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Anthelmintic Activities against *Haemonchus contortus* or *Trichostrongylus colubriformis* from Small Ruminants are Influenced by Structural Features of Condensed Tannins

*Condensed Tannin Structures and Anthelmintic Activities*

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ABSTRACT

Plants containing condensed tannins (CTs) may hold promise as alternatives to synthetic anthelmintic (AH) drugs for controlling gastrointestinal nematodes (GINs). However, the structural features that contribute to the AH activities of CTs remain elusive. This study probed the relationships between CT structures and their AH activities. Eighteen plant resources were selected based on their diverse CT structures. From each plant resource, two CT fractions were isolated and their in vitro AH activities were measured with the Larval Exsheathment Inhibition Assay, which was applied to Haemonchus contortus and Trichostrongyulus colubriformis. Calculation of mean EC\textsubscript{50} values indicated that H. contortus was more susceptible than T. colubriformis to the different fractions and that the F1 fractions were less efficient than the F2 ones, as indicated by the respective mean values for H.contortus F1 = 136.9 ± 74.1 µg/ml; and for H.contortus F2 = 108.1 ± 53.2 µg/ml and for T.colubriformis F1 = 233 ± 54.3 µg/ml and F2=166 ± 39.9 µg/ml. The results showed that the AH activity against H. contortus was associated with the monomeric subunits that give rise to prodelphinidins (\( P < 0.05 \)) and with CT polymer size (\( P < 0.10 \)). However, for T. colubriformis AH activity was correlated only with prodelphinidins (\( P < 0.05 \)). These results suggest that CTs have different modes of action against different parasite species.

KEY WORDS

Proanthocyanidins; larval exsheathment inhibition assay (LEIA); nematodes; ruminants; structure-activity relationships
INTRODUCTION

Gastrointestinal nematodes (GINs) represent a major threat for the breeding and production of grazing ruminants. Up to now, their control has been based mainly on the repeated use of synthetic AH drugs. However, worm populations in small ruminants have consistently developed resistance against all AH drugs. Therefore, the search for alternative solutions to such drug treatments is now a necessity for a more sustainable control of these parasites.

The last two decades have provided evidence that some plants possess natural AH bioactivity, which is based on the presence of condensed tannins (CTs) and flavonoids. Such plants, therefore, represent a promising alternative to chemotherapy especially when used as nutraceuticals that combine beneficial effects on health and nutrition in small and large ruminants.

The involvement of CTs in the observed anthelmintic (AH) effects against parasitic nematodes has been suggested from several results acquired in vitro using either plant extracts or purified CT fractions and from in vivo studies with tannin-containing resources.

Differences in AH effects have repeatedly been noticed between abomasal versus intestinal nematode species of both small ruminant and cattle parasite. These observations have been made in vitro and in vivo studies with the same CT-resources.

Some authors have suggested that different structural features of CTs are involved in their AH effects, namely: i) CT size; ii) the type of flavan-3-ol subunits that give rise to either prodelphinidin (PD) or procyanidin tannins (PC) or iii) the stereochemistry of the C-ring in these subunits (i.e. trans vs. cis flavan-3-ols). Taken together, these observations led us to hypothesize that there are quantitative and qualitative differences between CTs, which determine their activity against parasitic nematodes. There is thus a need to evaluate the structure-activity relationship between tannins and GINs. A better
understanding of these plant compounds is also required for a more rational use of these nutraceutical feeds under farm conditions.

Therefore, the objectives of the current study were: i) to examine the relationship between tannin structures and their anthelmintic activities by using 36 different tannin fractions that span CTs with a wide range of sizes and prodelphinidin/procyanidin and trans/cis-flavan-3-ol ratio, ii) to evaluate whether responses towards CTs differ between abomasal and intestinal small ruminant nematode species.

MATERIALS AND METHODS

Chemicals

Hydrochloric acid (37%, analytical reagent grade), butan-1-ol, acetic acid glacial (analytical reagent grade), acetone (analytical reagent grade), acetonitrile (HPLC grade), dichloromethane (laboratory reagent grade), hexane (GLC, pesticide residue grade) and methanol (HPLC grade) were obtained from ThermoFisher Scientific (Loughborough, UK); benzyl mercaptan (BM) from Sigma-Aldrich (Poole, UK); phosphate buffered saline (PBS) from Biomérieux (Marcy l’Etoile, France); Sephadex™ LH-20 from GE Healthcare (Little Chalfont, UK); ultrapure water (MQ H₂O) from a Milli-Q Plus system (Millipore, Watford, UK).

