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**Aesculus pavia foliar saponins: difensive role against the leafminer Cameraria ohridella.**

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*AESCULUS PAVIA* FOLIAR SAPONINS: DEFENSIVE ROLE AGAINST THE LEAFMINER  
*CAMERARIA OHRIDELLA*

Running title: Effect of a saponin against *Cameraria ohridella*

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1 **Abstract**

2 **BACKGROUND**

3 Recently the leafminer *Cameraria ohridella* Deschka & Dimic has caused heavy damage to the  
4 white-flowering horse chestnut in Europe. Among the *Aesculus* genus, *A. pavia* HBT genotype,  
5 characterized by red flowers, showed an atypical resistance towards the pest. Its leaves, shaken in  
6 water, originated a dense foam indicating the presence of saponins, unlike the common white horse  
7 chestnut tree. The aim of this work was to isolate and identify its leaf saponins and test their  
8 possible constitutive defensive role against *C. ohridella*.

9 **RESULTS**

10 NMR analyses of saponin mixture showed that *A. pavia* HBT genotype leaves contain a mixture of  
11 saponins, four of which based on the same structure of commercial escin saponins, the typical  
12 saponin mixture produced by *A. hippocastanum* and accumulated only within bark and fruit tissues.  
13 The mixtures showed a repellent effect on *C. ohridella* moth. The number of mines detected on the  
14 treated leaves was significantly lower than the control, and in many cases no mines were ever  
15 observed.

16 **CONCLUSION**

17 The results showed that the exogenous saponins are translocated from roots/stem to the leaf tissues  
18 and their accumulation in leaf tissues of the susceptible genotype seemed to ensure an appreciable  
19 degree of protection towards the leafminer.

20

21 **Key Words:** saponin; escin; *Cameraria ohridella*; *Aesculus pavia*; bioinsecticides.

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## 27 **1 INTRODUCTION**

28 Plants synthesise a diverse array of secondary metabolites, either as part of normal growth and  
29 development or in response to pathogen attack or stress. Glycosylated sterols or triterpenoids,  
30 known as saponins, are an important group of plant secondary metabolites that are widespread  
31 throughout the plant kingdom and have been identified in 80 plant families.<sup>1,2</sup>

32 The occurrence of saponins in higher plants has been reported by different authors concerning their  
33 isolation, structure determination, medicinal and pesticidal importance. In addition to their  
34 medicinal properties, plant saponins have also been reported to have toxic and feeding deterrent  
35 effects against phytophagous pests.<sup>3,4</sup> They are well known plant allelochemicals that form a  
36 chemical barrier for feeding of the phytophagous insects and their toxic effects to various organisms  
37 appear to be linked to their interaction with biological membranes, and may be related to their soap-  
38 like properties.<sup>5</sup> Saponins slow down the passage of food through the insects' alimentary canal,  
39 reduce its digestibility and inhibit food uptake by the herbivorous insects. Moreover, saponins can  
40 also block sterols uptake interfering with moulting.<sup>6</sup>

41 Seeds and bark of *Aesculus* spp., or horse chestnut tree, have been widely used in European  
42 traditional medicine since 16th century. The saponin mixture, "escin", obtained from the seeds is  
43 widely used above all in traditional medicine.<sup>7</sup>

44 The leafminer *Cameraria ohridella* Deschka & Dimic (Lepidoptera, Gracillariidae) is an invasive  
45 pest of *Aesculus* spp. Its main host is the white-flowering horse chestnut *A. hippocastanum*, but the  
46 moth can also complete its cycle and damage a range of other *Aesculus* species. The leaves are  
47 extensively mined by the larvae and the final result is a premature leaf fall in August and September  
48 depending on the intensity of the attack.<sup>8</sup> Since this tree is very common in parks, private gardens  
49 and streets, the new pest has attracted much attention, raising significant public concern, especially  
50 when it immediately breaks out in epidemic proportions after invading a new region.<sup>9</sup>

51 During field monitoring, a red-flowering horse chestnut tree was found in Turin. This tree,  
52 classified in the botanical garden and registered as *A. pavia* HBT (*Hortus Botanicus Taurinensis*)

