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5-Deoxyflavan-3-ol-based proanthocyanidins with antinutritional and antimicrobial properties from the forage legume *Acaciella angustissima*

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Summary

The proanthocyanidins of *Acaciella angustissima* (Mill.) Britton & Rose foliage have antinutritional and antimicrobial effects as proanthocyanidin-containing extracts reduced intake and weight gain of weanling rats when added to conventional diets and caused changes in fecal bacterial diversity and metabolic activity. Purified fractions of *A. angustissima* were shown to be more inhibitory to rat fecal bacteria than *Schinopsis* spp. (quebracho) proanthocyanidin extracts. In this study, it was determined that *A. angustissima* proanthocyanidins consist largely of 5-deoxyflavan-3-ols and proanthocyanidin dimers through hexamers, based on guibourtinidol, fisetinidol, and robinetinidol, with a predominance of fisetinidol units. These are accompanied by smaller amounts of higher oligomers (heptamers-decamers) and deoxyflavan-3-ols, dimers and trimers containing a *p*-hydroxybenzoate ester moiety.

Introduction

Many parts of the world have large tracts of arid and semi-arid lands that cannot be cultivated, but which can be used to raise livestock, mainly cattle, sheep and goats. It has often been suggested that leguminous, multi-purpose trees could be useful as livestock fodder in these regions (Osuji et al., 1995); however, secondary metabolites found in these potentially useful plants can be toxic to animals or cause a reduction in their productivity by reducing feed intake (Barry and Blaney, 1987). In many cases, the antinutritional compounds have not been identified and little is known about their specific effects on ruminant metabolism. One such leguminous shrub/tree *Acaciella angustissima* (Mill.) Britton & Rose [syn. *Acacia angustissima* (Mill.) Kuntze] has great agronomic and nutritional potential, but is limited by the presence of antinutritional compounds (Smith et al., 2001, 2003).

Proanthocyanidins from several species of the genus *Acacia sensu lato* and the segregate genus *Acaciella* have previously been examined. Best known are those from *Acacia mearnsii*, wattle or mimosa, widely cultivated in South Africa and Brazil for industrial tannin production (Roux, 1972; Roux et al., 1980; Seigler, 2003). In that instance, the condensed tannins are largely based on 5-deoxyflavan-3-ol units. The proanthocyanidins of another commercial tannin source, quebracho (*Schinopsis* spp., Anacardiaceae) are similar, but typically possess opposite configuration at positions 2 and 3 of the chain extending units to those from wattle. In some instances, 5-deoxyflavan-3-ols occur in combination with phloroglucinol-type A-rings (for example, catechin and epicatechin as in *Acacia baileyana*) (Foo, 1984). In a similar manner, the "upper" units of (4→8)- and (4→6)-linked prorobinetinidins from the stem bark of *Stryphnodendron adstringens* (Fabaceae: Mimosoideae) all are based on (-)-robinetinidol, but the lower or terminating units are based on (+)-gallocatechin and (-)-epigallocatechin (de Mello et al., 1996).

Proanthocyanidins based entirely on guibourtinidol/ epiguibourtinidol (258 Da), fisetinidol/epifisetinidol (274 Da), and robinetinidol/ epirobinetinidol (290 Da) (Fig. 1) also have previously been reported from legumes. Fisetinidinol and robinetidinol occur both as initiating or extender units and as terminal units (de Mello et al., 1996; Malan et al., 1988; Mathisen et al., 2002). (-)-Fisetinidol-(4β→6)-(-)-robinetinidol has been reported from the heartwood of the wild seringa, *Burkea africana* (Fabaceae: Caesalpinioideae) (Malan et al., 1988).

Both (2*R*,3*S*)- and (2*S*,3*R*)-absolute configurations for profisetinidins are known and, although almost all in legumes are (2*R*,3*S*)-, several instances with opposing configuration have now been documented (Ferreira et al., 2000). Nonetheless, profisetinidins based on units such as (+)-(2*S*,3*R*)-fisetinidol, (+)-(2*S*,3*R*)-robinetinidol and (2*S*,3*R*)-guibourtinidol] from legumes are rare (Ferreira et al., 2000; Nel et al., 1999; Steynberg et al., 1997). 2,3-*cis*-5-Deoxy compounds are even more uncommon. (2*R*,3*R*)-2,3-*cis*-Epifisetinidol chain extender units are known only from the bark of guamúchil, *Pithecellobium dulce* (Fabaceae: Mimosoideae), where they make up the major type of 5-deoxyflavan-3-ol present (Steynberg et al., 1997).

Acylated proanthocyanidins also have been reported from a number of legumes. Most of these involve gallic acid, typically attached as an ester at C-3 of one or both of the ring systems of the parent compound. For example, (-)-fisetinidol (4α→8)-(+)-catechin 3-*O*-galloyl ester has been reported from the heartwood of *Burkea africana* (Fabaceae: Caesalpinioideae) (Malan et al., 1988). In addition, several of the prorobinetinidins of *Stryphnodendron adstringens* (Fabaceae: Mimosoideae) occur as galloyl esters (de Mello et al., 1996).

In synthetic studies with (+)-mollisacacidin and (-)-fisetinidol, the principal dimeric products were (4α→6)- and (4β→6)-linked bis-profisetinidins (Steenkamp et al., 1983). The conspicuous absence of (4→8)-coupled analogs among this class of oligoflavanoids contrasts with the involvement of both C-6 and C-8 in interflavanyl bonding that occurs when the A-rings of the terminal or "lower" groups are of the phloroglucinol type (Foo, 1984). However, it should be noted that a series of 4α→6, 4β→6 and 4α→8 (-)-fisetinidol and (+)-*ent*-epifisetinidols were isolated in a later study from the heartwood of mopane, *Colophospermum mopane* (Fabaceae: Caesalpinioideae) (Steenkamp et al., 1988). The small amounts of (4→8)-linked-profisetinidins and proguibourtinidins in that instance were based on (-)-fisetinidol, (+)-*ent*-epifisetinidol, and (-)-guibourtinidol (Malan et al., 1990a, b).

Most proanthocyanidins, *p*-hydroxybenzoic acid, and *p*-hydroxy-benzoic acid esters possess antioxidant activity. However, because most proanthocyanidins have the ability to bind to protein, nucleic acids and carbohydrates to various degrees, they are at least weakly toxic in animal systems. They are known to inhibit some enzymes and not others (Zucker, 1983). In other instances, they exhibit more specific and well-defined effects. This may be related to stereospecific binding processes and in the case of larger (4→8)-linked oligomers, may involve helical forms of the proanthocyanidins (Haslam, 1979). There is compelling evidence that proanthocyanidins have quite selective interactions with proteins and, in particular, with known receptors (Zucker, 1983; Zhu et al., 1997). Certain proanthocyanidins arrest the cell cycle

in the G_0/G_1 phase (KOZIKOWSKI et al., 2003). Proanthocyanidins also have been shown to possess antimicrobial properties, possibly because of binding to proteins, interactions with membranes, and by deprivation of metal ions (SCALBERT, 1991; SMITH et al., 2005).

p-Hydroxybenzoic acid is widespread in nature. Esters of this compound (parabens) are commonly used as food additives and as common ingredients in cosmetics such as underarm deodorants to inhibit bacterial growth. This series of compounds is clearly antibiotic, but also has been shown to have estrogenic activity in mice and to appear in human breast cancer cells (DARBRE et al., 2003; LEMINI et al., 1997). *p*-Hydroxybenzoic acid also has been demonstrated to affect plant-water balance and has allelopathic activity (BARKOSKY and EINHELLIG, 2003).