Preparation of plant extracts and tannin fractions

Eighteen different plant materials were used: aerial plants of Onobrychis viciifolia (OV) were collected on 7 June 2012 (Barham, Kent, UK); Trifolium repens flowers were collected from at NIAB (Cambridge, UK; sample TRa) or purchased from Zioła z Kurpi (Jednorożec, Poland; sample TRb); Lespedeza cuneata (LC) pellets from Sims Brothers Seed Company (Union Springs, AL, USA); Betulae folium leaves (Betula pendula Roth and/or Betula pubescens Ehrh.; BP), Tiliae inflorescentia flowers (T; a mixture of Tilia cordata, T. platyphyllos and T. vulgaris L), Salicis cortex bark (SA) from various Salix spp. (including S. purpurea L.; S. daphnoides Vill.; S. fragilis L.), Ribes nigrum leaves (sample RNb) from Flos (Mokrsko, Poland); Corylus avellana (CA) pericarp from Société Inovfruit (Musidan, France);
Juglandis folium leaves of Juglans regia L. (JR) from Kawon (Gostyń, Poland); inner bark of Pinus sylvestris (PS) from University of Turku (Turku, Finland); Salix babylonica catkins (SB) collected on 26 May 2012 (Emmer Green, UK); Salix caprea (SCL and SCT) leaves and twigs harvested on 19 June 2012 (Goring-on-Thames, UK); Ribes nigrum leaves (sample RNa) and Ribes rubrum leaves (RR) collected on 13 August 2012 from Hildred PYO farm (Goring-on-Thames, UK); Theobroma cacao beans (TC) from Peru (Imported by “Detox your world” inc., Norfolk, UK); Vitellaria paradoxa (VP) meal (i.e. residue of VP nuts after fat extraction; AarhusKarlshamm Sweden AB, Sweden). Samples OV and TRa were lyophilized, samples PS, CA, SCL, SCT, RNa, RR were dried at room temperature for <10 days and then stored at room temperature. The different botanical families of each plants are indicated in the Table 2.

Extracts were prepared according to Stringano et al. with a few modifications. Plant samples (50 g; <1 mm sieve) were extracted with 70% acetone/H$_2$O (500 ml, 7:3, v/v) and filtered under vacuum. Chlorophyll and lipids were removed with dichloromethane (125 ml) by liquid-liquid extraction. The remaining solvents were removed from the aqueous phase on a rotary evaporator at 35 °C. The aqueous extracts were centrifuged for 3 min at 4500 rpm (Jouan CR3i Multifunction Centrifuge) to remove the remaining chlorophyll, insoluble particles and some precipitates. Extracts were freeze-dried and stored at -20 °C.

Extracts were purified on Sephadex™-LH-20 chromatographic columns to remove impurities (mainly sugars and small phenolics) with water. Elution with acetone/H$_2$O (3:7, v/v) yielded fraction 1 CTs (F1), a second elution with acetone/H$_2$O (1:1, v/v) fraction 2 CTs (F2). In total 36 (18 F1 and 18 F2) fractions were tested using Haemonchus contortus and Trichostrongylus colubriformis infective third stage larvae (L3).

Tannin analysis by thiolytic degradation and HPLC

The purified CT fractions were subjected to thiolytic degradation as described by Gea et al. with some changes in order to analyze CT contents (% CT) and features (size in terms of
mean degree of polymerization, mDP; percentage of prodelphinidins and procyanidins within CTs, % PD and % PC; and percentage of trans- vs cis-flavanols, % trans and % cis]).

Freeze-dried samples (4 mg) were weighed into 10 ml glass tubes, methanol (1.5 ml) was added, followed by acidified methanol (0.5 ml of 3.3 % HCl/ in MeOH), benzyl mercaptan (50 µl) and a magnetic stirrer. The tube was capped and heated at 40 °C for 1 h in a water bath. Water (2.5 ml) was added to stop the reaction and the internal standard (0.5 ml of taxifolin: 0.05 mg/ml) was added. Samples were analyzed within 48 h by RP-HPLC.

**Gastrointestinal nematodes**

The third-stage larvae (L3) were obtained from faeces of donor goats, kept indoors and infected monospecifically, with AH susceptible, strains of either *H. contortus* or *T. colubriformis*. The facilities hosting the animals and the trial was performed according to French ethical and welfare rules (agreement number C 31 555 27 of 19 August 2010).

Coprocultures were maintained for 12 days at 23 °C in order to obtain the third stage larvae. Larvae were then recovered from faeces using the Baerman technique and stored at 4 °C in a horizontally vented cap flask at a concentration of 1000 – 1500 L3/ml. Prior to use the larvae were checked to ensure that at least 90% of them were mobile and ensheathed.

**The larval exsheathment inhibition assay (LEIA)**

The larval exsheathment inhibition assay was performed as described by Bahuaud et al. to compare the inhibitory effects of the various tannin fractions (F1 and F2) on the exsheathment process of *H. contortus* and *T. colubriformis*. For both nematode species a batch of 2-month-old larvae was used to perform the *in vitro* assays.

Briefly, 1000 ensheathed L3 larvae (*H. contortus* or *T. colubriformis*) were first incubated for 3 hours at 20 °C with one of the fractions at serial dilutions from 600, 300, 150, 75 to 37.5 µg/ml in PBS (0.1 M phosphate, 0.05 M NaCl, pH 7.2). In addition to all the tested fractions, negative controls (L3 in PBS) were run in parallel. After incubation, the larvae were washed and centrifuged, 3 times in PBS, and then submitted to the artificial exsheathment process
by contact with a solution containing sodium hypochlorite (2% w/v) and sodium chloride (16.5 % w/v), which had been diluted 1 to 350 in PBS. The exsheathment kinetics were measured under a microscope at x 200 magnification by identifying the proportion of exsheathed larvae. Regular examination was performed at 0, 20, 40 and 60 min after contact with the exsheathment solution. The exsheathment percentage was calculated according to the formula: (number of exsheathed larvae) x 100 / (number of exsheathed larvae + ensheathed larvae). For each fraction, four replicates were run per concentration and observation time to examine the exsheathment kinetics.

