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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2013

Impact Factor: 2.91 · DOI: 10.1021/jf3046686 · Source: PubMed

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Evaluation of Antioxidant Activity and Characterization of Phenolic Constituents of *Phyllanthus amarus* Root

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ABSTRACT: The antioxidant property of the 70% aqueous ethanol extract of *Phyllanthus amarus* roots and its ether-soluble, ethyl acetate-soluble, and aqueous fractions were investigated by various *in vitro* assays. The root extracts showed higher DPPH, hydroxyl, superoxide, and nitric oxide radical scavenging and reducing power activity. Among all the samples, the ethyl acetate-soluble fraction demonstrated highest radical scavenging activity and total phenolics content. Twenty-eight different phenolic compounds were identified by LCMS/MS analysis of the ethyl acetate-soluble fraction. The majority of the compounds were found to exist as their glycosides, and many of these were gallic acid derivatives. Free epicatechin and gallic acid were also identified in the ethyl acetate-soluble fraction. The present investigation suggested that *P. amarus* root is a potent antioxidant and can be used for the prevention of diseases related to oxidative stress.

KEYWORDS: *antioxidant activity, gallic acid, LCMS/MS analysis, phenolic constituents, Phyllanthus amarus*

INTRODUCTION

Reactive oxygen species (ROS) are often generated as byproducts of biological processes/reactions or from exogenous factors.[1] The ROS overload causes oxidative stress, which is implicated in the etiology of many human pathogenic conditions and degenerative diseases.[2,3] A potent ROS scavenger may serve as a possible preventive as well as therapeutic agent against these diseases.[4] Currently herbal antioxidants have gained worldwide popularity as both drugs and food/drug supplements for the treatment of various diseases.[5,6] India has a rich plant biodiversity and a very long and safe history of using herbal drugs in the officially recognized alternative systems of medicines.

Phyllanthus amarus Schum. and Thonn. (family: Euphorbiaceae), widespread throughout the tropical and subtropical countries of the world, is most commonly used in the Indian Ayurvedic system of medicine to alleviate stomach, genitourinary system, liver, kidney, and spleen disorders.[7] It is credited with a host of medicinal attributes such as anti-inflammatory, antihepatotoxic, antilithic, analgesic, hypotensive, antispasmodic, antiviral, antibacterial, diuretic, antimutagenic and hypoglycemic properties.[8,9] However, its efficacy in treating hepatitic jaundice as such, or in conjunction with other drugs, is of major clinical importance.[10−12]

The diverse pharmacological profile of *P. amarus* has been attributed to its vast repertoire of phytochemicals, some of which are found only in the *Phyllanthus* genus.[13] Previous phytochemical investigations have focused on the whole plant extract of this species and the presence of lignans, alkaloids, and bioflavonoids.[14] Many of the flavones and steroids are present as glycosides in the plant and include niranthin, nirtetralin, phyltetralin, lintetralin, quercitrin, geranin, corilagin, astragalin, amarin, amariinic acid, amarulone, 1-galloyl-2,3-dehydrohexahydroxydiphenyl (DHHDP)-glucose, repandusinic acid, catechin, gallocatechin, and epigallocatechin as well as glycosides of quercetin, fisetin, and estradiol. Several hydrolyzable tannins such as amariin, amarulone, and terpenoids have also been isolated. Saponin is present in the aqueous, chloroform, and ethanol extracts of all parts of the plant except the seeds. The securinega-type alkaloids such as securinine, norsecurinine, and phyllanthine as well as two new compounds, isobubbialine and epibubbialine, were isolated from the leaves of *P. amarus*. The phytochemistry of *P. amarus* has been well reviewed.[15] In an interesting study on the phytochemicals and mineral contents of various parts of *P. amarus*, the presence of terpenoids, anthraquinones, tannins, and phlobatanins in various extracts of *P. amarus* roots has been ascertained.[16]

Very recently, we have observed that the 70% aqueous ethanol extract of *P. amarus* roots reduced the bilirubin level and oxidative stress in phenylhydrazine-induced neonatal jaundice in mice.[17] It is well known that the phytoconstituents depend on the genetic diversity of the plants, as well as other factors such as time of collection and growth conditions (soil nature, vegetation period climate, etc.). Hence, fingerprinting of the chemical constituents of herbal drugs is very important to ensure their quality control. The primary aim of the present study was to characterize some of the chemical constituents of the 70% aqueous ethanol extract of *P. amarus* roots and correlate these with the *in vitro* antioxidant property of the extract. To this end, the root extract and its various fractions were analyzed by HPLC, followed by LCMS/MS analysis of the

Received: November 19, 2012
Revised: February 25, 2013
Accepted: March 4, 2013
Published: March 4, 2013

dx.doi.org/10.1021/jf3046686 | *J. Agric. Food Chem.* 2013, 61, 3443−3450

most active fraction. Major emphasis was given to the polyphenolic profile of the fraction, showing maximum antioxidant activity.

MATERIALS AND METHODS

Plant Materials and Chemicals. *P. amarus* plants were collected in the months of July to September 2010 from the fields near Kolkata, West Bengal, and were authenticated at the Department of Botany, University of Calcutta, Kolkata, India (voucher specimen no. CUH09052012). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, quercetin, butylated hydroxytoluene (BHT), and pyrogallol were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Deoxyribose, xylenol orange, ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), and Folin-Ciocalteu reagent were purchased from E. Merck, Mumbai, India. Ferric chloride ($FeCl_3$), sodium nitroprusside (SNP), sulfanilic acid, napthylethylenediamine dihydrochloride (NED), and sodium pyruvate were purchased from SRL, Mumbai, India. All reagents used in this work were of analytical grade, and solvents were redistilled before use.

