



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



O. Tibe<sup>a,\*</sup>, I.A. Sutherland<sup>c</sup>, L. Lesperance<sup>d</sup>, D.R.K. Harding<sup>b</sup>

<sup>a</sup> Botswana College of Agriculture, Private Bag 0027, Gaborone, Botswana

<sup>b</sup> Institute of Fundamental Sciences, Massey University, Turitea Campus, Palmerston North 4442, New Zealand

<sup>c</sup> AgResearch, The Hopkirk Research Institute, Private Bag 11008, Palmerston North 4442, New Zealand

<sup>d</sup> 25 St Hill Lane, Havelock North, Hawkes Bay, New Zealand

## ARTICLE INFO

### Article history:

Received 19 November 2012

Received in revised form 19 June 2013

Accepted 4 July 2013

### Keywords:

Anti-parasitic

Condensed tannins

Gastrointestinal nematodes

## 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.

© 2013 Elsevier B.V. All rights reserved.

## 1. Introduction

Infection with gastrointestinal nematodes (GIN) significantly reduces protein utilisation and productivity in small ruminants, and animals can suffer from diarrhoea, inappetence, 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.

\* Corresponding author. Tel.: +267 3650281; fax: +267 3928753.

E-mail addresses: [Olekile.Tibe@hotmail.com](mailto:Olekile.Tibe@hotmail.com), [otibe@bca.bw](mailto:otibe@bca.bw) (O. Tibe).

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 herbage 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<sub>2</sub>O, B=0.1% formic acid in CH<sub>3</sub>CN, 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  $\mu$ L sub-sample on a reverse phase (C-18) Phenomenex Jupiter RP column (250  $\times$  4.6 mm, particle size: 5  $\mu$ m) at a flow rate of 0.3 mL/min.

#### 2.4. Condensed tannin content

The extractable, protein-bound and fibre-bound CT concentrations were determined by the butanol-HCl colorimetric method described by Jackson et al. (1996). All CT contents were determined using CT extracted from *Lotus pedunculatus* as a reference standard.

#### 2.5. Anti-parasitic assays

##### 2.5.1. Parasites

Lambs were infected with, either, *Haemonchus contortus*, *Trichostrongylus colubriformis* or *Teladorsagia circumcincta* and were housed indoors, fed lucerne and given free access to water.

##### 2.5.2. Experimental design

Initially, high-throughput anti-parasitic screening of all the crude extracts and purified fractions at 500  $\mu$ g/mL from the plants collected in 2009 and 2010 was conducted against *H. contortus* (data not shown). From this screening, the effective CT containing fractions (represented by SF6-I/II) from each plant at 100 and 500  $\mu$ g/mL were confirmed for anti-parasitic effects against *H. contortus*, *T. colubriformis* and *T. circumcincta*. Finally a serial dilution (1:2) of the positive fractions was carried out using *H. contortus*, which had a high yield of eggs sufficient for the assays.

##### 2.5.3. Preparation of eggs

Faecal samples were collected from housed lambs for faecal egg count (FEC) and assays were carried out once positive patent infections were achieved. The faecal matter was mashed into a slurry. The slurry was strained through 250  $\mu$ m and 100  $\mu$ m sieves to remove debris and washings containing the eggs were collected in a 20  $\mu$ m sieve. The eggs were obtained through flotation in saturated sodium chloride solution (Madibela and Jansen, 2003). They were centrifuged at 200 g (Centrifuge Eppendorf 5810R, Washington, USA) for 2 min to concentrate them. The concentrated eggs were diluted in distilled water to ensure that 60  $\mu$ L of the egg solution contained about 50 eggs.

##### 2.5.4. Egg hatch assay (EHA)

Each crude extract or purified LH-20 fraction was incubated with the eggs (60  $\mu$ L containing 50 eggs) of each gastrointestinal nematodes (*H. contortus*, *T. colubriformis* and *T. circumcincta*) at 25 °C for 26 h at 500  $\mu$ g/mL in triplicate. Distilled water was used as the negative control. At the end of the incubation period, a larval scoring method by McGaw et al. (2007) was adopted and modified: 0-hatched larvae and 1-completely unhatched larvae. This scoring method was found suitable for high-throughput screening.

##### 2.5.5. Larval development assay (LDA)

The assay was carried out in 96-well microtitre plate according to the method described by Molan et al. (2002). The nutrient medium was prepared from 1% yeast solution (Y-1000; Sigma) in 90 mL phosphate buffered saline (0.01 M; pH 7.3, P4417 Sigma) and 10 mL of Earle's balanced salt solution (E7510; Sigma), 0.015% *E. coli* (strain W (ATCC) 9637, Sigma) in distilled water and amphotericin B solution (A-9525, Sigma, 5 mg in 1 mL distilled water). The growth medium was prepared by mixing the yeast solution (45 mL), *E. coli* solution (45 mL) and 125  $\mu$ L of the amphotericin B solution (antifungal).

