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NUTRITIONAL VALUE, SECONDARY METABOLITES, ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTIBACTERIAL ACTIVITIES OF THREE WOODY FORAGE PLANTS

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ABSTRACT

Thirty-six leaf samples of *Sophora davidii*, *Indigofera amblyantha*, and *Broussonetia papyrifera* were collected during different growth periods. The dynamic changes in nutritional components and secondary metabolites were analysed, and the biological activities of the secondary metabolites were evaluated. The optimal utilization stage of each plant was comprehensively determined. The results showed that *S. davidii* (the crude protein (CP) content 24.82%, relative feed value (RFV) 186.52) and *I. amblyantha* (CP 24.28%, RFV 177.54) were premium forage materials and that *B. papyrifera* (CP 21.65%, RFV 133.84) was a first-level forage. The best period for using the leaves of these three plants as animal feed was the flowering stage. As the growth progressed, the contents of CP, crude fibre (CF), dry matter (DM), and nitrogen-free extract (NFE) decreased, while the CF, neutral detergent fibre (NDF), acid detergent fibre (ADF), and crude ash (Ash) increased. The *S. davidii*, *I. amblyantha*, and *B. papyrifera* contains total flavonoid (0.36-0.70, 1.07-2.87, and 0.59-0.78 mg/g, respectively), tannin (5526.21-7717.77, 4284.03-5818.73, and 6390.66-7640.60 nmol/g, respectively), total phenol (205.62-298.69, 163.22-214.22, and 143.87-214.34 mg/g, respectively), total saponin (0.11-0.22, 0.20-0.39, and 0.10-0.16 ng/g, respectively) and total alkaloid (0.20-0.32, 0.23-0.38, and 0.20-0.36 ng/g, respectively). Moreover, the tannins, total phenols, saponins, flavonoids, and alkaloids in these woody forages scavenged hydroxyl radicals and superoxide anions to varying degrees, and their ability to reduce Fe^{3+} gradually increased with concentration. These plants inhibited hyaluronidase and albumin denaturation. The alkaloid and flavonoid extracts of *S. davidii* significantly ($P\leq0.05$) inhibited *Staphylococcus aureus* with antibacterial diameters of 14.48 and 14.44 mm, respectively. The flavonoid (11.29 mm) and tannin (11.17 mm) extracts of *S. davidii* significantly ($P\leq0.05$) inhibited *Escherichia coli.* The alkaloid (12.29 mm) and tannin (13.15 mm) extracts of *B. papyrifera*, and the flavonoid extract of *S. davidii* (13.44 mm) and *I. amblyantha* (10.32 mm) inhibited *Bacillus subtilis*. The secondary metabolites of these plants possess antioxidant, anti-inflammatory and antibacterial activities. In conclusion, these findings provide new perspectives on the nutritional value and biochemical characteristics of leaves of *S. davidii*, *I. amblyantha*, and *B. papyrifera*, emphasizing their use as valuable feed source for ruminants.

Keywords: biological activity, secondary metabolite, *Broussonetia papyrifera*, *Indigofera amblyantha*, *Sophora davidii*

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INTRODUCTION

Due to the rapid development of animal husbandry, the demand for feed is increasing, and traditional grass and grain feed are in short supply (Wang *et al*., 2022). Thus, the identification of supplements to traditional feed is urgently needed to ensure the vigorous and stable development of animal husbandry. Woody forages are rich in resources, with a high yield of branches and leaves, high quality, good palatability, a long green period and useful life, and strong regeneration ability. In the winter and early spring, woody feed can replace pasture or other feeds as a source of protein, thereby increasing feed value and decreasing feeding costs (Obour *et al*., 2017). However, woody feed also has several shortcomings, such as a higher cellulose content and limited edible parts of some feed plants (Hejcmanová *et al*., 2014). Secondary metabolites in plants are endogenous active substances with antioxidant, antibacterial, anti-inflammatory, antitumour and other biological activities (Christoph and Zotchev, 2021). These compounds also increase the egg production rate of poultry and the productivity and immunity of livestock and poultry and improve meat quality (Hager-Theodorides *et al*., 2014). However, some antinutritional components, such as the polyphenolic compounds tannins, which lead to poor feed palatability, are present

among the secondary metabolites of some plants (Ali *et al*., 2022). Therefore, exploring the secondary metabolites and nutritional value of woody forages is highly important for ensuring the healthy development of animal husbandry.

Sophora davidii is a perennial shrub belonging to the legume family. Its leaves, flowers and fruits have been used as fodder and medicines by local people (Huang *et al*., 2018). *Indigofera amblyantha* is a perennial shrub of the Leguminosae family that has the advantages of tolerance to drought and acidic, shallow, and low fertility soils and high yield and nutritional value (Zhao *et al*., 2023). *Broussonetia papyrifera* is a deciduous tree in the Moraceae family. It is a high-quality protein feed resource with medicinal and feed value (Peng *et al*., 2019). In recent years, many studies have focused on the properties and composition of extracts obtained from the roots, bark and leaves of woody plants (Kiran *et al*., 2023). However, few comprehensive studies have assessed the nutritional value and antioxidant, anti-inflammatory and antibacterial activities of the leaves of these three woody forage plants.

In view of the limited information on these three woody forages, the present research was conducted to determine the nutrient compositions and secondary metabolite contents at different growth stages and to determine the best growth stages for livestock feeding. In addition the antioxidant, anti-inflammatory and antibacterial activity of secondary metabolite extracts were also evaluated.

MATERIALS AND METHODS

Biomass sampling: In this study, *S. davidii*, *I. amblyantha*, and *B. papyrifera* were selected. Sampling was conducted at the common garden experimental site (26°40′ N, 106°40′ E, 1100 m a.s.l.) located on the southern campus of Guizhou University, Guiyang, Guizhou Province.

From February to November 2019, leaf samples were collected from the selected woody species at different stages of the plant growth period, including the nutritional stage, flowering stage, pod-setting stage, and maturation stage. Two kilograms of leaf sample was collected from at least three individuals of each species. Thirty-six biological leaf samples were collected (3 types, 4 stages and 3 repetitions). The collected plant samples were dried at 105°C for 30 min, baked to a constant weight at 65°C, cooled, ground into a powder, passed through a 40-mesh sieve, and packed into self-sealing bags for storage at room temperature.

Nutritional value determination

Nutrient composition and Relative feed value (RFV): The dry matter (DM) content was measured after drying at 65°C. The crude protein (CP) content was determined using the Kjeldahl method. For the analysis of the crude fibre (CF) contents, acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents were measured using the polyester mesh bag method. The crude fat (EE) content was measured using the Soxhlet extraction method. The crude ash (Ash) content was measured using the ashing method, and the nitrogen-free extract (NFE) was calculated (AOAC, 2010).

