# Condensed Tannins and Flavonols of Selected Forage Plants from Botswana

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**ABSTRACT---** ***The characterization of the phenolic constituents of Viscum verrucosum, Tapinanthus oleifolius and Grewia flava forage plants, consisting of leaves and small stems, were investigated. The plants were harvested over two summers (February 2009 and 2010). Each plant sample was extracted and purified by a step (conventional) and linear gradient method on a Sephadex LH-20 column. The chemical composition of the condensed tannin (CT)-containing fractions in these plants was studied by the $^{13}$C-NMR spectroscopy. Further purification of the thiolysis products of the CT polymers from these plants led to the isolation of (-)epicatechin which was found to be the dominant compound in the extender units. Fractionation of the fractions from V. verrucosum and T. oleifolius yielded flavonols (kaempferol and quercetin derivatives), which were successfully purified and characterized by LC-ESI-MS/MS. The analytical techniques employed revealed that CT from V. verrucosum, T. oleifolius and G. flava were procyanidin (PC) and cis dominant, with variable chain lengths. In addition, low molecular weight phenolics were isolated and characterized.***

**Keywords----** forage **,** isolation, condensed tannins, flavonols,

## 1. INTRODUCTION

Forage plants are characterized by a large diversity of secondary metabolites and amongst this diversity is an array of compounds with potential biological activity. The secondary metabolites such as condensed tannins (CT) are widely distributed in the leaves, stems, barks, roots and seeds of these plants (Bravo, 1998). Therefore, alternative forage species that can reduce nutritional and health problems in ruminants and improve animal performance need to be evaluated and integrated into farming systems. Condensed tannins are oligomers and polymers of flavan-3-ol units (Figure 1). CT structures may differ at many levels. The flavan-3-ol units in several forage species consist of procyanidin (PC) monomers, catechin (C, 2,3-*trans*) and epicatechin (EC, 2,3-*cis*) and/or prodelphinidin (PD) monomers, gallocatechin (GC, 2,3-*trans*) and epigallocatechin (EGC, 2,3-*cis*) isomers. Many polymers are mixtures containing prodelphinidin and procyanidin units in differing ratios. The flavan-3-ol units are usually identified with the use of the alphabets A, B and C rings. In addition, CT may differ in the degree of polymerization, carbon-carbon interflavan linkages, and galloylation (Spencer et al., 2011). Hydroxylation, galloylation and the extent of oligomerisation (n=2 to 10) and stereochemistry play a significant role in the structure-activity relationships.

While the chemistry of CT and low molecular weight phenolics from a number of temperate forage plants and their biological activity have been extensively investigated (Foo et al., 1996; Foo et al., 1997), detailed studies on these substances from forage plants from Botswana are unknown. Condensed tannins of forage plants such as *Viscum verrucosum*, *Tapinanthus oleifolius* and *Grewia flava* from Botswana have been demonstrated to reduce the problems of parasitism in sheep (Tibe et al., 2013), and to stimulate innate immunity in goats *in vitro* (Tibe et al., 2012). Smallholding farmers in Botswana have reported feeding these plants as supplement to sheep and goats during the dry season to meet their nutritional requirements and to improve their health and wellbeing (Madibela et al., 2000). The biological effects of CT in ruminants depend on the chemical structure. Detailed investigation of the latter in forage plants from Botswana is lacking despite their purported nutritional and health properties. In order to fully understand the biological effects of

these compounds upon ruminants, it is necessary to identify their chemical structures. Hence, the isolation and characterization of CT and flavonols from *V. verrucosum*, *T. oleifolius* and *G. flava* is discussed.

## 2. MATERIALS AND METHODS

### *2.1 Plant Material*

Fresh samples of five different plants (*Viscum rotundifolium* (BT/08/05), *Viscum verrucosum* (BT/08/01) and *Grewia flava* (BT/08/02) leaves were collected from the Botswana College of Agriculture (BCA) farm in Gaborone, Botswana over two summers (February 2009 and 2010). In addition, voucher specimens of the plants (shown above) were deposited at the BCA herbarium.

### *2.2 Plant Extraction*

Freeze-dried and ground leaves and small stems (100 g) from each plant were extracted with acetone:water (7:3; v/v; 3L). The filtered extract was concentrated *in vacuo* at 40 $^{o}$C using a rotary evaporator (Rotavapor, Buchi, Switzerland) to remove acetone, and the aqueous solution was subsequently defatted with dichloromethane. The aqueous layer yielded a brown aqueous acetone crude extract solution.

### *2.3 Purification of Condensed Tannin Polymers*

#### *2.3.1 Step Fractionation (SF)*

CT crude extracts from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius* and *G. flava* and *I. sinensis* were fractionated according to the method described by Meagher et al. (2004). Each CT extract (6 g) was dissolved in methanol:water (1:1, 30 mL). The extract was then loaded onto an XK 26/40 Sephadex LH-20 column. Four fractions (SF1-SF4; 150 mL each) were obtained after elution with methanol:water (1:1). The final elution with acetone:water (7:3) yielded two fractions: SF5 (150 mL) and SF6 (350 mL), which were concentrated *in vacuo* and freeze-dried.

