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Concentration and composition of condensed tannins in leaves from fodder trees and shrubs in Pakistan

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Abstract

Background: The lack of dependable, high-quality forage and animal feedstuffs in many areas of the world hampers livestock production. In these regions, farmers often rely on native fodder tree and shrub leaves as a forage source for their animals with little or no information available on nutritional factors of these feedstuffs. In addition, there is a growing need to improve nitrogen use efficiency (NUE) in ruminants, especially in agricultural areas where livestock feedstuffs are limited in quantity and of lower quality. Condensed tannins (CTs) show capability of protecting protein from degradation during both the ensiling process and rumen digestion, producing greater rumen undegradable protein translating into higher ruminant livestock productivity.

Results: Determination of the concentration of CTs in the leaves of four fodder tree species (*Salix nigra*, *Salix alba*, *Populus nigra*, and *Elaeagnus angustifolia*), and two shrub species (*Hippophae rhamnoides* and *Rosa webbiana*) across seasons from three locations in Pakistan is detailed below. The highest levels of CTs were found in *S. nigra* and *S. alba* (seasonal averages of 17.9 and 18.5 mg/g, respectively) and were targeted for purification and characterization. ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectra of purified CTs indicated the following composition for *S. nigra*, procyanidin/prodelphinidin ratio = 59/41; *cis*/*trans* ratio = 59/41; estimated mean degree of polymerization (mDP) = 8.1. For *S. alba*, procyanidin/prodelphinidin ratio ≥99/1; *cis*/*trans* ratio = 85/15; estimated mDP = 6.9. The CT content of both *Salix* species trended higher with seasonal growth, with the CT content of *S. nigra* across seasons statistically significant. The CT content in *P. nigra* and *H. rhamnoides* trended lower across all three locations tested but did not reach the level of statistical significance. Insignificant levels of CTs were detected in *E. angustifolia* and *R. webbiana*.

Conclusion: Based on CT literature reports, *S. nigra* and *S. alba* likely contain adequate CT levels to bolster NUE in ruminants without exhibiting a detrimental effect on dry matter intake.

KEYWORDS
condensed tannins, fodder, livestock, NMR, nuclear magnetic resonance, proanthocyanidins, seasonal variation

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INTRODUCTION

Agricultural systems in many countries of the world suffer from the lack of adequate access to high-quality feedstuffs required for maintenance and production of ruminant animals. The development of methods or techniques to improve the nutritional quality of feedstuffs in these agricultural systems is highly desired. Livestock is one of the major sources of livelihood in Pakistan and due to the shortage of arable land for fodder, most of the ruminants are grazed on summer pastures and winter rangelands. Rangeland is the major feed resource with most of the rangelands grazed beyond their carrying capacity.[1–3] During severe and prolonged winters, animals are stall-fed on stored crop residues, hay, and dry fodder tree leaves. The lack of available feeds and forages with adequate nutrients has resulted in low milk production, poor growth, high mortality rates, and poor reproduction performance.[4–7]

As a result of low forage availability, fodder tree leaves and shrubs contribute to the ration of ruminant livestock during different parts of the year. These browse species are increasingly recognized as important components of animal feed especially for sheep and goats. Their shoots are either cut and carried to the barn to feed in the fresh form or the livestock are allowed to freely browse. Also, as a normal practice, leaves of many trees are collected in autumn when they are shed and stored as hay by mixing with wheat straw and dry roughages, and are used to feed all categories of livestock during winter. The utility of the leaves, pods, and edible twigs of shrubs and trees as animal feed is potentially limited by the presence of anti-nutritional factors.[8] Plants contain thousands of compounds that can have a beneficial or deleterious effect on organisms consuming them. These compounds, except nutrients, are referred to as 'allelochemicals'. Traditionally, condensed tannins fall under the allelochemical classification as secondary metabolites which are not identified with any necessary plant function but may have stimulatory or inhibitory effects on animal health and productivity. However, CTs have recently been classified as specialized metabolites with specific biological roles.[9,10] The consumption of CT-containing plants has shown beneficial effects in ruminant animals such as improved nitrogen utilization, prevention of bloat, mitigation of methane and ammonia production, and can lessen parasite burdens. However, they can reduce animal productivity and may also cause toxicity during periods of scarcity, when feed rich in these substances are consumed by animals in large quantities.[11,12]

