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**Does catechin make the Mediterranean palm tree *Chamaerops humilis* L. an unsuitable host for *Rhynchophorus ferrugineus*?**

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Does catechin make the Mediterranean palm tree *Chamaerops humilis* L. an unsuitable host for *Rhynchophorus ferrugineus* (Olivier)?

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1 **Can catechin make the Mediterranean palm tree *Chamaerops humilis* L. an unsuitable**  
2 **host for *Rhynchophorus ferrugineus* (Olivier, 1790)?**

3  
4 Short title

5 **Toxic effect of catechin against *R. ferrugineus***

6  
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19  
20 **Abstract**

21 The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), is an  
22 insect pest native to Southeast Asia, which has become the major threat of palms in the  
23 Mediterranean Basin, mainly due to the movement of infested planting material. Once  
24 infested, palms are difficult to manage and often die. In the literature, *Chamaerops humilis*  
25 palms are reported as resistant to RPW, but until now, no investigation has been performed on

26 *C. humilis* tissues to detect the possible presence of toxic endogenous metabolites on RPW. In  
27 the present study, we focused on the potential toxic effect of catechin against RPW larvae. In  
28 laboratory bioassays, the purified catechin was provided to two- and five-year old RPW  
29 larvae at three different concentrations. Our data showed how catechin can impair the survival  
30 of RPW, causing toxicity at concentrations as lower as 0.03  $\mu\text{g g}^{-1}$ . Larval mortality was dose-  
31 dependent, and furthermore larval age influenced the effect of catechin, being older larvae  
32 more susceptible than younger ones. The observed toxicity of catechin on RPW larvae agrees  
33 with the hypothesis that this compound is a component of the chemical defense mechanism of  
34 *C. humilis* against this insect pest.

35

### 36 **Keywords**

37 Red palm weevil, Curculionidae, exotic insect pest, toxicity, palm resistance

38

### 39 **Introduction**

40 The invasive curculionid *Rhynchophorus ferrugineus* (Olivier, 1790), otherwise known as the  
41 Red Palm Weevil (RPW), has become in the last three decades the most important pest  
42 affecting palms all over the world (El-Mergawy and Al-Ajlan 2011). This insect, native to  
43 Southeast Asia, has quickly colonized wide geographical areas in Asia, Africa, America and  
44 Europe and now its diet encompasses more than 40 different palm species, almost all  
45 Areaceae, although this pest originally subsisted on just four species in its native range  
46 (Nirula 1956).

47 Damage to host plants is mainly caused by the larval stage feeding within the trunk of palms  
48 and destroying the vascular system. Infestation can adversely affect fruit yield, lower the  
49 growth rate of the palm, and eventually cause its collapse and death (Blumberg 2008). A  
50 variable degree of tolerance to RPW has been observed in host palm species, ranging from a

51 minimum in *Phoenix canariensis* Chabaud (Barranco et al. 2000) to a significant degree in  
52 *Washingtonia filifera* (Lindl.) H.Wendl. (Cangelosi et al. 2016). *C. humilis*, native to Europe,  
53 is considered resistant to RPW (Barranco et al. 2000) and for this palm species, an antixenotic  
54 mechanism of resistance has been postulated (Dembilio and Jacas 2012). According to this  
55 hypothesis, the leaf rachis –a place frequently chosen by the females to lay their eggs—  
56 would be too tough and fibrous, being therefore unsuitable for oviposition. This sole  
57 antixenosis-based mechanism proved to be, in some cases, not enough to prevent RPW from  
58 invading palm tissues (Dembilio et al. 2009), suggesting the presence of a supplementary  
59 defensive mechanism in *C. humilis*. In a similar way, in *W. filifera* a complementary  
60 antibiosis defensive mechanism was first postulated (Dembilio et al. 2009) and then  
61 demonstrated (Cangelosi et al. 2015). Until now, however, no investigation has been  
62 performed on *C. humilis* tissues to detect the possible presence of endogenous metabolites  
63 with a significant degree of toxicity on RPW.

