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Efficacy of condensed tannins against larval *Hymenolepis diminuta* (Cestoda) *in vitro* and in the intermediate host *Tenebrio molitor* (Coleoptera) *in vivo*

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Abstract

Natural anti-parasitic compounds in plants such as condensed tannins (CT) have anthelmintic properties against a range of gastrointestinal nematodes, but for other helminths such effects are unexplored. The aim of this study was to assess the effects of CT from three different plant extracts in a model system employing the rat tapeworm, *Hymenolepis diminuta*, in its intermediate host, *Tenebrio molitor*. An *in vitro* study examined infectivity of *H. diminuta* cysticercoids (excystation success) isolated from infected beetles exposed to different concentrations of CT extracts from pine bark (PB) (*Pinus sps*), hazelnut pericarp (HN) (*Corylus avellana)* or white clover flowers (WC) (*Trifolium repens*), in comparison with the anthelmintic drug praziquantel (positive control). In the *in vitro* study, praziquantel and CT from all three plant extracts had dose-dependent inhibitory effects on cysticercoid excystation. The HN extract was most effective at inhibiting excystation, followed by PB and WC. An *in vivo* study was carried out on infected beetles (measured as cysticercoid establishment) fed different doses of PB, HN and praziquantel. There was a highly significant inhibitory effect of HN on cysticercoid development (p=0.0002). Overall, CT showed a promising anti-cestodal effect against the metacestode stage of *H. diminuta.*

Keywords:

Condensed tannins, praziquantel, cestodes, invertebrate-parasite model.

1. Introduction

For decades, parasite control in livestock has relied intensively on prophylactic treatment with synthetic anthelmintics, but increasing resistance to such drugs and consumer requests for organic animal products increases the need for alternative control strategies. Bioactive plants may offer potential alternatives for parasite control in vertebrates (Waller and Thamsborg, 2004). Condensed tannins (CT) are a group of secondary metabolites commonly found in tropical and temperate plants (Jansman, 1993). They vary widely in their molecular weights and the identity of the monomeric flavan-3-ol units, which make up the tannin polymers. Procyanidins (PC) consist of catechin or epicatechin, whereas prodelphinidins (PD) are comprised of gallocatechin or epigallocatechin flavan-3-ols (Williams et al., 2014). More complex CT structures may occur as heteropolymers (Mueller-Harvey and McAllan, 1992; Molan et al., 2003). Several laboratory and field experiments have shown that plant CT may control gastro-intestinal nematodes (Hoste et al., 2006; Novobilský et al., 2011; Novobilský et al., 2013). Besides anthelminthic properties, these bioactive plant products can also have beneficial effects on animal health and production (Hoskin et al., 2000; Ramírez-Restrepo et al., 2004; Hoste et al., 2005; Hoste et al., 2006), and reduce the level of host infection (Hoste et al., 2012). Although, *in vitro* anthelmintic efficacy of natural plant cysteine proteinases has also been reported against excysted scolices and adult worms of the rodent cestodes *Hymenolepis diminuta* and *Hymenolepis microstoma* (Mansur et al., 2014), the effect of CT against helminth taxa other than nematodes has not yet been investigated.

Due to close resemblance of drug effects between animals and humans (Lin, 1995), mammals are often used in pre-clinical pharmacological and toxicological assessment of new compounds (Baumans, 2004). International awareness on animal experimentation has enforced the focus on the "3Rs" to Reduce, Replace and Refine (nc3rs, 2014). An insect model could present an alternative to a range of experimental studies, e.g. as a model for human microbiology (Tan, 2002; Kavanagh and Reeves, 2004) and immunology (Pursall and Rolff, 2011). Further, invertebrate models may simplify and reduce costs of laboratory maintenance (Scully and Bidochka, 2006; Vokřál et al., 2012) and ease concerns associated with animal experimentation (Kemp and Massey, 2007).

In the present study, a host-parasite model employing the flour beetle *Tenebrio molitor* (Coleoptera) and the rat tapeworm *Hymenolepis diminuta* (Cestoda) was used to investigate the anti-cestodal effects of CT in three different plant extracts. *Tenebrio molitor- H. diminuta* is a well-known host-parasite model for studies on ecological and evolutionary host-parasite relationships (Shostak, 2014), and Woolsey, (2012) studied its potential for pre-clinical screening of anthelmintics (praziquantel, levamisole hydrochloride and mebendazole).