The RFV was calculated as DMI (%BW) × DDM (%DM)/1.29 (Rohweder *et al*., 1978). Here, dry matter intake (DMI) is the *ad libitum* intake of dry matter for roughage (units %BW), and digestible dry matter (DDM) is the digestible dry matter (units %DM). The prediction models for were DMI (%BW) = 120/NDF and DDM (%DM) = 88.9-0.779 × ADF. According to the USA import alfalfa evaluation standard, the RFV feed classification of the three woody forages was evaluated as described in Table 1.

Secondary metabolite extraction: *Tannin extraction*: Approximately 0.05 g of sample was weighed and placed into a 1.5 mL centrifuge tube. One millilitre of extraction solution was added, and sample extraction was performed in a 70°C water bath for 30 min with occasional shaking. After extraction was complete, high speed refrigerated centrifugation was performed at 12000 rpm and 25°C for 10 min. The supernatant was collected in a centrifuge tube and diluted to 1 mL with extraction solution for testing (Hagerman, 1988).

Flavonoid extraction: One gram of sample was weighed and placed into a 250 mL conical flask. The *B. papyrifera* leaf powder was mixed with a 50% ethanol solution at a material-to-liquid ratio of 1:30 (g:mL). Ultrasonication was performed for 30 min at 50°C. *S. davidii* and *I. amblyantha* were extracted using 70% ethanol at a material-to-liquid ratio of 1:20 (g:mL). Ultrasonication was performed for 30 min at 60°C. Each sample was filtered under reduced pressure with a suction filter bottle to remove the filter residue, after which the extract was collected and centrifuged in a centrifuge tube and adjusted to a volume of 100 mL for testing (Sun *et al*., 2021).

Saponin extraction: One gram of sample was weighed and placed into a 50 mL centrifuge tubes, and 20 mL of 70% ethanol was added for a material-to-liquid ratio of 1:20 (g:mL). Ultrasonic disruption was performed for 30 min at 20°C. After centrifugation, the supernatant (extract) was collected and degreased. The ethanol extract was concentrated under reduced pressure with a rotary evaporator to prepare the dry extract, which was dissolved in 20 mL of distilled water and filtered under reduced pressure to remove insoluble matter. An equal volume of petroleum ether was added for two-phase extraction (Hajimohammadi *et al*., 2017). N-butanol was added to the organic phase for saponin extraction. Then,

15 mL of n-butanol was added three times, and each time the sample was shaken until well mixed and allowed to separate. A rotary evaporator was used to concentrate the sample as a dry extract, which was dissolved in 10 mL of distilled water and stored until further testing.

Alkaloid extraction: Two grams of sample was weighed and placed into different 50 mL centrifuge tubes. Then, 40 mL of 30% acidic ethanol was added, and the mixture was vortexed for 30 s. Ultrasonic disruption was applied for 30 min at 50°C, followed by centrifugation at 5000 r/min for 15 min. The supernatant was removed, 40 mL of 30% acidic ethanol was added to the residue, and the extraction was repeated once. The extracts were combined, concentrated and brought to a volume of 25 mL under reduced pressure with a rotary evaporator. Then, HPD100 macroporous adsorption resin was used to adsorb the extract for 30 min, after which the sample was eluted with ultrapure water, concentrated, evaporated under reduced pressure, and dissolved in 10 mL for testing (Bugatti *et al*., 1991).

Phenol extraction: Approximately 0.10 g of sample was weighed and placed into different 5 mL centrifuge tubes. Then, 2.5 mL of 60% ethanol was added, and ultrasonication was applied for extraction at a power of 300 W, temperature of 60°C for 30 min with a break period of 5 s in 8 s intervals. Each sample was centrifuged for 10 min in a high speed refrigerated centrifuge at 12000 rpm and 25°C. The supernatant was diluted to 2.5 mL with 60% ethanol for testing (Wallace, 1987).

Determination of secondary metabolite contents and activity: The contents of tannins, flavonoids, alkaloids, saponins and phenols were determined using commercial kits purchased from Beijing Solabao Technology Co., Ltd.

Antioxidant activity: The hydroxyl radical scavenging capacity, superoxide anion scavenging capacity, and total antioxidant capacity were determined using kits according to the manufacturer's instructions (Beijing Solabao Technology Co., Ltd.).

Anti-inflammatory activity: (1) Hyaluronidase inhibition test: The Elson-Morgan method was used with slight modifications. Tubes labelled A, B, C, and D were established. Then, 0.5 mL of hyaluronidase (500 U/mL) was added to tubes A and C, and 0.5 mL of acetate buffer (0.1 mol/L, pH 5.0) was added to tubes B and D. Next, 0.1 mL of $CaCl_2$ (0.25 mmol/L) was added to each tube, and the tubes were placed in a constant temperature 40°C water bath for 20 min. Afterwards, 0.5 mL of sample was added to tubes C and D, 0.5 mL of distilled water was added to tubes A and B, and the tubes were incubated at 40°C for 20 min. Next, 0.5 mL of sodium hyaluronate solution (0.6 mg/mL) was added to each tube, and the tubes were incubated at 40°C for 20 min. Afterwards, 0.2 mL of boric acid solution was added, and the mixtures were boiled in a water bath for 5 min. After cooling, 3 mL of P-DAB developer was added. After standing for 5 min, the absorbance was measured at 585 nm, using diclofenac sodium as a positive control (Han *et al*., 2015). The hyaluronidase inhibition rate was calculated as follows.

$$\text{Hyaluronidase inhibition rate (\%)} = \left(1 - \frac{A - B}{C - D}\right) \times 100$$

(2) Inhibition of albumin denaturation test: Bovine serum albumin (2.0 g) was dissolved in acetate buffer (pH 5.0, 0.1 mol/L). Three millilitres of bovine serum albumin (2%) was placed in a 5 mL centrifuge tube in a 37°C incubator for 20 min, after which 0.5 mL of sample was added. The solution was diluted to 5 mL with acetic acid buffer (pH 5.0, 0.1 mol/L). After incubation in a water bath at 70°C for 10 min, the sample was quickly cooled to room temperature, and the turbidity of the sample was measured at 660 nm. Distilled water was used as a control, the absorbance of which was recorded as Ac, the absorbance of the test group was recorded as As, and diclofenac sodium was used as a positive control (Grabowska *et al*., 2016). The inhibition of albumin denaturation was calculated as follows.