64         The aim of this study was to characterize part of the chemical mechanism that is  
65 supposed to contribute to the observed resistance of *C. humilis* against *R. ferrugineus*. To do  
66 this, we analysed the chemical composition of the leaf tissues of *C. humilis* looking for  
67 compounds with a potential toxic effect against the larvae of *R. ferrugineus*. Due to its  
68 structural similarity to the chalconoid compound that is supposed to contribute to the  
69 chemical resistance mechanism in *W. filifera* (Cangelosi et al. 2015), we focused on the  
70 potential toxic effect of catechin. We used several chromatographic techniques in order to  
71 separate and purify this compound from the base of the leaves of *C. humilis*. The purified  
72 catechin was provided in laboratory bioassays at diverse concentrations to RPW larvae.  
73 Finally, we discussed the observed results in the context of the emerging importance of the  
74 chemical resistance of palms against this invasive insect pest.

75

76 **Material and Methods**

77 *Plant material*

78 Leaves of *C. humilis* were collected from healthy individuals of about 20 years old located in  
79 the historical garden of Villa Ormond, Sanremo, Italy (43°49'20'' N, 7°47'24'' E). The leaf  
80 basal part, i.e., the first 25 cm from leaf insertion on the palm stem, was harvested and  
81 immediately submitted to the laboratory. The basal part of the leaves is considered a target  
82 zone where the RPW females can lay their eggs (Dembilio and Jacas 2012). Some samples  
83 were kept as vouchers at -20 °C in the biochemistry laboratory of the CREA at Sanremo.

84 *Extraction, isolation and quantification of the selected compound*

85 Fresh leaf material (1 Kg) from several leaves was cut in small pieces (0.8-1.0 cm<sup>3</sup>) and  
86 extracted with H<sub>2</sub>O kept boiling for 3 h. The extracts were then concentrated to 100 mL at  
87 reduced pressure, filtered on Whatman paper and kept at -20 °C until needed. Aliquots (5  
88 mL) of the aqueous extract of *C. humilis* leaf basal tissues were chromatographed through a  
89 column (30 × 5 cm) filled with silica gel RP-18, packed with 200 mL MeOH and eluted  
90 according to a linear gradient elution profile from 90% to 0% A in B, where solvent A (150  
91 mL) was HCOOH 5% in H<sub>2</sub>O and solvent B (150 mL) was absolute EtOH. Fractionation of  
92 the elution was performed by a Gilson 203 Micro Fraction Collector. The obtained fractions  
93 (3 mL volume) were checked for purity on Merck precoated cellulose 20 × 20 cm glass TLC  
94 plates, eluted with propanol:H<sub>2</sub>O:acetic acid (20:80:1, v/v/v) or propanol:H<sub>2</sub>O (20:80, v/v)  
95 according to Vovk et al. (2005). TLC plates were inspected under visible and UV light at 254  
96 and 366 nm wavelength.

97 Chromatographic fractions presumably containing catechin were pooled and its UV spectrum  
98 was obtained using a Hitachi 150-20 spectrophotometer. The concentration of catechin in the  
99 analysed tissues of the palm was initially calculated via its molar attenuation coefficient ( $\epsilon$ ),  
100 by dissolving known amounts of the purified compound in MeOH.

101 The purity of the isolated catechin fraction was checked via reversed-phase liquid  
102 chromatography (RP-HPLC). The analysis was performed using a Supelco Discovery C18  
103 column 25 cm × 4.6 mm, 5µm particle size (Sigma Aldrich, USA). Solvents used were 0.1%  
104 H<sub>3</sub>PO<sub>4</sub> in water (A) and acetonitrile (B); the solvent gradient method was 0-30 min from 10%  
105 to 58% B, 1 min from 58% to 90% B, 5 min isocratic 10% A / 90% B, 1 min from 90% to  
106 10% B and 25 min isocratic 90% A / 10% B. Sample injection volume was 20 µL; solvent  
107 flow rate was 1 mL min<sup>-1</sup>. The exact identity of the isolated catechin was further verified via  
108 nuclear magnetic resonance (NMR), following the procedure used by Cangelosi et al. (2015).

