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Accepted Version

Ropiak, H. M., Desrues, O., Williams, A. R., Ramsay, A., Mueller-Harvey, I. and Thamsborg, S. M. (2016) Structure-activity relationship of condensed tannins and synergism with trans-cinnamaldehyde against Caenorhabditis elegans. Journal of Agricultural and Food Chemistry, 64 (46). pp. 8795-8805. ISSN 1520-5118 doi: https://doi.org/10.1021/acs.jafc.6b03842 Available at https://centaur.reading.ac.uk/67938/

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To link to this article DOI: http://dx.doi.org/10.1021/acs.jafc.6b03842

Publisher: American Chemical Society

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Structure-activity relationship of condensed tannins and synergism with *trans*-cinnamaldehyde against *Caenorhabditis elegans*

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ABSTRACT

Parasitic gastrointestinal nematodes (GIN) of livestock are increasingly developing resistance to synthetic nematocidal drugs. Moreover, the use of nematocides can induce ecotoxicity by affecting free-living nematodes. Condensed tannins (CT) are a structurally diverse group of bioactive plant compounds possessing anthelmintic activity against GIN. We investigated the relationship between the chemical structure of contrasting, purified CT and nematocidal effects using *Caenorhabditis elegans*. We also explored whether the nematocidal activity of CT could synergize with *trans*-cinnamaldehyde (CIN). A non-significant correlation was evident between the ability of CT fractions to inhibit *C. elegans* motility and the molar proportion of prodelphinidin subunits in purified CT samples. Synergistic inhibition of motility was achieved by combinations of CT and CIN. Galloylation of procyanidins was also a key factor for synergy. To increase the nematocidal effect of CT, plant sources containing CT with specific structural features could be selected and combined with compounds acting in synergy.

KEYWORDS

condensed tannins, proanthocyanidins, procyanidins, prodelphinidins, cinnamaldehyde, nematode, *Caenorhabditis elegans*, scanning electron microscopy

INTRODUCTION

The resistance of gastrointestinal nematodes (GIN) in livestock to anthelmintic (AH) drugs is an increasing and wide spread phenomenon.[1, 2] The efficacy of a new AH drug can decline within a decade.[1, 3, 4] In many livestock production systems, AH resistance has reached levels that cause compromised animal welfare and serious economic losses due to mortality and decreased productivity.[5, 6] Phytoparasitic nematodes are also an important economic factor in crop production[7] and are typically controlled by crop rotation and/or treatment with synthetic nematocidal agents.[8] Widespread use of synthetic nematocides in both crop and animal production has raised concerns in terms of adverse effects on human health, other non-target species, environmental impact and ecotoxicity by residues.[9-11]

There is thus a pressing need for alternative control options for GIN and phytoparasitic nematodes, one of which could be the use of natural products,[7, 8, 12, 13] some of which have already been recognized by ethno-veterinary medicine.[13, 14] Bioactive plant compounds such as condensed tannins (CT) can be found in some forages[13] and many medicinal plants.[15] Some crops containing CT are also resistant to parasitic nematode infections, e.g. banana roots against *Radopholus similis.*[16] Nematode-resistant crops may need less or no application of nematocides and can thereby reduce collateral damage, as even some natural product based nematocides may affect non-targeted species.[8] It is well known that some CT have AH activity against various life stages of GIN that infect livestock.[17, 18] Therefore, CT may potentially contribute to sustainable GIN control through their use as nutraceuticals.[13]

Research is still ongoing to pinpoint the structure-activity relationships of CT[19-21] and direct (pharmacological-based) or indirect (immunological-based) mechanisms of their AH action,[22-24] as CT have a variety of different structures. The oligomers and polymers of CT (Figure 1) are composed of monomeric subunits (flavan-3-ols), which can vary in relation to: a) the B-ring, where hydroxylation gives rise to procyanidins (PC) or prodelphinidins (PD);

b) the C-ring, i.e. *cis*-/*trans*-flavan-3-ol stereochemistry or the presence of galloyl groups;[25] or c) the presence of additional A-type linkages between flavan-3-ols.[26] Our group has recently conducted *in vitro* studies on purified CT fractions, where CT structure-AH activity has been investigated on various life stages of GIN affecting cattle [first stage larvae (L1) and adult *Ostertagia ostertagi* and *Cooperia oncophora*],[21] small ruminants (L3 *Haemonchus contortus*[19, 27] and *Trichostrongylus colubriformis*[19]) and pigs (L3 and/or L4 *Ascaris suum*[25, 28]). These studies have demonstrated that CT size (mean degree of polymerization, mDP), galloylation and PC/PD ratio are important factors for bioactivity. Activity also varies between nematode species, life stages (larvae[19, 21, 25, 27, 28] or adults,[21] with the latter generally less commonly studied) and type of assay.

The interactions of CT with other bioactive plant compounds on AH activity are not commonly studied, although these compounds often co-exist in the same plant materials or feed mixtures and may affect the potency of the plant material. It was recently reported that bioactive plant compounds found in the water/acetone extract of wild tamarind leaves or cocoa husks interacted with polyphenols and limited their AH effects on egg hatching of *H. contortus*.[29] In contrast, interactions between CT and some flavonoid monomers resulted in synergistic inhibition of L3 *H. contortus* exsheathment.[27] However, the interactions between CT and other bioactive plant compounds, such as essential oil components, have not yet been investigated. Cinnamaldehyde (CIN) is the main component of the essential oil of cinnamon bark, and is well-known for its anti-bacterial properties and nematocidal activity against root-knot nematodes (*Meloidogyne incognita*[7] and *M. javanica*[30]). Recently, we also demonstrated that CIN has strong AH properties *in vitro* against larvae of pig GIN (*A. suum*, *Oesophagostomum dentatum* and *Trichuris suis*), with the mechanism-of-action qualitatively different to that of CT.[26] Structurally, CIN is chemically distinct from CT and as aldehydes

have shown nematocidal activity before[7, 30] we tested whether CIN could represent a useful tool for assessing synergistic AH effects of CT in combination with this model aldehyde. Our overall aim in the present study was to investigate the relationship between chemical structure of CT and nematocidal effects against the free-living nematode *Caenorhabditis elegans*, and to assess any synergy between selected CT and CIN. *C. elegans* has been used in pharmacological studies of synthetic AH drugs,[31] natural products[32-35] and even high-throughput screening.[36] Studies on the AH properties of CT against adult GIN are scarce[21, 37, 38] and culturing of adult *C. elegans* allowed us to avoid the sacrifice of experimental ruminants or monogastric animals. We screened nematocidal activity of CT in purified fractions derived from model plant materials.[32, 33] These well-defined and highly contrasting CT in terms of size and structure allowed us to directly address the structure-nematocidal activity relationships of CT against an adult-stage nematode in unprecedented detail. The CT were selected to match PC against PD of a similar average size in order to reveal the CT structural characteristic that influence nematocidal activity the most. Additionally, we used a subset of CT fractions to evaluate synergistic effects with CIN.[39]

MATERIALS AND METHODS

Reagents

Sephadex LH-20 was obtained from GE Healthcare (Little Chalfont, UK); acetone (analytical reagent grade) from ThermoFisher Scientific (Loughborough, UK); select yeast extract (BioReagent), peptone from *Glycine max* (soybean), D-(+)-glucose (≥99.5%, GC), cholesterol (95%), monopotassium phosphate, sodium phosphate dibasic dehydrate, sodium chloride, magnesium sulfate, potassium hydroxide, sodium hydroxide, *trans*-cinnamaldehyde (99%), polyvinylpolypyrrolidone (PVPP, cross-linked) and levamisole hydrochloride (≥99%) from Sigma-Aldrich (Brøndby, Denmark); hemoglobin (from bovine blood, lyophilized,

≥90%) from Fluka (Sigma-Aldrich, Brøndby, Denmark); ethanol (96%) from CHEMSOLUTE® (Th. Geyer GmbH & Co., Renningen, Germany); and household bleach (5% solution of sodium hypochlorite) from døgnNetto (Copenhagen, Denmark).