Extraction and Fractionation. The roots, separated from the whole plant, and dried for 7 days at room temperature, were macerated in an electric blender. The powder (100 g) was extracted three times with 70% aqueous ethanol (total volume 1000 mL) and filtered using a Whatman No. 1 paper. The filtrate was concentrated using a rotary evaporator at 30 °C and lyophilized to obtain the crude extract (designated as CRD). A portion of CRD (6 g) was redissolved in distilled water (250 mL) and then partitioned sequentially with petroleum ether (250 mL) and ethyl acetate (250 mL) to obtain an aqueous fraction (AF) and two organic extracts. The organic extracts were concentrated under vacuum to yield the ether fraction (EF) and ethyl acetate fraction (EAF). The samples were stored in the dark at −20 °C for further analyses. The extract and fractions were evaporated until constant weight.

***In Vitro* Antioxidant Activity.** Six different tests were used to evaluate the antioxidant activity of CRD and its fractions. BHT, gallic acid, and quercetin, dissolved in 95% methanol, were used as the positive controls for all the assays, while sodium pyruvate was used for the H_2O_2 scavenging assay.

DPPH Radical Scavenging Assay. The assay was done as described previously, with minor modifications.[18] An aliquot of the samples was mixed with an equal volume of 0.04 mM DPPH (in 95% MeOH) and incubated for 30 min at room temperature in the dark. The absorbance at 517 nm was read, using MeOH as the blank.

Hydroxyl Radical Scavenging Assay. The assay was carried out according to a previously described method, with minor modifications.[19] The reaction mixture containing 100 μL of 28 mM 2-deoxyribose, 500 μL of sample in phosphate buffer, 200 μL of 200 μM $FeCl_3$ in 1.04 mM aqueous EDTA (1:1, v/v), 100 μL of 1 mM H_2O_2, and 100 μL of 1 mM ascorbic acid was incubated at 37 °C for 1 h. The reaction was terminated by the addition of 1.0 mL of 2.8% TCA. 1.0 mL of 1.0% TBA was added, and the mixture was incubated on a water bath at 80 °C for 20 min. After cooling, the absorbance at 532 nm was measured against an appropriate blank.

Superoxide Radical Scavenging Assay. The superoxide radical scavenging activity was assayed by measuring the autoxidation inhibition rate of pyrogallol according to a previously described method with minor modifications.[20] The sample (100 μL, 1 mg/mL) and 800 μL of 50 mM Tris-HCl buffer (pH 8.2) were added to a freshly prepared 100 μL solution of pyrogallol (3 mM, in 10 mM HCl). The inhibition rate of pyrogallol autoxidation was measured by monitoring the absorbance at 325 nm every 30 s over a period of 3 min.

Nitric Oxide Radical Scavenging Assay. The assay was carried out according to a previously described method, with modifications.[21] The reaction mixture contained 10 mM SNP, phosphate-buffered saline (pH 7.4), and the various concentrations of the samples. After incubation for 150 min at 25 °C, 1 mL of sulfanilic acid (0.33% in 20% glacial acetic acid) was added to 0.3 mL of the incubated solution, and the mixture allowed to stand for 5 min. NED (0.5 mL, 0.1% w/v) was added, and the mixture was incubated for 30 min at 25 °C. The pink chromophore, generated during diazotization of nitrite ions with sulfanilic acid and subsequent coupling with NED, was measured from the absorbance at 540 nm, using an appropriate blank.

H_2O_2 Scavenging Assay. Following a reported method, with some modifications, the H_2O_2 scavenging activity was determined.[22] H_2O_2 (50 μL, 1 mM) and various concentrations of the samples (each 100 μL) were incubated for 30 min at room temperature. FOX reagent (0.85 mL, prepared from 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, and 25 mM H_2SO_4 in water) was added into the mixtures, which were allowed to stand for 30 min at room temperature. The absorbance of the ferric–xylenol orange complex at 560 nm was measured against an appropriate blank.

Reducing Power Assay. The Fe^{3+}-reducing power of the extract was assayed following a reported method, with minor modifications.[23] Different concentrations of the sample were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide (0.5 mL) and incubated at 50 °C on a water bath for 20 min. TCA (0.5 mL, 10%) was added to the mixture, which was centrifuged at 3000 rpm for 10 min. Finally, 0.5 mL of the upper layer was mixed with an equal volume of distilled water and 0.1 mL of 0.1% $FeCl_3$ solution. The reaction mixture was left for 10 min at room temperature, and the absorbance at 700 nm was measured against an appropriate blank.

Determination of Total Phenolics Content. The total phenolics content was determined according to a reported method, with minor modifications.[24] Briefly, a mixture of the sample (40 μL, 1 mg/mL), Folin-Ciocalteu reagent (200 μL), and distilled water (1160 μL) was incubated for 3 min. Aqueous sodium carbonate (600 μL, 20%) solution was added to the mixture, which was kept in the dark for 2 h at room temperature, and the absorbance at 756 nm was measured. Gallic acid was used as the standard, and the results are expressed as mg of gallic acid equivalents (GAE) per gram of extract.

