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1 Winter Aconite (*Eranthis hyemalis*) Lectin as a cytotoxic
2 effector in the lifecycle of *Caenorhabditis elegans*

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17

18 **Abstract**

19 The lectin found in the tubers of the Winter Aconite (*Eranthis hyemalis*) plant is an N-acetyl-
20 D-galactosamine specific Type II Ribosome Inactivating Protein (RIP); Type II RIPs have
21 shown anti-cancer properties, and hence have potential as therapeutic agents. Here we present
22 a modified protocol for the extraction and purification of the *E. hyemalis* lectin (EHL) using
23 affinity chromatography. *De novo* amino acid sequencing of EHL confirms its classification
24 as a Type II Ribosome Inactivating protein. The biocidal properties of EHL have been
25 investigated against the nematode *Caenorhabditis elegans*. Arrested first stage larvae treated
26 with EHL have shown some direct mortality, with surviving larvae subsequently showing a
27 range of phenotypes including food avoidance, reduced fecundity, developmental delay and
28 constitutive dauer larvae formation. Both inappropriate dauer larvae development and failure
29 to locate to bacterial food source are consistent with the disruption of chemosensory function
30 and the ablation of amphid neurons. Further investigation indicates that mutations that disrupt
31 normal amphid formation can block the EHL-induced dauer larvae formation. In
32 combination, these phenotypes indicate that EHL is cytotoxic and suggest a cell specific
33 activity against the amphid neurons of *C. elegans*.

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39 Introduction

40

41 Lectins are a class of carbohydrate binding proteins ubiquitously expressed in plants, animals,
42 bacteria and viruses, characterised by their ability to agglutinate erythrocytes (Peumans &
43 Van Damme, 1995), a property that enabled the development of the ABO system of blood
44 typing. The second characteristic common to all lectins is the ability to bind carbohydrates
45 selectively based on the individual sugar specificity of the lectin. This also results in lectins
46 binding to the carbohydrate moieties of extracellular glycoconjugates specifically and
47 reversibly without introducing conformational changes to the mono- or oligosaccharides to
48 which they bind (Sharon & Lis, 2004). Plant lectins are involved in a wide range of
49 processes including carbohydrate transport, cell-cell signalling, cell surface binding and
50 recognition, pathogenic defence and in potentially mediating symbiotic relationships (Sharon
51 & Lis, 2004). Plant lectins play a key role in defence, with many specifically binding to
52 epithelial cells of herbivore and nematode guts (Schubert et al., 2012; Delatorre et al., 2007).
53 Insecticidal, antifungal and antiviral qualities have also been widely described (e.g. Kumar et
54 al., 1993; Rao et al., 1998; Peumans, Hao & Van Damme, 2001; Edwards & Gatehouse,
55 2007). For example, Balsamin, from *Momordica balsamina*, demonstrates potent anti-HIV
56 activity (Kaur et al., 2013).

57

58 In recent years the potential of lectins for use in cancer therapies has become a significant
59 research focus due to their ability to preferentially bind to specific carbohydrates, and
60 differentiate between glycosylation patterns. Moreover, a number of plant derived lectins
61 have been shown to have potent *in vitro* and *in vivo* anti-cancer effects (e.g. Voss et al., 2006;
62 Otsuka et al., 2014) inducing autophagous and apoptotic pathways in tumour cells, and some
63 are already used therapeutically. For instance, the recombinant mistletoe lectin rViscumin

64 has been through phase 1 clinical trials and a number of other native mistletoe lectin
65 preparations such as Lektinol and Iscador are prescribed widely throughout Europe as
66 adjuvant therapies although their efficacy is not readily quantified (Horneber et al., 2008).