In the natural lifecycle, rats excrete infective *H. diminuta* eggs which are ingested by the flour beetles where they encyst as larvae (cysticercoid) in the hemocoel (Burt, 1980). The life cycle completes when an infected beetle is eaten by a rat, in which the cysticercoid excysts, attaches to the intestinal wall and develops into an egg producing tapeworm (Chappell et al., 1970). Since the development of egg into cysticercoid and its excystation plays a crucial role in maintaining the life-cycle of *H. diminuta*,

interruption of these processes with CT would indicate that CT contains anti-cestodal properties.

The objective of the present study was to assess the anti-cestodal effects of three different CT types against cysticercoids of *H. diminuta* both freely exposed (*in vitro*) and within their intermediate host (*in vivo*), at a range of concentrations.

2. Materials and Methods

The *in vitro* experiments were performed with cysticercoids dissected from experimentally infected beetles. The effect of CT was measured as a reduction of excystation of cysticercoids, which serves as an important measure (proxy) for infectivity to rats. The *in vivo* study was conducted in live beetles in order to measure the establishment of cysticercoids in the presence of CT.

2.1. Condensed tannins and praziquantel

Condensed tannins were extracted and purified from three different plant sources. These were pine bark (PB) (*Pinus sp*), hazelnut pericarp (HN) (*Corylus avellana)* and white clover flowers (WC) (*Trifolium repens*). Most tannin-rich plants contain complex mixtures of procyanidins (PC) and prodelphinidins (PD), however our previous work has demonstrated that these three plants contain narrower tannin profiles, i.e. mainly PC or PD (Williams et al. 2014). Therefore, we used these plants as a source of well-defined model tannins that would allow us to investigate whether the molecular structure of the tannins influenced possible anti-parasitic activity. Tannins were extracted and analyzed as previously described (Williams et al., 2014). Briefly, 50 g of plant material was extracted

105 with acetone/water (7:3; v/v) at room temperature, concentrated and freeze-dried. Tannin
106 analysis was carried out by thiolytic degradation of the polymers and subsequent HPLC
107 analysis of the reaction products, providing information on CT content in the extract, the
108 mean degree of polymerization (mDP, i.e. average CT polymer size) and the PC)/ PD ratio
109 (Williams et al., 2014). Pine bark contained 50.8 g CT/100 g extract, with an mDP value of
110 2.5 and a PC/PD ratio of 64.2. Hazelnut pericarp contained 73.8 g CT/100 g extract with an
111 mDP value of 9.6 and a PC/PD ratio of 79.5. White clover contained 33.8 g CT/100 g
112 extract with an mDP value of 4.4 and, in contrast to the other extracts, its tannins were
113 almost exclusively comprised of PD, i.e. the PC/PD ratio was 0.8/99.2 (Williams et al.,
114 2014). The well-known anti-cestodal drug praziquantel (99.7 %, VETRANAL^{TM}) was used
115 as a positive control for both *in vitro* and *in vivo* studies.

116 2.2. Management of the beetles

117 *Tenebrio molitor* larvae (obtained from Avifauna ApS, Denmark) were propagated
118 in plastic containers (30×21×20 cm), placed in a dark incubator (26 °C), and provided
119 with fresh oatmeal and fresh slices of potato. The potato slices were changed twice a
120 week. After 2 weeks of incubation, pupae started to develop and these were then
121 transferred into another plastic container (30×21×20 cm) and kept in a dark incubator
122 (26 °C) until emergence of adults. Newly emerged adults were transferred to new
123 plastic containers (30×21×20 cm) twice a week and held under the conditions described
124 above.

125 2.3. Infection of beetles

126 Feces was collected from *H. diminuta* infected rats (*Rattus norvigecus* – Wistar
127 strain) stabled at the Veterinary Institute, Technical University of Denmark (Animal
128 permission no. 2010/561-1914 –section C10) and stored at 10 °C until use (two weeks
129 maximum). Ten g of fecal pellets were soaked 1 h in 25 ml of tap water and then stirred
130 with a wooden stick to make a uniform paste. The fecal paste was poured through a
131 double layer of cotton gauze (1×1 mm pore size) into a 200 ml plastic cup, and the
132 gauze was rinsed thoroughly with approximately 75 ml of tap water to increase egg
133 recovery. The resulting suspension was equally transferred into two 50 ml centrifuge
134 tubes. The tubes were centrifuged (Universal 16R) at 1148 g for 7 min. The supernatant
135 was removed and the sediment was again stirred with a wooden stick. This fecal paste
136 containing the *H. diminuta* eggs was used to infect the beetles.

