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1 **Repellency and toxicity of azadirachtin against granary weevil *Sitophilus granarius* L.**  
2 **(Coleoptera: Curculionidae)**

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7  
8  
9 **Abstract:** The granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), is  
10 known as a primary pest; and is able to feed on whole and undamaged cereal grains. This pest  
11 is probably one of the most destructive stored-product insect pests throughout the world  
12 affecting the quantity as well as quality of the grains. We have evaluated the fumigant and  
13 contact toxicity and the repellent property of azadirachtin a neem-based insecticide against *S.*  
14 *granarius* adults. Azadirachtin was found to exhibit fumigant and contact toxicity and the  
15 mortality increased as function the concentration and exposure time. In addition, the obtained  
16 results revealed an increase in the percent repellency as a function of concentration.  
17 Biomarker measurements in treated adult (LC<sub>25</sub> and LC<sub>50</sub>) revealed, activation of  
18 detoxification system as showed by an increase in CAT and GST activity and also a decrease  
19 in GSH rate. Moreover, nutrition depletion index was found to be concentration dependent  
20 depicting maximum reduction at LC<sub>50</sub> concentration. The biochemical compositions show that  
21 azadirachtin affected the energy reserves of adult of *S. granarius*. The results of persistence  
22 testing of azadirachtin applied by fumigation showed that their toxicity decrease as function  
23 the time. This study has highlighted the bioinsecticide activity of azadirachtin against granary  
24 weevil.

25  
26 **Keywords:** *Sitophylus granarius*, Azadirachtin, Toxicity, Repellent activity, Biomarkers, Nutrition  
27 index.

28  
29 **Introduction**

30 Insects are considered as the basis of problems in agricultural products storage since they  
31 affect the quality and quantity of the products. Due to the high potential and wide host range  
32 of products such as wheat, barley, rice and oats, granary weevil, *Sitophilus granarius* (L.) is  
33 ranked among the important stored grain pests. It was a primary pest in the past.<sup>[1]</sup> Insect pest  
34 control in stored grain products heavily relies on the use of gaseous fumigants and residual  
35 contact insecticides.<sup>[2]</sup> Moreover, the use of potentially toxic synthetic insecticides lead to  
36 serious problems such as residue threats and health hazard.<sup>[3,4]</sup> Protection of agricultural  
37 products from pest infestations is in the concern of scientists and the agrochemical industries  
38 worldwide. Plant products are being used to control many insect pests in the field and also in  
39 storage.<sup>[5,6]</sup> This highlights the importance to develop eco-friendly materials and methods with  
40 slight adverse effects on the environment and on consumers.<sup>[7,8]</sup>

41 Among the bioactive plant compounds, azadirachtin, abundantly found in *Azadirachta indica*  
42 A. Juss (Meliaceae) (a plant commonly known as neem), is the most studied and used plant  
43 species due to its high efficacy and very low toxicity to humans and antifeedant properties.<sup>[9,10]</sup>

44 It is demonstrated high potential for use against pests of agricultural importance in different  
45 production systems due to its high insecticide and acaricide activities and rapid degradation in  
46 the environment.<sup>[11-13]</sup>

47 In recent decades, *A. indica* has been extensively studied because it contains terpenoids with  
48 powerful insecticidal activity.<sup>[14]</sup> Azadirachtin, a limonoid with different modes of action, acts  
49 mainly in numerous species of economic pests such as antifeedancy, growth regulation,  
50 fecundity suppression and sterilization, oviposition repellency or attractancy, and changes in  
51 biological fitness.<sup>[15-17]</sup> Azadirachtin acts as a growth regulator with an antagonistic action of  
52 both juvenile hormone (JH) and moulting hormone (ecdysteroids)<sup>[10,18,19]</sup> but the mechanism of  
53 action of this pesticide remains unknown.<sup>[20]</sup>

54 In order to determine the action of the AZ on oxidative stress and to confirm the intervention  
55 of GST in the mechanism of its detoxication of azadirachtin<sup>[21]</sup>, we have chosen to follow the  
56 enzyme activities of two enzymes, CAT and GSTs and GSH rate.

57 Glutathione S-transferases (GST, EC 2.5.1.18) are multifunctional enzymes involved in many  
58 cellular physiological activities, such as detoxification of endogenous and xenobiotic  
59 compounds, biosynthesis of hormones and protection against oxidative stress.<sup>[22]</sup> In insects,  
60 three classes of GSTs have been identified namely delta, sigma, and epsilon classes<sup>[23]</sup>, and  
61 have GSH-dependent peroxidase activities, for the detoxification metabolism of insecticide.<sup>[24]</sup>

62 Catalase (CAT, EC 1.11.1.6) plays a vital role in reducing reactive oxygen-free radicals and  
63 maintaining cellular homeostasis in organisms<sup>[25]</sup>. It is the initial line of defense in antioxidant  
64 systems due to their significant function against oxidative stress.<sup>[26]</sup>

65 The aim of this study was to examine the insecticidal activity of azadirachtin and its  
66 repellency against *S. granarius* adults. Then, we investigated its effects on nutritional and  
67 biochemical profile of *S. granarius* adults and tested its residual activity. In order to give  
68 additional information on its mode of action, selected biomarkers (CAT, GST, GSH) were  
69 also measured.