Each CT extract stock solution (1000  $\mu$ g/mL) was prepared by dissolving 1 mg in 1 mL of dimethyl sulphoxide (DMSO). Briefly, each CT fraction was then cultured with the eggs for each GIN (*H. contortus*, *T. colubriformis* and *T. circumcincta*) at 100 and 500  $\mu$ g/mL in triplicate. The ingredients added in 96 well microtitre plates included the following to make final CT concentrations of 100  $\mu$ g/mL and 500  $\mu$ g/mL: agar (2%, 100  $\mu$ L), nutrient media (40  $\mu$ L), CT extracts (2  $\mu$ L for 100  $\mu$ g/mL and 10  $\mu$ L for 500  $\mu$ g/mL), or dimethyl sulphoxide (DMSO) as the negative control, and the eggs of each GIN (60  $\mu$ L containing 50 eggs). The culturing was carried out at 25 °C for seven days. At the end of the incubation period, a larval scoring method by McGaw et al. (2007) was adopted and modified: 0-active larvae and 1-all dead larvae. Lastly, serial dilution (1:2) of the active CT-containing fractions was performed in the concentrations ranging between 500 and 0.488  $\mu$ g/mL using *H. contortus* eggs. Dose-titration was carried out only on *H. contortus* since a high yield of eggs was recovered from the faecal samples. Low yield of eggs of *T. colubriformis* and *T. circumcincta* was obtained and the eggs were not adequate for serial dilution.

##### 2.5.6. Larval migration inhibition (LMI) assay

The assay was carried out according to the method described by Molan et al. (2000). The method involved preparations of CT test solutions and of L3 larvae which were combined and cultured in 48-well microtitre plates (Costar, Cambridge, MA). Each purified LH-20 fraction stock solution (1000  $\mu$ g/mL) was prepared by dissolving 1 mg in 1 mL of phosphate buffered saline (PBS; 0.1 M, 0.05 M NaCl; pH 7.2). The L3 larvae were exsheathed in sodium hypochlorite (0.025% chlorine) and concentrated to 1500 larvae per mL of PBS. Each purified LH-20 fraction or phosphate buffered saline (negative control) was incubated with larvae (L3) of each exsheathed gastrointestinal nematode species (*H. contortus*, *T. colubriformis* and *T. circumcincta*) at 37 °C for 2 h at concentrations of 500, 250, 125, and 62.5  $\mu$ g/mL ( $n=8$ ).

After the incubation, the solutions were transferred to sieves (7 mm ID with 20  $\mu$ m mesh at the bottom) placed in a 48 culture plates and left overnight (18 h) to enable the active larvae to migrate through the sieves. Larval counting was then performed using a microscope and LMI (%) was calculated.

For calculation of data and statistical analysis, the number of larvae which had migrated through the sieves was

counted using 40× magnification and the LMI (%) was determined using the following equation;

$$\% \text{ inhibition} = \frac{(A - B)}{A \times 100},$$

where *A* is the number of L3 larvae migrated through the sieves in the LMI assay in control incubations, and *B* is the number of L3 larvae migrated in incubations containing different concentrations of crude extracts and LH-20 fractions. Differences between treatments were analysed using GLM (general linear model; Minitab, version 15).

### 3. Results

#### 3.1. Phytochemical screening of the LH-20 fractions

Purified LH-20 fractions from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis* were screened for the presence of CT using RP-HPLC, which was coupled to a PDA detector. The methanol:water (1:1) fractions isolated from *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis* showed sharp peaks with retention times between 10 and 45 min. In contrast, the acetone:water (7:3) fractions from the same plants with broad, unresolved absorbance (hump) with retention times between 25 and 65 min (Fig. 1). However, a small hump was obtained for the methanol fractions from *V. rotundifolium*, suggesting the presence of CT with the Sephadex LH-20 gel operating in a size exclusion manner. The sharp peaks were indicative of the presence of the low molecular weight phenolics, while the broad peaks were indicative of the presence of polymeric CT. Broad humps were found in the acetone:water fractions from *V. verrucosum*, *T. oleifolius* and *G. flava*.

