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Full Length Research Paper

Cell protective antioxidants from the root bark of *Lannea velutina* A. Rich., a Malian medicinal plant

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Antioxidants (radical scavengers and 15-lipoxygenase inhibitors) in semipolar extracts of root bark from the Malian medicinal plant *Lannea velutina* have been investigated. A series of proanthocyanidins with degree of polymerization from 2 to more than 12 have been isolated, in addition to the monomeric substance, catechin. The major constituents have a degree of polymerization of ten or more. Most structures are proposed to be derived from a common biosynthetic route in which catechin is introduced as the terminal unit and epicatechin units are extenders. Catechin, dimeric, trimeric, decameric and dodecameric proanthocyanidins have been assayed as scavengers of the stable free radical diphenylpicrylhydrazyl and as inhibitors of the peroxidizing enzyme 15-lipoxygenase. All of them were shown to be effective radical scavengers (50% radical scavenging at concentrations of 5-7 microgram/mL) and 15-lipoxygenase inhibitors (50% inhibition at 10 - 18 microgram/mL). When epicatechin and trimeric proanthocyanidin were tested as antioxidants in cells, they gave a significant reduction in endogenously produced reactive oxygen species (ROS).

Key words: *Lannea velutina*, Anacardiaceae, medicinal plant, antioxidant, radical scavenging, 15-lipoxygenase, proanthocyanidins, electrospray mass spectrometry.

INTRODUCTION

Peroxidative processes and free radicals have been the subject of intense research in recent years, since they have been implicated in numerous diseases, including cardiovascular disease, some forms of cancer, inflammatory ailments, neurodegenerative disorders etc. (Bagchi et al., 2000; Coyle et al., 1993; Halliwell, 1999; Thomas and Kalyanaraman, 1997; Vendemiale et al., 1999). This has led to a widespread search for new antioxidants and radical scavengers, since compounds with this activity might help in the prevention and cure of important illnesses. Many medicinal plants have been shown to contain large amounts of effective antioxidants, and the search for antioxidants and radical scavengers from natural sources is pursued worldwide, and is a key focus of our research group (Maiga et al., 2006; Malterud et al., 1993; Malterud and Rydland, 2000; Mathisen et al., 2002; Wangensteen et al., 2004) . African medicinal plants, which are often more or less unknown scientifically and therefore of potential interest as new sources of medicinally active antioxidants, have recently been reviewed in this respect (Atawodi, 2005).

Lannea species (Anacardiaceae) are native to tropical Africa. Some *Lannea* species yield timber that is used locally; others are employed for a variety of purposes in indigenous medicine (Watt and Breyer- Brandwijk, 1962). *L. velutina* Rich. is a shrub or tree 15 m high of the wooded savanna from Senegal to Ghana. The leaves are very fluffy, pubescent (hairy) on top and with dense tomenta on the lower surface. The roots and bark are recorded to be used against diarrhoea and for treatment of rachitic children and strained muscles (Kerharo and Adams, 1974). In the Ivory Coast the bark is used for treatment of

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diarrhoea, oedema, paralysis, epilepsy, and insanity (Burkhill, 1997) . In Mali *L. velutina* is used in treatments for chest pain, gastric ulcer, wounds, skin diseases, respiretory tract diseases and fever (Maiga et al., 2006).

In our earlier studies (Maiga et al., 2006) on the radical scavenging and 15–lipoxygenase (15-LO; an enzyme which catalyzes peroxidation of polyunsaturated fatty acids) inhibitory activity of some medicinal plants from Mali, ethanol and methanol extracts of *L. velutina* and *Diospyros abyssinica* showed the highest radical scavenging and 15- LO inhibition (IC50 <20 microgram/mL) activities. These plants have been chosen for further investigation. In this paper we present ethnopharmacological information on the medicinal uses of *L. velutina* in Mali. We describe our studies of the constituents of the alcohol extracts of the root bark of *L. velutina*, and also report on the radical scavenging and 15-LO inhibitory activities of the extracts. Cellular antioxidant activity was shown in cultured cerebellar granule neurons, a cell type which produces free radicals in response to glutamate, a trigger of neurodegenerative disorders in humans.

MATERIALS AND METHODS

Ethnomedical information

Interviews with traditional healers were carried out in November 2002 in two different areas of Mali. The study areas were Dioila and Bandiagara. Another study was made in Kolokani in 2004. A multidisciplinary team consisting of an agro-forester, a medical doctor or nurse, a pharmacist and an interpreter was involved in the interviews. Local names, traditional uses, preparation, and administration method of the plant were recorded.

Plant materials

The root bark of *L. velutina* was collected in Blendio, Mali, in February 2002. Plant materials were identified in the Department of Traditional Medicine, air dried and pulverized. Herbarium voucher samples (voucher number 1014/DMT) are deposited in the Department of Traditional Medicine, Bamako.

General chemical methods

^{1}H-NMR and ^{13} C-NMR spectra were recorded on a Varian Gemini-200 instrument (Varian, Palo Alto, CA, USA) at 200 MHz and 50 MHz, respectively, in deuterated acetone with two drops of deuterium oxide.

For UV and absorbance measurements, a Shimadzu 160A instrument equipped with a Shimadzu CPS240A thermostatted cell changer (Shimadzu, Kyoto, Japan) was employed.

Analytical HPLC was carried out on a Varian ProStar system equipped with two ProStar Model 210 pumps, a ProStar 510 column thermostat and a ProStar 325 UV/V is detector, using a Varian Chrompack column (RP, C-18 on Microsorb- MV 100, 250 x 4.6 mm) . Aliquots of 20 microL of aqueous methanol solutions were injected after filtration through a Millex^{R}–HA 0.45 micrometer filter (Millipore, Ireland). A water/1% formic acid (A)- acetonitrile (B) grad-ient was employed for component elution {0 - 2 min, 95%A - 5%B, 2 - 32 min, 5 - 17%B in A, 32 - 50 min, 17 - 70% B, 50 - 60 min, 70%B, followed by reconditioning of column (Karonen et al., 2004); flow rate 1 mL/min, detection at 280 nm}.

ES-MS experiments were performed on an Applied Biosystems QStar pulsar *I* quadrupole orthogonal acceleration Time-of-Flight mass spectrometer (Foster City, CA, USA). The samples were solvated in methanol:water:formic acid (50:50:1, v/v/v) and were infused into the source of the mass spectrometer at a rate of 0.1 micro L/min (spray voltage -4kV). All experiments were performed in the negative mode. Nitrogen was used as the collision gas in all product ion experiments.

The LC-MS system consisted of a Tsp SCM1000 vacuum degasser, Tsp SpectraSystem P4000 quaternary gradient pump and a Tsp SpectraSystem AS3000 auto-sampler. Detection was using a Finnigan LCQ^{duo} ion trap mass spectrometer. Xcalibur™ version 1.3 software was used to control this system and to perform data acquisition (all Thermo Electron Corporation, Waltham, MA, USA).