53 genotype, surrounded by other white flowering horse chestnut trees heavily infested by *C. ohridella*,  
54 showed since the beginning an atypical resistance towards the moth. Even if *A. pavia* has been  
55 reported to be very susceptible to *C. ohridella* attacks, no mines have ever been found on *A. pavia*  
56 HBT since its identification, and this was the only case of resistance ever found in Turin and  
57 neighbouring areas.<sup>10,11</sup> Preliminary investigations, based also on the simple observation that only  
58 the *A. pavia* leaves shaken in water originate a dense persisting foam, indicated the presence of  
59 saponins within leaf tissues of the red-flowering horse chestnut tree. On the contrary, as reported in  
60 literature,<sup>12</sup> saponins were not detected in the leaves of the *C. ohridella*-susceptible *A.*  
61 *hippocastanum* white flowering trees, but only in fruits.

62 The aim of this work was to isolate and identify *A. pavia* HBT genotype leaf saponins and test their  
63 possible constitutive defensive role in this plant against *C. ohridella* by assaying their effect on the  
64 common *C. ohridella*'s susceptible *A. hippocastanum* in order to prevent a leafminer attack.

65

## 66 **2 MATERIALS AND METHODS**

### 67 **2.1 Extraction, isolation, purification and identification of *Aesculus pavia* HBT genotype**

#### 68 **saponins**

69 The leaves to be extracted were collected during springtime from healthy *A. hippocastanum* white  
70 flowering trees, planted in Turin streets as ornamental species, and from *A. pavia* HBT genotype.  
71 Leaves were allowed to dry on shelves at room temperature and then they were stored in a cool  
72 place until needed. 5 g dry samples were extracted for both plant species, by boiling for 5 min the  
73 crushed material suspended in ddw. The solutions, after filtration, were brought to 50 ml volume  
74 under reduced pressure, added with EtOH to precipitate sugars and extracted three times in a  
75 separation funnel with n-BuOH. The alcoholic phases were pooled and brought to dryness in a  
76 rotatory evaporator under reduced pressure at low temperature.

77 The dry residues, obtained from each different sample, were dissolved again with a few drops of  
78 HCOOH 5% in ddw and utilized in the further chromatographical analyses. With this purpose, a

79 glass chromatography column, 50 x 2 cm, was packed with Silica gel 100 C<sub>18</sub> reverse phase resin,  
80 40-63 μm particle size (Fluka, Germany), previously swollen in EtOH 80%; before  
81 chromatographic runs, the alcoholic phase was carefully replaced by HCOOH 5% in ddw and  
82 floating fines removed. After sample application, column was eluted according to an elution linear  
83 gradient profile where A = HCOOH 5% in ddw and B = CH<sub>3</sub>OH, from 0% to 80% B in A. 160 ml  
84 of both A and B were utilized, and 3 ml fractions were collected.

85 The presence of the investigated foliar natural saponin complex (NSC) was detected in the eluted  
86 fractions following upon its positive reaction, on TLC silica gel plates, to the Pancaldi reagent  
87 diagnostic for saponins [21g (NH<sub>4</sub>)<sub>6</sub>MoO<sub>4</sub> and 1g Ce(SO<sub>4</sub>)<sub>2</sub> in 469 ml H<sub>2</sub>O acidified with 31 ml  
88 H<sub>2</sub>SO<sub>4</sub>, by stirring at 50°C until solubilization].

89 After chromatographic purification, the structures of the components of the foliar NSC were  
90 determined by means of NMR and MS analyses by comparing the sample spectra with both those of  
91 reference compounds obtained under the same experimental conditions and those already available  
92 in literature. FABMS (glycerol matrix, CsI) were measured on a VG Prospec Fisons mass  
93 spectrophotometer. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded at 500 and 125 MHz, respectively, on a  
94 Bruker AMX-500 spectrometer.

95 A further confirmation of the saponin structures was obtained by HPLC, according to the following  
96 procedure. Aqueous samples were ultra filtered through 0.22 mm diameter hole filter and then used  
97 in the analyses. A HPLC Perkin-Elmer system was used, that included a binary model lc 200 pump,  
98 a rheodyne injector with a 20 μl sample loop, a model 785 A UV-vis detector and a Milton Roy  
99 refractive index detector (RID). A PE Nelson NCI900 interface and a Turbochrom (version 1.2)  
100 computer software were used. A Supelcosil™ LC<sub>18</sub> column (150 x 4.6 mm i.d., 5 μm particles)  
101 equipped with a Supelco™ pre-column (30 x 4.6 mm, i.d.) filled with the same stationary phase was  
102 employed.

