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Isolation, purification and identification of ellagic acid derivatives, catechins, and procyanidins from the root bark of *Anisophyllea dichostyla* R. Br.

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Abstract

The root bark of *Anisophyllea dichostyla* R. Br. is traditionally used in the Democratic Republic Congo for the treatment of several conditions such as anorexia, fatigue and intestinal infections. We have identified and quantitated several polyphenol antioxidants in the methanol extract of the root bark (120 g). The polyphenol content (3.32 g/kg) was predominantly ellagitannins (25%) and polyhydroxyflavan-3-ols (catechins and procyanidins, 75%) with 3′-*O*-methyl-3,4-methylenedioxo ellagic acid 4′-*O*-β-D-glucopyranoside and (–)-epicatechin as the major species in each class. These two compounds and the following species were identified unequivocally by NMR spectroscopy: (+)-catechin, (−)-epicatechin 3-*O*-gallate, 3-*O*-methyl ellagic acid, 3,3′-di-*O*-methyl ellagic acid, 3′-*O*-methyl-3,4-methylenedioxo ellagic acid, 3′-*O*-methyl-3,4-methylenedioxo ellagic acid 4′-*O*-β-D-glucopyranoside, and 3′-*O*-methyl ellagic acid 4-*O*-β-D-xylopyranoside. The following additional compounds were purified by semi-preparative HPLC and tentatively identified on the basis of UV spectra, HPLC–ESI-MS and nano-ESI-MS–MS: (+)-catechin-3-*O*-β-D-glucopyranoside, epicatechin-(4β → 8)-catechin (procyanidin B_1), epicatechin-(4β → 8)-epicatechin (procyanidin B_2), an (epi)catechin trimer, 3-*O*-methyl ellagic acid 4-*O*-β-D-glucopyranoside, (–)-epicatechin 3-*O*-vanillate, 3,4-methylenedioxo ellagic acid 4′-*O*- β-D-glucopyranoside, and 3,3′-di-*O*-methyl ellagic acid 4-*O*-β-D-xylopyranoside. Fractionation of the raw extract by column chromatography on silicic acid yielded 10 fractions. In the hypoxanthine/xanthine oxidase antioxidant assay system, CC-9 which contained a range of polyphenols dominated by (−)-epicatechin-*O*-gallate proved to be the most potent antioxidant fraction ($IC_{50} = 52$ μg/mL) in terms of ROS scavenging. In terms of XO inhibition CC-8, dominated by (epi)catechin trimer and which also contained appreciable amounts of 3′-*O*-methyl ellagic acid 4′-*O*-β-D-xylopyranoside, as well as the catechins (+)-catechin-3-*O*-β-D-glucopyranoside, epicatechin-(4β → 8)-catechin (procyanidin B_1), and (−)-epicatechin 3-*O*-gallate, proved to be the most potent ($IC_{50} = 36$ μg/mL).
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Keywords: *Anisophyllea dichostyla*; Antioxidant capacity; Chemoprevention; Ellagitannins; HPLC; HPLC–ESI-MS; NMR; Procyanidins; Xanthine oxidase

Abbreviations: BSTFA, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide; CC, Column chromatography; COLOC, Correlation spectroscopy for long-range couplings; COSY, Correlation spectroscopy; DCM, Dichloromethane; DEPT, Distortionless enhancement polarization transfer; DMSO, Dimethyl sulphoxide; D_2O, Deuterium oxide; nano-ESI-MS, Nano-electrospray mass spectrometry; NOE, Nuclear Overhauser effect; HPLC, High-performance liquid chromatography; HPLC–ESI-MS, High-performance liquid chromatography electrospray ionization mass spectrometry; NMR, Nuclear magnetic resonance; ROESY, Rotating-frame Overhauser-effect spectroscopy; ROS, Reactive oxygen species; TFA, Trifluoroacetic acid; TMS, Tetramethylsilane; XO, Xanthine oxidase.

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1. Introduction

Polyhydroxyflavan-3-ols such as the catechins and their oligomers, the procyanidins, are present in many commonly consumed foods (fruits, cereals, beans, nuts, spices) and drinks such as grape juice, tea, red wine and beer (Prior and Gu, 2005). On the other hand, ellagitannins, which are widespread in traditional medicinal plants (Okuda, 2005), are poorly represented in common foods and dietary intake is limited to a few fruit and nut species (strawberries, raspberries, blackberries and walnuts; Daniel et al., 1989; Narayanan et al., 1999). Evidence is now accumulating that polyhydroxyflavan-3-ols and ellagitannins taken in the diet may be beneficial to health.

In preclinical studies many groups have shown that catechin oligomers (mainly in the form of green and black tea extracts) inhibit carcinogenesis in the skin, lung, liver, small intestine, pancreas, colon and mammary gland (Laskin et al., 1992; Cao et al., 1996; Landau et al., 1998; Suganuma et al., 2001; Majima et al., 1998; Caderni et al., 2000; Weisburger et al., 1997). Furthermore tea catechins, especially epigallocatechin gallate, have been shown to cause growth inhibition and apoptosis in a number of human tumor cell lines in vitro, e.g., leukaemia and cancers of the prostate, breast, liver and colon (Chung et al., 2001; Valcic et al., 1996; Lea et al., 1993; Uesato et al., 2001). Tea catechin administration has also been shown to inhibit oxidative stress induced by carcinogens and tumor promoters (Huang et al., 2003; Xu et al., 1992).

The relatively low toxicity of ellagic acid and the ubiquitous occurrence of ellagitannins throughout the plant kingdom has led to many investigations related to cancer chemoprevention. In preclinical studies, ellagic acid was reported to have a wide range of chemopreventive activities, inhibiting chemically induced carcinogenesis in the esophagus, lung, tongue, colon and skin (Mandal and Stoner, 1990; Boukharta et al., 1992; Tanaka et al., 1993; Rao et al., 1991; Mukhtar et al., 1986).

Synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and propyl gallate have been used since the beginning of the 20th century. However, restrictions on the use of these compounds are now being imposed because of their carcinogenicity (Mahdavi and Salukhe, 1995). Therefore, there is growing interest in the cancer chemopreventive properties of natural secondary plant metabolites and their potential use as antioxidants in the food and pharmaceutical industries.

In our continuing investigations into chemopreventive agents from medicinal plant sources, ethnobotanic investigations of the species *Anisophyllea dichostyla* R. (*Rhizophoraceae*) were conducted in collaboration with local physicians in the province of Lemfu in the Democratic Republic of Congo (DRC) between 1981 and 1999. *Anisophyllea dichostyla* R. (*Rhizophoraceae*) has different names in the various ethnic regions: Mfungu (Kintandu), Mundulu (Kipende), Mbila esobe (Lingala). *A. dichostyla* is used as a medication against anorexia, fatigue and intestinal infections, among other maladies. Two spoonsful of the infused root bark powder in a glass of water is administered three times a day before meals.

However, in spite of the medicinal value and high consumption of *A. dichostyla* in tropical Africa, literature data are extremely sparse with regard to the medicinal chemistry of this plant. To date, no studies have been conducted to identify the therapeutic compounds present, or to investigate their cancer chemopreventive potential. Here we describe the isolation, identification and quantitation of a number of polyphenol compounds (flavanols, ellagic acid derivatives) found in methanolic extracts of *A. dichostyla* root bark. The extracts were analyzed by gradient reversed-phase HPLC with diode array detection followed by HPLC–ESI-MS. Preparative HPLC was carried out to purify all of the compounds for nano-ESI-MS. When sufficient sample quantities were obtained, detailed NMR spectroscopic analysis was performed. The antioxidant capacity of the raw extract and fractions obtained by column chromatography was also evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Acetic acid, acetonitrile, dichloromethane (DCM), hypoxanthine, xanthine oxidase [EC 1.1.3.22], salicylic acid and anhydrous sodium sulphate were obtained from E. Merck (Darmstadt, Germany); methanol, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (BSTFA) and trifluoroacetic acid (TFA) from Sigma (Deisenhofen, Germany); silica gel 60 from ICN Biomedicals (Eschwege, Germany) and ellagic acid, (−)-epicatechin, (+)-catechin, (−)-epicatechin-*O*-gallate, epicatechin-(4β → 8)-catechin (procyanidin B_1), and epicatechin-(4β → 8)-epicatechin (procyanidin B_2) from Extrasynthese (Lyon Nord, Genay, France).

