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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Abstract

BACKGROUND

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

RESULTS

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

CONCLUSION

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

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

1 INTRODUCTION

Plants synthesise a diverse array of secondary metabolites, either as part of normal growth and development or in response to pathogen attack or stress. Glycosylated sterols or triterpenoids, known as saponins, are an important group of plant secondary metabolites that are widespread throughout the plant kindom and have been identified in 80 plant families.[1,2]

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

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

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

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

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

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

2 MATERIALS AND METHODS

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

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

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

glass chromatography column, 50 x 2 cm, was packed with Silica gel 100 C_{18} reverse phase resin, 40-63 μm particle size (Fluka, Germany), previously swollen in EtOH 80%; before chromatographic runs, the alcoholic phase was carefully replaced by HCOOH 5% in ddw and floating fines removed. After sample application, column was eluted according to an elution linear gradient profile where A = HCOOH 5% in ddw and B = CH_3OH, from 0% to 80% B in A. 160 ml of both A and B were utilized, and 3 ml fractions were collected.

The presence of the investigated foliar natural saponin complex (NSC) was detected in the eluted fractions following upon its positive reaction, on TLC silica gel plates, to the Pancaldi reagent diagnostic for saponins [21g $(NH_4)_6MoO_4$ and 1g $Ce(SO_4)_2$ in 469 ml H_2O acidified with 31 ml H_2SO_4, by stirring at 50°C until solubilization].

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

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

The mobile phase consisted of A = 100% H_3PO_4 0.2 mM solution and B = 100% CH_3CN, while the elution profile was: 2 min, isocratic, 80% A and 20% B; 5 min, linear gradient to reach 40% A and

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

2.2 Plant Material and Experiment Setting

Fifty-four potted plants of the common *C. ohridella*-susceptible *A. hippocastanum* (white horse 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 outdoor conditions at the CRA-FSO Research Institute, Sanremo. Plants, 60-70 cm high, potted in 30 cm diameter clay pots, were placed in the cage according to a completely randomized distribution. A first series of treatments was set using 125 or 250 mg natural saponin complex (escin, Merck, Darmstat, Germany) dissolved in 500 ml double distilled water (ddw).

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

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

To evaluate the actual translocation towards leaves of the exogenously applied molecule, additional potted plants were used as leaf donors after the treatments. With this purpose, the applied treatments were: watering or stem brushing once and twice, (only the 250 mg dosage) respectively, the second treatment being carried out 17 days after the first one. Three plants per treatment were set.

A second series of experiments was carried out with the aim of comparing the effect of the foliar NSC and the commercially available escin. With this purpose, both NSC and escin were sprayed on leaves of potted white-flowering horse chestnut plants, of the same size and age as above mentioned. The two saponin complexes NSC and escin were respectively sprayed at 125 mg/l concentration in ddw, each on ten different plants, 12 days before adult release; control was represented by ten further plants sprayed with only ddw. The observations were performed, 45 days after adult release, on ten different leaves per plant: a hundred observations per treatment were thus carried out.

2.3 Insect Culture

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

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

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

Statistical Analyses. Data represent the mean ± standard error (SE) and were analyzed by one-way analysis of variance (ANOVA) and followed by *Dunnett's post test* for multiple comparisons. Differences were considered to be significant when the probability value was less than 0.01.

All the analyses were performed using the software SPSS® 13.0.

