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1 **Toxic effect of the novel chiral insecticide IPP and its biodegradation**
2 **intermediate in nematode *Caenorhabditis elegans***

3

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13

14 **ABSTRACT**

15 *Caenorhabditis elegans*, a kind of model organism, was used to investigate
16 biodegradation pathway of IPP and M1 in nematodes, *in vivo* toxicity from IPP and
17 M1 and the possible underlying molecular mechanism. The results showed that both
18 IPP and M1 could decrease lifespan, locomotion behavior, reproductive ability and
19 AChE activity. During IPP biodegradation process, three intermediates (M1-M3) were
20 monitored and identified. Based on the identified metabolites and their biodegradation
21 courses, a possible biodegradation pathway was proposed. IPP was probably
22 transformed to different three metabolites in nematodes through oxidation and

23 elimination of methyl and propyl etc. Under the same concentration, IPP had more
24 severe toxicity than M1 on nematodes. IPP and M1 might reduce lifespan and
25 decrease reproductive ability through influencing insulin/IGF signaling pathway and
26 TOR signaling pathway. They could decrease expression levels of *daf-16*, *sgk-1*,
27 *aak-2*, *daf-15* and *rict-1* genes, which involved in IGF and TOR signaling pathway.

28

29 **KEYWORDS:** Paichongding; Biodegradation intermediate; Toxic effect; Nematode;

30 *Caenorhabditis elegans*

31

32 1. Introduction

33 Paichongding (IPP, 1-((6-chloropyridin-3-yl) methyl)-7-methyl-8-nitro-5
34 -propoxy-1,2,3,5,6,7-hexahydroimidazo[1,2- α]-pyridine), is a novel chiral insecticide
35 with independent intellectual property rights developed in China (Cai et al., 2015a,
36 2015b, 2016a; Fu et al. 2013). It has higher insecticidal activity (40-50 times)
37 compared to imidacloprid-resistance pests, and also has low toxicity to human. In
38 China IPP was used for pesticide control and sprayed for almost 3.3 million hectares
39 (Cai et al. 2016a; Chen et al. 2017).

40 Insecticides residue in environment was accumulated and accelerated with the
41 increase of insecticides application. Many studies reported that insecticides and their
42 residue in soil environment have potential risk to the soil balance (soil biochemical
43 properties and fertility, *etc*), they also can be transferred through food chain and make
44 ecosystems deterioration (Zabaloy et al. 2012; Zhang et al. 2014). Previous studies
45 have emphasized on the biodegradation pathway of IPP, its behavior in soils, and its
46 effect on microbial community, soil enzyme activity *etc*. (Cai et al. 2016a, 2016b,
47 2016c). However, little information is available on the toxicity of IPP and its
48 biodegradation intermediates on protozoan in soils.

49 The nematodes are a diverse animal phylum and live in complex microbial
50 environments that present many potential challenges to their health and viability.
51 *Caenorhabditis elegans* is a kind of nematodes and the model organism, which has
52 widely been used in drug discovery, drug toxicity, ageing mechanism research etc.,
53 due to its relatively short lifespan and conserved mechanisms for regulation of

54 antioxidant response (Avila et al. 2012). *C. elegans* has been widely accepted and
55 utilized as an important alternative animal model for toxicity testing (Avila et al. 2012;
56 Leung et al. 2008; Sprando et al. 2009). Many studies used *C. elegant* for
57 toxicological research including organic compounds, drugs and nanomaterials etc.,
58 and found that toxicity is similar to that observed in mammals (Ju et al. 2013; Li et al.
59 2013; Zhao et al. 2013). In this study, we first studied and compared the toxicity of
60 IPP and its metabolites, M1, to nematodes *Caenorhabditis elegans*, and IPP and M1
61 degradation pathway in nematodes were also studied and proposed. Moreover, there
62 are limited information about toxicological mechanism for IPP and M1, we examined
63 the relative gene expression for toxicity of IPP and M1. The results of this study will
64 be useful for further understanding IPP degradation characteristics and its toxicity to
65 animals.

66

67 **2. Materials and methods**

68

69 *2.1. Chemicals and worm strain*

70 Paichongding (IPP, 1-((6-chloropyridin-3-yl) methyl)-7-methyl-8-nitro-5-propoxy-
71 1,2,3,5,6,7-hexahydroimidazo [1,2- α]-pyridine, chemical purity 98.3%) was obtained
72 from Jiangsu Kesheng Company Ltd.. The biodegradation intermediate of IPP,
73 1-((6-chloropyridin-3-yl) methyl)-7-methyl-8-nitro-5-hydroxyl-
74 1,2,3,5,6,7-hexahydroimidazo [1,2- α]-pyridine (M1), was synthesized in our lab and
75 its chemical purity was 97.2%. M1 was prepared and stored in the lab using a protocol

76 described previously (Studzinski et al. 2017). Briefly, IPP was dissolved in
77 dichloromethane and added boron tribromide at -78 °C, then reacted for overnight at
78 room temperature. M1 was extracted by dichloromethane. Other chemical reagents
79 are A.R. grade and purchased from Sinoreagent Company, China.

80 The wild type nematode, *Caenorhabditis elegans*, was used and maintained on
81 nematode growth medium (NGM, pH 7.2) plates seeded with *Escherichia coli* OP50
82 at 20 °C as described previously (Sulston et al. 1974; Zhang et al. 2015; Tissenbaum
83 et al. 2001). Age synchronous populations of *C. elegans* were obtained according to
84 the previous reports (Zhao et al. 2013; Zhang et al. 2015). Different concentration of
85 IPP and M1 (10mg L⁻¹, 5 mg L⁻¹ and 2.5 mg L⁻¹) were added to the NGM plates just
86 before inocubation.