137 Before administration of the paste, a group of 50 beetles were left without feed for
138 72 h in plastic containers (30×21×20 cm) with filter paper at the base, and stored in a
139 dark incubator (26 °C). For infection, a 10 µl fecal suspension was deposited on a
140 coverslip (1.5 × 1.5 cm) placed on filter paper in a series of petri dishes (5.5 cm
141 diameter, 1.42 cm depth). A starved beetle was placed inside each Petri dish covered
142 by a lid, and placed in a dark room for an hour. For assessment of eventual evaporation
143 of the fecal suspension, one petri dish setup (without a beetle) was left an hour. Only
144 beetles that had consumed the entire 10 µl of fecal suspension after 1 h were considered
145 successfully inoculated and were included in the experiments.

146 2.4. *In vitro* study with praziquantel and pine bark extract

147 At 15 days post inoculation, beetles were dissected and cysticercoids were
148 recovered using a Pasteur pipette under a dissection microscope (40×). A maximum of
149 10 cysticercoids (first observed) from each beetle were transferred to a watch-glass (33
150 mm diameter, 7 mm deep) containing phosphate buffered saline (PBS) and a total of 80
151 cysticercoids were collected. From these, 10 cysticercoids (first observed) were placed
152 in each of 8 wells (2 wells from 4 different 48 multi-well plastic plates). Then each well
153 was treated with 150 µl of either praziquantel dissolved in 2 % dimethyl sulfoxide
154 (DMSO) with a final concentration of 10^{-2} (high concentration), 10^{-3} (medium
155 concentration) or 10^{-4} mg/ml (low concentration), or CT extracts from PB dissolved in
156 Milli-Q^{TM} water at a final concentration of 0.1, 0.5 or 2.5 mg CT/ml. Control consisted
157 of 2 % DMSO or Milli-Q^{TM} water. All plates were subsequently kept in an incubator at
158 37 °C for 1 h.
159 After 1 h of incubation, the 10 cysticercoids along with the respective treatment
160 solution from each well were transferred separately to a watch glass and the treatment
161 solution was then removed with a Pasture pipette under a dissection microscope. One
162 ml of HCl-pepsin solution [2 ml 37 % HCl, 20 ml warm 0.9 % saline, 0.8 g pepsin
163 powder from porcine gastric mucosa (1:2500, Sigma Life Science)] was added and
164 placed in an incubator (37 °C). After 10 min of incubation, all the HCl-pepsin solution
165 was removed. The cysticercoids were washed three times with 1 ml warm (37 °C) PBS
166 and 1 ml of trypsin-taurocholate solution [0.1 g sodium taurocholate hydrate powder,
167 0.1 g trypsin powder from porcine pancreas, (97 %, Sigma Life Science), 10 ml warm
168 PBS] was added to the watch glass and placed in the incubator at 37 °C for 2.5 h. The
169 cysticercoids were then observed under the dissection microscope (40×) and recorded

as excystated (complete evagination and emergence of scolex and body part from the cyst) or non excystated (absence of the above) (Roberts and Janovy, 2008). This experiment was repeated five times.

2.5. *In vitro* study with pine bark, hazelnut and white clover extracts

Concentrations of PB, HN, and WC extracts were adjusted such that each extract contained equal final concentrations of CT in the assay. Three different concentrations: 2.5 (high concentration), 0.25 (medium concentration) and 0.025 mg CT/ml (low concentration) in Milli-Q^{TM} water from each were prepared. Pure Milli-Q^{TM} water was used as a control. The procedure was as described above (see: *In vitro* study of praziquantel and PB) and was repeated five times.