Separation was performed on a 250 x 4.6 mm (100 Å, 5 micrometer) Varian Microsorb® C18 column coupled to a precolumn at a flow rate of 1.00 mL/min. Gradient elution was carried out. Mo-bile phase A consisted of 1% formic acid, mobile phase B of acetonitrile. From t = 0 min to t = 2 min the mobile phase composition was constant at 95% mobile phase A / 5% mobile phase B. From t = 2 min to t = 32 min the mobile phase composition changed linear-ly from 95% mobile phase A / 5% mobile phase B to 83% mobile phase A / 17% mobile phase B. From t = 32 min to t = 50 min, the mobile phase composition changed linearly from 83% mobile phase A / 17% mobile phase B to 30% mobile phase A / 70% mobile phase B. After isocratic elution with 30% mobile phase A / 70% mobile phase B (from t = 50 min. to t = 60 min) the mobile phase composition changed back to starting conditions. The start/end composition was held for at least 5 column volumes to re-equilibrate the stationary phase.

The HPLC was connected to the MS equipped with an APCI interface. The MS was operated in both positive and negative ion modes using alternating scan events. Sheath gas (N_2) flow was set at 60 units, auxiliary gas (N_2) flow at 10 units. The APCI vaporizer was set at 450°C, the capillary temperature at 150°C. A discharge current of 5 microA was used. Full scan mass analysis was carried out over the range m/z 300 - 2000.

Oxidative depolymerisation of proanthocyanidins was carried out as described by Porter et al., 1986, using NH_4 Fe $(SO_4)_2$ .12 H_2O and HCl in n-butanol. After depolymerisation samples were subjected to two-dimensional TLC on cellulose coated foils (Polygram CEL300, 0.1mm thickness, Macherey-Nagel, Düren, Germany). In the first dimension, conc. HCl –formic acid –water (7:71:22, v/v) was used as mobile phase, in the second dimension methanol-conc. HCl-water: 109:1:10 was used (Mathisen et al., 2002). Cyanidin chloride (Sigma- Aldrich, St. Louis, MO, USA) was used for comparison. For some samples, absorbance at 550 nm was Registered as a semi-quantitative measure of proanthocyanidin content.

Determination of total phenolics (as gallic acid equivalents) was carried out with Folin-Ciocalteu reagent as described by Singleton and Rossi (1965). Test solutions were measured at concentrations of 10 and 20 mg/mL, and results were compared to a standard curve constructed by measurements of gallic acid at concentrations from 0 to 5 mg/mL.

Phloroglucinol degradation was carried out as described by Foo et al. (1996). Proanthocyanidin samples (usually 20 mg) were reacted with phloroglucinol (28 mg) in 1% HCl in Et OH (700 microL) with continuous shaking until complete dissolution. For hig-her polymers, larger amounts were used with corresponding increa-ses in the other reagents. The time taken for complete dissolution was *ca* 30 min. The resulting solution was fractionated on a Sepha-dex LH20 column (30 x 200 mm) using EtOH as eluent. Fractions were combined according to monitoring by TLC and visualization by UV irradiation and by spraying with methanolic DPPH solution. ^{1}H and in some cases ^{13}C NMR spectra were recorded.

Extraction and separation of the crude extracts

The dried pulverized materials (0.30 - 0.37 kg) were extracted in a Soxhlet apparatus with different solvents, followed by extraction

with water 50 and 100°C as described previously (Maiga et al., 2006).

The methanolic fraction and ethanolic fraction (ca. 20 g of each) were dissolved in 40 mL of methanol /water (50/50) and chromatographed on a 44 x 530 mm Diaion HP20 (Supelco, Bellefonte, PA, USA) column with methanol-water (50/50), methanol 100%, and acetone. Fractions were combined as indicated by their DPPH radical scavenging activity (TLC) or UV spectra to give 11 fractions. The fractions were assayed for DPPH scavenging activity, and subjected to ^{1}H NMR and ^{13}C NMR.

Purification and isolation of the proanthocyanidins

Fractions selected for further work, based on fraction weight, DPPH scavenging ability and NMR data, were submitted to further column chromatography on the same Diaion column, or a Sephadex LH20 column (Pharmacia, Uppsala, Sweden), 30 x 450 mm, or a reverse phase Si gel column (LiChroprep RP-18; Merck, Darmstadt, Germany), 30 x 300 mm. As eluent, water- methanol gradients (from 1:1 to pure methanol) followed by acetone were employed. In some experiments, crude extracts were applied directly to the Sephadex column.

Fractions from the columns were taken to dryness *in vacuo* and weighed. They were monitored by qualitative DPPH scavenging assay, ^{1}H and ^{13}C NMR spectroscopy and, for selected fractions, by quantitative DPPH scavenging assay and 15-lipoxygenase inhibit-ion assay.

Assays for radical scavenging and inhibition of 15-lipoxygenase

Qualitative determination of radical scavenging on TLC plates

The samples were applied to pre-coated plates (Silica gel 60F254, Merck) and visualized by spraying with a methanolic 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution at a concentration sufficient to give a violet color to the plate (approx. 0.4 mg/mL). Radicals scavengers were visible as yellow spots.

Quantitative determination of radical scavenging activity (Malterud et al., 1993; Mathisen et al., 2002).

The dried extracts or fractions were dissolved in dimethyl sulfoxide to a concentration of 10 mg/mL, and dilutions were made to 5, 2.5, 1.25 and 0.625 mg/mL.

To 2.95 mL of a methanolic solution of DPPH (A_{517} 1.0) was added 50 microL of the test solution. The mixture was stirred and the decrease in absorbance at 517 nm was measured over a period of 5 min. Per cent radical scavenging was calculated from the decrease in A_{517}. Calculation of results was carried out as previously described.

Inhibition of 15-LO

Inhibition of 15-lipoxygenase (15-LO) was carried out as described by Lyckander and Malterud (1992), using soybean lipoxygenase type 1-B (Sigma, or Fluka, Büchs, Switzerland) . Measurements of increase in absorbance at 234 nm for 30 to 90 s after enzyme addition were carried out in 0.2 M borate buffer (pH 9.00) with linoleic acid (134 microM) as substrate and an enzyme concentration of 167 U/mL, using test substance solutions in DMSO or (for blanks) DMSO alone. Enzyme inhibitory activity was calculated from the values for absorption increase per time unit, as supplied from the software of the spectrometer. Six or more parallels for blanks and three or more parallels for samples were measured. Calculations were carried out as previously described (Malterud et al., 1993; Lyckander and Malterud, 1992).

Assays in cellular systems

Cerebellar granule neurons were obtained from 7 – 8 days old albi-no rats (Gallo et al., 1987; Ciani et al., 1996). Neurons were seeded on plastic dishes coated with 20 microgram/mL poly-L-lysine (cell density of 2 x 10^{5} cells/cm^{2}) and cultured in basal Eagle's medium supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM gluta-mine, and 100 microgram/mL gentamicin. To prevent growth of non-neuronal cells, cytosine arabinofuranoside (10 microM final concentration) was added to the cultures 16 - 18 h after seeding. After 6 - 7 days *in vitro*, cultures were washed in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM HEPES, pH 7.4) and pre-incubated in the same buffer for 15 min before they were exposed to 100 microM gluta-mate (in the presence of 10 microM glycin as coagonist) at room temperature for 15 min in Mg^{2+}- free Locke's buffer. Control cultu-res were either untreated or exposed to physiological buffer in the absence of glutamate. At the end of glutamate exposure, cultures were washed and incubated in serum-free basal medium (25 mM KCl). The substances aimed at neuroprotection (3 microgram/mL epicatechin or 9 microgram/mL P.A. trimer) were present for the pre-incubation time of 15 min as well as during glutamate exposure. Cell death was measured after 24 h by a trypan blue assay.

