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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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## 1 **ABSTRACT**

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

## 18 **KEY WORDS**

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

## 21 INTRODUCTION

22 Gastrointestinal nematodes (GINs) represent a major threat for the breeding and production  
23 of grazing ruminants. Up to now, their control has been based mainly on the repeated use  
24 of synthetic AH drugs. However, worm populations in small ruminants have consistently  
25 developed resistance against all AH drugs.<sup>1</sup> Therefore, the search for alternative solutions  
26 to such drug treatments is now a necessity for a more sustainable control of these parasites.<sup>2</sup>  
27 The last two decades have provided evidence that some plants possess natural AH  
28 bioactivity, which is based on the presence of condensed tannins (CTs) and flavonoids.  
29 Such plants, therefore, represent a promising alternative to chemotherapy especially when  
30 used as nutraceuticals that combine beneficial effects on health and nutrition in small and  
31 large ruminants.<sup>3-6</sup>

32 The involvement of CTs in the observed anthelmintic (AH) effects against parasitic  
33 nematodes has been suggested from several results acquired *in vitro* using either plant  
34 extracts or purified CT fractions<sup>7-10</sup> and from *in vivo* studies with tannin-containing  
35 resources.<sup>11-15</sup>

36 Differences in AH effects have repeatedly been noticed between abomasal versus intestinal  
37 nematode species of both small ruminant and cattle parasite.<sup>12</sup> These observations have  
38 been made in *in vitro*<sup>9,10,16</sup> and *in vivo* studies with the same CT-resources.<sup>13,15,17,18</sup>

39 Some authors have suggested that different structural features of CTs are involved in their  
40 AH effects, namely: i) CT size<sup>7,10,19,20</sup>; ii) the type of flavan-3-ol subunits that give rise to  
41 either prodelphinidin (PD) or procyanidin tannins (PC)<sup>8,20,21,22</sup> or iii) the stereochemistry of  
42 the C-ring in these subunits (i.e. *trans* vs. *cis* flavan-3-ols)<sup>19,22</sup>. Taken together, these  
43 observations led us to hypothesize that there are quantitative and qualitative differences  
44 between CTs, which determine their activity against parasitic nematodes. There is thus a  
45 need to evaluate the structure-activity relationship between tannins and GINs. A better

46 understanding of these plant compounds is also required for a more rational use of these  
47 nutraceutical feeds under farm conditions.

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

## 53 **MATERIALS AND METHODS**

### 54 **Chemicals**

55 Hydrochloric acid (37%, analytical reagent grade), butan-1-ol, acetic acid glacial (analytical  
56 reagent grade), acetone (analytical reagent grade), acetonitrile (HPLC grade),  
57 dichloromethane (laboratory reagent grade), hexane (GLC, pesticide residue grade) and  
58 methanol (HPLC grade) were obtained from ThermoFisher Scientific (Loughborough, UK);  
59 benzyl mercaptan (BM) from Sigma-Aldrich (Poole, UK); phosphate buffered saline (PBS)  
60 from Biomérieux (Marcy l'Etoile, France); Sephadex™ LH-20 from GE Healthcare (Little  
61 Chalfont, UK); ultrapure water (MQ H<sub>2</sub>O) from a Milli-Q Plus system (Millipore, Watford, UK).

### 62 **Preparation of plant extracts and tannin fractions**

63 Eighteen different plant materials were used: aerial plants of *Onobrychis viciifolia* (OV) were  
64 collected on 7 June 2012 (Barham, Kent, UK); *Trifolium repens* flowers were collected from  
65 at NIAB (Cambridge, UK; sample TRa) or purchased from Ziola z Kurpi (Jednoróżec,  
66 Poland; sample TRb); *Lespedeza cuneata* (LC) pellets from Sims Brothers Seed Company  
67 (Union Springs, AL, USA); *Betulae folium* leaves (*Betula pendula* Roth and/or *Betula*  
68 *pubescens* Ehrh.; BP), *Tiliae inflorescentia* flowers (T; a mixture of *Tilia cordata*, *T.*  
69 *platyphyllos* and *T. vulgaris* L), *Salicis cortex* bark (SA) from various *Salix* spp. (including *S.*  
70 *purpurea* L.; *S. daphnoides* Vill.; *S. fragilis* L.), *Ribes nigrum* leaves (sample RNb) from Flos  
71 (Mokrsko, Poland); *Corylus avellana* (CA) pericarp from Société Inovfruit (Musidan, France);

72 *Juglandis folium* leaves of *Juglans regia* L. (JR) from Kawon (Gostyń, Poland); inner bark of  
73 *Pinus sylvestris* (PS) from University of Turku (Turku, Finland); *Salix babylonica* catkins (SB)  
74 collected on 26 May 2012 (Emmer Green, UK); *Salix caprea* (SCL and SCT) leaves and  
75 twigs harvested on 19 June 2012 (Goring-on-Thames, UK); *Ribes nigrum* leaves (sample  
76 RNa) and *Ribes rubrum* leaves (RR) collected on 13 August 2012 from Hildred PYO farm  
77 (Goring-on-Thames, UK); *Theobroma cacao* beans (TC) from Peru (Imported by “Detox your  
78 world” inc., Norfolk, UK); *Vitellaria paradoxa* (VP) meal (i.e. residue of VP nuts after fat  
79 extraction; AarhusKarlshamm Sweden AB, Sweden). Samples OV and TRa were  
80 lyophilized, samples PS, CA, SCL, SCT, RNa, RR were dried at room temperature for <10  
81 days and then stored at room temperature. The different botanical families <sup>23</sup>of each plants  
82 are indicated in the **Table 2**.