67

68 Using a modified extraction protocol developed from previously published studies (Cammue,
69 Peeters & Peumans 1985, Kumar *et al.* 1993, George *et al.* 2011). This paper focuses on the
70 lectin found in the tubers of Winter Aconite, *Eranthis hyemalis*, (EHL). To date, EHL is the
71 sole lectin representative described from the *Ranunculaceae* and has been identified as a
72 Type II Ribosome Inactivating Protein (RIP) (Kumar et al., 1993). EHL preferentially binds
73 N-acetyl-galactosamine, but also binds galactose, galacto-pyranosyl-D-glucose, and to a
74 lesser degree D-ribose (Kumar et al., 1993). Type II RIPs are classified as chimerolectins
75 and cytotoxic N-glycosidases and consist of either one or two heterodimers linked by
76 disulphide bonds. The B chain subunit is a sugar specific lectin containing the highly
77 conserved ricin B domain and will bind to extracellular glyconjugates. This mediates entry to
78 the intracellular environment for the attached cytotoxic A chain by endocytosis (Virgilio et
79 al., 2010). The toxin is then subjected to retrograde transport via the Golgi complex; to the
80 endoplasmic reticulum where the disulphide bonds are reduced and the A chain is free in the
81 cytosol to refold into an enzymatically active form (Hartley & Lord, 2004). The A chain acts
82 as an inhibitor of eukaryotic protein synthesis by cleaving a single adenosine (A₄₂₃₄) in the
83 28s rRNA subunit preventing Elongation Factor 2 from binding and resulting in immediate
84 and absolute cessation of peptide elongation (Hartley & Lord, 2004).

85

86 Type II RIPs are an area of increasing interest due to their antineoplastic properties, and their
87 glycomic binding profile can be used to target specific glycans of biological molecules. For
88 instance, the GalNac specific RIP *Ximenia americana* (Riproximin) (Voss et al., 2006),

89 *Sambucas sp* (Ferrerias et al., 2011) and ML1 from *Viscum album* (Tonevitsky et al., 1996)
90 show higher binding affinity for tumour cells than for healthy cells. This can be partly
91 attributed to the expression of particular surface saccharide groups in the changing glycomics
92 of malignant cells (Peumans, Hao & Van Damme, 2001; Bayer et al., 2012). The use of
93 lectins and Type II RIPs is also indicated in some studies for marking metastatic proliferation
94 of tumour cells due to excessive glycosylation in metastatic cell lines (Zhou et al., 2015).

95

96 Herein we present a modified extraction and purification protocol for EHL. Protein
97 sequencing of EHL further supports the findings of earlier work that EHL is a Type II RIP.

98 We also investigate the effect of EHL on the free living nematode *Caenorhabditis elegans*. *C*
99 *elegans* is a well-established model organism for initial toxicological studies due to the

100 conserved nature of its biological and biochemical processes including stress response and
101 disease pathways (Boyd, Smith & Freedman, 2012). Our research has revealed a range of
102 phenotypes including direct mortality and a constitutive dauer formation phenotype that is
103 consistent with neuronal ablation.

104

105 **Material and Methods**

106 **Preparation of affinity chromatography column**

107 An Amersham chromatography column was packed at room temperature with a final bed
108 volume of 8 ml of Fetuin-Agarose in solution with 0.5 M NaCl and immobilised on cross-
109 linked 4 % beaded agarose (Sigma-Aldrich Company ltd, UK). Prior to use the column was
110 equilibrated with 8 column volumes of Phosphate buffered solution (PBS).

111

112 **Extraction of EHL**

113 60 g of *E. hyemalis* tubers supplied by Eurobulbs Ltd (UK) were prepared using a modified
114 method from those described in previous studies (Cammue, Peeters & Peumans 1985, Kumar
115 *et al.* 1993, George *et al.* 2011) with adaptations as follows. The tubers were finely sliced
116 before being homogenised with 250 ml of ice cold PBS containing 5 mM thiourea and left to
117 settle on ice for 30 minutes. The homogenate was removed and stored and the remaining
118 slurry was mixed with a further 250 ml of PBS. The two fractions were then combined and
119 stirred at 4°C for 4 hours. The homogenised mixture was then centrifuged (Sorvall RC6 plus
120 HSC) at 20,000 g for 30 minutes. The supernatant was retained and frozen at -80°C overnight
121 in order to induce aggregation of any remaining lipid content in sample. The sample was then
122 defrosted and filtered through 3MM Whatman filter paper before undergoing a further
123 centrifuge cycle of 20,000 g for 20 minutes. The clarified supernatant then underwent
124 ammonium sulphate precipitation.