103 The mobile phase consisted of A = 100% H<sub>3</sub>PO<sub>4</sub> 0.2 mM solution and B = 100% CH<sub>3</sub>CN, while the  
104 elution profile was: 2 min, isocratic, 80% A and 20% B; 5 min, linear gradient to reach 40% A and

105 60% B ratio and further 12 min, isocratic with the same ratio. Flow rate was 0.7 ml/min. Due to the  
106 lowest UV Abs of both the foliar NSC and the reference commercial compound, both UV-vis ( $\lambda =$   
107 203 nm) detector and RID were connected to the system, in this sequence, and integration was  
108 performed on RI data while UV-vis detector was used as a control instrument. After the  
109 identification of the *A. pavia* HBT genotype NSC components, standard solutions of the  
110 corresponding commercial reference saponin complex (escin, Merck, Darmstat, Germany) were set,  
111 in the range 10 to 200  $\mu\text{g/ml}$  , to perform suitable HPLC calibration curves useful to assess the  
112 response linearity. With this aim, the curve slope and the other statistics were calculated by linear  
113 regression, to determine the detection and quantitation limits for escin under our experimental  
114 conditions.

## 115 **2.2 Plant Material and Experiment Setting**

116 Fifty-four potted plants of the common *C. ohridella*-susceptible *A. hippocastanum* (white horse  
117 chestnut tree) were isolated in a big cage (6.0 x 4.0 x 2.0 m) set up with an insect-proof net kept in  
118 outdoor conditions at the CRA-FSO Research Institute, Sanremo. Plants, 60-70 cm high, potted in  
119 30 cm diameter clay pots, were placed in the cage according to a completely randomized  
120 distribution. A first series of treatments was set using 125 or 250 mg natural saponin complex  
121 (escin, Merck, Darmstat, Germany) dissolved in 500 ml double distilled water (ddw).

122 Eight treatments were set and applied: watering and stem brushing, once or twice (each time, 125 or  
123 250 mg mixture administered per plant) respectively, the second administration being carried out 17  
124 days after the first one. Six plants per treatment were set. The control plants were treated only with  
125 water.

126 Twelve days after the first watering and stem brushing treatments, the insects, collected as  
127 described hereafter, were released. The programmed repeated treatments were carried out six days  
128 after the insect release. From then, plants were watered daily to keep continuously wet the soil of  
129 each pot.

130 To evaluate the actual translocation towards leaves of the exogenously applied molecule, additional  
131 potted plants were used as leaf donors after the treatments. With this purpose, the applied treatments  
132 were: watering or stem brushing once and twice, (only the 250 mg dosage) respectively, the second  
133 treatment being carried out 17 days after the first one. Three plants per treatment were set.  
134 A second series of experiments was carried out with the aim of comparing the effect of the foliar  
135 NSC and the commercially available escin. With this purpose, both NSC and escin were sprayed on  
136 leaves of potted white-flowering horse chestnut plants, of the same size and age as above  
137 mentioned. The two saponin complexes NSC and escin were respectively sprayed at 125 mg/l  
138 concentration in ddw, each on ten different plants, 12 days before adult release; control was  
139 represented by ten further plants sprayed with only ddw. The observations were performed, 45 days  
140 after adult release, on ten different leaves per plant: a hundred observations per treatment were thus  
141 carried out.

### 142 **2.3 Insect Culture**

143 Field collections were undertaken in Turin (northwestern Italy; 45°03' N, 7°40' E) in June on  
144 plants located in public parks and avenues. White horse chestnut trees attacked by *C. ohridella* were  
145 sampled and leaves were collected at random using lopping shears, then they were put in plastic  
146 bags and taken to the laboratory.

147 The parts of the leaves with the mines were cut out and kept in cardboard boxes. Each box had two  
148 holes on a side connected to glass test tubes to ease *C. ohridella*'s adults collection. The boxes were  
149 kept outdoors to ensure temperature and humidity conditions similar to those present in nature.

150 Once emerged, the adults were taken from the glass tubes and freed inside the big cage. Plants were  
151 carefully checked twice a week to detect the presence of mines.