70

## 71 **Materials and methods**

72

### 73 ***Insects rearing***

74 The insect species used in this study i.e. granary weevil *S. granarius* was procured from a  
75 farmer (Tébessa, Algeria). The insects were not affected by any material primarily. Cube  
76 containers (60x60x60cm) covered by a fine mesh cloth were used for insect rearing. The  
77 rearing was conducted as described by Aref & Valizadegan<sup>[27]</sup>, at  $27 \pm 1$  °C and  $65 \pm 5\%$   
78 relative humidity. Experiments were done between January and May 2018, and adult insects  
79 aged as 7 to 14 old days were used.

80

### 81 ***Azadirachtin***

82 Neem Azal-TS, a commercial formulation of azadirachtin (1% EC; Trifolio-M GmbH,  
83 Lahnau, Germany) was used in all experiments. Azadirachtin (AZ) is a triterpenoid isolated  
84 from the kernels of the neem tree, *Azadirachta indica* A. Juss.

85

### 86 ***Fumigant bioassay***

87 The fumigant toxicity of azadirachtin on *S. granarius* adults was tested in glass vials (60 mL).  
88 In each of them 10 adults (both sexes, male or female, 7-14 days old) were released. No.2  
89 Whatman filter paper disks were cut to 2.5 cm in diameter and attached to the undersurface of  
90 glass vial screw caps. Filter papers were impregnated with series of pure concentrations of  
91 essential oil: 20, 40, 80, 100, 200 and 400 µl/l air. Control insects were kept under the same  
92 conditions without essential oil. Each dose was replicated five times. After 24, 48 and 72  
93 hours from the beginning of exposure, numbers of dead and alive insects were counted. In  
94 these experiments, those insects incapable of moving their heads, antennae and body were  
95 considered as dead. Lethal concentrations (LC<sub>10</sub>, LC<sub>25</sub> and LC<sub>50</sub>) with their respective  
96 confidence limits (95% FL) were determined by a non-linear regression.

97

### 98 **Contact toxicity**

99 Azadirachtin dissolved in acetone has been tested at different concentrations (4, 8, 16, 20, 30  
100 and 60 µl/ml) on *S. granarius* adults in plastic vials with a capacity of 60 ml and containing 10  
101 g of wheat. Five replicates were run for each concentration and for the control. Numbers of  
102 dead insects were also counted after 12 and 24 hours from the start of exposure treatment.  
103 Control insects were kept under the same conditions with acetone. The lethal concentrations  
104 (LC<sub>10</sub>, LC<sub>25</sub> and LC<sub>50</sub>) were determined together with their corresponding 95% fiducial limits  
105 (95% FL) by a non-linear regression.

106

### 107 **Repellent activity**

108 The repellent effect of azadirachtin against adults of *S. granarius* was evaluated using the  
109 method of the preferred area on filter papers as described by Jilani & Saxena<sup>[28]</sup> Thus, the  
110 filter paper discs of 9 cm in diameter used for this purpose have been cut into two equal parts.  
111 Four doses were prepared (1, 2, 4 and 8 µl/ml) and diluted with ethanol. Then, 0.5 mL of each  
112 solution thus prepared was spread evenly over one-half of the disc. After 15 min, the two  
113 halves of the discs were glued together using adhesive tape. The filter paper disc was restored  
114 and placed in a box and kneaded a batch of 10 adult insects was placed in the center of each  
115 disk. The percentages of insects present on treated (P) and control (G) areas were recorded  
116 after 30 min. The repulsion percentage (RP) was calculated using Mc Donald et al.<sup>[29]</sup>  
117 formula: **RP = [(P-G) / (P+G)] × 100**

118 The average values were calculated and assigned as ranked by McDonald et al.<sup>[29]</sup> by a  
119 repulsive different classes varying from 0 to V [Class 0 (RP < 0.1%), class I (RP = 0.1% -  
120 20.0%), class II (RP = 20.1% - 40.0%), class III (RP = 40.1% - 60.0%), class IV (RP = 60.1%  
121 - 80.0%) and class V (RP = 80.1% - 100.0%)].

122

### 123 **Biomarker assays**

124 The LC<sub>25</sub> (15.26 µl/ml) and LC<sub>50</sub> (74.83 µl/ml) at 72h, were applied by fumigation on adult of  
125 *S. granarius* and its effects examined on CAT and GST activities and GSH concentration  
126 measured at various times (24, 48 and 72 h) following treatment.

127 CAT activity was measured by determining the decomposition of its substrate H<sub>2</sub>O<sub>2</sub> as  
128 described by Claiborne.<sup>[30]</sup> Each sample (3 pools each containing 10 individuals) was  
129 conserved in buffer phosphate (100 mM; pH 7.4). After sonication and centrifugation (15 000  
130 rpm for 10 min), the supernatant was collected and used for the determination of the CAT

131 activity. The protein amount in the total homogenate was quantified according to Bradford.<sup>[31]</sup>  
132 The absorbance was read at 240 nm. The assay was conducted with 6–8 repeats and data  
133 expressed as mmol/min/mg protein.

134 The assay of GST was carried out according to Habig et al.<sup>[32]</sup> previously described<sup>[33]</sup> with  
135 use of GSH (5 mM). Larvae decapitated body was homogenized in 1ml phosphate buffer (0.1  
136 M, pH 6). The homogenate was centrifuged (14000 rpm for 30 min). 200µl of the resulting  
137 supernatant was added to 1.2 ml of the mixture GSH-CDNB in phosphate buffer (0.1, pH 7).  
138 Changes in absorbance were measured at 340 nm every minute for a period of 5 min.