Condensed tannins exist in nature as a diverse mixture of oligomeric and polymeric substances constructed from a common set of building blocks, called flavan-3-ols, similar to how proteins are constructed from a defined set of amino acids and complex carbohydrates are assemblages of a defined set of monosaccharides. The CTs constructed from these flavan-3-ol subunits are characterized based on hydroxylation pattern, stereochemistry, functional groups, and interflavan linkages.[13] Most of the CTs occurring in common forage and browse species are primarily composed of the four flavan-3-ol subunits (Figure 1), catechin and epicatechin, which give rise to procyanidin tannins (PC), and gallocatechin and epigallocatechin, possessing an additional hydroxyl group at C-5 of the B-ring (Figure 1), which give rise to prodelphinidin tannins (PD). Catechin and epicatechin differ only in their stereochemistry (spatial orientation) of the hydroxyl (OH) group at the C-3 carbon. Similarly, gallocatechin and epigallocatechin differ from each other by the stereochemistry of the hydroxyl group at C-3. The relative stereochemistry of the C-2 and C-3 substituents in the C-ring of these subunits also comprise a defining structural feature of these polyphenols, with C2/C3 possessing a *cis*

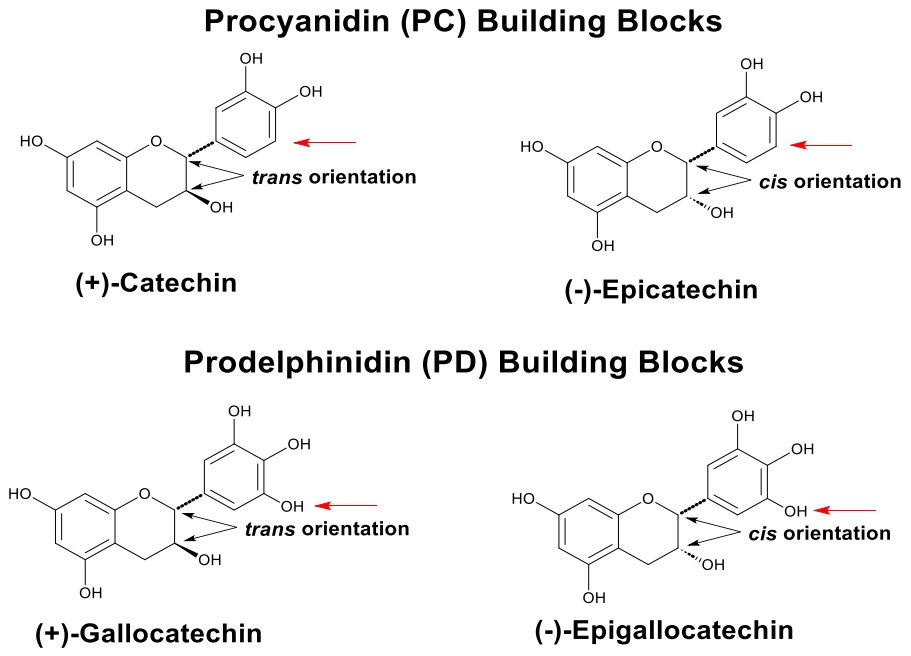


FIGURE 1 Flavan-3-ol subunits used as building blocks in production of condensed tannins in common forages.

orientation in epicatechin and epigallocatechin and a *trans* orientation in catechin and gallocatechin. As stated before, CTs exist as a mixture of different lengths, varying from 2 up to greater than 50 flavan-3-ol subunits linked together, collectively forming a vast mixture of CT components. Condensed tannins isolated as this type of mixture are commonly characterized by the average number of flavan-3-ol subunits contained in the components of this mixture or mean degree of polymerization (mDP).