Plants of *Acaciella angustissima* (Miller) Britton and Rose, can produce biomass yields as high as 5 tons per hectare per year. Further, the nitrogen content of the leaves of this plant is about 3%, i.e., almost 19% crude protein (AHN et al., 1989). Results of a study comparing supplementation of sheep maize stover diets with various legume forages indicated that *A. angustissima* may be a suitable alternative to *Leucaena leucocephala*, an established high-quality tree forage, that is not appropriate, however, for all agroecological conditions (MASAMA et al., 1997). Despite these outstanding features, air-dried *A. angustissima* plant material has been shown to be highly toxic to Ethiopian highland sheep at levels higher than 50 grams per day when fed without adaptation. Symptoms in the sheep suggested that the antinutritional factor(s) is a neurotoxin and that the cardiovascular system also is targeted (ODENYO et al., 1997). Further evidence of toxicity involves suppression of fermentation by mixed rumen organisms *in vitro* (OSUJI and ODENYO, 1997*)* and suppression of growth of pure bacterial cultures by aqueous extracts of *A. angustissima* (OSUJI and ODENYO, 1997; EL HASSAN et al., 1995). Samples of *A. angustissima* plant material contain 6.5% proanthocyanidin according to the vanillin-HCl method, but no tannins were detectable using the butanol-HCl method (AHN et al., 1989). These workers speculated that catechin gallates, which react positively in the vanillin-HCl method, but not the butanol-HCl method, are present in *A. angustissima*. However, because the proanthocyanidins of woody legumes often are based on 5-deoxyflavan-3-ols and others are acylated with other than galloyl units, it seems probable that these tannins are insensitive to the butanol-HCl reaction for other reasons. Gravimetric determination of soluble phenolics using trivalent ytterbium allowed other investigators to estimate the tannin content of *A. angustissima* (ILRI accession no. 15132, the same accession as used in this study), to be between 21 and 24% (ODENYO et al., 1997). In our laboratories, the tannin content of four *A. angustissima* accessions from Texas and Mexico ranged from 1.9 to 14.7% as determined by casein precipitation; proanthocyanidins (vanillin-HCl method) varied from 2-11.0% and hydrolysable tannins from 1.4-25.4% (iodate method) (READEL et al., 2001). The plant material used in this study was found to contain 17.8% total phenolics (Folin-Denis method) and 17.4% proanthocyanidins (vanillin-HCl method). Gallic acid, used as an indication of hydrolysable tannin after acid hydrolysis, was below detectable limits using the rhodanine assay (SMITH et al., 2001).

HAMMER and COLE (1965) found the proanthocyanidins of *A. angustissima* to be based primarily on fisetinidinol as evidenced by conversion to fisetinidin. They also reported the presence of the corresponding leucoanthocyanidin, 7,3',4'-trihydroxyflavan-3,4-diol.

Although the most obvious potentially toxic secondary metabolites identified from *A. angustissima* are non-protein amino acids (SEIGLER, 2003), based on the results of previous studies summarized in Tab. 1 (SMITH et al., 2001, 2003; SMITH and MACKIE, 2004), the deleterious substances appear to be proanthocyanidins. The ethyl acetate soluble portion from partition of lyophilized 70% acetone extract from milled air-dried foliage of *A. angustissima* between ethyl acetate and water (AA5) was previously used in a rat bioassay developed to evaluate palatability and toxicity and to determine the identity of the antinutritional factor(s) contained in the extract (SMITH et al., 2001). The major components involved in the antinutritional effects of this extract in rat diets were phenolics (SMITH et al., 2003), in particular, proanthocyanidins. Hydrolysable tannins were not detected. Inhibition of intake and weight gain with diets containing these phenolic substances was reversed by polyethylene glycol, a substance that complexes with proanthocyanidins (SMITH et al., 2003; MAKKAR et al., 1995).

Tannin resistant bacteria have been isolated from gastrointestinal

Tab. 1: Summary of biological effects of *Acaciella angustissima* proanthocyanidin-containing extracts.

A. angustissima extract	Major biological effects	Reference
70% acetone (similar to AA1)	2.3% proanthocyanidins in rat diets resulted in intake and average daily gain reduction (37% and 94% respectively). Increases in fecal nitrogen excretion and fecal proline, glycine and glutamic acid were most likely due to production of tannin-binding salivary gland proteins.	(Smith et al., 2001)
Ethyl acetate extract (similar to AA5)	0.7% proanthocyanidins in rat diets resulted in intake and average daily gain reduction (20% and 42% respectively). Increases in fecal nitrogen excretion and fecal proline, glycine and glutamic acid were detected.	(Smith et al., 2001)
Ethyl acetate extract (similar to AA5)	The negative effects of 4.2% proanthocyanidins in rat diets were neutralized by polyphenolic-binding polyethylene glycol.	(Smith et al., 2003)
Ethyl acetate extract (AA5)	The proportion of rat fecal tannin-resistant bacteria increased significantly from 0.3% to 25.3% and 47.2% with 0.7% and 2% proanthocyanidin-containing diets respectively. Predominant bacteria shifted towards tannin-resistant *Enterobacteriaceae* and *Bacterioides* species with a corresponding decrease in the *Clostridium leptum* group.	(Smith and Mackie, 2004)
Sephadex LH-20 extract (AA1.2)	The *A. angustissima* extract was more inhibitory to the growth of bacteria than a quebracho extract with a similar concentration of phenolics.	(Smith et al., 2005)

tract ecosystems where they are thought to prevent detrimental effects of dietary tannins on the animal. The mechanism of action of the protective effect of tannin-resistant bacteria in animals exposed to proanthocyanidins is unknown, but is likely to involve shifts in bacterial populations and modifications to their metabolic activity. After feeding *A. angustissima* extracts for three weeks, there was a significant shift in the proportion of tannin-resistant bacteria in the feces of rats (Smith and Mackie, 2004). 16S rDNA sequence analyses indicated that tannins selected for Gram-negative *Enterobacteriaceae* and *Bacteriodes* species, whereas there was a corresponding decrease in Low GC Gram-positive groups.

The present work was done to determine the structures and the antimicrobial effects of the proanthocyanidins present in foliage of the legume *A. angustissima*.

Materials and methods

General

All solvents were from Fisher Scientific (Pittsburgh, Pa.) and were reagent grade or better. TLC analyses were carried out on silica gel TLC plates (Sigma Chemical Co., St. Louis, MO, Silica Gel 60 F 254 on aluminum, 0.20 mm) and developed with ethyl acetate:methanol: water (79:11:10) or on cellulose plates (Fisher Scientific, Fairlawn, NJ, Avicel, 0.20 mm) with 2% acetic acid unless otherwise stated. Proanthocyanidins were visualized on TLC plates with vanillin-HCl reagent followed by brief heating at 110 C (pink spots).

Measurement of total phenolics in plant extracts was performed using the Folin-Denis method (Readel et al., 2001; Seigler et al., 1986). Quantification was based on a standard curve generated with tannic acid (Fisher Scientific Co., A-310, Fairlawn, NJ). In this instance, the vanillin reaction (Price et al., 1978) was used to determine the proanthocyanidin concentration of plant extracts, but purified *A. angustissima* tannin, obtained by absorption of tannins to Sephadex LH-20 and elution with 50% acetone (Strumeyer and Malin, 1975), was used to prepare the standard curve. Appropriate amounts of extract were dissolved in methanol. Sample and reagent volumes were reduced so reactions could be performed in Eppendorf tubes.

Mass Spectrometry

Mass spectra were determined by fast atom bombardment (FAB) on a Micromass ZAB-SE spectrometer and by matrix-assisted laser desorption/ionization (MALDI) on a Micromass TofSpec spectrometer in the Mass Spectrometry Laboratory of the School of Chemical Sciences, University of Illinois, Urbana, IL. FAB measurements were obtained in negative ion or positive ion modes. ES-MS analyses were made on an LCQ Deca XP mass spectrometer, Thermo Finnigan Corp., San Jose, CA.)

NMR spectrometry

NMR spectra were recorded on a Varian 400 spectrometer operating at 400 MHz for ^{1}H-spectra and 100 MHz for ^{13}C-spectra in the Nuclear Magnetic Resonance Laboratory of the School of Chemical Sciences, University of Illinois, Urbana, Ill.

Extraction of Proanthocyanidin-containing Fractions from *Acaciella angustissima*

Milled air-dried foliage from *A. angustissima* (ILRI accession #15132, 1 mm screen), grown in the Ethiopian Highlands was obtained from the International Livestock Research Institute (ILRI), Debre Zeit, Ethiopia. The extraction procedures can be visualized in Fig. 1-2.

Isolation of Purified Proanthocyanidin Extracts AA1 and AA1.2 (Fig. 1)

The plant material (100 g) was immersed six times in 70% acetone at room temperature for 1-2 hours and subsequently filtered (Whatman 4 paper). The extracts were combined and acetone removed under vacuum. Material that was insoluble in the remaining aqueous portion of the extract was removed by filtration (Whatman 4 paper) and the filtrate lyophilized to yield a blackish-brown amorphous substance (26.5 g, 26.5% yield). The TLC on silica gel gave a series of poorly resolved spots ranging between R_f 0.8-0.1 when visualized with vanillin-HCl reagent.