2.2. Plant material

Root bark of *Anisophyllea dichostyla* R. Br. was collected in the province of Lemfu in Kinshasa, DRC, in July 1999. The bark (120 g) from several aged plants was dried at ambient temperature and thereafter stored in the dark prior to processing. Plant identification was conducted at the herbarium in the Department of Sciences and Health, Institute of Agronomic Research Studies (INERA, Kinshasa, Democratic Republic of Congo) where duplicate voucher specimens of *A. dichostyla* R. Br. are deposited.

2.3. Extraction protocol

A. dichostyla root bark (120 g) was lixiviated with methanol (1.5 L) over a period of 3 h after defatting with hexane (500 mL). The extract was evaporated to dryness on a rotary evaporator in vacuo at 35 °C.

2.4. Column chromatography on silicic acid

The dried residue (25.3 g) was suspended in methanol (50 mL), immobilized on silicic acid (20 g) by lyophilisation, and subjected to column chromatography (CC), using a 38 × 4.5 cm glass column filled with silica gel 60 (mesh size: 70–230) in DCM to a level ca. 5 cm from the top. The immobilised extract was added to the free volume at the head of the column. After bedding down of the gel material, fractionation was conducted by successive applications of hexane, DCM, and methanol (1%, 2%, 5%, 10%, 20%, 30%, 50% and 100%) in DCM (250 mL). Fractions (250 mL) were collected, and the solvent was removed by rotary evaporation in vacuo at 35 °C. Dried fractions were suspended in methanol (5.0 mL) and diluted

when necessary prior to analytical high-performance liquid chromatography (HPLC). Phenolic compounds in the relevant fractions were purified by semi-preparative HPLC for spectroscopic analysis.

2.5. Analytical HPLC

Analytical HPLC was conducted on a Hewlett-Packard HP 1090 liquid chromatograph fitted with a C_{18} reversed-phase column (250 × 4.0 mm, 5 μm; Latek, Eppelheim, Germany). Samples of *A. dichostyla* extracts were dissolved in methanol (5.0 mL) and, when necessary, further diluted prior to injection (20 μL) into the HPLC. The mobile phase consisted of 2% acetic acid in water (solvent A) and methanol (solvent B) with the following gradient: 95% A for 2 min, to 75% A in 8 min, to 60% A in 10 min, to 50% A in 10 min and 0% A until completion of the run (Owen et al., 2000a, 2003). The flow rate was 1 mL/min. Phenolic compounds in the eluate were detected at 250, 278 and 340 nm with a diode array UV detector (HP 1040M). Quantitation of known catechins and ellagitannins in the raw extract and fractions obtained by CC was performed against standard curves of ellagic acid, (−)-epicatechin, (+)-catechin, (−)-epicatechin-*O*-gallate, epicatechin-(4β → 8)-catechin (procyanidin B_1), and epicatechin-(4β → 8)-epicatechin (procyanidin B_2) respectively, whereas other polyphenols with either a catechin or ellagic acid type basic structure were quantitated using standard curves prepared with (−)-epicatechin and ellagic acid. These were all conducted in the concentration range 0–4 mM.

2.6. HPLC–ESI-MS

HPLC–ESI-MS was conducted on an Agilent 1100 HPLC, coupled to an Agilent single quadrapole mass-selective detector (HP 1101; Agilent Technologies, Waldbronn, Germany). Chromatographic separation of methanolic extracts was conducted using a C_{18}, reversed-phase (5 μm) column (250 × 4 mm I.D.; Latek, Eppelheim, Germany). The mobile phase consisted of 2% acetic acid in water (solvent A) and acetonitrile (solvent B) with the following gradient: 95% A for 10 min, to 90% A in 1 min, to 60% A in 9 min, to 80% A in 10 min, to 60% A in 10 min, to 0% A in 5 min and continuing at 0% A until completion of the run. Detection of phenolic compounds was by means of UV absorbance (A) at 278 and 340 nm at room temperature. Mass spectra in the negative-ion mode were generated under the following conditions: fragmenter voltage = 100 V, capillary voltage = 2500 V, nebulizer pressure = 30 psi, drying gas temperature = 350 °C, mass range = 50–3000 D. The mobile phase (A + B solvent gradient) was employed as for analytical HPLC over a total run time of 50 min. Instrument control and data handling were performed with the same software as for analytical HPLC.

2.7. Semi-preparative HPLC and fraction collection

Semi-preparative HPLC was conducted on an Agilent 1100 liquid chromatograph fitted with a reversed-phase C_{18} column (10 mm I.D.; Latek, Eppelheim, Germany) similar to that used for analytical HPLC. Acetonitrile was used instead of methanol as mobile phase with a flow rate of 3 mL/min. Peaks eluting from the column were collected on an Agilent HP 220 Microplate Sampler. Each purified fraction was pooled, and solvent was removed by lyophilisation.

2.8. Nano-ESI-MS

Samples purified by semi-preparative HPLC were dissolved in methanol, and spectra were recorded on a Finnigan MAT TSQ 7000 triple-quadrupole mass spectrometer (Finnigan, San Jose, California, USA) equipped with a nanoelectrospray source (EMBL, Heidelberg, Germany), using both the positive- and negative-ion modes. Argon was used as collision gas at a nominal pressure of 2.5 mTorr (1 Torr = 133.3 Pa). Gold-plated glass capillaries for sample spraying were prepared in house using a microcapillary puller (Type-87-B, Sutter Instruments, Novato, California, USA). The applied voltage was 400–700 V, and the mass scan range was 20–2600 D.

2.9. Acid hydrolyses

Dried residues were taken up in 0.5 N H_2SO_4, incubated for 3 h at 37 °C and processed as described previously (Owen et al., 2000b).

2.10. NMR spectroscopy

For compounds that could be purified in sufficient quantities (1–5 mg), ^{1}H and ^{13}C NMR spectra were recorded at 500.13 and 125.76 MHz, respectively, using 5-mm sample tubes on a Bruker AM-500 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Typically, CD_3OD was used as solvent for measurements at 30 °C. However, for the ellagitannins solvent mixtures were sometimes used, with a trace of TFA added to reduce line broadening due to proton exchange, as noted in the tables. Conventional 1D Fourier transform techniques were employed for all compounds (^{1}H observe, ^{13}C observe with broadband ^{1}H decoupling, ^{13}C DEPT). For structure elucidation and complete spectrum analysis, the following additional experiments were performed as necessary: ^{13}C observation with selective ^{1}H decoupling, 2D H,H-COSY, 2D CH correlation via $^1J_{CH}$ couplings (polarization transfer with remote ^{1}H decoupling) or via long-range $^nJ_{CH}$ (COLOC or COLOC-S). Stereochemical assignments were made with the aid of 1D nuclear Overhauser (NOE) difference spectra or 2D ROESY experiments. Analysis of chemical shifts and spin–spin couplings was performed with Bruker's WIN-NMR software for PCs. Molecular modeling with the MM2 force field was performed with ChemOffice 2004 (CambridgeSoft, Cambridge, MA). ^{1}H and ^{13}C chemical shifts δ are reported in ppm relative to TMS with an internal reference in mixed solvents or indirectly via pure solvent signals: CHD_2OD = 3.31 ppm for ^{1}H; CD_3OD = 49.05 ppm for ^{13}C; DMSO-d_5 = 2.49 ppm for ^{1}H; DMSO-d_6 = 39.50 for ^{13}C. With very few exceptions (noted in the tables) all NMR assignments are unequivocal.