To detect reactive oxygen species (ROS), neurons were loaded with dihydroethidium (HE, 0.2 microM for 60 min) directly in the medium prior to glutamate exposure. Media were aspirated and the cultures were washed with Locke's buffer before glutamate exposure. Immediately after glutamate exposure, neurons were harvested and fluorescence was read on a fluorescence plate reader (Perkin Elmer HTS 7000 Plus, Bio Assay Reader, Perkin Elmer, Boston, MA, USA) for 30 min at 37°C. Oxidation of HE by ROS was read at excitation 485 nm and emission 595 nm. Blanks in the absence of fluorescent probe were subtracted from readings, and a BCA™ Protein Assay (BCA™ Protein Assay Kit, Pierce, IL, USA) was carried out in order to correct for the amount of protein. The slopes were calculated and then presented as percentage of the slope for the oxidation of HE in the glutamate-exposed cultures.

Statistical treatment

All values are given as averages ± SD or SEM. Student´s t-test or one way ANOVA on ranks followed by Dunn's posthoc test were was employed for calculation of statistical significance, using a *P* value of less than 0.05 as a significance criterion.

RESULTS

Ethnopharmacological information

L. velutina is widely used as a medicinal plant in Mali. In our survey we noted that 40 of the 50 healers interviewed used *L. velutina* for a multitude of ailments. Among those are: gastrointestinal tract disease [dysentery (5 reports), gastric ulcer (4), flatulence (2), abdominal pain (1) diarrhea (1)] ; skin diseases [dermatitis (3)]; pains [chest pain (3), back pain (2), muscular pain (3), tooth ache (1)]; eye diseases [3]; parasites [(malaria (1), blinding sick-ness (1), toxoplasmosis (1)]; sprains (2); others [cough (1), gonorrhea (1), unspecified diseases (2)]. All the parts (leaves, bark, fiber, roots) as well as *Loranthus* spp. growing

parasitically on the tree were used alone or in addition to other plants in different ways and in different forms (decoction, macerate, powders) for these purposes. In a survey performed in 2004 in the Kolokani area (Mali) the following uses were recorded: dermatitis, dysentery, wound, chronic gastric ulcer, boils, inflammations and haemorrhoids, infertility, and convulsions with fever. However, during our survey in the Bandiagara area (Dogonland) the species *L. velutina* appeared to be unknown.

The different uses of *L. velutina* in traditional medicine in the Dioila and Kolokani areas are summarized in Table 1.

Studies on the crude extracts

The methanol and 80% ethanol extracts of the root bark of *L. velutina* are rich in antioxidants, both radical scavengers and 15- lipoxygenase inhibitors (Maiga et al., 2006). Assay for total phenolic compounds with Folin-Ciocalteu reagent yielded values of 80% (calculated as gallic acid equivalents) of the total weight for the MeOH extract and 64% for the 80% EtOH extract. From the UV, ^{1}H NMR and ^{13}C NMR spectra of the extracts, it appeared that the major part of both consisted of proanthocya-nidins.

Depolymerization of the extracts with Porter´s reagent (Porter et al., 1986) furnished an intensely red-coloured solution. $E^{1\%}_{1\ cm}$ at 550 nm (calculated as averages of measurements at 5 - 30 microgram/mL) was determined as 252 ± 13 and 207 ± 9 for the MeOH and the 80% EtOH extracts, respectively. This corresponds to a proanthocyanidin content of 50 - 80%, dependent on the degree of polymerization (DP) (Porter et al., 1986; Porter, 1989).

^{13}C NMR is well suited for determination of the average DP of proanthocyanidins, since the starter unit of the molecule exhibits a C-3 signal at ca. 67 ppm, while C-3 in extender units resonates at ca. 72 ppm. Likewise, information about stereochemistry is obtained, since catechin-type monomers (or, more precisely, 2,3- trans configured monomers) show C-2 signals at 82 - 83 ppm, while epicatechin-type monomers (2,3-*cis*) have this signal at 76 - 77 ppm (Eberhardt and Young, 1994). Assuming that the C-3 atoms in starter and extender units have similar relaxation times and nuclear Overhauser factors, and that this is the case for the epimeric C-2 carbons as well, integration of the signals between 65 and 85 ppm will give quantitative information both about average DP and the amount of each steroisomer in the molecule. For the crude *Lannea* extracts, it appears that epicatechin-type stereochemistry is predominant and that the average DP is fairly large (>6), but the presence of interfering signals in this region which probably come from carbohydrates makes exact calculations impossible.

Isolation and identification of substances

Initially, the extracts were fractionated by repeated column chromatography (CC) using Diaion HP20, followed by CC on Sephadex LH-20 and/or reverse phase (C_{18}) Si gel. In the following, fractions are denoted by the letters D (Diaion), S (Sephadex) and R (reverse phase) and numbers, so that fraction D2D3S4 means that fraction 2 from a Diaion column has been chromatographed on another Diaion column, and fraction 3 from this second column has been rechromatographed over Sephadex LH20, giving fraction 4. In some later experiments, fractionation on Diaion HP20 was omitted. An example of the isolation procedure is shown in Figure 1.

Chromatographic fractions were combined based on absorbance and radical scavenging activity determined qualitatively by application of the fraction to a TLC plate and spraying with a methanolic DPPH solution (Glavind and Holmer, 1967). Combined fractions were taken to dryness in a rotary evaporator and monitored by ^{1}H and ^{13}C NMR spectroscopy, and most of them were assayed quantitatively for radical scavenging activity and inhibition of 15-lipoxygenase. Some fractions (D1S6, D2D3S3 and D2D8S4) were subjected to Porter degradation followed by 2-D TLC on cellulose (Mathisen et al., 2002). In all cases, only one red spot was obtained. This corresponded to cyanidin chloride (used as standard for comparison), demonstrating that catechin and/or epicate-chin are the main monomers, although the presence of minor amounts of other catechins (below the detection limit) cannot be excluded.

Identification of major constituents

From spectroscopic data, it appeared that the following species were present in the fractions from *L. velutina* extracts:

Catechin (in fractions (from MeOH extract) S3R2, 31.4 mg and S3R4, 75.7 mg) was identified by direct comparison (TLC, ^{1}H NMR, ^{13}C NMR) with the authentic substance. From ^{1}H NMR, it appeared that several other fractions contained smaller amounts of catechin in mixture with other polyphenols.

