

Proanthocyanidins inhibit Ascaris suum glutathione-S-transferase activity and increase susceptibility of larvae to levamisole and ivermectin in vitro

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1 **SHORT COMMUNICATION**

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3 **Proanthocyanidins inhibit *Ascaris suum* glutathione-S-transferase activity and increase susceptibility of**
4 **larvae to levamisole *in vitro*.**

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6 Tina V.A. Hansen¹, Christos Fryganas², Nathalie Acevedo³, Luis Caraballo³, Stig M. Thamsborg¹, Irene
7 Mueller-Harvey², Andrew R. Williams^{1*}

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9 ¹Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of
10 Copenhagen, Frederiksberg, Denmark.

11 ²Chemistry and Biochemistry Laboratory, School of Agriculture, Policy and Development, University of
12 Reading, Reading, United Kingdom.

13 ³Institute for Immunological Research, University of Cartagena, Cartagena, Colombia.

14 *arw@sund.ku.dk

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26 **Abstract**

27 Proanthocyanidins (PAC) are a class of plant secondary metabolites commonly found in the diet that have
28 shown potential to control gastrointestinal nematode infections. The anti-parasitic mechanism(s) of PAC
29 remain obscure, however the protein-binding properties of PAC suggest that disturbance of key enzyme
30 functions may be a potential mode of action. Glutathione-S-transferases (GSTs) are essential for parasite
31 detoxification and have been investigated as drug and vaccine targets. Here, we show that purified PAC
32 strongly inhibit the activity of both recombinant and native GSTs from the parasitic nematode *Ascaris*
33 *suum*. As GSTs are involved in detoxifying xenobiotic substances within the parasite, we hypothesised that
34 this inhibition may render parasites hyper-susceptible to anthelmintic drugs. Migration inhibition assays
35 with *A. suum* larvae demonstrated that the potency of levamisole (LEV) and ivermectin (IVM) were
36 significantly increased in the presence of PAC purified from pine bark (4.6-fold and 3.2-fold reduction in IC₅₀
37 value for LEV and IVM, respectively). Synergy analysis revealed that the relationship between PAC and LEV
38 appeared to be synergistic in nature, suggesting a specific enhancement of LEV activity, whilst the
39 relationship between PAC and IVM was additive rather than synergistic, suggesting independent actions.
40 Our results demonstrate that these common dietary compounds may increase the efficacy of synthetic
41 anthelmintic drugs *in vitro*, and also suggest one possible mechanism for their well-known anti-parasitic
42 activity.

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51 Gastrointestinal nematodes represent a major threat to sustainable and profitable livestock production
52 worldwide. The current reliance on a small arsenal of synthetic anthelmintic drugs has serious limitations
53 due to the threat of drug resistance, which has already reached crisis levels in small ruminant production
54 (Sargison, 2012), and has also been detected in nematodes of pigs and cattle (Cotter et al., 2015; Gerwert
55 et al., 2002). A complementary approach is the identification of bioactive diets that contain natural plant
56 compounds with anti-parasitic activity, and which can be used as nutraceuticals (Hoste et al., 2015). Such
57 an approach may slow the threat of drug resistance by reducing the frequency of drug interventions, as
58 well as potentially boosting the host's natural immunity (Ramírez-Restrepo et al., 2010).

59
60 Diets that are rich in proanthocyanidins (PAC - syn. condensed tannins) have been demonstrated to be
61 effective in reducing nematode fecundity and/or burdens in a variety of livestock species (Hoste et al.,
62 2015). Moreover, *in vitro* assays have confirmed that PAC have direct effects on parasite survival, with
63 electron microscopy studies demonstrating direct physical damage to both external and internal parasite
64 structures (Brunet et al., 2011; Williams et al., 2014). However, the mechanisms that lead to parasite death
65 have not yet been elucidated. As PAC have a strong protein-binding affinity, interference with key enzymes
66 is an attractive hypothesis. Consistent with this, Fakae et al. (2000) have shown that extracts from some
67 traditional Nigerian medicinal plants inhibit the function of glutathione-S-transferases from the swine
68 nematode *Ascaris suum*. This inhibition was speculated to be due to, at least in some cases, the presence of
69 PAC. Glutathione-S-transferases play a key role in detoxification of reactive oxygen species as well as
70 xenobiotics, and have been proposed as helminth vaccine targets (Goud et al., 2012) . Thus, interference
71 with GST function may result in endogenous toxicity to the parasite and also potentially increase the
72 susceptibility of parasites to xenobiotics such as synthetic drugs. Indeed, Whitney et al. (2013) recently
73 reported that ivermectin (IVM) treatment of *Haemonchus contortus* in lambs was more effective when the
74 lambs consumed PAC-containing red juniper berries.