2.11. Measurement of antioxidant capacity

The hypoxanthine/xanthine oxidase system in 1.0 mL phosphate buffer was used as described in detail previously (Owen et al., 2000a, 2003; Sudjaroen et al., 2005) to assay the antioxidant capacity of aliquots (range = 1–500 μL) of the raw methanol extract of *A. dichostyla*, taken up in a volume of 50 mL of methanol, or the individual CC fractions dissolved in 5.0 mL methanol. Scavenging of ROS generated by xanthine oxidase was measured as the inhibition of the hydroxylation of salicylic acid to produce 2,3- and 2,5-dihydroxybenzoic acids (DHBA), which were analysed by HPLC via their absorbance at 325 nm. Inhibition results are expressed, where possible, as IC_{50} values (50% inhibition) in μg/mL for the tested solution of raw extract or CC fraction. In addition, the direct inhibition of xanthine oxidase by phenolics was monitored by the decrease in the hydroxylation of xanthine to form uric acid, as detected by HPLC and the absorbance at 278 nm. The products of the enzymic or free-radical reactions were quantitated using appropriate standard curves prepared with the pure compounds.

2.12. Statistics

For the antioxidant assay described above the volume of phenolic extracts producing a 50% inhibition of oxidation (IC_{50}) was determined using the table curve program (Jandel Sciencific, Chicago, IL).

3. Results

3.1. Isolation and identification of polyphenols

The analytical HPLC chromatograms obtained from the raw methanol extract of *A. dichostyla* root bark and from various fractions obtained by column chromatography of the extract are compared in Fig. 1. A total of 16 polyphenol

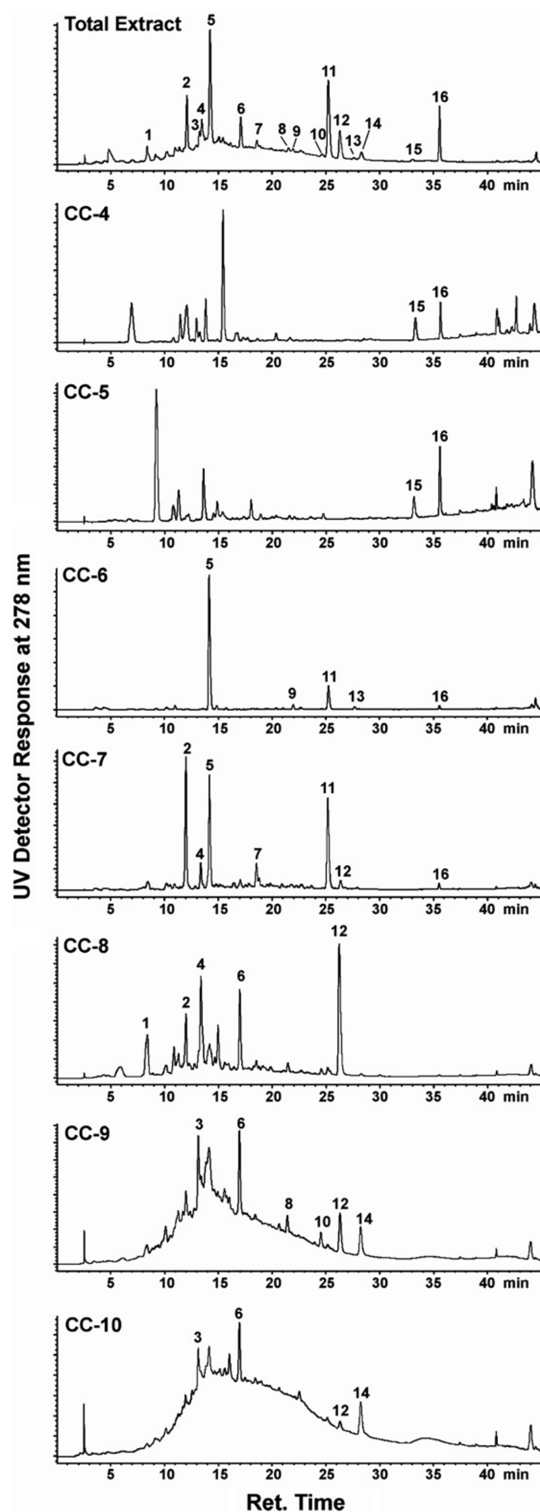


Fig. 1. Analytical HPLC chromatograms as monitored by UV absorption at 278 nm for the total methanolic extract of the root bark of *A. dichostyla* (top) and for the fractions CC-4 to CC-10 in methanol, as obtained by column chromatography of the extract on silicic acid. The peaks numbered with arabic numerals in the order of increasing retention time (Table 1) correspond to the compounds labelled with roman numerals in Schemes 1 and 2.

compounds in two substance classes have been identified: catechins and derivatives or oligomers (procyanidins) as shown in Scheme 1 and the ellagic acid derivatives (ellagitannins) shown in Scheme 2. The compounds are numbered with corresponding roman (structures) or arabic numerals (chromatography peaks) according to their retention times in the analytical HPLC (Fig. 1). The 16 compounds shown were also identified in the raw extract by HPLC–ESI-MS, as illustrated in Fig. 2, where variation in solvent system and HPLC conditions compared to Fig. 1 led to different retention times and reversal of the order of compounds 2 and 3.

The majority of the isolated phenolics were tentatively identified by their UV spectra and their analytical HPLC retention times in comparison with reference compounds by HPLC–ESI-MS in the negative-ion mode and nano-ESI-MS–MS in both the negative- and positive-ion modes. The relevant analytical data are summarized in Table 1. For compounds **III**, **V**, **VI**, **XI**, **XII**, and **XIV–XVI** sufficient quantities were purified by HPLC for definitive identification by NMR as described below.