152 *Statistical Analyses.* Data represent the mean  $\pm$  standard error (SE) and were analyzed by one-way  
153 analysis of variance (ANOVA) and followed by *Dunnnett's post test* for multiple comparisons.

154 Differences were considered to be significant when the probability value was less than 0.01.

155 All the analyses were performed using the software SPSS<sup>®</sup> 13.0.

156 **3. RESULTS**

157 **3.1 Identification of the components of the *Aesculus pavia* HBT Genotype foliar NSC**

158 NMR mono and bi-dimensional analyses showed that *A. pavia* HBT genotype leaves contain a  
159 complex of saponins (NSC) based on polyhydroxyoleanene pentacyclic triterpenoid structure. The  
160 four components of NSC saponin complex have been identified as the common saponin  
161 components of the commercial escin, the typical saponin mixture produced by *A. hippocastanum*  
162 and accumulated only within bark and fruit tissues.<sup>12</sup> In particular, based on Mass spectrum FAB  
163 negative, we obtained the following data: m/z 1130 [M-H]<sup>-</sup> corresponded to Ia, Ib and IIIa escin,  
164 m/z 1100 [M-H]<sup>-</sup> corresponded to IIa and IIb escin, with a ratio for the commercial product of 70%  
165 for type I escin (Ia + Ib), 30% for type II escin (IIa + IIb) and 0% for type III escin, respectively.  
166 The ratios observed for foliar HBT genotype saponin complex (NSC) were instead: 50% type I  
167 escin (Ia + Ib), 40% type II escin (IIa + IIb) and 10% type III escin (IIIa). The differences between  
168 NSC and commercial escin are therefore based both on component percentage and presence, since  
169 type III saponin can be found only in NSC and type I and II saponins are represented in different  
170 concentrations. To resume, a comparison between commercial escin complex and HBT genotype  
171 saponin (NSC) mixture can be represented as follows:

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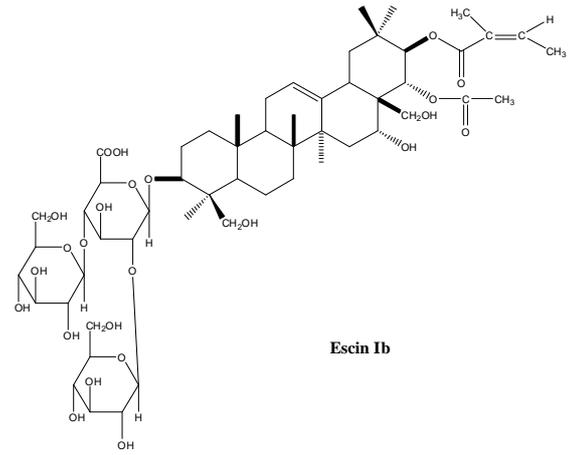
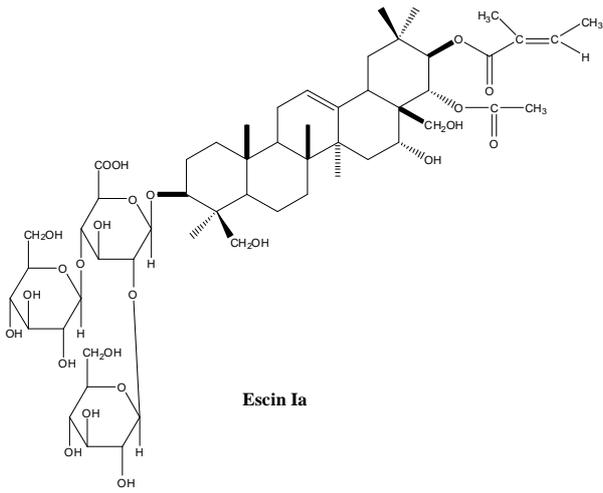
	Saponin type		
	I	II	III
% in commercial complex (escin)	70	30	–
% in HBT genotype saponin complex (NSC)	50	40	10