83 Extracts were prepared according to Stringano et al.<sup>24</sup> with a few modifications. Plant  
84 samples (50 g; <1 mm sieve) were extracted with 70% acetone/H<sub>2</sub>O (500 ml, 7:3, v/v) and  
85 filtered under vacuum. Chlorophyll and lipids were removed with dichloromethane (125 ml)  
86 by liquid-liquid extraction. The remaining solvents were removed from the aqueous phase  
87 on a rotary evaporator at 35 °C. The aqueous extracts were centrifuged for 3 min at 4500  
88 rpm (Jouan CR3i Multifunction Centrifuge) to remove the remaining chlorophyll, insoluble  
89 particles and some precipitates. Extracts were freeze-dried and stored at -20 °C.

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

#### 95 **Tannin analysis by thiolitic degradation and HPLC**

96 The purified CT fractions were subjected to thiolitic degradation as described by Gea et al.<sup>25</sup>  
97 with some changes in order to analyze CT contents (% CT) and features [(size in terms of

98 mean degree of polymerization, mDP; percentage of prodelphinidins and procyanidins  
99 within CTs, % PD and % PC; and percentage of *trans*- vs *cis*-flavanols, % *trans* and % *cis*].  
100 Freeze-dried samples (4 mg) were weighed into 10 ml glass tubes, methanol (1.5 ml) was  
101 added, followed by acidified methanol (0.5 ml of 3.3 % HCl/ in MeOH), benzyl mercaptan  
102 (50 µl) and a magnetic stirrer. The tube was capped and heated at 40 °C for 1 h in a water  
103 bath. Water (2.5 ml) was added to stop the reaction and the internal standard (0.5 ml of  
104 taxifolin: 0.05 mg/ml) was added. Samples were analyzed within 48 h by RP-HPLC<sup>25</sup>.

### 105 **Gastrointestinal nematodes**

106 The third-stage larvae (L3) were obtained from faeces of donor goats, kept indoors and  
107 infected monospecifically, with AH susceptible, strains of either *H. contortus* or *T.*  
108 *colubriformis*. The facilities hosting the animals and the trial was performed according to  
109 French ethical and welfare rules (agreement number C 31 555 27 of 19 August 2010).  
110 Coprocultures were maintained for 12 days at 23 °C in order to obtain the third stage larvae.  
111 Larvae were then recovered from faeces using the Baerman technique and stored at 4 °C  
112 in a horizontally vented cap flask at a concentration of 1000 – 1500 L3/ml. Prior to use the  
113 larvae were checked to ensure that at least 90% of them were mobile and ensheathed.

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

115 The larval exsheathment inhibition assay was performed as described by Bahuaud et al.<sup>26</sup>  
116 to compare the inhibitory effects of the various tannin fractions (F1 and F2) on the  
117 exsheathment process of *H. contortus* and *T. colubriformis*. For both nematode species a  
118 batch of 2-month-old larvae was used to perform the *in vitro* assays.

119 Briefly, 1000 ensheathed L3 larvae (*H. contortus* or *T. colubriformis*) were first incubated for  
120 3 hours at 20 °C with one of the fractions at serial dilutions from 600, 300, 150, 75 to 37.5  
121 µg/ml in PBS (0.1 M phosphate, 0.05 M NaCl, pH 7.2). In addition to all the tested fractions,  
122 negative controls (L3 in PBS) were run in parallel. After incubation, the larvae were washed  
123 and centrifuged, 3 times in PBS, and then submitted to the artificial exsheathment process



124 by contact with a solution containing sodium hypochlorite (2% w/v) and sodium chloride  
125 (16.5 % w/v), which had been diluted 1 to 350 in PBS. The exsheathment kinetics were  
126 measured under a microscope at x 200 magnification by identifying the proportion of  
127 exsheathed larvae. Regular examination was performed at 0, 20, 40 and 60 min after contact  
128 with the exsheathment solution. The exsheathment percentage was calculated according to  
129 the formula: (number of exsheathed larvae) x 100/ (number of exsheathed larvae +  
130 ensheathed larvae). For each fraction, four replicates were run per concentration and  
131 observation time to examine the exsheathment kinetics.

### 132 **Statistical analyses of the results**

133 The EC<sub>50</sub> (effective concentration that causes 50 % exsheathment inhibition) for each tannin  
134 fraction was calculated at 60 min (using the software Probit Polo Plus®). First a  
135 nonparametric rank correlation of Spearman was calculated using a 2 by 2 correlation in  
136 order to evaluate the relationship between the structural parameters characterizing the  
137 tannin fractions, and also the relationship between the *in vitro* AH activity (EC<sub>50</sub> of each  
138 fraction) and quantitative (% CT) and qualitative parameters (mDP, % PD and *trans*) of the  
139 respective F1, F2 and the combined F1 and F2 (F1+F2) fractions. Significant values ( $P <$   
140 0.05) and (close to significance) values ( $P < 0.10$ ) are reported.