#### *2.3.2 Linear Gradient Fractionation*

The fractionation of the crude extracts from *V. verrucosum*, *T. oleifolius* and *G. flava* on Sephadex LH-20 was carried out according to the linear gradient method described by Meagher et al. (2004). Each freeze-dried crude extract (17 g) from the plant samples collected in 2009 was dissolved in 100 mL water, then centrifuged at 4500 g for 10 minutes. The extracts were loaded onto a Sephadex LH-20 column (XK 26/100, column volume: 400 mL). The column was eluted first with water (two fractions, GF1 and GF2, 500 mL each), then with MeOH:$H_2O$ (GF3,1:3, v/v, GF3, 250 mL), followed by MeOH:$H_2O$ (1:1, GF4, 250 mL), MeOH:$H_2O$ (3:1, GF5, 250 mL), 100% MeOH (four fractions, GF6- GF9, 250 mL each), and finally with acetone:$H_2O$ (2 fractions, GF10, GF11, 500 mL each) at a flow rate of 7 mL/min. The GF1 fraction from *G. flava* was further fractionated on a Sephadex LH-20 column. The presence of CT (broad absorption peak at 280 nm) of the initial water gradient fraction from *G. flava* collected in 2009 was detected by RP-HPLC-PDA. An initial elution with water (100 mL, GF1-1) was followed by a wash with MeOH:$H_2O$ (1:1, 200 mL, GF1-2) and lastly with acetone:water (7:3, v/v, 200 mL, GF1-3). The fractions were concentrated *in vacuo* and freeze-dried.

### *2.4 Phytochemical Screening of Step and Linear LH-20 Fractions*

Phytochemical screening of the purified LH-20 fractions was performed by RP-HPLC (Alliance HT Waters 2790, Milford, MA, USA), which was equipped with a PDA detector (Waters 996A, Milford, MA, USA). The detector was set to monitor the chromatogram at 280 nm and the UV spectra were acquired between 240 and 360 nm. The following chromatographic conditions were used: mobile phase: A= 0.1% formic acid in $H_2O$, B= 0.1% formic acid in $CH_3CN$, acetonitrile, gradient elution conditions: 0-7 min; 10% B, 7-23 min; 10-18% B, 23-28 min; 18-23% B, 28-69 min; 23-41%, 69-72 min; 41-95% B, 72-80 min; 95-10%, 80-95 min; 10-10%. The samples were analysed by elution of a 25 µL sub-sample on a reverse phase (C-18) Phenomenex Jupiter RP column (250 x 4.6 mm, particle size: 5 µm) at a flow rate of 0.3 mL/min.

### *2.5 Isolation and Characterization of CT*

#### *2.5.1 CT Analysis by $^{13}$C-NMR*

The step fractions (SF6) isolated with acetone:water (7:3) from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 and 2010 were analyzed by $^{13}$C-NMR spectrometer (Rheinstetten, Karlsruhe, Germany). Each CT fraction (50 mg) was dissolved in acetone-$d_6$ (0.6 mL) and a minimum amount of $D_2O$ (0.1 mL).

### *2.5.2 Thiolysis of Condensed Tannins*

Thiolysis was carried out for the quantitative determination of the individual units in the CT polymers from the fractions of plants collected in 2009 and 2010 (*T. oleifolius*, *V. verrucosum* and *G. flava*). Each CT-containing fraction (SF6) from *V. verrucosum*, *T. oleifolius* and *G. flava* was used. A condensed tannin solution (4 mg/mL in methanol) was prepared for each fraction. A sub-sample (50 µL) was placed into a vial and to this was added concentrated hydrochloric acid in methanol (3.3 %; v/v 50 µL). The solution was heated to 40 $^{o}$C for 30 min in a heating block and then cooled to room temperature. An internal standard (IS), dihydroquercetin in water (100 µL, 5.2 x $10^{-2}$ mg/mL solution), was added and a 25 µL sub-sample was analysed immediately by RP-HPLC. The following chromatographic conditions were used: mobile phase: A= 0.1% formic acid in $H_2O$, B= 0.1% formic acid in acetonitrile, gradient elution conditions: 0-7 min; 10-18% B, 7-23 min: 18-23% B, 28-44 min; 23-31% B, 44-53 min; 31-85% B, 47-53 min; 85-85% B, 53-58 min; 85-10% B, 58-70 min; 10-10% B, stationery phase: a RP (C-18) Alltima column (250 x 2.1 mm, Alltech, particle size: 5 µm with 25 µL injections), flow rate: 0.3 mL/min. The UV absorbance was monitored between 240 and 360 nm and detection at 280 nm using a Waters PDA detector.

