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Isolation and Quantitative Analysis of Phenolic Antioxidants, Free Sugars, and Polyols from Mango (*Mangifera indica* L.) Stem Bark Aqueous Decoction Used in Cuba as a Nutritional Supplement

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An aqueous decoction of mango (*Mangifera indica* L.) stem bark has been developed in Cuba on an industrial scale to be used as a nutritional supplement, cosmetic, and phytomedicine. Previously we reported its antioxidant activity, and we concluded that the product could be useful to prevent the production of reactive oxygen species and oxidative tissue damage in vivo. A phytochemical investigation of mango stem bark extract has led to the isolation of seven phenolic constituents: gallic acid, 3,4-dihydroxy benzoic acid, gallic acid methyl ester, gallic acid propyl ester, mangiferin, (+)-catechin, (−)-epicatechin, and benzoic acid and benzoic acid propyl ester. All structures were elucidated by ES−MS and NMR spectroscopic methods. Quantitative analysis of the compounds has been performed by HPLC, and mangiferin was found to be the predominant component. Total polyphenols were assayed also by the Folin−Ciocalteu method. The free sugars and polyols content was also determined by GC−MS.

KEYWORDS: ***Mangifera indica* L.; nutritional supplement; phytomedicine; phenolic constituents; free sugars; polyols; antioxidant activity; 1D and 2D NMR; HPLC; GC−MS**

INTRODUCTION

Mango (*Mangifera indica* L.), which belongs to the family Anacardiaceae, is widely found in Cuba and in many other tropical and sub-tropical regions, and is one of the most popular edible fruits in the world. Mango stem bark has been traditionally used for the treatment of menorrhagia, scabies, diarrhea, syphilis, diabetes, cutaneous infections, and anemia, using an aqueous extract obtained by decoction as reported in the Napralert Data Base (*1*). An extract of this plant has been developed in Cuba on an industrial scale to be used as a nutritional supplement, cosmetic, and phytomedicine (*2*). The industrial extract obtained by decoction and drying of mango stem bark is a homogeneous brown powder which melts with decomposition from 215 to 218 °C and has a particle size of 30−60 μm. Previously, we compared the protective abilities of the following at the indicated concentrations: extract, 50−250 mg/kg; mangiferin, 50 mg/kg; vitamin C, 100 mg/kg; vitamin E, 100 mg/kg; and vitamin C + E, 100 mg/kg each; and β-carotene, 50 mg/kg body weight, respectively, in mice, against the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative damage in serum, liver, and brain, as well as in the hyperproduction of reactive oxygen species (ROS) by peritoneal macrophages. We concluded that mango stem bark extract could be useful to prevent the production of ROS and oxidative tissue damage in vivo, and it is more active than vitamin C, vitamin E, mangiferin, and β-carotene (*3*).

In this study, we report the isolation, structure characterization, and HPLC quantitative determination of phenolic constituents from the *n*-BuOH soluble portion of *Mangifera indica* stem bark. In the past few years there has been an increased interest in the study of mango phenolics, due to their antioxidant activity and health-promoting properties that make consumption of fruits and derived processed products from fruits, seed kernel, and stem bark a very healthy habit. Previously, alkylgallates were isolated from the blossoms of *M. indica* (*4*), whereas galloyl, hydroxybenzoyl esters, and epicatechin were isolated from the leaves (*5*). Saleh et al. (*6*) reported the polyphenolic composition of leaves, twigs, fruits, and seeds. The phenolic composition of mango stem bark is reported here for the first time. Quantitative determination of phenolics was essential for estimating their content in the extract and for quality control

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purposes. Quantitative evaluation of free sugars and polyols from the water-soluble fraction was also performed by GC−MS.