Condensed Tannin Fractions

Plant material was obtained and prepared as previously described.[25, 26, 28, 40] Briefly, acetone/water extracts were fractionated on Sephadex LH-20. The resulting CT fractions F1, F2, F3 (eluted by 30, 50, 80% acetone/water, respectively[40]) were analyzed by derivatization with benzyl mercaptan, RP-HPLC and LC-MS.[15] The freeze-dried fractions used in this study were cocoa bean F2,[28] *Tilia* flower F2, white clover flower F2, birch leaf F2, blackcurrant leaf (no. 1) F2, blackcurrant leaf (no. 2) F2 and F3, redcurrant leaf F2, yellow iris leaf F2, sainfoin aerial part F2 and great water dock root F2.[40]

Maintenance and Isolation of *C. elegans*

C. elegans strain N2 (wild type) was obtained from Biotech Research & Innovation Centre (BRIC), University of Copenhagen, Denmark. Nematode eggs were collected as described previously.[41] Briefly, agar confluent plates were washed with sterile dH_2O, and the liquids were collected in a sterile 15 mL Falcon tubes and diluted with dH_2O up to 3.5 mL. A fresh mixture of 0.5 mL 5 M NaOH with 1 mL bleach was added, vortexed for a few sec every 2 min for a total of 10 min. Tubes were centrifuged for 30 sec at 1300 x g to pellet released eggs, the supernatant was removed to 0.1 mL and sterile dH_2O was added to 5 mL and vortexed (wash was done twice). Eggs were transferred to a sterile liquid medium.[33]

C. elegans were maintained in axenic culture medium as described previously,[33] with following changes: during the isolation step (see below) of the young adult and adult nematode stages (henceforward referred to as adults) the larvae were kept, replenished with fresh culture medium and cultured in 50-100 mL sterile Duran bottles for 6-7 days prior to experiments.

Adults were isolated as described previously,[33] with changes e.g. to the sieves, nylon mesh sizes or wash steps. In brief, sieves were composed of a modified 50 mL Falcon tubes and nylon mesh sizes: 28 µm (sieve A) and 50 µm (sieve B), as nematodes in this study were smaller (~35 x 1000 µm). Sieves were disinfected by soaking in 70% ethanol. On average, six Duran bottles were combined and passed through the sieve A, filtrate was collected and larvae were cultured further (see above). The sieve A was washed with sterile dH_2O to remove any remaining larvae. Retained adult worms were placed onto fresh sieve A and washed with sterile dH_2O. Adult worms were then transferred onto a sieve B, which was immobilized by pegs in a 100 mL sterile beaker containing 50 mL of filter-sterilized M-9 medium. After 15 min, the adult nematodes migrated into the beaker and were passed through a fresh sieve B. Filtrate with adult nematodes was placed in 50 mL Falcon tube for sedimentation for 15 min. The upper phase was removed and 1 mL of suspension was left and used for the nematocidal assay.

Adult *C. elegans* Motility Inhibition Assay

The assay was performed as described previously.[33] In brief, filter-sterilized M-9 medium[33] was used as a measurement media and diluent for the tested CT fractions. The assay was performed in a 48-well plate with a total volume of 250 µL/well, i.e. 20 µL of nematode suspension (~50-200 nematodes per well) and corresponding tested CT fraction or medium. CT fractions were used in a range of dilutions at 0, 0.0625, 0.125, 0.25, 0.5, 1.0, 1.5 mg/mL; with 3 replicates per treatment. All CT fractions were soluble under the assay conditions. The negative control was M-9 medium and the positive control was 8 mg/mL of levamisole.[32] The 48-well plates were sealed with parafilm to avoid evaporation and incubated at 24 ºC for 24 h in the dark. CIN was used in a range of concentrations 0-3 mM in three independent experiments (with 3 replicates per treatment) to evaluate EC_{25} and EC_{50} (Figure S.1). The concentrations related to EC_{25} (0.605 mM) and EC_{50} (0.712 mM) were further used in

synergy experiments, and EC_{25} and at 2 mM (where the complete motility inhibition was observed) in scanning electron microscopy (SEM) imaging.

Plates were placed at ambient conditions for 10 min prior to measurement and read by using an inverted microscope. Non-motile nematodes had no tail, head, or pharyngeal movements during 5 sec of observation.[33] False positive results were avoided by differentiation of movement caused by larvae hatched from eggs inside a dead nematode.[33] The negative control was between 80-100% motile and the positive control was 0% motile.

Condensed Tannin Depletion by PVPP Pre-treatment. PVPP pre-treatment was used to check for the sample matrix effect on nematode motility,[42] with changes to remove small PVPP particles. In brief, 150 mg of PVPP was mixed with 2 mL of measurement buffer and purified by centrifugation (3 min, 3000 rpm). The supernatant (0.9 mL) was removed and 0.9 mL of fresh buffer was added, vortexed, centrifuged and these steps were repeated 3 times after which PVPP was left in 1 mL of buffer. Corresponding CT fractions were dissolved in 1 mL of buffer at a final concentration of 1.5 mg/mL. Dissolved CT fractions, PVPP pre-treated CT fractions and control (buffer only) were incubated at 4 °C overnight.[42] Solutions were vortexed, centrifuged and the supernatant was used in motility inhibition studies. PVPP pre-treated and untreated CT fractions in triplicates were used in one independent experiment at assay conditions.

Synergy Experiments

The 48-well plate was prepared at the same assay conditions as described above. The treatments in the synergy experiment consisted of: a) a range of dilutions of a CT fraction in triplicates (3 wells at each concentration), b) a range of dilutions of a CT fraction (3 wells at each concentration) combined with a concentration of CIN at the EC_{25} value (equal to 0.605 mM) or the EC_{50} value (equal to 0.712 mM), c) CIN alone at the EC_{25} or EC_{50} values (5

168 wells), d) negative controls (6 wells), e) a positive control (1 well). The experiment was
169 repeated and showed similar trends as in Figure 5-A, C (data not shown).

170 **Scanning Electron Microscopy Imaging**

171 The nematodes were incubated at assay conditions with the selected CT fractions at 1.5
172 mg/mL with and without CIN at EC_{25}, negative control, CIN at EC_{25} and 2 mM in 24-well
173 plates. After 24 h of incubation the nematodes were washed twice with PBS, fixed in 2%
174 glutaraldehyde in 0.05 M phosphate buffer[28] and kept at 4 °C until processed for SEM
175 imaging as described previously.[43] The effect of each compound was evaluated by observing
176 20 nematodes per treatment. The semi-quantitative evaluation was based on comparison of
177 the severity of damage to circumferential ridges (annuli) and furrows of the cuticle and
178 ranking it against the control nematodes.