$$\text{Rate of albumin denaturation ihibition (\%)} = \left(1 - \frac{As}{Ac}\right) \times 100$$

Antibacterial activity was assessed following the methodology of Marasini *et al*. (2015) as follows; The culture medium was prepared by dissolving 5 g of NaCl, 3.0 g of beef extract, and 10 g of peptone in 1000 mL of distilled water. The mixture was sterilized at 121°C for 20 min. The Petri dishes were inverted for three days. The culture medium was poured into a 10 mL test tube to prepare slant culture medium. For the purpose of strain activation, the S. aureus, B. subtilis and E. coli were purchased from Beijing Solabao Technology Co., Ltd. A small amount of the three bacterial strains were inoculated on the corresponding slant media with an inoculation loop using the streaking method. The cells were placed in a constant temperature incubator at 35-37°C for 18-24 hours. The bacterial suspension was prepared by mixing 10 mL of sterile water with a small number of bacterial cells, and each mixture was placed in a constant temperature culture shaker for 12 hours. For bacterial inoculation, the bacterial suspension (100 μL) was placed on the plate and spread evenly with a triangular glass spreader. The antibacterial effects were determined by using filter paper soaked in the extract and placing it on a plate coated with bacteria. This process was repeated 3 times for each strain. The inoculated plate was placed upside down in a constant temperature incubator for 48 hours.

Comprehensive evaluation of the membership function: The membership function value was calculated as follows: formula (1) is the membership function value that is positively correlated to the nutritional value of the forage and formula (2) is the membership function value that is negatively correlated to the nutritional value of the forage.

$R(X_{ij}) = (X_{ij} - X_{imin})/ (X_{imax} - X_{imin})$ (1);

$R(X_{ij}) = 1 - (X_{ij} - X_{imin})/ (X_{imax} - X_{imin})$ (2)

Here, X_{ij} is the membership function value of the index of variety i j, and X_{imax} and X_{imin} are the maximum and minimum values of a particular index for all tested materials, respectively.

Data analysis: One-way ANOVA and Duncan's Multiple comparisons tests were performed to analyse the measured data indicators using the software SPSS 20.0. SigmaPlot 14.0 was used to graph the data.

RESULTS

Dynamic changes in the nutrient contents of the three woody forage plants: As growth progressed, the DM contents of *S. davidii*, *I. amblyantha,* and *B. papyrifera* increased firstly and then decreased. The highest to lowest DM contents followed the order *B. papyrifera* > *I. amblyantha* > *S. davidii* (Table 2). The EE contents of *S. davidii* and *B. papyrifera* first increased and then decreased as growth progressed, while the EE content of *I. amblyantha* decreased (Table 2). The *S. davidii* and *B. papyrifera* had the highest EE contents at the flowering stage (4.07%) and pod-setting stage (13.56%), whereas lowest EE contents were noted for *S. davidii* (2.72%) and *B. papyrifera* (2.08%). The dynamic changes in the CF, NDF, and ADF contents tended to increase with an obvious positive correlation with advancing growth stage. The contents of the three indicators were greatest during the maturation stage in the order *B. papyrifera* > *I. amblyantha* > *S. davidii* (Table 2). As growth progressed, the ash contents of *S. davidii* and *B. papyrifera* increased, while that of *I. amblyantha* decreased gradually. The highest ash contents of *S. davidii* and *B. papyrifera* were detected in the maturation stage (7.52% and 15.19%, respectively), and the lowest ash contents were observed in the nutritional stage (5.09% and 11.21%, respectively). For *I. amblyantha*, the ash content was highest in the nutritional stage (11.73%) and the lowest in the pod-setting stage and maturation stage (10.96%) The NFE contents of *S. davidii* and *I. amblyantha* first increased and then decreased as growth progressed, and both presented the highest values in the flowering stage (22.79% and 17.81%, respectively) and the lowest values in the maturation stage (6.88% and 6.20%, respectively). The highest NFE content was detected in the flowering stage (34.30%), and the lowest was observed in the pod-setting stage (19.78%). The dynamic changes in CP and RFV were similar and decreased with growth stage The *S. davidii* and *I. amblyantha* are premium forage grasses, while *B. papyrifera* is a first-level forage, but all three plants have high feeding value (Table 1).

Dynamic changes in the secondary metabolite contents of three woody forage plants: The flavonoid contents in *S. davidii* and *I. amblyantha* gradually increased with growth stage, and the total flavonoid content decreased in the order maturation stage > pod-setting stage > flowering stage > nutritional stage. The flavonoid content in *B. papyrifera* increased in a wave-like manner, first increasing and then decreasing before increasing again, with the highest contents in the flowering stage (0.78 mg/g) and maturation stage (0.78 mg/g) and the lowest content in the nutritional stage (0.59 mg/g) (Table 3).

The tannin contents of *S. davidii* and *I. amblyantha* exhibited wave-like trends. The highest tannin contents were detected in the nutritional stage (7717.77 nmol/g and 5818.73 nmol/g, respectively). The tannin content of *B. papyrifera* first increased and then decreased in the order pod-setting stage > flowering stage > maturation stage > nutritional stage (Table 3). As growth progressed, the total phenol contents in *S. davidii* and *I. amblyantha* decreased gradually. The maximum contents were observed during the nutritional stage (298.70 mg/g and 214.22 mg/g, respectively). The total phenol content in *B. papyrifera* first increased and then decreased in the order pod-setting stage > flowering stage > nutritional stage > maturation stage. The total saponin contents changed in a V-shaped manner by first decreasing and then increasing. The lowest total saponin contents were observed in *S. davidii* and *I. amblyantha* in the pod-setting stage (0.11 ng/g and 0.20 ng/g, respectively). The total saponin content in *B. papyrifera* was the lowest in the flowering stage (0.10 ng/g). The alkaloid contents first decreased and then increased. The lowest alkaloid contents in *S. davidii* and *I. amblyantha* were detected in the flowering stage (0.20 ng/g and 0.23 ng/g, respectively), and the highest contents were observed in the maturation stage (0.32 ng/g and 0.38 ng/g, respectively). The alkaloid contents in *B. papyrifera* decreased in the order nutritional stage > flowering stage > maturation stage > pod-setting stage.

Feeding value analysis: The optimal utilization periods for *I. amblyantha* and *B. papyrifera* followed the order flowering stage > nutritional stage > pod-setting stage > maturation stage. The optimal utilization periods for *S. davidii* followed the order flowering stage > pod-setting stage > nutritional stage > maturation stage (Table 4). The optimal utilization stage for each plant was the flowering stage. Therefore, the next experiment used samples from the three woody forage plants taken during the flowering stage.