#### 3.2. Condensed tannin concentration

CT content results from five forage plants are shown in Table 1. The total CT concentrations ranged from 0.2 to 12.7 (g/100 g DM). *V. rotundifolium* and *I. sinensis* plants collected in 2009 and 2010 contained trace amounts of CT (<0.5 g/100 g DM). *V. verrucosum*, consisting of leaves and small stems, collected in 2009 and 2010 contained 6.0% and 5.3% CT, respectively. *T. oleifolius* leaves and small stems collected in 2009 contained 5.1% CT while those collected in 2010 contained 11.8% CT. *G. flava* leaves collected in 2009 had the highest CT concentration of 12.7% while *G. flava* collected in 2010 had 9.7%. The butanol-HCl results indicated that significant amount of CT was found in *V. verrucosum*, *T. oleifolius* and *G. flava*, and these results were supported by the qualitative results from RP-HPLC-PDA chromatograms.

#### 3.3. In vitro anti-parasitic assays

##### 3.3.1. Larval development assay using crude extracts

The results of the larval development assay of the ten crude extracts from plants collected in 2009 and 2010 are shown in Tables 2 and 3, although there was lack of activity in most extracts. The acetone:water (7:3) crude extracts (A-01) isolated from *G. flava* leaves were effective in inhibiting larval development of *T. circumcincta* at 100 µg/mL. However, anthelmintic effects were not exhibited at 500 µg/mL.

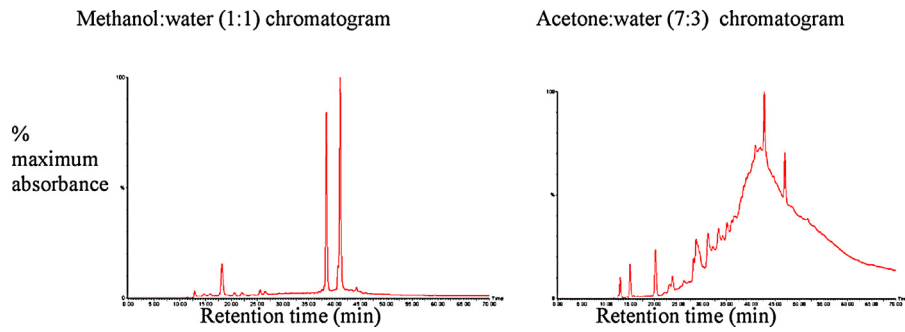
The crude extracts from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius* and *I. sinensis* did not exhibit anti-parasitic activity at either of these concentrations. None of the crude extracts and ethyl acetate extracts from plants collected in 2010 inhibited larval development. The ethyl acetate extract (E-02) isolated from *V. rotundifolium* plant showed anti-parasitic activity at 100 µg/mL against *H. contortus*. Overall, only the crude extract from *G. flava* (A-01) showed exhibit activity against one the tested species of gastrointestinal nematodes. There was no inhibition of larval development in the negative control.

Overall, the larval development assay results of the crude extracts isolated from 2009 and 2010 plants were similar because there was a lack of activity. Furthermore, the ethyl acetate extracts did not exhibit any inhibition of larval development despite activity observed in E-02 fraction from *V. rotundifolium*.

##### 3.3.2. Larval development assay using step LH-20 CT containing fractions

Fractions from *V. rotundifolium* and *I. sinensis* did not inhibit larval development at 100 and 500 µg/mL. Thus, these data were not shown due to lack of activity. The *V. verrucosum* fractions (SF6-I) derived from plants collected in 2009 completely inhibited (100% inhibition) larval development of *H. contortus*, *T. colubriformis* and *T. circumcincta* strains at both concentrations. However, *V. verrucosum* extracts of plants collected in 2010 (SF6-I and SF6-II) did not inhibit larval development. *T. oleifolius* fractions (SF6-I and SF6-II) from plants collected in 2009 completely inhibited development of all the tested GIN species at both concentrations. However, fractions from plants collected in 2010 did not inhibit larval development of all the tested GIN species. Conversely, inhibition of larval development was found in *G. flava* with high CT content. The fractions of *G. flava* leaves (SF6-I and SF6-II) collected in 2009 were more potent and completely inhibited development of *H. contortus*, *T. colubriformis* and *T. circumcincta* strains to the L3 stage. However, minimal to no inhibition of larval development was attained in the fractions from plants collected in 2010. Overall, no pattern was observed in inhibition of larval development with increasing CT content in the forage plants. However, forages containing traces of CT did not inhibit larval development. The results of inhibition of larval development of GIN species by the purified LH-20 fractions from plants collected in 2009 and 2010 are shown in Table 4. None of the tested fractions inhibited egg hatch *in vitro*.