#### 109 *Insect colonies*

110 Adults of *R. ferrugineus* were collected from infested palm trees in Sanremo (Italy) and  
111 transferred to the laboratory of the DISAFA of the University of Torino. The insects were  
112 surface sterilized with 2% formalin (37% formaldehyde) and left to dry in Petri dishes (15 cm  
113 diameter). Then they were transferred to rearing cages (20 cm length × 13 cm width × 12 cm  
114 height) in order to set up a mass rearing. All the insects were fed on banana (*Musa*  
115 *paradisiaca* L., Chiquita<sup>®</sup>'s organic banana) and apple slices (*Malus domestica* Borkh.,  
116 Golden Delicious<sup>®</sup> organic apple) and kept in climatic chamber (29 ± 1.5 °C, 65 ± 0.8% RH,  
117 16:8 L:D, 4000 lux illumination). The artificial diet was replaced every two days, carefully  
118 dissected, and the eggs removed according to the methods described by Weissling and Giblin-  
119 Davis (1994). The eggs were cleaned with 2‰ formalin solution for 10 min to prevent viral  
120 disease (Singh *et al.*, 1985) and then were transferred to Petri dishes (9 cm diameter) lined  
121 with moistened filter paper, using deionized water, sealed with parafilm, and stored in  
122 climatic chamber (29 ± 1.5 °C, 65 ± 0.8% RH, 16:8 L:D, 4000 lux illumination) until larval  
123 emergence.

#### 124 *Laboratory bioassays*

125 Two- and five-week old RPW larvae were individually placed in sterilised Petri dishes lined  
126 with humidified filter paper and sealed with Parafilm®. Larvae were fed with cylinder-shape  
127 pieces (1 cm height × 2.5 cm diameter) of organic apple, dipped for 1 min in a water solution  
128 of the selected flavonoid at three different concentrations. To do this, the pooled fraction  
129 containing the isolated compound was evaporated under reduced pressure; the resulting  
130 powder was resuspended in deionized water and adjusted to an initial concentration of 0.12  
131  $\mu\text{g mL}^{-1}$  (42 mM). Afterwards, two-fold dilutions in demineralized water were carried out in  
132 order to get the other two experimental concentrations, namely 0.06 and 0.03  $\mu\text{g mL}^{-1}$  (21 and  
133 10.5 mM, respectively). Controls were represented by RPW larvae fed with apple cylinders  
134 dipped for the same time duration in a filtered raw extract of basal leaf parts of *P. canariensis*,  
135 since this extract has no negative effect on the growth and survival of *R. ferrugineus* larvae  
136 (Cangelosi et al. 2016).

137 The effect of each concentration was tested on the two selected cohorts of RPW larvae for  
138 192 h ( $n = 10$ ). Every 24 hours the number of dead larvae was recorded. The test was carried  
139 out in controlled conditions at  $29 \pm 1.5$  °C,  $65 \pm 0.8\%$  RH, 16:8 L:D, 4000 lux illumination.

#### 140 *Statistical analysis*

141 Data were analysed using the R environment (R Core Team, 2019). The relationship between  
142 exposure time and larval survival at different concentrations for each cohort was determined  
143 via generalized linear models (GLMs) with binomial error distribution (dead / alive), using  
144 the function `glm()`. Lethal times ( $LT_{50}$  and  $LT_{95}$ ) of catechin for two- and five-week old *R.*  
145 *ferrugineus* larvae were calculated from the respective glm models using the function `dose.p()`  
146 ) from the MASS package. Statistical difference between model slopes and lethal times at  
147 different catechin concentrations for each larval age was calculated by means of z-tests (Zar  
148 2010). The statistical significance of the observed differences in lethal times between the two



149 age groups of larvae was calculated using a *t*-test. Data presented through the text are means  $\pm$   
150 SE.

151

## 152 **Results**

### 153 *Occurrence of catechin in the leaves of C. humilis*

154 TLC spots of the purified catechin fraction were uncoloured under visible light and appeared  
155 as dark violet areas under UV light (254 nm). No colour was observed under UV light at 366  
156 nm wavelength. Its UV spectrum in MeOH revealed a main peak of absorbance at 274 nm.

157 The NMR analysis of the molecular structure of the purified fraction confirmed that it was the  
158 widespread flavan-3-ol catechin (C<sub>15</sub>H<sub>14</sub>O<sub>6</sub>; molar mass = 290.271 g mol<sup>-1</sup>; IUPAC name  
159 (2*R*,3*S*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol). Catechin can be  
160 found in some plants as two enantiomers, called (+)-catechin (2*R*,3*S*) and (-)-catechin (2*S*,3*R*),  
161 which differ significantly in their biological activity (Bais et al. 2002). Since our study did not  
162 consider the use of chiral chromatography, it was not possible to determine the relative amount  
163 of the two enantiomers in the leaf samples of *C. humilis*. Based on a log  $\epsilon$  value, calculated in  
164 MeOH at 274 nm wavelength, the overall concentration of catechin within *C. humilis* leaf  
165 basal tissues was estimated to amount up to 0.238  $\mu\text{g g}^{-1}$  fresh material, a concentration in the  
166 same order of magnitude of those we used in the laboratory bioassays with RPW larvae,  
167 considering a correspondence between g and mL. Previous HPLC analyses carried out on leaf  
168 basal tissue extracts of both RPW-resistant *W. filifera* and RPW-susceptible *P. canariensis*  
169 individuals revealed that catechin was more abundant in *C. humilis* tissues (data not shown).