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

## 148 **RESULTS**

### 149 **Tannin analysis and relationships between structural parameters**

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

158 When the Spearman correlation test was applied to the combined F1+F2 data ( $n = 36$   
159 samples), there were positive correlation coefficients between % CT and mDP values ( $r =$   
160  $0.696$ ;  $P < 0.01$ ;  $df = 34$ ). A non-significant negative correlation existed between % CT and  
161 % *trans* ( $r = -0.261$ ; NS;  $df = 34$ ) and between % PD and mDP values ( $r = 0.270$ ; NS;  $df =$   
162  $34$ ). This absence of a link between % PD and mDP is important, because column  
163 chromatography of CTs from the same plant material tends to lead to fractions, where % PD  
164 and mDP are positively correlated (unpublished observations). Therefore, these F1 and F2  
165 fractions enable the investigation of relationships between CT structures and AH activities.  
166 Trends were observed for % PD and % *trans* ( $r = 0.300$ ;  $P < 0.08$ ;  $df = 34$ ).

### 167 **Anthelmintic activity**

168 The different fractions affected the larval exsheathment process in a dose-dependent way.  
169 The  $EC_{50}$  values for each of the F1 and F2 fractions per plant sample were used to  
170 characterize the AH activity and are shown for *H. contortus* and *T. colubriformis* in **Table 2**.  
171 For both parasites,  $EC_{50}$  values were generally lower with F2 than with F1 fractions. In  
172 addition, overall,  $EC_{50}$  values calculated for *H. contortus* were lower than those of *T.*  
173 *colubriformis*, suggesting that *H. contortus* was more susceptible to these fractions. Thus,  
174 the calculation of Spearman's correlation coefficients between  $EC_{50}$  values, obtained  
175 respectively for F1 and F2, showed significant and positive values for both species

176 separately, i.e. *H. contortus* ( $r = 0.642$ ;  $P < 0.05$ ;  $df = 15$ ) and *T. colubriformis* ( $r = 0.688$ ;  $P$   
177  $< 0.01$ ;  $df = 16$ ). However, there were no correlations between the EC<sub>50</sub> values of the F1  
178 fractions between *H. contortus* and *T. colubriformis* ( $r = -0.056$ ; NS;  $df = 15$ ) and also not for  
179 the F2 fractions ( $r = 0.397$ ; NS;  $df = 16$ ). Finally, there were also no correlations between the  
180 EC<sub>50</sub> values of both parasite species with the F1+F2 combined data ( $r = 0.164$ ; NS;  $df = 33$ ).  
181 **Figure 1** shows the EC<sub>50</sub> score values in rank order for *H. contortus* and *T. colubriformis*,  
182 respectively. The 25% of the most effective plants against both GIN species (i.e. lowest EC<sub>50</sub>  
183 values) were *Vitellaria paradoxa*, *Trifolium repens*, *Lespedeza cuneata*, *Ribes nigrum*,  
184 *Theobroma cacao* and *Betula* spp. In addition, *Onobrychis viciifolia* was active against *H.*  
185 *contortus* and *Ribes rubrum* and *Salix babylonica* were active against *T. colubriformis*.  
186 **Table 3** presents the Spearman's correlation coefficients between the EC<sub>50</sub> values and the  
187 various CT parameters for both nematode species in terms of the F1, F2 and the combined  
188 (F1+F2) data. For *H. contortus*, there were negative trends between EC<sub>50</sub> and mDP and %  
189 PD of the F1 fraction and between EC<sub>50</sub> and mDP of the (F1+F2) data. The correlation  
190 between EC<sub>50</sub>; and % PD was negative and significant for the (F1+F2) data. Somewhat  
191 surprisingly, a significant positive correlation was noticed for EC<sub>50</sub> values and % CT of the  
192 F2 fractions.  
193 In contrast, for *T. colubriformis* there were no correlations with mDP or % CT. Instead,  
194 negative correlation coefficients between EC<sub>50</sub> and % PD were close to significance for F1  
195 ( $r = -0.453$ ;  $P < 0.10$ ;  $df = 16$ ); F2 ( $r = -0.439$ ;  $P < 0.10$ ;  $df = 16$ ) and were significant for the  
196 combined (F1+F2) fractions ( $r = -0.403$ ;  $P < 0.05$ ;  $df = 34$ ).  
197 When PCA was applied separately to either *H. contortus* or *T. colubriformis*, the two main  
198 components of axis 1 were mDP and % CT. For axis 2, % PD appeared as the key  
199 component. The plane defined by the combination of axes 1 and 2 (**Figure 2**) represented  
200 67 % of the overall variability for *H. contortus* and close to 70 % for *T. colubriformis*.

201 The main objective of the PCA was to analyze the overall combined relationships between  
202 the different variables and the effects on exsheathment as assessed by the EC<sub>50</sub> values  
203 (**Figure 2**). Variables that are positively related are located on the same side of the plane.  
204 In contrast, variables that are negatively related are located in diagonally opposed  
205 quadrants. Analyses of these planes for both GIN species tend to confirm the 2 by 2  
206 Spearman's correlation results. For *Haemonchus*, the EC<sub>50</sub> values were in opposition to %  
207 PD and mDP values, and to a lesser extent to the % CT. For *Trichostrongylus*, the EC<sub>50</sub>  
208 values were mainly in opposition to % PD.

