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The effect of purified condensed tannins of forage plants from Botswana on the free-living stages of gastrointestinal nematode parasites of livestock

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ABSTRACT

The effect of condensed tannins (CT) extracted from forage plants from Botswana on the free-living stages of a number of species of gastrointestinal nematode parasites derived from infected sheep were investigated using *in vitro* assays. Fresh samples of five different plants (*Viscum rotundifolium*, *Viscum verrucosum*, *Tapinanthus oleifolius*, *Grewia flava* and *Ipomoea sinensis*) were collected over two summers (February 2009 and 2010). Fractionation of each crude extract on a Sephadex LH-20 column yielded low molecular weight phenolics and CT-containing fractions. The effect of each purified CT fraction on parasites was evaluated using either egg hatch, larval development or larval migration inhibition assays. Three gastrointestinal nematode species (*Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*) derived from infected sheep were evaluated in the study. CT from *V. rotundifolium* and *I. sinensis* fractions from samples collected in 2009 and 2010 did not inhibit larval development. However, CT isolated from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 completely inhibited the development of all parasite species. These CT fractions were more potent in inhibiting larval development of *H. contortus* than fractions from the same plant species collected in 2010. However, a slight effect on larval migration was observed with some CT extracts. The results suggest that CT extracts of some forage plants from Botswana have anti-parasitic properties *in vitro*, and that further research is required to determine any *in vivo* efficacy from feeding the plants to goats in a field situation.

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1. Introduction

Infection with gastrointestinal nematodes (GIN) significantly reduces protein utilisation and productivity in small ruminants, and animals can suffer from diarrhoea, inappetance, anaemia, and loss of body-weight leading to death (Dalton, 2006). One of the widely employed control strategies for GIN is the use of anthelmintic drugs. However, the sustained use of these drugs usually results in the development of resistance (Sutherland and Leathwick, 2011). There are also problems with potential unavailability and/or unaffordability of the drugs in some regions, as well as improper and inappropriate use, particularly in developing countries (Sutherland and Scott, 2009). For these reasons, alternative control strategies, including the use of forage plants with putative anthelmintic activity, may have value in managing parasitism in grazing ruminants.

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Numerous studies have reported either direct or indirect effects of condensed tannins (CT) from plants on GIN (Molan et al., 2002; Mupeyo et al., 2010). Direct effects of CT may involve binding of CT to external and internal proteins of parasites and modifying either their parasitic and/or free living stages. Alternatively, CT may affect parasites indirectly by protecting dietary proteins from degradation during passage through the rumen, enabling more proteins to dissociate in the small intestine, with the resulting increased absorption of amino acids leading to enhancement of the immune system (McNabb et al., 1998). In New Zealand, lambs grazing sulla (*Hedysarum coronarium*) containing CT (3.5 g/100 g DM) had lower worm burdens and faecal egg counts than those grazing lucerne (CT 0.04 g/100 g DM) (Niezen et al., 2002, 1998). Similarly, sheep fed *Lotus pedunculatus* showed reduced worm numbers, egg excretion, and increased liveweight gain compared to sheep fed perennial ryegrass (Niezen et al., 1998). In these studies, the effects of CT from forages on GIN were not shown to be either direct or indirect. However, when extracts are administered to the animals as a drench, anti-parasitic effects have been described as direct. For example, sheep infected with GIN and drenched with quebracho extracts had reduced faecal egg counts (Athanasiadou et al., 2001), although the effects may still have been mediated *via* immunity or by physiological changes in the gut.

The clearest indication of direct effects of CT on GIN has been observed using *in vitro* assays. According to Molan et al. (2002), purified CT extracts from seven herbages had direct anti-parasitic effects on *T. colubriformis*, in a larval development assay. In this case, there was a correlation between the levels of CT present and the proportion of eggs able to develop to infective-stage larvae (L3).

While the effects of CT derived from a number of temperate (Molan et al., 2002) and tropical forages (Madibela and Jansen, 2003) on GIN have been investigated, the *in vitro* effects of CT of forage plants from Botswana are unknown. In Botswana, smallholding farmers, feed a range of parasitic plants, also known as mistletoes, as supplement to cattle, sheep and goats temporarily to meet their nutritional requirements, and to putatively enhance their health and wellbeing (Madibela et al., 2000). In an *in vivo* study, goats fed *Viscum verrucosum* leaves and small stems from Botswana had reduced faecal egg counts which was attributed to ingestion of CT (Madibela and Jansen, 2003). In an *in vitro* study, Tibe et al. (2012) also demonstrated that CT extracted from *Grewia flava* and *T. oleifolius* from Botswana stimulated $\gamma\delta$ T cells derived from the circulating blood of young goats, and may therefore enhance innate immune responses.

The objective of this study, therefore, was to investigate whether CT extracts from Botswanan forage plants adversely affect GIN development and motility *in vitro*.

2. Materials and methods

2.1. Plant collection

Fresh samples of five different plants (*Viscum rotundifolium*, *Viscum verrucosum*, *Tapinanthus oleifolius*, *Grewia flava* and *Ipomoea sinensis*) were collected from the Botswana College of Agriculture (BCA) farm in Gaborone, Botswana over two summers (February 2009 and 2010). The first three plants are mistletoes while the latter two are a shrub and a legume, respectively. The plant samples, consisting of leaves and small stems, were freeze–dried and couriered to New Zealand under Ministry of Agriculture and Forestry (MAF) approval. In addition, voucher specimens of the plants 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; 3 L) containing ascorbic acid (1 g/L) and strained through cheesecloth to remove plant debris. The filtered extract was concentrated *in vacuo* at 40 °C using a rotary evaporator to remove acetone, and the aqueous solution was subsequently defatted with dichloromethane. The aqueous layer was partitioned three times with ethyl acetate (3 × 200 mL). The ethyl acetate solution was concentrated *in vacuo* to yield an ethyl acetate crude extract (represented by E-02), while the aqueous layer yielded a brown aqueous acetone crude extract solution (represented by A-01). Both extracts were then freeze–dried.

2.3. Step fractionation (SF)

CT crude extracts were fractionated according to the method described by Meagher et al. (2004). Briefly, 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 (Pharmacia, Uppsala, Sweden), which was equilibrated with methanol:water (1:1). Four fractions (SF1-SF4; 150 mL each) were obtained after elution with methanol:water (1:1). The first batch elution with acetone:water (7:3) yielded two fractions: SF5-I (150 mL) and SF6-I (350 mL), which were concentrated *in vacuo* and freeze–dried. The second batch (6 g CT extract/30 mL methanol:water 1:1) was fractionated from each plant to yield two CT fractions (SF5-II and SF6-II).

In summary, first and second batch fractionation yielded two purified CT-containing fractions (represented by SF6-I, SF6-II) from *V. verrucosum* (acetone:water; 7:3), *T. oleifolius* (acetone:water; 7:3) and *G. flava* (acetone:water; 7:3), which were sufficient to be used in the assays. Fraction 5 could not be used due to insufficient yield. For *V. rotundifolium*, CT were present in the first fraction (SF1, methanol:water; 1:1).

2.3.1. Phytochemical screening of step 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 fractions were analysed by RP-HPLC with PDA detection at 280 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;

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