139 The assay of GSH was conducted according to the method of<sup>[34]</sup> previously used.<sup>[35]</sup> Larvae  
140 bodies were homogenized in 1ml of EDTA (0.02 M, pH 6). The homogenate was subjected to  
141 a deproteinisation with sulfosalysilic acid (SSA) at 0.25 %. The optical density was measured  
142 at 412 nm.

### 143 *Extraction and estimation of energy reserves*

144 Proteins, carbohydrates and lipids were extracted following the procedure of<sup>[36]</sup> and quantified  
145 as previously described.<sup>[37]</sup> Briefly, for body biochemical analyses, newly molted adults from  
146 were collected. Pooled samples (10 individuals per pool) were weighed and extracted in 1 ml  
147 of trichloroacetic acid (20%). In brief, quantification of proteins was carried following the  
148 Coomassie Brilliant Blue G-250 dye-binding method<sup>[31]</sup> with bovine serum albumin as a  
149 standard. The absorbance was measured at 595 nm. Carbohydrates were determined following  
150 the anthrone method<sup>[38]</sup> using glucose as standard. Lipids were measured by the vanillin  
151 method.<sup>[39]</sup> Data were expressed in µg per individual. The amount of carbohydrate, lipid and  
152 protein in each sample was calculated in µg per adult by using standard graphs. The values of  
153 carbohydrate, lipid and protein in µg were converted into joules.<sup>[40]</sup>

154 Where: 1mg of carbohydrate or protein has an energy value = 16.74 J

155 1mg of lipid has an energy value = 37.65 J

156

### 157 *Nutrition depletion index*

158 The total nutrition (carbohydrates + lipids) depletion index (NDI) was calculated as follows:

$$159 \quad \text{NDI} = [(C - T) / (C + T)] \times 100$$

160 Where: **C** is the control total energy reserve and **T** is the total energy reserves present in  
161 treated adult. The NDI is considered important when it is greater than 75%, moderate when it  
162 is between 50 and 75%, and low when it is less than 50%.

163

### 164 *Evaluation of the residual activity*

165 Persistence of insecticidal activity of AZAD was evaluated as described by Ngamo et al.<sup>[41]</sup>

166 The fumigation LC<sub>50</sub> values of essential oils were pipetted onto filter paper discs (2.5 cm  
167 diameter) in plastic vials. Six hours later, 10 adults were introduced separately into vial and  
168 then numbers of dead insects were recorded 24h after commencement of the exposure. This  
169 procedure was also conducted at 6 h intervals (i.e. 12, 18, 24, 30h). For each interval, separate  
170 series were set up with ten replications.

171

172

173

174 *Statistical analysis*

175

176 Data are presented as the mean  $\pm$  SEM. Repetitions and numbers of individuals were also  
177 cited. One-way analysis of variance (ANOVA at  $P \leq 0.05$ ) followed by a post-hoc honestly  
178 significant difference (HSD) Tukey's test were used to compare between the different series.

179

180 **Results and discussion**

181

182 *Insecticidal activity*

183 Azadirachtin, produced as secondary metabolite, is the principal active constituent in neem  
184 extracts.<sup>[18]</sup> As reported by published reviews<sup>[18,42]</sup>, it is able to induce multiple effects in  
185 numerous species of economic pests such as antifeedancy, growth regulation, fecundity  
186 suppression and sterilization, oviposition repellency or attractancy, and changes in biological  
187 fitness. However, its effects depend on the species, stages of the insect, concentration and the  
188 method of application (contact, ingestion and injection.<sup>[43,44]</sup> Azadirachtin has been shown to  
189 exhibit insecticidal activity against >400 insect species such as *Helicoverpa armigera*,  
190 *Spodoptera litura*, *Plutella xylostella*, *Sitophilus oryzae*, *Sitophilus zeamidis*, *Earis vitella*,  
191 *Aphis gossypii*, *Bemisia tabaci*, *Pectiniphora gossypiella*, nematodes like *Cosmopolitism*  
192 *sordidus* etc.<sup>[45]</sup> The toxicity of this growth regulator is related to its high retention and  
193 stability.<sup>[46]</sup>

194 Figure 1 shows the percent mortality of *S. granarius* after exposure to different concentrations  
195 of azadirachtin applied by fumigation method. The highest percentage mortality was seen at  
196 100  $\mu\text{l/liter}$  air concentrations of AZAD. We calculated  $\text{LC}_{25}$  and  $\text{LC}_{50}$  values of azadirachtin  
197 and their fiducial limits (Table 2). Otherwise, application of azadirachtin by contact with the  
198 highest dose induces a 100% mortality rate at 12h (Fig. 1). Indeed, the  $\text{LC}_{25}$  and  $\text{LC}_{50}$  values  
199 decrease as a function of time (Table 1).