Epicatechin (4beta->8) catechin (procyanidin B1) was found in MeOH extract fraction S3R1 (19.5 mg) and EtOH extract fractions S3 (64.1 mg) and D8S8 (16.2 mg), and less pure in other fractions. Integration of the ^{13}C NMR spectrum in the 65-85 ppm region showed a 1:1 ra-tio between starter and extender units and between catechin and epicatechin-type monomers. The substance was identified by comparison of NMR spectra with literature data (Foo et al., 1997). This was corroborated by data from HPLC, which showed a t_R value relative to catechin that was consistent with procyanidin B1 and not in accord with the published value for epicatechin(4beta->6) catechin (procyanidin B7) (Ricardo da Silva et al., 1991). Phloroglucinol degradation of fraction S4R2 from the EtOH extract (which had this dimer as a major constituent) yielded catechin and epicatechin (4beta->2) phloroglucinol, separated by column chromatography and identified by ^{1}H NMR spectroscopy. This shows that catechin

Table 1a. Traditional uses of *Lannea velutina* in the Dioila region.

Part used	Preparation / administration		Traditional indications
Leaves	Decoction	Body bath	Dermatitis
		Steam bath of chest and body bath	Chest pain
		Steam bath and massage of the back	Back pain
		Face washing	Eye infections
		Drunk	Fever, dentition, gastric ulcer
Leaves	Macerate	Body bath	Dermatitis
Leaves and roots	Decoction	Drunk and as body bath	Unknown disease
Leaves mixed with *Parinaria curatellifolia* and *Entada africana*	Decoction	Drunk with body bath	Colitis
Leaves with salt	Decoction	Drunk	Flatulence
Leaves with those of *Saba senegalensis*	Decoction	Drunk	Dysuria, unknown disease
Leaves with droppings of goat	Decoction	Steam bath of the head	Migraine
Stem bark	Powder	Eaten	Cough, toxoplasmosis
	Macerate + salt	Drunk	Gastric ulcer
	Macerate + salt	Drunk	Gastric ulcer
	Decoction	Drunk	Dysentery, gastric ulcer, chest pains, abdominal pains, prolonged menstruation
		Drunk, steam and body bath	Dermatitis, weakness, back pain
		Bath	Dermatitis
Stem bark and leaves	Decoction	Bath	Dermatitis
Stem bark and *Strychnos spinosa*	Decoction	Bath	Diarrrhoea in children
Fibres of trunk	Powder + sugar	Eaten	Flatulence
	Decoction +grease or butter	Massage and bath	Weakness, sprains, muscular pains
	Decoction	Drunk	Gonorrhoea, dysentery
	Decoction	Anal bath	Anal prolepsis
	Macerate	Drunk	Dysentery, flatulence
Root bark	Macerate	Drunk	Malaria
	Decoction	Wash	Eye infections
	Decoction + pork meat	Eaten	Onchocerciasis
Loranthus spp.	Powder	Applied topically	Dermatitis
	Macerate	Drunk and body bath	Measles

is the starter unit and epicatechin the extender unit, in accord with the results above. A trimeric proanthocyanidin was isolated from MeOH extract fraction D4S(4-12)S(2-4)R4 (50.6 mg) and from MeOH extract fraction S5 (247.5 mg). The ^{13}C NMR spectrum showed starter: extender and catechin:epicatechin ratios of 1:2. Phloroglucinol degradation gave catechin and epicatechin (4 beta->2) phloroglucinol, demonstrating that the mole-cule is epicatechin->epicatechin->catechin. From the NMR data, however, it appears difficult to assign the bonds between monomers to either the 4->6 or 4->8 type (Shoji et al., 2003).

Similarly, tetramer (MeOH fraction D3D4, 82.9 mg), hexamer (MeOH fraction D5, 853.5 mg), heptamer (MeOH fraction D4, 1519.6 mg; EtOH fraction S(6+7)S3, 564.3 mg) and nonamer fractions (EtOH fraction D7S11, 129.4 mg) were isolated, in all instances seeming to contain one catechin unit, the rest of the oligomer being epicatechin. Phloroglucinol degradation of fraction D7S11 from the EtOH extract yielded catechin andepicatechin(4beta->2)phloroglucinol, so it might seem that in general, the proanthocyanidins of *L. velutina* have catechin as a starter unit and epicatechin as extender units.

A considerable proportion of the total tannins in the extracts, however, appear to be high MW compounds with average degree of polymerization (DP) between 10 and 17, although determination of DP becomes progressively less exact with increasing molecular size. Some of these

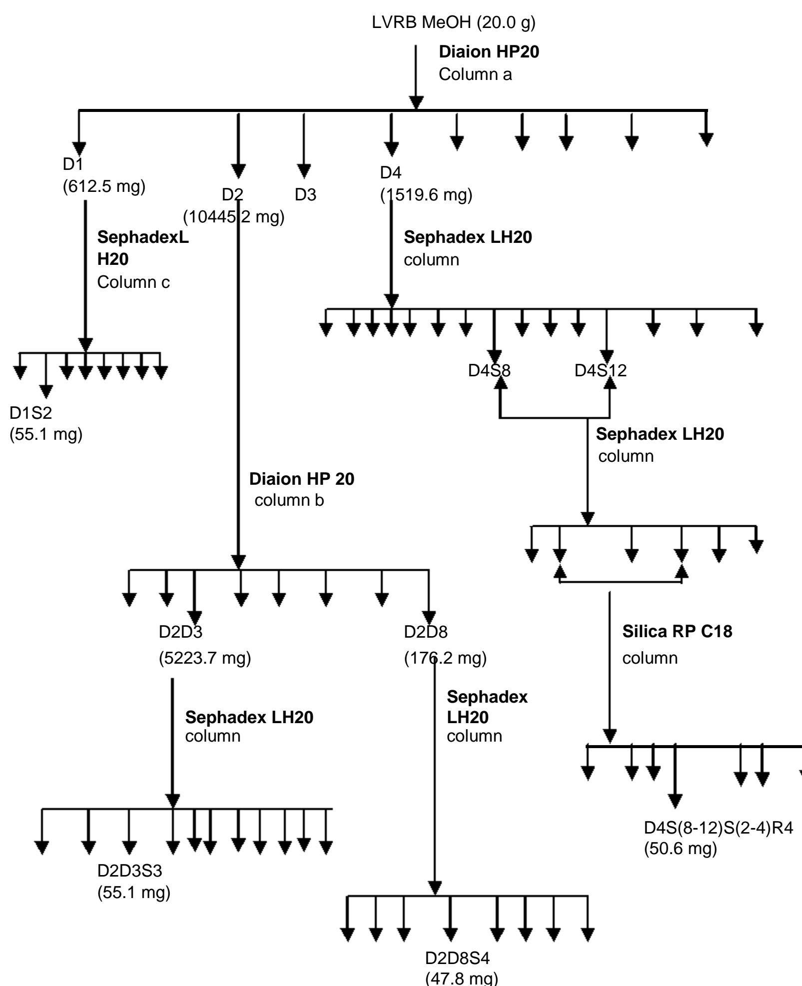

Figure 1. Fractionation and purification of *Lannea velutina* root bark MeOH proanthocyanidins, shown as an example of the fractionation and purification process.