Concentrations of terminal flavan-3-ol units and extender flavan-3-ol adducts from full thiolysis were estimated by peak area integration at 280 nm relative to dihydroquercetin (DHQ) as the internal standard. Responses relative to DHQ determined from standards were 0.09 for the terminal PC and 0.29 for the extender PC. Response factors of the extender units agreed with those of Sivakumaran et al. (2006). However, the response factor of the terminal units was lower which could have been due to the low signal intensity of the standard (DHQ). In addition, the low signal intensity could be due to a carbonyl functional group present in this compound which is not present in the CT polymers.

### *2.5.3 Purification of Thiolysis Adducts*

Each CT-containing fraction (SF6) from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 was used. Each CT containing fraction (240 mg) was dissolved in MeOH (30 mL) and reacted with 3.3% HCl in MeOH (30 mL) and 5% benzyl mercaptan in methanol (60 mL). The solution was heated at 40 $^{o}$C for 30 min in a heating block and cooled to room temperature. The reaction mixture (25 µL) was analysed by RP-HPLC-PDA. The solvents were removed by a rotary evaporator. The residue was dissolved in milli-Q water (75 mL) and partitioned with diethyl ether (25 mLx6). The aqueous extract was concentrated under reduced pressure and dissolved in water, while the diethyl ether extract was concentrated under reduced pressure. The oil residue from the diethyl ether extract was dissolved in methanol and loaded onto a C-18 column (12 x 3 cm, 50 g silica packing material, 5 µm particle size), pre-conditioned with MeOH:$H_2O$ (1:1) at a flow rate of 2.5 mL/min.

### *2.5.4 Characterization of Thiolysis Adducts*

The thiolysis adducts of the CT from *V. verrucosum*, *T. oleifolius* and *G. flava* were purified by open column chromatography (silica gel, C-18 column). The purified isolates were screened with RP-HPLC-PDA and subsequently characterized by ESI-MS and $^{1}$H NMR and $^{13}$C-NMR to study in more detail the structures that make up the CT polymer in both the terminal and extender units. The gradient elution of the thiolysis of the CT-containing fractions (SF6) from *V. verrucosum* with MeOH:$H_2O$ (1:1) to MeOH:$H_2O$ (7:3) yielded the following: SF6-1 and SF6-2 (50% MeOH, 50 mL, trace), SF6-3 (50-55% MeOH, 50 mL, trace), SF6-4 (55% MeOH, 50 mL, 6.8 mg), SF6-5 (55% MeOH, 20 mg), SF6-6 (55% MeOH, 50 mL, 43 mg), SF6-7 (55-60% MeOH, 50 mL, 60 mg) and SF6-8 (60% MeOH, 50 mL, 10.6 mg).

### *2.6 LC/MS/MS of Phenolics*

The LC-MS/MS data were acquired on a Surveyor HPLC connected to a [-LTQ] linear ion trap mass spectrometer (Thermo Finnigan, San Jose, USA) equipped with an electrospray interface operated in the positive ion mode. The column used was a 150 mm x 2.1 mm, i.d., 5 µm, Phenomenex RP (C-18), and elution was run with injection volume of 10 µL, flow rate was 0.2 mL/min and the mobile phase and gradients were as above. The analysis was performed under the following settings: temperature of the heated capillary was set at 275 °C, spray voltage of +4.5 kV, and normalised collision of 35%. The mass spectrometer was programmed to perform a full MS scan (*m/z* 100 to 900) followed by three MS/MS scans on the most intense ions from the MS1 spectrum that were run with the following dynamic exclusion settings: repeat count 2, repeat duration 0.5 min, exclusion duration 2.0 min. An aqueous acetonitrile solution (50:50, v/v) containing flavonoid fractions (100 µg/mL) was used in the analysis.

## 3. RESULTS AND DISCUSSIONS

### *3.1 Phytochemical Screening of Step LH-20 Fractions*

The step LH-20 fractions obtained from *V. verrucosum*, *T. oleifolius*, and *G. flava* were screened for the presence of CT by RP-HPLC-PDA. This detection confirmed the presence of CT in *V. verrucosum*, *T. oleifolius*, *G. flava*, and fraction 6 (SF6) from these plants was sufficient for CT characterization by NMR and thiolysis degradation reaction.