## 209 **DISCUSSION**

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

220 Three *in vitro* assays are available to explore the interactions between tannins and infective  
221 third stage larvae of gastro-intestinal nematodes<sup>30</sup>; i.e. the Larval Migration Inhibition Assay  
222 (LMIA), the Larval Feeding Inhibition Assay (LFIA) and the LEIA which has been used in the  
223 current study. The LEIA has been widely used to screen the AH activity of either plant  
224 extracts,<sup>26,30</sup> tannin fractions<sup>8,10</sup> or flavan-3-ol monomers.<sup>21,22</sup> The LEIA has proved to be  
225 simple and reproducible and like the LFIA it also has the advantage that it allows calculation  
226 of EC<sub>50</sub>-values, which is rarely the case for the LMIA. Moreover, LEIA has been related to

227 similar *in vivo* processes.<sup>31</sup> The LEIA was performed with 2-month-old larvae for both  
228 nematode species in order to allow comparison of EC<sub>50</sub> values obtained with the F1 and F2  
229 fractions of each plant sample and between the 2 nematodes species.

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

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

249 This discrepancy in relationship between dose and AH activity obtained with either CT-  
250 containing extracts or fractions could perhaps be related to other compounds that are also  
251 present in extracts.<sup>7,21</sup> Indeed Molan et al.<sup>22</sup> also reported deleterious effects of flavan-3-ol  
252 monomers against *T. colubriformis* at different life cycle stages, i.e. eggs (EHA) and larvae

253 (LDIA, LMIA). The highest AH effect occurred with the epigallocatechin gallate (EGCG)  
254 monomer. This observation was confirmed by further studies with green-tea fractions that  
255 were tested against *Teladorsagia circumcincta* and *T. colubriformis*, where higher EGCG  
256 content was linked with a higher AH effect.<sup>8</sup> Similarly, when monomeric subunits of CT were  
257 tested in the LEIA on *H. contortus* and *T. colubriformis*,<sup>21</sup> a higher AH activity was observed  
258 with i) the monomeric subunits of PDs (i.e. gallic catechin, epigallocatechin) and ii) the galloyl  
259 derivatives of both PDs and procyanidins.

260 Beside the possible contribution of CT concentration towards explaining antiparasitic  
261 activities, several authors have also suggested that CT structures (or quality) could explain  
262 some of the observations.<sup>8-10,19,20,22</sup> For instance, it has been proposed that the biological  
263 activity is affected by the hydroxylation at the B-ring in flavan-3-ol monomers and in  
264 polymers, where the presence of an additional hydroxyl group (OH) increases the interaction  
265 with proteins. This could explain the generally higher activity of PDs compared to PCs. In  
266 addition, activity is also increased when galloyl groups are present.<sup>21,32-34</sup>

267 Results of the 2 by 2 calculations of Spearman's correlation coefficients as well as  
268 multivariate analyses (PCA) tended to confirm that the *in vitro* AH activity in terms of EC<sub>50</sub>  
269 was related to CT structural features for both *H. contortus* and *T. colubriformis*. In addition,  
270 our results suggest that different mechanisms appear to be involved for each nematode  
271 species. For *H. contortus*, AH activity appeared stronger for CTs with higher PD contents  
272 and larger sizes (mDP values). Although, as described by Williams et al.<sup>20</sup> there was no  
273 effect of mDP or % PD within F2 fractions on the EC<sub>50</sub> values. For the F1 fractions, lower  
274 EC<sub>50</sub> values were associated with higher % PD and larger tannins (higher mDP values).  
275 Novobilský et al.<sup>10</sup> suggested that mDP was a key factor in the LFIA against L3 of *O.*  
276 *ostertagi* and *C. oncophora* after testing *O. viciifolia* extracts and fractions.

277 However, Naumann et al.<sup>19</sup> found no clear evidence for CT size and inhibition of *H. contortus*  
278 motility. However, only a narrow range of CT sizes was investigated. Conversely to the

279 present data, Manolaraki<sup>35</sup> found that lower mDP values were correlated with higher AH  
280 activity when extracts from 40 *O. viciifolia* accessions were tested by LEIA against *H.*  
281 *contortus*. Similarly, Barrau et al.<sup>7</sup> found that a fraction that contained CTs (< 2000 Da) plus  
282 flavonol glycosides had higher AH effects against *H. contortus* larvae than a fraction that  
283 contained only CTs (>2000 Da). At this stage, it is important to note that the complexity of  
284 plant extract compositions and difficulties in purifying CTs are likely to account for some of  
285 these apparent contradictions. Acetone/water extracts from CT-containing plants consist of  
286 CTs plus low molecular phenolic compounds (e.g. flavones, flavonols, flavonol glycosides,  
287 etc). In addition, CTs usually occur as complex mixtures that contain low to high molecular  
288 weight tannins and the mDP-value simply describes the average 'tannin size' rather than  
289 the distribution profile of all CTs. In fact, we recently discovered that mixtures of CTs and  
290 flavonoids had higher AH activities than CTs on their own.<sup>36</sup> Kozan et al.<sup>37</sup> also reported that  
291 flavonol glycosides (luteolin-7- $\beta$ -O-gucopyranoside and quercetin-3-O- $\beta$ -glucopyranoside)  
292 from *Vicia pannonica* var. *purpuracens*, might also participate in the modulation of bioactivity  
293 of the highly AH extract and fractions against trichostrongylid larvae. This underlines that  
294 the proximity of biochemical structure between flavonol glycosides and CT (which are flavan-  
295 3-ols' polymers) could suggest a similar or close mechanism of action for both types of  
296 compounds. Taken together, the presence of non-CT compounds (such as flavonoid  
297 monomers) could, therefore, explain the apparently contradictory observations by  
298 Manolaraki<sup>35</sup> and Barrau et al.<sup>7</sup> The F1 fractions had only half the CT contents of F2 fractions  
299 (**Table 1**). However, the combination of F1+F2 data gave a close to significant correlation  
300 of EC<sub>50</sub> and mDP values (**Table 3**).