125

126 Ammonium sulphate precipitation.

127 Solid ammonium sulphate was added slowly to the crude extract initially to a saturation point
128 of 40%, and after one hour of stirring at 4°C was centrifuged at 10,000g for 15 minutes. The
129 pellet was re-suspended in 15mls of PBS. Ammonium sulphate was then added to the
130 supernatant to a final saturation point of 80%, with a further hour of stirring at 4°C. The
131 resulting pellet was also re-suspended in PBS. Agglutination activity was found to be
132 retained in the 40% pellet and absent from the 80% pellet. An SDS-PAGE gel confirmed the
133 presence of target protein in the 40% fraction. Samples were dialysed against PBS in 3
134 buffer changes consisting of 200 x sample volume each including a final overnight exchange.

135

136 Purification of EHL

137 The crude dialysed extract was applied to a Fetuin-agarose affinity chromatography column
138 at a rate of 1 ml per minute using ÄKTA Express protein purification system (GE
139 Healthcare), non-target proteins were then eluted with PBS until absorbance at λ_{280} was
140 restored to base line value circa 40 mAU. The affinity matrix was then equilibrated with PBS
141 and subsequently EHL was eluted with 40 mM 1,3 diaminopropanol (DAP) and peak
142 fractions were collected in 0.5ml aliquots. The oligosaccharide structure of Fetuin has been
143 well defined and shown to have Gal and GlcNAc branched residues present. Fetuin has been
144 purified using the lectin RCA I which has specificity for Galactose and N-
145 acetylgalactosamine (Green *et al.*, 1988). Its use, therefore as an affinity chromatography
146 media for the lectin/type II RIP purification is based on this complementary interaction. The
147 eluant was neutralised with 2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-
148 HCl) at pH 7.0. Peak fractions were applied to pre-equilibrated PD-10 desalting columns
149 (GE Healthcare) and buffer exchanged into PBS.

150

151 Analysis of EHL

152 Purified EHL was tested for agglutination ability using defibrinated rabbit erythrocytes (TCS
153 Biosciences), with 20 μ l of post column eluant, or control, added to a 20 μ l sample of
154 erythrocytes in a wellled microscope slide. The purified EHL was also analysed by SDS-
155 PAGE, with both reduced and non-reduced samples electrophoresed on 12 % gels and
156 subsequently stained with Coomassie Brilliant Blue. Concentration was measured using a
157 Qubit flourometric protein assay.

158

159 Purified EHL was commercially sequenced. In-gel tryptic digestion was performed after
160 reduction with DTE and S-carbamidomethylation with iodoacetamide. Gel pieces were
161 washed two times with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium

162 bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min.
163 Sequencing-grade, modified porcine trypsin (Promega) was dissolved in the 50 mM acetic
164 acid supplied by the manufacturer, then diluted 5-fold with 25 mM ammonium bicarbonate to
165 give a final trypsin concentration of 0.02 $\mu\text{g}/\mu\text{L}$. Gel pieces were rehydrated by adding 10
166 μL of trypsin solution, and after 10 min enough 25 mM ammonium bicarbonate solution was
167 added to cover the gel pieces. Digests were incubated overnight at 37°C.

168

169 A 1 μL aliquot of each peptide mixture was applied to a ground steel MALDI target plate,
170 followed immediately by an equal volume of a freshly-prepared 5 mg/mL solution of 4-
171 hydroxy- α -cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% ,
172 trifluoroacetic acid (v:v).