Secondary metabolite activities of the three woody forage plants

Antioxidant activities of the secondary metabolites: The ability of the *S. davidii* tannin extract to scavenge hydroxy radicals significantly differed from that of the other two plants ($P \leq 0.05$) (Fig. 1a). At 0.025 mg/mL, the difference between *I. amblyantha* and the other two plants was significant ($P \leq 0.05$) (Fig. 1a). The scavenging of superoxide radicals increased with increasing extract concentration. The difference in the scavenging of superoxide radicals by the tannins from the three plants was not significant ($P > 0.05$), except at 0.025 mg/mL (Fig. 1f). The scavenging of superoxide radicals by the flavonoids of *B. papyrifera* was the lowest, except at 0.025 mg/mL (Fig. 1g). The highest scavenging of superoxide radicals was observed for the alkaloid and the saponin extracts of *B. papyrifera* (Fig. 1h, i). No significant difference ($P > 0.05$) in the scavenging of superoxide radicals by the total phenols of *I. amblyantha* or *B. papyrifera* was observed at concentrations of 0.025-0.1 mg/mL, but these results were significantly different from those for *S. davidii* ($P \leq 0.05$) (Fig. 1j). At concentrations of 0.2 mg/mL or greater, significant differences were observed among the three plant species ($P \leq 0.05$) (Fig. 1j).

The ferric ion reducing antioxidant power (FRAP) increased with increasing extract concentration. At 0.025 mg/mL, the difference in FRAP values of the tannins from *S. davidii* and *I. amblyantha* was not significantly different ($P > 0.05$) from each other but they were significantly ($P \leqslant 0.05$) greater than that of *B. papyrifera*. At 0.05 mg/mL and higher concentrations, significant differences ($P \leq 0.05$) in the FRAP values of the tannins were observed between the three plants (Fig. 1k). Significant differences ($P \leq 0.05$) in the FRAP of the flavonoid extracts of the three plants were detected at 0.025, 0.05, and 0.2 mg/mL (Fig. 1l); at 0.1 and 0.4 mg/mL, no significant difference ($P > 0.05$) was observed between the FRAP of the flavonoids of *S. davidii* and *I. amblyantha*, but the FRAP of the *B. papyrifera* flavonoids was significantly different ($P \leq 0.05$) (Fig. 1l). At 0.025 and 0.4 mg/mL, there were significant differences ($P \leq 0.05$) in the FRAP values of the saponin extracts of the three plants (Fig. 1n). At 0.05 and 0.2 mg/mL, the FRAP values of the saponins from *I. amblyantha* and *B. papyrifera* were significantly different from that of *S. davidii* ($P \leq 0.05$) (Fig. 1n). At 0.025 and 0.1 mg/mL, the FRAP values of the total phenol extracts of the three plants significantly differed ($P \leq 0.05$), and at 0.05 mg/mL, *S. davidii* and *I. amblyantha* exhibited significantly different values from that of *B. papyrifera* ($P \leq 0.05$) (Fig. 1o). At 0.2 mg/mL, the FRAP values of *B. papyrifera* and *I. amblyantha* were significantly different from that of *S. davidii* ($P \leq 0.05$) (Fig. 1o), while at 0.4 mg/mL, no significant difference ($P > 0.05$) in the FRAP values of the three woody forage plants was observed (Fig. 1o).

Anti-inflammatory activities of the secondary metabolites: The anti-denaturing activities of the three plants were weaker than that of the control (Fig. 2a, b, c, d, e), except for *S. davidii*; for this species, the saponin concentration was 0.05 mg/mL (Fig. 2d). Except for *S. davidii* and *I. amblyantha*, which had flavonoid concentrations of 0.025 and 0.05 mg/mL, respectively, the anti-denaturing activities increased with increasing extract concentration (Fig. 2b). The anti-denaturing activities of the tannins and total phenols followed the order *I. amblyantha* > *B. papyrifera* > *S. davidii* (Fig. 2a, e); the total flavones followed the order *B. papyrifera* > *I. amblyantha* > *S. davidii* (Fig. 2b); the alkaloids followed the order *S. davidii* > *B. papyrifera* > *I. amblyantha* (Fig. 2c); and the saponins followed the order *S. davidii* > *I. amblyantha* > *B. papyrifera* (Fig. 2d). Compared with that of the positive control, the anti-hyaluronidase activity of the three plant extracts was weaker, and the extracts strongly inhibited hyaluronidase activity (Fig. 2f, g, h, i, j). The tannin, flavonoid, and alkaloid extracts of the three plant species had anti-hyaluronidase activity in the order *I. amblyantha* > *B. papyrifera* > *S. davidii* (Fig. 2f, g, h); saponins showed activity in the order *S. davidii* > *I. amblyantha* > *B. papyrifera* (Fig. 2i); and hyaluronidase inhibition by total phenols followed the order *S. davidii* > *I. amblyantha* > *B. papyrifera* (Fig. 2j).

Antibacterial activities of the secondary metabolites: The extracts of secondary metabolites inhibited the tested three bacteria with different effects (Table 5). The alkaloids and flavonoids of *S. davidii* exhibited the greatest inhibitory activity, with inhibitory diameters of 14.48 mm and 14.44 mm, respectively. The flavonoid extract of *S. davidii* and the tannin extract of *B. papyrifera* displayed the greatest inhibitory activity against *E. coli*, with inhibitory diameters of 11.29 mm and 11.17 mm, respectively. The best inhibitory activity against *B. subtilis* was achieved with the alkaloid extract of *B. papyrifera* (12.29 mm); tannin (13.15 mm) and flavonoid extracts of *S. davidii* (13.44 mm); and flavonoid extract of *I. amblyantha* (10.32 mm).