87

88 2.2. *Lifespan and reproduction*

89 During the lifespan assay, the worms transferred daily for the first several days of
90 adulthood. The surviving worms were measured, and recorded every day and would
91 be scored as dead when they did not respond to the stimulation of a platinum wire.

92 The results were treated with three replicates. The reproduction was assayed by the
93 brood size, which was determined as the number of offspring at all stages beyond the
94 egg. Twenty replicates were performed.

95

96 2.3. *Locomotion behavior*

97 For the locomotion behavior assay, different concentration of IPP and M1 treatment

98 were performed throughout the lifespan from L1-larvae. Head thrash and body bend
99 were used as endpoints for locomotion behavior. Head thrashes are defined as a
100 change in the direction of bending at the mid body. Body bends are defined as a
101 change in the direction of the part of nematodes corresponding to the posterior bulb of
102 the pharynx along they-axis, assuming that nematode was traveling along the x-axis.

103 During the locomotion behavior assay, the examined nematodes were transferred
104 into the assay plate containing K medium on top of the agar. After a recovery time of
105 1 min, head thrashes, and body bends were counted for 1 min and 20 s respectively.
106 Twenty replicates were performed for each experiment.

107

108 *2.4. Acetylcholinesterase (AChE, EC 3.1.1.7) activity analysis*

109 Inhibition of AChE in the LC50-treated worms was monitored after 24 and 48 h
110 of exposure. Similarly, the normal worms were used to study the in vitro evaluation of
111 AChE activity. 100 worms were chosen to estimate AChE activity (Ache, EC 3.1.1.7)
112 and were ground with liquid nitrogen, then added 1.5ml of 0.1 M phosphate buffer
113 (containing 0.1% of TritonX-100, pH 7.5) and mixed thoroughly. The mixture was
114 centrifuged at 12,000 rpm for 30 min at 4 oC. The supernatant was used for
115 estimation of AChE activity and protein content. Protein was estimated by the method
116 of Lowry *et al.* (1951). AChE was assayed as described by Ellman *et al.* (1961).
117 The procedures of enzyme assay were as follows: the blank consists of phosphate
118 buffer (0.1 M, pH 8.0), substrate (0.075 M of acetylthiocholine iodide) and DTNB
119 solutions (Dithiobisnitrobenzoic acid, 0.01M, 39.6mg of DTNB were dissolved in

120 10ml pH 7.0 phosphate buffer and 15 mg of sodium bicarbonate were added). 0.1 ml
121 of supernatant and 0.5 ml of substrate were mixed together and kept at 37°C for 6 min,
122 then 0.01 ml of DTNB solution and 4% of SDS solution were added in the reaction
123 solution. The absorbance was measured at 421 nm. Enzyme activity was determined
124 graphically using double-reciprocal plots of Lineweaver and Burk transformations
125

126 *2.5. IPP/M1 degradation in nematodes and metabolite extraction*

127 The wild type worms were grown for two generations on NGM plates seeded with
128 OP50. The worms in six crowded plates were washed into 50 ml solution of NGM
129 medium contained 10 mg L⁻¹ of IPP or M1, grown at 22 °C and 220 rpm.
130 Concentrated OP50 from 500 ml bacterial cultures were given on day 1, day 3 and
131 day 5. The culture was harvested on day 7 by centrifugation at 5000 rpm. The
132 supernatant was lyophilized and the residue was extracted with 95% ethanol for 10 h.
133 the extraction was evaporated at RT to yield the crude extracts.

134 A Dionex U3000 HPLC system coupled with Bruker maXis 4G ion trap mass
135 spectrometer with an electrospray ionization source (ESI) was used for LC-MS/MS
136 analysis. The separate conditions were in accordance with those used for HPLC
137 analysis. The ion source temperature was controlled at 250 °C, and the capillary
138 voltage was -4.5 kV. The analysis mode of ionization was electrospray ionization (ESI,
139 positive). The operation conditions were as follows: collision energy, 10.0 eV; ISCID
140 energy and ion energy, 5.0 eV; dry gas, 6 L·min⁻¹; dry temperature, 180 °C; gas
141 pressure, 1.5 bar. The continuous full scanning from m/z 50 to 500 Da was performed

142 in positive ion mode.

143

144 2.6. RNA isolation and quantitative real-time PCR

145 Total RNA was isolated using Triazol RNA kit (Life technology) from worms

146 treated with or without 5 mg L⁻¹ of IPP or M1 for 48 h. Total RNA was then

147 reverse-transcribed using PrimeScript 1st strand cDNA synthesis kit (Takara).

148 Quantitative real-time-polymerase chain reaction (RT-PCR) was used to determine the

149 relative quantification of the targeted genes (*sod-2*, *daf-2*, *sgk-1*, *aak-2*, *age-1*, *daf-16*

150 and *aak-2* etc.) in comparison to the reference *act-1* gene, and the results were

151 expressed as the relative expression ratio. The primers used in this study were

152 referenced the previous reports by Zhang *et al* and Zhuang *et al*. (Zhang *et al*. 2015;

153 Zhuang *et al*. 2014).

154

155 3. Results and discussion

156

157 3.1. Effect of IPP and M1 on lifespan of *C. elegans*.

158 Nematodes were treated with different concentration of IPP and M1 (2.5 mg L⁻¹, 5

159 mg L⁻¹ and 10 mg L⁻¹) from L1-larvae stage in order to investigate IPP and M1's

160 effect on lifespan of *C. elegans*. The results were shown in Figure 1. Both IPP and M1

161 decreased nematodes lifespan significantly under the concentration of 10 mg L⁻¹, and

162 IPP has higher toxicity to nematodes than M1 under the same condition. Lifespan was

163 only 21 days in the 10 mg L⁻¹ of IPP solution, while it reached 23 days in M1 solution.