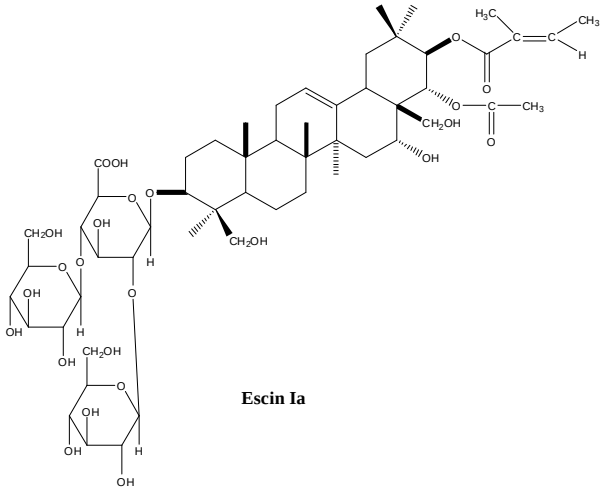
3. RESULTS

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

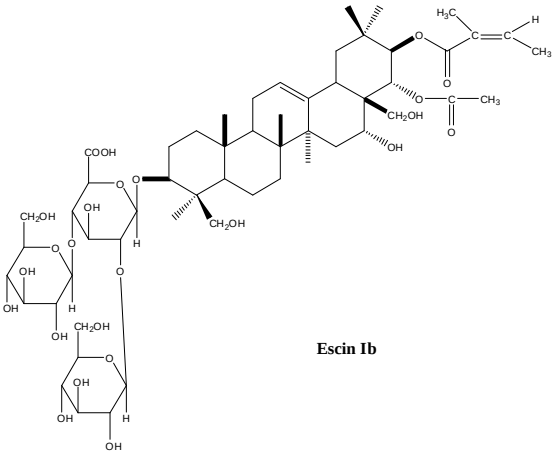
NMR mono and bi-dimensional analyses showed that *A. pavia* HBT genotype leaves contain a complex of saponins (NSC) based on polyhydroxyoleanene pentacyclic triterpenoid structure. The four components of NSC saponin complex have been identified as the common saponin components of the commercial escin,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

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

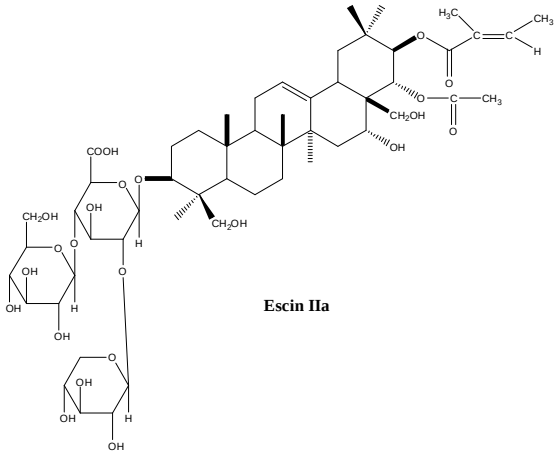
The structure formulae of the five different saponins detected during the analyses are reported below:



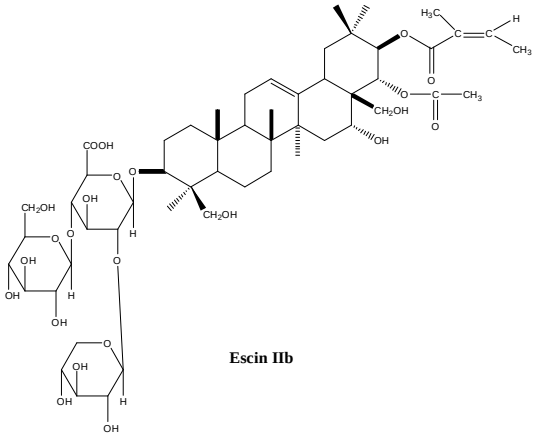
Escin Ia



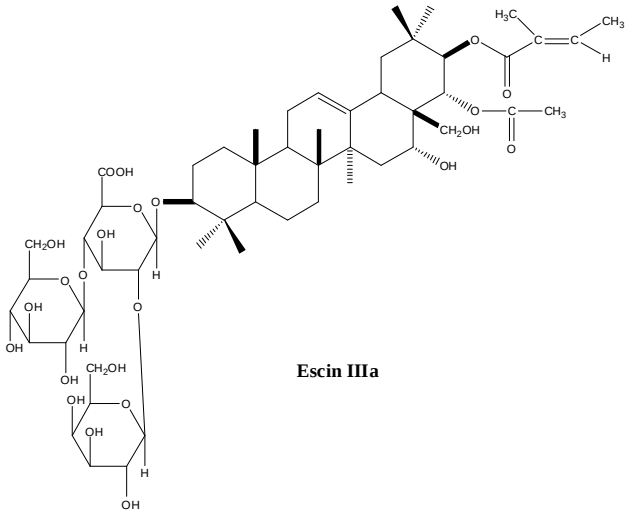
Escin Ib



Escin IIa



Escin IIb



Escin IIIa

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 ± 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 (*Dunnett'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.