Table 1b. Traditional uses of *Lannea velutina* in the Kolokani district

Part used	Preparation / administration		Traditional indications
Leaves	Decoction	Topical application after washing	Small boils
		Steam bath and head washing	Headache
		Face washing	Eye infections
Stem bark	Powder mixed with salt	Eaten	Gastric ulcer
	Powder	Topical application	Wounds
	Powder mixed in water	Drunk	Female infertility
	Juice	Topical application	Inflammation
	Decoction	Drunk	Dysentery, hemorrhoids
		Drunk and bath	Boils, itching
		Bath	Dermatitis, variola, itching, boils on the body
Stem bark and leaves	Decoction	Drunk and body bath	Disease with fever and convulsions

these seem to diverge from the figure suggested above: MeOH fraction D1S2 (12-mer, 55.1 mg) appears to contain only epicatechin, while another 12-mer, EtOH fraction S7 (743 mg) contained one catechin unit. MeOH fraction S9 (12-mer, 2180.2 mg) gave on phloroglucinol degradation mostly epicatechin(4beta->2) phloroglucinol, but in addition yielded minor amounts of catechin (starter unit) and epiafzelechin(4beta->2)phloroglucinol. Epiafzelechin is the 3´-deoxy analogue of epicatechin, so this proanthocyanidin can be regarded as a propelargonidin,

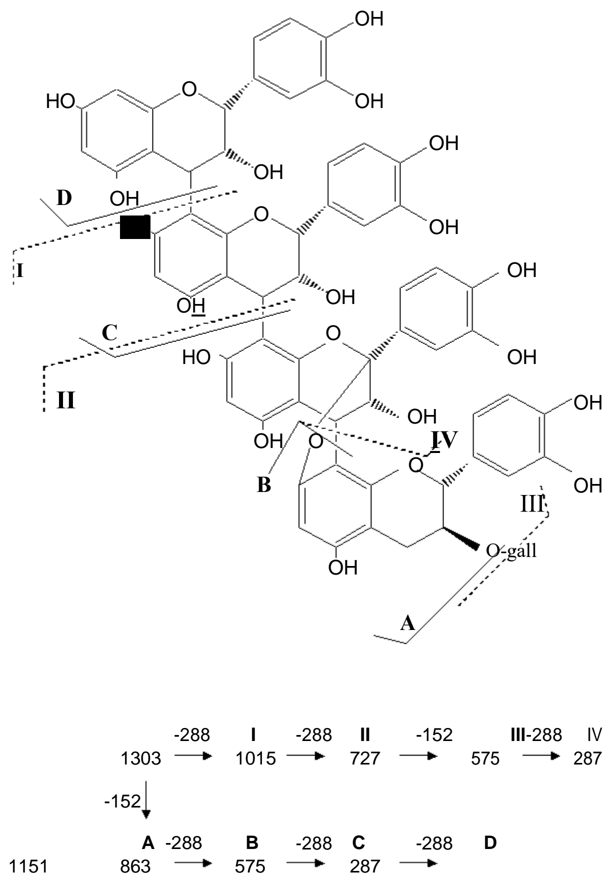

Figure 2. Possible fragmentation routes for the compound P4G1 ($M-H^-$ at m/z =1303). The different mass losses are suggested to take place as shown and give rise to fragments **I-IV** and **A-D**, see figure 1.

a less common type of proanthocyanidin than the procyanidins, which only contain catechin and epicatechin.

It should be realized that, especially for the higher polymers, the above data represent average values. It is highly likely that the fractions are heterogenous and that one fraction may contain several closely related substances, both structural isomers and homologues and rotamers. It may also contain small or trace amounts of other polyphenols, occurring in amounts below the detection limit for ^{13}C NMR spectroscopy.

Dimeric, trimeric and tetrameric proanthocyanidins are well known natural products. Procyanidins with a catechin starter unit and epicatechin extender units have been reported up to a DP of 10 (Awika et al., 2003). Apparently, none of the dodecamers isolated by us ((epicatechin)$_{11}$ – catechin; (epicatechin)$_{12}$ and (epicatechin)$_{11-n}$ (epiafzelechin)$_n$ –(catechin)) have been reported previously. As mentioned above, however, at this high degree of polymerization, isolates represent an average and not one specific structure.

Studies on minor constituents

From mass spectrometry, the presence of numerous minor constituents in the fractions was demonstrated (Figure 3a). A highly complex pattern of substances was evident (Table 2), comprising regular proanthocyanidin oligomers, A-type proanthocyanidins (with an additional ether bond between monomer units and thus a mass of two less than the corresponding B- type proanthocya-nidins which form the major part of the fractions), desoxy compounds (16 mass units less than the major compounds – epiafzelechin, reported above, is one example of this), methyllated compounds (14 mass units more than the major compounds), and galloylated compounds (152 units more). An ES mass spectrum of fraction MeOH D4S (8-12) S (2-4)R4 is shown in Figure 3a. A proposed structure of one galloylated oligomer containing A-type structure with the MS/MS data based on the fragmentation pattern shown in Figure 3b, is given in Figure 2.

Several of the fractions obtained from *L. velutina* were analyzed by LC -MS revealing different types of products, amongst these two different dimers, both giving ions at m/z 579 in the positive mode and 577 in the negative, and one galloylated dimer (m/z 729 in the negative mode). The dimers are most probably epimers as they are eluted at different retention times in the LC system. In addition, some of the fractions revealed by HPLC one in homogeneous peak that may comprise closely related compounds or a population of rotamers and numerous minor ones, which are likely to represent the compounds demonstrated by ES-MS experiments. In a study of proanthocyanidins in *Lannea coromandelica*, a related plant species (Islam et al., 2002), a similar complex pattern of proanthocyanidins as described above was observed.

DPPH radical scavenging and 15-LO inhibition activities

We have shown previously (Maiga et al., 2006) that the MeOH and 80% EtOH extracts of *L. velutina* root bark show high activity in both the DPPH radical scavenging assay and the 15-lipoxygenase inhibition assay. In Table 3, the concentrations of extracts and fractions that give 50% radical scavenging and 50% enzyme inhibition are shown. Activities of different substances are shown in Figures 4 and 5, and concentration-activity dependence in Figures 6 and 7.

It appears that all substances show high activity both as radical scavengers and 15-LO inhibitors. The DPPH scavenging activity of proanthocyanidins is well known (Mathisen et al., 2002; Rao et al., 2004), and 15-LO inhibition for this class of substances has also been reported previously (Mathisen et al., 2002). Thus, it seems reasonable to assume that the antioxidant properties of *L. velutina* root bark can be explained mainly based on the basis of its proanthocyanidin content.