### *3.2 Phytochemical Screening of Linear Gradient LH-20 Fractions*

A series of the gradient LH-20 fractions from *V. verrucosum*, *T. oleifolius*, and *G. flava* plants, which were collected in 2009, were screened for the presence of CT by RP-HPLC with PDA detection. Sharp HPLC peaks were obtained for the water and methanol fractions (GF1 to GF6), while broad humps were found in some methanol and aqueous acetone fractions (GF7 to GF10) from *V. verrucosum*. Few peaks were obtained in fractions GF5 and GF6, suggesting that the compounds were well purified. RP-HPLC-PDA showed the presence of CT in fraction GF8, GF9 and GF10 and were characterized by thiolysis. For *T. oleifolius*, sharp peaks were observed in fractions (GF1 to GF7), while broad peaks were observed in the methanolic (100% MeOH) fractions (GF8 and GF9) and the acetone:water (7:3) fraction (GF10). Fraction GF10 and GF11 were sufficient for CT characterization by NMR. Fraction GF1 (water) from *G. flava* showed a broad hump, which suggested the presence of CT and Sephadex LH-20 matrix operating in a size exclusion manner. The sharp peaks were observed in the ensuing fractions (GF2 to GF8), while broad peaks were obtained for the last eluted methanol (100%, GF9) and acetone:water (7:3, GF10) fractions. Fraction GF9, GF10 and GF11 were sufficient for CT characterization by thiolysis. Fraction GF1 of *G. flava* was purified on a Sephadex LH-20 column and yielded a CT fraction GF1-3. This fraction was further characterized by $^{13}$C-NMR spectroscopy and a thiolysis degradation reaction.

### *3.3 Analysis of CT Containing Step LH-20 Fractions by $^{13}$C-NMR*

The CT fractions isolated from *V. verrucosum*, *T. oleifolius* and *G. flava* were analysed by $^{13}$C-NMR to provide information on the oxidation pattern of the B-ring extender units (procyanidin to prodelphinidin ratio) and the stereochemistry of CT polymers. The assignment of signals was made in comparison with published data in the literature (Sivakumaran et al., 2006). The representative $^{13}$C-NMR spectrum of the fraction SF6 (acetone:water, 7:3) from *V. verrucosum*, *T. oleifolius* and *G. flava* is illustrated in Figure 2. Unassigned peaks could be attributed to impurities. The peaks of CT (δ ppm) from these plants were observed at 28.1, 30.8, 37.6, 54.6, 69.3, 72.6, 76.6, 82.6, 96.0, 114.6, 116.4, 119.7, 130.8, 144.6, 145.0, 154.0, 155.8 and 156.9.

### *3.4 Thiolysis*

This is the first report on the characterization of the step and linear gradient CT fractions from *V. verrucosum*, *T. oleifolius* and *G. flava* by a thiolysis degradation reaction (Figure 3). The relative amounts of various flavan-3-ols (from the terminal units) and their thioether derivatives (from the extension units) were measured by RP-HPLC to provide quantitative information on the mean degree of polymerization (mDP), procyanidin:prodelphinidin (PC:PD) ratio and *cis*:*trans* ratio (Table 1). The representative chromatograms of the degraded CT from *V. verrucosum*, *T. oleifolius* and *G. flava* are illustrated in Figure 4.

The individual units within the CT polymers of the step LH-20 fractions consisted of catechin and epicatechin (Table 2). However, the prodelphinidin units were not observed in any of the tested fractions. Catechin (94%) was the predominant terminal unit in the step fraction (SF6) of *V. verrucosum* collected in 2009. In addition, the same compound (93%) was the most abundant terminal unit in the step fraction (SF6) isolated from the same plant collected in 2010. Epicatechin was the dominant extender unit (100%) in all fractions. The fractions (SF6) isolated from *T. oleifolius* showed a similar pattern. Thus, catechin was the predominant terminal unit (95%) while epicatechin was the dominant extender unit (100%). In contrast, epicatechin (92%) was the predominant terminal unit in CT from fraction (SF6) from *G. flava* collected in 2009. The same compound (100%) was the dominant terminal unit in CT fraction (SF6) from *G. flava* collected in 2010. In the external unit in CT fractions (SF6) from *G. flava* collected in 2009 and 2010, epicatechin was dominant (100%), with the overall characteristic of a procyanidin-type CT. The thiolysis reaction also provided information on the relative stereochemistry at C2 and C3 of the CT polymer. The *cis*:*trans* ratio of CT from the forage species ranged from 84:16 to 100:0. Thus, CT from *V. verrucosum*, *T. oleifolius* and *G. flava* were PC and 2,3-*cis* stereochemistry dominant. Similarly, the individual units within the CT polymers of the linear gradient fractions consisted of catechin and epicatechin (Table 1).

The mean degree of polymerization (mDP) can be estimated from the ratio of the terminal to the extender units in the CT polymer. In the step LH-20 fractions, the mean DP of CT from the forage species collected in 2009 and 2010 ranged between 3 and 6.3. The highest mean DP was observed in the fractions from *V. verrucosum* and *T. oleifolius*, with a mDP

of 6.3 to 6.1, respectively. However, the lowest mean DP of 3.0 was obtained in CT from SF6 from *G. flava*. Thus, the step LH-20 fractions from these plants contained a low molecular weight CT (LMWCT).