Farmers in Pakistan and other countries dealing with limited arable land may need to rely on tree and shrub leaves as a forage source, however, there is little information available on nutritional factors of native fodder tree and shrub leaves across seasons. In addition, there is a growing need to improve nitrogen use efficiency (NUE) in ruminants, especially in agricultural areas where livestock feedstuffs are limited in quantity and of lower quality. Condensed tannins have shown the capability of protecting protein from degradation during both the ensiling process[14,15] and during rumen digestion,[16–19] which can result in greater rumen undegradable protein and higher ruminant livestock productivity.

The purpose of the present study was to investigate the seasonal variation of condensed tannin concentration in tree and shrubs leaves commonly used as livestock feed in Pakistan. This information may also identify plants as a tool to improve NUE in various ruminant diets.

MATERIALS AND METHODS

Study area

The study was carried out in Hopper Valley, Pakistan, to investigate the seasonal variations of condensed tannins of commonly used fodder tree leaves and shrubs. The Hopper Valley is located in northern Pakistan about 10 km away from Nagar Khas (36° N, 74° E) with an altitude of 2900 m above sea level. The valley is in a single cropping zone and remains snow-bound throughout the winter.

Sample collection

The trees and shrubs sampled in this study (2016) were part of an existing plantation, comprising both naturally regenerated and species that had been planted several years prior. Leaf samples of four species of commonly used fodder trees (*Salix nigra*, *Salix alba*, *Populus nigra*, and *Elaeagnus angustifolia*) and two shrubs (*Hippophae rhamnoides* and *Rosa webbiana*) were collected randomly (not from the same individual plants) in the same area during each of the three seasons (spring, summer, and autumn). To create a representative composite sample, the handpicking was conducted from randomly selected plants (15–20) of each species and combined into one composite sample, harvesting at least 10–15 leaves per plant from the top, middle, and bottom of the plant and collected from three different Pakistan village locations (Holshal+Ghashushal, Baroshal, and Hakalshal).

Processing of samples

The samples were air-dried in shade for 4–7 days. Samples were packed in pre-weighed empty paper bags with their identification and date of collection. The air-dried samples were further dried in an oven at 60°C for 24 h. The samples were ground in a Wiley mill to pass through a 1 mm screen and stored in clean and dry labeled bottles.

Quantification of total condensed tannins

Leaf samples were analyzed for total condensed tannins by using the modified acetone-butanol-HCl-iron method,[20] using purified condensed tannins from sanfoin as the reference standard.

Condensed tannins from *S. nigra* and *S. alba* contained the highest total CT concentrations and were purified following the method described in Thompson et al.[21] and analyzed by ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy.[22]

Purification of condensed tannins from *S. nigra* and *S. alba*

Dried and ground *S. nigra* leaves (3.5 g, autumn harvest, Baroshal) were transferred to a 125 mL Erlenmeyer flask equipped with a magnetic stir bar and 7:3 acetone/water (50 mL) was added. The suspension was stirred rapidly for 30 min and then vacuum filtered through a Buchner funnel equipped with a filter paper. The solids were returned to the Erlenmeyer flask and the material was extracted in a similar manner with 7:3 acetone/water (50 mL) two additional times. The combined extracts were concentrated under reduced pressure (rotary evaporator) to remove the acetone. The resulting aqueous layer was gently stirred (to avoid emulsion formation) with dichloromethane (50 mL) for 2 h, the layers were separated, and the aqueous layer was extracted again with dichloromethane (50 mL) in a manner totally analogous to the first dichloromethane extraction. The layers were separated, the aqueous layer was briefly concentrated under reduced pressure (rotary evaporator) to remove any traces of dichloromethane, and the aqueous layer was freeze-dried to afford 607 mg of a residue. A portion of the resulting residue (0.596 g) was dissolved in a 1:1 methanol/water solution (50 mL) and Sephadex LH-20 was added in small portions with stirring until the mixture reached the consistency of wet sand (13.2 g of Sephadex LH-20 added). The mixture was placed in a 100 mL sintered glass funnel equipped with a filter paper, and was suspended in a methanol/water mixture (1:1, 50 mL), allowed to stand for 5 min, and then vacuum filtered. The methanol/water washings (1:1, 50 mL) were repeated 14 additional times and the washings were pooled. The CT-laden resin was then washed with 3:7 acetone/water (50 mL × 4 times), and the washings were pooled, concentrated under reduced pressure (rotary evaporation) to remove the acetone, and freeze-dried to give 11 mg of an off-white colored solid.