In order to prepare a purified proanthocyanidin fraction, lyophilized 70% acetone extract (1 g) dissolved in ethanol was added to a column of Sephadex LH-20 (25 g) packed from a slurry in 80% ethanol (100 ml) (Asquith and Butler, 1985). The column was washed with 95% ethanol until no further material was eluted as judged by TLC, followed by washing with 50% aqueous acetone until elution of material ceased. The combined acetone washes were evaporated and extracted with an equal volume of EtOAc to obtain a solid sample (AA1.2) that consisted of 97% phenolics, as determined by the Folin-Denis method. TLC analysis of this fraction indicated the presence of three spots (R_f 0.76, 0.81 and 0.83). Two compounds that were visible, but that did not react with vanillin-HCl also were present (R_f 0.47, and 0.54). Six spots were visible on cellulose TLC plates (R_f 0.02, 0.07, 0.15, 0.47, 0.60 and 0.73). The three spots with lowest R_f values reacted with this reagent.

NMR Spectra. ^{1}H-NMR δ (acetone-d_6) 7.4-8.4 (broad multiplet), 6.2-6.8 (broad multiplet), 6.0-6.15 (multiplet), 5.1-5.6 (multiplet), 4.4-5.0 (multiplet), 4.1 (broad singlet), 4.0 (broad singlet), 3.8 (broad singlet), 3.7 (broad singlet), 3.5 (singlet), 3.38 (broad singlet, H_2O), 3.1 (singlet), 3.05 (singlet, H_2O), 2.4-2.8 (broad multiplet), 1.9-2.4 (multiplets), 2.0-2.1 (acetone methyl group), 1.3 (singlet), 1.25 (singlet). ^{13}C-NMR δ (acetone-d_6) 206.870, 206.673 (carbonyl groups), 156.880 (1), 152.316 (1) 145.729 (4), 133.352 (1), 132.329 (1), 128.761 (1), 118.192 (<1), 115.298 (1), 108.844 (1), 105.742 (3), 105.649 (3), 104.004 (1), 103.584 (1), 79.982 (1), 78.622 (1), 78.363 (1), 78.105 (1), 76.124 (1), 75.515 (<1), 73.185 (<1), 49.354

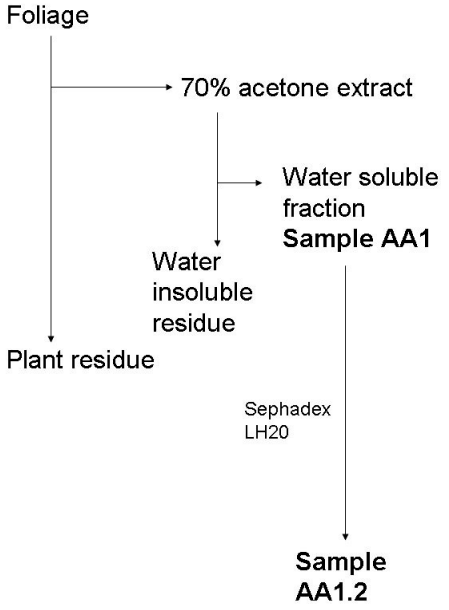

Fig. 1: Flow chart of solvent extraction of *Acaciella angustissima* foliage resulting in proanthocyanidin-enriched fractions AA1 and AA1.2. Details of solvent extractions are in the text under Materials and methods.

(1), 35.910 (1), 30.384 - 29.223 (methyl groups of acetone), 19.491 (1), 14.326 (1). Approximate carbon integrals estimated from the spectrum.

Mass Spectrum. FAB-MS (positive ion spectrum) (*m/z*) 329 (50), 361.1 (66), 393.1 (55) $[C_{22}H_{16}O_7+H]^+$, 411.1 (72%) $[C_{22}H_{18}O_8+H]^+$, 447.1 (20), 547.1 (29) $[C_{30}H_{26}O_{10}+H]^+$, 563.2 (46) $[C_{30}H_26O_{11}+H]^+$, 667.2 (34) $[C_{37}H_{30}O_{12}+H]^+$, 683.1 (100) $[C_{37}H_{30}O_{13}+H]^+$, 699.2 (28), $[C_{37}H_{30}O_{14}+H]^+$, 818.0 (20) $[C_{45}H_{38}O_{15}+H]^+$, 834.3 (46) $[C_{45}H_{38}O_{16}+H]^+$, 857.2 (14) $[C_{45}H_{38}O_{16}+Na]^+$, and 874.3 (12) $[C_{45}H_{38}O_{17}+Na]^+$, 939 (12) $[C_{52}H_{42}O_{17}+H]^+$, 955.3 (20) $[C_{52}H_{42}O_{18}+H]^+$, 971.3 (8) $[C_{52}H_{42}O_{19}+H]^+$, 1107.4 (5) $[C_{60}H_{50}O_{21}+H]^+$. MALDI-MS (*m/z*) 563.22 (80% of base peak) $[C_{30}H_{26}O_{11}+H]^+$, 583.22 (90) $[C_{30}H_{26}O_{11}+Na]^+$, 683.31 (97) $[C_{37}H_{30}O_{13}+H]^+$, 699.32 (78) $[C_{37}H_{30}O_{14}+H]^+$, 857.37 (100) $[C_{45}H_{38}O_{16}+Na]^+$, 873.35 (96) $[C_{45}H_{38}O_{17}+Na]^+$, 955.3 (83) $[C_{52}H_{42}O_{18}+H]^+$, 978.26 (53) $[C_{52}H_{42}O_{18}+Na]^+$, 1114.43 (82) $[C_{60}H_{50}O_{20}+Na]^+$, 1130.35 (94) $[C_{60}H_{50}O_{21}+Na]^+$, 1146.38 (85) $[C_{60}H_{50}O_{22}+Na]^+$, 1266.80 (40) $[C_{67}H_{54}O_{24}+Na]^+$, 1386.66 (50) $[C_{75}H_{62}O_{25}+Na]^+$, 1402.74 (74) $[C_{75}H_{62}O_{26}+Na]^+$, 1419.19 (63) $[C_{75}H_{62}O_{27}+Na]^+$, 1674.13 (43) $[C_{90}H_{74}O_{31}+Na]^+$, 1691.27 (46) $[C_{90}H_{74}O_{32}+Na]^+$, and smaller series of peaks corresponding to heptamers, octamers, nonamers, and decamers.

Ethyl Acetate Soluble Materials (AA5) from Partition of Lyophilized 70% Acetone Axtract with Water (Fig. 2)

An acetone soluble fraction was derived by extraction of dried foliage (5 kg) from *A. angustissima* (ILRI accession #15132, 1 mm screen) in 1 kg batches with 70% acetone (Smith et al., 2003). Acetone was removed and the resulting material lyophilized. Analysis by Folin-Denis reaction indicated that the lyophilized extract consisted of 60% proanthocyanidins. This solid residue was dissolved in the minimum amount of ethyl acetate needed to effect solubility and partitioned with an equal volume of water. Ethyl acetate was removed from the organic phase and the remaining aqueous material lyophilized to yield an amorphous solid (720 g, 14.4% yield). TLC analysis of this fraction (AA5) on silica gel revealed the presence of two proanthocyanidin-rich spots (R_f 0.84 and 0.81). Three additional spots (R_f 0.58, 0.54, and 0.51) were visible, but did not react with the vanillin-HCl reagent. Analysis on cellulose plates indicated the presence of at least six compounds (R_f values: 0.8, 0.46, 0.13, 0.4, 0.06, and 0.03).

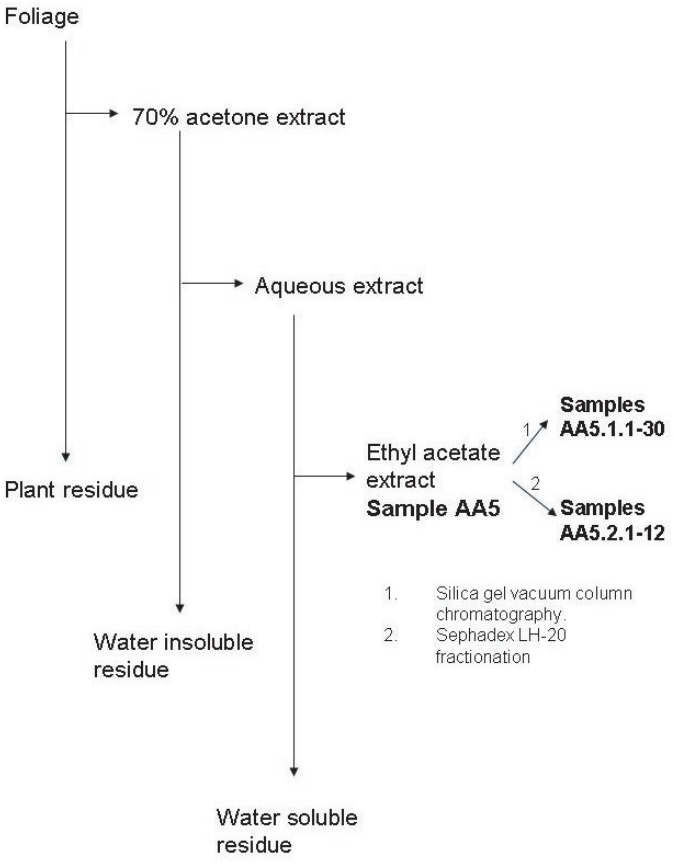

Fig. 2: Flow chart of solvent extraction of *Acaciella angustissima* foliage resulting in ethyl acetate extracted sample AA5 and further proanthocyanidin-enriched samples. Details of solvent extractions are in the text under Materials and methods.