179 **Data Analysis and Statistical Analysis**

180 Results from motility inhibition assay were normalized to the negative control at 100%
181 motile. CT study: all measurement data were corrected to CT content (CT g/100 g of fraction)
182 and fitted to a single sigmoid function to calculate effective concentrations to inhibit
183 nematode motility at 50% (EC_{50}) for each CT fraction (Table 1). Standard deviations were
184 calculated from two or three independent experiments. One independent experiment was
185 composed of triplicate measurements at each CT concentration treatment. CIN study:
186 measurement from three independent experiments was averaged and fitted to a single sigmoid
187 function. EC_{25} and EC_{50} values were derived from the fit (Figure S.1). All fitting was done
188 using Pro-Data™ Software Suite (Applied Photophysics Ltd, Leatherhead, UK).
189 Shapiro-Wilk test of normality was used (p>0.05; df=9) for mDP-values, molar percentages
190 of PC and *cis*-flavan-3-ols, EC_{50} (mg/mL) which excluded PC and EC_{50} as normally
191 distributed (natural log transformation did not change the outcome) and Spearman's rho was

192 determined to test for statistical significance (2-tailed test, $p<0.05$) using the IBM® SPSS®
193 Statistics version 21 software.
194 The predicted inhibitory effects of CT with CIN at EC_{25} or EC_{50} were calculated by Bliss
195 independence[44] and compared to the observed effects as previously described.[27, 45] In general,
196 an observed result with a significantly greater effect than the additive effect indicates
197 synergy. A significantly lower effect indicates antagonism. The effect of synergy was
198 assessed by a two-way ANOVA with Bonferroni post-hoc testing. Graphpad Prism version 6
199 (GraphPad Software Inc., USA) was used for the statistical analysis.
200
201 **RESULTS AND DISCUSSION**
202 **Motility Inhibition by Condensed Tannins**
203 The CT characteristics in purified fractions represented a broad range in terms of a) size:
204 mDP values ranged from 5.1-16.6 and b) structure: PD contents ranged from 0-99%, *cis*-
205 flavan-3-ol content ranged from 12-96% and included one PC with 54% galloylation (Table
206 1). Among these samples, PC content was not correlated with mDP (r_s=-0.464; p=0.205;
207 df=9); however, it was correlated with *cis*-flavan-3-ol content (r_s=0.767; p=0.016; df=9),
208 therefore this sample set was suitable for testing the mDP and PC effects separately. The use
209 of a range of dilutions of CT fractions allowed us to obtain motility inhibition curves for adult
210 *C. elegans* (Figures 1 and 2) and estimation of EC_{50} values (Table 1). An inhibition of
211 motility of 100% was reached for CT of high and moderate PD content from redcurrant leaf
212 F2 at 1.4 mg/mL, blackcurrant leaf (no. 2) F2 at 1.3 mg/mL, birch leaf F2 at 0.64 mg/mL and
213 yellow iris leaf F2 at 0.8 mg/mL (Figure 2). PC from cocoa bean F2 and *Tilia* flower F2 were
214 clearly less potent than PD at similar CT concentrations (Figure 3-A, B). A two-fold increase
215 of the CT concentration did not induce further inhibition of motility for PC from cocoa bean
216 F2 (2.3 mg/mL, Figure 3-D) and only up to ~80% inhibition of motility was reached for PC

from *Tilia* flower F2 (to 2.8 mg/mL, Figure 3-D). The galloylated PC from great water dock root F2 (at 1 mg/mL, Figure 3-C) were more effective in inhibiting the motility than the non-galloylated PC (Figure 3-A, D); however, they were not as effective as CT with moderate or high PD percentages (Figure 2). These CT results do not corroborate findings from a report on the ability of flavan-3-ols at 0.5 mg/mL to inhibit the migration of *T. colubriformis* L3; the monomeric subunits of PC, i.e. catechin and epicatechin, gave almost the same result as epicatechin gallate[46] (a subunit of galloylated PC from great water dock root F2[40]). The inhibition effect of epicatechin gallate was lower than epigallocatechin and higher than gallocatechin, which are the subunits of PD.[46]

The fit of the EC_{50} values plotted versus PD content showed a clear but non-significant negative relationship (R^2=0.98; r_s=-0.617; p=0.077; df=9; Figure S.2). This indicated that the motility of adult *C. elegans* was affected more by fractions containing PD-rich CT compared to PC-rich CT, as seen previously with adult and L1 of *C. oncophora*.[21]

It was previously reported that CT from Japanese red pine had increasing mortality effects on *C. elegans* with increasing polymer size, whereas PC dimers and a trimer affected only motility.[47] We did not observe any correlation between EC_{50} values and other CT characteristics such as mDP (r_s=-0.317; p=0.406; df=9). Furthermore, CT derived from the same plant material, blackcurrant leaf (no. 2) F2 and F3 with similar PD and *cis*-flavan-3-ol contents but of different mDP-values (mDP 7.8 and 16.6, respectively), resulted in a similar motility inhibition profile (Figure 2-D) and EC_{50} values (0.19 and 0.20 mg/mL, respectively). This indicated that nematode motility was not affected by CT size, although higher mDP values of 11.0-16.6 achieved more consistent results. The absence of a size effect agrees with the literature as CT F1 and F3 fractions from shea meal with comparable PC content (23.7 and 26.8 %, respectively) and galloylation (28.8 and 37.5 %, respectively) had similar potency against the migration of *A. suum* L3 despite their average size differences (mDP 2.2

and 7.7, respectively).[25] This can also be supported by a report on L4 and young adult *C. elegans*, which had been exposed to a range of PC oligomers at 1 mM for 72 h. Similar survival rates were observed for tetrameric to decameric and polymeric PC.[48] The *cis*-flavan-3-ols also did not contribute to the nematocidal effect (r_s=-0.183; p=0.637; df=9).

Next, we investigated if nematode motility inhibition was due to CT. Selected CT fractions at 1.5 mg/mL [birch leaf F2, great water dock root F2, blackcurrant leaf (no. 1) F2, *Tilia* flower F2, used later in synergy studies] were pre-treated with PVPP to check for the sample matrix effect as PVPP removes CT selectively.[42] It is known that PVPP can also bind to flavonol glycosides;[49] however, these compounds were not detected in the purified CT fractions (data not shown). The resulting preparations did not reduce the motility of nematodes significantly (92-100% motile), compared to untreated CT fractions (0-22% motile) indicating that the inhibition of nematode motility was due to the CT in the fractions. For example, birch leaf F2 inhibited nematode motility by 100% and there was no inhibition of motility observed after pre-treatment of this CT fraction with PVPP (Figure 4).