164 IPP and M1 both had gentle toxicity to *C. elegans* under the lower concentration (2.5
165 mg L⁻¹).

166

167 3.2. Effect of IPP and M1 on the locomotion and reproduction of *C. elegans*.

168 *C. elegans* shows gradually impaired locomotion ability during its aging process.

169 The effect of IPP and M1 on the locomotion ability of nematodes was investigated.

170 Two important endpoints for locomotion ability, head thrash and body bend, were

171 recorded every 1 minute during its lifespan after treated with different concentration

172 of IPP or M1 solution for 48 hours. As shown in Figure 2a and 2b, higher

173 concentration of IPP and M1 solution significantly decreased locomotion ability

174 during its aging process compared with untreated nematodes, which indicated that IPP

175 and M1 can decrease life quality of nematodes. The body bent frequency are 30 and

176 26 per minute after treated with 5 and 10 mg L⁻¹ of IPP, while they are 39 and 36 per

177 minute after treated with same concentration of M1, respectively. These results

178 showed that under IPP has more toxicity than M1 under the same concentration.

179 Number of progeny of nematodes was used for investigating the effect of IPP and

180 M1 on reproduction of *C. elegans*. Nematodes were pretreated with different

181 concentration of IPP or M1 solution for 48 h, then moved to the plates at 35 °C. The

182 number of progeny of nematodes were counted after 4 days and the results were

183 showed in Figure 2c. There are significant difference on the numbers of progeny with

184 or without IPP or M1 treatment, which indicated that the toxic effect of IPP and M1

185 on *C. elegans* existed by damaging the reproductive system of *C. elegans*. IPP shows

186 more toxicity to the reproductive system of *C. elegans* compared with M1 under the
187 condition of same concentration.

188

189 3.3. Effect of IPP and M1 on AChE activity of *C. elegans*

190 *Acetylcholinesterase* (AChE) is the key enzyme in biological nerve conduction. Its
191 enzymatic degradation of acetylcholine could terminate the effects of
192 neurotransmitters epicuticular on postsynaptic membrane excitation, for ensuring the
193 neural transmission of nerve signals in vivo. It could be concluded from Figure 2d
194 that higher concentration of IPP and M1 strongly inhibit AChE activity. 2.5 mg L⁻¹ of
195 IPP has almost no toxicity to AChE, however AChE activity was significantly inhibit
196 by 5 mg L⁻¹ of IPP. M1 has higher toxicity to AChE activity than IPP, especially under
197 the condition of lower concentration.

198 IPP is a novel chiral neonicotinoid insecticide with four stereoisomers (*RR*, *SS*, *RS*
199 and *SR*-IPP) and it was widely used in China for insecticide control in agriculture. Cai
200 *et al.* reported that IPP could decrease soil enzyme activities, change soil microbial
201 population, diversity and composition (Cai et al. 2015a, 2016b, 2016c). The activities
202 of microbial protease, catalase, urease and dehydrogenase in soil are both impacted
203 after IPP application in crop (Cai et al. 2015a, 2016b, 2016c). The relative abundance
204 of genus of *Bacillus*, *Pseudomonas*, *Azohydromonas* and *Paenibacillus* in soils with
205 IPP application increased, the genus of *Brevundimonas*, *Xanthomonadaceae*, *Massilia*,
206 *Pedobacter*, and *Hydrogenophaga* were newly appearance. The bacterial species
207 diversity and community structure in soils were remarkably different responding to

208 IPP-sprayed (Cai et al. 2016b, 2016c). Neonicotinoids have been considered to
209 increase mortality in honey bees by impairing their homing ability and to reduce the
210 reproductive success of bumble bees and solitary bees (Henry et al. 2012; Whitehorn
211 et al. 2012; Woodcock et al. 2017). Until now, there was little information about
212 neonicotinoids toxicity on other animals. In this study, *Caenorhabditis elegans*,
213 regarded as the model organism, was used as an alternative animal model for IPP
214 toxicity testing. The results showed that IPP and M1 can decrease nematodes lifespan,
215 inhibit locomotion ability and reproductivity. Based on these results, it can be
216 concluded that IPP had higher toxicity to *C. elegans* than M1 in nematodes. They also
217 inhibit AChE activity significantly, AChE was more sensitive to M1. The toxicity
218 differences between IPP and M1 at relatively high concentrations also occurred with
219 the aid of head thrash, body bend, locomotion and lifespan. M1 is one of the
220 intermediates of IPP biodegradation in environment, and also determined in soil after
221 IPP application (Cai et al. 2015a, 2015b, 2016a, 2016b, 2016c; Chen et al. 2017; Fu et
222 al. 2013). The toxicity of toxicants in *C. elegans* is similar to the toxic results in
223 mammals, the results in this study imply the possible potential more severe toxicity of
224 IPP than M1 in mammals. Woodcock *et al.* reported that reproduction in wild bees
225 was negatively correlated with neonicotinoid residues and neonicotinoids can caused
226 a reduced capacity of bee species to establish new populations (Woodcock et al. 2017).
227 Neonicotinoid insecticides are high toxicity to vertebrates, and its use has been partly
228 restricted for their effects on pollinators (Henry et al. 2012; Whitehorn et al. 2012).
229 These researches are accordance with the results in our research.

230

231 *3.4. IPP and M1 degradation characteristics by C. elegans.*

232 During the degradation of IPP in nematode body, three degradation metabolites
233 were determined by HPLC analysis with retention times of 2.2 min (M1), 19.3 min
234 (M2) and 12.2 min (M3). These intermediates appeared with the decrease of IPP. And
235 these three biodegradation intermediates of IPP were analyzed and identified by
236 high-performance chromatography-time-of flight mass spectrometry and LC-MS/MS.