Mass Spectrum. FAB-MS (negative ion spectrum) (*m/z*) 529.1 (12) $[C_{30}H_{26}O_9-H]^-$, 545.1 (29) $[C_{30}H_{26}O_{10}-H]^-$, 561.2 (82) $[C_{30}H_{26}O_{11}-H]^-$, 575.1 (100%) $[C_{31}H_{28}O_{11}-H]^-$, 591.2 (17) $[C_{31}H_{28}O_{12}-H]^-$, 607.2 (42) $[C_{30}H_{24}O_{14}-H]^-$, 785.2 (4) $[C_{45}H_{38}O_{13}-H]^-$, 801.2 (10) $[C_{45}H_{38}O_{14}-H]^-$, 817.2 (17) $[C_{45}H_{38}O_{15}-H]^-$, 833.2 (50) $[C_{45}H_{38}O_{16}-H]^-$, and 849.2 (2) $[C_{30}H_{26}O_{17}-H]^-$, 1089 (2) $[C_{60}H_{50}O_{20}-H]^-$ and 1105.4 (4) $[C_{60}H_{50}O_{21}-H]^-$.

Vacuum Column Chromatography (VLC). A portion of the ethyl acetate soluble material from partition with water (AA5) (1 g) was dissolved in ethyl acetate and mixed with silica gel (5 g, Merck, Rahway, NJ, GF-254 Type 60). The ethyl acetate was permitted to evaporate and the silica gel mixture placed onto a vacuum column containing silica gel (60 g) that had been flushed with petroleum ether (50 ml). Thirty fractions were collected: 1 (petroleum ether), 2 (20 % EtOAc in petroleum ether), 3 (40 %), 4 (60 %), 5 (80 %), 6 (EtOAc), 7 (1% of 1:1 MeOH:H_2O in EtOAc), 8 (2 %), 9 (3 %), 10 (4 %), 11 (5 %), 12 (6 %), 13 (7 %), 14 (8 %), 15 (9 %), 16 (10 %), 17 (12 %), 18 (14 %), 19 (16 %), 20 (18 %), 21 (20 %), 22 (25 %), 23 (30 %), 24 (35 %), 25 (40 %), 26 (45%), 27 (50 %), 28, 29, and 30 (100 % MeOH-H_2O, 1:1). Fractions 1-6 were 50 ml, all others 100 ml. After removal of solvents under vacuum, the fractions were analyzed by TLC (silica gel, vanillin-HCl reagent). Fractions 1-6 contained only traces. TLC of fractions 7 and 8 revealed the presence of two proanthocyanidin-containing spots (R_f 0.65 and 0.75) in each instance, Fraction 9 also had two spots (R_f 0.53 and 0.65); fractions 7-9 were kept separate. Fractions 10-13 had one proanthocyanidin (R_f 0.75), as well as other spots that did not react with vanillin-HCl reagent (R_f 0.35, 0.42 and 047), fractions 16-17 had one vanillin-HCl positive spot (R_f 0.24), as did fractions 23-25 (R_f 0.05). Based on TLC, fractions 10-11, 12-13, 14-15, 16-17, 18-22, 23-25, and 26-30 were combined.

Mass Spectrum of Fraction 7 from VLC (AA5.1.7). FAB-MS (positive ion spectrum) (*m/z*) 309.0 (Magic Bullet), 329.0 (70), 345.0 (36), 371.0 (54), 387.0 (52), 395.1 (48) $[C_{22}H_{18}O_7+H]^+$, 411.1 (100) $[C_{22}H_{18}O_8+H]^+$, 427.0 (30) M-152, reverse Diels-Alder fragment, 443 (36), 530.1 (18) $[C_{30}H_{26}O_9+H]^+$, 547.1 (41) $[C_{30}H_{26}O_{10}+H]^+$, 563.1 (76) $[C_{30}H_{26}O_{11}+H]^+$, 651.1 (12) $[C_{37}H_{30}O_{11}+H]^+$, 667.2 (22) $[C_{37}H_{30}O_{12}+H]^+$, 683.1 (32) $[C_{37}H_{30}O_{13}+H]^+$, 699.1 (12) $[C_{37}H_{30}O_{14}+H]^+$, 803.1 (7) $[C_{45}H_{38}O_{14}+H]^+$, 819.2 (12) $[C_{45}H_{38}O_{15}+H]^+$, and 835.1 (7) $[C_{45}H_{38}O_{16}+H]^+$.

NMR Spectrum of Fraction 7 from VLC (AA5.1.7). ^{1}H-NMR δ (acetone-d_6) 7.4-8.4 (broad multiplet); 6.2-7 (multiplet), 5.9-6.1 (multiplet), 5.2-5.4 (multiplet), 4.4-5.1 (multiplet), 3.0-3.5 (broad singlet, H_2O), 2.4-2.9 (multiplet), 1.8-2.4 (multiplet, includes acetone peak), 1.3-1.4 (broad singlet), 1.2 (singlet). ^{13}C-NMR δ (acetone-d_6) 206.552 (acetone carbonyl), 157.505 (1), other smaller peaks 153-159, 146.352 (6), 134.108 (2), 132.962 (2), 129.472 (2), 115.771 (3), 109.277 (2), 108.556 (2), 105.999 (4), 105.787 (4?), 105.476 (4?), 104.019 (2), 103.579 (2), 79.545 (2), 77.406 (2), 76.055 (2), 73.409 (1), 30.377-29.216 (acetone methyl groups), 20. The number of carbon atoms is estimated from the spectrum.

Fractionation of the Ethyl Acetate Soluble Material from Partition with Water (AA5) on Sephadex LH-20 (AA5.2.1-12) (Fig. 3).

A purified fraction of *A. angustissima* proanthocyanidins was obtained by chromatography of the ethyl acetate soluble material from partition with water (AA5) on Sephadex LH-20 (HAGERMAN, accessed 2011). A sample of this substance (1 g) was dissolved in 95% ethanol and applied to a Sephadex LH-20 column (50 g) (ASQUITH and BUTLER, 1985; HAGERMAN, accessed 2011). The column was washed with 95% ethanol (fraction 1) until material was no longer eluted as indicated by TLC. Fractions 2-7 were then eluted with 80% ethanol, fraction 8 with 60% ethanol, and fractions 9-12 with a mixture of acetone, ethanol and water (2:1:1). The column was finally washed with acetone (fraction 13) until no additional material was eluted.

Fraction 6 from this separation contained primarily one proanthocyanidin-rich spot (R_f 0.67). Based on TLC results, fractions 6-8 were combined and used for further analyses (320 mg). This sample (AA5.2.6-8) consisted of 72% condensed tannins by the vanillin-HCl assay.

NMR Spectra. ^{1}H-NMR δ (DMSO-d_6) 7.4-9.6 (broad multiplet), 5.8-7.0 (multiplets), 5.2-5.4 (multiplet), 4.2-5.0 (multiplet), 3.66 (broad singlet, H_2O), 3.459 (singlet), 3.445 (singlet), 3.431 (multiplet), 2.5-3.0 (broad multiplet), 2.471 (singlet), 2.096 (singlet), 1.6-2.05 (broad multiplet), 1.131 (singlet). ^{13}C-NMR δ (acetone-d_6) 206.954 (carbonyl groups of acetone), 160.645 (<1), 158.172 (2), 157.435 (1), 153.522, 146.245 (4), 134.168 (1), 132.856 (1), 129.305 (1), 123.107 (<2), 115.786 (1), 109.262 (1), 108.519, 106.068 (3), 105.764 (3), 103.974 (1), 79.538 (1), 76.101, 57.673, 36.446 (1), 30.377-29.223 (methyl groups of acetone). Number of protons estimated by peak height from spectrum.