Synergistic Effect of Condensed Tannins with Cinnamaldehyde

To evaluate the importance of structural features of CT on a potential synergistic effect with CIN we selected B-type CT with high, moderate and low PC contents: *Tilia* flower F2 had 99% of CT as PC, birch leaf F2 had 41% as PC and blackcurrant leaf (no. 1) F2 had 5% as PC. As we evaluated above, the CT size was not the main nematocidal factor in this experimental set up and, therefore, all selected B-type CT samples were chosen with moderate mDP-values (7.9, 8.3 and 11.8, respectively). Additionally, we included PC with galloylated subunits (54%) from great water dock root F2, to evaluate effects of galloylation. We used CIN as a model for an essential oil component, as these are commonly used as feed additives in order to achieve higher intakes or digestibility of organic matter.[39] The CIN

selection for synergistic studies was driven by our previous findings, where cinnamon bark extract at 0.5-2 mg/mL inhibited the motility of L4 *A. summ* after 24 h exposure *in vitro*.[26] This extract was composed mainly of PC with A- and B-type linkages and CIN (24.2 g/100 g and 7.8 g/100 g of the extract, respectively). A high AH potency was attributed to the CIN. We showed that pure CIN had *in vitro* AH activity against larvae of *A. suum* (L3, L4), *O. dentatum* (L3) and *T. suis* (L1)].[26] Additionally, CIN nematocidal activity affected second stage juveniles motility of *M. incognita*;[7] and mobility of *M. javanica*;[30] and doses of >0.01 mM might induce nematocidal toxicity in adult *C. elegans*.[50]

The observed effect of the CT/CIN mixture on motility was compared to the predicted additive effect. A clear synergy was observed between galloylated PC from water dock root F2 and CIN against motility of *C. elegans* (Figure 5). The observed effect was significantly higher than the predicted additive effect at CT concentrations of 0.16-0.96 mg/mL with CIN at EC_{25} (Figure 5-A), which demonstrated a significant synergy (e.g. 0.96 mg/mL, $P<0.001$). No synergy was observed at lower concentrations of CT. When the concentration of CIN was increased to the EC_{50}, synergy was observed even for lower concentrations of CT (0.08 mg/mL, $P<0.05$), Figure 5-B. The synergistic effect at the highest CT concentration (0.96 mg/mL) used was close to the predicted additive range; the likely reason is that synergy was not apparent due to the high concentrations of CT and related high motility inhibition.

A non-galloylated CT with high PC content from *Tilia* flower F2 (99% PC) did not exhibit synergy with CIN at EC_{25} (Figure 5-C), even though the CT concentration range was higher (up to 1.4 mg/mL). This is consistent with previous work in which the presence of A-or B-type PC (100% PC) did not enhance the AH effect of CIN in cinnamon bark extract.[26] Therefore, it can be concluded that galloylation of PC played a crucial role in the synergy with CIN. However, it is possible that the 11% of PD in CT from great water dock roots F2

(Table 1) may also have contributed to this synergistic effect, which should be investigated further.

The other two B-type CT of low and moderate PC from blackcurrant leaf (no.1) F2 and birch leaf F2 exhibited similar pattern in terms of inhibition of motility (EC_{50}=0.14 mg/mL, n=3, Table 1) and interaction with CIN at EC_{25}. There was a synergistic effect at low concentrations of CT from blackcurrant leaf (no. 1) F2 (0.19 mg/mL, $P<0.05$) and birch leaf F2 (0.16 mg/mL, $P<0.001$) with CIN at EC_{25} (Figure S.3).

There was no antagonistic effect of any of the CT tested with CIN at EC_{25} or EC_{50}. Overall, the most promising effect in synergy experiments was seen with purified CT from great water dock root F2 (Figure 5-A).

AH activity of CIN *in vivo* could not be demonstrated against the intestinal parasite *A. suum* in pigs,[26] which could be due to degradation or adsorption to matrix components as shown in *in vitro* simulations of gut fermentation.[51] However, CIN could still prove useful against other parasites if formulated to increase its stability and thereby reducing its disappearance from the digestive tract.[51] At high dose levels (above 1.6 g/day) CIN compromises the food intake or ruminal digestion[39] and this will need to be taken into account when developing strategies for CIN-CT applications.

CT bioavailability was higher in the abomasum of ruminants than in the intestine and was linked to an AH effect against *O. ostertagi* residing only there (in the abomasum).[52] Pepsin-resistant microencapsulation of CT[53] could potentially be employed to increase the availability of CT in the intestine. Furthermore, synergistic effects of CT and CIN could perhaps be used to lower the doses of both CT and CIN while maintaining an AH effect.

Changes to Cuticle and Sensilla of Lip Region Observed by Scanning Electron Microscopy

316 SEM investigation showed that all treatments with CT fractions (1.5 mg/mL) induced
317 structural changes to the cuticle and sensilla of the lip region. For example, the surface of the
318 cuticle became shriveled with moderately uniform ridge formations compared to the smooth
319 cuticle of the negative control (Figures 6 and 7-C). As the damage to the sensilla of the lip
320 region varied greatly between each CT treatment, we decided to use circumferential ridges
321 (annuli) and furrows of the cuticle as a comparative factor to evaluate the visible changes.
322 The severity of the damage to the cuticle was ranked in the following order: *Tilia* flower F2
323 (1.38 CT mg/mL, Figure 6-C) < blackcurrant leaf (no. 1) F2 (1.16 CT mg/mL, Figure 6-G) <
324 birch leaf F2 (0.95 CT mg/mL, Figure 6-E) < great water dock root F2 (0.96 CT mg/mL,
325 Figure 7-C). Although *Tilia* flower F2 had the highest CT concentration it was the least
326 effective, which agrees with the motility studies, where PC-rich CT were also least effective
327 (Table 1). However, the birch leaf F2 with a moderate PD (59%) level had the most potent
328 effect on the cuticle among these B-type samples (Figure 6-E). The severe changes to the
329 cuticle are in line with a previous report, where lesions were present on the cuticle of female
330 adult *H. contortus* after 24 h *in vitro* exposure to 1.2 mg/mL of sainfoin leaf extracts,[54] which
331 are known to be PD-rich.[55] Surprisingly, the highest level of disruption to cuticle integrity
332 was seen with galloylated PC (from great water dock root F2, Figure 7-C), despite the fact
333 that this sample was among the least effective in the motility study even at the highest
334 concentration (Figure 3-C), which was the same concentration used in SEM study (0.96 CT
335 mg/mL). It was shown previously that galloylation of flavan-3-ols enhanced AH effects; for
336 example, epicatechin gallate was more effective at inhibiting the exsheathment of *H.*
337 *contortus* and *T. colubriformis* L3 than epicatechin,[56] and gallocatechin gallate or
338 epigallocatechin gallate were slightly more effective in reducing the feeding of an *O.*
339 *ostertagi/C. oncophora* L1 mixture than gallocatechin or epigallocatechin.[21] Galloylated PD

340 from shea meal F2 were also the most potent CT at inhibiting the exsheathment of *T.*
341 *colubriformis* L3 compared to other non-galloylated CT.[19]
342 In the present study we observed that CT with an increasing molar percentage of PD provided
343 greater inhibition of the motility of adult *C. elegans*, which is in line with a previous report
344 on L1 and adult *C. oncophora* motility. However, mDP affected feeding inhibition of L1 of
345 *O. ostertagi* and *C. oncophora*.[21] CT have a high affinity to proline-rich proteins[40] and the
346 nematode cuticle is mostly composed of collagen-like, proline-rich proteins and structural
347 proteins such as cuticlins.[57, 58] The buccal cavity cuticle is lined with for example, prostom,
348 mesostom or arcade cuticles and has a different protein composition than the body cuticle,
349 which also covers the lip region.[59, 60] Given that the protein composition of the cuticle differs
350 between the developmental stages of nematodes,[60] it is conceivable that the importance of
351 specific CT features varies between life cycle stages as the CT-protein affinities may be
352 altered. The epicuticle, i.e. the outer layer of the cuticle, contains cuticlins with a minimal
353 consensus peptide motif rich in proline in *C. elegans* or *A. suum*.[61] In addition, the epicuticle
354 is lipid-rich with negatively charged glycoprotein-rich surface coat[57] and the composition of
355 lipid-rich layer varies among nematode species.[62] CT can also interact with lipid bilayers[63] or
356 lipid rafts mainly by binding to cholesterol,[64] which can be found in cuticle of some
357 nematodes.[62] Therefore, the primary interactions with CT are likely to occur with cuticlines
358 and by CT insertion into the (glycosylated) lipid layer.[63] Secondary interactions with the
359 proline-rich layers of collagen would further disturb the hypodermal cells as already observed
360 in *A. suum* after CT treatment.[28]
361 CIN induced a less pronounced change to the cuticle than CT and there was a dose dependent
362 effect as seen after treatment with CIN at the EC_{25} or 2 mM (Figures 7-E, G); however, there
363 were no noticeable changes to the mouth region (Figures 7-F, H). Our group reported
364 previously ultrastructural changes to the cuticle of *A. suum* L4 after exposure to CIN by

transmission electron microscopy and the effect on furrows[26] was more subtle compared to changes after exposure to CT from hazelnut pericarp.[28] In the present study we used 3 and 15 times higher CIN concentrations and doubled the exposure time before SEM analysis, which still resulted in less pronounced changes to the cuticle (Figure 7-E, G) compared to nematodes treated with CT (Figure 7-C and S.3).