Determination of Total Flavonoids Content. The total flavonoids content was determined according to a previously described method, with minor modifications.[24] An aliquot of 2% aluminum chloride (in 95% methanol) and an equal volume of the sample were incubated for 10 min, and the absorbance at 415 nm was measured. Quercetin was used as the standard, and the results are expressed as milligrams of quercetin equivalents (QE) per gram of extract.

HPLC Analysis of CRD and Other Fractions. HPLC analysis was carried out with a Jasco HPLC system, Jasco Corporation (Tokyo, Japan), equipped with a C-18 reverse phase HYPERSIL (Chromatopack, Mumbai, India) column (250 mm × 4.6 mm, 10 μ) and a UV detector set at a wavelength of 280 nm. The HPLC gradient was methanol in 0.1% formic acid/H_2O as follows: 5 to 15% in 15 min, 15 to 30% from 15 to 35 min, 30 to 40% from 35 to 40 min, 40 to 50% from 40 to 50 min, 50 to 60% from 50 to 55 min, 60 to 75% from 55 to 60 min, and finally reaching 95% in 65 min at a flow rate of 1.0 mL/min.

LCMS/MS Analysis of EAF. The chemical constituents of the root of *P. amarus* were identified by LCMS/MS analysis of the phenolic-enriched ethyl acetate fraction obtained from the crude extract. Mass spectra were recorded by atmospheric pressure chemical ionization in the negative mode using a Varian Ion Trap MS (410 Prostar Binary LC with 500 MS IT PDA detectors) equipped with a C-18 reverse phase stainless steel column (30 cm × 0.46 cm). All samples were filtered through a 0.45 μm filter (Millipore Corp.) before injection. The capillary voltage was kept at 80 V, and the air (nebulizing gas) pressure was 35 psi. Full scan data acquisition was performed by scanning from m/z 100 to 900. The presence of major phenolic compounds was confirmed by their molecular ion peak and base peak. The HPLC gradient was methanol in 0.1% formic acid/H_2O as follows: 5 to 15% in 7.5 min, 15 to 30% from 7.5 to 17.5 min, 30 to 40% from 17.5 to 20 min, 40 to 50% from 20 to 25 min, 50 to 60% from 25 to 27.5 min, 60 to 75% from 27.5 to 30 min, and finally reaching 95% in 32.5 min at a flow rate of 0.5 mL/min.

Statistical Analysis. All antioxidant data are expressed as means ± SD of three independent analyses each carried out in triplicate.