A dose-titration experiment to determine the lowest toxic concentration against *H. contortus* was also investigated by a larval development assay. The results of this experiment are shown in Table 5. The lowest inhibitory concentration for larval development in the fractions from *G. flava* leaves collected in 2009 (SF6-I and SF6-II) was 62.5 µg/mL. In contrast, the lowest inhibitory concentration of the fractions from *G. flava* (2010) was 250 µg/L. This suggests that CT from *G. flava* (2010) were less potent than those from *G. flava* collected in 2009. The lowest inhibitory concentration of CT from *T. oleifolius* and *V. verrucosum* was 125 µg/mL.



**Fig. 1.** HPLC-PDA chromatograms of LH-20 methanol:water (1:1) fractions with sharp peaks indicating the presence of low molecular weight phenolics and aqueous acetone:water (7:3) fractions with a broad peaks indicating the presence of condensed tannins in *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis*.

**Table 1**

Condensed tannin concentration (g/100 g DM) in the leaves and stems of the forage plants by the butanol-HCl colorimetric assay.

Plant name	Year	Free	Protein-bound	Fibre-bound	Total
<i>V. rotundifolium</i>	2009	0.1	0.4	<0.1	0.5
<i>V. rotundifolium</i>	2010	0.1	0.3	<0.1	0.4
<i>V. verrucosum</i>	2009	3.9	2.0	0.1	6.0
<i>V. verrucosum</i>	2010	3.2	2.0	0.2	5.3
<i>T. oleifolius</i>	2009	2.0	2.9	0.2	5.1
<i>T. oleifolius</i>	2010	9.2	2.4	0.3	11.8
<i>G. flava</i>	2009	6.9	5.0	0.7	12.7
<i>G. flava</i>	2010	5.0	4.1	0.5	9.7
<i>I. sinensis</i>	2009	<0.1	0.1	0.1	0.2
<i>I. sinensis</i>	2010	<0.1	0.1	0.1	0.2

**Table 2**

Effects of ten aqueous acetone crude extracts against three species of gastrointestinal nematodes using a larval development assay *in vitro* at 100 and 500 µg/mL relative to the negative control in triplicate.

Sample name	Date of collection	Aqueous crude extract	<i>H. contortus</i>		<i>T. circumcincta</i>		<i>T. colubriformis</i>	
			100	500	100	500	100	500
<i>Viscum rotundifolium</i>	27/02/2009	A-01	0	0	0	0	0	0
	10/02/2010	A-01	nd	0	0	0	0	0
<i>Viscum verrucosum</i>	27/02/2009	A-01	0	0	0	0	0	0
	12/02/2010	A-01	nd	0	0	0	0	0
<i>Tapinanthus oleifolius</i>	27/02/2009	A-01	0	0	0	0	0	0
	2/02/2010	A-01	nd	0	0	0	0	0
<i>Grewia flava</i>	27/02/2009	A-01	0	0	1	0	0	0
	02/02/2010	A-01	nd	0	0	0	0	0
<i>Ipomoea sinensis</i>	03/03/2009	A-01	0	0	0	0	0	0
	02/02/2010	A-01	0	0	0	0	0	0

Abbreviations: larval scoring: 0 – not dead; 1 – 100% inhibition or completely dead; nd – not determined; A – aqueous.

**Table 3**

Effects of ten ethyl acetate crude extracts against three different species of gastrointestinal nematodes using a larval development assay *in vitro* at 100 µg/mL and 500 µg/mL in triplicate.

Sample name	Date of collection	Ethyl acetate extract	<i>H. contortus</i>		<i>T. circumcincta</i>		<i>T. colubriformis</i>	
			100	500	100	500	100	500
<i>Viscum rotundifolium</i>	27/02/2009	E-02	1	0	0	0	0	0
	10/02/2010	E-02	nd	0	0	0	0	0
<i>Viscum verrucosum</i>	27/02/2009	E-02	0	0	0	0	0	0
	12/02/2010	E-02	nd	0	0	0	0	0
<i>Tapinanthus oleifolius</i>	27/02/2009	E-02	0	0	0	0	0	0
	02/02/2010	E-02	nd	0	0	0	0	0
<i>Grewia flava</i>	27/02/2009	E-02	0	0	1	0	0	0
	02/02/2010	E-02	nd	0	0	0	0	0
<i>Ipomoea sinensis</i>	03/03/2009	E-02	0	0	0	0	0	0
	02/02/2010	E-02	0	0	0	0	0	0

Abbreviations: larval scoring: 0 – not dead; 1 – 100% inhibition or completely dead; nd – not determined; E – ethyl acetate.

**Table 4**

Effects of CT extracts from *V. verrucosum*, *T. oleifolius* and *G. flava* (70% acetone from first and second batch) on three different species of GIN using an egg hatch assay (EHA) and larval development assay (LDA) *in vitro* at 100 and 500 µg/mL in triplicate.