After combining great water dock root F2 (0.96 CT mg/mL) and CIN at EC_{25} there was a slight alteration to the lip region (Figure 7-J), which was not as pronounced as after treatment with CT on its own (Figure 7-D). The cuticle shriveled (Figure 7-I) in the same way as observed after CT treatment (Figure 7-C) and additional ruptures between circumferential ridges were visible (Figure 7-K). Thus, these results demonstrated that the nematocidal mode of action of CT and CIN occurred via distinct mechanisms, and treatment of nematodes with both CT and CIN causes a combination of the ultrastructural damage observed with each treatment individually. This apparent difference in mechanistic activity between CT and CIN may partly explain the possible synergistic anthelmintic effects of these molecules.

In conclusion, we demonstrated that CT containing a higher proportion of PD had the most pronounced effect on the motility of adult nematodes which may provide practical information for plant breeding programs that seek to reduce the use of nematocides. We showed that nematocidal synergy can occur at low concentrations of both galloylated PC and CIN. Based on this, it may be possible to develop better nutraceuticals, which offer a balance between AH and nutritional effects. This could be especially relevant for bioactive plant components with a moderate range of AH activities (e.g. 50-70%), as higher CT or CIN concentrations can lead for example, to lower food intake.[39, 65] Alternatively, bioactive plant compounds could be used as feed supplements targeting one particular life cycle stage of one particular species, which could reduce general AH resistance of other species. It would be

worthwhile to investigate further the mode of nematocidal action or associated cellular pathways of CT and CIN by employing *C. elegans* drug resistant strains[34] or mutants.[36] Screening of CT in the presence of other plant compounds could help to identify, which combinations can act synergistically towards nematocidal effects. The use of *C. elegans* can further reduce the number of experimental ruminants or monogastric animals that would need to be sacrificed for drug development, e.g. for *in vivo* AH studies against adult GIN.[38, 66] Also, donor animals used to obtain parasitic specific nematode eggs for any initial *in vitro* studies would not need to be maintained. This approach promotes the 'three Rs' concept (replacement, refinement and reduction of animals in research)[67] and should result in decreased time and costs of future developments in the field of parasite control.

ABBREVIATIONS USED

AH – anthelmintic; ANOVA – analysis of variance; CIN – cinnamaldehyde; CT – condensed tannins; EC_{25} and EC_{50} – effective concentration to inhibit nematode motility at 25 and 50%; GIN – gastrointestinal nematodes; L1, L3 and L4 – first stage larvae, third stage larvae and fourth stage larvae; mDP – mean degree of polymerization; PC – procyanidins; PD – prodelphinidins; PVPP – polyvinylpolypyrrolidone; SEM – scanning electron microscopy.

AUTHOR CONTRIBUTIONS

HMR, OD, ARW, IMH and SMT conceived the study. HMR and OD designed the study. HMR and ARW designed the synergy study. HMR prepared and analyzed fractions, carried out the study and analyzed the data. ARW contributed to statistical analysis and performed electron microscopy imaging. AR prepared and analyzed cocoa bean F2. HMR wrote the manuscript with inputs from ARW, IMH and SMT. All authors critically read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank Teresa D. Ropiak, Marianne Oraviita, Christos Fryganas and Peter Davy for help in obtaining plant material; Teresa Rojo Romanos for obtaining *C. elegans*; researchers at the Core Facility for Integrated Microscopy, University of Copenhagen; Lise-Lotte Christiansen and Helena Mejer for technical help; Peter Nejsum, Peter Lachmann and Sundar Thapa for useful discussions; Chaweewan Klongsiriwet, Christopher Drake and Parwin H Majid for general help.

Supporting Information description

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Estimation of EC_{25} and EC_{50} values for cinnamaldehyde (Figure S.1); The relationship between the molar percentage of prodelphinidins (PD) of condensed tannins and EC_{25} (effective concentration at 25% reduction of *C. elegans* motility) (Figure S.2); Synergistic effects of CT with high or moderate molar percentages of PD on *C. elegans* motility in the presence of CIN (Figure S.3).

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Acknowledgment for funding sources

This study was supported by an EU Marie Curie Initial Training Network (PITN-GA-2011-289377, "LegumePlus") and the Danish Council for Independent Research (Technology and Production Sciences, Grant # 12–126630).

Notes

The authors declare no competing financial interest.

643 **Figure captions**

644 **Figure 1.** Example of B-type condensed tannin. Substitution of R_4 or R_5 by galloyl group
645 results in galloylation of condensed tannin.

646

647 **Figure 2.** Effects of condensed tannins (CT) rich or moderately rich in prodelphinidins on
648 adult *C. elegans* motility; (A) white clover flower F2 (n=2), (B) redcurrant leaf F2 (n=2), (C)
649 blackcurrant leaf (no. 1) F2 (n=3), (D) blackcurrant leaf (no. 2) F2 and F3 (n=1, for each
650 sample), (E) birch leaf F2 (n=3), (F) sainfoin aerial part F2 (n=2), (G) yellow iris leaf F2
651 (n=1); where, n is the number of independent experiments shown with different
652 markers/lines; the error bars show the standard deviation from triplicate measurements at
653 each CT treatment of one independent experiment; data have been corrected to CT content
654 (CT g/100 g of fraction).

655

656 **Figure 3.** Effects of procyanidin-rich condensed tannins (CT) on adult *C. elegans* motility;
657 (A) results of three independent experiments (n=3) with cocoa bean F2, (B) *Tilia* flower F2
658 (n=3), (C) great water dock root F2 (n=3), (D) cocoa bean F2 and *Tilia* flower F2 (n=1, for
659 each sample at double the concentration of that used in A and B); where, n is the number of
660 independent experiments shown with different markers/lines; the error bars show the standard
661 deviation from triplicate measurements at each CT treatment of one independent experiment;
662 data have been corrected to CT content (CT g/100 g of fraction).

663

664

Figure 4. Motility of adult *C. elegans* after exposure to condensed tannin (CT) fractions (1.5 mg/mL) with or without polyvinylpolypyrrolidone (PVPP) pre-treatment; negative control – 100% motile; data from one independent experiment (number of wells: n=6 for negative control, n=6 for PVPP control, n=3 for PVPP/CT fraction or CT fraction).