173

174 Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron
175 mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a range of
176 800-5000 m/z . Final mass spectra were externally calibrated against an adjacent spot
177 containing 6 peptides (des-Arg¹-Bradykinin, 904.681; Angiotensin I, 1296.685; Glu¹-
178 Fibrinopeptide B, 1750.677; ACTH (1-17 clip), 2093.086; ACTH (18-39 clip), 2465.198;
179 ACTH (7-38 clip), 3657.929.). Monoisotopic masses were obtained using a SNAP averagine
180 algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

181

182 For each spot the ten strongest precursors, with a S/N greater than 30, were selected for
183 MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction
184 of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-
185 subtracted and smoothed (Savitsky-Golay, width 0.15 m/z , cycles 4); monoisotopic peak
186 detection used a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H

187 7.7583) with a minimum S/N of 6. Bruker flexAnalysis software (version 3.3) was used to
188 perform spectral processing and peak list generation.

189

190 *De novo* sequencing of tandem mass spectra was performed by hand, with *a*-, *b*-, *b*⁰-, *y*-, *y*⁰-
191 and *y**-ions considered as possible fragment ions. *De novo* derived peptides sequences were
192 matched to homologous protein sequences using the on-line MS-BLAST service provided by
193 Washington University. The results of which were consistent with the in-house homology
194 search results conducted on confidently assigned sequences using the University of Virginia
195 UVa FASTA Server, the FASTS and SSearch algorithms were used for homology searching
196 against the SwissProt (NCBI) and PDB databases.

197

198 Activity against *C. elegans*

199 Worms were obtained from the *Caenorhabditis* Genetics Center and maintained using
200 standard methods (Stiernagle, 2006), on nematode growth media plates (NGM) using
201 *Escherichia coli* OP50 strain food source. N2 was used for initial testing and as a control in
202 other assays. To assess the effect of various mutations on EHL-induced dauer larvae
203 formation, the following strains were used: CX2065, *odr-1(n1936)*; CX2205 *odr-3(n2150)*;
204 PR671, *tax-2(p671)*; PR672, *che-1(p672)*; PR813, *osm-5(p813)*; SP1205, *dyf-1(mn335)*; and
205 SP1709, *dyf-10(e1383)*. In all experiments, treatments and genotypes were blind coded, the
206 position of plates within experimental blocks was randomised, and contaminated plates
207 excluded from all analysis.

208

209 For all assays, arrested and synchronised *C. elegans* first stage larvae (L1s) were obtained by
210 allowing eggs, isolated from gravid hermaphrodites by hypochlorite treatment (Stiernagle,
211 2006), to hatch on NGM plates in the absence of food for 24 hours at 20°C. For experiment 1,

212 arrested N2 L1s were washed from plates, resuspended in M9 with a series of EHL
213 concentrations from 3.92 to 0 mg/ml, incubated at 20°C for 6 hours, and 15 worms per
214 treatment were picked for analysis. For experiment 2, arrested N2 L1s treated as above
215 except treatments were 2.94 mg/ml and 0 mg/ml EHL and a greater number of worms per
216 treatment were analysed (n = 55 and n = 33 for the 2.94 and 0 mg/ml treatments,
217 respectively). After incubation, worms were washed 3 times in water. For the analysis of
218 development and fecundity (experiments 1 and 2), worms were transferred in a small volume
219 of liquid to NGM plates without food, then individually transferred from this plate to NGM
220 plates with *E. coli* OP50 strain food source and maintained at 20°C. Standard methods were
221 then used to analyse the reproductive schedule and lifetime fecundity (Hodgkin & Doniach,
222 1997). These data were then used to assess the effect of EHL on reproduction as assessed by
223 lifetime reproductive success (LRS), the total number of progeny produced (experiments 1
224 and 2), and the intrinsic rate of increase (r) (experiment 2), calculated by iteration from $\sum e^{-rx}l_xm_x = 1$, where l_x represents the age specific survivorship to day x and m_x represents the
225 fecundity on day x (Vassilieva & Lynch, 1999).