Plant sample	Date of collection	Fraction code	<i>H. contortus</i>			<i>T. circumcincta</i>			<i>T. colubriformis</i>		
			EH		LD	EH		LD	EH		LD
			500	100	500	500	100	500	500	100	500
Negative control			0	0	0	0	0	0	0	0	0
<i>V. verrucosum</i>	27/02/2009	SF6-I	0	1	1	0	1	1	0	1	1
		SF6-II	0	0	0	0	0	0	0	0	0
	2/02/2010	SF6-I	0	0	0	0	0	0	0	0	0
		SF6-II	0	0	0	0	0	0	0	0	0
<i>T. oleifolius</i>	27/02/2009	SF6-I	0	1	1	0	1	1	0	1	1
		SF6-II	0	0	1	0	1	1	0	0	1
	2/02/2010	SF6-I	0	0	0	0	0	0	0	0	0
		SF6-II	0	0	0	0	0	0	0	0	0
<i>G. flava</i>	27/02/2009	SF6-I	0	1	1	0	1	1	0	1	1
		SF6-II	0	1	1	0	1	1	0	1	1
	2/02/2010	SF6-I	0	0	1	0	0	0	0	0	1
		SF6-II	0	0	0	0	0	0	0	0	0

Abbreviations: larval scoring: 0 – not dead; 1 – 100% inhibition or completely dead; SF6-I – step fraction 6–first batch; SF6-II – step fraction 6–batch 2; nd – not determined; EHA – egg hatch assay; LDA – larval development assay; CT – condensed tannins.

### 3.4. Larval migration inhibition assay *in vitro*

The primary screening for the inhibition of larval migration of the LH-20 fractions from plants collected in 2009 was performed at 500 µg/mL (data not shown). A limited effect on larval migration was observed with some CT extracts. The results for the effect of CT on the inhibition of larval migration of *H. contortus* are shown in Fig. 2.

At 500 µg/mL, CT purified from *G. flava* (SF6-I) and *T. oleifolius* (SF6-I, 2009) markedly inhibited 17.2% and 14.6% ( $p < 0.05$ ) of the larvae (L3), respectively from migrating through the sieves compared to the negative control (PBS). The CT from *V. rotundifolium* and *V. verrucosum* did not inhibit the migration of the larvae at any tested concentration ( $p > 0.05$ ). Similar larval migration inhibition results were obtained with CT extracted from plants collected in 2010.

In summary, CT isolated from *G. flava* and *T. oleifolius* were more inhibitory to larval migration than those from *V. rotundifolium* and *V. verrucosum*.

## 4. Discussion

The results obtained in this study demonstrate that CT extracts from *V. verrucosum*, *T. oleifolius* and *G. flava*, forage

plants from Botswana, can completely inhibit the *in vitro* larval development of GIN species.

Previous studies have also demonstrated the *in vitro* anti-parasitic effect of CT on GIN development (Molan et al., 2002). For example, CT extracted from *Hedysarum coronarium*, *Lotus pedunculatus*, *Lotus corniculatus*, *Onobrychis viciifolia*, *Dorycnium rectum* and *Dorycnium pentaphyllum* each showed anti-parasitic effects against *T. colubriformis* (Molan et al., 2002, 1999). In addition, CT from *Lotus* spp. and from sainfoin (*Onobrychis viciifolia*) also had negative effects on cattle nematodes (Novobilsk et al., 2011). While few studies have been able to demonstrate corresponding *in vivo* anti-parasitic effects, it is interesting to note that goats fed *V. verrucosum*, one of the plants used in the current study, had lower faecal egg counts compared to relevant control animals (Madibela and Jansen, 2003).

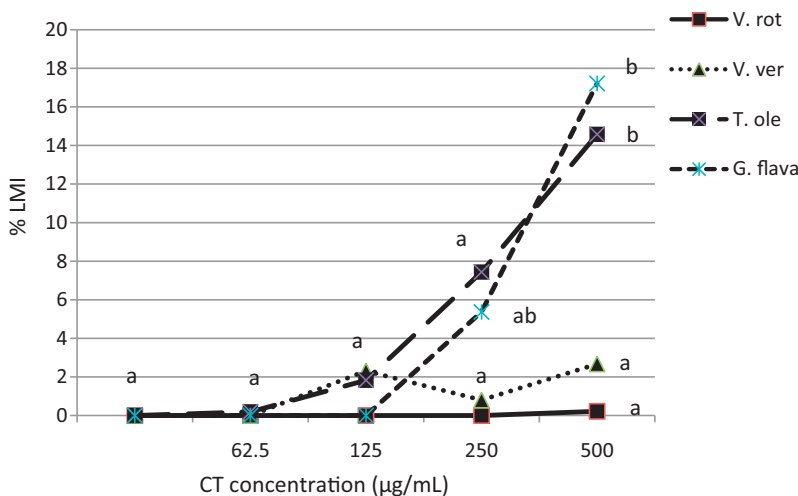
The mechanisms responsible for the inhibition of larval development *in vitro* are not well understood. However, the high affinity of CT for proteins suggests that they may either bind directly to external proteins of the larvae, or they may interact with the internal proteins of the larvae following feeding. It is also possible that CT may bind to nutrients in the growth medium, thus impeding their availability and inducing larval mortality. In addition, CT may bind to and prevent bacterial growth, hence starving the larvae.