Figure 5. Synergistic effects of condensed tannins (CT) and cinnamaldehyde (CIN) added at effective concentrations (EC) on *C. elegans* motility: (A) great water dock root F2 and CIN at EC_{25}; (B) great water dock root F2 and CIN at EC_{50}; and (C) *Tilia* flower F2 and CIN at EC_{25}. One independent experiment, where the error bars show the standard deviation from triplicate measurements at each CT treatment; data are corrected to CT content (CT g/100 g of fraction). Asterisks indicate that the predicted additive and observed values differed significantly (*** $P<0.001$; ** $P<0.01$; * $P<0.05$ by two-way ANOVA with Bonferroni post-hoc testing). Repetition of experiment (A) and (C) gave a similar pattern (data not shown).

Figure 6. Scanning electron microscopy of cuticle surface (left column: A, C, E, G) and sensilla of the lip region (right column: B, D, F, H) of adult *C. elegans*, after treatment with condensed tannin (CT) fraction (1.5 mg/mL); (A, B) control (buffer), (C, D) *Tilia* flower F2 (1.38 CT mg/mL), (E, F) birch leaf F2 (0.95 CT mg/mL), (G, H) blackcurrant leaf F2 (1.16 CT mg/mL); scale bars 5 μm; magnification: 3500x for D, E, G; 5000x for A-C, F, H.

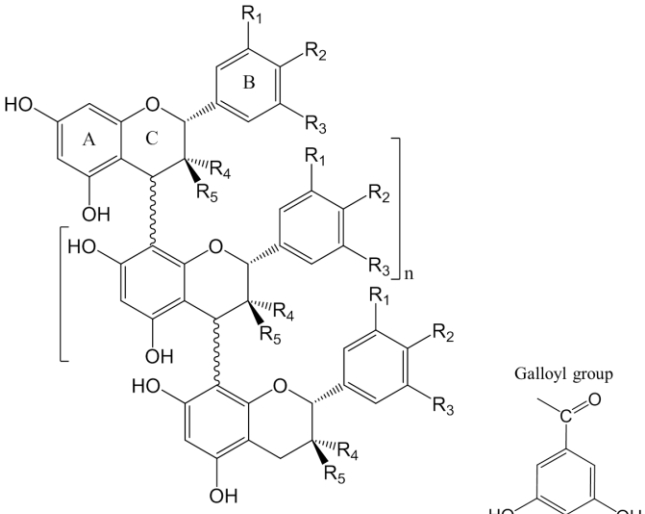
Figure 7. Scanning electron microscopy of cuticle surface (left column: A, C, E, G, I, K) and sensilla of the lip region (right column: B, D, F, H, J) of young adult and adult *C. elegans*, after treatment with condensed tannin (CT) fractions (1.5 mg/mL) or/and cinnamaldehyde (at EC_{25} or 2 mM), where (A, B): control (buffer), (C, D): great water dock root F2 (0.95 CT mg/mL), (E, F): cinnamaldehyde at EC_{25}, (G, H): cinnamaldehyde at 2 mM, (I-K): great water dock root F2 (0.95 CT mg/mL) and cinnamaldehyde at EC_{25} (0.605 mM); scale bars 5

μm (or 2 μm for D-H; 10 μm for K); magnification: 3500x for (K); 5000x for (A-C, I); 6500x for (D-G, J); and 8000x for (H).

Table 1. Condensed tannin (CT) content; mean degree of polymerization (mDP); molar percentages of: procyanidin, *cis*-flavan-3-ols and galloylation in fractions from various plant materials and estimated effective concentration of CT to reduce *C. elegans* motility by 50% (EC_{50}).

CT fraction	CT (g/100 g of fraction)		mDP		PD (%)		*cis* (%)		% galloylation		ref.	EC_{50} (mg/mL) [a]		
														n
great water dock root F2	63.7	(±1.7)	5.1	(±0.1)	11.4	(±0.1)	95.6	(±0.1)	54.3	(±0.1)	[40]	-	-	3
cocoa bean F2	75.5	(±2.9)	5.4	(±0.1)	0.0	(±0.0)	96.3	(±0.1)	*nd*		[19, 28, 40]	-	-	3
Tilia flower F2	91.7	(±3.8)	7.9	(±0.1)	0.9	(±0.1)	95.6	(±0.1)	*nd*		[19, 21, 37, 40]	1.34	-	1
white clover flower F2	82.4	(±2.0)	12.7	(±0.0)	98.8	(±0.0)	61.8	(±0.0)	*nd*		[19, 21, 37, 40]	0.12	(±0.03)	2
birch leaf F2	63.6	(±2.5)	8.3	(±0.1)	58.9	(±0.1)	70.7	(±0.1)	*nd*		[19, 21, 40]	0.14	(±0.02)	3
blackcurrant leaf (no. 1) F2	77.1	(±3.9)	11.8	(±0.1)	95.3	(±0.0)	18.8	(±0.1)	*nd*		[19, 21, 37, 40]	0.14	(±0.03)	3
blackcurrant leaf (no. 2) F2	86.6	(±2.7)	7.8	(±0.2)	94.9	(±0.0)	12.0	(±0.1)	*nd*		[40]	0.19	-	1
blackcurrant leaf (no. 2) F3	69.9	(±0.9)	16.6	(±0.1)	94.4	(±0.1)	19.8	(±0.1)	*nd*		[40]	0.20	-	1
redcurrant leaf F2	91.5	(±4.2)	11.0	(±0.1)	92.5	(±0.0)	65.3	(±0.1)	*nd*		[40]	0.15	(±0.01)	2
sainfoin aerial part F2	82.6	(±2.0)	12.5	(±0.1)	68.3	(±0.1)	82.6	(±0.1)	*nd*		[40]	0.15	(±0.04)	2
yellow iris leaf F2	85.1	(±2.8)	9.2	(±0.1)	30.2	(±0.1)	63.3	(±0.1)	*nd*		[40]	0.45	-	1

Note: data are presented for clarity purposes and literature references are provided, F2 – fraction 2, F3 – fractions 3; CT content has been reported[37] (calculated with mass response factor calculations); ([a]) data have been corrected for CT content (CT g/100 g of fraction); (*n*) number of independent experiments (composed of triplicate measurements at each CT concentration treatment); (*nd*) not detected; (-) not determined. Standard deviation in parentheses.



Condensed tannin	Flavan-3-ol subunit	Stereochemistry	R_1	R_2	R_3	R_4	R_5
procyanidin	catechin	*trans*	OH	OH	H	H	OH
	epicatechin	*cis*	OH	OH	H	OH	H
prodelphinidin	gallocatechin	*trans*	OH	OH	OH	H	OH
	epigallocatechin	*cis*	OH	OH	OH	OH	H

Figure 1.