Mass Spectrum. FAB-MS (positive ion spectrum) (*m/z*) 563.1 (20% of base peak) $[C_{30}H_{26}O_{11}+H]^+$, 573.1 (12), 665.1 (25) $[C_{37}H_{28}O_{12}+H]^+$, 683.2 (100) $[C_{37}H_{30}O_{13}+H]^+$, 699.2 (28) $[C_{37}H_{30}O_{15}+H]^+$, 817.3 (14) $[C_{45}H_{38}O_{15}+H]^+$, 835.3 (50) $[C_{45}H_{38}O_{16}+H]^+$, 857.2 (40) $[C_{45}H_{38}O_{16}+Na]^+$, 874 (22) $[C_{45}H_{38}O_{17}+Na]^+$, 937.2 (10) $[C_{52}H_{42}O_{17}+H]^+$, 955.2 (18) $[C_{52}H_{42}O_{18}+H]^+$, 971.3 (4) $[C_{52}H_{42}O_{20}+H]^+$, 1129 (4) $[C_{60}H_{50}O_{21}+Na]^+$.

Results and discussion

Based on previous studies, it was known that sizable quantities (6-25%) of proanthocyanidins are found in forage material of *A. angustissima* (SMITH et al., 2001, 2003; AHN et al., 1989; ODENYO et al., 1997; READEL et al., 2001). It has been established that these compounds are toxic to livestock and have antibacterial activity (SMITH and MACKIE, 2004) (Tab. 1). However, the composition of these proanthocyanidins has not been described previously.

Extracts of *A. angustissima* and their Purification

In order to evaluate the relative effectiveness of various chromatographic methods, lyophilized 70% acetone extracts of *A. angustissima* were fractionated in several ways. None of these extraction and chromatographic methods was completely effective in resolving the mixtures of proanthocyanidins from *A. angustissima*. However, prior silica gel or Sephadex LH-20 fractionation was important for carrying out subsequent mass spectrometric analyses. Many mass spectrometric peaks cannot be seen in crude mixtures and are only detectable in purified fractions (PRIOR et al., 2001; TAYLOR et al., 2003).

Crude Deoxyflavan-3-ols and Proanthocyanidins

Lyophilization of a 70% acetone extract of air-dried foliage from *A. angustissima* yielded an amorphous solid that consisted of 60% condensed tannins (READEL et al., 2001; SEIGLER et al., 1986) (26.5% yield). TLC of this material visualized with vanillin-HCl reagent indicated the presence of a complex series of proanthocyanidins. A purified proanthocyanidin sample (AA1.2) was prepared by absorption of the lyophilized material above on Sephadex LH-20, washing the column with 95% ethanol, and finally elution with 50% aqueous acetone. This fraction (AA1.2) consisted of 97% phenolics, as determined by the Folin-Denis method. Based on analysis by TLC (silica gel) and comparison to the original 70%

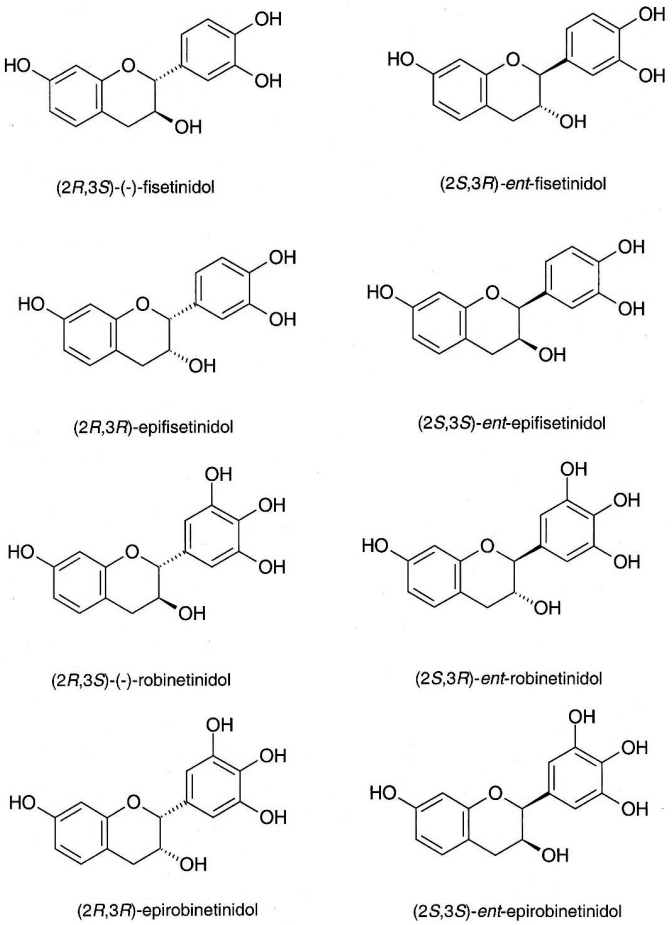

Fig. 3: 5-Deoxyflavan-3-ols

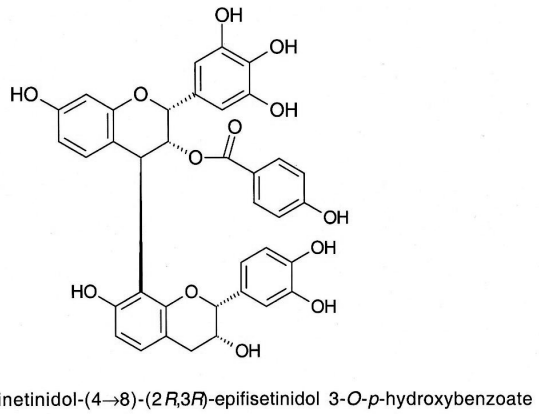

Fig. 4: A representative acylated proanthocyanidin.

acetone extract, this product appears to contain a full complement of the proanthocyanidins found in *A. angustissima*. The series of fractions terminated with those consisting mostly of acylated proanthocyanidins.

Positive ion FAB- and MALDI-MS, indicated that this material contains a complement of proanthocyanidins consisting mostly of dimers through hexamers (*m/z* 547.1, 563.2, 818.0, 834.3; sodium adducts *m/z* 857.2, 874.3, 1114, 1130.4, 1146.4, 1386.7, 1402.7, 1419.2, 1674.1, 1691.3) based on monomeric units of *m/z* 258, 274, and 290. In addition, peaks greater by 120 Da were present at 411.1, 683.1, 699.2, 939, 955.3, and 971.3; a Na adduct at 1266.8 or 120 Da greater than the acylated tetramer also was observed. These signals were accompanied by smaller peaks corresponding to proanthocyanidin heptamers through decamers. Few signals other than those associated with proanthocyanidins were present.

Because of atropisomerism (Shoji et al., 2003), the ^{1}H-NMR spectrum of this fraction (AA1.2) contains a series of poorly defined multiplets between δ 6-7 ppm (2'-, 5'- and 6'-protons of 3',4'- and 3',4',5'-substituted B-rings, and 5-, 6-, and 8-protons of 5-deoxyflavan-3-ol A-rings (Steynkamp et al., 1988; Malan et al., 1990b; Qa'Dan et al., 2003). Peaks in the ^{1}H-NMR spectrum corresponding to the presence of both robinetinidol (a singlet at 6.58 ppm) and fisetinidol (multiplet, 6-7 ppm) are present. Signals for 2-, 3-, and 4-C-ring protons of flavan-3-ols are found between 5.0 and 3 ppm. Multiplets in the region between 2 and 3 ppm corresponding to unsubstituted 4-protons (methylene groups) are largely obscured by solvent and impurity peaks in these samples. Because of the complex nature of the compounds and mixtures involved, the resonances of most protons in the ^{1}H-NMR spectrum cannot be assigned exactly.

The ^{13}C-NMR spectrum is more informative: The peak at 156.880 ppm coincides with that expected for C-7 (Mathisen et al., 2002; Czochanska et al., 1980). Signals centered at 145.729 ppm correspond to carbons at the 3'- and 4'-positions of 3',4'-disubstituted B-rings and 3', 5'-carbons of 3',4',5'-trisubstituted B-rings. The intensity of this peak (approximately 4 times greater than the peak for C-7) supports this assignment. Resonances at 133.352 and 132.329 ppm correspond to those expected for 1'-positions of 3',4'-disubstituted B-rings and 4'-positions of 3',4',5'-trisubstituted B-rings (Czochanska et al., 1980; Behrens et al., 2003; Morais et al., 1999). Signals at 118.192 ppm are compatible with those produced by 6'-carbons of 3',4'-disubstituted B-rings. Peaks at 115.298 ppm can be assigned to 2'- and 5'-carbons of 3',4'-disubstituted B-rings (Behrens et al., 2003; Porter et al., 1982).