Statistical analyses of the results

The EC$_{50}$ (effective concentration that causes 50 % exsheathment inhibition) for each tannin fraction was calculated at 60 min (using the software Probit Polo Plus®). First a nonparametric rank correlation of Spearman was calculated using a 2 by 2 correlation in order to evaluate the relationship between the structural parameters characterizing the tannin fractions, and also the relationship between the in vitro AH activity (EC$_{50}$ of each fraction) and quantitative (% CT) and qualitative parameters (mDP, % PD and trans) of the respective F1, F2 and the combined F1 and F2 (F1+F2) fractions. Significant values ($P < 0.05$) and (close to significance) values ($P < 0.10$) are reported.

Then multivariate analyses, principal component analyses (PCA), were performed separately for each nematode species based on the combined data of F1+F2 to obtain an overall synthesis of the relationships between the effects on larval exsheathment and the main CT features. The five variables composing the column of the 2 PCA matrices included quantitative (% CT) and qualitative parameters (mDP, % PD and % trans values) plus the EC$_{50}$ per species. The 36 rows of the matrix corresponded to the F1 and F2 data of the 18 plant samples. All statistical analyses were performed using Systat® 9 software (SPSS Ltd).

RESULTS

Tannin analysis and relationships between structural parameters
The parameters, which characterized the 18 CT samples are provided in Table 1. The average % CT, mean degree of polymerization (mDP) and % prodelphinidins (PD) values were higher in the F2 compared with F1 fraction, whereas the mean % trans values were lower for F2. The Spearman correlation coefficients were positive and significant between the F1 and F2 fractions for mDP \( (r = 0.583, P < 0.05, df = 16) \), % PD \( (r = 0.975, P < 0.01, df = 16) \), % trans \( (r = 0.728, P < 0.05, df = 16) \), which is due to the fact that these 15 plant species produce different CT types. There was no correlation for the % CT in both fractions \( (r = 0.082, NS, df = 16) \).

When the Spearman correlation test was applied to the combined F1+F2 data \( (n = 36 \) samples), there were positive correlation coefficients between % CT and mDP values \( (r = 0.696; P < 0.01; df = 34) \). A non-significant negative correlation existed between % CT and % trans \( (r = -0.261; NS; df = 34) \) and between % PD and mDP values \( (r = 0.270; NS; df = 34) \). This absence of a link between % PD and mDP is important, because column chromatography of CTs from the same plant material tends to lead to fractions, where % PD and mDP are positively correlated (unpublished observations). Therefore, these F1 and F2 fractions enable the investigation of relationships between CT structures and AH activities.

Trends were observed for % PD and % trans \( (r = 0.300; P < 0.08; df = 34) \).

**Anthelmintic activity**

The different fractions affected the larval exsheathment process in a dose-dependent way.

The EC\(_{50}\) values for each of the F1 and F2 fractions per plant sample were used to characterize the AH activity and are shown for *H. contortus* and *T. colubriformis* in Table 2.

For both parasites, EC\(_{50}\) values were generally lower with F2 than with F1 fractions. In addition, overall, EC\(_{50}\) values calculated for *H. contortus* were lower than those of *T. colubriformis*, suggesting that *H. contortus* was more susceptible to these fractions. Thus, the calculation of Spearman’s correlation coefficients between EC\(_{50}\) values, obtained respectively for F1 and F2, showed significant and positive values for both species.
separately, i.e. *H. contortus* (*r* = 0.642; *P* < 0.05; df = 15) and *T. colubriformis* (*r* = 0.688; *P* < 0.01; df = 16). However, there were no correlations between the EC$_{50}$ values of the F1 fractions between *H. contortus* and *T. colubriformis* (*r* = -0.056; NS; df = 15) and also not for the F2 fractions (*r* = 0.397; NS; df = 16). Finally, there were also no correlations between the EC$_{50}$ values of both parasite species with the F1+F2 combined data (*r* = 0.164; NS; df = 33).

**Figure 1** shows the EC$_{50}$ score values in rank order for *H. contortus* and *T. colubriformis*, respectively. The 25% of the most effective plants against both GIN species (i.e. lowest EC$_{50}$ values) were *Vitellaria paradoxa*, *Trifolium repens*, *Lespedeza cuneata*, *Ribes nigrum*, *Theobroma cacao* and *Betula* spp. In addition, *Onobrychis viciifolia* was active against *H. contortus* and *Ribes rubrum* and *Salix babylonica* were active against *T. colubriformis*.

**Table 3** presents the Spearman’s correlation coefficients between the EC$_{50}$ values and the various CT parameters for both nematode species in terms of the F1, F2 and the combined (F1+F2) data. For *H. contortus*, there were negative trends between EC$_{50}$ and mDP and % PD of the F1 fraction and between EC$_{50}$ and mDP of the (F1+F2) data. The correlation between EC$_{50}$; and % PD was negative and significant for the (F1+F2) data. Somewhat surprisingly, a significant positive correlation was noticed for EC$_{50}$ values and % CT of the F2 fractions.

In contrast, for *T. colubriformis* there were no correlations with mDP or % CT. Instead, negative correlation coefficients between EC$_{50}$ and % PD were close to significance for F1 (*r* = -0.453; *P* < 0.10; df = 16); F2 (*r* = -0.439; *P* < 0.10; df = 16) and were significant for the combined (F1+F2) fractions (*r* = -0.403; *P* < 0.05; df = 34).