200 Our results indicate that azadirachtin exhibit an interesting insecticidal activity with dose-  
201 response relationship against *S. granarius* adults. Similar results were found with the same  
202 insecticide applied against *Drosophila melanogaster*<sup>[47,48]</sup> and *Ceraeochrysa claveri*<sup>[49,50]</sup>  
203 reported that this compound presented fumigant toxicity against *Rhyzopertha dominica*.  
204 However, the lethal concentrations ( $\text{LC}_{50}$  and  $\text{CL}_{90}$ ) recorded in our study are higher to those  
205 found in this work ( $\text{LC}_{25} = 7.41 \mu\text{l/liter}$  air and  $\text{LC}_{50} = 21.33 \mu\text{l/liter}$  air). *Azadirachta indica*  
206 showed high toxicity (35.61%, 29.31% and 34.48%) when applied by contact on *R. dominica*,  
207 *Trogoderma granarium* and *Tribolium castaneum*, respectively<sup>[51]</sup> Various studies  
208 demonstrated the lethal effects of azadirachtin on different insect species.<sup>[43,52,53]</sup> Topical  
209 application of azadirachtin on *G. mellonella* induced lethal concentrations of 16,564 and  
210 3191,307 ppm corresponding to the  $\text{LC}_{50}$  and  $\text{LC}_{90}$ , respectively.<sup>[52,54]</sup> The toxicity of  
211 Azadirachtin (NeemAzal®) has been reported in different species of mosquito, *Culex*  
212 *pipiens*<sup>[55-57]</sup>, *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi*.<sup>[58]</sup> [19] showed  
213 the efficacy of Azadirachtin against Lepidoptera, such as *Shistocerca gregaria*, where the  
214  $\text{LC}_{50}$  has a very low value (0.007 ppm), whereas in the Hemiptera and Coleoptera species, the  
215  $\text{LC}_{50}$  is 100 ppm. The obtained results by Zhong et al.<sup>[59]</sup> indicated that AZ had a strong  
216 stomach and contact toxicity to *Tirathaba rufivena* (Lepidoptera: Pyralidae) larvae, and that  
217 the contact toxicity was greater than the stomach toxicity.



218

### 219 **Repellent Activity**

220 The repellent activity is a physiological phenomenon that occurs in insects as a defense  
221 mechanism against toxins secreted by plants.<sup>[50]</sup> An insect repellent has been defined as a  
222 chemical substance that causes the insect to make oriented movements away from the  
223 source.<sup>[60]</sup> The strong repellency of azadirachtin and neem concentrates in Xie et al.<sup>[61]</sup> study  
224 was reflected by reduced numbers of insects on treated wheat. This reduction is presumably  
225 caused by chemosensory effects of these products, either olfactory or gustatory.

226 In this study, this test was applied on *S. granarius* adult. The percent repellency of *R.*  
227 *dominica* adult after 30min of treatment with AZ (1, 2, 4 and 8 µl/ml) are presented in table 2.  
228 The obtained results revealed an increase in the repellency percentage as a function the  
229 concentration. The maximum repellency rate is 60% recorded with a dose of 8µl/ml.  
230 According to Mc Donald et al. <sup>[29]</sup>, this product belongs to the repellent class III.

231 These pesticides have shown contact, fumigant, antifeedant, repellent activity and growth  
232 regulating properties against insects.<sup>[62]</sup> AZ is a powerful behavior-modifying agent for a  
233 number of phytophagous insect species.<sup>[19,43,63]</sup> Various azadirachtin-based commercial  
234 formulations, applied at different concentrations, caused strong repellent and oviposition  
235 deterrent effects on *T. urticae* females.<sup>[64-67]</sup> Hanif et al.<sup>[51]</sup> reported a repellent activity of  
236 azadirachtin against *T. castaneum* and *Rhyzopertha dominica* with maximum of 77.66% and  
237 81.48% repulsive potential, respectively.

238

### 239 **Biomarker assays**

240 The lethal concentrations (LC<sub>25</sub>: 15.26 µl/ml and LC<sub>50</sub>: 74.83 µl/ml) of azadirachtin at 72h  
241 were applied by fumigation on adult of *S. granarius* and its effects examined on CAT and  
242 GST activities, and on GSH rate measured at various times (24, 48 and 72 h) following  
243 treatment (Table 6). Results show a significant increase in CAT activity for the two tested  
244 concentrations only at 72h (control vs LC<sub>25</sub> p= 0.0412 and control vs LC<sub>50</sub> p=0.0153) (Fig.  
245 2A, B and C), while GST activity measurements revealed a significant increase in the treated  
246 series (LC<sub>25</sub> and LC<sub>50</sub>) respectively compared to control at 48 (p= 0.0196 and p= 0.0015) and  
247 72 h (p= 0.0178 and p= 0.0032) without dose-response relationship. Finally, a significant  
248 decrease of glutathione rate was observed in treated series (LC<sub>25</sub> and LC<sub>50</sub>) (p= 0.0133 and  
249 0.0035) respectively at 72h as compared to control series.

250

251 The present results revealed a significant induction in glutathione S- transferase activity in *S.*  
252 *granarius* adult treated with AZ. This is in accordance with the literature as reported in  
253 *Choristoneura rasaceana*<sup>[68]</sup>, in *Xanthogaleruca luteola* (Müller) (Coleoptera)<sup>[69]</sup>, in  
254 *Helicoverpa armigera* larvae (Hübner) (Lepidoptera)<sup>[70]</sup> and in *Drosophila melanogaster* <sup>[71]</sup>;  
255 Or various insecticides such as neem oil in *Xanthogaleruca luteola*.<sup>[69]</sup>

256 Sometimes, the GST activities could be not affected by azadirachtin in *Choristoneura*  
257 *rasaceana*.<sup>[72]</sup> But the results of<sup>[73]</sup> have confirmed the intervention of GST in the mechanism  
258 of the detoxication of azadirachtin. Increased GST activity results in the detoxification  
259 process, is a form of insect defense against pesticide.<sup>[74]</sup>