237 The ion fragments of IPP were at m/z 367.1534 ($C_{17}H_{23}ClN_4O_3$) $[M+H]^+$ and m/z
238 321.1578 (Figure 3a), and the ion fragment of m/z 367.1500 includes daughter ions of
239 306.1368 (100), 308.4442 (15) and 137.1069 (62), which were accordance with the
240 structure of standard IPP (Cai et al. 2015a,2015b; Chen et al. 2017). M1 showed m/z
241 325.1052 $[M+H]$, and the daughter ion showed m/z 264.0904. Metabolite M1 was
242 identified as 1-((6-chloropyridin-3-yl) methyl)-7-methyl- 8-nitro-5-hydroxy-
243 1,2,3,5,6,7- hexahydroimidazo [1,2- α -] pyridine according to its LC-MS/MS and MS²
244 spectra (Figure 3d), which indicated that M1 was generated through hydrolysis of
245 propoxyl group of IPP. M2 appeared after M1 and its m/z was 276.0887 (M+H, Figure
246 3c), the M2 ions matched the formula $C_{14}H_{14}ClN_3O$, M2 was identified as
247 1-((6-chloropyridin-3-yl)methyl)-2,3- dihydro-5-one-7- methylimidazo [1,2- α]
248 pyridine.

249 Figure 3d shows that the m/z of metabolite M3 was 297.1144, which should be
250 the transformation of the group of 8-nitroso on M1 to the group of 8-amino, and the
251 nitroso group on M1 transformed to hydroxyl. On the basis of these characteristics,

252 M3 was preliminarily identified as 1-((6-chloropyridin-3-yl)methyl)-5,7-diol-8-
253 amino -1,2,3,5,6,7-hexahydroimidazo [1,2- α]pyridine.

254 During the degradation of M1 in nematode body, two degradation metabolites
255 were determined by HPLC analysis with retention times of 19.3 min (M2) and 12.2
256 min (M3). M2 and M3 were same intermediates of IPP. The results showed that IPP
257 and M1 have the same biodegradation intermediates and pathway in nematode.

258 In nematodes, there were three metabolites, M1, M2 and M3 were determined,
259 M1 appeared first, and then M2 and M3 formed almost simultaneously. The propyl
260 group of IPP was de-esterified and hydrolyzed to form M1, which was accordance
261 with the previous study (Cai et al. 2015a,2015b; Chen et al. 2017). M1 was
262 determined during the process of IPP biodegradation. The nitro group of M1 could be
263 aminated to form amino, methyl was hydrolyzed to hydroxyl group, thus M3 was
264 generated. M2 was formed through loss of nitro group from M1. Therefore the
265 biodegradation pathway in nematodes was concluded in Figure 5. M1 biodegradation
266 pattern was also described in Figure 5. IPP and M1 degradation pathway in nematodes
267 is similar with IPP biodegradation in aquatic and soil environment (Cai et al.
268 2015a,2015b; Chen et al. 2017).

269

270 *3.5. Effect of IPP and M1 on relative genes expression in nematodes*

271 There are three major signaling pathways controlling nematodes' aging process,
272 IGF (insulin/insulin-like growth factor), TOR (target of rapamycin) and germline
273 signaling pathways. In order to illustrate the mechanism of IPP and M1 toxicity to the

274 nematodes lifespan, expression of several genes involved in three signaling pathways
275 were investigated. After exposure in 5mg L⁻¹ of IPP or M1, expression level of *sod-2*,
276 *daf-16*, *sgk-1* and *aak-2* genes decreased, and *daf-2* and *age-1* genes increased (Figure
277 6). IPP has more toxicity for inhibiting the expression levels of *daf-16*, *sgk-1* and
278 *aak-2* than M1. IPP and M1 did not increase expression level of some genes involved
279 in TOR pathways, however, they both inhibited *daf-15* and *rict-1* gene expression (Fig.
280 6). Both IPP and M1 did not significantly influence expression level of some genes
281 involved in germline signaling pathway. The results indicated that IPP and M1 should
282 affect the lifespan through mixed IGF and TOF signaling pathways in nematodes.

283 The chemical structure of IPP and M1 is similar, the data of this study showed
284 that IPP and M1 inhibit lifespan and reproduction of nematodes possibly through
285 same molecular mechanism. They might reduce the lifespan and reproduction through
286 affecting both the IGF signaling pathway and TOR signaling pathway. In *C. elegans*,
287 insulin/IGF-1 receptor (DAF-2) can activate tyrosine kinase and initiates
288 phosphorylation process, which make some kinase active, including
289 phosphatidylinositol 3-kinase (PI3K/AGE-1), 3-phosphoinositide-dependent kinase-1
290 (PDK-1) and serin/theonine-protein kinase (SGK-1) (Zhuang et al. 2014). SGK-1
291 phosphorylates and inactivates the FOXO transcription factor DAF-16, which can
292 block the transcription of targeted genes (Zhuang et al. 2014). TOR C1 and TOR C2
293 are different complexes in TOR signaling pathway, and they have different
294 coactivators, DAF-15 Raptor and RICT-1 Rictor. In this study, IPP and M1 can
295 decrease lifespan and reproduction of nematodes, which probably due to the induction

296 of severe change of genes required for aging and reproductive control, and even
297 alteration in signaling pathways. The results illustrated that *daf-16*, *sgk-1*, *aak-2*,
298 *daf-15* and *rict-1* genes decreased after exposed to IPP and M1.