2.6. *In vitro* condensed tannin depletion assay

As the CT extracts used in this experiment were not 100 % pure, CT depletion experiments were performed, to investigate whether inhibition of cysticercoid excystation was exclusively due to the effect of CT. A total volume of 250 µl solution with concentration 2.5 mg CT/ml of Milli-Q^{TM} water was prepared separately from three types of CT extracts (PB, HN, and WC). For each solution, 12.5 mg of polyvinylpolypyrrolidone (PVPP) (at a dose rate of 50 mg PVPP/ml of solution) was added to precipitate CT, and was incubated (4°C) overnight. After centrifugation at 3000 g for 5 min, supernatant (CT depleted extract) was removed and used in the test assay (Novobilský et al., 2011). As a control, each CT solution was also incubated

(4°C) overnight. The above procedure (see: *In vitro* study of praziquantel and PB) was then followed and was repeated three times.

2.7. *In vivo* study with praziquantel, pine bark and hazelnut extracts

Eighty uninfected beetles (7- 14 days after eclosion) were randomly selected and depleted feed for 72 h as described above. Starved beetles were then randomly allocated into 8 groups, each with 10 beetles. The beetles of each group were presented individually to a droplet of 5 µl containing one of the following treatments: praziquantel (25, 50 and 100 mg/kg body weight of beetle), or PB (125, 250 and 500 mg CT/kg body weight of beetle), or 2 % DMSO, or Milli-Q^{TM} water for 15 min. Doses were formulated by measuring the average weight (+SE) of the beetles (103 ± 4.4 mg), which was calculated by weighing randomly allocated 25 beetles in 6 different groups. After 15 min, beetles that consumed the entire treatment solution were infected and maintained individually as described earlier (see: *In vitro* study).

After 15 days of incubation, 4 beetles were selected randomly from each treatment group for quantification of the establishment of cysticercoids. All cysticercoids in the haemocoel of the beetle were counted and recorded. The experiment for praziquantel and PB was repeated five and three times, respectively.

A separate study tested the effects of HN on cysticercoid establishment. A single dose of HN (500 mg CT/kg body weight) or a control (Milli-Q^{TM} water) were fed to infected beetles and all the procedures were done as described above, and was repeated three times.

211 2.8. Data analysis

212 All statistical analyses were performed using SAS® version 9.3 (SAS institute Inc,
213 Cary, North Carolina). Data from the *in vitro* study (except the CT depletion assay)
214 fulfilled all three assumptions of ANOVA. So, the proportions of cysticercoid
215 excystation in treatment groups were analyzed using PROC GLM fitting repetitions of
216 experiments as a random variable. When an overall significant effect was seen, pair-
217 wise comparisons were done using a post-hoc Tukey test. The data from the CT
218 depletion assay were analyzed by using a non-parametric Wilcoxon rank sum test. In
219 the *in vivo* study, the numbers of cysticercoids established in the treatment groups were
220 analyzed using PROC GENMOD, fitting negative binomial distributions. When overall
221 differences were observed individual comparisons were done using least square means.

222 **3. Results**

223 3.1. *In vitro* study with praziquantel and pine bark extract

224 All concentrations of praziquantel and PB significantly reduced the mean
225 percentage of cysticercoid excystation compared to their respective controls (Figs. 1A
226 & B) and a significant concentration dependent effect was observed for both treatments
227 with the highest concentrations having the strongest inhibitory effect on excystation
228 ($F_{2,4}$ = 26.87, p = 0.0003 for praziquantel and $F_{2,4}$ = 25.57, p = 0.0003 for PB). The
229 cysticercoid excystation inhibitory effect was the same for the praziquantel and PB
230 treatments ($F_{1,4}$ = 0.49, p = 0.4887).

231 3.2. *In vitro* study with pine bark, hazelnut and white clover extracts

232 The mean percentages of excystation of cysticercoids after exposure to the three CT
233 were significantly different among the treatment groups ($F_{3,4} = 28.37$, $p < 0.0001$) and
234 concentrations ($F_{2,4} = 110.58$, $p < 0.0001$; Fig. 2) and there was an interaction between
235 treatment groups and concentrations ($F_{4,4} = 5.48$, $p = 0.0015$). Concentration was found
236 to be a significant parameter for all three CT with the highest concentrations having the
237 strongest negative effect on excystation ($F_{2,4} = 66.48$, $p < 0.0001$ for PB, $F_{2,4} = 9.97$, $p =$
238 0.0067 for HN and WC).