Among the peaks at higher field are those for 6- or 8-carbons involved in linkage of proanthocyanidin ring systems. Signals at 104.004 and 103.584 may correspond to either C-8 in (4→6)-linked systems or to C-6 in (4→8)-linked systems based on 5-deoxyflavan-3-ols (Foo, 1984). Resonances assignable to either 6- or 8-carbons in (4→6)- or (4→8)-linked oligomers involving the linkage usually are found between 106-108.5 ppm (Porter et al., 1982). The lack of C-6 and C-8 resonances at 96-97 ppm and of a peak for C-5 (~ 152-155 ppm) for phloroglucinol-type proanthocyanidins in these spectra confirm that catechin/epicatechin, gallocatechin/epigallocatechin, or afzelechin/epiafzelechin are not present as either the "upper" initiating or "lower" terminal unit (Czochanska et al., 1980; Porter et al., 1982; Sun et al., 1988).

Peaks at 79.982, 78.622, 78.363, 78.105, 76.124, and 75.515 ppm in the condensed tannin sample (AA1.2) correspond to carbons at the 2- and 3-positions of C-rings and similar rings in oligomers. The peaks immediately below 80 ppm may be attributed to C-2 absorptions of 2,3-*cis*-units (Foo, 1984; Czochanska et al., 1980), whereas those at higher field are of C-3 carbons. In contrast, resonances above 80 ppm, corresponding to C-2 of 2,3-*trans*-units, are absent. Peaks for 1' (~ 121 ppm), 4a (~ 102 ppm), and 8a (~ 157 ppm) are often weak or not observed as in published spectra (Qa'Dan et al., 2003; Sun et al., 1988). Because the materials examined consist mainly of oligomers and acylated species in which the peak for C-3 is shifted both by acylation and by substitution at position 4, the peak for C-3 at 67-68 ppm (Czochanska et al., 1980) should be low in intensity and is not seen in most spectra. The C-4 peak of terminal or "lower" units of acylated monomers (28-29 ppm) in the ^{13}C-NMR of the purified condensed tannin sample (AA1.2) is reduced in intensity for the same reasons, but in addition often is obscured by solvent absorptions.

Proanthocyanidins from Partitioning between Water and Ethyl Acetate

Another purified proanthocyanidin extract was prepared by partitioning lyophilized 70% acetone extract between water and ethyl acetate (AA5 14.4% yield). The ethyl soluble portion (AA5) consisted largely of low molecular weight proanthocyanidin oligomers. Although TLC (silica gel) of this fraction revealed only the presence of two spots that reacted strongly with vanillin-HCl reagent (R_f 0.84 and 0.81), TLC analysis (cellulose) indicated the presence of at least six compounds.

A negative ion FAB-MS of the ethyl acetate soluble materials from partition (AA5) has peaks at *m/z* 529, 545.1, 561.2, 785.2, 801.2, 817.2, 833.2, 849.2, 1089 and 1105.4, corresponding to dimers, trimers, and tetramers based on monomeric units of *m/z* 258, 274, and 290. Peaks at 575.1, 591.2, and 607.2 are unidentified, but may correspond to flavonoid glycosides.

Proanthocyanidins from VLC

Fractionation of the material obtained by partitioning between water and ethyl acetate by vacuum chromatography (VLC, silica gel) yielded a series of proanthocyanidin-rich fractions as indicated by positive vanillin-HCl reactions on TLC. Based on the relative intensity of peaks in an FAB-MS, the major component of AA5.1.7 from this fractionation had *m/z* 411.1 (290 + 120), but this substance was accompanied by a series of dimeric and trimeric proanthocyanidins. A positive ion FAB-MS (fraction AA5.1.7) had peaks at *m/z* 411.1 (290 monomer + 120), 547.1, 563.1 (dimers based on monomers of 274 and 290). These peaks correspond to masses at 545.1 and 561.2 seen in the negative ion FAB-MS of sample AA5. Peaks of lower intensity corresponding to the proanthocyanidin trimers of AA5 occur at *m/z* 803.1, 819.2 and 835.1. Peaks at 667.2 and 683.1 (dimers + 120) in this sample correspond to acylated proanthocyanidin dimers. MS data suggest that at least 14 compounds were present. However, based on peak intensities, this mixture contained at least 50% acylated monomers and dimers.

Proanthocyanidins from Partition between Water and Ethyl Acetate Purified on Sephadex LH-20

An even more-highly purified fraction of proanthocyanidins was obtained by chromatography of the ethyl acetate soluble material from partition with water (AA5) on Sephadex LH-20 (Asquith and Butler, 1985; Hagerman, accessed 2011). As indicated by TLC (silica gel), the material eluted with 80% ethanol (AA5.2.6-8) contained primarily one proanthocyanidin-rich spot (R_f 0.7), but as indicated by MS, this fraction also consisted of a mixture of several proanthocyanidins. In a positive ion FAB-MS spectrum, peaks corresponding to dimers (low intensity, 563.1) and trimers (higher intensity, 817.3, 835.3; Na adduct peaks *m/z* 857.2, 874, 1129) based on the same series of monomeric units were observed. Signals with mass 120 Da greater were found at *m/z* 665.3, 683.2, 699.2, 937.2, and 955.2.

Structure of Proanthocyanidins of *A.* angustissima

The condensed tannins of *A. angustissima* consist of a series of 5-deoxyflavan-3-ols and dimers to hexamers based on monomeric units of 258, 274, and 290 Da (guibourtinidol, fisetinidol, and robinetinidol) and a series of compounds greater in mass by 120 Da based on the same series of monomeric units, probably corresponding to acylation. The presence of smaller amounts of oligomers as large as decamers is indicated by FAB-MS and MALDI-MS spectra.

Several common and widely distributed flavan-3-ols in legumes (plants of the Fabaceae) have identical mass values in mass spectra, but peaks in the ^{1}H-NMR spectra corresponding to H-6 and H-8 of catechin/epicatechin, gallocatechin/epigallocatechin, or afzelechin/epiafzelechin-based proanthocyanidins (~5.9, ~6.1 ppm) (DE MELLO et al., 1996; SHOJI et al., 2003; TANAKA et al., 2000), or the AA'BB' patterns of the B-ring of afzelechin/epiafzelechin (7.5 and 7.0 ppm, J = 8 Hz) (TANAKA et al., 2000) do not occur in the spectra of proanthocyanidins from *A. angustissima*, precluding the presence of phloroglucinol A-ring-based proanthocyanidins. Further, peaks at ~157 ppm characteristic for C-5 and 96-97 ppm characteristic for C-6 and C-8 of phloroglucinol-type A rings are absent from ^{13}C-NMR spectra. Proanthocyanidins based on oritin or epioritin (5-deoxy-7,8-dihydroxy substitutions) may be ruled out (BENNIE et al., 2002).

In previous studies, it has been found that (4β→6)-linkages predominate in condensations of 5-deoxyflavan-3-ols and other appropriate precursors (STEENKAMP et al., 1988; MALAN et al., 1990a, b), although small amounts of (4β→8)-, (4α→6)-, and (4α→8)-linkages are sometimes encountered (STEENKAMP et al., 1988). These observations suggest that the proanthocyanidins of *A. angustissima* contain largely (4β→6)-linkages, although this remains to be established.

The presence of a number of signals 120 Da greater than the monomers, dimers and trimers in mass spectra, strongly suggests the presence of acyl derivatives. This is supported by corresponding shifts in the positions of C-2, C-3, and C-4. The presence of an acyl group (gallate) at position C-3 of a phloroglucinol-type system with 2,3-*cis*-configuration effects an upfield shift in the position of C-2 of approximately 2.5 ppm in the "upper" ring system (77.1 to 75.8 ppm) (SUN et al., 1988). This corresponds to peaks at 79.050 and 76.124 that are present in the ^{13}C-NMR spectra of purified proanthocyanidins from *A. angustissima* (AA1.2).