**Table 5**

Lowest inhibitory concentration of the positive acetone:water (7:3) CT extracts from *V. verrucosum*, *T. oleifolius* and *G. flava* against *H. contortus* using serial dilution ranging from 500 to 0.488 µg/mL (1:2) in larval development assay.

Plant name	Year of collection	Sample code	Extract serial dilutions (concentration in µg/mL)				
			500	250	125	62.5	31.25–0.488
<i>V. verrucosum</i>	2009	SF6-I	1	1	1	0	0
		SF6-II	0	0	0	0	0
<i>T. oleifolius</i>	2009	SF6-I	1	1	1	0	0
		SF6-II	1	1	1	0	0
<i>G. flava</i>	2009	SF6-I	1	1	1	1	0
		SF6-II	1	1	1	1	0
<i>G. flava</i>	2010	SF6-I	1	1	0	0	0
		SF6-II	1	1	0	0	0

Abbreviations: larval scoring: 0 – not dead; 1 – 100% inhibition or completely dead; SF6-I – step fraction 6–first batch; SF6-II – step fraction 6–batch 2.



**Fig. 2.** Larval migration inhibition of *H. contortus* by LH-20 fractions (SF6-I) from Botswanan forage plants (*V. verrucosum*, *V. rotundifolium*, *T. oleifolius* and *G. flava*) in 2009 at 62.5–500 µg/mL. Each point represents the mean of four experiments in duplicates ( $n = 8$ ). Standard error of the mean = 2.06. Means with the same letter are not significantly different.

Putative external interactions involve the induction of toxicity from CT on the surface of the larvae (Geary et al., 1993). Our results have indicated that CT were more potent in inhibiting larval development than egg hatching and larval migration. These differences in activity could have been attributed to differences in the concentration and structure of CT. A similar pattern was obtained with CT from forages grown under temperate conditions (Molan et al., 2002), in which CT extracted from *H. coronarium*, *L. pedunculatus*, *L. corniculatus* and *O. vicifolia*, expressed on an average effect, decreased larval development by 91%, egg hatch by 34% and inhibited larval migration by 30%. This suggested that these CT may be ineffective in penetrating the cuticle of the eggs, larvae and/or adult worms. Another study by Marie-Magdeleine et al. (2010), suggested that transcuticular diffusion was a common route for the uptake of anthelmintic drugs. However, the drugs in question were lipophilic, suggesting they may more readily penetrate the external surface of helminths compared with hydrophilic CT. While there was a less marked effect of CT on larval migration in this and previous reports, what impact there was of CT may have been due to interference with the neurophysiology and neuromusculature of the larvae, resulting in paralysis, immobility and mortality (Molan et al., 2003, 2004).

CT from *G. flava* were more effective in inhibiting larval development than those from the other forages tested. It is noted that the lowest active concentration of 62.5 µg/mL was significantly less than previous reports of anti-parasitic activity, in which concentrations in excess of 200 µg/mL were required (Molan et al., 2002). The relatively high activity of *G. flava* CT suggests an investigation of potential *in vivo* efficacy should be undertaken.

In addition to the relatively high efficacy of *G. flava* CT in the current study, there were further observable differences in anti-parasitic activity between CT from different plant species. CT extracts from *G. flava* and *T. oleifolius* induced significant inhibitory effects while those from *V. rotundifolium* and *V. verrucosum* did not. In addition, CT

from plants collected in 2009 were more potent in inhibiting larval development than those from plants collected in 2010. This variability could be due to differences in the chemical structure and the concentration of CT in different plant species (Meagher et al., 2004; Min et al., 2003). Furthermore, soil type, climate, cultivar, season (Waghorn et al., 2006) and other environmental differences may affect the bio-synthetic pathway of CT, which may impact their chemical structure and concentration (Athanasiadou et al., 2007). The chemical structure of CT plays an important role in determining overall biological activity (Hoste et al., 2006). Structure-activity relationships can be deduced once the building blocks of CT polymers have been investigated. The chemical structures of CT have demonstrated that the compounds from *V. verrucosum*, *T. oleifolius* and *G. flava* were predominantly procyanidin (PC)-type (Tibe et al., 2013), with epicatechin as the prevalent component.