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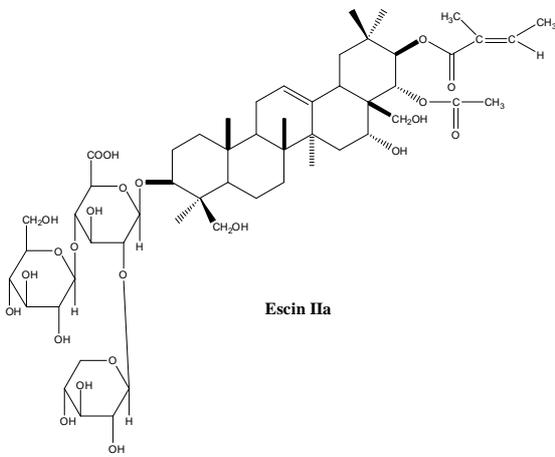
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176 The structure formulae of the five different saponins detected during the analyses are reported  
177 below:

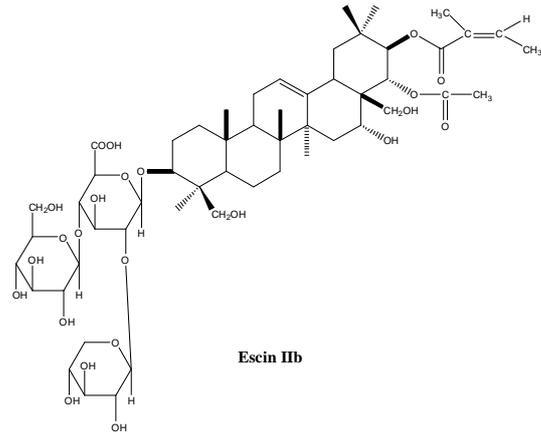
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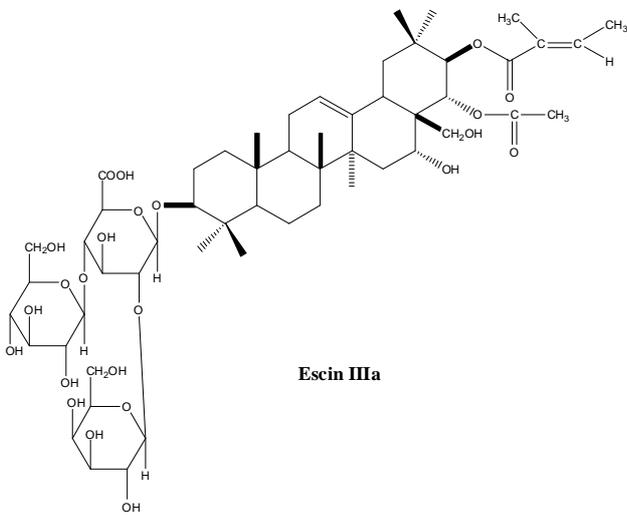
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186 Interestingly, no saponin was detectable in the *A. hippocastanum* leaves under the same  
187 experimental conditions used for the analysis of *A. pavia* HBT genotype leaves.

188 *Quantitation by HPLC of Leaf Saponin.* For a standard saponin quantitation, commercial escin  
189 useful range proved to be 15 to 150 µg/ml, range within which the calibration curve was linear. Due  
190 to the absence of a chromophoric portion in the saponin escin, its detection at 203 nm needed to be  
191 corroborated by the data obtained by the RID. Crossing these complementary results, we could  
192 calculate the NSC saponin concentration in *A. pavia* HBT genotype leaves (average from 10  
193 observations) of  $26 \pm 1.5$  mg/g dry tissue. The  $R_t$  value recorded for commercial reference escin was  
194 10.48 min.

195 The same analyses were carried out on *A. hippocastanum* leaves after the administration to plants of  
196 commercial escin by plant watering or stem brushing. The analytical results show that the  
197 exogenous saponins are translocated from roots/stem to the leaf tissues. The results are presented in  
198 the Table 1.

### 199 **3.2 Effect of the molecule**

200 As shown in Figure 1, the natural saponin complex obtained from the *A. pavia* HBT genotype, had a  
201 repellent effect on *C. ohridella* moth. The number of mines detected on the treated leaves was  
202 significantly lower than the control values (*Dunnnett's post test*,  $P < 0.01$ ), and in many cases no  
203 mines were ever observed. On the control plants on average about 147 mines per plant were  
204 counted, and on two plants more than 200 mines were recorded. On the contrary, the infestation  
205 recorded on the treated plants was about 94% lower than the control, and the highest value ever  
206 detected on the treated leaves was 62 mines counted on a plant brushed twice with the saponin at  
207 the lowest concentration.