The presence of an acyl group (gallate) at C-3 of a phloroglucinol-type system with 2,3-*cis*-configuration causes a downfield shift of approximately 1.5 ppm in the "upper" ring system (73.2 to 74.7 ppm) (SUN et al., 1988). The shift for a C-3 of the "lower" ring system with gallate attached is 69.2 ppm and is relatively independent of whether a gallate is attached to C-3 of the "upper" ring system or not (MATHISEN et al., 2002; SUN et al., 1988). Peaks at 73.185 and 75.515 ppm in the *A. angustissima* spectra correspond to the peaks predicted for acylated and non-acylated forms of the proanthocyanidins. A peak at 69.254 appears in some spectra, corresponding to the "lower" unit of oligomers, but this peak is weak in most instances.

The observed *m/z* of the most prominent peak of one of the peaks differing by 120 *m/z* from high resolution mass spectra (683.176300 observed, calculated for $C_{37}H_{31}O_{14}$: 683.176467) confirms the assigned empirical formula and strongly supports identification of this compound as the *p*-hydroxybenzoate derivative of a proanthocyanidin dimer.

Proanthocyanidins acylated by *p*-hydroxybenzoic acid have previously been reported in a type-A proanthocyanidin from *Prunus armeniaca* (PRASAD et al., 1998) and type-B proanthocyanidins from *Camellia sinensis* (HASHIMOTO et al., 1987). The ^{13}C-NMR of *p*-hydroxybenzoate moieties includes peaks at 166.4 (ester carbonyl), 121.7 (1'-), 132.2 (2'- and 6'-), 116 (3'- and 5'-), and 162.8 (4'-) ppm (HASHIMOTO et al., 1987). Although peaks corresponding to 2'-, 3'-, 5'-, and 6'- were observed, peaks corresponding to the carbonyl 1'- (~121.7 ppm), and 4'-carbons (~162.8 ppm) of proanthocyanidins of *A. angustissima* were not observed in the spectra. Signals for the AA'BB'-pattern of the *p*-hydroxybenzoate moiety (7.76-7.50 and 6.84-6.73 ppm, depending on the solvent, temperature, and composition of the mixture examined) of proton spectra were weak and difficult to observe in all fractions.

The presence of gallate esters can be ruled out by the absence of a singlet at 7.03-7.05 ppm (HASHIMOTO et al., 1987; DAUER et al., 1998, 2003) in the ^{1}H-NMR spectrum, a characteristic peak at 139 ppm in the ^{13}C-NMR spectrum (MATHISEN et al., 2002), and peaks with an increase of *m/z* 152 in the mass spectra of proanthocyanidins.

In summary, the proanthocyanidins of *A. angustissima* leaf material consist largely of 5-deoxyflavan-3-ols, and dimers through hexamers accompanied by smaller amounts of higher molecular weight oligomers (heptamers-decamers). These are accompanied by 5-deoxyflavan-3-ols, dimers and trimers that contain a *p*-hydroxybenzoate ester moiety.

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References

AHN, J.H., ROBERTSON, B.M., ELLIOT, R., GUTTERIDGE, R.C., FORD, C.W., 1989: Quality assessment of tropical browse legumes: Tannin content and protein degradation. Anim. Feed Sci. Tech. 27, 147-156.

ASQUITH, T.N., BUTLER, L.G., 1985: Use of dye-labelled protein as spectrophotometric assay for protein precipitants such as tannin. J. Chem. Ecol. 11, 1535-1544.

BARKOSKY, R.R., EINHELLIG, F.A., 2003: Allelopathic interference of plant-water relationships. Bot. Bull. Acad. Sin. 44, 53-58.

BARRY, T.N., BLANEY, B.J., 1987: Secondary compounds of forages. In: Hacker, J.B., Ternouth, J.H. (eds.), Nutrition of herbivores. Academic Press Australia: Sydney, Australia, 91-119.

BEHRENS, A., MAIE, N., KNICKER, H., KÖGEL-KNABNER, 2003: I. MALDI-TOF mass spectrometry and PSD fragmentation as means for the analysis of condensed tannins in plant leaves and needles. Phytochemistry 62, 1159-1170.

BENNIE, L.J., COETZEE, J., MALAN, E., FERREIRA, D., 2002: (4-6)-Coupled proteracacidins and promelacacidins from *Acacia galpinii* and *Acacia caffra*. Phytochemistry 60, 521-532.

BOTHA, J.J., FERREIRA, D., ROUX, D.G., 1981: Synthesis of condensed tannins. Part 4. A direct biomimetic approach to [4,6]- and [4,8]-biflavonoids. J. Chem. Soc. Perkin Trans. I 1981, 1235-1245.

CZOCHANSKA, Z., FOO, L.Y., NEWMAN, R.H., PORTER, L.J., 1980: Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. J. Chem. Soc. Perkin Trans. Part I. 1980, 2278-2286.

DARBRE, P.D., BYFORD, J.R., SHAW, L.E., HALL, S., COLDHAM, N.G., POPE, G.S., SAUER, M.J., 2003: Oestrogenic activity of benzylparaben. J. Appl. Toxicol. 23, 43-51.

DAUER, A., METZNER, P., SCHIMMER, O., 1998: Proanthocyanidins from the bark of *Hamamelis virginiana* exhibit antimutagenic properties against

nitroaromatic compounds. Planta Med. 64, 324-327.

DAUER, A., RIMPLER, H., HENSEL, A., 2003: Polymeric proanthocyanidins from the bark of *Hamamelis virginiana*. Planta Med. 69, 89-91.

DE MELLO, J.P., PETEREIT, F., NAHRSTEDT, A., 1996: Prorobinetinidins from *Stryphnodendron adstringens*. Phytochemistry 42, 857-862.

EL HASSAN, S.M., LAHLOU-KASSI, A., NEWBOLD, C.J., WALLACE, R.J., 1995: Antimicrobial factors in African multipurpose trees. In: Wallace, R.J., Lahlou-Kassi, A. (eds.), Rumen Ecology Research Planning. International Livestock Research Institute (ILRI): Nairobi, Kenya, 43-54.

FERREIRA, D., LI, X.C., 2000: Oligomeric proanthocyanidins: naturally occurring *O*-heterocycles. Nat. Prod. Rep. 17, 193-212.

FOO, L.Y., 1984: Condensed tannins: co-occurence of procyanidins, prodelphinidins, and profisetinidins in the heartwood of *Acacia baileyana*. Phytochemistry 23, 2915-2918.

HAGERMAN, A.E., Accessed 2011: Tannin chemistry: Hagerman laboratory methods. http://www.users.muohio.edu/hagermae/

HAMMER, R.H., COLE, J.R., 1965: Phytochemical investigation of *Acacia angustissima*. J. Pharm. Sci. 54, 235-239.

HASHIMOTO, F., NONAKA, G., NISHIOKA, I., 1987: Tannins and related compounds. LVI. Isolation of four new acylated flavan-3-ols from oolong tea. Chem. Pharm. Bull. 35, 611-616.

HASLAM, E., 1979: Vegetable tannins. In: Swain, T., Harborne, J.B., van Sumere, C.F. (eds.), Recent Advances in Phytochemistry. Plenum: New York, Vol. 12, 475-524.

KOZIKOWSKI, A.P., TÜCKMANTEL, W., BÖTTCHER, G., ROMANCZYK JR., L.J., 2003: Studies in polyphenol chemistry and bioactivity. 4. Synthesis of trimeric, tetrameric, pentameric, and higher oligomeric epicatechin-derived procyanidins having all 4β,8-interflavan connectivity and their inhibition of cancer cell growth through cell cycle arrest. J. Org. Chem. 68, 1641-1658.

LEMINI, C., SILVA, G., TIMOSSI, C., LUQUE, D., VALVERDE, A., GONZALEZ-MARTINEZ, M., HERNANDEZ, A., RUBIO-POO, C., CHAVEZ LARA, B., VALENZUELA, F., 1997: Estrogenic effects of *p*-hydroxybenzoic acid in CD1 mice. Environ. Res. 75, 130-134.

MAKKAR, H.P., BLUMMEL, M., BECKER, K., 1995: Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in *in vitro* techniques. Br. J. Nutr. 73, 897-913.

MALAN, J.C.S., YOUNG, D.A., STEENKAMP, J.A., FERREIRA, D., 1988: Oligomeric flavonoids. Part 2. The first profisetinidins with dihydroflavonol consitituent units. 1988. J. Chem. Soc. Perkin Trans. I 1988, 2567-2572.

