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The effect of maturity on concentration and biological activity of protein precipitating polyphenolics in ground juniper is dependent upon species

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ABSTRACT

Nutritional characteristics of *Juniperus* species are now well known and suitability as an abundant, low-cost feedstuff for ruminant animal production is a possibility. *Juniperus* species produce plant secondary metabolites including condensed tannins (CT) and volatile oils. Maturity of *Juniperus* species has been shown to affect the concentration of plant secondary metabolites produced. The objectives of this study were to determine the effects of *Juniperus* species (*Juniperus ashei, J. monosperma, J. pinchotii* and *J. virginiana*) and maturity on protein precipitating polyphenolic (PPP) compounds, their biological activity and relatedness of these effects to rumen digestibility characteristics. We found a species by maturity interaction for PPP ($P < 0.001$) and for the amount of protein bound (PB; $P < 0.001$) by PPP. Protein precipitable phenolic concentrations were greater ($P < 0.001$) for all species in the immature stage as compared to the mature stage except for *J. virginiana*, for which PPP were unaffected by maturity. The PB by *J. pinchotii* and *J. monosperma* was greater ($P < 0.001$) in the immature stage as compared to the mature stage whereas that of *J. ashei* and *J. virginiana* remained unchanged by stage of maturity. Linear regression within species and maturity of 48-h IVTDMD and total gas production on PPP suggested a positive correlation exists for immature *J. virginiana* ($R^2 = 0.71$) and mature *J. pinchotii* ($R^2 = 0.68$) for IVTDMD, and *J. virginiana* ($R^2 = 0.81$) for total gas production. There was no relationship for PB within species and maturity of juniper and 48-h IVTDMD or total gas production, suggesting that biological activity of protein binding by PPP is unrelated to overall *in vitro* digestion of *Juniperus* species. The effect of maturity on concentration and biological activity of PPP in juniper is dependent upon species.

1. Introduction

Nutritional characteristics of *Juniperus* species have been characterized (Stewart et al., 2015) and suitability of biomass as an effective low-cost roughage ingredient for small ruminant animal production has been demonstrated (Whitney and Muir, 2010;

Abbreviations: CT, Condensed tannis; PPP, protein precipitating polyphenolic; IVTDMD, *in vitro* true dry matter digestibility; PB, protein bound; AA, amino acid; RUP, rumen undegradable protein; PEG, polythethylene glycol

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Whitney et al., 2014, 2017). *Juniperus* species are known to produce plant secondary metabolites including an assortment of volatile oils as well as polyphenolic compounds including CT (Stewart et al., 2015; Whitney et al., 2014), and plant maturity has been shown to affect the concentration of plant secondary metabolites produced (Stewart et al., 2015).

Condensed tannins are polyphenolic compounds composed of polymerized flavan-3-ol monomers that exhibit a large variability in chemical structure characteristics (Naumann et al., 2017). The most notable effects of these compounds on ruminant nutrition include mitigation of ruminal methane production, anthelmintic effects in small ruminants and ruminal protein binding; the latter associated with commonly hypothesized effects on rumen bypass protein (Kariuki and Norton, 2008; Patra and Saxena, 2011). While CT may bind to minerals, carbohydrates and lipids, among other organic and inorganic substances, they are generally recognized for their characteristic binding to proteins. However, not all CT are biologically active and they may not demonstrate the ability to bind proteins. Measuring protein precipitable phenolics (PPP; biologically active tannins) and quantifying the amount of PB by PPP allows for more accurate prediction of the possible effects that forages and feedstuffs will have on ruminants that consume them. Thus, the objectives of this study were to determine the effects of *Juniperus* species (*Juniperus ashei, J. monosperma, J. pinchotii* and *J. virginiana*) and stage of maturity on concentration and biological activity of PPP, as well as the relationships of these variables to *in vitro* rumen digestibility characteristics. We hypothesized that condensed tannin concentration and bioactivity would vary depending on *Juniperus* species and that both would be greater in immature species as compared to mature species as a function of greater leaf to stem ratios, and related plant defense mechanisims.