MATERIAL AND METHODS

Chemicals. All of the pure standards were purchased from Sigma Chemicals (Milan, Italy). All organic solvents were products of Carlo Erba, Milano (Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

Plant Material. The stem bark of *Mangifera indica* L. (Anacardiaceae) was collected from plants grown in a fruit farm (Alquizar, Havana, January, 1998) without affecting the ecosystem. Bark was collected free of microbial contamination and subsequently dried and milled to obtain particles of around 5 cm. The industrial extract obtained by decoction and drying of the same plant material, hereafter coded as Vimang, was a homogeneous brown powder, which melted from 215 to 218 °C with decomposition, with a particle size of 30−60 μm, and was provided by the Center of Pharmaceutical Chemistry, Havana, Cuba.

Preparative Reversed-Phase (RP) HPLC Condition. Separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, a U6K injector, and a Waters μ-Bondapak C-18 column (30 cm × 7.8 mm i.d., 4 mm) using MeOH−H_2O (30:70 v/v, flow rate 2.5 mL/min) to yield compounds **1** (8.5 mg, R_t = 10.1 min), **2** (9.0 mg, R_t = 11.8 min), **3** (8.1 mg, R_t = 12.7 min), **4** (18.4 mg, R_t = 20.6 min), **5** (21.5 mg, R_t = 28.4 min), **6** (323.1 mg, R_t = 33.3 min), **7** (66.1 mg, R_t = 39.4 min), **8** (36.4 mg, R_t = 40.2 min), and **9** (20.1 mg, R_t = 41.2 min).

Sample Preparation for Analytical Determination. For sample preparation, 500 mg of pulverized plant material was partitioned twice with 80 mL of *n*-BuOH/H_2O (1:1, v/v) at room temperature. The aqueous extract contained only free sugars and polyols that were analyzed by GC−MS. A portion (3 mg) of the dried BuOH-soluble material (87 mg) was diluted to a volume of 3 mL (1 mg/mL) in a volumetric flask. Standard solutions containing 1 mg/mL of gallic acid, (−)-epicatechin, and benzoic acid in methanol were also prepared.

Quantitative HPLC Conditions. HPLC analyses were performed on a Waters 600 E-Multisolvent Delivery System liquid chromatograph, equipped with a U6-K injector (fitted with a 20-μL loop), a Waters 486 tunable UV−Vis spectrophotometric detector, and a Waters 746-data module integrator. Following are the operating conditions used. The column was a C18 μ-Bondapak (300 × 4.0 mm i.d.). Chromatographic separation was carried out using isocratic elution with two solvents [A = acetonitrile; B = water/2% acetic acid (98:2 v/v)] in the ratio 15:85 (v/v). Detection wavelength was 278 nm. Flow rate was 0.7 mL/min, and the injection volume was 10 μL. The t_R values for gallic acid (**1**), 3,4-dihydroxy benzoic acid (**2**), benzoic acid (**3**), methyl gallate (**4**), propyl gallate (**5**), mangiferin (**6**), (+)-catechin (**7**), (−)-epicatechin (**8**), and propyl benzoate (**9**) were 3.40, 3.98, 4.30, 6.95, 9.61, 11.25, 13.65, 16.17, and 22.02 min, respectively. Gallic acid, 3,4-dihydroxy benzoic acid, benzoic acid, methyl gallate, (+)-catechin, and (−)-epicatechin, for which standards were available, were identified by chromatograms according to their retention times. Propyl gallate, mangiferin, and propyl benzoate were identified by comparing the retention times of the peaks in the extracts with those of the same compounds previously isolated and characterized by NMR analysis.

Quantitation. Quantitation was performed by reporting the measured integration area in the calibration equation of the corresponding standard. Gallic acid, 3,4-dihydroxy benzoic acid, methyl gallate, and propyl gallate were assayed as gallic acid equivalent. Mangiferin, (+)-catechin, and (−)-epicatechin were assayed as (−)-epicatechin equivalent. Benzoic acid and propyl benzoate were assayed as benzoic acid equivalent.

Linearity. Linearity was determined for standards and for the constituents **1**−**9** in Vimang. Linearity of responses was determined on six levels of concentration with three injections for each level. A linear relationship between peak area and concentration (5−25 μg/mL) was observed for each standard with a correlation coefficient r = 0.9997. The minimum detection limit was 0.2 ng, which resulted in a signal-to-noise ratio of 3:1.