The condensed tannins from *S. alba* were purified from the ground plant material (6.014 g, autumn harvest, Hakalshal) in a

manner totally analogous to that described for the isolation of CTs from *S. nigra* with the following changes: The initial extractions (3) were performed with 60 mL of 7:3 acetone/water to give, after concentration, 1.398 g of a residue. Most of this residue (1.374 g) was dissolved in 1:1 methanol/water (65 mL), and Sephadex LH-20 was added in small portions with stirring until the mixture reached the consistency of wet sand (16.5 g of Sephadex LH-20 added). As described above, the resin was placed in a 100 mL sintered glass funnel equipped with a filter paper, and the resin was suspended in 1:1 methanol/water (65 mL), let stand for 5 min, and then vacuum-filtered 15 times and then washed with 3:7 acetone/water (65 mL × 4 times), and the washings were pooled, concentrated, and freeze-dried to provide 60 mg of an off-white colored solid. The purified CTs from *S. nigra* and *S. alba* were submitted for NMR analysis.

NMR spectroscopy

NMR spectra were obtained on the purified condensed tannin samples. ^{1}H, ^{13}C, and ^{1}H–^{13}C HSQC NMR (2D NMR) spectra were recorded at 27°C on a Bruker Biospin DMX-500 (^{1}H 500.13 MHz, ^{13}C 125.76 MHz) instrument equipped with Top Spin 3.5.2 software and a cryogenically cooled 5 mm TXI 1H/13C/15N gradient probe in inverse geometry. Spectra were recorded in DMSO-d_6 and were referenced to the residual signals of DMSO-d_6 (2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C spectra). ^{13}C NMR spectra were obtained using 5000 scans (acquisition time of 4 h and 30 min each). For ^{1}H–^{13}C HSQC experiments, spectra were obtained using 128 scans (acquisition time of 18 h and 30 min each) obtained using the standard Bruker pulse program with the following parameters: acquisition, TD 1024 (F2), 320 (F1); SW, 10.0 ppm (F2), 160 ppm (F1); O1, 2500.65 Hz; O2, 11,318.20 Hz; D1 = 1.50 s; CNST2 = 145; acquisition time, F2 channel, 102.55 ms; F1 channel, 7.9511 ms; processing, SI = 1024 (F2, F1), WDW = QSINE, LB = 1.00 Hz (F2), 0.30 Hz (F1); PH_mod = pk; baseline correction ABSG = 5 (F2, F1), BCFW = 1.00 ppm, BC mod = quad (F2), no (F1); linear prediction = no (F2), LPfr (F1). Samples sizes used for these spectra ranged from 5 to 15 mg providing NMR sample solutions with concentrations of 10–30 mg/mL.