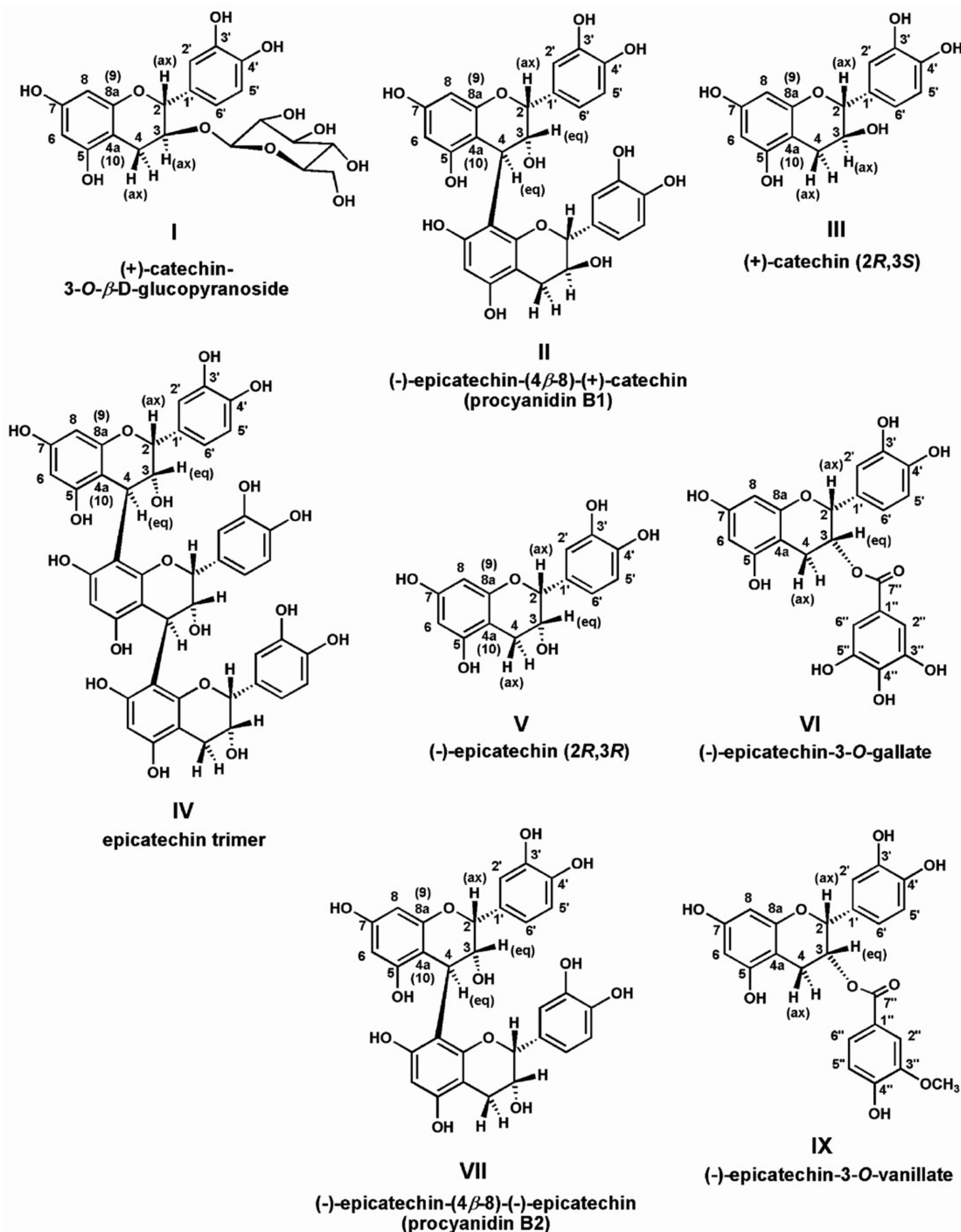
Acid hydrolysis and identification of the products by HPLC–ESI-MS provided additional supporting evidence for several compounds. The following products were obtained from the compounds given: catechin from **I**; 3-*O*-methyl ellagic acid from **VIII**; epicatechin and vanillic acid from **IX**; 3,4-methylenedioxo ellagic acid from **X**; 3, 3′-*O*-dimethyl ellagic acid from **XIII**. However, final confirmation of the precise structural details of the original phenolics (substituent positions, glycoside configuration and linkage) requires NMR analysis when sufficient amounts of material have been isolated.

3.2. Quantitation of polyphenols

The 16 identified polyphenols in the methanol extract of *A. dichostyla* root bark were quantified as described in Section 2, and the results are summarized in the last two columns of Table 1. The total yield of identified polyphenols in root bark of *A. dichostyla* was 3.32 g/kg dry weight. Of the compounds identified, 25% by weight were ellagic acid derivatives and 75% were polyhydroxyflavan-3-ols, comprising catechins and procyanidins. The major component in the ellagitannin class was 3′-*O*-methyl-3,4-methylenedioxy ellagic acid glucoside **XI** (12.8% of total polyphenols) while the major flavanol was (−)-epicatechin **V** (31.2% of total polyphenols).

3.3. Antioxidant capacity

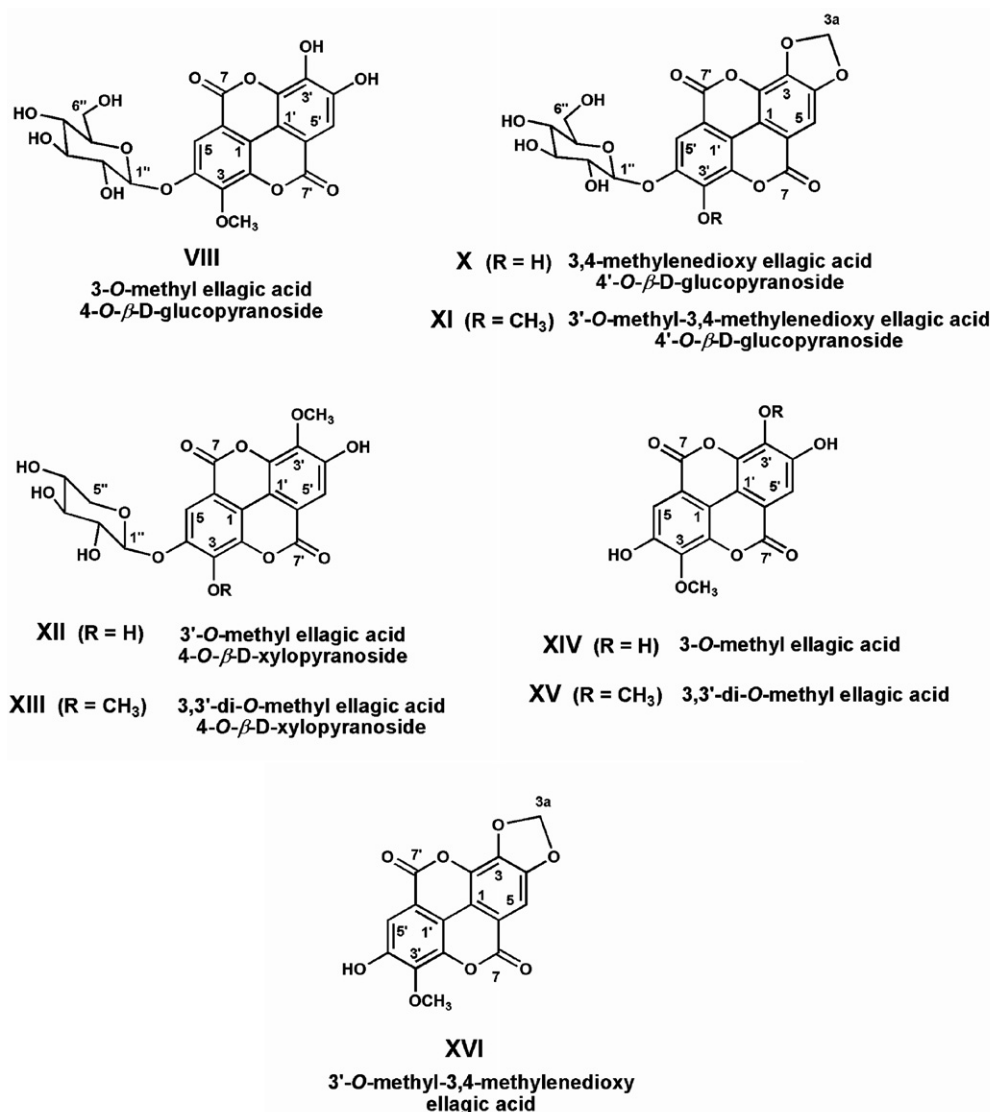
The raw methanol extract from the root bark of *A. dichostyla* exhibited an extremely potent antioxidant capacity, as illustrated in Fig. 3. The hypoxanthine/xanthine oxidase system (see Section 2) was used to assay scavenging of reactive oxygen species (ROS) such as hydroxyl radicals, i.e., the inhibition of the hydroxylation of salicylic acid to form dihydroxybenzoic acids (DHBA). In an assay volume of 1.0 mL, an IC_{50} of 262 μg/mL was obtained for the raw extract dissolved in 50 mL of methanol. For direct inhibition of xanthine oxidase (XO)



Scheme 1. Structures of the polyhydroxyflavan-3-ols and their derivatives identified in the methanolic extract of the root bark of *A. dichostyla*. The roman numerals correspond to the arabic numbers labelling the peaks in the analytical HPLC chromatograms of Fig. 1. The notations ax and eq denote protons in approximately axial or equatorial positions, respectively. The structure for epicatechin trimer represents one possible, as yet unconfirmed structure.

and the formation of uric acid, an IC_{50} of 42 μg/mL was determined.