When PCA was applied separately to either *H. contortus* or *T. colubriformis*, the two main components of axis 1 were mDP and % CT. For axis 2, % PD appeared as the key component. The plane defined by the combination of axes 1 and 2 (**Figure 2**) represented 67% of the overall variability for *H. contortus* and close to 70% for *T. colubriformis*.
The main objective of the PCA was to analyze the overall combined relationships between the different variables and the effects on exsheathment as assessed by the EC$_{50}$ values (Figure 2). Variables that are positively related are located on the same side of the plane. In contrast, variables that are negatively related are located in diagonally opposed quadrants. Analyses of these planes for both GIN species tend to confirm the 2 by 2 Spearman’s correlation results. For *Haemonchus*, the EC$_{50}$ values were in opposition to % PD and mDP values, and to a lesser extent to the % CT. For *Trichostrongylus*, the EC$_{50}$ values were mainly in opposition to % PD.

**DISCUSSION**

The study evaluated 36 CT fractions from 18 sources (15 plant species). These plants were chosen because they present a wide range of different CT features in terms of mDP, % PD and % trans values. It was expected that this variation would allow exploring the relationships between CTs and their AH activities. These particular CT parameters have been described previously as being involved in their biological activities.$^{10,19,20,22,27-29}$ From these 15 plant species 18 tannin extracts were obtained that yielded two related CT fractions (i.e. F1 and F2 fractions). These 36 samples were used to test the effects of quantitative and qualitative differences between CTs. The range of CT concentrations tested with these fractions was chosen based on previous *in vitro* data, which had been obtained with plant extracts of known CT concentrations.$^{16,26,27}$

Three *in vitro* assays are available to explore the interactions between tannins and infective third stage larvae of gastro-intestinal nematodes$^{30}$; i.e. the Larval Migration Inhibition Assay (LMIA), the Larval Feeding Inhibition Assay (LFIA) and the LEIA which has been used in the current study. The LEIA has been widely used to screen the AH activity of either plant extracts,$^{26,30}$ tannin fractions$^{8,10}$ or flavan-3-ol monomers.$^{21,22}$ The LEIA has proved to be simple and reproducible and like the LFIA it also has the advantage that it allows calculation of EC$_{50}$-values, which is rarely the case for the LMIA. Moreover, LEIA has been related to
similar \textit{in vivo} processes.\textsuperscript{31} The LEIA was performed with 2-month-old larvae for both nematode species in order to allow comparison of EC\textsubscript{50} values obtained with the F1 and F2 fractions of each plant sample and between the 2 nematodes species.

Overall, CT contents (\% CT) were higher in the F2 than the F1 fractions and the EC\textsubscript{50} values for F2 calculated for both nematodes were, in most cases, lower than for F1 fractions. This suggests a role for the \% CT in the antiparasitic effect. Similar results were obtained by Williams et al.\textsuperscript{20} for the AH effects against \textit{Ascaris suum} with a subset of these F1 and F2 fractions. Many studies, based on different \textit{in vitro} tests, have reported a dose-dependent AH effect when using tannin-containing plant extracts. For example, for some legume forages such dose-dependent effects have been described for i) \textit{O. viciifolia} (sainfoin) with the larval migration inhibition assay (LMIA),\textsuperscript{7} LEIA\textsuperscript{31}, egg hatch assay (EHA),\textsuperscript{28} and larval development inhibition assay (LDIA),\textsuperscript{28} and for ii) \textit{L. pedunculatus} and \textit{L. corniculatus} extracts with the LMIA and LDIA,\textsuperscript{27,28} the larval feeding inhibition assay (LFIA) and LEIA.\textsuperscript{9}

Although surprisingly, there was a significant positive correlation between CT content and AH activity of the F2 fractions for \textit{H. contortus}, there was, no significant correlation when combining the F1+F2 data. Similarly, Naumann et al.\textsuperscript{19} also found no relation between CT content and the AH activity against \textit{H. contortus} L3 when comparing fractions from three legumes (\textit{Lespedeza stuevei}, \textit{L. cuneata} and \textit{Arachis glabrata}). Novobilský et al.\textsuperscript{10} compared the effects of different CT fractions from \textit{O. viciifolia} on cattle nematodes of either the abomasum (\textit{Ostertagia ostertagi}) or the small intestine (\textit{Cooperia oncophora}). These authors also did not obtain consistent correlations between the CT contents and the \textit{in vitro} AH activity as measured by LFIA.

This discrepancy in relationship between dose and AH activity obtained with either CT-containing extracts or fractions could perhaps be related to other compounds that are also present in extracts.\textsuperscript{7,21} Indeed Molan et al.\textsuperscript{22} also reported deleterious effects of flavan-3-ol monomers against \textit{T. colubriformis} at different life cycle stages, i.e. eggs (EHA) and larvae
The highest AH effect occurred with the epigallocatechin gallate (EGCG) monomer. This observation was confirmed by further studies with green-tea fractions that were tested against Teladorsagia circumcincta and T. colubriformis, where higher EGCG content was linked with a higher AH effect. Similarly, when monomeric subunits of CT were tested in the LEIA on H. contortus and T. colubriformis, a higher AH activity was observed with i) the monomeric subunits of PDs (i.e. gallocatechin, epigallocatechin) and ii) the galloyl derivatives of both PDs and procyanidins. Beside the possible contribution of CT concentration towards explaining antiparasitic activities, several authors have also suggested that CT structures (or quality) could explain some of the observations. For instance, it has been proposed that the biological activity is affected by the hydroxylation at the B-ring in flavan-3-ol monomers and in polymers, where the presence of an additional hydroxyl group (OH) increases the interaction with proteins. This could explain the generally higher activity of PDs compared to PCs. In addition, activity is also increased when galloyl groups are present.