2. Materials and methods

2.1. Study design and harvesting protocol

The following study design and harvest protocol used was previously described in Stewart et al. (2015). The experiment was conducted as a 4 × 2 factorial consisting of 4 *Juniperus* species (*Juniperus ashei, J. monosperma, J. pinchotii* and *J. virginiana*) and 2 stages of maturity (mature and immature), each replicated 4 times (n = 32 plots). *Juniperus* species were collected over a 4-week period in March 2012 at 4 separate geographic locations. *Juniperus pinchotii* was collected in Tom Green County, TX (31°36′54.73″N, 100°32′24.48″W) on a Cho Association loamy, capionatic, thermic, shallow Petrocalcic Calciustolls site. *Juniperus ashei* was collected in Edwards County, TX (30°17′08.18″N, 100°32′46.30″W) on an Eckrant, clayey, skeletal, smectitic, thermic Lithic Haplustolls site. *Juniperus virginiana* was collected in Bastrop County, TX (29°55′18.69″N, 97°15′3.28″W) on a Silstid loamy, siliceous, semi-active, thermic Arenic Paleustalfs site. *Juniperus monosperma* was collected in Torrance County, NM (34°16′01.03″N, 105°25′26.21″W) on Pinon, loamy, mixed, super active, mesic Lithic Ustic Haplocalcids site. At each of the 4 sites, 4 plots, each separated by 165 m were designated as harvest sites. Plots for each species were the experimental unit and were maintained separate throughout the trial.

One mature male and one mature female tree (height: > 3 m), and 2 male and 2 female immature plants (height: 1 to 1.8 m) from each plot were mechanically harvested and transported to a central location and processed within 72 h. Immature trees were chipped using an Echo Bear Cat chipper (West Fargo, ND). Due to the large amount of biomass, mature plants were initially chipped through a Vermeer X1500) (Pella, IA) and then a 90-kg random sample was chipped once more using the Bear Cat chipper. All chipped material from immature and mature plants was then subsampled, fine-ground through a hammermill to pass a 4.76-mm sieve (Sentry, model 100, Mix-Mill Feed Processing Systems, Bluffton, IN), dried at 55 °C in a forced-air oven for 48 h, ground through a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) to pass a 2-mm screen, and stored at −20 °C.

2.2. Laboratory analyses

Three juniper plant extracts from each of two plant material sub samples from each plot, representing each combination of *Juniperus* species and maturity, were analyzed and averaged for protein-precipitable phenolics (n = 32) in *Juniperus* species and protein bound (n = 32) by *Juniperus* PPP using the scaled-down method of Hagerman and Butler (1978) as described by Naumann et al. (2014). Extracts were prepared by combining 50 mg of plant tissue with 1 mL of 50:50 (v/v) methanol:water and rotating for 15 min followed by centrifugation at 16,000 x g for 5 min at 4 °C.

A 50-ml aliquot of supernatant from plant extracts was combined with 250 mL Buffer A (0.20 M acetic acid, 0.17 M NaCl, pH 4.9), 50-mL BSA (5 mg/mL in Buffer A), and 50-mL 50:50 (v/v) methanol:water, and incubated at room temperature for 30 min before centrifuging at 16,000 x g for 5 min at 48 °C. Supernatants were removed by vacuum aspiration and the protein-phenolic pellet was washed in 100 mL Buffer A before re-centrifuging and aspirating.

To determine PPP, the pellets were dissolved in 800 mL of sodium dodecyl sulfate (1% w/v) triethanolamine (5% v/v) before adding 200 mL of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01 M HCl). Absorbances at 510 nm were measured after 15 min and PPP were quantified using a species-specific standard curve.

To determine PB, the precipitation reaction was carried out exactly as described above, but the BSA protein-PPP pellet was isolated, and analyzed for N to determine precipitated protein. To accomplish this, the protein-PPP pellet was vortexed in 500 mL Buffer A and the solution was transferred into a pre-weighed foil cup and allowed to dry. The dried protein-phenolic residue was analyzed for N using an Elementar Vario Macro C:N analyzer (Elementar Americas, Inc., Mt. Laurel, NJ, USA). Percent N was converted to protein by multiplying by a factor of 6.25. The ratio of PB to PPP was calculated and evaluated as an index for biological activity of PPP for each species. Values for 48-hour IVTDMD and total gas production were obtained from Stewart et al. (2015) and used for regression analyses with PPP and PB.