Table 1 Relative feed value grading standard

Grade	Premium	Level 1	Level 2	Level 3	Level 4	Level 5
CP/%	>19	17-19	14-16	11-13	8-10	≤8
RFV	>151	125-151	103-124	87-102	75-86	≤75

Table 2 Dynamic changes in the nutrient contents of the three woody forage plants (%)

Index	Species	Harvest time				Average
		Nutritional stage	Flowering stage	Pod-setting stage	Maturation stage	
DM	*S. davidii*	66.21±0.76^{b}	81.43±2.92^{a}	74.58±10.08^{ab}	67.06±4.48^{b}	72.32
	I. amblyantha	73.80±1.16^{b}	82.06±3.95^{a}	72.03±4.13^{b}	68.12±0.99^{b}	74.00
	B. papyrifera	83.91±2.74^{b}	92.40±7.48^{a}	80.66±5.89^{b}	84.31±1.36^{b}	85.32
EE	*S. davidii*	3.52±1.89^{b}	4.07±2.21^{a}	3.32±1.28^{b}	2.72±0.86^{c}	3.41
	I. amblyantha	4.23±2.93^{a}	3.47±1.92^{b}	3.08±0.89^{b}	2.08±1.32^{c}	3.22
	B. papyrifera	9.84±0.04^{b}	12.54±0.43^{ab}	13.56±0.38^{a}	10.88±0.45^{c}	11.71
CF	*S. davidii*	20.82±0.49^{c}	23.57±0.81^{b}	26.41±1.09^{a}	27.02±1.58^{a}	24.46
	I. amblyantha	17.61±0.60^{b}	24.59±0.58^{a}	25.30±0.55^{a}	25.49±1.28^{a}	23.25
	B. papyrifera	7.96±0.98^{b}	10.64±1.02^{ab}	9.70±0.79^{ab}	12.31±1.92^{a}	10.15
NDF	*S. davidii*	30.75±1.25^{b}	34.27±2.41^{ab}	36.74±1.77^{a}	38.96±3.63^{a}	35.18
	I. amblyantha	34.03±5.25^{b}	34.66±1.80^{b}	36.89±4.43^{ab}	42.95±9.66^{a}	37.13
	B. papyrifera	32.40±3.05^{b}	56.02±0.63^{a}	45.79±2.57^{ab}	60.3±13.13^{a}	48.63
ADF	*S. davidii*	18.83±0.74^{b}	25.98±2.38^{a}	26.38±0.70^{a}	27.57±0.99^{a}	24.69
	I. amblyantha	22.08±0.92^{b}	25.75±1.17^{ab}	26.95±0.72^{ab}	30.43±3.25^{a}	26.30
	B. papyrifera	21.78±5.68^{b}	35.43±1.36^{a}	34.00±1.97^{a}	36.63±2.88^{a}	31.96
Ash	*S. davidii*	5.09±0.16^{c}	5.85±0.26^{b}	6.14±0.44^{b}	7.52±0.07a	6.15
	I. amblyantha	11.73±0.28^{a}	11.21±0.11^{ab}	10.96±0.35^{b}	10.96±0.17^{b}	11.22
	B. papyrifera	11.21±0.19^{c}	14.92±0.13^{ab}	14.33±0.46^{b}	15.19±0.13^{a}	13.91
NFE	*S. davidii*	12.34±2.37^{ab}	22.79±1.05^{b}	15.29±8.66^{ab}	6.88±3.31^{b}	14.33
	I. amblyantha	14.47±3.30^{ab}	17.81±2.79^{a}	9.36±4.58^{b}	6.20±1.80^{b}	11.96
	B. papyrifera	27.16±1.71^{a}	34.30±8.69^{a}	19.78±6.08^{b}	27.47±2.37^{a}	27.18
RFV	*S. davidii*	224.65±5.62^{a}	186.42±6.13^{b}	173.96±5.53^{c}	161.06±4.05^{d}	186.52
	I. amblyantha	200.48±2.57^{a}	185.22±8.00^{ab}	173.54±7.03^{ab}	150.90±8.13^{b}	177.54
	B. papyrifera	207.29±7.57^{a}	101.79±1.06^{c}	127.03±3.93^{b}	99.24±6.81^{c}	133.84
CP	*S. davidii*	27.78±1.11^{a}	25.14±0.68^{ab}	23.42±0.57^{b}	22.93±0.56^{c}	24.82
	I. amblyantha	25.75±0.41^{a}	24.97±0.05^{b}	23.66±0.12^{c}	22.72±0.19^{d}	24.28
	B. papyrifera	24.73±0.93^{a}	20.73±0.96^{bc}	22.66±0.76^{b}	18.48±0.62^{c}	21.65

^{abcd}Values with different superscripts in the same row indicate significant differences between the different growth periods (*P≤0.05*). The data are presented as the mean±standard deviation.

Table 3 Dynamic changes in the secondary metabolite contents in the three woody forage plants

Index	Species	Harvest time				Average
		Nutritional stage	Flowering stage	Pod-setting stage	Maturation stage	
Flavones (mg/g)	*S. davidii*	0.36±0.06^{b}	0.44±0.11^{a}	0.55±0.08^{a}	0.7±0.09^{a}	0.5136
	I. amblyantha	1.07±0.23^{b}	2.66±0.23^{a}	2.82±0.04^{a}	2.87±0.04^{a}	2.3558
	B. papyrifera	0.59±0.02^{b}	0.78±0.03^{a}	0.6±0.03^{b}	0.78±0.03^{a}	0.6610
Tannins (nmol/g)	*S. davidii*	7717.77±312.87^{a}	5526.21±392.87^{b}	6084.54±112.21^{b}	5706.00±728.76^{b}	6836.72
	I. amblyantha	5818.73±484.92^{a}	4427.83±203.43^{b}	4673.22±340.98^{b}	4284.03±55.02^{b}	4614.48
	B. papyrifera	6390.66±512.77^{b}	7144.71±579.18^{b}	7640.60±803.81^{a}	7047±182.85^{b}	7065.30
Phenols (mg/g)	*S. davidii*	298.69±31.20^{a}	223.71±25.11^{b}	208.80±11.21^{b}	205.62±11.83^{b}	234.21
	I. amblyantha	214.22±11.66^{a}	190.48±14.85^{ab}	163.22±3.80^{c}	167.45±10.18^{bc}	183.84
	B. papyrifera	181.15±4.65^{b}	190.62±7.04^{b}	214.34±9.95^{a}	143.87±2.11^{c}	182.49
Saponins (ng/g)	*S. davidii*	0.22±0.04^{b}	0.13±0.02^{a}	0.11±0.01^{b}	0.17±0.03^{b}	0.16
	I. amblyantha	0.28±0.06^{a}	0.39±0.02^{b}	0.20±0.02^{b}	0.28±0.04^{ab}	0.29
	B. papyrifera	0.16±0.03^{a}	0.10±0.01^{b}	0.12±0.02^{ab}	0.16±0.03^{a}	0.14
Alkaloids (ng/g)	*S. davidii*	0.25±0.04^{ab}	0.20±0.01^{b}	0.21±0.02^{b}	0.32±0.07^{a}	0.24
	I. amblyantha	0.26±0.01^{b}	0.23±0.06^{b}	0.28±0.02^{ab}	0.38±0.07^{a}	0.29
	B. papyrifera	0.36±0.02^{a}	0.33±0.02^{a}	0.20±0.02^{b}	0.30±0.07^{a}	0.30

^{abcd}Values with different superscripts in the same row of the table indicate significant differences between the different growth periods (*P*≤0.05). The data are presented as the mean±standard deviation.