299

300 **4. Conclusions**

301 IPP and its metabolite, M1, were selected to study their toxicity to the model
302 organism, *Caenorhabditis elegans*. The effect on lifespan, locomotion, reproduction
303 and AChE activity were investigated using different concentration of IPP and M1.
304 And the biodegradation pathway of IPP and M1 were also proposed through
305 identifying their intermediates by LC/LC-MS. In order to discuss molecular
306 mechanism of reduction of lifespan and reproduction in nematodes, the expression
307 level of some genes related to three signaling pathways were illuminated. The data
308 showed that both insulin/IGF-1 signaling pathway and TOR signaling pathway were
309 involved in the regulation of toxicity induction from IPP or M1 in nematodes. We also
310 hypothesized that IPP and M1 induced the toxicity on nematodes through same
311 molecular mechanism, which will be helpful for better understanding the potential
312 damage on health of animals.

313

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318

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399 **TABLE AND FIGURE CAPTIONS**

400

401 **Figure 1.** Effect of IPP and M1 on lifespan of *C. elegans*. (A. 10 mg L⁻¹; B. 5 mg L⁻¹;
402 C. 2.5 mg L⁻¹).

403 **Figure 2.** Effect of IPP and M1 on locomotion, reproduction and AChE activity of *C.*
404 *elegans*. (A:Body bent frequency; B Head swing frequency; C: Number of progeny; D: AchE
405 activity).

406 **Figure 3.** Mass spectra of IPP and metabolites (M1, M2 and M3) .(a:IPP; b:M1; c:M2;
407 d:M3).

408 **Figure 4.** HPLC chromatograms with the retention time of degradation molecules

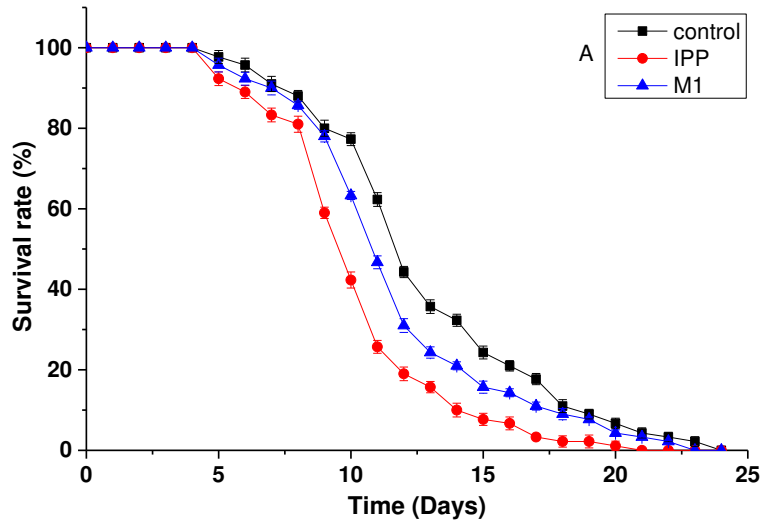
409 **Figure 5.** Proposed pathways of IPP and M1 degradation in nematode *C. elegans*.

410 **Figure 6.** Expression level of some genes involved in signaling pathways in nematode
411 *C. elegans*.

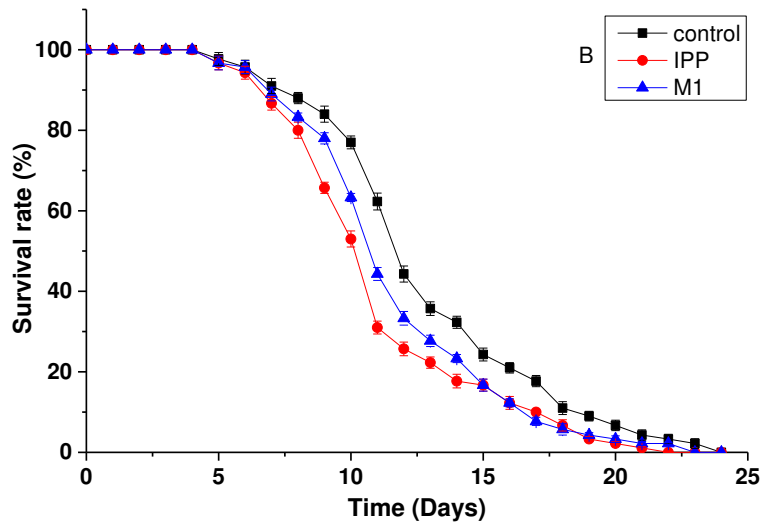
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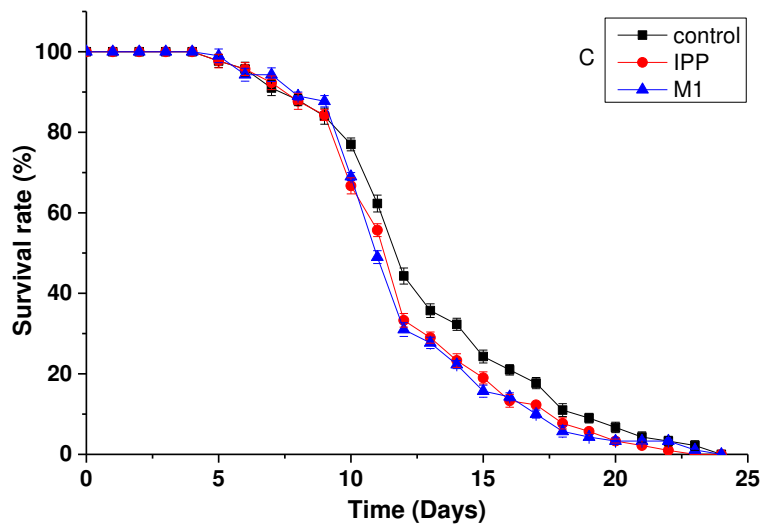
414 **Figure 1.**