Statistics

The data were analyzed with the analysis of variance procedure by using the GLM procedure in Statistical Analysis System (SAS). The model included the effects of species and season, with village considered only for sample replication and was not statistically analyzed. Differences between least-square means were determined using the PDIFF option with Tukey adjustment for multiple comparisons. Results are presented as least-square means with standard error of means, with differences only presented when the main effect or interaction of main effects was found to be significant. Treatment effects were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

There have been many methods developed for the determination of CT content in plant materials.[23] One of the most popular is the HCl-butanol-iron assay. Several improvements of the HCl-butanol-iron assay have been introduced but it was not until the improvements described by Grabber et al.[20] that CT researchers could be sure that the total CT content was being accounted for from these modifications of the assay. Although it is highly recommended that the reference standard CT for the HCl-butanol-iron assay be purified from the same plant undergoing CT content determination,[20,24–29] in the current case, a limited amount of available plant material coupled with the relatively low level of CT occurring in these plant materials rendered following this protocol unfeasible. In lieu of using the same plant-derived reference standards, we opted to use a surrogate purified CT with a highly similar structure and composition. During initial purifications of CT in this study, we analyzed a small portion of purified CT from *S. nigra* by 2D NMR spectroscopy.[22] Using this information, we selected a surrogate CT sample from our condensed tannin library[21] which most closely mirrored the structure and composition of the *S. nigra* sample. A purified sample of CT from *Onobrychis viciifolia* (sainfoin) was identified to serve as an adequate replacement for the same plant-derived reference standard. This sample was analyzed by thiolytic degradation and was shown to have a procyanidin (PC)/prodelphinidin (PD) ratio of 51.7/48.3, a *cis*/*trans* ratio of 83.5/16.5 with an estimated mean degree of polymerization (mDP) of 9.3. The 2D NMR spectrum of this sample, with the cross-peaks assignments labeled, is provided in Figure 2. It was decided to use this particular sample as the HCl-butanol-iron assay reference standard for all of the CT concentration determinations in this study.

Concentrations of CT of fodder tree and shrub leaves were affected by species ($p < 0.01$) and are presented in Table 1. *S. nigra* and *S. alba* showed the highest mean values of 17.90 and 18.53 mg/g, respectively, and *P. nigra* and *H. rhamnoides* contained intermediate levels of CT (4.5 and 8.5 mg/g, respectively). Insignificant levels of CT were detected in *E. angustifolia* and *R. webbiana*.

Condensed tannin concentrations were affected by a species × season interaction ($p < 0.01$) with different trends for CT in individual species across the seasons (Table 1). *H. rhamnoides* showed low CT values which declined with seasonal progression while *P. nigra* CT values tended to exhibit a less progressive decline. Samples from *E. angustifolia* and *R. webbiana* showed negligible values of CT across all seasons. The CT content of *S. nigra* and *S. alba* showed the highest values and trended upward with the onset of autumn, and hence, the CTs from these samples were targeted for purification. ^{1}H–^{13}C HSQC NMR spectra of purified CTs from *S. nigra* (black willow) and *S. alba* (white willow) with the assignments of 2D NMR cross-peaks labeled are shown in Figures 3 and 4, respectively. Although these samples were not optimally pure, the ^{1}H–^{13}C HSQC NMR spectra were of sufficient quality to provide a clear assessment of the composition of the CTs in the isolated fractions.

Compositional analysis of the CTs purified from *S. nigra* was ascertained through volume integration of the respective cross-peaks