Reproducibility. The reproducibility of the injection integration procedure was determined for standards and for the Vimang constituents **1**−**9**. The solutions were injected 10 times and the relative standard deviations were calculated (gallic acid, 1.01%; 3,4-dihydroxy benzoic acid, 0.98%; benzoic acid, 1.04%; methyl gallate, 2.5%; propyl gallate, 1.7%; mangiferin, 1.9%; (+)-catechin, 3.2%; (−)-epicatechin, 4.9%; and propyl benzoate, 2.6%). Relative standard deviation for retention times was less than 1%.

Repeatability. To evaluate the repeatability of the method, three solutions at different concentrations (0.7 mg/mL, 1.0 mg/mL, and 1.3 mg/mL of dry extract in methanol) of Vimang BuOH extract were prepared; each solution was injected three times. The contents of constituents **1**−**9** were calculated in order to estimate the RSD (gallic acid, 1.12%; 3,4-dihydroxy benzoic acid, 1.93%; benzoic acid, 2.43%; methyl gallate, 2.01%; propyl gallate, 1.64%; mangiferin, 3.11%; (+)-catechin, 1.87%; (−)-epicatechin, 1.23%; and propyl benzoate, 2.32%).

Total Polyphenols Assay. Estimation of the global polyphenol content in the extracts was performed by the Folin−Ciocalteu method. Vimang (1.15 mg) was dissolved in MeOH (2 mL), and the extract was diluted 10-fold with water. Folin−Ciocalteu reagent (0.5 mL; Merck) was added to the diluted solutions (0.5 mL), then 0.5 mL of a 100 g/L solution of Na_2CO_3 was added. The absorbance was measured at 720 nm (Shimadzu UV-2101 spectrophotometer) with a blank sample (water plus reagents) in the reference cell. Quantification was obtained by reporting the absorbances in the calibration curve of gallic acid used as standard phenol.

GC−MS Analysis of Free Sugars and Polyols. The aqueous extract, obtained as described above, was dried, and 10 mg of residue was diluted with 40 μL of dry pyridine. The diluted residue was then directly silylated at 80 °C for 30 min with 50 μL of *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane as catalyst. The TMS derivatives were kept in isooctane and analyzed by GC−MS as follows: Shimadzu MS-QP5050 system; column, DB1 J&W, 25m × 0.2 mm i.d., 0.33 μm; injection, 1 μL; split ratio, 2; oven temperature, 100° for 1 min, to 180 °C at 4 °C/min, then to 290 °C at 10 °C/min, and held for 20 min.; injector temperature, 290 °C; detector EMV 1.35−1.5 kV; carrier gas, helium; flow, 1 mL/min. Individual sugars and polyols were identified by comparison of MS spectra and retention times of peaks with those of TMS derivatives of authentic compounds. The results represents the mean ± SD of 10 determinations. The concentrations of each compound in the extract were calculated from the experimental peak areas by interpolation to standard calibration curves.

Spectroscopic Apparatus. The ES−MS spectra were determined on a Fisons Platform spectrometer both in the positive (90 V) and negative (100 V) modes. The sample was dissolved in MeOH and injected directly.

UV spectra were measured with a HP 8472-A spectrometer in MeOH, (c = 1) and IR spectra with a Nicolet Impact 400, in KBr.

A Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 for ^{13}C, with the UXNMR software package, was used for NMR experiments measured in CD_3OD. The DEPT experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH_3 and negative ones for CH_2. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. ^{1}H−^{1}H DQF−COSY (*7*), ^{1}H−^{13}C HSQC, and HMBC (*8*, *9*) experiments were obtained using the conventional pulse sequences as described in the literature, and 1D TOCSY (*10*) was acquired using waveform generator-based GAUSS shaped pulse, mixing time ranging from 80 to 100 ms, and a MLEV-17 spin-lock field of 10 kHz was preceded by a 2 ms trim pulse. CD measurement was performed on a Jasco J-7140 spectropolarimeter.