For the linear gradient fractions, the mean DP of CT from the plant species ranged from 2.7 to 12.9. Fractions GF9 and GF10 from *V. verrucosum* consisted of low molecular weight CT (LMWCT), with a mDP of 4.8 and 7.5, respectively. Fractions from GF10 and GF11 from *T. oleifolius* had a mDP of 6.2 and 6.5, respectively. Furthermore, fractions GF9 and GF10 consisted of LMWCT with a mDP of 3.3 and 2.7, respectively. A medium molecular weight CT (MMWCT) of 12.9 was isolated from GF8 from *V. verrucosum*. Fraction GF1-3, purified from GF1 from *G. flava*, had a LMWCT with a mDP of 5.5.

Condensed tannins differ at many levels. The flavan-3-ol units in several forage plants consist of procyanidin (PC) monomers, catechin (C, 2,3-*trans*) and epicatechin (EC, 2,3-*cis*) and/or prodelphinidin (PD) monomers, gallocatechin (GC, 2,3-*trans*) and epigallocatechin (EGC, 2,3-*cis*) isomers. The main constituent in the extender units of CT from *T. oleifolius*, *V. verrucosum* and *G. flava* was comprised predominantly of epicatechin, whereas catechin and epicatechin were the predominant terminal unit. The findings from this study on thiolytic cleavage of CT from *T. oleifolius*, *V. verrucosum* and *G. flava* are comparable to those reported by Meagher et al. (2004). Previous studies have also demonstrated that procyandin-type CT are the most abundant in forage plants. CT in forage plants such as birdsfoot trefoil (*L. corniculatus*) are PC predominant (Foo et al., 1996). Procyanidin-type CT have also been reported from a number of plant-derived food substances. For example, PC-type CT are present in grape seeds (Da Silva et al., 1991), apple (Guyot et al., 2001), and lowbush blueberry and cranberry (Gu et al., 2002). However, the PC:PD and *cis*:*trans* ratio can be variable. Marais et al. (2000) reported that sainfoin CT had a highly variable composition with *cis*:*trans* ratios ranging between 47:53 and 90:10 and PC:PD ratios between 64:36 and 7:93. The composition of the terminal and extender units in sainfoin CT appeared to be cultivar specific. The biological activity of the CT from sainfoin is usually attributed to the predominance of *cis* stereochemistry (Marais et al., 2000). Thus, CT from *G. flava*, *T. oleifolius* and *V. verrucosum* consisting of (-)epicatechin units with *cis* stereochemistry were potent in antiparasitic (Tibe et al., 2013) and immunostimulatory (Tibe et al., 2012) activities.

### *3.5 Purification and Characterization of Thiolysis Adducts*

The purified adducts of CT from *V. verrucosum*, *T. oleifolius* and *G. flava* were characterized by ESI-MS, $^{1}$H NMR (Figure 5) and $^{13}$C-NMR (Figure 6). Each fraction was analysed by RP-HPLC-PDA. The same compound present in fractions SF6-5 to SF6-8 was also found in CT from *G. flava* and *T. oleifolius*. Signal assignment was made in accordance with published data by Cai et al. (1991).

#### *3.5.1 (-)epicatechin-4β-benzylthioether (1)*

Freeze-dried light brown powder (60 mg). UV $\lambda_{max}$ (nm) MeOH 280. HPLC $R_t$ 49.10 min. ESI-MS *m/z* 411 $[M+H]^+$. $^{1}$H-NMR ($d_4$-methanol): Ph 7.20-7.45 (5H, m). 2′ 7.07 (1H, d, *J* = 2.0 Hz), 5′ 6.76-6.83 (1H, m), 6′ 6.76-6.83 (1H, m), 6.06 (1H, m, 6-H), 5.92 (1H, d, J = 2.3 Hz, 8-H), 2-H 5.26 (s), 3-H 4.07-3.86 (1H, m), 4-H 4.07-3.86 (1H, m), S-$CH_2$ 4.09-4.00 (1H, m). $^{13}$C-NMR δ 36.6 -$SCH_2$, 42.6 (C4), 70.2 (C3), 74.2 (C2), 94.4 (C8), 95.5 (C6), 98.8 (C4a), 113.9 (C2′), 114.6 (C5′), 117.9 (C6′), 126.8 (C4″), 127.9 (C3″ and C5″), 128.8 (C2″ and C6″), 130.7 (C1′), 139.0 (C1″), 144.4 (C3′), 144.6, (C4′), 155.9 (C5), 157.6 (C8a), 157.7 (C7).