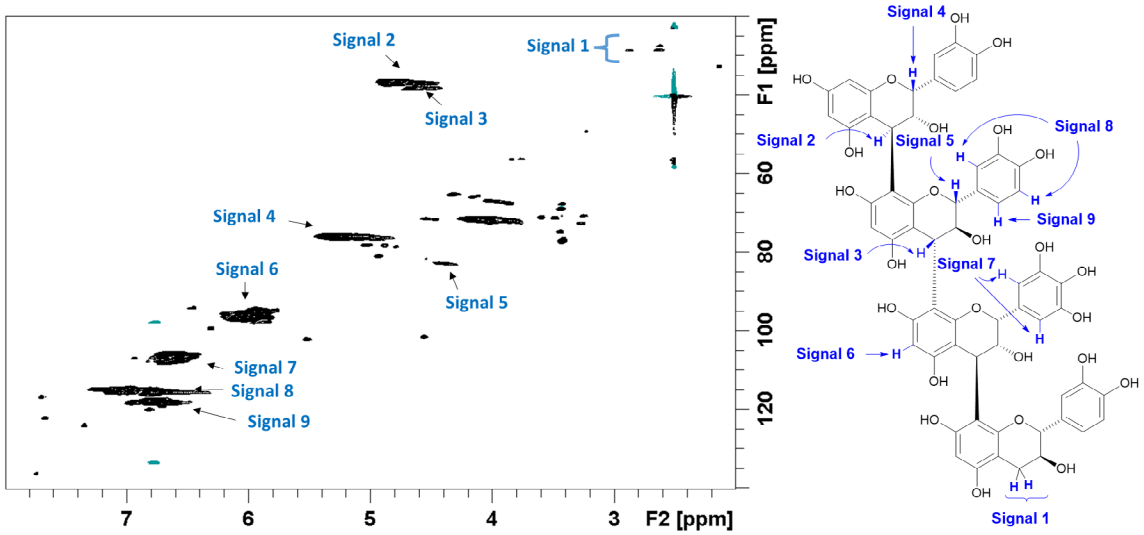


FIGURE 2 $^{1}H–^{13}C$ heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectrum of purified condensed tannins from *O. viciifolia* (sainfoin).

TABLE 1 Seasonal variation and mean concentrations of condensed tannins (mg/g DM) in leaves of four fodder tree species and two shrub species across three seasons and three locations (least-square mean ± standard deviation).

1	2	3	4	5
Fodder species	**Spring**	**Summer**	**Autumn**	**CT, mg/g**
Salix nigra	11.8 ± 4.1[b]	20.0 ± 5.0[a]	21.9 ± 2.9[a]	17.9[A]
Salix alba	16.6 ± 0.8	17.9 ± 0.4	21.1 ± 1.8	18.5[A]
Populus nigra	6.69 ± 1.49	4.31 ± 0.50	2.46 ± 0.66	4.5[C]
Elaeagnus angustifolia	0.25 ± 0.36	0.49 ± 0.42	0.12 ± 0.10	0.3[D]
Hippophae rhamnoides	16.0 ± 1.4[a]	5.73 ± 3.01[b]	3.75 ± 0.66[b]	8.5[B]
Rosa webbiana	0.17 ± 0.14	0.72 ± 0.50	0.10 ± 0.17	0.3[D]
Mean				8.3
SEM	1.135			0.65

Note: Least-square means within species (across rows 2–4) with the same lower-case letters are not significantly different. Species × season interaction $p < 0.01$. Means with the same upper-case letters (Column 5) are not significantly different. Main effect of species $p < 0.01$.
Abbreviations: CT, condensed tannins; SEM, standard error of mean.

in the $^{1}H–^{13}C$ HSQC NMR spectrum (Figure 3) to assign procyanidin (PC)/prodelphinidin (PD) and *cis*/*trans* ratios,[22] and an estimate of the mean degree of polymerization (mDP) of the isolated material.[30] The sample isolated from *S. nigra* had a PC/PD ratio of 59/41, a *cis*/*trans* ratio of 59/41 with an estimated mDP of 8.1. Compositional analysis of the CT purified from *Salix alba* was ascertained similarly to *S. nigra*, and had a PC/PD ratio of >99/1, a *cis*/*trans* ratio of 85/15 with an estimated mDP of 6.9 (Figure 4).

Willow varieties have been shown to contain CTs with a wide range of composition. Ayres et al. showed the composition of CTs between seven different Salix species exhibited PC/PD ratios ranging from 34/66 to 83/17, *cis*/*trans* ratios from 30/70 to 74/26, and mDPs between 4.8 (*Salix novae-angliae*) and 14.6 (*Salix monticola*).[31] Falchero et al.[32] examined leaves from 18 accessions of willow whose mDPs varied from 4.1 to 26.1, PC/PD ratios varied from 14.8/85.2 to 97.7/2.3, and *cis*/*trans* ratios ranged from 1.7/98.3 to 81.3/18.7. There are also reports of CT occurring in *H. rhamnoides* with levels as high as 18.5 mg/g dry matter.[33]