260

261 Glutathione (GSH) plays an important role in the detoxification and excretion of  
xenobiotics.<sup>[75]</sup> In our study, AZ induces a significant greater decrease in GSH rate in *S.*

262 *granarius* adult. Similar effects observed by Kiran et al.<sup>[76]</sup> who mentioned that *Boswellia*  
263 *carterii* essential oil on *Callosobruchus chinensis* and *C. maculatus* increased significantly the  
264 concentration of GSH. This cofactor in *S. oryzae* and *R. dominica* was also increased after  
265 treatment (CL<sub>50</sub>) with *Gaultheria procumbens* essential oil<sup>[77]</sup>, and in *C. pipiens* with *T.*  
266 *vulgaris*.<sup>[78]</sup> In contrast, the adult of *S. oryzae* treated with anhydride 2,3-diméthylmaléique  
267 displayed an increase in the GSH activity. The decrease of glutathione could be explained by  
268 an increased consumption of this cofactor by the GSTs in order to detoxify the organism and a  
269 reduction of the non-enzymatic antioxidant system.

270

271 Our finding shows a significant increase of CAT activity. Similar results were found with  
272 azadirachtin applied in *Drosophila melanogaster*.<sup>[47]</sup> <sup>[76]</sup> was found also an increase in CAT  
273 levels of 30.29% and 38.82% after 24 h exposure to the LC<sub>50</sub> of *Boswellia carterii* essential  
274 oil on *C. chinensis* and *C. maculatus* respectively. The increase in activity of CAT reflects an  
275 establishment of the process of detoxification, which is a form of defense of the insect against  
276 the pesticide.<sup>[79]</sup> In contrast, a decrease in CAT activity was observed in *S. oryzae* and *R.*  
277 *dominica* treated with *Gaultheria procumbens*<sup>[77]</sup>, which could be explained by an increased  
278 production of the radical superoxide anion.<sup>[80,81]</sup> This decrease in CAT activity results  
279 accumulation of toxic H<sub>2</sub>O<sub>2</sub> in the cell, leading to peroxidation of membrane lipids.<sup>[82]</sup> The  
280 induction of the GST system in *D. melanogaster* is correlated with an increase in specific  
281 CAT activity after treatment with Neem Azal.<sup>[47]</sup> This oxidative stress could be explained by  
282 the antagonist action of azadirachtin on endogenous 20E and its antioxidant activity.<sup>[47]</sup>

283

#### 284 ***Estimation of energy reserves and protein content***

285

286 Changes in main biochemical components (carbohydrates, lipids and proteins) were estimated  
287 in the whole body of the control and treated adult of *S. granarius* at different times following  
288 treatment (Table 3). Results show a significant decrease ( $p < 0.001$ ) in the protein content in  
289 treated series (LC<sub>25</sub> and LC<sub>50</sub>) as compared to controls during the tested period: 24 (control vs  
290 LC<sub>25</sub>:  $p < 0.001$  ; control vs LC<sub>50</sub>:  $p < 0.001$  ; LC<sub>25</sub> vs LC<sub>50</sub>:  $p = 0.006$ ), 48 (control vs LC<sub>25</sub>:  
291  $< 0.001$  ; control vs LC<sub>50</sub>:  $< 0.001$ ) and 72 hours (control vs LC<sub>25</sub>:  $< 0.001$  ; control vs LC<sub>50</sub>:  
292  $< 0.001$  ; LC<sub>25</sub> vs LC<sub>50</sub>:  $p = 0.008$ ).

293 Concerning the total energy (Table 3) , the results revealed a significant decrease in the  
294 treated series (LC<sub>25</sub> and LC<sub>50</sub>) respectively compared to control at 24h (control vs LC<sub>25</sub>:  
295  $p < 0.001$  ; control vs LC<sub>50</sub>:  $p < 0.001$  ; LC<sub>25</sub> vs LC<sub>50</sub>:  $p = 0.001$ ), 48 (control vs LC<sub>25</sub>:  $< 0.001$  ;  
296 control vs LC<sub>50</sub>:  $< 0.001$ ; LC<sub>25</sub> vs LC<sub>50</sub>:  $p < 0.001$ ) and 72 hours (control vs LC<sub>25</sub>:  $< 0.001$  ;  
297 control vs LC<sub>50</sub>:  $< 0.001$  ; LC<sub>25</sub> vs LC<sub>50</sub>:  $p = 0.02$ ).

298

#### 299 ***Nutrition depletion index***

300 Nutrition depletion index (NDI %) in treated adult was determined in order to investigate the  
301 effectiveness of azadirachtin (Table 4). The decrease was concentration-dependent with a  
302 maximum depletion in LC<sub>50</sub> treated series at different periods after treatment: 24 ( $p = 0.003$ ),  
303 48 ( $p < 0.001$ ) and 72hours ( $p = 0.022$ ). Azadirachtin induced a moderate nutritional depletion.

304

305 All types of insecticides have some negative impact on the growth and development of the  
306 insect, and also affect the metabolic and biochemical processes.<sup>[83]</sup> This investigation shows



307 that after treatment of azadirachtin, the protein level and energy reserves of *S. granarius*  
308 larvae decreased during the tested period.