Results of the 2 by 2 calculations of Spearman’s correlation coefficients as well as multivariate analyses (PCA) tended to confirm that the in vitro AH activity in terms of EC_{50} was related to CT structural features for both H. contortus and T. colubriformis. In addition, our results suggest that different mechanisms appear to be involved for each nematode species. For H. contortus, AH activity appeared stronger for CTs with higher PD contents and larger sizes (mDP values). Although, as described by Williams et al. there was no effect of mDP or % PD within F2 fractions on the EC_{50} values. For the F1 fractions, lower EC_{50} values were associated with higher % PD and larger tannins (higher mDP values). Novobilský et al. suggested that mDP was a key factor in the LFIA against L3 of O. ostertagi and C. oncophora after testing O. viciifolia extracts and fractions. However, Naumann et al. found no clear evidence for CT size and inhibition of H. contortus motility. However, only a narrow range of CT sizes was investigated. Conversely to the
present data, Manolaraki\cite{35} found that lower mDP values were correlated with higher AH activity when extracts from 40 *O. vicifolia* accessions were tested by LEIA against *H. contortus*. Similarly, Barrau et al.\cite{7} found that a fraction that contained CTs (< 2000 Da) plus flavonol glycosides had higher AH effects against *H. contortus* larvae than a fraction that contained only CTs (>2000 Da). At this stage, it is important to note that the complexity of plant extract compositions and difficulties in purifying CTs are likely to account for some of these apparent contradictions. Acetone/water extracts from CT-containing plants consist of CTs plus low molecular phenolic compounds (e.g. flavones, flavonols, flavonol glycosides, etc). In addition, CTs usually occur as complex mixtures that contain low to high molecular weight tannins and the mDP-value simply describes the average ‘tannin size’ rather than the distribution profile of all CTs. In fact, we recently discovered that mixtures of CTs and flavonoids had higher AH activities than CTs on their own.\cite{36} Kozan et al.\cite{37} also reported that flavonol glycosides (luteolin-7-β-O-gucopyranoside and quercetin-3- O-β-glucopyranoside) from *Vicia pannonica* var. *purpuracens*, might also participate in the modulation of bioactivity of the highly AH extract and fractions against trichostrongyloid larvae. This underlines that the proximity of biochemical structure between flavonol glycosides and CT (which are flavan-3-ols’ polymers) could suggest a similar or close mechanism of action for both types of compounds. Taken together, the presence of non-CT compounds (such as flavonoid monomers) could, therefore, explain the apparently contradictory observations by Manolaraki\cite{35} and Barrau et al.\cite{7} The F1 fractions had only half the CT contents of F2 fractions (Table 1). However, the combination of F1+F2 data gave a close to significant correlation of EC$_{50}$ and mDP values (Table 3).

In contrast, for *T. colubriformis*, % PD was consistently (F1, F2, and combined F1+F2) related to AH activity. This agrees with other reports on *T. colubriformis* larvae, which found higher AH *in vitro* effects of PD- compared with PC-rich tannins.\cite{21,22}
Interestingly, there were different susceptibilities between the two parasite species, which suggested that *H. contortus* was more susceptible than *T. colubriformis*. This is indicated by the overall lower EC\textsubscript{50} values for the abomasal species with both types of CT fractions. Molan et al.\textsuperscript{8} also pointed out that the abomasal nematode *T. circumcincta* was more susceptible than *T. colubriformis* to the AH effects of flavan-3-ol monomers and oligomeric CTs in the LMIA. The same conclusion was drawn from *in vitro* studies that examined extracts from different woody plants (*Rubus fructicosus*, *Quercus robur* and *Corylus avellana*) against *H. contortus*, *T. circumcincta* and *T. colubriformis* based on LMIA and LEIA tests.\textsuperscript{16} However, other authors found no such differences in the response to quebrachao or *O. viciifolia* extracts\textsuperscript{11,31} between abomasal or intestinal species. Moreno-Gonzalo et al.\textsuperscript{38,39} even found a higher *in vitro* susceptibility of *T. colubriformis* compared to *H. contortus* and *T. circumcincta* when measuring the AH activity of extracts from different heather species (*Calluna vulgaris*, *Erica cinerea* and *E. umbellata*). It remains to be seen whether differences in assay conditions could account for some of these contradictory results. Moreover, it will be worth exploring whether exist species specific differences in the quality of larval sheath proteins between the abomasal vs the intestinal species in order to better understand the mode of actions of polyphenols against the different GIN species.