The antioxidant capacities of the various CC fractions (see Fig. 2) obtained from the raw extract by column chromatography on silicic acid and dissolved in 5.0 mL methanol are summarized in Table 2. Fractions CC-6 to -9 exhibited significant ROS scavenging with IC_{50} values in the range 52–241 μg/mL, however, only fractions CC-7 and -8 showed significant inhibition of XO with IC_{50} values in the range 36–106 μg/mL. Fraction CC-9 which contained a range of polyphenols dominated by (−)-epicatechin-*O*-gallate proved to be the most potent antioxidant fraction ($IC_{50} = 52$ μg/mL) in terms of ROS scavenging. Fraction CC-8, dominated by (epi)catechin trimer and which also contained appreciable amounts of 3′-*O*-methyl ellagic acid 4′-*O*-β-D-xylopyranoside, as well as the catechins (+)-catechin-3-*O*-β-D-glucopyranoside, epicatechin-(4β → 8)-catechin (procyanidin B_1), and (−)-epicatechin



Scheme 2. Structures of the ellagic acid derivatives identified in the methanolic extract of the root bark of *A. dichostyla*. The roman numerals correspond to the arabic numbers labelling the peaks in the analytical HPLC chromatograms of Fig. 1.

3-*O*-gallate, proved to be the most potent ($IC_{50} = 36$ μg/mL).

3.4. Structure elucidation and NMR signal assignments

Tables 3–6 summarize the NMR data and signal assignments obtained for compounds that were obtained in sufficient quantity and purity, as noted in Table 1. These results provided unequivocal determination of structures and stereochemistry. The key evidence and arguments used to define the structures shown are briefly described in the following sections.

3.4.1. Catechin and epicatechin

The data from the spectra obtained for compounds **III** and **V** in CD_3OD are listed in Tables 3 and 4 and show excellent agreement with the spectra obtained from authentic (+)-catechin and (−)-epicatechin, respectively, in the same solvent. The vicinal J_{HH} couplings for protons at positions 2–4 confirmed the relative stereochemistry at these centers (see Scheme 1). For catechin (**III**) irradiation of H2′ gave a weak but measurable NOE of 0.3% for the signal assigned to H8 and no NOE at H6, as expected from modeling. The assignments for H6 and H8 led to the assignments of carbon shifts in the order C6 > C8, in agreement with literature data (Bae et al., 1994), and long-range J_{CH} correlations gave the assignments shown for C5, C7, C8a. For epicatechin (**V**) the initial assumption that $\delta_{C6} > \delta_{C8}$ led to the assignments shown for H6, H8, C5, C7 and C8a, in agreement with the relative shifts given by Cui et al. (1992). After about 3 weeks in CD_3OD both catechin and epicatechin exhibited substantial deuteration at C6 and C8. The resulting patterns of ^{13}C shifts for the four possible isotopomers and the associated multiple-bond H,D isotope shift effects provided further confirmation for the ^{13}C assignments for C5, C7 and C8a.

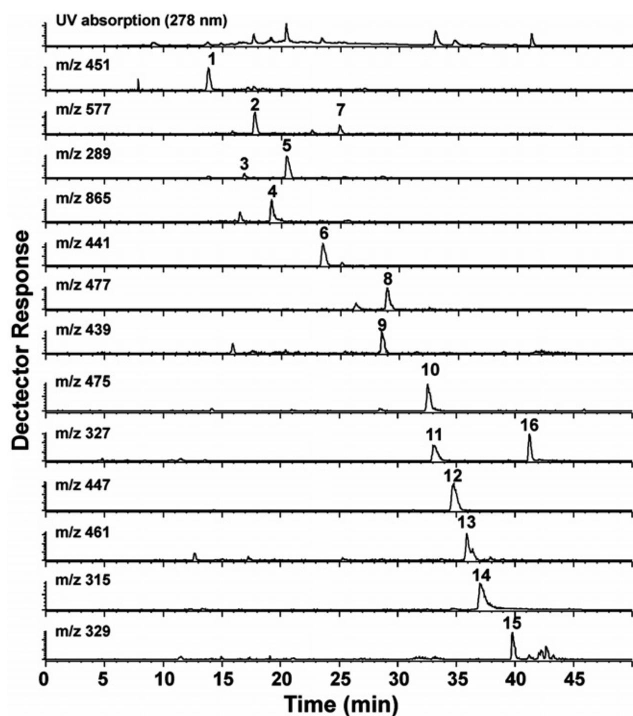


Fig. 2. HPLC–ESI-MS chromatograms (negative-ion mode) of a methanolic extract of the root bark of *A. dichostyla*. Top: UV chromatogram (278 nm) under the conditions used for HPLC–ESI-MS. The traces labelled with individual *m*/*z* values for $[M–H]^-$ ions represent ion-selective chromatograms for the compounds listed in Table 1. The arabic numerals correspond to the roman numerals in Table 1 and Schemes 1 and 2. *Note:* the chromatographic conditions and retention times differ from those for analytical HPLC (Fig. 1), leading to a reversal of the order for peaks 2 and 3.

3.4.2. Epicatechin 3-*O*-gallate

For compound **VI** less than 2 mg of substance were available, and substantial deuteration at C6 and, in particular, C8 after 2–3 weeks in CD_3OD hampered extensive ^{13}C experiments with long measurement times. The ^1H spectrum showed the spectral patterns typical for epicatechin and gallate fragments (e.g. 1-*O*-gallate in glucose-based ellagitannins), and the large positive shifts at H3 and C3 relative to epicatechin provided sufficient evidence for the identification of **VI** as the 3-*O*-gallate derivative.

3.4.3. Ellagitannins

The NMR data for the ellagitannins studied are summarized in Tables 5 and 6, together with our own reference data for ellagic acid, including the long-range C–H couplings that were the key to assigning quaternary carbon signals. Since the ellagic acid molecule has C_2 symmetry (2×7 carbon sites), the corresponding primed and unprimed sites in the two halves of the molecule (see Scheme 2) have the same chemical shifts. These are listed in Tables 5 and 6 for completeness and ease of comparison across rows of the tables. Note that C1 and C6 can be readily distinguished on the basis of the magnitude of their ^3J and ^2J couplings with H5, respectively, and the presence of a ^4J coupling between C1 and H5′. Our carbon assignments for ellagic acid in $CD_3OD/CDCl_3$ agree with the relative shifts given by Li et al. (1999) and Nawwar et al. (1994) for a DMSO-d_6 solution. However, the assignments for C1,C6 are reversed in some earlier studies (Khac et al., 1990). The various substances examined in our study were not uniformly soluble in methanol. Therefore, mixed solvents were used in a number of cases.

3.4.4. 3-*O*-methyl ellagic acid

Compound **XIV** exhibited a doubling of the characteristic ellagic acid signals (14 carbon sites) due to the presence of one –OMe substituent, breaking the C_2 symmetry of the parent compound. Initially, the spectra were plagued with broad linewidths, but addition of a trace of TFA to the samples sharpened the signals dramatically and facilitated 2D long-range CH correlation experiments. The ^{13}C shift of ca. 61 vs. 57 ppm for the –OMe group was typical for substitution at C3 rather than C4 of ellagic acid (Sato, 1987). C3 exhibited ^3J CH correlations with H5 and the –OMe group. This established the proton shift order H5 > H5′, and the quaternary carbons in each half of the ellagic acid moiety could be completely assigned via the long-range CH correlations to the corresponding H5 or H5′ proton. Noteworthy are the chemical shift increments of 5.1, 3.7 and 4.2 ppm for C2, C4 and C6, respectively, in the 3-*O*-methyl derivative compared to ellagic acid (including solvent effects).