239 The mean percentage of cysticercoid excystation with all three CT depleted
240 solutions showed significantly more cysticercoid excystation compared to their
241 respective controls ($\chi^2 = 4.09$, df = 1, $p = 0.043$ for PB and WC, $\chi^2 = 3.97$, df = 1, $p =$
242 0.043 for HN; Fig. 3).

243 3.3. *In vivo* study with praziquantel, pine bark and hazelnut extracts

244 There was a significant effect of treatment (praziquantel and PB) on cysticercoid
245 establishment ($\chi^2 = 133.1$, df=3, $p<0.0001$) but the effect was not dose dependent (χ^2
246 $=1.92$, df=2, p=0.382). All three doses of praziquantel reduced cysticercoid
247 establishment to almost zero while each beetle in the control treatment had +SE 27.1 ±
248 6.65 cysticercoids (Fig. 4A), whereas the effect of CT from PB at all doses did not
249 differ from the control, although there was a trend of reduced establishment (p=0.841,
250 0.374 and 0.098 for low, medium and high doses respectively; Fig. 4B).

251 The separate experiment with HN at 500 mg CT/kg beetle body weight revealed
252 significantly lower cysticercoid establishment (mean cysticercoids per beetle +SE: 25.5

± 2.54) in comparison to the control group (mean cysticercoids per beetle +SE: 36.3 ± 2.33), (χ^2 = 10.48, df = 1, p = 0.0012).

4. Discussion

The results from the *in vitro* study suggest that treatment with CT from three different plants (PB, HN and WC) and praziquantel can substantially reduce the excystation of *H. diminuta* cysticercoids in a concentration-dependent manner. A similar concentration-dependent inhibitory effect of praziquantel has been previously shown using the same model (Woolsey, 2012). Our *in vivo* results showed that PB did not significantly inhibit cysticercoid establishment, but praziquantel and HN were associated with a reduction in the number of established cysticercoids. The cysticercoid excystation inhibitory effect of CT from the three plant extracts disappeared in the presence of tannin-inhibitor polyvinylpolypyrrolidone (PVPP)(Hagerman and Butler, 1981), confirming that CT are the major active compounds for the observed inhibition. The observed effects of CT in this model are most likely due to their direct anti-parasitic activity, although additional, indirect effects by increasing host resistance may occur in mammals (Hoste et al., 2006).

The cysticercoid capsule, scolex and other cellular structures contain protein with polysaccharides and lipids (Burt, 1980). As CT are able to bind to proteins (Hoste et al., 2006), they might interact with the protein portion of the cystic capsule and alter its physical and chemical properties, as reported previously for *Trichostrongylus colubriformis,* where direct damage to the cuticle was observed after incubation with CT (Hoste et al., 2006). Condensed tannins might also interfere with enzyme activities,

which are involved in metabolic pathways responsible for the development and functioning of parasites (Athanasiadou et al., 2001). Furthermore, due to the presence of pores and vesicles in the cystic capsule (Burt, 1980), bioactive compounds may reach the internal structures of the scolex and other cellular proteins. Taken together, all of these changes might interfere with the cysticercoid structures and metabolic pathways, which are essential in cysticercoid functioning and excystation. However, the exact mode of action of CT and the active compounds responsible for the anthelmintic activity are still unknown (Novobilský et al., 2013) and could differ depending on the species of parasite, its developmental stage, and possibly the biochemical characters and structures of the forage species (Min and Hart, 2003).

The different potency of CT from these plant extracts may be associated with the percentage of procyanidin monomer units or the mDP of the CT polymer, as the CT content in all three applied samples was standardized in the assays. However, HN also contains a small percentage of galloylated CTs (Irene Mueller-Harvey and Christos Fryganas, unpublished results), which may also influence anti-parasitic activity (Brunet and Hoste, 2006). The preliminary conclusion from this CT series is that the procyanidin tannins were more effective than prodelphinidin tannins in the *in vitro* and *in vivo* experiments. This is an unexpected finding as most other studies ascribed higher anthelmintic activities to the prodelphinidins (Brunet and Hoste, 2006). There may be two possible reasons: 1) the presence of galloylated CT in HN or 2) the fact that the pH values of insect guts tend to be alkaline (Gullan and Cranston, 2010) and are therefore quite different from the rumen or abomasum of ruminants. Relatively little is known about the reactivity of PC and PD tannins under alkaline conditions with constituents of