Table 4 Evaluations of the subordinate function indices of each nutrient and the active compound compositions of the three species at different growth stages

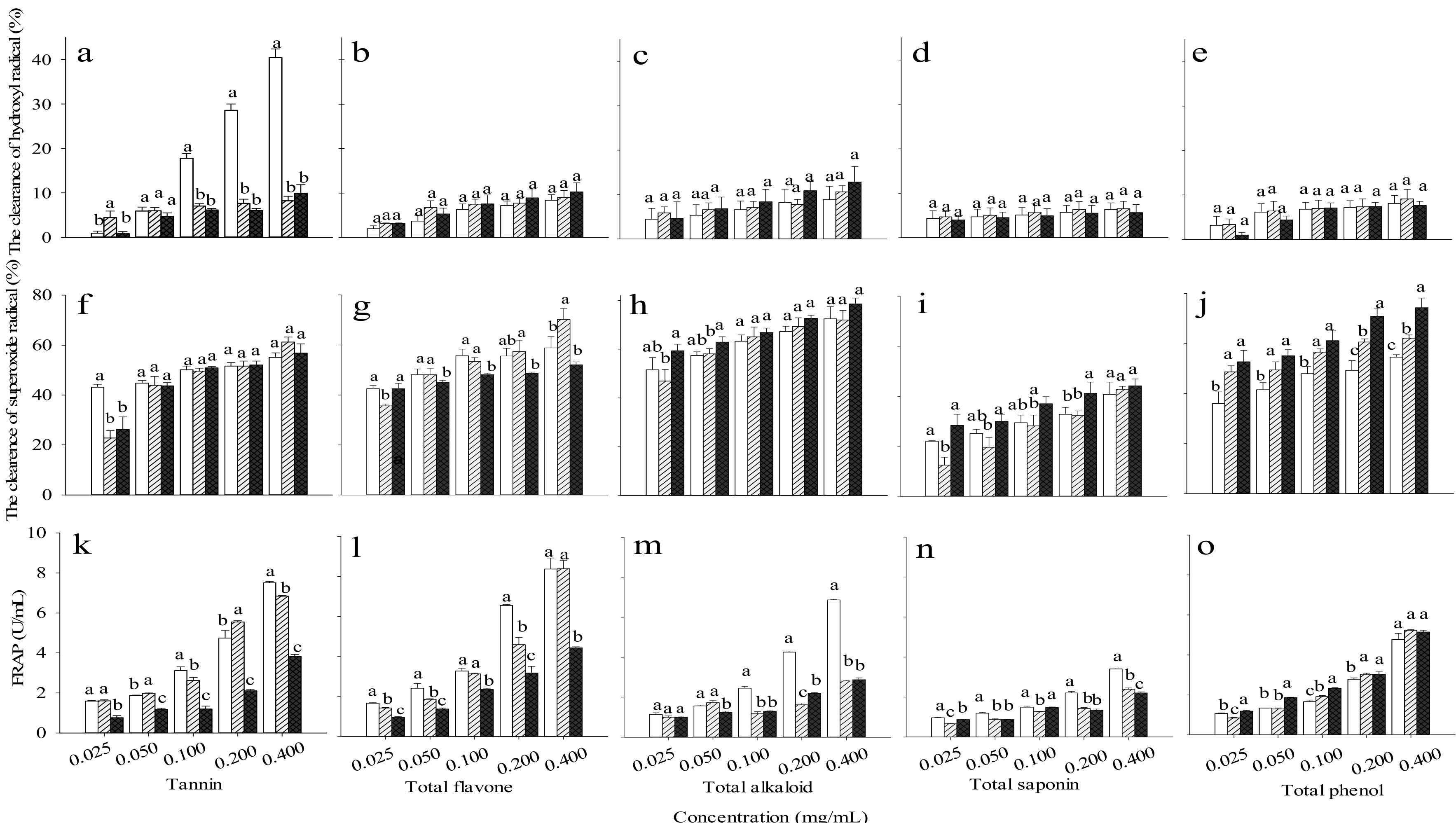
Index	*Sophora davidii*				*Indigofera amblyantha*				*Broussonetia papyrifera*			
	N	F	W	M	N	F	W	M	N	F	W	M
X1	0.00	1.00	0.55	0.06	0.41	1.00	0.28	0.00	0.28	1.00	0.00	0.31
X2	1.00	0.46	0.10	0.00	1.00	0.74	0.31	0.00	1.00	0.36	0.67	0.00
X3	0.59	1.00	0.44	0.00	1.00	0.65	0.47	0.00	0.00	0.73	1.00	0.28
X4	1.00	0.56	0.10	0.00	1.00	0.11	0.02	0.00	1.00	0.38	0.60	0.00
X5	1.00	0.57	0.27	0.00	1.00	0.93	0.68	0.00	1.00	0.15	0.52	0.00
X6	1.00	0.18	0.14	0.00	1.00	0.56	0.42	0.00	1.00	0.08	0.18	0.00
X7	0.00	0.31	0.43	1.00	1.00	0.32	0.00	0.00	0.00	0.93	0.78	1.00
X8	0.34	1.00	0.53	0.00	0.71	1.00	0.27	0.00	0.51	1.00	0.00	1.00
X9	0.00	0.24	0.56	1.00	0.00	0.88	0.97	1.00	0.00	1.00	0.05	1.00
X10	0.00	0.92	0.75	0.92	0.00	0.91	0.75	1.00	1.00	0.40	0.00	0.47
X11	0.00	0.81	0.97	1.00	0.00	0.47	1.00	0.92	0.47	0.34	0.00	0.00
X12	0.00	0.82	1.00	0.52	0.58	0.00	1.00	0.58	0.00	1.00	0.67	0.00
X13	0.58	1.00	0.92	0.00	0.8	1.00	0.67	0.00	0.00	0.19	1.00	0.41
Average	0.42	0.69	0.52	0.34	0.65	0.66	0.53	0.30	0.48	0.58	0.42	0.34
Rank	3	1	2	4	2	1	3	4	2	1	3	4

Notes: X1: DM; X2: CP; X3: EE; X4: CF; X5: NDF; X6: ADF; X7: ash; X8: NFE; X9: flavone content; X10: tannin content; X11: phenol content; X12: saponin content; X13: alkaloid content; N: nutritional stage; F: flowering stage; W: pod-setting stage; and M: maturation stage