Although it is difficult to extrapolate from *in vitro* to *in vivo* results, our current data provide a screening of CT-containing plants, whose AH properties will need to be explored further in controlled *in vivo* studies in order to develop their potential for on-farm exploitation. It is also worth noting that the CT fractions from three legumes ranked amongst the most effective ones (i.e. having the lowest EC\textsubscript{50} values): *L. cuneata* pellets, *O. viciifolia* plants and *T. repens* flowers (Figure 1). The last decade has seen an accumulation of *in vivo* results that confirm the AH effects of *L. cuneata* and *O. viciifolia* against the main GIN species whether offered to small ruminants in the form of freshly grazed pasture,\textsuperscript{40,41} as hay,\textsuperscript{15,17,42} as silages\textsuperscript{42} or as pellets.\textsuperscript{18}
As far as *T. repens* is concerned, no other data are available because the genus *Trifolium* *sp* is usually considered as a tannin-free legume and consequently the various *Trifolium* species have received little attention for their antiparasitic potential. However, Carlsen and Fomsgaard provided an extensive review of the secondary metabolites in *T. repens* and pointed out the high CT content in flowers. The current study found that CTs from *T. repens* flowers had a strong AH effect and confirmed the dose-dependent inhibition effects of *T. repens* tannins observed for *C. oncophora* in the LFIA.

The CT fractions of *V. paradoxa* were also ranked as highly effective against both nematode species and suggested that some agro-industrial by-products could be of interest for their antiparasitic properties. It is worth noting that AH effects on *H. contortus* and *T. colubriformis* were recently also described not only for cocoa seed but also for husk extracts using the EHA.

In conclusion, our results showed that structural features of condensed tannins are key factors that impact on the anthelmintic effects against gastro-intestinal nematodes of ruminants. In addition, there were differences in the susceptibilities of the abomasal intestinal nematode species. These differences have been described previously in the literature and could be related to the fact that the nematode sheath proteins differ in these parasite species. This could perhaps affect their interactions with the tannins. It is worth also to underline that the current results have been acquired on infective larvae and that other assays that target other parasitic stages might have different outcomes. Further studies will be needed to explore these interactions at the molecular level.

**ABBREVIATIONS USED**

Gastrointestinal nematodes, (GINs); condensed tannins (CT); anthelmintic (AH); mean degree of polymerization, (mDP); prodelphinidins, (PD); procyanidins, (PC); phosphate buffered saline, (PBS); larval exsheathment inhibition assay, (LEIA); infective stage nematode larvae, (L3); effective concentration for 50% inhibition of larvae’s exsheathment
EC50; larval development inhibition assay, (LDIA); larval feeding inhibition assay, (LFIA); egg hatch assay, (EHA); larval migration inhibition assay, (LMIA).

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We thank Alison and Dave Prudence for the S. caprea leaves and twigs, Hildred’s PYO Farm for the R. nigrum and R. rubrum leaves and Mr P. Davy for the O. viciifolia plants. The V. paradoxa meal sample was kindly provided by AarhusKarlshamm Sweden AB, Sweden.

We also deeply appreciate to all LegumePlus’ fellows for help collecting plant samples.

SUPPORTING INFORMATION

Origin and supplier of each tannin-containing resource tested. This material is available free of charge via the Internet at http://pubs.acs.org.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.
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(33) Li, C.; Leverence, R.; Trombley, J.D.; Xu, S.; Yang, J.; Tian, Y.; Reed, J.D.; Hagerman, A.E. High molecular weight persimmon (*Diospyros kaki* L.) proanthocyanidin, a highly


**Figure Legends**

**Figure 1**: EC_{50} values (and 95% confidence interval) scores for A) *Haemonchus contortus* and B) *Trichostrongylus colubriformis* using F1 and F2 fractions from the 18 tannin-containing plant resources
Figure 2: Multivariate principal component analyses (PCA) explained to condensed tannins for each parasite species: A) H. contortus, B) T. colubriformis. For both nematode species, the matrix was composed of 5 variables and 36 lines corresponding to 2 fractions (F1 and F2) of a range of 18 tannin-containing samples. Abbreviations: EC<sub>50</sub> values based on LEIA (low values reflect high anthelmintic activities), CT (condensed tannins content, units g CT/100 g fraction); mDP (mean degree of polymerization of tannins); PD (% of prodelphinidins) trans (% of trans flavan-3-ols). The planes represent 67 % of the variability for H. contortus and 70 % for T. colubriformis, respectively.
Table 1: Chemical characterization of two tannin fractions from 18 plant resources (F1, and F2 fractions; % PC = 100 - % PD; % cis = 100 - % trans).