Gallic Acid (1). White amorphous solid; ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 169 $[M-H]^-$ (100).

3,4-Dihydroxy Benzoic Acid (2). White amorphous solid; ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 153 $[M-H]^-$ (100).

Benzoic Acid (3). White amorphous solid; ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 121 $[M-H]^-$ (100).

Methyl Gallate (4). White amorphous solid; ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 183 $[M-H]^-$ (100).

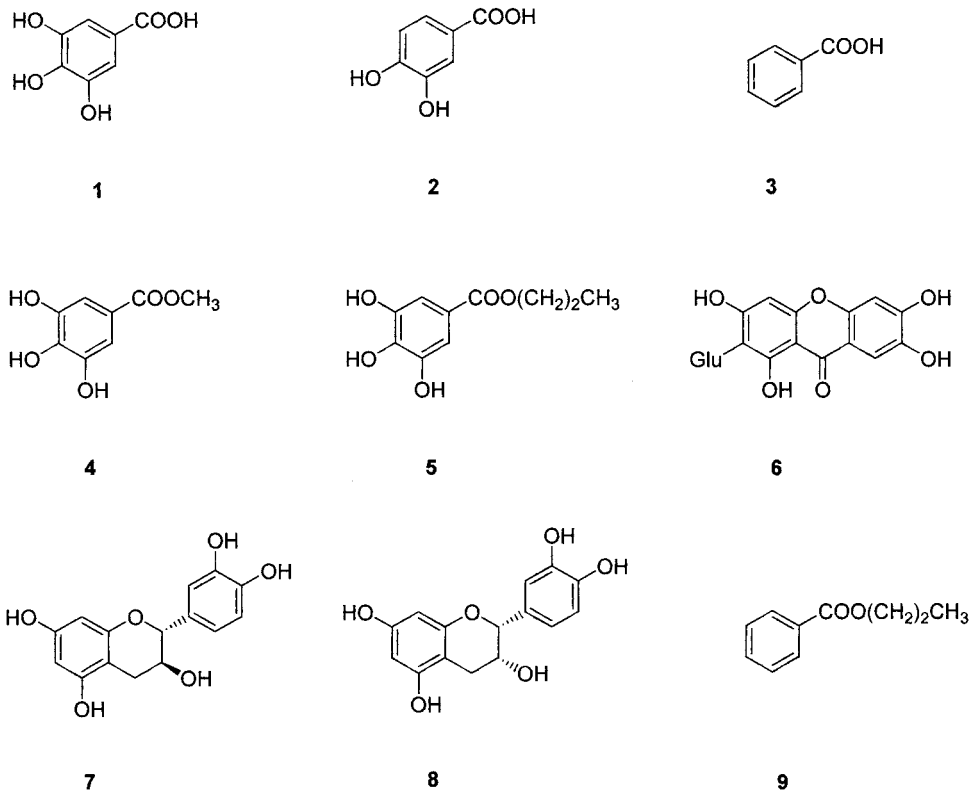

Figure 1. Compounds isolated from *Mangifera indica* L. stem bark extract: gallic acid (**1**), 3,4-dihydroxy benzoic acid (**2**), benzoic acid (**3**), methyl gallate (**4**), propyl gallate (**5**), mangiferin (**6**), (+)-catechin (**7**), (–)-epicatechin (**8**), and propyl benzoate (**9**). Glc = β-D-glucopyranosyl.

Propyl Gallate (5). White amorphous solid; ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 211 $[M-H]^-$ (100).

Mangiferin (6). Colorless amorphous solid; UV λ max (MeOH): 237, 254; 268, 312, 364; +KOH: 235, 268, 298, 341; +$AlCl_3$: 235, 268 321sh; 352, 392; +$AlCl_3$ + HCl: 226, 258, 276sh, 316, 330 395; + NaOAc: 237, 263, 301sh. ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 421 $[M-H]^-$ (100).

(+)-Catechin (7). Colorless amorphous solid; ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 289 $[M-H]^-$ (100).