75

76 We have previously shown that *A. suum* third-stage larvae (L3) are susceptible to the anti-parasitic activity
77 of PAC (Williams et al., 2014). In the present study, we derived highly purified PAC from two plant sources
78 to investigate 1) whether *A. suum* GST function was inhibited by PAC, and 2) whether exposure of *A. suum*
79 larvae to PAC *in vitro* would result in synergistic increases in the efficacy of IVM and levamisole (LEV).
80

81 We first purified native *A. suum* GST (nGST) from adult worms collected from the small intestine of pigs at a
82 local slaughterhouse (Danish Crown, Ringsted, Denmark). Worms were pulverised mechanically using liquid
83 nitrogen and the powder was then dissolved in 15 mL cold Binding Buffer (140 mM NaCl, 2.7 mM KCl, 10
84 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and centrifuged for 10 min at 3134g. The supernatant was filtrated through
85 a 0.20 µm syringe filter (Corning) and nGST isolated on glutathione columns (GSTrap HP[®], GE Healthcare)
86 following the protocol of the manufacturer. The eluate was concentrated to 500 µL and subsequently
87 exchanged with PBS using Amicon Ultra-4 centrifugal filter units (MWCO 10 kDa). Protein concentration
88 was determined by the BCA assay using BSA as a standard. In addition, recombinant GST1 (rGST1) from *A.*
89 *suum* was produced as described elsewhere (Acevedo et al., 2013). Isolation of nGST was confirmed by
90 coomassie stain using rGST1 as a reference. SDS-PAGE was performed in a 10% polyacrylamide (NuPAGE[®]
91 Novex[®] 10% Bis-Tris Midi Gels, Life Technologies) according to the manufacturer's recommendations
92 except that 0.5 µL DL- Dithiothreitol (Sigma-Aldrich) was used as the reducing agent. An amount of 1.4 µg
93 nGST and 1.05 µg rGST1 was applied. After electrophoresis, proteins were stained with SimplyBlue™
94 SafeStain (Life Technologies) for 1 hour, and visualized using Odyssey FC Imager (Li-Cor Biotechnologies). As
95 shown in Figure 1A, nGST was successfully isolated as indicated by two bands (23 and 24 kDa)
96 corresponding to the GST1 and GST2 isoforms previously described by Liebau et al. (1994), and consistent
97 with the 25 kDa single band obtained with rGST1 (Acevedo et al., 2013).
98

99 In order to test whether PAC inhibited GST function, PAC were extracted from white clover flowers (WCF;
100 *Trifolium repens*) and pine bark (PB; *Pinus sylvestris*), purified on Sephadex-LH20 columns, and analysed by

101 HPLC-MS as previously described (Gea et al., 2011; Williams et al., 2014). These plant samples were chosen
102 as they represented the two most common classes of PAC, these being procyanidins (found in PB) and
103 prodelphinidins (found in WCF). The second fraction to elute from the column, containing high molecular
104 weight PAC of high purity (84% for PB, 100% for WCF), was used in these experiments. GST activity was
105 assayed at 26°C using the GST Detection Module (GE Healthcare Life Sciences) with a final concentration of
106 5 µg/mL protein. The assay was conducted in 96 well plates and read at 340 nm (Spectra Max Plus 384,
107 Molecular Devices) using 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM) as GST substrate and reduced
108 glutathione as the reducing agent (0.308 µg/mL). Enzyme activity (nGST) was significantly reduced in the
109 presence of PAC (Figure 1B). Similar vales were obtained with rGST1 (data not shown). The IC₅₀ values were
110 0.96 and 0.20 µg/mL for PB and WCF, respectively. Thus, both procyanidin and prodelphinidin type-PAC
111 efficiently inhibit GST activity from *A. suum*.

