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Risks assessment of two acaricides (fluvalinate and oxalic Acid) in *Apis mellifera intermissa* (Hymenoptera, Apidae): Acetylcholinesterase and glutathione S-transferase activities

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Abstract
The ectoparasitic mite *Varroa destructor* [1] (Acari, Varroidae) is one of the most serious pests of honey bees *Apis mellifera* L., (Hymenoptera, Apidae) worldwide. It has being linked with the death of millions of colonies and several acaricides are used by beekeepers to control *V. destructor*. The objective of the present study was to determine possible negative effects of two acaricides fluvalinate, a synthetic compound, and oxalic acid, a natural substance by measuring acetylcholinesterase (AChE) and glutathione S-transferases (GSTs) activities in newly emerged workers bees, nurses and foragers of *A. mellifera intermissa.* Two groups of five hives each were treated with these acaricides and one group was left as control. Data showed that fluvalinate led to increase GST activity and decrease AChE activity in emerged and nurse bees as compared to controls. In the forager bees, the enzymatic activities were similar in all groups of honey bees. However, oxalic acid has no significant effect on AChE and GST activities in the emerged, nurse and forager bees.

Keywords: Apis mellifera intermissa, *Varroa destructor*, Acaricides, Secondary effects, Acetylcholinesterase, Glutathione S-transferase.

1. Introduction
Honey bees, *Apis mellifera* L. (Hymenoptera, Apidae), are ecologically and economically important insects. They ensure the pollination of many wild flowers, and thus contributing to plant biodiversity [2, 3]. The economic value of honey bees result not only from the hive products (honey, royal jelly, propolis and wax) but also from pollinating activity on crop plants [4, 5]. Honey bee colonies are host to a variety of parasites that can seriously affect their growth and survival [6]. *Varroa destructor* [1], is the most serious parasitic mite of honey bees worldwide [7, 8] that influences the development and performance of colonies [9-11]. In Algeria, *V. destructor* is also the major pest of honey bees affecting its physiology [12] and it is generally controlled by synthetic acaricides such as flumethrin, fluvalinate and amitraz [13]. Early control of the mite is necessary to prevent bee colonies from dying of secondary infections [14, 15]. Several chemical substances were used to reduce or eliminate the damages caused by *V. destructor* [16, 10, 17]: organophosphates (coumaphos); formamidine (amitraz) and pyrethroids (flumethrin and fluvalinate) [18-20] and natural substances such as organic acids (formic, lactic, and oxalic acid) [21, 22] and components of essential oils (thymol, eucalyptol) [23-25, 13]. Little has been reported on the side-effect of acaricides in honeybees [26-30] and few information based on the enzymatic aspects of the host after exposure to varroacides, has been given in literature [31, 32, 27, 33]. Likewise, depth studies on these acaricides are needed to evaluate their risks on the honeybee populations. Biomarkers have been used to reveal the exposure of organisms to various chemicals in the environment [34]. In previous reports, we have shown the efficacy of some synthetic and natural acaricides (flumethrin, amitraz, thymol and thymol blended with essential oils) against *V. destructor* [13] and evaluated their impact on the nutritional biochemistry of *A. mellifera* [29] and on their physiological enzymes: acetylcholinesterase (AChE) and glutathione S-transferase (GST) [33].
AChE represents a biomarker of neurotoxicity widely used for identifying exposure to chemicals such as organophosphorus and carbamate insecticides [35, 36] and some pyrethroids [37, 38]. To protect against the effects of oxidative stress, organisms have a variety of

Detoxifying enzymes at their disposal, such as catalase, glutathione reductase, glutathione peroxidase and GST, all of which have been reported to occur in insects [39-41]. Honey bees have active detoxifying enzyme systems [42-44] playing an important role for eliminating harmful substances [43, 45].

Therefore, the objective of this study was to investigate the probable effects of two potent acaricides fluvalinate, a synthetic compound, and oxalic acid, a natural substance against *A. mellifera intermissa* during the adult stages by measuring the AChE activity. To test the detoxification system, the GST activity was also determined. The attempt results permit us to evaluate the possible use of these two acaricides for *V. destructor* control.