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Family</th>
<th>Common name/sample</th>
<th>% CT ± SD</th>
<th>mDP ± SD</th>
<th>% PD ± SD</th>
<th>% Trans ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onobrychis vicifolia</td>
<td>Leguminosae</td>
<td>Sainfoin/ whole plant</td>
<td>F1: 37.2 ±4.5</td>
<td>F2: 100±4.1</td>
<td>F1: 2.8±0.1</td>
<td>F2: 8.7±0.0</td>
</tr>
<tr>
<td>Trifolium repens*</td>
<td>Leguminosae</td>
<td>White clover/ flower</td>
<td>F1: 11.7±0.4</td>
<td>F2: 100±2.4</td>
<td>F1: 1.8±0.0</td>
<td>F2: 8.6±0.0</td>
</tr>
<tr>
<td>Trifolium repens†</td>
<td>Leguminosae</td>
<td>White clover/ flower</td>
<td>F1: 13.4±0.4</td>
<td>F2: 82.4±2.0</td>
<td>F1: 3.1±0.1</td>
<td>F2: 12.7±0.0</td>
</tr>
<tr>
<td>Lespedeza cuneata</td>
<td>Leguminosae</td>
<td>Sericea lespedeza/ pellets</td>
<td>F1: 42.1±0.2</td>
<td>F2: 82.6±1.4</td>
<td>F1: 5±0.0</td>
<td>F2: 11.3±0.0</td>
</tr>
<tr>
<td>Betula spp</td>
<td>Betulaceae</td>
<td>Birch/ leaf</td>
<td>F1: 12.9±0.3</td>
<td>F2: 63.6±2.5</td>
<td>F1: 2.2±0.0</td>
<td>F2: 8.3±0.1</td>
</tr>
<tr>
<td>Corylus avellana</td>
<td>Corylaceae</td>
<td>Hazelnut/ pericarp</td>
<td>F1: 49.2±1.1</td>
<td>F2: 67.5±0.6</td>
<td>F1: 4.6±0.1</td>
<td>F2: 9.2±0.1</td>
</tr>
<tr>
<td>Juglans regia</td>
<td>Juglandaceae</td>
<td>Walnut/ leaf</td>
<td>F1: 21.8±1.4</td>
<td>F2: 69.0±1.7</td>
<td>F1: 2.9±0.0</td>
<td>F2: 12.3±0.1</td>
</tr>
<tr>
<td>Pinus sylvestris</td>
<td>Pinaceae</td>
<td>Pine/ inner bark</td>
<td>F1: 54±2</td>
<td>F2: 79±2.4</td>
<td>F1: 2.3±0.0</td>
<td>F2: 6.6±0.2</td>
</tr>
<tr>
<td>Tilia L.</td>
<td>Tiliaceae</td>
<td>Lime tree/ flower</td>
<td>F1: 47.5±2.8</td>
<td>F2: 91.7±3.8</td>
<td>F1: 2.0±0.0</td>
<td>F2: 7.9±0.1</td>
</tr>
<tr>
<td>Salix babylonica</td>
<td>Salicaceae</td>
<td>Weeping willow/ catkins</td>
<td>F1: 23.1±1.7</td>
<td>F2: 83.3±0.6</td>
<td>F1: 2±0.0</td>
<td>F2: 9.9±0.0</td>
</tr>
<tr>
<td>Salix caprea</td>
<td>Salicaceae</td>
<td>Goat willow/ leaf</td>
<td>F1: 51.5±0.1</td>
<td>F2: 83.8±1.8</td>
<td>F1: 2.1±0.1</td>
<td>F2: 5.3±0.1</td>
</tr>
<tr>
<td>Salix caprea</td>
<td>Salicaceae</td>
<td>Goat willow/ twigs</td>
<td>F1: 72±1.1</td>
<td>F2: 93.2±11</td>
<td>F1: 2.1±0.1</td>
<td>F2: 5.3±0.1</td>
</tr>
<tr>
<td>Ribes nigrum*</td>
<td>Grossulariaceae</td>
<td>Black currant/ leaf</td>
<td>F1: 59.8±1.3</td>
<td>F2: 100±1.7</td>
<td>F1: 2.5±0.0</td>
<td>F2: 6.5±0.1</td>
</tr>
<tr>
<td>Ribes nigrum†</td>
<td>Grossulariaceae</td>
<td>Black currant/ leaf</td>
<td>F1: 55.5±3.2</td>
<td>F2: 77.1±3.9</td>
<td>F1: 3.8±0.0</td>
<td>F2: 11.8±0.1</td>
</tr>
<tr>
<td>Ribes rubrum</td>
<td>Grossulariaceae</td>
<td>Red currant/ leaf</td>
<td>F1: 57.7±9.1</td>
<td>F2: 68.2±1.1</td>
<td>F1: 4.9±0.0</td>
<td>F2: 10±0.1</td>
</tr>
<tr>
<td>Theobroma cacao</td>
<td>Malvaceae</td>
<td>Cocoa/ seed</td>
<td>F1: 58.5±2.9</td>
<td>F2: 75.5±8.1</td>
<td>F1: 2.3±0.0</td>
<td>F2: 5.4±0.1</td>
</tr>
<tr>
<td>Vitellaria paradoxa</td>
<td>Sapotaceae</td>
<td>Shea/ meal</td>
<td>F1: 33.0±0.6</td>
<td>F2: 44.9±0.8</td>
<td>F1: 2.2±0.1</td>
<td>F2: 4.1±0.1</td>
</tr>
<tr>
<td>Mean values</td>
<td></td>
<td></td>
<td>F1: 40.2±9.2</td>
<td>F2: 81.1±7.4</td>
<td>F1: 2.8±0.5</td>
<td>F2: 8.4±1.3</td>
</tr>
</tbody>
</table>

*sample a; †sample b
Table 2: EC$_{50}$ values by parasite and by fraction (F1 or F2) from each tannin-containing resource tested