298 the insect gut. Anthelminthic activities of CT from different plants or plant extracts are
299 known to have markedly different effects on parasites. For example, grazing of sheep
300 on *Lotus penduncul*atus reduced nematode fecal egg counts more effectively than
301 grazing on *L. corniculatus* (Niezen et al., 1998), as *L. pedunculatus* has a higher PD/
302 PC ratio than *L. corniculatus* (Foo et al., 1997). Thus, the multitude of different CT
303 structures (Mueller-Harvey and McAllan, 1992) may influence their biological
304 activities (Athanasiadou et al., 2001). Further experiments are needed to determine the
305 relative contributions of mDP and PD/PC ratio on cysticercoid excystation.

306 Availability of free CT in the intestine may be important factor for CT to be
307 effective. Formation and dissociation of the protein-CT complex is highly pH
308 dependent. Stable protein-CT complexes are formed at pH 5 - 7, but the complexes
309 easily dissociate and release proteins at higher and lower pH (Mueller-Harvey and
310 McAllan, 1992). Optimum complex formation occurs at the isoelectric point of the
311 protein, but little is known about the isoelectric point of proteins in the beetle gut. There
312 is thus a possibility of lack of formation of protein-CT complex in the intestine of
313 beetles or CT being subjected to oxidative changes.

314 In summary, our *in vitro* results indicate concentration dependent inhibitory effect
315 of all tested CT in plant extracts on cysticercoid excystation, the HN extract being most
316 potent, followed by PB and WC. Anti-excystation activity appeared to be positively
317 linked to the presence of procyanidin tannins. The *in vivo* treatment with HN reduced
318 cysticercoid establishment, and is the first observation on anti-cestodal properties of CT
319 from plant extracts. Although the invertebrate-parasite model is not fully representative
320 of the biological action of CT in the mammalian system, this model could be useful for

a first screening of potentially interesting compounds. This invertebrate model has several advantages over vertebrate models as far as, ethical clearance, legislation, time and cost are concerned. Future studies will need to address the mechanism of CT action and include comparative studies with vertebrate animals in order to explore their effects against the different lifecycle stages and species of tapeworms.

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Fig. 1. *In vitro* mean percentage (n=5, ±SEM) of cysticercoids excystation treated with praziquantel (A) and condensed tannin (CT) extract from pine bark (B) at different concentrations. Ten cysticercoids were used for each concentration. Control refers to 2% DMSO for (A) and to Milli-Q^{TM} water for (B). Different letters within each figure represent statistical significance ($\alpha = 0.05$).

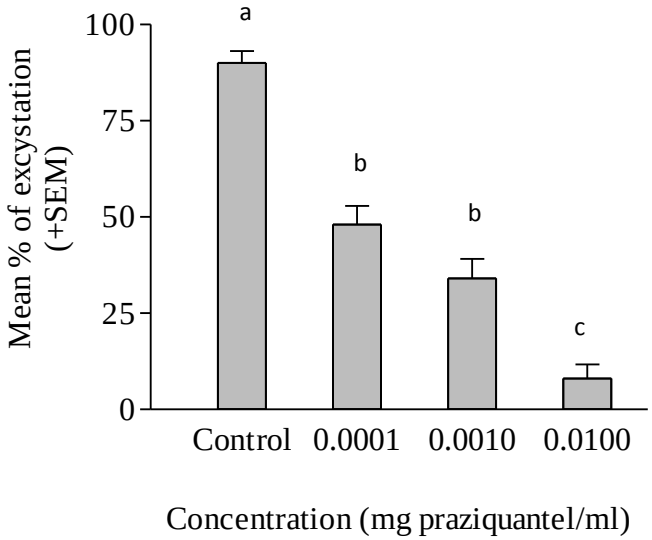
Fig. 2. *In vitro* mean percentage (n=5, ±SEM) of cysticercoids excystation treated with three different condensed tannin (CT) extracts [white clover flower (WC), pine bark (PB), and hazelnut skin (HN)] at different concentrations. Ten cysticercoids were used for each concentration. Control refers to Milli-Q^{TM} water. Different letters within each figure represent statistical significance ($\alpha = 0.05$).