227

228 Based on phenotypes observed in the initial screen, the ability of EHL to induce constitutive
229 dauer larvae formation (a dauer-constitutive, or Daf-c, phenotype) was investigated in greater
230 detail. Here, worms were treated with 0.98, 1.96 or 2.94 mg/ml EHL, as described above,
231 except that after washing, worms were transferred *en masse* to plates with food. After four
232 days at 20°C plates were visually scored to assess the proportion of worms that had
233 developed as dauer larvae (number of dauer larvae/total number of worms). After counting,
234 worms were washed from plates and incubated in 1 % SDS for one hour, a treatment that kills
235 all *C. elegans* stages except dauer larvae (Cassada & Russell, 1975), worms were washed
236 once in M9, transferred to fresh NGM plates with food and the number of dauer larvae again

237 counted. These dauer larvae were then transferred individually to NGM plates with food at
238 20°C and monitored for the next 14 days to determine if they were capable of resuming
239 development. To further analyse EHL-induced dauer larvae formation, N2 and mutant
240 worms were treated, as described above, with 0, or 1.54 mg/ml EHL, washed, and transferred
241 *en masse* to plates (n = 3 per combination of treatment and genotype) with food. Plates were
242 then incubated at 20°C for four days at which time the proportion of worms that had
243 developed as dauer larvae was scored.

244

245 Dauer larvae formation in PR672, *che-1(p672)*, was further analysed both in standard dauer
246 larvae formation assays and in assays of growing populations. For assays of dauer larvae
247 formation in response to defined amounts of pheromone, assays were performed as
248 previously described (Golden & Riddle 1984; Green et al., 2014), with worms allowed to lay
249 eggs on assay plates containing dauer pheromone extract and limited amounts of food, and
250 progeny scored after two days at 25°C. Dauer larvae formation in growing populations was
251 assessed as previously described (Green et al., 2013), with populations initiated with single
252 worms and a defined amount of food allowed to grow to food exhaustion, except that assays
253 were performed at 25°C.

254

255 Results

256 Purification and characterisation of EHL

257 Qubit fluorometric measurement showed a typical concentration of 2.5 mg/ml using our
258 revised and improved purification strategy. This shows an approximately 5 to 6-fold increase
259 in recovery in comparison to the previously reported yield of 380 µg/ml in George *et al.*
260 (2011). Non-reducing SDS-PAGE analysis produced characteristic reduced protein bands at
261 circa 31 and 28 kDa as well as an unreduced band circa 50kDa (Fig. 1); these values are

262 consistent with those previously reported in the literature (Kumar et al., 1993). An intense
263 agglutination response of rabbit erythrocytes was exhibited and thus confirmed the presence
264 of EHL (Fig. 2).

265

266 De novo sequencing analysis.

267 De novo sequence analysis of fragmentation spectra was carried out and suggested two

268 confidently assigned peptide fragment sequences, with the following tags,

269 QQWA[L/I]YSDST[L/I]R and NWNNGNP[L/I]Q[L/I]WQCTQQQNQR (Fig. 3). Peptide

270 fragment homology searches produced matches to various Type II Ribosome Inactivating

271 Proteins (RIPS) all within ricin-b domain (carbohydrate binding) regions. This result

272 confirms the status of EHL as a Type II RIP, as has been reported in previously published

273 sequence data (Kumar et al., 1993). Sequence tags QQWA[L/I]YSDST[L/I]R and

274 NWNNGNP[L/I]Q[L/I]WQCTQQQNQR were matched to two regions in Nigrin-b (SNA-

275 V; *Sambucas nigra agglutinin-V*) UniProtKB P33183.2 correlating to residues 470-481 and

276 325-345 respectively using the FASTS search facility against the SwissProt database. Close

277 homology to other Type II RIPS was also matched within the ricin b domain of Abrin-a

278 (P11140) and Ricin (P02879). PDB structures were searched using SSearch algorithm and

279 also showed matches to SNA-II (3C9Z) (*Sambucas nigra agglutinin II*), Abrin-a (1ABRB)

280 and Ricin (1RZOB) in the ricin b domain as well as ML1 from *Viscum album* (1QNKB).