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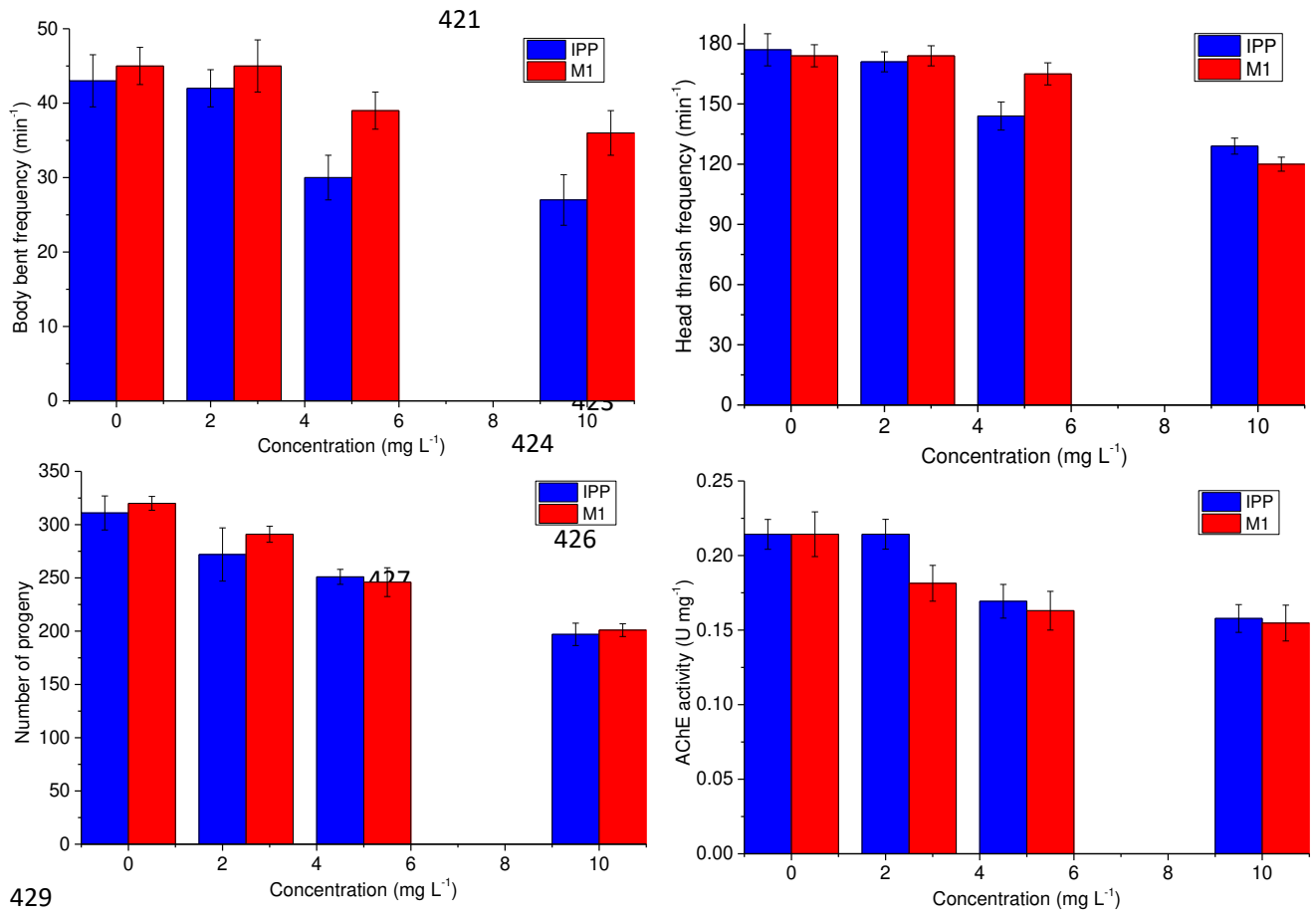