### 170 *Laboratory bioassays*

171 Purified catechin from *C. humilis* had a significant negative effect on the survival of RPW  
172 larvae (Table 1). Larval mortality was dose-dependent, being doses 1 and 2 (42 and 21 mM,  
173 respectively) more toxic than dose 3 (10.5 mM, Figg. 1A-B). Larval age also influenced the

174 negative effect of catechin; overall, older larvae were more susceptible than younger larvae,  
175 with an estimated  $LT_{50}$  1.9 times shorter (Welch two sample  $t$ -test,  $t = 5.073$ ,  $df = 2.74$ ,  $P =$   
176  $0.009$ , see Table 1). Average  $LT_{95}$  was also significantly shorter in five-week old larvae,  
177 where catechin led to 95% mortality 2.1 times faster than in the two-week old group (Welch  
178 two sample  $t$ -test,  $t = 3.978$ ,  $df = 2.05$ ,  $P = 0.028$ , see Table 1). Accordingly, GLMs slopes  
179 were significantly steeper in the group of five weeks old larvae (Welch two sample  $t$ -test,  $t =$   
180  $4.122$ ,  $df = 3.90$ ,  $P = 0.008$ , Figg. 1A-B).

181

## 182 **Discussion**

183 The impact of exotic pests varies considerably depending on the host plant species and the  
184 area being invaded. Control strategies, namely chemical, cultural, and biological strategies are  
185 often time- and cost-effective, and for many insect pests inapplicable due to the endophytic  
186 habit during the early stages of infestation and the potential adverse effects on the  
187 environment and public health.

188 In the search for alternative solutions to face pest outbreaks, the interest in studying plant  
189 species compound as a defense tool against pests has increased. Secondary plant metabolites,  
190 such as alkaloids, glycoalkaloids, terpenoids, organic acids or alcohols, are regarded as  
191 promising sources of plant-protecting substances (Chowański et al. 2016). Literature reports  
192 have indicated that many of these compounds have important implications in the agricultural  
193 and forestry environment. For example, the pesticidal importance of saponins in higher plants  
194 has been reported for *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae), an  
195 invasive leafminer of *Aesculus* spp. (Ferracini et al. 2010), as well as the antifeedant activity  
196 of *Ginkgo biloba* secondary metabolites against *Hyphantria cunea* (Lepidoptera: Arctiidae)  
197 larvae (Pan et al. 2016), and the antifeedants compounds isolated from the bark of the  
198 lodgepole pine effective against the pine weevil *Hylobius abietis* (L.) (Coleoptera:

199 Curculionidae) (Bratt et al. 2001). In this study, we found that catechin, an abundant phenolic  
200 compound present in the raquis of the *C. humilis* palm, can impair the survival of RPW  
201 larvae. Since this compound significantly contributes to the leaf phenolic profile of *C.*  
202 *humilis*, its observed toxic effect strongly points at its involvement in the chemical defense  
203 mechanism of this palm against *R. ferrugineus*. In our leaf samples, catechin was present at  
204 concentrations higher than previously reported by other Authors in *C. humilis* (0.08 – 0.16  $\mu\text{g}$   
205  $\text{g}^{-1}$ , Delle Monache et al. 1972), although we found that this compound can cause toxicity in  
206 RPW larvae at concentrations as low as 0.03  $\mu\text{g g}^{-1}$ . Considering its relative abundance in  
207 the basal part of the leaves, this compound likely makes part of a constitutive, unspecific  
208 defence mechanism of this palm species against herbivore insects and pathogens in general.  
209 Actually, catechin has been reported to cause feeding deterrence and/or toxicity not only in  
210 curculionid species (Khatun et al. 2011; Hammerbacher et al. 2019), but also in insect species  
211 belonging to the Pyralidae, Chrysomelidae, Scarabaeidae, Tenebrionidae and Culicidae  
212 families (Potter and Held 2002; Pavela 2007; Barboza-Silva et al. 2009; Khatun et al. 2011;  
213 Elumalai et al. 2016). In addition, catechin is known to display antimicrobial activity against  
214 plant pathogenic fungi (Yamaji and Ichihara 2011; Ullah et al. 2017). Although our leaf  
215 samples were collected from apparently healthy individuals, variation in catechin  
216 concentration as a plant response to the presence of insect eggs or larval stages cannot be  
217 excluded and needs to be checked in further studies.