208 In some cases, as a side effect of saponinic treatments, leaves turned yellow when the 250 mg  
209 dosage was used. The number of mines of the leaves treated with the extract was lower in  
210 proportion to the concentration of saponin used, except for the brushing once treatment.

211 When commercial escin was compared to NSC (Fig. 2), despite the composition analogy of the two  
212 saponin mixtures, some differences in their respective effect were observed. NSC indeed gave the  
213 best results, statistically higher than those of the commercial escin. Notwithstanding, both the two  
214 saponin mixtures showed an appreciable effect against the leaf miner.

#### 215 **4. DISCUSSION**

216 Saponins are generally considered to be defensive against herbivorous insect when present in plants  
217 in sufficient concentration. This has been confirmed in the case of alfalfa saponins, which were  
218 shown to reduce larval growth and cause mortality, in the flower beetle, *Tenebrio molitor*, the  
219 European grape moth, *Lobesia botrana*, the European corn borer, *Ostrinia nubilalis*, and in a  
220 number of other insects.<sup>2,13,14</sup>

221 Due to the need in preserving the landscape and aesthetic value of the *Aesculus* trees, innovative  
222 control options have been pursued. Since parasitism rates are still very low,<sup>15</sup> and chemical control,  
223 using trunk injections, cannot be used routinely in urban situations, the removing of the fallen  
224 leaves, applied in autumn or winter, was the only advisable method to slow down the infestation  
225 during spring.

226 In the light of these recent results the possibility of limiting the severe infestation having recourse to  
227 saponins clear the way to a new possibility in containing the pest.

228 The field experiments showed that the tested molecule, when exogenously supplied, was easily  
229 transported from roots to leaves where it was then safely stored. The accumulation in adequate  
230 concentrations of the saponin in the leaves of the saponin-deprived susceptible genotype seems to  
231 ensure an appreciable degree of protection towards the leafminer. In effect, the fundamental  
232 findings of Kukula-Mlynarczyk,<sup>16</sup> evidenced the constitutive presence of saponins, in appreciable  
233 concentration, in the leaves of *Aesculus x carnea*, a genotype resistant to *C. ohridella*: this  
234 discovery shed light on the possible defensive role of this class of molecules against leaf miners in  
235 the *Aesculus* genus and it seems likewise to corroborate our hypothesis based upon our  
236 experimental data. From this point of view, the quali-quantitative differences observed between

237 NSC and commercial escin could explain the respective different effect recorded for the two  
238 saponin complexes. Interestingly, beside a different quantitative ratio of components, also the  
239 exclusive presence in NSC of the type III saponin could play some role in determining a higher  
240 biological effect.

241 It should be however taken into account that the chemical approach against herbivore insects is  
242 today conceived as a multi-factorial system, based on the interaction of different elements which  
243 induce first an antinutritional indirect effect, cooperating with a direct toxic activity to give an  
244 effective defensive response.<sup>17</sup>

245 After this promising results further investigations will be carried out to study in detail the influence  
246 of this saponin on *C. ohridella*, with particular care to its possible antifeedant, growth inhibitory or  
247 oviposition-deterrent activity.

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314 **Table 1.** Different escin concentrations found in treated leaves of the saponin-deprived *A.*  
315 *hippocastanum* plants according to different methods and concentrations

	Watering (once)		Watering (twice)		Stem brushing (once)		Stem brushing (twice)	
	125	250	250	500	125	250	250	500
Quantity (mg) administered per plant								
Cncn found in leaves (mg/g dry tissue)	4.5	8.0	4.7	12.8	2.3	2.6	4.6	5.3