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420 **Figure 2**



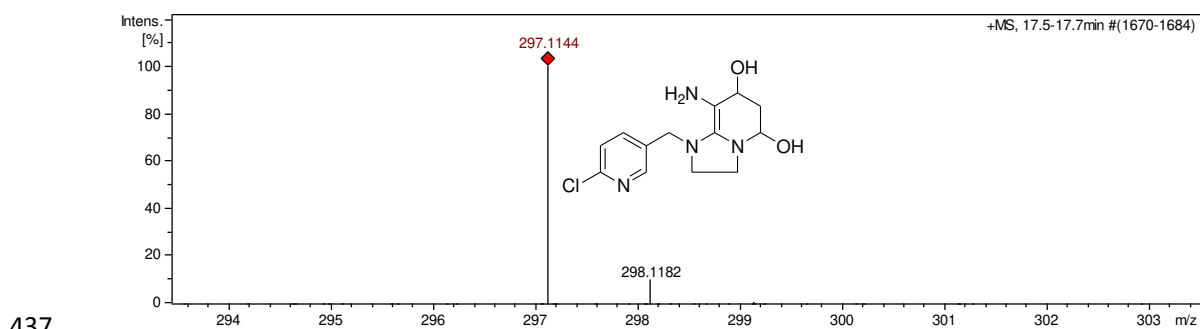
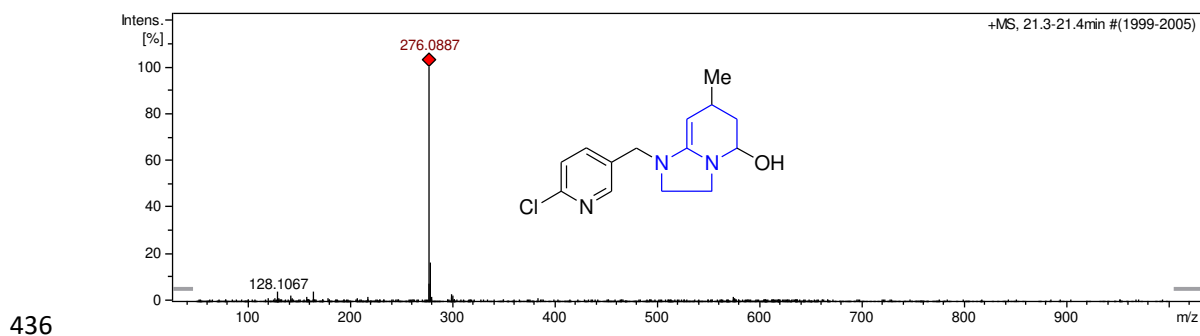
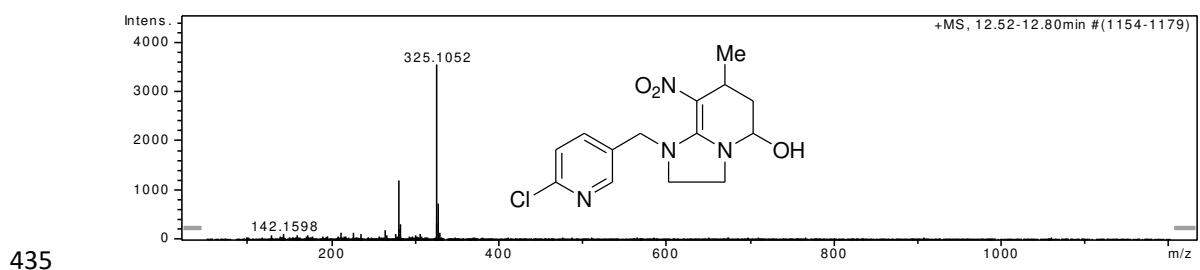
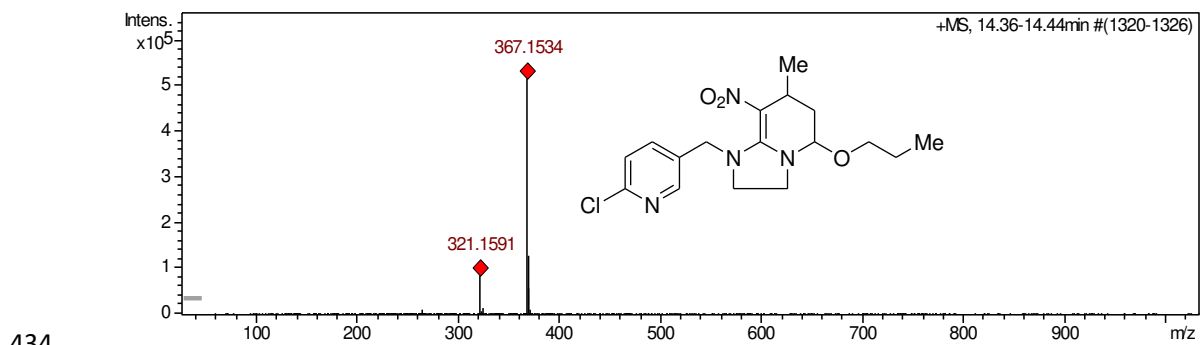
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433 **Figure 3.**

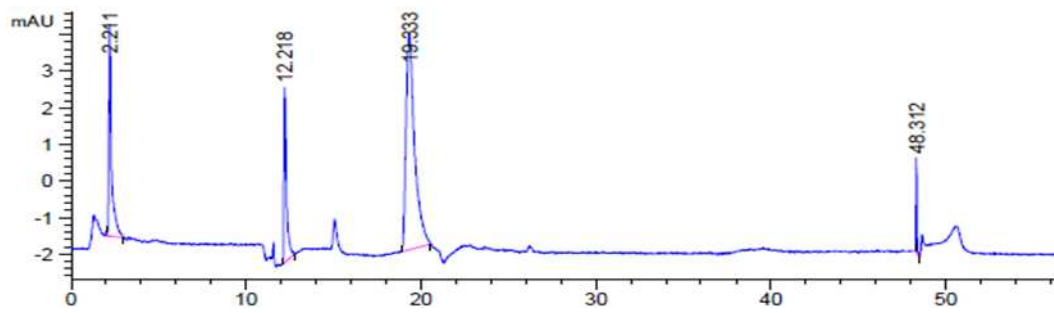


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440 **Figure 4.**

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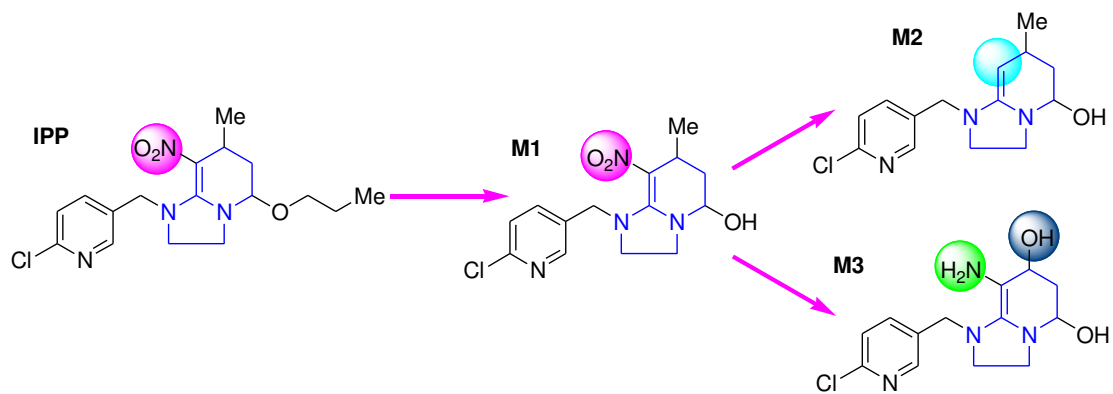
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447 **Figure 5.**