### *3.6 LC/ESI-MS/MS of Flavonoids*

Identification of the individual compounds in *T. oleifolius* and *V. verrucosum* was performed by comparison of their PDA UV/Vis and ESI-MS/MS spectrometric data with published data by Vallejo et al. (2004). LC-ESI-MS/MS analysis in the positive ion mode was used to identify the molecular weight ($M^+$) of the flavonoid glycosides, molecular weight information on the aglycone moiety and to ascertain whether there were sugar conjugates attached as evidenced by $M^+$-162 or $M^+$-146 or $M^+$-132 loss for glucose or galactose, rhamnose and a pentose (arabinose or apiose), respectively. The results obtained are summarised with the UV $\lambda_{max}$ absorbance below and in Table 2. The ESI/MS/MS data showed the protonated aglycone $[A+H]^+$ ions at *m/z* 303, 287, 305 respectively, corresponding to quercetin, kaempferol and dihyroquercetin with the serial loss from the $[M+H]^+$ ion of rhamnose or glucose or apiose units to yield intermediate ions $[I+H]^+$ then further loss of a single hexose (glucoside) unit. This indicated that the hexose (glucose or rhamnose) was attached to the aglycone moiety, and the other sugars were attached on the hexose rather than to the aglycone.

Five flavonol glycosides were identified from *T. oleifolius*. The full scan of fraction SF4 from *T. oleifolius* showed the presence of quercetin-3-*O*-diglucoside or sophoroside at *m/z* 626.99 (relative abundance (r.a) 100%, $R_t$ 31.97, UV $\lambda_{max}$ 230, 250, 355). This compound exhibited a neutral loss of 324 corresponding to sophoroside, a $MS^2$ fragment at *m/z* 465 $[M+H\text{-glucose}]^+$, a $MS^3$ fragment ion at *m/z* 303. The formed fragment at *m/z* 447.07 $[M+H\text{-}18]^+$ suggested that the sugars were attached at C3 of the aglycone moiety. The dimeric form $[2M+H]^+$ of this compound was shown at *m/z* 1252. The other fragments for $MS^3$ were shown at *m/z* 399, 369 and 345. The aglycone and sugar linkages were confirmed in the NMR data (not shown). Trace amounts of quercetin-3-*O*-rutinosides (rutin) at *m/z* 611 (r.a 100%, $R_t$ 34.11 min, UV

$\lambda_{max}$ nm 230, 255, 355) were also detected from this fraction. This compound showed a loss of 308 corresponding to glucose-rhamnose, and a $MS^2$ fragment at *m/z* 465 $[M+H\text{-glucose}]^+$, a $MS^3$ fragment at *m/z* 303. The other fragments for $MS^3$ were shown at m/z 369 and 345. The dimeric form $[2M+H]^+$ of this compound showed at *m/z* 1220.

The full scan spectrum $[M+H]^+$ of fraction SF5 from *T. oleifolius* exhibited a compound at *m/z* 758.99 ($R_t$ 30.49, UV $\lambda_{max}$ nm 265, 355), which was assigned to quercetin-glucosyl-apiosyl-glucoside. This compound exhibited a loss of 18 $[M+H\text{-}18]^+$ (m/z at 741), and a $MS^2$ fragment at *m/z* 627 $[M+H\text{-pentose}]^+$, 465 and 303, and a $MS^3$ fragment at *m/z* 597, 507, 477, 447 and 345. The dimeric form $[2M+H]^+$ of this compound showed at *m/z* 1516. Quercetin-diglucoside or sophoroside at *m/z* 626.99 (relative abundance (r.a) ($R_t$ 33.65, UV $\lambda_{max}$ nm 230, 255, 355) was also isolated. The dimeric form $[2M+H]^+$ of this compound showed at *m/z* 1252. Kaempferol-3-*O*-rutinoside at *m/z* 611.01 ($R_t$ 33.47 min) was tentatively identified from *Tapinanthus oleifolius*. The–$MS^2$ $[M\text{-}H]^-$ fragmentation of 3-sophorosides is the typical for diglycosides at C3 position.

Five flavonol glycosides were isolated and identified from *V. verrucosum*. Fraction GF5 yielded quercetin-3-*O*-rhamnosyl-apiosyl-glucoside at *m/z* 742.99 (relative abundance (r.a) 100%, $R_t$ 32.44, UV $\lambda_{max}$ nm 230, 255, 355). This compound exhibited a loss of 18 $[M+H\text{-}18]^+$ (m/z at 725), and a $MS^2$ fragment at *m/z* 611 $[M+H\text{-pentose}]^+$, 465 and 303, and a $MS^3$ fragmentation for the precursor ions at *m/z* 285, 257, 229, 219, 165, 137 and 111. The dimeric form of this compound was detected at *m/z* 1484. Quercetin-3-*O*-dirhamnosyl-glucoside at *m/z* 756.90 (r.a) 100%, $R_t$ 34.24, UV $\lambda_{max}$ nm 230, 265sh, 355 was also detected. This compound showed a loss of $COH_2$ $[M+H\text{-}30]^+$ at *m/z* at 727 and 610 for the full scan, and a $MS^2$ fragment at *m/z* 593, 465, 447 and 303 $[M+H\text{-pentose}]^+$, and a $MS^3$ fragment at *m/z* 303, 285, 229, 183, 165, 153 and 93. The NMR data showed that the sugars were attached at position 3 of the aglycone moiety (not shown). The following compounds were isolated and identified from GF6: Quercetin-3-*O*-apiosyl-glucoside at *m/z* 596.95 ($R_t$ 34.01, UV max nm 230, 255, 355) were tentatively identified from *Tapinanthus oleifolius*. Furthermore, this fraction produced a condensed tannin (CT) dimer such as epicatechin-4β→8-epicatechin at *m/z* 577.00 ($R_t$ 16.62, UV $\lambda_{max}$ 235, 280) and dihydroquercetin at *m/z* 305.16 at ($R_t$ 33.14, UV $\lambda_{max}$ 235, 285). Purified fraction GF5-3 isolated from GF5 yielded quercetin-3-O-rhamnosyl-glucosyl-apioside.