The minor constituents observed in the mass spectra were not isolated. From the relatively scant literature on antioxidant activity of these subtypes of proanthocyanidins, it appears that antioxidant activity is decreased by B-ring methylation, while galloylation increases activity

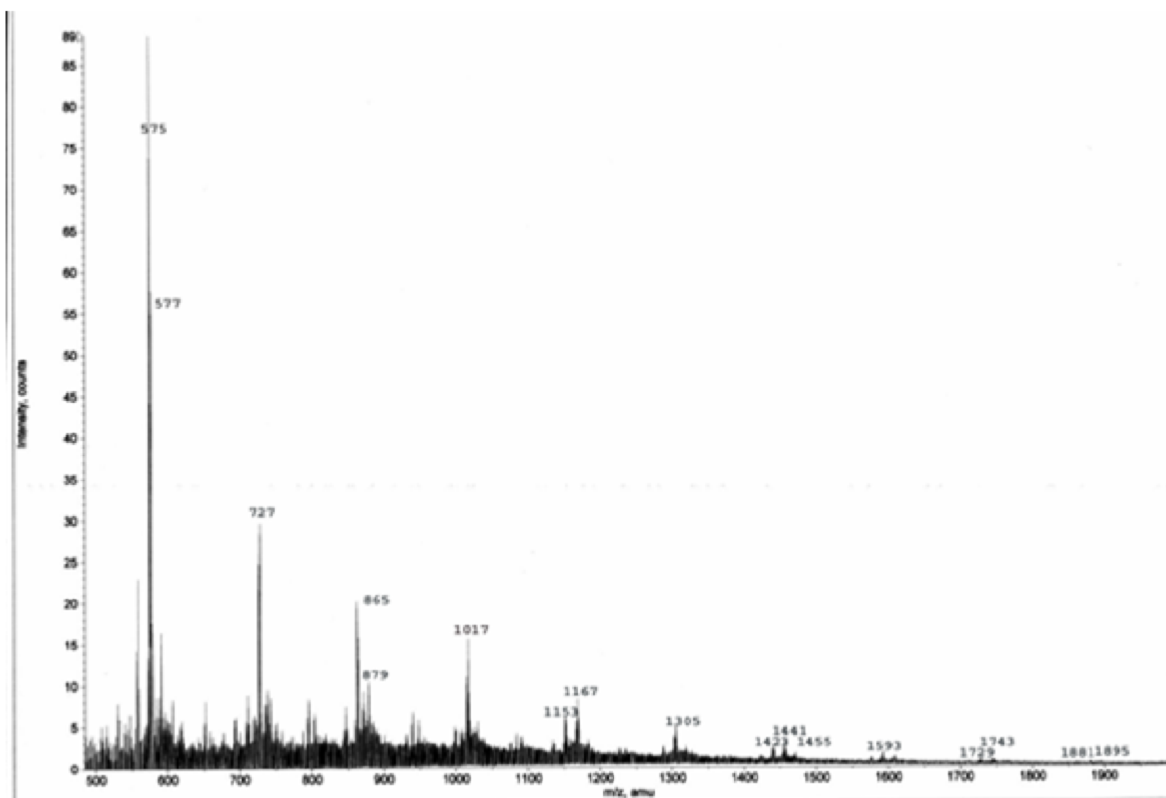

Figure 3a. Negative ion mass spectrum (ES-MS) of fraction MeOH D4S(8-12)S(2-4)R4.

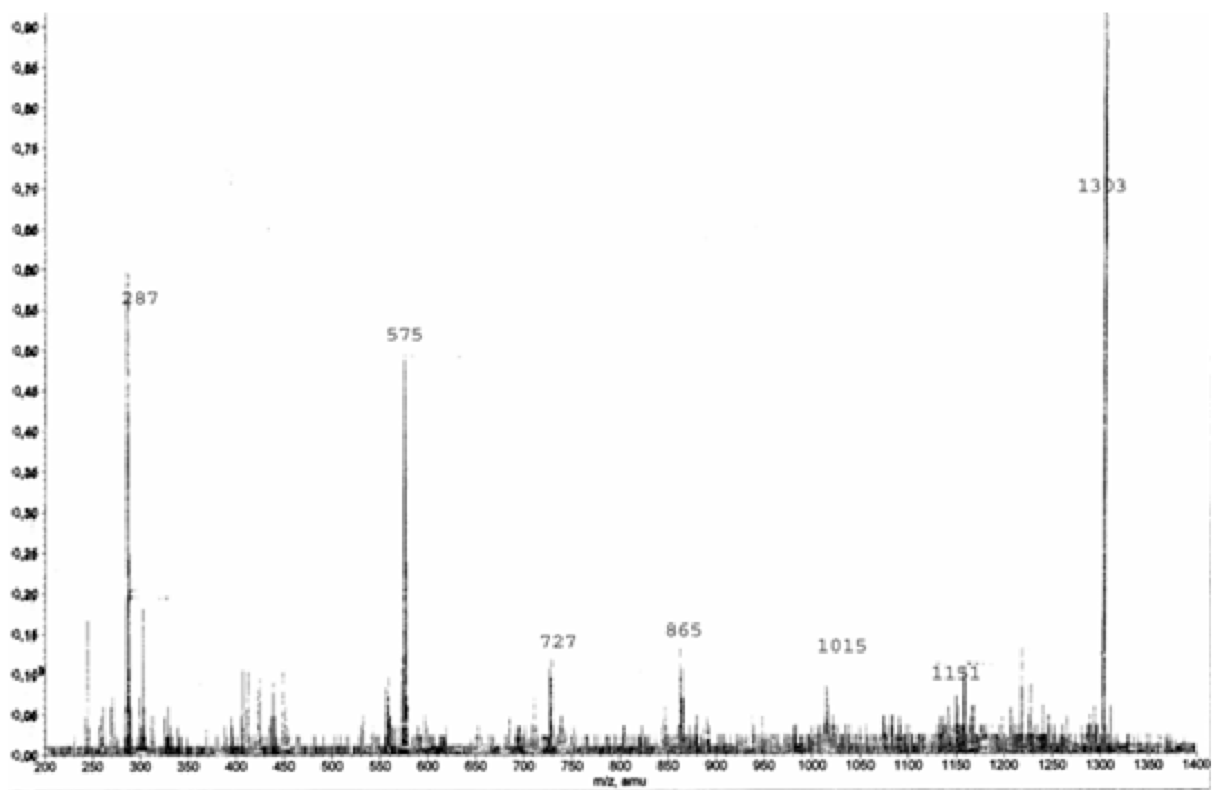

Figure 3b. Negative ion ES-MS/MS of compound with M-H$^-$ at m/z = 1303 in figure 1a.

Table 2. Tentative assignments of structures of the proanthocyanidins present as minor components in *L. velutina* bark based on molecular weights. All species are singly charged.

	Proposed identity [1]						
m/z-1			G1	-2	G2	-2	
287	P1-2						
289	P1						
559		P2-2-16					1A1B
575		P2-2					
577	P2						
591	P2+14						
727				P2G1-2			
729			P2G1				
845		P3-2-2-16					1A2B
863							
865	P3						
879	P3+14						
1015				P3G1-2			
1017			P3G1				
1135		P4-16-2					1A3B
1137	P4-16						1A3B
1151		P4-2					
1153	P4						
1167	P4+14					P3G2-2	
1169					P3G2		
1287				P4G1-2-16			1A3B
1289			P4G1-16				1A3B
1303				P4G1-2			
1305			P4G1				
1315							
1319			P4G1+14				
1423		P5-16-2					1A4B
1425	P5-16						1A4B
1437		P5-2-2					
1439		P5-2					
1441	P5						
1455	P5+14						
1457	P5+16				P4G2		
1459					P4G2+2		
1471		P5+30(2O-2)			P4G2+14		
1559			P5G1-32-2				
1577				P5G1-14-2			
1591				P5G1-2			
1593			P5G1				
1729	P6						
1743	P6+14						
1745					P5G2		
1759					P5G2+14		
1881			P6G1				
1883			P6G1+2				
1895			P6G1+14				

[1]Abbreviations: P1- proanthocyanidin momomer, $M-H^-$ at m/z=289, P2 – proanthocyanidin dimer etc., G1 – monogallate mass increment of 152 Th, etc., A = P – 16, i.e. –OH, B = P $M-H^-$ at m/z 289.