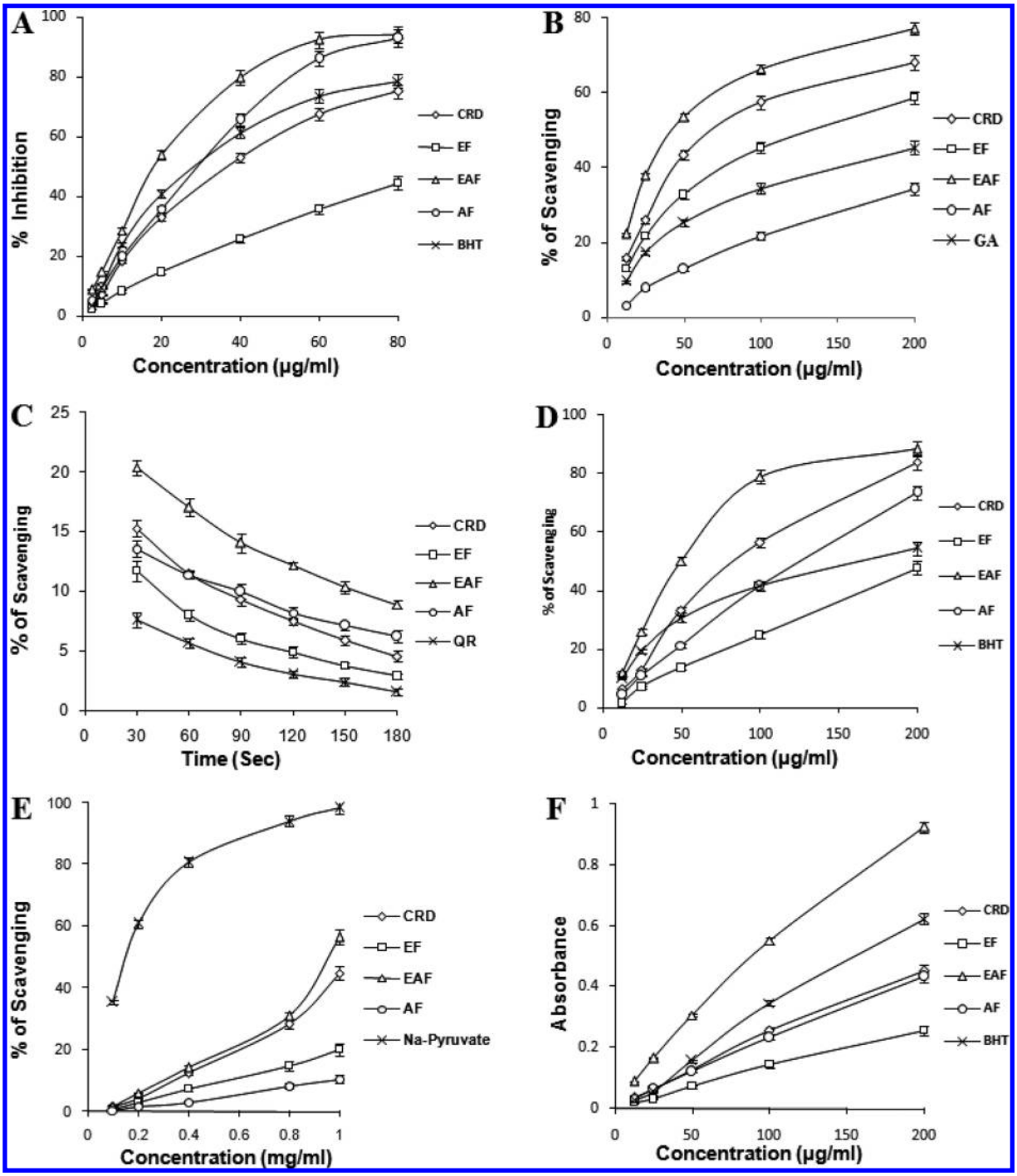

Figure 1. *In vitro* antioxidant assays of the crude extract of *P. amarus* root and its various fractions. (A) DPPH radical scavenging activity. (B) Hydroxyl radical scavenging activity. (C) Superoxide radical scavenging activity. (D) Nitric oxide radical scavenging activity. (E) Hydrogen peroxide scavenging activity. (F) Reducing power. BHT: Butylated hydroxytoluene. GA: Gallic acid. QR: Quercetin. Values are expressed as mean ± SD (n = 9).

Statistical analysis was carried out using the analysis of variance method (Origin 6.1 software), and means were expressed as significantly different or not at the 5% level of confidence.

RESULTS AND DISCUSSION

Extraction Yield of the Crude Extract and Its Various Fractions. The present study was conducted with aqueous 70% ethanol extract of *P. amarus* root in view of its curative efficacy against neonatal jaundice in mice.[17] The yield of CRD was 6.5 ± 0.7% (w/w). Among the fractions, the yield of AF was maximum (74.5 ± 4.5%, w/w). The yield of EF (12.7 ± 1.2%, w/w) was significantly greater than that of EAF (7.0 ± 0.8%, w/w).

***In Vitro* Antioxidant Activity.** Due to the chemical diversity of the antioxidant compounds present in plants, a single antioxidant assay does not reflect their total antioxidant capacity (TAC), which also depends on the synergic and redox interactions among the different plant constituents. Several methods, differing in their chemistry (generation of different radicals and/or target molecules), and detection of end points

dx.doi.org/10.1021/jf3046686 | J. Agric. Food Chem. 2013, 61, 3443−3450

Table 1. Antioxidant Activity of CRD, Its Various Fractions, and Standards

scavenging assay	CRD	EF	EAF	AF	BHT	gallic acid	Na-pyruvate
DPPH•[a]	35.48 ± 0.86	96.05 ± 3.52	18.43 ± 0.52	29.52 ± 1.65	30.20 ± 1.19		
OH•[a]	77.94 ± 1.27	155.67 ± 3.22	44.15 ± 1.02	289.92 ± 5.78		216.67 ± 3.94	
NO•[a]	86.21 ± 0.69	198.41 ± 1.27	59.24 ± 0.41	120.2 0 ± 0.92	105.48 ± 0.85		
H_2O_2[b]	1.18 ± 0.03	2.45 ± 0.07	1.0 ± 0.03	4.5 ± 0.13			0.11 ± 0.005

[a]IC_{50} values (μg/mL). [b]IC_{50} values (mg/mL).

have been developed for measuring the TAC of herbal products.[25] The antioxidant action of a test sample is mediated by hydrogen atom transfer (HAT) and single electron transfer (SET) reactions as well as their combinations (sequential proton loss and electron transfer (SPLET)). Hence, we selected both SET-based (total phenol assay, Fe(III)-reducing power) and SPLET-based (DPPH) methods in the present studies. The ET-based assays measure the reducing capacity, while the SPLET-based assays quantify hydrogen atom and electron-donating capacity of the antioxidants. During this process, antioxidants can function as free radical scavengers and reducing agents.[26] In addition, scavenging of several physiologically important radical and nonradical ROS by the test samples was also ascertained.

DPPH Free Radical Scavenging Activity. The bleaching of DPPH absorption by a test sample is representative of its capacity to scavenge free radicals, generated independent of any enzymatic or transition metal based systems.[27] CRD and its fractions scavenged the DPPH radical concentration-dependently (Figure 1A). Among the samples, EF showed the least scavenging activity (IC_{50} = 96.1 μg/mL), while EAF with IC_{50} = 18.4 μg/mL showed a better result than the positive control BHT (30.2 μg/mL). The activity of AF (29.5 μg/mL) was comparable to that of BHT, but CRD (IC_{50} = 35.5 μg/mL) was slightly less potent. The results are summarized in Table 1. These suggested that CRD is an efficient radical scavenger, its activity being primarily confined to the ethyl acetate-soluble fraction.

Hydroxyl Radical Scavenging Activity. Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage.[28] The OH• scavenging activity of the crude extract and its various fractions was determined by measuring their ability to prevent oxidative degradation of 2-deoxyribose (Figure 1B). All the samples showed dose-dependent OH• scavenging activities at the test concentrations. In this case also, EAF (IC_{50} = 44.2 μg/mL) showed the highest scavenging activity. Interestingly the activities of even CRD (IC_{50} = 77.9 μg/mL) and EF (IC_{50} = 155.7 μg/mL) were better than that of gallic acid (IC_{50} = 216.7 μg/mL). As expected, AF showed the least activity, with an IC_{50} value of 289.9 μg/mL (Table 1).