301 In contrast, for *T. colubriformis*, % PD was consistently (F1, F2, and combined F1+F2)  
302 related to AH activity. This agrees with other reports on *T. colubriformis* larvae, which found  
303 higher AH *in vitro* effects of PD- compared with PC-rich tannins.<sup>21,22</sup>

304 Interestingly, there were different susceptibilities between the two parasite species, which  
305 suggested that *H. contortus* was more susceptible than *T. colubriformis*. This is indicated by  
306 the overall lower EC<sub>50</sub> values for the abomasal species with both types of CT fractions.  
307 Molan et al.<sup>8</sup> also pointed out that the abomasal nematode *T. circumcincta* was more  
308 susceptible than *T. colubriformis* to the AH effects of flavan-3-ol monomers and oligomeric  
309 CTs in the LMIA. The same conclusion was drawn from *in vitro* studies that examined  
310 extracts from different woody plants (*Rubus fruticosus*, *Quercus robur* and *Corylus*  
311 *avellana*) against *H. contortus*, *T. circumcincta* and *T. colubriformis* based on LMIA and LEIA  
312 tests.<sup>16</sup> However, other authors found no such differences in the response to quebracho or  
313 *O. viciifolia* extracts<sup>11,31</sup> between abomasal or intestinal species. Moreno-Gonzalo et al.<sup>38,39</sup>  
314 even found a higher *in vitro* susceptibility of *T. colubriformis* compared to *H. contortus* and  
315 *T. circumcincta* when measuring the AH activity of extracts from different heather species  
316 (*Calluna vulgaris*, *Erica cinerea* and *E. umbellata*). It remains to be seen whether differences  
317 in assay conditions could account for some of these contradictory results. Moreover, it will  
318 be worth to explore whether exist species specific differences in the quality of larval sheath  
319 proteins between the abomasal vs the intestinal species in order to better understand the  
320 mode of actions of polyphenols against the different GIN species.

321 Although it is difficult to extrapolate from *in vitro* to *in vivo* results, our current data provide a  
322 screening of CT-containing plants, whose AH properties will need to be explored further in  
323 controlled *in vivo* studies in order to develop their potential for on-farm exploitation. It is also  
324 worth noting that the CT fractions from three legumes ranked amongst the most effective  
325 ones (i.e. having the lowest EC<sub>50</sub> values): *L. cuneata* pellets, *O. viciifolia* plants and *T.*  
326 *repens* flowers (**Figure 1**). The last decade has seen an accumulation of *in vivo* results that  
327 confirm the AH effects of *L. cuneata* and *O. viciifolia* against the main GIN species whether  
328 offered to small ruminants in the form of freshly grazed pasture,<sup>40,41</sup> as hay,<sup>15,17,42</sup> as  
329 silages<sup>42</sup> or as pellets.<sup>18</sup>



330 As far as *T. repens* is concerned, no other data are available because the genus *Trifolium*  
331 *sp* is usually considered as a tannin-free legume<sup>43</sup> and consequently the various *Trifolium*  
332 species have received little attention for their antiparasitic potential. However, Carlsen and  
333 Fomsgaard<sup>44</sup> provided an extensive review of the secondary metabolites in *T. repens* and  
334 pointed out the high CT content in flowers. The current study found that CTs from *T. repens*  
335 flowers had a strong AH effect and confirmed the dose-dependent inhibition effects of *T.*  
336 *repens* tannins observed for *C. oncophora* in the LFIA.<sup>45</sup>

337 The CT fractions of *V. paradoxa* were also ranked as highly effective against both nematode  
338 species and suggested that some agro-industrial by-products could be of interest for their  
339 antiparasitic properties. It is worth noting that AH effects on *H. contortus* and *T. colubriformis*  
340 were recently also described not only for cocoa seed but also for husk extracts using the  
341 EHA.<sup>46</sup>

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

#### 351 **ABBREVIATIONS USED**

352 Gastrointestinal nematodes, (GINs); condensed tannins (CT); anthelmintic (AH); mean  
353 degree of polymerization, (mDP); prodelphinidins, (PD); procyanidins, (PC); phosphate  
354 buffered saline, (PBS); larval exsheathment inhibition assay, (LEIA); infective stage  
355 nematode larvae, (L3); effective concentration for 50% inhibition of larvae's exsheathment

356 (EC<sub>50</sub>); larval development inhibition assay, (LDIA); larval feeding inhibition assay, (LFIA);  
357 egg hatch assay, (EHA); larval migration inhibition assay, (LMIA).

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### 363 **SUPPORTING INFORMATION**

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

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### 369 **CONFLICT OF INTEREST**