309 Protein synthesis is necessary particularly for the maintenance of body growth and  
310 reproduction. They enter in various reactions such as the hormonal regulation and they  
311 integrated in the cell as a structural element at the same time as the carbohydrates and the  
312 lipids.<sup>[84,85]</sup> In the present investigation, after treatment of *S. granarius* adults with AZ, an  
313 inhibitory action on proteins was generally exhibited.<sup>[86]</sup> reported that stress due to insecticide  
314 exposure might interfere with insect physiology, consequently resulting in a decrease in total  
315 protein leading to low amino acids formation in Krebs cycle. This further leads to insufficient  
316 fatty acid required for synthesis of Adenosine Triphosphate (ATP) energy, thus reduction in  
317 ATP energy triggers stress in insects leading to death.<sup>[72]</sup> Nevertheless, Ebadollahi et al.<sup>[87]</sup>  
318 reported a decrease in the carbohydrates, proteins, and lipids content in *T. castaneum* larvae  
319 treated with *Agastache foeniculum* EO. The same observations were reported by Tarigan and  
320 Harahap<sup>[88]</sup> after treatment of *Tribolium castaneum* with *Cinnamomum aromaticum*, *Elettaria*  
321 *cardamomum* and *Myristica fragrans* EOs, with efficacy of the cinnamon oil. This depletion  
322 might be due to their degradation for metabolic purposes or to an impaired incorporation of  
323 amino acids into polypeptide chains or inhibition of protein synthesis<sup>[83]</sup> or to the breakdown  
324 of these proteins into amino acids used in the compensatory mechanism as energy source to  
325 compensate stress.<sup>[89]</sup>

326 Neem extract contains azadirachtin that has been known to affect protein amount and  
327 expression. For instance, azadirachtin have been known to interfere with protein synthesis in  
328 *Schistocerca gregaria*<sup>[90]</sup> and *Spodoptera litura*.<sup>[91]</sup> Further, it is reported that protein  
329 expression in *S. litura* was significantly lowered under azadirachtin treatment.<sup>[44]</sup> Rao &  
330 Subrahmanyam<sup>[92]</sup> found disturbance in the hormone that regulates protein synthesis due to  
331 azadirachtin in *Schistocerca gregaria*. The decrease in total protein in the adult of *S.*  
332 *granarius* was postulated as an indicator of toxic exposure to insecticides. According to  
333 Mordue et al.<sup>[18]</sup>, AZ alters or prevents the formation of new assemblages of organelles or  
334 cytoskeleton resulting in the disruption of cell division, blocked transport and release of  
335 neurosecretory peptides. It also inhibits protein synthesis in cells that are metabolically active.

336  
337 The carbohydrates are considered as important energy elements playing a crucial role in the  
338 insect physiology, such as the molt process and the reproduction.<sup>[93]</sup> In the present study, AZ  
339 decreases the carbohydrate contents in *S. granarius* adults. Glucose level of the larvae treated  
340 with *A. annua* extract was decreased by 24.65%. The reduction in glucose content was more  
341 significant in larvae exposed to *Az. indica* extract by 58.96% decline over control. This  
342 depletion in glucose content may be due to utilization of the reserved glucose sources of larval  
343 tissues as a result of insecticidal stress.

344 AZAD derivatives also lead to a decrease in the concentration of carbohydrates in  
345 *Ctenoparyngodon idella*.<sup>[94]</sup> Tine et al.<sup>[95]</sup> show a significant decrease in ovarian protein, lipid  
346 and carbohydrate contents in *B. orientalis* treated by azadirachtin. Treatment may have caused  
347 possible disturbance in the vitellogenesis process via the nervous, neuroendocrine and/or  
348 endocrine system. In another study, Tine et al.<sup>[50]</sup> found that azadirachtin induced negative  
349 effects on energy contents compared with control in *Ryzopertha dominica*. The carbohydrate  
350 content was reduced in larvae of *Spodoptera littoralis* after treatment with essential oil of *A.*

351 *indica* and *Citrullus colocynthis* methylene chloride extract and was increased with garlic and  
352 lemon Eos.<sup>[96]</sup>

353  
354 Lipids are also an important source of acetyl groups needed to synthesize the enzymes from  
355 constitutive amino acids.<sup>[97]</sup> This reduction in lipid profile indicates a negative effect of the  
356 extract on lipid metabolism and peroxidation. This observation is similar to the findings of  
357 Lohar & Wright<sup>[98]</sup>, who found that *Tenebrio molitor* suffered lipid depletion in haemolymph,  
358 fat bodies and oocytes when exposed to malathion. Sak et al.<sup>[99]</sup> reported the decline in lipid  
359 content due to shift in energy metabolism to lipid catabolism due to insecticidal stress induced  
360 by *Pimpala turionellae*.

### 361 362 **Residual activity of azadirachtin**

363  
364 During the 30h treatment periods; the results of persistence testing of azadirachtin applied by  
365 fumigation showed that their toxicity decrease as function the time. The toxicity of AZAD  
366 decreased with time; after 6h its toxicity was 32 % and decreased to 6 % after 24 h to  
367 disappear after 30h of exposure (Fig. 3).