Fig. 3. *In vitro* mean percentage (n=3, ±SEM) of cysticercoid excystation treated with three different condensed tannin (CT) depleted solutions (depleted) and condensed tannin extract solutions (control) at 2.5mg CT/ml concentration. Ten cysticercoids were used for each solution of white clover flower (WC), pine bark (PB) and hazelnut pericarp (HN). *refers to a significant difference relative to the respective controls.

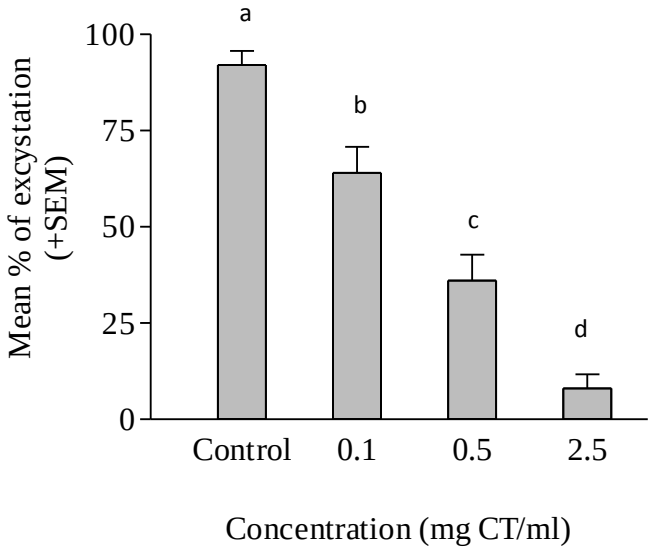
Fig. 4. *In vivo* mean number of cysticercoids (n=5 for A and n=3 for B, ±SEM) treated with praziquantel (A), and a condensed tannin (CT) extract from pine tree bark (B) at different concentrations. Average numbers of cysticercoids from four beetles were used for each treatment. Control refers to 2 % DMSO in (A) and to Milli-Q^{TM} water in (B).

Fig. 1.