112

113 We next investigated whether exposure of *A. suum* third-stage larvae (L3) to PAC purified from PB would
114 improve the *in vitro* efficacy of LEV and IVM. Pine bark PAC were chosen for these experiments as
115 procyanidins are more commonly found in the diet than prodelphinidins. Third-stage larvae were obtained
116 by mechanically hatching embryonated eggs as described (Williams et al., 2014). The larvae were then pre-
117 treated for 60 minutes with either 20 or 10 µg/mL of purified PAC, or PBS as a control. Then, concentration
118 gradients of either LEV or IVM (both obtained from Sigma-Aldrich, Stellenbosch, Germany) were added to
119 the PAC- or PBS-treated larvae and incubated overnight. Additional groups of larvae were incubated
120 overnight with either PAC or PBS alone. The pre-treatment time for PAC of 60 minutes was chosen as this
121 time-frame allows irreversible binding of PAC to *A. suum* larvae (A.R. Williams, unpublished data), whilst
122 the concentrations of PAC were chosen as preliminary experiments demonstrated that they achieved
123 approximately 15% inhibition of larval migration, thus allowing the possibility to test for synergistic effects
124 between PAC and the synthetic drugs. Migratory ability was assessed by an agar-based assay as previously

125 described (Williams et al., 2014). Inhibition of migration was expressed relative to L3 incubated in media
126 only.

127

128 Incubation of larvae in LEV or IVM alone resulted in a dose dependent inhibition of migration (Figure 2A).
129 For both drugs, the addition of PAC increased the efficacy, resulting in a 4.6-fold and 3.2-fold reduction in
130 IC₅₀ value for LEV and IVM, respectively, when combined with 20 µg/mL PAC. To assess whether these
131 increase in efficacy represented a synergistic or additive interaction, predicted additive values for the
132 percentage of migration inhibition were calculated from the observed inhibitory effects of the individual
133 treatments (each concentration of drug or PAC) according to Bliss' definition of independent action
134 (Klongsiriwet et al., 2015). The observed effect of the combined PAC/drug treatments were then compared
135 to these calculated vales, with efficacy greater than the predicted additive effect defined as synergy. This
136 approach demonstrated that the relationship between PAC and LEV tended to be synergistic, with
137 consistently higher observed values for the combination than the additive values predicted by independent
138 action (Figure 2B). The effect was particular noticeable at low concentrations of LEV and 10 µg/mL PAC. For
139 IVM, the relationship was better described as additive (Figure 2B), indicating that PAC tend to enhance the
140 activity of LEV, but in the case of IVM the two agents seem to act independently of each other to inhibit
141 larval migration. This differential interaction of PAC with LEV and IVM is perhaps consistent with the
142 distinct anthelmintic mechanisms of these two drugs, whereby LEV acts on nicotinic acetylcholine receptors
143 (Sarai et al., 2015) and IVM acts by binding to glutamate-gated chloride channels (Hibbs and Gouaux,
144 2011).

145

146 We have thus demonstrated that *A. suum* GST function is efficiently inhibited by PAC, which may offer a
147 mechanistic explanation to their well-documented anthelmintic activity. However, the high affinity that
148 PAC have for proteins means it is highly unlikely that any one parasite metabolic pathway is specifically
149 targeted. Instead, it is more plausible that a range of enzymatic functions are inhibited by PAC. In addition,

150 previous studies using electron microscopy to observe nematodes exposed to PAC have noted aggregates
151 of material forming around the buccal cavities (Martínez-Ortíz-de-Montellano et al., 2013), and have
152 proposed that a 'coating' effect whereby PAC form complexes with external parasite proteins leads to an
153 inhibition of parasite feeding and subsequent mortality. Furthermore, PAC are likely to interact *in vivo* with
154 both host proteins as well as the parasite, adding further complexity to the situation.

155

156 Whilst it is clear that no one single mechanism may be responsible for the anthelmintic activity of PAC,
157 inhibition of GST function raises the possibility that the parasite's detoxification mechanisms may be
158 impaired, which may result in increased susceptibility to drugs, or, *in vivo*, reactive oxygen species
159 produced by host phagocytes. Our data suggest that the efficacy of drugs (particular LEV) may be increased
160 when larvae are co-incubated with PAC, which is in agreement with some previous *in vitro* and *in vivo*
161 studies involving *H. contortus* (Armstrong et al., 2013; Whitney et al., 2013). Further studies will be
162 necessary to determine the mechanisms behind these combinatorial effects. Given the rapid binding of PAC
163 to proteins (Mueller-Harvey, 2006), we speculate that in our experiments key parasite proteins were
164 neutralised and/or destroyed during the pre-incubation with PAC, leaving the larvae more susceptible to
165 the subsequent addition of levamisole. In addition to inhibition of GST function, other plausible
166 mechanisms include decreased cuticle integrity due to PAC-binding, which may result in increased diffusion
167 of drugs, and inhibition of other detoxification mechanisms such as xenobiotic efflux pumping by p-
168 glycoproteins, or activity of gluconyl transferases. Thus, we cannot conclude that the synergistic effects of
169 PAC and levamisole are due only to the GST inhibition, and the effect of PAC on the activity of these other
170 parasite pathways is worthy of further investigation.