Finally, purified fraction GF6-3 from GF6 confirmed the presence of epicatechin-4β→8-epicatechin at *m/z* 577.00 ($R_t$ 16.62, UV $\lambda_{max}$ 235, 280) and dihydroquercetin at *m/z* 305.16 at (Rt 33.14, UV $\lambda_{max}$ 235, 285).

## 4. CONCLUSION

The results of this study demonstrated that CT from *V. verrucosum*, *T. oleifolius* and *G. flava* contained marked variations in chain lengths, which were procyanidin-type and dominated by *cis* stereochemistry. Procyanidin-type CT have been isolated for the first time. The presence of CT and other flavonoids in these plants indicates that their domestication and integration into pastoral agricultural systems to supplement traditional forages and improve animal performance is paramount.

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**Table Legends**

Table 1 Condensed tannin composition of the step and linear gradient LH-20 fractions from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 and 2010 by thiolysis reaction products for the mean degree of polymerisation, % contributions of terminal and extender units of CT and ratio of prodelphinidin (PD) to procyanidin (PC) units.

Table 2 Identified compounds, *m/z* of parent ions, *m/z* of intermediate ions, *m/z* of fragment ions, and retention times as determined by LC-DAD using ESI/MS/MS in positive ion mode for flavonols from *T. oleifolius* and *V. verrucosum*

**Table 1** Condensed tannin composition of step and linear gradient LH-20 fractions from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 and 2010 by thiolysis reaction products for the mean degree of polymerisation, % contributions of terminal and extender units of CT and ratio of prodelphinidin (PD) to procyanidin (PC) units.

| Plant samples and fractions | Eluting solvent | | Terminal (%) | | | | Extender (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | [a]mDP | [b]GC | [c]EGC | [d]C | [e]EC | GC | EGC | C | EC | [f]PC:[g]PD | *cis*:*trans* |
| **Step LH-20 fractions** | | | | | | | | | | | | |
| *V. verrucosum* | | | | | | | | | | | | |
| SF6 (2009) | Acetone:$H_2O$ (7:3) | 6.3 | 0 | 0 | 94 | 6 | 0 | 0 | 0 | 100 | 100:0 | 87:13 |
| SF6 (2010) | Acetone:$H_2O$ (7:3) | 4.9 | 0 | 0 | 93 | 7 | 0 | 0 | 0 | 100 | 100:0 | 84:16 |
| *T. oleifolius* | | | | | | | | | | | | |
| SF6 (2009) | Acetone:$H_2O$ (7:3) | 6.1 | 0 | 0 | 95 | 5 | 0 | 0 | 0 | 100 | 100:0 | 87:13 |
| SF6 (2010) | Acetone:$H_2O$ (7:3) | 6.1 | 0 | 0 | 95 | 5 | 0 | 0 | 0 | 100 | 100:0 | 87:13 |
| *G. flava* | | | | | | | | | | | | |
| SF6 (2009) | Acetone:$H_2O$ (7:3) | 4.7 | 0 | 0 | 8 | 92 | 0 | 0 | 0 | 100 | 100:0 | 99:1 |
| SF6 (2010) | Acetone:$H_2O$ (7:3) | 3.0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 100 | 100:0 | 100:0 |
| **Linear Gradient LH-20 (2009)** | | | | | | | | | | | | |
| *V. verrucosum* | | | | | | | | | | | | |
| GF8 | 100% MeOH | 12.9 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 | 100:0 | 93:7 |
| GF9 | 100% MeOH | 4.8 | 0 | 0 | 96 | 4 | 0 | 0 | 0 | 100 | 100:0 | 83:17 |
| GF10 | Acetone:$H_2O$ (7:3) | 7.5 | 0 | 0 | 94 | 6 | 0 | 0 | 0 | 100 | 100:0 | 89:11 |
| *T. oleifolius* | | | | | | | | | | | | |
| GF10 | 100% MeOH | 6.2 | 0 | 0 | 9 | 91 | 0 | 0 | 0 | 100 | 100:0 | 99:1 |
| GF11 | Acetone:$H_2O$ (7:3) | 6.5 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 | 100:0 | 87:14 |
| *G. flava* | | | | | | | | | | | | |
| GF9 | 100% MeOH | 3.3 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 100 | 100:0 | 100:0 |
| GF10 | Acetone:$H_2O$ (7:3) | 2.7 | 0 | 0 | 25 | 75 | 0 | 0 | 0 | 100 | 100:0 | 100:0 |
| GF1-3 | Acetone:$H_2O$ (7:3) | 5.5 | 0 | 0 | 47 | 53 | 0 | 0 | 0 | 100 | 100:0 | 93:7 |