Differences in the extent of the inhibitory effects of CT were also observed between the different species of parasite tested, with activity of each extract exhibiting activity against the abomasal parasite *H. contortus*. These findings are in agreement with those of Ademola et al. (2007), who found that *H. contortus* was very sensitive to plant extracts, although this study was conducted in infected goats. Other studies, which investigated the activity of plant extracts against *T. colubriformis* and *T. circumcincta* using a larval migration inhibition assay, demonstrated that the effects of CT from *Quercus robur*, *Rubus fruticosus* and *Corylus avellana* varied between the nematode species (Paolini et al., 2004). However, in an *in vitro* study, there was no difference in the response of *T. circumcincta*, *H. contortus* or *Trichostrongylus vitrinus* to a quebracho CT extract. This was despite observed differences in susceptibility to quebracho CT between the species when tested *in vivo* (Athanasiadou et al., 2001).

The effect of CT from *T. oleifolius*, *V. verrucosum* and *G. flava* plants against different species of GIN from goat

has not been investigated. However, as goats and sheep have broadly similar species of parasites, it is expected that similar results would be obtained as using parasites from infected sheep.

The potential impact of CT extracts from *T. oleifolius* and *G. flava* on parasite burdens or egg production has not been investigated *in vivo*. However, given the *in vitro* data presented in this study, in which CT from these two species had marked activity against a range of GIN species, such an experiment will be of value.

## 5. Conclusions

The results of this study demonstrate that extracts of forage plants from Botswana collected in 2009 have anti-parasitic effects *in vitro*, which may translate into a role for these plants in reducing the effects of parasitism in young ruminants.

## Acknowledgements

The authors would like to thank the Parasitology Team, the Hopkirk Research Institute, AgResearch for their valuable contributions and assistance in assisting O. Tibe to perform the assays. Mrs. Mary Gaddam is thanked for her contributions on the egg hatch and larval development assays, while Mrs. Sheralée Cleland is appreciated for provision of sheep faecal samples. Dr. Tania Waghorn and Dr. Ross Bland are also thanked for critiquing the manuscript. Finally, O. Tibe would like to thank Botswana of Agriculture (BCA) for providing funding for the study.