368  
369 The biopotency is negatively correlated with time. Ngamo et al.<sup>[41]</sup> and Heydarzade &  
370 Moravvej<sup>[100]</sup> reported that the persistency of *Lippia rugosa* Hochs (Lamiales: Verbenaceae)  
371 and *Satureja hortensis* (L.) (Lamiales: Lamiaceae) EOs were probably the result of its high  
372 content in oxygenated monoterpenes which attribute more stability in the biological activity  
373 of EOs. *Securidaca longepedunculata* has preserved toxicity while for *B. grandifolia* plant  
374 powder, the toxicity decreases rapidly.<sup>[101]</sup> This decrease is similar to this obtained with  
375 *Xylopiya aethiopica* against *Callosobruchus maculatus*.<sup>[102]</sup> These results can be explained  
376 through chemical active component of the species plant used. Investigations on the EO of  
377 several aromatics plants in Northern Cameroon<sup>[41,103]</sup> had proven that plant species has more  
378 persistence toxic effect when they contained higher proportion of oxygenated molecules such  
379 as oxygenated monoterpenes and sesquiterpenes. The persistence of insecticidal activity was in  
380 relationship with the sensitivity of the major target pest to active compound.<sup>[41,103,104]</sup> In the  
381 experiment of Akami et al.<sup>[105]</sup>, when tested individually, none of the isolated major  
382 constituents had produced as higher effects as the crude EO not even their complex mixture.  
383 The crude EO is the most persistent. This situation could be the result of many factors: the  
384 high volatility of the compounds, the rapid degradation of low single compounds, and the  
385 potential oxidation of Sesquiterpene hydrocarbons.<sup>[105]</sup> Regnault-Roger et al.<sup>[106]</sup> showed the  
386 lower volatility of oxygenated molecules because of their higher molecular weight.

### 387 388 389 **Conclusion**

390 Azadirachtin exhibited fumigant toxicity against *S. granarius* adults confirming its potential  
391 as a natural alternative to synthetic insecticides for the control of stored-product pests. In  
392 addition, a strong repellent activity. Moreover, azadirachtin was found to exhibit a residual  
393 toxicity on *S. granarius*. The bioinsecticide caused the activation of the system of  
394 detoxification, traduced by an increase of the specific activity of GST and Catalase and a

395 decrease of GSH rate. Our results provide an interesting opportunity to develop  
396 bioinsecticides and repellent formulations.

397

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399

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730 **Legends of Table and Figures**

731 **Table 1.** Fumigant ( $\mu\text{l/l}$  air) and Contact ( $\mu\text{l/ml}$ ) toxicity of azadirachtin against the adult of *S.*  
 732 *granarius*: Determination of lethal concentrations and their confidence intervals (95%), LCL - Lower  
 733 confidence Limit, UCL - Upper confidence Limit)  
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Toxicity	Times (hours)	R <sup>2</sup>	Lethal concentrations (LCL-UCL)	
			LC <sub>25</sub> (95% FL)	LC <sub>50</sub> (95% FL)
Fumigation ( $\mu\text{l/l}$ air)	24	0.93	<b>113.70</b> (74.10 - 164.60)	<b>549.10</b> (343.30 - 1480)
	48	0.91	<b>57.71</b> (27.34 - 92.94)	<b>335.70</b> (213.20 - 852.90)
	72	0.75	<b>17.29</b> (0.02 - 55.15)	<b>72.01</b> (12.52 - 185.90)
Contact ( $\mu\text{l/ml}$ )	12	0.96	<b>5.84</b> (4.15 - 7.60)	<b>30.35</b> (22.72 - 48.07)
	24	0.94	<b>1.04</b> (0.36 - 1.83)	<b>3.16</b> (1.92 - 4.38)

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736 **Table 2.** Repellent Percentage (RP) of azadirachtin against *S. granarius* adults at different  
 737 concentrations.

Concentrations	RP (%)	Class
1 $\mu\text{l/ml}$	25	II
2 $\mu\text{l/ml}$	35	II
4 $\mu\text{l/ml}$	50	III
8 $\mu\text{l/ml}$	60	III

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742 **Table 3.** Effect of azadirachtin (LC<sub>25</sub> and LC<sub>50</sub>) on proteins content and total energy (joule  
 743 /individual) in *S. granarius* adults at different time after treatment (mean  $\pm$  SE, n = 3 pools  
 744 each containing 10 individuals).  
 745

Times (hours)	Components	Control	LC <sub>25</sub>	LC <sub>50</sub>
24	Proteins	3.61 $\pm$ 0.04 a	0.92 $\pm$ 0.02 b	0.74 $\pm$ 0.02 c
	Total energy	8.30 $\pm$ 0.17 a	4.54 $\pm$ 0.13 b	3.53 $\pm$ 0.05 c
48	Proteins	3.61 $\pm$ 0.05 a	0.83 $\pm$ 0.01 b	0.73 $\pm$ 0.04 b
	Total energy	8.18 $\pm$ 0.11 a	4.31 $\pm$ 0.08 b	2.95 $\pm$ 0.00 c
72	Proteins	3.61 $\pm$ 0.00 a	0.78 $\pm$ 0.02 b	0.65 $\pm$ 0.03 c
	Total energy	7.87 $\pm$ 0.27 a	3.19 $\pm$ 0.06 b	2.39 $\pm$ 0.19 c

The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test ( $p < 0.05$ ).