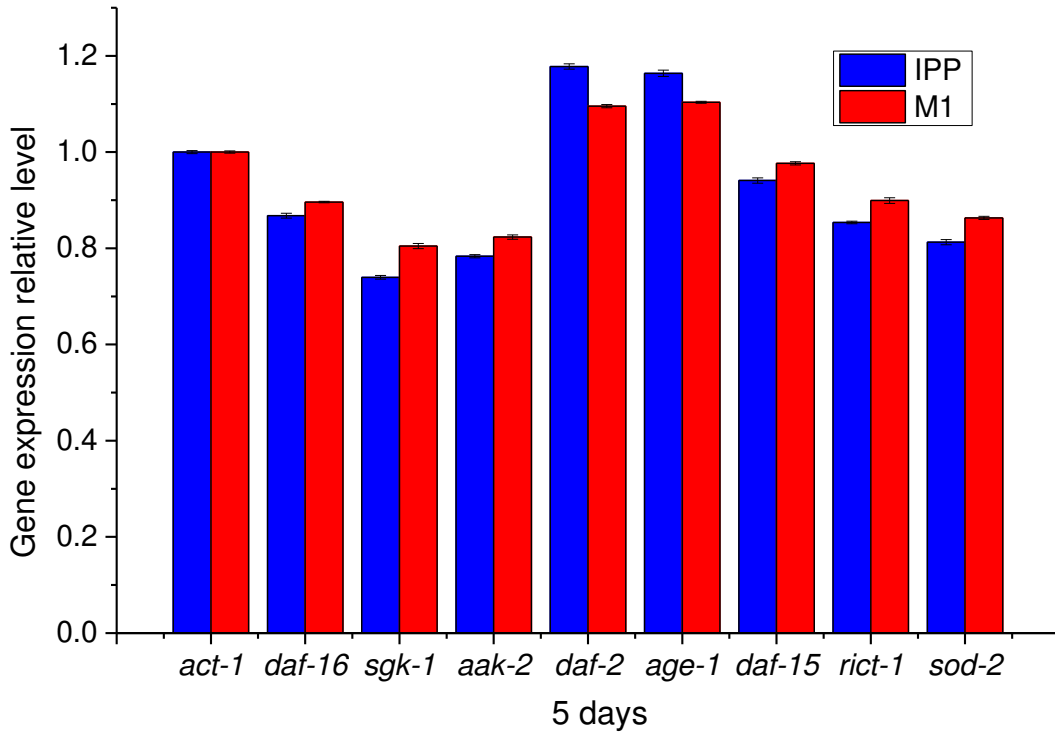


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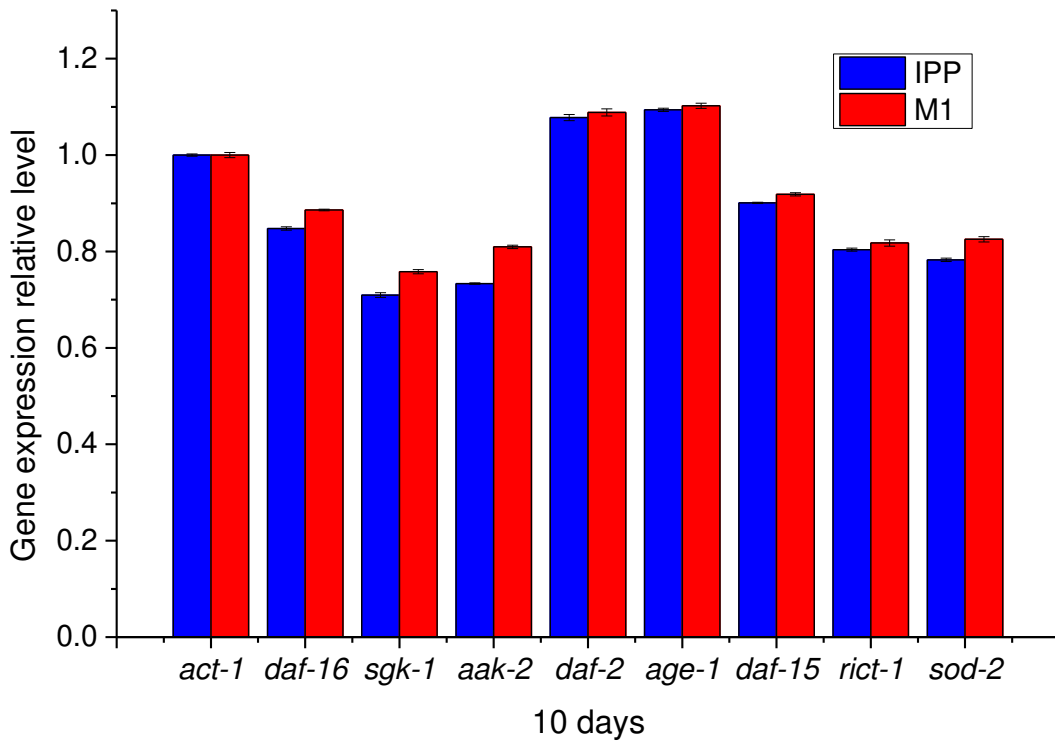
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451 **Figure 6**



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