## References

- Ademola, I., Fagbemi, B., Idowu, S., 2007. Anthelmintic activity of *Spigelia anthelmia* extract against gastrointestinal nematodes of sheep. *Parasitol. Res.* 101, 63–69.
- Athanasiadou, S., Githiori, J., Kyriazakis, I., 2007. Medicinal plants for helminth parasite control: facts and fiction. *Animal* 1, 1392–1400.
- Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R., 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: *in vitro* and *in vivo* studies. *Vet. Parasitol.* 99, 205–219.
- Dalton, C., 2006. *Internal Parasites of Sheep and their Control- Now and in the Future: Background Information for Farmers*, second edition. Reward Publishing, Hamilton, New Zealand.
- Geary, T., Sims, S., Thomas, E., Vanover, L., Davis, J., Winterrowd, C., Klein, R., Ho, N., Thompson, D., 1993. *Haemonchus contortus*: ivermectin-induced paralysis of the pharynx. *Exp. Parasitol.* 77, 88–96.
- Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S., Hoskin, S., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends Parasitol.* 22, 253–261.
- Jackson, F.S., Barry, T.N., Lascano, C., Palmer, B., 1996. The extractable and bound condensed tannin content of leaves from tropical trees, shrubs and forage legumes. *J. Sci. Food Agric.* 71, 103–116.
- Madibela, O.R., Boitumelo, W.S., Letso, M., 2000. Chemical composition and *in vitro* dry matter digestibility of four parasitic plants (*Tapinanthus lugardii*, *Erianthum ngamicum*, *Viscum rotundifolium* and *Viscum verrucosum*) in Botswana. *Anim. Feed Sci. Technol.* 84, 97–106.
- Madibela, O.R., Jansen, K., 2003. The use of indigenous parasitic plant (*Viscum verrucosum*) in reducing faecal egg counts in female Tswana goats. *Livestock Res. Rural Dev.* 15 (9) <http://www.lrrd.org/lrrd15/9/madi159.htm> (accessed 26.08.11)
- Marie-Magdeleine, C., Udino, L., Philibert, L., Bocage, B., Archimede, H., 2010. *In vitro* effects of Cassava (*Manihot esculenta*) leaf extracts on four development stages of *Haemonchus contortus*. *Vet. Parasitol.* 173, 85–92.
- McGaw, L., Jäger, A., Van der Merwe, J., Eloff, J.N., 2007. *In vivo* anthelmintic, antibacterial and cytotoxic extracts from plants used in South African ethnoveterinary medicine. *Vet. J.* 173, 366–372.
- McNabb, W.C., Peters, J.S., Foo, L.Y., Waghorn, G.C., Jackson, F.S., 1998. Effect of condensed tannins prepared from several forages on the *in vitro* precipitation of ribulose-1,5-bisphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC 2.4.21.4) and chymotrypsin (EC 2.4.21.1). *J. Sci. Food Agric.* 77, 201–212.
- Meagher, L.P., Lane, G., Sivakumaran, S., Tavendale, M.H., Fraser, K., 2004. Characterization of condensed tannins from *Lotus* species by thiolitic degradation and electrospray mass spectrometry. *Anim. Feed Sci. Technol.* 117, 151–163.
- Min, B.R., Barry, T.N., Attwood, G.T., McNabb, W.C., 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Anim. Feed Sci. Technol.* 106, 3–19.
- Molan, A., Waghorn, G., McNabb, W., 2002. Effect of condensed tannins on egg hatching and larval development of *Trichostrongylus colubriformis* *in vitro*. *Vet. Rec.* 150, 65–69.
- Molan, A., Waghorn, G., Min, B., McNabb, W., 2000. The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration *in vitro*. *Folia Parasitol. (Praha)* 47, 39–44.
- Molan, A.L., Meagher, L.P., Spencer, P.A., Sivakumaran, S., 2003. Effect of flavan-3-ols on *in vitro* egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis*. *Int. J. Parasitol.* 33, 1691–1698.
- Molan, A.L., Sivakumaran, S., Spencer, P.A., Meagher, L.P., 2004. Green tea flavan-3-ols and oligomeric proanthocyanidins inhibit the motility of infective larvae of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* *in vitro*. *Res. Vet. Sci.* 77, 239–243.
- Molan, A.L., Waghorn, G.C., McNabb, W.C., 1999. Condensed tannins and gastro-intestinal parasites in sheep. *Proc. N. Z. Grassland Assoc.* 61, 57–61.
- Muñoz, B., Barry, T., Pomroy, W., Ramírez-Restrepo, C., López-Villalobos, N., Perntner, A., 2010. Effects of feeding willow (*Salix* spp.) upon death of established parasites and parasite fecundity. *Anim. Feed Sci. Technol.* 164, 8–20.
- Niezen, J., Charleston, W., Robertson, H., Shelton, D., Waghorn, G., Green, R., 2002. The effect of feeding sulla (*Hedysarum coronarium*) or lucerne (*Medicago sativa*) on lamb parasite burdens and development of immunity to gastrointestinal nematodes. *Vet. Parasitol.* 105, 229–245.
- Niezen, J., Robertson, H., Waghorn, G., Charleston, W., 1998. Production, faecal egg counts and worm burdens of ewe lambs which grazed six contrasting forages. *Vet. Parasitol.* 80, 15–27.
- Novobilsk, A., Mueller-Harvey, I., Thamsborg, S.M., 2011. Condensed tannins act against cattle nematodes. *Vet. Parasitol.* 182, 213–220.
- Paolini, V., Fouraste, I., Hoste, H., 2004. *In vitro* effects of three woody plant and sainfoin extracts on 3rd-stage larvae and adult worms of three gastrointestinal nematodes. *Parasitology* 129, 69–77.
- Sutherland, I., Scott, I., 2009. *Gastrointestinal Nematodes of Sheep and Cattle*. Wiley- Blackwell, West Sussex, UK.
- Sutherland, I.A., Leathwick, D.M., 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? *Trends Parasitol.* 27, 176–181.
- Tibe, O., Perntner, A., Sutherland, I., Lesperance, L., Harding, D., 2012. Condensed tannins from Botswanan forage plants are effective priming agents of  $\gamma\delta$  T cells in ruminants. *Vet. Immunol. Immunopathol.* 146, 237–244.
- Tibe, O., Sutherland, I., Lesperance, L., Harding, D., 2013. Isolation and characterisation of phenolics and condensed tannins from a selected range of Botswanan forage plants. *Phytochemistry* unpublished.
- Waghorn, T., Molan, A., Deighton, M., Alexander, R., Leathwick, D., McNabb, W., Meagher, L., 2006. *In vivo* anthelmintic activity of *Dorycnium rectum* and grape seed extract against *Ostertagia (Teladorsagia) circumcincta* and *Trichostrongylus colubriformis* in sheep. *N. Z. Vet. J.* 54, 21–27.