The variation on PC/PD ratios of CTs from *S. alba* and *S. nigra* are considerable and require discussion. Several studies have shown that CTs containing a significant level of PD flavan-3-ol subunits more effectively interact with proteins, translating into greater impacts on ruminant health and nutrition. Jones et al.[34] demonstrated through comparison of CTs purified from six different plants that a general trend of ability of CTs to bind to and precipitate proteins (astringency) increases with increasing PD content of the CT. A comparison of CTs from *Lotus corniculatus* (high PC content) and *Lotus pedunculatus* (high PD content) showed similar ability to precipitate Rubisco protein.[35] However, the CT from *L. pedunculatus* was more effective at reducing the degradation of the Rubisco-CT complex in an in vitro rumen fluid environment. In an in vivo study, two *Calliandra calothyrsus* provenances (San Ramon, CT with high PD content) and Patulul (CT with

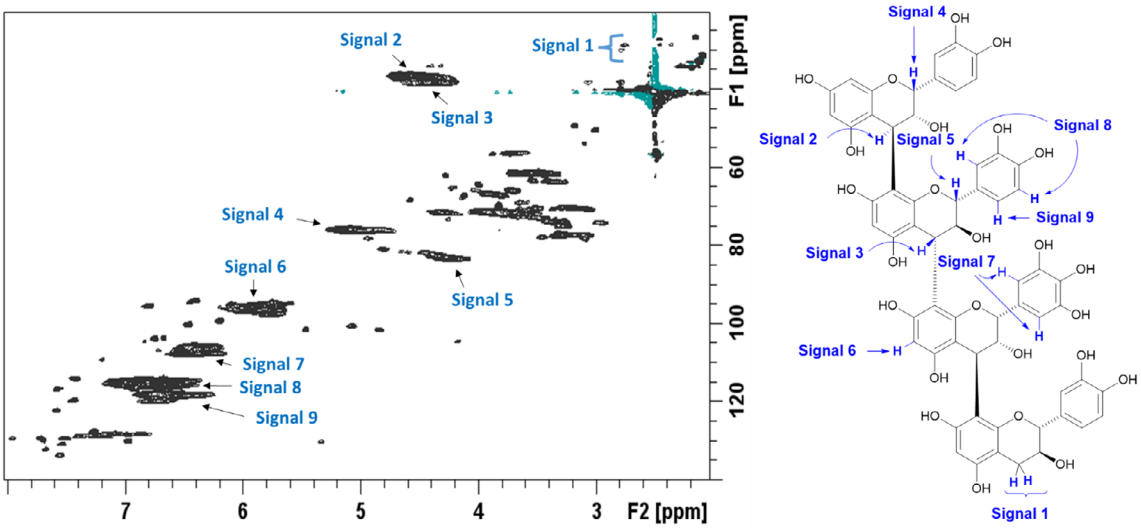


FIGURE 3 $^{1}H–^{13}C$ heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectrum of purified condensed tannins from *Salix nigra* (black willow).