2. Materials and methods

2.1. Bees

The trials were carried out in an apiary of honeybees derived from *Apis mellifera intermissa* [46] during September–October 2010 in eastern Algeria (Annaba: 30°50'N5°36'E). Three groups of five colonies of honey bees each (two treated and one control) were used. Before the experiment, the colonies were examined for diseases and were found to be healthy and no treatment was applied prior to our studies. Acaricides were applied just before egg laying. The adult bees (newly emerged, nurses and foragers) were sampled then from each colony.

2.2. Acaricides and treatment

Two acaricides, currently used against *Varroa* mites in honeybee colonies, were tested. They were applied after the honey had been harvested and as recommended by the manufacturers. Fluvalinate is available under the trade name Apistan (Wellmark International, Dallas, TX) was impregnated in plastic strips. Two plastic strips of Apistan (800 mg per strip) were inserted in the brood chamber of each hive. Strips of Apistan were left for four weeks and then removed from the treated hives. Oxalic Acid used at 3%OA/32% sucrose solution (w/w) was prepared from 6.5 g OA dihydrate (Kemika, Zagreb, Croatia) and 50g sucrose in 100 ml deionised water. Oxalic Acid treatment was applied by trickling 50 mL of the prepared solution which would be distributed with a syringe-5ml into each of the 9 spaces between the combs and 2.5 ml into the 2 spaces formed between the 1st and 10th combs and the sides of the hives. When the treatments had been on the colonies for 21 days (the duration of development of a worker bee), 12–18 adult bees were sampled from each treatment group when they reached 0, 7, and 21 days of age.

2.3. Enzyme assays

The acaricides were evaluated on enzyme activities in honeybees according to procedure previously described by Habes *et al.* [47]. The AChE activity was carried out following the method of Ellman *et al.* [48]. Using acetylthiocholine as a substrate. Pooled head were homogenized in the following solution containing 38.03 mg ethylene glycol tetra acetic acid (EGTA), 1ml Triton X-100, 5.845 g NaCl and 80 ml Tris buffer (10Mm, pH 7). After centrifugation (5000g, 5 min), the AChE activity was measured in aliquots (100µl) of resulting supernatants added to 100 µl of 5-5' dithiobis-(2-nitrobenzoic acid) (DNTB) in Tris buffer (0.01 M, pH 8) and 1 ml Tris (0.1 M, pH 8).After 5 min,100µl acetylthiocholine was added. Measurements were conducted at a wavelength of 412 nm with a run time of 20 minutes.

GST activities were determined with the soluble fraction as enzyme source. GST activities toward 1-chloro-2, 4-dinitrobenzene (CDNB) were measured according to Habig *et al.* [49]. Bees were sampled from control and treated groups and the sting apparatus and venal glands of adults were carefully removed [50]. Each decapitated body was homogenized in sodium phosphate buffer (0.1 M, pH 6) and centrifuged (14000 g, 30 min). 200 µl of the resulting supernatant was added to 1.2 ml of reaction mixture containing 1Mm CDNB and 5 mM reduced glutathione (GST) in the homogenization buffer. Changes in absorbance were recorded at 340 nm. Total protein content was determined according to method of Bradford [51]. Using bovine serum albumin as a standard. Enzyme activities were expressed as µM/min/mg proteins.

2.4. Statistical analysis

Results are expressed as mean ± Standard Deviation (m ± SD). The age and numbers of samples tested per series are given with the results. All data were subjected to one–way analysis of variance (ANOVA). The homogeneity of variances was controlled by the Levene method [52]. All data were subjected to one–way analysis of variance (ANOVA). Means were separated with a post-hoc Tukey test (p= 0.05) in [53] version 13 (Minitab Inc., State College, PA).