(−)-Epicatechin (8). Colorless amorphous solid; ES−MS, *m*/*z* (rel int.) (100 V, negative mode): 289 $[M-H]^-$ (100).

Benzoic Acid Propyl Ester (9). White amorphous solid; EI−MS, *m*/*z* (rel int.) (100 V, negative mode): 163 $[M-H]^-$ (100).

RESULTS AND DISCUSSION

Vimang obtained by decoction and drying of *Mangifera indica* L. stem bark was extracted at room temperature with MeOH. The MeOH extract (5 g) was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH soluble portion (0.87 g) which was submitted to preparative reversed-phase HPLC to yield phenolic compounds gallic acid (**1**), 3,4-dihydroxy benzoic acid (**2**), benzoic acid (**3**), methyl gallate (**4**), propyl gallate (**5**), mangiferin (**6**), (+)-catechin (**7**), (−)-epicatechin (**8**), and propyl benzoate (**9**) (**Figure 1**). The structures and molecular formulas of compounds **1**−**9** were determined from their ES−MS spectra, as well as from 1D and 2D ^1H and ^{13}C NMR data.

Compound **1** was identified as gallic acid from the ^1H NMR spectrum and further confirmed by comparison of its chromatographic behavior with that of an authentic sample. Compounds **2** and **3** showed spectroscopic data identical with those of 3,4-dihydroxy benzoic acid and benzoic acid, respectively. The NMR spectra of compounds **4** and **5** also showed the presence of a gallic acid moiety, but in addition there was a methyl ester signal in **4**, and resonances for a propyl ester in **5**. Compounds **4** was methyl gallate and **5** was propyl gallate. This assignment was also corroborated by comparison with published data (*3*).

Compound **6** was the most abundant phenolic and the ^{13}C NMR spectrum showed 19 carbon atom signals. The presence of a glycosyl moiety was clearly suggested from the analysis of the 2D COSY, HSQC, and HMBC experiments. The sugar was identified as a β-D-glucopyranose on the basis of its ^1H and ^{13}C NMR data (*11*). The mass spectra did not show the usual fragmentation pattern for *O*-glycoside derivatives, and these data together with the chemical shifts of H-1 (δ 4.86) and C-1 (δ 73.4) unambiguously showed the C-linkage of the sugar. ^1H and ^{13}C NMR chemical shifts of the aglycon were in accordance with those reported in the literature for tetrahydroxyxanthones (*12*). The glycosidic linkage of **6** was determined to be at the C-4 position based on the cross-peaks due to ^3J long-range coupling between the anomeric-H (δ 4.86, H-1) and C-2 (δ 107.9), C-1 (162.1), and C-3 (164.1) in the HMBC spectrum. From these considerations the structure of mangiferin (*13*) was assigned to **6**.

Compounds **7** and **8** were identified as catechin and epicatechin by HPLC comparison with authentic samples, and their identities were also apparent from their ^{13}C NMR spectra which showed similar carbon signals for the phloroglucinol A-ring and catechol B-ring, but slight difference in signals for the pyran ring. The absolute configuration of C-2 and C-3 for (+)-catechin and (−)-epicatechin were deduced as 2S from the CD spectroscopy measurement in comparison with the data reported in the literature (*14*).

Compound **9** was identified easily as propyl benzoate from its NMR and also by comparison with compound **5**.

The quantitative analysis of the phenolic compounds from mango stem bark was performed by HPLC. The concentrations of each compound in the extract, calculated from the experimental peak areas by interpolation to standard calibration curves,

were 1.12% for compound **1**, 1.30% for **2**, 1.14% for compound **3**, 2.56% for **4**, 2.74% for **5**, 41.06% for **6,** 7.52% for **7**, 4.64% for **8**, and 2.29% for **9**, corresponding to 208.0 mg/100 g dry weight for compound **1**, 226.2 mg for **2**, 198.6 mg for **3**, 445.2 mg for **4**, 476.2 mg for **5**, 7140.1 mg for **6**, 1308.0 mg for **7**, 807.4 mg for **8**, and 398.7 mg for **9**. The sum of all phenolic compound concentrations obtained by quantitative HPLC (10.61 g/100 g dry weight) was compared with the results obtained from the Folin−Ciocalteu assays, generally considered as the method of choice to estimate total phenol contents in plant extracts (*15*). No significant difference was found between the two methods, as the total polyphenols in Vimang determined by the Folin−Ciocalteu method, and expressed as gallic acid equivalents, was 9.4 g/100 g dry weight.