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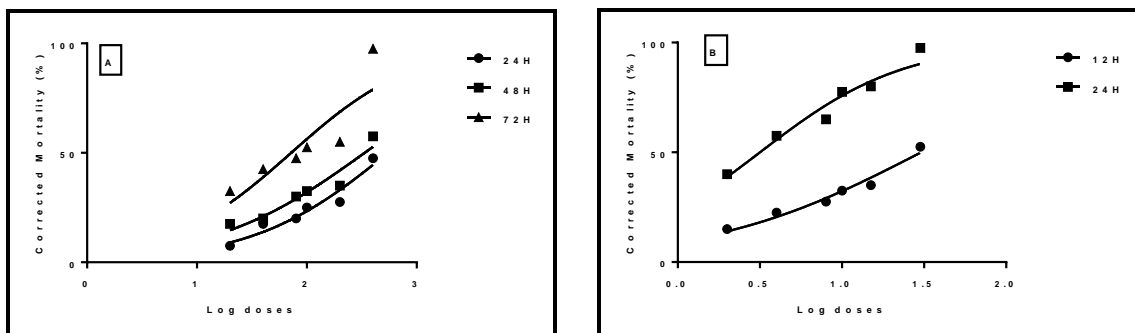
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**Table 4.** Nutrition Depletion Index (%) of *S. granarius* adult after treatment with AZAD at different time after treatment. Data represented as mean  $\pm$  SE (n =3 pools each containing 10 individuals).

Times (hours)	LC <sub>25</sub>	LC <sub>50</sub>	P value
24	29.26 $\pm$ 2.07 <sup>a</sup>	40.31 $\pm$ 0.93 <sup>b</sup>	0.003
48	30.98 $\pm$ 0.58 <sup>a</sup>	46.93 $\pm$ 0.59 <sup>b</sup>	<0.001
72	42.21 $\pm$ 2.21 <sup>a</sup>	53.36 $\pm$ 3.32 <sup>b</sup>	0.022

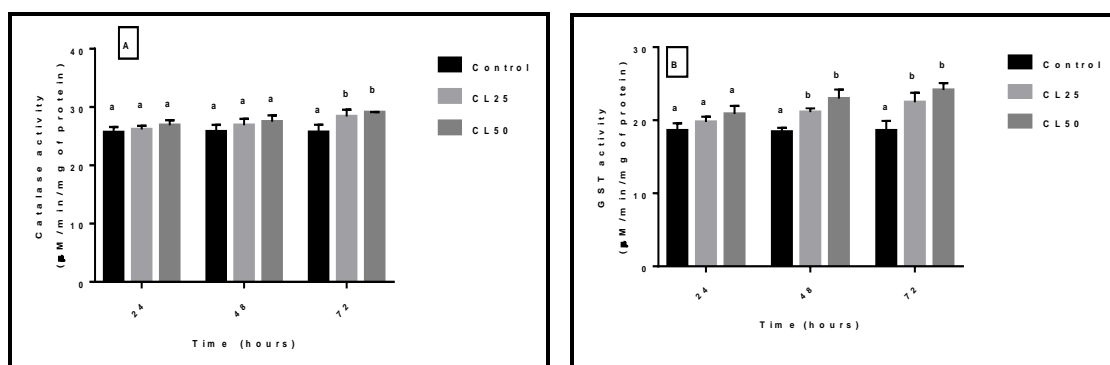
Different small letters indicate a significant difference between treated individuals in the same period.

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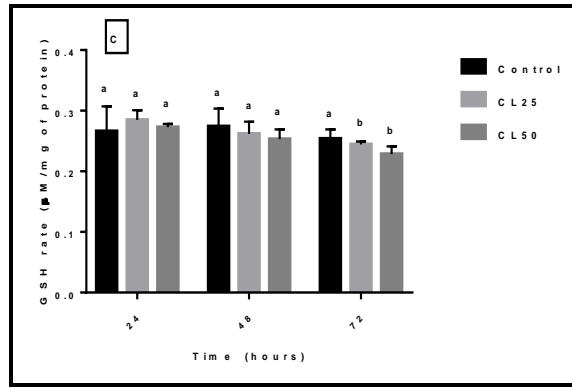


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**Figure 1.** Efficacy of azadirachtin applied by fumigation (A) and by contact (B) on adult as function the exposure time (hours) (mean  $\pm$  SEM, n = 5 replicates each containing 10 adults).

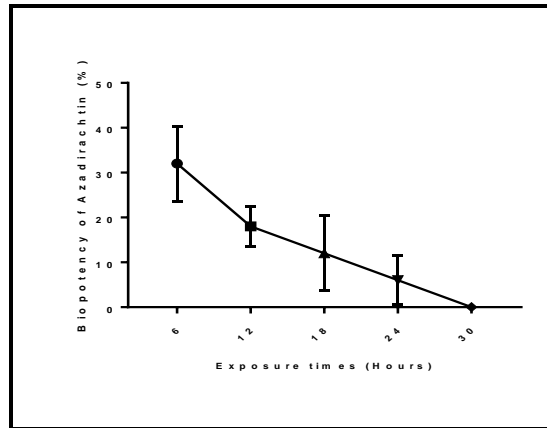


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**Figure 2.** Effect of azadirachtin (LC<sub>25</sub> and LC<sub>50</sub>) on CAT (A) and GST (B) activities (µM/min/mg of protein) and GSH (C) rate (C) (µM/mg of protein) in *S. granarius* adults at different time after treatment (mean ± SEM, n= 3 pools each containing 10 individuals). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test ( $p < 0.05$ ).



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**Figure 3.** Residual activity of azadirachtin (LC<sub>50</sub>) by fumigation against *S. granarius* adults for a delay of 30h (mean ± SD, n = 4 pools each containing 10 individuals)