Table 5 Antibacterial activities of the tannins, total flavonoids, total alkaloids, total saponins, and total phenols from the three woody forage plants

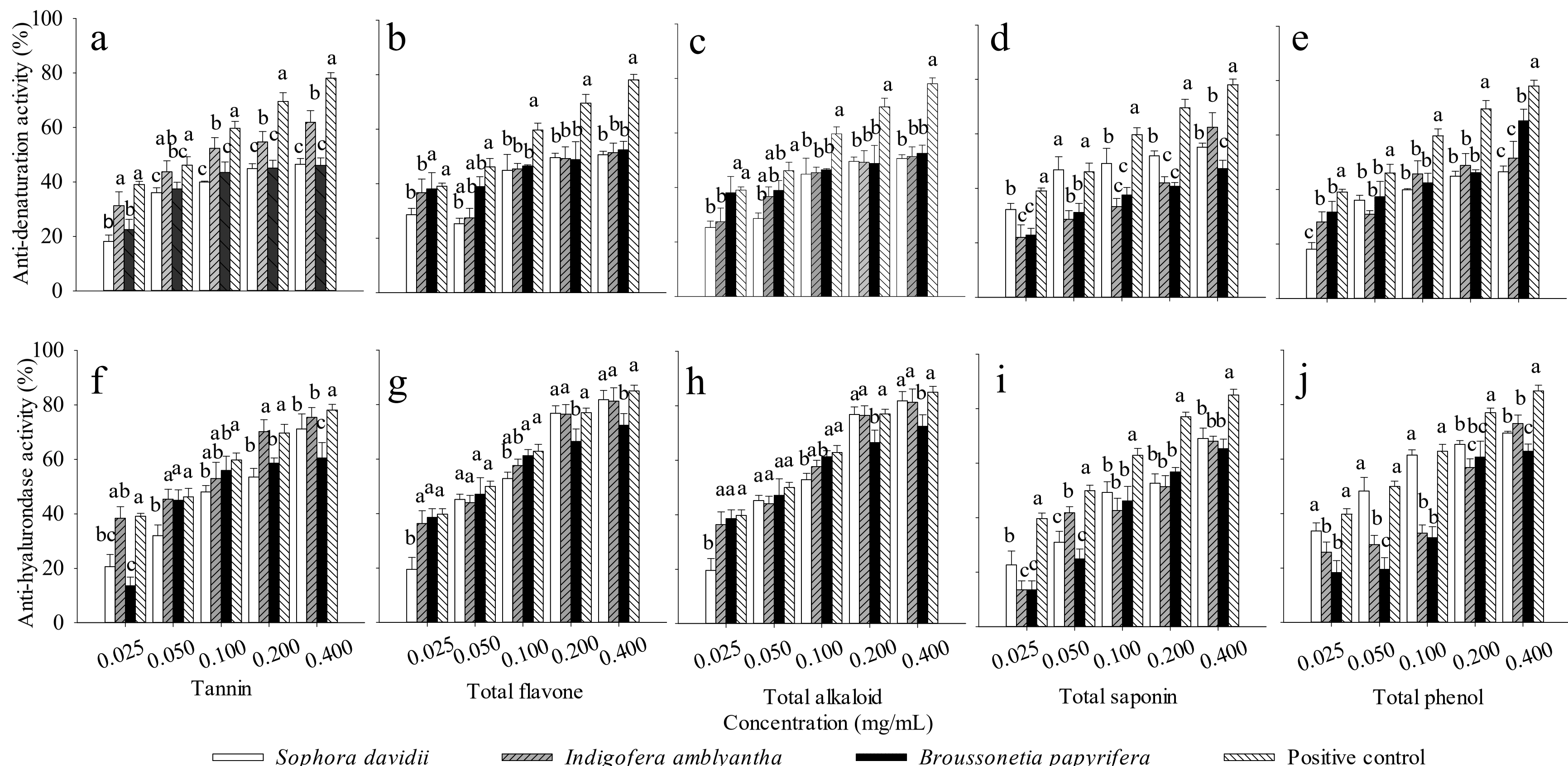
Extract	Species	Bacteriostatic diameter (mm)		
		S. aureus	*E. coli*	*B. subtilis*
Tannin	*S. davidii*	6.51±0.07[a]	8.76±0.05[b]	8.55±0.26[b]
	I. amblyantha	7.83±0.24[a]	7.59±0.10[b]	6.98±0.34[c]
	B. papyrifera	6.69±0.44[a]	11.17±0.78[a]	13.15±0.64[a]
Flavone	*S. davidii*	14.44±0.16[a]	11.29±0.12[a]	13.44±0.16[a]
	I. amblyantha	11.33±0.81[b]	8.56±0.21[b]	10.32±0.16[b]
	B. papyrifera	11.40±0.46[b]	7.67±0.26[b]	8.65±0.15B[c]
Saponin	*S. davidii*	7.68±0.08[b]	8.30±0.08[a]	8.10±0.20[a]
	I. amblyantha	8.94±0.35[a]	7.88±0.55[b]	9.78±0.46[a]
	B. papyrifera	8.69±0.09[a]	6.50±0.11[b]	9.15±0.64[a]
Alkaloid	*S. davidii*	14.48±0.54[a]	6.53±0.10[b]	8.15±0.28[b]
	I. amblyantha	7.97±0.27[c]	7.44±0.07[a]	6.38±0.18[c]
	B. papyrifera	9.49±0.52[b]	8.77±0.16[a]	12.29±0.13[a]
Total phenol	*S. davidii*	9.59±0.24[a]	8.85±0.30[a]	9.46±0.24[a]
	I. amblyantha	7.91±0.39[b]	7.28±0.22[b]	8.38±0.25[a]
	B. papyrifera	8.83±0.38[b]	8.86±0.77[a]	7.59±0.04[b]

[abcd]Values with different superscripts in the same column of the table indicate significant differences between different plants ($P \leq 0.05$). The bacteriostatic zone included the diameter of the filter paper (6 mm). The data are presented as the mean±standard deviation.



Note: Different lowercase letters indicate significant differences between different plants ($P \le 0.05$).