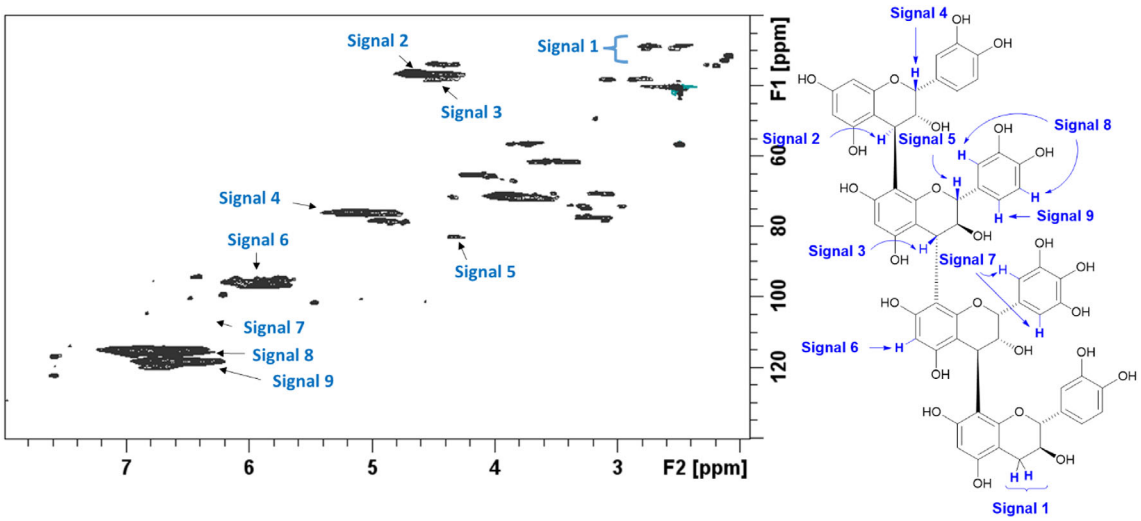


FIGURE 4 $^{1}H–^{13}C$ heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectrum of purified condensed tannins from *Salix alba* (white willow).

high PC content)[36] were fed to ruminal and intestinal cannulated sheep. The San Ramon *C. calothyrsus* provenance proved superior in delivery of total nitrogen to the duodenum resulting in a greater ruminal escape dietary nitrogen over the Patulul *C. calothyrsus* provenance, affirming ruminant nutritional benefit. Lastly, it is also well documented that PD-rich CTs serve as better anthelminthic agents than CTs composed of mainly PC flavan-3-ol subunits.[37] Thus, *S. nigra* will likely have a greater impact on ruminant health and nutrition than *S. alba* in subsequent feeding studies.

Different species of tree foliage respond differently to seasonal variations concerning their tannin concentration,[38] and also differ in their response to physiological changes.[39] The CT content of *Salix* species were significantly higher than those of the other species examined. The CT concentration in the present study (0.1–21.9 mg/g DM) was in the range of 0.2–28 mg/g DM observed in acacia species[40] and above the levels of 0.15 mg/g to 0.39 mg/g DM of other browse trees and shrubs.[41] *Salix* species (17.9–18.5 mg/g) showed highest levels of CT among fodder tree species, but all browse species were considerably lower than the total CT contents of forages ranging from 60 to 100 mg/g DM that depress the growth of animals and feed intake such as *L. pedunculatus*.[42,43] Consumption of plant species containing more than 50 mg/g DM of CT can lead to significant declines in voluntary feed intake, while consumption of plants having less than 50 mg/g DM of CT does not typically affect voluntary intake.[42,44,45] It has been mentioned in the literature that at low to moderate diet levels of CT, the amount of dietary protein absorbed increases due to greater flow of essential amino acids to the small intestine in ruminant animals. The levels of CT shown in

the selected browse species in this study were low and safe, likely not to exhibit anti-nutritional effects or decrease dry matter intake.[46] Factors such as the age of the plant, maturity of leaves, soil type, and availability of water are known to influence CT concentration and other potentially defensive characteristics such as toughness, fibrosity, and thorns.[47,48] Tannin concentration in plants is increased by high temperatures, water stress, extreme light intensities, and poor soil quality.[49,50]

CONCLUSION

In summary, *S. nigra* and *S. alba* likely contain adequate condensed tannin levels to bolster nitrogen use efficiently in ruminants without exhibiting a detrimental effect on dry matter intake.[46] The results of this study, although only designated trending for *S. alba* and *P. nigra*, suggest that the greatest impact on ruminant nutrition would be when the *Salix* species leaves are harvested in the fall and the *P. nigra* and *H. rhamnoides* leaves were harvested in the spring. Following these recommendations, producers will likely exploit the benefits of these condensed tannin-containing fodder species to maximize use of the protein present in these feedstuffs.[46]

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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