<table>
<thead>
<tr>
<th>Plant</th>
<th>Abbreviation</th>
<th>Family$^{26}$</th>
<th>H. contortus EC$_{50}$ (95% CI) (µg/ml)</th>
<th>T. colubriformis EC$_{50}$ (95% CI) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>F1</td>
<td>F2</td>
</tr>
<tr>
<td>Onobrychis vicilifolia</td>
<td>OVF1/OVF2</td>
<td>Leguminosae</td>
<td>62.7 (49.9-76.5)</td>
<td>212 (182-250)</td>
</tr>
<tr>
<td>Trifolium repens (a)</td>
<td>TRaF1/TRaF2</td>
<td>Leguminosae</td>
<td>287 (249-328)</td>
<td>177 (131-239)</td>
</tr>
<tr>
<td>Trifolium repens (b)</td>
<td>TRbF1/TRbF2</td>
<td>Leguminosae</td>
<td>37.5 &lt; (0.7 -74.4)</td>
<td>37.5 &lt; (0.08-42.4)</td>
</tr>
<tr>
<td>Lespedeza cuneata</td>
<td>LCF1/LCF2</td>
<td>Leguminosae</td>
<td>78.2 (28.1-157)</td>
<td>37.5 &lt; (2.5-55.3)</td>
</tr>
<tr>
<td>Corylus avellana</td>
<td>CAF1/C1F2</td>
<td>Corylaceae</td>
<td>166 (82.5-441)</td>
<td>143 (104-170)</td>
</tr>
<tr>
<td>Juglans regia L.</td>
<td>JRF1/JRF2</td>
<td>Juglandaceae</td>
<td>94.7 (65.5-115)</td>
<td>70.6 (46.9-106)</td>
</tr>
<tr>
<td>Betula spp</td>
<td>BPF1/BPF2</td>
<td>Betulaceae</td>
<td>62.8 (58.6-82)</td>
<td>62.6 (19-90.3)</td>
</tr>
<tr>
<td>Pinus sylvestris L.</td>
<td>PSF1/PSF2</td>
<td>Pinaceae</td>
<td>236 (192-290)</td>
<td>144 (125-167)</td>
</tr>
<tr>
<td>Tilia L. spp.</td>
<td>TF1/TF2</td>
<td>Tiliaceae</td>
<td>113 (82-157)</td>
<td>88.7 (66.1-107)</td>
</tr>
<tr>
<td>Salix spp</td>
<td>SAF1/SAF2</td>
<td>Salicaceae</td>
<td>188 (137-241)</td>
<td>138 (117-154)</td>
</tr>
<tr>
<td>Salix babylonica</td>
<td>SBF1/SBF2</td>
<td>Salicaceae</td>
<td>174 (120-206)</td>
<td>128 (69.8-166)</td>
</tr>
<tr>
<td>Salix caprea (twigs)</td>
<td>SCTF1/SCTF2</td>
<td>Salicaceae</td>
<td>195 (142-266)</td>
<td>132 (97.6-184)</td>
</tr>
<tr>
<td>Salix caprea (leaves)</td>
<td>SCLF1/SCLF2</td>
<td>Salicaceae</td>
<td>196 (86-217)</td>
<td>161 (133-191)</td>
</tr>
<tr>
<td>Ribes nigrum (sample a)</td>
<td>RNf1/RNaF2</td>
<td>Grossariaceae</td>
<td>145 (85-259)</td>
<td>157 (124-203)</td>
</tr>
<tr>
<td>Ribes nigrum (sample b)</td>
<td>RNbF2/RNbF2</td>
<td>Grossariaceae</td>
<td>48.7 (78.1-158)</td>
<td>59.2 (18.5-111)</td>
</tr>
<tr>
<td>Ribes rubrum</td>
<td>RRf1/RRF2</td>
<td>Grossariaceae</td>
<td>-</td>
<td>97.8 (85.4-305)</td>
</tr>
<tr>
<td>Theobroma cacao</td>
<td>TCF1/TCF2</td>
<td>Malvaceae</td>
<td>208 (168-246)</td>
<td>65.2 (34.1-95.7)</td>
</tr>
<tr>
<td>Vitellaria paradoxa</td>
<td>VP1/VP2</td>
<td>Sapotaceae</td>
<td>37.5 &lt; (0.7-29.1)</td>
<td>37.5 &lt; (0.48-36.5)</td>
</tr>
</tbody>
</table>

Mean values 136.9 ± 74.1 108.1 ± 53.2 233 ± 54.3 166 ± 39.9

* the calculation of the EC$_{50}$ values relying on the Polo Plus software gave the following values for the effects against *H. contortus* for *T. repens* (b) fraction F1 = 33.2 µg/ml and fraction F2 = 14.5 µg/ml; for *Lespedeza cuneata* fraction F2 = 29.4 µg/ml, for *Vitellaria paradoxa* fraction F1 = 13, 6 µg/ml and fraction F2 = 16.5 µg/ml

Table 3: Spearman’s correlation coefficients for anthelmintic activity by nematode species according to tannin content and structural parameters in F1 and/or F2 fractions

25
<table>
<thead>
<tr>
<th>Variable</th>
<th>Haemonchus contortus</th>
<th>Trichostrongylus colubriformis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1</td>
<td>F2</td>
</tr>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
</tr>
<tr>
<td>Degree of freedom (df)</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>% CT</td>
<td>0.30</td>
<td>0.44</td>
</tr>
<tr>
<td>mDP</td>
<td>-0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44</td>
</tr>
<tr>
<td>% PD</td>
<td>-0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44</td>
</tr>
<tr>
<td>% trans</td>
<td>0.08</td>
<td>0.44</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05, <sup>b</sup> P < 0.10
Figure 1

A) EC\textsubscript{50} (µg/ml) values for *Haemonchus contortus*

B) EC\textsubscript{50} (µg/ml) score values for *T. colubriformis*
Figure 2

A) PCA values for *H. contortus*

B) PCA for *T. colubriformis*
1st: Condensed tannins (CTs) extraction from tannin-containing resources. 2nd: CTs fractionation. 3rd: F1 and F2 are yielded. 4th: bioassay. Larval Exsheathment Inhibition Assay (LEIA). 5th: interpretation.