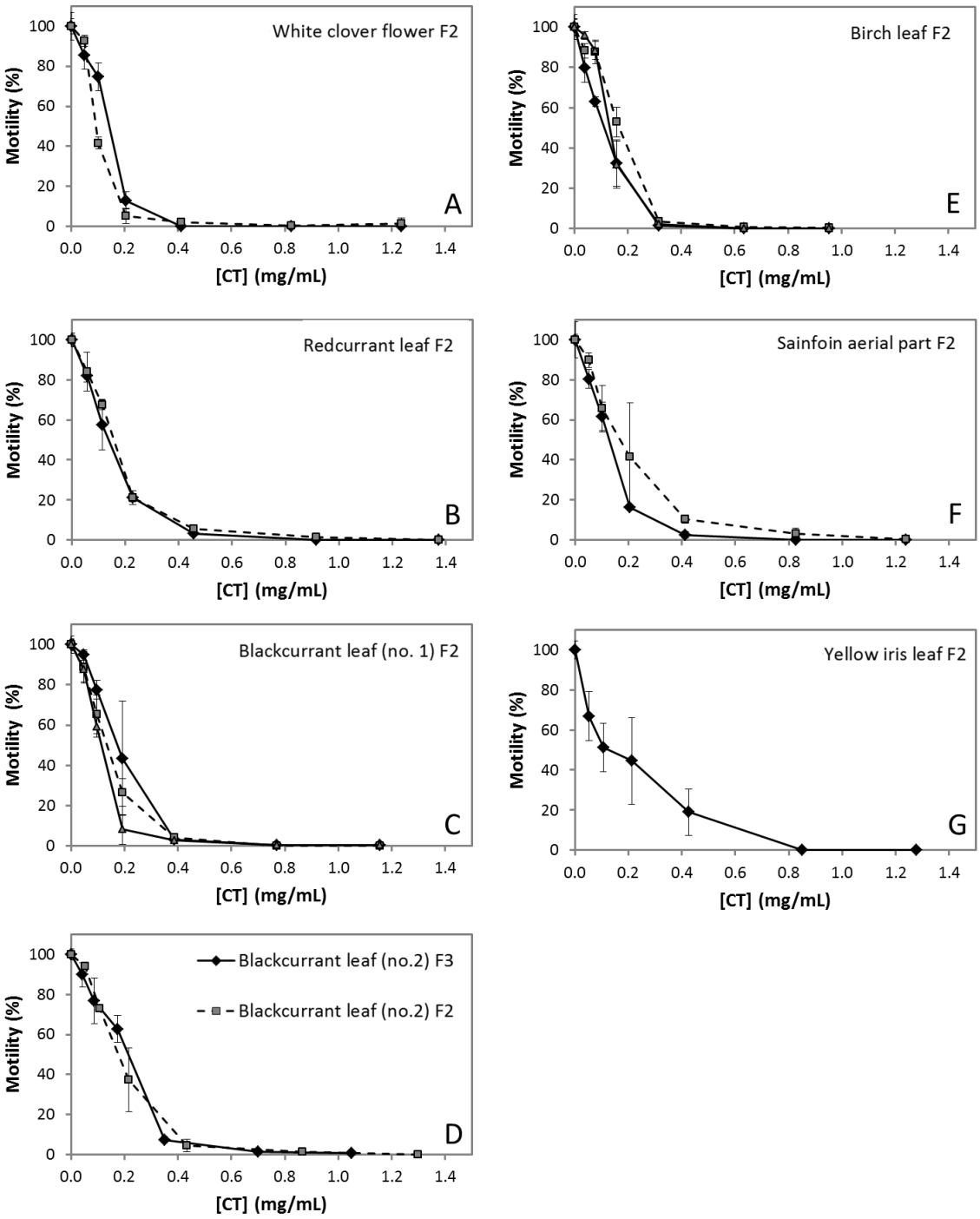

White clover flower F2
A
Birch leaf F2
E
Redcurrant leaf F2
B
Sainfoin aerial part F2
F
Blackcurrant leaf (no. 1) F2
C
Yellow iris leaf F2
G
Blackcurrant leaf (no.2) F3
Blackcurrant leaf (no.2) F2
D
Motility (%)
[CT] (mg/mL)

Figure 2.

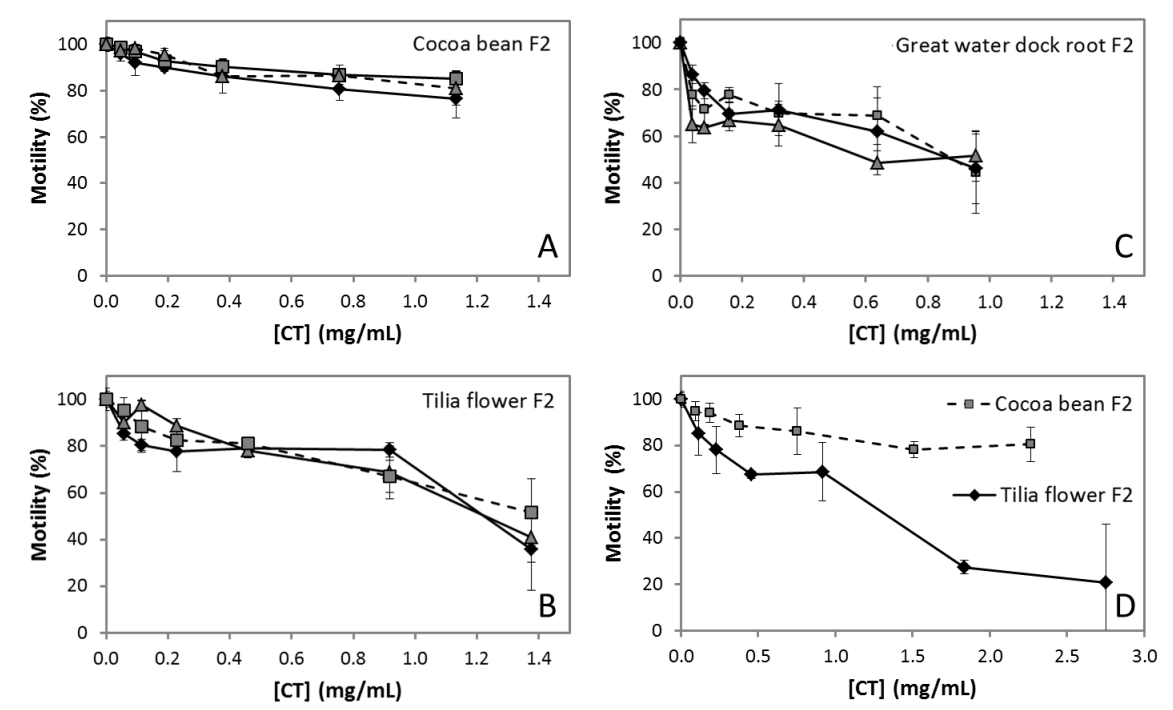

Cocoa bean F2
Great water dock root F2
Tilia flower F2
Cocoa bean F2
Tilia flower F2
Motility (%)
[CT] (mg/mL)
A
B
C
D

Figure 3.

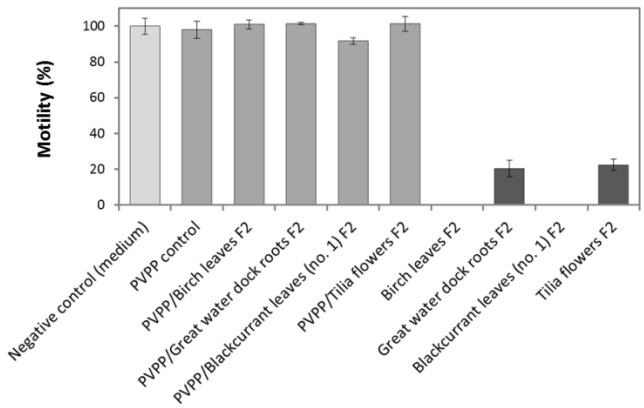

Motility (%)
100
80
60
40
20
0
Negative control (medium)
PVPP control
PVPP/Birch leaves F2
PVPP/Great water dock roots F2
PVPP/Blackcurrant leaves (no. 1) F2
PVPP/Tilia flowers F2
Birch leaves F2
Great water dock roots F2
Blackcurrant leaves (no. 1) F2
Tilia flowers F2

Figure 4.