3.4.5. 3,3′-di-*O*-methyl ellagic acid

Compound **XV** exhibits NMR spectra consistent with C_2 symmetry and two equivalent –OMe substituents at C3,C3′. Compared to the monomethyl derivative, the dimethyl derivative exhibits shift increments for C2′, C4′ and C6′ of 4.6, 3.6 and 4.0, respectively. Our results agree very closely with data in the literature (Nawwar et al., 1982; Sato, 1987).

3.4.6. 3′-*O*-methyl-3,4-methylenedioxo ellagic acid

Compound **XVI** was measured in DMSO-d_6, and the spectra are consistent with a non-symmetrical ellagic acid moiety with one –OMe substituent at a C3 position and one unusual methylene group with C/H shifts of 104.16/6.368, corresponding to the bridging $–O–CH_2–O–$ moiety. As for the monomethyl ellagic acid derivative **XIV**, it was necessary to use long-range CH correlations to determine which aromatic proton signal corresponded to position 5 in the ring bearing the –OMe group at position 3. For all of the ellagitannins studied it was not possible to detect such a connectivity via NOE. Modelling indicates that the internuclear distances between H5 and 3-OMe protons will be more than 4.9 Å so that NOEs should be undetectable. The ring in question is labelled with primes in Tables 5 and 6 and exhibits a set of ^{13}C shifts and shift increments (relative to ellagic acid) at C2′, C4′ and C6′ due to the

Table 1
Analytical data for the polyphenolic compounds isolated from a methanolic extract of *A. dichostyla* root bark

Substance	NMR[a]	Formula	Exact mass (M)	HPLC–ESI-MS[b] (*m*/*z*)		Nano-ESI-MS[c] (*m*/*z*)			Quantitation	
				Rt (min)	$[M–H]^-$	$[M–H]^-$	$[2M–H]^-$	$[M + Na]^+$	mg/kg dry wt.	% of total
I (+)-catechin-3-*O*-β-D-glucopyranoside	–	$C_{21}H_{24}O_{11}$	452.132	8.5	451.1	451.2	903.2	n.d.	176	5.3
II epicatechin-(4β → 8)-catechin (procyanidin B_1)	–	$C_{30}H_{26}O_{12}$	578.142	12.1	577.1	576.9	n.d.	601.2	662	19.9
III (+)-catechin	+	$C_{15}H_{14}O_6$	290.079	13.2	289.1	288.9	579.0	313.1	69	2.1
IV epicatechin trimer	–	$C_{45}H_{35}O_{18}$	866.206	13.5	865.1	865.2	n.d.	889.4	484	14.6
V (–)-epicatechin	+	$C_{15}H_{14}O_6$	290.079	14.3	289.1	288.9	579.0	313.1	687	20.7
VI (–)-epicatechin 3-*O*-gallate	+	$C_{22}H_{18}O_{10}$	442.090	17.1	441.1	440.9	n.d.	n.d.	281	8.5
VII epicatechin-(4β → 8)-epicatechin (procyanidin B_2)	–	$C_{30}H_{26}O_{12}$	578.142	18.6	577.1	576.9	n.d.	601.2	98	3.0
VIII 3-*O*-methyl ellagic acid 4-*O*-β-D-glucopyranoside	–	$C_{21}H_{18}O_{13}$	478.075	21.6	477.1	477.0	955.1	n.d.	17	0.5
IX (–)-epicatechin 3-*O*-vanillate	–	$C_{23}H_{20}O_9$	440.111	22.0	439.1	439.0	n.d.	n.d.	29	0.9
X 3,4-methylenedioxo ellagic acid 4′-*O*-β-D-glucopyranoside	–	$C_{21}H_{16}O_{13}$	476.059	24.8	475.1	474.9	951.1	n.d.	16	0.5
XI 3′-*O*-methyl-3,4-methylenedioxo ellagic acid 4′-*O*-β-D-glucopyranoside	+	$C_{22}H_{18}O_{13}$	490.075	25.3	489.1	488.9	n.d.	n.d.	458	13.8
XII 3′-*O*-methyl ellagic acid 4-*O*-β-D-xylopyranoside	+	$C_{20}H_{16}O_{12}$	448.064	26.5	447.1	446.9	895.1	n.d.	158	4.8
XIII 3,3′-di-*O*-methyl ellagic acid 4-*O*-β-D-xylopyranoside	–	$C_{21}H_{18}O_{12}$	462.080	27.8	461.1	460.9	923.1	485.3	8	0.2
XIV 3-*O*-methyl ellagic acid	+	$C_{15}H_8O_8$	316.022	28.4	315.1	314.9	630.9	n.d.	33	1.0
XV 3,3′-di-*O*-methyl ellagic acid	+	$C_{16}H_{10}O_8$	330.038	33.1	329.1	328.9	658.9	n.d.	13	0.4
XVI 3′-*O*-methyl-3,4-methylenedioxo ellagic acid	+	$C_{16}H_8O_8$	328.022	35.5	327.1	326.9	654.9	n.d.	140	4.2
Total polyphenolics									3320	100

[a] For compounds without NMR confirmation (–), the precise stereochemistry, substituent linkage, and glycoside identity as shown are assumed.
[b] Online HPLC–MS with neg.-ion detection; Rt = retention time.
[c] Neg.- and pos.-ion ESI-MS after purification by semi-preparative HPLC; n.d. = not detected.

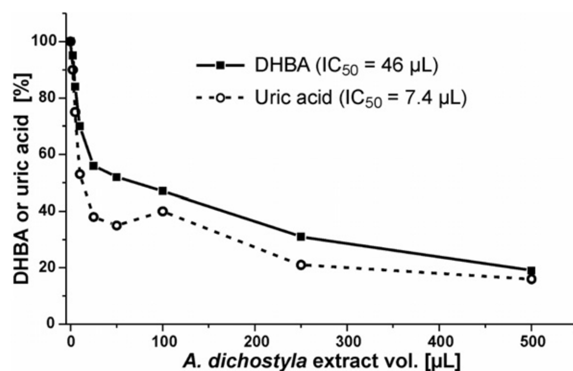


Fig. 3. ROS inhibition assays for the methanolic extract of the root bark of *A. dichostyla*. The solid squares represent the attack of reactive oxygen species on salicylic acid to form dihydroxy benzoic acids (DHBA), as monitored by absorbance at 325 nm. The open circles represent the formation of uric acid by xanthine oxidase, as monitored at 278 nm.

–OMe at C3′ that are very similar to the increments described above. The other (unprimed) half of the ellagic acid moiety also showed considerable ^{13}C shift increments (3.3, −5.7, −7.0, 2.8 ppm for C1, C2, C5, C6), consistent with a methylene bridge between the oxygens at positions 3 and 4. Thus, compound **XVI** was identified as a 3,4-methylenedioxo ellagic acid derivative with –OMe substituent at the 3′ position. The complete set of ^{13}C shifts shows good agreement with the literature data obtained with pyridine-d_5 as solvent (Atta-Ur-Rahman et al., 2001).