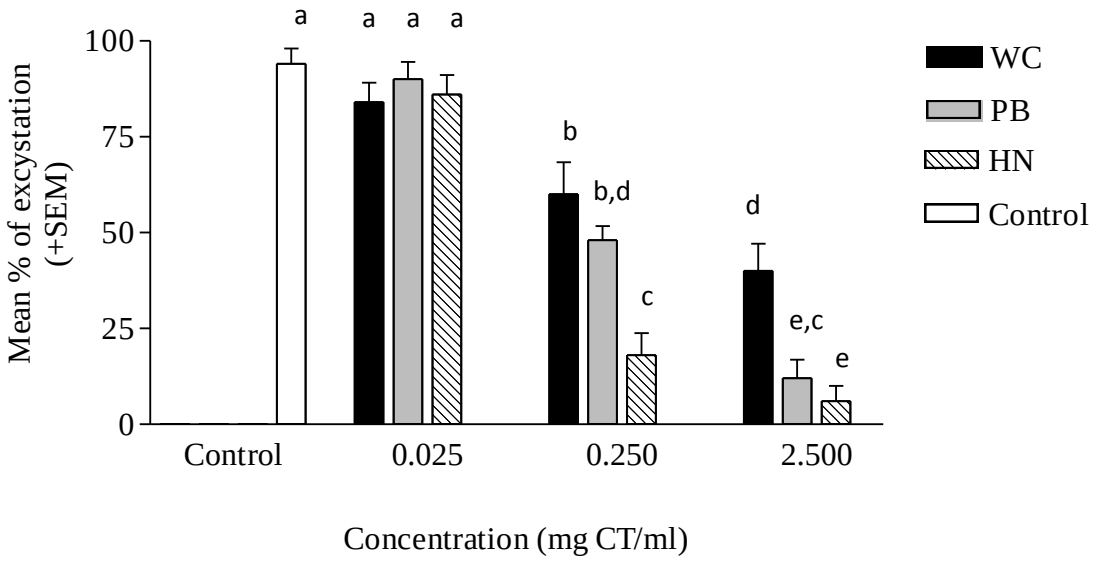
A. Praziquantel



B. Pine tree bark (PB)



456 Fig. 2.



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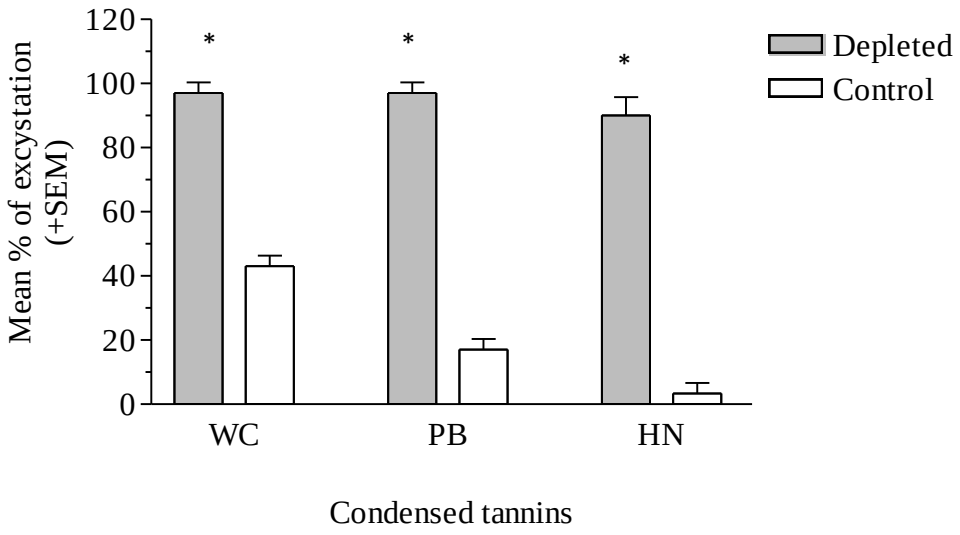
459 Fig. 3.



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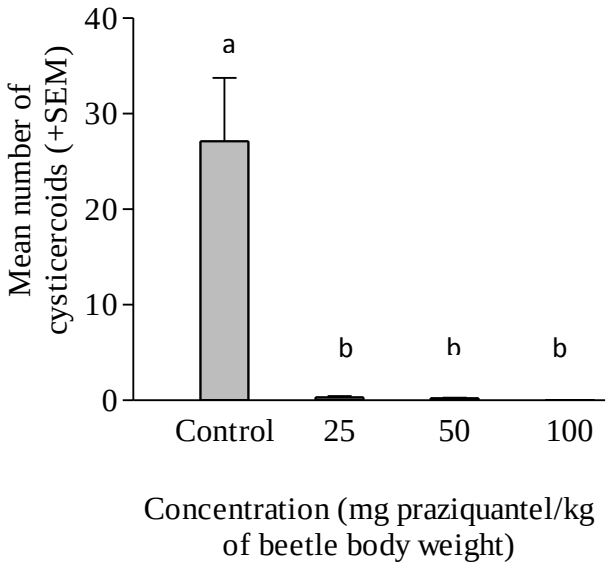
462 Fig. 4.

463 A. Praziquantel



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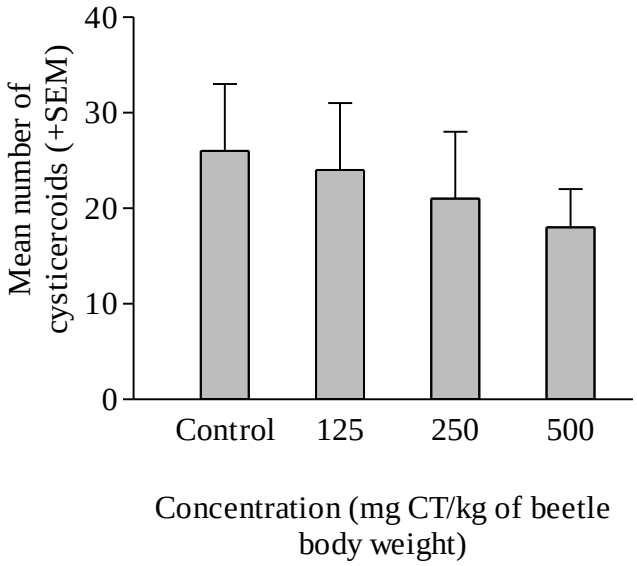
465 B. Pine tree bark (PB)



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