MALAN, J.C.S., STEENKAMP, J.A., STEYNBERG, J.P., YOUNG, D.A., BRANDT, E.V., FERREIRA, D., 1990a: Oligomeric flavonoids. Part 8. The first profisetinidins and proguibourtinidins based on C-8 substituted (-)-fisetinidol units and related C-ring isomerized analogues. J. Chem. Soc. Perkin Trans. I 1990, 209-218.

MALAN, J.C.S., YOUNG, D.A., STEYNBERG, J.P., FERREIRA, D., 1990b: Oligomeric flavonoids. Part 10. Structure and synthesis of the first tetrahydropyrano[3,2-*g*]chromenes related to (4,6)-bis-(-)-fisetinidol profisetinidins. J. Chem. Soc. Perkin Trans. I 1990, 227-234.

MASAMA, E., TOPPS, J.H., NGONGONI, N.T., MAASDORP, B.V., 1997: Effects of supplementation with foliage from the tree legumes *Acacia angustissima, Cajanus cajan, Calliandra calothyrsus* and *Leucaena leucocephala* on feed intake, digestibility and nitrogen metabolism of sheep given maize stover *ad libitum*. Anim. Feed Sci. Tech. 69, 233-240.

MATHISEN, E., DIALLO, D., ANDERSEN, Ø.M., MALTERUD, K.E., 2002: Antioxidants from the bark of *Burkea africana*, an African medicinal plant. Phytother. Res. 16, 148-153.

MORAIS, S.A.L., NASCIMENTO, E.A., QUEIROZ, R.A.A., PILÓ-VELOSO, D., DRUMOND, M.G.,1999: Studies on polyphenols and lignin of *Astronium urundeuva* wood. J. Brazil. Chem. Soc. 10, 447-452.

NEL, R.J.J., MTHEMBU, M., COETZEE, J., VAN RENSBURG, H., MALAN, E., FERREIRA, D., 1999: The novel flavan-3-ol, (*2R,3S)*-guibourtinidol and its diastereomers. Phytochemistry 52, 1153-1158.

ODENYO, A.A., OSUJI, P.O., KARANFIL, O., ADINEW, K., 1997: Microbiological evaluation of *Acacia angustissima* as a protein supplement for sheep. Anim. Feed Sci. Tech. 65, 99-?

OSUJI, P.O., ODENYO, A.A., 1997: The role of legume forages as supplements to low quality forages – ILRI experience. Anim. Feed Sci. Tech. 69, 27-38.

OSUJI, P.O., FERNÁNDEZ-RIVERA, S., ODENYO, A.A., 1995: Improving fibre utilization and protein supply in animals fed poor quality roughages: ILRI nutrition research and plans. In: Wallace, R.J., Lahlou-Kassi, A. (eds.), Rumen Ecology Research Planning. International Livestock Research Institute (ILRI): Nairobi, Kenya, 1995, 1-22.

PORTER, L.J., NEWMAN, R.H., FOO, L.Y., WONG, H., HEMINGWAY, R.W., 1982: Polymeric proanthocyanidins. Part? ^{13}C-NMR studies of procyanidins. J. Chem. Soc. Perkin Trans. I 1982, 1217-1221.

PRASAD, D., JOSHI, R.K., PANT, G., RAWAT, M.S., INOUE, K., SHINGU, T., HE, Z.D., 1998: An A-type proanthocyanidin from *Prunus armeniaca*. J. Nat. Prod. 61, 1123-1125.

PRICE, M.L., VAN SCOYOC, S., BUTLER, L.G., 1978: A critical evaluation of the vanillin reaction as an assay for tannin in grain sorghum. J. Agric. Food Chem. 26, 1214-1218.

PRIOR, R.L., LAZARUS, S.A., CAO, G., MUCCITELLI, H., HAMMERSTONE, J.F., 2001: Identification of procyanidins and anthocyanins in blueberries and cranberries (*Vaccinium* spp.) using high-performance liquid chromatography/mass spectrometry. J. Agric. Food Chem. 49, 1270-1276.

QA'DAN, F., PETEREIT, F., NAHRSTEDT, A., 2003: Prodelphinidin trimers and characterization of a proanthocyanidin oligomer from *Cistus albidus*. Pharmazie 58, 416-419.

READEL, K., SEIGLER, D.S., HWANG, K., KEESY, J., SEILHEIMER, S., 2001: Tannins from mimosoid legumes of Texas and Mexico. Econ. Bot. 55, 212-222.

ROUX, D.G., 1972: Recent advances in the chemistry and chemical utilization of the natural condensed tannins. Phytochemistry 11, 1219-1230.

ROUX, D.G., FERREIRA, D., BOTHA, J.J., 1980: Structural considerations in predicting the utilization of tannins. J. Agric. Food Chem. 28, 216-222.

SCALBERT, A., 1991: Antimicrobial properties of tannins. Phytochemistry 30, 3875-3883.

SEIGLER, D.S., 2003: Phytochemistry of *Acacia – sensu lato*. Biochem. Syst. Ecol. 31, 845-873.

SEIGLER, D.S., SEILHEIMER, S., KEESY, J., HUANG, H.F., 1986: Tannins from four common *Acacia* species of Texas and northeastern Mexico. Econ. Bot. 40, 220-232.

SHOJI, T., MUTSUGA, M., NAKAMURA, T., KANDA, T., AKIYAMA, H., GODA, Y., 2003: Isolation and structural elucidation of some procyanidins from apple by low-temperature nuclear magnetic resonance. J. Agric. Food Chem. 51, 3806-3813.

SMITH, A.H., MACKIE, R.I., 2004: Effect of condensed tannins on bacterial diversity and metabolic activity in the rat gastrointestinal tract. Appl. Environ. Microbiol. 70, 1104-1115.

SMITH, A.H., ODENYO, A.A., OSUJI, P.O., WALLIG, M.A., KANDIL, F.E., SEIGLER, D.S., MACKIE, R.I., 2001: Evaluation of toxicity of *Acacia angustissima* in a rat bioassay. Anim. Feed Sci. Tech. 91, 41-57.

SMITH, A.H., WALLIG, M.A., SEIGLER, D.S., ODENYO, A.A., MCSWEENEY, C.S., MACKIE, R.I., 2003: Amelioration of toxic effect of *Acacia angustissima* with polyethylene glycol in rats. Anim. Feed Sci. Tech. 106, 165-174.

SMITH, A.H., ZOETENDAL, E., MACKIE, R.I., 2005: Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microb. Ecol. 50, 197-205.

STEENKAMP, J.A., FERREIRA, D., ROUX, D.G., HULL, W.E., 1983: Synthesis of condensed tannins: Part 8. The first "branched" [4,6:4,8:4,6]-tetraflavonoid. Coupling sequence and absolute configuration. J. Chem. Soc. Perkin Trans. I 1983, 23-28.

Steenkamp, J.A., Malan, J.C.S., Roux, D.G., Ferreira, D., 1988: Oligomeric flavonoids. Part 1. Novel dimeric profisetinidins from *Colophospermum mopane*. J. Chem. Soc. Perkin Trans. I 1988, 1325-1330.

Steynberg, P.J., Steynberg, J.P., Brandt, E.V., Ferreira, D., Hemingway, R.W., 1997: Oligomeric flavonoids. Part 26. Structure and synthesis of the first profisetinidins with epifisetinidol constituent units. J. Chem. Soc. Perkin Trans. I 1997, 1943-1950.

Strumeyer, D.H., Malin, M.J., 1975: Condensed tannins in grain sorghum: Isolation, fractionation, and characterization. J. Agric. Food Chem. 23, 909-914.

Sun, D., Zhao, Z., Wong, H., Foo, L.Y., 1988: Tannins and other polyphenolics from *Myrica esculenta* bark. Phytochemistry 27, 579-583.

Tanaka, T.K., Kondou, K., Kouno, I., 2000: Oxidation and epimerization of epigallocatechin in banana fruits. Phytochemistry 53, 311-316.

Taylor, A.W., Barofsky, E., Kennedy, J. A., Deinzer, M. L., 2003: Hop (*Humulus lupulus* L.) proanthocyanidins characterized by mass spectrometry, acid catalysis, and gel permeation chromatography. J. Agric. Food Chem. 51, 4101-4110.

Zhu, M.J.D., Phillipson, P.M., Greengrass, P.M., Bowery, N.E., Cai, Y., 1997: Plant polyphenols: Biologically active compounds or non-selective binders to protein? Phytochemistry 44, 441-447.

Zucker, W.V., 1983: Tannins: Does structure determine function? An ecological perspective. Am. Nat. 121, 335-365.

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