Superoxide Radical Scavenging Activity. Superoxide radical is formed in viable cells during several biochemical reactions, and its effect can be magnified because it produces other types of free radicals and oxidizing agents that can induce cell damage.[29] Presently, CRD and its different fractions showed higher superoxide radical scavenging activity than the positive control, quercetin (Figure 1C). Among the fractions, EAF showed the strongest superoxide anion scavenging activity followed by AF and EF.

Nitric Oxide Radical Scavenging Activity. Nitric oxide (NO) has an important role in various inflammatory processes. Its sustained production is toxic to tissues and can contribute to the vascular collapse associated with septic shock. Chronic generation of nitric oxide is associated with carcinomas and various inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis.[30] Our results showed dose-dependent NO scavenging activity by all the test samples (Figure 1D). The IC_{50} values (Table 1) of CRD (86.2 μg/mL), EAF (59.2 μg/mL), AF (120.2 μg/mL), and EF (198.4 μg/mL) for scavenging NO revealed that CRD and EAF were more potent than the positive control, BHT (IC_{50} = 105.5 μg/mL). In this case also, EAF was the most active, while AF was a poor NO scavenger.

H_2O_2 Scavenging Activity. H_2O_2 is a weak oxidizing agent that can directly inactivate some enzymes, usually by oxidation of the essential thiol group. As a neutral small molecule, it can cross cell membranes and generate the highly reactive hydroxyl radicals inside the cells by a superoxide-driven Fenton reaction.[31] Hence scavenging even a low level of H_2O_2 is important. The dose-dependent H_2O_2 scavenging activities of the test samples are shown in Figure 1E. The individual IC_{50} values (Table 1) of CRD, EF, EAF, AF, and the positive control, sodium pyruvate, were found to be 1.2, 2.5, 1.0, 4.5, and 0.11 mg/mL, respectively. Thus, the test samples were significantly less potent than sodium pyruvate, EAF showing the highest activity among the plant extracts.

Reducing Power. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity.[32] Hence, the Fe^{3+}-reducing powers of CRD as well as its various fractions were investigated, and the results compared with that of the reference compound, BHT. The reducing powers of the samples were found to increase concentration-dependently (Figure 1F). In this assay, a higher absorbance of reaction mixture indicates higher reducing power of the sample. Presently, the absorbances of the assay mixtures containing a fixed concentration (200 μg/mL) of CRD, EF, EAF, AF, and BHT were found to be 0.43, 0.92, 0.26, 0.43, and 0.62, respectively. The order of the reducing powers of the test samples was EAF > CRD ~ AF > BHT > EF. Thus, most of the test samples showed better reducing powers than BHT.

Total Phenolics and Flavonoids Contents. The results of the total phenolics and total flavonoids contents of CRD along with its fractions are shown in Table 2. CRD showed higher phenolics and flavonoids content. Among the various fractions, the highest phenolics and flavonoids content were observed in EAF. The results demonstrated that most of the

Table 2. Total Phenolics and Flavonoids Contents of CRD and Its Various Fractions[a]

extract	total phenolic content (μg of GAE/mg of extract)	total flavonoid content (μg of QE/mg of extract)
CRD	99.6 ± 2.9	16.6 ± 0.9
EF	57.9 ± 2.9	12 ± 0.6
EAF	298.9 ± 6.5	60.9 ± 1.4
AF	87 ± 3.7	16.1 ± 0.6

[a]All data are expressed as mean ± SD (n = 9).