218 The chemical structure of catechin significantly resembles that of the other metabolite hitherto  
219 isolated from palms and likely related to their resistance to *R. ferrugineus*, the chalconoid  
220 filiferol (Cangelosi et al. 2015). Contrary to filiferol, catechin cannot induce protein  
221 precipitation (Hagerman and Butler 1978), excluding direct inactivation of digestive enzymes  
222 and access limitation to ingested proteins as its mechanism of action. In addition, many insect  
223 species show protective mechanisms, such as alkalinisation of the gut fluids and the presence

224 of surfactants, as adaptations to prevent protein binding in the gut lumen (Martin et al. 1985;  
225 Zimmer 1997). The mechanism of action of catechin might instead rely on its oxidation under  
226 alkaline conditions to form *o*-benzoquinones (Han et al. 2019), that can lead to the production  
227 of reactive oxygen species (ROS) with significant cytotoxic and immunotoxic effects (Bolton  
228 and Dunlap 2017). Although catechin is also present in RPW susceptible species, such as  
229 *Phoenix dactylifera* L. and *P. canariensis* (Ziouti et al. 1996; Hifnawy et al. 2016), little is  
230 known about the distribution of this compound in the different parts of the plants and about its  
231 availability as a function of the type of tissue. In addition, there is evidence that the  
232 performance of herbivore insects does not necessarily follows a linear and dose-dependent  
233 relationship with the concentration of potentially toxic metabolites produced by plants  
234 (Lehrman et al. 2012), suggesting that catechin toxicity might depend on the presence of other  
235 compounds. These hypothesized interactions would support our previous idea that chemical  
236 defense of palms against RPW is likely based on a diversity of substances and mechanisms  
237 (Cangelosi et al. 2016).

238         The delayed mortality of young larvae treated with catechin revealed an unexpected  
239 pattern of response to a plant toxic compound. Except for some cases (Sáenz-de-Cabezón  
240 Irigaray et al. 2005), old and large larvae are usually more resistant to toxicants than young  
241 larvae (Schoonhoven et al. 2005). This tolerance is related to factors such as age-related  
242 changes in the physiology of the digestive apparatus (Schultz and Lechowicz 1986; Keller et  
243 al. 1996) and the ability to immobilize toxic compounds in storage tissues (Nishida, 1994).  
244 Our results indicate that in RPW larvae none of these mechanisms operate to reduce the toxic  
245 effect of catechin, suggesting that *R. ferrugineus* is poorly adapted to the toxicity caused by  
246 this kind of compound. The delayed mortality in young RPW larvae points also to a post-  
247 ingestive mode of action of catechin, excluding toxicity effects by contact. This kind of  
248 exposure effects depends on the body surface area / volume ratio and are typically stronger in

249 young/small individuals (Traas and Van Leeuwen 2007). The observed feeding rate of the  
250 larvae during our experiments also excluded the functioning of catechin as a feeding  
251 deterrent. Since at the subcellular level catechins are known to accumulate not only in the  
252 chloroplasts of the soft palisade cells, but also in the vessel wall (Liu et al. 2009), we  
253 hypothesize that age-related differences in food digestion efficiency may determine the  
254 greater susceptibility of older larvae to catechin. In RPW larvae, the relative sizes of the gut  
255 parts change and the overall gut length increases with age (Monroy and Ferracini,  
256 unpublished data), suggesting that increased gut transit times with age may favour the  
257 absorption of cell wall-bound phenolic compounds. As far as we know, no studies addressing  
258 this possible factor are available neither for *R. ferrugineus* nor for other insect larvae.  
259 In conclusion, the observed toxicity of catechin on RPW larvae agrees with the hypothesis  
260 that this compound is a component of the chemical defense mechanism of *C. humilis* against  
261 this insect pest. Based on their relative abundance in the analysed leaf tissues, catechin may  
262 be considered as a constitutive defense compound, and therefore its concentration likely  
263 depends on biotic (sex, age) and abiotic (environment) factors. Finally, the identification of  
264 different toxic compounds to *R. ferrugineus* in different palm species supports the view of a  
265 rich, so far hidden array of chemical resistance mechanisms against this pest in some of its  
266 potential hosts.