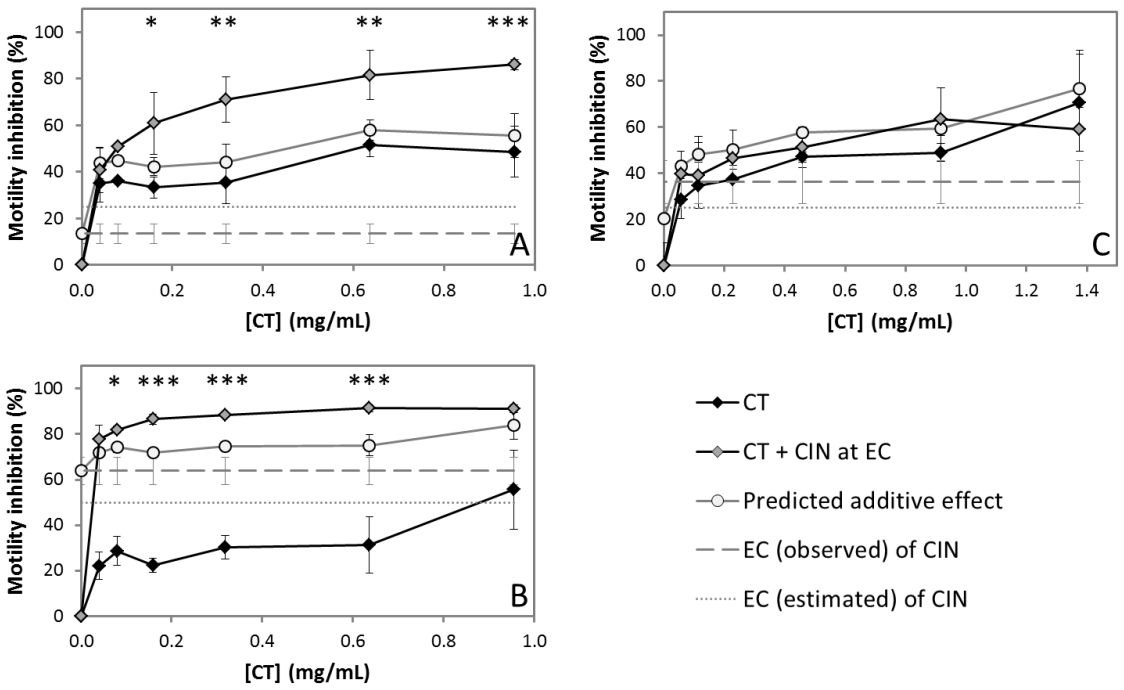

Motility inhibition (%)
[CT] (mg/mL)
A
B
C
* ** ** ***
* *** *** ***
CT
CT + CIN at EC
Predicted additive effect
EC (observed) of CIN
EC (estimated) of CIN

Figure 5.

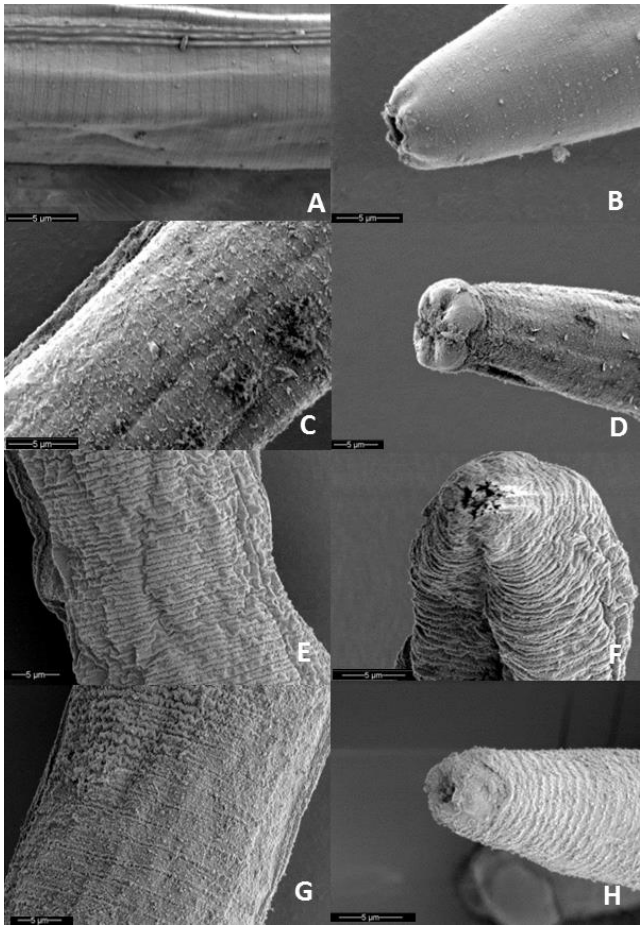


Figure 6.

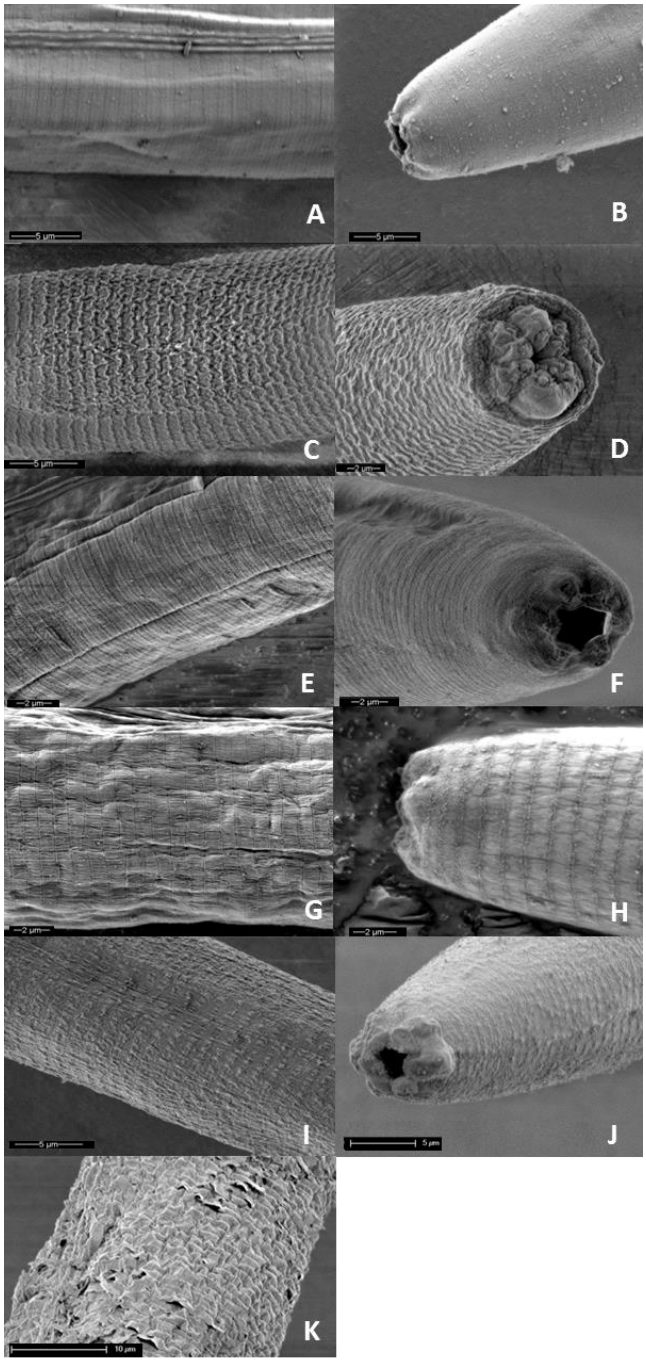


Figure 7.

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