Table 3. Concentrations (in microgram/mL) to give 50% scavenging of the DPPH radical and 50% inhibition of 15-lipoxygenase. *Data from Maiga et al. (2006) . In all cases, the effect is statistically significant (P<0.05) at all concentrations measured (down to 2.6 microgram/mL).

Extracts and compounds	DPPH scavenging	15-LO inhibition
MeOH crude extract	12±2	14±1
80% EtOH crude extract	17±2	18±2
Catechin	6.2±0.1	17±1
Dimer (procyanidin B1; EtOH fr. D8S8)	4.6±0.1	16±2
Trimer (MeOH fr. D4D(8-12)S(2-4)R4)	5.1±0.1	14±2
10-mer (MeOH fr. D2D3S3; catechin + epicatechin)	6.3±0.3	18±2
10-mer (EtOH fr. D7S11; catechin + epicatechin)	5.4±0.4	10±1
12-mer (MeOH fr. S9; catechin + epicatechin)	4.9±0.1	12±1
Quercetin (positive control)*	3.4±0.3	11.5±0.6

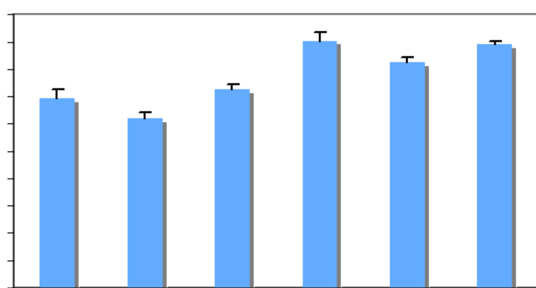

Figure 4. Scavenging of the diphenylpicrylhydrazyl radical by *Lannea velutina* root bark (LVRB) crude extracts and purified proanthocyanidin at a concentration of 21 microgram/mL. 1:LVRB MeOH crude extract, 2: LVRB EtOH crude extract, 3: Procyanidin B1 (dimeric), 4: Trimeric proanthocyanidin, 5: 10-meric proantho-cyanidin (epicatechin/catechin 9:1), 6: 12-meric proanthocyanidin (only epicatechin).

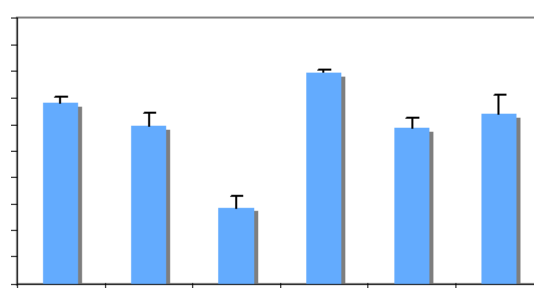

Figure 5. Inhibition of 15-lipoxygenase by *Lannea velutina* root bark (LVRB) crude extracts and purified proanthocyanidin at a concentration of 21 microgram/mL. 1:LVRB MeOH crude extract, 2: LVRB EtOH crude extract, 3: Procyanidin B1 (dimeric), 4: Trimeric proanthocyanidin, 5: 10-meric proanthocyanidin (epicatechin/catechin 9:1), 6: 12-meric proanthocyanidin (only epicatechin).

(Cren- Olivé et al., 2003; Plumb et al., 1998; Pollard et al., 2006). This is in accord with general considerations for antioxidant properties of proanthocyanidins (Bors et al., 2001). These effects differ, however, in different assays. A-type and B-type proanthocyanidins appear to be fairly similar as radical scavengers (Hatano et al., 2002).

Very little is known about the influence of galloylation and methylation of proanthocyanidins on 15-lipoxygenase inhibition. By comparing data from our previous investigations (Mathisen et al., 2002; Utenova et al., 2007), it does not appear that the enzyme inhibitory activity is drastically changed by such substitution.

Considering the investigations discussed above, it appears unlikely that the minor proanthocyanidin constituents in *L. velutina* are influencing the radical scavenging and lipoxygenase inhibiting properties of the extracts to any significant degree.

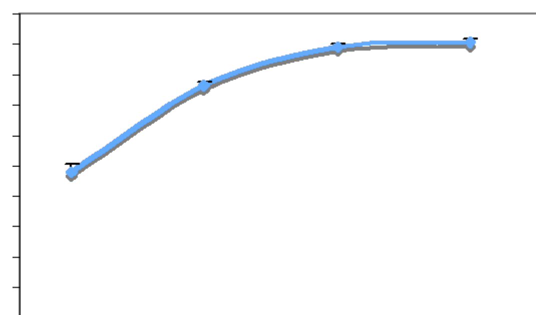

Figure 6. Concentration dependence of diphenylpicrylhydrazyl radical scavenging by proanthocyanidin 10-mer (epicatechin / catechin 9:1)

Epicatechin and trimeric proanthocyanidin as antioxidants in cerebellar granule cells

The biological activity has been tested for epicatechin, the major building block of proanthocyanidins, and trimeric proanthocyanidin, present in root bark of *L. velutina* and small enough to cross the plasma membranes of

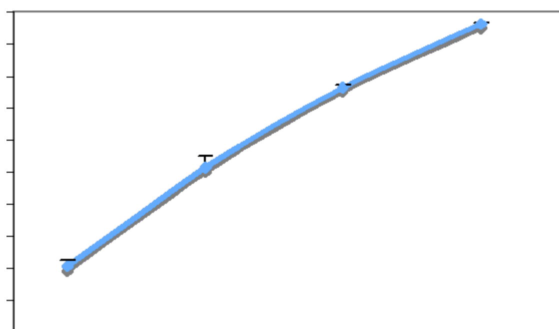

Figure 7. Concentration dependence of 15-lipoxygenase inhibition by proanthocyanidin 10-mer (epicatechin / catechin 9:1).