281

282 EHL affects survival, development and reproduction in *C. elegans*

283 Acute treatment of arrested *C. elegans* L1s for 6 hours with EHL at different concentrations

284 resulted in a subsequent range of developmental, fertility and survival defects (Fig. 4), with

285 all EHL concentrations reducing lifetime reproductive success (LRS) (Fig. 4A, pairwise,

286 Bonferroni corrected, Mann–Whitney U tests against N2 showing reduced fecundity in all

287 EHL treatments). Much of this decrease in fecundity is however a consequence of worms not
288 reproducing in the EHL treatments (comparison of Fig. 4A and B), although LRS does still
289 decrease over the range of EHL concentrations tested when only those worms that
290 reproduced are considered (Fig. 4B, Pearson product-moment correlation of LRS against
291 EHL concentration: including the 0 mg/ml group, $r = -0.52$, $p < 0.001$; excluding the 0 mg/ml
292 group, $r = -0.38$, $p = 0.013$). EHL-treated worms that did reproduce showed a delay in
293 development, with many treated worms starting reproduction a day or more after the control
294 worms. Of the worms that did not reproduce, some showed no movement from the point at
295 which they were placed on the plate and no response to stimulus after 24 hours and therefore
296 died as a consequence of the EHL treatment. Other EHL-treated worms were observed to
297 remain as arrested L1s or to develop as dauer larvae, a non-feeding developmentally arrested
298 stage. Many of the non-reproducing worms were found not on a food source, and more than
299 would be expected under these conditions were found to have climbed the sides of the plate;
300 both behaviours are indicative of a disruption to chemosensory ability and an inability to
301 detect the bacterial food.

302

303 To investigate these reproductive effects and the survival in more detail, a larger number of
304 worms were assayed (Fig. 5). Here, EHL treatment resulted in immediate mortality of 41%
305 of EHL treated individuals and again EHL-treated worms were observed to remain as L1s
306 and to arrest as dauer larvae. These results suggest that EHL treatment affects the sensory
307 neurons. Overall, EHL treatment reduced subsequent LRS (Fig. 5A, control vs all EHL
308 treated worms, $W = 2376.0$, $p < 0.001$), with the subset of EHL treated worms that did
309 reproduce producing a greatly reduced number of progeny (control vs reproducing EHL
310 treated worms, $W = 792.0$, $p < 0.001$). A similar pattern was observed in the analysis of the
311 effects of EHL treatment on the estimated rate of increase (Fig. 5B, control vs all EHL treated

312 worms, $W = 2376.0$, $p < 0.001$; control vs reproducing EHL treated worms, $W = 792.0$, $p <$
313 0.001).

314

315 To further characterise the development of EHL-treated worms as dauer larvae, an additional
316 set of arrested L1s were analysed. As in the assay for reproductive effects (above), some of
317 these EHL treated worms developed as dauer larvae (Fig. 6). These worms were then SDS
318 treated, with survival confirming that they were indeed dauer larvae. Fifty of these dauer
319 larvae were then transferred to plates with food and maintained at 20°C, with only one worm
320 out of the fifty recovering and completing development as a reproductive adult after four
321 days, and a second recovering after a total of fourteen days. Under these conditions dauer
322 larvae normally recover rapidly and would be expected to have commenced reproduction
323 approximately 2 days after transfer to food (Green & Harvey, 2012).

324

325 Analysis of mutant strains indicates that the ability of EHL treatment to induce dauer larvae
326 formation varied across the genotypes (Table 1). All genotypes were however observed to
327 show a developmental delay in response to EHL treatment, with reproduction of most EHL-
328 treated worms not commencing until day 5 after treatment. Analysis of dauer larvae
329 formation in PR672, *che-1(p672)* showed that this mutation does not block dauer larvae
330 formation in response to defined amounts of pheromone and that similar numbers of dauer
331 larvae are formed in N2 and PR672 in growing populations ($F_{1,33} = 0.05$, $p = 0.82$).