171

172 In conclusion, we have confirmed that PAC strongly inhibit GST function from an important parasitic
173 nematode, and we also have demonstrated that PAC can synergistically improve the efficacy of LEV and

174 also act additively with IVM *in vitro*. Further studies will focus on the mechanisms involved and whether
175 PAC-rich diets can improve drug efficacy *in vivo*.

176

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199 **Figure Legends**

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201 **Figure 1 – Isolation of *Ascaris suum* glutathione-S-transferase (GST) and inhibition by proanthocyanidins**
202 **(PAC)**

203 A) *A. suum* GST was isolated from adult worms and visualised by SDS-PAGE. Lane 1 – isolated native GST,
204 Lane 2 – recombinant GST1

205 B) Inhibition of native GST activity by PAC purified from pine bark (PB) and white clover flowers (WCF).

206 Results are the mean (\pm S.E.M) of two independent experiments, each performed in duplicate.

207

208 **Figure 2 – Proanthocyanidins (PAC) increase the efficacy of levamisole and ivermectin *in vitro***

209 A) Percentage migration of *Ascaris suum* larvae in the presence of levamisole (LEV) and ivermectin (IVM)
210 with or without 10 (PB10) or 20 (PB20) $\mu\text{g}/\text{mL}$ of PAC isolated from pine bark. Results are the mean (\pm
211 S.E.M) of two independent experiments, each performed in duplicate. Also shown are IC_{50} values calculated
212 by non-linear regression. For each drug, values followed by different subscripts indicate significantly
213 ($P < 0.0001$) different IC_{50} values.

214 B) Synergy analysis of levamisole (LEV) or ivermectin (IVM) combined with 10 or 20 $\mu\text{g}/\text{mL}$ PAC from pine
215 bark (PB). Shown is the percentage inhibition of larval migration achieved by the drug alone and in
216 combination with PAC, and the additive values predicted by the assumption of independent action of the
217 drug and PAC (see text). Combined data from two independent experiments is presented.

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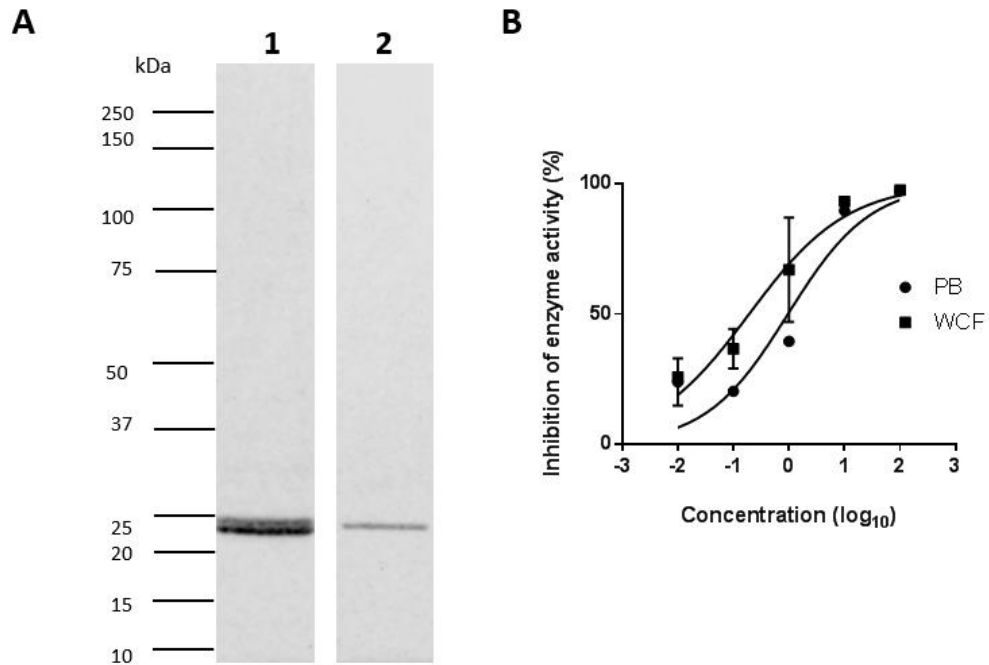
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281 Figure 1

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