dx.doi.org/10.1021/jf3046686 | J. Agric. Food Chem. 2013, 61, 3443−3450

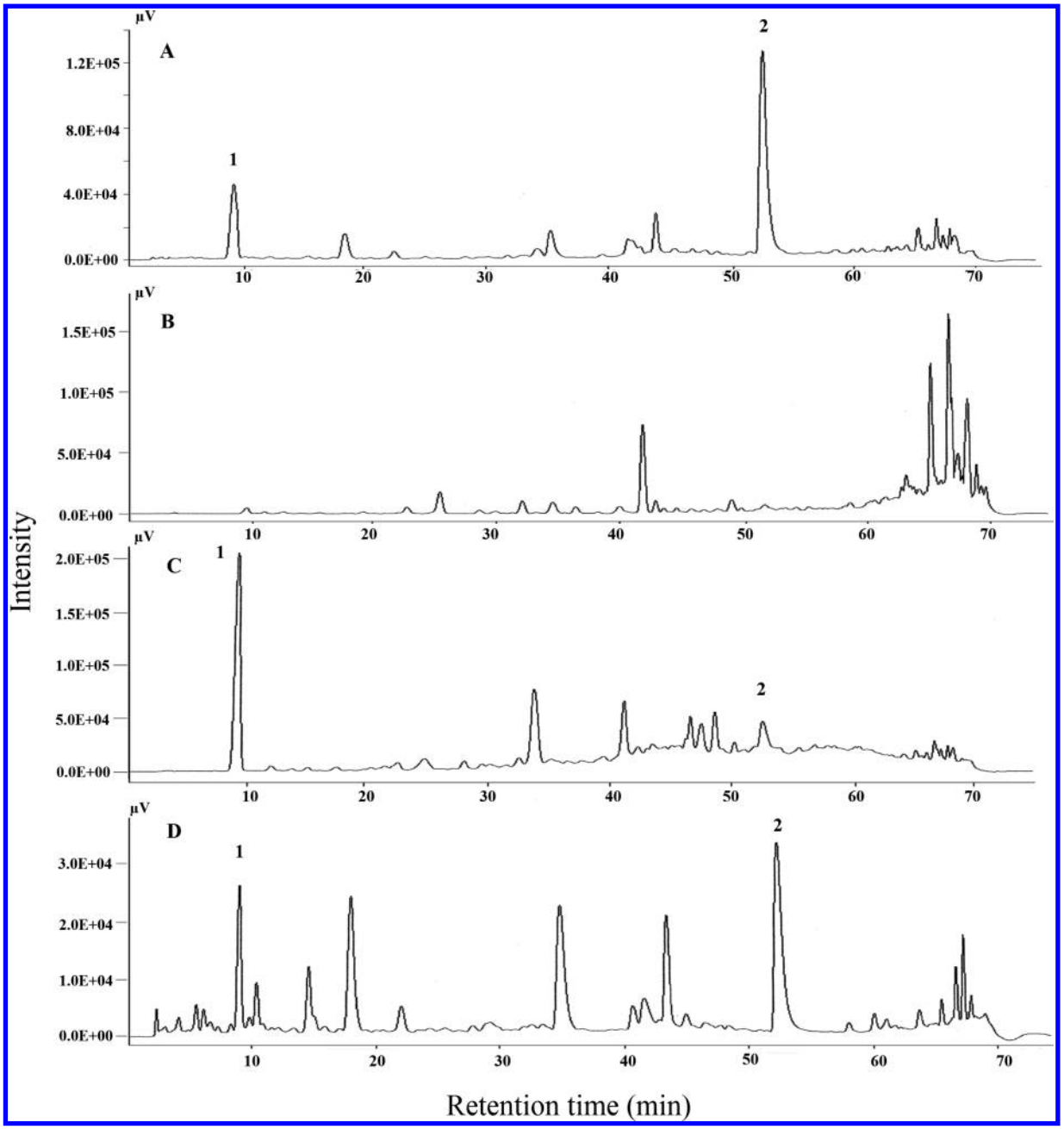

Figure 2. HPLC profile of (A) ethanol extract of *P. amarus* root, (B) ether fraction, (C) ethyl acetate fraction, and (D) aqueous fraction. 1: gallic acid.

phenolic and flavonoid compounds in CRD were extracted into the ethyl acetate fraction. This was also evident from the very low total phenolics and flavonoids contents in the other fractions, compared to that of EAF. Antioxidant activity of plant materials is well correlated with the content of their phenolic compounds.[33] Our results matched with their respective radical scavenging abilities.

Characterization of Phenolics in the Extract. Extensive previous studies revealed that *P. amarus* whole plant is a rich source of different types of secondary metabolites.[14,15] However, the phytoconstituents of the root extracts have not been studied extensively. In view of this, we analyzed the chemical composition of the crude root extract as well as its fractions by HPLC to characterize and quantify some of the major peaks. In particular, we were interested in the phenolic constituents, which are well-known antioxidants. It is also noteworthy that the antioxidant properties of *P. amarus* extracts depend on the methods of extraction and drying.[35] The characterization and quantification of the major phenolics were done using authentic commercially available samples. However, natural polyphenols generally occur as conjugates of sugars, usually *O*-glycosides, which are often identified using the LC/MS/MS technique.[36−38] Hence, we also used HPLC-APCI-MS/MS for identification of many of the compounds.

HPLC Analysis. The chromatogram of CRD showed two major peaks (1 and 2) along with several other peaks with retention times around 18−20, 22−24, 35−36, 42−46, and 65−70 min (Figure 2A). Among its various fractions, EAF (Figure 2C) and AF (Figure 2D), but not EF (Figure 2B) contained both these components. Peak 2, the major

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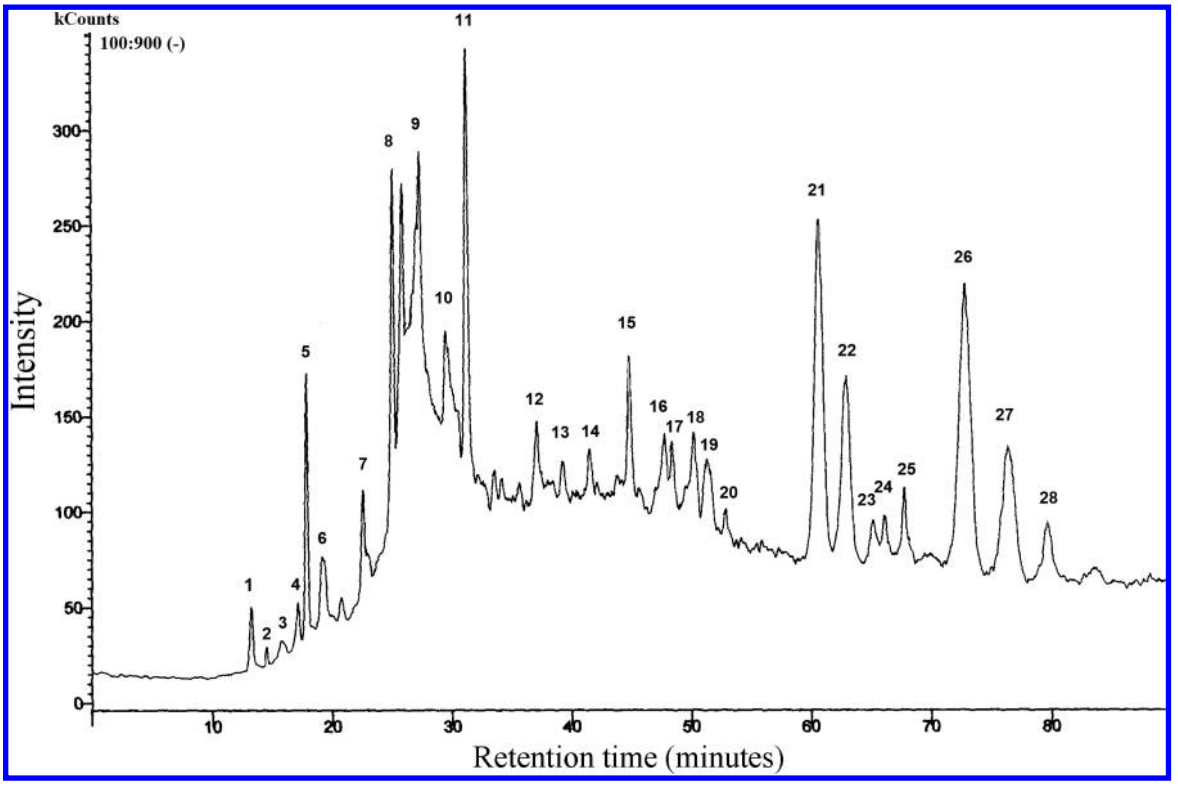