267

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273

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### 391 **Figure legend**

392 Figure 1. Negative effect of three catechin concentrations on the survival of two-week-old  
393 larvae (A) or five-week old larvae (B) of *R. ferrugineus* ( $n = 10$ ). The control treatment  
394 consisted of a non-toxic extract of leaf basal tissues from the palm *P. canariensis*, a  
395 susceptible, non-resistant host of *R. ferrugineus*. Dose 1: 42 mM; dose 2: 21 mM; dose 3: 10.5  
396 mM.

397 **Table 1.** Comparison of dose-response model slopes and lethal times (LT<sub>50</sub> and LT<sub>95</sub>) of the toxicity tests with (±)-catechin from *C. humilis* on 2- and 5-week-  
 398 old *R. ferrugineus* larvae. Data are presented as estimates ± SE. Different letters indicate statistically significant differences at α = 0.05 using a z-test.

399

*2-week-old larvae*

Dose	Model slope	LT <sub>50</sub> (days)	LT <sub>95</sub> (days)
1 (42 mM)	- 0.779 ± 0.202 <sup>a</sup>	10.55 ± 0.62 <sup>a</sup>	14.33 ± 1.39 <sup>a</sup>
2 (21 mM)	- 0.509 ± 0.145 <sup>ab</sup>	11.83 ± 1.07 <sup>b</sup>	17.62 ± 2.53 <sup>b</sup>
3 (10.5 mM)	- 0.349 ± 0.121 <sup>b</sup>	14.02 ± 2.21 <sup>c</sup>	22.45 ± 4.96 <sup>c</sup>

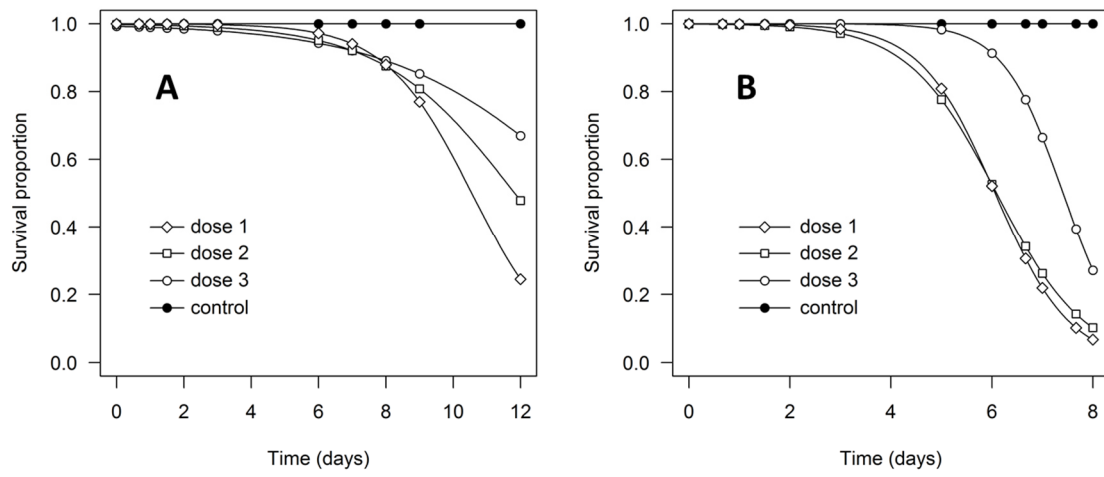
*5-week-old larvae*

Dose	Model slope	LT <sub>50</sub> (days)	LT <sub>95</sub> (days)
1 (42 mM)	- 1.357 ± 0.303 <sup>ab</sup>	6.06 ± 0.25 <sup>a</sup>	8.23 ± 0.48 <sup>a</sup>
2 (21 mM)	- 1.140 ± 0.242 <sup>a</sup>	6.09 ± 0.27 <sup>a</sup>	8.67 ± 0.56 <sup>ab</sup>
3 (10.5 mM)	- 1.673 ± 0.471 <sup>b</sup>	7.41 ± 0.21 <sup>b</sup>	9.17 ± 0.59 <sup>b</sup>

400

401

402 Figure 1



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