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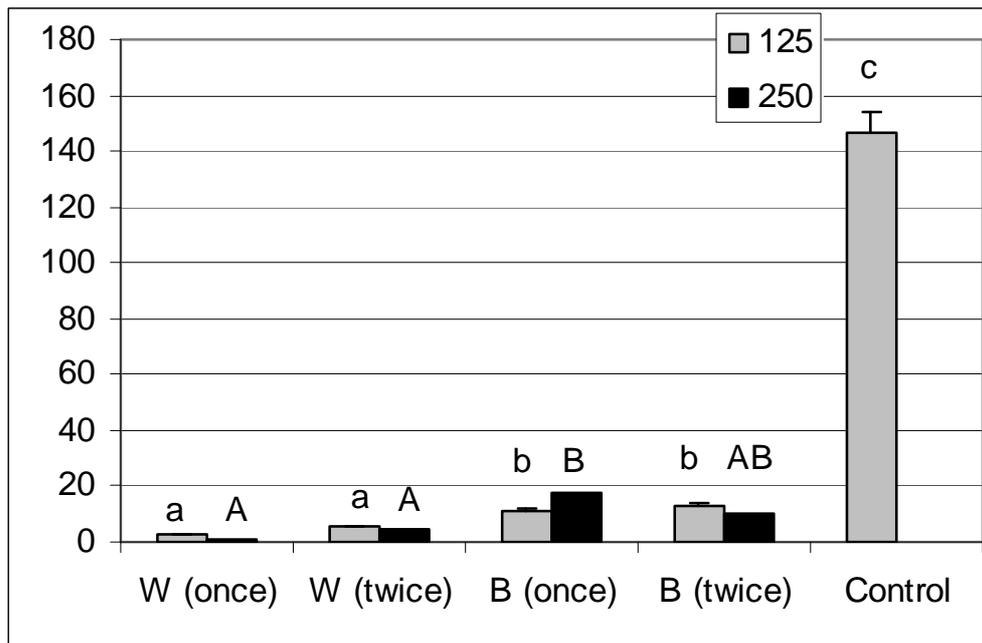
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348 **Figure 1.** Mean number of mines ( $\pm$  SE) of *Cameraria ohridella* Deschka & Dimic recorded on the  
349 leaves of the common *C. ohridella*-susceptible *A. hippocastanum* plants, treated with NSC (natural  
350 saponin complex) with different methods (W = watering; B = stem brushing) and concentrations.  
351 Bars with a common letter are not significantly different (Dunnnett's post test,  $P < 0.01$ ).

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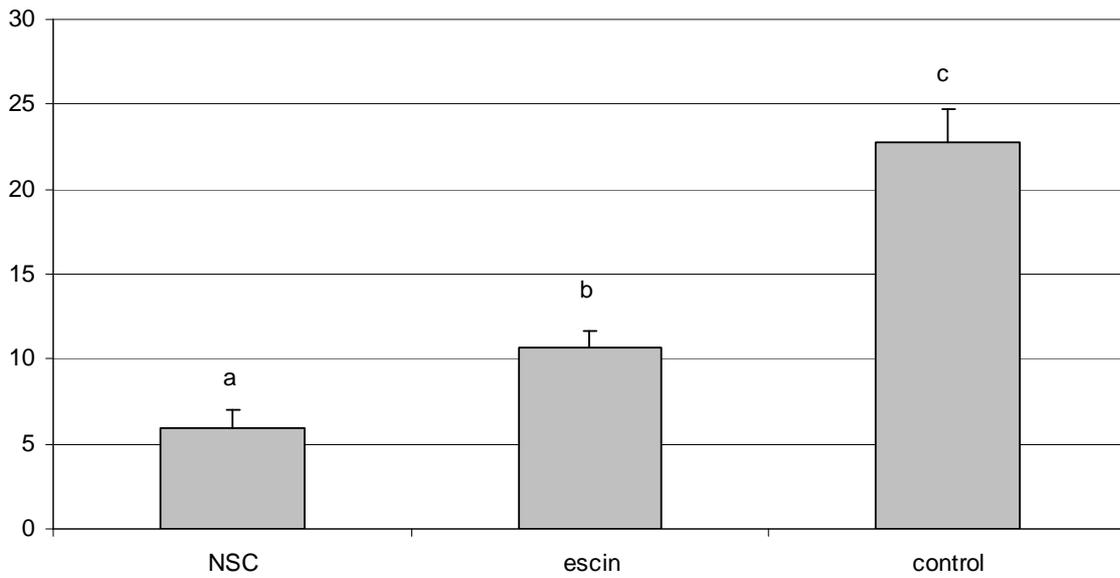
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362 **Figure 2.** Mean number of mines ( $\pm$  SE) of *Cameraria ohridella* Deschka & Dimic recorded on the  
363 leaves of the common *C. ohridella*'s susceptible *A. hippocastanum* plants, sprayed with both NSC  
364 (natural saponin complex) and commercial escin. Bars with a common letter are not significantly  
365 different (Dunnett's post test,  $P < 0.01$ ).