370 The authors declare no competing financial interest.

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## Figure Legends

**Figure 1:** EC<sub>50</sub> 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*).

Scientific name	Family <sup>23</sup>	Common name/sample	% CT ± SD		mDP ± SD		% PD ± SD		% <i>Trans</i> ± SD	
			F1	F2	F1	F2	F1	F2	F1	F2
<i>Onobrychis viciifolia</i>	Leguminosae	Sainfoin/ w hole plant	37.2 ±4.5	100±4.1	2.8±0.1	8.7±0.01	72±0.3	64.9±0.1	33.3±0.2	20.9±0.3
<i>Trifolium repens</i> *	Leguminosae	White clover/ flow er	11.7±0.4	100±2.4	1.8±0	8.6±0.0	98.3±0.3	98.7±0.0	82.2±0.1	41.1±0.6
<i>Trifolium repens</i> †	Leguminosae	White clover/ flow er	13.4±0.4	82.4± 2.0	3.1±0.1	12.7±0.0	98.1±0.1	98.8±0.0	74.2±0.2	38.2±0.0
<i>Lespedeza cuneata</i>	Leguminosae	Sericea lespedeza/ pellets	42.1±0.2	82.6±1.4	5±0.0	11.3±0.3	92.4±0.1	92.3±0.0	34.7±0.0	24.8±0.2
<i>Betula</i> spp	Betulaceae	Birch/ leaf	12.9 ±0.3	63.6±2.5	2.2±0.0	8.3±0.1	44.7±0.1	58.9±0.1	59.3±0.1	29.3±0.1
<i>Corylus avellana</i>	Corylaceae	Hazelnut/ pericarp	49.2±1.1	67.5±0.6	4.6±0.1	9.2±0.1	18.3±0.9	20.9±0.8	59±0.11	52.2±0.35
<i>Juglans regia</i> L.	Juglandaceae	Walnut/ leaf	21.8±1.4	69.0±1.7	2.9±0.0	12.3±0.1	9.3±0.4	30.9±0.0	56.1±0.0	23.7±0.0
<i>Pinus sylvestris</i> L.	Pinaceae	Pine/ inner bark	54±2	79±2.4	2.3±0.0	6.6±0.2	15.1±0.6	11.2±1.7	51.9±0.7	21.9±1.8
<i>Tilia</i> L.	Tiliaceae	Lime tree/ flow er	47.5±2.8	91.7±3.8	2.0±0.0	7.9±0.1	1.1±0.0	0.9±0.1	16.4±0.0	4.4±0.1
<i>Salix</i> spp	Salicaceae	White w illow / bark	23.1±1.7	83.3±0.6	2±0.1	9.9±0.0	0.0±0.0	6.0±0.0	63.0±0.2	21.9±0.0
<i>Salix babylonica</i>	Salicaceae	Weeping w illow / catkins	40.2±2.8	97.4±2.2	2.9±0.0	8±0.3	24.6±0.1	33±1.7	44.5±0.2	42.3±1.2
<i>Salix caprea</i>	Salicaceae	Goat w illow / leaf	51.5±0.1	83.8±1.8	2.1±0.1	5.3±0.1	5.8±0.3	4.8±0.6	93.2± 0.3	95.8±0.2
<i>Salix caprea</i>	Salicaceae	Goat w illow / tw igs	72±1.1	93.2±11	2.1±0.0	5.3±0.1	15.6 ±0.9	21.3±0.71	59.4±0.1	37.2±0.4
<i>Ribes nigrum</i> *	Grossilariaceae	Black currant/ leaf	59.8±1.3	100±1.7	2.5±0.0	6.5±0.1	93.7±0.07	94.5±0.11	87.2±0.1	93.0±0.1
<i>Ribes nigrum</i> †	Grossilariaceae	Black currant/ leaf	55.5±3.2	77.1±3.9	3.8±0.0	11.8±0.1	94.0±0.0	95.3±0.0	91.5±0.1	81.2±0.1
<i>Ribes rubrum</i>	Grossilariaceae	Red currant/ leaf	57.7±9.1	68.2±1.1	4.9±0.0	10±0.1	85.8±0.4	90.4±0.1	55.7±1.1	35.6±0.9
<i>Theobroma cacao</i>	Malvaceae	Cocoa/ seed	58.5±2.9	75.5±8.1	2.3±0.0	5.4±0.1	0.0±0.2	0.0±0.0	8.7±0.2	3.7±0.1
<i>Vitellaria paradoxa</i>	Sapotaceae	Shea/ meal	33.0±0.6	44.9±0.8	2.2±0.1	4.1±0.1	76.3±0.1	72.5±0.1	41.4±0.3	40.2±0.1
		Mean values	40.2±9.2	81.1±7.4	2.8±0.5	8.4±1.3	44.6±20.2	49.7±19.4	56.2±12.4	39.3±13.3