332

333 Discussion

334 We have successfully isolated the type II RIP found in the tubers of the Winter Aconite, *E.*
335 *hyemalis*, by modification of a previously published protocol (Cammue, Peeters & Peumans,
336 1985; Kumar et al., 1993). Analysis indicates EHL is a heterodimeric protein consisting of

337 two chains of molecular weights of approximately 28 and 31 kDa (Fig. 1). Protein sequencing
338 confirms that EHL is a Type II RIP with the cytotoxic potential for depurination of
339 eukaryotic ribosomes.

340

341 EHL was used to study potential lectin-mediated toxicity against *C. elegans*. The bioassays
342 performed indicate that EHL has biocidal properties against *C. elegans*. Four phenotypic
343 effects were identified: reduced fecundity (Fig. 4 and 5), developmental delay, chemosensory
344 disruption and constitutive dauer formation (Fig. 6 and Table 1). *C. elegans* physiology is
345 such that at the arrested L1 larval stage, the only cells which are not enclosed by a largely
346 impermeable cuticle are the amphids and phasmids. These are bilaterally symmetrical
347 sensory organs that contain the sensory neurons: each amphid containing twelve neurons and
348 each phasmid containing two (Ward et al., 1975). Of the twelve amphid neurons, the ciliated
349 nerve endings of eight are exposed to the external environment via the amphid pore (Ward et
350 al., 1975). These neurons control a range of phenotypes, including egg-laying and the
351 decision to develop as a dauer larvae (Albert, Brown & Riddle, 1981). Laser ablation of the
352 ASI, ADF and ASG cells is sufficient to result in constitutive dauer larvae development, with
353 ablation of the ASJ cell resulting in an inability to recover from dauer arrest (Bargmann &
354 Horvitz, 1991). Our observations of inappropriate dauer larvae formation and the failure of
355 most such dauer larvae to resume development indicate that EHL is interacting with these
356 neurons and are consistent with EHL resulting in neuron death.

357

358 In general, biocidal assays with lectins involve ingestion of the lectin by the target organism.
359 For example, EHL had previously been tested against the coleopteran pest *Diabrotica*
360 *undecimpunctata howardii*, resulting in a high mortality rate and an 80% reduction in body
361 size of survivors; there was however no previous data on reproductive effects (Kumar *et al.*,

362 1993). A study of the toxic effects of the CCL2 lectin from *Coprinopsis cinerea* (Ink Cap
363 mushroom) on *C. elegans* reported a phenotype of severe developmental delay; the lectin was
364 adsorbed in the epithelial cells of the intestine, potentially degrading the membrane and
365 preventing growth (Schubert *et al.*, 2012). The absence of a food source in our assay has
366 therefore enabled the observation of entirely new lectin-mediated *C. elegans* phenotypes
367 induced by EHL, including a Daf-c phenotype which has not been reported before.

368

369 The cause of the developmental delay in EHL-treated worms is not clear. Possibilities would
370 include damage to the pharynx and a subsequent reduction in pumping (feeding) ability, or, if
371 some feeding is initiated, damage to the epithelial cells of the intestine as observed in
372 response to the *C. cinerea* CCL2 lectin (Schubert *et al.*, 2012). A further possibility is that
373 differences in body size and development are also a consequence of damaged neurons (see
374 Fujiwara, Sengupta & McIntire, 2002).

375

376 Wild-type *C. elegans* take up dyes such as DiI and FITC into the amphid neurons AWB,
377 ASH, ASJ, ASK, ADL and ASI (Hedgecock *et al.*, 1985). Given the likely mode of action of
378 EHL, we reasoned that mutations that disrupt the normal formation of sensory amphids
379 would block EHL-induced dauer larvae formation. Consistent with this, disruption of *osm-5*,
380 *dyf-1* and *dyf-10*, all mutations in which the amphid neurons cannot take up dyes, result in no
381 EHL-induced dauer larvae formation (Table 1). In both *odr-1* and *odr-3* mutants, where dye
382 filling is not affected and EHL would be expected to be able to access the neurons normally,
383 there is no reduction in dauer larvae formation in response to EHL treatment (Table 1). In
384 contrast, dauer larvae formation is reduced in PR671, but some are still formed (Table 1),
385 indicating that disruption of *tax-2* only partially blocks the effect. TAX-2 forms, with TAX-4,
386 a cyclic nucleotide-gated cation channel that is required for chemotaxis in response to AWC-

