hz, H-2), 4.91 (1H, d, J=12 Hz, H-3), 5.94 (1H, d, J=4.6 Hz, H-6), 5.96 (1H, d, J=4, H-8), 7.17 (2H, d, J=8.4 Hz, H-2',6'), 6.53 (2H, d, J=8.4 Hz, H-3',5'), 12.65 (1OH, s, OH-5) 6.47 (1H, s, H-3''), 6.73 (2H, s, H-3''), 7.25 (1H, s, H-2'''), 6.93 (1H, d, J=8.4, H-5'''), 7.59 (1H, d, J=8, H-6'''), 5.15 (1H, d, J=8, H-1'''), 3.3-3.8 (5H, m, H-2''',3''',4''',5''',6'''), 12.08 (1OH, s, OH-5''). ¹³C NMR (100 MHz, δ-ppm): δ 82.5 (C-2), 50.7 (C-3), 195.0 (C-4), 164.5 (C-5), 96.5 (C-6), 165.7 (C-7), 97.7 (C-8), 167.0 (C-9), 103.5 (C-10), 130.3 (C-1'), 129.6 (C-2'/6'), 115.5 (C-3'/5'), 158.0 (C-4'), 165.8 (C-2''), 103.5 (C-3''), 182.0 (C-4''), 162.0 (C-5''), 100.0 (C-6''), 161.2 (C-7''), 103.5 (C-8''), 155.0 (C-9''), 106.4 (C-10'') 123.7 (C-1'''), 114.9 (C-2'''), 146.0 (C-3'''), 152.5 (C-4'''), 114.9 (C-5'''), 120.6 (C-6'''), 101.6 (C-1'''), 76.1 (C-2'''), 77.5 (C-3'''), 69.6 (C-4'''), 79.1 (C-5'''), 60.9 (C-6''').

Figure S1: LC-MS/MS analysis of 7-epi-nemorosone

Figure S2: LC-MS/MS analysis of garcinol

Figure S3: HPTLC derivatised plate (A) and densitogram (B) of *Garcinia travancorica* leaf methanol extract (tracks 6,7 and 8) and standard fukugiside (0.5 to 1.5 μ g, tracks 1,2,3,4 and 5) at 580nm.

Figure S4: IC_{50} values of DPPH and superoxide radicals scavenging assay (**3**-GB-1a, **4**- GB-1, **5**- GB-2, **7**- Fukugiside, **GTL**- *G. travancorica* leaf methanol extract, **ASA**- standard ascorbic acid)

Figure S5: Reducing power assay (**3**-GB-1a, **4**- GB-1, **5**- GB-2, **7**- Fukugiside, **GTL**- *G. travancorica* leaf methanol extract, **ASA**- standard ascorbic acid)

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