\*sample a; †sample b

**Table 2:** EC<sub>50</sub> values by parasite and by fraction (F1 or F2) from each tannin-containing resource tested

Plant	Abbreviation	Family <sup>26</sup>	<i>H. contortus</i> EC <sub>50</sub> (95% CI) (µg/ml)		<i>T. colubriformis</i> EC <sub>50</sub> (95% CI) (µg/ml)	
			F1	F2	F1	F2
<i>Onobrychis viciifolia</i>	OVF1/OV2	Leguminosae	62.7 (49.9-76.5)	212 (182-250)	203 (131-322)	147 (99-230)
<i>Trifolium repens</i> (a)	TRaF1/TRaF2	Leguminosae	287 (249-328)	177 (131-239)	110 (82.1-145)	152 (109-210)
<i>Trifolium repens</i> (b)	TRbF1/TRbF2	Leguminosae	37.5 < (0.7 -74.4) *	37.5 < (0.08-42.4) *	132 (92.3-186)	110 (63.2-166)
<i>Lespedeza cuneata</i>	LCF1/LCF2	Leguminosae	78.2 (28.1-157)	37.5 < ( 2.5-55.3) *	198 (108-366)	94.9 (50.5-140)
<i>Corylus avellana</i>	CAF1/C1F2	Corylaceae	166 (82.5-441)	143 (104-170)	351 (287-441)	329 (209-671)
<i>Juglans regia</i> L.	JRF1/JRF2	Juglandaceae	94.7 (65.5-115)	70.6 (46.9-106)	258 (130-386)	243 (169-384)
<i>Betula</i> spp	BPF1/BPF2	Betulaceae	62.8 (58.6-82)	62.6 (19-90.3)	226 (163-335)	125 (86.7-169)
<i>Pinus sylvestris</i> L.	PSF1/PSF2	Pinaceae	236 (192-290)	144 (125-167)	184 (121-305)	135.91 (112-163)
<i>Tilia</i> L. spp.	TF1/TF2	Tiliaceae	113 (82-157)	88.7 (66.1-107)	459 (353-660)	297 (258-335)
<i>Salix</i> spp	SAF1/SAF2	Salicaceae	188 (137-241)	138 (117-154)	300 (271-333)	191 (126-294)
<i>Salix babylonica</i>	SBF1/SBF2	Salicaceae	174 (120-206)	128 (69.8-166)	181 (152-214)	108 (83.8-132)
<i>Salix caprea</i> (twigs)	SCTF1/SCTF2	Salicaceae	195 (142-266)	132 (97.6-184)	385 (296-459)	125 (94.9-159)
<i>Salix caprea</i> (leaves)	SCLF1/SCLF2	Salicaceae	196 (86-217)	161 (133-191)	377 (316-435)	316 (243-420)
<i>Ribes nigrum</i> (sample a)	RNaF1/RNaF2	Grossilariaceae	145 (85-259)	157 (124-203)	145 (123-169)	89.5 (70.1-111)
<i>Ribes nigrum</i> (sample b)	RNbF2/RNbF2	Grossilariaceae	48.7 (78.1-158)	59.2 (18.5-111)	315 (212 -592)	209 (140-344)
<i>Ribes rubrum</i>	RRF1/RRF2	Grossilariaceae	-	97.8 (85.4-305)	130 (84.5-199)	124 (99.5-152)
<i>Theobroma cacao</i>	TCF1/TCF2	Malvaceae	208 (168-246)	65.2 (34.1-95.7)	76.1 (24.3-130)	122 (94.8-200)
<i>Vitellaria paradoxa</i>	VP1/VP2	Sapotaceae	37.5 < (0.7-29.1) *	37.5 < (0.48-36.5) *	169 (115-288)	76 (65.7-86.7)
Mean values			136.9 ± 74.1	108.1 ± 53.2	233 ± 54.3	166 ± 39.9

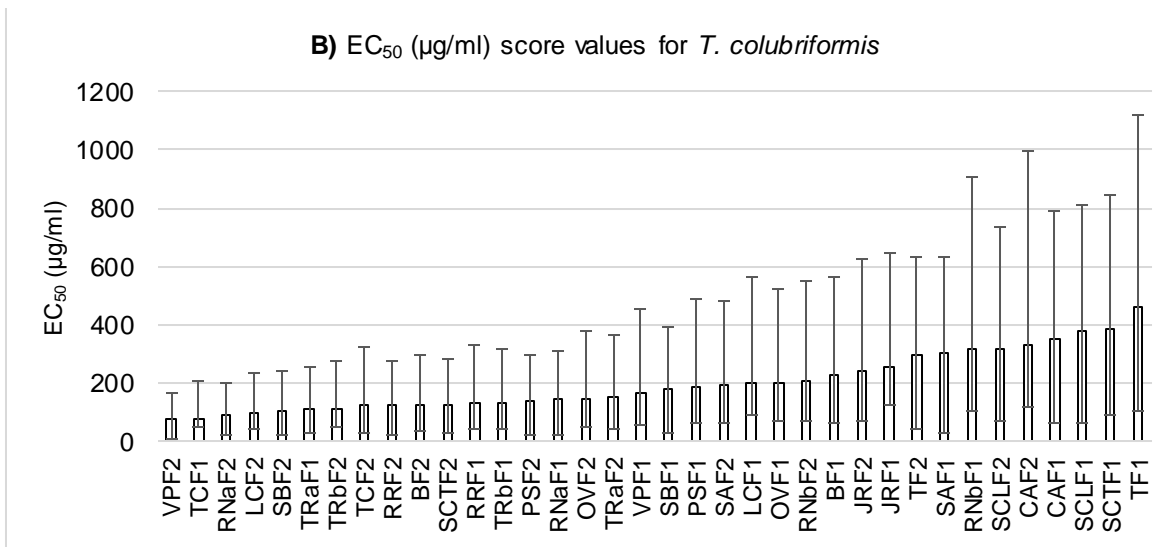
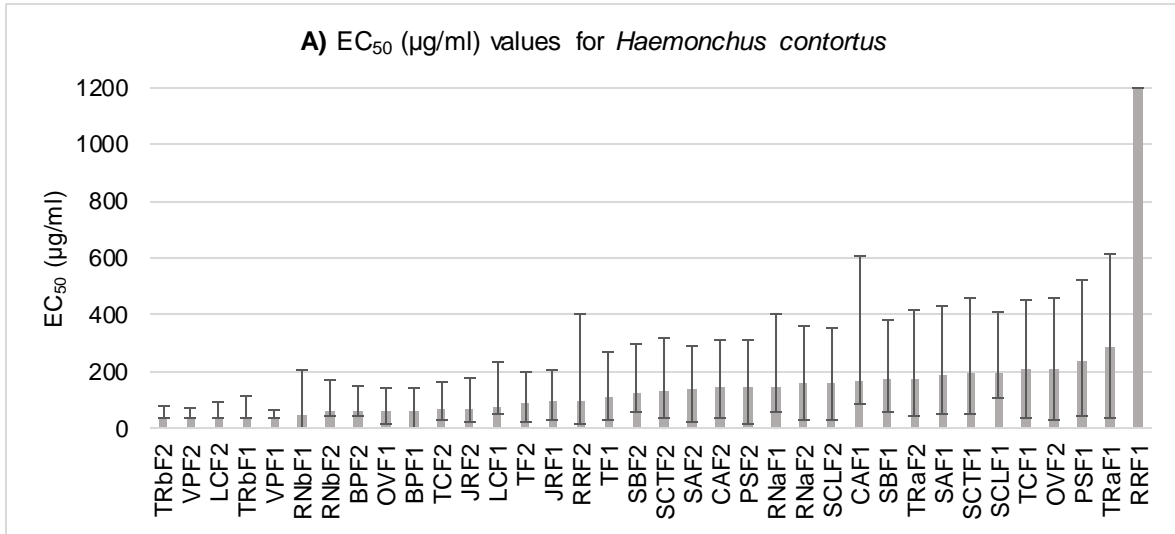
\* the calculation of the EC<sub>50</sub> values relying on the Polo Plus software gave the following values for the effects against *H. contortus* for *T. repens* (b) fraction F1 = 33.2 µg/ml and fraction F2 = 14.5 µg/ml; for *Lespedeza cuneata* fraction F2 = 29,4 µg/ml, for *Vitellaria paradoxa* fraction 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