Fig. 1 Antioxidant activities of the tannins (the ability of the tannin extracts to scavenge hydroxy radicals and superoxide radicals and the ferric ion reducing antioxidant power (FRAP) values are shown in a, f, and k, respectively), total flavonoids (the ability of the flavonoid extracts to scavenge hydroxy radicals and superoxide radicals the FRAP values are shown in b, g, and l, respectively), total alkaloids (the ability of the alkaloid extracts to scavenge hydroxy radicals and superoxide radicals and FRAP values are shown in c, h, m, respectively), total saponins (the ability of the saponin extracts to scavenge hydroxy radicals and superoxide radicals and FRAP values are shown in d, i, and n, respectively), and total phenols (the ability of the phenol extracts to scavenge hydroxy radicals and superoxide radicals and FRAP values are shown in e, j, and o, respectively) from the three woody forage plants.



Note: Different lowercase letters indicate significant differences between different plants ($P \leq 0.05$).

Fig. 2 Anti-inflammatory activities of the tannins (the anti-denaturing and anti-hyaluronidase activities of the tannin extracts are shown in a and f, respectively), total flavonoids (the anti-denaturing and anti-hyaluronidase activities of the flavonoid extracts are shown in b and g, respectively), total alkaloids (the anti-denaturing and anti-hyaluronidase activities of the alkaloid extracts are shown in c and h, respectively), total saponins (the anti-denaturing and anti-hyaluronidase activities of the saponin extracts are shown in d and i, respectively), and total phenols (the anti-denaturing and anti-hyaluronidase activities of the phenol extracts are shown in e and j, respectively) from the three woody forage plants.

DISCUSSION

***Nutrient composition*:** In the present study, all three woody forage plants had higher DM contents as growth progressed that indicates better storage potential (Ogungbenle and Omosola, 2015) and more biomass. The total CP content of feed plants is an indicator of their nutritional value for ruminants. In this study, the CP contents of the three woody forage plants reached 20-30%. Studies have shown that CF and nutrient digestibility are inversely related, (Shiawoya *et al*., 2012). In the present study, *B. papyrifera* had the lowest CF content, especially during the nutritional stage. Although fats are a concentrated source of energy, they do not constitute a major source of energy from forages (Pamo *et al*., 2007). NDF is negatively correlated with palatability and feed intake. ADF is negatively correlated with feed digestibility. In the present study, the ADF and NDF contents were the highest in *B. papyrifera*.

Dynamic changes in secondary metabolite contents: For ruminant herbivores, the nutritional quality of forage may be determined by its digestibility (Robbins *et al*., 1987). Phenolic compounds may directly affect dry matter digestibility (Iason and Palo, 1991). Tannins are important antinutritional factors in plant feed and are widely present in multiple plant organs and tissues (Sinha and Amresh *et al*., 2018). Alkaloids are an important class of natural organic compounds that exhibit antiparasitic (Risticevic *et al*., 2020), antiplasmodial (Pereira *et al*., 2017), antiseptic (Gonzales and Tolentino, 2014), antioxidant (Supong *et al*., 2016), antibacterial (Onyema *et al*., 2016) and insecticidal properties. Excess saponins causes adverse effects on animals, resulting in a decrease in growth rate, inhibition of enzyme activity and reduced absorption of nutrients in the digestive tract (Glencross, 2016). The results of this study showed that the secondary metabolite contents in the three plants were different. Therefore, systematically studying the components of these forage plants and the influencing factors is important for their rational utilization.

Secondary metabolite activities

Antioxidant activity: As shown in this study, the tannins, total phenols, total saponins, total alkaloids, and total flavonoids of the three plants had different degrees of hydroxyl free radical scavenging effects. Within the same concentration range, the extracts had less of an inhibitory effect on the hydroxyl radical OH· than vitamin C. As the extract concentration increased, the inhibitory activity increased. Among them, at 0.4 mg/mL, the scavenging effects of the total phenols and alkaloids from *B. papyrifera* and the alkaloids from *S. davidii* reached more than 70%. In addition to the saponins, the other extracts from *I. amblyantha* exhibited a superoxide anion scavenging effect of more than 60% and strong antioxidant capacity. In general, the reducing and antioxidant abilities of the secondary metabolites are correlated. The flavonoids from *I. amblyantha* (8.39%) and *S. davidii* (8.38%) exhibited strong total antioxidant capacities.

Anti-inflammatory activity: The five extracts of the three plants inhibited hyaluronidase, and the effect increased as the concentration increased. Except for the *S. davidii* alkaloids, the other extracts of the three plants inhibited hyaluronidase activity by more than 60%. Studies have confirmed that tannins and flavonoids inhibit hyaluronidase activity, but their mechanism of action remains unclear. Protein denaturation may be used as an effective measure of anti-inflammatory activity (Ainalis *et al*., 2006). The five extracts of the three plants inhibited albumin denaturation in a concentration-dependent manner, and albumin denaturation inhibition was greater than 40% at 0.4 mg/mL. Among them, the saponins and tannins of *I. amblyantha*, the total phenols of *B. papyrifera*, and the alkaloids of *S. davidii* had inhibition rates greater than 60%.

Antibacterial activity: In the present study, the five extracts of the three plants showed potential antibacterial activities against *S. aureus, E. coli* and *B. subtilis*, but the effects were different on each bacterial species. The alkaloids and flavonoids of *S. davidii* exerted greater inhibitory effects on *S. aureus*; the flavonoid extract of *S. davidii* and the tannin extract of *B. papyrifera* exerted greater inhibitory effects on *E. coli*; and the alkaloids and tannins of *B. papyrifera*, the flavonoids of *S. davidii*, and the flavonoids of *I. amblyantha* had good inhibitory effects on *B. subtilis*. Many previous studies have shown that the same active ingredients exhibit very different inhibitory effects on different bacterial species. For example, the anthocyanin extract of *Prunus cerasus* strongly inhibited *S. aureus* but its inhibitory activity against gram-negative bacteria and yeast was weak (Liegiute *et al.*, 2009). This difference may be due to differences in the content of active ingredients or other impurities, or it may be related to differences in the mechanism of action of antibacterial active substances in different extracts against different pathogens (Li *et al*., 2017).

Recent studies have shown that *S. davidii* (Huang *et al*., 2018), *I. amblyantha* (Zhao *et al*., 2023) and *B. papyrifera* (Peng *et al*., 2019) have considerable nutritional value and are potentially excellent forage plants in arid and semiarid areas. The best stage for utilizing these three woody forage plants is the flowering stage, the stage during which these plants exhibit the highest nutritional value. In addition, these three woody plants possess significant antioxidant, anti-inflammatory and antibacterial activities. However, further research is needed to evaluate the antinutritional factors of the woody plants studied here and their palatability and effects on productivity.

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Conflicts of Interest: None of the authors have conflicts of interest to declare.

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