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

4. DISCUSSION

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

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

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

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

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

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

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

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

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

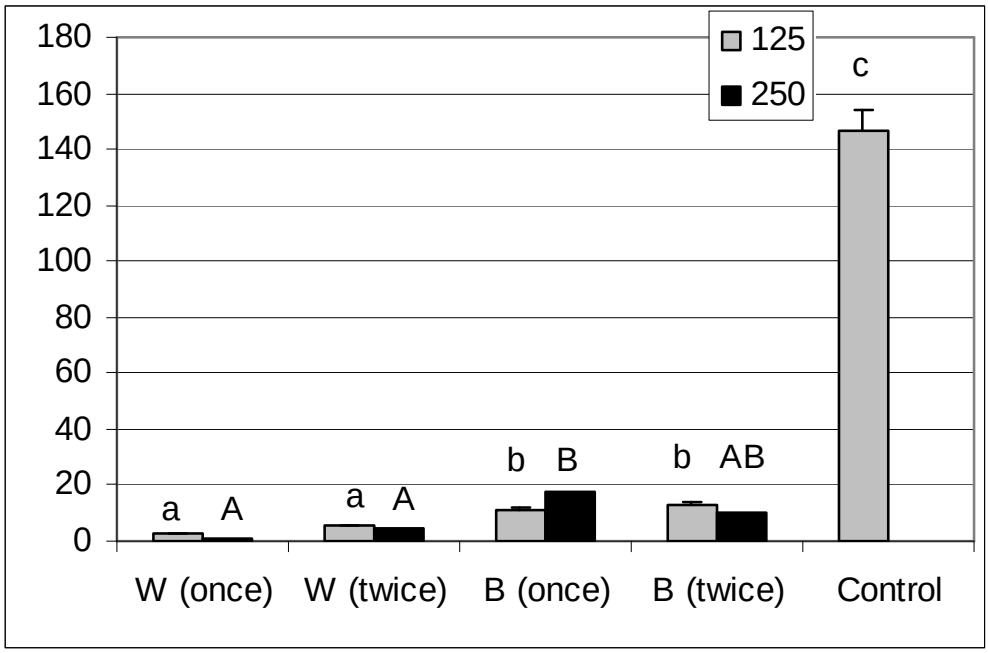

Figure 1. Mean number of mines (± SE) of *Cameraria ohridella* Deschka & Dimic recorded on the leaves of the common *C. ohridella*-susceptible *A. hippocastanum* plants, treated with NSC (natural saponin complex) with different methods (W = watering; B = stem brushing) and concentrations. Bars with a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

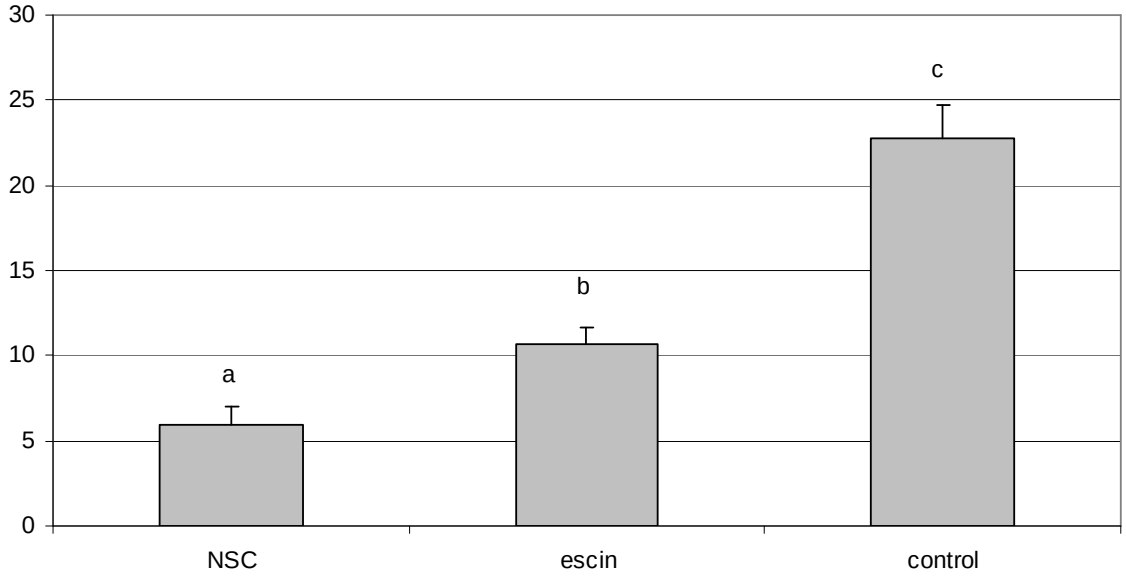

Figure 2. Mean number of mines (± SE) of *Cameraria ohridella* Deschka & Dimic recorded on the leaves of the common *C. ohridella's* susceptible *A. hippocastanum* plants, sprayed with both NSC (natural saponin complex) and commercial escin. Bars with a common letter are not significantly different (Dunnett's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