387 sensed odorants (Coburn & Bargman, 1996). Axon outgrowth defects have however been
388 noted in *tax-2* mutants, with c. 80% of *tax-2(p671)* animals observed to have abnormal ASJ
389 axons (Coburn & Bargman, 1996). It is not clear if the reduction in the EHL-induced dauer
390 larvae formation observed in the *tax-2* mutants is a consequence of the axon guidance defects
391 or the channel disruption.

392

393 That EHL-induced dauer larvae formation is also blocked in *che-1* mutants further supports
394 the hypothesis that EHL is disrupting neurons. CHE-1 is a C2H2-type zinc-finger
395 transcription factor that is required for the identity of ASE neurons (Uchida *et al.*, 2003).
396 Loss of CHE-1 expression eliminates the function of ASE neurons and *che-1* mutations have
397 previously been shown to suppress *Daf-c* phenotypes (Reiner *et al.*, 2008). No significant
398 structural defects have been observed in *che-1* mutants (Lewis & Hodgkin, 1977) and our
399 results indicate that dauer larvae formation does not appear altered in either standard dauer
400 larvae assays or in growing populations. This thereby implies that the mutation is
401 specifically blocking EHL-induced constitutive dauer larvae formation.

402 It is well established that lectins bind to glycoconjugates on cell surfaces and that toxicity in
403 RIPs is due to lectin mediated entry to the cell; this mode of action is consistent with the
404 results presented here. In the case of *C. elegans* the only cells exposed are the amphid
405 neurons. As a Type II RIP EHL can be subject to retrograde transport from the cell surface
406 along the neuronal processes, at which point the ribosomes are inactivated, causing
407 translation to cease (Wiley, Blessing & Reis, 1982). As no post-embryonic somatic division
408 occurs in mature individuals, and multiple chemoreceptors are expressed in a single neuron,
409 ribosome inactivation of the neurons within the amphids would affect many functions derived
410 from chemosensation (Sulston & Horvitz, 1977). Toxicity variables can be attributed to
411 differing carbohydrate specificities but there is also evidence of the role of individual cell

412 types in how they interact with lectins, indicating that any effects are characteristic of both
413 variables (Battelli *et al.*, 1997).

414

415 In conclusion, successful extraction using affinity chromatography has enabled assays to be
416 conducted for biocidal properties against *C. elegans*. The results obtained demonstrate a
417 significant reduction in fecundity, development, growth and a high incidence of abnormal
418 dauer development when arrested L1 larvae were treated in the absence of food. The
419 occurrence of dauer formation and a failure to recover in the presence of food supports the
420 hypothesis that EHL is binding specifically to amphid neurons. Mutant screening has
421 demonstrated that EHL can act as a neuronally specific cytotoxin, an effect which has
422 previously been described with Ricin and other RIPs on mammalian sensory neurons (Wiley,
423 Blessing & Reis, 1982; Tong *et al.*, 2012). Further studies will aim to determine if those
424 individuals that remained as arrested L1s were doing so as a consequence of an inability to
425 perceive the food or if an additional mechanism is at work.

426

427 Our research shows that EHL has biocidal and potential cytotoxic activity. Moreover, EHL
428 shows specificity for GalNac, an overexpressed sugar in the Tn (GalNac clustered) antigen
429 which characterises cancer linked O-glycans (Ju, Otto & Cummings, 2011). Other GalNac
430 Type II RIPs such as Mistletoe Lectin (ML1) and Riproximin have demonstrated promising
431 therapeutic relevance as anticancer agents (Voss *et al.*, 2006; Bayer *et al.*, 2012; Adwan *et*
432 *al.*, 2014. These factors suggest that EHL is a viable candidate for further study in respect of
433 antineoplastic characteristics.

434

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440 References

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