Abbreviations: [a]mDP = mean degree of polymerisation. [b]GC = gallocatechin. [c]EGC = epigallocatechin. [d]C= catechin. [e]EC = (epi) catechin. [f]PC= procyanidin. [g]PD = prodelphinidin).

**Table 2** Identified compounds, *m/z* of parent ions, *m/z* of intermediate ions, *m/z* of fragment ions, and retention times as determined by LC-DAD using ESI/MS/MS in positive ion mode for flavonols from *T. oleifolius* and *V. verrucosum*

| Plant name and fraction | Flavonoids | Rt (min) | UV λmax (nm) | [M+H]+ (m.u) | [I+H]+ (m.u) | Fragments [F+H]+ (m.u) |
|---|---|---|---|---|---|---|
| SF4 *T. oleifolius* | Quercetin-3-O-diglucoside or sophoroside | 31.97 | 230, 250, 355 | 626.99 | 465.07 | 447.06, 399.11, 369.11, 345.13, 303.15 |
| | Quercetin-3-O-rhamnosyl-glucoside (rutin) | 34.11 | 230, 255, 355 | 611.01 | 465.11 | 447.14, 369.27, 345.14, 303.20 |
| GF5 *T. oleifolius* | Quercetin-glucosyl-apiosyl-glucoside | 30.49 | 265, 355 | 758.99 | 627.08 | 597.07, 507.13, 489.14, 465.17, 435.16, 387.26, 345.16, 303.12 |
| | Quercetin-diglucoside | 32.02 | 230, 255, 355 | 627.00 | 465.07 | 447.06, 399.10, 369.20, 345.26, 303.25 |
| | Kaempferol-diglucoside | 33.47 | 230, 265, 345 | 611.01 | 449.04 | 431.09, 383.17, 353.11, 329.16, 299.26, 287 |
| GF6 *T. oleifolius* | Quercetin-diglucoside | 31.90 | 225, 255, 355 | 626.95 | 465.17 | 447.08, 399.12, 369.08, 345.17, 303.13 |
| | Quercetin-apiosyl-glucoside | 33.65 | 230, 255, 355 | 596.96 | 465.08 | 4435.08, 345.18, 303.15 |
| | Quercetin-diglucoside | 34.16 | 230, 255, 355 | 610.99 | 465.05 | 303.1 |
| GF5 *V. verrucosum* | Quercetin-3-O-rhamnosyl-apiosyl-glucoside | 32.44 | 230, 255, 355 | 742.99 | 610.87 | 597.08, 579.36, 464.97, 345.06, 303.16 |
| | | | | | | |
| | Quercetin-3-O-dirhamnosyl-glucoside | 34.24 | 230, 265sh, 355 | 756.90 | 610.98 | 465.19, 303.09 |
| GF5-3 | Quercetin-3-O-rhamnosyl-glucosyl-apioside | 32.39 | 230, 265sh, 355 | 742.96 | 597.08 | 477.09, 434.999, 345.22, 303.19 |
| GF6-3 *V. verrucosum* | Epicatechin-4β→8-epicatechin | 16.62 | 235, 280 | 577.00 | 451.06 | 415.06, 409.13, 391.16, 289.12 |
| | dihydroquercetin | 33.41 | 235, 285 | 305.16 | 286.9 | 259.06, 195.04, 153.04 |
| | Quercetin-3-O-rhamnosyl-glucoside (rutin) | 34.55 | 230, 255, 355 | 610.97 | 465.12 | 447.09, 345.35, 303.10 |
| GF6-3 | 2,3-*cis* -dihydroquercetin | 33.14 | 235, 285 | 305.19 | 286.9 | 259.11, 195.06, 153.02 |
| | Quercetin-3-O-apiosyl-glucoside | 34.01 | 230, 265sh, 355 | 596.95 | 465.06 | 435.05, 345.13, 303.09 |
| | Quercetin-3-O-rhamnosyl-glucoside (rutin) | 34.35 | 230, 255, 355 | 611.02 | 465.02 | 447.19, 345.17, 303.13 |
| | | | | | | |

**Abbreviations:** Rt-retention time, m-mass units, $[M+H]^+$ parent ion and fragment ions, $[I+H]^+$intermediate ion, $[A+H]^+$ aglycone ion