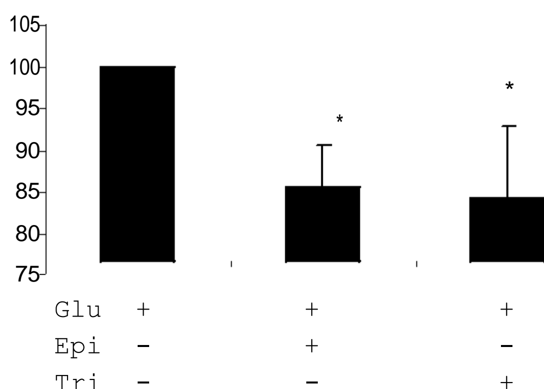

Figure 8. ROS production in cerebellar granule neurons induced by glutamate in the absence or presence of epicatechin (Epi, 3 microgram/mL) or proanthocyanidin trimer (Tri, 9 microgram/mL). Readings in untreated cells were 76%, as shown on the y axis. The results are mean +/- SEM, N = 8 (4 independent experiments with duplicates). * P< 0.05, One way ANOVA on ranks followed by Dunn's posthoc test, comparing antioxidant treatments to glutamate (glu) alone.

cells, as antioxidants in cultures of cerebellar granule neurons (Figure 8). This cell type produces free radicals in response to glutamate, which is a trigger of neurodegenerative disorders in humans. Glutamate-induced production of ROS, as measured by its ability to oxidize HE to a fluorescing compound, was strongly reduced by both antioxidants. The concentrations of the antioxidants were chosen to lie on the dose-effect curve for antioxidative effect *in vitro* (Figure 6). Since superoxide is involved in mediating the toxicity induced by glutamate, we measured cell death induced by glutamate in the absence or presence of the antioxidants (Figure 9) . The best protective effect was seen with the trimeric proanthocyanidin, consistent with its in vitro antioxidant capacity which is three times higher than that of the epicatechin dose given.

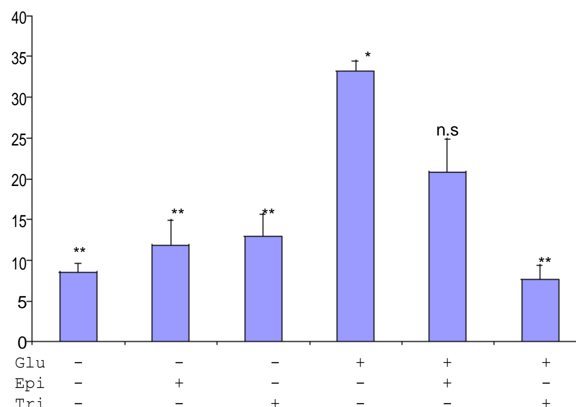

Figure 9. Cell death in percent of the total cell count in cerebellar granule neurons treated with glutamate (glu) in the absence or presence of epicatechin (Epi, 3 microgram/mL) or proanthocyanidin trimer (Tri, 9 microgram/mL) from the root bark of *Lannea velutina.* The results are mean +/- SEM, N=8 (4 independent experiments with duplicates). P< 0.05, one way ANOVA on ranks followed by Dunn's posthoc test, comparing treatments to control (first bar, *) or to glutamate treatment (**). n.s = not significantly different from control or glutamate treatments.

DISCUSSION

There is considerable interest in the therapeutic potential of antioxidants and radical scavengers and in their ability to take part in the prevention or amelioration of several degenerative and other diseases. Cellular pro-oxidant states with increased concentrations of reactive oxygen species (ROS) and free radicals are believed to be implicated in the pathology of several major chronic diseases such as atherosclerosis, some forms of cancer, inflammatory diseases, and also in the aging process. The body normally controls oxidative stress through protective enzymes such as superoxide dismutase, catalase and glutathione peroxidase, in addition to antioxidants which are constituents of the diet, such as vitamin C and vitamin E. If the natural antioxidant defense of the body is overwhelmed, however, illness may result. Peroxidation induced by 15-lipoxygenase may be important in the formation of atherosclerotic lesions, since 15-LO is able to oxidize low density lipoprotein, a process that is believed to be a key step in the atherosclerotic process (Steinberg, 1999).

Many naturally occurring compounds, such as flavonoids and carotenoids, are antioxidants. Proanthocyanidins and other flavonoids are polyphenols, a class of substance that seems particularly effective in this respect, and conceivably may play an important role as dietary antioxidants. It has been suggested that in many cases, proanthocyanidins are the active principles in medicinal plants (De Bruyne et al., 1999). For these reasons, polyphenols have attracted widespread attention in the fields of nutrition and health, and it has been suggested that they may modulate key biological pathways *in vivo* in mammals (Rice-Evans and Packer, 2003).

Proanthocyanidins have been reported to have a broad spectrum of biological, pharmacological and therapeutic activities against free radicals and oxidative stress (Ariga, 2004; Cos et al., 2004; Dixon et al., 2005; Fine, 2000; Prior and Gu, 2005; Scalbert et al., 2000; Scalbert et al., 2002). Dysentery and diarrhoea are among the major indications for *L. velutina* bark preparations (Table 1). It is therefore noteworthy that proanthocyanidin-rich plants have a long tradition of use as antidiarrhoeals in folk medicine (Lewis and Elvin-Lewis, 2003). The efficacy of this has been shown in several recent investigations (Adzu et al., 2003; Agbor et al., 2004; Atta and Moneir, 2004; Bizimenyera et al., 2005), and a proanthocyanidin preparation from *Croton lechleri* has been in development as a commercial drug (Fischer et al., 2004). Interestingly, *Shigella dysenteriae* toxin (Kaur et al., 1998) and *Entamoeba histolytica* lectin (Rawal et al., 2004) have been reported to increase oxidative stress in intestinal cells. Both of these organisms are well known inducers of dysentery. A similar pro- oxidative effect has been reported for chronic diarrhoea (Nieto et al., 2000).

Gastric ulcer is also among the diseases that are treated with *L. velutina* bark preparations. An antioxidant polyphenol mixture, thearubigin from black tea (Maity et al., 2003), bark extracts from *Rhizophora mangle*, which are rich in antioxidant tannins (Berenguer et al., 2006), tempol, a radical scavenger (Cuzzocrea et al., 2000) and synthetic antioxidants (Choudhary et al., 2001) have all been shown to counteract gastric ulcers and colitis. Purified proanthocyanidins show this effect as well (Iwasaki et al., 2004).

Ailments such as dermatitis and itching are treated topically with *L. velutina* bark preparations. Proanthocyanidins (Deters et al., 2001) as well as other antioxidants, e.g. vitamin E (Thiele et al., 2005) are used extensively in therapy of skin diseases.

Conclusion

The 80% ethanol and methanol extracts of root bark of *L. velutina* are excellent radical scavengers in non-cellular and cellular systems. They are also effective 15–lipoxygenase inhibitors. This effect is mainly due to their high content of proanthocyanidins. These compounds have not been previously reported in *L. velutina*. From the above discussion, it appears reasonable that some of the traditional medicinal usages of this plant in Mali can rationally be ascribed to its content of proanthocyanidins with strong antioxidant properties. From the studies referred to above, it appears that our results can explain the use of *L. velutina* in ailments such as gastric ulcer, dysentery and dermatitis (Tables 1a and 1b). Efficacy in other reported use, such as against malaria and gonorhea, will probably depend on the uptake of active constituents from the gastrointestinal tract, as will activity against diseases in which oxidative stress is involved, such as atherosclerosis. So far, no studied on bioavailability of *L. velutina* preparations have been performed.

Controlled clinical studies are needed to elucidate the efficacy of *L. velutina* bark preparations and constituents *in vivo*.

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