Figure 3. LCMS/MS profile of the ethyl acetate fraction.

constituent of CRD, also dominated the chromatographic profile of AF. Peak 1 was identified as gallic acid by comparing its retention time with that of an authentic standard. The relative distribution of gallic acid in CRD was 0.48%, but much higher (2.06%) in EAF, which showed the highest antioxidant capacity among the test samples. This suggested that gallic acid is the most potent antioxidant in CRD. However, other phenolic components may also act individually or synergistically and contribute to the antioxidant property of CRD. This was also substantiated by the fact that despite their much lower gallic acid contents, AF (0.3%) and EF (0.04%) showed less, but significant antioxidant activity. Previously, hypophyllanthin was identified as the major ingredient of the whole plant extract of *P.* amarus.[39] But in our studies the alkaloid was absent in CRD, as reported earlier.[14]

HPLC-APCI-MS/MS. During MS analysis, the glycosidic linkage of the phenolic glycosides gets cleaved. Hence, mass fragments at m/z 162 amu (hexose, glucose, or galactose), 146 amu (deoxyhexose, rhamnose), 132 amu (pentose, xylose, or arabinose), and 176 amu (glucuronic acid) or loss of these fragments is observed. Usually the distribution pattern of the sugars and acids remains unchanged from that of the precursor flavonoids. Likewise, the presence of gallic acid can be inferred by the mass fragments at m/z 169 ($[M - H]$ ion of gallic acid) and/or a peak at m/z 125, derived by the loss of a CO_2 unit from the $[M - H]^-$ ion peak.

Presently, EAF was analyzed to obtain several peaks (Figure 3). For convenience the same HPLC peak numbers were assigned to the corresponding compounds. All the LC-separated compounds furnished their respective pseudomolecular ions $[M - H]^-$ peaks. Many of these also showed characteristic signatures of glycosidic and gallic acid moieties. The mass fragments of some of the compounds also showed fragmentation ions due to malonyl (m/z 86) and methoxycinnamic acid units.[40] Twenty-eight compounds were completely/partially identified using the $[M - H]^-$ ion peaks and fragmentation patterns, discussed above. Table 3 shows the pseudomolecular ion and base peaks along with the mass peaks of the characteristic fragmentations.

Fourteen compounds (peak nos. 1, 2, 9, 10, 12−15, 17−19, 21, 22, 24) showed mass fragmentation peaks due to the gallic acid moiety, indicating them to be gallic acid derivatives. Peak 1 was identified as gallic acid by comparison of its mass spectrum with that of standard gallic acid. Peaks 2 and 13 were assigned to two different monogalloyl hexose derivatives, based on the above criteria and reported mass fragmentation of monogalloyl glucose.[41] Peak 9 was a galloyl methoxycinnamic acid hexoside. Peaks 10, 14, and 24 could not be completely identified, while peaks 21 and 22 may be some isomeric gallic acid derivatives, because their mass spectra were similar. Peak 12 was assigned to a gallic acid trimer derivative. The HPLC peaks 15, 17, and 18 showed a mass fragmentation at m/z 255, accounting for a gallic acid (m/z 169) and a malonyl (m/z 86) moiety. This suggested them to be malonyl gallate derivatives. Further, peak 17 is likely to be the rhamnoside of peak 18, because the difference in their molecular weights was 146 amu. The HPLC peak 19 was assigned as galloyl HHDP glucopyranose by comparing its mass fragmentation pattern with those reported earlier.[37]

On the basis of reported[42] mass fragmentation and the loss of 148 mass units from the parent ion $[M - H]^-$, peak 3 could be either gentisic acid rhamnoside or protocatechuic acid rhamnoside. Comparison of the R_f values of the hydrolyzed products with those of standard compounds confirmed it as gentisic acid rhamnoside. Peak 5 could be an epicatechin or its isomer, catechin.[43] On the basis of retention times of the standard compounds, it was identified as epicatechin. For peak 4, the fragmentation ions at m/z 169 and at m/z 305