3. Results

3.1. Acetylcholinesterase activity

Data on the specific activity of AChE recorded at 0, 7 and 21 days following treatment of workers showed that there was no significant difference among colonies treated with oxalic acid and untreated colonies (P>0.05 for 0-, 7- and 21-days-old workers). In contrast, the activity of AChE were reduced in colonies treated with fluvalinate at day 0 (p<0.0001) and 7 (p<0.001) during the adult stage. However, treatment had no significant effect (p = 0.331) at day 21 (Fig. 1).

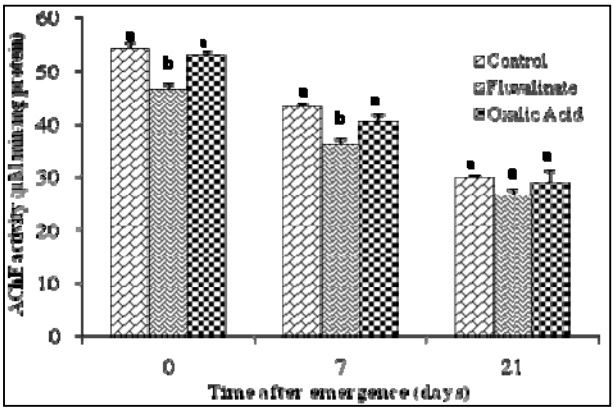

Fig 1: Effects of fluvalinate and oxalic acid on the activity of acetylcholinesterase (µmol/minute/mg protein) in the adult stage (0, 7 and 21 days old) of *Apis mellifera intermissa.* Data are expressed as means ± S.D. (n=12-18). For each age group, different letters above bars indicate significant differences at P<0.05 (ANOVA followed by a post-hoc Tukey test).

3.2. Glutathione S-transferase activity

The GST activity in treated and untreated groups is presented in figure 2. A significant increase in the specific activity of GST was observed after treatment by fluvalinate in newly emerged workers (p<0.0001) and in workers of 7 days old (p<0.05) as compared to controls. But at 21days during the adult life, the GST activity between treated and untreated ones was not significantly different (p>0. 05). Oxalic acid treatment had no significant effect (p>0. 05) on the GST activity at the different exposure times during the adult life (0, 7 and 21 days old).

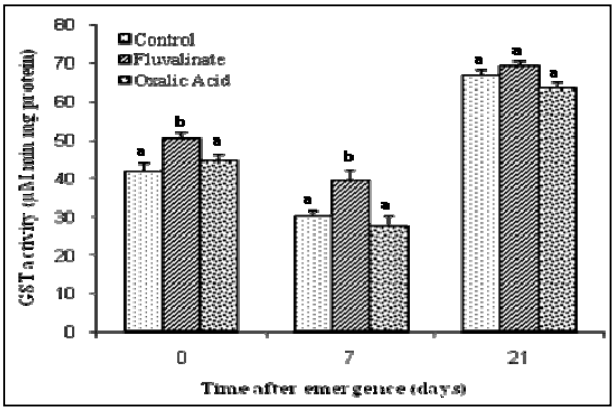

Fig 2: Effects of fluvalinate and Oxalic Acid on the activity of glutathione S-transferase (µmol/minute/mg protein) in the adult stage (0, 7 and 21 days old) of *Apis mellifera intermissa.* Data are expressed as means ± S.D. (n=12- 18). For each age group, different letters above bars indicate significant differences at $P<0.05$ (ANOVA followed by a post-hoc Tukey test).

4. Discussion

In the present investigation, our purpose was to determine the specific activities of acetylcholinesterase and glutathione-S transferase in the adult stage of honeybees from treated colonies with acaricides and control ones. AChE is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter, acetylcholine, in the nervous system in various organisms [54, 55]. In insects, AChE is confined to the central nervous system [56]. It is largely distributed in the bee brain [57-61]. AChE is of interest because it is the target site for organophosphorus and carbamate insecticides in the central nervous system, and its role in cholinergic synapses is essential for insects [62]. It has been demonstrated that some pyrethroids, which act mainly on the voltage dependent Na+ channels of the nerve cell membrane, can have secondary effects underlying the neurotoxicity like effects on AChE activity [36, 38]. Also, other classes of environmental contaminants such as complex mixtures of pollutants, detergents, and metals are also involved in AChE reduction [63, 38, 64, 35]. Inhibition of AChE causes accumulation of ACh at the synapses, so that the post-synaptic membrane is in a state of permanent stimulation, which results in paralysis and eventual death [65, 66].