3.4.7. 3′-O-methyl-3,4-methylenedioxy ellagic acid 4′-O-β-D-glucopyranoside

The NMR spectra of compound **XI** in a mixture of CD_3OD and DMSO-d_6 revealed a 3,4-methylenedioxo ellagic acid parent with one –OMe and one β-glucosyl substituent, which must be attached at positions 3′ and 4′, respectively. The glucosyl linkage at C4′ was confirmed by a downfield shift of H5′ by ca. 0.3 ppm, a 6.7% NOE from H5′ to Glc-H1″ ($r = 2.6$ Å in the energy-minimized model) and a weak NOE of ca. 1% for 3′-OMe to H2″ (min. $r = 4.1$ Å). Our data show good basic agreement with the ^{1}H and ^{13}C NMR data presented by Li et al. (1999) for the glucoside in DMSO-d_6; there is complete agreement on the order of chemical shifts. On the other hand, the available ^{13}C data for a pyridine-d_5 solution. as presented by Atta-Ur-Rahman et al. (2001), show several differences in the order of chemical shifts, and there are obvious errors in the assignments for the glucosyl fragment.

3.4.8. 3′-O-methyl ellagic acid 4-O-β-D-xylopyranoside

The NMR data for **XII** in CD_3OD indicated a 3-*O*-methyl ellagic acid glycoside. The carbohydrate fragment had five carbon signals (4 CH, 1 CH_2) and a series of *trans* axial J_{HH} couplings (7–10 Hz) that were consistent only with β-Xyl in the pyranose (six-membered) ring form. The following NOEs were detected: H5 to H1″ = 1.6%; H1″ to H5 = 2.9%, H1″ to H5″a = 1.8%; 3-OMe to any site <0.5%. The modelled structures had $r = 2.7$ Å for H5 to

Table 2
Antioxidant capacity of the raw extract from *A. dichostyla* root bark or column chromatography fractions as tested in the hypoxanthine/xanthine oxidase assay system[a]

Compound	Polyphenolic content (µg/ml)							
	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	Raw extract
I					199			302
II				745	259			1135
III						57	31	119
IV				224	716			830
V			666	345				1178
VI					257	113	52	482
VII				180				168
VIII						12		29
IX			40					49
X						11		27
XI			108	242				785
XII				26	201	30	5	271
XIII			12					14
XIV						25	17	56
XV	7	5						21
XVI	7	11	7	7				240
Total[b]	14	16	833	1769	1632	248	105	5706
ROS (IC_{50})[c]	n.d.	n.d.	117	241	59	52	n.d.	262
XO (IC_{50})[c]	n.d.	n.d.	n.d.	106	36	n.d.	n.d.	42

IC_{50} values in µg/mL correspond to 50% inhibition; n.d. = minimal inhibition, IC_{50} not determinable.
[a] A solution of raw extract dissolved in 50 mL methanol or an equivalent CC fraction in 5.0 mL methanol was tested for inhibition of formation of dihydroxybenzoic acids (DHBA) from salicylic acid (scavenging of ROS) and inhibition of xanthine oxidase (XO) catalysis of uric acid formation.
[b] Total for fractions = 4617 µg/mL.
[c] µg/mL.

Table 3
500 MHz ^{1}H NMR data of selected catechins in CD_3OD at 30 °C[a]

Compound[b]	Cat (III)			Epicat (V)			Epicat-3-*O*-G (VI)		
Position	δ_H	Mult.	J_{HH} (partner)	δ_H	Mult.	J_{HH} (partner)	δ_H	Mult.	J_{HH} (partner)
2ax	4.578	dm	7.49 (3ax)	4.810	m	Not resolved	5.030	m	Not resolved
3ax	3.986	ddd	8.10 (4ax); 5.42 (4eq)						
3eq				4.173	ddd	4.55 (4ax); 3.0 (4eq); 1.4 (2ax)	5.525	ddd	4.65 (4ax); 2.46 (4eq); 1.3 (2ax)
4ax	2.516	dd	−16.1 (4eq)	2.864	dd	−16.6 (4eq)	2.994	ddm	−17.4 (4eq)
4eq	2.852	dd		2.743	dd		2.852		
6	5.939	d	2.32 (8)	5.958	d	2.32 (8)	5.962	AB[c]	2.3 (8)
8	5.870	d		5.932	d		5.959	AB[c]	
2′	6.845	ddd	2.05 (6′); 0.50 (2ax); 0.3 (5′)	6.984	dd	1.98 (6′); 0.4 (2ax)	6.931	dd	2.05 (6′); 0.4 (2ax)
5′	6.768	dd	8.08 (6′)	6.766	d	8.15 (6′); 0.53 (2′)	6.695	d	8.20 (6′)
6′	6.723	ddd	0.55 (2ax)	6.802	ddd	0.5 (2ax)	6.807	ddd	0.6 (2ax)
2″,6″							6.948	s	

[a] Chemical shifts in ppm relative to CHD_2OD at 3.31 ppm (TMS = 0); *J* couplings in Hz.
[b] *Abbreviations*: cat = (+)-catechin; epicat = (–)-epicatechin; G = gallate.
[c] Tight AB system with intense, overlapping inner lines.

Table 4
125.76 MHz ^{13}C NMR data of selected catechins in CD_3OD at 30 °C[A]

Compound[B] Position	Mult.	Cat (**III**) δ_C	Epicat (**V**) δ_C	Epicat-3-*O*-G (**VI**) δ_C
2	D	82.85	79.85	78.70
3	D	68.82	67.48	70.04
4	T	28.49	29.25	26.91
4a (10)	S	100.89	100.13	99.46
5	S	157.58	157.97	157.91[a]
6	D	96.37	96.46	96.64
7	S	157.82	157.63	157.86[a]
8	D	95.58	95.95	95.97
8a (9)	S	156.93	157.36	157.32
1′	S	132.25	132.29	131.54
2′	D	115.29	115.34	115.19
3′	S	146.23	145.91	146.04[b]
4′	S	146.25	145.75	146.02[b]
5′	D	116.15	115.95	116.08
6′	D	120.09	119.46	119.45
1″	S			121.53
2″,6″	D			110.30
3″,5″	S			146.39
4″	S			139.92
7″	S			167.68

[a,b] Pairs of assignments may be interchanged and could not be confirmed by 2D NMR due to significant deuteration at C6,C8.
[A] Chemical shifts relative to CD_3OD at 49.053 ppm (TMS = 0); multiplicity: S, D, T, Q = 0, 1, 2, 3 attached protons.
[B] *Abbreviations:* cat = (+)-catechin; epicat = (–)-epicatechin; G = gallate.

H1″, $r = 2.5$–2.6 Å for H1″ to H3″ and 5″a, min. $r =$ ca. 4 Å for H2″ to a putative 3-OMe, and $r > 4.9$ Å for H5 to 3-OMe protons. Thus, the absence of NOEs from the –OMe group suggest that it is attached at the C3′ site remote from the glycoside at C4. More direct proof for this structure is provided by measurement and assignment of the long-range CH couplings with the help of selective ^{1}H decoupling. The data in Table 6 show that the –OMe protons and H5′ are coupled to the same carbon C3′ while the xylose moiety is attached to C4 and interacts with H5 (NOE). A detailed analysis of CH couplings was achieved for the ellagic acid ring labelled with primed numbers, while the signals for C2, C3, C4 and C6 showed some exchange broadening so that long-range CH couplings could not be resolved.

4. Discussion

The results of this study show that the root bark of *A. dichostyla* contains a complex mixture of polyphenols consisting mainly of polyhydroxyflavan-3-ols (catechins, procyanidins) and ellagic acid derivatives. Polyhydroxyflavan-3-ols were detected as the simple catechins and their dimers and trimers, with (–)-epicatechin representing the dominant species. The only flavanoid glycoside detected was (+)-catechin-3-*O*-β-D-glucopyranoside.