Table 3. Retention Times (t_R), Pseudomolecular Ions, and Fragment Ions of EAF[a]

peak no.	t_R (min)	MW	$[M-H]^-$ m/z	MS^2 $[M-H]^-$ m/z	compound
1	13.33	170	169	**125**	gallic acid
2	15.8	350	349	271, 211, 169, **125**	monogalloyl glucose derivative
3	15.85	302	301	**153**, 109	gentisic acid rhamnoside
4	17.12	458	457	331, 305, 169, **125**	epigallocatechin gallate
5	17.9	290	**289**	245, 205, 179	epicatechin
6	19.1	442	441	289, 169, **125**	epicatechin gallate
7	22.6	462	**461**	285, 257, 169,	kaempferol glucuronide
8	24.99	584	583	**437**, 285, 125	kampferol- galloyl rhamnoside
9	27.25	492	491	339, **177**, 125	galloyl methoxycinnamic acid hexoside
10	29.45	526	525	327, 293, 236, 213, **125**	gallic acid derivative
11	31.12	710	**709**	447, 285	2′-hydroxygenistein 4′, 7-*O*-glucoside malonylated
12	37.05	586	585	473, **125**	derivative of gallic acid trimer
13	39.29	388	387	271, **125**	monogalloyl glucose derivative
14	41.4	666	665	279, 169, **125**	gallic acid derivative
15	44.78	282	281	**255**, 125	malonyl gallate derivative
16	47.8	602	601	271, 221, **125**	genistein- monogalloyl glucose
17	48.4	632	631	487, 364, 255, **125**	malonyl gallate derivative rhamnoside
18	50.3	488	487	441, 255, **125**	malonyl-galloyl derivative
19	51.38	634	633	463, 301, 284, **125**	gallol HHDP glucopyranose
20	52.8	464	463	**301**, 179, 151	quercetin 3-*O*- glycoside
21	60.67	839	838	748, **712**, 257, 125	gallic acid derivative
22	63.07	839	838	754, **712**, 125	isomer of 839
23	65.2	551	550	463, **301**	quercetin 3-malonylglucoside
24	66.2	727	726	621, 312, 212, **125**	gallic acid derivative
25	68.1	759	758	550, 463, **301**	quercetin 3-malonylglycoside derivative
26	72.88	887	886	776, **740.6**, 579, 271, 125	naringin glycoside derivative
27	76.7	887	886	776, **740.6**, 579, 271, 125	isomer of naringin glycoside derivative
28	79.86	580	**579**	459, 271, 235	naringin

[a]Obtained by HPLC-APCI-MS/MS analysis. The base peaks are indicated in bold font.

corresponded to gallic acid and epigallocatechin, respectively. Therefore, it was identified as epigallocatechin gallate, which had an identical mass fragmentation pattern with the standard molecule.[42] Peak 6 was identified as epicatechin gallate by comparing with the reported fragmentation pattern of an authentic sample.[42] The HPLC peak 7 was attributed to kaempferol glucuronide, as its mass spectrum showed a loss of 176 mass units due to glucuronic acid as well as the reported mass spectral features of kaempferol.[38] Likewise, peak 8 was kampferol galloyl rhamnoside. The HPLC peak 11 was due to the known compound 2′-hydroxygenistein 4′,7-*O*-glucoside malonylated,[44] while peak 16 was identified as genistein monogalloyl glucose considering the mass spectrum of peak 11 and that of monogalloyl glucose. The mass spectra of peaks 20, 23, and 25 showed identical fragmentation patterns as that of standard quercetin (m/z 301) and additional fragmentation peaks due to hexose and malonyl moieties. Hence these were identified as quercetin 3-*O*- glycoside, quercetin 3-malonylglucoside, and a quercetin 3-malonylglucoside derivative, respectively. The mass spectrum of peak 28 matched very well with that of naringin.[43] The mass spectra of peaks 26 and 27 were identical, showing a mass fragmentation peak at m/z 579 (naringin) along with breakdown of a hexose. Hence these were identified as two isomeric naringin glycoside derivatives.

To the best of our knowledge, the presence of galloyl methoxycinnamic acid hexoside, gallol HHDP glucopyranose, kampferol galloyl rhamnoside, genistein monogalloyl glucose, quercetin 3-malonylglucoside, naringin, epicatechin, and epicatechin gallate in *P. amarus* is reported here for the first time. Although *P. amarus* is known to contain epigallocatechin, the presence of epicatechin and epicatechin gallate in the plant is reported for the first time. Earlier, the presence of quercetin and quercetin 3-*O*-glycoside was reported in *P. amarus*.[7] Presently we identified quercetin 3-malonylglucoside also as one of the plant constituents. Besides gallic acid, compounds such as epicatechin and quercetin can effectively reduce oxidative stress.[41,42] Numerous preclinical studies have shown that kaempferol and some of its glycosides have a wide range of pharmacological activities.[37] On the basis of our results, it is tempting to propose *P. amarus* root as a potent antioxidant, and this warrants further *in vivo* studies to ascertain its prophylactic/curative property against various diseases.

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Funding

We are grateful to Board of Research in Nuclear Sciences (2008/37/19/BRNS) for funding this scientific work. We would like to thank UGC-CAS and DST-FIST for providing infrastructural support in the Department of Biochemistry, University of Calcutta, India.

Notes

The authors declare no competing financial interest.

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