GST belongs to phase II detoxification system involved in conjugation reactions and may also detoxify a number of toxic ligands by acting as a non-catalytic intracellular binding protein [67]. It is believed that these enzymes play essential role in the survival of insects exposed to endogenous or exogenous xenobiotics [50]. Induction of GST activity has been reported not only after exposure of insects to organophosphate [68, 69]. and organochlorine insecticides [70], but also to pyrethroids [43, 71]. These chemicals may act alone or in concert, in ways currently unknown, to create a toxic environment for the honey bee [8].

It is well known that the activity of AChE in honeybees increase progressively in the pupae stage to reach a maximal value at emergence [72]. Then, the activity of AChE decrease in the forager bees [72, 73]. However, the activity of GST is higher in forager than newly and nurse bees [45].

This study demonstrates that fluvalinate led to decrease AChE activity in emerged and nurse bees. Similar result was observed by [37] when deltamethrin (Pyrethroid) induced reduction in AChE activity in the honeybees. Other pyrethroids present similar effects on AChE activity [36, 38, 74-76]. However, treatment with natural substance (oxalic acid) has not caused any significant alteration in AChE activity. The same result has been observed after treatment with some acaricides tested in larval, pupae and adult stage of honeybee [33]. Weick and Thorn [27]. Observed similar results with other acaricides and reported that externally applied coumaphos showed no significant effect on bee brain AChE activity. However, since acaricides are used in many countries without supervision by the authorities, beekeepers sometimes leave the strips or treat the colonies with the new strips without removing the used ones. It is possible that high doses of acaricides will elicit AChE activity decrease.

Induction of GST activity has been reported in many insects following insecticide treatment [69-71]. Our data for GST activity indicate that only treatments with synthetic compound (fluvalinate) caused an increase in GST activity in emerged and nurse honeybees compared with the control group. This observation could be explained by induction of GST by toxic stress in the hive. However, GST activity in the forager bees showed no difference between treated and control group indicating that forager bees were exposed to a lesser degree of toxicant than were the individuals confined to the hive [32]. Loucif-Ayad *et al.* [33]. reported a similar effect when the treatment with synthetic compounds (Flumethrin and Amitraz) led to increase GST activity in the larval instars, pupae emerged and nurse bees. Other research effectuated by Nielson *et al.* [32]. Showed that identical effects after treatment with flumethrin. The difference in GST activity of treated and non-treated colonies could be an indication of a physiological effect on individuals in the treated colonies [31]. In contrast, a treatment of colonies with oxalic acid does not seem to have a difference in GST activity in all colonies tested in emerged, nurse and forager bees. Brosgaard *et al.* [31]. Also reported that oxalic acid does not affect GST activity in pupae and newly emerged adults. As well, Loucif-Ayad *et al.* [33]. Showed similar effects of two natural substances used as acaricides (Thymol and Thymol blended with essential oils) on GST activity in nurse and forager bees.

5. Conclusion

In conclusion, the current study demonstrates that fluvalinate has an effect on AChE and GST activities in honeybees. A reduction of AChE activity reveals that AChE could be used as a biomarker of neurotoxicity after exposing honeybee to fluvalinate. The increase of GST activity indicated that bees are exposed to toxic stress when fluvalinate was used in hives. However, the oxalic acid has not caused any significant alteration of the AChE and GST activities in the emerged, nurse and forager bees. Therefore, the oxalic acid, a natural substance, can be recommended as treatment but synthetic compound as fluvalinate should be minimized. Also, efforts are necessary to optimize the diversity and availability of mite control strategies and to use acaricides under supervision by the authorities in order to preserve bee health.

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