The aqueous extract, containing free sugars and polyols, was also analyzed by GC−MS. The major sugars were galactose, glucose, and arabinose (839.7, 495.6, and 483.7 mg/100 g dry weight, respectively); fructose was detected at the low concentration of 155.9 mg/100 g. Additionally, Vimang contained three polyols: sorbitol, present in appreciable quantities (685.2 mg), myoinositol (303.2 mg), and xylitol (52.5 mg/100 g dry weight).

Vimang contained a variety of polyphenols, which included phenolic acids (gallic acid, 3,4-dihydroxy benzoic acid, benzoic acid), phenolic esters (methyl gallate, propyl gallate, propyl benzoate), flavan-3-ols (catechin and epicatechin), and a xanthone (mangiferin). These phenolics made up approximately 11% of Vimang. The phenolic composition of mango stem bark is reported here for the first time.

There is now much interest in polyphenolic products of the plant phenylpropanoid pathway as they have considerable antioxidant activity in vitro and are ubiquitous in our diet. Rich sources include tea, wine, fruits and vegetables (although levels are affected by species), light, degree of ripeness, processing, and storage. There is little convincing epidemiological evidence that intakes of polyphenols are inversely related to the incidence of cancer. In contrast, numerous cell culture and animal models indicate potent anticarcinogenic activity by certain polyphenols mediated through a range of mechanisms including antioxidant activity, enzyme modulation, gene expression, apoptosis, up-regulation of gap junction communication and P-glycoprotein activation (*16*).

In a previous work, we tested mango bark extract against vitamins (C, E, and C + E) and β-carotene in vivo, in terms of lipid peroxidation inhibition (LPI) and protection to oxidative damage (POD). LPI values for Vimang were similar to those of vitamins in plasma, significantly higher than vitamin C and β-carotene in liver, and similar to the combination of vitamins C + E in brain. POD values for Vimang were significantly higher than those for all products tested in plasma, macrophages, liver, and brain, being the most significant difference in brain (*3*).

We also evaluated the protective abilities of Vimang against the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative damage in blood serum, liver, and brain, as well as in the hyper-production of reactive oxygen species (ROS) by peritoneal macrophages (*3*).

Mangiferin, the predominant component (7%), has been reported to have multiple biological effects such as hypolipidemic activity in cholesterol-fed mice (*17*), antidiabetic activity (*18*), inhibitory effects in bowel carcinogenesis of male F344 rats (*19*), and antitumor, immunomodulatory, and anti-HIV effect (*20*). Its mechanism as an anti-oxidant has been described elsewhere (*21*). The metabolism of mangiferin yields norathyriol after cleavage of the C−C linkage of the glucose moiety, which exhibits a potent iron chelating effect, and an inhibitory effect of the induced-respiratory burst in rat neutrophils (*22*). In our experiments, mangiferin did not show higher biological activity than the whole extract; and we therefore hypothesized that the strong antioxidant activity of the mango stem bark extract may be due to the presence of a combination of polyphenolic compounds such as catechins, and galloyl esters derivatives, known for their antioxidant activities (*23*, *24*), together with important microelements (copper, zinc, and selenium) determined by ICP spectrometric techniques in a previous study (*25*). Those elements play important roles in the activity of enzymes involved in the endogenous antioxidant defense mechanism in the human organism, such as superoxide dismutase (SOD) and gluthation peroxidase (GPx). The mineral content of mango stem bark extract was 40 mg/100 g dry weight.

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