The potential health benefits of monomeric catechins are well documented, especially in preclinical studies (Crespy

Table 5
500 MHz ^{1}H NMR data of selected ellagitannins[a]

Compound[b]	Ellagic acid	3-*O*-Me-EA (XIV)	3,3′-*O*-Me-EA (XV)	3′-*O*-Me-3,4-mdEA (XVI)	3′-*O*-Me-3,4-mdEA 4′-*O*-β-Glc*p* (XI)		3′-*O*-Me-EA 4-*O*-β-Xyl*p* (XII)	
Solvent (Temp.)[c]	A (37 °C)	B (30 °C)	C (30 °C)	DMSO-d_6 (37 °C)	D (30 °C)		CD_3OD (30 °C)	
Position	δ_H	δ_H	δ_H	δ_H	δ_H	J_{HH} (partner)	δ_H	J_{HH} (partner)
5	7.600	7.536	7.527	7.521	7.472		7.640	
3a				6.368	6.350			
3-OMe		4.081	4.053					
5′	7.600	7.489	7.527	7.536	7.880		7.484	
3′-OMe			4.053	4.044	4.162		4.159	
1″					5.157	7.4 (2″)	4.876	7.3 (2″)
2″					3.486	9.1 (3″)	3.568	8.7 (3″)
3″					3.457	9.1 (4″)	3.508	9.0 (4″)
4″					3.362	9.7 (5″)	3.625	10.0 (5″a), 5.3 (5″b)
5″					3.492	5.4 (6″a) 2.3 (6″b)	3.455 (a) 4.016 (b)	−11.3 (5″)
6″					3.653 (a) 3.819 (b)	−12.1 (6″)		

[a] Chemical shifts rel. TMS; *J* couplings in Hz.
[b] *Abbreviations:* EA = ellagic acid, mdEA = 3,4-methylenedioxo ellagic acid.
[c] *Solvents:* A = 1:1 $CD_3OD/CDCl_3$; B = 7:3 DMSO-d_6/CD_3OD + TFA; C = DMSO-d_6 + 5% CD_3OD + TFA; D = 2:1 CD_3OD + DMSO-d_6.

and Williamson, 2004) whereas the biologic significance of the oligomeric procyanidins is less clear. Nevertheless, the procyanidins are effective scavengers of superoxide radicals ($O_2^{\cdot-}$) generated by the xanthine–xanthine oxidase system and hydroxyl radicals generated in the Fenton reaction, as reported by da Silva et al. (1991).

Free ellagic acid was not detected in the extracts of the root bark of *A. dichostyla*, but rather its various *O*-methyl derivatives, with or without a 3,4-methylenedioxo bridge, and either as the free phenols or as 4-*O*-glycoside conjugates. The major ellagic acid derivative detected was 3′-*O*-methyl-3,4-methylenedioxo ellagic acid 4′-*O*-β-D-glucopyranoside (**XI**).

The biologic activities of ellagic acid and its derivatives have been studied extensively. In addition to their effects as antioxidants and antimutagens, ellagic acids strongly inhibit a number of important enzymes such as HIV-1 reverse transcriptase and polymerases α, β, (Take et al., 1989) as well as human DNA topoisomerases I and II (Constantinou et al., 1995). It is known that ellagic acid can inhibit the mutagenic effects of many carcinogens such as *N*-nitrosodimethylamine (Wilson et al., 1992), aflatoxin B-1 (Mandal et al., 1987) and *N*-methyl-*N*-nitrosourea (Dixit and Gold, 1986).

Consumption of natural plant-derived products is associated with many health benefits, including the prevention of cancer. Based on in vitro experiments, many mechanisms have been proposed to account for cancer chemopreventive activity. These include inhibition effects on cell proliferation, Phase I enzymes, Cox-1 and -2, iNOS and angiogenesis, or the induction of Phase II enzymes, apoptosis, and binding to oestrogen receptors. Scavenging of reactive oxygen species and inhibition of xanthine oxidase are also considered to be important mechanisms of cancer chemoprevention.

In this study the methanolic extract from the root bark of *A. dichostyla* and fractions obtained by chromatography on silicic acid have been evaluated by a HPLC-based method utilising the hypoxanthine/xanthine oxidase assay. The methanolic extract exhibited strong antioxidant and enzyme inhibition capacities, whereas of the column chromatography fractions, CC-9 which contained a range of polyphenols, dominated by (−)-epicatechin-*O*-gallate, proved to be the most potent antioxidant fraction (IC_{50} = 52 μg/mL) in terms of ROS scavenging. In terms of XO inhibition CC-8, dominated by (epi)catechin trimer and which also contained appreciable amounts of 3′-*O*-methyl ellagic acid 4′-*O*-β-D-xylopyranoside, as well as the catechins (+)-catechin-3-*O*-β-D-glucopyranoside, epicatechin-(4β → 8)-catechin (procyanidin B_1), and (−)-epicatechin 3-*O*-gallate proved to be the most potent (IC_{50} = 36 μg/mL).

Studies are in progress to evaluate the antioxidant and enzyme inhibition capacities of the major individual purified compounds, which will also be subjected to a battery of additional in vitro tests similar to those described by Gerhäuser et al. (2003).

Table 6
125.76 MHz ^{13}C NMR data of selected ellagitannins[a]

Compound[b]	Mult.	Ellagic acid		3-*O*-Me-EA (XIV)	3,3′-*O*-Me-EA (XV)	3′-*O*-Me-3,4-mdEA (XVI)	3′-*O*-Me-3,4-mdEA 4′-*O*-β-Glc*p* (XI)	3′-*O*-Me-EA 4-*O*-β-Xyl*p* (XII)	
Solvent[c] (Temp.)		A (37 °C)		B (30 °C)	C (30 °C)	DMSO-d_6 (37 °C)	D (30 °C)	CD_3OD (30 °C)	
Position		δ_C (ppm)	J_{CH}	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)	J_{CH}
1	S	113.65	7.2 (H5), 1.0 (H5′)	112.45	111.71	116.04	116.22	116.60	7.20 (H5), 0.7 (H5′)
2	S	136.90	1.0 (H5)	141.99	141.22	131.00	132.39	138.40	br
3	S	140.34	7.0 (H5)	140.64	140.19	138.25	139.33	150.72[d]	vbr
4	S	148.76	3.0 (H5)	152.47	152.03	149.95	151.62	150.67[d]	br
5	D	111.65		111.81	111.40	103.78	104.70	113.49	164.6
6	S	108.81	1.7 (H5)	113.04	112.14	110.91[d]	112.73	103.19	vbr
7	S	161.24	4.0 (H5)	159.39	158.46	158.2	159.06	161.68	3.8 (H5)
3a	T					104.16	105.23		
3-OMe	Q			61.24	60.94				
1′	S	113.65		112.74	111.71	110.98	114.53	113.70	7.10 (H5′), 0.7 (H5)
2′	S	136.90		136.65	141.22	141.55	142.25	142.99	0.8 (H5′)
3′	S	140.34		139.90	140.19	140.24	142.97	141.57	7.05 (H5′), 3.6 (OMe)
4′	S	148.76		148.48	152.03	152.91	152.88	153.62	2.7 (H5′)
5′	D	111.65		110.82	111.40	112.10	113.49	112.56	165.6
6′	S	108.81		108.13	112.14	112.58[d]	113.28	114.90	1.33 (H5′)
7′	S	161.24		159.30	158.46	157.57	158.40	161.48	4.0 (H5′)
3′-OMe	Q				60.94	60.84	62.24	62.00	
1″	D						102.37	104.56	
2″	D						74.20	74.64	
3″	D						77.39	77.26	
4″	D						70.38	71.08	
5″	D/T						78.04	67.14	
6″	T						61.51		

[a,b,c] See footnotes to Table 5.
[d] Assignments tentative, long-range CH correlation or couplings not detected.

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