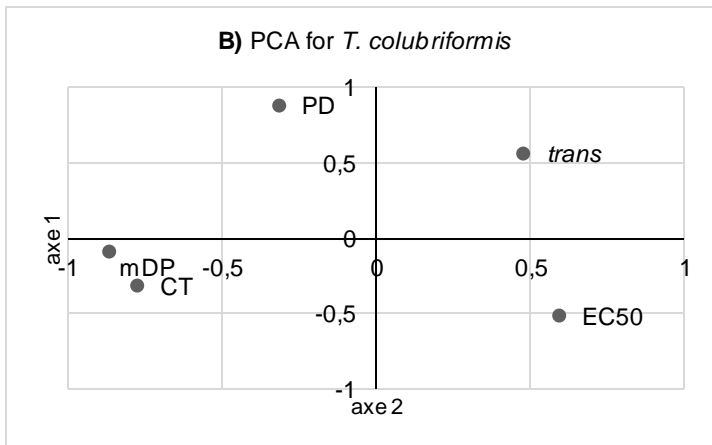
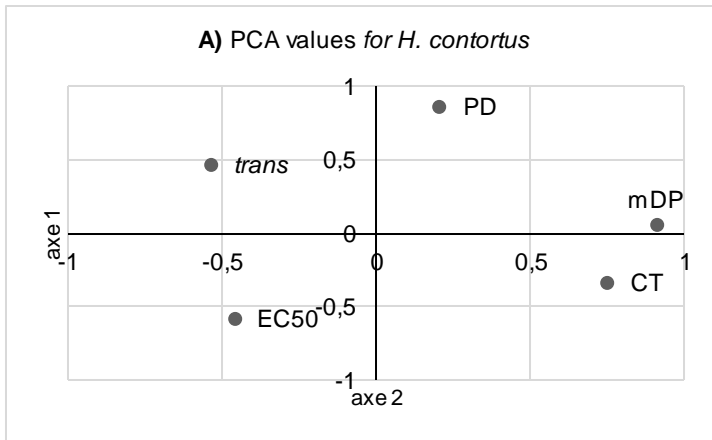
Variable	<i>Haemonchus contortus</i>						<i>Trichostrongylus colubriformis</i>					
	F1		F2		F1 + F2		F1		F2		F1 + F2	
	EC <sub>50</sub> (µg/ml)		EC <sub>50</sub> (µg/ml)		EC <sub>50</sub> (µg/ml)		EC <sub>50</sub> (µg/ml)		EC <sub>50</sub> (µg/ml)		EC <sub>50</sub> (µg/ml)	
	15		16		33		16		16		34	
Degree of freedom (df)	<i>r</i> -value	<i>P</i> -value	<i>r</i> -value	<i>P</i> -value	<i>r</i> -value	<i>P</i> -value	<i>r</i> -value	<i>P</i> -value	<i>r</i> -value	<i>P</i> -value	<i>r</i> -value	<i>P</i> -value
% CT	0.30	0.44	0.61 <sup>a</sup>	0.50	0.12	0.29	0.10	0.43	0.01	0.43	-0.22	0.28
mDP	-0.46 <sup>b</sup>	0.44	-0.28	0.43	-0.33 <sup>b</sup>	0.29	-0.17	0.43	0.19	0.43	-0.26	0.28
% PD	-0.44 <sup>b</sup>	0.44	-0.22	0.43	-0.35 <sup>a</sup>	0.34	-0.46 <sup>b</sup>	0.43	-0.43 <sup>b</sup>	0.43	-0.40 <sup>a</sup>	0.34
% <i>trans</i>	0.08	0.44	0.12	0.43	0.18	0.29	0.12	0.43	-0.01	0.43	0.24	0.28

<sup>a</sup>  $P < 0.05$ , <sup>b</sup>  $P < 0.10$

**Figure 1**



**Figure 2**



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